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ISOLATION AND CHARACTERIZATION OF HOST SPECIFIC BACTERIOPHAGES AND DETERMINATION OF THEIR POTENCY TO INHIBIT MULTIPLICATION OF MULTIPLE DRUG RESISTANT (MDR) BIOFILM FORMING *PSEUDOMONAS AERUGINOSA* ISOLATES

THESIS

SUBMITTED TO
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
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LUCKNOW

For the Degree of
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BY

SADHANA SINGH SAGAR
Under the Supervision of

DR. RAJESH KUMAR

Associate Professor

DEPARTMENT OF ENVIRONMENTAL MICROBIOLOGY

SCHOOL FOR ENVIRONMENTAL SCIENCES

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

(A CENTRAL UNIVERSITY)

VIDYA VIHAR, RAI BARELI ROAD, LUCKNOW-226 025, INDIA

Babasaheb Bhimrao Ambedkar University
(A Central University)
Vidya Vihar, Raebareli Road Lucknow - 226 025
बाबा साहेब भीमराव अम्बेडकर विश्वविद्यालय,
विद्या विहार रायबरेली रोड, लखनऊ – **226025**

Dr. Rajesh Kumar
Associate Professor
Department of Environmental Microbiology
School for Environmental Sciences

Letter No :----------

Date:…….

CERTIFICATE

This is to certify that the thesis entitled **"Isolation and characterization of host specific bacteriophages and determination of their potency to inhibit multiplication of multiple drug resistant (MDR) biofilm forming *Pseudomonas aeruginosa* isolates"** submitted in the partial fulfillment of the requirement for the degree of **Doctor of Philosophy** in **Environmental Microbiology** is a record of bona fide research carried out by **"Ms. Sadhana Singh Sagar"** under my guidance and no part of this thesis is submitted for any other degree or diploma.

(Dr. Rajesh Kumar)
Associate Professor
Dept. of Environmental Microbiology

Email: rajesh4971@yahoo.com, Mobile no-+919412090052; +919621329477

DECLARATION

I, Sadhana Singh Sagar, hereby declare that the thesis work entitled "**Isolation and characterization of host specific bacteriophages and determination of their potency to inhibit multiplication of multiple drug resistant (MDR) biofilm forming *Pseudomonas aeruginosa* isolates"** is my own work carried out under the guidance of **Dr. Rajesh Kumar, Associate Professor, Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University, (A Central University) Vidya Vihar, Rae Bareli Road, Lucknow.** The matter embodied in this thesis work is written by me and has not been submitted to any other university for the fulfillment of the requirement of any other Degree or Diploma.

Place: - **Lucknow** (**Sadhana Singh Sagar**)

Date: -

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Sadhana Singh Sagar

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ABBREVIATIONS

µg	Microgram
µl	Microliter
bp	Base pair
CFU	Colony forming units
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
EPS	Extracellular polymeric substances
FDA	Food and Drug Administration
FESEM	Field emission scanning electron microscope
gm	Gram
h	hours
ICTV	International committee on the taxonomy of viruses
L	Liter
min	Minutes
MOI	Multiplicity of infection
OD	Optical density
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
pfu	Plaque forming unit
SD	Standard deviation
SR	Static biofilm formation with media renewal
TEM	Transmission electron microscope
TSA	Tryptic soy agar
TSB	Tryptic soy broth
V	Volume

Introduction

CHAPTER 1 INTRODUCTION

Pseudomonas aeruginosa is a gram negative, opportunistic pathogen, which is an inhabitant in broad host range and diverse environments due to its vast genome size (Palleroni 1984; Stover *et al.,* 2000). *P. aeruginosa* is associated with a number of diseases in immuno-compromised hosts such as septicemia, myocarditis, pneumonia, chronic lung infection, endocarditis, dermatitis and osteochondritis. Several studies, illustrate that *P. aeruginosa* is equipped with a number of virulence factors which enable them to penetrate the host immune system and intoxicate host cells leading to chronic infections. These virulence factors include extracellular toxins (extracellular A, phospholipase C and elastase), which cause severe damage of host tissue by their enzymatic activity (Bitter, 2003). Other virulence factors are flagella, pili and alginate which are responsible for the adherence of cells to the substratum (Arora *et al.,* 1998). Alginate helps in the formation of expolysaccharide, which provides protection to cells from host clearance and from environmental stresses (Arora *et al.,* 1998; Govan, and Deretic. 1996; Manson *et al.,* 1998; Mattick, 2002).

P. aeruginosa often exist in the form of biofilm in nature on biotic as well as on the abiotic surfaces. Biofilm can also be formed on indwelling medical devices and subcutaneous biomedical implants such as cardiac pacemakers, heart valves, urinary tract prosthesis, peritoneal membranes and peritoneal dialysis catheters, vascular grafts and stents, joint prostheses and cerebrospinal fluid stunts (O'Toole *et al.,* 2000; Mah and O'Toole 2001; Donlan and Costerton 2002). Biofilm is a complex community of microorganisms in which microbes are embedded inside the matrix of exopolymeric substances (Costertone *et al.,* 1999; Davies 2003). Biofilm is made up of exopolymeric substances (polysachharides, humic substances, nucleic acids, proteins and water) and microbes (Casterton, 1978). Microbes acquire such kind of living style to protect themselves from environmental stresses. Biofilm provide 100-1000 times more protection to biofilm cells than the planktonic cells (Donlan and Costertone, 2002).

Pseudomonas aeruginosa is well known for its ability to develop resistance against most of the commercially available antibiotics. Several studies have suggested that resistance could arise through several mechanisms, including antibiotic-modifying enzymes and intrinsic resistance mechanisms (such as decreased outer membrane permeability and up-regulation of multidrug efflux pumps). *P. aeruginosa* have lower outer membrane permeability than any other gram negative bacteria and this feature provides slow diffusion of drug through the outer membrane (Hancock, and Speert, 2000).

Another mechanism employed by *P. aeruginosa* is to pump out undesirable chemicals, including anionic detergent and various antibiotics through a group of membrane-localized multidrug efflux pumps which is found in various gram-negative bacteria. To date, four such efflux pumps have been characterized in *P. aeruginosa*: MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM; along with several important regulatory factors (MexR/NalB, NfxB, NfxC/MexT, and MexZ), which controls the expression of these genes (Hocquet *et al.*, 2003).

Antibiotic modifying enzymes such as β-lactamase and aminoglycoside plays an important role in antibiotic resistance mechanism of *P. aeruginosa* directly by modifying or breaking down the antibiotic substrates. These enzymatic genes are commonly carried by mobile genetic elements such as plasmids and transposons, and in other cases these are chromosomally encoded (Bryan,1988).

However, the planktonic mode of life of these organisms can be controlled by the application of the latest class of antibiotics with high dose, but biofilm cells cannot be controlled. As we know biofilm embedded microbes are one thousand times more resistant for any antimicrobial compound than the planktonic cells (Foley and Gilbert 1996; Donlan and Costerton, 2002). Thus, it is difficult to remove biofilm cells with commercially available antimicrobials. Several protective mechanisms have been proposed to explain the biofilm resistance, including impaired penetration of antibiotics into the biofilm matrix, reduced growth rates of the bacteria within the biofilm, and an induced expression of specific resistance genes (Costerton 1999; Gilbert *et al.*, 2002;

Mah *et al.,* 2003). Thus, the failure of antibiotics in the control of biofilm forming pathogens has encouraged the researcher to search for the alternative methods.

Lytic bacteriophage has drawn the attention of researchers to control such type of pathogens (Theil 2004; Skurnik *et al.,* 2007). Bacteriophages are most abundant obligate bacterial parasite with an estimate of about 10^{31} phage over 10^{30} bacteria (Whitman *et al.,* 1998; Rohwer and Edwards 2002). Bacteriophages were first observed by de'Herelle in 1919, and he introduced phages in the medical practices for the control of bacterial pathogens. Hence, bacteriophage is not a new idea for the control of pathogens; it was common in practice in the early 19^{th} century in many countries such as France, Georgia, Soviet united and Russia. France was the pioneer country for the introduction of phages in medical practices. Afterwards, many countries accepted that phage can help in controlling pathogens until the discovery of antibiotics. But after the discovery of antibiotics the use of phage therapy got limited due to lack of knowledge about the pharmacokinetics and pharmacodynamics. Smith and Huggins rediscovered bacteriophage therapy in 1980. Thereafter, phage therapy was accepted by Europe and Asia in controlling pathogens. To date, phages and its cocktails are commercially available.

Bacteriophage can control planktonic as well as sessile form of microbial growth. Phage associated enzymes like dispersin B, depolymerases and alginases are helpful in degrading the biofilm matrix (Hughes *et al.,* 1998, Harper *et al.,* 2014). Phages are self-replicating with lot of advantages over antibiotics i.e. antibiotic acts only upon the actively dividing cells while phages can lyse cells at any stage. Use of phage therapy is an ecofriendly and economical technique.

Hence, in the present research, attempt has been made to control the biofilm formation of multiple drug resistant *P. aeruginosa* isolates with isolated bacteriophages. To see the impact of *P. aeruginosa* on the biofilm, first of all five multiple drug resistant *P. aeruginosa* isolates were isolated from patients with burn wound infections. Pathogenicity tests were performed to know about the pathogenicity of isolates. All the isolates were high biofilm former; hence to control the biofilm of pathogens six lytic bacteriophages were isolated from different sewage sources such as hospital drainage and sewage treatment plant around Kanpur city and Ganges river water, Kanpur, Uttar

Pradesh, India. All the phages were characterized for their stability to pH, and temperature. Furthermore, electron microscopy of phages was performed to know the morphology of phages. After characterization, phages were applied on the different stages of biofilm formation and found that all the phage has potential to inhibit *P. aeruginosa* biofilm at any stage, as verified by SEM and TEM studies.

Review of Literature

CHAPTER 2 REVIEW OF LITERATURE

2.1. About the *P. aeruginosa*

P. aeruginosa is a ubiquitous microorganism, can inhabit in broad environmental conditions. It is an opportunistic pathogen, can cause number of infections in a wide range of eukaryotic organisms including plants, invertebrates and vertebrates (Vasil, 1986; Rahme *et al*., 1995; Walker *et al*., 2004). Infections caused by *P. aeruginosa* can either be acute or chronic (Furukawa *et al*., 2006). Acute infections such as ventilator-associated pneumonia and urinary tract infections are characterized by rapid bacterial growth, eventually followed by sepsis, and if untreated, frequently death of the host may occur (Parrillo *et al*., 1990). Chronic infection such as cystic fibrosis, which is caused by *P. aeruginosa* (Lee *et al*., 2005; Smith *et al*., 2006; Jelsbak *et al*., 2007). Infections are often severe, life threatening and are very difficult to control because of the limited susceptibility to antimicrobials, consequence of that emergence of high number of antibiotic resistant microorganisms (Carmeli *et al.,* 1999*;* Garner *et al.,* 1988). Emergence of antibiotic resistance in *P. aeruginosa* is a severe threat to the medical society. Multiple drug resistance in pathogens is most common in fashion, which is a consequence of uptake of irregular and intermittent dose of antibiotics. Details about the drug resistance and their mechanism in bacteria have been discussed in the next section.

2.2. Emergence of Antibiotic resistance in *P. aeruginosa*

According to the survey of NNIS (National Nosocomial Infection Surveillance) organization in 2004, the problem of emergence of antibiotic resistant *P. aeruginosa* is increasing. High level of drug resistance is a consequence of de novo emergence of resistance after exposure to a specific organism (Carmeli *et al.,* 1999) as well as of patient to patient spread of resistant organisms (Fridkin and Gaynes, 1999). Accumulation of resistance after exposure to various antibiotics and cross-resistance between agents may result in multidrug-resistance (MDR) *P. aeruginosa*. Usually, *P.*

aeruginosa employs a different type of mechanism to escape themselves from commercial antibiotics.

2.3.Mechanism of antibiotic resistance in *P. aeruginosa*

Excessive and irregular use of antibiotics causes the emergence of multiple drug resistance in bacteria. Microorganisms employ a number of mechanisms to defend themselves from antibiotics. Resistance mechanism in bacteria can be acquired or intrinsic in nature. In *P. aeruginosa,* intrinsic resistance mechanisms have been evolved to protect themselves from antibiotics. According to Hancock and Speert (2000), efflux mechanism in *P. aeruginosa* is the only important element of the intrinsic resistance mechanism. Two efflux systems have been described, which have a role in the intrinsic antibiotic resistance mechanism based on their apparent constitutive expression and the influence of knockout mutations and inhibition. The first of these to be studied in the MexAB-OprM system (Zhao, 1998). This system is a prototype of RND (resistance-nodulation-division) system with a cytoplasmic pump protein, MexB, a periplasmic linker protein, MexA, and an outer-membrane protein, OprM. Mutations that prevent expression of any or all of these genes results in hyper-susceptibility to quinolones, tetracyclines, chloramphenicol, sulfamethoxazole, trimethoprim, and some β-lactams, but not aminoglycosides, erythromycin, polymyxins or imipenem and other β-lactams (Nikaido, 1994: Zhao, 1998). On the other hand, another efflux pump operon, MexXY, in apparent collaboration with OprM, was recently discovered and it has have the capability to efflux many of the same substrates as MexAB-OprM, but have a primary role in intrinsic resistance to aminoglycoside antibiotics and erythromycin (Aires *et al.,* 1999; Westbrock-Wadman 1999). Thus, mutation of MexXY led to increased susceptibility to aminoglycoside and erythromycin antibiotics.

P. aeruginosa strains produce an AmpC-like inducible chromosomal β-lactamase that can inactivate β-lactams by hydrolysis (Hancock and Speert, 2000). Induction of this β-lactamase, which occurs upon exposure to some β-lactams can result in increased resistance to the other β-lactams. However, not all β-lactams are strong inducers. According to some studies, the susceptibility of *P. aeruginosa* to some β-lactams (e.g.

ceftazidime, cefepime, piperacillin and aztreonam) is more strongly influenced by efflux, whereas susceptibility to others (imipenem and panipenem) is more strongly affected by the presence of β-lactamase, while a third group (ceftriaxone, meropenem and moxalactam) is influenced only by knockout of both efflux and β-lactamase (Masuda, 1999; Nakae, 1999). In contrast, in depressed mutants, knockout of efflux has no apparent effect. Acquired resistant mechanism in bacteria is a trait in which bacteria previously sensitive to an antibiotic, display resistance further, either by mutation or acquisition of DNA or a combination of the two (Vogel *et al.,* 2003).

Methods of acquiring antibiotic resistance in bacteria are further given below:

Krasovec and Jerman, (2003), reported that mutations in bacteria can be either spontaneous or adaptive. Spontaneous mutation may be either by replication error or due to DNA damage in actively dividing cell responsible for antibiotic resistance (Martinez and Baquero, 2000). Several studies reported that mutations in the genes encoding the targets of rifamycins and fluoroquinolones, i.e. RpoB and DNA-topoisomerases respectively, results in resistance against those compounds (Mascaretti, 2003; Schwarz et al., 1995). Prolonged exposure of bacterial species to sublethal concentration of antibiotic switches a small population of bacteria to generate a brief state of high mutation (Blazquez *et al.,* 2012; Gullberg *et al.,* 2011). This stage of mutation in bacteria is called 'hypermutation' in which they acquire to relieve the selective pressure, they grow, reproduces and exits the state of high mutation rate (Dalhoff, 2012; Macia, 2005). Krosovec and Jerman (2003) reported that, bacteria overcome such selective pressure related problem by induction of a special type of SOS inducible mutator DNA polymerase (pol) IV.

Hypermutation in bacteria play a significant role in the evolution of antibiotic resistance and may also be responsible for the multi-resistant phenotype which has been reported in several literature (Blazquez, 2003; Chopra *et al.,* 2003; Malik *et al.,* 2006; Mascaretti, 2003). These mutations, known as adaptive mutations, have been associated with the evolution of antibiotic resistant mutants under natural conditions (Bjedov, *et al.,* 2003; Lewin *et al.,* 1991; Tomasz and Munaz, 1995). Adaptive mutagenesis is regulated by the

stress responsive error prone DNA polymerase V (umuCD) and IV (dinB) (Patel *et al.*, 2010; Sutton *et al.*, 2003; Rosche, 2009).

Bacterial biofilm also provide protection from environmental stresses and make the embedded cells resistant to antibiotic 1000 fold more than planktonic cells (Donlan, 2002). Biofilm is the most preferable lifestyle of microbial cells; more than 99% of all bacteria exist in nature as biofilms (Costerton, 1987). Biofilms are defined as an assembly of microbial cells that are irreversibly associated with a surface (either inert or living) and enclosed in a matrix of primarily polysaccharide material, which allow to grow bacterial cells in that environment. The term biofilm was coined by Costerton in 1978. Since then, it has been well documented that biofilm-associated microbes differ from their planktonic relatives in terms of the genes that are transcribed (Donlan, 2002). In nature, biofilms constitute a protected growth modality that allows the bacteria to survive in hostile environments. Bacteria can develop biofilm on a number of surfaces such as indwelling medical devices and subcutaneous biomedical implants - cardiac pacemakers, heart valve, urinary tract prosthesis, peritoneal membrane and peritoneal dialysis catheters, vascular graft and stent, joint prostheses and cerebrospinal fluid stunts living tissues, medical devices (Donlan, 2001; Flemming and Wingender, 2001). Biofilm of medical implants is a serious cause of concern for medical practitioners, and it is very difficult to control the biofilm of pathogens because biofilm cells are highly resistant than planktonic cells for antimicrobial compounds. Along with these resistant mechanisms, biofilm also contribute in the emergence of a multiple drug resistance in bacteria against a range of antimicrobials. The details about the biofilm and its role in antimicrobial resistance are given below.

2.4. Biofilm and its structure:

Formation of biofilm is characterized as the most prominent survival strategy of microbes under the stressed condition. Nevertheless, the detailed studies of sessile communities in different environments have led the conclusion that planktonic growth of microbes rarely exists in nature (Costerton, 1999). Most of bacteria live in microenvironment known as biofilm. EPS of biofilm, allow biofilm cells to grow by

rendering it resistant against harsh environments, biofilm associated microbes differ from planktonic cells with respect to their physical and molecular working mechanism (Donlan, 2002).

Biofilm is composed of microbial cells and EPS. EPS of biofilm contains polysaccharides, uronic acids, proteins, nucleic acids, lipids, phospholipids and humic substances (Jahn and Nielsen, 1998; Sutherland, 2001; Wingender *et al.*, 1999). According to Tsuneda (2003), proteins and polysaccharides account for 75–89% of the biofilm EPS composition, indicating that they are the major components. Bacterial population in biofilms is accompanied by extracellular DNA (eDNA) (Dubnau, 1999; Lorenz *et al.*, 1991) and because most bacterial species bind to DNA, it appears that eDNA may serve as a cell-to-cell interconnecting compound in many different biofilms. However, exopolymers, outer membrane proteins and a variety of cell appendages such as pili and flagella may also function as a part of the biofilm matrix. The components of the biofilm matrix are usually, but not always, produced by the bacteria themselves. This is why biofilm formation in natural and industrial environments allows bacteria to develop resistance to amoebae, chemically diverse biocides, host immune responses and antibiotics (Costerton, 1999). Biofilm science and engineering is an emerging field which helps us in understanding the complexity of biofilm structure and their characteristic (Costerton *et al.*, 2003).

2.5. Steps of biofilm formation:

Biofilm formation is carried out in following steps as shown in Figure. 1

i) Attachment to the surface
ii) Formation of microcolonies (Facilitated by quorum sensing)
iii) Formation of Matrix (Facilitated by quorum sensing and molecular signaling)
iv) Formation of Macrocolony (Maturation of biofilm)

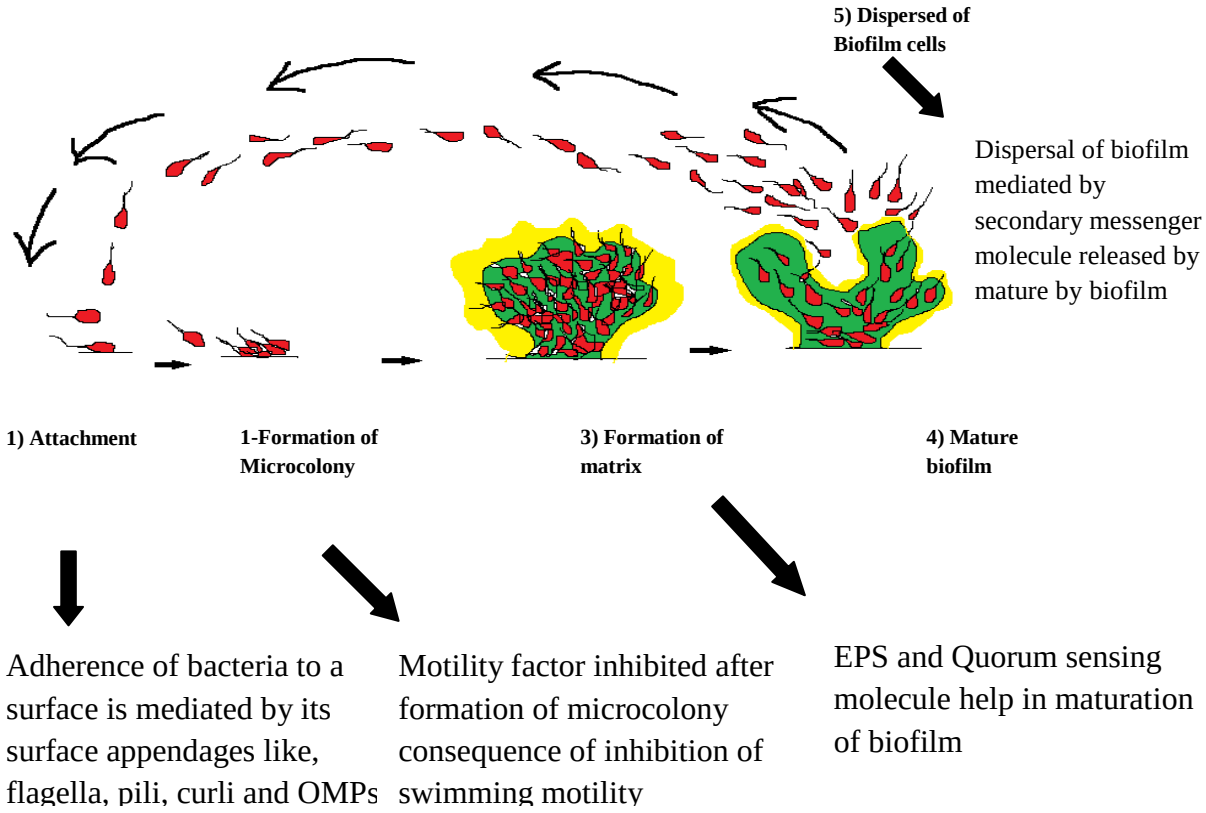


Figure.1 Steps of biofilm formation

(i) Attachment to the surface:

The initial stage of biofilm formation is the attachment of planktonic cells to a surface. The process of attachment is reversible and dynamic. During this stage bacteria may rejoin or detached from the surface (Anderson *et al.,* 2004; Banin *et al.,* 2005; Wu and Outten, 2009). This process is facilitated by repulsive and weak interaction forces. Eventually the attachment of cells to surface gets irreversible. In some cases, pili is involved in attaining the irreversible attachment to the surface. In *P. aeruginosa* type IV pili facilitate the irreversible attachment (O'Toole and Kolter, 1987; Klausen *et al.,* 2003).

EPS: EPS provides strength and hydrodynamic environment to the biofilm. EPS of biofilm is very complex in nature and it is composed of humic substances, polysaccharides, lipids, proteins and nucleic acids. It impart the three dimensional

structure to the biofilm. EPS of biofilm is act as diffusion barrier which prevents the penetration of any type of harmful substance inside the biofilm (Donlan, 2002).

(ii) Formation of microcolonies: After attachment to the surface, bacteria start the formation of microcolony. Microcolony facilitated by quorum sensing. Details about the quorum sensing in bacteria is given bellow.

Quorum sensing: Bacterial biofilm is just like a house. As in a house, people use different kind of language to communicate with each other, in the same manner bacteria use quorum sensing based mechanism for communication. Quorum sensing is a density based mechanism in which bacteria interact with each other through the chemical signal. Quorum sensing is a vital component for the formation of biofilm. At high density, microbes secrete autoinducer molecules in the extracellular environment, which turn on the quorum sensing system (Kjelleberg and Molin 2002). Activation of quorum sensing circuit occurs when the concentration of autoinducer exceeds beyond the threshold (Fig.2). The *luxI/luxR* system is a prototype of a quorum-sensing system used by many gram-negative bacteria (Henke and Bassler, 2004). The quorum sensing system is quite different in gram positive and gram negative bacteria (figure. 2).

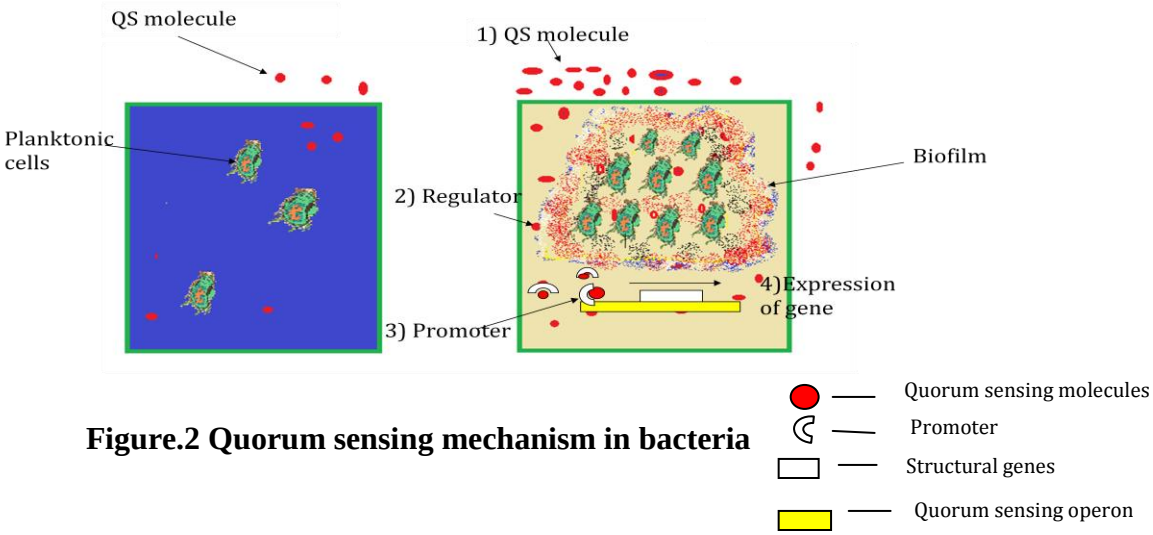


Figure.2 Quorum sensing mechanism in bacteria

The details of this system were first elucidated in the luminescent marine bacterium *Vibrio fischeri*, in which quorum sensing regulates light production (Fuqua *et al.,* 2001; Losick *et al.,* 2001). Bioluminescence in such bacterium is achieved when the cells reach a threshold density in the system. Bioluminescence in *V. fischeri* is controlled by *lux* genes (Henke and Bassler, 2004; Fuqua *et al.,* 2001; Losick *et al.,* 2001). At right side in Lux operon, genes present which are required for the synthesis of autoinducer (LuxI) and for bioluminescence. Lux gene is responsible for the production of autoinducer which synthesizes N-(3 oxohexanoyl)-L-homoserine lactone (OHHL) (Losick *et al.,* 2001). Binding of the autoinducer to the N-terminal regulatory domain enhances multimer formation by LuxR, which inducing the C-terminal domain to activate transcription from both the Lux operons (Koch *et al.,* 2005). Here the Lux CDE encode products that form the multi enzyme complex which synthesizes aldehyde substrate utilized by luciferase. Lux G gene encodes flavin reductase and is followed by a transcription termination site (Nealson and Hastings, 1979).

Hybrid quorum sensing system:

Vibrio harveyi uses hybrid systems with components of both the gram-positive and gram-negative prototype of quorum-sensing systems (Bassler, 2002; Bassler *et al.,* 1993). Hybrid quorum sensing system cause bioluminescence in *V. harveyi* (Bassler *et al.,* 1994), type III secretion in *E. coli* (Sperandio *et al.,* 1999), production of virulence factor vir B in *Shigella* (Day *et al.,* 2001) and recently it has been proposed that dental bacteria use this system for biofilm formation (Li *et al.,* 2002; McNab *et al.,* 2003). In this systems, Acyl Homoserine Lactone (AHL) -type autoinducers are present. In addition to the AHL-type autoinducers, which is species specific, an interspecies autoinducer called AI-2, a furonosyl borate ester, has been also identified as a signaling molecules for hybrid quorum-sensing systems (Henke and Bassler, 2004).

Quorum sensing system in Gram Positive bacteria:

Gram positive bacteria use secretary proteins as an autoinducer molecule for activation of quorum sensing system. Different bacterial species such as *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus pneumonia* employ quorum sensing system to

regulate phenotypic expression (Novick, 2003). When the density of microbial cells reaches at a threshold level, then the secreted autoinducing peptide (AIP) signal is detected by two component sensor kinase. This activates the response regulator, facilitating DNA binding and alters the transcription of target genes involved in quorum sensing (Novick *et al.*, 1995).

AIP-deficient mutants form more robust biofilms than the wild-type strain (Vuong *et al.*, 2004; Yarwood *et al.*, 2004), leading to the conclusion that the AIP quorum-sensing system negatively regulates biofilm formation. It has been observed that biofilm formation in *S. aureus* is highly dependent on the glucose availability in culture medium. Regassa *et al.*, 1992 reported that *S. aureus* biofilm has gone detached in glucose deficiency by increasing AIP level and activating *agr* quorum-sensing system. This may be partially supported that glucose is necessary supplement carbon source for *S. aureus* biofilm formation (Boles and Horswill, 2008).

Quorum sensing in Gram Negative Bacteria

In gram negative bacteria, quorum sensing system play vital for expression of virulence traits. In *P. aeruginosa* quorum sensing system comprises two circuits Las and Rhl which used N-acyl homoserine lactone signal molecules 3OC12-HSL and C4-HSL respectively (Chugani and Greenberg, 2015) for signalling. Acyl homoserine lactone (AHL) type of quorum sensing system is involved in direct interaction with plants and animals (Parsek *et al.*, 1999). AHLs are synthesized by enzymes, which are member of LuxI family of acyl-HSL synthases (Fuqua *et al.*, 1994; Schaefer *et al.*, 1996; Parsek *et al.*, 1999; Fuqua *et al.*, 2001). Different LuxI homologs generate different acyl-HSLs. *P. aeruginosa* produces RhlI, primarily catalyzes the synthesis of N-butyryl- HSL (C4-HSL), and LasI directs the synthesis of N-(3-oxododecanoyl)-HSL (3OC12-HSL) (More *et al.*, 1996). Acyl-HSL signaling is critical for virulence in the plant pathogen *Erwinia carotovora* (Schaefer *et al.*, 2001) and for virulence of *P. aeruginosa* in mouse models of lung (Pirhonnen *et al.*, 1993), in burn infections (Tang *et al.*, 1996), in invertebrates (Rumbaugh *et al.*, 1999), and in plants (Tan *et al.*, 1999). LasR is a transcriptional regulator that responds primarily to LasI generated signal and 3OC12-HSL, while RhlR responds best to the RhlI generated signal, C4-HSL (More *et al.*, 1996). RhlR then

activates expression of genes required for production of a variety of secondary metabolites such as hydrogen cyanide and pyocyanin (Rahme *et al.,* 1995). Quorum sensing plays a very important role in biofilm formation in *P. aeruginosa*. Mutation in LasI imparts a dramatic change in biofilm formation by suppressing the synthesis of 3OC12-HSL. LasI mutant's biofilm formation is arrested after microcolony (More *et al.,* 1996).

Signaling inside biofilm at molecular level:

C-di GMP: Cyclic di-GMP is a secondary messenger molecule which controls biofilm formation as well as virulence expression in a bacterial cell. Cyclic di-GMP was first discovered as a regulator of cellulose synthesis in *Glucoacetobacter xylinus* and it emerged as a major bacterial secondary messenger (Ross *et al.,* 1990; Huang *et al.,* 2003; Rice *et al.,* 2005; Romling *et al.,* 2005). The intracellular concentration of cyclic di-GMP is controlled by the GGDEF domain proteins with diguanylate cyclase (DGC) activity and the EAL domain proteins with cyclic di-GMP-specific phosphodiesterase activity (Huang *et al.,* 2003). GGDEF domains catalyze the condensation of two molecules of GTP to generate cyclic di-GMP, while the EAL domain catalyzes the hydrolysis of cyclic di-GMP to generate dinucleotide 5′-pGpG.

EAL domain contains a large number of proteins. Several proteins which contain EAL and GGDEF domains regulate virulence gene transcription, biofilm formation, motility and adhesion in various pathogenic bacteria. VieA and CdgC proteins, which are involved in biofilm formation, motility and virulence factor production in the human pathogen *Vibrio cholera* (Tamayo *et al.,* 2007; Jenal and Malone, 2006; Karatan *et al.,* Lim *et al.,* 2006). EAL domain protein was found to control lateral flagellar-gene expression and swarming behaviour in *Vibrio parahaemolyticus* (Tischler and Camilli, 2004). In *Salmonella enterica*, the disruption of the EAL domain protein CdgR weakens bacterial resistance to hydrogen peroxide and accelerates bacterial killing by macrophages (Kim and McCarter, 2007). In the opportunistic pathogen *P. aeruginosa*, the EAL domain-containing protein FimX controls twitching motility and biofilm formation (Rice *et al.,* 2005; Hisert *et al.,* 2005) and the BifA protein controls biofilm formation and swarming (Kazmierczak, *et al.,* 2006). A systematic analysis of the GGDEF and EAL domain proteins in *P. aeruginosa* identified several other EAL domain

proteins as being involved in virulence expression and biofilm formation (Hisert *et al.*, 2005; Kuchma *et al.*, 2007).

(IV) Maturation:

Microcolony of bacterial biofilm is converted to mature form by multiplication of cells and secretion of polymeric substances. Quorum sensing also play a very important role in the maturation of biofilm. Klausen *et al.,* (2003) showed non-motile and a migrating subpopulation of *P.aeruginosa,* which formed stalks, while the motile subpopulation which formed mushroom-shaped caps on these stalk by migrating with type IV pili. Boles *et al.,* (2004) reported functional diversification of *P. aeruginosa* cells in the biofilm, with a subpopulation which form a wrinkle colony on the agar surface while another form a mini colony. The matured biofilm is characterized by a complex architecture (Davey *et al.,* 2003; Boles *et al.,* 2004; Wood *et al.,* 2010).

V) Dispersal of biofilm:

There are several factors which are responsible for the dispersal of biofilm for eg. oxygen and , nutrient depletion and presence of toxins (Bridier *et al.,* 2011; Sauer *et al.,* 2004; Rowe *et al.,* 2010; Hong *et al.,* 2010). Biofilm can be dispersed in the form of bulk and in the form clumps from the attached surface. Nutrient depletion can also be cause of dispersal of biofilm in *Pseudomonas* species (Karatan and Watnick, 2009; Hunt *et al.,* 2004). Bacteriophage associated enzyme can also dissolved the matrix of biofilm, which eventually leads to dispersal of biofilm (Gjermansen *et al.,* 2005). Secondary messenger molecule cyclic-di GMP plays a pivotal role in the formation of biofilm but reduced the level of these molecule switch the cells sessile form to planktonic form (Hanlon *et al.,* 2001; Boles *et al.,* 2004; Morgan *et al.,* 2006; Pruss *et al.,* 2006).

2.6. Significance of Biofilm:

Persistent infection is a global problem for medical practitioners, and it is reported that biofilm play a significant role in persistant infections. Bacterial cells that cause

infections, affect internal organs such as *P. aeruginosa* affects lungs (cystic fibrosis; Barraud *et al.,* 2009), *E. coli* causes urinary tract infections (Klausen *et al.,* 2003) and *Mycobacterium tuberculosis* causes human tuberculosis (Ojha *et al.,* 2008) have been associated with biofilm formation. A spectrum of indwelling medical devices or other devices used in the healthcare have been shown to harbor biofilms, resulting in measurable rates of device associated infections (Donlan, 2001). The role of biofilms in the contamination of medical implants has been well established. Early electron microscopic studies of medical implants revealed the signs of bacteria residing in the form biofilms (Neu *et al.,* 1994; Busscher *et al.,* 1998; Gristina *et al.,* 1988; Singh *et al.,* 2000).

It is evident that bacterial biofilms on prosthetic valves are the leading cause of endocarditis in patients who have undergone heart valve replacement. Amongst patients who develop these infections have high mortality rate of about 70% (Nickel *et al.,* 1987). Millions of catheters (e.g., central line, intravenous and urinary catheters) used in the medical field are potential source of infection and ideal surfaces for the formation of biofilm. Biofilm formation can also occur on contact lenses and these biofilms are thought to contribute keratitis (Hyde *et al.,* 1998; Gorlin *et al.,* 1996; Elder *et al.,* 1995). Another example of a biofilm-mediated infection is chronic ear infection (otitis media). These infections are often caused by biofilm bacteria (McLaughlin-Borlace *et al.,* 1998). Thus, biofilms are the major cause of damage in medical and social resources every year.

2.7. Resistance Mechanism of biofilm against antimicrobial compounds:

The bacterial cells enclosed within the biofilm matrix are extremely resistant to antibiotic treatment. Such resistance can be explained hypothetically given by (Figure.3).

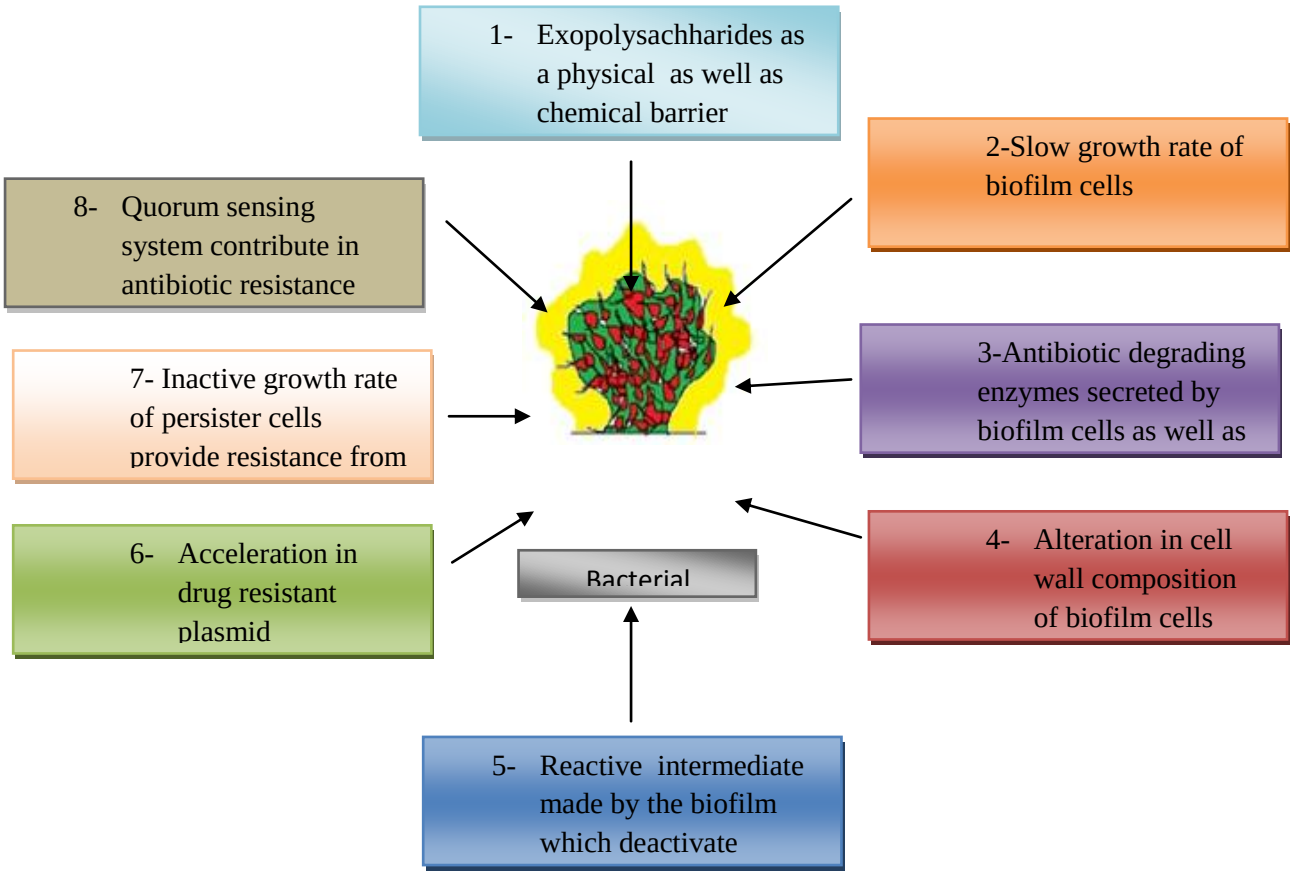


Figure.3 Biofilm resistance mechanisms

1) EPS of biofilm acts as a physical/chemical barrier, thus preventing many antimicrobials (Dingman *et al.*, 1998; Costerton *et al.*, 1995; O'Toole *et al.*, 2000; Lewis, 2001). Moreover, EPS is negatively charged due eDNA and functions as an ion-exchange resin which is capable of binding a large number of the antibiotics such as streptomycin, gentamicin, tobramycin and neomycin and tetracycline (Chopra *et al.*, 1992) etc. have positive charge that are attempting to reach the embedded biofilm cells.

2) Embedded biofilm bacteria generally not divide rapidly, virtually antibiotics only act on rapidly dividing cells (Thien and O'toole, 2001; Brown *et al.*, 1998; Wingender *et al.*, 1999). Slow growth rate inactivates the RelA protein which regulates synthesis of ppGpp. In bacterial cells, ppGpp inhibits the anabolic processes as well as suppresses the activity of autolysin, SLT, which makes cells resistant to antibiotic and autolysin respectively (Wentland *et al.*, 1996; Betzner *et al.*, 1990; Cashel *et al.*, 1996).

3) EPS of biofilm matrix contains the β lactamase enzyme in the immobilized form, which degraded β lactame antibiotics. Biofilm of *P. aeruginosa* contain 32 times more β lactamase enzyme than the planktonic cells (Prakash *et al.,* 2003).

4) Up to 40% of the cell-wall protein composition of bacteria in biofilms is altered from planktonic cells (Tuomanen *et al.,* 1986; Costerton *et al.,* 1995). The membranes of bacteria embedded in biofilm might be better equipped to pump out antibiotics to protect themselves from the antimicrobials.

5) Biofilm matrix deactivates antimicrobials faster than its diffusion, for example, reactive oxidants such as hypochlorite and hydrogen peroxide (Costerton *et al.,* 1995; Lewis, 2001; Potera, 1999; Chen and Stewart, 1996; De Beer *et al.,* 1994). These antimicrobial oxidants are product of the oxidative burst of phagocytic cells and poor penetration of these may partially account for the inability of phagocytic cells to destroy biofilm microorganisms.

6) Biofilms also provide an ideal environment for the exchange of extrachromosomal DNA (Plasmid) responsible for antibiotic resistance, virulence factors and survival capabilities at accelerated rates, all these things make it prefect milieu for emergence of drug resistance pathogens (Donlan, 2002; Collet *et al.,* 2007; Xu *et al.,* 1996).

7) Formation of persister cells inside the biofilm provides resistance against antibiotics (Hausner, and Wuertz, 1999). Persister cells are characterized as non growing cells. However, the target of antibiotics is actively dividing cells (Lewis, 2010). They are extremely resistant to antibiotics as well as host immune response (Jayaraman, 2008).

9) Quorum sensing system in bacterial biofilm contributes in antibiotic tolerance and resistance to the host immune system (Lewis, 2008).

2.8. Characteristics of biofilm that can be important in infectious disease processes include-

a) detachment of cells or biofilm aggregates may result in bloodstream or urinary tract infections or the production of emboli (Dufour *et al.,* 2012).

b) increased resistance to antimicrobials and immune system: Cells may additionally exchange resistance plasmids within biofilm leading to exaltation of virulence factors,

or antibiotic resistance (Raad *et al.*,1992; Murga *et al.*, 2001; Sedor *et al.*, 1999; Donlan, 2000).

c) production of toxins: Gram negative bacteria within the biofilm produce endotoxins (Sedor and Mulholland, 1999). Endotoxins can transport in the dialysis membrane of dialyzer through dialysate in hemodialysis patients (Vincent *et al.*, 1989). Measurement of the level of endotoxin in the hemodailysis tube indicates a correlation with bacterial colonization (Holland *et al.*, 2000). Endotoxin release from the biofilm increase the biocompatibility of dialysis fluid and leads to chronic inflammatory complications in patients (Marion-Ferey *et al.*, 2005).

d) Quorum sensing systems contribute in expression of multiple virulence factors in microorganism (Lewis, 2008).

2.9. Strategies used for the control of biofilm:

Bacterial biofilm are very difficult to control or eradicate from surfaces, therefore for the removal of biofilm alternative approaches of conventional antibiotics have been used.

There are a range of small molecular compounds which can inhibit the biofilm formation of gram positive as well as gram negative bacteria. Opperman *et al.* (2009), screened a number of molecules and found that aryl rhodanines inhibit the early stage of biofilm formation in gram positive and gram negative bacteria. Sambanthamoorthy *et al.*, (2011) reported that benzimidazole inhibit the biofilm formation by inhibition of second messenger molecule C-di GMP. Davies and Marques, 2009, reported dispersal of biofilm by cis-2-decenoic acid.

Brominated furanones is a natural compound isolated from the marine algae *Delisea pulchra* that have very good antibiofilm property against *S. aureus, P. aeruginosa, Enterococcus feacalis, S. mutan, S. epidermidis* (Pereira *et al.*, 2014). cis-9-octadecenoic acid also exhibits very good antibiofilm activity, which is isolated from rhizospheric bacterium *Stenotrophomonas maltophilia* BJ01 (Singh *et al.*, 2013).

Many heavy metals also have antimicrobial activity and can inhibit biofilm formation, for example, silver ion inhibits the biofilm formation; but biofilm matrix proteins

degrade silver ions. Application of silver nanoparticles for the control of bacterial biofilm is a good approach. Thuptimdang *et al.,* 2015 reported that biofilm is more susceptible for nanoparticle when EPS is stripped from it. Kalishwarlal et al., 2010 reported that biofilm formation of *P. aeruginosa* and *S. epidermidis* are controlled by silver nanoparticles. Mohanty *et al.,* 2012 reported the control of biofilm of *P. aeruginosa* with starch mediated synthesized silver nanoparticles. Habash *et al.,* 2014 reported control of biofilm of *P. aeruginosa* PA01 biofilm with citrate capped nanoparticles. Gum Arabic capped silver nanoparticle control biofilm formation of clinical isolates *P. aeruginosa* in concentration dependent manner.

Furanones is a chemical compound isolated from the red alga *Delisea pulchra;* it inhibits the biofilm formation (Khan *et al.,* 2002). Coating of such biofilm inhibitor can limit the biofilm formation in the medical implants. Dispersin B produced by a gram negative *Actinobacillus actinomycetemcomitans,* disperses the biofilm by targeting the matrix of biofilm (Kaplan *et al.,* 2004).

In addition of these chemical compounds, there are some biological agents which can limit the biofilm formation. These include bacteriophage and their associated enzyme. Phage control biofilm by production of depolymerases, dispersin B and alginases enzymes which help in the breakdown of the biofilm EPS (Yan *et al.,* 2013; Harper *et al.,* 2014; Sharp *et al.,* 2006). There are two strategies employed by bacteriophage for control biofilm is blocking of biofilm development and eradication and removal of existing biofilm cells (Różalska *et al.,* 2010). The most advantageous feature of bacteriophage is that they are host specific, self replicating entities, and once administered they self replicate in the host and destroyed them (Parasion *et al.,* 2014). Moreover, bacteriophage cocktails can remove mixed biofilms of bacteria (Lu and Collins, 2007; Lehman and Donlan, 2015). Use of bacteriophage is more economical, ecological and ecofriendly.

2.10. Control of Biofilm of *P. aeruginosa*:

As discussed above that emergence of multiple drug resistance pathogens are very fast and development of new antibiotics for their control is under pipeline. Lytic

bacteriophages have drawn special attention in controlling the MDR superbugs. Bacteriophage are obligate parasite of bacteria are ubiquitous in nature.

2.10.1. What are bacteriophages?

Bacteriophages are obligate parasite of bacteria, are found wherever their host is present such as water, soil, air, sewage, ocean, feces, urine, saliva, rumen and serum (Higgins *et al.*, 2007; Gantzer *et al.*, 2002; Caroli *et al.*, 1980; Bachrach *et al.*, 2003; Nigutová *et al.*, 2008; Keller and Traub, 1974). It is estimated that approximately 10 bacteriophage exist for one bacterial cell on the earth (Suttle, 2005; Fuhrman and Noble, 1995; Fuhrman, 1999). Phages are highly specific to their host. Due to host specific nature, phages can be a good antibacterial agent. In India, people have faith that by taking bath in the holy river (Ganges) all the skin diseases get cured. The actual tenet working behind this tradition is that Ganges river is rich source of phage and on exposure to its water bacterial pathogens are killed by phages (Fig.4 shows the tradition of taking bath in Ganges river).

Figure. 4 People are taking bath in Ganges river

2.10.2. Historical background of phage and their implication in control of infections:

In 1896, a British scientist, Ernest Hanbury Hankin demonstrated that Indian river Ganges and Yamuna contained some kind of agent that causes lysis of bacterial pathogen Cholera. He also demonstrated that destructive agent can pass through filter membrane and can reside in bacteria. In 1915, British Microbiologist, Twort

demonstrated that bacterial culture may associate with filter pass substances which is transparent and can destroy the bacterial cell. This demonstration was performed on the *Micrococcus* bacteria isolated from vaccinia (material of some colonies which could not be cultured but can infect new cells). According to him the bacteria associated non-filterable agent are able to infect pure culture for several generations. Twort described that these agents are as ferment which secreted by bacteria for some purpose and it was not clear at that time. After two year of Twort finding, Felix d'Herelle independently described similar finding, while studying on patients suffering or recovering from the bacillary dysentery. d'Herelle isolated that agent from the shigellosis patient and named them shiga-toxins, he filtered shiga-toxin and incubated for 18 h after that applied them on the fresh culture, and found that shiga-toxin can limit the growth of bacteria by lysis of bacterial cells. He demonstrated these agents as obligate parasite of bacteria. d'Herelle introduced this in medical practices and published non-randomized trials all over the world (figure-5), while Twort due to lack of fund could not pursue his study in the same domain. He even introduced treatment with intravenous phage for invasive infections, and he summarized all these findings and observations in 1931 (d'Herelle, 1931).

Several contributions of other investigators supported the d'Herelle idea that phage are the living entity and they can replicate inside of the bacterial cell. In 1925, Bordet and Bail proposed the phenomenon of phage lysogeny. Frank Macfarlane was an Australian scientist awarded Noble Prize in 1960, described the lysogeny as well as presented that phage is viral particle which can interact with their host (Sankaran, 2010). Schlesinger confirmed the biochemical nature of phages nucleoproteins (Pennazio, 2006; Schlesinger, 1934; Ruska, 1940), and allowed the existing theories to join together: phages are viral particles that are made of nucleoproteins.

After the invention of Electron Microscope by Helmut Ruska, it was demonstrated that spherical shaped as well as sperm shaped particle found to adhere on bacterial membrane are actually bacterial parasites (Kruger, 2000). After one year description of phage by electron microscopy, Luria and Anderson in Camden, New Jersey, described roughly spherical shape particle associated with a tail like structure (Luria

et al., 1943; Smith and Huggins, 1982). They also described different stages of bacteria on phage infection: adsorption which increases with time, extensive bacterial damage and appearance of a large number of newly formed bacteriophages.

Figure. 5 Bacteriophage injection now commercially available in the market was launched by George Eliavia Institute, Georgia

2.10.3. Structural morphology and classification of bacteriophage:

Bacteriophage are made up of nucleic acid (DNA or RNA), capsid and envelope. Bacteriophages have been classified into 13 families, three of them are well known (Myoviridae, Siphoviridae and Podoviridae). The classification of phage into families was done with regard to their morphology and size as shown in Table 1 and figure-6 respectively. About 96% of them are tailed, but filamentous and pleomorphic phages may exist as well (Ackermann, 2007; Hendrix, 2002). Generally, the phage virion consists of two basic components: nucleic acid (double- or single-stranded RNA or DNA) and a protein envelope. Some have lipids as components of the envelope (Ackermann, 2003).

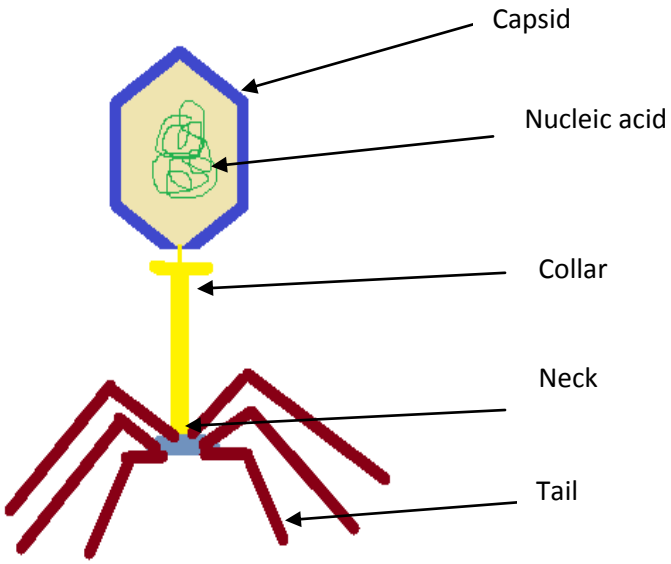


Figure.6 Phage morphology

Table 1 Classification of bacteriophages

Family[a]	Members[a]	Nucleic acid structure[a]
Myoviridae	T2	Linear dsDNA
	T4	Non-enveloped, contractile
	ΦgspC	tail, consisting of a Sheath
	PFpW-3	and a central tube
	CP-51	
	C16	
	MB08	
Siphoviridae	Λ	Linear dsDNA
	T1	Non-enveloped, long non-contractile tail
	T5	
	PFpW-8	
	TSP4	
	P001	

	P008	
	MB07	
Podoviridae	T3	Linear dsDNA
	T7	Non-enveloped, short
	PFpW-6	non-contractile tail
	28B	
	Kpn5	
	N22	
Microviridae	ΦX174	Circular sDNA Non-enveloped, isometric
Corticoviridae	PM2	Superhelical, circular dsDNA Non-enveloped, isometric, lipid layer in capsid
Tectiviridae	MS2	
	PP7	
	Qβ	
	f2	
Leviviridae	MS2	Linear ssRNA
	Qβ	Non-enveloped, isometric
	f2	
	PP7	
Cystoviridae	Phi6	Linear segmented Lipoprotein envelope, spherical
Inoviridae	M13	Circular ssDNA Non-enveloped, filamentous
	Fd	
	Pf1	
	Vf33	
Lipothrixviridae	TTV1	Linear dsDNA Enveloped, rod-shaped
	SIFV	
Rudiviridae	AFV-1	Linear dsDNA Non-enveloped, rod-shaped
	SIRV1	

Plasmaviridae	L2	Circular superhelical dsDNA Enveloped, pleomorphic
Fuselloviridae	SSV1	Circular superhelical dsDNA
	SSV2	Non-enveloped, lemon
	SSV3	shaped, short spikes at one end
	His1	

[a]Based on Ackermann, 2003

2.10.4. Bacteriophage Life cycle:

Bacteriophages have an alternative option to complete their life cycle in their host. These are lytic and lysogenic option, to complete their life cycle by lysis of host cells (lytic life cycle), by incorporating their genome in the host cells or dividing with host cells (lysogenic), pseudo-lysogenic and in chronic infections they carry out both types of life cycle (Weinbauer, 2004; Drulis-Kawa *et al.,* 2012). Phages can be divided in to three groups on the basis of their life cycle-lytic, lysogenic and pseudolysogenic. Bacteriophage life cycle starts from the attachment with the host cells. On exposure to the host a series of events occurs which leads to the infection of host cells.

a. Attachment:

The first event of lytic life cycle is attachment of phage to the host cell surface receptors. Attachment is common for all type of cycle of phage. There are a variety of receptors available on the surface of bacteria such as lipopolysaccharides, flagella and different type of proteins such as Lam B proteins which is used by Lamda bacteriophage for attachment (Chatterjee and Rothenberg, 2012). Some phages also are able to synthesize specific enzymes (such as hydrolases or polysaccharidases and polysaccharide lyases), which are able to mortifying exopolysaccharide structure or capsules (Drulis-Kawa *et al.,* 2012).

b. Adsorption:

After attachment to the host cells, the second event comes up, which is adsorption, by the binding to receptor of host, phage induces hole in the bacterial cell wall and injects DNA into the host.

c. Expression of phage genome and assembly:

This is followed by the expression of phage early genes, in which the lytic phages redirects the bacterial synthetic machinery to the reproduction of viral nucleic acids and proteins (Wittebole *et al.*, 2014). Assembly and packing of phages is then scrutinized before bacterial cell lysis and release of phage progeny occurs.

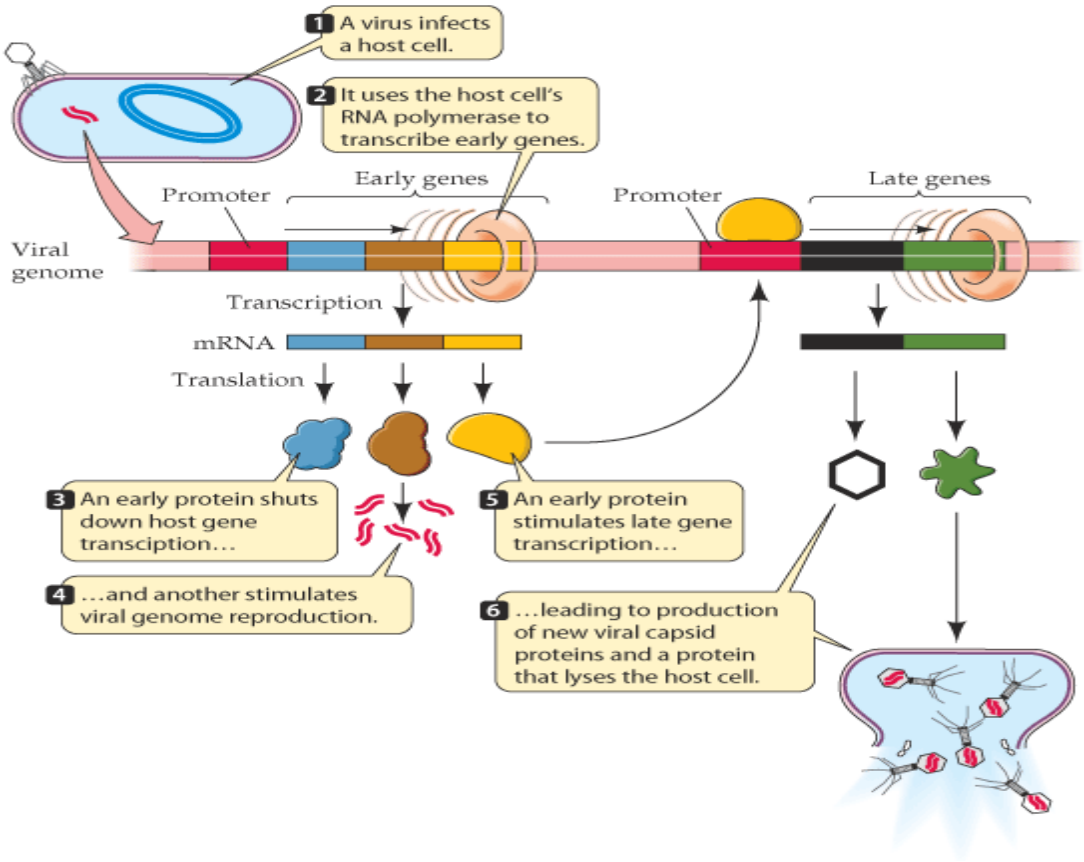


Figure-7 Expression of gene in phage life cycle

d. **Lysis of host cell and release of phage progeny:**

Phage's late enzymes such as lysins, holins, and murein are employed for the release of virion in the extracellular environment (Wittebole *et al.*, 2014). The number of released viral particles is (burst size) greatly varies according to the phage, the state of the bacterial host, and other environmental factors such as nutritive components affect the host lyis and release of phage (Weinbauer, 2004).

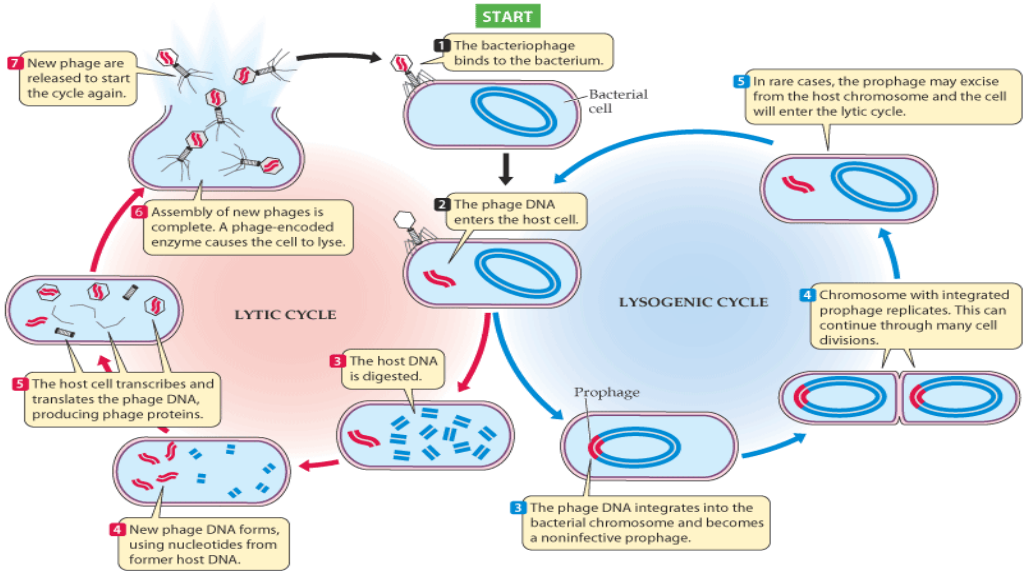


Figure.8 Life cycle of bacteriophage

2.10.4.1. Lysogenic life cycle:

Lysogenic life cycle, is also known as temperate life cycle, operate by temperate bacteriophage by incorporation of phage genome with bacterial chromosome, where it remain silent and replicate along with bacteria and pass to the progeny. This prophage DNA is vertically transmitted along with the whole bacterial genome to its progeny until the lytic cycle is induced (Weinbauer, 2004). Fig.2 depicted lysogenic life cycle of bacteriophage:

2.10.5. Role of bacteriophages against multiple drug resistant bacteria

The limitation of therapeutics focused the researcher to search for alternative agent for the control of (MDR) bacteria, known by the acronym as the ESKAPE pathogens (for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* spp.), which has now become a looming health care crisis in many ICUs worldwide (Pendleton *et al.,* 2013).

Antibiotic resistance is now recognized as a health care emergency and appeals for the development of novel strategy to combat, it have been voiced by many people. However, antibiotics are developed on the basis of free market criteria, rather than on the basis of direct benefit to the public (Aiello *et al.,* 2006).

The need for phage applications certainly exceeds due to its use in human infections. Indeed the use of bacteriophages has been described in various situations, including (but not limited to): food safety (Mahonty *et al.,* 2007), agriculture (Haq *et al.,* 2012), veterinary applications (Atterbury *et al.,* 2009), industry (Mahonty *et al.,* 2007), and clinical diagnostic applications for detection and typing of bacteria in human infections.

2.10.5.1. Use of bacteriophage in food industry:

There are five main microorganisms which cause spoilage of food material in the food industries. These are *E. coli*, *Campylobacteria*, *Salmonella, Listeria monocytogens* and *S. aureus*. Food industries are going through a huge economical loss every year due to the spoilage and contamination of food.

Listeria monocytogens is a gram positive bacterium which causes spoilage of food and therefore for their control, bacteriophages are administered on the approval of US food and drug administration (FDA) as a name ListshieldT^{M}, Intralytix (Golkar *et al.,* 2014).

E. coli is a gram negative bacterium causes a number of disease associated with its toxins. The most common way of transmission of infection is via undercooked, contaminated food, while water and milk contamination are related to direct or indirect contact with feces. *E. coli* multiply in ruminants and because they can survive better in intestine and if proper care not taken during slaughtering, the contents of the intestines,

fecal material, or dust on the hide may contaminate meat (Kaper, 1999). *E. coli* contamination can be lethal some time, because they can't be prevented by commercially available antibiotics. Therefore, for the control of such deadly pathogen, phage therapy came into play. Phage therapy is successfully employed against *E. coli* in ruminants, cattle and to poultry (Huff *et al.*, 2002, 2003).

Campylobacter is a genus of gram-negative, spiral, motile, and microaerophilic bacteria. It is most common in zoonotic in Europe (EFSA, 2004). *Campylobacter* commonly multiplied in the intestine of poultry and cattle, though infection most commonly associated with fecal contamination (Evans and Sayers, 2000; Oliver *et al.,* 2005). *Campylobacter* causes campylobacteriosis, typically characterized by fever, bloody diarrhoea and acute abdominal pain (Humphrey *et al.*, 2007). The World faces every year handsome amount of economical loss and agricultural loss due this microorganism. Therefore, for the control of *Campylobacter*, phage therapy has been introduced (Carrillo *et al.* ,2005; El-Shibiny *et al.,* 2009; Carvalho *et al.*, 2010; Reich *et al.,* 2008). Phage therapy is very successful against drug resistant bacteria and it has no side effects.

Salmonella is a gram negative facultative intracellular bacterium; it is a principal cause of zoonotic disease worldwide. *Salmonella* serovars can colonize and persist within the gastrointestinal tract and so human salmonellosis is commonly associated with consumption of contaminated foods of animal origin (Sillankorva *et al.*, 2012). *Salmonella enteric* serovars, Enteritidis and Typhimurium, are responsible for the majority of *Salmonella* outbreaks, and most events are reported to be due to consumption of contaminated eggs and poultry, pig, and bovine meats, respectively (EFSA, 2009). *Salmonella* is also a known spoilage bacterium in processed foods. Once ingested, this microorganism can cause fever, diarrhoea, abdominal cramps, and even life-threatening infections (Barbara *et al.,* 2000). Phage therapy became very successful against *Salmonella*. Valuable outputs have been made in the last couple of years in terms of using phages for *Salmonella* biocontrol. Today, two phage products are available: (1) **BacWash** from OmniLytics Inc. which received, in 2007, USDA's Food Safety and Inspection Services approval to be commercialized and applied as a mist, spray, or wash on live animals prior to slaughter; (2) **BIOTECTOR S1** phage product from Cheil Jedang Corporation that is to be applied on animal feed to control *Salmonella* in poultry.

S. aureus is a gram positive bacterium which is a major threat to food safety and also causes mastitis in cow's milk (Le Loir *et al.*, 2003). The contamination of food by this organism can be by human as well as animal activity. It causes toxin oriented disease on the consumption of contaminated food. The annual estimated loss worldwide due to mastitis in adult dairy cows is about 35 billion US dollars (Wellenberg). Phage research has focused on the treatment of mastitis in lactating dairy cows and mostly in dairy food products. Use of bacteriophage against *S. aureus* has been proved as beneficial and economical. Phage therapy significantly reduces the chances of spoilage of dairy products without any side effect. Therefore, phage therapy has proved as magical biocontrol element, which do not have any side effects on consumption.

2.10.5.2. Use of bacteriophage in agriculture:

Food demand is increasing with the increment in human population. Plant pathogens such as bacteria can reduce the plant growth as well as crop yield. For instance, citrus greening of orange, which is caused by bacteria belonging to genus *Candidatus* (Scortichini *et al., 2012)* while canker of kiwifruit caused by *Pseudomonas syringae* causes disease in two crops (Kiwifruit Vine health, 2012). Both diseases cause major loss of crop and result in an economical loss (Cooksey, 1990). However, these pathogens can be controlled with copper and antibiotics. There are a stacks of methods reported for control of these pathogens such as pesticides, insect vectors vector control, antibiotics (e.g., tetracycline and streptomycin), and copper (McManus *et al.*, 2002; Dye, 1953). Copper has been used over 100 years for the control of pathogens. Antibiotic streptomycin is used against *Pseudomonas spp*.. But the increasing use of such antimicrobials cause the emergence of resistant microorganisms as well as accumulation of metals in the environment which eventually has reached a toxic level. Hence the introduction of bacteriophage is more effective and advantageous for control of such pathogens.

Phages are viruses that specifically infect bacteria, yet have no direct negative effects on animals or plants (Gilligan, 2008).. Infection of a bacterium by a virulent phage typically results in rapid viral replication, followed by the lysis of the bacterium and the release of numerous progeny phages. These phages can then proceed to infect neighbouring

bacteria. Therefore, the numbers of phage will expand when target pathogens are encountered and the therapy will essentially be amplified in response to the bacterial infection.

Revival of interest in phage therapy for the control of phytopathogens has been seen in recent years (Balogh *et al.,* 2010; Jones *et al.,* 2007; Svircev *et al.,* 2010). Apparently, this renewed interest is due to the nontoxic nature of phages and their ability to infect antibiotic or heavy metal resistant bacteria. Successful phage therapy is being applied commercially to processed and packaged foods by Intralytix and Micreos Food Safety (formerly EBI Food Safety) and to agricultural crops by Omnilytics. There has been enhancement in interest in the use of phage in the detection of phytopathogens. Many of the phages against plant pathogens have been isolated for diagnosis and strain typing of plant pathogens (Klement, 1959; Petty *et al.,* 2007; Schofield *et al.,* 2012). Interestingly, in the EU (Commission decision, 2004) and the USA Listex (Listex*TM,* 2012) has been employed for the plant pathogens. In the agricultural sector Omnilytics has developed AgriPhage and a range of phage products for the control of *Xanthomonas campestris* pv. *vesicatoria*, for the treatment of bacterial spot of tomatoes and peppers, and *Pseudomonas syringae* which is the causative agent of bacterial speck on tomatoes (Frampton *et al.,* 2012).

Phage in agriculture is being applied as a biopesticide to control plant pathogens of tomato (Jones *et al.,* 2012), citrus (Balogh *et al.,* 2008), and onion (Lang *et al.,* 2007). Phages have potential to control fire blight infections, as lytic phages have been highly infective against plant pathogens.

2.10.5.3. Use of bacteriophage in veterinary:

As humans, animals also suffer from microbial infections and some time these infections become fatal. Slanetz and Jawetz in 1941 first time published report of isolation of bacteriophage against *S. aureus* from cow's milk. Slanetz and Jawetz studied about the effectiveness of phage against mastitis. *Salmonella* and *E. coli* are most important animal pathogens, and the improper treatments of infected animal are major cause of mortality. *Salmonella* is a major food contaminant which is severe public health concern. In

commercial poultry, including chickens (Fiorentin *et al* 2005, Toro *et al* 2005, Atterbury *et al* 2007, Filho *et al* 2007, Borie *et al.,* 2008a, Borie *et al.,* 2008b), turkeys (Higgins *et al.,* 2007) and laying hens (Borie *et al.,* 2011), they are most common intestinal microorganisms.

Campylobacter is the most commonly reported zoonotic in Europe (EFSA 2011), and *C. jejuni* estimated to cause approximately 845,000 illnesses, 8,400 hospitalizations, and 76 deaths each year in the USA(Carrillo, et al., 2005). This widespread infection is explained because ingestion of low doses (400–500 cells) (El-Shibiny *et al.,* 2009) can cause campylobacteriosis typically characterized by fever, bloody diarrhea, and acute abdominal pain (Carvalho *et al.,* 2010). *Campylobacter* is capable of colonizing the intestine of poultry and cattle, and thus infection is mostly acquired by fecal-oral contact, ingestion of contaminated foods (i.e., raw meat and milk contaminated through feces), and waterborne through contaminated drinking water (Evans *et al.,* 2000; Oliver, *et al.,* 2005; Reich *et al.,*).

Hence, bacteriophage is an alternative biocontrol agents which are employed by veterinarian to control the deadly pathogens of animals.

2.10.5.4. Use of bacteriophage in clinical field:

Pathogenic microbes are spreading their claws every day to capture their host worldwide. Many of bacterial pathogens, which are associated with epidemics are highly Multiple Drug Resistant (MDR) (Davies and Davies, 2010). Some threat for the clinical society are *Acinetobacter baumannii*, *Burkholderia cepacia*, *Campylobacter jejuni*, *Citrobacter freundii*, *Clostridium difficile*, *Enterobacter spp.*, *Enterobacter faecium*, *Enterococcus faecalis*, *E. coli*, *Haemophilus influenzae*, *Klebsiella pneumonia*, *Proteus mirabilis*, *P. aeruginosa*, *Salmonella spp.*, *Serratia spp.*, *S. aureus* and *Streptococcus pneumonia* (Davies and Davies, 2010*)*. These microbes have evolved resistant mechanism for different class of antibiotics and they called as 'superbugs'. Superbugs enhanced mortality and morbidity by acquiring multiple mutations to get resistant against different class of antibiotics. The therapeutic options for the control of such microbes are limited, while the time period of hospital care as well as cost are usually extended. Hence, to

overcome from the stress of microbial drug resistance, medical practitioners have diverted their attention from antibiotics to bacteriophage. Bacteriophage therapy has become successful in controlling the multiple drug resistant microbes. After the rediscovery of phage therapy by Smith and Huggins in 1980, phage therapy employed by many countries for controlling multiple drug resistant microbes.

If we look upon the history of phages, then we found that in France from 1919, phage therapy was in practice. France was the leading country for phage therapy by 1978. Theodore Mazure, son in law of d'Herelle was produced several types of phage cocktails, which were commercially available named Bacte-Coli-Phage, Bacte-Intesti-Phage, Bacte-Dysenterie-Phage, Bacte- Pyo-Phage and Bacte-Rhino-Phage.

Mikeladze was discussed in his review in 1936, about the treatment of diverse disease such as typhoid fever, acute colitis, peritonitis, prostate and urinary tract infections, furunculosis, sepsis and otolaryngology with bacteriophage. He described the phage treatment of acute colitis caused *Shigella* or *Salmonella*. In 1990, Lyon, Chief Clinical Microbiologist of Pasteur institute and with his team prepared an antistaphylococcal vaccine and therapeutic phage. He had stated in his review published in 2002 that phage therapy is safe for human and it is without side effect, at that time phage therapy was boom in Georgia and Russia.

Phage therapy is not just started from the 19th century, however, it is reported in Biblical book about the recovery of prophet Elisa from Naaman's disease by getting seven time bath in the Jordan river. France was the pioneer country which applied phages for the clinical purposes, aftert that United State started the use of phage therapy against animal and human. In 1959, after completion of safety trials, SPL was licensed for the human therapeutic (Salmon and Symonds, 1963) and was administrated for several different routes, for example intranasal application by aerosol, topically, orally, subcutaneously, and intravenously.

In US, one of the first study of subcutaneous phage administration was carried out at the Michigan Department of Health, where Larkum reported treatment of 208 patients with chronic furunculosis; 78% of the patients had no recurring infections for at least six months after treatment with phage and only 3% showed no improvement. They have been reported remarkable success with *staphylococcal* septicemia and meningitis.

Additionally, several studies (Schless, 1932) reported the treatment of MRSA using phages, which can be accomplished by local application for local infections. If necessary, more caution, more systemic dosing, including intraperitoneally for systemic infections should be administered (Straub and Applebaum, 1933).

Another type of major infectious agent *Klebsiella pneumoniea* causes respiratory problem in United States was limited by phage therapy. *K. pneumonia* was associated with community acquired infection was first reported in Asia (Podschun and Ullmann, 1998) and after that worldwide (Fung *et al.,* 2002). *K. pneumonia* causes nosocomial diseases such as septicemia, pneumonia, and urinary tract infection in immunocompromised individuals (Abuladze *et al.,* 2008).

In the initial 19^{th} century phage therapy was boomed in curing of bacterial infection. After the Second World War when the antibiotic was discovered the craze of phage therapy was got down. But still some countries continued their practices with phage therapy. In the next section, the countries which continued their practices with phage therapy after the discovery of antibiotic will be discussed.

France was the pioneer country for the discovery of phages and its application in controlling microbial infections. d'Herelle who discovered the phage in 1919 was from the France. Since then, d'Herrele carried out his work on the phage, he treated many children who were suffering from diarrhea and typhoid (Sulakvelidze and Kutter, 2005; Kutter, 2008; Pirnay *et al.*, 2011). He wrote many books on the combating of infection with bacteriophage (d'Herrele, 1921, 1922, 1923, 1924, 1926, 1926, 1929, 1930, 1933, 1938). The first trial of phage therapy against *Staphylococcus* was carried out by him and this article was reported in 1921 (Bruynoghe *et al.*, 1921).

Grorgia or Russia, in the mid 1990, started production of phage cocktail (Dublanchet, 2009). Mikeladze cured typhoid patients by intravenous injection of phage; he also described the complete treatment of acute colitis by phage therapy in Georgia (Mikeladze *et al.,* 1936). In addition, they noted that phages caused a decrease in temperature and an improvement in the patient's feeble and rapid pulse, intestinal pain and tenesmus (Mikeladze *et al.,* 1936).

Gougerot and Peyre, (1936) described the treatment of skin infection by applying the phage; they rubbed the phage on the lesion and apply the phage bandage on the infected area and they found that skin infection get cured eventually. In cases of bacterial infection of the dermis and epidermis, such as impetigo, they recommended unroofing the lesions, removing the crusts, opening bullous lesions and rubbing a bit roughly in order to introduce the phages into the skin, then applying a large compress moistened with the same bacteriophages and they found that after some time the infection get cured.

In Poland phage was successfully cured thousands of patients. Slopek *et al.,* (1985) reported that phage therapy given 84.6% positive results from severe disease. Since 2005, the Hirszfeld Institute of Immunology and Experimental Therapy in Wrocław (founded in 1954) itself has had a phage therapy center dedicated especially to treating antibiotic resistance infections. Poland is now a member of the European Union, this clinical phage application is being conducted officially within the purview of a western medical regulatory system.

Schultz, (1929) and Schless, (1932), reported remarkable success with *Staphylococcal septicemia* and meningitis, respectively. Squibb and Sons and a division of Abbott Labs were started production commercial therapeutic phage. These all study from the United States which also started using phage therapy for controlling bacterial infection. But unfortunately, they found that all of them had problems with quality control, stability and establishment of efficacy. Eaton and Bayne-Jones, (1934) were explored over the 100 studies of Journal of American Medical Association (JAMA) thoroughly about the phage therapy. Their discouraging JAMA report found consistent, convincing data only for the treatment of localized *Staphylococcal* infections and for cystitis, and the review generally had a dramatically negative impact on the opinions of the medical and scientific communities (Sulakvelidze and Kutter, 2005).

In 1941, JAMA was reviewed by Krueger and Scribner (1941, 1941a) and it's all reflected the lack of care in the research of available data, it is not outright personal bias in the conclusions. Further review reports refuted the JAMA many more reviews conclusions. Even after the discovery of antibiotic in 1940, phages were still used successfully to treat typhoid fever, which was refractory to the antibiotics available at that time. MacNeal *et al.,* 1942 subsequently reported very positive results in the cumulative

treatment of 500 patients with *Staphylococcal* bacteremia, using cocktails of phages that were lytic in vitro. More details of the early work on *Staphylococcus* are included in the sections on MRSA and on purulent infections under Treatment of Specific Diseases. In the initial 1990s renewed realizes in the US that Phages can be an alternative solution for the antibiotic, therefore formation of several companies was occurred at that time. Among these companies were Intralytix and Exponential Biotherapies; both initially targeted Vancomycin Resistant Enterococcus (VRE) (Abedon *et al.,* 2011).

In Georgia, Phage therapy came after the foundation of Eliava Institute in Tbilisi by George Eliava in 1930s in association with Felix d'Herelle. At that time several infections associated with bacteria was cured by phage cocktail at Eliava institute. Georgian military employed canister of phage cocktail during 1991 and 1992 war for battle infections to cure injured soldier. Phage treatment was again used extensively during the battles between Georgia and Russia. Instiphage and Pyophage were publically available without any prescription in Georgia and Russia. Instiphage can almost control 20 pathogenic gastrointestinal microbes while Pyophage can control *Staphylococcus, Streptococcus, Pseudomonas, Proteus* and *E. coli* produced by a second Georgian company, Biochimpharm, and in several cities in Russia, such as Ufa and Perm, where factories also flourished.

In addition, phage therapy was also use to treat the burn wound infection for example Abdul-Hassan et al. 1990, reported the treatment of 30 cases of burn-wound associated antibiotic-resistant *P. aeruginosa* sepsis. Recently, a group of Belgian surgeons and scientists have developed an extensive collaboration with phage biologists in both Moscow and Tbilisi to explore the possibilities of using phages in burn applications. In preparation, they carried out a year-long study of *P. aeruginosa* colonization and infection during which a total of 441 patients were treated at the 32-bed Burn Wound Centre of the Queen Astrid Military Hospital in Brussels.

On the basis of above studies it is clear that phage therapy is a prominent alternative of antibiotics and it is an economical and ecofreindly strategy to control pathogens.

Objectives

CHAPTER 3 OBJECTIVES

The proposed study entitled "**Isolation and characterization of host specific bacteriophages and determination of their potency to inhibit multiplication of multiple drug resistance biofilm forming *Pseudomonas aeruginosa* isolates"** had been taken with the following objectives.

- **Isolation of host cell *P. aeruginosa* and their characterization.**
- **Isolation and purification of host specific *(P. aeruginosa)* bacteriophage from sewage and river water.**
- **Characterization of isolated bacteriophage.**
- **Interaction of bacteriophage with host cell *P. aeruginosa* and their biofilms.**

Materials & Methods

CHAPTER 4 MATERIALS AND METHODS

This chapter provides the detail discussion about the methodology to address the objectives of the current study. The objectives of the present study were control of biofilm formation of *P. aeruginosa* with bacteriophages which has been accomplished using the materials and methods as discussed in the given sections in this chapter.

All the media used were of high quality obtained from Hi Media, India while the chemicals and reagents used were of analytical grade and obtained from Merck, India, molecular grade chemicals were obtained from Bangalore Genei and Sigma.

Methods and protocols used in the study have been described in the present chapter and most of them are standard protocols used as such or with slight modifications which are mentioned in the concerned sections. Chapter also describes the source of biological materials used in the present study and their isolation techniques and characterization.

- **Composition of media:** Different media's used in the present study with their composition are listed below alphabetically. All the quantities mentioned are in g l^{-1} and distilled water used is 1000 ml, unless mentioned otherwise.

- **Pseudomonas agar medium**

Casein enzymic hydrolysate	10.0
Pancreatic digest of gelatin	16.0
Potassium sulphate	10.0
Magnesium chloride, anhydrous	1.4
Agar	15.0
pH	7.0
Distilled Water	1000.0

Use in the present study: This medium was used for the isolation of bacteria

- **Tryptone Soy Agar Medium:**

Trypticase	20.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
Distilled water	1000.0

Use in the present study: This medium was used for the maintenance of isolated culture and for further study

- **Mueller Hinton Agar**

Beef, infusion medium	300
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00

Use in the present study: Used for antibiotic sensitivity test and for MIC test

- **Blood Agar Medium**

Infusion from beef heart	500.0
Tryptone	10.0
NaCl	5.0
Agar	15.0

Distilled water	1000.0
pH	7.3

Use in the present study: Medium was used for determination of hemolytic activity of isolates

- **Congo Red Agar (for 100 ml):**

Brain heat infusion Agar medium	5.2gm
Sucrose	5gm
Congo red	0.08gm
Agar	1.5gm

Use in the present study: Medium was used for the determination of presence of amyloid fiber for on the cell wall of bacteria for the formation of biofilm

Media and chemicals used for biochemical characterization of isolates: Biochemical characterization of isolates were done by using following medium and reagents

- **Glucose Phosphate Broth**:

Peptone	7.0
Glucose	5.0
Potassium	5.0
Distilled Water	1000.0
pH	6.9

- **Starch Agar Media**:

Starch	20.0
Beef Extract	3.0

Peptone	5.0
Agar	15.0
Distilled Water	1000.0

- **Gelatin Media**:

Gelatin	120.0
Beef Extract	3.0
Peptone	5.0
Distilled Water	1000.0

- **Triple Sugar Iron Medium:**

Peptone	20.0
Lactose	10.0
Sucrose	10.0
Dextrose	10.0
NaCl	5.0
Ferrous ammonium sulphate	0.2
Na-thiosulphate	0.2
Phenol Red	0.025
Agar	15
Distilled Water	1000.0
pH	7.3

- **Simmon's Citrate Agar:**

Sodium citrate	2.0
$MgSO_4$	0.2
$(NH_4)H_2PO_4$	1.0
NaCl	5.0
Bromothymol Blue	0.08
K_2HPO_4	1.0
Agar	15.0
Distilled Water	1000.0
pH	7

- **Tryptone Broth**:

Tryptone	10.0
NaCl	5.0
Distilled Water	1000.0

- **Swimming Motility medium**:

Peptone	5.0
Beef Extract	3.0
NaCl	5.0
Agarose	3.0
Distilled Water	1000.0

- **Swarming Motility medium**:

Peptone	5.0

Beef Extract	3.0
NaCl	5.0
Agarose	5.0

Reagent's used in Biochemical characterization:

- **Kovac's reagent**:

p-Dimethyleaminobenzaldehyde	5.0
Isoamyl Alcohol	150 ml
HCl	75 ml

- **Barritt's Water**:

VP (1) reagents	5% n-naphthol dissolved in alcohol
VP (2) reagents	40% KOH solution

- **Methyl Red Reagents**:

Methyl Red	50ml
Ethanol	300ml
Distilled Water	200ml

Material used in the biofilm formation of isolates:

- Phosphate buffer solution 100 ml
- Crystal violet 0.3%
- DMSO solution
- 96 wells polystyrene microtiter plate

Distilled Water 1000.0 ml

For Determination of minimal inhibitory concentration (MIC) of isolates following antibiotics were used:

Ciprofloxacin stock solution	500mg in 1ml water
Imipenem stock solution	1gm in 1ml water

Material used for the phage isolation:

- **Soft agar:**

Trypticase	20.0
Peptone	5.0
Sodium chloride	5.0
Agar	8.0
Distilled water	1000.0

Use in the present study: This medium was used for the Double layer agar assay.

- **SM buffer (1L)**

NaCl	5.8 g
$MgSO_4.7H_2O$	2.0 g
1M TrisHCl(pH-7.4)	50 ml
2% gelatin	5 ml

Use in the present study: This buffer was used for the enrichment of isolated bacteriophage

- **Phosphate buffer saline (PBS)**

NaCl	0.4gm
KCl	0.2gm

$NaHPO_4$	1.44gm
KH_2PO_4	0.24gm
Distilled water	1000.0ml
pH	7.8

Use in the present study: This buffer was used for the enrichment of isolated bacteriophage with host cells for the short duration

4.1.1. Materials used for the characterization of phages:

Phage protein isolation by SDS PAGE:

- **Stacking gel for SDS-PAGE (12 ml)**

H_2O	3.07ml
0.5M Tris-HCl	1.25ml
20% (w/v) SDS	0.025ml
Acrylamide/Bisacrylamide (30% / 0.9% w/v)	0.67ml
10 %(w/v) APS (ammonium per sulphate)	0.025ml
TEMED	0.005ml
pH	6.8

- **Running buffer**

Tris HCl	3.0
Glycine	14.4
SDS	1.0
Millipore water	1000 ml

- **Coomassie staining solution for SDS-PAGE**

Methanol	15ml
Acetic acid	10ml
CBBG	0.125gm
Water	75ml

For isolation of phage DNA:

- **Agarose gel electrophoresis**:

  Agarose (1%)

  TAE buffer

  Sample loading buffer

  Ethidium bromide dye

  Gel casting trays and combs

  DNA samples

Equipments used in the present study: Different instruments used in the present study are listed below along with their make/model:

- Autoclave (MAC)
- Spectrophotometer (THERMO)
- Phase contrast microscope(OLYMPUS)
- Incubator shaker (REMI)
- Bacterial incubator (REMI)
- ELISA Reader (THERMO)
- Hot air oven (MAC)
- Refrigerator (REMI)
- Biosafety hood (YORCO)
- Centrifuge (GENEI)

- Electrophoresis unit (GENEI)
- UV Transilluminator (GENEI)

4.2. Isolation of bacterial host

Bacterial isolates used in the present study were isolated from patients with burn wound infections. A total of 12 isolates were isolated and based on antibiotic resistance pattern, five isolates were selected for further study.

Pus samples from burn wound infections of three patients (Lala Lajpat Rai Hospital, Kanpur, India) were collected using sterilized cotton swab in the year 2012. These swabs were used for spreading the sample on Pseudomonas Agar medium as *Pseudomonas* was the target organism for the present study. Medium with sample was incubated at 37^0C for 24 hours. Shining and transparent colonies appearing after 24 hours were selected and purity checked using Gram staining reaction.

a) Maintenance and storage of culture:

All the twelve isolates were maintained on Tryptone Soy Agar (TSA) or Tryptone Soy Broth (TSB) medium (Hi Media, India) at 4 °C for further study. While for the long term storage cultures were mixed with glycerol stock (1:1 in phosphate buffer saline) and kept at -20 °C.

b) Morphological characterization of bacterial isolates:

Isolated cultures were characterized for their morphological features as well as to ascertain the purity of cultures. Two features were studied- staining and culture characteristics.

Staining: Gram staining (Gram's Method) is an empirical method for the differentiation of bacteria into two large groups (gram positive and gram negative) based on their physical and chemical properties of the cell wall (Holt *et al*., 1994). The method is named after the Danish scientist Sir Hans Christian Gram (1853-1938), who developed the technique to differentiate bacteria with similar clinical symptoms.

Procedure:

Smear of culture was made on glass slide

↓

Smear was stained with the crystal violet

↓**45 seconds**

After the incubation, stain was removed by washing with water

↓

After washing, a drop of iodine was put on the crystal violet stained smear

↓**45 seconds**

After incubation iodine was removed by washing with the water

↓

After using iodine, decolourizer was used for 10 seconds

↓

Decolourizer was removed by washing with water

↓

Safranin was used to counterstain the bacterial cells for 10 seconds

↓

After staining procedure, sample was observed using immersion oil lens and mineral oil under the light microscope

c) **Culture characterization:** A loop full culture was streaked on Pseudomonas agar plate and incubated for 37 °C for 24 h. After incubation, the colony morphology of all twelve isolates was recorded.

4.3. Determination of antibiotic sensitivity profile of the isolates:

Antibiotic sensitivity test was performed to know the resistant profile of isolates for the various classes of antibiotics. Antibiotic sensitivity test was performed by Kirby Bauer disk diffusion method. Culture of $1X10^8$ CFU/ml was spread on Mueller Hinton Agar (MHA) plates and then antibiotic discswere placed and incubated for 18 h at 37 ^oC .A total of 14 antibiotics upto third generation antibtioics were used to assess the multiple

drug resistance pattern of the bacterial isolates. The antibiotics used in the current study were- Azithromycin (15 mcg), Cefaxime (5mcg), Ceftriaxone (15mcg), Chloramphenicol (30 mcg), Co-Trimoxazole (25 mcg), Ciprofloxacin (5mcg), Moxifloxacin (5mcg), Gentamicin (10 mcg), Imipenem (10mcg), Levofloxacin (30 mcg), Meropenem (10 mcg), Oflaxacin (5mcg), Streptomycin (10 mcg), Tobramycin (10mcg). After incubation, inhibition zone by antibiotics was recorded.

Classes of the tested antibiotics are as given:

Azithromycin (15 mcg)	- Advance generation antibiotic
Cefixime (5mcg),	- Third generation antibiotic
Ceftriaxone (15 mcg),	- Third generation antibiotic
Chloramphenicol (30 mcg),	- Third generation antibiotic
Co-Trimoxazole (25 mcg),	- Third generation antibiotic
Ciprofloxacin (5mcg),	- Second generation antibiotic
Moxifloxacin(5mcg),	- Fourth generation antibiotic
Gentamicin (10 mcg),	- Second generation antibiotic
Imipenem (10mcg),	- First generation antibiotic
Levofloxacin (30 mcg),	- Third generation antibiotic
Meropenem (10 mcg) ,	- Second generation antibiotic
Oflaxacin (5mcg),	- Second generation antibiotic
Streptomycin (10 mcg),	- First generation of drug
Tobramycin (10 mcg).	- Third generation antibiotic

Selection of five bacterial isolates for further study

Based on antibiotic resistance pattern, five bacterial isolates having multiple drug resistance as seen in the above experiment were selected for further study.

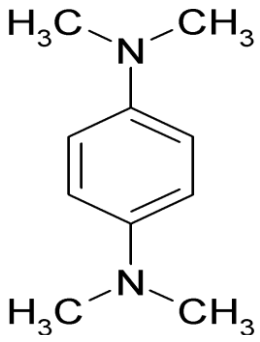
4.4. Biochemical characterization of the selected isolates:

For confirmation of isolates whether they belong to Pseudomonad group or not, different biochemical tests were performed.

4.4.1 Catalase Test: This test was performed to detect for the presence or absence of catalase enzyme. Most of aerobes and facultative anaerobes have characteristic catalase activity. Aerobic microbes usually utilize oxygen and produce hydrogen peroxide, which are toxic to nucleic acid and many cellular proteins. Hence, this test is used to detect for the presence of catalase enzyme in bacteria. A few drops of 0.3% H_2O_2 solution was poured on bacterial colony. Presence of bubbles is an indication of the catalase production.

4.4.2. Oxidase Test: To differentiate certain groups of bacteria, oxidase test is generally performed. It uses disks impregnated with a dye tetramethyl-p-phenylenediamine (TMPD) dihydrochloride. The reagent is a dark-blue/purple colored when oxidized, and colorless when reduced. Oxidase-positive bacteria possess cytochrome oxidase or indophenol oxidase (an iron-containing hemoprotein). These both catalyze the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). The test dye, TMPD dihydrochloride acts as an artificial electron donor for the enzyme oxidase. The oxidized reagent forms the colored compound indophenol blue. The cytochrome system is usually only present in aerobic organisms that are capable of using oxygen as the terminal

Fig.: TMPD



electron acceptor. The end-product of this metabolism is either water or hydrogen peroxide.

4.4.3. Gelatin hydrolysis Test: Gelatin is a polymer of amino acid. Protein is used as a nitrogen and carbon source for microorganisms. Gelatin is generally broken down into peptides of short amino acid polymer and amino acid which can be transported easily. The enzyme that acts on Gelatin is protease (Gelatinase). The property of gelatin is to remain solid below 22^0C while the degraded form loses its consistency and turns into semi-solid to liquid form below 22^0C. For the analysis of presence of Gelatinase enzyme in the isolates this test was performed. Gelatin medium was prepared (20% gelatin in nutrient broth) and poured into test tubes. Test tubes were stabbed with culture and then incubated for 48 h at 37^0C. After incubation, liquefaction of media was analyzed. If the medium become liquid, then test is positive, if there is no change in consistency of the medium then the test is negative.

4.4.4. Starch hydrolysis Test: Starch is insoluble polymer of glucose. Some bacteria have ability to degrade it into maltose with the help of enzyme amylase. Degraded form of maltose is transported across the cell membrane. For detection of amylase enzyme, plates containing starch agar media were prepared. Culture was streaked on the plate and incubated for 24 h at 37 ^{o}C. After incubation, iodine was poured on the streaked zone; appearance of blue to black color around streak zone indicates the formation of complex starch and iodine. If area around the streak zone is clear than this test is positive.

4.4.5. Indole production test from tryptophan by bacteria: Some enteric bacteria that produce indole during the hydrolysis of tryptophan this production is facilitated by tryptophanase enzyme. Indole production can be detected by using Kovac's reagent that give cherry red color layer of reagents. For detection of indole production by bacteria, a broth medium was prepared which contains tryptone. Tryptone broth was inoculated with culture and incubated at 37 ^{o}C for 48 h. After

incubation, 0.5 ml of reagent was added and appearance of red color indicates presence of tryptophanase enzyme.

4.4.6. Methyl red and Voges-Proskaur (MR-VP) test: All enteric bacteria initially produce pyruvic acid from glucose metabolism. Some enterics subsequently use the mixed acid pathway to metabolize pyruvic acid to other acids, such as lactic, acetic and formic acids. These bacteria are called methyl-red positive while the other group subsequently uses the butylenes glycol pathway to metabolize pyruvic acid to neutral end products and are termed as methyl red negative bacteria. If the test bacteria produces substantial amount of organic acid as the end product, on addition of methyl red, red color develops indicating positive reaction and if the medium turns yellow, it's an indication of a negative test; it is all because of pH of medium increased due to production of ethylene. To perform this test, peptone water broth was prepared and inoculated with the culture and incubated it at 37 ^{o}C for 48 h. After incubation, 10 drops of VP-1 reagent (Barritt's reagent with 5% alpha-napthol in 95% methyl alcohol) and VP-2 (40% KOH in distilled water) reagent were added, after that the mixture was shaken gently. Appearance of red color indicates positive test. If MR test is positive, then VP should be negative. Sometime MR and VP both become positive. Methyl red (2-(N,N-dimethyl-4-aminophenyl) azobenzenecarboxylic acid), also called C.I. Acid Red 2, is an indicator dye that turns red in acidic solutions. It is an azo dye, and is a dark red crystalline powder. Methyl red is a pH indicator; it is red in pH under 4.4, yellow in pH over 6.2, and orange in between, with a pKa of 5.1

4.4.7. Citrate utilization test: Microorganism utilizes citric acid as sole carbon source. Citric acid is intermediate metabolite of Kreb's cycle which oxidizes pyruvic acid to carbon dioxide. The bacteria must have ability to transport across the membrane. For this experiment, slants were prepared containing Simmon's citrate agar medium and were streaked with culture and incubated at 37 ^{o}C for 48 h. After incubation, appearance of change in color of medium was observed. Simmons' Citrate Agar is a defined, enrichment medium that tests for an

organism's ability to use citrate as a sole carbon source and ammonium ions as the sole nitrogen source. The medium contains citrate, ammonium ions, and other inorganic ions needed for growth. It also contains bromothymol blue, a pH indicator. Bromothymol blue is green at pH below 6.9, and then turns blue at a pH of 7.6 or greater.

Organisms growing on Simmons Citrate Agar are capable of using citrate as the sole carbon source and they can metabolize the ammonium salt in the medium. Use of citrate results in the creation of carbonates and bicarbonates as byproducts, thus increasing the pH of the medium. The increase in pH then causes color change in the bromothymol blue indicator, turning it blue. Under acidic condition it changes to yellow color. This color change is useful because growth on Simmons Citrate Agar is often limited and would be hard to observe if it were not for the color change. Sometimes, it is possible to detect growth on the Simmons Citrate Agar without the accompanying color change to blue due to insufficient incubation but is also considered as positive.

4.5. Molecular Characterization of the test isolates using 16sRNA sequencing:

Molecular characterization of the five bacterial isolates established to be *Pseudomonas aeruginosa* by biochemical characterization were further sequenced partially using 16S rDNA sequencing. For 16S rDNA sequence analysis, isolated genomic DNA was amplified using PCR consensus primers (Bangalore Genei, India). The DNA sequences obtained were analyzed using National Center for Biotechnology Information server and BLASTn tool and corresponding sequences downloaded (www.ncbi.nih.nlm.in). Phylogenetic tree was constructed using the neighbor joining method in MEGA 3.1. All the five sequences have been submitted to NCBI database and accession number is due.

4.6. Pathogenicity test-

4.6.1 Blood agar test for hemolytic activity: Blood agar test was performed to check the hemolytic activity of isolates. Briefly, a blood plate was prepared with 5%

(v/v) of blood in blood agar, culture were streaked on the blood agar plate and observed after 24 h. Appearance of clear zone around the streak area indicates the β hemolytic activity, while appearance of slightly green area indicates α hemolytic activity.

4.6.2 Congo red binding assay: Congo red dye binds with the proteinaceous amyloid curli fibers. Amyloid fibers present on the extracellular surface of bacteria, help in attachment of bacteria and also cause infection by adhering on the epithelial cells. For detection of amyloid fibers, congo red binding assay was performed (Kay et al., 1985). Congo red agar plate was prepared with 0.3% congo red dye in Brain Heart Infusion (BHI) Agar with 5% (v/v) sucrose, cultures were streaked on the plate and incubated for24 h at 37 °C. Appearance of reddish black color shows that bacteria have high content of amyloid fiber.

4.6.3 Biofilm formation assay:

a.Crystal violet assay-Static biofilm formation assay was done in 96 well polystyrene microtiter plate as per O'Toole *et al*., 1998. In brief, 24 h old log culture of isolates were grown in the microtiter plate containing 200 µl tryptone soy broth (TSB) for 24 h at 37 ^{o}C. After 24 h incubation, planktonic cells were removed using sterile microtips. Subsequently, the adherent biofilm (including biofilm forming bacterial cells) were washed with 0.75% saline to remove any trace of planktonic cells, followed by staining with 0.3% crystal violet for 20 minutes. Washed thrice with 0.75% saline, to remove unbound crystal violet. This was followed by detaching of stained and adhering cells using 200 µl of dimethyl sulfoxide and solubilized biofilm was measured by using ELISA microreader (Rayto, USA) at 630 nm.

b. MTT assay- Biofilm formation was also checked using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as per Abate *et al*.,

(1998). Herein, 24 h log culture of isolates were grown in the microtiter plate containing 200 µl tryptone soy broth (TSB) for 24 h at 37^{o}C. After 24 h incubation, planktonic cells were removed using sterile microtips. Subsequently, the adherent biofilm was washed with 0.75% saline to remove any trace of planktonic cells, followed by staining with 0.3% MTT dye in PBS for 2 h. After staining, excess MTT was removed by washing thrice with saline. Stained and adhering cells were then removed using 200 µl of dimethyl sulfoxide and solubilized biofilm was measured by using ELISA microreader (Rayto, USA) at 620 nm.

MTT assay-In this research work viability test of phage or antibiotic treated biofilm was done by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay as per Abate *et al*.,(1998). Herein, 24 h phage or antibiotic treated biofilm cells were washed with PBS, and washed biofilm were incubated in 0.3% MTT dye in PBS for 2 h after incubation biofilm cells was detached and solubilized by using dimethylesulfoxide and solubilized biofilm were measured using ELISA microreader at A540. MTT is a tetrazolium redox dye, metabolically active cells turned in to purple color in the presence of dye.

4.6.2. Motility test of planktonic as well as biofilm cells: Motility test was performed for determination of motility in the cells which are engaged in biofilm formation and compared with the planktonic cells. Static biofilm was formed on the microtiter plate for 24 h, after incubation planktonic cells were separated and biofilm was dissolved with 1M-10mM Na-EDTA solution. For the swimming and swarming motility test, media was prepared with 0.3 and 0.5% agarose respectively, planktonic and dissolved biofilm cells were inoculated on the poured plated containing swimming and swarming medium and then incubated for 24 h. Results were observed after incubation in the form of zone size of motile area of bacteria on the agar plate.

4.7. Isolation of host(bacterial) specific phages

4.7.1 River Ganges water sample for isolation of phage: Water sample was collected from river Ganges in sterile water collection bottles in the from Kanpur region during the month of April, 2012.

Sewage water: Sewage water for isolation of phages was collected from hospitals and Panki Power House sewage treatment plant, Kanpur, India during April, 2012 in sterile containers.

Water and sewage water samples were transported to the laboratory within one hour of collection for further isolation.

Procedure:

The enrichment method of Cerveny *et al*., (2000) was adopted for the isolation of phages specific to *P. aeruginosa* from sewage as well as river water samples. All the phages were isolated in separate experiments from independent samples.

Samples were centrifuged (10,000 rpm, 10 min, 4^oC) and supernatants were filter sterilized (0.45 μm pore size Millipore filter). 5 ml filtrate, 5 ml sterile SM buffer and 5.0 ml overnight culture of *P. aeruginosa* were mixed and incubated at 37^oC for 24 h. The bacteria were removed by centrifugation (12,000 rpm, 10 min, 4^oC); supernatant was filter sterilized and checked for the presence of phages.

4.7.2. Phage propagation and purification

All the isolated phages were purified by successive single-plaque isolation until homogenous plaques were obtained by the standard procedure described by Sambrook *et al*., (1989). Briefly, one well separated phage was picked with sterile micro tip with the surrounding cell mass and inoculated into 5.0 ml Tryptone Soy Broth (TSB), in which overnight culture of 10^9 host cells was added and incubated at 37^oC for 24 h with agitation at 140 rpm. After complete lysis, the mixture was centrifuged (10,000 rpm, 10 min, 4^oC), filter sterilized and treated with chloroform (1% v/v) to remove any bacterial contamination. Purified phages were stored in 60% glycerol at -20^oC for long term storage. Short term stock preparations were maintained at 4^oC.

4.7.3. Spot test for lytic activity of isolated bacteriophages:

To detect the presence of phages in supernatant, spot test was carried out as described by Chang *et al*., (2005) with some modifications. Briefly overnight culture of isolated bacteria was mixed with soft agar and poured on the TSA plate and after solidifying of soft agar isolated bacteriophages were spotted on the upper layer of plate containing soft agar and culture.

4.7.4. Plaque assay for determination of purity of bacteriophages:

The phage titer was determined by plaque assay by employing double layer agar (DLA) overlay technique. Briefly, each of the phage suspension was serially diluted. 10 µl diluted phage were spot inoculated on molten agar (0.8% agar, w/v) containing host cells of 10^7 CFU/ml. Clear zones of plaques were observed after incubating the plates overnight at 37^oC.

4.7.5. Titration of bacteriophages: Phages were concentrated according to the method of Yamamoto *et al*., (1979) with some modifications. Briefly, *P. aeruginosa* host cells were added to phage preparation at a MOI (Multiplicity of Infection) of 0.1 and vigorously shaken for 24 h at 37^oC, resulting in complete lysis of bacteria. The lysate was centrifuged (10,000 rpm for 10 min, 4^oC) and filter sterilized. 1 M NaCl and 10% polyethylene glycol (PEG) 8000 were added to filtered lysate and kept at 4^oC overnight. The precipitates were collected by centrifugation, re-suspended in 5 ml SM buffer and treated with equal volume of chloroform to remove PEG 8000 and bacterial cell debris from the bacteriophage suspension.

4.7.6. Determination of host range of phage for bacteria: To determine the infectivity of phages for the various hosts, this test was performed. In this experiment, lawn of host bacteria *Pseudomonas aeruginosa, Escherichia coli, Enterobacter aerogens, Salmonella typhi, Staphylococcus epidermidis, Staphylococcus arlettae, Burkholderia cepacia* and *Staphylococcus aureus* was formed by pouring soft

agar with host (100 µl of 10^7 cfu/ml) on the agar medium to determine bacterial susceptibility to phages. After drying of the host amended soft agar, a drop of the phage suspension (10^7pfu/ml) was spotted over it, incubated at 37 °C for 24 h and then lysis of bacteria was analyzed at the spotted place. Spot testing is a rapid and efficient method for determining the host range in large collection of bacteria, Clokie *et al*., 2009.

4.8 Characterization of the phages

Features studied in this section are mentioned in subsequent sections.

4.8.1. Burst size determination by one step growth curve: A one step growth curve of isolated phages was performed to determine the various stages of phage such as eclipse period, latent period and burst size. This test was performed according to the method of Pajunen *et al*., (2000) with modifications. The density of a mid exponential bacterial culture (TSB) was adjusted to 1×10^8cfu/ml. An equal volume of bacterial suspension and phage diluted to 10^8-10^9 pfu/ml were incubated at 37^0C for 5 min, after that time the mixture was diluted to 10^5, and samples in triplicate were taken at 2 min interval for titration.

4.8.2. Phage adsorption procedure: The adsorption of phages to bacterial host cells was examined as previously described with some modifications as per Ackermann and Du Bow, 1987. Overnight cultures of *P. aeruginosa* grown on TSA were used. Cells from the agar medium were suspended in enrichment broth (TSB) to an optical density at 620 nm of approximately 0.9–1.0. An equal volume of bacterial suspension and phage diluted to 10^8 - 10^9 pfu/ml were incubated at 37 °C for 5 min. After incubation, the culture was filtered (0.22 µm) and the numbers of free phages were determined at every 2 minute time interval, in duplicate, by double agar layer method. The reduction in phage titer was observed which is an indication of absorbance of phages to the cells.

4.8.3. Determination of stability of phages to temperature and pH: This test was performed to determine the stability of phages for variable temperature and pH. To perform this experiment, filter-sterilized bacteriophages (10^7 pfu/ml) were incubated at 37, 55, 75 and 95^{o}C respectively for 10 min with intermittent shaking. After incubation at different temperatures, 10^7 cfu/ml cells were added to each bacteriophage preparation and DLA (Double Layer Agar) assay was performed and plates were observed after 24h. For pH stability, phages (10^7pfu/ml) were incubated at pH 3.0, 5.0, 7.0, and 9.0 respectively for 30 min at 37°C with intermittent shaking. Post incubation DLA was performed with 10^7cfu/ml host cells. After all these experiments, the bacteriophage titer was assessed, Adams, 1959.

4.8.4. SDS-PAGE of bacteriophages: For separation of proteins on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE),ultracentrifuged *P. aeruginosa* specific phages were mixed with equal volume of sample buffer (0.0625 M Tris-HCl; pH 6.8, 1% SDS, 15% glycerol, 1% Beta-mercaptoethanol and bromophenol blue), heated in a boiling water bath for 3-5 min (Sen and Ghosh, 2005) and loaded on a SDS polyacrylamide gel (12%) by the standard method (Chopra *et al.*, 1996). After loading, sample was run for 6 h at 75 V. After completion of the process, protein bands were visualized by staining the gel with coomassiebrilliant blue. Medium range protein molecular weight marker (Bangalore Genei, India) was used for determination of molecular weight of phage proteins.

4.8.5. DNA isolation from Bacteriophage and its restriction digestion analysis: Purified phage particles (10^{10} pfu/ml) were treated with 1 µg of DNase I and RNase A (Bangalore Genei, Bangalore, India) at 37^{o}C for ½ h. To the mixture, Proteinase K (Bangalore Bangalore Genei, India) and SDS were added at a final concentration of 0.05 mg/ml and 0.5% respectively and incubated at 65^{o}C. After 1 h incubation, an equal volume of phenol: chloroform was added to remove

proteinaceous material. The extraction was repeated thrice with phenol-chloroform (1:1). The nucleic acid was precipitated with chilled 70 % ethanol and suspended in 20 µl of TE buffer (10 mM Tris-HCl, pH 7.0, 1.0 mM EDTA, and pH 7.0) according to standard procedure by Sambrook *et al*., (1989). Restriction enzyme digestion of isolated phage DNA was carried out by following the instructions provided by suppliers. Type II restriction endonucleases, PsutYl and BamHI (MBI Fermentas and Bangalore Genei, Bangalore, India, USA) were added to purified bacteriophage DNA. The restriction digests were separated on 1% agarose gel in 1× TAE buffer (40 mM tris-acetate and 1 mM EDTA, pH 8.0) containing 0.5 µg/ml of ethidium bromide (Hi- Media) at 75 V for 2 h.

4.8.6. Morphological characterization of phages using Transmission Electron microscopy: To observe phage morphology, transmission electron microscopy of *P. aeruginosa* PA01 specific phages was performed as described by Goodridge *et al*., (2003) with some modification. Drops of ultracentrifuge phage samples were dropped on copper coated grids (diameter, 3 mm; 300 meshes). After 5 min, the phage particles were stained with 2% (w/v) phosphotungstic acid (PTA) for 10 s. The grids were allowed to dry for 20 min and examined under a transmission electron microscope (FEI Tecnai S Twin) at 200 Kv.

4.9 Determination of MIC of ciprofloxacin for biofilm of *Pseudomonas* isolates by MTT assay: Minimum inhibitory concentration of *Pseudomonas* isolates were determined for ciprofloxacin, cells were treated with 2 fold dilutions (100, 50, 25, 12.5, 6.25, 3.125, 1.5 and 0.75 µg/ml) respectively. For biofilm formation assay, antibiotic treated isolates cells were harvested post 24 h incubation at 37 ^{o}C. For MIC of biofilm of isolates, 24 h log culture of isolates were treated with varying concentrations of antibiotic and incubated in the microtiter plated for the formation of biofilm formation under static condition for 24 h. After incubation planktonic cells were removed and their absorbance was taken at 540 nm and attached biofilm cells were washed with PBS, and incubated in 0.3% MTT dye in PBS for 2 h.

After incubation, biofilm cells were solubilized by using dimethyl sulfoxide and solubilized biofilm were measured using ELISA microreader at 540 nm.

4.10 Determination of susceptibility of *Pseudomonas* isolates biofilm to phages by viability assay (MTT assay): Since the potent antibiotic ciprofloxacin, having low molecular weight and therefore better penetration power could not completely inhibit the biofilm cells as well as the planktonic cells even at the 100 mcg concentration ml^{-1}, test phages were employed in the present experiment to test for the efficacy of phages to inhibit bacterial hosts Ps. 1 to Ps. 5 (planktonic as well as biofilm cells).

In order to determine effect of phage treatment, 10 µl of different MOI (0.01) was used for the treatment. For biofilm formation assay, phage treated isolated cells were harvested post 24 h incubation at 37 ^{o}C. After incubation phage treated biofilm cells were washed with PBS, and washed biofilm were incubated in 0.3% MTT dye in PBS for 2 h. After incubation, biofilm cells were detached and solubilized by using dimethyl sulfoxide and solubilized biofilms were measured using ELISA microreader at 540 nm.

4.11 Scanning Electron Microscopy of bacteriophage treated biofilm: For the qualitative analysis of killing of biofilm by phage, scanning electron microscopy was performed**.** For this experiment, biofilm of isolates was formed on the cover slip and then treated with 0.1MOI of bacteriophage. After treatment, cover slip containing biofilm was prepared for scanning electron microscopy. Biofilm formed on cover slip was rinsed gently in sterile PBS and then fixed in 5% (v/v) glutaraldehyde in PBS buffer (pH 6.2) for 2 h followed by post fixation by 1% osmium tetroxide for 1h, dehydrated through a graded series of 10-min ethanol immersions (30, 50, 70, 90 and 100%). Specimens were mounted on aluminum stubs, coated with gold (JFC 1600, Auto Fine Coater, JEOL, Japan), and observed on SEM (JEOL-JSM-6490 LV, Japan). The entire cover slip surface was examined, and images were chosen that represented the typical field of view.

Interaction of Phage P1 with most promising host *Pseudomonas aeruginosa* Ps. 5

4.12 Electron microscopy of phage treated biofilm: Biofilm formed by the test bacterial isolates on the microtiter plate was taken, treated with bacteriophage for 24 h at 37 ^{o}C. After incubation, biofilm was detached using sodium EDTA solution and was analysed using Transmission Electron Microscopy as described by Goodridge *et al*., (2003) with some modifications. Drops of phage treated biofilm samples were dropped on copper coated grids (diameter, 3 mm; 300 meshes). After 5 min, the sample was stained with 2% (w/v) phosphotungstic acid (PTA) for 10 s. The grids were allowed to dry for 20 min and examined under a transmission electron microscope (FEI Tecnai S Twin) at 200 Kv.

Results

CHAPTER 5 RESULTS

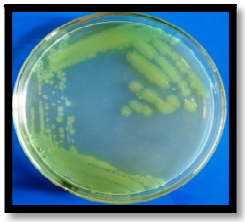
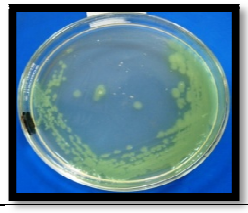
This chapter embodies the results obtained in the present study entitled "**Isolation and characterization of host specific bacteriophages and determination of their potency to inhibit multiplication of multiple drug resistant (MDR) biofilm forming *Pseudomonas aeruginosa* isolates".**

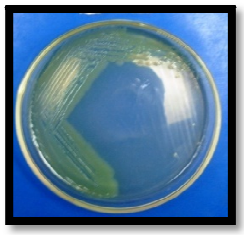
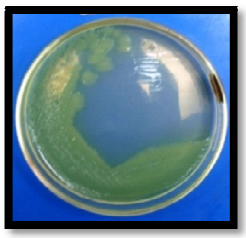
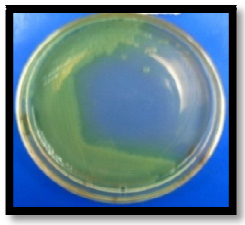
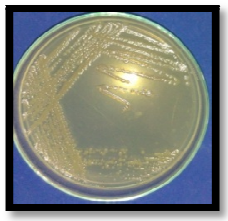
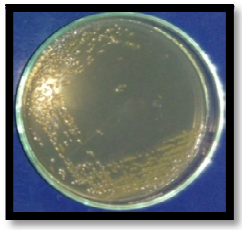
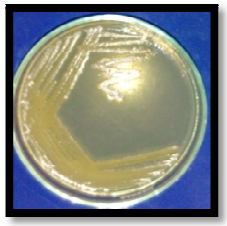
5.1) Isolation of bacterial host: A total of twelve bacterial isolates were isolated from wound samples of patients on *Pseudomonas* isolation agar medium. Transparent and sticky consistency colonies giving green fluorescence were selected and further purified.

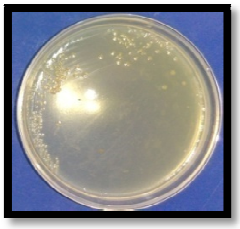
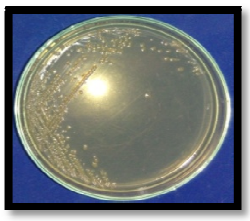
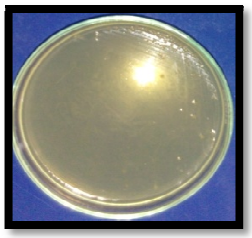
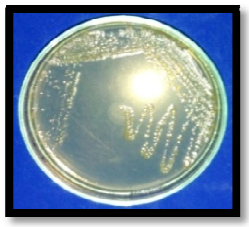
All the bacterial isolates were maintained on Tryptone soy agar (TSA) at 4 ^{o}C. The colony characteristics of bacteria are provided in table 1.

5.1.1) Morphological characterization of isolated bacteria: Cultural characteristics of all the twelve isolates on *Pseudomonas* isolation agar incubated at 37 ^{o}C for 24 h is mentioned in Table-1. Furthermore, the isolates were characterized for their morphology by gram staining method. On the basis of gram staining it was confirmed that all the isolates were gram negative (Table-1).

Table-1) Bacterial isolates and their morphological characteristics

S. No.	Name of isolates	Colony morphology	Gram's Characteristics	Photograph of colony characteristics
1.	**Ps.1**	Green fluorescent , transparent and sticky	Gram negative	
2.	**Ps.2**	Green fluorescent, transparent and sticky	Gram negative	

3.	**Ps.3**	Green fluorescent, transparent and sticky	Gram negative	
4.	**Ps.4**	Green fluorescent, transparent and sticky	Gram negative	
5.	**Ps.5**	Green fluorescent, transparent and sticky	Gram negative	
6.	**Ps.6**	Shiny, transparent and sticky	Gram negative	
7.	**Ps.7**	Shiny, transparent and sticky	Gram negative	
8.	**Ps.8**	Green fluorescent, transparent and sticky	Gram negative	

9.	**Ps.9**	Shiny, transparent and sticky	Gram negative	
10.	**Ps.10**	Shiny, transparent and sticky	Gram negative	
11.	**Ps.11**	Shiny, transparent and sticky	Gram negative	
12.	**Ps.12**	Green fluorescent , transparent and sticky	Gram negative	

5.2) Antibiotic susceptibility of isolates: Out of 12 isolates tested for antibiotic sensitivity, only five isolates were having multiple drug resistance for the tested antibiotics. Isolate Ps.1 was resistant to chloramphenicol, co-trimoxazole, ceftriaxone and cefixim while sensitive to azithromycin, ciprofloxacin, gentamicin, imipenem, moxifloxacin, ofloxacin and levofloxacin and intermediate to streptomycin. Isolate Ps.2 was resistant to chloramphenicol, co-trimoxazole, ceftriaxone, streptomycin and cefixim and sensitive to azithromycin, ciprofloxacin, gentamicin, imipenem, moxifloxacin, ofloxacin, levofloxacin and meropenem. Ps.3 was resistant to chloramphenicol, co-trimoxazole, ceftriaxone and cefixim while sensitive to azithromycin, gentamicin, imipenem, ofloxacin, levofloxacin and meropenem while intermediate to ciprofloxacin, moxifloxacin and streptomycin.

Table- 2) Antibiogram of isolates

Sr. No.	Antibiotics	Generation of antibiotic	Concentration of Antibiotics	*Ps.* 1	*Ps.* 2	*Ps.* 3	*Ps.* 4	*Ps.*5	*Ps.*6	*Ps.*7	*Ps.*8	*Ps.*9	*Ps.*10	*Ps.*11	*Ps.*12
				(zone size in mm)											
1	**Azithromycin**	Advance	15 mcg	29 (S)	28 (S)	25 (S)	20 (S)	15(R)	30(S)	32(S)	27(S)	36(S)	23(S)	24(S)	26(S)
2	**Cefixime**	IIIrd	5 mcg	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	19(S)	28(S)	21(S)	10(R)	22(S)	28(S)	26(S)
3	**Ceftriaxone**	IIIrd	15 mcg	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	22(S)	10(R)	19(S)	27(S)	12(R)	24(S)	20(S)
4	**Chloramphenicol**	IIIrd	30 mcg	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	19(S)	20(S)	18(S)	21(S)	26(S)	28(S)	10(R)
5	**Ciprofloxacin**	IInd	5 mcg	35(S)	38 (S)	30 (I)	30 (I)	30(I)	39(S)	40(S)	42(S)	38(S)	43(S)	46(S)	38(S)
6	**Cotrimoxazole**	IIIrd	25 mcg	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	10(R)	19(S)	12(R)	10(R)	16(R)	20(R)	24(S)
7	**Gentamicin**	IInd	10 mcg	19 (S)	21 (S)	22 (S)	20 (S)	15(R)	24(S)	29(S)	28(S)	26(S)	30(S)	18(S)	29(S)
8	**Imipenem**	Ist	10 mcg	30 (S)	25 (S)	25 (S)	27 (I)	10 (R)	39(S)	40(S)	42(S)	38(S)	38(S)	30(S)	36(S)
9	**Levofloxacin**	IIIrd	30 mcg	30 (S)	28 (S)	27 (S)	24 (I)	12 (R)	29(S)	28(S)	32(S)	30(S)	34(S)	29(S)	38(S)
10	**Meropenem**	IInd	10 mcg	27 (S)	28 (S)	28 (S)	24 (I)	12 (R)	30(S)	28(S)	32(S)	27(S)	39(S)	40(S)	36(S)
11	**Moxifloxacin**	IVth	5 mcg	27 (S)	26 (S)	24 (I)	22 (I)	11 (R)	30(S)	32(S)	28(S)	26(S)	38(S)	40(S)	39(S)
12	**Ofloxacin**	IInd	5 mcg	28 (S)	28 (S)	25 (S)	23 (S)	0 (R)	25(S)	20(S)	26(S)	28(S)	30(S)	29(S)	27(S)
13	**Streptomycin**	Ist	10 mcg	24 (I)	20 (R)	24 (I)	24 (I)	12 (R)	20(R)	22(I)	21(R)	28(S)	23(S)	19(R)	18(R)
14	**Tobramycin**	IIIrd	10 mcg	30 (S)	ND	ND	ND	20(R)	ND	ND	ND	ND	ND	ND	ND

Note: S- Sensitive, I- intermediate, R- resistant, ND- not done

Ps.4 was resistant to chloramphenicol, co-trimoxazole, ceftriaxone, cefixim and sensitive to azithromycin, gentamicin and ofloxacin, while intermediate for ciprofloxacin, moxifloxacin, streptomycin, levofloxacin and meropenem, Ps.5 was intermediate for ciprofloxacin and resistant for rest of the antibiotics which were used in the current study. Rests of the seven isolates were susceptible for almost all antibiotics. Susceptibility zone of the bacterial isolates to different antibiotics is depicted in Table-2.

Based on the above susceptibility pattern, it can be seen that only five bacterial isolates (Ps. 1, Ps. 2, Ps. 3, Ps. 4, Ps. 5) were having resistance to more than three class of antibiotics and were selected for further studies.

5.3) Biochemical characteristics of isolates:

All the five selected isolates were characterized biochemically. Results are depicted in Table 3. All the five isolates were positive for catalase, oxidase, gelatinase production and citrate utilization while negative for methyl red, voges proskaur and amylase (Table-3).

On the basis of morphological characteristics as depicted in Table 1 and biochemical characteristics as seen in table 3, it can be concluded that all the five isolates may be *P. aeruginosa.* For further confirmation about the isolates, molecular characterization was performed wherein 16S rRNA sequencing was undertaken for the bacterial isolates.

Table-3 Biochemical characterization test of isolates:

Biochemical tests	Ps. 1	Ps. 2	Ps. 3	Ps. 4	Ps.5
Catalase test	+	+	+	+	+
Oxidase test	+	+	+	+	+
Methyl Red test	–	–	–	–	–
Voges–Proskauer	–	–	–	–	–
Citrate Utilization test	+	+	+	+	+
Amylase test	–	–	–	–	–
Gelatinase test	+	+	+	+	+

+ = Positive, - = Negative

5.3.1) 16S rRNA sequencing of isolated bacteria: For identification of isolates, 16S rRNA sequencing was carried out. 16S rRNA fragment was amplified using universal primers and sequenced. The sequences showed 98% similarity to *P. aeruginosa*. Figure 2 shows the phylogenetic tree (drawn using the neighbor joining programme in MEGA 3.1) between different members of the *Pseudomonas* genus and the isolates. On the basis of the 16S rRNA gene sequence analysis, the isolates (labeled Ps.1, Ps.2, Ps.3, Ps.4 and Ps.5) were identified as *P. aeruginosa* (Figure 1a-e).

a) **Phylogenetic tree of isolate *P. aeruginosa* Ps.1:** Phylogenetic tree illustrate that the isolate Ps. 1 is *P. aeruginosa*

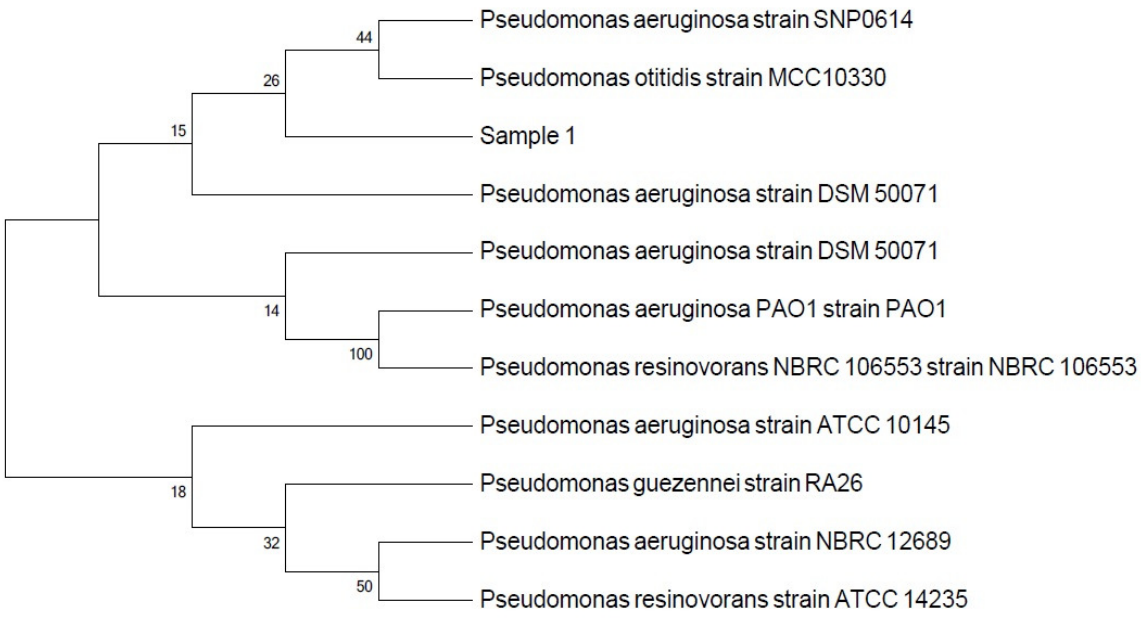


Figure-1.a) Phylogenetic tree of Ps.1 Isolate

b) Phylogenetic tree of isolate *P. aeruginosa* Ps.2: Phylogenetic tree illustrate that the isolate Ps. 2 is *P. aeruginosa*

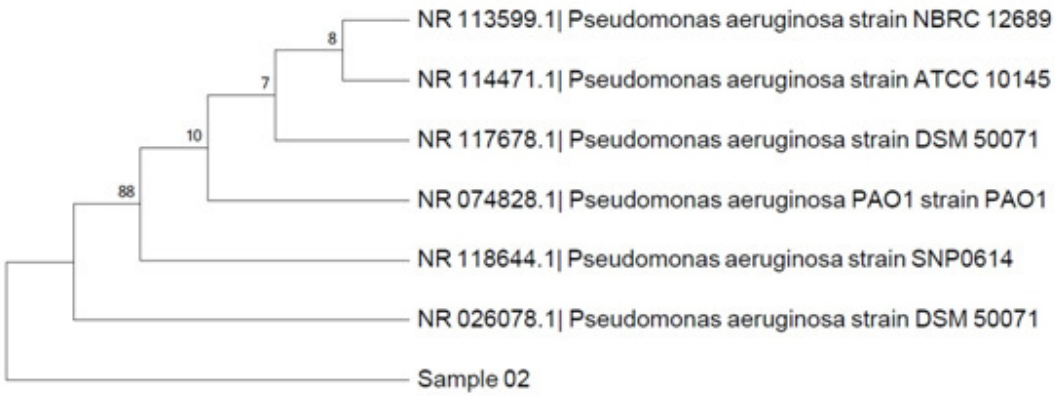


Figure-1.b) Phylogenetic tree of Ps.2 Isolate

c) Phylogenetic tree of isolate *P. aeruginosa* Ps.3: Phylogenetic tree illustrate that the isolate Ps. 3 is *P. aeruginosa*

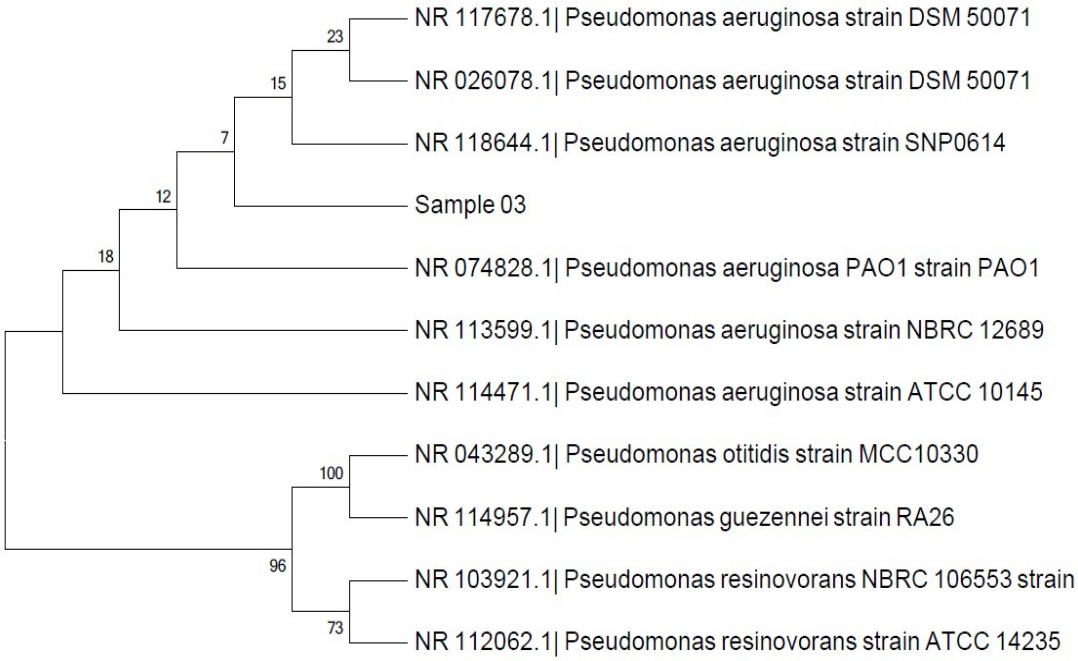


Figure-1.c) Phylogenetic tree of Ps.3 Isolate

d) Phylogenetic tree of isolate *P. aeruginosa* Ps.4: Phylogenetic tree illustrate that the isolate Ps. 4 is *P. aeruginosa*

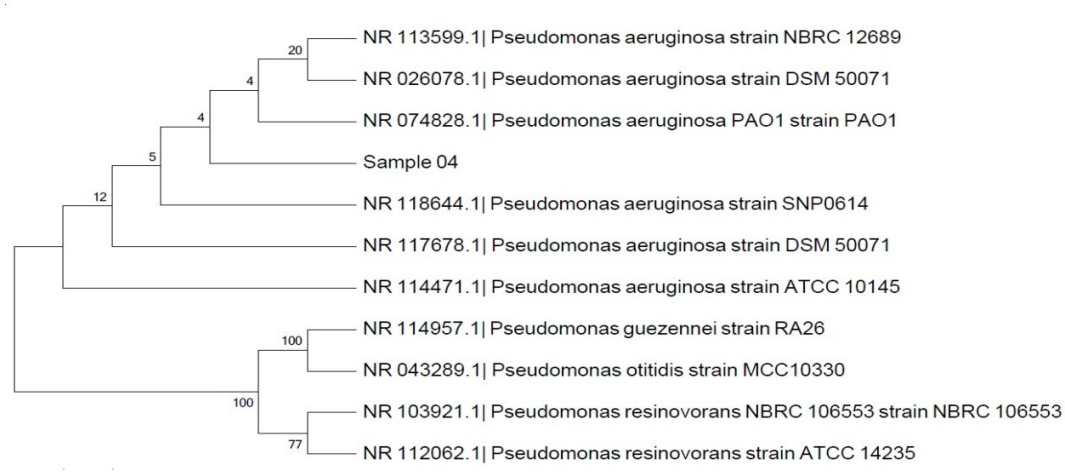


Figure-1.d) Phylogenetic tree of Ps.4 Isolate

e) Phylogenetic tree of isolate *P. aeruginosa* Ps.5: Phylogenetic tree illustrate that the isolate Ps. 5 is *P. aeruginosa*

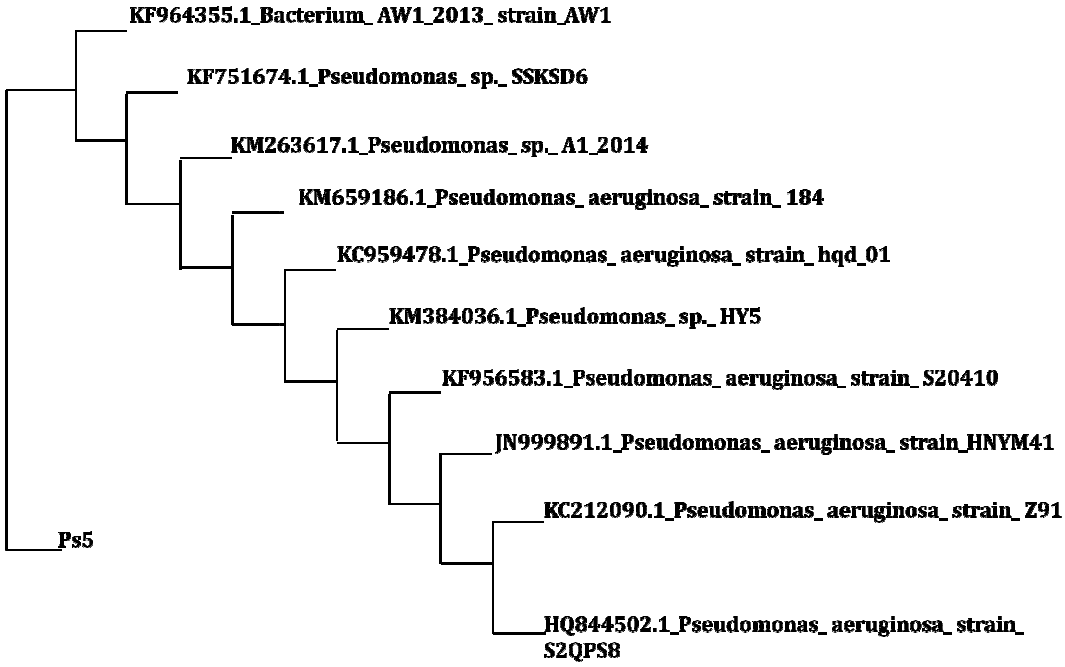


Figure-1.e) Phylogenetic tree of Ps.5 Isolate

5.4) Pathogenicity results of the bacterial host Ps.1 to Ps. 5: Pathogenicity tests were performed to determine the virulence characteristics of the *Pseudomonas* strains by using hemolytic activity test, congo red binding assay and biofilm assay (result shown in table-4 and figure-2, 3 and 4).

5.4.1) Blood agar test for hemolytic activity: All the five bacterial host strains were found to have β hemolytic activity (zone of clearance) as compared to control strain *Pseudomonas aeruginosa* PA01 (Table-4 and Figure-2).

Figure-2) Hemolytic activity test of isolates on Blood Agar

5.4.2) Congo red binding assay for amyloid fiber presence: Congo red binding assay was done for determination of presence of amyloid fiber on the extracellular surface of bacteria. All the five strains appeared dark reddish in color as compared to control strain, confirming presence of high amyloid fiber with high adhering efficiency (Figure-3). Presence of high amyloid fiber not only helps in attachment of cell to the surface but also is an indication of good biofilm forming capability.

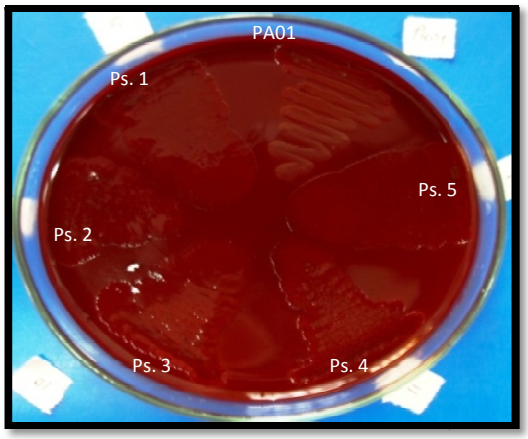


Figure-3 Congo red binding assay of isolates on Blood Agar

Table-4) Pathogenicity tests of isolates:

Pathogenicity tests		
Isolates	**Blood Agar test**	**Congo Red test**
Ps.1	+	+
Ps.2	+	+
Ps.3	+	+
Ps.4	+	+
Ps.5	+	+

+ = Positive

5.4.3) Biofilm formation assay:

5.4.3.1) Crystal violet assay- Absorbance of crystal violet dye by the test isolates is shown in Figure-4, More the biofilm formation, more will be the absorption of the dye by the biofilm of the bacterial strains. As seen in the Figure-4, all the five strains absorbed crystal violet dye better than the control strain PA01.

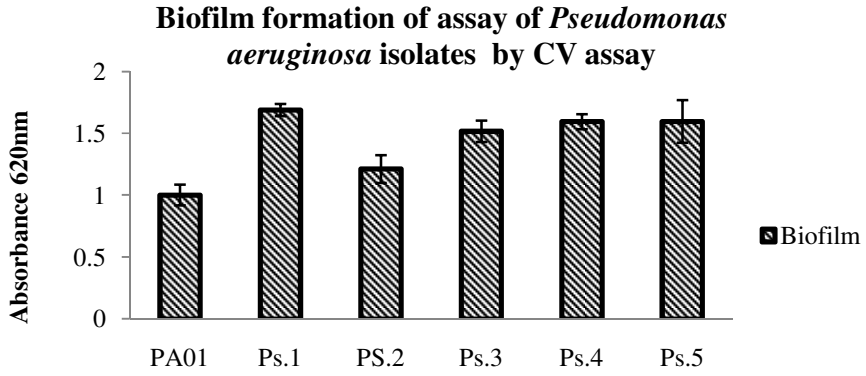


Figure-4 Biofilm formation assay of isolates

5.4.3.2) MTT assay: MTT assay is more specific and therefore was performed to further confirm the biofilm formation. MTT dye is absorbed by the biofilm formed by the host cells. Here, all the strains produced biofilm and the amount of biofilm formed by all the test strains Ps.1 to Ps.5 except Ps.2 was more as compared to the control culture PA01 (Figure-5).

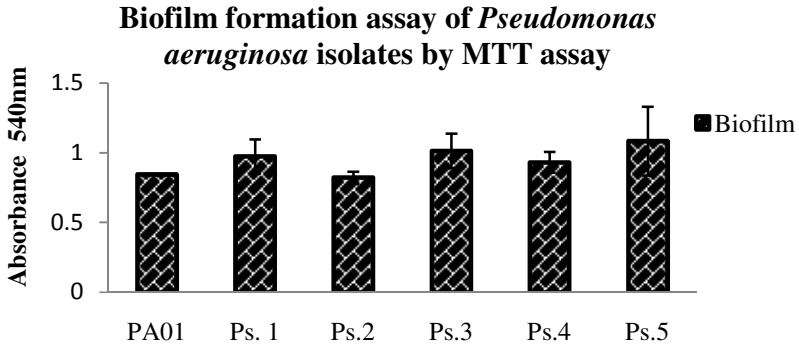


Figure-5 Biofilm formation assay of isolates

5.5) Motility test: This test was done to know the swimming and swarming motility of isolates results shown in Table-5 and 6.

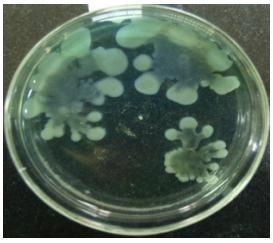
5.5.1) Swimming motility: In TSB with 0.3% agarose, all the strains were highly motile with swimming motility, results shown Table-5

Table-5) Swimming motility by *Pseudomonas* strains:

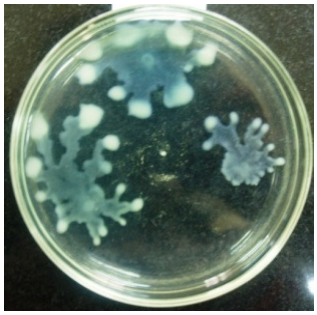
Swimming Motility

Planktonic cells motility

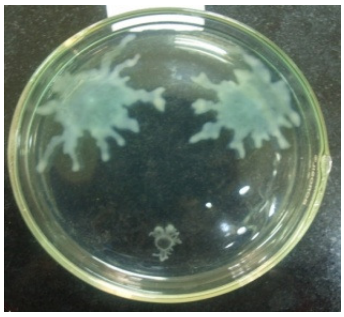
Ps.1



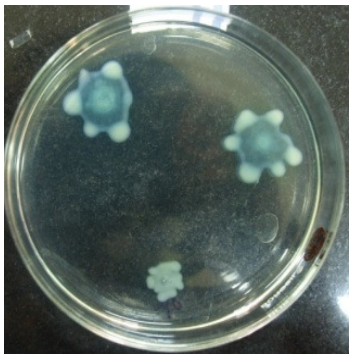
Ps.2



Ps.3

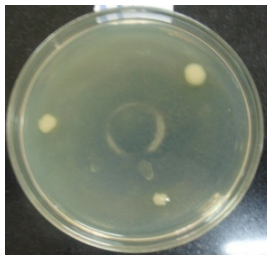


Ps.4

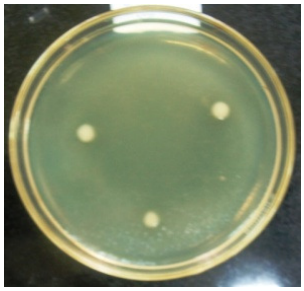


Biofilm cells motility

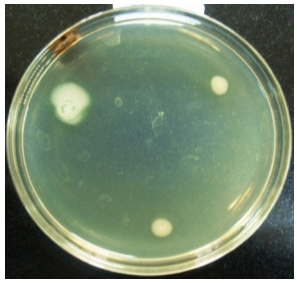
Ps.1



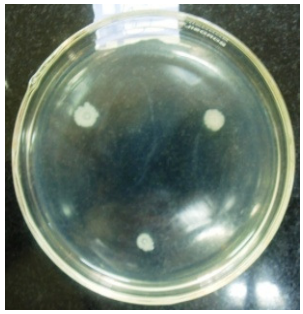
Ps.2



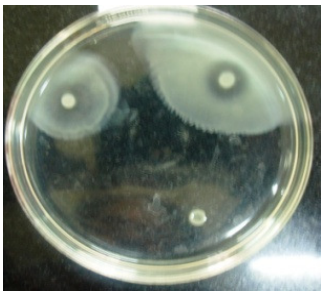
Ps.3



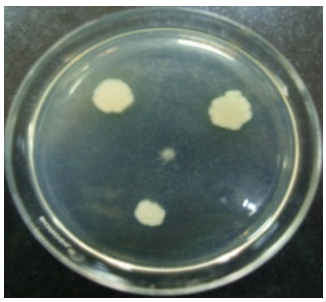
Ps.4



Ps.5



Ps.5



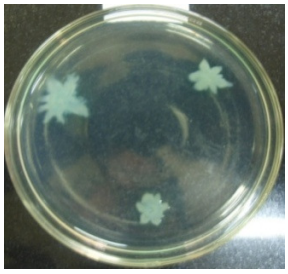
5.5.2) Swarming motility: In TSB with 0.5% agarose, all the strains were highly motile with swarming motility

Table-6) Swarming motility of *Pseudomonas* strains:

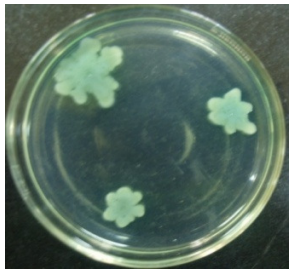
Swarming Motility

Planktonic cells motility **Biofilm cells motility**

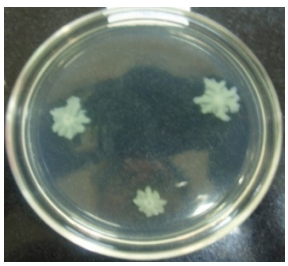
Ps.1



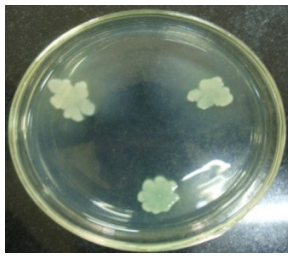
Ps.1



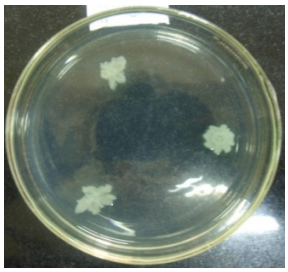
Ps.2



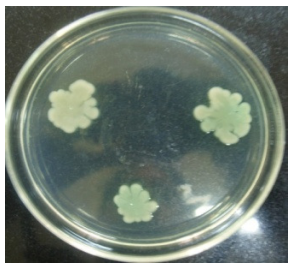
Ps.2



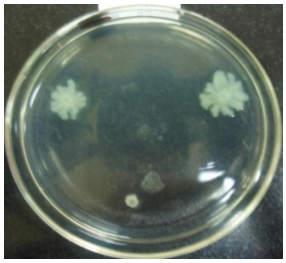
Ps.3



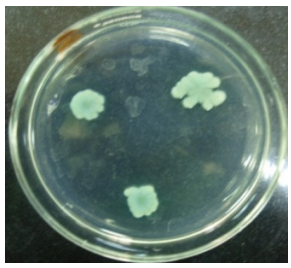
Ps.3



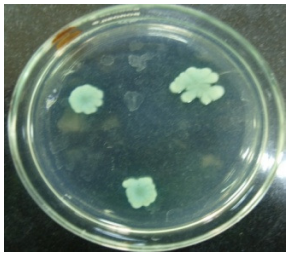
Ps.4



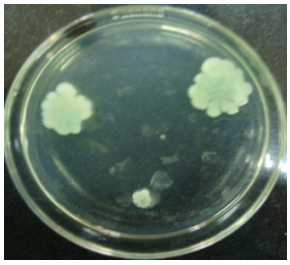
Ps.4



Ps.5



Ps.5



As seen in the above tables, swimming motility was observed in planktonic cells while the cells which formed biofilm depicted swarming motility. Swimming motility is necessary for the initial step of biofilm formation and attachment to a substrate. Therefore, the planktonic cells showed swimming motility, while as they formed biofilm, they started showing swarming motility which is helpful in dispersal of biofilm.

5.6) Isolation of host specific (bacterial) Bacteriophages:

Bacteriophages (phages) are obligate parasites of bacteria and are highly specific to their host. Hence in the present study *Pseudomonas* specific phages were isolated from Ganges river water, Panki sewage treatment plant and hospitals, all sites in the

Kanpur region of Kanpur District, Uttar Pradesh, India. Nine phages as shown in Table-7 and were isolated from different sites.

Table-7 Sources of phage collection and their plaque characteristics:

Sample source for phage isolation	Type of phage	Number of type of plaque
Ganges water	T, C	4
Lala Lajpat Rai Hospital, Kanpur	T	2
Rama Dental Hospital, Kanpur	Nil	0
LPS institute of Cardiology, Kanpur	Nil	0
Panki Sewage treatment Plant Kanpur	C	3

Note-C- Clear plaque, T- Turbid plaque

5.6.1) Spot test for lytic ability: Lytic ability of pure phages was tested by spot and plaque assay.

Spot assay: Six bacteriophages with the lytic ability as shown in Figure-6 (showing clear zone on bacterial lawn PA01) were selected for the present study and numbered as P1, P2, P3, P4, P5 and P6. While the other three phages were without any clear zone i.e. depicted turbidity, hence not shown in the figure.

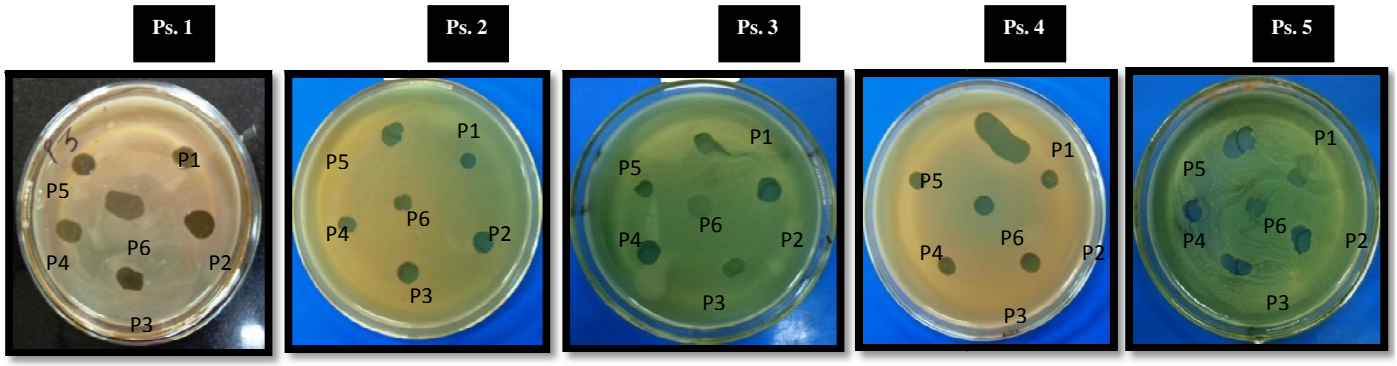


Figure-6 Spot assay of phages for lytic activity

Plaque assay for purity testing: Plaque assay was performed on TSA to test for the purity. All the phages if seen are of same size then they are assumed to be pure. A plaque assay figure for phage P6 is shown in the figure-7 where all phages of same size, which establishes its purity.

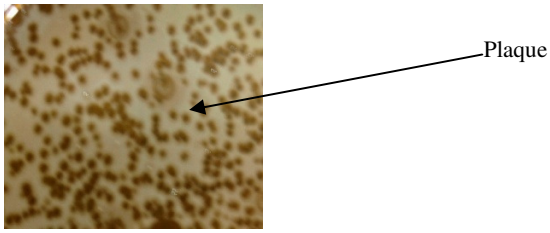


Figure-7 Plate showing plaque test of P6 phage

5.6.2) Titer value determination for isolated phages: Isolated bacteriophages were concentrated by enrichment method. Isolated phages were concentrated and titer value was determined by using double layer agar overlay method. Phages P4 and P5 had the highest titer value of 10^{15} (Table-8).

Table-8- Titration of phages

S. No.	Name of isolated phages	Titer
1.	P1	10^{13}
2.	P2	10^{14}
3.	P3	10^{13}
4.	P4	10^{15}
5.	P5	10^{15}
6.	P6	10^{12}

5.6.3) Host range of isolated phages: Host range was determined to check the lytic activity of test phages for different hosts other than their own host. Table-9 represents the % host range of isolated bacteriophage for *P. aeruginosa, Burkholderia cepacia, Escherichia coli, Enterobacter aerogens, Salmonella typhi, Staphylococcus aureus, Stphylococcus epidermidis,* and *Staphylococcus arlettae*. Presence of clear plaque (C) indicates phages ability to lyses of the host, while turbid plaque (-) is indication of lysogeny i.e. phage is in-effective against that host. Of the eight different hosts tested, two strains *S. aureus* and *S. arlettae* were reference strains while *P. aeruginosa* was standard/control strain in the present study. Percentage of host infection with the test phages varied from 62.5 to 75.

Table-9) Host range of phages:

Host	Phages					
	P1	**P2**	**P3**	**P4**	**P5**	**P6**
Pseudomonas aeruginosa	C	C	C	C	C	C
Escherichia coli	C	C	C	C	C	C
Enterobacter aerogens	C	C	C	C	C	C
Salmonella typhi	C	C	C	C	C	C
Staphylococcus epidermidis	C	_	_	_	_	_
Staphylococcus arlettae	_	C	_	_	_	_
Burkholderia cepacia	C	C	C	C	C	C
Staphylococcus aureus	_	_	_	_	_	_
Percentage of host range	**75**	**75**	**62.5**	**62.5**	**62.5**	**62.5**

C- clear plaque

5.7) Characterization of the phages- characterization of phage was done by following methods-

5.7.1) Burst size determination using one step growth curve: The burst size was determined by one step growth curve which indicates the average number of phage progeny per infected cell at the end of one infection cycle. The existence of an optimal lysis time can be understood as a consequence of the trade-off between generation time and burst size. Longer the generation time, larger is the burst size and the number of progeny's will also be more, but their ability to cause infection in

bacteria will be delayed while the ones with shorter cycle will have faster infection ability and less burst size. Burst size of P1, P2, P3, P4, P5, and P6 was 314, 228, 226, 153, 289 and 275 pfu/infected cell respectively. The burst size of phage P5 is very low 157 while phage P1 had very large burst size. Hence, phage P1 has great potential to infect cells very frequently than other phages (figure 8 a-f).

a) One step growth curve of P1

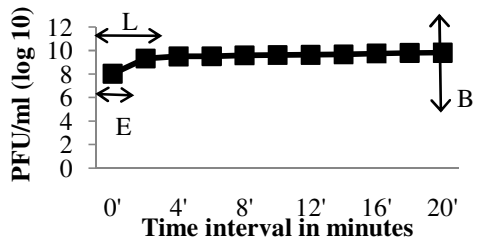


Figure-8a one step growth cure

b) One step growth curve of P2

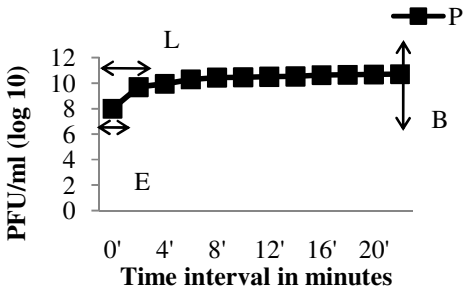


Figure-8b one step growth cure

c) **One step growth curve of P3**

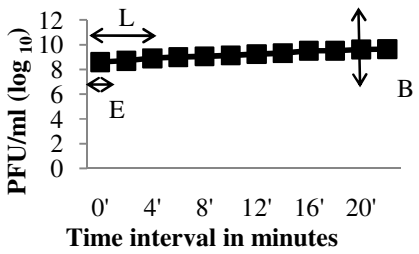


Figure-8c one step growth cure

d) One step growth curve of P4

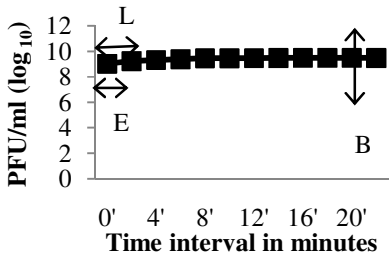


Figure-8d one step growth cure

e) **One step growth curve of P5**

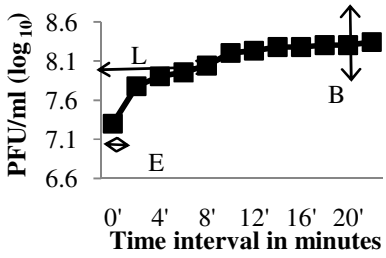


Figure-8e one step growth cure

f) One step growth curve of P6

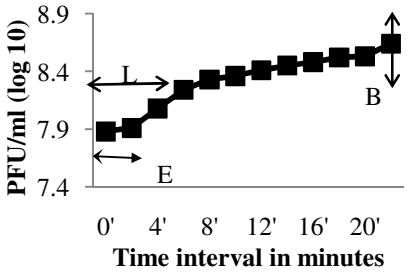


Figure-8f one step growth cure

5.7.2) Adsorption of isolated phages: Adsorption is the first event in the life cycle of phages. It has been divided into two stages. Reversible and irreversible attachment. In this study, irreversible adsorption rate was defined. Phage, which has minimum adsorption time has high adsorption rate i.e. in less time more phages will be adsorbed. Here, phage P2 has the minimum adsorption time i.e. 6 minutes while phage P5 and P6 have 8 minutes and rest phages have 10 minutes adsorption time (Figure-9 a-f).

(a) Adsorption of phage P1

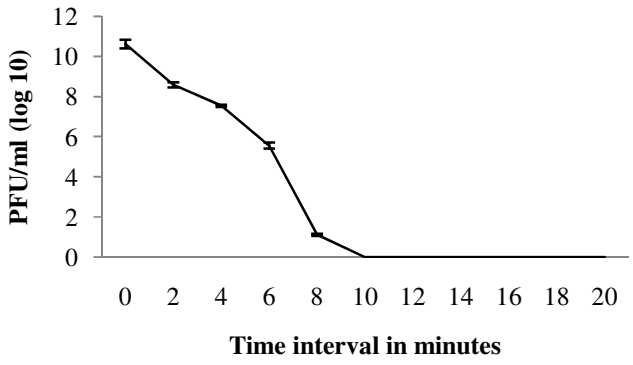


Figure-9a Adsorption of phage P1

b) Adsorption of phage P2

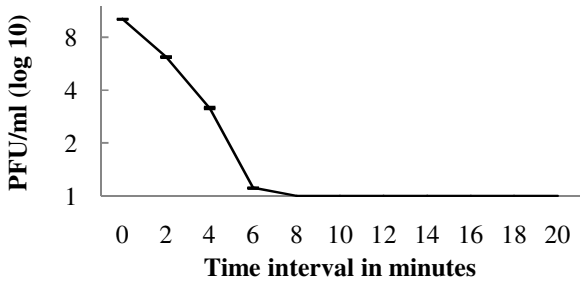


Figure-9b Adsorption of phage P2

c) Adsorption of phage P3

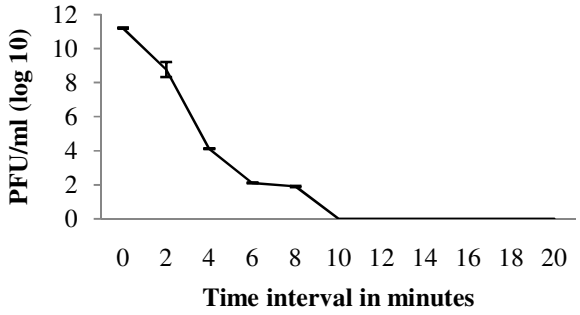


Figure-9c Adsorption of phage P3

d) Adsorption of phage P4

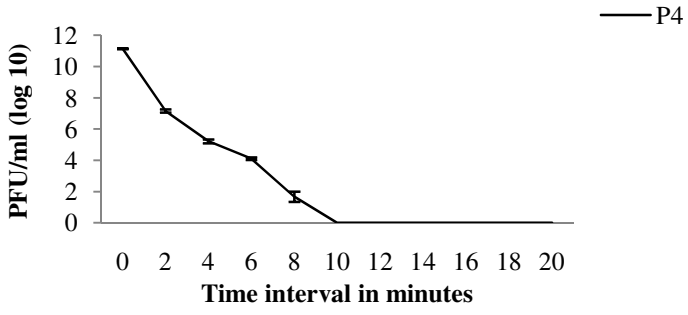


Figure-9d Adsorption of phage P4

e) Adsorption of phage P5

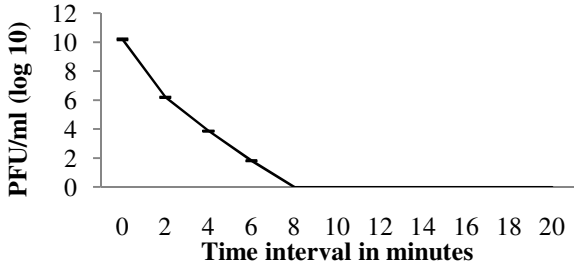


Figure-9e Adsorption of phage P5

(f) Adsorption of phage P5

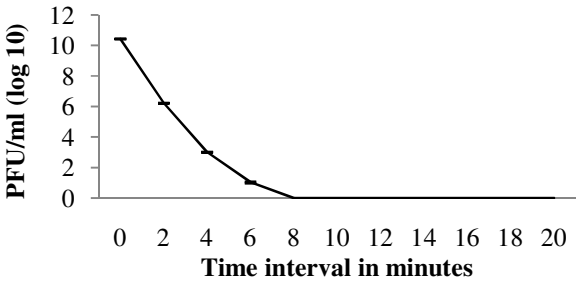


Figure-9f Adsorption of phage P6

5.7.3) Determination of stability of phages to physic-chemical stresses:

Phages were subjected to variation in temperature and pH to see their effectiveness at different temperature and pH.

5.7.3.1) Temperature tolerance of test phages: This test was performed to determine the sensitivity of isolated phages towards the variation in temperature. Assessment was made using the changes in the titer value on the basis of double layer agar assay. Phage P1, P3, P4 and P6 were able to maintain their multiplication at 95^{o} C, with P1 phage (isolated from river Ganges) being the best while others showed minor loss in titer value. This is something very important as the temperature of the river water never goes to such a high temperature but still phages with high

thermo-tolerance limit are present in water. At 75^{o} C, all phages except P5 (isolated from Panki, Kanpur thermal power plant sewage water) were able to multiply. At 37 and 55 ^{o}C, all the phages were able to multiply (Figure-10). This variation in tolerance limit is important for the phages P2 and P5 were unable to survive at this temperature. All the phages were stable at 75 ^{o}C with minor loss in titer, while phage P5 was unable to survive at this temperature as shown in Figure-10.

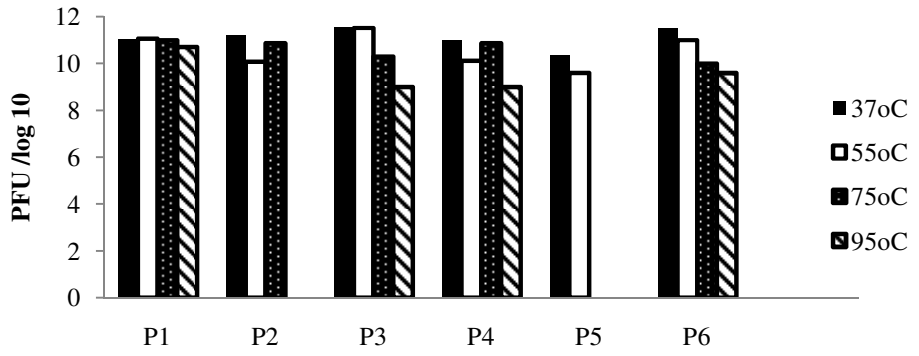


Figure-10 Temperature tolerance of phages

5.7.3.2) pH sensitivity of phages: On looking for activity of phages at varying pH, all phages were stable at pH 9 except phage P5. Better survival rate of phages was observed at neutral and less acidic conditions while at highly acidic conditions, only phage P1 and P3 were able to survive and multiply (Figure- 11).

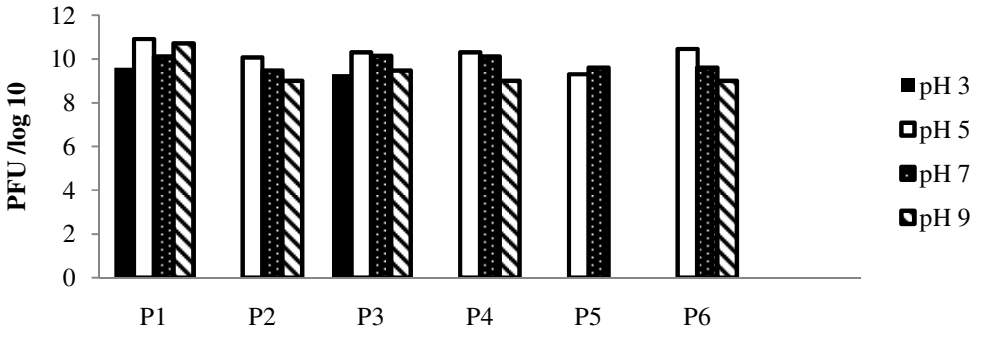


Figure-11 pH sensitivity of phages

5.7.4) SDS-PAGE of phages proteins:

Phages were analyzed for structural proteins composition and it was found that P1 phage had structural proteins of 40 kDa, 20 kDa and ~14.3 kDa, Phage P2 had 40 kDa, 20.1 kDa, 15 kDa, 14.3 kDa and minor protein of 3.5 kDa, Phage P3 contained 30 kDa, 14.3 kDa and 10 kDa, P4 phage had 35 kDa and 10 kDa, while phage P5 had 30 kDa, 20 kDa, 14.3 kDa and 10 kDa, Phage P6 had proteins of 35 kDa, 20.1 kDa and 10 kDa molecular weight (Figure-12). Structural protein analysis of phages reveals that all six isolated bacteriophages are six different entities having different protein composition.

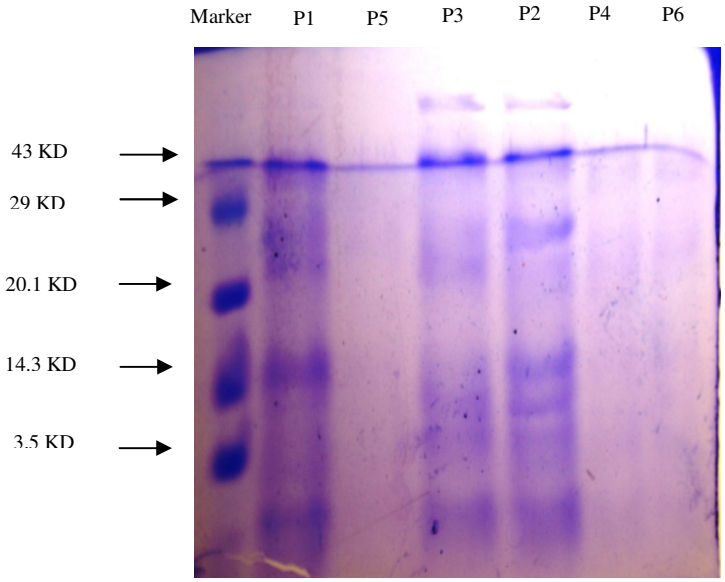


Figure-12 Image of SDS PAGE of phage proteins

5.7.5) Isolation and restriction digestion of bacteriophage: DNA of bacteriophages was isolated and electrophoresed on 1% agarose gel along with DNA / EcoRl / HindIII double digested DNA marker. Molecular weight of all the phage DNA was found to be ranging from 21.0 kb to 35.0kb.

For molecular characterization of phages, the isolated phage DNA samples were further subjected to digestion with restriction enzymes BamHl and PsutYl. The results revealed that all phages were sensitive to BamHl and PsutYl except P1 and P4, exhibited different banding patterns, which confirming that all phages were genetically different and harbored ds DNA as genetic material shown in (Figure

13). However, lane 4 and 12 were loaded with RE digested DNA of phage P2 and P6 respectively, which clearly indicate that BamHl and PsutYl completely digested the DNA, while Lane 10 of phage P5 RE digested DNA had shown 6 bands ranging from 17 kb to 564bp, Lane 12 of phage P6 loaded DNA shown 1 band of 5kb.

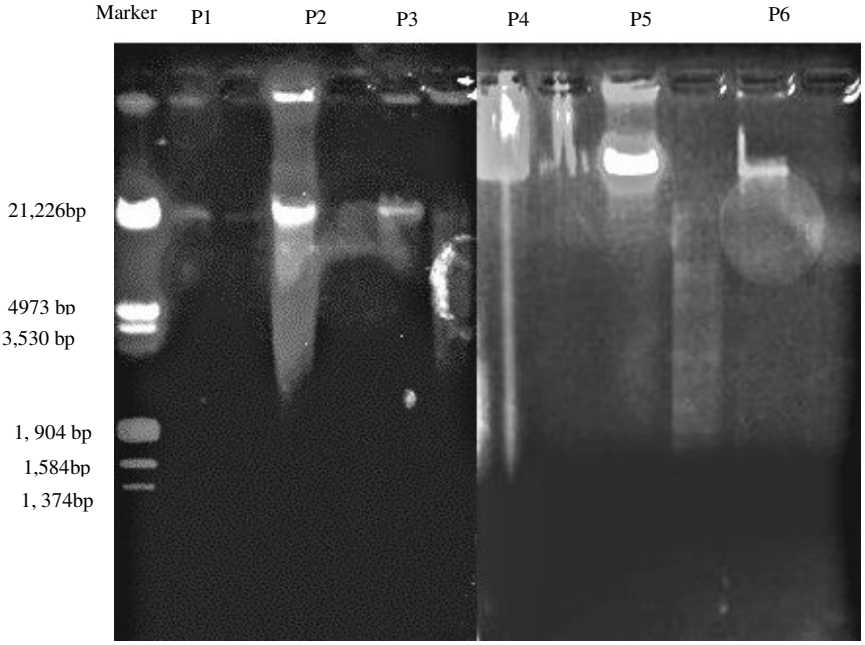


Figure-13 Picture of phages DNA and their restriction digestion

5.7.6) Morphological characterization of isolated phage by Electron Microscopy: Morphological characterization is generally regarded as one of the important criteria used to place phages into specific families owing to the fact that clear differences in the size and structure of tailed phages which can be easily established and there is a great deal of information available for comparison purposes. Some of the challenges which are however faced with this criterion are: structures such as collars may be present in one instance and absent in another, related phages may have varying tail dimensions, the majority (about 61%) of tailed phages belong to the *Siphoviridae* family (Ackermann et al., 1992). Measurement of isolated phages according electron micrograph revealed that phage P1, P2, P3 P4, P5 and P6 had head of 49±2.14, 45±1.89, 28±2.86, 30±1.86,

31±1.97, and 24±3.14 nm respectively while they had tail of 15±1.56, 65±2.14, 43±2.45, 60±2.76, 50±3.14 and 45±1.96 nm respectively. Phage P1 belonged to *Pyoviridae* because it had short tail while rest of the phages belonged to *Myoviridae* family because they had long tail (figure-14).

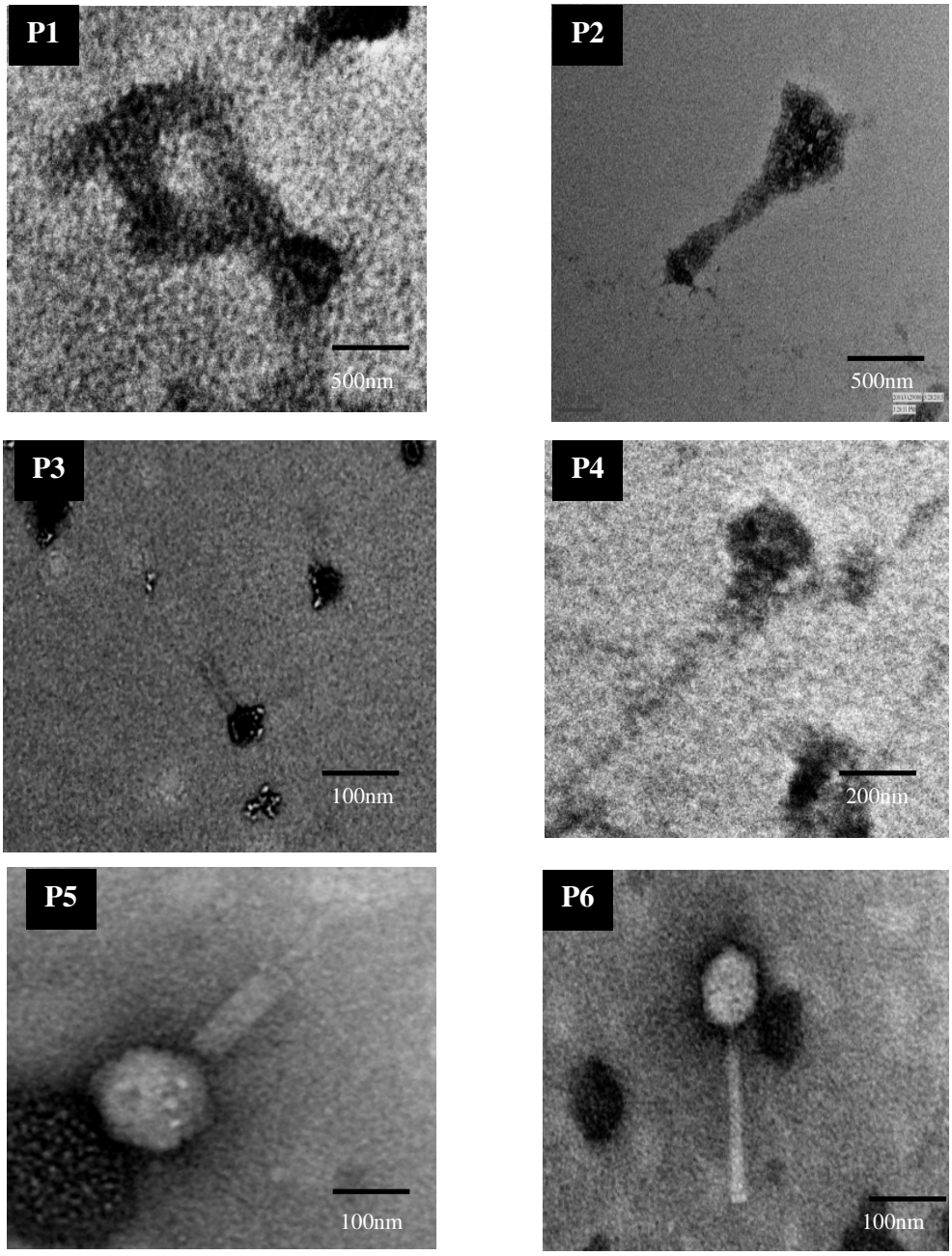


Figure-14 Electron microscopy of isolated bacteriophage

5.8) Minimum Inhibitory Concentration (MIC) determination of ciprofloxacin antibiotic for inhibition of *Pseudomonas aeruginosa* biofilm formation:

Biofilm is a complex structure and it protects microbial cell from antimicrobial compounds. Therefore, for the control of biofilm formation, ciprofloxacin antibiotic was selected because this antibiotic is low in molecular weight and therefore can penetrate the biofilm matrix easily. All the isolates were sensitive for this antibiotic; therefore this antibiotic was chosen for the inhibition of biofilm formation of isolates. For the inhibition of biofilm formation, variable ranges (1.56-100 µg/ml) of ciprofloxacin were used.

When host *P. aeruginosa* (Ps.1) was treated with varying concentration (1.56 mcg to100 µg/ml) of ciprofloxacin, it was observed that high concentration of antibiotic inhibits / kills the planktonic cell as well as biofilm cells (Figure-15a). MIC in the present case is beyond 100 µg/ml. Similarly, MIC of ciprofloxacin for other test hosts Ps2, Ps.3, Ps.4, Ps.5 was also tested in the same way. In all cases planktonic as well as biofilm cells were inhibited to the maximum by 100 µg/ml concentration of ciprofloxacin (Figure-15a-e).

Graph of MIC of ciprofloxacin antibiotic for inhibition of *Pseudomonas aeruginosa* biofilm formation

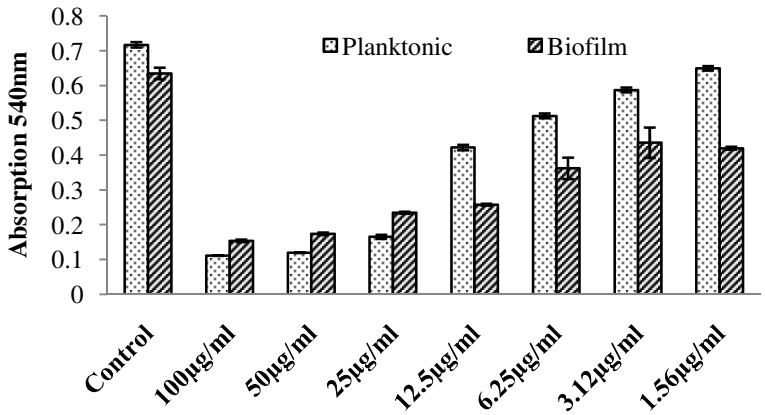


Figure 15 (a). At initial stage of biofilm formation, Ps.1 treated with varying concentration of ciprofloxacin

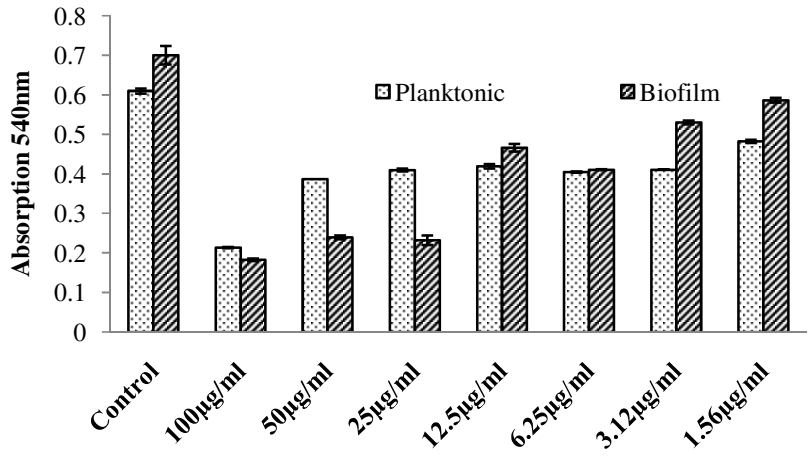


Figure 15(b). At initial stage of biofilm formation, Ps.2 was treated with varying concentration of antibiotic ciprofloxacin

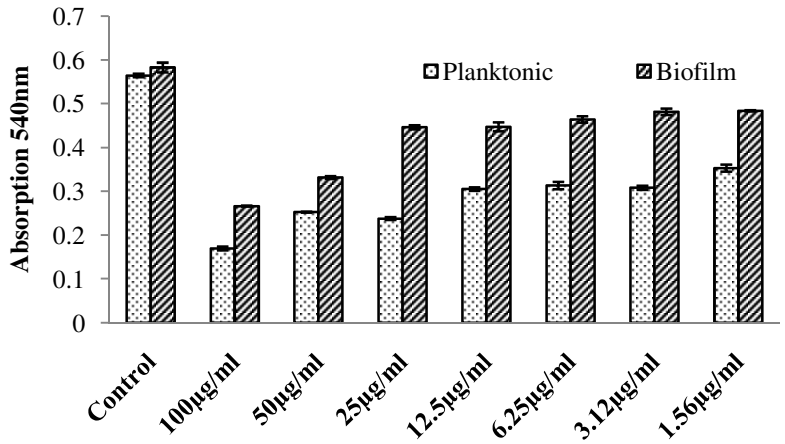


Figure 15(c). At initial stage of biofilm formation Ps.3 was treated with varying concentration of ciprofloxacin

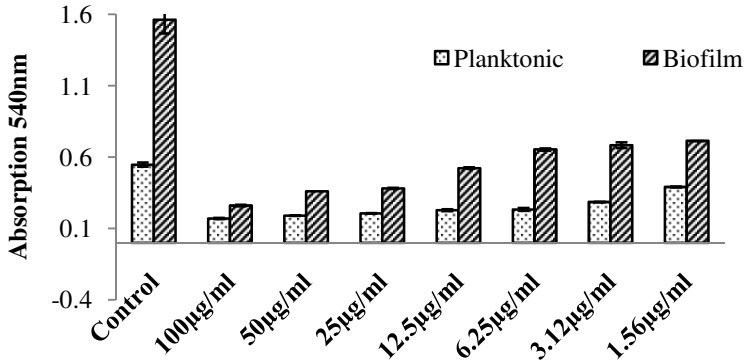


Figure 15(d). At initial stage of biofilm formation Ps.4 was treated with varying concentration of antibiotic

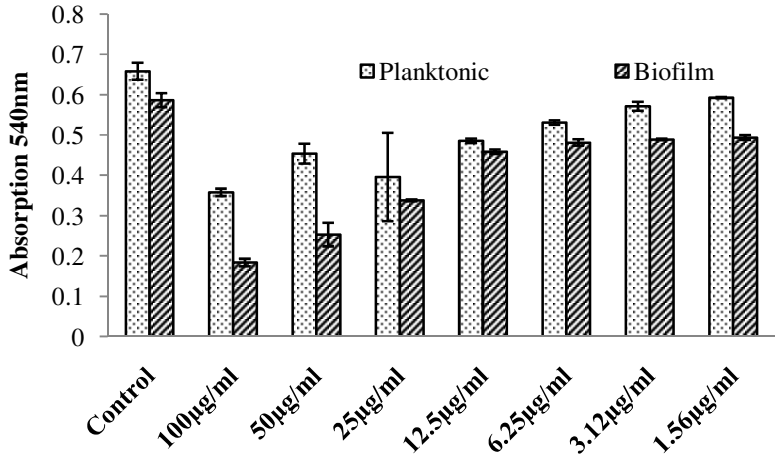


Figure 15(e). At initial stage of biofilm formation Ps.5 was treated with varying concentration of ciprofloxacin

5.9) Control of biofilm formation of *P. aeruginosa* strains with isolated bacteriophages: From the above results, it is clear that high concentration of antibiotic can control biofilm formation to some extent, but even a very high dose of 100 µg/ml was able to inhibit only upto a certain extent and not completely therefore test phages were employed in the present experiment to test for the efficacy of phages to inhibit bacterial hosts Ps.1 to Ps.5 (planktonic as well as biofilm cells).

Phage mediated control of biofilm formation and inhibition of *Pseudomonas* isolates was performed on the microtiter plate. After phage treatment, live cell of biofilm was analyzed by MTT dye. MTT is a redox dye which turns purple coloured formazone compound by metabolically active cells, while dead cells are not able to form formazone compounds and appear yellow as shown in Figure- 16.

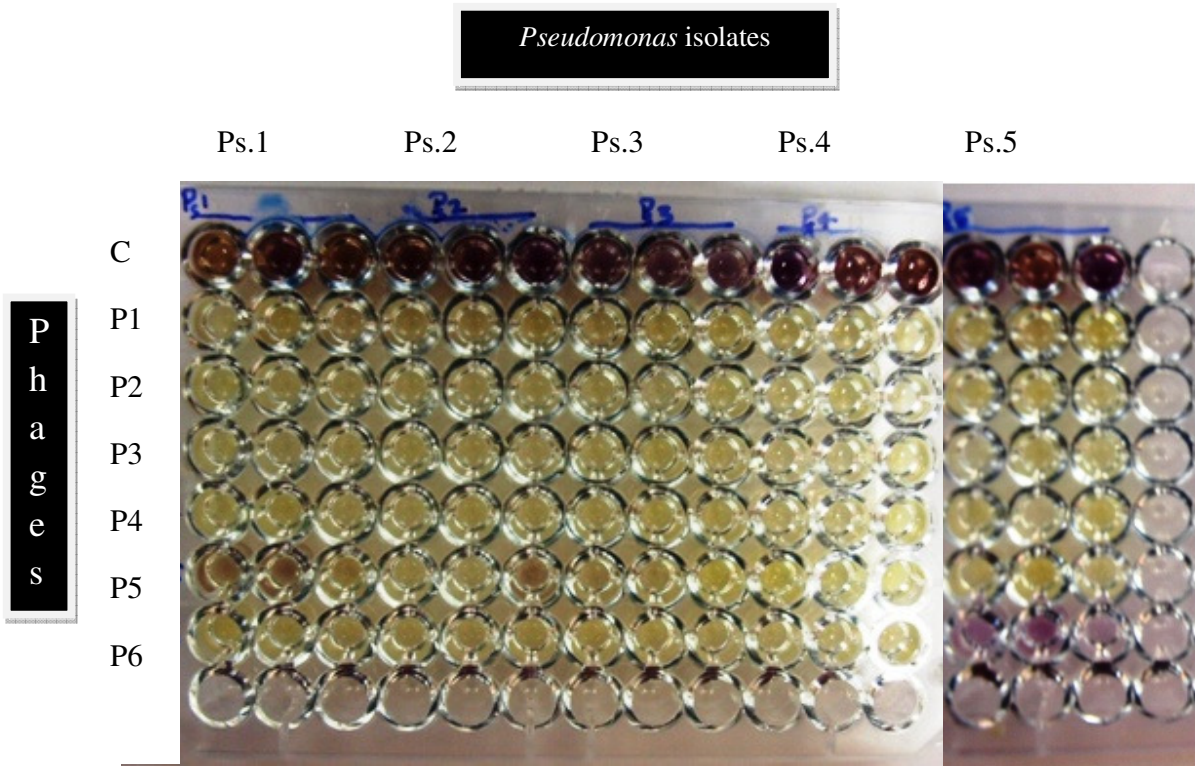


Figure- 16 Microtiter plate containing biofilm of host stained with MTT dye

The detailed result about the effect of phage on the *Pseudomonas* strains and biofilm inhibition are given below:

a) When the host Ps.1 was exposed at the initial stage of biofilm formation with 0.01MOI of phages then it was found that all the phages efficiently controlled biofilm of isolate Ps.1. Phage P1 was more potent (94% killing) as compared to other phages with phage P5 being the least effective (Figure-17 a).

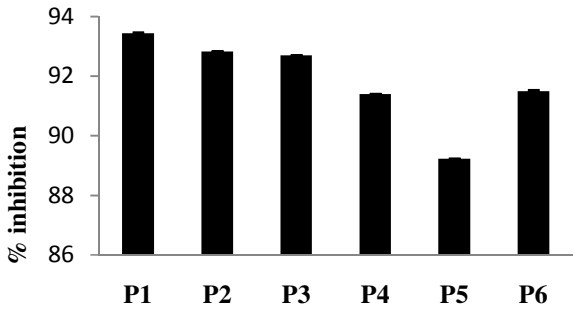


Fig 17 a) Inhibition of biofilm formation of Ps.1 with isolated bacteriophage P1, P2, P3, P4, P5 and P6

b) In this experiment, Ps2 was exposed to phages at the initial stages of biofilm formation. In this case also, Phage P1 was more effective controller as compared to other phages (Figure-17 b).

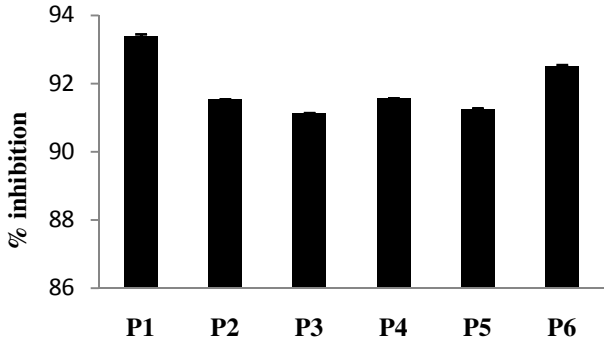


Fig 17 b) Inhibition of biofilm formation of Ps.2 with isolated bacteriophage P1, P2, P3, P4, P5 and P6

c) In case of Ps. 3, all the phages were effective in controlling biofilm formation with P1 being the best and P6 almost comparable to P1 in its efficacy (Figure-17c).

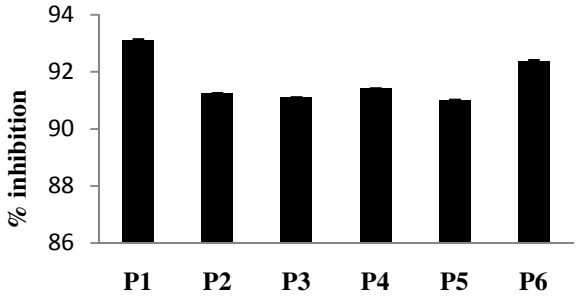


Fig 17 c) Inhibition of biofilm formation of Ps.3 with isolated bacteriophage P1, P2, P3, P4, P5 and P6

d) All the phages were able to control biofilm forming cells approximately by 92% (Figure-17d).

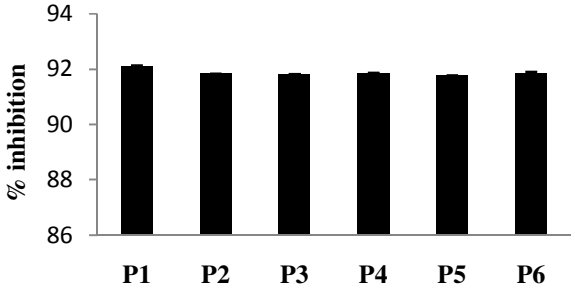


Fig 17 d) Inhibition of biofilm formation of Ps.4 with isolated bacteriophage P1, P2, P3, P4, P5 and P6

e) Ps.5 is highly resistant isolates for different class of antibiotics and when this strain was exposed to phages, it was found that phage P1 inhibits the cells and biofilm formation by 92% while rest of the phages killed biofilm cells up to 91% (Figure-17e).

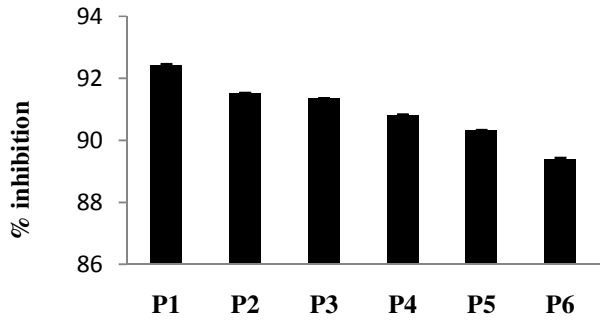


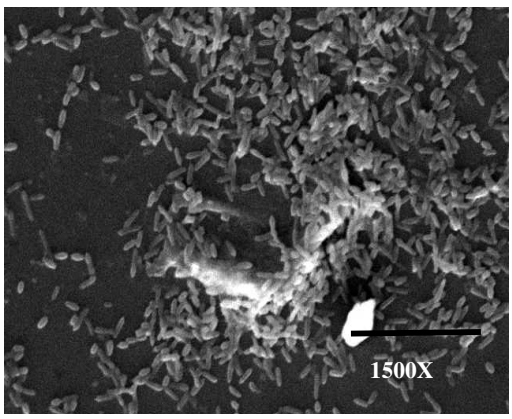
Fig 17 e) Inhibition of biofilm formation of Ps.5 with isolated bacteriophage P1, P2, P3, P4, P5 and P6

In all the above findings, it was observed that Phage P1 was the best (upto 94% efficiency) in controlling biofilm forming cells and inhibited biofilm formation by these cells better as compared to other phages P2-P6.

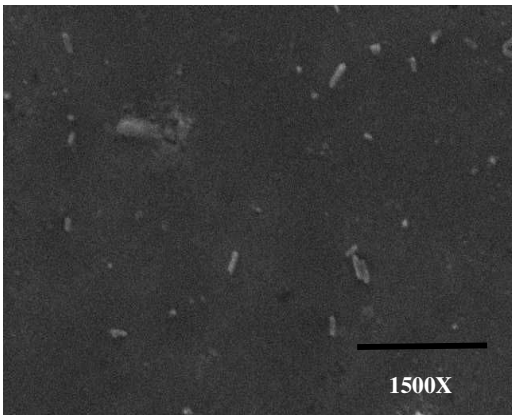
5.10) SEM analysis of *Pseudomonas* isolates biofilm treated with bacteriophages:

From the above experiments it was completely clear that phage P1 is most potent because it had high potential to inhibit biofilm formation of the entire host *Pseudomonas* Ps.1, Ps.2, Ps.3, Ps.4 and Ps.5. Therefore, qualitative analysis of killing efficiency of phage P1 against biofilm of host Ps.1-Ps.5 was done by SEM.

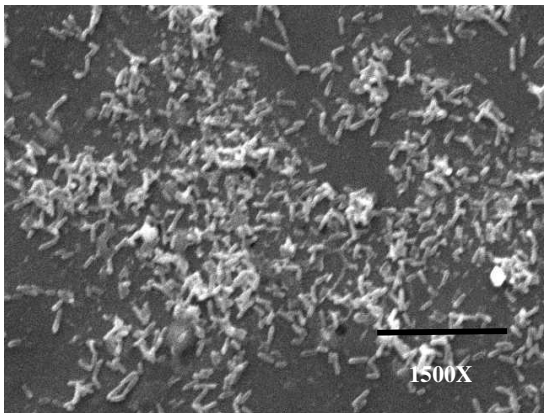
SEM images of biofilm treated with phage P1 indicated that P1 phage is most potent phage and had high efficiency to control the biofilm of multiple drug resistant *Pseudomonas* isolates as shown in figure-18 (a-e).



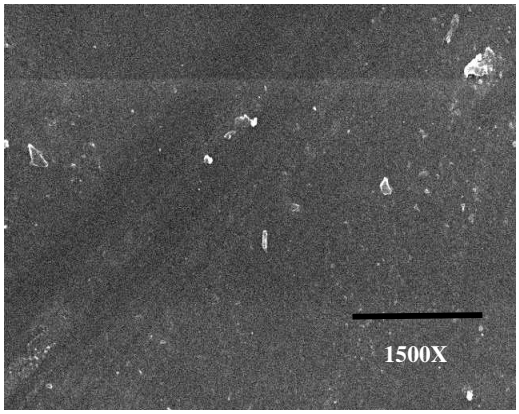
Untreated biofilm of Ps.1



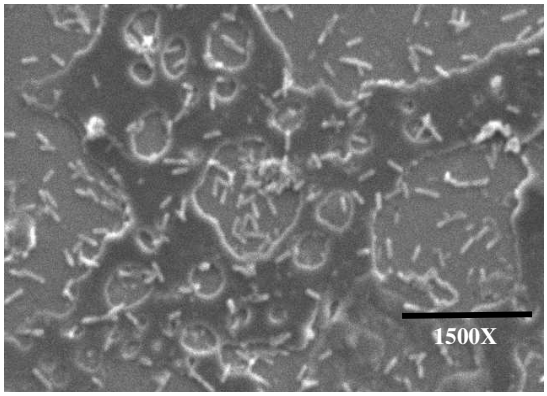
Biofilm of Ps.1 treated with bacteriophage P 1



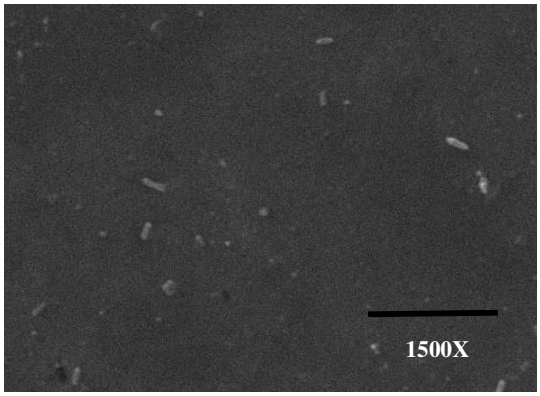
Untreated biofilm of Ps.2



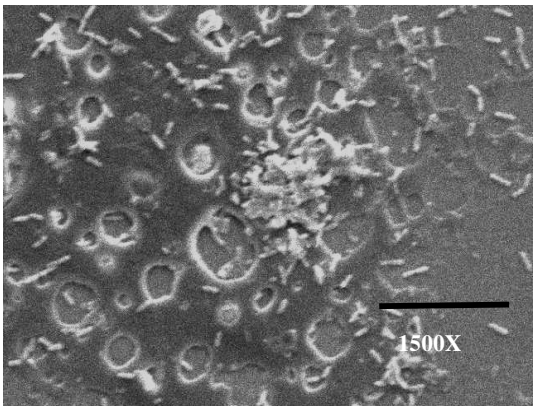
Biofilm of Ps.2 treated with bacteriophage P 1



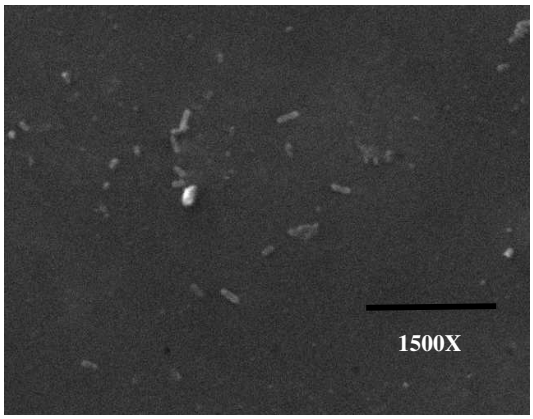
Untreated biofilm of Ps.3



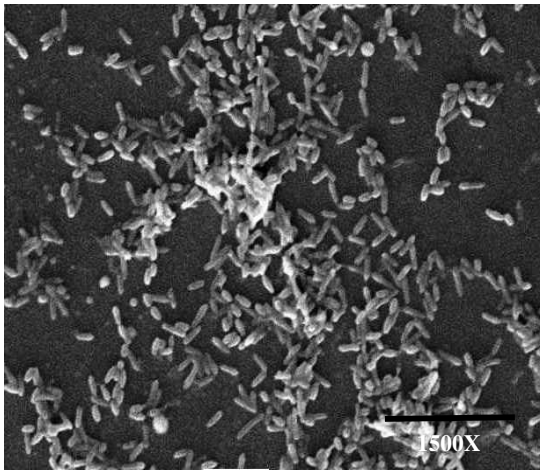
Biofilm of Ps.3 treated with bacteriophage P 1

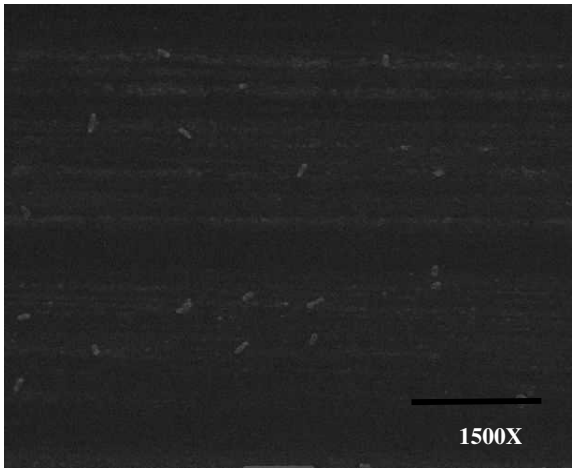


Untreated biofilm of Ps.4



Biofilm of Ps.4 treated with bacteriophage P 1





Untreated biofilm of Ps.5

Biofilm of Ps.5 treated with bacteriophage P 1

Figure-18 SEM analysis of P1 phage mediated inhibition of biofilm formation of Ps.1, Ps.2, Ps.3, Ps.4 and Ps.5

Interaction of Phage P1 with most potent *Pseudomonas aeruginosa* Ps. 5 strain for confirmation of above SEM results

5.11) Electron Microscopy of initial stage of biofilm formation of Ps.5: In this experiment, at the initial stage, attachment of biofilm forming cells was observed. EPS matrix started surrounding the cell which protects cells from antimicrobial compounds and environmental stresses as shown in figure-19.

At the initial stage of biofilm formation, planktonic cells started adhering to the substratum and after that they formed microcolony (Fig. 19). Microcolony led to the formation of mature biofilm which later on dispersed.

Again the repetition of above cycle started with the dispersed biofilm cells.

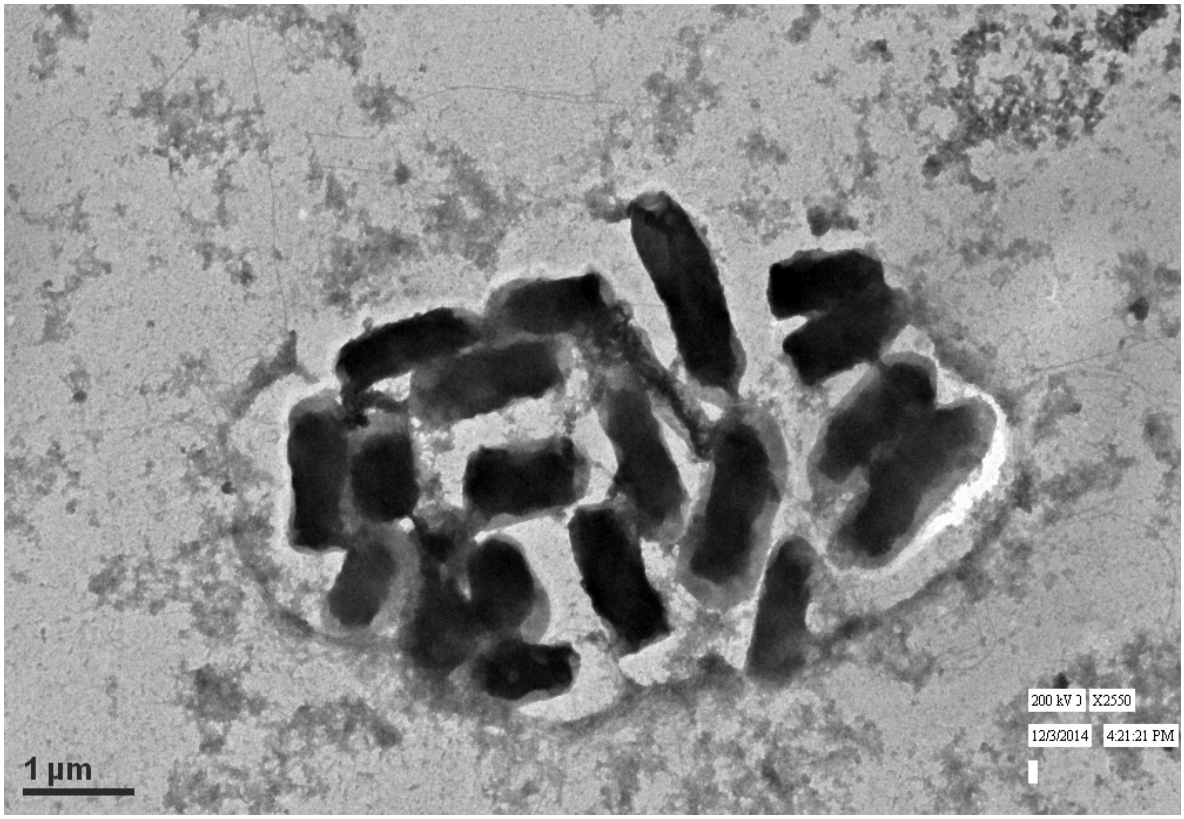


Figure-19 Electron Microscopy of initial stage of biofilm formation of Ps.5

5.11.1) Interaction of phage P1 and their host Ps.5: Attachment of phage with the host is carried out with the help of receptors, which are present on the cell surfaces such LPS, pilli and antigens etc. After the successful attachment of phages onto the host surface, adsorption of phage DNA takes place eventually leading to initiation of phage life cycle in the host cell (Fig. 20).

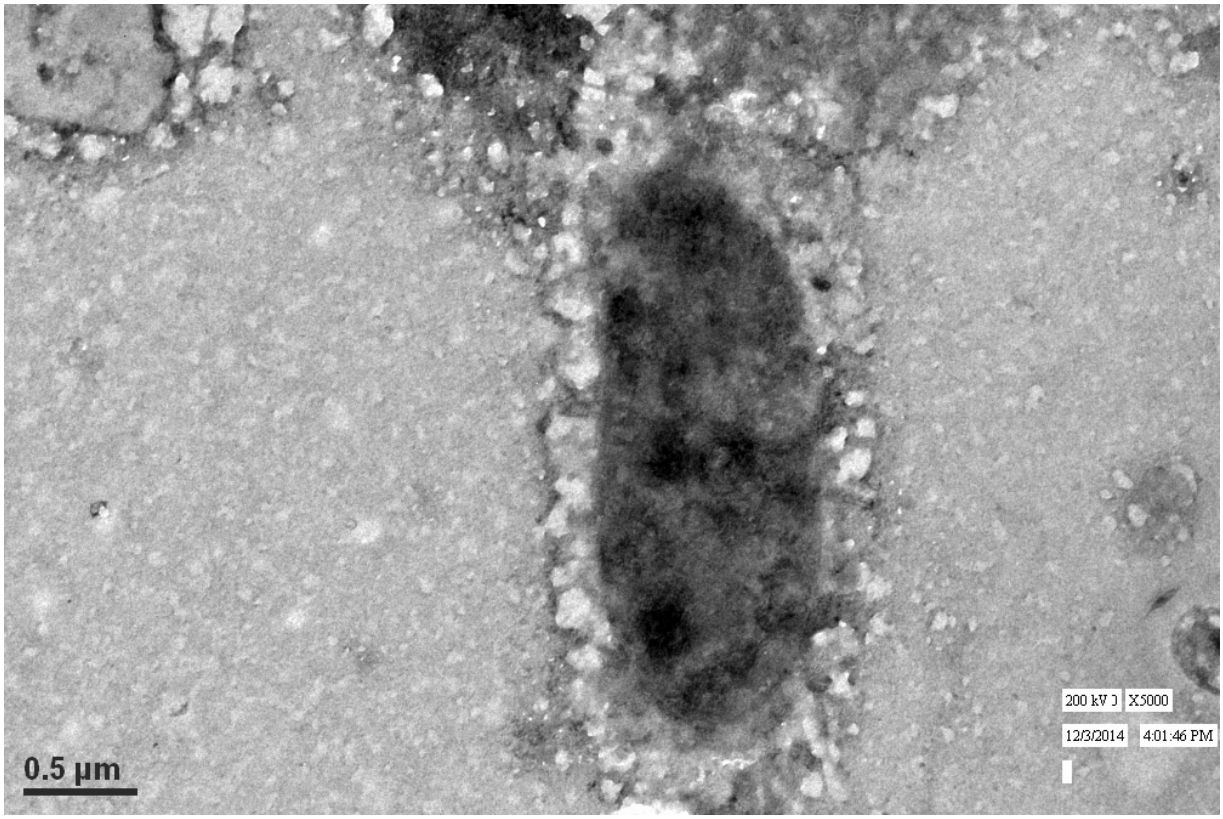


Figure-20 Electron Microscopy of initial stage of biofilm formation of Ps.5 isolate

5.11.2) Lysis of infected P1 infected Ps.5 host cell:

The last stage of infection cycle of phage is lysis of the host cell and release of virion (Fig. 21). After lysis of the host, release of phage particle is the last step of phage lytic life cycle, in the figure-21, phage particle released by lysed host cell can be seen.

5.11.3) Release of phage particle after lysis of host Ps.5:

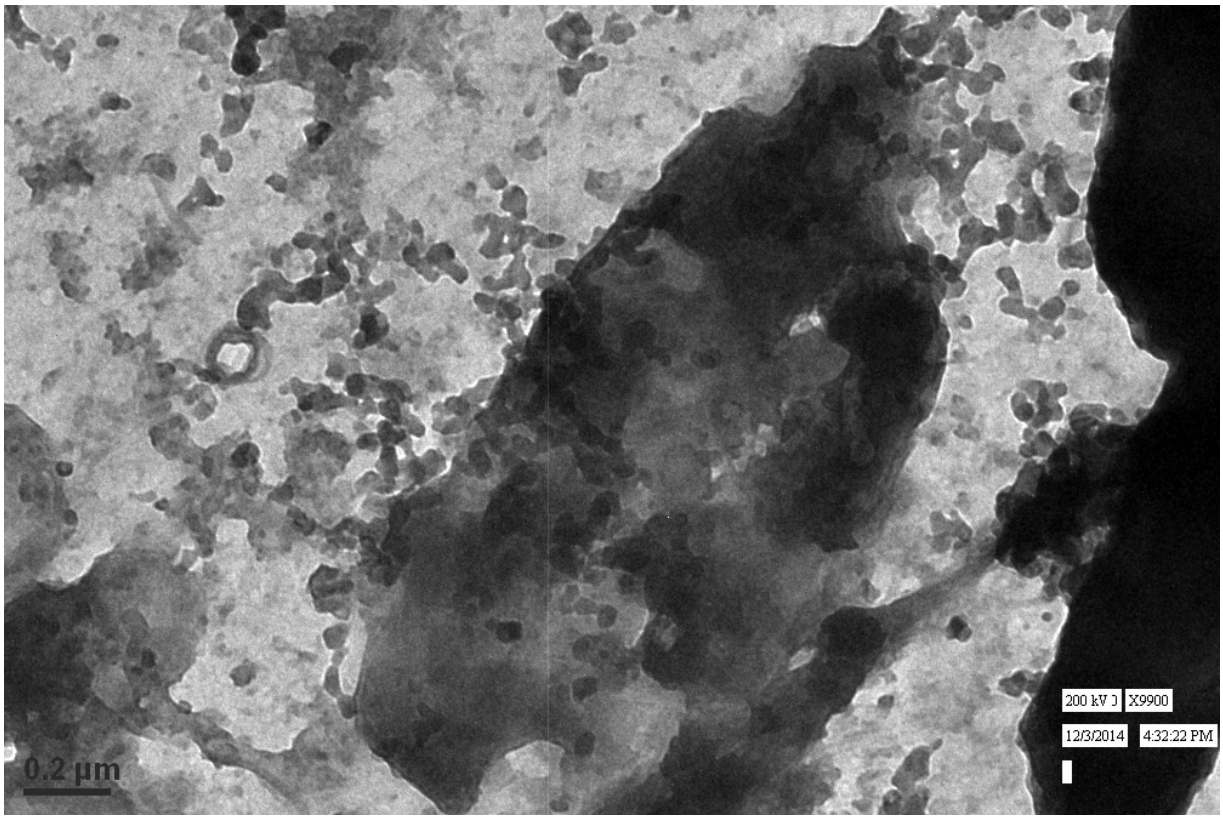


Figure- 21 Electron Microscopy of released of phage P1 after lysis of host Ps.5

The results obtained in this chapter are discussed in detail in the next chapter entitled "Discussion".

Discussion

CHAPTER 6 DISCUSSION

P. aeruginosa is an opportunistic pathogen which causes infections such as common acute septicemia in burn or surgical wound, urinary tract infection, corneal ulceration (from wearing contact lenses), endocarditis (caused by intravenous drug use, etc.), and pneumonia (from use of ventilator and endotracheal tube) (Bodey *et al.*, 1983). *P. aeruginosa* is well known microorganism for its expertise to resist commercially available antibiotics and this resistance could arise through several mechanisms such as efflux pump, enzymatic degradation of antibiotics and by alteration in membrane permeability. In addition, *P. aeruginosa* exist in nature in the form of biofilm. Biofilm embedded *P. aeruginosa* cells are 1000 times more resistant for any antimicrobial than planktonic cells. These features make *P. aeruginosa* highly resistant for commercially available antibiotics and therefore *P. aeruginosa* is a major cause of concern for the medical practitioners.

In the current research work, 12 bacterial strains isolated from patients with burn wound infections were isolated, characterized for their antibiotic resistance pattern and other features. Based on antibiotic resistance potential, five *Pseudomonas* strains having multiple drug resistance against most of the antibiotics tested were selected for further studies. The strains were characterized morphologically, biochemically, 16SrRNA sequencing, their pathogenicity tested for determination of virulence trait on blood agar, congo red binding assay, biofilm formation using crystal violet and MTT assay. All the strains were found to be MDR strains and possessed pathogenicity and biofilm forming ability which conferred resistance in them that could not be controlled by commercially available therapeutic molecules. So, in quest of searching safe and effective technique to control such MDR strains, bacteriophage therapy was employed.

So, primary concern was to target different habitats for isolation of phages which could target these strains as their host. In this quest, six different phages from phages from diverse environments (river Ganges and sewage water, Kanpur, Uttar Pradesh, India) were isolated, characterized and tested as parasite on these hosts (*Pseudomonas*

strains). Ability of these phages to control biofilm formation in the hosts was checked by interaction between phages and the host using quantitative (MTT assay of biofilm treated with bacteriophage) and qualitative (SEM and TEM of phage treated biofilm). Results obtained already described in Chapter 5 (Results) are discussed here in this chapter.

Twelve different bacterial strains supposed to be *Pseudomonas* were isolated on Pseudomonas agar as has been used earlier by LaBauve *et al.*, 2012. Isolated strains were morphologically characterized and tested for determination of multiple drug resistance. The goal of current study was the selection of multiple drug resistant bacteria and their control, so further study was on selection of MDR strains from the 12 isolates. Hence antibiotic susceptibility test was performed using Azithromycin (15 mcg), Cefixime (5 mcg), Ceftriaxone (15 mcg), Chloramphenicol (30 mcg), Co-Trimoxazole (25 mcg), Ciprofloxacin (5 mcg), Moxifloxacin (5 mcg), Gentamicin (10 mcg), Imipenem (10 mcg), Levofloxacin (30 mcg), Meropenem (10 mcg), Ofloxacin (5 mcg), Streptomycin (10 mcg) and Tobramycin (10 mcg) for sensitivity test. On the basis of antibiotic sensitivity test five multiple drug resistant isolates (Ps.1, Ps.2, Ps.3, Ps.4 and PS.5) were chosen for the further study (Table-5.2). Biochemical tests were performed for identification of isolates (table-3). On the basis of biochemical results, it was clear that isolates were *Pseudomonas sp*. as per Bergey's manual of systematic bacteriology for identification of bacteria. A similar biochemical result for *Pseudomonas* has been reported earlier by Ranjbar *et al.*, 2011. For further confirmation of isolate's species, 16S rRNA sequencing of all five isolates was done using universal primer. On the basis of 16S rRNA, it was confirmed that all the isolates were *P. aeruginosa*. 16S rRNA sequencing and comparison with NCBI database has been performed earlier also to establish the identity of the strain (Kumar *et al.*, 2014).

Furthermore, the determination of pathogenic characteristics of *P. aeruginosa* strains (Ps.1, Ps.2, Ps.3, Ps.3, Ps.4 and Ps.5), hemolytic test, congo red binding and biofilm assay were done (table-4 and figure-2, 3 and 4). These tests were performed to know whether the isolates are pathogenic or not. Pathogenicity tests defined that all five isolates possessed virulence traits and can cause disease in immuno-compromised host. These pathogenicity tests have been used by earlier workers to ascertain the virulence of

the strains (Satoh *et al.*, 2004; Sharma and Sihag 2013). In addition, motility test was performed to know the biofilm forming efficiency of strains. In reality, for the biofilm formation, bacteria should be motile, therefore two type of motility, swimming and swarming were analyzed. Bacteria use swimming motility for the initial stage of biofilm formation which is mediated by fimbriae, while swarming motility is used for the dispersal of biofilm cells. In the current study, planktonic cells possessed swimming motility i.e. had high biofilm forming efficiency (shown in table-5). Whereas, once the biofilm is formed, cells have poor swimming motility but dominant swarming motility. This swarming motility helps the biofilm cells to disperse as planktonic cells / biofilm associated planktonic cells. These cells can move from one place to another and can form biofilm (Table 6). Similar findings with respect to motility has been reported by Murray *et al.*, 2010; Caiazza *et al.*, 2007.

On the basis of above analysis, it was confirmed that all the five *P. aeruginosa* strains were multiple drug resistant biofilm forming pathogens. Thus, the infection caused by such bacteria can be fatal. *P. aeruginosa* have evolved resistance for currently available antimicrobials (Bentzmann and Plésiat, 2011). Therefore, for control of such biofilm forming pathogens, other strategies have to be used.

Phage therapy has been used since long to control the pathogenic infections in the wounds more effectively than most of the antimicrobials as evident from the review and therefore was used for control of pathogenic strains (test strains) of *Pseudomonas aeruginosa* in the present study.

Bacteriophages are an alternate to antibiotics, which can control microbial growth. Several studies have shown about the potential of phages to treat infectious diseases in animals (Loc, 2005; McVay *et al*., 2007; Nakai and Park, 2002; Wagenaar *et al*., 2005) and humans (Weber *et al*., 2001), even those caused by antibiotic resistant bacteria (Weber *et al*., 2003). Phages arose as a good alternative of antibacterial agents to cure such type of pathogenic bacteria. Phages are obligate parasite of bacteria and they multiply along with lysis of their host. Thus, it can be an ideal strategy for the control of microorganisms.

Thus, for the control of biofilm forming multiple drug resistant *P. aeruginosa* strains, phages were isolated from Ganges river and sewage water form hospital and

Panki sewage treatment plant, Kanpur, Uttar Pradesh, India in the year 2012. In all, six different lytic phages were isolated and their host range was determined using spot assay against various hosts. Six lytic *P. aeruginosa* specific phages were found and named as P1, P2, P3, P4, P5 and P6 phages. Phages had ability to increase in number during the infectious process, and this property make them excellent diagnostic and therapeutic agent for fighting against bacterial diseases. According to Adam, 1959, appearance of big clear plaques is characteristic feature of lytic virulence phages, and in the present study, all the isolated phages exhibited clear plaque activity (figure-7.

Most of the phages are restricted by their host and the defense mechanism includes adsorption resistance (which results in reduced interaction between phage and the bacterium) and restriction including abortive infection. In restriction, bacteria (host) survive while the phages die. In case of abortive infection, both bacteria and the phages die. Adsorption resistance includes loss of phage receptor molecules on hosts as well as physical barriers hiding receptor molecules (e.g. capsules). Restriction mechanisms include phage-genome uptake blocks, superinfection immunity, restriction modification, and CRISPR (cluster regulatory interspaced palindromic repeats). CRISPR regions have been implicated in bacterial resistance to lytic bacteriophage infection (Barrangou *et al.,* 2007; Sorek *et al.,* 2008). Barrangou and colleagues recently described the association of acquired immunity to a lytic bacteriophage infection with the presence of DNA sequences derived from the infecting phage in the spacers of CRISPRs and furthermore demonstrated that mutations in those spacers resulted in a loss of phage resistance (Barrangou *et al.,* 2007). They also found that mutation of certain cas genes resulted in the loss of resistance to lytic phage in previously resistant strains, while mutations in different cas genes produced strains that were unable to acquire phage resistance (Barrangou *et al.,* 2007). The mechanism by which phage DNA is incorporated into CRISPR spacers, and how these spacers confer resistance to infection by the same phage, have not been established (Hyman and Abedon, 2010; Zegans *et al.,* 2009; Barrangou *et al.,* 2007). Due to these mechanisms, phages are specific for their host.

In the present study, Phage P1 and P2 had host lytic activity against gram positive bacteria *S. epidermidis* and *S. arlettae* respectively. Hence phage P1 and P2 exhibited broad host range.

Growth characteristic of phages were determined by one step growth curve as well as by adsorption rate, shown in figure-8 and 9. The burst size was determined by one step growth curve which determines the average number of phage progeny that will be released per infected cell at the end of one infection cycle. According to Wang *et al*., (1996), phage which has longer generation time has large burst size. But a good phage is that which have short generation time and large burst size, feature is responsible for high efficiency to infect host cells frequently. In the present study, phage P1 had the large burst size 314 pfu/infected cell, while phage P4 had the smallest burst size 153 pfu/infected cell (figure-8). Adsorption of phage to the host is the first event in the infection process. Here in, irreversible adsorption rate was studied and it was found that phage P2 has minimum absorption time of 6 min while rest of the phages had absorption time up ranging from >6 to 10 min (fig. 9a-e).

P. aeruginosa is an euritherm organism that can grow in temperature range from 10 to 44 ^{o}C, with an optimum around 35 ^{o}C (Pitt and Simpson, 2006). With respect to physic-chemical activity of phages, Knezevic *et al*., (2011) reported that phages can absorb to *P. aeruginosa* in a temperature range from 7- 44 ^{o}C. In the present study, multiplication of phages was observed at different temperature ranging from 37 to 95 ^{o}C. Phage P1, P3, P4 and P6 maintained their multiplication at 37, 55, 75 and 95 ^{o}C, while phage P2 and P5 were unable to sustain beyond 75 ^{o}C (Figure-10). If we look for pH stability of phages, then it was observed that all phages were stable at pH 9 except phage P5. Present findings with respect to growth at neutral to alkaline pH are in conformity to the earlier findings of Adams, 2009, Amin and Day, 1988 except in case of phage P1 and P3 in the present study. Phages P1 and P3 were able to survive at pH 3 (figure-11).

Capsid protein analysis of isolated phages was undertaken to detect the types of protein present in the phages to protect themselves from the environmental stresses. SDS PAGE data shows that the proteins carried by the test phages ranged from 14.3 Kd to ~45Kd as shown in figure-12. Various workers have reported different sized proteins in the phages, Kumari *et al.,* 2007 reported 20kDa to 205kDa proteins in *P. aeruginosa* specific phages, while Radhakrishnan *et al.,* 2012 reported phage proteins ranged from 14 to 97 kDa.

Restriction digestion of isolated phages was done to determine the similarity between different phages. Although the gel figure is not very clear but it depicts different banding pattern leading to conclusion that the isolated/test bacteriophages are different with different properties (figure- 13).

Morphological characteristics of phage are usually seen under the electron microscope and these observations are very important to classify the taxonomy of a phage (Ackermann, 1996). Approximately 96% of all investigated phages in the last 45 years have come in the members of *Siphoviridae*, the *Myoviridae,* and *Podoviridae* (Ashelford *et al.,* 1965). Thus it was not surprising that isolated phages come in the categories of these three morphological families. In the present study, head and tail dimension of Phage P1 closely resembled *Podoviridae* family, Kumari *et al.,* 2009 reported the similar type of *Pseudomonas* specific phages in their study, while rest of the isolated phages belonged to *Myoviridae* family according to Ackerman, 1996 classification of phages, because all the phages exhibited long tail (figure-14).

On the basis of above characterization of phages, it is confirmed that all the phages are highly stable and have high potential to control their host *P. aeruginosa*. Therefore, these phages were further subjected against the biofilm formation of their hosts i.e. the interaction studies.

For the control of biofilm formation of *P. aeruginosa* isolates, ciprofloxacin antibiotic was used because it is a low molecular antibiotic and can penetrate matrix of biofilm (Nesar *et al*., 2012). Nesar *et al.,* 2012 reported about the emergence of resistance to fluoroquinolones in *P. aeruginosa* clinical isolates. In the present study, different concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.5, 0.75 µg/ml) of ciprofloxacin which is commonly used in practices for the control of pathogens were used for the screening of dose of antibiotic. From this experiment, it was clear that high concentration of ciprofloxacin (100 µg/ml) can control the biofilm at some level but as the concentration of antibiotic decreases, persistence of biofilm cells increases. Thus, current data about the antibiotic mediated control of biofilm illustrate that currently available commercial antibiotics cannot remove biofilm completely and discovery of new antibiotic is still under pipeline. Therefore for the control of pathogens, phage therapy can be employed as they are effective and are from natural sources and environment friendly.

Bacteriophage emerges as an ideal antimicrobial agent for the control of biofilm formation. Phages are obligate parasite of bacteria and they are host specific in nature. Hence, keeping this phenomenon in mind, the isolation and characterization of phages were performed.

In addition to characterization, phages were further subjected to study the control of biofilm formation of *P. aeruginosa* strains by quantitative (MTT assay) and qualitative (SEM and TEM) tests.

When Phages were applied on initial stage of biofilm formation of host, it was found that Phage P1 had high efficiency to inhibit biofilm formation up to 94% in case of all the five isolates while rest of the phages though inhibited biofilm formation but the percentage of inhibition was less as compared to phage P1 as shown in figure-18 a-e. According to Pires *et al*., 2011, 50% inhibition of biofilm formation of *P. aeruginosa* PA01 biofilm with bacteriophage was found, while Alves *et al*., 2015 reported 95% killing of biofilm cells of *P. aeruginosa* with 10 MOI of phage cocktail. Knezevic *et al*., 2011 reported 95% killing of *P. aeruginosa* biofilm as well as planktonic cells with 10 MOI of isolated bacteriophage. However, in this study killing of biofilm cell was achieved by just 0.01MOI of phage, which is a very low concentration, which can be effectively used for killing of biofilm cells.

Furthermore, the Scanning electron microscopy (SEM) of biofilm treated with P1 was done. P1 phage is a very potent phage that has high efficiency to inhibit biofilm formation of isolates at any stage with a very low MOI 0.01. Hence, to see the impact of P1 phage on the biofilm, scanning electron microscopy was performed to study the interaction. From the SEM images it is absolutely clear that P1 can remove biofilm cells of *P. aeruginosa* strains as shown in figure-19 a-e.

In addition to SEM, transmission electron microscopy of Phage P1 treated Ps.5 isolate was done. This experiment shows that how bacteriophage attacks the host cell, for adsorption and after completion of lytic life cycle, lysis of the host cells take place (figure-20-22). No one has reported this type of study till date. Here, only one host and only one phage was used for the study, to confirm the above findings of SEM for the adsorption of phage on the host, its multiplication and lysis of the host cell.

There are two strategies employed bacteriophage in fighting biofilm, which are by blocking of biofilm development and eradication and removal of existing biofilm cells by using enzymes dispersinB, depolymerase and alginase for the degradation of EPS of biofilm (Yan et al., 2013; Harper et al., 2014). The most advantageous feature of bacteriophage are that they are host specific self replicating entities, once administered they self replicate in the host and destroyed them (Parasion et al., 2014).

Conclusion

CHAPTER 7 CONCLUSIONS

Antibiotic resistant bacteria have evolved before the discovery of antibiotics, since then resistant bacteria have been reported worldwide. There are six bacteria ESKAPE (*E. coli*, *S. aureus*, *Klebsiella*, *Acinetobacter*, *P. aeruginosa*, *Enterobacter*) identified as multiple drug resistant bacteria reported by CDC (Center for disease control and preservation). These bacteria are highly pathogenic and cause fatal diseases. Therefore, *P. aeruginosa* was chosen for the current study. In the present study, 5 Multiple drug resistant *Pseudomonas* bacteria were isolated and characterized. All the isolated bacteria were drug resistant biofilm forming pathogens. Ps.5 *P. aeruginosa* was highly resistant to the fourth generation moxifloxacin. When MIC test was performed with ciprofloxacin that it was found that MIC of biofilm of isolates (Ps.1, Ps. 2, PS.3, Ps.4 and Ps.5) was greater than 100µg/ml. For the control of such fourth generation drug resistant bacteria another strategy was employed which was the use of lytic bacteriophage for the control of multiple drug resistant bacteria.

In this study, 6 bacteriophages (P1, P2, P3, P4, P5 and P6) were isolated from Ganges river water, sewage water from sewage treatment plant Panki and hospital drainage system around the Kanpur city. All phages had very good lytic activity. Bacteriophages were morphologically characterized to determine the shape, on the basis of electron microscopy it was confirmed that all phages were tailed phages with icosahedral head and belonged to *Pyoviridae* and *Myoviridae* family. Furthermore, bacteriophages were characterized to check their efficiency, after characterization, it was confirmed that phages were highly stable at different pH and temperature and have high burst size. Phage P1 gave best efficiency at 95 °C and was functional in both acidic and alkaline pH.

Isolated phages were used to test their efficacy for inhibition of biofilm formation by *P. aeruginosa* strains and it was found that all the phages caused killing of biofilm cells with phage P1 giving the maximum killing of all the host upto 94% at 0.01MOI. Hence, this study illustrates how bacteriophage therapy can be an effective technique for

control of MDR strains and inhibition of their biofilm forming ability which confers upon them more stability or resistance.

Due to limitation of discovery of new antibiotics, phage therapy can be a good choice for clinicians. Phage therapy can be a better choice than that of any antimicrobial because it is pathogen specific, self replicating, ecofreindly, economical and without any side effect. Phages have the potential to control the biofilm of multiple drug resistant bacteria. Thus, phages hold ideal position in the medical world for the control of drug resistant bacteria.

Summary

Summary

Pseudomonas aeruginosa is a motile, Gram negative, facultative, rod-shaped bacterium resistant to high concentration of salts and dyes, and many commonly used antibiotics. *P. aeruginosa* produces two types of soluble pigments, pyoverdin, and pyocyanin. Pyocyanin plays a vital role in iron metabolism and is produced in abundance in low-iron content media.

P. aeruginosa produces cell-surface polysaccharides which serve as a barrier between the cell wall and the environment, mediates host-pathogen interactions, and form structural components of biofilm. Lipopolysaccharide (LPS) plays a central role in maintaining the integrity of cell and acts as an important mediator of host pathogen interactions during disease. *P. aeruginosa* is a nosocomial pathogen and is described as an opportunistic human pathogen which can evade host defense to initiate infection. It causes infection in immune-compromised host such as those with cystic fibrosis, cancer, or AIDS and produces endocarditis, respiratory infections, bacteremia, septicemia, infections in central nervous system, ear and eye, bone and joints, urinary tract, gastrointestinal, and skin and soft tissues.

P. aeruginosa is classified as a dangerous pathogen because it is resistant to therapeutics. The resistance in *P. aeruginosa i*s due to the impermeability of its outer membrane to antibiotic penetration; its ability to form an exopolysaccharide protected (EPS) biofilm, the presence of multidrug efflux pumps in the cytosol, and chromosomally encoded antibiotic resistance genes. In addition to these intrinsic resistance factors, *P. aeruginosa* can easily acquire resistance by mutation of its chromosomally encoded genes or through horizontal gene transfer of antibiotic resistance determinants from plasmids. Recent studies have shown that phenotypic resistance associated with biofilm formation and the emergence of small-colony variants also may be important factors in its development of resistance. Thus, for the control of such pathogens, more specific, economical, and environment friendly therapy is required for management of this pathogen and the infections caused by it. Phage therapy, which has been used since long can be an effective

therapy against this MDR pathogen and other pathogens which are becoming resistant due to injudicious use of antibiotics.

Phages are obligate parasite that infects bacteria. Advantages of phage therapy include continuous self replication at the site of infection and host specificity that leaves other bacterial and body cells undisturbed. Some phages have depolymerases as tail or spike fibers and this enables them to reach the bacterial cell wall when an EPS coating is present. Through their interaction with host cells, phages may cause biofilm disruption, lysis, and degradation of the EPS. Phage therapy is potentially broad in its application and can be particularly important in the treatment of bacterial infections that display multidrug resistance. It is currently being used in the food industry for the prevention of poultry infection, wound healing therapy, allergy prevention therapy, and in the control of opportunistic bacterial infections present in immuno-compromised mice.

The current research work entitled "**Isolation and characterization of host specific bacteriophages and determination of their potency to inhibit multiplication of multiple drug resistant (MDR) biofilm forming *Pseudomonas aeruginosa* isolates**" was carried out by isolation of host *P. aeruginosa* and their characterization. Isolation of host bacteria was done from the patient with burn wound infections. During the isolation 12 bacteria isolates were isolated from the samples collected from patients. Morphological characterization of 12 isolates was done by gram staining and from gram staining data it was clear that all the isolates were gram negative.

After morphological characterization, antibiotic sensitivity test was performed and this test determined that from 12 isolates only 5 isolates were multiple drug resistant. Therefore, further study was continued with these 5 multiple drug resistant isolates numbered as Ps.1, Ps.2 Ps.3, Ps. 4, and Ps. 5. Biochemical tests were performed for the identification of 5 multiple drug resistant bacteria. 16S rRNA sequencing was performed for further confirmation of characterized isolates. 16S rRNA sequencing data illustrated that all 5 isolates were *P. aeruginosa.*

Apart from the identification of *P. aeruginosa* strains, pathogenicity traits were also determined by hemolysis test on blood agar, by congo red binding assay and by biofilm formation assay. Blood agar test determined that all five strains Ps.1, Ps.2, Ps.3, Ps.4 and

Ps.5 had β hemolytic activity, and this can be fatal to those who come in contact with such microbes. Congo red binding assay was done for the detection of presence of amyloid fiber. Curli proteins are matrix-associated proteins which are assembled in to the fiber known as amyloid fiber. Amyloids contribute to the attachment of bacteria to surfaces and the raising of aerial structures, and they have a role in pathogenic processes such as adhesion to host cells and induction of toxicity to host cells. Amyloid protein has been shown to be an important functional component of a biofilm matrix. These amyloid fibers render resistance to biofilm embedded cells to environmental attacks such as proteolysis as well as the ability to bind certain dyes for example congo red, and this property has been commonly exploited to detect amyloids *in vitro* and *in vivo*. In the present study, Congo red binding assay elucidates that all the five strains have very high content of amyloid fiber. Biofilm is a complex community of microorganisms and it provides protection to the cells from the environmental conditions. Therefore biofilm forming capability of *P. aeruginosa* strains was analyzed by the crystal violet assay and MTT assay and these both tests determined that all the five *P. aeruginosa* were high biofilm former. Furthermore, motility test was also performed for the assessment of presence of motility factor responsible for the biofilm formation. Herein, swimming and swarming motility test was performed. Motility test described that planktonic cells of all five strain have very good swimming motility while biofilm cells have very good swarming motility. Swimming motility is responsible for the formation of biofilm, while swarming motility is responsible for the dispersal of biofilm. By this experiment it was clear that planktonic cells had the capability to form the biofilm by using swimming motility while biofilm cells had capability to disperse by swarming motility factor and further forms biofilm at other place. Above analysis about the *P. aeruginosa* strains illustrate that all the five strains are multiple drug resistant biofilm forming pathogens and they cannot be controlled with 4^{th} generation of antibiotics because biofilm is 100-1000 more resistant for the commercial antibiotics.

Thus for the control of such pathogenic bacteria, phage therapy was employed. For the isolation of bacteriophage, Ganges river water, which has been shown to possess good antimicrobial properties and phages specific to *Vibrio* was selected for isolation of phages, while sewage water from hospital waste was selected so that the chances of isolating

phages parasitic to drug resistant bacterial hosts are more. In all, six bacteriophages named P1, P2, P3, P4, P5, and P6 were isolated from the collected samples of Kanpur, India as discussed above. These phages had a good lytic activity for their host *P. aeruginosa.* Isolated phages were further purified by single plaque isolation method and their concentration was defined by titration of phages. Phage P4 and P5 had highest titer while rest of phage had low titer. Furthermore, host range was also analyzed for the determination of infectivity range of phages for the variable hosts, and this test illustrated that all phages had infectivity for gram negative bacteria while phage P1 and P2 had lytic activity for *Staphylococcus epidermidis* and *Staphylococcus arlettae* respectively. Characterization of isolated phages was done by one step growth curve, adsorption assay, temperature and pH stability test, SDS PAGE analysis of their proteins and by electron microscopy. One step growth curve determined that phage P1 had large burst size ~ 314 pfu/infected cells, while phage P2, P3, P4, P5 and P6 had the burst size ~ 228, 226, 153, 289 and 275 pfu/infected cell respectively. Adsorption test determined that phage P2 had the minimum adsorption time i.e. 6 minutes while phage P5 and P6 have 8 minutes and rest phages have 10 minutes adsorption time. Temperature stability test concludes that phage P1, P3, P4 and P6 had high potential to multiply at high temperature 55, 75 and 95 ^{o}C while phage P2 and P5 were unable to maintain their multiplication at 95 ^{o}C. pH sensitivity data explained that phage P1 was able to maintain its multiplication at variable pH range (3, 5, 7 and 9) while rest of phages were unable to survive in acidic pH and could survive at neutral to alkaline pH. SDS PAGE analysis determined that all the phages posses proteins with variable molecular weight. This variability in protein provides the evidence for survival of phages in varied environments from which they were isolated. Phage DNA analysis explained that all the phages possesed different molecular weight DNA which accounts for protection in harsh environmental conditions. Electron microscopy was done for the determination of phage morphology. In this study phage electron micrograph revealed that all the isolated phages were the tailed phages with icosahedral head and all the phages belonged to two different families with phage P1 to *Pyoviridae* because it had short tail while phage P2, P3, P4, P5 and P6 to *Myoviridae* family because they had long tail.

After characterization of phage it was confirmed that all the phages were very potent phages with high lytic activity to their host and therefore these phages were applied for the control of biofilm formation of their host *P. aeruginosa.* As it was mentioned in the earlier section that *P. aeruginosa* strains were multiple drug resistant biofilm forming pathogens and their biofilm cannot be controlled with commercial available antibiotics with routine dose of antibiotics. In this study, the effect of different range of ciprofloxacin antibiotic for the control the biofilm formation of *P. aeruginosa* strains was also examined and it was found that high dose (100 µg/ml) of ciprofloxacin although reduced the biofilm formation to some extent but could not completely inhibit it, while with the decreasing antibiotic concentration of ciprofloxacin, killing efficiency of antibiotic reduced. Hence, isolated phage were employed for the control of biofilm formation and it was found that phage P1 had high potential to control of biofilm formation of all the strains up to 94% while rest of the phage had the potential to control the biofilm formation with maximum upto 92% and that too varied from host to host. Thus this data suggests that Phage P1 had high potential to control the biofilm formation better than the other phages.

Overall, this study concludes that *P. aeruginosa* is an opportunistic pathogen and has got ability to form biofilm which cannot be controlled effectively even by advance antimicrobials, but phage therapy in low doses of phages can control these pathogenic strains by producing enzymes such as depolymerase, dispersin B and alginase for the degradation of biofilm matrix, which confers on them more resistance. The current study illustrates that bacteriophages can be an ideal alternative of antibiotics in controlling the pathogens and their biofilm. Phage therapy is devoid of side effects, economical and environment friendly.

References

Abate, G., Mashana R.N., Miorner, H. 1998. Evaluation of a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for the rapid detection of rifampicin resistance in *M. tuberculosis* . *Int J Tuber Lung Dis.*, 2:1011-1016.

Abdul-Hassan, H.S., El-Tahan, K., Massoud, B., Gomaa, R. 1990. Bacteriophage therapy of *Pseudomonas* burn wound sepsis. *Ann Med Burn Club*., 3:262-4.

Abedon, S.T., Kuhl, S.J., Blasdel, B.G., and Kutter, E.M. 2011. Phage treatment of human infections. *Bacterio.*, 1:66-85.

Abuladze, T., Li, M., and Marc, Y. 2008. Bacteriophages reduce experimental contamination of hard surface: Tomato, Spinach, Broccoli and ground beef by *E.coli* 0157:H7. *Appl Environ Microbio*., l74: 6230–6238.

Ackermann, H.W. 1996. Frequency of morphological phage descriptions in 1995. *Arch Virol*., 141: 209 – 218.

Ackermann, H.W. 2003. Bacteriophage observations and evolution. *Res Microbiol*., 154:245–251.

Ackermann, H.W. 2007. 5500 phages examined in the electron microscope. *Arch Virol.*, 152:227–243.

Ackermann, H.W., DuBow, M.S., 1987. Viruses of prokaryotes, *CRC Boca Raton* 143–172

Adams, M. 1959. Bacteriophages. *Interscience N.Y.* 137–159.

Aiello, A.E., King, N.B., Foxman, B. 2006. Ethical conflicts in public health research and practice: antimicrobial resistance and the ethics of drug development. *Am J Public Health*, 96:1910-1914.

Aires, J.R., Köhler, T.H., Nikaido, H., Plésiat, P. 1999. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicro Agents Chemother*., 43: 2624–2628.

Alves, D.R., Perez-Esteban, P., Kot, W. , Bean, J.E., Arnot, T., Hansen, L.H., Enright, M.C., Tobias, A., Jenkins, A. 201. 5A novel bacteriophage cocktail reduces and disperses *Pseudomonas aeruginosa* biofilms under static and flow conditions. doi:10.1111/1751-7915.12316

Amin, M.K., Day, M.J. 1988. Influence of pH value on viability and transduction frequency of *Pseudomonas aeruginosa* phage F116. *Lett Appl Microbiol.*, 6: 93–96.

Anderson, G.G., Dodson, K.W., Hooton, T.M., Hultgren, S.J. 2004. Intracellular bacterial communities of uropathogenic *Escherichia coli* in urinary tract pathogenesis. *Trends Microbiol.*, 12: 424–430.

Arora, S.K., Ritchings, B.W., Almira, E.C., Lory, S., and Ramphal R. 1998. The *Pseudomonas aeruginosa* flagellar cap protein, FliD, is responsible for mucin adhesion. *Infect Immun.*, 66:1000-7.

Ashelford, K.E., Norris, S. J. , Fry, J.C., Bailey, M.J., Day, M.J. 2000. Seasonal population dynamics and interactions of competing bacteriophages and their host in the rhizosphere. *Appl Environ Microbiol.*, 66:4193-4199.

Atterbury, R.J. 2009. Bacteriophage biocontrol in animals and meat products. *Microb Biotechnol*., 2:601-612.

Atterbury, R.J., Van Bergen, M.A.P., Ortiz, F., Lovell, M.A., Harris, J.A., De Boer, A., Wagenaar, J.A., Allen, V.M., Barrow, P.A., Fiorentin, L., N.D., Vieira, Barioni, W. 2005. Oral treatment w bacteriophages reduces the concentration of *Salmonella* Enteritidis PT4 in caecal contents of broilers. *Avia Pathol.*, 34:258-263.

Balogh, B., Canteros, B.I., Stall, R.E., Jones, J.B. 2008. Control of citrus canker and citrus bacterial Spot with Bacteriophages. *Plant Dis*., 92:1048–1052.

Balogh, B., Jones, J.B., Iriarte, F.B., and Momol, M.T. 2010. Phage therapy for plant disease control. *Curr Pharmac Biotechnol.*, 11(1):48–57.

Banin, E., Vasil, M.L., Greenberg E.P. 2005. Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci USA.*, 102: 11076–11081.

Barbara, G., Stanghellini, V., Berti-Ceroni C. *et al.*, 2000. Role of antibiotic therapy on long-term germ excretion in faeces and digestive symptoms after *Salmonella* infection. *Aliment Pharmacol and Therap.*, 14(9):1127–1131.

Barrangou R., *et al.*, 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Sci.*, 315:1709–1712.

Barraud, N., Storey, M.V., Moore, Z.P., Webb, J.S., Rice, S.A. and Kjelleberg, S. 2009. Nitric oxide-mediated dispersal in single- and multi-species biofilms of clinically and industrially relevant microorganisms. *Microb Biotechnol*., 2:370–378.

Bassler, B.L. 2002. Small talk. Cell-to-cell communication in bacteria. *Cell,* 17;109:421-424.

Bassler, B.L., Wright, M. and Silverman, M.R. 1994. Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Mol Microbiol.*, 13:273–286.

Bassler, B.L., Wright, M., Showalter, R.E., and Silverman, M.R. 1993. Intercellular signaling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Mol Microbiol*., 9:773–786.

Bentzmann S., and Plésiat P. 2011. The *Pseudomonas aeruginosa* opportunistic pathogen and human infections. *Environ Microbiol.*, 13:1655–1665

Betzner, A.S., Ferreira, L.C., Höltje, J.V., and Keck, W.1990. Control of the activity of the soluble lytic transglycosylase by the stringent response in *Escherichia coli*. *Lett FEMS Microbiol*., 55: 161–164.

Bitter, W. 2003. Secretions of *Pseudomonas aeruginosa*: large holes in the outer membrane. *Arch Microbiol.*, 179:307-14.

Bjedov, I., Tenaillon, O., Gerard, B., Souza, V., Denamur, E., Radman M., Taddei F., Matic I. 2003. Stress-induced mutagenesis in bacteria. *Sci.*, 300:1404–1409.

Blazquez, J., Couce, A., Rodriguez-Beltran, J., Rodriguez-Rojas, A. 2012. Antimicrobials as promoters of genetic variation. *Curr Opin Microbiol.*, 15:561–569.

Bodey, G.P., Bolivar, R., Fainstein, V., and Jadeja, L. 1983. Infections caused by *Pseudomonas aeruginosa*. *Rev Infect Dis*., 5**:**279-313.

Boles, B.R., Thoendel, M., and Singh, P.K. 2004. Self-generated diversity produces "insurance effects" in biofilm communities. *PNAS*., 103:166330-16635.

Boles, B.R. and Horswill, A.R. 2008. Agr-mediated dispersal of *Staphylococcus aureus* biofilms. *PLoS Pathog*., 4(4): 4e1000052.

Borie, C., Albala, I., Sanchez, P., Sanchez, M.L., Ramirez, S., Navarro, C., Morales M.A., Retamales, J., Robenson, J. 2008a. Bacteriophage treatment reduces *Salmonella* colonization of infected chickens. *Avian Dis.*, 52:64-67.

Borie, C., Hauva, C., Quiroga, J., Bravo, V., Retamal, P., Retamales, J., and Robeson. J. 2011. Uso de bacteriofagos en gallinas de postura infectadas con *Salmonella enterica* serotipo Enteritidis: prevencion de la colonizacion intestinal y reproductiva. *Arch Med Vet*., 43:85-89.

Borie, C., Zurita, P., Sanchez, M.L., Rojas, V., Santander, J., Robeson, J. 2008b. Prevencion de la infeccion por *Salmonella enteric* subespecie *enterica* serotipo Enteritidis (*Salmonella* Enteritidis) en pollos mediante un bacteriofago. *Arch Med Vet.*, 40:197-201.

Bridier, A., Briandet, R., Thomas, V., and Dubois-Brissonnet, F. 2011. Resistance of bacterial biofilms to disinfectants: a review. *Biofouling*., 27:1017-1032.

Brown, M.R., Allison, D.G. and Gilbert, P. 1988. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *J Antimicrob Chemother*., 22:777–780.

Bruynoghe, R., and Maisin, J. 1921. Essais de therapeutique au moyen du bacteriophage du Staphylocoque. *Compt Rend Soc Biol*., 85:1120-1.

Bryan, L.E. 1988. General mechanisms of resistance to antibiotics. *J Antimicrob Chemother*., 22 Suppl A:1-15.

Busscher, H.J., Bruinsma, G., Weissenbruch, R.V., Leunisse, C., vander Mei, H.C., Dijk F, Albers, F.W. 1998. The effect of buttermilk consumption on biofilm formation on silicone rubber voice prostheses in an artificial throat. *Eur Arch Otorhinolaryngol*., 255:410–413.

Carmeli, Y., Troillet, N., Karchmer, A.W., and Samore, M.H. 1999. Health and economic outcomes of antibiotic resistant *Pseudomonas aeruginosa*. *Arch Intern Med.*, 159:1127-1132.

Carrillo, C.L., Atterbury, R.J., El-Shibiny, A. *et al.,* 2005. Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. *Appl and Environ Microbiol*., 71(11):6554–6563.

Carvalho, C.M., Gannon B.W., Halfhide, D.E. *et al.,* 2010. The in vivo efficacy of two administration routes of a phage cocktail to reduce numbers of Campylobacter coli and *Campylobacter jejuni* in chickens. *BMC Microbiol*., 10:232.

Cashel, M., Gentry, D.R., Hernandez, V.J., and Vinella, D. 1996.*Escherichia coli and Salmonella*: Cellular and Molecular Biology, Washington, D.C., pp. 1400–1416.

Cerveny, K.E, DePaola, A., Duckworth, D.H., Gulig, P.A. 2002. Phage therapy of local and systemic disease caused by *Vibrio vulnificus* in iron-dextran-treated mice. *Infect Immun.,* 70:6251-62.

Chang, H.C., Chen, C.R., Lin, J.W., Shen, G.H., Chang, K.M., Tseng, Y.H., *et al.,* 2005. Isolation and characterization of novel giant *Stenotrophomonas maltophilia* phage ΦSMA5. *Appl. Environ Microbiol.,* 71:1387–1393.

Chatterjee, S, Rothenberg, E. 2012. Interaction of bacteriophage l with its *E. coli* receptor, Lam B. *Virus.,* 4:3162-3178.

Chen, X., and Stewart, P.S. 1996. Chlorine penetration into artificial biofilm is limited by a reaction-diffusion interaction. *Environ Sci Technol*., 30:2078-2083.

Chopra, I, O'Neill, A.J., Miller, K. 2003. The role of mutators in the emergence of antibiotic resistant bacteria, *Drug Resist Update.*, 6:137–145

Chopra, I., Hodgbon, J., Metcalf, B., Porte, G., 1996. New approaches to the control of infections caused by antibiotic-resistant bacteria: An industry perspective. *J Am Med Assoc*10: 401-4.

Chopra, I., O'Neill, A.J., Miller, K. 2003. The role of mutators in the emergence of antibiotic resistant bacteria. *Drug Resist Update*, 6:137–145.

Clokie, M.R.J., Kropinski, A. 2009. Isolation, Characterization, and Interactions Protocols: Methods and Protocols Springer 1.

Collet, A., Vilain, S., Cosette, P., Junter, G.A., Jouenne, T., Phillips, R.S., Di Martino, P. 2007. Protein expression in *Escherichia coli* S17-1 biofilms: impact of indole. *Antonie van Leeuwenhoek.*, 91:71-85.

Commission decision of 30 January 2004 concerning thenon-inclusion of certain active substances in Annex I toCouncil Directive 91/414/EEC and the withdrawl of authorizationsfor plant protection products containing thesesubstances," Official Journal of the European Union, 2004,http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:037:0027:0031:EN:PDF

Cooksey, D.A. 1990. Genetics of bactericide resistance in plant pathogenic bacteria. *Annu Rev of Phytopathol*., 28:201–219.

Costerton, J.W. 1999. Introduction to biofilm. *Int J Antimicrob Agents*, 11: 217-221.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., Marrie, T.J. 1987. Bacterial biofilms in nature and disease. *Annu Rev Microbiol.*, 41: 435-464.

Costerton, J.W., Geesey, G.G., Cheng, K.J. 1978. How bacteria stick? *Sci Am*., 238:86-95.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Scott, L.H.M. 1995. Microbial biofilms. *Annu Rev Microbio*l., 49:711–745.

Costerton, W., Veeh, R., Shirtliff, M., Pasmore, M., Post, C., Ehrlich, G. 2003. The application of biofilm science to the study and control of chronic bacterial infections. *J Clin Invest.*, 112:1466-1477.

d'Herelle, F. 1924. Drie Voordrachten over het Verschijnsel der Bacteriophagie. Groningen: J.B. Wolters.

d'Herelle, F. 1926. Le Bacteriophage et son Comportement. Paris: Masson et Cie.

d'Herelle, F. 1929. Etudes sur le Cholera. Alexandrie: Impr A. Serafini.

d'Herelle, F. 1931. Bacteriophage as a treatment in acute medical and surgical infections. *Bull N Y Acad Med*; 7:329-48.

d'Herelle, F. 1933. Le Bacteriophage et ses Applications Therapeutiques. Paris: Doin.

d'Herelle, F. 1938. Le Phenomene de la Guerison dans les Maladies Infectieuses. Paris: Masson et cie.

d'Herelle, F.1921. Le Bacteriophage: Son Role dans l Immunite. Paris: Masson et cie,.

d'Herelle, F.1922. Der Bakteriophage und seine Bedeutung fur die Immunitat; nach einem erweiterten und verbesserten. Braunschweig: F. Vieweg & Sohn.

d'Herelle, F.1923. Les Defenses de l'Organisme. Paris: Flammarion.

d'Herelle,F., Smith, G.H. 1930. The Bacteriophage and its Clinical Application. Springfield, IL: Charles C. Thomas, Publisher,.

Dalhoff, A. 2012. Global Fluoroquinolone Resistance Epidemiology and Implictions for Clinical Use.*Interdisciplinary Perspectives on Infectious Diseases*, vol. 2012, Article ID 976273, 37 pages.

Davey, M.E., Caiazza, N.C., and O'Toole, G.A. 2003. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol*., 185:1027-1036.

Davies, D. 2003. Understanding biofilm resistance to antibacterial agents. *Nat Rev Drug Disco*., 2: 114–122.

Davies, D.G., and Marques, C.N. 2009. A fatty acid messenger is responsible for inducing dispersion in microbial biofilms. *J Bacteriol*., 191:1393-403.

Davies, J., and Davies, D. 2010. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev*., 74: 417-433.

Day, W.A., Jr, and Maurelli, A.T. 2001. *Shigella flexneri* LuxS quorum sensing system modulates virB expression but is not essential for virulence. *Infect Immun.*, 69:15–23.

De Beer, D., Srinivasan, R., and Stewart, P.S. 1994. Direct measurement of chlorine penetration into biofilms during disinfection. *Appl Environ Microbiol*., 60:4339.

Dingman, J.R., Rayner, M.G., Mishra, S., Zhang, Y., Ehrlich, M.D., Post, J.C., and Ehrlich, G.D. 1998. Correlation between presence of viable bacteria and presence of endotoxin in middle-ear effusions. *J Clin Microbiol*.,36:3417– 3419.

Donlan, R., Costerton, J.W. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev.*, 15:167–193.

Donlan, R.M. 2001. Biofilms and device-associated infections. *Emerg Infect Dis*., 7: 277–81.

Donlan, R.M. 2002. Biofilms: Microbial life on surfaces. *EmergInfectDis.,* 8:881–890.

Donlan, R.M. 2000. Role of biofilms in antimicrobial resistance. *ASAIO J*., 46:S47–52.

Drulis-Kawa, Z., Majkowska-Skrobek, G., Maciejewska B., Delattre A.S., Lavigne R. 2012. Learning from bacteriophages - advantages and limitations of phage and phage-encoded protein applications. *Curr Protein Pept Sci.,* 13:699-722.

Dublanchet, A. 2009. Des Virus Pour Combattre les Infections: La Phagotherapie. Favre.

Dubnau, D. 1999. DNA uptake in bacteria. *Annu Rev Microbiol.,* 53:217–244.

Dufour, D., Leung, V.and Lévesque, C.M. 2012. Bacterial biofilm: structure, function, and antimicrobial resistance. *Endodontic Topics*., 22: 2–16.

Dye, D.W. 1953. Control of *Pseudomonas syringae* with streptomycin. *Nature*, 172(4380):683–684.

Eaton, M.D., and Bayne-Jones, S. 1934c. Bacteriophage therapy: Review of the principles and results of the use of bacteriophage in the treatment of infections (III). *J Am Med Assoc*.,103:1934-1939.

Eaton, M.D., Bayne-Jones, S. 1934a. Bacteriophage therapy: Review of the principles and results of the use of bacteriophage in the treatment of infections (I). *J Am Med Assoc*., 103:1769-1776.

Eaton, M.D., Bayne-Jones, S. 1934b. Bacteriophage therapy: Review of the principles and results of the use of bacteriophage in the treatment of infections (II). *J Am Med Assoc*; 103:1847-1853.

EFSA, 2011. "The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2009," *The Europ Food Saf Autt J*., 9(3) p. 2090.

Elder, M.J., Stapleton, F., Evans, E., and Dart, J.K.G. 1995. Biofilm related infections in ophthalmology. *Eye*., 9:102–109.

El-Shibiny, A., Scott, A., Timms, A., Metawea, Y., Connerton, P., and Connerton, I. 2009. Application of a group II campylobacter bacteriophage to reduce strains of *Campylobacter jejuni* and campylobacter coli colonizing broiler chickens. *J of Food Protec.*, 72(4):733–740.

Evans, S.J. and Sayers, A.R. 2000. A longitudinal study of campylobacter infection of broiler flocks in Great Britain. *Prevent Veterin Medic.*, 46(3):209–223.

Filho, R., Higgins, J., Higgins, S., Gaona, G., Wolfenden, A., Tellez, G., Hargis, G,B. 2007. Ability of bacteriophages isolated from different sources to reduce *Salmonella enterica* serovar Enteritidis *in vitro* and *in vivo*. *Poultry Sci.,* 86:1904-1909.

Fiorentin, L., Vieira, N.D., Barioni Júnior, W. 2005. Oral treatment with bacteriophages reduces the concentration of Salmonella Enteritidis PT4 in cecal contents of broilers. *Avian Pathol* ., 34:1-6.

Flemming, H.C., Wingender, J. 2001. Relevance of microbial extracellular polymeric substances (EPSs) – Part I: Structural and ecological aspects. *Water Sci. Technol.,* 43:1-8.

Foley, I., Gilbert, P. 1996. Antibiotic resistance of biofilms. *Biofoul.*,10:331–346.

Frampton, R.A., Pitman, AR., and Fineran, P.C. 2012. Advances in Bacteriophage-Mediated Control of Plant Pathogens. *Int J of Microbiol.,* Volume 2012, Article ID 326452, 11 pages.

Fridkin, S.K., and Gaynes, R. 1999. Antimicrobial resistance in intensive care units. *Clin Chest Med*., 20:303-316.

Fuhrman, J.A. 1999. Marine viruses and their biogeochemical and ecological effects. *Nature*, 399:541-8.

Fuhrman, J.A., and Noble, R.T. 1995. Viruses and protists cause similar bacterial mortality seawater. *Limnol Oceanogr.,* 40:1236-42.

Fung, C.P., Chang, F.Y., Lee, S.C., Hu, B.S., Kuo, B.I., Liu, C.Y., Ho, M., and Siu, L.K. 2002. A global emerging disease of *Klebsiella pneumonia* liver abscess: is serotype K1 an important factor for complicated endophthalmitis? *Gut*., 50: 420-424.

Fuqua, C., Parsek, M.R. and Greenberg, E.P. 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet*., 35:439–468.

Fuqua, W.C., Winans, S.C. 1994. A LuxR-LuxI type regulatory system activates Agrobacterium Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *J Bacteriol*., 176:2796–2806.

Furukawa, S., Kuchma, S.L., and O'Toole, G.A., 2006. Keeping their options open: acute versus persistent infections. *J Bacteriol*., 188: 1211–1217.

Ghigo, J.M. 2001. Natural conjugative plasmids induce bacterial biofilm development. *Nature*, 412:442-445.

Gilbert, P., Allison, D.G., McBain, A.J. 2002. Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? *Symp Ser Soc Appl Microbiol*., 98S-110S.

Gilligan, C.A. 2008. Sustainable agriculture and plant diseases: an epidemiological perspective. *Philosoph Transact of the Royal Soc B.,* 363(1492):741–759, 2008.

Gjermansen, M., Ragas, P., Sternberg, C., Molin, S., and Tolker-Nielsen, T. 2005. Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms. *Environ Microbiol*., 7:894–906.

Golkar, Z., Bagasra, O., Pace, D.G. 2014. Bacteriophage therapy: a potential solution for the antibiotic resistance Crisis. *J Infect Dev Ctries.*, 8(2):129-136.

Gonzalez, B.A.F., Zuo, R., Hashimoto, Y., Yang, L., Bentley, W.E., Wood, T.K. 2006. Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). *J Bacteriol*., 188:305-316.

Goodridge, L., Gallacci, A., Griffiths, M.W. 2003. Morphological, host range and genetic characterization of two coliphages. *Appl Environ Microbiol.*, 69:5364-5371.

Gorlin, A.I., Gabriel, M.M., Wilson, L.A., andAhearn, D.G. 1996. Effect of adhered bacteria on the binding of *Acanthamoeba* to hydrogel lenses. *Arch Ophthalmol*., 114:576–580.

Gougerot, H., and Peyre, E. 1936. Le bacteriophage dans la traitement des affectiones cutanees. *La Medecine*., 17:45-8.

Govan, J.R., and Deretic, V. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev*., 60:539-74.

Gristina, A.G., Dobbins, J.J., Giammara, B., Lewis, J.C., and DeVreies, W.C. 1988. Biomaterial-centered sepsis and the total artificial heart. *JAMA*.,259:870– 874.

Gullberg, E., Cao, S., Berg, O.G., Ilback, C., Sandegren, L., Hughes, D., Andersson, D.I. 2011. Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog.*, 7:e1002158.

Habash, M.B., Park, A. J., Vis, E.C., Harris, R.J., and Khursigara, C.M. 2014. Synergy of silver nanoparticles and aztreonam against *Pseudomonas aeruginosa* PAO1 biofilms. *Antimicrob agents and chemoth.*, 58:5818-5830.

Hancock, R.E., and Speert, D.P. 2000. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug Resist Updat*., 3:247-255.

Hanlon, W.G., Denyer, Ps., Olliff, J.C., and Ibrahim, J.L. 2001. Reduction in exopolysaccharide viscosity as an aid to bacteriophage penetration through *Pseudomonas aeruginosa* biofilms. *ASM*., 67: 2746-53

Haq, I.U., Chaudhry, W.N., Akhtar, M.N., Andleeb, S., Qadri, I. 2012. Bacteriophages and their implications on future biotechnology: a review. *Virol J.*, 9:9.

Harper, D.R., Parracho, H.M.R.T., Walker, J., Sharp, R., Werthén, H.G., Lehman, S., and Morales, S. 2014. Bacteriophages and Biofilms. *Antibio.*, 3:270-284.

Hausner, M., and Wuertz, S. 1999. High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Appl Environ Microbiol.*, 65:3710–3713.

Hendrix, R.W., Lawrence J.G., Hatfull, G.F., Casjens, S. 2000. The origins and ongoing evolution of viruses. *Trend Microbiol.*, 8:504–508.

Henke, J.M., and Bassler, B.L. 2004. Bacterial social engagements. *Trends Cell Biol.*, 14:648–656

Higgins,S.E., Higgins, J.P., Bielke, L.R., and Hargis, B.M.2007. Selection and application of Bacteriophages for Treating *Salmonella enteritidis* Infections in Poultry. *Int J Poult Sci*., 6:163-168.

Hisert, K.B., MacCoss, M., Shiloh, M.U., Darwin, K.H., Singh, S., Jones, R.A., Ehrt, S., Zhang, Z.Y., Gaffney, B.L., Gandotra, S., Holden, D.W., Murray, D., and Nathan, C. 2005. A glutamate-alanine-leucine (EAL) domain protein of *Salmonella* controls bacterial survival in mice, antioxidant defense and killing of macrophages: role of cyclic di-GMP. *Mol Microbiol*., 56:1234-1245.

Hocquet, D., Vogne, C., El Garch, F., Vejux A., Gotoh, N., Lee, A., Lomovskaya, O., and Plesiat, P. 2003. MexXY-OprM efflux pump is necessary for a adaptive resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob *Agents Chemother*., 47:1371-5.

Holland, S.P., Mathias, R.G., Morck, D.W., Chiu, J., and Slade, S.G. 2000. Diffuse lamellar keratitis related to endotoxins released from sterilizer reservoir biofilms. *Ophthalmology*, 107:1227-1233.

Holt, J.G., Krieg, N.R., Sneath, P.H. A., Staley, J.T., and Williams, S.T. 1994. Aerobic chemolithotrophic bacteria and associated organisms. *Bergey's manual of determinative bacteriology, 9th ed. Williams & Wilkins, Baltimore, USA*, 427-455.

Hong, S.H., Lee, J., and Wood, T.K. 2010. Engineering global regulator Hha of *Escherichia coli* to control biofilm dispersal. *Microb Biotechnol*., 3:717–728.

Huang, B., Whitchurch, C.B., and Mattick, J.S. 2003. FimX, a multidomain protein connecting environmental signals to twitching motility in *Pseudomonas aeruginosa*. *J Bacteriol*., 185:7068-7076.

Huff, W.E., Huff, G.R., Rath, N.C., Balog, J.M., and Donoghue, A.M., 2002. Prevention of *Escherichia coli* infection in broiler chickens with a bacteriophage aerosol spray. *Poul Sci.*, 8(10):1486–1491.

Hughes, K.A., Sutherland, I.W., Jones, M.V. 1998. Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiol-SGM.,* 144:3039-3047.

Humphrey, T., O'Brien, S., and Madsen, M. 2007. Campylobacters as zoonotic pathogens: a food production perspective. *Int J of Food Microbiol.,* 117(3):237–257.

Hunt, S.M., Werner, E.M., Huang, B., Hamilton, M.A., and Stewart, P.S. 2004. Hypothesis for the role of nutrient starvation in biofilm detachment. *ApplEnvironMicrobiol*., 70:7418-7425.

Hyde, J.A.J., Darouiche, R.O., and Costerton, J.W. 1998. Strategies for prophylaxis against prosthetic valve endocarditis: a review article. *J Heart Valve Dis*.,7:316–326.

Jahn, A., & Nielsen, P.H. 1998. Cell biomass and exopolymer composition in sewer biofilms. *Water Sci and Technol.,* 37:17–24.

Jayaraman, R. 2008. Bacterial persistence: some new insights into an old phenomenon. *J Biosci*., 33:795–805.

Jelsbak, L., Johansen, H.K., Frost, A.L., Thogersen, R., Thomsen, L.E., Ciofu, O. *et al.*, 2007. Molecular epidemiology and dynamics of *Pseudomonas aeruginosa* populations in lungs of cystic fibrosis patients. *Infect Immun*., 75: 2214-2224.

Jenal, U., and Malone, J. 2006. Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet.,* 40:385-407.

Jones, J.B., Jackson, L.E., Balogh, B., Obradovic, A., Iriarte F.B., and Momol, M.T., Bacteriophages for plant disease control. Ann Rev of Phytopathol., 45:245– 262.

Jones, J.B., Vallad, G.E., Iriarte, F.B., Obradović A., Wernsing, M.H., Jackson L. E., *et al.*, 2012.Considerations for using bacteriophages for plant disease control. *Bacteriophage,* 2:208–214.

Kalishwaralal, K., Barath, M.K.S., Pandian, S.R.K., Deepak, V., and Gurunathan, S. 2010. Silver nanoparticles impede the biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus* epidermidis. *Colloids Surf B.*, 79:340-344.

Kaper, J.B. 1998. Enterohemorrhagic *Escherichia coli*. *Currn Opin. Microbiol*., 1: 103–108.

Kaplan, J.B., Ragunath, C., Velliyagounder, K., Fine, D.H., and Ramasubbu, N. 2004. Enzymatic detachment of *Staphylococcus* epidermidis biofilms. *Antimicrob agents and chemoth.,* 48: 2633-2636.

Karatan, E., and Watnick, P. 2009. Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol Mol Biol Rev.,* 73(2):310-47.

Karatan, E., Duncan, T.R., and Watnick, P.I. 2005. NspS, a predicted polyamine sensor, mediates activation of *Vibrio cholerae* biofilm formation by norspermidine. *J Bacteriol*., 187:7434-7443.

Kay, W.W., Phipps, B.M., and Ishiguro, E.E. 1985. Porphyrin binding by the surface array virulence protein of Aeromonas salmonicida. *J of bacterial.*, 164: 1332-1336.

Kazmierczak, B.I., Lebron, M.B., and Murray, T.S. 2006. Analysis of FimX, a phosphodiesterase that governs twitching motility in *Pseudomonas aeruginosa. Mol Microbiol.*, 60:1026-1043.

Khan, M.S., and Husain, A. 2002. Syntheses and reactions of some new 2-arylidene-4-(biphenyl-4-yl)-but- 3-en-4-olides with a study of their biological activity. *Pharmazie*, 57:448–452.

Kim, Y.K., and McCarter, L.L. 2007. ScrG, a GGDEF-EAL protein, participates in regulating swarming and sticking in *Vibrio parahaemolyticus*. *J Bacteriol*., 189:4094-4107.

Kiwifruit Vine Health," 2012, http://www.kvh.org.nz/.

Kjelleberg, S. and Molin, S. 2002. Is there a role for quorum sensing signals in bacterial biofilms? *Curr Opin Microbiol*., 5:254–258.

Klausen, M., Aaes-Jorgensen, A., Molin S., Tolker-Nielsen, T. 2003. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Mol Microbiol.*, 50:61–68.

Klement, Z. 1959. Some new specific bacteriophages for plant pathogenic *Xanthomonas* spp," *Nature*, 184(4694):1248–1249.

Knezevic, O.D., Curcin, S., Petrusic, M., Aleksic, V., Kostanjsek, R., Petrovic,O. 2011. Phages of *Pseudomonas aeruginosa:* response to environmental factors and *in vitro* ability to inhibit bacterial growth and biofilm formation. *American Microbiology*, 111: 245–254.

Knezevic, O.D., Curcin, S., Petrusic, M., Aleksic, V., Kostanjsek, R., Petrovic, O. 2011. Phages of *Pseudomonas aeruginosa:* response to environmental factors and *in vitro* ability to inhibit bacterial growth and biofilm formation. American Microbiology, 111: 245–254.

Koch, B., Liljefors, T., Persson, T., Nielsen, J., Kjelleberg, S., and Givskov, M. 2005. The LuxR receptor: the sites of interaction with quorum-sensing signals and inhibitors. *Microbiol.,* 151:3589–3602.

Krasovec, R. and Jerman, I. 2003. Bacterial multicellularity as a possible source of antibiotic resistance. *Med Hypoth.*, 60:484–488.

Krueger, A.P., and Scribner, E.J. 1941a. The bacteriophage: Its nature and its therapeutic use. *J Am Med Assoc*; 116:2160-7.

Krueger, A.P., Scribner, E.J. 1941b. The bacteriophage: Its nature and its therapeutic use. *J Am Med Assoc*., 116:2269-77.

Kruger, D. H., Schneck, P., and Gelderblom, H. R. 2000. Helmut Ruska and the visualisation of viruses. *The Lan.*, 355: 1713-1717.

Kuchma, S.L., Brothers, K.M., Merritt, J.H., Liberati, N.T., Ausubel, F.M., and O'Toole, G.A. 2007. BifA, a cyclic-di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. *J Bacteriol*., 189:8165-8178.

Kumari, S., Harjai, K., and Chhibber, S. 2009.Characterization of *Pseudomonas aeruginosa* PAO Specific Bacteriophages Isolated from Sewage Samples. *Am J Biomed Sci.*, 1: 91-102.

Kutter, E., Phage Therapy: Bacteriophages as naturally occurring antimicrobials. In: Goldman,E., Green LH,eds. Practical Handbook of Microbiology. Boca Raton, FL: CRC Press, 2008:713-30.

Lang, J.M., Gent, D.H., Schwartz, H.F. 2007. Management of *Xanthomonas* leaf blight of onion with bacteriophages and a plant activator. *Plant Dis.*, 91:871–878.

Le Loir, Y., Baron, F., and Gautier, M. 2003. *Staphylococcus aureus* and food poisoning. *Genet and Mole Res.*, 2(1):63–76.

Lee, B., Haagensen, J.A., Ciofu, O., Andersen, J.B., Hoiby, N., and Molin, S. 2005. Heterogeneity of biofilms formed by nonmucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *J Clin Microbiol.*, 43:5247-5255.

Lewin C.S., Amyes S.G.B., The role of the SOS response in bacteria exposed to zidovudine or trimethoprim, *J Med Microbiol*., 34(6):329–332.

Lewis, K. 2001. Riddle of biofilm resistance. *Antimicrob Agents Chemother*., 45:999–1007.

Lewis, K. 2008. Multidrug tolerance of biofilms and persister cells. *Curr Top Microbiol Immunol*., 322:107–131.

Lewis, K. 2010. Persister cells. *Annu Rev Microbiol*., 64: 357–372.

Li, Y.H., Tang, N., Aspiras, M.B., Lau, P.C., Lee, J.H., Ellen, R.P., Cvitkovitch, D.G. 2002. A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. *J Bacteriol.,* 84:2699-708.

Lim, B., Beyhan, S., Meir, J., Yildiz, F.H. 2006. Cyclic-di-GMP signal transduction systems in *Vibrio cholerae*: modulation of rugosity and biofilm formation. *Mol Microbiol*., 60:331-348.

Loc, C.C., Atterbury, R.J., Shibiny, E.A., Connerton, P.L., Dillon, E., Scott, A., Connerton, I.F. 2005. Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of Broiler Chickens. *Appl Env Microbio.*,71: 6554-6563.

Lorenz, M.G., Gerjets, D., and Wackernagel, W. 1991. Release of transforming plasmid and Chromosomal DNA from two cultured soil bacteria. *Arch Microbiol.*, 156:319–326.

Losick, R. and Kaiser, D. 1997. Why and how bacteria communicate. *Sci Am.*, 276:68–73.

Luria, S.E., Delbruck, M., and Anderson, T.F. 1943. Electron Microscope Studies of Bacterial Viruses. *J Bacteriol*., 46:57-77.

Macia, M.D., Blanquer, D., Togores, B., Sauleda, J., Perez, J.L., Oliver, A. 2005. Hypermutation is a key factor in development of multipleantimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. *Antimicrob Agents Chemother.*, 49:3382–3386.

MacNeal, W.J., and Frisbee, F.C. 1936. One hundred patients with *Staphylococcussepticemia* receiving bacteriophage service. *Am J Med Sci*., 191:179-95.

Mah, T.F., O'Toole, G.A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trend Microbiol*., 9:34–39.

Mah, T.F., Pitts, B., Pellock, B., Walker, G.C., Stewart, P.S., O'Toole, G.A. 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature*, 426: 306-310.

Malik, M., Zhao, X., Drlica, K., Lethal fragmentation of bacterial chromosomes mediated by DNA gyrase and quinolones. *Mol Microbiol.*, 61(3):810–825.

Manson, M.D., Armitage, J.P., Hoch, J.A., and Macnab, R.M. 1998. Bacterial locomotion and signal transduction. *J Bacteriol.*, 180:1009-22.

Marion-Ferey, K., Leid, J.G., Bouvier, G., Pasmore, M., Husson, G., and Vilagines, R. 2005. Endotoxin level measurement in hemodialysis biofilm using "the whole blood assay". *Artificial Organs*., 29:475-81.

Martinez, J.L., and Baquero, F. 2000. Mutation frequencies and antibiotic resistance. *Antimicro Agents Chemother*., 44:1771–1777.

Mascaretti, O.A. 2003. Bacteria versus Antibacterial Agents. An Integrated Approach, ASM Press, Washington, DC USA, 2003.

Masuda, N., Gotoh, N., Ishii, C., *et al.,* Interplay between chromosomal β -lactamase and the MexAB-OprM efflux system in intrinsic resistance to β -lactams in *Pseudomonas aeruginosa*. *Antimicro Agents Chemother*., 43: 400–402.

Mattick, J.S. 2002. Type IV pili and twitching motility. *Annu Rev Microbiol*., 56:289-314.

McLaughlin-Borlace, L., Stapleton, F., Matheson, M., and Dart, J.K.G. 1998. Bacterial biofilm on contact lenses and lens storage cases in wearers with microbial keratitis. *J Appl Microbiol*., 84:827–838.

McManus, P.S., Stockwell, V.O., Sundin, G.W. and Jones, A.L. 2000. Antibiotic use in plant agriculture. *Annu Rev of Phytopathol*., 40:443–465.

McNab, R., Ford, S.K., El-Sabaeny, A., Barbieri, B., Cook, G.S., and Lamont, R.J. 2003. Lux s based singnallingin *Streptococcus gordanii*: autoinducer 2 control carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. *J Bact.,* 185:274-284

McVay, C.S., Velasquez, M., Fralick, J.A. 2007. Phage therapy of *Pseudomonas aeruginosa* infection in a mouse burn wound model. *Antimicrob Agents Chemother.,* 51: 1934-1938.

Mikeladze, C., Nemsadze, E., Alexidze, N., Assanichvili, T. 1936. Sur le traitement de la fievre typhoide et des colites aigues par le bacteriophage de d'Herelle. *La Medecine*., 17:33-8.

Mohanty, B.R., & Sahoo, P.K. 2007. Edwardsiellosis in fish: a brief review. *J of biosci.,* 32(7);1331-1344.

Mohanty, S., Mishra, S., Jena, P., Jacob, B., Sarkar, B., and Sonawane, A. 2012. An investigation on the antibacterial, cytotoxic, and antibiofilm efficacy of starch-stabilized silver nanoparticles. *Nanomed: Nanotec Biol and Med*, 8:916-924.

More, M.I., Finger, D., Stryker, J.L., Fuqua, C., Eberhard, A. and Winans, S.C. 1996. *Sci*., 272: 1655–1658.

Morgan, R., Kohn, S., Hwang, S.H., Hassett, D.J.,and Sauer, K. 2006. BdlA, a chemotaxis regulator essential for biofilm dispersion in *Pseudomonas aeruginosa*. *J Bacteriol*., 188: 7335– 7343.

Murga, R., McAllister, S., Miller, J.M., Tenover, F., Bell, M. and Donlan, R.M. 2001. Effect of vancomycin treatment of methicillin-resistant *S. aureus* (MRSA) biofilms on central venous catheters in a model system. Poster No. C276 presented at the 2001 American Society for Microbiology Annual Meeting, Orlando, FL.

Nakae, T., Nakajima, A., Ono, T., Saito, K., Yoneyama, H. 1999. Resistance to β -lactam antibiotics in *Pseudomonas aeruginosa* due to interplay between the MexAB-OprM efflux pump and β-lactamase. *Antimicro Agents Chemother*., 43:1301–1303.

Nakai, T., Park, S.C. 2002. Bacteriophage therapy of infectious diseases in aquaculture. *Res. Microbiol*., 153: 13-18.

National Nosocomial Infection Surveillance System (NNISS). National Nosocomial Infection Surveillance (NNIS) System report, data summary from January 1992 through June 2004, issued October 2004. *Am J Infect Control*, 32:470-485.

Nealson, K.H. and Hastings, J.W. 1979. Bacterial bioluminescence: its control and ecological significance. *Microbiol Rev.,* 43:496–518.

Nesar,S., Shoaib, M.H., Rahim, N., Rehman, R. 2012. Emergence of resistance to fluoroquinolones among gram positive and gram negative clinical isolates. *Pak J Pharm Sci*., 25:877-81.

Neu, T.R., Verkerke, G.J., Herrmann, I.F., Schutte, H.K., vander Mei, H.C., and Busscher, H.J. 1994. Microflora on explanted silicone rubber voice protheses: taxonomy, hydrophobicity and electrophoretic mobility. *J Appl Bacteriol*., 76:521–528.

Nickel, J.C., Downey, J.A., and Costerton, J.W. 1989. Ultra structural study of microbiologic colonization of urinary catheters. *Urol.*, 34: 284–291.

Nikaido, H. 1994. Prevention of drug access to bacterial targets: Permeability barriers and active efflux. *Sci.*, 264: 382–387.

Novick, R.P. 2003. Autoinduction and signal transduction in the regulation of *staphylococcal* virulence. *Mol Microbiol.,* 48:1429-1449.

O'Toole, G., Kaplan, H.B., Kolter, R. 2000. Biofilm formation as microbial development. *Annu Rev Microbiol*., 54: 49–79.

O'Toole, G.A., Kaplan, H.B., and Kolter, R. 2000. Biofilm formation as microbial developments. *Annu Rev Microbio*., l54:49–79.

Ojha, A.K., Baughn, A.D., Sambandan, D., Hsu, T., Trivelli, X., Guerardel, Y., Alahari, A., Kremer, L., Jr. Jacobs, W.R., and Hatfull. G.F. 2008. Growth of *Mycobacterium*

tuberculosis biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Mol. Microbiol.*, 69:164–174.

Oliver, S.P., Jayarao, B.M., and Almeida, R.A. 2005. Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Food borne Pathog and Dis.*, 2(2):115–129.

Opperman, T.J., Kwasny, S.M., Williams,J.D., Khan, A.R., Peet, N.P., Moir, D.T., and Bowlin, T.L. 2009. Aryl rhodanines specifically inhibit staphylococcal and enterococcal biofilm formation. *Antimicrob. Agents Chemother.*, 53:4357–4367.

O'Toole, G.A. and Kolter, R. 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mole Microbiol.*, 30: 295–304

O'Toole, G.A., Kolter, R. 1987. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol*., 28(3):449-61.

Pajunen, M., Kiljunen, S., Skurnik, M., 2000. Bacteriophage φYeO3-12, specific for Yersinia enterocolitica serotype O: 3, is related to coliphage T3 and T7. *Journal of Bacteriology,*182:5114–5120.

Palleroni, N.J., 1984. Pseudomonadaceae Kreig, R, Holt, J.G. (Eds.) In Bergey's manual of systematic bacteriology Baltimore M D: Williams & Wilkins Co pp. 141–199.

Parrillo, J.E., Parker, M.M., Natanson, C., Suffredini, A.F., Danner, R.L., Cunnion, R.E., and Ognibene, F.P. 1990. Septic shock in humans. Advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy. *Ann Intern Med*., 113: 227-242.

Parsek, M.R., Val, D.L., Hanzelka, B.L., Cronan, J.E., Jr. Greenberg, E.P. 1999. Acyl homoserine-lactone quorum-sensing signal generation. *Proc. Natl. Acad. Sci.* USA 96: 4360–4365.

Patel, M., Jiang, Q., Woodgate, R, Cox, M.M., Goodman, M.F. 2010. A new Model for SOS-induced mutagenesis: how RecA protein activates DNA polymerase V. *Critic Rev Biochem Mole Biol.*, 45(3):171–184.

Pendleton, J.N., Gorman, S.P., Gilmore, B.F. 2013. Clinical relevance of the ESKAPE pathogens. *Expert Rev Anti Infect Ther.*, 11:297-308.

Pennazio, S. 2006. The origin of phage virology. *Riv Biol*., 99:103-29.

Pesci, E. C., and Iglewski, B. H. 1997. The chain of command in Pseudomonas quorum sensing. *TIM., 5:* 132-134.

Petty, N.K., Evans, T.J., Fineran, P.C., and Salmond, G.P.C. 2007. Biotechnological exploitation of bacteriophage research. *Tren in Biotechnol.*, 25(1):7–15.

Pires, D., Sillankorva, S., Faustino, A., Azeredo, J. 2011. Use of newly isolated phages for control of *Pseudomonas aeruginosa* PA01 and ATCC 10145 biofilms. *J Res Microbiol.*, 162:798-806.

Pirhonnen, M., Flego, D., Heikiheimo, R., and Palva, E.T. 1993. *EMBO J*., 12:2467–2476.

Pirnay, J.P., De, V.D., Verbeken, G., Merabishvili, M., Chanishvili, N., Vaneechoutte, M., et al. 2011. The Phage Therapy Paradigm: Pret-a-Porter or Sur-mesure?. *Pharm Res*., 28:934-7.

Pitt, T.L., Simpson, J.H. 2006. *Pseudomonas* and *Burkholderia* spp. In Principles and Practice of Clinical Bacteriology ed. Gillespie SH Hawkey PM Chichester UK John Wiley & Sons, Ltd. 427– 444.

Podschun, R., and Ullmann, U. 1998. *Klebsiella spp*. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev*., 11: 589-603.

Potera, C. 1999. Forging a links between biofilms and diseases. *Science*., 283:183–184.

Prakash, B., Veeregowda, B.M. and Krishnappa, G. 2003. Biofilms: a survival strategy of bacteria. *Current sci*., 85:1299-1307.

Pruss, B.M., Besemann, C., Denton, A., and Wolfe, A.J. 2006. A complex transcription network controls the early stages of biofilm development by *Escherichia coli*. *J Bacteriol*., 188:3731–3739.

Raad, I.I., Sabbagh, M.F., Rand, K.H., Sherertz, R.J. 1992. Quantitative tip culture methods and the diagnosis of central venous catheter-related infections. *Diagn Microbiol Infect Dis*., 15:13–20.

Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, J., Tompkins, R.G., Ausubel, F.M. 1995. Common virulence factors for bacterial pathogenicity in plants and animals. *Sci.*, 268: 1899–1902.

Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, J., Tompkins, R.G., and Ausubel, F.M. 1995. Common virulence factors for bacterial pathogenicity in plants and animals. *Sci.*, 268: 1899–1902.

Regassa, L.B., Novick, R.P. and Betley, M.J. 1992. Glucose and non maintained pH decrease expression of the accessory gene regulator (agr) in *Staphylococcus aureus*. *Infect Immun*., 60:3381-3388.

Reich, F., Atanassova, V., Haunhorst, E., & Klein, G. 2008. The effects of Campylobacter numbers in caeca on the contamination of broiler carcasses with Campylobacter. *Int J of food microbial.*, 127: 116-120.

Reisner, A., Holler, B.M., Molin, S., and Zechner, E.L. 2006. Synergistic effects in mixed *Escherichia coli* biofilms: conjugative plasmid transfer drives biofilm expansion. *J Bacteriol.*,188:3582-3588.

Rice, S.A., Koh, K.S., Queck, S.Y., Labbate, M., Lam, K.W., and Kjelleberg, S. 2005. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. *J Bacteriol*., 187:3477-3485.

Rohwer, F., Edwards, R. 2002. The phage proteomic tree: A genome-based taxonomy for phage. *J Bacteriol*., 184: 4529–4535.

Romling, U., Gomelsky, M., and Galperin, M.Y. 2005. C-di-GMP: the dawning of a novel bacterial signaling system. *Mol Microbiol*., 57:629-639.

Rosche, B., Li, X.Z., Hauer, B., Schmid, A., Buehler, K. 2009. Microbial biofilms : a concept for industrial catalysis ? *Trends in biotechnol*, 27: 636-643.

Ross, P., Mayer, R., Weinhouse, H., Amikam, D., Huggirat, Y., Benziman, M., Vroom de, E., Fidder, A., Paus de, P., and Sliedregt, L.A. 1990. The cyclic diguanylic acid regulatory system of cellulose synthesis in *Acetobacter xylinum*. Chemical synthesis and biological activity of cyclic nucleotide dimer, trimer and phosphothioate derivatives. *J Biol Chem*., 265:18933-18943.

Rowe, M.C., Withers, H.L., and Swift, S. 2010. Uropathogenic *Escherichia coli* forms biofilm aggregates under iron restriction that disperse upon the supply of iron. *FEMS Microbiol Lett*., 307:102–109.

Różalska, B., Walecka, E. and Sadowska, B. 2010. Wykrywanie biofilmów stanowiących problemy medyczne i perspektywy ich eradykacji. *Zakażenia*, 10:13–21.

Rumbaugh, K.P.**,** Griswold, J. and Hamood, A. 1999. Contribution of the regulatory gene lasR to the pathogenesis of *Pseudomonas aeruginosa* infection of burned mice. *J Burn Care Rehabil*., 20:42-49.

Ruska, H. 1940. Die Sichtbarmachung der bakteriophagen Lyse im Ubermikroskop. *Naturwissenschaften*, 28:45-46.

Salmon, G.G., and Symonds, M. 1963.Staphlilo phage lysate therapy in chronic *Staphylococcal* infections.*J Med Soc N J*., 60:188-193.

Sambanthamoorthy, K., Gokhale,A.A., Lao,W., Parashar,V.,Neiditch,M.B., Semmelhack ,M. F., Lee,I. and Waters C. M. 2011. Identification of a novel benzimidazole that

inhibits bacterial biofilm formation in a broad-spectrum manner. *Antimicrob. Agents Chemother*., 55:4369–4378.

Sambrook, J., Fritsch, E.F., Maniatis, T. Molecular Cloning: A Laboratory Manual. 2nd ed. :*Cold Spring Habor Lab Press*; 1989.

Sankaran, N. 2010. The bacteriophage, its role in immunology: how Macfarlane Burnet's phage research shaped his scientific life. *Stud Hist Philos Biol Biomed Sci*., 41:367-75.

Sauer, K., Cullen, M.C., Rickard, A.H., Zeef, L.A., Davies, D.G., and Gilbert, P. 2004. Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *J Bacteriol*., 186:7312–7326.

Schaefer, A.L., Greenberg, E.P., Parsek, M.R. 2001.Acylatedhomoserine lactone detection in *Pseudomonas aeruginosa* biofilms by radiolabel assay. *Method Enzymol*., 336: 41–47.

Schaefer, A.L., Val, D.L., Hanzelka, B.L., Cronan, J.E., Jr Greenberg, E.P. 1996. Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified Vibrio fischeriLuxI protein. *Proc Natl Acad Sci U S A.* 93:9505–9509.

Schlesinger, M. 1934. Zurfrage der chemischenzusammensetzung des bakteriophagen. *Biochem Z*., 273:306-11.

Schless, R.A. 1932. *Staphylococcus aureus*meningitis: treatment with specific bacteriophage. *Am J Dis Child*., 44: 813-822.

Schless, R.A. 1932. *Staphylococcus aureus* meningitis: treatment with specific bacteriophage. *Am J Dis Child*., 44:813-822.

Schofield, D.A., Bull, C.T., Rubio, I., Wechter, W.P., Westwater, C., and Molineux, I.J. 2012. Development of an engineered "bioluminescent" reporter phage for the detection of bacterial blight of crucifers. *Appl and Environ Microbiol.*, 78(10):3592–3598.

Schultz, E.W. 1929. The bacteriophage as a therapeutic agent.*Cal West Med*., 31:5-10

Schwarz, S., Werckenthin, C., Pinter, L., Kent, L.E., Noble, W.C. 1995.Chloramphenicol resistance in *Staphylococcus intermedius*from a single veterinary centre, Evidence for plasmid and chromosomal location of the resistance genes. *Veterin Microbiol*., 43:151- 159.

Scortichini, M., Marcelletti, S., Petriccione, P. F. and Firrao G. 2012. *Pseudomonas syringae*pv. *actinidiae*: a re-emerging, multi-faceted, pandemic pathogen. *Molecul Plant Pathol.*,13:631–640.

Sedor, J., Mulholland, S.G. 1999. Hospital acquired urinary tract infections associated with the indwelling catheter. *Urol Clin North Am*., 26:821–8.

Sen, A., Ghosh, A.N., 2005. Physicochemical characterization of Vibriophage N5.*Virology* 2: 27.

Sharp, R., Hughes, G., Hart, A., and Walker, J.T. 2006. Bacteriophage for the treatment of bacterial biofilms. U.S. Patent 7758856 B2.

Sillankorva, S.M., Oliveira, H., and Azeredo, J. 2012. Bacteriophages and their Role in Food Safety. *Int J of Microbiol*.vol. 2012, Article ID 863945, 13 pages.

Singh, P.K., Schaefern, A.L., and Parsek, M.R. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature.*, 407:762–764.

Skurnik, M., Pajunen, M., Kiljunen, S. 2007. Biotechnological challenges of phage therapy. *Biotechnol Lett*., 29: 995–1003.

Slanetz, L., Jawetz, E. 1941. Isolation and characteristics of bacteriophages for *Staphylococci* of bovine mastitis. *J Bacteriol*., 41:447-455.

Slopek, S., Kucharewicz-Krukowska, A., Weber- Dabrowska, B., and Dabrowski, M. 1985.Results of bacteriophage treatment of suppurative bacterial infections. IV. Evaluation of results obtained in 370 cases. *Arch Immunol Ther Exp (Warsz).*, 33:219-240.

Smith, E.E., Buckley, D.G., Wu, Z., Saenphimmachak, C., Hoffman, L.R., D'Argenio, D.A. *et al.*, 2006. Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. *Proc Natl Acad Sci USA.*, 103: 8487-8492.

Smith, H.W., and Huggins, M.B. 1982. Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. *J Gen Microbiol.*, 128:307-318.

Sperandio, V., Mellies, J.L., Nguyen, W., Shin, S., and Kaper, J.B. 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proc Natl Acad Sci USA.*, 96:15196–15201

Stevens, K.N., Crespo-Biel,O., van den Bosch, E.E., Dias, A.A., Knetsch, M.L., Aldenhoff, Y.B., van der Veen, F.H., Maessen, J.G., Stobberingh, E.E., and Koole, L.H. 2009. The relationship between the antimicrobial effect of catheter coatings

containing silver nanoparticles and the coagulation of contacting blood. *Biomaterials.*,30:3682–3690.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J. *et al.*, 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature.*, 406: 959–964.

Straub, M.E., and Applebaum, M. 1933.Studies on commercial bacteriophage products.*J Am Med Assoc*., 100: 110-113.

Sulakvelidze, A., Kutter, E. 2005. Bacteriophage therapy in humans. In: Kutter E, Sulakvelidze A, eds.Bacteriophages: Biology and Application. Boca Raton, FL: CRC Press, 381-436.

Sutherland, I.W. 2001. The biofilm matrix – an immobilized but dynamic microbial environment. *Trends in Microbiol.,* 9:222–227.

Suttle, C.A. 2005. Viruses in the sea.*Nature*, 437:356- 61.

Sutton, M.D., Smith, B.T., Godoy, V.G., Walker, G.C. 2003. The SOS response: Recent insights into *umuDC*-dependent mutagenesis and DNA damage tolerance. *Annu Rev Genet*., 4:479–497.

Svircev, A.M., Castle, A.J., and Lehman, S.M. 2010. Bacteriophages for control of phytopathogens in food production systems," in *Bacteriophages in the Control of Food- and Waterborne Pathogens*, P. M. Sabourand M.W.Griffiths, Eds., ASM Press, Washington, DC, USA, pp. 79–102.

Tamayo, R., Pratt, J.T., and Camilli, A. 2007.Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annu Rev Microbiol*., 61:131-148.

Tan, M.W., Rahme, L.G., Sternberg, J.A., Tompkins, R.G., and Ausubel, F.M. 1999. *Pseudomonas aeruginosa* killing of *Caenorhabditiselegans* used to identify *P. aeruginosa* virulence factor. *Proc Natl Acad Sci USA*., 96:2408–2413.

Tang, H.B., DiMango, E., Bryan, R., Gambello, M., Iglewski, B.H., Goldberg, J.B., and Prince, A. 1996. Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. *Infect Immun*., 64:37–43.

Theil, K. 2004. Old dogma, new tricks—21st century phage therapy.*Nat Biotechnol*., 22: 31–36.

Thien, F.C.M., and O'toole, G.A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol*., 9:34–39.

Thuptimdang, P., Limpiyakorn, T., McEvoy, J., Prüß, B. M., and Khan, E. 2015. Effect of silver nanoparticles on *Pseudomonas putida* biofilms at different stages of maturity. *J Hazard Mater.*, 290:127-133.

Tischler, A.D., and Camilli, A. 2004. Cyclic diguanylate (c-di-GMP) regulates *Vibrio cholerae* biofilm formation. *Mole Microbiol*., 53:857-869.

Tomasz A, Munaz R. 1995. ß-lactam antibiotic resistance in Grampositive bacteria pathogens of upper respiratory tract: a brief overview of mechanism, *Microb Drug Resist.,* 1:103–109.

Toro, H., Price, S.B., McKee, A.S., Hoerr, F.J., Krehling, J., Perdue, M., Bauermeister, L. 2005. Use of bacteriophages in combination with competitive exclusion to reduce Salmonella from infected chickens. *Avi Dis.,* 49:118-24.

Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A., & Hirata, A. 2003.Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *Lett FEMS Microbiol.,* 223:287–292.

Tuomanen, E., Durack, D.T., and Tomasz, A. 1986. Antibiotic tolerance among clinical isolates of bacteria.*Antimicrob Agents Chemother*., 30:521–527.

Vasil, M.L. 1986. *Pseudomonas aeruginosa*: biology, mechanisms of virulence, epidemiology. *J Pediatr Res.,* 108:800-805.

Vincent, F.C., Tibi, A.R., Darbord, J.C. 1989. A bacterial biofilm in a hemodialysis system.Assessment of disinfection and crossing of endotoxin. *ASAIO Trans*., 35:310–3.

Vogel, F., Naber, K.G., Adam, D., Bodmann, K.F., Lebert, C., Rodloff, A., Sörgel, F. 2005. Aktuelle, Bewertung der Fluorchinolone, *Arzneimitteltherapie,* 23:130–136.

Vuong, C., Voyich, J.M., Fischer, E.R., Braughton, K.R., Whitney, A.R., DeLeo, F.R., Otto, M. 2004. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. *Cell Microbiol*., 6:269–275.

Wagenaar, J.A., Bergen, M.A.P.V., Mueller, M.A., Wassenaar, T.M., Carlton, R.M. 2005. Phage therapy reduces *Campylobacter jejuni* colonization in broilers. *Veterin Microbiol.,* 109:275-283.

Walker, T.S., Bais, H.P., Déziel, E., Schweizer, H.P., Rahme, L.G., Fall, R., and Vivanco, J.M. 2004. *Pseudomonas aeruginosa*–plant root interactions. Pathogenicity, biofilm formation, and root exudation. *Plant Physiol*., 134: 320– 331.

Wang, N., Dykhuizen, D.E., and Slobodkin, L.B. 1996. The evolution of phage lysis timing. *Evolut Ecol.*, 10: 545-558.

Weber, D.B., Mulczyk, M., Gorski, A. 2001. Bacteriophage therapy for infections in cancer patients. *Clin Appl Immunol Rev.*, 1:131-134.

Weber, D.B., Mulczyk, M., Gorski, A. 2003. Bacteriophages as an efficient therapy for antibiotic-resistant septicemia in man. *Transplan Proceed.*, 35:1385-1386.

Weinbauer, M.G. 2004. Ecology of prokaryotic viruses. *FEMS Microbiol Rev*., 28:127-181.

Wellenberg, G.J., Van der Poel, W.H.M., and Van Oirschot, J.T. 2002. Viral infections and bovine mastitis: a review. *Veter Microbiol*., 88(1):27–45.

Wentland, E.J., Stewart, P.S., Huang, C.T., and McFeters, G.A. 1996. Spatial variations in growth rate within *Klebsiellapneumoniae* colonies and biofilm. *Biotechnol Prog*., 12:316–321.

Westbrock-Wadman, S., Sherman, D.R., Hickey, M.J. *et al.*, 1999.Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. *Antimcro Agents Chemother*., 43:2975–2983.

Whitman, W.B., Coleman, D.C., Wiebe, W.J. 1998. Prokaryotes: The unseen majority. *Proc Natl Acad Sci*., 95:6578–6583.

Wingender, J., Neu, T. R., Flemming, H.C. 1999. What are bacterial extracellular polymeric substances, in: Microbial extracellular polymeric substances – Characterization, structure and function. pp.1–19.

Wittebole, X., De Roock, S., and Opal, S.M. 2014.A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Virul.*, 5:226-235.

Wood, T.K., Hong, S.H., and Ma, Q. 2010. Engineering biofilm formation and dispersal. *Trends Biotechnol*., 29:87–94.

Wu, Y., Outten, F.W. 2009. IscR controls iron-dependent biofilm formation in *Escherichia coli* by regulating type I fimbria expression. *J Bacteriol.*, 191:1248–1257.

Xu, X., Stewart, P.S., and Chen, X. 1996. Transport limitation of chlorine disinfection of *Pseudomonas aeruginosa*entrapped in alginate beads. *Biotechnol Bioeng*., 49:93-100.

Yamamoto, K.R., Alberts, B.M., Benzinger, R., Lawhorne, L., Treiber, G. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large scale virus purification.*Virol.*, 40:734-744.

Yan, J., Mao, J., and Xie, J. 2013. Bacteriophage polysaccharide depolymerases and biomedical applications. *Bio Drugs.*, 28:265–274.

Yarwood, J.M., Bartels, D.J., Volper, E.M., Greenberg, E.P. 2004. Quorum sensing in *Staphylococcus aureus* biofilms. *J Bacteriol.*, 186:1838-1850.

Zegans, M.E., *et al.*, 2009. Interaction between bacteriophage DMS3 and host CRISPR region inhibits group behaviors of *Pseudomonas aeruginosa*. *J. Bacteriol*., 191:210–219

Zhao, Q., Li X.Z., Srikumar, R., Poole, K. 1998. Contribution of outer membrane efflux protein OprM to antibiotic resistance in *Pseudomonas aeruginosa* independent of MexAB. *Antimicro Agents Chemother*., 42:1682–1688.