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

Summary

THESIS

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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 4th 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.