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Isolation and Partial Characterization of Virulent Phage Specific against Pseudomonas Aeruginosa

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen, frequently associated with nosocomial infections worldwide. Multiple drug resistance has been reported in previous studies against pathogenic P. aeruginosa and the biofilm which makes use of antibiotics futile. Bacteriophages specific for P. aeruginosa can prove to be a new therapeutic approach for controlling infections and biolfilm contamination against this pathogen. The aim of our study was to isolate and partially characterize virulent phage specific for P. aeruginosa from sewage water. Different parameters of which make phages as suitable candidate towards future therapeutics were also investigated. Phages having lytic life cycle, high burst size and thermally more stable proposed themselves as effective therapeutic candidates. In this study, a virulent phage was isolated from sewage water having burst size of 1036 and latent period of 21 minutes. This phage has narrow host range and shows the remarkable thermal resistance and is viable up to 60 ºC. Sincere efforts in term of identification, isolation, purification and characterization of multiple types of phages against Pseudomonas aeruginosa ; and development of cocktail with pool of lytic phages against it can prove to be a promising strategy to overcome frequent bacterial proliferation.

Index terms— pseudomonas aeruginosa, phage, isolation, partial characterization.

1 Isolation and Partial Characterization of Virulent

Phage Specific against Pseudomonas Aeruginosa Zahra Zahid Piracha ? , Umar Saeed ? , Aqsa Khurshid ? & Waqas Nasir Chaudhary ? Pseudomonas aeruginosa is an opportunistic pathogen, frequently associated with nosocomial infections worldwide. Multiple drug resistance has been reported in previous studies against pathogenic P. aeruginosa and the biofilm which makes use of antibiotics futile. Bacteriophages specific for P. aeruginosa can prove to be a new therapeutic approach for controlling infections and biolfilm contamination against this pathogen. The aim of our study was to isolate and partially characterize virulent phage specific for P. aeruginosa from sewage water. Different parameters of which make phages as suitable candidate towards future therapeutics were also investigated. Phages having lytic life cycle, high burst size and thermally more stable proposed themselves as effective therapeutic candidates. In this study, a virulent phage was isolated from sewage water having burst size of 1036 and latent period of 21 minutes. This phage has narrow host range and shows the remarkable thermal resistance and is viable up to 60 ºC. Sincere efforts in term of identification, isolation, purification and characterization of multiple types of phages Introduction seudomonas aeruginosa is Gram negative, rod shaped opportunistic pathogen of animals, ubiquitous in nature [1]. According to United States National Nosocomial Infection Surveillance System, it accounts for 16.1% of all nosocomial infections and ranked second among Gram negative pathogens [2]. Healthy adults rarely encounter its infection but main target is people having compromised immune system including HIV infections. The infection ranges from self-limiting folliculitis to life threatening bacteremia, wound related morbidity, septicemia, skin infections, otitis media,

ecthymegangrenosum, the black necrotic lesion, endocarditis, corneal ulceration and device related infections [3] ??4]. It is the third leading cause of 12 % of hospital-acquired urinary tract infections, upper and lower respiratory tract infections like cystic fibrosis that is associated with high mortality rate in immune-compromised patients ??4]. Gender-wise prevalence showed 61.78% male and 38.22% females were infected by P. aeruginosa [3]. Souli and colleagues (2008) [5] published data from 23 countries on the European Antimicrobial Resistance Surveillance System and it was shown that 18% of all isolates were multidrug resistant P. aeruginosa strains. Aman Ullah and colleagues (2012) [6] carried out a study in Islamabad and showed that P. aeruginosa is 94% resistant to Chloramphenicol, 88% to Colistin /sulphate, 73% to Tetracycline and 3% to Imipenem. The resistance against the newly tested drugs is still evolving as P. aeruginosa is highly resistant to antibiotics, both at the genetic level and as a result of living in multilayered and complex biofilm [7].

Bacteriophages are bacterial viruses that cause lethal effect on bacteria. They have genetic material in the form of either DNA or RNA (single or doublestranded), encapsidated by a protein coat [8]. Therapeutic role of the phages was interrupted by the advent of antibiotics however the emergence of multidrug resistant bacteria and adverse effects of antibiotics led to renewed interest in phages as therapeutic agent. [9]. Phages have several advantages over antibiotics and other antimicrobial agents, such as host specificity, no side effects, and multiplication in the presence of their hosts [8]. Phages are highly specific and as they are living entity so they evolve with the evolving bacteria [10]. The specificity of phages sometimes may be considered as a possible disadvantage because there are much more pathogenic bacteria than expected to be targeted. To address this problem, a cocktail of phages should be prepared [8]. The Food and Drug Administration of the United States of America recently approved some phages as safe for food products to control Listeria infections [11]. Animal tests of phage therapy are being conducted for treatments of various bacterial infections, and many lytic phages have been isolated and tested for such applications. Phages have been used to treat the Escherichia coli infections and P. aeruginosa infections in mouse models and in guinea pigs and efficient results have been obtained [12].

Approximately 10^30 bacteriophages are present in the environment but only about 300 phages have been characterized [13][14]. In the pre antibiotic era, phage therapy was failed because uncharacterized phages were used so in order to use them fully, it is important to isolate and characterize new phages especially in light of the observation that most of the disease-causing organisms live in matrix-enclosed environments called biofilms that inherently show increased resistance toward all antibiotics [15][16]. In the present study, we have reported the isolation and partial characterization of a virulent phage specific for P. aeruginosa from sewage water in Islamabad and evaluated the different parameters of phage that makes it suitable candidate for future therapeutics.

2 II.

3 Materials and Methods

4 a) Identification of bacterial isolate

Pure cultures of bacterial strain were obtained from Microbiology laboratory of Kahuta research lab hospital, Islamabad. The bacteria were already resistant to Chloramphenicol, Colistin, Cotrimoxazole, Tetracycline and Aztreonam. After overnight incubation of bacterial strain, microbiological methods such as colony morphology and Gram staining were used for identification of bacterial strain [17].

5 b) Phage enrichment and isolation

Sewage water was taken from Sewage treatment plant I-9 Islamabad. Sewage water was centrifuged at 10,000 rpm for 10 min to remove algal cell and sewage debris. To enrich the phage population, above prepared sample concentrates (5 ml) were added to a 30 ml log phase P. aeruginosa. Enriched cultures were incubated overnight at 37 °C with shaking at 150 rpm. Chloroform (1%) was added to 1.5 ml of sample to disrupt bacterial cell and release phages and then centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was filtered using 0.45 and 0.20 mm (Minisart, SalotriusStedim Biotech) syringe filters and transferred to a new tube. Phage isolation and detection was carried out by plaque assay on LB agar plate (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, agar 15 g/L) with soft agar (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, agar 0.7%) . Overnight bacterial culture and phage sample were mixed in 0.7% LB soft agar (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, agar 0.7%; at a temperature of 45 °C) and poured over LB plates. On solidification plates were incubated overnight at 37 °C and examined for plaques the following day. For a negative control, phage alone was added to the molten agar. Well isolated plaques were serially propagated until a single phage type was obtained. The purified phages were then stored in SM buffer (100 mMNaCl, 8 mM MgSO4, 50 mM, Tris-HCl [pH 7.5]), and 0.002% w/v gelatin at 4 °C with the addition of 7% dimethyl suloxide (DMSO) at -80 °C. [18] c) One step growth experiment One step growth curve experiment was performed to determine latent period and burst size of phage [19][20]. In brief, 50 ml of P. aeruginosa culture was incubated to mid exponential phase having O.D 600 0.6 and cells were harvester via centrifugation. The pellet was re-suspended in 0.5 ml of LB media and mixed with 0.5 ml of the phage solution having plaque forming unit (pfu) of 2.75x10^9. This mixture was allowed to stand for 3 minutes at 37 °C so that phages adsorbed to the host cells. Mixture was then centrifuged at 13,000 rpm for 2 minutes to remove the free phage particles. The pellet was re-suspended in 100 ml of LB medium and

101 culture was incubated at 37 °C with shaking. Samples were taken after every 3 minutes for 45 minutes and after
102 centrifugation at 13,000 rpm for 1 minute, subjected to determination of phage titer via plaque assay.

103 6 d) Analysis of calcium ion effect on phage adsorption

104 In order to measure the effect of divalent metal ions on phage adsorption rate CaCl 2 was used [21]. The 50 ml
105 of P. aeruginosa overnight culture having O.D 600 0.6 was divided in two autoclaved flask 25 ml each. One flask
106 was inoculated with phage 500 ?l having pfu of 2.75x10^9 (control), while the second flask was inoculated both
107 with 500 ?l phage (pfu 2.75x10^9) and 250 ?l of 1 M CaCl s and incubated with constant shaking at 90 rpm
108 at 37 °C. Samples were taken from both flasks at different time intervals of 0, 10, 20, and 30 minutes. Samples
109 were centrifuged at 13,000 rpm for 3 minutes to sediment the phage adsorbed bacteria. The supernatant was
110 assayed for unabsorbed phages via double layer plaque assay method and counts were compared with the titer of
111 control. e) Thermal stability analysis Thermal stability tests were performed according to the method described
112 by Suarez [21]. Phage filtrates were taken in microcentrifuged tubes and treated under different temperatures
113 at 37 °C (control), 40 °C, 50 °C, 60 °C and 70 °C for 20 minutes, 40 minutes and for 1 hour. After incubation
114 plaque assay was performed for each treated sample. Results were compared with control. P. aeruginosa culture
115 1 ml was inoculated in 2 flasks having 150 ml LB media. The phage supernatant 500 ?l was introduced in one
116 flask and other flask was treated as control and both the flasks were incubated in shaking incubator at 37 °C.
117 The O.D 600 readings were taken after every 2 hours for 24 hours using spectrophotometer. The values were
118 compared with control.

119 7 g) Host range

120 The host range of the isolated phage was checked on the range of clinical pathogenic bacterial L, latent phase;
121 R, rise phase; P, plateau phase.

122 8 d) Calcium Ion Effect on the Adsorption Rate of Phage

123 Divalent ions often affect the adsorption of phages. In order to check this, CaCl 2 1mM was added in P. aeruginosa
124 phage mixture and number of free phages left in solution were detected after 10 minutes interval for 30 minutes
125 by using plaque assay. Data analysis showed that significant differences existed between the control and calcium
126 ion treated group. Results obtained show that calcium ions stabilize the process of adsorption and infectivity is
127 increased due to addition of CaCl 2 . Infection of bacteria with the phages was monitored for 24 hours. The
128 bacterial reduction caused by phages was compared with control. Phage infection produced a drastic decrease of
129 the P. aeruginosa culture as compared to control as it is evident from the graph. However, a constant increase in
130 OD 600 was seen after 16 hours this is most probably due to growth resistant phages. The susceptibility of the
131 phage was investigated with clinical strain of P. aeruginosa isolated from various sources and results show that
132 the phage hasnarrow host range.

133 9 Discussion

134 P. aeruginosa is a nosocomial pathogen and leading cause of health care associated infections. It is an emerging
135 multidrug resistant pathogen around the globe and also in Asia [2]. Way of acquiring resistance specifically the
136 chromosome encoded efflux and low outer membrane permeability accounts for its high resistance and makes use of
137 antibiotics futile. The biofilm induces the resistance and restricts the diffusion of the antibiotic which makes most
138 of the antimicrobial agents less effective. It can also acquire additional resistance genes from other organisms via
139 plasmids, transposons and bacteriophages [7]. Imipenem was considered one of the effective antibiotics however
140 Lautenbech and colleagues in 2010 detected the imipenem resistant P. aeruginosa strains. All these evidence
141 showed an alarming situation and demands an alternative treatment for P. aeruginosa infections [6, 23].
142 In this study, our results showed that a newly isolated bacteriophage can lyse actively growing P. aeruginosa.
143 Phages are found with the natural habitat of their host cells such as sea water, sewage water/ sludge ponds etc
144 [1]. They are host specific and evolve along with their host [10]. Phage was isolated from sewage water. Sewage
145 in general, contains a large diversity of microbes due to contamination from fecal and hospital drainage water.
146 This newly isolated phage was highly lytic and produced large plaque of 4 mm. Phage establishes an infection in
147 the host when it gains entry into the host after interaction with the bacterial receptor [24]. Many phages have
148 been found to be greatly specific for their receptors present on the host cell surface. They show no interaction
149 with receptors having a different structure. The specificity turns out to be the basis of phage typing methods
150 used for the identification of bacterial strains. The results obtained clearly showed that our virulent phage for P.
151 aeruginosa was highly specific. Results indicated that the phage may be using a common receptor.
152 One step growth curve explains all the stages involved in multiplication of phages. From this curve, latent
153 period and burst size are determined. Latent period or incubation period is the time which the phage requires
154 inside the bacterial cell and is 21 minutes in case of our isolated phage. The optimum latent period leads to
155 high phage fitness. Rise phase is the time when infected cell begin to lyse and plateau phase is time when the
156 infectious centers is due to only single virion [19]. Phage burst size is average number of phage bacterial cells
157 produced per bacterial cells upon infection. The burst size of phage is 1036 particles per bacterial cell. Wang [25]
158 described that the duration of the latent phase correlates with the burst size. The longer is the latent phage the

159 bigger is the burst size of the phage. The number of phages liberated upon infection is quite high as compared
160 to early reported phages.
161 The factors which influence the infectivity of phage in-vitro are temperature and divalent cations. The
162 physiological state of host also plays an important role in reproduction of phage. The infection of the virion
163 starts when it binds to host bacterial cell. In phage host interaction, calcium might be playing an important role
164 in infection cycle [19] .Calcium ion stabilizes the weak interaction of virion with receptors or they may change
165 the structure of the receptor to make the accessibility of phages smoothly. Ions have an electrostatic effect in the
166 interaction of phage bacterium systems [26]. Different amount of calcium gives different infectivity.
167 The physical parameters like the temperature not only accelerate the adsorption of phages but also have direct
168 effect on metabolic activities of the host [26]. Extremely thermal resistant phages have been isolated from thermal
169 habitats and also in other aquatic environments [27]. Our results implied that phage we have isolated is active
170 on high temperatures. The phage remained active till four months at 4 °C and it remains viable up to 60 °C
171 after 1 hour incubation. The maximum infectivity was observed at 37 °C and least infectivity was observed at
172 60 °C.
173 The phage we isolated also causes decrease in turbidity of P. aeruginosa culture and this behavior was obvious
174 for 16 hours as compared to control. There was maximum bacterial destruction during this time period but some
175 bacterial cells show resistance to virus infection. This may be because of host bacterium adaptation as bacterial
176 replication was going on meanwhile and bacterial resistant cells survived and though they were less in number
177 but they started multiplying after about 16 hours and it was time when turbidity of culture increases as shown
178 in figure 6. This
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181 type of behavior can be a hindrance in phage therapy. Some studies shown that phage resistant bacteria losses
182 their virulence factor because those virulence can be a site for phage infections. Such loss of virulence factor in
183 a phage resistant bacteria mutant has been demonstrated in fish pathogens [28]. Due to resistance, the fitness of
184 bacteria reduces and it renders it competing unfavorably with its phage sensitive ancestors. The gradual increase
185 in optical density can also be attributed to some other factors like bacterial debris and this might be a hindrance
186 in exposition of actual results.
187 V.

188 11 Conclusion

189 Viral diseases are increasing day by day in world and it is anticipated that soon hepatitis viruses would emerge
190 as most dangerous viral pathogens [29,30]. But on the other hand nature has selected many viruses to kill
191 various other pathogens. . Nature has many hidden remedies against multiple disorders such as cardiovascular
192 diseases, metabolic disorders, cancer, chronic inflammation and many others); there is strong need to discover
193 therapeutic potentials of natural items. Many studies showed elevated activation of various cellular proteins
194 cause cancer proliferation, which can be further inhibited by potential inhibitors **??**31-Partial characterization
195 of the virulent phage showed that it is highly efficient in lysing Pseudomonas aeroginosa, as it has shown some
196 outstanding aspects including rapid growth nature, high thermal stability and optimum latent period. All these
197 characteristics make this phage very promising for possible application in eradication of Pseudomonas aeroginosa
198 contaminations and treatment of Pseudomonas aeroginosa infections. Phage has shown narrow host range, so for
199 the broad-spectrum elimination of bacteria; a "cocktail" with a pool of lytic phages might be more useful against
200 present and other bacterial strains. A better understanding of phages and lytic enzyme biology could facilitate
201 development of novel future therapeutics against multiple drug resistant bacteria.
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Figure 1: C

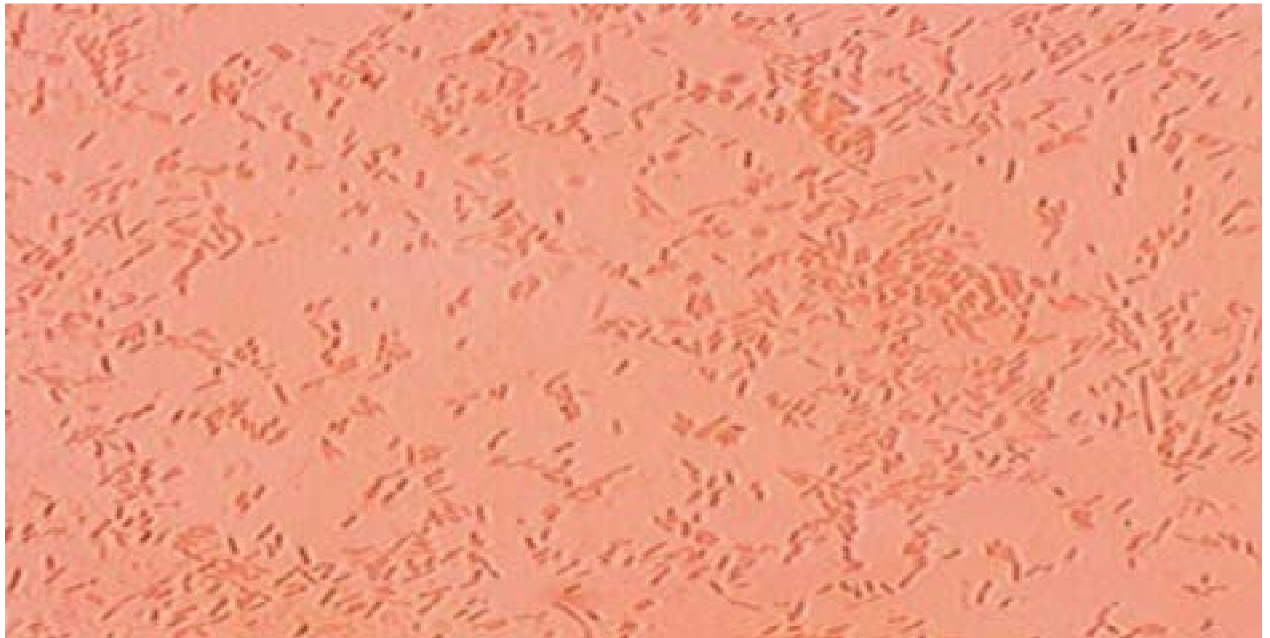

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Figure 2: Figure 1 :

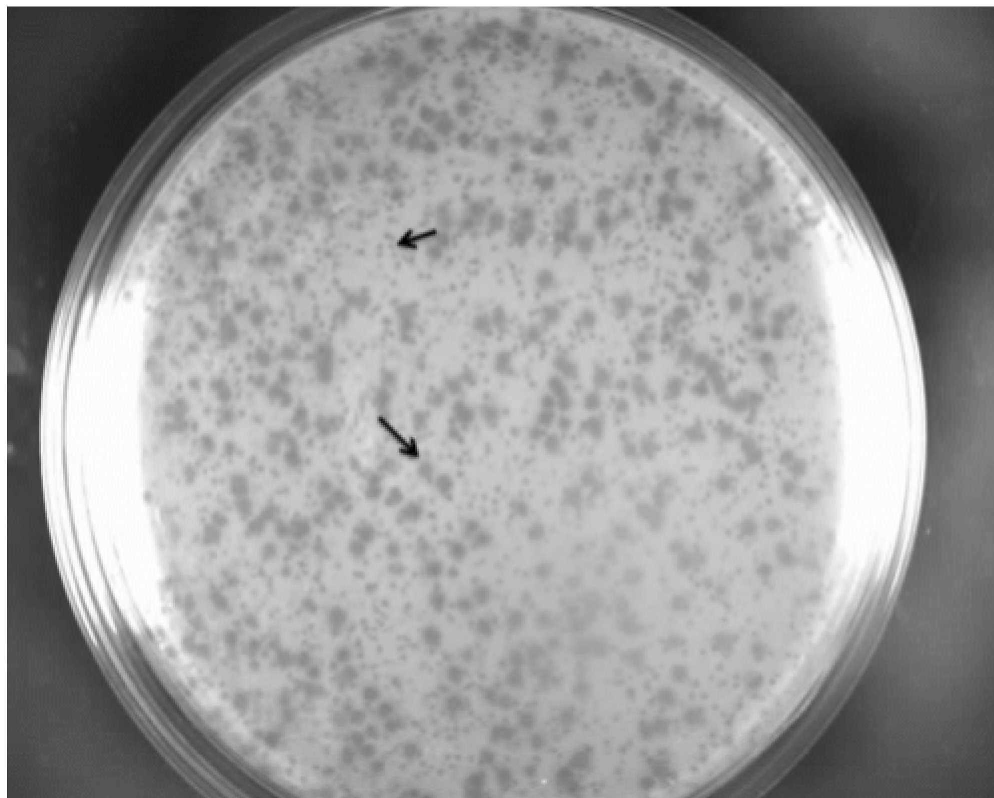
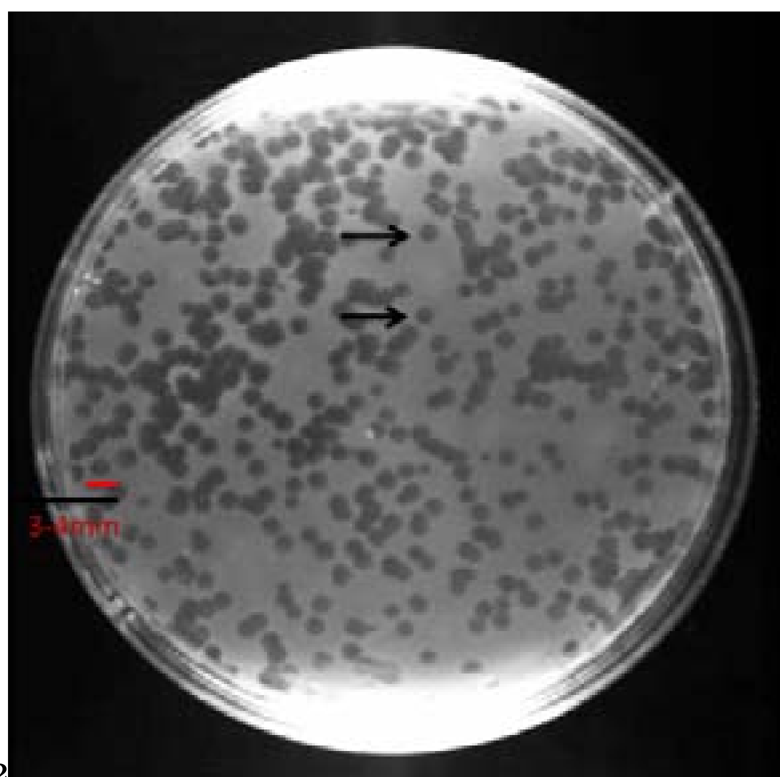


Figure 3:



2

Figure 4: Figure 2 :

1

Sr. No	Color Margin Shape Elevation Luminous behavior	Texture Size

Figure 5: Table 1 :

2

of surviving PFU (%) Percentage	100.00% 0.00% 40.00% 80.00%	20.00% 60.00%						37 Degree 40 Degree 50 Degree
			10	20 30 Incubation time in minutes	40	50	60	
Bacterial O.D600	0.000 0.100 0.300 0.400 0.600 0.700	0.200 0.500	0	5 Control Flask Having Phage 10	15	20	25	30

Sr. No Source of bacteria		Time (Hours) Clinical strain	Host range (+,-)
1.	Pus sample	P. aeruginosa	+
2.	Blood sample	P. aeruginosa	-
3.	Urinary tract sample	P. aeruginosa	_

Figure 6: Table 2 :

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