Bacteriophages can be used as infection control agents: A proof-of concept study involving anti-*Acinetobacter baumannii* phage

Aamir Hussain[1,2,3], Amna Manzoor[2], Ihsan Ullah[3], Atif Aziz[2], Mubashar Aziz[2], Muhammad Qamar Saeed[2*]

[1]Combined Military Hospital, Multan, Punjab, Pakistan

[2]Dr. Ghulam Nabi Chaudhry Laboratory of Microbial Technologies, Department of Microbiology, Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, Punjab, Pakistan

[3]Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Khyber PakhtunKhah, Pakistan

*Corresponding author

mqamarsaeed@bzu.edu.pk (MQS)

Abstract

Hospital acquired infections are responsible for morbidity and mortality worldwide. *Acinetobacter spp.* infections are particularly notorious for complicating patient management in ICU settings. Extremely high mortality rates are associated with *Acinetobacter* infections because of their resistance to first- and second-line drugs. There is imminent need to develop infections control systems that are specific and environment friendly. Here, we report a proof-of-concept anti-*Acinetobacter spp.* bacteriophage-based infection control assay that is very target specific as well as innocuous to environment. extensively drug resistant (XDR) *Acinetobacter baumannii* strain was inoculated at various solid surfaces. A bacteriophage, enriched in the same strain, was applied on the inoculated surfaces. Phenol (carbolic acid) was used as a positive control. We show that bacteriophages can be used as infection control agents. In our assay, they killed XDR *Acinetobacter baumannii* present on solid surfaces. Our bacteriophage was extremely effective at reducing the CFU of inoculated strain to almost undetectable levels.

Introduction

25

26 Different surfaces in healthcare facilities may serve as reserves of pathogens. These pathogens are

27 originally contributed by infected hospitalized patients [1]. Bacteria are well known to be promiscuous in

28 terms of exchange of genetic material. They can donate or accept genes even from distantly related

29 species and strains directly or indirectly [2,3]. Under selective evolutionary pressures in hospitals, these

30 pathogens, having genetic capability of horizontal gene transfer, gradually evolve increased virulence and

31 antibiotic resistance— the characteristic of hospital acquired infections [2]. Critical first step of infection

32 control, therefore, is providing aseptic environment to patients so that cost of treatment as well as

33 transmission of multidrug resistant (MDR) infections can be avoided.

34 There are many routes of transmission of infection in hospital settings. Direct contact between patients is

35 a rare occurrence. A health care worker who may carry some pathogen on his/her skin or clothing may be

36 the source of infection [1,4]. Similarly, patients are in direct contact with ward and ICU surfaces.

37 Pathogens can also hide in medical devices, which, when applied to patients, can transmit infections [1].

38 MDR *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Acinetobacter baumannii* infections are

39 mostly acquired in hospital settings [5].

40 Clinically isolated strains of *Acinetobacter spp*. are particular notorious for their extreme drug resistance.

41 This organism has the ability to cause different systemic infections which include bacteremia, pneumonia,

42 urinary tract infections, superficial and deep wound infections, and meningitis etc. [3]. Ability to cause

43 multiple types of infections, coupled with drug resistance greatly limits treatment options. The best

44 options against it is to develop targeted infection control regimes which are effective at clearing

45 *Acinetobacter spp.* from various surfaces acting as their reservoirs.

46 Many hospitals rely on carbolic acid (phenol) as their method of disinfection. However, phenolics are

47 damaging to both environment and humans [6,7]. These compounds may cause skin corrosion, germ cell

48 mutagenicity and organ toxicity on repeated exposures. Moreover, use of phenolics in nurseries is highly

49 discouraged. In one study, phenolics exposed infants were found to have higher bilirubin levels as

50 compared to non-exposed, even when disinfectant was prepared according to manufacturer's

51 recommendation [6]. Therefore, such methods must not be used in medical instruments such as

52 ventilators. There is need of bactericidal agents that are effective as well as non-toxic to patients.

53 Bacteriophages are natural bacterial parasites. They have the benefit of being extremely specific to their

54 host species and are innocuous to environment and humans [8]. Additionally, as they reproduce in their

55 host, they keep on growing on the surfaces where their host is available. Thirdly, they are self-limiting.

56 They survive only as long as their host survives [9]. Use of bacteriophages in critical health care

57 instrument, therefore, can be very useful in controlling infections in health care settings.

58 We designed a proof-of-concept study to show that bacteriophage raised against *XDR Acinetobacter*

59 *baumannii* could effectively reduce bacterial load on solid surfaces. Our assay shows that it was almost as

60 effective as phenol in reducing the colony forming units on inoculated surfaces.

Materials and Methods

Selection of Acinetobacter spp. for Phage Isolation

63 A patient isolated strain of *Acinetobacter baumannii* was selected because it was resistant to all the tested

64 drugs. This strain was provided by Armed Forces Institute of Pathology, Rawalpindi. This strain was used

65 to isolate bacteriophage from sewage samples.

66 *Acinetobacter baumannii* was cultured on Blood and MacConkey agar plates a day before the start of

67 bacteriophage isolation. For the isolation of bacteriophages, sewage water samples were collected from

68 multiple sites in sterile pre-labeled bottles. Sampling sites included effluents from combined military

69 hospital (CMH) Multan, Nishtar Hospital Multan, Children Hospital Multan and some dairy farms

70 effluent from Multan region.

Phage enrichment

72 A heavy inoculum of sub cultured *Acinetobacter baumannii* was prepared from a randomly selected

73 colony on MacConkey agar in a 10 ml tube by overnight culture. Pre-labeled glass flasks were poured

74   with 10 ml of 2x Luria-Bertani (LB) broth (Oxoid, Catalog no. 0264) followed by addition of 10 ml

75   sterile sewage samples filtrate and 500 μl of bacterial culture. Sewage samples were sterilized using 0.2 μl

76   syringe filters (Corning, Catalog no. 411224). All these flasks were incubated at 37°C for 24 hours.

77   After 24 hours, 12-13 ml suspension from each flask was transferred into pre labelled 15 ml tubes. After

78   centrifugation of 10 minutes at 4200 RPM, the supernatant from each tube was syringe filtered. This

79   filtrate was used for two more rounds of enrichment to increase titers of possible anti-acinetoviruses.

80   **Plaque assay**

81   After three enrichments, suspected bacteriophage containing filtrates were taken and serially diluted ten

82   times (10^{-1} through 10^{-10}) in sterile saline solution in ten tubes. An early log phase bacterial inoculum was

83   prepared by adding 300 μl of bacterial suspension in 30 ml LB broth in a flask, followed by the

84   incubating it for 2-4 hours at 37°C. After that, a mixture of bacterial isolate, bacteriophage dilution and

85   soft LB agar (normal solid media contain 1.5% agar) was prepared by adding 0.6% agar in LB broth. For

86   this purpose, 100 μl of early log phase inoculum was added into 20 ml glass tube followed by the addition

87   of 10μl of each of the ten phage dilutions. Then 20 ml of soft LB agar was added. All these tubes were

88   incubated at 37°C for 20 minutes for adsorption to take place.

89   After that about 8 ml of the mixture from each tube was poured on pre labeled LB agar plates and

90   incubated at 37°C for 24 hours or until appearance of plaques on phage positive plates. Plaques were

91   observed and virus titer was calculated by counting plaques on 10^{-7} plate (Fig 1).

92   **Acinetobacter Clearance Assay**

93   A 0.5 McFarland inoculum of *Acinetobacter baumannii* was prepared in 10 ml tube. Three circles of one-

94   inch diameter each were drawn on a pre-sterilized lab bench surface (Fig 2) and labeled as "phenol",

95   "phage" and "saline". Then 2ml of bacterial inoculum was poured in each circle. After 20-25 min, 250 μl

96   of 90% phenol, normal saline, and bacteriophage suspension was added on respective inoculated circles.

97   Phage was added at 10 MOI (multiplicity of infection: ratio between no. of infectious phage particles and

98   no. of host bacterial cells). Same procedure was adopted for two more surfaces; top of the incubator and

99   office table.

100   After overnight exposure of inoculated circles with phenol (positive control), saline (negative control) and

101   phage, any remaining bacteria were collected with a moistened sterile swab by rolling it over each circle

102   thoroughly. Then cotton part of swab was aseptically cut into 10ml sterile saline and vortexed to collect

103   bacteria in the saline. Subsequently, 1 µl calibrated loop (SPL Life Sciences, Catalog no. 90001) was

104   dipped into the saline containing inoculum from circles and LB agar plates were semi-quantitatively

105   streaked. Colonies from phenol exposed, saline exposed and phage exposed circles were counted to

106   determine the bactericidal effects of phage (Table 1).

Results and Discussion

Phage Isolation and Titer

109   We collected some 50 sewage samples from various sites and one sample from Children Hospital Multan

110   yielded bacteriophage. For purification of single phage, a single well isolated plaque was picked from the

111   plate and subjected to enrichment. After every round of enrichment isolated plaque was collected and

112   process was repeated three time in order to get a pure single type of phage (Fig 1). Titer was measured in

113   terms of plaque forming units per milliliter (PFU/ml). There are many ways of measuring virus

114   concentration in a sample. Some of them are based on measuring quantity of physical particles based on

115   viral proteins or antibodies (Such as ELISA and Western blotting). Others measure concentration of virus

116   nucleic acid by qPCR methods. Many particles in a given sample may be incapable of causing productive

117   infection. Most reliable approach, therefore, is measurement of infectious particles because methods

118   based on physical measurements are not always linearly correlated to infectivity [10]. Therefore, PFU/ml

119   gives us infectious titer and hence approximates number of "physically fit" particles. Plates containing 10^{-7}

120   dilution of phage were used to count plaques as it had isolated and hence countable plaques (Fig 1C).

121   All work was done in triplicate and averages were calculated. PFU/ml were extrapolated from the plaque

122   count to be 2×10^{12}.

123 *Fig 1: Virus plaques are visible on the lawn of XDR Acinetobacter baumannii strain. Ten plates were prepared but lysis became*
124 *observable from 10^{-5} dilution and plaques became countable on plate containing 10^{-7} phage dilution. For reference, plate*
125 *containing 10^{-5} to 10^{-10} dilutions are shown. Note decreasing plaque number from A through F.*

Use of Phage as Bactericidal Agent

127 Hospital acquired infections are increasingly becoming a great challenge due to drug resistance and

128 augmented virulence. Several hospital strains hide themselves on solid surfaces such as work benches of

129 hospital staff, side tables of patient beds, and medical instruments such as ventilators [1,4]. Fortunately,

130 more and more health establishments are implementing infection control regimes in their hospitals. Most,

131 however, use phenolics to decontaminate environment due to their low cost, ready availability and great

132 microbicidal potential. But it is not an agent of choice when it comes to its undesired effects. Many

133 studies have shown it to be toxic to animals and humans and especially infants [7]. Its unfriendliness to

134 environment and patients precludes its use in disinfection of medical devices.

135 In this backdrop, we set out to determine potential of our acinetophage isolate in clearing clinically

136 relevant XDR *Acinetobacter* strain from solid surfaces. For our experiment, we decided to mimic

137 conditions that are common to hospital inhabiting pathogens (Fig 2). We inoculated different solid

138 surfaces with XDR *Acinetobacter baumannii* strain in known quantities and applied our phage on those

139 surfaces to determine its clearing potential. We infected our bacterial inoculums at 10 MOI because

140 calculations based on Poisson distribution model predict that at 10 MOI almost all bacterial cells receive

141 at least one virus particle. Same volumes of 90% phenol and normal saline were used to serve as positive

142 and negative controls respectively. Purpose was to observe effectiveness of phage as compared to phenol

143 which is an established bactericidal.

144 *Fig 2: A: Representative illustration of the circled solid surfaces where XDR Acinetobacter baumannii strain was inoculated.*
145 *Three different surfaces were selected and inoculated as explained in the text and table 1. B: Representative plates showing*
146 *clearance of Acinetobacter from surfaces exposed with either phenol (B1, positive control) or Phage (B2). Plate streaked with*
147 *saline (B3, negative control) shows much higher growth of bacteria. Note that plates B1 and B2 only have sporadic colonies*
148 *which do not necessarily represent specific growth originating from the inoculated surfaces.*

149 After overnight exposure to phage, phenol and saline, we collected any remaining bacteria from those

150 surfaces and cultured on LB agar plates in quantitative manner using calibrated 1μl loops. Therefore,

151 colony count represented CFU per microliter after the exposure of our bactericidal agents.

152   We observed that phenol, quite expectedly, was the most efficient antibacterial as its exposure resulted in

153   least number of CFU from all three surfaces; 3, 5 and 2 from lab bench, incubator top and office table

154   respectively (Table 1). Saline exposed surfaces gave much higher colony count (78, 103 and 91 CFU/µl

155   for three surfaces in above mentioned order). Again, it was an expected observation as saline has no

156   antimicrobial potential. Phage exposed surfaces gave 15, 13 and 11 CFU/µl which, although, was higher

157   number when compared to phenol but is significantly smaller than in case of saline treatment (Table 1).

158   *Table 1: Colony forming units (CFU/µL) of Acinetobacter baumannii from phenol, saline and phage exposed surfaces*

Surface	Phenol	Phage	Saline
Lab Bench	3	15	78
Top of Incubator	5	13	103
Office Table	2	11	91
Avg±SD	3.33±1.5	13±2	90.67±12.5

159

160   Colonies appearing from phenol and phage exposed samples were sporadic (Fig 2: B1 and B2). They

161   were not neatly present on the streaked area, rather, they were randomly located on the plates. It can

162   therefore, be concluded that those colonies did not necessarily originate from inoculated surfaces and may

163   have grown from accidental trapping of bacteria from incubator environment. Why, then, we did not see

164   similar number of nonspecific colonies in both phage and phenol exposed inoculums? This apparent

165   anomaly can be explained on the basis of broad-spectrum effect of phenol but not phage. As swabs were

166   used to collect bacteria from inoculated surfaces (as explained in materials and methods), they could have

167   also carried traces of phenol and phages along with bacteria. When these were subsequently cultured on

168   LB plates, phenol and phages (albeit, in small quantities) would have entered in the plates. Any bacteria

169   entering from incubator environment would have been killed by broad-spectrum effect of phenol but not

170   by a narrow-spectrum phage; resulting in more colonies in latter case.

Conclusion

This experimental assay provides a proof of concept that phages can be used as infection control agents in hospitals. In contrast to phenolics, they have the advantage of being environment friendly, very specific to their bacterial host and completely safe for humans. Their safety is such that they are being investigated systematically for use as human therapeutics worldwide [11,12]. However, their extreme specificity also affords certain limitations. Unlike broad spectrum chemical agents, one type of phage can usually target one bacterial species. Effective broad range phage based bactericidals, therefore, must contain more than one type of phage. Such phage cocktails can be used to kill a spectrum of hospital inhabiting pathogens while being environment, healthcare worker and patient friendly. An effective phage cocktail must contain phages against pathogens implicated in causing hospital acquired infections (e.g. *Acinetobacter baumannii*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* etc.) [5].

Competing Interests: None to declare

References

1. Mehta Y, Gupta A, Todi S, Myatra S, Samaddar DP, Patil V, et al. Guidelines for prevention of hospital acquired infections. Indian J Crit Care Med Peer-Rev Off Publ Indian Soc Crit Care Med. 2014;18: 149–163. doi:10.4103/0972-5229.128705

2. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJV. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol. 2014;13: 42–51. doi:10.1038/nrmicro3380

3. Lee C-R, Lee JH, Park M, Park KS, Bae IK, Kim YB, et al. Biology of Acinetobacter baumannii: Pathogenesis, Antibiotic Resistance Mechanisms, and Prospective Treatment Options. Front Cell Infect Microbiol. 2017;7. doi:10.3389/fcimb.2017.00055

4. Vanhems P, Barrat A, Cattuto C, Pinton J-F, Khanafer N, Régis C, et al. Estimating Potential Infection Transmission Routes in Hospital Wards Using Wearable Proximity Sensors. PLoS ONE. 2013;8. doi:10.1371/journal.pone.0073970

5. Ali II, Khan IA, Munir MK. Frequency of Multi Drug Resistant Nosocomial Pathogens in Intensive Care Units of a Tertiary Care Hospital in Karachi. Ann King Edw Med Univ. 2017;23. Available: http://www.annalskemu.org/journal/index.php/annals/article/view/1543

6. Doan HM, Keith L, Shennan AT. Phenol and Neonatal Jaundice. : 4.

7. pubmeddev, al CM et. Association of exposure to phenols and idiopathic male infertility. - PubMed - NCBI. [cited 31 Oct 2019]. Available: https://www.ncbi.nlm.nih.gov/pubmed/23435201

8. Gohar M, Ali HA, Saeed MQ. Use of lytic bacteriophages in controlling multi drug resistant staphylococcus aureus. J Pak Med Assoc. 2018;68: 2.

9. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. Bacteriophage. 2011;1: 66–85. doi:10.4161/bact.1.2.15845

10. Qamar Saeed M, Dufour N, Bartholmae C, Sieranska U, Knopf M, Thierry E, et al. Comparison Between Several Integrase-defective Lentiviral Vectors Reveals Increased Integration of an HIV Vector Bearing a D167H Mutant. Mol Ther Nucleic Acids. 2014;3: e213. doi:10.1038/mtna.2014.65

11. Loc-Carrillo C, Abedon ST. Pros and cons of phage therapy. Bacteriophage. 2011;1: 111–114. doi:10.4161/bact.1.2.14590

12. Jault P, Leclerc T, Jennes S, Pirnay JP, Que Y-A, Resch G, et al. Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by Pseudomonas aeruginosa (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. Lancet Infect Dis. 2019;19: 35–45. doi:10.1016/S1473-3099(18)30482-1
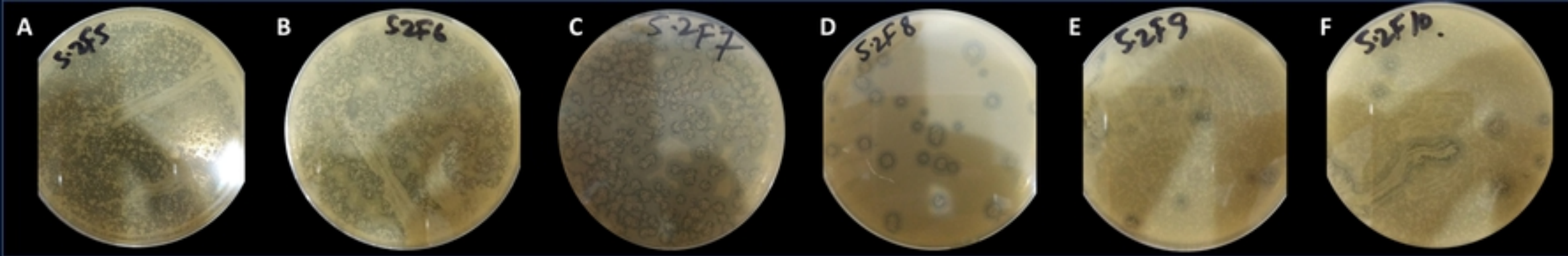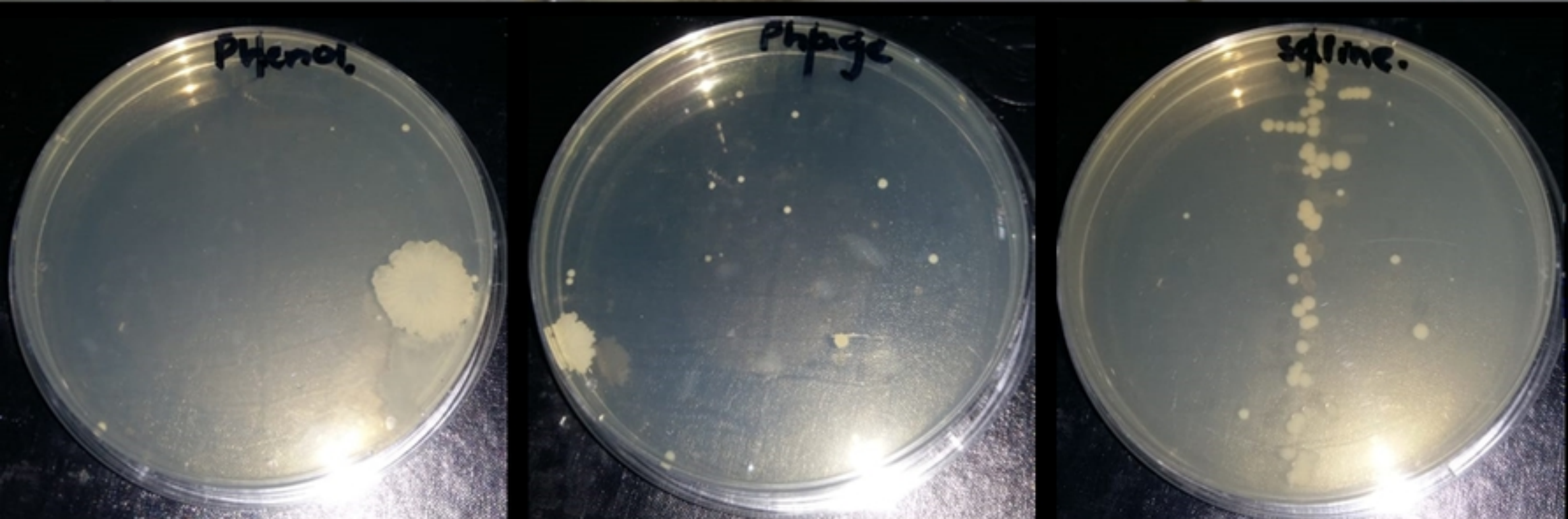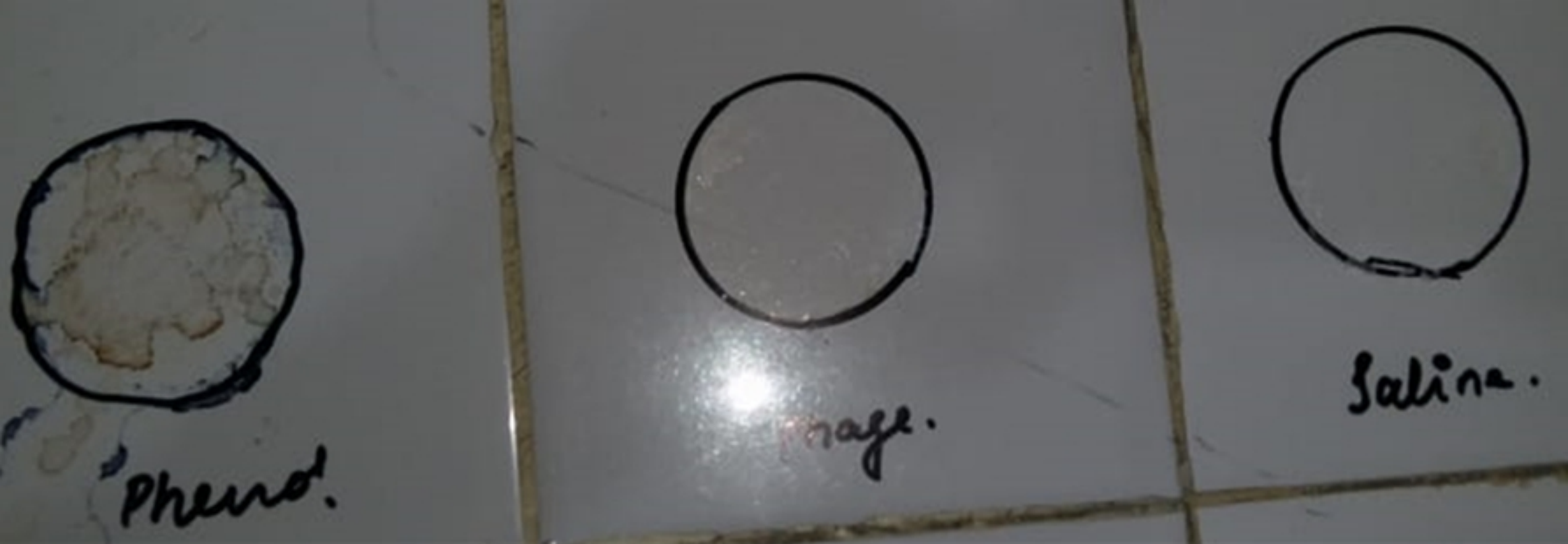
Fig1

Fig2