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2 **BACTERIOPHAGE THAT INFECTS *Escherichia coli***

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9 Running head: Morphotype C3 *Escherichia coli* bacteriophage

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15 **BACTERIOPHAGE THAT INFECTS *Escherichia coli***

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20

21 **ABSTRACT**

22 It has been isolated a lytic bacteriophage specific to *Escherichia coli*, which can infect at least
23 one different bacterial group. Phage Φ GF1 was isolated from a wastewater treatment plant. It
24 is resistant to the effect of chloroform and is stable at 40 and 50 °C. In addition, it is stable in
25 the range of pH 5-8. Its host range is wide, infecting even strains from another genus such as
26 *Shigella*. The one-step growth curve yielded a short latent period of 15 minutes and a burst
27 size of 85 PFU per infected cell. Under the electron microscope, this phage presents the C3
28 morphotype, extremely rare among members of the Podoviridae family. Phage Φ GF1 shows
29 some characteristics that could be considered useful in biocontrol applications against *E. coli*.

30 Keywords: Bacteriophage, *Escherichia coli*, morphotype C3, *Podoviridae*.

31

32 **IMPORTANCE**

33 Wastewater throughout the world is a heavy carrier of potential pathogens that live in their
34 environment along with other biological agents, such as bacteriophages, which play a
35 controlling role of the bacterial populations there, as in soil. The description of the diversity
36 of such bacteriophages is of paramount importance since they could be used to intentionally
37 reduce or remove those pathogens from that environment. Our work describes a
38 bacteriophage that lives primarily in this type of water.

39 INTRODUCTION

40 Bacteriophages are viruses that infect bacteria but no other cellular forms and whose
41 discovery is attributed both to British microbiologist Frederick W. Twort (1915) and to
42 French-Canadian bacteriologist Felix d'Herelle in 1917 (X. Wittebole *et al.*, 2014; D.H.
43 Duckworth, 1976). While the initial interest of the scientific community about bacteriophages
44 was its use as tools to fight bacterial diseases, over time this idea was left aside and its study
45 presented new perspectives, becoming the onset of new techniques today considered
46 elemental in molecular biology, thanks to Schlesinger studies, as well as those of Hershey and
47 Chase (R. Sharp, 2001). Furthermore, although it is very common for bacteriophages to have
48 a high specificity for their host, there are some cases in which the host range is broad and
49 could indicate some utility in biocontrol applications and their ability to model microbial
50 distribution in natural environments (B. Koskella and S. Meaden, 2013; Ross *et al.*, 2016). In
51 any case, this specificity depends on the ability of the virus to bind to bacterial receptors (J.
52 Bertozzi *et al.*, 2016).

53 *Escherichia coli* is a gram-negative rod-shaped bacterium that can cause a wide range of
54 diseases, mainly in the intestinal tract, although pathogenic strains have also been found that
55 affect the urinary tract, the bloodstream and the central nervous system (J.B. Kaper *et al.*
56 2004; M.A. Croxen and B.B. Finlay, 2010).

57 Some strains of *E. coli*, in addition to produce cytotoxins, can also produce other virulent
58 factors such as intimin and hemolysin (P.K. Fagan *et al.*, 1999); for these reasons *E. coli* is
59 considered a pathogen capable of causing diarrhea outbreaks, hemolytic uremic syndrome,
60 hemorrhagic colitis and dysentery, mostly in children (M.A. Croxen *et al.*, 2013) and many of
61 these diseases are transmitted by food.

62 Foodborne diseases are a threat to public health worldwide, mainly because many of these
63 bacteria have become more aggressive and resistant to antibiotics (O.A. Odeyemi and N.A.

64 Sani, 2016). *E. coli* is among the most common pathogenic bacteria that are transmitted by
65 food (T. González and R. Rojas, 2005), in addition generates great economic losses in the
66 food industry, where antibiotics cannot be used because they generate bacterial resistance and
67 physical and chemical treatments to inactivate these bacteria affect the organoleptic properties
68 of food (P. Garcia *et al.*, 2010). It is why non - thermal alternatives are sought for the
69 elimination or reduction of the bacterial load, without affecting the organoleptic
70 characteristics of the food (M. Somolinos *et al.*, 2008).

71 The development of the use of bacteriophages for therapy is long term, since it requires many
72 regulations in the western world and therefore many companies have opted for the application
73 of these viruses in the field of food safety (T.K. Lu and M.S. Koeris, 2011), as the proteins
74 derived from these viruses (endolysins) for the biocontrol of pathogenic bacteria in foods,
75 without altering their organoleptic properties (P. García *et al.*, 2010). Added to this, several
76 studies have shown that bacteriophages can lyse multidrug-resistant bacteria taking advantage
77 of the fact that their mechanism of action is different from that of antibiotics (I. Haq *et al.*
78 2012; C. Verraes *et al.*, 2013; A. Nilsson, 2014).

79 Bacteriophages have already been used for the reduction or elimination of the bacterial load
80 of different pathogens in different types of food, but not all bacteriophages are efficient
81 eliminating in the process of bacterial reduction or elimination. Therefore, it is necessary to
82 know the microbiological, physicochemical and molecular characteristics of the
83 bacteriophage to be used (G.A. Gonçalves *et al.*, 2015).

84 The usefulness of bacteriophages depends both on their own biological properties and on the
85 environment where they will be used, so the goal of this study was the determination of the
86 microbiological and physicochemical properties of the lytic bacteriophage Φ GF1 that infects
87 *Escherichia coli*.

88

89 **RESULTS**

90 *Isolation and purification of bacteriophage*

91 Bacteriophage Φ GF1 was isolated from a positive sample in broth (Figure 1A). Successive
92 double soft layer agar assays led to the isolation of a pure phage, and through the spot test it
93 was possible to demonstrate the lytic activity of this phage against *Escherichia coli* ATCC[®]
94 25922[™] (Figure 1B).

95

96 *Sensitivity to chloroform*

97 The viability of phage Φ GF1 was not affected after 1 hour of exposure to chloroform
98 compared to the control test, in both cases a very similar PFU/mL value was observed (Figure
99 2).

100

101 *Thermal stability*

102 Thermal stability of phage Φ GF1 is maintained at temperature ranging from 40 to 50 °C for
103 up to 1 hour, and the phage is completely inactivated after 30 minutes at 70 °C and 5 minutes
104 at 80 °C, as showed in Figure 3.

105

106 *pH stability*

107 Φ GF1 is stable in the range of pH 5 to 8, while at pH of 9 and 10 its viability decreases up to
108 54 %. Acid environments of pH 3 and 4, significantly affects the viability of the phage,
109 causing a 3-log decrease (Figure 4). However, none of the assayed pH variations completely
110 inactivates Φ GF1 after 1 hour of exposure.

111

112 *Determination of the lysis spectrum of Φ GF1*

113 Thirty one strains were evaluated, among them, other enterobacteria and gram-positive
114 bacteria. Sensitive strains to phage Φ GF1 were *E. coli* GF1, GF2 and EC3 (laboratory wild
115 type strains), along with *Escherichia coli* ATCC[®] 13706[™], *Escherichia coli* ATCC[®] 25922[™]
116 and *Shigella sonnei* ATCC[®] 25931[™].

117

118 ***Multiplicity of infection (MOI) and one-step growth curve***

119 Phage Φ GF1 showed an optimal MOI of 0.01, a value that was used to carry on the one-step
120 curve assay. A burst size of 85 PFU/cell was observed with a latent period of 15 minutes
121 (Figure 5).

122

123 ***Transmission electron microscopy***

124 Ultrastructure of phage Φ GF1 was studied by TEM. According to this, Φ GF1 belongs to the
125 Podoviridae virus family, characterized by an elongated head and a very short tail (Figure 6).
126 In addition, morphological similarities with the viral genus *Phi*eco32virus are observed,
127 including a 125 nm length and a 41 nm width a capsid and a tail with a length of 20 nm and a
128 width of 11 nm.

129

130 **DISCUSSION**

131 Wastewater is a good place to obtain a large number of bacterial strains, namely *Escherichia*
132 *coli*, as well as non-pathogenic, pathogenic and antibiotic-resistant bacteria with great genetic
133 diversity (E. Franz *et al.*, 2015) and therefore along with them, a great diversity of specific
134 bacteriophages. Φ GF1 was isolated from the wastewater treatment plant "La Taboada", in
135 Lima, Peru, showing lytic activity and certain features suitable for future applications.

136 Φ GF1 is resistant to chloroform, which is an organic compound with solvent capabilities, and
137 although the sensitivity to chloroform is associated with the presence of lipids in the viral

138 structure, one third of tailed phages that do not have lipids as part of the particle are sensitive
139 to chloroform (H. W. Ackermann, 2006). In fact, chloroform can act as a destabilizing factor,
140 and in many trials where chloroform is applied to remove the bacterial fraction after phage
141 challenging, filtration is the preferred and only treatment (I.H. Basdew and M.D. Laing,
142 2014).

143 Φ GF1 is very stable at 40 to 50 °C. This is very common in phages such as PA5oct, which
144 infects bacteria in an optimum temperature of 37 °C (Z. Drulis-Kawa *et al.*, 2014). Phage has
145 to be adapted to its host and its environmental conditions. Va1 is a specific bacteriophage to
146 *Vibrio alginolyticus*: the particle is stable at 20 to 30 °C, because *Vibrio* is a bacterial genus
147 usually found in marine environments (C. Fernandez *et al.*, 2017). It is pertinent to add that in
148 certain pathogenic bacteria, such as *Burkholderia thailandensis*, temperature determines the
149 fate of the phage inside the bacterial cell, carrying out a lytic cycle at 37 °C, but going
150 through lysogenic cycle at 25 °C (J. Shan *et al.*, 2014).

151 Phage Φ GF1 is stable between pH 5 and 8, as reported by N. Jamalludeen *et al.* (2007) and
152 N.X. Hoa *et al.* (2014) who determined stability of *E. coli* specific phages in a pH range of 5
153 to 9. Even M.K. Taj *et al.* (2014) have found stable coliphages at pH 4. In a review by E.
154 Jończyk *et al.* (2011), *E. coli* T7 phages can remain stable in pH ranges of 3 to 11, at very low
155 temperatures. Very acidic pHs significantly decrease the viral concentration, but do not
156 entirely eliminate the phage fraction.

157 The latent period of this phage extends to 15 minutes, close to the 19 minutes latent period
158 reported for phage Ω 8 by K. Jann *et al.* (1971), and significantly shorter in comparison to the
159 25 minute latent phase of phage T4 (A.H. Doermann, 1952). Although the burst size of Φ GF1
160 was 85 plaque-forming units per infective center (PFU/IC), not as high as the 100-150
161 PFU/IC for T4 (A.H. Doermann, 1952), its shorter latency period and considerably high burst
162 size make it a good candidate for future applications (M. Middelboe *et al.*, 2010).

163 Φ GF1 has a wide host range, infecting some wild strains of *E. coli*, *E. coli* ATCC[®] 13706[™]
164 and *E. coli* ATCC[®] 25922[™]. The particular thing about this phage is that it is not only
165 infective for strains of the same genus, it is also infective for *Shigella sonnei* ATCC[®]
166 25931[™]. This coincides with previous reports of common infection of *E. coli* and *Shigella*
167 such as phage Φ 24_B and phage CA933P (C.E. James *et al.*, 2001; C. Dini, 2011). This is
168 probably due to the fact that the genus *Shigella* is closely related to enteroinvasive *E. coli*
169 (EIEC) (R. Lan *et al.*, 2004), as well as the direct relationship between the *E. coli*
170 bacteriophages and the acquisition of the Shiga toxin that EIEC strains present there (A.D.
171 O'Brien *et al.*, 1984). Determination of the phage host range is important. P.E₁ phage only
172 infects some pathogenic strains of *E. coli*, which is why it is ideal for phage therapy (Z. Bibi
173 *et al.*, 2016). However, when the spectrum is broad, it can affect the intestinal natural flora, if
174 it is used to this end (J.J. Gill and P. Hyman, 2010). On the contrary, phages with a broad
175 spectrum, such as Φ GF1, have different uses for surface decontamination and for the
176 treatment of superficial infections (I.T. Kudva *et al.*, 1999, S. O'Flaherty *et al.*, 2009) or else
177 as food additives for preventing foodborne diseases (D. Jorquera *et al.*, 2016). Currently uses
178 include biocontrol in wastewater treatment (S.A.A. Jassim *et al.*, 2016).

179 Morphology of Φ GF1 was determined by transmission electron microscopy (TEM). It has a
180 C3 morphotype, characterized by a capsid length that exceeds its width by several times.
181 Phages with this morphotype are extremely rare among members of the *Podoviridae* family
182 (Y. Li *et al.*, 2012) and when they are specific for enterobacteria, they are usually related by
183 serology and DNA homology (F. Grimont and P.A.A. Grimont, 1981). Φ GF1 could belong to
184 the genus *Phi*co32virus that has only 6 bacteriophage species, all infective for *E. coli*. This
185 will only be confirmed by genome sequencing.

186 In conclusion, bacteriophage Φ GF1 has a short latency period, a considerable burst size, and
187 has a wide host range, characteristics that make it a good candidate for a diversity of

188 biocontrol of applications *E. coli*, besides presenting an uncommon morphology to other
189 bacteriophages already reported.

190

191 **MATERIALS AND METHODS**

192 *Bacterial strain*

193 The strain used as a host for the isolation of bacteriophage Φ GF1 was a wild type *Escherichia*
194 *coli*, isolated from "La Taboada" wastewater treatment plant, in Lima, Peru. After checking its
195 infectivity against *Escherichia coli* ATCC[®] 25922TM, the characterization tests were carried
196 out with this strain, which was maintained in Heart Brain Infusion broth (BHI, MerckTM) at
197 37 °C for 24 hours.

198

199 *Phage Isolation, purification and propagation*

200 Phage Φ GF1 was isolated from wastewater prior processing at the treatment plant. A 300 mL
201 sample was filtered through Whatman grade 1 paper. It was then filtered again in a vacuum
202 pump (Boeco TM R-300) using 0.45 μ m nitrocellulose membranes (Durapore[®], MerckTM).

203 To demonstrate the presence of bacteriophages in the sample and increase their number, a
204 qualitative method was carried out; modifying what was done by S. George *et al.* (2014).
205 Briefly, in 10 mL of BHI broth, 1 mL of the filtrate was added along with 100 μ L of a log
206 phase *Escherichia coli* ATCC[®] 25922TM broth culture. A control assay was made by adding
207 10 mL of BHI broth, 1 mL of phosphate buffered saline (PBS) and 100 μ L of the same
208 bacterial strain. Both tubes were incubated at 37 °C for 8 hours.

209 The mix showing a significant clearance against the control test tube was centrifuged at 8,000
210 rpm for 8 minutes and the supernatant was filtered through a 0.45 μ m nitrocellulose
211 membrane. To evaluate the presence of bacteriophages, a "spot test" was applied by plating
212 100 μ L of a *Escherichia coli* ATCC[®] 25922TM overnight culture over a lawn of Tryptic Soy

213 Agar (TSA, Merck Millipore™), and then adding 100 µL of the virus filtrate in each of three
214 thirds of the plate.

215 The positive spot test filtrate was mixed with the host strain using the double agar layer
216 technique (M.H. Adams, 1959), to produce lysis plaques with similar morphology, according
217 to N. Jamalludeen et al. (2007). The isolated lysis plaques were transferred to phosphate
218 buffered saline. This viral suspension was mixed with the bacterial host two more times in the
219 same manner until the lysis plaques were uniform in shape and diameter, ensuring the purity
220 of our bacteriophage. This last lysis plaque was cut from the agar layer, resuspended in
221 phosphate buffered saline, filtered and mixed with a culture of *Escherichia. coli* ATCC®
222 25922™ in BHI broth and incubated at 37 °C for 8 hours. Finally, this mixed solution was
223 centrifuged at 4,400 $\times g$ (Thermo Scientific ST8R) for 30 minutes and filtered through
224 nitrocellulose membranes (0.45 µm). This bacteriophage suspension was stored at -4 °C.

225

226 ***Effect of chloroform***

227 To estimate sensitivity to chloroform, C. Chenard *et al.* (2015) methodology was slightly
228 modified. Briefly, 500 µL of phage suspension (2×10^{10} PFU·mL⁻¹) was mixed with 500 µL
229 of extra pure chloroform (Merck Millipore™) and kept under 250 rpm·min⁻¹ for 1 hour. It
230 was then centrifuged at 4 100 $\times g$ for 5 minutes and the supernatant was transferred to a
231 microcentrifuge vial; then it was incubated for 6 hours at room temperature to remove any
232 chloroform residue. As a control, the same procedure was performed with 500 µL of the
233 phage suspension and 500 µL of saline solution (NaCl 0.9 % w/v). Each assay was performed
234 in duplicate and the concentration was determined by the agar double layer technique (M.H.
235 Adams, 1959).

236

237 ***Effect of temperature***

238 The thermal stability of the Φ GF1 phage was tested at 40, 50, 60, 70 and 80 °C for 0, 5, 15,
239 30, 45 and 60 minutes using a phage titer of 2×10^{10} PFU·mL⁻¹ (Z. Drulis-Kawa *et al.*, 2014).
240 Each experiment was performed in triplicate and the phage titer was determined by the agar
241 double layer technique.

242

243 ***Effect of pH***

244 To determine the stability of the Φ GF1 phage against pH variations, pH ranging from 3 to 10
245 for 1 hour was assayed, slightly modifying what N.X. Hoa *et al.* (2014) proposed. 100 μ L of
246 phage suspension (1.3×10^8 PFU·mL⁻¹) was added to 900 μ L of saline solution (NaCl 0.9 %
247 w/v), set to a specific pH and incubated at 37 °C for 1 hour. As a control test, 100 μ L of phage
248 suspension was inoculated in 900 μ L of saline solution (NaCl 0.9 % w/v) without changing
249 pH. After incubation, each sample was adjusted to pH 7 (N.X. Hoa *et al.*, 2014). Each test of
250 pH stability was carried out in triplicate and the phage titer was determined by the double agar
251 overlay technique (M.H. Adams, 1959).

252

253 ***Host range***

254 Bacterial susceptibility to Φ GF1 phage was demonstrated by a spot test. 100 μ L of an
255 overnight culture of each bacterial strain were tested against 100 μ L of phage (2×10^{10}
256 PFU·mL⁻¹) on TSA, spotted in three different regions of a plate, following incubation at 37 °C
257 for 24 hours (C. Dini and P.J. de Urza, 2010).

258

259 ***Determination of multiplicity of infection (MOI) and one-step growth curve***

260 The optimal multiplicity of infection (MOI) of the bacteriophage was determined following L.
261 Li and Z. Zhang (2014), infecting *Escherichia coli* strain ATCC[®] 25922TM at 3 different MOI
262 (0.01, 0.1 and 1) at 37 °C for 4 hours. For the one-step growth curve experiment, 100 μ L of an

263 overnight culture of *Escherichia coli* ATCC® 25922™ was inoculated to 10 mL of BHI broth
264 and incubated at 37 °C to reach a 10⁸ CFU mL⁻¹ titer (0.5 McFarland standard). Later 1 mL of
265 this broth was mixed with 1 mL of ΦGF1 phage suspension, at the optimal MOI previously
266 determined, following incubation at 37 °C for 10 minutes and then centrifuged at 4 000 x g
267 for 3 minutes. Pellet was resuspended in 2 mL of BHI broth. 100 µL of this broth were
268 transferred to 50 mL of BHI broth and incubated at 37 °C (M. Middelboe et al., 2010).
269 Samples (in duplicate) were taken every 5 minutes for 60 minutes and assayed by the double
270 agar overlay technique (M.H. Adams, 1959).

271

272 *Electron microscopy of ΦGF1*

273 A concentrated phage sample was negatively stained with 2 % (w/v) uranyl acetate (pH 4.0)
274 on a Formvar-coated copper grid and examined by Transmission Electron Microscopy (JEOL
275 JEM-1400 Plus) (N. Jamalludeen *et al.*, 2007). Phage size was determined from the average
276 of three independent measurements.

277

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285

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413

414 **TABLE LEGEND**

415

416 Table 1. Bacterial strain susceptibility against Φ GF1.

417

418 **FIGURE LEGENDS**

419

420 Figure 1. (A) Broth clearance after 8 hours incubation suggests the presence of
421 bacteriophages. (B) Positive "spot test" of Φ GF1 against *Escherichia coli* ATCC® 25922™,
422 where three lysis spots are observed.

423

424 Figure 2. The effect of chloroform on Φ GF1 stability after 60 minutes of exposure.

425

426 Figure 3. Effect of temperature on bacteriophage stability checked at 40 °C, 50 °C, 60 °C, 70
427 °C and 80 °C after 5, 15 30, 45 and 60 minutes.

428

429 Figure 4. Effect of pH on bacteriophage stability. Φ GF1 lysate was treated at different pH
430 values (3, 4, 5, 6, 7, 8, 9 and 10) for one hour at 37 °C and followed by calculating phage titer
431 by the double agar overlay technique.

432

433 Figure 5. One-step growth curve of phage Φ GF1. The graph shows the plaque-forming units
434 at different times (in minutes). The length of the latent period is 15 minutes and the burst size
435 was estimated to be 85 PFU per each infected cell.

436

437 Figure 6. TEM image of Φ GF1. Morphology corresponds to the C3 morphotype from the
438 viral family Podoviridae (order Caudovirales), with a rare elongated head connected to a short
439 contractile tail by a short neck. Scale bar represents 100 nm.

440

441

442

443 **TABLE**

Species	Strain	Φ GF1
<i>E. coli</i> GF1	Wild type	+
<i>E. coli</i> GF2	Wild type	+

<i>E. coli</i> AR	Wild type	-
<i>E. coli</i> EC1	Wild type	-
<i>E. coli</i> EC2	Wild type	-
<i>E. coli</i> EC3	Wild type	+
<i>E. coli</i> EC5	Wild type	-
<i>E. coli</i> EC6	Wild type	-
<i>E. coli</i> EC7	Wild type	-
<i>E. coli</i> EC8	Wild type	-
<i>Escherichia coli</i>	ATCC® 13706™	+
<i>Escherichia coli</i>	ATCC® 25922™	+
<i>Shigella sonnei</i>	ATCC® 25931™	+
<i>Shigella flexneri</i>	ATCC® 12022™	-
<i>Salmonella</i> Typhimurium	ATCC® 14028™	-
<i>Salmonella enterica subsp. enterica</i> <i>serovar Abortusequi</i>	ATCC® 9842™	-
<i>Salmonella enterica subsp. enterica</i> <i>serovar Enteritidis</i>	ATCC® 13076™	-
<i>Proteus vulgaris</i>	ATCC® 6380™	-
<i>Proteus mirabilis</i>	ATCC® 12453™	-

<i>Enterobacter aerogenes</i>	ATCC® 13048™	-
<i>Pseudomonas aeruginosa</i>	ATCC® 15442™	-
<i>Vibrio cholerae</i>	Wild type	-
<i>Vibrio parahaemolyticus</i>	ATCC® 17802™	-
<i>Enterococcus faecalis</i>	ATCC® 29212™	-
<i>Streptococcus agalactiae</i>	ATCC® 12386™	-
<i>Staphylococcus epidermidis</i>	ATCC® 12228™	-
<i>Bacillus cereus</i>	ATCC® 14579™	-
<i>Listeria monocytogenes</i>	ATCC® 19114™	-
<i>Listeria monocytogenes</i>	ATCC® 19115™	-
<i>Listeria ivanovii</i>	ATCC® 19119™	-
<i>Listeria innocua</i>	ATCC® 33090™	-

444 + = susceptible strain

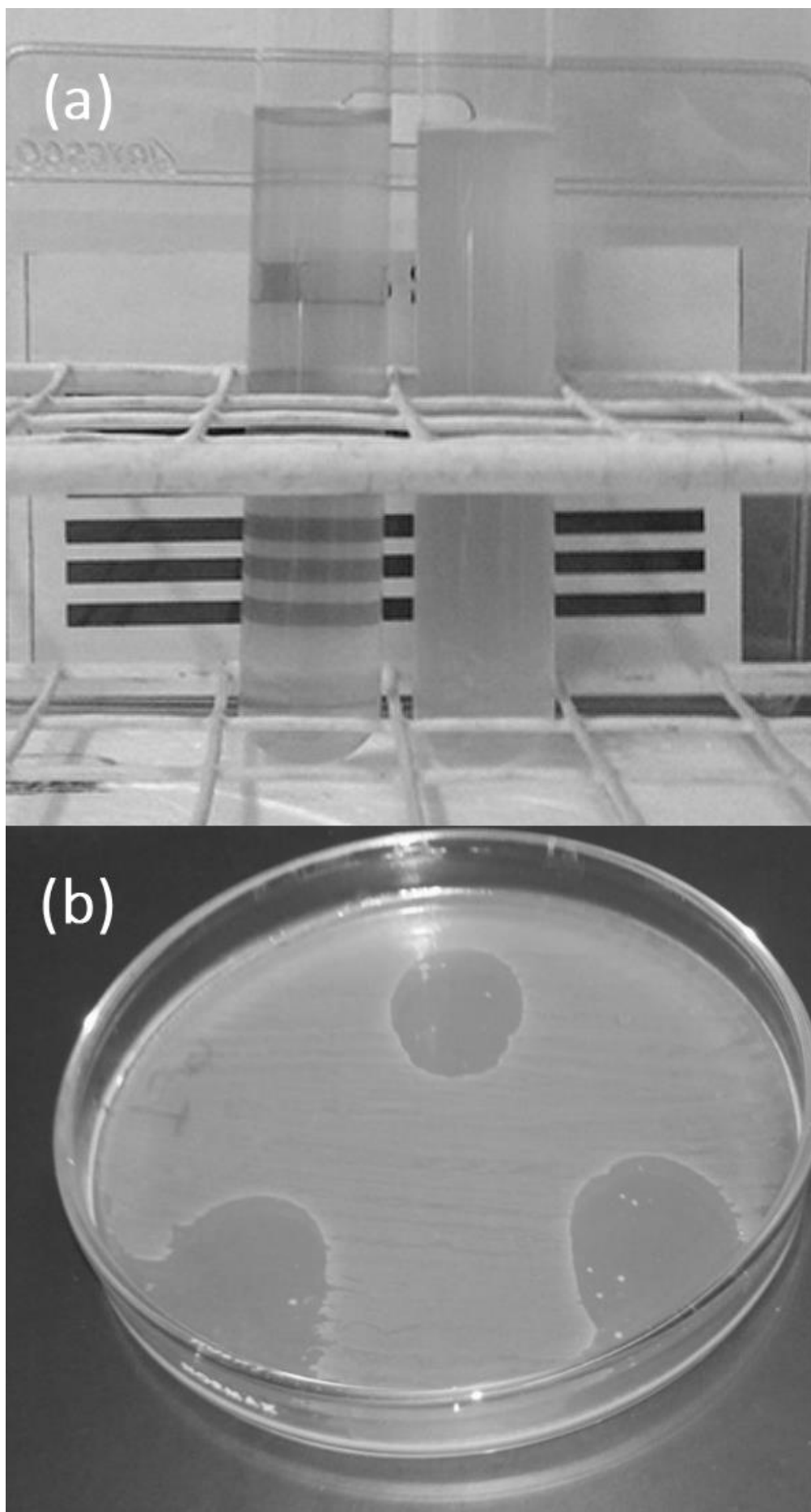
445 - = non-susceptible strain

446

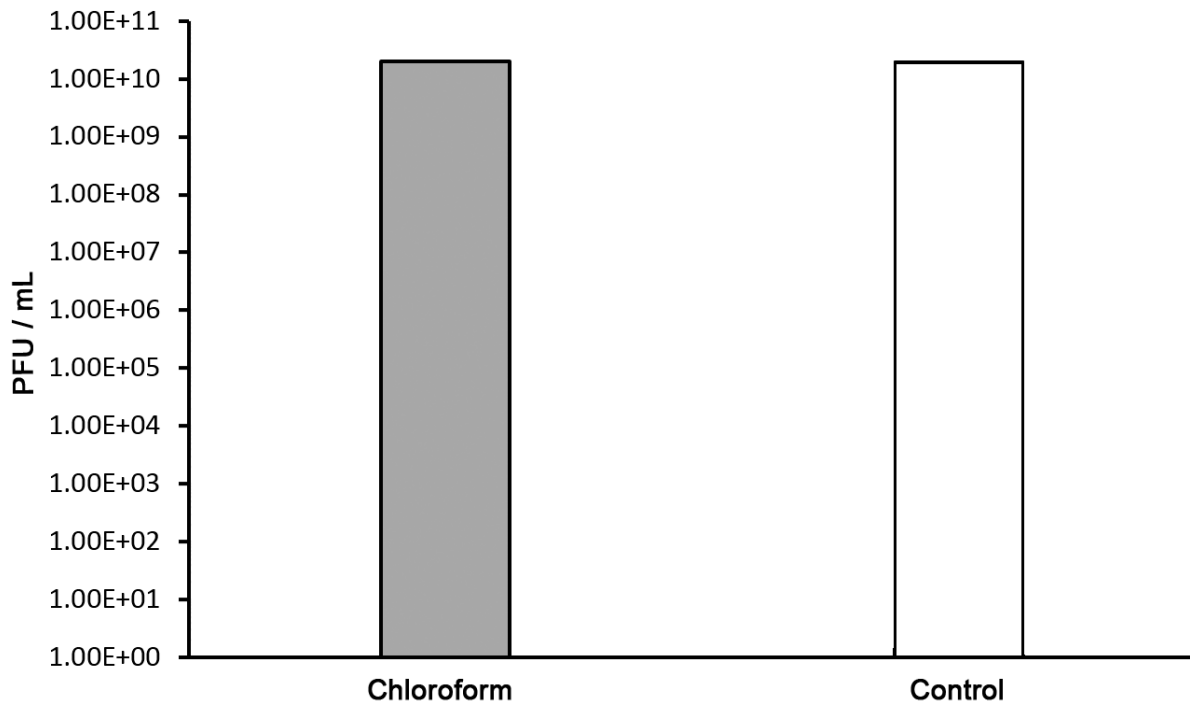
447 **FIGURES**

448

449 **FIGURE1**



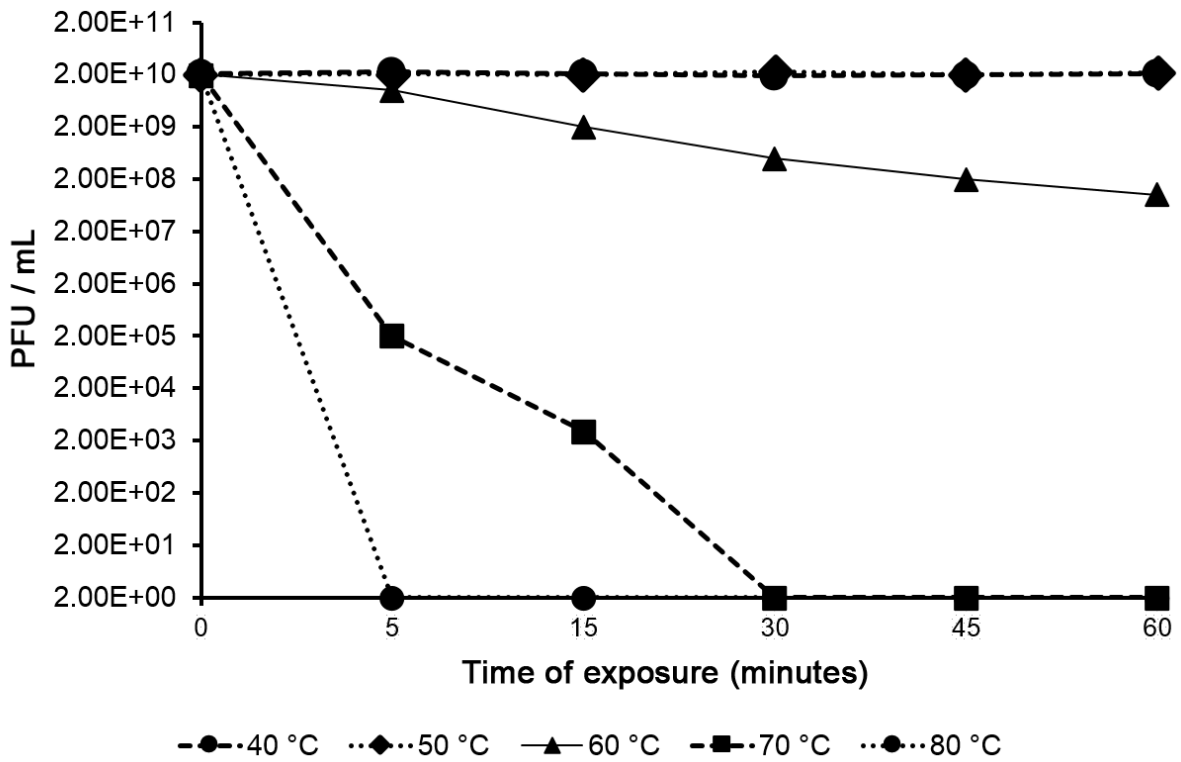
451 **FIGURE 2**



452

453

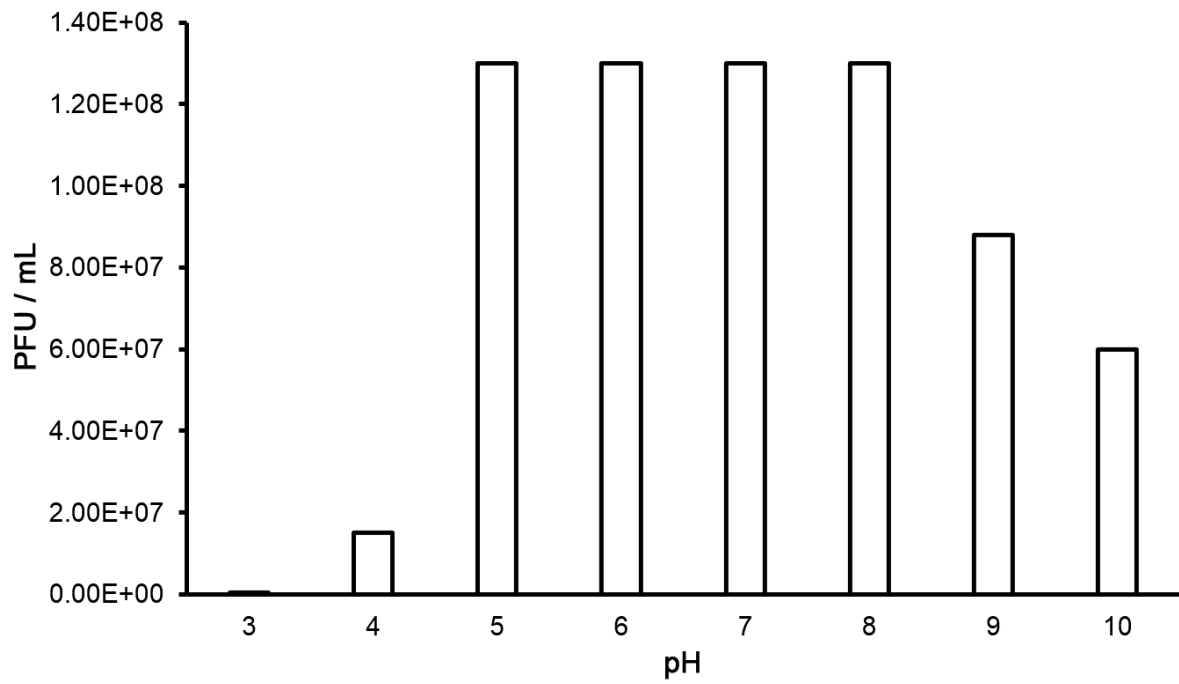
454 **FIGURE 3**



455

456

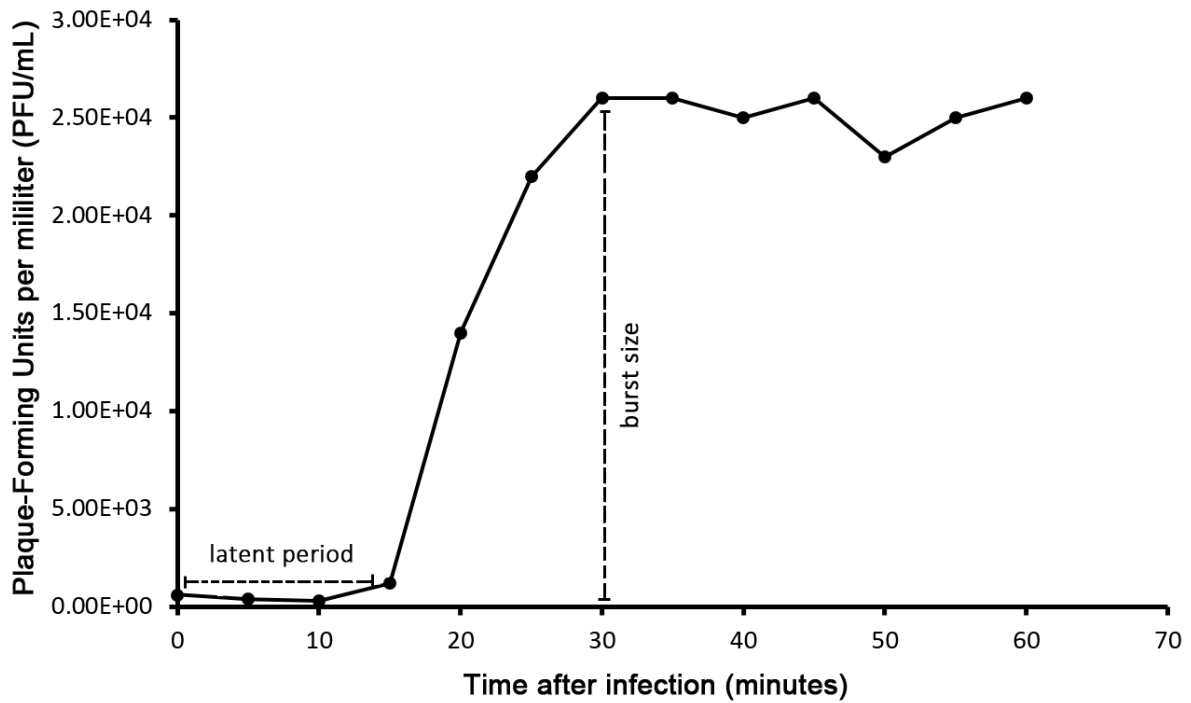
457 **FIGURE 4**



458

459

460 **FIGURE 5**

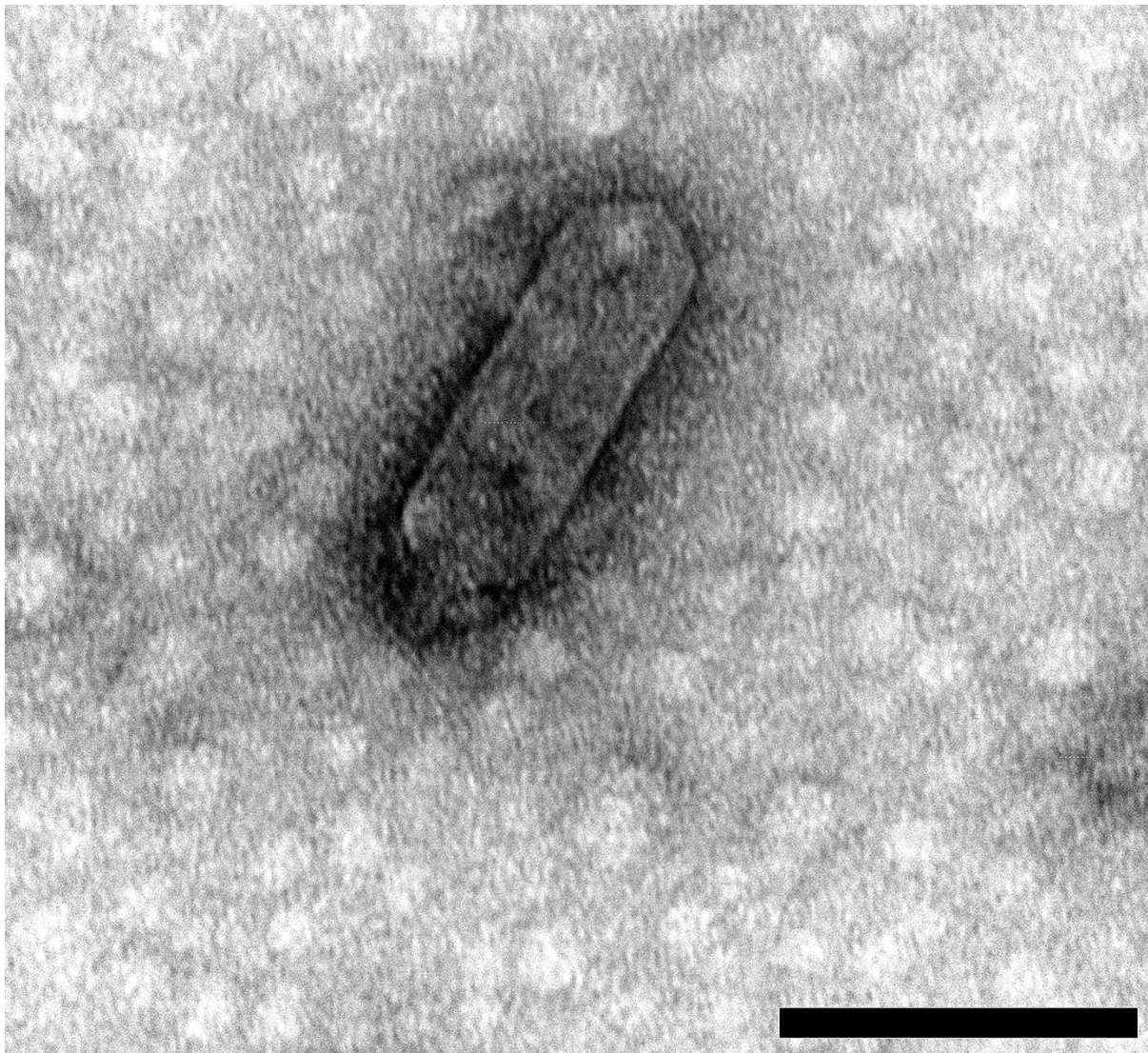


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464 **FIGURE 6**



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