Determination of the Optimal Bacteriophage Dose to Control *Pseudomonas aeruginosa* using evolutionary programming and stochastic kinetics

Diana C Ardila [1], **Juan D Castro** [1], **Angela V Holguín** [2], **Viviana Clavijo** [2], **Catalina Prada** [2], **Martha J Vives** [2], **Andres F Andres Gonzalez Barrios** [Corresp. 1]

[1] Department of Chemical Engineering, Universidad de Los Andes, Bogota, CUNDINAMARCA, Colombia

[2] Department of Biological Sciences, Universidad de Los Andes, Bogota, CUNDINAMARCA, Colombia

Corresponding Author: Andres F Andres Gonzalez Barrios
Email address: andgonza@uniandes.edu.co

Phage-therapy is a promising alternative against pathogenic, multiple drug resistant bacteria. In this work we propose an algorithm to determine the optimal bacteriophage dose able to minimize a population of *Pseudomonas aeruginosa.* Reverse engineering was used to determine the kinetic parameters; subsequently, a bi-level optimization platform was implemented for a model based on evolutionary programming. Our prediction of optimal dose was tested *in vitro* with planktonic cultures of *P. aeruginosa*. From the data obtained, we conclude that reverse engineering and stochastic simulations are a useful approach to find optimal phage doses against pathogenic bacteria, an important step for the implementation of phage-therapy.

Determination of the Optimal Bacteriophage Dose to Control *Pseudomonas aeruginosa* using evolutionary programming and stochastic kinetics

Diana Catalina Ardila[1], Juan David Castro[1], Ángela Victoria Holguín[2], Viviana Clavijo[2], Catalina Prada[2], Martha Josefina Vives[2] and Andrés Fernando González-Barrios[1*]

1. Department of Chemical Engineering, Carrera 1E N 19 A - 40, Universidad de los Andes, Bogotá, Colombia. Tel (571) 3394949
2. Department of Biological Sciences, Universidad de los Andes, Carrera 1E N 18A- 10, Bogotá, Colombia. Tel (571) 3394949

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2359v1 | CC BY 4.0 Open Access | rec: 15 Aug 2016, publ: 15 Aug 2016

ABSTRACT

Phage-therapy is a promising alternative against pathogenic, multiple drug resistant bacteria. In this work we propose an algorithm to determine the optimal bacteriophage dose able to minimize a population of *Pseudomonas aeruginosa.* Reverse engineering was used to determine the kinetic parameters; subsequently, a bi-level optimization platform was implemented for a model based on evolutionary programming. Our prediction of optimal dose was tested *in vitro* with planktonic cultures of *P. aeruginosa*. From the data obtained, we conclude that reverse engineering and stochastic simulations are a useful approach to find optimal phage doses against pathogenic bacteria, an important step for the implementation of phage-therapy.

Keywords: Mathematical modeling, Phage-therapy, Optimal dose, *Pseudomonas aeruginosa*,

1. INTRODUCTION

The treatment of intra-hospital infections (nosocomial infections) has turned out to be an enormous challenge as the current microorganisms display high capability to resist multiple varieties of antibiotics. A number of bacterial resistance mechanisms against antibiotics exist

with some examples including: diffusional limitation provided by an exopolysaccharide matrix from biofilm assembly,[1] active site mutations where an antibiotic is no longer capable of binding to the cell wall and toxic molecule ejection through efflux pumps and transmembrane proteins,[2] among others.

Hospitals and more specifically Intensive Care Units are generally considered epicenters of antibiotic resistance and the principal sources of outbreaks of multiple drug-resistant bacteria.[3] For this reason, biomedical communities have been urged to investigate new anti-bacterial treatments such as the next generation drugs and therapies with improved spectrums against resistant microorganisms.[4] Approaches based on bacteriophages (or phages), known as phage-therapy, constitute an interesting alternative due to the ease of isolating phages capable of targeting antibiotic resistant bacteria. Also, viruses evolve with their host allowing them to infect phage-resistant cells when they would appear. Regarding the diffusional obstacles, antibiotics in general have to overcome several hurdles due to the presence of exopolysaccharides when the biofilm phenotype is present; phages, on the other hand, are in theory capable of efficiently penetrating the biofilm so the infection would be deeply inhibited.[5] The mechanism exploited in phage therapy is the obligatorily lytic life cycle when the viral particles recognize the bacterial cell surface, followed by reversible and irreversible binding and the injection of its DNA or RNA from phage capsid into the host. Once the phage genome has shut down, most of the host's proteins are amplified inside the cell using its host's molecular machinery and consequently viral progeny are formed. Finally, cell lysis occurs resulting in progeny exiting the cell and repeating a new infective cycle.[6] *P. aeruginosa* is one of the principal causes of acquired infections and mortality in hospitals.[7] This microorganism is highly adaptive, considered an opportunist nosocomial pathogen and constitutes a high risk microorganism because of its virulence and

resistance to most antibiotics currently available.[8] Moreover, it affects different organs and anatomical sites such as the upper respiratory tract, lungs, heart valves, urinary tract, surgical tract and open wounds.[7]

The advent of mathematical biology has allowed a greater understanding of the underpinnings of several biological events with important applications for disease control. In order to get a better comprehension of the dynamics of the infection process deterministic and stochastic modeling was applied to predict the behavior of the phages infecting a planktonic population of several bacterial species.[9-12] Deterministic approaches are commonly used and assume that the species in the system change continuously and deterministically over time and are based on ordinary differential equations (ODES). These models describe the dynamics of the system in terms of the species present, and parameters related with the rates of change in the concentration of these species.[12] From this approximation, it's possible to make an evaluation of the parameters that have an influence on the rate of the infective process (kinetic parameters). Stochastic models are based on random collisions among reacting species, making them useful to simulate or predict events in biological systems such as metabolic regulation systems and genetic networks. The stochastic approach allows modeling phage-host infection in which the effects of noise, variations, and uncertainty are reflected in the system dynamics.[13]

Once the dynamics of the infection are understood, the search space or feasible region can be predicted to find the optimum therapeutic dose that would allow for controlling the pathogen and mitigate the occurrence of phage-resistance.[14] To do so, it's necessary to establish an optimization algorithm with an efficient searching capacity, satisfactory robustness and low computational demand. Our group has prior experience with using Monte Carlo-based algorithms to find the global optimum and one such example was simulated annealing to find the

quencher dose in *P. aeruginosa* quorum sensing networks.[15] Contrary to deterministic optimization, most of stochastic methods are not gradient based so either stochastic or deterministic models can be used as a template to find the optimum.

Different authors have studied the bacteriophage-host dynamics from only one or the other of the two approaches. Cairns *et al.* studied *Campylobacter* and bacteriophage interactions using a kinetic model based on ODES.[16] Jain et al used five different deterministic models to evaluate the infection dynamics of phage MS2 in its host *Escherichia coli.*[12] Arkin *et al* used a stochastic kinetic analysis to investigate the mechanism that causes the lysis-lysogeny decision circuit of phage λ in *E. coli.*[10] In a more recent study, Bardina *et al* made a stochastic formulation to study bacteriophage treatments for infections.[17] All these works have been made to understand the dynamics of phage-bacteria systems and calculate the parameters that affect the specific case of study.

Here in this study we proposed and applied an optimization platform, based on deterministic and stochastic-derived models built from previous experimental data to find the optimal dosage able to diminish a *P. aeruginosa* population. The experimental validation of the predicted dose was performed in three different ways: First, elucidating the behavior *in vitro* of the infection process. Second, evaluating the extrapolable characteristic of the dose to other phage-bacteria systems. And third, corroborating the optimal value of the dose performing a challenge test.

2. MATERIALS AND METHODS

2.1. Microorganism, growth medium and experimental curves acquisition

In this study, three *Pseudomonas aeruginosa* strains resistant to multiple antibiotics (named *P. aeruginosa* P1, P3 and P4, kindly donated by Dr. Claudia Echeverri from Hospital Federico

LLeras Acosta, Ibagué, Colombia) were used. The three strains were used to isolate lytic bacteriophages; among them, three were selected for the assays (named F1, F2 and F3) (unpublished results). Bacterial strains were conserved by ultra-low freezing at -80°C in 10% glycerol. Bacteriophages were maintained in Salt Medium buffer, SM (Composition: 0.05 mol/L of Tris-HCl pH 7.5, 0.1 mol L^{-1} of NaCl, 10 mol/m^3 $MgSO_4$, gelatin at 1% w/v) at 4°C.

In order to establish the bacterial growth kinetics, two different sets of experiments were done in triplicate; first, individual growth curves for strains of *P. aeruginosa* P1, P3 and P4 were performed. Each strain was inoculated in 3 cm^3 of minimal salt medium (MSM) (composition per liter: KH_2PO_4 3.5 g, $(NH_4)_2HPO_4$ 1.0 g, $MgSO_4$ 1.2 g, glucose 5.0 g, trace elements solution 12.0 cm^3; trace elements solution composition per liter: iron citrate III 60 mg, EDTA III 8.4 mg, $CoCl_2.6H_2O$ 2.76 mg, $MnCl_2.4H_2O$ 15 mg, zinc acetate 8.4 mg, $Na_2MoO_4.2H_2O$ 2.67 mg, H_3BO_3 3.3 mg, $CuCl_22H_2O$ 1.5 mg). Cultures were grown overnight at 200 rpm and 37°C. Three hundred µl of the overnight culture was transferred to 30 cm^3 of MSM; these day cultures were incubated for 20 hours at 200 rpm and 37°C. Then, 3 cm^3 of the day cultures were transferred to 30 cm^3 of MSM and were incubated at 37°C and 200 rpm, and for the duration of 18 hours optical density, colony forming units per cubic centimeter (CFU/cm^3) and glucose concentrations (*BioSystems*® glucose kit) were measured. This data corresponds to what is will now be referred to as uninfected cultures. Second, to determine the effect of bacteriophages on the bacterial strains, each strain was grown as described above to the logarithmic phase; then, 200 µl of phage F1 at a concentration of 10^5 PFU/cm^3approximately (PFU, plaque forming units), was added to the bacterial cultures. Cultures were followed for 18 hours in triplicate where the optical density, viable cell concentration and glucose concentration, as already described for the uninfected cultures, were measured.

2.2. Deterministic mathematical models

In order to model the infection process in *P. aeruginosa*, six deterministic models were formulated, each one making different assumptions about the system.

Model 1. This model classifies bacteria cells as uninfected and infected. The population density increases only due to the growth of uninfected cells.[12]

$$\frac{dx}{dt} = \mu_x x - k_1 P x - k_2 x \quad (1)$$

$$\frac{dy}{dt} = k_1 P x - k_3 y \quad (2)$$

$$\frac{dP}{dt} = k_4 y - k_1 P x - k_5 P \quad (3)$$

$$\frac{ds}{dt} = -\mu_x x \frac{1}{y_{x/s}} \quad (4)$$

$$\mu_x = \frac{\mu_{\max,x} s}{K_{s,x} + s} \quad (5)$$

Where x is the density of uninfected cells/bacterial survivors, y is the density of phage-pregnant cells , p is the supernatant or free phage density, s is the substrate (glucose) concentration, μ_x is the growth rate of uninfected cells, k_1 is the infection rate, k_2 is the death rate of uninfected cells, k_3 is the rate of lysis of infected cells, k_4 is the rate at which progeny phage are produced, k_5 is

the rate at which supernatant phage particles degrade, $y_{x/s}$ is the yield factor relating production of uninfected bacterial cells to substrate consumed, $\mu_{max,x}$ is the maximal growth of uninfected cells, and $K_{s,x}$ is the Monod constant for uninfected cells. The uninfected cell density corresponds to the counts of viable cells in culture, measured in CFU/cm^3; the infected cell density is the difference between the counts in CFU/cm^3 of the infected culture and those of uninfected cultures (comparing a phage infected flask to a control flask). Free phage densities for the different cultures were obtained based on the profiles provided during the solution of the set of differential equations for each model.

Model 2. Here, moreover uninfected cells, infected cells display growth at the same rate. This model is derived from Model 1.

$$\frac{dx}{dt} = \mu_x x - k_1 P x - k_2 x \tag{6}$$

$$\frac{dy}{dt} = \mu_x y + k_1 P x - k_3 y \tag{7}$$

$$\frac{dP}{dt} = k_4 y - k_1 P x - k_5 P \tag{8}$$

$$\frac{ds}{dt} = -\mu_x x \frac{1}{y_{x/s}} - \mu_x y \frac{1}{y_{x/s}} \tag{9}$$

$$\mu_x = \frac{\mu_{\max,x} s}{K_{s,x} + s} \quad (10)$$

Model 3. In this model, uninfected and infected cells grow at different rates; equations derived from this assumption follows:

$$\frac{dx}{dt} = \mu_x x - k_1 P x - k_2 x \quad (11)$$

$$\frac{dy}{dt} = \mu_y y + k_1 P x - k_3 y \quad (12)$$

$$\frac{dP}{dt} = k_4 y - k_1 P x - k_5 P \quad (13)$$

$$\frac{ds}{dt} = -\mu_x x \frac{1}{y_{x/s}} - \mu_y y \frac{1}{y_{y/s}} \quad (14)$$

$$\mu_x = \frac{\mu_{\max,x} s}{K_{s,x} + s} \quad (15)$$

$$\mu_y = \frac{\mu_{\max,y} s}{K_{s,y} + s} \quad (16)$$

Where μ_y is the growth rate of infected cells, $\mu_{max,y}$ is the maximal growth rate of infected cells, $K_{s,y}$ is the Monod constant for infected cells, and $y_{y/s}$ is the yield factor relating production of infected bacterial cells to substrate consumed.

Model 4. Here, only uninfected cells grow; and are split in two different populations: susceptible to the phage (z), and resistant to the phage (R). Resistant cells display growth.

$$\frac{dz}{dt} = \mu_z z - k_1 P z - k_2 z \quad (17)$$

$$\frac{dR}{dt} = \mu_R R - k_6 R \quad (18)$$

$$\frac{dy}{dt} = k_1 P z - k_3 y \quad (19)$$

$$\frac{dP}{dt} = k_4 y - k_1 P z - k_5 P \quad (20)$$

$$\frac{ds}{dt} = -\mu_z z \frac{1}{y_{z/s}} - \mu_R R \frac{1}{y_{R/s}} \quad (21)$$

$$\mu_z = \frac{\mu_{\max,z} s}{K_{s,z} + s} \quad (22)$$

$$\mu_R = \frac{\mu_{\max,R} s}{K_{s,R} + s} \quad (23)$$

$$x = R + z \quad (24)$$

Model 5. In this model, only uninfected cells grow, and are split in two different populations: susceptible to the phage (z), and resistant to the phage. Resistant cells display growth. The lysis of infected cells is unleashed after 480 min.

$$\frac{dz}{dt} = \mu_z z - k_1 P z - k_2 z \quad (25)$$

$$\frac{dR}{dt} = \mu_R R - k_6 R \quad (26)$$

$$\frac{dy}{dt} = k_1 Pz - k_3 yU \quad (27)$$

$$\frac{dP}{dt} = k_4 yU - k_1 Pz - k_5 P \quad (28)$$

$$\frac{ds}{dt} = -\mu_z z \frac{1}{y_{z/s}} - \mu_R R \frac{1}{y_{R/s}} \quad (29)$$

$$\mu_z = \frac{\mu_{\max,z} s}{K_{s,z} + s} \quad (30)$$

$$\mu_R = \frac{\mu_{\max,R} s}{K_{s,R} + s} \quad (31)$$

$$x = R + z \quad (32)$$

Where U is 0 when $t \leq 480$ min and 1 when t > 480min.

Model 6. Finally, for model six, uninfected and infected cells grow at different rates; and lysis of infected cells occurs after 480 min.

$$\frac{dx}{dt} = \mu_x x - k_1 P x - k_2 x \quad (33)$$

$$\frac{dy}{dt} = \mu_y y + k_1 P x - k_3 y U \quad (34)$$

$$\frac{dP}{dt} = k_4 y U - k_1 P x - k_5 P \quad (35)$$

$$\frac{ds}{dt} = -\mu_x x \frac{1}{y_{x/s}} - \mu_y y \frac{1}{y_{y/s}} \quad (36)$$

$$\mu_x = \frac{\mu_{\max,x} s}{K_{s,x} + s} \quad (37)$$

$$\mu_y = \frac{\mu_{\max,y} s}{K_{s,y} + s} \quad (38)$$

Simulated annealing and genetic algorithms were used to perform the parametric regression from experimental data with an estimated phage dose of $1x10^5$ PFU/cm^3 using the System biology toolbox in Matlab®.[18] Finally the three individual models that best described each system *in vitro* (*P. aeruginosa* P1, P3 and P4 infected with phage F1) were merged to create a petri net capable of describing the infection dynamics of F1 infecting these three strains in unison (Fig. 1).

2.3. Determination of the optimal dose

The optimization problem consisted of finding the optimum initial phage dose and the time in which bacteria must be exposed to phages to minimize the population of each strain tested

(initial time of infection) using the global model built from each interaction analyzed. The optimization problem can be posed as:

$$\min PD_{ti,x_{p1(0)},x_{p3(0)},x_{p4(0)}} \tag{34}$$

$$s.t 0.1 < x_{p1(t_i)}, x_{p3(t_i)}, x_{p4(t_i)} \tag{35}$$

where t_i is the time where the total bacterial population is minimized, $x_{p1(0)}$, $x_{p3(0)}$, $x_{p4(0)}$ are the initial conditions of living cells for each *P. aeruginosa* strain and $x_{p1(t_i)}$, $x_{p3(t_i)}$, $x_{p4(t_i)}$ represent the cell population for each strain at t_i and PD is the initial phage dose.

2.4. Stochastic modeling for optimal dose validation in silico

We previously emphasized the need for validating the predicted dose in biological systems due to the presence of uncertainty and variation. It is known that this noise could lead to the appearance of dispersion in the population besides the objective function dispersion, so the dose reported by our platform cannot represents the actual dose to eradicate the presence of the pathogen.

To assess this, we performed stochastic simulations by numerically solving the master equation based on the Gillespie algorithm. A review of the main features of the master equation formalism and the Gillespie algorithm is given by Gillespie, 1977.[13] Briefly, the master equation is used to describe the evolution of a system over time that can be in one particular state at a given time point and then switch between states and be treated probabilistically. With the Gillespie

algorithm it's possible to simulate the temporal behavior of a system by calculating the probability of each discrete event occurring, and the resulting changes in the number of each species participating. For each simulation that was done, it gave a representative case of the timing and the sequence of events of phage infection in individual bacteria. In order to obtain statistical significance and a more accurate representation of the system multiple runs with the same initial conditions were done. For our purposes petri nets were derived from a merged deterministic model (Fig. 1) and one hundred thousand runs were performed with the optimum dose predicted by the genetic algorithm. For this, we deployed two different virtual clusters in three computer rooms with 35 computers each. All computers had an Intel Core 2 Duo 1.8 GHz processor and 4GBs of RAM. Virtual machines were assigned with both cores and 1 GB of RAM.

2.5. Experimental validation of the optimal dose

The predicted dose was experimentally validated *in vitro* in order to test the accuracy of the computational models. To achieve this, each strain was grown in MSM to logarithmic phase, established by the deterministic models: *P. aeruginosa* P1 was grown up to 10^9 CFU/cm^3, *P. aeruginosa* P3 up to 10^8 CFU/cm^3, *P. aeruginosa* P4 up to 10^6 CFU/cm^3. At the desired cell density, each bacteriophage was added, separately, at a concentration of 10^7 PFU/cm^3. The cultures were monitored for 1100 minutes where optical density and bacterial survivors were measured.

Additional tests were also performed with the P4 strain and the phage F1 to assess the potential difference in kinetics with varied phage doses above and below the optimal dose. P4 was grown in MSM to logarithmic phase, as described earlier, in two different cultures and the phage were

added at a final concentration of 10^6 PFU/cm^3 and 10^8 PFU/cm^3, respectively. Cultures were monitored for 1100 min and optical densities and bacterial survivors were measured.

All the experiments explained in this section were carried out in triplicate.

3. RESULTS AND ANALYSIS

3.1. Phage-strain individual models

Models 1 through 6 were tested against experimental data using least squares sum, to determine which assumptions described the dynamics best (Table 1). Interestingly, we found kinetic parameters and model fitness were dependent on the strain analyzed. For *P. aeruginosa* P1 and P3 infected with phage F1, the model that best described the infection process was Model 6. , Model 2 best described the infection process of *P. aeruginosa* P4 infected with phage F1 however. This is likely because *P. aeruginosa* P1 and P3 uninfected cells grew at different rates (infected cells had a different growth rate since the number of infected cells is calculated as the total cell population minus the uninfected cells), and a decline in the population of uninfected cells of *P. aeruginosa* P4 wasn't observed (Fig. 2), meaning infected cells weren't as prevalent. Model selection and parameterization is strongly influenced by the strain due to differences in interaction dynamics, the mechanisms of infection and/or bacterial resistance to phage F1. According to our data, P4 continues to grow as an uninfected culture, indicating that this strain had weaker respond to phage F1 (Fig. 2). We believe that this difference is based on dissimilar adsorption rates and parameters, DNA injection, DNA replication, progeny assembly, among others, which were not taken into account in this case. We observed that the infection process is also dependent on the length of the eclipse phase, which suggested that it was dependent on the bacterial strain. For example, *P. aeruginosa* P3 displayed a clear lytic process after 1500 minutes

(Fig. 2B) whereas lysis in *P. aeruginosa* P1 took place in a shorter period of time (around 500 minutes) (Fig. 2A). No lysis was observed with *P. aeruginosa* P4 and the cells continued to grow.

In Table 2 the kinetic parameters for each system shown in accordance with the model that best fit. These parameters were used later to feed the model for optimal dose calculations.

Additionally an indentifiability analysis of all six models was made using GenSSI, a Matlab toolbox for studying structural identifiability using iterative lie derivatives and identifiability tableaus.[19] With this analysis we could determine that models 1, 2, 3, 4, and 6 are globally identifiable (results not shown), which means there is only one solution for the parameters of each model using our experimental data. The model five has two non-identifiable parameters: k_2 and k_6. However, these parameters are easy to obtain directly from experimental data as is described in Jain et al, 2006. [12] Furthermore, this model didn't fit any of the experimental data and was not used for calculating the optimized phage dose.

3.2. Optimal phage dose

Evolutionary programming was used to calculate the optimum dose. This algorithm was chosen for a number of reasons including being less demanding of computational resources and its natural relation with biological systems, as it is based on Darwinian evolution. The system assumed the presence of the three *P. aeruginosa* strains, aiming to simulate a clinical infection caused by any one of the three strains. Genetic algorithm parameters were heuristically determined using a gene probability of 0.5 and a tolerance of 0.001. We initially obtained the model for each strain in order to elucidate the mechanism without considering the possible interactions among strains. Then, we fused them together and proposed a Petri net that represents

the communication between each model (Fig. 1). We obtained thirteen ordinary differential equations, which relate the initial population for each strain, the initial phage dose, and the length of infection. The differential equation system was used to feed kinetic parameters previously found and was coupled with the genetics algorithm platform and the optimal dose was evaluated. With a treatment time of 1,100 min the genetic algorithm predicted an optimal dose for the phage concentration of 6.50×10^7 PFU/cm^3. Numerical integration of the ordinary differential equations using the Runge Kutta fourth order method predicts that the bacterial survivors of *P. aeruginosa* P1 would decrease from 1.2×10^5 to 8.4×10^2 CFU/ cm^3, *P. aeruginosa* P3 from 5×10^8 CFU/cm^3 to 2×10^3 CFU/cm^3, *P. aeruginosa* P4 from 9×10^6 CFU/cm^3 to 3×10^3 CFU/cm^3 (results not shown). The mixed population model (P1, P3, and P4) would be reduced from 1.4×10^9 CFU/cm^3 to 2.16×10^5 CFU/cm^3 (Fig. 3).

3.3. Phage dose stochastic validation

Master equations of each biological system were numerically solved based on the Guillespie algorithm with a software, developed by our group, called Bacterium Simulator Grid.[20] 1,000 simulations were performed aiming to obtain a representative sample and statistical distribution of the population. Due to a change in the scale (from macro-scale to micro-scale) it was mandatory to modify the initial conditions for each strain; resulting in 150 individuals being selected as the initial conditions for each strain. Optimal phage dose was also scaled to 430 free phage particles. Population histograms displayed a unimodal distribution of the population (results not shown) and an efficient action of the phage based on the small dispersion obtained (Standard deviation= ± 5.12 cells at time= 2,000 s). The host population decreased by an order of magnitude after an infection time of 2,000 s (equivalent to 33.33 min) (Fig. 4). Interestingly, we

found a positive correlation between the uninfected cells dispersion and time, suggesting that the viral inoculation should take place in early stages, similar to a prophylactic application, in order to avoid dispersion of the bacteria. The early presence of the phage would also lower the phage dose needed to eliminate the pathogen.

3.4. Phage dose experimental validation

In order to elucidate the behavior of the system *in vitro* using the optimum phage dose predicted infection curves were assayed. *P. aeruginosa* P1, P3, and P4 strains were grown as explained before and phage F1 was added in the optimum dose in early stages of bacterium growth based on our stochastic predictions. After 1,100 min, the bacterial survivors of all strains were reduced similarly to the predictions of our model. *P. aeruginosa* P1 cells decreased from 2.95 $\times 10^6$ CFU/cm^3 to 4.25$\times 10^3$ CFU/cm^3, *P. aeruginosa* P3 decreased from 1.17 $\times$ 10^8 CFU/cm^3 to 2.1 $\times$ 10^3 CFU/cm^3, *P. aeruginosa* P4 from 3.17 $\times$ 10^7 CFU/cm^3 to 3.87$\times 10^2$ CFU/cm^3. These results, as well as simulations, indicate that the phage is most effective against P3 strain and it is able to diminish populations of the other two strains. In Table 3 a comparison between simulation results and the experimental data with optimal dose is shown. The simulation results were corroborated and the optimum phage dose was validated; by using reverse engineering and utilizing stochastic simulations it is possible to find optimal phage doses against pathogenic bacteria. This optimization approach can help reduce wet laboratory trials, saving time and resources.

To test if the obtained optimal dose could be extrapolated to different phages, infection curves of P1, P3 and P4 were conduct using phages F2 and F3 (Results not shown). The total population reduction was calculated as the difference between the total population of the control curve

(uninfected) at 1100min and the total population of the infection curve at 1100 min (Table 4). Results showed that, with these phages when added to the culture at an optimal dose of $6x10^7$ PFU/cm^3, the population of all three *P. aeruginosa* strains was reduced by seven orders of magnitude or mores.

To challenge the optimal dose we assayed two additional phage doses, one above and one below the optimal dose, using strain *P. aeruginosa* P4 and phage F1 (Fig. 5). With a phage dose of 10^6 PFU/cm^3, one log unit below the predicted optimal dose, the population of bacteria was reduced as expected. Similar behavior is also observed in infections using a phage dose one log unit above the optimal dose predicted (10^8 PFU/cm^3). In this scenario, our hypothesis pointed to a bigger reduction of bacteria population, or at least one obtained with the optimal dose. Nevertheless, the results indicate at the beginning of an infection a reduction of the bacteria population was experienced but at around 420 min phage-resistance occurred. It has been demonstrated in previous phage-host systems that a threshold exists where the overabundance of phage puts selective pressure on their host to resist them, in turn causing the phages to be ineffective.[21, 22, 23] This can be explained by different mechanisms: first, due the high number of viral particles, the capacity of them to be adsorbed on the bacteria surface is reduced.[21] As a consequence, the phage dies in the early stages of the infection process resulting in no viable phage progeny being assembled. This phenomenon is called lysis from without.[24] Second, at high PFU the bacteria can rapidly acquire certain resistance to the phages resulting in an abortive infection.[21] This phenomenon is due in part because using higher MOIs (MOI, multiplicity of infection) results in one generation of progeny being successfully produced which can result in a rapid development of resistance.[25] This resistance is caused by specific factors and avenues that

437 are required for productive phage infection being altered in such a way that inhibit phage-
438 mediated lysis of the host.[21]

439 Previous publications have reported phage densities utilized for effective bacterial treatments:
440 shouldn't be too low, less than 10^7 PFU/cm^3, because there won't be enough phage particles to
441 lyse enough of their host growing in logarithmic phase;[26, 27] and shouldn't be too high, higher
442 than 10^8 PFU/cm^3, which often results in poor pharmacodynamic effects.[26, 27]

443 According to our study we can say computer models coupled with *in vitro* testing are indicative
444 that predicted optimal dose is consistent with the behavior of the infection curves that have been
445 previously reported. [21, 26, 27]

446

447 **4. CONCLUSIONS**

448 The mathematical modeling of viral infections in *P. aeruginosa* allowed us to analyze the
449 dynamics and kinetics of our phage-host system. The combined use of deterministic and
450 stochastic kinetics helped us to elucidate the evolution of the population with two frameworks:
451 the law of mass action and stochastic kinetics. The optimal dose predicted for phage F1
452 effectively reduced bacterial populations, and it also was applied to other two other phages (F2
453 and F3); which indicates that our models can describe different phage-host systems. The optimal
454 dose is in the range of effectiveness reported, which was in the range of 10^7-10^8. Our results
455 validate that phage therapy is a viable alternative to control *P. aeruginosa* and with appropriate
456 mathematical modeling the behavior of the phage-host interaction can be predicted. Our data was
457 shown to be effective using a single phage type and a future project our group should study is the
458 behavior of a system using phage cocktails to delay the appearance of phage-resistant cells.

459

6. ACKNOWLEDGEMENTS

We wish to thank Zachary Hobbs, Research Associate of the T4 Bacteriophage Lab at The Evergreen State College, Olympia, WA, USA, for his valuable comments and edits to this manuscript.

This work was supported by Universidad de los Andes under the interfaculty grant "Programa de consolidación de la interacción entre las Facultades de Ciencias e Ingeniería, Bioprospección de microorganismos y análisis de su aplicación por medio de técnicas de modelamiento", 2007.

REFERENCES

[1] J. W. Costerton, *Science.* **1999**, 284, 1318.

[2] K. Poole, K. Krebes, C. McNally, and S. Neshat, *J. Bacteriol.* **1993**,175, 22.

[3] M. H. Kollef, and V. J. Fraser, *Ann. Intern. Med.* **2001,** 134, 4.

[4] W. C. Summers, *Annu. Rev. Microbiol.* **2001,** 55, 1.

[5] J. Azeredo, and I. W. Sutherland, *Curr. Pharm. Biotechnol.* **2008,** 9, 4.

[6] M. Skurnik, and E. Strauch, *Int. J. Med. Microbio.* **2006,** 296, 1.

[7] Y. Lebeque Pérez, H. J. Morris Quevedo, and N. Calás Viamonte, *Rev. Cubana Med.* **2006**, 45, 1.

[8] E. L. P. Rojas, D. P. de León Pandolfi, and R. R. Ponce, *Acta Méd. Peruana.* **2008***,* 25, 3.

[9] D. C. Ardila, M. Vives-Florez, and A. F. G. Barrios, *Proceedings 8th congress of chemical engineering*. **2009,** 1, 1**.**

[10] A. Arkin, J. Ross, and H. H. McAdams, *Genetics*. **1998***,* 149, 4.

[11] A. F. Gonzalez Barrios, and L. E. K. Achenie, *Biosystems.* **2010**, 99, 1.

[12] R. Jain, A. L. Knorr, J. Bernacki, and R. Srivastava, *Biotechnol. Progr.* **2006***,* 22, 6.

[13] D. T. Gillespie, *J. Phys. Chem.* **1977**, 81, 25.

[14] S. J. Labrie, J. E. Samson, and S. Moineau, *Nature Rev. Microbiol.* **2010**, 8, 5.

[15] A. F. González, V. Covo, L. M. Medina, M. Vives-Florez, and L. Achenie, *Bioprocess Biosyst. Eng.* **2009**, 32, 4.

[16] B. J. Cairns, A. R. Timms, V. A. A. Jansen, I. F. Connerton, R. J. H. Payne, *PLoS Phathog.* **2009**, 5,1.

[17] X. Bardina, D. Bascompte, D. Rovira, S. Tindel. *arXiv*. **2011**

[18] H. Schmidt, and M. Jirstrand, *Bioinformatics.* **2005**, 22, 4.

[19] O. Chis, J. R. Banga, E. Balsa-canto, *Bioinformatics.* **2011**, 27, 18

[20] M. Villamizar, H. Castro, and A. F. Gonzalez Barrios, *Proceedings Of The First Eela-2 Conference.* **2009**, 1, 1.

[21] A. Curtright, and S. Abedon, *J. Bioanal. Biomed.* **2001**, 6, 2.

[22] R. J. Payne, and V. A. Jansen, *J. Theor. Biol.* **2001**, 208, 1.

[23] R. J. Payne, and V. A. Jansen, *Clin. Pharmacol. Ther.* **2000**, 68, 3.

[24] S. T. Abedon. *Bacteriophage,* **2011**, 1, 1.

[25] C. K. Mathews, E. M. Kutter, G. Mosig, P. B. Berget, *Bacteriophage T4*. 1st edition, American Society for Microbiology, Washington D. C, **1993**.

[26] S. T. Abedon, and C. Thomas-Abedon, *Curr. Pharm. Biotechnol. 2010*, 11, 1.

[27] S. T. Abedon. *Adv. Appl. Microbiol.* **2011**, 77.

Figure captions

Figure 1. Global petri net for *P. aeruginosa* P1, P3 and P4 merged model. *Kab* represents the kinetic parameter a for strain b (e.g., K31 means kinetic parameter K_3 for *P. aeruginosa* P1). x*a* and y*a* means uninfected and infected cells for strain *a* respectively. (e.g., x1 uninfected cells for *P. aeruginosa* P1 and y1 is infected cells for *P. aeruginosa* P1). Circles and rectangles represent coins and transitions respectively.

Figure 2. Model predictions in comparison with experimental data for uninfected cells of *P. aeruginosa* P1 (A), *P. aeruginosa* P3 (B), *P. aeruginosa* P4 (C). The infections were performed with phage F1.

Figure 3. Total uninfected cells profile obtained by the simulation for the merge model mixing *P. aeruginosa* P1, P3 and P4 strains. Optimal dose of phage F1 was used to achieve infection.

Figure 4. Uninfected cells profile for the merged model (*P. aeruginosa* P1, P3, and P4) based on the stochastic kinetics model. Mean and standard deviation was calculated from 100 000 runs.

Figure 5. Challenge test with a dose above and below the optimal dose predicted. *P. aeruginosa* P4 infected with a dose of 10^6 PFU/cm^3 of phage F1compared with the positive control (P4 without any phage) (A). *P. aeruginosa* P4 infected with a dose of 10^8 PFU/cm^3 of phage F1 compared with the positive control (P4 without any phage) (B).

Nomenclature:

F1 Phage F1

F2 Phage F2

F3 Phage F3

538 k_1 Infection rate *(min· phage particles/cm^3)$^{-1}$*

539 k_2 Death rate of uninfected cells *(min)$^{-1}$*

540 k_3 Rate of lysis of infected cells *(min)$^{-1}$*

541 k_4 Rate at which the phage progeny was produced *(PFU/cell min)*

542 k_5 Rate at which the free phage particles were degraded *(min)$^{-1}$*

543 k_6 Dead rate of resistant cells *(min)$^{-1}$*

544 $K_{s,R}$ Monod constant for uninfected cells resistant to the phage *(mol/m^3 glucose)*

545 $K_{s,x}$ Monod constant for uninfected cells *(mol/m^3 glucose)*

546 $K_{s,y}$ Monod constant for infected cells *(mol/m^3 glucose)*

547 $K_{s,z}$ Monod constant for uninfected cells nonresistant to the phage *(mol/m^3 glucose)*

548 ***P1*** *Pseudomonas aeruginosa* P1

549 ***P3*** *Pseudomonas aeruginosa* P3

550 ***P4*** *Pseudomonas aeruginosa* P4

551 ***P*** Free phage density *(PFU/cm^3)*

552 ***PD*** Initial phage dose *(PFU/cm^3)*

553 ***R*** Uninfected cells resistant to the phage *(CFU/cm^3)*

554 ***s*** Substrate density *(mol/m^3)*

555 t_i Time where the bacterial population is minimized *(min)*

556 U Step function. U is 0 when $t \leq 480$ min and 1 when t > 480min
557

558 x Uninfected cell density *(CFU/cm³)*

559 x_1 cell population of P1 at t_i *(CFU/cm³)*

560 x_3 cell population of P3 at t_i *(CFU/cm³)*

561 x_4 cell population of P4 at t_i *(CFU/cm³)*

562 $x_{p1(0)}$ Initial condition of living cells for *P. aeruginosa* P1 *(CFU/cm³)*

563 $x_{p3(0)}$ Initial condition of living cells for *P. aeruginosa* P3 *(CFU/cm³)*

564 $x_{p4(0)}$ Initial condition of living cells for *P. aeruginosa* P4 *(CFU/cm³)*

565 y Infected cell density *(CFU/cm³)*

566 $y_{R/s}$ Yield factor relating production of uninfected cells resistant to the phage, to substrate
567 consumed (*CFU/grams of glucose)*

568 $y_{x/s}$ Yield factor relating production of uninfected bacterial cells to substrate consumed
569 (*CFU/grams of glucose)*

570 $y_{y/s}$ Yield factor relating production of infected bacterial cells to substrate consumed
571 (*CFU/grams of glucose)*

572 $y_{z/s}$ Yield factor relating production of uninfected cells nonresistant to the phage, to substrate
573 consumed (*CFU/grams of glucose)*

574 z Uninfected cells nonresistant to de phage *(CFU/cm^3)*

575

576 **Greek symbols**

577 $\mu_{max,R}$ Maximal growth of uninfected cells resistant to the phage *(min^{-1})*

578 $\mu_{max,x}$ Maximal growth of uninfected cells *(min^{-1})*

579 $\mu_{max,y}$ Maximal growth of infected cells *(min-1)*

580 $\mu_{max,z}$ Maximal growth of uninfected cells nonresistant to the phage *(min^{-1})*

581 μ_R Growth rate of uninfected cells resistant to the phage *(min^{-1})*

582 μ_x Growth rate of uninfected cells *(min^{-1})*

583 μ_y Growth rate of infected cells *(min^{-1})*

584 μ_z Growth rate of uninfected cells nonresistant to the phage *(min^{-1})*

585

Table 1(on next page)

Table 1. Experimental- simulation fitness evaluation for each *P. aeruginosa* strain organized form the best fit to the worst fit for each strain.

1 **Table 1**. Experimental- simulation fitness evaluation for each *P. aeruginosa* strain organized form the best fit to the worst fit for each strain.

P1		*P3*		*P4*	
Model	**Squares sum**	**Model**	**Squares sum**	**Model**	**Squares sum**
6	1.01×10^{10}	6	2.69×10^{17}	2	4.19×10^{16}
3	1.74×10^{10}	2	3.37×10^{17}	1	7.94×10^{16}
1	1.77×10^{10}	1	4.62×10^{17}	5	1.32×10^{17}
2	1.85×10^{10}	3	4.85×10^{17}	3	4.17×10^{17}
5	2.26×10^{10}	5	9.21×10^{18}	6	5.85×10^{17}
4	2.8×10^{10}	4	9.53×10^{18}	4	1.40×10^{19}

2

Table 2(on next page)

Table 2. Kinetic parameters for the best fit deterministic phage-strain models for *P. aeruginosa* P1, P3 and P4

Table 2. Kinetic parameters for the best fit deterministic phage-strain models for *P. aeruginosa* P1, P3 and P4

Parameter	*P1*	*P3*	*P4*
	Model six	Model six	Model two
k_1 *(min· phage particles/cm³)*$^{-1}$	1.000 65	1	1.5
k_2 *(min)*$^{-1}$	0	0	0
k_3 *(min)*$^{-1}$	0.01	0.01	0.01
k_4 *(PFU/cell x min)*	0.16	0.16	0.16
k_5 *(min)*$^{-1}$	0	0	0
k_6 *(min)*$^{-1}$	0	0	0
$K_{s,x}$ *(mol/m³ glucose)*	0.007	0.007	1
$K_{s,y}$ *(mol/m³ glucose)*	10	10	14.99
$Y_{x/s}$ *(cells/grams of glucose)*	20 001.1	257 877	257 877
$Y_{y/s}$ *(cells/grams of glucose)*	969 000	688 938	688 938
$\mu_{max,x}$ *(min*$^{-1}$*)*	0.004	0.016	0.003
$\mu_{max,y}$ *(min*$^{-1}$*)*	0.001	0.002	0.005

Table 3(on next page)

Comparison between the results in the reduction of *Pseudomonas aeruginosa* P1, P3, and P4 strains population in the simulation and in the experimental validation at time point 1100 min. The total population reduction was calculated as the difference

Table 3. Comparison between the results in the reduction of *Pseudomonas aeruginosa* P1, P3, and P4 strains population in the simulation and in the experimental validation at time point 1100 min. The total population reduction was calculated as the difference between the total population of the control curve (cells growing without phage infection) at 1100min and the total population of the infection curve at 1100 min

Strain	*Total population reduction (CFU/cm³) in simulation results*	*Total population reduction (CFU/cm³) in experimental validation results*	*Difference in order of magnitude between results*
P1	1.19×10^5	2.95×10^6	1
P3	5×10^8	1.17×10^8	0
P4	9×10^6	3.17×10^7	1

Table 4(on next page)

Table 4. Total population reduction of *Pseudomonas aeruginosa* P1, P3 and P4 with phages F2 and F3 (Optimal dose extrapolation) at time point 1100 min. The total population reduction was calculated as the difference between the total population of th

Table 4. Total population reduction of *Pseudomonas aeruginosa* P1, P3 and P4 with phages F2 and F3 (Optimal dose extrapolation) at time point 1100 min. The total population reduction was calculated as the difference between the total population of the control curve (cells growing without phage infection) at 1100min and the total population of the infection curve at 1100 min

Strain	*Phage*	*Total population reduction (CFU/cm³)*
P1	F2	5.03×10^7
	F3	4.51×10^7
P3	F2	4.85×10^8
	F3	3.15×10^9
P4	F2	4.41×10^8
	F3	6.7×10^8

Figure 1

Global petri net for *P. aeruginosa* P1, P3 and P4 merged model. *Kab* represents the kinetic parameter a for strain b (e.g., K31 means kinetic parameter K_3 for *P. aeruginosa* P1). x*a* and y*a* means uninfected and infected

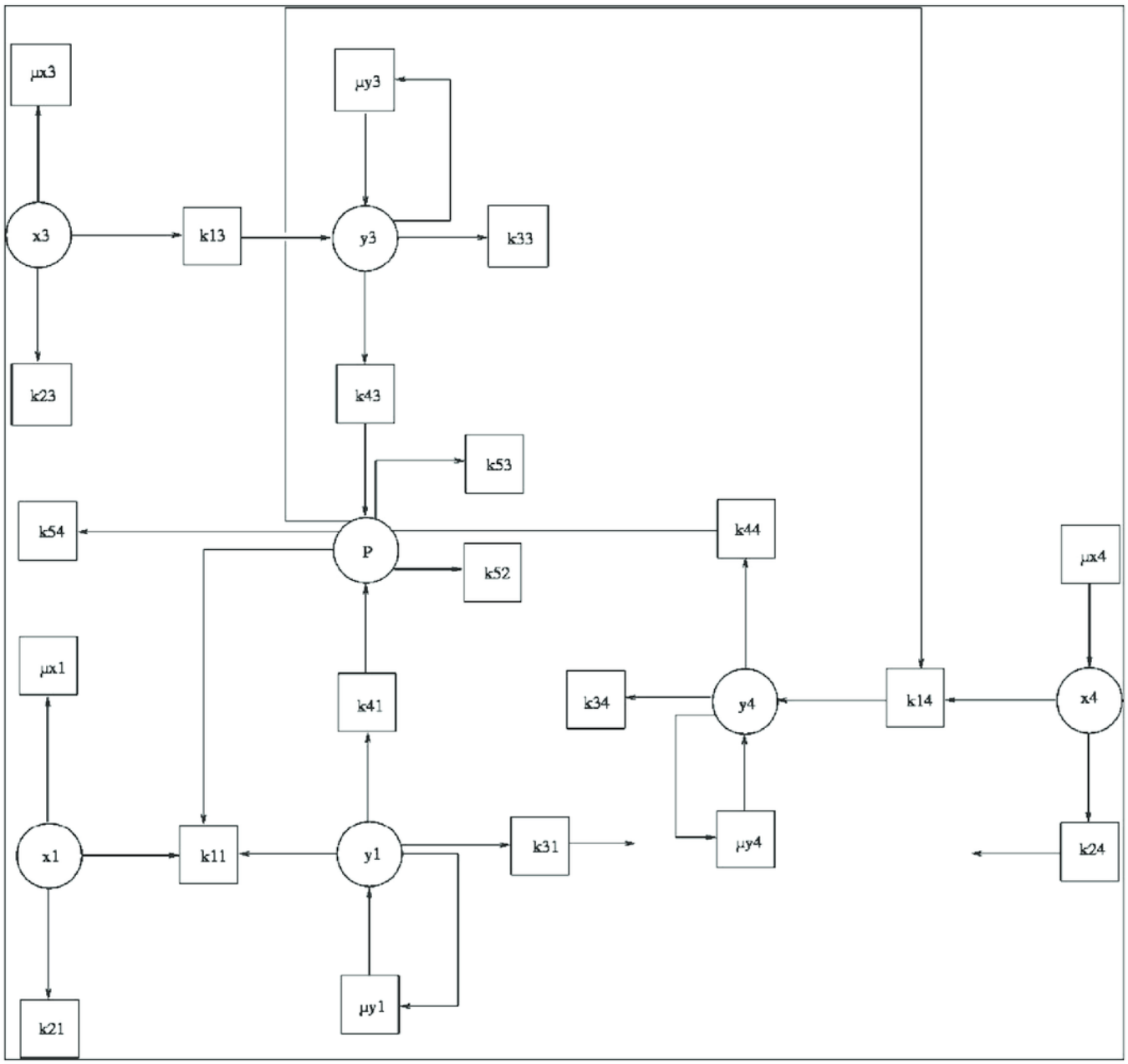

Figure 2

Model predictions in comparison with experimental data for uninfected cells of *P. aeruginosa* P1 (A), *P. aeruginosa* P3 (B), *P. aeruginosa* P4 (C). The infections were performed with phage F1.

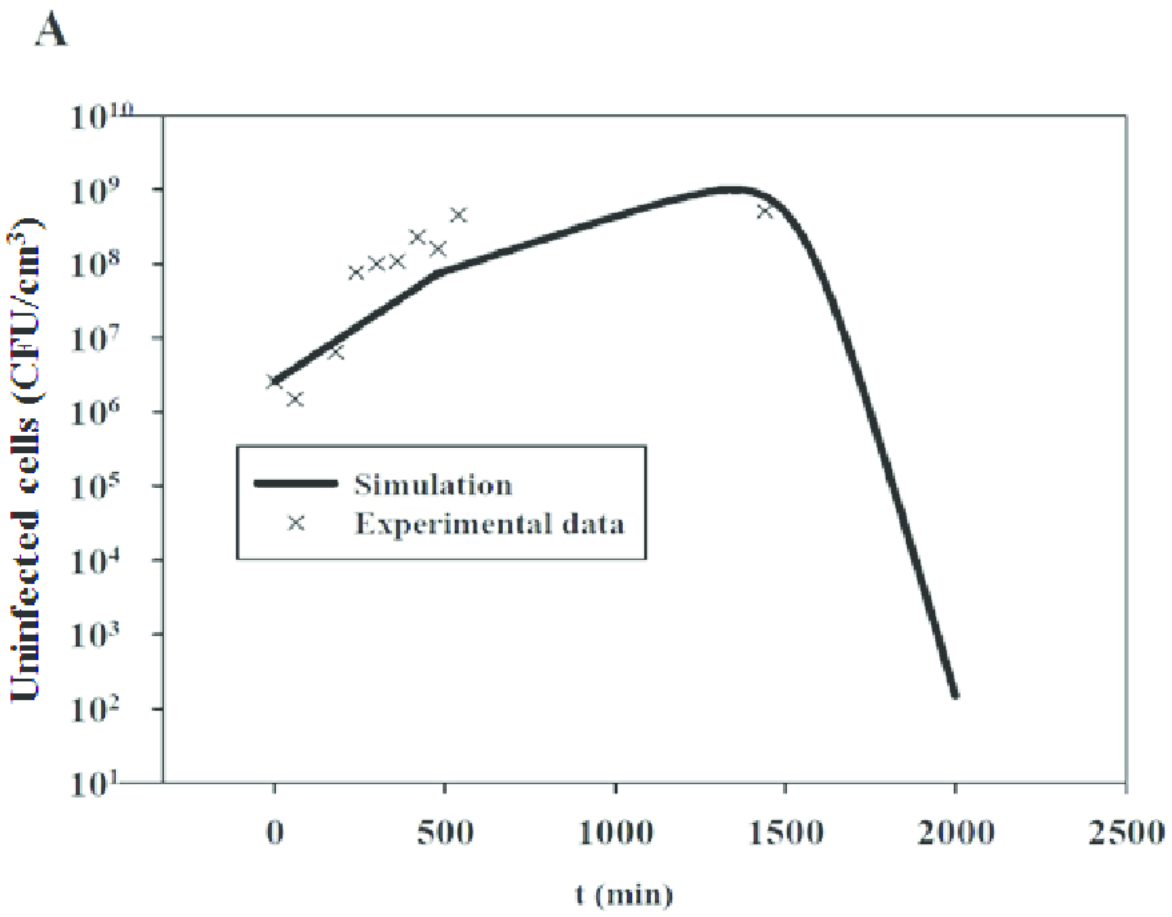

Figure 3

Model predictions in comparison with experimental data for uninfected cells of *P. aeruginosa* P1 (A), *P. aeruginosa* P3 (B), *P. aeruginosa* P4 (C). The infections were performed with phage F1.

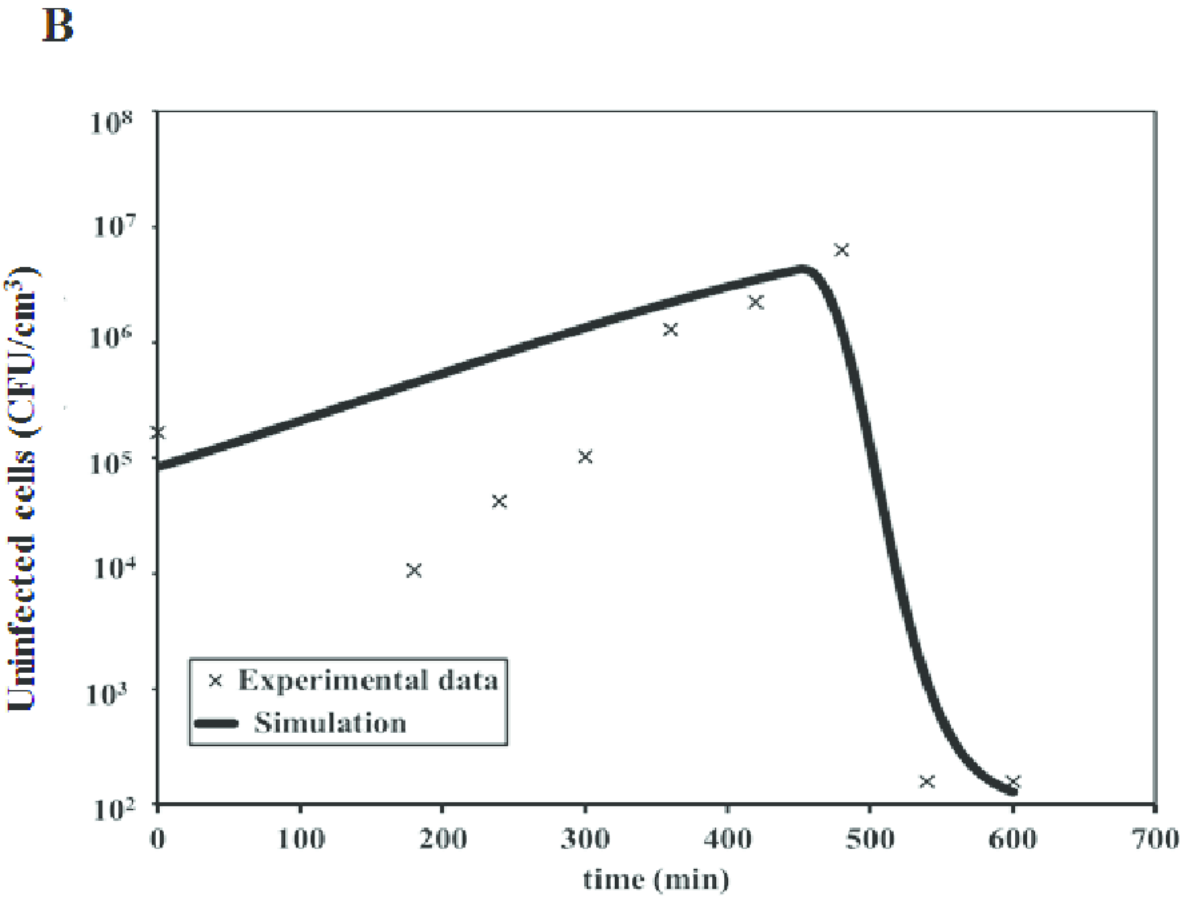

Figure 4

Model predictions in comparison with experimental data for uninfected cells of *P. aeruginosa* P1 (A), *P. aeruginosa* P3 (B), *P. aeruginosa* P4 (C). The infections were performed with phage F1.

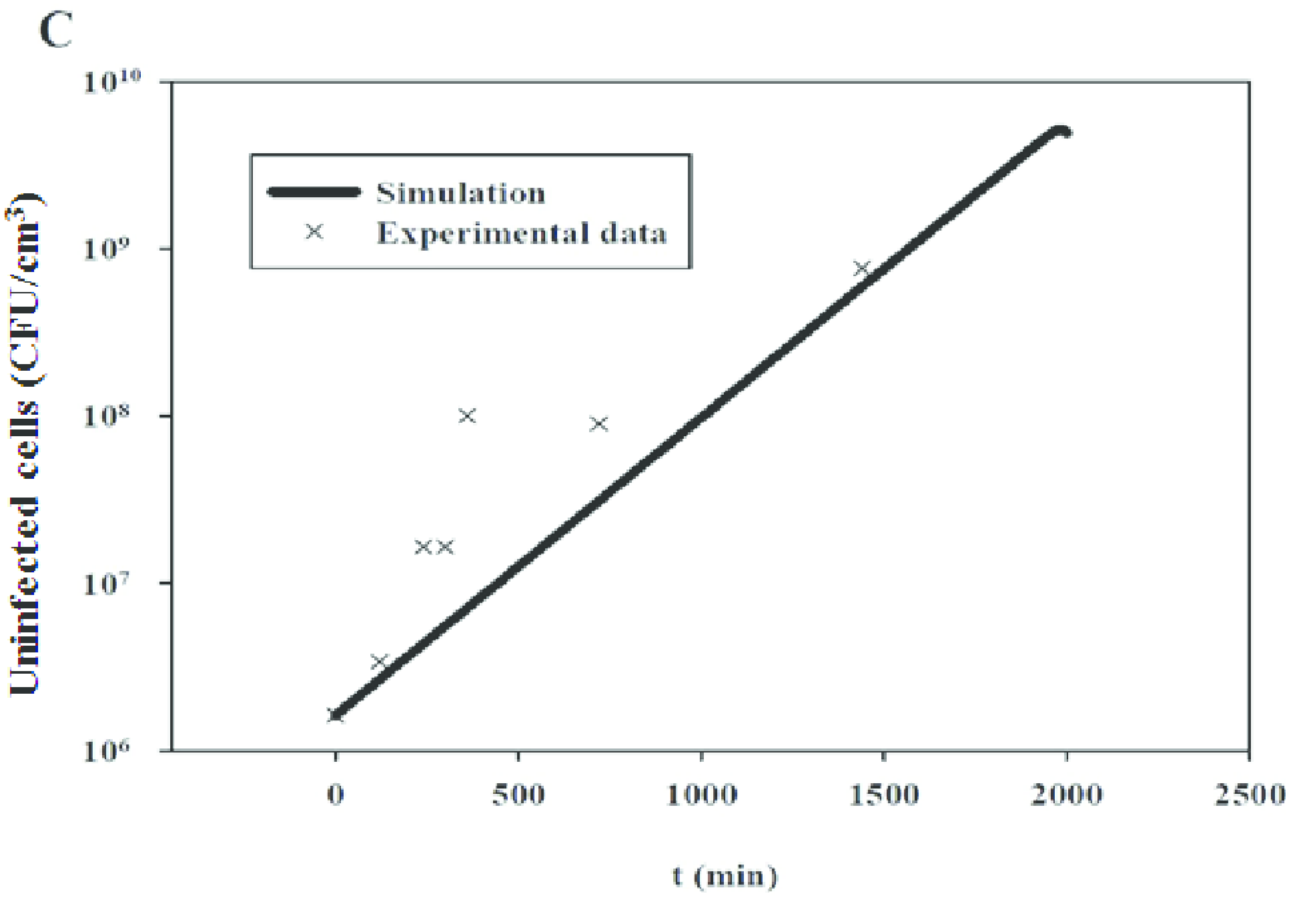

Figure 5

Total uninfected cells profile obtained by the simulation for the merge model mixing *P. aeruginosa* P1, P3 and P4 strains. Optimal dose of phage F1 was used to achieve infection.

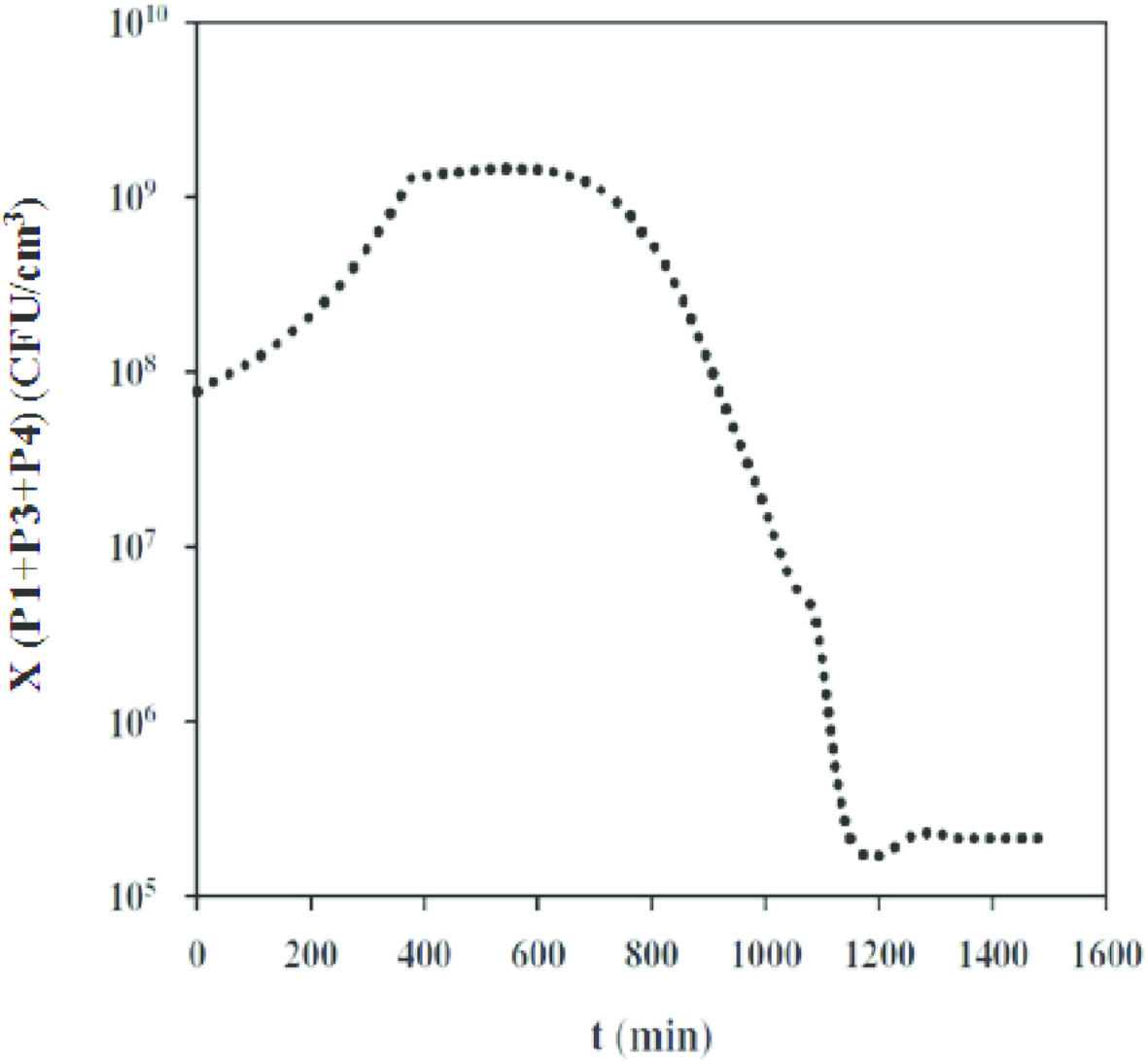

Figure 6

Uninfected cells profile for the merged model (*P. aeruginosa* P1, P3, and P4) based on the stochastic kinetics model. Mean and standard deviation was calculated from 100 000 runs.

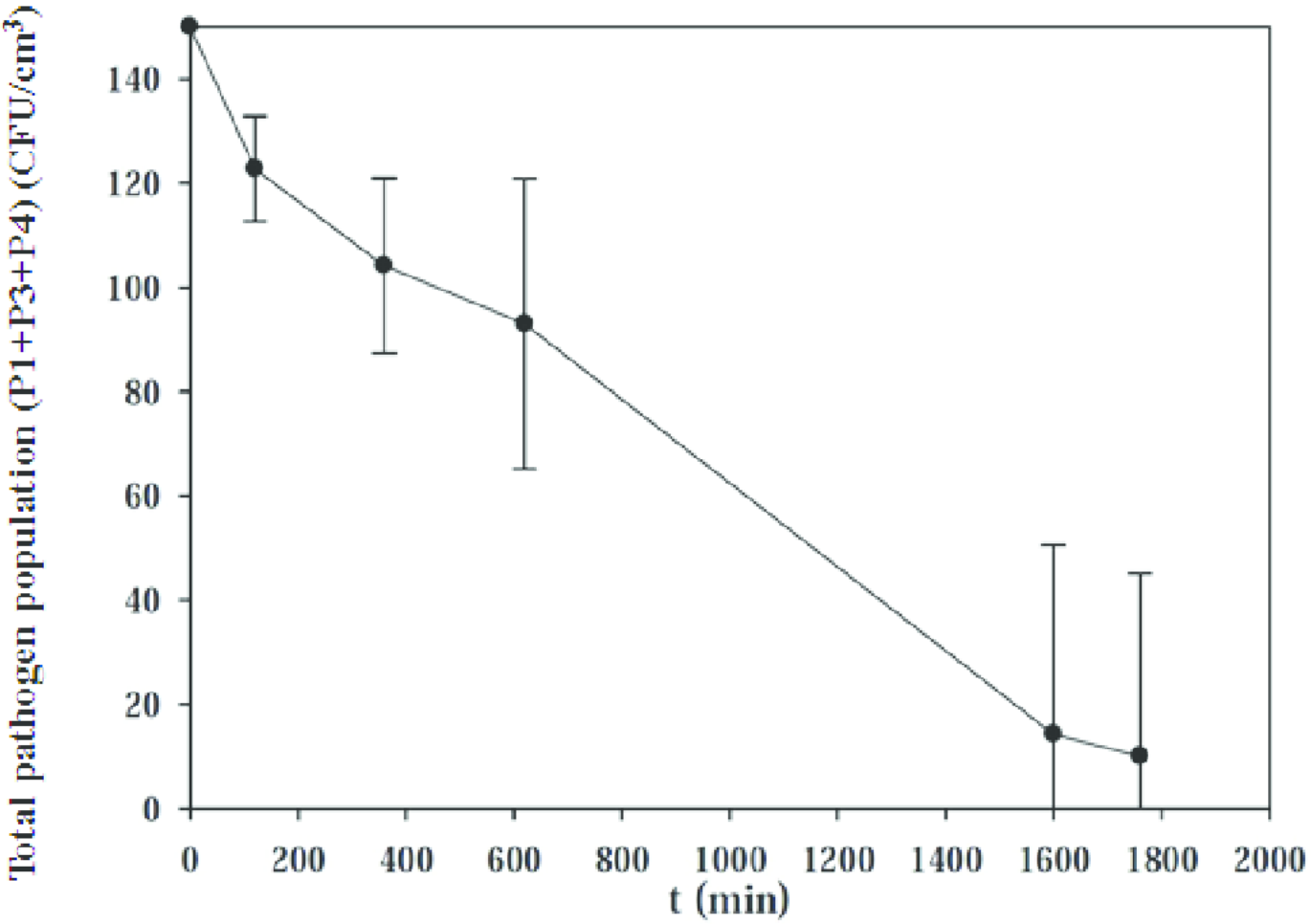

Figure 7

Challenge test with a dose above and below the optimal dose predicted. *P. aeruginosa* P4 infected with a dose of 10^6 PFU/cm^3 of phage F1compared with the positive control (P4 without any phage) (A). *P. aeruginosa* P4 infected

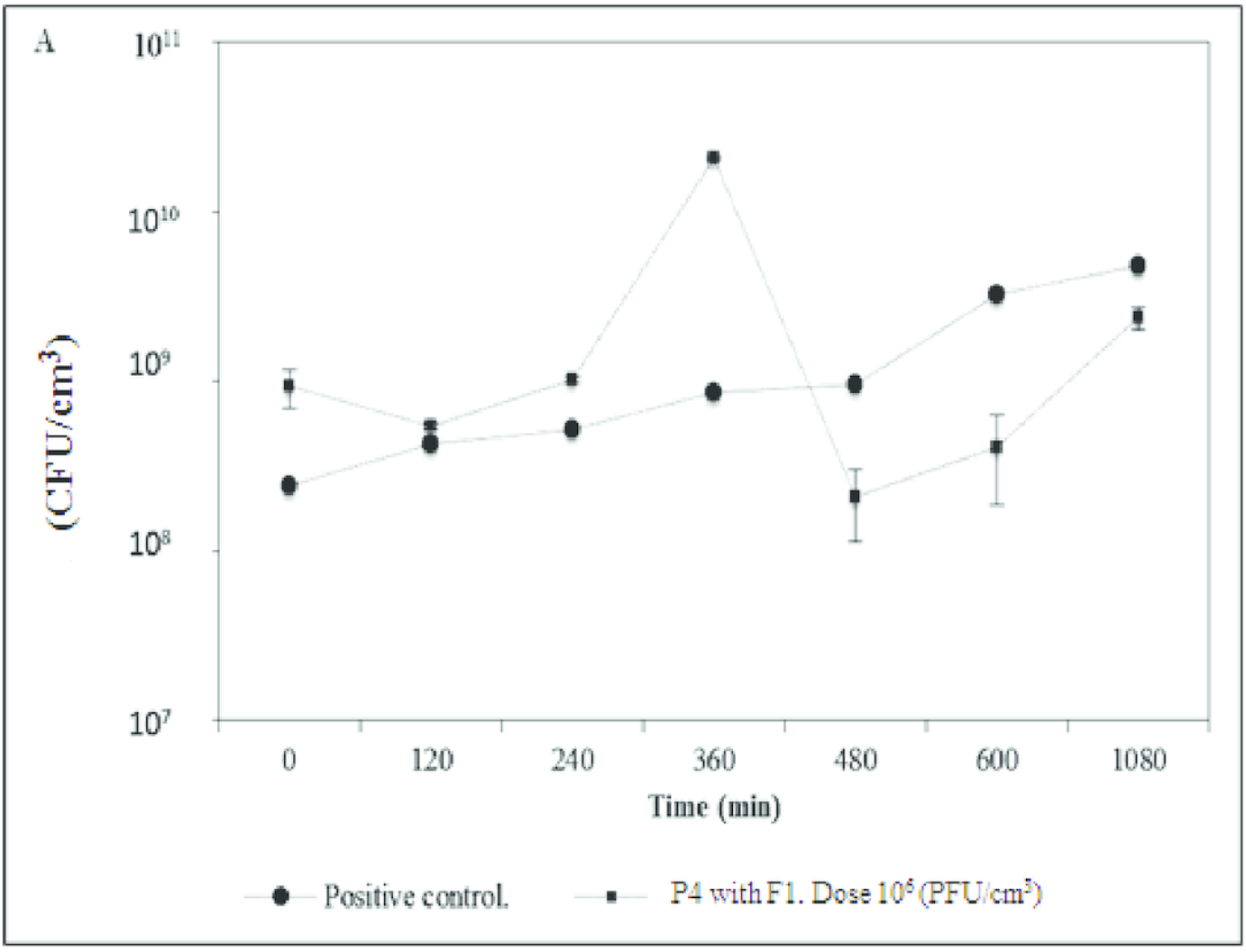

Figure 8

Challenge test with a dose above and below the optimal dose predicted. *P. aeruginosa* P4 infected with a dose of 10^6 PFU/cm^3 of phage F1compared with the positive control (P4 without any phage) (A). *P. aeruginosa* P4 infected