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Statistical approaches to understanding between and within host dynamics of infectious disease agent replication

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Statistical Approaches to Understanding Within Host and Between Host Dynamics of Infectious Agent Replication

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Statistical approaches to understanding between and within host dynamics of infectious disease agent replication

By Jessica Coates

Despite the design of highly effective antibiotics and vaccines, infectious diseases still cause a major burden on public health. Infectious diseases account for three of the ten major causes of death in the United States and globally infectious diseases can be attributed to over 17 million deaths per year. Because of an increase in antibiotic resistance and decreased vaccine effectiveness for multiple vaccine preventable diseases, there is a need to better understand the dynamics of infectious disease replication under antibiotic stress and in highly vaccinated populations. To better understand these dynamics, this work aimed to explore two unique cases: (1) the within host dynamics of E. coli replication in the presence of bacteriostatic and bactericidal antibiotics and (2) the between host dynamics of mumps transmission in US populations with a high vaccine coverage. Given the usefulness of statistical approaches to understanding infectious disease dynamics, we utilized data derived from traditional experimental biology to construct a mathematical model describing the birth and death processes of bacteria exposed to antibiotics. This work demonstrated that bactericidal and bacteriostatic antibiotics have differing effects on the growth dynamics of bacteria that can be manipulated to increase the likelihood of bacterial clearance. In a second study, we utilized observational data collected through a systematic review to estimate the effective reproduction number for mumps outbreaks in the United States post the introduction of a two-dose vaccination recommendation. This work highlighted the heterogeneity in transmission dynamics for different populations with similar vaccination coverage and provided foundational evidence about the potential effectiveness of non-pharmaceutical interventions in controlling mumps outbreaks. Together this work adds to the body of work focused on understanding the importance of within and between host dynamics of infectious agent replication. Additionally, the work provides novel insight that may aid in the design of future studies. This is particularly important because as we develop a better understanding of the dynamics of infectious agent replication, it improves our ability to reduce the burden caused by infectious diseases on human health.

Statistical Approaches to Understanding Within Host and Between Host Dynamics of Infectious Agent Replication

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Authorship Contributions

I co-first authored the publication in chapter II, which was published in eLife in March of 2018. I contributed by characterizing trends in bacteriostatic and bactericidal drugs through plating efficiency assays and analysis of within-population dynamics. Data from these experiments are featured in Figures 1-4 and the corresponding supplemental figures. I wrote a draft of the methods section for experiments, and I performed revisions with guidance from Minsu Kim. I also participated in the approval of the final draft.

I am the sole author of the research described in chapter III. I contributed to project development, data collection, interpretation, and statistical analysis. I decided on the scope of the project, collected all data, and performed statistical analyses for findings in all figures and tables. I completed all transmission analyses under the guidance of Katia Koelle. I wrote the original draft of all sections of the manuscript and participated in editing and reviewing the final draft with advice from Katia Koelle, Carole Hickman, Anice Lowen, Nic Vega, Pat Marsteller and Paul Rota.

Chapter I. Introduction

Jessica Coates

Abstract

Infectious diseases (IDs), or diseases caused by the transmission of microorganisms, cause a major burden on public health in the United States. Public health strategies utilizing antibiotics and vaccines can be effective for reducing within-host and between host replication of infectious diseases agents. In the introduction chapter of this dissertation, the author introduces background information on the within-host and between host transmission dynamics of infectious diseases and how public health strategies can be used to reduce transmission. Lastly, the author presents the gaps in knowledge, describes the questions asked in the dissertation, and provides a summary of the remaining research chapters.

Introduction

Infectious diseases (IDs), or diseases caused by microorganisms, -- such as bacteria, viruses, fungi, or parasites – are major causes of morbidity and mortality in the United States and globally. In 2020, three out of the ten major causes of death in the United States were the result of infectious diseases[1]. In addition to causing mortality, infectious diseases can be associated with the onset of negative long-term effects, such as, increased risk of cancer[2], paralysis[3], and intellectual deficiencies[4]. Lastly, illness due to infectious disease can have major economic impacts as a result of reduced human production, decreased desirability in investment, and the financial costs of healthcare[5]. Due to the high burden associated with infectious diseases, it is essential to understand the dynamics associated with infectious disease agent replication in the United States and to design effective strategies for the control and prevention of infectious diseases.

For infectious diseases to be maintained within a population, two levels of reproduction must occur: (1) within-host and (2) between-host. The infectious disease process begins with within-host reproduction, or the microorganism of interest (bacteria, virus, or parasite) encountering a host and circumventing the host's immune response to complete a series of replication cycles. As a result, of each replication cycle, the host may experience symptoms and become infectious to other hosts. This process can cause significant damage to the host, sometimes resulting in death. Therefore, to sustain infectious diseases at the population level, there must be a successful act of transmission between an infectious host and a susceptible host, or between-host reproduction. Transmission between two hosts can occur through multiple different mechanisms. Transmission mechanisms include direct (i.e., skin-to-skin contact, kissing, and sexual intercourse), droplet spread (short-range aerosols produced by sneezing, coughing, or talking) and indirect transmission (infectious agent from a reservoir to a host by suspended air particles). Additionally, infectious disease agents can be

transmitted via inanimate objects (e.g., unsanitary food, impure water, surfaces) and animate intermediaries (vectors))[6].

Several public health and medical interventions have been devised to prevent pathogen replication at each level. Within-host and between host replication, can be mitigated, or prevented using social distancing, barrier methods (i.e., condoms and masks), or vaccinations[7]. Once within the host, antimicrobials and vaccine mediated immune responses can be used to prevent entry into the cellular target, reduce pathogen replication, and minimize harm done to the host[8,9]. Overall, these methods have been highly effective as demonstrated by the decreased infectious disease incidence and burden following their introduction. Following the discovery of antibiotics and vaccines, the United States observed a shift from infectious diseases being the major cause of death to chronic diseases – such as heart disease, cancer, and diabetes until the COVID-19 pandemic[10]. Two exemplary examples of the effectiveness of antibiotics and vaccines are the case of penicillin and the measles vaccine. Prior to the implementation of the measles vaccination program in 1963, an estimated 3 to 4 million measles cases happened annually in the United States. Since the vaccination program started, widespread use of the measles vaccines has led to >99% decrease in measles cases compared with the pre-vaccine era[11]. Part of the United States' success in World War II can be attributed to efforts by the United States government to produce and use penicillin for soldiers. During World War II, the use of penicillin reduced soldier mortality from 18% to 1%[12]. However, in the case of some infectious diseases, there has been an observed decrease in the ability of antibiotics and vaccines to prevent or control replication.

The Gaps in Knowledge

For this dissertation, I focused on two cases where effective preventative and control measures have been introduced but infectious disease agent replication persists at the between and within host levels. Specifically, I start with the case of failed antibiotic treatment despite perceived population susceptibility and proceed to discuss the increased incidence of mumps cases in the United States despite a high two-dose vaccine coverage since 1989.

Random (stochastic) processes can affect the course of an epidemic in many ways. For example, at the beginning of an epidemic when the number of initial infected hosts is low if a host randomly dies or experiences a life event that prevents them from being around other susceptible hosts the epidemic may die out before tremendous harm is done to the population. This phenomenon also holds true for the within host dynamics of infectious agent replication. At the beginning of an infection and following treatment with antimicrobials, the agent load is presumably small and vulnerable to stochastic processes that could result in the eradication, or clearance of infection, from the host.

The effect of stochasticity on within host replication can be best depicted utilizing a statistical theory called branching process. Branching process, is a type of mathematical object that can be utilized to conceptualize the process of bacterial replication within a host[13]. In this process, every bacterial cell can undergo binary fission or die at a predetermined time step with some probability. Therefore, the process of going from the initial bacterial load to the final bacterial load can be illustrated by a tree whose root is the first bacterium. On average, the probability of a bacterium undergoing binary fission in this tree might add up to an average probability greater than one. However, chance events might lead to a situation where most cells do not undergo binary fission and ultimately die. In this instance, the infection also will die out preventing the opportunity

for between host transmission. The likelihood of such events happening is greater when the population size is small and chance events have a greater effect.

In an ideal scenario, antibiotics could be used to reduce the bacterial load to a small population size that is highly susceptible to a stochastic breakdown in the branching process, or extinction. However, clinically this is not always observed. Instead, antibiotic treatments have been observed to fail to clear 100% of bacteria during an infection[14]. In this instance, as described later in this dissertation, there is a need to better understand the population dynamics of small bacterial populations exposed to antibiotics.

Since the early 1900s, statistical methods have been used to better understand the between and within host dynamics of infectious agent replication and to design effective strategies for preventing and controlling replication. Between-host models of infectious diseases follow the dynamics of disease spread at the level of the host population by tracking the number of susceptible and infected individuals of different types[15]. Susceptible-Infected-Recovered, or SIR, models are some of the most used models in epidemiology to explore between host dynamics of infectious agent replication. SIR models attempt to compartmentalize individuals within a population based on their disease status where susceptible individuals can become infected[15]. Following infection, individuals can transition to recovered depending on the longevity of immunity. When an infected individual enters the population, it can infect on average R_0 others, who can in turn transmit the infectious disease agent to other susceptible individuals. R_0, or the basic reproductive number, is the number of newly infected hosts resulting from one already infected host in a population of all susceptible hosts[16]. R_0 can take on a range of values when observed within real epidemics such as in the cases of hyper-endemic malaria in Nigeria ($R_0 = 80$) in the 1970s and HIV in male homosexuals in England and Wales from 1981-1985[17] ($R_0 = 2$). When is $R_0 \geq 1$, the population is considered to

be in an epidemic state, or an unexpected increase in the number of disease cases in a specific geographical area. Eventually as the infectious agent continues to replicate within a population the number of susceptible hosts decreases. As the pool of susceptible hosts becomes smaller, between host transmission also slows, and the R_0 begins to decrease.

In an ideal scenario, SIR models could be utilized to devise rational vaccination strategies to shift R_0 until the value reaches zero indicating eradication of the disease from the population. Through the use of statistical methods, investigators can estimate the p_{crit}, or the proportion of the population that needs to be vaccinated to achieve an $0 \geq R_0 \leq 1$[18]. However, in the case of some diseases – such as mumps, R_0 never reaches zero and the disease continues to circulate within a vaccinated population. As described later in this dissertation, mumps is a vaccine preventable infection caused by the mumps virus that affects the respiratory system and salivary glands[19]. However, despite the design of an effective vaccine and high vaccine coverage in the United States, cases of mumps continue annually in historically marginalized communities (i.e., Marshall Island and Jewish descendants), universities, and detention centers[20]. In this instance, it is more appropriate to utilize the R_e, or effective reproduction number, to explore between host dynamics of infectious agent replication. The R_e is defined as the average number of secondary cases per infectious case in a population made up of both susceptible and non-susceptible hosts[21]. If $R_e > 1$, the number of cases will increase similar to when $R_0 > 1$. Where $R_e = 1$, the disease is endemic, or constantly maintained at a baseline level in a geographic. The Re value, as described later in this dissertation, can be used similarly to the R_0 value to devise effective strategies to control and prevent infectious agent replication in vaccinated populations.

Given the large impact of antibiotic treatment failure and mumps disease on public health, this dissertation sought to utilize statistical methods to understand the within-host and between host

dynamics of infectious agent replication. Specifically, this work describes the combined use of experimental biology methods and stochastic models to investigate changes in the population dynamics of a small population of E. coli bacteria once exposed to bactericidal and bacteriostatic antibiotics. Additionally, I present data describing the R_e value for mumps disease in the United States despite having a high vaccine coverage for a perceived vaccine preventable disease. Finally, work concludes to offer recommendations for the improvement of future studies and the design of strategies to prevent and control the spread of infectious diseases. Herein, I describe the questions asked in this dissertation and provide a summary of the remaining dissertation chapters.

The Questions

To evaluate the within-in and between-host dynamics of infectious disease agent reproduction under antibiotic stress and in vaccinated populations, this dissertation attempted to ask the following questions:

Questions on within-host dynamics of infectious disease reproduction

1.1.1. How frequently does E. coli reproduction occur in the presence of antibiotics?

1.1.2. Is the frequency of E. coli reproduction associated with a previously acquired resistance? If not, what reproduction characteristic (growth rate vs death rate) is correlated with the reproduction frequency?

1.1.3. Can the characteristic(s) associated with reproduction frequency be manipulated to decrease the likelihood of reproduction using currently available antibiotics?

Questions on between-host dynamics of infectious disease reproduction

1.1.4. What is the mean effective reproduction numbers for mumps outbreaks occurring in the United States?

1.1.5. Is there a relationship between setting and the effective reproduction number for mumps?

Outline of The Thesis and Chapter Summaries

In Chapter 2, I present a joint experimental and theoretical study on the dynamics of bacterial reproduction under antibiotic stress. In this investigation, we exposed E. coli K-12 MG1655, a commonly used laboratory strain with no known antibiotic resistance, and mutant strains with increased antibiotic resistance to antibiotics to estimate the frequency of pathogen reproduction. This bacteria strain serves as the ideal model organism because of the availability of information about the genetic background, few genetic manipulations, and the opportunity to investigate antibiotic treatment failure.

In this study, we utilized single-cell microscopy, conventional plating assays, and a stochastic birth-death processes model to evaluate the growth and death rate of E. coli in the presence of bactericidal and bacteriostatic antibiotics. The results of our experiments suggest that: (I) population level survival varies at sub-inhibitory concentrations of bactericidal antibiotics in a concentration dependent manner, (II) survival at sub-inhibitory concentrations of bactericidal antibiotics is not associated with acquired resistance, and (III) probability of population extinction is associated with stochastic fluctuations in death rate.

To explore the potential implications of the experimental results for the design and evaluation of antibiotic treatment regimens, we used a Markovian birth-death model to account for the stochastic extinction dynamics. The results of our model suggest that when bactericidal antibiotics are used in combination with bacteriostatic antibiotics at concentrations where the death rate is greater than the growth rate the frequency of population extinction can be increased. This provides a potential alternative antibiotic treatment strategy using currently available antibiotics that would not have been identified using a deterministic approach.

In Chapter 3, I present a systematic review to understand the dynamics of mumps transmission in different settings in the United States following the 2006 resurgence. Mumps outbreaks serve as an excellent model for exploring between-host reproduction under current prevention measures because of the continued outbreaks observed in universities, mass gatherings, detention centers, and close-knit communities despite a high two dose mumps, measles, and rubella (MMR) vaccination coverage.

To assemble the data necessary to estimate the Re value for mumps in the United States, we used a MeSH term search to obtain observational data about mumps outbreaks reported to MedLine as an alternative to collecting experimental data. Due to the lack of a non-human primate experimental model organism for mumps and ethical concerns for performing human challenge studies, this method allows for sample analysis of mumps transmission that would be otherwise difficult. Due to a small dataset, we were unable to estimate the mean Re value for mumps in the United States post the 2006 resurgence. However, we were able to generate point estimates for the Re values that suggest mumps transmission can be reduced by non-pharmaceutical interventions (NPIs). The results of our analysis suggest that NPIs may be useful in reducing the spread of mumps in combination with vaccination but further studies are necessary to validate that claim.

To conclude the main portion of this study, I briefly summarize the general conclusions of the investigations contained in this dissertation in Chapter 4. I discuss additional questions and lines of inquiry that the studies herein have generated and consider potential future direction.

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Chapter II: Antibiotic-induced population fluctuations and stochastic clearance of bacteria

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Abstract

Effective antibiotic use that minimizes treatment failures remains a challenge. A better understanding of how bacterial populations respond to antibiotics is necessary. Previous studies of large bacterial populations established the deterministic framework of pharmacodynamics. Here, characterizing the dynamics of population extinction, we demonstrated the stochastic nature of

eradicating bacteria with antibiotics. Antibiotics known to kill bacteria (bactericidal) induced population fluctuations. Thus, at high antibiotic concentrations, the dynamics of bacterial clearance were heterogeneous. At low concentrations, clearance still occurred with a non-zero probability.

These striking outcomes of population fluctuations were well captured by our probabilistic model. Our model further suggested a strategy to facilitate eradication by increasing extinction probability. We experimentally tested this prediction for antibiotic-susceptible and clinically isolated resistant bacteria. This new knowledge exposes fundamental limits in our ability to predict bacterial eradication. Additionally, it demonstrates the potential of using antibiotic concentrations that were previously deemed inefficacious to eradicate bacteria.

Introduction

The frequent failure of antibiotic treatments is a serious public health threat. A recent study projects treatment failures caused by antibiotic resistance will lead to 300 million deaths and a healthcare burden of $100 trillion by 2050 (O'Neill, 2016). This epidemic is further exacerbated by our inability to reliably eradicate antibiotic-susceptible bacteria. For example, antibiotic treatments of infections caused by antibiotic-susceptible bacteria never achieve a success rate of 100%, often failing to eradicate them unexpectedly (Doern and Brecher, 2011; Weidner et al., 1999; Gopal et al., 1976; Ficnar et al., 1997; Forrest et al., 1993). To design effective treatments and avoid antibiotic failure, there is a strong need to better understand the dynamics of bacterial populations exposed to antibiotics.

Previously, laboratory studies have extensively characterized how large bacterial populations (e.g., ~10^8 cells in a culture) decline under antibiotic treatment, e.g., see (Nielsen et al., 2011; Ferro et al., 2015; Regoes et al., 2004). These studies have led to the current, deterministic model of the pharmacodynamics, that is, the population dynamics of bacteria exposed to antibiotics follows a predetermined course and can be predicted deterministically a priori; see (Regoes et al., 2004; Czock et al., 2009) and references therein. This deterministic framework successfully captures the reproducible dynamics of a large bacterial population declining to a small population under antibiotic treatments. However, due to their experimental detection limits (e.g., >>100 cells [Nielsen et al., 2011; Ferro et al., 2015]), the dynamics of a small population undergoing extinction have not been directly characterized.

Inoculum size as small as a few cells can produce infections (Jones et al., 2006; Haas and Rose, 1994; Jones et al., 2005; Tuttle et al., 1999; DuPont et al., 1989; Hara-Kudo and Takatori, 2011; Kaiser et al., 1992). Thus, if antibiotics manage to reduce a large bacterial population to a very

small population but fail to eradicate it, the survivors may replicate and restore infections. Additionally, these survivors are more likely to develop antibiotic resistance, making subsequent antibiotic treatment of the restored population more difficult (Gullberg et al., 2011; Kohanski et al., 2010; Lopatkin et al., 2016; Dagan et al., 2001; Allen et al., 2014). Thus, in many cases, including life threatening infections or even minor infections in immuno-compromised patients, treatment success depends on complete clearance of the infection-causing bacteria. To effectively clear bacteria using antibiotics, it is critical that we understand not only how a large population of bacteria declines to a small population, but also how a small population eventually goes extinct. Extensive studies focused on the former process (discussed above). The present study focuses on the latter process.

By employing a conventional plate assay, single-cell microscopy, and quantitative modeling, we directly characterized the extinction dynamics of antibiotic-susceptible Escherichia coli populations. We found that antibiotics known to kill bacteria (i.e., bactericidal drugs) induce population fluctuations. At high drug concentrations, all populations go extinct (as expected), but the extinction time is highly variable and cannot be deterministically predicted a priori. Even at low drug concentrations, due to these fluctuations, populations go extinct with a non-zero probability. We found that the Markovian birth-death model quantitatively accounted for the probabilistic occurrence of population extinction. Informed by the model, we then altered the extinction probability by manipulating cell growth and showed that a bacterial population could be eradicated at low drug concentrations that were previously deemed inefficacious. Our work demonstrates that the deterministic knowledge obtained from previous studies of large bacterial populations cannot be extrapolated to population extinction. Our findings also have significant implications for the prediction of treatment outcomes, development of innovative therapies, and assessment of antibiotic efficacy.

Results

Contrasting trends in plating efficiency for bacteriostatic and bactericidal drugs

Previous studies of large populations have established the 'minimum inhibitory concentration' (MIC; the lowest concentration of the drug that inhibits population growth) as the most critical parameter for characterizing the dynamics of a bacterial population under antibiotics (Regoes et al., 2004; Czock et al., 2009; Craig, 1998; Falagas et al., 2012). The dynamics of bacterial populations exposed to different concentrations of antibiotics have been examined and modeled deterministically in relation to the MIC, as follows. First, without drugs, the growth rate of cells, l, is higher than the death rate, f (i.e., $l > f$), and thus a bacterial population always grows. When drug concentration increases, if the concentration remains below the MIC (i.e., sub-MIC), growth rate is higher than death rate ($l > f$), and thus a population still grows, albeit at slower rates. When the drug concentration increases further and reaches the MIC, growth rate becomes equal to death rate ($l = f$), and the population size is maintained at a constant level. Only at drug concentrations above the MIC does a bacterial population decline. Extrapolating this deterministic knowledge to population extinction, studies often claimed that maintaining drug concentrations above the MIC was absolutely essential to eradicate bacterial populations. As will become evident later, our data challenge this deterministic framework.

As a first step to examine the dynamics of a small population in relation to the MIC, we used a plate assay and characterized how single E. coli cells grew and formed colonies at various antibiotic concentrations. Antibiotic-susceptible, wild-type (WT) E. coli cells were cultured in liquid LB medium without antibiotics and then spread on LB agar plates containing increasing concentrations of antibiotics. After 18 hr of incubation, the number of colony-forming units

(NCFU) was determined. By normalizing the NCFU to that for an antibiotic-free plate (N0CFU), we then obtained the plating efficiency (=NCFU/N0CFU), which indicates the fraction of cells forming colonies. Following the definition of MIC as the lowest drug concentration that inhibits population growth, the lowest concentration yielding no visible colonies on the plates was defined as the MIC here. See Figure 1—figure supplement 1 for a detailed illustration of this procedure and Supplementary file 1 for the MIC values for all of the antibiotics examined.

When we performed this plate assay for various antibiotic drugs, we observed two strikingly distinct trends, which depended on whether the drug used was bacteriostatic (which suppresses cell growth) or bactericidal (which induces cell death). For bacteriostatic drugs, at increasing concentrations, the plating efficiency remained nearly constant and abruptly dropped to zero when the drug concentration reached the MIC (Figure 1A); the grey line was obtained from a linear regression analysis of the whole data set below 0.75 MIC (see Figure 1A caption for details). This trend indicates that almost every single cell spread on the plate grew and formed colonies at a wide range of subMIC drug concentration, and no cells formed colonies at (and above) MIC. This observation, suggesting homogeneous population dynamics, agrees with the deterministic prediction discussed above. Additionally, we observed a decrease in colony size at increasing drug concentrations (Figure 1—figure supplement 2).

For bactericidal drugs, at increasing concentrations, the plating efficiency decreased gradually from 1 to 0 (Figure 1B); the grey line was obtained from a linear regression analysis of the whole data set (see Figure 1B caption for details). This trend contrasts with our finding for bacteriostatic drugs (compare the grey lines in Figure 1A and B) and cannot be explained by the deterministic model. In the literature, other studies have reported a similar gradual decrease in the plating efficiency (Liu et al., 2011; Ernst et al., 2002; Dong et al., 2000). However, those studies primarily

concerned how to better determine the MIC in the face of such a gradual decrease and have not characterized population dynamics underlying the gradual decrease.

A subsequent plate assay reveals a lack of heritable resistance

The plating efficiency between 0 and 1 indicates heterogeneous colony formation. In the plate assay above, we found that at ~0.6 MIC, the plating efficiency was ~0.5, meaning that approximately 50% of the cells plated formed colonies and 50% did not. One possible explanation is that the colony-forming cells were intrinsically more resistant to the drugs than the cells that did not form colonies, subsequently giving rise to resistant daughter cells (i.e., heritable resistance). To examine this possibility, for each bactericidal drug used in the experiment (for which the results are shown in Figure 1B), we picked colonies from agar plates exhibiting a plating efficiency of ~0.5 (near 0.6 MIC), suspended them in liquid medium, and immediately plated them on fresh agar plates containing various concentrations of the same drug. The results were plotted in Figure 1C and Figure 1— figure supplement 3. Contrary to our expectation, the plating efficiency of the second plating was about the same as that of the first plating, or in some cases, marginally lower (possibly because cells were challenged with drugs twice consecutively). This observation rules out heritable resistance as an explanation for heterogeneous colony formation at sub-MIC drug concentrations.

Bactericidal drugs induce stochastic fluctuations in population dynamics

Our data above showing the absence of heritable resistance in the surviving populations suggest the possible involvement of stochasticity. That is, bactericidal drugs might induce stochastic fluctuations in the bacterial population size. To investigate this possibility, we performed a plate assay as above and followed the population dynamics of growing micro-colonies at single-cell resolution; we spread cells on agar plates, and rather than waiting for 18 hr and counting colonies

visible to the naked eye, we examined how isolated single cells grew to form micro-colonies using time-lapse microscopy. First, as a control, we examined the dynamics in the absence of antibiotics (Video 1). We counted the number of growing cells in each colony and plotted the number versus time (Figure 2A). The colonies proliferated homogeneously, meaning that different colonies grew similarly. We then repeated the experiment using a bacteriostatic drug (chloramphenicol, thiolutin, or tetracycline) at a sub-MIC level. Visually inspecting the image sequences, we found that cells stably grew, albeit at lower rates (Video 2). The number of growing cells in each micro-colony increased homogeneously and similarly (Figure 2B and Figure 2—figure supplement 1). Previously, we developed a microfluidic chemostat for cell culture (Kim et al., 2012; Deris et al., 2013). When we repeated these experiments using this device, we again observed the same homogeneous population dynamics (Figure 2—figure supplement 2A).

Next, we characterized population dynamics for a bactericidal drug (cefsulodin, ofloxacin, kanamycin, or 6-APA), at a sub-MIC level. We found that the population dynamics were highly stochastic (Video 3). Visual inspection of such image sequences indicated that within a given population, some cells were killed stochastically, whereas other cells survived and divided. Such demographic stochasticity would lead to random fluctuations in the population size. Indeed, the number of growing cells in each colony fluctuated randomly over time (Figure 2C and Figure 2—figure supplement 3A–C). These fluctuations led to dramatically different dynamics for different colonies, even though they originated from genetically identical cells and were cultured under homogeneous antibiotic conditions. When we repeated these experiments using the microfluidic chemostat, we again observed significant population fluctuations (Figure 2—figure supplement 2B–D).

Importantly, the fluctuations drove some colonies into extinction (the light, red-shaded area in Figure 2C). We counted the number of colonies that went extinct and plotted the probability of colony extinction at various drug concentrations; here, we are interested in colony extinction because it is equivalent to bacterial clearance, which means treatment success. Figure 2—figure supplement 4 showed that the extinction probability increased with increasing drug concentrations. This increase in the extinction probability agrees with the trend of decreasing plating efficiency we found above (Figure 1B).

The effects of bactericidal drugs on cell growth and death

A population will undergo extinction if cells die more frequently than divide. Because bactericidal drugs induce cell death, an increase in extinction probability at higher drug concentrations is expected to be due to an increase in the rate of cell death, f. Additionally, bactericidal drugs inflict damage on cells (Belenky et al., 2015; Lobritz et al., 2015). Thus, the rate of cell growth, l, might decrease at higher drug concentrations, which could also contribute to colony extinction. We next sought to determine how bactericidal drugs affect f and l. Previous studies of population growth have shown that at a higher concentration of bactericidal drugs, the 'net growth rate', which is equal to l f, decreases (Regoes et al., 2004). But, to separately resolve changes in f and l, the growth and death of cells must be examined at single cell resolution. We analyzed the single-cell-level image sequences we obtained above (see Figure 2—figure supplement 5 for details of the Video 1. The growth of micro-colonies in the absence of a drug. We characterized how cells grew and formed micro-colonies on LB agar using time-lapse microscopy. An example image sequence is shown here. The time interval between each frame is 20 min. analysis) and determined l and f at various concentrations of bactericidal drugs. We found that at increasing drug concentrations, f

increased (Figure 2—figure supplement 6A). But l changed little, remaining nearly constant (Figure 2—figure supplement 6B). Therefore, bactericidal drugs have significant effects on cell death, but not on cell growth.

A simple, stochastic model of the population dynamics accounts for stochastic clearance of bacterial populations

Our data above suggest that an increase in the probability of population extinction at higher drug concentrations (Figure 2—figure supplement 4) is likely due to an increase in the rate of cell death (Figure 2—figure supplement 6). To quantitatively understand the relationship between the extinction probability and death rate, we employed a stochastic model, known as the Markovian birth-and-death process, that has been widely used to study the basic features of stochastic population dynamics (Novozhilov et al., 2006; Pavel Krapivsky and Ben-Naim, 2010; Kendall, 1948). This model contains two parameters, the rate of cell growth and death, l and f, respectively. Each individual cell can divide or die stochastically with the probabilities determined by these parameters. Due to this demographic stochasticity, the number of cells within a population, n, fluctuates over time. Thus, n cannot be predicted deterministically but only probabilistically, and the probability is described as follows,

$$P_n = \lambda(n-1)P_{n-1} - (\lambda + \phi)nP_n + \phi(n+1)P_{n+1}$$

where Pn refers to the probability of n cells being present in a population. The key boundary condition in this model is that once n reaches 0, it cannot change afterward. Known as an 'absorbing boundary', this condition reflects the biological reality that once a population goes

extinct, it cannot revive. Therefore, a solution of this model for n = 0 (i.e., $P_{n=0}$ or simply P_0) describes the dynamics of population extinction. Please see Appendix 1 for the detail and solution.

We first considered the extinction probability P_0 at low drug concentrations in which the death rate is lower than the growth rate ($f < l$). In this range, P_0 is given by their ratio, $P_0 = f / l$; see Equation A6. Thus, if the death rate is zero ($f = 0$), the extinction probability P_0 is 0, meaning n always increases (this makes intuitive sense). As the death rate increases ($0 < f < l$), P_0 increases and becomes non-zero, meaning that n may stochastically reach the absorbing boundary, agreeing with our observation of stochastic population extinction at sub-MIC drug concentrations; Figure 2C shows that some populations reached n = 0 (marked by the light red shaded area), while other populations thrived (also see Figure 2—figure supplement 3A–C). The solution $P_0 = f / l$ predicts that the extinction probability increases linearly with death rate, with the slope being 1 / l (the solid line in Figure 2D). We sought to test this prediction quantitatively by comparing it with experimental data. Above, analyzing time-lapse microscope images, we obtained the probability of population extinction (Figure 2—figure supplement 4), and the death rate (Figure 2—figure supplement 6), at different concentrations of bactericidal drugs. Using these data, we obtained the relationship between the probability of population extinction and the death rate and plotted it in Figure 2D. We found good agreement between the model prediction and experimental data (compare the solid line and symbols in Figure 2D).

Next, using the quantitative relationship we found above ($P0 = f / l$), we will specify the condition for the MIC. In our plate assay (Figure 1B), we observed that the plating efficiency decreases at higher drug concentrations (in the sub-MIC range) and reaches zero at the MIC. Also, the quantitative relationship we found above showed that extinction probability increases at higher drug concentrations (consistent with a decrease in the plating efficiency), reaching one when the

death rate is equal to the growth rate (see the dashed line in Figure 2D); thus, P0 = 1 at f = l. The extinction probability equal to one (P0 = 1) means that all colonies go extinct, which corresponds to zero plating efficiency. The drug concentration at which the plating efficiency reaches zero is the MIC (Figure 1B). Taken together, at the MIC, the plating efficiency is zero because extinction probability is one (P0 = 1), and the extinction probability is one because the growth rate and death rate are equal (f = l). In short, f = l at the MIC.

Next, we considered drug concentrations above the MIC, where the death rate is higher than the growth rate (f > l). In this range, the model predicts that all populations eventually go extinct (P0 = 1 in Equation A6); this makes intuitive sense. Importantly, due to population fluctuations, populations are expected to go extinct at various times (Equation A5), meaning that the number of live populations (the populations that have not undergone extinction yet) decreases gradually over time. The model predicts that this decrease can be approximated by an exponential decay in the long time limit (t 1=jf lj); see Equation A7. We tested these model predictions by repeating timelapse microscope experiments at drug concentrations above the MIC. All the populations indeed went extinct at various times (Figure 3A and Figure 2—figure supplement 3D–F). When we counted the number of live colonies, this number decreased gradually over time (Figure 3B). In this semi-log plot, the decrease was linear (compare it with the dashed line), consistent with the model prediction of an exponential decay (Equation A7).

A population with large inoculum size is subject to stochastic clearance at sub-MIC drug concentrations

Our findings above indicate that the simple stochastic model can adequately capture the extinction dynamics of populations exposed to bactericidal drugs. What is striking in our findings is that, due to drug-induced population fluctuations, a bacterial population may undergo extinction

even at subMIC concentrations, the concentrations that were previously deemed inefficacious to clear bacteria. We have established this result by examining the dynamics of colonies originated from single bacterial cells, the smallest possible inoculum size. Clinical studies have characterized the bacterial inoculum size that can produce infections (i.e., infectious dose). The infectious dose can be as low as one (Jones et al., 2006; Haas and Rose, 1994; Jones et al., 2005), but is generally 10–100 (Tuttle et al., 1999; DuPont et al., 1989; Hara-Kudo and Takatori, 2011; Kaiser et al., 1992) or larger (Kothary and BABU, 2001; Gama et al., 2012). It is expected that, with larger inoculum size, a population experiences less fluctuations (because demographic stochasticity gets averaged out). When we estimated the magnitudes of fluctuations by the coefficient of variation (CV), that is, the standard deviation divided by the mean, using our model, Equation A16 shows decreasing CV with increasing inoculum size, supporting the expectation above. Interestingly, Equation A16 also predicted that the magnitude of population fluctuations depends on rates of cell growth and death as well (Equation A16); CV increases as the death rate approaches the growth rate, meaning that population fluctuations become intensified when neither growth nor death is a dominant event. This prediction, together with our finding above that the death rate approaches the growth rate as the drug concentration increases to the MIC (Figure 2D), suggests that near the MIC, a population with relatively large inoculum size may still be prone to stochastic extinction.

To test this possibility, we experimentally characterized stochastic clearance of a bacterial population starting with different inoculum sizes. First, we prepared a large volume of a cell culture with the cell density of ~640 cells/ 200 mL, supplemented the culture with a low concentration of cefsulodin (23 mg/ml), and then distributed 200 mL of the cell culture equally to 12 isolated chambers in a microtiter plate. Here, an isolated cell culture in each chamber represents a separate population. We repeated this procedure using higher cefsulodin concentrations (but maintaining the

inoculum size). We then incubated the microtiter plate overnight. We found that, at low cefsulodin concentrations (to the left of the dashed line in Figure 3C), all chambers became turbid, meaning all populations grew. Thus, the probability of population extinction was zero (P0 = 0). In Figure 3C, we used a heat map to graphically represent the probability (we also added the values of the probability in the graph for additional clarification). At high concentrations (to the right of the solid line), all chambers were clear (P0 = 1); thus, the solid line indicates the MIC. At intermediate concentrations (between the dashed and solid lines), we observed heterogeneous population growth; some chambers were clear while others were turbid (0 < P0 < 1). Subsequent plating of the clear cultures on drug-free LB agar plates yielded no colonies, indicating population extinction.

We then repeated this experiment by using different inoculum sizes and bactericidal drugs. The results were plotted as heat maps in Figure 3D; here, the Y axis represents different inoculum sizes. As above, a solid line represents the MIC, above which all chambers were clear (P0 = 1). MIC values were higher at higher cell densities. We note that the higher MIC at higher cell density (inoculum effect) has been observed previously and is being actively studied by others in the field (Artemova et al., 2015; Brook, 1989; Tan et al., 2012; Karslake et al., 2016). Thus, it is not the focus of our study; rather, we focus on stochastic clearance below the MIC. As above, a dashed line represents the concentrations below which all chambers were turbid (P0 = 0). The area between the dashed and solid lines indicates the range of drug concentrations and inoculum sizes that exhibited heterogeneous population growth (0 < P0 < 1), meaning stochastic clearance. Figure 3D shows that stochastic clearance occurs even for a population starting with as large as ~20,000 cells, inoculum size much larger than infectious doses for many infectious diseases.

Alteration of the extinction probability to facilitate bacterial eradication at sub-MIC drug concentrations

Previously, antibiotic treatment at sub-MIC levels was not considered a viable option for bacterial eradication, because the deterministic model predicts that all bacterial populations should grow at sub-MIC levels (i.e., antibiotic treatment failure). However, our experimental results and stochastic model above indicate that at sub-MIC levels of bactericidal drugs, a population might undergo extinction stochastically. An increase in this probability while keeping the drug concentration low would be therapeutically useful; with an increased probability of extinction, sub-MIC ranges of drugs could be used to eradicate bacteria reliably. We therefore employed our model to explore how the extinction probability can be altered by means other than changing the bactericidal drug concentration.

Our model indicates that the extinction probability is determined by the ratio of the death and growth rates ($P0 = f/l$; Equation A6). Thus, based on the model, a reduction in growth rate (denominator) should lead to an increase in the extinction probability. Growth rate can be reduced by using poor growth media, or alternatively using bacteriostatic drugs. This means, for a sub-MIC concentration of a bactericidal drug (for which the extinction probability is less than 1), either a switch to poor growth media or addition of a sub-MIC level of a bacteriostatic drug would lead to an increase in the extinction probability. We note that the latter represents combination therapies, and other studies have characterized bacterial responses to combination therapies (Bollenbach, 2015). However, these studies primarily concerned deterministic changes in the MIC of a large population. Conversely, our study focuses on how combination therapies affect stochastic occurrence of population extinction. Another difference is our focus on sub-MIC drug ranges, an

important point given previous research showing that the effects of drug combinations at the MIC might differ from those at sub-MIC levels (Ocampo et al., 2014).

To characterize the extinction probability, we introduced the plating inefficiency (= 1 – plating efficiency); the plating efficiency reflects the probability that a bacterial cell forms a population of a bacterial colony, and therefore, the plating inefficiency reflects the probability of population extinction. We first calculated the plating inefficiency using the plate assay results in Figure 1B and plotted it as white columns in Figure 4A; thus, white columns represent the extinction probability in rich growth media (LB) with bactericidal drugs alone. To test the effect of our growth reduction strategy, we repeated the plate assay, either by replacing LB with casamino acids (a poor nutrient source that leads to slower growth than LB; see Figure 4—figure supplement 1), or by adding a sub-MIC concentration of chloramphenicol or tetracycline. The plating inefficiency obtained with these treatments was plotted as solid columns in Figure 4A. The rise of solid columns above white columns indicates that growth reduction indeed led to an increase in the extinction probability, in agreement with our prediction.

We next examined how generally such an increase in the extinction probability might occur. The extinction probability depends on the growth rate (l) and death rate (f), which we assumed to be independent. Because the probability is equal to their ratio (f/l; Equation A6), this growth reduction strategy might not work when f is not independent but decreases in response to a decrease in l. Such coupling between f and l could occur for bactericidal drugs that kill only growing cells, possibly because these drugs target processes critical for cell growth. This means that, for bactericidal drugs that exhibit a killing rate of zero for non-growing cells (i.e., ffi 0 when lfi 0), neither the switch to poor growth medium nor addition of bacteriostatic drugs would increase the extinction probability. To test this possibility, we first identified such bactericidal drugs; we stopped cell growth in cultures

by depriving the cells of nutrients, added bactericidal drugs at concentrations capable of eradicating growing cells, and then determined which drugs were no longer capable of killing the bacteria. As shown in Figure 4—figure supplement 2, killing was completely abolished for kanamycin and 6-APA, indicating that ffi 0 when lfi 0. As expected, for these drugs, neither the switch to poor growth medium nor addition of bacteriostatic drugs resulted in an increase in the extinction probability (Figure 4—figure supplement 3).

Furthermore, our model does not consider specific drug-drug interactions. For example, a previous study showed bacteriostatic translation-inhibiting drugs and bactericidal quinolone drugs affect gene expression in a way to negate their effects (Bollenbach et al., 2009). Consistent with this study, we failed to observe significant changes in the extinction probability when ofloxacin or ciprofloxacin (quinolone drugs) is used with and without bacteriostatic translation-inhibiting drugs (Figure 4—figure supplement 4).

Extending the growth-reduction strategy to antibiotic-resistant strains

Above, we tested the growth reduction strategy for a WT (antibiotic-susceptible) E. coli strain. Although the strategy did not work for some drugs due to their complex effects on cells, for those that worked, the strategy substantially increased the chance of bacterial clearance at sub-MIC drug concentrations. We wondered if this strategy could be applicable to antibiotic-resistant strains. Resistant strains are difficult to eradicate because their MICs are very high, to levels that are toxic to hosts. Therefore, the development of therapies that utilize sub-MIC doses would be highly useful. To test if our growth-reduction strategy would work for antibiotic-resistant bacteria, we repeated a plate assay using resistant strains. Laboratory evolution of rifampicin resistance has been frequently reported in the literature (Goldstein, 2014). By plating WT (antibiotic-susceptible) E. coli cells on a

LB agar plate containing the rifampicin concentration equal to 2x MIC, we isolated a rifampicin resistant (Rifr) mutant; the MIC of this mutant was ~10 fold higher than that of the parent strain. We then plated the mutant on LB agar plates containing various rifampicin concentrations. Additionally, we acquired clinically isolated E. coli strains that were resistant to either cefsulodin or vancomycin (see Methods), and plated them on LB agar plates with increasing concentrations of cefsulodin or vancomycin. These resistant strains exhibited non-zero plating inefficiency at sub-MIC concentrations of bactericidal drugs (white columns in Figure 4B), indicating stochastic clearance. We then repeated the experiments either by replacing LB with casamino acids or by adding a sub-MIC concentration of a bacteriostatic drug. As with the antibiotic-susceptible strain, these treatments led to an increase in the plating inefficiency (Figure 4B), indicating that our growth reduction strategy facilitated the clearance of antibiotic-resistant bacteria at sub-MIC concentrations.

Discussion

Antibiotic treatment typically targets mature infection which contains a large number of bacterial cells (e.g., 10^8) (Smith and Wood, 1956; Palaci et al., 2007; Feldman, 1976; Canetti, 1965; Canetti, 1956). To clear infections and avoid post-treatment relapse, not only the reduction of a large population of bacteria to a small population, but also the complete extinction of the small population is desired (Tomita et al., 2002; Wilson et al., 2013; Bayston et al., 2007); this is especially so for immuno-compromised hosts and also for infections involving bacteria with a low infectious dose. Previous studies of large bacterial populations adequately accounted for the former process. This study focused on the latter process. Our results directly revealed that antibiotics induce significant fluctuations in population size, leading to stochastic population extinction. Modeling population fluctuations using a probabilistic model, we then established a quantitative understanding

of stochastic extinction. This model further predicted how the extinction probability could be manipulated to facilitate bacterial eradication at sub-MIC drug concentrations. We experimentally tested how amenable the extinction probability is to manipulation.

One possible molecular-level mechanism that gives rise to population fluctuations could be cell-to-cell variability in gene expression. Previous studies showed that variation in expression of antibiotic-resistance genes, marA, cat, kagG, ompC, or bla, results in variation in antibiotic susceptibility to carbenicillin, chloramphenicol, isoniazid, kanamycin or ceftriaxone, respectively (Deris et al., 2013; El Meouche et al., 2016; Wakamoto et al., 2013; Sanchez-Romero and Casadesus, 2014; Wang et al., 2014). Although our study mostly focuses on antibiotic-susceptible bacteria, a similar mechanism might play a role, leading to heterogeneous growth/death of bacterial cells and eventually population fluctuations. We note that there were attempts to stochastically model large populations of antibiotic-susceptible bacteria (e.g., see [Ferrante et al., 2005]). But, given the deterministic nature of observed dynamics, the need for stochastic models was not clear, and the model prediction of stochasticity was not tested in the work. On the other hand, previous theoretical studies of the evolution of antibiotic resistance typically modeled the growth and death of newly emerged mutants as stochastic processes, showing how fluctuations in the size of small mutant populations affect evolutionary dynamics; e.g., see (Hermsen et al., 2012; Nissen-Meyer, 1966). Our study validates this modeling approach.

Our findings expose fundamental limits in our predictive ability for bacterial clearance. Clinical studies of antibiotic therapies have often reported unexpected failures of eradicating antibiotic-susceptible bacteria (Doern and Brecher, 2011; Weidner et al., 1999; Gopal et al., 1976; Ficnar et al., 1997; Forrest et al., 1993). Laboratory studies of simple model organisms such as worms have reported similar observations (Moy et al., 2006; Needham et al., 2004; Kaito et al.,

2002). The variability in host environments could certainly contribute to such unexpected antibiotic failures. For example, a recent study showed the effects of variability in host immunity on infection course (Duneau et al., 2017). Our study demonstrates that even in the absence of host variability, bacterial clearance occurs stochastically due to antibiotic-induced population fluctuations. At subMIC drug concentrations, bacterial populations may or may not go extinct. At drug concentrations above the MIC, all populations eventually go extinct, but not all at once. Rather, the extinction time is highly variable, meaning that in some cases, it can take significantly longer to eradicate bacteria. This inherent stochasticity, together with host variability, makes it difficult (or even impossible) to deterministically predict antibiotic-mediated clearance of bacterial infection.

More studies are needed to elucidate the impact of these population fluctuations on treatment outcomes in clinical settings. However, we observed such fluctuations even for a relatively large population (~10^4 cells). In comparison, previous in vivo studies showed that the population size needed to establish infections (i.e., infectious dose) can be as small as 1–100 cells (Jones et al., 2006; Haas and Rose, 1994; Jones et al., 2005; Tuttle et al., 1999; DuPont et al., 1989; Hara-Kudo and Takatori, 2011; Kaiser et al., 1992), which is well within the stochasticity range. This means that if tens of cells (or even a few cells) happen to stochastically survive a fixed course of antibiotic treatment, this small population can re-establish infections once antibiotics are removed, leading to treatment failure. Importantly, a recent article raised an issue regarding the conventional wisdom of 'complete the prescribed course', and argued for re-consideration of antibiotic duration (Llewelyn et al., 2017). We believe that our observation of stochastic extinction dynamics, especially inherent variability in extinction time, has significant bearing on this issue.

Furthermore, our study may guide the design of new therapeutic strategies. Based on the deterministic understanding of population dynamics, it has been generally accepted that only at drug

concentrations above the MIC, bacterial populations go extinct. Contrarily, we observed that stochastic population fluctuations drive a population to extinction even at sub-MIC drug concentrations. This observation suggests an intriguing possibility that sub-MIC drug ranges can be used as a clinical option to clear bacteria. We acknowledge that the stochastic nature in the population dynamics is a double-edged sword. Stochasticity can be advantageous because it can drive a population to extinction even at low drug concentrations. But, stochasticity makes it impossible to pre-determine whether the bacterial population of particular interest will go extinct or not; this is disadvantageous because we cannot predict a priori if a specific treatment will work or not. However, this disadvantage can be minimized by manipulating population fluctuations and thereby increasing the probability of extinction. In the present study, we explored this possibility with the help of the probabilistic model, showing that it is possible to increase the probability of clearance. This idea of using subMIC drug concentrations to clear bacteria is particularly attractive in the context of antibiotic resistance. Antibiotic-resistant bacteria have very high MICs, often above the levels that are toxic to hosts. Thus, antibiotic concentrations above the MIC cannot be administered, which is why antibiotic resistance is a serious public concern worldwide (O'Neill, 2016). Our plate assay using bactericidal drugs revealed that, like antibiotic-susceptible bacteria, resistant bacteria exhibited non-zero plating inefficiency below the MIC, indicating a non-zero probability of clearance at sub-MIC drug concentrations. We also showed that this probability of clearance could be manipulated, facilitating the clearance of antibiotic-resistant bacteria. For comparison, we note a recent study in the field of viral infections, which showed that stochastic noise in HIV gene expression may be used to treat HIV infections (Dar et al., 2014). This study further supports the idea that stochasticity can be advantageous and be used to combat infections. We believe that the time is ripe for the development of clinical treatment strategies to take advantage

of stochasticity, especially so given recent advances in our understanding of stochasticity in biological processes (Jones et al., 2014; Ackermann, 2015; Tanouchi et al., 2015; Banerjee et al., 2017; Scott et al., 2007; Schmiedel et al., 2015; Sigal et al., 2006; Blount et al., 2008; Ray and Igoshin, 2012).

Our study will also have positive impacts on in vitro assessment of antibiotic efficacy. MIC is the most critical parameter to assess antibiotic efficacy. In the deterministic framework, MIC is defined as the drug concentration at which the population size is maintained, which is realized when the death rate is equal to growth rate (f = l); see Equation A2. Accordingly, in a broth dilution method, the drug concentration that yields no change in the turbidity of bacterial cultures is defined as the MIC. Above, we found that in the stochastic framework, at the MIC, the rates of death and growth are equal (f = l; see Figure 2D) and the extinction probability is 1 (Equation A6), meaning all populations go extinct at the MIC. Therefore, in both deterministic and stochastic frameworks, at the MIC, the condition, f = l, is satisfied, but population dynamics are very different (population maintenance versus population extinction). This clarification can reconcile two common ways to determine the MIC, a plate assay based on complete colony extinction and a broth method based on no change in culture turbidity (population maintenance); although the MICs were determined based on different population dynamics in these two cases, both methods identify the drug concentration at which growth rate is equal to death as the MIC. This clarification is particularly important in light of recent efforts to increase the efficiency of the broth method by using small culture volumes (which include a few or tens of bacterial cells) (Avesar et al., 2017). With such small volumes, MIC should be defined based on population extinction, not population maintenance.

Lastly, our findings have implications on bacterial persistence. Dormant cells are refractory to antibiotics, persisting through antibiotic treatments (Allison et al., 2011; Balaban et al., 2004).

They are present in very low frequencies (typically 10^{-5}, meaning 1 out of 10^5 cells) (Lewis, 2010), and thus have little effects on population dynamics in small populations considered here ($<<10^5$). However, a study of persister formation requires the enrichment of persisters. To enrich them, studies often treat a large population using antibiotics and characterize a small population of survivors as persisters. The inherently stochastic nature of a small population may lead to variability in this process of enrichment and characterization, complicating studies of persisters. In fact, such variability was reported by a recent quantitative study of persistence (Brauner et al., 2017). Therefore, our findings on the dynamics of small populations could be useful for a better understanding of persistence.

Materials and methods

Bacterial strains and culture

Experiments were conducted using E. coli strain NCM3722 (Soupene et al., 2003; Lyons et al., 2011; Brown and Jun, 2015). Bacteria were grown in 5 mL of Lysogeny Broth (LB, Fisher Bioreagents) in 20 mL borosilicate glass culture tubes at 37° C with shaking (250 rpm). Our typical experimental procedure is as follows. Cells were first cultured in LB broth overnight (pre-culture). The next morning, the cells were sub-cultured into pre-warmed LB broth at the optical density (OD600) of ~0.001 (measured using a Genesys20 spectrophotometer, Thermo-Fisher) and allowed to grow exponentially. The culture at the OD600 of ~0.4 was used for a plate assay or for microscope experiments.

We evolved a rifampicin-resistant mutant from our WT strain (NCM3722). We plated ~10^8 WT cells on a LB agar plate containing 20 mg/ml (2 x MICWT). 18 colonies were formed next day. We chose one colony and purified it by re-streaking. The mutant (EMK32) had the MIC of 160

mg/ml. We obtained the cefsulodin-resistant strain (EMK35, MIC = 170 mg/ml) and vancomycin-resistant strain (EMK36, MIC = 250 mg/ml) from Georgia Emerging Infections Program MuGSI collection (their MuGSI strain numbers were Mu519 and Mu107, respectively.)

Plate assay

Cells were spread on LB agar plates containing different concentrations of various antibiotics (see below). Through serial dilutions (using 1.16% (w/v) NaCl solution), we ensured the number of colonies to be between 50 and 250 on a plate (100x 15 mm Petri dish). The plates were then incubated at 37°C. After 18 hr of incubation, the number of visible colonies on plates was determined. As indicated in the main text, we also used casamino acids agar plates. We dissolved 2% casamino acids in N-C- minimal medium (Csonka et al., 1994) and filtered the medium. We separately autoclaved agar, and when agar was cooled and felt warm to the touch, the casamino acids medium was added.

Antibiotics

Stock solutions of ciprofloxacin (1 mg/ml), kanamycin (50 mg/ml), 6-APA (2 mg/ml), streptomycin (25 mg/ml), ofloxacin (1 mg/ml), erythromycin (10 mg/ml), vancomycin (100 mg/ml), and cefsulodin (30 mg/ml) were prepared in sterilized water. Stock solutions of tetracycline (50 mg/ml) and chloramphenicol (10 mM) were prepared in methanol. Stock solutions of thiolutin (2 mg/ml) and rifampicin (50 mg/ml) were prepared in DMSO. Antibiotics were purchased from Biobasic Inc (Canada), Acros Organics, or Sigma-Aldrich (St. Louis, MO.).

Time-Lapse microscopy

Cells were cultured as described above without antibiotics first. At OD600 of ~0.4, 2 mL of aliquot from a culture was loaded into a pre-warmed 35 mm glass-bottom Petri dish (InVitro

Scientific). A pre-warmed LB agarose pad containing antibiotics was placed over them (this procedure marked time zero). The dish was then moved into a pre-warmed (at 37°C) inverted microscope (Olympus IX83), and appropriate stage positions (typically ~50 positions) were selected. Selection of multiple state positions and setting up the software for automatic image acquisition normally took 1 ~ 2 hr. The microscope had an automated mechanical XY stage and auto-focus and was controlled by the MetaMorph software (Molecular Devices). Also, it was housed by a microscope incubator (InVivo Scientific) which maintained the temperature of samples at 37°C during experiments. An oil immersion phase-contrast 60X objective was used for imaging. Images were captured using a Neo 5.5 sCMOS camera (Andor). We also cultured cells in a microfluidic chemostat (using LB broth as growth medium). The detailed procedure for the microfluidic experiments was published in our previous articles (Kim et al., 2012; Deris et al., 2013). Images were analyzed using MicrobeJ, a plug-in for the ImageJ software (Ducret et al., 2016), and the analysis results were validated manually. In our experiments, all cells that stopped growing eventually underwent lysis. Although dormant cells that survive antibiotics (i.e., persisters) may complicate our analysis, their frequency is very low, ~10^{-5} (i.e., 1 in 10^5 cells) (Lewis, 2010). Because our study involves small populations (less than 100 cells), dormancy has little relevance to our study.

Replicate culture using a microtiter plate

We first cultured cells in 5 mL LB medium to the OD600 of ~0.4, as described above. Previously, using a plate assay, we determined that OD600 of 0.4 contains 2 108 cells per 1 mL. Using this relation, we diluted the culture in 10 mL LB broth such that there were ~640 cells per 200 mL (we separately confirmed this density by plating cells on LB agar plates containing no antibiotics and counting colony-forming units). We supplemented this culture with various

concentrations of antibiotics. We then equally distributed 200 mL of this culture to 12 chambers in a microtiter plate. We repeated this procedure by varying cell density. Next day, we measured the OD600 of each chamber.

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Figure Captions

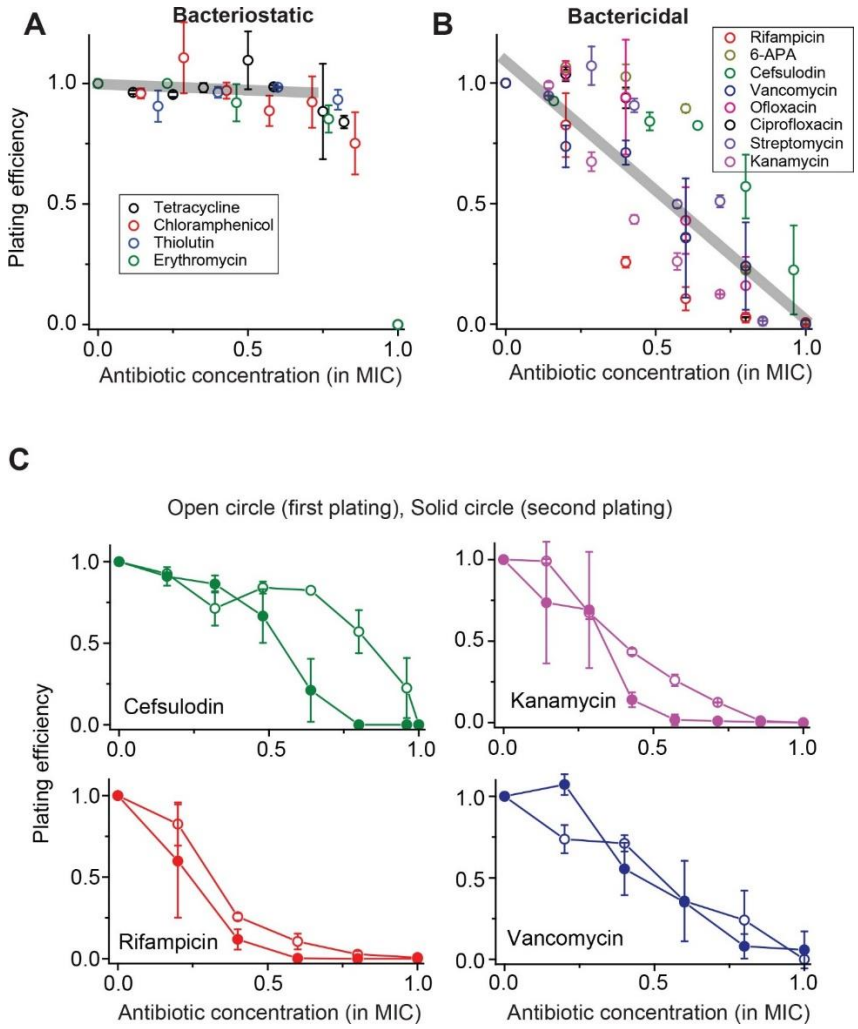


Figure 0-1: Contrasting trends in plating efficiency at increasing concentrations of bacteriostatic and bactericidal drugs.

A) When we performed the plate assay using bacteriostatic drugs, N_{CFU} was generally maintained at increasing drug concentrations. See Figure 1-figure supplement 1 for a detailed illustration of how this plot was made. A linear regression analysis was performed for the whole data set below 0.75× MIC, resulting in the grey line (slope = −0.05, intercept = 0.9942, R-squared = 0.99).

Colony size decreased at increasing drug concentrations (Figure 1-figure supplement 2). B) For bactericidal drugs, N_{CFU} decreased at increasing drug concentrations, indicating heterogeneous population dynamics of bacteria. A linear regression analysis was performed for the whole data set, and the result was plotted as a grey line (slope = −1.07, intercept = 1.10, R-squared = 0.79). For both groups of drugs, the lowest concentration yielding no colonies was defined as the MIC. The Supplementary File 1 lists the MICs for all drugs examined in this study. We plotted plating efficiency for each antibiotic in separate panels in Figure 1-figure supplement 4. C) For each bactericidal drug used in the experiment (for which the results were shown in Fig. 1B), we picked a few colonies from the agar plate exhibiting a plating efficiency of ~0.5 (e.g., near 0.6x MIC) and plated them immediately on fresh agar plates containing various concentrations of the same drug. The plating efficiency was similar or marginally lower on the second plating, possibly because exposure to the antibiotics on the first plate adversely affected the cells and rendered them more susceptible to the antibiotics on the second plating. See Figure 1-figure supplement 3 for similar results for other drugs. Therefore, the ability of bacteria to grow and form colonies on plates containing bactericidal drugs was not heritable. We performed at least two biological repeats for all the experiments and plotted the mean here. The error bars represent one standard deviation from the repeats.

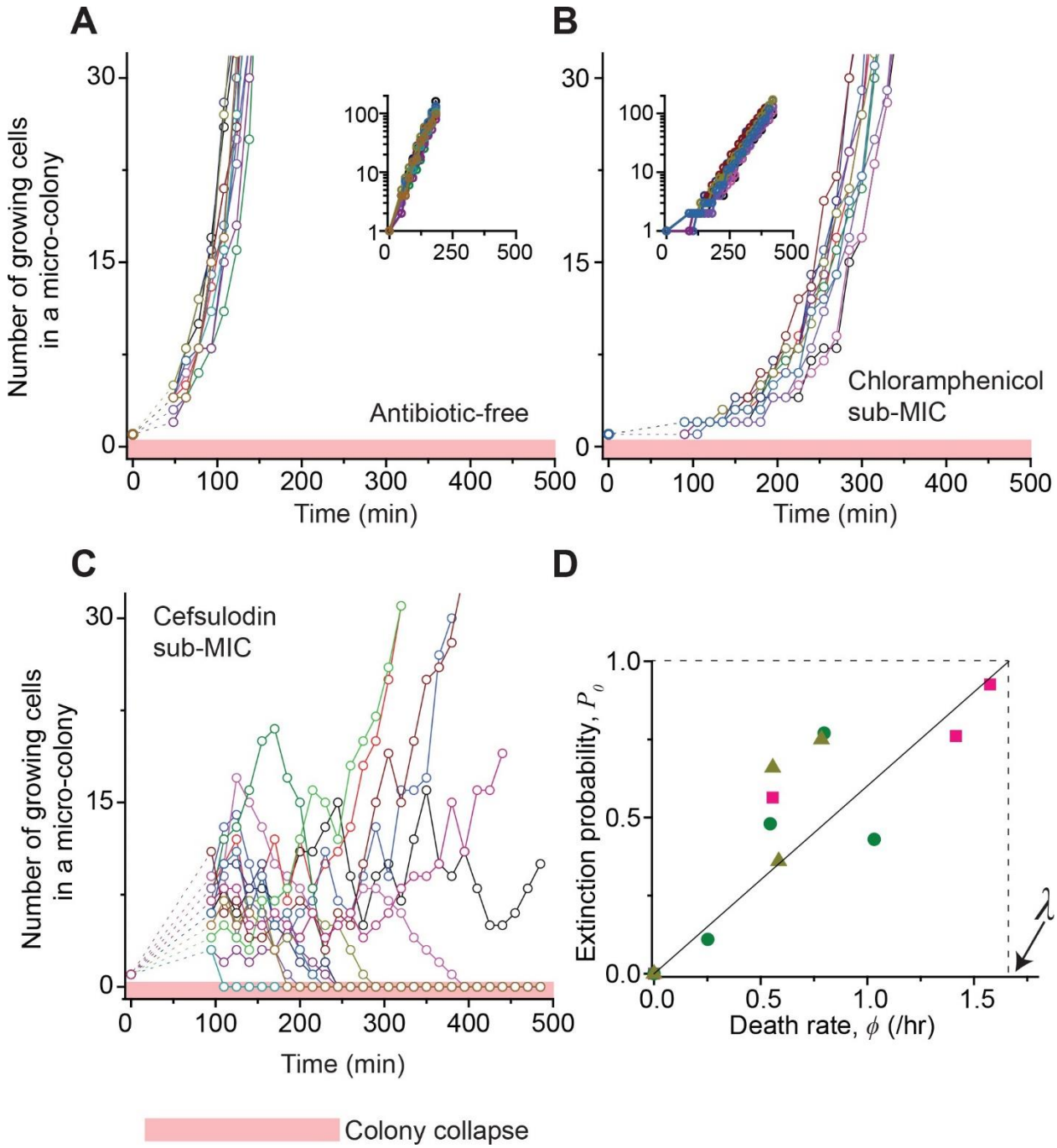


Figure 0-2 Stochastic population dynamics of bacteria exposed to bactericidal drugs.

We characterized how cells grow and form micro-colonies on LB agar plates containing different concentrations of antibiotics using time-lapse microscopy. At time zero, we transferred cells

growing in antibiotic-free LB liquid medium to a LB agar plate, and confirmed that individual cells were spread out and isolated. Setting up a time-lapse imaging experiment after the transfer took 1~2 hours, which is why there is a gap in the data immediately after time zero (dashed lines). The experiments were independently repeated twice (biological repeats) and the data from one experiment are shown here. A, B) We counted the number of growing cells in each micro-colony (represented by a different color). In the absence of antibiotics (panel A) or with a sub-MIC level of a bacteriostatic drug (panel B, 0.7x MIC of chloramphenicol), the number increased similarly for different colonies, revealing homogeneous population growth. Example image sequences are shown in Video 1 and 2. The data are replotted on a semi-log scale (insets). Such homogeneous population growth was observed for other bacteriostatic drugs (thiolutin and tetracycline) as well; see Figure 2-figure supplement 1. When we repeated the experiment using a microfluidic device, we again observed the same homogeneous population dynamics (Figure 2; figure supplement 2A). C) In contrast, the population dynamics of bacteria exposed to a bactericidal drug were highly heterogeneous. An example image sequence was shown in Video 3. The number of growing cells within micro-colonies at 0.8x MIC of cefsulodin is plotted here; the result shows stochastic population fluctuations. Such population fluctuations were again observed when experiments were repeated using other bactericidal drugs (ofloxacin, kanamycin, and 6-APA); see Figure 2-figure supplement 3A-C. The light, red-shaded region indicates the number equal to zero (i.e., population extinction). When we repeated these experiments using a microfluidic device, we again observed population fluctuations (Figure 2-figure supplement 2B-D). D) Our model predicts that the probability of population extinction increases linearly with death rate, with the slope being 1 (the solid line). We experimentally characterized the extinction probability (Figure 2-figure supplement 4), and the death rate (Figure 2-figure supplement 5-6), at different concentrations of bactericidal

drugs. Using these data, we obtained the relationship between the extinction probability and the death rate, and plotted it here (green circles: cefsulodin, pink squares: ofloxacin, and grey triangles: 6-APA). We found good agreement between the model prediction and experimental data. Note that at increasing drug concentrations, death rate changed little (Figure 2-figure supplement 6B), and thus was taken as a constant in the analysis here.

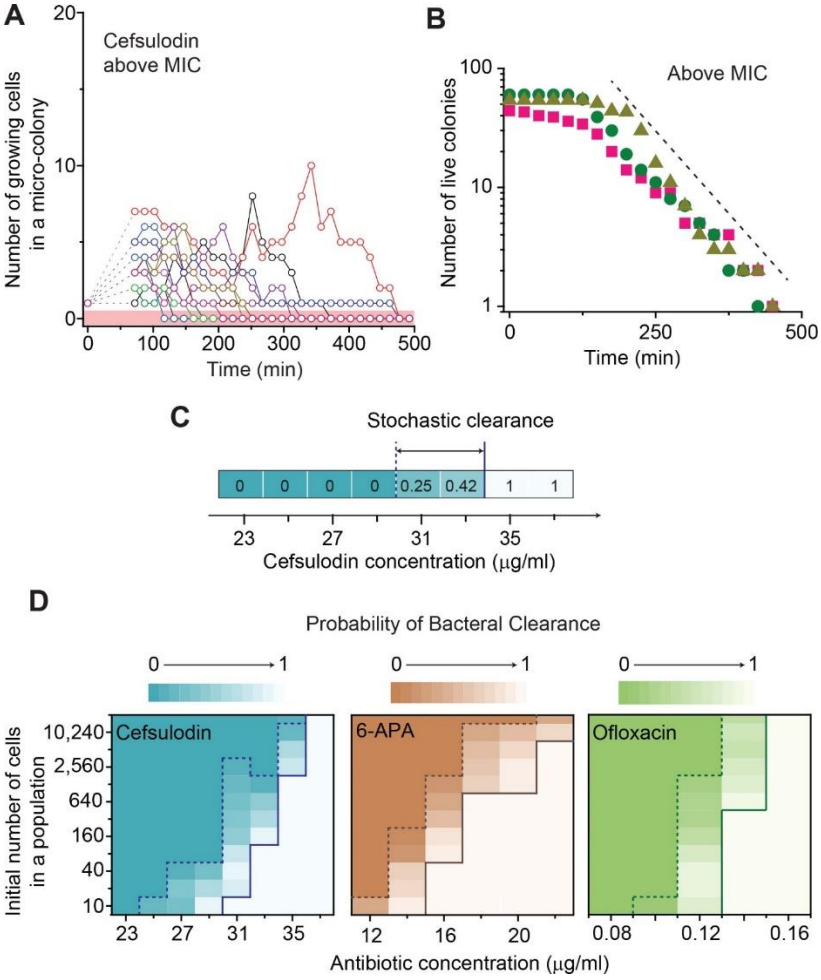


Figure 0-3 . Further characterization of stochastic population dynamics.

A, B) In Fig. 2C and Figure 2-figure supplement 3A-C, we showed the population dynamics of bacteria exposed to sub-MIC levels of bactericidal drugs. Here, we show the dynamics at drug

concentrations above MIC (1.2x MIC). The number of growing cells within micro-colonies for cefsulodin is plotted in panel A. The results for other bactericidal drugs were plotted in Figure 2-figure supplement 3D-F. We observed that all populations went extinct at various times. We then counted the number of live colonies (colonies that have not undergone extinction yet) at various times (~150 colonies monitored). The number decreased gradually over time (green circles: cefsulodin, pink squares: ofloxacin, and grey triangles: 6APA); see panel B. The decrease was linear in a semi-log plot, consistent with the model prediction of an exponential decay (Eq. S7). C) We characterized the extinction probability of populations starting with ~640 cells. We prepared a large volume of a cell culture with the cell density of ~640 cells/ 200 μL, supplemented the culture with a low concentration of cefsulodin (23 μg/ml), and then distributed 200 μL of the cell culture equally to 12 isolated chambers in a microtiter plate. We repeated this procedure using higher cefsulodin concentrations (but maintaining the inoculum size). We then incubated the microtiter plate overnight. By counting the chambers that became turbid or clear, we calculated the extinction probability. We used a heat map to graphically represent the probability; for illustration purpose, we also added the values of the probability to the plot. At low cefsulodin concentrations (to the left of the dashed line), all chambers became turbid ($P_0 = 0$). At high concentrations (to the right of the solid line), all chambers were clear ($P_0 = 1$); thus, the solid line indicates MIC. At intermediate concentrations (between the dashed and solid lines), we observed heterogeneous results (only some chambers were clear, $0 < P_0 < 1$). D) We repeated this experiment using different inoculum sizes and bactericidal drugs. Please note that although we prepared a large volume culture and distributed it equally to chambers, the number of cells in each chamber might vary. We found that the variation was ~10% or less. See Appendix 3 for details.

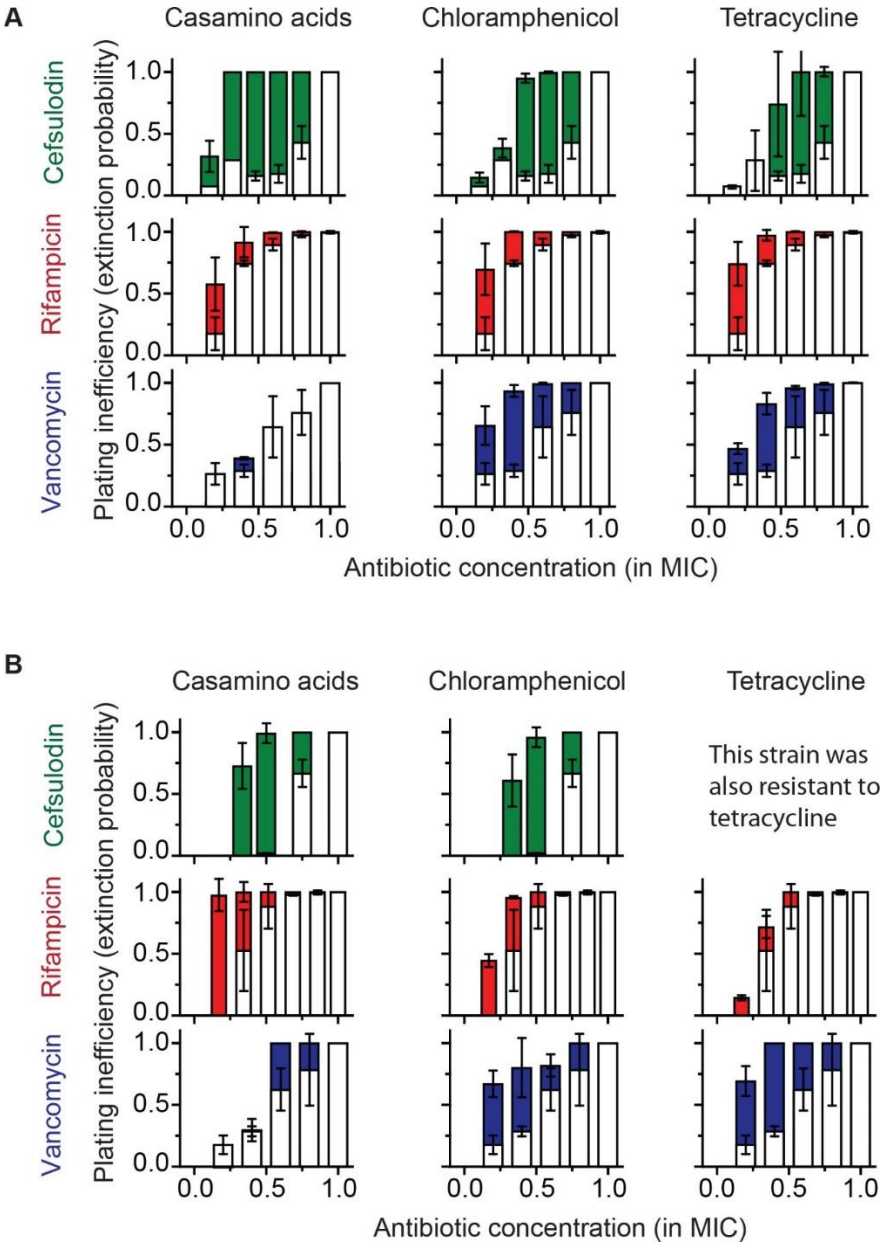


Figure 0-4 Increasing the population-extinction probability by a growth reduction strategy.

(A, B) Previously, we plated WT (antibiotic-susceptible) E. coli cells on LB agar plates at various concentrations of cefsulodin and plotted the plating efficiency in Fig. 1B. Here, we plotted the plating inefficiency (1- plating efficiency) as white columns in the top row; the plating inefficiency

represents the extinction probability. We then repeated a plate assay, either by using casamino acids agar plates (left), or by adding a sub-MIC concentration of a bacteriostatic drug, chloramphenicol (0.7x MIC, middle) or tetracycline (0.5x MIC, right), to LB agar plates. Note that casamino acids lead to slower growth than LB; see Figure 4-figure supplement 1. We plotted the plating inefficiency obtained with these treatments as solid columns in panel A. We repeated this procedure for rifampicin (middle row) and vancomycin (bottom row), for kanamycin and 6-APA (Figure 4-figure supplement 3), and for ciprofloxacin and ofloxacin (Figure 4-figure supplement 4). Note that how generally growth reduction leads to an increase in the extinction probability depends on whether growth rate is dependent on death rate or not; see the main text and Figure 4-figure supplement 2. We then used antibiotic-resistant strains and repeated these experiments (panel B). Please note that the MICs of these mutants were five to ten-fold higher than those of the WT strain. The rise of solid columns above white columns indicates an increase in the extinction probability. We performed at least two biological repeats for all the experiments and plotted the mean. The error bars represent one standard deviation from the repeats.

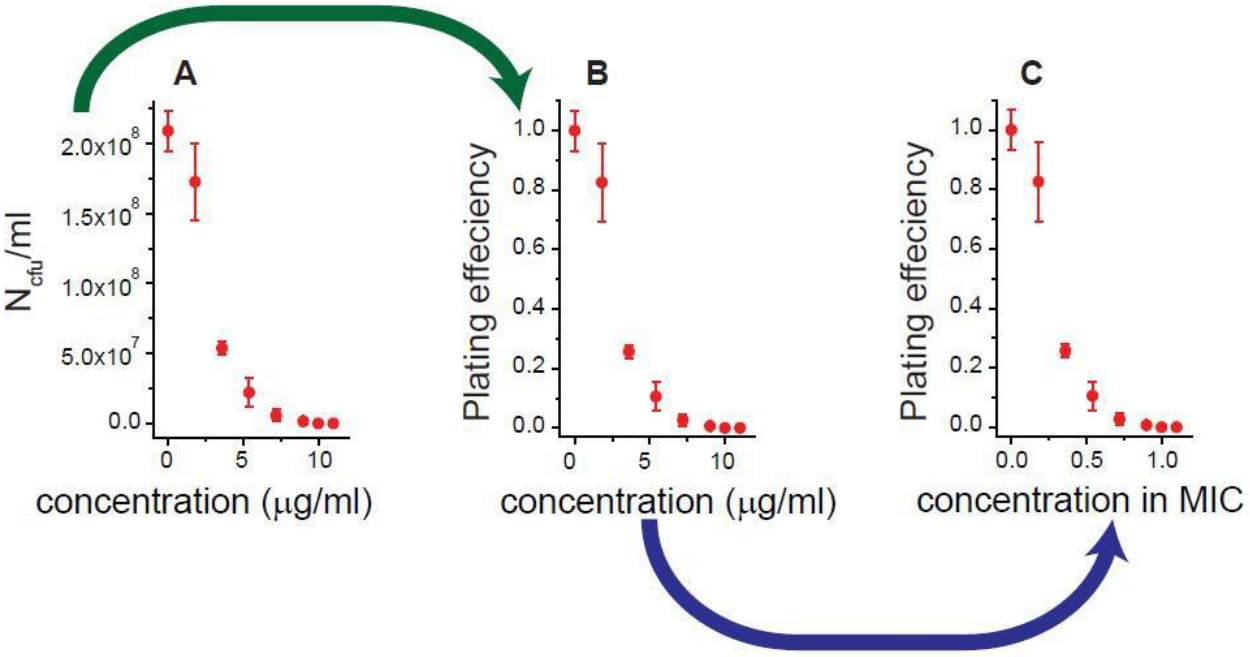


Figure 0-5 - figure 1 supplement 1

We illustrated how we obtained Fig. 1A and 1B, using the results for rifampicin as an example. Antibiotic-susceptible, wild-type E. coli cells were spread on agar plates containing increasing concentrations of rifampicin. After 18 hours of incubation, the number of colony forming units (N_{CFU}) per milliliter was determined; see panel A. By normalizing N_{CFU} to that for an antibiotic-free plate (N^0_{CFU}), we obtained the plating efficiency ($=N_{CFU}/N^0_{CFU}$); see panel B. We defined the lowest drug concentration at which the plating efficiency was zero as the MIC: MIC=10 μg/ml for rifampicin. We normalized the drug concentration to the MIC value; see panel C. We repeated this procedure for various antibiotics and plotted the results in Fig. 1A and 1B.

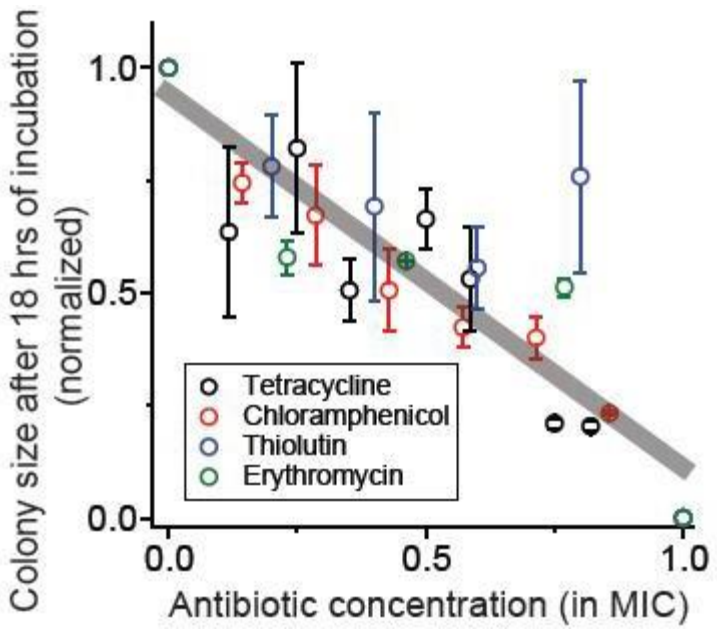


Figure 0-6 -figure 1 supplement 2

We spread cells on agar plates containing various concentrations of bacteriostatic drugs, and after 18 hours of incubation, measured the size of the colonies (using ImageJ software). At increasing concentrations, the size of the colonies decreased. The figure shows the sizes of the colonies normalized to the size on the plate without antibiotics. We performed at least two biological repeats for all the experiments and the error bars represent one standard deviation from the repeats. The grey line was obtained from a linear regression analysis of the whole data set: slope = −0.83, intercept = 0.95, R-squared = 0.80.

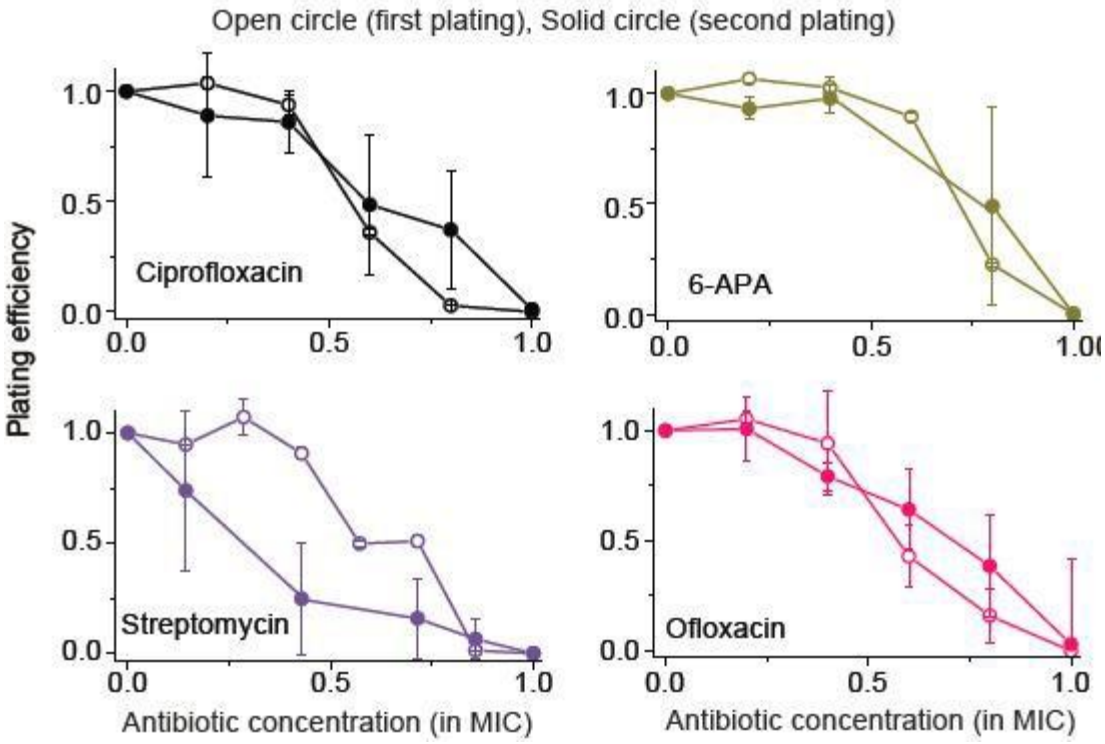


Figure 0-7 Figure 1-figure supplement 3

For each bactericidal drug used (for which the results were shown in Fig. 1B), we picked a few colonies from an agar plate exhibiting the plating efficiency of ~0.5 (e.g., near 0.6x MIC) and plated them immediately on fresh agar plates containing various concentrations of the same drug. The data for ciprofloxacin, 6-APA, streptomycin, and ofloxacin were reported here. (The data for cefsulodin, vancomycin, kanamycin, and rifampicin were reported in Fig. 1C.)

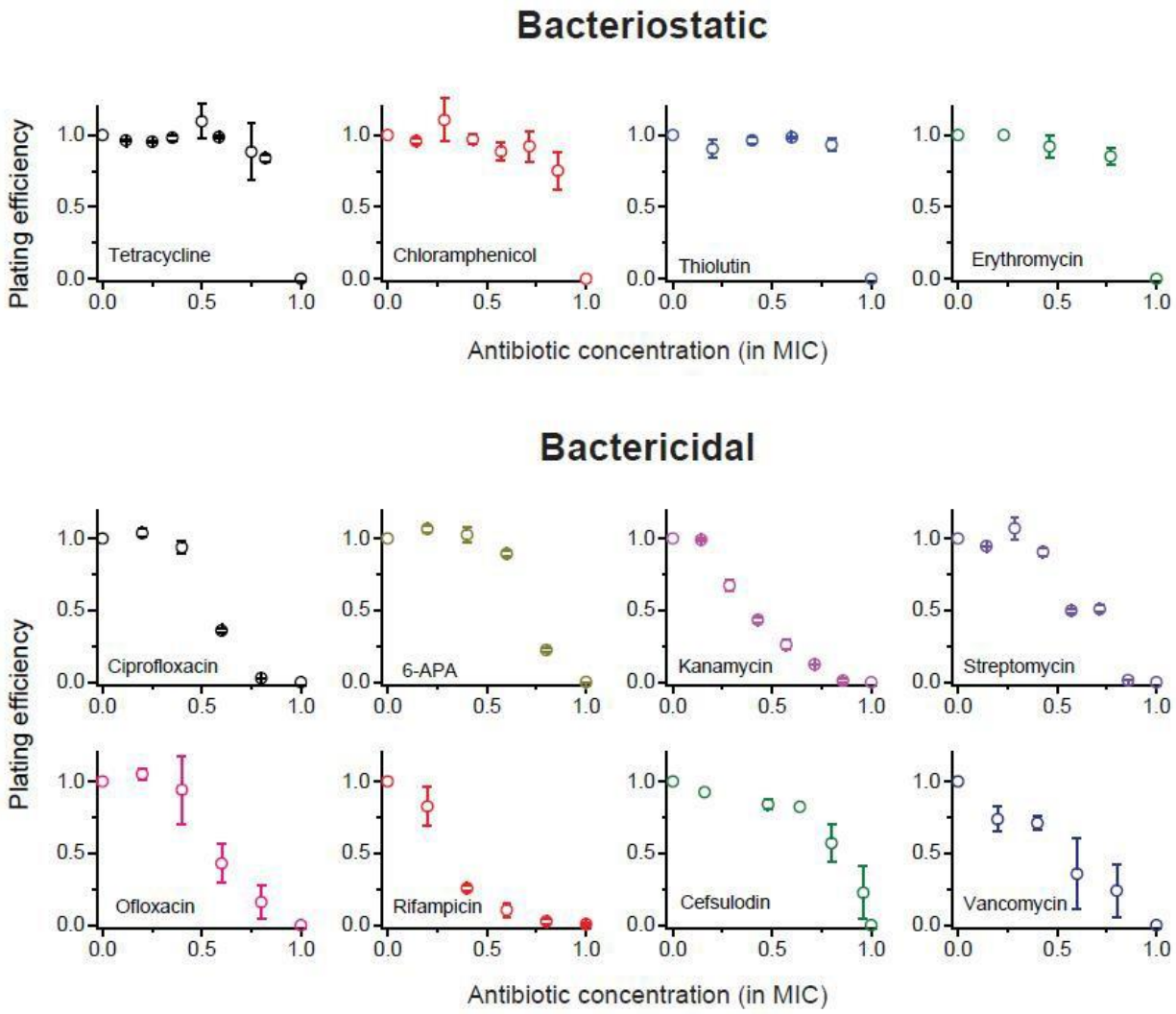


Figure 0-8 Figure 1-figure supplement 4.

We previously plotted plating efficiency for all the antibiotics tested in two panels (Fig. 1A-B). Here, we plot it for each antibiotic in separate panels.

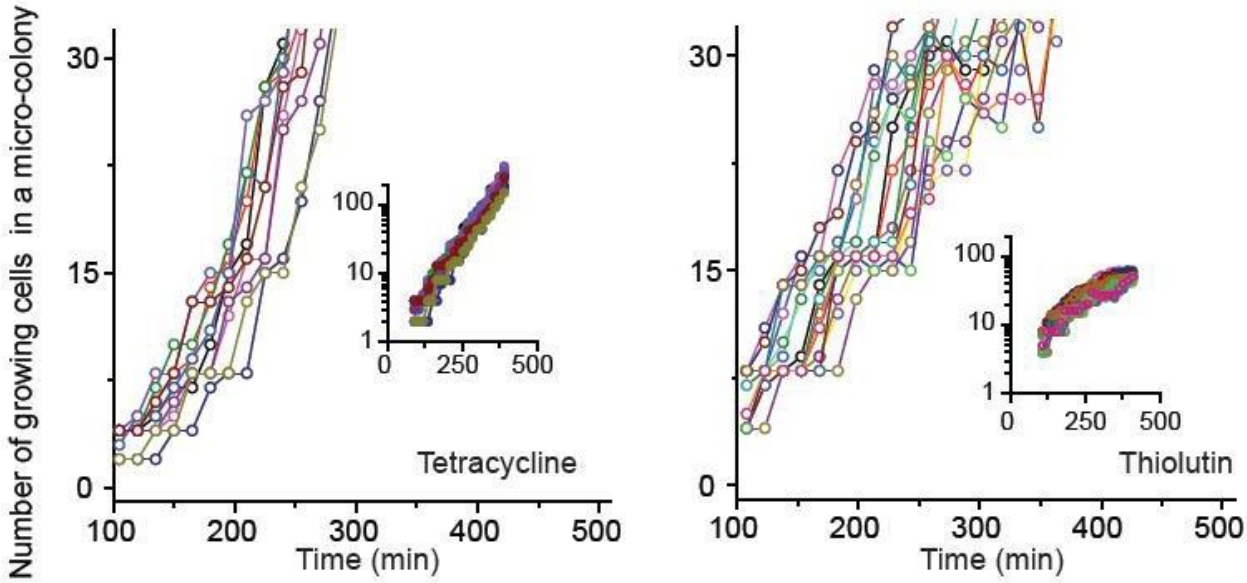


Figure 0-9 Figure 2-figure supplement 1.

We characterized how cells grew and formed micro-colonies at sub-MIC levels of bacteriostatic drugs (chloramphenicol, thiolutin, and tetracycline) at single-cell resolution. See Fig. 2 and its caption for details. The data for chloramphenicol were plotted in Fig. 2B. The data for tetracycline (0.7x MIC) and thiolutin (0.5x MIC) were plotted here. The data were replotted on a semilog scale (insets).

In our plate assay (data shown in Fig. 1A), we plated cells on agar containing increasing concentrations of bacteriostatic drugs and, after 18 hours of incubation, counted the number of visible colonies. The lowest drug concentrations yielding no visible colonies were defined as the MIC. When we performed our microscope experiments at the MICs of bacteriostatic drugs, we observed that some cells still grew, albeit at very low rates, and micro-colonies expanded very slowly. After 18 hours, the colonies reached the size less than 200 μm (estimated from the microscope image). This is too small and difficult to detect with the naked eye, which was why we failed to notice in our plate assay. Also, at these concentrations of chloramphenicol and tetracycline,

we observed filamentation of some cells. Some of these filamentous cells divided and produced normal-looking daughter cells, which grew properly afterwards. There were also filamentous cells that did not resume normal growth within our observation window (~24 hrs).

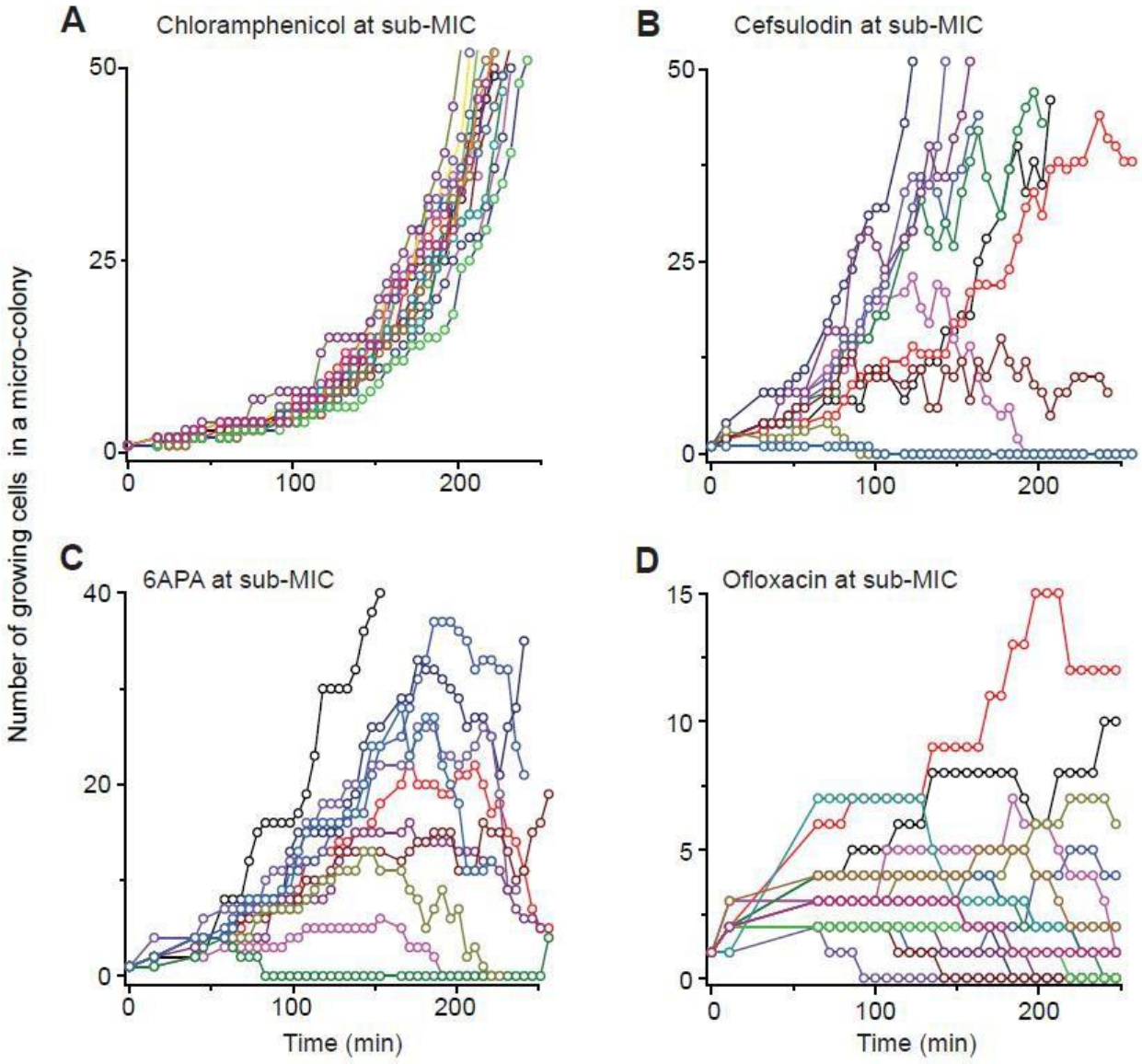


Figure 0-10 Figure 2-figure supplement 2.

Previously, we developed a microfluidic chemostat for cell culture (28, 29). We cultured E. coli cells in this device. Our microfluidic chemostat has about one hundred microchambers. We inoculated

these chambers by loading single cells into them and then exposed them to 0.8x MIC of various drugs. Then, using time-lapse microscopy, we recorded how one cell in each chamber grew and formed a population. (There were chambers that were initially inoculated with multiple cells. We did not record cell growth in such chambers). By counting the number of cells at different times in each chamber, we determined the trajectory of population size for each population.

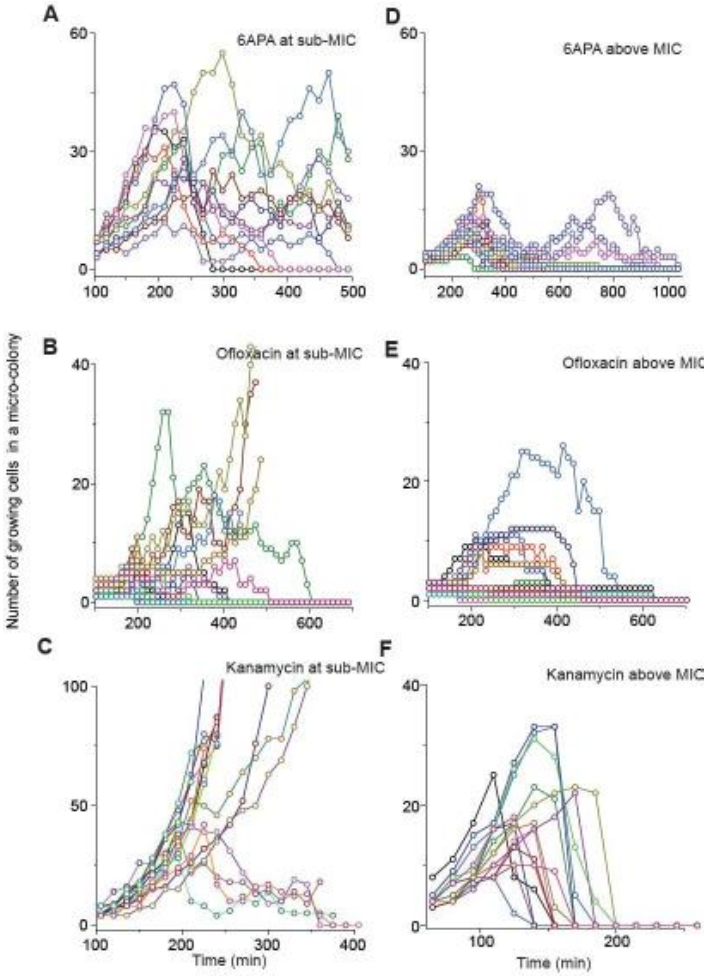


Figure 0-11 Figure 2-figure supplement 3.

We show population dynamics of bacteria exposed to bactericidal drugs (0.8x MIC or 1.2x MIC).

See Fig. 2C and 3A, and their captions for details.

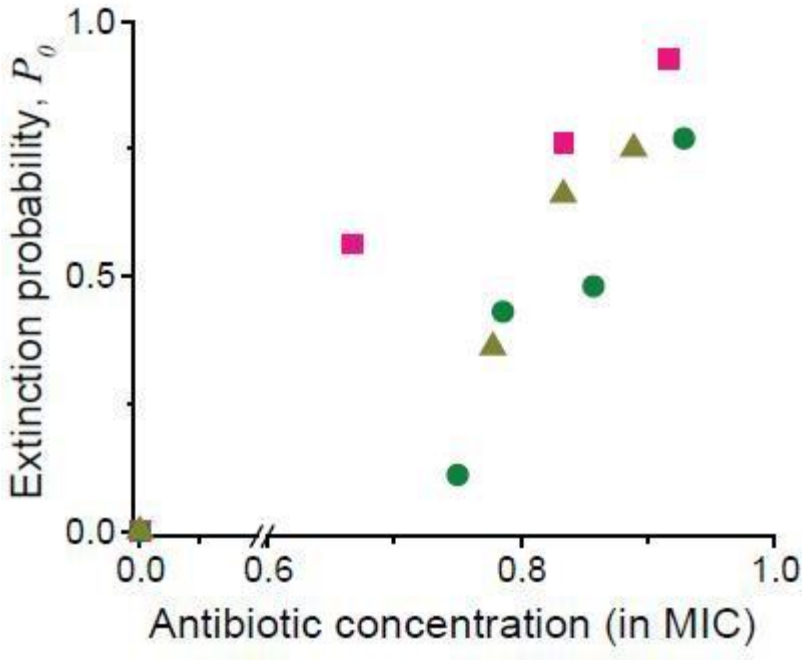
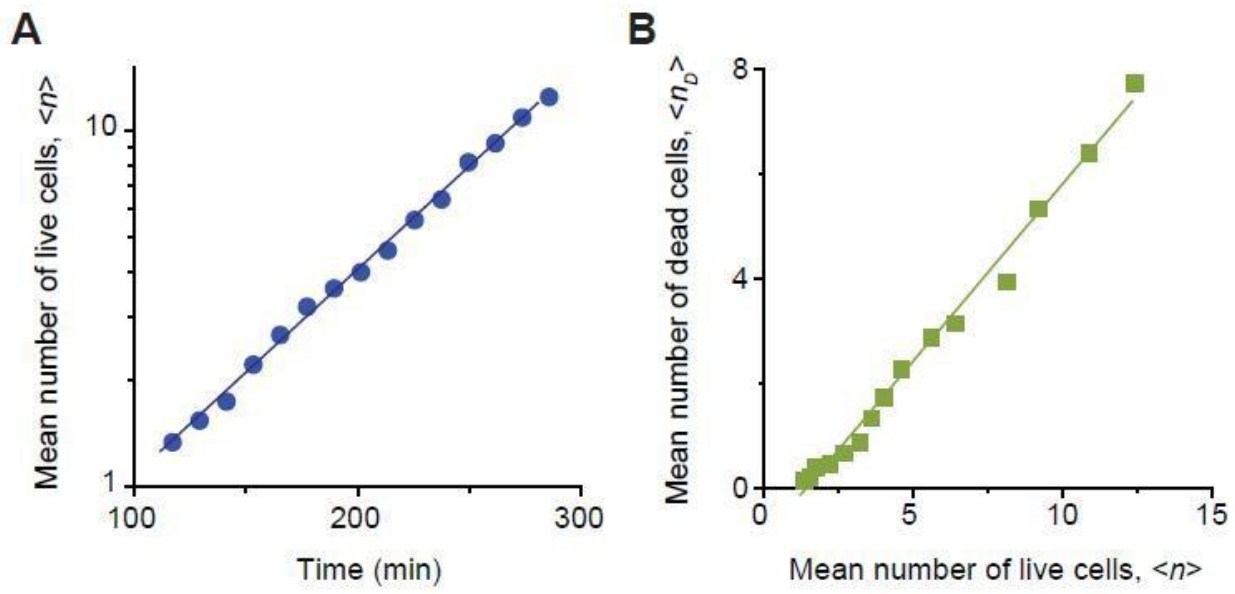


Figure 0-12 Figure 2-figure supplement 4.

As discussed in the main text, we analyzed single-cell-level image sequences, and counted the number of colonies that went extinct. Here, we plotted the probability of colony extinction (P_0) at different drug concentrations (green circles: cefsulodin, pink squares: ofloxacin, and grey triangles: 6-APA).



We determined the rates of cell growth λ and death ϕ at sub-MIC drug concentrations, by analyzing the time-lapse microscopy images of colony growth.Here, we described our determination procedure using the data from 0.8 × MIC of cefsulodin as an example. As shown in the Video 3, live cells continued to elongate and divide. Every now and then, these cells underwent lysis (i.e., cell death). We counted the number of live cells,n, and the number of dead cells, nD, in each colony. We then averaged them across ~20 colonies, which yielded a temporal trajectory for ⟨n(t)⟩ and another trajectory for ⟨nD(t)⟩. Equation A9 predicted that ⟨n⟩ increases exponentially over time, and this prediction was supported by a linear pattern in the temporal semi-log plot; see panel A (a linear regression analysis was performed, resulting in the blue line (slope = 0.0058, intercept = −0.556, R-squared = 0.9971)). The slope of this linear increase is determined by λ−ϕ (See Equation A9). Equation A12 predicted a linear relation between ⟨n⟩ and ⟨nD⟩, and this prediction was supported by a linear pattern in panel B (a linear regression analysis was performed, resulting in the green line

(slope = 0.6770, intercept = −0.998, R-squared = 0.9896)). The slope of this linear pattern is equal to$\phi\lambda-\phi$(see Equation A12). Analyzing these slopes, we determined λ and ϕ to be 1.35/hr and 0.54/hr. We repeated this procedure for various drug concentrations, as well as for other drugs. The results were plotted in Figure 2—figure supplement 6. linear pattern in panel B (a linear regression analysis was performed, resulting in the green line (slope = 0.6770, intercept = -0.998, R-squared = 0.9896)). The slope is this linear line is equal to (see Eq. S12). Analyzing these slopes, we determined to be 1.35 /hr and 0.54 /hr. We repeated this procedure for various drug concentrations, as well as for other drugs. The results were 864 plotted in Figure 2-figure supplement 6.

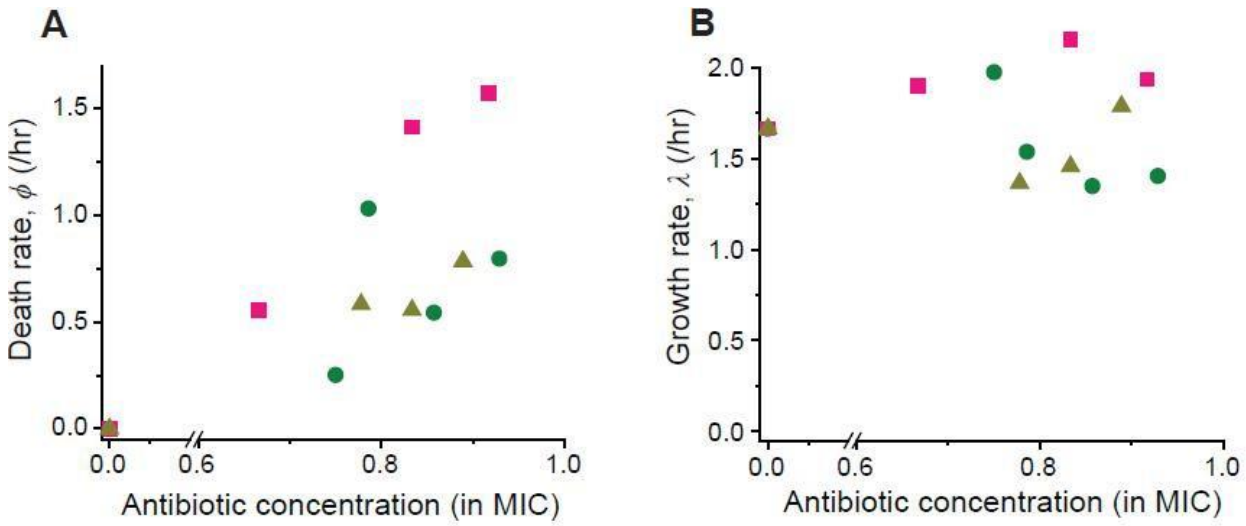


Figure 0-13 Figure 2-figure supplement 6

Following the procedure described in Figure 2-figure supplement 5, we determined the rates of cell growth and death at different drug concentrations (green circles: cefsulodin, pink squares: ofloxacin, and grey triangles: 6-APA).

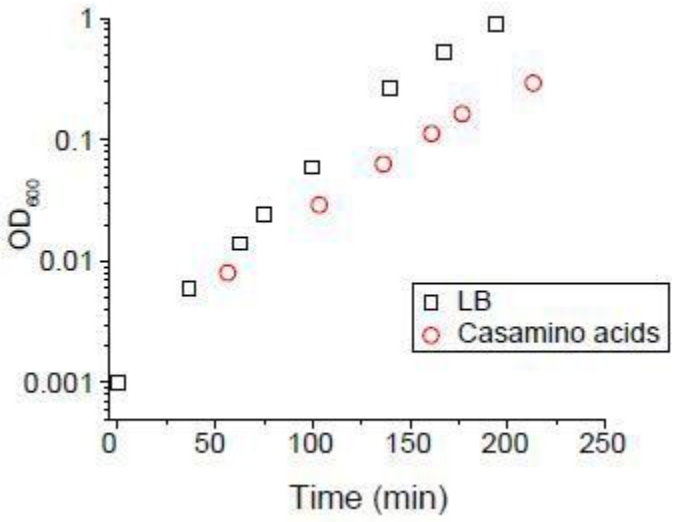


Figure 0-14 Figure 4-figure supplement 1.

We cultured cells in either LB medium or N-C- medium supplemented with 2% of casamino acids (83). The doubling time was 18 mins and 33 min respectively.

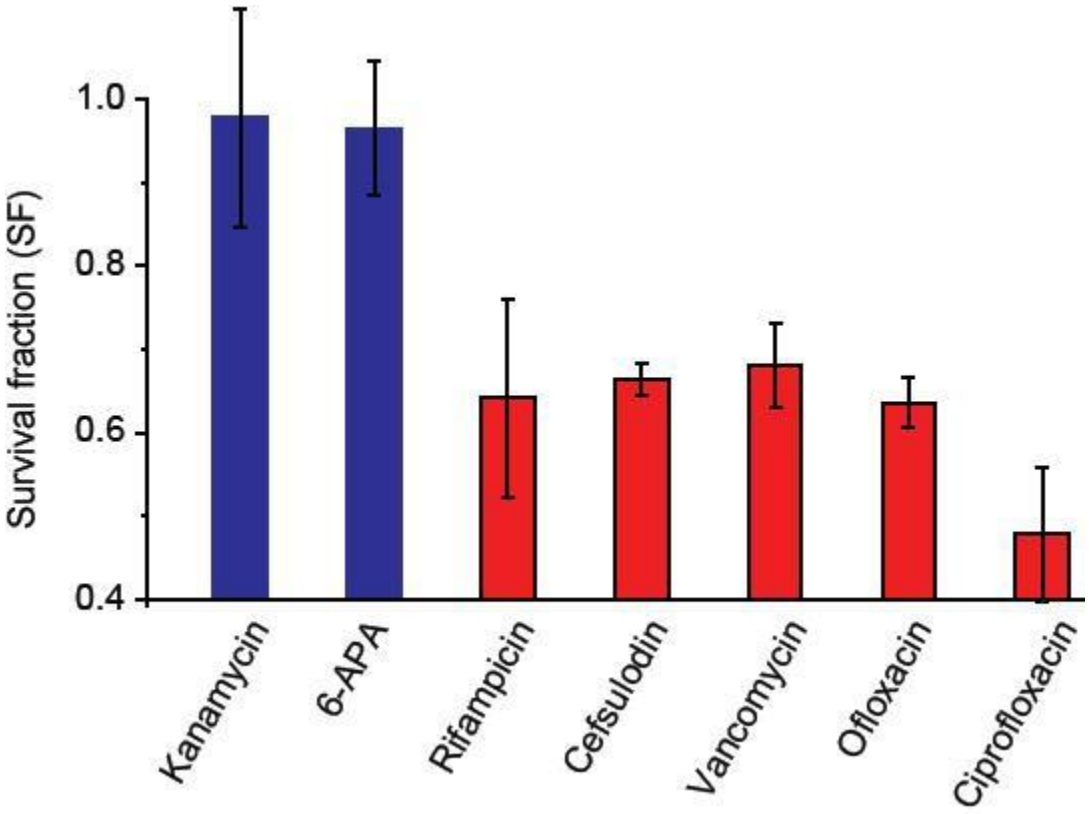


Figure 0-15 Figure 4-figure supplement 2.

We measured MICs of bactericidal drugs using exponentially-growing cultures and confirmed that at 1.5× MICs, all growing cells were eradicated; i.e., survival fraction (SF) was equal to zero. We then stopped cell growth in cultures by depriving the cells of nutrients, added 1.5× MICs, and measured SF. SF was 1 for kanamycin and 6-APA (blue columns), indicating that they were not capable of killing non-growing cells. Other drugs (red columns) could kill non-growing cells, but with lower efficacy as SF was not zero. Lower susceptibility of non-growing bacteria to drugs has been previously documented (e.g., see (85)). We performed two biological repeats and plotted the 877 mean. The error bars represent one standard deviation from the repeats.

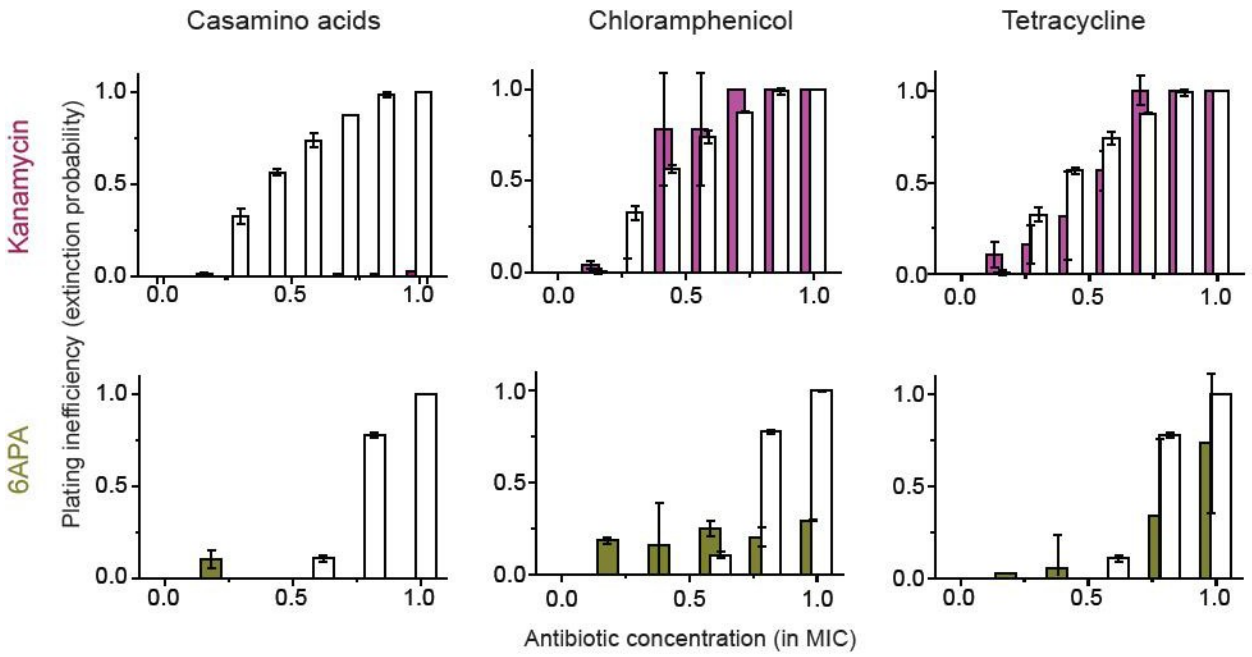


Figure 0-16 Figure 4-figure supplement 3.

In Figure 4-figure supplement 2, we found that kanamycin and 6-APA do not kill non-growing cells. Here, we show that the growth reduction strategy does not work for these two antibiotics. White columns indicate the plating inefficiency without the growth reduction treatment. The solid columns are with treatment. In the figure, overlapping columns were from the same drug concentrations, but their centers were shifted to visualize both white and solid columns. Please see the main text and the caption of Fig. 4 for detail. We performed two biological repeats and plotted the mean. The error bars represent one standard deviation from the repeats.

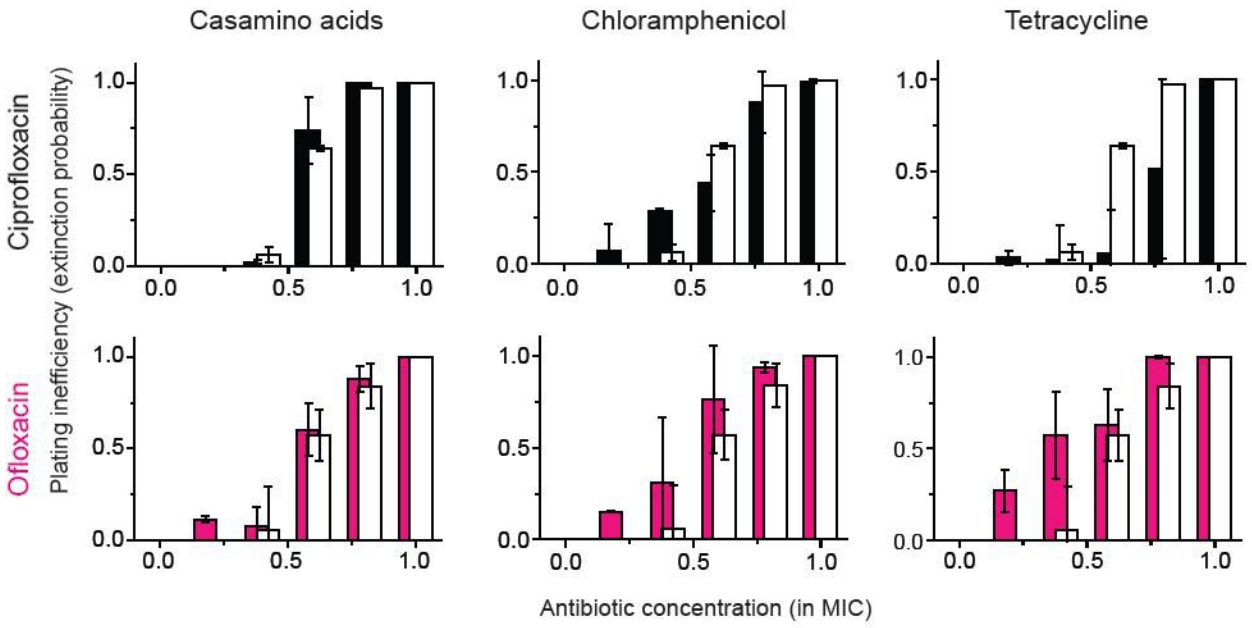


Figure 0-17 Figure 4-figure supplement 4.

A previous study showed bacteriostatic translation-inhibiting drugs and bactericidal quinolone drugs affect gene expression in a way to negate their effects (43). The figure shows that for ofloxacin or ciprofloxacin (quinolone drugs), the growth reduction strategy did not lead to a significant change in the extinction probability, consistent with the previous study. We performed two biological repeats and plotted the mean. The error bars represent one standard deviation from the repeats.

Appendix 2

To rule out heritable resistance as an explanation for heterogeneous colony formation (Fig. 1B), we performed two rounds of plating at sub-MIC drug concentrations (Fig. 1C and Figure 1-figure supplement 3). We picked a few colonies from the agar plate exhibiting a plating efficiency of ~0.5 (e.g., near 0.6x MIC) and plated them immediately on fresh agar plates containing various concentrations of the same drug. The plating efficiency was similar or marginally lower on the second plating, possibly because exposure to the antibiotics on the first plate adversely affected the

cells and rendered them more susceptible to the antibiotics on the second plating. This observation rules out heritable resistance as an explanation for heterogeneous colony formation at sub-MIC drug concentrations. Of course, if we perform multiple rounds of plating, we might eventually select for antibiotic-resistant mutants. However, for each round, we collect ~10^5 cells from a previous plate and plate ~100 cells on a fresh plate. This procedure creates a bottleneck effect, which reduces genetic variation and effectively weakens selection pressure for antibiotic resistance. Therefore, we expect that the evolution of antibiotic resistance is slow.

Appendix 3

We characterized the fluctuations in inoculum size by determining standard deviation and percent variation; see Appendix-Table 1. The variation was ~10% or less. In Fig. 3D, which shows the extinction probability at various (average) inoculum size, the probability changes only gradually for two-fold increment in inoculum size (which is equivalent to a 100% increase). Therefore, we do not believe that these fluctuations in inoculum size have a significant effect on the results.

Appendix-Table 1

Average inoculum size	Standard deviation	Percent variation
11.4	1.49	13%
58.4	3.7	6.3%
135.8	7.8	7.8%

Video 1: The growth of micro-colonies in the absence of a drug.

We characterized how cells grew and formed micro-colonies on LB agar using time-lapse microscopy.

An example image sequence is shown here. The time interval between each frame is 20 minutes.
https://doi.org/10.7554/eLife.32976.014

Video 2: The growth of micro-colonies with a bacteriostatic drug

We characterized how cells grew and formed micro-colonies on LB agar using time-lapse microscopy.

0.7x MIC of chloramphenicol was used. An example image sequence is shown here. The time interval 991

between each frame is 19 minutes. https://doi.org/10.7554/eLife.32976.015

Video 3: The growth of micro-colonies with a bactericidal drug

We characterized how cells grew and formed micro-colonies on LB agar using time-lapse microscopy. 994

0.8x MIC of cefsulodin was used. An example image sequence is shown here. The time interval between

each frame is 15 minutes. https://doi.org/10.7554/eLife.32976.016

Chapter III: Mumps transmission in United States in the post-vaccine era: A systematic review

Jessica Coates

Abstract

Despite a high two-dose coverage of measles-mumps-rubella (MMR) vaccine in the United States, a resurgence of mumps cases has been observed in university, close knit, mass gathering, and detention center settings since 2006. We aimed to utilize a systematic review with the following criteria to estimate differences in mumps transmission by setting in the United States after the resurgence in 2006. We searched the PubMed/MEDLINE database ending August 2021 to identify any observational studies reporting mumps cases in the United States after the licensure of the Jeryl Lynn mumps vaccine. We included interventions such as any mitigation strategies that encouraged isolation of sick patients, mask wearing, and vaccination. Results included in our analysis were self-reported, or provider-reported estimates of mumps cases, vaccination coverage, and population size for the affected settings that allowed us to estimate the effective reproduction (Re) number for mumps in the United States. Two reviewers independently appraised 331 citations for inclusion. One reviewer independently appraised 97 full texts for inclusion and methodological quality. We identified 14 articles describing 1,395 cases of mumps occurring after the licensure of the Jeryl Lynn mumps vaccine. Six cases occurring after the 2006 resurgence and were included in further quantitative analysis. Outbreaks after the resurgence took place in military barracks (1), an elementary school (1), and universities (4). Due to reporting bias and data unavailability, we were unable to estimate the mean effective Re value for mumps in the United States, but we did observe point estimates between 0.05 and 1.22. The lower Re values observed may be the consequence of

non-pharmaceutical interventions used in combination with vaccination campaigns to reduce the transmission of mumps, but further studies are necessary to verify the validity of that claim.

Introduction

The mumps virus, a single-stranded, negative-sense RNA virus belonging to the Paramyxovirus family, is the causative agent of mumps. Following a 15-to-24-day (median 19 days) incubation period, infection is characterized by unilateral or bilateral parotid swelling, fever, and headaches in up to 70% of cases [1]. One-third of mumps infections are asymptomatic but in severe cases, orchitis, oophoritis, pancreatitis, aseptic meningitis and encephalitis can occur [2].

Mumps is a moderately to highly contagious infection in humans [3]. Transmission results from a susceptible individual coming in contact with saliva or the respiratory droplets from the mouth, nose, or throat of an infectious individual [4,5]. On average, individuals are infectious from two days before to five days after parotitis onset [6]. Asymptomatic individuals can also contribute to the transmission of mumps; up to one-third of individuals infected exhibit no symptoms but are contagious [7]. The risk for mumps infection increases with longer and closer contact highlighting the importance of understanding the role of epidemiologic setting in mumps transmission [8].

Mumps is preventable through routine childhood vaccination with measles-mumps-rubella (MMR) vaccine which is available in most developed countries. A one-dose vaccination strategy was implemented in the United States in 1977 leading to a 99% reduction of mumps cases from a pre-vaccine era incidence of 251/100,000 in 1968 to 2.5/100,000 cases in 1982 [9]. However, despite the early success of the mumps vaccination program, a resurgence of mumps cases was observed between 1983 and 1992 with a national reported peak of 12,848 cases in 1987, corresponding to an incidence rate of 5.4/100,000 that year [9]. A two-dose vaccination strategy for MMR vaccine was implemented in 1989 in response to a major resurgence of measles cases. Mumps activity decreased to an incidence of 0.1/100,000) until a series of outbreaks began in 2006 (incidence 2.2/100,000).

Sustained mumps outbreaks have continued to be reported in a variety of epidemiologic settings including close-knit communities[10–12], universities[13], mass gatherings[14], and detention centers[15].

Most recent mumps outbreaks in the United States occurred in populations with high two dose vaccine coverage and in settings with high population density or prolonged person-to-person contact [9]. Lower vaccine effectiveness has been reported in congregate exposure settings, such as household contacts and close-knit religious communities [16]. Such densely populated environments with prolonged face-to-face contact can facilitate larger doses of mumps virus exposure during social interactions, which may overcome vaccine-mediated protection and result in secondary vaccine failure [17].

Understanding how mumps transmission dynamics differ by setting can provide useful information for mumps outbreak control strategies. A systematic literature review can be a useful tool for guiding control strategies. Here, we investigate the MEDLINE database to estimate differences in mumps transmission by setting using the effective reproduction number derived from data reported in peer-reviewed studies. We first conducted a systematic literature search with consultation from Centers for Disease Control and Prevention epidemiologists to assemble manuscripts describing US mumps cases in the post-vaccine era (1968-2019). Then, we assembled a dataset describing the outbreak dynamics to complete a narrative synthesis comparing reports before and after the 2006 resurgence. Using data extracted from the reports after the 2006 resurgence and deemed of low risk for bias, we established point estimates of the effective reproductive number (Re) for each outbreak of 0.05 and 1.22. However, we were unable to make conclusive statements about how mumps transmission varies by setting. To successfully investigate how mumps transmission differs by setting, additional investigations are needed.

Objective and Overview of Systematic Review

The objective of the systematic review was to estimate differences in mumps transmission (Re) in the United States by setting after the 2006 resurgence.

The interventions explored in the systematic review were any efforts to reduce mumps transmissions including previous vaccination, isolation of patients, vaccination campaigns, and wearing face masks. The population of interest was anyone exposed to mumps in the United States after the introduction of MMR vaccine. Participants were narrowed down to anyone exposed to mumps in the United States after 2006 for quantitative analysis. Our primary outcome measure was the potential for epidemic spread at a specific time t under the control measures in place calculated, or the effective reproduction number (R_t). The ability to calculate our outcome measure was based on population level reports of the affected population size and vaccine coverage.

Methods

We included only epidemiologic studies that reported mumps outbreaks in the United States in the post vaccine era. These included descriptive observational studies and analytic observational studies. We defined descriptive observational studies as those concerned with characterizing the amount and distribution of health and disease within a population in the absence of a control group. Any observational studies including a control group (i.e., case control, cohort), were defined as analytic observational studies. Any other study design was excluded from the analysis. Limiting the review to observational studies was not ideal but practical due to the ethical concerns associated with conducting mumps transmission studies in humans.

We included studies comprised of any participants that were exposed to a mumps outbreak in the United States after the licensure of the Jeryl Lynn vaccine (1967). Participants did not need to be born in or hold citizenship in the United States, but they must have been present in the United States when the outbreak took place. Because our focus was understanding differences in

transmission by setting, studies that only reported the location (e.g., state, country) of the participants and not the setting (e.g., clinic, school, work) were excluded. Additionally, we excluded participants that were pregnant or had a known co-infection. No exclusions were made by gender, age, sexual orientation, language, occupation, racial or ethnic group, or other characteristics.

Studies reporting outcome measures needed to estimate the effective reproduction number were included. Examples of outcome measures included count population vaccine data, population vaccine coverage, and population size. If reports included an ambiguous measure, authors were not contacted for further clarification and the study was excluded from the analysis.

Search methods for identification of studies

We performed a MeSH (Medical SubHeadings) term search to identify studies that have reported mumps outbreaks in the United States. We searched the Boolean phrase:

(Mumps [MeSH Terms]) AND (United States [MeSH Terms]).

Only studies published in English were included. No restrictions on journal of publication were imposed.

Our search was purely limited to the Medline Embase database and ended in August 2021. We did not search any other electronic databases, relevant government websites (i.e., Centers for Disease Control and Prevention, National Institute of Health), hand-search, peer-reviewed primary literature sources provided by experts in the field, or cross references.US (population) originating from a single setting and detailing information on vaccine coverage (outcome 1) and size of the affected population (outcome 2) thereby enabling calculation of the effective reproduction (Re) number by setting. We selected the Re value as our outcome of interest because it is a standard measure of transmission dynamics when a population is not fully susceptible like in the instance of mumps.

We applied the following exclusion criteria to categorize excluded articles.

1) Wrong patient population: the affected population was outside of the United States or occurred before the licensure of the Jeryl Lynn mumps vaccine
2) Wrong outcomes: authors failed to report vaccine coverage and size of the affected population
3) Wrong study design: articles were not primary literature sources
4) Wrong setting: outbreak was not confined to single identifiable setting
5) No full-text available

Citations were merged using Covidence 1.0 [1818] to facilitate management. Two reviewers independently applied the inclusion criteria to all retrieved articles and records of mumps cases in an unmasked standardized manner, evaluated by title, abstract, and full text. Disagreements between reviewers were resolved by a senior reviewer. No efforts were made to address missing data. Characteristics of excluded articles are available on Github (link: https://github.com/jcoatesmicrobiologist/Mumps-Systematic-Review). Articles that did not mention mumps in the United States in the title or abstract were deemed irrelevant can also be found on Github and are detailed in table S2.

For each included study, all reviewers extracted identification information including country of outbreak, setting, author contact information, region of the country, and area type (rural or urban). Additionally, one reviewer extracted information on study design, population characteristics and interventions.

Threats to Internal Validity

The internal validity of outcomes based on observational data can be threatened by systematic bias. Observational studies of mumps transmission are particularly vulnerable to problems with the comparability of participants or populations in a study (selection bias), factors other than the intervention or exposure of interest that influence the effect estimate (performance bias or confounding), problems with measurement or classification of exposure or outcomes (detection bias), and missing information attrition bias or reporting bias) [19]. To assess the risk of

systematic bias in the included studies, we utilized the Joanna Briggs Institute (JBI) checklists [20] for cohort, case control, and case series studies to critically appraise each article by answering a series of questions to evaluate the possibility for selection, performance, detection, attrition, or reporting bias specific to the study design.

Measures of transmission dynamics

The goal of our systematic review was to estimate transmission dynamics or the Re value for each outbreak described in the literature. The Re calculation is dependent upon the estimated number of susceptible individuals in the population. To estimate the number of susceptible individuals (S), two reviewers extracted population size (N), and dose dependent vaccination coverage (p_n) for each study. We estimated the number of susceptible individuals using the equation:

$$S = N[\frac{p_0}{p_0 + p_1 + p_{2+}}(1 - v_0) + \frac{p_1}{p_0 + p_1 + p_{2+}}(1 - v_1) + \frac{p_{2+}}{p_0 + p_1 + p_{2+}}(1 - v_{2+})]$$

where N is the population size, p_0, p_1, and p_{2+} denote the fraction of the population that has been vaccinated with zero, one, or two or more doses, respectively, and v_0, v_1, and v_{2+} denote vaccine effectiveness of individuals with zero, one, or two or more vaccine doses, respectively. S is rounded to the nearest whole number. We assume unvaccinated individuals are fully susceptible to infection ($v_0 = 0$). We set vaccine effectiveness for individuals who have received one dose to $v_1 = 0.78$ and for those who have received two or more doses to $v_{2+} = 0.88$ [6].

To calculate the effective reproduction number and the standard errors (SE) for each outbreak, we used a similar approach to a previous study that compared differences in transmission by setting [21] that use the final epidemic size

$$R_e = \frac{S-1}{C} \sum_{i=S-C+1}^{S} \frac{1}{i}$$

$$S_e(R_e) = \frac{S-1}{C} \sqrt{\sum_{i=S-C+1}^{S} \frac{1}{i^2} + \frac{CR_e^2}{(S-1)^2}}$$

where C is the number of cases in the outbreak, and S is the number of susceptible persons at the start of the outbreak. The final size method assumes a susceptible-infected-recovered infection with a closed, homogeneously mixing population.

To investigate the feasibility of a meta-analysis, all eligible studies were summarized in Covidence to the best extent possible. A narrative synthesis was provided for all results, but we determined that a statistical meta-analysis was not appropriate for this review due to reporting bias and lack of available data. Had the studies been comparable and more completely reported, our meta-analysis would have measured the average effect size of the effective reproduction value for mumps in the United States.

Where statistics were missing (e.g., number of participants per group, vaccine coverage, means and standard deviations or percentages), we excluded studies from future analysis and made no attempts to contact investigators for missing data.

Results

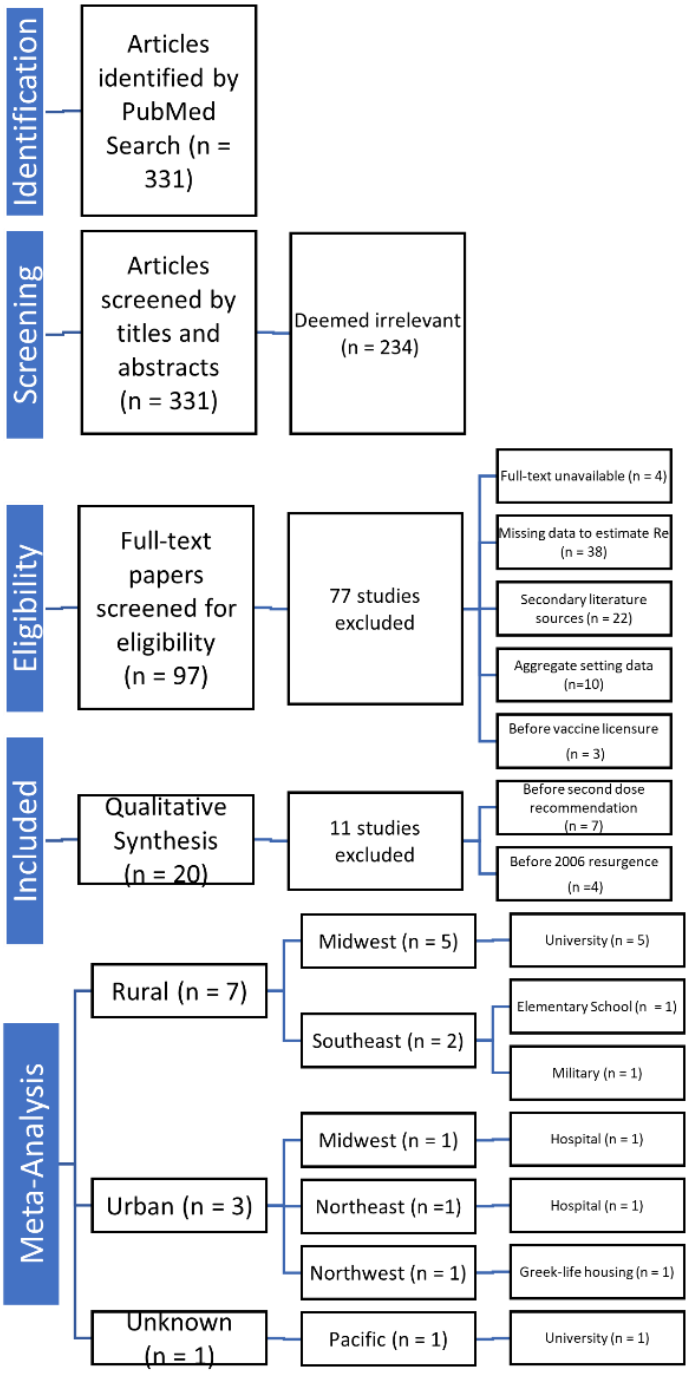


Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram of study selection. Outcomes of interest include population size, vaccine coverage, and the number of mumps cases.

To collect data for statistical analysis, we utilized a mumps specific Medical Subheadings (MeSH) term search strategy to identify manuscripts describing US mumps cases in the post-vaccine era (1968 - 2019). The results of our search strategy yielded 331 articles. After screening the titles and abstracts, we deemed 234 articles irrelevant or not related to mumps in the United States. Ninety-seven papers were deemed potentially relevant for further evaluation. Full text copies for 93 of these citations were obtained.

Of the 93 papers for which the full text was obtained, 73 were excluded for the following reasons: 38 were missing data necessary to estimate the effective reproduction number, 22 were secondary literature sources, 10 were reports of mumps cases not originating from a single setting and 3 were reports occurring before vaccine licensure.

After these studies were excluded, the narrative synthesis was limited to 20 separate papers, comprising 22 separate reports of mumps cases originating from a single setting. Two reports described outbreaks in multiple settings [22,23]. Four articles described the same outbreak: two reports described an outbreak in a rural Ohio middle school [24,25] and two reports described an outbreak in an Iowa University [26,27].

Since incidence rates occurring during the first resurgence were reduced with administration of additional dose of MMR vaccination, studies occurring before the 2006 resurgence were excluded from the quantitative analysis to focus our quantitative efforts on understanding mumps transmission in two dose era. Quantitative analysis was limited to nine papers, comprising eleven separate reports of mumps cases originating from a single setting. Two reports described two settings [22,23].

Narrative Synthesis

Detailed information about individual manuscripts may be found on Github in the Table of Included Articles (Table S3).

Eighty-two percent (18) of the included studies were cohort studies. The remaining four studies included two case series reports [28,29] and two case-control studies [30,31]. Sixteen of the author institutions were the Centers for Disease Control and Prevention. The remaining two author institutions were medical centers [32,33]. Author institution was not reported for four studies [22,34,35].

Together, the 20 articles reported 22 studies. No studies were missing population data. The population sizes ranged from 28 [22] to 36,000 [29]. The median population size was 1066. All 22 studies took place in the United States. Most studies took place in the Midwest, Southeast, and Northwest. Ten studies took place in the Midwest. Four studies reported in three articles took place in the Southeast [22,31,36]. Three studies took place in the Northeast [33,37,38]. One study took place in each of the following regions: non-contiguous United States [39], Northwest [28], Rocky Mountains [32], Southwest [40] and Pacific [29]. Area type (rural versus urban) was reported for twenty studies. 73% (16) of the included studies took place in rural areas. Four studies took place in exclusively urban settings [28,33,34,37]. The location of the outbreaks was usually an educational setting (universities or K-12 schools). Other cases took place in a hospital [32–34], Greek Life social organizations [28], close-knit communities [35,39], military [22], and a summer camp [38].

Participants ranged from newborns [32–34] to middle age [22]. No studies included information on the elderly. Mean age for the population was only reported for one study [22] (military; mean age 24 years, range 20 to 37 years). When age was reported, it was often reported in a format that made estimating the mean age impossible. For example, one study reported case data by age with bins (<14, >18) for students outside "typical" high school ages [36], and another reported age only as a binary outcome (>6 months or <6 months) [32].

Information on vaccination status was self-reported or reported by parents for individuals under eighteen for three studies. Two studies relied on information reported by parents [25]. The remaining study relied on self-reporting surveys [30,34]. Information on vaccination status was also obtained through reviewing school and medical records. Six studies relied on school records [26,27,29,37,40,41] and four studies used medical records [22,32,33,38]. Two studies used two methods to obtain vaccine data: Briss et.al, [36] used school and medical records, and Wharton et al. [31] used school record and self-reporting, to obtain vaccine data. One study utilized a combination of methods to report vaccine data including school records, medical records, immunization databases, and self-reporting [30]. One manuscript reporting two studies relied on electronic medical data [23]. Five studies reported over four manuscripts included information on vaccine status or an estimate of population vaccine coverage but did not provide information on how the values were obtained [22,28,35,39].

Fourteen studies in thirteen manuscripts reported an attempt to assess the validity of vaccination records [22,23,26,31,33,34,36–41]. Vaccination records were validated by comparing school immunization records to healthcare providers [31,36,40,41] or parental records [37]. Schaffzin et al. [38] also relied on healthcare provider recorders to validate vaccine status but only validated a subset of the records [38]. Eight studies did not report how vaccine data were validated [22,24,25,28–30,32,35,39]. Maynard et al. [39], did not report how vaccine data were obtained but assumptions on the validity can be justified based on historical data suggesting a lack of vaccination in the area.

Clinical and laboratory diagnosis was the primary method for confirming mumps cases. Five studies, all before the second dose recommendation, relied solely on clinical diagnosis on the basis of parotid swelling [24,25,31,35,37]. The remaining studies used a combination of clinical and laboratory diagnosis. Common laboratory diagnostic methods included detection of viral genomic material (i.e., RT-PCR) and detection of mumps specific IgM via enzyme-linked immunosorbent assays (ELISA) or immunofluorescence assays (IFA).

Seven studies reported in six manuscripts did not report information on interventions used to decrease the spread of mumps [24,25,38,40,41]. The remaining studies utilized vaccination campaigns to reduce transmission. Three of the remaining studies utilized isolation of cases in addition to vaccination to reduce transmission [30,32,34]. Contact tracing in addition to vaccination and case isolation was used in three studies reported over two manuscripts [22,35]. Only one study utilized surgical masks, contact tracing, patient isolation, and vaccination to reduce spread of mumps [33].

Outbreak year was reported for all the studies. Seven studies occurred before the second dose recommendation [24,25,31,35,37,39,41]. Fifteen studies occurred after the second dose recommendation. Four studies occurred after the second dose recommendation but before the 2006 resurgence [32,36,38,40]. The remaining eleven studies occurred after the 2006 resurgence. Two studies described the same outbreak [26,27]. Shah et al. [27], was excluded from further analysis because the manuscript lacked detailed information on vaccination coverage.

Narrative Synthesis for studies after the 2006 resurgence

The 10 studies conducted after the 2006 resurgence (reported over 9 manuscripts) were retained for risk of bias assessment and future quantitative analysis. Seven of these studies (reported over 6 manuscripts) were retrospective cohort studies [22,26,26,33,34]. Two included studies were case series reports [28,29]. One study was a case-control study [30].

Two studies (reported in one manuscript) were affiliated with the Department of Preventive Medicine, Blanchfield Army Community Hospital in Fort Campbell Kentucky [22]. The Centers for Disease Control and Prevention was the author institution for the remaining studies.

The total population size and range did not differ significantly from the narrative synthesis including cases before the 2006 resurgence.

Most cases occurred in the Midwest. The most common location for studies was universities. Other cases took place in an elementary school [22], Greek life housing [28], hospitals [33,34], and the military [22].

Three studies did not report their method for obtaining vaccine data. School records were the most common method for obtaining vaccine data. Other methods included self-reported surveys [34] and medical records [22].

All studies utilized laboratory diagnostic methods in line with the CSTE standards for detecting mumps. Epi-linkage to a confirmed case was also considered in detecting mumps cases.

Three studies reported over two manuscripts [23,38] did not report intervention strategies used to control mumps transmission. The remaining studies all reported vaccination campaigns as a means for decreasing mumps spread. Two studies only reported vaccination strategies to reduce transmission [26,28]. Three studies combined vaccination and patient isolation to control spread within the community [29,30,34]. Studies reported by Downs et al[22]., used contact tracing in addition to vaccination as an intervention to decrease mumps transmission. Only one study reported more than three interventions; in addition to contact tracing, patient isolation, and vaccination, Gilroy et al. [33], reported the use of surgical masks to reduce transmission.

Risk of Bias

Threats to Internal Validity

Case series reports were deemed high risk for bias because of incomplete reporting of relevant demographic data and lack of reporting on the outcome of interventions. Due to the high risk for bias, these studies were excluded from future analysis [28,29]. Ultimately five manuscripts reporting seven studies were deemed low risk for bias and included in point estimations of the effective reproduction number [22,23,26,30,34]. University settings comprised four of the studies. Remaining studies occurred in a hospital [34], elementary [22], or military [22] setting. The lack of data

reported in settings other than universities limited our ability to perform sub-group analysis and determine how mumps transmission differs by setting.

Estimating how transmission dynamics differ by setting

The 'effective reproduction number' (Re; the average number of secondary cases per infectious case in a population made up of both susceptible and non-susceptible individuals) has been established as the most critical parameter for characterizing the transmission dynamics in populations where some immunity exists [42].[42]. As a first step to examine if heterogeneity existed in mumps transmission dynamics between different settings, we used the Becker method [4343] to estimate the R_e value for each outbreak included in our dataset. The R_e was calculated based on 3 variables, the total population size of the outbreak (N), the total number of cases in the outbreak (C), and the number of susceptible persons at the start of the outbreak (S). Ten studies reported enough reliable data to estimate the Re value and were included in the analysis.

Cases ranged from 1 [22] to 259 [26]. Vaccine coverage ranged from 0.2% [26] to 19% [22], 1% [23] to 91% [22], and 0% [22] to 99.5% [26] for zero, one, and two plus doses of MMR, respectively. Vaccine coverage was unknown for 0% [26] to 93% [34] of individuals. Due to the large percentage of unknown vaccination status in Bonebrake et al. [34], it would have been excluded from a meta-analysis. The proportion of susceptible individuals ranged from 12% [23,26,30] to 29% [22]. Point estimates for the effective reproduction number ranged from 0.05 [22] to 1.22 [23] (Table 1).

Comparison of these values was deemed statistically incomparable because of a limited sample size, preventing meta-analysis. By not completing a meta-analysis, we are unable to make statistical conclusions about the nature of mumps transmission in the post-vaccine era or compare the differences in transmission by setting. However, preliminary evidence that heterogeneity in mumps transmission may be the result of combining non-pharmaceutical interventions (NPIs) with vaccination. The two outbreaks described in Downs et al [22]. had significantly lower Re values than

those observed in the university setting despite having a lower two-dose vaccination coverage. Other unique characteristics included early detection of the initial case, a lower percentage of individuals with unknown vaccination, and required quarantines for infected and exposed individuals. Further studies are necessary to determine the validity of this claim.

Table 1. Studies included in the systematic review

Title	Setting	N	punknown	p0	p1	p2+	Cases	Susceptible	Re
Risk factors for mumps at a university with a large mumps outbreak	University	19155	0.03			0.97	97	2299	1.04
Effectiveness of a Third Dose of MMR Vaccine for Mumps Outbreak Control	University	20496	0	0.002	0.003	0.995	259	2504	1.05
Public health response to imported mumps cases - Fort Campbell, Kentucky, 2018	Elementary School	77	0.01	0.08	0.91	0	1	17	0.05
Public health response to imported mumps cases - Fort Campbell, Kentucky, 2018	Military	28	0	0.19	0.81	0	1	8	0.11
Mumps vaccination coverage and vaccine effectiveness in a large outbreak among college students - Iowa, 2006	University	1550	0.12	0.003	0.01	0.87	46	193	1.13
Mumps vaccination coverage and vaccine effectiveness in a large outbreak among college students - Iowa, 2006	University	1199	0.19	0.008	0.17	0.19	63	178	1.22

Discussion

By using the MEDLINE database, we investigated differences in transmission dynamics by setting for mumps outbreaks. While we were unable to estimate Re values by setting, our analysis led to three key findings: (1) the number of publications describing mumps cases in the United States available on PubMed/MEDLINE is too limited to estimate the differences in mumps transmission

by setting, (2) point estimates for the effective reproduction number in the United States since the 2006 resurgence range from 0.05 to 1.22, and (3) further research is needed to confirm the impact of NPIs on differences in mumps transmission by setting.

Our finding that the data necessary to estimate an aggregate effective reproduction number by setting for mumps using the MEDLINE database were limited is not surprising. In a previous study, Winchester et al., [44] found that MEDLINE database search limits can inadvertently eliminate an important number of research articles. Additionally, mumps cases in the United States are primarily reported to the Centers for Disease Control and Prevention (CDC) and not published as primary literature articles. A thorough literature search of multiple relevant databases (ex. Embase, CINAHL, Google Scholar) and relevant organizational websites (ex. Centers for Disease Control and Prevention, World Health Organization, National Foundation for Infectious Diseases, and National Institute of Allergy and Infectious Diseases) is necessary to assemble a large enough dataset from published studies.

The large number of case series reports found on MEDLINE also limited our ability to build a dataset large enough for statistical analysis due to the potential for bias. A case series is a descriptive study that does not test a hypothesis but follows a group of patients who have a similar diagnosis or who are undergoing the same procedure over a certain period [45]. As previously mentioned, cases of mumps are required to be reported to the CDC but the information necessary to estimate transmission dynamics by setting are not required to be reported to the CDC. The primary method for obtaining data needed to estimate the Re value for mumps is the Morbidity and Mortality Weekly Report (MMWR), a weekly publication composed mostly of epidemiological case reports because of the need to quickly disseminate public health information in the event of an outbreak. Since the CDC is considered a primary credible source for mumps data, there is a need for future efforts to improve the quality of case reports published in MMWR. Public health officials can

improve case series reports by including a clearly defined research question (inclusive of a study population, intervention, and primary outcome), case definition, and clearly defined methods for data collection with a description of the methodological limitations [46]. The inclusion of these elements will allow investigators to assess the potential more accurately for bias in each report and potentially increase the number of usable studies.

Despite the limited number of publications available, data from MEDLINE on mumps' transmission in the United States allowed us to estimate Re values for multiple outbreaks in university settings and two outbreaks in Kansas stemming from an imported case. While our findings were not significantly different from Re values found in previous studies [47], they did provide interesting insight for future research. Given the success of the public health response in Downs et al. [22], it is worth investigating how NPIs can be used in combination with vaccination campaigns to reduce the transmission of mumps in the United States.

NPIs are actions like wearing a face mask and staying away from sick people that people and communities can take apart from getting vaccinated and taking medicine, to help slow the spread of illnesses [48]. NPIs were found to significantly reduce the spread of disease during the 1918 influenza pandemic [48,49] and the COVID-19 pandemic [50]. To the author's knowledge, there is no formal study on the relationship between NPIs and mumps transmission. Given the continued transmission of mumps despite a highly effective vaccine and increased vaccine hesitancy [51,52], it is worth exploring the impact of NPIs on reducing mumps transmission.

We attempted to utilize a systematic review to quantify the differences in mumps transmission by setting in the United States. However, limitations in the number of useable reports inhibited our ability to do so. Instead, we report the point estimates for six outbreaks. Our results suggest that NPIs may play a role in the differences in mumps transmission, but further analysis is necessary to determine the validity of that claim. Additionally, our work highlights the need for

improved reporting of mumps cases in the United States, which heavily influences the number of reliable studies to include in estimates of Re for mumps.

Conclusions

Implications for practice

Given the limitations of the evidence, this review cannot draw conclusions about settings-based differences in mumps transmission in the United States. Our search only yielded reports in university settings. Therefore, we cannot comment on mumps transmission outside of undergraduate students in university settings in the rural Midwest.

Our results suggest a need for strategies to improve the internal validity of observational studies, with a focus on case series reports. Specifically, we find a need for improved reporting of key methodological, clinical, and statistical information concerning confounding factors, as well as more complete reporting of vaccination data at the population level. These improvements will support better estimation of Re in different settings, in part by informing analyses that account for time since vaccination and age at vaccination.

Implications for research

Future research could address the following questions raised by this review:

- How does the effective reproduction number change between affected setting types (close-knit communities, mass gatherings, universities, and detention centers) and settings where mumps transmission is less frequent?
- How effective is the current vaccine in close contact settings?
- What practices, including NPIs, are best for reducing transmission for existing high-risk settings?

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Chapter IV: Discussion

Jessica Coates

Introduction

Antibiotics and vaccines are considered two of the greatest medical discoveries during the 20th century because of the dramatic reduction in deaths associated with infectious diseases (IDs) following their discovery. However, despite the early success of antibiotics and vaccines there has been a continued spread of infectious diseases within the United States. In 2020, three of the top ten leading causes of deaths in the United States were due to infectious diseases [1]. While many efforts are underway to improve the effectiveness of vaccines and to design more potent antibiotics, there is an immediate need to address the public health burden caused by IDs. Without the design of strategies that address immediate public health needs, infectious diseases will continue to impact the economic well-being of the United States[2,3] and the health of the American people. Data-driven approaches that incorporated within and/or between-host reproduction dynamics have been previously used to aid in the eradication of diseases like smallpox[4] and have the potential to reduce the modern-day burden of infectious diseases likes mumps and bacterial infections caused by E. coli. For the purposes of this dissertation, I present data providing insight on within-host infectious disease replication in Chapter 2 by exploring stochastic bacterial population growth dynamics during treatment with bactericidal vs. bacteriostatic antibiotics. I also present data providing insight on between-host infectious dynamics in Chapter 3 by utilizing a systematic literature search to estimate the effective reproduction number for mumps outbreaks in the United States after the 2006

resurgence. In the final chapter of this work, I summarize the major observations of Chapters 2 and 3 and provide recommendations for improving observational data reporting in microbiology and public health to ensure future investigators can accurately estimate the R value of infectious diseases impacting public health.

Summary of Major Findings

To explore the within-host dynamics of infectious disease reproduction, we utilized a combination of microbiology and biophysics approaches to characterize bacterial population dynamics when E. coli bacteria are exposed to increasing concentrations of bactericidal and bacteriostatic antibiotics. Our experiments yielded unique results compared to previously conducted studies that suggested bacterial population dynamics under antibiotic stress were deterministic [5,6]. Our results, however, suggested that bacterial eradication by bactericidal antibiotics is a stochastic, heterogeneous process. Specifically, we observed that at sub-MIC concentrations, the impact of bactericidal antibiotics on E. coli population dynamics differed from the impact of bacteriostatic antibiotics. Bactericidal antibiotics functioned in a heterogeneous, concentration dependent manner to kill bacterial cells while bacteriostatic antibiotics functioned in a homogenous, concentration independent manner to slow down the growth of bacterial cells. The heterogenous nature of bacterial killing observed under bactericidal stress was not associated with previously acquired resistance traits, which suggested instead that killing was a stochastic process. A simple stochastic model was able to predict stochastic clearance of bacterial populations as antibiotic concentration approached the MIC; this model further suggested that stochastic fluctuations would increase as drug concentration approached the MIC, allowing us to predict stochastic extinction of a large bacterial inoculum at near-MIC concentrations of drug. Further, based on a model-selected growth-reduction approach to maximizing stochastic extinction, we used combinations of bactericidal and bacteriostatic antibiotics to promote stochastic population eradication even in genetically drug-

resistant strains. This work contributes to the field by adding further evidence on the importance of considering population size when evaluating with-in host dynamics of infectious agent replication. By understanding the dynamics of small population sizes, future studies could be potentially developed to revitalize currently available antibiotics and address the current antibiotic public health crisis.

The second part of this thesis focused on between-host dynamics of ID reproduction. Here we used a systematic literature review to estimate the effective reproduction number for Mumps outbreaks in the United States after reaching a high two dose measles, mumps, rubella (MMR) vaccine coverage. Our literature search yielded 5 articles describing 6 outbreaks and 496 cases of Mumps in the United States after 2006. Due to biases in the settings represented and limitations associated with a small dataset, we were unable to estimate the mean effective reproduction number for mumps in the United States, but we were able to calculate point estimates that lead to three major findings: (1) Re values for mumps in the United States range from 0.05 to 1.22. (2) Outbreak responses that utilized non-pharmaceutical interventions were associated with lower transmission, but future studies are needed to validate that claim. (3) There is a need for improvement to data reporting in public health to enable investigators to accurately estimate the effective reproduction value for diseases like mumps, COVID-19, and seasonal influenza that are primarily reported using observational data.

Recommendations for future studies

These results highlight the importance of high-quality data in drawing inferences about infectious disease dynamics. Our ability to describe and model antibiotic-associated population dynamics for E. coli in Chapter 2 was largely due to the benefits of utilizing experimental data. By utilizing experimental data, we could control for potential biases that may lead to an over or underestimation of the reproduction metric and accurately measure population-wide and colony-

specific growth rates for E. coli exposed to bactericidal and bacteriostatic antibiotics. However, in Chapter 3, we were unable to estimate the mean Re value for mumps in part because of biases associated with using observational data to draw conclusions.

Most data for infectious disease transmission are from observational studies, for ethical as well as practical reasons, and well-known issues exist for observational data of this kind. Observational data, or data obtained from a study in which investigators obtain outcome measurements solely from observing the population of interest without attempting to influence the outcome, is highly vulnerable to bias because of the inability to control for external factors and therefore is often avoided for drawing conclusions for guiding intervention strategies. However, in the instance of mumps and other infectious diseases – such as COVID-19[7], seasonal influenza[8], and HIV[9] – there is no other option than to utilize observational data to understand the real world effectiveness of prevention methods. Therefore, given the importance of reducing infectious disease spread, it is necessary to improve observational data reporting by establishing a standardized reporting manner that allows investigators and policymakers to accurately assess the potential bias present in the data reported. To allow investigators and policymakers to accurately assess the potential bias present in observational data, future reports of observational data should include full descriptions of the affected population and the setting where disease spread occurred to account for all possible confounding factors that may impact the reliability of R value estimates. The ability to accurately estimate the R value for vaccinated populations relies on precise estimates for the number of susceptible individuals, the intensity of the exposure, and the number of cases. Any future infectious disease observational study reports should include detailed reporting of confounding factors that impact the ability to accurately estimate the number of susceptible individuals, the intensity of exposure, and the number of cases in the affected population. By providing this information in reports, investigators and policymakers can accurately assess the reliability of

outcomes needed to design effective control and prevention strategies possibly leading to the eradication of detrimental infectious diseases.

Despite the limitations in this work, we were able to highlight the importance of understanding within host and between host dynamics to successfully design public health strategies to prevent or control infectious agent replication. This phenomenon isn't unique to work focused on bacteria and mumps but is also relevant to other diseases that cause a major impact on human health and public health, including malaria[10] and influenza[11]. As the field progresses, there is a need to not only understand these dynamics better but to understand the link connecting within host dynamics and between host dynamics of infectious agent replication. Previous studies have attempted to use sophisticated quantitative models to establish the link between within host dynamics and between host dynamics but have failed to capture new information about these dynamics[12]. This work could potentially provide insight into how to reform studies dedicated to quantitatively linking the two different levels of transmission by showcasing the importance of considering stochasticity for within host dynamics and intervention mediated methods for increasing susceptibility rather than relying on traditional deterministic approaches and focusing on vaccine campaigns to control outbreaks.

The goal of the studies described in this dissertation was to utilize statistical approaches to understand within and between host dynamics in the transmission of infectious diseases. These studies provide novel insight into the nature of bacterial replication under antibiotic stress and mumps transmission in highly vaccinated populations. Through this work, we demonstrated the stochastic nature of bacterial replication under antibiotic stress and the importance of considering small populations in infection dynamics. Additionally, this work highlights the importance of understanding the impact of vaccine-mediated versus intervention-based heterogeneity in susceptibility to mumps and transmission in population with a high two dose vaccine coverage.

Results from these studies lay the foundation for future research on between and within host dynamics of infectious disease transmission and provides insight for the design of future public health control and prevention strategies.

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