

DISSERTATION

CANDIDATE RESERVOIR UNDERLYING RE-EMERGENT PLAGUE OUTBREAKS

Submitted by

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ABSTRACT

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Modern outbreaks of highly lethal pathogens (including plague, anthrax, Ebola, Nipah, and hantavirus) are often characterized by sporadic and re-occurring outbreaks interspersed by periods of apparent absence or dormancy. Perhaps the largest barrier to global prevention and eradication of these diseases lies in understanding how and where they are maintained in the environment prior to subsequent outbreaks and spillover into human populations. In many of these disease systems, a complete understanding of transmission and persistence dynamics has been obscured by complex interactions among hosts, vectors, pathogens, the environment, and putative reservoirs. This complexity has produced numerous competing hypotheses for the persistence of plague within host colonies over inter-outbreak periods. Herein, I explore the plausibility of a disease reservoir in driving cryptic persistence and sporadic re-emergence of plague and provide recommendations to predict and prevent future plague outbreaks.

In chapter one, I provide a broad overview of the eco-epidemiology of plague. I discuss the history of *Yersinia pestis*, the etiologic agent of plague, from ecological, evolutionary, and epidemiological perspectives. This review encompasses classically recognized disease transmission routes, hosts, vectors, and plague biogeography.

In chapter two, I critically review candidate mechanisms that may facilitate local plague persistence and sporadic outbreak re-emergence. These candidate mechanisms are broken down into four classical hypotheses including I) long-range re-introduction events, II) host metapopulation dynamics, III) enzootic transmission dynamics, and IV) maintenance in bacterial

reservoirs. Within hypothesis IV, I critically review candidate reservoirs including resistant mammalian hosts, flea vectors, soil environments, protozoan amoebae, and other telluric microorganisms. I provide evidence suggesting that distant re-introduction events via source-sink dynamics are exceedingly rare and incapable of explaining the majority of plague re-emergence in established foci. Further, I conclude that maintenance of plague in regional metapopulations via extinction-recolonization dynamics and local enzootic dynamics are plausible explanations for re-emergence following short periods of inter-outbreak quiescence. However, these mechanisms do not adequately explain re-emergence dynamics over long inter-outbreak periods. Local persistence in reservoirs is the only classical hypothesis capable of explaining long-term inter-outbreak periods.

Plague ecology is characterized by sporadic epizootics, then periods of dormancy. Building evidence suggests environmentally ubiquitous amoebae act as feral macrophages and hosts to many intracellular pathogens. In chapter three, we conducted environmental genetic surveys and laboratory co-culture infection experiments to assess whether plague bacteria were resistant to digestion by five environmental amoeba species. First, we demonstrated that *Yersinia pestis* is resistant or transiently resistant to various amoeba species. Second, we showed that *Y. pestis* survives and replicates intracellularly within *Dictyostelium discoideum* amoebae for >48 hours post-infection, whereas control bacteria were destroyed in <1 hour. Finally, we found that *Y. pestis* resides within amoeba structures synonymous with those found in infected human macrophages, for which *Y. pestis* is a competent pathogen. Evidence supporting amoebae as potential plague reservoirs stresses the importance of recognizing pathogen-harboring amoebae as threats to public health, agriculture, conservation, and biodefense.

Chapter four, examines the long-term eco-epidemiological dynamics occurring within a model plague foci comprised of susceptible hosts, flea vectors, and amoebae reservoirs, as well as variable climate conditions. I use an epidemiological model of plague affecting populations of Black-tailed prairie dogs to explore the role of a long-term reservoir in enabling local maintenance of plague during inter-epizootic periods. I demonstrate that natural variation in eco-epidemiological conditions, including host immunity and reservoir life-history, can drive the emergence of both epizootic and pseudo-enzootic outbreak dynamics. This empirically-informed model is concordant with 25 years of field observation and suggests a unified explanation for cryptic plague persistence and heterogeneous outbreak dynamics.

Amoebae are known reservoirs for numerous pathogens and are themselves the etiologic agents of numerous diseases. Amoebae cause up to 3.2 million combined annual cases of blindness, cutaneous ulcers, liver abscesses, diarrheal dehydration, and encephalitis, with case fatality rates reaching as high as 97%. The speed of clinical intervention is inhibited by a shortage of comprehensive and rapid diagnostic tools capable of identifying and differentiating between major pathogenic amoebae genera. In chapter five I developed five end-point simplex PCR assays that can be combined into one multiplex PCR that targets five of the most clinically important amoebae genera that exhibit primary pathogenicity or significant associations with amoeba-resistant pathogens (ARP). This multiplex assay rapidly and specifically identifies clinically significant amoebae that have been cultured from clinical or environmental samples and could improve patient diagnosis and treatment in a wide range of settings.

Collectively, this dissertation advances the field of plague ecology by evaluating mechanisms by which *Y. pestis* may persist and re-emerge in natural environments. Identifying which maintenance mechanisms are predominantly active within individual foci will enable

development of more effective disease forecasting and prevention strategies without misappropriating increasingly limited resources towards unlikely causes of reemergence in highly lethal disease systems.

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DEDICATION

To the scientific pursuit of order and understanding amidst chaos and ignorance.

“Only entropy comes easy.” — Anton Chekhov (c.1900)

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CHAPTER 1: THE ECO-EPIDEMIOLOGY OF PLAGUE

***Yersinia pestis*, the etiologic agent of plague**

Yersinia pestis is the etiologic agent of plague, the bacterial disease that dramatically shaped human history beginning at least 5,000 years ago (Rasmussen *et al.* 2015, Rascovan *et al.* 2018) but most notably during three global pandemics. In modern times, *Y. pestis* continues to emerge during sporadic outbreaks afflicting both human and wildlife populations around the globe. The first recorded pandemic, referred to as the Plague of Justinian, ravaged the Byzantine Empire and virtually all Mediterranean port cities from 541CE – 750CE, resulting in 25-50 million deaths, representing approximately 13-26% of the global population (Rosen 2007). Following a nearly 600 year period of relative quiescence in Europe and the Mediterranean region, the second plague pandemic, infamously referred to as The Black Death, spread from central Asia westward along trade routes arriving in Europe in 1347. Death estimates range from 75-100 million, representing 17-22% of the known global population (Gottfried 1983). Recurrent outbreaks continued at varying intervals throughout Europe and Western Asia until the early 19th century. The third and final recognized pandemic began in 1855 in China's Yunnan province and disseminated globally as a result of increased human connectivity via ocean trade routes causing over 12 million deaths in China and India within three decades (Orent 2004). No official end of the third pandemic has been declared (WHO 2016). Delineation of these pandemics is somewhat imprecise given the heterogeneity associated with clinical disease features, eco-epidemiology, and continual sporadic reoccurrence in between the generally recognized pandemics.

Despite common perception of plague as an ancient disease, it is still active globally. The time span from 2010 to 2015 saw 3,248 cases of plague with 584 fatalities (case fatality rate

18%)(WHO 2016). In late 2017 a single outbreak in Madagascar produced 2,348 reported cases, 202 deaths, and a case fatality rate of 8.6 %. The Western U.S. has an average of 7 cases annually over the past 20 years, with 17 and 16 reported in 2006 and 2015. *Y. pestis* continues to pose a public health and bio-terrorism concern (Inglesby *et al.* 2000).

Y. pestis is taxonomically in the family *Enterobacteriaceae* and a congener with two other recognized pathogens, *Y. pseudotuberculosis* and *Y. enterocolitica*, and numerous non-pathogenic species. *Y. pseudotuberculosis* is an enteric pathogen found predominantly in soil that infects mammalian macrophages via the oral-fecal route (Chain *et al.* 2004). *Y. pestis* is thought to have evolved from *Y. pseudotuberculosis* 5,000-28,600 years ago based on molecular clock estimates of mutation rates (Achtman *et al.* 1999, Riehm *et al.* 2012, Rasmussen *et al.* 2015, Rascovan *et al.* 2018). *Y. pestis* maintains 98% DNA sequence similarity with *Y. pseudotuberculosis* (Skurnik *et al.* 2000) and possesses numerous pseudogenes that remain functional in *Y. pseudotuberculosis* (Kukkonen *et al.* 2004). It is hypothesized that *Y. pestis* originated as a telluric organism and later shifted to from oral-fecal to vector-born transmission (Drancourt *et al.* 2006, Ayyadurai *et al.* 2008, Zeppelini *et al.* 2016).

Geographic regions experiencing plague are often differentiated into discrete foci, which we define as the spatial extent that a *Y. pestis* clone persists and is transmitted among a group of specific mammalian hosts, their fleas, and possibly amoeba (Maher *et al.* 2010, Smith *et al.* 2010, Giles *et al.* 2011, Ben-Ari *et al.* 2012, Lowell *et al.* 2015). This definition of foci is useful for understanding the extent of pathogen dispersal and admixture with other clones, whereas more traditional definitions of plague foci are more ecologically based and do not distinguish between disease caused by separate bacterial clones in the same geographical region. Additionally, *Y. pestis* is sometimes classified into three biovars, *Y.p. medievalis*, *Y.p. orientalis*,

and *Y.p. antiqua*, which are classically associated with each of the three global pandemics in chronological order (Prentice & Rahalison 2007). Biovar classification is an evolving concept and is progressively being replaced by more nuanced classification schema following the identification of numerous sub-species and plasmidovars (Anisimov *et al.* 2004, Haensch *et al.* 2010, Cui *et al.* 2013). The plague bacterium's genome is comprised of a ~4.6-5 million bp chromosome, and three plasmids: ~10k bp pPCP1 (also referred to as pPla or pPst), ~70k bp pCD1, and ~110k bp pMT1 (or pFra). The first plasmid is *Y. pestis*-specific, whereas the second and third are shared by all *Yersiniae*. For a more in-depth review of *Yersiniae* genome organization and virulence determinants, we refer readers to (Perry & Fetherston 1997, Prentice & Rahalison 2007, Zhou & Yang 2009, Abbot & Rocke 2012) and for gene-specific analyses we recommend (Straley 1993, Hinnebusch *et al.* 1996, Parkhill *et al.* 2001, Darby *et al.* 2002, Kukkonen *et al.* 2004, Huang *et al.* 2006, Felek & Krukoni 2009, Morelli *et al.* 2010, Connor *et al.* 2015).

Yersinia pestis is identified as a gram-negative, rod-shaped coccobacillus and facultative anaerobe, measuring from 0.5-0.8mm in diameter and 1-3mm long (Perry & Featherston 1997). It replicates optimally at 28-30°C and pH 7.2-7.6, although replication is possible from 4-40C and from pH 5-9.6. In mice, the LD50 at 25C is lower than at 37C indicating temperature regulation of virulence factors (Cavanaugh & Williams 1980). Plague is clinically described by the location of bacterial replication and manifests in mammals as bubonic, septicemic, or pneumonic, and less commonly as pharyngeal, meningial, cutaneous, abortive, or asymptomatic. We refer readers to (Perry & Fetherston 1997, WHO Plague Manual 1999, Prentice & Rahalison 2007) for a comprehensive review of plague's clinical disease symptoms and treatment.

Disease Transmission

Transmission of *Y. pestis* bacteria leading to disease in a mammalian host can occur via numerous pathways (Figure 1.1) with the prototypical transmission route being via flea vectors (Gage & Kosoy 2005). Fleas are common ectoparasites of mammalian plague hosts and can become infected by feeding on hosts with bacterial titers of at least 10^6 per milliliter of blood (Burroughs 1947, Pollitzer 1954, Engelthaler *et al.* 2000, Lorange *et al.* 2005, Boegler *et al.* 2016). Direct physical exposure to bodily fluids of septic hosts and contaminated soil or surfaces can cause disease, especially if bacteria enter through open wounds on a naïve host. Fecal-oral transmission can result from ingestion of contaminated food or water sources (Cavanaugh 1972, Saeed *et al.* 2005), occasionally occurring in wildlife via predation or cannibalism of infected hosts or carcasses, though this route of transmission is thought to be rare (Perry & Featherston 1997). Alternatively, infectious respiratory droplets by hosts with pneumonic plague may be subsequently inhaled leading to infection. Each of the above transmission routes can require the successful delivery of fewer than 10 bacteria to naïve hosts to illicit disease (Perry & Featherston 1997).

Flea species are differentially capable of transmitting plague following ingestion of an infectious blood-meal. One mechanism of onward transmission is referred to as “early-phase transmission” (EPT) and the other more classical route involves blocked or partially-blocked transmission. EPT-capable fleas are infectious from 3-96 hours post feeding without requiring the formation of a biofilm that blocks the flea’s proventriculus (Burroughs 1947, Eisen *et al.* 2006, Eisen *et al.* 2007, Eisen *et al.* 2008, Wilder 2008a, Wilder 2008b). Alternatively, some fleas transmit plague after approximately five days via blocked transmission, which requires that the ingested *Y. pestis* bacilli form a biofilm that partially or completely blocks the flea’s

proventriculus. When a blocked flea attempts to feed, it regurgitates saliva containing a mixture of anti-coagulant compounds and *Y. pestis* bacilli that slough off the biofilm. When completely blocked, the flea is unable to ingest subsequent blood-meals and under threat of starvation, engages in repeated biting behavior that increases the likelihood of transmission by exposing new hosts to bacilli ejected by the flea during each feeding attempt. The lifespan of fleas following complete blockage is usually five days (Burroughs 1947), whereas fleas with a partial blockage survive longer periods and are often able to continue to transmit until the blockage is cleared or progresses to a complete blockage (Gratz 1999, Gage & Kosoy 2005, Abbot & Rocke 2012).

Vector-borne transmission is hypothesized to select for greater bacterial virulence and higher bacteremia in mammalian hosts, as high bacteremia is required to infect the inefficient flea vectors and enable onward transmission (Zhou & Yang 2009). Infectivity can depend on the route of transmission due to expression, or lack thereof, of mammalian virulence factors. Direct contact with infected mammalian hosts permits transmission of fully virulent bacteria with activated mammalian virulence factors between 26°C and 37°C (Straley & Perry 1995, Perry & Fetherston 1997). Conversely, several genes are only activated at the lower temperatures experienced by fleas. For example, *Yersinia murine toxin*, found on the *pMT1* plasmid, aids in the colonization of the flea mid-gut and is toxic to some murid rodent hosts.

The primary disease presentation in mammals depends on route of infection and can often progress to secondary forms of disease if left untreated. Infection by direct contact that results in *Y. pestis* bacteria entering the blood stream can cause primary bubonic or less frequently primary septicemic plague. Inhalation of *Y. pestis* bacteria typically presents as primary pneumonic plague. Ingestion of *Y. pestis* has been shown to cause pharyngeal plague in

rare human cases, whereas predation, scavenging or cannibalism among wild hosts can also lead to disease. Infection via flea bite most frequently presents as primary bubonic plague. A prototypical course of infection begins following a flea bite, where *Y. pestis* bacilli migrate to the nearest lymph nodes and subsequently replicate, resulting in iconic buboes or swollen lymph nodes. Infection can then disseminate into the bloodstream and bodily organs, increasing the likelihood of secondary septicemic or pneumonic plague. The likelihood of subsequent vector-borne transmission is increased dramatically if fleas feed on highly bacteremic/septicemic hosts after the infection has spread to the peripheral blood supply.

Hosts

In the wildlife disease literature, various host classes and characteristics have been inconsistently or nebulously defined based on the host's role in the disease system. Host susceptibility is perhaps one of the broadest categories and distinguishes between hosts that do or do not develop clinical signs of disease following exposure and infection. We use the following terminology with respect to host population dynamics in response to plague. The term enzootic host describes mammalian host populations that experience continuous low-level transmission and low-intermediate mortality (perhaps due to increased host immunity). The term epizootic host describes mammalian host populations that experience sporadic and widespread transmission conferring high host mortality. Epizootic dynamics are frequently interspersed by periods of apparent dormancy, wherein plague may be maintained by numerous candidate mechanisms described later.

Over 250 mammalian species across 73 genera are capable of becoming naturally infected by *Y. pestis*. with rodents being classically regarded as the most important epizootic hosts for plague because of their abundance, population structure, generally high susceptibility,

and vector-borne transmission pathways (Gage & Kosoy 2005, Abbot & Rocke 2012). Additional mammalian host groups with recorded or presumed infections include lagomorphs, insectivores, mustelids, carnivores, ungulates, primates, and marsupials, as ordered by susceptibility to plague. Immune or resistant hosts have been hypothesized to play a role in the maintenance or transmission of plague, but resistant hosts are unable to contribute to vector-borne transmission because of sub-clinical bacteremia (Burroughs 1947, Pollitzer 1954, Engelthaler *et al.* 2000, Lorange *et al.* 2005, Boegler *et al.* 2016) and transmission via direct contact or ingestion is thought to be exceedingly rare (Perry & Featherston 1997). Additionally, many species within these diverse groups have not been observed with clinical signs of disease or have not demonstrated the capability to transmit infectious *Y. pestis* to subsequent hosts. The inclusion of many of these hosts is largely based on the detection of *Y. pestis* antibodies identified in serological studies. Positive serology does not necessarily indicate transmission and persistence, but solely indicates prior exposure and resistance or recovery (Salkeld & Stapp 2006). For these reasons, serology may be a poor tool for distinguishing between hosts that are incidentally infected and hosts that drive the maintenance or transmission of plague. For specifics of which plague hosts are found in plague foci around the globe and their individual susceptibility to plague, we refer readers to (Gratz 1999, Gage & Kosoy 2005, Abbot & Rocke 2012).

In addition to host species, host population structure impacts plague transmission and may also contribute to long-term plague persistence. Black-tailed prairie dogs and great gerbils are model epizootic plague hosts that exhibit classical metapopulation structure (Hoogland 1995, Davis *et al.* 2007). Metapopulations are an assemblage of spatially distinct local populations, each with independent dynamics, that are coupled by some degree of migration or gene flow

(Hanski & Gaggiotti 2004). Host metapopulation structure may help buffer against complete host extinction through source-sink migration dynamics that enable recolonization of extinct sub-populations (Boots *et al.* 2004).

Vectors

The predominant transmission route for plague occurs between diseased and naïve hosts via flea vectors. Fleas are common ectoparasites of mammals and can become infected by feeding on bacteremic hosts (Burroughs 1947, Pollitzer 1954, Engelthaler *et al.* 2000, Lorange *et al.* 2005, Boegler *et al.* 2016). Over 1,500 species of fleas have been identified, with approximately 30 proven to be capable plague vectors (Abbot & Rocke 2012). Flea vectors exhibit differential infectivity time-lags, host preferences, transmission efficiencies, life history, and environmental tolerances. This heterogeneity contributes to a diversity of observed and hypothesized flea-mediated disease dynamics.

These sources of heterogeneity also compound with heterogeneity described in other portions of the plague system and exacerbate the difficulty of accurately measuring vector competency and determining the relative importance of particular fleas in plague transmission and persistence. For example, the time-lag until an infected flea becomes infectious is primarily dependent on its capability to perform early-phase transmission, but various environmental conditions also influence infectious time-lags (Wilder *et al.* 2008a, Williams *et al.* 2013). Fleas maintain different host-preferences with some fleas specializing as ectoparasites of a single host species, whereas other flea species are less fastidious and act as generalist ectoparasites across a wide diversity of mammalian host species. Varying flea host-preferences have been used to explain how plague infection spreads between different species. Narrower host ranges for fleas provide an explanation for why only a single host species is affected by plague despite co-

localization of other susceptible hosts. Transmission efficiency, an approximation of the bacterial abundance required for a flea to become infected and subsequently transmit that infection, varies and is correlated with the transmission modes available to that particular species as well as the flea's host-preferences (Wilder *et al.* 2008b). Finally, fleas demonstrate variability in life history traits and tolerances to extreme environmental conditions including temperature and desiccation (Williams *et al.* 2013).

Biogeography

Instances of human and sylvatic plague outbreaks span enormous swaths of time and space, encompassing the last 5,000 years (Rasmussen *et al.* 2015, Rascovan *et al.* 2018), five continents, and at least 117 countries (Figure 1.2). While plague is primarily a pathogen of ground-dwelling rodents in temperate and relatively dry regions, seasonal and environmental correlations with plague outbreaks vary across plague foci and further contribute to the heterogeneity described in prior sections. In some instances, multiple plague outbreaks occur synchronously in a particular region, which is thought to be driven by an environmental trigger (Ben-Ari *et al.* 2011, Savage *et al.* 2011, Lowell *et al.* 2015). In the U.S. the majority of plague outbreaks occur from May through September, which correlate with many ecological events of potential significance, including rodent host burrow construction and recolonization, breeding/birthing periods, and peak vegetation growth. Numerous other environmental correlates have also been investigated including, temperature, precipitation, soil type and nutrient content (Enscore *et al.* 2002, Stapp *et al.* 2004, Snäll *et al.* 2008, Maher *et al.* 2010, Ben-Ari *et al.* 2011, Savage *et al.* 2011). Die-offs of black-tailed prairie dogs on the Great Plains in the western US (Stapp *et al.* 2004) are correlated with El Niño–Southern Oscillation events that cause above average precipitation, warmer spring temperatures, and cooler summers. A variety of cascade

models have determined that wet springs tend to yield increased plague occurrence in subsequent months (Stapp *et al.* 2004, Ben-Ari *et al.* 2011), but other studies have found no evidence of environmental cascade effects on host/flea abundance (Savage *et al.* 2011). The direct positive effect of precipitation and direct negative effect of hot temperatures on plague persistence or transmission is supported by additional modeling studies (Snäll *et al.* 2008). Outside the US, temperature and rainfall variation similarly triggers plague in gerbils in Kazakhstan (Stenseth *et al.* 2006) and human plague cases in Vietnam (Cavanaugh & Marshall 1972). The effects of climate on plague occurrence are regional and not, in general, broadly applicable for predicting plague incidence globally.

It is classically believed that plague persists in sylvatic foci in four continents, North America, South America, Asia, and Africa, with historical spillover into Europe. However, the extent of probable sylvatic foci is likely underestimated because of lack of reporting in certain regions (WHO 2000). In North America, *Y. pestis* was introduced into the continental United States on several occasions, but the only introduction that resulted in establishment likely occurred in San Francisco, California, US in 1899 and was first recorded in wildlife populations in 1904 (Wherry 1908, Gage & Kosoy 2005). Other studies have suggested Los Angeles, California, US and Seattle, Washington, US as possible introduction points (Adjemian *et al.* 2007). Its range expanded to encompass approximately 90% of counties west of longitude 103° W by 1940 and devastated populations of susceptible host species (Antolin *et al.* 2002). The factors enabling or preventing further Eastward expansion of plague in the continental U.S. are not definitively known, but are likely related to increased precipitation and lack of abundant ground-dwelling rodent populations like prairie dogs and ground squirrels. Some hypotheses stipulate insufficient host/vector compatibility and/or distribution, varying soil-types and

moisture regimes, or impacts from intensive agricultural operations prevalent in the Midwest (Zeppelini *et al.* 2016). Plague ranges are predicted to be subtly shifting northward due to gradual climate change (Nakazawa *et al.* 2007).

Generalizable geographic correlates with plague foci are sparse, and include an inverse association with topographic slope and soil sand content, primarily due to host habitat preferences (Augustine *et al.* 2016). Additional correlates have been proposed for individual plague regions, including Uganda where plague correlates with elevations greater than 1300m (Eisen *et al.* 2010). Unsurprisingly, proximity to plague-positive colonies is correlated with an increased likelihood of plague occurrence, whereas barriers inhibiting movement and connectivity of hosts and vectors like water bodies or roads are negatively correlated with plague occurrence (Collinge *et al.* 2005). Several studies suggest that landscape context (e.g. barriers to migration and proximity to plague-positive host colonies) is more important to local plague occurrence than characteristics of host and vector assemblages (Brinkerhoff *et al.* 2010). At regional inter-colony scales, host population structure is thought to impact plague transmission and possibly enable long-term maintenance of the disease (Zeppelini *et al.* 2016).

Figures

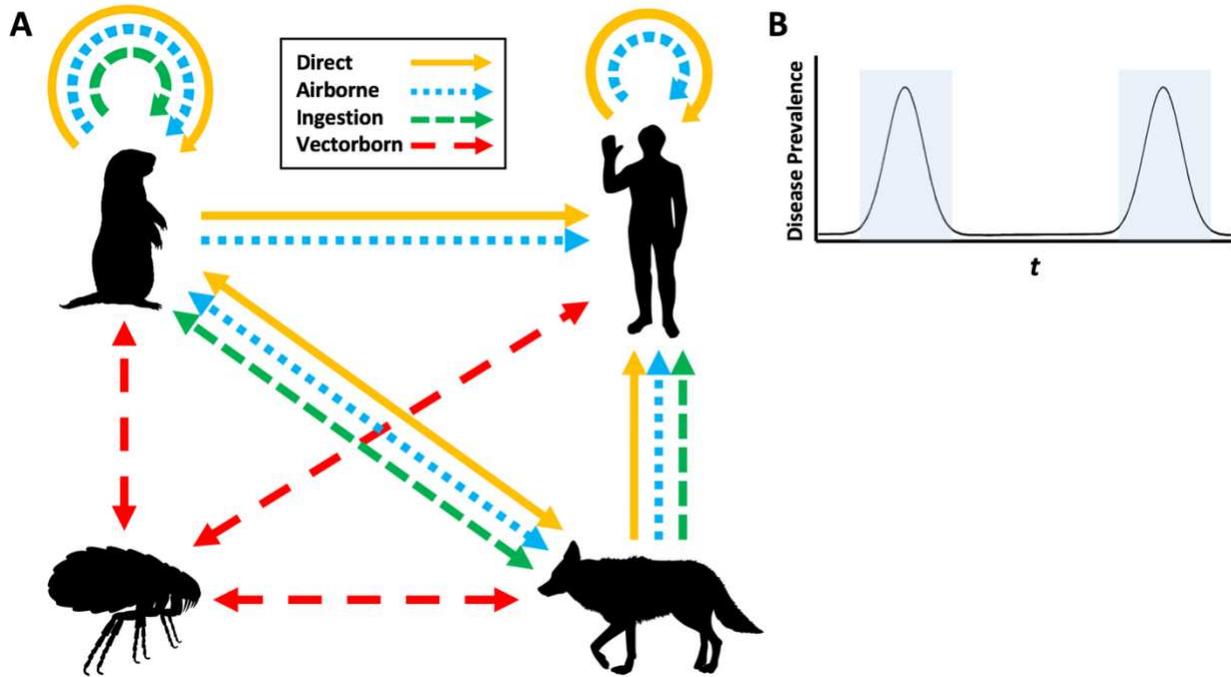


Figure 1.1: A) Recognized *Y. pestis* transmission pathways between epizootic hosts (e.g. prairie dog in top-left), alternate hosts (e.g. fox in bottom-right), vectors (e.g. flea in bottom-left), and occasionally humans (top-right). The vast majority of plague transmission occurs through the vector-borne route, especially during large outbreaks or epizootics (blue regions in 1B). However, several other transmission pathways exist and may be relevant for the transmission and persistence of *Y. pestis* during the periods interspersing outbreaks.

CHAPTER 2: MAINTENANCE MECHANISMS FACILITATING RE-EMERGENT PLAGUE OUTBREAKS

Introduction

Highly lethal pathogens (including plague, anthrax, Ebola, Nipah, and hantavirus) are often characterized by sporadic and re-occurring outbreaks interspersed by multi-year periods of apparent absence or dormancy (Gage & Kosoy 2005, Salkeld *et al.* 2016). Perhaps the largest barrier to global prevention and eradication of these re-emergent diseases lies in understanding how and where they are maintained during these quiescent periods. In many of these disease systems, a thorough understanding of transmission and persistence dynamics has been obscured by heterogeneous outbreak dynamics driven by complex interactions among hosts, vectors, pathogens, reservoirs, and the environment.

Plague dynamics are often characterized by transiently high disease prevalence within susceptible host populations (epizootics, Table 2.1) that results in rapid and substantial host mortality. This is often followed by multi-year periods of apparent disease quiescence prior to subsequent epizootic re-emergence (Girard *et al.* 2004, Webb *et al.* 2006, Snäll *et al.* 2008, Eisen & Gage 2009, Gibbons *et al.* 2012, Lowell *et al.* 2015, Salkeld *et al.* 2016). These dynamics occur across a hierarchy of spatial scales including: the broader landscape that is comprised of multiple independent plague foci (Table 2.1), individual foci that are often comprised of numerous host colonies, and individual host colonies that are comprised of numerous host burrows.

Epizootic re-emergence after quiescent periods likely require an initial reintroduction or spillover event from a maintenance source in combination with adequate host/vector susceptibility, host/vector connectivity (correlated with abundance and density), and climatic

conditions. Following initial re-emergence, various combinations of heterogeneous pathogen, host, vector, reservoir, and environmental factors may help to amplify or perpetuate an epizootic. For example, increased vector abundance driven by increased host abundance (Tripp *et al.* 2009) may increase transmission and exacerbate the severity of an initial spillover or reintroduction event and lead to the emergence of epizootic dynamics. Epizootic dynamics have been well described (Gage & Kosoy 2005, Abbot & Rocke 2012) largely because high mortality in host populations is easily observed. By contrast, little is known about the dynamics occurring over inter-epizootic periods, specifically regarding pathogen maintenance. Numerous hypotheses have been proposed to explain how plague is maintained over inter-epizootic periods that range from 1-300 years (Barreto *et al.* 1995, Chanteau *et al.* 1998, Tikhmorov 1999, Arbaji *et al.* 2005, Bertherat *et al.* 2007, Tarantola *et al.* 2009, Seifert *et al.* 2016).

Here we compile the most compelling evidence for and against four classical hypotheses thought to describe the maintenance and re-emergence of plague including I) Long-range reintroduction, II) Metapopulation dynamics, III) Enzootic transmission, and IV) Reservoir maintenance (Figure 2.1). Each of these hypotheses have received varying degrees of investigation yielding equally variable amounts of convincing evidence. Distant reintroduction events are exceedingly rare and appear incapable of explaining the majority of plague re-emergence in established foci. Maintenance of plague in host metapopulations via extinction-recolonization dynamics or maintenance via enzootic transmission may be plausible explanations for maintenance over short periods of inter-epizootic quiescence. However, these mechanisms do not adequately explain re-emergence dynamics over long inter-epizootic periods. Bacterial persistence in reservoirs appears capable of explaining long-term inter-epizootic periods, yet has been subject to the least research. Several of these hypotheses remain prominent in the literature

despite a lack of cogent supporting evidence (Gage & Kosoy 2005, Eisen & Gage 2009, Stapp *et al.* 2008, Abbot & Rocke *et al.* 2012) from over a century of research (Wherry 1908, Gage & Kosoy 2005).

We conclude these maintenance mechanisms are not mutually exclusive and likely co-occur with shifting predominance over space and time. We suggest that reservoirs may serve as a basal persistence mechanism across many plague foci that can give rise to other maintenance and transmission mechanisms under specific eco-epidemiological conditions. Re-evaluating the plausibility of these maintenance mechanisms and identifying which ones predominate within individual plague foci will enable development of more effective disease forecasting and prevention strategies without misappropriating increasingly limited resources towards unlikely causes of re-emergence. This possibility motivates the pursuit of a comprehensive understanding of inter-epizootic plague dynamics despite challenges posed by the massive heterogeneity observed within the plague system.

Inter-epizootic hypotheses

We present a reductive framework that categorizes putative explanations for sylvatic plague maintenance based on transmission dynamics required to sustain plague across varying spatial scales (Figure 2.1). Given immense variation and complexity within the plague system, we selectively focus on the requirements necessary for *Y. pestis* to persist and re-emerge as opposed to the myriad of correlated factors that may contribute to amplifying transmission following initial re-emergence. This framework reduces prominent classical explanations into four candidate hypotheses for plague maintenance. For each hypothesis we present supporting and detracting evidence and summarize its power to explain observed inter-epizootic dynamics across plague foci.

Hypothesis I: Long-range reintroduction

Hypothesis I stipulates that *Y. pestis* does not persist within an individual host colony or the surrounding plague foci over inter-epizootic periods because of insufficient maintenance conditions in host, vector, and reservoir populations or the environment. Hypothesis I suggests that subsequent epizootics in the region result from discrete long-range reintroduction events from transmission between distant plague foci (e.g. >50km). This encompasses any series of events or interactions that causes the translocation of infectious *Y. pestis* across large spatial scales and results in re-emergence of plague in regions where it was historically present, but recently absent. Examples of such events include translocation of hosts, associated vectors, or infectious material via natural migration or anthropogenic transport.

Discrete long-range transmission events have frequently preceded the establishment of novel plague foci (Wherry 1908, Link 1955, Girard *et al.* 2004, Gage & Kosoy 2005), but evidence suggesting this mechanism is responsible for re-occurring epizootics in existing foci is largely speculative. Potential examples include transmission of *Y. pestis* by hosts with large migratory ranges such as coyotes, foxes, or owls in the Western U.S. (McGee *et al.* 2006, Salkeld *et al.* 2007, Holt *et al.* 2009). In this example, the migration of infectious hosts could transmit the infection to dense populations of susceptible rodent hosts via multiple routes including infectious vectors they carry, via direct antagonistic interactions with susceptible hosts, or via susceptible hosts consuming an infected carcass of a migratory host (Harrison *et al.* 2003, Salkeld & Stapp 2006, McGee *et al.* 2006, Salkeld *et al.* 2007, Holt *et al.* 2009). These infrequent long-range transmission events have been proposed to explain repeated plague outbreaks in Europe (Schmid *et al.* 2015) and are potentially explanatory for the lack of plague detection by bio-surveillance efforts over inter-epizootic periods in susceptible hosts populations.

This hypothesis is also compelling where recurrent epizootics are observed in regions with few of the requirements characteristic of natural plague foci.

However, investigations into gene-flow between distant plague foci and the natural behaviors exhibited by proposed transmission agents provide compelling evidence opposing this mechanism as a predominant explanation for sporadically recurrent plague epizootics. A study in Colorado U.S. investigated the geographic extent of individual plague clones, as identified by a SNP based genotyping chip (Lowell *et al.* 2015). Bacterial population genetic structure of isolates from within and between individual metapopulations indicated localized persistence of individual clones across inter-epizootic periods (Girard *et al.* 2004, Lowell *et al.* 2015). This suggests that long-range transmission and gene flow between distant foci are not predominant drivers of plague re-emergence. Instead, more substantial evidence suggests intra-foci persistence and diversification of *Y. pestis* lineages following an initial long-range introduction (Girard *et al.* 2004, Lowell *et al.* 2015). Genetic evidence further indicates that epizootic occurrence is often caused by independent emergence of geographically-distinct *Y. pestis* clones, which contrasts with long-range reintroduction events (Girard *et al.* 2004, Snäll *et al.* 2008, Gibbons *et al.* 2012, Viana *et al.* 2014, Lowell *et al.* 2015). Prior studies have also demonstrated limited host dispersal distances (Hoogland 2013), thereby further reducing the likelihood for successful long-range reintroduction. Additionally, an epidemiological analysis suggests a strain of plague that re-emerged in Oran, Algeria after 53 years of quiescence was locally maintained and not imported (Bertherat *et al.* 2007).

The life history of known plague hosts generally do not support long-range migration and subsequent transmission. Natural reintroduction events would require an infected source (e.g. vector or host) to traverse large distances and elicit onward transmission, which may be difficult

for hosts experiencing clinical plague symptoms. If the host were resistant, then vector-borne transmission would not be possible because of insufficient bacteremia and transmission via direct exposure or ingestion are thought to be rare (Cavanaugh 1972, Perry & Featherston 1997). It is also likely that habitat interspersing plague foci would be less hospitable which further reduces the likelihood of successful long-range migration by infected hosts or vectors. Long-range transmission is clearly required for novel plague epizootics in historically disease-free regions. However, as an explanation for recurrent plague epizootics, discrete long-range translocation of plague appears exceedingly rare because of the natural behaviors exhibited by candidate transmission agents and lack of gene-flow between distant plague foci. This supports the notion that plague foci appear to be self-sustaining at intra-foci scales and do not require external reintroductions to illicit recurrent plague epizootics.

Hypothesis II: Metapopulation dynamics

Hypothesis II stipulates that *Y. pestis* is not maintained within an individual host colony following an epizootic, yet is able to persist at an inter-colony (i.e. metapopulation) scale. Susceptible host-colonies within a metapopulation may experience asynchronous cycles of plague quiescence and epizootic re-emergence respectively caused by extinction and recolonization of the hosts. Interactions between sub-populations of susceptible hosts and their associated vectors may incidentally serve as transmission routes and are thought to facilitate reintroduction of the pathogen and initiate a new epizootic cycle (Viana *et al.* 2014). It is clear that metapopulation dynamics play a defining role in the transmission of plague during ongoing epizootics, but the contribution of metapopulations in maintaining plague over inter-epizootic periods is uncertain. Under the strictest interpretation of this hypothesis, no period of quiescence exists from the perspective of the plague foci. In other words, this hypothesis entails sequential

chains of epizootics distributed across the foci resulting in the simultaneous appearance of epizootic cycles within individual colonies and perpetual epizootics across the regional foci (Figure 2.1B). A more nuanced version of this hypothesis includes the potential for transient periods of low-level (i.e. sub-epizootic) transmission to occur before and after detectable epizootics (St. Romain *et al.* 2013, Salkeld *et al.* 2016), thereby increasing the duration time where onward transmission could plausibly be sustained despite no obvious epizootics occurring within the host metapopulation. An alternate explanation for plague maintenance and re-emergence involves perpetual sub-epizootic transmission among more resistant hosts (enzootic transmission) and is examined further in Hypothesis III.

Evidence that metapopulation dynamics within a single foci represent the predominant mechanism for maintaining plague is difficult to disentangle from evidence supporting the involvement of metapopulations in the epizootic spread that occurs after maintenance and re-emergence by some other mechanism. Continual transmission among regional metapopulations is often thought to occur via host extinction-colonization dynamics (Zeppelini *et al.* 2016). Theoretically, pathogens with high virulence are able to persist within host metapopulations, as long as inter-colony transmission is high enough to infect other colonies before the local host population becomes extinct (Boots *et al.* 2004). Further, this hypothesis requires that extinct host colonies are recolonized at a rate greater than the sum of between-population transmission and the epizootic's R_0 (Antolin 2008), otherwise the host metapopulation would trend towards total extinction. Therefore, successful maintenance of plague via metapopulations relies heavily on high transmission and high inter-colony host migration, each of which can be encompassed by measures of inter-colony connectivity. The above conditions validate the potential for plague maintenance via sequential detectable epizootics within a susceptible host metapopulation,

however, an analysis of *Y. pestis* isolates from around the world indicated that rates of SNP accumulation were lower than what would be expected if persistence was a result of a continuous chain of epizootics occurring within a plague foci (Cui *et al.* 2013). In the absence of detectable epizootics across a host metapopulation, plague persistence may be possible via low-level (sub-epizootic) transmission (St. Romaine *et al.* 2013, Salkeld *et al.* 2016). Sub-epizootic transmission among highly susceptible host metapopulations requires a balance between conditions that deterministically lead to epizootic emergence or conditions that lead to pathogen extinction. In other words, the maintenance transmission chain could be easily broken and is unlikely able to explain multi-year periods of quiescence across entire foci.

Prevailing sentiment for North American plague systems in prairie dogs (Antolin *et al.* 2006, Snäll *et al.* 2008) and plague in Kazak gerbils (Davis *et al.* 2007) suggests that the degree of connectedness between susceptible host colonies in a metapopulation enhances the likelihood of persistence and the emergence of cyclical epizootic dynamics at the colony level (Keeling & Gilligan 2000, Stapp *et al.* 2004, Davis *et al.* 2004, Davis *et al.* 2007, Davis *et al.* 2008). It is also widely accepted that alternate host species may play an important role for increasing inter-colony connectivity and enabling onward transmission through transport of infectious vectors (Gog *et al.* 2002, Salkeld *et al.* 2010). Observation and experimental evidence supporting the sufficiency of metapopulations to maintain plague within individual foci is scarce, but a quantitative modeling study has suggested that rodent metapopulations and their associated flea vectors were able to maintain plague (Keeling & Gilligan 2000). However, this study assumes a host a susceptibility of 25-50%, which is much lower than susceptibility levels derived from other studied host species (Abbot & Rocke 2012, Rocke *et al.* 2015). Subsequent quantitative analyses have suggested that maintenance of plague among host and vector metapopulations was

not possible without the inclusion of a reservoir (Webb *et al.* 2006, Buhnerkempe *et al.* 2011, Richgels *et al.* 2016, Markman *et al.* Unpublished). Other models using historical datasets from North American plague foci have indicated that inter-colony connectivity was not a strong driver of plague epizootics and host migration is unlikely to be a primary driver of plague transmission (Snäll *et al.* 2008, George *et al.* 2013). The metapopulation hypothesis is also not adequately able to explain re-emergent plague within susceptible hosts that do not have a metapopulation structure.

Numerous plague foci have been subject to intensive bio-surveillance during inter-epizootic periods in attempt to detect *Y. pestis* and validate the metapopulation maintenance hypothesis. Despite thorough examination, a scarcity of empirical evidence supports the ability of susceptible host metapopulations to explain temporal plague dynamics including synchronous epizootic re-emergence and multi-year periods of quiescence across entire foci (Girard *et al.* 2004, Webb *et al.* 2006, Salkeld & Stapp 2008, Eisen & Gage 2009, Brinkerhoff *et al.* 2010, Ben Ari *et al.* 2011, Savage *et al.* 2011, Lowell *et al.* 2015). Synchronous epizootics, characteristic of plague, lend support to emergence via an environmental trigger (Snäll *et al.* 2008, Ben Ari *et al.* 2011, Savage *et al.* 2011, Lowell *et al.* 2015) and are at odds with perpetual circulation within a foci's host metapopulation. It is increasingly unlikely that plague is maintained solely via continuous transmission within susceptible host metapopulations given that periods of quiescence can span multiple decades without yielding any detectable signs of host mortality or transmission in intensively studied plague foci (Drancourt *et al.* 2006). This has resulted in disagreement over the sufficiency and necessity of host metapopulations for maintaining plague over long periods of apparent quiescence.

We suggest a portion of this disagreement results from difficulties determining whether metapopulation dynamics are responsible for plague maintenance and re-emergence or whether metapopulation dynamics predominate during the epizootic that occurs after maintenance and re-emergence by some other mechanism. Much of the evidence supporting the metapopulation maintenance hypothesis fails to explicitly address this distinction while also ignoring several eco-epidemiological characteristics of plague epizootics like seasonality and synchronicity. Given existing evidence, we advance the idea that metapopulations of susceptible hosts could plausibly maintain plague over shorter inter-epizootic periods, whereas maintenance over longer quiescent periods would require another mechanism.

Hypothesis III: Enzootic transmission

Hypothesis III proposes that *Y. pestis* can be sustained via continuous low-level transmission among mammalian hosts and their associated vectors over inter-epizootic periods without external reintroduction (Barnes *et al.* 1993). This hypothesis suggests that transmission is continuously occurring at levels below the threshold that would initiate an epizootic, also referred to as a percolation threshold (Salkeld *et al.* 2010, Viana *et al.* 2014). Conditions like host connectivity and host resistance may define the threshold above which a pathogen is able to percolate through a host population and manifest as an epizootic (Richgels *et al.* 2016). Proposed enzootic maintenance dynamics often involve resistant alternate hosts that may facilitate sub-epizootic maintenance over inter- or intra-colony spatial scales. Hypothesis III focuses on the potential for resistant hosts to facilitate ongoing transmission at sub-epizootic levels, whereas we consider non-transmitting resistant hosts as reservoirs and discuss them in Hypothesis IV.

For a mammal to be classified as resistant, it would require sufficient protective immunity to *Y. pestis* to survive initial infection and subsequent exposure to plague, though

acquired immunity may decrease over time (Graham *et al.* 2014). Additionally, if Hypothesis III requires that resistant hosts be able to facilitate onward transmission of *Y. pestis*. Immunity is not commonly observed in North American prairie dogs as indicated by several species of prairie dog suffering nearly 100% mortality following infection (Lechleitner *et al.* 1968, Rayor 1985, Ubico *et al.* 1988, Cully *et al.* 1997, Pauli *et al.* 2006) and in a separate experiment, only ~3% developed significant V-antigen titers post-infection (Rocke *et al.* 2012). Several other rodent species have evolved increased levels of immunity, as found in Kazak gerbils (Davis *et al.* 2004). There also remains significant debate over the degree to which resistant hosts could transmit *Y. pestis* given that resistant hosts would not have bacterial titers high enough for vector-borne transmission (Burroughs 1947, Pollitzer 1954, Engelthaler *et al.* 2000, Lorange *et al.* 2005, Boegler *et al.* 2016). However, host populations that are predominantly resistant may still include several susceptible individuals through which onward transmission may be possible. It is further possible that more resistant alternative host populations coexisting with more susceptible host populations could rarely enable transmission via predation or scavenging (Cavanaugh 1972, Perry & Fetherston 1997). In total, little evidence exists to support the notion that a separate enzootic cycle of plague occurs in coexisting but more resistant rodent or carnivore hosts in the western US foci (Salkeld and Stapp 2008, Stapp *et al.* 2008, Thiagarajan *et al.* 2008, Richgels *et al.* 2016).

Similar to Hypothesis II, successful maintenance of plague via enzootic transmission requires a balance between conditions that inhibit ongoing transmission and conditions that rapidly and frequently lead to epizootics. Two of the most important factors thought to facilitate the emergence of epizootic dynamics are correlated and include host connectivity and host abundance. (Snäll *et al.* 2008, Salkeld *et al.* 2010, George *et al.* 2013). Measures of host

connectivity encompass encounter and transmission rates for hosts and vectors. Transmission rates are further influenced by pathogen virulence, host resistance, vector efficiency, vector abundance, and the potential for transmission lags such as those caused by infection reservoirs (discussed further in Hypothesis IV). Host abundance has been measured differently in past studies, often consisting of net number of hosts, host density relative to colony area, or percentage of occupied burrows within rodent host colonies (Hoogland 1995). Several quantitative studies have evaluated the specific levels of intra-colony connectivity and host abundance required to achieve homeostatic dynamics where plague could persist at low levels for over one year without causing a colony-wide epizootic (Davis *et al.* 2008, Salkeld *et al.* 2010, George *et al.* 2013). While specific thresholds for connectivity and abundance vary based on colony attributes, it is evident that host connectivity must remain at intermediate levels to prevent frequent epizootics or pathogen extinction respectively caused by high and low measures of connectivity (Salkeld *et al.* 2010). Additionally, it is possible to observe pseudo-enzootic patterns if host connectivity increases while the duration of the quiescent period is reduced (Viana *et al.* 2014). This would give the appearance of enzootic maintenance despite actually requiring continual inputs of bacteria from other sources. Natural observations of host colonies that grow exponentially in abundance or density without triggering epizootics appear to contradict to the enzootic hypothesis. If plague was continually circulating at low levels within the foci, dramatically increased host connectivity should be sufficient to facilitate epizootic emergence.

Many studies have debated the importance of alternate host species for modulating intra-colony connectivity and plague persistence (Salkeld & Stapp 2008, Stapp *et al.* 2008, Eisen *et al.* 2008, Eisen & Gage 2009, Salkeld *et al.* 2010). One modeling approach determined that alternate

host species were not required for sub-epizootic maintenance within a host colony, but they were significantly associated with increasing host connectivity which drives the emergence of epizootics (Salkeld *et al.* 2010). Another quantitative study indicated that alternate hosts were required to yield epizootic frequencies that mirror natural systems (Markman *et al.* Unpublished). It is generally accepted that alternate hosts increase connectivity among primary hosts, thereby amplifying transmission (Salkeld & Stapp 2008, Stapp *et al.* 2008, Eisen *et al.* 2008, Eisen & Gage 2009, Salkeld *et al.* 2010). However, few studies distinguish whether alternate hosts are responsible for enzootic plague maintenance or whether the role of alternate host dynamics only becomes relevant for transmission after maintenance and re-emergence by some other mechanism.

While these studies agreed alternate hosts were relevant for amplifying transmission (shifting from sub-epizootic to epizootic conditions), they presented contradicting evidence on whether enzootic transmission is capable of maintaining plague over varying periods of quiescence. The median duration of inter-epizootic quiescence within host colonies in one intensively studied North American plague foci is approximately 6 years (encompassing the duration of an initial host population crash, colony vacancy, and time from recolonization to subsequent epizootic)(Markman *et al.* Unpublished). Whereas, other studies only provided theoretical support for enzootic maintenance over inter-epizootic periods of 3.3 years (Salkeld *et al.* 2010).

Outside of modeling studies, experimental or observational evidence indicating alternate hosts are involved in the maintenance of plague is scarce. Numerous prior studies have found no viable *Y. pestis* in alternate host rodents before, during, or after epizootics despite intensive sampling (Salkeld & Stapp 2008, Stapp *et al.* 2008, Eisen *et al.* 2008). Evidence of antibody

seropositivity in alternate hosts has seldom been found outside of epizootic periods (Stapp *et al.* 2008). Even so, seropositivity does not entail active infection or infectiousness and therefore the role that seropositive hosts play in maintenance and re-emergence remains uncertain despite extensive research. It is possible that prior sampling efforts were still insufficient given the difficulties associated with thoroughly sampling underground host populations. Nonetheless, it is also improbable that alternate hosts maintain sufficient vector abundance over inter-epizootic periods given the predominantly low vector efficiency of their associated fleas (Eisen & Gage 2009). While alternate hosts are unlikely to contribute to enzootic plague persistence, their ability to modulate intra-colony connectivity and increase infection percolation may be a necessary requirement for epizootic dynamics to emerge from sub-epizootic maintenance conditions (e.g. Hypothesis III or Hypothesis IV). This is supported by observations that epizootics in prairie dogs were more likely following years where grasshopper mice were more abundant (Stapp *et al.* 2009).

Some of the most compelling evidence in support of enzootic persistence is from research on flea vector abundance, transmission rates, and survival. Fleas that were PCR positive for plague have been collected slightly over a year prior to observed epizootics in host populations (St. Romain *et al.* 2013). Similarly, numerous prairie dog colonies in Montana, U.S. were sampled during periods of apparent plague quiescence. 63% of those colonies contained plague positive fleas. Within positive colonies, 23% of host burrows yielded positive flea samples (Hanson *et al.* 2007). Infected *O. hirsuta* and *O. tuberculata cynomuris* fleas were among those collected from burrows. These fleas are primarily found on prairie dogs, but have also been found on alternate host species (Harrison *et al.* 2003, McGee *et al.* 2006, Salkeld *et al.* 2007). These flea species do not readily form proventricular blockages though they may form a

blockage if they survive for long enough (Burroughs 1947). Several modeling studies indicate that blocked fleas are incapable of maintaining plague (Webb *et al.* 2006, Buhnerkempe *et al.* 2011, Richgels *et al.* 2016), however fleas exhibiting partial blockage may survive for longer periods and improve transmission rates. *O. hirsuta* and *O. tuberculata cynomuris* flea species are capable of early phase transmission (EPT) (Wilder *et al.* 2008a, Wilder *et al.* 2008b), however the period where EPT can efficiently occur only lasts for four days post-infection and requires feeding on a host with high bacteremia (Wilder *et al.* 2008b), which is incompatible with resistant alternate hosts.

An environmental hurdle for maintaining plague over inter-epizootic periods is temperature. *O. montana* fleas have been shown to demonstrate efficient transmission of *Y. pestis* when maintained at temperatures as low as 6°C (Williams *et al.* 2013) and it is thought *Y. pestis* is able to overwinter within the flea gut and potentially cause infection during the following transmission season (Williams *et al.* 2013). The potential for infectious fleas to overwinter and re-ignite transmission the next spring provides a plausible explanation for short inter-epizootic periods, but, fleas appear incapable of explaining multiple years of quiescence that intersperse re-emergent epizootics.

It has also been suggested that variation in virulence across *Y. pestis* strains may indicate the bacterium is capable of alternating its virulence profile to facilitate enzootic persistence (Rosqvist *et al.* 1988, Thomas *et al.* 1990). Evidence of phenotypic plasticity in virulence traits is demonstrated by a lack of expression of mammalian virulence factors below 26°C, which is characteristic of flea vector or telluric environments, and increased expression of virulence factors when temperatures increase to 37°C, characteristic of mammalian host environments (Lorange *et al.* 2005, Zhou & Yang 2009). Phenotypic plasticity may play a larger role when

bacteria enter new environments that require differing survival strategies (Straley & Perry 1995, Breneva *et al.* 2005, Sokurenko *et al.* 2006, Bearden & Brubaker 2010). With one exception, no evidence exists to indicate *Y. pestis* down-regulates its expression of virulence traits within hosts enabling persistent infections and enzootic transmission. Intra-host attenuation of *Y. pestis* was observed in hibernating ground squirrels and marmots. Host body temperatures can reach 5°C during hibernation (Ortmann & Heldmaier 2000, Kauffman *et al.* 2004) which inactivates multiple bacterial virulence factors (including CafI, Pla, PsaA, Yops, and Yscs) (Han *et al.* 2005). Additionally, *Y. pestis* strains lacking the Fraction 1 antigen can cause chronic infections in rats (Williams & Cavanaugh 1983) but no evidence exists that *Y. pestis* can down-regulate F1 capsular protein production at mammalian host temperatures. This may reduce pathogenicity and facilitate overwintering of the bacteria until the host returns to an active physiological state causing the bacteria to regain pathogenicity. This example offers a potential explanation for short-term persistence in some mammalian hosts.

In summary, plague maintenance via enzootic transmission appears plausible over inter-epizootic periods spanning one or two years, but models disagree on the possibility of sub-epizootic persistence over longer periods (Salkeld *et al.* 2010, Markman *et al.* Unpublished). Enzootic persistence is difficult to reconcile with longer inter-epizootic periods or instances where humans have contracted pneumonic plague by excavating plague corpses in Madagascar, suggesting potential for long-term survival of *Y. pestis* outside of the canonical disease system (Ayyadurai *et al.* 2008). Finally, we reiterate the importance of distinguishing between factors relevant to plague maintenance during inter-epizootic periods and factors relevant to the transmission of plague during epizootics. For example, specific levels of intra-colony

connectivity may either maintain plague at low levels or instead may be an important driver of transmission that follows plague re-emergence via another mechanism (Figure 2.2).

Hypothesis IV: Reservoir maintenance

Bacterial persistence in the absence of disease transmission is hypothesized to involve reservoirs. We define a reservoir as an environment enabling multi-year survival and possibly amplification of plague bacteria without requiring transmission to susceptible hosts or vectors. Under this mechanism, epizootics would initially re-emerge via spillover of pathogens from reservoirs directly into susceptible host populations. The reservoir hypothesis is compelling because it is concordant with: genetic evidence indicating local bacterial persistence, synchronous climate-driven epizootics, and addresses the scarcity of evidence found in support of other hypotheses. Hypotheses regarding the existence of a plague maintenance reservoir have circulated for at least 125 years, predominantly within the literature from Russia and the former Soviet Union (Yersin 1894, Pavlovsky 1964, Eisen & Gage 2009, Lowell *et al.* 2015, Salkeld *et al.* 2016). Relative to other hypotheses, there has been little investigation into the merits of the reservoir hypothesis for explaining recurrent plague epizootics. Thus far there has been no direct empirical evidence of plague persistence in reservoirs lasting long enough to account for inter-epizootic periods that span years (Pavlovsky 1964, Girard *et al.* 2004, Webb *et al.* 2006, Snäll *et al.* 2008, Eisen & Gage 2009, Lowell *et al.* 2015, Salkeld *et al.* 2016).

The existence of a plague reservoir is generally supported by genomic evidence, quantitative modeling, and empirical observations of the plague system. Genetic evidence includes the unusually high conservation of functional genes from related telluric bacteria, *Y. pseudotuberculosis* and *Y. enterocolitica*, which are unrelated to survival in the flea or mammalian host environments. This may suggest that *Y. pestis* experiences evolutionary

pressures from environments outside of the vector-host cycle (Drancourt *et al.* 2006, Easterday *et al.* 2012). *Y. pestis* exhibits phenotypic plasticity in response to changing environmental conditions and upregulates gene expression that is hypothesized to aid in extra-host and extra-vector survival (Straley & Perry 1995, Breneva *et al.* 2005, Zhou & Yang 2009, Bearden & Brubaker 2010). Further, the existence of a plague reservoir is suggested by the recent molecular detection of the same unique *Y. pestis* clone during sequential epizootics with no detection in host or vector populations during the inter-epizootic quiescence period, indicating local cryptic persistence (Girard *et al.* 2004, Lowell *et al.* 2015). Historic molecular research provides evidence of genetically identical *Y. pestis* bacteria found in the dental remains of victims who died in the 14th and 17th centuries during the second plague pandemic (Seifert *et al.* 2016). This suggests long-term and local persistence without significant accumulation of mutations which would be expected after 300 years of sporadic epizootic transmission with numerous bacterial population bottle-necks and amplification phases (Morelli *et al.* 2010, Cui *et al.* 2013, Seifert *et al.* 2016).

Several quantitative modeling studies have indicated the necessity of a short-term reservoir to drive epizootics (Webb *et al.* 2006) and an off-host long-term reservoir to maintain *Y. pestis* over inter-epizootic periods (Buhnerkempe *et al.* 2011, Markman *et al.* Unpublished). However, the majority of modeling studies focus on conditions necessary for epizootic emergence, but do not address mechanisms enabling bacterial persistence over the inter-epizootic period (Webb *et al.* 2006, Buhnerkempe *et al.* 2011, Richgels *et al.* 2016). To our knowledge only one modeling study has examined plague persistence over multi-year inter-epizootic periods (Markman *et al.* Unpublished) and it suggests a long-term reservoir is necessary to mirror

disease dynamics described by a 25-year natural dataset from a natural plague focus in CO, USA (Markman *et al.* Unpublished).

The persistence of *Y. pestis* in absence of apparent transmission among vector or mammalian host species has been demonstrated many times and has been attributed to numerous candidate reservoirs (Yersin 1894, Pollitzer 1954, Baltazard *et al.* 1963, Karimi 1963, Mollaret 1963, Mollaret 1965, Mollaret 1968, Mollaret 1969, Mollaret 1971, Bazanova *et al.* 1997, Gage & Kosoy 2005, Webb *et al.* 2006, Drancourt *et al.* 2006, Eisen *et al.* 2008, Ayyadurai *et al.* 2008). Observations of synchronous epizootics supports the existence of a direct environmental trigger (Snäll *et al.* 2008, Ben-Ari *et al.* 2011, Savage *et al.* 2011, Lowell *et al.* 2015) which may increase rates of spillover from reservoirs to susceptible host species. However, not all susceptible host populations experience plague epizootics following conducive climatic events, suggesting that either *Y. pestis* does not remain at low levels in all host populations (Eisen & Gage 2009) or that spillover from reservoirs is a necessary but insufficient condition to initiate an epizootic after multi-year inter-epizootic periods of apparent dormancy.

Given the complexity inherent to the plague system, various reservoir environments and persistence mechanisms have been proposed, namely: resistant non-transmitting mammals, survival within susceptible mammalian host tissues, host carcasses, flea vectors, soil, and telluric organisms such as nematodes or protozoan amoebae (Ayyadurai *et al.* 2008, Eisen *et al.* 2008, Eisen & Gage 2009, Lambrecht *et al.* 2013, Gengler *et al.* 2015, Lambrecht *et al.* 2015, Santos-Montañez *et al.* 2015, Markman *et al.* 2018). These hypothesized reservoirs may enable the cryptic persistence and re-emergence of *Y. pestis*, in regions where the pathogen was ostensibly absent (Figure 2.2). Below we evaluate the most compelling evidence supporting and opposing each candidate reservoir.

Mammals

Hypothesized mammalian reservoirs include individuals that are not actively transmitting plague to susceptible hosts during inter-epizootic periods. Plague-resistant mammals have been hypothesized to develop long-term subclinical bacteremia and therefore act as durable reservoirs of infection (Baltazard & Mofidi 1950). Later field observations and experiments by the same researchers led the authors to conclude that resistant species are not able to sustain plague (Baltazard *et al.* 1963). However, sub-clinical maintenance of *Y. pestis* has been demonstrated for at least 30 days in inguinal buboes of grasshopper mice, which are characterized as a moderately resistant host species (Thomas *et al.* 1988, Eisen & Gage 2009). Outside this single study describing bacterial sequestration in the tissue of three individual grasshopper mice (Thomas *et al.* 1988), little evidence suggests that vertebrate hosts exhibit long-term subclinical infections or exhibit prolonged courses of disease enabling increased opportunity for vector-borne transmission before death. Resistant hosts are unlikely to act as reservoirs of *Y. pestis* infection (Eisen & Gage 2009) primarily because of the rarity of successful onward transmission from resistant hosts and the progression of infected resistant hosts towards clearance of the infection over time periods that are too rapid to explain maintenance over multi-year inter-epizootic periods. Plague immunity is not commonly observed in North American prairie dogs as indicated by several species of prairie dog suffering nearly 100% mortality following infection (Lechleitner *et al.* 1968, Rayor 1985, Ubico *et al.* 1988, Cully *et al.* 1997, Pauli *et al.* 2006) and in a separate experiment, only ~3% developed significant V-antigen titers post-infection (Rocke *et al.* 2012). Several other rodent species have evolved increased levels of immunity, as found in Kazak gerbils (Davis *et al.* 2004). However, resistant hosts could only transmit via direct contact or ingestion because vector transmission requires bacterial titers of at least 10^6 per milliliter of

infected host blood (Burroughs 1947, Pollitzer 1954, Engelthaler *et al.* 2000, Lorange *et al.* 2005, Boegler *et al.* 2016). Transmission by direct contact and ingestion are generally not thought to play a role in the maintenance of *Y. pestis* in animal reservoirs (Cavanaugh 1972, Perry & Fetherston 1997) but the first reported case of human plague in Jordan in over 80 years was caused by consumption of infected camel meat (Arbaji *et al.* 2005). How this camel became infected and the importance of transmission via ingestion for explaining long-term plague maintenance remains uncertain.

It is clear that varying levels of resistance in host populations will impact the response to pathogen exposure during epizootics, however, the role resistant hosts play in maintaining plague over inter-epizootic periods is questionable. Despite the persistence of the resistant host theory, there has been little evidence to support the existence of resistant reservoir hosts (Gage & Kosoy 2005, Stapp *et al.* 2008, Eisen & Gage 2009, Abbot & Rocke 2012). This is compounded by under-reporting bias for instances where no infected or resistant hosts were found during inter-epizootic periods.

Infected host carcasses have been hypothesized to serve as plague reservoirs, but numerous observational and modeling studies have indicated that host carcasses are frequently scavenged or naturally decompose more rapidly than would be required to maintain plague over inter-epizootic periods (Webb *et al.* 2006, Godbey *et al.* 2006, Boone *et al.* 2009, Buhnerkempe *et al.* 2011, Markman *et al.* Unpublished). The duration of plausible plague maintenance may be increased if infected host carcasses are able to infect questing flea vectors. A cycle of host infection, death, partial decay, and re-infection of fleas may enable maintenance of plague for longer periods than carcasses or fleas could explain in isolation. This hypothetical maintenance

mechanism combines short-term reservoir capabilities with the enzootic dynamics discussed in Hypothesis III.

Fleas

The majority of flea vectors are short-lived, but there have been numerous accounts of infected fleas surviving for months or years after their initial infectious blood feed (, Golov & Ioff 1928, Kartman *et al.* 1962, Sharets *et al.* 1958, Bazanova & Maeviskii 1996, Tripp *et al.* 2009, Salkeld *et al.* 2010, St. Romaine *et al.* 2013, Graham *et al.* 2014). Fleas must remain infectious in the absence of transmission to adhere to our definition of a reservoir, whereas if transmission is ongoing, the fleas would be considered a link in the chain of enzootic transmission outlined in Hypothesis III. Infected prairie dog fleas have been collected from burrows in Colorado up to one year after an epizootic of plague wiped out the host colony (Kartman *et al.* 1966, Lechleitner *et al.* 1968). In many instances, it is exceedingly difficult for field studies to determine whether transmission is actively occurring throughout the inter-epizootic period. Despite these factors, one compelling field study collected infected fleas approximately 14 months after plugging the openings of marmot burrows in Kazakhstan (Sharets *et al.* 1958) and another detected infected fleas up to 13 months after an epizootic (St. Romaine *et al.* 2013).

Flea vectors exhibit differential responses to plague infection ranging from developing a complete biofilm-induced proventricular blockage to remaining entirely unblocked. The degree of blockage is hypothesized to contribute to a flea's reservoir potential (Gage & Kosoy 2005, Vetter *et al.* 2010). Complete blockage is uncommon among capable flea vectors despite its status the prototypical outcome of infection. Several species of infected fleas exhibiting partial blockages have survived for up to 220 days when held between 14-27C and another species

survived for 396 days when held between 0-15C (Burroughs 1947). Additional studies have indicated that temperature regulates the formation and stability of proventricular biofilms (Perry & Fetherston 1997, Gage & Kosoy 2005). Higher temperatures down-regulate expression of the *hmHFRS* operon responsible for biofilm formation resulting in fleas clearing the plague infection (Gage & Kosoy 2005, Williams *et al.* 2013). This may detract from the reservoir capacity of tropical flea species, while supporting reservoir potential for fleas at more temperate latitudes (Williams *et al.* 2013).

Despite evidence that fleas can act as infectious reservoirs for up to 14 months, this time period is not explanatory for longer inter-epizootic periods without incorporating other reservoirs or transmission dynamics (e.g. alternation between persistence in short-term reservoirs and enzootic transmission). It is likely that infected fleas would require maintenance feeds to survive for longer periods and subsequent feeding on uninfected hosts has been shown to clear plague biofilms from infected fleas (Eisen *et al.* 2007, Williams *et al.* 2013). Additional research has determined that *Y. pestis* is not transmitted transovarially, and artificially infected flea larvae often clear the bacteria within 24 hours (Perry & Fetherston 1997, Bitam *et al.* 2010). Quantitative transmission models indicate that fleas are insufficient to explain local, dormant persistence in plague foci for the median inter-epizootic period of six years observed in studies of black-tailed prairie dogs in Colorado (Webb *et al.* 2006, Buhnerkempe *et al.* 2011, Richgels *et al.* 2016, Markman *et al.* Unpublished). It is likely fleas serve as infectious reservoirs for shorter inter-epizootic periods.

Soil

Survival of *Y. pestis* in particular soil types and conditions has been suggested as a possible mechanism explaining maintenance over inter-epizootic periods and as a factor that

temporally and spatially defines plague foci (Pollitzer 1954, Baltazard *et al.* 1963, Bazanova *et al.* 1997, Gage & Kosoy 2005, Drancourt *et al.* 2006, Webb *et al.* 2006, Eisen *et al.* 2008, Eisen & Gage 2009, Savage *et al.*, 2012). In this scenario, soil serves as a maintenance reservoir and facilitates spillover to burrowing rodents via direct contact. Flea vectors may subsequently amplify and transmit the pathogen to other susceptible hosts and initiate an epizootic. Reported instances where humans have contracted pneumonic plague by excavating plague corpses in Madagascar suggest survival of *Y. pestis* in the soil (Ayyadurai *et al.* 2008). Despite the inability of *Y. pestis* to persist for greater than 5 days on glass, steel, polyethylene, and paper surfaces (Rose *et al.* 2003), substantial empirical evidence indicates that infectious *Y. pestis* persists in soil environments without hosts or vectors present. Numerous studies have demonstrated the survival of *Y. pestis* in soil under diverse natural and experimental conditions including low temperatures, protection from UV light, and various nutrient concentrations (Baltazard *et al.* 1963, Karimi 1963, Mollaret 1963, Mollaret 1965, Breneva *et al.* 2005, Drancourt *et al.* 2006, Eisen *et al.* 2008). Notably, the persistence and transmission of *Y. pestis* may differ between natural and experimental conditions.

In 1894, viable *Y. pestis* was isolated from soil in a home where the inhabitants had previously died of plague (Yersin 1894). Other experiments showed *Y. pestis* survived for at least 16 months in sterilized soil and easily infected naïve rodents living in the soil (Mollaret 1963). More recently, *Y. pestis* was shown to survive in sterile soil for at least 40 weeks (Ayyadurai *et al.* 2008). Separate experiments demonstrated that rodent hosts, including mice and guinea pigs, could contract plague from *Y. pestis* in the soil (Drancourt *et al.* 2006, Boegler *et al.* 2012). One study isolated viable *Y. pestis* from a natural soil environment 24 days after a host died of plague infection (Eisen *et al.* 2008). Another study conducted under natural conditions isolated *Y. pestis*

from soil in a rodent burrow several years after any animal had actually lived there (Karimi 1963), though it is difficult to be certain there were no transient animal inhabitants that could have reintroduced the bacteria. Further, *Y. pestis* was demonstrated to persist in the nest substrate of the long-tailed suslik (Bazanov *et al.* 1997). Isolation of *Y. pestis* from soil with high salt content in Algeria (Malek *et al.* 2017) indicates that *Y. pestis* is able to tolerate a wider range of environmental conditions than previously thought.

It has been hypothesized that *Y. pestis* can alter its gene expression to survive in telluric environments (Baltazard 1964, Levi 1997). Further experiments have shown that low temperature conditions enabled *Y. pestis* to enter a viable but non-culturable state after 21 days in a tap water microcosm (Pawlowski *et al.* 2011). Some researchers believe selection for vector-borne transmission has resulted in the loss of many genes enabling long-term persistence outside of the host or vector (Achtman *et al.* 1999, Gage & Kosoy 2005). Genetic evidence suggests *Y. pestis* shares numerous genes and pseudogenes with other telluric organisms that were likely acquired by ancestral strains of *Y. pseudotuberculosis*. Evolutionary history in telluric environments may provide support for maintenance in soil or some other telluric reservoir (Easterday *et al.* 2012). Evidence also indicates *pst* and *pim* genes are likely to play a role in environmental persistence (Easterday *et al.* 2012). Many pseudogenes in *Y. pestis* have functioning homologs in related *Y. pseudotuberculosis* and *Y. enterocolitica* that code for pathogenesis and general physiology for the telluric and enteric lifestyle (Straley & Perry 1995, Chain *et al.* 2004, Zhou & Yang 2009, Connor *et al.* 2015). Several studies have concluded that neutral SNPs become rapidly fixed during epizootics (Cui *et al.* 2013). However an analysis of *Y. pestis* isolates from around the world indicated that rates of SNP accumulation were lower than what would be expected if epizootics were continually occurring within plague foci (Cui *et al.*

2013). This suggests *Y. pestis* persists locally in the absence of continuous epizootics. Despite reductive evolution in *Y. pestis*, indicated by ~13% loss of *Y. pseudotuberculosis* genes, several genes related to survival in telluric environments are maintained without accumulating deleterious SNPs (Chain *et al.* 2004, Zhou & Yang 2009, Cui *et al.* 2013). This is consistent with positive selection to maintain function in those genes and suggests a telluric phase may constitute a portion of modern *Y. pestis* life history.

Quantitative modeling has suggested a relationship between epizootic likelihood and past host abundance (Davis *et al.* 2004) and that colony size is a stronger determinant of plague persistence than inter-colony connectivity (George *et al.* 2013). These findings may be explained by mass host mortality and carcass decay generating large influxes of infectious material into soil-borne reservoir populations, thereby creating a highly localized infectious reservoir pool. As host recolonization occurs, susceptible individuals re-excavate vacant burrow systems and could potentially become exposed to pockets of infectious soil or soil-borne reservoirs. Irrespective of this theoretical infection cycle, it is empirically evident that *Y. pestis* can persist in diverse soil environments for long periods of time. Epidemiological and modeling studies have identified a wealth of environmental factors correlated with the survival and persistence of *Y. pestis*, which may help to more rigorously define global plague foci (Eisen *et al.* 2008, Snäll *et al.* 2008, Bearden & Brubaker 2010, Savage *et al.* 2011, Malek *et al.* 2017). Notably, the numerous lines of evidence supporting soil as a suitable reservoir environment are not mutually exclusive from other proposed telluric reservoirs including microorganisms living in the soil.

Protozoan amoebae

The ability of *Y. pestis* to survive intracellularly within soil-borne protozoan reservoirs is receiving increasing support (Nikul'shin *et al.* 1992, Domaradsky 1999, Pushkareva 2003,

Steinert 2011, Anacarso *et al.* 2012, Lambrecht *et al.* 2013, Lambrecht *et al.* 2015, Santos-Montañez *et al.* 2015, Benavides-Montañó & Vadyvaloo 2017, Markman *et al.* 2018, Markman *et al.* Unpublished). Amoebae in particular have been identified as competent reservoirs in other disease systems and over 200 animal and plant pathogens are reportedly able to exploit the intracellular amoeba environment for survival or replication (Barker & Brown 1994, Brown & Barker 1999, Schuster & Visvesvara 2004, Greub & Raoult 2004, Thomas *et al.* 2008, Salah *et al.* 2009, Thomas *et al.* 2010, Siddiqui & Khan 2012b, Lambrecht *et al.* 2015, Markman *et al.* 2018). Only recently has *Y. pestis* been confirmed as an amoeba-resistant bacteria (Benavides-Montañó & Vadyvaloo 2017, Markman *et al.* 2018) thereby renewing interest in the role amoebae play in plague's eco-epidemiology.

Many amoeboid organisms are protozoans in the phylum *Amoebozoa* and are predominantly unicellular and free-living. The majority of amoebae cycle between two life stages: a mobile, phagocytic trophozoite stage and a dormant cyst or spore stage. Amoebae are the dominant bacterial consumers in terrestrial environments and are responsible for up to 60% of the total reduction in bacterial population (Sinclair *et al.* 1981, Siddiqui and Khan 2012a), but numerous bacteria have evolved the ability to avoid digestion following phagocytosis by amoebae and are termed resistant. During suboptimal conditions, defined by a lack of bacterial food resources or lack of sufficient moisture, many trophozoites will encyst within a secreted rigid double outer-wall. Amoebae cysts are able to survive in extreme conditions for greater than 5 years, despite the absence of nutrient supplementation, while maintaining the pathogenicity of intracellular bacteria. (Khunkitti *et al.* 1996, Khan 2006). When conditions improve (e.g. increased moisture following precipitation events), cysts will revert into a trophozoite state, begin rapid proliferation, and eject vesicles containing waste products including resistant bacteria

(Lambrecht *et al.* 2013, Lambrecht *et al.* 2015). It is believed that amoebae spend most of their life in the encysted state (Esteban *et al.* 2006).

Several lines of evidence support amoebae as natural plague reservoirs, including the molecular similarity between amoebae and macrophages, genomic similarities between *Y. pestis* and related *Yersinia spp.* that persist in amoebae, direct experimental evidence of short-term *Y. pestis* persistence in amoebae, quantitative modeling, and numerous eco-epidemiological correlates between amoebae and plague dynamics.

Y. pestis is a facultative intracellular pathogen that exploits mammalian macrophages in order to proliferate within mammalian hosts. Amoebae maintain a wide array of functional homology with macrophages, specifically their vesicular trafficking, phagocytic machinery, and permissiveness to certain resistant intracellular pathogens (Siddiqui & Khan 2012b). In macrophages and amoebae, the primary mechanism for degradation and digestion of intracellular pathogens is the acidification of phagolysosomes, or less commonly, a specialized autophagic process called xenophagy which targets and acidifies intracellular pathogens in the host cell's cytosol (Pujol *et al.* 2009, Ke *et al.* 2013). Amoeba-resistant bacteria are often able to create an intracellular replicative niche suitable for bio-amplification outside of traditional hosts (Greub & Raoult 2004). Many previously identified amoeba-resistant bacteria are also competent intracellular pathogens in macrophages resulting from similar cellular machinery and bactericidal processes found in both phagocytic cell types (Greub & Raoult 2004, Thomas *et al.* 2010, Salah *et al.* 2010). It is thought that the same selective pressures that yield amoebae resistance may also confer resistance to phagocytic macrophages. For this reason amoebae are occasionally portrayed as evolutionary "training grounds" for microbes which later develop into human pathogens (Greub & Raoult 2004, Cosson & Soldati 2008, Adiba *et al.* 2010, Steinert 2011, Wheat *et al.*

2015). *Y. pestis* is able to recruit and exploit several host molecular factors, including Rab1B GTPases, to escape the phagolysosomal pathway in macrophages (Connor *et al.* 2015). Several amoebae species have orthologous Rab1B sequences, potentially indicating that *Y. pestis* could also exploit the intracellular amoebae environment (Connor *et al.* 2015, Markman *et al.* 2018). Several other genetic factors of interest include the *Y. pestis* *hms* locus which aids in iron acquisition, increases uptake of bacteria into eukaryotic cells, and helps to deactivate bactericidal factors released by phagocytic cells (Hinnebusch *et al.* 1996, Darby *et al.* 2002). Additionally, *Y. pestis* *Ail* protein helps to initiate an anti-phagocytic response and cause immune suppression (Felek & Krukonis, 2009). The *Ail* protein is present in both *Y. pestis* and *Y. pseudotuberculosis*, and could play a similar role in facilitating intra-amoeba survival (Connor *et al.* 2015).

Beyond molecular similarities between amoebae and macrophages, abundant evidence indicates closely related *Yersinia* species are able to persist within amoebae. *Y. pseudotuberculosis* and *Y. enterocolitica* are able to escape the amoeba's phagolysosome, replicate intracellularly, and survive amoeba encystment cycles, suggesting the possibility for *Y. pestis* to exhibit similar behavior (Pujol & Bliska 2003, Moreau *et al.* 2010, Lambrecht *et al.* 2015, Santos-Montañez *et al.* 2015, Connor *et al.* 2015, Benavides-Montañón *et al.* 2017). It has also been shown that related *Yersinia* are adapted for survival in nutrient deficient conditions (Somova *et al.* 2006) and may enter viable, but not culturable states similar to *Y. pestis* (Pawłowski *et al.* 2011). Reviews of the evolutionary history between *Y. pestis* and related *Yersinia* (Chain *et al.* 2004, Zhou & Yang 2009, Connor *et al.* 2015) indicate that *Y. pestis* has undergone significant reductive evolution as suggested by the inactivation of ~13% of functional genes from *Y. pseudotuberculosis* (Chain *et al.* 2004, Zhou & Yang 2009, Cui *et al.* 2013). Some of the changes in gene structure and expression in *Y. pestis* are attributed to positive selection for

the flea-borne and macrophage-infecting lifestyle, with others attributed to neutral mutation accumulation. For example, inactivation of several *Y. pseudotuberculosis* genes were proposed to increase vector transmission in *Y. pestis* (Chain *et al.* 2004), whereas loss of several other genes appeared to have no impact on *Y. pestis* survival. Evidence of genome compaction in *Y. pestis* has been used to support contradicting hypotheses regarding the ability of *Y. pestis* to survive in environments outside of mammalian hosts and vectors (Chain *et al.* 2004). Interestingly, genome compaction is also observed in the evolution of other pathogens such as *Salmonella typhi* (Parkhill *et al.* 2001) and *Mycobacterium leprae* (Cole *et al.* 2001), both of which exhibit long-term survival and replication in amoebae (Gaze *et al.* 2003, Wheat *et al.* 2015).

Perhaps the strongest support for amoebae reservoirs is found in recent experimental evidence indicating that five strains of *Y. pestis*, encompassing biovars Antiqua, Mediaevalis, and Orientalis, survived intracellularly within the pervasive *Acanthamoebae castellanii* amoebae for at least five days (Benavides-Montaña *et al.* 2017). It was also shown that *Y. pestis* and five clinically relevant and intensively studied amoebae species co-occur in soils of prairie dog burrows undergoing plague epizootics (Markman *et al.* 2018). Following co-culture of the model amoebae species and *Y. pestis*, it was determined that *Y. pestis* bacterium could survive and replicate inside *Dictyostelium discoideum* amoebae for >48 hours post infection (Markman *et al.* 2018). These timespans indicate amoebae resistance given that susceptible bacteria, *Escherichia coli*, were successfully digested by amoebae in less than one hour after infection (Markman *et al.* 2018). The durations of intracellular persistence that have been experimentally demonstrated thus far are insufficient to assume persistence over inter-epizootic periods, but neither the upper limit on intra-amoeba survival nor the ability of *Y. pestis* to survive amoeba encystment have been tested. These represent compelling next steps in the pursuit of evaluating amoeba reservoir

potential. At least one study has attempted to detect *Y. pestis* in amoebae isolated from plague-positive prairie dog burrows, but no evidence of natural intracellular bacteria was observed because of limitations imposed by current isolation methodologies and few sampling attempts (Markman *et al.* 2018).

Y. pestis has acquired genes via horizontal gene transfer from other telluric *Enterobacteriaceae* species at some point in its evolutionary history (Parkhill *et al.* 2001, Wren 2003). Acquisition of these genes may have occurred ancestrally prior to divergence from *Y. pseudotuberculosis*, or may indicate more recent interaction with telluric bacteria in host, vector, or reservoir environments. For example, *Y. pestis* experimentally acquired antibiotic resistance genes following coinfection of fleas with *Y. pestis* and antibiotic resistant *E. coli* (Hinnebusch *et al.* 2002). Analysis across global *Y. pestis* isolates indicated evolutionary divergence, measured by rates of SNP accumulation, were lower than expected if epizootics were continually occurring (Cui *et al.* 2013). The heterogeneity in mutation rates that exists among individual isolates is likely driven by demographic factors and may indicate which persistence mechanisms predominate in individual foci (Zhou & Yang 2009, Cui *et al.* 2013). Additional studies have proposed molecular mechanisms enabling pathogens to switch between reservoir and amplification environments, but this has not been explicitly evaluated in the plague system (Sokurenko *et al.* 2006). Bacterial persistence in telluric reservoirs may explain the hyper-localized and slowly evolving genetic structure observed among global plague foci (Girard *et al.* 2004, Seifert *et al.* 2013, Cui *et al.* 2013, Lowell *et al.* 2015).

A stochastic epidemiological model evaluated the capacity for soil-borne microorganisms to serve as plague reservoirs and provided theoretical support for maintenance in amoebae (Markman *et al.* Unpublished). The model's median duration of inter-epizootic period, 6 years,

matched natural values derived from a 25 year dataset describing prairie dog population responses to plague in one Colorado, US foci (Savage *et al.* 2011, George *et al.* 2013, Markman *et al.* Unpublished). Additional eco-epidemiological correlates between amoebae and plague dynamics include direct dependence on seasonal temperature, soil moisture, and nutrient availability (Greub & Raoult 2004, Khan 2006, Snäll *et al.* 2008, Savage *et al.* 2011). Amoeba excystment and subsequent ejection of vesicles containing resistant bacteria occurs in response to precipitation events and may explain the synchronicity of plague epizootics that have a lagged association with rainfall (Khan *et al.* 2006, Snäll *et al.* 2008, Savage *et al.* 2011, Lambrecht *et al.* 2013, Lambrecht *et al.* 2015). Lagged association is to be expected given the time required for burrowing rodent hosts to become infected and initiate a novel transmission chain, perhaps involving mechanisms previously discussed, before leading to an epizootic. In summary, numerous lines of evidence support amoebae as reservoirs of *Y. pestis* and encourage additional research into the mechanisms enabling *Y. pestis* and related *Yersinia*e to survive and replicate in amoebae and macrophages. Future research on the ability of *Y. pestis* to survive periods of dormancy in encysted amoeba species may explain the long-term cryptic persistence of plague in between observable epizootics.

Other telluric microorganisms

Alternative telluric microorganisms have been suggested as candidate reservoirs of *Y. pestis*, including other protozoans, nematodes, and mycorrhizae. Several species of nematodes are capable of parasitizing insects because of a symbiotic association with bacteria inside the nematode's digestive tract (Gengler *et al.* 2015). Experiments have demonstrated that *Y. pseudotuberculosis* is able to co-opt this symbiotic relationship to establish a protected replicative niche inside the nematode while also increasing dissemination (Gengler *et al.* 2015).

Genomic analysis indicates *Y. pestis* obtained genes from bacterial symbionts found in telluric nematodes that code for homologous insecticidal toxin complexes (Parkhill *et al.* 2001, Wren 2003, Drancourt *et al.* 2006). Experimental infection of the nematode *C. elegans* with *Y. pestis* and *Y. pseudotuberculosis* resulted in successful biofilm formation in the nematode gut (Darby *et al.* 2002, Joshua *et al.* 2003), but the biofilm was only transiently persistent (Darby *et al.* 2002).

Gregarines, a group of worm-like Apicomplexan protozoans, routinely inhabit the gut of flea vectors and are thought to be similar to protozoans in their permissiveness to *Y. pestis* (Iv & IuM 2012). Gregarines are hypothesized to serve as reservoirs capable of re-infecting flea vectors without involving mammalian hosts (Iv & IuM 2012). *Tetrahymena*, a genus of free-living protozoan ciliates were experimentally infected with several virulent and avirulent strains of *Y. pestis*. Virulent *Y. pestis* strains persisted intracellularly for 10 months at 4-8C and for 3.5 months at ~22C (Breneva & Maramovich 2008). The duration of intracellular survival was positively correlated with *Y. pestis* virulence, with the avirulent strains of *Y. pestis* being quickly eliminated. These results suggest a virulence-associated mechanism for phagocytic avoidance and intracellular survival that is generalized across diverse protozoans and perhaps mammalian macrophages. However other research indicates traditional *Y. pestis* virulence determinants are not required for bacteria to proliferate in rodent macrophages (Straley & Harmon 1984). Finally, one experiment demonstrated the ability of *Y. pestis* to penetrate the roots of *Impatiens walleriana* and disseminate into the stem of the plant (Rivkus & Bochkarev 2000). Some mycorrhizae associated with plant roots assist with iron acquisition and it is hypothesized that *Y. pestis* may form extracellular biofilms around the plant roots because of similar iron affinity (Rivkus & Bochkarev 2000).

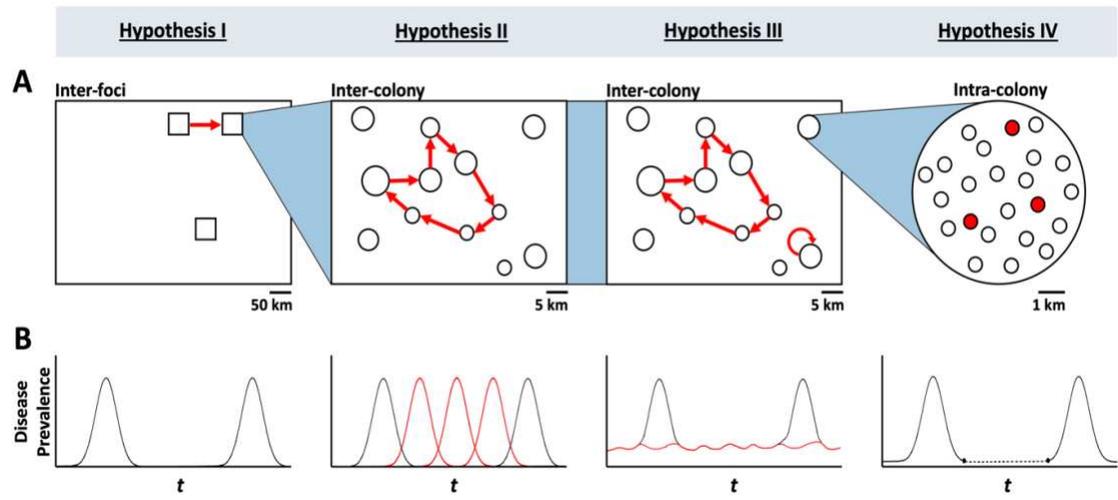
In summary the reservoir hypothesis offers a compelling explanation for epidemiological phenomena observed in the plague system, specifically over periods of inter-epizootic quiescence. Supporting lines of evidence include: intra-colony persistence of *Y. pestis*, little indication of continuous transmission among sylvatic hosts or vectors during inter-epizootic periods, similarities between amoebae and macrophages, the ability of related *Yersinia* to persist in amoebae, experimental persistence of *Y. pestis* in amoebae, empirical modeling, synchronous climate-driven epizootic emergence, and frequent amoeba-associations within other cryptically persistent disease systems. However, the reservoir hypothesis lacks direct evidence of long-term *Y. pestis* persistence *in situ*. Future research on the ability of *Y. pestis* to survive periods of dormancy in encysted amoeba species may explain the long-term cryptic persistence of plague in between observable epizootics. Combining such experiments with field surveys for naturally infected amoebae would confirm the ability of *Y. pestis* to persist in long-term reservoir environments prior to subsequent epizootic re-emergence.

Conclusion

Given the observed heterogeneity across global plague foci, it is unlikely a single mechanism can explain observed inter-epizootic plague dynamics. The hypotheses reviewed here are not mutually exclusive and likely represent dynamics that (co-)occur in the plague system with transient and shifting predominance. Given the accumulated evidence, we suggest that reservoirs may serve as a basal persistence mechanism across most plague foci, which can give rise to other maintenance and transmission mechanisms under specific conditions. We expect the active maintenance mechanisms within a particular focus to vary in accordance with the environmental conditions present in the foci. For example, it is probable that plague foci characterized by short inter-epizootic periods exhibit vastly different maintenance dynamics as

compared to foci exhibiting multi-decadal inter-epizootic periods. The former scenario is more amenable to persistence via extinction-recolonization dynamics within metapopulations or enzootic transmission, whereas the latter scenario necessarily relies on persistence in reservoirs or discrete long-range reintroduction events. We conclude that long-term maintenance of *Y. pestis* is likely driven by reservoir dynamics, whereas subsequent epizootic re-emergence is likely driven by spillover into host and vector populations leading to amplification and transmission via other mechanisms (e.g. extinction-recolonization dynamics in host metapopulations). Ergo, none of the reviewed classical hypotheses are able to explain the cryptic persistence and re-emergence of plague in isolation. This theoretical framework as well as the compiled genetic, experimental, and observational evidence may be used alongside observations from individual plague foci to identify which maintenance and transmission mechanisms are active within individual foci. This will enable development of more effective disease forecasting and prevention strategies without misappropriating increasingly limited resources towards unlikely causes of re-emergence in highly lethal disease systems.

Figures



Inter-epizootic dynamics

Maintenance mechanism	not maintained	extinction-recolonization in metapopulation	enzootic transmission	persistence in reservoir
Minimum area for maintenance	inter-foci	inter-colony	Inter-colony or intra-colony	intra-colony
Hosts/vector transmission frequency	none	continuous	continuous	None

Figure 2.1: Putative explanations for recurrent plague epizootics based on the transmission dynamics required to sustain plague across varying spatial scales and quiescent periods. A) Diagrams depict transmission of plague (red arrows) over inter-epizootic periods at different spatial scales. Red circles indicate pathogen maintenance without transmission. B) Graphs depict disease prevalence over time relative to the spatial scales in 1A. Red-lines indicate disease prevalence in adjacent colonies within the same metapopulation. Table) Description of key inter-epizootic dynamics across four maintenance hypotheses.

Hypothesis I) Long-range reintroduction: Following an epizootic, *Y. pestis* goes extinct (e.g. all hosts die) and is truly absent from the host colony and surrounding focus during the inter-epizootic period. Long-range (e.g. >50km) reintroduction from another plague focus initiates a subsequent epizootic. **Hypothesis II) Metapopulation dynamics:** Following an epizootic, *Y. pestis* goes extinct in an individual host colony, but is maintained within the larger metapopulation of susceptible hosts via extinction-recolonization dynamics that drive inter-colony transmission and enable recolonization of original host colony prior to reintroduction of plague. **Hypothesis III) Enzootic transmission:** Following an epizootic, *Y. pestis* is maintained via continuous transmission among alternate (resistant) mammalian hosts and vectors at sub-epizootic levels. **Hypothesis IV) Reservoir maintenance:** Following

an epizootic, *Y. pestis* persists within a the area of a host colony without being transmitted among host/vector populations during inter-epizootic periods (e.g. maintenance in reservoir prior to spillover into host populations).

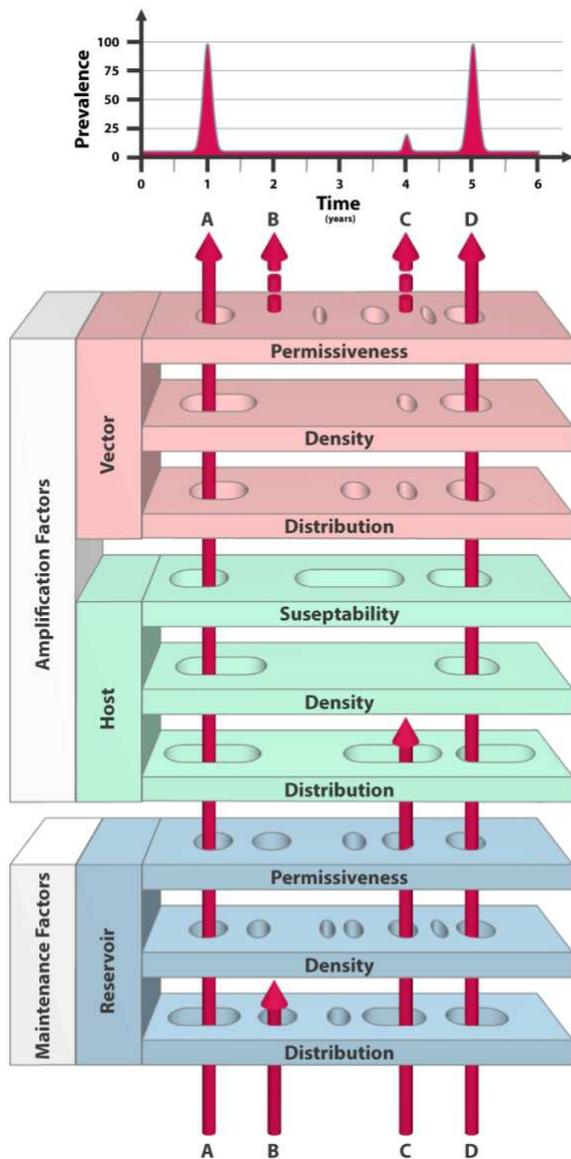


Figure 2.2: Conceptual diagram of pathogen maintenance and epizootic emergence under Hypothesis IV. Red curves in graph indicate disease prevalence over six years to encompass epizootic and inter-epizootic periods. Solid horizontal layers represent barriers to pathogen maintenance or amplification and gaps in the layers represent conditions that are conducive to pathogen maintenance or amplification. Layers are segmented into maintenance and amplification factors. Maintenance factors consist of reservoir (blue) traits that influence pathogen persistence, including reservoir spatial distribution, density, and permissiveness to the pathogen. Amplification factors consist of host (green) and vector (pink) traits including distribution, density, and either susceptibility or permissiveness. This diagram depicts reservoirs as a basal maintenance mechanism from which hosts and vectors may amplify the pathogen above basal levels depending on eco-environmental conditions. When numerous suitable conditions (gaps) align, pathogens (red arrows) may

permeate all layers resulting in epizootic emergence (red arrows A and D). Under conditions that limit amplification, perhaps from insufficient host density, it may be possible to observe sub-epizootic levels of disease (arrow C). Therefore initial spillover from reservoirs into host populations may be a necessary but insufficient condition to initiate an epizootic. If spillover events are frequently permitted and pathogen amplification is frequently prohibited (e.g. highly resistant host population) pseudo-enzootic dynamics may emerge where the pathogen is continuously at low-levels but not maintained via enzootic transmission (Hypothesis III).

Table 2.1: Definitions

Primary host, amplifying host	Mammalian hosts that generally exhibit high mortality following infection often because of low resistance (e.g. 1% resistance in US black-tailed prairie dogs). We will use the term primary host.
Alternate host, secondary host, maintenance host	Mammalian hosts that generally exhibit low or intermediate mortality following infection often because of increased resistance (e.g. 50% resistance in Kazakh great gerbils). We will use the term alternate host.
Metapopulation	Metapopulations are an assemblage of spatially distinct local populations, each with independent dynamics, that are coupled by some degree of migration or gene flow (Hanski & Gaggiotti 2004). Host metapopulation structure may help buffer against complete host extinction through source-sink migration dynamics that enable recolonization of extinct sub-populations (Boots <i>et al.</i> 2004).
Plague focus	Geographic regions where plague is found are often differentiated into discrete foci (plural of focus), defined by the spatial extent that a <i>Y. pestis</i> clone persists and is transmitted among a specific group of mammalian hosts, vectors, and possibly reservoirs (Maher <i>et al.</i> 2010, Smith <i>et al.</i> 2010, Giles <i>et al.</i> 2011, Ben-Ari <i>et al.</i> 2012, Lowell <i>et al.</i> 2015). This definition of foci is useful for understanding the extent of pathogen dispersal and admixture with other clones, whereas more traditional definitions of plague foci are more ecologically based and do not distinguish between disease caused by separate bacterial clones in the same geographical region. Plague foci are likely to geographically correlate with the spatial extent of host metapopulations.
Epizootic dynamics	Periods of sporadic and widespread transmission of plague among mammalian hosts with low resistance causing high mortality. Both primary and alternate hosts may be involved in transmission during epizootics. Black-tailed prairie dogs and great gerbils are model epizootic plague hosts that exhibit classical metapopulation structure (Hoogland 1995 Davis <i>et al.</i> 2007).
Inter-epizootic dynamics	Periods of apparent plague quiescence that intersperse epizootics. Apparent quiescence does not preclude the possibility of maintenance or transmission occurring below the threshold of detection or the threshold necessary to initiate an epizootic.
Enzootic transmission	Continuous low-level transmission among mammalian hosts that exhibit low-intermediate mortality, often resulting from high resistance. Enzootic hypotheses often involve a suite of alternate hosts and vectors that are responsible for perpetually maintaining plague at levels below the threshold that would initiate an epizootic.
Reservoir	We define a reservoir as an environment enabling the long-term (e.g. greater than one year) survival and possibly amplification of plague bacteria that does not involve active transmission among hosts or vectors. Candidate reservoirs include non-transmitting resistant hosts, soil, or telluric microorganisms (e.g. amoebae). Re-emergent epizootics would theoretically be caused by pathogen spillover directly from reservoirs into susceptible host populations.

CHAPTER 3: *Yersinia pestis* SURVIVAL AND REPLICATION IN POTENTIAL AMOEBIA RESERVOIR¹

Introduction

Plague ecology is characterized by sporadic epizootics, then periods of dormancy. Building evidence suggests environmentally ubiquitous amoebae act as feral macrophages and hosts to many intracellular pathogens. We conducted environmental genetic surveys and laboratory co-culture infection experiments to assess whether plague bacteria were resistant to digestion by 5 environmental amoeba species. First, we demonstrated that *Yersinia pestis* is resistant or transiently resistant to various amoeba species. Second, we showed that *Y. pestis* survives and replicates intracellularly within *Dictyostelium discoideum* amoebae for >48 hours post infection, whereas control bacteria were destroyed in <1 hour. Finally, we found that *Y. pestis* resides within amoeba structures synonymous with those found in infected human macrophages, for which *Y. pestis* is a competent pathogen. Evidence supporting amoebae as potential plague reservoirs stresses the importance of recognizing pathogen-harboring amoebae as threats to public health, agriculture, conservation, and biodefense.

The etiologic agent of plague, *Yersinia pestis*, is a gram- negative coccobacillus and a facultative intracellular pathogen. *Y. pestis* exhibited the highest overall mortality rate of any infectious disease from its earliest recorded emergence through 1941 (Perry & Fetherston 1997). During 2010–2015, a mean of 650 cases were reported globally each year, with a case fatality rate of 23%–41% (depending on manifestation as bubonic, pneumonic, or septicemic plague),

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rising to 66%–100% when adequate medical care was not promptly received (Bertherat 2016). *Y. pestis* primarily infects small ground-dwelling mammals, specifically of the taxonomic order Rodentia, but maintains high spillover potential to other vertebrates, including humans, caused by its high virulence and flea-borne transmission. Epizootic plague is typically vectored by multiple flea species and is transmitted within and between meta-populations of hosts by flea bites (Figure 3.1).

Plague ecology is characterized by sporadic epizootics, followed by 2–5-year cryptic dormancy periods (Girard *et al.* 2004, Webb *et al.* 2006, Snäll *et al.* 2008, Eisen & Gage 2009, Gibbons *et al.* 2012, Lowell *et al.* 2015, Salkeld *et al.* 2016). Despite much information on epizootic transmission mechanisms, little is known about the origin of re-emergent plague cases in wild animal populations (Figure 3.1). Plague among wild animals commonly re-emerges in plague foci after multiple years of inactivity, despite ongoing bio-surveillance and attempts at detection during interepizootic periods. The existence of environmental plague reservoirs has been theorized for >80 years (Pavlovsky 1966, Girard *et al.* 2004, Gage & Kosoy 2005, Webb *et al.* 2006, Ayyadurai *et al.* 2008, Snäll *et al.* 2008, Eisen & Gage 2009, Gibbons *et al.* 2012, Lowell *et al.* 2015, Salkeld *et al.* 2016, Benavides-Montaña & Vadyvaloo 2017). Various avenues of recent research suggest that soil-dwelling amoebae may be competent environmental reservoirs of *Y. pestis*. Amoebae are a taxonomically diverse group of phagocytic organisms residing in every major lineage of eukaryotes. Amoebae are pervasive in soil and water environments and are recognized for their ability to harbor pathogens that drastically affect ecologic communities (Greub & Raoult 2004, Hilbi *et al.* 2007, Bichai *et al.* 2008, Salah *et al.* 2009, Thomas *et al.* 2010, Wheat *et al.* 2014). Free-living amoebae cycle between 2 distinct life-

states: trophozoites, an active, mobile, feeding state; and cysts or spores, a robust dormant state induced in part by adverse environmental conditions.

Ameba reservoir potential for *Y. pestis* is indicated by 4 major factors: the ability of related *Y. enterocolitica* and *Y. pseudotuberculosis* bacteria to persist in protozoan amebae (Pujol & Bliska 2003, Lambrecht *et al.* 2015, Santos-Montañez *et al.* 2015); correlative data indicating plague epizootics temporally follow periods of increased precipitation known to reanimate ameba cysts (Collinge *et al.* 2005, Snäll *et al.* 2008, Savage *et al.* 2011); the demonstrated ability of *Y. pestis* to express various proteins enabling escape of the phagolysosome in a diverse array of phagocytic cells including human macrophages (Easterday *et al.* 2012, Ke *et al.* 2013, Connor *et al.* 2015); and prior associations between *Y. pestis* and the soil amebae, *Vermamoeba* (formerly *Hartmanella*) *rhysodes* and *Acanthamoeba castellanii*, that demonstrate intracellular persistence up to 5 days (Nikul'shin *et al.* 1992, Pushkareva 2003, Benavides-Montañó & Vadyvaloo 2017). Amebae display a high degree of functional homology with mammalian macrophages, leading to the description of amebae as feral macrophages. The ameba reservoir hypothesis is compelling for many pathogens with unexplained sporadic occurrence and cryptic dormancy periods as supported by a growing catalog (>225) of intra- cellular pathogens capable of surviving and/or replicating within amebae under diverse conditions (Barker & Brown 1994, Greub & Raoult 2004, Salah *et al.* 2009, Thomas *et al.* 2010).

We tested the hypothesis that 5 species of environmentally ubiquitous amebae demonstrate reservoir potential for the maintenance of *Y. pestis*. We implemented field and laboratory investigations to assess environmental co- occurrence of study ameba species with plague epizootics; experimental infection prevalence in amebae; experimental infection intensity; intra-ameba bacterial location; bacterial viability post-phagocytosis; and bacterial replication

inside trophozoite amebae. We discuss the potential for *D. discoideum* ameba to act as interepizootic reservoirs, the functional homology between phagocytic amebae and mammalian macrophages, and the ability of ameba to exert selective pressure on the evolutionary trajectory of pathogen virulence and transmission mode. Further, we stress the importance of recognizing pathogen-harboring amebae as potential threats to global health, agriculture, conservation, and biodefense.

Methods

By using field experiments, we molecularly assessed the co-occurrence of amebae and *Y. pestis* in prairie dog burrows in the Pawnee National Grassland of northeastern Colorado, USA. This grassland is an established plague foci that has exhibited recurrent plague epizootics since \approx 1940 (Link 1955). We used molecular analyses of soil and amebae cultured from the soil to identify candidate ameba species that may act as reservoirs for plague persistence.

Plague-endemic soil isolates

We selected 24 prairie dog burrows from 8 prairie dog colonies, which can contain hundreds of animals, on the basis of suspected plague presence indicated by sustained decreases in population size during a 3-week observation period in August 2016 (Appendix 1, Figure S3.1). We selected individual burrows within the colony boundaries on the basis of apparent prairie dog activity (feces, freshly excavated soil, and non-collapsed burrow structure) and along a gradient from the center of the colony to the periphery. We collected soil by attaching 50-mL conical tubes to a 6-m flexible metal probe, maneuvering the probe into the prairie dog burrow to maximum achievable depth, and using the probe to scrape soil into the tubes. We sealed viable soil samples (>20 mL from > 3 m deep) and stored them at 22°C until processing within 12 hours.

Cultivation of amoebae from soil

We isolated amoebae from soil in plague-affected prairie dog burrows by using modified culture methods (Lagkouvardos *et al.* 2014) (Appendix 1, Figure S3.2), incubated culture plates at 28°C, and observed for changes daily. We supplemented liquid medium with gentamicin (200 µg/mL) after 72 hours or at earliest detection of any bacterial growth. We aseptically transferred amoeba cultures without bacterial contamination to 25-cm² tissue culture flasks in amoeba-specific media containing penicillin/streptomycin. We identified amoeba by using multiplex and endpoint PCR after extracting DNA by using a QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) (Charette & Cosson 2004, Le Calvez *et al.* 2012) (Appendix 1, Figure S3.3).

Bacterial Strains and Culture Conditions

We cultured *Y. pestis* strains from frozen stocks in lysogeny broth (LB) medium. We used 2 strains of *Y. pestis* throughout the study: a non-transformed prototypical strain of *Y. pestis* CO92 and a recombinant *gfp*-expressing strain, *Y. pestis* CO92 *pgm+*, *pCD1*, *pGFPuv*, *amp+*, from the Centers for Disease Control and Prevention (Fort Collins, CO, USA). We cultured the transformed strain by using 100 µg/mL carbenicillin to maintain selective pressure for retention of *gfp* plasmids. Culture conditions simulated a mammalian host environment (37°C for 24 h to stationary phase) and then an extra-host environment (28°C for 24 h) to activate phenotypically plastic expression profiles. We monitored bacterial growth spectrophotometrically at OD₆₀₀.

Amoeba strains and culture conditions

We obtained stocks of *A. lenticulata* (ATCC 30841), *A. castellanii* (ATCC 30234), *A. polyphaga* Linc-Ap1 (CCAP 1501/18), and *V. vermiformis* (ATCC 50237) from the American Type Culture Collection (Manassas, VA, USA) and the Culture Collection of Algae and

Protozoa ([https:// www.ccap.ac.uk/](https://www.ccap.ac.uk/)) and *Dictyostelium discoideum* (NC4A2) from DictyBase (<http://dictybase.org/>). We axenically cultivated amoeba stocks with genera-specific media in T25 tissue culture flasks at 28°C and verified them to be axenic by using standardized methods (Schuster 2002, Thomas *et al.* 2006, Fey *et al.* 2013, Wheat *et al.* 2014).

Co-culture experiments

Intra-amoeba infection prevalence and intensity assays

We individually co-cultured laboratory amoeba species with *Y. pestis* by using established methods (Lambrecht *et al.* 2015). We adjusted viable amoeba trophozoite densities to 5×10^5 trophozoites/mL in triplicate 25-cm² tissue culture flasks and combined *Y. pestis* (CO92 *pgm+*, *pCD1*, *pGFPuv*, *amp+*) cultures with amoeba flasks (excluding amoeba controls), resulting in 5×10^7 viable *Y. pestis* cells/mL and a multiplicity of infection (MOI) of 100 on the basis of OD 600 calculations. We incubated co-cultures at 28°C for 4 h before removing infected amoebae, amoeba controls, and bacteria controls from the surface of the flasks and washing them 3 times with Page amoeba saline (PAS) at $100 \times g$ for 5 min (Thomas *et al.* 2006). We then exposed amoebae to gentamicin (100 µg/mL) for either 1 or 4 h to eliminate residual extracellular bacteria, then washed them 3 more times to remove antibiotic drug residue. Finally, we removed the supernatant from the last wash, concentrated it via centrifugation ($4,400 \times g$ for 10 min), then plated it on LB agar to ensure no extracellular bacteria persisted.

We fixed aliquots of each infected amoeba treatment in 4% paraformaldehyde for 15 min before washing ($4,400 \times g$, 5 min) and resuspending in $1 \times$ PAS for microscopic analysis. We used a fluorescent confocal microscope (Zeiss LSM 510 with ZEN 2009 SP2 software (Carl Zeiss GmbH, Thornwood, NY, USA)) to determine mean infection prevalence (the percentage of amoebae containing >1 intracellular *Y. pestis* bacterium across 16 fields of view per treatment

replicate). We determined mean infection intensity and its distribution by quantifying the number of intracellular bacteria per individual infected amoeba, verified by z-stack confocal microscopy across 16 fields of view per treatment replicate. We used 1-way measured analysis of variance (ANOVA) on prevalence and intensity means across all 5 amoebae species. We log-transformed data as necessary to meet model assumption and used least-squared mean analyses with Tukey's adjustments for pairwise comparisons.

Ultrastructural description of intra-amoeba bacterial location

We used *Y. pestis* (CO92 *pgm+*, *pCD1*, *pGFPuv*, *amp+*) in similar co-culture infection assays with *A. castellanii* (MOI 100 in PAS at 28°C). We co-cultured bacteria for durations of 10 min, 30 min, and 24 h to assess proximal and delayed effects of phagocytosis on bacterial cell viability and intra-amoeba bacterial location. After co-culture, mixtures were fixed in standard electron microscopy fixative for 2 h, then washed 2 times in 0.1 M cacodylate buffer. We then shipped fixed samples in 0.1 M cacodylate buffer to the Electron Microscopy Laboratory in the Department of Pathology at the University of Texas Medical Branch (Galveston, TX, USA) for transmission electron microscopy (TEM) according to standardized procedures. We determined bacterial location within amoebae by ultrastructural analysis of transmission electron micrographs and studied intra-cellular bacterial morphology to assess amoeba-mediated bacterial lysis as measured by cell size, shape, and apparent membrane integrity.

Intra-amoeba bacterial survival and quantification of intra-amoeba bacterial replication

We performed intra-amoeba bacterial survival and replication assays in triplicate across 5 amoeba species (*A. castellanii*, *A. lenticulata*, *A. polyphaga*, *D. discoideum*, and *V. vermiformis*); 2 bacteria species (*Y. pestis* CO92 and *Escherichia coli*); 5 post-infection time points (0, 1, 4, 24,

and 48 h); and 3 antibiotic drug exposure periods (0, 1, and 4 h) for removing extracellular bacteria post-infection. We used *E. coli* as an ameba-susceptible control bacterium. We performed co-cultures in 200- μ L volumes within 96- well plates at a MOI of 100 in 1/2 \times dilute ameba growth medium at 28°C for 1 h and used control ameba and bacteria as monocultures. After initial infection, we removed the supernatant of each well, washed wells 3 times with PAS, exposed them to gentamicin (100 μ g/mL), washed 3 times more, and incubated them in PAS. PAS was verified to be bacteriostatic to *Y. pestis*, thereby precluding extracellular bacterial replication and continuous ingestion by amebae. We lysed infected ameba trophozoites selectively with 100 μ L 0.5% sodium deoxycholate for 5 min before serially diluting and plating on LB agar. We incubated plates at 28°C for 48 h before counting CFUs to determine bacterial survival and replication after phago- cytosis by amebae. The 0.5% sodium deoxycholate lysing detergent had no effect on CFU counts in bacterial monoculture controls (data not shown).

Results

Y. pestis and five species of amebae co-occur in soils of prairie dog burrows undergoing plague epizootics. We cultured a wide diversity of amebae from soil within plague-positive prairie dog burrows in northeastern Colorado and identified live amebae of each study species (Appendix 1, Figure S3.3). Among 8 prairie dog colonies, 24 burrows sampled yielded 15 heterogeneous ameba cultures free of bacteria or fungi. We identified >1 *Acanthamoeba spp.* from 86.6% of cultures (n = 13), *D. discoideum* from 53.3% of cultures (n = 8), and *V. vermiformis* from 6.6% of cultures (n = 1).

Y. pestis is phagocytosed by amebae with heterogeneous prevalence and intensity. Representative fluorescent confocal micrographs of *A. castellanii* and *D. discoideum* illustrate differences in infection intensity and prevalence (Figure 3.2). ANOVA F-test results indicate

significant differences in infection prevalence (or phagocytic efficiency) among amoeba species ($p = 0.0231$) (Table 3.1). Repeat experiments maintained relative ranking of mean infection intensity and infection prevalence across amoeba species (*A. castellanii*, $n = 1,441$; *A. lenticulata*, $n = 1,156$; *A. polyphaga*, $n = 737$; *D. discoideum*, $n = 624$; and *V. vermiformis*, $n = 528$). Pairwise comparisons indicate *V. vermiformis* has significantly lower infection prevalence than *A. lenticulata* ($p = 0.0344$). Infection prevalence ranged from 24.07% in 1 replicate of *V. vermiformis* to 54.83% in 1 replicate of *A. lenticulata*.

Infection intensity was also significantly different among amoeba species ($p = 0.0014$) (Table 3.1). Pairwise comparisons showed *V. vermiformis* has a significantly lower infection intensity than both *A. lenticulata* ($p = 0.0014$) and *A. polyphaga* ($p = 0.0082$) and that *D. discoideum* has a significantly lower infection intensity than *A. lenticulata* ($p = 0.0455$). These findings demonstrate genus-level differences in infection intensity. Infection intensity frequencies followed a strong negative binomial distribution (Figure 3.3). Each amoeba species had several high-intensity outliers ranging up to a maximum of 84 intracellular bacteria observed in 1 *A. lenticulata* amoeba (Figure 3.3).

Y. pestis resides in digestive and central vacuoles of both *D. discoideum* and *A. castellanii* amoebae. Green fluorescent protein expressed by intracellular *Y. pestis* co-localizes with amoeba vacuoles (Figure 3.2). TEM micrographs depict intracellular *Y. pestis* maintaining cellular shape and apparent membrane integrity inside *A. castellanii* amoeba for <24 h post-infection (Figure 3.4). Ultrastructural analysis of TEM images reveals *Y. pestis* persistence within the niche of a tight-fitting vacuolar membrane visually similar to Yersinia-containing vacuoles (YCVs) observed in infected macrophages (Connor *et al.* 2015).

Y. pestis can survive inside *D. discoideum* amoebae for >48 hours, but we found genus-level differences in intra-amoeba survival of *Y. pestis* (Table 3.1). The bacterium did not survive beyond 24 h post-infection in *A. castellanii*, *A. lenticulata*, *A. polyphaga*, or *V. vermiformis*. However, *Y. pestis* co-cultured with *D. discoideum* exhibited consistent intracellular survival for >48 h post-infection under variable treatment conditions (Table 3.1; Figure 3.5). *Y. pestis* exhibited significantly higher survival/recoverability when co-cultured with amoebae as compared to *Y. pestis* monoculture controls ($p < 0.001$). *Y. pestis* monoculture controls yielded a mean of 17 CFUs after 1 h of gentamicin exposure with no recoverable control bacteria across all other treatments. Conversely, *E. coli* bacteria did not significantly persist under any treatment conditions when co-cultured with amoeba ($p < 0.001$). Uninfected amoeba control lysates consistently yielded zero bacteria across all amoeba species and treatments (data not shown). All *Y. pestis* co-cultures exposed to antibiotics had supernatants free of extracellular bacteria. *Y. pestis* had no cytopathic effect on any of the tested amoeba species as verified by comparing amoeba abundance between co-culture treatments and amoeba monoculture controls (data not shown).

Y. pestis replicates intracellularly in *D. discoideum* amoebae for >48 hours post-infection (Table 3.1; Figure 3.5). In *D. discoideum*, the abundance of viable intracellular *Y. pestis* was significantly greater at each successive time point (24 and 48 h post-infection) after 1 h of antibiotic drug exposure ($p = 0.01$ and $p = 0.002$, respectively). Additionally, the abundances of viable *Y. pestis* in *D. discoideum* at 24 and 48 h post-infection were significantly greater than immediately after the 4-h antibiotic treatment ($p = 0.008$ and $p = 0.001$, respectively). After 48 h post-infection, viable intracellular *Y. pestis* was only recovered from *D. discoideum* treatments. Because the data did not meet standard ANOVA assumptions of normality despite

transformation attempts, we used a nonparametric Kruskal-Wallis rank-sum test to compare treatment means by species. Results indicated that the increased abundance of *Y. pestis* in *D. discoideum* was significant compared with all other species at 48 h post-infection ($p < 0.001$).

Discussion

We demonstrate that *Y. pestis* (CO-92) can survive and replicate intracellularly within the social, heterogamous amoeba *D. discoideum*, whereas *Y. pestis* is only transiently resistant to 4 species of free-living and cyst-forming amoebae (*A. castellanii*, *A. lenticulata*, *A. polyphaga*, and *V. vermiformis*). Relative to *E. coli* controls, *Y. pestis* demonstrated significantly increased survival and replication within amoebae despite the 4 cyst-forming amoebae successfully killing the bacteria by using unidentified mechanisms.

Amoebae cultured from soil in prairie dog colonies with active plague epizootics confirm that amoeba species used in our experiments co-occur spatially and temporally with *Y. pestis* under natural conditions. Interactions between amoebae and *Y. pestis* could select for increasingly amoeba-resistant phenotypes, considering the transient resistance already observed in 4 cyst-forming amoeba species. Other research has demonstrated the potential for amoebae to affect pathogen transmission mode, alter virulence, and act as training grounds for intracellular pathogens by selecting for traits enabling macrophage invasion or avoidance (Molmeret *et al.* 2005, Salah *et al.* 2009).

Genus-level differences in amoeba infection intensity and infection prevalence confirm that various amoeba species have greater reservoir potential than others. In accordance with super-spreader theories, a minority of individual amoeba harboring atypically high numbers of intracellular bacteria may be disproportionately causative for pathogen maintenance and re-emergence.

We observed a shorter duration of survival for *Y. pestis* in *A. castellanii* compared with prior experiments (24 h vs. 5 d in 13), likely from differing co-culture conditions and amoeba strains. Incubation temperatures differed between this and prior experiments (28°C vs. 4°C and 25°C in 13). Many *Y. pestis* virulence factors are temperature regulated and may differentially facilitate cellular invasion, inhibition of the phagolysosomal pathway, and intracellular persistence (Perry & Fetherston 1997, Molmeret *et al.* 2005, Salah *et al.* 2009). Additionally, *A. castellanii* (ATCC 30234) used in this study was originally derived from yeast cultures in London in 1930, whereas *A. castellanii* (ATCC 30010), used by Benavides-Montaña & Vadyvaloo (Benavides-Montaña & Vadyvaloo 2017), was originally isolated from California soil in 1957 and enabled longer intracellular survival of *Y. pestis*. Intracellular survival may be affected by traits acquired by co-evolution between amoebae and resistant bacteria in soil environments (Salah *et al.* 2009).

In macrophages, *Y. pestis* recruits host Rab1b protein to the phagosome, resulting in inhibition of phagosome acidification and disruption of the remaining phagolysosomal metabolic pathway (Straley & Perry 1995, Grabenstein *et al.* 2006, Pujol *et al.* 2009, Ke *et al.* 2013, Connor *et al.* 2015). *Y. pestis* then establishes a replicative niche within the YCV, characterized by a tight-fitting vacuole that expands commensurately with bacterial replication (Connor *et al.* 2015). Examination of TEM micrographs shows that intracellular bacteria are localized within form-fitting vacuolar membranes, similar to the YCVs found in macrophages (Figure 3.4).

The successful intracellular survival of *Y. pestis* in *D. discoideum* for >48 h demonstrates that *Y. pestis* is an amoeba-resistant bacterium. This classification supports the potential for *D. discoideum* or related amoeba species to be environmental reservoirs of plague. Intracellular survival of the observed duration is consequential given that typical interactions between bacteria

and phagocytic cells result in bacterial death in <40 min (Connor *et al.* 2015). Most phagocytosed bacteria cannot survive digestive processes characteristic of phagocytic cells including phagolysosome fusion and acidification, or the subsequent recruitment of endosomal lytic factors (Pujol *et al.* 2009, Ke *et al.* 2013, Connor *et al.* 2015). Ongoing research assesses the maintenance of viable *Y. pestis* through the entire *D. discoideum* life cycle, including transmissible dormant spores.

Ameba-resistant pathogens often replicate in vacuoles before escaping into the cytosol or outside of the phagocytic cell entirely. In addition to viable intracellular persistence, we observed active intracellular replication of *Y. pestis* (CO-92) in *D. discoideum* (Figure 3.5) and possible, but unconfirmed, replication of *Y. pestis* (CO92 pgm+, pCD1, pGFPuv, amp+) in *A. castellanii* (Figure 3.4A). Analysis of TEM micrographs proved inconclusive for confirming central constriction in elongated bacterium; thus, only *D. discoideum* has conclusively demonstrated intracellular replication of *Y. pestis*. *Y. pestis* CFUs recovered from within *D. discoideum* increased significantly ($p = 0.001-0.01$; Figure 3.5) in successive post-infection time points across both antibiotic treatment conditions (except in one instance where *Y. pestis* increased non-significantly ($p = 0.1624$; Figure 3.5)). The consistent absence of extracellular bacteria in all *D. discoideum* co-cultures indicates resistance to digestion and the exploitation of an intra-ameba replicative niche.

Intracellular replication of *Y. pestis* in macrophages occurs within YCVs, and the formation of YCVs requires metabolic pathway inhibition by recruitment of Rab1b GTPases. Orthologous mechanisms are likely the cause for observed *Y. pestis* replication and survival within amoebae. We searched for macrophage Rab1b by using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) against full genome sequences of each study amoeba

species and located homologous genetic sequences (99.8% similarity) within *A. castellanii* and *D. discoideum* (GenBank accession nos. XM_004347056.1 and XM_637217.1, respectively (42,43)). Future research should attempt to establish whether these ameba sequences are functionally orthologous to those identified in macrophages and whether the presence of particular host GTPases is diagnostic of ameba permissiveness to intracellular bacteria.

Results of this study support the reservoir potential of environmental ameba but do not definitively prove that this mechanism occurs in situ. Further research is necessary to determine if the maximum duration of intra-ameba *Y. pestis* survival corresponds with the durations of cryptic interepizootic persistence that are characteristic of plague dynamics. Increasing evidence for dormant or viable but nonculturable forms of *Y. pestis* may provide explanations underlying hypothesized multiyear survival in ameba spores or cysts (Buzoleva & Sidorenko 2005, Somova *et al.* 2006, Ayyadurai *et al.* 2008, Pawlowski *et al.* 2011, Easterday *et al.* 2012). Outcomes of this research prompt questions regarding evolutionary selection imposed by amebae on environmental pathogens and applications of the ameba reservoir model for other cryptic environmental pathogens. Further research into ameba-mediated pathogenesis and persistence will offer practical insights for public health, conservation, agricultural management, and biodefense.

In conclusion, the mechanisms underlying plague re- emergence following dormancy have eluded researchers for centuries (Perry & Fetherston 1997, Gage & Kosoy 2005). Plague persistence within soil-borne microorganisms has been hypothesized as an elusive maintenance mechanism (Eisen & Gage 2009, Gage & Kosoy 2005, Ayyadurai *et al.* 2008, Easterday *et al.* 2012). We demonstrated spatiotemporal co-occurrence of plague bacterium and various ameba species during an active plague epizootic. Further, we observed the persistence of viable and

replicative *Y. pestis* in *D. discoideum* amoebae for >48 hours post-infection and persistence of *Y. pestis* in 4 cyst-forming amoeba species for <24 hours post-infection, whereas amoeba-susceptible control bacteria were eliminated by amoebae in <1 hour. Thus, *Y. pestis* are respectively amoeba-resistant and transiently amoeba-resistant under the tested infection conditions. *Y. pestis* bacteria resided within amoeba structures that were visually analogous to YCVs observed in infected macrophages. These results encourage research into the eco-evolutionary interactions between pathogenic bacteria, amoebae, and host immune factors. The reservoir potential of amoebae and their shared infection-permissiveness with phagocytic macrophages show promise in explaining the cryptic properties underlying interepizootic plague transmission and persistence.

Figures

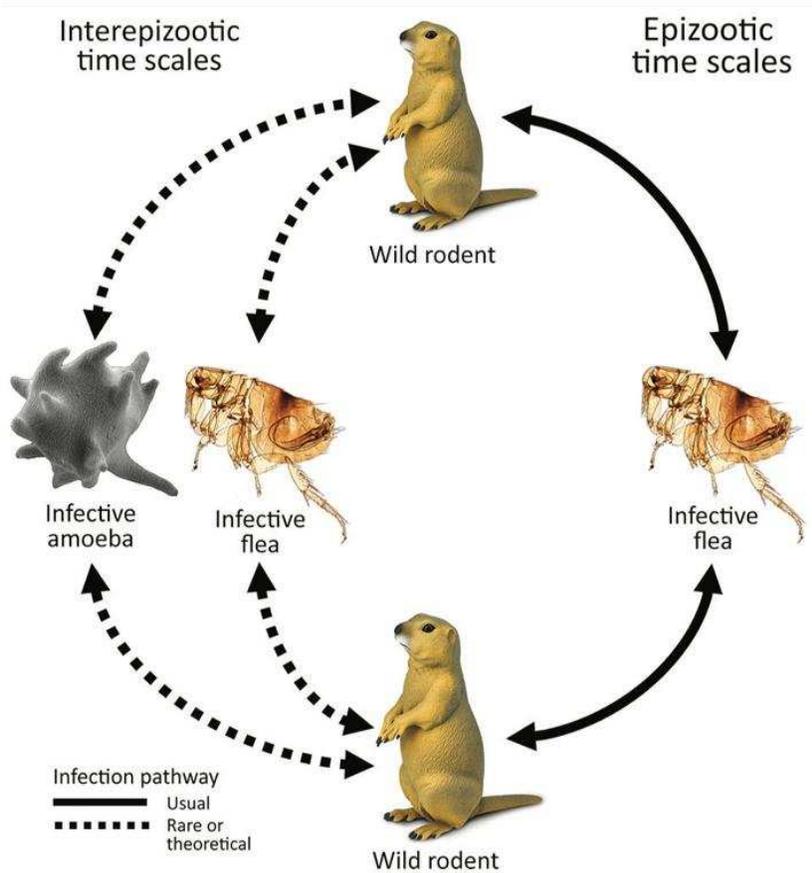


Figure 3.1: Infection pathways for plague. During plague epizootics, transmission occurs through flea vectors within meta-populations of ground-dwelling rodents. It is unknown by what route or mechanism *Yersinia pestis* is maintained during interepizootic periods of plague quiescence. Previous research on fleas has not strongly supported their reservoir potential across interepizootic periods (Webb *et al.* 2006). The experiment and analysis of this study test the hypothesis that ameboid species demonstrate reservoir potential for *Y. pestis*. If *Y. pestis* is maintained within ameba reservoirs, we suspect that epizootic recrudescence may occur when infected soil-borne amoebae enter the bloodstream of naive rodent hosts (by entering wounds from antagonistic host-to-host interactions or burrowing activities). Amebae typically lyse when incubated at 37°C and simultaneously release their intracellular cargo, potentially initiating an infection.

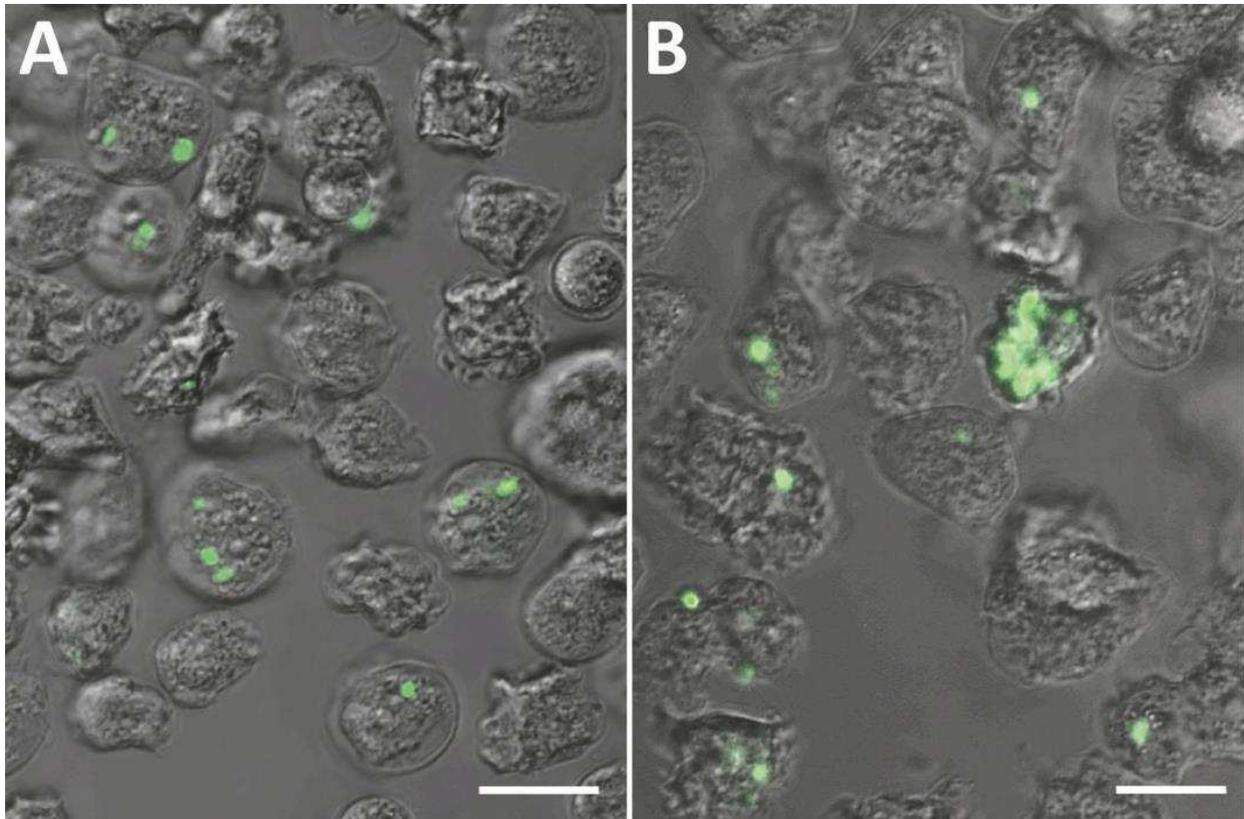


Figure 3.2: Representative fluorescent confocal images of (A) *Acanthamoeba castellanii* (B) and *Dictyostelium discoideum* after experimental co-culture with *Yersinia pestis* (CO92 pgm+, pCD1, pGFPuv, amp+) and removal of extracellular bacteria. After co-culture of ameba trophozoites and *Y. pestis*, we determined the prevalence and intensity of bacterial uptake by manual counting of amebae by using z-stack fluorescent confocal microscopy and averaging across 15 fields per replicate of each ameba species. Confocal count data represent the minimum prevalence/intensity values. Bacteria adherent to the outside of ameba or those with uncertain intracellular status were discarded. The minimum count threshold to reduce random count bias to accepted levels was determined to be 500 per ameba species. Scale bars indicate 30 μm .

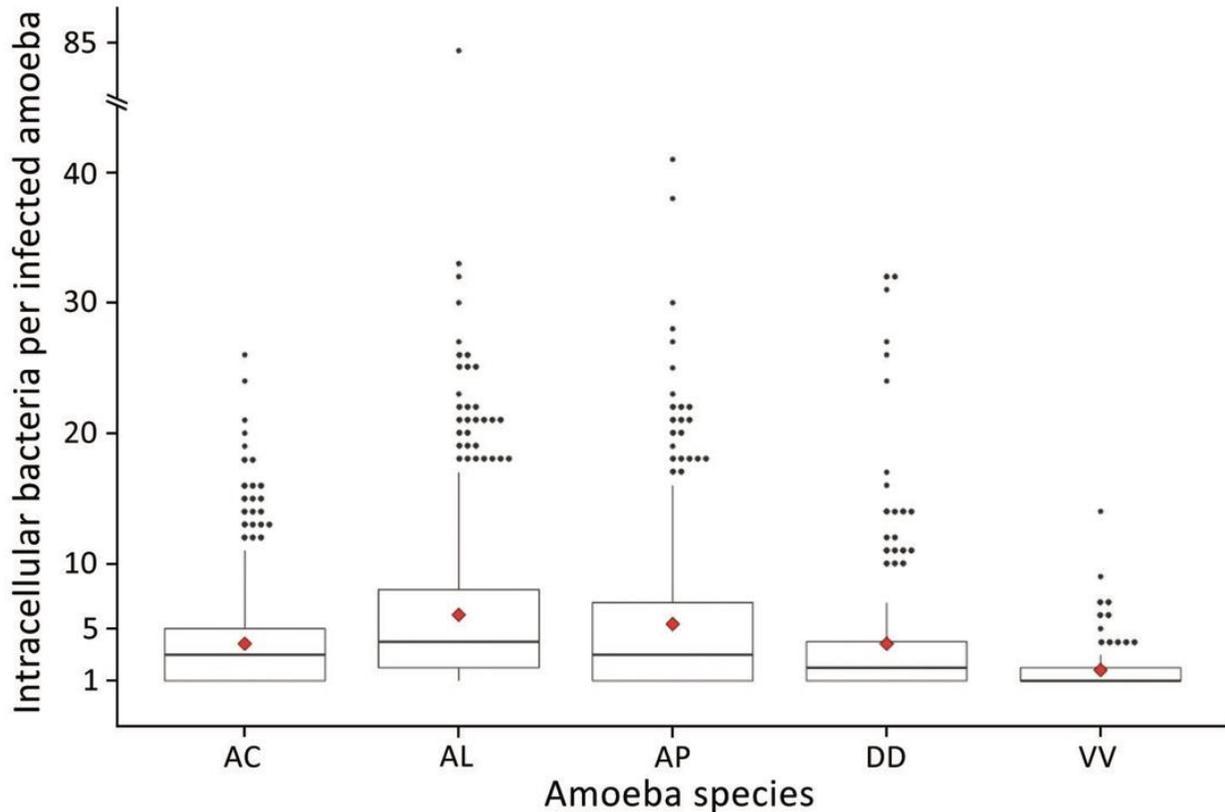


Figure 3.3: Boxplots of infection intensity across amoeba species after experimental infection with *Yersinia pestis*. Infection intensity frequencies followed a strong negative binomial distribution. Median infection intensities (horizontal lines inside boxes): AC = 3, AL = 4, AP = 3, DD = 2, VV = 1. Red diamonds denote mean infection intensity (Table 3.1). Each amoeba species had several high-intensity outliers ranging up to a maximum of 84 intracellular bacteria observed in 1 *A. lenticulata* amoeba (note broken y-axis). AC, *Acanthamoeba castellanii* (n = 1,441); AL, *A. lenticulata* (n = 1,156); AP, *A. polyphaga* (n = 737); DD, *Dictyostelium discoideum* (n = 624); VV, *Vermamoeba vermiformis* (n = 528).

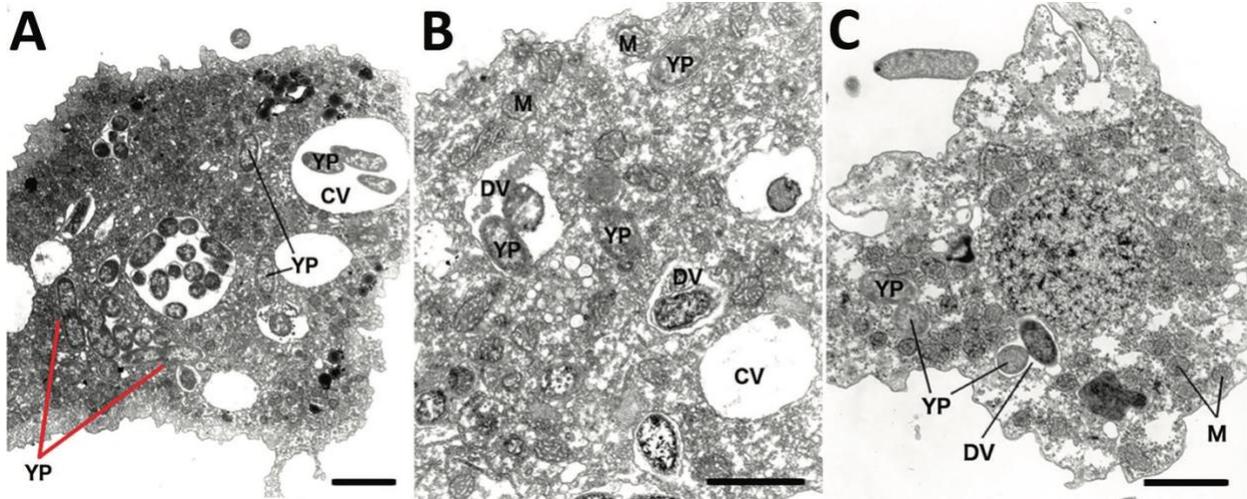


Figure 3.4: Representative transmission electron micrographs (TEM) depict *Acanthamoeba castellanii* amoebae during A) 10-minute, B) 30-minute, and C) 24-hour co-cultures (multiplicity of infection 100) with *Yersinia pestis* (CO92 pgm+, pCD1, pGFPuv, amp+). Red arrows in panel A indicate potential intra-amoeba binary fission of *Y. pestis* bacterium. Visual analysis of TEM micrographs proved inconclusive for identifying constriction in elongated bacterium. *Y. pestis* resides within the potential replicative niche of a tight-fitting vacuolar membrane, similar to *Yersinia*-containing vacuoles observed in macrophages. YP, *Y. pestis*; CV, central vacuole; DV, digestive vacuole; M, mitochondria. Scale bars indicate 3 μm .

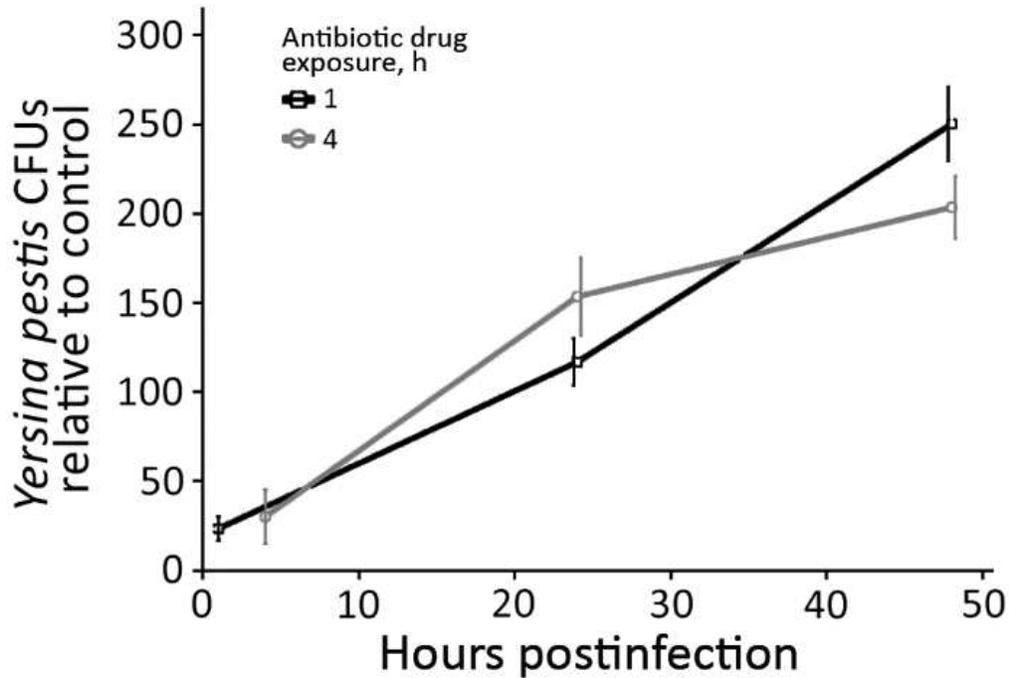


Figure 3.5: Intra-ameba *Yersinia pestis* abundance in *Dictyostelium discoideum* across 2 post-infection antibiotic drug exposure periods, 1 hour and 4 hour. In *D. discoideum*, the abundance of viable intracellular *Y. pestis* was significantly greater at each successive time point (24 and 48 hours) after the 1-hour antibiotic drug treatment ($p = 0.01$ and $p = 0.002$, respectively). After the 4-hour antibiotic drug treatment in *D. discoideum*, the abundance of viable intracellular *Y. pestis* at 24 and 48 hours was significantly greater than at 4 hours ($p = 0.008$ and $p = 0.001$, respectively). The abundance of viable *Y. pestis* within *D. discoideum* at 48 hours post-infection was not significantly different between the 1-hour and 4-hour antibiotic drug treatments ($p = 0.1624$). Viable intracellular *Y. pestis* abundance was significantly greater in *D. discoideum* compared with all other species at 48 hours post-infection ($p < 0.001$).

Table 3.1: Properties and kinetics of five amoebae species after experimental *Yersinia pestis* infection

Table. Properties and kinetics of 5 amoebae species after experimental *Yersinia pestis* infection

Species	Dormant state	Infection prevalence*		Infection intensity†		Intracellular survival‡				Intracellular replication
						24 h		48 h		
		Mean, %	SEM	Mean, %	SEM	Mean, %	SEM	Mean, %	SEM	
<i>Acanthamoeba castellanii</i>	Cyst	33.63	5.21	4.22	0.61	0	0	0	0	Inconclusive§
<i>A. lenticulata</i>	Cyst	51.66	3.17	6.41	0.43	10	11.55	0	0	No
<i>A. polyphaga</i>	Cyst	49.08	5.41	5.36	0.37	31.66	22.04	0	0	No
<i>Dictyostelium discoideum</i>	Spore	39.24	3.13	3.57	0.97	270	19.92	226.67	22.71	Yes
<i>Vermamoeba vermiformis</i>	Cyst	29.61	3.4	1.84	0.13	10	9.66	0	0	No

*Mean percentage of amoebae containing ≥ 1 intracellular bacterium.

†Mean no. of intracellular bacteria per individual infected amoeba.

‡Mean no. of surviving intracellular bacteria (relative to control) in experiments with 1 and 4 h of antibiotic drug exposure.

§We observed no replication in the intraamoeba replication assay, which we used to count intraamoeba bacterial colony-forming units before and after co-culture. However, we observed probable but nondefinitive mitotic bacterial replication in the TEM micrographs (Figure 4, panel A).

CHAPTER 4: ECO-EPIDEMIOLOGICAL TRAITS EXPLAIN PLAGUE PERSISTENCE AND VARIABLE OUTBREAK DYNAMICS

Summary

Yersinia pestis, the causative agent of plague, is a pathogen of ground-dwelling rodents, mainly in temperate regions, with capacity for spillover to other mammals through multiple transmission routes. Plague is further characterized by cryptic local persistence over multi-year periods of apparent dormancy that intersperse sporadic and severe outbreaks (epizootics). Two prominent explanations for local persistence include continuous sub-epizootic transmission among resistant hosts and flea vectors (i.e. enzootic transmission), and latent persistence in reservoirs with occasional spillover into host populations. Drivers of plague persistence and heterogeneous outbreak dynamics have been obscured by complex and idiosyncratic interactions among hosts, flea vectors, putative reservoirs, and the environment. We use an epidemiological model of plague affecting populations of Black-tailed prairie dogs to explore the role of a long-term reservoir in enabling local maintenance of plague during inter-epizootic periods. We show that natural variation in eco-epidemiological conditions, including host immunity and reservoir life-history traits, can drive the emergence of both epizootic and pseudo-enzootic outbreak dynamics. This empirically-informed model is concordant with 25 years of field observation and suggests a parsimonious explanation for cryptic plague persistence and heterogeneous outbreak dynamics.

Introduction

Infectious diseases like Plague, Ebola, Nipah, anthrax, leprosy, and hantavirus have zoonotic origins, where human cases arise from pathogens maintained in wildlife (Zeppelini *et al.* 2016). The likelihood of human exposure, or spillover events, grows when wild host

populations experience disease outbreaks (Gage & Kosoy 2005). However, the mechanisms by which many of these zoonotic pathogens persist in between obvious outbreaks is not well understood. Understanding mechanisms that facilitate sylvatic pathogen maintenance and re-emergence is critical for reducing human incidence of zoonotic disease.

As a case study, we investigate plague, a severe zoonotic disease that sporadically causes large-scale epizootics and mortality in ground-dwelling rodent populations when eco-epidemiological conditions (e.g. host immunity or seasonality) among hosts, flea vectors, and the environment are favorable (Barnes 1993, Cully & Williams 2001, Gage & Kosoy 2005). Epizootics are interspersed by periods of apparent quiescence, characterized by a lack of apparent transmission or apparent disease among host and vector populations, and can reportedly last up to 300 years (Barreto *et al.* 1995, Chanteau *et al.* 1998, Tikhmorov 1999, Arbaji *et al.* 2005, Bertherat *et al.* 2007, Tarantola *et al.* 2009, Seifert *et al.* 2016). Substantial genetic evidence indicates that clonal isolates of plague persist locally during these inter-epizootic quiescent periods before re-emerging and causing subsequent epizootics (Girard *et al.* 2004, Bertherat 2007, Cui *et al.* 2013, Lowell *et al.* 2015, Seifert *et al.* 2016).

Two prominent hypotheses put forth to explain local inter-epizootic maintenance include continuous sub-epizootic circulation among resistant mammalian hosts (enzootic maintenance) and latent persistence in reservoirs. The enzootic hypothesis is classically characterized by the existence of a separate assemblage of resistant hosts and vectors that perpetually and asymptotically maintain plague (Barnes 1993, Cully & Williams 2001, Gage & Kosoy 2005). When conditions enable pathogen spillover into susceptible host populations an epizootic may re-emerge (Figure 4.1A). Despite widespread adoption and over a century of investigation, a

dearth of convincing evidence supports this hypothesis (Gage & Kosoy 2005, Salkeld *et al.* 2016).

An alternative explanation for inter-epizootic persistence involves transient sequestration of *Y. pestis* in latent reservoirs that can facilitate spillover into both resistant and susceptible host populations (Figure 4.1B) (Yersin 1894, Pavlovsky 1964, Brown & Barker 1999, Greub & Raoult 2004, Drancourt *et al.* 2006, Eisen *et al.* 2008, Eisen & Gage 2009, Ayyadurai *et al.* 2008, Benavides-Montaña & Vadyvaloo 2017, Markman *et al.* 2018). Proposed reservoirs include telluric microorganisms like protozoan amoebae that are pervasive in soil and water, and have previously been recognized for harboring other cryptic zoonotic pathogens including *Legionella spp.*, *Mycobacterium spp.*, *Francisella spp.*, and other *Yersinia spp.* (Brown & Barker 1999, Greub & Raoult 2004, Khan 2006, Lambrecht *et al.* 2013, Wheat *et al.* 2014, Benavides-Montaña & Vadyvaloo 2017, Markman *et al.* 2018). Many free-living amoebae cycle between two life stages: a mobile, feeding, trophozoite stage and a robust, dormant, cyst stage depending on environmental conditions (Khan 2006). Various species of trophozoite amoebae naturally cohabit with *Y. pestis* and have demonstrated permissiveness to intracellular *Y. pestis* survival and replication in laboratory studies (Benavides-Montaña & Vadyvaloo 2017, Markman *et al.* 2018). The same amoeba species have also been shown to persist for over 20 years in cyst form (Mazur 1995, Sriram 2008), indicating the potential for long-term maintenance of intra-cellular bacteria. Finally, the timing of peak amoebae abundance and their ejection of pathogenic cargo exhibit the same correlation with periods of relatively high rainfall that are frequently observed preceding or during plague outbreaks (Stapp *et al.* 2004, Khan 2006, Snäll *et al.* 2008, Savage *et al.* 2011).

Here we implement mathematical models, parameterized using both epidemiological traits and ecological conditions based on empirical measurements, to explore the plausibility of alternative endogenous mechanisms (those not relying on external import of plague) that can account for local persistence of plague in Black-tailed prairie dogs. Eco-epidemiological traits include transmission between populations of mammalian hosts, within host carcasses, flea vectors, and putative reservoirs. Our models simulate interactions among host, vector, and reservoir populations alongside seasonal environmental conditions over 5,000 days, and represent the first multi-population analysis of the plague system that spans both outbreaks and inter-outbreak periods. We evaluate eight model variants with respect to their endogenous maintenance potential, including: 1) susceptible host populations, 2) resistant host populations, 3) susceptible hosts with amoeba reservoirs, 4) resistant hosts with amoeba reservoirs, 5) susceptible hosts with amoeba trophozoites, but not amoeba cysts, 6) resistant hosts with no amoeba cysts, 7) susceptible hosts with amoeba reservoirs but no host immigration, and 8) susceptible hosts with amoeba reservoirs and decreased intra-colony connectivity.

This quantitative framework may be used to predict host population responses to disease and inform host vaccination campaigns or other intervention strategies attempting to induce eco-epidemiological conditions that lead to pathogen extinction. This model may also apply to other disease systems that involve heterogeneous outbreak dynamics, variable host resistance, and hypothesized environmental reservoirs.

Methods

Stochastic Model

In order to evaluate dynamics across multiple epizootic and inter-epizootic periods, we modified existing continuous ordinary differential equation models (Webb *et al.* 2006,

Buhnerkempe *et al.* 2011, Richgels *et al.* 2016) to characterize host, vector, and reservoir population responses to plague over 5,000 days (50,000 model time steps). Our simulations assumed no import of *Y. pestis* (i.e. immigration of infectious hosts or associated vectors) in order to evaluate endogenous maintenance potential. This model features three interacting sub-compartments representing discrete classes found in host, flea vector, and reservoir populations (Figure 4.2, Table 4.1). Hosts may transition through susceptible, exposed, infectious, resistant, and deceased states. Flea vectors can transition through six discrete states described by level of infectiousness (susceptible, highly infectious, or less infectious) and host association (on-host or questing). The amoeba sub-model consists of two discrete infected states based on trophozoite or cyst life stages. Infected amoebae trophozoites result from influxes of infectious leachate into soil from decomposing host carcasses.

Further modifications were made to existing models by implementing a deterministic sinusoidal seasonal climate function (Figure 4.3, Equation 14) representing natural environmental fluxes that drive interactions between and within different host, flea vector, and reservoir classes. Notable dynamics include transitions between amoebae life-stages and an annually-pulsed reproduction and immigration function for susceptible host populations, to realistically approximate host breeding and migratory patterns over multi-year periods (Peel *et al.* 2014, Hayman 2015).

To simulate the stochastic dynamics of the model, we considered each event separately, where events are births, deaths, and class transitions among host, vector, and reservoir populations (i.e. a susceptible host becomes an exposed host). We assume that during a short time window, only one event will occur, and within that window the single event is independent of other events. We also assume that this time window is short enough such that time-varying

rates (i.e. those rates that depend on the weather) do not change. Under such circumstances, the probability of a given event occurring during this short time window is exponentially distributed according to its corresponding rate in equations 1-13 (Figure 4.3). We then simulate the dynamics of a given population using the tau-leaping approximation to the Gillespie algorithm (Gillespie 1976, Gillespie 2001). See Appendix 3 for code repository.

Variables and parameters

Model variables and parameters were largely derived from prior studies (Webb *et al.* 2006, Buhnerkempe *et al.* 2011, Richgels *et al.* 2016) and reflect the plague system in Northern Colorado with Black-tailed prairie dogs as hosts and their associated fleas as vectors. Variables and parameters are briefly described in Table 4.1 and Table 4.2 and are more thoroughly described in source materials. Variables that are unique to this model are for the plague reservoir and include infected amoeba trophozoites (A_i) and infected amoeba cysts (A_{ic}). Parameters that are unique to this model include: seasonal birth pulses in host populations (k, s, b) (Peel *et al.* 2014), pulsed annual immigration of susceptible hosts (w), transmission rate from infected amoeba to susceptible hosts (β_d), and all amoebae parameters.

Values for new parameters were derived from published sources, or in the case of parameters with high variability, values were chosen from the center of ranges reported in literature. It is assumed that natural mortality and growth rates of amoebae are \log^{-1} scaled relative to rates reported under optimal laboratory conditions. Obtaining accurate measurements of growth and mortality rates *in situ* is difficult, but available data suggest that laboratory measures provide a reasonable approximation (Khan 2006, Markman *et al.* 2018). The ability of *Y. pestis* to survive and replicate within amoebae has been demonstrated in laboratory experiments (Benavides-Montañó & Vadyvaloo 2017, Markman *et al.* 2018) and we assume that

this occurs under natural conditions allowing infected amoebae to remain infected after mitotic division. In models that include amoebae cysts, we assume intracellular survival of *Y. pestis* is possible, as seen in related *Yersinia spp.* (Nikul'shin *et al.* 1992, Lambrecht *et al.* 2013, Santos-Montañez *et al.* 2015), but this has to be empirically verified for *Y. pestis*. Finally, we assume infected amoebae maintain equal fitness compared to naïve amoebae in the surrounding soil environment. Laboratory experiments have indicated amoebae infected by *Y. pestis* and *Y. pseudotuberculosis* do not experience increased mortality relative to uninfected amoebae and that amoeba reproductive rates are also unaffected (Lambrecht *et al.* 2013, Benavides-Montaño & Vadyvaloo 2017, Markman *et al.* 2018).

Identification of sensitive eco-epidemiological traits

To test the sensitivity of the model with respect to individual parameters, we selected a subset of 23 parameters that exhibit natural variation. Each parameter was randomly sampled from a log normal distribution of values spanning +/- one order of magnitude relative to the baseline parameter value in Table 4.2. Each parameter was randomly perturbed 100 times and 200 simulations were performed for each perturbation to obtain a distribution of mean inter-outbreak period (IOP, described below). We regressed mean IOP values against perturbed parameter values that were natural log transformed. Then we defined sensitivity as the absolute value of the first order derivative of the line of best fit from the regression. Additionally, we obtained partial regression correlation coefficients (PRCC) for each parameter's effect on IOP duration to determine strength of association between parameters and IOP duration (Figure S4.1).

Model variations

Parameter values were varied to simulate dynamics under eight separate conditions of interest, and to test sufficiency of each model variation to endogenously maintain plague over realistic inter-epizootic periods. Stochastic model variants included: 1) susceptible hosts, no transmission from the amoeba reservoir ($\beta_d = 0$), 2) resistant hosts, no reservoir transmission ($\beta_d = 0$, $\rho = 0.6$, $\varphi = 0.005$)(Quan 1985, Biggins & Kosoy 2001, Gage & Kosoy 2005), 3) susceptible hosts with amoeba reservoir (no change from Table 4.2), 4) resistant hosts with amoeba reservoir ($\rho = 0.6$, $\varphi = 0.005$), 5) susceptible hosts, no amoeba cysts ($\varepsilon_c = 0$), 6) resistant hosts, no amoeba cysts ($\rho = 0.6$, $\varepsilon_c = 0$), 7) susceptible hosts with amoeba reservoir, no host immigration ($w = 0$), 8) susceptible hosts with amoeba reservoir, low intra-colony connectivity ($\beta_r = 0$).

Model 5 and 6 were implemented to determine the theoretical ability of *Y. pestis* to persist in amoebae trophozoite populations without being able to latently reside in amoebae cysts. Model 5 and 6 differ by including either susceptible or resistant host populations. Model 7 examines the role of susceptible host immigration in population recovery following an epizootic-induced population crash. Model 8 evaluates the impact of excluding alternate mammalian hosts that could have a role in pathogen maintenance or transmission by modulating levels of intra-colony connectivity.

Inter-outbreak period

To determine whether model variations could maintain viable *Y. pestis* over observed inter-epizootic periods, we calculated the duration of inter-outbreak periods (IOP) from model outputs. We defined inter-outbreak period (IOP) as the duration in days between outbreaks in host populations. IOP is used to approximate periods of apparent plague quiescence and to

evaluate the sufficiency of candidate mechanisms to maintain plague over those timespans. We define an outbreak as a population of susceptible hosts that drops below ten animals while there are > 0 infected hosts present during the previous ten days. For a given host population trajectory, multiple outbreaks may occur, and the time between each outbreak was recorded as the IOP. If outbreak conditions were met within 150 days of the last outbreak, those events were considered to be part of the same outbreak. All population trajectories were simulated for 5,000 days and IOP values were computed for 500 trajectories for each of the stochastic model variations described above.

Natural dataset

We used 25 years of observational data collected from 92 prairie dog colonies on the Pawnee National Grassland in north-central Colorado, USA where plague naturally occurs (Savage *et al.* 2011, George *et al.* 2013). This dataset measured colony occupancy, size, spatial connectedness, and outbreak occurrence, which allows inference of host population dynamics. Natural IOP values were measured as the time from first recorded colony re-establishment to subsequent outbreak. In addition to the 25-year dataset on epizootic host populations, we used published estimates of population metrics and relevant ecological and epidemiological traits from enzootic plague regions to provide parameter values for the model (Table 4.2).

Statistical methods

We compared IOP duration across model variants and against field values. Because the data did not meet standard ANOVA assumptions of normality despite transformation attempts, we used a nonparametric Kruskal-Wallis rank-sum test to compare treatment means by species. This was followed by pairwise comparisons with Tukey adjustments. A probability of < 0.05 was considered statistically significant. We performed statistical analyses using R version 3.5.2.

Results

In the model, we found that plague can persist locally and cryptically across inter-epizootic quiescent periods under eco-epidemiological conditions that mirror natural plague foci (Figure 4.4). We explored the plausibility of various endogenous maintenance mechanisms (those not relying on external import of plague) including maintenance within hosts and host carcasses (Figure 4.4A and 4.4B), flea vectors (Figure 4.4C and 4.4D), or putative reservoirs (Figure 4.4E and 4.4F). We also identified three key eco-epidemiological traits that strongly influence plague maintenance and re-emergence dynamics (Figure 4.5A) and used those traits to explore eight model variations to isolate conditions necessary for endogenous maintenance. Model output indicates persistence in reservoir populations is necessary for endogenous maintenance to occur and that subsequent spillover into host populations drives epizootic emergence or pseudo-enzootic dynamics, depending on host resistance and traits driving reservoir transmission and abundance.

Sensitivity analysis

Sensitivity analysis was performed relative to duration of inter-outbreak period (IOP) and highlights parameters that are influential in enabling subsequent outbreaks and the frequency with which they occur. The IOP in our stochastic model is sensitive to measures of intra-colony connectivity (B_n and B_r), host immigration (w), host resistance (ρ), and a suite of amoebae life-history traits including, transmission rate to susceptible hosts (β_d), amoeba fecundity (r_a), carrying capacity (K_a), and natural mortality rates (μ_{t1} and μ_{t2}) (Figure 4.6). Large increases in intra-colony connectivity (B_n and B_r) resulted in nearly continuous plague exposure that prevented host population recovery and suppressed total susceptible host abundance. Immigration of susceptible hosts (w) strongly influenced host population recovery following an

outbreak and IOP duration. IOP was also sensitive to host resistance (ρ), which is highly variable across various plague systems. Influential amoebae life-history traits were also highly variable in the literature and contributed to infectious reservoir abundance and transmission.

We explored model output, as measured by IOP duration, in response to variation in ρ and r_a across bio-realistic values ranging from 0-1 in increments of 0.05 (Figure 4.5A and 4.5B). Model output indicates that these traits can drive the emergence of epizootic or pseudo-enzootic dynamics and revealed a predominantly inverse relationship between IOP and outbreak frequency. Infrequent outbreaks coupled with longer IOPs and low host resistance characterize epizootic conditions, whereas frequent outbreaks with shorter IOPs and high host resistance characterizes enzootic conditions.

Comparison of candidate models

The mechanism(s) by which plague persists in between obvious outbreaks is not definitively known. We tested eight variant models to determine which epidemiological trait values and ecological conditions were necessary to support endogenous maintenance of plague that causes sporadically re-occurring outbreaks. We then compared output from these models, which included maximum duration of plague persistence and duration of inter-outbreak periods (IOP), to field IOP estimates derived from a 25-year dataset of susceptible Black-tailed prairie dogs dynamics in a Northern Colorado, US, plague foci (Table 4.3).

Models 1 & 2: Susceptible and resistant hosts with no reservoir

Endogenous (intra-colony) maintenance of *Y. pestis* was not possible over naturally observed inter-epizootic dormancy periods (median 1095 days) without the inclusion of a long-term reservoir. In model 1, using susceptible hosts and excluding amoebae reservoirs, *Y. pestis* did not persist long enough to cause any subsequent epizootics (Table 4.3). The maximum

persistence of infectious material following the initial simulated outbreak was 234 days for fleas and 291 days for host carcasses. Similarly, in model 2, using resistant hosts and excluding amoeba reservoirs, *Y. pestis* was not sustained long-term in host or vector populations. Under model 2 conditions ($\beta_d = 0$, $\rho = 0.6$, $\varphi = 0.005$), re-occurring plague outbreaks were initially rare ($n = 32$ across 500 simulations) with short inter-outbreak periods (mean 289.72 days)(Table 4.3) and low host mortality. Additionally, no outbreaks were observed beyond 525 days. All simulations of model 1 and 2 progressed to complete pathogen extinction and host populations returned to carrying capacity.

Models 3 & 4: Susceptible and resistant hosts with amoeba reservoirs

Model 3, which included amoeba reservoirs with susceptible host populations, yielded endogenous plague maintenance and re-emergent outbreaks that mirror field observations from a 25-year independent dataset on susceptible black-tailed prairie dog populations. IOP duration (Table 4.3) was not significantly different from field-derived IOP values (mean 1553 days, median 1095 days, 95% CI +/- 278 days, Kruskal-Wallis $X^2 = 962.4621$, $p = 0.75$). Additionally, outbreak occurrence was consistent with natural seasonal patterns (Figure 4.4B and Figure 4.5B).

Model 3 mean stochastic trajectories of host, vector, and reservoir populations over 5000 days produce epizootic dynamics (Figure 4.4A, 4.4C, 4.4E) similar to prior modeling studies (Webb *et al.* 2006, Buhnerkempe *et al.* 2011, Richgels *et al.* 2016) while also displaying long-term endogenous persistence over inter-epizootic quiescence periods (Figure 4.4B, 4.4D, 4.4F).

Model 4, characterized by amoeba reservoirs with resistant host populations, yielded pseudo-enzootic dynamics. *Y. pestis* was maintained in reservoir and host populations through more frequent spillover events across the entire simulation period and exhibited significantly

shorter IOP duration relative to model 3 with susceptible hosts and amoeba reservoirs (Table 4.3, $p < 0.001$).

Epizootic and pseudo-enzootic dynamics emerged in response to variation in key traits, including host resistance level (ρ) and infectious amoebae fecundity (r_a) (Figure 4.5A). Simulations using susceptible hosts traits (model 3, Table 4.2) were characterized by longer IOPs, fewer net outbreaks, and greater host mortality, whereas simulations using resistant trait values (model 4, $\rho = 0.6$, $\varphi = 0.005$) yielded shorter IOPs, more frequent outbreaks, and lower host population mortality (Table 4.3). Variation in these key traits revealed a predominantly inverse relationship between IOP and outbreak frequency (Figure 4.5A). Exceptions to this pattern occur under trait combinations that are poorly suited for endogenous maintenance of plague, as signified by infrequent or non-existent re-occurring outbreaks (Figure 4.5A).

Models 5 & 6: Susceptible and resistant hosts with amoeba trophozoites but no amoeba cysts

Simulations of susceptible host populations with amoeba trophozoites but no amoebae cysts (model 5, $\varepsilon_c = 0$) indicated that no subsequent epizootics occurred. At these simulated trait values long-term plague persistence was reliant on the ability of *Y. pestis* to survive within amoeba cysts. Whereas, in simulations of resistant host populations with amoeba trophozoites but no amoebae cysts (model 6, $\rho = 0.6$, $\varepsilon_c = 0$), trophozoites were sufficient to enable persistence of *Y. pestis* for the entire duration of the simulation resulting from reciprocal re-infection of host and reservoir populations that generated pseudo-enzootic dynamics (Table 4.3).

Models 7 & 8: Susceptible hosts and amoeba reservoirs with host immigration excluded or alternate hosts excluded

Our model also provides insight into the role of host demographics and life-history on disease dynamics. Model 7 excluded immigration of susceptible hosts following the initial

outbreak and resulted in dramatic reductions in host abundance for the entire simulation period relative to populations where immigration was permitted. Immigration of susceptible hosts was positively correlated with successful host recolonization following the initial simulated outbreak. Model 7, which prevented immigration of susceptible hosts (reducing host abundance) also resulted in far fewer outbreaks than model 8, which excluded alternate hosts (reducing intra-colony connectivity) (Table 4.3). Model 8 excluded alternative hosts that could aid in pathogen maintenance or transmission by increasing intra-colony connectivity. Simulations indicated significant differences in IOP relative to model 3, which included alternative hosts ($p < 0.001$). Absence of alternate hosts increased IOPs and decreased the frequency of outbreaks by reducing intra-colony connectivity.

Discussion

Plague is maintained at intra-colony spatial scales across multi-year periods of disease quiescence (Bertherat *et al.* 2007, Cui *et al.* 2013, Lowell *et al.* 2015, Seifert *et al.* 2016), thereby prompting mechanistic explanations of how and where this endogenous maintenance occurs. Historically, one of the most prominent hypotheses used to explain the diverse outbreak dynamics observed within the plague system involved separate enzootic and epizootic periods, consisting of different hosts, vectors, environments, and processes (Barnes 1993, Cully & Williams 2001, Gage & Kosoy 2005). Given over a century of investigation and little convincing evidence, it is highly improbable that plague is cryptically maintained in separate assemblages of mammalian hosts and flea vectors over periods of apparent quiescence (Gage & Kosoy 2005, Salkeld *et al.* 2016).

As an alternative, metapopulation models (Keeling & Gilligan 2000, Snäll *et al.* 2008, George *et al.* 2013, Salkeld *et al.* 2016) with local extinctions and recolonization have also been

posited as an explanation for re-occurrent outbreaks following periods of quiescence. However, explanations that *exclusively* rely on imported infections are inconsistent with evidence for endogenous maintenance. Neither of these proposed models adequately explain persistence of plague clones across quiescent periods where it becomes undetectable followed by re-emergence in the same location several years (or decades) later.

Here we advance a competing hypothesis that endogenous plague persistence can be explained by a long-term pathogen reservoir (e.g. amoebae) and that heterogeneous disease dynamics, including epizootic emergence and apparent enzootic maintenance, may result from host interactions with amoeba reservoirs under particular ecological conditions and epidemiological trait values. The existence of an environmental plague reservoir was hypothesized as early as 1894 (Yersin 1894), and has recently been supported by laboratory evidence indicating *Y. pestis* can survive and replicate in trophozoite amoebae (Benavides-Montaña & Vadyvaloo 2017, Markman *et al.* 2018). Additional studies demonstrate the ability of related *Y. enterocolitica* and *Y. pseudotuberculosis* to survive within amoebae cysts (Nikul'shin *et al.* 1992, Lambrecht *et al.* 2013, Santos-Montañez *et al.* 2015). Our findings are consistent with extensive laboratory and field research, including an independent 25-year dataset, and offer a unified framework that is capable of explaining plague maintenance over long quiescent periods and emergence of both epizootic and pseudo-enzootic dynamics.

Models 1-4

Our results suggest that telluric microorganisms similar to amoebae can serve as a long-term plague reservoir over multi-year periods and subsequently facilitate outbreak re-emergence via spillover into susceptible host populations. No other natural mechanisms considered in our

model (resistant hosts, host carcasses, or questing vectors) were able to endogenously maintain plague over multi-year periods of disease quiescence.

Model dynamics indicated that amoeba reservoirs were predominantly responsible for maintenance and initial re-emergence (spillover), whereas the majority of subsequent pathogen amplification and transmission occurs within host and vector populations as driven by host-connectivity (Snäll *et al.* 2008, Salkeld *et al.* 2010, Savage *et al.* 2011, Salkeld *et al.* 2016), host abundance (Davis *et al.* 2004, Salkeld *et al.* 2010), flea abundance (Tripp *et al.* 2009), vector competence of fleas (Wilder *et al.* 2008), and climatic conditions (Snäll *et al.* 2008, Savage *et al.* 2011, Ben-Ari *et al.* 2012). This finding supports results from prior modeling studies (Buhnerkempe *et al.* 2011, Richgels *et al.* 2016) that indicate enzootic dynamics rely on non-host reservoirs and that epizootic amplification relies on vector-mediated transmission and amplification among host populations. Additionally, the ability for realistic values of reservoir fecundity and host resistance to produce both epizootic and pseudo-enzootic dynamics (Figure 4.5A) are in agreement with several prior works that also suggest trait-based rationale for heterogeneous disease dynamics (Gage & Kosoy 2005, Buhnerkempe *et al.* 2011, Salkeld *et al.* 2016).

It is perhaps intuitive to see how infectious reservoirs enable spillover into highly susceptible populations and produce epizootic dynamics. However, it may be less intuitive to discern how infectious reservoirs contribute to pseudo-enzootic dynamics. Simulations using high host resistance in the absence of amoebae reservoirs were unable to endogenously maintain plague. In order to generate dynamics that appear enzootic (characterized by continuous low-level infection among resistant host populations), spillover events must occur more frequently. We found that simulations including amoebae reservoirs and high host resistance resulted in

frequent outbreaks, short IOPs, and low host mortality (Figure 4.5A), thereby generating the appearance of enzootic dynamics. In this case, while the pathogen is maintained, infections arise from frequent spillover from infectious reservoir populations (Figure 4.1B). This dynamic may have the appearance of true enzootic persistence, which relies on low levels of transmission among mammalian hosts and flea vectors. However, the model depends upon continual spillover from the reservoir itself, and thus we call it “pseudo-enzootic. The inability of resistant hosts to endogenously sustain plague and the ability of key eco-epidemiological traits to elicit epizootic and pseudo-enzootic dynamics suggests that the same entities and processes may underlie heterogeneous plague dynamics. This appears contrary to the classical enzootic hypothesis that involves separate host and vector assemblages.

Models 5-6

Simulations of susceptible host populations with amoeba trophozoites and no amoeba cysts were unable to sustain *Y. pestis* long enough to cause subsequent epizootics in host populations. However, model 6, parameterized in the same way but with resistant hosts was able to sustain *Y. pestis* for the entire simulation duration. These results further indicate a relationship between host resistance and infectious reservoir fecundity in driving plague heterogeneous maintenance dynamics. Model dynamics indicate a reciprocal cycle of re-infection between partially resistant host populations ($\rho = 0.6$) and amoeba trophozoites. We hypothesize maintenance was possible under these conditions because the IOP in resistant host populations was shorter than the lifespan of infected amoebae trophozoites, thus enabling spillover to occur. Conversely, in model 5, with susceptible host populations, the IOP was longer than the maximum survival of an infected trophozoite and spillover could not occur. Laboratory evidence demonstrates that infected amoebae maintain equal fitness with uninfected amoebae, which

suggests that other eco-environmental conditions (e.g. temperature extremes) may be predominantly responsible for trophozoite mortality when they are artificially prevented from encysting as in these two model simulations.

Models 7-8

Simulations where there was no immigration of susceptible hosts (resulting in low host abundance) (model 7) had far fewer subsequent outbreaks than simulations that had low intra-colony connectivity (model 8). This may indicate that endogenous persistence is more heavily reliant on abundance than intra-colony connectivity. However, it is difficult to disentangle these related metrics.

In model 8, we removed alternate hosts to reduce intra-colony connectivity. However, we did not explicitly examine the impact of broader inter-colony connectivity among host meta-populations in order to evaluate endogenous maintenance potential. While host meta-populations may be important for plague transmission during epizootics and may be sufficient to maintain plague over shorter quiescent periods, we demonstrate that host meta-population structure is not a requirement for endogenous plague persistence when amoeba reservoirs are considered. This may indicate a mechanisms by which plague can persist in host populations that don't exhibit meta-population structure.

Prior studies have also suggested a relationship between outbreak likelihood and past host abundance (Davis *et al.* 2004) and prior quantitative modeling has indicated that colony size is a stronger determinant of plague persistence than inter-colony connectivity (George *et al.* 2013). Each of these findings is concordant with large-scale epizootics amplifying plague bacteria into soil via carcasses of killed hosts, thereby seeding amoebae trophozoites and creating a highly localized infectious reservoir pool. As host recolonization occurs, individuals re-excavate vacant

burrow systems and may encounter pockets of infectious soil-borne amoebae. Host abundance and density may provide predictive insight into when recolonizing populations are at increased risk of exposure to infectious amoebae reservoirs and facilitating onward transmission.

We suggest future investigations prioritize empirical evaluation of *Y. pestis* survival in amoeba cysts and development of isolation techniques capable of detecting *Y. pestis* inside amoebae in natural environments. This model may be improved by implementing a spatially-explicit metapopulation or gravity model to more realistically approximate the impact of host inter-colony connectivity on long-term plague persistence. Additionally, implementation of a climate function that uses historical temperature and precipitation data may enhance model comparison with recorded outbreak dynamics. This modeling framework may also be used to investigate disease dynamics in response to host vaccination campaigns or other intervention strategies attempting to induce eco-epidemiological conditions that leading to pathogen extinction.

Conclusion

Our findings simultaneously indicate that endogenous (intra-colony) plague persistence requires a long-term reservoir and that natural variation in eco-epidemiological traits can drive both the emergence of epizootics and pseudo-enzootic disease dynamics that are consistent with laboratory experiments and 25 years of independent field data. These results suggest a unified explanation for plague persistence over long quiescent periods and emergence of both epizootic and pseudo-enzootic dynamics. This quantitative framework may be applied to numerous zoonotic disease systems, especially those hypothesized to involve an environmental reservoir, and may enable development of more accurate outbreak forecasting and effective prevention

strategies without misappropriating limited resources towards unlikely causes of re-emergence of highly lethal zoonotic pathogens.

Figures

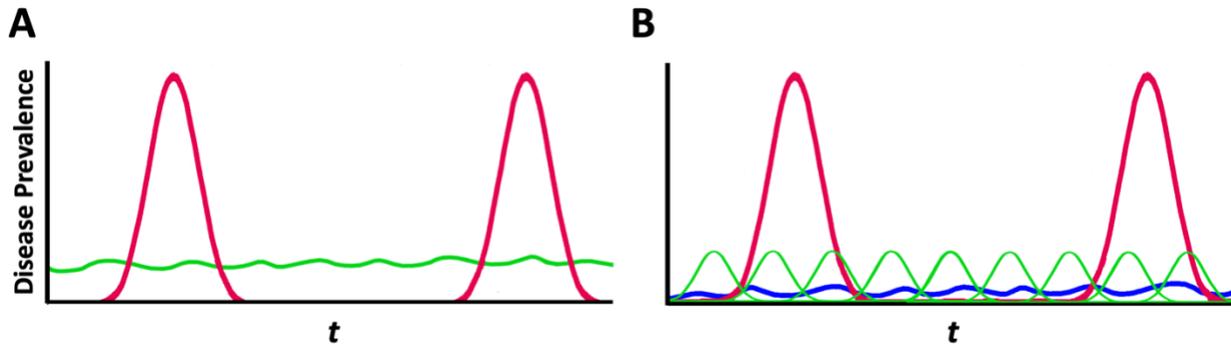


Figure 4.1: Conceptual diagram of hypothesized endogenous maintenance dynamics. A) The enzootic maintenance hypothesis is characterized by continuous low-level transmission among resistant host populations (green) with occasional spillover into highly susceptible host populations which causes epizootic emergence (red). **B)** The reservoir hypothesis is characterized by latent pathogen persistence in reservoirs (blue) with potential for spillover into both susceptible (red) and resistant (green) host population, respectively causing high or low mortality. Pseudo-enzootic dynamics (green) may emerge under eco-epidemiological conditions where reservoir spillover events are frequent and host resistance is sufficiently high to prevent mass mortality. Field sampling bias may give the appearance that enzootic dynamics are present, when in fact they are pseudo-enzootic and the result of frequent spillover from reservoir populations.

indicated by solid red arrows. Variables that directly impact other variables without involving a class transition are indicated by green dashed arrows. Additional variable and parameter description is available in Table 4.1, Table 4.2.

Figure 4.3: Equations

Host sub-model

$$\frac{dS}{dt} = \phi RS \left[\mu + (\beta_e F_{eq} + \beta_l F_{lq}) \left(1 - e^{-aN/(B_n - B_r)} \right) + \frac{1}{B_n - B_r} (\beta_m M + \beta_i I + \beta_d A_i/Q) \right] + r_n(t)(S + R + w)(1 - N/K_n)$$

1. $r_n(t) = ke^{-s \cos^2(\pi t - b)}$

2. $\frac{dE}{dt} = S \left[(\beta_c F_{eq} + \beta_i F_{lq}) \left(1 - e^{-aN/(B_n - B_r)} \right) + \frac{1}{B_n - B_r} (\beta_i \psi + \beta_d A_i/Q) \right] - (\sigma + \mu)E$

3. $\frac{dI}{dt} = \sigma(1 - p)E - \alpha I$

4. $\frac{dR}{dt} = \sigma p E - (\phi + \mu)R$

5. $\frac{dM}{dt} = \alpha I - \lambda_m M$

Vector sub-model

6. $\frac{dF_{sh}}{dt} = F_{sq} \left(1 - e^{-aN/(B_n - B_r)} \right) - F_{sh} (\mu_{fh} + \delta + (\gamma I/N)) + \theta_l F_{lh} (S + E + R)/N + r_n(t)(S + R + w)(1 - N/K_n)r_w$

7. $\frac{dF_{sq}}{dt} = \delta F_{sh} + r_f F_0 (N/(1 + M + F_0)) - F_{sq} (\mu_{fq} + (1 - e^{-aN/(B_n - B_r)})) + \zeta_l F_{lq}$

8. $\frac{dF_{ch}}{dt} = F_{eq} \left(1 - e^{-aN/(B_n - B_r)} \right) - F_{ch} [\mu_{fh} + \delta + \alpha + \theta_e + ((S + E + R)/N)] + F_{sh} \gamma (I/N) + F_{lh} \gamma (I/N)$

9. $\frac{dF_{eq}}{dt} = F_{ch} (\delta + \alpha) - F_{eq} [\mu_{fq} + (1 - e^{-aN/(B_n - B_r)})] - \zeta_e F_{eq}$

10. $\frac{dF_{lh}}{dt} = F_{lq} \left(1 - e^{-aN/(B_n - B_r)} \right) + \theta_e F_{ch} (S + E + R)/N - F_{lh} [\mu_{fh} + \delta + \alpha + \gamma (I/N) + \theta_l (S + E + R)/N]$

11. $\frac{dF_{lq}}{dt} = (\delta + \alpha) F_{lh} - F_{lq} [\mu_{fq} + (1 - e^{-aN/(B_n - B_r)})] + \zeta_e F_{eq} - \zeta_l F_{lq}$

Reservoir sub-model

12. $\frac{dA_i}{dt} = r_a A_i (1 - A_i/K_a) G(t) + \lambda_m M Q + \epsilon_t G(t) A_{ci} - \mu_t A_i G(t) - (\mu_{t2} + \epsilon_c) (1 - G(t)) A_i$

13. $\frac{dA_{ci}}{dt} = \epsilon_c (1 - G(t)) A_i - \epsilon_t G(t) A_{ci} - \mu_c A_{ci}$

Environmental driver

14. $G(t) = -\frac{\cos(\pi t/90 + 1)}{2}$

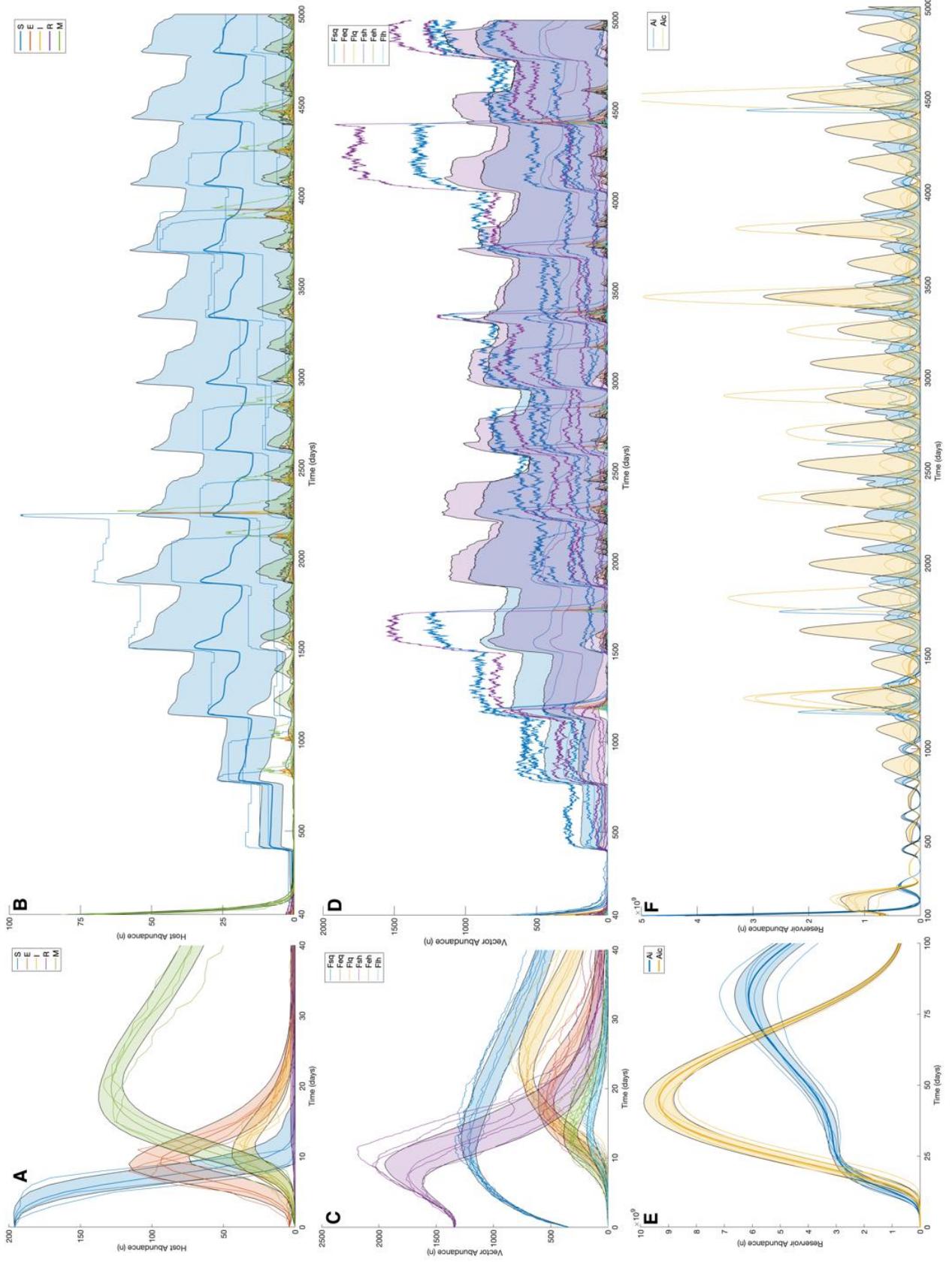


Figure 4.4: Mean stochastic trajectories of susceptible host (A, B), flea vector (C, D), and amoeba reservoir populations (E, F) are indicated by bold lines(model 3: susceptible hosts with reservoirs). Epizootic dynamics are shown in panels A, C, and E, whereas long-term dynamics encompassing epizootic and inter-epizootic dynamics are shown in panels B, D, and F. Shaded regions indicate +/- one standard deviation of the mean and example individual trajectories are depicted by thin lines.

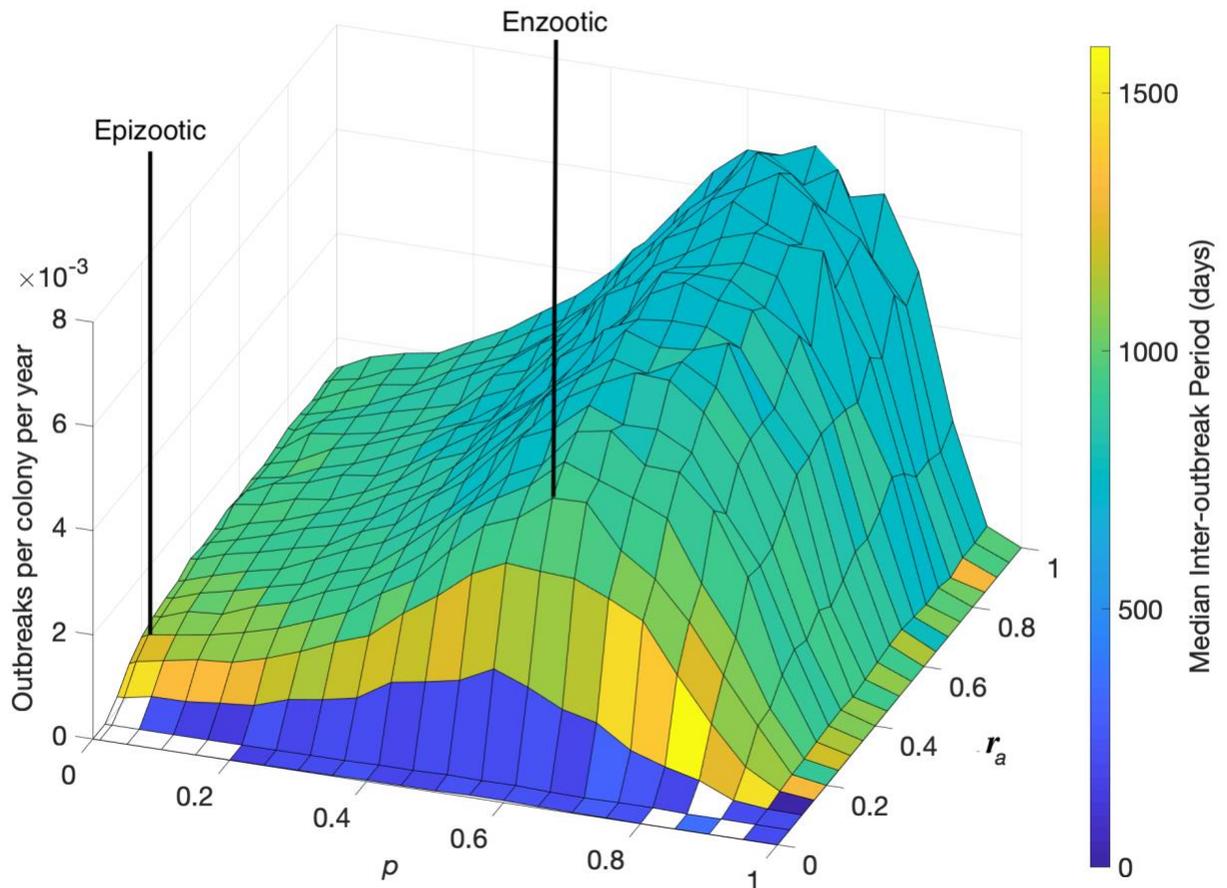


Figure 4.5A: Relationship between infectious reservoir fecundity (r_a), host resistance (ρ), and outbreaks per colony per year. The color of each square on the three-dimensional grid indicates the median inter outbreak period (IOP) for each parameter combination. Variation in both host resistance and infectious reservoir fecundity drive the emergence of epizootic or pseudo-enzootic disease dynamics and revealed a predominantly inverse relationship between IOP and outbreak frequency. Infrequent outbreaks coupled with longer IOPs and high host-mortality (low host resistance) characterize epizootic conditions experienced by black-tailed prairie dogs (e.g. lower left corner of the plot), whereas frequent outbreaks with shorter IOPs and high host resistance characterizes enzootic conditions (the central ridge in the plot). As host resistance approaches 60% and as infectious reservoir fecundity increases, enzootic conditions predominate. When infectious reservoir fecundity is low and host resistance is either low or extremely high, epizootic conditions predominate. Re-occurrent outbreaks are rare or non-existent (white squares) whenever reservoir fecundity is extremely low ($r_a < 0.1$) or when host resistance is extremely high ($\rho > 0.95$). Host population responses to variation in these parameters may explain heterogenous outbreak dynamics observed in natural populations and inform host vaccination campaigns or other intervention strategies attempting to induce eco-

epidemiological conditions that lead to pathogen extinction. These eco-epidemiological traits may also be applied to determine host population responses in other disease systems characterized by heterogenous host resistance and infectious reservoir fecundity.

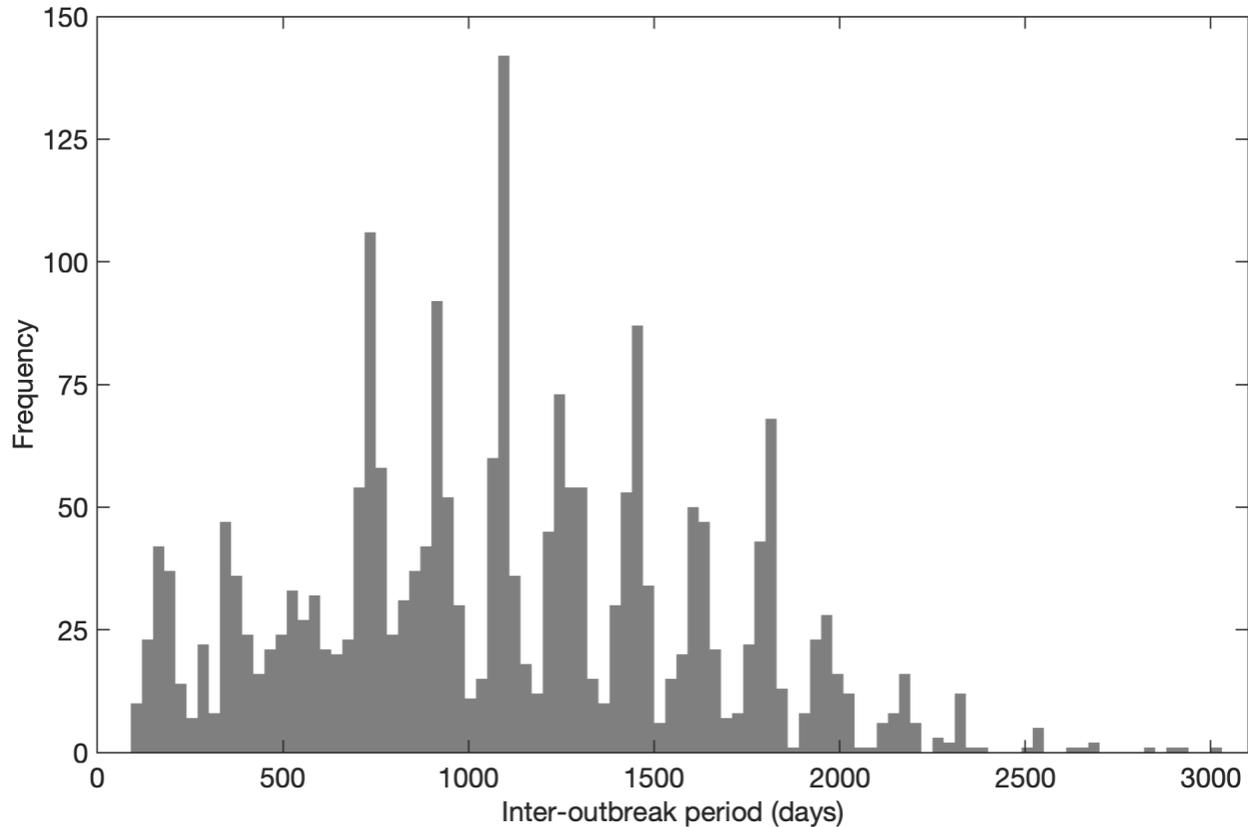


Figure 4.5B: The distribution of inter-outbreak periods (IOP) observed across 500 simulations corresponds with naturally occurring outbreak seasons (e.g. epizootics do not frequently occur in winter or mid-summer). IOP distribution is centered around a mean of 1,105 days (median 1,090 days) and the distribution within each seasonal peak is approximately normally distributed. These dynamics approximate natural conditions as indicated by IOP estimates from a 25-year field dataset on susceptible Black-tailed prairie dogs in a Northern Colorado, US, plague foci (median IOP 1,095 days, Table 4.3).

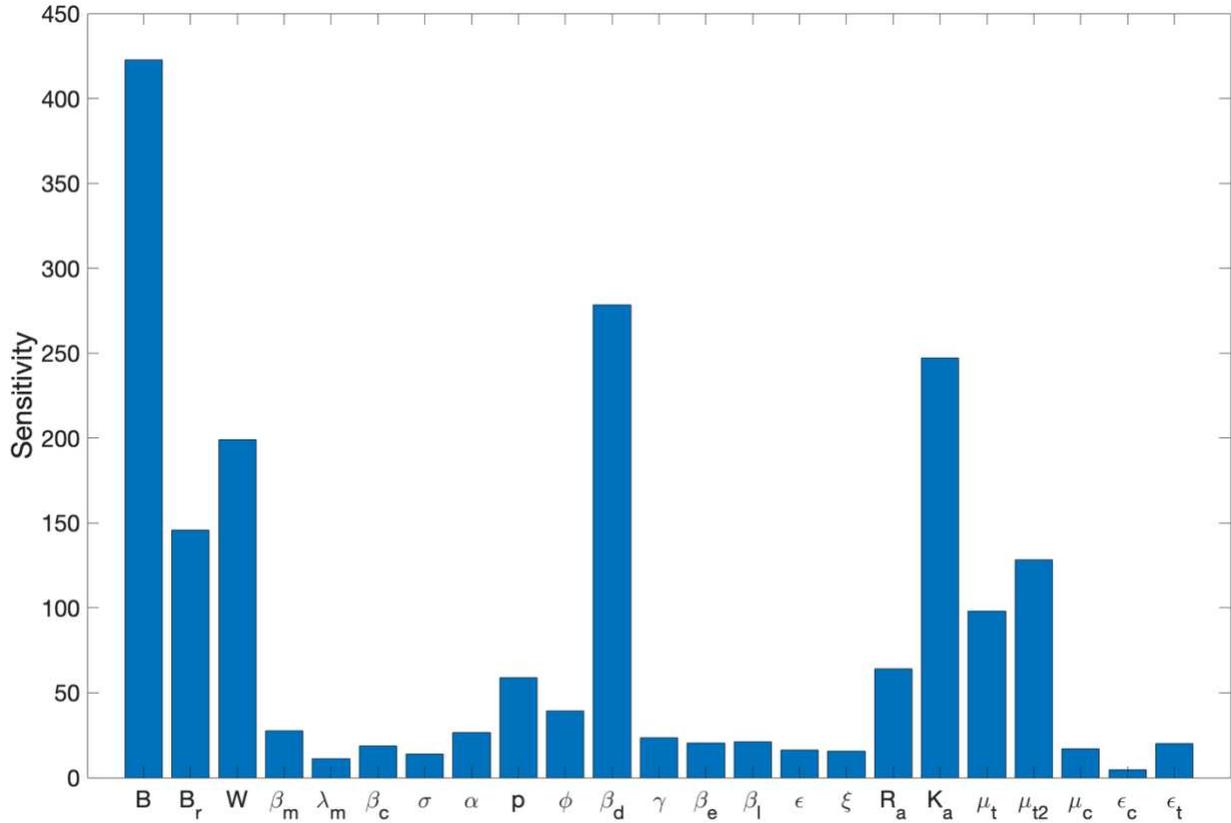


Figure 4.6: Sensitivity of inter-outbreak period (IOP) to variation in individual model parameters. 23 parameters (x-axis) were selected based on their natural variability (i.e. model parameters that do not vary were excluded) and were individually analyzed for their relative impact on IOP. Each parameter was randomly sampled from a log normal distribution of values spanning +/- one order of magnitude relative to the baseline parameter value in Table 4.2. Each parameter was randomly perturbed 100 times and 200 simulations were performed for each perturbation to obtain a distribution of mean IOP. We regressed mean IOP values against perturbed parameter values that were natural log transformed. Then we defined sensitivity (y-axis above) as the absolute value of the first order derivative of the line of best fit from the regression. The duration of IOP in the stochastic model is sensitive to measures of intra-colony connectivity (B_n and B_r), host immigration (w), host resistance (ρ), disease transmission rate from amoebae to susceptible hosts (β_d), and a suite of amoebae traits including reproductive rate (R_a), carrying capacity (K_a), and natural mortality rates (μ_t and μ_{t2}).

Table 4.1: Model variables to simulate plague system

Variable	Initial Value (individuals)	Description	Reference
S	196	Susceptible host	Webb <i>et al.</i> 2006
E	4	Exposed host	Webb <i>et al.</i> 2006
I	0	Infectious host	Webb <i>et al.</i> 2006
R	0	Resistant host	Buhnerkempe <i>et al.</i> 2011, Richgels <i>et al.</i> 2016
M	0	Infectious carcass	Buhnerkempe <i>et al.</i> 2011
N	$S+E+I+R$	Total hosts	Richgels <i>et al.</i> 2016
F_{su}	336	Susceptible, questing fleas	Webb <i>et al.</i> 2006, Tripp <i>et al.</i> 2009, Buhnerkempe <i>et al.</i> 2011
F_{sh}	1344	Susceptible, on-host fleas	Webb <i>et al.</i> 2006, Tripp <i>et al.</i> 2009, Buhnerkempe <i>et al.</i> 2011
F_{su}	0	Highly infectious questing fleas	Webb <i>et al.</i> 2006, Tripp <i>et al.</i> 2009, Buhnerkempe <i>et al.</i> 2011
F_{eh}	0	Highly infectious on-host fleas	Webb <i>et al.</i> 2006, Tripp <i>et al.</i> 2009, Buhnerkempe <i>et al.</i> 2011
F_{lu}	0	Moderately infectious questing fleas	Webb <i>et al.</i> 2006, Tripp <i>et al.</i> 2009, Buhnerkempe <i>et al.</i> 2011
F_{lh}	0	Moderately infectious on-host fleas	Webb <i>et al.</i> 2006, Tripp <i>et al.</i> 2009, Buhnerkempe <i>et al.</i> 2011
F_0	$F_{sh} + F_{eh} + F_{lh}$	Breeding on-host fleas yielding F_{su}	Buhnerkempe <i>et al.</i> 2011
A_i	0	Infected amoeba trophozoites	
A_{ci}	0	Infected amoeba cysts	

Table 4.2: Model parameters to simulate plague system

Parameter	Value	Description	Reference
Host Life History			
K_n	200, ind	Carrying capacity of prairie dogs	Hoogland 1995
μ_n	0.0002, days ⁻¹	Natural host mortality rate	Evans & Holdenried 1943, Hoogland 1995, Hoogland 2001
β_n	20, days ⁻¹	Number of burrows hosts use per day	Linsdale 1946, Hoogland 1995, Webb <i>et al.</i> 2006
β_r	4.25, days ⁻¹	Number of active burrows entered per day by alt-hosts	Kraft & Stapp 2013
k	0.0336, unitless	Scale of the seasonal birth pulse	King 1955, Hoogland 2001, Peel <i>et al.</i> 2014
s	150, unitless	Birth pulse duration	Hoogland 2001, Peel <i>et al.</i> 2014
b	$-\pi/3$, unitless	Phase of birth pulse	Hoogland 2001, Peel <i>et al.</i> 2014
w	10, ind	Annual host immigration	Knowles 1985, George <i>et al.</i> 2013
Host Infection			
β_m	0.073, days ⁻¹	Transmission rate of infectious carcass	Gani & Leach 2004, Webb <i>et al.</i> 2006
λ_m	0.04, days ⁻¹	Decay rate of infectious carcass	Putnam 1978, Webb <i>et al.</i> 2006, Godbey <i>et al.</i> 2006, Boone <i>et al.</i> 2009
β_c	0.073 days ⁻¹	Transmission rate of direct contact	Gani & Leach 2004, Webb <i>et al.</i> 2006
ψ	0.79, unitless	Proportion of pneumonic infections	Busch <i>et al.</i> 2013, Richgels <i>et al.</i> 2016
σ	0.22, days ⁻¹	Exposed incubation period	Quan <i>et al.</i> 1985, Cully & Williams 2001
α	0.5, days ⁻¹	Mortality rate of diseased hosts	Cully & Williams 2001, Lorange <i>et al.</i> 2005
ρ	0.01, unitless	Proportion of exposed hosts that become resistant	Cully & Williams 2001
φ	0.011, days ⁻¹	Rate resistance is lost	Quan <i>et al.</i> 1985
β_d	0.00096, days ⁻¹	Transmission rate from A_i to S	Boegler <i>et al.</i> 2012
Flea Life History			
r_f	1.5, ind	Flea births per blood meal	Webb <i>et al.</i> 2006
μ_m	0.01 days ⁻¹	Natural on-host flea mortality rate	Eskey & Haas 1940
μ_{fa}	0.07 days ⁻¹	Natural off-host flea mortality rate	Krasnov <i>et al.</i> 2002, Wilder <i>et al.</i> 2008a
δ	0.059 days ⁻¹	Rate of leaving hosts	Hartwell <i>et al.</i> 1958
a	0.02 days ⁻¹	Questing efficiency	Hartwell <i>et al.</i> 1958, Webb <i>et al.</i> 2006
r_w	8.4-12.2, ind	Number of fleas on immigrating hosts	Webb <i>et al.</i> 2006, Tripp <i>et al.</i> 2009
Flea Infection			
γ	0.84, days ⁻¹	Transmission rate from host to flea	Eisen <i>et al.</i> 2006, Wilder <i>et al.</i> 2008a, Wilder <i>et al.</i> 2008b
β_o	0.044, days ⁻¹	Transmission rate of EP1 flea	Eisen <i>et al.</i> 2006, Wilder <i>et al.</i> 2008a, Wilder <i>et al.</i> 2008b
β_i	0.01, days ⁻¹	Transmission rate of EP2 flea	Eisen <i>et al.</i> 2006, Wilder <i>et al.</i> 2008a, Wilder <i>et al.</i> 2008b
θ_o	1, unitless	Conversion rate from EP1 to EP2 while feeding	Eisen <i>et al.</i> 2006, Wilder <i>et al.</i> 2008a, Wilder <i>et al.</i> 2008b
θ_i	1, unitless	Conversion rate from EP2 to susceptible while feeding	Eisen <i>et al.</i> 2006, Wilder <i>et al.</i> 2008a, Wilder <i>et al.</i> 2008b
ζ_o	0.125, days ⁻¹	Conversion rate from EP1 to EP2 while not feeding	Eisen <i>et al.</i> 2007
ζ_i	0.033, days ⁻¹	Conversion rate from EP2 to susceptible while not feeding	Engelthaler <i>et al.</i> 2000
Amoeba Life History			
r_a	0.2, days ⁻¹	Reproductive rate of trophozoite amoeba	Khan 2006
K_a	$4*10^8$, ind	Carrying capacity of A_i in soil impacted by carcass	Khan 2006, Koshel' <i>et al.</i> 2015, Markman <i>et al.</i> 2018
μ_t	0.04762, days ⁻¹	Natural mortality of A_i under optimal conditions	Markman <i>et al.</i> 2018 (unpublished data)
μ_{t2}	0.16666, days ⁻¹	Natural mortality of A_i under sub-optimal conditions	Lambrech <i>et al.</i> 2015, Markman <i>et al.</i> 2018
μ_c	0.00013, days ⁻¹	Natural mortality of cysts	Sriram <i>et al.</i> 2008
ϵ_c	0.2 days ⁻¹	Conversion rate from A_i to A_{ci}	Lambrech <i>et al.</i> 2015
ϵ_t	0.08 days ⁻¹	Conversion rate from A_{ci} to A_i	Foissner <i>et al.</i> 1999
Amoeba Infection			
q	$182*10^6$, ind	Number of A_i produced per carcass	Khan 2006, Koshel' <i>et al.</i> 2015, Markman <i>et al.</i> 2018

Table 4.3: Summary outputs of model variations

Table 3. Summary output of model variants

	1) susceptible hosts, no reservoir	2) resistant hosts, no reservoir	3) susceptible hosts, reservoir	4) resistant hosts, reservoir	5) susceptible hosts, no cysts	6) resistant hosts, no cysts	7) susceptible hosts, reservoir, no immigration	8) susceptible hosts, reservoir, low connectivity	9) field data
Subsequent outbreaks (n)	-	32	2208	2637	-	3747	108	1602	70
Max persistence of <i>Y. pestis</i> (days)	291	525	5000	5000	329	5000	5000	5000	-
IOP (days)									
Mean	-	289.72	1104.76	754.50	-	770.17	1216.95	1222.80	1553.86
Median	-	195.00	1090.50	614.00	-	712.00	1270.50	1235.50	1095.00
SD	-	144.97	517.27	531.48	-	424.17	713.13	579.69	1185.08
CI (+/-)	-	50.23	21.58	20.29	-	13.58	134.49	28.39	277.62

CHAPTER 5 – SIMPLEX AND MULTIPLEX PCR TARGETING CLINICALLY SIGNIFICANT AMOEBAE

Introduction

Amoebae are the etiologic agents of numerous diseases, globally causing up to 3.2 million combined annual cases of blindness, cutaneous ulcers, liver abscesses, diarrheal dehydration, and encephalitis, with case fatality rates reaching as high as 97%. The speed of clinical intervention is inhibited by a shortage of comprehensive and rapid diagnostic tools capable of identifying and differentiating between major pathogenic amoebae genera. We developed five end-point simplex PCR assays that can be combined into one multiplex PCR that targets five of the most clinically important amoebae genera that exhibit primary pathogenicity or significant associations with amoeba-resistant pathogens (ARP). This multiplex assay rapidly and specifically identifies clinically significant amoebae that have been cultured from clinical or environmental samples and could improve patient diagnosis and treatment in a wide range of settings.

Many amoeba species have two different developmental stages: the trophozoite, a mobile, phagocytic vegetative form, and the cyst, a dormant persistent form that allows the amoebae to survive for over 20 years in extreme environments (Mazur *et al.* 1995, Sriram *et al.* 2008). Several species have additional stages including aggregate social stages that produce germinating spores in *Dictyostelium spp.* (Paquet & Charette 2016) and flagellate stages in *Naegleria* (Visvesvara *et al.* 2007). The majority of known amoebae are not pathogenic themselves, but many associate with a subset of over 200 pathogens that are resistant to being digested by the amoebae (Greub & Raoult 2004, Schuster & Visvesvara 2004a, Schuster & Visvesvara 2004b, Thomas *et al.* 2008, Salah *et al.* 2009, Thomas *et al.* 2010, Siddiqui & Khan

2012, Wheat *et al.* 2014, Markman *et al.* 2017, Strassman & Shu 2017). These amoebae-resistant pathogens (ARPs) exploit the intracellular amoeba environment for survival and possibly replication (Barker & Brown 1994, Brown & Barker 1999).

Some amoebae genera are ubiquitous in natural soil and water bodies with the added potential for aerosolization in dust or water droplets (Esteban *et al.* 2006). The environmental diversity and relative abundance of many amoeba species scales linearly over spatial area (Esteban *et al.* 2006), indicating relatively equal potential for human-amoeba encounters across the globe. Some amoeba trophozoites or cysts can survive broad and extreme environmental conditions including: 0-100% moisture, pH 1.0-12, 0-55°C, γ irradiation (250 rads), UV irradiation (800 mJ/cm²), and numerous modern disinfecting chemicals (Schuster & Visvesvara 1996, Aksozek *et al.* 2002, Sheehan *et al.* 2003, Khan 2006, Visvesvara *et al.* 2007, Coulon *et al.* 2010). Amoebae are also commonly found in domestic and clinical niches that contain water including, ventilation, air conditioning, drinking water systems, dialysis machines, and dental machinery (Greub & Raoult 2004, Schuster & Visvesvara 2004a, Schuster & Visvesvara 2004b, Khan 2006, Thomas *et al.* 2008, Salah *et al.* 2009, Thomas *et al.* 2010).

Global case burden of pathogenic amoeba likely exceeds 3.2 million per year (Perez & Bush 2007, Farrar *et al.* 2013, Schlossberg 2015) while the case burden resulting from amoeba-associated ARPs is unknown. Some amoebic diseases are non-fatal with clinical intervention (e.g. amoebic keratitis) (CDC 2018), whereas others may be characterized by extremely high case mortality rates despite intervention (e.g. >97% for primary amoebic meningoencephalitis (CDC 2018)) because of both awareness and effective drugs are lacking (Siddiqui & Khan 2008). The case burden of amoebic diseases is likely underestimated for the same reasons. Current diagnostic assays include cerebrospinal fluid (CSF) smear, culture, immunofluorescence

or polymerase chain reaction (PCR) (Qvarstrom 2006). Advancements enabling more rapid detection and identification will enhance the likelihood of successful clinical intervention (Siddiqui & Khan 2008).

Amoebae genera identified by this multiplex PCR assay include, *Acanthamoeba*, *Balamuthia*, *Dictyostelium*, *Entamoeba*, and *Naegleria*. The pathological conditions associated with select species in each genera are described in Table 5.1.

Acanthamoeba is comprised of at least 15 distinct genotypes, eight of which are pathogenic (Schuster & Visvesvara 2004a). *Acanthamoeba spp.* annually cause between 1.2 and 3 million cases of amoebic keratitis (Schlossberg 2015) and several hundred estimated cases of fatal granulomatous amoebic encephalitis (Khan 2006). *Acanthamoeba spp.* also exhibit permissiveness to many amoeba-resistant pathogens (Greub & Raoult 2004, Schuster & Visvesvara 2004a, Schuster & Visvesvara 2004b, Khan 2006, Thomas *et al.* 2008, Thomas *et al.* 2010).

Balamuthia contains one pathogenic species, *B. mandrillaris*, with over 200 identified cases since initial discovery in 1993, 70 of which occurred in the US (Perez & Bush 2007). No associations between *B. mandrillaris* and ARPs have been reported.

The genus *Dictyostelium* is comprised of numerous species, including *D. discoideum*, which is most notable as a model research organism. *D. discoideum* is not usually classified as pathogenic but it caused one recorded case of amoebic keratitis (Reddy *et al.* 2010) and facilitates the survival, replication, and dissemination of many high-consequence ARPs (Hilbi *et al.* 2007, Cosson & Soldati 2008, Steinert 2011, DiSalvo *et al.* 2015, Paquet & Charette 2016, Taylor-Mulneix *et al.* 2017, Markman *et al.* 2017).

The genus *Entamoeba* contains at least 20 species, all of which are non-pathogenic with the exception of *E. histolytica* and rarely *E. gingivalis* (Schuster & Visvesvara 2004a, Jian *et al.* 2008). *E. histolytica* is responsible for over 110,000 deaths annually (Farrar *et al.* 2014, WHO 1997), whereas *E. gingivalis* is associated with cases of gingivitis, though it is disputed if it is the etiological agent. *E. histolytica* infection is not always lethal, but remains a large source of secondary bacterial infections and has a mutualistic relationship with several other pathogens, including HIV (Hung *et al.* 2005, Visvesvara 2013).

The genus *Naegleria* consists of at least 47 species, of which only one, *N. fowleri*, is pathogenic (De Jonckheere 2002). *N. fowleri* boasts a case fatality rate over 97%, one of the highest recorded across all human pathogens (CDC 2018). Only 5 out of 143 known infected individuals in North America from 1962 to 2017 have survived due to delayed detection and lack of universally effective drug therapies (CDC 2018). *N. fowleri* also serves as a permissive host to ARPs, including several *Legionella spp.* (Huang *et al.* 2011).

Several genera of amoebae were intentionally omitted in this multiplex PCR assay, including *Vermamoeba* (formerly *Hartmanella*) because of its rarity as an etiologic agent of disease (Abedkhozasteh *et al.* 2013) and infrequent association with ARPs (Hsu *et al.* 2011). *Sappinia spp.* were omitted due to only one attributed case of primary amoebic meningoencephalitis worldwide, from which the patient fully recovered (Gelman *et al.* 2001). Several genera of non-pathogenic amoeba that facilitate the survival, amplification, and dissemination of ARPs with lesser clinical significance have also been omitted (Barker & Brown 1994, Brown & Barker 1999, Greub & Raoult 2004, Schuster & Visvesvara 2004a, Schuster & Visvesvara 2004b, Thomas *et al.* 2008, Salah *et al.* 2009, Thomas *et al.* 2010, Siddiqui & Khan 2012b, Wheat *et al.* 2014, Markman *et al.* 2017, Strassman & Shu 2017).

We focused on five of the most clinically important amoebae genera that exhibit primary pathogenicity or significant associations with ARPs. We developed five end-point simplex PCR assays, which we combined into one multiplex PCR intended for use with unknown amoeba species cultured from clinical or environmental samples. This multiplex assay rapidly and specifically identifies clinically significant amoebae and could improve patient diagnosis and treatment.

Methods

Amoeba DNA samples

Stocks of *Acanthamoeba lenticulata* (ATCC 30841), *A. castellanii* (ATCC 30234), and *A. polyphaga* Linc-Ap1 (CCAP 1501/18) were obtained from the American Type Culture Collection (Manassas, VA, USA) and the Culture Collection of Algae and Protozoa (<https://www.ccap.ac.uk/>). *D. discoideum* (NC4A2) was obtained from DictyBase (<http://dictybase.org/>). Amoeba stocks were axenically cultivated with genera-specific media in T25 tissue culture flasks at 28°C and verified to be axenic using standardized methods (Schuster 2002, Thomas *et al.* 2006, Fey *et al.* 2013, Wheat *et al.* 2014, Markman *et al.* 2018). Isolation of genomic DNA from amoeba cultures was performed following standard protocol for cultured cells included with QIAamp DNA Mini Kit (Qiagen) and extracted DNA concentrations were determined with a Qubit 2.0 Fluorometer (ThermoFisher). Reference DNA for *N. fowlerii*, *B. mandrillaris*, and *E. histolytica* was generously provided by the US Centers for Disease Control Free-Living and Intestinal Amebas Lab in Atlanta, GA.

in silico reference templates and primer design

Primer design was performed by identifying complete mitochondrial genome sequences (Hwang & Kim 1999) for each target species in GenBank and individually entering each into

NCBI Primer-BLAST (Ye *et al.* 2012) as the template sequence for comparison against all available whole genome sequences for amoebae in the following genera: *Acanthamoeba*, *Vermamoeba*, *Hartmannella*, *Entamoeba*, *Balamuthia*, *Dictyostelium*, *Naegleria*, *Echinamoeba*, *Vahlkampfia*, *Paravahlkampfia*, *Sappinia*, and *Willertia*. Candidate primers were then selected based on desired amplicon length, desired melting temperature, maximum genus-level specificity, and maximum template complementarity (Table 5.1). Additional *in silico* analysis was performed using the Multiple Primer Analyzer (ThermoFisher) to estimate potential for primer-dimer formation. Primer specificity was also confirmed by independently performing pairwise simplex PCR reactions between each DNA isolate and each primer pair with positive and negative controls. All combinations were tested and negative for cross-amplification. Final *Acanthamoeba* primers required inclusion of degenerate bases to capture species diversity present within the genus.

PCR conditions

Individual simplex reactions were performed in 50 ul volumes using Platinum Taq DNA polymerase (Invitrogen, 1 U), 1x reaction buffer, 3 mM of MgCl₂, 0.2 mM of each dNTPs, 0.5 uM of each primer, and 2 ul of genomic DNA template (between 5 and 50 ng per reaction). PCR was then performed using a PTC-200 thermal cycler (MJ Research) with an initial denaturation step at 95C for 120 seconds, then 30 cycles of 95C for 30 seconds, 60C for 30 seconds, and 68C for 60 seconds, followed by a final elongation step at 68C for 300 seconds. 6ul of each PCR product was run on a 2% agarose gel using SYBR Safe DNA gel stain (Invitrogen) and detected via ultraviolet light (302 nm) in a Molecular Imager Gel Doc XR System (Bio-Rad Laboratories). Gel images were obtained using Image Lab software (Bio-Rad Laboratories version 6.0.1).

Multiplex reactions were optimized in 50 ul volumes using Platinum Taq DNA polymerase (Invitrogen, 2 U), 1x reaction buffer, 4 mM of MgCl₂, 0.2 mM of each dNTPs, 2 ul of each genomic DNA template (between 10 and 60 ng per reaction), and varied concentrations of each primer set (ENTA_F/R 3.5 uM, NAEG_F/R 0.3 uM, ACAN_F/R 5 uM, DICT_F/R 0.3 uM, BALA_F/R 0.1 uM). Multiplex PCRs using equimolar primer concentrations of 0.5 uM resulted in uneven amplification even after optimizing other reagents and cycling conditions. Empirically adjusting the concentration of each primer set resulted in equal amplification intensity across target loci (Henegariu *et al.* 1997). Taq DNA polymerase and MgCl₂ concentration was also increased to optimize multiplex PCRs (Henegariu *et al.* 1997). All remaining PCR and analysis conditions were identical to simplex reactions.

Results & Discussion

PCR Specificity

Amplicon length obtained in simplex and multiplex reactions matched *in silico* predictions (Figure 5.1, Table 5.1). Degenerate primers were used for *Acanthamoeba* to capture species diversity within the genus. This resulted in bright 510 bp amplicons and two additional faint amplicons at ~250bp and ~130 bp. Faint amplicons did not occur with any other tested amoeba species and amplicon length did not overlap with any other tested amoeba species. Absence of unexplained bands in agarose gels indicates there was no cross-amplification in the final simplex or multiplex reactions and that primer pairs are specific among tested amoeba isolates.

Simplex PCR products were individually purified and Sanger sequenced using their respective forward PCR primers (Table 5.1). Trace files were analyzed using 4Peaks (v1.8) and consensus FASTA sequences were aligned to known reference sequences using the NCBI

Nucleotide BLAST (BLASTn) search algorithm. All PCR product sequences matched intended targets with sequence identities ranging from 97.72% - 99.61% while also displaying no significant similarity to non-target sequences in target or non-target species.

PCR Sensitivity

Simplex reactions were more sensitive as indicated by the multiplex PCR requiring slightly increased concentrations of template DNA despite optimizing other reaction conditions. We hypothesize that the multiplex PCR was less efficient because of increased competition for reaction components, resulting in slightly reduced sensitivity. This was empirically verified by conducting multiplex reactions with varying quantities and concentrations of template DNA. Reduced sensitivity was only observed in multiplex reactions that contained all five genera of amoebae template DNA, whereas multiplex reactions containing only four template samples had approximately the same sensitivity as in simplex reactions. This further suggests that increased volume and concentration of template DNA reduced reaction efficiency through inhibition or competition. Despite a slight decrease in sensitivity, this multiplex assay functions with concentrations of template DNA that can be realistically isolated from unknown amoeba cultures from clinical or environmental samples.

Conclusion

Herein we developed simplex and multiplex end-point PCR protocols alongside five primer sets enabling the rapid and specific detection of clinically significant amoebae genera. The majority of amoebae-related disease occurs in impoverished regions characterized by scarce and rudimentary laboratory resources. These assays were optimized for simplex and multiplex end-point reactions to enable detection in a wider range of clinical and research settings than allowed by qPCR or ddPCR. The development of genus-level primer pairs enables detection of

primarily pathogenic amoeba species as well as other species in the same genus that may contribute to secondary pathogenesis via association with ARPs. These molecular assays could be used in as an alternative to, or in conjunction with, conventional culture methods to more rapidly inform clinical diagnosis and treatment.

Figures

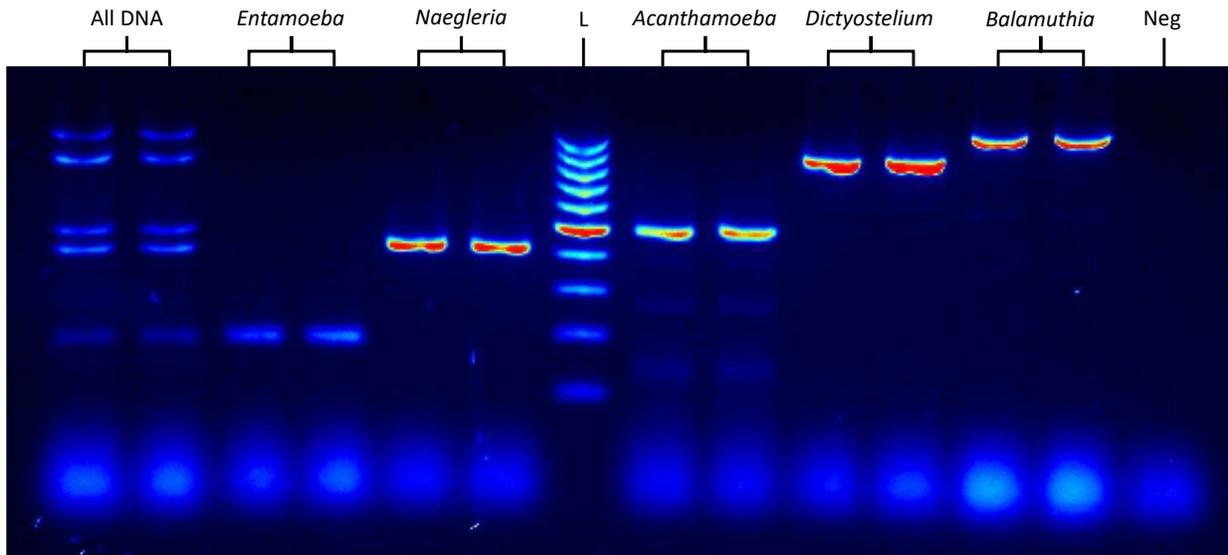


Figure 5.1: Multiplex PCR amplification using all primer pairs for multi-template DNA and single template DNA samples. Each reaction was run in duplicate. L: 100 bp DNA ladder (Invitrogen), Neg: control without template DNA.

Table 5.1: Reference species with associated clinical pathologies, primer sequences, and amplicon length. Pathology key: 1: amoebiasis (amoebic dysentery), 2: amoebic liver abscesses, 3: primary amoebic meningoencephalitis, 4: cutaneous amoebiasis, 5: granulomatous amoebic encephalitis, 6: amoebic keratitis, 7: generalized disseminated infection, 8: permissive to ARPs (Hung *et al.* 2005, Visvesvara *et al.* 2007, Siddiqui & Khan 2008, da Rocha-Azevedo *et al.* 2009, Visvesvara 2013, CDC 2018a,b,c,d,e).

Amoeba genera	Reference species	Pathology	Primer name	Primer sequence	Amplicon length
<i>Entamoeba</i>	<i>E. histolytica</i>	1, 2, 7	ENTA_F ENTA_R	TGGTTGTGTTGTACGCCTACCGA TTGTTGTGCTCGTGGTCCTTCGAT	184 bp
<i>Naegleria</i>	<i>N. fowleri</i>	3	NAEG_F NAEG_R	TCCCATGAACGAACGAGACCCTCA TGTTCCAGCCACAGATTCCTCTACG	437 bp
<i>Acanthamoeba</i>	<i>A. castellanii</i>	3, 4, 5, 6, 7, 8	ACAN_F ACAN_R	ACKGCRCAKGCMTTGTATGATT AGGATCKCCYCCGCCTGATGG	510 bp
<i>Dictyostelium</i>	<i>D. discoideum</i>	6, 8	DICT_F DICT_R	TGTTGGTTCCAATGCCCTAAGCGG TGTGCTGCAGGATGTTGTGGTCC	891 bp
<i>Balamuthia</i>	<i>B. mandrillaris</i>	3, 5, 7	BALM_F BALM_R	CGACCTCGTTGTGTTGTTGTGT GCCGCATCAGAAGCAGTAGC	1073 bp

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APPENDICES

Appendix 1: Supplementary Figures and Tables

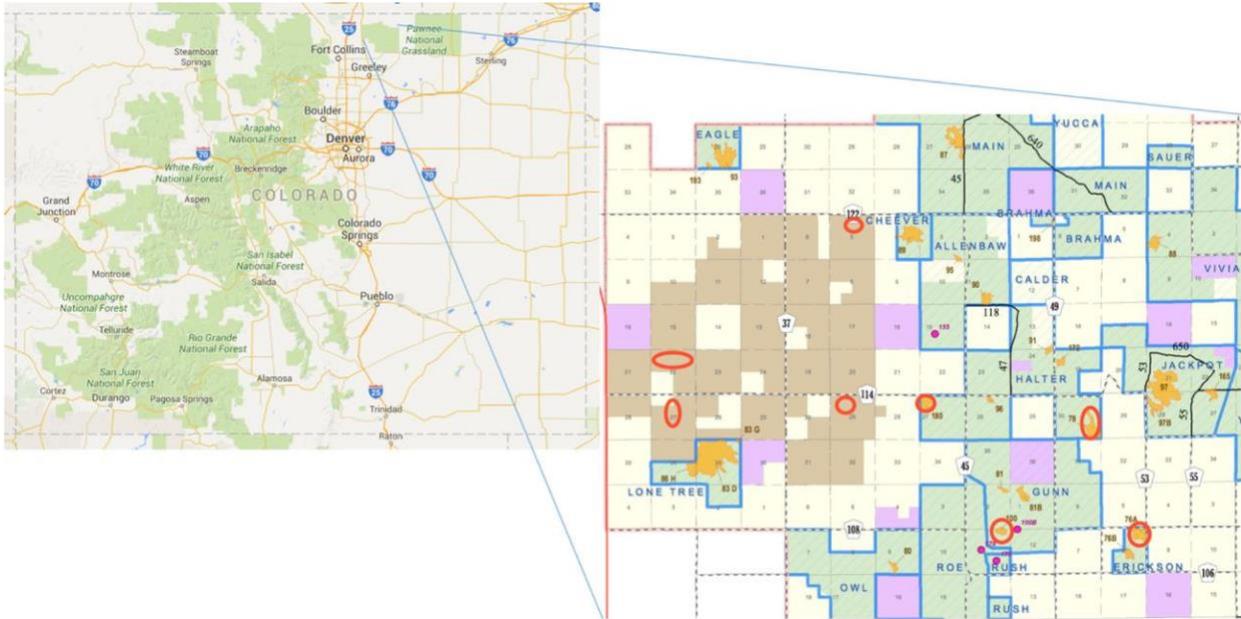


Figure S3.1: Pawnee National Grassland, Weld County, Colorado, US. Red circles indicate the burrows of 8 prairie dog colonies where plague epizootics were identified during 2015 and 2016 (Naylor 2016). Amebae were cultured from soil samples and identified to species by multiplex and endpoint PCR assays.

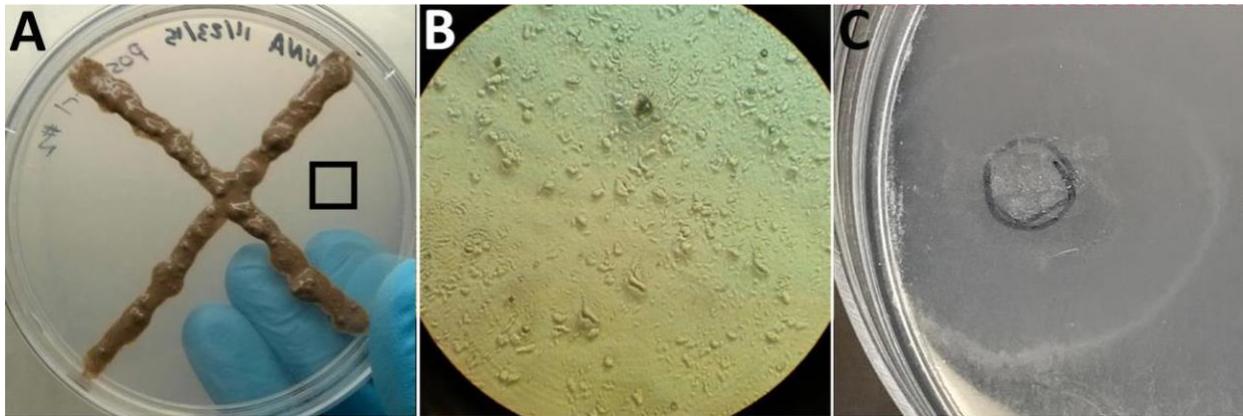


Figure S3.2: Amoebae isolated from soil samples by using modified methods (Bertherat 2016). A) Soil obtained from within a prairie dog burrow with an ongoing plague outbreak plated on ameba isolation agar that was pre-coated with heat-killed *Escherichia coli*. Black square indicates region of plate depicted at higher magnification in 2B. B) Trophozoite amoebae demonstrate faster motility than most soil microorganisms with the exception of fungal hyphae proliferation. Amoebae migrate away from the soil and associated contaminants while digesting the *E. coli* spread across the agar surface. Amoebae are characterized by their irregular shape with a large internal vacuole. Other diverse soil microorganisms are present on initial isolation plates. C) Ameba isolation agar depicting the migration of amoebae and clearance of *E. coli* lawn. Black circle indicates where amoebae excised from 2B were re-plated to support further purification by migration. Not pictured are the transfer and acclimation of amoebae to liquid cultures in genera specific media.

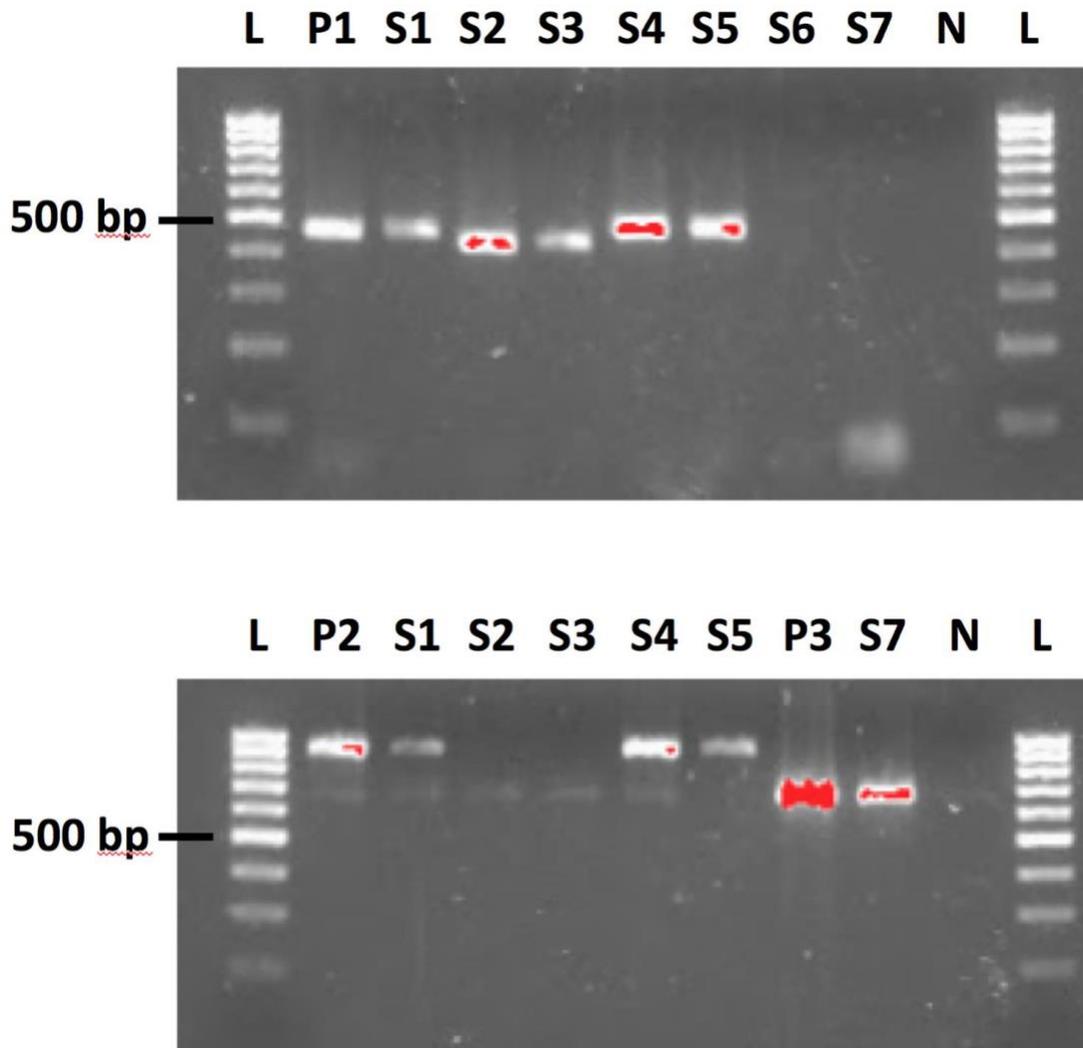


Figure S3.3: Representative gel after PCR was performed on amebae DNA extracted from soil isolates. The species of amebae isolated from the soil were used in subsequent laboratory experiments to test how environmental amebae and *Yersinia pestis* interact. Basepair ladder (L), *Acanthamoeba* spp. positive control (P1, 423–551bp) (Schroeder *et al.* 2001); *Dictyostelium discoideum*–positive control (P2, 900bp) (Charette & Cosson 2004); and *Vermamoeba vermiformis*–positive control (P3, 700bp) (Lasjerdi *et al.* 2011); soil samples (S1–S5).

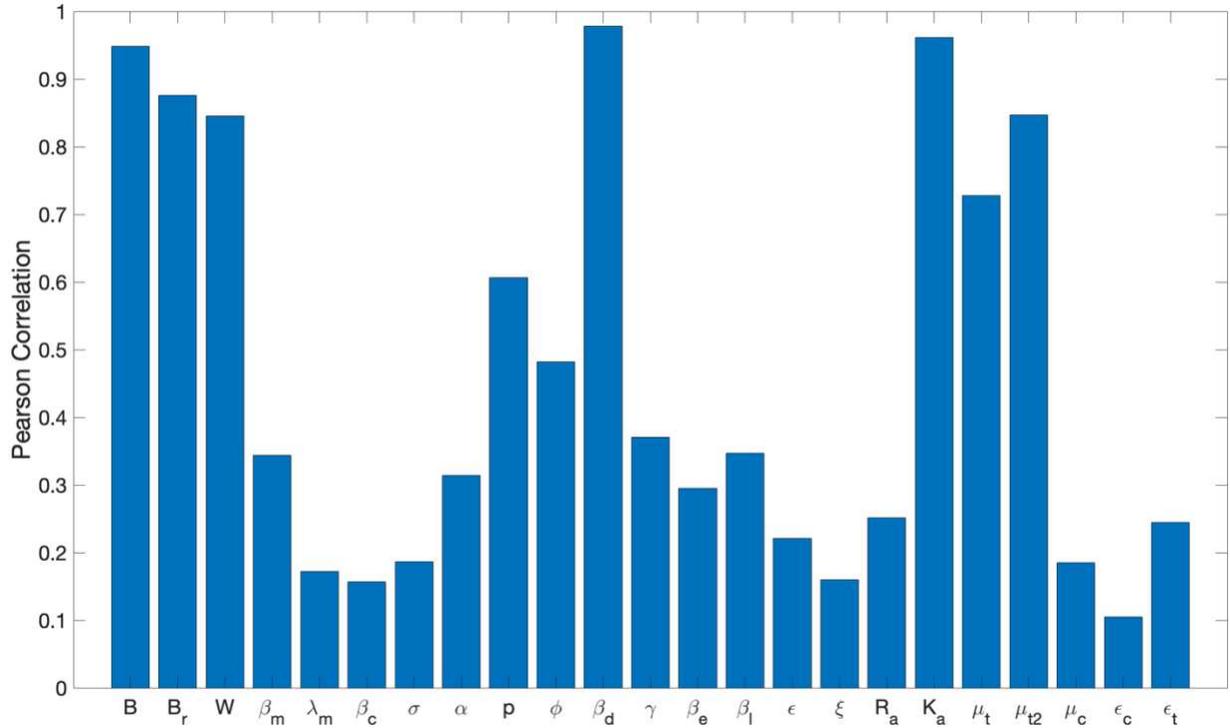


Figure S4.1: Partial regression correlation coefficients (PRCC) were calculated to quantify the strength of association of parameters with respect to inter-outbreak period (IOP). Several parameters exhibit strong correlation with model output, including: measures of intra-colony connectivity (B_n and B_r), host immigration (w), host resistance (p), disease transmission rate from amoebae to susceptible hosts (β_d), and a suite of amoebae traits including carrying capacity (K_a), and natural mortality rates (μ_t and μ_{t2}).

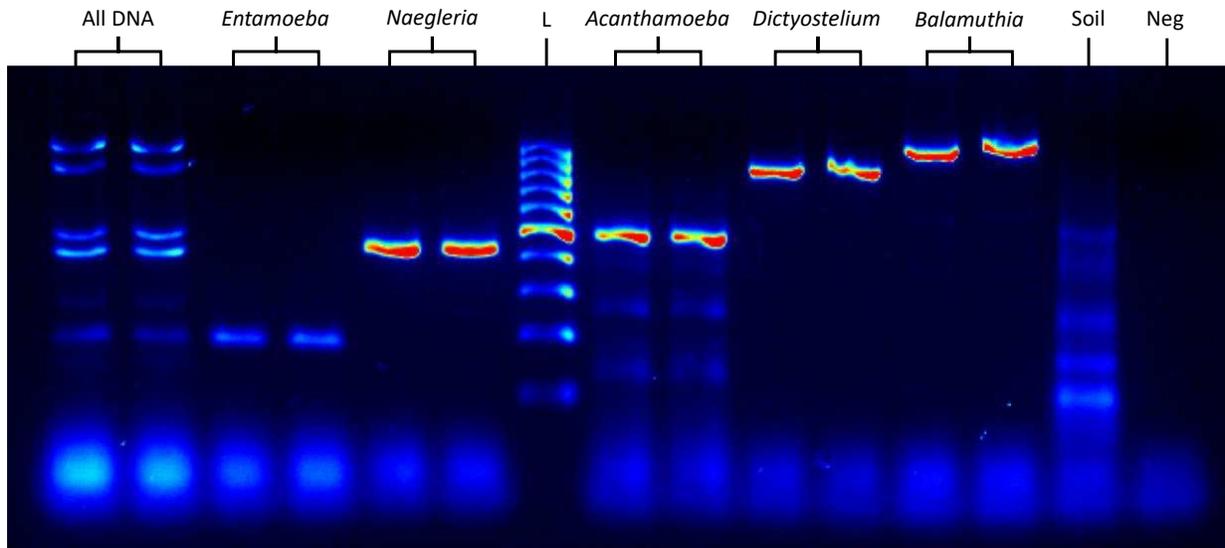


Figure S5.1: Total DNA was extracted from soil using a ZR Soil Microbe DNA Miniprep kit (Zymo Research) then used in the full multiplex assay (50 ng per reaction). Compared to the laboratory cultured samples, the soil sample reaction produced noticeable smearing without any bright amplicons. L: 100 bp DNA ladder (Invitrogen), Soil: total genomic DNA isolated from soil sample, Neg: control without template DNA.

Table S1.1: Modern and historic plague distribution. References: 1) WHO 2016, 2) WHO 2000, 3) Lotfy 2015, 4) CBC 2010, 5) Ministry 2012, 6) Plague 1900, 7) Schmid *et al.* 2015, 8) Cesana *et al.* 2017, 9) Benedictow 2006, 10) Melikishvili 2006, 11) Schneider *et al.* 2014.

Supplementary Table 1.1 Modern and historic plague distribution

Country	Plague presense Pre/Post-2000 CE *	Reference	Country (continued)	Plague presense Pre/Post-2000 CE *	Reference
Albania	Pre	8, 9	Lithuania	Pre	8, 9
Algeria	Post	1	Luxembourg	Pre	8, 9
Andorra	Pre	8	Madagascar	Post	1
Angola	Post	2	Malawi	Post	1
Argentina	Pre	2	Malta	Pre	8, 9
Armenia	Post	1	Mauritania	Pre	3
Australia	Pre	6	Mexico	Pre	11
Austria	Pre	8, 9	Moldova	Pre	10
Azerbaijan	Post	1	Monaco	Pre	8, 9
Belarus	Pre	8, 9	Mongolia	Post	1
Belgium	Pre	8, 9	Morocco	Post	7
Bolivia	Post	1	Mozambique	Post	1
Bosnia and Herzegovina	Pre	8, 9	Myanmar	Post	1
Botswana	Post	2	Namibia	Post	2
Brazil	Post	1	Nepal	Post	1
Bulgaria	Pre	8, 9	Netherlands	Pre	8, 9
Burkina Faso	Pre	2	New Zealand	Pre	5
Cambodia	Pre	2	Nigeria	Pre	3
Cameroon	Pre	2	Norway	Pre	8, 9
Canada	Post	4	Palestine	Pre	8, 9
Chile	Pre	11	Panama	Pre	11
China	Post	1	Paraguay	Pre	11
Croatia	Pre	8, 9	Peru	Post	1
Cuba	Pre	11	Philippines	Pre	2
Czech Republic	Pre	8, 9	Poland	Pre	8, 9
Denmark	Pre	8, 9	Portugal	Pre	8, 9
DR Congo	Post	1	Puerto Rico	Pre	11
Ecuador	Post	1	Romania	Pre	8, 9
Egypt	Post	7	Russia	Post	1
El Salvador	Pre	2	San Marino	Pre	8, 9
Equatorial Guinea	Pre	3	Saudi Arabia	Post	1
Estonia	Pre	8, 9	Senegal	Pre	3
Finland	Pre	9	Serbia and Montenegro	Pre	8, 9
France	Pre	8, 9	Slovakia	Pre	8, 9
Georgia	Post	1	Slovenia	Pre	8, 9
Germany	Pre	8, 9	Somalia	Pre	3
Ghana	Pre	3	South Africa	Post	1
Greece	Pre	8, 9	South Sudan	Pre	3
Guinea	Pre	2	Spain	Pre	8, 9
Hungary	Pre	8, 9	Sweden	Pre	8, 9
Iceland	Pre	9	Switzerland	Pre	8, 9
India	Post	1	Syria	Pre	8, 9
Indonesia	Post	1	Tajikistan	Post	1
Iran	Post	1	Tanzania	Post	1
Iraq	Pre	8, 9	Tunisia	Post	7
Ireland	Pre	8, 9	Turkey	Post	7
Israel	Pre	8, 9	Turkmenistan	Post	1
Italy	Pre	8, 9	Uganda	Post	1
Jordan	Post	1	Ukraine	Pre	8, 9
Kazakhstan	Post	1	United Kingdom	Pre	8, 9
Kenya	Post	1	United States	Post	1
Kuwait	Pre	8, 9	Uruguay	Pre	11
Kyrgyzstan	Post	1	Uzbekistan	Post	1
Laos	Pre	2	Venezuela	Pre	2
Latvia	Pre	8, 9	Vietnam	Post	1
Lebanon	Pre	8, 9	Western Sahara	Post	2
Lesotho	Pre	2	Zambia	Post	1
Libya	Post	1	Zimbabwe	Post	2
Liechtenstein	Pre	8, 9			

* At least one reported case of human or sylvatic plague pre or post-2000 C.E. within modern country boundaries. Modern (post-2000) presense does not preclude historic (pre-2000) presence.

Appendix 2: Code repository

Code repository: <https://github.com/davidwmarkman/Plague-Modeling>

Appendix 3: Amoeba reference genome sequences

- *Entamoeba histolytica* HM-1:IMSS scf_1104750518587 genomic scaffold, whole genome shotgun sequence (GenBank accession: DS571145.1)
- *Naegleria fowleri* mitochondrion, complete genome (GenBank accession: NC_021104.1)
- *Acanthamoeba castellanii* mitochondrion, complete genome (GenBank accession: U12386.1)
- *Dictyostelium discoideum* mitochondrion, complete genome (GenBank accession: NC_000895.1)
- *Balamuthia mandrillaris* strain CDC-V039 mitochondrion, complete sequence, whole genome shotgun sequence (GenBank accession: CM003363.1)