Yersinia pestis Survival and Replication in Potential Ameba Reservoir

David W. Markman, Michael F. Antolin, Richard A. Bowen, William H. Wheat, Michael Woods, Mercedes Gonzalez-Juarrero, Mary Jackson

Plague ecology is characterized by sporadic epizootics, then periods of dormancy. Building evidence suggests environmentally ubiquitous amebae 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 ameba species. First, we demonstrated that Yersinia pestis is resistant or transiently resistant to various ameba species. Second, we showed that Y. pestis survives and replicates intracellularly within Dicyostelium discoideum amebae for ≥48 hours postinfection, whereas control bacteria were destroyed in <1 hour. Finally, we found that Y. pestis resides within ameba structures synonymous with those found in infected human macrophages, for which Y. pestis is a competent pathogen. Evidence supporting amebae as potential plague reservoirs stresses the importance of recognizing pathogen-harboring amebae as threats to public health, agriculture, conservation, and biodefense.

The etiologic agent of plague, Yersinia pestis, is a gram-negative cocobacillus and a facultative intracellular pathogen. Y. pestis exhibited the highest overall mortality rate of any infectious disease from its earliest recorded emergence through 1941 (1). During 2010–2015, a mean of 650 cases were reported globally each year, with a case fatality rate of 23%–41% (depending on manifestation 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), rising to 66%–100% when adequate medical care was not promptly received (2). 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 fleaborne transmission. Epizootic plague is typically vectored by multiple flea species and is transmitted within and between meta-populations of hosts by flea bites (Figure 1).

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**Yersinia pestis** in Potential Ameba Reservoir

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; intraameba bacterial location; bacterial viability postphagocytosis; 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.

**Materials and 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 ≈1940 (31). 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 (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/24/2/17-1065-Techapp1.pdf). We selected individual burrows within the colony boundaries on the basis of apparent prairie dog activity (feces, freshly excavated soil, and noncollapsed 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 Amebae from Soil**

We isolated amebae from soil in plague-affected prairie dog burrows by using modified culture methods (32) (online Technical Appendix Figure 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 ameba cultures without bacterial contamination to 25-cm² tissue culture flasks in ameba-specific media containing penicillin/streptomycin. We identified ameba by using multiplex and endpoint PCR after extracting DNA by using a QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) (33,34) (online Technical Appendix Figure 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 nontransformed 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.

**Ameba 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. vermiciformis* (ATCC 50237) from the American Type Culture Collection (Manassas, VA, USA) and the Culture Collection of Algae and Protozoa (https://www.ccap.ac.uk/) and *Dictyostelium discoideum* (NC4A2) from DictyBase (http://dictybase.org/). We axenically cultivated ameba stocks with genera-specific media in T25 tissue culture flasks at 28°C and verified them to be axenic by using standardized methods (19,35–37).

**Co-culture Experiments**

**Intraameba Infection Prevalence and Intensity Assays**

We individually co-cultured laboratory ameba species with *Y. pestis* by using established methods (21). We adjusted viable ameba trophozoite densities to 5 × 10^6 trophozoites/mL in triplicate 25-cm² tissue culture flasks and combined *Y. pestis* (CO92 pgm+, pCD1, pGFPuv, amp+) cultures with ameba flasks (excluding ameba 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 amebae, ameba controls, and bacteria controls from the surface of the flasks and washing them 3 times with Page amoeba saline (PAS) at 100 × g for 5 min (36). We then exposed amebae 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 × g for 10 min), and plated it on LB agar to ensure no extracellular bacteria persisted.

We fixed aliquots of each infected ameba treatment in 4% paraformaldehyde for 15 min before washing (4,400 × g, 5 min) and resuspending in 1× 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 amebae 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 ameba, 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 amebae 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 Intraameba 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 intraameba 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 Cryo-electron Microscopy Laboratory at the University of Texas Medical Branch (Galveston, TX, USA) for transmission electron microscopy (TEM) according to standardized procedures. We determined bacterial location within amebae by ultrastructural analysis of transmission electron micrographs and studied intracellular bacterial morphology to assess ameba-mediated bacterial lysis as measured by cell size, shape, and apparent membrane integrity.

**Intraameba Bacterial Survival and Quantification of Intraameba Bacterial Replication**

We performed intraameba bacterial survival and replication assays in triplicate across 5 ameba species (*A. castellanii*, *A. lenticulata*, *A. polyphaga*, *D. discoideum*, and *V. vermiciformis*); 2 bacteria species (*Y. pestis* CO92 and *Escherichia coli*); 5 postinfection time points (0, 1, 4, 24, and 48 h); and 3 antibiotic drug exposure periods (0, 1, and 4 h) for removing extracellular bacteria postinfection. We used *E. coli* as an ameba-susceptible control bacterium. We performed co-cultures in 200-μL volumes within 96-well plates at a MOI of 100 in 1/2× 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 μg/mL), washed them 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 μL 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 phagocytosis 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 5 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 (online Technical Appendix Figure 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 2). ANOVA F-test results indicate significant differences in infection prevalence (or phagocytic efficiency) among ameba species (p = 0.0231) (Table). Repeat experiments maintained relative ranking of mean infection intensity and infection prevalence across ameba 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 ameba species (p = 0.0014) (Table). 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). Each ameba species had several high-intensity outliers ranging up to a maximum of 84 intracellular bacteria observed in 1 A. lenticulata ameba (Figure 3).

Y. pestis resides in digestive and central vacuoles of both D. discoideum and A. castellanii amebae. Green fluorescent protein expressed by intracellular Y. pestis co-localizes with ameba vacuoles (Figure 2). TEM micrographs depict intracellular Y. pestis maintaining cellular shape and apparent membrane integrity inside A. castellanii ameba for ≥24 h postinfection (Figure 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 (27).

Y. pestis can survive inside D. discoideum amebae for ≥48 hours, but we found genus-level differences in intracellular survival of Y. pestis (Table). The bacterium did not survive beyond 24 h postinfection 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 postinfection under variable treatment conditions (Table; Figure 5). Y. pestis exhibited significantly higher survival/recoverability when co-cultured with amebae 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 ameba \((p<0.001)\). Uninfected ameba control lysates consistently yielded zero bacteria across all ameba 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 ameba species as verified by comparing ameba abundance between co-culture treatments and ameba monoculture controls (data not shown).

*Y. pestis* replicates intracellularly in *D. discoideum* amebae for \(>48\) hours postinfection (Table; Figure 5). In *D. discoideum*, the abundance of viable intracellular *Y. pestis* was significantly greater at each successive time point (24 and 48 h postinfection) 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 postinfection were significantly greater than immediately after the 4-h antibiotic treatment \((p = 0.008\) and \(p = 0.001\), respectively). After 48 h postinfection, 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 postinfection \((p<0.001)\).

**Discussion**

We demonstrate that *Y. pestis* (CO-92) can survive and replicate intracellularly within the social, heterogamous ameba.

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Dormant state</th>
<th>Infection prevalence*</th>
<th>Infection intensity†</th>
<th>Intracellular survival‡</th>
<th>Intracellular replication</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean, % ± SEM</td>
<td>Mean, % ± SEM</td>
<td>Mean, % ± SEM</td>
<td></td>
</tr>
<tr>
<td>Acanthamoeba castellanii</td>
<td>Cyst</td>
<td>33.63 ± 5.21</td>
<td>4.22 ± 0.61</td>
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<td>0 ± 0</td>
</tr>
<tr>
<td>A. lenticulata</td>
<td>Cyst</td>
<td>51.66 ± 3.17</td>
<td>6.41 ± 0.43</td>
<td>10 ± 1.15</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>A. polyphaga</td>
<td>Cyst</td>
<td>49.08 ± 5.41</td>
<td>5.36 ± 0.37</td>
<td>31.66 ± 2.24</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Dictyostelium discoideum</td>
<td>Spore</td>
<td>39.24 ± 3.13</td>
<td>3.57 ± 0.97</td>
<td>270 ± 19.92</td>
<td>226.67 ± 22.71</td>
</tr>
<tr>
<td>Vermamoeba vermiformis</td>
<td>Cyst</td>
<td>29.61 ± 3.4</td>
<td>1.84 ± 0.13</td>
<td>10 ± 0.966</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

*Mean percentage of amebae containing >1 intracellular bacterium.†Mean no. of intracellular bacteria per individual infected ameba.‡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 intraameba replication assay, which we used to count intraameba 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.).
D. discoideum, whereas Y. pestis is only transiently resistant to 4 species of free-living and cyst-forming amebae (A. castellanii, A. lenticulata, A. polyphaga, and V. vermiformis). Relative to E. coli controls, Y. pestis demonstrated significantly increased survival and replication within amebae despite the 4 cyst-forming amebae successfully killing the bacteria by using unidentified mechanisms.

Amebae cultured from soil in prairie dog colonies with active plague epizootics confirm that ameba species used in our experiments co-occur spatially and temporally with Y. pestis under natural conditions. Interactions between ameba and Y. pestis could select for increasingly ameba-resistant phenotypes, considering the transient resistance already observed in 4 cyst-forming ameba species. Other research has demonstrated the potential for amebae to affect pathogen transmission mode, alter virulence, and act as training grounds for intracellular pathogens by selecting for traits enabling macrophage invasion or avoidance (17,38).

Genus-level differences in ameba infection intensity and infection prevalence confirm that various ameba species have greater reservoir potential than others. In accordance with super-spreader theories, a minority of individual ameba 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 ameba 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 (1,17,38). 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-Montaño et al. (13), 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 ameba and resistant bacteria in soil environments (17).

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 (26,27,39–41). *Y. pestis* then establishes a replicative niche within the YCV, characterized by a tight-fitting vacuole that expands commensurately with bacterial replication (27). Examination of TEM micrographs shows that intracellular bacteria are localized within in-form-fitting vacuolar membranes, similar to the YCVs found in macrophages (Figure 4).

The successful intracellular survival of *Y. pestis* in *D. discoideum* for ≥48 h demonstrates that *Y. pestis* is an ameba-resistant bacterium. This classification supports the potential for *D. discoideum* or related ameba 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 (27). Most phagocytosed bacteria cannot survive digestive processes characteristic of phagocytic cells including phagolysosomal fusion and acidification, or the subsequent recruitment of endosomal lytic factors (26,27,41). 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 5) and possible, but unconfirmed, replication of *Y. pestis* (CO92 pgm+, pCD1, pGFPuv, amp+) in *A. castellanii* (Figure 4A). Analysis of TEM micrographs proved inconclusive for identifying the bacterial division septum; 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 5) in successive postinfection time points across both antibiotic treatment conditions (except in 1 instance where *Y. pestis* increased nonsignificantly [p = 0.1624; Figure 5]). The consistent absence of extracellular bacteria in all *D. discoideum* co-cultures indicates resistance to digestion and the exploitation of an intrameba 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 amebe. We searched for macrophage Rab1b by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against full genome sequences of each study ameba 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 GTGases is diagnostic of ameba permisiveness 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 intraameba *Y. pestis* survival corresponds with the durations of cryptic interepizootic persistence that are characteristic of plague dynamics. Increasing evidence for dormant or viable but nonculrable forms of *Y. pestis* may provide explanations underlying hypothesized multyear survival in ameba spores or cysts (12,25,44–46). Outcomes of this research prompt questions regarding evolutionary selection imposed by ameba 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 (1,11). Plague persistence within soilborne microorganisms has been hypothesized as an elusive maintenance mechanism (6,11,12,25). 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* amebe for ≥48 hours postinfection and persistence of *Y. pestis* in 4 cyst-forming ameba species for ≤24 hours postinfection, whereas ameba-susceptible control bacteria were eliminated by amebe in <1 hour. Thus, *Y. pestis* are respectively ameba-resistant and transiently ameba-resistant under the tested infection conditions. *Y. pestis* bacteria resided within ameba structures that were visually analogous to YCVs observed in infected macrophages. These results encourage research into the eco-evolutionary interactions between pathogenic bacteria, amebe, and host immune factors. The reservoir potential of amebe and their shared infection-permisiveness with phagocytic macrophages show promise in explaining the cryptic properties underlying interepizootic plague transmission and persistence.
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References


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