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Short Report: Exposing Laboratory-Reared Fleas to Soil and Wild Flea Feces Increases Transmission of *Yersinia pestis*

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Abstract. Laboratory-reared *Oropsylla montana* were exposed to soil and wild-caught *Oropsylla montana* feces for 1 week. Fleas from these two treatments and a control group of laboratory-reared fleas were infected with *Yersinia pestis*, the etiological agent of plague. Fleas exposed to soil transmitted *Y. pestis* to mice at a significantly greater rate (50.0% of mice were infected) than control fleas (23.3% of mice were infected). Although the concentration of *Y. pestis* in fleas did not differ among treatments, the minimum transmission efficiency of fleas from the soil and wild flea feces treatments (6.9% and 7.6%, respectively) were more than three times higher than in control fleas (2.2%). Our results suggest that exposing laboratory-reared fleas to diverse microbes alters transmission of *Y. pestis*.

The majority of vector-borne pathogen transmission studies rely on laboratory-reared insects and other arthropod vectors. In addition to providing the large number of these organisms necessary for research, rearing vectors in the laboratory is the only way to ensure that individuals are pathogen free at the start of the study. However, laboratory conditions cannot mimic conditions in nature and the applicability of transmission studies to transmission under natural conditions is somewhat compromised. One consequence of rearing insects in the laboratory is the substantial loss of microbial diversity harbored by their wild counterparts.[1–5] Given that the ability of pathogens to successfully invade and persist within insects is likely influenced by the native insect microbiota,[6,7] lower microbial diversity in laboratory-reared vectors may affect transmission competency and efficiency.

In *Oropsylla montana*, a common squirrel flea, the abundances of lineages within the Actinobacteria, Bacteroidetes, and Firmicutes are significantly reduced within 96 hours of being kept under laboratory conditions.[2] *Oropsylla montana* is the vector responsible for the majority of flea-borne plague cases in humans in North America.[8] The estimated vector competency and transmission efficiency of fleas for *Yersinia pestis* varies widely among flea species but also among studies of the same flea species, including *O. montana*.[9] It has been suggested that variance among studies may be the result of differences in *Y. pestis* concentration in blood fed to fleas, the type of animal blood used in experiments, temperature differences, and the time point fleas are tested post-infection.[9] An often overlooked factor that may help explain the fate of pathogens after ingestion by insects is the insect gut microbiota,[6] and interactions between *Y. pestis* and other bacteria within fleas may affect transmission efficiency of plague.

Oropsylla montana have been reared at the Division of Vector-Borne Disease since 1993 under the following conditions: incubation at 85% relative humidity and 23°C, in autoclaved media (sand, ground rat chow, and powdered dried blood have been used for more than 10 years), which provides a site for egg-laying by fleas, nutrition for developing flea larvae, and a place for pupation and adult emergence to occur. Adult fleas in the colonies are fed on live mice 2–3 times per week. Fleas kept under these conditions quickly lose their native bacterial diversity.[2] Here, our aim was to determine if exposing laboratory-reared fleas to diverse microbes that would be typically encountered under natural conditions affects transmission of *Y. pestis*. To assess this possibility, we exposed laboratory-reared fleas to two treatments: 1) soil from a plague-endemic area, and 2) feces of wild-caught fleas.

We collected ~500 mL of soil from the entrance of a black-tailed prairie dog, *Cynomys ludovicianus*, burrow in Fort Collins, CO. Laboratory-reared fleas were housed in this soil at 23°C and 85% humidity for 8 days before use in the infection experiment. We also collected hundreds of wild *O. montana* from a rock squirrel colony in Colorado Springs, CO. These wild-caught fleas were housed in the sterile flea-rearing media described previously, typically used for laboratory-reared fleas for 12 days and then completely removed. Laboratory-reared fleas were then placed in this contaminated substrate for 8 days before use in the infection experiment. It is very unlikely that any wild flea eggs developed into adults in the 20-day period of time, because this is not enough time for eggs to develop and hatch into larvae, cycle through the three stages of larval development, and complete pupation. Furthermore, cocoons are very easily detected and none were observed in the media.

Fleas from each treatment (control, exposure to wild flea feces, and exposure to soil) were fed a fully virulent strain of *Y. pestis* (strain: CO96-3188)[10] using artificial feeders as previously described.[11] *Yersinia pestis* was grown for 18 hours and added to defibrinated Sprague-Dawley rat blood (Bioreclamation, Jericho, NY) to a final concentration of 1×10^8 colony forming unit (CFU)/mL. Fleas were offered the *Y. pestis*-spiked blood meal and allowed to feed for 1 hour. After feeding, fleas were chilled on ice and examined to determine if they had consumed a blood meal. Fleas that consumed infectious blood were held for later transmission experiments.

Transmission experiments took place at 24, 72, and 120 hours after fleas were exposed to the *Y. pestis*-spiked blood meal using previously described methodology.[11] Ten specific pathogen free Swiss-Webster mice per treatment per time period were used to test transmission efficiency. Ten potentially infectious fleas per mouse were allowed

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to feed for 1 hour using feeding capsules attached to the mice (one capsule per mouse). The maintenance and care of experimental animals was thoroughly reviewed and approved by the Center for Disease Control and Prevention's Animal Care and Use Committee. After feeding, fleas were chilled, examined to determine if they fed, sexed, and then placed in a solution of 90 μL HIB and 10 μL glycerol, and macerated. This solution was cultured for 48 hours at 28°C on sheep blood agar plates to determine the viable *Y. pestis* concentration within each flea.

Mice were kept individually in filter-top cages and observed at least two times per day. When mice displayed clinical symptoms of plague (e.g., malaise, lethargy, ruffled fur, etc.), they were immediately killed; plague was confirmed by using a fluorescence antibody test targeting the F1 antigen on spleen and liver smears. After 21 days, all surviving mice were tested for antibody titers to the F1 antigen using passive hemagglutination and inhibition tests. Because we used specific pathogen free mice, we do not expect any cross-reactivity with other pathogens; mice with titers > 1:10 were considered positive for *Y. pestis* transmission. We define transmission as *Y. pestis* entering the host by the flea and causing an infection.

Fleas from each treatment transmitted *Y. pestis* to mice: 23.3% (7/30) of mice from the control treatment contracted plague; 40% (12 of 30) of mice from the wild flea feces treatment contracted plague; 50% (15 of 30) of mice from the soil treatment contracted plague (values are cumulative across the 24, 72, and 120 hours post feeding times tested). A χ^2 test of independence indicated that mice from the soil treatment infected significantly more mice than control mice ($\chi^2 = 4.593$; P value = 0.032) (Figure 1). Mice from the wild flea feces treatment also contracted plague at a higher rate, but it was not significantly higher than the control group ($\chi^2 = 1.926$; P value = 0.165) (Figure 1). Fleas from all treatments transmitted *Y. pestis* 24 and 72 hours after the infectious feed, but only fleas from the soil and wild flea feces treatments transmitted *Y. pestis* 120 hours after the infectious feed.

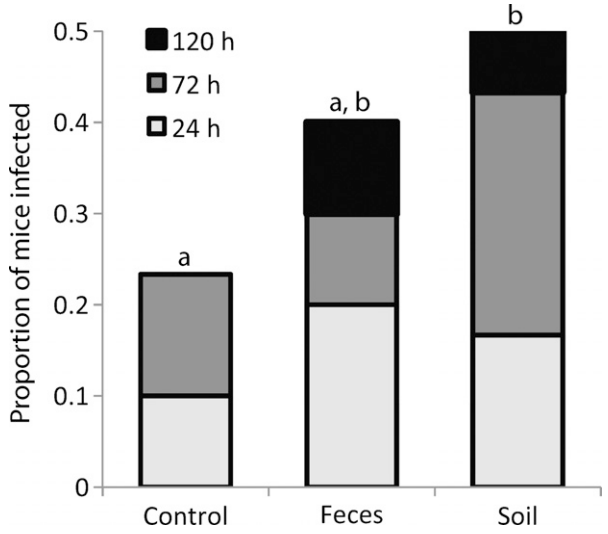

FIGURE 1. Transmission rates of *Yersinia pestis* from fleas exposed to soil, fleas exposed to wild flea feces, and control fleas. Fleas exposed to soil-transmitted *Y. pestis* at a significantly higher rate than control fleas ($\chi^2 = 4.593$; P value = 0.032). Transmission rates of fleas exposed to wild flea feces did not significantly differ from rates of the control fleas ($\chi^2 = 1.926$; P value = 0.165). Different letters above the columns indicate a significant difference between treatments. *Yersinia pestis* was transmitted by fleas from all three treatments at 24 hours and 72 hours post-infectious feed, but only fleas exposed to soil or wild flea feces transmitted *Y. pestis* at the 120 hours post-infectious feed.

TABLE 1

Number of infectious fleas and *Yersinia pestis* loads for plague-positive mice

	Hours	Infected fleas/ fed fleas	Median bacterial load	Bacterial load (range)
Control				
Mouse C5-1	24	7/7	4.55×10^4	9.00×10^1–8.65×10^5
Mouse C6-1	24	8/10	7.78×10^5	0–3.40×10^6
Mouse C10-1	24	7/8	1.93×10^5	0–2.35×10^6
Mouse C2-3	72	9/10	5.60×10^3	0–7.70×10^6
Mouse C8-3	72	9/10	6.40×10^4	0–4.00×10^5
Mouse C9-3	72	7/8	5.60×10^2	0–3.75×10^4
Mouse C10-3	72	7/8	1.65×10^3	0–2.95×10^4
Wild flea feces				
Mouse F1-1*	24	6/6	1.06×10^6	5.80×10^4–7.10×10^6
Mouse F3-1*	24	6/7	8.90×10^5	0–3.50×10^6
Mouse F5-1	24	6/6	6.08×10^5	1.30×10^5–5.10×10^6
Mouse F6-1	24	4/10	0	0–1.20×10^6
Mouse F7-1	24	6/6	1.23×10^6	1.35×10^5–8.30×10^6
Mouse F9-1	24	7/7	3.45×10^5	8.00×10^4–4.40×10^6
Mouse F6-3*	72	4/5	2.70×10^2	0–1.60×10^4
Mouse F7-3	72	6/8	3.83×10^2	0–3.10×10^3
Mouse F8-3	72	4/4	1.55×10^3	9.00×10^1–6.50×10^3
Mouse F1-5	120	5/7	1.95×10^4	0–3.45×10^5
Mouse F2-5	120	6/7	5.45×10^4	0–7.50×10^5
Mouse F8-5	120	5/7	1.55×10^3	0–4.65×10^4
Soil				
Mouse S5-1	24	7/7	7.00×10^5	2.50×10^5–2.30×10^6
Mouse S7-1	24	4/6	1.06×10^5	0–2.25×10^6
Mouse S8-1	24	8/8	1.43×10^6	3.15×10^5–2.80×10^6
Mouse S9-1	24	8/8	7.00×10^5	1.10×10^4–4.45×10^6
Mouse S10-1	24	9/9	5.60×10^5	2.35×10^5–2.15×10^6
Mouse S3-3	72	5/8	1.85×10^2	0–6.45×10^4
Mouse S4-3*	72	10/10	4.00×10^3	4.90×10^2–3.85×10^4
Mouse S5-3*	72	7/10	3.65×10^3	0–2.60×10^5
Mouse S6-3	72	10/10	3.60×10^3	3.00×10^1–4.55×10^4
Mouse S7-3	72	9/10	1.28×10^4	0–1.57×10^6
Mouse S8-3	72	8/10	6.40×10^3	0–2.60×10^5
Mouse S9-3*	72	6/8	5.03×10^3	0–2.40×10^6
Mouse S10-3	72	8/9	1.40×10^4	0–9.50×10^4
Mouse S4-5	120	7/9	1.55×10^4	0–1.18×10^6
Mouse S6-5	120	7/8	5.15×10^3	0–1.50×10^4

*Mouse was determined to be plague-positive by testing for antibody titers to the F1 antigen using passive hemagglutination and inhibition tests.

We quantified the concentration of *Y. pestis* in fleas from mice that contracted plague (Table 1). The number of CFUs declined significantly after 24 hours but did not differ among treatments within any given time period (Figure 2). The percentage of fleas infected with *Y. pestis* did not differ among treatments.

We determined the minimum transmission efficiency of fleas for each mouse by dividing the transmission rate (100% for plague-positive mice or 0% for plague-negative mice) by the number of fed fleas (e.g., if five fleas fed on a plague-positive mouse, the minimum transmission efficiency = 20%). Fleas exposed to soil and exposed to wild flea feces had significantly higher minimum transmission efficiency (6.89% and 7.59%, respectively) than control fleas (2.20%) (Figure 3A). We also used PooledInfRate v4 (http://www.cdc.gov/ncidod/dvbid/WestNile/resources/PooledInfRateV4-2007.zip) to

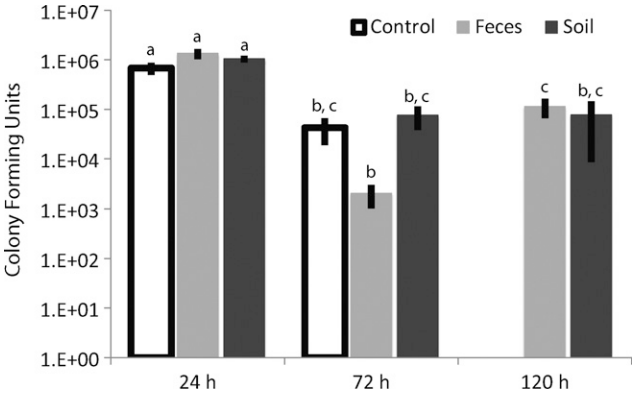

FIGURE 2. Colony forming units of *Yersinia pestis* from fleas exposed to soil, fleas exposed to wild flea feces, and control fleas. The number of viable *Y. pestis* declined after the 24-hour post-infectious feed but did not differ among treatments within a time period (*t* test; $P > 0.05$). No fleas from the control treatment transmitted *Y. pestis* in the 120-hour post-infectious feed time point; therefore, no data exist for that group. Error bars represent standard error.

determine maximum likelihood estimates of transmission efficiency of fleas from the three treatments.[12] The maximum likelihood approach also suggested that soil and feces treatments increased transmission efficiency, but treatments did not significantly differ because of the large confidence intervals (Figure 3B).

Our results suggest that replacing the sterile substrate of laboratory-reared fleas with soil or contaminating the sterile substrate with the introduction (and subsequent removal) of wild fleas can substantially alter the ability of fleas to transmit *Y. pestis*.

We found the mechanism responsible for the increases in transmission to be unclear. Concentration of *Y. pestis* does not differ among treatments (Figure 2), indicating that bacterial loads within fleas do not affect the ability of a flea to transmit plague. Numerous other studies have also found that flea bacterial load does not correlate with transmission.[13–18] Given the small infectious does of *Y. pestis* (< 100 cells),[10,19–22] the overall number of *Y. pestis* bacteria is less important than the ability of a small number of these organisms to invade the mouse. It has been suggested that very small numbers of *Y. pestis* residing in a flea's proventriculus could cause infection through regurgitation during feeding.[9]

Thus, a possible mechanism to explain our findings is that the treatments increase the ability of *Y. pestis* to remain in the proventriculus (or elsewhere in the foregut) rather than move through this structure and pass into the flea's midgut where the likelihood of transmission would be expected to decrease. This could occur because of 1) a higher likelihood of biofilm formation in the proventriculus in the presence of environmentally acquired bacteria (though complete blockage would be unlikely given the limited time between the infectious feed and transmission), 2) a physiological response of fleas to the presence of other microorganisms that alters the physiology and movements of the flea's gut so that *Y. pestis* is more likely to be retained in the foregut and perhaps regurgitated, 3) the presence of other microorganisms alters the expression of as yet unknown transmission factors by *Y. pestis* in a way that favors transmission, or 4) the presence of non-biological micro-particles in the media that affects the physiology of the flea.

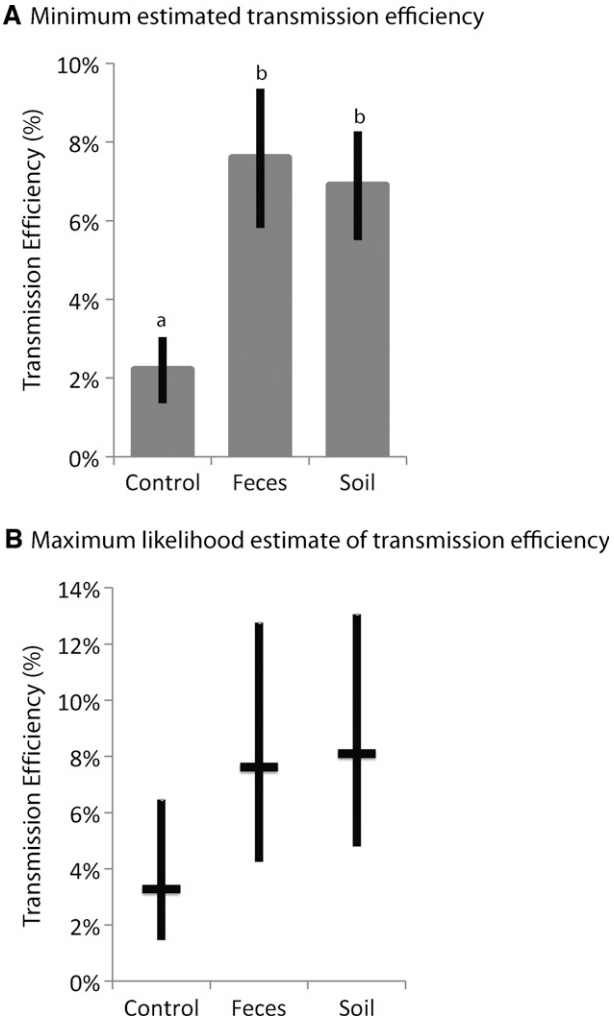

FIGURE 3. Transmission efficiency of *Yersinia pestis* from fleas exposed to soil, fleas exposed to wild flea feces, and control fleas. Minimum estimated transmission frequency is significantly greater in fleas exposed to soil and wild flea feces than in control fleas (*t* test; $P = 0.009$ and 0.006, respectively) but does not differ between fleas exposed to soil and fleas exposed to wild flea feces (*t* test; $P = 0.758$). (**A**) Error bars represent standard error. (**B**) Maximum likelihood estimates of transmission efficiency show no significant differences between treatments (error bars represent 95% confidence interval).

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