Applied and Environmental Microbiology, Dec. 2006, p. 7909–7911 Vol. 72, No. 12
0099-2240/06/$08.00+0 doi:10.1128/AEM.01471-06
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

Survival of *Campylobacter* spp. in Darkling Beetles (*Alphitobius diaperinus*) and Their Larvae in Australia[▽]

Jillian M. Templeton,* Amanda J. De Jong, P. J. Blackall, and Jeanette K. Miflin

Department of Primary Industries and Fisheries (Queensland), Animal Research Institute, Yeerongpilly 4105, Australia

Received 26 June 2006/Accepted 25 September 2006

***Campylobacter* infection is the most frequently reported notifiable food-borne disease in humans in Australia. Our studies investigated the persistence of *Campylobacter* spp. in or on darkling beetles (*Alphitobius diaperinus*) and their larvae. Our results in analyses with chickens confirm that, unless very short turnaround times are used (<72 h), beetles colonized in one production cycle (i.e., one batch of chickens) are most unlikely to still be colonized during the next cycle of chickens.**

Campylobacter jejuni is recognized as one of the leading causes of food-borne disease in most parts of the developed world (1). Epidemiological evidence implicates poultry as the most important source of sporadic cases of *Campylobacter* infection (3). Horizontal transmission is generally considered to be the most significant mode of *C. jejuni* acquisition by broiler flocks, although confirmatory studies using genotyping techniques are needed (8).

Darkling beetles and their larvae are known to be reservoirs of a range of viruses, bacteria (including *Campylobacter*), and protozoa (4, 6, 10). Studies around the world have shown that beetles and/or larvae are only *Campylobacter* positive if the chickens are positive (2, 5, 6). Despite this, suggestions that the darkling beetle can play a role in the entry of *Campylobacter* into a broiler flock continue (2).

The purpose of the present study was to investigate the survival of *Campylobacter* spp. in or on darkling beetles and larvae.

Beetle culture. Darkling beetle adults and larvae were maintained in the laboratory in lidded plastic containers on a substrate of 76% bran, 17% chicken feed pellets, and 7% nonfermenting yeast and provided with half an apple for moisture. In experiments where laboratory-bred beetles were used, samples taken from the laboratory colony before the start of all experiments were always *Campylobacter* negative.

***Campylobacter* isolation.** Beetles and larvae were aseptically removed, anesthetized on ice, and crushed. The swab was streaked onto Karmali *Campylobacter* agar base (CM935; Oxoid, Melbourne, Australia) containing *Campylobacter* selective supplement (SR167E) and incubated for 48 h in a modified atmosphere of 85% N_2, 10% CO_2, and 5% O_2. For enrichment, 1.5 ml of Preston broth (nutrient broth no. 2; Oxoid CM67) supplemented with Preston *Campylobacter* selective supplement (Oxoid SR117E) and *Campylobacter* growth supplement (SR084E) was added, and the broths were incubated for 18 to 24 h at 42°C and then subcultured on Karmali agar. Individual chicken fecal samples were cultured on Karmali agar. Presumptive *Campylobacter* colonies were confirmed by microscopy for motility, and a subset was confirmed by PCR (11).

Prevalence and duration of colonization in naturally infected field-sourced beetles and larvae. Two broiler farms (farm 91 and farm 77) were selected for the study. On each farm, poultry fecal samples, beetles, and larvae were collected from all sheds on the farm. On the day of collection the beetles and larvae were transferred to laboratory culture. Beetles and larvae were withdrawn every 24 h for *Campylobacter* isolation by direct culture and enrichment.

On farm 91, all 30 chicken fecal samples tested from each shed were *Campylobacter* positive. All beetles and larvae tested from sheds 1, 2, and 3 were *Campylobacter* positive at 0 h and negative from 24 to 120 h, except for one group of larvae from shed 2 that tested positive at 24 h. Beetles from shed 4 tested *Campylobacter* negative at all time intervals from 0 to 120 h, except at 48 h when one sample tested positive. In contrast, the larvae were determined to be *Campylobacter* positive from 0 h to 72 h and *Campylobacter* negative at 96 and 120 h.

On farm 77, all 30 chicken fecal samples from each shed tested *Campylobacter* positive. All beetles and larvae from shed 1 tested *Campylobacter* positive at 0 h and negative from 24 to 144 h, except for one group of beetles that tested positive at 72 h by direct culture and by enrichment. All beetles and larvae from shed 2 tested *Campylobacter* positive at 0 and 24 h and negative from 48 to 144 h except for one group of beetles that tested positive at 72 h by direct culture and by enrichment. The beetles from shed 3 tested *Campylobacter* positive at 0, 24, and 48 h and tested negative from 72 to 144 h. The larvae tested negative from 96 to 168 h, except for one group of larvae at 120 h that tested positive by enrichment only.

Simulated natural infection of laboratory-bred beetles using *Campylobacter*-positive poultry feces. Beetles were divided into two groups. Group 1 was deprived of apple for 4 days, and group 2 was deprived of apple for 7 days. The beetles were then given freshly collected chicken fecal material every 48 h for a total of 144 h. The beetles were then transferred to fresh containers and tested at this time and subsequently every 24 h for *Campylobacter* isolation by direct culture and enrichment.

Group 1 beetles were *Campylobacter* negative on all sam-

* Corresponding author. Mailing address: Animal Research Institute, Department of Primary Industries and Fisheries (Queensland), Locked Mail Bag No. 4, Moorooka, Queensland 4105, Australia. Phone: 61 7 3362 9520. Fax: 61 7 3362 9429. E-mail: jillian.templeton@dpi.qld.gov.au.
▽ Published ahead of print on 29 September 2006.

TABLE 1. Results of *Campylobacter* isolation of laboratory-bred beetles exposed by either spray inoculation or feeding on infected apple as the artificial infection method

Time point (h)	No. of positive samples/no. of samples tested[a]			
	Group 1 (spraying)		Group 2 (apple fed)	
	D	E	D	E
0	10/10	10/10	8/10	10/10
24	7/10	10/10	3/10	10/10
48	3/10	10/10	0/10	7/10
72	1/10	8/10	0/10	0/10
96	0/9[b]	1/9	0/10	1/10

[a] D, direct culture to Karmali agar (each sample consisted of 10 beetles); E, enrichment in Preston broth and culture on Karmali agar.
[b] A lower number of groups was tested due to insufficient beetles remaining.

pling occasions from 0 to 96 h. Group 2 beetles were *Campylobacter* positive only by enrichment at 0 and 24 h and *Campylobacter* negative from 48 to 96 h.

Artificial infection of laboratory-bred beetles using bacterial cultures. A 5-h mixed culture of the two *Campylobacter* isolates of different *fla A* types (7) was prepared containing 2.6×10^8 CFU/ml. Beetles in group A were spray inoculated with 20 ml of bacterial culture from a pump-spray bottle. Beetles in group B were allowed to feed for 24 h on apple soaked in 20 ml of the same bacterial culture for 20 min. The beetles were tested every 24 h for *Campylobacter* isolation by direct culture and enrichment.

The results of artificial inoculation techniques are summarized in Table 1. Group 1 showed a very high carriage rate at 0 h by direct culture and by enrichment. By 48 h all 10 groups were still testing as *Campylobacter* positive by enrichment. At 72 h 8 of 10 groups were *Campylobacter* positive; however, by 96 h only 1 of 9 groups was still positive. The beetles in group 2 also showed a high carriage rate initially. At 48 h 7 of 10 groups were found to be *Campylobacter* positive by enrichment only. At 72 h all 10 groups were *Campylobacter* negative, and at 96 h 1 of 10 groups was positive by enrichment only.

It has been well documented that darkling beetles have the potential to play a role in the epidemiology of *Campylobacter* on broiler farms (2, 6, 10). The purpose of the present study was to determine how long *Campylobacter* persists in or on beetles and larvae once they are removed from the organism's source, for example, *Campylobacter*-positive chicken feces.

Our study of naturally infected beetles and larvae collected in chicken sheds and transferred to the laboratory was hampered by the lower-than-expected prevalence of *Campylobacter* spp. in the insects collected on both study farms. The majority of beetles and larvae from both farms tested positive for a maximum of 72 h after collection. The exception was a single isolation from larvae from shed 3 by enrichment at 120 h.

These results indicated that *Campylobacter* generally did not survive more than about 72 h in beetles and larvae once they were removed from the source of *Campylobacter*. We wanted to verify this result by starting with a population of beetles with higher initial levels of colonization, so we decided to artificially infect beetles by feeding them *Campylobacter*-positive chicken feces, mimicking how they acquire the organism in the field. However, with this method, even when beetles were deprived of moisture for 7 days, a very low infection rate was achieved. All samples were negative by 48 h.

At this point we were unable to determine whether the apparent short survival time was representative of what occurs in the field or if our inoculation technique was failing. Therefore, artificial infection methods were used in an attempt to achieve a higher *Campylobacter* carriage rate in the beetles, allowing us to study how long the beetles maintain the organism.

We developed two new inoculation methods, spraying beetles with *Campylobacter* broth culture and allowing beetles to feed on apple pieces that had been soaked in *Campylobacter*-positive broth. Our findings demonstrated that these two methods of inoculation achieved a much higher colonization rate than fecal exposure. Not only were more samples positive, but samples were positive by direct culture too, indicating that there were higher numbers of bacteria per beetle. These exposure methods are clearly an experimental phenomenon and are not representative of what happens in the field. Despite this, after spray inoculation, only one of nine sample lots remained positive at 96 h and only by enrichment. In the apple inoculation group, all of the beetles tested negative at 72 h, and only one of ten sample lots tested positive at 96 h. It was unfortunate that another sampling event was not included in the experimental design. However, it is unlikely that any organisms would have remained since only one sample tested positive, and only by enrichment, in the last sampling occasion, indicating that very low levels of bacteria were present.

Our experimental results suggest that *C. jejuni* does not survive for extended periods on/in darkling beetles or larvae. However, continuous exposure to *Campylobacter*-positive food sources enables them to become short-term carriers during the production cycle. For this reason they could play a role in horizontal transmission. Our experimental studies on naturally and artificially infected beetles detected *Campylobacter*-positive beetles at greater than 72 h postexposure on only one occasion. This matches with the findings of Strother et al. (10), who reported a maximum survival time of 72 h in the interior of the larvae. This suggests that unless very short turnaround times are used—less than 72 h—beetles colonized in one cycle are most unlikely to be still colonized at the placement of the next cycle of chickens. These findings agree with those of Skov et al. (9) that the darkling beetle is not a likely source of the carryover of *Campylobacter* from flock to flock due to the short duration of bacterial carriage by the beetle.

We thank Trevor Lambkin, Steven Rice, and Justin Bartlett (Department of Primary Industries and Fisheries, Queensland, Australia) for their supply of laboratory-cultured darkling beetles and their assistance in beetle culture for our studies.

This research has been financially supported by the Chicken Meat Sub-Program of the Australian Government Rural Industries Research and Development Corp. We are grateful for the cooperation of the participating companies and especially their growers.

REFERENCES

1. **Altekruse, S. F., M. L. Cohen, and D. L. Swerdlow.** 1997. Emerging foodborne diseases. Emerg. Infect. Dis. **3:**285–293.
2. **Bates, C., K. L. Hiett, and N. J. Stern.** 2004. Relationship of *Campylobacter* isolated from poultry and from darkling beetles in New Zealand. Avian Dis. **48:**138–147.

3. **Corry, J. E. L., and H. I. Atabay.** 2001. Poultry as a source of *Campylobacter* and related organisms. J. Appl. Microbiol. **90:**96S–114S.
4. **Goodwin, M. A., and W. D. Waltman.** 1996. Transmission of *Eimeria*, viruses, and bacteria to chicks: darkling beetles (*Alphitobius diaperinus*) as vectors of pathogens. J. Appl. Poultry Res. **5:**51–55.
5. **Gregory, E., H. Barnhart, D. W. Dreesen, N. J. Stern, and J. L. Corn.** 1997. Epidemiological study of *Campylobacter* spp. in broilers: source, time of colonization, and prevalence. Avian Dis. **41:**890–898.
6. **Jacobs-Reitsma, W. F., A. W. van de Giessen, N. M. Bolder, and R. W. A. W. Mulder.** 1995. Epidemiology of *Campylobacter* spp. at two Dutch broiler farms. Epidemiol. Infect. **114:**413–421.
7. **Nachamkin, I., K. Bohachick, and C. M. Patton.** 1993. Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. J. Clin. Microbiol. **31:**1531–1536.
8. **Newell, D. G., and C. Fearnley.** 2003. Sources of *Campylobacter* colonization in broiler chickens. Appl. Environ. Microbiol. **69:**4343–4351.
9. **Skov, M. N., A. G. Spencer, B. Hald, L. Petersen, B. Nauerby, B. Carstensen, and M. Madsen.** 2004. The role of litter beetles as potential reservoir for *Salmonella enterica* and thermophilic *Campylobacter* spp. between broiler flocks. Avian Dis. **48:**9–18.
10. **Strother, K. O., C. D. Steelman, and E. E. Gbur.** 2005. Reservoir competence of lesser mealworm (Coleoptera: Tenebrionidae) for *Campylobacter jejuni* (Campylobacterales: Campylobacteraceae). J. Med. Entomol. **42:**42–47.
11. **Wang, G., C. G. Clark, T. M. Taylor, C. Pucknell, C. Barton, L. Price, D. L. Woodward, and F. G. Rodgers.** 2002. Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. J. Clin. Microbiol. **40:**4744–4747.