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Survival of *Salmonella enterica* in Freshwater and Sediments and Transmission by the Aquatic Midge *Chironomus tentans* (Chironomidae: Diptera)

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Survival of a nalidixic acid-resistant strain of *Salmonella enterica* serovar Typhimurium mr-DT-104 in water and sediments was tested using artificially contaminated aquaria. Water samples remained culture positive for salmonella for up to 54 days. Sediment samples were culture positive up to 119 days. In addition, potential mechanisms for spreading salmonella in the environments by chironomid larvae and adults were tested. We evaluated the acquisition of mr-DT-104 by chironomids from contaminated aquatic sediments and subsequent spread to uncontaminated sediments. Larval chironomids raised in contaminated sediments became culture positive, and the bacteria were carried over to adults after emergence. Contamination of clean sediments by chironomid larvae was not demonstrated. These findings clearly suggest that mr-DT-104 serovar organisms can survive in aquatic sediments for at least several months. Uptake of salmonellae by chironomid larvae and adults suggests that they are possible vectors of mr-DT-104 in both aquatic and terrestrial environments, although the role of larval defecation in movement of bacteria to new sediments was not demonstrated.

Recent outbreaks of an antibiotic-resistant strain of *Salmonella enterica* serovar Typhimurium, termed multiresistant DT-104 (mr-DT-104), have been found in Pacific Northwest livestock by Besser et al. (2, 3). Molecular characterization and resistance in mr-DT-104 are described by Cloeckaert and Schwarz (8). Of special concern are reports that human infection by this particular strain of salmonella is more likely from contact with infected animals than for other serovars (2, 6, 33, 34). Isolations from specimens submitted to the Washington Animal Disease Diagnostic Laboratory (unpublished data) have shown mr-DT-104 infections in a wide range of species, including cows, horses, cats, goats, emus, dogs, elk, coyotes, ground squirrels, raccoons, chipmunks, and birds. Factors responsible for the distribution of the serovar across multiple species in widely diverse habitats are unknown. Identification of mr-DT-104 in wildlife in rural environments in the Pacific Northwest has raised questions regarding its spread and appearance in wildlife populations, some of which are not typically in close contact with humans or domestic animals. Transmission of *Salmonella* strain mr-DT-104 in the environment is an important health concern for people and livestock (27). Elucidating the pathways for spread of this strain is critical for finding means to stop or slow its distribution in the environment.

Numerous studies have investigated the survival of enteric bacteria in aquatic ecosystems (5, 11, 12, 14, 15, 18, 20, 23, 24, 32). *Salmonella* spp. are usually found in higher concentrations in sediments than in overlying water (14, 15, 20, 23). This has been attributed to sedimentation, sorption, and the extended survival in sediments (5). The evidence provided by these studies suggests that aquatic sediments are reservoirs for pathogens, creating a potential health hazard from resuspension, transmission, and subsequent ingestion by humans and wildlife. If *Salmonella* spp. are capable of surviving, concentrating, or multiplying in aquatic hosts, water pollution may be further accentuated by fecal excretion or shedding (21).

Chironomidae (chironomids), or midges, are good examples of prospective aquatic vectors for mr-DT-104. These insects live their entire larval stage, usually 3 to 4 weeks in duration, in sediments and then emerge as adults. The adults live a few hours to a few days in terrestrial environments, where they mate, and the female deposits egg masses back into the aquatic habitat. Not only do they live in intimate contact with sediments, but also many species are detritivores, potentially ingesting bacteria as they feed. In turn, chironomids at all stages of their life cycle provide food for fish, waterfowl, and other animals. Chironomids are found under a wide range of conditions; many species are tolerant of polluted environments, and as a group they are considered pollution tolerant. Chironomids are often found in large numbers in farm ponds, watering troughs, and other freshwaters associated with livestock facilities. All of these factors contribute to a high potential that larvae in these environments would be exposed to enteric bacteria such as mr-DT-104, making them potential vectors for spreading bacteria.

There are multiple potential pathways for mobilization of bacteria via aquatic vectors, such as chironomids. For example, larvae are often washed downstream; infected organisms may contaminate new sediments through exfoliation of externally attached bacteria and through their defecation. Contaminated larvae may pass bacteria to feeding predators. Emerging adults shed their larval exoskeletons in the water, and these contam-

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inated exuvia may infect new environments. Adults themselves are often preyed upon by avian species and thus may provide a pathway from aquatic to terrestrial environments. Avian species feeding upon chironomid adults might provide even greater mobility, potentially contaminating food sources and other possible habitats via defecation.

To identify the possibility of chironomids as hosts and vectors for the movement of mr-DT-104 through some of these pathways, we conducted a laboratory study to investigate (i) survival of mr-DT-104 in water and sediments; (ii) if mr-DT-104 organisms are ingested and survive in chironomid larvae; (iii) the ability of chironomids to carry and transmit mr-DT-104 from a contaminated to a clean environment; and (iv) the larvae-adult carry-over of mr-DT-104.

MATERIALS AND METHODS

General experimental method. An initial experiment was conducted to test survival of mr-DT-104 in water and sediments. One test tank (tank B) was inoculated with mr-DT-104 at a concentration of about 10,000 cells/ml. Water and sediment samples were taken for bacteria enumeration every other day through 125 days.

To test potential uptake of mr-DT-104 by chironomids, experiments were initiated by adding chironomid egg masses to five tanks (tanks C though G). Bacteria were added to the tanks in a food–mr-DT-104 slurry on day 4. Water, sediment, and larvae were sampled periodically from each tank until emergence of chironomid adults. Time to emergence varied from about 40 to 80 days. Adult chironomids were collected as they emerged from these tanks and were tested for mr-DT-104.

A final experiment was performed to assess transfer of bacteria by larval chironomids. Larvae from tanks C through G were transferred to a clean, salmonella-free tank. After 5 and 10 days, water and sediments from the clean tank were tested for mr-DT-104. Experimental procedures are detailed in the following paragraphs.

Experimental tanks. Studies were conducted in hexagonal, 7-liter acrylic aquaria. All tanks and tank accessories (air stone, tubing, and lid) were sterilized prior to each experiment with a 20% benzyl ammonium chloride solution (ROCAL-D; Pharmacia) and then rinsed with glass-distilled water. For the test, each aquarium was filled with approximately 4 liters of sterilized well water and 2 to 3 cm of test sediments. Tanks were maintained at 23 ± 2°C, on a 16:8 h light-dark photoperiod, with a dissolved oxygen concentration of 5 to 7 mg/liter, and a pH of 7 to 8. Tanks were placed randomly on the same countertop to account for ambient room factors.

Test sediments. Sediments were composed by milling playground sand to a consistent grain size of less than 200 μm. The milled sediment was rinsed in glass-distilled water, autoclaved, and allowed to settle in the test tanks for 24 to 48 h prior to chironomid egg mass additions.

Chironomid cultures. *Chironomus tentans* adults were captured from stock lab cultures and held in flasks for 24 h to allow mating and oviposition (1). One egg mass was transferred into each test aquarium. The time of egg mass addition was designated day zero for each specific test aquarium. Egg masses were monitored for the first 3 days to assure hatching and dispersal of the resulting first-instar larvae. On day 4 and every third day thereafter, 2 ml of Tetra-Min fish food slurry (10 g of Tetra-Min in 100 ml of water) was added.

Test bacteria. Stock cultures of mr-DT-104 were grown overnight at 37°C in Luria-Bertani agar (LB). Tank inoculations were made by first adding 0.4 ml of actively growing stock mr-DT-104 to an aliquot of Tetra-Min fish food. Then, 2 ml of the resulting mr-DT-104-positive food slurry was added on day 4 to each tank to achieve a final tank concentration of about 10,000 cells per ml in the aquaria water.

Enumeration of bacteria in water and sediment. Water and sediment samples were taken every other day following inoculation. Water samples were taken by pipette from the water column, taking care to avoid sediment entrainment. Sample volumes (1 ml and 100 μl) were spread plated on 150-mm-diameter plates. For sediments, 1 g of sample was taken and then suspended in 9 ml of LB diluent. Subsamples (1 ml and 100 μl) of this diluent-sediment mix were plated as described for water. Enumeration was performed using spread-plate counts on MacConkey agar containing 20 μg of nalidixic acid (MacNal)/ml. We regularly culture *Salmonella* strain mr-DT-104 in this medium, and the stock bacteria from which the test cultures were derived were maintained in this medium. *Salmonella* spp. isolates were confirmed using biochemical reactions and salmonella-specific antisera (16).

Bacteria in chironomids. Chironomid samples were taken from mr-DT-104-positive tanks periodically during their entire life cycle, from egg to adult stage. The number of chironomids sampled varied, depending on the populations in each test tank. Collected larvae were rinsed four times in distilled water and separated into three groups. Rinse waters numbers 1 and 4 were plated on MacNal and enumerated for mr-DT-104 to assess bacterial contamination on larva external surfaces. All chironomid samples were enumerated using MacNal plates following incubation for 18 to 24 h at 37°C.

One group of chironomids was homogenized and mixed with 1 to 2 ml of LB diluent and plated for enumeration. A second group was held in clean water for 48 h to allow depuration of the gut. These larvae were then mixed with 1 to 2 ml of diluent, homogenized, and plated for mr-DT-104 enumeration. Holding waters were also tested for mr-DT-104 at the end of the 48 h to test for excreted bacteria. The third chironomid group was transferred to a clean tank (salmonella negative) to test transmission of mr-DT-104 by infected larvae.

Adult chironomids were collected from tanks C through G upon emergence. Adults from all tanks were combined and stored at 4°C until plating was conducted.

RESULTS

Survival of mr-DT-104 in water and sediment. In tank B, mr-DT-104 was present in overlying water through 20 days thereafter (Table 1). Bacterial densities in the water column decreased with time. Negative results were first observed in the water on day 21 and remained negative through day 119 at the lower, 100-μl sample volumes. The 1-ml samples were intermittently culture positive and negative through day 54, at which time only 1 CFU/ml was detected. From day 55 onward, the 1-ml water samples were culture negative for mr-DT-104.

Sediments in tank B showed greater than 10,000 CFU/g at 4 to 12 days postinoculation and remained detectable until day 119 (Table 1). The CFU per gram of sediment values ranged from as low as 100 CFU/g on day 51 to as high as 12,200 CFU/g on day 64. All samples taken on days 120 to 125 were negative for mr-DT-104.

mr-DT-104 in chironomids. Chironomid samples were taken from tanks C through G. Table 2 shows larvae numbers and mr-DT014 enumeration data for water, sediments, and larvae sampled for each tank. Prior to plating, all chironomids collected were rinsed in glass-distilled water four times, and the rinse water was plated to MacNal agar to evaluate numbers of mr-DT-104 that might be attached to exteriors of the insects. Rinse waters were all negative except on three occasions where very low (1 to 2 CFU/ml) numbers were detected. Colonies observed in the chironomid samples were expressed as CFU per larva. Each test tank produced at least 1 mr-DT-104-positive individual, with contamination rates ranging from 0.04 to 82 CFU/larva.

On three separate occasions, a total of 85 chironomids were held in a vial containing glass-distilled water for 48 h to allow depuration of the gut. In all cases, larvae tested positive for mr-DT-104 (Table 3). Colonies ranged from 230 CFU/ml to >10,000 CFU/ml of homogenized larvae. Holding water tested positive for mr-DT-104, ranging from 28 to >10,000 CFU/ml.

Transmission of mr-DT-104 by positive larvae. A total of 161 mr-DT-104-positive larvae were placed in clean (salmonella-free) test tanks in four replicates (tanks H through K). In these experiments, water and sediment samples were negative for mr-DT-104 when tested on days 5 and 10 (Table 4).

TABLE 1. *Salmonella* strain mr-DT-104 in tank B water and sediments[a]

Day	CFU in sample			
	100 μl of water	1 ml of water	1 ml of sediment	100 μl of sediment
1	5,400	3,747	7,696	ND
4	1,690	1,094	TMTC	ND
5	3,40,262	TMTC	ND	
6	11,085	TMTC	ND	
7	280,213	TMTC	ND	
8	360,227	TMTC	ND	
12	22,025	TMTC	ND	
13	30	9	ND	67,900
14	60	93	ND	58,200
16	570	449	ND	57,200
19	10	25	ND	18,900
21	0	0	ND	33,200
22	0	7	ND	35,800
26	0	0	ND	3,300
28	200	7	ND	47,300
33	0	0	ND	600
35	0	0	ND	20,600
40	0	0	ND	27,600
47	0	0	ND	13,100
51	0	0	ND	100
54	0	1	ND	5,400
55	0	0	ND	7,100
56	0	0	ND	6,000
58	0	0	ND	8,000
62	0	0	ND	TMTC
64	0	0	ND	12,000
68	0	0	ND	1,000
70	0	0	ND	200
75	0	0	ND	300
81	0	0	132	ND
83	0	0	ND	300
85	0	0	ND	0
88	0	0	ND	0
90	0	0	95	ND
96	0	0	4	ND
98	0	0	46	ND
105	0	0	10	ND
119	0	0	0	ND
121	0	0	0	ND
123	0	0	0	ND
125	0	0	0	ND

[a] Data are CFU per milliliter of water sample or CFU per gram of sediment sample. TMTC, too many to count; ND, not determined.

Larvae-to-adult transmission of mr-DT-104. Adult chironomids were collected from the five mr-DT-104-positive test tanks. Groups of 1, 15, 50, and 70 adults for a total of 136 were tested for mr-DT-104 on four different occasions. Adults from all four groups tested positive for mr-DT-104 (Table 5). Colony numbers ranged from 4 CFU/chironomid to too numerous to count.

DISCUSSION

Survival of mr-DT-104 in water and sediments. This study indicates that mr-DT-104 can survive for several months in aquatic environments, with enhanced survival in sediments relative to overlying water. These results are similar to studies on other bacteria species, many of which are typically found in higher numbers and which survive longer in aquatic sediments than in overlying waters (14, 22, 25, 31). Our data support previous observations that enteric bacteria are able to survive more readily in sediments than in overlying water (7, 17, 20, 30). Although the initial inoculum density of 10^5 CFU/ml used in the present study is higher than that usually found in natural systems, it is similar to the levels of enteric bacteria in human and animal feces. Consequently, our study would reflect conditions in a relatively heavily contaminated aquatic environment, as might be found around feedlots, farm yards, or other situations with high livestock densities.

mr-DT-104 in chironomids. The first part of this study provided evidence indicating that mr-DT-104 has the ability to survive for several months in aquatic sediments. As a result, benthic organisms in contaminated aquatic sediments have increased potential to be exposed to these bacteria. Because of their benthic habitat, feeding habits, widespread distribution, pollution tolerance, and ecological roles, chironomids in par-

TABLE 2. mr-DT-104 in water, sediments, and chironomids[a]

Tank	Time (days)	Water (CFU/ml)	Sediment (CFU/g)	Chironomids (CFU/larva)	Chiro-dep (CFU/ml)	No. of larvae
C	10	10	372	164	898	11
	20	317	1,227	17	630	40
	30	30	242	85	1,033	20
	40	0	151	0	0	10
Avg		89.3	498	66.5	640.3	20.3
SD		152.3	494.4	74.7	458.5	13.9
D	10	1,706	TNTC	62	732	10
	20	8	167	50	153	6
	30	1	22	0	0	25
	40	2,296	TNTC	1	20	35
	50	62	115	0	0	27
	60	3	77	0	0	10
Avg		679.3	95.3	18.8	150.8	18.8
SD		1,040.9	61.2	29.0	290.9	11.7
E	10	0	1,000	1	7	10
	20	55	128	0.1	30	15
	30	2	0	0.3	5	15
	40	13	2,800	7	105	15
	50	0	334	0.24	6	25
	60	1	8	1	28	28
	70	160	523	1	20	20
	80	45	266	0	0	10
Avg		34.5	632.4	1.33	25.1	17.3
SD		55.2	934.5	2.3	34.2	6.6
F	10	0	59	12	59	5
	20	17	200	3	54	18
	30	0	334	0	0	25
	40	0	203	0.4	10	25
	50	151	1,028	6	120	20
	60	1	120	0	0	10
Avg		28.2	291.3	3.6	40.5	17.2
SD		60.5	378.7	4.8	47.0	8.1
G	10	8	400	0	0	15
	20	11	65	0	0	25
	30	73	155	0.04	1	25
	40	101	1,214	1	20	20
	50	3	21	0	0	10
	60	ND	258	0	0	15
Avg		39.2	352.2	0.2	3.5	18.3
SD		44.8	443.8	0.4	8.1	6.1

[a] Chiro-dep, depuration water sample; TNTC, too numerous to count.

TABLE 3. Larvae collected from mr-DT-104-contaminated tanks (C, D, and E,) and mr-DT-104 counts in chironomids and in holding water after 48 h[a]

Tank	No. of larvae	CFU in larvae			CFU in holding water		
		10 μl	100 μl	500 μl	100 μl	500 μl	1 ml
C	20	ND	TNTC	TNTC	ND	TNTC	TNTC
D	30	TNTC	TNTC	TNTC	TNTC	ND	TNTC
E	35	230	680	ND	0	ND	28

[a] Data are CFU per milliliter. TNTC, too numerous to count; ND, not determined.

TABLE 5. Enumeration of mr-DT-104 colonies in adult chironomids[a]

No. of adults tested	CFU/organism per vol tested		
	100 μl	500 μl	1 ml
1	ND	ND	4
15	15	15	9
50	323	63	TNTC
70	TNTC	TNTC	ND

[a] ND, not determined; TNTC, too numerous to count.

ticular are strong prospective candidates as vectors for mr-DT-104 mobility in the environment.

Data from tanks C through G were pooled, and a Pearson correlation coefficient was run between bacteria densities in sediments and chironomids. At a significance level of 0.05, the calculated *P* value was 0.053 and the *r* value was 0.357, indicating a marginally significant correlation. As shown in Table 2, standard deviations in the data are large, making any rigorous linear correlation between actual numbers of bacteria in chironomids and sediments questionable. However, previous studies have shown a general relationship between bacterial densities in water or sediments and in a variety of organisms living in those media. For example, Greenberg et al. (13) reported an increase in *Salmonella* excreted by the housefly with every increase of *Salmonella* sp. exposure. Kopanic et al. (19) report that the density of salmonella in guts of cockroaches is dependent on inoculum load, and Rowse and Fleet (29) showed that release of bacteria in oyster feces was dependent on contamination levels. For the most part, contaminated sediments in our experiments produced contaminated chironomids. Lack of colonies in the rinse waters indicated that bacteria were actually in the larvae guts and not attached externally. It is reasonable to conclude that chironomids ingest mr-DT-104 organisms present in the sediment and that the probability of contamination with salmonella is to some degree a function of the degree of sediment contamination.

Cross-contamination by infected chironomids was not established in the present study. We did detect mr-DT-104 in the chironomid holding waters (Table 3), and this would indicate that carrier larvae do shed bacteria. For the aquaria experiments, the number of infected chironomids may have been too small, or the numbers and viability of salmonella cells excreted may have been insufficient to infect the clean tanks or to be detected. Roszak et al. (28) reported that simple nutrient additions were insufficient to resuscitate cells of *Salmonella enterica* serovar Enteritidis after their apparent die-off. In the present study, nutrients were added to the clean test tanks at 3-day intervals as long as chironomid larvae were present. However, we do not know whether the amount was enough to maintain nutritional requirements of potentially stressed mr-DT-104 organisms that may have been released by infected larvae, or if any released bacteria were simply nonviable.

Data from the 48-h holding experiment (Table 3) did show that chironomids were infected and were excreting mr-DT-104. It was not determined how long mr-DT-104 survived in the holding water and how fast densities declined. The holding water (50 ml) may have been more conducive to the survival of mr-DT-104, compared to the tank environments. We can reasonably speculate that mr-DT-104 organisms were not multiplying in the chironomid guts. Overall, the question of spread of contamination by bacteria excreted from chironomid larvae, although not supported by our study, cannot be ruled out either.

Results from the larvae-to-adult transmission trials illustrate that mr-DT-104 is carried from the larval to adult stage. In all four trials in which adults were tested, mr-DT-104 was found in high numbers, and there was a consistent increase in CFU per chironomid as the number of adults tested increased. It is possible that the bacteria were able to multiply in the adult digestive system. Greenberg et al. (13) found *Salmonella* was able to increase in numbers in adult houseflies, compared to lower survival in housefly maggots. These authors suggested that the gut of larval houseflies may contain natural flora that present antagonism towards the survivability of *Salmonella* sp. If this is so, salmonella numbers in larvae may be a function of time of exposure to bacteria and may explain some of the variability of bacterial results in the larvae.

One potential issue concerns viable-but-not-culturable (VBNC) bacteria that may be present in the experiments and could have been carried over to water and sediments with chironomids. The presence of VBNC bacteria would mean that actual organism numbers were underestimated by the culturing methods employed here, as some have suggested (for example, see Domingo et al. [9]). However, several recent papers contend that bacteria do not enter VBNC states (for example, see Bogosian et al. [4]). Even for *Vibrio cholerae*, the organism for which there seems to be the most extensive literature on VBNC, a recent review casts doubt on the importance of this mechanism as a reservoir (10). As a recent exhaustive review on the subject has pointed out, "the existence of the VBNC

TABLE 4. Larvae collected from mr-DT-104-contaminated tanks (C, D, E, and F) and pooled results of mr-DT-104 in water and sediment samples from *Salmonella*-free tanks (H, I J, and K) after 10 days[a]

Source tank	No. of larvae	mr-DT-104 (CFU/mL) in sample		
		100 μl of water	1 ml of water	1 ml of sediment
C	45	0	0	0
D	58	0	0	0
E	50	0	0	0
F	8	0	0	0
Total	161			

[a] All samples were negative for mr-DT-104.

state is in debate, since there is no direct conclusive information about the underlying molecular processes or the genetic factors involved" (26). Culturing methods remain widely accepted and constitute the mainstay of microbiological detection for health departments throughout the country. Evaluating the relevance of VBNC for mr-DT-104 was beyond the scope of this study.

In summary, our results have demonstrated that mr-DT-104 can survive for long periods in water and even longer in sediments. Chironomid larvae do appear to take up mr-DT-104 from contaminated sediments, but the larval digestive system does not appear to enhance salmonella growth and reproduction. The issue of larval chironomids as a vector for these bacteria in aquatic environments remains an open question. *Salmonella* strain mr-DT-104 can survive in chironomid adults, and it may even multiply, indicating a strong potential that they are vectors for transmission of *Salmonella* sp. from aquatic to terrestrial environments.

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