Escape of Salmonellae from Chlorination during Ingestion by *Pristionchus lheritieri* (Nematoda: Diplogasterinae)[1]

S. M. SMERDA,[2] H. J. JENSEN, AND A. W. ANDERSON[3]

Abstract: *Salmonella typhi* and *S. wichita* survived 15 min in 10 ppm free chlorine solution during ingestion by the free-living nematode, *Pristionchus lheritieri*, and were viable when defecated in suitable media. *S. typhi* and *S. wichita* were recovered in 13.5 and 46.6% of the trials on five different media. *Key Words:* Bacteriophagous nematode, *Salmonella typhi* and *S. wichita*, Chlorine, Ingestion, Defecation.

Free-living bacteriophagous nematodes are routinely found in both raw and treated domestic water supplies (1, 2, and author's unpublished data). These usually are associated with decomposing organic material and soil before reaching raw water supplies, providing ample opportunity for ingestion of bacteria. Bacteria also can be obtained directly from raw and waste water. Thus, occurrence of bacteriophagous nematodes in water may be important when enteric organisms are present.

The threat of waterborne pathogenic bacteria in metropolitan drinking water has largely been eliminated by standardized water treatment procedures. Many streams, however, are used simultaneously for water supply and for carrying effluent sewage. Also, an expanding population requiring a more frequent reuse of water may increase the incidence of waterborne pathogenic bacteria. Unusual situations occur where treatment facilities might be hampered by natural disasters. The immediate remedy would probably be introduction (or increase) of free chlorine in the water supply. In such conditions, bacteriophagous nematodes in water supplies survive routine chlorination procedures and may contribute to the peril of waterborne bacterial diseases.

Occurrence of nematodes in treatment plants and in drinking water is not a new or rare phenomenon. Cobb (5) found free-living nematodes in slow-sand filter beds and aroused public attention to this association. It has been determined that pathogenic enteric bacteria are ingested by free-living nematodes and while in the nematode gut are protected from very high concentrations of free chlorine. Chang, Woodward, and Kabler (2) and Chang and Kabler (4) have shown bacteria survive in 100% of nematodes, 90% of which have been immobilized by chlorination. They were, however, not able to prove that viable, infective bacteria were defecated by the surviving nematodes (2, 4).

The purpose of the present study was to determine whether viable *Salmonella typhi* or *S. wichita* survive chlorination after ingestion by *Pristionchus lheritieri* and whether these pathogens remain viable (and potentially infective) following passage through and defecation from the nematode.

MATERIALS AND METHODS

The organisms used were *Pristionchus lheritieri*, Maupas, the nematode carrier, and *Salmonella typhi* and *S. wichita*, the enteric pathogens. Salmonellae from stock cultures maintained on nutrient agar slants at 5 C, were transferred to non-selective agar plates and incubated 24 hr at 35 C. Nematodes,

Received for publication 20 May 1970.

[1] From a M.S. thesis submitted by the senior author at Oregon State University. Technical Paper No. 2892, Oregon Agricultural Experiment Station.

[2] Present address of senior author: Microbiology Laboratory, Kraftco Corporation; 801 Waukegan Road, Glenview, Illinois 60025.

[3] Professor of Nematology and Professor of Microbiology, Oregon State University, Corvallis, Oregon 97331.

growing on nutrient agar plate cultures of *Pseudomonas* sp., were added to the *Salmonella* plates and incubated for two days at room temperature. Individual nematodes were placed in sterile petri plates or sterile glass vials containing 5 ml of 10 ppm free chlorine solution. A 15-min exposure in 10 ppm of free chlorine was sufficient to remove Salmonellae from the nematodes' cuticle as shown by Chang *et al.* (3). The following preliminary test indicated that external *S. typhi* and *S. wichita* were as susceptible to this concentration × time exposure as those tested by Chang *et al.* (3) and yet had no obvious effect upon the nematodes.

Individual nematodes which had fed on *Salmonella* for two days were transferred into 5 ml of 10 ppm free chlorine solution. At 10, 20 and 30 min intervals, five 0.1-ml aliquots were removed and placed on nutrient agar plates. After 24 hr at room temperature, the nutrient agar plates were streaked with a sterile loop, transferred to *Salmonella-Shigella* (SS) agar plates and incubated at 35 C. Although no Salmonellae were recovered at these time intervals, the contact time was kept consistent with Chang's work and a 15-min exposure was used for the remainder of the tests. Ppm free chlorine remaining after the designated contact time was titrated by the Iodometric Method for determining the ppm of the chlorine solution. The decrease was very slight indicating that there was enough available chlorine to destroy exposed Salmonellae.

After neutralization of the chlorine by sterile sodium thiosulfate, the nematodes in the petri plates were transferred to non-selective agar plates and incubated at room temperature for 10 hr. Individual nematodes from glass vials were transferred to another set of vials containing one of four liquid media; a non-selective broth, 0.1% peptone-water, unsterile tap water and sterile tap water and stored at room temperature for 10–24 hr depending on the type of media.

Following the 10 hr incubation period the nematodes were transferred to fresh agar plates and incubated as before. Original plates (those initially containing surface-sterilized nematodes) were streaked with a sterile loop which was then used to streak an SS agar plate.[4] Both plates were incubated at 35 C for 48 hr. The streaking procedure was repeated again in 24 hr.

Liquid media suspensions received a slightly different treatment. After 10 hr (for sterile and unsterile tap water vials) and 24 hr (for the broth and peptone-water vials) of incubation, sterile loop transfers were made from each vial onto separate plates of SS agar and nutrient agar for additional incubation of 48 hr and 35 C. Typically positive colonies on SS agar plates were transferred directly to Triple Sugar Iron (TSI) slants and incubated 24 hr at 35 C. If no growth was evident on SS agar plates but could be detected on nutrient agar plates, streaks were made from the latter to SS plates and incubated as before. If typical colonies were present, they were transferred to TSI agar slants. The sole criterion for identification of viable Salmonellae was the ability of positive TSI slants to agglutinate polyvalent "0" antisera.

Grinding was used by Chang to determine the nematode's internal population of Salmonellae. The body wall of the nematodes in liquid preparations could conceivably rupture causing recovery results to be measures of internal and not defecated Salmonellae. Thus, following incubation periods, the vials were emptied into petri dishes and examined

[4] Dr. Chang (Chief, Etiology Bureau of Water Hygiene, Dept. Health, Education, and Welfare. Cincinnati Laboratory) recently informed us (personal communication) that the amperometric titration is more efficient for free-chlorine determinatioin and that SS agar is toxic to *Salmonella-Shigella* organisms although toxicity is less than to other bacteria. He also suggested that XLD or Hectone agar are better selective media for *Salmonella* and *Shigella* organisms.

TABLE 1. Defecation of viable *Salmonella typhi* by *Pristionchus lheritieri.*[a]

	Hartsell's agar plates	Hartsell's broth	Peptone water	Tap water	Sterile tap water
Total No. trials	40	40	40	40	40
No. Hartsell's plates with growth	40	40	40	0	10
No. presumed-positive SS[b] plates	32	40	37	0	0
No. presumed-positive TSI slants[c]	8	19	0	0	0
No. positive agglutination tests	8	19	0	0	0
% trials positive	20	47.5	0	0	0

[a] Following chlorination (ten ppm free chlorine for 15 min) and neutralization (0.1 N sterile sodium thiosulfate), the nematode was transferred to a Hartsell's agar plate or suspended in one of the liquid environments listed.
[b] *Salmonella-Shigella* agar.
[c] Triple Sugar Iron agar.

TABLE 2. Defecation of viable *Salmonella wichita* by *Pristionchus lheritieri.*[a]

	Hartsell's agar plates	Hartsell's broth	Peptone water	Tap water	Sterile tap water
Total No. trials	30	60	40	60	40
No. Hartsell's plates with growth	30	60	38	30	12
No. presumed-positive SS[b] plates	29	60	30	11	4
No. presumed-positive TSI slants[c]	28	56	11	7	3
No. positive agglutination tests	28	56	11	7	3
% trials positive	93.3	93.3	27.5	11.7	7.5

[a] Following chlorination (ten ppm free chlorine for 15 min) and neutralization (0.1 N sterile sodium thiosulfate), the nematode was transferred to a Hartsell's agar plate or suspended in one of the liquid environments listed.
[b] *Salmonella-Shigella* agar.
[c] Triple Sugar Iron agar.

microscopically for the presence of the intact nematode. In all instances only intact nematodes were found.

RESULTS AND DISCUSSION

Major differences in recovery of the two Salmonellae spp. were noted (Tables 1 & 2). *S. typhi* was recovered in 20% of the trials in which nematodes were added to agar plates while *S. wichita* recovery was 93.3% under the same conditions. Positive trials in the non-selective broth were not as obvious but still favored *S. wichita* over *S. typhi* 93.3% to 47.5%, respectively. It also was noted that *S. typhi* was not recovered from the other liquid suspensions while *S. wichita* occurred in all suspensions.

It should be noted that both *Salmonella* spp. were cultured in optimum conditions where their behavior and development are similar. Differences then could be due to the quantity of organisms ingested, preferential digestion of ingested Salmonellae or mutation and survival during passage through the digestive tract. No direct comparison of quantities of ingested *Salmonella* spp. was made, although microscopic examination of the intestinal contents did not reveal marked differences. Preferential digestion, mutation and survival may account for major differences. Nematodes are known to possess various digestive enzymes which could affect specific cell walls interfering with development and survival of ingested bacteria during passage through the alimentary canal (6).

Another major difference was the effect of various liquid media upon defecation of Salmonellae by *P. lheritieri.* Defecation of *S. typhi* more than doubled in nutrient broth (47.5%) over nutrient agar plates (20%) with none recovered from peptone water, sterile tap water or unsterile tap water (Table 1). *S. wichita* showed equally high recovery (93.3%) on nutrient agar plates and in

nutrient broth but somewhat less in the other three media (Table 2).

A major consideration is that defecation of viable *S. wichita* occurred in tap water, thus approaching conditions that could be encountered by the public. Although opportunity for transmission of pathogens by a nematode carrier may be slight, it is sufficient to justify concern. Nematodes easily tolerate chlorination, a standard procedure in treating municipal water supplies and for emergency water treatment. When the nematodes also have access to bacterial pathogens, chlorination will not be effective against ingested organisms. We support Chang's recommendation that finished water be examined for nematodes and when their number exceeds 10 per gallon, an investigation as to the water source and remedial measures should be developed.

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