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SUSAN MAE SMERDA for the M. S.
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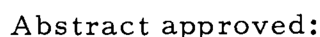

The role of nematodes (*Diplogaster lheritieri* Maupas 1919) as excretors of pathogenic organisms was studied. *Salmonella typhi* and *Salmonella wichita* were fed to nematodes for two days. The nematodes were surface sterilized with free chlorine. The activity of the chlorine was stopped by the addition of sodium thiosulfate followed by transfer of the worms to one of four liquid environments or to a non-selective agar plate. Recovery of excreted salmonellae was determined by the presence or absence of these organisms in the liquid environments or on the agar plate.

Salmonella wichita was recovered in more trials than was *Salmonella typhi*.

As the nutrient value of the suspending media decreased, it was noted that excretion of the organisms by the nematodes decreased.

Excretion of Viable Salmonellae by *Salmonella*-Fed, Chlorine-Treated Nematodes

by

Susan Mae Smerda

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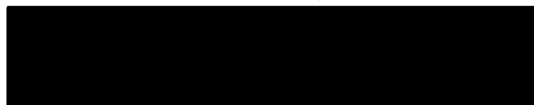
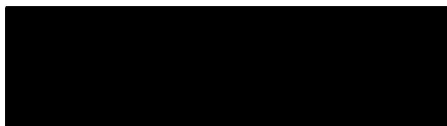


Professor of Microbiology
in charge of major



Chairman of Department of Microbiology

Dean of Graduate School

Date thesis is presented November 21, 1969

Typed by Marion F. Palmateer for Susan Mae Smerda

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EXCRETION OF VIABLE SALMONELLAE BY *SALMONELLA*-FED, CHLORINE-TREATED NEMATODES

INTRODUCTION

The fear of waterborne bacterial diseases has been eliminated due to the excellent treatment procedures employed by sanitation authorities. Many streams, however, are now being used alternately for water supply and for carrying effluents from sewage treatment plants. In some cases, there is a relatively frequent reuse of water which might increase the occurrence of waterborne bacterial diseases (12).

In addition, consider some abnormal conditions that one may encounter where normal treatment facilities might be hampered. This could include an individual living in an area recently devastated by flood, tornado or hurricane, or a community inflicted with an enteric epidemic, or a soldier in an underdeveloped country. The immediate remedy would undoubtedly be an introduction (or an increase) of free chlorine into the water supply.

Under the above conditions, the nematode, which feeds on bacteria, has access to water supplies and survives routine chlorination procedures, may contribute to the peril of waterborne bacterial diseases. It is the intent of the author to examine the role of nematodes as potential carriers of enteric pathogenic organisms.

LITERATURE REVIEW

Nematodes, belonging to the phylum Nemata, are unsegmented roundworms that can be divided into fresh water, marine, soil or parasitic groups (10). It is to free-living soil nematodes that our attention is drawn.

Occurrence of these organisms in drinking water and in treatment plants is by no means a new or rare phenomenon. Cobb (9), as early as 1918, found free-living nematodes in slow sand filter beds and estimated that in the upper three inches of a bed hundreds of millions of nematodes could be recovered per acre. Their occurrence in a large city water supply in 1955 (6) revealed the existence of the nematode problem in water treatment plants employing rapid sand filtration. Jensen (13) can routinely recover nematodes from Corvallis drinking water while Chang, Woodward and Kabler (5) have isolated these organisms from 10 out of 22 finished water samples studied. It should be noted, also, that disinfection (presumably chlorination) was the final step in the treatments studied by the latter investigators--slow sand filtration, rapid sand filtration, lime or lime-soda softening being initial steps. Chang cites elsewhere (3, 7) that the most remarkable feature of these free-living nematodes is their high resistance to free chlorine.

The majority of these nematodes isolated belong to a group

associated with decomposing or rotting plant tissues in contact with the soil, or any site where bacteria are plentiful, for these nematodes are saprophages (11). Therefore, the opportunity exists for the nematodes to pick up the bacteria, before the nematodes reach the raw water supply. In order to reach domestic water, these soil nematodes are washed into the supply or originate from sewage treatment plants (4). Occurrence of bacteria-feeding nematodes in water is important when the presence and persistence of fecal organisms in water can be demonstrated. In laboratory experiments, the viability of *Salmonella typhi* in raw water has been shown by A. C. Houston (17) to extend to three weeks preceded by an initial kill of 99.9% in one week. In addition, there exists the possibility that the nematodes may ingest the bacteria following treatment should post-contamination of the water occur or at the site of treatment since both the nematodes and the microorganisms can be present. When *Salmonella typhi* and *Salmonella paratyphi B* were added to purified drinking water, Thresh, Beale and Suckling (17) obtained an initial rapid decrease in viable organisms followed by complete kill after three weeks' storage. Shrewsbury and Barson (16) inoculated large numbers of typhoid organisms into tap water, distilled water and normal saline solution and were able to recover viable *Salmonella typhi* for the longest period of time in distilled water (443 days) followed by tap water (211 days) and, finally, normal

saline (153 days). The above experiments point out an important fact; pollution of pure or purified waters at treatment plants, in reservoirs or in mains is particularly dangerous.

It can be seen that nematodes can and do exist in pre- and post-chlorinated water, that salmonellae organisms can survive for long periods in finished drinking water after obtaining access to such water and, finally, that nematodes can withstand disinfection procedures. Therefore, the question must be raised as to the feasibility of nematodes acting as both protectors and excretors of pathogenic organisms following chlorination procedures.

It must be noted that previous work in this area has been conducted in its entirety by Chang and his colleagues (4, 7). The objectives of these investigators were to find out how effective nematodes fed on human enteric pathogens, how long the organisms survived in the worm gut, how much protection against chlorination the carrier worms offered to the ingested pathogens and whether any viable pathogens were excreted by the worms.

The first three objectives were achieved. The nematodes were found to readily ingest salmonellae organisms on plate cultures. After 24 hours in sterile Ohio River water (preceded by 15 minutes in ten parts per million free chlorine), 5 to 6% of the ingested *Salmonella typhi* and 12 to 16% of the *Salmonella paratyphi* survived while 48 hours incubation showed a reduction in

survival to 0.1% and 1% respectively. The nematodes protected the ingested pathogens to the extent that the latter had complete survival even when 90% of the worms were immobilized by free chlorine. It is further stated that chlorination at any degree may be ineffective in eliminating the carrier status of the nematode, since the pathogens survived in the worm intestine even though the worm was dead. What these authors failed to show was that the nematodes excreted viable organisms. This was explained as due to the worms' dependence on proteinaceous material for their food supply and to their inability to ingest sufficient food to distend the intestine, so that they were breaking down the organisms into useable protein before they could be excreted intact.

The role of nematodes as protectors of salmonellae organisms has been well established by the above authors. Attention is subsequently focused onto the possibility of nematodes excreting viable salmonellae organisms following chlorination treatment.

MATERIALS AND METHODS

Nematode Employed

The nematode employed as the carrier worm, *Diplogaster lheritieri* Maupas 1919, was one of many saprozoic nematodes. Jensen (13) obtained the original population from rotten carrots approximately one and one-half years ago and has since made weekly transfers except during the hot weather when the time was shortened to five days. The genus *Diplogaster* had been consistently associated with work of this nature (4, 5, 7). Female and male adult nematodes were randomly chosen for experiments, their length varying from one to one and one-half millimeters. The worm is externally covered with a noncellular layered cuticle (10) which extends inward at the mouth, anus and vulva (8). The nematodes' resistance to chlorination treatment is attributed to the presence of this cuticle (13).

Microorganisms Studied

The two salmonellae species were obtained from the Oregon State University Microbiology Department stock culture collection and both have been associated with enteric diseases (18). They were *Salmonella typhi* and *Salmonella wichita*.

Culture Media

The medium used for nematode propagation consisted of 15 grams Difco Nutrient Agar, 5 grams peptone or tryptone, 3 grams beef extract and 1000 milliliters distilled water. Autoclaving for 20 minutes at 17 pounds per square inch pressure was sufficient for sterilization.

The salmonellae species were streaked onto poured Harsell's agar plates, a non-selective medium comprised of 5 grams proteose-peptone, 5 grams tryptone, 5 grams sodium chloride, 5 grams yeast extract, 2.5 grams veal infusion medium, 20 grams agar and 1000 milliliters distilled water. Following autoclaving at 15 pounds per square inch pressure for 20 minutes, the pH was 7.2.

After chlorination and neutralization, the nematodes were suspended in various liquid environments as well as being placed directly onto Harsell's agar plates. These liquid environments were:

1. Hartsell's broth: made as indicated above for Hartsell's agar with the omission of agar.
2. Peptone water: one gram proteose-peptone and 1000 milliliters distilled water. Autoclaving was at 15 pounds per square inch pressure for 20 minutes.
3. Sterile tap water: obtained from the Corvallis water supply and autoclaved for 20 minutes at 15 pounds per square inch pressure.

4. Tap water: obtained from the Corvallis water supply and used directly.

Recovery of salmonellae necessitated the use of differential media. For this purpose, Difco Salmonella-Shigella agar and Difco Triple Sugar Iron agar slants were used. Preparation of the media followed instructions provided on the containers. Serological confirmation of salmonellae was made with Difco Polyvalent "O" Antisera.

Feeding of Salmonellae to Nematodes

The salmonellae stock cultures were maintained on Hartsell's agar slants at 5°C. Transfers were made to Hartsell's agar plates and incubated for 24 hours at 35°C. Five to seven day cultures of nematodes were provided by Jensen upon request. Three small cubes of nematode-laden medium were aseptically removed and placed onto Hartsell's agar plates of salmonellae. Feeding was allowed to continue for two days at room temperature.

Surface Sterilization of Nematodes

Individual nematodes were aseptically placed into either sterile petri plates containing 5 milliliters of 10 parts per million free chlorine or into sterile glass vials (1.5 inches in diameter and 2.5 inches in height) containing the same amount of free chlorine. The

aid of a dissecting microscope was necessary to facilitate these transfers due to the nematodes' small size. The transfer was readily accomplished with a splintered piece of bamboo acting as transfer implement. The splinter was placed into chlorine when not in use or between transfers of nematodes to fresh Hartsell's agar plates. In addition, the splinter was frequently checked as to its sterility by streaking it along Hartsell's agar plates and Salmonella-Shigella agar plates. Ten parts per million free chlorine for 15 minutes were sufficient to remove clinging salmonellae from the nematode without immobilization of the nematode occurring. The chlorine solutions were prepared by diluting commercial Purex. The Iodometric Method (1) was followed for the determination of initial parts per million free chlorine and of residual chlorine. After the addition of an excess of sterile 0. 1 N sodium thiosulfate to stop the activity of the chlorine, those nematodes placed into the petri plates had to be removed to Hartsell's agar plates. This was done with a sterile pipette. The nematode with as little liquid as possible (usually no more than 0. 1 milliliter) was drawn into the pipette, released onto the agar plate and held at room temperature for various time periods. The transfer of the nematode to a liquid environment was not a problem, since the liquid was added to the vial containing the nematode and the neutralized chlorine solution. The vials were likewise held at room temperature for varying time periods.

Recovery of Viable Salmonellae

The petri plates containing nematodes were examined after ten hours at room temperature and then the nematodes were transferred again to a fresh Hartsell's agar plate and incubated as above. The original plate was streaked with a sterile loop which was used, in turn, to streak a *Salmonella*-*Shigella* agar plate. Both plates were incubated at 35° C. for no more than 48 hours. This procedure was repeated at 24 hours following the first transfer of the nematode to the Hartsell's agar plate.

After ten hours (for sterile and unsterile tap water vials) and 24 hours (for Hartsell's broth and peptone water vials) in the suspending fluid, sterile loop transfers were made from each vial onto separate plates of *Salmonella*-*Shigella* agar and Hartsell's agar. Incubation was again for no longer than 48 hours at 35° C.

Typically positive colonies on *Salmonella*-*Shigella* agar plates were directly picked to Triple Sugar Iron agar slants and incubated for 24 hours at 35° C. If no growth was evident on the *Salmonella*-*Shigella* agar plates but could be detected on Hartsell's agar plates, streaks were made from the latter plates to *Salmonella*-*Shigella* agar plates and incubated as above. Typically positive colonies were again sought and, if present, picked to Triple Sugar Iron agar slants.

The sole criteria used for determining the presence of viable

salmonellae was the ability of positive Triple Sugar Iron agar slants to agglutinate Polyvalent "O" Antisera. The agglutination procedure accompanying the antisera was followed.

RESULTS AND DISCUSSION

The experimenter must have confidence in the method employed in order to draw reliable conclusions. To gain this confidence, preliminary tests were indicated to determine the amount of chlorine and contact time needed for killing external salmonellae, the residual chlorine after contact time and the stability of the nematode in the suspending media.

Individual nematodes, following feeding on salmonellae plates for two days, were dropped into five milliliters of ten parts per million free chlorine. At 10, 20 and 30 minute intervals, five 0.1 milliliter aliquots were removed and placed onto Hartsell's agar plates. After 24 hours at room temperature, the Hartsell's agar plates were streaked with a sterile loop and transferred to *Salmonella-Shigella* 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 15 minutes were used for the remainder of the tests.

The free chlorine remaining after the designated contact time was titrated according to the method already referred to for determining the initial parts per million of free chlorine (Iodometric Method). The decrease was very slight indicating that there was enough available chlorine to destroy free salmonellae.

Nematodes had been found to be very resistant to repeated thawing and freezing. Their digestive tracts were also found to be resistant to blending (4). Grinding was, therefore, used by Chang (4, 7) to determine the internal population of salmonellae. Suspension in various liquid environments, however, could conceivably break up the nematode and the recovery results would then be qualitative ones of internal and not of excreted salmonellae. Therefore, following incubation periods, the vials were emptied into petri plates and examined with the dissecting microscope for the presence of the intact worm. In all instances, the intact worm was found.

The choice of medium and reproducibility of results are two other considerations to be made regarding the method. Salmonellae methodology remains one of the most complicated in analytical microbiology. Quantitative recovery of even pure cultures on selective media was reduced several logs when compared to a non-selective medium.[1] Because the method that was used in this research was a qualitative test and because a non-selective medium (Hartsell's agar) was used in conjunction with Salmonella-Shigella agar, the possibility of missing salmonellae was lessened. In several instances (in sterile tap water and tap water), initial streaking onto Salmonella-Shigella agar plates proved negative while secondary streaking, from

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[1] Author's personal observation of these two organisms on both Salmonella-Shigella agar plates and on Hartsell's agar plates.

Hartsell's agar plates, proved positive.

Although false positive colonies appeared regularly on Salmonella-Shigella agar plates, this was not the case with presumed-positive Triple Sugar Iron agar slants. This was, in part, due to the fact that the reactions of the organisms on Triple Sugar Iron agar slants were known beforehand and readily recognized. Nevertheless, all presumed-positive slants were agglutinated in Polyvalent "O" Antisera before establishing their identity as salmonellae.

The experiments were repeated in duplicate sets to determine reproducibility of results. In only one instance, Salmonella wichita and sterile tap water, was reproducibility not achieved, i. e., one set of 20 identical vials proved negative while the second set of 20 showed three positives. Reasons for this discrepancy remain unknown to the author.

As is evident from Tables 1 and 2, differences existed in two directions. The first was the difference between the two salmonellae species. Salmonella typhi was recovered in 20% of the trials when the nematode was placed onto Hartsell's agar plates while Salmonella wichita yielded 93. 3% positive trials under the same conditions. In Hartsell's broth, the percent of positive trials were 47. 5% and 93. 3% for Salmonella typhi and Salmonella wichita respectively. These findings could be due to one of two things. There was either a preferential selection of organisms for ingestion or a preferential digestion

Table 1. Excretion of viable *Salmonella typhi* by *Diplogaster lheritieri*.

	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** plates	32	40	37	0	0
No. presumed-positive TSI** slants	8	19	0	0	0
No. positive agglutination tests	8	19	0	0	0
% . trials positive	20	47.5	0	0	0

*Following chlorination (ten parts per million free chlorine for 15 minutes) 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.

***Salmonella*-*Shigella* agar

***Triple Sugar Iron agar

Table 2. Excretion of viable Salmonella wichita by Diplogaster lheritieri.

	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** plates	29	60	30	11	4
No. presumed-positive TSI*** slants	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

*Following chlorination (ten parts per million free chlorine for 15 minutes) 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.

**Salmonella-Shigella agar

***Triple Sugar Iron agar

of ingested organisms by the nematode. The bacteria-feeding, free-living nematodes contain at least two known enzymes, amylase and protease (14). To date, however, little work has been done in the area of digestive enzymes for non-parasitic nematode groups. Nevertheless, it is possible that saprozoic nematodes contain an enzyme that digests specific salmonellae cell walls.

The second difference resulted from the type of suspending media employed. Salmonella typhi were excreted more in Hartsell's broth (47.5%) than they were on Hartsell's agar plates (20%) with no excretion of the organisms occurring in peptone water, sterile tap water or unsterile tap water. Salmonella wichita showed equal recovery amounts (93.3%) on Hartsell's agar plates and Hartsell's broth while the remaining three liquid environments were somewhat less. Their percentages of positive trials, in descending order, were peptone water (27.5%), unsterile tap water (11.7%) and sterile tap water (7.5%).

As stated previously, the nematode must depend on proteinaceous material for its food supply yet does not ingest sufficient food to distend the intestine. In addition, the excreta from the nematode was composed mainly of ammonia and carbonate (7). It was these observations that led to Chang's conclusion that the nematode digested the organisms to useable proteins before they could be excreted intact. It was surmised, therefore, that if a continuous protein supply was available to the nematode, then the nematode could utilize the protein

supplied in the medium and not obtain its supply from the organisms. Some organisms would then be expected to escape enzymatic break down and be excreted intact. The data supported this theory. As the nutrient supply was decreased in the suspending media, the excretion recoveries of Salmonella wichita decreased. It was to be expected that Hartsell's broth would give higher recovery percentages than Hartsell's agar plates since all nematodes, even the soil forms, require a moisture film in which to move and through which to breathe (10). This latter relationship is especially evident from Table 1.

The pH value for sterile tap water was 6.55 and that of unsterile tap water was 6.65. Because these differences were so slight, the pH was not considered to affect the survival in tap water. Sterilization was considered to alter the food nutrients in the water in some way accounting for the higher recovery percentages of Salmonella wichita in unsterile tap water.

The main consideration was that excretion occurred in tap water, thus approaching conditions that would normally be encountered by the public. Does a danger actually exist for the consumer? It must be stated that the opportunity for the transmission of pathogens by nematodes is slight. However, is it sufficient to conclude, as has been done in the past, that this possibility need not concern sanitation authorities? In light of the fact that chlorination is frequently the

remedy for emergency treatment, the author believes that concern is justified. Although the work to date has emphasized the remoteness of possible danger to the consumer under normal conditions, Chang does suggest that finished water be examined for the presence of nematodes. When the number of nematodes exceeds ten per gallon, an investigation into the source of water is recommended followed by remedial measures (5). This practice has not been adopted.

It is always ideal to work under conditions of closest approximation to the normal situation. Initial work must be conducted, however, in order to warrant more extensive examination of the problem. The author feels that the groundwork has been laid for recognition of a problem. Now is the time for a more detailed study into the possibility of danger under routine treatment conditions. It is suggested that pilot water and sewage treatment plants be set up under laboratory auspices and equal amounts of salmonellae-fed and E. coli-fed nematodes be exposed to said treatment and the excretion capabilities of the nematodes be determined. E. coli would act as the indicator organism in the above pilot study since they are presently the indicator organisms for water examination in the United States (1, 2, 15).

Since nematodes are so resistant to chlorination procedures, an investigation into other remedies that could be used in conjunction with or in place of free chlorine should be made.

SUMMARY

Nematodes (Diplogaster lheritieri Maupas 1919) were allowed to feed on one of two salmonellae species, surface sterilized with free chlorine (which was neutralized with sodium thiosulfate) and transferred to Hartsell's agar plates, Hartsell's broth, peptone water, unsterile tap water or sterile tap water. Recovery of excreted salmonellae was determined by the presence or absence of these organisms on the agar plate or in the liquid environments.

A difference between the nematodes' ability to excrete the two salmonellae species was noted. Salmonella wichita was recovered in more trials than was Salmonella typhi. A second finding indicated that the excretion potential of Salmonella wichita by the nematode increased as the nutrient value of the suspending media increased.

Although the chance of nematodes carrying bacterial diseases to the public through the water supply is slight, their ability to survive chlorination and to protect and excrete pathogenic organisms justifies further investigation into this possibility.

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