

EFFECT OF SODIUM HYPOCHLORITE ON THE BACTERIAL FLORA OF INFECTIVE LARVAE OF *NIPPOSTRONGYLUS BRASILIENSIS* AND *STRONGYLOIDES RATTI*

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In vitro experiments were conducted to determine if third-stage larvae of *Strongyloides ratti* (the rat threadworm) and *Nippostrongylus brasiliensis* (the rat hookworm) can carry and release bacteria incorporated during feeding in previous stages. Bacteriological sterilization of the larvae was by treatment with Clorox (5.25 percent sodium hypochlorite). The results demonstrate that first and second-stage larvae of *S. ratti* and *N. brasiliensis* incorporate bacteria during feeding and the third-stage larvae that develop have different capacities of protecting incorporated bacteria when such third-stage larvae are treated, *in vitro*, with Clorox.

INTRODUCTION

Transmission of pathogenic bacteria is generally accepted to be either by ingestion with water and food, inhalation, direct contact, or by arthropod vectors. Transmission of pathogenic bacteria by parasitic nematode larvae has been reported in several clinical cases involving *Escherichia coli*, *Bacillus viscosum* (1), *B. necrophorus* (2), and *B. anthracis* (3). Third-stage larvae of *Strongyloides papillosus* have been shown capable of carrying *Erysipelothrix rhusiopathie* through the skin of pigs (4) and third-stage larvae of *Strongyloides stercoralis* have been suspected of carrying *E. coli* (5). These studies did not use sterilized third-stage larvae exposed to a single bacterial species to determine whether the larvae could harbor bacteria internally or simply carry them by mechanical means, externally. Our experiments involving external sterilization of third-stage larvae containing incorporated bacteria are designed to serve as a model to demonstrate the potential of nematode larvae as carriers of pathogenic bacteria.

Several investigators have used solutions containing sodium hypochlorite (but not Clorox) for inactivation of bacteria carried by nematode larvae (6, 7, 8, 9) for the purpose of *in vitro* nematode cultivation. These investigators, however, did not report the minimal concentrations of sodium hypochlorite solution required for bacterial sterilization of larvae. Since sodium hypochlorite is also detrimental to the nematode larvae, the minimal amounts needed for bacterial sterilization in relation to larval tolerance must be known for *in vitro* and *in vivo* studies.

The purpose of this study was to evaluate Clorox (The Clorox Company, Oakland, California) as an antibacterial agent for the bacterial sterilization of infective nematode larvae. Experiments were performed to demonstrate the *in vitro* bacterial harboring capacities of two models of tissue-invasive third-stage larvae, *N. brasiliensis* and *S. ratti*, using Clorox as the sterilizing agent.

MATERIALS AND METHODS

S. ratti and *N. brasiliensis* cultures were prepared according to Graham (10) and Haley (11). Egg-laden feces and intestinal contents from infected rats were mixed with moist charcoal in culture dishes. The cultures were sprayed with a fine mist of tap water and maintained at 23 C (*N. brasiliensis*) and at 27 C (*S. ratti*) for 5 or more days to produce third-stage larvae for inoculation into rats.

Caesarean-originated-barrier-sustained rats from the Charles River Breeding Laboratories, Wilmington, Massachusetts, were used to maintain the nematode infections. Twenty-one-day-old male rats were inoculated subcutaneously, at biweekly intervals,

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with 1000 third-stage larvae from the preceding infection.

Viable larvae, raised in charcoal cultures, were Baermannized and washed 3 times with sterile physiological saline. Larvae (10^8 larvae per sample) were treated with 0, 2, 4, 6, 8, 10, 12, 14, and 16 percent Clorox-saline solutions, pH 7.0, for 20 minutes at 23 C. The larvae were aseptically washed 5 times with sterile physiological saline and both viable and nonviable larvae were placed into 10 ml of Thiol broth for 24 hours at 23 C. The decanted Thiol broth fluid from the larvae was then incubated at 37 C for 7 days and streaked onto brain-heart infusion agar plates and were incubated at 37 C for 7 days. The specific bacteria recovered from the plates were not identified.

Freeze-killed larvae were washed and treated in the same way as viable larvae to determine whether larval viability was necessary for protection of incorporated bacteria from the various Clorox solutions. The destruction of fecal bacteria from nematode cultures (as a control to demonstrate the effect of Clorox or larval culture bacteria) was investigated by subjecting rat feces (without larvae) to the same Clorox treatments as the viable and nonviable nematode larvae.

Determination of larval viability after treatment with various Clorox-saline solutions was done using the larvae before placing them into Thiol broth for demonstration of bacterial-carrying capacities. A sample from each Clorox treatment containing 1000 larvae was plated into a petri dish in 3 ml of buffer and incubated at 37 C for 20 minutes. Larvae that were moving after 20 minutes were considered viable. Percentage larval viability from each experiment was determined by counting six samples of 100 larvae.

RESULTS

Determination of the Bactericidal Effect of Clorox on Third-Stage Nematode Larvae

Viable larvae of *S. ratti* were found capable of harboring viable bacteria after subjection to as high as 14 percent Clorox-saline solutions (Table 1). Freeze-killed *S. ratti* larvae proved incapable of carrying viable bacteria after treatment with a Clorox-saline solution of greater than 4 percent Clorox, indicating that the bacteria carried by the live larvae were protected in the intestinal tract of the larvae. All Clorox solutions employed destroyed fecal bacteria not incorporated into larvae.

Viable *N. brasiliensis* larvae were found to be incapable of protecting bacteria from destruction by Clorox concentrations greater than 2 percent (Table 1). This is a marked difference from the bacterial carrying capability of *S. ratti* larvae (14 percent Clorox).

Freeze-killed *S. ratti* larvae and freeze-killed *N. brasiliensis* larvae protected incorporated bacteria from the lethal effects of Clorox in 4 and 2 percent dilutions, respectively.

TABLE 1. Bactericidal effect of Clorox on bacteria found in larval cultures and on bacteria found in viable and nonviable *S. ratti* and *N. brasiliensis* third-stage larvae. All experiments were repeated six times. (+) = viable bacteria, (-) = nonviable bacteria.

Sample	Percent Clorox Treatment								
	0	2	4	6	8	10	12	14	16
<i>S. ratti</i> Larval Culture Bacteria	+	-	-	-	-	-	-	-	-
Freeze-killed <i>S. ratti</i> Third-stage Larvae	+	+	+	-	-	-	-	-	-
Viable <i>S. ratti</i> Third-stage Larvae	+	+	+	+	+	+	+	+	-
<i>N. brasiliensis</i> Larval Culture Bacteria	+	-	-	-	-	-	-	-	-
Freeze-killed <i>N. brasiliensis</i> Third-stage Larvae	+	+	-	-	-	-	-	-	-
Viable <i>N. brasiliensis</i> Third-stage Larvae	+	+	-	-	-	-	-	-	-

Determination of the Nematocidal Effect of Clorox on Third-Stage Nematode Larvae

The viability of third-stage larvae of *S. ratti* and *N. brasiliensis* (10^8 larvae per sample) in different percentages of Clorox solutions (as previously described) is shown in Figure 1.

S. ratti larvae were shown to tolerate and survive in the 90 percent survival range in solutions containing up to 2 percent Clorox. Clorox was 100 percent lethal for *S. ratti* larvae at a 14 percent concentration. This 14 percent nematocidal concentration of Clorox was the same as the concentration required for bacterial sterilization of the larvae demonstrating the inability of Clorox to serve as a bacterial sterilization agent for viable *S. ratti* larvae.

Viable *N. brasiliensis* larvae survived in the 90 percent survival range in solutions containing up to 4 percent Clorox. The bactericidal concentration of Clorox of 2 percent is much less than the nematocidal concentration of 16 percent for *N. brasiliensis* larvae. Thus, Clorox concentrations of up to 2 percent can be used as a bacterial sterilization agent for viable *N. brasiliensis* larvae.

DISCUSSION

These experiments have demonstrated that larvae of *S. ratti* and *N. brasiliensis* are capable of acquiring bacteria in their intestinal tracts during their free-living feeding stages and of releasing the bacteria in a viable state after development to the third or infective stage.

The inability of *N. brasiliensis* larvae to protect bacteria from Clorox treatment or to release bacteria after Clorox treatment, in contrast to the bacterial carrying capacity of *S. ratti* larvae, indicates that different third-stage nematode larvae have different potentials as vectors of bacteria. The observed differences could be due to several different things. One difference is the variation in the cuticular integrity of the two species of larvae. Infective, non-sheathed, larvae of *S. ratti* have a very sturdy, protective cuticle (12) whereas *N. brasiliensis* infective larvae retain their cuticular sheath and are protected by it (13) until they penetrate the skin of the rat host. The sheath of *N. brasiliensis* larvae protects them against most environmental changes but the chemical changes due to oxidation with Clorox are not withstood; neither does the intestinal tract of the larvae provide this protection. This difference in cuticular morphology could result in the differences in the nematocidal effects of Clorox on the larvae and possibly on larval bacterial carrying capacities.

Another difference in bacterial carrying capacity may be due to the different growth requirements of the bacteria harbored by the larvae. The bacteriological media used in the assay system could have selected for the bacteria from one type of nematode larvae over those from the other type of nematode larvae.

This simple bacterial sterilization technique for third-stage nematode larvae, serves as a useful tool in the determination of pathogenic bacterial carrying capacities of infective nematode larvae.

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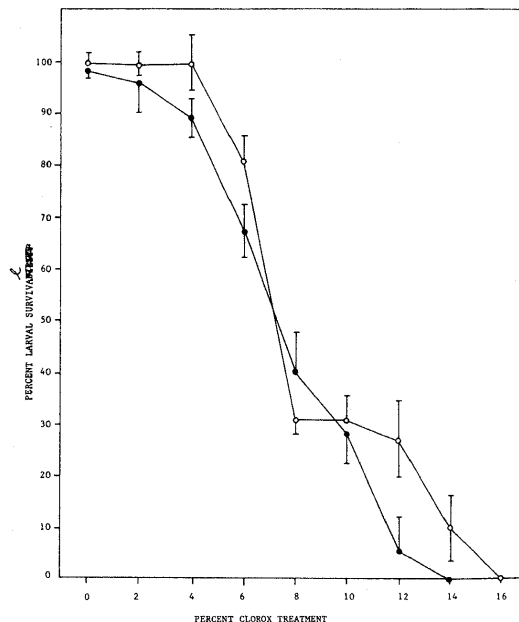


FIGURE 1. Survival of viable third-stage larvae of *N. brasiliensis* (o) and *S. ratti* (●) after treatment with various Clorox-saline solutions. The points represent the mean (± 1 S.D.) of six experiments.

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