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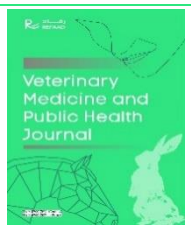

Optimal Application of Sodium Hypochlorite to Maintain the Viability of Exsheathed Nematode Larvae

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Abstract:

Objectives: Sodium hypochlorite (NaOCl) is widely recognized for its unique ability to dissolve tissues, making it a common choice for the exsheathment of nematode larvae in various studies. This chemical has frequently been utilized for larval exsheathing at different concentrations, with exposure durations varying significantly across studies. However, concerns have been raised regarding the impact of this compound on the viability of exsheathed larvae. The current study aimed to identify the optimal chemical concentration and the shortest exposure time that can be used without compromising larval viability, using third-stage strongylid larvae cultured from donkey fecal material as a model.

Methods: Larvae were treated with NaOCl concentrations of 0.025%, 0.05%, 0.1%, 0.2%, 0.3%, or 3% and observed for 3-, 5-, or 10 minutes post-treatment. Visual immobility and noticeable morphological changes were used as criteria for assessing viability.

Results: The results indicate that a concentration of 0.025% is optimal, as it does not adversely affect larval viability even after 10 minutes of exposure. In contrast, a significant reduction in the proportion of motile larvae was observed when the concentration was increased to 0.05%, with 6.02% of the 415 larvae analyzed exhibiting sluggishness. Higher concentrations were associated with decreased motility or immobilization of the larvae. At the extreme concentration of 3%, the larvae exhibited significant damage.

Conclusions: Given the widespread use of NaOCl as an artificial exsheathing agent for nematode larvae and the variety of protocols employed, the current findings provide a valuable reference for researchers utilizing NaOCl in nematode studies.

Keywords: *Sodium hypochlorite; exsheathment; viability; strongylid larvae.*

1 Introduction

The ionic compound sodium hypochlorite (NaOCl) possesses a unique ability to dissolve tissues (Echeverri and Acuña, 2012; Dutta and Saunders, 2012; de Almeida *et al*., 2013; Khoshroo *et al*., 2020). It has been utilized as an exsheathment medium for nematode larvae in numerous studies, including those evaluating the effectiveness of anthelmintic drugs, determining nematode resistance to anthelmintics, assessing plant bioactive products as anthelmintics, and studying nematode viability. Coles *et al.* (1980) compared various media—namely acid pepsin, ox bile, sodium tetraborate, and NaOCl—as exsheathing agents for nine species of nematodes. They found that NaOCl was the only satisfactory exsheathing medium. Conversely, Conder and Johnson (1996) employed the Mongolian jird (*Meriones unguiculatus*) as an experimental model to evaluate the viability of exsheathed larvae of ruminant parasites after exposure to different media (distilled water, Earle's balanced salt solution + CO2, nematode washing buffer + CO2, NaOCl), using infectivity as a measure of viability. They discovered that, although NaOCl could achieve a high percentage of exsheathment (≥ 98.5%), the infectivity of the exsheathed larvae was significantly lower than that of larvae exposed to other media. Consequently, they cautioned that when using exsheathing agents, results should be interpreted with care, as techniques known to be highly effective may also reduce viability. This chemical has frequently been utilized for larval exsheathing at various concentrations, with exposure durations varying significantly across studies. Given the unique tissue-dissolving properties of NaOCl, the widespread application of this compound as an artificial exsheathing agent for nematode larvae, and the diverse protocols employed, optimizing its use to minimize the risk of reduced viability is crucial. In a prior study (Elowni *et al*.,2022), *Strongyloides papillosus*, a nematode whose larvae naturally lack a protective sheath, was used as a model to investigate the potential negative effects of NaOCl on larval viability. The results indicated that the impact of the compound depended on both concentration (C) and exposure time (T), with specific limits (0.3% > C > 0.2%; 10 min > T > 5 min) beyond which viability was compromised. This research aimed to identify the optimum concentration of the compound and the shortest exposure time that can be utilized without compromising larval viability, guided by the findings from the *Strongyloides* model. Third-stage strongylid nematode larvae cultured from the feces of donkeys were used for this purpose. Generally, nematode viability is assessed by observing various phenotypic characteristics of the parasites, including gross morphology, developmental processes, and motility (O'Grady and Kotze, 2004; Smout *et al*., 2010; Andre *et al*., 2016; Preston *et al*., 2016) with motility being the most commonly adopted criterion (Bennett and Pax, 1986), typically assessed through visual means (Mackenzie *et al*.,2017). In this study, visual larval motility and gross morphological changes were employed to evaluate the effects of NaOCl on viability. This article is based on content previously published in a preprint (Elowni *et al.*, 2024 a).

2 Materials and Methods

2.1. Parasite Source and Culture of Larvae

Fresh feces were collected from naturally infected working donkeys and transported to the Parasitology Laboratory at the Faculty of Veterinary Medicine, University of Khartoum. The samples were screened for strongylid-type eggs (Roeber *et al*., 2013) using a flotation technique with a hypertonic sodium chloride solution. Due to the challenges associated with morphologically distinguishing between the eggs of various strongylid species (Khan *et al*., 2015), fecal samples were cultured to facilitate development to the third larval stage. Fecal masses containing eggs were crushed, moistened with tap water, and cultured at room temperature (30–33 °C) for 9 to 10 days. Larvae were then extracted from the culture using the Baermann technique (Gibbons *et al*., online source). Random larval samples were examined microscopically, and the generic and species composition of the culture was tentatively determined by examining the morphology of intestinal cells (Santos *et al*., 2018; Maestrini *et al*., 2020; Amer *et al*., 2022).

2.2. Experimental Procedures

The larvae harvested from the eluent culture fluid were centrifuged at 2,000 rpm for 2 minutes. The supernatant was decanted, leaving a small volume of liquid at the bottom of the tubes. A drop of the suspended larvae was placed on a glass slide, and 10 µL of NaOCl (AMCLEAN, Al Fayrouz Dental and Medical Equipment, Sharjah, UAE) at varying concentrations (0.025%, 0.05%, 0.1%, 0.2%, 0.3%, or 3%) was added. The mixture was then covered with a glass coverslip and examined at ×40 and ×100 magnifications using an OPTIKA Srl B-193

compound microscope. The viability of the larvae was assessed by evaluating individual specimens, regardless of species, at 3, 5, or 10 minutes after treatment. The trials were replicated using larvae from different cultures. The larvae were classified as actively motile, in comparison to untreated controls (Supplementary File 1; Elowni, 2024), sluggish (regardless of degree; Figure 1), immotile (Figure 2), or damaged (Figure 3).

Figure 1: A typical posture of sluggish wriggling third-stage strongylid larvae exposed to NaOCl treatment.

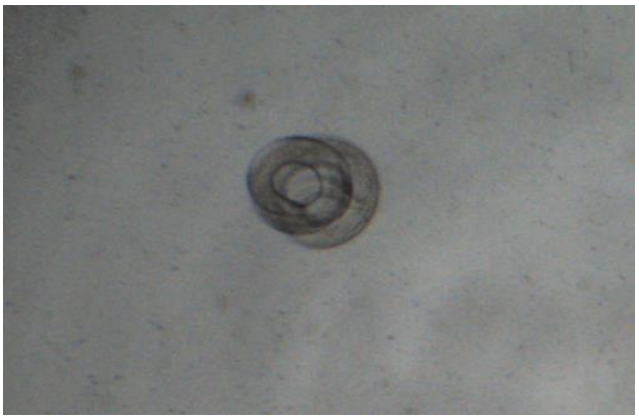

Figure 2: A dead third-stage strongylid larva exposed to NaOCl treatment. Typically, dead larvae exhibit a coiled shape and show a complete cessation of motility.

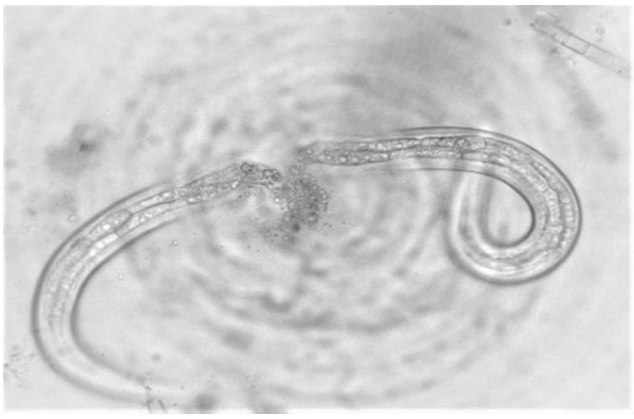

Figure 3: Damaged third-stage strongylid larvae exposed to NaOCl treatment.
(Source: Preprint, https://doi.org/10.21203/rs.3.rs-2748755/v1).

3 Results

3.1. Cultured Larvae

The larvae retrieved from the culture comprised a mixture of strongylid species (Figure 4). This was evidenced by the presence of larvae exhibiting well-defined triangular intestinal cells (Figure 5), rectangular or elongated cells (Figure 6), trapezoidal cells (Figure 7), and poorly defined cells (Figure 8). Additionally, the cultures contained *Strongyloides westeri* larvae, identifiable by their distinctive long filariform esophagus, which

accounts for nearly half of the total length of the larva, and by the absence of a sheath.

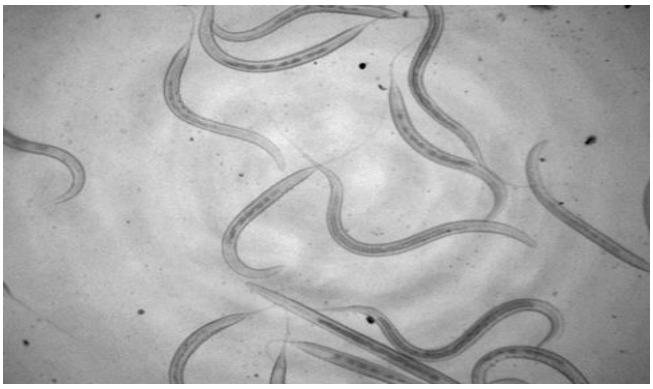

Figure 4: Third-stage strongylid larvae of mixed species recovered from the culture. (Source: Preprint, https://doi.org/10.21203/rs.3.rs-2748755/v1).

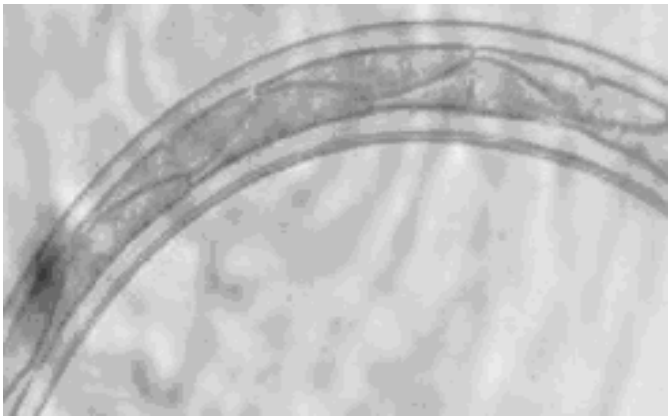

Figure 5: Larva with triangular intestinal cells. (Source: Preprint, https://doi.org/10.21203/rs.3.rs-2748755/v1).

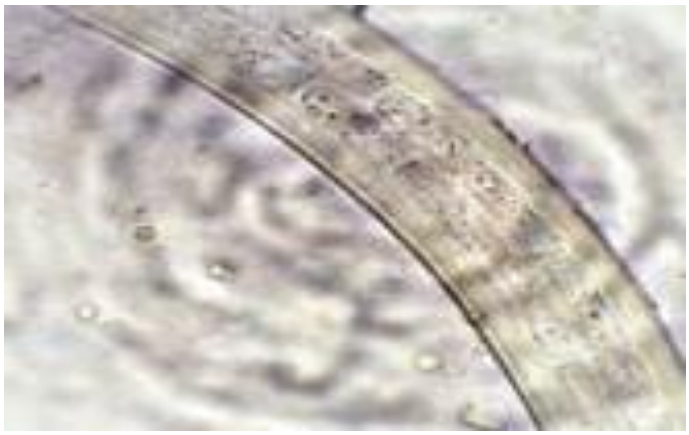

Figure 6: Larva with elongated intestinal cells. (Source: Preprint, https://doi.org/10.21203/rs.3.rs-2748755/v1).

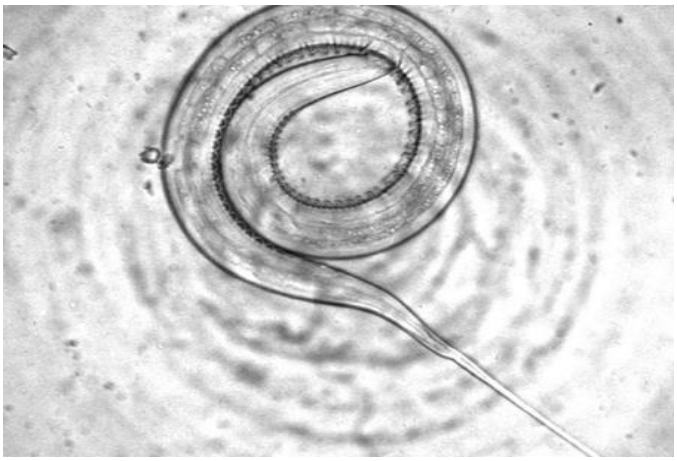

Figure 7: Larva with trapezoidal intestinal cells.
(Source: Preprint, https://doi.org/10.21203/rs.3.rs-2748755/v1).

Figure 8: Larva with poorly defined intestinal cells.
(Source: Preprint, https://doi.org/10.21203/rs.3.rs-2748755/v1).

3.2 Pilot Tests

Larvae were treated on microscope slides with 10 μL of a 3% NaOCl solution as a benchmark to evaluate the initial dissolution properties of the compound, its exsheathing capacity, its effect on larval motility, and its impact on larval tissues. Exsheathment was a rapid process, occurring within one minute after treatment (Figure 9A). The dissolution capacity was demonstrated through a progressive dissolving effect on the cast sheath (Figures 9A-C). After five minutes, the sheath became nearly indiscernible. The chemical was found to reduce motility and induce structural damage.

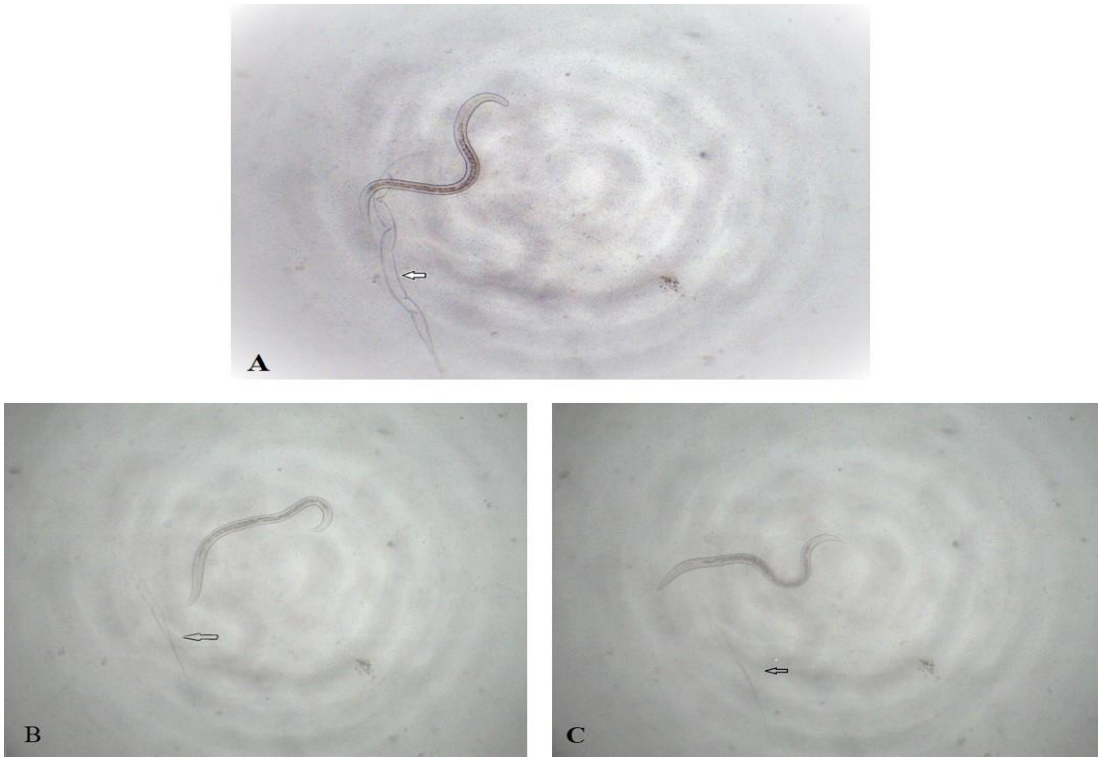

Figure 9: Exsheathment of 3rd-stage strongylid larva following treatment with 3% NaOCl solution at 2 min (A), 4 min (B), or 5 min (C) after chemical treatment (cast sheath; arrows).
(Source: Preprint, https://doi.org/10.21203/rs.3.rs-2748755/v1).

3.3 Assessment of Viability

There was no effect on motility when larvae were exposed to 0.025% chemical concentrations for 3, 5, or 10 minutes (Table 1). However, a significant reduction in the proportion of motile larvae was observed when the concentration was increased to 0.05% ($z = -5.0774$; $p < 0.01$), with 6.02% of the 415 analyzed larvae exhibiting sluggishness. Further increases in chemical concentration above 0.05% were associated with decreased motility or immobilization of the larvae. At the extreme concentration of 3%, viability was significantly compromised ($z = -16.5325$; $p < 0.01$); the majority of larvae (84.2%) were sluggish or immotile, while 10.5% remained motile, and 5.3% exhibited structural damage.

Table 1: Viability of 3rd-stage nematode larvae at different times following treatment with NaOCl

Exposure time (min)	NaOCl concentration					
	0.025%	0. 05%	0.1%	0.2%	0.3%	3.0%
3	(+) [3]	(+, ±) [5]	(+, ±) [3]	(+, ±) [3]	(+, ±, -) [3]	(±, -) [3]
5	(+) [4]	(+) [4]	(+, ±) [4]	(+, ±) [4]	(+, ±, -) [5]	(+, ±, -) [4]
10	(+) [4]	(+, ±) [4]	(+, ±) [4]	(+, ±) [5]	(+, ±, -) [7]	(+, ±, -) [4] *
n	249	415	171	250	335	169

(+) Actively motile, (±) Sluggish, (-) Immotile, (*) Damaged. [?] Replicates, (n) Total number of larvae examined.

4 Discussion

Due to its exceptional ability to dissolve organic tissues (Echeverri and Acuña, 2012; Dutta and Saunders, 2012; de Almeida *et al.*, 2013; Khoshroo *et al.*, 2020), NaOCl has been widely utilized as an artificial exsheathing agent for nematode larvae. This chemical has frequently been employed for larval exsheathing at concentrations ranging from 0.005% in some studies to 3.5% in others. The duration of exposure also varied significantly

between studies, ranging from less than a minute to 18 hours. Nematodes represent a diverse phylum that encompasses numerous species. The outermost layer of cells is protected by an extracellular matrix known as the exoskeleton or cuticle, primarily composed of proteins with trace amounts of lipids and carbohydrates (Fetterer and Rhoads, 1993; Page *et al.,* 2014). Therefore, it is anticipated that a compound such as NaOCl, recognized for its effective tissue-dissolution properties, could adversely affect overexposed exsheathed larvae. Garduño *et al.* (2010) investigated the effects of NaOCl on the infective larvae of sheep nematodes, specifically *Haemonchus contortus* and *Cooperia curticei*. At concentrations of 1.3% and 0.53%, the larvae were killed after 1 hour and 2-3 hours, respectively. Conder and Johnson (1996) demonstrated that after exposure to NaOCl for either 1 minute or 18 hours, the infectivity of exsheathed *H. contortus, Ostertagia ostertagi,* and *Trichostrongylus colubriformis* larvae to the Mongolian jird was significantly reduced. Campbell and Gauler (1992) studied the effects of NaOCl on the entomopathogenic nematode *Heterorhabditis bacteriophora*. They found that nematodes exposed to a 1% solution for 5 minutes exhibited reduced motility compared to normal ensheathed larvae, suggesting that this decrease in motility may be due to the adverse effects of the compound on the larvae. Results from a previous study utilizing *Strongyloides papillosus* larvae (Elowni *et al.,* 2022) indicated specific concentration and exposure time limits beyond which viability decreases, and excessive exposure to the compound could cause significant damage. The objective of the current study was to determine the optimal concentration of NaOCl and the shortest exposure time that can be utilized without compromising larval viability, using third-stage strongylid larvae cultured from donkey fecal material. Nematodes that parasitize equids comprise nearly 83 species, most of which belong to the Strongylidae family (Lichtenfels *et al.,* 2008; Nielsen *et al.,* 2014). Identifying the larvae of these nematodes at the generic or species level necessitates the killing and chemical staining (Zajac and Conboy, 2012) to facilitate morphometric analysis using identification keys (Thienpont *et al.,* 2003; Lichtenfels *et al.,* 2008; Amer *et al.,* 2022). Various criteria, including the number and morphology of intestinal cells, the morphology of the tail sheath, body length, and the body-to-tail ratio, have been employed for this purpose. Initial morphological screening of the larvae used in the current study revealed a mixed strongylid species, as indicated by the differences in the morphology of intestinal cells (Santos *et al.,* 2018; Maestrini *et al.,* 2020; Amer *et al.,* 2022). The larvae are characterized by their distinctive, active wriggling movements, and when examined microscopically, they can traverse the field of view (Supplementary Figure 1). Consequently, it has been challenging to determine the diagnostic features of the larvae, complicating the attribution of treatment results to a specific species. To address this challenge, we conducted replicated trials with larvae from different cultures to assess the responses of individual larvae, regardless of their species, within the microscope field. The results indicated that a concentration of 0.025% had no demonstrable effect on larval motility, even after a treatment duration of 10 minutes. Results from a previous study (Elowni *et al.,* 2024 b) using NaOCl concentrations of 0.1%, and 0.05% indicated that exsheathment of strongylid larvae from donkeys could be achieved within 8 and 10 minutes, respectively, without compromising larval viability. In this context, it would be valuable to determine whether a concentration as low as 0.025%, which has been established as optimal in this study, is sufficient to induce exsheathment within the 10-minute exposure limit.

5 Conclusions

Given the widespread use of NaOCl as an artificial exsheathing agent for nematode larvae and the variety of protocols employed, the current findings serve as a valuable reference for researchers utilizing NaOCl in nematode studies. The observed results align with previous research on the effects of NaOCl on nematode larvae, highlighting the potential risks associated with excessive exposure to this chemical. The study's conclusions were based on the assumption that various strongylid species respond similarly to NaOCl exposure. To obtain more definitive results, researchers could utilize single-species larvae, such as those of *Haemonchus contortus* obtained from eggs extracted from adult worms recovered from the abomasum, or infective *Trichostrongylus colubriformis* larvae derived from fecal cultures of sheep or other small ruminants with monospecific infections.

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