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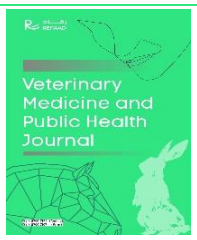

Sodium Hypochlorite: Optimization of Application as an Exsheathing Agent for Nematode Larvae to Minimize the Risk of Reduced Viability

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Abstract:

Objective: Nematode larvae exsheathing is a crucial procedure employed in experimental studies such as nematode viability assays, evaluating nematodes' resistance to anthelmintics, and evaluating the efficacy of anthelmintic medications. The exsheathment procedure allows for quantifying the direct impact on larvae when the protective sheath is removed. Sodium hypochlorite (NaOCl) is a commonly used artificial exsheathment medium. However, concerns have been raised about its potential impact on the viability of exsheathed larvae. A previous study utilized Strongyloides papillosus larvae, a nematode species without a protective sheath, to investigate the effects of NaOCl on parasite viability. The findings revealed that there are specific concentration (C) and exposure-time (T) thresholds (0.3% > C > 0.2%; 10 min > T > 5 min) beyond which viability decreases, and excessive exposure can cause significant damage. The current study aims to determine the optimal concentration of NaOCl that can induce exsheathment in ensheathed-type larvae without compromising their viability.

Methods: Third-stage strongylid larvae of donkey origin were utilized for this study, with chemical concentrations of 3%, 0.3%, 0.2%, 0.1%, or 0.05%. Larval motility served as the criterion for evaluating viability.

Results: NaOCl can exsheath larvae at any of the five concentration levels evaluated, depending on the duration of exposure to the compound. A concentration of 0.2% is considered optimal, inducing exsheathment as early as 2 minutes. Extended exposure for up to 10 minutes did not impact larval viability. A 3% solution causes larval damage.

Conclusions: Regarding the effect of NaOCl on nematode larvae, the results validate the earlier findings with Strongyloides papillosus and those by other authors employing nematodes such as Haemonchus contortus and Cooperia curticei, and the entomopathogenic nematode Heterorhabditis bacteriophora.

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

1 Introduction

Sodium hypochlorite (NaOCl) is an ionic chemical compound known for its tissue dissolution properties (Dutta and Saunders, 2012; Echeverri and Acuña, 2012). It has been widely employed as an artificial exsheathment medium for nematode larvae in various research studies, including the evaluation of anthelmintic drug effectiveness, resistance assessments, and testing of plant-based anthelmintics. Coles *et al.* (1980) compared different exsheathing agents for nine nematode species, highlighting NaOCl as the most effective medium. Conversely, Conder and Johnson (1996) utilized the Mongolian jird (*Meriones unguiculatus*) as a model to assess the viability of exsheathed larvae exposed to various media, including NaOCl. They observed that while NaOCl induced substantial exsheathment, the infectivity of resulting larvae was lower compared to other media, cautioning against interpreting results from studies using NaOCl-exsheathed larvae due to potential viability reduction. In a prior investigation (Elowni *et al.*, 2022), *Strongyloides papillosus* larvae naturally lacking a protective sheath were utilized to investigate NaOCl's impact on parasite viability. The study revealed concentration (C) and exposure time (T) thresholds beyond which viability decreased (0.3% > C > 0.2%; 10 min > T > 5 min). Optimizing the application of NaOCl as an exsheathing agent for nematode larvae, therefore, is crucial to minimize the risk of reduced viability. It is essential to carefully determine the appropriate concentration and exposure time to maintain larval viability while effectively exsheathing the larvae. This current study aims to determine the optimal NaOCl concentration and exposure time for inducing exsheathment in ensheathed-type larvae without compromising viability, utilizing third-stage strongylid larvae of donkey origin.

2 Materials and Methods

2.1 Parasite source and culture of larvae

Rectal faecal samples were collected from donkeys brought to a local Sudanese market and were transported unpreserved to the parasitology laboratory at the Faculty of Veterinary Medicine, University of Khartoum. Samples were screened for strongylid eggs by centrifugal flotation. Faecal masses were thoroughly crumbled, the material was sufficiently moistened with tap water, and cultured in wide-mouth glass jars at room temperature (30-33 °C) for 9-10 days to obtain third-stage larvae. Larvae were recovered from culture by the Baermann standard technique as described in "The Royal Veterinary College/FAO Guide to Veterinary Diagnostic Parasitology" (*https://www.rvc.ac.uk /static/review/ parasitology//review/parasitology/Baermann/Purpose.htm#).* The eluent fluid was transferred to centrifuge tubes and centrifuged at 1500 rpm for 2 minutes. The supernatant was decanted, leaving a trickle of fluid at the bottom of the tubes. Sedimented larvae were transferred by a micropipette to microscope slides soon after recovery and treated with 2 uL of NaOCl (AMCLEAN, Al Fayrouz Dental and Medical Equipment, Sharjah, UAE) of different concentrations (Table 1). Larvae were examined using an OPTIKA Srl B-193 optical microscope (Ponteranica, Italy) fitted with a digital camera (OPTIKAM 4083.B1) with an image setting at a 60-second capture configuration. Generally, nematodes' viability is assessed by observing different parasite phenotypic characteristics, including gross morphology, developmental processes, or motility (Mackenzie *et al.* 2017). Both visual motility and gross morphological changes were used in the present study to assess the performance of NaOCl as an exsheathing agent for nematode larvae. Larvae were designated as actively motile (Additional movie file 1; Elowni, 2024), sluggish (regardless of the degree of sluggishness), or immotile. Assignment to a category was made following scanning larvae *en masse* within the coverslip area. Individual larvae were classified accordingly. An overall judgment was based on the results of 3 replicates.

2.2 Pilot tests

Normal larvae from the culture (Fig. 1 & 2) were treated with 2 uL of a 3% NaOCl stock solution and examined microscopically to determine the initial tissue-dissolution capacity of the compound. A decrease in motility was noted, and by 10 minutes, the larvae were completely immotile (Table 1). Occasional larval damage was observed (Fig. 3). Exsheathment (Fig. 4) was a rapid process occurring within the first 2 minutes of exposure.

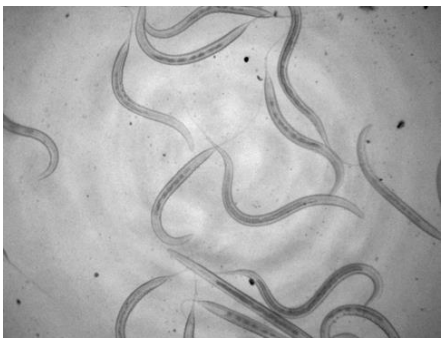

Fig. 1: Normal ensheathed 3rd stage strongylid larvae retrieved from culture

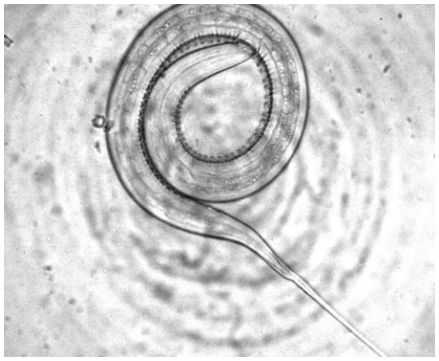

Fig. 2: A typical ensheathed 3rd stage strongylid larva showing the extended sheath beyond the larval tail

Fig. 3: Damage of 3rd-stage larva

Fig. 4: Exsheathment of strongylid 3rd stage larva following exposure to NaOCl 3% solution for 2 min (arrow, stripped larval sheath)

3 Results

The results indicate that NaOCl has the potential to induce exsheathment at any of the five concentration levels tested, depending on the duration of exposure to the compound (Table 1). A concentration of 0.2% was optimal for treatment, with exsheathment achieved as early as 2 minutes of exposure without affecting motility. Prolonged

exposure of up to 10 minutes at this concentration did not negatively impact motility. In contrast, exposure to lower chemical concentrations (0.1%, 0.05%) resulted in delayed exsheathment, occurring at 8 and 10 minutes, respectively.

Table 1: Effect of NaOCl treatment on strongylid 3rd-stage larvae

Exposure time (min)	NaOCl concentration				
	3%	0.3%	0.2%	0. 1%	0. 05%
2	±*	+*	+*	+	+
4	±*	+*	+ *	+	+
6	±*	±*	+ *	+	+
8	±*	±*	+ *	+*	+
10	- *	±*	+ *	+*	+*

(+) motile; (±) sluggish, regardless of sluggishness; (-) immotile. (*) exsheathment of larvae

4 Discussion

NaOCl has been widely used as an artificial exsheathment agent for nematode larvae in research due to its significant tissue dissolution capacity (Dutta and Saunders, 2012; Echeverri and Acuña, 2012). Previous studies have utilized NaOCl at concentrations ranging from 0.08% to 2.0% and treatment times varying from <1.0 min to 18 hours. Typically, larvae are washed multiple times to eliminate chemical residues before experimentation, considering the compound's potent tissue-dissolving properties. Therefore, it was crucial to determine the minimum effective concentration (C) and exposure time (T) for inducing exsheathment without risking prolonged exposure. Our findings demonstrate that a specific threshold (C, 0.2%; T, 2 min) can trigger exsheathment without affecting larval motility, highlighting the importance of removing any residual compound before utilizing the larvae for further experiments. In this respect, the findings may influence current practices and protocols in research where NaOCl is used as an exsheathing medium for nematode larvae.

For comparisons, Coles *et al.* (1980) examined the effectiveness of several media, namely, sodium tetraborate, ox bile, acid pepsin, and NaOCl, as exsheathing agents for nine different nematode species. They reported that the only effective exsheathing medium was NaOCl. Conversely, Conder and Johnson (1996) evaluated 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 to the Mongolian jird as a measure of viability. They found that while NaOCl could achieve a high exsheathing percentage (≥ 98.5%), the exsheathed larvae's infectivity was much lower than that of larvae exsheathed in other media. They concluded that the use of exsheathing agents may result in reduced viability. The effects of NaOCl on the infectious larvae of *Haemonchus contortus* and *Cooperia curticei*, two sheep nematodes, were investigated by Garduño *et al.* (2010). The larvae were killed after 1 or 2-3 hours when treated at concentrations of 1.3% or 0.53%, respectively. Campbell and Gauler (1992) investigated the impact of NaOCl on *Heterorhabditis bacteriophora*, an entomopathogenic nematode. They found that larvae exsheathed with a 1% NaOCl solution for five minutes were less motile than the untreated controls, suggesting that this decrease in motility may be due to an adverse effect of the compound on the larvae.

5 Conclusions

Our study confirms that NaOCl is an effective exsheathing agent for nematode, with 0.2% concentration and 2 minutes of exposure identified as optimal. These conditions ensure effective exsheathment while maintaining larval viability. Given the widespread use of NaOCl as an artificial exsheathing agent for nematode larvae and the diverse protocols employed, these findings offer a valuable reference for researchers using NaOCl in nematode studies.

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Data availability:

All the data on which the conclusions have been drawn were included in the text.

Conflicts of interest:

The authors declare that no competing interests exist.

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