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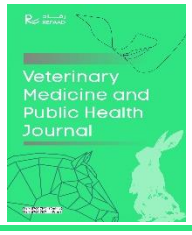

Assessment of the Effect of Sodium Hypochlorite, an Artificial Exsheathment Medium, on the Viability of Nematode Larvae

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Abstract: Sodium hypochlorite (NaOCl) is used extensively as an artificial exsheathment medium of nematode larvae in various studies, such as comparing the efficacy of drugs, and assessment of resistance to anthelmintics or evaluation of plant extracts as anthelmintics due to its unique capacity for tissue dissolution. Studies with NaOCl by other authors indicate that the compound, although highly effective as an exsheathing agent, significantly lowers the infectivity of the exsheathed larvae produced, suggesting reduced viability. We used *Strongyloides papillosus* larvae, a nematode that naturally lacks a protective sheath and a potentially highly motile organism, as a model to exclude or confirm possible negative effects on the viability of the parasite. Motility was taken as a viability assay. Larvae were designated as actively motile, sluggish, or immotile. Results were presented as additional supplementary movie files. The viability of larvae is dependent on both the concentration and the time of exposure to the compound. There are certain concentration (C) and time (T) limits beyond which viability is impaired (0.3% > C > 0.2%; 10 min > T > 5 min). It is concluded that results from studies where NaOCl is used as an exsheathment medium should be interpreted with caution as the compound is capable of reducing the viability of larvae. It may even induce structural damage.

Keywords: *Viability; nematode larvae; Strongyloides papillosus; sodium hypochlorite.*

1. Introduction

Sodium hypochlorite (NaOCl) is an ionic chemical compound commonly used in many fields such as agriculture, chemical industries, food and pharmaceutical industries, and water treatment as a disinfectant. In medicine, the chemical is widely used as an irrigating solution in endodontics due to its unique capacity for tissue dissolution, a property that has been proven experimentally when bovine and porcine muscle tissue and bovine dental pulp were exposed to the compound (Dutta and Saunders, 2012; Echeverri and Acuña, 2012). Regarding such an effective dissociative property, the compound has largely been used as an exsheathment medium for nematode larvae in a diversity of studies such as comparing the efficacy of drugs, assessment of resistance to anthelmintics, evaluation of plant extracts as anthelmintics as well as the effects of extracts on larval exsheathment processes and susceptibility to extracts. In a comparative study on the efficacy of different exsheathment media (*viz* acid pepsin, ox bile, sodium tetraborate, and NaOCl) involving nine species of nematodes (Coles *et al.*, 1980), NaOCl has been described as the only satisfactory exsheathing agent. In this regard, Conder and Johnson (1996) used infectivity to the Mongolian jird *(Meriones unguiculatus)* as a measure for the viability of exsheathed larvae of ruminant parasites following exposure to different media (distilled water, Earle's balanced salt solution + CO_2, nematode washing buffer + CO_2, and NaOCl). They reported that NaOCl was capable of producing high exsheathment levels (≥ 98.5%), however, the infectivity of the exsheathed larvae was significantly lower than that of larvae exsheathed in other media. They concluded that caution must be observed when concluding from *in vitro* studies where exsheathed larvae are used since the techniques commonly recognized to be highly effective in exsheathment "also appear to reduce viability". Regarding the wide application of NaOCl as an exsheathment medium in nematode-based research, the present study was undertaken to assess the viability of larvae using *Strongyloides papillosus* as the test organism. For this purpose, we adopted motility as a viability assay taking advantage of the capacity of the larva for active movement upon retrieval from culture. The use of this parasite also provides another advantage in that it allows for the effect of the compound to be directly measured in a parasite that naturally lacks a protective sheath. Generally, NaOCl is used as an exsheathing agent at different concentrations ranging from 0.08% to a 2.0 % solution diluted 1:300, 1:250 or 1:330 up to an absolute 2.0% solution. Likewise, the period of application of the compound also varied considerably from < 1.0 min to 60 min in some studies up to 18 hours in others. Experiments were, therefore, undertaken to establish concentration and exposure time limits where no effect on viability is expected to take place.

2. Materials and Methods

2.1. Source of the parasite, culture, and identification of larvae

Feces were collected from the rectum of sheep slaughtered in a government-owned slaughterhouse in Khartoum. Samples were transported in screw-cap bottles to the Parasitology laboratory at the Faculty of Veterinary Medicine, University of Khartoum. Using centrifugal flotation, random subsamples of feces were screened anticipating the presence of thin-shelled embryonated *S. papillosus* eggs, a parasite known to be highly prevalent among desert sheep in this country (Abdelnabi, 2000). Fecal pellets were thoroughly crumbled in a mortar, moistened sufficiently with tap water, and transferred to wide-mouth glass jars for the culture of eggs to the infective third larval stage (L3). Jars were loosely covered with lids and were stored in a cabinet for 3-5 days at room temperature (30 - 33 °C). The developing larvae were retrieved from culture by the Baermann standard technique. Samples of harvested larvae were immobilized by gently heating the larval suspension for 2 minutes in a water bath to enable examination of the larvae in an extended position. The taxonomic identity of the larvae was established according to the criteria described by Gibbons *et al.* (2010) and Hansen and Perry (1994).

2.2. Pilot tests

Pilot tests were conducted to determine the extreme concentration of the compound that completely immobilizes larvae within an 8-minute set time limit. Larvae in the eluent culture fluid were transferred to centrifuge tubes and centrifuged at 1500 rpm for 2 minutes. The supernatant was discarded leaving a trickle of fluid at the bottom of the tube. Larval sediments were transferred to microscope glass slides and treated with a few drops of NaOCl 3% (AMCLEAN, Al Fayrouz Dental and Medical Equipment, Sharjah, UAE) and were examined using an OPTIKA Srl B-193 optical microscope (Ponteranica, Italy) fitted with a digital camera (OPTIKAM 4083.B1). Larval motility was movie recorded at 1, 2,4, or 8 minutes with image setting at 60-seconds capture configuration. Movie files were presented as additional supporting files and can be retrieved online at *Figshare https://doi.org/10.6084/m9.figshare.18421136*

2.3. Effect of treatment with different concentrations of NaOCl and for different durations

Based on the results of pilot tests, the compound was used at concentrations of 0.03%, 0. 3%, or 3.0 %. Motility was movie recorded at 1, 2,5, or 10 minutes for each of the concentration levels tested. Larvae were designated as actively motile, capable of traversing the microscope field, sluggish, or immotile. The experimental procedure required the tracing of individual larvae for the assessment of treatment;

examination of larvae *en masse* was found to be difficult regarding the interception of different species of larvae normally present in the culture which are also actively motile (see Additional File A). To overcome this shortcoming and validate the results, the assessment of a specific treatment was based on 3 replicates involving individual larvae each time.

2.4. Critical tests

Regarding the findings of tests on the effects of different concentrations, experiments were performed to establish critical concentration and exposure time limits where no effect on motility is expected to be produced using concentrations lower than 0. 3 % but higher than 0.03% (0.2, 0.1, 0.05%) and an exposure time of 1, 2,5, or 10 minutes in each case.

3. Results

Parasites retrieved from culture were found to be predominantly *S. papillosus* larvae verified by the slender body, the distinctive long filariform oesophagus of almost 1/2 the total length of the larva, and the absence of a sheath (Figs 1 and 2).

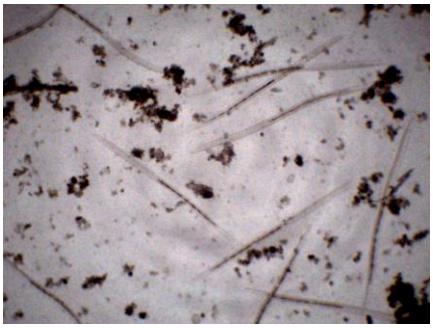

Fig (1): Heat-immobilized larvae from faecal culture. Larvae were predominantly *S. papillosus*

Fig (2): Living *S. papillosus* larva retrieved from culture ×100

The larvae retrieved from the culture were actively motile showing swift wriggling movements while traversing the microscope field (Additional file 1). Effects of different levels of treatment were shown in Tables 1 and 2.

Table (1): Motility of *S. papillosus* larvae exposed to different concentrations of NaOCI and for different exposure periods.

Exposure time (min)	NaOCI concentration		
	3%	0. 3%	0. 03%
1	± (3a)	+ (4a)	+ (5a)
2	± (3b)	+ (4b)	+ (5b)
5	- (3c) *	+ (4c)	+ (5c)
10	- (3d) *	± (4d)	+ (5d)

(+) actively motile; (±) sluggish, regardless of the degree of sluggishness; (-) immotile. (*) Dissolution of larval tissue.
Figures in brackets: Additional files retrievable online at *https://doi.org/10.6084/m9.figshare.18421136*

Table (2): Motility of *S. papillosus* larvae exposed to different concentrations of NaOCI and for different periods of exposure

Exposure time (min)	NaOCI concentration		
	0.2%	0.1 %	0.05%
1	+ (6a)	+ (7a)	+(8a)
2	+ (6b)	+ (7b)	+(8b)
5	+ (6c)	+ (7c)	+(8c)
10	± (6d)	± (7d)	±++ (8d) (i)(ii)(iii)

(+) actively motile; (±) sluggish, regardless of the degree of sluggishness.
Figures in brackets: Additional files retrievable online at *https://doi.org/10.6084/m9.figshare.18421136*

4. Discussion

Nematodes from a diverse phylum including parasitic species which are of great medical, veterinary, and agricultural importance. All species are encased in an exoskeleton (known as the cuticle), a complex protective extracellular matrix covering the outermost layer of cells composed primarily of proteins with trace amounts of lipids and carbohydrates (Page *et al.*, 2014; Fetterer and Rhoads,1993). NaOCl received wide application as an artificial exsheathment substance for larvae in nematode-based research by its remarkable tissue dissolution capacity (Dutta and Saunders, 2012; Echeverri and Acuña, 2012). On such occasions, the larvae are repeatedly washed to remove traces of the compound before the larvae are used. The question, therefore, arises whether a substance with such an effective disintegrative property would have an extended effect on the exsheathed larvae before the residues are completely removed. Generally, nematodes' viability is assessed by observing different phenotypic characteristics of the parasite, including gross morphology, developmental processes, or motility, to serve different purposes such as evaluation of anthelmintic activity or detection of anthelmintic resistance. Results of the present study indicate that NaOCl can affect the viability of *S. papillosus* negatively when the concentration and exposure time exceeds certain limits. The compound was also capable of dissolution of larval tissue when these levels are exceeded. These findings support the conclusion from infectivity tests (Conder and Johnson,1996) that, had NaOCl been used as an exsheathment medium, the results should be interpreted with caution as the compound is capable of reducing the viability of larvae. In this respect, and regarding the popular use of exsheathed nematode larvae in research, it will be of interest to know if the level of NaOCl tolerated by *S. papillosus* described in this study is sufficient to induce exsheathment in ensheathed-type larvae without the risk of reducing viability. Using ensheathed larvae, for instance, those of *Haemonchus contortus* pure cultured from abomasal worms (Zangueu *et al.*, 2018) may provide an answer.

Generally, various techniques have been used for studying the motility of nematodes based on different principles such as larval migration inhibition, computer-based systems such as processing of digital video imaging, motion-electrical waveform conversion, and an automated “INVAP” system for screening compounds that affect motility and development of parasitic worms (Wagland *et al.*, 1992; Demeler *et al.*, 2012; Demeler *et al.*, 2013; Storey *et al.*, 2014; Nutting *et al* ., 2015; Partridge *et al* ., 2018). Other techniques describe the use of infrared light-interference relying on the measurement of motility of nematode larvae, and drug microfluidic real-time sensitivity screening chip for studying the locomotion behavior of free-living and parasitic nematodes Carr *et al.,* 2011; Risi *et al.*, 2019; Taki *et a*l., 2021). The use of *S. papillosus* provides a simple and cost-effective model for the evaluation of the viability of larval nematodes where such advanced techniques are not accessible. The use of this model also has other advantages (i) it enables the effect of substances to be measured directly in a parasite that naturally lacks a protective sheath (ii) the larvae are potentially highly motile if motility is to be taken as a criterion for assessment of viability (iii) the larvae can easily be distinguished morphologically amid mixed-species if the faecal culture was from a multiple natural field infection (Additional file 9).

5. Conclusions

The results indicate that the viability of *S. papillosus* depends on both the concentration and the time of exposure to NaOCI. There are certain concentration (C) and time (T) limits beyond which viability is impaired (0.3% > C > 0.2%; 10 min > T > 5 min). The chemical can induce structural damage if these levels are exceeded.

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Conflict of Interest:

The authors declare that they have no conflict of interest.

Competing interests:

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Authors' contributions:

EE conceived, designed, and performed the experiments, wrote the original manuscript, and used photomicrography. GHA MFA contributed and prepared study materials, performed the experiments, and interrupted the results. All authors read and approved the final manuscript.

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