Caenorhabditis elegans as a model to screen plant extracts and compounds as natural anthelmintics for veterinary use

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ABSTRACT

The most challenging obstacles to testing products for their anthelmintic activity are: (1) establishing a suitable nematode in vitro assay that can evaluate potential product use against a parasitic nematode of interest and (2) preparation of extracts that can be redissolved in solvents that are miscible in the test medium and are at concentrations well tolerated by the nematode system used for screening. The use of parasitic nematodes as a screening system is hindered by the difficulty of keeping them alive for long periods outside their host and by the need to keep infected animals as sources of eggs or adults when needed. This method uses the free-living soil nematode Caenorhabditis elegans as a system to screen products for their potential anthelmintic effect against small ruminant gastrointestinal nematodes, including Haemonchus contortus. This modified method uses only liquid axenic medium, instead of agar plates inoculated with Escherichia coli, and two selective sieves to obtain adult nematodes. During screening, the use of either balanced salt solution (M-9) or distilled water resulted in averages of 99.7 ($\pm$0.73)% and 96.36 ($\pm$2.37)% motile adults, respectively. Adult worms tolerated DMSO, ethanol, methanol, and Tween 80 at 1% and 2%, while Labrasol® (a bioenhancer with low toxicity to mammals) and Tween 20 were toxic to C. elegans at 1% and were avoided as solvents. The high availability, ease of culture, and rapid proliferation of C. elegans make it a useful screening system to test plant extracts and other phytochemical compounds to investigate their potential anthelmintic activity against parasitic nematodes.

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1. Introduction

Caenorhabditis elegans is a free-living nematode naturally found in temperate climate soils. Experimentation with this nematode began in 1960 when researchers were looking for a multicellular organism, with a few cells, easy to raise and reproduce for embryonic developmental studies. Since then, C. elegans has become one of the most studied nematodes in many areas of biology.

The Order Rhabditida, to which C. elegans belongs, is closely associated with the Order Strongylida, which contains the important trichostrongyle parasites of ruminants, including Haemonchus contortus and Trichostrongylus spp. The rhabditid and strongylid nematodes have been placed in Clade V based on genetic analysis. Other common nematodes of domestic animals and humans are less closely

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related and have been placed in other clades. For example, ascarid and filarial worms are in Clade III, and *Trichinella* and *Trichuris* in Clade I (Geary and Thompson, 2001).

Simpkin and Coles (1981) examined the effect of commercial anthelmintics using *C. elegans* as an experimental model and concluded that this nematode satisfies many of the criteria needed for an *in vitro* test because it is cheap, readily available, and easy to work with. Since then, other parasitologists have also used this model to screen anthelmintic drugs (McGaw et al., 2007). Besides the nematocidal effect, the mode of action of anthelmintic drugs can be evaluated *in vitro* through nematode behavior, locomotion, and reproduction. If tested drugs are effective in *C. elegans* cultures at low concentrations, it is reasonable to assume that they may have anthelmintic activity against related nematodes, including *H. contortus* (Thompson et al., 1996).

Gastrointestinal parasitism is a serious problem in small ruminant production due to high morbidity and high mortality caused by *H. contortus* and related nematodes. This problem has been aggravated by the growing reports of multi-drug resistant gastrointestinal parasites worldwide (Jackson and Coop, 2000; Zajac and Gipson, 2000; Kaplan, 2004). The best test to determine if a compound has anthelmintic activity for veterinary use would be to use infections in the natural ruminant host. However, this requires livestock facilities and large amounts of plant material, making extensive screening not feasible. To facilitate initial screening of products, several *in vitro* assays have been used extensively to assess potential anthelmintic activity of natural compounds, including the egg hatch assay, the larval motility assay, and others (Hoste et al., 2006; Eguale et al., 2007; Brunet et al., 2008). However, these assays do not involve testing products against adult stages, which are the most harmful to the definitive host. Tests with adult *H. contortus* involve the killing and dissecting of an animal host to provide subjects for the adult worm motility (AWM) assay (Marie-Magdeleine et al., 2009). Using a *C. elegans* model for screening provides the advantages of a low cost *in vitro* laboratory method combined with the ability to examine activity of compounds against adult parasitic stages in related nematode species. The objectives of this work were to test a model system using liquid, axenic cultures of *C. elegans* to (1) propagate the organism, (2) select (with sieves) adult worms for testing, and (3) evaluate different solvents for their tolerability to *C. elegans*. This improved method is designed to screen plant extracts and compounds for their anthelmintic activity.

2. Materials and methods

2.1. Solvents

Dimethyl sulfoxide (DMSO), ethanol, methanol, acetone were all reagent grade (Fisher Scientific, www.Fishersci.com). Labrasol®, a bioenhancer composed of caprylcaproyl polyoxyl-8 glycerides of both vegetable and petrochemical origin was donated by Gatefossé (Paramus, New Jersey, USA), and both Tween 20 and Tween 80 were provided by Sigma–Aldrich (www.Sigma-Aldrich.com).

2.2. Maintenance of C. elegans

Strain N2 (wild type) acquired from the USDA Nematology Laboratory, Beltsville, MD, was raised in an axenic culture medium (Chitwood and Feldlaufer, 1990), composed of 90 ml distilled water, 3.0 g yeast extract (catalog No. Y1625, Sigma–Aldrich Corp., St. Louis, MO), 3.0 g soy peptone (Sigma P-0521), 1.0 g dextrose, 0.25 ml cholesterol (Sigma cat. No. C8667) solution (5 mg cholesterol per 1.0 ml 95% ethanol). The medium was autoclaved and then supplemented with 10 ml of a hemoglobin stock solution containing 0.5% hemoglobin (Sigma cat. No. H-2500) in 100 ml 0.001 M KOH filter-sterilized through a 0.45 μm sterile filter, then through a 0.22 μm sterile filter, and frozen until needed. Worms were sub-cultured each week by transferring two drops from a one-week-old culture to a sterile scintillation vial containing 1.0 ml of fresh medium, and incubated at 24 °C.

2.3. Isolation of adult nematodes

Nematodes were identified as adults, young adults, L_4 or juveniles (L_1–L_3) by size and the presence and extent of vulvar development (Wood, 1988). The L_1–L_3 stages were identified by their smaller size. The development of the vulva in young adults and adults was observed with an inverted microscope. The L_4 lacked the vulva and only presented a clear area with a semi-circular shape in the genital area midway along the length of the nematode. During the change from L_4 to adult stage, the vulva becomes prominent (young adult) and the clear area disappears (Bull et al., 2007). The tests were performed with young adults and adults with intact cuticle. Young adults are morphologically similar to adults, but smaller, and do not bear eggs.

In order to select only young adults and adults, four vials containing 1.0 ml of *C. elegans* axenic culture 5–7-day old were transferred to a 50-ml tube and the volume was completed with sterile distilled water. All stages of the life cycle were present and the tube was placed in a rack for 3 min to allow settling of the largest nematodes. The surface water containing smaller floating nematodes was then removed to reduce the volume to approximately 0.5 ml. Nematodes were separated according to their size using two sieves. Sieves were created by inserting nylon filter cloth between 2 pieces of PVC pipe of 2.1 and 1.9 cm diameter. First, we used a sterile 38 μm sieve to retain the largest nematodes and allow smaller nematodes to migrate through the sieve. This procedure retained young adults, adults, and large dead nematodes, which were non-motile and straightened (Fig. 1). Approximately 10 ml of distilled water was used to facilitate the migration.

Nematodes retained in the 38-μm sieve were transferred with 50 ml sterile water onto a larger mesh 53-μm sieve. In this procedure all active nematodes (adults and young adults) passed through the mesh and all dead or inactive nematodes were retained by the 53-μm sieve (Fig. 2).

The selection process was performed twice with each sieve, resulting in a suspension in which approximately 100% of the adult nematodes were alive and active.
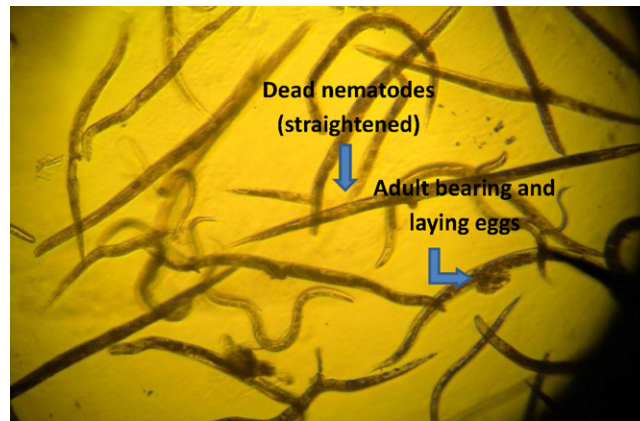
Fig. 1. Adults, young adults and dead (straightened) nematodes retained by the 38-μm mesh sieve. Small nematodes (L4 and smaller) were not retained by this sieve. Approximate lengths of young adults and late adults raised on agar plates seeded with *E. coli* were 1.0 mm and 1.4 mm, respectively (Knight et al., 2002). Our nematodes, raised entirely in liquid cultures, were approximately 40% longer than agar plate-raised ones. Based on this information, the approximate magnification = 28$\times$.

The final solution had a concentration of approximately 50 nematodes/20 μl.

2.4. Assay preparation

Tests were performed using a balanced salt solution (M-9) as the diluent for solvents and M-9 was also used as the medium to prepare a nematode stock with 50 nematodes/20 μl. M-9 solution was composed of 1.5 g KH_2PO_4, 3 g Na_2HPO_4, 2.5 g NaCl, 0.5 ml 1 M $MgSO_4$, and sterile distilled water to bring the volume to 500 ml (Brenner, 1974). Tests were performed in 24-well plates containing a total volume of 250 μl/well, with 6 replicates per treatment. The 24-well plates were covered with transparent plastic, and incubated at 24 °C for 24 h. The M-9 medium produced better nematode survival than using

distilled water, perhaps because the medium better preserved the nematode's osmotic balance.

2.5. Assay evaluation

After incubation at 24 °C for 24 h, plates were read using an inverted microscope and all nematodes counted and determined as motile or non-motile. They were considered motile when they exhibited any movement, and as non-motile when there were no tail, head, or pharyngeal movements during 5 s of observation (Skantar et al., 2005). It is important to differentiate motility in adult nematodes from movement caused by larvae hatched from eggs inside the body of dead *C. elegans*. The negative control group consistently showed 95–100% motile nematodes and the positive control (levamisole) 0% motile nematodes 24 h after incubation. To facilitate counting of
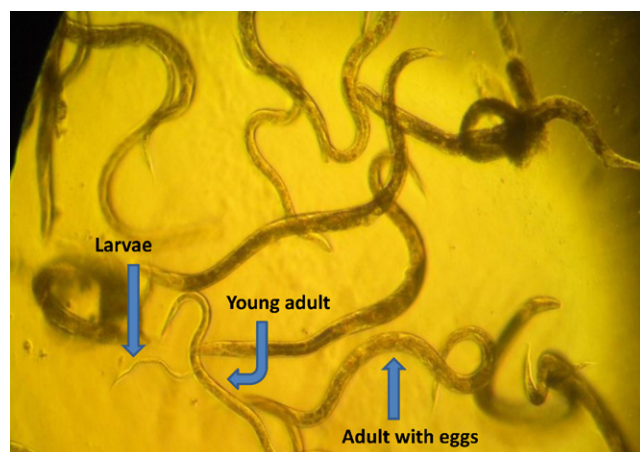
Fig. 2. Nematodes collected after passage through the 53-μm mesh sieve. Dead or inactive adults could not migrate and were excluded. Only active adults and young adults of *C. elegans* went through. Adults bearing eggs, young adults, and one larva (probably L_1) can be seen in this picture. Remaining young larvae were eliminated by repeating the procedure of passing the nematodes through both sieves two to three times. Based on size differences between nematodes raised in liquid cultures and on agar plates, and on the size of 0.25 mm for L1 larvae, 1.0 mm for young adults, and 1.4 mm for late adults (Knight et al., 2002), the approximate magnification = 32$\times$.

Table 1
Effect of solvents (DMSO, ethanol, methanol, acetone, Labrasol®, Tween 20 and Tween 80) in the adult *C. elegans* motility test. Results are the averages (%) of two tests, with six replicates each, of motile worms observed at each solvent concentration. The average % motile adult larvae for the M-9 control medium was 99.70 ± 0.73% 24 h after incubation. Standard errors are presented in between parenthesis.

Solvent	Concentration			
	1%	2%	4%	6%
DMSO	91.89 (2.07)	82.86 (3.88)	45.74 (5.23)	33.77 (2.76)
Ethanol	95.85 (5.15)	87.45 (5.34)	82.32 (3.17)	44.05 (6.27)
Methanol	90.76 (3.28)	81.11 (3.1)	83.98 (2.72)	54.08 (2.15)
Acetone	90.71 (4.67)	82.52 (3.19)	73.58 (2.2)	56.25 (3.89)
Labrasol	0	0	0	0
Tween 20	29.55 (4.73)	9.23 (1.47)	9.28 (3.81)	9.53 (1.55)
Tween 80	94.28 (3.03)	94.10 (5.33)	70.30 (2.45)	44.14 (2.54)

50 nematodes/well, horizontal lines were drawn at the bottom of the plates at 0.1 mm distance intervals.

3. Results and discussion

Our work indicates that the method using liquid *C. elegans* cultures is a fast and reliable way to propagate and synchronize *C. elegans*. This method can be used to screen crude plant extracts and compounds for their potential anthelmintic activity. The method differs from previously described screening tests (Stiernagle, 2006) in that it uses: (1) an axenic (no *E. coli*) liquid medium throughout, instead of agar plates inoculated with *E. coli* to maintain and propagate *C. elegans*, (2) nylon sieves of 38 and 53 μm pore size to select adult nematodes for testing, instead of life cycle synchronization, and (3) only early adult and adult nematodes, rather than multiple stages, to carry out the tests (Fig. 2). The main advantages of avoiding agar plates with *E. coli* lawns are: (1) time and effort saved on plate preparation and inoculation with *E. coli* as a food source for *C. elegans*, (2) reduction in contamination and elimination of antibiotics during the screening tests, and (3) prevention of bacterial metabolism of phytochemicals from the extracts (Simpkin and Coles, 1981). Additional time was gained from selecting worms through sieving and avoiding the synchronization process.

During the development of this method, we noticed that worms could tolerate up to 2% DMSO, ethanol, methanol, acetone or Tween 80 without loss of motility, but 2% Tween 20 and 1% Labrasol (Gatefoseé, France) were very toxic to *C. elegans* (Table 1). However, even 2% DMSO or Tween 80 could not dissolve lyophilized extracts made with low polarity solvents (e.g., dichloromethane, chloroform, pure ethanol) or non-polar solvents (e.g., hexane, petroleum ether). Thus, these solvents should be avoided for extract preparation. The bioenhancer Labrasol® was very effective in dissolving lyophilized plant extracts for anthelmintic testing without toxicity in gerbils at 25% (Squires et al., 2011), and in mice at 66% (Ribnicky et al., 2009). Unfortuantely, Labrasol killed all nematodes in the *C. elegans in vitro* system even at 1% (Table 1) and could not be used for plant extract dissolution.

The high survival rates of *C. elegans* in 1% DMSO (91.89%), ethanol (95.85%), methanol (90.76%), acetone (90.71%), and Tween 80 (94.28%), indicate that any of these solvents used at a concentration of 1% or 2% (Table 1), can be used to help dissolve crude plant extracts with low toxicity to *C. elegans*. However, one should consider survival rates of solvent controls when calculating survival rates in response to tested plant extracts. This will prevent an over-estimation of anthelmintic activity of the plant extracts. Although data related to tests with plant extracts in different media are not presented here, it is important to mention that the axenic (basal) medium containing hemoglobin and soy peptone is a good maintenance medium, but these proteins react with tannins making it unsuitable for screening. Also, while both M-9 and distilled water both resulted in average motile adults of 99.7 (±0.73)% and 96.36 (±2.37)%, respectively, nematodes seemed more adjusted to the isotonic M-9 medium than on distilled water, with the former being better suited for plant extract screening.

4. Conclusion

C. elegans cultured in medium containing heme (hemoglobin), replaces the use of agar plates with an *E. coli* lawn. While this axenic culture method is a simpler and faster method for providing large numbers of clean, active adult nematodes, the isotonic M-9 medium has no protein and was more suitable to keep *C. elegans* for screening purposes. By using the selective sieves described here, it is possible to collect many adults within one week in a small volume of medium. Freezing the culture medium in small volumes makes it rapidly available when needed, in any quantity, compared to preparation of *E. coli* inoculated agar plates. As a result, one person could screen at least six tests with results available in 48 h instead of 72–96 h using agar plates with *E. coli*.

Conflict of interest statement

The authors declare no conflict of interest. Mention of trade names or commercial products in this publication is solely for the convenience of the reader and does not imply endorsement of the U.S. Department of Agriculture

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