MethodsX 12 (2024) 102743

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/methodsx

Evaluating anthelmintic activity through *Caenorhabditis elegans* egg hatching assay ☆

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A R T I C L E I N F O

Method name:
Caenorhabditis elegans egg hatching assay

Keywords:
Anthelmintic drug
Natural compound
Synthetic compound
Parasitic nematode
Model organism
Egg hatching inhibition

A B S T R A C T

The *Caenorhabditis elegans* egg hatching methodology is a valuable tool for assessing the anthelmintic activity of drugs and compounds and evaluating anthelmintic drug efficacy.

Isolated eggs from gravid adults are exposed to different concentrations of selected drugs and the percentage of egg hatching is determined with respect to the control condition. The assay allows the construction of concentration-response curves and determination of EC_{50} or EC_{90} values for egg hatching inhibition. Also, it allows measurements of inhibition as a function of time of exposure.

This approach addresses the urgent need for new anthelmintics, as resistance to current treatments poses a significant challenge in parasitic nematode infection. This resistance not only affects humans but also animals and plants, causing significant economic losses in livestock farming and agriculture.

By using the free-living nematode *C. elegans* as a parasitic model organism, researchers can efficiently screen for potential treatments and assess drug combinations for synergistic effects. Importantly, this assay offers a cost-effective and accessible alternative to traditional methods, eliminating the need for specialized infrastructure, hosts, and trained animal maintenance personnel. Additionally, the methodology closely mimics natural conditions, providing insights into egg development and potential therapeutic targets.

This method allows for evaluating the direct negative impact of drugs on egg hatching, which correlates with long-term anthelmintic effects, offering advantages in preventing or reducing the transmission and spread of worm infections by eggs.

Overall, this approach represents a significant advancement for anthelmintic discovery, offering both practical applications and avenues for further scientific research.

☆ **Related research article For a published article**: Hernando G, Turani O, Bouzat C. *Caenorhabditis elegans* muscle Cys-loop receptors as novel targets of terpenoids with potential anthelmintic activity. PLoS Negl Trop Dis. 2019;13(11):e0007895. Published 2019 Nov 25. doi:10.1371/journal.pntd.0007895.

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https://doi.org/10.1016/j.mex.2024.102743
Received 24 February 2024; Accepted 2 May 2024
Available online 9 May 2024
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- The *C. elegans* egg hatching assay is a robust and effective method for assessing the anthelmintic potential of various drugs and compounds, allowing the generation of concentration-response curves.
- By leveraging the free-living nematode *C. elegans* as a parasitic model organism, this method facilitates efficient screening of potential treatments and evaluation of drug combinations.
- The method addresses the urgent need for new anthelmintics, offering a cost-effective and accessible alternative to traditional approaches.

Specifications table

Subject area:	Pharmacology, Toxicology and Pharmaceutical Science
More specific subject area:	Anthelmintic drug, drug discovery
Name of your method:	***Caenorhabditis elegans* egg hatching assay**
Name and reference of original method:	N.A.
Resource availability:	N.A.

Method details

Caenorhabditis elegans *strains and culture*

The nematode strains utilized for the development of this method included the N2 Bristol wild-type and PD4251: *ccIs4251;dpy-20(e1282)*. All strains were obtained from the *Caenorhabditis* Genetic Center, supported by the National Institutes of Health - Office of Research Infrastructure Programs (P40 OD010440).

Nematodes were maintained at 18–25 °C on freshly prepared Nematode Growth Medium (NGM) petri dishes spread with *Escherichia coli* (OP50) as a food source, following established protocols [1]. Maintenance procedures for *C. elegans* strains were carried out according to the protocol described by Stiernagle detailed in WormBook [1].

Assay procedure

1. Agar plates for egg hatching assay:

1.1. Use agar plates consisting solely of agar and distilled water (1.7 g agar in 100 mL H2O). To prepare drug-free agar plates, prepare them ideally 12 to 24 h before use. It is essential not to exceed the 24-hour timeframe to prevent excessive drying, which could adversely affect worm development. Divide the plates into quadrants to facilitate egg and L1 (Larva 1) larvae counting.

Note: If agar plates are prepared in advance, they should be stored at 8–10 °C.

2. Test compound and drug preparation:

2.1. Prepare drugs and compounds from pure stock in M9 buffer (22 mM KH2PO4, 42 mM Na2HPO4, 86 mM NaCl, and 1 mM MgSO4) or sterile distilled water, do so on the same day as the experiment [2]. Additionally, prepare respective controls, including a M9 buffer-only control and controls with respective vehicles such as DMSO or ethanol. Prepare at least 5 different concentrations of each desired drug to construct concentration-response curves in order to estimate EC_{50} and EC_{90} values.

3. *C. elegans* egg obtention:

3.1. Obtain *C. elegans* eggs following the worm synchronization protocol described by Christensen [3]. Wash 3 to 5 NGM plates mostly full of gravid worms with distilled water and transfer them into a 15 mL conical tube. Centrifuge worms at 1200 rpm to clean sediment from bacteria, and repeat the washing procedure three times (hereinafter, x3).

3.2. Chemical treatment: Expose the pellet containing worms to a solution of 1.2 % NaOCl and 0.5 M NaOH for five minutes (5 mL final volume). Use strong agitation to break worms' bodies into small parts and liberate eggs. After 5 min, stop the reaction with sterile distilled water to a final volume of 14 mL and centrifuge at 1200 rpm. Wash the pellet containing eggs from this reaction with 14 mL sterile distilled water (invert the tube several times) and centrifuge at 1200 rpm (washing procedure x3). Then, add 5 mL sterile M9 (or sterile water) to the bacteria-clean egg pellet and homogenize.

3.3. Generate egg stock solution: Estimate 100–140 eggs in 100 μL M9 buffer and aliquot 500 μL per condition. To estimate the desired number of eggs, put 20 μL of buffer containing eggs on a slide, and count eggs for correct dilution (x3), calculating the average number of eggs per volume. Correct the number of eggs by adding or removing buffer from the egg solution to achieve a final concentration of 100–140 eggs in 100 μL M9.

Note: An additional step to clean the egg pellet from remaining worms' bodies may be performed prior to dilution. Add a solution of 30 % sucrose to the pellet containing eggs, mix well, and centrifuge at 1200 rpm. Transfer the supernatant containing eggs to a new 15 mL conical tube with 10 mL sterile water. Collect the supernatant as soon as the centrifuge stops, as eggs start to fall from the ring formed at the top of the sucrose gradient solution. Body worms and undesired residue remain in the pellet. Finally, mix eggs well, wash with sterile water, and centrifuge at 1200 rpm (x2) [3].

4. *C. elegans* egg hatching assay:

4.1. Egg treatment: Transfer 500 μL egg stock solution to a 1.5 mL Eppendorf tube per condition. Add an equal volume of the compound of interest (treatment) or buffer (control and control with vehicle if applicable) to the Eppendorf tube containing eggs.

Incubate both control groups and experimental groups for 4–8 h at 22 °C. The duration of the incubation period depends on the stability of the drug, the concentration required to achieve a desirable effect, and the realistic timeframe during which contact between the drug and eggs would be feasible in a real environment. Set a desired time, such as 6 h and use it between experiments to avoid variability.

4.2. Recover eggs after the incubation period. Centrifuge the 1.5 mL Eppendorf tube at 6000 rpm, wash the pellet with 1 mL M9 buffer (x3), and resuspend to a final volume of 240 μL M9. Place both control groups and experimental groups into fresh agar plates divided into quadrants (20 μL per quadrant, three agar plates per condition) for development at 16–18 °C to complete 24 h from egg obtention.

4.3. Image Collection: After 24 h from obtaining *C. elegans* eggs, take images of the respective quadrants of both control groups and experimental groups' agar plates under a Stereoscopic Zoom Microscope. Acquire images of quadrants with a digital camera such as ToupCam UCMOS 05100KPA (ToupTek Photonics), and determine the number of eggs and L1 larvae using an image processing software such as ImageJ.

Note: Use at least three agar plates for each condition per experiment.

4.4. Incubate agar plates for an additional 12–24 h at 16–18 °C after image collection to determine whether the inhibition of egg hatching is irreversible.

4.5. For drugs that are less effective in buffer (e.g., small molecules such as serotonin and betaine), use distilled water for better results. Should that be the case, the incubation period should be shorter (4–6 h) because water negatively affects eggs and L1 larvae viability.

4.6. Estimate the incubation period beforehand in a pilot test to establish the shortest exposure time according to the stability of compounds to be used and the lowest possible concentrations for a real *in vivo* situation.

5. Data Analysis:

5.1 Calculate The ability of drugs to inhibit egg hatching according to the following equation:

Fraction of unhatched eggs = number of unhatched eggs / (number of hatched larvae L1 + number of unhatched eggs).

Collect data from at least three to five independent experiments (from different egg batches). Analyze the data using appropriate software such as SigmaPlot.

Demonstration of assay procedure using a reference drug

1. Agar plates for egg hatching assay: We prepared agar plates consisting solely of agar and distilled water 24 h prior to the experiment. Typically, we use a 35 mm dish with 3 mL of agar solution. This helps maintain proper focus on the stereoscopic microscope when changing plates and capturing images for the final count. Plates were labelled according to each condition, quadrants I-IV were marked, and plates were reserved for later use.
2. Test compound and drug preparation: Selection of the drug of interest is driven by the goal, which may encompass investigations into compounds with negative, positive, or neutral effects on the hatching and/or viability of eggs. In this experiment, we opted for thymol as our aim is to evaluate the anthelmintic capacity of various natural compounds [2]. Thymol mother solution was prepared in DMSO from pure stock on the day of the experiment, thymol dilutions and respective controls are prepared in sterile distilled water. We prepared 6 different thymol concentrations, thymol: 0.75 mM, 1 mM, 1.25 mM, 1.5 mM, 1.75 mM and 2 mM to construct concentration-response curves.
3. *C. elegans* egg obtention and stock solution: To obtain eggs, we followed the worm synchronization protocol described by Christensen [3]. We started with five 5 cm NGM plates full of gravid worms. We added 5 mL sterile water to the bacteria-clean egg pellet and homogenized. To estimate the desired number of eggs, we put tree drops (20 μL each drop) of homogenized solution containing eggs on a slide, we counted eggs for correct dilution, (example: 26, 30 and 28 eggs per drop, prom 28 ± 2 eggs in 20 μL). Egg solution final concentration: ~140 eggs in 100 μL sterile water.
4. *C. elegans* egg hatching assay: We transferred 500 μL egg stock solution to a 1.5 mL Eppendorf tube per condition (in triplicate), and then added 500 μL of respective solution. For experimental groups the thymol final concentration were 0.75 mM, 1 mM, 1.25 mM, 1.5 mM, 1.75 mM and 2 mM, and the respective controls were sterile water (control 1) and DMSO/sterile water (0.41% DMSO in sterile water, control 2). We incubated both the control and experimental groups for 6 h at 18–20 °C.
   After treatment, we recovered eggs by centrifugation and washed the pellet with 1 mL sterile distilled water (x1) and M9 buffer (x2), and resuspended to a final volume of 240 μL M9. We placed all conditions in its respective agar plates prepared in advance, and seeded 80 μL per agar plate (20 μL per quadrant, 3 agar plates per Eppendorf tube, 3 tubes per condition). We let agar plates at 16–18 °C to complete 24 h from egg obtention.
   After 24 h from obtaining *C. elegans* eggs, we acquired images of all quadrants with a digital camera, and determined the number of eggs and L1 larvae using an image processing software. Also, we incubated agar plates for an additional 24 h at 16–18 °C after image collection to determine whether the inhibition of egg hatching was irreversible.
5. Data Analysis: We collected data from three independent experiments to construct concentration-response curve. For thymol dose-response curve (Fig. 1), the EC_{50} value was calculated as the concentration at which the curve passes through the 50% inhibition level. Thymol EC_{50} value: 1.23 ± 0.12 mM. Likewise, we verified that there were no significant differences between control 1 and control 2 (0.104 ± 0.012 and 0.106 ± 0.0132, respectively; $P = 0.806$).
   Overall, the technique serves as a valuable tool for evaluating the effects of various drugs and compounds. Different studies have employed various techniques to assess egg hatching with different objectives, whether the focus is on assessing hatching

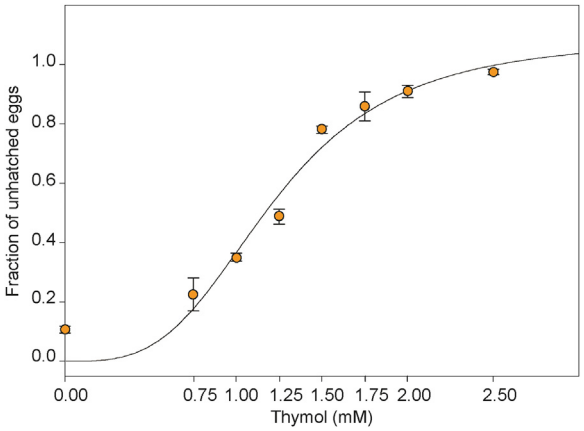

Fig. 1. Concentration-response curves for *Caenorhabditis elegans* egg hatching inhibition exerted by thymol. Representative experiment showcasing the fraction of unhatched eggs exposed to the indicated thymol concentrations for 6 h.

inhibition, proper egg viability, or testing developmental effects (negative, positive, neutral) [4–6]. We propose a simple and reliable technique for obtaining reproducible results.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Guillermina Hernando: Conceptualization, Investigation, Methodology, Software, Funding acquisition, Writing – original draft, Writing – review & editing. **Cecilia Bouzat:** Funding acquisition, Writing – review & editing.

Data availability

No data was used for the research described in the article.

Acknowledgments

This work was supported by Argentine grants from Agencia Nacional de Promoción de la Investigación, el Desarrollo Tecnológico y la Innovación (PICT 2019-01751 to GH and PICT 2020-00936 to CB), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP11220200102356) and Universidad Nacional del Sur (PGI 24/B340 to GH).

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