In vitro comparison of treatments and commercially available solutions on mortality of *Angiostrongylus cantonensis* third-stage larvae

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Abstract

On Hawai'i Island, an increase in human neuroangiostrongyliasis cases has been primarily associated with the accidental ingestion of *Angiostrongylus cantonensis* L3 in snails or slugs, or potentially, from larvae left behind in the slug's slime or feces. We evaluated more than 40 different treatments *in vitro* for their ability to kill *A. cantonensis* larvae with the goal of identifying a safe and effective fruit and vegetable wash in order to reduce the risk of exposure. Our evaluation of treatment lethality was carried out in two phases; initially using motility as an indicator of larval survival after treatment, followed by the development and application of a propidium iodide staining assay to document larval mortality. Treatments tested included common household products, consumer vegetable washes and agricultural crop washes. We found minimal larvicidal efficacy among consumer-grade fruit and vegetable washes, nor among botanical extracts such as those from ginger or garlic, nor acid solutions such as vinegar. Alkaline solutions, on the other hand, as well as oxidizers such as bleach and chlorine dioxide, did show larvicidal potential. Surfactants, a frequent ingredient in detergents that lowers surface tension, had variable results, but dodecylbenzene sulfonic acid as a 70% w/w solution in 2-propanol was very effective, both in terms of the speed and the thoroughness with which it killed *A. cantonensis* L3 nematodes. Thus, our results suggest promising directions for future investigation.

Introduction

Angiostrongylus cantonensis, the rat lungworm, is a parasitic nematode responsible for angiostrongyliasis or rat lungworm disease, and the leading cause of human eosinophilic meningitis worldwide (Hochberg *et al.*, 2007; Graeff-Teixeira *et al.*, 2009; Prociv and Turner, 2018). Infections of humans by *A. cantonensis* can occur through several different routes. While in many countries, angiostrongyliasis is often associated with the consumption of traditional foodstuffs, including poorly cooked or uncooked gastropods (Punyagupta, 1965; Lv *et al.*, 2009; Eamsobhana *et al.*, 2010), in the USA, infection results primarily from the accidental ingestion of gastropods, often parts of slugs or as small neonates within uncooked plant material, i.e. vegetables and fruits, which have been inadequately cleaned (Lindo *et al.*, 2002; Slom *et al.*, 2002; Waugh *et al.*, 2005; Cowie, 2013; Hollingsworth *et al.*, 2013; Yeung *et al.*, 2013). Human infection has also been reported from drinking raw vegetable juices (Tsai *et al.*, 2004, 2013) and home-prepared kava (a plant-based drink common in the Pacific islands) in which the semi-slug *Parmarion martensi* was subsequently found (Office of the Governor of Hawaii, 2017).

The potential for acquiring angiostrongyliasis from *A. cantonensis* third-stage larvae (L3) shed through gastropod mucus or feces is another possible route of transmission, although its epidemiological significance is unknown (Cowie, 2013; Kramer *et al.*, 2017). For example, while studies using both PCR and microscopy have detected *A. cantonensis* in the mucus of both naturally infected *P. martensi* semi-slugs and experimentally infected *Austropeplea lessoni* snails, larvae have only been found in relatively low numbers (Qvarnstrom *et al.*, 2007; Jarvi *et al.*, 2012; Chan *et al.*, 2015). Another possible source of infective larvae is from gastropod feces (personal observation) or dead and decomposing gastropods. Larvae from such sources might be disseminated throughout produce from soaking salad greens or through the reuse of contaminated water for vegetable cleaning (Crook *et al.*, 1971; Waugh *et al.*, 2005; Howe *et al.*, 2019). Another potential route is through the use of rainwater catchment, which is a primary source of household or agricultural water in many areas of Hawai'i, including east Hawai'i Island (Howe *et al.*, 2019).

A review of the literature finds a limited number of papers that have investigated methods of rendering food safe from transmitting *A. cantonensis* infection. Alicata (1967) investigated

the use of freezing or boiling to kill *A. cantonensis* L3 within giant African snails (*Achatina fulica*) and freshwater prawns (*Macrobrachium rosenbergi*) and found that larvae were no longer infective when snails or prawns had been exposed to −15°C for 12 or 24 h. Similarly, he found that larvae were rendered noninfective after boiling snails for 2 or 3 min or prawns for 1 min and third-stage larvae died in water heated to between 50 and 55°C. Crook *et al.* (1971) tested iodine solution (16 mg quart⁻¹), a chemical commonly used in water treatment, against *A. cantonensis* L3 isolated from giant African snails (*A. fulica*). While larvae were reduced in number, they were not eliminated. Working with the closely related nematode *Angiostrongylus costaricensis*, Zanini and Graeff-Teixeira (2001) compared infectivity in Swiss mice after incubating isolated larvae for 1 h in wine vinegar, saturated cooking salt and a 1.5% bleach solution. Of the three treatments, 1.5% bleach showed the greatest efficacy as it killed 97% of infective larvae. Yeung *et al.* (2013) also compared vinegar, bleach and salt but focused on their efficacy in removing snails and slugs from lettuce. They found none of the experimental solutions capable of removing all gastropods and none to be more effective than simply washing and rinsing with tap water.

Given the continued unmet need to reduce or eliminate rat lungworm transmission and the potential severity of angiostrongyliasis as a disease, we developed tests that allowed us to compare the efficacy of commercially available solutions and treatments in killing infectious stage (L3) *A. cantonensis* larvae through *in vitro* testing. In addition, we investigated how long isolated L3 can survive in the environment, e.g. on counters or benchtops, and under the effects of changing temperature and moisture level.

Materials and methods

Slugs were collected from multiple localities on the east side of Hawai'i Island in either 2012 (March–October) or 2019 (May–December).

2012 Larval preparation and treatment

For tests completed in 2012, *P. martensi* were placed in Petri dishes where the head was removed by sterile, disposable single edge razor and an approximate 50 mg tip of the tail was collected, divided into two and put into individual DNA lysis buffer tubes (0.1M Tris HCl, 0.1M EDTA, 2% SDS) and stored at −80°C until tested by qPCR. Both head and body were minced and placed in 5 mL HCl (0.7%) – pepsin (0.5%) buffer (∼2 h, 37°C) (Jarvi *et al.*, 2012). This digestion solution was filtered twice through a modified Baermann funnel (Baermann, 1917; Jarvi *et al.*, 2012). Briefly, this consists of a double-layer of KimWipes® placed in the opening of the funnel with a clamped rubber hose attached to the bottom. The solution containing nematodes was poured through the KimWipe®/funnel and allowed to sit at room temperature for at least 6 h to allow the larvae to accumulate above the clamp, and larvae were then collected.

Tubes of larvae were centrifuged at 750 rpm for 10 min, after which the supernatant was removed, and replaced with 2 mL of wash treatment of a defined dilution. The digestion buffer of HCl-pepsin was added to one microcentrifuge tube out of six to serve as a live untreated control. Tubes of larvae with treatment were then transferred to 50 mm Petri dishes, and the number of moving *vs* immobile larvae were visualized by ×10–×40 microscopy and recorded at 5 min intervals for a total of 20–30 min to document treatment effects on larval mobility.

2019 Larval preparation and treatment

Parmarion martensi collected for the 2019 studies were drowned in 50 mL of tap water in Falcon® tubes for a minimum of 60 h, after which slugs were removed and the bottom 15 mL from each tube was pipetted into a culture plate (60 mm × 15 mm) (Howe *et al.*, 2019). Tap water was verified to have a chlorine content <10 ppm using Precision Chlorine Test Paper strips (Bartovation, LLC, Queens, NY, USA). Larvae exhibiting the *S* and *Q* swimming movements attributed to *A. cantonensis* L3 (Lv *et al.*, 2009) were visualized by ×10–×40 microscopy (Leica S9 D and Wild Heerbrugg M4A APO) and were transferred by pipette into 35 mm × 10 mm culture plates containing approximately 2 mL of tap water. Additional washes were carried out as necessary to thoroughly separate larvae from debris.

Each treatment run included nine tubes of at least 50 larvae each: one tube of killed untreated, three tubes of live untreated, one tube of killed treated, three tubes of live treated and one tube of 50 larvae saved for later genetic analysis. Live larvae for each treatment run were prepared by adding 50 live larvae into 20 µL of tap water in three 1.5 mL microcentrifuge tubes. Live untreated larvae served as negative controls. Killed larvae served as positive controls for the propidium iodide (PI) assay and were prepared by transferring 100 larvae into 20 µL tap water, adding 500 µL of 100% methanol (BDH; VWR International, Radnor, PA, USA), followed by freezing at −80°C for a minimum of 24 h, although in most cases killed larvae were actually frozen at least 1 week (Ferreira *et al.*, 2015; Jarvi *et al.*, 2019). Tubes of killed larvae controls were thawed, poured into small glass Petri dishes containing 10 mL of tap water, and the methanol allowed to evaporate for 1 h with gentle rocking. Killed control larvae (*n* = 50) were then transferred into two 1.5 mL microcentrifuge tubes per treatment run (one treated, one untreated). A single tube of 50 larvae was transferred into DNA lysis buffer (0.1M Tris HCl, 0.1M EDTA, 2% SDS) and stored at −80°C for later genetic analysis *via* real-time PCR to verify the presence of *A. cantonensis* larvae (Jarvi *et al.*, 2012).

One killed control tube, and three live larvae tubes were exposed to a chemical or commercial wash product at a specific concentration (including manufacturer's recommended concentration) with gentle rocking for 60 min at room temperature (unless the duration was otherwise noted, e.g. some of the salt water treatments). Following the treatment, the wash solution was removed from larvae by rinsing at least twice with 500 µL of tap water, vortexing gently, centrifuging for 5 min at 7000 rpm and removing 500 µL of the supernatant. Rinsed larvae were then transferred into culture plates containing 2 mL of tap water. Untreated larvae were vortexed and centrifuged (but not rinsed) as with the treatment washes.

Death assay: 2019

A critical difference in our determination of mortality, between 2012 and 2019, was the use of PI as a death assay in the latter year as described by Jarvi *et al.* (2019). Thus, after treatment and wash protocols were completed, larvae were transferred into individual wells of a black, transparent-bottom view plate (#384, PerkinElmer, Waltham, MA, USA). A 1.25% solution of PI (Biotium, Freemont, CA, USA) in water was prepared for the differential staining of dead larvae (Jarvi *et al.*, 2019). Twenty microlitres of PI was added to each well along with 1.2 µL of 100x penicillin-streptomycin and sufficient water to reach a final volume of 120 µL in each well. The solutions were then mixed on a 3D rotator mixer for 1 h. Plates were imaged using an Operetta high-content imaging system (Perkin Elmer Life Sciences, Boston, MA, USA) as described (Jarvi *et al.*, 2019) and images collected using a ×20 long working distance lens with brightfield, PI (535 nm excitation, 617 nm emission) and fluorescein (494 nm excitation and 512 nm emission) filters. The fluorescein filter on the Operetta allowed the detection of

autofluorescent live larvae, which appeared green, while under the filter for detecting PI-stain, dead larvae appeared bright yellow (Jarvi *et al.*, 2019). Only larvae showing the maximum level of PI fluorescence were counted as dead. The total number of larvae and the number of larvae highly stained with PI were recorded 1 h post-staining and every 24 h for 7 days to follow the progression in L3 mortality.

Testing effects of pH, drying and temperature

We evaluated several products with various alkaline pH levels (Pinnacle M530 pH meter, Corning, Woburn, MA, USA) and compared their effectiveness in killing *A. cantonensis* L3 against solutions of sodium hydroxide (Cat. S318-1, Fisher Chemical, Fair Lawn, NJ, USA), buffered to pH 12.2 through 13 as described above.

Angiostrongylus cantonensis L3 were also tested for their ability to survive desiccation; either alone or after applying ethanol (Decon Labs, King of Prussia, PA, USA) or 2-propanol (Target, Minneapolis, MN, USA) as follows: 25 live and visibly moving larvae were transferred in 20 μL of water to a depression slide, then the slide water allowed to completely dry with the time recorded. When testing ethanol or 2-propanol, sufficient alcohol was added to the water on the slide to create a 70% solution, which again, was allowed to dry. Following drying, 20 μL of tap water was added to each slide according to the following times; slide 1 was allowed to dry for one additional minute before adding water, slide 2 for 2 min before adding water, and so on for up to 5 min. Within 5 min of adding water, and every 5 min thereafter, the number of both mobile and immobile larvae was counted and recorded as percentage immobility.

To test the effects of cold temperatures on larval survival, four tubes of larvae (3 live and one killed control) ($n = 60$ larvae/tube) were placed independently in a -15°C freezer and a 0°C refrigerator for four time periods: 0°C for 24 h; 0°C for 7 days; -15°C for 12 h; -15°C for 24 h. Refrigerator and freezer temperatures were recorded hourly for the duration of all tests using an Onset Hobo Pro v2 Humidity and Temperature Data Logger (Onset Computer Corporation, Bourne, MA, USA). After all treatments, larvae were visualized as described above and were counted every 24 h for 7 days.

Genetic analysis of larvae

Larvae were homogenized in a bead mill homogenizer as described (Niebuhr *et al.*, 2020) and DNA extracted using a DNeasy® Blood & Tissue Kit (QIAGEN, Valencia, California, USA) per the manufacturer's Animal Tissue protocol with minor modification (Jarvi *et al.*, 2012; Howe *et al.*, 2019). Extracted DNA was analysed by real-time PCR using species-specific primers in a TaqMan Gene Expression Assay, as described in Jarvi *et al.* (2012) with modification of increasing the number of cycles to 50 (Niebuhr *et al.*, 2020).

Results

Gastropod collection

Due to its high rate of infection, the semi-slug *P. martensi* (Hollingsworth *et al.*, 2007; Jarvi *et al.*, 2012; Kim *et al.*, 2018) was the only gastropod collected as a source of *A. cantonensis* L3 for this study. Sources of *P. martensi* collected from Hawai'i Island are provided in Table 1.

Table 1. Sources of *P. martensi* collected from Hawai'i Island and drowned to obtain L3 for 2012 and 2019 trials

	District	Latitude	Longitude	Elevation (m)
Locale in 2012				
Leilani Estates	Puna	19.4397 N	154.9513 W	354
Pahoa	Puna	19.4700 N	154.8337 W	10
Pahoa	Puna	19.5231 N	154.8591 W	55
Hawai'i Paradise Park	Puna	19.5844 N	155.0069 W	85
Hawai'i Paradise Park	Puna	19.5880 N	154.9685 W	40
Hilo	Hilo	19.7239 N	155.0965 W	52
Kamuela	Waimea	20.0160 N	155.6531 W	841
Locale in 2019				
Leilani Estates	Puna	19.4397 N	154.9513 W	354
Pahoa	Puna	19.4635 N	154.9734 W	341
Pahoa	Puna	19.5231 N	154.8591 W	55
Keaau	Puna	19.5440 N	154.9877 W	158
Waiakea	South Hilo	19.6339 N	155.0891 W	134
UH Hilo	North Hilo	19.6974 N	155.0895 W	95
Paukaa	Hāmākua	19.7710 N	155.1012 W	118

Study criteria

Treatment test results from both 2012 and 2019 were independently compiled, and results from replicated tests are provided as an average. The minimum concentration used to get the highest mortality is reported when multiple tests of the same treatment were repeated. Standard durations are listed as 30 min for tests done in 2012, and typically 60 min for those completed in 2019, except for salt water (sodium chloride), which was also tested for 24 and 48 h. The criteria used to measure treatment efficacy differed between the trials run in 2012 and 2019.

2012 Wash solution study

The 2012 tests used larval mobility as an indicator of potential larval mortality; therefore, efficacy was measured in terms of 'Percent Immobility', meaning the number of larvae that were not moving, out of the total number of larvae being evaluated (Table 2). Of the wash products tested in 2012, the majority resulted in <10% immobility including vinegar (an acid), and other common household products such as hydrogen peroxide, as well as botanical extracts, including garlic or ginger juice, oils from neem, lemongrass and peppermint. Products that did induce immobility in a high percentage of *A. cantonensis* included bleach (10 and 15% of the stock solution of 6% sodium hypochlorite), the vegetable wash *EpiClean* (Pace International, Wapato, WA, USA), the surfactant dodecylbenzene sulfonic acid (DBSA) 70% (w/w) in 2-propanol (Cat. 522953, Sigma-Aldrich, St. Louis, MO, USA) and 10% salt solution. These solutions shown to be effective in 2012 were retested in 2019 using PI differential stain as an *in vitro* death assay.

2019 Wash solution study

Efficacy in the 2019 trials is expressed in terms of 'Percent Mortality' as a function of the number of fluorescing larvae

Table 2. Effect of treatments on larval mobility in 2012

Wash Trade Name	Concentration	% Immobility
Bleach (6% sodium hypochlorite)	6.25%	97
Difluoromethylornithine (DFMO)	10 mM	5
DBSA (Dodecylbenzene sulfonic acid, 70% (w/w) in 2-propanol)	0.2%	100
EpiClean	33%	100
Fit® Organic™ Fruit & Veggie Wash	1:2	0
Garlic juice	100%	0
Ginger juice	100%	0
GSE (Grapefruit Seed Extract)	1:200	2
Hydrogen peroxide	3%	7
Insecticidal Soap	1:2	4
Lemon Grass Oil	1:2	9
MMS (Miracle Mineral Supplement)	1:2	1
Neem oil	ND	2
Peppermint Oil	1:2	31
Salt water	10%	85
Salt water	15%	100
Tween 80	ND	0
Vinegar	30%	2

ND, not determined.
All test results from incubating L3 *A. cantonensis* larvae in each treatment for up to 30 min, in 5 min observation increments.

divided by the total number of larvae and is given for the live treated group days 0, 1 and 7 post-treatment in Tables 3 and 4. Day 0 results were collected 1 h after adding PI, Day 1 results collected 24 h post-staining, and Day 7 results illustrate the progression of mortality for 7 days. Although larvae exhibited variation in both the rate and extent of staining over the course of death, only those larvae showing PI fluorescence over the entire length of their body were counted as dead.

The results from the 2019 tests are presented in two tables: Table 3 provides results of wash products (*n* = 18) showing <50% kill rate by Day 7, and Table 4 provides those showing >50% efficacy by Day 7 (*n* = 11). Wash products showing <50% efficacy included all consumer-grade fruit and vegetable washes along with the three solutions which had appeared effective in the 2012 immobility assays: the crop wash *EpiClean*, the 15% salt solution for 1 h exposure and the 5% salt solution for 24 and 48 h exposure. Treatments showing more than 50% effectiveness at 1 h exposure included three commercial crop washes *Field Clean*, *Real Clean* and *Sooty Mold Cleaner* (Pace International), all of which were highly alkaline (pH 12.86, 12.21 and 12.45, respectively). Other effective treatments included 10 and 15% salt water for 24 h exposure, the commercial nematicide *Nemitol*™ (Champon Millennial Chemicals, Alexandria, VA, USA), as well as the oxidizers bleach and *Keeper® Pro Post-Harvest* (chlorine dioxide; Bio-Cide International, Norman, OK, USA). Surfactants, a frequent ingredient in detergents that lowers surface tension, had variable results overall, but DBSA as a 70% (w/w) solution in 2-propanol was very effective, both in terms of the speed and the thoroughness with which it killed *A. cantonensis* L3 nematodes.

pH, drying, alcohol and cold temperatures

pH
Because the Pace International products *Field Clean*, *Real Clean* and *Sooty Mold Cleaner* all demonstrated greater killing rates and had highly alkaline active ingredients, the effect of alkaline pH was tested directly on *A. cantonensis* L3 survival using NaOH at multiple high pH values and substantial mortality was only found at pH > 12.8 (Table 5).

Drying and alcohol
Tests of L3 survival after desiccation (replicated four times) demonstrated that while an average of 85.7% of L3 could survive 1 min of drying, the number of surviving larvae dropped off sharply thereafter (Table 6). In two out of four tests, no nematodes survived longer than 3 min under desiccation conditions. Applying alcohol (70% ethanol or 2-propanol), then drying, more than doubled the number of *A. cantonensis* rendered immobile in the first minute compared with drying alone, although differences in mortality between all treatments were relatively minor by the third minute of drying (Table 7).

Temperature
In comparing the survival of *A. cantonensis* L3 in a refrigerator (0°C) *vs* a freezer (−15°C), it took ∼7 days at 0°C, and an entire subsequent week of observation for *A. cantonensis* to exhibit 99% mortality (Table 8). On the other hand, freezing L3 at −15°C for 12 h did result in death rates >50%, although there was a 2-day delay before achieving 75% mortality (Table 8).

Genetic analysis

Genetic analysis, using real-time PCR of nematodes isolated for treatment testing, showed positive results with C_T values ranging 19.621–22.276, indicating the presence of *A. cantonensis* in all wash trials conducted in 2019.

Discussion

One of our main goals in testing a variety of wash solutions and other treatments was to compare their relative efficacy in killing *A. cantonensis* third-stage larvae as a means of reducing accidental infection. In 2012, we evaluated the effectiveness of wash treatments based on the degree to which they inhibited larval mobility. Use of mobility assumes that larvae that are moving must at the very least be alive, and thus potentially infective. However, lack of movement in nematodes may indicate temporary quiescence in response to environmental stress or result from sub-lethal doses of toxic or anaesthetic substances from which the larvae may eventually recover (Chandel, 2009; Eamsobhana et al., 2010; Jarvi et al., 2019). In light of these concerns, in 2019, we used an alternative *in vitro* assay, validated in our laboratory, which uses PI stain as an indicator of L3 *A. cantonensis* larval death (Jarvi et al., 2019). In comparing our 2012 immobility and 2019 PI assays, we found that many treatments that had produced complete and long-lasting immobility in 2012 also exhibited high L3 mortality when using the PI assay. There were two exceptions: *EpiClean* produced 100% immobility in 2012 but, when tested with PI in 2019, showed 0% mortality. We suspect this discrepancy to be due to the significant viscosity of *EpiClean*, which may have inhibited larval movement. The second exception was salt water, which, when tested in 2012, produced 85% larval immobility at 10% concentration and 100% immobility at 15% (Table 2), but then <10% mortality in the 2019 PI assay (Table 3). However, this discrepancy was only found when incubating larvae in salt water for 60 min. When we exposed larvae to

Table 3. Wash products showing <50% mortality rate by Day 7 using PI death assay in 2019

Manufacturer	Wash Trade Name	Conc	Day 0 (%)	Day 1 (%)	Day 7 (%)
Beaumont Products	Veggie Wash	1:4	0	16	31
Biokleen	Produce Wash	1:1	1	1	1
EatCleaner	Fruit & Vegetable Wash	1:4	0	0	0
EcoLab®	Antimicrobial Fruit & Veg. Treatment	3:1	0	0	4
Envirotech	Bioside™ HS 15%	30%	0	16	14
Fit Organic	Fit® Organic™ Produce Wash	1:4	0	1	0
Frank T Ross & Sons Ltd.	Nature Clean Fruit & Veggie Wash	1:4	0	0	0
Medicinal Foods	Chaga Max. Potency Mushroom Powder	0.8%	0	1	0
Morton Coarse Kosher	Salt water	15%	0	0	9
'household salt'	Salt water (24 h)[a]	5%	0	NA	NA
'household salt'	Salt water (48 h)[a]	5%	0	NA	NA
Nutraneering	Veg Wash	1:15	2	2	11
Pace International	EpiClean	1:6	0	0	0
Pace International	Nature's Shield® 110-NCL	1:10	1	1	1
Pace International	Nature's Shield® 220-ACL	1:10	0	0	0
Pace International	Nature's Shield® 330-ALK	1:10	0	0	0
Pace International	PacFoam Plus	1:250	0	0	0
Pace International	Tri-Circ	1:250	0	3	16
Rebel Green	Fruit & Veggie Clean	1:1	0	0	0
Young Living Essential Oils	Thieves Fruit & Veggie Spray	1:4	0	1	18

All test results from incubating L3 *A. cantonensis* larvae in each treatment for 60 min and counting fluorescent L3 1 h (Day 0), 24 h (Day 1) and 7 days (Day 7) after adding PI.
[a]Salt water results based upon incubation for 24 and 48 h and fluorescent L3 counted only on Day 0 (thus Day 1 and Day 7 are not applicable).

Table 4. Wash products showing >50% mortality rate by Day 7 using PI death assay in 2019

Manufacturer	Wash Trade Name	Conc (%)	Day0 (%)	Day1 (%)	Day7 (%)	Mode of action (active ingredient)
Champon MC, Inc.	Nemitol™	1.18	1	13	98	Irritant (capsaicin & allyl isothiocyanate)
Generic	Bleach (6% sodium hypochlorite)	1.5[a]	100	100	100	Oxidizer (sodium hypochlorite)
Bio-Cide International	Keeper® Pro Post-Harvest	0.005	8	52	66	Oxidizer (chlorine dioxide)
Pace International	Field Clean	5	47	60	69	Alkaline (potassium hydroxide)
Pace International	Real Clean	0.78	33	59	71	Alkaline (sodium hydroxide)
Pace International	Sooty Mold	5.26	22	41	71	Alkaline (sodium hydroxide)
'household salt'	Salt water (24 h)[b]	10	100	NA	NA	Dehydration/Denaturation
'household salt'	Salt water (24 h)[b]	15	100	NA	NA	Dehydration/Denaturation
'household salt'	Salt water (48 h)[b]	10	100	NA	NA	Dehydration/Denaturation
'household salt'	Salt water (48 h)[b]	15	100	NA	NA	Dehydration/Denaturation
Sigma-Aldrich	Dodecylbenzene sulfonic acid, 70% (w/w) in 2-propanol	0.3	98	100	100	Surfactant

All test results from incubating L3 *A. cantonensis* in each treatment for 60 min and counting fluorescent L3 1 h (Day 0), 24 h (Day 1) and 7 days (Day 7) after adding PI.
[a]25% dilution of the 6% stock concentration.
[b]Salt water results based upon incubation for 24 and 48 h and fluorescent L3 counted only on Day 0 (thus Day 1 and Day 7 are not applicable).

10 or 15% salt solution for 24 or 48 h, we achieved 100% mortality based on the PI assay (Table 4). Therefore, our recommendations for making and using 'slug-jugs' (https://pharmacy.uhh.hawaii.edu/rat-lung-worm-activity-book) include leaving any potentially infected gastropods in 15% salt water for at least 24 h or longer to

kill *A. cantonensis* escaping from their dying hosts (Howe *et al.*, 2018).

Particular wash treatments were chosen for testing for a variety of reasons. Because angiostrongyliasis is both a newly emerging and potentially severe disease in Hawai'i (Jarvi *et al.*, 2017,

Table 5. Percent mortality in L3 *A. cantonensis* treated with NaOH to test the effects of alkaline pH on survival using PI death assay in 2019

pH	Day0 (%)	Day1 (%)	Day7 (%)
12.2	0	1	0
12.4	0	2	34
12.6	4	11	23
12.8	39	52	74
13.0	77	97	99

Table 6. Percent immobility of L3 after drying on hard surfaces to determine the effect of desiccation on *A. cantonensis* survival

Run	Dry 1 min (%)	Dry 2 min (%)	Dry 3 min (%)	Dry 4 min (%)
1	20	64	95	95
2	16	100	100	100
3	21	92	100	100
4	0	36	60	100
AVE	14.3	73.0	88.8	98.3

Study conducted in 2019.

Table 7. Percent immobility of L3 after applying 70% alcohol, then drying on hard surfaces to determine the effect of alcohol plus desiccation on *A. cantonensis* survival

Run	Dry 1 min (%)	Dry 2 min (%)	Dry 3 min (%)	Dry 4 min (%)	Dry 5 min (%)
Ethanol I	72	84	80	96	100
Ethanol II	16	80	96	100	100
AVE	44	82	88	98	100
2-propanol I	24	54	100	100	100
2-propanol II	40	88	96	100	NA
AVE	32	71	98	100	100

NA, data not available.
Study conducted in 2019.

2018; Kim *et al.*, 2018; Johnston *et al.*, 2019), there are well-warranted concerns among the general population which has led to a variety of washes being recommended based on anecdotal evidence and disseminated through social media and hearsay. Many of these recommendations involve the use of common household products or 'alternative therapies', and we judged it important to test such claims. Another broad category of treatments in our tests includes soaps and mild detergents, generally sold to the consumer market as fruit and vegetable washes, which claim to remove bacterial and viral contamination as well as waxes and pesticide residues. Yet another category of treatments includes sprays, dips and washes used in agriculture and commercial food production. Finally, we investigated treatments that, based upon our review of the literature, showed anthelminthic potential.

Based on their mode of action, our most efficacious treatments include alkaline solutions, oxidizing agents such as bleach and chlorine dioxide, alcohols, and surfactants (compounds that lower surface tension and are frequently used in detergents). Relative to these categories, our results show general patterns. Acid solutions, such as vinegar, appear to have little effect in killing *A. cantonensis* L3. Given that third-stage larvae infect their rat host by passing through the acidic environment of the host stomach on to the small intestine as part of the *A. cantonensis* life cycle (Mackerras and Sandars, 1955; Alicata, 1965), such resistance to acid solutions is predictable. On the other hand, alkaline solutions, particularly when compounded with surfactants as in the case of *Field Clean*, *Sooty Mold Cleaner* and *Real Clean* were found to be some of the most effective treatments in killing L3-stage *A. cantonensis*. These three products all contain highly alkaline ingredients (potassium hydroxide in *Field Clean*, sodium hydroxide in the others) and measure pH 12.86, 12.45 and 12.21, respectively. In testing *A. cantonensis* L3 in solutions of sodium hydroxide ranging from pH 12.2 to 13, we only found substantial larvicidal effect at pH 12.8 and higher (Table 5), suggesting that the lethality of these commercial products may be due to other constituents in addition to their alkalinity.

Some of the oxidizers tested also showed considerable larvicidal capability, particularly bleach and the chlorine dioxide product *Keeper*® *Pro Post-Harvest*. Interestingly, the minimum effective concentration of household bleach in our tests was 1.5%, which is the same as that found by Zanini and Graeff-Teixeira (2001).

Another treatment to show promise in our tests was the insect control concentrate *Nemitol*™. While *Nemitol*™ was slow to show effect compared to other treatments, it did result in close to complete mortality by Day 7. The active ingredients of *Nemitol*™ – capsaicin and allyl isothiocyanate (derived from chilies and mustard oil, respectively) – are both irritants and may function as membrane disruptors (Agricultural Marketing Service, 2014; Omolo *et al.*, 2018; Sharma *et al.*, 2019).

As a broad category, surfactants showed a variable larvicidal effect although DBSA as a 70% (w/w) solution in 2-propanol (Sigma-Aldrich) was very effective, both in terms of the speed and the thoroughness with which it killed *A. cantonensis* L3 nematodes. Surprisingly, treatments containing DBSA as an ingredient such as *PacFoam Plus*, *EpiClean* and *EcoLab*® *Antimicrobial Cleaner* generally showed little larvicidal capability.

Finally, our tests in both 2012 and 2019 showed very little larvicidal efficacy among consumer-grade fruit and vegetable washes as a class, nor on the part of botanical extracts such as those from ginger, garlic or lemongrass.

In addition to testing treatments as potential fruit and vegetable washes, we were interested in investigating other means of reducing infection from *A. cantonensis*. For example, there is considerable evidence that many gastropods shed *A. cantonensis* larvae when submerged in water (Cheng and Alicata, 1965; Howe

Table 8. Mortality rates in L3 *A. cantonensis* from refrigeration *vs* freezer using PI death assay in 2019

Temperature	Duration	Day0 (%)	Day1 (%)	Day 2 (%)	Day 3 (%)	Day 4 (%)	Day 5 (%)	Day 6 (%)	Day7 (%)
0°C	24 h	1	1	1	2	3	2	5	7
0°C	7 days	38	45	72	86	88	92	98	99
−15°C	12 h	4	75	92	47	35	36	6	5
−15°C	24 h	0	59	87	68	38	47	2	1

et al., 2019), that these larvae are capable of surviving for considerable lengths of time in water (Richards and Merritt, 1967; Howe *et al.*, 2019) and that shed *A. cantonensis* larvae are capable of infecting rats (Crook *et al.*, 1971). What is less well documented is the fate of larvae after the water they are in has dried. Thus, we were interested in determining whether *A. cantonensis* L3 would remain viable after desiccation and, if so, for how long. We found that while only an average of 14.3% of L3 were immobilized by 1 min of drying, the number of surviving larvae dropped off an additional 58%, following another minute of drying. However, the greatest variability in immobility rates also occurred at the 2 min mark; it ranged from as little as 36% to as much as 100%. These results, which showed that the majority (an average of 73%, *n* = 4) of L3 die after 2 min of drying agree with previous findings in the literature. Campbell and Little (1988) found that L3 died within minutes outside their intermediate and paratenic hosts without sufficient moisture. Richards and Merritt (1967) tested the viability of third-stage larvae 2 and 10 min after allowing their surrounding water to evaporate and found that while all larvae survived for 2 min, none survived for 10 min. Thus, it appears clear that *A. cantonensis* L3 are unable to survive desiccation for more than a few minutes on hard, impermeable surfaces such as benchtops and doors of incubators and freezers. On the other hand, larval survival might well be extended in moist environments such as sinks and food preparation areas or possibly within snail or slug mucus.

Nevertheless, even short-term survival of L3 *A. cantonensis* on hands, laboratory benches and household counter-tops presents the possibility of surface-borne transmission. Therefore, we tested the effectiveness of ethanol and 2-propanol for sanitizing such surfaces. Since alcohol is typically sprayed on, then either wiped off or allowed to dry, we repeated our tests of the effect of drying but with the addition of 2-propanol or ethanol; both applied at 70% dilution. We found that the use of either alcohol more than doubled the number of *A. cantonensis* rendered immobile in the first minute compared with drying alone, with ethanol showing somewhat faster results. Surfaces dried before using either alcohol type would probably see even more immediate reductions in larval mobility, lending support to the utility of using alcohol in reducing the risk of transmission.

Temperature also plays a role in larval survival, with refrigeration having been previously suggested as a means of reducing infection from eating foods containing either *A. cantonensis* or the closely related nematode *A. costaricensis* (Alicata, 1967; Morera, 1973; Richinitti *et al.*, 1999). In comparing the survival of *A. cantonensis* L3 in a refrigerator *vs* a freezer, we found the effect of 0°C refrigeration on larval mortality to be ambiguous; 24 h at 0°C resulted in minimal mortality whereas 7 days at 0°C did result in steadily mounting death rates, although it took the entire subsequent week to achieve close to 100% mortality. While it is problematic to compare with Richinitti *et al.* (1999), who refrigerated *A. costaricensis* at 5°C, it is notable that they found their L3 to remain infective after 17 days. Both of these results suggest that refrigeration by itself does not result in a sufficient death rate to serve as a reliable method of preventing *Angiostrongylus* infection due to ingestion of produce and undercooked food. On the other hand, freezing at −15°C, both for 12 and 24 h, clearly did result in high death rates, although again, there was a 2-day delay before achieving close to complete mortality. This agrees with the results found by Alicata (1967), who demonstrated that L3 were no longer infective when exposed to −15°C for 12 or 24 h within giant African snails (*A. fulica*) and freshwater prawns (*M. rosenbergi*). One confounding observation for our L3 frozen at −15°C in the freezer was that PI results for percent mortality peaked at Day 2, then fell dramatically in the days thereafter. Because PI fluorescence is dependent upon

intercalation within the bases of intact DNA (ThermoFisher Scientific, 2020), freezing and thawing are well known to have deleterious effects on DNA integrity (Srinivasan *et al.*, 2002). Given that we froze these larvae in water, that is, with no fixative such as alcohol, as was the case with the negative controls, and then stored them for 7 days at room temperature after being defrosted, we surmise that this drop-off in the percentage of PI staining to be due to decomposition of DNA from freeze–thaw.

There are inherent limitations to our study; for example, given the broad range of wash treatments tested in this study, interference and possible retardation of PI staining must be expected, and thus our measurements of mortality can only be approximations. For example, while the average mortality by day 7 post-treatment for all killed untreated was 95.2% and for live untreated 1.3%, as would be expected; day 7 average mortality for all killed treated was 87.5%, thus lower than expected. We found that many of the treatments we tested, while not capable of inducing mortality in live L3, did appear to actively degrade killed larvae, which may have resulted from the protein denaturation brought on by using methanol used to kill our nematodes. In addition, there are several other issues of a more practical nature that our study does not address; for example, the duration of time that a treatment has to be left in place to be effective in killing sufficient larvae to prevent infection may be too long for a typical consumer. As our tests were carried out *in vitro*, they necessarily simplify the interaction of treatments on *A. cantonensis* L3 mortality. Future experiments need to investigate real-world variables such as how gastropod mucus or fruit and vegetable surfaces might affect treatment efficacy. It is important that our results, particularly the more efficacious treatments, be validated in rat models (*in vivo*). In addition, future studies should address such questions as the extent to which our more effective treatments may affect the palatability of the fruits and vegetables with which they come in contact, and whether these treatments and the concentrations which are effective in killing *A. cantonensis* larvae are equally safe for human consumption. With further research into the mechanisms by which our most efficacious treatments kill nematodes, we hope to contribute towards the goal of developing a truly effective fruit and vegetable wash that would minimize risk or prevent infection by *A. cantonensis*.

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Conflict of interest. The authors declare there are no conflicts of interest.

Ethical standards. Not applicable.

References

Agricultural Marketing Service, USDA (2014) Technical Evaluation Report: *Allyl Isothiocyanate Crops*. Available at http://www.ams.usda.govsitesdefaultfiles mediaAllyl Isothiocyanate TR.pdf (Accessed 12 May 2020).

Alicata JE (1965) Biology and distribution of the rat lungworm, *Angiostrongylus cantonensis*, and its relationship to eosinophilic meningoencephalitis and other neurological disorders of man and animals. *Advances in Parasitology* **3**, 223–248.

Alicata JE (1967) Effect of freezing and boiling on the infectivity of third-stage larvae of *Angiostrongylus cantonensis* present in land snails and freshwater prawns. *Journal of Parasitology* **53**, 1064–1066.

Baermann G (1917) Eine einfache Methode zur Auffingung von Ankylostomum-(Nematoden)-larvan in Erdpropben. *Geneeskunding Tijdschrift voor Nederlandsch-Indie* **57**, 131–137.

Campbell BG and Little MD (1988) The finding of *Angiostrongylus cantonensis* in rats in New Orleans. *The American Journal of Tropical Medicine and Hygiene* **38**, 568–573.

Chan D, Barratt J, Roberts T, Lee R, Shea M, Marriott D, Harkness J, Malik R, Jones M, Aghazadeh M, Ellis J and Stark D (2015) The prevalence of *Angiostrongylus cantonensis/Mackerrasae* complex in molluscs from the Sydney Region. *PLoS ONE* **10**, e0128128.

Chandel SJ (2009) Saffron stain for differentiating live and dead nematodes. *Nematologia Mediterranea* **30**, 137–138.

Cheng TC and Alicata JE (1965) On the modes of infection of *Achatina fulica* by the larvae of *Angiostrongylus cantonensis*. *Malacologia* **2**, 267–274.

Cowie RH (2013) Pathways for transmission of angiostrongyliasis and the risk of disease associated with them. *Hawai'i Journal of Medicine & Public Health* **72**, 70–74.

Crook JR, Fulton SE and Supanwong K (1971) The infectivity of third stage *Angiostrongylus cantonensis* larvae shed from drowned *Achatina fulica* snails and the effect of chemical agents on infectivity. *Transactions of the Royal Society of Tropical Medicine & Hygiene* **65**, 602–605.

Eamsobhana P, Yoolek A and Yong H-S (2010) Effect of Thai 'koi-hoi' food flavoring on the viability and infectivity of the third-stage larvae of *Angiostrongylus cantonensis* (Nematoda: Angiostrongylidae). *Acta Tropica* **113**, 245–247.

Ferreira SR, Mendes TAO, Bueno LL, de Araújo JV, Bartholomeu DC and Fujiwara RT (2015) A new methodology for evaluation of nematode viability. *BioMed Research International* **2015**, 879263.

Graeff-Teixeira C, da Silva ACA and Yoshimura K (2009) Update on eosinophilic meningoencephalitis and its clinical relevance. *Clinical Microbiology Reviews* **22**, 322–348.

Hochberg NS, Park SY, Blackburn BG, Sejvar JJ, Gaynor K, Chung H, Leniek K, Herwaldt BL and Effler PV (2007) Distribution of eosinophilic meningitis cases attributable to *Angiostrongylus cantonensis*, Hawaii. *Emerging Infectious Diseases* **13**, 1675–1680.

Hollingsworth RG, Kaneta R, Sullivan JJ, Bishop HS, Qvarnstrom Y, da Silva AJ and Robinson DG (2007) Distribution of *Parmarion* Cf. *martensi* (Pulmonata: Helicarionidae), a new semi-slug pest on Hawai'i Island, and its potential as a vector for human angiostrongyliasis. *Pacific Science* **61**, 457–467.

Hollingsworth RG, Howe K and Jarvi SI (2013) Control measures for slug and snail hosts of *Angiostrongylus cantonensis*, with special reference to the semi-slug *Parmarion martensi*. *Hawai'i Journal of Medicine & Public Health* **72**, 75–80.

Howe K, Bach J, DeCoito M, Frias S, Hatch R and Jarvi S (2018) Reducing rat lungworm disease in Hawai'i through a collaborative partnership with K-12 school garden and agriculture projects. *Frontiers in Public Health* **6**, 1–9.

Howe K, Kaluna L, Lozano A, Torres Fischer B, Tagami Y, McHugh R and Jarvi S (2019) Water transmission potential of *Angiostrongylus cantonensis*: larval viability and effectiveness of rainwater catchment sediment filters. *PLoS ONE* **14**, e0209813–20.

Jarvi SI, Farias MEM, Howe K, Jacquier S, Hollingsworth R and Pitt W (2012) Quantitative PCR estimates *Angiostrongylus cantonensis* (rat lungworm) infection levels in semi-slugs (*Parmarion martensi*). *Molecular and Biochemical Parasitology* **185**, 174–176.

Jarvi SI, Quarta S, Jacquier S, Howe K, Bicakci D, Dasalla C, Lovesy N, Snook K, McHugh R and Niebuhr CN (2017) High prevalence of *Angiostrongylus cantonensis* (rat lungworm) on eastern Hawai'i Island: a closer look at life cycle traits and patterns of infection in wild rats (*Rattus* spp. *PLoS ONE* **12**, e0189458.

Jarvi SI, Howe K and Macomber P (2018) Angiostrongyliasis or rat lungworm disease: a perspective from Hawai'i. *Current Tropical Medicine Reports* **5**, 59–66.

Jarvi SI, Jacob J, Sugihara RT, Leinbach IL, Klasner IH, Kaluna LM, Snook KA, Howe MK, Jacquier SH, Lange I, Atkinson AL, Deane AR, Niebuhr CN and Siers SR (2019) Validation of a death assay for *Angiostrongylus cantonensis* larvae (L3) using propidium iodide in a rat model (*Rattus norvegicus*). *Parasitology* **146**, 1421–1428.

Johnston DI, Dixon MC, Elm JL, Calimlim PS, Sciulli RH and Park SY (2019) Review of cases of angiostrongyliasis in Hawaii, 2007–2017. *The American Journal of Tropical Medicine and Hygiene* **101**, 608–616.

Kim JR, Wong TM, Curry PA, Yeung NW, Hayes KA and Cowie RH (2018) Modelling the distribution in Hawaii of *Angiostrongylus cantonensis* (rat lungworm) in its gastropod hosts. *Parasitology* **146**, 42–49.

Kramer KJ, Posner J and Gosnell WL (2017) Role of gastropod mucus in the transmission of *Angiostrongylus cantonensis*, a potentially serious neurological infection. *ACS Chemical Neuroscience* **9**, 629–632.

Lindo JF, Waugh C, Hall J, Cunningham-Myrie C, Ashley D, Eberhard ML, Sullivan JJ, Bishop HS, Robinson DG, Holtz T and Robinson RD (2002) Enzootic *Angiostrongylus cantonensis* in rats and snails after an outbreak of human eosinophilic meningitis, Jamaica. *Emerging Infectious Diseases* **8**, 324–326.

Lv S, Zhang Y, Liu HX, Zhang CW, Steinmann P, Zhou XN and Utzinger J (2009) *Angiostrongylus cantonensis*: morphological and behavioral investigation within the freshwater snail *Pomacea canaliculata*. *Parasitology Research* **104**, 1351–1359.

Mackerras MJ and Sandars DF (1955) The life history of the rat lung-worm, *Angiostrongylus cantonensis* (Chen) (Nematoda: Metastrongylidae). *Australian Journal of Zoology* **3**, 1–21.

Morera P (1973) Life history and redescription of *Angiostrongylus costaricensis* Morera and Céspedes, 1971. *The American Journal of Tropical Medicine and Hygiene* **22**, 613–621.

Niebuhr CN, Jarvi SI, Kaluna L, Torres Fischer BL, Deane AR, Leinbach IL and Siers SR (2020) Occurrence of rat lungworm (*Angiostrongylus cantonensis*) in invasive coqui frogs (*Eleutherodactylus Coqui*) and other hosts in Hawaii. *Journal of Wildlife Diseases* **56**, 203–207.

Office of the Governor of Hawaii (2017) DOH News Release: *Hawaii Department of Health confirms two cases of rat lungworm on Hawaii Island with four probably cases*. Available at https://governor.hawaii.gov newsroomlatest-newsdoh-news-release-hawaii-department-of-health-confi rms-two-cases-of-rat-lungworm-on-hawaii-island-with-four-probable-cases (Accessed 12 May 2020).

Omolo MA, Zen-Zi W, Weih GB, Grant AH, Kamal D and David JB (2018) Comparative analysis of capsaicin in twenty nine varieties of unexplored Capsicum and its antimicrobial activity against bacterial and fungal pathogens. *Journal of Medicinal Plants Research* **12**, 544–556.

Prociv P and Turner M (2018) Neuroangiostrongyliasis: the 'subarachnoid phase' and its implications for anthelminthic therapy. *The American Journal of Tropical Medicine and Hygiene* **98**, 353–359.

Punyagupta S (1965) Eosinophilic meningoencephalitis in Thailand: summary of nine cases and observations on *Angiostrongylus cantonensis* as a causative agent and *Pila ampullacea* as a new intermediate host. *The American Journal of Tropical Medicine and Hygiene* **14**, 370–374.

Qvarnstrom Y, Sullivan JJ, Bishop HS, Hollingsworth R and da Silva AJ (2007) PCR-based detection of *Angiostrongylus cantonensis* in tissue and mucus secretions from molluscan hosts. *Applied and Environmental Microbiology* **73**, 1415–1419.

Richards CS and Merritt J (1967) Studies on *Angiostrongylus cantonensis* in molluscan intermediate hosts. *Journal of Parasitology* **53**, 382–388.

Richinitti LM, Fonseca NA and Graeff-Teixeira C (1999) The effect of temperature on mobility of *Angiostrongylus costaricensis* third stage larvae. *Revista do Instituto de Medicina Tropical de Sao Paulo* **41**, 225–228.

Sharma N, Phan HTT, Yoda T, Shimokawa N, Vestergaard MC and Takagi M (2019) Effects of capsaicin on biomimetic membranes. *Biomimetics* **4**, 17–12.

Slom TJ, Cortese MM, Gerber SI, Jones RC, Holtz TH, Lopez AS, Zambrano CH, Sufit RL, Sakolvaree Y, Chaicumpa W, Herwaldt BL and Johnson S (2002) An outbreak of eosinophilic meningitis caused by *Angiostrongylus cantonensis* in travelers returning from the Caribbean. *The New England Journal of Medicine* **346**, 668–675.

Srinivasan M, Sedmak D and Jewell S (2002) Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *The American Journal of Pathology* **161**, 1961–1971.

ThermoFisher Scientific (2020) Propidium iodide https://www.thermofisher.com/us/en/home/life-science/cell-analysis/fluorophores/propidium-iodide.html (Accessed 12 May 2020)

Tsai H-C, Lee SSJ, Huang C-K, Yen C-M, Chen E-R and Liu Y-C (2004) Outbreak of eosinophilic meningitis associated with drinking raw vegetable juice in southern Taiwan. *The American Journal of Tropical Medicine and Hygiene* **71**, 222–226.

Tsai H-C, Chen Y-S and Yen C-M (2013) Human parasitic meningitis caused by *Angiostrongylus cantonensis* infection in Taiwan. *Hawai'i Journal of Medicine & Public Health* **72**, 26–27.

Waugh CA, Shafir S, Wise M, Robinson RD, Eberhard ML and Lindo JF (2005) Human *Angiostrongylus cantonensis*, Jamaica. *Emerging Infectious Diseases* **11**, 1977–1978.

Yeung NW, Hayes KA and Cowie RH (2013) Effects of washing produce contaminated with the snail and slug hosts of *Angiostrongylus cantonensis* with three common household solutions. *Hawai'i Journal of Medicine & Public Health* **72**, 83–86.

Zanini GM and Graeff-Teixeira C (2001) Inactivation of infective larvae of *Angiostrongylus costaricensis* with short time incubations in 1.5% bleach solution, vinegar or saturated cooking salt solution. *Acta Tropica* **78**, 17–21.