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In vitro study of disinfectants on the embryonation and survival of *Toxascaris leonina* eggs

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Research Paper

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Abstract

The effect of six available and commercial disinfectants on the embryonation and larval development of *Toxascaris leonina* eggs was studied. Dettol® and Virkon® both induced a 100% reduction in larval development ($P \leq 0.05$). Dettol® resulted in deformed eggshells and a halt in embryonal development at 1 week post exposure. All Virkon®-treated eggs showed an early embryonic lysis 24 h post exposure. TH4+ and 70% ethanol both significantly ($P \leq 0.05$) affected larval development, with 58.8 and 85.8% reduction, respectively. Neither sodium hypochlorite nor phenol significantly affected larval development (2.8 and 21.0%, respectively). Sodium hypochlorite treatment caused a visible decortication of the eggshell; however, phenol-treated embryonated *Toxascaris* eggs appeared more or less morphologically normal. In conclusion, the disinfectants tested induced variable degrees of decortication and suppression of larval development. Virkon®S was the most effective disinfectant against *Toxascaris* eggs, suggesting that it is the most advisable one to use. To the best of our knowledge, this is the first report of the use of Virkon®S as an ovicide and/or larvicide of helminths, particularly *Toxascaris leonina*.

Introduction

Stray cats (*Felis catus*) harbour a variety of parasitic nematodes of both veterinary and zoonotic significance (Okulewicz *et al.,* 2012; Wright *et al.,* 2016). Among these nematodes are *Toxascaris leonina*, *Toxocara cati* and *Toxocara canis*, in the family Ascarididae (Urquhart *et al.,* 1996; Gibbons *et al.,* 2001; Pawar *et al.*, 2012), which are important intestinal helminths (Parsons, 1987). *Toxocara cati* and *T. canis* are more pathogenic for kittens, puppies and children, with the potential for visceral larva migrans (VLM) and ocular larva migrans (OLM) in the latter. Potentially, *T. leonina* may also emerge as a zoonotic agent causing VLM in humans (Prokopic & Figallova, 1982; Kim & Huh, 2005; Tarsitano *et al.*, 2010; Okulewicz *et al.,* 2012).

Unlike *Toxocara*, where infective larvae migrate through the lungs, the entire life cycle of *T. leonina* takes place in the gut of the definitive host (dog, cat or other carnivore) and the eggs are shed with the faeces of the host. Outside the host, infective second-stage larvae (L2) develop inside the eggs within approximately 1 week under optimal climatic conditions (Soulsby, 1982). Eggs may remain infective for several months in cold and humid climates, but die rapidly in dry and hot seasons. Both felids and canids become infected by ingesting infective eggs in contaminated food or water (Labarthe *et al.,* 2004; Dalimi *et al.,* 2006; Dubna *et al.,* 2007; Reperant *et al.,* 2007; Itoh *et al.,* 2011). The second-stage larvae hatch from the eggs in the intestine of definitive hosts and penetrate the gut wall, where they undergo two cycles of moulting. The larvae then return to the gut lumen, moulting to adults, and become sexually mature.

When paratenic rodent hosts ingest *T. leonina* eggs, the hatched larvae migrate through the tissues and may persist in the tissues for long periods of time (Wright, 1935; Epe, 2009; Traversa, 2012). When another paratenic host ingests the infected rodent tissues, the infective larvae (L2) migrate to different organs, where they undergo encystment, but development to adult worms will not take place. When a definitive host then ingests such infected rodents, the tissue cysts disseminate the infective larvae (L2), which move directly to the gut where they complete development to adult worms (Sprent, 1959).

Toxascaris leonina eggs are colourless and oval, with a maximum size of 85 × 75 µm, and possess a smooth shell about 2 µm thick with no striations or albuminous coat (Dunn, 1978; Gonzales *et al.,* 2007). The eggs are resistant to both climatic and chemical exposure, which allows them to remain viable in the environment for long periods. Ambient temperature,

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humidity and soil features are the predominant factors affecting the duration until the eggs moult into the larval stages (Sommerfelt *et al.,* 2006). *Toxascaris leonina* eggs are able to adapt to various climatic conditions, such as temperature, and are able to embryonate in the dark (Okoshi & Usui, 1968; Feney-Rodriguez *et al.,* 1988; Anderson, 2000).

As a part of control programmes for zoonotic agents, commercial disinfectants are widely used. Numerous disinfectants are available, categorized on the basis on their active ingredients and ability to kill various micro-organisms (Zeweil *et al.,* 2015). Currently, more than 5000 antimicrobial products are used to destroy or suppress the growth of pathogens. Alcoholic compounds are fast acting and highly effective against both bacteria and *T. canis* eggs (Aycicek *et al.,* 2001; Turpin, 2013). Sodium hypochlorite has wide antibacterial activity (Grooms, 2003) and is effective against *T. canis* eggs (Aycicek *et al.,* 2001). Virkon®S has broad-spectrum disinfectant activity against viruses, bacteria and some fungi (Scott & Swetnam, 1993; Gasparini *et al.,* 1995). To date, there is little information regarding its antiparasitic effects, although activity against *Gyrodactylus salaris* in salmon has been reported (Koski *et al.,* 2016).

There is a scarcity of information regarding the use of disinfectants against ascarids of pets, particularly *T. leonina* eggs, in Egypt, although comparisons among a few disinfectants against infective *T. cati* and *T. canis* eggs have been reported in Turkey and Spain, respectively (Aycicek *et al.,* 2001; Morrondo *et al.,* 2006). Therefore, the present study was designed to evaluate the efficacy of six commercially available disinfectants against the embryonation and larval development of *T. leonina* eggs obtained from adult female worms in necropsied cats, to consider whether they might be useful in reducing the zoonotic potential of worms of pets.

Materials and methods

Collection of eggs

Toxascaris leonina eggs were obtained from adult female worms recovered from the small intestines of naturally infected cats. The eggs were either collected by incubating gravid female worms in 0.15 M sodium chloride at 37°C for 24 h to allow egg deposition (Barriga & Omar, 1992) or by washing gravid female worms with normal saline and then grinding the worms to release the eggs from the uteri. The eggs were then sieved, washed and precipitated several times using 1% formol-saline as described previously (Sabry, 1999).

Exposure of unembryonated eggs to disinfectants

For the *in vitro* assay, approximately 7000 unembryonated *T. leonina* eggs were pooled and divided into seven equal groups of approximately 1000 eggs, which were treated in plates as follows. (1) Control: eggs were incubated in 6 ml 1% formol-saline solution to assess normal embryonic development. (2) Sodium hypochlorite: eggs were incubated in 6 ml solution containing 2.5 ml sodium hypochlorite 0.5% + 2 ml egg suspension + 1.5 ml 1% formol-saline. (3) Ethanol: eggs were allowed to settle, centrifuged, the supernatant was removed and then the eggs were incubated in 6 ml 70% ethanol. (4) Virkon®S (DuPont, Wilmington, USA; active ingredients potassium peroxymonosulphate 21.41%, sodium chloride 1.50% and other ingredients 77.09%): eggs were incubated in 6 ml of diluted Virkon (5 g/l distilled water or 30 mg/6 ml distilled water). (5) TH4+ (quaternary ammonium compounds and glutaraldehyde): eggs were incubated in 6 ml of diluted TH4+ (1 ml/200 ml distilled water or 30 µl/6 ml distilled water). (6) Phenol (carbolic acid): eggs were incubated in 6 ml of diluted phenol (100 ml/20 l distilled water or 30 µl/6 ml distilled water). (7) Dettol® (chloroxylenol): eggs were incubated in 6 ml of diluted Dettol (25 ml/l distilled water or 130 µl/6 ml distilled water).

All plates were incubated for 7–10 days at 28°C and 80% relative humidity, and shaken daily to allow oxygenation.

Exposure of embryonated eggs to disinfectants

Approximately 7000 unembryonated eggs were allowed to undergo embryonation in 1% formol-saline at 28°C and 80% relative humidity with daily agitation, as above. The embryonated eggs were divided into seven groups of approximately 1000 eggs and then exposed to the treatments described above for 7–10 days.

Evaluation of effects on larval development

Dishes were examined daily to check for alterations in the eggshell and survival of larvae. After 7–10 days, the percentage of both non-developed and larvated eggs was recorded. The reduction in larval development for the treated groups was determined according the equation:

$$\frac{(\text{Larvated eggs of the control group}) - (\text{Larvated eggs of the treated group})}{\text{Larvated eggs of the control group}} \times 100$$

Statistical analysis

Data were analysed statistically using Statistical Package for Social Science (SPSS for Windows (IBM), version 22; SPSS Inc., Chicago, Illinois, USA) to determine if variables differed among treatments. Data were analysed using analysis of variance (ANOVA) tests and subsequent Duncan's multiple range test to determine the differences of means. Results were expressed as means ± SD. Probability values of less than 0.05 ($P \leq 0.05$) were considered to be significant.

Results

The effects of disinfectants on unembryonated eggs

Both sodium hypochlorite and phenol had non-significant effects on unembryonated toxascarid eggs compared to the control untreated eggs (table 1), resulting in 2.8 and 21.0% reductions in larval development, respectively.

TH4+ and ethanol had significant ($P \leq 0.05$) effects on unembryonated toxascarid eggs compared to the control untreated eggs (table 1), resulting in 58.8 and 85.8% reductions in larval development, respectively (table 1).

Both Dettol® and Virkon®S treatment resulted in 100% reduction in larval development ($P \leq 0.05$) (table 1). Virkon®S induced early embryonic lysis in treated eggs after 24 h (fig. 1).

The effects on embryonated eggs

Concomitant with the effects of disinfectants on the unembryonated eggs, the tested disinfectants induced variable degrees of effects on the eggshells and/or larvae. Sodium hypochlorite elicited a marked decortication of treated eggs. The eggs of the

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Table 1. The effect of various disinfectants on the embryonation of *Toxascaris leonina* eggs.

Group	Percentage of unembryonated eggs (mean ± SD)	Percentage of larvated eggs (mean ± SD)	Reduction of larval development (%)
Control untreated	$60.85^{a} \pm 10.33$	$39.14^{c} \pm 10.33$	0
Sodium hypochlorite	$61.93^{a} \pm 9.68$	$38.06^{c} \pm 9.68$	2.77
Phenol	$69.07^{a} \pm 3.4$	$30.92^{c} \pm 3.41$	21.01
TH4+	$83.86^{b} \pm 3.38$	$16.13^{b} \pm 3.38$	58.79
Ethanol 70%	$94.44^{c} \pm 3.57$	$5.55^{a} \pm 3.57$	85.79
Dettol	$100.00^{c} \pm 0.00$	$0.00^{a} \pm 0.00$	100
Virkon®S	$100.00^{c} \pm 0.00$	$0.00^{a} \pm 0.00$	100

Superscripts of the same letters in the same column mean non-significant findings.
Superscripts of different letters in the same column mean significant ($P \leq 0.05$) findings.

phenol-treated group appeared more or less morphologically normal. TH4+ and 70% ethanol had no apparent effect on the larvae from treated eggs.

Dettol®-treated eggs showed a deformity in the shell 1 week post exposure. Virkon®S-treated eggs showed a complete degeneration of most of the larvae, and loss of their features 24 h post exposure (table 1 and fig. 2).

Discussion

The present study examined the effects of various commercially available disinfectants on embryonation, larval development and eggshell structure of *T. leonina* eggs. Although the disinfectants tested serve as bactericidal, virucidal and fungicidal agents, their effect on *T. leonina* eggs had not yet been investigated. In our study, differences among the various disinfectants in their effects on *T. leonina* eggs were observed.

Sodium hypochlorite and ethanol have considerable efficacy against infective eggs of *T. canis* and, therefore, veterinarians recommend these disinfectants for dog kennels, cages and dog houses (Morrondo *et al.*, 2006; Verocai *et al.*, 2010) due to their effectiveness, low cost and ready availability. Although it would seem that they might be effective against *T. leonina* eggs, since both *T. canis* and *T. leonina* belong to the family Ascarididae,

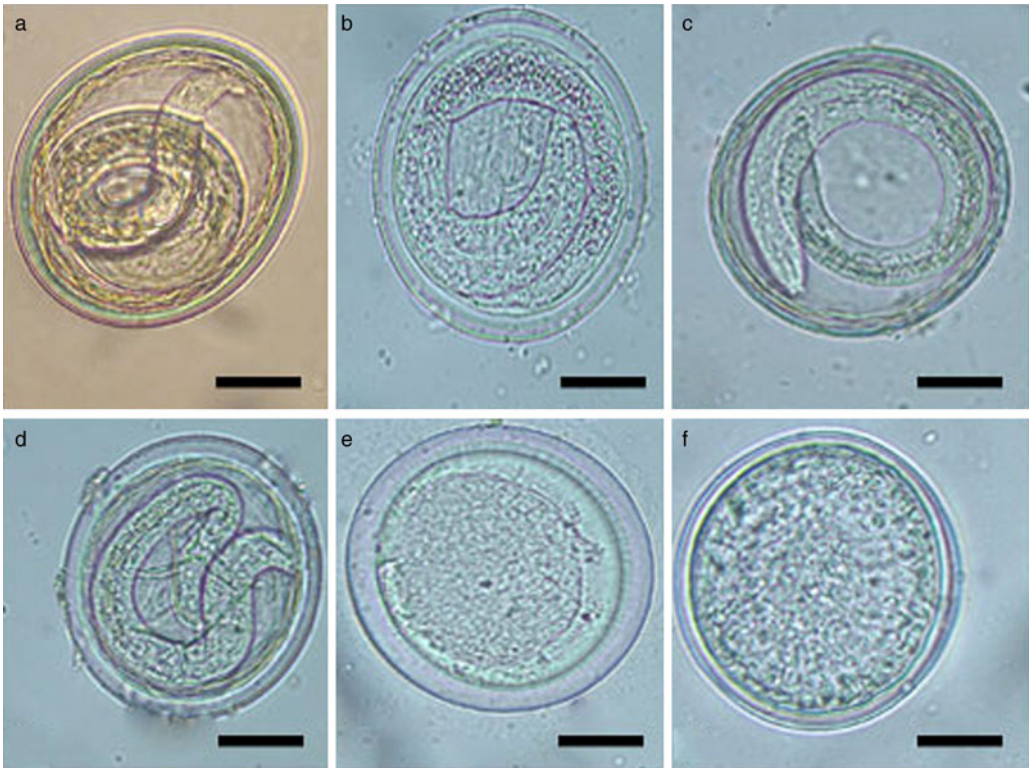

Fig. 1. Morphology of unembryonated eggs from *Toxascaris leonina* adult females obtained from necropsied cats and exposed to various disinfectants with incubation period 7–10 days. (a) An untreated egg (control). Scale bar: 25 µm. (b) An egg treated with 70% ethanol. Note the clearance of the eggshell and slight degeneration of the larval structure. Scale bar: 25 µm. (c) An egg treated with phenol. Both the eggshell and the larval structure appeared more or less normal. Scale bar: 25 µm. (d) An egg treated with TH4+. Note the thinning of the eggshell and compression of the contained larva. Scale bar: 25 µm. (e) An egg treated with Dettol. Note the great disappearance of the eggshell and cessation of embryonal development. Scale bar: 25 µm. (f) An egg treated with Virkon®S. Note the absolute decortication of the egg associated with a complete cessation and atrophy of the embryonal cells. Scale bar: 25 µm.

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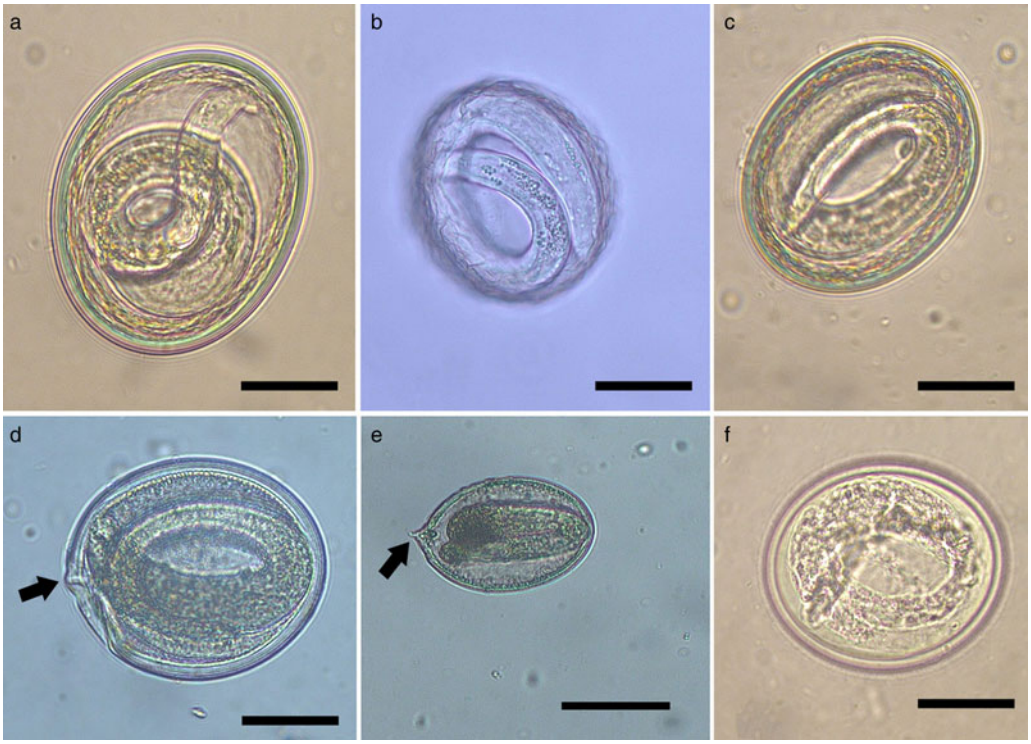

Fig. 2. Morphology of embryonated eggs from *Toxascaris leonina* adult females obtained from necropsied cats and exposed to some disinfectants. (a) An intact egg, not treated with disinfectants, showing a well-defined eggshell and fully formed larva. Scale bar: 25 µm. (b) An egg treated with sodium hypochlorite. Note the complete disappearance of the outer layer of eggshell with a still intact larva. Scale bar: 25 µm. (c) An egg 24 h post treatment with Dettol. Note a slight thinning of the eggshell together with more or less normal larval structure. Scale bar: 25 µm. (d) An egg 7 days post treatment with Dettol. Note the prominent destruction (arrow) of the eggshell. Scale bar: 25 µm. (e) An egg 7 days post treatment with Dettol. Note a prominent projection (arrow) of the eggshell. Scale bar: 50 µm. (f) An egg treated with Virkon®S. A distinct thinning/disappearance of the eggshell associated with a marked degeneration of the larva can be seen, denoting the effect of the disinfectant. Scale bar: 25 µm.

sodium hypochlorite was not effective in reducing *T. leonina* larval development in the current study. However, it did cause a marked decortication in *T. leonina* eggs. On the other hand, ethanol did induce a significant reduction in larval development. On the contrary, Oh *et al.* (2016) showed that sodium hypochlorite suppressed the development of *Ascaris suum* eggs within 5 min of exposure, whereas ethanol did not inhibit embryonation of decorticated eggs even 1 h post exposure.

Ethanol is known to be effective against most bacteria, viruses and fungi, with a few reports on inhibition of sporulation (Yasuda-Yasuki *et al.*, 1978; McDonnell & Russell, 1999). The antimicrobial activity of ethanol is optimal at a concentration of 60–90%. Ethanol causes proteins in the cell wall to denature, leading to membrane damage, interrupted metabolism and, eventually, cell lysis (Morton, 1983). Similarly, Dettol® (chloroxylenol) is mainly used as an antibacterial, acting on cell surfaces (Russell & Furr, 1977). In the current study, Dettol® suppressed embryonal development, with a significant reduction in larval development. This might be due to the germicidal effect of Dettol® on *T. leonina* eggshells. TH4+ is a widely used disinfectant with bactericidal, virucidal and fungicidal action. While demonstrating a significant reduction in *T. leonina* larval development, TH4+ was not as effective as ethanol, Dettol® or Virkon®S in reducing larval development in our study.

In the current study, phenol-exposed *T. leonina* eggs did not show a significant reduction in larval development and the eggs appeared more or less normal externally, despite some embryonal degeneration. This might be attributed to phenol, a protoplasmic poison, inducing coagulation of protoplasmic organelles with irreversible cell damage (Sharma, 1997; McDonnell & Russell, 1999).

Virkon®S is a unique cleaning and disinfecting agent used in all animal and industrial purposes as a bactericide, virucide and fungicide (Møretrø *et al.*, 2009). Interestingly, in this study, Virkon®S caused complete embryonic death together with an absolute cessation of larval development in mature *T. leonina* eggs, suggesting that it has lethal effects on both eggshell and embryonic cells. To the best of our knowledge, this is the first report revealing both the ovicidal and larvicidal activities of the commercial disinfectant Virkon®S against *T. leonina* eggs.

Okoshi & Usui (1968) determined that the development of *T. leonina* eggs was affected by temperature and climatic conditions. When eggs were exposed to −15°C, they remained viable for 40 days, and when exposed to 25°C, approximately all eggs completed development to the infective stages. Thus, the effectiveness of any particular disinfectant against *T. leonina* is likely to be influenced by these factors as well.

In conclusion, our study shows that several types of available and commercial disinfectants could be used to stop embryonation and/or larval development of *T. leonina* eggs. However, in practice, Virkon®S or Dettol® might outperform others, based on the results of this current study. Their usage is highly recommended against other potentially zoonotic parasites of pets, particularly helminths such as *T. cati* and *T. canis*. In the future, multidisciplinary studies should be undertaken to determine the pharmacology of Virkon®S as an antiparasitic agent.

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