Effect of reagents used during detection and quantification of *Ascaris suum* in environmental samples on egg viability
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**ABSTRACT**

Soil-transmitted helminths (STHs) are a major health concern globally. Infection is mostly through contact with contaminated water, food or soil. Therefore to break the cycle of viable transmission STH eggs must be quantitatively detected in the environment. The effect of different reagents on the viability of *Ascaris suum* eggs during laboratory detection and quantification was assessed and different incubation solutions compared. Sulphuric acid gave a slightly higher recovery percentage of viable eggs (91.2%) than distilled water (90.0%) and 0.5% formalin (87.6%), although the difference was not statistically significant (*p* > 0.05). Acetoacetic acid, ethyl acetate, ammonium bicarbonate, zinc sulphate, magnesium sulphate and Tween 80, are reagents widely used in test protocols for the detection and quantification of STH eggs. Eggs were exposed to these reagents for different time durations. Acetoacetic acid resulted in the highest loss of viability (3.4 ± 0.7% viable), while magnesium sulphate resulted in the least effect (88.5 ± 1.2% viable). In conclusion the use of the selected reagents in the detection of these eggs was found to affect the viability of exposed eggs, especially during prolonged exposures. Therefore we recommended that eggs be exposed for ≤5 minutes, to reduce the risk of viability loss.

**Key words** | *Ascaris suum*, inactivation, sludge, soil-transmitted helminths, viability, wastewater

**INTRODUCTION**

In soil-transmitted helminth (STH) infection endemic regions it is estimated that wastewater may contain up to ~3,000/L (Kamizoulis 2008; Mara & Sleigh 2010) of STH eggs, while documented counts in faecal sludge from public toilets are between 2,500 and 60,000 eggs/L (Yen-Phi et al. 2010). From septic tanks the concentrations are reported to be from 600 to 16,000 eggs/L (Yen-Phi et al. 2010). Trönnberg et al. (2010) assessed the concentrations of STHs in the faecal vaults of urine-diverting (UD) toilets in the KwaZulu Natal Province of South Africa. Counts varied from below detection limit to a maximum of 1,425 eggs per gram (EPG) of faeces for *Ascaris lumbricoides*, 147 EPG for *Trichuris trichiura* and 703 EPG for *Taenia* spp. These STH eggs can survive for a long period of time in the environment, where fertilized eggs of *Ascaris* spp. for instance have been reported to survive for up to 7 years under favourable temperatures of 25 °C and humidity greater than 55% (WHO 2015). Due to the highly varying numbers, the detection and quantification of STH eggs is important for the determination of health risk due to exposure during the collection, conveyance, treatment and possible reuse of faecal waste. It is also important in the validation of wastewater treatment technologies (Peterson et al. 2011).

Ingestion of fertilized eggs of STHs may result in asymptomatic diarrhoea (Brownell & Nelson 2006). The most common STH infection is ascariasis, infecting 771.7–891.6 million people worldwide (Strunz et al. 2014), with the majority of these infections occurring in tropical and sub-tropical countries (Stolk et al. 2016). Although infection with STHs is not lethal it may result in impairment of physical and cognitive development (Naidoo et al. 2016).

The greatest risk to public health in relation to STH infections is the reuse of wastewater, sludge, compost etc.
in agriculture (Habbari et al. 1999; Amahmid & Bouhoun 2005; Ensink et al. 2005). To ensure public safety several international and national agencies have recommended guidelines for wastewater reuse in agriculture (USEPA 1992; WHO 2006). These guidelines have been adopted by various national and regional bodies, such as the Union of National Associations of Water Suppliers and Waste Water Services from countries within the EU (Angelakis & Bontoux 2001). The guidelines account for not only the quality for reuse but also the treatment steps needed to achieve the desired quality. The World Health Organization (WHO 2006) recommends ≤1 helminth egg per litre for unrestricted agriculture as against no detectable helminth egg per litre of irrigation water by the US Environmental Protection Agency (USEPA 1992). To determine the suitability of wastewater or sludge etc. for reuse or the validation of treatment technologies there is the need for accurate determination of viable STH eggs in the various samples (wastewater, sludge etc.). However, there are a variety of methods, with the major ones being the United States Environmental Protection Agency (USEPA) (Schwartzbrod 1998) and the World Health Organization (WHO) methods (Ayers & Mara 1996), or variants of these (Amoah et al. 2017).

The variety in the techniques used is influenced by several factors, with key among these being the variability in the sample matrices, reagents available and personal preferences of the researcher (Mes 2005). Sludge, compost and UD wastes are often blended with a detergent solution, which could be Tween 20 or Tween 80, ammonium bicarbonate or others with the intention to increase the separation of the egg from solid materials in the samples (Zenner et al. 2002; Trönnberg et al. 2010). In addition to these detergents, there are several other reagents used with different purposes. For instance flotation solutions, such as zinc sulphate, magnesium sulphate and sodium chloride, are used to further separate the eggs from other particulate matter based on differential flotation of these materials (Maya et al. 2006; Rosa Xavier et al. 2010). A phase extraction step is included in some of the methods for the removal of lipid-soluble and ether-absorbing material from the samples (Rude et al. 1987; Nelson & Darby 2001). Diethyl ether, ethyl acetate and acetoacetic buffer are commonly used for this purpose.

This variety of reagents used may inadvertently affect the viability of the STH eggs. Long-term exposure to ZnSO₄ has been shown to be toxic to STH eggs (Gaspard et al. 1996); in addition, soaking eggs in MgSO₄ overnight may also inactivate embryonated eggs (Smith 1991). One per cent of formalin is mainly used as incubation solution for STH eggs but Oksanen et al. (1990) stated that it retarded their development as compared to water or 0.1 N H₂SO₄ (Nelson & Darby 2001). Phase extraction with acid-alcohol and diethyl ether also decreases egg viability (Nelson & Darby 2001). Inactivation of eggs may be due to the damage of the egg shell of the parasites, which protect the egg and enhance the survival ability of STHs. A change in the permeability of the cell-wall layer of eggs may result in the loss of viability (de Souza et al. 2011).

Despite the perceived effect of different chemicals/reagents on the viability of helminth eggs, few quantitative assessments of the effects of the various reagents have been made. As part of an ongoing project aimed at developing a uniform method for the detection and quantification of STH eggs in environmental samples, a series of experiments were carried out to determine the effect of selected reagents (commonly used) on the viability of STH eggs, using Ascaris suum eggs as a surrogate.

**METHODOLOGY**

**Preparation of stock solution**

*Ascaris suum* eggs were purchased from Excelsior Sentinel Inc. (Ithaca, NY, USA). The eggs were collected from the intestinal contents of infected pigs and concentrated through a series of sieves. A working egg solution with approximately 1,000 eggs/mL was prepared by suspending the eggs in distilled water. Stock solution of eggs was stored at 4°C to prevent any egg development. Individual samples were prepared by placing 1.0 mL of the working solution in 50-mL centrifuge tubes.

**Initial screening and comparison of incubation solutions**

Viability of the eggs was determined, after initial screening, to ascertain their stage of development. Initial viability was determined by incubation in three different solutions: distilled water, 0.5% formalin and 0.1 N sulphuric acid. Eggs were viewed microscopically (×40) after 28 days of incubation and categorized as potentially viable or non-viable based on morphology. Undeveloped (at different cell stages) and embryonated eggs with a visible motile larva were considered potentially viable. Eggs with broken egg shell, visible internal globules and necrotic or immotile larvae were considered to be potentially non-viable (Moodley et al. 2008). Percentage viability was calculated for each incubation solution by dividing viable eggs by the...
RESULTS AND DISCUSSION

Effect of different incubation solutions on viability of *A. suum* eggs

Initial microscopic analysis of the eggs showed that the eggs generally were in the pre-larval stage prior to any...
experiment. After 28 days of incubation viable eggs were quantified based on stated criteria (‘Methodology’ section). Figures 1 and 2 show the stages of egg development. Incubation with the three solutions (distilled water, 0.5% formalin and 0.1 N sulphuric acid) gave viability of between 90.0 and 91.2% of the analysed eggs. The highest percentage of viability after the 28 days of incubation was recorded with 0.1 N sulphuric acid (91.2 ± 0.6%), followed by distilled water (90.0 ± 3.7%) and the least percentage viability was recorded with 0.5% formalin solution (87.6 ± 0.5%) (Figure 3). The difference in the percentage viabilities between these three solutions was not statistically

Figure 1 | Images of potentially viable eggs at different stages of development. (a) Egg at the one cell stage; (b) decorticated egg undergoing embryonation; (c) decorticated egg at multicellular stage (>7 cells); (d) eggs with visible larvae (indicated by the arrow).

Figure 2 | Images of non-viable eggs after incubation. (a) Decorticated eggs with visible globules (indicated by arrows); (b) dead egg.
Effect of reagents on viability of A. suum eggs spiked into distilled water

Exposure of A. suum eggs to the different reagents had effect on the viability of the egg as determined by incubation. Out of the nine reagents or combination of reagents, the highest negative effect in viability was observed due to exposure to acetoacetic acid, with only $3.4 \pm 1.68\%$ of the eggs remaining viable, followed by combination of acetoacetic acid and ethyl acetate ($15.2 \pm 14.4\%)$. Ethyl acetate as well as the combination of acetoacetic acid and formalin also gave statistically significant loss in viability as compared to the mean viability determined by incubation in sulphuric acid without any treatment (Table 1). Statistically, ethyl acetate, acetoacetic acid, combination of ethyl acetate and acetoacetic acid and then the combination of acetoacetic acid and formalin resulted in mean percentage viabilities that were significantly lower than the mean percentage viability of untreated (un-exposed) eggs (Table 1). This might be due to the effect of the acid on the lipid layer of the eggs, thereby increasing permeability of the egg shell, which subsequently led to the inactivation (Clarke & Perry 1988). Although formalin and ethyl acetate did not significantly affect the viability of the eggs as individual components, their combinations with acetoacetic acid led to a significant reduction in viability. However, the individual effect of acetoacetic acid was higher. Table 1 shows that the reagents used as detergents for the dissociation of eggs from particulate matter (Tween 80 and ammonium bicarbonate) as well as solutions used for the flotation steps (magnesium sulphate and zinc sulphate) did not significantly affect the viability of the A. suum eggs. However, reagents used during the phase extraction step (ethyl acetate and acetoacetic acid) significantly affected the egg viability. These phase extraction reagents are mostly lipid-soluble and ether-absorbing chemicals and their negative effect might be due to the degradation of the lipid layer of the egg (which is the last defence for the eggs), thereby inactivating them.

The dose of a toxic substance that an organism is exposed to, as well as the duration of exposure to the said substance, is very important in the determination of the dose–response relationships (Nelson & Darby 2001). This is used here to evaluate the inactivation of A. suum eggs as a result of prolonged exposure to these reagents. With a fixed concentration of these reagents the duration of exposure is the main factor in the inactivation of A. suum eggs. It was found that prolonged exposure had a destructive effect on the viability of the A. suum eggs. There was a negative correlation for ethyl acetate, acetoacetic acid,
magnesium sulphate, Tween 80 and then the combination of ethyl acetate and acetoacetic acid as well as the combination of acetoacetic acid and formalin with percentage viability (shown in Table 2). Although Tween 80 and magnesium sulphate did not show any significant reduction in mean percentage viability, an increase in the duration of exposure results in a reduction in viable eggs. This makes it necessary to reduce the exposure of eggs to the reagents to the shortest time possible. The best correlation between duration of exposure and inactivation was found for the combination of acetoacetic acid and ethyl acetate, probably due to the individual effect of acetoacetic acid, as treatment with this reagent alone resulted in the highest loss of viability, with only 3.4 ± 1.68% of eggs remaining viable. This is confirmed by the correlation results presented in Table 2.

Although ethyl acetate recorded a mean percentage of viable eggs of 73.4 ± 8.3%, which was not significantly different from the untreated eggs (determined during the initial screening phase with sulphuric acid, formalin and distilled water), an increase in the duration of exposure led to a greater reduction in viability. Increase in duration of exposure correlated negatively with percentage viability ($/C0.63$), which was the highest negative correlation for all the reagents studied. This shows that although exposure for short periods ($/C20$ minutes) might not affect the viability of exposed eggs, prolonged exposure ($/C21$ 30 minutes) might result in the loss of viability. The loss in viability might be due to the increase in permeability during the hatching of the eggs. Permeability of egg shells increases during hatching, which might be the reason for the increase in the inactivation effect of the reagents with prolonged duration (Clarke & Perry 1988). A similar relationship between prolonged exposure and percentage viability was seen for exposure to the combination of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Detailed descriptive analysis of the mean percentage of viable eggs for each treatment ($N = 200$)</th>
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<tbody>
<tr>
<td></td>
<td>Ethyl acetate</td>
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<tr>
<td>Minimum</td>
<td>60.8</td>
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<tr>
<td>25% percentile</td>
<td>67.5</td>
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<tr>
<td>Median</td>
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<td>75% percentile</td>
<td>80.4</td>
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<tr>
<td>Maximum</td>
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<tr>
<td>Mean (SD)</td>
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<td>95% confidence interval of mean</td>
<td>64.1–84.7</td>
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<td>t-test*</td>
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*p value < 0.05 was considered as statistically significant.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Correlation between duration of exposure and the mean percentage viability</th>
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<tbody>
<tr>
<td></td>
<td>Time</td>
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<tr>
<td>Time</td>
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</tr>
<tr>
<td>Ethyl acetate</td>
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</tr>
<tr>
<td>Acetoacetic acid</td>
<td>−0.47</td>
</tr>
<tr>
<td>Formalin</td>
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<tr>
<td>Ammonium bicarbonate</td>
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</tr>
<tr>
<td>Zinc sulphate</td>
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<tr>
<td>Magnesium sulphate</td>
<td>−0.18</td>
</tr>
<tr>
<td>Tween 80</td>
<td>−0.28</td>
</tr>
<tr>
<td>Acetoacetic acid + ethyl acetate</td>
<td>−0.72</td>
</tr>
<tr>
<td>Acetoacetic acid + formalin</td>
<td>−0.61</td>
</tr>
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acetoacetic acid and formalin, with acetoacetic acid seen as the main contributor to the effect. This is based on the correlation between the effects of the combined treatment against treatment with the reagents individually (Table 2). Although the exposure to magnesium sulphate did not show a significant loss in viability as compared to the untreated eggs, prolonged exposure negatively correlated with the percentage of viability (−0.53) which means that as duration of exposure increases there is a decrease in the percentage of eggs that retain their viability. Magnesium sulphate is used as a flotation solution in most literature as recommended by the USEPA method (USEPA 1999), but there is no maximum time of exposure recommended. In this case the duration of exposure of samples (which presumably contain the *Ascaris* spp. eggs or other helminths) is left to the discretion of the researcher. The results obtained indicate that with prolonged exposure there is a decrease in the number of viable eggs recovered, thereby resulting in false results. The same trend was seen for Tween 80, but its influence was very weak (−0.0027). Acetoacetic acid exposure for 5 minutes (the minimum time of exposure studied) resulted in only 5.4 ± 1.7% of the eggs remaining viable and the prolonged exposure had a negative correlation with the percentage of viable eggs recovered. Based on this the use of acetoacetic acid or reagents that contain this chemical should not be recommended as it results in the loss of over 95% of viable eggs, which might result in inaccurate results if viability is determined after exposure of eggs.

For every 1 minute increase in duration of exposure, the viability of the exposed *A. suum* eggs reduces. The greatest decrease in percentage viability with every 1 minute increase in exposure time was for the combined exposure to acetoacetic acid and ethyl acetate (−0.72), followed by exposure to acetoacetic acid (−0.47), then a combined exposure to acetoacetic acid and formalin (−0.61). The reduction in percentage viability for the six treatments with negative correlation between duration of exposure and reduction in percentage viability is shown in Figures 4–6.

The exponential regression model was the best fit model for treatments with acetoacetic acid and the combined treatment with acetoacetic acid and ethyl acetate (Figure 4). Exponential models are best in describing a process where the rate of an event occurring depends on the amount present (Shestopaloff 2010). Therefore inactivation of *A. suum* eggs with prolonged exposure to these two reagents (acetoacetic acid and the combination of acetoacetic acid and ethyl acetate) may depend on the amount of these remaining at a given time. This exponential rate of inactivation of the eggs might be due to reduction in the potency of the reagents as a result of evaporation or other unknown factors. Polynomial regression models were found to be the best fit model for treatments with ethyl acetate (cubic), magnesium sulphate (fourth order), Tween 80 (cubic), and combined treatments with acetoacetic acid and formalin (cubic). Polynomial models best predict the relationship between variables based on data present but they have poor interpolatory properties (Shestopaloff 2010). However, none of the regression models fitted for the data as explained above were statistically significant at 95% confidence level.

**Inactivation of eggs spiked into wastewater and sludge**

The percentage of viability for eggs spiked into the wastewater samples as compared to the eggs spiked into the distilled water
was not significantly different (at 95% confidence interval), except for exposure to acetoacetic acid ($p$ value was 0.0001). The eggs in wastewater would thus be affected in a similar manner to eggs in the distilled water. Acetoacetic acid was found in the previous sections to result in the highest loss of viability (Table 1), but the results from the wastewater spiking tests indicate that its effect might be reduced by the properties of the wastewater (Table 3). Wastewater contains a lot of particles (Bougrier et al. 2003) that might have had a shielding effect against the impact of this reagent. In addition there might be components within the wastewater that may be antagonistic to the effect of acetoacetic acid. For instance, wastewater from domestic sources, as well as some industrial sources, may contain basic compounds, such as soaps and other detergents (Lajeunesse et al. 2008; Gracia-Lor et al. 2010), that react with the acetoacetic acid.

![Figure 5](image1.png) Reduction in percentage viability of eggs after prolonged exposure to treatments of ethyl acetate (EA) and Tween 80 (T8).

![Figure 6](image2.png) Reduction in percentage viability of eggs after prolonged exposure to treatments of magnesium sulphate (MS) and combination of acetoacetic acid and formalin (AA + F).
A major shielding effect was seen for eggs spiked into the sludge samples, with a higher percentage of viable eggs in these samples as compared to distilled water spiked eggs. Reagents that had a negative impact on eggs spiked in distilled water, such as ethyl acetate, acetoacetic acid, combination of these two and combination of acetoacetic acid and formalin, still resulted in the loss of egg viability but not as much as was seen in the distilled water (Table 4). The impact of these reagents was mostly severe when exposure time exceeded 30–60 minutes. After 30 minutes of exposure to acetoacetic acid, about 64.2% (Table 4). The impact of these reagents was mostly severe when exposure time exceeded 30–60 minutes. After 30 minutes of exposure to acetoacetic acid, about 64.2% of the eggs were still viable, which is higher than the viability of eggs in the distilled water (see Table 4).

A similar trend was seen for almost all the other reagents indicating a shielding effect. The shielding effect found in the sludge tests could be attributed to several factors; one major factor could be the presence of larger particles within the samples (Shimizu et al. 1997; Zorpas et al. 2002; Bougrier et al. 2005) with the potential to protect the eggs from the reagents, limiting their impact. In addition, the heterogeneity of sludge means that there may be particles within the sludge that might absorb the reagents (Hörsing et al. 2011; Stevens-Garmon et al. 2011), thereby reducing the concentration of these in the samples. A comparison of the number of viable eggs for each exposure time for eggs spiked into the wastewater and sludge showed that sludge has a much higher shielding effect than wastewater. Eggs spiked into wastewater and sludge and exposed to ethyl acetate, acetoacetic acid, combination of these two and combination of acetoacetic acid and formalin recorded significantly different percentage of viable eggs after incubation (p values <0.05). The increase in loss of viability with increase in exposure time might be due to the increase in contact between the eggs and reagents as the duration of exposure increase.

### CONCLUSION

Even though sulphuric acid had the best performance and was used in the further inactivation tests, it is important to note that the difference in percentage viability as assessed by sulphuric acid and distilled water was not statistically
significant. Distilled water performed very well (90.0 ± 3.7%) as an incubation solution, better than 0.5% formalin (87.6 ± 0.5%) which is widely used. However, there could be the loss of egg viability due to bacterial or fungal growth when distilled water is used but this effect was not determined in the current study. Therefore in conclusion distilled water may be used to reduce the cost involved in STH determination in the current study. Therefore in conclusion distillation of water is used but this effect was not be the loss of egg viability due to bacterial or fungal (87.6 ± 3.7%) as an incubation solution, better than 0.5% formalin.

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