Viability determination of *Ascaris* ova in raw wastewater: a comparative evaluation of culture-based, BacLight Live/Dead staining and PMA-qPCR methods

Vivek B. Ravindran, Esmaeil Shahsavari, Sarvesh K. Soni and Andrew S. Ball

**ABSTRACT**

Accurate evaluation of viable *Ascaris* ova in wastewater is the key to mitigating *Ascaris* reinfections in endemic regions. In this study, the viability of *Ascaris* ova in raw wastewater was determined using three different detection methods: culture-based, BacLight Live/Dead staining and propidium monoazide–quantitative polymerase chain reaction (PMA-qPCR). Furthermore, comparative assessment of viability utilising the aforementioned detection methods was performed using seeded experiments in wastewater. The percentage of viability was: culture-based (82%), BacLight Live/Dead staining (87%) and PMA-qPCR (85%) respectively. Despite the fact that no statistical difference was shown in the viability determination among the three methods, PMA-qPCR-based viability determination would be preferable over the other two methods for evaluating potential public health risks with *A. suum* ova due to its accuracy, being least subjective and its rapid reaction time.

**Key words** | *Ascaris* ova, baclight live/dead staining, PMA-qPCR, viability, wastewater

**INTRODUCTION**

Wastewater use is expected to increase globally to meet rapidly growing demands for potable and non-potable water supplies due to growing population and changing climate (Hanjra *et al.* 2012). It has been estimated that around 20 million ha of agricultural lands are irrigated with treated as well as raw wastewater (Jiménez & Asano 2008). In addition, direct application of raw wastewater into agricultural land is a common practice in developing countries, an activity that is rarely reported. However wastewater represents a reusable water source; yet it also has significant potential to act as a reservoir for waterborne disease transmission (Sidhu & Toze 2009; Li *et al.* 2014). The extent of the health risk from the reuse of wastewater depends on several factors including the numbers of pathogens present, dose, exposure routes and the susceptibility of exposed individuals (Navarro *et al.* 2009). Among the disease-causing microbial pathogens in wastewater, soil-transmitted helminths, especially the round worm (*Ascaris lumbricoides*), whip worm (*Trichuris trichiura*) and hook-worms (*Ancylostoma duodenale* and *Necator americanus*) pose a significant health risk to humans (Hotz *et al.* 2017).

*Ascaris lumbricoides* pose a major public health risk globally, with an estimated prevalence of over 1.2 billion people (da Rocha *et al.* 2016; Amoah *et al.* 2017). Diseases caused by these parasites lead to gastroenteritis, anaemia, intestinal obstruction, and poor physical and cognitive development in children (Bethony *et al.* 2006). Infections are prevalent in sub-Saharan Africa, Latin America and South-East Asia (Brooker *et al.* 2006). Poor sanitation and hygiene, inadequate water supply and reuse of wastewater and sludge for agriculture contribute to infections caused by *A. lumbricoides* (Stolk *et al.* 2016). A female worm can shed over 200,000 ova per day in the faeces of infected hosts. Due to the low infective dose required, ingestion of even one ovum in food or water contaminated with the embryonated larvae may cause disease (Raynal *et al.* 2012; Wardell *et al.* 2017). *Ascaris* species constitute 84% of the helminth ova in wastewater (Jiménez *et al.* 2016); they are also more resistant to environmental stresses than other...
helminths and microbes (Mara & Sleight 2010). For this reason Ascaris spp. are recommended as indicators of the effectiveness of wastewater treatment processes in terms of eliminating infectious organisms from treated wastewater and sludge (Steinbaum et al. 2017).

To reduce this risk, wastewater treatment processes are designed to inactivate viable Ascaris ova and pathogens (Jiménez 2007; Stevens et al. 2017), however, these are not feasible in developing countries due to the lack of advancements in technology. Due to high standards of sanitation and wastewater treatment processes in developed countries, ascariasis is not endemic in humans and Ascaris ova are rarely detected in wastewater systems (O’Connor et al. 2017). However, migrants from the endemic regions in addition to the increased rates of travel to developing countries could possibly increase the incidence of Ascaris infections in developed countries. Hence, the removal of Ascaris ova from wastewater in non-endemic countries remains essential for the safe use of sludge or treated water in agriculture (Pritchard et al. 2010). For unrestricted use in agriculture and irrigation, the World Health Organisation (WHO) guidelines recommend ≤1 viable Ascaris ova/L of wastewater sample (Shahsavari et al. 2017). State controls in Victoria, Australia, require >2 log10 reduction in Ascaris ova viability during wastewater treatment for the verification of wastewater and sewage sludge treatment processes for unrestricted use (EPA 2004). A better understanding of the health risk posed by Ascaris ova in wastewater matrices requires accurate identification and quantification (Collender et al. 2015).

Conventional monitoring for helminth ova in these environments relies on culture-based methods such as incubation and optical microscopy, which have many obvious limitations, such as being time-consuming (up to 4 weeks) and laborious, which limit their usefulness as a detection tool (Bowman et al. 2003; de Victorica & Galván 2003; Collender et al. 2015). Despite the fact that improvements to traditional methods have been implemented, resulting in reductions in incubation duration and the utilisation of vital stains for enhanced accuracy in counting, they remain cumbersome (Gyawali et al. 2016; Shahsavari et al. 2017). Accordingly, approaches involving quantitative polymerase chain reaction (qPCR) have been utilised for pathogen detection resulting in the development of a more rapid, sensitive and specific alternative method (Basuni et al. 2011). However, accurate quantification of Ascaris ova using qPCR can be challenging due to the presence of varying numbers of gene copies in different development stages.

The inability to differentiate viable and non-viable cells by qPCR results in an overestimation of ova concentration and thereby a misleading magnification of the health risk (Li et al. 2014; Randazzo et al. 2018). Propidium monoazide (PMA) coupled with real-time PCR has been utilised to selectively detect viable bacteria, viruses, protozoa and hookworm ova (Dąbrowska et al. 2014; Gyawali et al. 2017). PMA is a photo-reactive DNA intercalating dye that is able to penetrate the compromised membranes/cell wall of non-viable cells and subsequently combines with extracellular DNA or DNA from non-viable cells via exposure to bright visible light (Randazzo et al. 2018). Once combined with PMA, DNA will be incapable of being amplified in the subsequent PCR reaction, whereas only DNA protected by intact membranes of viable cells will normally be detected by qPCR (Kibbee & Ormeci 2017). However, there is limited information regarding whether PMA can distinguish between viable and non-viable Ascaris ova in wastewater. Also, considering that culture-based methods have been used as the ‘gold standard’, a consistent relationship between culture-based methods, BacLight Live/Dead viability staining (Karkashan et al. 2015) and PMA-qPCR should be developed in order to effectively monitor Ascaris ova within wastewater treatment plants (WWTPs).

The objective of the present study was to assess PMA-qPCR for monitoring viable Ascaris ova in wastewater. PMA-qPCR, BacLight Live/Dead viability staining and culture-based methods were simultaneously used to quantify the ova concentration. Detection results obtained by the aforementioned methods were compared and the relationship between PMA-qPCR, BacLight viability staining and culture-based methods was evaluated.

MATERIALS AND METHODS

Source of Ascaris suum ova

Ascaris suum ova were used as surrogate as it exhibits 98.1% genomic similarity to A. lumbricoides (human roundworm) and is morphologically indistinguishable (Ravindran et al. 2019). Faecal samples from pigs infected with A. suum were obtained from an abattoir in Laverton, Australia, and the ova were isolated by modifying the settling time, flotation time and centrifugation steps in the Tulane method (Bowman et al. 2003; Ravindran et al. 2019). Enumeration of ova was performed by optical microscopy (×200 magnification) using Whitlock universal four-chamber worm-egg counting slides (J. A. Whitlock & Co, Australia). The enumerated ova were
 aliquoted into 1 mL volumes of 5% potassium dichromate (Chem-Supply, Australia) in 1.5 mL Eppendorf tubes and stored at 4 °C for spiking/seeding experiments.

**Production of non-viable ova by heat treatment**

Approximately 500 (±20) Ascaris suum were suspended in 200 μL of 1% phosphate buffer saline (Sigma-Aldrich, Australia) in 1.5 mL tubes (Eppendorf, Australia) and incubated at 100 °C for 10, 15, 20 and 30 minutes in triplicate in a dry block heater. The supernatant was discarded, and the ova were stained in 0.5% methylene blue (Chem-Supply, Australia) and observed under an optical microscope (Olympus, Australia). Incubation at 100 °C for 15 min was found to produce 100% non-viable ova.

**Conventional incubation-microscopy for culture-based viability determination**

Approximately 1 mL of the stored suspension (in triplicate) containing 1,000 ova (±50) for untreated and 500 (±20) for heat-treated ova was placed in a 90 mL Petri dish, covered with 1% formalin and incubated at 25 °C for up to 4 weeks. Following incubation, the contents of the Petri dish were centrifuged at 800 g for 3 minutes and the deposit washed with MilliQ water and made up to 1 mL. Using the Whitlock egg counting chamber, 500 μL of the ova suspension was added into two chambers of the Whitlock slide and was counted in triplicate using optical microscopy. Embryonated ova with larvae were considered as viable and unembryonated ova were considered as non-viable ova.

**Viability determination with BacLight Live/Dead staining method**

The BacLight Live/Dead staining kit (Thermofisher, Australia) was used to stain ova following (i) recovery from pig faeces (without incubation) and (ii) recovery and heat treatment (without incubation). An aliquot (0.7 μL) of each BacLight component (A and B) was added to 200 μL of suspension containing 500 ova (±20) in an Eppendorf tube. The tubes were thoroughly mixed by hand-shaking to distribute the ova evenly and the mixture was maintained at 25 °C in the dark for 15 minutes. Following incubation 20 μL volumes of the suspension were examined using confocal laser scanning microscopy (CLSM). The procedure was repeated several times for each sample of recovered ova. The stained suspensions of ova were viewed and captured using a Nikon A1R confocal microscope with a ×20 objective and using NIS-Element advanced software. Green fluorescence was observed with a 488 nm excitation filter and 500–550 nm emission filter for Syto 9. Red fluorescence was observed with a 588 nm excitation filter and 570–620 nm emission filter for propidium iodide. Merged images from each of the two individual channels were captured. A. suum ova were classified as viable when the inner membrane was stained green and non-viable if stained red.

**BacLight Live/Dead staining to determine ova viability in wastewater**

The BacLight Live/Dead staining method (Dąbrowska et al. 2014) was applied to ova suspended in a sample of raw wastewater obtained from Lang Lang wastewater treatment run by South-East Water in Victoria, Australia. An aliquot of 1 mL of unheated (200 ± 10) and heat-treated ova (500 ± 20) in triplicate was added to 2 mL of wastewater and mixed by hand-shaking to ensure the ova were thoroughly distributed in the tube and centrifuged at 800 g for 3 minutes. The supernatant was discarded and 0.2 mL of the deposit with the seeded wastewater was stained by adding 0.7 μL of BacLight component (A and B) and incubated in the dark at 25 °C for 15 minutes. Volumes (20 μL) of the reaction mixture were examined by CLSM using the same procedure as described above in the preceding subsection. The total numbers of viable and non-viable ova were counted several times in the total volume of stained material.

**PMA-qPCR method**

**Optimisation of PMA concentration**

Stock solution (2 mM) of PMA (phenanthridium, 2-amino-8-azido-5-[3-(diethylmethylammonio)propyl]-6-phenylchloride) (Biotium, USA) was prepared by dissolving 1 mg of PMA into 978 μL of 20% dimethyl sulfoxide (Sigma-Aldrich, Australia). The stock solution was stored at −20 °C. Approximately 500 heat-killed A. suum ova (triplicate samples) were transferred into 1.5 mL Eppendorf tubes and varying concentrations of PMA (0, 50, 100 and 200 μM) added. PMA-treated non-viable ova were incubated at 25 °C in the dark for 10 minutes with periodic mixing to allow penetration of PMA into the damaged cell membrane of the ova. The cross-linking of PMA due to photo-induction was achieved using exposure to a PMA-Lite™ LED Photolysis device (Biotium, USA) for 15 minutes. The tubes were then incubated on ice for 10 minutes. DNA isolation was performed from PMA-treated non-viable Ascaris ova using
Isolate II genomic kits (Bioline, Australia). Minor modifications such as using glass beads were performed to the manufacturer’s protocol. Amplification of the DNA samples from non-viable ova using qPCR revealed that a PMA concentration of 100 μM did not permit amplification and this value was selected for the discrimination of viable and non-viable Ascaris ova using the PMA-qPCR assay.

Optimisation of qPCR

A quantitative PCR assay was designed to detect the internal transcribed spacer gene of A. suum using previously published primers and probes (Llewellyn et al. 2016) (Table 1). For the standard curve, ten-fold serial dilutions of A. suum ova were prepared in triplicate ranging from 3,000 to 3 ova/mL and were subjected to DNA extraction using Isolate II Genomic DNA Spin Column kits (Bioline, Australia). The performance of qPCR amplification was assessed by criteria such as efficiency (E), slope, intercept, R² and lower limit of quantification (LLOQ) which were determined using standard curves. The amplification reaction mixture consisted of 10 μL 2X GoTaq buffer (Promega, USA), 2.4 μL of 10 μM for each primer and probe, and 1 μL Equine Herpes virus (EHV) primers and probe, 1 μL template DNA, made to 20 μL with nuclease-free water. A negative control without target DNA, a positive control containing A. lumbricoides DNA and EHV DNA were included in the amplification reaction. Viral DNA internal control was added to analyze DNA extraction and amplification efficiency. A magnetic induction cycler (Bio Molecular Systems, Sydney, Australia) was utilised for PCR analysis.

To overcome contamination, preparation of the master mix, DNA isolation and qPCR were performed in different rooms within the laboratory. The cycler program consisted of 95 °C for 10 minutes, followed by 95 °C for 30 seconds, 60 °C for 60 seconds, repeated for 40 cycles. A magnetic induction cycler (Bio Molecular Systems, Australia) was used to analyze cycling reactions, absolute quantification and for preparation of the standard curve. The cycle threshold or Ct value is the total number of amplification cycles needed to detect fluorescence of the amplified products to exceed the set threshold value. Consequently, the Ct value is inversely proportional to DNA concentration in the sample. The assay was determined to be negative if the Ct value exceeded 40 cycles (no amplification curve). The least concentration of copies from the standard series detected in all replicates was considered to be the qPCR LLOQ.

Development of PMA-qPCR method

For the quantitative detection of viable A. suum ova: (i) 500 (+20) untreated ova, (ii) 50% untreated (n = 250) + 50% heat-treated (n = 250), and (iii) 500 (+20) heat-treated ova were treated with 100 μM PMA as described in the preceding subsection. Genomic DNA was isolated using Isolate II Genomic Spin Column kits (Bioline, Australia) and the subsequent DNA examined by qPCR.

Validation of PMA-qPCR for raw wastewater

To validate the PMA-qPCR method in terms of determining the viability of A. suum ova in raw wastewater, 9 mL of raw wastewater (Lang Lang WWTPs, Victoria, Australia) was seeded with 1 mL of: (i) 500 (+20) untreated ova, and (ii) 500 (+20) heat-treated ova. The ova-seeded samples were transferred into 50 mL sterile Falcon tubes and centrifuged at 800 g for 5 minutes. The resulting supernatant was discarded and ova were separated from the sediment (pellet) using magnesium sulphate (using the flotation method) using a modified Tulane method (Bowman et al. 2003; Ravindran et al. 2019). The recovered ova were treated with 100 μM PMA and DNA was isolated using Isolate II Genomic Spin

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers/probes (5’-3’)</th>
<th>Product size (bp)</th>
<th>Gene</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. suum</td>
<td>Forward-GTAATAGCAGTCGGCGGTTCCTT</td>
<td>88</td>
<td>ITS1</td>
<td>Llewellyn et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>Reverse-GCCCAACATGCCACCTATTC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Probe</td>
<td></td>
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<tr>
<td></td>
<td>ROX-TTGCGGACAATTTCGATGAT-IBRQ</td>
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<tr>
<td>EH Virus</td>
<td>F-GATGACACTAGCGATTTTCA</td>
<td>81</td>
<td>gB</td>
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<tr>
<td></td>
<td>R-CAGGCGAGAACCATAGACA</td>
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<td>Probe</td>
<td></td>
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<tr>
<td></td>
<td>CY5/FAM-TCGCCGTGCCCATCCAG-IBFQ</td>
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Column kits (Bioline, Australia) and the resulting DNA examined using qPCR. The Cq values conversion to viable ova was calculated as previously described by Gyawali et al. (2016).

RESULTS

Conventional incubation–microscopy for culture-based viability determination

Viable and non-viable ova were distinguished using a culture-based incubation–microscopy method. Of the 1,000 (±50, n = 3) ova for the culture-based method, each replicate was enumerated and the number of viable (embryonated) ova (Figure 1(a) and 1(b)) were determined to be 829, 851 and 792 with an average of 824 ± 24 (82% recovery).

The numbers considered as non-viable (unembryonated) ova (Figure 1(a)) were 176 ± 24 (18%). Furthermore, there was no embryonation or larval development in heat-treated ova (data not shown).

Viability determination with BacLight Live/Dead staining method

Viability determination of A. suum ova recovered from pig faeces using the BacLight Live/Dead staining and observation by CLSM showed that the inner lipoprotein layer of most of the ova was stained with both Syto 9 and PI (propidium iodide); however, the internal structures were unstained, denoting that these ova were viable. In contrast, the internal structures stained only by PI were considered to be non-viable (Figure 2). Unfertilised ova exhibited a similar response in terms of staining as non-viable ova. Out of the 500 (±20) untreated ova (n = 3), 397 ± 6 (79%) were considered viable and 94 ± 9 (19%) were non-viable; the remaining 9 ± 2 (2%) were partially stained and considered as uncertain (Table 2).

Similarly, out of 500 (±20) heat-treated ova, 498 ± 1 (99%) were considered as non-viable and 2 ± 1 (1%) were partially stained and were considered ‘uncertain’ (Table 2); however, no viable ova were present. The results confirm
the efficacy of the heat treatment of A. suum ova at 100 °C for 15 minutes. The total number of ova (heated and untreated) seeded in wastewater was 200 ± 20 (n = 3). Despite the presence of debris, both viable and non-viable ova were readily identified based on their size and morphology. Based on the BacLight Live/Dead staining method, 174 ± 5 (87%) were found to be viable, 21 ± 3 (11%) were non-viable and 4 ± 2 (2%) were partially stained and termed uncertain (Table 2). However, heat-treated ova seeded in wastewater were all observed to be non-viable.

**Optimisation of PMA concentration**

Amplification of non-viable ova was observed at the PMA concentration of 50 μM and in the untreated sample; however, PMA concentrations of 100 μM and 200 μM did not undergo amplification. This result confirms that a PMA concentration of 50 μM was insufficient to discriminate viable and non-viable ova while PMA concentrations of 100 μM and 200 μM were capable of discriminating between viable and non-viable ova (Figure S1, Supplement). The least concentration (100 μM) possessing the ability to inhibit amplification of non-viable ova was selected for further validation. The qPCR standard curves for target A. suum resulted in a linear range of quantification from 3 × 10³ to 3 × 10⁶ gene copies per microlitre of DNA. The qPCR amplification efficiency was 90% and the correlation coefficient (r²) was 0.99 with −3.91 as the slope of the line (data not shown). The qPCR LLOQ was 3 × 10⁰ gene copies (36 cycles).

**PMA-qPCR assay for determination of viability**

The assay was used to differentiate viable A. suum ova from a mix with non-viable ova. The mean Cq values for 100% viable (without PMA), 100% viable with PMA, 50% viable and 50% non-viable ova with PMA, and non-viable ova with PMA were 27, 28, 31, 40 and 28 respectively. These data reflected that the amplification of DNA from non-viable ova was almost completely inhibited (above 40 cycles) by the PMA treatment while there was no effect of PMA treatment on viable ova. Furthermore, the Cq values of the mixed (viable and non-viable) with PMA increased, suggesting that PMA had selectively inhibited the PCR reaction with DNA of non-viable ova (Figure 3).

**Validation of PMA-qPCR for raw wastewater**

Culture-based and BacLight Live/Dead staining methods provided information on the numbers of viable and non-viable A. suum ova in raw wastewater evaluated by culture-based, BacLight Live/Dead staining and PMA-qPCR

<table>
<thead>
<tr>
<th>Methods (viability determination)</th>
<th>Viable</th>
<th>Non-viable</th>
<th>Uncertain</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture-based</td>
<td>824 ± 24 (82%)</td>
<td>176 ± 24 (18%)</td>
<td>0 (0)</td>
<td>1,000</td>
</tr>
<tr>
<td>BacLight staining (wastewater)</td>
<td>397 ± 6 (79%)</td>
<td>84 ± 9 (19%)</td>
<td>9 (2%)</td>
<td>500</td>
</tr>
<tr>
<td>BacLight staining (wastewater)</td>
<td>174 ± 5 (87%)</td>
<td>21 ± 3 (11%)</td>
<td>4 (2%)</td>
<td>200</td>
</tr>
<tr>
<td>PMA-qPCR</td>
<td>424 ± 17 (85%)</td>
<td>76 ± 17 (15%)</td>
<td>0</td>
<td>500</td>
</tr>
</tbody>
</table>

**Table 2**

PMA-qPCR assay for determination of viability

The assay was used to differentiate viable A. suum ova from a mix with non-viable ova. The mean Cq values for 100% viable (without PMA), 100% viable with PMA, 50% viable and 50% non-viable ova with PMA, and non-viable ova with PMA were 27, 28, 31, 40 and 28 respectively. These data reflected that the amplification of DNA from non-viable ova was almost completely inhibited (above 40 cycles) by the PMA treatment while there was no effect of PMA treatment on viable ova. Furthermore, the Cq values of the mixed (viable and non-viable) with PMA increased, suggesting that PMA had selectively inhibited the PCR reaction with DNA of non-viable ova (Figure 3).

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Culture-based and BacLight Live/Dead staining methods provided information on the numbers of viable and non-viable A. suum ova in raw wastewater evaluated by culture-based, BacLight Live/Dead staining and PMA-qPCR.
non-viable ova. Hence a relation between gene copy numbers and corresponding ova was established. The number of viable ova quantified by PMA-qPCR was 424 ± 17 and the mean numbers of non-viable ova were 76 ± 17 (Table 2).

**Comparative assessment of the three methods**

The mean percentage of viability determined using the BacLight live/dead staining method for untreated ova in raw wastewater was 87%, slightly higher, although not statistically significant (Fisher exact test, \( p > 0.05 \)), than the viability determined using either the culture-based method (82%) or PMA-qPCR (85%) (Table 2).

**DISCUSSION**

Determination of the viability of *Ascaris* ova is necessary to assess the potential public health risks associated with the reuse of wastewater in agriculture in order to meet the guidelines of the WHO (McCarty et al. 2012). Few studies have assessed viability determination using PMA-qPCR, culture-based and vital stains for hookworm ova (Gyawali et al. 2017). Here we studied the utilisation of PMA-qPCR for viability determination of *Ascaris* ova in wastewater and for the first time, the accuracy was comparatively evaluated with the culture-based and BacLight Live/Dead staining methods for determining the viability of *Ascaris* ova.

The culture-based method would normally show the least accuracy when enumerated directly from wastewater samples. The low accuracy can be attributed to factors such as: (i) damage of ova during transport of wastewater samples to a central processing laboratory, (ii) storage methods, (iii) lack of a universal method to recover higher yield of ova, and (iv) identification of ova based on visual observation consequently leading to variation in the number and identity of ova (Verweij et al. 2007). Since infections with *Ascaris* species are not common in Australia due to good sanitation systems and high standards of urban wastewater treatment (Stevens et al. 2017), a seeding experiment was performed to assess viability using the culture-based incubation–microscopy method. This method involves various steps and chemicals that can distort the structural integrity of the ova cell wall (Bowman et al. 2003).

Our results on the viability determination of *A. suum* ova using the BacLight Live/Dead staining method are in general agreement with various studies utilised to distinguish viable and non-viable helminth ova (Dąbrowska et al. 2014). Syto 9 and PI saturated the lipoprotein layer of viable ova while staining the internal components of the non-viable ova. The reason for this differential staining may be the tough outer shell and an impermeable lipoprotein layer that act as barriers to the diffusion of stains into the viable ova (Wharton 1983). Since the *A. suum* ova used for this study were recovered from pig faeces, they generally had mature outer coats compared with the immature outer coats of ova collected from the uterine content of worms. In this study, the unembryonated and the heat-killed *A. suum* ova were considered as non-viable ova. Both Syto 9 and PI penetrated the lipoprotein layer and stained the internal nuclear material of all these types of non-viable ova. Also, Syto 9 and PI had no apparent toxic effects on viable ova as would normally occur with some of the vital stains (Karkashan et al. 2015).

The percentage viability of seeded *A. suum* ova in raw wastewater using the BacLight Live/Dead staining showed good agreement with the culture-based method. However, further work will be required before an assumption can be made that this method would be reliable in treated wastewater samples or sludge that normally undergoes several treatment processes in WWTPs. For instance, treatment of wastewater with UV and gamma radiation can damage the internal components of helminth ova with minor effect on the lipoprotein layer (Brownell & Nelson 2006). *A. suum* ova exposed to hydrostatic pressure in food processing exhibited no changes in morphology when examined using optical microscopy. However the ova were unable to undergo embryonation, possibly due to damage to proteins that are required for cell division (Rosypal et al. 2011). Ultracentrifugation also damages the internal components of *A. suum* ova without distorting the lipoprotein layer. In such a case, even non-viable *A. suum* ova that underwent the aforementioned treatment processes appear viable when examined using the BacLight Live/Dead staining method (Karkashan et al. 2015). Although the BacLight Live/Dead staining method showed promise as an alternative to culture-based methods for evaluating the viability of *A. suum* ova, it is time-consuming, requires accurate identification of helminth ova using CLSM and the efficiency of determining viability depends on the environmental sample.

To overcome the limitations of culture-based and BacLight Live/Dead staining methods, PMA-qPCR has been developed to detect and quantify viable bacteria, protozoa and viruses in environmental samples. Currently, there is limited knowledge on the comparative assessment of methods to determine the viability of *A. suum* ova using culture-based, BacLight Live/Dead staining and PMA-qPCR. Various studies have utilised PMA-qPCR to overcome the issue of quantitatively detecting viable cells (Gyawali et al. 2016; Randazzo et al. 2018). Variable PMA concentration and exposure to light may affect the viability of cells in
wastewater. A low concentration of PMA may not be efficient as unwanted debris may absorb PMA; however, a high PMA concentration may infiltrate viable cells and inhibit the PCR reaction leading to an underestimation of viable ova (Gyawali et al. 2016).

For photoactivation, PMA Lite™ (Biotium, USA) was used as it improves reproducibility and avoids variations that occur while performing manual photoactivation. The system is thermally stable with a uniform light source and allows 18 samples to be photoactivated simultaneously. In addition, photoactivation times are programmable, allowing efficient optimisation. Based on optimisation, a PMA concentration of 100 μM was chosen for this study. This value is in agreement with various studies that have used PMA for determining the viability of cells (Li et al. 2014). In this study, PMA only inhibited amplification of non-viable ova, due to a loss of structural integrity of the ova that allowed PMA to enter the cytoplasm.

Direct determination of the viability of A. suum ova in raw wastewater using PMA-qPCR might have some limitations such as prevention of PMA photoactivation due to sample turbidity. Samples have to be diluted to enable light to reach the PMA (Li et al. 2014). Nevertheless, in this study, PMA-qPCR measured only viable A. suum ova. However, the generated gene copy numbers need to be converted to ova. There is currently a lack of information regarding how many gene copies are present in a single ovum. Therefore, to determine the gene copy numbers, a separate qPCR experiment was needed to determine the standard. A kinetic study for the determination of the gene copy numbers of an ovum in every cell development stage would significantly improve the PMA-qPCR method (Pecson et al. 2006).

A comparison of assessed viability revealed that there was no statistical difference in the percentage viability of A. suum ova when assessed using culture-based, BacLight Live/Dead staining and PMA-qPCR. Despite the difficulty in estimating accurate gene copy numbers per ovum, the PMA-qPCR method is faster, sensitive and can overcome the limitations of the other two methods and therefore could be used for the rapid quantification of viable A. suum ova. Since the PMA-qPCR method can differentiate helminth ova to the species level, it could potentially be used for in-vitro viability assessment of soil-transmitted helminth ova.

CONCLUSION

The accurate quantitative detection of viable A. suum ova in wastewater is essential for evaluating the potential risks to public health. The culture-based and BacLight Live/Dead staining methods are based on microscopy whereby inbuilt errors occur such as the loss of ova during enumeration in addition to them being time-consuming. The PMA-qPCR was sensitive and specific and amplified only the viable ova thus preventing overestimation of infection intensity. Based on the speed, sensitivity, specificity and viability determination of A. suum in wastewater, PMA-qPCR outperformed the other two methods and could be applicable for evaluation of viable A. suum ova in raw wastewater.

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SUPPLEMENTARY DATA

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