# Comparison of culture-based, vital stain and PMA-qPCR methods for the quantitative detection of viable hookworm ova

P. Gyawali, J. P. S. Sidhu, W. Ahmed, P. Jagals and S. Toze

## ABSTRACT

Accurate quantitative measurement of viable hookworm ova from environmental samples is the key to controlling hookworm re-infections in the endemic regions. In this study, the accuracy of three quantitative detection methods [culture-based, vital stain and propidium monoazide-quantitative polymerase chain reaction (PMA-qPCR)] was evaluated by enumerating 1,000 ± 50 *Ancylostoma caninum* ova in the laboratory. The culture-based method was able to quantify an average of 397 ± 59 viable hookworm ova. Similarly, vital stain and PMA-qPCR methods quantified 644 ± 87 and 587 ± 91 viable ova, respectively. The numbers of viable ova estimated by the culture-based method were significantly ($P < 0.05$) lower than vital stain and PMA-qPCR methods. Therefore, both PMA-qPCR and vital stain methods appear to be suitable for the quantitative detection of viable hookworm ova. However, PMA-qPCR would be preferable over the vital stain method in scenarios where ova speciation is needed.

**Key words** | culture-based, detection methods, hookworm ova, PMA-qPCR, public health, vital stains

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## INTRODUCTION

Hookworm ova can remain viable for an extended period of time in the environment due to their multi-layered cell wall. Thus, rapid inactivation may not occur through basic and non-sophisticated wastewater and sludge treatment processes. As a result, higher numbers of hookworm ova may be found in the environment due to the application of partially treated wastewater and sludge on agricultural land (Karkashan *et al.* 2015; Bastos *et al.* 2013). The ability to quantify the levels of viable hookworm ova in the sources (wastewater and sludge) and receiving environment where they can potentially develop into infective larvae ($L_3$) is therefore an important process. This will aid in the hookworm disease control operations such as mass drugs administration and water, sanitation and hygiene interventions.

The numbers of viable hookworm ova in environmental samples including wastewater and sludge are usually enumerated by a culture-based method. This method involves incubating the ova at 28–30 °C for up to a week in an incubator (US EPA 2003; WHO 2006). The viable ova become larvae ($L_1$), and then they are enumerated with the aid of a microscope. The process is lengthy and may not be suitable for scenarios where rapid results are warranted (de Victorica & Galvan 2003; Nocker & Camper 2008; Gyawali *et al.* 2015a, 2016a, 2016b). Another shortcoming is that the culture-based method does not differentiate between various species of hookworm (Traub *et al.* 2004; Verweij *et al.* 2007).

To overcome these limitations, a vital stain method has been developed. The advantage of this method is that it is rapid, less technical and inexpensive (de Victorica & Galvan 2003). This method relies on the structural integrity of viable and non-viable ova. The cell wall of a viable ovum has three layers that act as an alternative barrier and prevent the vital stains from entering into the cytoplasm (Matthews 1986; Gregori *et al.* 2001). Once the ovum becomes non-viable, the integrity of the cell wall is compromised and it becomes permeable to the vital stain (Bae & Wuertz 2009). The cell wall, however, does not become permeable to vital stains immediately after inactivation (Gyawali *et al.* 2016b). Therefore, this method has the potential to overestimate the numbers of viable ova in a sample. In addition, this method also relies on skilled personnel to differentiate hookworm ova from ova of other helminths (Cabaret *et al.* 2002; Traub *et al.* 2004; Verweij *et al.* 2007). Despite this, the application of molecular-based methods has not been extensively used for the quantitative detection of hookworm ova. It has been reported that the propidium monoazide-quantitative

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polymerase chain reaction (PMA-qPCR) method can be used to detect and quantify viable hookworm ova in a one-step closed-tube reaction with high sensitivity, specificity, and accuracy by amplifying a specific gene (Gyawali *et al.* 2016c).

In this study, we compared the accuracy of the three quantitative methods (i) culture-based, (ii) vital stain, and (iii) PMA-qPCR for the enumeration of viable hookworm ova. All three methods required isolation of hookworm ova from environmental samples. Therefore, the experiments were conducted using *A. caninum* ova that were recovered from dog feces. Despite low risk of public health associated with the procedure, *A. caninum* ova were used due to the unavailability of sufficient human hookworm ova in Australia.

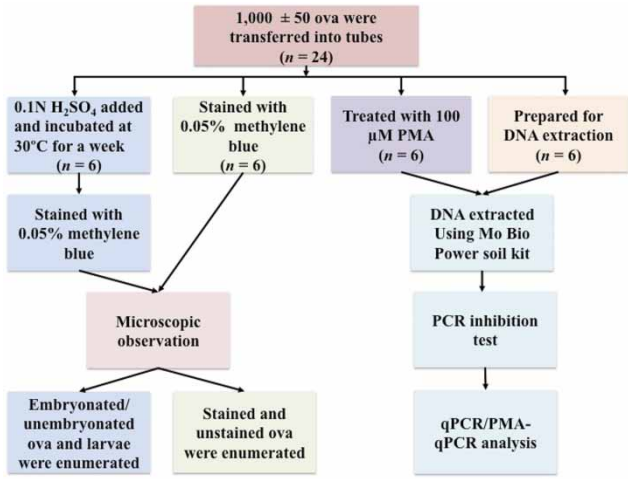


**Figure 1** | Flow chart showing the procedure for quantitative detection of hookworm ova.

## MATERIALS AND METHODS

### Isolation of ova from dog feces and enumeration

Fresh dog fecal samples were collected from the School of Veterinary Science, The University of Queensland, Gatton, Australia. *A. caninum* ova were isolated from approximately 20 *g* of dog fecal samples using a flotation method described elsewhere (Bowman *et al.* 2003). After isolation, ova were transferred into 2 mL microcentrifuge tubes containing 500 µL 0.5% formalin and enumerated. To enumerate the *A. caninum* ova, 20 µL of formalin solution containing ova was loaded onto a Sedgewick-Rafter Counting Chamber (PYSER – SGI, UK), and enumerated using a microscope (Olympus, USA). The numbers of ova were enumerated in each grid at ×10 magnification in triplicates.

### Methods used for the quantification of *A. caninum* hookworm ova

#### Culture-based method

The quantification methods used in this study are shown in Figure 1. The culture-based method is a standard method for quantifying viable hookworm ova from environmental samples (Bowman *et al.* 2003; US EPA 2003; WHO 2006). The procedure used in this study has been described in detail elsewhere (Bowman *et al.* 2003). In brief, approximately 1,000 ± 50 ova (50 µL solution) were transferred into a 5 mL tube. Culture fluid [(0.1 N $H_2SO_4$), (3.95 mL)] was added into each tube. The level of liquid in the tubes was marked on the outside using a permanent marker. Replicate uncapped tubes ($n = 6$) containing ova were incubated at 30 °C in an incubator for 1 week. The level of the liquid in the tubes was checked daily for the loss due to evaporation. The culture fluid was added to compensate for the evaporated volume as required. After 1 week of incubation, the tubes were centrifuged at $800 \times g$ for 5 min. 3.5 mL supernatant was discarded from each tube. The remaining volume (0.5 mL) of fluid was vortexed and transferred (20 µL) onto a slide. Embryonated ova/larvae and unhatched ova were enumerated using a microscope (Figure 2). Each sample was enumerated three times and the total numbers of viable ova were estimated using Equation (1).

$$N_{VO} = \frac{O_{EO}}{O_V} \times T_V \quad (1)$$

where $N_{VO}$ is the number of embryonated ova/larvae in a sample, $O_{EO}$ is the observed embryonated ova/larvae on a slide, $T_V$ is the total volume of the sample and $O_V$ is the observed volume of the sample.

Similarly, the total numbers of non-viable ova were estimated using Equation (2).

$$N_{NVO} = \frac{O_{NVO}}{O_V} \times T_V \quad (2)$$

where $N_{NVO}$ is the number of non-viable ova in a sample, $O_{NVO}$ is the observed non-viable ova on a slide, $T_V$ is the total volume of the sample and $O_V$ is the observed volume of the sample.

#### Vital stain method

The vital stain method used in this study has been described elsewhere (de Victorica & Galvan 2003). In brief,

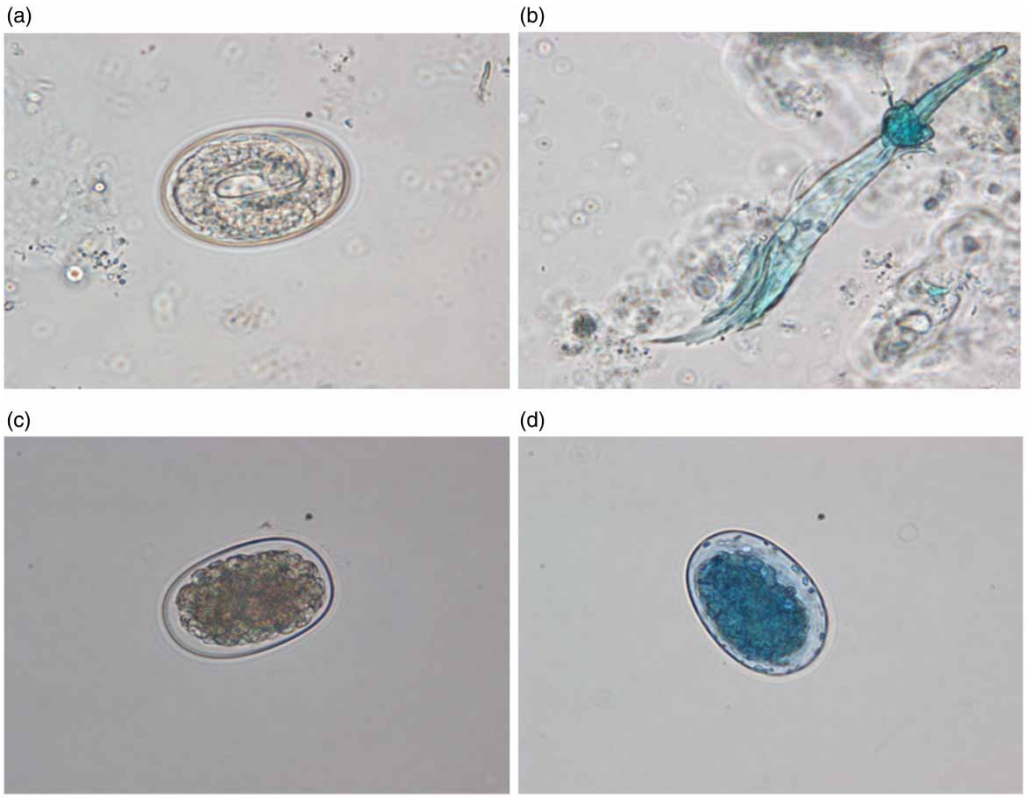


**Figure 2** | Photomicrographs of (a) embryonated ova, (b) larva, (c) viable ova and (d) non-viable ova of hookworm (*A. caninum*) obtained using culture-based and vital stain methods.

approximately 1,000 ± 50 ova (50 μL solution) were transferred into a 1.5 mL centrifuge tube ($n = 6$) containing 400 μL UltraPure™ DNase/RNase-free water (Life Technologies, Australia). The ova were stained with 50 μL methylene blue (0.05%) for 5 min at room temperature (Figure 1). The tube was vortexed, and 20 μL of liquid was transferred onto a slide. Viable (unstained) and non-viable (stained) ova were observed under a microscope and their numbers were recorded (Figure 2). Each sample was enumerated three times and the total numbers of viable and non-viable ova were estimated using Equations (1) and (2), respectively.

## PMA-qPCR method

The PMA-qPCR method used in this study has been described elsewhere (Gyawali *et al.* 2016c). In brief, 2 mM PMA working solution was obtained by dissolving 1 mg PMA (Biotium, Inc., USA) into 978 μL 20% dimethyl sulfoxide (Sigma-Aldrich, Australia). Approximately 1,000 ± 50 ova (50 μL solution) were transferred into 2 mL transparent centrifuge tubes ($n = 6$) and treated with 100 μM PMA (Figure 1). The ova were incubated in the dark for 5 min to allow PMA to penetrate into the non-viable ova. Photo-induced cross-linking of PMA to DNA was achieved by using a PhAST Blue light (GeniUL, Spain) for 15 min. The tubes were further incubated on ice for 5 min to minimize the effect of excessive heat on photoactivation. DNA from each sample was extracted using a Mo Bio Power Soil DNA Isolation Kit (Mo Bio, Carlsbad, USA). PCR inhibition test was also conducted as described elsewhere (Gyawali *et al.* 2015a).

## qPCR standard and cycling parameters

A 101 base pair (bp) of the 5.8S rRNA gene of ITS-1 region of *A. caninum* plasmid DNA sequence (TTTGCTAACGTG CACTGAATGACAGCAAACTCGTTGTTGCTGCTGAATC GTTTACCGACTATAAAACGTTTTGGCAGTGGCTAGTA TGACAACGGTGTTTC) was purchased from Integrated DNA Technologies (IDT) (IDT Technology, USA). Upon arrival, dry plasmid DNA was suspended into 100 μL UltraPure™ DNase/RNase-free water (Life Technologies, Australia) to obtain approximately 40 ng per μL of DNA. Gene copy numbers were determined by multiplying the DNA concentration by Avogadro's number and dividing by the product of the plasmid size (bp) and an average

weight of a base pair (Ahmed *et al.* 2014; Gyawali *et al.* 2015b). Serial dilutions were prepared ranging from $10^6$ to $10^0$ gene copies per μL of DNA.

Previously published primer and probe were used for the qPCR and PMA-qPCR assays (Gyawali *et al.* 2015a). The qPCR amplifications were performed in 25 μL reaction mixtures containing 12.5 μL iQ™ Supermix (Bio-Rad Laboratories, USA), 3 μL of template DNA, 250 nM of each primer, 400 nM of probe and UltraPure™ DNase/RNase-free water (Life Technologies, Australia). The cycling parameters were as follows: 95 °C for 15 min, 95 °C for 15 s, 59 °C for 1 min for 40 cycles. The qPCR and PMA-qPCR analyses were performed using the Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, USA). All qPCR and PMA-qPCR reactions were performed in triplicate.

### Estimation of gene copies

Culture-based and stain-based methods provide information on the ova numbers, whereas PMA-qPCR generates gene copy numbers. Therefore, it is important to establish a relation between gene copy numbers and corresponding ova. Since no information is available on the gene copy numbers per ovum, we extracted DNA directly from 1,000 ± 50 ova (50 μL solution) using a Mo Bio Power Soil DNA Extraction Kit (Mo Bio, USA) as described elsewhere (Gyawali *et al.* 2015b). Replicates samples ($n = 6$) were used for DNA extraction in order to capture the variability that may occur due to the presence of various cell-staged ova.

### Estimation of ova from gene copy numbers

The PMA-qPCR estimated gene copy numbers were converted to viable ova by Equation (3).

$$N_{VO} = \frac{NG_{PMA(qPCR)}}{NG_{qPCR}} \times N_O \quad (3)$$

where $N_{VO}$ is numbers of viable ova, $NG_{PMA(qPCR)}$ is average gene copy numbers estimated by PMA-qPCR, $NG_{qPCR}$ is average gene copy numbers estimated by qPCR and $N_O$ is total numbers of ova. Similarly, the numbers of non-viable ova were obtained by Equation (4).

$$N_{NVO} = \frac{NG_{qPCR} - NG_{PMA(qPCR)}}{NG_{qPCR}} \times N_O \quad (4)$$

where $N_{NVO}$ is numbers of non-viable ova, $NG_{PMA(qPCR)}$ is average gene copy numbers estimated by PMA-qPCR, $NG_{qPCR}$ is average gene copy numbers estimated by qPCR and $N_O$ is total numbers of ova.

### Quality control

To minimize qPCR and PMA-qPCR contamination, DNA extraction and qPCR set-up were performed in separate laboratories. A reagent blank was also included during DNA extraction to account for any carryover contamination during extraction. For each qPCR and PMA-qPCR experiment, corresponding positive (standards) and negative controls (UltraPure™ DNase/RNase-free water) were included.

### Statistical analysis

Microsoft Excel ver. 2010 (Microsoft, USA) was used to conduct the statistical analysis. A T-test for equal means was performed to determine the significant difference between the numbers of viable and non-viable ova estimated by the different methods (culture-based, vital stain and PMA-qPCR). A T-test for equal means was also performed to determine the significant different between culture and vital stain methods for unaccounted ova (could not be detected using these methods).

## RESULTS

### Numbers of viable and non-viable ova determined using three different methods

Of the 1,000 ± 50 ova, it was determined that an average of 694 ± 27 ova were quantifiable using the culture-based method. Approximately 306 ± 27 ova were unaccounted for due to inbuilt analytical errors associated with the method. Among the quantified ova, 397 ± 59 were found to be viable and 296 ± 52 non-viable (Figure 3). Similarly, the vital stain method quantified an average of 751 ± 50 ova, with 249 ± 50 ova were unaccounted. Among the quantified ova using the vital stain method, 644 ± 57 were viable and 107 ± 31 were non-viable (Figure 3). Similarly, PMA-qPCR quantified an average of 595 ± 74 viable and 405 ± 75 non-viable ova (Figure 3). There were no unaccounted ova for the PMA-qPCR method.

The numbers of viable ova estimated by the culture-based method were significantly ($P < 0.05$) lower than that estimated by both vital stain and PMA-qPCR methods. T-test for equal means indicated that the numbers of viable

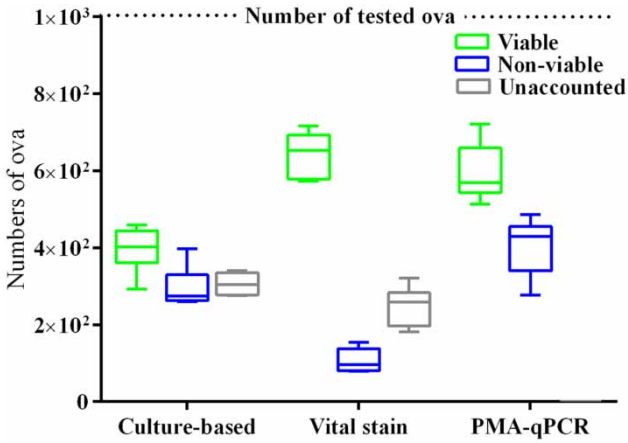


**Figure 3** | Numbers of viable and non-viable ova detected using three different quantitative methods. The numbers of ova that were unaccounted for while using culture and vital stain methods.

ova estimated by vital stain and PMA-qPCR methods were not statistically significant ($P > 0.05$). T-test for equal means indicated that the difference between non-viable ova estimated by all three methods was statistically ($P < 0.05$) significant to each other.

### Comparison of viability assessment

The PMA-qPCR method estimated an average of 54 ± 6% viability of *A. caninum* ova in the tested samples (Figure 4). This was 19% lower than the percentage of viability of ova (64 ± 5%) estimated by the vital stain method. In comparison, the percentage of viability estimated by the PMA-qPCR was 38% higher than the viability of ova estimated by the culture-based method (40 ± 6%).

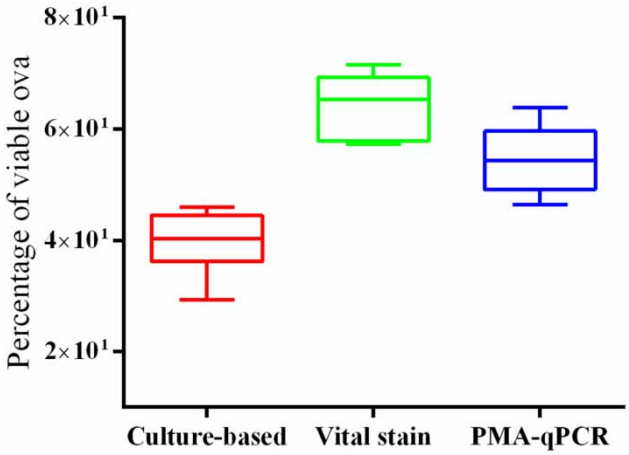


**Figure 4** | Box and Whisker plots of an average percentage of viability assessed by three different detection methods for *A. caninum* ova. The inner box lines represent the medians while the outer box lines represent 5th and 95th percentiles ($n = 6$).

## DISCUSSION

Quantitative detection and differentiation between viable and non-viable hookworm ova in soil, wastewater, sludge and contaminated food samples is necessary in order to implement strategies to mitigate re-infections in endemic regions (McCarthy *et al.* 2012; Gyawali *et al.* 2016a). Despite the advances in PCR/qPCR methods, the viability of hookworm ova has been determined by either culture-based or vital stain methods. This is because PCR/qPCR methods are unable to differentiate between viable and non-viable ova and not readily available in the developing countries. The recent development in the PMA-qPCR method overcomes such limitation and allows quantitative detection of viable hookworm ova from environmental samples (Gyawali *et al.* 2016c). In this study, the accuracy of a PMA-qPCR method was compared to the most commonly used culture-based and vital stain methods.

Among the three methods compared, the culture-based method had the lowest accuracy compare to the PMA-qPCR and vital stain methods. This may be because hookworm ova enumerated in this study were recovered from dog fecal samples using a flotation method. This method involves various steps and chemicals that can compromise the structural integrity of the ova cell wall (Nelson & Darby 2001). Incubating those cell wall compromised ova at 28–30 °C for a week might result in the disappearance or disintegration of some ova, which were not observed under a microscope. In addition, microscopic observation has inbuilt errors that may reduce the sensitivity of the method (Weber *et al.* 1991; Verweij *et al.* 2007).

Despite inbuilt errors (the low detection threshold of a microscope) associated with the microscopic observation, the vital stain method was more accurate compared to the culture-based method. In this study, ova were stained for 5 min as recommended prior to the microscopic observation, which is standard for the vital stain method. However, Gyawali *et al.* (2016b) reported that inactivated ova may take up to 12 h to become permeable to vital stains. Such time variation may lead to the overestimation of viable ova counts. Overestimation of viable ova using the vital stain method has also been reported by others (Kato *et al.* 2001; Nelson & Darby 2001; Schlosser *et al.* 2001; O'Grady & Smith 2002).

The accuracy of the vital stain and PMA-qPCR methods were similar. However, the major limitation of

the PMA-qPCR method is that the generated gene copy numbers need to be converted to ova. There is 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 needs to be undertaken parallel to PMA-qPCR. This increases the workflow and diagnostic time by 2–3 h. A kinetic study for the determination of the gene copy numbers of an ovum in every cell development stage (starting with fresh feces and not ending before all larvae are hatched) would significantly improve the PMA-qPCR method. Despite the difficulty in estimating accurate gene copy numbers per ovum, the PMA-qPCR method is faster, sensitive and could be used for the rapid quantification of viable hookworm ova. Since the PMA-qPCR method can differentiate hookworm ova to the species level, it could potentially be used for in-vitro viability assessment of hookworm ova.

## CONCLUSIONS

In conclusion, specific detection/quantification of viable hookworm ova from environmental samples is fundamental to assess the potential human health risks. Culture-based and vital stain methods rely on the sensitivity of a microscope. The sensitivity of a microscope is associated with the inbuilt error, such as loss of ova during observation. The PMA-qPCR method provided more viable hookworm ova in the tested samples than the culture-based method and fewer viable ova than the vital stain method. Since the vital stain method has a tendency to overestimate viable ova in a particular sample, the viability of hookworm ova estimated by PMA-qPCR could be applicable for assessing the potential health risks.

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