Enumeration of viable and non-viable larvated *Ascaris* eggs with quantitative PCR

Maria Raynal, Eric N. Villegas and Kara L. Nelson

ABSTRACT

The goal of this study was to further develop an incubation-quantitative polymerase chain reaction (qPCR) method for quantifying viable *Ascaris* eggs by characterizing the detection limit and number of template copies per egg, determining the specificity of the method, and testing the method with viable and inactivated larvated eggs. The number of template copies per cell was determined by amplifying DNA from known numbers of eggs at different development stages; the value was estimated to be 32 copies. The specificity of the method was tested against a panel of bacteria, fungi, protozoa and helminths, and no amplification was found with non-target DNA. Finally, fully larvated eggs were inactivated by four different treatments: 254 nm ultraviolet light, 2,000 ppm NH_3-N at pH 9, moderate heat (48 °C) and high heat (70 °C). Concentrations of treated eggs were measured by direct microscopy and incubation-qPCR. The qPCR signal decreased following all four treatments, and was in general agreement with the decrease in viable eggs determined by microscopy. The incubation-qPCR method for enumerating viable *Ascaris* eggs is a promising approach that can produce results faster than direct microscopy, and may have benefits for applications such as assessing biosolids.

Key words | biosolids, helminth eggs, polymerase chain reaction, sludge, viability

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INTRODUCTION

It is estimated that about 25% of the world's population is infected with the intestinal helminth *Ascaris lumbricoides* (Crompton 2001). The largest number of infections occurs in tropical and subtropical regions of the developing world where poor sanitation conditions persist. Each female worm produces around 200,000 eggs per day; these single-celled eggs are excreted by infected individuals. In the single-celled state, the eggs are not infective, but can develop into infective eggs containing larvae after approximately 18–30 d in warm (above ~28 °C), aerobic and humid environments (Crompton 2001). To prevent the transmission of *Ascaris* eggs via feces, wastewater or sludge, the eggs must be removed or inactivated. Typically, the concentration of viable eggs is regulated if treated wastewater or sludge (biosolids) is to be used beneficially, for example as irrigation water or a soil amendment (WHO 2006).

Current standard methods for detecting and determining the concentration of viable *Ascaris* eggs in environmental samples involve laborious multi-step procedures to clean the sample and concentrate the eggs, followed by an incubation period of roughly 4 weeks, and a final step to identify and count the larvated eggs under a light microscope (US EPA 1994). An advantage of this standard method is that specialized or expensive equipment is not needed. However, the long incubation time, high labor requirements and specialized knowledge necessary for identifying *Ascaris* eggs present challenges for monitoring treated effluents and biosolids, as well as for researchers studying disinfection. Enumeration methods that produced results faster, using common laboratory procedures, could have advantages in some settings.

In a previous paper, our research group reported the development of a quantitative polymerase chain reaction

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(qPCR) method for *Ascaris* eggs (Pecson *et al.* 2006) that has the potential to produce faster results than direct microscopy. The method in Pecson *et al.* (2006) targets a region in the ITS-1 rRNA gene that was designed to be specific to *Ascaris* based on the sequence information in GenBank. Importantly, the method was able to distinguish between viable and non-viable eggs, if a 10 d incubation step was included in the procedure prior to DNA extraction. The principle is that a single-celled viable egg develops into the infective larval stage, which has approximately 600 cells, when incubated (Roussell *et al.* 1994); conversely, a non-viable single-celled egg does not develop a larvae and remains at the single-cell stage. The qPCR signal thus increases by a factor of ~600 for viable eggs upon incubation. The ability of the qPCR method to quantify inactivation of single-celled eggs was compared to the traditional microscopy method following four different types of treatments to inactivate eggs: 254 nm ultraviolet light (UV_{254}), ammonia at high pH, moderate heat (48 °C) and high heat (70 °C; Pecson *et al.* 2006). The qPCR and traditional microscopy methods produced similar inactivation profiles. The demonstration that the incubation-qPCR method could distinguish viable and inactivated eggs is promising, as the persistence of DNA in inactivated microorganisms has been a major limitation to the application of qPCR for the detection of indicator and pathogenic viruses, bacteria and protozoa in water and sludge (Bae & Wuertz 2009; Brescia *et al.* 2009; Rodríguez *et al.* 2009). Indeed, DNA from the inactivated single-celled eggs persisted after most treatments, but because the DNA from the inactivated eggs did not replicate, as it did in the viable eggs, the incubation-qPCR method could detect up to approximately 2.8 log inactivation ($\log_{10}(600)$).

This previous research did not address the fact that partially or fully larvated eggs may exist in wastewater, sludge, or environmental samples if sufficient time has elapsed under aerobic conditions prior to sampling. Although eggs in the larvated state may remain viable for some time, they may also be inactivated by treatment processes or environmental conditions. Additionally, the specificity and exclusivity of the incubation-qPCR was only partially addressed. Therefore, to develop the incubation-qPCR method further, it is necessary to determine whether it can distinguish between viable and inactivated larvated eggs and determine the cross reactivity of the qPCR assay to other waterborne microorganisms.

The overall goal of this research was to develop further the incubation-qPCR method for quantification of viable *Ascaris* eggs by resolving a number of key concerns with its application to testing water and sludge samples. The specific objectives were to determine: (1) the quantitative relationship between ITS-1 copy number and egg number for single-celled and larvated eggs; (2) the specificity of the qPCR method; and (3) whether viable and inactivated larvated eggs could be distinguished.

MATERIALS AND METHODS

Ascaris egg samples

Ascaris suum eggs were purchased from Excelsior Sentinel (Ithaca, NY); the company collected the eggs from the intestinal contents of pigs, using sequential sieving to concentrate and clean the eggs. Eggs were shipped at a concentration of 10^5 ml^{-1} and stored at 4 °C in 0.5% formalin. The stock solution was verified by microscopy to contain only single-celled eggs. To prepare larvated eggs, an aliquot of the stock solution was washed three times with 0.1 mol l^{-1} H_2SO_4 and diluted in the same acidic solution to the desired working concentration. The eggs were incubated at 27 °C for 18 d in a water bath to develop larvae.

Detection limit and copy number of the qPCR method

Single-celled *Ascaris* eggs from the stock solution were diluted with 0.1 mol l^{-1} H_2SO_4 to obtain 1 ml samples containing 1,000, 500, 100, 50 and 10 eggs. Samples were incubated at 27 °C in a water bath. Duplicate samples were removed at 0, 6, 12 and 26 d. DNA was extracted for each time point and analyzed with the qPCR method as described below. Control samples (200 μl) were stored at 4 °C and were removed at the same time points for quantification by microscopy.

In a preliminary test, it was observed that there was very little recovery in the DNA extractions from the samples containing 100, 50 and 10 eggs. To improve recovery from these samples, 20 μg salmon sperm DNA (Invitrogen, Carlsbad,

CA) was added during DNA extraction (before and after lysis of the cells) to reduce sorption of target DNA to the walls of sample tubes and pipet tips. Salmon sperm DNA was also added to the *Ascaris suum* plasmid DNA standard stock solution to preserve our DNA from degradation in the −20 °C freezer. The addition of salmon sperm DNA to our plasmid standards did not increase or decrease the qPCR signal (data not shown).

Bacteria, fungi, protozoa and helminths

Bacillus thuringiensis (35646-D), *Bacillus cereus* (10987-D), *Shigella flexneri* (29903-D) and *Escherichia coli K-12* (10798-D) genomic DNA were purchased from American Type Culture Collection (Manassas, VA). *Cryptosporidium parvum* (Iowa strain), *Encephalitozoon hellem* (CDC:0291: V213), *Encephalitozoon intestinalis* (50502, ATCC), *Encephalitozoon cuniculi* (50502, ATCC) *Giardia muris, Giardia duodenalis* (H3; Assemblage B) and *Toxoplasma gondii* (RH strain) were propagated at the US Environmental Protection Agency (EPA; Cincinnati, OH). *Cryptosporidium hominis* (TU502) oocysts were obtained from Dr Giovanni Widmer, Tufts University, School of Veterinary Medicine, N. Grafton, MA. *Ancylostoma caninum, Schistosoma mansoni, Schistosoma japonicum, Schistosoma haematobium and Strongyloides stercoralis* were obtained from the Department of Pathobiology, University of Pennsylvania, School of Veterinary Medicine.

Specificity test for the qPCR method

The ITS-1 primers and probe were tested with DNA isolated from the protozoa, bacteria and helminths listed above (see the section on the qPCR method for details on reaction mixtures and amplification conditions). Genomic DNA was extracted using either a proteinase K based QIAamp DNA mini kit (Qiagen, Valencia, CA) or a mechanical lysis-based Mobio Ultraclean Fecal DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA). DNA concentration was determined using a Nano Drop 3300 (Thermo Scientific, Wilmington, DE), and the presence of genomic DNA from each organism was tested using 10 ng of template DNA and universal primers for the 16S (U514F (5′-GTGCCAGCMGCCGCGG-3′) and U1492R (5′-ACCTTGTTACGACTT-3′)), 18S (E528F (5′-CGGTAAT TCCAGCTCC-3′) and U1492R) or Alu gene (both 5′ GTG GAT CAC CTG AGG TCA GGA GTT TC 3′) regions, as previously described (Edgcomb *et al.* 2002; Veilleux 2011). Sample inhibition was tested by spiking a replicate PCR tube with 1,000 copies of the *Ascaris* ITS-1 plasmid standard (after DNA extraction) and quantifying amplification of the spiked DNA by qPCR.

Incomplete inactivation experiments

Fully larvated eggs were exposed to four different treatments: high heat, moderate heat, ammonia and low-pressure UV_{254} following the same procedures described in Pecson *et al.* (2006). The specific treatment conditions were: 70 °C (in 0.1 mol l^{-1} HEPES, pH 7) for 0.5, 1, 2 and 3 min; 48 °C (in 0.1 mol l^{-1} HEPES, pH 7) for 6, 15, 18 and 24 h; 42 °C + 2,000 mg l^{-1} NH_3–N at pH 9 (in 0.1 mol l^{-1} CHES) for 18, 36, 54 and 72 h; and UV_{254} fluences of 250, 500, 750, 1,000 and 10,000 Jm^{-2} (in 0.1 mol l^{-1} HEPES, pH 7).

These four treatment conditions were the same as those used previously to develop incomplete inactivation curves for single-celled eggs (Pecson *et al.* 2006). The initial egg concentration was 100 eggs ml^{-1}. For the heat treatments, 15 ml samples (1,500 eggs) were incubated in 50 ml plastic centrifuge tubes in a water bath. For the UV treatment, egg solutions were placed in 50 mm diameter glass Petri dishes and gently stirred under a quasi-collimated UV beam (Brownell & Nelson 2006).

Following treatment, all samples were placed on ice. The experimental solutions were replaced with 5 ml of 0.1 mol l^{-1} H_2SO_4 and all samples were incubated at 27 °C with loose caps in a water bath for 10 d. Control samples were egg solutions that were incubated without any treatment. A decrease in the number of eggs recovered from the treated samples compared to the control samples was interpreted as physical destruction of eggs.

Microscope method

The concentration and viability of the eggs in each sample treated by the four methods described above was also measured by microscopic examination. Briefly, eggs were incubated for 10 d and viewed at 20 × magnification using

brightfield microscopy (Olympus BH-2 microscope). Eggs that had a motile larva were considered viable, while all others were classified as inactivated. While this definition of viability is used by the US EPA (1994), it should be noted that it is possible that some non-motile larvated eggs were also viable. The fraction of inactivated eggs was calculated as the number of inactivated eggs divided by the total number of eggs counted in the control. Duplicate aliquots of 100 μl were analyzed from each sample.

qPCR method

DNA samples for specificity tests were prepared as described above. DNA from *Ascaris* eggs samples was isolated from 1 ml aliquots using the Mobio Ultraclean Fecal DNA isolation kit (Carlsbad, CA). ITS-1 rDNA levels were quantified by qPCR using the method described in Pecson *et al.* (2006) using a StepOnePlus™ real-time PCR machine (Applied Biosystems, Foster City, CA). Each 12.5 μl reaction contained 10 μl of 2.5 × Taqman Fast Universal PCR master mix (Applied Biosystems, Foster City, CA), 0.125 μl 15 mmol l^{-1} Mg^{+2}, 0.125 μl of both forward and reverse primers (0.7 μmol l^{-1}), 0.175 μl of the TaqMan probe (0.2 μmol l^{-1}) and 1 μl of the DNA extract from each sample (template). The cycling conditions used were as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 59 °C for 1 min. The standards were developed using plasmid of the whole ITS-1 region, as described in Pecson *et al.* (2006). The plasmid concentration was determined using a Nano Drop ND-3300 fluorospectrometer (Thermo Scientific). A standard curve was analyzed with each 96 well plate and was constructed using six points: 10^7, 10^5, 10^3, 10^2, 10 and 1 ITS-1 DNA copies. The amplification efficiency for the standard curves was always 99%. Non-template controls were processed with each 96 well plate and no amplification was observed.

RESULTS

Detection limit and copy number per egg

The results for the detection limit test are presented in Figure 1(a). The copy number of ITS-1 PCR targets per egg

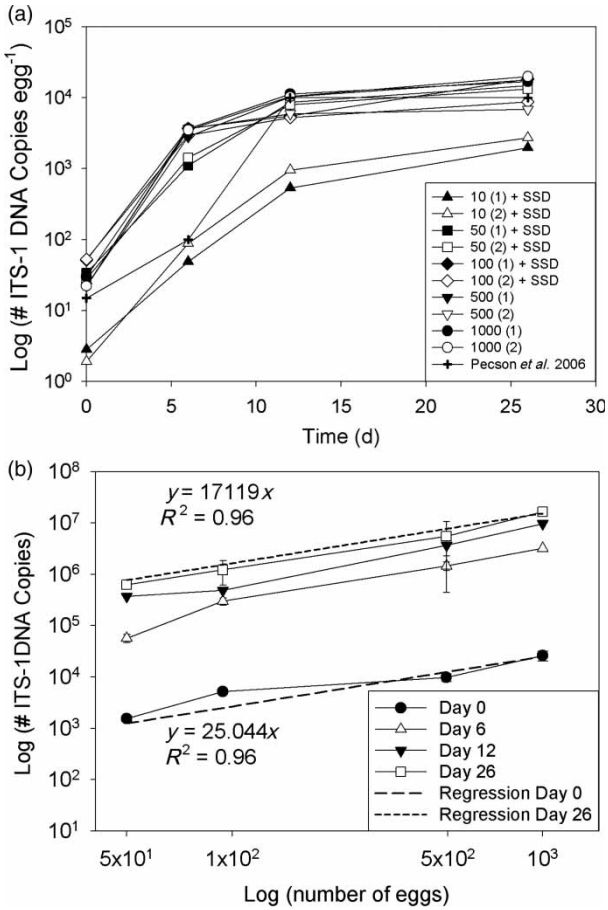

Figure 1 | Relationship between ITS-1 copy number and number of eggs, as a function of the developmental stage of the eggs. (a) Increase in ITS-1 copy number per egg as a function of incubation time. (b) Correlation between ITS-1 DNA copies and number of eggs at different time points. SSD = Salmon sperm DNA.

is shown as a function of the incubation period, during which the eggs matured from single cells into larvae. As expected, the ITS-1 copy number per egg increased over the first 12 d, and then remained fairly constant. It was confirmed by microscopy that the majority of eggs had matured to larvae by 12 d (data not shown), consistent with Pecson *et al.* (2006). During the first set of experiments, the DNA recovery was low from the samples containing 100 eggs and fewer (data not shown). To reduce losses of DNA due to potential sorption to the membrane, or the walls of the sample tubes and pipet tips, 20 μg of salmon sperm DNA was added to each sample during the isolation step (Rensen *et al.* 2002; Schiffner *et al.* 2005; Kishore *et al.* 2006). After this modification, the number of ITS-1 copies measured per egg was similar for samples containing 50

eggs or more. However, recovery from samples containing 10 eggs remained low. Interestingly, the ITS-1 copy number was low in the 10 egg samples, even for larvated eggs, which suggests that another reason for the low copy number was poor recovery of the eggs themselves, which may also stick to plastic surfaces (causing losses during preparation of samples as well as during incubation and DNA extraction). These losses would be expected in the samples with higher egg numbers as well, but would have less effect on the recovery efficiency.

The results obtained by Pecson *et al.* (2006), for which a sample size of 1,000 *Ascaris* eggs was used, are also shown in Figure 1(a). Those results are quite comparable to the new results, except for the value at 6 d. The method detection limit under the conditions studied thus appears to be between 10 and 50 eggs.

The number of ITS-1 copies measured in each sample is plotted against the number of eggs in the sample for each time point (0, 6, 12, 26 d) in Figure 1(b). These are the same data as in Figure 1(a), just analyzed in a different manner (and excluding the data from samples with 10 eggs due to low recovery). As expected, the ITS-1 copy number increased as the eggs were incubated longer; in this plot, it is apparent that there is still a slight increase in ITS-1 copy number between day 12 and day 26. On the log–log scale in Figure 1(b), the slope of the line for each sampling time is very close to unity, indicating that the number of ITS-1 copies per cell is constant. The value of this constant can be estimated by calculating the slope of the line using the untransformed values and then dividing by the number of cells per egg. For example, for the single-celled eggs, the value is: (25.04 ITS-1 copies per egg)/(1 cell per egg) = 25.0 ITS-1 copies cell^{-1}. For the fully larvated eggs at day 26, the value is: (17,119 ITS-1 copies per egg)/(600 cells per egg) = 28.5 ITS-1 copies cell^{-1}. Note that it was not possible to perform the calculation for eggs on day 6 or 12 because we did not have a good estimate for the number of cells per egg during intermediate stages of egg development.

A small source of error is introduced when analyzing larvated eggs, because 100% of the eggs do not develop to the larval stage. The percentage of non-viable eggs in stock egg solutions varies from batch to batch and experiment to experiment, which will affect the estimate of ITS-1 copies cell^{-1}. In the experiments in Figure 1(a) and (b), about 10% of the eggs were observed to remain at the single-cell stage (results not shown). If we account for the lower number of larvated eggs, then our revised value for the 26 d eggs is 31.7 ITS-1 copies cell^{-1}.

The values determined from Figure 1(b) are lower than the value reported by Pecson *et al.* (2006) of 42.9 ITS-1 copies cell^{-1}. Nonetheless, we consider the agreement between the values obtained in this study and those obtained by Pecson *et al.* to be reasonable, given that different batches of *Ascaris* eggs were used, as well as different DNA extraction methods, qPCR instruments and master mixes. The value in Pecson *et al.* was based on qPCR analysis of four replicate samples containing ~1,440 single-celled eggs each, whereas in this research, we based the calculation on eight samples of larvated eggs (duplicate samples at four different concentrations). It is recommended that other research groups measure this value to determine how consistent it is across laboratories. If the value is observed to vary, and the incubation-qPCR method is adopted for enumerating *Ascaris* eggs in unknown environmental samples, it may be advisable to determine the ITS-1 copies cell^{-1} by processing a set of larvated control eggs in parallel with each batch of environmental samples.

Specificity test

The microorganisms chosen for specificity testing were selected because they could potentially be present in environmental, wastewater or sludge samples. Two sets of samples were analyzed: DNA samples extracted with a proteinase K-DNA isolation kit (Table 1) and with the Ultraclean Mobio DNA isolation kit (Table 2). For the first set of samples (Table 1), all of the samples were either below the detection limit (10 ITS-1 copies) or did not amplify at all (cycle threshold $Ct > 40$). Conversely, PCR products were detected when these samples were amplified using universal primers for the 16S, 18S or Alu genes, confirming that DNA was present (data not shown). Unexpectedly, one of two replicates of the *C. parvum* sample amplified with a result of 34 copies (per 10 ng of DNA). This was likely due to accidental contamination by the ITS-1 plasmid since no amplification occurred in subsequent experiments using *C. parvum* genomic DNA (Table 2). We also observed very poor recovery of the

Table 1 | Results from specificity testing. The DNA of the other bacteria, eukaryotes and protozoa was extracted using a proteinase K-DNA isolation kit. '+' indicates detection, '−' indicates below detection limit (10 ITS-1 DNA copies) or no detection (*Ct* > 40). The *Ct* value of the blank was 40

Organism	No. copies detected (0 ITS-1 added)	No. copies detected (1,000 ITS-1 added)	Recovery, %
Bacteria			
Bacillus cereus	–	68	6.8
Bacillus thuringiensis	–	–	–
Escherichia coli K12	–	63	6.3
Shigella flexneri	–	89	8.9
Protozoa			
Cryptosporidium hominis	–	66	6.6
Cryptosporidium muris	–	94	9.4
Cryptosporidium parvum	+[a]	87	8.7
Cryptosporidium parvum 2	–	55	5.5
Giardia duodenalis	–	73	7.3
Giardia muris	–	89	8.9
Toxoplasma gondii	–	55	5.5
Fungi			
Encephalitozoon cuniculli	–	92	9.2
Encephalitozoon hellem	–	78	7.8
Encephalitozoon intestinalis	–	180	18
Nematodes and trematodes			
Ancylostoma caninum	–	82	8.2
Schistosoma haematobium	–	68	6.8
Schistosoma japonicum	–	107	10.7
Schistosoma mansoni	–	–	–
Schistosoma mansoni 2	–	88	8.8
Strongyloides stercorales	–	67	6.7

[a]*C. parvum* sample amplified with a result of 34 copies (per 10 ng of DNA) in the absence of the ITS-1 plasmid, which was likely due to the presence of *Ascaris* DNA.

Table 2 | Results from specificity test. The DNA of the protozoa was extracted using the Mobio Ultraclean Fecal DNA isolation kit. '+' indicates detection, '−' indicates below detection limit (10 ITS-1 DNA copies) or no detection (*Ct* > 40). The *Ct* value of the blank was >40

Organism	No. copies detected (0 ITS-1 added)	No. copies detected (1,000 ITS-1 added)	Recovery, %
Cryptosporidium muris	–	654	65
Cryptosporidium parvum	–	1,149	115
Giardia duodenalis	–	895	89

Ascaris standard for all of these samples (range 0–26%). We believe the poor recovery of ITS-1 plasmid during these tests was due to inhibition coming from proteinase K (Burkhart 2002) since qPCR inhibition was also observed when we used other proteinase K based DNA isolation kits (results not shown).

To overcome the observed inhibition using the proteinase K-DNA extraction procedure, we conducted additional

analyses of *C. parvum* oocysts, *C. muris* and *G. duodenalis* cysts. This time, the Mobio Ultraclean Fecal DNA isolation kit, a mechanical bead-beating lysis-based DNA extraction kit was used (Table 2). Results revealed that the number of copies amplified by the qPCR method was below the detection limit (<10 ITS-1 copies) for all non-target samples. Further, the recovery of the *Ascaris* plasmid standard mixed with the DNA from these three protozoa increased to an average value of ~89%.

Additional bioinformatic analyses using the BLAST tool of the National Institute of Health database to search and identify non-*Ascaris* spp. sequences homologous to the incubation-qPCR primers and probe set produced no matches (data not shown). Based on the BLAST results and the qPCR results, we conclude that the primers and probe are highly specific to *Ascaris* spp.

Inactivation measured by microscopy

The results from the inactivation experiments as determined by conventional direct microscopy are presented in Table 3. All of the treatments were effective at achieving high levels of inactivation, with >99% inactivation achieved by all except UV. For UV fluences from 250 to 1,000 Jm^{-2}, the inactivation appeared to decrease with increasing fluence, which is opposite to the expected trend. It is suspected that the

Table 3 | Inactivation of larvated *Ascaris* eggs by four different types of treatment, as determined by direct microscopy. Two aliquots were counted for each time point and the mean values are reported. The inactivation for each treatment was calculated as 1 – (number active eggs/total number of active eggs of the control)*100. The percentage of destroyed eggs was calculated as 1 – (total number of eggs/total number of eggs in the control)*100

	Day 0					Day 10				
Treatment	Inactive eggs	Active eggs	Total no. of eggs	Inactivation, %	Destruction, %	Inactive eggs	Active eggs	Total no. of eggs	Inactivation, %	Destruction, %
UV, Jm^{-2}										
250	36	10	46	89	50	42	2	44	98	52
500	30	8	38	91	59	48	0	48	>99	48
750	38	4	42	96	55	36	0	36	>99	61
1,000	38	20	58	78	38	32	18	50	81	46
10,000	16	2	18	98	81	26	3	32	97	65
70 °C										
30 s	70	2	72	98	22	68	0	68	>99	26
1 min	82	0	82	>99	11	78	0	78	>99	16
2 min	81	0	81	>99	12	76	0	76	>99	18
3 min	40	0	40	>99	57	36	0	36	>99	61
48 °C										
6 h	36	0	36	>99	61	42	0	42	>99	55
5 h	34	0	34	>99	63	38	0	38	>99	59
18 h	38	0	38	>99	59	44	0	44	>99	52
24 h	16	0	16	>99	83	20	0	20	>99	78
42 °C; 2,000 mg l^{-1} NH_3-N, pH 9										
18 h	22	0	22	>99	76	24	0	24	>99	74
36 h	20	0	20	>99	78	12	0	12	>99	87
54 h	18	0	18	>99	81	12	0	12	>99	87
72 h	36	0	36	>99	61	40	0	40	>99	57
Control	0	90	90	0.00	NA	0	95	95	0.00	NA

NA, not applicable.

stirring conditions pushed some eggs to the edge of the Petri dishes, which prevented the eggs from receiving uniform exposure to the light. Microscopic observations revealed that the 48 and 70 °C heat treatments caused small fissures in the egg shells, including breakage of some shells, although the larvae were not released from the eggs. In the eggs treated with ammonia, granules were visible inside the larva; with the incubation time of 10 d, many larvae were released from the eggs and seemed to start degrading. With UV treatment (which included stirring), the larvae were released from the eggs immediately after UV treatment; however, the larvae seemed to remain intact (data not shown).

For all the treatments, the total number of eggs recovered was lower than the approximately 100 eggs in each sample before treatment. As the total numbers of eggs recovered from the treatments were lower than the number of eggs recovered from the control samples (90 eggs at day 0 and 95 at day 10), we believe that some of the larvated eggs were destroyed during all the treatments. The highest destruction of eggs was observed for the treatment with ammonia (~80%).

qPCR method results for the incomplete inactivation experiments

The results obtained for the inactivation test using fully larvated eggs with the qPCR method are presented in Figure 2. The results obtained by qPCR are in general agreement with those obtained by direct microscopy (Table 4). Both methods reported about 99% inactivation for treatment at 48 °C, although the inactivation measured by direct microscopy may have been higher, since 99% was the highest level of inactivation that could be measured. For treatment by ammonia at 42 °C, the inactivation measured by qPCR was about 99.9%, and all measurements by microscopy were >99%. For UV treatment, the levels of inactivation measured by both methods were similar and did not increase consistently with dose, suggesting that the UV dose was not applied evenly to the sample, as mentioned above. Finally, for treatment at 70 °C, the inactivation measured by qPCR was lower than that measured by microscopy.

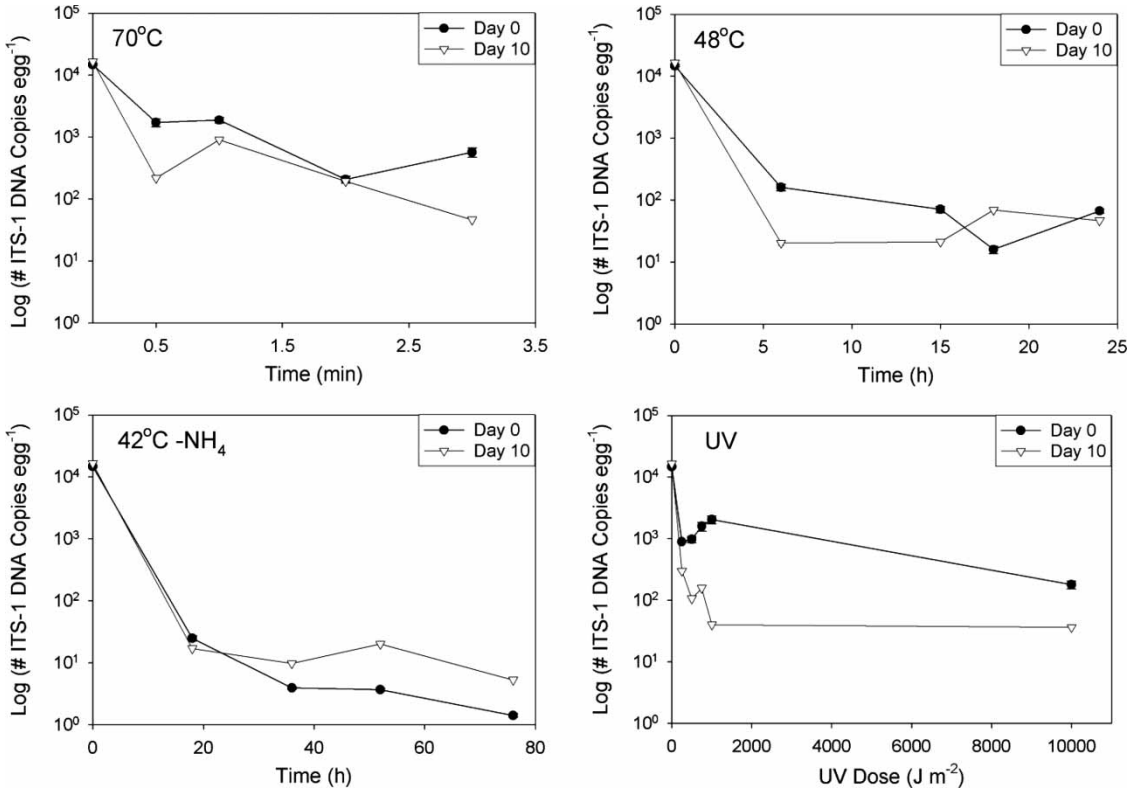

Figure 2 | Results for the qPCR method for the four treatments studied. qPCR was performed at two time points, immediately after inactivation treatment (0 d) and after incubation for 10 d.

Table 4 | Number of viable, larvated, eggs in treated samples determined by microscopy and by qPCR. The eggs presented for the microscopy method are the active eggs (same as Table 3). The number of eggs for the qPCR method was calculated by dividing the number of ITS-1 copies by 19,021 copies per larvated eggs (600 cells l per larvated egg times 31.70 copies per cell)

	Microscopy		qPCR	
Treatment	**Day 0**	**Day 10**	**Day 0**	**Day 10**
UV, Jm^{-2}				
250	10	2	6.5	1.5
500	8	0	5.7	0.5
750	4	0	9.2	0.5
1,000	20	18	11.9	0.4
10,000	2	3	1.1	0.2
70 °C				
30 s	2	0	10.0	1.3
1 min	0	0	1.2	1.2
2 min	0	0	10.9	5.2
3 min	0	0	3.3	0.3
48 °C				
6 h	0	0	1	0
15 h	0	0	0	0
18 h	0	0	0	0
24 h	0	0	1	0
42 °C, 2,000 mg l^{-1} NH_3				
18 h	0	0	0.7	0.5
36 h	0	0	0.1	0.3
52 h	0	0	0.1	0.5
76 h	0	0	0.0	0.2
Control	90	95	85.5	95.4

For the egg samples treated by UV, the qPCR signal measured after incubating the eggs for 10 d was lower than that measured immediately after treatment (0 d). These results suggest that the DNA continued to degrade during the 10 d after treatment. For the other treatment methods, however, there was not a consistent decrease in the qPCR signal after incubating the eggs for 10 d, suggesting that the DNA was fairly stable.

DISCUSSION

The main goals of this research were to build on an incubation-qPCR method that was developed using single-celled *Ascaris* eggs by extending the approach to larvated eggs, as well as to confirm the specificity of the PCR target. As shown in Table 1 and 2, the *Ascaris* ITS-1 qPCR method did not amplify DNA extracted from a range of organisms that may be present in environmental samples. An important and encouraging result was that the qPCR signal decreased significantly in larvated eggs that were exposed to four different inactivating treatments (Figure 2). The qPCR signal persisted for 10 d after treatment, except in the case of UV treatment (Ma *et al.* 1994; Rudi *et al.* 2005; Yaradou *et al.* 2007; de Roda Husman *et al.* 2009). Nonetheless, the results suggest that viable larvated *Ascaris* eggs can be distinguished from inactivated larvated eggs by qPCR, under the conditions that were studied.

Several important differences were observed in the behavior of larvated versus single-celled *Ascaris* eggs, as reported previously (Pecson *et al.* 2006). With the single-celled *Ascaris* eggs, a decrease in the qPCR signal following inactivation was not observed, and the qPCR signal persisted for several weeks after treatment (except for 70 °C heat treatment). The larvated eggs were found to be more susceptible to inactivation by treatment at 48 °C, 70 °C and 42 °C + 2,000 mg l^{-1} NH_3–N at pH 9 (compare Table 4 results with Figure 5 in Pecson *et al.* (2006)). The different behavior of the larvated eggs and single-celled eggs could be due to changes in the eggshell that occur when the eggs mature (e.g. the eggshell may become more permeable and susceptible to mechanical stress; Barrett 1976). Another possibility is that the DNA in the larva is more vulnerable to inactivation due to the higher copy numbers and its physical packaging.

An important step in making the qPCR method quantitative is correlating the ITS-1 copy number (determined by qPCR) with the number of *Ascaris* eggs. There are two pieces of information needed to make the conversion. The first is the number of ITS-1 copies per cell. Based on the results presented in Figure 1, it appears that a good estimate of this value is ~32 ITS-1 copies $cell^{-1}$. The second is the number of cells per *Ascaris* egg. The incubation step of the qPCR method allows all viable eggs to develop to the larvated stage, so that it can be assumed that there are approximately 600 cells egg^{-1} (see Pecson *et al.* (2006) for further discussion).

In summary, the incubation-qPCR method involved the following steps: (i) clean and concentrate sample; (ii) incubate sample for 10 d at ~27 °C to allow viable eggs to develop to larval stage; (iii) extract DNA and perform qPCR analysis using standard curve based on ITS-1 plasmid; and (iv) convert qPCR signal to an egg count. The ability of the method to measure only viable eggs is based on two rationales: (1) non-viable single-celled eggs will not develop larva during the incubation stage, and their contribution to the qPCR signal will be minimal compared to the signal from the larvated eggs and (2) non-viable larvated eggs that are present in the original sample will not contribute significantly to the qPCR signal (based on the results in Figure 2). A remaining question is how eggs that are at an intermediate stage of development will influence the method.

The detection limit of the incubation-qPCR method needs to be determined in real matrices. In this study, we found that egg recovery was low in samples containing 10 eggs, whereas recovery was consistently high in samples containing 50 eggs or more. However, in an actual sample matrix, fewer eggs may be lost during the transfer steps or due to sorption to the surfaces of sample containers and pipet tips. In wastewater or sludge samples, on the other hand, other types of interferences are expected to dominate the detection limit (recovery during cleaning and concentration steps, as well as inhibition of PCR reaction due to compounds in matrix).

In conclusion, we believe the incubation-qPCR method is a promising method for quantifying the number of viable *Ascaris* eggs in environmental samples. Additional research is needed to determine how this assay will perform with detecting infectious *Ascaris lumbricoides*, which may be specific to human hosts and other near-neighbor ascarids, as well as to understand the effects of other types of inactivation (e.g. composting) and real-world treatments with actual sample matrices on the qPCR signal. Adequate methods for concentrating and extracting *Ascaris* DNA from environmental samples, and removing PCR inhibitors are also needed.

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