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Short communication

Validation of droplet digital Polymerase Chain Reaction for the detection and absolute quantification of *Taenia solium* eggs in spiked soil samples

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ABSTRACT

To enable the detection of taeniid eggs in environmental samples, a sensitive technology is required. In this study, we validated the effectiveness of a digital droplet Polymerase Chain Reaction (ddPCR) assay for detection, identification and absolute quantification of taeniid DNA from artificially contaminated soils with varying numbers of taeniid eggs using a set of universal primers, JB3 & JB4.5. The results showed that the number of *cox1* copies detected increased gradually for both species with the number of taeniid eggs added to the different soil types. The highest *cox1* DNA copies recovery for *Taenia solium* and *T. lynciscapreoli* was from the sand soil with lowest recovery being observed in clay soils. Therefore, ddPCR is a promising technology for screening of taeniid eggs from soil samples collected in the environment irrespective of the soil type and the number of eggs. The potential of the ddPCR protocol to detect taeniid egg DNA in spiked soil samples has great practical application for taeniid egg screening in soils from endemic areas. However, when universal primers are used in screening environmental samples, the identity of ddPCR positive samples must be confirmed by sequencing. In addition, more validation studies using species-specific primers and field soil samples is recommended.

1. Introduction

The pork tapeworm, *Taenia solium*, is a neglected zoonotic parasite, which continues to be a major public health challenge in endemic regions (Assana et al., 2013; Okello et al., 2015). In small scale pig farming industry, *T. solium* infection causes huge economic loss because of devaluation or total condemnation of pork (Praet et al., 2009). Environmental contamination represents the main features for epidemiology of the parasite. Pigs and humans get infected by the larval form of the parasite, *T. solium* cysticercosis, following ingestion of viable eggs of the parasite from contaminated environment. Human carriers are the sole source for environmental contamination with faeces, in turn contaminated drinking water, food and/or soil are ingested by both pigs and humans (Sharma et al., 2005).

The World Health Organization (WHO) considers that *T. solium* infection to be preventable and so can be eradicated, though it has proved difficult through improved meat inspection, sanitary installations and health education. So far, there are only occasional successful examples at village level in Peru (Garcia et al., 2016); no endemic country has so far eradicated the parasite on a nationwide basis. One of the main obstacles might be lack of a reliable and sensitive diagnostic tool for the detection of various stages of *T. solium* not only in soil but also in both human and pig hosts (Chacha et al., 2013; Johansen et al., 2016). On one hand, the diagnostic tools used today have low sensitivity and specificity, and they result into either misdiagnosis or underestimation of the problem (Katabarwa et al., 2008). Similarly, those with high sensitivity are sophisticated and hardly would be available to the endemic poor communities. Lack of success in eliminating *T. solium* in endemic areas may also be that the environmental transmission route have been overlooked (Maganira et al., 2018). This calls for a reliable and effective detection tool for *T. solium* eggs in environmental samples including the soil, which may contribute to more successful control and ultimately elimination of the parasite.

Molecular methods have frequently been used as a confirmatory test for taeniid cysts (Abuseir et al., 2006; Chembensofu et al., 2017) and eggs or proglottids in human stool (Eom et al., 2011). Applications of the technology for environmental screening are rare. Droplet digital Polymerase Chain Reaction (ddPCR) is a third generation PCR

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technology for precise quantification of target nucleic acids (Pinheiro et al., 2012). With ddPCR, the total number of individual target molecules is quantified in a digital format with improved sensitivity (Pinheiro et al., 2012). One of the advantages compared to other PCR methods is the ability for absolute quantification without the need of running standard curves and hence minimizing the different effects between the reference and test sample (Demeke and Dobnik, 2018; Pinheiro et al., 2012). In addition, the presence of PCR inhibitors counts little in ddPCR (Demeke and Dobnik, 2018). The use of ddPCR technology has been observed in the detection and quantification of genetically modified organisms (Dobnik et al., 2015), adulteration of meat species in both raw and processed food (Ren et al., 2017; Shehata et al., 2017), human malaria and schistosomiasis (Koepfli et al., 2016; Weerakoon et al., 2017b) and gastrointestinal nematodes of grazing livestock (Elmahalawy et al., 2018). This technology has also been used in the detection of soil-transmitted helminths eggs such as *Ascaris lumbricoides* in reclaimed water and examination of bacterial dynamics in soil (Kim et al., 2014; Soto et al., 2017). The suitability of ddPCR in detecting contamination by taeniid eggs in soils is not reported so far. We report the effectiveness of a ddPCR protocol for detection and absolute quantification of taeniid eggs in soil using *T. solium* and *T. lynciscapreoli* eggs, which served as a proxy.

2. Material and methods

2.1. Source of soil and taeniid eggs

Clay, silt and sand soil were collected from domestic animal free premises in Morogoro Municipality, Tanzania. Loam soil was made available by mixing clay, silt and sand in the ratio of 1:2:2 by weight (Foth, 1990), respectively. Taeniid eggs were recovered from adult *T. solium* and *T. lynciscapreoli* worm proglottids obtained from the Sokoine University of Agriculture, Tanzania and the National Veterinary Institute, Sweden, respectively. Prior to egg recovery, the proglottids were inactivated for biosecurity reasons at −80 °C for seven days and then stored −20 °C until use. After recovery, the eggs were then stored at −20 °C before the spiking experiment.

2.2. Spiking of soil with taeniid eggs

Before spiking soil with taeniid eggs, each soil type was sterilized at 130 °C for 60 min in a hot oven. Then, about 5 g of each soil was artificially spiked in triplicates with approximately 10, 50, 100 and 500 eggs. In addition, a positive control was prepared by spiking approximately 10, 50, 100 and 500 eggs in triplicates in 100 µl of distilled water without soil. In a similar manner, unspiked soil served as negative controls. The eggs-soil spiking exercise consisted of two rounds for each of the four soil types and was repeated with eggs from either *T. solium* or *T. lynciscapreoli*.

2.3. Recovery of taeniid eggs from soil

Recovery of taeniid eggs from spiked soil (5 g) was carried out by sequential sieving of the supernatant using zinc chloride (Acros Organics, Geel, Belgium) solution (Miller et al., 2016) with minor modifications. Briefly, approximately 20 ml of Phosphate Buffered Saline (Sigma Aldrich, Stockholm, Sweden) containing 0.3% Tween 20 (Merck Schuchardt, Hohenbrunn, Germany) was added to the sample, thoroughly mixed by vortexing and incubated in the refrigerator at 4 °C overnight. After centrifugation at 1600 × g for 10 min, the supernatant was discarded and about 15 ml of saturated zinc chloride solution (density 1.45 g/ml) was added to the content and thoroughly mixed by vortexing. After centrifugation at 1000 × g for 30 min, the supernatant solution was collected. The eggs were then isolated using modified 50 ml Falcon tubes fitted with nylon sieves with 41/100 µm and 20/100 µm mesh size (Sefar AG, Switzerland). All the meshes were pre-wetted with water containing 0.2% Tween 20. Modifications of the Falcon tubes involved cutting the bottom end of the tube using a fine saw followed by drilling a 2.5 cm diameter hole in the tube lid. The lid with a hole was used to fix in place the nylon sieve onto the modified Falcon tube. The supernatant solution containing taeniid eggs was passed through the modified Falcon tube fitted with 41/100 µm sieve using the modified Falcon tube lid allowing the solution to pass through with taeniid eggs. The 41/100 µm sieve flow-through solution was again passed through the modified Falcon tube fitted with 20/100 µm lid, which retains any taeniid eggs. The 20/100 µm nylon sieve with fine particles and taeniid eggs was then inverted in a funnel on a 50 ml collecting ordinary Falcon tube and the contents collected by thoroughly washing the sieve with a jet stream of water (approximately 25 ml). This was followed by centrifugation at 200 × g for 10 min in which the eggs settled down and most of the supernatant was sucked off and discarded. Using a micropipette about 100 µl of the remaining final suspension volume (sample pellets) was transferred into a 1.5 ml microcentrifuge tube.

2.4. DNA extraction from recovered taeniid egg

DNA was extracted from the sample pellets recovered from the egg-soil spiking experiment (Section 2.3) using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer's protocol with minor modifications. Prior to DNA extraction, alkaline lysis and neutralization procedures were performed on the sample pellets (Miller et al., 2016; Stefanic et al., 2004). The extracted DNA was stored at −20 °C in labelled microcentrifuge tubes before further analysis.

2.5. Droplet digital PCR assay optimization

Determination of optimal annealing temperature was performed in a MyCycler thermal cycler (Bio-Rad Laboratories) and utilized DNA extracted from *T. solium* cysticerci from pigs or proglottids of adult worms of *T. lynciscapreoli* using the QIAamp DNA Mini Kit following manufacturer's protocol (DNA purification from tissues). The thermal gradient ranged between 50 °C to 62 °C with 2 µl of template DNA from *T. solium* or *T. lynciscapreoli* DNA extracts. DNA samples from both species were also tested for sensitivity against two-fold serial dilutions. Samples were run in biological triplicates and tested in technical duplicates.

The identity of parasite species were confirmed by using JB3/JB4.5 forward (F) and reverse (R) universal primers (Bio-Rad Laboratories), which amplified the mitochondrial cytochrome c oxidase subunit I (*cox1*) in DNA from the cysts and worms. Sequence of JB3 and JB4.5 were F-TTTTTTGGGCATCCTGAGGTTTAT and R-TAAAGAAAGAACAT AATGAAAATG (Bowles et al., 1992; Bowles and McManus, 1994). Conventional PCR was run in a Kallur thermal cycler (Applied Biosystems) and then the mitochondrial cytochrome c oxidase I (*cox1*) gene was sequenced. The PCR reactions were performed in a total volume of 25 µl containing 3 µl template DNA, 0.5 µM each primer, 2.5 µl 10x PCR buffer, 2 µl MgCl2, 0.65 µl dNTPs, 15.7 µl H2O and 0.13 µl AmpliTaq Gold DNA polymerase. The following PCR protocol was used: 95 °C 10 min followed by a set of 40 cycles, 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min and final elongation at 72 °The amplified PCR product, 5 µl, was then subjected to 1% agarose gel electrophoresis. Positive samples were purified using Illustra ExoProStar 1-step and sent to Macrogen (Amsterdam, Netherlands) for sequencing. Editing and trimming of the obtained sequences were performed using CodonCode Aligner version 7.1.2 (https://www.codoncode.com/aligner/) and then blasted in GenBank to determine the species identity.

2.6. Droplet digital PCR for detection of taeniid eggs

To carry out ddPCR reaction, JB3/JB4.5 primers were used to amplify *cox1* in DNA from the different soil types. The ddPCR reaction

mixtures (22 µl each) were prepared for each sample containing 10 µl QX200 ddPCR EvaGreen Supermix (Bio-Rad Laboratories), 1 µl primer (250 nM), 9 µl molecular-grade H_2O (Sigma Aldrich, Stockholm, Sweden) and 2 µl DNA template and loaded in a 96 well plate. Prior to PCR amplification, a QX200 Automated Droplet Generator (Bio-Rad Laboratories) partitioned samples into approximately 20,000 nanoliter-sized droplets. After droplet generation, the plate containing the samples was heat-sealed with pierceable foil at 180 °C for 5 s (PX1 PCR Plate Sealer, Bio-Rad Laboratories). PCR amplification of the droplets was performed in a MyCycler thermal cycler (Bio-Rad Laboratories). The thermal cycling conditions for PCR reaction mixture amplification were an initial enzyme activation at 95 °C for 5 min, followed by 80 cycles of denaturation at 95 °C for 30 s and annealing at 54 °C for 1 min and final enzyme deactivation at 4 °C for 5 min and 90 °C for 5 min with a ramping rate of 2 °C /sec. Results of target DNA amplification in the droplets were read using a QX200 Droplet Reader (Bio-Rad Laboratories).

2.7. Data analysis

Data generated by the QX200 Droplet Reader were analysed using QuantaSoft version 1.7.4 software (Bio-Rad Laboratories). Analysis of variance (ANOVA) was performed to compare *cox1* detection results between species, soil types or egg concentrations. When ANOVA results were statistically significant ($p < 0.05$), Tukey's multiple comparisons *post hoc* test was used to compare the groups. Statistical data analyses employed Microsoft Excel 2016, R version 3.4.3 (R Foundation, Vienna, Austria) and GraphPad Prism version 8.0.0 for Windows (GraphPad Software, La Jolla California, USA) software.

3. Results

3.1. Assay optimization

The optimal annealing temperature was found to be 54 °C. The concentration of *cox1* copies in both species declined gradually relative to serial dilution.

3.2. Detection of cox1 among egg concentrations

The number of *cox1* copies increased with the number of *Taenia* eggs per gram in the spiked soil samples (Fig. 1). This increase was highly significant for both *T. solium* ($F_{3,32} = 29.160$, $p < 0.0001$) and *T. lynciscapreoli* eggs ($F_{3,32} = 44.17$, $p < 0.0001$).

3.3. Detection of cox1 among soil types

The detection range of *cox1* by ddPCR in the spiked soil samples was between 10 and 14,220 copies per ddPCR reaction mixture (Fig. 2). There were also significant differences in the total number of *cox1* copies in the spiked soil for both *T. solium* ($F_{3,32} = 3.091$, $p = 0.0408$) and *T. lynciscapreoli* ($F_{3,32} = 37.61$, $p < 0.0001$) detected by ddPCR. The highest *cox1* DNA copies recovery was detected in sand ($n = 31,317$, 36% for *T. solium* and $n = 28,775$, 58% for *T. lynciscapreoli*) and the lowest was detected in clay ($n = 15,438$, 18% for *T. solium* and $n = 6203$, 13% for *T. lynciscapreoli*).

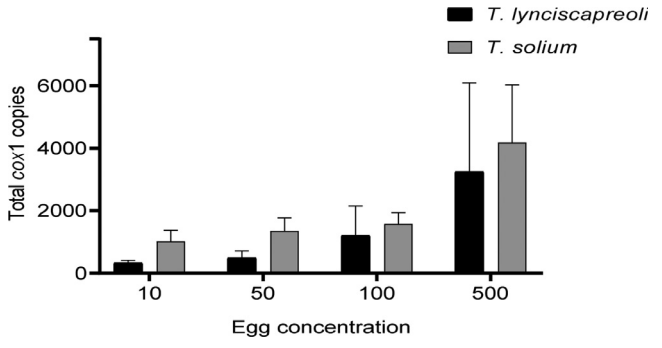


Fig. 1. Copies of *cox1* detected by ddPCR per eggs spiked in soil samples for the two taeniid species. The error bars represent standard deviation (SD).

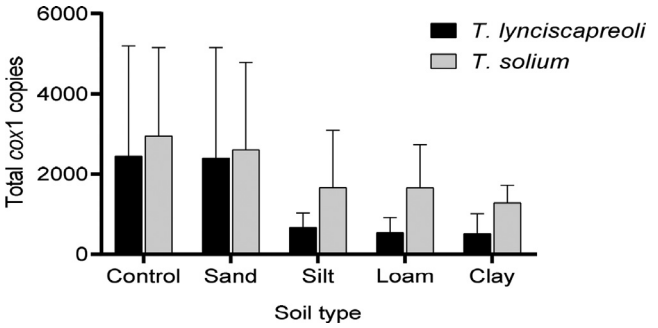


Fig. 2. Copies of *cox1* detected by ddPCR per soil sample for the two taeniid species. The error bars represent standard deviation (SD).

4. Discussion

In the present study, we tested for the first time a novel ddPCR technology for the detection of taeniid eggs in four different types of soil after egg recovery according to a protocol originally developed for detection of eggs of the closely related species *Echinococcus multilocularis* in fox faeces (Miller et al., 2016). Our results revealed that ddPCR technology is a promising tool capable of detecting DNA copies of *cox1* of different taeniids eggs after recovery from different types of soil.

For both species, the number of *cox1* copies increased with the number of eggs added to all types of soil. The increasing number of taeniid eggs recovered may explain the observed gradual increase in *cox1* copy numbers in proportional to the number of eggs spiked in soil samples and subsequently increasing the amount of taeniid DNA recovered from the eggs. Similarly the ddPCR technology have been reported to detect *A. lumbricoides* eggs in water samples (Soto et al., 2017) and *Schistosoma japonicum* in an experimental mouse model (Weerakoon et al., 2017a). In the present study, quantification of *cox1* in soil samples at a low taeniid egg concentration suggests an acceptable egg recovery efficiency of zinc chloride as a flotation fluid (density 1.45 g/ml), egg double sieving technique, DNA extraction protocol and detection efficiency of ddPCR protocol regardless of the types of soil.

Meanwhile, the recovery rates of taeniid eggs from soils with different texture spiked with varying egg concentrations for both taeniid species was statistically different. The sand soil gave a higher number of *cox1* than clay and all other types of soil for both *T. solium* and *T. lynciscapreoli* revealing the importance of considering the soil type in taeniid egg recovery. In addition, there was less variation in the number of *cox1* copies detected in loam, silt and clay soils. Differences of *cox1* copies detected between different soil types might be explained by differences in the number of eggs recovered from each soil type. Our findings are similar to a study by Nunes and others which recorded a higher *Toxocara canis* eggs recovery rates in sand compared to other soil types using the flotation method (Nunes et al., 1994). In addition higher recovery rates of soil-transmitted helminths eggs including *A. lumbricoides* and *Trichuris trichiura* were also observed in sandy than loamy soils (Steinbaum et al., 2017). Differences in soil types may determine *Taenia* infection dynamics in the pig and human population. However, the detection of taeniid DNA in soil per se does not necessarily indicate the presence of viable, infectious eggs, thus it is important to consider the viability of the eggs in the environment for assessing the risk of

transmission of *Taenia* infection in endemic areas.

The reported study is limited by the experimental soil samples, which were used in the laboratory, which could not be compared with field soil samples from *T. solium* endemic areas. In addition, we used universal primers, which can amplify DNA from a range of cestodes and trematodes. Therefore, the identity of positive samples must be confirmed by sequencing, for examples when screening environmental samples. More studies need to be done in the future to confirm the applicability of ddPCR protocol in screening *T. solium* eggs from environmental soil. It is also important to design species-specific primers in future studies for direct detection of *T. solium* DNA.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2019.105175.

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