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LIMIT OF MOLECULAR DETECTION OF *STRONGYLOIDES STERCORALIS* PARASITE PERFORMING TWO PCR PROTOCOLS

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Abstract

Low parasite density in chronic infection with *S. stercoralis* is a challenging diagnostic issue. Generally, molecular techniques don't depend on parasite viability while copro-culture or Baermann concentration method relies on the presence of living larvae in fecal samples. Therefore, evaluation of PCR-based methods is important to increase the detection rates in light or chronic infection. This study was designed to analyze the sensitivity of quantitative PCR (qPCR) and nested PCR (nPCR) regarding the minimum amount of *S. stercoralis* DNA template that can be reliably detected by each molecular technique. Strongyloides larvae were collected from cultured stool samples from suspected infected Egyptian children. After counting larvae present in a known volume under the microscope, DNA extraction was done and serial dilution of genomic materials was prepared. Then, qPCR and nPCR targeting the small subunit of the rRNA gene were performed. Regarding qPCR, the limit of detection was 0.0005 *S. stercoralis* larvae/µl, with crossing threshold (Ct) value ranged from 17.8 to 38.7 while, nPCR did not detect from (0.002 to 0.00012 *S. stercoralis* larvae/µl) with minimum limit 0.004 *S. stercoralis* larvae/µl. Real-time quantitative PCR is very sensitive technique that can detect very low genomic load up to about 10 (9.765) genome copies/reaction compared to nested PCR which started positivity from 78.125 genome copies/reaction. Therefore, qPCR is recommended to detect chronic strongyloidiasis especially in high-risk groups to prevent life-threatening spread of such infection.

Keywords: *Strongyloides stercoralis,* Limit of detection, Nested PCR, Quantitative PCR.

Introduction

Strongyloides stercoralis is a geohelminthic intestinal nematode, endemic in tropical and subtropical areas of the world due to favorability of ecological conditions in such areas. The recorded number of infected individuals is between 30 to 100 million people (Paula *et al,* 2015). The prevalence rate in Egypt was reported to be from 1.5 to 11% (Salem *et al,* 1990; Rayan *et al,* 2012). This nematode is known to affect lower socioeconomic individuals especially those living in the rural communities and walking bare feet (CDC, 2013a). The parasitic stages inhabit the sub mucosa of the intestine. The infection is chronic and can be maintained asymptomatically for long times through cycles of internal or external autoinfection (Mejia and Nutman, 2012). Strongyloidiasis may progress into a very serious condition if infected patients become immunosuppressed in view of the fact that *S. stercoralis* disseminates through body (Schär *et al,* 2013**)**. Geneidy and El-Hamshary (2012) reported a fatal chronic case in an immunocompromized Egyptian patient with pulmonary infection. Transmission of *S. stercoralis* is also known to occur through organ transplantation. The majority of *Strongyloides* infections in organ transplant recipients are thought to arise when patients receive immunosuppressive therapy and the fatality rate among these patients is greater than 50% (CDC, 2013b). Therefore, detection of chronic strongyloidiasis in high-risk groups is pivotal to prevent such life-threatening infection (Paula and Costa-Cruz, 2011).

Due to the chronic nature of the hidden parasitic stages, the parasite density of *S. stercoralis* is usually low, thus requires highly sensitive diagnostic methods to be discovered. Standard dia-gnostic procedures

in humans include both stool concentration and culture methods. A combination of these methods donated high sensitivity especially if multiple stool samples were analyzed and always laborious and time-consuming (Siddiqui and Berk, 2001). Also, Massoud *et al*. (2006) reported that *S. stercoralis* diagnosis must be considered as a challenge to clinician and laboratory technician due to difficult eradicated auto-infective larvae. Many serological methods are available with variable sensitivities and specificities depending on the antigen used, immunoglobulin isotypes and nature of the studied groups (Ravi *et al,* 2002; Silva *et al,* 2003; Rodrigues *et al,* 2007). But, cross-reactivity between the different nematode species was recorded to be a major limitation in such tests, in addition to low sensitivity when testing travelers returning from endemic areas (Sudarshi *et al,* 2003; Kramme *et al,* 2011).

DNA detection is increasingly used to recognize parasitic stages and it could help solving current problems in human strongyloidiasis diagnosis (ten Hove *et al,* 2009). But, the advantages and limitations of conventional polymerase chain reaction (cPCR) and real-time quantitative PCR (qPCR) in diagnosis of human strongyloidiasis was not fully discussed in the literature especially items concerning their analytical sensitivity.

This study aimed to analyze the sensitivity of nPCR and qPCR regarding the minimum amount of *Strongyloides stercoralis* DNA template that can be reliably detected by each molecular technique.

Subjects, Materials and Methods

Collection of samples: A total of 60 stool samples from children suspected to have *S. stercoralis* were collected from outpatient clinic of Pediatric Department, Faculty of Medicine, Fayoum University. For detecting *S. stercoralis* infected samples, parasitological examination was performed by individual stool cultures on agar plate medium (Arakaki *et al,* 1990). Three grams of each fecal sample was placed in the center of nutrient agar plate. Then the plates were incubated for 3 days at room temperature (26-30°C). The plates were examined every day for three successive days under the microscope for the presence of moving larvae or free-living adults or their tracks on the surface of the agar. According to Markell (2006), the surface of the agar related to the positive samples was washed with PBS (Phosphate buffer saline) solution to collect filariform larvae of *S. stercoralis* using Baermann apparatus. The supernatant containing larvae and/or adults were fixed in 10% formalin for identification. Considering morphological characters of the L3 larvae, differential diagnosis from other possible nematodes, especially *Rhabditis* spp. and *Trichostrongylus* spp., was performed (Inatomi *et al,* 1981).

Calculation of limit of detection (LOD): LOD refers to the minimum number of gene copies in a sample that can be measured with a PCR reflecting the accurate sensitivity of the molecular assay. To achieve this, the study followed the method of Bustin *et al.* (2009), in which serial dilution of target nucleic acid was prepared after counting the larvae present in a known volume under the microscope. The estimation of the number of gene copies in the eluted volume was made according to the postulation of Alberts *et al.* (1994). It was estimated that one *S. stercoralis* larva consists of approximately 2000 cells. Taking into consideration that each cell of *S. stercoralis* is diploid means that two copies of the genome are present in each cell, so; the genomic copies in each cell were 4000. If there was one larva in one microlitre, the genome number copies/µl would be 4000 and in 5µl reaction be 20,000.

DNA extraction and preparation of serial dilutions: Sample was first subjected to grinding by liquid nitrogen, and then WIZARD genomic DNA purification kit was used for DNA extraction. For preparation of serial dilutions, DNA was dissolved in DNA solution Ultra-Pure™ (Applied Biosystem) to block non-specific hybridization. Based on the previous estimation of amount of lar-

vae per volume, a dilution with 1 larva/ µl was prepared. This solution was serially diluted from 0.5larva/µl to 0.00012 larva/µl and genome copies/ reaction were calculated (Tab. 1) to assess the limit of detection in both molecular techniques.

Table 1: Properties of each of eleven dilutions of nucleic acids extracted from one *S. stercoralis* larva/µl. 5 µl of template DNA added to one reaction

Dilution factor		no of larvae/µl	no of genome copies/reaction (5µl)
1	1:2	0.5	10000
2	1:8*	0.125	2500
3	1:32*	0.031	625
4	1:64	0.016	312.5
5	1:128	0.008	156.25
6	1:256	0.004	78.125
7	1:512	0.002	39.06
8	1:1024	0.001	19.5
9	1:2048	0.0005	9.76
10	1:4096	0.00025	4.88
11	1:8192	0.00012	2.44

*Some dilutions excluded to decrease number of diluted samples to reach lowest limit of dilution for molecular detection.

Nested PCR: Following the protocol of Nilforoushan *et al.* (2007), with minor modifications, nested PCR primers for *Strongyloides* were used; SS-F0 Forward: (5`-ATC CTT CCA ATC GCT GTT GT- 3`) and SS-R0 Reverse: (5`-TTT CGT GAT GGG CTA ATT CC-3`) to amplify product of 750 bp containing internal transcribed spacer (ITS) regions ITS-1 5.8S and ITS-2 of the ribosomal DNA gene, for each set of PCR reaction. Nested PCR was performed in a 25µl volume containing 2×PCR Master mix, primers (25pmol each) & DNA template (5µl). Cycling condition started by an initial denaturation step at 95°C for 7 min, 30 cycles of denaturation at 94°C for 45s, annealing at 55°C for 90s, extension at 72°C for 90s followed by a final extension at 72°C for 5 min. Consequently, 5µ of 1/10 diluted of first run amplicon was subjected to a second amplification round, using nested primers; Forward SS-F1(5`-GTA ACA AGG TTT TCG TAG GTG AA-3`) and SSR-1 Reverse: (5`- ATT TAG TTT CTT TTC CTC CGC TT-3`) resulted in a product of 680bp. Cycling conditions were in form of an initial denaturation step at 94°C for 3 min, 30 cycles of denaturation at 94°C for 45s, annealing at 60°C for 45s, extension at 72°C for 1min, followed by a final extension at 72°C for 5min. Negative controls were used as no template control (distilled water & DNA extracted of negative stool samples). Products of nested PCR were loaded on 1.5% TBE (Tris 0.09M- borate 0.09M-EDTA 0.02M) agarose gels. Gel contained 0.5µg/ml ethidium bromide for staining. Electrophoresis was done in 45 min at 85V. Ultraviolet light visualized stained DNA.

Real-time PCR: Fluorescence resonance energy transfer (FRET)-based real-time PCR assay for *Strongyloides* species targeting the 28S rRNA gene was performed using *S. stercoralis*-specific primers and probes. A 20µl reaction contained 2µl PCR buffer (Applied Biosystems), 200µM of each dNTP (Applied Biosystems), 2.5mM MgCl2, 750 nM StroS (5"-TTA GAG TCG TGT TGC TTG GAA-3"), 1µM StroAS (5"-GTG CAA CTG GCT CTG TAT GC-3"), 50nM StroP3 (5"-CTG TGA AGG AAA ATT GCA AAG TAC TCC GGA-FAM-3"), 200 nM StroP4 (5"-LC640-GAG AGT TAA AGA GGA CGT GAA ACC GAT ACG-PH-3"), 200 nM StroP5 (5"-LC705-ATC GTT CGT TGA GCG ATT AGC AGTTPH-3"), 0.8µg BSA & 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems). Five microliters of DNA solution were added to each reaction. Thermal cycling in a Light Cycler 1.5 Instrument (Roche Diagnostics) included denaturation at 95°C for 15min, followed by

45 cycles of 95°C for 5s, 50°C for 20s & 72°C for 30s. Fluorescence was measured at 50°C step on detection channel F2 (640nm). Non-template controls were used with every batch of reaction solution to scan for cross-contamination or template free amplification (Kramme *et al,* 2011).

Ethical considerations: Ethical permission was obtained from the manager of the Pediatric outpatient clinic, Faculty of Medicine, Fayoum University. After informing about the study purpose, written consent was taken from all parents to participation optional.

Results

Regarding qPCR, fluorescent signals were detected in first 9 dilutions (Fig. 1), thus, the limit of molecular detection was 0.0005 *S. stercoralis* larvae/µl. Cycle threshold (crossing threshold Ct) value ranged from 17.8 to 38.7 and it was inversely proportional with the samples' dilution (Higher the concentration, the lower Ct). Nested PCR failed to detect last 5 dilutions from 0.002 to 0.00012 *S. stercoralis* larvae/µl (fig. 2). Thus, limit of molecular detection was 0.004 *S. stercoralis* larvae/µl.

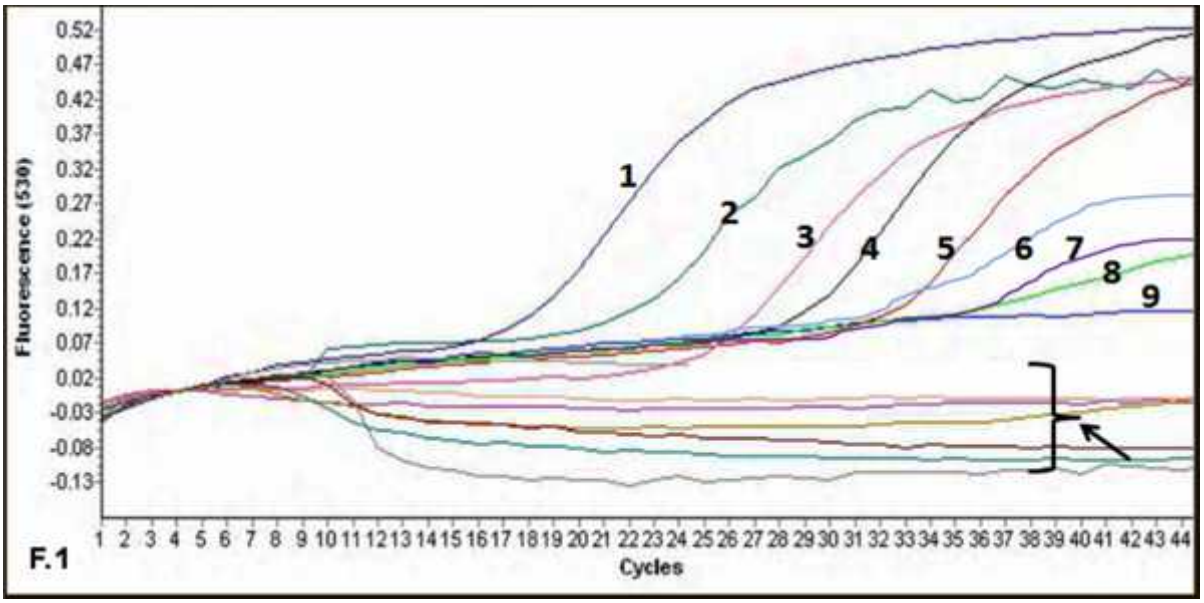

Fig. 1: Quantitative real time PCR shows positive curves for first serially diluted 9 samples. Arrow below directed to negative results; samples with lowest dilutions and negative control samples.

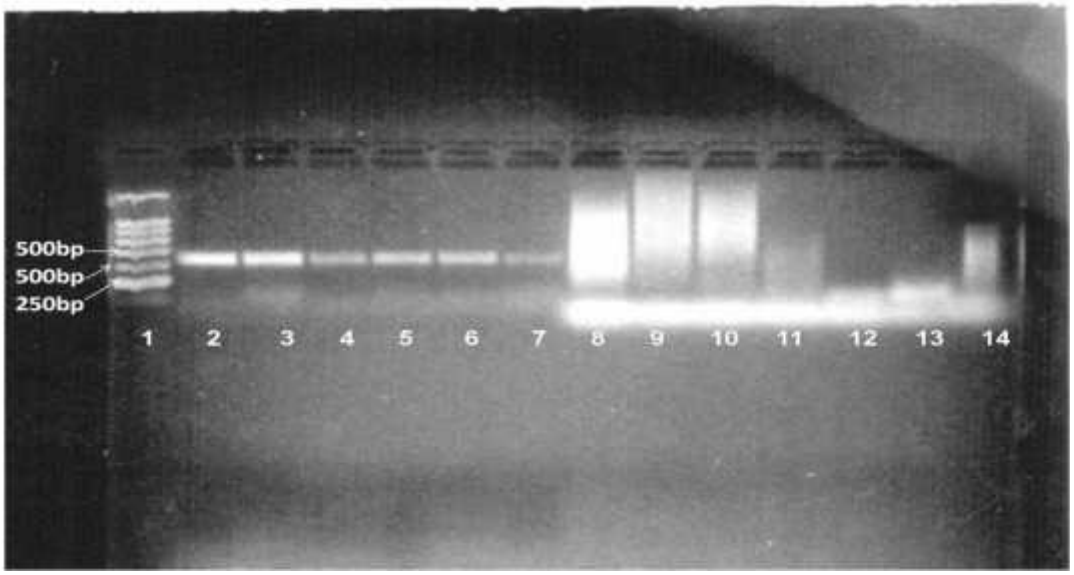

Fig. 2: Nested PCR; lane 1 = DNA marker, 250bp; lane 2 to7 showed positive reactions represents the nested primers with 680bp products for first 6 dilutions, lane 8 to 12 (last 5 dilutions), last 2 lanes = negative control samples (no template negative control).

QPCR detected very low genomic load up to about 10 (9.765) genome copies/reaction. While bands were visualized in nPCR in the first 6 dilutions only up to 78.125 genome copies/ reaction. No signals (qPCR) or bands (nPCR) were observed in negative control samples. The high dynamic range of qPCR accurately differentiated the variable concentration. Cycle threshold discriminated accurately between different samples with different genomic equivalents while more or less similar bands' intensity was visualized in nPCR. Bands 2 & 3 were akin, in addition to bands 5 and 6 which were analogous as well and, band 4 looks weaker than 5 and 6 despite its higher concentration (Fig. 2).

Discussion

In general, failure of the parasitological methods to detect many *Strongyloides* infected cases could be explained either by the intermittent excretion of larvae due to chronic infections or by the presence of dead larvae in stool (Sing *et al,* 1999). In contrast to PCR, which does not depend on parasite viability, detection of *S. stercoralis* in culture or Baermann concentration method depends on the presence of living larvae in fecal samples (Garcia, 2009). Therefore, evaluation of PCR-based methods was suitable alter-native method to increase the detection rates especially in cases with light infection are needed. In this study, the results of the analytical sensitivity showed that qPCR was able to detect an estimated 0.0005 *S. stercoralis* larvae/µl of extracted DNA; equivalent to 9.76 number of genome copies/ reaction. While nested PCR was able to detect lower number of serially diluted samples with the lowest limit of detection 0.004 *S. stercoralis* larvae/µl.

In studies conducted by ten Hove *et al*. (2008) and Calderaro *et al.* (2010) the qPCR assay was developed for the simultaneous detection of *Schistosoma* spp. and *Dientamoeba fragilis* respectively and revealed very low detection limits for this molecular assay. Previously, Weiss (1995) demonstrated that qPCR assay detected as few as 10 copies of target sequence present in a specimen and proved the ability of real-time qPCR to detect the DNA of all stages of the parasite. Rayan *et al.* (2012), qPCR identified all *S. stercoralis* positive samples diagnosed by conventional parasitological techniques, with a sensitivity of 100%. The higher sensitivity of qPCR in relation to parasitological methods was reported (ten Hove *et al,* 2009) as well in diagnosis of *Strongyloides* and other intestinal parasites in returning travelers. Also, Verweij *et al.* (2009) reported a high sensitivity of real-time qPCR compared to other parasitological techniques. Rayan *et al.* (2012) in comparison between molecular and conventional parasitological methods they reported that real-time PCR was able to detect 5 *S. stercoralis* positive samples not detected by any other conventional techniques. However, lack of genomic concentration and analytical sensitivity in the previous work did not allow us to compare our results with theirs. Though, the authors explained that the use of a highly specific Taq-Man probe and the use of one step closed tube qPCR method prevented the possibility of false-positive results (Smith *et al,* 2004). In addition, the higher Ct-values obtained from *S. stercoralis* negative samples compared to the samples tested positive by conventional techniques indicated that they contained a low parasite load (Verweij *et al,* 2009). Paula *et al.* (2015) compared the results showed that qPCR was more sensitive than cPCR. However, disagreements in the results were observed in all groups; several samples were negative by cPCR, but were positive by qPCR and the opposite was observed, as well. This supports the present authors' suggestion that more studies on qPCR are needed to improve the sensitivity of the molecular technique that showed great discrepancies between published articles onj molecular diagnosis of *S. stercoralis.*

In the present work, PCR cycle threshold (Ct) values ranged from 17.8 to 38.7 reflecting parasite-specific DNA loads in samples.

The more the sample was diluted, the higher the Ct value. The results agreed with Supali *et al.* (2010) and Knopp *et al.* (2014) who showed highly significant correlation between the ova count or the number of larvae found in the samples and Ct values in intestinal parasitic-specific qPCR. Therefore, it was concluded that the Ct values were inversely proportional to the amount of parasite-specific DNA in the sample (ten Hove *et al,* 2009) and the lower the number of larvae in the sample, the harder to be diagnosed by conventional methods, but the parasite DNA could be detected by real-time PCR giving higher Ct values (Rayan *et al,* 2012). Besides, the excretion and distribution of parasite DNA in the stool is expected to be less variable than the number of parasitic stages (ten Hove *et al,* 2009).

On the other hand, in this study, a nested PCR targeting ITS regions of the ribosomal DNA gene was used to amplify *S. stercoralis* DNA. ITS region showed to be useful for species identification due to its lower sequence variety within the same species (Nilforoushan *et al,* 2007). The region was relatively short and repetitive in nature, resulting in a more sensitive PCR assay suitable for diagnostic use (Gasser, 2001). Nested PCR technique was able to detect the first 6 sample dilutions in the current work with a minimum limit of detection of 0.004 larvae/µl of extracted DNA**;** equivalent to 78.125 numbers of genome copies/reaction. The relative lack of sensitivity of the nPCR technique for the detection of light helminthic infections was reflected in our study with its failure to detect the last three dilutions (from 0.002 to 0.0005) that could be detected by qPCR.

Ahmad *et al.* (2013) investigated serological and molecular approaches for the detection of *S. stercoralis* infection among an indigenous community in Malaysia. The study revealed amplification of *S. stercoralis* DNA of three individuals, which were negative by microscopic examination but positive for anti-parasite antibody. The authors cleared that nested PCR used involved two separate amplification reactions using two sets of primers to raise the detection sensitivity compared to cPCR, which involved only a single amplification reaction (Nilforoushan *et al,* 2007). Therefore, even low numbers of *S. stercoralis* DNA present in stool samples could be amplified in the secondary PCR reaction using enough template DNA obtained from the primary PCR. Moreover, the use of the secondary set of primers would also raise the specificity of the nPCR method by allowing more target-specific genetic region within the primary PCR product to be amplified (Van Pelt-Verkuil *et al,* 2008). On the contrary, Moghaddassani *et al.* (2011) revealed that the sensitivity of a single PCR was higher than nested PCR. In nested PCR, only in 75% of infected samples (12/16) *S. stercoralis* DNA were amplified; while in single PCR all positive samples were detected. The false negative results of nested PCR might be due to the size of amplified fragment which was very small in single PCR.

Conclusion

The real-time PCR method is considered as one of the most sensitive and specific tests available for the evaluation of the presence of *S. stercoralis* larvae in stool samples. It can detect as few as 10 copies of target DNA present in a specimen. Therefore, in cases that the worm burden is too low or dead larvae are present and could not be detected on agar plate culture, the application of PCR will be useful. The technique is rapid, easy to perform and interpret the data besides the ability to visualize the reaction and catch positive samples at the moment of the crossing point.

Despite that qPCR is relatively costly method but it is becoming essential where screening for infectious agents is essential in many circumstances including organ transplantation. The ability to detect multiple parasites simultaneously in one setting using multiplex real-time PCR certainly will save time, effort and cost in such situations. Molecular techniques have increasingly been used as diagnostic tools for parasites; however, more studies in the future are of essential importance for discovering new target sequences and also for improving the interpreta-

tion of results. This would contribute to a more efficient and less time-consuming diagnostic procedure.

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