Conventional Multiplex PCR For Identify Soil Transmitted Helminthes (*Ascais Lumbricoides, Trichuris Trichura* And *Ancylostoma Duodenale*) In Fecal Specimens

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Abstract

Ascaris lumbricoides, Trichuris trichiura, and *Ancylostoma duodenale* are soil-transmitted helminths (STHs) and medically neglected in Iraq country in spite of their effect on the public health. A cross-sectional study was performed in the Maternity and Childhood Teaching Hospital and General Education Hospital in Al-Dewanyia province, included 850 tool samples collected from patients who attended to the O&P lab. General stool examination (GSE), Direct wet mount method DWMM and Kato-Katz were using for diagnosis of STH infections through detected the adult and the ovum of the helminthes. A conventional multiplex PCR assay was used for detection of STHs in fecal samples. Base on microscopic examination. The results showed that 275/ 850 range among triple, double and single infection on other hand was 365/ 850 range among triple, double and single infection. In conclusions the investigative sensitivity of DWMM is notable for STH, in exception, it is capable to identify patients with the intention of highest required of management, and therefore contributes to the universal target to reduce STH as a community healthiness trouble.

Introduction

Soil transmitted helminthes (STH) are a group related to nematode helminthes responsible for high morbidity in many of the world's with a low economically status. With growing stress and a new strain on disease mapping and eradication, infections mostly remain undiagnosed due to lack of trained personnel and appropriate technologies[1]. Intermittent shedding of eggs or larvae further makes the diagnosis difficult Thus, there is a dire need of rapid and accurate tests for the diagnosis of STHs [2]. The diagnostic methods include conventional and molecular methods for the accessibility of the exact and low coast diagnostic measures is of dominant importance to worldwide control and suppression efforts [3, 4]. The soil transmitted helminthes are recurrently occurs in distant and lack distress areas where lowest hygiene, soil transmitted helminthes *Ascaris lumbricoides, Trichuris trichiura*, and hookworms (*Necator americanus* and *Ancylostoma duodenale*) are the most helminthes infestations worldwide[5, 6].

Material And Methods

Samples: Eight hindered and fifty stool samples were collected from patient who attended to the O & P lab in the Maternity and Childhood Teaching Hospital and General Education Hospital in Al-Dewanyia province from the beginning of May 2019 to the ending of may2021, Ethical agreement of the project was obtained from the Medical Ethics Committee.

General stool examination (GSE) were done and also approach is the Kato-Katz were used for detects the egg and adult of the worms[7]. formal-ether concentration (FEC) method which has lower specific gravity than the helminthes, thus concentrating the worms' eggs in the sediment [8]. About two gram of all samples were conserved in five ml of 70% ethanol alcohol.

Diagnosis of *Ascaris lumbricoides, Trichuris trichiura*, and *Ancylostoma duodenale*. By detected an egg in a wet mount through making a smooth, thin preparation (with saline and iodine) and cover with a cover glass[9].

For extract the DNA from stool samples, 0.5 ml of conserved ethanol alcohol stool samples were twice washed with deionizer sterile distal water and was freezing rapidly at -20ºC, DNA was extracted using Stool DNA separation Mini-Kit (Bioneer. Korea). Concentrations of DNA were resolute via NanoDrop® spectrophotometer ND-1000 (NanoDrop Technologies, Wilington, DE) and the extracted DNA was situate at -20ºC for analyzed.

Primers: Primers for detection *Ascaris lumbricoides, Trichuris trichiura, and Ancylostoma duodenale* [10, 11] were used as in Table 1

Table 1
Specific primers for detection of *Ascaris lumbricoides, Trichuris trichiura* and *Ancylostoma duodenale* using in conventional multiplex PCR [11].

Primer of helminthes spp. target DNA	**Sequence 5'-3'**		**Accession number**	**Length (nt)**	**Amplification**
A. lumbricoides **COI**	**F**	**5’ GGAGGTTTTTGGGTCTTTGG 3**	**EU582499**	**20**	**192bp**
	R	**5’ CCAAACAAGGTAGCCAACCA 3’**		**20**	
A. duodenale **ITS2 r DNA**	**F**	**5´-GAT GAG CAT TGC WTG AAT GCC G-3´**	**KC896800**	**20**	**330bp**
	R	**5´-GCA AGT RCC GTT CGA CAA ACA G-3**		**20**	
T. trichiura **18rS DNA**	**F**	**5’ CTGCGAGGATTGACAGATCA 3’**	**GQ352548**	**20**	**498bp**
	R	**’ GTACAAAGGGCAGGGACGTA 3’5**		**20**	
hGPDH		**’5 GCATCCTGGGCTACACTGAG 3’**	**XM005253678**	**20**	**150**
		’ TGCTGTAGCCAAATTCGTTG 3”5		**20**	

Statistical Analysis

Data were translated into a computerized database structure. An expert statistical advice was sought for. Statistical analyses were computer assisted using SPSS version 21 (Statistical Package for Social Sciences). Frequency distribution for selected variables was done first. The statistical significance, direction and strength of linear correlation between 2 quantitative variables, one of which being non-normally distributed was measured by Spearman's rank linear correlation coefficient. P value less than the 0.05 level of significance was considered statistically significant [12].

Results And Discussion

General stool examination and staining methods result:

The studied cases were classified into three categories based on results of microscope and conventional multiplex PCR into single, double and triple infection.

The microscopically result shows that a total patients infected with STH **(*Ascais lumbricoides, Trichuris trichura and Ancylostoma duodenale*)** was 275/ 850 range among triple, double and single infection as in table 2image 1.

Table 2
The percent of single, double and triple infection of STH based on microscopical examination.

Microscopical examination	Positive results	Percent	P value
Ascais lumbricoides	64	0.07%	**NS**
Trichuris trichura	55	0.06%	
Ancylostoma duodenale	35	0.04%	
Total single infection	**154**	**18.11%**	**P < 0.05**
Ascais lumbricoides and *Trichuris trichura*	43	0.05%	**NS**
Ascais lumbricoides, and *Ancylostoma duodenale*	33	0.03%	
Trichuris trichura and *Ancylostoma duodenale*	30	0.03%	
Total double infection	**106**	**12.47%**	**P < 0.05**
Total of triple infection Ascaris lumbricoides, Trichuris trichiura, **and** Ancylostoma duodenale	**15**	**1.17%**	
Total infection	275	32.35%	**P < 0.01HS**

HS: High significant association (P <0.01)

NS: no significant

As shown in Table 2 and image 1, the single infection of *Ascais lumbricoides* highest among those with *Trichuris trichiura*, and *Ancylostoma duodenale*, while the lowest was *Ancylostoma duodenale*. The percent of double infection was lower than single infection while the triple infection was the lowest. The difference in P value among three helminthes was no significant while among three category (single, double and triple) infection was statistically significant.

It could not found a field consist a triple infection and also could not found an image consist a double infection with *Trichuris trichura* and *Ancylostoma duodenale* .

The standard method for STH diagnosis in these programs is the Kato-Katz thick smear. Beyond these programs, Kato-Katz is rarely used. Instead, the direct wet mount microscopy (DWMM) is the most frequently used routine diagnostic method for STH and other intestinal parasitic infections in health care facilities in Iraq country [13–15].

approximately the health care services in developing countries utilize DWMM because its lowest cost, simple methods and can be to identify a wide range of GIT parasites, including STH and protozoa [15, 16]. Even if its sensitivity is poor is in general accepted, there are few researches had assessed the diagnostic sensitivity of DWMM for STH[17, 18].

The present study confirmed the poor sensitivity, but it also painted significant differences both among soil-transmitted helminths. The diagnostic sensitivity of DWMM for *Ascaris* infection was significantly higher compared to that for both *Trichuris* and *Ancylostoma*, and the sensitivity better with heavy infections [18, 19].

Multiplex PCR methods result:

The result shows that a total patients infected with STH **(** Ascais lumbricoides, Trichuris trichura **and** Ancylostoma duodenale **)** was 365/ 850 range among triple, double and single infection as in Table 3.

Table 3

The percent of single, double and triple infection of STH based on multiplex PCR examination.

Multiplex PCR methode	Positive results	Percent	P value
Ascais lumbricoides	**81**	**0.09%**	**NS**
Trichuris trichura	**70**	**0.08%**	
Ancylostoma duodenale	**48**	**0.05%**	
Total single infection	**199**	**23.41%**	**P < 0.05**
Ascais lumbricoides and *Trichuris trichura*	**58**	**0.06%**	**NS**
Ascais lumbricoides, and *Ancylostoma duodenale*	**49**	**0.05%**	
Trichuris trichura and *Ancylostoma duodenale*	**39**	**0.04%**	
Total double infection	**146**	**19%**	**P < 0.05**
Total of triple infection *Ascaris lumbricoides, Trichuris trichiura*, and *Ancylostoma duodenale*	**20**	**0.02%**	
Total infection	**365**	**42.41%**	**P < 0.01HS**

HS: High significant association (P <0.01)

The speedy, high sensitive of molecular techniques, predominantly multiplex PCR make it suitable for diagnose STH. Until now, molecular revealing of STH was mostly limited to the research and study, on other hand, there is agreement of adopting molecular tests in the World Health Organization STH elimination programs[20]. Thus, STH infections are important public health problems and should be correctly diagnosed and treated to reduce the mortality and morbidity significantly[21].

Conventional multiplex PCR Validity for revealing of STH infestations:

Table 4
Validity parameters for conventional multiplex PCR and microscopically examination.

Multiplex PCR				PPV at pretest probability =		NPV at pretest probability =	
Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	10%	50%	10%	50%
Single infection *Ascaris lumbricoides,*	95.0	87.0	78.9	64.9	97.3	76.5	98.4
Single infection *Trichuris trichiura,*	90.0	85.0	89.3	56.8	92.2	100.0	100.0
Single infection *Ancylostoma duodenale*	94.5	100.0	97.6	89.0	100.0	88.9	47.1
double infection *Ascaris lumbricoides, Trichuris trichiura*	89.0	87.8	93.5				
Double infection *Ascaris lumbricoides, Ancylostoma duodenale*	100.0	98.5	98.8	98.5	99.8	100.0	100.0
Double infection *Trichuris trichiura, and Ancylostoma duodenale*	81.3	100.0	96.4	100.0	100.0	84.2	37.3
Total of triple infection *Ascaris lumbricoides, Trichuris trichiura, and Ancylostoma duodenale*	87.0	100	96.0	91.0	100.0	86.3	38.8

The multiplex PCR was detected in the form *A. lumbricoides* COI, *T. trichiura* 18rS DNA and *A. duodenale* ITS2 r DNA, genes, the results showed that a single infection were 0.09%,0.08% and 0.05% respectively while a double and triple infection were 19% and 0.02%, The test was highly specific with a single infection(100) while in the double and triple infection were (87 and 98) respectively and resulting in low false positive test results (7.1%). Testing positive productive value (PPV) would establish the diagnosis of soil transmitted helminthes with a single infection of *Ascaris lumbricoides, Trichuris trichiura, and Ancylostoma duodenale* (97.3,92.2 and 100.0) respectively confidence at the highest pretest probability of 50%, while it would be unreliable at the low pretest probability of 10% (37% confidence). Testing negative productive value(NPV) on the other hand would exclude a possible diagnosis of with 93% confidence a single infection of *Ascaris lumbricoides, Trichuris trichiura, and Ancylostoma duodenale* at the lowest pretest probability, while it would of low usefulness at the 50% pretest probability (NPV = 59.8%).

The Receiver Operating Characteristics (ROC) curve and area results

To test the validity of the molecular technique in the presence of the infection STH, two receiver operating characteristics (ROC) were obtained for the four genes .As shown all the 4 gens were highly useful (of high validity) in predicting pathogenic STH (ROC area > 0.9) among cases group.

The microscopic examination still used as a usual method for diagnosing STH infections in Iraq country hospitals. even if, this technique is easy and cheap but it was low sensitivity, particularly in patients who are carrier. One option is PCR assay, which it's a sensitive and specific and has a elevated throughput ability that be able to be used for finding of STH [22].

Conclusion In conclusion, sensitivity and specificity of DWMM is poor for STH, especially when the infection was low intensity. On the other hand, DWMM is capable to distinguish those subjects that were in the heavy infection and need a management, and therefore contributes to the universal aim to eradicate STH as a community problem.

Declarations

Conflict of Interests

The authors declare that no competing interest exists.

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Figures

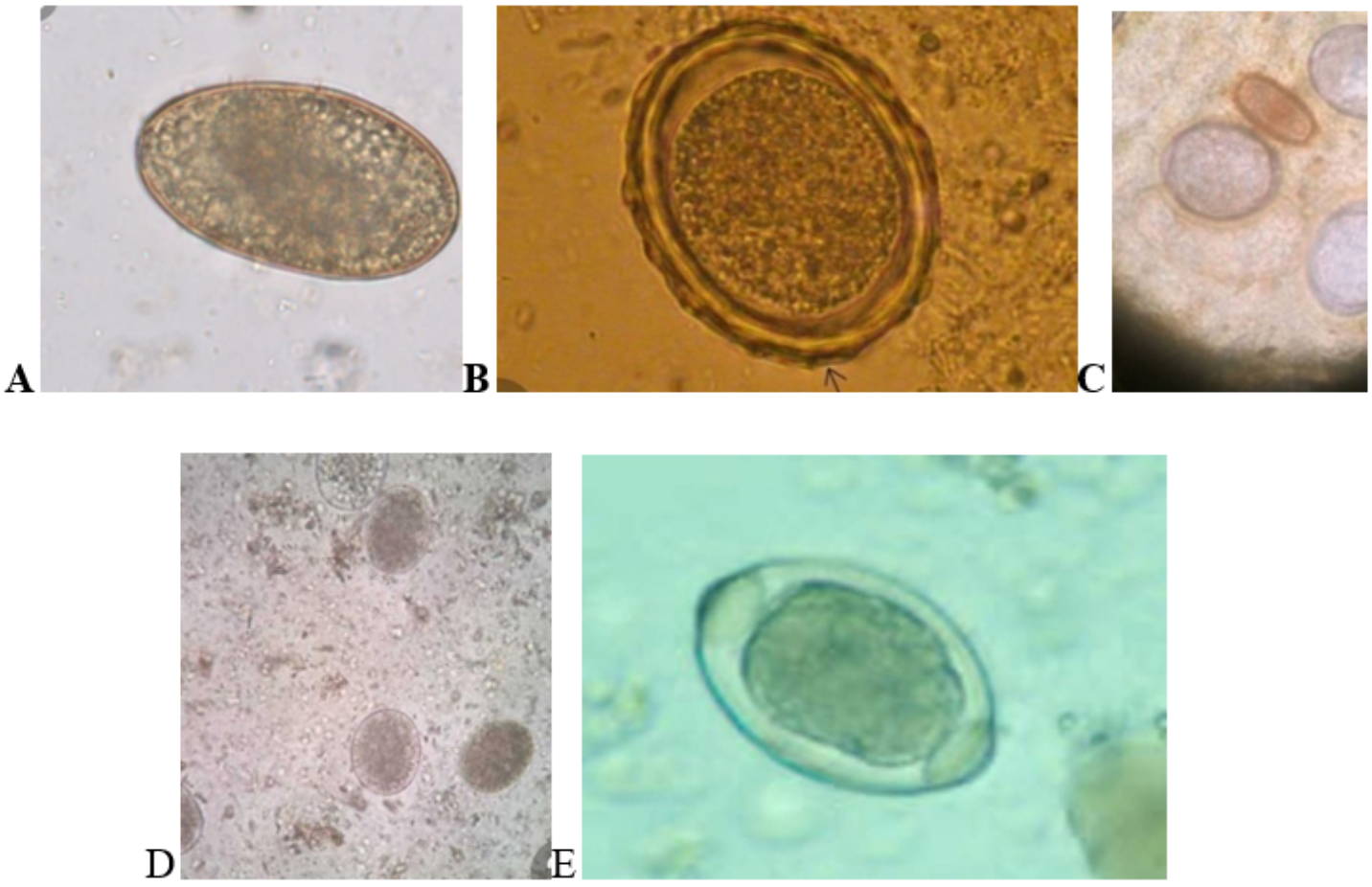

Figure 1

A. Ancylostoma douodenale ova , B. Ascaris lumbrecoides ova, C.double infection Ascais lumbricoides and Trichuris trichura ovum, D.double infection Ascais lumbricoides, and Ancylostoma duodenale ovum, E. Trichuris trichura ova. X=400

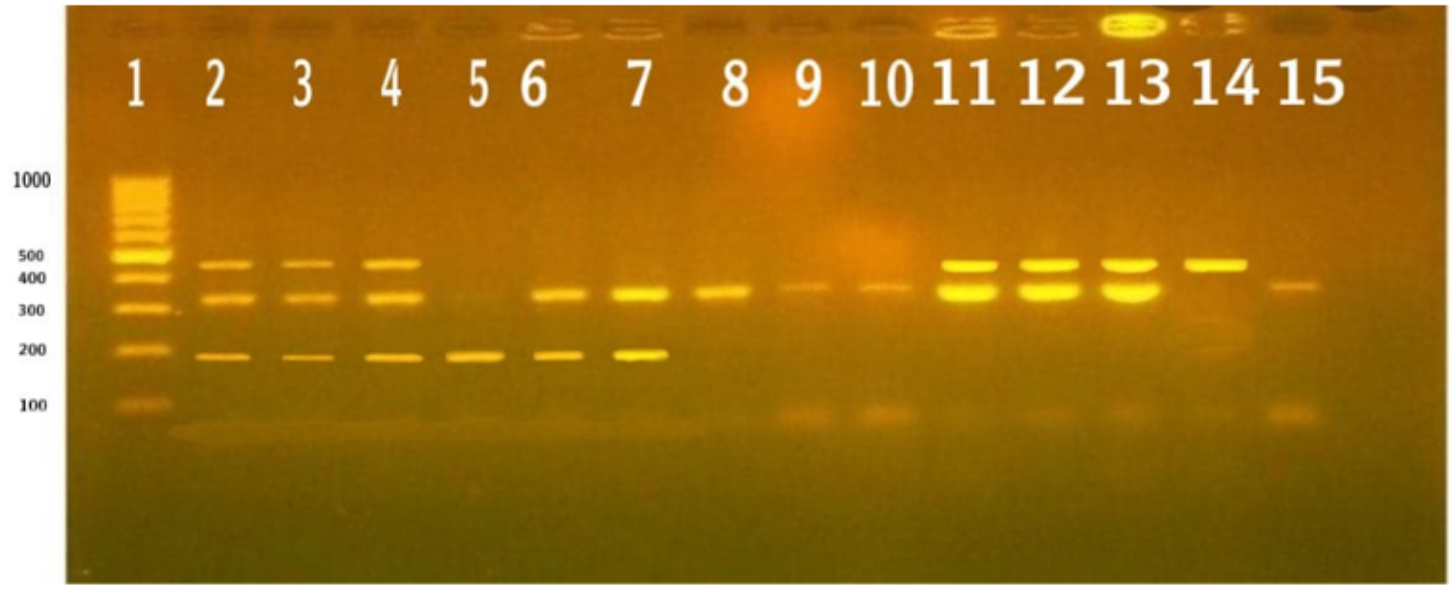

Figure 2

Multiplex PCR detection of STHs. Staining by ethidium bromide stain- separated by 2% agrose. lane 1 =DNA marker (100-1000), Lane 2,3,and 4 = triple infection (192bp Ascais lumbricoides, , 330bp and Ancylostoma duodenale, , 498bp Trichuris trichura) Lane 5 = single infection (192bp Ascais lumbricoides), Lane 6,7= double infection (192bp Ascais lumbricoides and 330bp Ancylostoma duodenale), Lane 8,9,10,15 = single infection (330bp Ancylostoma duodenale), Lane11,12,13 = double infection (330bp Ancylostoma duodenale, and 498bp Trichuris trichura), Lane 14= single infection (498bp Trichuris trichura)

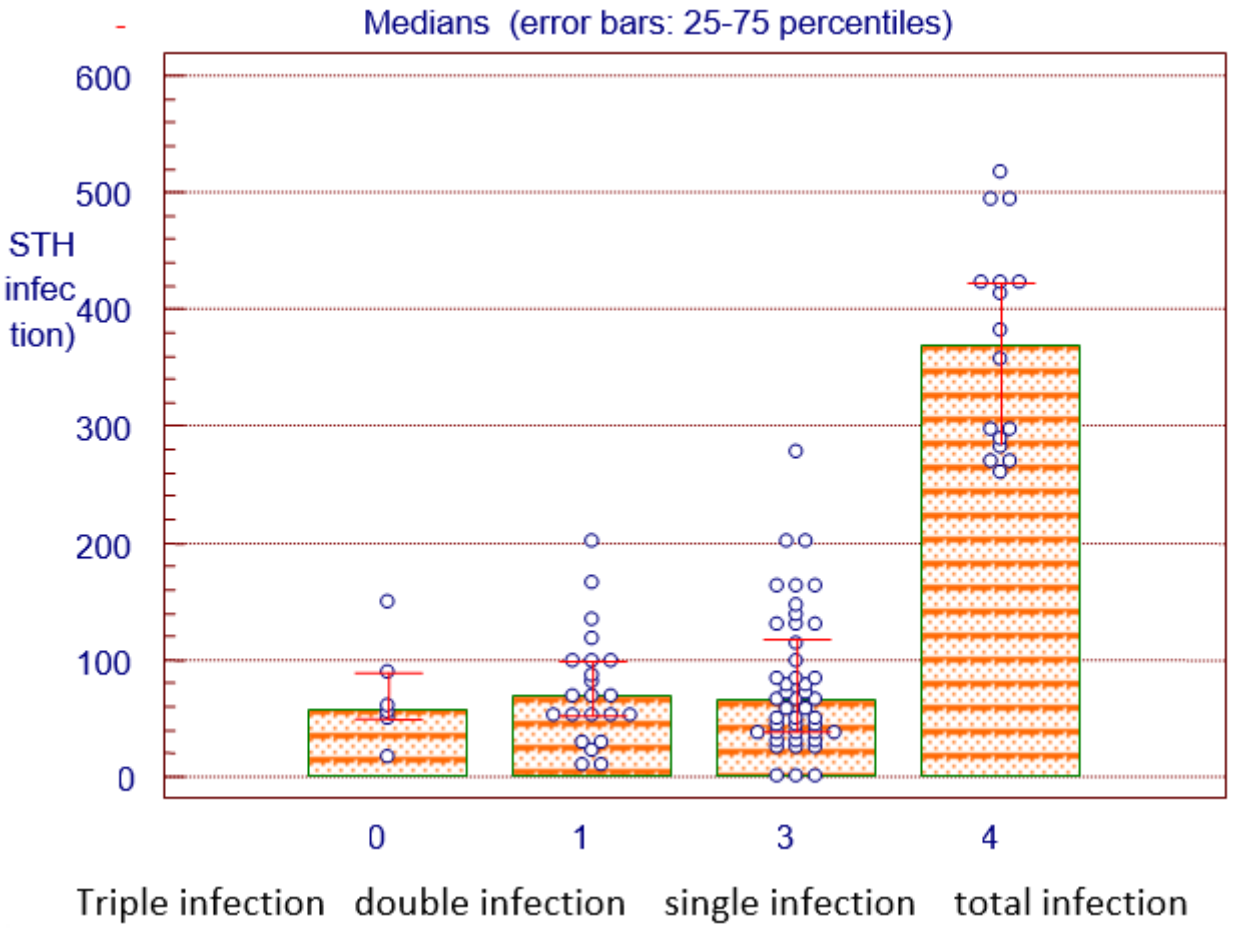

Figure 3

Dot diagram with error bars showing the median (with its inter-quartile range) of multiplex PCR among 4 groups as classified study designed.