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ORIGINAL RESEARCH ARTICLE

Prevalence of *Capillaria philippinensis* in diarrheic patients using the small subunit ribosomal DNA (ssurDNA) gene

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Abstract. *C. philippinensis* is a parasite of the small intestine that causes a severe enteropathy and at times death in cases of delayed diagnosis or inappropriate treatment. This study aims to detect the molecular prevalence of *C. philippinensis* among diarrheic patients at Beni-Suef, Egypt. Stool samples were collected from 121 diarrheic patients and subjected to copro-parasitological examination and copro-nested PCR (nPCR) assays using small subunit ribosomal DNA (ssurDNA) gene. *C. philippinensis* was detected microscopically in diarrheic stool samples of 7 (5.8%) cases and PCR product of *Capillaria* was detected in 14 samples (11.6%), all positive samples by microscopy were detected by PCR. Durations of diarrhea and number of motions/day were correlated with the infection. Recurrence and severity of diarrhea, borborgymi, lower limb edema and presence of charcot Leyden crystals in stool were significantly associated with capillariasis *(p value*=0.0001, 0.0001, 0.006, 0.0001, 0.0001 respectively). Detection of capillariasis using nPCR targeting ssurDNA gene is a specific and accurate method and can identify infection avoiding the delay in management and possible complications.

Keywords: *Capillaria philippinensis;* Intestinal Capillariasis; Chronic diarrhea; Nested PCR; Malabsorption; Borborgymi; Charcot Leyden crystals.

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Introduction

Human intestinal capillariasis is a parasitic disease caused by *C. philippinensis,* a nematode parasite of fish-eating birds. It belongs to the subfamily *Capillariinae* (Okulewicz and Zalesny, 2005). Man is accidentally infected by this parasite (Soukhathammavong et al., 2008), which is first documented in the Philippines in 1963 by Chitwood et al. (1964). Later, it has been reported in Philippines and Thailand, some sporadic cases were diagnosed in other areas (Austin et al., 1999). In Egypt, Youssef et al. (1989) reported the first case of capillariasis, then some cases appeared sporadically in middle Egypt (Khalifa and Othman, 2014). Intestinal capillariasis leads to weight loss, chronic diarrhea, abdominal pain, malabsorption, borborygmi, muscle wasting,

cachexia, weakness, edema, ascites and/or pleural effusion due to the hypo-albuminaemia caused by the disease. The irreversible hypokalemia and/or concomitant bacterial infection may ends by death (Cross, 1998; El-Dib et al., 1999; El-Dib and Doss, 2002; Lu et al., 2006 and Saichua et al., 2008). The irregular shedding of the parasite in feces and confusion of clinical pictures with other intestinal parasitic infections causing delayed diagnosis of capillariasis (Cross, 1992; Bair et al., 2004 and Saichua et al., 2008; Soukhathammavong et al., 2008 and Fan et al., 2008). Diagnosis of capillariasis is difficult and uncommon in non-endemic areas (Vasantha et al., 2012). A definitive diagnosis is based on the recovery of eggs, larvae and/or adult worms from stool, but multiple stool samples may be needed (Bair et al., 2004). Using copro-antigen detection of the parasite has been successfully developed (El-Dib et al., 2004), however it isn't easy to be used as a routine method for detection due to difficulties in its preparation. In addition, intestinal biopsies or aspirations may be needed to confirm the diagnosis (Sangchan et al., 2007). Detection of the parasite DNA is useful in diagnosis of even low intensity infections (Szöllősi et al., 2008) and help to enhance diagnosis of the disease (El-Dib et al., 2015).

The delay in diagnosis highlights the importance of detecting the parasite by a specific and accurate method, thus the present study aims to screen prevalence of *C. philippinensis* among diarrheic patients at Beni-Suef, Egypt by using nPCR techniques.

Material and methods

Study design and population

A cross sectional study was carried out over 121 patients attending the outpatient's clinics of Internal, Tropical and Pediatric Medicine Departments at Beni-Suef University hospital. All patients were suffering from diarrhea.

Copro-parasitological examination

All samples were examined macroscopically for consistency, color, presence of blood and/or mucus. Microscopic examination of concentrated and un-concentrated fecal samples using direct wet smear and Formalin-ethyl acetate sedimentation method was done.

Extraction of genomic DNA

All samples were subjected to genomic DNA extraction using the Favor Prep Stool DNA Isolation Kit (Favorgen Biotech corporation ping-Tung 908, Taiwan) following manufacturer's instructions with modification in the form of thermal treatment of samples by prolongation of the incubation period from 1 hour to overnight at 56°C followed by incubation at 95°C for one hour.

DNA amplification using Nested PCR

DNA amplification of ssu rDNA gene sequence was done according to El-Dib *et al*. (2015). For the primary reaction, the CAP18S Forw-1(5-CGA CGA TGC TTT GAA ATG ACT TGC TC-3) and CAP18S Rev-1(5-GCT CGG TCG TTC CGG TAA C -3) primers were used. While CAP18S Forw-2 (5-CAA CTG TCG ATG GTA GGT TAC G -3) and CAP18S Rev-2 (5-GTC TCA TCG AGA TAC GTT C-3) were used for the secondary reaction. The PCR components consisted of 75 mM Tris-HCL (pH 9), 2mM $MgCL_2$, 50mM KCL, 20 mM $(NH_4)_2$ SO_4, 200 µM each of dNTPs, 1.25U of the PCR primers (200 n*M*), 0.75U DNA Taq polymerase (1U/µl) for 1st and 2nd PCR respectively in a final volume of 25µl. The optimized PCR conditions for 1ry and 2ry PCR assays reaction components were processed. Visual inspection of amplified products was done after electrophoresis separation on 1.5% agarose gel using ethidium bromide stain.

Statistical analysis of the results

Data were statistically coded and entered in a Microsoft Access using the SPSS computer software, version 17 (Chicago, IL, USA). Descriptive data were analyzed as frequency. The univariate and multivariate logistic regression analysis were used to study the significance of the different variables as regards prevalence of capillariasis which was the dependent variable. Statistical significant was considered if p value ≤ 0.05. All significant risk factors by univariate analysis were

included in multivariate analysis and odds ratio (OR) and 95% confidence interval (CI) were computered to detect risk factors for the disease.

Results

Results of microscopy

Different stages of *Capillaria* were detected by microscopy in 7 samples (5.8%), adult female was detected in 3 cases, mixed egg and larva in 2 cases, while egg and mixed egg and adult were detected in one sample for each (table 1).

Table 1. Diagnostic yield of microscopy and nPCR in detection of *C. philippinensis* among studied group

			Study group (n=121) nPCR		
			+ve	–ve	Total
Microscopy	+ve	Adults	3	0	3
		Eggs	1	0	1
		Adults & Eggs	1	0	1
		Eggs & Larvae	2	0	2
		Total	7	0	7
	-ve		7	107	114
	Total		14	107	121

Data presented as n.

Table 2. Kappa agreement of microscopy results among studied group

	Microscopy	(κ)* Interpretation	
Sensitivity	50.0%	< 0:	Poor agreement
Specificity	100%	0.01 – 0.20:	Slight agreement
PPV	100%	0.21 – 0.40:	Fair agreement
NPV	93.9%	0.41 – 0.60:	Moderate agreement
Accuracy	94.2%	0.61 – 0.80:	Substantial agreement
Kappa (κ)*	0.64	0.81 – 1.00:	Almost perfect agreement

Results of nPCR

The expected molecular size of *Capillaria* DNA was detected in all positive *Capillaria* samples by microscopy in addition to further 7 samples which were previously negative by microscopy. The total number of molecular positive samples for *Capillaria* was 14 (11.6%). Considering nPCR as a gold standard, microscopy gave sensitivity and specificity (50%, 100%, respectively) with substantial kappa agreement (0.64) (tables 1, 2).

Results of demographic and clinical variables among positive cases of Capillaria

Female was predominant (71.4%) than male. The mean of age of infected patients was 25.14±12.99, however infection was prominent in young adult (50%). All infected patients were complaining of recurrent diarrhea, mostly chronic (85.7%) with motion ranged from 3 to 10 times/day (tables 3, 4). Liquid pattern of stool was prevalent in 78.6% of infected samples, while microscopic examination revealed Charcot Leyden crystals in 57.1% of samples. Using multivariate analysis revealed that, among different variables (age, gender, diarrhea, clinical symptoms, stool pattern and microscopic examination of stool) recurrent diarrhea is significantly associated with positive cases of *Capillaria* (OR=2.9, 95% CI=0, *P*=0.0001) (table 5).

Table 3. Distribution of demographic and clinical variables among positive cases of *Capillaria*

		Frequency				*P* value*
		PCR +ve n=14		PCR –ve n=107		
		n	%	n	%	
Age Group	**children**	4	28.5	67	62.6	0.073
	young	7	50	31	28.9	
	middle	3	21.4	8	7.4	
	old	0	0	1	0.9	
Gender	**Male**	4	28.5	53	49.6	0.14
	Female	10	71.4	54	50.4	
Recurrent diarrhea	Yes	0	0	63	58.8	0.0001*
	No	14	100	44	41.1	
Severity of diarrhea	Acute	1	7.1	68	63.5	0.0001*
	Persistent	1	7.1	20	18.7	
	Chronic	12	85.7	19	17.7	
Dysentery	**Yes**	2	14.2	20	18.6	0.69
	No	12	85.7	87	81.3	
Vomiting	**Yes**	12	85.7	69	64.4	0.11
	No	2	14.2	38	35.5	
Alternating diarrhea and constipation	**Yes**	4	25.5	12	11.2	0.07
	No	10	71.4	95	88.7	
Abdominal Pain	**Yes**	12	85.7	92	85.9	0.98
	No	2	14.2	15	14	
Flatulence	**Yes**	9	64.3	70	65.4	0.93
	No	5	35.7	37	34.5	
Borborygmus	**Yes**	10	71.4	33	30.8	0.003*
	No	4	28.5	74	69.2	
Fever	**Yes**	5	35.7	63	58.8	0.10
	No	9	64.3	44	41.2	
Lower Limb odema	**Yes**	6	42.8	2	1.8	0.0001*
	No	8	57.1	105	98.2	
Weight Loss	**Yes**	11	78.6	63	58.8	0.16
	No	3	21.4	44	41.2	

Table 4. Results of microscopic examinations among positive cases of *Capillaria*

			Frequency				*P* value*
			PCR +ve n=14		PCR –ve n=107		
Microscopic examination	Mucous	Yes	2	14.2	13	12.1	0.82
		No	12	85.7	94	87.9	
	Pus	>5	1	7.1	5	4.6	0.69
		0-5	13	92.8	102	95.4	
	RBCs	Yes	1	7.1	8	7.4	0.97
		No	13	92.8	99	92.6	
	Charcot Leyden Crystals	Yes	8	57.1	5	4.6	0.0001*
		No	6	42.8	102	95.4	
Stool pattern	Liquid		11	78.6	59	55.1	0.10
	Soft		3	21.4	48	44.9	
	Formed		0	0	0	0	

Table 5. Multivariate analysis for PCR *C. philippinensis* positive cases

			OR	95% CI	*P* value*
Diarrhea	**Recurrent**	Yes/No	2.9	0	0.0001*
	Severity	Persistent/acute	0.294	0.02-4.92	0.28
		Chronic/acute	0.023	0.01-0.19	0.0001*
Borborygmus		Yes/No	5.606	1.64-19.18	0.006*
Lower Limb Odema		Yes/No	7.643	0	0.0001*
Charcot Leyden Crystals in stool		Yes/No	5.2	0	0.0001*

Data presented as n, CI = Confidence interval, with (*) *P* value for OR (Odds` Ratio) < 0.05 is significant.

Table 6. Mean of age, duration of diarrhea and daily motions of *C. philippinensis* cases in the studied group

	Minimum	Maximum	Mean	SD*
Age (Years)	5	47	25.14	±12.99
Duration of diarrhea (day)	3	730	216.5	±245.82
Motions/day	3	10	5.5	±2.07

Data presented as mean, ± SD= Standard deviation.

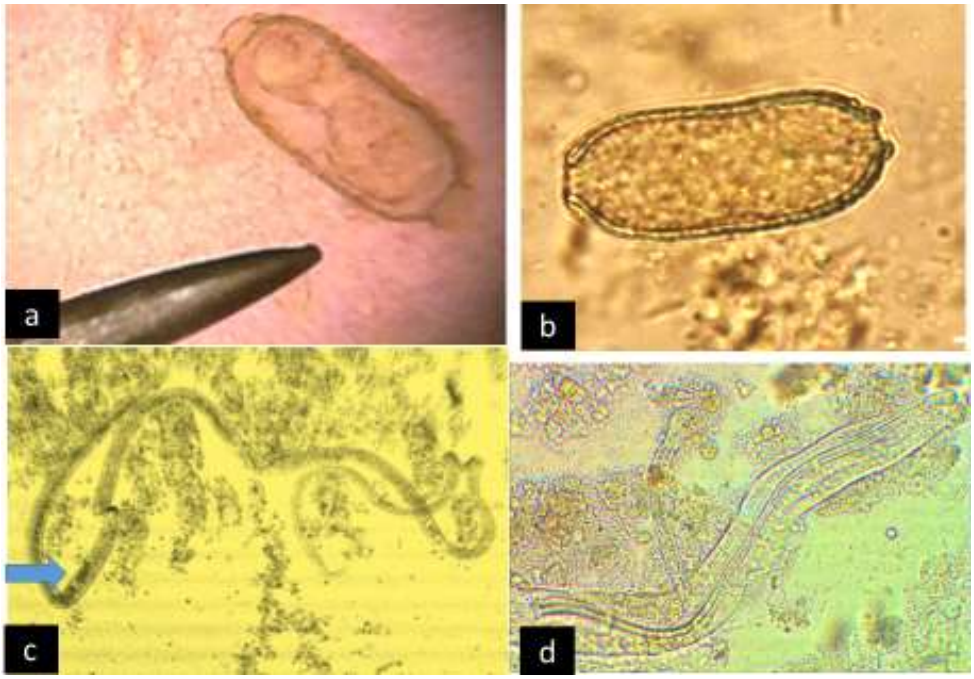

Figure 1a, b. Peanut-shaped egg of *C. philippinensis* with flattened bipolar mucoid plugs and shell striations (×400).
c. Adult female *C. philippinensis* showing eggs in the uterus (the blue arrows) (×100).
d. Adult male *C. philippinensis* showing the spicule (×400).

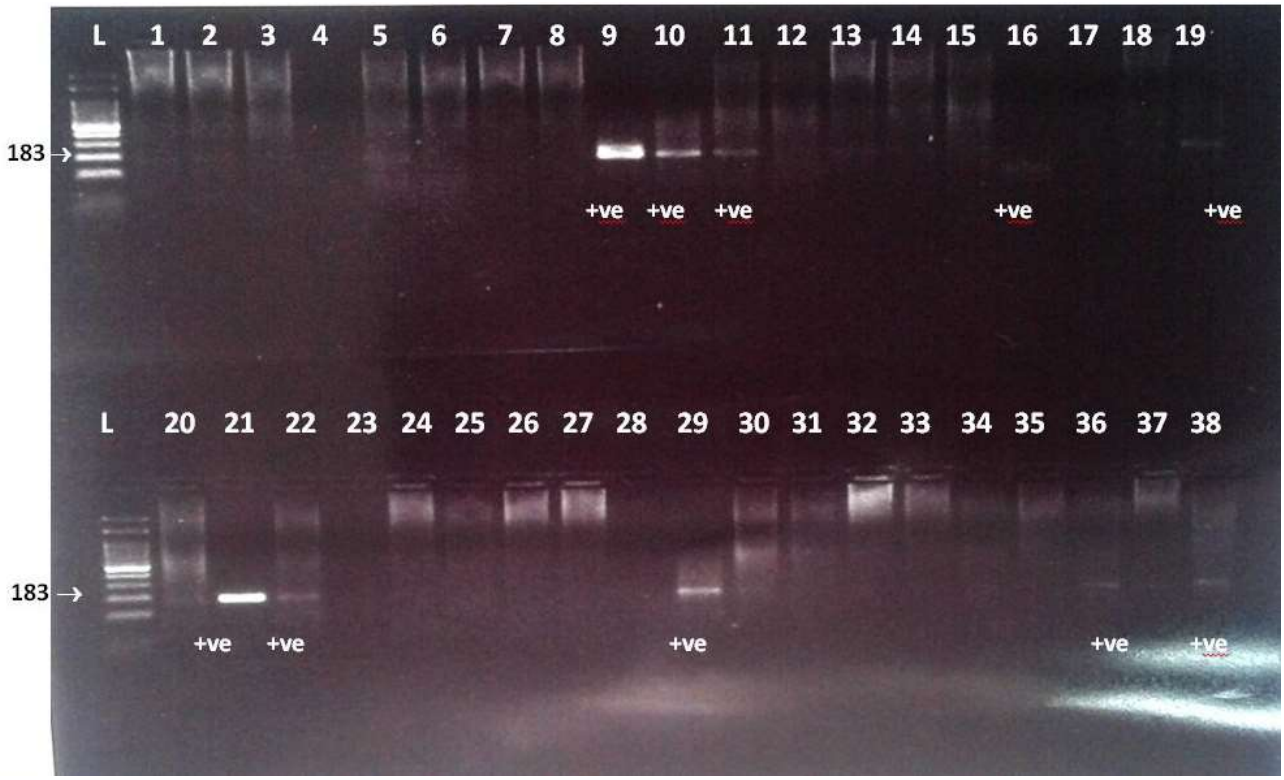

Figure 2. Agarose gel electrophoresis for the products of the PCR targeting CAP 18S gene of *C. philippinensis* at 183 bp, L: 100 bp DNA molecular weight marker, Lane 8: negative control, Lane 9: positive control, Lanes 10,11, 16, 19, 21, 22, 29, 36, and 38: Positive samples.

Discussion

The present study recorded a high molecular prevalence (11.6%, 14/121) among symptomatic cases of diarrhea. There are no previous data to compare, however the high rate of infection detected by this study could be due to the selection of patients involved population that were clinically symptomizing with criteria suggestive to *Capillaria* infection and the use of nPCR which was greatly helpful in detecting 50% of the positive cases among *Capillaria* negative cases by microscopy. Some authors reported that, the severity of intestinal capillariasis is growing in Egypt which is a country outside the endemic area (El-Dib and Doss, 2002; Amin et al., 2011; Attia et al., 2012).

In this study we targeted CAP 18S gene using nPCR. The amplified region chosen was a region of the ssrDNA high conserve for *C. philippinensis*. The nPCR was able to detect *C. philippinensis* copro-DNA in all positive cases with microscopic examination. Similar results were obtained by El-Dib et al. (2015), in their report, all infected cases by microscopy gave the expected size of capillariasis.

The present study showed that, most of infected patients were females 71.4% (10/14) and the age of nPCR positive cases ranged from 5 to 47 years with a mean age of 25.14 (±12.99). The prevalence of *C. philippinensis* was high among young adult and middle-aged females. This comes in agreement with Ahmed et al. (1999); El-Dib and Doss (2002); Amin et al. (2011) and Attia et al. (2012). Age of 25 years represents the active age of a house wife during which the female carries most of the household demands including food preparation (El-Dib and Doss, 2002). Females usually prepare or help in preparation of food for the family, so they have a more chance to catch infection than males. They usually contaminate their fingernails after fish evisceration and then they transmit the larvae which cause infection to themselves during food consumption.

All *Capillaria* positive cases had diarrhea ranged from 3 days to 2 years with mean

duration of 7 months. Number of motions/day ranged from 3 times in some patients and up to 10 times in others with mean number of 5 times/day. This is in agreement with those of Pradatsundarasar et al. (1973) and Cross, (1992). By using the multivariate analysis, there was a significant association between prevalence of *C. philippinensis* with borborgymi and lower limb edema *(P value* = 0.006, 0.0001 respectively). Charcot Leyden crystals were significantly detected *(P* value = 0.0001, 95% CI = 0.00, OR = 5.2) in stool of infected cases which is a common and important finding. This coincides with those of Garcia (2001); Saichua et al. (2008) and Amin et al. (2011).

Diagnosis of *Capillaria* using nPCR targeting CAP 18S gene offers a relatively promising, practical, acceptable and alternative method for diagnosis of infection in clinically suspected patients. It helps to determine the true prevalence, epidemiology of the disease.

Conflict of Interest

The authors declare that they have no reported conflicts of interest.

Informed consent

Informed consent was obtained from all patients and stool samples are noninvasive were obtained with their agreement after informing them by the purpose of the study.

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