# DEVELOPMENT OF NESTED PCR FOR IDENTIFICATION OF *ENTAMOEBA COLI* IN HUMAN FECAL SAMPLES

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**Abstract.** Microscopic examination is the gold standard for detecting *Entamoeba* spp in stool specimens, although it is often not adequately sensitive or specific. Here, a nested PCR assay was developed for the detection of *E. coli* in fecal samples with a limit of detection of 1 pg/20 mg of sample and good specificity (no cross-amplification of other intestinal *Entamoeba* spp or protozoa). The nested PCR assay employs two set of primers, one set for amplifying *Entamoeba* genus DNA and the other specific for *E. coli* small subunit rDNA. Applying this technique to stored fecal samples ($n$ = 55) from school children in western Thailand, 33% of the samples were positive for *E. coli* compared to 29% by microscopic examination, the latter method also showing 29% mixed infections, all among *Entamoeba* sp-positive samples. Sensitivity and specificity of the in-house nested PCR assay compared to microscopy was 100% and 95% respectively, with positive and negative predictive value of 0.9 and 1.0 respectively. Kappa analysis ($\kappa$ = 0.9) indicated an excellent agreement of nested PCR assay and microscopy. This in-house nested PCR assay is suitable for both laboratory screening and epidemiology of *E. coli* infection in human population.

**Keywords:** *Entamoeba coli,* fecal sample, molecular diagnosis, nested PCR, light microscopy

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## INTRODUCTION

Nine species of genus *Entamoeba* have been found in humans, namely, *E. bangladeshi, E. chattoni, E. dispar, E. coli, E. gingivalis, E. hartmanni, E. histolytica, E. moshkovskii,* and *E. polecki* (Ali *et al*, 2008; Royer *et al*, 2012; Ngobeni *et al*, 2017). Approximately 500 million people are infected with *Entamoeba* spp worldwide, 10% with pathogenic *E. histolytica* (causing amebiasis) and the remaining with nonpathogenic species (Anonymous, 1997).

*E. coli* is distributed globally, with the highest prevalence in rural areas and regions with poor sanitation (Jones *et al*, 2019) and is most commonly found in the human intestinal tract, transmitted via oral-anal route (Pritt and Clark, 2008; Stensvold *et al*, 2011; Feng *et al*, 2018). In some rural school communities in Colombia, prevalence of *E. coli* is 25.7-35.0% (Hernández *et al*, 2019; Peña-Quistial *et al*, 2020). Interestingly, *E. coli* can phagocytose red blood cells, efficiency depending on strain, but this ingestion is rarely seen in fecal samples (Trissl *et al*, 1978). *E. coli* is associated with diarrhea and gall bladder disease (Kalk and Wildhirt, 1954; Geyer, 1959; Corcoran *et al*, 1991; Wahlgren, 1991) and patients with high loads of *E. coli* infection may experience indigestion, gastritis, and dyspepsia, but, on the whole, *E. coli*-infected patients have gastrointestinal presentations, such as colicky abdominal pain, flatulence and loose stool (Ali *et al*, 2008; Wahlgren, 1991). In addition, *E. coli* infection is an indicator of poor nutritional status in school children (Boeke *et al*, 2010).

Human *Entamoeba* spp can be diagnosed using microscopy, on the basis of size and number of nuclei, but difficulties can arise when an inexperienced laboratory technician performs such examination (Tanyuksel and Petri, 2003; Fotedar *et al*, 2007). Moreover, morphological features of *Entamoeba* spp are similar among *E. bangladeshi*, *E. dispar*, *E. histolytica*, and *E. moshkovskii*, making it difficult for inexperienced laboratory personnel to differentiate these four *Entamoeba* species from other amoebae including *E. coli* of the human colon, often leading to misdiagnosis (Carrero *et al*, 2020).

Pre-cyst form of *E. coli* begins with the trophozoite becoming more spherical and binucleated, and a glycogen vacuole is formed (Fotedar *et al*, 2007). These nuclei further divide during development to a mature cyst containing eight nuclei, the most distinguishing feature of *E. coli*, but the nucleus may occasionally divide into 16 nuclei (Fotedar *et al*, 2007). Tetra nucleated cysts of *E. coli* can be mistaken for mature cysts of *E. histolytica*. *E. coli* cyst size is 10-35 μm in diameter (Fotedar *et al*, 2007; Haidar and De Jesus, 2021), which overlaps with that of *E. bangladeshi*, *E. dispar*, *E. histolytica*, and *E. moshkovskii*.

Trichrome or iron-hematoxylin staining is used to identify *E. coli* trophozoite (Tanyuksel and Petri, 2003; Fotedar *et al*, 2007), but this method is time-consuming, requires a well-trained technician, and is not suitable for a large sample size. In addition, such factors as storage condition, time spent on sample processing, and parasite density, affect the outcome of microscopic examination (Goñi *et al*, 2012), resulting in low sensitivity and specificity (Krogstad *et al*, 1978; Haque *et al*, 1995; Huston *et al*, 1999; Fotedar *et al*, 2007; Carrero *et al*, 2020). Furthermore, the ability to identify *Entamoeba* spp in fecal samples is essential in understanding their epidemiology as the prevalence of amoeba infection varies with populations in different countries and among areas with different socioeconomic status within the same region of a country (Tanyuksel and Petri, 2003). Up to 50% of the population can be infected where poor sanitary conditions exist (Caballero-Salcedo *et al*, 1994).

Although several molecular methods, such as conventional PCR, nested PCR, nested PCR-RFLP, real-time PCR, and LAMP, have been established to diagnose *E. histolytica* and differentiate it from *E. dispar* and *E. moshkovskii* (Roy *et al*, 2005; Hamzah *et al*, 2006; Khairnar and Parija, 2007; Khairnar *et al*, 2007; Liang *et al*, 2009; Hamzah *et al*, 2010), few methods are available for detecting *E. coli* in fecal samples, such as reverse line hybridization (Verweij *et al*, 2003), PCR combined with nucleotide sequencing (Santos *et al*, 2010; Chihi *et al*, 2019) and PCR using *E. coli*-specific primers (Tachibana *et al*, 2009; Jirků-Pomajbíková *et al*, 2016; Pattanawong *et al*, 2021). However, the limits of detection of these methods were not documented.

Here, we employed a nested PCR targeting *E. coli* small subunit (SSU) rDNA and determined sensitivity and specificity of infected stored fecal samples. Sensitivity and specificity of the assay should provide an accurate diagnosis of human *E. coli* infection sufficient to support epidemiological studies.

## MATERALS AND METHODS

### Source of fecal samples

Fecal samples (*n* = 55) were previously collected from school children in Tak and Ratchaburi provinces, western Thailand during 2017-2018 and stored at -80°C at the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

The research protocols were approved by the Ethics Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok (TMEC 19-002).

### Microscopic examination

Fecal samples were prepared using a wet mount and examined by microscopy (Fotedar *et al*, 2007).

Samples positive for protozoan and suspected *Entamoeba* spp infection were chosen for subsequent nested PCR analysis.

**Nested PCR assay protocol**

DNA from fecal samples (200 mg) was extracted using a QIAamp stool DNA extraction kit (QIAGEN, Hilden, Germany) and stored at -20°C until use. For nested PCR, two sets of primers were used: genus *Entamoeba*-specific primers as previously described (forward primer Entam1 (5' -GTTGATCCTGCCAG-TATTATATG-3') and reverse primer Entam2 (5' -CACTATTGGAGCTG-GAATTAC-3') (Verweij *et al*, 2001) and *E. coli*-specific primers, designed based on SSU rDNA gene sequence (GenBank accession no. AF149915) and using a non-business primer 3 software (Rozen and Skaletsky, 2000), namely, [EcoliF (5'-CTAAGCACAAA-GTCCTAGTATGATG-3') and EcoliR (5'- CCTCATCGATTACACTCCCA-GAG-3'). First round nested PCR mixture (30 μL) containing 1X PCR buffer (GE Healthcare, Amersham, UK), 1.5 mM $MgCl_2$, 200 μM each dNTP, 25 pmol each of *Entamoeba* genus-specific primers, 1 U *Taq* DNA polymerase (GE Healthcare, Amersham, UK), and 2 μl of DNA. *E. coli* (07-286 strain) DNA, kindly provided by Dr Graham Clark (London School of Hygiene and Tropical Medicine, London, UK), was used as positive control. Thermocycling (conducted in a *Px2* Thermal Cycler (ThermoHybaid, Middlesex, UK) was carried out as follows: 94°C for 5 minutes; 35 cycles of 94°C for 60 seconds, 55°C for 60 seconds and 72°C for 60 seconds; with a final step of 72°C for 7 minutes. Amplicon (550 bp) was analyzed by 1.5% agarose gel-electrophoresis and stained with ethidium bromide. Second round nested-PCR was performed in 20 μl mixture containing 1X PCR buffer (GE Healthcare, Amersham, UK), 1.5 mM $MgCl_2$, 50 μM each dNTP, 0.5 μM each *E. coli*-specific primer, 0.5 U *Taq* polymerase (GE Healthcare, Amersham, UK), and 1 μl of post-1$^{st}$ round PCR solution. Thermocycling was carried out as described above but using the following conditions: 94°C for 5 minutes; 25 cycles of 94°C for 30 seconds, step-down 68-60°C for 30 seconds and 72°C for 30 seconds; 10 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds; with a final step of 72°C for 2 minutes. Amplicon (166 bp) was analyzed by 2.0% agarose gel-electrophoresis and stained with ethidium bromide. Amplicons were excised from the gel and directly sequenced (Macrogen, Seoul, South Korea) to assure that PCR products belonged to the *E. coli SSU rRNA* sequence which had already been deposited with GenBank (accession no. AF149915).

**Sensitivity and specificity determination of nested-PCR assay**

Sensitivity of the nested-PCR assay was determined using a 10-fold serial dilution (100, 10, 1, and 0.1 pg) of *E. coli* genomic DNA as described above.

*E. coli* DNA concentration was measured by a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA). Specificity was tested employing DNA extracted from other intestinal protozoa and bacteria, namely, *Blastocystis hominis, Cryptosporidium* spp, *E. dispar, E. histolytica, E. moshkovskii, Escherichia coli, Giardia duodenalis, Salmonella* sp, and *Shigella* sp given by the Department of Protozoology and Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University. A parasite-free stool sample was used as a negative control.

## RESULTS

### Microscopic examination

Microscopic examination of the fecal samples revealed 29/55 (53%) stool samples were positive for protozoan infection [*Blastocystis hominis* (*n* = 13/29, 45%), *Endolimax nana* (*n* = 13/29, 45%) and *Giardia intestinalis* (*n* = 3/29, 10%)] and 19 (34%) for *Entamoeba* spp, comprising suspected *E. coli* (*n* = 16/19, 84%), *E. hartmanni* (*n* = 2/19, 10%) and *E. histolytica* (*n* = 1/19, 6%) (Table 1). Twenty-nine percent (16/55) of samples contained mixed infections, all among *Entamoeba* sp-positive samples.

### Sensitivity and specificity of nested PCR assay for *E. coli*

A nested PCR assay for the detection of *E. coli* was developed in which the 1st round PCR employed an *Entamoeba* genus-specific primer set (Verweij *et al*, 2001) generating a 550-bp amplicon, followed by a 2nd round PCR using an in-house primer set targeting *E. coli*-specific SSU rDNA to produce a 166-bp amplicon.

Table 1

Identification of protozoa in fecal samples by microscopic examination and of *Entamoeba* genus and *Entamoeba coli* by nested PCR

| Sample no. | Microscopic examination[a] | *Entamoeba* genus-specific PCR[b] | *E. coli*-specific PCR[c] |
|---|---|---|---|
| 1 | Negative | - | - |
| 2 | *Blastocystis hominis, Endolimax nana* | - | - |
| 3 | Negative | + | + |
| 4 | Negative | - | - |
| 5 | *B. hominis, En. nana* | + | + |
| 6 | Negative | - | - |
| 7 | Negative | - | - |

Table 1 (cont)

| Sample no. | Microscopic examination[a] | *Entamoeba* genus-specific PCR[b] | *E. coli*-specific PCR[c] |
|---|---|---|---|
| 8 | Negative | - | - |
| 9 | Negative | + | + |
| 10 | *B. hominis, E. coli, En. nana* | + | + |
| 11 | Negative | + | - |
| 12 | Negative | - | - |
| 13 | Negative | - | - |
| 14 | Negative | + | - |
| 15 | *B. hominis* | - | - |
| 16 | *B. hominis, E. coli, En. nana* | + | + |
| 17 | Negative | - | - |
| 18 | Negative | + | - |
| 19 | *B. hominis* | - | - |
| 20 | Negative | + | - |
| 21 | Negative | - | - |
| 22 | *En. nana* | - | - |
| 23 | Negative | - | - |
| 24 | Negative | - | - |
| 25 | Negative | - | - |
| 26 | Negative | - | - |
| 27 | Negative | - | - |
| 28 | *B. hominis, E. coli* | + | + |
| 29 | Negative | - | - |
| 30 | *B. hominis, En. nana* | - | - |
| 31 | *E. coli, En. nana, Giardia duodenalis* | + | + |
| 32 | *B. hominis* | - | - |
| 33 | Negative | - | - |

Table 1 (cont)

| Sample no. | Microscopic examination[a] | *Entamoeba* genus-specific PCR[b] | *E. coli*-specific PCR[c] |
|---|---|---|---|
| 34 | *B. hominis, E. coli, En. nana* | + | + |
| 35 | *B. hominis, En. nana* | - | - |
| 36 | *B. hominis, E. coli, E. hartmanni, En. nana* | + | + |
| 37 | Negative | - | - |
| 38 | Negative | + | - |
| 39 | *En. nana* | + | + |
| 40 | *B. hominis, E. coli* | - | - |
| 41 | *E. coli, B. hominis, En. nana* | + | + |
| 42 | Negative | + | - |
| 43 | Negative | - | - |
| 44 | *B. hominis* | - | - |
| 45 | *E. coli, E. hartmanni, E. histolytica, En. nana,* | + | - |
| 46 | *E. coli* | + | + |
| 47 | *G. duodenalis* | + | - |
| 48 | *E. coli* | + | + |
| 49 | *E. coli, G. duodenalis* | + | + |
| 50 | *En. nana* | + | - |
| 51 | *E. coli* | + | + |
| 52 | *E. coli, En. nana* | + | + |
| 53 | *G. duodenalis* | + | - |
| 54 | *E. coli* | + | + |
| 55 | *B. hominis, Cryptosporidium* sp., *E. coli, G. duodenalis* | + | + |

[a]Wet-mount examination; [b]1st round nested-PCR using *Entamoeba* genus-specific primers; [c]2nd round nested-PCR using *E. coli*-specific primers

+ refers to positive result while – refers to negative result

Using control *E. coli* DNA, limit of detection of the nested PCR assay was 1 pg (Fig 1A) and there was no cross-reaction with fecal samples infected with other *Entamoeba* spp (*E. dispar*, *E. histolytica* and *E. moshkovskii*) or other protozoa and bacteria (*Blastocystis hominis, Cryptosporidium* sp, *Escherichia coli, Giardia duodenalis, Salmonella* sp, and *Shigella* sp) (Fig 1B).

### Detection of *E. coli* in fecal samples by nested PCR

Using a set of *Entamoeba* genus-specific primers, 1st round of the nested PCR assay generated a 550-bp amplicon (Fig 2A) in 28/55 (51%) fecal samples, and the 2nd round yielded an *E. coli*-specific 166-bp amplicon (Fig 1B) in 18 samples (33%). The nested PCR assay detected four *E. coli* false-negative and two false-positive samples by microscopic examination (Table 1).

Sensitivity of nested PCR assay for detection of *E. coli* in fecal samples was 100% and its specificity was 95% while positive and negative predictive value was 0.9 and 1.0 respectively (Table 2). Kappa analysis, a statistical measure of inter-rater reliability for categorical variables, revealed 90% agreement between nested PCR assay and microscopic examination ($\kappa = 0.9$).

## DISCUSSION

*Entamoeba* spp in fecal samples is normally identified using a microscopic examination of morphological features (Tanyuksel and Petri, 2003; Fotedar *et al*, 2007). However, light microscopy can only differentiate *Entamoeba* spp at the genus level and similar morphological features pose problems in routine examination, leading to misdiagnosis. Previous reports showed that microscopic method is not adequately sensitive and specific to precisely identify *Entamoeba* spp in fecal specimens (Gonzalez-Ruiz *et al*, 1994, Petri *et al*, 2000).

*E. coli* comprises two major clades, ST1 and ST2, the former found in humans and the latter more common in nonhuman hosts (Stensvold *et al*, 2011). Based on sequence divergence, *E. coli* ST1 and ST2 may be considered as two distinct species; however, no other differences between the two subtypes were detected (Stensvold *et al*, 2011; Elsheikha *et al*, 2018). Here, *E. coli*-specific primers were designed from *E. coli* ST1 SSU rRNA gene sequences. The use of these primers in a nested PCR assay showed no cross-detection of other human intestinal *Entamoeba* spp or protozoa. It was not possible to compare the sensitivity of the in-house nested PCR assay as sensitivities of other PCR-based assays were not reported (Tachibana *et al*, 2009; Santos *et al*, 2010; Jirků-Pomajbíková *et al*, 2016). Nevertheless, the detection limit of our assay was 10-fold lower than those of nested PCR assay for *E. histolytica* and *E. moshovskii* (10 pg) and 20-fold lower than that for *E. dispar* (20 pg) (Hamzah *et al*, 2006). Based on Kappa analysis, the in-house nested

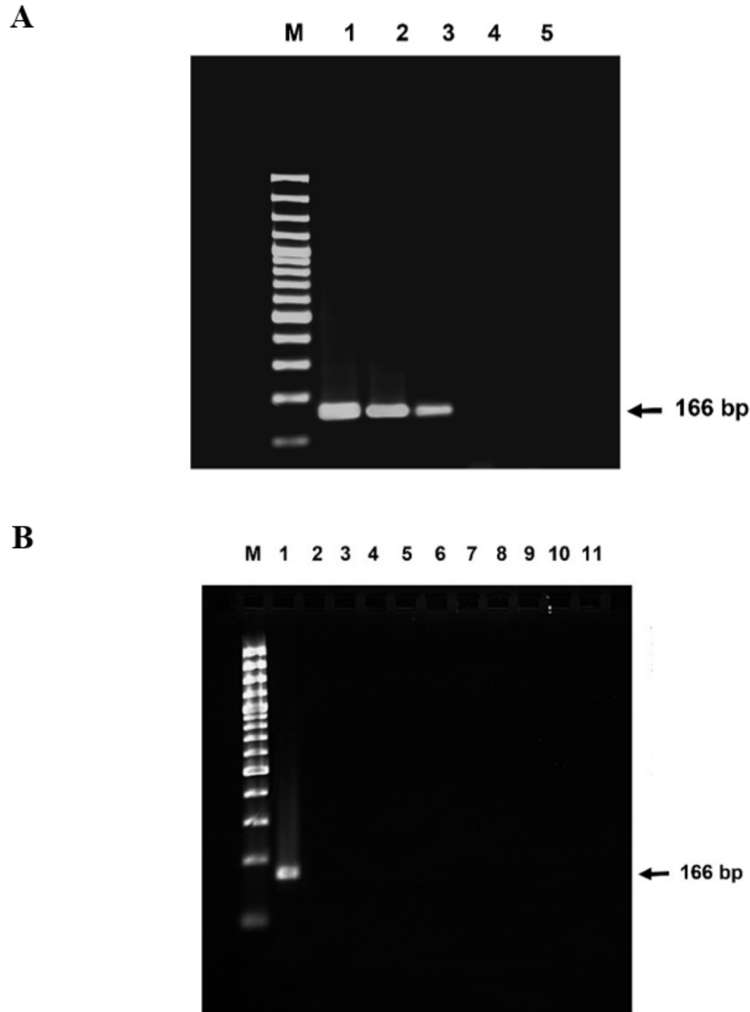


Fig 1 - Determination of detection limit (A) and specificity (B) of nested PCR assay for *Entamoeba coli*

Nested PCR was carried out as described in legend to Fig 1.

Fig 1A - Lane M: 100-bp DNA markers; Lane 1: 100 pg of *E. coli* DNA; Lane 2: 10 pg of *E. coli* DNA; Lane 3: 1 pg of *E. coli* DNA; Lane 4: 0.1 pg of *E. coli* DNA; Lane 5: water negative control.

Fig 1B - DNA was extracted from infected human fecal samples and subjected to nested PCR. Lane M: 100 bp ladder DNA marker; Lane 1: *E. coli* DNA; Lane 2: parasite-free sample; Lane 3: *E. histolytica*; Lane 4: *E. dispar*; Lane 5, *E. moshkovskii*; Lane 6: *Salmonella* sp; Lane 7: *Shigella* sp; Lane 8: *Escherichia coli*; Lane 9: *Blastocystis hominis*; Lane 10: *Giardia duodenalis*; Lane 11, *Cryptosporidium* sp

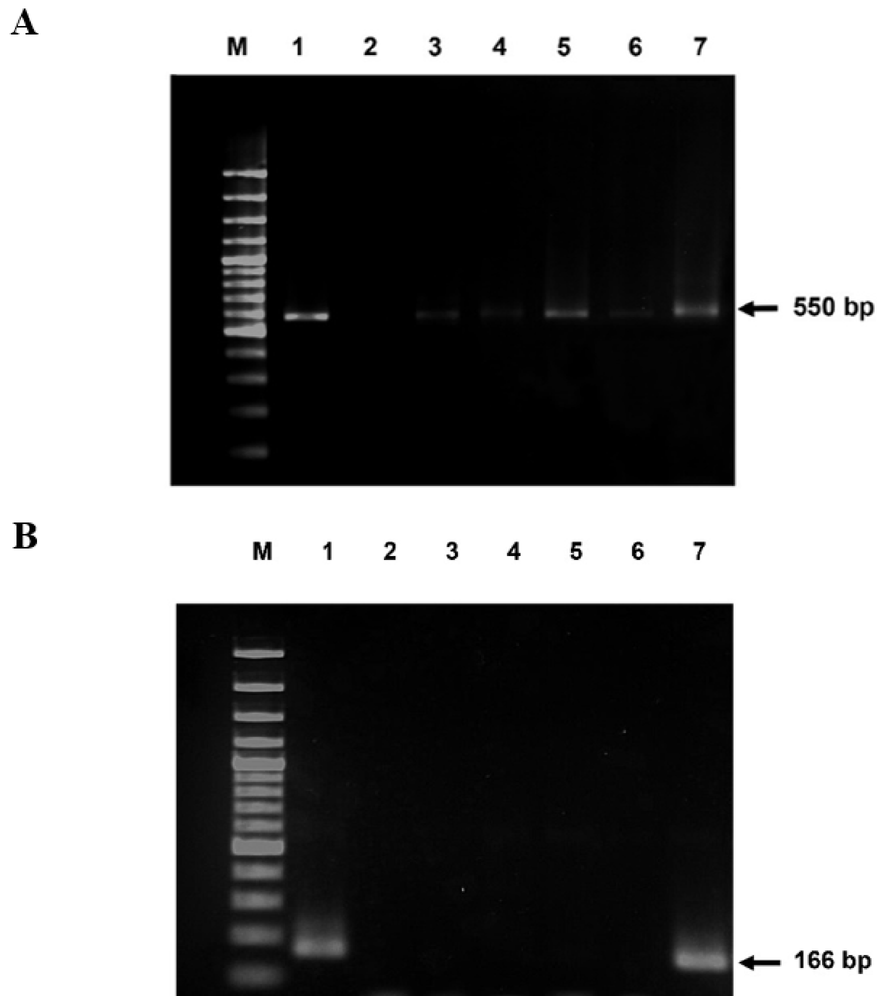


Fig 2 - Nested PCR detection of *Entamoeba coli* in human fecal samples

Amplicons were separated by 1.5% (A) or 2.0% (B) agarose gel-electrophoresis and stained with ethidium bromide.

Fig 2A - 1st round nested PCR. *Entamoeba* genus-specific primer pair was used to generate a 550-bp amplicon (Verweij et al, 2001). Lane M: 100-bp DNA markers; Lane 1: *E. coli* DNA positive control; Lane 2: water negative control; Lanes 3-7: DNA from fecal samples

Fig 2B - 2nd round nested PCR. In-house *E. coli*-specific primer pair targeting small subunit rDNA was used to generate a 166-bp amplicon. Lane M: 100-bp DNA markers; Lanes 1-7: reaction solutions from (A)

Table 2

Positive predictive value, negative predictive value, sensitivity, and specificity of nested PCR assay compared to gold standard microscopy for *Entamoeba coli* detection in fecal samples

| Nested PCR | Microscopy | | Predictive value |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 16 | 2 | 0.9 (16/18) |
| Negative | 0 | 37 | 1.0 (37/37) |
| Sensitivity (%) | 100 (16/16) | | |
| Specificity (%) | 95 (37/39) | | |

PCR assay was comparable to the gold standard microscopy method. It is worth noting microscopy detected only 57% of *Entamoeba* spp-infected samples positive by *Entamoeba* genus-specific PCR. Pattanawong *et al* (2021) recently reported that detection of *E. coli* by PCR is superior to microscopic examination of fecal specimens from Thai school children at the Thai-Myanmar border region of northwest Thailand.

*E. coli* was the most common *Entamoeba* sp present in fecal samples from school children in western regions of Thailand, in keeping with previous reports demonstrating that in India and Mexico, *E. coli* is more commonly found in fecal samples compared to other *Entamoeba* spp (Rayan *et al*, 2010, Zavala *et al*, 2016). Moreover, contamination of raw vegetables and fruits with *Entamoeba* spp cysts has been documented worldwide (Li *et al*, 2020), with *E. coli*, *E. dispar* and *E. histolytica* the most commonly detected species (Shahnazi and Jafari-Sabet, 2010; Duedu *et al*, 2014; Gabre and Shakir, 2016; Mohamed *et al*, 2016; Li *et al*, 2020).

*E. coli* is understudied as it is designated a nonpathogenic parasite showing no symptoms in infected individuals (Speich *et al*, 2013). However, *E. coli* infection can cause malabsorption and promote a (type 1) pro-inflammatory response (Tong and Lv, 2014). A moderate to heavy infection with *E. coli* contributes to fat deposition and a larger waist circumference through inflammatory response mechanisms (Ahmed *et al*, 2011), and, thereby, have long-term consequences on human health (Zavala *et al*, 2016). The impact of *E. coli* infection on immune and metabolic consequences warrants further investigation. In addition, *E. coli* may alter the microbiota homeostasis as found with other intestinal protozoa, resulting in changes to appetite, food

intake and metabolism (Sanchez *et al*, 2014; Wilhelm and Yarovinsky, 2014). For example, increased caloric uptake from diet arising from altered gut microbiota can modulate host genes, which regulate fat deposition in adipocytes, thereby increasing the risk of diet-induced obesity (Gangarapu *et al*, 2014). On the other hand, high *E. coli* infection load may lead to micronutrient deficiencies in the digestive tract, also a risk factor for obesity (Garcia *et al*, 2013).

In children, moderate or heavy infection of *E. coli* may adversely affect waist-to-height ratio and waist circumference caused by abdominal distention that intestinal infection promotes (Zavala *et al*, 2016). However, microbiota profile of children with *E. coli* infection demonstrates an increase in the relative abundance of the beneficial bacterium *Akkermansia,* as opposed to the case of *Blastocystis* spp-infected children, who showed the reduction of this bacterium in the gut (Alzate *et al*, 2020). In future epidemiological studies, all intestinal *Entamoeba* spp should be identified to understand the prevalence of individual species and how their presence affects human health, in particular, the role of nonpathogenic *Entamoeba* spp on gut microbiota.

In conclusion, the study shows that a new set of *Entamoeba coli*-specific primers together with a set of *Entamoeba* genus-specific primers applied in a nested PCR assay were capable of detecting ST1 *E. coli* in human fecal samples with high sensitivity and specificity, suitable for application both in routine laboratory screening and in epidemiological investigations. Incorporation of this in-house *E. coli*-specific primer set (and primers for other human intestinal *Entamoeba* spp) into a quantitative PCR platform should reduce labor and time required for high through-put assays necessary in large scale epidemiological research to understand amoeba infections in humans.

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## CONFLICTS OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

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