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Original article

Real-time PCR: Benefits for Detection of Mild and Asymptomatic *Giardia* Infections

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Abstract: The majority of *Giardia* infections are transmitted by the fecal-oral route and cause giardiasis. Children who live in crowded conditions or low socio-economic areas are the risk group for *Giardia* infection. Interestingly, most of them are asymptomatic or only mildly infected and can shed the *Giardia* cysts in the environment. Thus, the diagnosis of *Giardia* infection in asymptomatic or mild infection plays an important role in achieving control of *Giardia duodenalis* transmission. The objective of this study was to examine parasitic infections using microscopy and to develop a real-time PCR method for detection of *Giardia* infection in the stool samples of children living on the Thai-Myanmar border. Both species-specific primers and fluorescent labeled *G. duodenalis* probe were designed using small-subunit ribosomal RNA (*ssrRNA*). The results showed that 10 (7.69%) and 40 (30.77%) of 130 stool samples were positive for *G. duodenalis* by microscopy and real-time PCR respectively. Only 3 out of 9 liquid stools revealed *G. duodenalis* positive using microscopy, but all of them were *G. duodenalis*-positive using real-time PCR. The detection limit of real-time PCR for *G. duodenalis* was 0.1 pg/25 μl reaction. It can detect both mild and asymptomatic *Giardia* infections in children living on the Thai-Myanmar border.
Key words: *Giardia duodenalis*, asymptomatic or mild infection, real-time PCR, small-subunit ribosomal RNA, Thai-Myanmar border

INTRODUCTION

Giardia duodenalis (synonyms: "*G. lamblia*" or "*G. intestinalis*") is a flagellate protozoan considered to be the most common intestinal protozoan infecting humans worldwide. Many countries, especially developing countries, show a high infection rate of giardiasis [1]. It is believed that giardiasis is still a significant health problem. Most infected persons are children who suffer and experience growth retardation. In Thailand, studies on the prevalence of human giardiasis have increased significantly [2–4]. Popruk *et al.* reported that the prevalence of *Giardia* infection was 11.67% in Thai orphans [4]. The clinical manifestations of symptomatic giardiasis include greasy stools, flatulence, diarrhea, abdominal cramps, epigastric tenderness and malabsorption [5]. Asymptomatic giardiasis-infected persons, who show neither signs nor symptoms, can shed cysts in their living environment, and unwittingly cause transmission of *G. duodenalis* to other individuals.

G. duodenalis infects humans and other mammals [6]. Furthermore, isolates of *G. duodenalis* are classified into seven assemblages: A, B, C, D, E, F and G, based on the characterization of the glutamate dehydrogenase (*gdh*), small-subunit ribosomal RNA (*ssrRNA*) and triosephophate isomerase (*tpi*) gene [7]. The *ssrRNA* gene is a more conserved sequence and useful for screening *Giardia* infection in humans.

Diagnosis of *G. duodenalis* is usually based on microscopic examination. However, this achieves only about 60% sensitivity and depends greatly on the experience and skill of the microscopist [8]. Antigen detection methods are also available for detecting *Giardia* infection, but unfortunately these methods have limitations and therefore may not be efficient for the detection of low parasite levels in stool samples [9].

Nowadays, molecular techniques provide a more sensitive and rapid means for pathogenic detection. Additionally, they have provided useful tools in several fields of study. They are effective for the detection of *Giardia* in clinical samples as well as epidemiological studies of giardiasis in humans and animals, particularly in low parasite level infection [10].

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Along the border of Thailand, children are at a high risk for parasitic infections, especially *G. duodenalis* and soil-transmitted helminthes, because of the poor quality of life, lack of clean water, and improper personal hygiene. Many infected persons are asymptomatic or mildly (few symptoms) infected and can be carriers who transmit the diseases and spread pathogens. That is the reason why parasitic infections persist and remain difficult to control. In this situation, detecting both mild and asymptomatic *Giardia* infections plays a key role in managing *G. duodenalis* transmission because the infected persons will receive prophylaxis, thus helping to prevent the shedding of cysts in the environment. Therefore, the objective of the present study was to examine parasitic infections using microscopic examination and to develop a real-time PCR method for detection of *Giardia* infection in the fresh stool samples of children living on the Thai-Myanmar border.

MATERIALS AND METHODS

Study design & Study area

Conducted in June 2011, this study was cross-sectional in design. The study area is located at Ban Bong Ti Lang in Sai Yok district, Kanchanaburi province (Thai-Myanmar border) in the western part of Thailand. The population density is 17 people per km^2. Agriculture is the predominant occupation. The temperature is relatively constant throughout the year (averaging 20–35°C) with a relative humidity of 62.3%. This area is made up of long mountain ranges with plateaus, 300–600 meters above sea level.

Sample collection

The subjects included 75 female and 55 male children ranging in age from 6 to 12 years and studying at Bong Ti Lang School, Ban Bong Ti Lang, Kanchanaburi province. Physical examinations were conducted by the same physician throughout the study. A stool sample was obtained from each subject or 130 in total. Written informed consent was obtained from all participants and the guardians of the children prior to stool sample collection, and the collected data were kept confidential to assure privacy. All of the children were apparently healthy (without signs or symptoms of illness) at the time of the study. The specific instructions for collecting and avoiding contamination of stool samples were clearly explained to the children. Fresh stool samples were collected early in the morning and transported immediately to the laboratory. All stool samples were examined microscopically to detect *G. duodenalis* and other intestinal parasites. These samples were further screened for *Giardia* infection using real-time PCR.

Laboratory analyses

Microscopic examination for *Giardia duodenalis* and other intestinal parasites

The 130 stool samples were examined microscopically for the presence of intestinal parasites, and the consistency (gross examination) was recorded as well: soft stool = stool can be cut with an applicator stick, hard stool = stool cannot be punctured with an applicator stick, and liquid stool = stool shapes to the container. Saline and iodine-stained slides were observed under a microscope. The presence of intestinal parasites was confirmed by two expert microscopists independently.

DNA extraction and real-time PCR

DNA extractions were performed using commercial PSP® Spin Stool DNA Kit. Briefly, the stool samples were lysed with lysis buffer under high temperatures. After the lysis procedure, PCR inhibitors present in the sample were absorbed efficiently to the InviAdsorb matrix for the removal of undissolved particles, followed by Proteinase K digestion to ensure high yields of DNA. DNA samples were stored at –20°C until use.

The detection limit and standard curve (plot of cycle threshold (CT) values/crossing points of different standard dilutions against log of amount of standard) of the real-time PCR were performed using 10-fold serial dilution of *G. duodenalis* trophozoite DNA, starting from 10 ng to 0.1 pg. Additionally, the specificity of the primers and probe was checked in this study to distinguish other intestinal protozoa such as *Cryptosporidium parvum*, *Cyclospora cayetanensis*, and *Entamoeba histolytica*.

All 130 extracted DNA stool samples were tested for the presence of *G. duodenalis* using real-time PCR. They were screened by small-subunit ribosomal RNA (*ssrRNA*), with primer GiarF (5'-GACGCTCTCCCCAAGGAC-3'), primer GiarR (5'-CTGCGCACGCTGCTCG-3') [11] and the probe GiarP (FAM-5'-TGTCCTGAGCCGTCCGCCG-3'-BHQ). The assay was performed in a 25 µl PCR mixture containing 12.5 µl of TaqMan 2x SensiMix™, 0.32 µmol/L of each *G. duodenalis* specific primer, and 0.12 µmol/L of *G. duodenalis* specific double-labeled probe. Amplification consisted of a step of 5 min 98°C followed by 45 cycles of 10 s at 98°C, and 30 s at 60°C in an Qiagen rotor gene 6000 instrument. The detection of *G. duodenalis* in each stool sample was conducted in duplicate. The negative (distilled water) and positive (*G. duodenalis* trophozoite DNA) controls were included in each real-time PCR reaction. Amplification curves are graphs plotting the normalized fluorescence signals of a real-time PCR reaction against the PCR cycle number. Amplification fluorescence signal was presented in positive control but not in negative control.

Increasing fluorescence during amplification suggests the presence of *G. duodenalis* in the sample examined. The CT (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold. CT levels are inversely proportional to the amount of *G. duodenalis* DNA in the stool samples.

Statistical Analysis

Descriptive analysis was used with percentages to demonstrate the positive cases of *G. duodenalis* and other intestinal parasites by microscopic examination and/or real-time PCR.

RESULTS

Most participants (93.07%; 121/130) showed either soft or hard stools. Only 9 cases (6.93%; 9/130) showed liquid stools. The 130 stool samples were examined microscopically for intestinal parasites. In addition, real-time PCR method was performed to detect *G. duodenalis* in the 130 stool samples. The overall prevalence of intestinal parasitic infection was 41.53% (54 out of 130 children), and 10 cases of *Giardia* infection (7.69%) were disclosed by microscopic examination. Other intestinal parasites including *Blastocystis hominis*, *Entamoeba coli*, *Endolimax nana*, *Entamoeba histolytica*-like, *Chilomestix mesnili*, *Ascaris lumbricoides*, and Hookworm were observed, but the results of real-time PCR indicated that 40 of the stool samples were positive for *G. duodenalis* (Table 1). It is interesting that only 3 out of 9 liquid stools were positive for *G. duodenalis* by microscopic examination while all of these were *G. duodenalis* positive by the real-time PCR method. The most frequently found protozoa were *G. duodenalis*, as shown in this study.

No amplification curves were present using DNA from other intestinal protozoa or negative control (sterile water), except *G. duodenalis*, as shown in Fig. 1. Thus, no cross-reactivity was seen using this method, indicating that the assay identified *G. duodenalis* accurately. The detection limit of real-time PCR method for detecting *G. duodenalis* was determined with 10-fold serial dilution of *G. duodenalis* trophozoite DNA. The lowest detectable concentration was 0.1 pg (Fig. 2).

In this study, all *Giardia* positive samples were calculated directly based on DNA weight in nanogram from the standard curve (Fig. 3). Moreover, the number of trophozoites or cysts in *Giardia* positive stool samples was calculated by the following equation: X ng/0.144 and X ng / 0.313, respectively (X: Quantity of *G. duodenalis* DNA sample obtained from the standard curve; each individual *G. duodenalis* trophozoite and mature cyst contained ~0.144 pg and 0.313 pg of DNA, respectively) [12]. In fact, it is difficult to differentiate the original source of these DNA of *G. duodenalis* (trophozoite or cyst stage). Thus, we assumed

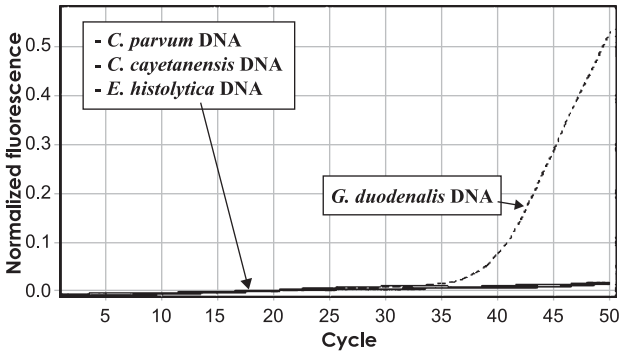

Fig. 1. Amplification curve showing increases in fluorescence from *Giardia duodenalis* DNA. No fluorescent signals presented in other intestinal protozoa (*Cryptosporidium parvum*, *Cyclospora cayetanenis* and *Entamoeba histolytica*)

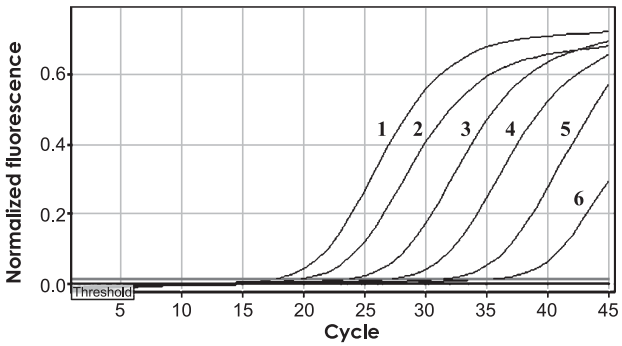

Fig. 2. Amplication curve of 10-fold serial dilution of *Giardia duodenalis* trophozoite DNA. 1 = 10 ng, 2 = 1 ng, 3 = 0.1 ng, 4 = 0.01 ng, 5 = 1 pg, 6 = 0.1 pg

Table 1. Comparison between microscopic examination and real-time PCR method for detection of *Giardia duodenalis* (n = 130)

Method (for detecting *G. duodenalis*)		Microscopic examination		Total
		Positive	Negative	
Real-time PCR	Positive	10	30	40 (30.77%)
	Negative	0	90	90 (69.23%)
	Total	10 (7.69%)	120 (92.31%)	130 (100%)

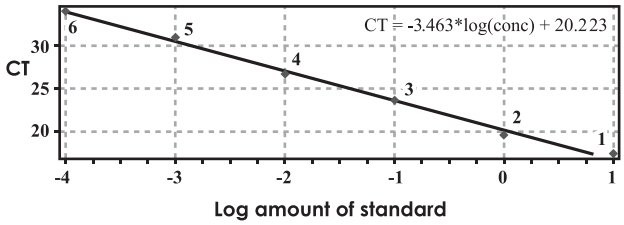

Fig. 3. Standard curve of 10-fold serial dilution of *Giardia duodenalis* trophozoite DNA. 1 = 10 ng, 2 = 1 ng, 3 = 0.1 ng, 4 = 0.01 ng, 5 = 1 pg, 6 = 0.1 pg; R = 0.997

Table 2. The number of *Giardia duodenalis* cysts among 3 different types of stool

Type of Stool	No. of cysts Median (range)
Liquid (n = 9)	700 (500–2,000)
Soft (n = 21)	250 (75–400)
Hard (n = 10)	100 (6–300)

that the number of *G. duodenalis* in stools can be calculated from the cyst stage. The results revealed that liquid stools contained higher amounts of *G. duodenalis* than soft or hard stools (liquid stool, 500–2,000 cysts/25 µl of reaction; soft stool, 75–400 cysts/25 µl of reaction; and hard stool, 6–300 cysts/25 µl of reaction), as shown in Table 2.

DISCUSSION

In Thailand, intestinal parasitic infections still pose serious public health problems [13, 14]. It is difficult to eradicate them from our country. Several factors present a risk for intestinal parasitic infections, such as geographic area and personal and community hygiene [15]. Soil-transmitted helminth (STH) infections are more common in moist climates (similar to the study area), low-income communities, and places where hygiene and sanitation are poor. STH are transmitted through contaminated soil and consist of *A. lumbricoides*, whipworm (*Trichuris trichiura*), Hookworm (*Ancylostoma duodenale* and *Necator americanus*) and *Strongyloides stercoralis* [16]. The majority of STH infections occur in school-age children [17–19] who are at risk for malnutrition and anaemia. Our results also demonstrated *A. lumbricoides* and Hookworm in the stool samples of school-age children.

G. duodenalis is the most common protozoa found worldwide [20]. Major infections result from fecal-oral transmission and produce non-specific symptoms (asymptomatic to symptomatic). The clinical manifestations depend on host immunity and parasite load. There are two important assemblages (A and B) of *G. duodenalis* that can cause human giardiasis [21, 22]. The results of this study showed that *Giardia* infection was the most frequently occurring pathogenic intestinal parasitic infection among children living in the study area.

Several methods can be used to examine intestinal parasitic infections in humans, ranging from conventional to modern. The conventional diagnostic method is microscopic examination of stool samples. This is still commonly used in many countries including Thailand. The disadvantages of this method include a low sensitivity and the need for expert technicians, leading to misdiagnosis in mild infection and failure to treat infected persons who can easily and unwittingly spread cysts to other individuals or communities [23]. As a result, microscopic examination may not be a suitable method for detecting low levels of parasite infection. To increase the sensitivity of microscopic examination, three samples should be collected on different days, but the collection of a large number of stool samples is not appropriate because it is a labor-intensive and time-consuming method. Interestingly, most *Giardia*-positive cases in our study are asymptomatic and/or show a low number of *Giardia* cysts in stool samples. In this study, some liquid stools were *Giardia*-positive by microscopy while all of them were *Giardia*-positive by real-time PCR. This is attributable to the fact that liquid stools are diluted and difficult to examine microscopically while real-time PCR is a highly sensitive method [24–26]. That is why we detected *G. duodenalis* at a level of less than 1 trophozoite (the lowest detectable concentration = 0.1 pg). Moreover, we selected the *ssrRNA* gene for this study because it presents multiple copies, allowing easier detection [27].

At present, the real-time PCR method is an alternative method for detecting pathogenic protozoa including *G. duodenalis*. It is a rapid method with high sensitivity and specificity and also a quantitative technique. Detection of parasitic DNA by PCR is more sensitive than that by microscopy. It can detect both mild and asymptomatic *Giardia* infections in children living on the Thai-Myanmar border.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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