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임상미생물학 LMO

한국인 환자에서 장관 감염성 원충의 검출을 위한 BD MAX Enteric Parasite Panel과 Seegene Allplex Gastrointestinal Parasite Assay의 비교 평가

Detection of Intestinal Protozoa in Korean Patients Using BD MAX Enteric Parasite Panel and Seegene Allplex Gastrointestinal Parasite Assay

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Background: Intestinal protozoan infection is one of the main causes of gastrointestinal diseases. Protozoa are usually detected by direct smear microscopy, concentration techniques, or special stains; however, these techniques are labor-intensive and require well-trained technicians. Therefore, molecular techniques involving polymerase chain reaction (PCR) have been developed to satisfy the need for unbiased and rapid analytical methods with high sensitivity and specificity. In this study, the BD MAX™ Enteric Parasite Panel (EPP) (Becton, Dickinson and Company, USA), designed to detect *Cryptosporidium parvum* and/or *hominis*, *Giardia lamblia*, and *Entamoeba histolytica*, and the Allplex™ Gastrointestinal Parasite Assays (AGPA) (Seegene Inc., Korea), designed to detect *Cryptosporidium* species, *G. lamblia, E. histolytica, Blastocystis hominis, Dientamoeba fragilis*, and *Cyclospora cayetanensis* were compared to determine whether any of these assays could become a useful tool for detecting intestinal protozoan infections in Korea.
Methods: We investigated 295 fecal samples using EPP and AGPA. Then we confirmed the positive results with the conventional and nested PCR. Consistent detection by conventional PCR, nested PCR, and one of the multiplex panels was considered "true positive."
Results: Out of 295 samples, 17 were true positives for *B. hominis* and 2 were true positives for *E. histolytica*. EPP detected parasites in only two samples owing to its design; however, its true positive detection rate was 100% (2/2). AGPA detected parasites in 24 samples with 79.2% (19/24) true positives.
Conclusions: The incidence of protozoan, especially *B. hominis*, infection may be more prevalent than expected. AGPA could be an effective tool for screening protozoan infections.

Key Words: Parasite infection, Real-time PCR, *Blastocystis hominis*

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INTRODUCTION

Intestinal parasite infection is one of the main causes of gastrointestinal diseases [1]; however, parasitic infections in affected patients have been underreported compared to bacterial or viral infections [2]. Intestinal protozoan parasites cause various gastrointestinal symptoms, ranging from asymptomatic to life-threatening watery or hemorrhagic diarrhea [3, 4]. Most laboratories use the microscopic ova and parasite examination for stool parasite testing. Usually direct smear microscopy, concentration techniques [5], or special stains such as Modified Fields' stain, Giemsa stain, or iron hematoxylin stain are needed to detect protozoans [6]; however, these techniques are labor-intensive and require well-trained and highly skilled technicians for optimal interpretation. Furthermore, protozoan parasites are difficult to identify especially when they are present in low numbers; therefore, microscopic examination is not effective for accurate detection of the parasites due to low diagnostic sensitivity and specificity [3, 7-10]. Hence, molecular techniques involving polymerase chain reaction (PCR) have been developed for detecting protozoa to satisfy the need for unbiased and rapid analytical methods with high sensitivity and specificity [11, 12].

Cryptosporidium, Blastocystis, Entamoeba, Giardia lamblia, and *Dientamoeba fragilis* are the predominant species associated with intestinal infection etiology globally [1]. The commercially available multiplex panels, BD MAX™ Enteric Parasite Panel (EPP) (Becton, Dickinson and Company, Sparks, MD, USA) and Allplex™ Gastrointestinal Parasite Assays (AGPA) (Seegene Inc., Seoul, Korea), are designed to detect protozoans. EPP is designed to detect *Cryptosporidium parvum* and/or *hominis, G. lamblia*, and *Entamoeba histolytica*, which are the most common parasites in developed countries. AGPA is designed to detect *Cryptosporidium parvum* and/or *hominis*, *G. lamblia*, *Entamoeba histolytica*, *Blastocystis hominis*, *D. fragilis* and *Cyclospora cayetanensis*.

In this study, we compared EPP and AGPA to determine whether any of these assays could become a useful tool for detecting intestinal protozoan infections from fecal samples in Korean laboratory settings.

MATERIALS AND METHODS

1. Sample collection

A total of 295 fecal samples, including both loose and formed stool, from patients with symptoms of gastroenteritis or patients visiting for regular health check-up were collected from September 2017 to May 2018. Most of the 264 fecal samples from patients with gastroenteritis were not examined for intestinal parasites but were tested for bacterial or viral infections. The samples were stored at -80°C until used for DNA extraction. This study was approved by the Institutional Review Board of the Severance Hospital (IRB no. 4-2016-0946).

2. DNA extraction

Genomic DNA from 295 fecal samples was extracted using the QIAamp DNA mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The DNA samples were stored at -20°C until tested.

3. Parasites detection using multiplex real-time PCR

The fecal samples were processed using fully automated real-time PCR systems; EPP designed to detect *Cryptosporidium parvum* and/or *hominis, G. lamblia*, and *Entamoeba histolytica*, and AGPA designed to detect *Cryptosporidium* species, *G. lamblia*, *E. histolytica*, *Blastocystis hominis*, *D. fragilis*, and *Cyclospora cayetanensis*. The fecal samples were processed in accordance with the manufacturers' instructions. For EPP, the stool sample was directly applied using inoculation loop; it took about 3–4 hours to show the ct value, and the type of parasite was identified using an exclusive program. AGPA requires the extracted DNA for the real-time PCR, and it takes about 2–3 hours to get the result.

4. Confirming positive results of EPP and AGPA through conventional and nested PCRs

The conventional and nested PCRs were designed to confirm the positive results of EPP and AGPA, and the primers used are shown in Table 1. We originally designed the first primer for *B. hominis* using Primer 3 software. Then we designed the second primers for the nested PCR using a free trial of Genious prime version 2019.1.1, downloaded from the website http://geneious.com (Biomatters Ltd. Auckland, New Zealand). Each of the second primers for amplification was selected with size ranging from

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Table 1. Conventional and nested PCR primers

Target Organism		Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)	Target	Accession number of target sequence
Blastocystis hominis	First primer	GGAGAGGGAGCCTGAGAGAT	ACTTGCCCTCCAATTGTTTATCG	18s rRNA	KX908213.1
	Second primer	GAGATGGCTACCACATCCAA	GGATTGGGTAATTTACGCGC		
Cryptosporidium parvum	First primer*	TGTGTTCAATATCTCCCTGCAAA	GCATGTCGATTCTAATTCAGCTG	Cowp1	AB089292.1
	Second primer	TGTGTTCAATATCTCCCTGCA	CTGGTGCCATACATTGTTGT		
Giardia lamblia	First primer*	GAGGTCAAGAAGTCCGCCG	CAAGGGACTTGCGGAAGTTT	betagiardin	XM_001705373.1
	Second primer	CGCCGACAACATGTACCT	CATGGTGTCGATCTCCTCC		
Entamoeba histolytica	First primer*	GCGGACGGCTCATTATAACA	TGTCGTGGCATCCTAACTCA	18s rRNA	X65163.1
	Second primer	CGGACGGCTCATTATAACAG	ACAAACTGGATCGTCTCAAG		
Dientamoeba fragilis	First primer*	TTAGACCTTAGACAACGGATGTCTTG	TGTGCATTCAAAGATCGAACTTATC	18s rRNA	JQ677163.1
	Second primer	CAACGGATGTCTTGGCTCT	TGCAACGTTCTTCATCGTG		

*The first primers of the parasites are from Won et al. [2].

30 to 50 base pairs. PCR reaction was established with the following conditions: pre-denaturing at 95°C for 5 minutes, 35 cycles of denaturation at 96°C for 30 seconds, gradient annealing at 57°C for 30 seconds, and extension at 72°C for 1 minute, and a final extension for 5 minutes at 72°C. Consistent detection of parasites through conventional PCR, nested PCR, and one of the commercial multiplex panels was considered "true positive." Any other results not confirmed by both conventional and nested PCR were considered "false positive."

RESULTS

As shown in Table 2, EPP detected two *E. histolytica* (No. 101 and No. 220) from 295 fecal samples (0.67%). AGPA detected parasites from 24 fecal samples out of 295 (8.14%) as follows: two *E. histolytica* (No. 101 and No. 220), one sample with *E. histolytica*, *C. parvum/hominis*, and *G. lamblia* (No. 163), one sample with *B. hominis* and *D. fragilis* (No. 32), two *G. lamblia*, and 18 *B. hominis*.

The 24 positive samples were re-tested using laboratory-designed conventional and nested PCR methods (Table 2). Overall, 19 out of 295 samples were positive, including 17 *B. hominis* and 2 *E. histolytica*. EPP only detected 2 parasites due to its design, but it was 100% true positive (2/2). AGPA detected parasites from 24 samples and 79.2% of the results were true positive (19/24). Five samples were considered false positive (No. 11, No. 22, No. 32, No. 163, and No. 269). Among those, three samples (No. 22, No. 163, and No. 269) showed negative results. *B. hominis* was detected in sample No. 11 and No. 32; in sample 11, *G. lamblia* was originally detected by AGPA. In sample No. 32, *B. hominis* was detected without the presence of *D. fragilis*.

Table 2. Comparison of two real-time PCR, conventional PCR, and nested PCR results

Sample No.	EPP	AGPA	Conventional PCR	Nested PCR
3	Negative	BH	BH	BH
5	Negative	BH	BH	BH
11	Negative	GL	BH	BH
15	Negative	BH	BH	BH
19	Negative	BH	BH	BH
22	Negative	GL	Negative	Negative
23	Negative	BH	BH	BH
32	Negative	BH, DF	BH	BH
38	Negative	BH	BH	BH
42	Negative	BH	BH	BH
65	Negative	BH	BH	BH
77	Negative	BH	BH	BH
89	Negative	BH	BH	BH
90	Negative	BH	BH	BH
91	Negative	BH	BH	BH
97	Negative	BH	BH	BH
101	EH	EH	EH	EH
115	Negative	BH	BH	BH
118	Negative	BH	BH	BH
163	Negative	GL, EH, CR	Negative	Negative
220	EH	EH	EH	EH
238	Negative	BH	BH	BH
268	Negative	BH	BH	BH
269	Negative	BH	Negative	BH

Abbreviations: EPP, BD MAX™ Enteric Parasite Panel; AGPA, Allplex™ Gastrointestinal Parasite Assay; PCR, polymerase chain reaction; EH, *Entamoeba histolytica*; BH, *Blastocystis hominis*; GL, *Giardia lamblia*; CR, *Cryptosporidium parvum/hominis*; DF, *Dientamoeba fragilis*.

DISCUSSION

In this study, we compared the performance of two commercial multiplex panels in detecting intestinal protozoa using clinical stool samples (N=295) and confirmed the results with the con-

ventional and nested PCRs. Many studies from outside of Asia have already approved the detection ability of EPP to be highly sensitive and specific [10, 13, 14]. In this study, EPP effectively detected *E. histolytica* (sample No. 101 and No. 220), which were also detected by AGPA. Additionally, EPP showed negative results from sample No. 22 and No. 163, which were consistent with the results of conventional and nested PCRs. Nevertheless, it is difficult to conclude that EPP's specificity is excellent since negative results may be shown due to its inability for detecting other types of parasites.

Despite the high sensitivity of EPP, AGPA could detect parasites from more fecal samples in this study since it is designed to detect *B. hominis*, *G. lamblia*, *D. fragilis*, *E. histolytica*, *C. caytanensis*, and *Cryptoporidium* spp. AGPA demonstrated high efficacy in detecting the parasites, especially *B. hominis*. Due to the kit's design, AGPA can primarily be used to detect protozoan parasites more effectively than other kits [15]. Interestingly, AGPA detected *B. hominis* from 19 fecal samples with 2 false positives and 17 true positives. In brief, *B. hominis* was detected from 5.8% of fecal samples (17/295 samples).

B. hominis is a gastrointestinal eukaryotic parasite found in human and many animals [16, 17]. Even though the prevalence of *B. hominis* in Korea is not well-known, it may be the most common human intestinal protozoan in the world [18], with a reported prevalence of more than 50% in developing countries [19]. It causes gastrointestinal symptoms, but in most cases, it is asymptomatic [4]. According to Salim et al., animal handlers have a higher risk of *B. hominis* infection [20].

In this study, three *B. hominis* positive samples (No. 38, No. 42, and No. 97) were from patients who submitted their stool for regular health check-up. Unfortunately, we could not identify if the patients were animal handlers and what gastrointestinal symptoms they might have experienced. Although treating asymptomatic patients colonized with *B. hominis* may not be needed [21], still, detecting *B. hominis* is clinically important in Korea due to the increase in the number of people raising pets in households, and the rising interest in fecal microbiota transplantation in hospitals. Detecting *B. hominis* is one of the exclusion criteria for selecting donors for fecal microbiota transplantation [22]. Not only microscopic examination but also culturing *B. hominis* is possible; however, since there are many difficulties in using in vitro culture for diagnosing *B. hominis* [23], PCR methods can be helpful in situations such as selecting donors for fecal microbiota transplantation.

Therefore, despite EPP's detection ability, it may not be the right choice for detecting intestinal parasites in Korean clinical setting; however, AGPA could possibly be used for screening purposes since it can help the laboratory to detect *B. hominis* from fecal samples. We could not examine all the fecal samples microscopically, and further investigations could not be implemented due to the deterioration of the samples. Fresh fecal samples for both microscopic examination and RT-PCR assays may improve the detection of intestinal parasites. Additionally, sequencing PCR products may help in confirming the type of parasites, therefore, further studies comparing RT-PCR and sequencing results are needed.

Meanwhile, owing to the lack of prevalent parasites in Korea for both assays, complementary microscopic examination would be needed to increase the detection rate of parasites. Ironically, however, low prevalence of parasites makes it more difficult to create new detection methods. It is the reason why AGPA could not receive approval from the South Korea Ministry of Food and Drug Safety (MFDS), and the development of new multiplex PCR to detect prevalent parasites such as *C. sinensis* and *M. yokogawai* is difficult. Despite the difficulties, PCR-based methods, which can be effectively used in laboratories in Korean hospitals, should be developed to manage parasitic infections.

요 약

배경: 장내 원충 감염은 위장관계 질환의 주요 원인 중 하나이다. 일반적으로 직접도말법, 집란법, 또는 특별한 염색법 등을 사용하여 현미경으로 원충 감염을 진단한다. 그러나 이러한 방법은 노동집약적이며 매우 숙련된 검사자가 필요하다는 단점이 있다. 따라서 높은 민감도와 특이도로 빠르면서도 편견 없는 결과가 필요함에 따라 PCR 등을 이용한 분자생물학적 진단방법이 발전되어왔다. 이 연구에서는 국내 검사실에서 장내 원충류 검사를 위해 *Cryptosporidium parvum/hominis, G. lamblia* 그리고 *Entamoeba histolytica*를 검출하는 BD MAX™ Enteric Parasite Panel (EPP) (Becton, Dickinson and Company, USA)와 3개의 원충류에 추가적으로 *Blastocystis hominis, D. fragilis* 그리고 *Cyclospora cayetanensis*를 검출하는 Allplex™ Gastrointestinal Parasite Assays (AGPA) (Seegene Inc., Korea) 중 어느 것이 유용할지 비교해보고자 하였다.

방법: 저자들은 295개의 대변 검체들을 EPP와 AGPA를 사용하여

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검사하였고 이 중 양성 결과를 확인하기 위해 일반 PCR 및 nested PCR을 이용하여 재검하였다. EPP 또는 AGPA의 결과 및 일반 PCR, Nested PCR에서 동일한 결과를 진양성으로 간주하였다.

결과: 총 295개의 대변 검체에서 17개의 *B. hominis* 및 2개의 *E. histolytica*가 검출되었다. EPP는 검출 가능한 원충류 종류로 인한 한계로 비록 2개의 원충만 검출하였으나 이는 100% (2/2) 진양성이었다. AGPA는 총 24개의 검체에서 원충류를 검출하였으나 이 중 진양성 및 위양성은 각각 79.2% (19/24), 20.8% (5/24)였다.

결론: 원충류 감염, 특히 *B. hominis*의 감염은 예상보다 많을 수 있다. 따라서 AGPA는 원충류 감염의 스크리닝 목적으로 사용될 수 있을 것으로 판단된다.

Conflicts of Interest

None declared.

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