Osong Public Health Res Perspect 2013 4(3), 146–151
http://dx.doi.org/10.1016/j.phrp.2013.04.006
pISSN 2210-9099 eISSN 2233-6052

ELSEVIER

ORIGINAL ARTICLE

Development of a Diagnostic Kit to Detect *Cryptosporidium parvum* and *Giardia lamblia*

Hyeng-Il Cheun [a,c], Byung-Suk Chung [a], Da-Won Ma [a], Bo-La Goo [a], Shin-Hyeong Cho [a], Mi-jung Ji [b], Won-Ja Lee [a,*]

[a]*Division of Malaria and Parasitic Diseases, Korea National Institute of Health, Osong, Korea.*
[b]*Biotech Laboratory, Standard Diagnostics Inc., Yongin, Korea.*
[c]*Division of Epidemic Intelligence Service, Korea Centers for Disease Control and Prevention, Osong, Korea.*

Received: March 1, 2013
Revised: March 25, 2013
Accepted: April 5, 2013

KEYWORDS:
Cryptosporidium parvum, *Giardia lamblia*, immunochromatography, rapid diagnostic kit

Abstract
Objectives: This study aims to develop a high-sensitivity antibody diagnostic kit that will enable a rapid and accurate detection of *Cryptospofidium parvum* and *Giardia lamblia* in patients with diarrhea.
Methods: The cultivated *C. parvum* oocysts and *G. lamblia* cysts in each calf and dog were injected to mice to obtain antibodies, which were titrated. Spleen cells of the immunized mouse were separated and blended with myelomas to produce hybrid cell lines that form monoclonal antibodies. Using ELISA method, antibodies that specifically respond to *C. parvum* and *G.lamblia* were then selected. The cells were injected into the abdominal cavity of a BALB/c mouse to isolate hydrops abdominis containing high level of antibodies. The IgG antibody was purified using protein G gel.
Results: The detection limit of monoclonal antibodies for *Cryptosporidium parvum* and *Giardia lamblia* was 125 oocysts/mL and 1250 cysts/mL, respectively. In addition, during testing they did not show cross-reactivity to viruses ($n = 15$), bacteria ($n = 17$), and parasites ($n = 9$).
Conclusion: The rapid diagnostic antibody kit developed in this study, which specifically responds to *C. parvum* and *G. lamblia*, will be useful in detecting and monitoring diarrheal infections.

1. Introduction

Cryptosporidium parvum and *Giardia lamblia* have been recognized as the causative agents of diarrhea in humans worldwide [1]. These protozoans are transmitted by the fecal–oral route and most commonly by the consumption of contaminated food and water [2]. Infections are mostly seen in young children and immunocompromised patients. These infections are seen in both developing countries and developed countries.

*Corresponding author.
E-mail: wonjalee@gmail.com

However, especially in developing countries, there is an increased risk of transmission, due to urban crowding and poor sanitation facilities [3].

Among the Korean population, *C. parvum* and *G. lamblia* account for less than 1% of diarrheal cases; however, the rate of *C. parvum* infection has been on the rise, and one case of infection by *G. lamblia* was reported in Jinan-gun, Jeollabuk-do Province, among people who drank water from a nearby valley [4,5].

Traditional diagnostic methods for treating these parasitic infections include testing fecal samples for the pathogen and must include concentration procedures along with specific staining techniques for proper microscopic detection and identification of the parasite [6]. These methods are laborious, take a long time, and require specialized and trained personnel. Although other techniques such as immunofluorescence microscopy improve sensitivity, they are expensive and laborious, and are not routinely available in all laboratories [7]. In addition, molecular techniques to detect *Cryptosporidium*, *Giardia* include polymerase chain reaction (PCR) and real-time PCR that provide high sensitivity and specificity, but these techniques are time consuming and require expensive specialized equipment [8,9].

Therefore, there is a need for a simple yet accurate method of detection for rapid and effective treatment of diarrheal infection. The aim of this study was to develop a new antigen diagnostic kit and evaluate its efficiency in detecting *C. parvum* and *G. lamblia* infections. In addition, the usefulness of this rapid diagnostic kit was compared with enzyme-linked immunosorbent assay (ELISA) and other diagnostic kits that are commercially available.

2. Materials and Methods

2.1. Preparation of immunogen

C. parvum oocyst was purchased from MEGACOR (MEGACOR Diagnostik GmbH, Hoerbranz, Vorarlberg, Austria) and orally injected to a month-old calf. From the 2nd day onward, the stool samples were examined with a Crypto-Strip (Coris BioConcept, Gembloux, Belgium). The samples were floated on saline solution, kept still for 2 hours, and the upper layer was extracted. The collected fluid was centrifuged and the precipitation was cleansed three times with sterilized saline solution to retrieve *C. parvum* oocysts.

G. lamblia cyst was purchased from American Type Culture Collection (Manassas, VA, USA; catalog number: PRA-242) and orally injected to a 2-month-old beagle dog. From the 2nd day onward, the stool samples were examined with a Giardia-Strip (Coris BioConcept). The cysts were retrieved similar to the procedure described for *C. parvum*.

2.2. Administration of adjuvant emulsions

Samples of *C. parvum* and *G. lamblia* were separately mixed with complete Freund's adjuvant (Sigma Aldrich). Approximately 200 μg of the emulsion was injected four times into the tail vein of a mouse at a 2-week interval. While complete adjuvant was used for the first injection, incomplete adjuvant was used for the rest of the injections. Overall, three intravenous injections were administered into the tail vein of the mouse.

2.3. Serum collection, titration, and cell fusion

A small amount of blood was drawn from the tail of the immunized mouse; subsequently, the serum was separated and ELISA was used for titration of the serum sample. The immunogen was adhered to the ELISA plate at a concentration of 1 μg/mL. An antiserum was then diluted in ten stages (10, 100, 1000 times, and so on) by adding 1% bovine serum albumin for reactivity test. Secondary reactivity test was conducted using the goat antimouse immunoglobulin G (IgG) peroxidase, and 3,3′,5,5′-tetramethylbenzidine substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) was added for color development. The cut-off rate was set at three times the absorbance level of a normal mouse serum. At a dilution factor greater than 1000, if the sample shows antibody titer above the cut-off value, cell fusion was performed. Spleen cells of the immunized mouse were separated and blended with myelomas to produce hybrid cell lines that form monoclonal antibodies. Using ELISA method, antibodies that specifically respond to *C. parvum* were then selected. The cell lines were cultured in a large

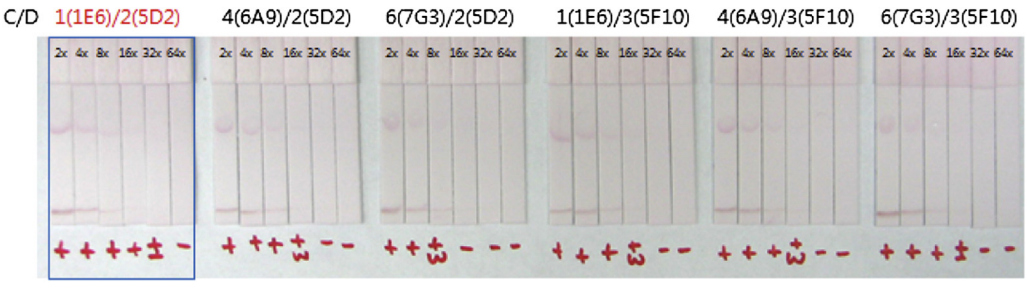

Figure 1. Selection of optimal pair for anti-*Cryptosporidium parvum* by the detection limit test.

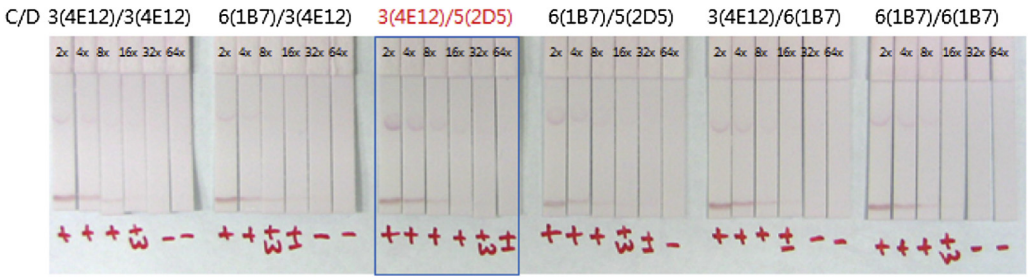

Figure 2. Selection of optimal pair for anti-*Giardia lamblia* by the detection limit test.

quantity, and the cells were injected into the abdominal cavity of a BALB/c mouse to isolate hydrops abdominis containing high level of antibodies. The IgG antibody was purified using protein G gel.

2.4. Pharmaceutical engineering test

A solution containing 40-nm gold nanoparticles was prepared. To 1 mL of this solution, 20 μg of the antibody was added. The pH levels were properly adjusted for each antibody and at the end of the preset time, a solution of polyethylene glycol was added to stop the reaction and obtain gold conjugates. A matching test was then carried out for the purified antibody and the gold conjugates to identify the optimal pair of antibodies for the test and the gold conjugates.

3. Results

3.1. Development of *C. parvum*-specific antibody

From the serum of the immunized mouse, three antibodies with the highest level of anti-*Cryptosporidium* titers were selected for fusion (data not shown); among the hybridomas produced, seven cells (numbers 1−7) with the highest anti-*Cryptosporidium* titers in the supernatant were selected to test detection limit by antibody dilution. According to the test results, cell number 1 (1E6) and cell number 2 (5D2) were selected as antibodies for the final test and the gold conjugates, respectively (Figure 1).

3.2. Development of *G. lamblia*-specific antibody

From the serum of the immunized mouse, five antibodies with the highest level of anti-*Cryptosporidium* were selected for fusion (data not shown); among the hybridomas produced, eight cells (numbers 1−8) with the highest anti-*Cryptosporidium* titer in the supernatant were selected to test detection limit by antibody dilution. According to the test results, cell number 3 (4E12) and cell number 5 (2D5) were selected as antibodies for the final test and the gold conjugates, respectively (Figure 2).

3.3. Detection limit test for *C. parvum* and *G. lamblia*

In order to test the detection limit of *C. parvum*, the samples were tested with two imported rapid detection kits, namely, Crypto-Strip and *Cryptosporidium* Antigen Detection ELISA (Diagnostics Automation, Inc.). The results of the comparative test were that the solution developed in this study, Crypto-Strip, and *Cryptosporidium* Antigen Detection ELISA showed positive concentration for 12.5 oocysts/0.1 mL, 50 oocysts/0.1 mL, and 12.5 oocysts/0.1 mL, respectively (data not shown). In case of *G. lamblia*, the detection limit was 125 cysts/0.1 mL, 250 cysts/0.1 mL, and 125 cysts/0.1 mL, respectively (data not shown).

3.4. Sensitivity and specificity test for field sample

The sensitivity and specificity tests for the field samples obtained from Professor Dr Yeong-Min Lee (Department of Internal Medicine, Veterinary College of Jeju National University) were also conducted. The antibody developed in this study showed the same level of sensitivity and specificity when compared with the imported kits (tested using cow stool samples for *C. parvum* and dog stool samples for *G. lamblia*) (Table 1).

Table 1. Comparison of imported detection kits and antibody developed in this study

		CORIS BioConcept Crypto-Strip		CORIS BioConcept *Giardia*-Strip		*Cryptosporidium* antigen detection ELISA		*Giardia lamblia* antigen detection ELISA	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Antibody developed in this study	Positive	8	0	9	0	8	0	9	0
	Negative	0	30	0	30	0	30	0	30

ELISA = enzyme-linked immunosorbent assay.

Table 2. Results of cross-reactivity testing for *Cryptosporidium parvum* and *Giardia lamblia* antigen rapid kit against 15 viruses

Panel	Culture	Strain	Concentration (mg/mL)	Result	Reference
Virus	Adenovirus 3	GB	1	—	ATCC VR3
	Adenovirus 6	Tonsil 99	1.2	—	ATCC VR6
	Adenovirus 21	AV-1645/128	1	—	ATCC VR256
	Cytomegalovirus	Towne	1.5	—	ATCC VR977
	Echovirus 2	Cornelis	1.3	—	ATCC VR1039
	Echovirus 5	Noyce	0.9	—	ATCC VR1043
	Echovirus 11	Gregory	1	—	ATCC VR41
	Herpes simplex virus 1	F	0.25	—	ATCC VR733
	Herpes simplex virus 2	G	0.115	—	ATCC VR734
	Mumps virus	Enders	1.8	—	ATCC VR1379
	Parainfluenza virus 1	Sendai	1.5	—	ATCC VR105
	Parainfluenza virus 2	CA/Greer	3.9	—	ATCC VR92
	Parainfluenza virus 3	C243	1.93	—	ATCC VR93
	Respiratory syncytial virus	A-2	1	—	ATCC VR1540
	Respiratory syncytial virus	Long	0.42	—	ATCC VR26

ATCC = American Type Culture Collection.

3.5. Cross-reactivity test

A cross-reactivity test was conducted for 15 viruses, 17 bacteria, and nine parasites. The samples were diluted 100 times by extracting the stool samples. The samples did not show any cross reactivity (Tables 2–4).

4. Discussion

The World Health Organization ranks diarrheal disease as the second most common cause of morbidity and mortality in children in the developing world. The

Table 3. Results of cross-reactivity testing for *Cryptosporidium parvum* and *Giardia lamblia* Ag rapid test kit against 17 different bacteria

Panel	Culture	Concentration (cells/mL)	Result (dilution ratio 1:100)	Reference
Bacteria	*Bordetella pertussis*	1×10^8	—	ATCC 8467
	Enterococcus faecalis	1×10^8	—	ATCC 4079
	Escherichia coli	1×10^8	—	ATCC 25922
	Haemophilus influenzae	1×10^8	—	ATCC 8142
	Klebsiella pneumoniae	1×10^8	—	ATCC 4208
	Legionella pneumophila	1×10^8	—	ATCC 33153
	Mycobacterium avium	1×10^8	—	ATCC 15769
	Mycobacterium intracellulare	1×10^8	—	KTCC 9514
	Mycobacterium tuberculosis	1×10^8	—	ATCC 27294
	Mycoplasma pneumoniae	1×10^8	—	ATCC 15492
	Neisseria gonorrhoeae	1×10^8	—	ATCC 31953
	Neisseria meningitidis	1×10^8	—	ATCC 6250
	Proteus vulgaris	1×10^8	—	ATCC 6361
	Pseudomonas aeruginosa	1×10^8	—	ATCC 27853
	Staphylococcus aureus	1×10^8	—	ATCC 29213
	Streptococcus pneumoniae	1×10^8	—	ATCC 19615
	Streptococcus pyogenes	1×10^8	—	ATCC 4012

ATCC = American Type Culture Collection; KTCC = Korean Type Culture Collection.

Table 4. Results of cross-reactivity testing for the *Cryptosporidium parvum* and *Giardia lamblia* Ag rapid test kit against nine different parasites

Panel	Culture	Concentration (parasite/μL)	Result (dilution ratio 1:100)	Reference
Parasite	*Entamoeba histolytica*	1×10^4	—	ATCC 30190
	Microsporidia	1×10^4	—	ATCC 50040
	Cyclospora cayetanensis	1×10^4	—	Positive sample in this study
	Plasmodium vivax	1×10^4	—	Positive sample in this study
	Plasmodium falciparum	1×10^4	—	Positive sample in this study
	Toxoplasma gondii	1×10^4	—	ATCC 50174
	Trypanosoma cruzi	1×10^4	—	ATCC 50791
	Leishmania donovani	1×10^4	—	ATCC 50127
	Leishmania infantum	1×10^4	—	ATCC 50134

ATCC = American Type Culture Collection.

etiological agents of diarrhea are viruses, bacteria, and parasites. Among parasites, *Entamoeba histolytica*, *G. lamblia*, and *Cryptosporidium* spp. are considered to be the most common and important [3].

G. lamblia is considered as one of the main nonviral causes of diarrhea in developed countries [10]. Cryptosporidiosis is a frequent cause of diarrheal disease in humans. In developing countries, *Cryptosporidium* spp. infections occur mostly in children younger than 5 years of age, with a peak in children younger than 2 years of age [11]. In immunodeficient humans, especially individuals with human immunodeficiency virus infection/acquired immunodeficiency syndrome, cryptosporidiosis can be associated with chronic, potentially life-threatening diarrhea [11].

In Korea, *C. parvum* and *G. lamblia* infection is on the rise, which was not the case before [4,5,12]. And yet, there is no standard test method for the detection and diagnosis of the protozoan that causes acute diarrhea.

For these two kinds of parasites, diagnosis by analyzing microscopic results is neither sensitive nor specific. Individual antigen detection tests and PCR tests are now available for these parasites, but multiplex antigen detection tests are still under development [13]. The PCR assay for the detection of *G. lamblia* and *Cryptosporidium* spp. developed in this study is a useful alternative for diagnosis of these parasites.

In recent years, antigen-detection assays, such as enzyme immunoassays, various PCR methods, and immunochromatography, to detect *Cryptosporidium* and *Giardia* have been developed [13−15]. A number of products with a good range of sensitivity and specificity are commercially available. For the diagnosis of *Cryptosporidium*, discrepancies found between microscopy results and PCR can be interpreted as follows: in samples that were positive by microscopy and negative by PCR, this technique will fail because of the low number of oocysts present in the

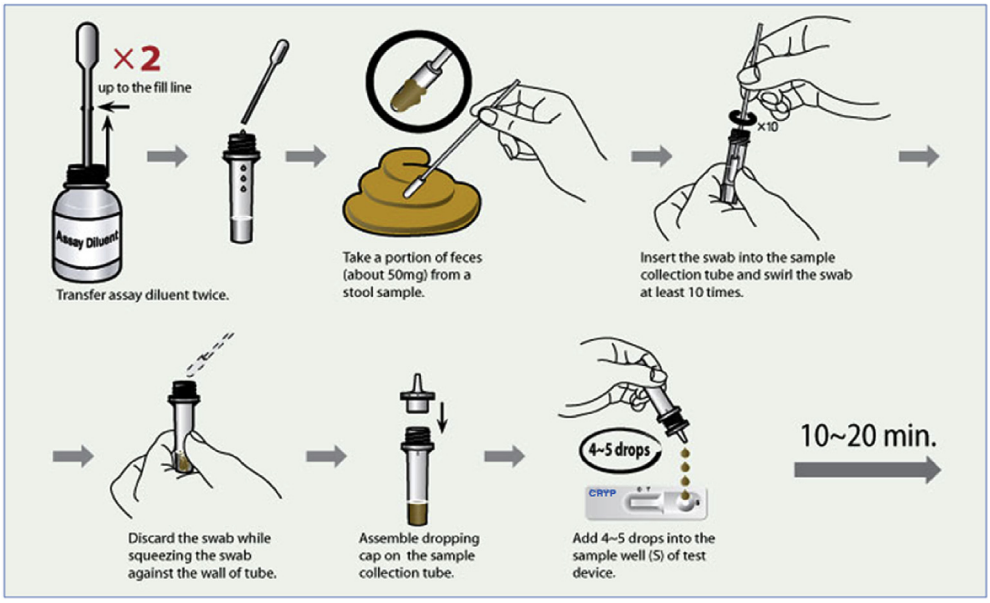

Figure 3. Test procedure for *Cryptosporidium parvus* and *Giardia lamblia* rapid test kit.

samples and/or because of the presence of PCR-inhibitory substances. By contrast, in samples that were negative by microscopy, with a low number of oocysts, and without the presence of PCR-inhibitory substances, the greater sensitivity of this technique permits the detection of *Cryptosporidium* [8].

In the case of *Giardia* detection, microscopy was the reference technique, but PCR was found to be 100 times more sensitive than ELISA in detecting this parasite [9].

In this study, an antibody diagnostic kit for *C. parvum* and *G. lamblia* was developed and compared with commercial kits. The kit will enable early detection and diagnosis of diarrheal infections, thereby preventing collective waterborne protozoans.

Appendix

For the testing method, stool (feces) samples were used. The testing steps are as follows (Figure 3):

- Open the tube containing the solution.
- Draw the assay diluents up to the fill line marked on the dropper, and transfer them to the sample-collection tube.
- Take a portion of the sample with a swab and dip it into the tube; swirl the swab at least 10 times.
- Discard the swab.
- Cap the tube.
- Take the test device out of the silver foil, and place it on an even surface.
- Add four to five drops of the diluted sample into the sample well.
- Analyze the outcome after 10−20 minutes. A positive reactivity can be seen immediately in some cases.

Acknowledgments

This work was supported by a grant from the Korea National Institute of Health (Grant No. NIH-091-4800-4847-300), National Research and Development Program, Ministry of Health and Welfare, the Republic of Korea.

References

1. Marshall MM, Naumovitz D, Ortega Y, Sterling CR. Waterborne protozoan pathogens. Clin Microbiol Rev 1997 Jan;10(1):67−85.
2. Fleckenstein JM, Bartels SR, Drevets PD, et al. Infectious agents of food- and water-borne illnesses. Am J Med Sci 2010 Sep; 340(3):238−46.
3. Savioli L, Smith H, Thompson A. *Giardia* and *Cryptosporidium* join the 'Neglected Diseases Initiative'. Trends Parasitol 2006 May;22(5):203−8.
4. Lee MY, Cho EJ, Lee JH, et al. A survey of *Cryptosporidium* oocysts in water supplies during a 10-year period (2000−2009) in Seoul. Korean J Parasitol 2010 Sep;48(3):219−24.
5. Lee MY, Cho EJ, Lee JH, et al. A ten-year survey of *Giardia* cysts in drinking water supplies of Seoul, the Republic of Korea. Korean J Parasitol 2011 Mar;49(1):9−15.
6. Garcia LS. Diagnostic medical parasitology. Washington, DC: ASM Press; 1997.
7. Garcia LS, Shimizu RY. Evaluation of nine immunoassay kits (enzyme immunoassay and direct fluorescence) for detection of *Giardia lamblia* and *Cryptosporidium parvum* in human fecal specimens. J Clin Microbiol 1997 Jun;35(6):1526−9.
8. Kehl KS, Cicirello H, Havens PL. Comparison of four different methods for detection of *Cryptosporidium* species. J Clin Microbiol 1995 Feb;33(2):416−8.
9. Lee JH, Lee J, Park SJ, et al. Detection and genotyping of *Giardia intestinalis* isolates using intergenic spacers(IGS)-based PCR. Korean J Parasitol 2006 Dec;44(4):343−53.
10. Ali SA, Hill DR. Giardia intestinalis. Curr Opin Infect Dis 2003 Oct;16(5):453−60.
11. Dillingham RA, Lima AA, Guerrant RL. Cryptosporidiosis: epidemiology and impact. Microbes Infect 2002 Aug;4(10): 1059−66.
12. Park JH, Kim HJ, Guk SM, et al. A survey of cryptosporidiosis among 2,541 residents of 25 coastal islands in Jeollanam-Do (Province), Republic of Korea. Korean J Parasitol 2006 Dec; 44(4):367−72.
13. Goñi P, Martín B, Villacampa M, et al. Evaluation of an immunochromatographic dip strip test for simultaneous detection of *Cryptosporidium* spp, *Giardia duodenalis*, and *Entamoeba histolytica* antigens in human faecal samples. Eur J Clin Microbiol Infect Dis 2012 Aug;31(8):2077−82.
14. Haque R, Roy S, Siddique A, et al. Multiplex real-time PCR assay for detection of *Entamoeba histolytica, Giardia intestinalis*, and *Cryptosporidium* spp. Am J Trop Med Hyg 2007 Apr;76(4): 713−7.
15. Minak J, Kabir M, Mahmud I, et al. Evaluation of rapid antigen point-of-care tests for detection of *Giardia* and *Cryptosporidium* species in human fecal specimens. J Clin Microbiol 2012 Jan; 50(1):154−6.