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

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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 Cryptosporidium 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.

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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 antiseraum 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.](image-url)
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 \textit{C. parvum}-specific antibody

From the serum of the immunized mouse, three antibodies with the highest level of anti-\textit{Cryptosporidium} titers were selected for fusion (data not shown); among the hybridomas produced, seven cells (numbers 1—7) with the highest anti-\textit{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 \textit{G. lamblia}-specific antibody

From the serum of the immunized mouse, five antibodies with the highest level of anti-\textit{Cryptosporidium} titers were selected for fusion (data not shown); among the hybridomas produced, eight cells (numbers 1—8) with the highest anti-\textit{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 \textit{C. parvum} and \textit{G. lamblia}

In order to test the detection limit of \textit{C. parvum}, the samples were tested with two imported rapid detection kits, namely, Crypto-Strip and \textit{Cryptosporidium} Antigen Detection ELISA (Diagnostics Automation, Inc.). The results of the comparative test were that the solution developed in this study, Crypto-Strip, and \textit{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 \textit{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 \textit{C. parvum} and dog stool samples for \textit{G. lamblia}) (Table 1).

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

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

ELISA = enzyme-linked immunosorbent assay.
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
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

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### 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 × 10⁴                     | —                            | ATCC 30190              |
|       | *Microsporidia*        | 1 × 10⁴                     | —                            | ATCC 50040              |
|       | *Cyclospora cayetanensis* | 1 × 10⁴                     | —                            | Positive sample in this study |
|       | *Plasmodium vivax*     | 1 × 10⁴                     | —                            | Positive sample in this study |
|       | *Plasmodium falciparum* | 1 × 10⁴                     | —                            | Positive sample in this study |
|       | *Toxoplasma gondii*    | 1 × 10⁴                     | —                            | ATCC 50174              |
|       | *Trypanosoma cruzi*    | 1 × 10⁴                     | —                            | ATCC 50791              |
|       | *Leishmania donovani*  | 1 × 10⁴                     | —                            | ATCC 50127              |
|       | *Leishmania infantum*  | 1 × 10⁴                     | —                            | ATCC 50134              |

ATCC = American Type Culture Collection.

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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.

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