APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2007, p. 5949–5950 Vol. 73, No. 18
0099-2240/07/$08.00+0 doi:10.1128/AEM.00668-07

Sensitivity of Nested PCR in the Detection of Low Numbers of *Giardia lamblia* Cysts[▽]

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Received 23 March 2007/Accepted 13 July 2007

Nested PCR was performed on individually isolated *Giardia lamblia* cysts in replicates of 50 for sets of 1, 2, 3, 4, 5, 7, and 10 cysts. Amplification ranged from 80% for 1 cyst to 100% for 10 cysts. The results suggest that nested PCR is well adapted for *G. lamblia* single-cyst detection.

Giardia lamblia (also known as *Giardia duodenalis* and *Giardia intestinalis*) is an intestinal protozoan parasite and a leading cause of diarrheal disease in humans worldwide (2, 4, 7, 9). Transmission occurs via the fecal-oral route, with consumption of contaminated water as a major cause of infection (1, 2, 3, 5, 6, 8, 10, 12). Possible symptoms of infection include malabsorption, loose or watery stools, dehydration, and abdominal cramping (2, 3, 5). Giardiasis is generally self-limiting in healthy individuals, with most cases being asymptomatic (3, 5). However, infection can pose a serious health risk to the immunocompromised and to children whose immune systems have difficulty clearing the infection (3). In addition, prolonged giardiasis causing chronic malnutrition in children up to 2 years old has been correlated with poor cognitive function later in life (4).

Of the six genotypes of *G. lamblia* characterized thus far, two (A and B) are known to infect humans (2, 3, 5, 13). Therefore, when performing epidemiological and risk assessment studies, it becomes important to be able to differentiate between the human and nonhuman genotypes (6). Currently, various PCR assays are able to distinguish between the different recognized *G. lamblia* genotypes and are being used to assist with epidemiological and waterborne-disease-outbreak studies as well as to monitor water systems (1, 3, 5, 6, 8, 10, 12, 13). Also, due to the increased usage of PCR as a detection assay, it has become necessary to assess the sensitivity of the technique for singly isolated *Giardia* cysts due to the low infectious dose and the low naturally occurring numbers in the environment (1, 6, 7). Previous studies evaluating the sensitivity of PCR in the detection of low numbers of cysts have used dilution methods after hemocytometer enumeration from a purified cyst stock solution (1, 8, 10). Performing dilutions to obtain low cyst numbers to the point of a single cyst causes a standard deviation, and thus, the true number of cysts is not being evaluated. This process also does not account for the potential loss of DNA through extraction and purification techniques. The purpose of this project was to accurately determine how efficiently nested PCR could amplify the DNA from low numbers of *G. lamblia* cysts.

In order to obtain data on the sensitivity of nested PCR in the detection of confirmed low numbers of cysts, a micromanipulation technique was utilized to isolate and transfer single cysts (11). *Giardia lamblia* cysts obtained as purified suspensions from Waterborne, Inc., were isolated through micromanipulation, followed by detection with nested PCR. The cysts, previously sequenced as genotype B (data not shown), were initially fluorescent antibody labeled using a Merifluor *Cryptosporidium*/*Giardia* direct immunofluorescence detection kit. To ensure that single cysts were isolated at one time, microscopic isolation was completed on 75 single cysts, which were transferred to microscope slides and visually detected at 20× microscopy (11). Validation of our micromanipulation technique revealed an 82.7% success rate, with the capture and distribution of 62 of 75 individual cysts. On 5 separate tries, 2 cysts were captured at once, and 8 of the 75 attempts yielded no delivery of cysts to the microscope slides.

To evaluate the detection efficiency of nested PCR, micromanipulated cysts were isolated and transferred, in sets of 1, 2, 3, 4, 5, 7, or 10 cysts, into 50 separate PCR tubes containing approximately 2 μl of 1× PCR buffer. The isolated cysts were subsequently subjected to five freeze/thaw cycles (2 min in liquid nitrogen followed by 2 min in a 98°C water bath), followed by nested PCR detection. The external primers (AL3543 and AL3546) and the nested primers (AL3544 and AL3545) used in this study created 605-bp and 530-bp amplicons, respectively (13). The above-mentioned primers were chosen for their abilities to amplify a region of the highly conserved triosephosphate isomerase gene for *G. lamblia* genotypes A and B. The external PCR mixture consisted of 2.0 μl of template DNA, 200 μM of each deoxynucleoside triphosphate (Fermentas), 1× PCR buffer (Eppendorf), 3 mM $MgCl_2$ (Invitrogen), 10% dimethyl sulfoxide (Sigma), 0.1 μl bovine serum albumin (Sigma), 1.0 U of Hotmaster *Taq* polymerase (Eppendorf), and 200 nM of each primer for a 50-μl total volume per reaction. The nested PCR mixture consisted of the same components listed above, including 2.0 μl of the external PCR product instead of template DNA. Both external and nested PCR mixtures were subjected to an initial temperature of 94°C for 3 min, followed by 40 cycles of 94°C for 20 s, 50°C for 10 s, and 72°C for 1 min, and a final extension period of 72°C for 10 min in an Eppendorf Mastercycler gradient. PCR products and a 100-bp ladder were visualized on a 1% ethidium bromide-stained agarose gel for confirmation of amplification.

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▽ Published ahead of print on 20 July 2007.

This nested PCR protocol was demonstrated to be very sensitive with low numbers of *G. lamblia* cysts. For each of the seven different sets (consisting of 1, 2, 3, 4, 5, 7, and 10 cysts) of *G. lamblia* cysts isolated, in replicates of 50, the amplification results were as follows: 100% for 10, 7, 5, and 4 cysts; 94% for 3 cysts; 90% for 2 cysts; and 80% for 1 cyst.

In a previous study, Sturbaum et al. performed PCR on singly isolated *Cryptosporidium parvum* oocysts to evaluate the sensitivity of the technique (11). *C. parvum* is a highly infectious intestinal parasite commonly found along with *Giardia* in contaminated water and soil (6, 7, 9, 12). Collectively, these two intestinal parasites constitute major protozoal threats to human populations worldwide via waterborne transmission routes (7, 9). As PCR can be highly specific and sensitive for both *Cryptosporidium* and *Giardia* species detection, it can be used to obtain a better understanding of how the organisms can be controlled through advances in water treatment (6). To our knowledge, this is the first study showing the sensitivity of nested PCR in the detection of *G. lamblia* cysts at a confirmed single-cyst level.

We thank Gregory D. Sturbaum, B. Helen Jost, and Margarethe A. Cooper for all their assistance and helpful discussions as well as Nina S. Castro and Kathryn E. Lancaster for their support.

This project was funded in part by USDA grant ARZT-136034-H-02-124.

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