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Construction and characterization of a full-lengh cDNA library from non-fresh *Giardia lamblia*

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ABSTRACT

Objective: To construct rapidly a full-length cDNA library from nanogram amounts total RNA of *Giardia lamblia (G. lamblia)* trophozoites stocked in RNA stabilization reagent. **Methods:** Total RNA of *Giardia* was extracted using Trizol reagent. A full-length cDNA library of *G. lamblia* trophozoites was constructed by a long-distance PCR (LD-PCR) method. The recombinant rate and the coverage rate of full-length clones of the library were evaluated. The inserted fragments were identified and sequenced by PCR amplification. **Results:** The titer of cDNA library was 3.85 $\times 10^7$ pfu/mL. The length of inserted fragments ranged from 0.4 to 2.5 kb, and the recombination efficiency accounted for 100% (20/20). The coverage rate of full-length clones is high (17/20). **Conclusions:** The RNA stabilization reagent may be used to fix the cells and prevent the RNA in cells even though delivered under normal atmospheric temperature. The long-distance PCR can be used to construct a full-length cDNA library rapidly and it needs less RNA than the traditional method from mRNA.

1. Introduction

Giardia duodenalis [synonyms: *Giardia lamblia* (*G. lamlia*) and *Giardia intestinalis*)] is an enteric protozoan parasite of mammals, birds, amphibians and reptiles. In many countries, giardiasis is the most commonly reported intestinal infection in humans and in many domesticated and wild mammals[1,2]. Usually, the infections are chronic or reoccurring with intermittent diarrhea and substantial weight loss. On immunocomprised population, it can cause server and lethal diarrhea. So diagnosis and prevention of *Giardia* infection are major concerns.

Scanning a special cDNA library is a kind of basic method to find a canditate molecular, which can be used as diagnostic or preventable purpose[3-5]. But the construction of a full-length cDNA library is limited by number of the cell and qulification of RNA. In addition, it needs to cost a lot of money and time to culture the *Giardia* trophozoites and gain available cell number. Therefore, this study develops a new-used and high-effective method to construct a full-length cDNA library.

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2. Materials and methods

2.1. *Giardia lamblia* trophozoites culture, stock and delivery

Trophozoites of *G. lamblia* isolates C_2 derived from a patient in Southwest China was anemically cultured in

modified TYI-S-33 medium as described previously[6]. The medium was supplemented with 10% heat-inactivated bovine serum (Hangzhou Sijiqing Biological Engineering Materials, Hangzhou, China) and 0.05% bovine bile (Sigma, America) in borosilicate glass screw-cap culture tubes which was incubated without shaking at 37 ℃. Subcultures (2×10^5 trophozoites per 4 mL tube) were made three times a week. The culture was chilled on ice for 20 min to detach trophozoites from tube wall. Trophozoites for delivery were collected in a 1.5-mL tube with 1 mL RNAlater RNA Stabilization Reagent (QIAGEN, America) by centrifugation at room temperature for 10 min with 2 000 rpm speeding. The sample was delivered under normal atmospheric temperature and stocked at -80 ℃ at destination lab.

2.2. RNA isolation

The fixed cells were moved from -80 ℃ and collected by centrifugation with 2 000 rpm for 10 min at 4 ℃ after dissolution at 37 ℃ water bath. The total RNA was isolated using Trizol® reagent (Invitrogen, USA) followed by the purification step of Phenol-chloroform extraction according to the manufacturer's instructions. The purificated RNA was measured at 260 and 280 nm using a spectrophotometer (Therma, NANODROP2000) and the 260 nm reading was used to estimate the concentration of total RNA. The 4 μL of the RNA were run in a 1% agarose-formaldehyde gel stained with ethidium bromide to confirm the RNA quality.

2.3. cDNA synthesis

Full-length cDNA synthesis was done with the method of a long-distance PCR as described previously. 3 μL of the RNA solution (400 ng of total RNA) was added to a reaction mixture containing 2.4 μM CDSⅢ and 2.4 μM SMART Oligo Ⅳ oligonucleotide (SMART™ cDNA library construction kit, Clontech Laboratories, USA) in a 0.2 mL prechilled PCR tube. Deionized H_2O was added to bring the total volume up to 5 μL. Afterwards, the mixture was incubated at 72 ℃ for 2 min and then kept on ice for 2 min to remove RNA secondary structures. Then, the mixture was added 1mM of dNTP mix, 2 mM of DTT and 100 units SMARTScribe MMLV reverse transcriptase (SMART™ cDNA library construction kit, Clontech Laboratories, USA). The total volume of reaction was brought up to 10 μL with first-strand buffer. The reaction tube was then incubated at 42 ℃ for 1 h. Subsequently, the reaction system was placed on ice to terminate first-strand synthesis.

2 μL of the first-strand solution was took and put in a clean, prechilled PCR tube. Keep the tube on ice and add the following components in the reaction: 80 μL of deonized H_2O, 10 μL of 10× advantage 2 PCR buffer(Advantage® 2 PCR Kit, Clontech Laboratories, USA), dNTP at final concentration of 0.4 μM, 5′PCR primer (SMART™ cDNA library construction kit, Clontech Laboratories, USA) at final concentration of 0.48 μM, CDSⅢ/ 3′PCR primer (SMARTTM cDNA library construction kit, Clontech Laboratories, Inc) at final concentration of 0.48 μM, and 4 units advantage 2 polymerase mix (Advantage® 2 PCR Kit, Clontech Laboratories, USA). Start the thermal cycling immediately from 95 ℃ using the following program: initial denaturation at 95 ℃ for 1 min followed by 18 cycles of 95 ℃ for 15 s, 62 ℃ for 6 minutes.

2.4. cDNA size fractionation

The ds cDNA produce from cDNA synthesis was digested by proteinase K and the restriction endonuclease Sfil. cDNA size fractionation was processed by CHROMA SPON-400 (SMART™ cDNA library construction kit, Clontech Laboratories, USA) according the manufacturer's instructions. On a 1% agarose/ EtBr gel, 3 μL of each fraction was run alongside 0.1 μg of a 1 kb DNA marker at 150 V for 10 min. Pool the first four fractions containing cDNA and precipitate the cDNA with 2.5 volumes of alcohol, 26 μg glycogen and 1/10 volumes of sodium acetate. The pellet of cDNA was resuspended in 7 μL of deionized H_2O.

2.5. cDNA library construction

λ TriplEx2 Vector were provided by Clontech (SMART™ cDNA library construction kit, Clontech Laboratories, USA). A 5-μL ligation system was constructed in a 200-μL PCR tube containing 1.5 μL of the cDNA from the fractionation step, 1 μL of λ TriplEx2 Vector (500 ng/μL), 0.5 μL of 10 ×ligation buffer, ATP with final concentration of 1 mM, and 200 U of T4 DNA ligase. The reaction tube was incubated at 16 ℃ overnight. The packaging protocol was done according to the manufacturer's instructions (Gigapack Ⅲ 200201-12, STRATAGENE, USA).

2.6. Tittering the library and PCR insert screening

The plating/tittering protocol was used to determine the titter of unamplified library and the percentage of recombinant clones. Transformation of 1 μL of the ligation reaction into *Escherichia coli* XL1-Blue competent cells was accomplished by chemical method in 1×lambda dilution buffer at 37 ℃ for 15 min. The mixture of the transformed bacteria and melted LB/$MgSO_4$ soft top agar was poured onto LB/$MgSO_4$ agar plates. Then, cooled plates were culture at 37 ℃ for 16-18 h. Plaques on the plates were calculated to determine the titter of the library. 20 clones were pick up from the plates randomly and boiled in 50 μL deionized

H_2O. 1 μL of dissolved phage solution was used as template to do PCR with vector-specific primers (λ TriplExF and λ TriplExR) to check the insert size and the recombination rate. All 20 of PCR products were sequenced (Lone Star labs DNA Sequencing, America).

3. Results

3.1. RNA quality

The absorbance ratios of the RNA at 260/280 nm and 230/260 nm were 2.00 and 2.01, respectively, and the concentration of RNA was 0.133 μg/μL. The result of total RNA running on a denaturing gel showed a most intense smear (no showed) between 400 bp and 4 kb indicating that the RNA was of the highest quality and therefore useful for the construction of the cDNA library.

3.2. cDNA synthesis and size fractionation

When 18 cycles of LD PCR were completed, 5-μL sample of the PCR product was analyzed in 1% agarose/EtBr gel. The ds cDNA appeared as a 0.1-4 kb smear and had a few distinct bands on the gel (Figure 1). It suggested that the ds cDNA was complete for the next step experiment.

On a 1% agarose/EtBr gel, 3 μL of each fraction was run alongside 0.1 μg of a 1 kb DNA marker (Figure 2). Totally, the cDNAs were fractionated into 12 tube with 35 μL(two drop). According the size of cDNA in each tube, the fractions from tube 5 to tube 8 were pooled and concentrated in 7 μL deionized H_2O.

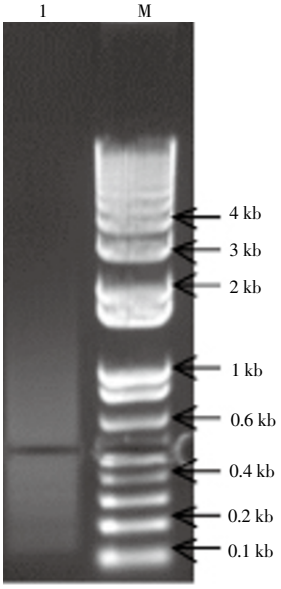

Figure 1. ds cDNA from the total RNA of Giardia with LD PCR method.
1. dsDNA; 2. 1 kb DNA ladder maker.

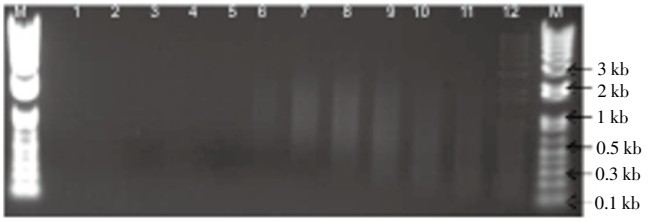

Figure 2. ds cDNA fractions.
M: 1kb DNA marker. 1-12: ds cDNA fractions.

3.3. cDNA library quality

The titer of the cDNA library yielded 3.85×10^7 pfu/mL. Overall, size of the PCR products was between 0.4 and 2.5 kb, and the recombination rate of the library was 100%(20/20) (Figure 3).

The total 20 PCR products from 20 clones randomly picked up were sequeced. 17 different sequences were identified by Blastn in Genbank (Table 1).

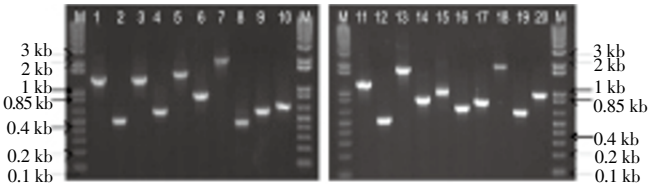

Figure 3. PCR insert screening of the library.
M:1 kb DNA maker.1-20: phage clone.

Table 1
Identification of cDNA library by PCR and sequencing.

No. of clone	Gene ID
1	Gl50803_114573
2	Gl50803_119598
3	Gl50803_114921
4	X
5	Gl50803_2107
6	Gl50803_8955
7	Gl50803_10969
8	Gl50803_114609
9	Gl50803_12949
10	X
11	X
12	Gl50803_112103
13	Gl50803_4547
14	Gl50803_11487
15	Gl50803_102813
16	Gl50803_9707
17	Gl50803_003622
18	Gl50803_17056
19	Gl50803_24412
20	Gl50803_5942

4. Discussion

Trophozoite of *G. lamlia* is sensitive to temperature and will die soon under external environment[7]. It increases the risk of RNA degradation during delivery. Enven though transportation of RNA can be carried in liquid nitrogen or dry ice to protect RNA from degradation, it is very expensive. RNAlater buffer is a kind of RNA stabilization reagent and can quickly permeates tissues, stabilizing and protecting the RNA expression pattern[8]. In current study, we fixed the trophozoites with RNAlater buffer and deliver the sample under normal atmospheric temperature to destination lab where the fixed trophozoites were stocked at −80 ℃. Further, we gained complete and high quality total RNA for construction of cDNA library.

cDNA library is a common tool. SMART Oligo Ⅳ oligonucleotide supplied by (SMART™ cDNA library construction kit) in the system of the first-strand synthesis serves as a short, extended template at the 5′ end of the mRNA (SMART anchor). Only those ss cDNA having a SMART anchor at the 5′ end can serve as a template and can be exponentially amplified. The selective amplification supplied an available method to construct a cDNA library using nanogram amounts of either total RNA[9]. In present study, we constructed a full-length cDNA library of *G. lamblia* trophozoite, which was started from 400 ng total RNA and gained the cDNA library with high titter of 3.85×10^7 pfu/mL (unamplicated) with a high percentage of full-length clones (17/20). It can save the cost on money and time to do a large-scale culture and increase the efficiency of cDNA library construction and finding the diagnostic methods.

In conclusion, the cell sample for RNA extraction can be stock in RNAlater to decrease the cost of traditional low temperature methods and LD-PCR method is an ideal choice when the amount of available RNA starting material is limited.

Conflict of interest statement

We declare that we have no conflict of interest.

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