academicJournals

Vol. 7(12), pp. 1035-1040, 19 March, 2013
DOI: 10.5897/AJMR12.1930
ISSN 1996-0808 © 2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Construction of a yeast two-hybrid cDNA library from second-generation merozoites of *Eimeria tenella*

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Accepted 8 March, 2013

To construct a yeast two-hybrid cDNA library from second-generation merozoites of *Eimeria tenella* (*Et*sMZ), total RNA of *Et*sMZ was extracted using Trizol reagent and mRNAs were isolated from total RNA. The first-strand cDNAs were synthesized by reverse transcription using MMLV. The dscDNAs acquired by Long-distance Polymerase Chain Reaction (LD-PCR) and purified by Chroma Spin TE-400 Column were transformed into AH109 yeast competent cells with pGADT7-Rec, a yeast expression vector, according to the screening method of yeast mating. All clones were harvested and the yeast two-hybrid cDNA library of the *Et*sMZ was constructed. The results show that the capacity and titer of the library were 3.22×10^{15} cfu and 8.05×10^{12} cfu/mL, respectively. PCR amplification revealed that the library contained approximately 96% recombinant clones, and the inserted cDNA fragments were between 300 and 2000 bp. In addition, five specific genes of *E. tenella* were amplified from the constructed cDNA library. It could be concluded that a yeast two-hybrid cDNA library of the *Et*sMZ was successfully constructed. The library can provide a foundation for screening invasion-related interaction proteins of *E. tenella* merozoite by yeast two-hybrid method.

Key words: Coccidia, *Eimeria tenella*, second-generation merozoite, yeast two-hybrid, cDNA library.

INTRODUCTION

Avian coccidiosis, a worldwide parasitic disease of poultry, is caused by the protozoan *Eimeria* spp that infect poultry in a host-specific manner. Intensively-reared poultry provide excellent opportunities for *Eimeria* spp to accumulate in large numbers and to be transmitted easily among individual hosts. Infection with *Eimeria* spp especially *E. tenella* which is the most pathogenic to chicken cost serious financial losses all over the world (Shirley et al., 2007). *Eimeria* spp have complex developmental life cycles with an exogenous phase in the environment during which oocysts excreted from the chicken undergo differentiation (sporulation) and become during which there are two or more (depending on the infective, and an endogenous phase in the intestine species) rounds of discrete, expansive asexual reproduction (schizogony) followed by sexual differentiation, fertilisation and shedding of unsporulated oocysts (Lal et al., 2009). The second-generation merozoites of *E. tenella* (*Et*sMZ) is the progeny from the most pathogenic endogenous life cycle stage of the parasite (Wan et al., 1999). Although some proteins such as apical membrane antigen 1, rhoptry neck proteins, micronemes play important roles in cell invasion, it is still not clear how merozoites invade intestine cells so far (Jenkins, 1988; Han et al., 2007; Lal et al., 2009). Therefore, identification of the invading-related proteins of merozoites will help understand the invasion mechanism,

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which will benefit for discovering new targets of coccidian invasion and for developing coccidiosis vaccine and new treatment drugs for coccidiosis control (Han et al., 2007).

Construction of the cDNA library, a modern molecular biology method, is necessary for analyzing the expression, structure and regulation of the whole genes of one stage, and for studying protein functions and interactions (Ying, 2004; Wan et al., 2006). Several cDNA libraries of *Eimeria* spp have been constructed up to now, such as subtractive cDNA libraries of unsporulated oocysts, sporulated oocysts, and sporozoites of *E. tenella* (Han et al., 2010), cDNA libraries of sporulated oocysts of *E. tenella* (Li et al., 2006), *E. necatrix* (Bian et al., 2008) and *E. acervulina* (Yan et al., 2007), and cDNA library of sporozoites of *E. tenella* (Klotz et al., 2007). Moreover, yeast two hybrid cDNA library is mainly used to screen interaction proteins and widely used to study the protein interaction in apicomplexan parasites, such as *Toxoplasma gondii* (Ueno et al., 2011; Nallani et al., 2005). p23 co-chaperone protein which interacts with a bradyzoite-specific DnaK-tetratricopeptide repeat (DnaK-TPR) protein was obtained by screening the yeast two hybrid cDNA library of *T. gondii* , and the research indicated that p23 co-chaperone protein may play an important role in the tachyzoite-tobradyzoite differentiation (Ueno et al., 2011). Some proteins associated with *Tg*SRCAP (*T. gondii* Snf2-related CBP activator protein) were identified during the pre-cyst stage and 10 genes, which may participate in the cyst development, were detected from yeast two hybrid cDNA library of *T. gondii* (Nallani et al., 2005). However, the application of yeast two hybrid cDNA library in *E. tenella* is not reported yet. To identify some invasion-related proteins of *E. tenella* merozoites, a yeast two-hybrid cDNA library of *Et*sMZ was constructed, which could provide foundations for screening invasion-related interaction proteins and for exploring cell invasion mechanism of *E. tenella* merozoite.

MATERIALS AND METHODS

Animals

One-day old broiler chickens, purchased from one broiler hatchery of Shanghai suburbs, were kept under the coccidia-free condition in Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The licence number of using experiment animals was SYXK (Hu) 2011-0611.

Parasites

E. tenella was provided by parasitic-disease control laboratory of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The serial number is CAAS21111601.

Universal primer of cDNA library

The universal primers were designed to amplify the inserted fragments of cDNA library according to vector pGADT7-Rec sequence and synthetized by Shanghai Saibaisheng Company: Forward primer: 5'-CTATTCGATGATGAAGATACCCCACCAAACCCA-3'; Downstream primer: 5'-GTGAACTTGCGGGGTTTTTCAGTATCTACGATT-3'.

Collection and purification of the *Et*sMZ

Two-week old coccidia-free chickens were orally inoculated with 5×10^4 sporulated oocysts of *E. tenella* per bird. *Et*sMZ were collected from ceca at 112 h after inoculation. Isolation and purification were carried out as previously reported (Liu et al., 2006).

Isolation of total RNA and mRNA

Total RNA was extracted from *Et*sMZ using Trizol reagent (Takara, Japan). mRNAs were isolated using mRNA Isolation Kit (Promega, USA). Total RNA was quantified by UV spectrophotometry, and the purified total RNA and mRNAs were assessed on a 1% agarose / EtBr gel. All procedures were handled according to the provided instructions strictly.

Construction of cDNA library

The yeast two hybrid cDNA library of *Et*sMZ was construced by the Matchmaker™ Library Construction and Screening Kit (Clontech, USA). The first-strand cDNAs (sscDNAs) were synthesized by separated mRNAs using CDIII primer (5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d$(T)_{30}$VN-3'), MMLV reverse transcriptase and SMART (switching mechanism at the 5'-end of the RNA transcript) II oligonucleotide (5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3'). The double-strand cDNAs (dscDNAs) were acquired by 22 cycles of long-distance PCR using the Advantage 2 Polymerase Mix (Clontech) with the presence of 2 µL sscDNAs product, and the reaction parameters were as follows: denaturation at 95°C for 30 s, followed by 21 cycles of 95°C for 10 s, 68°C for 6 min (increase the extension time by 5 s with each successive cycle) and a final extension at 68°C for 5 min. To remove the fragments less than 200 bp, dscDNAs were purified by Chroma Spin TE-400 Column (Clontech) and resuspended in 20 µL DEPC-water. 5 µL of total RNA, mRNAs and dscDNAs were quantified by electrophoresis on 1% agarose gel stained with ethidium bromide before going to the next step, respectively. Purified dscDNAs and pGADT7-Rec, a yeast expression vector, were transformed to AH109 yeast competent cells according to the screening method of yeast mating. Then well-distributed yeast cells were cultured on 100 dropout media (SD/-Leu) 150-mm plates and incubated at 30°C until clones appeared (4 days). All clones were harvested with freezing medium (YPD medium with 25% glycerol) and stored at -70°C for further use.

Calculation of the transformation efficiency

1 mL transformed yeast cells were diluted with YPDA medium, and 100 µL of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilutions were spread on 100-mm SD/-Leu plates, respectively and incubated at 30°C until clones appeared (4 days). These clones (cfu) were counted to calculate the transformation efficiency. The expected results should be more than 1×10^6/3 µg pGADT7-Rec.

Calculation of the library titer

1 mL cDNA library was diluted with YPDA medium, then 100 µL of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} dilutions were spread on 100-mm SD/-Leu plates, respectively and incubated at 30°C until clones appeared (4 days). The clones (cfu) were counted to calculate the library titer.

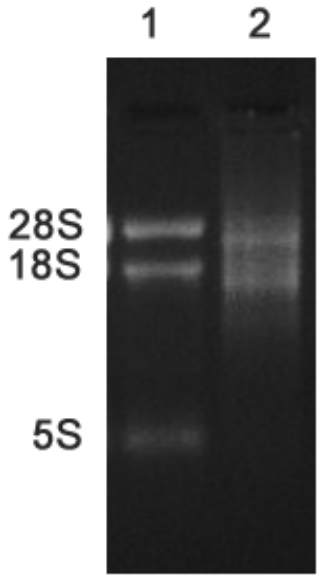

Figure 1. Agarose gel electrophoresis of total RNA and mRNA from second-generation merozoites of *Eimeria tenella,* Lane 1, total RNA ; lane 2, mRNA.

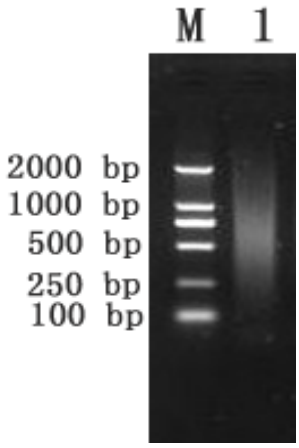

Figure 2a. First-strand cDNAs of second-generation merozoites of *Eimeria tenella* were analyzed on a 1% agarose/EtBr gel. Lane M, DNA molecular marker; Lane 1, first-strand cDNAs.

Detection of inserted cDNAs and recombination frequency

24 single clones were picked with toothpick into 50 µL ddH_2O randomly. These clones were freeze-thawed three times in liquid nitrogen and boiling water, then centrifuged. The supernatants remained as the templates. The universal primers were used in PCR reaction. The PCR conditions included an initial denaturation at 94°C for 5 min followed by 34 cycles of 94°C for 30 s, 55°C for 30 30 s, 72°C for 2 min, and a final extension at 72°C for 10 min, and then held at 4°C. The PCR products were analyzed by 1% agarose gel electrophoresis to determine the recombination frequency and the size of fragments.

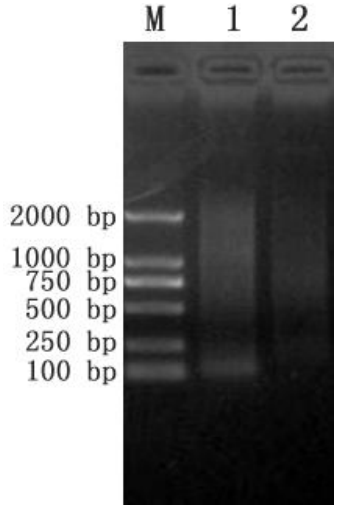

Figure 2b. The double-strand cDNAs of second - generation merozoites of *Eimeria tenella* were analyzed on a 1% agarose/EtBr gel before and after Chroma Spin TE-400 Column purification. Lane M, DNA molecular marker; LANE 1, unpurified double-strand cDNAs; lane 2, purified double-strand cDNAs.

Amplification of *E. tenella* genes with special primers from cDNA library

The templates were obtained from repeatedly frozen and thawed 1 mL cDNA library. Five ESTs of specific genes of *E. tenella* were amplified by PCR and analyzed by 1% agarose gel electrophoresis. The five ESTs were ZB7-B08 (Genbank accession number: ES351380), ZB2-DO7 (Genbank accession number: ES351367), BW4-C03 (Genbank accession number: ES346910), RON2 (Genbank accession number: AM773998), and ZB1-H07 (Genbank accession number: ES351365).

RESULTS

Total RNA and mRNA isolation

The ratio of OD_{260}/OD_{280} of total RNA isolated from *Et*sMZ was 2.06. Identified by 1% agarose gel electrophoresis, total RNA appeared as three specific bands (28s ribosomal RNA, 18s ribosomal RNA and 5s ribosomal RNA) and the ratio of intensities of 28s and 18s rRNA bands was about 2, which indicated that the total RNA had high purity and was undergraded. mRNAs appeared as a smear by 1% agarose gel electrophoresis (Figure 1). So both total RNA and mRNAs were high quality to construct cDNA library.

Construction of cDNA library

5 µL of sscDNAs and dscDNAs were analyzed by electrophoresis on 1% agarose gel, respectively. The results show that the bands of sscDNAs appeared as a smear between 100 to 2000 bp (Figure 2a). The size of dscDNAs unpurified was 100 to 2000 bp, and the size of dscDNAs changed to 200 to 2000 bp after purification by Chroma Spin TE-400 Column (Figure 2b). The purified dscDNAs

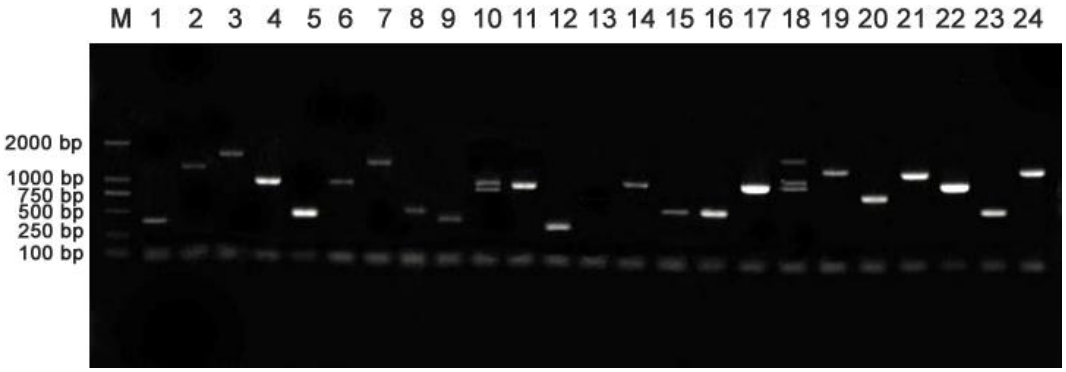

Figure 3. 24 clones were randomly picked from SD/-Leu plate and amplified by PCR with universal primers . The PCR products were analyzed by 1% agarose gel electrophoresis to determine the recombination frequency and the size of fragments. Lane M, DNA molecular marker; lanes 1-24, amplifications by PCR from 24 randomly picked clones with universal primers.

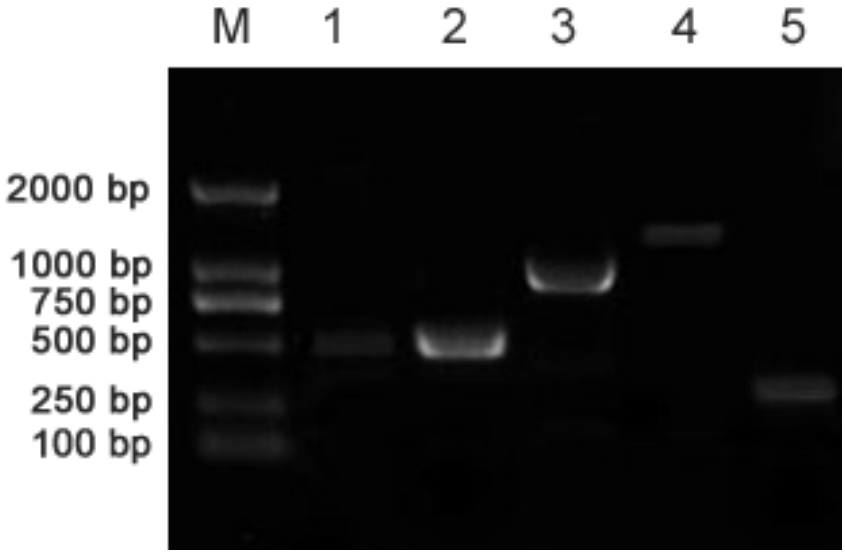

Figure 4. Five ESTs of *Eimeria tenella* were amplified by PCR with specific primers from the cDNA library of second-generation merozoites of *Eimeria tenella*. Lane M, DNA molecular marker; Lanes1-5, ZB7-B08, ZB2-DO7, BW4-C03, RON2, ZB1-H07, respectively.

could avoid invalid restructuring of small fragments cDNA and shorten the workload for the later screening.

Identification of cDNA library

Based on the number of clones on SD/-Leu plates, the transformation efficiency was 5.03×10^6/3 μg pGADT7-Rec, the library titer was 8.05×10^{12} cfu/mL and the capacity of cDNA library was 3.22×10^{15} cfu. PCR amplification revealed that the cDNA libraries contained approximately 96% recombinant clones. The inserted fragments obtained by PCR were mainly distributed between 300 and 2000 bp. All lanes appeared as one single band except lanes 10, 13 and 18 (Figure 3). More than one bands amplified in a single clone may be caused by several plasmids, which possessed the same replication origin and transformed into one yeast cell together. These results indicate that the cDNA library could be used for further research.

Amplification of *E. tenella* genes with special primers from cDNA library

E. tenella five genes (ZB7-B08, ZB2-DO7, BW4-C03, RON2, ZB1-H07) amplified by PCR indicated that the cDNA library constructed contained special expressed genes of *E. tenella* in the second-generation merozoites stage (Figure 4).

DISCUSSION

Since full-length cDNA clones provide information about intron and exon structures, splice junctions, and 5'- and 3'-untranslated regions (UTRs), obtaining full-length cDNA clones is essential for analyzing the expression, structure and regulation of genes, and for studying protein functions and interactions (Wan et al., 2006). But it is not an easy-going job to construct a high quality full-length cDNA library unless high quality mRNAs are gotten first. In this research, we acquired three specific bands of total RNA on 1% agarose gel electrophoresis, and isolated mRNAs which presented diffuse bands by 1% agarose gel electrophoresis, so both total RNA and mRNAs were high quality enough to construct cDNA library. Evaluating the quality of a cDNA library depended on the capacity of the library, the size of inserted cDNA fragments and recombination frequency of library (Duan et al., 2011). Moreover, the capacity of a good cDNA library was 1.7×10^5 pfu/ml at least (Sambrook and Russell, 2001). So, in this study, the capacity, the titer and the inserted cDNA fragment of the yeast two hybrid cDNA library from *Et*sMZ were all at high levels. Five specific genes of *E. tenella* were amplified from this constructed cDNA library; it was another way to evaluate the guality of the cDNA library. All these information indicated that a high quality cDNA library of *Et*sMZ was constructed and could be used for the further research of interaction proteins by yeast two hybrid.

Up to now, several methods have been developed to construct cDNA library and all of them were based on 5' cap of mRNA on eukaryote in order to obtain full-length cDNA. For example, mRNA cap retention procedure (CAPture) was developed by using a fusion protein containing the murine cap-binding protein and a solid support matrix to generate cDNA library (Edery et al., 1995). A full-length cDNA library was construced by using biotinylation of the cap structure (the CAP-trapper method) (Sugahara et al., 2001). The oligo-capping method was used to construct the full-length cDNA libraries of clear cell renal cell carcinoma (ccRCC) and normal kidney tissues (Tang et al., 2006) and an oil-rich race B strain of Botryococcus braunii (BOT-70) (Ioki et al., 2012). To identify promoters from the large volumes of genomic sequences, Suzuki et al (2001) used mRNA start sites determined by a large-scale sequencing of the cDNA libraries constructed by the "oligo-capping" method as well.

However, these methods were laborious and contained several enzymatic steps that must be performed on 5' cap of mRNA, so they were sensitive to quality loss through RNA degradation and likely to form the structure of cDNA - RNA duplexes. Furthermore, all the methods required high quality and quantity of starting mRNA because of the low efficiency of the enzymatic enrichment steps. All of these disadvantages limited the application of these methods largely (Wellenreuther et al., 2004). In contrast, using SMART technology for full-length enrichment of cDNA was very straightforward and have high-efficiency which required only a little starting mRNAs. This technology utilized the property of some MMLV reverse transcriptases to prolong template by reverse transcription, so dscDNAs can indicate mRNA abundance of original sample (Wellenreuther et al., 2004; Zhu et al., 2001). Thus, in this report, SMART technology was used to construct yeast two hybrid cDNA library from *Et*sMZ.

High quality cDNA libraries have been constructed successfully with SMART technology among different species. For example, these capacities of cDNA libraries of different *Eimeria* spp constructed by SMART method were all above 10^6 pfu/mL, and the recombination efficiencies were more than 95% (Yan et al., 2007; Bian et al., 2008). Li et al (2009) also constructed a cDNA library which had the genetic diversity and good representative of *Schistosoma japonicum* with SMART method. Duan et al. (2011) constructed a cDNA library with chicken embryo fibroblast (CEF) in which the average inserted cDNA size was 1.3 kb. Lu et al (2005) screened 2.37×10^6 clones from appressorium stage cDNA library of *Magnaporthe grisea* and PCR results show that the average size of inserted cDNAs was 660 kb. Compared with these cDNA libraries, the constructed *Et*sMZ cDNA library possessed these advantages of higher capacity, higher recombination frequency and better representative.

Yeast two hybrid system was first used in eukaryotes transcriptional regulation by Fields and Song (Fields and Song, 1989). Now, the system is widely applied and developed in the world because it is rapid and sensitive to screen interaction proteins. The technology have been already used in *T. gondii* but not reported in *Eimeria* spp. The present study was the first construction of high quality yeast two hybrid cDNA library from *Et*sMZ, which provided foundations for further screening invasion-related interaction proteins of *E. tenella* merozoites as well as studying the mechanism of coccidian invasion.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (Grant No.31201699) and Central Public-interest Scientific Institution Basal Research Fund (2011JB01).

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