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【论著】

Gene-cloning, Expression and Antigenicity Analysis of Rhoptry Protein 17 of *Toxoplasma gondii*

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【**Abstract**】 **Objective** To clone and express the rhoptry protein 17 (ROP17) gene of RH strain of *Toxoplasma gondii*, analyze the antigenicity of recombinant protein. **Methods** Total RNA was extracted from tachyzoites of RH strain of *T. gondii*. The open reading frame of *Tg*ROP17 gene was amplified with a pair of specific primers which was designed according to the coding sequence of *Tg*ROP17 gene (GenBank accession No. AM075203.1), the product of RT-PCR was digested with double restriction enzyme and ligated into a pGEX-6P-1 vector. The recombinant pGEX-6P-1-*Tg*ROP17 plasmid was transferred into *E. coli* DH5α and the positive clones were selected through the colony-PCR and confirmed by the double restriction enzyme digestion and sequencing. The constructed pGEX-6P-1-*Tg*ROP17 was transformed into *E. coli* Rosetta (DE3) and induced with IPTG for expression. The expression products were analyzed through SDS-PAGE followed by Coomassie blue staining. Western blotting assay with GST primary antibody and rabbit anti-*T. gondii* serum was used to confirm the expression of GST-ROP17 and analyze its antigenic properties. **Results** The product of RT-PCR was with 1 850 bp. The recombinant plasmid pGEX-6P-1-*Tg*ROP17 was confirmed by colony-PCR, double restriction enzyme digestion and sequencing. A soluble recombinant protein with relative molecular weight of 96 000 was analyzed by SDS-PAGE followed by Coomassie blue staining. The GST tag in GST-ROP17 and the antigenicity of ROP17 were detected efficiently by Western blotting with the GST primary antibody and with the prepared antiserum against *T. gondii*, respectively. **Conclusion** The recombinant GST-ROP17 protein has been produced in *E. coli* and shows specific antigenicity.

【**Key words**】 *Toxoplasma gondii*; Rhoptry protein 17; Prokaryotic expression; Antigenicity

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刚地弓形虫棒状体蛋白 17 基因的克隆、表达及抗原性分析

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【**摘要**】 **目的** 克隆、表达刚地弓形虫(*Toxoplasma gondii*) RH 株棒状体蛋白 17(*Tg*ROP17)基因, 并分析其抗原性。 **方法** 制备弓形虫 RH 株速殖子总 RNA, 根据 *Tg*ROP17 基因全长编码序列(GenBank 登录号为 AM075203.1)的开放阅读框设计引物并进行逆转录 PCR(RT-PCR)扩增, 扩增产物经双酶切后连接入 pGEX-6P-1 载体, 重组质粒转化大肠埃希菌(*E. coli*)DH5α, 阳性菌落经 PCR 和双酶切鉴定, 并测序。将重组质粒 pGEX-6P-1-*Tg*ROP17 转化至 *E. coli* Rosetta (DE3)并加入异丙基-β-*D*-硫代半乳糖苷(IPTG)诱导表达, 十二烷基磺酸钠-聚丙烯酰胺凝胶电泳(SDS-PAGE)结合考马斯亮蓝染色检测表达产物。分别以兔抗弓形虫血清和抗谷胱甘肽 S 转移酶 (GST) 标签抗体为一抗, 采用蛋白质印迹(Western blotting) 分析重组蛋白及其抗原性。 **结果** RT-PCR 扩增产物约为 1 850 bp。菌落 PCR、双酶切及测序结果显示重组质粒 pGEX-6P-1-*Tg*ROP17 构建成功。SDS-PAGE 结果显示, 经 IPTG 诱导获得相对分子质量(M_r)约 96 000 的可溶性重组蛋白。Western blotting 结果表明, 诱导表达的蛋白质为带 GST 标签的重组蛋白, 且能被兔抗弓形虫血清识别。 **结论** 获得刚地弓形虫重组 ROP17 蛋白, 且具有抗原性。

【**关键词**】 **刚地弓形虫; 棒状体蛋白 17; 原核表达; 抗原性**

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Toxoplasma gondii is an obligate intracellular parasite that can infect virtually any nucleated cell and adopts several forms. The tachyzoite is a rapidly dividing haploid form of *T. gondii*, which is able to infect a wide range of mammalian host cells including immune and non-immune cells[1]. In immunocompromised patients, *T. gondii* may cause severe degeneration in the central nervous system and congenital transmission of tachyzoites could also result in grave consequences for infected fetuses and newborns[2], though healthy individuals are rarely affected by *T. gondii* infection since the immune system could recognize and eliminate the parasites that differentiate back to tachyzoites[3]. Cell invasion by tachyzoites is a crucial event that shapes parasite survival, replication, and manipulation of the host cell, during which the parasitophorous vacuole membrane (PVM) will be formed surrounding the intracellular parasites and providing a stable environment for parasite multiplication.

The tachyzoite proteins are a complex of proteins including rhoptry proteins (ROPs), surface antigen proteins (SAGs), dense granule proteins (GRAs) and microneme proteins(MICs). ROP2-superfamily is the largest family of proteins that have been known to be secreted by *Toxoplasma* into its host cell[4,5]. Two virulence factors have been defined as the members of this superfamily, *i.e.* ROP16, a soluble kinase that could translocate to the host nucleus and disturb the gene expression in the host[6,7] and ROP18, that can enhance the virulence of *T. gondii* through phosphorylation of host immunity-related GTPase (IRG), disrupting its functions in destroying the PVM[8,9].

ROP17, another member of the ROP2-superfamily, shares a high similarity in structure with ROP16 and ROP18 featured by the C-terminal kinase-like domain[10,11]. In this report, ROP17 gene was cloned from *T. gondii* and ROP17 protein was induced to express as a fusion form successfully, this would pave the way for further investigation of the kinase activity of ROP17 protein.

MATERIALS AND METHODS

1 Parasite, bacterial strains, plasmid and reagents

Tachyzoites of the highly virulent RH strain of *T. gondii* (type Ⅰ) were used in this study. The parasites were maintained by serial intraperitoneal passages in BALB/c mice. *E. coli* strains [DH5α and Rosetta (DE3)] and the pGEX-6P-1 vector were preserved in the authors' laboratory. The restriction enzymes and DNA ligase were purchased from TaKaRa. Trizol reagent, HiFi-MMLV cDNA kit, DNA polymerase, DNA and protein molecular weight markers, gel extraction kit, plasmid mini kit and GST primary antibody were purchased from Beijing CoWin Biotechnology Company. Horseradish peroxidase (HRP)-labeled goat anti-rabbit/mouse IgG was purchased from Zhongshan Goldenbridge Biotechnology Company.

2 Preparation of rabbit anti-*T. gondii* serum

T. gondii tachyzoites were stored in liquid nitrogen until being revived. Antisera were raised against *T. gondii* tachyzoites in two male New Zealand albino rabbits with body weight of 2.5–3.0 kg. Before challenging the rabbits, a 5 ml peripheral blood sample was collected from an ear vein of each animal for preparation of pre-immune serum. Then live *T. gondii* tachyzoites (1.0×10^5) were hypodermically injected in the neck of the rabbits. *T. gondii* at the same dose was boosted every 10 days thereafter for four times. Blood samples were taken from the marginal ear vein on the 10th, 20th, 30th and 40th day after the first challenge, and on the 10th day after the final challenge. Meanwhile, a blood sample was obtained from the jugular vein for monitoring the generation of a specific antibody with ELISA based on STAg as a coating antigen. The antisera were separated by centrifugation at $2\,300\times g$ for 10 min at 4 ℃. The final serum IgG antibody titer was 1∶4 096, and the samples were stored at −80 ℃.

3 Amplification of the open reading frame (ORF) of ROP17 gene

Total tachyzoite RNA was extracted from 5×10^8 tachyzoites according to Trizol reagent instructions. The coding region of ROP17 was amplified by reverse transcription polymerase chain reaction (RT-PCR). The first strand of cDNA was synthesized using the HiFi-MMLV cDNA kit, and the PCR reactions were carried out with a PTC-100 Peltier Thermal Cycler. Considering the order of ROP17 of RH strain of *T. gondii*

(GenBank Accession No. AM075203.1). The primers for amplification of the ORF of ROP17 gene was designed and synthesized by TaKaRa. The forward primer was 5′-CGCGGATCCATGGAGTTGGTGTTGTGCTTTGT-3′, the reverse primer was 5′-CCGCTCGAGTTACTCCTTC-TGTAATAAAGCCGCCT-3′, containing the *Bam*H Ⅰ and *Xho* Ⅰ restriction sites (underlined), respectively. The condition of PCR amplification was initial denaturation at 94 ℃ for 5 min followed by 30 consecutive cycles of denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 30 s, and extension at 72 ℃ for 90 s, and then a final extension at 72 ℃ for 10 min. The amplified products were analyzed by electrophoresis on a 1% (w/v) agarose gel.

4 Construction of the pGEX-6P-1-*Tg*ROP17 recombinant plasmid

Plasmid pGEX-6P-1 was digested with *Bam*H Ⅰ and *Xho* Ⅰ and then purified from agarose gel using the gel extraction kit. A recombinant plasmid was constructed by inserting the digested amplified fragments into pGEX-6P-1 *via* the sites of *Bam*H Ⅰ and *Xho* Ⅰ and transformed into *E. coli* strain DH5α. The transformants (pGEX-6P-1-*Tg*ROP17) were selected by colony PCR, and confirmed by restriction enzyme digestion and DNA sequencing.

5 Expression of GST-ROP17

A single colony resulted from successfully *E. coli* Rosetta (DE3) transformants was picked up and cultured overnight at 37 ℃ in LB medium supplemented with 100 μg/ml ampicillin. The culture mixture was then inoculated to fresh LB medium (1∶50 dilution) plus ampicillin and grown at 37 ℃ under continuous shaking, until the absorbance (A_{600} value) reached the range between 0.5–0.8. To optimize the conditions for protein expression, different doses of isopropyl-β-*D*-thiogalactopyranoside (IPTG)(1, 0.5 and 0.1 mmol/L) were added into the medium before continuous culture at 37 ℃ or 25 ℃ for another 8 h, respectively.

After 8 h of induction, the cells were harvested by centrifugation at 6 000×*g* for 15 min at 4 ℃. The supernatants were discarded and the pellets were resuspended in PBS (pH 7.4), and lysed by using lysozyme, and incubated for 30 min at room temperature with gentle shaking to assure thorough cell lysis. The cell lysate was further disrupted by sonication on ice with six 10 s pulses at high intensity with a 20 s cooling period between each burst. The supernatant and pellet were separated by centrifuging at 12 000×*g* for 30 min. The degree of expression was evaluated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue R-250 staining.

6 Western blotting

The products of GST-ROP17 expressed in *E. coli* Rosetta (DE3) were boiled at 95 ℃ for 5 min and centrifuged at 12 000×*g* for 10 min. The supernatants were separated in a 10% SDS-PAGE gel and then electrophoretically transferred to a PVDF membrane. The membrane was blocked in 5% skim milk for 1 h at room temperature and then incubated with the GST primary antibody (1∶1 000) or the rabbit anti-*T. gondii* serum (1∶200) at 4 ℃ overnight[12]. The membrane was incubated with anti-mouse (or rabbit) HRP-IgG for 1 h at room temperature. Chemiluminescence was detected using an enhanced chemiluminescence (ECL) blot detection system.

RESULTS

1 Cloning of the ORF of ROP17 gene

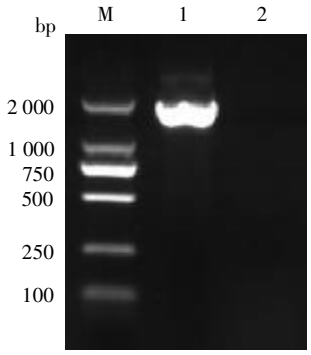
The nucleotide sequence encoding ROP17 protein was amplified from the template of RH strain of *T. gondii* cDNA using the gene-specific primers. Amplification of the ROP17 gene by PCR produced a single amplified 1 850 bp DNA fragment on 1% agarose gel (Fig. 1).

2 Construction of expression plasmid pGEX-6P-1-TgROP17

The PCR product was ligated into the pGEX-6P-1 vector and transformed into the competent *E. coli* DH5α cells. The clones were screened by PCR and restriction analysis (Fig. 2). And a positive clone was then sequenced, the result of which confirmed that the ORF of ROP17 gene inserted into the pGEX-6P-1 was in frame with N-terminal GST tag of the vector.

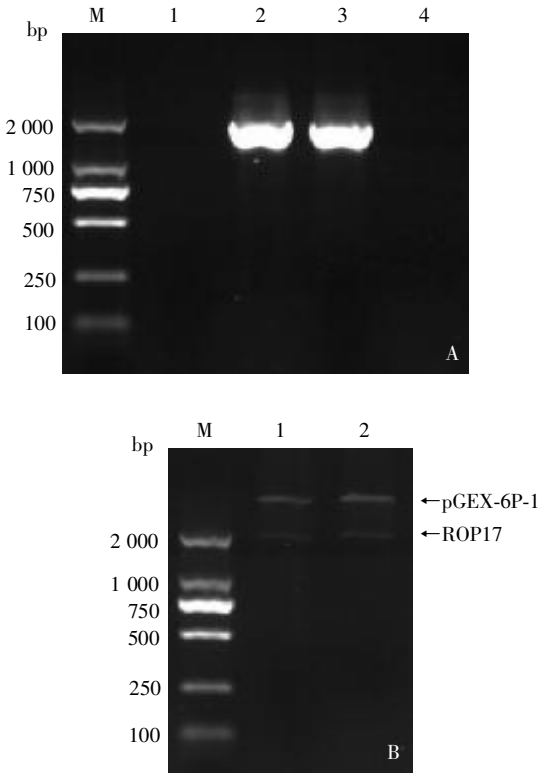
3 Expression of the recombinant protein

The recombinant vector was transformed into



M: DNA marker (DL2000); 1: Product of ROP17 RT-PCR; 2: Negative control.

Fig. 1 PCR amplification of ROP17 gene from *T. gondii*



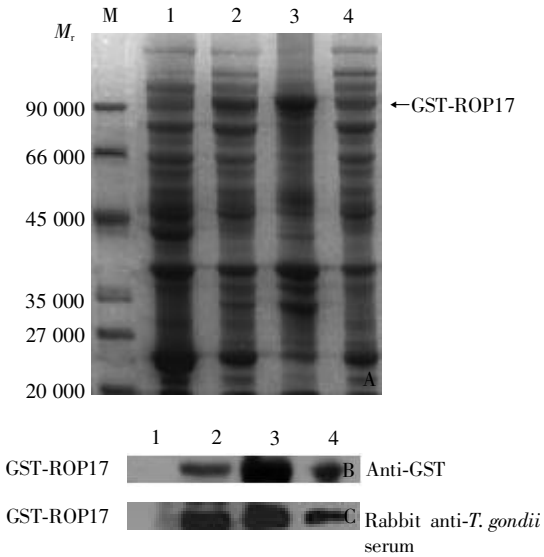
A: M: DNA marker (DL2000); 1–3: Three different single clone from LB agar plate; 4: Negative control. B: M: DNA marker (DL2000); 1, 2: Product from pGEX-6P-1-ROP17 digested by *Bam*H Ⅰ and *Xho* Ⅰ.

Fig. 2 Identification of the recombinant plasmid pGEX-6P-1-ROP17 by Colony-PCR (A) and restriction enzyme digestion (B)

E. coli Rosetta (DE3) cells. In order to obtain the soluble recombinant protein maximally, we optimized the expression conditions as described in 'Materials and Methods'. As shown in Fig. 3A, a recombinant protein with relative molecular weight (M_r) of 96 000 was expressed in the transformed *E. coli* Rosetta (DE3) cells rather than in non-induced cells transformed with the same vector. The recombinant protein could be induced at either 37 ℃ or 25 ℃, but the highest level of the expression in soluble form was only achieved under an optimized condition, *i.e.* inducing with 0.1 mmol/L IPTG for 8 h at 25 ℃. The expression of GST-ROP17 was then confirmed by Western blotting with GST primary antibody (Fig. 3B).

4 Antigenicity of ROP17

To verify the antigenic properties of the recombinant ROP17, the recombinant proteins were immunoblotted with anti-serum obtained from tachyzoites of the highly virulent RH strain of *T. gondii*-infected rabbit. The recombinant proteins (soluble and insoluble) were recognized by the rabbit anti-*T. gondii* serum in immunoblot analysis (Fig. 3C).



A: SDS-PAGE; B: Recombinant GST-ROP17 confirmed by Western blotting with GST primary antibody; C: Antigenicity of recombinant GST-ROP17 confirmed by Western blotting with the rabbit anti-*T. gondii* serum. M: Protein marker; 1: Product of uninduced *E. coli*; 2: Product of induced *E. coli*; 3: Pellet product of induced *E. coli*; 4: Supermatant product of induced *E. coli*.

Fig. 3 Analysis of the recombinant protein GST-ROP17 by SDS-PAGE and Western blotting

DISCUSSION

Rhoptry proteins secreted by rhoptry play an important role on invasion, nutrition and virulence for *T. gondii*. ROP2 has been shown to traffic to the cytoplasmic face of the PVM where it is believed to mediate association of host cell mitochondria and endoplasmic reticulum[13,14]. ROP16 influences host gene expression, for example activating some host genes

but suppressing immune responses such as the induction of IL-12 by prolonging the phosphorylation of signal transducer and activator of transcription 3 (STAT3) and STAT6 (STAT3/6)[15]. ROP18 enhances the survival and virulence of *T. gondii* by phosphorylation of immunity-related GTPases such as IRGb6 and IRGb10 which could disrupt the PVM [9]. ROP17 gene located on chromosome Ⅶ just like ROP16 and ROP18 [16], its ORF was 1 827 bp and coded 609 amino acid. Moreover, ROP17, like ROP16 and ROP18, containing a C-terminal kinase-like domain and carrying key residues linked to kinase activity[10,11].

In this study, we successfully cloned the ROP17 gene from RH strain of *T. gondii*. pGEX-6P-1 vector was chosen for expression of ROP17 because it will produce the recombinant proteins with a N-terminal GST tag which could not only increase the solubility of the expressed proteins but also be easily purified with glutathione sepharose 4B and removed by PreScission™ protease after purification to obtain pure protein without tag. In addition, *E. coli* strain Rosetta (DE3) that could code some rare codons was also used as host cell in our study to improve the production of the recombinant proteins. pGEX-6P-1-*Tg*ROP17 recombinant plasmid was constructed and the nucleotide sequence of ROP17 was confirmed by sequencing. Under optimized inducing conditions, the higher ratio of recombinant GST-ROP17 proteins were expressed in soluble form and also maintained intact antigenicity, which was indicated by Western blotting using rabbit anti-*T. gondii* serum.

As there is a relatively high similarity in the protein sequence of the kinase-like domain between ROP16, ROP18 and ROP17, a high possibility could be raised that they might work as functional kinases in regulating virulence of *T. gondii*. At this point, the expression of ROP17 protein might provide the foundation for the future study.

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