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RESEARCH ARTICLE

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Construction and expression of the eukaryotic expressed plasmid of *MIC3* gene from *Toxoplasma gondii* in IBRS-2 cells

Abstract The sequence encoding *MIC*3 was obtained by amplification from genomic DNA of *Toxoplasma gondii* RH strain and cloned into the vector pMD18-T. The target gene was subcloned into the eukaryotic vector pcDNA3.1 after the identification of pMD18-T-*MIC*3 by enzyme digesting, PCR amplification and sequencing. Then the target recombinant plasmids pc*MIC*3 were transfected into IBRS-2 cells, and the positive cells containing pc*MIC*3 plasmids were obtained under the selection of G418. The expressed proteins from the positive cells were detected by SDS-PAGE, Western blot and ELISA. The results showed that the DNA sequence identity was 99.9% between amplified MIC3 and that from GenBank. The molecular weight of the recombinant MIC3 protein with good immuno-activity was about 39.2 ku. These available data would lay the foundation for further studies on DNA vaccine against *Toxoplasma gondii*.

Keywords *Toxoplasma gondii*, *MIC3*, gene clone, eukaryotic expression

1 Introduction

Toxoplasmosis (*Toxoplasma gondii*) causes reproductive failure in pregnant women and pregnant animals, manifested as puny and abnormal embryo, abortion and stillbirths. *Toxoplasma gondii* is ubiquitous among people and animals throughout the world and is highly infective in most herds. It is the enemy of human prepotency and one of the four factors leading to abnormal embryo in pregnant women (Joiner and Dubremetz, 1993). In animals, toxoplasmosis is of great economic importance worldwide because it causes abortions, stillbirth, hyperpyreticness, fleshlessness and neonatal loss in all types of livestock, especially in pigs (Buxton, 1998). In addition, the tissue cysts of *T. gondii* in meat of infected livestock are an important source of infection for humans (Tenter et al., 2000; Choi et al., 1997; Dias et al., 2005; Mendonca et al., 2004).

So far, there is a lack of effective vaccine and medication for controlling and curing toxoplasmosis. The only developed vaccine is the live attenuated tachyzoite. However, this vaccine is not widely accepted because of its side effects, short shelf life, and high cost. Live vaccines also carry a risk of accidental infection of humans (Zhou and Chen, 1998; Jiang and He, 2004). DNA vaccine is a novel one with potent, long-lasting humoral and cell-mediated immunity (Zhou, 1999) since the 20th century. Therefore, the development and exploitation of *T. gondii* DNA vaccine have been taken as the important goal and hotspot for effectively controlling toxoplasmosis. In this work, the eukaryotic expressed plasmid pc*MIC*3 was constructed and transfected into IBRS-2 cells, the immunogen activity of its expressed products was identified. This result will lay a foundation for the DNA vaccine.

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2 Methods

2.1 Cloning, screening and identification of eukaryotic expressed recombinant plasmid pc*MIC*3

According to the methods of Jiang et al. (2006) and Yang et al. (2003), *Toxoplasma gondii* RH was resuscitated and inoculated to mice, the parasites were collected from peritoneal fluid, and then the total DNA was isolated.

A pair of oligonucleotides primers were designed as follows: P1: 5′-CCCAAGCTTAAAATGGCGCTGT-TGCACGCGC-3′ and P2: 5′-TCTAGATCACTGCTT-AATTTTCTCACAGC-3′. The primer sequences corresponded to the conserved amino acids at 714–1775 base site of *T. gondii MIC*3 gene (GenBank Acc. AJ132530), and the primer's 5′ terminal was restrictive enzyme *Hin*dIII, and 3′ terminal was *Xba*I and protective bases with PCR segment 1062 bp. The primers were synthesized by Beijing AuGCTbiotech Co., Ltd.

*MIC*3 DNA was amplified by PCR using a pair of P1 and P2. PCR reactions were performed by being denatured at 95°C for 5 min, followed by 35 cycles at 94°C for 45 s, 62°C for 45 s and 72°C for 1 min, with another extension at 72°C for 8 min. The amplified fragments of 1062 bp were cloned into vector pMD18-T. DNA sequencing was carried out by Beijing Sunbiotech Co. Ltd..

The identified *MIC*3 DNA from pMD18-T- *MIC*3 were cloned into vector pcDNA3.1(+). Eukaryotic expressed recombinant plasmid pc*MIC*3 was identified by restrictive enzyme digestion.

2.2 Mass-preparation and detection of pc*MIC*3 plasmids

Mass pc*MIC*3 plasmids were prepared from *E. coli* and its concentration and quality were measured and analyzed using a Beckman DU800 spectrophotometer at $OD_{260\ nm/280\ nm}$.

2.3 Transfection of pc*MIC*3 to IBRS-2 cell

Purified pc*MIC*3 plasmids were transfected into eukaryotic IBRS-2 cell using Lipofectin Transfection Kit, the positive IBRS-2cells with pc*MIC*3 were screened under the selection of G418 as described by Zhao (2002).

2.4 ELISA analysis of expression products of pc*MIC*3 in IBRS-2 cell

IBRS-2 cells and culture liquid were collected from monolayer anti-G418 cells respectively; and the IBRS-2 cells were washed with PBS, then frozen, melted, and crashed by ultrasonic. The lysis of cells was centrifuged at 10000 r·min^{-1} for 10 min to get supernatant. According to the ELISA method of Wang (2005), the culture liquid and supernatant were detected for the expression levels of MIC3 proteins in IBRS-2 cells by anti-*Toxoplasma gondii* Ab as the first antibody and HRP-conjugated goat against rabbit IgG as the second antibody.

2.5 SDS-PAGE and Western blot analysis of expression products of pc*MIC*3 in IBRS-2 cell

Expression products of pc*MIC*3 in IBRS-2 cell were resolved on 10% SDS-PAGE. The proteins were transferred to the nitrocellulose membrane and probed with anti-*Toxoplasma gondii* Ab as the first antibody with 1:400 dilution. The MIC3 protein bands were visualized on tetrahydrochloride (DAB) after incubation with HRP-conjugated second antibody with 1:5000 dilution (goat antibody against rabbit IgG). Negative contrast was treated the same as above.

3 Results

3.1 Cloning and identification of pc*MIC*3 eukaryotic expressed recombinant plasmids

3.1.1 PCR amplification of *MIC*3

*MIC*3 DNA was amplified from *T. gondii* RH total DNA and the PCR products were analyzed on a 0.7% agarose gel. Special segment was detected with a 1000-bp nucleotide band (Fig. 1 lane 1), which was in good agreement with the expectation. No band was found in the negative control (Fig. 1 lane 2).

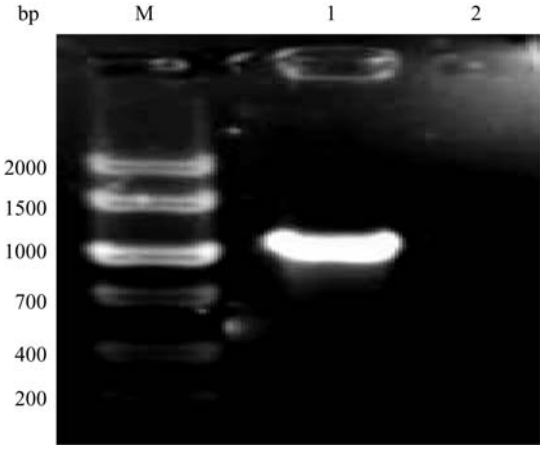

Fig. 1 The result of PCR amplification of *MIC*3
Note: Lane M represents DNA ladder; lane 1 represents PCR products; lane 2 represents negative control (ddH_2O).

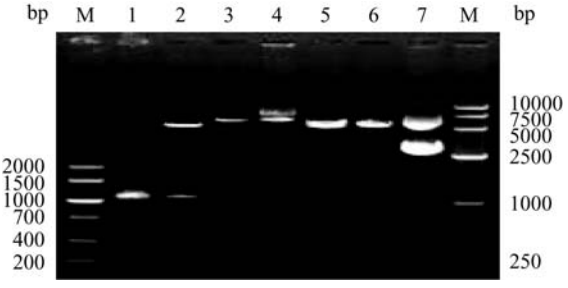

Fig. 2 Identification of pc*MIC*3 recombinant plasmids with restrictive enzyme digestion and PCR amplification
Note: Lane M represents DNA ladder. Lanes 1–7 represent PCR products of pc*MIC*3, digested products of pc*MIC*3 by *Hind*III + *Xba*I, digested products of pc*MIC*3 by *Hind*III, pc*MIC*3 plasmid, digested products of pcDNA3.1 by *Hind*III, digested products of pcDNA3.1 by *Xba*I, and pcDNA3.1 plasmid, respectively.

3.1.2 Cloning and identification of pc*MIC*3

Blast analysis showed that *MIC*3 nucleotide sequence from pMD18-T-*MIC*3 had a 99.9% similarity to one from AJ132530 on GenBank except for T mutation to C (praline → leucine) in 730 sites. Then the *MIC*3 cDNA from pMD18-T-*MIC*3 was cloned to the pcDNA3.1 plasmid and identified by restrictive enzyme digestion and PCR amplification. As shown in Fig. 2, a 1000-bp nucleotide band (Fig. 2 lanes 1, 2) of pc*MIC*3 was in good agreement with the expectation, indicating eukaryotic expressed recombinant plasmid pc*MIC*3 was constructed successfully.

3.2 ELISA analysis of the expressed products of pc*MIC*3 plasmids in IBRS-2 cells

Cell products of recombinant plasmid pc*MIC*3 and pcDNA3.1 from IBRS-2 were prepared and the expression level of the MIC3 proteins was analyzed on ELISA. As shown in Table 1, high expressed products of the *MIC*3 gene was detected in IBRS-2 and it existed inside IBRS-2. Statistical analysis showed that a significant expressed level was obtained among the inside IBRS-2 and the pcDNA3.1 and other contrast ($P < 0.01$).

Table 1 Assay of the expressed products of pc*MIC*3 plasmids in IBRS-2 cells by ELISA

plasmids	transfected cells	supernatant of culture
pc*MIC*3	0.697 ± 0.039	0.317 ± 0.034
pcDNA3.1	0.254 ± 0.026	0.222 ± 0.018
normal IBRS-2 cells	0.263 ± 0.035	0.253 ± 0.023

Note: OD was detected at 630 nm.

3.3 Identification of the expressed products of pc*MIC*3 plasmid from IBRS-2 cells

Cell products of pc*MIC*3 and pcDNA3.1 from IBRS-2 were prepared and identified by Western blot. The anti-*Toxoplasma gondii* Ab reacted strongly with a 39.2 ku polypeptide band (Fig. 3(b) lane 3). The pcDNA3.1 and the other contrast did not show any visible band (Fig. 3(b) lanes 1, 2, 4–6).

4 Discussion

For the *MIC*3 gene was single copied without intron (Cèrède et al., 2002), the 714–1775 base site of MIC3 DNA was chosen to be cloned. This fragment was 1062 bp with 354 amino acids. The expressed MIC3 protein contains full antigen surface of T and B lymphocyte, and specific structure closely correlated with the function of MIC3 protein except for part signal sequence.

Blast analysis showed that *MIC*3 nucleotide sequence from pc*MIC*3 had a 99.9% similarity to one from AJ132530 on GenBank, with an existing dot mutation, T to C (praline → leucine) at 730 site. Because of dot mutation occurring in front of the pro-peptide cleavage site of N terminal protein, it was presumed that the dot mutation of MIC3 may have no effect on its biologic activity, which was confirmed by ELISA (Table 1) and Western-blot (Fig. 3).

The eukaryotic expressed plasmid pcDNA3.1 containing high efficiency promoter/enhancer element, BGH polyadenylation and AmpR CpG sequence of Human Cytomegalo virus (HCMV) is a high efficiency expression vector for cloning genes in eukaryotic cell, which is widely used in construction and research of DNA vaccine (Mao

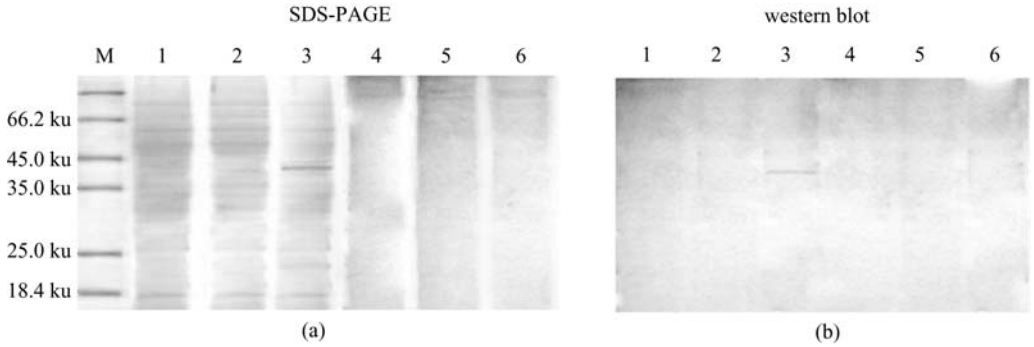

Fig. 3 SDS-PAGE and western blot analysis of the expressed products of pc*MIC*3 plasmid from IBRS-2 cells
Note: (a) represents products of pc*MIC*3 resolved on 10% SDS-PAGE. Lane M represents protein molecular weight standard markers. Lanes 1–6 represent Lysis of normal IBRS-2 cells, Lysis of IBRS-2 cells transfected with the pcDNA3.1 plasmid, Lysis of IBRS-2 cells transfected with the pc*MIC*3 plasmid, Supernatant of the culture medium for normal IBRS-2 cells, Supernatant of the culture medium for IBRS-2 cells transfected with the pcDNA3.1 plasmid, and Supernatant of the culture medium for IBRS-2 cells transfected with the pc*MIC*3 plasmid, respectively. (b) represents western blot of the same samples as in (a). The proteins were transferred to the nitrocellulose membrane and probed anti-*Toxoplasma gondii* as the first antibody. The MIC3 protein bands were visualized on DAB after incubation with HRP-conjugated second antibody (goat antibody against rabbit IgG).

et al., 2004). Microneme protein MIC3 of *Toxoplasma gondii* is related with recognition and combination of host-cell as parasites do, and plays an important role in parasites invading host-cell (Soldati et al., 2001). The *MIC*3 gene is regarded as a potent vaccine candidate against toxoplasmosis because of its expression in tachyzoites, bradyzoites, and sporozoites of *Toxoplasma gondii* (Cèrède et al., 2002; Ismael et al., 2003). In this research, the constructed eukaryotic expressed plasmid pc*MIC*3 was transfected in IBRS-2 cell; the expression was confirmed by ELSA and Western-blot. These would lay a firm foundation for immunizing Balb/c mice by the above eukaryotic pc*MIC*3 and for further studies on the effect of DNA vaccine.

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