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Sequence Variation in *Toxoplasma gondii* MIC4 Gene and Protective Effect of an MIC4 DNA Vaccine in a Murine Model Against Toxoplasmosis

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Abstract: *Toxoplasma gondii* infects nearly one third of the total population of the world, as well as warm blooded animals causing serious public health problems and economic losses in the world. Micronemes plays a key role in the invasion process of *T. gondii* which are used for host cell recognition, binding and motility. In this research, the researchers examined sequence variation in the microneme protein 4 (MIC4) gene sequences of 12 *T. gondii* isolates and reference strains from different hosts and geographical locations, then constructed the DNA vaccine expressing MIC 4 of *T. gondii* evaluated its immune response induced in Kunming mice. The results demonstrated that sequence variation in MIC 4 among different *T. gondii* isolates was low, which is a useful feature as a vaccine candidate. Immunization of mice with pVAX-MIC4 induced strong immune responses in mice as shown by significant lymphocyte proliferation, cytokine production and antibody responses as well as increased survival time of the immunized mice after challenge with tachyzoites of the virulent *T. gondii* RH strain, demonstrating that *T. gondii* MIC4 is a potential vaccine candidate against toxoplasmosis.

Key words: *Toxoplasma gondii*, toxoplasmosis, Microneme protein 4 (MIC4), immunity, DNA vaccine, mice, variation

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan capable of infecting humans and a variety of mammals and birds (Montoya and Liesenfeld, 2004; Weiss and Dubey, 2009) with a worldwide distribution. *T. gondii* is considered the most successful parasite because it infects one-third of human world population, causing considerable diseases in immuno-compromised patients and pregnant women (Montoya and Liesenfeld, 2004). Moreover, toxoplasmosis can result in serious economic losses and causes abortions, stillbirths and neonatal deaths in all kinds of livestock (Buxton, 1998).

Despite the current knowledge of immunology, pathology and genetics related to the parasite, the effective treatment and a safe vaccine for prevention of the infection in both humans and animals are still not available (Zhang *et al*., 2007). Thus, the development of an effective vaccine against *T. gondii* would be extremely valuable for the effective control of *T. gondii* infection in humans and animals. Micronemal proteins (MICs) are thought to be critical ligands determining host cell specificity at the time of invasion.

MIC adhesive proteins contribute to binding host cell receptors and blocking micronemal secretion dramatically reduces invasion (Carruthers *et al*., 1999). These proteins, which often cluster into multiunit complexes are actively discharged from parasites upon the apical attachment to a host cell and play a role in host cell attachment and penetration (Tomley and Soldati, 2001).

Several studies of DNA-based vaccines against toxoplasmosis have been conducted, mainly with mice and various *T. gondii* antigens such as membrane associated surface antigen SAG1 (Angus *et al*., 2000; Nielsen *et al*., 1999), rhoptry proteins ROP1 (Chen *et al*., 2001) and ROP2 (Leyva *et al*., 2001; Vercammen *et al*., 2000), excreted-secreted dense-granule proteins GRA1

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(Scorza *et al*., 2003; Vercammen *et al*., 2000), GRA4 (Desolme *et al*., 2000) and GRA7 (Vercammen *et al*., 2000), Micronemal proteins MIC1, MIC3, MIC4 and MIC6 (Lourenco *et al*., 2006; Peng *et al*., 2009; Xiang *et al*., 2009; Wang *et al*., 2009). All of them showed the protective effect to some extent. The objectives of the present study were to examine sequence variation in the MIC 4 gene and evaluate the protective effect of a MIC4 DNA vaccine (expressed in the eukaryotic expression vector pVAX I) in a murine model against *T. gondii*.

MATERIALS AND METHODS

Mice, parasites, isolation of genomic DNA and preparation of Soluble Tachyzoite Antigens (STAg): Female Kunming mice used in this experiment were purchased from Southern Medical University Laboratory Animal Center in China. All experimental procedures were conducted according to institutional guidelines for animal ethics. All mice were maintained under specific-pathogen free conditions and were at 5-6 weeks of age when immunizations were initiated. Tachyzoites of *T. gondii* (RH strain) were conserved well in liquid nitrogen in the laboratory. The researchers dissolved them in 38°C water-bath and infected Kunming mice intraperitoneally and harvested tachyzoites from the peritoneal fluid of mice after 72 h. After they were washed by centrifugation and then suspended in sterile Phosphate-Buffered Saline (PBS), the tachyzoites of *T. gondii* were used for challenge of immunized mice. Genomic DNA (gDNA) was extracted from the tachyzoites stored at -80°C by sodium dodecyl-sulphate/proteinase K treatment (Zhu *et al*., 2002), column-purified using Wizard™ DNA Clean-Up System (Promega) and eluted into 50 μL H_2O according to the manufacturer's recommendations. DNA samples were stored at -20°C until use.

For the preparation of STAg, the tachyzoites were collected from the peritoneal fluids, washed by centrifugation and then suspended in sterile Phosphate Buffered Saline (PBS) and sonicated. The sonicate was centrifuged at 2100× g for 15 min and the supernatant containing STAg was kept at -80°C until further use.

Enzymatic amplification, sequencing and analysis: The MIC4 gene was amplified with a pair of primers (MIC4, forward primer: 5'-GAATTCATGAGAGCGTCGCTC-3' and reverse primer: 5'-CTCGAGTCATTCTGTGTCTTTCGC-3'. PCR reactions (25 μL) were performed in 2 mM of $MgCl_2$, 2.5 μM of each primer, 2.5 μL Ex Taq buffer, 0.2 mM of each dNTPs, 1.25 U of Ex Taq DNA polymerase (TAKARA), 2 μL of DNA sample in a thermocycler (Biometra) under the following conditions: initial denaturation at 94°C for 4 min followed by 36 cycles of 94°C, 1 min (denaturation), 62°C, 1 min (annealing), 72°C, 2 min (extension) and a final extension of 72°C for 7 min. Samples with host DNA were included in each amplification run as host controls. Samples without genomic DNA were included in each amplification run as negative controls. Each amplicon (5 μL) was examined by agarose gel electrophoresis to validate amplification efficiency. The DL2000 marker (TAKARA) was used to estimate the size of MIC4 amplicons.

PCR products were purified using spin columns (Wizard™ PCR-Preps DNA Purification System, Promega) and the purified PCR products were ligated with pGEM-T Easy plasmid vector (Promega) according to manufacturer's instructions. The recombinant plasmid was transformed into JM109 competent cells (Promega), positive transformants containing recombinant plasmids were selected and the plasmid DNA extracted using Wizard™ Plus Minipreps DNA Purification System (Promega). Cell cultures with confirmed recombinant plasmid were sequenced using an ABI 377 automated DNA sequencer (BigDye Terminator Chemistry).

Sequences of the MIC4 gene were aligned using the computer program ClustalX 1.81 (Thompson *et al*., 1997), genetic distance calculation was performed using PUZZLE 4.1 (Strimmer and Von Haeseler, 1996), sequence homology analysis was performed and the nucleotide composition, transition and transversion were accounted using the Megalign program in the software DNA Star version 5.0.

Construction of the eukaryotic expression plasmid: After generation of pGEM-MIC6 as earlier, the fragment of MIC6 was inserted into the eukaryotic expression vector pVAX I by double digestion with restriction enzymes EcoR I/Xho I and ligation was finally done using T_4 DNA ligase (Promega). The plasmid that gained was named pVAX-MIC4. The concentration of pVAX-MIC4 was determined by spectrophotometer at OD_{260} and OD_{280}.

Expression of MIC4 *in vitro*: Marc-145 cells were transfected with a kind of eukaryotic expression plasmid or an empty vector (control plasmid) using lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's recommendations. After 48 h, the cells that transfected were fixed with cool acetone for 30 min and MIC4 expression was detected using the indirect Immunofluorescence Assay (IFA) with anti-*T. gondii* polyclonal antiserum (Goat) and a FITC-labeled donkey-anti-goat IgG (Proteintech Group Inc, Chicago, USA). Evans blue (Fisher) was included in the secondary antibody solution as a counterstain. Coverslips were

rinsed three times with PBS. The monolayers binding the marker were covered with glycerine and examined for specific fluorescence under a Zeiss Axioplan fluorescence microscope.

DNA immunization: Eighty Kunming mice were randomly divided into four groups of 20 mice per group. Mice in Group I was injected intramuscularly with PBS as control, group II with empty pVAX I vector also as control, group III with 100 μg of pVAX-MIC4 DNA suspended in 100 μL sterile PBS. Group IV served as non-treatment control. Mice were immunized using the same protocol on weeks 0, 2 and 4. The blood of mice in each group was collected by retro-orbital plexus puncture on days 13, 27 and sera were stored at -20°C until use.

IgG determination: Serum samples from immunized mice were tested using anti-*T. gondii* IgG ELISA Kit according to the manufacture's recommendations (Combined Biotech CO., LTD, Shenzhen, China). Plates were coated overnight at 4°C with 1 μg mL^{-1} solution of STAg in carbonate buffer pH 9.2. After washing three times (each time for 3 min) with PBS containing 0.05% Tween-20 (PBST-20), sera diluted in 1% BSA-PBST-20 (1:100) were applied to the wells and incubated for 30 min at 37°C. After washing, bound antibodies were incubated with 50 μL horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000 dilution). Immune complexes were revealed by incubating with orthophenylene diamine (Sigma) and 0.15% H_2O_2 for 30 min. The reaction was stopped by the addition of 1M H_2SO_4 and the absorbance was measured at 450 nm using an ELISA reader (Bio-TekEL × 800, USA). All samples were run in triplicates.

Lymphoproliferation assay: Two weeks after the final inoculation, spleen cells were harvested from the seven immunized mice of each group under aseptic conditions and the erythrocytes were lysed by using RBC lysis solution (Sigma). Splenocytes were re-suspended in DMEM medium supplemented with 10% FCS, cells were plated in 96-well costar plates at a density of 5×10^5 cells per well and cultured with STAg (μg mL^{-1}) or concanavalin A (ConA; 5 μg mL^{-1}; Sigma; positive control) or medium alone (negative control) at 37°C with 5% CO_2. The proliferative activity was measured using a 3-(4, 5-dimethylthylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 5 mg mL^{-1}, Sigma) dye assay, the Stimulation Index (SI) was calculated as the ratio of the average OD_{570} value of wells containing antigen stimulated cells to the average OD_{570} value of wells containing only cells with medium. All assays were performed in triplicate.

Cytokine assays: Cells were stimulated by incubation with STAg, ConA or with the medium alone. Cell-free supernatants were harvested and assayed for interleukin-2 (IL-2) and interleukin-4 (IL-4) activities at 24 h for interleukin-10 (IL-10) activity at 72 h and for gamma-interferon (IFN-γ) activity at 96 h. The concentrations of IL-2, IL-4, IL-10 and IFN-γ were evaluated by using commercial ELISA Kits according to the manufacturer's instructions (DuoSet, Genzyme). All assays were performed in triplicate. Cytokine concentrations were determined by making reference to the standard curves constructed with known amounts of mouse recombinant IFN-γ, IL-2, IL-4 or IL-10.

Challenge infection: Kunming mice of all groups were challenged intraperitoneally with 1×10^3 tachyzoites of virulent *T. gondii* (RH strain) 2 weeks after the last immunization and the mice were observed and the time of the death was recorded.

Statistical analysis: All data were compared using the Statistical Package for the Social Sciences SPSS 13.0 Data Editor (SPSS Inc., Chicago, Illinois, USA). $p<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Sequence variation in the MIC4 gene sequences of *T. gondii* isolates: The MIC4 fragments obtained from all examined isolates were approximately 1740 bp in length (Fig. 1). Sequencing of purified amplicons obtained a 1743 bp sequence for all examined isolates. Sequence comparison revealed 12 (0.69%) variable nucleotide positions among the examined isolates. The A + T and C + G contents of the sequences were 49.05-49.28 and 50.72-50.95%, respectively. For the MIC4 gene, intra-specific nucleotide variation was related mainly to

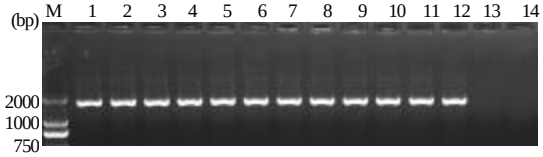

Fig. 1: Analysis of the MIC4 PCR products of *Toxoplasma gondii* isolates by agarose gel electrophoresis. Lanes 1 and 2 represent RH strain and QHO strain, respectively. Lanes 3-10 represent isolates TgCtGZ1~TgCtGZ8, respectively. Lanes 11 and 12 represent isolate TgPgPYS and strain TgCgCa1, respectively. Lanes 13 and 14 represent host (mouse) and negative control, respectively. M represents a DNA size marker

Table 1: Geographical origins, genotypes, hosts of *Toxoplasma gondii* isolates and the GenBank accession numbers of their MIC 4 genes

Sample codes	Host	Geographical origin	Genotype	Reference	Accession number for MIC 4
RH	Human	USA	I	Zhou *et al.*, 2009	FJ785459
QHO	Sheep	Qinghai Province in China	II	Zhou *et al.*, 2009	FJ785460
TgCtGZ1	Cat	Guangzhou city in China	II	Zhou *et al.*, 2009	FJ785462
TgCtGZ2	Cat	Guangzhou city in China	II	Zhou *et al.*, 2009	FJ785463
TgCtGZ3	Cat	Guangzhou city in China	II	Zhou *et al.*, 2009	FJ785464
TgCtGZ4	Cat	Guangzhou city in China	II	Zhou *et al.*, 2009	FJ785465
TgCtGZ5	Cat	Guangzhou city in China	II	Zhou *et al.*, 2009	FJ785466
TgCtGZ6	Cat	Guangzhou city in China	II	Zhou *et al.*, 2009	FJ785467
TgCtGZ7	Cat	Guangzhou city in China	II	Zhou *et al.*, 2009	FJ785468
TgCtGZ8	Cat	Guangzhou city in China	II	Zhou *et al.*, 2009	FJ785469
TgPgPYS	Pig	Guangzhou city in China	II	Zhou *et al.*, 2009	FJ785470
TgCgCa1	Cougar	Canada	I/II/III	Zhou *et al.*, 2009	FJ785461

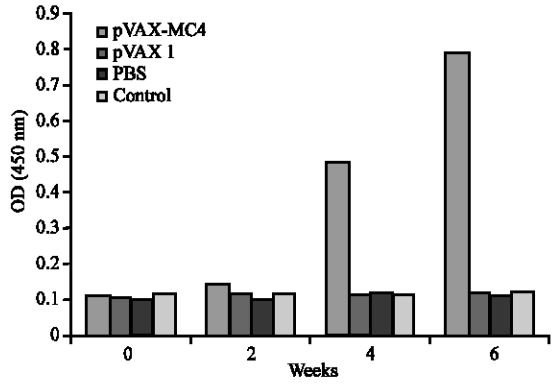

Fig. 2: Determination of specific anti-MIC4 antibody titers in the sera of Kunming mice immunized with 100 μg of pVAX I or PBS alone or combined with 100 μg of MIC4 on weeks 0, 2 and 4. Sera were collected on 1 day prior to each immunization and tested by ELISA using STAg. The titer is given as the reciprocal of the highest dilution with an OD_{450} that was 2.5-fold greater than the OD of untreated mouse sera at the same dilution

changes at the first and second codon positions while fewer changes were detected at the third codon position (Table 1).

Identification of the expressed product by IFA: *In vitro* expression of pVAX-MIC4 was evaluated by IFA at 48 h post-transfection. The green fluorescence was observed in Marc-145 cells transfected with pVAX-MIC4.

Humoral immune responses: All sera were tested by ELISA to detect the significant *T. gondii* specific IgG production in vaccinated mice (Fig. 2). A strong antibody response was found in mice immunized with pVAX-MIC4, which were significantly higher than those of the control groups ($p<0.05$), versus other groups).

Cytokine production: The levels of IFN-γ, IL-2, IL-4 and IL-10 produced in splenocytes from immunized mice are shown in Table 2. Significant high levels of IFN-γ, IL-2 and IL-12 were observed in spleen cell cultures from mice immunized with pVAX-MIC4 compared with mice immunized with PBS and empty plasmid. Besides, levels of IL-4 in supernatants of spleen cells from mice immunized with pVAX-MIC4 were lower than other three groups.

Protection of mice against challenge with virulent strain following DNA vaccination: To test whether immunization of pVAX-MIC4 induced effective protection against *T. gondii* infection, the immunized mice were intraperitoneally challenged with tachyzoites of the virulent RH strain at 2 weeks after last immunization (Fig. 3). The survival rate of the mice immunized with pVAX-MIC4 was higher than other groups ($p<0.05$). However, immunization of Kunming mice with pVAX-MIC4 only increased the survival time, which did not induce complete protection against challenge with the RH strain of *T. gondii*.

Previous studies have shown that MIC4 is a component of the micronemes in *T. gondii* and the deduced amino acid sequence of MIC4 predicts a protein that contains six conserved apple domains (Brecht *et al.*, 2001). Microneme proteins are mainly used for host cell recognition, binding and motility. Besides, MIC4 binds efficiently to host cells and the adhesive motif maps in the most C-terminal apple domain.

The present study examined nucleotide variation in MIC 4 sequences among different *T. gondii* isolates and found that the sequence variation was very low, demonstrating that MIC4 can be considered as the candidate of DNA vaccines against *T. gondii* infection.

As an attractive alternative to conventional vaccines, DNA vaccines show promise in inducing protection against infectious diseases. Lourenco *et al.* (2006) showed that the vaccination of C57BL/6 mice with *T. gondii* MIC1 and MIC4 induced protective immunity against infection by *T. gondii*. In the present study, the researchers used a highly-biosafety plasmid pVAX I as the vector to express MIC4, this vector contains appropriate genetic elements (e.g., promoter) required for

Table 2: Cytokine production by splenocytes of immunized kunming mice after stimulation by STAg

Groups	Cytokine production (pg mL^{-1}) IFN-γ	IL-2	IL-4	IL-10	Proliferation SI
pVAX-MIC4	657±32.74^{a}	614.33±30.92^{a}	281.33±14.29^{a}	608.33±17.01^{a}	1.61±0.05^{a}
pVAX I	52.33±11.02^{b}	48.67±20.55^{b}	52±15.52^{b}	53.67±8.50^{b}	0.19±0.03^{b}
PBS	47.33±5.13^{b}	41.00±7.55^{b}	50.67±4.73^{b}	50.67±6.81^{b}	0.20±0.06^{b}
Control	49.33±10.50^{b}	45.33±8.02^{b}	47.67±13.58^{b}	50.00±14.18^{b}	0.19±0.08^{b}

SI stands for stimulation index; Splenocytes from mice were harvested 2 weeks after the last immunization; Values for IFN-γ are for 96 h, values for IL-2 and IL-4 are for 24 h, values for IL-10 are for 72 h; The same letter means no difference ($p>0.05$) whereas different letters mean significant difference ($p<0.05$)

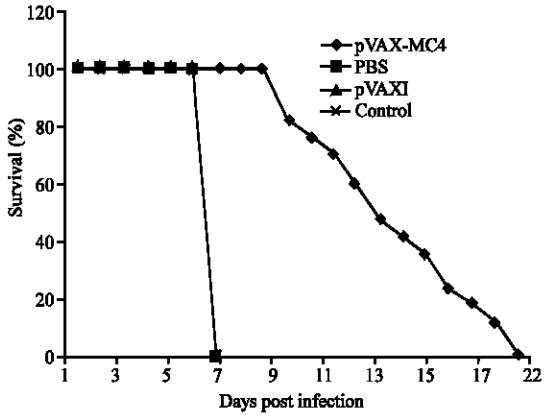

Fig. 3: Survival curves of immunized Kunming mice after lethal challenge with 1×10^{3} tachyzoites of *T. gondii* RH strain 2 weeks after the last immunization

in vivo expression of the antigen gene of interest in the target organism and meets U.S. Food and Drug Administration (FDA) guidelines for design of DNA vaccines.

The researchers observed significant production of serum IgG antibodies (Fig. 2), a strong increase of proliferative response of splenocytes ($p<0.05$) and significant levels of IFN-γ, IL-2 and IL-10 ($p<0.05$) but low production of IL-4 compared with mice immunized with PBS or pVAX I alone (Table 2).

In this study, it is evaluated the protection induced by pVAX-MIC4 by intraperitoneally infecting the vaccinated mice with tachyzoites of the virulent RH strain at 2 weeks after the final immunization. An effective and highly significant degree of protection was obtained in mice immunized with DNA vaccine compared to the other groups. Mice immunized with pVAX I alone or PBS died on the fifth day. However, pVAX-MIC4 vaccination resulted in longer survival time (Fig. 3).

CONCLUSION

The present study indicated that the recombinant pVAX-MIC4 was able to elicit a significant humoral and cellular immune response, as well as increased survival time of immunized mice. These results demonstrate that that *T. gondii* MIC4 is a potential vaccine candidate against toxoplasmosis.

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