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Research Brief

# Identification and characterization of Tu88, an antigenic gene from *Theileria uilenbergi*

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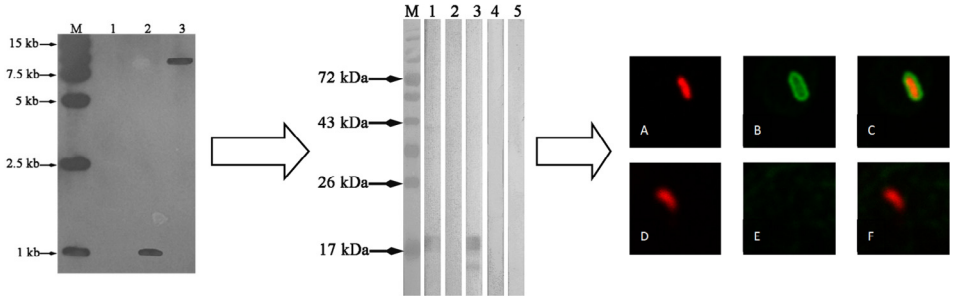
## HIGHLIGHTS

- An antigenic gene termed Tu88 was identified in a *Theileria uilenbergi* merozoite cDNA library.
- Characterization of Tu88 showed that it is a single copy gene expressed primarily in the cytoplasm of merozoites.
- Tu88 is an immunodominant molecule, which could be potentially used to develop sero-diagnostic tests for *Theileria uilenbergi*.

## GRAPHICAL ABSTRACT

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## ABSTRACT

*Theileria uilenbergi* is a pathogen that causes ovine theileriosis. Prevention and control of theileriosis relies on its diagnosis at early stages of occurrence and requires understanding of proteins with antigenic properties from the pathogen. Despite its prevalence in China, only a few molecules with antigenic properties have been characterized from *T. uilenbergi*. In this study, we identified a cDNA named Tu88 by immunoscreening a *T. uilenbergi* merozoite cDNA library with *T. uilenbergi*-positive sera from infected sheep. Recombinant Tu88 (rTu88) expressed in bacteria reacted strongly with the positive sera of *T. uilenbergi* in western blot analysis indicating its potential as an antigen. Southern blot analysis showed that it is a single copy gene. Protein localization by immunostaining blood smears from an infected sheep demonstrated the presence of native Tu88 in merozoites. These findings suggest that Tu88 is a potential candidate antigen for the development of a sero-diagnostic tool.



## 1. Introduction

*Theileria uilenbergi* is an apicomplexan intracellular protozoan parasite that causes theileriosis in sheep and goats. Ovine theileriosis is prevalent in the sheep and goat farms in many provinces of China including Qinghai, Gansu, Ningxia, Inner Mongolia, Shaanxi, Sichuan, and Xinjiang (Li et al., 2011; Luo and Yin, 1997) and leads to severe economic losses in endemic regions (Ahmed et al., 2002, 2006). The disease is caused by *T. uilenbergi* and its closely related counterpart, *T. luwenshuni*, which are transmitted by the tick species, *Haemaphysalis qinghaiensis* and *H. longicornis* (Li et al., 2007, 2009; Yin et al., 2002). Clinical manifestations of the disease include high fever, loss of appetite, enlargement of lymph nodes, jaundice, weight loss, and even death (Luo and Yin, 1997; Yin et al., 2007). Currently, prevention and control of the disease depends on the elimination of vector ticks from the animal body and chemical therapy against infections.

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Recently, some parasite-specific molecules were identified that are being assessed for their immunogenicity against infections and for the development of diagnostic tools (Liu et al., 2008b; Schnittger et al., 2003; Seitzer et al., 2008). Notably, the 18S rRNA gene has been used as a key marker to differentiate *T. uilenbergi* from *T. luwenshuni* and the other closely related parasites, and for the development of nucleic acid-based diagnostic tools (Schnittger et al., 2003, 2004; Yin et al., 2004, 2008). In addition, the *Theileria* sp. (China) merozoite heat shock protein 70 (TcHSP70), *T. uilenbergi* immunodominant protein (TuIP) and clone-9 have been expressed as recombinant antigens for the development of enzyme-linked immunosorbent assays (ELISA) for the diagnosis of ovine theileriosis (Abdo et al., 2010; Liu et al., 2010; Miranda et al., 2006a). In the *Theileria* sp. (China) merozoite cDNA, two proteins, which induced both cellular and humoral immunity in tick-infected or blood-infected sheep, have been identified (Miranda et al., 2006b; Seitzer et al., 2008). One is a homolog of *T. annulata* surface protein (TaSP) termed TcSP, and the second is a homolog of putative *T. annulata* membrane protein (TaD). However, identification of more functional molecules from the parasites is needed to better understand their pathogenicity and for the development of more efficient control methods.

In the present study, we identified a gene termed Tu88 by immunoscreening a cDNA library from the *T. uilenbergi* merozoites using *T. uilenbergi* positive sera from infected sheep. Tu88 was characterized based on its molecular structure and functions. The results indicate that Tu88 is a candidate antigen for the development of serological diagnostic tools or vaccines.

## 2. Materials and methods

### 2.1. Animals and serum samples

Animal experiments involving sheep and rabbits were performed only after formal ethics approval. Animals were housed and fed in accordance with institutional guidelines for the care and use of research animals. Negative serum sample (Sample No. 2201) and *T. uilenbergi* positive serum samples (Sample Nos. 1240 and 1229) from sheep were as reported in our previous study (Liu et al., 2010).

### 2.2. Identification of the Tu88 gene

The *T. uilenbergi* positive sera (Nos. 1240 and 1229) were used to screen a cDNA library prepared from *T. uilenbergi* merozoites as reported previously (Liu et al., 2008a). Screening was performed using the picoBlue™ immunoscreening kit following the manufacturer's instructions (Stratagene, USA) and as described in our previous study (Liu et al., 2010). After repeated screenings, we identified one plaque that positively reacted with the serum. This plaque was isolated and sequenced (Sangon Biotech, Shanghai, China).

To clone the full-length Tu88 gene, 5′ rapid amplification of cDNA end (RACE) was conducted using a BD SMART™ RACE cDNA amplification kit (BD Biosciences Clontech, USA) according to the manufacturer's instruction and using the cDNA from *T. uilenbergi* merozoites as template. Gene-specific primers, Tu88 R1 (5′-GCCGTTCGTCGTCCAGGTTCAGGG G-3′) and Tu88 R2 (5′-GGGCG TCGTTGACTTTGATCCTGT-3′), were designed based on the partial Tu88 gene sequence identified from the cDNA library. The amplified products were cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced (Sangon Biotech, Shanghai, China). The full-length sequence of Tu88 was assembled manually using the Lasergene SeqMan software (DNAStar, Madison, WI) and then analyzed using bioinformatics tools including SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/), TopPred (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=toppred) and TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP/).

### 2.3. Southern blot analysis

A 729 bp DNA fragment was amplified by PCR from the genomic DNA of *T. uilenbergi* using primers F (5′-ATGATTGTTGCCATCAACCTGC-3′) and R (5′-TTAGGGCT TAGGGCTTTCCCACTT-3′). The reactions were performed in a final volume of 50 μl, containing 1 mM concentrations of each primer, 5 μl of PCR buffer, 4 μl of deoxynucleoside triphosphates, 0.25 μl of TaKaRa Taq (5 U/ml) (TaKaRa, China), and 1 μl of DNA template. Reactions were conducted in an automated DNA C1000 thermal cycler (Bio-Rad, Beijing, China). The cycling conditions were initiated with denaturation for 4 min at 94 °C, followed by 30 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s. The amplified DNA fragment was labeled with digoxigenin-11-dUTP and used as the probe for southern blot analysis performed using the DIG high prime DNA labeling and detection Starter kit according to the manufacturer's protocol (Roche, Germany). About 10 μg of genomic DNA from *T. uilenbergi* was digested separately with 30 U each of *Hind* III and *Kpn* I at 37 °C for 16 h. About 10 μg of genomic DNA from healthy sheep was digested with *Hind* III as the negative control. Then, the DNA was separated on a 0.8% agarose gel and transferred to a nylon membrane (Hybond-$N^+$, Amersham) and hybridized with 25 ng/ml of the DIG-labeled DNA probe.

### 2.4. Protein expression and purification

In order to remove the signal peptide at the N-terminus of the T88 gene, the C-terminus of the gene with the complete ORF was amplified using primers Tu88F containing the *Kpn* I restriction site (5′-GGGGTACCCAAACTGGCGAAAA CAAGTACAAGG-3′) and Tu88R containing the *Bam*H I restriction site (5′-GGAGGATCCTTAGGG CTTAGGGCTTTCCCACTT-3′). The amplified product was sub-cloned into the pET-30(a) vector (Qiagen, Hilden, Germany) to create the recombinant expression plasmid, pET30 (a)-Tu88. The plasmid was then transformed into BL21 (DE3) pLysS cells and expressed at 37 °C for 4 h after induction with isopropylthio-β-galactopyranoside (IPTG). Aliquots of the cell culture collected before and after induction with IPTG were centrifuged and analyzed on SDS–PAGE to detect recombinant Tu88 expression. The histidine tagged recombinant protein (rTu88) was then purified using Ni–NTA agarose beads under native conditions following the QiaExpressionist protocol (Qiagen, Hilden, Germany).

### 2.5. Western blot analysis

Purified rTu88 was separated on a 12% SDS–PAGE, transferred to a nitrocellulose membrane and blocked in blocking buffer (5% skimmed milk, 0.1% Tween 20 in TBS, 0.05M Tris Base, 0.9% NaCl, pH 7.4) overnight. Then, the membrane was cut to strips, and one strip was incubated with RGS-His™ mouse anti-histidine antibody (1:4000, Qiagen, Hilden, Germany) followed by incubation with alkaline phosphatase (AP) conjugated goat anti-mouse IgG + IgM (H + L) antibody (1:10,000, Dianova, Hamburg, Germany) to detect the His-tagged rTu88 protein. The other strips were first incubated with *T. uilenbergi* positive serum 1229 (1:100), negative serum 2201 (1:100) or blocking buffer as the blank control, and then incubated with 1:5000 diluted alkaline phosphatase (AP) conjugated anti-sheep IgG (Sigma, USA) to detect the reactivity and specificity of rTu88. All serum samples, primary antibodies and secondary antibodies were diluted in dilution buffer (1% BSA, 0.1% Tween 20 in TBS, 0.05M Tris Base, 0.9% NaCl, pH 7.4). Binding of secondary antibody was detected with a BCIP/NBT substrate.

### 2.6. Preparation of rabbit anti-rTu88 anti-serum

Three rabbits received a first dose of rTu88 by a subcutaneous injection of 0.2 mg purified rTu88 emulsified in complete Freund's