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*Full Length Research Paper*

# Screening of *Theileria parva* apicomplexan antigen homologs for induction of MHC CD4$^+$ and CD8$^+$ T-cell responses

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***Theileria parva*, an intracellular apicomplexan pathogen transmitted by *Rhipicephalus appendiculatus* ticks, infects and transforms lymphocytes of cattle and African buffalo causing the disease called East Coast fever (ECF). The genome of of *T. parva* was sequenced in order to facilitate research on parasite biology, assist the identification of schizont antigens for vaccine development and extend comparative apicomplexan genomics. In the present study, eight putative apicomplexan antigens were identified from literature and their homologs purified from a *T. parva* Muguga schizont cDNA library. These were immunoscreened for MHC CD4$^+$ and CD8$^+$ CTL response. The results showed that of the expressed proteins, only the *T. parva* T-complex 1 protein zeta subunit ortholog was found to elicit CD4$^+$ response; none elicited CD8$^+$ response. The elicitation of CD4$^+$ T cell response by the *T. parva* T complex Protein-1 zeta subunit homolog indicates that it is a candidate T-helper cell target antigen.**

**Key words:** Theileria parva, apicomplexan antigen, East Coast fever (ECF), major histocompatibility complex (MHC), cytotoxic T lymphocyte.

## INTRODUCTION

*Theileria parva*, an apicomplexan pathogen causing economic losses to small holder farmers in Africa, infects and transforms lymphocytes of cattle and African buffalo causing the disease called East Coast fever (ECF). Transmitted by *Rhipicephalus appendiculatus* ticks, the parasite causes a severe lymphoproliferative disease of cattle in eastern, central, and southern Africa (Katzer et al., 2006). It is an intracellular parasite that infects and transforms bovine lymphocytes. This disease, which kills over 1 million cattle each year in sub-Saharan Africa, results in economic losses exceeding $200 million annually (Norval et al., 1992).

The genome of *T. parva* was sequenced in order to facilitate research on parasite biology, assist the identification of schizont antigens for vaccine development (Graham et al., 2006) and extend comparative apicomplexan genomics, in particular with *Plasmodium falciparum*, which causes malaria. The haploid *T. parva* nuclear genome is 8.3 x 10$^6$ base pairs (Mbp) in length and consists of four chromosomes. Gardner et al. (2005) also sequenced the parasite apicoplast and mitochondrial genomes (Kairo et al., 1994). The *T. parva* chromosomes contain one extremely A+T-rich region (>97%) about 3kbp in length that may be the centromere. The telomeric repeats are short. The *T. parva* nuclear genome contains about 4035 protein-encoding genes, 20% fewer than *P. falciparum*, but exhibits higher gene density, a greater proportion of genes with introns, and shorter intergenic regions.

Mining of sequence data has proved useful in the search for candidate vaccine antigens (Graham et al., 2006). Two approaches have been previously adopted for antigen identification in *T. parva* (Graham et al., 2006). Both techniques depend on screening of transiently transfected antigen-presenting cells with fully characterized cytotoxic T lymphocyte (CTL) (Taracha et al., 1995; Goddeeris and Morrison, 1988) from live vaccine-immunized cattle of diverse bovine leukocyte

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**Table 1.** Primers for amplifying *T. parva* apicomplexan homologs.

| Putative antigen | Apicomplexan parasite | Primer name | Primer sequence |
|---|---|---|---|
| 12D3 antigen | *Babesia* spp. | Forward | GCCGCCACCATGGGCTACTACCCACTTCCTTCAAC |
| | | Reverse | TTAAAGTTCATTCACCGTTTGAACACTT |
| 110kDa antigen | *Plasmodium knowlensi* | Forward | GCCGCCACCATGAAAGATGAAGAGTTGCATCATGC |
| | | Reverse | TTAACAAGTTACTTCCGTTCCGCTCTGA |
| T-complex protein1 | *Plasmodium falciparum* | Forward | GCCGCCACCATGTCGAAGAGTATGCCATCTATTGA |
| | | Reverse | TTACTCTGTACCAATTAGCGTAACTTGA |
| Apical membrane antigen (AMA1) | *Plasmodium chaubadi* | Forward | GCCGCCACCATGAGTTTTAGCCCTAACACTGCTGA |
| | | Reverse | TTATATGAATGGTCCTGAAGAAACGGGT |
| 22.4.1 protein | *Cryptosporidium parvum* | Forward | GCCGCCACCATGAGTTCAAGGTATAAAAGAAATTA |
| | | Reverse | TTAGGAGGGTGTGTATTCAGGGCAGTTG |
| T-complex protein zeta subunit | *Plasmodium falciparum* | Forward | GCCGCCACCATGGCAGTCAATATCTTAAATAGCAG |
| | | Reverse | TTACGAAGGAGCGTTATGCATAGACCTT |
| T-complex protein 1, delta subunit | *Babesia microti* | Forward | GCCGCCACCATGCCACCGCCTTCTAATAATTCTGT |
| | | Reverse | TTACTCGAACATGTCAGAGATGCTATTC |
| Ring-infected erythrocyte surface antigen | *Plasmodium falciparum* | Forward | GCCGCCACCATGTCCGAATGTGATACCATGGAGAT |
| | | Reverse | TTAGTAAAAGGGCTCGTTTGAGTAGTGT |
| HSP-70 | *Plasmodium berghei* | Forward | GCCGCCACCATGAGTTGCATTTTAAAGTGTAATGA |
| | | Reverse | TTAATTTTGATCATTATTATTATCAATG |

antigen (BoLA) major histocompatibility complex (MHC) class I genotypes. First, in a targeted gene approach, genes that were predicted by using preliminary sequence data from one of the four *T. parva* chromosomes (Gardner et al., 2005) to contain a secretion signal were immunoscreened. The approach was based on the observation that the schizont lies free in the host cell cytoplasm (Shaw, 2003) whereby signal peptide-containing parasite proteins would directly access the host cell MHC class I antigen processing and presentation pathway. In the second approach, a random immunoscreen of schizont cDNA clones was conducted because secretion of proteins that do not contain signal sequences has been reported (Nacer et al., 2001).

However, gene homologs encoding antigens from other apicomplexan parasites constitute a source of possible vaccine candidate antigens. This complimentary homolog screen approach to antigen identification would be to screen proteins with related amino acid sequence from related apicomplexan parasites that are known to be antigenic (Tonukari and Kangethe, 2009a, b). Here, eight putative apicomplexan antigens were identified from literature and their homologs purified from a *T. parva* Muguga schizont cDNA library (Graham et al., 2006). These were immunoscreened for MHC $CD4^+$ and $CD8^+$ CTL response.

## MATERIALS AND METHODS

### Cloning of targeted genes

The homologs of apicomplexan antigens described as antigenic in literature were selected for analysis (Tonukari and Kangethe, 2009b). The selected gene sequences were used to perform a BLAST search (Altschul et al., 1990; Altschul et al., 1997) of the *T. parva* genome database to identify homologous genes. Those identified in the *T. parva* database were translated to their amino acid sequences and analyzed using Signal P-2.0 and TMHMM software for the presence of signal peptides, glycosyl-phosphatidyl-inositol (GPI) anchor (which can be equated as a signal for their expression and possible antigenicity) and transmembrane domains. This would identify secreted proteins or proteins located on the surface of the parasite. Eight putative antigen identified (Table 1) were amplified from a purified *T. parva* Muguga schizont cDNA library (Graham et al., 2006) and cloned as previously described (Tonukari and Kangethe, 2009a, b).

### PCR and cloning

Polymerase chain reaction (PCR) was performed with a thermocycler (MJ Research, Watertown, MA) using *Taq* DNA polymerase (Promega) and two primers based on the sequences

identified in *T. parva* using cDNA library as template (Graham et al., 2006). The PCR product generated above was cloned into pGEM T-easy vector (Promega, Madison and WI). Vector specific primers both forward and reverse were synthesized (Table 1). These primers were then used to amplify the ortholog genes from cDNA. All the PCRs were performed as described using the following conditions: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 1 min; annealing at 55°C for 1 min and polymerization at 72°C for 2 min. A final round of polymerization at 72°C for 10 min was performed at the end of the 35 cycles. Aliquots (10 µl) of PCR products with 5 µl loading buffer were loaded onto a 0.8% TAE agarose gel, stained with ethidium bromide and visualized on a UV transilluminator.

PCR products were extracted and purified using QIA quick gel DNA extraction kit protocol (QIAGEN Co.) and the purified PCR products ligated into pGEM-T Vector (Promega Co., USA) according to the manufacturer's instructions. 1 µl of ligation reaction was transformed into *Escherichia coli* strain DH5-α competent cells. DNA nucleotide sequences were determined by gel based sequencing at the International Livestock Research Institute sequencing unit in Nairobi, Kenya. Sequences were analyzed using various basic alignment search tools (BLAST) served at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/).

**Expression and purification of *T. parva* proteins**

After sequence verification, the cloned fragment or part of it was excised from pGEM-T by digestion with Hind III and XhoI and cloned into pET 28b, a bacterial based expression vector. The cloning sites used were generated by digesting with Hind III and XhoI. The constructs were transformed into *E. coli* BL21 (DE3). For expression using *E. coli* cells harbouring pET 28b with the cloned fragment, 500 ml of 2xYT bacterial broth was inoculated with 50 ml of overnight cultures of the respective *E. coli* transformed with homolog clones and grown in a 3 litre conical flask, in a 37°C rotatory incubator at 255 rpm (Becton Dickinson, Franklin Lakes, NJ, USA) to an optical density of 0.6 at 600 nm. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 2 mM and incubated for 24 h with time-lapse samples picked after 0, 3, 5 and 24 h. Bacterial cells were harvested, pelleted and weighed. Each batch of cells was then lysed with 5 ml of 8 M Urea, 0.1 M phosphate buffer at pH 8.0 per 1 g of pellet, by gently stirring overnight at 4°C. This was then spun down at 10,000 g in a J100 Sorval rotor to get rid of cell debris. The supernatant was then mixed with 2 ml of 50% Ni-NTA agarose affinity matrix for 1 h, and the resin washed three times with 8 M urea-100 mM $Na_2HPO_4$-50 mM NaCl at pH 8.0 and once with the same solution at pH 7.5. Elution of bound recombinant proteins was performed by addition of 50 mM ethylenediaminetetraacetic acid (EDTA). Eluted proteins were analyzed for purity and molecular mass on a 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blot analysis. The samples were then dialyzed against phosphate-buffered saline (PBS), passed through a 0.2 µm filter, and protein concentrations determined by a bicinchoninic acid protein assay (Pierce).

**Western blot analysis**

Expressed and purified proteins were confirmed using western blotting. 5µg of each sample was run on a 12.5% SDS-PAGE gel at 35 mA in a 1 X running buffer (25 mM Tris, 250 mM glycine (electrophoresis grade) pH 8.3, 0.1% SDS prepared from a 10x running buffer stock solution). The transfer of proteins onto nitrocellulose sheets was performed as described by Towbin et al. (1979). After electrophoresis, the gel was equilibrated in transfer buffer (10% methanol, 24 mM Tris, 194 mM glycine) for 30 min to avoid any change in its size during transfer. The samples were electro-transferred onto nitrocellulose paper (0.45 µm protan nitrocellulose, Schelcher and Schuell, Dassel, Germany) at 70 V for 1 h at 4°C, or 15 V overnight, in the transfer buffer. The blot was then stained with ponceau S (Sigma Aldrich, St. Louis MO, USA) to monitor the transfer, and then destained in transfer buffer and several rinses with water. The nitrocellulose filter was then blocked with blocking solution (TBS-Tween: 20 mM Tris-HCl, 200 mM NaCl (pH 7.4) containing 5% non-fat dry milk) for 1 h. The primary antibody, anti-His-tag protein (His-tag monoclonal antibody, ovagen; EMB Sciences, SanDiego CA, USA), was added to the blocking solution at a dilution of 1:2000 with 0.02% $NaN_3$ and incubated overnight. The next morning, the filter was washed 4 times with PBS/0.1% Tween 20, for 10 min each. The second antibody, rabbit anti-mouse IgG HRP conjugate (Amersham International PLC, Aylesbury Buckinghamshire, England), was added at a dilution of 1:1500 in blocking solution and incubated for 3 h. The filter was then washed two times with PBS/0.1% Tween 20, then twice with PBS. The blot was developed by the addition of the chromogenic substrate 3, 3-diaminobenzidine (DAB; Sigma) in PBS at a concentration of 1 mg/ml in the presence of 0.1% (v/v) hydrogen peroxide. The filter was rinsed rapidly with water and dried sandwiched between two Whatmann 3 mm filters.

**SDS-PAGE**

Total proteins as well as the purified *T. parva* proteins from the *E. coli* cells harbouring pET 28b with the cloned fragment were analysed by SDS-PAGE according to Laemmli (1970) using 12% polyacrylamide gels followed by staining with coomassie brilliant blue. Protein quantitative analysis was determined by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

**Transfection of iSF and COS-7 Cells**

iSF were immortalized by using standard procedures (Jha et al., 1998) with modifications (unpublished observations). iSF were transfected in 96-well plates with clones or pools of schizont cDNA (100 ng per well) by using FuGENE 6 (Roche Diagnostics, Mannheim, Germany) and cultured for 24 h. COS-7 cells were cotransfected with 100 ng of each clone per well or pooled together with 50 ng of pcDNA3 BoLA-N*00101 (Bensaid et al., 1991) or BoLA-N*01301 cDNA per well (Graham et al., 2006).

**IFN-γ ELISpot**

Transfectants were cocultured with schizont-specific CTL, and recognition was assessed by using an IFN-γ ELISpot assay (Taracha et al., 2003; Graham et al., 2006).

## RESULTS AND DISCUSSION

Eight *T. parva* homologs of apicomplexan antigens were isolated and sub-cloned. Recombinant proteins were generated using *E. coli* expression vector system. The expressed proteins were tested by ELIspot. Only the *T. parva* T-complex 1 protein zeta subunit ortholog was found to elicit $CD4^+$ response; none elicited $CD8^+$ response (Table 2 and Figure 1).

The *T. parva* ortholog of the zeta subunit of T-complex protein 1 (TCP-1) which plays a role in protein folding,

**Table 2.** Apicomplexan antigen homologs identified in *Theileria parva*.

| Putative antigen | Apicomplexan parasite | *T. parva* homolog ORF (bp) | *T. parva* homolog $CD4^+$ response | *T. parva* homolog $CD8^+$ response |
|---|---|---|---|---|
| 12D3 antigen | *Babesia* spp. | 1218 | – | – |
| 110kDa antigen | *Plasmodium knowlensi* | 936 | – | – |
| T-complex protein1 | *Plasmodium falciparum* | 489 | – | – |
| Apical membrane antigen (AMA-1) | *Plasmodium chaubadi* | 1425 | – | – |
| 22.4.1 protein | *Cryptosporidium parvum* | 576 | – | – |
| T-complex protein zeta subunit | *Plasmodium falciparum* | 1647 | + | – |
| T-complex protein 1, delta subunit | *Babesia microti* | 1479 | – | – |
| Ring-infected erythrocyte surface antigen | *Plasmodium falciparum* | 1710 | – | – |
| HSP-70 | *Plasmodium berghei* | 792 | – | – |

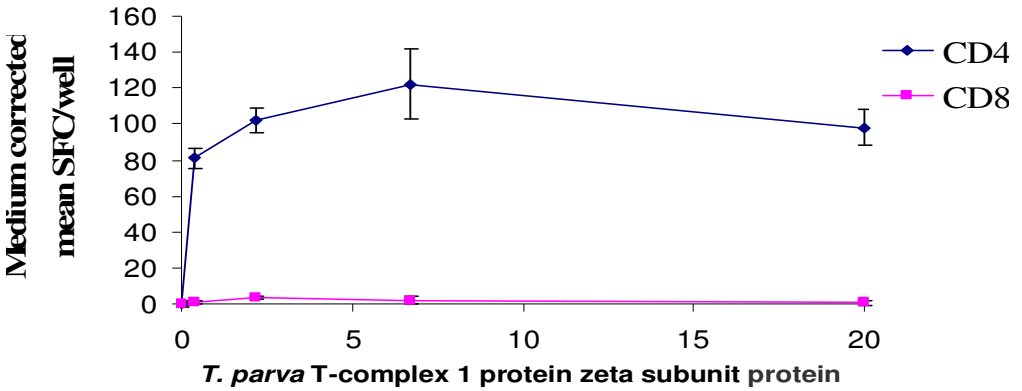


**Figure 1.** Response of *T. parva* specific $CD4^+$ and $CD8^+$ polyclonal T cells to recombinant *T. parva* T-complex 1 protein zeta subunit protein. *T. parva* specific $CD4^+$ and $CD8^+$ polyclonal T cell lines isolated from ITM immunised cattle BW014 were co-cultured with autologous monocytes and stimulated with a titration of truncated recombinant *T. parva* T-complex 1 protein zeta subunit protein. Responses are presented as mean numbers of spot-forming cells (SFC)/well.

assembly and transport has been previously reported (Tonukari and Kangethe, 2009a). The deduced amino acid sequence of the *T. parva* ortholog has a 55% identity and 77% similarity to the same protein in a number of eukaryotes such as *Danio rerio* (zebra fish) and man. T-complex protein 1 is a chaperonin-containing protein (Kubota et al., 1995) and is abundant in the eukaryotic cytosol. It is involved in the folding of actin and tubulin concomitant with ATP hydrolysis *in vitro.* One of the characteristics that distinguish TCP-1 from other chaperonins is its hetero-oligomeric nature, which in general comprises of eight different polypeptide species (Kubota et al., 1994).

The major mechanism responsible for the elimination of cells infected with the parasite is MHC-class 1-restricted (parasite specific) $CD8^+$ CTL activity. However, CTL activity requires the input of specific $CD4^+$ T-cell help for induction. Katzer et al. (2006) observed that protective immunity against *T. parva* has considerable impact on the emergence of targeted parasites following challenge but fails to prevent their differentiation into transmissible forms. Furthermore, the genotypic compositions of the transmitted parasites arising from a given challenge vary between immune individuals with distinct MHC phenotypes.

Five candidate vaccine antigens that are the targets of MHC class I-restricted $CD8^+$ CTL from immune cattle have been previously identified (Graham et al., 2006). Schizont-infected cell-directed $CD8^+$ cytotoxic T lymphocytes (CTL) constitute the dominant protective bovine immune response after a single exposure to infection. $CD8^+$ T cell responses to these antigens were boosted in *T. parva*-immune cattle resolving a challenge infection and when used to immunize naïve cattle, it induced CTL responses that significantly correlated with survival from a lethal parasite challenge. These data provide a basis for developing a CTL-targeted anti-East Coast fever subunit vaccine (Graham et al., 2006).

According to Graham et al. (2006), the ultimate vaccine against *T. parva* will almost certainly need to incorporate

multiple antigens and epitopes in order to confer protection in the genetically diverse outbred cattle population exposed to challenge by antigenetically diverse parasite populations in the field. Furthermore, the major challenge in the development of a vaccine against *T. parva* based on the induction of T-cell responses will be to generate responses that are effective against all parasite strains. The elicitation of $CD4^+$ T cell response by the *T. parva* T Complex Protein-1 zeta subunit homolog indicates that it is a candidate T-helper cell target antigen. Incorporation and further evaluation of this antigen for vaccine design in East Coast fever disease should be of interest.

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