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Original article

Babesia microti thioredoxin 3 is an effective antioxidant and involved in the response to antiprotozoal drugs

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A B S T R A C T

The intra-erythrocytic apicomplexan *Babesia microti* is the predominant pathogen that causes human babesiosis, an infectious disease that occurs worldwide. *B. microti* relies on the antioxidant including thioredoxin system to maintain the redox balance during the erythrocytic stage. In the present study, the full-length *B. microti* thioredoxin 3 (BmTrx3) gene was cloned, expressed *in vitro*, and its response to antiprotozoal drugs were tested. The full-length BmTrx3 was 663 bp and contained an intact open reading frame of 567 bp. The encoded polypeptide was 188 amino acids and the predicted molecular weight of the protein was 21.7 kDa. A conserved thioredoxin-like family domain was found in BmTrx3. The expression of BmTrx3 was upregulated on both the third and eighth day post-infection in mice, whereas expression was downregulated during the beginning and later stages. Western blot analysis showed that mouse anti-BmTrx3 serum could recognize the native BmTrx3 in parasite lysates and that the mouse anti-*B. microti* serum could recognize the recombinant BmTrx3 protein. Immunofluorescence microscopy showed that BmTrx3 localized in the cell cytoplasm of *B. microti* merozoites in *B. microti*-infected red blood cells. The results of bovine insulin reduction assay indicated the enzyme activity of the purified recombinant BmTrx3 protein. The anti-malaria drug chloroquine significantly inhibited the expression of BmTrx3, however, another anti-malaria drug qunine, and a known anti-babesiosis drug clindamycin, induced significantly higher upregulation of BmTrx3 mRNA. The results of the present study demonstrate that BmTrx3 is a functional enzyme with antioxidant activity and may be involved in the response of *B. microti* to anti-parasite drugs.

1. Introduction

Babesia microti is a protozoan parasite belonging to the phylum Apicomplexa that invades and replicates in the red blood cells of hosts, including rodents and human, causing the emerging zoonotic babesiosis (Brennan et al., 2016). The clinical manifestations of babesiosis infection range from subclinical form that involves symptoms likechills accompanied by headache, myalgia, anorexia, nonproductive cough, arthralgia, and nausea, to acute symptoms that might lead to death (Vannier and Krause, 2012). In humans, Babesiosis infection is majorly transmitted by hard ticks, but can also be encountered on blood transfusion. The majority of cases in the United States are caused by *B. microti* (Vannier et al., 2008). *B. microti* is also responsible for the sporadic human babesiosis cases in many regions of the People's Republic of China (P.R. China) including Heilongjiang, Zhejiang and Fujian provinces (Zhou et al., 2014). However, to date, no effective methods with clear control and prevention measures have been developed against babesiosis. On the basis of the recent emergence of tick-borne and transfusion-transmitted babesiosis, the identification of a drug target on *B. microti* and the development of effective drug treatment are urgent.

Thioredoxin system whicn includes thioredoxin (Trx), thioredoxin reductase (TrxR) and nicotinamide adenine dinucleotide phosphate (NADPH) has been identified in multiple eukaryotes and prokaryotes to

Abbreviations: Trx, thioredoxin; TrxR, thioredoxin reductase; NADPH, nicotinamide adenine dinucleotide phosphate; BmTrx3, *B. microti* thioredoxin 3; RACE, rapid amplification of cDNA ends; IFA, Immunofluorescence assay; IPTG, Isopropyl β-D-1-Thiogalactopyranoside; PBS, Phosphate-buffered saline; qRT-PCR, Quantitative real time PCR; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; LB, Luria-Bertani; Hoechst 33342, 2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole trihydrochloride trihydrate

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maintain the redox balance (Holzerova et al., 2016). Over a long period of evolution, the malaria parasite *Plasmodium*, as well as *Babesia*, have developed efficient antioxidant defense systems to cope with the oxidative stress during the erythrocytic stage of infection (Fabbri et al., 2013; Bosch et al., 2015). Since the lack of catalase and glutathione peroxidases, thioredoxin reductase has been confirmed with vital importance in the thioredoxin system and is essential for the survival of intraerythrocytic *P. falciparum* (Krnajski et al., 2002). The *Babesia* spp., like the malaria parasite *Plasmodium*, parasitize the RBCs of mammalian hosts and are particularly susceptible to oxidative challenge, therefore antioxidative proteins of the thioredoxin system represent promising targets for anti-parasitic drug design (Kanzok et al., 2000; Esmaeilnejad et al., 2012). Targeting PfTrx2 pharmacologically might interfere with protein secretion into the host cell and offers a new strategy for antimalarial drug discovery (Peng et al., 2015). However, u thioredoxins of *Babesia* spp. did not receive much attention as compared to malaria parasites. In the present study, we described the biochemical characterization of *B. microti* Trx3 (BmTrx3) gene and its role in the response to drugs was tested.

2. Materials and methods

2.1. Parasites

B. microti strain ATCC® PRA-99TM was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in our laboratory by serial passage in four-week old females BALB/C mice (SLAC Laboratory Animal Co., Ltd., Shanghai, China) using the method described elsewhere (Zhang et al., 2016).

2.2. The transcriptome sequencing of B. microti at erythrocytic stage

To characterize transcriptional activity, total RNA was sequenced by BGI (The Beijing Genomics Institute, Shenzhen, China) using Illumina Hiseq4000 as described previously (Zhang et al., 2016).

2.3. The cloning and sequence analysis of cDNA of BmTrx3

In the present study, BmTrx3 was selected from the transcriptome library obtained previously, for further investigation. The total RNA of *B. microti* merozoites was extracted using E.Z.N.A. Total RNA Kit II (Omega bio-tek, GA, USA) according to the manufacturer's instructions. For the synthesis of complementary DNAs (cDNAs), the reverse transcription PCR (RT-PCR) was performed using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the instructions. The partial coding sequence of BmTrx3 (GenBank: XM_021481756.1) was present in the genome of *B. microti* (Cornillot et al., 2012), however the complete coding sequence of BmTrx3 was not available. The rapid amplification of cDNA ends (RACE) was employed to generate the complete sequence of BmTrx3 using SMARTer RACE cDNA amplification kit (Clontech, USA) according to the manufacturer's instructions. Primer sets designed based on the sequence that was generated by RACE were used to amplify the full length open reading frame (ORF) of BmTrx3 as shown in Table 1. Then, the obtained PCR products were subsequently purified and ligated into the pMD-19T vector (TaKaRa) and confirmed by sequencing. Finally, the identified sequence was submitted to the GenBank.

The Genetyx software (Software Development Co., Ltd., Tokyo, Japan) was used for the analysis of BmTrx3 nucleotide and amino acid sequences. The Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was used for sequence alignment. Phylogenetic tree was constructed using ClustalW alignment and neighbor-joining method of the software Mega 6.06.

2.4. Prokaryotic (in vitro) expression and purification of recombinant BmTrx3 protein

In the present study, e *in vitro* xpression of recombinant BmTrx3 protein (rBmTrx3) was conducted using a technique described previously (Wang et al., 2016). Briefly, the ORF of BmTrx3 was amplified using the BmTrx3-ORF primer pairs (Table 1) and subsequently subcloned into the pET-30a(+) vector (Novagen, USA). After confirmed by sequencing, the recombinant expression plasmid pET-30a(+)-BmTrx3 was transferred into *Escherichia coli* BL21 (DE3) (Novagen, USA) and induced with the isopropyl β-D-thiogalactoside (IPTG).

The inclusion bodies of rBmTrx3 were denatured using 8 M urea prior to the purification using His-Trap FF Columns (GE Healthcare, USA) according to the manufacturer's instructions. The purified rBmTrx3 protein was washed off with elution buffer pH 8.0 (20 mM PBS, pH 7.4; 0.5 M NaCl; 20 mM imidazole and 8 M urea,) and refolded using re-naturing buffer (20 mM Tris-Cl, 500 mM NaCl, 1 mM glutathione [GSH], 0.1 mM oxidized glutathione [GSSG], pH8.0) containing different concentrations of urea (8, 6, 4, 3, 2, 0 M) as described previously (Narayan et al., 2000). Finally, the rBmTrx3 were dialyzed in phosphate buffered saline (PBS) for three times. All the dialysis processes were performed at 4 °C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to check the purity of the purified protein and a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA) was used to determine the concentration. A Pierce High Capacity Endotoxin Removal Spin Columns (Thermo Fisher Scientific, USA) was utilized to remove the endotoxin from protein samples used for *in vivo* trials. The purified protein was aliquoted and stored at −80 °C for further use.

2.5. Immune serum production

A polyclonal antibody against rBmTrx3 was obtained using the method described elsewhere (Hai et al., 2017) except rBmTrx3 was used in the present study. The serum was collected 14 days after the last immunization. Enzyme-linked immunosorbent assay (ELISA) was employed to assess the antibody titers following a protocol developed previously (Wang et al., 2016).

Anti- *B. microti* serum was collected from BALB/C mice 21 days post infection with 1×10^8 *B. microti*-infected RBCs (iRBCs) with parasitemia of 25%. The immune serum was aliquoted and stored at −80 °C until further use.

2.6. Western blot analysis of native and recombinant BmTrx3 protein

Western blot analysis was performed as described previously (Wang et al., 2017). Briefly, in the present study, samples including five batches of somatic extract of iRBCs (5, 6, 7, 8 days post-infection and non-infected erythrocytes) and the purified rBmTrx3 were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Then, primary antibodies, mouse anti-rBmTrx3 sera (1:200) or mouse anti- *B. microti* sera (1:200) were applied, respectively. Finally, a secondary antibody (horseradish peroxidase [HRP]-conjugated goat anti mouse IgG [dilutions 1:2000]) (Sigma-Aldrich, USA) was added and the proteins were detected using a Pierce DAB Substrate Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

2.7. Relative expression analysis of BmTrx3 post infection

The determination of BmTrx3 expression post infection was performed as described previously (Huang et al., 2017) except the 18 s ribosomal RNA of *B. microti* (18S) (GenBank: XM_021481625.1) was used as an internal reference in this study. Briefly, mice were injected with 1×10^8 iRBCs and the blood was collected from 1 to 10 days post-injection. Total RNAs were extracted from iRBCs at different post-

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