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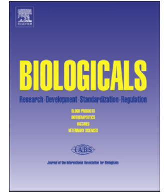

Serodiagnosis of *Toxoplasma gondii* infection in bovines from Kerala, India using a recombinant surface antigen 1 ELISA

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ABSTRACT

Data on the prevalence of toxoplasmosis in farm animals from India is scanty. Though a few reports exist on prevalence of toxoplasmosis in small ruminants, information on toxoplasmosis in large ruminants is virtually nonexistent from India. An antibody detection recombinant ELISA specific for *Toxoplasma gondii* was laboratory standardized using recombinant surface antigen 1 (SAG1) protein. A 958-bp truncated sequence coding for tachyzoite stage specific SAG1 protein was amplified and expressed in *Escherichia coli* BL21(DE3) cells. A high-level expression of the histidine-tagged thioredoxin fusion protein was obtained after 8 h of incubation. The recombinant protein was affinity purified by Ni-NTA agarose chromatography and characterized by SDS-PAGE and Western blot. Subsequently, the diagnostic potential of the recombinant protein was assessed with 258 cattle sera samples from field by a laboratory standardized recSAG1 ELISA. Sera from 71.8% of the cattle showed sero positivity for *T. gondii* specific IgG. The sensitivity and specificity of the recSAG1 ELISA were 84.38% and 87.88%, respectively in comparison to indirect fluorescent antibody test (IFAT). This is the first report on sensitive serodetection of *Toxoplasma* infection in bovines from India.

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1. Introduction

Toxoplasma gondii, an apicomplexan obligate parasite, has global distribution with minimal host specificity. The unique ability of *T. gondii* to infect any nucleated cell of vertebrates and its subsequent encystment in various host tissues has assigned it a special zoonotic importance. Toxoplasmosis is a serious issue for animal and human health, especially for immunocompromised or pregnant individuals or subjects undergoing immunosuppressive therapy. The economic impact of the disease from India is difficult to assess because information on prevalence of the parasite among various livestock species is scanty [1–3] and no report is available on prevalence of *T. gondii* in bovines from India. The cattle population in India is the largest in the world (199 million), which contribute significantly to the annual milk production of 122 million tonnes that is the highest annual milk production of any country. Though several parasitic infections are responsible for poor livestock health and associated production losses, baseline prevalence data for many of them, including *T. gondii* infection in bovines, is absent from India. *T. gondii* is difficult to detect using standard parasitological techniques because none of the biological stages are demonstrable in the blood and/or natural excretions or secretions. PCR is useful in detection of *T. gondii* during the acute phase of infection but fails to detect infection in chronic cases due to the lack of *T. gondii* DNA in blood [4]. Serological tests, therefore, play a crucial role in diagnosis of toxoplasmosis. Serodiagnosis of infection relies on detection of *Toxoplasma* specific IgG and IgM molecules in blood or CSF. Several serological tests using either particulate tachyzoites or native soluble antigens have been developed, however, these tests though sensitive suffers from limitation of specificity [3]. For a high throughput sero-surveillance of toxoplasmosis, continuous availability of a native diagnostic protein in abundant quantities is a constraint and, therefore, a purified recombinant diagnostic protein based test is desirable. The surface antigens of *T. gondii* are relevant for both immunodiagnosis and immunoprophylaxis owing to their initial presentation to the

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host immune system. Among the various surface antigen molecules, surface antigen 1 (SAG1) is the most immunodominant and stage-specific antigen present on tachyzoites [5].

The present communication deals with the primer-directed amplification of the open reading frame (ORF) of SAG1 gene of *T. gondii*, RH strain, followed by the cloning and expression of recombinant SAG1 in a heterologous prokaryotic system. In order to generate baseline data on the prevalence of toxoplasmosis in Indian cattle populations, an ELISA was laboratory standardized with the purified heterologously expressed recombinant SAG1protein. Bovine sera samples were procured from Kerala, India. There are about 2.1 million cattle extant in Kerala, the southernmost state of India, and 84.3% of cows are crossbred (as per 2003 animal census); in addition to resident cattle, movement of cattle from the adjoining states occurs. The sensitivity and specificity of the rSAG1-ELISA was determined with reference to the benchmark indirect fluorescent antibody test (IFAT).

2. Materials and methods

2.1. In vivo propagation of T. gondii tachyzoites in mice

The animal experimentations were conducted in compliance with the ethical considerations and guidelines issued by CPCSEA and Institutional Animal Ethics Committee (IAEC) on laboratory animals. Tachyzoites of *T. gondii* RH strain maintained as cryostock in the Protozoology Laboratory, Division of Parasitology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India were propagated in Swiss-albino mice through intra-peritoneal inoculation and used in the study.

2.2. Bacterial strain and growth conditions

Escherichia coli BL21(DE3)pLysS (Novagen) cells were used as prokaryotic bacterial host for experiments pertaining to plasmid DNA manipulation. The cells were grown in either Luria Bertani (LB) broth or LB agar, supplemented with ampicillin (100 mg/ml) and chloramphenicol (32 mg/ml).

2.3. PCR amplification of SAG1 gene

For PCR amplification of the SAG1 coding sequence, complementary DNA (cDNA) was synthesized using oligo dT primer from the total RNA extracted from tachyzoites of *T. gondii* following a standard protocol [7]. PCR primers for amplification of SAG1 were designed incorporating suitable restriction sites based on the sequence information generated earlier [8]. A truncated 958 bp SAG1 fragment was PCR amplified using the forward primer 5′-GGTTGTATGTCGGTTTCGCTGCAC-3′ and a reverse primer 5′-CGTCAAGCTTCAGCCGATTTTGCTGAC-3′. The reverse primer contained a *Hind III* restriction site to facilitate directional cloning into the expression plasmid vector pET-32b(+) (Novagen). The PCR conditions were optimized in 25 μl reaction volume containing the following: 30 ng cDNA; 20 pmol each forward and reverse primer; *Pfu* polymerase buffer containing 1.5 mM $MgCl_2$; 200 μM dNTP mix; and, 1U *Pfu* polymerase. Cycling conditions were initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 1 min. Thereafter, a final elongation was given at 72 °C for 10 min. The amplification was confirmed by visualization of the specific 958 bp product in relation to a marker under UV light following electrophoresis on 1.25% agarose gel stained with ethidium bromide. Purification of the PCR product was done using commercially available gel extraction kit (Qiagen, Germany), following the manufacturers protocol.

2.4. Construction of recombinant plasmid and transformation of competent E. coli cells

The purified DNA fragment (958 bp) was double digested with *Bam*HI and *Hin*dIII at 37 °C for 4 h and ligated to the corresponding cloning sites of pET-32b(+) vector (following standard protocol [6]). The competent *E. coli* BL21(DE3)pLysS (Novagen) cells were transformed with recombinant plasmid and plated on LB agar containing ampicillin. Confirmation of the recombinant clones was done by colony PCR as well as restriction enzyme analysis for release of insert following standard protocol [6].

2.5. Expression of recombinant protein

Five positive recombinant clones were selected randomly for induction. The colonies were grown overnight in 5 ml of LB broth containing ampicillin at 37 °C in a shaking water bath. One hundred microlitres of the overnight grown culture was isolated and further grown in 10 ml of fresh LB broth containing ampicillin at 37 °C with constant shaking until mid-log phase (for 4 h when the OD reached to 0.6). One ml of the culture was separated from each tube and kept as an uninduced control. To the rest of the culture, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM and incubated at 37 °C with constant shaking at 140 rpm. One millilitre of the induced culture was collected at hourly interval starting from 3 h onwards and the cells were pelleted by centrifugation at 13,800 × g in a refrigerated centrifuge at 4 °C.

2.6. Protein harvest and purification of recombinant His6-tagged fusion protein

Purification of expressed His-tagged thioredoxin fusion proteins was carried out using Ni-affinity chromatography following the manufacturer's protocol (Qiagen, USA). In brief, cells from 1 L of the induced culture were pelleted and resuspended in 15 ml of lysis buffer containing 8 M urea (pH 8.0). The cell suspension was kept at room temperature for 2 h on a rotatory shaker with intermittent vortexing. Following lysis, the debris was pelleted by centrifugation for 10 min at 7600 × g and the clarified supernatant was transferred to a clean tube. Then 800 μl of Ni-NTA agarose slurry (Qiagen, Germany) containing 15 mM imidazole (Amresco, USA) and 20 mM β-mercaptoethanol (Amresco, USA) was mixed thoroughly with the supernatant and kept on a rotatory shaker for 1 h with intermittent mixing. The lysate-resin mixture was then loaded on to an empty 5 ml polypropylene column (Qiagen, Germany) equilibrated with 1 × Tris-phosphate buffer (pH 8.0). The flow-through of the column was collected. The column was washed with 15 ml of wash buffer (pH 7.0) to which 5 mM imidazole (pH 7.0) was added and finally the bound protein was eluted as 500 μl fractions with 4 ml of elution buffer (pH 4.2–4.5). For renaturation, the His-tagged thioredoxin fused rSAG1 was diluted to a final concentration of 100 mg/ml in dilution buffer (pH 8.0) containing 50 mM sodium dihydrogen phosphate and 100 mM sodium chloride. The diluted sample was dialysed first against buffer I (urea 0.5 M, Tris–HCl (pH 8.0) 20 mM, EDTA 1 mM) followed by buffer II (Tris–HCl (pH 8.3) 20 mM, EDTA 1 mM, reduced glutathione 2 mM, oxidized glutathione 0.2 mM) at 4 °C for 24 h. About 30 ml of dialysed sample was concentrated in a dialysis sac (10 kDa cutoff) using solid PEG-20,000 to one-third of its original volume. The resultant rSAG1 was designated as refolded rSAG1. Any debris formed during the renaturation was removed by centrifugation at 8200 × g for 10 min in a refrigerated centrifuge. Concentration of the purified recombinant protein was assayed by modified Lowry protein assay kit (Pierce, USA) according to the manufacturer's protocol. The protein was aliquoted in 0.5 ml

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