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Research paper

Design and characterization of a recombinant colorimetric SAG1–alkaline phosphatase conjugate to detect specific antibody responses against *Toxoplasma gondii*☆

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ABSTRACT

The purpose of this study was to design a novel *in vitro* tool by using recombinant protein technology to detect specific antibody responses against *Toxoplasma gondii* in a simple, rapid and highly sensitive reagent. The surface *T. gondii* SAG1 protein is an important immunodominant target, which provides a great interest as a diagnostic antigen. To further exploit its immunodetection capacity, in the present study, the full length *sag1* gene was inserted into the pLIP6 prokaryotic expression vector so as to produce a SAG1 antigen genetically fused to the bacterial alkaline phosphatase (AP). After expression optimization, the recombinant fusion protein folded correctly in soluble form in the periplasmic space and preserved both the AP enzymatic activity and the SAG1 immunoreactivity. Subsequently, direct-ELISA and dot-blot immunoassays were designed, using crude preparation SAG1–AP conjugate, to explore its value in serodiagnosis of human toxoplasmosis. We demonstrate that the recombinant SAG1–AP can detect specific *T. gondii* antibodies in one-step procedure and can successfully discriminate between *T. gondii* immune and non-immune patients, in agreement with the standard gold test. In conclusion, the present study shows that the genetic fusion protein provides a new tool for one-step *T. gondii* immunodetection, which was easily, quickly and reproducibly produced as homogeneous bi-functional reagent. Thus, this recombinant immunoconjugate is a promising marker for *Toxoplasma* serodiagnosis, requiring further evaluation on a larger series and could provide the basis for direct antibody capture enzyme-immunoassay for specific immunoglobulin M and G detection.

1. Introduction

Toxoplasma gondii (*T. gondii*) is an intracellular protozoan parasite that infects a large variety of domestic and wild mammals, including humans. In humans, infection with *T. gondii* is generally asymptomatic but during pregnancy, it can result in congenital infection with severe sequelae or late onset eye disease and is a frequent cause of encephalitis in severely immune suppressed patients with AIDS (Araujo and Remington, 1987). Toxoplasmosis is also a serious complication following organ transplantation (Aubert et al., 1996).

So, detection of *T. gondii* infection by sensitive and specific methods is a key step towards treating and managing patients with suspected toxoplasmosis. Serological assays are the initial and primary tests routinely used for toxoplasmosis diagnosis (Montoya, 2002). Most of the commercially available kits detect specific anti-*Toxoplasma* immunoglobulins by means of native antigens originating from *T. gondii*. The main disadvantage

Abbreviations: T. gondii, *Toxoplasma gondii*; SAG1, surface antigen-1; AP, alkaline phosphatase; IPTG, isopropyl-β-D-thiogalactoside; PBS, phosphate-buffered saline; NBT, nitro-blue tetrazolium; BCIP, 5-bromo-4-choro-1-indolylphosphate; SDS-PAGE, sodium dodecyl sulfate poly-acrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay

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of using the parasite whole soluble extract as the antigen in serology tests is its inconstant quality. The use of recombinant proteins obtained *via* molecular biology is an alternative for the detection of serum antibodies that allow better standardization of the immunoassays and may enhance the sensitivity of an antibody-based assay (see review, Kotresha and Noordin, 2010). Besides, current detecting methods using enzyme-labeled conjugates present several advantages such as, stability, safety of the reagents, intrinsic amplification, and the various methods available to measure enzyme activity (Guesdon, 1992). However, the immunoconjugates are obtained by chemical labeling, which present different drawbacks, such as a random cross-linking chemical reaction, partial denaturation of both components and heterogeneity of coupling (non-uniform antibody or antigen/enzyme stoichiometries) (Porstmann and Kiessig, 1992; Avrameas, 1983). To overcome these problems, while preserving the advantage of using enzyme-linked proteins, gene fusion technology which allows direct production of enzyme tagged recombinant proteins in a bacterial expression system (Lindbladh et al., 1993) might constitute an interesting approach. *Escherichia coli* (*E. coli*) alkaline phosphatase (EC 3.1.3.1) (AP) which displays substrate specificity similar to the calf intestinal enzyme was efficiently expressed in *E. coli* when coupled at its amino terminus to different antibody fragments (Carrier et al., 1995; Muller et al., 1999; Mousli et al., 2007) or antigens (Gillet et al., 1993; Chanussot et al., 1996; Butera et al., 2003) without loss of activity. In addition, AP and AP-fusions are secreted into the bacterial periplasm (Michaelis et al., 1983); thus, disulfide bonds required for target proteins can be formed and fusion proteins readily extracted from bacteria by periplasmic lysis using cold osmotic shock. Finally, multiple chromogenic and fluorogenic substrates exist, allowing direct quantification of the amount of fusion protein bound to a target protein with high sensitivity (Brickman and Beckwith, 1975). Thus, recombinant tracers constitute an alternative way of providing homogeneous and stable immunoconjugates for use in diagnostic assays.

The surface antigen 1 (SAG1, also named P30) is the major *T. gondii* component being expressed on the surface of intra- and extra cellular tachyzoïtes (Dubremetz et al., 1985) and was suggested to be the most immunogenic constituent of the invasive form (Rodriguez et al., 1985). It is a non-variant antigen which is well conserved immunologically and in amino acid sequence levels (Nagel and Boothroyd, 1989). *T. gondii* SAG1 gene has been expressed in various heterologous systems and the recombinant antigen was widely used in *Toxoplasma* serodiagnosis (Kotresha and Noordin, 2010). Recently, we have demonstrated that the recombinant SAG1 antigen, produced in bacterial system, shows a high capacity to screen anti-*Toxoplasma* IgG antibodies in sera as well as in saliva samples from pregnant women using ELISA system (Chahed Bel-Ochi et al., 2013). In the present study, to further exploit its immunodetection capacity, we proposed to design a recombinant SAG1 protein genetically fused to *E. coli* alkaline phosphatase for use in rapid, sensitive and specific *Toxoplasma* serodiagnosis tests. After bacterial expression optimization, the bi-functionality of the SAG1–AP immunoconjugate was characterized, and then it was applied in one-step detection immunoassays such as direct-ELISA and dot-immunoblotting for *Toxoplasma* serodiagnosis.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* DH5α strain (Invitrogen, Carlsbad, CA) was used for the preparation of plasmids and cloning. The *E. coli* XL1-Blue (Stratagene, La Jolla, USA) and W3110 strains (American Type Culture Collection, no. 27325) were applied to the expression of recombinant fused antigen.

The pLIP6-GN vector was kindly provided by Dr Ducancel F. (Laboratoire d'Ingénierie des Anticorps pour la Santé CEA-Saclay, France). This vector presents a *Sfi*I/*Not*I cloning site between codons coding for residues +6 and +7 of mature alkaline phosphatase. In the empty pLIP6-GN vector, the *AP* gene is out of frame and advantageously restored upon cloning of the foreign DNA insert, permitting a visual selection of blue cloned colonies on BCIP agar plates (Gillet et al., 1992). The presence of both the signal peptide and the first six amino acid residues of AP facilitate export of the hybrid into the periplasmic space of *E. coli*, after induction of the *tac* promoter with IPTG.

2.2. Construction of sag1–AP fusion gene expression vector

The DNA sequence of the gene encoding the *T. gondii* SAG1 antigen was obtained from the GenBank (accession no. X14080). The entire *sag1* gene was amplified by PCR from the pET22-*sag1-His* plasmid (Chahed Bel-Ochi et al., 2013) with the following primers: P1: 5′-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCA**TCGGATCCCCCTCTTGTTG**-3′ and P2: 5′-ATGATGTGCGGCCGC**CGCGACACAAGCTGCCG**-3′, which introduced the underlined *Sfi*I and *Not*I recognition sites at the 5′ and 3′ ends of the PCR product, respectively. The bold text within the primer sequences indicates complementarity to the nucleotide sequences of the *sag1* gene, whereas 5′ overhanging ends of primers were designed to facilitate cloning.

Specific 867 bp PCR product was digested with *Sfi*I and *Not*I restriction enzymes (Amersham Biosciences, France) and then, isolated from an agarose gel band using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, France). This DNA fragment was ligated into pLIP6-GN vector previously linearized with the same enzymes and used to transform *E. coli* DH5α strain. The transformed bacteria were seeded in Luria-Bertani (LB)-Agar plates, containing 100 μg/ml ampicillin, 100 μM IPTG (Isopropylthio-β-galactoside, Sigma-Aldrich, Inc., Germany), and 40 mg/ml BCIP (5-Bromo-4-chloro-3′-indolyphosphate, Sigma-Aldrich, Inc., Germany). The phosphate in the media inhibits the expression of the constitutive DH5α *AP* gene, while the IPTG induces the *tac* promoter, allowing the expression of AP fusion proteins that hydrolyses the BCIP substrate resulting in blue colonies (Boulain and Ducancel, 2004). Three independently positive ampicillin-resistant blue colonies containing *sag1* gene fragment were selected and analyzed by sequencing using the ABI PRISM Cycle Sequencing kit (ABI, Applied Biosystems).

2.3. E. coli cultures and fusion protein expression

A single clone pLIP6-*sag1–AP* was used to transform fresh competent *E. coli* XL-Blue and W3110 strains (Sambrook and Russel, 2001). Colonies were grown in LB medium supplemented