Biotechnology Reports 23 (2019) e00360

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Enhancing the detection of *Toxoplasma gondii via* an anti-SAG1 scFv-alkaline phosphatase immunoconjugate

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ARTICLE INFO

Article history:
Received 2 May 2019
Received in revised form 29 June 2019
Accepted 30 June 2019

Keywords:
Alkaline phosphatase
Fluorescent immunodetection
Recombinant conjugate
Single-chain antibody fragment variable (scFv)
Toxoplasma gondii

ABSTRACT

The purpose of this study was to implement a fluorometric method for enhancing the detection sensitivity of *Toxoplasma gondii* in biological fluids. To address this challenge, we designed and produced a recombinant immunoconjugate tool based on a single-chain antibody fragment anti-*T. gondii* SAG1 antigen (scFvSG15) genetically fused to the bacterial alkaline phosphatase (AP) using 4-methyl-umbelliferyl-phosphate as fluorogenic substrate. The anti-SAG1 scFv-AP conjugate was fully bifunctional and was used successfully in different assays including immunoblot, fluorometric ELISA and direct immunofluorescence. The fluorometric immunoassay afforded an extremely low detection limit (1 tachyzoite/well), which was in agreement with the real-time PCR control test. The immunofluorescence imaging has provided captivating visual evidence of *T. gondii* detection. These results strongly suggest that the recombinant anti-SAG1-AP conjugate generated here might serve as useful and highly sensitive immunoassay probe to direct detect *T. gondii* in a one-step procedure, opening up new perspectives for diagnosis of toxoplasmosis.

1. Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular pathogen within the phylum Apicomplexa. This protozoan parasite is one of the most widespread, with a broad host range including many birds and mammals and a geographic range that is nearly worldwide [1]. Infection in humans is usually asymptomatic in immunocompetent hosts. However, this infectious disease can cause congenital toxoplasmosis when maternal infection occurs during pregnancy and severe infections in immunocompromised patients (mainly transplant and AIDS patients) [2,3]. In addition, toxoplasmosis is responsible for major economic losses in most classes of livestock through early embryonic death, abortion, stillbirth, and neonatal death [4].

So, detection of *T. gondii* infection by sensitive and specific methods is a key step towards treating and managing patients with suspected toxoplasmosis. There are a various of different strategies used for decades to diagnose the Toxoplasmosis disease, including serologic tests, amplification of specific Toxoplasma DNA, histologic detection of the parasite and/or its antigens, or by isolation of the organism, as has been reviewed by investigators [5,6,7]. However, each assay has its own set of advantages and disadvantages regarding cost, sensitivity, specificity, ease of use, or technical expertise [8]. Currently, numerous serological kits are available and widely used to detect Toxoplasma-specific antibodies in the serum samples of infected patients. Unfortunately, they may be inconclusive or unreliable in some specific clinical situations, such as patients receiving immunosuppressive therapy or certain cases of congenital toxoplasmosis infection [7,9]. In taking all these considerations into account, a rapid method for the direct detection of whole *T. gondii* parasite or antigenic components in body fluids or tissues might offer a valuable aid for rapid and specific diagnosis of human Toxoplasmosis.

Since their discovery by Köhler and Milstein in 1975, monoclonal antibodies (mAbs) have proven to be effective biological reagents in several areas and more particularly in the immunodiagnostic of various microbial or parasitic diseases or as reagent to directly demonstrate the causative agent [10]. Due to their high specificity and selectivity, today, mAbs are used in a variety of medical diagnostic applications as immunoassays, immunohistochemistry, western blotting, and magnetic cell sorting or flow cytometry. Currently, the analyte detection in immunoassays is carried out with primary or secondary antibodies that are chemically cross-linked with sensitive reporter molecules, such as colorimetric enzymes and fluorescent/luminescent proteins [11]. However, the conventional chemical

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https://doi.org/10.1016/j.btre.2019.e00360

conjugationapproach present some drawbacks, such as a random linking chemical reaction that produce heterogeneous complexes with different coupling molar ratios and leads to side reactions that damage the combining site and reduce assay sensitivity [12]. To address these problems, recombinant gene fusion techniques have provided new facilities and have opened a path for preparing fusion proteins as single molecular species with a definite molar ratio [13]. In this context, several attempts to produce recombinant bifunctional molecules in which the variable domains of an antibody were expressed as a single-chain antibody (scFv) genetically linked to a protein tracer have been successfully reported to develop robust immunoassays [14,15,17]. This approach presents several attractive advantageous. First, it allows to yields a homogeneous and stable bifunctional product with reduced size. Next, Due to the reduction of steric hindrance, the immunoconjugate should be able to bind epitopes with poor accessibility more efficiently. Lastly, it can be produced in large quantity in bacterial expression system at low cost [18,19].

The level of sensitivity of the immunoassay depends on a number of factors especially the enzyme marker and the substrate used. Varieties of enzymes are used as markers in immunoassays; and the most commonly conjugated are horseradish peroxidase, alkaline phosphatase (AP), β-galactosidase, urease and glucoamylase [11]. AP is frequently used, due to its number of advantages: its high catalytic activity, good enzymic stability, high affinity and high-turnover for a large range of substrates, and easy conjugation to antibodies at cost efficient way [20]. In addition, variety of chromogenic and fluorogenic AP substrates are available, allowing direct quantification of the amount of immunoreagent bound to a target protein with high sensitivity, and are also nontoxic and relatively stable reagents [21]. P-nitrophenyl-phosphate (PNP), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) are a commonly used chromogenic substrate systems for AP reporter enzyme and the 4-methyl-umbelliferyl-phosphate (4-MUP) is the most popular used from a range of fluorogenic substrates [22].

In this study, we sought to design of a novel class of a recombinant immunotracer to direct detect and quantify *T. gondii* parasites, using genetic engineering approaches. For this purpose, an scFvSG15 directed against *T. gondii* major surface antigen (SAG1) was genetically fused to alkaline phosphatase derived from an *Escherichia coli* (*E. coli*) strain into a soluble expression vector pLIP6-GN [23]. After bacterial expression optimization and purification process on Protein-L matrix, we illustrated the benefit of this bioconjugate system in a number of common assays including immunoblot, ELISA and direct immunofluorescence assay for a one-step specific detection of the *T. gondii* parasites measured fluorometrically.

The antigen target, the surface antigen 1 (SAG1, also named P30), is the first component to contact with the host cells and is the major and the important immune protein which provides a strong humoral and cellular immune response against the invasive tachyzoites [24]. In such a context, SAG1 has been widely studied as a vaccine candidate in animal models [25]. In addition, SAG1 is considered as an important candidate for the development of effective diagnostic reagents. Indeed, SAG1 is one of the first antigens recognized by Toxoplasma-specific IgM antibodies, who are one of the most important serological markers to identify acute toxoplasmosis [26], and is suitable for detecting anti-SAG1-specific IgG to determine immune status and susceptibility to Toxoplasma infection during pregnancy [27]. Furthermore, this molecule belongs also to a group of excreted-secreted antigens and has been evaluated as diagnostic marker that can be detected, by Western blot, in cerebrospinal fluids and serum of patients, during toxoplasmosis [28]. Consequently, because of his direct accessibility to the immune system, SAG1 represent the candidate target for the direct detection of the parasite.

2. Materials and methods

2.1. Parasites and antigens preparation

T. gondii RH strain tachyzoites were maintained by serial passage in Swiss mice for 48–72 h. The parasites were collected by peritoneal exudate washing with phosphate-buffered saline (PBS, 10 mM sodium phosphate containing 0.15 M NaCl, pH 7.2) as previously described [29], counted and stored at 10^7 tachyzoites/ml/aliquots at −80 °C until use.

Toxoplasma Lysate Antigen (TLA) was prepared according to the method described before [30] with a slight modification. Briefly, purified tachyzoites were sonicated for 4 cycles of 1.0 A/min, for 2 min with 20 s intervals, in lysis buffer (1.5 mM Tris-HCl at pH 7.6, 0.15 mM EDTA, 4% glycerol and 0.8% SDS) supplemented with protease inhibitor mixture (Product N°. S-8820, Sigma-Aldrich Co., St. Louis, MO, USA) and then centrifuged at 6000 rpm for 15 min. The supernatant was collected and filtered through a 0.22-μm pore filter (Sartorius-Minisart). The TLA concentration was determined using the BCA assay kit (Pierce, Rockford, IL), adjusted to 1 mg/ml with physiological saline and then stored at −20 °C until further use.

2.2. Bacterial strains and plasmids

The *E. coli* Top10 strain (Invitrogen, Carlsbad, CA) was used in cloning work. The *E. coli* W3110 strain (American Type Culture Collection, N°. 27325) was applied to the expression of recombinant fusion protein.

The pLIP6-GN plasmid was kindly provided to us by Dr Frédéric Ducancel (Department of ImmunoVirology, CEA centre, Fontenay-aux-Roses, France). This vector presents successively in the open reading frame the AP signal sequence and a *Sfi*I/*Not*I cloning site located between codons +6 and +7 of the *E. coli AP* gene, thus enabling the recombinant proteins to be directed to the periplasmic space and folding into their native conformation, after induction of the *tac* promoter with isopropyl-β-D-thiogalactoside (IPTG) [23].

2.3. Construction of a plasmid encoding an scFvSG15-AP conjugate

The scFvSG15 anti-SAG1 selected for this study was derived from a murine hybridoma secreting well-characterized mAb 4F11E12 (IgG1, κ chain). 4F11E12 recognizes a highly conserved conformational epitope on the surface of the D1 domain in native *T. gondii* SAG1 antigen. The Poly(A)$^+$ RNA sequences encoding the heavy-chain (V_H) and the light-chain (V_L) fragments of Fab 4F11E12 were determined by cloning, sequencing of cDNAs and the deduced amino acid sequences have been stored in the Brookhaven Protein Data Bank under the accession code **1YNT_B** and **1YNT_A**, respectively [31]. The synthetic gene scFvSG15 was chemically synthesized (GeneArt-Life Technologies, Germany), in which the V_H domain was fused to the V_L domain through a flexible linker of 15 residues $(Gly_4Ser)_3$ and harbored into pMA (AmpR) cloning vector (unpublished data), was generously provided by Dr Matthieu Juste (UMR-INRA 1282 Infectiologie et Santé Publique, University of Tours, France).

The entire *scFvSG15* gene was amplified by PCR from the pMA-scFvSG15 plasmid using primers: SG1FwSfi (5'-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCC**CAGGTTCAGCTGCAGCAGA**-3') and SG1RvNot (5'-GAGTCATTCTGCGGCCGC**GCTAGCACCACCGCCTTTAATTTCCA**-3'). The SG1FwSfi and SG1RvNot have introduced *Sfi*I and *Not*I recognition sites (underlined) at the 5 and

3 ends of the PCR product, respectively, for directed cloning into vector. The bold text within the primer sequences indicates complementarity to the nucleotide sequences of the *scFvSG15* gene.

The amplified fragment was cut with the restriction enzymes *Sfi*I and *Not*I (Roche, Mannheim, Germany), inserted into the pLIP6-GN vector cleaved with the same enzymes and used to transform calcium chloride-competent *E. coli* Top10 strain. All standard molecular biology procedures were performed as described [32].

The positive recombinant clones were directly screened by visualizing blue-white colonies on the Luria-Bertani (LB)-Agar plates supplemented with 100 μg/ml ampicillin (Amp), 100 μM IPTG (Sigma-Aldrich), and 40 mg/ml BCIP (Sigma-Aldrich). The ampicillin-resistant clones expressing the AP fusion protein hydrolyzed the BCIP substrate, resulting in blue colonies [23]. Three positive clones containing *scFvSG15* gene fragment were selected and analyzed by sequencing using the ABI-PRISM Cycle Sequencing kit (ABI, Applied Biosystems 3500 genetic analyzer).

2.4. Site-directed mutagenesis

A mutagenesis approach to confer Protein-L binding ability to any scFv for affinity purification was recently described by Lakhrif and co-workers [33]. Based on that, single mutation in the framework region (FR-1) of scFvSG15 κ-V_L domain was introduced by PCR amplification of the pLIP6-scFvSG15-AP vector using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and the following complementary mutagenic primers permitting amino-acid substitution from threonine to proline at position 8 (T8P) on the κ-V_L (the mutated nucleotides are underlined): VLT8PFor: 5'-CAGGTTACCCAGACC**C**C**G**TCTAGCCT-GAGC-3' VLT8PRev: 5'-GCTCAGGCTAGA**C**G**G**GGTCTGGGTAACCTG-3'. The PCR reaction was performed with the *Pyrococcus furiosus Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) as follows: 94 °C for 30 s, 55 °C for 1 min; 68 °C for 8 min, for 14 cycles. After digestion by the *Dpn*I restriction enzyme (New England Biolabs), the mutated gene construct carried in the pLIP6-scFvSG15-AP vector was subcloned into competent *E. coli* Top10. Finally, the mutation was confirmed by DNA sequencing. The obtained construction is named pLIP6-scFvSG15(V_LT8P)-AP.

2.5. Expression and purification of the fusion protein

Fresh competent *E. coli* W3110 strain were transformed with recombinant plasmids pLIP6-scFvSG15-AP and pLIP6-scFvSG15 (V_LT8P)-AP. Of each construction, single clone was picked and cultured in LB medium at 37 °C supplemented with 100 μg/ml Amp. When bacteria were grown until A_{600} reached 0.6-0.8 absorbance units, protein expression was induced by adding 0.5 mM IPTG and the temperature was shifted to 16 °C for a further 18 20 h with shaking at 200 rpm.

After expression, periplasmic proteins were extracted by cold osmotic shock protocol as previously described [34] with a slight modification. The bacterial culture was harvested by centrifugation (1500 rpm/10 min/at 4 °C) and the periplasmic extract was then prepared by resuspending the pellet in 1:20 vol of ice-cold TES buffer (300 mM Tris-HCl, pH 8, 1 mM EDTA, 20% Sucrose) containing 10 μg/ml lysosyme and incubated on ice for 30 min. The re-suspended extract was collected by centrifugation at 9500 rpm for 30 min at 4 °C to obtain the supernatant containing recombinant fusion proteins. The periplasmic proteins were dialyzed overnight against Tris Buffered Saline (TBS, 20 mM Tris-HCl, 150 mM NaCl at pH 7.4).

For purification steps, each construction of the scFv-AP fusion protein periplasmic extract was clarified through a 0.45-μm pore filter membrane and then was applied onto a column of Protein-L-Agarose (0.5 ml; Pierce, USA) pre-equilibrated in standard buffer following the manufacturer's recommendations. The column was washed with 10 ml of TBS and the recombinant protein was eluted in 0.5 ml fractions with 0.1 M glycine (pH 2) and neutralized immediately with 1 M Tris-HCl (pH 9, 50 μl). Elution fractions containing the scFv-AP were pooled, dialyzed against TBS at 4 °C, and quantitated using BCA assay kit.

2.6. Analysis of scFv-AP fusion proteins by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Analysis of the purity and integrity of the purified recombinant fusion proteins was performed *via* homogeneous 12% SDS-PAGE with subsequent coomassie blue staining and two Western blots. They were processed as described before [19]. For the first blot, the recombinant proteins were visualized using anti-bacterial AP mAb (Product N°. B-6804, Sigma-Aldrich) and peroxidase-labeled anti-mouse IgG conjugates (Product N°. A-4416, Sigma-Aldrich), and developed by enhanced chemiluminescence (ECL) HRP substrate (Amersham). For the second, the nitrocellulose (NC) membrane (Amersham Biosciences, France) was directly revealed using the BCIP/NBT AP substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, containing 0.3 g/l NBT and 0.15 g/l BCIP) to visualize the scFv-AP fusion protein.

2.7. Functional tests of the scFv-AP immunoconjugate

2.7.1. Direct immunoblot assay and detection limits of TLA

Five micrograms of TLA were subjected to 12% SDS-PAGE conditions (lacking β-mercaptoethanol) to separate proteins [32]. After electroblotting onto a NC membrane and a blocking step using 5% (w/v) skim milk in TBS-T (TBS containing 0.1% Tween-20), the membrane was directly incubated with purified recombinant scFvSG15(V_LT8P)-AP fusion protein at room temperature (RT) for 1 h. Then, the immunoblot was revealed in BCIP/NBT detection solution. Thrice washes were performed with TBS-T between each step.

A positive control was effectuated under the same conditions but in indirect detection to target the antigen. The anti-*T. gondii* SAG1 mAb (Product N°. 11-132, Argene SA, Verniolle; France) diluted at 1:1000 in TBS-T was firstly used as specific primary antibody and followed by incubation with secondary antibody AP-conjugated anti-mouse Fc produced in goat (Product N°. A-1418, Sigma-Aldrich). The immunoblot was revealed as above.

Minimum detectable value was made by the serial antigen dilution. Briefly, serial 5-fold dilutions of TLA with TBS, starting from 5 μg, were prepared and were electrophoresed on discontinuous SDS-polyacrylamide 12% under non-reduced condition. Western blot was then performed with purified recombinant scFvSG15(V_LT8P)-AP fusion protein as described above.

2.7.2. Immunoreactivity by Direct-ELISA

TLA preparation was diluted in coating buffer (0.2 M sodium carbonate/bicarbonate, pH 9.4) and used to coat wells (5 μg/well/100 μl) of a 96-microtiter plate (Nunc, Roskilde, Denmark) for 16 h at 4 °C. Wells were saturated with blocking solution (5% skim milk in TBS-T) for 1 h at 37 °C. Subsequently, 100 μl of a serial two-fold dilution of each periplasmic extract of the scFv-AP fusion protein (crude or purified (starting from 50 μg/ml)) was added and incubated for 2 h at 37 °C. Immunocomplexes were directly revealed by adding a fluorogenic AP substrate solution (1 M diethanolamine, 1 mM $MgCl_2$ at pH 9.25) containing 0.2 mM 4-MUP (Product N°. M-8883, Sigma-Aldrich) and incubated in the dark for 30 min at 37 °C. The reaction was stopped with 100 μl of a 1 M K_2HPO_4-KOH buffer (pH 10.4). The fluorescence intensity

was measured through excitation at 360 nm and emission at 440 nm using a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific Laboratory Equipment). Thrice wash steps were carried out with TBS-T between each step. Background noise was controlled by replacing the fusion protein in the incubation with irrelevant proteins (non-induced periplasmic extract preparation). The test was performed in triplicate.

2.7.3. Tachyzoites-binding assay

One hundred μl of 10-fold serial dilution of tachyzoites, starting from 10^6/ ml, was fixed onto 24-well polystyrene tissue-culture plates (Zellkultur Testplatte, TPP, Switzerland) in cold methanol for 10 min on an ice bath. After rinsing with TBS-T, the endogenous AP activity was inhibited with 20 mM levamisole hydrochloride (Product N°. L-9756, Sigma-Aldrich) in 50 mM bicarbonate buffer (pH 9.6) containing 0.5 mM $MgCl_2$ for 10 to 15 min at 37 °C followed by saturation step with blocking buffer (5% skim milk, for 1 h at 37 °C). Thereafter, the purified scFvSG15(V_LT8P)-AP conjugate (50 μg/ml) was added and incubated for 1 h at 37 °C. Finally and after washing steps, the immunoassay was revealed using the fluorogenic 4-MUP AP substrate as described above. Background was carried out with non-induced periplasmic extract preparation as irrelevant proteins. The immunoassay was conducted in triplicate.

2.7.4. Quantification of T. gondii *tachyzoites* via *quantitative real-time PCR method*

The 10-fold serial dilution of tachyzoites used above was quantified further *via* quantitative real-time PCR (qRT-PCR) method as previously described [35]. One ml of a media suspension containing different concentrations of tachyzoites 10^6, 10^5, 10^4, 10^3, 10^2, 10, 1 and 10^{-1} was prepared. DNA was extracted using the QIAamp DNA Mini-Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's recommendations using approximately 100 μl of diluted tachyzoites for the first buffer step. DNA concentration was determined using a NanoDrop spectrophotometer. qRT-PCR assay was performed in the Laboratoire de Parasitologie Médicale, Institut Pasteur de Tunis, routinely used for molecular diagnosis of toxoplasmosis as previously described [36]. The PCR assay targeted the highly repetitive sequence REP-529 (Genbank accession no. AF487550) consists of 200 to 300 copies in the genome of *T. gondii*. qRT-PCR was conducted in a final volume of 25 μl by using a Taq-Man universal master mixture (Applied Biosystems, USA) containing 600 nM of each primer (F: 5 -AGAGACACCGGAATGCGATCT) and (R: 5 -TTCGTCCAAGCCTCCGACT), 200 nM of probe (FAM: 5 -TCGTGGTGATGGCGGAGAGAATTGA) and 5 μl of DNA extract. The DNA was amplified in an Applied Biosystems® (Applied Biosystems, Foster City, CA) for 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Each sample was tested in duplicate, and each run included a no template control (NTC). A standard curve plotted from a ten-fold dilution series of *T. gondii* DNA (extracted from 5.10^6 tachyzoïtes), allowed parasite quantification. Real-time PCR was considered positive for *T. gondii* tachyzoites when the threshold cycle (C_t) was to 34.37 which corresponded to 0.02 parasites per reaction tube.

2.7.5. Identification of T. gondii *tachyzoites by direct immunofluorescence assay*

10^4 tachyzoites were fixed with chilled methanol onto microscopic slides. After blocking step, the endogenous AP was inhibited with 20 mM levamisol as described above, and then, 100 μl of the purified scFvSG15(V_LT8P)-AP (50 μg/ml) was added and incubated at 37 °C for 1 h. The specific immunocomplexes were detected using the fluorogenic 4-MUP AP substrate (30 min at 37 °C, in the dark). A fluorescence microscope (INV100-FL; BEL Engineering®, Milan, Italy) was used for the imaging interaction between parasites and the scFvSG15(V_LT8P)-AP conjugate. The slides were washed three times with TBS-T between each step. A positive control was performed in the same conditions with the anti-*T. gondii* SAG1 mAb (diluted 1:500) but followed by incubation with secondary antibody FITC-conjugated goat anti-mouse IgG (diluted 1:500, Product No. F-0257, Sigma-Aldrich). The negative control was used in the same conditions with non-induced periplasmic extract preparation.

3. Results

3.1. Cloning and expression of a recombinant conjugate scFvSG15-AP

The full length scFvSG15 coding gene was PCR-amplified as 735 bp including *Sfi*I/*Not*I clamp sequences (data not shown) and then inserted between codons +6 and +7 of *AP* gene into pLip6-GN expression vector. Top10 competent cells were transformed firstly and the positive transformants were selected by blue-white colony selection in LB-agar plates containing IPTG and BCIP. The blue appearance of cones allows indicating that only the inserted foreign DNA between codons +6 and +7 of the *AP* gene restores the initial frame of the *AP* gene in the vector and an additionally confirms that the molecule inserted into the N-terminus extremity of the bacterial AP does not perturb significantly the enzymatic activity [23]. The resulting recombinant plasmids were also confirmed by sequencing and the deduced amino acid sequences of the scFvSG15 variable domains were in agreement with the heavy-chain and the light-chain fragments of Fab 4F11E12 sequences [31].

In order to examine the expression of the recombinant fusion protein, the pLIP6-scFvSG15-AP vector was transformed into the *E. coli* host strain W3110, cultured and induced with IPTG. After extraction of the periplasmic fraction, proteins were subjected to SDS-PAGE and Western blot analysis (using an anti-bacterial AP mAb). Analyses showed a protein band with an apparent molecular weight of 78 kDa (data not shown), in agreement with the scFv-AP predicted molecular mass. However, no band was observed in non-induced conditions.

3.2. Mutation in the FR-1 (κ-V_L) domain of scFvSG15 to confer Protein-L binding ability and purification of the fusion protein

The plasmid encoding the recombinant fusion protein pLip6-GN is without specific Flag-Tag system for purification of the immunoconjugate from flask cultures or fermentation broth. We used, first, Protein-L-coupled chromatography resins, that binds specifically the κ-V_L domain of many immunoglobulins [37], to purified the scFvSG15-AP preparation. Unfortunately, despite several attempts, the recombinant immunoconjugate could not be purified (data not shown); we deduced that the scFvSG15 does not interact with Protein-L. In order to provide it Protein-L binding capacity, a single amino acid substitution in the FR-1 of scFvSG15 κ-V_L domain, proline replacing threonine at position 8, was generated by site-directed mutagenesis. Therefore, the obtained mutant pLIP6-scFvSG15(V_LT8P)-AP vector was used to transform *E. coli* W3110 strain and after expression and periplasmic extraction, the Protein-L binding ability was inspected *via* affinity chromatography using a column of Protein-L-Agarose. Indeed, the expected scFvSG15(V_LT8P)-AP fusion protein band (78 kDa) was obtained in eluted fraction (Fig. 1A, lane 1), analyzed with a 12% continuous SDS-PAGE gel stained with coomassie blue and further confirmed by Western blotting (Fig. 1B, lane 1) revealed with the anti-bacterial AP mAb. Moreover, no proteolytic degradation and no endogenous AP were detected according to Western blot analysis, emphasizing the stability of the fusion protein.

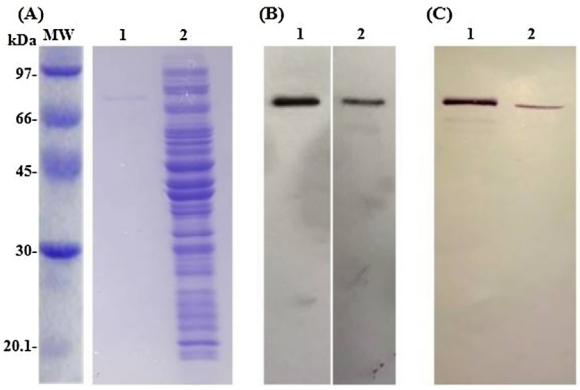

Fig. 1. scFvSG15(V_LT8P)-AP fusion protein expression and purification analysis. SDS-PAGE analysis of bacterial periplasmic extract and Coomassie Brilliant Blue staining (Panel A) and Western blot treated with anti-AP antibody (Panel B), or directly revealed with BCIP/NBT AP substrate (Panel C). Five micrograms periplasmic proteins were applied in the gels. Numbers correspond to purified fusion protein onto Protein-L-agarose that appears as a band at 78 kDa (lane 1) and induced crude periplasmic preparation (lane 2). MW: molecular weight markers in kDa are indicated on the left.

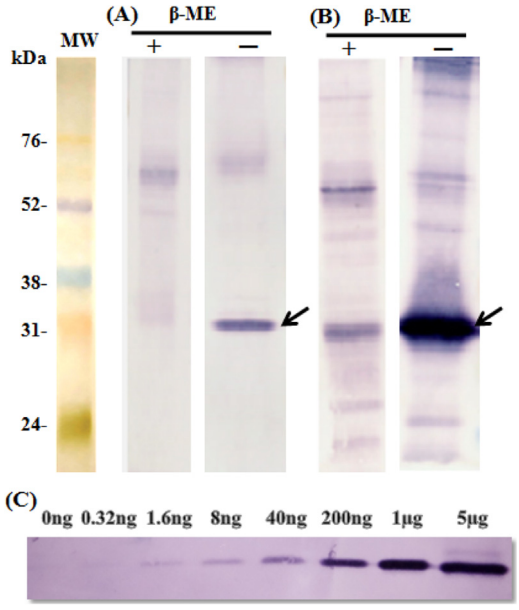

Fig. 2. SAG1-specific binding activity of the recombinant scFvSG15(V_LT8P)-AP immunoconjugate and sensitivity. The antigen-specificity of the recombinant immunoconjugate was analyzed by immunoblot assay after an electrophoretic separation 5 µg of the whole TLA under both reduced and non-reduced conditions (lacking β-Mercaptoethanol (β-ME)). The SAG1/scFvSG15(V_LT8P)-AP immunocomplex was directly detected with the colorimetric BCIP/NBT AP substrate (Panel A). A positive control was used in the same conditions with anti-*T. gondii* SAG1 mAb and followed with secondary antibody AP conjugate (Panel B). The target SAG1 antigen, with a molecular weight of approximately 30 kDa, is shown with arrows. MW: molecular weight markers in kDa are indicated on the left. To assess the lower limit of detection, serial 5-fold dilutions of TLA starting from 5 µg were run on SDS-PAGE under non-reducing conditions and were subjected to Western blot analysis with the scFvSG15(V_LT8P)-AP fusion protein for direct detect SAG1 antigen (Panel C). The colorimetric signal intensity is proportional to the protein quantity on the blot.

The final yield of soluble recombinant protein averaged approximately 0.5 mg scFvSG15(V_LT8P)-AP/liter of bacterial culture. This amount largely exceeds the requirement for conventional biological activity tests.

3.3. Bifunctional characterization of the scFv-AP conjugate

Purified recombinant immunotracer was investigated for both AP and scFv activities.

3.3.1. Assessment of AP-linked catalytic activity

Crude and purified periplasmic extracts obtained previously in Fig. 1A were transferred to NC membranes and we checked directly the AP catalytic activity using BCIP/NBT colorimetric AP substrate. The purple precipitate provided by the AP substrate, at 78 kDa band level (Fig. 1C) indicates firstly that the AP remains fully active under fusion protein format and secondly that the whole periplasmic extract can be directly used as a marker.

3.3.2. Direct immunoblot analysis

In order to investigate the bifunctionality of the recombinant fusion protein, an immunoblot was performed by transblotting the SDS-PAGE-separated whole TLA under both reduced and non-reduced conditions, and then treated with preparations containing the purified scFvSG15(V_LT8P)-AP conjugate. Immunocomplexes were directly revealed with the colorimetric AP substrate. As shown in Fig. 2A under the non-reduced conditions, the *T. gondii* SAG1 (MW, 30 kDa) was directly recognized by the scFvSG15 (V_LT8P)-AP immunoconjugate, into its native conformation. In contrast, no band was appeared under the reduced conditions (Fig. 2A). This first result strongly suggests that the recombinant immunoconjugate is effectively bifunctional, preserving both *T. gondii* SAG1-binding speci city and AP catalytic activity. The SAG1 immunoreactivity was confirmed by Western blot analysis with the anti-*T. gondii* SAG1 mAb (Fig. 2B) used as positive control. In addition, a serial whole TLA dilution was immunoblotted with the scFvSG15(V_LT8P)-AP immunoconjugate for estimating the lowest detectable antigen concentration. Thus, referring to Fig. 2C, it seem to appear a linear range in which the colorimetric signal intensity is proportional to the protein quantity on the blot and the deduced lowest quantity of *T. gondii* SAG1 antigen is 1.6 ng approximately, performed in one-step reaction procedure.

3.3.3. One-step ELISA based on the scFvSG15(V_LT8P)-AP conjugate

A range of crude periplasmic extracts and Protein L-purified scFv-AP fusion protein was incubated in microtiter plates precoated with TLA. After washing steps, the antigen-binding complex detection was revealed by means of fluorescent marker, 4-MUP AP substrate. Indeed, we found that the corresponding immunoanalytical test showed dose-dependent increase in the fluorescent signal with increasing amounts of the purified scFvSG15(V_LT8P)-AP (Fig. 3B), which suggests that this immunoconjugate bound specifically to SAG1 contained in the whole TLA in a dose-dependent manner. And further, the fluorescent signal intensity was shown to linearly decrease with the increasing crude periplasmic dilutions similarly for the both constructions (wild type *vs* mutant) of the scFv-AP fusion proteins (Fig. 3A). This result confirms that the mutation T8P introduced in FR-1 of scFvSG15 κ-V_L domain, restoring the Protein-L interaction, does not affect the antigen-binding activity against the SAG1 antigen. And no significant background was detectable using with irrelevant proteins.

3.3.4. Using a fluorometric immunoassay to detect and quantify T. gondii tachyzoites with a scFvSG15(V_LT8P)-AP fusion protein

We developed fluorometric system to detect *T. gondii* parasites using conventional Fluorescence plate reader. After incubation fixed successive dilutions of tachyzoites with purified scFvSG15 (V_LT8P)-AP conjugate, the immunocomplex was directly revealed using a fluorogenic 4-MUP AP substrate. As can be seen in Fig. 4A, the corresponding fluorescent signal decrease in a dose-dependent manner with decreasing amounts of the tachyzoites within the dilutions tested and in addition, the minimal detectable quantity

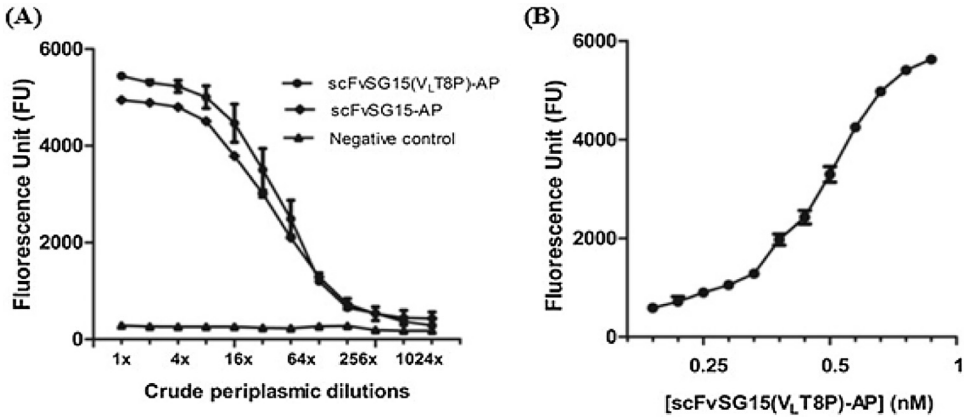

Fig. 3. Direct fluorometric ELISA to evaluate the bifunctionality of the scFvSG15(V_LT8P)-AP conjugate. 96-well ELISA plates were coated with TLA (5 μg) and treated with increasing crude periplasmic dilutions (Panel A) or with various purified concentrations of the scFv-AP (from 50 μg/ml to 10 ng/ml) (Panel B) in a one-step procedure. The immunoconjugate activity was determined by measuring the fluorescence intensity (FU) (λex =360 nm, λem =440 nm) after incubation with a fluorogenic AP substrate solution containing 0.2 mM 4-MUP. The background was assessed with the non-induced periplasmic extract. The error bars indicate the standard deviation in the triplicate experiments.

reached the level of 1 parasite/well approximately. Thus, the fluorescent reagent used allowed us to detect low concentrations of tachyzoites and hence to improve the sensitivity of the assay. In order to test the compliance of this limit of detection, *T. gondii* tachyzoites DNA obtained from three higher dilutions (10, 1 and 10^{-1}) was moreover quantified using qRT-PCR assays based on the 529-bp repetitive element. The real-time PCR amplification plot was shown in Fig. 4B and the deducing of quantity of parasites *versus* the C_t values was resumed in Table 1. Thus, the ten-fold serial dilutions of tachyzoites used in the fluorometric immunoassay correlate well with the amount of parasites deduced by qRT-PCR analysis. Hence, this confirms that the sensitivity of the scFvSG15(V_LT8P)-AP immunoconjugate for the detection of *T. gondii* parasites using a fluorometric immunoassay is found to be single parasite /well approximately.

3.3.5. *Immunostaining of* T. gondii *parasites with a scFvSG15(V_LT8P)-AP using a fluorogenic 4-MUP AP substrate*

This experiment was conducted to visualize interaction between parasites preparation and the recombinant immunoprobe. The purified scFvSG15(V_LT8P)-AP preparation was incubated onto methanol-fixed tachyzoites microscopic slides. After washing, the specific immunocomplex was detected using the fluorogenic 4-MUP AP substrate and the slides were observed under a fluorescence microscope. The bright green fluorescence shown in Fig. 5, panel A, reveals that the scFvSG15(V_LT8P)-AP fusion protein specifically reacted with *T. gondii* on outer cell periphery. However, no fluorescence signal was observed when the tachyzoites were directly treated with the non-induced periplasmic extract preparation (Fig. 5D) or with the fluorogenic 4-MUP AP substrate (Fig. 5B) used as negative controls to determine the level background fluorescence. As a result of the fluorescent microscopic examination, only scFvSG15(V_LT8P)-AP immunoprobe interacted with tachyzoites and there were no non-specific interactions between parasites and the fluorogenic 4-MUP AP substrate. Because SAG1 is a surface protein of *T. gondii* tachyzoites, the fluorogenic immunoconjugate scFvSG15(V_LT8P)-AP specific to SAG1 covered the outer surface of the parasites, and the fluorescence image was compatible with the natural shape of the parasites when the anti-*T. gondii* SAG1 mAb was used as positive control in two-steps, stained with secondary antibody FITC-conjugated (Fig. 5C). Thus, the scFvSG15 (V_LT8P)-AP fusion protein has dual activity and can be used for rapid immunostaining and specific detection of *T. gondii* parasites by means of fluorescent substrate in a one-step procedure.

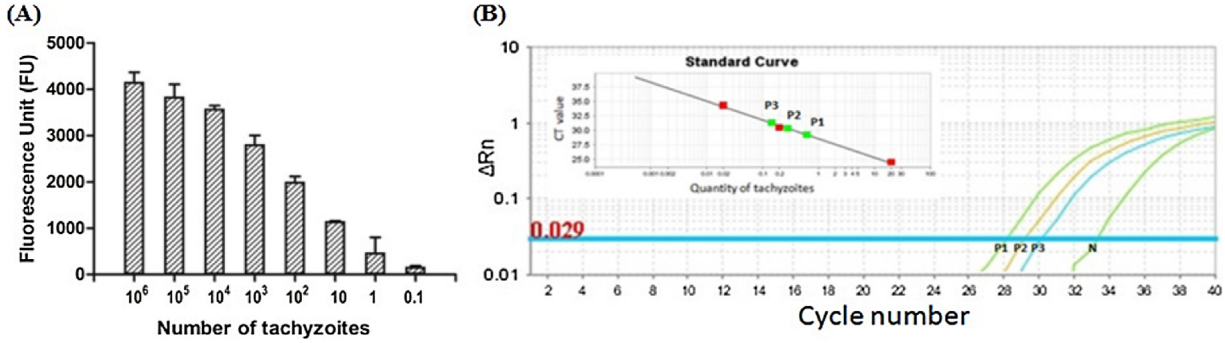

Fig. 4. Fluorometric immunoassay to detect and quantify *T. gondii* tachyzoites using the scFvSG15(V_LT8P)-AP conjugate. Panel A: 24-well polystyrene tissue-culture plates were coated with serial decimal dilutions of tachyzoites (from 10^6/ml to 10^{-1}/ml), the endogenous AP was blocked with 20 mM levamisole, and then incubated with purified scFvSG15(V_LT8P)-AP immunoconjugate (50 μg/ml). The immunocomplex was directly revealed using a fluorogenic 4-MUP AP substrate solution (λex =360 nm, λem =440 nm). Results are expressed by a decrease of fluorescence units (FU) with decreasing parasites amount. The error bars indicate the standard deviation in the triplicate experiments. Panel B: Control *T. gondii* tachyzoites quantification *via* qRT-PCR assay. Real time PCR amplification curves obtained from the ten-fold dilutions of *T. gondii* DNA described in Table 1 as well as negative control (N) are also shown. Left rectangle indicates the standard curve established with ten-fold dilutions of *T. gondii* DNA (■), ranging from 20 to 0.02 parasites, assessed under similar conditions. C_t values were plotted against log (quantity of tachyzoites) and parasites pools were considered positive for a C_t 34.37. The horizontal line indicated the threshold line determined for drawing up the standard curve, Cut-off = 0.029 tachyzoite/PCR reaction. C_t: threshold cycle; ΔRn: normalized reporter.

Table 1
Threshold cycle (C_t) values for each decimal dilution of *T. gondii* DNA tested in duplicate. Similar results were obtained in double independent experiments. NTC, no-template control.

Test	Number of tachyzoites	Test C_t
P1	10	29.3
P2	1	30.39
P3	0.1	31.34
N	NCT	40

4. Discussion

Several conventional antibodies, polyclonal (pAbs) or monoclonal antibodies, with immunodiagnostic value have been produced against many important protozoan and parasitic diseases. They have greatly increased the specificity of diagnosis by eliminating cross reactions between closely related parasite spp., without suffering a significant loss of sensitivity, and/or directly demonstrating the presence of the causative organism [10,38]. Despite these attractive features, the use of antibodies as immunoanalytical tools suffer from several disadvantage such as multiple animals are prone to be immunized against the same antigen which induces a high degree of batch-to-batch variability for pAbs production or time consuming, high-cost, and needs good technical expertise to generate specific mAbs. Fortunately, the advent of recombinant DNA technology has provided new paths to produce recombinant antibodies and their derivatives [39]. One of the most popular types of recombinant antibodies are scFvs as they have been successfully engineered into a number of different antibodies formats and are easily expressed by several heterologous expression systems. From a unique set of genes, recombinant antibody production is controlled, reliable and several problems with hybridoma production can be avoided, such as gene loss, gene mutations, and cell-line drift. This leads to antibodies with very little batch-to-batch variability, giving highly reproducible results and saving millions of dollars [40]. The small size of the scFvs allows improving pharmacokinetic properties, such as better tissue penetration, high-level of diffusion and limits steric hindrance effects. In addition, having access to coding sequences, scFvs can be tailored to other functional domains to produce molecules with multivalent or multifunctional formats in minimal size [41]. So, producing recombinant versions of the antibodies or immunoconjugates offers the opportunity to create well defined and more effective molecules, than conventional antibodies, could be immuno-biotechnologically profitable. In such a context, we designed recombinant immunoconjugate tool based an anti-SAG1 scFv antibody fragment fused to the AP of *E. coli* with the purpose to implement a fluorometric method for direct detection and quantification of *T. gondii* in biological samples. After production and purification steps, the bifunctionality of the recombinant immunotracer was evaluated using chromogenic and fluorogenic enzyme substrates. Finally, its value as valuable tool to direct detect *T. gondii* tachyzoites in a one-step procedure was also established.

We expressed the scFvSG15 coding gene in the pLIP system as fusion antibody into the bacterial periplasm. This approach enabled the production of a soluble and bifunctional fusion protein formed of an antigen-binding against SAG1 inserted between residues +6 and +7 of the mature form of wild-type bacterial AP. Indeed, the presence of both the signal peptide and the first six amino acid residues of AP promote the efficient export of hybrids into the periplasm of *E. coli*, where their cysteines are oxidized into disulfide bonds and their native-like structure is

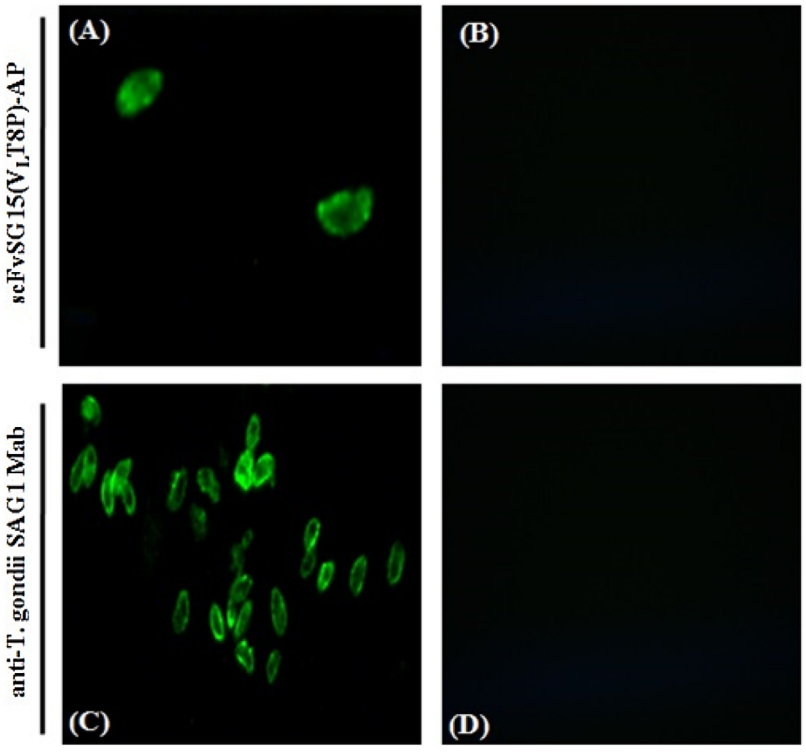

Fig. 5. Direct immunostaining of *T. gondii* tachyzoites with the scFvSG15(V_LT8P)-AP conjugate. Tachyzoites were fixed onto microscopic slides, endogenous AP was blocked with 20 mM levamisol and then incubated with the purified scFvSG15(V_LT8P)-AP immunoconjugate (50 μg/ml). The specific immunocomplex was directly visualized using the fluorogenic 4-MUP AP substrate (Panel A). A positive control was used in the same conditions with anti-*T. gondii* SAG1 mAb and followed with secondary antibody FITC conjugate (Panel C). Negative controls directly incubated with the fluorogenic 4-MUP AP substrate (without the immunoconjugate) or with the non-induced periplasmic extract are shown on the right panels B and D respectively. Tachyzoites were imaged with a fluorescence microscope at 40X magnification.

likely to be formed. Other scFvs have been also fused successfully to AP and have been reported, using the same or a similar approach [16,17,42]. In all cases, the sequence encoding the signal peptide of AP precedes the inserted gene resulting in periplasmic localization and correct folding. And the activity of endogenous bacterial proteases against the recombinant molecule is limited if bacteria are induced in optimized conditions.

The recombinant anti-SAG1-AP conjugate created through genetic engineering approach represents a better alternative methodology to conventional chemical cross-linking, which lead frequently to immunoconjugates with highly reduced activity even under mild conditions [43]. In addition, the recombinant procedure of production is simple, reproducible and can be adapted in various host systems such as bacteria, yeast, plants, or mammalian cells, even on an industrial scale. Furthermore, the recombinant immunoconjugate could be purified in one-step using universal purification systems. Indeed, variety of standard affinity chromatography methods or fusion tag strategies that can be used to purify scFvs or its conjugate derivatives formats from crude extracts have been reported [44], but some of them are not adapted to direct purify the anti-SAG1-AP conjugate. First, the pLIP6-GN expression vector, used here, is lacks of target peptide for recombinant protein purification. Genetic grafting an appropriate peptide tag either at the N- or C- terminus of the recombinant immunoconjugate is possible. However, fusion tag can lead to many undesirable situations such as it may interfere with the correct folding, it may also tend to promote aggregation or to induce a non-functional protein, as has been reported in the literature [45,46]. Secondly, as all scFvs, scFvSG15 is lacks of the constant domains of antibodies, using protein A from *Staphylococcus aureus* or protein G from *Streptococcus sp*. for the direct affinity chromatography purification is not feasible. Given all these considerations, only protein that can be used as a tool for the affinity purification of scFvSG15 as immunoconjugate is Protein-L from the anaerobic species *Peptostreptococcus magnus*, interacting mainly with FR-1 of V-kappa [37]. We have matured the Protein-L binding capacity onto anti-SAG1 scFvSG15-AP by simple site-directed mutagenesis, which initially does not interact significantly with this protein. We performed a T8P mutation in the FR-1 (κ-V_L) to obtain the mutant scFvSG15(V_LT8P)-AP fusion protein. It seems like that this proline at position 8 plays a crucially important role because it interacts with many residues on Protein-L, particularly with tyrosine 34 and tyrosine 32 [47] and furthermore, it probably enables a conformational modification of the κ-VL that would favor the interaction with Protein-L [33]. Thus, this substitution provided us the possibility to purify scFvSG15 (V_LT8P)-AP through a one-step Protein-L-Agarose immunochromatographic procedure. Additionally, both the quantity and purity of the recombinant conjugate obtained were sufficient to investigate its value as immunoanalytical tool to direct detect and quantify *T. gondii* parasites.

In order to reach this goal, first, we have investigated the scFvSG15(V_LT8P)-AP conjugate ability to detect SAG1 antigen by a Western blot procedure. Owing to its advantages of high sensitivity and simple procedure, this analytical technique can theoretically serve as an effective early diagnostic tool in patient samples [48]. As shown in Fig. 2, the immunoconjugate binds specifically to the native form of SAG1 under non-reducing conditions in one-step procedure, and provides a limit of detection approximately equal to 1.6 ng. This value is in the nanograms range of the Western blots sensitivity, usually performed with pAbs or mAbs for antigen quantification using chromogenic substrates and in two-steps. A greater sensitivity of detection in immunoblots and other membrane-based detections can be improved through the use of the recently developed AP chemifluorescent substrates [49].

In a second stage, one-step fluorometric immunoassay procedure was established using the recombinant immunoconjugate anti-SAG1-AP for detecting and quantifying *T. gondii* parasites. After coating with serial dilutions of parasites, the tachyzoites/scFvSG15(V_LT8P)-AP fusion protein complex was directly revealed with AP 4-MUP fluorogenic substrate. The fluorogenic assay appears to be specific for SAG1 and accurate with a limit of detection as low as 1 parasite/well approximately. This quantification is in agreement with the C_t values deduced by qRT-PCR assay targeting the reference 529-bp repetitive fragment in taking into account the relative concentration-dilution of *T. gondii* DNA (Table 1). Almost similar degree of sensitivity was noted by other groups describing the quantitative real-time PCR method using hybridization probes for the detection of *T. gondii*, where they were able to detect 1 parasite in biological samples such as amniotic fluid, blood or tissues [50,51]. A combination of several factors contributes to this high level of sensitivity, which might allow the recombinant immunoconjugate anti-SAG1-AP to be used as a diagnostic tool for toxoplasmosis. Firstly, the bivalent nature of the immunotracer: the bacterial AP is only active as a homodimeric form. Thus, the resulting scFv-AP fusion protein mimics the structure of the natural IgG molecule in which all the constant domains have been substituted with the dimeric form of the AP protein. This leads to a bivalent antigen-binding molecule, which result in a higher apparent affinity (avidity) of the fused scFv. This may play a beneficial role in significantly increasing the sensitivity of detection. In addition, the gene fusion technique employed here, where the structural genes of the both partners were fused in tandem, in a strict 1:1 ratio, results a uniform structure of the immunoconjugate with no affected the antigen-binding site or the enzyme activity, often obtained in using bifunctional chemical crosslinking agents. Secondly, detection mode: it has been well known for many years that the fluorometric methods generally are more sensitive than colorimetric methods. AP is the most widely used enzyme for fluorescent applications. The major fluorogenic substrate, 4-MUP, is usually paired up with AP which is suited for fluorometric assay offering high-sensitivity of the immunocomplex at a specific wavelength. Indeed, the fluorogenic substrates are able to detect or measure even in extremely low concentrations of analytes. They offer up to 1000 times greater sensitivity than chromogenic substrates, leading to greater limits of detection, while potentially using less antigen, making this a useful powerful system for many different immunoassays [52]. In addition, chromogenic substrates are, generally, powders which must be individually weighed into the wells of microplates and undissolved substrate particles can interfere with subsequent measurement of colored supernatants causing an increase in background [53]. Thus, using a fluorometric substrate eliminates the interferences and non-specificity encountered in colorimetric assays that occurs in some biological samples. And finally, pre-treating *T. gondii* parasites at levamisol for efficiently inactivating endogenous AP activity or from different sources is fundamental for eliminating or reducing nonspecific background signal, which may leads to remove false positive and to avoid biased results.

Encouraged by the high sensitivity of the fluorometric immunoassay we apply the scFvSG15(V_LT8P)-AP fusion protein to implement a direct immunofluorescence assay to detect fixed *T. gondii* tachyzoites onto microscopic slides. Immunofluorescence assay is one of the most powerful techniques in the diagnosis of infectious diseases and it has proven to be a widely useful tool in the diagnosis of parasitic diseases. For this purpose, the experimental conditions (including incubation times, reagent concentration, temperature, levamisol pretreatments, washing time) were held constant in the immunofluorescence technique as the fluorometric immunoassay s description, guaranteeing certain homogeneity amongst replicas and experiments. The

immunostaining was revealed by means of the fluorogenic 4-MUP AP substrate and the slides were observed under a fluorescence microscope. The specific green fluorescence around the tachyzoites membranes, as shown in Fig. 5, indicates that the scFvSG15 (V_LT8P)-AP fusion protein binds to the native form of SAG1 antigen, abundantly present on the *T. gondii* surface. This well affirms that the recombinant immunotracer anti-SAG1-AP can be successfully used in a rapid visual immunofluorescence assay for the specific detection *T. gondii* parasites. Similar ascertainment was recently reported by Sert et *al*. in using a fluorescein isothiocyanate (FITC) labeling of the SAG1-specific chicken egg yolk antibody (IgY) to show the potential for immunofluorescence imaging of *T. gondii in vitro* [54]. Therefore, the recombinant conjugate scFvSG15(V_LT8P)-AP presented here has the potential to be used as diagnostic tools to confirm the presence of *T. gondii* parasites in biological samples by means of direct immunofluorescence assay. Only just additional evaluation using various clinical specimens (e.g. amniotic fluid, blood, cerebrospinal fluid, etc.) is needed in order to provide it a more value and a more reliable detection of this pathogen.

In summary, in this study, recombinant immunoconjugate anti-SAG1-AP was successfully constructed, and the expressing product was purified using Protein-L immunoaffinity chromatography. Both the specific antibody binding capacity and the AP enzyme activity were retained in the scFv-AP fusion protein. The proposed recombinant immunoreagent scFvSG15(V_LT8P)-AP conjugate proved to be a good alternative tool for quick and highly sensitive immunodetection of *T. gondii* parasites in a fluorometric immunoassay using AP 4-MUP fluorogenic substrate. This fusion protein has some advantages, such as can be prepared reproducibly, rapidly and at low cost. In addition, using a doubly functional protein, this makes possible single-step immunoassay which does not require a secondary immunoconjugate and/or multiple incubation steps frequently employed. Nevertheless, although the anti-SAG1-AP conjugate designed in this article is promising, further work is needed before an immunoassay with recombinant reagent product will be available for diagnostic purposes.

Acknowledgments

We are very appreciative of the excellent technical assistance provided by Dr. Makram Essafi (Laboratory of Transmission, Control and Immunobiology of Infections LR16-IPT02, Pasteur Institute of Tunis) in fluorometric immunoassay analysis. The authors thank Mrs. Olfa Souiss (Department of Medical Parasitology, Pasteur Institute of Tunis) for her outstanding technical assistance in quantitative real-time PCR. This work was supported by the Ministry of Higher Education and Scientific Research in Tunisia and carried out within the framework of the Laboratoire de Parasitologie Médicale, Biotechnologies et Biomolécules LR11-IPT06, Institut Pasteur de Tunis.

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