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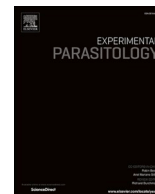
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Production and evaluation of a new set of recombinant antigens for the serological diagnosis of human cysticercosis

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ABSTRACT

Human cysticercosis caused by *Taenia solium* (*T. solium*) is endemic in certain areas of Latin America, Asia and Sub-Saharan Africa. Neurocysticercosis (NCC) is mainly diagnosed by neuroimaging, which, in most cases, is unavailable in endemic areas. Due to their high sensitivity and specificity, serological tests such as enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) based on the glycosylated fraction of the cyst CS50 are widely used for the detection of the anti-cysticercus IgG antibodies despite their significant cost and the need of cysticercus material. Given their cost-effectiveness and simplicity, immunoassays based on recombinant proteins could provide new alternatives for human cysticercosis diagnosis: such tests would be aimed at screening those people living in remote areas who need further examination. To date, however, no test using recombinant antigens is commercially available.

Herein, five recombinant proteins (R14, R18, R93.1, R914.1, and R915.2) were produced, three of which (R93.1, R914.1, and R915.2) were newly identified from the cyst fluid. Evaluation of the diagnostic performance of these recombinant antigens by ELISA was done using sera from 200 epileptic and non-epileptic individuals in comparison with the WB-CS50 as the reference serological method.

Recombinant proteins-based ELISA showed a level of diagnostic performance that is inferior than the reference serological method, but similar to that of the native antigen ELISA for human cysticercosis (commonly used for screening). Further optimization of expression conditions is still needed in order to improve proteins solubility and enhance diagnostic performance for human cysticercosis detection. However, this preliminary evaluation of the recombinant antigens has shown their potential valuable use for screening cysticercosis in patients with epilepsy attending dispensaries in remote areas. Future studies should be conducted to evaluate our recombinant antigens in a large group of patients with different stages of NCC, and in correlation with imaging findings.

1. Introduction

Human cysticercosis is a parasitic infection caused by *Taenia solium* (*T. solium*) *Cysticercus*. In humans, the parasite commonly affects the central nervous system causing Neurocysticercosis (NCC). In the brain parenchyma, the cyst progressively evolves through various stages (Escobar et al., 1985; Singhi, 2011). During the first vesicular stage, the cyst can remain viable for months without eliciting a host inflammatory

reaction (Flisser, 1994; Garcia et al., 2014). In the colloidal vesicular stage of NCC (Escobar's stage), the parasite begins to show degenerative changes. There is loss of immune tolerance of the parasite with the alteration in vitality resulting in osmotic imbalance of the cyst membrane. The cyst shows hyaline degeneration and early mineralization (Obregón-Henao et al., 2003; de Aluja and Vargas, 1988; Venkat et al., 2016). The cyst fluid is extruded out, inciting an inflammatory reaction in the surrounding tissues. The cyst wall becomes thicker and the fluid

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becomes turbid with increased protein content (Venkat et al., 2016). This liquid is highly antigenic and inflammatory, but it is subsequently released late in the life-time of the parasite, especially when the parasite is dying. At this stage, epileptic crisis tend to occur and thus patients with epilepsy usually attend dispensary for examination (Carabin et al., 2011; Singh et al., 2013). Neuroimaging is the gold standard for NCC diagnosis (Del Brutto et al., 2001; García et al., 2003). However, in developing countries, availability and accessibility of neuroimaging are a major public health concern. Serodiagnostic tests, i.e. enzyme-linked immunosorbent assay (ELISA) and Western blot (WB) using crude extract or purified antigen, have been developed to improve the management of NCC- infected or suspected patients (Tsang et al., 1989; da Silva et al., 2000). The WB, which is based on glycosylated purified antigen (CS50) extracted from *T. solium* cysticerci is considered as the reference serological method (Tsang et al., 1989). Today, seven antigenic glycoproteins (50-, 39–42, 24-, 21-, 18-, 14-, 13-KDa) from cyst membrane, are specific for the diagnosis of human cysticercosis. Fractions 18- and 14 kDa are associated with active cysts. In 1999, the two antigens T14 and T18 were originally purified, characterized, and used for the diagnosis of *T. solium* cysticercosis (Greene et al., 1999; Plancarte et al., 1999). One year later, TS14 and TS18 were synthesized and expressed in *Escherichia coli* (*E. coli*) for evaluation by ELISA (Greene et al., 2000). In patients with multiple cystic lesions, the WB-CS50 was reported to be highly specific (100%) and sensitive (98%) (Tsang et al., 1989; Kramer et al., 1989), although it has been observed that its sensitivity decreases (60%–70%) in cases with a single lesion or late degenerative cyst (Wilson et al., 1991; García et al., 1994; Rodriguez et al., 2012). Moreover, for rural populations living in poor endemic areas, the accessibility of ELISA- and WB-CS50 is very limited due to the lack of laboratory facilities and native purified antigens (Lee et al., 2011; Handali et al., 2010; Mubanga et al., 2021). Therefore, recombinant antigens have been used as an alternative for native antigens. Yet, no tests using recombinant antigens have been commercialized and no lateral flow tests (LFT) have been developed.

The purpose of this study was to produce and evaluate new recombinant proteins derived from proteins that are found in the liquid of the cyst of the parasite and not from the membrane. Antibodies against the new recombinant antigens may be detected at a stage of the infection which is later than with other methods: this may add new insights in the diagnosis of cysticercosis.

Through bioguided fractionation, new antigenic targets from the liquid of cysticerci have been identified at the Institut Pasteur de Madagascar (Nativel et al., 2016). In the present study, a new set of recombinant antigens was developed to evaluate their performance in single formula and using a statistical combination (alternative and cumulative) of ELISA results to detect anti-cysticercus IgG antibodies in serum as compared with WB-CS50 (the reference serological method). This evaluation is part of the research program "PasteurInnov RADCYST – rapid diagnostic test for cysticercosis" co-funded by Institut Pasteur and Institut Carnot Pasteur MS which is aimed at developing a rapid diagnostic test (RDT) for cysticercosis by detecting specific antibodies.

2. Materials and methods

2.1. Serum samples

A total of 200 serum samples were collected from a case-control study conducted in 2015–2017 in Abidjan, Côte d'Ivoire (Soumahoro et al., 2021). Each positive subject was matched by age and sex to three negative control cases. Indeed, out of the 200 subjects selected, 150 were seronegative nonepileptic, 50 were seropositive (34 epileptic and 16 nonepileptic patients). Positive controls are defined as sera positive for cysticercosis serology by both ELISA and WB using native antigen CS50. Negative controls are defined as negative in both tests. Negative sera were collected from individuals living in non-endemic areas and were confirmed to be negative by ELISA- and WB-CS50. During this

process, patients were not submitted to CT scan, which was not available. All study participants had provided their prior written informed consent before being involved.

2.2. Native antigens

For native antigens preparation, cysticerci of *T. solium* were collected from infected pigs in slaughterhouses in Madagascar, washed repeatedly in a phosphate-buffered saline solution, and stored at -80°C until use. The extraction of the native antigens was conducted at the Institut Pasteur Paris, according to Tsang et al. (1989).

2.3. Production and purification of recombinant proteins

Five recombinant antigens were prepared and evaluated in this study: R14, R18, R93.1, R914.1 and R915.2. These antigens were identified using a bio-guided approach. Identification of the proteins in cysticercus fluid was performed in Madagascar according to Nativel et al. (2016). Proteins were analyzed using ion exchange chromatography and 2D separation and were selected by WB analysis using sera from *T. solium* on infected and non infected pigs. Spots from the Coomassie-stained gel corresponding to these proteins were then analyzed by mass spectroscopy and proteins were identified using a bank of Expressed Sequence Tags (EST) of *T. solium*. Eighteen new proteins of interest were preliminarily identified, nine of which showed a high reactivity with sera from infected pigs and were therefore subsequently selected for further development. During this present study, production and evaluation of these antigens presented; Recombinant 93.1, 914.1 and 915.2 were expressed in *E. coli*; Recombinant 14 and 18 were expressed in Baculovirus-insect cells. Recombinant proteins production and purification were performed at the Institut Pasteur Paris.

2.3.1. Recombinant antigens: R93.1, R914.1 and R915.2

The genes coding R93.1, R914.1 and R915.2 proteins were subcloned into pET-15b vectors and expressed in the *E. coli* BL21 (DE3) strains. A sequence encoding for 10xHis-Streptavidin-Binding Peptide (SBP)-Small Ubiquitin-like Modifier (SUMO) tag was inserted into recombinant vectors. Cloning and transformation were performed by Eurofins Genomics. Each transformed strain BL21 (DE3) pET15b was grown on Luria-Bertani (LB)-Agar plates supplemented with 100 mg/mL ampicillin at 30°C . After overnight growth, recombinant colonies were inoculated in 100 mL LB liquid medium and grown at 30°C , 180 rpm in a Multitron shaking incubator. When the OD600 of the culture reached 0.2, 50 mL of the 100 mL preculture were transferred to each of two 2-liter erlenmeyer flasks containing 1L of NZYtech autoinduction medium and incubated at 37°C with 240 rpm agitation. After 4H, the culture temperature was switched to 16°C and the culture was further incubated for additional 24H. Cells were harvested by centrifugation at 14000 rpm for 15 min, then resuspended in 100 mL lysis buffer (50 mM Tris, pH 8, 500 mM NaCl, 10 mM Imidazole, 1 mM DTT, Protease inhibitor cocktail Tablet (Roche)). The lysate was disrupted using the frenchpress (Constant System), then the disrupted extract was centrifuged for 30 min at 19000 rpm. For each recombinant protein, the clarified supernatant (soluble fraction) and the pellet (insoluble fraction) were assessed using SDS-PAGE stained by Coomassie blue (Fig. 1). The resulting supernatant was loaded onto 5 mL of a nickel-nitrilotriacetic acid (Ni-NTA) resin-based column (GE Healthcare) pre-equilibrated with buffer A (50 mM Tris, pH 8, 500 mM NaCl, 10 mM imidazole, 1 mM DTT) at 2 mL/min flow rate and eluted with buffer B (buffer A containing imidazole 500 mM) using a linear gradient. The pooled eluate fractions were dialyzed against 2L of 50 mM Tris buffer, 300 mM NaCl, 1 mM DTT, pH 7.5 at 4°C , then incubated overnight with SUMO protease at a final SUMO to protein ratio of 1:10. The recombinant protein was purified by applying the reaction mixture to Ni-NTA column as described previously to remove 10xHis-SBP-SUMO tag (17 kDa). Pooled fractions containing R914.1 and R93.1 were further

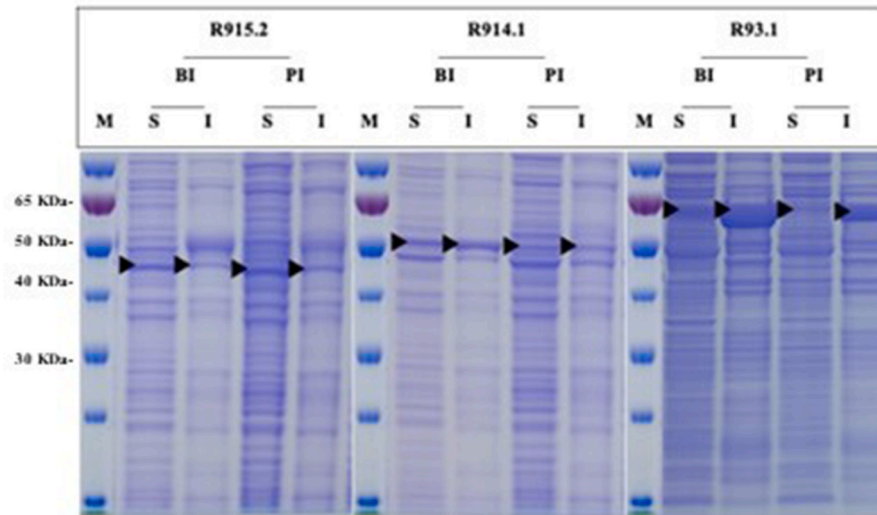


Fig. 1. SDS-PAGE of soluble (S) and insoluble (I) fractions of *E. coli* cultures expressing each of the recombinant proteins: R915.2 (44 kDa), R914,1 (47 kDa) and R93.1 (56 kDa). M: Molecular weight marker in kDa. BI: Before induction. PI: Post induction.

purified and subjected to Gel-filtration chromatography using superdex 75 pg Hiload 16/60 column preequilibrated with 50 mM Tris buffer, 500 mM NaCl, pH 7.5 and eluted with the same buffer at 0.5 ml/min flow rate.

2.3.2. Recombinant antigens: R14 and R18

R14 and R18 were produced in the baculovirus-insect cell expression system to assure glycosylation of the final protein. Recombinant baculoviruses were obtained using the plasmid pVL1393 (Expression Systems, CA USA) containing the polyhedrin promoter for the *Spodoptera frugiperda*, SF9, insect cell expression, the gene encoding the recombinant protein and the 6xHis-SUMOstar-TEV tag (Ki and Pack, 2020). The cloning of the genes encoding R14 and R18 into the baculovirus (AcMNPV) vectors and the generation of the recombinant baculoviruses were performed by Eurofins Genomics. Transfection and culture of the two recombinant baculoviruses into SF9 insect cells, derived from *Spodoptera frugiperda* ovaries were carried out with the BestBac 2.0 kit Baculovirus Cotransfection kit. Following the production of each baculovirus, purification steps were necessary to obtain a pure and concentrated product. 25 mL of cell pellet from culture expressing the recombinant protein was resuspended in 25 mL of lysis buffer (300 mM NaCl, 10 mM Tris, 100 mM Triton, pH 7.5) and incubated for 1H at 4 °C. The lysate was centrifuged for 45 min at 20000 rpm. The supernatant obtained containing the recombinant protein was concentrated and then diafiltered with Akta flux (Cytiva, Velizy France). The diafiltered recombinant protein was loaded onto 1 mL HisTrap HP column (Akta pure-GE Healthcare) pre-equilibrated with Buffer A (50 mM NH₂PO₄, 300 mM NaCl, 10 mM Imidazole, pH 7.5) at 0.5 mL/min flow rate and eluted by buffer B (50 mM NH₂PO₄, 300 mM NaCl, 500 mM Imidazole, pH 7.5) at 0.8 mL/min flow rate. The pooled eluate fractions were incubated with TEV protease a 1:10 ratio overnight for 4 °C and dialyzed against 50 mM NH₂PO₄, 300 mM NaCl buffer at 4 °C. The Dialysed eluate was applied to Ni-NTA column (Akta pure-GE Healthcare) to remove tags (16.3 kDa).

Purified protein, whether expressed in *E. coli* or SF9 cells, was detected by measuring the UV absorbance at 280 nm. Fractions peak were collected from chromatography columns and analyzed by SDS-PAGE (Fig. 2). Fractions with large amounts of recombinant antigen and high apparent purity were pooled and stored in liquid nitrogen at –80 °C.

2.4. Western blot

Western blot based on the glycosylated fraction of the cyst CS50 was conducted by the Department of Parasitology in the Institut Pasteur de Côte d'Ivoire, as previously described by Tsang et al. (1989). To summarize, the proteins were transferred from the gel following SDS-PAGE onto a nitrocellulose membrane with a 0.2- μ m-pore-size using Semi-Dry Electrophoretic system (Trans-Blot transfer system-Bio-Rad Laboratories, based in Hercules, California) for 1H 30 at 30 V and 4 °C. The membrane was cut longitudinally, and each strip was blocked with 5% skim milk for 1H. After washing three times with Phosphate-Buffered Saline (PBS) containing 0.05% Tween-20 (Sigma, P3813; Sigma, P1379) PBST, the strips were incubated with 1:20 diluted patient serum for 2H and diluted 1:10.000 Alkaline Phosphatase conjugated anti-Human IgG for 1H at 37 °C under constant shaking, respectively. After adding of 1 mL salt substrate of NBT/BCIP (nitro-blue tetrazolium chloride 5-bromo-4-chloro-3'-indolylphosphate p-toluidine), the plates were incubated for 30 min at 37 °C. We stopped the enzyme-substrate reaction by rinsing the strips with distilled water. Positive reactions were identified by the appearance of clearly defined bands.

2.5. ELISA

ELISA was used to detect IgG antibodies against R915.2, R914.1, R93.1, R18 and R14. 96-well microtiter plates Nunc MaxiSorp (Thermo Fisher Scientific) were coated with 100 μ L per well of recombinant antigen diluted to a final concentration of 1 μ g/mL in PBS pH 7.4 (Sigma, P3813). The plates were incubated overnight at 4 °C then washed four times with PBST then blocked with 1% non-fat dry milk in PBST (150 μ L/well) for 1H at 37 °C. After washing the plates three times with PBST, 100 μ L serum samples diluted at 1 : 100 in 0.3% non-fat dry milk in PBST were added to each well. After incubation for 1H at 37 °C, the plates were washed three times with PBST and 100 μ L of Horseradish Peroxidase conjugated diluted anti-Human IgG antibody (Sigma, A8419) were added to each well. The plates were incubated for 1H at 37 °C and washed again with PBST five times. Subsequently, 100 μ L of o-phenylenediamine dihydrochloride (OPD) substrate solution (Sigma, P5412) was added to each well, and the plates were incubated for 20 min at 37 °C. Afterwards, the reaction was stopped by adding 50 μ L of 2.5 N H₂SO₄ (Sigma, 258105), and the optical density (OD) was measured at 492 nm and 630 nm with the ELISA plate reader.

All ELISAs were conducted in duplicate. Positive and negative

controls sera were included in each plate. The cut-off was calculated as the mean of the negative controls plus three standard deviations.

2.6. Statistical analysis

Since an extensive diagnostic exploration of the patients was not possible, the goal of this study was not to evaluate the diagnostic performance of the antigens, but to compare the performance of recombinant proteins-based ELISA to WB (as the gold standard). ELISA data were entered in Excel (2010) and analyzed using XLSTAT 2014.5.03.

Sensitivity, specificity and 95% confidence interval (95% CI) were calculated (Altman and Bland, 1994). Statistical combinations of ELISA results were performed in duplex (2 antigens) and triplex (3 antigens) using: (i) alternative method: serum sample was considered positive when at least one antigen was reported as positive; (ii) cumulative method: serum sample was considered as positive when it was positive with at least two antigens. In both cases, these were statistical combinations of ELISA results and not results from ELISA using a mix of antigens. Agreement between CS50 and recombinant antigens was analyzed for both ELISA and WB used as the reference serological

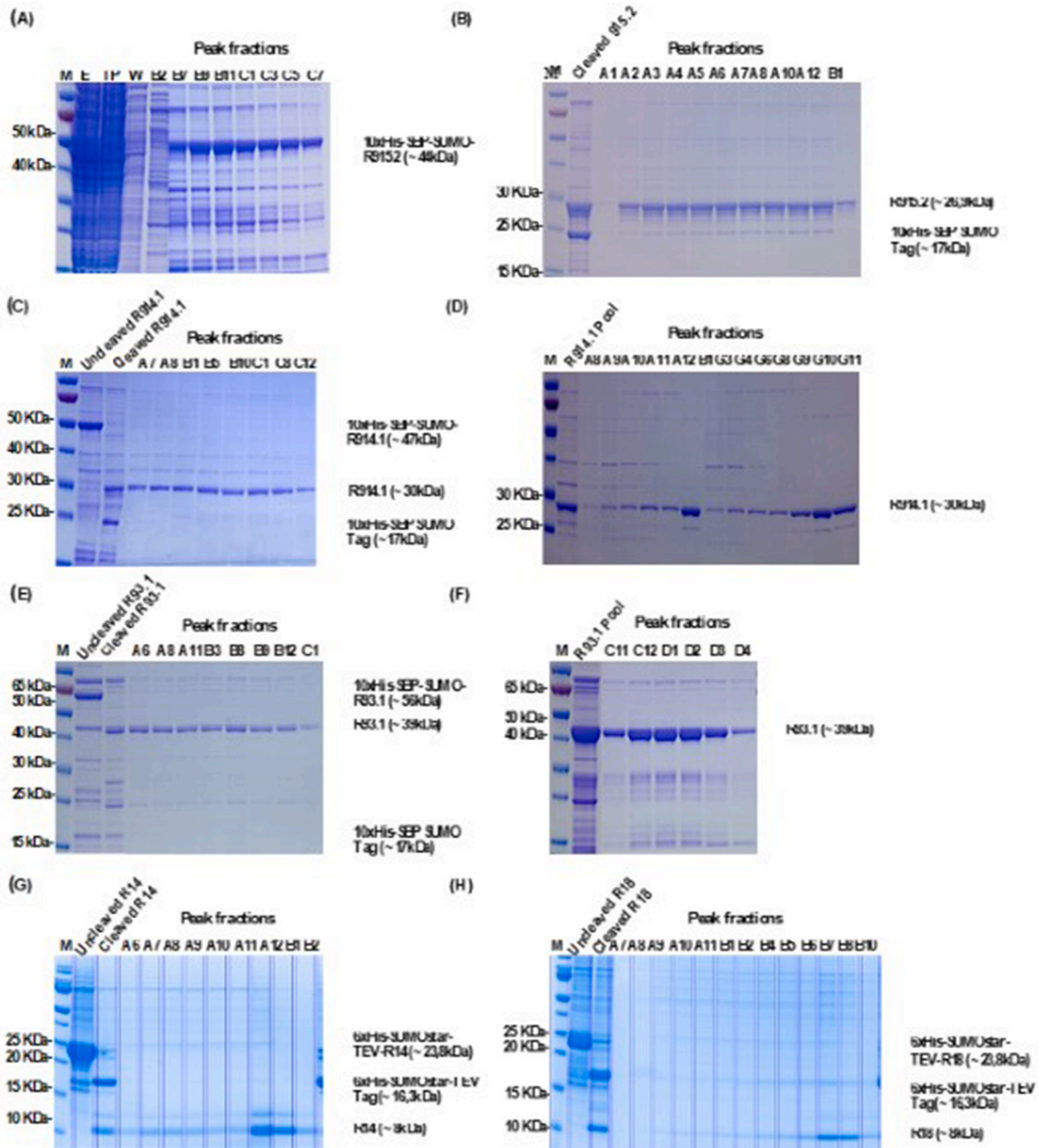


Fig. 2. SDS-PAGE analysis of the main peak fractions of the eluted recombinant proteins before and after cleavage. The gel was stained with Coomassie. (A) Poly-histidine tagged R915.2 (44 kDa) were purified from the *E. coli* extract by Ni-NTA chromatography. Fraction numbers (B2–C7) are shown above each lane. M: Molecular weight marker in kDa, E: Crude extract, TP: Through pass, W: Wash. (B) The Pooled fractions containing untagged R915.2 (29,6 kDa) were loaded on a Ni-NTA column to remove 10xHis-SBP-SUMO tag. (C) Cleavage of His-tagged R914.1 (47 kDa) with SUMO protease and purification of R914.1 (30 kDa) by Ni-NTA chromatography. (D) R914.1 eluted fractions from Ni-NTA (A7–C12) were pooled and further purified with gel filtration. (E) Cleavage of His-tagged R93.1 (56 kDa) with SUMO protease and purification of R93.1 (39 kDa) by Ni-NTA chromatography. (F) R93.1 eluted fractions from Ni-NTA (A6–C1) were pooled and further purified with gel filtration. (G) Following TEV protease cleavage of 6xHis-tag, R14 (8 kDa) was purified by Ni-NTA chromatography. (H) Following TEV protease cleavage of 6xHis-tag, R18 (8 kDa) was purified by Ni-NTA chromatography.

method.

2.7. Ethics

All the participants or their parents were informed about the objectives and the procedures of the study. Written informed consent was obtained for each patient before inclusion. The study was approved by the National Ethics and Research Committee (CNER) of the Ministry of Health of Côte d'Ivoire on May 29, 2015 under the number 022/MSLS/CNER-dkn.

3. Results

3.1. Expression and purification of R915.2, R914.1, R93.1, R14 and R18

The R915.2, R914.1 and R93.1 encoding genes were cloned into expression vector pET15b to construct recombinant plasmids which were transformed into *E. coli* BL21 (DE3) and induced by NZYtech autoinducible media. SDS-PAGE analysis of the soluble and insoluble fractions obtained from *E. coli* cultures highlighted the presence of bands in 44-, 47- and 56-kDa corresponding to the theoretical molecular weight of the proteins R915.2, R914.1 and R93.1, respectively (Fig. 1). Although the target proteins were poorly soluble and mostly found in insoluble form, proteins were purified from the supernatant after centrifugation. In the first purification step by Ni-NTA IMAC column, tagged R915.2 was eluted using imidazole gradient and an additional purification on Ni-NTA IMAC column was needed to remove the 10xHis-SBP-SUMO tag (17 kDa) from the cleaved protein. SDS-PAGE analysis of the corresponding fractions (A2-B1) revealed the presence of a band at approximately 26,9 kDa which is the expected molecular weight of the cleaved R915.2 (Fig. 2A; Fig. 2B). The purification of the cleaved R914.1 et R93.1 consisted of three chromatographic steps: two Ni-NTA IMAC column chromatography and size exclusion chromatography (gel filtration). Protein pool collected by the second Ni-NTA column after tag cleavage was applied on gel filtration (Fig. 2C; Fig. 2E). The peak fractions from each elution step were run separately in 12% SDS-PAGE gels which indicated the presence of cleaved R914.1 and R93.1 bands at the expected molecular weight 30- and 39-kDa, respectively (Fig. 2D; Fig. 2F).

R14 and R18 tagged proteins were expressed in the baculovirus system in soluble form and purified using two chromatographic steps: HisTrap HP column and Ni-NTA IMAC column chromatography. As shown in Fig. 2G and Fig. 2.H, the cleaved forms of R14 and R18 migrate to predicted molecular weight of 8 kDa.

3.2. Evaluation of recombinant antigens performance in single and in combination

Performances of ELISA- R915.2, R914.1, R93.1, R14 and R18 were compared with the reference serological method, WB-CS50, using a set of 200 serum samples (epileptic and nonepileptic). Sensitivity, specificity, agreement, and concordance are shown in Table 1. In this study, the sensitivity, specificity, concordance, and positive likelihood ratio of the standard ELISA-CS50 in comparison with the WB, were respectively 45.5% (34.1–57.2), 87% (79.7–92.4), 71% and 3.494. In single, recombinants showed low sensitivity values, ranging from 21% (11.7–33.2) to 38.1% (27.2–50). Specificity ranged from 58% (44.9–63) to 78% (69.2–84.8). The recombinant R915.2 gave the highest sensitivity value 38.1% (27.2–50) and the lowest specificity value 58% (44.9–63). The agreement with WB ranged from 48% to 60%, but the positive likelihood ratio was greater than 1.475 for only one recombinant antigen, R93.1.

To improve recombinants performance, two combination strategies were used, i.e. alternative (one antigen OR the other) and cumulative (one AND the other). The former strategy aims to increase sensitivity

Table 1

Performance evaluation of ELISAs with CS50 and recombinant antigen in single formula as compared to WB-CS50.

Antigens	Diagnostic index			
	Sensitivity (%)	Specificity (%)	Agreement (%)	Positive likelihood ratio
	(CI95%)	(CI95%)		
ELISA-CS50	45,5 (34,1–57,2)	87 (79,7–92,4)	71	3494
R14	25 (15–37,4)	62,2 (52,8–70,9)	49,2	0,661
R18	21 (11,7–33,2)	74,1 (65,2–81,9)	56	0,811
R93.1	33 (22,7–44,4)	78 (69,2–84,8)	60	1475
R914.1	28,6 (18,8–40)	61,8 (52,6–70,4)	49	0,748
R915.2	38,1 (27,2–50)	58 (44,9–63)	48	0,83

while the latter supports specificity (Table 2; Table 3). For this analysis, only results were statistically combined and recombinant antigens were not mixed during ELISA. For the first alternative strategy, sensitivity and specificity ranged from 29.6% (18.6–42.6) to 35.7% (27–45.1) and from 58.2% (45.6–70.2) to 60.8% (52–69.6) respectively. In the same line, concordance and positive likelihood ratio ranged from 39.3% to 55% and 0.69 to 1.1. The combination R14+R18+R915.2 gave the lowest concordance value (39.3%), while the combination R18+R93.1 showed the highest one (55%). As for the intrinsic parameters, the combination R14+R18 gave the lowest value of sensitivity and the combination R18+R93.1 gave the highest value of specificity. For the cumulative combination strategy, sensitivity and specificity ranged from 8.3% (3.1–17.3) to 35.1% (24.5–46.8) and from 51% (40.8–61.1) to 90.1% (83.3–94.8) respectively. For likelihood, it ranged from 0.49 to 1.452. The combination of R18+R914.1 yielded the lowest value for sensitivity and the combination of R18+R93.1 yielded the largest value for specificity.

From the 200 individuals included in this study and described above, a subgroup of 34 epileptic patients with positive serology in ELISA-CS50 was selected (Table 4). Most patients were male and more than 5 years. Up to 83% of cases had generalized seizures with clonic-tonic manifestation (87%–100%). However, most epileptic patients had no brain CT scan available. ELISA positivity was 44.22% (15/34), 38.24% (13/34), 29.41% (10/34), 24% (8/34) and 32.4% (11/34) with R915.2, R914.1, R93.1, R18 and R14, respectively.

4. Discussion

The aim of this study was to identify new recombinant antigen candidates for the development of a rapid easy-to handle test for cysticercosis diagnosis in epileptic patients from endemic areas (World Health Organization, 2022). This test should serve as a first screening tool for epileptic positive serology patients who may then undergo imaging tests to confirm diagnosis. The reference method for the detection of anti-cysticercus IgG is the WB using the CS50 glycosylated fraction of membrane proteins (Tsang et al., 1989). In daily practice, WB is used to confirm ELISA positive results; in the present study, the sensitivity of the ELISA-CS50 compared with the WB was only 45.5%. The new set of antigens tested during this study includes five recombinant proteins: R915.2, R914.1, and R93.1, originating from the liquid of cyst, and two new preparations of recombinant 14kD and 18kD proteins (R14 and R18). Blood samples were obtained from a pool of 200 epileptic/non-epileptic individuals. Antigens were evaluated in single and in combination for ELISA compared to the reference method. R14 and R18 were expressed in the *Baculovirus-Infected Insect Cell System*. Unlike *E. coli*, the eukaryotic system can perform the post-translational

Table 2
Performance evaluation of ELISAs using alternative combination of recombinant antigens as compared to WB-CS50.

Antigens	Diagnostic index			
	Sensitivity (%) (CI95%)	Specificity (%) (CI95%)	Agreement (%)	Positive likelihood ratio
R18+R914.1	37,7 (26,9–49,4)	45,5 (36–55,2)	42,3	0,69
R14+R18	29,6 (18,6–42,6)	59,5 (50–68,5)	49,1	0,728
R14+R18+R914.1	47,8 (35,4–60,4)	42,5 (33,5–51,9)	44,4	0,831
R14+R914.1	46,3 (34–59)	44 (35–53,1)	45	0,823
R18+R914.1+R915.2	51 (38,1–63,4)	43 (34–52,3)	46	0,89
R14+R915.2	50 (37,5–62,6)	40 (31,1–49,4)	44	0,833
R14+R914.1+R915.2	54,6 (41,9–66,9)	36,7 (28,1–46)	43	0,861
R18+R915.2	47 (34,6–59,7)	46,6 (37,4–56)	46,7	0,88
R914.1+R915.2	42,1 (31–54)	49,2 (40,1–58,3)	46,5	0,829
R14+R18+R93.1	50 (37,6–62,4)	48 (38,7–57,2)	48,7	0,96
R14+R18+R915.2	44,7 (33,4–56,6)	35,7 (27–45,1)	39,3	0,695
R18+R93.1	43,5 (31,6–56)	60,8 (52–69,6)	55,0	1,1
R93.1+R914.1	34,5 (25,1–44,6)	53,3 (44,1–62,4)	44,8	0,735
R93.1+R915.2	42,9 (31,6–54,6)	50,4 (41,3–59,5)	47,5	0,864
R14+R93.1	46,4 (34,3–58,8)	48,7 (39,5–58,1)	47,9	0,905
R14+R93.1+R914.1	58,2 (45,6–70,20)	36,7 (28,1–46)	44,4	0,919
R14+R93.1+R915.2	54,4 (41,9–66,5)	36,7 (28,1–46)	43,1	0,859
R18+R93.1+R914.1	55,2 (32,6–67,4)	44,2 (35,11–53,6)	48,1	0,989
R18+R93.1+R915.2	51,5 (39,1–63,8)	43,5 (34,5–52,7)	46	0,908
R93.1+R914.1+R915.2	46,8 (35,3–58,5)	47,2 (38,1–56,4)	47	0,885

modifications of proteins (phosphorylation, acetylation and glycosylation) necessary for protein activity. After cleavage, in SDS-PAGE, R14 and R18 migrated to 8 kDa, which is probably due to the above-mentioned post-translational modifications (Plancarte et al., 1999). Compared with WB, the performances of R18 and R14 in ELISA were, respectively, 21% and 25% for sensitivity, and 74.1% and 62.2% for specificity. Scheel et al. (2005) showed in a study of patients with NCC that the sensitivity of the ELISA-TS14 decreases in case of old infestations where the cysticerci were degenerated (42%) or calcified (30%). Specificity was also low (76%), probably on account of cross-reactivity with other parasites such as *Echinococcus granulosus* and *Schistosoma spp* in individuals free of NCC (Scheel et al., 2005). In the same line, based on an evaluation concerning subjects from Peru with NCC, the QuickELISA™ using the synthetic TS18var1 had a sensitivity that varied according to the stage of development of the parasite and the number of cysticerci in the brain: sensitivity was 96.4% in the presence of multiple cysts; 20.8% in the presence of a single viable cyst; 40% in the case of degenerated cysticerci; and 33.5% in the case of calcified cysticerci (Lee et al., 2011). The diagnostic performances of the recombinant antigens obtained in this study were in the same range as those found in previous studies. Incidentally, it was observed that the baculovirus-Insect cell expression system didn't seem to enhance performance.

The recombinant antigens R915.2, R914.1 and R93.1 were expressed in *E. coli*. Given its fast growth, high yield of the product, and cost-

effectiveness, *E. coli* is the widely used expression host for the heterologous proteins production (Baeshen et al., 2015). However, the overproduction of recombinant proteins often leads to protein misfolding and insoluble aggregates formation (Carrió et al., 1998; De Marco et al., 2007). To overcome this problem, several strategies have been developed to improve protein solubility in *E. coli* such as using fusion partners and affinity tags, optimizing the culture conditions, and co-expressing of recombinant proteins with molecular chaperones (Studier, 2005; Vera et al., 2007; Blommel et al., 2007; Marblestone et al., 2006; Ki and Pack, 2020; De Marco et al., 2007). In this study, a dual affinity tags (10xHis and SBP) and a fusion partner (SUMO) were incorporated into the pET expression vector. Once R915.2, R914.1 and R93.1 have been expressed, the tags were removed as they can interfere with the protein structure and affect its biological activity (Arnaud et al., 2006; Randolph, 2012). The *E. coli* strains were cultivated at 37 °C for 4H, followed by auto-induction at 16 °C overnight. Studies have shown that reducing temperature and using auto-induction media increase the yield of soluble proteins (Studier, 2005; Vera et al., 2007). Herein, R915.2, R914.1 and R93.1 were produced almost in the insoluble form. Neither affinity tags nor culture conditions optimization enhanced proteins solubility.

The recombinant antigens R915.2, R914.1 and R93.1, derived from cysticercus fluid, were supposed to allow detection of late stage of NCC infection, to reduce diagnostic delay for patients attending dispensaries. Their performances in ELISA were globally in the same range as R18 and R14 (sensitivity <38.1% and specificity <78%). By combining three

Table 3
Performance evaluation of ELISAs using cumulative combination of recombinant antigens as compared to WB-CS50.

Antigens	Diagnostic index			
	Sensitivity (%) (CI95%)	Specificity (%) (CI95%)	Agreement (%)	Positive likelihood ratio
R18+R914.1	8,3 (3,1–17,3)	86 (78,1–91,5)	56,55	0,583
R14+R18	18 (9,4–30)	75,9 (67–83,3)	56	0,747
R14+R18+R914.1	23 (13,1–35,5)	68,4 (59,1–76,7)	52,8	0,726
R14+R914.1	10,1 (4,2–19,8)	80,8 (72,8–87,3)	55,7	0,528
R18+R914.1+R915.2	24 (16–33,6)	51 (40,8–61,1)	37,5	0,49
R14+R915.2	16,7 (9–27,3)	75,8 (67,2–83,2)	53,7	0,69
R14+R914.1+R915.2	31,2 (21,1–42,7)	59,3 (50,1–68,1)	49	0,767
R18+R915.2	15,3 (7,9–25,7)	80,8 (72,6–87,4)	56,3	0,797
R914.1+R915.2	24,7 (15,6–35,8)	68,3 (59,3–76,4)	52	0,778
R14+R18+R93.1	18 (9,4–30)	72,4 (65,3–80,3)	53,7	0,654
R14+R18+R915.2	23 (13,2–35,5)	69 (59,7–77,2)	53,1	0,74
R18+R93.1	11,1 (4,9–20,7)	90,1 (83,3–94,8)	60,6	1,12
R93.1+R914.1	15,6 (8,3–25,6)	85,4 (77,9–91,1)	58,5	1065
R93.1+R915.2	26 (16,6–37,2)	82,1 (74,2–88,4)	60,5	1452
R14+R93.1	11,3 (5–21)	89,2 (82,2–94)	60,2	1,04
R14+R93.1+R914.1	25 (15,8–36,2)	71,3 (62,4–79,1)	53,5	0,871
R14+R93.1+R915.2	31,6 (21–43,3)	65,3 (56,1–73,7)	52,3	0,91
R18+R93.1+R914.1	23,7 (14,7–34,8)	73,8 (65–81,3)	54,6	0,903
R18+R93.1+R915.2	30,3 (20,2–41,9)	70,6 (61,3–78,2)	54,9	1017
R93.1+R914.1+R915.2	35,1 (24,5–46,8)	58,5 (49,3–67,3)	49,5	0,846

Table 4
Main features of the epileptic patients (n = 34) with positive serology in ELISA-recombinant antigen.

Antigen	ELISA-recombinant positivity (%) in PWE	Sex (M/ F)	Seizures				CT scan		
			Focal	Generalized	Tonic-clonic	Tonic or clonic	N	A	Unk
R915.2	15/34 (44,12%)	10/5	1/15 (7%)	14/15 (93%)	13/15 (87%)	2/15 (13%)	2/15 (13%)	2/15 (13%)	11/15 (74%)
R914.1	13/34 (38,24%)	9/4	0	100%	12/13 (92%)	1/13 (8%)	4/13 (31%)	2/13 (15%)	7/13 (54%)
R93.1	10/34 (29,41%)	8/2	0	100%	9/10 (90%)	1/10 (10%)	1/10 (10%)	2/10 (20%)	7/10 (70%)
R18	8/34 (25%)	7/1	1/8 (17%)	7/8 (83%)	100%	0	1/8 (17%)	–	7/8 (83%)
R14	11/34 (33,33%)	9/2	1/11 (9%)	10/11 (91%)	100%	0	–	1/11 (9%)	10/11 (91%)

*PWE: Patient with epilepsy, M: Male, F: Female, N: Normal (absence of lesion), A: Abnormal (presence of lesion), UnK: Unknown.

proteins (R14+R93.1+R914.1) using the alternative method, the sensitivity increased to 58.2%. Usually very high sensitivity is required for human cysticercosis screening test since the goal of the latter is to detect epileptic patients with cysticercosis. In contrast, in a cumulative approach, the combination of two proteins (R18+R93.1) had significantly lower sensitivity (11.1%) but higher specificity (90.1%). Specificity is the proportion of true negative individuals who do not have human cysticercosis (Altman and Bland, 1994). However, since the test

is not completely specific (100%), it does produce some false-positive results (i.e. the screening test will be positive in patients free of infection).

In a study conducted by Nhancupe et al. (2016) in Mozambique, in patients with NCC, sensitivity and specificity of TSOLp27 immunoblot compared with the reference serological method, WB, were estimated to be 85.71% and 96.69% respectively (Nhancupe et al., 2016). Recently, *Ts26GST* and *TsTrx-1* recombinant antigens from *T. solium* were evaluated

in EITB to detect anti-cysticercosis antibodies in a small group of thirty three NCC patients (Jiménez et al., 2022). Ts26GST and TsTrx-1 had a sensitivity of 79.5% and 88.6%, respectively. Moreover, the specificity of TsGST26 was 86%, while that of TsTrx-1 was 98% (Jiménez et al., 2022). In addition to the level of antibodies present in the patient blood, recombinant protein conformation was essential to develop sensitive diagnostic tests. Carbohydrate complex played a key role in the antigenicity of glycoproteins (Obregón-Henao et al., 2003). In contrast, previous research on recombinant antigen TS14 has demonstrated that the absence of glycosylation does not affect the antigenicity and specificity of the proteins (da Silva et al., 2006). In small scale studies, some recombinants have reached better level of sensitivity and specificity. Three recombinant antigens TsF78, TsP43 and TsC28 evaluated by ELISA to detect cysticercosis, were reported to have a sensitivity and specificity of 93.8% and 93.3%, 95.0% and 85.4%, and 91.7% and 93.3%, respectively. Despite the good results, these recombinant antigens have not yet been evaluated in open-label population studies (Morillo et al., 2020).

In this study, the new set of recombinant antigens evaluated using human serum showed moderate sensitivity and specificity, consistent with findings in the literature. However, their performance was lower than the WB reference test, as summarized by Rodríguez et al. (2012). This trend is common among available tests, particularly for diagnosing single-lesion NCC or cysts undergoing degenerative processes. Sensitivity of immunological tests for NC in humans is generally poor unless conducted on cerebrospinal fluid, which is uncommon in remote areas but feasible in hospitals. Testing CSF represents another investigative step. Additionally, while screening recombinant antigens is crucial, their performance in lateral flow assays (LFA) could differ due to differences in support. Evaluating the kinetics of test positivity is also important, as antigens from the liquid of the cyst may differ from those of the membrane.

Furthermore, most commercially available ELISAs use native antigens. One potential issue is the binding of recombinant antigens to plastic, which could alter their immunogenicity. Therefore, lateral flow tests (LFT) may significantly enhance the performance of antigens in diagnosing human cysticercosis (Sadaow et al., 2023). In this technique, antigens can be utilized in the mobile phase without binding to plastic (Omidfar et al., 2023; Zhou et al., 2021).

This study also has several key limitations. Firstly, due to financial constraints, CT scans, which are standard for detecting cysts, were not conducted for most epileptic cases. This situation is common in developing countries where patients often bear the cost of their own imaging. This underscores the need for the development of a highly sensitive rapid diagnostic test for human cysticercosis. However, it's important to note that epilepsy combined with positive serology doesn't always correlate with neuroimaging of NCC.

Secondly, the study did not explore the combination of multiple antigens (multiplex ELISA). While multiplex analysis could yield slightly different results due to interactions in the well, the ultimate goal is to develop lateral flow tests (LFT) where antigens are used in separate lines. Therefore, multiplexing antigens in the same well may not be relevant.

Finally, low solubility and the formation of inclusion bodies are common issues in recombinant protein expression in *E. coli*. Improving protein solubility remains a significant experimental challenge, as optimal expression conditions for each protein need to be tested. However, most results were obtained with antigens produced in a eukaryotic system, which is not subject to this solubilization problem.

5. Conclusions

In remote low-income areas, due also to the scarcity of adequate laboratory facilities, the diagnostic tests for human cysticercosis disease are often replaced with screening tests. Patients with suspected NCC need to travel out of their areas and pay costly neuroimaging to confirm the diagnosis. Evaluation of the performance of antigens based in a

lateral flow format will therefore be the subject matter of subsequent study.

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CRedit authorship contribution statement

Jihen Melki: Writing – original draft, Visualization, Investigation. **Thierry-Borel N'dri Kouadio:** Investigation, Data curation. **Mireille Nowakowski:** Resources. **Zara Razafiarimanga:** Resources. **Man-Koumba Soumahoro:** Writing – original draft, Validation, Methodology. **Stephane Peltres:** Resources. **Ronan Jambou:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

This manuscript is not accepted for publication or under editorial review for publication elsewhere. No potential competing interest has to be declared and all authors have approved the manuscript for submission.

Data availability

Data will be made available on request.

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