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# Isolation of a 14 kDa antigen from *Taenia solium* cyst fluid by HPLC and its evaluation in enzyme linked immunosorbent assay for diagnosis of porcine cysticercosis

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**Abstract**

A fraction with a major band of 14 kDa was obtained from crude cyst fluid of *Taenia solium* cysticerci by 2-step chromatography. A first fraction isolated by gel filtration (Sephacryl S-300 high resolution) was purified using an anion exchange column (Mono Q HR 5/5) on high performance liquid chromatography. Evaluation of the analytic sensitivity of this fraction (F3) was carried out in an antibody enzyme linked immunosorbent assay (Ab-ELISA-F3) using serum samples from pigs experimentally infected with different doses of *T. solium* eggs. The cross-reactivity of F3 was evaluated with serum samples from pigs that were naturally or experimentally infected with *Taenia hydatigena*, *Taenia saginata asiatica*, *Fasciola hepatica*, *Trichinella spiralis*, *Metastrongylus apri*, *Trypanosoma congolense* and *Sarcoptes scabiei*, and with serum samples of rabbits hyper-immunised with metacestode cyst fluid of *T. hydatigena* and *T. solium*. Antibody titres of lightly or heavily infected pigs differed in their kinetics. However, the increase in F3-specific antibodies could not be related to the infection level. Analysis of the specificity of the F3 showed that serum samples of pigs infected with other parasites did not recognise this antigen. Cross-reaction with *T. hydatigena* occurred in ELISA using cyst fluid as antigen, but the F3 antigen fraction was not recognized by rabbit hyper-immune serum samples to *T. hydatigena*. Evaluation of the diagnostic sensitivity and specificity of the Ab-ELISA-F3 was done by a non-parametric receiver operating characteristic (ROC) analysis using 66 serum samples from Zambian village pigs. The total number of cysticerci of these pigs was determined by dissection (28 pigs harboured *T. solium* cysticerci and 38 were negative at dissection). In addition, 58 serum samples from Cameroonian pigs (28 pigs from cysticercosis-free farms and 30 pigs with cysticerci at tongue inspection) were used in a separate ROC analysis. The results from the ROC analysis yielded a low diagnostic value (area under ROC curve = 0.48) with the sera from the Zambian pigs while a relatively high diagnostic value was obtained with the sera from Cameroonian pigs (area under ROC curve = 0.78). The main factor contributing to a low diagnostic value based on the Zambian serum samples seemed to be the false-positive reactions that were likely caused by the occurrence of transient antibodies in the non-infected animals.


*Keywords:* *Taenia solium*; Cyst fluid; Purification; ELISA; ROC; Evaluation; Sensitivity; Specificity

## 1. Introduction

Cysticercosis is a zoonotic disease caused by *Taenia solium* metacestodes in humans and pigs. Neurocysticercosis (NCC), resulting from the establishment of cysticerci in the human brain and the subsequent host reaction, is the greatest cause of acquired epilepsy worldwide (Commission on Tropical Diseases of the International League against Epilepsy, 1994). The development of improved diagnostic techniques has contributed to our knowledge on the importance of cysticercosis both in pigs and humans. Accurate

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diagnosis of human cysticercosis can be performed with magnetic resonance imaging (MRI) and computed tomography (CT), that are also useful to classify NCC in active, inactive or mixed forms (Sotelo et al., 1985; Carpio et al., 1998). However, these techniques are expensive and inaccessible for populations from areas at risk (Diaz et al., 1992). Immunodiagnosis (Engvall and Perlman, 1971) is more accessible and less expensive than the neuroimaging techniques. Many efforts have been made to improve the diagnostic capacities of immunodiagnostic tests in *T. solium* cysticercosis (Dorny et al., 2003; Ito and Craig, 2003).

The serological diagnosis of porcine cysticercosis includes antibody and antigen detection. Some tests for the detection of antibodies using crude antigen preparation showed a high sensitivity (Biagi and Tay, 1958; Herbert and Oberg, 1975; Pathak et al., 1984; Pinto et al., 2000). However, cross-reactions between crude antigens of *T. solium* and other helminths are a major problem (Kumar and Gaur, 1987; Cheng and Ko, 1991). Several studies have shown that low molecular weight glycoproteins (10–26 kDa) isolated from cyst fluid of *T. solium* cysticerci provide high specificity and sensitivity for serological diagnosis of cysticercosis (Gottstein et al., 1986; Tsang et al., 1989; Ito et al., 1998; Ko and Ng, 1998; Yang et al., 1998). Lentil–Lectin affinity chromatography and iso-electric focusing were the techniques used to isolate these low molecular weight antigens.

In this study, we attempted to purify cyst fluid of *T. solium* cysticerci by 2-step high performance liquid chromatography (HPLC) to obtain species-specific low molecular weight native antigens.

## 2. Materials and methods

### 2.1. Crude Cyst fluid (CF) preparation and purification

Fresh *T. solium* cysticerci were obtained from naturally infected pigs in Dschang, Cameroon. The host membrane tissues, which covered the cysticerci were gently removed. After washing 3 times in cold 0.15 M NaCl, the cysticerci were stored at −30 °C until fluid extraction. The cyst fluid was aspirated with a needle from the intact and thawed cysts, pooled and centrifuged at 18,000*g* for 30 min at 4 °C. The supernatant (CF) was collected, lyophilized and stored at 4 °C until further processing.

An automated liquid chromatography system (ÄKTA basic 100, Amersham Pharmacia Biotech, Uppsala, Sweden) with a fraction collector (Frac 901, Amersham Pharmacia Biotech) was used to separate proteins from CF. The system was controlled by UNICORN Software (UNICORN V3, Amersham Pharmacia Biotech).

Forty milligrams of lyophilized CF were dissolved in 5 ml of Tris–HCl buffer (20 mM Tris–Base, pH 7.2) and loaded onto a gel filtration column (HiPrep 16/60 Sephacryl S-300 HR (120 ml), Amersham Pharmacia Biotech, Uppsala, Sweden) through the injection valve connected to a sample loop of the HPLC system. The system was run at room temperature at a flow rate of 0.5 ml $min^{-1}$. A Tris–HCl buffer (20 mM Tris–Base, pH 7.2) was used as elution buffer. A UV monitor (part of the HPLC system) was used to detect the eluted protein in the fractions (UV-1, 280 nm) Fractions of 2 ml were collected. The fractions corresponding to individual peaks were pooled, dialyzed and lyophilized. Analysis of the proteins was performed with 12% or 15% standard polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli (1970). Protein loads of 10 μg were used. The gel was stained with Coomassie Brilliant Blue R250 (SIGMA). Molecular weight markers from MBI Fermentas (Germany) and from Amersham Pharmacia Biotech (Uppsala, Sweden) were used as molecular weight reference for Coomassie Brilliant Blue and Silver-staining, respectively. After electrophoretic analysis the peak containing the major protein band of 14 kDa was retained for further purification.

The fraction containing the 14 kDa peak obtained by gel filtration was dialyzed against Tris–HCl buffer (20 mM Tris–Base, pH 7.2) and lyophilized. Then lyophilized fractions were dissolved to the appropriate concentration in Tris buffer on an anion exchange column (Mono Q HR 5/5, Amersham Pharmacia Biotech, Uppsala, Sweden). Buffer A (Tris–HCl buffer, pH 7.5) and buffer B (1 M NaCl in Tris buffer, pH 7.5) were used, respectively as equilibration buffer and elution buffer. The fraction was eluted by gradient concentration of buffer B (0–100% B) in buffer A at a flow rate of 1 ml $min^{-1}$ at room temperature. The gradient length of 20 ml was programmed using Unicorn software. The eluted protein was detected as described for the gel filtration. The size of collected fractions was 1.75 ml per tube. The collected fractions of the same peak were pooled and dialyzed against Tris 20 mM Tris buffer (overnight at 4 °C). A protein assay kit (SIGMA diagnostics) determined the protein concentrations of the fractions. The pooled fractions were also analyzed by SDS–PAGE. Protein loads of 1.5 μg were used and the gel was silver-stained. The pooled fraction showing apparent purity was selected as purified cyst fluid (F3).

### 2.2. Evaluation of the analytic sensitivity and specificity of the purified fraction (F3)

The purified antigen was evaluated and compared with CF antigen by enzyme-linked immunosorbent assay (ELISA). The optimal dilutions of antigens, test serum samples and conjugate were determined by checker-board titration. ELISA was performed on polystyrene plates (Nunc® Maxisorp). The antigens (CF or F3) were diluted at 0.5 μg/ml in a carbonate buffer (0.06 mol, pH 9.6) for coating (1 h at 37 °C and overnight at 4 °C). Washing between steps was done with phosphate buffered saline containing 0.05% Tween 20 (PBS-T20). Blocking and incubation of serum samples and conjugate were done on a shaking plate for 1 h at 37 °C. PBS-T20 + 2% gelatine was used for blocking. Serum samples were diluted at 1/

500 in PBS-T20 + 0.1% gelatine + EDTA 5 mM. A rabbit anti-pig IgG peroxidase conjugate (SIGMA) was used at a dilution of 1/30,000. The 3,3′5,5′tetramethylbenzidine (TMB) chromogen solution (KPL, Gaithersburg, USA) was used as the substrate. The reaction was stopped by adding 1 M phosphoric acid ($H_3PO_4$) to each well. Optical densities were measured at 450 nm with a microplate reader (Multiscan EX, Termo Labsystem).

Serum samples from five pigs, experimentally infected with *T. solium* eggs (34 sera at different weeks post-infection) obtained from Cameroon, were used to determine the ability of the purified fraction to detect antibodies against *T. solium*. The test conditions of these experimental infections were described by Nguekam et al. (2003). Those pigs had been slaughtered and necropsied for total cysticerci counts at the end of the experiment. In pigs IV-1 and IV-2, infected with whole gravid proglottids, more than 3000 viable cysticerci per kg of thigh muscle were counted; whereas in pigs I-4 and II-3, infected with 1000 and 10,000 eggs respectively, only three cysticerci were found in the whole carcass. In pig III-4, infected with 100,000 eggs, only one cysticercus was found at necropsy (Nguekam et al., 2003).

The cross-reactivity of F3 was evaluated with serum samples from pigs that were naturally (NI) or experimentally infected (EI) with different parasites: *Taenia hydatigena* (10 NI pigs), *Taenia saginata asiatica* (8 EI pigs), *Fasciola hepatica* (2 NI pigs), *Trichinella spiralis* (3 EI pigs), *Metastrongylus apri* (2 EI pigs), *Trypanosoma congolense* (6 EI pigs) and *Sarcoptes scabiei* (5 NI pigs). In addition, serum samples of rabbits hyper-immunised with metacestode cyst fluid of *T. hydatigena* (1 rabbit) and *T. solium* (1 rabbit) were also used. Negative serum samples from 16 Cameroonian pigs and serum samples from the above five experimentally infected pigs obtained at week 16 post infection were used as controls. The mean optical density of the negative controls plus three standard deviations was taken as the cut-off value.

For the serum of hyper-immunised rabbits, the standardised ELISA was similar to that used for pigs, but with some minor modifications: the conjugate anti-rabbit IgG (Sigma) was diluted 1/20,000; blocking was done with PBS-Tween 20–2% new born calf serum.

### 2.3. Evaluation of the diagnostic sensitivity and specificity of F3 antigen

The diagnostic sensitivity was defined as the proportion of pigs infected with *T. solium* cysticerci identified as positive by ELISA, and the diagnostic specificity was defined as the proportion of non-infected pigs identified as negative by ELISA.

For diagnostic evaluation of F3, serum samples of Zambian village pigs ($N = 66$; 38 non-infected pigs and 28 pigs harbouring cysticerci at carcass dissection) (Dorny et al., 2004b) and serum samples of pigs originating from Cameroon ($N = 58$; 28 pigs from cysticercosis – free commercial farms and 30 pigs with cysticerci at tongue inspection (Pouedet et al., 2002)) were used.

The diagnostic ability of the ELISA test was determined with non-parametric receiver operating characteristic (ROC) analysis (StataCorp, 2001. Stata Statistical Software, Release 7.0. Stata Corporation 2001, College Station, TX).

## 3. Results

### 3.1. Purification of CF of T. solium

Three major peaks were resolved by gel filtration of the CF (Fig. 1). The fractions, corresponding to each peak, were collected separately (Peak 1, Peak 2, Peak 3).

The SDS–PAGE profile of Peak 1 and Peak 2 showed several protein bands ranging between 10 and 116 kDa, while Peak 3 showed a single prominent band of about 14 kDa. Peak 3 was selected for a second purification by anion exchange chromatography.

Peak 3 showed a small amount of bound proteins on the anion exchange column (Fig. 2a). Silver-staining following SDS–PAGE (Fig. 2b) of the unbound fractions (F3) showed a prominent protein band at 14 kDa. Based on the apparent purity of this protein fraction, F3 was selected as antigen for further evaluation in an ELISA for antibody detection. Due to the small eluted quantity, the fraction that bound to the anion exchange column was not analysed by SDS–PAGE.

### 3.2. Analytic sensitivity and specificity evaluation of CF and F3 antigens

The results of the ELISA using CF and F3 as antigens for detecting antibodies in serum samples of experimentally infected pigs are shown in Fig. 3. Using CF as antigen, the absorbance values of serum samples from heavily infected pigs (IV-1 and IV-2) were higher compared to those of lightly infected pigs (I-4 and II-3). Pig III-4 that had been infected with 100,000 eggs but, in which at autopsy only one viable cyst was detected, showed increased absorbance values in serum from weeks 8 and 12 post-infection. In contrast, with the use of F3 there was an increase in optical densities in serum from lightly infected pigs I-4 and II-3 between weeks 2 and 4 post-infection, while optical densities in serum from the heavily infected pigs (IV-1 and IV2) and in pig III-4 showed only a slight increase after week 12. With the ab-ELISA-F3 antibody levels of all animals returned to almost pre-infection levels at week 24.

Fig. 4 shows the results of the ELISA's with CF and F3 as antigens in which the reactivity of serum samples from pigs infected with other parasites was assessed. No cross-reactions were recorded with 36 serum samples from pigs infected with other parasites using the F3 antigen. With CF, cross-reactions were observed with some sera of pigs naturally infected with *T. hydatigena* cysticerci, *F. hepatica* or *M. apri*. No cross-reaction was observed with F3 antigen

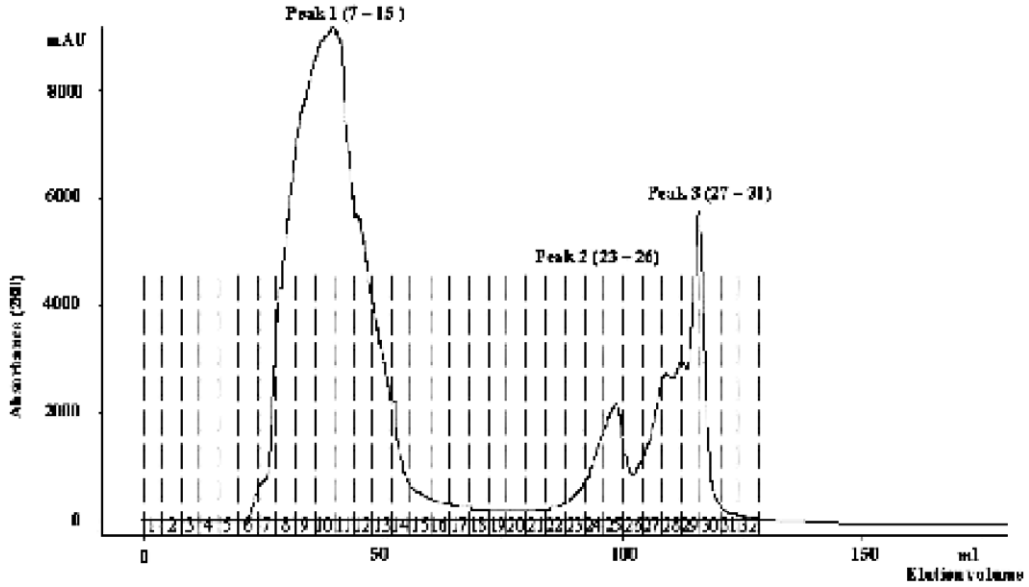


Fig. 1. Fractionation of CF of *T. solium* by gel filtration and anion exchange chromatography: The absorbance value is depicted on the *Y*-axis and is relative to the protein concentration in the fractions. The *X*-axis shows the eluted volume in relation to the number of the fraction. Plain line: Absorbance at 280 nm (UV1); dashed lines: fractions.

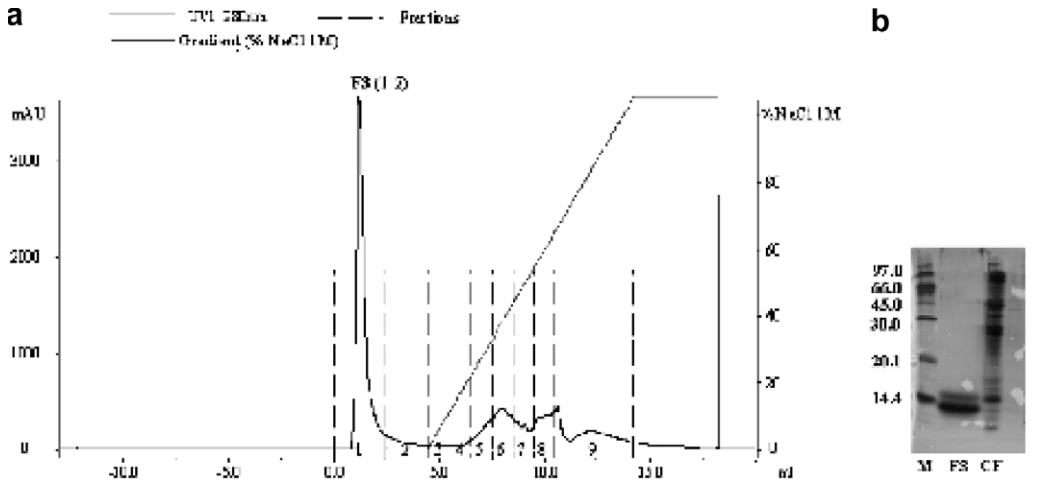


Fig. 2. Purification of Peak 3 using an anion exchange column (Mono Q HR) on HPLC. (a) Elution profile at 280 nm of the anion exchange purification (Mono Q HR) of Peak 3 fraction of *T. solium* cyst fluid. (b) Silver-stained SDS–PAGE (15% gel) profiles of F3 fraction of *T. solium* cyst fluid purified by anion exchange chromatography (final purified protein) (1.5 μg of protein was loaded).

in serum of rabbits that were hyper-immunized with crude cyst fluid of *T. hydatigena* metacestodes (Fig. 5).

### 3.3. Diagnostic sensitivity and specificity of CF and F3 antigens

The ROC curves and area under the curve (AUC) for Ab-ELISA-CF and Ab-ELISA-F3 on serum samples from Zambian village pigs are shown in Fig. 6a. The AUC can be interpreted as the probability that the ELISA result of a randomly drawn sample event is correct with regard to the infection status. The AUC for CF (0.69) is higher than for F3 (0.48). At a selected cut-off value where the sensitivity = specificity, more than 50% of negative Zambian pigs were false positive in the Ab-ELISA-F3 (data not shown).

Fig. 6b shows the ROC curves for the Ab-ELISA-CF and Ab-ELISA-F3 calculated from the data of Cameroonian pigs. The area under the curve (AUC) for both antigens in Fig. 6b is similar (0.81 and 0.78, respectively for CF and F3).

## 4. Discussion

Glycoproteins of *T. solium* metacestode extracts have been characterised and showed evidence of specificity for the diagnosis of *T. solium* cysticercosis (Grogl et al.,

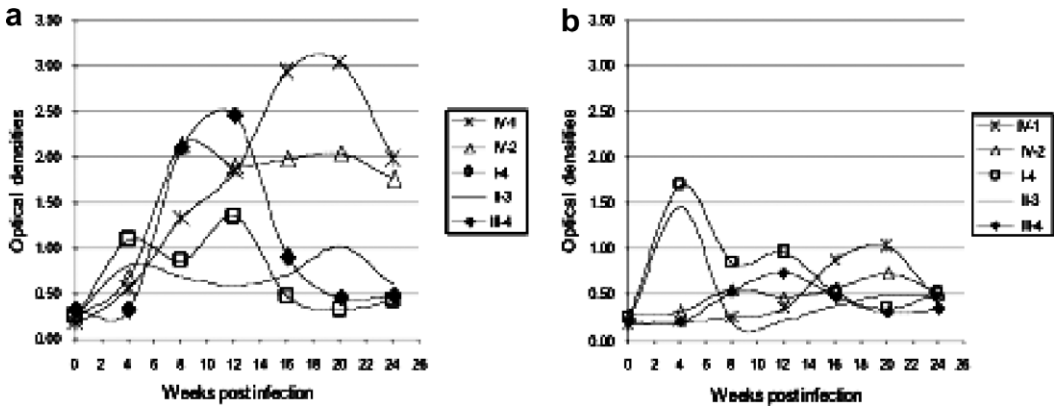


Fig. 3. Comparison of kinetics of circulating antibodies detected by CF and F3 antigens in serum samples of heavily or lightly *T. solium cysticerci* infected pigs IV1, IV2: heavily infected pigs (>3000 cysticerci $kg^{-1}$); I4, II3 and III4: lightly infected pigs (1–3 cysticerci in whole carcass) (Nguekam et al., 2003).

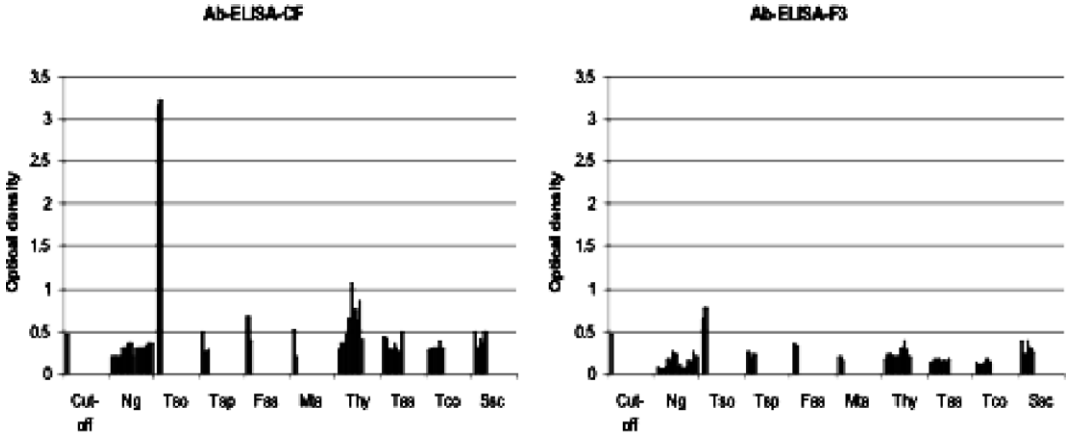


Fig. 4. Testing the cross-reactivity of cyst fluid of *T. solium* in the Ab-ELISA-CF and of the purified fraction in the Ab-ELISA-F3 using serum samples of pigs infected with heterologous parasitic infections. Ng = Negative control; Tso = *Taenia solium*; Thy = *Taenia hydatigena*; Tas = *Taenia saginata asiatica*; Fas = *Fasciola hepatica*; Tsp = *Trichinella spiralis*; Mta = *Metastrongylus apri*; Tco = *Trypanosoma congolense*; Ssc = *Sarcoptes scabiei*.

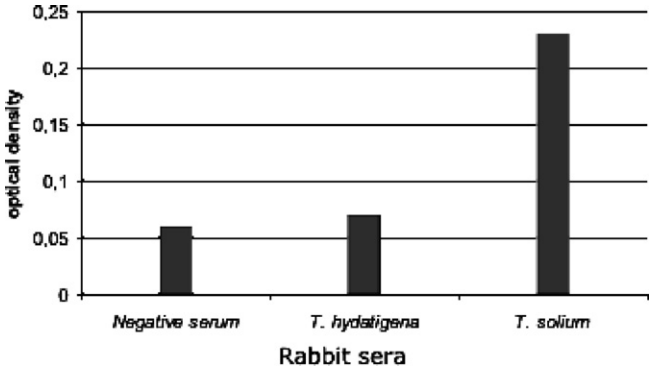


Fig. 5. Testing the cross-reactivity of the F3 fraction of *T. solium* using polyclonal rabbit antiserum against *T. hydatigena* cyst fluid.

1985). Furthermore, several studies have demonstrated that the 10–26 kDa glycoprotein components of cysticerci are the ones showing the highest specificity for serodiagnosis of cysticercosis (Gottstein et al., 1986; Tsang et al., 1989; Ito et al., 1998). The purpose of this work was to isolate *T. solium*-specific antigens with a molecular weight between 10 and 26 kDa from crude *T. solium* cyst fluid by HPLC. Using Sephacryl S-300 HR gel filtration in combination with anion exchange, a protein fraction with a major band of 14 kDa on SDS–PAGE was isolated and considered in this work as a purified fraction (F3).

In the ELISA's using CF and F3 as antigens, antibodies were detected from week 4 to 8 post-infection in serum samples from experimentally infected pigs (Fig. 3). This agrees with the results of Sato et al. (2003), who observed in an ELISA using glycoproteins purified by iso-electric focusing as antigen, that the antibody response of heavily or lightly infected pigs started 30 and 60 days after infection, respectively. In the Ab-ELISA-CF, antibody response was more or less related to the infection dose and/or the establishment rate of the parasites. In contrast, the optical densities in the Ab-ELISA-F3 were higher in two lightly infected pigs than in the heavily infected pigs between weeks 2 and 6 after infection. This difference is difficult to explain, but may be related to the infection dose. It is known that high doses of antigen might induce immunotolerance (Tizard, 1992), which could explain the lower antibody levels in the heavily infected animals. However, for

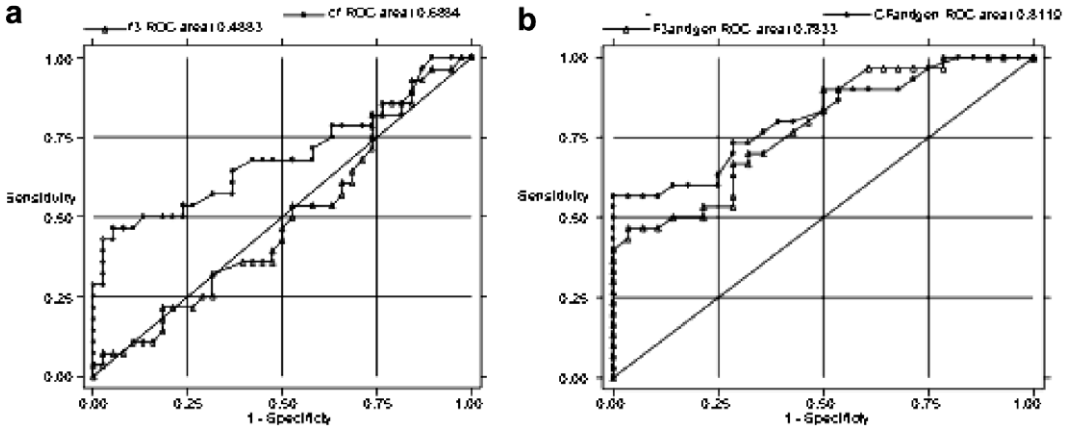


Fig. 6. (a) Comparison of non-parametric ROC curves of ELISA's using CF and F3 as antigen on 66 reference serum samples from Zambian pigs infected or not with *T. solium*. (b) Comparison of non-parametric ROC curve for ELISA using CF and F3 as antigen on 58 serum samples from Cameroonians pigs infected or not with *T. solium*.

all animals in this study, including IV-1 and IV-2 that showed a high parasitic load at autopsy, antibody levels to F3 decreased to pre-infection levels between 2 or 3 months after their appearance. This finding is in contrast with the often-reported problem, encountered with antibody-detecting tests, of persistence of antibodies for months after disappearance of viable cysticerci (Dorny et al., 2004a). F3 might be an antigen expressed early during metacestode development, and hence, inducing an early antibody response. While a test that fails to detect active infection is not desirable for diagnosis or sero-prevalence studies, the Ab-ELISA-F3 might be useful in epidemiological studies to measure the incidence of *T. solium* cysticercosis by the detection of recently infected animals.

In addition, no cross-reaction was observed with the other parasites used in this study suggesting that F3 has a good analytic specificity (Jacobson, 1998). However the species-specificity of this purified fraction should be further examined by testing the cross-reactivity with the other species of *Taenia.*

In this study we used a non-parametric receiver operating characteristic (ROC) analysis to evaluate the diagnostic potential of the Ab-ELISA-F3. In practice, cut-off values of ELISA's and other quantitative serodiagnostic tests are often established as the mean plus two- or three-fold standard deviation of the results observed with the sera from a negative reference population to estimate the sensitivity and specificity of a test (Richardson et al., 1983). This procedure is not adequate if the test values follow a skewed or multinomial distribution (Barajas-Rojas et al., 1993). Cut-off values can also be calculated by comparing the optical density of each sample with the mean of a series of eight sera (Dorny et al., 2000). This assumption only holds true if the sample is represented by only a single variable, so it does not contribute to the degrees of freedom or to the estimate of the variance within groups (Sokal and Rohlf, 1981). ROC analysis (Zweig and Campbell, 1993) can describe the sensitivity and the specificity of a test as a function of the selected cut-off. Highly discriminatory tests have ROC curves that crowd toward the upper left corner with area under curve (AUC) approaching 1.

When using the serum samples from Zambia we found that the Ab-ELISA-F3 had a low diagnostic value: the area under curve (AUC) was lower than 0.5. We found a higher diagnostic value (AUC) using serum samples from Cameroonian pigs. This difference may be due to the selection of the serum samples in these two countries. In Cameroon, the positive samples originated from village pigs that were found positive on tongue palpation, suggesting heavy infection (Dorny et al. (2004b), whereas the negative samples were collected from pigs living in an area free of *T. solium*. The Zambian samples all originated from village pigs living in hyper-endemic areas, in which the total number of cysticerci was determined by dissection (Dorny et al., 2004b). This group comprised heavily and lightly infected pigs as well as negative pigs. In this group of animals it cannot be excluded that pigs, found uninfected at autopsy, had specific antibodies because they had been exposed to infection or had previously been infected. Pigs living in cysticercosis-endemic areas may develop a transient antibody response following exposure to oncospheres. This phenomenon was reported in human cysticercosis by Garcia et al. (2001). The difference in test performance when applied on different series of samples confirms that the sensitivity and specificity of a test vary with characteristics of the population to which they are applied (Greiner and Gardner, 2000). Because a ROC curve plots the sensitivity versus the false positive rate (1-specificity) of a test to select an optimal cut-off to distinguish between infected and non-infected animals, the use of a population from an endemic cysticercosis area could lead to a lower diagnostic specificity at any selected cut-off. A similar observation was reported in the diagnosis of bovine brucellosis: the specificity of a serologic test for *Brucella abortus* is higher when the test is used in a non-vaccinated population (Dohoo et al., 2003). The absence of antibodies (no immune response against *B. abortus*) may explain this difference. However, another drawback of our study is the low sample size used

for the determination of the diagnostic sensitivity and specificity, which in turn might influence the outcome of the ROC analysis. The F3 fraction should be evaluated on a larger number of samples before definitive conclusions can be drawn.

In conclusion, this study demonstrated that the purified 14 kDa fraction (F3) performed well in ELISA when considering the analytical sensitivity and specificity. When applied on field samples the performances of the F3-ELISA were however, lower than these of a crude cyst fluid antigen and dependent of the sample population.

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