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CHARACTERIZATION AND OPTIMIZATION OF BOVINE *Echinococcus granulosus* CYST FLUID TO BE USED IN IMMUNODIAGNOSIS OF HYDATID DISEASE BY ELISA

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SUMMARY

The aim of this work was to assess the influence in the diagnostic value for human hydatid disease of the composition of bovine hydatid cyst fluid (BHCF) obtained from fertile (FC) and non-fertile cysts (NFC). Eight batches from FC and 5 from NFC were prepared and analysed with respect to chemical composition: total protein, host-derived protein, carbohydrate and lipid contents. No differences were observed in the first two parameters but carbohydrate and lipid contents were shown to be higher in batches from FC than in those from NFC. Bands of 38 and 116 kD in SDS-PAGE profiles were observed to be present in BHCF from FC only. Two pools were prepared from BHCF batches obtained from FC (PFC) and NFC (PNFC), respectively. Antigen recognition patterns were analysed by immunoblot. Physicochemical conditions for adsorption of antigens to the polystyrene surface (ELISA plates) were optimized. The diagnostic value of both types of BHCF as well as the diagnostic relevance of oxidation of their carbohydrate moieties with periodate were assessed by ELISA using 42 serum samples from hydatid patients, 41 from patients with other disorders, and 15 from healthy donors. Reactivity of all sera against native antigen were tested with and without free phosphorylcholine. The best diagnostic efficiency was observed using BHCF from periodate-treated PFC using glycine buffer with strong ionic strength to coat ELISA plates.

KEYWORDS: *Echinococcus granulosus*; Bovine hydatid cyst fluid; Hydatidosis; Immunodiagnosis

INTRODUCTION

Unilocular hydatidosis is a disease caused by infection with the metacestode stage of the dog tapeworm, *Echinococcus granulosus*. This is recognised as one of the world's relevant zoonoses, affecting both humans and their domestic animals[20].

Immunodiagnosis of this disease in humans can be achieved by one or a combination of different techniques, e.g. indirect haemagglutination[26,38], immunoelectrophoresis (IEF)[27,39], latex agglutination[2,27,31], immunoblotting[15,33], counterimmunoelectrophoresis[12], ELISA[1,11,14]. The usual source of antigen for immunodiagnosis of hydatid disease is the fluid from sheep cysts. In Uruguay, cattle are infected as well as sheep and bovine cysts, which are frequently fertile, are generally larger than those from sheep. Therefore, availability of bovine cysts is higher than that of sheep cysts[2].

Hydatid cyst fluid is a complex mixture of parasite-derived and host-derived molecules. It contains several antigens derived from the metabolism of the parasite together with many components from the host[28]. Therefore, the type and concentration of parasite-derived molecules are likely to be very different in fertile and non-fertile cysts.

One of the major parasite antigens in hydatid fluid is antigen 5 (Ag5). Ag5 cross-reacts with human antibodies to other cestode, trematode and nematode parasites and part of this cross-reactivity is associated with the presence of phosphorylcholine bound to its 38 kD subunit[17,32]. As in the case of other helminths cross-reaction may also be associated with carbohydrate epitopes[7], thus reducing specificity and sensitivity of diagnostic assays. Data obtained in our laboratory suggest that carbohydrate epitopes in hydatid cyst fluid antigen are immunodominant both in natural[34] and experimental hydatid infection[9].

Sensitivity and reliability of ELISA depend on composition, concentration and stability of the adsorbed antigen. The use of an appropriate antigen concentration may be necessary to saturate the polystyrene surface with relevant antigen[22]. Moreover, antigen concentration is crucial to avoid artifacts which may be observed due to differences in antibody affinity, if an inappropriate concentration of antigen is employed[24,25].

In this context, the aim of this work was to assess the usefulness as well as the influence of the composition of bovine hydatid cyst fluid (BHCF) for the immunodiagnosis of human hydatid disease by ELISA. Physicochemical conditions necessary for adsorption of antigens to

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polystyrene surface was optimized. In order to improve the diagnostic sensitivity and specificity, the effects on ELISA of oxidation of carbohydrate epitopes and inhibition by free phosphorylcholine were also assessed.

MATERIALS AND METHODS

Antigens

Hyaline hydatid cysts were collected from cattle livers and lungs within 24 h of slaughter and processed according to CAROL *et al*., 1989 with slight modifications, sodium azide (NaN3) (1 g/l) and EDTA (Ethylenediaminetetraacetic acid) (10 mM) were immediately added to the BHCF, the former as a conservative and the latter as a protease inhibitor[19]. A total of 13 batches of BHCF were prepared and characterized, 8 from fertile cysts (FC) and 5 from non-fertile cysts (NFC). FC indicates that viable protoscoleces (PSC) were found in the fluid. Viability was evaluated by 5% eosin exclusion[29] and flame cell activity[10].

Two pools were prepared from BHCF batches by mixing equal volumes of each batch, one from the 8 FC (PFC) 11,3 l and the other one from 5 NFC (PNFC) 8.7 l, respectively. These pools were dialysed against distilled water through a kidney dialysis cartridge, freeze-dried and stored at 4 °C.

Sera

The serum samples were collected from 42 surgically confirmed hydatid patients, 15 healthy donors showing no bands in immunoelectrophoresis (IEF)[40], and 41 patients with other pathologies, including *Ascaris lumbricoides* (n=1), *Treponema pallidum* (n=3), *Giardia lamblia* (n=1), *Aspergillus* spp (n=4), *Salmonella typhis* (n=1), *Trypanosoma cruzi* (n=2), *Trichuris trichiura* (n=2), *Echinococcus multilocularis* (n=5), *Taenia solium* (n=20), and rheumathoid factor (n=2).

A pool of serum samples from 6 hydatid patients was prepared as positive serum control. A pool of 6 healthy donors also was prepared as negative serum control. These pools were tested by IEF according to VARELA-DÍAZ & COLTORTI, 1979[40].

All serum samples were stored at –20 °C until used.

Enzyme conjugated antibodies

One rabbit was immunized with human gammaglobulins according to DRESSER, 1986. Rabbit immunoglobulins were precipitated with 50% saturated ammonium sulphate, then reprecipitated with 40% saturated ammonium sulphate and extensively dialysed against PBS[13]. Those rabbit anti-human gammaglobulins were conjugated with: a) peroxidase (Type VI-A, Sigma, St Louis MO, USA) according to TIJSSEN, 1985[35], and b) with alkaline phosphatase according to WOODWARD *et al*., 1985[44].

Analysis of the chemical composition of BHCF

Estimations of total protein and lipid contents were assessed using commercial kits (from Pierce Labs. and Wiener labs., respectively). Bovine serum albumin (BSA, marker of host proteins) was determined by simple radial immunodiffusion[5] and carbohydrate content was determined by a modified resorcinol-sulphuric acid assay[43] according to MIGUEZ *et al*., 1996[21].

Polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by LAEMLI, 1970[16] using 12.5% acrylamide gels and 1% SDS. Antigens were separated under reducing conditions (5% 2-mercaptoethanol). The antigen bands were visualized by silver staining according to TSANG *et al*., 1983[37].

Plate coating

Solutions of different concentrations (5-100 µg/ml) of native and denatured (urea 6 M and KSCN 2 M) antigens were used in ELISA. Solutions were made up in the following buffers: PBS pH 7.5, PBS containing 0.5 M NaCl, 0.1 M glycine pH 8.2, 0.1 M glycine pH 8.2 containing 0.5 M NaCl. The antigen solutions were incubated (100 µl/well, moist chamber) during 1, 2, 4 and 8 days at room temperature and 4 ºC in polystyrene microtitre plates (Nunc, Denmark).

Enzyme-linked immunosorbent assay (ELISA)

After the coating solution had been discarded, ELISA was carried out as described by FERRAGUT & NIETO, 1996[9], using peroxidase-conjugated rabbit anti-human immunoglobulins.

Western blot

Antigens were resolved by SDS-PAGE as described above. Western blot was done according to TOWBIN *et al*., 1979[36]. The membranes of nitro-cellulose were cut into strips which were blocked with 5% (w/v) skimmed milk in PBS for 30 min with rocking. Then, they were washed 3 times with PBS-T (10 min) and once with PBS (5 min). Blots were developed according to FARR & NAKANE, 1981[8]. Serum samples were diluted in PBS-T-BSA and incubated with the strips for 2 h with rocking. After washing as above, the strips were incubated for 2 h with alkaline phosphatase-conjugated rabbit anti-human appropriately diluted in PBS-T-BSA. Then, they were washed and substrate solution containing BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt) and NBT (p-nitro-blue-tetrazolium chloride) was added according to the manufacturer's instructions (Bio Rad).

Sodium metaperiodate treatment of native antigens

ELISA plates or the nitro-cellulose strips coated with either of two antigens (FC or NFC, respectively) were treated with 20 mM sodium m-periodate in 50 mM sodium acetate buffer, pH 4.5 according to WOODWARD *et al*., 1985[44]. After this treatment the ELISA and immunoblot were performed as described by STERLA *et al*., 1996[34].

ELISA and immunoblot with free phosphorylcholine

ELISA and immunoblot were performed as described above, but PBS buffers were substituted for 20 mM Tris-HCl, pH 7.3. All sera were tested with and without 50 mM free phosphorylcholine as described by STERLA *et al*., 1996[34].

Data analysis

The Student's t-test for pairs of data was used to determine the significance of differences between arithmetic means ($p < 0.001$).

Relative molecular weights (Mr) of BHCF components were estimated using a logarithmic plot of the migration of a set of molecular weight standards included in every gel.

Adsorption to the polystyrene surface was evaluated by comparing the OD_{600} observed in ELISA for each antigen concentration adsorbed, by reacting, on one hand with a positive serum control and on the other with a negative serum control, using the differences (D) of the mean of such readings as a measure of the antigen adsorbed.

Human anti-hydatid reference serum[30] was kindly provided by Dr. Guisantes (Vitoria, Spain). Antibody concentrations expressed as arbitrary units per ml (au/ml) and OD_{600} corresponding to dilutions of the reference serum were correlated by linear regression. ELISA data (OD_{600}) from each sample were converted to antibody concentrations equivalent to this reference for analytical consistency[18]. Thirty sera from healthy donors were used to determine the cut-off value for antibody detection calculated as the mean plus 3 standard deviations. Sensitivity, specificity and diagnostic efficiency were calculated as described by BARBIERI *et al*., 1998[3].

RESULTS

Chemical composition of antigenic preparations

Total protein, lipids, BSA as marker of host protein, and carbohydrate concentrations were estimated in each batch prepared from BHCF (8 from FC and 5 from NFC). The results obtained are shown in Table 1. Total protein of batches from FC and NFC were not significantly different while carbohydrate and lipids were significantly different. Host protein contents were higher in NFC than in FC batches.

Antigenic characterization

Comparison of the SDS-PAGE patterns showed (Figure 1) larger number of bands in batches from FC than in batches from NFC. Additionally, 38 and 116 kD bands were observed on batches from FC only.

Table 1
Chemical composition of antigens

	Fertile cysts (n=8)	Non-fertile cysts (n=5)
Total protein (g%)	1.10 ± 0.98	1.29 ± 0.80
Host protein (g%)	0.23 ± 0.13	0.57 ± 0.35
Carbohydrate (mg/ml)	5.13 ± 1.00	1.70 ± 0.25
Lipids (mg/ml)	1.84 ± 0.21	0.49 ± 0.08

Western blot

Immunoblotting of PFC and PNFC using pooled hydatid human sera and pooled healthy donors sera showed different pattern of antigenic recognition (Figure 2, lanes 2 and 4). Pooled hydatid human sera recognised 116, 50, 38, 26 and 16 kD bands of PFC and 50, 26 and 16 kD bands of PNFC. Pooled human sera from healthy donors showed reaction with bands of 50 and 26 kD of PFC and PNFC (Figure 2, lanes 4). A strongly decreased recognition of antigens of M_r lower than 38 kD in PFC and in the band of 26 kD in PNFC was observed when those pools of antigens were periodate treated (Figure 2, lanes 1).

Western blot analysis after addition of free phosphorylcholine in diluted pooled sera showed an inhibition in the recognition of the 38 kD band while an enhanced recognition of low molecular weight bands were observed in PFC. The latter also was observed in PNFC (Figure 2, lanes 3).

Optimization of physicochemical conditions of coating antigen in ELISA

An increased value of D associated with increased incubation time as well as with the antigen concentration used to coat the plates in PBS (data not shown) was observed.

An increased value of D when the antigen solutions were prepared in presence of SCN and also in presence of urea compared with PBS alone were observed (Figure 3). Also an increased value of D was observed when the pH 7.2 (PBS) was increased to 8.2 (glycine) and when the ionic strength was increased (0.15 M to 0.5 M in NaCl). The greatest value of D was observed when antigen concentration was 30 µg/ml.

D values associated with different concentrations of PFC diluted both in buffer glycine 0.1 M pH 8.2-0.5 M NaCl and in the same buffer but containing 6 M urea at different incubation periods and temperatures

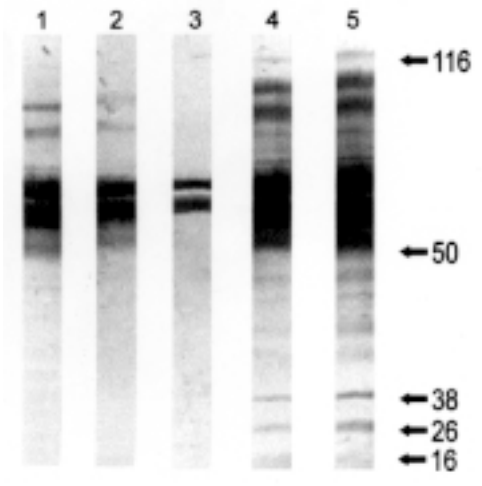

Fig. 1 - Electrophoretic analysis of antigen composition. Comparison of SDS-PAGE patterns of batches from non-fertile cysts (lanes 1, 2, 3) and from fertile cysts (lanes 4, 5). M_r are indicated in kD.

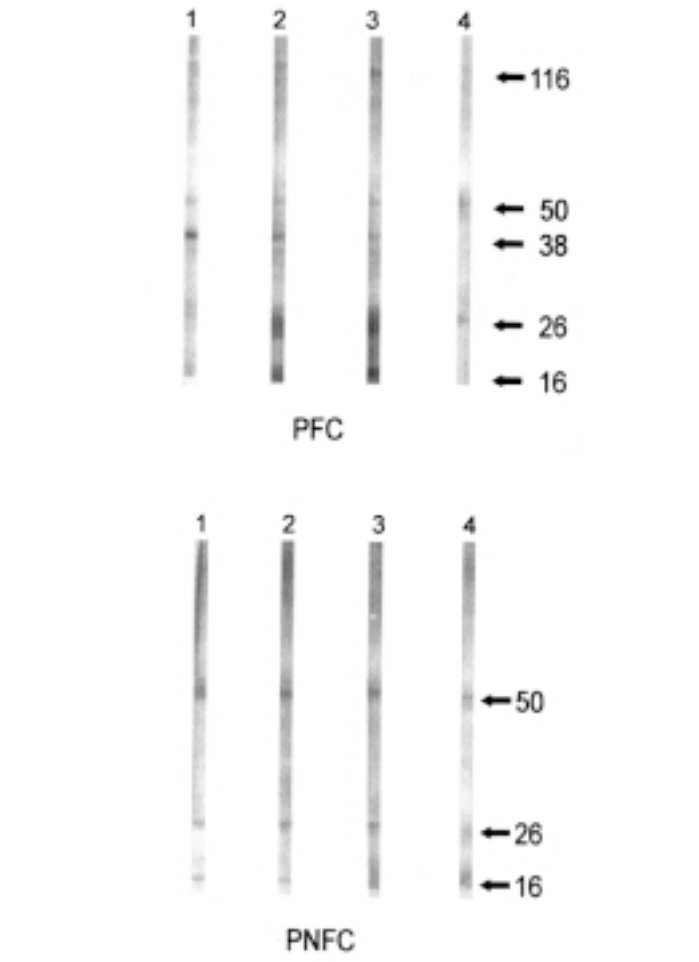

Fig. 2 - Immunoblotting pattern of PFC and PNFC as antigen. Lane 1 - Reaction with reduced native and periodate-treated antigens of pooled hydatid human sera. Lane 2 - Reaction with reduced native antigens of pooled hydatid human sera. Lane 3 - Reaction with reduced native antigens of pooled hydatid human sera with free phosphorylcholine. Lane 4 - Reaction with reduced native antigens of pooled healthy donors sera. M_r are indicated in kD.

were studied (Figure 4). Saturation was achieved with 10 µg/ml, 30 µg/ml, 40 µg/ml and 20 µg/ml for 1, 2, 4 and 8 days of incubation, respectively, in buffer glycine at room temperature (Figure 4 a). Using those coating conditions but at 4 °C, the D values were lower than those obtained at room temperature (Figure 4 c, d).

Diagnostic value of PFC and PNFC for antibody detection in ELISA

In ELISA using PFC 33 of 41 sera from hydatid patients and 2 of 5 sera from patients with *E. multilocularis* yielded positive results. When the antigen was periodate treated 36 of 41 sera from hydatid patients yielded positive results and also the 2 patients with *E. multilocularis* remained positive. In ELISA performed with free phosphorylcholine 36 hydatid patients, all sera from *E. multilocularis* and two of 20 *T. solium* sera showed positive results.

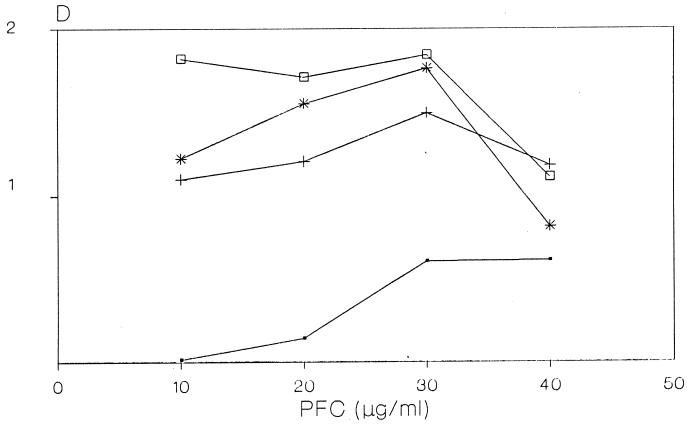

Fig. 3 - Influence of denaturing agents, pH and ionic strength on the adsorption of antigen to polystyrene plates. D values corresponding to PFC adsorption after overnight incubation at room temperature are shown in ordinates. Different concentration of PFC in PBS pH 7.2 (·), PBS pH 7.2 + 2 M SCN (+), PBS pH 7.2 + 6 M urea (*), glycine pH 8.2 0.5 M NaCl (□) are shown.

In ELISA using PNFC 18 of 41 hydatid patients, all *E. multilocularis* sera and 2 *T.solium* sera showed positive results. In ELISA with periodate treatment of PNFC 23 of 41 hydatid patients, all *E. multilocularis* sera 1 *T. solium* showed positive results. In ELISA performed with free phosphorylcholine 18 hydatid patients, all *E. multilocularis* sera and 12 of 20 *T. solium* sera showed positive results. Sensitivity, specificity and diagnostic efficiency are summarized in Table 2.

DISCUSSION

In our laboratory, while 1.6 l of hydatid fluid were obtained from ovine cysts, 19.6 l were obtained from bovine cysts. The latter were larger and their percentage of infection and/or calcification was 86% smaller than ovine cysts.

Because of the complex biochemical nature of the hydatid antigen, it is necessary to standardize hydatid fluid for satisfactory diagnosis in order to obtain reliable and reproducible results when crude bovine hydatid fluid is used.

We selected 8 batches from FC and 5 batches from NFC to analyse their chemical composition. Concentration of BSA was used as a marker of host protein in hydatid fluid since it has been described that albumin is its major host-derived impurity[41]. The highest concentration of antigenic protein was shown in batches from FC, no differences in total protein were observed in batches from FC and NFC while host protein contents were higher in NFC batches (Table 1). These results are in accordance with the electrophoretic analysis of antigen composition (Figure 1).

We submitted PFC and PNFC to western blot analysis in order to study the recognition of immunogenic components. The most diagnostically relevant difference between fertile and non-fertile BHCF is the presence of the 38 and 116 kD bands in the antigens from FC (Figure 2). The 38 kD band probably corresponds to one of the subunits of Ag5[32] and the 116 kD band was described as specifically recognized by hydatid patients[15]. The figure also showed that phosphorylcholine,

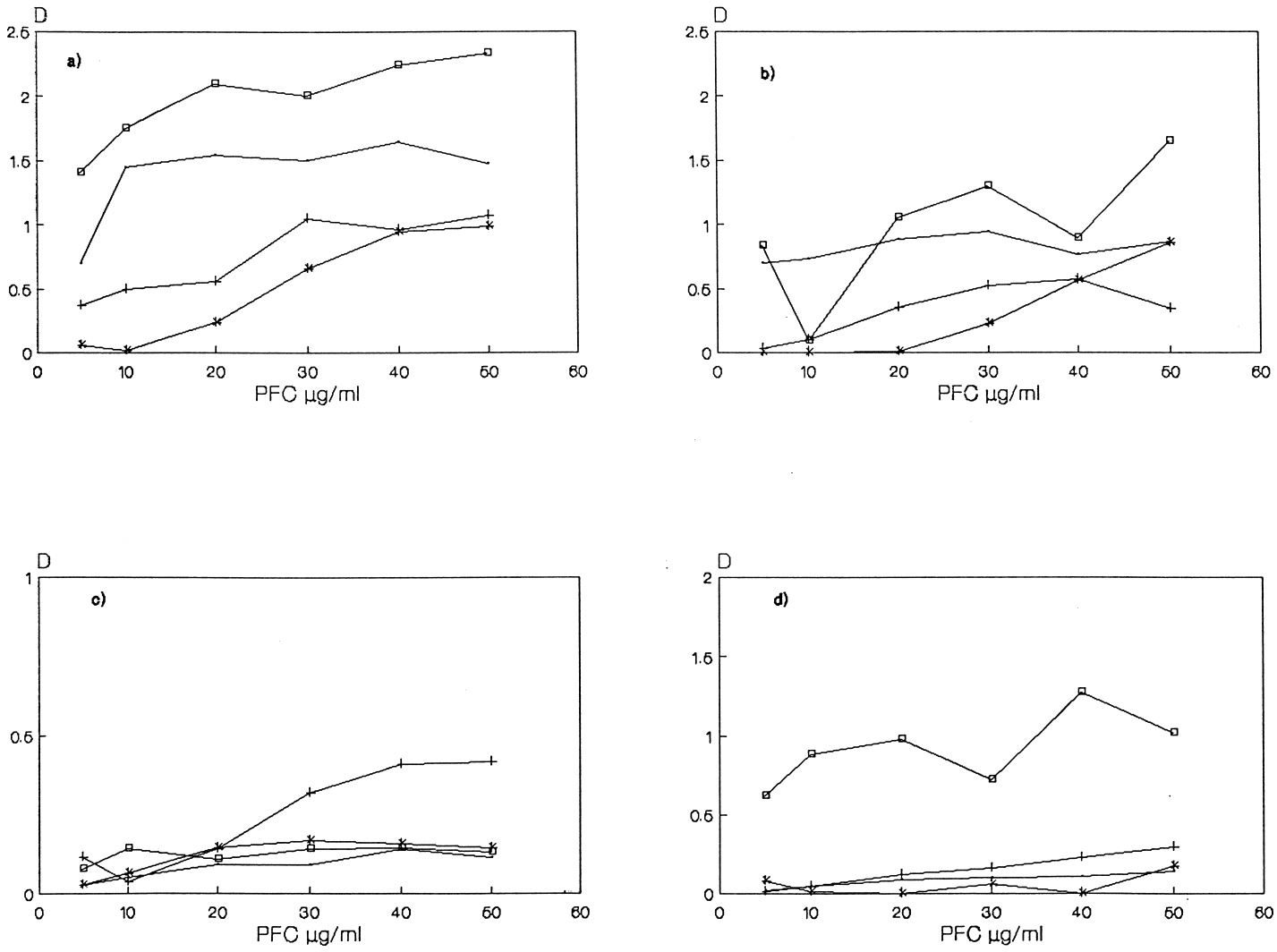

Fig. 4 - Influence of incubations periods and temperatures on antigen adsorption to ELISA plates. Plates were coated using solutions of different concentration of PFC (abscissae) in buffer glycine 0.1 M pH 8.2-0.5 M NaCl (a, c) and in the same buffer containing 6 M urea (b, d) during 1 (·), 2 (+), 4 (*) and 8 (□) days of incubation at room temperature (a, b) and 4 ºC (c, d). In ordinates are shown D values as indicator of the antigen adsorbed level in ELISA.

Table 2
Diagnostic value of PFC and PNFC in ELISA for diagnosis of hydatidosis

	Native antigen		Periodate treated antigen		Inhibition with free phosphorylcholine	
	PFC	PNFC	PFC	PNFC	PFC	PNFC
Sensitivity (%)	81	44	88	56	88	44
Specificity (%)	95	84	96	88	82	46
Diagnostic efficiency (%)	89	67	93	74	85	45

carbohydrate and peptidic epitopes are present in both, 38 and 116 kD bands. Our results show that BHCF composition and antigenic performance are largely affected by the fertility of the cyst, in agreement with what has been described for the cyst fluid from other intermediate hosts of *E. granulosus*[28]. It is interesting to note that FC has significantly higher concentrations of carbohydrates and lipids than NFC. This is consistent with the highest carbohydrate and lipid concentration found in the surface of protoscoleces[21]. To sum up, the majority of diagnostically relevant parasite antigens were present in PFC.

The appropriate selection of the optimum antigen concentration for ELISA plate coating may have influence either on the antibody titre or affinity determination[22], specially when a complex antigen is used and the antibodies during the infection can be recognizing different molecules on it. For that reason, analysis of the optimum physicochemical conditions for antigen adsorption to the polystyrene surface (coating buffer system, including its pH and ionic strength; antigen concentration; time and temperature of incubation) was made by evaluating ELISA results using D as indicator of the level of adsorbed antigen.

An increase of D when increasing both the incubation time and the antigen concentration in PBS was observed. For one day of incubation at room temperature the antigen adsorption is very low and its saturation value is 30 µg/ml instead of 20 µg/ml according to peroxidase saturation technique[22] (data not shown).

Comparison of the D values corresponding to overnight incubation of different concentrations of native and denatured (SCN and urea) antigen in PBS pH 7.2 at room temperature is shown in Figure 3. The adsorption of antigenic molecules increased when they were denatured. This suggests that the denaturation did not highly affect the relevant epitopes but instead it affected other moieties of those molecules. An increase on the D value is observed associated with increases in the pH and the ionic strength.

Buffer glycine with or without urea 6 M was selected to study the adsorption of different antigen concentrations, incubation periods and temperatures. ELISA results observed after increasing the incubation time at room temperature related to antigen adsorption suggest that there is a slow reordering of the antigenic molecules that are adsorbed to the plates. The saturation value also seems to depend on the antigen coating time (Figure 4 a). On the other hand, at this pH and ionic strength, the treatment with urea (Figure 4 b) seems to produce such a denaturation of the relevant epitopes that the D values are lower than in the case of the native antigen. The antigen adsorption is not well carried out at 4 °C (Figure 4 c, d).

In summary, it may be concluded that the optimum adsorption conditions are two days of incubation at room temperature with a solution of 30 µg of BHCF per ml of buffer glycine 0.1 M pH 8.2-0.5 M NaCl.

In these conditions we analysed the diagnostic value of PFC and PNFC for human hydatid disease (Table 2). When used in ELISA, BHCF from FC showed 81% sensitivity and 95% specificity, while 44% sensitivity and 84% specificity were observed with BHCF from NFC. The diagnostic efficiency of PFC and PNFC improved when ELISA was carried out with the periodate-treated antigens. In particular, specificity and sensitivity using both, native and periodate-treated PFC, increased from 95 to 96% and from 81 to 88%, respectively. The addition of free phosphorylcholine did not improve the specificity of the ELISA because the reactivity with patients with *E. multilocularis* and *T. solium* increased. This results are in agreement with what has been obtained by STERLA *et al.*, 1996[34]. Best diagnostic efficiency was obtained using periodate-treated PFC.

We can conclude that crude bovine hydatid fluid to be used in human immunodiagnosis by ELISA should be from fertile cysts and periodate-treated in the adsorption conditions described above.

RESUMO

Caracterização e otimização do líquido vesicular de *Echinococcus granulosus* bovino para utilização no imunodiagnóstico da hidatidose por ELISA

O objetivo do presente trabalho foi testar a composição química do líquido hidático bovino (BHCF) obtido de cistos hidáticos férteis (FC) e não férteis (NFC). Oito lotes de FC e 5 de NFC foram preparados e testados quanto à composição química, proteínas totais, proteínas derivadas do hospedeiro, conteúdo de carbohidratos e lipídeos. Não foram observadas diferenças entre os dois primeiros parâmetros sendo que o conteúdo de carbohidratos e lipídeos foi maior nos lotes FC do que nos NFC. Por SDS-PAGE foram observadas bandas de 38 e 116 kD somente nos BHCF do FC. Foram preparados dois «pools» de BHCF, um de FC (PFC) e outro de NFC (PNFC). Os padrões de reconhecimento dos antígenos foram analisados por imunoblot. As condições físico-químicas para adsorção dos antígenos na superfície das placas de poliestireno (ELISA plates) foram otimizadas. O valor de diagnóstico de ambos tipos de BHCF bem como a importância diagnóstica da oxidação das moléculas de carbohidratos com periodato foram analisadas por ELISA usando 42 amostras de soro de pacientes com hidatidose, 41 de pacientes com outras doenças e 15 de doadores aparentemente saudáveis. A reatividade de todos soros contra antígenos nativos foi analisada com e sem fosforilcolina livre. A melhor eficiência diagnóstica foi observada usando BHCF de PFC tratado com periodato usando tampão glicina com forte força iônica para sensibilizar as placas de ELISA.

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