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Diagnostic evaluation of a synthetic peptide derived from a novel antigen B subunit as related to other available peptides and native antigens used for serology of cystic hydatidosis

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SUMMARY

A synthetic peptide (GU4) derived from an antigen B (AgB) subunit was serologically compared with crude antigen (HCFA); immunopurified AgB and antigen 5 (Ag5), and two other synthetic peptides, for diagnosis of human cystic hydatidosis. GU4 was derived from the sequence of AgB/2, the novel AgB subunit described by us. The other two peptides: 65 (AgB mimotope) and 89–122 (Ag5 mimotope), were described by others. Antigens B and 5 showed higher diagnostic sensitivity than corresponding peptides. All sera reacting with peptides 89–122 and GU4 also reacted with 65. The latter provided three to four times higher sensitivity than the former two peptides, but 30% lower specificity. The diagnostic efficiency of AgB (82%) was higher than those of Ag5 (74%) and HCFA (71%). Interestingly, 89–122 only reacted with hydatid sera, some of which did not react with AgB. Considering positive those reacting with 89–122 or AgB, sensitivity increases from 77% (with AgB) to 82% (combined), while specificity is the same as with AgB (86%). Our results suggest that hydatid serology may be improved by: a) combining several defined antigens (including synthetic peptides), b) design of new E. granulosus*-specific mimotopes, which react with the false negative sera (16/90; 18%).*

Keywords *E. granulosus, hydatid serology, AgB, Ag5, synthetic peptides, ELISA*

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INTRODUCTION

Serology, either alone (Coltorti *et al*. 1988) or combined with imaging methods (Babba *et al*. 1994, Barbieri *et al*. 1994) has proved to be very useful to diagnose human cystic hydatid disease produced by *Echinococcus granulosus*. Several techniques using crude hydatid cyst fluid antigen (HCFA) have been used for that serology (Force *et al*. 1992, Gottstein 1992), but all of them show some lack of sensitivity and specificity.

It has been shown that up to 25% of the surgically confirmed cystic hydatid patients do not show serum anti-HCFA antibodies using any of the assays available (Wattre *et al*. 1980, Eckert *et al*. 1981, Schantz & Gottstein 1986; Barbieri *et al*. 1993, 1994, Lightowlers & Gottstein 1995). Higher incidence of false negative sera has been reported in patients with lung and intact hyaline cysts (Lightowlers & Gottstein 1995). In addition, it has consistently been reported the existence of anti-HCFA antibodies in sera from patients with other pathologies (Ben-Ismail *et al*. 1980, Dar *et al*. 1984, Rickard & Lightowlers 1986, Shepherd & McManus 1987, Lightowlers *et al*. 1989).

Therefore, identification of discrete parasite antigens that could provide a more sensitive and specific serology became a relevant research aim. One of the diagnostically relevant antigens in HCFA is a 60–400 kDa glycoprotein complex, named antigen 5 (Ag5) (Bout *et al*. 1974). It is composed of 62–67 kDa heterodimers which dissociate into two subunits of 38 and 23 kDa under reducing conditions (Lightowlers *et al*. 1989, Zhang & McManus 1996). Detection of Ag5-binding antibodies in serum showed that 10 to 40% of surgically confirmed hydatid patients have no detectable anti-Ag5 antibodies (Picardo & Guisantes 1981, Schantz & Gottstein 1986). Further to this, Ag5 has been reported to cross-react with antibodies in sera from patients with alveolar hydatidosis (Schantz & Gottstein 1986, Lightowlers *et al*. 1989),

filariasis (Rickard *et al*. 1984), schistosomiasis (Mattossian *et al*. 1979), neurocysticercosis (Lightowlers *et al*. 1989) and fascioliasis (Ben-Ismail *et al*. 1980).

Antigen B (AgB) is another diagnostically relevant antigen in HCFA. It is a complex 120 kDa thermostable lipoprotein (Oriol *et al*. 1971), showing 50% of alpha-helical conformation (Oriol & Oriol 1975). SDS-PAGE analysis of HCFA, using non reducing conditions, shows that AgB produces a ladder-like pattern consisting of subunits with 8, 16 and 24 kDa (Shepherd & McManus 1987, Lightowlers & Gottstein 1995). Furthermore, González *et al* (1996) showed that native immunopurified AgB is an oligomer of 8 kDa subunits exhibiting 3 more bands in SDS-PAGE with 32, 40 and 48 kDa.

Immunoblot detection of the smallest subunit (8 kDa) of AgB provides a good diagnostic tool for cystic hydatid disease (Maddison *et al*. 1989, Legatt *et al*. 1992). Nonetheless, 18% of sera from cystic hydatid patients did not react, and 39% of sera from alveolar hydatid patients cross-reacted with that subunit (Maddison *et al*. 1989).

The available data on the diagnostic use of recombinant Ag5 (Facon *et al*. 1991) and AgB (Helbig *et al*. 1993) suggest that they do not produce a relevant improvement of diagnostic sensitivity and/or specificity, as compared with the corresponding native antigens previously used. Thus it is possible that both antigens may contain epitopes recognized by sera from cystic hydatidosis patients as well as from patients with other parasitic diseases. Nonetheless, the possible occurrence in the same antigenic molecule of some epitopes solely recognized by antibodies in sera from cystic hydatidosis patients can not be ruled out. Therefore, the identification of *E. granulosus*-specific epitopes in each antigen and the design of the appropriate synthetic peptides to mimic them are relevant research aims in this area.

Chamekh *et al*. (1992) reported the use in serology of a 34 aminoacids long synthetic peptide (peptide 89-122), mimicking an epitope of Ag5. The authors described that this peptide yielded 85% sensitivity and 86% specificity. Whereas Legatt & McManus (1994) reported the diagnostic evaluation of a 27 aminoacids long synthetic peptide (peptide 65), mimicking an epitope from an 8 kDa subunit of AgB, and they found that this peptide yielded 68% sensitivity and 70% specificity. The panels of patients' sera used in both cases were different, thus precluding actual comparison of the diagnostic values of both peptides (Lightowlers & Gottstein, 1995).

Fernández *et al*. (1996) described an 8 kDa (AgB8/2) *E. granulosus* polypeptide that was shown by González *et al*. (1996) to be a novel subunit of AgB (an oligomer including the 2 types of 8 kDa subunits). This subunit shows an overall identity of 44% with the one (AgB8/1) reported by Frosch *et al*. (1994). The sequence of AgB8/1 corresponding to peptide 65 is located in an N-terminal extension showing 69% identity with AgB8/2. In this paper we analyse the diagnostic value of a 34 aminoacids long synthetic peptide (peptide GU4) the sequence of which corresponds to a C-terminal extension of AgB8/2 which shows only 29% identity with the corresponding region in AgB8/1. Hydrophobic cluster analysis (Lemesle-Varloot *et al*. 1990) predicts a putative epitope to be located in the AgB8/2 region corresponding to GU4 sequence.

The described lower diagnostic performance of synthetic peptides as compared with full antigens may be due to the loss of conformational epitopes occurring when the polypeptide sequences are moved from the environment provided by the rest of the native protein molecule to that provided by the plastic plate surface. Small and medium size synthetic peptides are likely to assume unordered conformations in water which may affect antibody binding. In fact, Gras-Masse *et al*. (1988) showed that antibody binding by synthetic peptides may be linked to their helical organization. Actually, Chamekh *et al*. (1992) showed that binding of an Ag5-specific monoclonal antibody as well as some cystic hydatidosis patients sera, to the epitope mimicked by the peptide 89-122 may be dependent on the alpha-helical conformation of the antigen.

Alternatives are available to experimentally address the problem created to serology by the loss of native epitopes conformation by synthetic peptides mimicking them. It has been reported that 2,2,2 trifluoroethanol (TFE) increases the alpha-helical organization of synthetic peptides (Lang e*t al*. 1994), thus it can be used for that aim in plate coating. In addition, coupling the synthetic peptides to bovine serum albumin (BSA) and coating the plates with those conjugates may preserve the conformation of some epitopes that otherwise would be altered due to peptide-plastic interactions, thereby impaired by the BSA arm.

A major weakness of the information available on the diagnostic value of the different native antigens as well as of peptides mimicking epitopes from them, is related to the different sera collections used to evaluate them and the appropriateness of their compositions (Lightowlers & Gottstein 1995). For that reason, it is so far not possible to compare them and select which antigen or appropriate combination of antigens provides the optimum diagnostic efficiency (DE).

Therefore, we report in this paper a direct serologic (ELISA) comparison of the novel GU4 synthetic peptide with the native immunopurified AgB and Ag5 as well as with two other synthetic peptides mimicking epitopes from the last two antigens, using the same comprehensive sera collection. Further to this, we also analyse the recovery of epitope conformation by the synthetic peptides in different

plate coating conditions, and its influence on their diagnostic performance.

MATERIALS AND METHODS

Human sera collection

A total of 206 human sera were used in the present study. Ninety sera were from surgically confirmed cystic hydatid patients, 65 of them exhibiting the following available record of cyst location: liver (40), lungs (11), bones (8) and multiple sites (6). Twenty-eight sera from healthy donors and 88 from patients with the following diseases: alveolar hydatid disease (27), *Taenia solium* cysticercosis (22), toxocariasis (10), schistosomiasis (6), onchocerciasis (6), rheumatoid arthritis (5), Chagas' disease (4), toxoplasmosis (3), filariasis (2), syphilis (2) and cancer (1).

Antigens

Complex antigens
HCFA was prepared from cattle cysts essentially as described by Barbieri *et al*. (1993).

AgB
The AgB-specific monoclonal antibody DC11 (González *et al*. 1996) was coupled to CNBr-activated Sepharose (Pharmacia, Sweden) and used to immunopurify AgB directly from HCFA according to the method described by González *et al*. (1996) for that step. Briefly, an appropriate amount of HCFA in PBS was applied to the DC11-Sepharose column and, after extensive washing with PBS-0·05% Tween (PBS-T), it was eluted with 0·1 M glycine buffer pH 2·5. The eluate was neutralized and extensively dialysed against PBS and protein concentration estimated according to its OD 280.

Ag5
It was prepared essential as above but using the Ag5-specific monoclonal antibody EG 02154/12 (Chamekh *et al*. 1990) kindly provided by Dr M. Chamekh.

Synthetic peptides
The peptide 89-122 (Chamekh *et al*. 1992) and the peptide 65 (Leggatt & McManus 1994) were prepared in the Molecular Biology Facility of the University of Newcastle (UK), according to the described aminoacid sequences.

The synthetic peptide GU4 was synthesized in Severn Biotech. Ltd. (UK), according to the following sequence, selected from the sequence of AgB8/2:

NDLTAICQKLQLKIREVLKKYVKNLVEEKDDDSK.

BSA-peptide conjugates
Each of the three peptides (1·5 mg of each) were coupled to BSA (2 mg) using a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride conjugation kit according to the instructions provided by PIERCE Chemical Company (USA).

Enzyme immunoassay (ELISA)

Polystyrene ELISA plates (NUNC, Denmark) were coated by overnight incubation at 4°C with the appropriate antigen solution (100 μl/well). After discarding coating solution, they were blocked 1 h at room temperature with 1% BSA in PBS (PBS-BSA) (200 μl/well) and washed with PBS-T. The serum samples were diluted 1/400 in PBS-T, incubated overnight at 4°C and washed with PBS-T. Peroxidase-conjugated rabbit anti human-IgG (Nordic) was appropriately diluted in PBS, incubated three h at room temperature and washed three times with PBS-T and once with PBS. A substrate solution containing H_2O_2 3-methyl, 2-benzothiazolinone hydrazone hydrochloride and 2-dimethylaminobenzoic acid (Ngo & Lenhoff 1980) (200 μl/well) was added and the plates incubated for 20 min at room temperature with shaking. The optical densities at 600 nm (OD_{600}) were measured with an ELISA reader (Labsystems Multiskan MS, Finland). A cut-off value was defined for each test, corresponding to the mean value of OD_{600} showed by the sera from the 28 healthy donors plus 3 SDs. OD_{600} readings above that cut-off value were considered as positive. Sera from cystic hydatidosis patients showing positive ELISA readings were considered true positives (tp) and those showing negative readings were considered false negatives (fn). Whereas sera from healthy donors or non cystic hydatidosis patients showing positive readings were considered false positives (fp) and those showing negative readings were considered true negatives (tn).

Data treatment
The following definitions were used to calculate the corresponding diagnostic parameters:

$$\text{Sensitivity } \% = \text{tp} \times 100/(\text{tp} + \text{fn})$$
$$\text{Specificity } \% = \text{tn} \times 100/(\text{tn} + \text{fp})$$
$$\text{DE } \% = (\text{tp} + \text{tn}) \times 100/(\text{tp} + \text{fp} + \text{tn} + \text{fn})$$

Statistical analysis
The verification of the homogeneity in the DE for the HCFA in TFE or PBS, the verification of the non-homogeneity in the DE: related to the coating conditions for each peptide, and between the following pairs of antigens: HCFA-AgB, Ag5-AgB, AgB-peptide 65-BSA, AgB-peptide GU4 in TFE and Ag5-peptide 89-122 in TFE, were run using the χ^2 test,

($P = 0{\cdot}05$) (Sachs 1978, Guttman *et al*. 1982). The verification that the DE of AgB combined with peptide 89-122 in TFE is higher than the DE of AgB was run using the test of the signs of Mc Nemar ($P = 0{\cdot}05$) (Sachs 1978).

TFE coating

Solutions with the appropriate concentration of each peptide dissolved in 100% TFE (Sigma-USA) were delivered into the plates (100 μl/well), and incubated overnight at 37°C. After this step, the ELISA protocol was continued as described above.

Determination of optimum antigen concentration for plate coating

ELISA tests were run essentially as described above, with the following modifications. Several antigen dilutions ranging from 0·1 μg/ml to 30 μg/ml were prepared in the corresponding buffer. PBS was used for native antigens and peptide-BSA conjugates, 0·1 M sodium bicarbonate buffer pH 9·6 (BCB) was used for free peptides, and TFE was used for free peptides and HCFA. These dilutions were used to coat different wells in the plates, according to the above described protocol. A pool of 28 sera from healthy donors (negative pool) and another of 25 randomly selected sera from cystic hydatid patients (positive pool) were incubated with every dilution of each antigen tested. Dose-response curves were made showing in ordinates the differences in OD_{600} observed between readings corresponding to positive and negative pools and in abscissae the corresponding antigen concentrations used for coating. The optimum antigen concentration was the lower one providing the maximum OD_{600} difference between the positive and negative pools.

RESULTS

Influence of antigen coating conditions on diagnostic efficiency

The dose-response curves corresponding to the ELISAs performed with peptide GU4 using the 3 different coating conditions are shown in Figure 1. Similar curves (not shown) were done with each antigen to select the corresponding optimum plate coating protocol.

The optimum coating conditions for HCFA, Ag5 and AgB were 20, 10 and 10 μg/ml in PBS, respectively. The optimum coating concentrations and corresponding diagnostic efficiencies of HCFA, using either PBS or TFE for coating, were compared to study the influence of TFE on the performance of the native antigens. To analyse DE, the complete sera collection was tested in both cases. The same optimum concentration (20 μg/ml) and the same DE (71%) were observed either with PBS or TFE ($\chi^2 = 1{\cdot}07$, critical region [7·81, ∞)). The optimum coating concentrations for each peptide in the different conditions are shown in Table 1, together with the corresponding diagnostic parameters observed when the complete sera collection was tested.

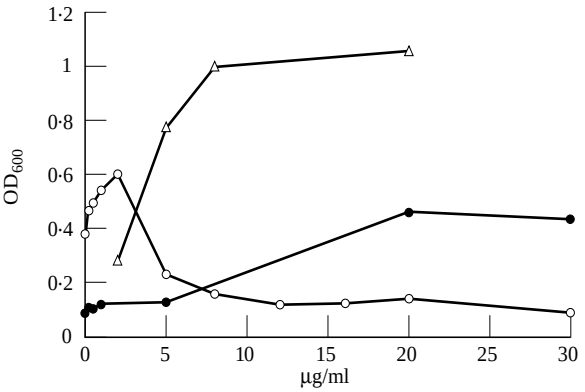

Figure 1 Influence of peptide concentration and plate coating conditions on specific antibody binding to GU4 synthetic peptide. Actual peptide concentration (μg/ml) used to coat the plates with GU4 synthetic peptide dissolved in bicarbonate buffer (BCB) or in trifluoroethanol (TFE) are shown in abscissa. The corresponding concentrations of the GU4-BSA conjugate are expressed referred to the concentrations of GU4-synthetic peptide in the PBS solutions used to coat the plates, considering a theoretical 100% yield of conjugation to BSA. The OD_{600} values in ordinates correspond to the difference between the OD_{600} values shown by the pools of sera from cystic hydatid patients and from healthy donors. ○ GU4-BCB; ● GU4-TFE; △ GU4-BSA.

The highest DE observed with peptide 65 was achieved when used conjugated with BSA compare with BCB and TFE ($\chi^2 = 24{\cdot}75$, critical region [12·59, ∞)). In the case of peptide 89-122 it was higher when it was dissolved in TFE ($\chi^2 = 35{\cdot}92$, critical region [12·59, ∞)). For the peptide GU4, there is no significant difference between the DE observed using the different coating conditions ($\chi^2 = 6{\cdot}72$, critical region [12·59, ∞)), but we select TFE as the best coating condition for the peptide because we have more tp sera with only 2 fp sera.

Diagnostic value of the native antigens

The reactivities of sera with the native antigens (HCFA, Ag5 and AgB) were analysed in ELISA using the same sera collection to be able to effectively compare their corresponding diagnostic values. The tests were run in similar conditions for all antigens, which were dissolved in PBS for coating. The results are shown in Tables 2 and 3. The DE of native immunopurified AgB (82%) was higher than those of Ag5 (74%) ($\chi^2 = 15{\cdot}98$, critical region [7·81,∞)) and HCFA (71%) ($\chi^2 = 21{\cdot}55$, critical region [7·81, ∞)).

Table 1 Diagnostic performance of the synthetic peptides

Antigen	Coating solvent	Concent. (μg/ml)	Sensitiv. (b) (%)	Specific. (b) (%)	DE (b) (%)
Peptide 65	BCB	20	43/90 (48%)	80/116 (69%)	123/206 (60%)
Peptide 65	TFE	20	31/90 (34%)	97/116 (84%)	128/206 (62%)
Pep.65-BSA (a)	PBS	8	36/90 (40%)	107/116 (92%)	143/206 (69%)
Pep.89-122	BCB	20	15/90 (17%)	106/116 (91%)	121/206 (59%)
Pep.89-122	TFE	20	14/90 (16%)	116/116 (100%)	130/206 (63%)
89-122-BSA (a)	PBS	8	21/90 (23%)	89/116 (77%)	110/206 (53%)
Peptide GU4	BCB	2	11/90 (12%)	116/116 (100%)	127/206 (62%)
Peptide GU4	TFE	20	16/90 (18%)	114/116 (98%)	130/206 (63%)
GU4-BSA (a)	PBS	8	15/90 (17%)	111/116 (96%)	126/206 (61%)

Concentrations are expressed referred to the amount of synthetic peptide conjugated to BSA in the solutions of conjugate used to coat the plates, considering a theoretical 100% conjugation to BSA. Concent. refers to the antigen concentration in the solution used to coat the plates. Sensitivity, specificity and DE were calculated according to the corresponding definitions described under Data analysis in Materials and Methods and expressed as percentage. The ratios of numbers of sera used for those calculations are also described (b).

Table 2 shows that 50% of sera from cystic hydatidosis patients showed positive results with the 3 antigens, 77% with all but Ag5 and 84% only with HCFA. There were 16% of these sera which showed negative results with the three antigens.

Interestingly, 8% of the cystic hydatidosis sera reacted with the crude antigen but not with the purified AgB or Ag5, thus suggesting that some diagnostically relevant antigens different from AgB and Ag5 may be present in cyst fluid.

Influence of cyst location on serology with native antigens

The influence of cyst location on serological results is shown by the fact that while 60% of the patients with known liver cysts showed positive reaction with the three antigens, only 45% of patients with known lung cysts showed similar reaction. In addition, 8% of the former sera and 45% of the latter showed negative results with the three antigens. Sera from 91% of the patients with known lung cysts showed similar reactivity with the three antigens. Twenty eight percent of the sera from patients with known liver cysts showed negative results with Ag5, although they were positive with the other two antigens.

Twelve percent of the sera from patients with bone cysts showed to be positive with the three antigens, while 62% were positive with all but Ag5 and 12% were negative with the three antigens.

Cross-reactivity with native antigens

Sera from patients with other pathologies showed high cross-reactivity with these antigens. The higher effect was observed with sera from patients with alveolar hydatidosis, as 30% of them showed positive results with the three antigens, 26% reacted with all but Ag5 and only 4% were negative with the three antigens. Thirty six percent of patients with *T. solium* cysticercosis, showed positive results only with HCFA and 64% were negative with the three antigens. Sera from patients with all other helminthiasis studied showed 71% of negative results with the three antigens.

Diagnostic value of the synthetic peptides

Table 4 shows the ELISA results obtained with all individual sera reacting with GU4 compared with the affinity-purified AgB and Ag5 as well as with two synthetic peptides mimicking epitopes of the last two antigens, all used in their corresponding optimum coating conditions.

Only 14% of hydatid patients sera showed positive results with GU4 and the other two peptides and 59% showed negative results with all of them.

Influence of cyst location in serology with peptides

Sixty two percent of sera from patients with known liver cysts were negative with GU4 and the other two peptides.

Table 2 Reactivities of individual sera with the native antigens

Group of sera	Number of sera per group	HCFA	AgB	Ag5
1	45	+	+	+
2	24	+	+	−
3	7	+	−	−
4	14	−	−	−
5	69	−	−	−
6	30	+	−	−
7	1	−	+	−
8	7	+	+	−
9	1	+	−	+
10	8	+	+	+

Patients sera with similar patterns of reactivity were included in each group to allow comparison among antigens. The relevant parasitological data corresponding to sera included in each group are the following: Group 1: cystic hydatidosis: hepatic cysts (24), lung cysts (5), bone cyst (1), multiple location cyst (1) and not recorded cyst location (14); Group 2: cystic hydatidosis: hepatic cysts (11), bone cysts (5), multiple location cysts (2) and not recorded cyst location (6) Group 3:. cystic hydatidosis: hepatic cysts (2), lung cyst (1), bone cyst (1), multiple location cysts (2) and not recorded cyst location (1): Group 4: cystic hydatidosis: hepatic cysts (3), lung cysts (5), bone cyst (1), multiple location cyst (1) and not recorded cyst location (4); Group 5: alveolar hydatidosis (1), *Taenia solium* cysticercosis (14), *Onchocerca volvulus* (2), *Schistosoma mansoni* (6), toxocariasis (8), filariasis (1), rheumatoid factor positive (2), Chagas' (1), toxoplasmosis (3), syphilis (2), cancer (1), healthy donors (28); Group 6: alveolar hydatidosis (10), *Taenia solium* cysticercosis (8), *Onchocerca volvulus* (4), toxocariasis (2), filariasis (1), rheumatoid factor positive (2), Chagas' (3); Group 7: rheumatoid factor positive (1); Group 8: alveolar hydatisodis (7) Group 9: alveolar hydatidosis (1); Group 10: alveolar hydatidosis (8). Each serum was tested by ELISA using plates coated with HCFA, AgB or Ag5. The symbols (+) or (−) indicate that the serum showed an OD_{600} higher or lower than the cut-off value for the corresponding test, respectively. The cut-off was defined as the mean value of the OD_{600} shown by the sera from healthy donors, plus 3 standard deviations.

Table 3 Diagnostic performances of native antigens

Antigen	Coating conc. (μg/ml)	Sensitivity (a) (%)	Specificity (a) (%)	DE (a) (%)
HCFA	20	76/90 (84%)	70/116 (60%)	146/206 (71%)
Ag5	10	45/90 (50%)	107/116 (92%)	152/206 (74%)
AgB	10	69/90 (77%)	100/116 (86%)	169/206 (82%)

Coating conc. refers to the antigen concentration in the PBS solution used to coat the plates. Sensitivity, specificity and DE were calculated according to the corresponding definitions described under Data analysis in Materials and Methods and expressed as percentage. The ratios of numbers of sera, used for those calculations are also described (a).

Table 4 Reactivities of individual sera with immunopurified native antigens and their corresponding synthetic peptides

Group of sera	Number sera/group	AgB (PBS)	65-BSA (PBS)	GU4 (TFE)	Ag5 (PBS)	89–122 (TFE)
1	5	+	+	+	+	+
2	3	+	+	+	−	+
3	2	+	+	+	+	−
4	1	+	+	−	+	+
5	17	+	+	−	+	−
6	5	−	+	+	−	+
7	1	+	−	+	+	−
8	19	+	−	−	+	−
9	3	+	+	−	−	−
10	18	+	−	−	−	−
11	16	−	−	−	−	−
12	92	−	−	−	−	−
13	8	+	−	−	−	−
14	6	−	+	−	−	−
15	1	−	−	−	+	−
16	1	−	−	+	−	−
17	5	+	−	−	+	−
18	2	+	+	−	+	−
19	1	+	+	+	+	−

Patients sera with similar patterns of reactivity were included in each group to allow comparison among antigens. The relevant parasitological data corresponding to sera included in each group are the following: Group 1: cystic hydatidosis: hepatic cyst (1), lung cysts (3), not recorded cyst location (1); Group 2: cystic hydatidosis: hepatic cysts (2), not recorded cyst location (1); Group 3: cystic hydatidosis: hepatic cyst (1), not recorded cyst location (1); Group 4: cystic hydatidosis: not recorded cyst location (1); Group 5: cystic hydatidosis: hepatic cysts (9), lung cysts (2), bone cyst (1), multiple location cyst (1) and not recorded cyst location (4); Group 6: cystic hydatidosis: hepatic cyst (1), not recorded cyst location (4); Group 7: cystic hydatidosis: hepatic cyst (1); Group 8: cystic hydatidosis: hepatic cysts (12), not recorded cyst location (7); Group 9: cystic hydatidosis: not recorded cyst location (3); Group 10: cystic hydatidosis: hepatic cysts (9), bone cysts (5), multiple location cysts (2) and not recorded cyst location (2); Group 11: cystic hydatidosis: hepatic cysts (4), lung cysts (6), bone cysts (2), multiple location cysts (3) and not recorded cyst location (1); Group 12: alveolar hydatidosis (10), *Taenia solium* cysticercosis (22), *Onchocerca volvulus* (2), *Schistosoma mansoni* (5), toxocariasis (9), filariasis (2), rheumatoid factor positive (4), Chagas' (4), toxoplasmosis (3), syphilis (2), cancer (1), healthy donors (28); Group 13: alveolar hydatidosis (7), rheumatoid factor positive (1); Group 14: *Onchocerca volvulus* (4), *Schistosoma mansoni* (1), toxocariasis (1); Group 15: alveolar hydatidosis (1); Group 16: alveolar hydatidosis (1); Group 17: alveolar hydatidosis (5); Group 18: alveolar hydatidosis (2); Group 19: alveolar hydatidosis (1). Each serum was tested by ELISA using plates coated with AgB, Ag5 and the 3 synthetic peptides using the corresponding optimum conditions. The symbols (+) or (−) indicate that the serum showed an OD_{600} higher or lower than the cut-off value for the corresponding test, respectively. The cut-off was defined as the mean value of the OD_{600} shown by the sera from healthy donors, plus 3 SDs.

Only 15% were positive with GU4, 10% with 89-122 and 35% with peptide 65.

Fifty-five percent of sera from patients with known lung cysts were negative with GU4 and the other two peptides, the remaining 45% were positive only with peptide 65. Twenty seven percent of the sera were positive both with GU4 and 89-122.

Eighty-eight percent of sera from patients with known bone cysts were negative with GU4 and the other two peptides and the remaining 12% were positive only with peptide 65.

Cross-reactivity with synthetic peptides

Eighty-nine percent of the sera from patients with alveolar hydatidosis and all the sera from patients with *T. solium* cysticercosis were negative with GU4 and the other 2 peptides. Interestingly, four out of six sera from patients infected with *Onchocerca volvolus* were positive only with peptide 65, while all these sera were negative with the other two peptides. One out of six sera from patients infected with *Schistosoma mansoni* and one out of ten with toxocariasis were positive only with peptide 65. All those sera were negative with peptide 89-122 and only two sera from patients with alveolar hydatidosis were positive with peptide GU4.

Comparison of native antigens with the peptides mimicking epitopes from them

Comparison of peptide 65-BSA and peptide GU4 in TFE with AgB show that the native antigen provides higher DE than each peptide ($\chi^2 = 27{\cdot}09$, critical region $[7{\cdot}81, \infty)$ and $\chi^2 = 74{\cdot}42$, critical region $[7{\cdot}81, \infty)$, respectively). The same between peptide 89-122 in TFE with Ag5 $\chi^2 = 33{\cdot}59$, critical region $[7{\cdot}81, \infty)$).

Although 69 out of 90 cystic hydatidosis sera showed positive results with AgB, there were 74 out of 90 showing those results with either AgB or peptide 65 and also 74 out of 90 with either AgB or peptides 89-122 or GU4 (see Table 4).

Similarly, 45 out of 90 hydatid sera showed positive results with Ag5, while 53 out of 90 showed those results with either Ag5 or peptides 89-122, or 65 or GU4.

The titres of all sera when tested with AgB and with peptide 89-122 TFE are shown in Table 5. If sera reacting with either AgB or peptide 89-122 TFE were to considered positive, the corresponding combined serology would increase DE from 82% (only with AgB) to 84% (combined), significantly higher ($P = 0{\cdot}03125$).

DISCUSSION

We have compared the relative diagnostic values in ELISA

Table 5 Titres showed by individual sera with AgB and peptide 89–122

Group of sera	Number of sera/group	Reactivity with AgB	Reactivity with 89-122 TFE
1	1	4	2
2	3	4	1
3	36	4	–
4	1	3	2
5	1	3	1
6	9	3	–
7	1	2	1
8	10	2	–
9	2	1	1
10	5	1	–
11	1	–	4
12	1	–	3
13	2	–	2
14	1	–	1
15	16	–	–
16	1	4	–
17	1	3	–
18	8	2	–
19	6	1	–
20	100	–	–

Patients sera were grouped according to the titres observed with both antigens to show their complementary reactivity. The relevant parasitological data corresponding to sera included in each group is the following: Group 1: cystic hydatidosis: not recorded cyst location (1); Group 2: cystic hydatidosis: hepatic cyst (1), lung cysts (2); Group 3: cystic hydatidosis: hepatic cysts (19), lung cysts (2), bone cysts (2), multiple location cyst (1) and not recorded cyst location (12); Group 4: cystic hydatidosis: lung cyst (1); Group 5: cystic hydatidosis: not recorded cyst location (1); Group 6: cystic hydatidosis: hepatic cysts (5), bone cysts (3), not recorded cyst location (1); Group 7: cystic hydatidosis: hepatic cyst (1); Group 8: cystic hydatidosis: hepatic cysts (5), bone cyst (1), multiple location cysts (2) and not recorded cyst location (2); Group 9: cystic hydatidosis: hepatic cyst (1), not recorded cyst location (1); Group 10: cystic hydatidosis: hepatic cysts (3), not recorded cyst location (2); Group 11: cystic hydatidosis: not recorded cyst location (1); Group 12: cystic hydatidosis: not recorded cyst location (1); Group 13: cystic hydatidosis: hepatic cyst (1), not recorded cyst location (1); Group 14: cystic hydatidosis: not recorded cyst location (1); Group 15: cystic hydatidosis: hepatic cysts (4), lung cysts (6), bone cysts (2), multiple location cysts (3) and not recorded cyst location (1); Group 16: alveolar hydatidosis (1); Group 17: alveolar hydatidosis (1); Group 18: alveolar hydatidosis (8). Group 19: alveolar hydatidosis (5), rheumatoid factor positive (1); Group 20: alveolar hydatidosis (12), *Taenia solium* cysticercosis (22), *Onchocerca volvulus* (6), *Schistosoma mansoni* (6), toxocariasis (10), filariasis (2), rheumatoid factor positive (4), Chagas' (4), toxoplasmosis (3), syphilis (2), cancer (1), healthy donors (28). The values in this table reflect the reactivities of each individual serum in ELISA using either AgB in PBS or peptide 89–122 in TFE to coat the plates. The reactivity values correspond to the OD_{600} readings in the corre-sponding ELISA tests are expressed according to the following definitions: Reactivity −: $OD_{600} < X + 3$ SD. Reactivity 1: $x + 3\,SD < OD_{600} < x + 5$ SD. Reactivity 2: $x + 5\,SD < OD_{600} < x + 7$ SD. Reactivity 3: $x + 7\,SD < OD_{600} < x + 9$ SD. Reactivity 4: $x + 9\,SD < OD_{600}$. In the above definitions x is the mean value of the OD_{600} shown by sera from healthy donors and SD the value of the corresponding standard deviation.

of the novel synthetic peptide (GU4) with HCFA, with purified AgB and Ag5 as well as with two already described synthetic peptides mimicking epitopes from the last two antigens. This was performed by testing these six antigens with a large and comprehensive sera collection. We also tested GU4 and the other two peptides with the whole panel of sera, using different plate coating conditions which may influence peptide-antibody binding, to improve mimicking of native epitopes by each peptide. As far as we know, serological comparison among native and synthetic hydatid antigens using the same defined panel of patients sera, as recommended by Lightowlers & Gottstein (1995), has not been previously described.

We have controlled both immunopurified native AgB and Ag5 using SDS-PAGE and Western blot (data not shown). Our results show that immunopurified native AgB is more efficient for diagnostic use than HCFA (see Table 3). Sensitivity shown by HCFA (84%) is consistent with what has already been reported (75%–94%) (Lightowlers & Gottstein 1995). Interestingly, 92%, 88% and 55% were the sensitivities observed with this crude antigen among patients with known liver, bone and lung cysts, respectively. The corresponding values for AgB were 88%, 75% and 45% and those for Ag5 were 60%, 12% and 45% (Table 2). These results are consistent with those reviewed by Lightowlers & Gottstein (1995), and suggest that Ag5 is very weakly immunogenic in patients with bone hydatid cysts, while in this case AgB shows high immunogenicity. Our results using immunopurified AgB in ELISA are consistent with those reported by Maddison *et al*. (1989) using immunoblot to identify serum antibodies reacting with the 8 kDa AgB subunit, rather than those of Leggat *et al*. (1992) who found cross-reaction of sera from patients with *T. solium* cysticercosis with the lower MW subunit of AgB. Maddison *et al*. (1989) showed that 82% of the sera from cystic hydatidosis patients and 39% of sera from patients with alveolar hydatidosis were positive to their test. In our test (see Table 2) 77% and 56% respectively were observed. They also reported that 52% of sera from patients with alveolar hydatidosis recognized a ladder of bands with MW ranging from 14 to 40 kDa. Interestingly, native immunopurified AgB shows a ladder-like SDS-PAGE profile with 6 bands of 8, 16, 24, 32, 40 and 48 kDa (González *et al*. 1996). AgB also showed higher DE than Ag5, consistently with the data reviewed by Lightowlers & Gottstein (1995). Although Ag5 showed increased specificity as compared with AgB, a much higher sensitivity was provided by the latter. In fact, none of the sera from hydatid patients which showed positive results with Ag5 was negative with AgB (see Table 2). We found no sera in this sera collection, from patients with parasitic diseases other than cystic or alveolar hydatidosis, which reacted either with AgB or Ag5. We showed (Table 2) that 33% of alveolar hydatid patients and 50% of cystic hydatid patients were positive with Ag5 as compared with 58% and 74%, respectively, previously reported using immunoelectrophoresis (Lightowlers & Gottstein 1995). The lower sensitivity with both kinds of sera may be reflecting the fact that the Ag5 recognized by MoAb EG02 154/12 would not be the only one precipitating in immunoelectrophoretic arc-5. This is consistent with the results reported by Zhang & McManus (1996) showing that an Ag5-specific MoAb (AGH2) reacted with several 38 kDa bands putatively corresponding to subunits of Ag5. Interestingly, these subunits did not show significant homology with the sequence of a fusion protein (FP6) reported by Facon *et al*. (1991) to react with EG 02 154/12 and with arc-5 positive human sera. Our results also confirm the diagnostic value of the native antigens bearing the epitopes recognized by MAb EG 02154/12. As shown in Table 2, seven out of 90 sera from cystic hydatid patients were positive with HCFA and negative with either AgB or Ag5. These results suggest that HCFA may contain antigens different from AgB and Ag5 which may be used to improve the diagnostic sensitivity provided by AgB in ELISA. This is consistent with the results described by Kanwar *et al*. (1992) and Shambesh *et al*. (1995).

Adsorption of GU4 onto the plastic surface using concentrations above 2 μg/ml in BCB, severely affects its antibody binding capacity (see Figure 1). Although this effect was not observed with GU4 in TFE, antibody binding to plates coated with 20 μg/ml of GU4 in TFE was lower than that shown by plates coated with 2 μg/ml of GU4 in BCB (see Figure 1). Therefore, coating conditions appear to be crucial for antibody binding to GU4. In spite of the fact that TFE did not affect the diagnostic performance of native HCFA it did influence that of the synthetic peptides (see Table 1). Comparison of the effect of TFE on GU4 and peptide 65, showed that this treatment increased the reactivity of peptide GU4 (both with hydatid and non-hydatid patients sera), while it decreased that of peptide 65. The effect of TFE on peptide 89-122 was similar to peptide 65, but weaker. Although Chamekh *et al*. (1992) showed that alpha-helical conformation may be crucial for antibody binding by 89-122, our results can not confirm this. They suggest that antibody binding by peptide GU4 may be more dependent on recovery of alpha-helical conformation than in the case of peptides 65 and 89-122. Prediction of secondary structure from sequence data (Garnier *et al*. 1978, PC Gene) showed that the sequences of AgB8/1 and AgB8/2 are consistent with 82% and 66% of alpha-helical organization, respectively. The region of AgB8/2 corresponding to the sequence of peptide GU4 is predicted to have only helical organization, while that of AgB8/1 corresponding to peptide 65 shares helical, turn and extended organizations.

Interestingly, predictions on the basis of the sequences of both isolated peptides suggested that peptide 65 may have high helical organization (85%), while no helical organization at all is predicted for isolated GU4. The latter predictions are consistent with the higher influence of coating with TFE on antibody binding by peptide GU4 as compared with peptide 65. Coating peptides directly to the plate, is likely to generate higher peptide-plastic interactions than coating them through a BSA arm. In fact, conjugation of GU4 and the other two peptides to BSA markedly increased in all cases the ELISA readings observed with all sera (data shown only for peptide GU4, see Figure 1). Nonetheless, seven sera from cystic hydatid patients which showed positive serological results with peptide 65 directly adsorbed to the plates were negative when it was conjugated with BSA, and this also happened with 27 non-cystic hydatid sera. In spite of that, although peptide 65 directly adsorbed to the plate showed higher sensitivity and lower specificity than its BSA conjugate, the opposite effect was observed with the other two peptides (Table 1). Therefore, signal strength should not be the only criterion considered to select the appropriate coating conditions to use synthetic peptides in serology. It is advisable to select them on the basis of reactivity with a panel of patients sera rather than with only some positive sera or a pool of them. No general rule concerning the optimum coating conditions for synthetic peptides in ELISA could be concluded from our results. In some cases, as in that of peptide 65, dramatic variations in reactivity were observed using different coating conditions, while this was not the case with GU4. Therefore, selection of the appropriate coating conditions appears as a crucial issue when using synthetic peptides in serology.

The relative diagnostic values shown by peptides 65 and 89–122 using the same sera collection were very different from those reported separately by Legatt & McManus (1994) and Chamekh *et al*. (1992), respectively, using different sera collections. Therefore our results support the suggestion, made by Lightowlers & Gottstein (1995), related to the necessary use of the same panel of sera to effectively compare the diagnostic values of the different antigens available.

Analysing the reactivities of individual sera, it may be concluded that if a serum would be considered positive when it reacts with AgB and/or 65-BSA, the corresponding serology would show 82% sensitivity, 81% specificity and 82% DE. A similar analysis with AgB and/or peptide 89-122 shows that sensitivity would be 82%, specificity 86% and DE 84%. According to Table 5, which shows the titres of all sera tested against AgB in PBS and peptide 89-122 in TFE, eight sera from cystic hydatidosis patients (groups 9, 10 and 14) showed titres in the range of 3–5 SDs above the diagnostic threshold. In addition, six sera (group 19) from patients with other pathologies showed titres in the same range, five of them being from patients with alveolar hydatidosis. All these six sera reacted with AgB but not with the peptide 89–122. These 14 sera showing titres near the diagnostic threshold are a clear indication of the sensitivity and specificity limitations of hydatid serology. Therefore, the best diagnostic results would be obtained if two ELISA tests were used for each serum, one with AgB and another with peptide 89–122 coated in TFE. With this approach, a serum reacting with AgB and/or peptide 89–122 should be considered positive.

Taken together, our results suggest: 1) that the data available on the diagnostic values of hydatid antigens using different sera collections do not allow an appropriate comparison of these antigens, 2) that cyst fluid contains diagnostically relevant antigens, different from Ag5 and AgB, 3) that new synthetic peptides mimicking conformational epitopes of AgB and Ag5 different from those epitopes mimicked by the three peptides used here, may be needed to achieve a relevant improvement in cystic hydatid serology, 4) that coating conditions possibly affecting the conformation of peptides may influence their serological performance on a non-predictable way.

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