Comparative Analysis of the Diagnostic Performance of Six Major *Echinococcus granulosus* Antigens Assessed in a Double-Blind, Randomized Multicenter Study

Carmen Lorenzo,¹ Henrique B. Ferreira,² Karina M. Monteiro,² Mara Rosenzvit,³ Laura Kamnetzky,³ Hector H. García,⁴ Yessika Vasquez,⁴ Cesar Naquira,⁵ Elizabeth Sánchez,⁵ Myriam Lorca,⁶ María Contreras,⁶ Jerry A. Last,⁷ and Gualberto G. González-Sapienza¹*

Cátedra de Inmunología, Facultad de Química, UDELAR, Instituto de Higiene, Montevideo, Uruguay¹; Laboratório de Biologia Molecular de Cestódeos, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Rio Grande do Sul, Brazil²; Departamento de Parasitología, Instituto Nacional de Enfermedades Infecciosas, ANLIS “Dr. Carlos G. Malbrán,” Buenos Aires, Argentina³; Departamento de Microbiologia, Universidad Peruana Cayetano Heredia, Instituto de Ciencias Neuroológicas, Lima, Peru⁴; Laboratorio de Zoonosis, División de Parasitología, Instituto Nacional de Salud, Lima, Peru⁴; Unidad de Parasitología, Facultad de Medicina, Universidad de Chile, Santiago de Chile, Chile⁵; and Department of Internal Medicine (Pulmonary), School of Medicine, University of California, Davis, California⁷

Received 11 November 2004/Returned for modification 7 January 2005/Accepted 10 February 2005

The serodiagnosis of hydatid disease is a valuable instrument for clinical diagnosis and epidemiological surveillance of high-risk populations. In the past decade a wealth of reports on the diagnostic performance of numerous antigens have been produced. However, their diagnostic value has been estimated under different conditions, using different serum collection, therefore precluding their direct comparison. Here we report an unbiased comparison of the same batch of six major *E. granulosus* antigens, namely, hydatid cyst fluid (HCF), native antigen B (AgB), two recombinant AgB subunits, an AgB-derived synthetic peptide, and recombinant cytosolic malate dehydrogenase from *E. granulosus* (EgMDH), against the same serum collection. The double-blind analysis was performed using a standardized protocol and receiver operating characteristic (ROC) data analysis by a network of six South American laboratories. High intercenter reproducibility was attained, and the intralaboratory analysis allowed the comparative ranking of the antigen panel. HCF, AgB, and its AgB8/1 subunit exhibited equivalent diagnostic efficiencies, 81.4% ± 0.5%, 81.3% ± 0.6%, and 81.9% ± 2.0%, respectively; with a more favorable balance toward specificity in the case of the last antigen. The diagnostic efficiencies for the other three antigens were 76.8% ± 6.8%, 69.1% ± 2.7%, and 66.8% ± 2.1%, for the peptide, the AgB8/2 subunit, and the EgMDH, respectively. The study also included an analysis of batch-to-batch variation in the diagnostic performance of different HCF regional preparations. Based on these results, a suggested recommendation on the use of these antigens was drawn.

The larval stage of the cestode parasite *Echinococcus granulosus* causes cystic hydatid disease, which affects humans and a range of livestock animals (17, 21). *E. granulosus* has a cosmopolitan distribution, and the disease is well know in Asia, Africa, South and Central America, the Mediterranean, and Eastern Europe, with some foci in the United Kingdom (3, 5). Hydatid disease is preventable; therefore, in places where efficient and sustained control campaigns have been implemented its prevalence has decreased dramatically (22). Unfortunately, this is not the general scenario, and numerous reports indicate that its incidence has increased in various regions of the world (4). The accurate assessment of its prevalence is therefore a major element to expose the magnitude of the problem and evaluate the success of the control strategy. This involves clinical diagnosis of the disease, but very importantly the epidemiological surveillance of high-risk populations. The most useful tools to monitor the incidence of the disease in asymptomatic high-risk populations are imaging techniques and serology. Imaging methods, such as sonography, are highly sensitive for inspection of the abdominal cavity; while serology, which is considered to be less sensitive, can be used regardless of cyst localization (16).

In the past decade major advances have been produced in the purification, cloning, and characterization of relevant *E. granulosus* antigens. A wealth of reports on the diagnostic evaluation of immunopurified components from hydatid cyst fluid and protoscoleces, as well as that of numerous recombinant *Echinococcus* antigens, are available (9, 10, 14, 15, 23). However, the diagnostic performance of these antigens has been assessed in different laboratories, using different serum collections and different techniques, which makes it difficult to draw conclusions. Indeed, in a recent review on this matter...
(24), it can be observed that the sensitivity and specificity obtained with hydatid cyst fluid (HCF), as reported by different laboratories, range from 31 to 96%, and 41 to 100%, respectively. Though less extreme, a wide variation in these parameters was also found for the diagnostic performance of native antigen B (AgB) and antigen 5. This lack of concordance generates confusion, and has hampered the transition towards a more standardized and consensual immunodiagnosis.

In order to join efforts and contribute to the standardization of hydatid disease immunodiagnosis, we recently established a network of South American laboratories (http://bilbo.edu.uy/~immuno/serology). In our initial study, which is reported here, we conducted a double-blind, multicenter study, where the same batch of six *E. granulosus* antigens was analyzed against a common serum collection. Our work produced a reliable comparison of these antigens and showed that, under controlled conditions, it is possible to obtain highly reproducible results in distant laboratories.

**MATERIALS AND METHODS**

**Human serum collection.** The serum collection used in this study comprised 59 serum samples from patients with surgically confirmed hydatid disease, collected in Argentina, Brazil, Chile, Peru, and Uruguay, with the following record of cyst location: liver, 33 samples; lungs, 15 samples; multiple sites, 11 samples. The serum samples were not preselected on the basis of previous serologic information and were drawn before surgery. In addition, 15 sera from healthy donors and 55 serum samples from patients with the following diseases were included: alveolar hydatid disease (n = 10), cysticercosis (n = 20), schistosomiasis (n = 8), fascioliasis (n = 7), and trichinosis (n = 10). The sera were preserved with 0.05% sodium azide and stored at −20°C until tested.

**Antigens.** A common panel constituted by the same batch of six *E. granulosus* antigens was evaluated by all participating laboratories and included bovine HCF (HCF1); native AgB; two recombinant AgB subunits, namely, AgB8/B1 (7) and AgB8/B2 (6); recombinant cytotoxic malate dehydrogenase from *E. granulosus* (18); and an AgB-derived synthetic peptide (p176) (10). HCF was obtained by aseptic aspiration from either bovine or ovine fertile cyst (2). Seven HCF preparations were used in this study, HCF1 to HCF4 and HCF5 to HCF7, from bovine and ovine origin, respectively. HCF was analyzed in parallel in all participating laboratories. AgB was immunopurified from HCF according to the method described by Gonzalez et al. (8). The recombinant antigens were prepared as glutathione S-transferase (GST) fusion proteins and affinity purified. The fusion protein antigen moieties were recovered by thrombin cleavage as described by Virgino et al. (23). Antigen concentration was determined by the bichinchoninic acid method (Pearce, Rockford, Ill.). p176 is a 38-mer peptide (DDGLT STRSVMKMFGEVKYFFERDPLQGKVVDLLKE) corresponding to the N-terminal extension of the AgB8/B1 subunit. The peptide was synthesized, purified by reverse-phase high-performance liquid chromatography (95%), and analyzed by mass spectrometry at Iris Biotech GmbH.

**Enzyme-linked immunosorbent assay (ELISA).** Antigen coating solutions were prepared in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.2 (25 µg/ml for HCF or 4 µg/ml for the other antigens). Then microtitration plates (NUNC Maxisorp or Greiner Microtiter High Binding) were coated by overnight incubation at 4°C with the appropriate antigen solution (100 µl/well). After the coating solution was discarded, the plates were blocked for 1 h at 37°C with 5% nonfat milk powder in phosphate-buffered saline (PBS) and washed with PBS–0.05% Tween 20 (PBS-T). The serum samples were diluted 1:200 in PBS-T containing 5% nonfat milk powder and tested in triplicate. After 90 min of incubation at 37°C the plates were washed three times with PBS-T. Then 100 µl of peroxidase-conjugated goat anti-human immunoglobulin G (Sigma, St. Louis, Mo.) diluted 1:3,000 was dispensed into each well and incubated 1 h at 37°C. After washing, a substrate solution containing H2O2 and 2,2′-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid was added in 50 mM citrate buffer, pH 4.0 (100 µl/well), and incubated for 15 min at room temperature with shaking. Optical densities were measured at 405 or 415 nm.

**Data analysis.** The cutoff value for positive scores was calculated in two ways. In the first case, the cutoff was defined as the mean absorbance value for the 15 healthy donors plus 3 standard deviations. Using this criterion, the hydatid patient sera were classified as true positives (tp) or false negatives (fn), on the basis of their positive or negative scores. Similarly, the rest of the sera were classified as false positive (fp) or true negatives (tn), depending on whether the readings were higher or lower than the cutoff, respectively. The following definitions were used to calculate the corresponding diagnostic parameters: sensitivity (se; %) = tp × 100/(tp + fn); specificity (sp; %) = tn × 100/(tn + fp); diagnostic efficiency (de; %) = (tp + tn) × 100/(tp + fp + tn + fn). Alternatively, the receiver operating characteristic (ROC) analysis (20) was utilized to analyze the data using the SPSS 10.0 software package (SPSS Inc., Chicago, Ill.). ROC curves were generated by plotting sensitivity versus 1−specificity, and the area under the curve was used to carry out a pairwise comparison of the diagnostic performance of the antigens.

**RESULTS**

**Characteristics of the study.** This study involved six laboratories in five countries: Laboratory of Molecular Biology of Hydatid Disease, Carlos Malbran Institute, Buenos Aires, Argentina; Laboratory of Cestode Molecular Biology, Biotechnology Center, UFRGS, Rio Grande do Sul, Brazil; Parasitology Unit, Faculty of Medicine, University of Chile, Santiago de Chile, Chile; Parasitology Unit, National Health Institute, Lima, Peru (Peru-1); Department of Microbiology, Cayetano Heredia Peruvian University, Lima, Peru (Peru-2); Immunology Department, Faculty of Chemistry, Universidad de la República, Montevideo, Uruguay. The serum collection utilized in this study was established with serum samples contributed by the participating laboratories. The antigens and sera were gathered in the coordinating laboratory in Montevideo, where they were randomly encoded, aliquoted, and submitted to the network centers for analysis. The size and composition of our serum collection was considered to be a good balance between representativeness and volume of ELISA work, which allow testing of each antigen using five ELISA plates. Similarly, in order to reduce the complexity of the study and facilitate the double-blind analysis of the different antigens, a common ELISA protocol was utilized. To diminish the sources of assay variations, critical components, such as ELISA plates, blocking agent, and secondary antibody, were shared among the participants.

**Determination of the cutoff value.** All serum samples were analyzed in triplicate, and low- and medium-titer standards (triplicates) were included in all ELISA plates and used for data normalization. The raw data were submitted to the coordinating laboratory in Montevideo, where serum samples and antigens were decoded and data processed. Figure 1 displays representative results produced in the different centers using HCF1, AgB, and AgB8/B1, although with lower readings, similar results were obtained with the other antigens (not shown). For each antigen, the cutoff value, which differentiates positive from negative results, was established by two methods. In the first case, we used the widespread approach of defining the cutoff as the mean value of the normal serum group plus three standard deviations. Alternatively, the cutoff was established by ROC analysis, defined as the absorbance value that gave the highest sum of sensitivity (%) and specificity (%). Figure 2 shows a pair of representative sets of ROC curves obtained in two of the participating laboratories, which are used to estimate the cutoff. In general, ROC analysis provided a better discrimination between true-positive and true-negative sera, particularly in the case of HCF1 and AgB (Table 1). Except when specifically stated, this criterion will be used throughout this study to compare the antigens.
FIG. 1. Reactivity of the serum collection against HCF, AgB, and AgB8/1 assessed by ELISA. The sera were grouped as follows: Eg, sera from patients with cystic hydatidosis; Ts, sera from patients with cysticercosis; Em, sera from patients with alveolar hydatidosis; Ns, sera from healthy donors; others, other sera used in this study. The cutoff estimated as the mean value plus three standard deviations and by ROC analysis are shown by dotted and solid lines, respectively. OD, optical density.
Intralaboratory results. Regarding the mean value of the diagnostic performance estimated in the different laboratories, HCF1, AgB, and AgB8/1 exhibited very similar diagnostic efficiencies, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1). Within this group, small differences were observed in sensitivities, which were the highest of the antigen panel (Table 1).

Interlaboratory results. A remarkable outcome of our study was the high reproducibility attained in the different centers. Again, this was especially true for HCF1, AgB, and AgB8/1 (de $\approx 81.4\% \pm 0.5\%, 81.3\% \pm 0.6\%$, and $81.9\% \pm 2.0\%$, respectively). This is in agreement with the pairwise comparison of the same antigens in different laboratories as shown in Table 3. No significant differences in the diagnostic precision were found for HCF1, AgB, and AgB8/1 (except for a small discrepancy in the evaluation of the latter between the Chilean and Argentinean and Chilean and Uruguayan laboratories). Larger differences among the participating laboratories were found for the determination of the diagnostic value of the remaining antigens, particularly for p-176.

Evaluation of batch and HCF origin influence on ELISA diagnostic performance. In order to evaluate possible variations in the diagnostic performance of different HCF prepaa-
tions, each laboratory analyzed an additional HCF batch, including three bovine HCF and three ovine HCF preparations. Despite significant differences in the cutoff values obtained (Fig. 3), there was a good agreement in the calculated parameters of sensitivity and specificity attained with the different HCF preparations. This is also evident from the pairwise comparison of the diagnostic performance of the antigens by ROC analysis. All HCF batches, regardless of their origin, performed without significant differences (Table 4).

**DISCUSSION**

The main goal of this work, to our knowledge the first of its kind, was the generation of a reliable comparison of relevant *E. granulosus* antigens. This is a major need, since it is impossible to contrast the diagnostic value of antigens that have been evaluated with different protocols, in different laboratories, and using different serum collections. That is so critical that, despite the availability of well-defined, purified, or recombinant antigens, their potential use is halted by the lack of sound criteria that may or may not justify their utilization. The antigens chosen for this study included HCF, which, due to its ease of preparation, is the most popular antigenic source, and native AgB, the major parasite component of HCF, which is regarded as one of the most valuable *E. granulosus* antigens. Our panel also comprised three AgB-related antigens, namely, AgB8/1, AgB8/2, and the peptide p-176, which have been reported as highly antigenic in human infections (7, 10, 19). Additionally, EgMDH was also included on the basis of its availability and previously reported diagnostic value, and as an alternative to AgB-related antigens. Other candidate antigens, which were on hand in the participating laboratories, were specifically excluded because they were considered inferior to those of the
selected panel. This was the case of native antigen 5, which had been previously compared to AgB against the same serum collection, showing a markedly reduced diagnostic performance (1).

As shown in Tables 1 and 2, HCF, AgB, and AgB8/1 exhibited the highest diagnostic value and behaved as equivalent antigens with no significant differences in their diagnostic performance when the data were analyzed by ROC. Moreover, the individual analysis of our serum collection showed that, using these antigens, there was an almost complete agreement in the intra- and interlaboratory classification of each individual serum as positive or negative, which reinforces the statistical findings. We speculated that based on the complex composition of HCF its de could be affected by a high degree of cross-reactivity; however the use of ROC to set up the cutoff for this antigen seems to solve this problem. Under these conditions HCF emerged as a valuable antigen, and for this reason we specifically addressed the issue of its batch-to-batch reproducibility. Strikingly, as can be seen from Table 4, no significant differences existed among HCF batches, even for those of different host species and geographical origins.

The other antigens in our panel, p-176, Ag8/2, and EgMDH, did not perform as well as would have been expected from the previous literature. We would speculate that these contradictions arise, mainly, because of use of a different serum collection. Regarding that, we still do not know the level of variation in the subunit composition of AgB at different stages of the metacestode development (12), or how the integrity of the cyst will determine the host exposure to EgMDH (a cytosolic component of *E. granulosus*), which certainly depends on the extent of parasite cell damage. In the case of the peptide, an intriguing fact was its differential behavior in the different centers. Indeed, the estimated mean value of p-176 de showed the largest interlaboratory variation (75.8% ± 6.3%). However, it can be observed that three centers (Argentina, Peru-2, and Uruguay) produced a mean value of 81.4% ± 2.1%, similar to that of HCF, AgB, and AgB8/1, and in agreement with the de previously reported for this antigen (10). On the other hand, the other centers (Brazil, Chile, and Peru-1) attained a markedly lower de value of 70.3% ± 1.2%. Since this is a highly reproducible reagent, it may be worth the additional effort to study the causes that negatively affected its performance in these laboratories.

One of the major challenges of hydatid serology is the definition of suitable tools for large-scale seroepidemiological studies. Due to its rather low prevalence, these studies require simple and inexpensive methods, allowing the parallel analysis of thousands of samples with high sensitivity. For this reason, our study was based in the use of ELISA to measure total immunoglobulin G responses. However, despite the fact that our panel of sera was tested against a selection of widely used and highly promising antigens none of them provided the desired sensitivity, and roughly one-fifth of the hydatid sera gave rise to false-negative results. Our previous experience indicates that when the serum collection is based upon samples that have not been selected on the basis of previous serological information, this is a common scenario and not a particular character-

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<th>Table 4. ROC z values corresponding to the interlaboratory pairwise comparison of different batches of HCF</th>
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![Graph](http://jcm.asm.org/)

FIG. 3. Reactivity of the serum collection against different preparations of HCF assessed by ELISA. The sera were grouped as follows: Eg, sera from patients with cystic hydatidosis; Ts, sera from patients with cysticercosis; Em, sera from patients with alveolar hydatidosis; Ns, sera from healthy donors; others, other sera used in this study. The cutoff estimated by ROC analysis is shown.
istic of the serum collection used in this study (1, 10). In that regard, we searched for complementarity among the antigens to explore the possibility that the combination of two or more antigens would improve sensitivity. However this was not the case and in general, once the proper cutoff has been established, each individual serum classified as either positive against all of HCF, AgB, and AgB8/1 or negative against all of them, with no intermediate situations. Although the potential role of novel antigens to improve this situation cannot be ruled out, it seems that for some patients and particular stages of the disease, the limiting factor is the actual existence of a measurable antibody response. Indeed, it is a well-established fact that the major evasion strategy of *Echinococcus* sp. parasites relies on their capacity to seclude themselves from the host immune response (11).

In conclusion, our collaborative work shows that, under controlled conditions, it is possible to perform serological studies in distant laboratories with comparable results. Through a double-blind objective study, this work demonstrated that HCF, AgB, and AgB8/1 are the most valuable antigens of the panel, with equivalent diagnostic performance. The fact that most of the serum samples that score positive using HCF also score positive using AgB or AgB8/1 offers the opportunity of a second confirmatory test that may improve specificity without significant loss of sensitivity. This provides a tool for the confirmation of the large fraction of weakly positive/negative serum samples that classified as doubtful in the initial screening, because their readings are close to the cutoff value, a common scenario in the serology of hydatid disease. Consequently, on the basis of its availability and little influence of batch-to-batch variations, we thus recommend the use of HCF for initial screening in large seroprevalence studies, utilizing a rather permissive cutoff value (such as the mean value of a group of normal sera plus 2 standard deviations). Further analysis of positive serum samples with AgB and AgB8/1 (using a properly estimate ROC cutoff) would allow the confirmation of true positives and eliminate a large fraction of false-positive sera, thus providing specificity.

**ACKNOWLEDGMENTS**

This work was supported by NIH Fogarty International Center Grant TW05718. Additional support was also provided by grants AI53894 and AI051976 from the NIH (United States) and the RIKDP network funded by the Swedish International Development Agency. K.M.M. is the recipient of a PIBIC-CNPq (Brazil) fellowship. Conicet and Cabbio y Roemmers (Argentina) are also acknowledged.

We thank Arnaldo Zaha for critical reading of the manuscript and Kimiaki Yamano from the Hokkaido Institute of Public Health for donation of the *E. multilocularis* sera.

This work was done as part of the PAHO South American Subregional Program for Control and Surveillance of Hydatid Disease and the WHO Informal Working Group on Immunodiagnosis of Human Cystic Echinococcosis.

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