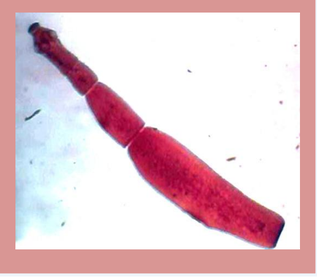
DIAGNOSTIC PERFORMANCE OF A NATIVE ANTIGEN 5 ELISA FOR HUMAN CYSTIC ECHINOCOCCOSIS



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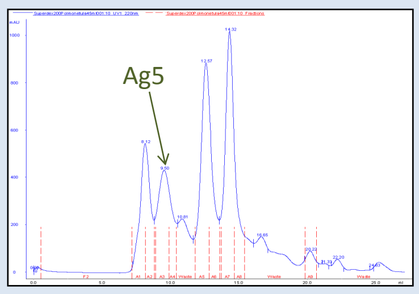
1. AIM

Antigen 5 (Ag5) is one of the most abundant and immunogenic proteins expressed by *Echinococcus granulosus*. After an initial interest in its use for cystic echinococcosis (CE) diagnosis, it has been neglected due to possible cross-reactivity issues in patients affected by other parasitoses, as well as to controversial results in terms of sensitivity and specificity. However, this variability might be due to the poor inter-laboratory reproducibility of antigenic preparations that often rely on outdated methodologies, improperly defined as "purifications". Recently we described a very easy, efficient and reproducible chromatographic method for the preparation of a highly enriched Ag5 fraction from HCF [1]. The high reactivity of patient sera against this preparation prompted us to further evaluate its use for CE serodiagnosis. Here, we present a large scale study (327 cases and 253 controls) aimed to evaluate diagnostic accuracy of two different Ag5 ELISA setups, compared with that of a commercially available ELISA routinely used in clinical laboratories. The influence of several clinical variables on the ELISA results was also assessed.

2. MATERIALS & METHODS

2.1 Ag5 preparation. Enriched Ag5 was obtained as described previously. Briefly, aliquots of sheep HCF were fractionated by Fast Protein Liquid Chromatography on a Superdex-200 column (10/300 GL, GE Healthcare); the fractions of interest were pooled and analyzed by mass spectrometry to verify the quality of the preparation.



HCF Size exclusion chromatography

2.2 Serology assays by Ag5 and Commercial ELISA. A total of 327 sera (283 with CE cysts and 44 who underwent surgery) from patients with heterogeneous clinical conditions (cyst stage, number, localization, previous treatments) and 253 sera from healthy controls were analyzed with an ELISA based on the Ag5 preparation in two different experimental setups (A and B) and, in parallel, by RIDASCREEN® Echinococcus IgG (R-biopharm) commercial ELISA. In order to compare results obtained from different assay plates, sample ratios (SR= (Sample mean-Negative control mean)/(Positive control mean-Negative control mean)) were calculated for the Ag5 tests, whilst sample indexes (SI) were evaluated for the commercial kit, according to the manufacturers' instructions.

2.3 Statistical analysis.

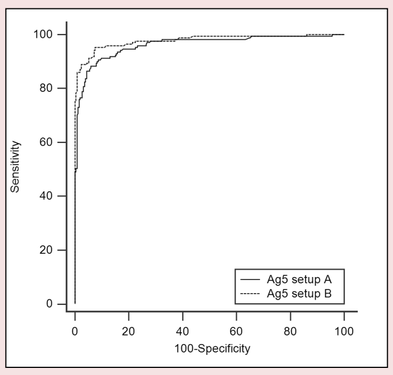
SR values from patients with CE1, CE2, CE3a and CE3b as positive group and healthy controls as negative group were used to build a receiver-operator characteristic (ROC) analysis to define optimal cut-off values for data evaluation. The area under the ROC curve (AUC) was used to define the antigen discriminatory power (between subjects with and without the disease). A *p*-value ≤0.05 was considered statistically significant. Chi-squared test was performed on the 580 ELISA results, to compare the sensitivities of the two in-house Ag5 setups and the commercial assays. The comparison among the Ag5 ELISAs was also described by box-and-whiskers plots. Differences in median SR or SI values between patients and healthy groups were analyzed by Kruskal-Wallis test, for the three ELISAs, independently, whilst Conover test, with Bonferroni correction, was applied for multiple comparisons.

In order to evaluate the effect of clinical variables such as cyst stage, number and chemotherapy on Ag5 ELISA results, a chi squared test, a bivariate logistic regression, and a multiple regression, were applied on the 283 CE patients. A *p*-value ≤0.05 was considered statistically significant.

3. RESULTS

3.1 ROC curves. At the best cut off value, the two Ag5 ELISA setups showed different behaviors, in terms of sensitivity and specificity. Setup A showed higher specificity, whilst setup B showed higher sensitivity. The areas under the ROC curve (AUC) were 0.962 and 0.978, respectively.

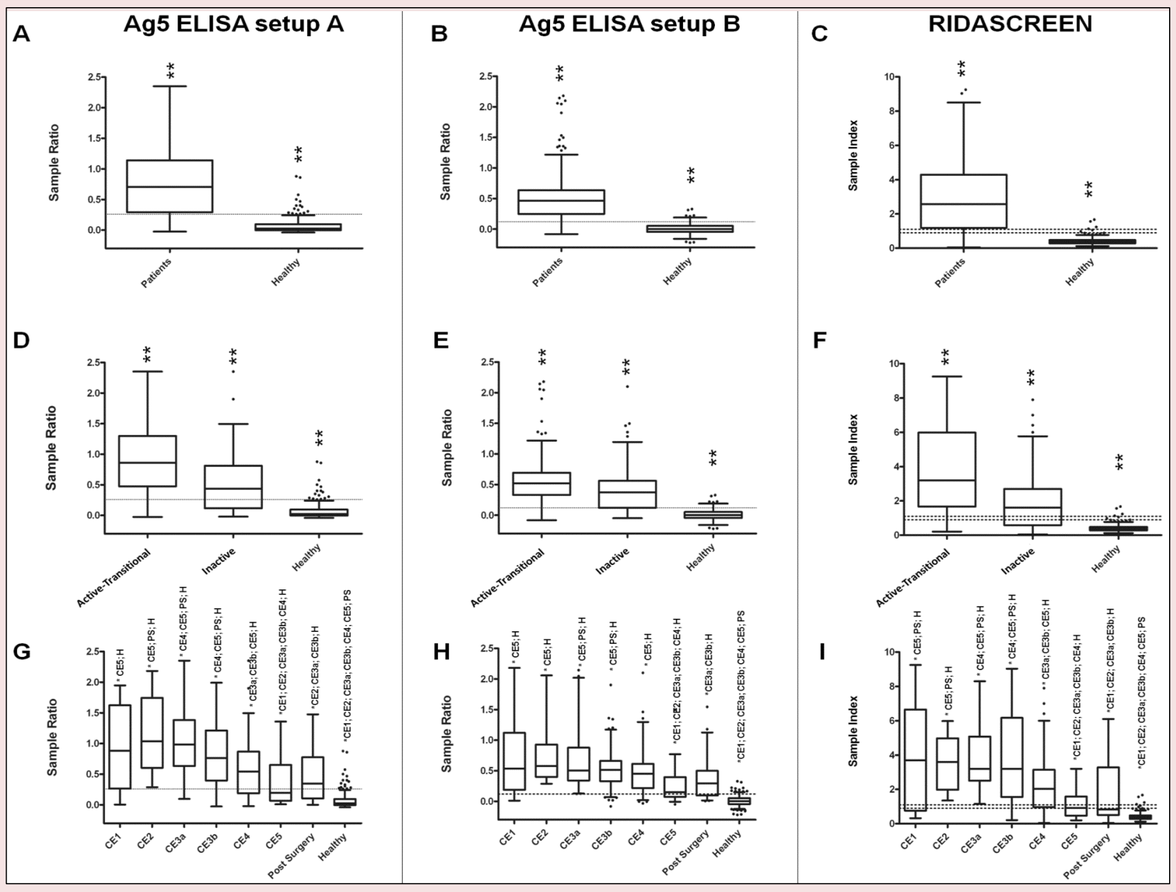
Ag5 ELISA setup B revealed an overall sensitivity (95.3%) significantly higher (p <0.05) than both Ag5 setup A (88.3%) and RIDASCREEN test (87.7%), while differences in specificity (94.1% for Ag5 setup A, 92.5% for Ag5 setup B, and 98.4% for the commercial assay) were not statistically significant .



Group		Number of patients		Positive by Ag5 ELISA Setup A		Positive by Ag5 ELISA Setup B		Positive by RIDASCREEN ELISA	
Active-Transitional	CE1	15	171	12 (80.0 %; R)	151 (88.3%; B, R)	14 (93.3%)	163 (95.3%; A, R)	10 (66.7%; A)	150 (87.7%; A, B)
	CE2	9		9 (100%)		9 (100%)		9 (100%)	
	CE3a	40		38 (95.0%;B, R)		40 (100%; A)		40 (100%; A)	
	CE3b	107		92 (86.0%; B, R)		100 (93.4%; A, R)		91 (85%; A, B)	
Inactive	CE4	76	112	54 (71.0; B, R)	71 (63.4%; B, R)	63 (82.9%; A, R)	84 (75%; A, R)	56 (73.7%; A, B)	71 (63.4%; A, B)
	CE5	36		17 (47.2%; B, R)		21 (58.3%; A, R)		15 (41.7%; A, B)	
Post-surgery		44		18 (40.9%; B, R)		32 (72.7%; A, R)		19 (43.2%; A, B)	
Healthy controls		253		15 (5.9%)		19 (7.5%)		4 (1.6%)	

Serological results. A, B, R indicate statistically significant differences between tests (A: different from Ag5 setup A; B: different from Ag5 setup B; R: different from RIDASCREEN).

3.2 Box-and-whiskers plots. All the three ELISAs were able to discriminate between patients and healthy controls (A, B, C); statistically different results were also obtained with the three ELISAs, when patients were grouped taking into account the active-transitional versus the inactive stages of CE (D, E, F).



****: p <0.05 in panels A, B, and C; **: p <0.017 in panels D, E, and F; * group: p <0.0018 in panels G, H, and I. P: patients; PS: post surgery; H: healthy donors.**

Finally, none of the three methods was able to completely discriminate among the CE single groups and post surgery patients; however, pairwise comparisons of the subgroups highlighted some differences (G, H, I).

According to the chi-squared test, patients with more than one cyst, and/or in the active or transitional stage, and/or under chemotherapy, were positive to Ag5 test more frequently than the other patients. The bivariate logistic regression and the multiple regression both highlighted an effect due to the pharmacological treatment and to the cyst activity, while the number of cysts maintained a statistical significance only when setup B was used, confirming the importance of these variables as reported in other previous works [2, 3].

4. CONCLUSION

The described serological assay, combining robustness, sensitivity, and easiness of execution, with the low cost, high reproducibility and rapidity of the Ag5 preparation method, makes this antigen a promising candidate for the serodiagnosis of CE. Further studies will be needed to evaluate the ability of our test to provide useful information on specific CE clinical traits.

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