# STRUCTURAL CHARACTERIZATION OF *ECHINOCOCCUS GRANULOSUS* IMMUNODOMINANT PROTEINS AND DEVELOPMENT OF A NOVEL IMMUNOASSAY FOR HUMAN CYSTIC ECHINOCOCCOSIS

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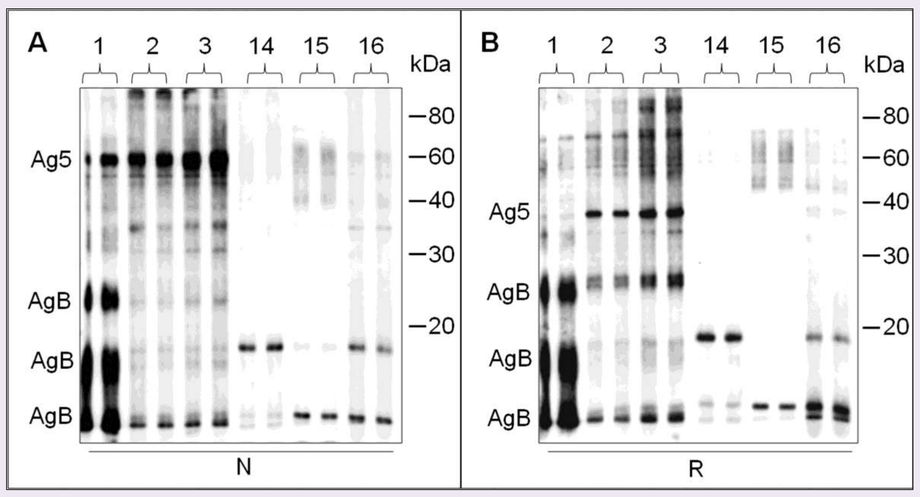
## 1 Introduction

Human cystic echinococcosis (CE) is a long-lasting infection caused by the cestode *Echinococcus granulosus*. Serological immunoassays should enable reliable diagnosis of CE, but currently available immunodiagnostic tests lack sensitivity and specificity, and their use needs standardizing. In fact, the serological response of patients with CE is ambiguous; moreover, the host antibody response may generate false positives due to cross-reactions with other parasites.

**Aim of this study was the structural and immunogenic characterization of the most abundant and immunogenic HCF proteins, in order to assess the antibody response to purified and synthetic antigens for the development of a sensitive and specific diagnostic kit** [1].

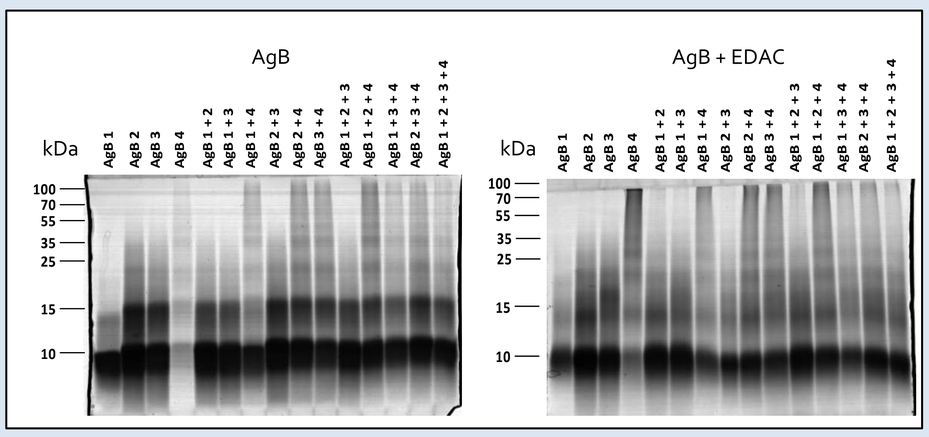
The most immunodominant proteins from *Echinococcus granulosus* are **Antigen 5** (Ag5) and **Antigen B** (AgB). Ag5 is a 67 kDa glycoprotein that under reducing conditions separates into two components, of 38 kDa and 22–24 kDa, respectively [2]. AgB is an oligomeric protein, composed of at least two different subunits of 8 kDa. On SDS-PAGE, AgB produces a characteristic ladder-like pattern, consisting of regularly spaced subunits with apparent molecular weights of 8, 16, and 24 kDa [3].



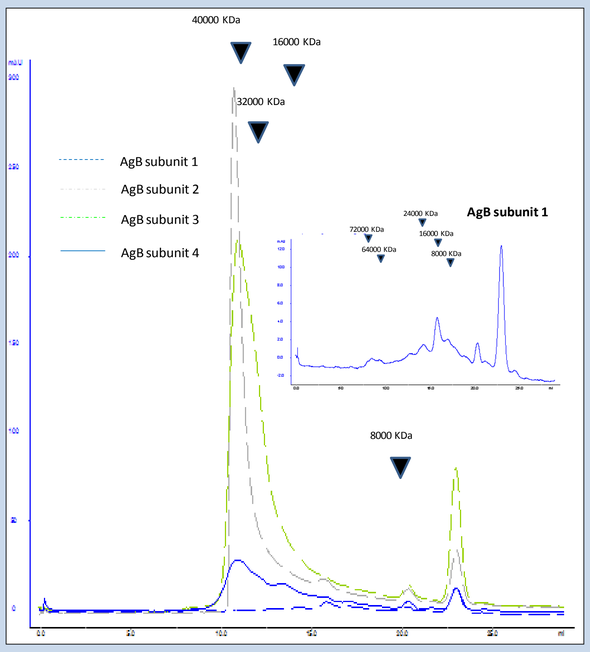

**Western Immunoblotting on non-reduced (N, panel A) and reduced (R, panel B) crude HCF** against CE patients (1-3) and healthy control (14-16) sera. Sera 2 and 3 do not recognize the typical ladder-like pattern of AgB in both conditions. Serum 1 recognizes only weakly the 38 kDa band putatively arising from Ag5 reduction (B). Moreover, control sera produce non specific responses toward some protein components.

## 2 AgB

AgB is considered the "Pandora's box" for the development of CE diagnostic tools. In order to explore the potential use of this oligomeric protein, four AgB subunits (AgB1, AgB2, AgB3 and AgB4) were chemically synthesized with a solid-phase Liberty microwave peptide synthesizer (CEM Corporation) and purified by RP-HPLC. Self-assembly properties of the subunits were studied by non reducing SDS-PAGE and size exclusion chromatography with AKTA Explorer 10 equipped with a Sephadex-75 column. The presence of supramolecular states was also investigated by means of the crosslinking agent 1–Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), showing a ladder-like pattern comparable to that of crude HCF. The recognition of the synthetic subunits by a pool of patient sera confirmed, in Western immunoblotting, the suitability of the four antigens to be used in immunological assays.

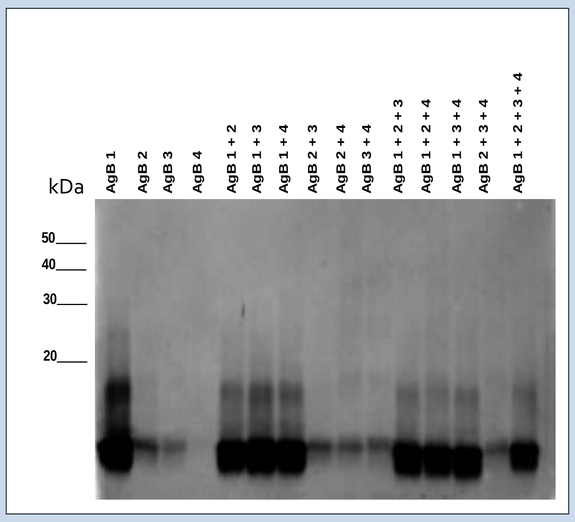



***SDS-PAGE characterization***
*Aliquots of 4 µg per well of each subunit, or their combination were run onto a 15% acrylamide gel in non-reducing conditions in the presence of EDAC (100/1 molar ratio EDAC/AgB, right panel) or its absence (left panel). As a result, the pretreatment with the crosslinker stabilizes the formation of the multimers.*



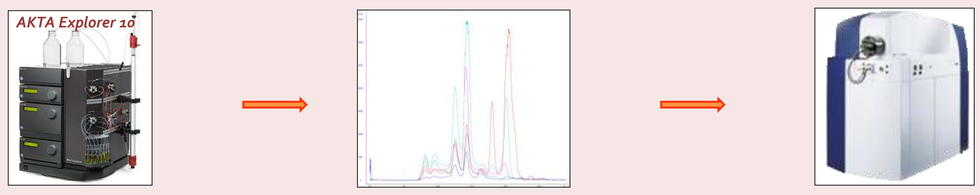

***Size exclusion chromatography characterization***
*An amount of 80 µg of each subunit was loaded, separately, onto a Superdex-75 column. The overlay of the chromatograms shows the formation of several oligomers for each subunit, ranging from 40 kDa (pentamer) to 8 kDa (monomer). The AgB1 elution profile, zoomed in the box, revealed a flat profile, probably indicating a more complex structural organization.*

***Immunoreactive profile of the subunits by Western immunoblotting***
*The immunoreactivity of the synthetic subunits AgB1, AgB2, AgB3 and AgB4 as single molecules or as their combination was confirmed by Western immunoblotting, in non-reducing conditions, against a pool of positive human sera. In particular, AgB1 gives a stronger signal compared to the other subunits, whilst AgB4 is only weakly recognized. The combination of two, three or four subunits does not seem to improve recognition.*



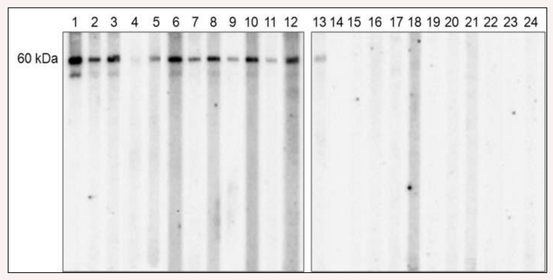

## 3 Ag5

The hydatid cyst fluid (HCF), the main source of antigens to be used for serological diagnosis, was aspirated from liver and lung cysts found in infected sheep and submitted to size exclusion chromatography on an AKTA Explorer 10 system with a Superdex-200 column (10/300 GL, GE Healthcare). The method is easy to perform and provides a very high increase in Ag5 content, going up to about 63% (evaluated by label free quantitation approach), when compared to the starting HCF material, where Ag5 represents only about the 3.8% of the total content. Moreover, the procedure is reproducible, since different hydatid cyst fluid (HCF) sources produced very similar chromatograms, notwithstanding the clearly evident and extreme heterogeneity of the starting material. Fractions from FPLC were digested and characterized by LC-MS/MS on a ESI-Q-TOF mass spectrometer equipped with a nano lock Z-spray source and coupled on-line with NanoAcquity system (Waters).



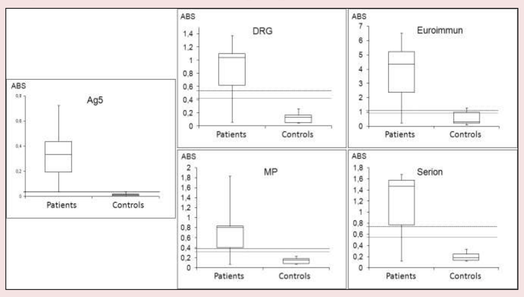

*Ag5 preparation by Size Exclusion Chromatography* — *Overlay of five HCF chromatograms* — *Q-Tof Mass Spectrometer*

To assess its diagnostic value, the chromatographic Ag5 preparation was assayed by immunoblotting on a Multiscreen Apparatus (Bio-Rad), with a collection of 24 human sera, 13 of which were from CE patients and the other 11 were from healthy controls. An unambiguous reactivity of patient sera against the non-reduced, enriched Ag5 fraction was observed, when compared to the heterogeneous results seen with crude HCF; moreover, a significant reduction in nonspecific signals was detected with control sera.




***Western immunoblotting of human sera against Ag5 enriched preparation.***
*Sera from CE patients (1–13) and control subjects (14–24) were tested against Ag5 based antigen. All CE patients sera react against the Ag5 protein band, although with a variable intensity probably depending on the antibody titer of each serum. Moreover, control sera do not give any non-specific response. The molecular weight region of Ag5 (60 kDa) is indicated on the left.*

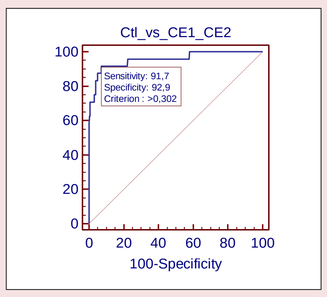
***Comparative evaluation of Ag5 and commercial ELISA kits***




The suitability of the enriched native Ag5 preparation was tested on an ELISA platform with all patients and control sera and compared with four commercial ELISA assays (DRG, Euroimmun, MP and Serion).

**Boxplots** demonstrate a better separation of the patient and control groups with the Ag5 enriched antigen, showing a greater sensitivity when compared to commercially available ELISAs.

**Validation of Ag5 ELISA**




ELISA assays on almost 700 sera (368 patients and 253 healty donors) were carried out for validation. Based on control and CE patients absorbance values, the optimal cut-off value was determined building a Receiver Operative Curve (**ROC**). The resulting curve revealed very high values of diagnostic sensitivity and specificity.

## 4 Conclusions

The methods and results reported open interesting perspectives for the development of sensitive diagnostic tools to enable the timely and unambiguous detection of cystic echinococcosis antibodies in patient sera.

**REFERENCES**

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[2] C. Lorenzo, J.A. Last, G.G. González-Sapienza, *Parasitology*, **131**, 669-77 (2005).
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