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Vaccination against hydatidosis: molecular cloning and optimal expression of the EG95NC^{-} recombinant antigen in *Escherichia coli*.

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Abstract

Background: Cystic echinococcosis (CE) is a widely distributed zoonosis that is highly endemic in the Mediterranean basin. The disease represents a serious public health threat and causes economic losses. The parasite life-cycle involves dogs and ruminants as definitive and intermediate hosts; humans are accidently infected, causing serious clinical issues. Vaccination of ruminants and dog treatments represent the most efficient measures to prevent parasite transmission. The recombinant protein vaccine, EG95, has been used successfully in sheep vaccine trials against Cystic echinococcosis (CE) in several countries.

Results: In this study, we expressed the modified antigen, EG95NC-GST, in *Escherichia Coli* for use as a vaccine against *Echinococcus granulosus*in ruminants. We tested three different media formulations for *E. coli* culture and established for each culture conditions for optimal levels of soluble EG95 expression.

Conclusion: The results demonstrate that SOC and TB media provided high yields in cell density and EG95 protein expression. Purification of the recombinant protein with affinity chromatography (using FPLC) was also performed to increase the purity of the EG95NC^{-}-GST antigen.

Keywords:

Echinococcus granulosus, Hydatidosis, Escherichia *coli*, Recombinant protein, vaccine.

Abbreviations:

CE: Cystic Echinococcosis ; GST : Glutathione S-transferase;
IPTG: isopropyl-β-D-thiogalactoside; LB: Luria broth:
TB: Terrific broth; SOC: Super broth; FPLC: Fast protein liquid chromatography; SDS-Page Electrophoresis: Sodium dodecyl sulphate-polyacrylamide gel Electrophoresis.

1. Background:

Echinococcus granulosus is cyclophyllid cestode causative agent of cystic echinococcosis (CE) or hydatidosis, a chronic and zoonotic infection that occurs worldwide and is caused by the larval stage of the parasite (1). It has a considerable socio-economic impact in endemic areas (1). It is assumed that 50 million people are at risk of acquiring the disease in Asia and Africa (2). Morocco is known as an endemic country (3); in 2012, 5.5 per 100,000 inhabitant of cystic echinococcosis (CE) were recorded in the country with 3% fatality (4).

In Morocco, 22% to 58.8% is the infection rate in dogs depending on the region (5). For livestock, Azlaf and Dakkak reported an infection rate of 10.6% in sheep, 1.9% in goats, 23% in cattle, 12% in camels and 17.8% in equines, mostly in the Middle Atlas (48.7% in cattle) and in the North West (37.6% in cattle and 31.6% in sheep) regions of Morocco (6) with a higher number of infections caused by the genotype G1 (96%) followed by G3 (3%) and G2 (1%) with similar rates of infection in cattle and sheep.(7)

Vaccination of sheep intermediate hosts against cystic echinococcosis (CE) is an interesting option that has evolved considerably in recent years with the development of a new recombinant vaccine (8). A similar strategy has been tested successfully using the recombinant protein EG95 as recombinant vaccine against *Echinococcus granulosus* showing an effective protection (8–10).

EG95 has been reported to be a highly effective sheep vaccine in Australia, New Zealand, Argentina and China (9, 11 and 12). Lightowlers et al. (8) used this protein for sheep vaccination; the vaccine provided a high degree of protection (96-98%) against experimental challenge with *E. granulosus*. Using a truncated cDNA encoding the EG95 antigen (EG95NC^{-}). Gauci et al., (2011) succeeded in producing a plasmid construct that achieved high level expression of immunogenic protein. The vaccine may prove to be a useful tool in the control of hydatidosis in endemic areas (12-13)

The objective of this study was to optimize production conditions of the truncated recombinant protein, EG95NC^{-}, in *Escherichia coli* using 3 different media formulations that favour veterinary vaccine preparation.

2. Materials and Methods

2.1. Strains and Plasmids

E. coli Top10F' (Invitrogen) was used as the host strain for recombinant plasmid preparation. *E. coli* BL21 DE3 (Invitrogen) was used as an expression host. The expression vector used

was plasmid pGEX (GE Healthcare). The pGEX vector contains the *tac* promoter and allows expression of recombinant protein fused to the C-terminus of Glutathione S-transferase (GST).

2.2. Gene synthesis and cloning

The truncated EG95NC^{-}cDNA was synthesised with codon optimisation for procaryotic expression (Fig.1) and cloned into *Bam*HI/*Xho*I restriction sites in the same reading frame with the GST tag in the pGEX vector. The recombinant plasmid was transformed into *E. Coli* Top10F'. The plasmid was extracted from the bacteria using the Plasmid MiniPrep DNA Extraction Kit, (Qiagen), and digested by *Bam*HI/*Xho*I enzymes to confirm ligation of this gene into the pGEX plasmid (Fig.2). Nucleotide sequencing was performed to confirm correct insertion of the target gene.

2.3. Expression of recombinant EG95NC^{-}-GST protein in *E.coli*

The plasmid pGEX-EG95NC^{-} was transformed into *E. coli* BL21(DE3). Culture of recombinant *E. coli* was performed in shake flasks. In order to provide the optimum conditions for aeration and mixing, the culture volume represented 20% of the total flask volume. Luria broth medium (LB) containing 100 µg/ml of ampicillin (Serva) was inoculated with the recombinant *E. coli* and incubated in a shaker incubator (MaxQ 4000, ThermoScientific) at 37°C, 200 rpm for 14 h. Cell density was measured until the OD (595nm) reached approximately 0.8-1 unit and dilution made for the second passage at OD 0.05 in LB medium containing ampicillin 100 µg/ml, followed by incubated with shaking.

Protein expression was induced by addition of isopropyl-β-D-thiogalactoside (IPTG, Promega) at a concentration of 0.5 mM. Assessment of levels of recombinant protein expression was performed for 5h every hour during cultivation, *E. coli* cell suspensions were centrifuged at 3500g (1-15PK; SIGMA). The culture supernatant was discarded and bacterial pellets suspended in loading buffer (Tris 50 mM, Glycine 200 mM, SDS 0.1 %, beta mercaptoethanol 5%, pH 8.3) and lysed in a water bath at 95°C for 5min before chilling on ice for SDS-PAGE analysis (Fig. 3).

2.4. Media trials for expression optimization

To evaluate the EG95NC^{-} recombinant protein production, media commonly used for *E. coli* culture, were tested. Duplicate cultures were carried out in three different media induced with IPTG at 0.5mM: Luria broth (LB), terrific broth (TB) and super broth (SOC). All media were

formulated as specified in the Handbook of Microbiological Media (Atlas 1997). An overnight culture of *E. coli* BL21 (DE3) containing the pGEX-EG95NC$^-$ plasmid in the three media was diluted to OD 0.05 with ampicillin. Expression kinetics were determined for all media with cell density control. Optimum cell culture conditions were selected for scale-up production of the antigen in a fermentor and affinity purification.

2.5. Purification of Recombinant Proteins by Affinity Chromatography

Pellets obtained from 2 liters of the *E. coli* cultures expressing the EG95NC-GST-tagged proteins were suspended in 50 ml PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) supplemented with 1 mM DTT, 10 µmM EDTA+1 mM PMSF. The cells were lysed by sonication with a MSE ultrasonic processor for 3 min, amplitude 50 %. The cell extract was kept on ice during sonication. Cell debris was removed by centrifugation at 28000 g, 4°C for 15 min. The supernatant was applied to a 0.45µm filter.

The following purification procedures were performed using fast protein liquid chromatography (FPLC) and Akta Purifier100 chromatography system. The columns, GSTPrep FF 16/10 (GE Healthcare) were equilibrated with 5 column volumes (CV) of PBS, pH 7.4 and the sample was applied to the column. The column was washed with 5 CV of PBS and the fusion protein was eluted with a gradient using 7 CV of Tris-HCl, pH 8.0 including 10 mM reduced glutathione. The purity of eluted proteins was analyzed by SDS-PAGE.

2.6. SDS-PAGE Analysis of Recombinant Protein

Proteins were resolved by SDS-PAGE, (10% w/v separating gel) under reducing conditions using the discontinuous buffer system (14) and a Protean II vertical electrophoresis system (Bio-Rad Laboratories, CA, USA).Protein calibration markers (Broad Range 2-212 kDa, Biolabs) were used as size standards. Proteins were visualized by staining with Coomassie brilliant blue R250 (Sigma, USA).

2.7. Protein expression estimation

Bovine serum albumin (BSA, Amresco) at concentrations from 2 to 25 µg/µl was loaded onto SDS-PAGE in parallel with different volumes of *E. coli* lysis products corresponding to samples obtained during culture. Gels were scanned and the percentage of the recombinant protein present in the sample was estimated and compared to BSA concentration, using GelQuant.NET (BiochemLabSolution.com). The pixel densities of the bands corresponding to

BSA protein concentration were used to plot a standard curve (pixel densities Vs BSA concentration), and used to approximate the recombinant protein concentration.

2.8. Purified protein assay

Protein concentration was estimated using the method of Bradford (15). A standard curve (concentration "µg" = f (absorbance at 595 nm) was determined using five dilutions of BSA protein with a range from 1 to 25 µg/ml. The absorbance measurement at 595 nm obtained with the diluted recombinant protein dosage was used for protein quantification according to the BSA standard curve.

3. Results

3.1. Gene synthesis and cloning

Digested products controlled with agarose gel electrophoresis revealed a DNA band at about 370bp (Fig. 2). Nucleotide sequencing (data not shown) results confirmed the successful construction of the recombinant expression plasmid pGEX-EG95NC.

3.2. Expression of recombinant EG95NC^{-}-GST protein

E. coli cultures expressing EG95NC^{-}-GST was analyzed in SDS-PAGE showing a protein band at approximately 40kDa, corresponding to the expected protein size. The EG95 protein expression was absent before induction with IPTG in the first lane and shows increasing yields during the induction of the culture (Fig.3).

3.3. Expression optimization with culture Media

The growth rates of the recombinant bacteria cultured on three media are shown in Fig.4. The final *E. coli* cell concentrations varied with the three media tested. The LB (Luria-Bertani) medium, although commonly used in cultivation of *E. coli* and expression of recombinant proteins (16-17), produced the lowest final cell density comparing to Soc medium and TB medium. As shown in Fig.4 the highest final cell density was obtained with SOC medium and simultaneously produced the highest level of EG95NC-_GST recombinant protein shown by highest densities of the bands compared to TB medium (Fig. 5) and LB medium (data not shown). Recombinant protein concentration was estimated using GelQuant software to be 200 mg/L from TB medium (4000 doses/L) versus 350 mg/L with SOC medium which represents 7000 doses/L (50µg/dose as recommended by Lightowlers et al., 1999 (11)).

3.4.Purification and protein assay

The substantial increase in yield of EG95NC^{-}-GST allowed scale-up of the antigen production using Fermentor with SOC medium. Following purification on a glutathione sepharose column using FPLC, fractions containing the protein EG95NC^{-}-GST (A1-B6) (Fig. 6-7) were pooled and concentrated. This provided significant amounts of purified antigen (260 mg/L) which represents 5200 doses/L to supply large scale vaccine formulation.

4. Discussion

Cystic echinococcosis is an important zoonotic disease caused by cestodes of the genus Echinococcus. Recombinant vaccine EG95 provide a very practical and cost-effective prevention strategy against hydatidosis.

Achieving the goal of expression optimization to increase the quantity of produced recombinant protein using three different medium and codon optimization that can improve the efficiency of protein expression, It has been reported that LB medium has no buffering system, with a low amount of carbon source, nitrogen and divalent cations which does not support growth to very high cell density (18). Consistent with the data shown in Fig.4, media such as terrific broth (TB) and Super broth (SOC) are generally considered better for obtaining high cell densities, more rich of yeast extract and phosphate salts which is very important during recombinant protein synthesis (19,20) with a good buffering capacity to prevent pH fluctuations that can affect bacterial growing. Proteins produced and purified using the GST fusion system can be used in numerous biological applications.

Conclusion

This study allows optimization of EG95NC-_GST expression using recombinant *E. coli* (BL21DE3) growth in SOC medium to produce the hydatidosis vaccine for ruminants including the genotype G1 of Echinococcus granulosus which represent the most prevalent genotype in Morocco. High yields of the recombinant protein were obtained in a fermentor that may reach up to 7000 sheep doses/L of culture. Purification of the antigen was possible using affinity chromatography to increase the quality of the final antigen that can be used for immunological studies and vaccine production.

Authors' contributions:

MH, KT and ML initiated the study, participated in experimental design. MJ conducted the experiment. All authors participated in data analysis; interpretation of the results, MJ, MH wrote the paper and ML review it. All authors read and approved the final manuscript.

Competing interests:
The authors declare that they have no competing interests.

Availability of data:
All data from this study are contained within the manuscript.

Ethics/consent:
No human or animal subjects were used in this study. Declarations of ethical treatment of human subjects or animals, or consent of human subjects are therefore not applicable.

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- **Fig. 1:** Amino acid sequences of full EG95 gene and truncated EG95NC- gene.
- **Fig. 2:** Agarose gel electrophoresis of recombinant plasmid pGEX-EG95NC double digested with (BamHI/XhoI). Lane1: double digestion of plasmid. Lane2: plasmid undigested. SM: DNA size marker.
- **Fig. 3: SDS** page analysis of *E. coli* culture samples expressing EG95NC-GST. S**:** sample before IPTG induction. **1-6:** 1h to 6h post induction with 0.5mM IPTG. **SM**: protein size marker.
- **Fig. 4**: Cell density of recombinant E. coli cultures using Luria broth medium (LB), terrific broth medium (TB) and Super broth medium (SOC).
- **Fig. 5: SDS** page analysis of recombinant bacteria expressing EG95NC-GST inTB and SOC medium. **1-3:** 4h, 5h and 6h post induction using TB medium. 4-6: 4h, 5h and 6h post induction using SOC medium. S**M**: protein size marker. Arrow denotes position of the recombinant proteins.
- **Fig. 6:** Purification chromatogram of the *EG95NC-GST* protein using GSTPrep FF 16/10 column.
- **Fig. 7:** SDS-PAGE analysis of the eluted fractions. S: Start material diluted 3x. A2-B6: eluted fractions. SM: protein size marker.

EG95
QLCLILFATSVLAQEYKGMGVETRTTETPLRKHFNLTPVGSQGIRLSWEVQHLSDLKGTDISLKAVNPSDPLVYKR
QTAKFSDGQLTIGELKPSTLYKMTVEAVKAKKTILGFTVDIETPRAGKKESTVMTSGSALTSAIAGFVFSCIVVVLT

EG95NC-
QEYKGMGVETRTTETPLRKHFNLTPVGSQGIRLSWEVQHLSDLKGTDISLKAVNPSDPLVYKRQTAKFSDGQLTI
GELKPSTLYKMTVEAVKAKKTILGFTVDIETPRAGKKESTVMTSGSA

Fig. 1: amino acid sequences of full EG95 gene and truncated EG95NC- gene

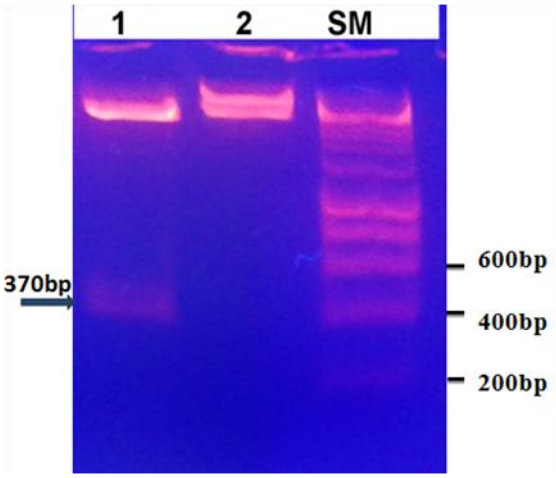

Fig. 2: Agarose gel electrophoresis of recombinant plasmid pGEX-EG95NC double digestion (BamHI/XhoI). Lane1: double digestion plasmid. Lane2: plasmid undigested. SM: DNA size marker

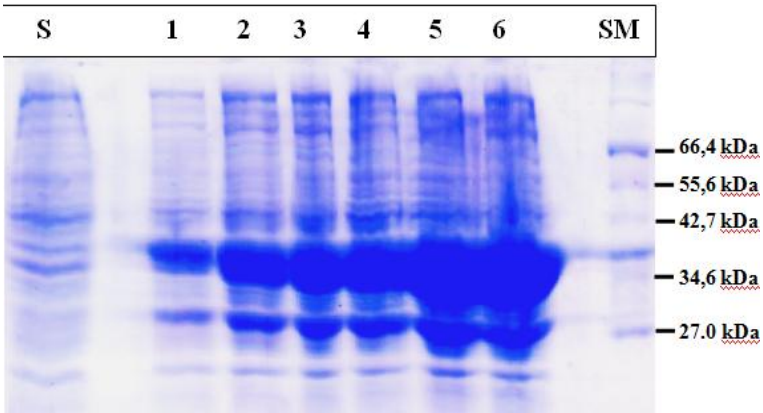

Fig. 3: SDS page analysis of *E. coli* culture samples expressing EG95NC-GST. **S:** sample before IPTG induction. **1-6:** 1h to 6h post induction with 0.5mM IPTG. **SM**: protein size marker.

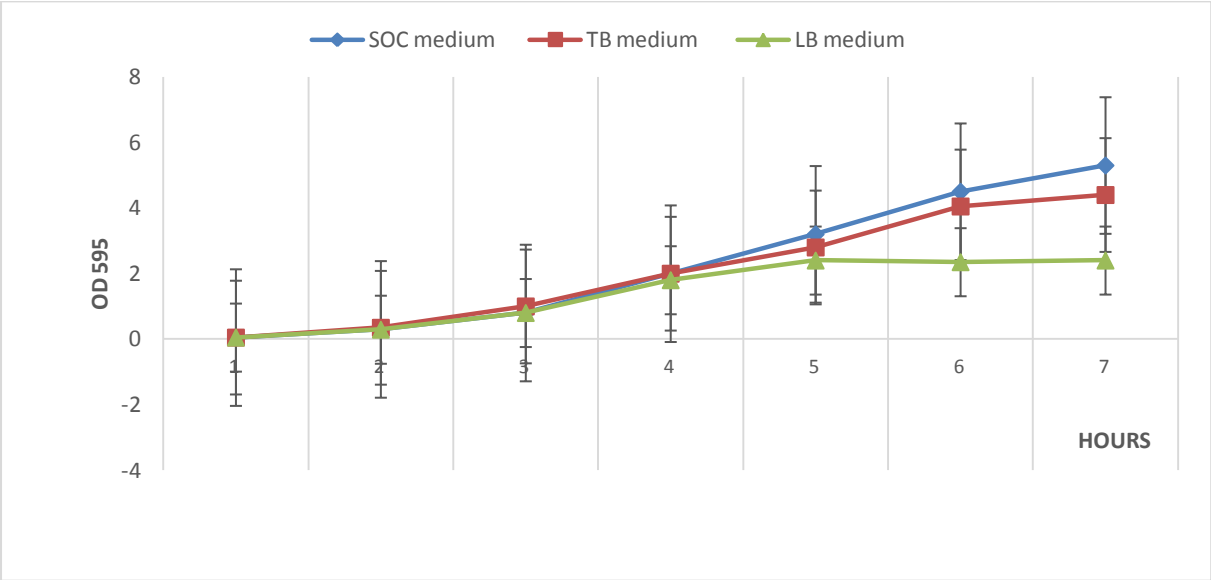

Fig. 4: Cell density of recombinant E. coli cultures using Luria broth medium (LB), terrific broth medium (TB) and Super broth medium (SOC).

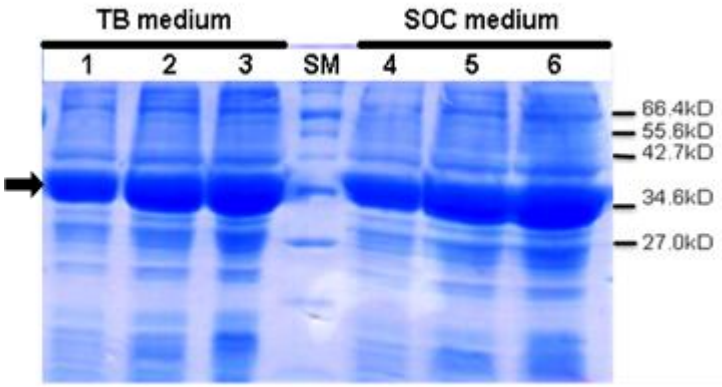

Fig. 5: SDS page analysis of recombinant bacteria expressing EG95NC-GST inTB and SOC medium. **1-3:** 4h, 5h and 6h post induction using TB medium. 4-5: 4h, 5h and 6h post induction using SOC medium. S**M**: protein size marker. Arrow denotes position of the recombinant proteins.

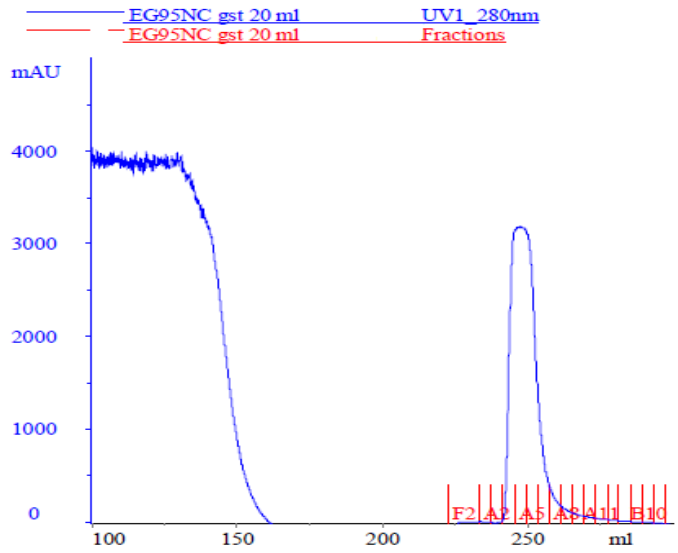

Fig. 6: Purification chromatogram of the *EG95NC-GST* protein using GSTPrep FF 16/10 column.

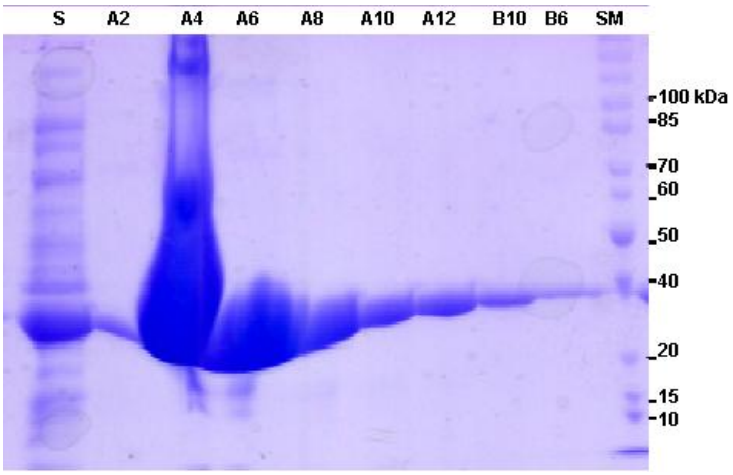

Fig. 7: SDS-PAGE analysis of the eluted fractions. S: Start material diluted 3x. A2-B6: eluted fractions. SM: protein size marker.