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Immunological responses and potency of the EG95NC⁻ recombinant sheep vaccine against hydatidosis

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ABSTRACT

Cystic echinococcosis (CE) is a zoonotic disease caused by the cestode parasite *Echinococcus granulosus*. The disease has an important impact on human health as well as economic costs including the cost of treatment as well as loss of productivity for the livestock industry. In many parts of the world where the disease is endemic, sheep and other livestock play an important role in the parasite's transmission. A vaccine to protect livestock against CE can be effective in reducing transmission and economic costs of the disease. A recombinant antigen vaccine has been developed against infection with *E. granulosus* (EG95) which could potentially be used to reduce the level of *E. granulosus* transmission and decrease the incidence of human infections. Further development of the EG95 recombinant vaccine as a combined product with clostridial vaccine antigens is one potential strategy which could improve application of the hydatid vaccine by providing an indirect economic incentive to livestock owners to vaccinate against CE. In this study we investigated the efficacy of the EG95 recombinant vaccine produced in Morocco by vaccination of sheep, including a combined vaccine incorporating EG95 and clostridia antigens. Vaccination with EG95 either as a monovalent vaccine or combined with clostridia antigens, protected sheep against a challenge infection with *E. granulosus* eggs and induced a strong, long lasting, and specific antibody response against the EG95 antigen.

Keywords: *Echinococcus granulosus*; hydatidosis; cystic echinococcosis; recombinant protein; EG95; vaccine.

1. Introduction.

Hydatidosis or cystic echinococcosis (CE) is an important parasitic disease of livestock with high zoonotic potential and public health threats worldwide [1, 2]. CE is caused by the development of larvae of *Echinococcus granulosus*, a cestode parasite having wild or domestic canids as the definitive host and herbivorous or omnivorous mammals serving as intermediate hosts. Humans and livestock become infected through eating infective eggs excreted with dog feces. CE is endemic in many parts of the world especially in countries of the temperate zones, including southern South America, the entire Mediterranean littoral, southern and central parts of the former Soviet Union, central Asia, China, Australia, and parts of Africa [3, 4]. CE has economic impacts through its effects on human health as well as livestock productivity [5, 6].

In endemic countries, the prevalence of CE in livestock is very variable depending on the regions and the farming methods. The infection does not commonly cause overt symptoms in sheep, goats, cattle, pigs, camelids and horses. However, the disease causes sometimes considerable economic losses due to the seizure of infested organs, 50% of which may be affected in certain areas [7]. Globally, the economic impact of this disease would be estimated at more than 1.65 billion euros / year in terms of animal production and more than 577 million euros / year in terms of public health [8].

To limit the economic impact on animal production and prevent disease in humans, vaccination of livestock has been tested in several countries with proved efficacy. Initial experiments demonstrated the presence of protective antigens in the egg and oncosphere of *E. granulosus* [9, 10], however technical constraints due to antigen availability and risk of handling the parasite prevented a practical vaccine being developed based on this source. Lightowlers et al. [11] subsequently cloned, identified, and expressed a protective recombinant protein (EG95) derived from activated *E. granulosus* oncospheres and showed it

could induce high levels of protection in sheep against a challenge infection with *E. granulosus*. The vaccine is currently being used in sheep to prevent naturally-acquired CE infections in Argentina and China.

In Morocco, hydatidosis is one of the diseases with mandatory notification (Order of the Minister of Health No. 1020-03 of May 23, 2003). To date, there are no reports describing the use of vaccination to control CE in Morocco despite the high prevalence and huge impact of the disease. In this study, we evaluated the EG95 vaccine manufactured in Morocco [12] against CE infection in sheep by vaccination of 4 groups of 20 animals, with follow up using antibody response and challenge infection with *E. granulosus* eggs. Results are discussed on the feasibility of livestock vaccination to protect humans and avoid lose in animal production in Morocco and other North African countries.

2. Materials and methods

2.1 Antigen preparation

The recombinant protein EG95NC was expressed as described by Jazouli et al. [12]. The total soluble protein was obtained after sonication of the fermentation harvest pellet [13] and quantification of EG95NC recombinant protein using GelQuant software. Vaccine formulation were realized mixing at equal volume the antigen at the desired concentration (50µg/dose) with SEPPIC oil adjuvant ISA11R. An emulsion oil in water (O/W) was obtained by moderate agitation at room temperature for 20 min. The vaccine was tested for sterility and stability before use. The same vaccine formulation technique was used to prepare monovalent as well as polyvalent vaccines containing 5 valences of Clostridia antigens as used in the VAXAID commercial vaccine marketed by MCI Sante Animale, Morocco and incorporating antigens of *Cl perfringens* (B and D toxins), *Cl. septicum*, *Cl sordelli*, *Clostridium chauvoei* and *Clostridium novyi*), but excluding the *Mannheimia* or *Escherichia*

coli antigens that form part of the VAXAID commercial vaccine. A control EG95NC⁻ vaccine was kindly supplied by the University of Melbourne in lyophilized vials together with Quil A as adjuvant and prepared as described by Gauci et al. [13]. The vaccine was rehydrated with sterile distilled water on the day of use.

2.2 Vaccination trial with recombinant antigens

The design of the vaccination and challenge trial in sheep is summarized in Table 1. Eighty sheep of local Timahdit breed, aged 5–8 months were divided into four groups of twenty sheep. One group was immunized with 50 µg of the EG95NC⁻ alone with SeppicISA11R adjuvant. The second group was vaccinated with the reference EG95NC⁻ preparation from Melbourne and the third group vaccinated with 50µg EG95NC⁻ formulated together with Clostridia antigens and SeppicISA11R adjuvant. The remaining twenty sheep served as unvaccinated negative controls. Immunizations were given by the subcutaneous route behind the elbow in a volume of 2 ml, other than from group 2 which received 1ml. Vaccinated animals received three injections. the first on day 0, a second injection one month later and a third vaccination given four months after the second injection. The trials was conducted in accordance with the International Guideline for care and handling of experimental animals. The study protocol was approved by the Internal Ethic Committee of MCI Santé Animale in Morocco.

Table 1

Sheep vaccination and challenge experimental design and results of challenge infection with *E. granulosus* eggs.

Group	Vaccine antigen(s)	Adjuvant	Number of animals	Number of animals infected with hydatid cysts following challenge	Numbers of cysts found at necropsy	% of protection
G1	EG95NC$^-$	Seppic ISA 11R	20	0	0	100%
G2	EG95NC$^-$	Quil A	20	0	0	100%
G3	EG95NC$^-$ + Clostridia antigens	Seppic ISA 11R	20	0	0	100%
G4	Nil	Nil	20	5**	10, 55, 80, 115, 155	-

* G1: group1, G2: group2, G3: group3, G4: group4; ** P<0.05: Fisher's Exact test in comparison with each of groups G1, G2 or G3

The site of the injection was monitored for at least two weeks after each injection, recording the size of the lump if one was present, and whether it caused pain on palpation. Body temperature measurements were taken for 14 days after each vaccination. Blood samples were collected for serology at regular intervals of time via the jugular vein.

2.3 ELISA

Enzyme-linked immunosorbent assays were performed on sera from vaccinated and control sheep using as antigen an EG95 maltose binding protein fusion protein expressed in *E. coli* (EG95±MBP) supplied by the University of Melbourne and prepared as described by Woollard et al. [14]. Wells of microtiter plates (Nunc MaxiSorp™) were coated with antigen at 2µg/ml in bicarbonate buffer at 4 C overnight. After blocking with 200µl/well of PBS+5% freeze-dried skimmed milk during two hours, the plate was washed five times using phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) at pH of 7.4. Sera

samples were diluted (1:1000) using phosphate-buffered saline containing 5% freeze-dried skimmed milk (PBS/milk). Diluted sheep serum (100µl) was loaded into the wells. Plates were incubated for 1 hour at 37°C, after which the wells were washed 5 times with PBST, and 100µl of anti-sheep IgG conjugated to HRP (Bethyl, Montgomery USA) optimally-diluted (1:10,000) in PBS/milk were added to each well and incubated for 1 hour at 37°C. The plate was washed 3 times as described above and 100µl of tetramethyl benzidine ("TMB", Medicago AB, Uppsala, Sweden) substrate added to each well, and incubated for 5 mins at room temperature. The reaction was then terminated by addition of 100µl of 0.5M (H_2SO_4) to each well. The absorbance was read in an ELISA reader (Sigma EIA multi-well reader) at 450 nm.

2.4 Challenge with E. granulosus *eggs and assessment of infection*

Three weeks after the second booster vaccination, all animals were challenged with 1000 *E. granulosus* eggs via stomach tube, prepared as described by Jazouli et al. (submitted for publication). Briefly, infected sheep organs from a local abattoir, were used to infect 3 dogs which were euthanized 6 weeks later and mature parasites collected for use as infection material for the challenge. Twelve months after the challenge, sheep were autopsied. Liver and lung were inspected visually and palpated to identify and enumerate hydatid cysts in each animal. Strict safety precautions were taken when handling *E. granulosus* adult worm parasites and their eggs. Personal protective equipment was worn including gloves and goggles. Equipment used was decontaminated by autoclaving.

3. Results

3.1 Post-vaccination observations

During the experimental period (18 months), the behavior of the vaccinated animals did not reveal any particular anomaly. No alterations of the health or appetite were observed. Hyperthermia was observed in some of the three vaccinated groups after the primary vaccination and after each booster vaccination, from day 1 to days 5 after vaccination, with a peak of 41°C (Fig. 1). After primary vaccination hyperthermia was observed in 50% of animals vaccinated with monovalent vaccine, 65% of animals vaccinated with the reference vaccine and 75% of animals injected with the multivalent vaccine (Fig1a). After the booster vaccination, hyperthermia was noted in 85% of the monovalent vaccine group, 70% of reference vaccine group and 55% of the multivalent vaccine group (Fig1b). Almost the same profile was obtained after the second boosting (data not show). Temperatures remained normal in control animals.

3.2 Local inflammation

Inflammation at the injection site was observed after the vaccine administration for the three groups, from day 2 to day 14 after vaccination. The diameter of the inflammation was between 1 and 3 cm, it was noted in 95% of the animals vaccinated with the monovalent and multivalent vaccines (G1 and G3) and 85% of the animals vaccinated with the reference vaccine (G2). After the booster 100% of G1 and G3 animals and 55% of G2 presented inflammation at the injection site.

3.3 Serological responses

Seroconversion was detected in 100% of vaccinated animals in the 3 groups after primary vaccination (Figure 2). Levels of serological activity in are shown in in Table 2 and

Fig 2. Antibody titers increased after the first booster immunization while the second booster induced a more substantial and long lasting increase in antibody response in all three vaccinated groups (Figure 2); the control animals remained seronegative. Immunogenicity of the clostridial antigens in the combined EG95-clostridial vaccine were assessed according to current commercial standards and all responses were found to meet or exceed the immunogenicity requirements of an effective vaccine [15-17].

Table 2

Optical density in ELISA of specific anti-EG95 IgG antibody responses in sheep vaccinated with various EG95 vaccine formulations and control, non-vaccinated animals. G1, EG95NC^{-} + ISA 11R; G2, EG95NC^{-} + Quil A; G3, EG95NC^{-} + Clostridial antigens + ISA 11R; G4 non-vaccinated controls. Primary vaccination day 0, first booster vaccination 1 month post primary and second booster given four months after the second immunization. Blood samples were treated and aliquots conserved at -20°C for ELISA control. (Mean peak OD +/- standard deviation obtained after three weeks post V1, V2 and V3 in the three vaccinated groups, with the final blood sample taken on the day of challenge with *E. granulosus* eggs.

Vaccination	Vaccine group			
	G1	G2	G3	G4
Primary	0.58 $\pm$ 0.2	1.62 $\pm$ 0.5	0.40 $\pm$ 0.2	0.12 $\pm$ 0.03
First booster	2.29 $\pm$ 0.4	2.8 $\pm$ 0.4	2.1 $\pm$ 0.6	0.12 $\pm$ 0.03
Second booster	3.37$\pm$0.2	3.43$\pm$0.1	3.08$\pm$0.5	0.25$\pm$0.07

3.4 Challenge infection

A summary of the results of the challenge infection in the four experimental groups of animals is shown in Table 1. Postmortem examination of the liver and lungs of each animal

in the 4 groups one year after the challenge infection showed no detectable lesions observed in the 3 vaccinated groups. In the unvaccinated control group, the presence of viable cysts was detectable in 25% of the animals. Cysts were present only in the liver of the control animals with no lesions observed in lungs. The number of cysts was between 10 and 155 (Table 1).

4. Discussion

In the experiment described here we tested two preparations of the EG95 antigen produced in Morocco for their ability to prevent cystic echinococcosis in sheep following a challenge infection with *E. granulosus* eggs. Both preparations achieved a statistically significant 100% protection against a challenge infection with *E. granulosus* eggs in comparison with non-vaccinated controls. One preparation was a monovalent vaccine with ISA 11R oil adjuvant. The second preparation was a combination EG95 together with five clostridia antigens. Result obtained show that both monovalent and multivalent preparations are safe in livestock and efficacious with moderate local inflammation and transient hyperthermia following the vaccinations. The impact of cystic echinococcosis is principally on public health, more than it is for livestock health/production. For this reason there is little incentive for livestock's breeders to purchase/use a monovalent vaccine. However, vaccination against clostridial diseases in sheep is very common in Morocco and regular programs of clostridial vaccination are carried out in many sheep rearing regions of the world. For this reason, a combination clostridial/EG95 vaccine would allow livestock owners to prevent important diseases of economic importance while at the same time helping to reduce transmission of an important zoonotic disease.

In our study, we used a reference preparation of the EG95 vaccine provided at the University of Melbourne together with the adjuvant Quil A. This vaccine has been used

extensively in experimental trials undertaken in several countries where it has achieved 95-100% protection [11, 18, 19]. In this respect, the high level of protective efficacy of the EG95 antigen which we describe here for the preparation produced in Morocco is consistent with previous studies undertaken with the original EG95 antigen [11, 18, 19] as well as with the slightly truncated EG95NC⁻ protein prepared from *E. coli* lysates as total soluble protein [13].

All animals vaccinated with EG95 showed 100% serological positivity after vaccination, regardless of the group. Our results are similar to those described from trials with the EG95 vaccine undertaken in other countries where two injections with EG95 were reported to induce positive serological responses in most or all vaccinated animals, with a third booster vaccination eliciting a greater response [20-22] The ELISA test was also used to monitor antigen production batches for potency. Potency was monitored by evaluating the serological response of vaccinated sheep in comparison with serum from animals known to have been protected by vaccination [21].

The results of the challenge infection in the control, non-vaccinated sheep revealed infections in only 25% of the animals, all of which were in the liver. These results suggest that the quantity of ingested *E. granulosus* eggs is not sufficient to induce a high percentage of infection. It may also be due to the poor sensitivity of sheep breed used (Timahdite), but no data is available on the Timahdite breed's susceptibility to CE. Our expectation was that these sheep would show a high susceptibility to infection because Timahdite is the most common breed of sheep in the region of the Atlas Mountains in Morocco [23] where cystic echinococcosis is hyperendemic [24]. Further studies are needed to assess differences in the susceptibility of different Moroccan breeds of sheep to infection with *E. granulosus*.

In the study described here we used an experimental infection in sheep to test the protective efficacy of the EG95 vaccine. An alternative method of demonstrating the vaccine's efficacy is to vaccinate young animals against a natural field infection in a region

where CE is highly endemic. This approach has been taken in the Rio Negro province of Argentina where annual vaccination of lambs plus a single booster vaccination has achieved a significant reduction in cystic echinococcosis in sheep [25-28]. In that trial, vaccinated sheep were shown to develop long-lasting antibody responses following a booster vaccination given approximately a year after the initial vaccination in lambs [25]. Our results were similar in that high levels of specific antibody were maintained in sheep following the second booster vaccination given at 4 months after the initial vaccination. This was also the case with the combination vaccine containing clostridia antigens. These results suggest that the clostridial combination vaccine would be capable of delivering long lasting protection, with high levels of antibody persisting for at least a year after the second booster vaccination.

Vaccination of livestock as the intermediate host is likely the best approach to interrupt the *E. granulosus* life cycle and indirectly prevent infection of humans. Interruption of the parasite's life cycle in the dog host can provide a complementary method for control by vaccination [27]. However, in Morocco there are difficulties to target the definitive host for treatment because the dogs are commonly uncontrolled in the endemic areas, with many stray dogs and other logistic difficulties of working with dogs.

In conclusion, an EG95 vaccine produced in Morocco was found to be protective against CE in sheep, providing a potential mechanism by which to break the parasite's life cycle. The vaccine could be used alone or in combination with other antigens such clostridia or, possibly, injectable anthelmintics. The impact of cystic echinococcosis on livestock production and public health is substantial. The protective responses seen here with the clostridial/EG95 combination vaccine warrant the implementation of field trials of the vaccine in zones of high endemicity in north Africa in order to demonstrate the potential of the vaccine to both reduce transmission of cystic echinococcosis while at the same time providing protection against important clostridial infections of sheep.

Conflict of interest

M. Jazouli, Z. Bamouh, K. Tadlaoui, and M. Elharrak are employees of the sponsor company, MCI Sante Animale, Morocco. Other authors declare no conflicts of interest.

Acknowledgments

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Figure legends

Fig. 1 Body temperature measurements of groups of 20 sheep following vaccination with monovalent EG95 vaccine produced in Morocco (G1), monovalent EG95 vaccine produced in Australia (G2) and polyvalent combination vaccine with EG95 and clostridia antigens (G3) (mean +/- standard deviation). Panel A – days following the initial immunization, Panel B – days following secondary immunization.

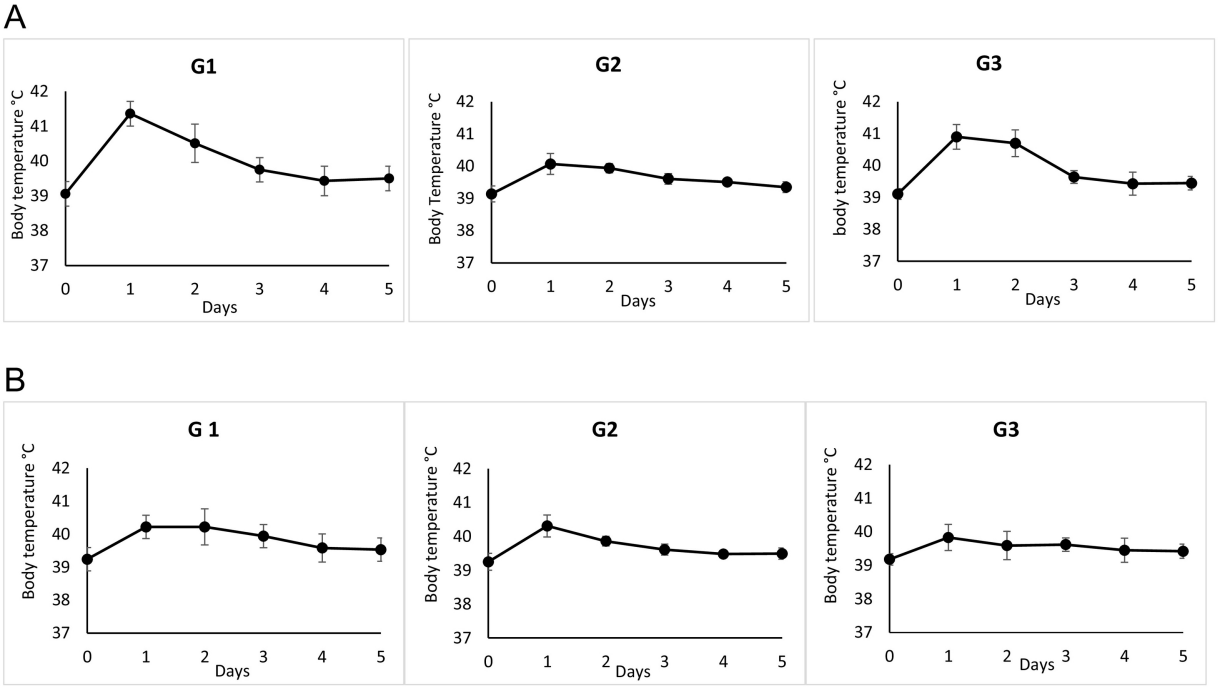
Fig. 2 Serological responses to EG95determined in ELISA in sheep vaccinated with monovalent EG95 vaccine produced in Morocco (G1), monovalent EG95 vaccine produced in Australia (G2), polyvalent combination vaccine with EG95 and clostridia antigens (G3) and unvaccinated controls (G4). V1; first injection, V2: 1st booster, V3: 2nd booster, C: challenge. Mean +/- standard deviation.

Highlights

- The EG95 recombinant vaccine against cystic echinococcosis was produced in Morocco and used to vaccinate sheep in combination with clostridial antigens.

- The combined EG95/clostridial vaccine induced specific anti-EG95 antibody responses in all vaccinated animals after two immunizations. A third vaccination induced high and long-lasting levels of specific antibody.

- Sheep vaccinated with EG95 as a stand-alone vaccine, or combined with clostridial antigens, were fully protected against a challenge infection with *Echinococcus granulosus* when challenged three weeks after a third immunization.


A
G1
Body temperature °C
42
41
40
39
38
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0 1 2 3 4 5
Days
G2
Body Temperature °C
42
41
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Days

Figure 1

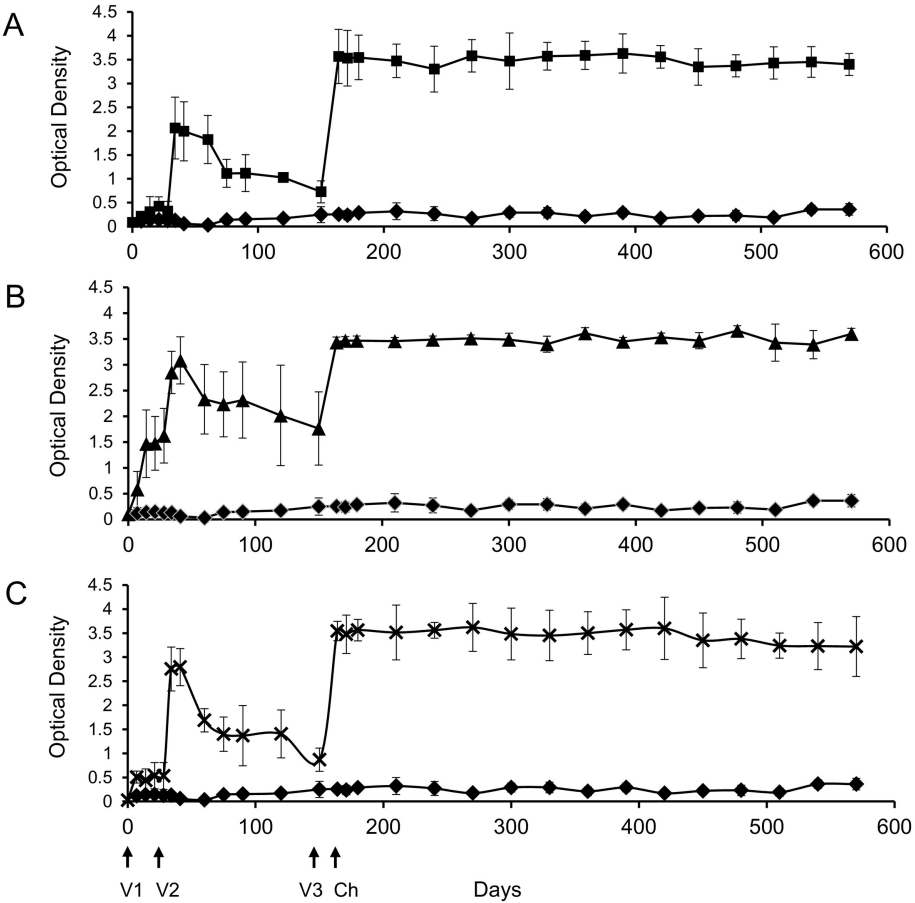
A
B
C
Optical Density
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
100
200
300
400
500
600
V1
V2
V3
Ch
Days

Figure 2