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SEROLOGY AND LONGEVITY OF IMMUNITY AGAINST *ECHINOCOCCUS GRANULOSUS* IN SHEEP AND LLAMA INDUCED BY AN OIL BASED EG95 VACCINE

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Running Title: Immunity induced by EG95 vaccine

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Abstract:

An oil-based formulation of the EG95 vaccine to protect grazing animals against infection with *Echinococcus granulosus* was formulated in Argentina. The efficacy of the vaccine was monitored by serology in sheep and llama (*Lama glama*), and was compared to the serology in sheep previously published using a QuilA-adjuvanted vaccine. Long-term efficacy was also tested in sheep by challenging with *E. granulosus* eggs of the G1 strain 4 years after the beginning of the trial. The serological results for both sheep and llama were similar to those described previously, except that there was a more rapid response after the first vaccination.

A third vaccination given after 1 year resulted in a transient boost in serology that lasted for about 12 months, which was similar to results previously described. Sheep challenged after 4 years with 3 vaccinations presented 84.2% reduction of live cysts counts compared with control group, and after a fourth vaccination prior to challenge this reduction was 94.7%. The oil-based vaccine appeared to be bio-equivalent to the QuilA vaccine.

Key Words: hydatidosis, *Echinococcus granulosus*, EG95 vaccination, protection, serology, sheep, llama.

1. Introduction

Cystic echinococcosis (CE), caused by infection with the larval stage of *Echinococcus granulosus* (*E. granulosus*), is one of the most prevalent parasitic zoonosis in Argentina. Significant foci of CE were detected especially in rural areas from the Provinces of Chubut and Rio Negro, Patagonia region where the disease is endemic in sheep and goats, and in the Northwest where the transhumant goat and llama (*Lama glama*) herding is the most important economic activity due to restricted ecological conditions of Quebrada and Puna [1].

Genetic variability of *E. granulosus* has been recognised for a long period of time and has been described as affecting characteristics such as host specificity as well as morphological and biochemical traits. With the advent of modern DNA-based methods, this genetic variability was confirmed and a number of genotypes were recognised (G1–10). Further revisions have proposed a new taxonomy for the genus, retaining the name *E. granulosus* sensu stricto (s. s.) for G1–3, giving species status to G4 (*E. equinus*), G5 (*E. ortleppi*), the genotypic cluster G6–10 (*E. canadensis*) and the 'lion strain' (*E. felidis*). This genetic

characterisation has been remarkably important in understanding the transmission pattern of the parasite between definitive and intermediate hosts, and humans [2]. It is well known that the G1 genotype is responsible for most of the cases of human cystic echinococcosis. We characterised the genetic status of the *E. granulosus* challenge infection described here.

Sufficient evidence has been gathered to indicate that the EG95 hydatid vaccine [3] has wide applicability as an effective control measure to reduce the level of transmission of *E. granulosus* in sheep, goat and cattle and, indirectly, reduce the burden of hydatid disease on human health [4-7]. The characteristics of the immunity stimulated by the EG95 vaccine have been summarised by Lightowlers et al., 1999 [8]. Two immunisations stimulate greater than 95% protection against hydatid infection in sheep. More than 50% of vaccinated animals have no viable hydatid cysts after challenge infection with *E. granulosus*. Immunity persists for at least a full year after two immunisations with the vaccine. Approximately 80% immunity is induced in sheep after a single vaccination. Solid immunity is transferred in colostrum from a vaccinated ewe to neonatal offspring. Furthermore, the EG95 vaccine demonstrated a high level of protection of both sheep and goats under field conditions in China [5] when it is used as an additional control tool. The evaluation of the outcomes of vaccination in field trials done in Argentina [9, 10] revealed a significant 62% decrease in the prevalence of hydatid infection in sheep vaccinated over a six year period. In previous trials in Argentina including sheep and goats, one vaccine dose of a laboratory-made inclusion body vaccine showed 82% protection after 12 months whereas 2 vaccine doses protected 98% after 11 months [8]. Similar results were obtained with a commercially-produced inclusion body vaccine in Xinjiang, China [5]. Two injections could induce about 85% protection against infection for 12 months in sheep and in the presence of field challenge this sort of protection level was maintained for at least another year. If a booster injection was

given 6 or 12 months after the 2nd, then the protective response could apparently last up to 3 or 4 years.

Serology had been shown to be partially associated with protection against an *E. granulosus* infection [11]. We used serology as the main monitor of putative protective antibodies, specifically comparing the serology induced by the Argentina commercial formulation with an unvaccinated control group, in both sheep and llama.

2. Materials and Methods

2.1 Vaccine

We compared the EG95 oil-based inclusion body vaccine [3] (approximately 50% pure) commercially manufactured by Tecnovax SA, (Providean Hidatil EG95®) with an unvaccinated control group.

For the commercial vaccine, a 1 mL/dose contained (70%) ISA70 VG (Seppic, France [Montanide ISA 70 VG mixed with a commercial saponin 0.125 mg (Ultra Dry 100-Q/Saponin QD 100 Ultra, 65% saponin, Natural Response S.A.) and 0.1% formaldehyde, combined with (30% volume) containing 50ug of EG95 of the solubilised inclusion bodies.

2.2 Ethics

All the trials were approved by the Instituto de Ciencia y Tecnología " Dr. Cesar Milstein" Ethics Committee and have been carried out in accordance with the Directive 2010/63/EU of the European Parliament.

2.3 Sheep

The trial was located in Peninsula de Valdez, Departament of Biedma, Province of Chubut (La Isla farm, Longitude: 64º 29' W, Latitude: 42º 26' S, Altitude/ elevation: 11 m (36 ft). SHEEP: Twenty Argentine Merino lambs born in the Spring of 2008 were allocated to groups of 10 animals when they were 6 months old. One group was not vaccinated. Animals in the other group were immunized with the oil-based *Escherichia coli* (*E. coli*) inclusion body commercial vaccine. They received 2 vaccinations, one month apart, in the Autumn of 2009. Injections were made subcutaneously in the neck region. V1 and V2 were given on opposite sides of the neck so that any injection reactions could be monitored. The serology in sheep was monitored for 1470 days. On Day 0, 30, 45, 465, 720, 1000, 1440 and 1470 a serum sample was obtained from the jugular vein to monitor the specific EG95 serological response by ELISA procedure. A booster vaccination was given 465 days later, and some animals received a fourth vaccination at 1440 days. An oral challenge with *E. granulosus* eggs was given to all surviving animals 30 days after the 4th vaccination. Necropsy took place 2 years later, which was 2170 days after the beginning of the trial (69 months or 5.7 years). The intention was to monitor the serology in sheep induced by the commercial vaccine and to demonstrate the correlation between ELISA absorbance and the protection after challenge previously validated by Heath and Koolaard [11].

Statistical analyses were performed by Kruskal-Wallis non parametric analysis of variance. Significance was determined as a P value <0.05.

2.3.1 Challenge infection

On Day 1470 sheep were each orally challenged with 1000 viable *E. granulosus* eggs prepared from mature worms obtained from thirty naturally infected farm dogs dewormed with arecoline bromohidrate in Trevelin, Departament of Futaleufu, Province of Chubut (Río Frio farm, Longitude 71º 42'W, Latitude 43º12'S, Altitude/ elevation: 704 m (2309 ft). The challenge infection was left to develop for 23 months.

2.3.2 Parasite Genotyping from cysts

Total parasite genomic DNA was prepared from 70% ethanol preserved hydatid cysts from challenged control animals by conventional techniques [12]. The DNA obtained was re-suspended in nuclease-free water and stored at −20 ◦C.

The *E. granulosus* species/genotype was determined by sequencing a fragment of the mitochondrial cytochrome c oxidase subunit 1 (CO1), as previously described [13-14]. The sequences were aligned with reference genotype sequences using Bioedit.

2.3.3 Necropsy of sheep.

Twenty three months after challenge infection the sheep were killed by stunning with a captive-bolt and then exsanguination. The liver, the heart, the spleen, lungs and kidneys of each animal were finely sliced (3mm for liver and 5mm for lungs) and each slice was inspected and palpated to find any cysts. All cysts were sliced through the middle with a sharp scalpel to confirm that they were *E. granulosus*, either with a fluid-filled central cavity

indicating viability, or a caseous centre with no cavity, indicating death during early development.

2.3.4 Statistical Analysis of cyst data

The natural logarithm of the total number of viable cysts for each treatment were analysed by analysis of variance (ANOVA). GenStat software was used for all analyses (VSN International (2014). GenStat for Windows 17th Edition.VSN International, Hemel Hempstead, UK. GenStat.co.uk)

2.4 Llama

For the llama, the animals at 8 months of age received the priming and boosting vaccinations (on days 0, 30 and 360) and then serology was monitored for a further 12 months. Llama were raised at Abra Pampa Experimental Station, Province of Jujuy (Longitude: 65º 42' W, Latitude: 22º 43' S, Altitude/ elevation: 3507 m (11505 ft). They were allocated to two groups of n = 10 animals.

One group received Placebo formulated with ISA70 VG (70w/30w) plus 0.125mg saponin without protein antigen EG95 and the other group was vaccinated with 1 mL dose of the commercial oil-based vaccine (Providean Hidatil EG95®).

Vaccination was done in the inner thigh subcutaneously. The left side was used for the first dose, right for the second dose. The vaccine was dispensed in individual disposable insulin syringes. On Day 0, 30, 66, 180, 439 and 764 a serum sample was obtained from the jugular vein to monitor the specific EG95 serological response by ELISA procedure. Statistical

analyses were performed by Student´s t- test. Significance was determined as a P value <0.05. Changes in body temperature, behaviour and reactions at the injection site were observed and collected.

2.5 ELISA Procedures.

The EG95 antigen for ELISA was prepared by expressing EG95-6HIS in *E. coli*, and purifying the construct with Protino Ni-TED/IDA (QIAGEN).

The antigen was titrated with chequer-board dilutions of positive and negative sera, and laid down at -80º C at a concentration where it could be diluted 1:1000 with coating buffer for coating Nunc Immunosorb ELISA plates. The antigen was thawed and diluted with carbonate/bicarbonate coating buffer pH 9.0 (Sigma). Wells were sensitised with 50uL of the antigen at room temperature (RT) overnight. The following day the coating buffer was poured off and plates were washed 3 times for 3 minutes each time with washing buffer (0.15M Phosphate-Buffered Saline containing 0.3% Tween 20 (Sigma). Plates were then blocked with 300 µl/well of blocking solution (900 mL Phosphate-Buffered Saline, 100 mL adult horse serum, 1% phenol red) for 1 hour at RT. The blocking buffer was poured off and plates washed 3 times. Then 100 µL of the test sera or control sera, dilution ranging from 200 to 25600 in blocking solution and was added to each well and plates were incubated at RT for 2 hours. Plates were then washed 3 times and 100µl of donkey anti-sheep IgG 1:3000 (Invitrogen) or goat polyclonal anti-llama 1:10000 (ABcam) IgG-HRP conjugate in blocking solution was added and plates were left for 1 hour at RT. Plates were washed 3 times and 100 µL of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), 0.5mg/ml in 70mM citrate phosphate buffer, pH 4.2) with 8µl of 30% hydrogen peroxide per 6 ml of substrate added at the last moment, was added to each well. Plates were incubated in the dark for 20

minutes. When the first hint of colour showed in the negative controls, the reaction was stopped by the addition of 50µl of 2% sodium fluoride to each well. Plates were then read at 405nm using an automated ELISA plate reader. The negative control sera from each trial were titrated with the test sera on the same plate, and provided a visual end-point for stopping the development of the colour reaction.

2.6 Statistical analysis:

Necropsies of 8 vaccinated and 7 control animals were performed at the end of the trial. Viable cysts were counted in each of them. The controls enabled the calculation of percentage of reduction of the viable cysts count, but were not included in the statistical analysis.

3. Results

3.1 Serology

The study looked at the immunogenicity induced by the EG95 oil-based vaccine (black square) and its comparison with an unvaccinated control group (open circle) in sheep (Fig.1) and llama (Fig. S2).

Figure 1 shows the average levels of antibodies obtained in sheep vaccinated with EG95 oil-based vaccine on day 0-30 (V1,V2), the differences between serum levels obtained in placebo group compared with higher values reached with the commercial vaccine were extremely significant ($P< 0.05$).

After an annual booster on day 465 (V3), sheep immunized with EG95 oil-based vaccine showed sustained long-term antibody levels that enhanced after V4 on day 1440. These values were significantly different ($P< 0.05$) to those obtained with the placebo (Fig.1).

Figure S2 shows the average levels of antibodies obtained in llama vaccinated with EG95 oil-based vaccine on day 0-30 (V1,V2), the differences between serum levels obtained in placebo group compared with higher values reached with the commercial vaccine were extremely significant ($P< 0.05$).

After an annual booster on day 365 (V3), llama immunized with EG95 oil-based vaccine showed enhanced antibody levels on day 439 and these values were significantly different to those obtained with the placebo.

These serum OD405nm levels followed a different pattern than that seen in sheep after two doses (Fig.1). In sheep the peak of serum antibodies was detected between 45 and 60 dpv.

3.2 Safety

All sheep remained non-febrile during the 7 days after the initial inoculation. No changes in behaviour and no local inflammation were noted at the immunization sites by direct observation.

Changes in behaviour and reactions at the injection site were not observed in llama after the injections. Slight swelling at the site of injection was detected 3 - 5 days post-vaccination. Body temperature remained according to animal species and season of the year.

3.3 Sheep necropsy results

Elisa absorbance and the inverse Log Eg95 antibody titre at time of challenge are shown in Table 1. All viable cysts were checked for a patent central cavity in order to confirm them.

3.4 Sequencing

Results show that all analysed cysts in liver, lung and kidney belong to *E. granulosus* sensu stricto G1 genotype (Fig. S3).

3.5 Viable cysts counts

Statistical analysis confirmed all the results, despite the small numbers of animals. Significant differences among treatments for total viable cysts were observed ($P < 0.05$). Both treated groups, 3 doses and 4 doses of vaccine, presented viable cysts counts significantly lower than the control group. Despite the 4 doses group showed counts lower than the 3 doses group, the differences were not significant (Table 1).

4. Discussion

Vaccination of potential intermediate hosts of *E. granulosus* with the EG95 recombinant vaccine [4, 5] could potentially be used to reduce the level of *E. granulosus* transmission and decrease the incidence of human infections [7, 15].

The commercial EG95 oil-based inclusion body vaccine is a recombinant protein non-infectious and non-toxic cloned from mRNA from the oncosphere life cycle stage of the parasite and expressed in *E. coli* [3, 5]. Two immunizations of the EG95/QuilA vaccine in young animals protects against a challenge infection with *E. granulosus* [3] by inducing specific antibodies against the oncosphere, eliminating it before it can establish and develop in the tissues of the intermediate host.

Torgerson [16, 17] and Torgerson and Heath [18] have used mathematical models to predict the impact of various options for control of CE and considered that a programme involving vaccination of intermediate hosts together with 6-monthly treatment of dogs with praziquantel would decrease the time needed to achieve control of disease transmission. It is noteworthy that in this current trial there was no deworming treatment for dogs. The area where the trial was conducted was relatively free of Echinococcus infection, being chosen because the farm was owned by the previous president of the International Association for Hidatidology (Jorge Iriarte). Nevertheless, our recommended approach for endemic regions is based on good 12 month immunity from V1 and V2, followed by annual boosting, of all available species that can contribute to the epidemiology and a minimum of 2 dog anthelmintic treatments per year.

In addition to some data about the use of the vaccine in China [5, 19], from Argentina there is some published information about the impact of the EG95 vaccine when used in field conditions, demonstrating that the EG95 vaccine is a valuable tool to assist with reducing *E. granulosus* transmission, even in circumstances where delivery of the program faces many practical difficulties [9, 10]. Since 1996 numerous trials have been undertaken in target species in different parts of Argentina [20].

An immunogenicity trial was carried out in llama vaccinated with 2 doses of the EG95 oil-based vaccine and an annual booster. On day 60 (Fig.S2), we detected that serum antibody levels were lower than those seen in sheep (Fig. 1) at the same time. It is possible that these observations would be associated to the particular kinetics of expression of functional antibodies in camelids without the antibody L chain (Fig. S2) [21].

Further trials are planned to determine whether the single chain antibodies are effective in preventing infection with *E. granulosus* in vaccinated animals.

It is remarkable that antibody OD levels observed in sheep showed a similar serological pattern to that previously published [5], but with enhanced reaction to V1.

In one publication for sheep [11] it was observed that if the EG95 vaccine induces specific antibody levels above OD 1.0, it would indicate that the vaccine induces protection and is of good quality and made in the correct concentration. In this trial the protective level of antibody OD detected by our ELISA system for sheep, is above OD of 1.0, in the face of a very large challenge with *E. granulosus* eggs. In addition, a higher protection (average 94%) was associated with ODs between 0.9 and 1.3, and the lesser protection (average 84%) was associated with ODs around to 0.7, compared to an OD for controls of 0.2. These results are consistent with that published previously [11], in that higher ODs are associated with a greater degree of protection. How this relates to the lower ODs observed with llama remains to be determined.

The similarity between the serology obtained with the New Zealand vaccine [4] and the Providean Hidatil EG95® vaccine is supported by the results of the challenge infection in sheep. Regarding the percentage of protection and serology, both regression analysis and analysis of variance showed that the link between serology and protection is tenuous but still significant.

In general one vaccination with EG95 plus QuilA adjuvant promotes a small OD response, and V2 then boosts this to a protective level. The OD then decreases to a lower level which is maintained for an extended period, and which may result in a significant degree of protection [19, Fig 2; 6]. After a V3 given 12 to 18 months following V2, a higher level of OD is observed, but this soon decreases, to a level generally above that achieved after V2, and this plateau is maintained for an extended period. Only in one trial in China, where regular natural challenges were occurring, was the level of OD after V3 maintained at a high level [19, Fig 3]. In the absence of challenge infection the pattern observed in the present trial was also followed after regular annual booster injections [19, Fig 4].

The difference observed using the Providean Hidatil EG95® vaccine is that V1 promotes a strong response that may be protective, although this has not yet been tested. Otherwise, the response to V2 and V3 are similar to that previously published.

It is not feasible to challenge animals at every point in time with *E. granulosus* eggs. Thus the fact that data is available that shows that 50% of the variation in protective antibody response is mirrored by the serological OD against EG95 [11] gives veracity to the current trials.

There is variability in the results of serology and the number of cysts in V3 group, this variability could also be due to different numbers of eggs hatching and oncospheres establishing, brought about by variations in intestinal physiology or the innate immune status of each individual animal, especially regarding complement levels [11].

Long-term immunity from 3 vaccinations (2 when young) and 1 a year later, or the addition of a V4 booster, is very useful in (a) protecting animals that may miss their annual shot and (b) in lowering significantly the biomass of potentially-infective cysts available for dogs.

Our sequencing results confirmed the presence of *E. granulosus* sensu stricto G1 genotype in all analysed cysts, the common sheep strain in Chubut Province and which is infective for humans [22].

In summary, the serological response to vaccination and the longevity of this response were clearly demonstrated. There were significant differences between responses to the oil/ saponin-based inclusion body vaccine in both sheep or llama versus non vaccinated animals. For the sheep, there were low numbers of surviving animals. In this 6 year trial, 7 and 8 sheep remained alive in control and vaccinated groups respectively. Livestock in this region were affected by a severe drought and ashes from a volcanic event.

There was a weak association between serology at the time of infection, and protection against establishment of viable *E. granulosus* cysts. Significant protection was evident 1000 days after V3, and this was enhanced by V4. These results confirm the efficacy of the registered commercial vaccine (Providean Hidatil EG95®) for practical use in the framework of the National Plan for Hydatidosis Control (http://www.senasa.gov.ar/prensa/Home/consulta_publica/2015/179/Plan%20Hidatidosis%20R.

.pdf) and its bioequivalence to the protective New Zealand formulation. This is the first report of a long term-immunity trial with experimental challenge in sheep lasting 2170 days (6 years) and also the first report of serological monitoring of South American camelid (*Lama glama*) vaccinated against hydatid disease.

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Figure Captions

Figure 1. Serological absorbances (OD425nm) of 3 groups of sheep: control (non-vaccinated), vaccinated 3 doses (0, 30 and 465 days) and vaccinated 4 doses (0, 30, 465 and 1440 days), mean values and 95%CI.

Table 1. Live cysts count at necropsy (23 months after challenge) and ELISA titre at challenge of all sheep included in the study, by group (control, vaccinated 3 doses and vaccinated 4 doses).

Supporting Information

Figure S2. Serological absorbances of vaccinated (at 0, 30 and 365 days) and control *Lama glama*, mean values and 95%CI.

Figure S3. Alignment of nucleotide sequences of a 373 bp fragment of the mitochondrial CO1 gene, for a sequence from a cysts and reference sequences from G1-G8 and G10 genotypes. Dots denote identity with the sequence of all cyst.

Group	Treatment	Animal ID	Live cysts			ELISA at time of challenge			
			Number	Mean 95%CI	Reduction %	Absorbance	Mean 95%CI	Log Inv titre	Mean 95%CI
EG95IB ISA70 + Saponin	3V	J 714	19			0.627		2.9	
		J 715	4	16.3	84.2	0.712	0.71	2.9	3.1
		J 718	22	8.2 - 24.3	76.3 - 92.1	0.834	0.62 - 0.80	3.2	2.92 - 3.34
		J 720	20			0.653		3.5	
	4V	J 713	2			0.979		3.2	
		J 716	8	5.5	94.7	1.296	1.10	3.8	3.4
		J 717	7	2.9 - 8.1	92.1 – 97.2	0.985	0.95 -1.24	3.2	3.22 - 3.64
		J 719	5			1.123		3.5	
Placebo		J 731	249			0.230		0.3	
		J 732	61			0.217		0.3	
		J 733	29			0.339		0.3	
		J 736	171	102.9 42.2 - 163.5	0	0.327	0.27 0.22 - 0.32	0.3	0.3
		J 737	36			0.282		0.3	
		J 739	55			0.337		0.3	
		J 740	119			0.173		0.3