

Utilization of EG95 vaccine for sheep immunization against cystic echinococcosis in Romania

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Abstract. Cystic echinococcosis is an important zoonotic disease. Reports from several eastern European countries provide documented evidence for the re-emergence of this parasite. To appreciate the cystic echinococcosis control opportunities the EG95 vaccine was tested. Studies were carried out on a private sheep flock from Bocsig village, Arad county, Romania. In April 2003, 10 lambs were vaccinated with EG95 vaccine and another 10 lambs (control group) were inoculated with glutathione S transferase, two times one month apart. Two weeks from the last vaccination all 20 lambs were experimentally infected with approximately 2000 viable eggs of *Echinococcus granulosus*. Blood samples were collected five times on 2-3 months interval, both from the experimentally infected lambs and from control group. Twelve months after the experimental infection, the lambs were euthanized, and the liver and the lungs were minutely examined to identify all the hydatid cysts. Every cyst was recorded from each individual and the average intensity was calculated. The efficacy of EG95 vaccine (protection degree) was calculated as a percent of reduction of the mean number of viable cysts developed in vaccinated lambs compared to the mean number of viable cysts from the control group. The protection degree in vaccinated lambs was 97%. No significant changes were recorded in the blood parameters.

Keywords: Cystic echinococcosis; EG95 vaccine; efficacy.

Received 04/01/2010. Accepted 16/03/2010.

Introduction

In many areas of the world, cystic echinococcosis (CE) is highly endemic (Ming et al., 1992; Cabrera et al., 1996; Orlando et al., 1999; Dalimi et al., 2002; Altintas, 2003; Cabrera et al., 2003; Seimenis, 2003; Sotiraki et al., 2003; Morariu et al., 2005).

The control of disease in these regions, including Romania (Morariu, 2004) is based mainly on the medical education of people, who must be taught not to feed dogs with raw offal and to treat them, periodically, with a cestocide product. But such campaigns last for years and, the rules are not applied strictly, and any interruption is a possible source for a new transmission of the parasite (WHO, 1981; Gemmell, 1990; Andersen et al., 1991; WHO, 1996; Mantovani, 1997; Economides et al., 1998; Pawlowski et al., 2001; Kachani et al., 2003). Even if the greatest part of the domestic animals is receptive, the sheep have the main role in transmitting the parasite.

The new molecular biology techniques have caused a revolution in parasitology. Within the last years there has been created a "library" of cDNA from the mRNA of the *Echinococcus granulosus* eggs, selected with the help of 23 and 25 kDa proteins (Heath and Lawrence, 1996). The clones have been expressed in the pGEX-3-EX system (Smith and Johnson, 1988), and, from all of these, the EG95 clone, a 24.5 kDa antigen, has proved to be the most effective. That is why a recombinant antigen, EG95 was successfully used in sheep from Australia, New Zealand, China and Argentina (Heath and Lightowers, 1997; Lightowers et al., 1999; Woolard et al., 1999; 2000; Chow et al., 2001; Dărăbuș et al., 2002; Heath et al., 2003) where it gave more than 94% protection. The EG95 molecule can be obtained in series, at low costs, from *Escherichia coli* lysates. After the bacteriological lyses, its inclusions are concentrated, purified and, then, rendered soluble. EG95 vaccine uses Quil A as adjuvant (Lightowers et al., 1996). The vaccine is presented under lyophilized form, in plastic sterile recipients. The vaccine can be stored 12 months, at least, at a temperature of +4°C, but it has a better action if preserved at -20°C. The vaccine must be diluted with sterile distilled

water. The reconstituted but not used vaccine cannot be preserved more than a month, not even at -20°C. Each charge of vaccine is tested before being launched on market.

Materials and methods

Vaccination

The trial was performed on a private sheep flock from Bocsig village, County of Arad, Romania. Two groups of two-month old, cross-breed lambs (Domestic Tigaia x Serbian-Somborska Tigaia) were considered for the study. The first group of 10 lambs of both sexes, was the control group. They were not vaccinated, but received GST (glutathione S transferase). The second group, also of 10 lambs of both sexes, was the experimental group and was vaccinated with EG95 antigen. Both GST inoculations and the vaccination were made twice, at one month interval. The first vaccination was made at the end of March 2003, and the second one at the end of April 2003. The vaccine was inoculated subcutaneously, on the neck left side, in a 2 ml dose. After vaccination we have recorded for all the individuals the temperature and the possible alterations of the tissues around the inoculation place (edema, nodules, etc.).

Experimental infection

In two weeks after the last vaccination, all the 20 lambs were experimentally infected, with about 2000 viable eggs of *E. granulosus*. The eggs were drawn from the gravid proglotids of adult *E. granulosus*, recently shedded by sheep dogs, following the purgation with arecoline hydrobromide. Proglotids were washed three times in phosphate buffer saline (PBS) and than prepared as described by Heath and Lawrence (1976). They were stored at +4°C for two days, before they were used. Based on previous studies carried out in the same region on larval cestodes obtained from slaughtered sheep and cattle, we considered that the circulating strain of *E. granulosus* was G1 (Morariu, 2004; Bart et al., 2006). Lambs were inoculated with a 1 ml dose by a thicker 16 cm long needle adapted to a 5 ml syringe, directly into the rumen. Prior to inoculation, the

accurate positioning of the needle into the rumen was checked.

Calculation of the EG95 vaccine efficacy

Twelve months after the experimental infestation, the lambs were euthanized, their lungs and liver collected and examined for the presence of hydatid cysts. The liver was sectioned in thin slices, of about 0.5 cm, and the lungs, in fragments of about 1 cm thickness. All the fragments were carefully palpated, in order to check for the presence of cysts. Only the liquid cysts were considered viable. The caseous and calcified hydatid cysts were considered non-viable. All the cysts found in each individual were counted and an average was calculated. The vaccine efficacy (its degree of protection) was calculated on the basis of the number of viable cysts, expressed as a percentage reduction of the mean number of hydatid cysts developed by the vaccinated lambs (VG), as compared with the mean number of viable vesicles found in the control group (CG): Efficacy (%) = [(CG-VG)/CG] x 100.

Blood sample collection

Blood samples were collected from the both lamb groups. Four milliliters of blood were collected from the jugular vein on anticoagulant, in sterile test tubes. The samples were examined in the lab of the Medical Pathology Department, with the automatic hematology analyzer MS 9 Vet.

Results

Efficacy of EG95 vaccine

The degree of protection calculated based on number of viable cysts and non-viable cysts in

vaccinated lambs as compared to the control group was 97%. The cysts dimensions have varied between 0.1 cm and 1.2 cm in diameter. In the control group, the viable cysts represented 62.46%, while in the vaccinated group they represented only 26.08% out of the total number of cysts.

The results of vaccination efficacy are presented in table 1.

Table 1. The efficacy of EG95 vaccine in lambs

Group	Cyst type	Average no. of cysts
Control	Viable	19.8
	Non-viable	11.9
Vaccinated	Viable	0.6
	Non-viable	1.7

Temperature, clinical signs and blood test values after vaccination

Table 2 presents the average of the three days temperature recordings, in the vaccinated lambs. The values of the temperatures recorded in all three days do not pass over the normal interval limits (38.5-40.5°C), even if, both when inoculating the vaccine and the GST, a one degree increasing was recorded, as compared with the initial temperature. Three of the vaccinated lambs have shown a slight increasing over the normal maximum value (40.7°C, 40.8°C, and 40.9°C, respectively). No clinical signs were recorded, but, in 6 from the vaccinated lambs, painless nodules with variable dimensions (0.7-2.2 cm diameter) were noticed. The nodules disappeared after 6 weeks from the last vaccination. The results of blood tests are shown in table 3.

Table 2. Temperature variation before and after EG95 vaccination in lambs

Vaccination	Group	Average body temperature in lambs before (BV) and after (AV) vaccination (°C)				
		BV	6h AV	24h AV	48h AV	72h AV
First	Control	39.1	39.5	39.7	39.1	38.8
	EG95	39.0	40	40.3	39.3	39.0
Second	Control	39.2	39.8	40	39.3	38.9
	EG95	39.1	40	40.4	39.4	39.1

Table 3. Hematology values before (BV) and after (AV) EG95 vaccination in lambs

Parameter	BV	2 weeks AV	5 months AV	8 months AV	1 year AV	Reference values
Erythrocytes (mil./mm ³)	11.71 ± 2.44	13.31 ± 2.44	11.41 ± 1.85	10.38 ± 1.09	10.48 ± 1.41	12 ± 4
Hemoglobin (g/dl)	11.37 ± 2.64	11.45 ± 1.91	10.22 ± 1.36	9.63 ± 0.92	9.60 ± 1.12	12 ± 2
PCV (vol. %)	38.66 ± 8.98	39.5 ± 7.21	36.11 ± 3.05	35.43 ± 3.56	35.87 ± 3.61	36 ± 4
MCV (μ ³)	33.01 ± 2.06	29.67 ± 1.46	31.64 ± 2.54	34.13 ± 1.05	34.22 ± 0.89	34 ± 6
MCH (pg/cell)	9.70 ± 0.49	8.60 ± 0.53	8.95 ± 0.57	9.27 ± 0.39	9.16 ± 0.44	8 - 12
MCHC (g/dl)	29.41 ± 0.77	28.98 ± 0.91	28.30 ± 0.92	27.18 ± 0.45	26.76 ± 0.53	32 ± 3
Leucocytes (tsd/mm ³)	6.48 ± 1.38	7.62 ± 2.64	6.58 ± 1.76	5.55 ± 1.62	5.84 ± 0.88	9 ± 3
Lymphocytes (%)	66.96 ± 3.49	66.35 ± 3,30	66.16 ± 5.61	61.27 ± 9.93	63.04 ± 5.21	58 ± 10
Monocytes (%)	3.81 ± 1.47	10.25 ± 2.40	10.42 ± 2.18	8.83 ± 2.20	9.38 ± 1.77	3 ± 3
Granulocytes (%)	29.22 ± 2.21	23.40 ± 2.86	23.41 ± 3.93	29.88 ± 9.86	29.00 ± 3.73	22 ± 5

Discussions

Comparing our results with those obtained in other countries where the same vaccine type was used, an intermediary value is obtained. Nevertheless, the value confers a solid protection to the vaccinated animals. In New Zealand, Lightowers et al. (1996) have obtained an average of 36.8 viable cysts in a control group of five lambs and no viable cysts in the vaccinated group, composed, also, of five lambs, which demonstrates a 100% protection. In Australia, an average of 4.7 viable cysts/infected lamb was obtained in the control group (n=9), and 0.2 viable cysts/animal in the vaccinated group (n=10). This corresponds to a protection of 96% (Lightowers et al., 1999; 2000; Lightowers and Gauci, 2001). The experiments carried out in Argentina have demonstrated a protection of 99%, with an average of 23.1 viable cysts/lamb in the control group (n=10) and 0.1 viable cysts/lamb in the vaccinated group (n=7). In China, the efficacy of vaccination was 97%, the same as in our experiment (Heath et al., 1999; Lightowers et al., 1999; 2000; Lightowers and Gauci, 2001; Heath et al., 2003).

Analyzing the average values of the blood test parameters (number of erythrocytes, hemoglobin, and PCV) as well as those of

derivate erythrocytic index (MCV, MCH, MCHC), we can notice that they are within the limits considered as normal for sheep, both before and after vaccination. The differences between the average values of the investigated parameters, depending on the moment of sampling were not significant (p>0.05). The number of leucocytes has recorded a slight increasing, in two weeks after the experimental infection. After this moment, it has gradually decreased, up to the last sampling. The differences between the initial values and those after vaccination have been insignificant (p>0.05). During all this period, the average values have maintained within physiological limits, but closer to the low limit of the referenced values. Percentage value of granulocytes has been diminished (p<0.001) but the monocyte number has increased (p<0.001). The lymphocytes proportion did not record differences (p>0.05) as compared with the initial value, with none of the other four blood tests. These findings are in contrast with those obtained by Vuitton (2003), because cellular immunity induced by Th1 type cytokine secretion could destroy the early stages of the larval cestodes. Studies carried out in humans have also demonstrate that the relative ratio of T lymphocyte subsets in the liver distort the efficiency of granulomas and, of course, the outcome of infection. The main

classes of T cells were CD4+ in normal granulomas, while CD8+ T cells were prevalent in severe cases (Vuitton et al., 1989). On the other hand, in progressive cysts obtained from cattle, CD8+ T cells were predominant in the adventitial layer, and in the regressive cysts infiltrating lymphocytes were CD4+ (Sakamoto and Cabrera, 2003).

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