

Short Communication

Preparation of concentrated blackleg vaccine

**Mossawi Shoshtary*, M., Pilehchian Langroudi, R., Abdolmohammadi, L.,
Jabbari, A.**

*Department of anaerobic bacterial vaccine production and research, Razi Vaccine and Serum Research Institute,
Karaj, Iran.*

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ABSTRACT

Blackleg in cattle has been recognized since 1938 in Iran that mostly affects cattle in enzootic farm. Main object of this study was to prepare and formulate a concentrated potent vaccine for immunization of cattle against blackleg in Iran. Experimental concentrated blackleg vaccine was prepared according to the method described by FAO. The medium (Modified medium for production of experimental *C. chauvoei* vaccine by fermenter) consisting of peptone, glucose, sodium chloride, cysteine hydrochloride and yeast extract was prepared by fermenter and inoculated by *Clostridium chauvoei* strain for preparation of blackleg vaccine. Aluminum hydroxide gel adjuvant was added to the high yield vaccine. The vaccine was also concentrated by the method of precipitation. None of tested animals showed any local or general adverse reactions. All of vaccinated guinea pigs resisted the challenge with 4 MLD of virulent *C. chauvoei*.

Keywords: Blackleg, *Clostridium chauvoei*, Concentrated Vaccine

INTRODUCTION

Blackleg is a fatal disease of young cattle. It produces an acute local infection with the resulting blood poisoning leading to rapid death. The name 'blackleg' derives from the fact that the site of infection is often a leg muscle with the affected muscle dark in color. Blackleg infection is caused by *Clostridium chauvoei* and is almost associated with wound infection in cattle. Most cases occur in young stock between 10 months and two years of age. Feet or legs and the tongue are often the

predilection site. Within 48 hours there is a high fever and if limb muscles are involved the animal becomes stiff and unwilling to move. Skin discoloration, subcutaneous oedema and gas production may be present and perineal oedema is sometimes seen. Infections of the head may produce marked oedema and even bleeding from the nose. Death usually follows a period of anorexia, profound depression and prostration (Miyashiro *et al* 2007, Uzal *et al* 2003, Blood *et al* 1983). Generally, the best-conditioned animals are affected with most losses occurring where there is an abundance of feed. Blackleg can occur at any time of the

* Author for correspondence. E-mail: mousavi_msh@yahoo.com

year though more losses are seen during hot, humid weather or following the sudden onset of cold periods. Blackleg is produced by spore-forming bacteria. The organism most commonly responsible is *Clostridium chauvoei* and, less frequently, *C. septicum*. Spores produced by the clostridia can lie dormant in the soil for years without losing their potency (Songer 1998). While the presumptive and diagnosis of blackleg can be achieved by clinical and pathological findings, confirmation of the disease is routinely performed by isolation and identification of *C. chauvoei* by conventional or molecular (PCR) methods (Kojima et al 2001). Blackleg is generally known to affect cattle but sheep, goats, swine, camels, deer and mink are also susceptible. Blackleg in cattle has been recognized since 1938 in Iran and mostly affects cattle in enzootic farm but in 1968, a severe and extensive outbreak occurred in a vast area among cattle flocks in fifteen villages in south part of Iran which killed 400 cattle (Ardehali et al 1975). The only effective means of controlling blackleg is by vaccination. Several makes of vaccine are available commercially and care should be taken to follow the manufacturers' instructions (Walker 1992). The blackleg anaculture vaccine has been prepared for immunization of cattle and sheep against the disease at Razi Institute using traditional manner (Moosawi et al 1992) and also by fermenter (Pilehchian et al 2002). The main object of this study was to prepare and formulate a concentrated potent vaccine for immunization of cattle against blackleg in Iran.

MATERIALS AND METHODS

Modified medium for production of experimental

Peptone	0.4 % W/V
Trypton	0.8 % W/V
Meat extract	0.2 % W/V
Casein hydrolysate	0.6 % W/V
Na ₂ HPO ₄	0.04 % W/V
Yeast extract	0.2 % W/V
Cystein hydrochloride	0.1 % W/V
pH	7-7.2

C. chauvoei vaccine by fermenter was as follows: Glucose solution was prepared separately in a bottle containing 12.5 liter of distilled water and autoclaved at 110 °C for 30 minutes. The above ingredients were measured and carried to the fermenter. It was dissolved in 380 liters distilled water in a mixer tank by means of circulation of steam. The medium was pumped into fermenter and the final volume was reached to 800 liters by adding distilled water. The sterilization of the medium was done at 121 °C. for 30 minutes. The medium was cooled down up to 37 °C. Sampling from the sterilized and cool medium was done. The sample was cultured in nutrient agar, broth and liver broth to control it. Direct observation by microscope by taking slides to check the purity is necessary.

Working seed. The contents of a lyophilized culture of *C. chauvoei* (CN 701) was transferred to a fresh tube of liver medium. The tube was incubated for 24 hours at 37 °C in Anoxomate anaerobic jar. After an active growth, it was transferred to two tubes of liver media and was incubated in Anoxomate anaerobic jar for 24 hours at 37°C. The original tube was used as mother pre-seed. All the tubes were kept in refrigerator.

Working seed culture. The two tube cultures were subcultured to two 250 ml of fresh liver infusion broth. The flasks were incubated at 37 °C for 24 hours in the anoxomate anaerobic jar. The seed cultures were controlled in broth and slope agar. The next day, after an active growth, the contents of flasks were transferred to five 500 ml flasks of fresh liver infusion broth and were incubated at 37 °C for 24 hours.

Subculture. A day before, the subculture medium was prepared in ten 20 liter capacity bottles, each containing 16 liters of the above mentioned medium.

Cultivation. The temperature of fermenter was adjusted to 37 °C and its pH regulated on 7.2. Cultivation was done early in the morning and the agitation was stopped till the growth started and was

adjusted at 50 RPM. The period of incubation was about 10 hrs. Sample was taken every hour during the growth and sent to Biotechnology Department for counting the number of bacteria by method of Spectrophotometer. Sample also was taken at the end of incubation for purity of culture and also for pathogenicity of *C. chauvoei* in guinea-pigs.

Toxoiding. After an active growth duration, detoxification and killing of bacteria was done by adding formaldehyde (37%) at the rate of 0.7% and pH of culture adjusted to 7.2. The prepared vaccine remained for 5 days in fermenter at 37 °C with agitation 50 RPM to complete detoxification. A sample of two liters of vaccine was taken and stored in refrigerator for further studies and the rest of vaccine was evacuated into sterile 250 liter deposit tanks and kept in cold room.

Concentration. Aluminum hydroxide gel as adjuvant was used for the *C. chauvoei* vaccine. The adjuvant was added slowly into the inactivated *C. chauvoei* culture in sufficient quantity to give 10 percent gel in final concentration. The adjuvant vaccine was stirred slowly at 30 minute interval for five minutes at 20 °C. The adsorption was carried in this manner for ten hours. The adjuvanted vaccine was stored in refrigerator at 4 °C .The vaccine was kept for three days at 4 °C to settle organisms. The supernatant was siphon off from the adsorbed vaccine to give 2.5 times concentration, i.e. from one liter of final adsorbed of experimental vaccine siphon off 640 ml supernatant. Merthiolate as a preservative was added in the adjuvanted vaccine at the rate of 0.01 percent.

Safety and potency trials. The quality control of the experimental vaccine was determined according to the European pharmacopeia veterinary 5th edition (2004). Three healthy cattle were selected for safety test of the vaccine. Each cattle was injected subcutaneously with 4 ml of the vaccine and observed for ten days. Ten healthy guinea pigs (300-400 g) were injected subcutaneously with 2ml of the vaccine as primary dose. Second injection was

carried out three weeks later with the same quantity. After two weeks the vaccinated and unvaccinated guinea-pigs were challenged intramuscularly with 4 MLD for vaccinated and 1MLD for unvaccinated with a virulent culture of *C. chauvoei* (Table 1).

Abnormal toxicity. Two guinea pigs and five mice were injected subcutaneously with 2ml and 5ml of experimental vaccine respectively and the animals were observed for 7 days.

Control of sterility .The vaccine was cultured on blood agar, broth, slope agar and sabourod and kept in incubator for 48 hrs.

RESULTS AND DISCUSSION

High yield of *C. chauvoei* cultures were obtained in fermenter after 10 hrs. The sterility tests showed that all of the bacterial suspensions were pure without any contamination. The cultures were highly pathogenic, 0.25ml killed guinea-pigs with typical lesions of *C. chauvoei* infection after 36 hrs. Each ml of vaccine contained 6×10^8 organisms. After precipitation by aluminum hydroxide, each ml of concentrated vaccine contained up to 1.5×10^9 organisms. None of injected cattle showed any general or local reactions. All vaccinated guinea-pigs resisted the challenge with the virulent culture of *C. chauvoei* while the unvaccinated (control) guinea-pigs died (Table 1).

Table 1: Results of challenge of vaccinated and control guinea pigs with virulent *C. chauvoei* strain.

Treatment Group (Number)	Dose of Challenge	Adverse Reaction	S/C*	Percent of Protection
Vaccinated (10)	4 MLD (1ml)	Nil	10/10	100
Control (2)	1 MLD (0/25ml)	Nil	0/2	0

*S: Number of survived animals after challenge, C: Number of challenged animals.

C. chauvoei is an etiological agent of blackleg in cattle. This bacteria is widespread in nature and is part of the normal bacterial soil flora. There is no consensus on the pathogenesis of blackleg infection that occurs in ruminant, but toxins and

neuraminidase produced by *C. chauvoei* are believed to play a significant role in the pathogenesis of the disease (Useh *et al* 2003). In cattle, blackleg infection is endogenous, in contrast to malignant edema. Lesions develop without any history of wounds, although bruising or excessive beef breeds, in excellent health, gaining weight, and usually the best animals of their group. Outbreaks occur in which a few new cases are found each day for several days. Most cases occur in cattle from 6 months to 2 years old, but thrifty calves as young as 6 weeks and cattle as old as 10-12 years may be affected. The disease usually occurs in summer and fall and is uncommon during the winter. In sheep, the disease is not restricted to the young, and most cases follow some form of injury such as shearing cuts, docking, crutching, or castration (Songer, 1998). It is 3 decades that Blackleg vaccine is produced in Razi institute and because of enhanced need of country we decided to improve the production procedure of this vaccine. Batch fermentation can be considered to be a closed system. At time $t = 0$ the sterilized nutrient solution in the fermentor is inoculated with microorganisms and incubation is allowed to proceed. In the course of the entire fermentation, nothing is added, except oxygen (in case of aerobic microorganisms), an antifoam agent, and acid or base to control the pH. The composition of the culture medium, the biomass concentration, and the metabolite concentration generally change constantly as a result of the metabolism of the cells. After the inoculation of a sterile nutrient solution with microorganisms and cultivation under physiological conditions, four typical phases of growth are observed. It was shown that the immunoprotective capacity of *C. chauvoei* was highest if the culture was inactivated at the stationary phase of growth (Mattar *et al* 2002). The most effective way of controlling clostridial diseases is by vaccination. The veterinary surgeon or the manufacturers' datasheets should be consulted for their proper use.

The organic standards permit the use of vaccination in cases where there is a known disease risk. Single vaccines are preferred to more complex multiple vaccines unless such cover is specifically required. Vaccine choice and use should be agreed with the nominated veterinary surgeon to ensure adequate disease protection during the conversion period with, where possible, progressive reductions in use as the organic unit become established. Only healthy animals should be vaccinated (Miyashiro *et al* 2007). The immunity to *C. chauvoei* is considered to be mainly anticellular, and for this reason there is scarce information about the immunogenicity of extra cellular proteins (Muttar *et al* 2007). Routine blackleg vaccine is a bacterin containing *C. chauvoei* that is safe and suitable for both cattle and sheep. Calves should be vaccinated at the age of 2- 6 months. In the high-risk areas, revaccination is necessary at one year later and every five years, thereafter. When outbreaks are encountered, all susceptible cattle should be vaccinated and treated prophylactically with penicillin to prevent new cases, which may develop for up to 10 days (Blood *et al* 1983). Reduced dose concentrated blackleg vaccine could be replaced of the routine blackleg vaccine because of long immunity in vaccinated animals, economic and of bottling of vaccine. The results of abnormal toxicity were quite satisfactory, none of the injected guinea-pigs and mice showed any local and general reactions. The results of sterility showed no any organism on cultured media.

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