

Full Article

Large scale production of Blackleg vaccine by fermenter and enriched culture medium in Iran

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ABSTRACT

In all biological systems growth is defined as increase of chemical compounds. Bacteria can achieve to balanced growth if they are growing in a medium, which are completely adapted to it. *Clostridium chauvoei*, (*Clostridium fesceri*) is an anaerobic, spore forming, motile, and polymorph bacteria, which its size varies from 0.5-1 to 3-8 micron and could be observed as individual bacterium, diplo, and rarely streptococcus. Blackleg is a fatal disease of young cattle. It produces an acute local infection, and the resulting blood poisoning leads to rapid death. *Clostridium chauvoei* and, less frequently, *Clostridium septicum* are the most commonly responsible organisms. Vaccination is the only effective means for controlling of blackleg disease. Several kinds of vaccine are available commercially. It is 4 decades that blackleg vaccine is produced in Razi institute and because of enhanced demand of country, decision was made to improve the production procedure of this vaccine using large-scale fermenter, so the aim of this study was adaptation of *Clostridium chauvoei* to growth and proliferation in fermenter for preparation of a potent vaccine. Accordingly attempts were made to prepare and formulate the ingredients in order to obtain high yield of *Clostridium chauvoei* in culture medium by fermenter. All experiments were done in two sets: A-growth in glass bottles using conventional culture medium and B-growth in fermenter using conventional culture medium similar to A and also enriched culture medium. Results showed high yield of *Clostridium chauvoei* suspension in fermenter after 10 hours, using enriched culture medium (more than 1,480,000,000 organisms/ml), but no significant changes was obtained in glass bottles conditions comparing to the fermenter conditions. The safety and potency of the prepared vaccine was determined in sheep and guinea pigs according to British pharmacopoeia (veterinary) with satisfactory results. Since this research has been successfully done in Razi research institute, so the mono valent inactivated blackleg vaccine, using the enriched culture medium is currently producing by fermenter and is used for immunization of cattles in Iran.

Keywords: Blackleg vaccine, *Clostridium chauvoei*, fermenter, enriched medium, conventional medium

INTRODUCTION

Developments in veterinary and medical bacterial vaccines are outlined. In the case of veterinary

vaccines, economic considerations are very important and less purified products of proven efficacy could be found in market but for human use vaccines, safety and absence of side effects are increasingly demanded (Walker 1992). Blackleg is a fatal disease of young

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cattle and is produced by spore-forming bacteria. *Clostridium chauvoei* and, less frequently, *Clostridium septicum* are the most commonly responsible organisms. Spores produced by the *Clostridia* can remain latent in the soil for years without losing their potency. Blackleg produces an acute local infection and the resulting blood poisoning can lead to rapid death (Robson & Wilson 2007). The name of blackleg derives from this fact that the site of infection is often a leg muscle and the affected muscle has a dark color (Blaha 1989). Blackleg infection is almost associated with wound infection in cattle. Although blackleg has been found in cattle as young as two months old, most cases occur in young stock between age of 6 months up to the end of two years old. Feet or legs and the tongue are often the predilection site. High fever will occur within 48 hours and if limb muscles are involved the animal becomes stiff and do not want to move. Nose bleeding, skin discoloration, subcutaneous oedema and gas production may be present and perineal oedema sometimes could be seen. Infections of the head may produce marked oedema. And finally death usually follows a period of anorexia, profound depression and prostration (Moosawi 1992).

Occasionally, losses are seen in adult cattle. The disease occurs world wide. A similar infection in man is not uncommon. 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 year though more losses are seen during hot, humid weather or following the sudden onset of cold periods. Uzal describes the epidemiological and pathological features of an outbreak of Clostridial myocarditis in calves due to *Clostridium chauvoei*. Four of seven two-month-old Hereford calves died in the course of a week. Their gross postmortem lesions were similar and consisted of irregular dark red areas of myocardial necrosis through the full thickness of the atrial and ventricular myocardium. No lesions were observed in skeletal muscle (Uzal 2003). Blackleg is generally known to affect cattle but sheep, goats, swine,

camels, deer and mink are also susceptible (Mackey 1979). There is no consensus on the pathogenesis of blackleg infection that occurs in ruminants, but toxins and neuraminidase produced by *Clostridium chauvoei* are believed to play a significant role in the pathogenesis of the disease (Useh 2003). Blackleg in cattle has been recognized since 1938 in Iran in enzootic farm (Rafyi & Ardehali 1966). In 1988, a severe and extensive outbreak occurred in a vast area among cattle flocks in fifteen villages in south part of Iran which resulted to 400 cattles death. Many strains of *Clostridium chauvoei*, has been isolated from samples received from different part of country, and studied at Razi institute (Ardehali et al 1971), (Ardehali et al 1975).

Vaccination is the only effective means of controlling of blackleg disease. Although there are a few references in the literature concerning production of blackleg vaccine (Reed & Reynolds 1977), (Walker 1992), (Songer 1998), but the best instruction could be find in FAO manual for the production of anthrax and blackleg vaccines (Misra 1991). Beside several kinds of vaccine are available commercially and care should be taken to follow the manufacturers' instructions. The formalized whole culture vaccine has been prepared for immunization of cattle and sheep against the disease at Razi Institute using conventional proceduers (Moosawi et al 1992). The aim of this study was adaptation of *Clostridium chauvoei* to growth and proliferation in fermenter to prepare a potent vaccine against blackleg of cattle.

MATERIALS AND METHODS

All experiments were done in two Sets:

A: growth in glass bottles. Conventional culture medium consisting of peptone, cystein hydrochloride, Na₂HPO₄, NaCl and glucose solution (Moosawi et al 1992).

1. Working seed: Lyophilized *Clostridium chauvoei* vaccinal strain (CN701) was obtained from anaerobic vaccine research and production department of Razi

research institute and transferred into a culture tube of fresh liver medium. The culture tube was incubated at 37 °C in the Anoxomate anaerobic jar for 24 hours. After an active growth period, it was transferred into two culture tubes of fresh liver medium and was incubated at 37 °C in the anaerobic jar for 24 hours. The contents of original culture tube was used as pre-seed. All the tubes were kept in refrigerator.

2. Working seed culture: The contents of two culture tubes were inoculated into two 250ml of fresh and sterilized liver infusion broth. The flasks were incubated at 37 °C in the Anoxomate anaerobic jar for 24 hours. The seed cultures were controlled using broth and slope agar. The next day, after an active growth period, the contents of flasks were transferred into five 500ml flasks of fresh and sterilized liver infusion broth and were incubated at 37 °C in the anaerobic jar for 24 hours.

3. Inoculation, culture period and detoxification: The above mentioned suspension was inoculated into each of 20 liters glass bottles (consisting 16 liters of culture medium) and left for 48 hours at 37 °C. then formaldehyde was added (0.6%) and detoxification was done for 10 days as described previously (Moosawi, M. et al 1992).

B: Growth in fermenter. 1. Conventional culture medium: similar to above mentioned (A-1).

2. Enriched culture medium consisting of tryptone (0.8% W/V), peptone (0.4% W/V), meat extract (0.2% W/V), Yeast extract (0.2% W/V), casein hydrolysis (0.6% W/V), L-cystein hydrochloride (0.1% W/V), Na₂HPO₄ (0.04% W/V) and glucose solution (Cameron C.M. et al 1986). Glucose solution was prepared separately in a glass bottle containing 12.5 liters of distilled water and autoclaved at 110 °C for 30 minutes and was added at the time of inoculation.

3. Working seed and Working seed culture, similar to above mentioned (A-2 & A-3).

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

5. Culture medium: The above mentioned ingredients (B-2) except of glucose solution were transferred into the mixer chamber and dissolved in 380 liters distilled water by means of circulation of clean steam, then transferred into fermenter chamber and the final volume of culture media was adjusted to 800 liters by adding distilled water. The sterilization of the culture medium was done at 121 °C for 30 minutes and then was cooled down to 37 °C. Sterility test was carried out by sampling of the sterilized culture medium and inoculating into nutrient agar, nutrient broth, liver broth and blood agar.

6. Cultivation: The temperature of fermenter was adjusted to 37 °C and its pH regulated on 7.2. Inoculation into fermenter chamber was done using peristaltic pump early in the morning and the agitation was stopped till the growth started and then was adjusted at 5 RPM. The period of incubation was about 10 hours. Sampling was carried out each hour during the growth and total count was done. At the end of incubation period another sample was taken for purity test of suspension and also for pathogenicity test of *Clostridium chauvoei* in guinea-pigs.

7. Toxoiding: after an active growth period, bacteria inactivation and detoxification was carried out by adding formaldehyde (0.6%), and the pH of the suspension was adjusted to 7. The prepared vaccine was left in the fermenter chamber at 37°C with 50 RPM agitation to complete detoxification. After 5 days two liters of vaccine was taken as sample and stored in refrigerator for further studies.

Quality control. Quality control was done for two kinds of vaccines that were produced in fermenter and in glass bottles but enriched culture medium was not used in glass bottles.

1. Control of sterility: each kind of vaccines was inoculated on the blood agar, nutrient broth, slope agar and sabouraud and kept in incubator for 48 hours.

2. Abnormal toxicity (residual toxicity test): Two guinea pigs and five mice were injected subcutaneously with respectively 2ml and 5ml of vaccines and the

animals were observed for 7 days according to European pharmacopeia Veterinary (2004 5th edition).

3. Potency test (challenge): The quality control of the vaccines was determined according to the European pharmacopeia Veterinary 5th edition (2004). Ten healthy guinea pigs (300-400 gr) were injected with 2ml of the vaccine (SC) as primary dose, followed by three weeks later injection of the same quantity of the vaccine as the secondary dose. After two weeks the vaccinated and unvaccinated guinea pigs were injected (IM) with fresh virulent suspension of *Clostridium chauvoei* (respectively 4 MLD for vaccinated and 1MLD for unvaccinated guinea pigs).

4. All of these steps were repeated 5 times, which in the 1st, 2nd and 3rd repeats conventional culture medium and in the 4th and 5th repeats, enriched culture medium was used.

5. Safety test: three healthy cattle and two healthy sheep were selected for safety test of the vaccine. Each cattle was injected 2ml of the vaccine (SC) and was observed for ten days, two sheep also were injected using 10 ml and 5 ml (over does) (SC) of prepared vaccine and were observed for one month.

RESULTS

High yield of *Clostridium chauvoei* suspension was obtained in fermenter after 10 hours, using enriched culture medium, but no significant changes was obtained in glass bottles conditions comparing to the fermenter conditions. The bacterial suspensions were highly pathogenic and the guinea-pigs died during 36 hours after injection of 0.25ml of the suspension. Typical lesions of *Clostridium chauvoei* infection was observed in the different organs of dead animals. The results of safety and potency tests on susceptible healthy cattle, sheep and guinea-pigs were quite satisfactory. None of the injected cattle and sheep showed any general or local reactions. All vaccinated guinea-pigs resisted to the challenge dose of *Clostridium chauvoei* virulent culture while the unvaccinated (control) guinea-pigs died of *Clostridium chauvoei* infection. Figure 1 shows

comparative growth curves of *Clostridium chauvoei* cultured in fermenter using conventional and enriched medium for five repeats.

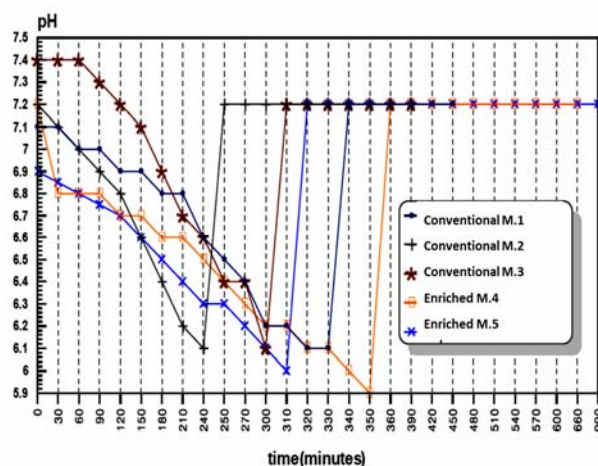


Figure 1. Comparative growth curves of *Clostridium chauvoei* cultured in fermenter using conventional and enriched

Table 1. total cell counts changes of the *Clostridium chauvoei* during culture period in fermenter using both conventional and enriched culture medium.

Time (min.)	Conventional culture medium 1 st repeat	Enriched culture medium 4 th repeat	Enriched I culture medium 5 th repeat
0*	22,500,000	30,000,000	30,000,000
270	155,000,000	-	-
300	567,500,000	-	148,000,000
390	650,000,000	160,000,000	-
450	675,000,000	485,000,000	230,000,000
510	-	700,000,000	-
600	-	1,120,000,000	-
660	-	1,480,000,000	1,050,000,000
750	-	-	1,120,000,000
990	-	-	1,400,000,000

Table 1 shows that total cell count at the end of culture period for 1st, 4th and 5th repeats respectively is 675000000, 1480000000 and 1400000000. Figures 2 & 3 show growth curves of three above-mentioned repeats. Table 2 shows Challenge results of *Clostridium chauvoei* vaccine in guinea-pigs.

DISCUSSION

Growth in all biological systems is defined as increase of chemical compounds. Bacteria can achieve to

balanced growth if they are growing in a medium, which are completely adapted to it. *Clostridium chauvoei* cultivation in bottle is specified with its low gas production and low turbidity.

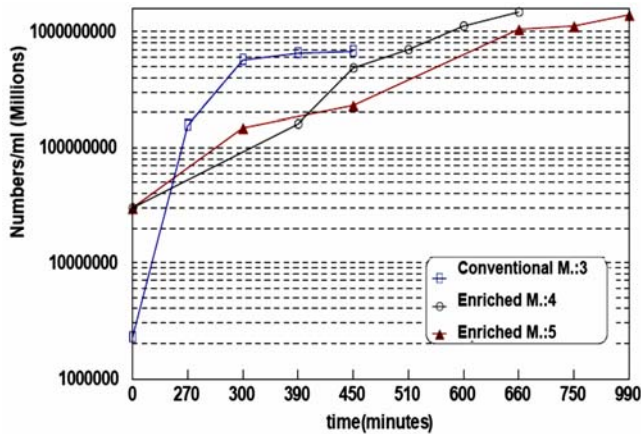


Figure 2. Growth of *Clostridium chauvoei* in fermenter using conventional and enriched medium. Comparison based on total cell count in different stages of bacterial growth

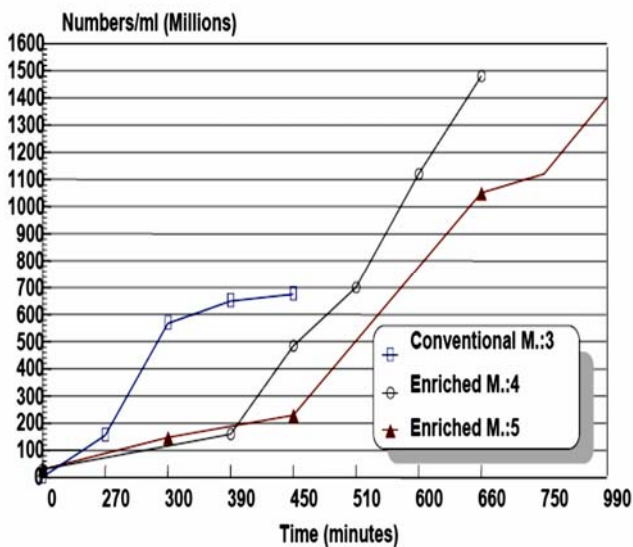


Figure 3. logarithmic growth of *Clostridium chauvoei* in fermenter using conventional and enriched medium. Comparison based on logarithmic numbers of total cell count in different stages of bacterial growth.

In this study *Clostridium chauvoei* was adapted to grow in fermenter, and a new culture medium was suggested for this purpose, which short duration of culture period, high gas production, high turbidity and high number of bacteria per/ml of suspension are its specificities. Furthermore cultivation of *Clostridium chauvoei* in fermenter is simpler than in bottle and there

would be less chance of contamination because all steps are done in bioreactor chambers and there is no transportation and direct impact in the air exposed area. The anaerobic bacillus *Clostridium chauvoei* is the causative agent of blackleg, a lethal disease that has an important impact on the sheep and cattle industry worldwide. Immunity to *Clostridium chauvoei* is considered mainly anticellular, and for this reason, there is scarce information about the immunogenicity of extracellular proteins (Smith 1975).

Table 2. Challenge of *C.chauvoei* vaccine in guinea-pigs.

No. Of guinea Pigs	Minimum vaccinal dose	Reaction	Challenge Material	Challenge Results
10	2ml	No	1ml (4MLD) 24hr <i>C.chauvoei</i> Virulent culture	Resisted
2 (control)	0	No	0.25ml (1MLD) 24 hr <i>C. chauvoei</i> Virulent culture	Died of <i>C.</i> <i>chauvoei</i> infection

The immunoprotective capacity of four *Clostridium chauvoei* strains at different growth stages had been tested. In all the strains tested, the cells coming from the stationary phase were those with the highest immunoprotective capacity and, depending on the strain, this protective capacity diminished or even disappeared in other phases. Protein profiles were similar in all the strains and few proteins were during growth (Mattar et al 2002, 2007). Blackleg is an acute and fatal disease of young cattle. It produces an acute local infection with the resulting blood poisoning leading to rapid death (Robson & Wilson 2007). The organism most commonly responsible is *Clostridium chauvoei* and, less frequently, *Clostridium septicum*. Spores produced by these clostridia can lie dormant in the soil for years without losing their potency. The only effective means of controlling blackleg is by

vaccination. Several kinds of vaccine are available commercially and care should be taken to follow the manufacturers' instructions. It is 4 decades that blackleg vaccine is produced in Razi institute and because of enhanced demand of country we decided to improve the production procedure of this vaccine using large-scale fermenter and enriched culture medium. Previously a semi-synthetic culture medium and method had been described for the production of a reduced dose *Clostridium chauvoei* vaccine. The vaccine gave excellent results in guinea pigs, and two injections of 2.0 ml protected cattle against challenge with two M.L.D. of a virulent culture for at least 12 months (Cameron 1986). In the present study 2 groups of experiments were set up. In the 1st group conventional vaccine production procedures in 20 liters glass bottles and conventional culture medium was used. In the 2nd group fermenter and both conventional culture medium and enriched culture medium was used and produced vaccines in these two systems were compared. Bacteria produce toxin during the growth in the glass bottles as well as acetic acid, butyric acid, butanol and also equivalent amount of carbon dioxide and hydrogen, so the suspension fall into the acidic phase. At the other hand because of no agitation there is no homogeneity in the suspension. Since free and bonded nitrogen are very important for bacterial growth and toxin production, so in the 1st experiment 12 different peptone from 12 different manufacturers were used but total cell count of bacteria suspension at the end of culture period for all of 12 different sources of pepton in this group remained unchanged and was 300,000,000. These data showed no significant difference when compared to the data and condition that was described previously by the same researchers (Moosawi *et al* 1992). In the 2nd group of experiments these situation clearly improved (because of removing of gases), by adding NaOH to neutralize acidic state and also agitation for homogeneity of medium ingredients and temperature. So the total cell count of bacteria for both conventional culture medium and enriched culture medium was increased and the culture

period for both conventional culture medium and enriched culture medium was decreased. The potency test (challenge) prescribed for *Clostridium chauvoei* vaccines in the British Veterinary Codex requires the complete survival of a group of immunized guinea pigs and death of all unvaccinated controls. Problems arises the influence of extraneous factors such as animal strain, diet, and seasonal variation (Knight & Kent 1976). As the last confirmation, potency test according E.Ph. with the special attention on the above mentioned problems by controlling different parameters was used (European pharmacopeia Veterinary 2004 5th edition) . In 1970 Chandler described a highly protective strain of *Clostridium chauvoei*, compared, and contrasted it with a number of less protective strains and strain variants. This strain was found to produce uniformly smooth colonies and was highly toxic to mice and pathogenic to guinea pigs. It possessed an O antigen common to all the strains of *Clostridium chauvoei* and an H antigen, which in this study was shared only by the standard challenge strain. Apart from flagella type, no single characteristic sets this strain clearly apart from other less protective strains. In the present study, different strains of *Clostridium chauvoei* were not compared to each other and only the vaccinal strain (CN701) was used. Crichton, R. et al in 1986 reported simultaneously testing of twelve commercial 5-component Clostridial vaccines with known variations in potency of the blackleg (*Clostridium chauvoei*) component, in sheep and guinea pigs. Controlled challenge experiments provided evidence of a highly significant correlation in the response of the two species. Based on this study the guinea pig laboratory model is considered a valid indicator of field performance for vaccines containing blackleg antigen. In the present study results of using guinea pigs which are shown on table 2, confirms these findings. Since this research has been successfully done in Razi research institute, so the monovalent inactivated blackleg vaccine, using the enriched culture medium currently is producing by fermenter and is used for immunization of cattles in Iran.

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