Abstract

The aim of this study was to investigate the vaccine properties of three Algerian strains of *Clostridium chauvoei*. Batch culture in laboratory fermenter was performed on CAM medium and Vaccine was obtained by formalin inactivation of whole stationary-phase culture. In *vitro* and *in vivo* toxicity of strains were evaluated, respectively, by the hemolytic activity and lethal effect on mice and guinea pigs of the culture supernatant. Pathogenicity of strains was assessed by injection of a culture to guinea pigs and mice. The passive protection assay was performed on mice inoculated with anti-serum and challenged with a virulent strain. Vaccine potency was tested on guinea pigs inoculated with vaccine and challenged with a virulent strain. The study of characteristics of growth distinguished the strain ALG2 from other strains by its greater growth rate (0.85 h^{-1}) and structural integrity. Pathogenicity and toxicity were observed for ALG2, while pathogenicity was lower and *in vivo* toxicity was absent in other strains. Passive protection tests showed broader protective effect of immune sera from strain ALG2. Active protection testing with the vaccine prepared from strain ALG2 showed that all vaccinated guinea pigs challenged with five wild strains have survived. This study highlighted the immunogenicity and protective capacity of *C. chauvoei* strain ALG2 isolated in Algeria, which may be a good candidate for vaccine production.

Keywords: *Clostridium chauvoei*, Blackleg, Local strain, Vaccine, Immunoprotection

INTRODUCTION

Blackleg is an acute disease that mainly affects young cattle and sheep, characterized by myositis and toxemia, and is often fatal. *Clostridium chauvoei*, the causative agent of the disease, is a Gram positive, rode shaped, strict anaerobic, gas producing and endospore forming bacterium. The spores have high toughness, they can survive in the soil and food for many years, and they contaminate animals if swallowed. The geographical distribution of blackleg is global, but with regional concentrations. This disease is common in Algeria and responsible for economic losses in livestock. Because of the acute nature of the disease, treatment is not always effective. The most appropriate
The objective of this study was to test the vaccine properties of three Algerian local strains of *Clostridium chauvoei*.

**MATERIAL and METHODS**

**Bacterial Strains**

Five strains of *Clostridium chauvoei* were used in this study, a reference vaccine strain CCM5735, (Harshey, Veterinary Research Institute, BRNO, Czechoslovakia), an Iranian strain CJ1R and three local strains, ALG1, ALG2 and ALG3, isolated from calf carcasses in 1993 during a blackleg epidemic in the region of Setif (Algeria). The local strains were identified based on cultural and morphological characteristics, biochemical profiles using classic tests [3], API 20A and Rapid ID 32A strips (BioMérieux, Marcy l’Etoile, France), immunofluorescence [4] and PCR-restriction fragment length polymorphisms of 16S ribosomal RNA gene [5,6].

Strains have been stored in a lyophilized form in sealed vials and isolated pure cultures were maintained in liquid TGY medium in bottle of 50 mL at 4°C.

The strains were also propagated *in vivo* using guinea pigs (SPF, Hartley, Charles River, France) of 250 g to 300 g. A volume of 1 mL of a culture of 18 h on TGY medium was injected intramuscularly and the state of the animal was monitored for 48 hours. Blood samples from the heart, liver, leg muscle and sero-fibrinous exudate at the inoculation site were used to seed the TGY medium. After incubation, microscopic examination, isolation on Columbia Blood Agar, and pathogenicity and toxicity tests were performed.

**Medium and Culture Conditions**

Strains of *C. chauvoei* were cultivated under standard anaerobic conditions at 37°C on Columbia agar with 5% sheep blood (GBA), and in Trypticase Glucose Yeast (TGY) and CAM [7] liquid media.

Fermentation for vaccine production was carried out on CAM medium. First, a preculture of the strain was carried out with the same medium. A vial of lyophilized strain was inoculated into a fresh tube of TGY medium and incubated 18 h at 37°C. Then the TGY culture was inoculated into 750 mL of CAM medium in 1 liter capacity glass bottle and incubated at 37°C for 18 h. The anaerobic atmosphere was maintained by introducing nitrogen gas through a sterilizing filter. Controls were carried out at t=0 and t = 18 h.

Batch culture in laboratory fermenter was performed on 7 L of CAM medium inoculated with the preculture corresponding to 10% of total volume of the medium, at 37°C, pH 7.2, agitation of 100 rpm and with the introduction of nitrogen and antifoam agent (if necessary). Samples were taken at stationary phase and at 24 h, for the controls.

The checks carried out were as follows:

- **Morphology**: direct microscopic examination of cultures and after Gram staining.
- **Microbiological purity**: isolation on GBA and incubation in aerobic and anaerobic conditions.
- **Antigenic specificity**: slide agglutination reaction with *Clostridium chauvoei* anti-serum.
- **Microbial growth**: by measuring the OD at 650 nm and direct enumeration via culture on GBA and Thomas cell method.
- **Pathogenicity testing**
  - **Toxicity testing in vitro and in vivo**

**Pathogenicity and Toxicity Testing**

The *in vitro* toxicity of strains was estimated by the hemolytic activity of the supernatant of a culture at concentration of 2 to 4x10⁶ organisms mL⁻¹ [8]. The supernatant was recovered by centrifugation at 6,500 × g for 20 min at 4°C, filtered on 0.22 µm pore size Milipore and successively diluted by a factor of 2. A volume of 0.5 mL of each dilution was added to 0.5 mL of 2% sheep erythrocytes suspension in physiological saline, and then incubated 10 min at 70°C or 2 h at 37°C. Hemolysin titer was the highest dilution that produces 50% hemolysis. The 50% hemolysis was valued through a control consisting of the mixing of 0.25 mL of 2% sheep erythrocytes suspension (0% hemolysis), 0.25 mL of 2% sheep erythrocytes completely hemolized (100% hemolysis) and 0.5 mL of physiological saline.

The *in vivo* toxicity of the strains was evaluated by the lethal effect of the filtrate of the culture supernatant administered intravenously to five Balb/c mice and one guinea pig. The animals were observed for 3 days.

Pathogenicity of strains was assessed by intramuscular injection of a culture alone or added with CaCl₂·2H₂O (final concentration of 0.225 M) to 2 guinea pigs (250 g to 300 g) at a dose of 1 mL and to 5 mice (18 g to 20 g) at a dose of 0.5 mL [9,10]. The controls were the culture medium alone and added with CaCl₂·2H₂O.

**Anti-O and anti-H Immune Sera Preparation**

O and H antigenic suspensions were prepared, respectively, by heating to 100°C for 1 h and by treatment with formalin (0.6%) [11] of an 18 h culture on TGY at concentration of 5x10⁸ bacteria mL⁻¹. After centrifugation and washing, the pellet was resuspended in physiological...
saline. The immunization was performed on two New Zealand albino rabbits weighing 2.5 to 3 kg (Pasteur Institute of Algeria) for each suspension according to the method of Micalizzi and de Guzman [12]. The anti-H serum was adsorbed with the antigen suspension O. The sera were stored at -20°C. Titrations of sera were performed by an agglutination test tube with dilutions from 1/10 to 1/1280.

**Assessment of Passive Protection by Anti-H and Anti-O Immune Sera**

The passive protection assay was performed according to the method of Micalizzi and de Guzman [12]. Mice in groups of 5 were inoculated intraperitoneally with 0.5 mL of anti-serum and of non-immunized rabbit serum. After 6 h, the mice were challenged by IM way with 0.5 mL of a culture of 18 h on TGY of a virulent strain. The animals were observed for 5 days, the survivors are considered fully protected.

A non-immunized control group of mice was directly inoculated with virulent strain, death must occur within 30 h.

**Vaccine Formulation**

Pure stationary-phase culture [13] was inactivated with formalin (0.6% final concentration) at 37°C for 14 days with stabilization of pH at 7 and frequent agitation. The adjuvant, aluminium hydroxide gel (Al(OH))3, was added at the concentration of 6 mg mL⁻¹ and leaving the adsorption for few hours at temperature of 12°C to 20°C with slow shaking. Then the vaccine was kept four days at 4°C to settle organisms. The vaccine composition was then as follows: Clostridium chauvoei anaculture, adjuvant and formaldehyde.

Sterility testing was performed by seeding GBA incubated for 48 h and TGY, Thioglycolate, nutrient broth, trypticase soja and Sabouraud media incubated for 14 days.

According to British Pharmacopoeia [14], abnormal toxicity was tested by inoculating 2 guinea pigs and 5 mice monitored for 7 days. No abnormal local or systemic reaction occurs during the test. The pH was also controlled as well as free formaldehyde and Al³⁺.

**Assessment of Vaccine Active Protection**

Vaccine potency was tested according to the method of Mhoma [13]. The test was performed on 6 white guinea pigs weighing 250 to 300 g, of which 5 were inoculated subcutaneously with 2 mL of vaccine and the sixth control guinea pig was not vaccinated. A re-inoculation was made 21 days after. Ten days after the second dose, the animals were challenged with 1 mL of a virulent strain culture of 18 h. The animals were observed for 10 days.

All experiments were performed on triplicate, values given are averages.

Animal experiments were conducted in Animals Unit of Bacterial Vaccine Laboratory with the approval of authorities of Pasteur Institute of Algeria (reference: 02/ DLRD/IPA).

**RESULTS**

Strains cultured in batch on the CAM medium showed growth in rates of 0.85 h⁻¹, 0.84 h⁻¹, 0.5 h⁻¹ and 0.4 h⁻¹ for ALG2, CCM5735, ALG1 and ALG3, respectively. The stationary phase was reached at about 10 h for ALG2 and CCM5735 and at about 15 h for ALG1 and ALG3. Morphologically, the strain ALG2 had spindle, citron and rod shapes and CCM5735 strain showed spindle and rod shapes, with sporulation. The ALG1 strains and ALG3 had irregular shapes. The antigenic structure was uniformly maintained as indicated by the agglutination titer of 640 of a specific anti-serum anti-C. chauvoei observed for all strains. Mobility was very important for ALG2, medium for CCM5735 and absent in ALG1 and ALG3. Pathogenicity was present for all strains; however it was more pronounced for ALG2, followed by CCM5735 and ALG1, and then ALG3. In vivo toxicity was observed for ALG2 while it was absent for other strains. In vitro toxicity was present for all strains with a Minimal Hemolytic Dose of >522 (Table 1). The evaluation of titers of anti-H and anti-O immune sera prepared from strains ALG2 and CCM5735 gave a titer of 1280 for anti-H whatever the strain test, whereas anti-O had a titer of 1280 with homologous strain and 160 with heterologous strain (Table 2). Passive protection of mice with immune sera anti-O and anti-H showed that anti-H prepared with ALG2 protected against ALG2, CCM5735 and ALG1 (5 survivors/5), while anti-H obtained from CCM 5735 partially protected against ALG2 and ALG1 (4 survivors/5). Anti-O from ALG2 fully protected against ALG1 and ALG2 and partially against CCM5735 (3 survivors/5). Anti-O from CCM5735 completely protected against CCM5735, very weakly against ALG1 (1 survivors/5) and not against ALG2 (0 survivors/5) (Table 3). Full protection was observed in animals (5 survivors/5) against four strains (ALG2, CCM5735, ALG1 and C.IR) in guinea pigs vaccinated with the vaccine made from ALG2 strain (Table 4).

**DISCUSSION**

The comparative study of characteristics of growth, micromorphology, mobility, antigenicity, pathogenicity and toxicity of strains distinguished the strain ALG2 from the other local strains ALG1 and ALG3. Indeed, ALG2 had a greater growth rate (0.85 h⁻¹), equivalent to that of reference strain CCM57335 and the stationary phase was reached after 10 h of growth for the two strains. The cell structural integrity, appraised by cell shape, antigenicity and mobility, was conserved for ALG2 and CCM5735 after 10 h and 24 h of growth; while a pleomorphism and lack of mobility were observed for ALG1 and ALG3. The occurrence...
of non-motile variants is common in *C. chauvoei* \[16\].

Pathogenicity and toxicity were observed for ALG2, while pathogenicity was lower and *in vivo* toxicity was absent in other strains. The virulence character is variable in *C. chauvoei*. The ability to produce toxins varies greatly in *C. chauvoei* strains \[17\] and *in vivo* toxicity can be absent, despite the detection of the hemolytic activity in the filtrate of the culture supernatant \[11,18\]. Because of the importance of cell structural integrity, fitness and virulence in the immunogenicity and protective efficacy of a strain \[11-13,19\], strain ALG2 was selected for the formulation of a vaccine.

It is known that the protection is mainly provided by the structural antigens of bacteria, parietal somatic antigens O and mostly the flagellar antigens \[20\]. Indeed, flagella have been described as associated with full expression of virulence and immunoprotection against blackleg \[16,21,22\]. However, studies have reported the role of exotoxins and exo-enzymes in virulence and immunoprotection, hence the interest of introduce the culture supernatant in vaccine formulation \[23\].

### Table 1. Characteristics of growth, morphology, antigenicity, mobility, pathogenicity and toxicity of *C. chauvoei* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth Rate</th>
<th>Morphology</th>
<th>Antigenicity (Slide Agglutination Titer)</th>
<th>Mobility</th>
<th>Pathogenicity</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCM 5735</td>
<td>0.84 h⁻¹</td>
<td>T=10 h: spindles, rods T=24 h: sporulated-spindles, rods</td>
<td>+++ (640)</td>
<td>+</td>
<td>++ (4 dead mice/5)</td>
<td>- (Minimal Hemolytic Dose₅₀ &gt;522)</td>
</tr>
<tr>
<td>ALG1</td>
<td>0.5 h⁻¹</td>
<td>T=15 h and 24 h: irregular shapes</td>
<td>+++ (640)</td>
<td>-</td>
<td>++ (4/5)</td>
<td>- (MHD₅₀ &gt;522)</td>
</tr>
<tr>
<td>ALG2</td>
<td>0.85 h⁻¹</td>
<td>T=10 h: spindles, citrons, rods T=24 h: sporulated-spindles and citrons, some rods</td>
<td>+++ (640)</td>
<td>++</td>
<td>+++ (5/5)</td>
<td>+ (MHD₅₀ &gt;522)</td>
</tr>
<tr>
<td>ALG3</td>
<td>0.4h⁻¹</td>
<td>T=15 h and 24 h: irregular shapes</td>
<td>+++ (640)</td>
<td>-</td>
<td>+ (2/5)</td>
<td>- (MHD₅₀ &gt;522)</td>
</tr>
</tbody>
</table>

### Table 2. Titers of anti-H and anti-O immune-sera made from ALG2 and CCM5735 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Test Immune-Serum Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALG2 Strain</td>
</tr>
<tr>
<td></td>
<td>Anti-O</td>
</tr>
<tr>
<td>ALG2</td>
<td>1280</td>
</tr>
<tr>
<td>CCM5735</td>
<td>160</td>
</tr>
</tbody>
</table>

### Table 3. Passive protection conferred by O and H antisera obtained with ALG2 and CCM5735 strains

<table>
<thead>
<tr>
<th>Immune Serum</th>
<th>Number of Surviving Challenged Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALG2</td>
</tr>
<tr>
<td>Anti-O from ALG2</td>
<td>5/5</td>
</tr>
<tr>
<td>Anti-H from ALG2</td>
<td>5/5</td>
</tr>
<tr>
<td>Anti-O from CCM 5735</td>
<td>0/5</td>
</tr>
<tr>
<td>Anti-H from CCM 5735</td>
<td>4/5</td>
</tr>
<tr>
<td>Control serum from non immunized rabbit</td>
<td>0/5</td>
</tr>
<tr>
<td>Control: non-immunized mice challenged with virulent strain</td>
<td>0/5</td>
</tr>
</tbody>
</table>

### Table 4. Active protection by vaccine from local strain ALG2

<table>
<thead>
<tr>
<th>Challenge Virulent Strains</th>
<th>Responses of Guinea-Pigs to Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Guinea-Pigs Vaccinated by ALG2 Strain</td>
</tr>
<tr>
<td>ALG₁</td>
<td>5 survivors/5</td>
</tr>
<tr>
<td>ALG₂</td>
<td>5 survivors/5</td>
</tr>
<tr>
<td>CJR</td>
<td>5 survivors/5</td>
</tr>
<tr>
<td>CCM 5735</td>
<td>5 survivors/5</td>
</tr>
</tbody>
</table>
Active protection testing with the vaccine prepared from the ALG2 strain showed that all vaccinated guinea pigs challenged with 4 wild strains ALG1, ALG2, CCM5735 and C.I.R were survived; in contrast to unvaccinated animal controls that were died. These results highlighted the high and broader protective capacity of ALG2 strain. Because of the high variability of the protective capacity of vaccines according to strains used in vaccine formulation and the enzootic nature of blackleg, it is appropriate to use local strains in vaccine production; they have greater protector effect [12,13,20,24]. The observation is that the level of protection is high when the vaccine strain is homologous to local field strains [20].

We report in this study the immunogenic and protective capacity against blackleg disease of a strain of C. chauvoei ALG2 isolated in Algeria, it would be a good candidate for vaccine production. Assessment of vaccine potency should be performed in cattle.

ACKNOWLEDGEMENT

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REFERENCES