Kafkas Univ Vet Fak Derg
21 (6): 825-829, 2015
DOI: 10.9775/kvfd.2015.13616

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Study of Vaccinal Properties of *Clostridium chauvoei* Strains Isolated During a Blackleg Outbreak in Cattle in Algeria

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Article Code: KVFD-2015-13616 Received: 20.04.2015 Accepted: 25.06.2015 Published Online: 05.08.2015

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*

Cezayir'de Sığırlarda Yanıkara Salgınında İzole Edilen *Clostridium chauvoei* Suşlarının Aşısal Özelliklerinin Araştırılması

Özet

Bu çalışmanın amacı üç farklı *Clostridium chauvoei* Cezayir suşunun aşı özelliklerini araştırmaktır. Laboratuvar fermenterinde bir parça kültürü CAM medyumda yapıldı. Durağan-faz kültürün tümü formalinde inaktive edilere aşı üretildi. Suşların *in vitro* ve *in vivo* toksisiteleri, kültür spernatantlarının fare ve kobaylarda hemolitik aktivite ve letal etkileri ile değerlendirildi. Suşların patojeniteleri kültürün kobay ve farelere enjeksiyonu ile değerlendirildi. Pasif koruma deneyi anti-serum inoküle edilen ve virulent suş uygulanan fareler üzerinde yapıldı. Aşı kapasitesi, aşı inoküle edilen ve virulent suş uygulanan kobaylarda test edildi. ALG2 suşu daha büyük büyüme oranı (0.85 h^{-1}) ve yapısal özellikleri ile diğer suşlardan ayırt edildi. ALG2 için patojenite ve toksisite gözlemlendi. Diğer suşlar için patojenite daha düşük ve *in vivo* toksisite yoktu. Pasif koruma testleri ALG2 den elde edilen immun serum için daha geniş koruyucu etki gösterdi. ALG2 suşundan hazırlanan aşı ile yapılan aktif koruma testi, 5 saha suşu ile muamele edilen aşılı kobayların hayatta kaldıklarını gösterdi. Bu çalışma Cezayir'de izole edilen *C. chauvoei* ALG2 suşunun immunojenik ve koruyucu kapasitelerini incelemiş ve aşı üretimi için uygun bir aday olabileceğini göstermiştir.

Anahtar sözcükler: *Clostridium chauvoei, Yanıkara, Lokal suş, Aşı, İmmunkoruma*

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

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measure of control of this disease is vaccination; the formalin-killed whole bacterial culture is the commonly used [1]. The complex antigenic composition of *C. chauvoei* may be responsible for the variability in the protective effect of the vaccine according to strains used; some strains have a broader spectrum of protection than others [2]. Therefore, to ensure maximum effectiveness of a vaccine, it is recommended to make it from local strains [2]. The objective of this study was to test the vaccine properties of three Algerian local strains of *C. 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 C.IR 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 4×10^9 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 Millipore 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 hemolyzed (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 $CaCl_2.2H_2O$ (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 $CaCl_2.2H_2O$.

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 5×10^8 bacteria mL^{-1}. After centrifugation and washing, the pellet was resuspended in physiological

saline. The immunization was performed on two a 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 Al^{3+}.

Assessment of Vaccine Active Protection

Vaccine potency was tested according to the method of Mhoma [15]. 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 $Dose_{50}$ 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

Table 1. *Characteristics of growth, morphology, antigenicity , mobility, pathogenicity and toxicity of C. chauvoei strains*
Tablo 1. *C. chauvoei suşlarının büyüme, morfoloji, antijenik, hareketlilik, patojenite ve toksisite özellikleri*

Strain	Growth Rate	Bacterial Structural Integrity			Pathogenicity	Toxicity	
		Morphology	Antigenicity (Slide Agglutination Titer)	Mobility		*In-vivo*	*In-vitro*
CCM 5735	0.84 h^{-1}	T=10 h : spindles, rods T=24 h : sporulated-spindles, rods	+ ++ (640)	+	++ (4 dead mice/5)	-	+ (Minimal Hemolytic $Dose_{50}$: >522)
ALG1	0. 5 h^{-1}	T=15 h and 24 h : irregular shapes	+++ (640)	-	++ (4/5)	-	+ (MHD_{50} >522)
ALG2	0.85 h^{-1}	T=10 h spindles, citrons , rods T=24 h: sporulated-spindles and citrons, some rods	+++ (640)	++	+++ (5/5)	+	+ (MHD_{50} >522)
ALG3	0.4h^{-1}	T=15 h and 24 h : irregular shapes	+++ (640)	-	+ (2/5)	-	+ (MHD_{50} >522)

Table 2. *Titers of anti-H and anti-O immune-sera made from ALG2 and CCM5735 strains*
Tablo 2. *ALG2 ve CCM5735 suşlarından üretilen anti-H ve anti-O immune-sera titreleri*

Strain Test	Immune-Serum Titer			
	ALG2 Strain		CCM 5735 Strain	
	Anti-O	Anti-H	Anti-O	Anti-H
ALG2	1280	1280	160	1280
CCM5735	160	1280	1280	1280

Table 3. *Passive protection conferred by O and H antisera obtained with ALG2 and CCM5735 strains*
Tablo 3. *ALG2 ve CCM5735 suşları ile sağlanan O ve H antisera ile elde edilen pasif koruma*

Immune Serum	Number of Surviving Challenged Mice		
	ALG2	CCM 5735	ALG1
Anti-O from ALG2	5 /5	3/5	5/5
Anti-H from ALG2	5/5	5/5	5/5
Anti-O from CCM 5735	0/5	5/5	1/5
Anti-H from CCM 5735	4/5	5/5	4/5
Control serum from non immunized rabbit	0/5	0/5	1/5
Control: non-immunized mice challenged with virulent strain	0/5	0/5	0/5

Table 4. Active protection by vaccine from local strain ALG2
Tablo 4. Lokal suş ALG2'den üretilen aşı tarafından sağlanan aktif koruma

Challenge Virulent Strains	Responses of Guinea-Pigs to Challenge	
	Guinea-Pigs Vaccinated by ALG2 Strain	Guinea Pigs not Vaccinated
ALG_2	5 survivors/5	1 dead/1
ALG_1	5 survivors/5	1 dead/1
C.IR	5 survivors/5	1 dead/1
CCM 5735	5 survivors/5	1 dead/1

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].

In order to know the protective capacity of O and H antigens, anti-O and anti-H immune-sera were prepared from ALG2 strain and reference strain CCM5735.The results showed the same titers for anti-H and anti-O sera (1/1280) for the two strains. However, the titers of the anti-O varied according to the test strain, they were reduced to 1/160 when testing with heterologous strain; while the titers of anti H remained (1/1280) whatever the test strain. This result suggests a greater uniformity of H antigens among strains, unlike O antigens seems more variable, this finding is not consistent with results reported by Chandler and Gulasekharam [11]. Passive protection tests, performed on mice challenged with ALG2 or CCM5735, showed a protective capacity of both O and H antigens; however protection is more pronounced with H. Moreover, we noted the broader protective effect of immune sera from the strain ALG2 against both the strain ALG2 itself and the reference strain CCM5735.

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.IR 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 [25].

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

This work was performed with means of Pasteur Institute of Algeria and University of Sciences and Technology Houari Boumediene. We thank Prof. Georges Daube from Faculty of Veterinary Medicine of University of Liège (Belgium), for his help in molecular identification of *C. chauvoei* strains.

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