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CHARACTERIZATION OF A *Clostridium chauvoei* STRAIN, CANDIDATE AS A VACCINE STRAIN

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Abstract

Clostridium chauvoei is the etiological agent of blackleg, a severe disease of cattle and small ruminants, characterized by necro-hemorrhagic myositis and a superacute evolution. The superacute evolution of the disease does not allow antibiotic therapy to be effective; therefore vaccination remains the main prophylactic measure. The aim of the present study was characterize a C. chauvoei isolate, with the purpose of producing a vaccine against blackleg for small ruminants. The bacterial strain, isolated from a case of blackleg, was identified based on morphological, cultural, biochemical characteristics and PCR. The alpha toxin of the 12 hour anaerobically cultured strain provided a hemolytic titer of 1/1024, and the biological value assessed in vivo on Balb/C mice, calculated as lethal dose 50%, of 16 LD50. The inactivated whole-cell culture absorbed onto aluminum hydroxide was successful in protecting 100% of vaccinated guinea pigs, against death and clinical disease, in the challenge test. The results of the experiments recommend the isolated strain as a candidate for blackleg vaccine production.

Key words: *alpha toxin, blackleg, immunogenic, Clostridium chauvoei, vaccine.*

INTRODUCTION

Anaerobic pathology, determined by members of the Clostridium genus, is a major cause of economic loss, especially in small ruminant herds, where other pathogens, such as retroviruses and mycoplasmas, often evolve simultaneously (Enache et al., 2017; Turcu et al., 2010). Blackleg is a worldwide disease of cattle and small ruminants, severe and fatal, caused by *Clostridium chauvoei,* a spore-forming, Gram-positive, anaerobic bacterium. The rapid progression and high mortality of the disease cause major losses in livestock production (Groseth et al., 2011; Frey and Falquet, 2015). Cattle aged 6 to 24 months are more susceptible to blackleg, while in sheep, the disease can occur at all ages. *C. chauvoei* spores persist in the environment in the soil of pastures, manure and perished animals. Animals can become infected via the digestive or the respiratory tract, and in the case of small ruminants, infections can occur through skin lesions, caused by shearing or castration (Hatheway, 1990). The spores are transported to muscle tissue, where they remain dormant until anaerobic conditions are generated via tissue trauma, promoting the germination, multiplication and toxin production of the bacteria. The *C. chauvoei* toxins cause necrosis and edema, fever and lameness (Uzal, 2012; Frey and Falquet, 2015). The disease progresses rapidly and the death of the affected animal occurs within 48 hours of clinical onset. Due to the brief evolution of blackleg, generated mainly by bacterial toxins, antibiotic therapy is generally not effective; therefore, vaccination represents the main tool of controlling the disease (Rychener et al., 2017). Vaccination against blackleg has been documented since 1882, and remains the safest course of action for disease control (Useh et al., 2006). The aim of this study was to obtain an effective vaccine against blackleg, using a field *C. chauvoei* strain , isolated from a case of blackleg in sheep.

MATERIALS AND METHODS

Bacterial culture and toxicity assessment. The *C. chauvoei* strain was previously isolated from

a case of blackleg case in a traditional flock of sheep.

The strain was multiplied using a medium prepared based on: 1% liver meat glucose cysteine broth, 1% cooked meat medium broth, 1.5% *C. perfringens* spore broth, 1.5% casein yeast peptone, 0.7% liver hydrolysate, 0.5% yeast extract, 0.5% glucose, 0.5% L-cysteine, 0.05% fresh liver extract. Incubation was carried out 12 hours, at 37°C, 200 rpm, at a pH of 7.2 (± 0.2). Samples were collected during incubation and before inactivation for bio-molecular analysis and potency tests.

The toxin was obtained by centrifugation at 4000 rpm, for 30 minutes at 4°C, followed by filtration of supernatant through Millipore® filters of 0.8 µ 0.45 µ, 0.2 µ and finally 0.1 µ. The toxicity evaluation was performed on dilute samples (1/6, 1/8, 1/10) in peptone water. Each dilution was inoculated intravenously to Balb/C mice, 0.5 ml / mouse. The control group (5 Balb/C mice) was inoculated with sterile peptone water. All the animals were monitored during 72 hours post-inoculation.

Biochemical properties of the *C. chauvoei* strain. The biochemical characterization of the isolate was performed using conventional methods.

Nucleic acids and proteins extraction. The ZR Fungal/Bacterial DNA Miniprep Kit (Zymo Research) was used for genomic DNA extraction and two others - the Ambion™ TRIzol™ Plus RNA Purification Kit (Thermo Fischer Scientific) and Direct-zol Miniprep (Zymo Research) - for mRNA isolation and purification. The soluble proteins in the culture broth were concentrated at +4°C by ammonium sulfate precipitation (0.476% w/v) and centrifugation (20,000 g/1 h), and stored at –85°C in the 4× native buffer (40% (v/v) glycerol, 0.5 M Tris, pH 6.8).

Toxins genes detection. Amplification of the toxins sequences for *Clostridium* species were performed according to the literature (Table 1). Primers for highly conserved gene regions o C. chauvoei were designed using Vector NTI Advance 11 (Table 1). The GeneAmp® PCR 9600 system (Applied Biosystems) and Fast Start High/High Fidelity PCR System kits (Roche) were used for the amplification according to the manufacturer's recommendations, using 10 µm for each primer. The amplicons were visualized with ethidium bromide on agarose gels (Sigma-Aldrich), at appropriate concentration, and photographed with a ChemiDoc XRS+ imager (Bio-Rad Laboratories, Inc.).

Table 1. Primers and results of *Clostridium chauvoei* identification and gene expression detection

No.	Primers name	Primers sequence	Gene identification / toxotype (PCR)	Expression detection/ toxotype (RT-PCR)	Reference
1	cctACC-F	TCCATCAGGATTATCACGTGTTGG	α toxin – 687bp	α toxin – 687bp	This paper
2	cctACC-R	CCTGCATGCTCAACAGTATGGTTT			
3	ccfF	ATCGGAAACATGAGTGCTGC	flagellin C/fliC – 460bp	flagellin C/fliC – 460bp	
4	ccfR	AGTCTTTATGCTTCCGCTAG			
5	nanACC-F	ATCAGCAATAGATACATC	sialidase/nanA – 438bp	sialidase/nanA – 438bp	Vilei et al., 2011
6	nanACC-R	TGACCTCTTCCTGGTCCTGT			

Gene expression. An analysis of gene expression at the mRNA level was performed for the main virulence factors according to the literature (Table 1) using a GeneAmp® PCR 9600 system (Applied Biosystems) and Titan One RT-PCR system and Transcript One Step RT-PCR kit (Roche), as recommended by the manufacturer. The amplification products were visualized with ethidium bromide on agarose gels (Sigma-Aldrich), at appropriate concentration, and images were taken with a ChemiDoc XRS+ imager (Bio-Rad Laboratories, Inc.).

Hemolytic activity. Hemolytic activity was assessed of two fold serial dilution (up to 1:2048) of clostridial filtrate incubated sheep red cells (with PBS prewashed). The reaction was developed after 1h at +37ºC and 12 h at +4ºC. The experiments were performed in triplicates of 2 replicates.

Culture inactivation and vaccine formulation. The bacterial culture inactivation was performed when the pH showed no more variations of the preset value using formaldehyde (solution 37%), added up to the final concentration of 0.6%. The inactivation process took place over

the course of 7 days at 37°C. The addition of formaldehyde caused a pH drop of 1 unit. To check the efficacy of the inactivation process, 1 ml of the inactivated culture was inoculated i.m. to guinea pigs in 10 replicates.

The vaccine against blackleg was formulated using an aluminum-hydroxide based adjuvant. The pH was adjusted to 7.2 with sodium hydroxide solution, and the final product was tested for sterility by propagation on 10% sheep blood agar, incubated at 37°C, for 72 hours.

Vaccine efficacy test. To investigate the efficacy of the *C. chauvoei* bacterin vaccine, a challenge test was performed on guinea pigs. Each of the 10 guinea pigs was inoculated subcutaneously with 2 ml of the vaccine. After 14 days, the animals received a booster inoculation, using the same dose of vaccine. The 10 guinea pigs used as negative controls, were inoculated with sterile 0.9% saline solution, via the same route. The animals were monitored for 5 days after each inoculation, in order to note and record any systemic or local side effects. At 21 days after the booster inoculation, the vaccinated and the control animals were challenged with 0.5 ml i.m. of *C. chauvoei* live culture. The guinea pigs were monitored for 72 hours post inoculation.

RESULTS AND DISCUSSIONS

Based on PCRs results, it was confirmed that the *C. chauvoei* strain belonged to the known species or toxotype (Table 2).

Toxin expression was identified at the transcriptional level too (Figure 1, Table 2).

Table 2. Analysis results for *Clostridium chauvoei*

Toxin/ techniques	PCR	RT-PCR	Hemolytic activity
α toxin/ chauvoei	poz.	poz.	poz.
fliC/chauvoei	poz.	poz.	n.a.
nanA/ chauvoei	poz.	poz.	n.a.

Legend: poz. - positive signal; n.a. - not applied

Worldwide virulent *C. chauvoei* strains have the same toxigenic pattern represented by cytotoxin A, aggressive (Frey et al., 2012) and immunogenic (Nicholson et al., 2019). This *C. chauvoei* isolate is no exception. The expression of the main antigens of the isolate was highlighted at translational (Figure 1, Table 2) and functional levels (Table 3).

Hemolytic activity of the *C. chauvoei* isolate toxin reached the highest titer at a dilution of 1:1024 (Table 3).

The biochemical properties of the *C. chauvoei* isolate are summarized in Table 4.

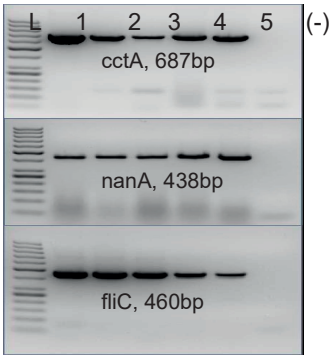

Figure 1. RT-PCR experiment/toxins expressions: *C. chauvoei:* 1 - 8 h 30' pi; 2 - 9 h 30' pi; 3 - 10 h 30' pi; 4 - 12 h pi; 5 - 13 h pi; RNA of strain was extracted from bacterial culture and amplified in order to detect specific genes expression. L - weight 50bp DNA ladder; numbers: - harvest time; (-) negative control

Table 3. Hemolytic activity of alpha toxin for *C. chauvoei* strain

Toxins dilution	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**1/2048**
C. chauvoei sample 1	+	+	+	+	+	+	+	+	+	+	-
C. chauvoei sample 2	+	+	+	+	+	+	+	+	+	+	-

Legend: +, red cells hemolysis; -, lack of hemolysis.

Table 4. Biochemical properties of the isolate

Glu	Fru	Gal	Mal	Lac	Inu	Sal	Man	Gli	Dex	Ind	Gel
+	+	+	+	+	-	-	-	-	-	-	+

Legend: GLU - glucose, Fru – fructose, Gal – galactose, Mal – maltose, , Lac-lactose, Inu – inulin, Sal – salicilin, Man- mannitol, Gli – glicerol, Dex – dextrose, Ind – indole formation, Gel - gelatin hydrolysis, + = positive result, - = negative result.

The toxicity test for the *C. chauvoei* isolate was performed on *BALB/c* male mice of 21-24 grams. The LD_{50} (lethal dose 50) was calculated for culture supernatant by a non-linear logistic regression with specific confidence intervals, depending on the minimal value necessary for the vaccine formulation. Each dilution was inoculated by caudal intravenous route 0.5 ml/mouse, in 5 replicates. The control group was represented by 5 mice inoculated with sterile peptone water. The animals were monitored for 72 hours after inoculation; moribund animals were humanely euthanized and considered as positive results (Table 5). The experiments were performed in duplicates. The biological value of the *C. chauvoei* toxins assessed *in vivo* was 16 LD_{50}.

Table 5. Toxicity tests design and results

Species/ Toxotypes	Toxin dilutions	Dead mice		
		24 h pi	48 h pi	72 h pi
C. chauvoei	1/6	5	-	-
	1/8	5	-	-
	1/10	**0**	**1**	**1**

Legend: numbers - media of duplicate experiments; bold letters - highest dilution inflicting death.

Culture inactivation tests demonstrated the efficacy of the formalin treatment. All 10 guinea pigs inoculated with the inactivated culture survived, and showed no local or systemic reactions following inoculation.

The efficacy test of the formulated blackleg vaccine was performed on guinea pigs. Post-vaccination side effects were limited to small local reactions at the site of inoculation, in the form of subcutaneous nodules, sized 0.2-0.4 cm. The animals in the control group showed no local or systemic reactions to the saline solution. All the animals in the vaccinated group survived the challenge with the virulent *C. chauvoei* culture, and none of the vaccinated animals developed clinical disease. Within the control group, all the guinea pigs died within 48 hours following the challenge. The results of the current study have demonstrated that the locally isolated strain of *C. chauvoei* can be an adequate candidate for vaccine production.

Anaerobes belonging to the *Clostridium* genus can cause numerous severe diseases, and their ability to sporulate cancels the eradication attempt. Clostridial bacterins are of high importance in livestock production, as immunization is the only option to avoid animal clostridiosis (Zaragoza et al., 2019). The selection, characterization and improvement of *Clostridium* strains are essential the development of an effective vaccine to control this burdensome pathology (Negru et al., 2021). Due to the complex antigenic structure of *C. chauvoei*, some researches recommend the use of local isolates to secure the vaccine's efficacy (Araujo et al., 2010). Other important factors are the use of appropriate culture media to ensure a positive impact on the antigenicity capacity of the *Clostridium* strain (Cortinas et al., 1994), and the stabilization / induction of *in vitro* multiplication capacity in large scale setting (Jabbari et al., 2012). Further research is required in order to determine the efficacy of *C. chauvoei* vaccines in protecting the target species against blackleg (Uzal, 2012).

CONCLUSIONS

A local *C. chauvoei* isolate was genetically and biochemically characterized, with the purpose of obtaining an effective vaccine against blackleg. Toxin potency value assessed *in vivo* for the *C. chauvoei* isolate was 16 LD_{50}. The alpha toxin of the isolate had a hemolytic titer of 1/1024. The vaccine prepared using the *C. chauvoei* strain was successful in protecting 100% of the vaccinated guinea pigs in the challenge test. The results of the experiments recommend the isolated strain as a candidate for blackleg vaccine production.

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