INCREASING THE IMMUNIZING VALUE OF A Clostridium septicum STRAIN

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Abstract
The pathology caused by bacteria belonging to the genus Clostridium is a major cause of economic loss in small ruminant herds. The clinical manifestations caused by these pathogens are the result of a wide and variable spectrum of toxins released during their multiplication. The aim of this study was to enhance the toxigenesis of one of the Clostridium septicum isolates and to select the most productive clone, being the closest to the field strain. The strain toxicity was improved by passages on guinea pigs. In vivo assays on balb/C mice were confirmed by in vitro experiments (Western Blott, semi quantitative dosing of cytopathic effect and quantification of hemolytic activity). Assessment of the immunizing value was carried out by seroneutralization in mice. The selected and improved strain produced a toxin with a biological value superior to the minimum recommended limits for the formulation of anaerobic vaccines for small ruminants.

Key words: α toxin, immunogenic, Clostridium septicum.

INTRODUCTION
The pathology caused by bacteria belonging to the genus Clostridium often associated with the specific pathology of the family Retroviridae, is a major cause of economic loss in small ruminants herds (Enache, 2017). Clostridium genus is a varied group of gram-positive anaerobic bacteria that produce thermosensitive endospores, critical criteria in epidemiology. Many members of this genus are equally pathogenic to humans and animals because of extracellular toxins. Clostridium septicum is recognized as a major contributor to clostridial infections (Kornbluth, 1989; Larson, 1995; Chew, 2001), respectively the primary aetiological agent of spontaneous gas gangrene or atraumatic myonecrosis (Hatheway, 1990; Abella, 2003). Major infection symptoms are represented by cyclic neutropenia, immunosuppression, diabetes mellitus and arteriosclerosis (Chew, 2001; Turcu, 2010; Stevens, 1990; Pelletier, 2000), plus a strong association (50% to 85%) between C. septicum infection and colorectal and hematological malignancy (Larson, 1995; Chew, 2001; Pelletier, 2000; Alpern, 1969; Koransky, 1979). Under such conditions the bacteria travel by the bloodstream and establish an infection (Abella, 2003), which is rapidly fulminating and often fatal, with mortality rates around 60% (Kornbluth, 1989; Larson, 1995; Abella, 2003). C. septicum produces four main toxins termed alpha, beta, gamma, and delta. Alpha toxin is the main protein involved in the pathological activities of C. septicum due to its biological properties (Popoff and Bouvet, 2009). The C. septicum lethal α-toxin is a necrotizing pore-forming cytolysin (Ballard, 1995). The toxin is secreted inactive and cleaved at an RGKR motif by host cell proteases in order to form the active monomers (Gordon, 1997). The C. septicum lethal α-toxin is a necrotizing pore-forming cytolysin (Ballard, 1995). The toxin is secreted inactive and cleaved at an RGKR motif by host cell proteases in order to form the active monomers (Gordon, 1997). The monomers bind to GPI-anchored proteins on the cell surface (Gordon, 1999), oligomerize into hexameric complexes generating 1.3-1.6 nm pores into the cell membrane (Sellman, 1997; Tweten, 2001) and inducing lytic and vacuolating effects (Ballard, 1992; Wichroski, 2002). The lethal effect of the α-toxin is tested
on mice (Tweten, 2001) and the cytotoxicity on CHO cells (Gordon, 1997). The aim of this study was to enhance the toxigenesis of a C. septicum isolate and to select the most productive clone, being the closest to the field strain. The strain toxicity was improved by passages on guinea pigs.

**MATERIALS AND METHODS**

The cultivation and toxicity evaluation of a C. septicum strain. The strain was cultured in anaerobic conditions, at 37°C, 200 rpm, at a pH of 7.5 (± 0.2). The culture medium was prepared after the following recipe: 1% Liver meat glucose cysteine broth, 1% cooked meat medium broth, 1.7% C. perfringens spore broth, 1.7% casein yeast peptone, 0.8% liver hydrolysate, 0.6% yeast extract, 0.7% liver extract, 0.6% glucose, 0.6% L-cysteine, 1% fresh liver extract. The toxin was obtained by the following protocol: culture centrifugation at 4000 rpm, for 30 minutes at 4°C, collection of supernatant and filtration through Millipore® filters of 0.8 µ, 0.45 µ, 0.2 µ and finally 0.1 µ. In order to test the alpha toxin titer, dilutions were prepared using peptone water. Each dilution was administered to a group of 5 Balb/C mice, intravenously, 0.5 ml/animal. All the animals in the control group survived, and showed no local or systemic reactions to the peptone water. The necropsy findings included hemorrhagic edema at the inoculation site, pink fluid in the peritoneal cavity, intestinal congestion, anemic lungs, necrotic spots on the liver and kidneys. Samples were collected from the liver, spleen, heart cavity and bone marrow of the guinea pigs. The samples were cultured anaerobically in VF culture medium. After 20 hours of incubation, the cultures were subcultured on 10% fresh sheep blood agar at 37°C for 24 hours in anaerobic conditions. After morphological and cultural examinations, colonies were selected and purified on blood agar plates.

### Biochemical identification

The biochemical properties of the bacterial isolate were determined using the API 20A test (Biomerieux). The API 20A strip included tests such as gelatinase, licitinase, lipase, nitrate, reductaze and amylase, and also motility, indol, sugar fermentation for glucose, sucrose, lactose, maltose, salicin mannitol and esculin. The identification was performed according to the producer’s instructions, with the help of the provided software.

### Cultivation of the C. septicum clone obtained by animal passage

The isolate was cultured in anaerobic conditions, at 37°C, 200 rpm, at a pH of 7.5 (± 0.2), for 20 hours. The culture medium was prepared after the following recipe: 1% Liver meat glucose cysteine broth, 1% cooked meat medium broth, 1.7% C. perfringens spore broth, 1.7% casein yeast peptone, 0.8% liver hydrolysate, 0.6% yeast extract, 0.7% liver extract, 0.6% glucose, 0.6% L-cysteine, 10% fresh liver extract.

### Nucleic acids and proteins extraction

The ZR Fungal/Bacterial DNA Miniprep Kit (Zymo Research) was used for genomic DNA extraction and others two - the Ambion™ TRIzol™ Plus RNA Purification Kit (Thermo Fischer Scientific) and Direct-zol Miniprep (Zymo Research) - for mRNA isolation and purification. Cellular membrane and soluble proteins were extracted on ice with the total lysis buffer (TLB: 62.5 mM Tris, pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 6 M urea, 0.01% (w/v) bromophenol blue, 0.01% (w/v)
phenol red, and 5% (v/v) beta-mercaptoethanol) from the bacterial pellet. The secreted proteins suspension was concentrated at +4°C by ammonium sulfate precipitation (0.476% w/v) and centrifugation (20,000 g/h), and stored at -85°C in the 4× native buffer (40% (v/v) glycerol, 0.5 M Tris, pH 6.8).

**Toxin genes detection**
Amplification of the representative toxins sequences for *Clostridium* species were performed according to the literature (Table 2). Primers for *C. septicum* genes were designed for conservative regions by Vector NTI Advance 11 (Table 2). A GeneAmp® PCR 9600 system (Applied Biosystems) and a Fast Start High/High Fidelity PCR System kits (Roche) were used for the amplification according to the manufacturer’s recommendations, using an universal concentration of 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 2. Primers and results of molecular *Clostridium septicum* identification and gene expression detection

<table>
<thead>
<tr>
<th>Nr. crt.</th>
<th>Primers name</th>
<th>Primers sequence</th>
<th>Gene identification/toxotype (PCR)</th>
<th>Expression detection/toxotype (RT-PCR)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. septicum:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>csa-F</td>
<td>CGGCAGTAGTACCACATGTACAA</td>
<td>α-toxin - 325bp</td>
<td>α-toxin - 325bp</td>
<td>This paper</td>
</tr>
<tr>
<td>2</td>
<td>csa-R</td>
<td>CGATACCCACCTTGATAAGGAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>hyaCS-F</td>
<td>ATGAAAGTGCAGTGCAAGAAGG</td>
<td>γ-toxin - 872bp</td>
<td>γ-toxin – 872bp</td>
<td></td>
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<tr>
<td>4</td>
<td>hyaCS-R</td>
<td>CCCTGCTGTAGCAAATATTGATCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>colCS-F</td>
<td>GAAAGGAGGTGCAACAGAAGG</td>
<td>collagenase A/colA - 2475bp</td>
<td>collagenase A/colA - 2475bp</td>
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</tr>
<tr>
<td>6</td>
<td>colCS-R</td>
<td>CGCTTTTTTCATCTTTCTCCA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Western-blot analysis**
After thermal (5 min, 95°C) and chemical (0.1% v/v beta-mercaptoethanol) denaturation, the total cellular and secreted proteins were separated by SDS-PAGE and blotted to a nitrocellulose membrane (Trans-Blot Semi-Dry Transfer Cell, Bio-Rad Laboratories, Inc.). Signal detection was based on a HRP system (Metal Enhanced DAB Substrate kit, Thermo Fischer Scientific).

**Hemolytic activity test**
Hemolytic activity was quantified by incubation of two fold serial dilution (up to 1:1024) of clostridial toxins incubated with PBS prewashed sheep red cells. The reaction was developed after 1h at +37°C and 12 h at +4°C. The experiments were performed in triplicates of 2 replicates.

**Cytopathic effect**
The cytotoxic effect of clostridial toxins was tested on *BHK-21* cells. Cells were split 1:4. The extracellular extracts were diluted with cell media (1:1000). Infected cells were incubated 21 h at 37°C into a 5% CO₂ atmosphere. The experiments were performed in duplicates of 2 replicates.

**Neutralization test in mice**
A 12 hours culture of the strain was filtered and then 0.5 ml of the obtained toxin was mixed with 0.2 ml nutrient broth and 0.1 ml specific antiserum. The serum toxin mixtures were kept at room temperature for 30 minutes and then 0.5 ml of the mixture was injected intravenously into a group of two mice per mixture. The inoculated mice were closely observed for 24 hours.

**The toxicity analysis of the *Clostridium* isolated strain**
The active extracellular proteins were prepared by culture centrifugation (4000 rpm, 30 minutes, 4°C), and supernatant filtration through a 0.8, 0.45, 0.22 and 0.1 μ Millipore filters. The potency of bacterial toxins was tested on *BALB/c* male mice of 21-24 grams.

**RESULTS AND DISCUSSIONS**
After 20 hours of incubation on blood agar, the *Clostridium septicum* culture appeared grayish, semi-translucent. The colonies were 2-3 mm in diameter, slightly raised, with markedly irregular to rhizoid margins (Figure 1).
Microscopic examination of the characterized C. septicum strain revealed long cylindrical bacilli, 0.8×3-5 μm or longer (Figure 2). The biochemical properties of the isolated strain are summarized in Table 3. Based on genes amplification, it was confirmed that the C. septicum strain belonged to the known species or toxotype (Table 4). Toxins expression was identified at transcriptional (Figure 3) or and translational level (Figure 4). The cytopathic effect of the C. septicum strain is shown in Figure 5 (21 h pi - 37°C, 5% CO2), compared to the non-infected cells (Figure 6) and the non-infected cell culture after 21 hours incubation (Figure 7). Hemolytic activity of the C. septicum isolate toxin reached the highest titer at a dilution of 1:512 (Table 5).

Figure 1. Colonies of Clostridium septicum after 20 hours on blood agar plate

Figure 2. Long Gram-positive cylindrical bacilli of Clostridium septicum after 20 hours on blood agar plate (1000x)

Figure 3. RT-PCR experiment/toxins expressions: Clostridium septicum-1 inoculum; 2 - 8 h 30’ pi; 3 - 9 h 30’ pi; 4 – 10 h 30’ pi; 5 - 12 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>
<thead>
<tr>
<th>INDOLE</th>
<th>UREASE</th>
<th>GLUCOSE</th>
<th>MANNOSE</th>
<th>LACTOSE</th>
<th>SACCHAROSE</th>
<th>XYLOSE</th>
<th>ARABINASE</th>
<th>GELATIN</th>
<th>ESCULENT</th>
<th>Glycerol</th>
<th>CELLOBIOSE</th>
<th>MANNITOL</th>
<th>MELEZITOSE</th>
<th>RAFFINOSE</th>
<th>SORBITOL</th>
<th>RHAMNose</th>
<th>TREHALose</th>
<th>CATALASE</th>
<th>MALTOSE</th>
<th>SALICIN</th>
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</table>


Table 3. Biochemical properties of the Clostridium septicum isolated strain

Table 4. Analysis results for the Clostridium septicum isolated strain

<table>
<thead>
<tr>
<th>Toxin/techniques</th>
<th>PCR</th>
<th>RT-PCR</th>
<th>WB</th>
<th>Hemolytic activity</th>
<th>Cytopathic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-toxin/septicum</td>
<td>poz.</td>
<td>poz.</td>
<td>poz.</td>
<td>poz.</td>
<td>poz (BHK-21 cells)</td>
</tr>
<tr>
<td>γ-toxin/septicum</td>
<td>poz.</td>
<td>poz.</td>
<td>poz.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>colA/septicum</td>
<td>poz.</td>
<td>poz.</td>
<td>poz.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Legend: uppercase letters – toxotypes; bold letters – strongest expression; poz. – positive signal; n.a. – not applied.
**Neuralatization test in mice**
Mice inoculated with mixtures of toxins and *C. septicum* antiserum survived, while the animals inoculated with similarly diluted toxin with nutrient broth, died within 24 hours post inoculation.

**Clostridium toxicity**
The potency of bacterial toxins was tested 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. 0.5 ml of each dilution was administered per mice in 5 replicates, by caudal intravenous route.

**Table 6. Toxicity tests design and results of the *Clostridium* isolated strain**

<table>
<thead>
<tr>
<th>Species/Toxotypes</th>
<th>Toxin dilutions</th>
<th>Dead mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. septicum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/400</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>1/500</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>1/600</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

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

By guinea pigs passage, we were able to create *Clostridium septicum* clone with a higher toxicity and virulence. The toxicity of the obtained strain was evaluated on Balb/C mice. The potency of the toxin produced by the revitalized strain was 50 times higher than that of the initial *Clostridium septicum* strain. The hemolytic activity of *Clostridium septicum* toxin after guinea pig passage increased from 1/32 to 1/512.

REFERENCES


