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Molecular characterization of virulence genes *cctA*, *nanA*, and *fliC* in *Clostridium chauvoei* from Rio Grande do Sul and São Paulo State, Brazil

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ABSTRACT: *Clostridium chauvoei toxin A (CctA), neuraminidase (NanA), and flagellin (FliC) proteins contribute to the pathogenicity of Clostridium chauvoei, the causative agent of blackleg in cattle. The aim of this study was to analyze the genetic variability of cctA, nanA, and* fliC genes *in C. chauvoei isolates from the Rio Grande do Sul and São Paulo state- Brazil, during different sampling periods. The presence of these genes was verified through PCR amplification and partial gene sequencing of 17 strains. Alignment of PCR amplicons combined with bioinformatics analysis was used in an attempt to study the variability across* C. chauvoei *solates. The similarity among the partial sequences of cctA and nanA* genes *was 100%. The sequencing of fliC revealed three different paralog alleles of flagellin, and two strains were seen to be polymorphic, with amino acid alterations in the predicted protein. Overall, this study indicates that strains of C. chauvoei isolated in Brazil are highly conserved with respect to the virulence factors evaluated.*
Key words: *blackleg, Clostridium chauvoei toxin A, neuraminidase, flagellin.*

Caracterização molecular dos genes de virulência *cctA*, *nanA* e *fliC* em *Clostridium chauvoei* isolados no Rio Grande do Sul e São Paulo, Brasil

RESUMO: *Toxina A de Clostridium chauvoei (CctA), neuraminidase (NanA) e flagelina (FliC) são proteínas que contribuem para a patogenicidade de Clostridium chauvoei, o agente causador do carbúnculo sintomático em bovinos. O objetivo deste estudo foi analisar a variabilidade genética dos genes cctA, nanA, e* fliC em *C. chauvoei isolados em diferentes períodos no Rio Grande do Sul e São Paulo. A presença destes genes foi verificada pela amplificação dos produtos da PCR e sequenciamento parcial dos genes de 17 cepas. Os alinhamentos da amplificação dos produtos da PCR combinados com a análise de bioinformática foram utilizados na tentativa de avaliar a variabilidade dos genes entre os isolados de C. chauvoei. A similaridade do sequenciamento parcial dos genes cctA e nanA* foi *100%. O sequenciamento do fliC revelou três alelos paralogos diferentes de flagelina e duas cepas mostraram polimorfismos, causando alterações na sequência de aminoácidos. As cepas de C. chauvoei isoladas no Brasil mostraram-se altamente conservadas em relação aos fatores de virulência avaliados neste estudo.*
Palavras-chave: *carbúnculo sintomático, Clostridium chauvoei toxina A, neuraminidase, flagelina.*

INTRODUCTION

Blackleg is a universal disease of cattle. It is an acute, endogenous/soil-borne infection caused by *Clostridium chauvoei*, a Gram positive, motile, histotoxic, and sporulating anaerobic bacterial species (USEH et al., 2006a). Current knowledge indicates that the toxins, DNAse, hyaluronidase, hemolysin, sialidase, and flagellar protein seem to play an important role in pathogenicity of *C. chauvoei* (FREY & FALQUET, 2014). At present, the only well characterized virulence factors of *C. chauvoei* are sialidase (HEUERMANN et al., 1991; USEH et al., 2006b; USEH et al., 2003; VILEI et al., 2011), *Clostridium chauvoei* toxin A (CctA) (FREY et al., 2012), and flagellin (KOJIMA et al., 2000; TAMURA et al., 1995; TANAKA et al., 1987).

The β-barrel protein porin, leukocidine CctA, has been shown to constitute the main virulence factor of *C. chauvoei*. It is likely that *C.*

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chauvoei is not able to cause disease without the *cctA* gene, which encodes the CctA protein. However, until now, the generation of a knockout mutant of *cctA* has not been reported. FREY et al. (2012) demonstrated that vaccination of guinea pigs with CctA, in the form of a fusion protein, protected animals against virulent *C. chauvoei* spores.

Neuraminidases or sialidases have been reported in viruses, bacteria, protozoa, fungi, and metazoans (VIMR et al., 2004), and they have been implicated in the pathogenesis of blackleg. These enzymes cleave N-acetylneuraminic acid from carbohydrate polymers, such as mucin, glycoproteins, gangliosides, and other sialoglycoconjugates located on many mammalian cell membranes (VILEI et al., 2011). VILEI et al. (2011) showed that antibodies produced against recombinant sialidase NanA of *C. chauvoei* neutralized the activity of the infectious agent to degrade sialic acid, indicating that NanA is a potential candidate antigen for the development of novel vaccines against blackleg.

Flagella of *C. chauvoei* drew particular attention towards characterization of this pathogen. Flagella are not directly involved in pathogenesis of blackleg (STEVENSON et al., 2015). However, they can contribute to the infectious process since they provide mobility to bacteria and enable them to reach the target tissue where the pathogen causes injury and disease. Moreover, flagellar antigens were studied as potential candidates for vaccines (KOJIMA et al., 2000; TAMURA & TANAKA, 1984; TAMURA & TANAKA, 1987). Flagella have also been reported to be important for immune response against other pathogenic bacteria, such as *Salmonella enterica* (KITAO & HATA, 2017), *Vibrio cholerae* (YANCEY et al., 1979), and *Pseudomonas aeruginosa* (MONTIE et al., 1987). In *C. chauvoei*, twenty genes involved in flagellar biosynthesis have been characterized (FREY & FALQUET, 2014). Flagellin is an immuno-dominant surface protein of the *C. chauvoei*, which may have a protective role as well as a diagnostic potential (USHARANI et al., 2015).

Full genome sequences of twenty strains of *C. chauvoei*, isolated from four different continents, over a period of sixty-four years, revealed that *C. chauvoei* genome is highly conserved in contrast to other *Clostridium* species (RYCHENER et al., 2017). Therefore, the aim of this study was to investigate genetic diversity of CctA (*cctA*), neuraminidase (*nanA*), and flagellin (*fliC*) across strains from different origins and periods of isolation in Brazil.

MATERIALS AND METHODS

Bacterial source, growth conditions and characterization

The seventeen *C. chauvoei* isolates used in this study were obtained from Brazilian blackleg cases from 2002 to 2016 (Table 1). In addition, we included the Brazilian reference strain (MT) used in official tests for control of blackleg vaccines, and the *C. chauvoei* type strain (ATCC 10092) from the American Type Culture Collection (ATCC). All strains were stored in Reinforced Clostridial Medium (Oxoid Microbiology products, Thermo Scientific, Waltham, MA, USA). Isolates were validated using polymerase chain reaction (PCR) amplification of the *fliC* gene according to the protocol proposed by SASAKI et al. (2002), and further validation was performed by 16S rRNA gene sequencing.

Immunoblot analysis of CctA

C. chauvoei strains were grown anaerobically in Brain Heart Infusion (BHI) broth (Oxoid Microbiology products, Thermo Scientific, Waltham, MA, USA) supplemented with 0.05% L-cysteine (Sigma-Aldrich chemical, St. Louis, MO, USA) at 37 °C for up to 72 hours to mid exponential growth phase. One milliliter aliquots of each culture were centrifuged at 10,000 x g for 30 min at 4 °C, and the supernatant and bacterial pellet were separated carefully. Supernatants (5 µL aliquots) from each *C. chauvoei* strain were mixed 1:1 (v/v) with SDS-PAGE loading buffer (65.8 mM Tris-HCl (pH 6.8), 26.3% (w/v) glycerol, 2.1% SDS, and 0.01% bromophenol blue) containing 5% β-mercapto-ethanol. Samples were separated by SDS-PAGE using 12% acrylamide gels. Proteins were electro-transferred onto polyvinylidene diflouride (PVDF) membranes (Millipore, IPVH10100) in transfer buffer (25 mM Tris, 192 mM glycine, and 20% (v/v) methanol). The membranes were blocked for 30 min using TBST (20 mM Tris, 137 mM NaCl, and 0.1% Tween-20 detergent (pH 7.6)) with 5% skimmed-milk powder, and then, probed overnight at 4 °C with rabbit anti-CctA antiserum (FREY *et al.*, 2012) diluted 1:1000 in TBST, followed by incubation for 2 h at room temperature with phosphatase-labelled goat anti-rabbit (KPL 4751-1516) diluted 1:5000 in TBST. Signals were detected by incubating the blot in fresh alkaline phosphatase substrate buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$) containing BCIP (Roche 11585029001) and NBT (Roche 10760994001) staining solutions.

Table 1 - Brazilian *Clostridium chauvoei* isolates from blackleg cases during 2002 to 2016 used in this study. SP: São Paulo, RS: Rio Grande do Sul. *Sequencing by Illumina HiSeq and Immunoblot analysis of CctA.

	Strain designation/Year	City (geographical coordinates)	State
1	2655/2003	Araçatuba (21º12'S/ 50º25'W)	SP
2	2828/2004*	Luiziânia (21º40'S/ 50º19'W)	SP
3	2974/2005*	Piracicaba (22º43'S/ 47º38'W)	SP
4	NF	Pelotas (31º46'S/ 51º20'W)	RS
5	SBP 07/2009*	São Martinho da Serra (29º32'S/ 53º51'W)	RS
6	SB 52/2011*	São Pedro do Sul (29º37'S/ 54º10'W)	RS
7	SB 65/2011	Santa Maria (29º41'S/ 53º48'W)	RS
8	SB 97/2011*	Jaguari (29º29'S/ 54º41'W)	RS
9	SB 105/2011	Jaguari (29º29'S/ 54º41'W)	RS
10	3270/2002*	Canguçu (31º23'S/ 52º40'W)	RS
11	SB 87/2013 286*	Toropi (29º28'S/ 54º13'W)	RS
12	SB 87/2013 285	Toropi (29º28'S/ 54º13'W)	RS
13	SB 131/2013	São Vicente do Sul (29º41'S/54º40'W)	RS
14	SB 46/2014	Jaguari (29º29'S/ 54º41'W)	RS
15	SBP 17/2015	Jaguari (29º29'S/ 54º41'W)	RS
16	SBP 43/2015*	Jaguari (29º29'S/ 54º41'W)	RS
17	SBP 59/2016	Ijuí (28º23'S/ 53º54'W)	RS

DNA extraction, PCR amplification, sequencing, and nucleotide analysis

Bacterial DNA was extracted according to the protocol proposed by TAKEUCHI et al. (1997). Pairs of primers were designed in the Primer-BLAST (NCBI, USA), targeting the specific region of the leukocidin toxin gene (*cctA)* and the neuraminidase gene (*nanA)* of *C. chauvoei*. For the amplification of the *fliC* gene, the primers were adapted from USHARANI et al. (2015) (Table 2).

DNA amplification using PCR was performed in a reaction volume of 25 µL consisting of 5 µL buffer 10X + mM magnesium chloride, 20 mM of each dNTP (Ludwig Biotec, Brazil), 1.25 U Taq DNA polymerase (Promega, USA), 10 mmol of each primer, ultrapure distilled water qsp (Invitrogen, USA), and 1 µL DNA. PCR was carried out in a Veriti 384-well thermal cycler (Applied Biosystems, USA). The cycling conditions used were as follows: initial denaturation of 94 ºC for 5 minutes; followed by 35 cycles of 95 °C for 50 s; 61 °C for 50 s (*cctA*), 62 °C for 50 s (*nanA*) or 68 °C for 50 s (*fliC*); and 72 ºC for 7 minutes. Final extension was carried out at 72 ºC for 4 min.

PCR products were sequenced by Sanger sequencing (ACTGene Molecular Analysis LTDA, Biotechnology Center, UFRGS, Porto Alegre, RS, Brazil). In addition, flagellin reads of eight strains (Table 1) were sequenced by Illumina HiSeq (Fasteris SA, Plan-les-Ouates, Switzerland), according to the

Table 2 - Forward (F) and reverse (R) primers used for sequencing of genes of *Clostridium chauvoei* strains (n=17).

Primer	Sequence 5' → 3'	Bp	Temperature	Reference
cctA-F cctA-R	TGCTTGCTTTAGCAACAACAACT GGATGCGTCAACAATTTCTCA	1120	61 ºC	JQ728486* Strain JF3703/1956
nanA-F nanA-R	TCTTTGGCATACACCGTGGG CATCCCAAGTTACCCCACCA	1100	62 ºC	FM213082* Strain JF4135/2004
fliC-F fliC-R	AGCTAACGATACAAACGTAG GCTGAATTTATAGTCTTTATGC	708	58 ºC	Adapted from (USHARANI et al., 2015)

*GenBank Database access number.

protocol described by RYCHENER et al. (2017) were used. The DNA sequences, including strain JF3703 (GenBank Database access number JQ728486), were analyzed by Geneious software version 7.1.5 (KEARSE et al., 2012).

RESULTS AND DISCUSSION

The specific fragments of the genes *cctA*, *nanA*, and *fliC* were amplified from all the strains. The identity of the *cctA* gene sequences from the 17 Brazilian *C. chauvoei* strains, the reference strain, MT, and the type strain ATCC 10092, was 100%. Minor differences were reported in the strain JF3703, a strain, which was isolated in 1956 in New Zealand (99.62% identity) (RYCHENER et al., 2017). In addition, the *nanA* gene sequences were 100% identical among the Brazilian strains, as well as the reference strains, MT and ATCC 10092. Minor differences were reported when the *nanA* gene sequences were compared to the New Zealand strain, JF3703; the sequence identity was 96.50%.

CctA, the major virulence factor of *C. chauvoei*, belongs to the leucocidin superfamily of bacterial toxins and is not reported in any other clostridia species (FREY et al., 2012). In the present study, no genetic variability was reported for the 17 Brazilian *C. chauvoei* strains investigated. FREY et al. (2012) reported that the strain JF3703/1956 showed a slightly different allele of *cctA*, which does not affect the predicted protein sequence (RYCHENER et al., 2017). This high degree of identity among strains suggested that *cctA* gene is a potential candidate that can be employed to identify *C. chauvoei* by PCR in clinical veterinary microbiology laboratories.

Immunoblot analysis of culture supernatants from eight Brazilian *C. chauvoei* strains revealed that CctA is expressed and secreted by all of the strains (Figure 1) at relatively equal amounts. No reactions with anti-CctA serum were observed on immunoblots containing the culture pellet material (not shown). These results indicated that all strains tested secrete most or all of the expressed CctA toxin.

Sialidases are considered virulence factors for several pathogenic organisms. The sialidase activity of the *C. chauvoei*, NanA, contains a CBM40 module that specifically binds sialic acid residues that has been previously reported for NanJ of *C. perfringens* (BORASTON et al., 2007; ROTHE et al., 1991; USEH et al., 2006a; USEH et al., 2003). The region amplified by the *nanA* primers used in this study consisted primarily of CBM40 domain. All strains showed a conserved DNA sequence revealing an identity of 100%, except for the strain JF3703 for which the observed differences were located in this domain (RYCHENER et al., 2017). Sialidase activity of *C. perfringens* type A has previously been detected by LLANCO et al. (2014), where the *nanH,*

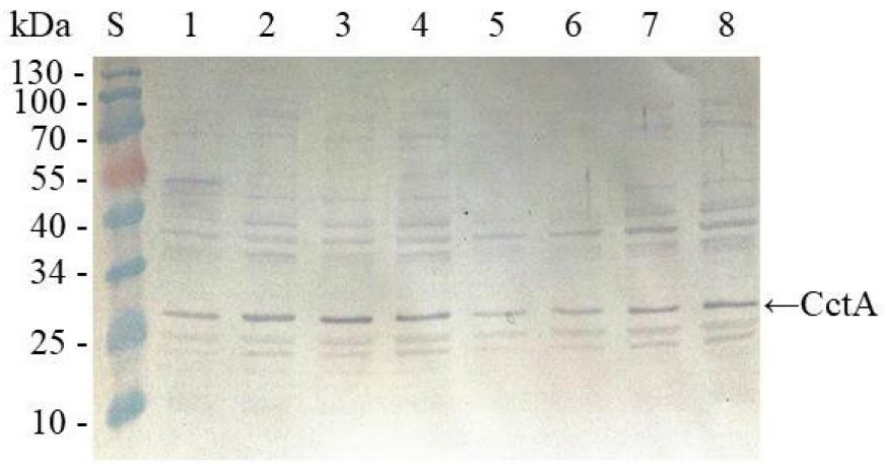

Figure 1 - Expression of CctA analyzed on immunoblots of culture supernatants from eight Brazilian *Clostridium chauvoei* strains. Minor bands can be observed in the background, which are also present in pre-immunization rabbit serum. S: molecular mass standard as indicated on the left; 1 - 8: Brazilian *C. chauvoei* strains: 1: 2828/2004; 2: 2974/2004; 3: SBP 07/2009; 4: SB 52/2011; 5: SB 97/2011; 6: 3270/2002; 7: SB 87/2013 286; and 8: SBP 43/2015.

nanI, and *nanJ* genes were investigated and different patterns were observed, indicating heterogeneity of the microorganism. However, *C. chauvoei***,** unlike a bacterial enteric pathogen, is highly specialized. The conserved gene sequences might reflect the restricted environment for survival of this pathogen and its adaptation to limited number of hosts (cattle, sheep, and goats) (FREY & FALQUET, 2014; RYCHENER et al., 2017).

The multiplex PCR protocol described by SASAKI et al. (2002) has been widely used for the identification and differentiation of *C. chauvoei*, *C. haemolyticum*, *C. novyi* types A and B, and *C. septicum*. Primers for *C. chauvoei* contain a conserved initial region of the flagellin gene, *fliC*. SASAKI et al. (2002) demonstrated that these clostridia have at least two copies of the flagellin gene (*fliC*) arranged in tandem (*fliA* and *fliB*). The deduced N and C terminal amino acid sequences of their FliC proteins are well conserved, but their central amino acid sequence is not. *Clostridioides difficile* strains also exhibit conservation in the N and C termini of the FliC protein, whereas their central regions are less conserved (TASTEYRE et al., 2000). RYCHENER et al. (2017), using the full genome Illumina HiSeq sequencing, observed three putative genes, *fliC1*, *fliC2*, and *fliC3*, encoding three different paralog genes. THOMAS et al. (2017) through single molecule real-time DNA (SMRT DNA) sequencing, also observed different copy number variations of these genes among the two strains studied.

The primer used for amplification of *fliC* was selected because USHARANI et al. (2015) reported that central region of the flagellin gene is unique for *C. chauvoei*. However, sequencing of PCR products for the *fliC* gene showed triple peaks for some sequences among all the seventeen *C. chauvoei* strains used in this study, suggesting that this primer anneals in a region of the flagellin that amplifies three alleles not reported by USHARANI et al. (2015). For this reason, eight strains were subjected to Illumina HiSeq sequencing. The sequences deposited on GenBank by USHARANI et al. (2015) are identical to the FliC sequence reported by KOJIMA et al. (2000) and FliA sequence reported by SASAKI et al. (2002).

The complete genome sequencing of the eight strains by Illumina HiSeq revealed three different paralog alleles of the flagellin (*fliC1, fliC2* and *fliC3*). Six out of the eight *C. chauvoei* strains contained the same three alleles as those observed for the strains JF 4335 (RYCHENER et al., 2017), 12S0467, and ATCC 10092 (THOMAS et al., 2017). The strain 2828 (isolated in 2004 in São Paulo state) showed a different allele of *fliC3* with one non-synonymous SNP (position 729), resulting in substitution of one aspartic acid to glutamic acid (Figure 2). Strain SB 52 (isolated in 2011 in Rio Grande do Sul state) showed two SNPs, one synonymous (GGT_{Gly} instead of GGC_{Gly}) and other non-synonymous, resulting in substitution of isoleucine for valine (Figure 2), both in *fliC3* allele. The central variable region of *fliC* is not easily resolved by sequencing techniques employing short reads, such as reversible terminator-based sequencing (Illumina). However, there was no assembly error because the sequences obtained by THOMAS et al. (2017) demonstrated 100% identity with most of those obtained by the Illumina sequencing.

Flagellar activity is characterized by motility; it is inherent in the wild-type strain and is not just a peculiarity of the original variants (TAMURA et al., 1995). Polymorphisms in flagellin genes might

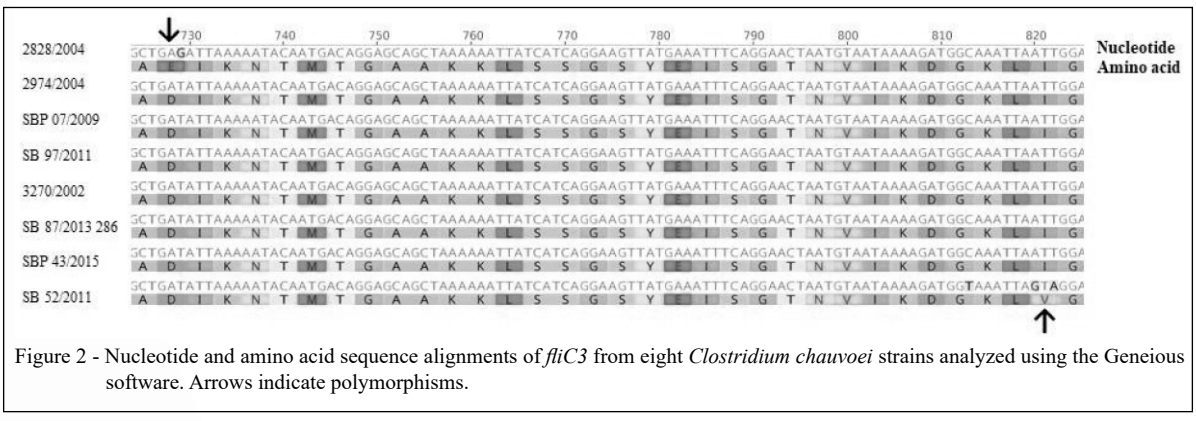

Figure 2 - Nucleotide and amino acid sequence alignments of *fliC3* from eight *Clostridium chauvoei* strains analyzed using the Geneious software. Arrows indicate polymorphisms.

alter the proteins encoded by them and influence flagellar activity that could be related to bacterial virulence (KOJIMA et al., 1999, 2000). A poor protective immunity was induced by the recombinant flagellin in mice, suggesting that a conformation-dependent epitope may play an important role in the development of immunity against blackleg (KOJIMA et al., 2000). Alternatively, antibodies against flagellin alone might not be sufficient to induce protective immunity as neutralizing antibodies against the main toxin.

A study by TAYASTERE et al. (2000) indicated that non-flagellated *C. difficile* serotypes retain the transcription of *fliC* genes, but the protein products have remained undetected. In *C. difficile*, the flagella may be important for colonization and adherence, but not sufficient to induce protective immunity (STEVENSON et al., 2015). As the pathogenesis of blackleg caused by *C. chauvoei* infection and of colitis caused by *C. difficile* infections are very different, the true involvement of the flagellum in the pathogenesis of blackleg, as well as how the transcription of the copies of *fliC* is regulated in *C. chauvoei* needs to be unraveled to elucidate their role in pathogenesis.

CONCLUSION

Overall, the virulence genes *cctA* and *nanA* were highly conserved among all Brazilian strains studied as well as the Brazilian reference strain MT and the type strain ATCC 10092. Due to the conservation of CctA and NanA among these strains, these soluble antigens are good candidates for the design and quality control of *C. chauvoei* vaccines. Moreover, genetic similarity might indicate that, currently, *C. chauvoei* is not being challenged to develop mutations. However, three different *fliC* alleles were detected among the strains studied, and they were related to different serotypes of *C. chauvoei*. Their role in infection and in inducing protective immunity remains to be elucidated.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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