African Journal of Microbiology Research Vol. 6(8), pp. 1685-1689, 29 February, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.886
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Sequencing and phylogenetic analysis of C*lostridium septicum* alpha toxin gene from Brazilian field and vaccine strains

Ronnie Antunes de Assis[1], Antonio Augusto Fonseca Júnior[1], Franciele Maboni Siqueira[2]*, Marcelo Fernandes Camargos[1], Felipe Masiero Salvarani[3], Prhiscylla Sadanã Pires[3], Rodrigo Otávio Silveira Silva[3], Ricardo Aurélio Pinto Nascimento[1], Agueda Palmira Castagna de Vargas[4] and Francisco Carlos Faria Lobato[3]

Laboratório Nacional Agropecuário de Minas Gerais, LANAGRO-MG. Av. Rômulo Joviano s/n, CP 35/50, CEP 33600-000, Pedro Leopoldo, MG, Brasil.
[2]Programa de Pós-Graduação em Bioquímica, Centro de Biotecnologia- Universidade Federal do Rio Grande do Sul. Av. Bento Gonçalves, 9500, prédio 4342, setor IV. CP 15005. CEP 91501970, Porto Alegre, RS, Brasil.
[3]Escola de Veterinária da Universidade Federal de Minas Gerais, Depto de Medicina Veterinária Preventiva. Av. Antônio Carlos 6627, CP 567, Campus da UFMG, CEP 30123-970. Belo Horizonte, MG, Brasil.
[4]Universidade Federal de Santa Maria, Laboratório de Bacteriologia, Depto de Medicina Veterinária Preventiva. Centro de Ciências Rurais. Prédio 44, sala 5137, CEP 97105-900, Santa Maria, RS, Brasil.

Accepted 8 February, 2012

In this study, sequencing and phylogenetic analysis of partial sequences of the gene that encodes alpha toxin from field and vaccine strains of *Clostridium septicum* from Brazil were performed to analyze genetic differences in these strains. Alpha toxin nucleotide and amino acid sequences from five field strains of *C. septicum* and four strains used in the production of vaccines against clostridiosis were compared. Based on this sequence analysis, the *C. septicum* strains can be classified in two groups. Group 1 presents the majority of the sequences; including two vaccine strains and is very conserved. Group 2 presents sequences with significative number of changes in the nucleotide and amino acid sequences, and contains the other two vaccine strains used in the production of vaccines in Brazil. Therefore, genetic diversity occurs in Brazilian *C. septicum* strains.

Key words: *Clostridium septicum*, alpha toxin, sequencing, phylogenetic analysis.

INTRODUCTION

Clostridium septicum is a microorganism capable of initiating the gas gangrene, a high lethal exogenous infection which occurs in ruminants and other animal species, attacking mainly muscles and subcutaneous tissues. For this infectious disease *C. septicum* can act alone or together with other agents, such as *Clostridium chauvoei, Clostridium sordellii, Clostridium novyi* type A and *Clostridium perfringens* type A.

Alpha toxin, which is necrotizing, hemolytic, and leukocidic, is a prime virulence factor for *C. septicum* and accounts for its extreme pathogenicity (Tweten, 2001; Hunley et al., 2008). Alpha toxin might have a primary cytotoxic effect on the vascular endothelium which could result in the loss of fluid from the circulatory system and may induce subsequent shock (Tweten, 2001). The resulting tissue destruction leads to clinical aspects characteristic of clostridial myonecrosis: pain, frequently out of proportion to physical findings, swelling, and blister formation progressing to serosanguinous bullae. Crepitus due to local gas

*Correspondig author. E-mail: franmaboni@gmail.com. Tel: +55 51 3308 6077. Fax: +55 51 3308 7009.

Table 1. Field isolates and vaccine strains of *C. septicum* used for sequencing.

Identification	Species	Age	Material to isolation	State/Country
19748	Bovine	[a]NS	Liver	Guaratinguetá-SP/Brazil
[c]16	[a]NS	[a]NS	[a]NS	[a]NS/ Brazil
421/355/98	Bovine	10 months	Bone marrow	Sto. Antônio de Pádua-RJ/ Brazil
421/701/97	Bovine	18 months	Bone marrow	Aperibé-RJ/ Brazil
421/250P/90	Bovine	5 months	Bone marrow	Itaperuna-RJ/ Brazil
[b]L-1	[a]NS	[a]NS	[a]NS	Brazil
[b]L-2	[a]NS	[a]NS	[a]NS	Brazil
[b]L-3	[a]NS	[a]NS	[a]NS	Brazil
[b]L-4	[a]NS	[a]NS	[a]NS	Brazil

[a] NS- no shown. [b] *Clostridium septicum* vaccine strains proceeding of four Brazilian laboratories. [c] Isolate gave by Instituto Biológico de São Paulo (IBSP).

formation may be felt (Hunley et al., 2008).

C. septicum produces four toxins called alpha (hemolytic, necrotic and lethal), beta (Dnase), gamma (hyaluronidase) and delta (Lobato et al., 2007), being alpha toxin the most important for pathogenicity, and according to some authors, for immunogenicity as well (Ballard et al., 1992; Ballard et al., 1993; Amimoto et al., 2002). The alpha toxin is a pore-forming toxin, member of the aerolysin family. After binding to a cellular receptor, the toxin is activated and oligomerize, originating a pre-pore complex before of its membrane insertion, with consequent osmotic lysis (Melton et al., 2004). The protein is constituted by three domains, being domains I and II responsible for binding to the receptor, while domain III contains the sites for activation and regulation of oligomerization (Melton et al., 2006).

The aim of this work was to perform the sequencing and phylogenetic analyses of the gene encoding alpha toxin from Brazilian field and vaccine strains of *C. septicum*, currently commercialized in this same country in order to verify the relation between these isolates and possible implications of specific mutations on the protein structure.

MATERIALS AND METHODS

Nine brazilian *C. septicum* isolates, being five isolated from field samples and four from vaccine strains were used in this work. The characteristics of the strains are shown in Table 1. The isolates and vaccine strains were identified by direct immunofluorescence (DFA) (Assis et al., 2001). The strains were cultivated in brain heart infusion (Difco, Maryland, EUA) and incubated at 37°C in anaerobiosis for 48 h. Behind the growth and purity verification by Gram stain, isolates were again cultivated in 15 ml of the same broth medium and incubated at the same conditions above described. For DNA genomic extraction the cells were centrifuged and the pellet was resuspended in 1.5 ml of TES buffer (10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl) containing 25% sucrose and 2 mg/ml lysozyme and incubated at 37°C for 1 h. Then, 150 µl of 10% SDS solution was added to the suspension and it was incubated at 37°C for 10 min. The lysed cell suspension was diluted with 3 ml of TES buffer before being extracted twice with phenol and once with phenol/chloroform/isoamyl-alcohol (50/48/2) and finally precipitated with ethanol. Deoxyribonucleic acid (DNA) was resuspended in 100 µl of TE buffer and used as template for polymerase chain reaction (PCR) amplification (Takeuchi et al., 1997).

The primers described by Takeuchi et al. (1997) for specific amplification of *C. septicum* alpha toxin gene were used in the present work. These primers amplify a specific product of 270 bp corresponding to positions from 611 to 880 of the nucleotide sequence, which encodes *loop* 1 of domain I of alpha toxin. This region is a binding site for the cellular receptor and, hence, essential for the lethal action of the protein (Melton et al., 2006).

The PCR was performed according to Takeuchi et al. (1997) with some modifications. The amount of DNA was 300 ng, and of dNTPs was 0.25 mM, with an initial denaturation cycle at 94°C for 5 min and a final extension cycle at 72°C for 7 min. *C. septicum* ATCC 12464 genomic DNA was used as a positive control, while the negative control was a PCR reaction without DNA. After amplification, products were subjected to electrophoresis on 1% (w/v) agarose gel stained with ethidium bromide (0.5 µg/mL).

The PCR products were purified by using the Wizard SV Gel and PCR Clean-Up System Kit (Promega) following producer recommendations, and sent for sequencing at Bioagro (UFV, Minas Gerais, and Brazil). Two sequencing reactions were made for every isolate, each one with a forward or a reverse primer. The sequences of this study were aligned, with alpha toxin gene sequences deposited in GenBank with access numbers: D17668, AB083433, AB083434, AB083435, AB083436, AB083437, AB083438, AB083439 and S75954 (Table 2), and compared to the consensus sequence for nucleotide analysis.

The phylogenetic analysis was performed based on the nucleotide alignments, by MEGA version 4.0 (Tamura et al., 2007). The Kimura 2-parameter model of nucleotide substitution and the neighbor joining tree construction method (Saitou and Nei, 1987) were applied in this work. The *Shewanella baltica* YP 001049948.1 and *Hahella chejuensis* YP 434730.1 sequences were used as outgroup to the tree.

The predicted amino acid sequences were subjected to other *in silico* analyses used to evaluate the substitutions and possible consequences. The Selecton software was utilized in the analysis of positive pressure to compare its influence in the protein differentiation (Stern et al., 2007). The hydrophilic profile was determined by Hopp and Woods (1981) method, using two stages to functional site detection and consequence of amino acid changes. Sequences were examined using ConSeq(Berezin et al., 2004) to verify the important residues in aerolysin family.

Table 2. The nucleotide sequences of *C. septicum* alpha toxin gene available in GenBank.

Isolate	*GenBank* access number	Country	Sequence size (nt)	Reference
NCTC547	D17668	Japan	2293	Imagawa et al. (1994)
Cellular lineage: 44	AB083433	Japan	1333	Amimoto et al. (1994)
Cellular lineage: Kagoshima 8	AB083434	Japan	1333	Amimoto et al. (1994)
Cellular lineage: Mie	AB083435	Japan	1333	Amimoto et al. (1994)
Cellular lineage: Yamaguchi 6335	AB083436	Japan	1333	Amimoto et al. (1994)
Cellular lineage: Kagoshima 1	AB083437	Japan	1333	Amimoto et al. (1994)
Cellular lineage: Fukushima 5	AB083438	Japan	1333	Amimoto et al. (1994)
Cellular lineage: Tokachi	AB083439	Japan	1333	Amimoto et al. (1994)
BX96	S75954	USA	1542	Ballard et al. (1992)

Table 3. Number and type of nucleotide (nt) and amino acid (aa) substitutions in sequences of *C. septicum* alpha toxin gene in relation to consensus.

Sequences	Number of substitutions (%)		Number of silent substitutions (%)	Nucletide substitution type	
	nt	aa		Transitions	Transversions
D17668	0	0	0	0	0
S75954	0	0	0	0	0
AB043435	0	0	0	0	0
AB043437	0	0	0	0	0
AB043438	0	0	0	0	0
AB043439	0	0	0	0	0
L-1	0	0	0	0	0
L-3	0	0	0	0	0
16	0	0	0	0	0
19748	0	0	0	0	0
Itaperuna	0	0	0	0	0
AB083433	18 (7,3)	8 (9,8)	5 (27,7)	12	6
AB083434	17 (6,9)	8 (9,8)	4 (23,5)	11	6
AB083436	1 (0,4)	1 (1,2)	0	1	0
Aperibé	1 (0,4)	1 (1,2)	0	1	0
L-2	16 (6,5)	8 (9,8)	4 (25,0)	11	5
L-4	16 (6,5)	7 (8,5)	5 (31,2)	10	6
S. Antônio	13 (5,3)	6 (7,3)	3 (23,1)	7	6

RESULTS AND DISCUSSION

No deletions or insertions were observed in the analyzed sequences. Among the nucleotide substitutions, 74.4% were non silent. The majority of nucleotide substitutions occurred by transition (64.6%) (Table 3) in agreement with the results of Fitch (1986). The characteristics of nucleotide and amino acid substitutions in sequences of *C. septicum* alpha toxin gene, in relation to consensus, are presented in Table 3.

According to the dendrogram obtained from phylogenetic analysis of *C. septicum* sequences (Figure 1), there were two phylogenetically distinct groups (1 and 2). Group 1 included most of the available sequences taken from field isolates, and was highly conserved. Group 2 presented sequences with a significative number of substitutions. This low similarity and formation of few groups phylogenetic has been reported in others Clostridium studies. For instance, the *Clostridium botulinum* type C avian strains analyze that found only three phylogenetic groups by randomly amplified polymorphic DNA analysis (RAPD) and pulsed-field gel electrophoresis (PFGE) (Skarin et al., 2010). Analyses of complete coding regions of *C. botulinum* toxins demonstrated a high degree of similarity between them (Fang et al., 2010).

Regarding the field isolates, from Rio de Janeiro state, they were distributed into both groups, suggesting the existence of genotypic variability between isolates from this state. The isolates 19748, from São Paulo State,

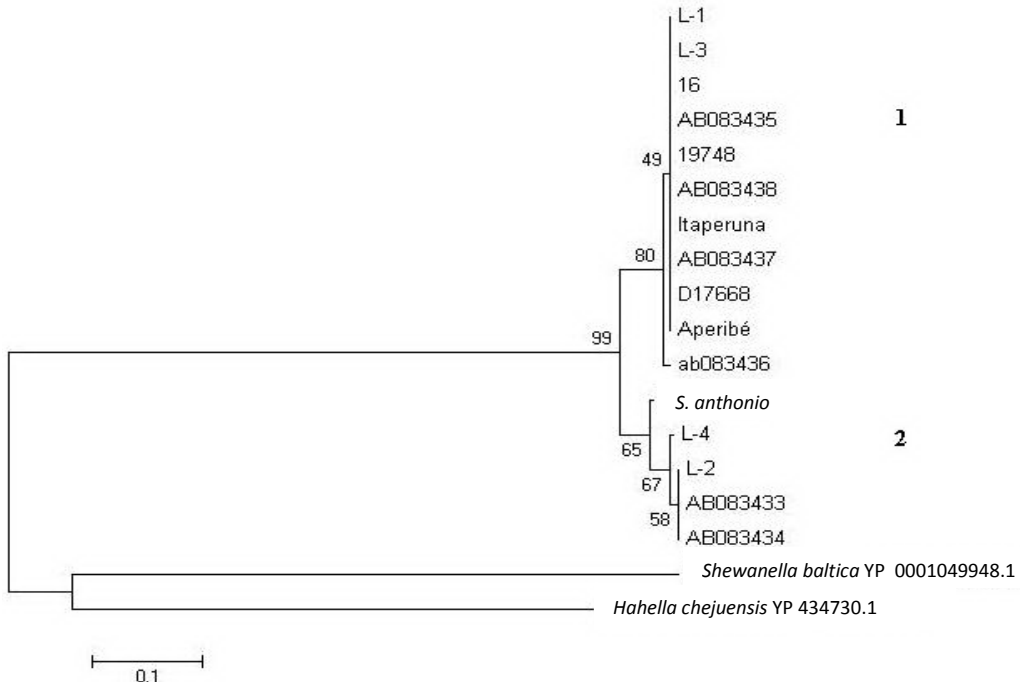

Figure 1. The phylogenetic analysis of partial sequence of *Clostridium septicum* alpha toxin gene. Neighbor joining construction method, 1000 *bootstrap*. The sequences *Shewanella baltica* YP 001049948.1 and *Habella chejuensis* YP 434730.1 were utilized as outgroup.

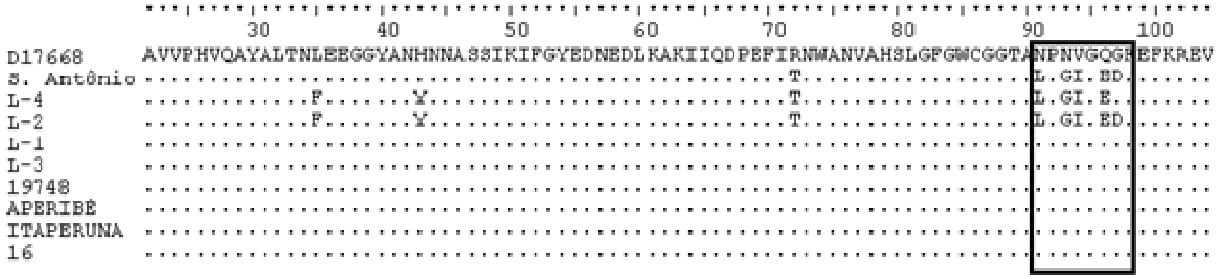

Figure 2. Alignment of amino acid sequences. The delimited amino acids (position 91 to 98) correspond to binding site in loop 1 of domain I of *Clostridium septicum* alpha toxin.

and 16, from the Instituto Biológico de São Paulo (IBSP) collection, belonged to the same group of L-1 and L-3 vaccine strains (Group 1), while homologous sequences from L-2 and L-4 strains belonged to Group 2. The analysis of the vaccine strain derived sequences showed that the strains from laboratories L-1 and L-3 did not show any substitution of amino acid when compared to the consensus sequence. However, L-2 and L-4 sequences presented respectively, eight and seven amino acid substitutions. Figure 2 shows the substitutions that caused the large divergence between group 1 and group 2 in phylogenetic tree. It was possible to observe that the majority of mutations were conserved (Figure 2).

The phylogenetic analysis indicated that Itaperuna and Aperibe strains had a high genetic similarity, while Santo Antônio de Pádua isolate was not directly genetically related with the previous. This result suggests a different origin for these outbreaks. In comparison with other strain alignments, the Santo Antônio de Pádua isolate showed high number of non silent mutations in a conserved region.

The *in silico* analysis of protein sequence enabled the study to make some considerations concerning the mutations. The Selecton software analysis showed that the sequenced region was not under significative positive pressure, indicating that substitutions have been

accumulated over time. This model was in agreement with the neutralist theory of evolution, which affirms that most of the mutations fixed in the evolution course are selectively neutral, and its fixation occur randomly (Kimura, 1983). Such mutations were not accumulated in short space of time. Thus, it seemed that the Santo Antônio de Pádua strain came from another region with high divergence.

Alpha-toxin genetic variability studies are important for understanding *C. septicum* clinical manifestation. This toxin is essential for the pathogenesis and little mutations may prevent some of its functions, for instance forming pores (Kennedy et al., 2009). The nucleotide non-synonymous substitutions lead to amino acids changes that may modify important biological characteristics, for instance, biological activity or action against the immune system. Animoto et al. (2006) studied alpha toxin of different genetic profile and there were no differences in the neutralization tests, indicating that no major epitope was modified by amino acids changes.

Amino acid substitutions may be relevant as the protein activity. The Hopp and Woods method (1981) demonstrated that relevant change in hydrophilic profile occurred on a hotspot located between the nucleotides 824 and 850. The main change was the substitution of a glycine by an aspartic acid (residue 97), which could interfere in pore complex forming, and consequently influence the toxin activity (Melton et al., 2006). The ConSeq server results confirmed the substitution of residue 97. Melton et al. (2006) described that a simple substitution by alanine in residues 94, 95, 97 or 98 can decrease toxin lethality in lineage cells at almost 100% when compared to the wild-type.

This work seemed to be the first in molecular epidemiology realized with Brazilian field and vaccine strains of *C. septicum*. Differences in the alpha toxin sequences of field and vaccine strains were been demonstrated. And, more investigations are necessary about these and others genetic differences for the future understanding of strains toxicity.

REFERENCES

Amimoto K, Ohgitani T, Sasaki O, Oishi E, Katayama S, Isogai M, Ota S (2002). Protective effect of *Clostridium septicum* alpha-toxoid vaccine against challenge with spores in guinea pigs. J. Vet. Med. Sci., 64: 67-69.

Amimoto K, Sasaki Y, Fukuyama S, Tamura Y (2006). Genetic variation and cross-reactivity of *Clostridium septicum* alpha-toxin. Vet. Microbiol., 114: 51-59.

Assis RA, Lobato FCF, Dias LD, Uzal FA, Martins NE, Silva N (2001). Producción y evaluación de conjugados fluorescentes para diagnóstico de mancha y gangrena gaseosa. Rev. Med. Vet., 82: 68-70.

Ballard J, Bryant A, Stevens D, Tweten RK (1992). Purification and characterization of lethal toxin (alpha-toxin) of *Clostridium septicum***.** Infect. Immun., 60: 784-790.

Ballard J, Crabtree J, Roe BA, Tweten RK (1995). The primary structure of *Clostridium septicum* alpha-toxin exhibits similarity with that of *Aeromonas hydrophila* aerolysin. Infect. Immun., 63: 340-344.

Ballard J, Sokolov Y, Yuan WL, Kagan BL, Tweten RK (1993). Activation and mechanism of *Clostridium septicum* alpha toxin. Mol. Microbiol., 10: 627-634.

Berezin C, Glaser F, Rosenberg J, Paz I, Pupko T, Fariselli P, Casadio R, Ben-Tal N (2004). ConSeq: the identification of functionally and structurally important residues in protein sequences. Informatics, 20: 1322-1324.

Fang PK, Raphael BH, Maslanka SE, Cai S, Singh BR (2010). Analysis of genomic differences among *Clostridium botulinum* type A1 strains. BMC Genomics, 23: 11-725.

Fitch WM (1986). The estimate of total nucleotide substitutions from pairwise differences is biased. Phil Trans R Soc Lond B, 312: 317-324.

Hoop TP, Woods KR (1981). Prediction of protein antigenic determinants from amino acid sequences. Proceed. Natl. Acad. Sci., 78: 3824-3828.

Hunley TE, Spring MD, Peters TR, Weikert DR, Jabs K (2008). *Clostridium septicum* myonecrosis complicating diarrhea-associated hemolytic uremic syndrome. Pediatr. Nephrol., 23: 1171-1175.

Imagawa T, Dohi Y, Higashi Y (1994). Cloning, nucleotide sequence and expression of a hemolysin gene of *Clostridium septicum.* FEMS Microbiol. Lett., 117: 287-292.

Kennedy CL, Lyras D, Cordner LM, Melton-Witt J, Emmins JJ, Tweten RK, Rood JI (2009). Pore-forming activity of alpha-toxin is essential for *Clostridium septicum*- mediated myonecrosis. Infect Immun., 77(3): 943-951.

Kimura M (1983). The Neutral Theory of Molecular Evolution. Cambridge University Press, Cambridge. 342 pages. ISBN 0-521-23109-4.

Lobato FCF, Salvarani FM, Assis RA (2007). Clostridial diseases of small ruminants. Rev. Portuguesa Ciências Vet., 102: 23-34.

Melton JA, Bentsen LM, Tweten RK (2006). Identification of functional domains of *Clostridium septicum* alpha toxin. Biochemistry, 45: 47-54.

Melton JA, Parker MW, Rossjohn J, Buckley JT, Tweten RK (2004). The identification and structure of the membrane-spanning domain of the *Clostridium septicum* alpha toxin. J. Biol. Chem., 279: 14315-14322.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406-425.

Skarin H, Lindberg A, Blomqvist G, Aspán A, Båverud V (2010). Molecular characterization and comparison of *Clostridium botulinum* type C avian strains. Avian Pathol., 39(6): 511-518.

Stern A, Doron-Faigenboim A, Erez E, Martz E, Bacharach E, Pupko T (2007). Selecton 2007: advanced models for detecting positive and purifying selection using a Bayesian inference approach. Nucl. Acids Res., 35: 506-511.

Takeuchi S, Hashizume N, Kinoshita T, Kaidoh T, Tamura Y (1997). Detection of *Clostridium septicum* Hemolysin Gene by Polymerase Chain Reaction. J. Vet. Med. Sci., 59: 853-855.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24: 1596-1599.

Tweten RK (2001). *Clostridium perfringens* beta toxin and *Clostridium septicum* alpha toxin: their mechanisms and possible role in pathogenesis. Vet. Microbiol., 82: 1-9.