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Sialidase Production and Genetic Diversity in *Clostridium perfringens* Type A Isolated from Chicken with Necrotic Enteritis in Brazil

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Abstract The sialidase activity and genetic diversity of 22 *Clostridium perfringens* strains isolated from chickens with necrotic enteritis were determined. Sialidase activity was detected in 86.4 % of the strains. All *C. perfringens* showed a high value of similarity (>96 %), and they were grouped into seven clusters clearly separated from the other reference bacterial strains. From these clusters four patterns were defined in accordance with their phenotypic (sialidase production and antibiotic resistance profile) and genotypic (presence of *nanI* and *nanJ* genes) characteristics. Our results showed heterogeneity among strains, but they were genotypically similar, and it is suggested further studies are needed to better understand the pathogenesis of necrotic enteritis.

Introduction

Clostridium perfringens is considered to be an important pathogen in human and animal diseases [28]. In the poultry industry, *C. perfringens* is the causal agent of necrotic enteritis causing high mortality, as well as great economic losses in several countries, mainly on those that ban the use of antibiotics [30].

Bacterial sialidases are considered virulence factors in several pathogenic organisms which colonize mucosal surfaces, since these enzymes catalyzes the sialic acid (*N*-acetyl neuraminic acid) hydrolysis from glycoproteins, glycolipids, and polysaccharides of the cell membrane, exposing the β-galactosyl determinant which acts in the adherence process among bacteria and the host mucosa. Several bacterial strains show a sialidase activity, and it has been suggested that this activity plays a role in the bacterial attachment to animal cells, as well as in the hemagglutination process [17, 19].

Clostridium perfringens harbor the sialidase genes *nanH, nanI*, and *nanJ*, but they are not present in all the strains [4]. Sialidase NanH is an intracellular enzyme involved in the short oligosaccharides cleavage; NanI and NanJ are extracellular enzymes which degrade sialoglyconjugates to free sialic acid that is then transported into the cell using a permease (NanT). In addition, intracellular enzymes, lyase (NanA), kinase (NanK), and epimerase (NanE) are capable of hydrolyzing sialic acid to *N*-acetylglucosamine-6-phosphate used for peptidoglycan synthesis [32].

The virulence of *C. perfringens* is related to toxin production, and isolates are divided into five types (A–E) based on the production of four lethal toxins (α, β, ε, and ι) [20]. The role of toxins in the development of intestinal lesions is well known; however, other factors such as adhesion, colonization and others, conferring some competitive advantage are still poorly studied [24].

Studies in vitro have shown that α-toxin (CPA) associated to NanI increases the *C. perfringens* virulence [9]. This α-toxin is a phospholipase type C with lethal and hemolytic effects, and also induces to the sphingomyelin and phosphotidylcholine degradation, as well as membrane destruction [21]. The α-toxin has an important role in gastrointestinal diseases and gas gangrenous [28], but other virulence factors and genetic markers are commonly evaluated to find any association with the clinical signs of these diseases.

A novel pore-forming toxin, NetB, has been identified in these virulent avian *C. perfringens* strains. Using a gene

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knockout mutant, it has been shown that NetB is a critical virulence factor in the pathogenesis of necrotic enteritis in broilers. In addition to toxin production, other factors have been described that contribute to the ability of certain *C. perfringens* strains to cause necrotic enteritis in broilers. It has been suggested that proteolytic enzymes play an important role in the initial stages of necrotic enteritis since the villi are first affected at the level of the basement membrane and the lateral domain of the enterocytes.

Because, it is important to find possible relationship between some phenotypic and genotypic characteristics, the determination of the sialidase production and the presence of the sialidase genes, as well as resistance to antimicrobial drugs, could collaborate to better understand the pathogenesis of these bacteria in the necrotic enteritis.

The genetic diversity of *C. perfringens* from avian origin with different health status have been performed using pulsed-field gel electrophoresis (PFGE), Multiple-locus variable-number tandem repeat analysis (MLVA), Multiloccus sequence typing (MLST), RAPD-PCR, and AFLP [10], but none of them have showed association between any specific genotype and clinical status.

In field outbreaks of necrotic enteritis, a single clone of *C. perfringens* is dominant in intestines of all affected birds, as opposed to the mixture of different *C. perfringens* strains that can be isolated from healthy bird intestines. It has been proposed that bacteriocin production is responsible for the dominance of a single strain in necrotic enteritis cases. Recent studies, using a large number of NetB positive and NetB negative *C. perfringens* strains to inoculate broiler chickens, showed a perfect match between necrotic enteritis induction and the ability to produce NetB [12, 15]. The role of the NetB toxin in the necrotic enteritis is still controversial because it has been detected in both healthy and sick animals and its prevalence varies in different countries [15].

Other important toxin has been reported in *C. perfringens* type C and type A, the TpeL toxin that comprise a large clostridial cytotoxins ranging in size from 250 to 308 kDa and it is suggested that TpeL may contribute significantly to the pathogenesis of necrotic enteritis [6].

In this study, the sialidase production, presence of *nan* genes, and antibiotic resistance in *C. perfringens* type A isolated from chicken with necrotic enteritis, as well as their genetic diversity, were determined.

Materials and Methods

Strains

Twenty two *C. perfringens* isolated from intestinal samples of nine chickens with necrotic enteritis were analyzed. All the strains were identified by biochemical tests [11], and in all of them, the presence of toxin genes was evaluated using PCR assays [2, 14] (Table 1).

Antimicrobial Susceptibility Testing

The bacterial susceptibility to 14 antibiotics was determined using agar dilution method with Wilkins-Chalgren agar [5]. The antibiotics used were as follows: amoxicillin, cephalexin, clindamycin, erythromycin, tetracycline (Luper Ind Farm Ltd, Sao Paulo, SP, Brazil), amoxicillin-clavulanic acid (Smithkline Beecham Brazil Ltd, Sao Paulo, SP, Brazil), cefoxitin (Merck, Sharp & Dohme, Sao Paulo, SP), metronidazole (Aventis Farm Ltd, Sao Paulo, SP, Brazil), bacitracin and chloramphenicol (Sigma Aldrich, Sao Paulo, SP, Brazil), enrofloxacin (Montana, Lima, Peru), oxytetracycline (GenFar, Cali, Colombia), penicillin–streptomycin (Univet, Ireland), and sulfaquinoxalin (Veterinaria Laboratorios, Lima, Peru). Plates containing two-fold serial dilutions of antimicrobial agents ranging from 0.25 to 512 μg/mL were used, and a final inoculum of 1.5×10^5 cfu/spot was delivered using a Steers replicator. Media without antibiotics were used as controls. All the plates were incubated in anaerobiosis (90 % N_2 + 10 % CO_2) at 37 °C for 48 h. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of each antimicrobial agent able to inhibit the macroscopic bacterial growth. The strain *C. perfringens* ATCC 13124 was included in each experiment to assess the reliability of the method. All the tests were done in duplicate.

Detection of Sialidase Activity

Sialidase activity was determined in microtitre plates using fresh normal chicken erythrocytes as described [19]. Briefly, erythrocytes were collected in Alsever's solution (10 %) and bacterial cells resuspended (1.5×10^8 bacteria/mL) in BHI broth. Then, 1 mL of bacteria was added to 10 μL of erythrocytes and incubated in anaerobiosis at 37 °C for 4 h. Serial dilutions of the lectin from peanut (2.5 mg/mL) (*Arachis hypogaea*; PNA, Sigma Chemical Co) in phosphate buffered saline (PBS) were performed, and 20 μL of each dilution was added to the mixture bacteria-erythrocytes (20 μL) and incubated at 4 °C, overnight. The presence of the sialidase was noticed when an agglutination (bacteria-erythrocytes-lectin) was observed. A mixture of bacteria-erythrocytes without lectin was used as negative control. The agglutination titer was defined as the reciprocal of the end-point dilution. The inhibition of the sialidase activity by the bacterial treatment with 1 mM galactose was also evaluated. The reference strains *C. perfringens* ATCC 13124 (type A, sialidase positive), *C. difficile* VPI 10468 (sialidase

Table 1 Oligonucleotides and PCR conditions used in the genes detection

Genes	Oligonucleotides 5′→3′	Annealing temperature (°C)	Amplicon (bp)	References
plc	AGTCTACGCTTGGGATGGAA	57	900	[2]
	TTTCCTGGGTTGTCCATTTC			
cpb	TCCTTTCTTGAGGGAGGATAAA	57	611	[2]
	TGAACCTCCTATTTTGTATCCCA			
cpe	GGGGAACCCTCAGTAGTTTCA	57	506	[2]
	ACCAGCTGGATTTGAGTTTAATG			
etx	TGGGAACTTCGATACAAGCA	57	396	[2]
	TTAACTCATCTCCCATAACTGCAC			
iap	AAACGCATTAAAGCTCACACC	57	293	[2]
	CTGCATAACCTGGAATGGCT			
cpb2	CAAGCAATTGGGGGAGTTTA	57	200	[2]
	GCAGAATCAGGATTTTGACCA			
netB	GCTGGTGCTGGAATAAATGC	50	384	[12]
	TCGCCATTGAGTAGTTTCCC			
tpeL	ATATAGAGGCAAGCAGTG GAG	55	466	[6]
	GGAATACCACTTGATATA CCTG			
nanH	ACAACGAAACGCTTATCGTGGCTAT	47	1,108	This study
	ACCTTGGCATCCAGAGCCCTT			
nanI	ATTACAAAGGGATAACTTTAATTTTAAC TTTATTAGCTCCACTCTC	50	2,060	[26]
nanJ	GCTATTATTGAAACTGCTATT ATCTTCATCTAAAACTTCAAT	50	2,400	[4]

negative), and *B. fragilis* ATCC 25285 (sialidase positive) were included in all the tests. All the assays were performed in duplicate.

Bacterial DNA

Total DNA was obtained as described [23]. Briefly, bacteria grown in BHI were harvested and twice washed with PBS (pH 7.2). Pellet was incubated with lysozyme (10 mg/mL) at 37 °C for 3 h. Then, 20 % SDS and 20 mg/mL proteinase K were added and incubated at 55 °C for 2 h. DNA was obtained using equal volumes of phenol–chloroform and centrifugation (14,000 g × 5 min). The supernatant was precipitated with sodium acetate and isopropanol. DNA was washed with 70 % ethanol and eluted in 100 µL of TE, and finally, stored at −80 °C until use.

Toxinotyping and Detecting Genes nanH, nanI, and nanJ

The presence of genes encoding the toxin (α, β, ε, i, β2, and enterotoxin) and sialidases production, as well as, the NetB and TpeL production were determined using 35 cycles and with their respective annealing temperatures, as described in Table 1. The DNA amplifications were performed using final volumes of 25 mL containing 10 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 U Platinum *Taq DNA* polymerase (Invitrogen), 0.4 mM each primer, and 1 ng DNA. A thermocycler (PE Applied Biosystems Gene Amp PCR System 9700) was programed to: 1 cycle of 95 °C (3 min), followed by 35 cycles of 95 °C (1 min), respective annealing temperature (1 min) and 72 °C (2 min) and a final cycle of 72 °C (5 min) to allow the final DNA extension.

PCR products were analyzed in 1 % agarose gel stained with 0.5 mg/mL ethidium bromide, and photographed using a Kodak Digital System DC-120. The strains *C. perfringens* ATCC 13124 (*plc* +, *nanH* +, *nanI* +, and *nanJ* +), *C. perfringens* CpB (*plc* +, *cpb* +, *etx* +), *C. perfringens* (*plc* +, *iap* +), *C. perfringens* EHE-NE-18 (*netB* +), and *C. perfringens* JGS-5369 (*tpeL* +) were used as controls.

Genetic Diversity

Genetic analysis was performed using arbitrary primed polymerase chain reaction (AP-PCR) technique with nine decamer oligonucleotides of random sequences (OPA-3: AGTCAGCCAC; OPA-9: GGGTAACGCC; OPA-12: TCGGCGATAG; OPA-17: GACCGCTTGT; OPA-18: AGGTGACCGT; OPF-5: CCGAATTCCC; OPI-1: ACCTGGACAC; OPI-4: CCGCCTAGTC; OPI-14:

Table 2 Phenotypic and genotypic features of 22 *C. perfringens* from chicken with necrotic enteritis

Isolates	Toxinotype	Neuraminidase (Titer)	Genes		Resistance profile	Genetic groups
			nanI	*nanJ*		
1a	A, *tpeL* (−)	2	+	+	B, CF, CL, ER, O, S, T	I
1b	A, *tpeL* (−)	2	+	+	CF, CL, ER, O, S, T	I
3c	A, *tpeL* (+)	8	+	+	B, CF, CL, ER, S	I
1d	A, *tpeL* (−)	0	−	−	CF, CL, ER, S	II
5a	A, *tpeL* (−)	16	+	+	CF, ER, S, T	II
8c	A, *tpeL* (−)	32	+	+	CF, ER, S	II
2a	A, *tpeL* (−)	16	+	+	CF, ER, S	II
6a	A, *tpeL* (−)	8	+	+	B, CF, ER, S	III
6b	A, *tpeL* (+)	4	+	+	B, CF, ER, S	III
6c	A, *tpeL* (+)	8	+	+	B, CF, ER, S	III
6d	A, *tpeL* (+)	16	+	+	B, CF, ER, S	III
8a	A, *tpeL* (−)	16	+	+	CF, ER, S	III
Cp	A, *tpeL* (−)	4	+	+	CF, CL, S	III
8b	A, *tpeL* (−)	16	+	+	CF, ER, S, T	III
8d	A, *tpeL* (−)	8	+	+	CF, ER, S	III
4a	A, *tpeL* (+)	4	+	+	CF, CL, ER, O, S, T	IV
9a	A, *tpeL* (−)	4	+	−	CF, ER, S	IV
9b	A, *tpeL* (+)	4	+	−	CF, ER, S, T	IV
7a	A, *tpeL* (−)	2	+	−	B, CL, S	V
3a	A, *tpeL* (−)	0	+	+	B, CF, CL, ER, S	VI
3b	A, *tpeL* (−)	0	+	+	B, CF, ER, O, S	VI
3d	A, *tpeL* (+)	8	+	+	B, CF, ER, O, S	VI
1c	A, *tpeL* (−)	16	+	+	B, CF, CL, ER, S	VII

TGAAGGCGGT), as described [8]. Amplifications were carried out in final volumes of 25 µL in a thermocycler programed to 1 cycle of 94 °C (5 min), followed by 35 cycles of 94 °C (1 min), 42 °C (2 min), and 72 °C (2 min), and a final cycle of 72 °C (10 min). The different bands were visually scored and coded in a binary matrix. Pairwise similarities were computed by the NTSYS program (Applied Biostatistic, inc. version 2.21) using UN1 coefficient of similarity and unweighted pair group method with arithmetic averages (UPGMA) clustering. *Clostridium perfringens* ATCC 13124, *C. sporogenes* ATCC 2413, *C. difficile* VPI 10468, *B. fragilis* ATCC 25285, and *E. coli* E-2369 were included in all the analyses.

Results

All the 22 *C. perfringens* belonged to type A, and they did not harbor the *netB* gene; however, in seven isolates the *tpeL* gene was observed. These seven *tpeL* + isolates were grouped in four different clusters (Table 2).

Sialidase activity was detected in 86.4 % of the strains with titers ranging from 2 to 32; and galactose was able to inhibit this activity in all the sialidase-producing strains (data not shown). Eighteen (81.8 %) out of 22 tested strains harbored both the *nanI* and *nanJ* genes, and three (16.6 %) strains carried only the *nanI* gene. None of the 22 strains harbored the *nanH* gene, and only the strain 1d harbored neither the *nanI* nor *nanJ* genes as well as no sialidase activity was observed.

All the tested strains showed resistance to 50 % of the used antimicrobials. In addition, bacterial resistance was observed to bacitracin (50 %), cephalexin (95 %), clindamycin (36 %), erythromycin (95 %), oxytetracycline (23 %), sulphaquinoxalin (100 %), and tetracycline (32 %). The resistance ranges of antimicrobials were bacitracin (64 to ≥512 µg/mL), cephalexin (8–64 µg/mL), clindamycin (32–256 µg/mL), erythromycin (8–64 µg/mL), oxytetracycline (8–128 µg/mL), sulphaquinoxalin (≥512 µg/mL), and tetracycline (8–32 µg/mL). All the strains were susceptible to amoxicillin, amoxicillin/clavulanic acid, cefoxitin, chloramphenicol, enorfloxacin, metronidazole, and penicillin/streptomycin.

Only the primer OPA-3 generated at least two polymorphic DNA patterns among the strains tested. Seven clusters were defined with more than 96 % of similarity, and as expected, all the *C. perfringens* were clearly separated from *C. difficile, C. sporogenes, E. coli*, and *B.*

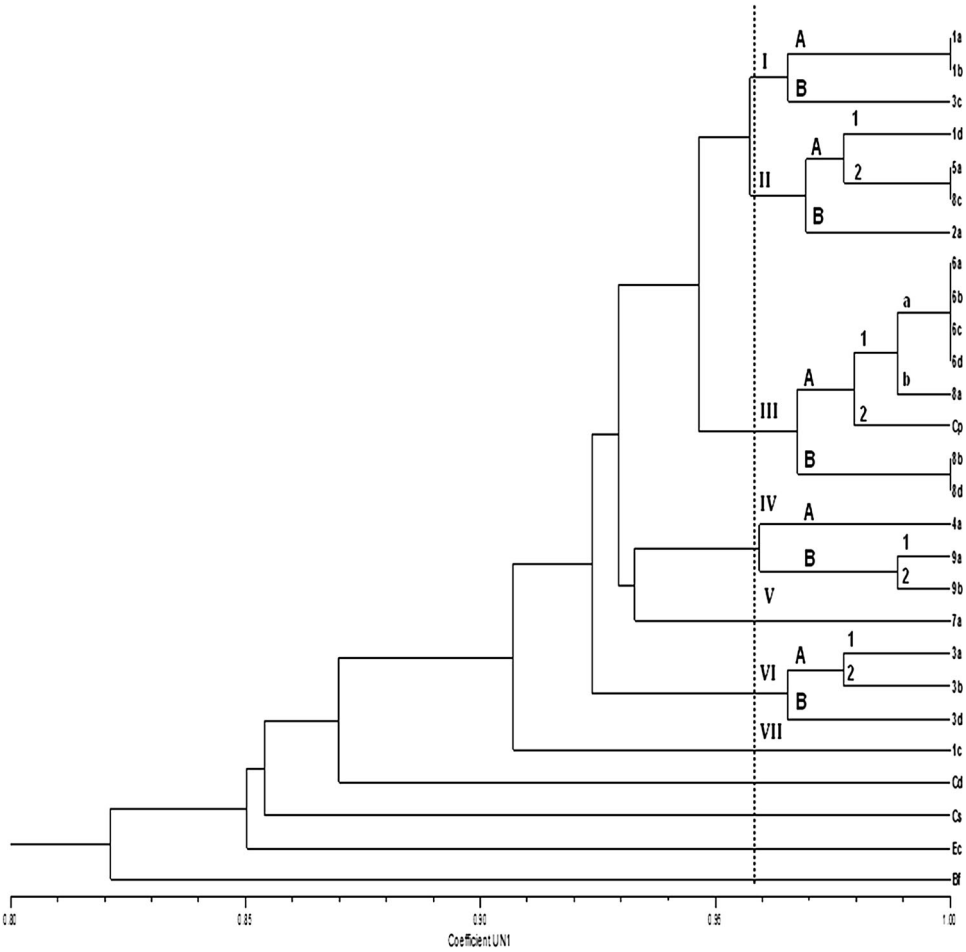

Fig. 1 Dendrogram of genotyping of 22 *Clostridium perfringens* type A isolated from chickens with necrotic enteritis. *Cp C. perfringens* ATCC 13124; *Cd C. difficile* VPI 10468; *Cs C. sporogenes* ATCC 2413; *Ec E. coli* E-2369; *Bf B. fragilis* ATCC 25285

fragilis, and they did not show any similarity (Fig. 1). The genotypic analysis showed seven clusters. Cluster I harbored three strains (two clones isolated from the same chicken) that were sialidase +, *nanI* +, *nanJ* +; and Cluster II harbored three sialidase-producing strains and they were *nanI* + and *nanJ* +. Also, one strain (1d) did not show these characteristics.

Cluster III was divided into sub-clusters A and B. In A1, it is possible to note the group A1a with four clones obtained from the same chicken, and group b with one strain, but all of them harbored the genes *nanI* and *nanJ*. Also, *C. perfringens* ATCC 13124 was grouped in cluster III A2. In addition, cluster III B grouped two strains with the same characteristic as other bacteria of this cluster.

Cluster IV grouped three strains, one sialidase-producing strain 4a (group A) harboring the genes *nanI* and *nanJ*; and strains 9a (group B1) and 9b (group B2) did not harbor the *nanJ* gene. Cluster V harbored only one strain (7a) with the same characteristics observed in strains 9a and 9b (cluster IV). Clusters VI showed groups A1 and A2 with strains harboring the genes *nanI* and *nanJ*, but they did not produce sialidase, different to the group B (strain 3d) harboring *nanI* and *nanJ* and producing sialidase. Cluster VII harbored only the strain 1c with sialidase, *nanI*, and *nanJ* positives.

Based on the sialidase production, presence of genes *nanI* and *nanJ*, and common antimicrobial resistance of the 22 strains evaluated here, four genetic patterns were observed (Table 3).

Discussion

The prevalence of *C. perfringens* type A observed in this study is in accordance with previous studies involving broiler chicken and other avian species, as well as, in humans, and it is considered as the most common genotype isolated from fecal and environmental samples.

The sialidase activity is required for colonization of some bacteria [31]; however, sialidase-producing *C. perfringens* are able to use mucopolysaccharides and glycoproteins from host's cells [22]. On the other hand, some pathogens, such as *Haemophilus influenza, Pasteurella multocida, Neisseria gonorrhoeae,* and *Streptococcus*

Table 3 Genetic patterns of *Clostridium perfringens* from chicken with necrotic enteritis

Isolates	Sialidase production	Presence of genes		Common resistance to antimicrobials	Patterns
		nanI	*nanJ*		
1a, 1b, 3c, 5a, 8c, 2a, 6a, 6b, 6c, 6d, 8a, 8b, 8d, 4a, 3d, 1c	+	+	+	Cx, Er, S	I
1d	−	−	−	Cx, Cl, Er, S	II
9a, 9b, 7a	+	+	+	S	III
3a, 3b	−	+	+	B, Cx, Er, S	IV

B bacitracin; *Cx* cephalexin; *Cl* clindamycin; *Er* erythromycin; *S* sulphaquinoxalin

agalactiae are able to incorporate the sialoglycan into capsule or LPS used as protection against host's immune cells, by inhibiting the opsonization and phagocytosis [16].

Studies have shown that *B. fragilis* recovered from pathological samples produce significantly higher levels of sialidases than non-pathological isolates [18]. Bacterial sialidases have the capacity to modify the host's immune response against infection [25]. In this study, lectin agglutinating neuraminidase-treated human erythrocytes was observed; although this hemagglutination method shows the neuraminidase activity in biological material, but does not quantify the amount of produced enzyme, due to the inherent problems in cell agglutination.

In *C. perfringens* isolated from human gas gangrene, an interaction between NanI and the extracellular environment from the host's tissue during infection have been observed, as well as a synergistic interaction with ε-toxin, which is required for *C. perfringens* type B- and D-mediated diseases [13].

Clostridium perfringens is a common inhabitant of the chicken's intestine and its presence is not enough to cause necrotic enteritis; although, some predisposing factors, and the high frequency of bacterial adherence to the damaged mucosa, as well as the bacterial proliferation and toxin production collaborate to the development of diseases [33].

The high sialidase production found in the evaluated *C. perfringens* suggests an ecological and pathogenic role in the chicken necrotic enteritis. In this study, most of *C. perfringens* harbored both *nanI* and *nanJ* genes, and of them two strains (3a and 3b) did not produce sialidase. Because no sialidases were produced, it is suggested that they were silent genes. Interestingly, a strain 1d produced neither sialidase nor harbored the genes *nanI* and *nanJ*. In addition, non-sialidase-producing *C. perfringens* also did not harbor the gene *nanJ*, and it is suggested that gene *nanI* is essential to the production of this enzyme.

Most of tested strains were resistant to bacitracin, cephalexin, clindamycin, erythromycin, oxytetracycline, sulphaquinoxalin, and tetracycline. Different countries use antimicrobials mixed to feed and water as effective method to prevent or to control the necrotic enteritis outbreak, although bacterial resistance to several drugs has been reported [27]. The use of tetracycline and other antibiotics as growth-promoting is often observed in Brazilian poultry production, and it may explain the bacterial resistance to the used antimicrobials in this study. It is suggested a continuous monitoring of the antimicrobial susceptibility of *C. perfringens* from animal origin, particularly, poultry, for a better prevention and treatment of the necrotic enteritis [14].

In this study, a close relationship among the tested strains was observed, and since all the strains were recovered from necrotic enteritis, our results suggest that the chicken's intestinal microbiota harbor a diverse population of pathogenic *C. perfringens* and they could be important to the intestinal lesions. The sialidase activity and the presence of genes *nanI* and *nanJ* did not appear to be factors to the cluster's organization, and it is suggested that other genotypic or phenotypic characteristics could be involved in the clonal diversity. On the other hand, on base to the sialidase production, presence of genes *nanI* and *nanJ*, and common resistance profile to antibiotics, four different patterns were observed (Table 3); this result shows the heterogeneity of this microorganism, and it can represent important ecological factors in the avian intestinal ecosystem, as well as in the development of necrotic enteritis.

Genetic analyses of *C. perfringens* isolated from different origins using RAPD-PCR, MLST, and PFGE methods have shown that some clones might be associated to enteritis necrotic and they can persist for long time at the diseased sites [1, 8]. The AP-PCR method has been used to detect genetic differences into whole genome [3]. Studies using DNA-23S rRNA pairing or full rRNA sequences have shown that *Clostridium* spp. can be clustered separated from others [29].

The genotypic and phenotypic characteristics of avian *C. perfringens* have been determined by the presence of

netB, cpe and *cpb2* genes, and α-toxin or bacteriocin production, but no relationship among them has been observed [7, 26]. Studies have shown that NetB toxin is an important virulence factor for the necrotic enteritis. In this study, no *C. perfringens* harbored the respective *netB* gene, and it suggests that the mere presence of the NetB toxin is not enough to produce the enteritis necrotic in broiler chicken. On the other hand, seven out of 22 isolates harbored the *tpeL* gene. In addition, the absence of *netB* gene, and the presence of the *tpeL* gene in some *C. perfringens* isolates suggest that the TpeL toxin or other toxin plays a role in the pathogenesis of the necrotic enteritis, in accordance with Li et al. [13] who showed that the genotype NetB-/TpeL + were present in toxigenic strains type A of *C. perfringens*.

Since that one strain (1d) did not produce sialidase and did not harbor both genes, our results suggest that more studies are necessary to determine the true role of these and other enzymes to better understand the intestinal homeostasis breaking or to determine other intestinal markers associated to the pathogenesis of necrotic enteritis in chicken.

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Conflict of interest The authors declare no conflict of interest.

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