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TOXINOTYPING AND ANTIMICROBIAL SUSCEPTIBILITY OF *Clostridium perfringens* ISOLATED FROM BROILER CHICKENS WITH NECROTIC ENTERITIS

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Abstract- The toxinotyping and the antimicrobial susceptibility of *Clostridium perfringens* strains isolated from chicken with necrotic enteritis were determined. All the 22 *C. perfringens* belonged to toxinotype A and the MIC values to 14 antimicrobial agents showed that all strains were susceptible to amoxicillin, amoxicillin-clavulanic acid, cefoxitin, chloramphenicol, enrofloxacin, metronidazole and penicillin-streptomycin. Most strains showed high rates of resistance to erythromycin, cephalexin and bacitracin and sulfaquinolaxin. Our results suggest an important role of the α-toxin in the pathogenesis of necrotic enteritis and new strategies for preventing and controlling the *Clostridium perfringens* infection in poultry need to be investigated.

Key words- *Clostridium perfringens*, Chicken, Necrotic enteritis, Toxinotypes, Antimicrobial susceptibility

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Introduction

Clostridium perfringens is a spore-forming gram positive anaerobic rod and a common inhabitant of the intestine of healthy broiler chickens belonging to the resident microbiota [1]; however, this microorganism along with predisposing factors, such as mucosal damage are requisites to developing of the disease. In addition, certain conditions as coccidiosis and consumption of feed with high fiber content can also collaborate to the overgrowth of *C. perfringens* and subsequent toxin production, causing the both clinical and sub-clinical disease [2].

This microorganism is grouped into five toxinotypes (A, B, C, D and E) producing α, β, ε and i toxins [3]. *Clostridium perfringens* type A has a ubiquitous habitat and is the main gangrene-producing toxinotype and food poisoning in humans. In animals, this bacterium can be associated with diarrhea in foals and pigs and necrotic enteritis in chicken. The toxinotype B is associated with newborn lambs' dysentery, neonatal calves' hemorrhagic enteritis and sheep enterotoxaemia. The type C produces necrotic enteritis in piglets, lambs, calves, foals and chickens and the toxinotypes D and E are responsible for enterotoxemia in lambs, sheep, calves and goats [3]. *Clostridium perfringens* also produces other potent toxins and enzymes, including NetB related to human and veterinary diseases [4].

Necrotic enteritis is an important clinical disease produced by *C. perfringens* that affects the poultry industry worldwide causing serious economic loss, about of two dollar billions/year [5]. This disease is characterized by severe necrosis of the small intestine mucosa in the proximal jejunum region and it is associated with high mortality rates [6]. On the other hand, subclinical disease leads to a decreased performance, due to the extensive mucosal damage [7].

Several studies have shown that *C. perfringens* type A is often isolated from poultry chicken; however, its presence producing poultry infections in different countries are still scarce. *Clostridium perfringens* toxinotype A produces a α-toxin, a phospholipase C that hydrolyzes phospholipids causing the production of inflammatory mediators and acute death [8].

The most effective method to prevent or to control the outbreak of necrotic enteritis is the use of antimicrobials mixed to feed and water, although, bacterial resistance to bacitracin, tetracycline, clindamycin, lincomycin and erythromycin has been reported in several countries, such as, Denmark, Switzerland, Norway, Belgium, Jordan and Brazil [9-11].

For decades, growth-promoting antibiotics have been used in broiler chicken to increase the weight and decrease food spending [12]. Although, in countries that have stopped of using growth-promoting antibiotics, the problems of diseases associated to *C. perfringens* in broiler chicken have increased [13].

In this study, the toxinotyping and the antimicrobial susceptibility of *C. perfringens* strains isolated from broiler chickens with necrotic enteritis were determinated.

Materials and Methods

Intestinal Samples

Intestinal pieces from 96 chickens with necrotic enteritis (marked depression, decreased appetite, ruffled feathers, enteritis and diarrhea); and 63 intestinal pieces from healthy chickens were collected. The ethic committee in animal experimentation of the Institute of Biomedical Science, University of Sao Paulo, SP, Brazil (Proc. No. 104) approved this study.

Bacterial Isolation and Identification

Approximately, 2 cm of intestine, showing severe injuries, were transferred to tubes containing broth meat (Difco Laboratories, USA) and incubated at 37°C for 48 h under anaerobic conditions (90% N_2, 10% CO_2,). Aliquots of 0.1 mL were streaked onto tryptic case soy agar (TSA, Difco Laboratories, USA) enriched with 5% defibrinated horse blood agar. Plates were incubated at 37°C for 48 h in anaerobiosis. Bacterial identification was performed by colonial and cell morphology and biochemical tests. Characteristic colonies displaying short gram-positive bacilli, dual haemolysis and gelatinase and lectinase producing, were isolated for identification by biochemical tests [14]. The reference strain *C. perfringens* ATCC 13124 was used as positive control. All the tested strains were stored in 10% skimmed milk at - 80°C until use.

Toxinotyping by PCR

Bacterial DNA was obtained from a colony grown in BHI according to Sambrook, etal. [15]. Briefly, bacteria were harvested by centrifugation and pellets were twice washes 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 extracted by using equal volumes of phenol-chloroform and centrifuged (14,000 g x 5 min). The supernatant was precipitated with sodium acetate and isopropanol and centrifuged (14,000 g x 10 min). DNA was washed with 70% ethanol and eluted in 100 mL of TE and then, stored at - 80° C until use.

Table 1- Primers used in toxinotype of Clostridium perfringens isolated from chickens with necrotic enteritis

Gene (Toxin)	Genetic localization	Sequence 5'→3'	Amplicon (bp)	Reference
cpa (a)	Chromosome	AGTCTACGCTTGGGATGGAA TTTCCTGGGTTGTCCATTTC	900	[16]
cpb (b)	Plasmid	TCCTTTCTTGAGGGAGGATAAA TGAACCTCCTATTTTGTATCCCA	611	[16]
cpe*	Chromosome/ Plasmid	GGGGAACCCTCAGTAGTTTCA ACCAGCTGGATTTGAGTTTAATG	506	[16]
etx (e)	Plasmid	TGGGAACTTCGATACAAGCA TTAACTCATCTCCCATAACTGCAC	396	[16]
iap (i)	Plasmid	AAACGCATTAAAGCTCACACC CTGCATAACCTGGAATGGCT	293	[16]
cpb2 (b2)	Plasmid	CAAGCAATTGGGGGAGTTTA GCAGAATCAGGATTTTGACCA	200	[16]
netB	Plasmid	GCTGGTGCTGGAATAAATGC TCGCCATTGAGTAGTTTCCC	384	[4]

**Enterotoxin.*

The presence of genes encoding the toxins α, β, ε, i, β2 and enterotoxin production were detected by a multiplex PCR assay [16]. The DNA amplifications were performed by using final volumes of 25 mL containing 10 X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 U Platinum *Taq DNA* polymerase (Invitrogen), 0.4 mM of each primer and 1 ng of DNA. A thermocycler (PE Applied Biosystems Gene Amp PCR System 9700) was programmed to: 1 cycle of 95°C (3 min), followed by 35 cycles of 95°C (1 min), 56°C (1 min) and 72°C (2 min) and a final cycle of 72°C (5 min) to allow the final DNA extension. The detection of the *netB* gene was performed using specific primers and annealing temperature of 55°C (1 min) with single PCR reactions. All the used primers are shown in Table 1.

PCR products were analyzed in 1% agarose gel stained with ethidium bromide (0.5 mg/mL) and photographed by using a Kodak Digital System DC-120. The reference strain *C. perfringens* ATCC 13124 (α-toxin positive) and *C. perfringens* EHE-NE-18 (*netB*-toxin positive) kindly provided by Dr. Rob Moore at the Monash University, Australia, were used as controls.

Antimicrobial Susceptibility Testing

The bacterial susceptibility to 14 antibiotics was determined by using an agar dilution method with Wilkins-Chalgren agar [17]. 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 the final inoculum of 1.5 x 10^5 cfu/spot was delivered by using a Steers replicator. Media without antibiotics were used as controls. All the plates were incubated in anaerobiosis 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* σ 215 was included in each experiment to assess the reliability of the method. All the tests were done in duplicate.

Results and Observations

In nine (9.4%) out of 96 intestinal samples with necrotic enteritis *C. perfringens* was found and 22 strains were recovered. All the isolated strains harbored only the *cpa* gene encoding the α-toxin production (Fig. 1) and they did not harbor the *cpe* gene encoding the enterotoxin production. In addition, none of these strains harbored the *cpb, etx, iap, cpb2* and *netB* genes. *Clostridium perfringens* strains were not isolated from the evaluated healthy chickens.

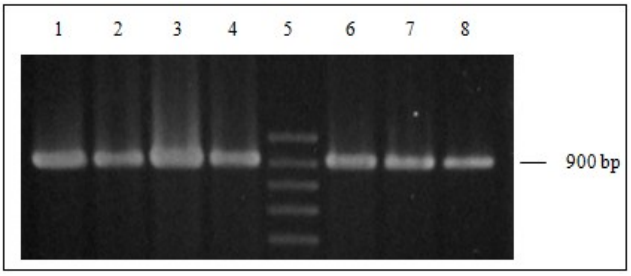

Fig. 1-

MIC values and the resistance rates to the different antibiotics against *C. perfringens* strains are shown in Table 2. Strains were susceptible to amoxicillin, amoxicillin-clavulanic acid, cefoxitin, chloramphenicol, enrofloxacin, metronidazole and penicillin-streptomycin. In addition, amoxicillin, amoxicillin-clavulanic acid, cefoxitin, clindamycin, enrofloxacin and penicillin-streptomycin showed the lowest MIC_{50} values (≤ 0.25 µg/mL - 0.5 µg/mL). High MIC_{90} values to sulfaquinoxalin, bacitracin, clindamycin, cephalexin and erythromycin (64 µg/mL - ≥ 512 µg/mL) were observed. Most of the strains were resistant to sulfaquinoxalin (100%), erythromycin (95%), cephalexin (95%), bacitracin (50%), clindamycin (36%), oxytetracycline (23%) and tetracycline (32%).

Table 2- Susceptibility to 14 antimicrobials of Clostridium perfringens Type A isolated of chickens with necrotic enteritis

Antibiotics	Breakpoint* µg/mL	MIC (µg/mL) Range	50%	90%	Resistance (%)
Amoxicillin	8	≤0.25 - 1	≤0.25	0.5	0
Amoxicillin- Clavulanic acid	8	≤0.25 - 4	0.5	4	0
Bacitracin	8	≤0.25- ≥ 512	4	128	50
Cephalexin	8	4-64	16	64	95
Cefoxitin	32	≤0.25	≤0.25	≤0.25	0
Clindamycin	4	0.5 - 256	0.5	128	36
Chloramphenicol	8	01-Apr	4	4	0
Enrofloxacin	8	≤0.25 - 4	≤0.25	4	0
Erythromycin	8	4-64	16	32	95
Metronidazole	16	≤0.25 - 8	4	4	0
Oxytetracycline	8	≤0.25 - 128	4	8	23
Penicillin-Streptomycin	8	≤0.25 - 8	0.5	4	0
Sulfaquinoxalin	ND**	≥ 512	≥ 512	≥ 512	100
Tetracycline	8	2-32	4	8	32

** Breakpoints used in accordance with CLSI (2007).*
*** ND: not defined.*

Discussion

It is known that alpha-toxin-producing *C. perfringens* is predominant in intestinal microbiota of diseased broilers. Our results showed that *C. perfringens* isolated from chicken with necrotic enteritis harbored the α-gene as predominant toxinotype. Although, studies have shown that the presence of alpha-toxin-producing *C. perfringens* is not a prerequisite for initiating and developing the disease, because predisposing factors are required. In addition, the absence of *C. perfringens* in healthy chicken suggests that this microorganism has an important role in necrotic enteritis or feed mixed with antibiotics can suppress bacterial growth.

The presence of α-toxin-producing *C. perfringens* in poultry farm with necrotic enteritis appears to be no association with any subtype of *C. perfringens* or disease [18]. Previous studies have shown the induction of intestinal damage when *C. perfringens* culture supernatant was inoculated in chickens; however, the relevance of the α-toxin was not determined, since other toxins may also be present [19].

Ours results showed the absence of *C. perfringens* in healthy poultry and it may indicate that both the presence of α-toxin-producing *C. perfringens* and the mucosal damage are prerequisite to cause the necrotic enteritis. Although, the presence of α-toxin gene does not represent a virulence factor of *C. perfringens* type A and other factors, such as adhesion and invasion, could be involved and it need to be investigated [20].

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 [21]. In this study, none strains harbored the *netB* gene; although, it is interesting to note that its presence does not necessarily determine the toxin production [22]. In addition, another important toxin has been reported in *C. perfringens* type C and type A [23]. This novel TpeL toxin 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 [24]; however, in this study, this toxin was not evaluated.

Antimicrobial drugs are still used in poultry as growth promoting and as preventing against several infectious diseases, however, their use have caused the spreading of bacterial resistance in different ecosystems. Antibiotics are used as growth promoting in Canada, but in European countries their use have been stopped [25]. It is well known that antimicrobial drugs can produce alterations on host's microbiota selecting resistant organisms, which can appear as opportunistic pathogens [26].

The modification of the host's intestinal bacterial population caused by external factors, such as diet or antibiotic, is not easily monitored using traditional methods [27]. In recent years, several molecular methods have been developed to evaluate population from different ecosystems [28].

The mechanisms by which antimicrobials improve growth performance is not well know, but it is suggested that nutrients are efficiently absorbed at the thinner small-intestine epithelium or microorganisms causing subclinical infections are reduced or eliminated; however, no explanation of this process has been observed.

Amoxicillin, amoxicillin/clavulanic acid and cefoxitin, as well as penicillin-streptomycin showed an excellent activity against the evaluated *C. perfringens* strains and it is in accordance with studies performed in other countries [10]. On the other hand, studies have shown that amoxicillin is effective against necrotic enteritis and its use is suggested for prevention of *C. perfringens* infection [29]. Cephalexin showed low activity against the tested strains and resistance rate of 95% was observed; it might be explained by its widespread use in broiler production and by the low cost [30].

Enrofloxacin showed a good activity against the tested strains, showing a value of MIC_{50} ≤ 0.25 µg/mL in accordance with Gholamiandehkordi, etal. [31]. On the other hand, our results divergent with those reported by Gharaibeh, etal. [10] showing a value of MIC_{50} = 8 µg/mL and suggesting that the resistance to this drug could be due to the prolonged use in avian infections. Moreover, in some countries the use of this drug in poultry production is not indicated due to the negative impact on human health and to the transmission of resistant to antibiotics via food chain [32].

The resistance to tetracycline is commonly observed in *C. perfringens* and it is codified by the *tetP* gene [9]. Although, studies have shown that oxytetracycline has an excellent activity against *C. perfringens* [10]. In this study, the resistant to oxytetracycline and tetracycline was observed, respectively, in 23% and 32% of the tested strains. The use of tetracyclines and other antibiotics as growth-promoting is often observed in Brazilian poultry production.

Chloramphenicol is considered a drug of choice used against *Sal-*

monella Typhimurium and other severe gastrointestinal diseases, due to its action on almost all the members of the intestinal microbiota causing a complete depletion of coliforms and lactobacilli [33]. Chloramphenicol showed an excellent activity against all the tested strains with MIC_{50} and MIC_{90} values of 4 µg/mL in accordance to Rood, etal. [34].

Similarly, MIC values of 4 µg/mL to metronidazole were also observed in accordance with Chalmers, etal. [23]. Metronidazole is a drug used only for the treatment of anaerobic infections in humans; although, because of sensitivity of the *C. perfringens* strains isolated from chicken with low MIC_{90} values, its use could be a choice for treatment or controlling infections in poultry.

In this study, 36% of the *C. perfringens* strains were resistant to clindamycin and it suggests that those genes were distributed in the evaluated avian industries. This drug produces a marked depletion of the intestinal microbiota, causing pseudomembranous colitis by *C. difficile*. Clindamycin and metronidazole are effective to control the acute symptoms but not the chronic process, maybe because they have not any effect neither on spores nor toxin [35].

Moreover, *C. perfringens* strains of animal origin often display a high resistant to clindamycin and erythromycin [36]. The resistance to the macrolide-lincosamide-streptogramin group has been attributed to the presence of the *ermQ* and *erm*B genes which codified enzymes responsible for the 23S rRNA dimethylation [37].

The use of bacitracin as a feed additive in poultry industry is often observed [32] and high resistance values in bacterial strains isolated from poultry have been reported [9,23]. Bacitracin is not absorbed from the gastrointestinal tract; however it produces no relief subclinical infection, as a primary mechanism of action for growth-permitting antibiotics [38]. In this study, 50% of the tested strains were resistant to this drug. It is known that bacteria become resistant due to the selective pressure in the intestinal ecosystem, however, the mechanisms of this resistance is not yet clear [23]. On the other hand, studies have shown that bacitracin was effective to decrease the morbidity and mortality in experimental models of necrotic enteritis [39].

Sulfonamide is another drug commonly used as feed additives and for the treatment of respiratory diseases in poultry. In this study, all the tested strains were resistant to sulfaquinoxalin in accordance with previous report [1].

A continuous monitoring of the virulence factors and the antimicrobial susceptibility profile of *C. perfringens* from animal origin, particularly, poultry are necessary for a better prevention and treatment of the necrotic enteritis in avian and new control and prevention strategies are needed.

Conflict of Interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence the content of the paper.

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