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, cephalaxin and bacitracin and sulfquinolaxin. 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 also 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 enterotoxaemia 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 determined.
Materials and Methods

Intestinal Samples

Intestinal pieces from 96 chickens with necrotic enteritis (marked depression, decreased appetite, ruffled feathers, enteritis and diarrhoea); and 83 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% N₂, 10% CO₂.). Aliquots of 0.1 mL were streaked onto tryptcase 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 lecinitase producing, were isolated for identification by biochemical tests [16]. 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.

Toxotyping by PCR

Bacterial DNA was obtained from a colony grown in BHI according to Sambrook, et al. [15]. Briefly, bacteria were harvested by centrifugation and pellets were twice washed with PBS (pH 7.2). Pellet was resuspended in 1 mL of buffer (10 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂) and incubated at 95°C (5 min) and 56°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, cephealin, 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 10⁴ 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 α 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 cpe, etx, iap, cpa2 and netB genes. Clostridium perfringens strains were not isolated from the evaluated healthy chickens.

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

<table>
<thead>
<tr>
<th>Gene (Toxin)</th>
<th>Genetic localization</th>
<th>Sequence (5'→3')</th>
<th>Amplicon Reference (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpa (a)</td>
<td>Chromosome</td>
<td>AGCTACGCTGGGATGGAATTTCTGGGTTGTCATTTC</td>
<td>900 [16]</td>
</tr>
<tr>
<td>cpb (b)</td>
<td>Plasmid</td>
<td>TCCTTTTTCGGAGGAGAATAAGTAGAATGATTAAT</td>
<td>611 [16]</td>
</tr>
<tr>
<td>cpe (e)</td>
<td>Chromosome</td>
<td>ACCACGCGGATTGATTTAATAATGGTGAAGTACACACACC</td>
<td>506 [16]</td>
</tr>
<tr>
<td>ect (e)</td>
<td>Plasmid</td>
<td>TAAATCTCCTCCACAATAATTCGAC</td>
<td>396 [16]</td>
</tr>
<tr>
<td>iap (i)</td>
<td>Plasmid</td>
<td>AAGCGATTTAAGCTCATACCAGCTGCCATACTCGCATACCATGAC</td>
<td>293 [16]</td>
</tr>
<tr>
<td>cpg (2b)</td>
<td>Plasmid</td>
<td>GCAGCATGAGCATTCCATCCATCCGATAGTGGGCT</td>
<td>200 [16]</td>
</tr>
<tr>
<td>netB</td>
<td>Plasmid</td>
<td>GGCGGATCGGAAATGATGCCGTGCAGGATTGTTGATTTCC</td>
<td>384 [4]</td>
</tr>
</tbody>
</table>

*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 MgCl₂, 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.

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<sub>90</sub> values (≤ 0.25 µg/mL - 0.5 µg/mL). High MIC<sub>90</sub> values to sulfamethoxaxin, bacitracin, clindamycin, cephalaxin and erythromycin (64 µg/mL - ≥ 512 µg/mL) were observed. Most of the strains were resistant to sulfamethoxaxin (100%), erythromycin (95%), clindamycin (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

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<sub>90</sub> ≤ 0.25 µg/mL in accordance with Ghomaei-Dehkordi etal. [31]. On the other hand, our results divergent with those reported by Gharaibeh etal. [10] showing a value of MIC<sub>90</sub> = 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 Salmonella.
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 MIC20 and MIC90 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 MIC90 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 ermB 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 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 sulfaguanxalin 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 inappropriate-ly influence the content of the paper.

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