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## Genotyping and antimicrobial susceptibility of *Clostridium perfringens* isolated from *Tinamidae*, *Cracidae* and *Ramphastidae* species in Brazil

Genotipagem e sensibilidade antimicrobiana de estirpes de *Clostridium perfringens* isolados de espécies de *Tinamidae*, *Cracidae* e *Ramphastidae* no Brasil

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### ABSTRACT

The aim of this study was to isolate, genotype and evaluate the antimicrobial susceptibility of *Clostridium perfringens* found in species *Tinamidae*, *Cracidae* and *Ramphastidae* in Brazil. *C. perfringens* was isolated in 13 (5%) out of 260 swab samples and five (8.3%) out of 60 stool samples. All strains were classified as *C. perfringens* type A, and nine (50%) were positive for the beta-2 toxin-encoding gene. No strains were positive for the necrotic enteritis toxin B-like (NetB)-encoding gene. All isolates were susceptible to penicillin, metronidazole and vancomycin, whereas four (22.2%), five (27.8%) and 13 (72.2%) strains were considered resistant to erythromycin, oxytetracycline and lincomycin, respectively.

**Key words:** necrotic enteritis, avian, toucan.

### RESUMO

O objetivo do presente estudo foi isolar, genotipar e avaliar a sensibilidade antimicrobiana de estirpes de *Clostridium perfringens* de espécies de *Tinamidae*, *Cracidae* e *Ramphastidae* no Brasil. *C. perfringens* foi isolado de 13 (5%) dos 260 suabes e de cinco (8,3%) das 60 amostras de fezes. Todos os isolados foram classificados como *C. perfringens*, tipo A, e nove (50%) foram positivos para o gene *cpb2*, responsável pela produção da toxina beta-2. Nenhuma estirpe foi positiva para o gene que codifica a produção da toxina NetB. Todos os isolados avaliados foram sensíveis à penicilina, metronidazol e vancomicina, enquanto que quatro (22,2%), cinco (27,8%) e 13 (72,2%) foram considerados resistentes à eritromicina, oxitetraciclina e lincomicina, respectivamente.

**Palavras-chave:** enterite necrótica, aves, tucanos.

### INTRODUCTION

*Clostridium perfringens* is a spore-forming anaerobic bacillus that is a common

environmental bacterium and can be isolated from the intestines of avian and mammals (SIQUEIRA et al., 2012). In broiler chicken, it is responsible for necrotic enteritis (NE) and hepatitis (HIBBERD et al., 2011). In addition to the economic importance of *C. perfringens* in poultry, it also constitutes a public health risk through the food chain because it is one of the most frequently isolated pathogens in foodborne disease outbreaks in humans (GOULD et al., 2013).

Despite some case reports of NE (MCORIST & REECE, 1992; ASAKA et al., 2004; HAGEN & BILDFELL, 2007; URIBE et al., 2008) the role of *C. perfringens* in wild birds is still unclear. For many avian species, it is unknown whether this microorganism is part of the normal microbiota; moreover, the specific genotypes present in each species or family have not been elucidated. In addition, NE pathogenesis is not well understood, in poultry and undetermined in wild birds (SLAVIĆ et al., 2011).

In broiler chicken, studies showed that a plasmid-encoded pore-forming toxin called necrotic enteritis toxin B-like (NetB) is an important virulence factor for the disease (KEYBURN et al., 2008). Due to the absence of studies with wild birds, the importance of NetB toxin of *C. perfringens* are unknown.

In addition, the treatment of NE is still based on oral antimicrobials (LENSING et al., 2010). Therefore, evaluating the minimal antimicrobials concentration that inhibits *C. perfringens* strains isolated from wild birds could be useful to guide the treatment of sporadic cases of enteric disease in this species.

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Despite the great diversity of the Brazilian bird fauna, little is known about pathogens that can inflict diseases in free range and captive native birds (CATÃO-DIAS, 2008). The aim of this study was to isolate, genotype *C. perfringens* strains found in Tinamidae, Cracidae and Ramphastidae species in Brazil, as well as to evaluate the antimicrobial susceptibility profile.

## MATERIAL AND METHODS

### Samples

Apparently healthy Ramphastids (n=128), Cracids (n=131) and Tinamids (n=61) from 13 captive facilities for hobbyist, commercial and conservational purposes in Minas Gerais, Brazil were sampled. All birds were kept in captivity for various lengths of time in single, pair or collective enclosures with access to the floor, and all were fed with a mix of fruits and commercial rations without antimicrobials or anticoccidian. Cloacal swabs were taken from 260 birds, while 60 stool samples were collected in sterile containers whenever birds defecated during physical examination or swabbing.

### Isolation and genotyping of *Clostridium perfringens*

For isolation, 0.08 to 0.12g of stool was serially diluted by a factor of 10 in the range from  $10^{-1}$  to  $10^{-6}$ . Aliquots of approximately 50µl of each dilution were plated on sulfite polymyxin sulfadiazine agar (SPS, Difco Laboratories, USA) (SILVA et al., 2013a). Swab samples were streaked directly onto the SPS agar plates. All plates were incubated anaerobically at 37°C for 24 hours. After incubation, characteristic colonies from each dilution were collected and suspended in 400µl of sterile Milli-Q water. After DNA extraction (BAUMS et al., 2004), genes encoding beta-2 toxin (*cpb2*), enterotoxin (*cpe*) and the major *C. perfringens* toxins (alpha, beta, epsilon and iota) were detected by multiplex PCR (VIEIRA et al., 2008) and NetB-encoding gene (*netb*) was detected as previously described monoplex PCR (KEYBURN et al., 2008). Amplifications were carried out in a thermocycler (Veriti 96 Well Thermal Cycler - Applied Biosystems, USA), and the products were visualized under UV light in a 2% agarose gel stained with ethidium bromide (Sigma-Aldrich, USA).

### Antimicrobial susceptibility

The minimal inhibitory concentration (MIC) was determined by the agar dilution method, as recommended by the *Clinical and Laboratory Standards Institute* (CLSI, 2011). The following

antimicrobials were evaluated: penicillin, lincomycin, oxytetracycline, erythromycin, vancomycin and metronidazole. For an assay control, the reference specimen *Bacterioides fragilis* (ATCC 25285) was used. For the antimicrobials, the following concentrations were tested: 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0, 128.0 and 256.0µg ml<sup>-1</sup>. Tests were performed on *Brucella* agar (Difco Laboratories, USA) supplemented with 5% horse blood, hemin and vitamin K (CLSI, 2011).

### Statistical analysis

Fisher's exact test was used to evaluate associations between variables. Significance was set at a p-value of <0.05 (STATA, College Station, Texas, EUA).

## RESULTS

Of the 320 samples tested, including 260 from swabs (81.2%) and 60 from stool (18.8%), *C. perfringens* strains were isolated from 18 (5.6%). The isolation rate was similar in stools (5/60; 8.3%) or in swabs samples (13/260; 5%) meaning no statistical difference ( $p=0.348$ ). All 18 strains were genotyped as *C. perfringens* type A, and nine of them (50%) were positive for the beta-2 toxin gene (*cpb2*); however, none were positive for the NetB-encoding gene or the enterotoxin-encoding gene (*cpe*). Table 1 summarizes the results of the isolation and genotyping analyses. All isolated strains were susceptible to penicillin, metronidazole and vancomycin, whereas four (22.2%), five (27.8%) and 13 (72.2%) strains were considered resistant to erythromycin, oxytetracycline and lincomycin, respectively (Table 2).

## DISCUSSION

To the best of the authors' knowledge, this is the first description of genotyping and antimicrobial susceptibility of *C. perfringens* in captive wild birds' population. Unfortunately, this lack of data hinders any comparisons, as there are only sporadic case reports about this microorganism (MCORIST & REECE, 1992; ASAOKA et al., 2004; HAGEN & BILDFELL, 2007; URIBE et al., 2008). The detection of *C. perfringens* type A corroborates previous studies involving birds and other species, including humans, as this genotype is the most commonly isolated from stool samples and from the environment (CRESPO et al., 2007; GOMES et al., 2008; VAN ASTEN et al., 2010; SIQUEIRA et al., 2012). The absence of strains positive for the enterotoxin-encoding gene (*cpe*) is

Table 1 - Isolation and genotyping of *Clostridium perfringens* from swabs and stool samples from the *Ramphastidae*, *Tinamidae* and *Cracidae* species in Brazil.

Family		-----Samples-----			Number of isolated strains (%) <sup>1</sup>	Number of positive <i>cpb2</i> strains (%) <sup>2</sup>
		Swabs	Stool	Total		
<i>Ramphastidae</i>	<i>Ramphastos toco</i>	73	1	74	10 (13.5)	3 (30)
	<i>R. dicolorus</i>	21		21	1(4.8)	0 (0)
	Other species	33		33	1 (3.0)	0 (0)
	Total	127	1	128	12 (9.4) <sup>a</sup>	3 (25)
<i>Tinamidae</i>		43	18	61	4 (6.6) <sup>a</sup>	2 (50)
<i>Cracidae</i>		90	41	131	2 (1.5) <sup>b</sup>	1 (50)
Total		260	60	320	18 (5.6)	9 (50)

<sup>1</sup> - Percentage relative to the number of samples per line.<sup>2</sup> - Percentage relative to the number of isolated strains per line.

Values within a column with unlike superscript letters were significantly different (P&lt;0.01)

not surprising because these strains are rarely found in avian samples (CRESPO et al., 2007; GOMES et al., 2008) although they are common in isolates from dog and human feces (MARKS et al., 2011; SANZ et al., 2011; SILVA et al., 2013b).

Despite the large number of samples, *C. perfringens* was found in only a few animals, raising the possibility that it is not commonly part of the microbiota of these three families, in contrast with domestic avian species (CRESPO et al., 2007; GOMES et al., 2008). This suspicion is stronger to Cracidae once the isolation frequency of this group were lower than for Tinamidae and Ramphastidae (P<0.001). Anyway, more studies are needed to clarify whether *C. perfringens* is commensal in these species. It also important to note that although the birds are wild species, these animals were kept in captivity, which could affect the isolation rate and could be a confounding factor in this study.

Ramphastids do not have ceca but those in Cracids and Tinamids are well developed, and, according to GERLACH (1994), *C. perfringens* colonizes mainly in the ceca and is rarely found in birds where this organ is residual or absent. In light of this, in contrast with the present results, one would expect to isolate the bacteria at higher rates from Cracids and Tinamids than from Ramphastids. Additionally, Gerlach's statement (GERLACH, 1994) could explain the low isolation rate for Ramphastids but not for the other families.

The presence of strains positive for the beta-2-encoding gene (*cpb2*) was previously reported in wild avian species (CRESPO et al., 2007; VAN ASTEN et al., 2010). Detection of the *cpb2* gene

is associated with the occurrence of disease in some domestic animals, such as horses and swine (SCHOTTE et al., 2004; VIEIRA et al., 2008; SILVA et al., 2013c). On the contrary, some authors reported that the *cpb2* gene is more common in healthy broiler chickens than in animals with NE (CRESPO et al., 2007; SLAVIĆ et al., 2011). Although the importance of the beta-2 toxin in the pathogenesis of avian NE is unclear, the percentage of *C. perfringens* carrying this gene (50%) is considerable among wild birds, and the role of this gene should be investigated further.

Another additional virulence factor of *C. perfringens* that has gained importance is the toxin NetB. Recent studies have shown a high correlation between the detection of the NetB-encoding gene and the occurrence of NE in broiler chickens and in turkeys (KEYBURN et al., 2008; ALLAART et al., 2012; LYHS et al., 2013). In the present study, there were no *C. perfringens* strains positive for

Table 2 - Classification (susceptible, intermediate and resistance) of the 18 *Clostridium perfringens* strains isolated from *Ramphastidae*, *Tinamidae* and *Cracidae* species in Brazil.

Antimicrobial	-----Classification (%)*-----		
	Susceptible	Intermediate	Resistance
Penicillin	18 (100)	0	0
Metronidazole	18 (100)	0	0
Vancomycin	18 (100)	0	0
Erythromycin	14 (77.8)	0	4 (22.2)
Oxytetracycline	7 (38.9)	6 (33.3)	5 (27.8)
Lincomycin	5 (27.8)	0	13 (72.2)

\*According to CLSI (2011) and EUCAST (2011).

the presence of this gene. This work is the first to investigate this gene in *C. perfringens* isolates from wild birds. However, only apparently healthy animals were included in the study. Thus, further studies with diseased are needed to evaluate the relationship between the presence of the NetB-encoding gene and the occurrence of NE in wild birds and the possibility of using it as a diagnostic marker.

Studies evaluating the antimicrobial susceptibility of *C. perfringens* isolates from avian species, even for broiler chickens, are scarce, and for wild avian species, these data are extremely rare. In the present report, all of the isolated strains were susceptible to penicillin, vancomycin and metronidazole, corroborating other studies with domestic avian species and other animal species (WATKINS et al., 1997; MARTEL et al., 2004; CHALMERS et al., 2008; SALVARANI et al., 2012; SILVA et al., 2013a). Despite the reported decreased sensitivity of *C. perfringens* isolates from cattle and swine to beta-lactam antimicrobials (SASAKI et al., 2001; SLAVIĆ et al., 2011) penicillin inhibited all of the strains at the lowest concentration tested (0.25 mg/L). Resistance of *C. perfringens* to metronidazole is also rare but has already been shown by other investigations in chicken (SLAVIĆ et al., 2011). Those results were interpreted as an inherent lower susceptibility to these drugs and not as resistance mediated by acquired genes or mutations.

In the present report, four (22.2%) strains were resistant to erythromycin. Resistance to erythromycin was previously reported in *C. perfringens* isolates from cattle and swine (SLAVIĆ et al., 2011). It is also important to note that a clear bimodal distribution could be observed: 14 (77.8%) strains were inhibited with 0.5mg L<sup>-1</sup> and the remaining four strains (22.2%) were inhibited only with 256mg L<sup>-1</sup>. This type of distribution suggests a genetic mechanism of resistance. This result is likely due to the presence of the *ermQ* gene, which encodes an erythromycin resistance methylase (BENNING & MATHERS, 1999; SLAVIĆ et al., 2011). This resistance gene was previously described in *C. perfringens* isolates from several domestic animals (SLAVIĆ et al., 2011) but until now it was not found in wild avian isolates.

For oxytetracycline and lincomycin, five (27.8%) and 13 (72.2%) strains were considered resistant, respectively. For both antimicrobials, similar results have already been described for *C. perfringens* isolates from various species including poultry, cattle, dogs, foals, swine and humans (WATKINS et al., 1997; MARTEL et al., 2004; SILVA

et al., 2009; SLAVIĆ et al., 2011; SALVARANI et al., 2012; SILVA et al., 2013a). It has previously been reported that *Clostridium* species can carry tetracycline resistance genes that encode a ribosome-protecting cytoplasmic protein (CHOPRA & ROBERTS, 2001). On the other hand, resistance gene studies for lincomycin are poorly reported, and, according to MARTEL et al. (2004), resistance to this compound may be due to as-yet unknown genes.

Three strains (16.7%) were considered multi-drug resistant because they had high MIC values for three different antimicrobials (oxytetracycline, erythromycin and lincomycin); two of these strains were isolated from two *Ramphastos toco* sampled in different sites, and one was isolated from a *Tinamidae*. There was no association between the presence of the *cpb2* gene and the resistance pattern, in contrast with previously reported for *C. perfringens* isolates from piglets (SALVARANI et al., 2012).

Evaluation of antimicrobial susceptibility of *C. perfringens* isolates might be useful in the treatment of this enteric disease in wild birds. In addition, the present report highlights the need for more studies to clarify the role of *C. perfringens* as commensal or pathogenic in these species. The next step of this study is to evaluate the similarity between *C. perfringens* isolated from wild avian species and broiler chicken, which may clarify differences between these strains and help to explain the role of wildlife in the dissemination and transmission of *C. perfringens* to domestic animals.

## BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

All procedures performed were approved by the Ethics Committee on Animal Experiments of the Universidade Federal de Minas Gerais (CETEA-UFMG, protocol number 014/11) and by Chico Mendes Institute for Biodiversity Conservation (SISBIO/ICMBio, protocol number 24619-1).

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