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14 **Abstract**

15 Necrotic enteritis in broiler chickens is associated with *Clostridium perfringens* type A , carrying
16 the NetB toxin. *C. perfringens* type A is also a member of the normal intestinal microbiota of
17 broilers. Clinically healthy chickens carry several different *Clostridium perfringens* clones in
18 their intestine. In flocks suffering from necrotic enteritis, however, mostly only one single clone
19 is isolated from the gut of all the diseased animals. Selective proliferation of these clinical
20 outbreak strains in the gut and spread within the flock seems likely, but an explanation has not
21 yet been given. The hypothesis that necrotic enteritis associated *Clostridium perfringens* strains
22 might suppress the growth of normal microbiota *Clostridium perfringens* strains, was therefore
23 tested. Twenty-six *Clostridium perfringens* strains isolated from healthy broilers and 24 clinical
24 outbreak isolates were evaluated for their ability to induce intra-species growth-inhibition in an *in*
25 *vitro* setup. A significantly higher proportion of the *Clostridium perfringens* clinical outbreak
26 strains inhibited the growth of other *Clostridium perfringens* strains compared to *Clostridium*
27 *perfringens* strains isolated from the gut of healthy chickens. It is proposed that, in addition to
28 toxin production, intra-species inhibition may be a virulence trait that contributes to the ability of
29 certain *Clostridium perfringens* strains to cause necrotic enteritis in broilers.

30

31 *Keywords:* *Clostridium perfringens* / broiler / necrotic enteritis / growth-inhibition

32

32 **1. Introduction**

33 Since the ban on growth-promoting antibiotics in animal feed in the European Union,
34 *Clostridium (C.) perfringens* associated necrotic enteritis is reemerging in broilers (Grave *et al.*,
35 2004; Van Immerseel *et al.*, 2004; Williams, 2005). Necrotic enteritis in poultry is associated
36 with *C. perfringens* type A, carrying the NetB toxin (Keyburn *et al.*, 2008; Van Immerseel *et al.*,
37 2008). *C. perfringens* type A is also a member of the normal intestinal microbiota of broilers.
38 Strains isolated from healthy broilers, however, do not induce necrotic enteritis in an
39 experimental model using predisposing factors, in contrast to strains isolated from outbreaks of
40 necrotic enteritis (Timbermont *et al.*, 2008).

41 In *C. perfringens* isolates from healthy birds, a high degree of genetic diversity is found,
42 even between isolates from the same animal. In contrast, different isolates from a flock suffering
43 from a clinical outbreak are generally of the same Pulsed Field Gel Electrophoresis (PFGE) type,
44 regardless of the animal or the part of the intestine from which the strain was isolated (Nauerby *et*
45 *al.*, 2003; Gholamiandehkordi *et al.*, 2006). The reason for the presence of a single clone in
46 necrotic enteritis outbreaks is not known. It is speculated that during an outbreak, certain
47 *C. perfringens* strains have a competitive advantage over other *C. perfringens* strains in the
48 broiler gut.

49 In the present study, strains isolated from healthy broilers and strains isolated from
50 broilers suffering from necrotic enteritis were compared with respect to their capacity of intra-
51 species growth-inhibition in an *in vitro* inhibition assay.

52

53 **2. Materials and methods**

54 *2.1. Bacteria*

55 Fifty *C. perfringens* type A strains belonging to different genotypes, as analyzed by
56 PFGE, were included. Thirty-five strains were isolated from broiler chickens in Belgium: 26
57 strains from clinically healthy broiler chickens and 9 strains from broilers suffering from necrotic
58 enteritis (Gholamiandehkordi et al., 2006). Fifteen Danish *C. perfringens* isolates from necrotic
59 enteritis cases were kindly provided by Dr. L. Bjerrum (Nauerby et al., 2003).

60 Strains of *C. perfringens* were grown on Colombia agar (Oxoid, Basingstoke, UK)
61 containing 5% defibrinated sheep blood. Plates were incubated at 37°C in an anaerobic working
62 cabinet (invivo₂500, Ruskinn Life Sciences, Bridgend, UK), in an atmosphere of
63 8%H₂:8%CO₂:84%N₂. Strains were stored at -80°C in lyophilisation medium (LYM) containing
64 7% glucose, 23% Brain Heart Infusion broth (BHI, Oxoid) and 70% defibrinated horse serum
65 (Invitrogen, Merelbeke, Belgium).

66

67 2.2. *In vitro* growth-inhibition assay

68 The 50 *C. perfringens* strains were used in a checkerboard test for intra-species growth-
69 inhibition. Each strain was cultured anaerobically in BHI broth (Oxoid) for 24 h at 37°C. The
70 overnight cultures were diluted in Phosphate Buffered Saline (PBS) to a density of McFarland
71 No. 0.5, and 200 µl of these suspensions were spread with a sterile swab on the whole surface of
72 BHI agar plates to obtain a bacterial lawn (12 cm x 12 cm). A single colony of each
73 *C. perfringens* isolate was transferred with a sterile toothpick to the agar plates seeded with the
74 different *C. perfringens* strains. Absence of growth of the bacterial lawn around a colony results
75 in an inhibition zone (Fig. 1). After overnight incubation under anaerobic conditions, diameters
76 of inhibition zones were measured in mm. The tests were performed in triplicate.

77

78 2.3. Statistical analysis

79 The data were analysed with SPSS 16 software using the chi-square test to compare the
80 number of strains of the normal microbiota group with number of strains of the necrotic enteritis
81 group that were able to inhibit other strains. The student t-test was used to compare the mean
82 inhibition zones of the healthy animal strains and the necrotic enteritis strains. Significance was
83 determined at $P < 0.05$.

84

85 **3. Results**

86 Sixty percent of all tested strains inhibited growth of at least one other strain of
87 *C. perfringens* (i.e., there was a zone of clearing around the stabbed colony). While some strains
88 inhibited growth of many other strains, others had a very limited inhibitory spectrum. Fifteen
89 (58%) of the 26 healthy animal strains and 4 (17%) of the 24 clinical outbreak strains were not
90 able to inhibit the growth of any other strain. In contrast, 46% (11/24) of the clinical outbreak
91 strains were able to inhibit more than 90% (more than 45/50) of the other strains while this was
92 only the case for 15% (4/26) of the healthy animal strains (Fig. 2). The number of strains that
93 were able to inhibit any number of other strains was significantly higher for the clinical outbreak
94 strains than for the healthy animal strains ($P < 0.05$). If strains were able to inhibit the growth of
95 other *C. perfringens* strains, they were able to inhibit both healthy animal strains and necrotic
96 enteritis outbreak strains.

97 In the strains that were able to inhibit, the zones of inhibition varied for the strains from
98 healthy broilers between 3 mm and 7 mm and for the necrotic enteritis strains between 3.5 mm
99 and 5.5 mm. There was no significant difference in the average sizes of the inhibition zones: 4.9
100 mm and 4.3 mm for healthy animal strains and clinical outbreak strains, respectively.

101

102 **4. Discussion**

103 Intra-species inter-strain growth-inhibition can explain the presence of a single clone of
104 *C. perfringens* in a broiler flock suffering from necrotic enteritis. Our results show that inhibition
105 of other *C. perfringens* strains is a trait that is significantly more developed in clinical outbreak
106 strains, compared with healthy animal strains. A minority of healthy animal strains were able to
107 inhibit other *C. perfringens* strains isolated from healthy birds and necrotic enteritis strains. In a
108 recent study of Barbara et al. (2008), none of the normal flora strains (n=17) could inhibit the
109 growth of other *C. perfringens* strains. Also a minority of necrotic enteritis outbreak strains did
110 not have the intra-species growth-inhibitory phenotype. Possibly in these strains other virulence
111 traits may compensate for the lack of inhibitory capacity. Another possible explanation for the
112 lack of *in vitro* inhibitory phenotype is that expression of the gene encoding for the growth-
113 inhibiting phenomenon can be down-regulated by certain signal transduction systems or
114 regulators (Dupuy and Mamouros, 2006). The gene for the growth-inhibiting factor might also be
115 located on a plasmid and the absence of growth-inhibition may be caused by the loss of this
116 plasmid after *in vitro* cultivation (Rood and Cole, 1991).

117 The results of the radial diffusion assay suggest that the intra-species inter-strain growth-
118 inhibition is caused by a substance secreted by the bacterium into its micro-environment. The
119 nature of this substance is hitherto unclear. It is known that *C. perfringens* is able to produce
120 bacteriocins capable of lysing other *C. perfringens* strains. Watson et al. (1982) observed that
121 79% of strains implicated in food poisoning outbreaks produced bacteriocins; however, only 18%
122 of 322 isolates from the feces of healthy persons, human and animal infections, various foods,
123 and the environment produced bacteriocins suggesting a relationship between bacteriocin
124 production and the ability to cause food poisoning.

125 In this study, secretion of intra-species growth-inhibitory substances by *C. perfringens* is
126 proposed to be an additional virulence trait that can possibly contribute to the pathogenesis of

127 necrotic enteritis by selective proliferation of one single clone in the broiler gut. Indeed, when
128 certain environmental predisposing factors, such as a coccidial infection, are present, nutrients
129 utilizable by *C. perfringens* are leaking in the lumen (Collier et al., 2008). Intra-species inter-
130 strain growth inhibition may constitute an important advantage for certain strains in the
131 competition for nutrients in the intestinal tract. It is well documented that *C. perfringens* requires
132 at least 11 different essential amino acids for its multiplication (Petit et al., 1999). Suppressing its
133 competitors may allow unlimited access to the nutrients and thus the possibility of explosive
134 multiplication. The massive production of specific toxins and enzymes, such as the NetB toxin
135 and proteolytic enzymes (Olkowski et al., 2006; Olkowski et al., 2008; Keyburn et al., 2008) at
136 sites of multiplication of these bacteria in the gut would then lead to necrotic lesions, and thus the
137 release of more nutrients, completing the cycle.

138 In conclusion, *C. perfringens* outbreak isolates are significantly more capable of intra-
139 species growth inhibition compared to strains isolated from healthy birds. It is proposed that
140 intra-species growth-inhibition by *C. perfringens* is a possible virulence trait in necrotic enteritis
141 in broilers.

142

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149

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- 197

197 **Figure 1**

198 Result of the radial diffusion assay. A colony of a *C. perfringens* isolate was stabbed through a
199 lawn of another *C. perfringens* strain and partially through the agar beneath. A. No inhibition B.
200 Clear inhibition zone

201

202

203 **Figure 2**

204 Percentage of *C. perfringens* strains secreting intra-species inhibitory substances. The percentage
205 of healthy animal strains (grey) and clinical outbreak strains (black) of *C. perfringens* that were
206 able to inhibit a number of *C. perfringens* strains (N = 50), as indicated on the X-axis, is shown.

207





