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**Intra-species growth-inhibition by *Clostridium perfringens* is a possible virulence trait in
necrotic enteritis in broilers**

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Abstract

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

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

1. Introduction

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

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

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

2. Materials and methods

2.1. Bacteria

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

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

2.2. *In vitro* growth-inhibition assay

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

2.3. Statistical analysis

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

3. Results

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

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

4. Discussion

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

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

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

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

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

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Figure 1

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

Figure 2

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