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Short communication

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 *C. 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 *C. perfringens* strains might suppress the growth of normal microbiota *C. perfringens* strains, was therefore tested. Twenty-six *C. 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 *C. perfringens* clinical outbreak strains inhibited the growth of other *C. perfringens* strains compared to *C. perfringens* strains isolated from the gut of healthy chickens. It is proposed that, in addition to toxin production, intra-species growth-inhibition may be a virulence trait that contributes to the ability of certain *C. perfringens* strains to cause necrotic enteritis in broilers.

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1. Introduction

Since the ban on growth-promoting antibiotics in animal feed in the European Union, *Clostridium* (*C.*) *perfringens* associated necrotic enteritis is re-emerging 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

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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 (invivo$_2$500, Ruskinn Life Sciences, Bridgend, UK), in an atmosphere of 8%H_2:8%CO_2:84%N_2. 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

Fifty *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 × 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 analyzed 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's *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.

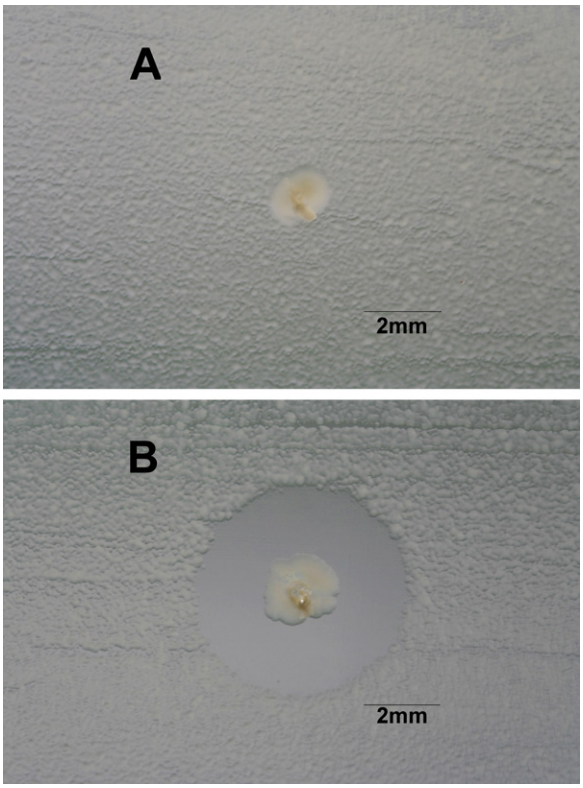

Fig. 1. Result of the radial diffusion assay. A colony of a *C. perfringens* isolate was stabbed through a lawn of another *C. perfringens* strain and partially through the agar beneath. (A) No inhibition. (B) Clear inhibition zone.

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

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