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Genetic diversity and prevalence of *netB* in *Clostridium perfringens* isolated from a broiler flock affected by mild necrotic enteritis

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ABSTRACT

This study was undertaken to examine the genetic diversity of *Clostridium perfringens* isolated from a single broiler flock reared without in-feed antimicrobials (antibacterial growth promoters and anticoccidials) and affected by mild necrotic enteritis (NE). We used pulsed-field gel electrophoresis (PFGE) to investigate the genetic diversity of *C. perfringens* isolates from broilers of varying disease status, and from litter. The prevalence of the toxin gene *netB* was also investigated. Altogether 32 PFGE genotypes were found among 88 isolates. Several genotypes were detected in *C. perfringens*-associated organ lesions from chickens that were sampled at random and alive without clinical symptoms, suggesting that these genotypes proliferated concurrently in such lesions. More than 90% of all isolates from NE-specific organ lesions carried *netB* which codes for a recently described pore-forming toxin. *NetB* positive isolates were less predominant in non-lesion samples from broilers affected by NE, and found infrequently or not at all in healthy birds and isolates from litter.

These findings show that the presence of *netB* in *C. perfringens* strains is associated with NE and suggest that mild NE differs from severe NE with regard to *C. perfringens* genotype diversity.

1. Introduction

C. perfringens is a common finding in the intestinal tract of healthy chickens. But this bacterium can also induce production loss and disease, which currently is of great concern in the poultry meat production. The severity of disease induced by *C. perfringens* varies on a continuous scale from no clinical symptoms but slightly impaired production and minor mucosal ulcers and pseudomembranes (subclinical necrotic enteritis) (Kaldhusdal and Hofshagen, 1992) to severe clinical outbreaks with high daily mortality (clinical necrotic enteritis) (Al-Sheikhly and Truscott, 1977a,b; Long et al., 1974). Flocks that have been affected by any degree of NE during the rearing period show increased frequencies of *C. perfringens*-associated liver lesions at slaughter (Lovland and Kaldhusdal, 1999; Randall, 1991). The most severe of such lesions may cause condemnation of not only of the liver but the whole carcass. Lovland and Kaldhusdal (1999) demonstrated that the level of such condemnations at slaughter may be a good indicator of NE occurrence during the rearing period, providing appropriate inspection routines are practised. Cases of subclinical NE and cases with mild clinical symptoms but normal mortality may be economically even more important than outbreaks causing increased mortality, because such disease is more prevalent, usually or often not detected during rearing and therefore not treated, and consequently allowed to induce significant production losses (Dahiyaa

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et al., 2006; Kaldhusdal and Hofshagen, 1992; Kaldhusdal et al., 2001; Stutz and Lawton, 1984).

Genetic diversity among *C. perfringens* isolates originating from poultry has previously been investigated by pulsed-field gel electrophoresis (PFGE) (Chalmers et al., 2008b; Engstrom et al., 2003; Gholamiandekhordi et al., 2006; Nauerby et al., 2003). Other molecular typing methods such as Multiple Locus Variable Number Tandem Repeat Analysis (MLVA) (Chalmers et al., 2008c) and multilocus sequence typing (MLST) (Chalmers et al., 2008a) have also been used. The results of these studies are not easily compared due to differences in methodology. Previous PFGE studies have shown a high degree of genetic diversity among isolates from healthy birds but little genetic diversity among isolates from clinical outbreaks of NE (Cooper and Songer, 2009; Timbermont et al., 2009). These studies apparently have not examined genotype diversity among isolates from cases of NE from flocks with normal mortality.

Recent studies indicate that alpha-toxin, which was believed to be the major virulence factor in NE, is not an essential factor in disease development (Keyburn et al., 2006; Van Immerseel et al., 2008). Furthermore, a new toxin designated NetB has been detected in *C. perfringens* strains isolated from birds affected by NE, and this toxin has been demonstrated to be associated with experimental NE (Keyburn et al., 2008). The prevalence of *netB* in *C. perfringens* isolates was recently shown to be substantially higher in samples from outbreaks of NE than in samples from outbreak free flocks (Chalmers et al., 2008b; Martin and Smyth, 2008).

Data presented in this study are based on samples from a commercial broiler flock with normal accumulated mortality, where necrotic enteritis was detected due to a close surveillance and not because of clinical symptoms. The aim of this investigation was to examine the genetic diversity of *C. perfringens* in the broiler flock. Further, the prevalence of the toxin gene *netB* of *C. perfringens* isolated from such a broiler flock was investigated.

2. Materials and methods

2.1. Study flock

The commercial broiler flock (Ross 208 hybrid) was started on May 11, 2004 and slaughtered on June 11, 2004. The broilers were reared on commercial feed without any coccidiostats or antibacterial growth promoters, but the chicks were vaccinated with an anticoccidial vaccine (Paracox 5®, Schering-Plough Animal Health) and given a competitive exclusion product (Broilact®, Orion Pharma Animal Health) at the hatchery. The broilers were reared on wood shavings in a rigorously controlled environment. The examined broilers were started 43 days after shipping of the previous flock to the slaughterhouse. This previous flock had been given feed supplemented with narasin (Elanco Animal Health) (declared inclusion rate 70 ppm). Between successive grow-outs, used litter was removed and the broiler house was cleaned and disinfected.

2.2. Sampling and cultivation

The flock was sampled on day 6, 14, 23, and 30. On each sampling day, 10 live chickens and one sample from five evenly distributed spots of surface litter were selected at random. On arrival at the laboratory, the live birds (in the following referred to as 'collected alive') were stunned (procedure approved by the governmental Norwegian committee for experimental animals, http://www.mattilsynet.no/fdu/) and post-mortem examination. Specimens for bacteriology were collected from 10 of the 40 birds collected alive during the rearing period (Table 1). Tissues examined included small intestinal pseudomembranes (D/J in Table 1; chickens with NE lesions only) caecal contents and mucosal surface of the gizzard.

The five litter samples from each sampling occasion were pooled prior to bacteriological examination.

If the farmer suspected disease, birds found dead in the morning were also collected and submitted to the laboratory on the same day, together with the live birds and the litter samples. Specimens from 4 birds found dead with NE or CPH (*C. perfringens*-associated hepatic change) were collected from tissues with lesions detected in the small intestine (mucosa with lesions) and the liver (the liver parenchyma and gall bladder), respectively. *C. perfringens* isolates were recovered from these samples.

The flock was slaughtered at 31 days of age; samples were collected from carcasses with CPH and NE lesions at

Table 1
PFGE genotypes found among *C. perfringens* isolates recovered from 14 broilers sampled (at random) during the rearing period. Chickens A–J were collected alive, K–N were found dead with necrotic enteritis or *C. pefringens*-associated hepatic disease.

Chicken	Age (days)	Tissue[a]	PFGE genotype[b]	NE[c]	CPH[d]
A	6	G	25, 26, 26	0	0
A	6	C	25, 25, 25	0	0
B	6	C	24, 24, deg[e]	0	0
C	14	G	28, 28, 28	0	0
C	14	C	25, **14**, 25	0	0
D	23	D/J	16, **16, 16**	1	0
D	23	G	15, 21,18	1	0
E	30	G	17, 17, 17	0	0
E	30	C	18, 18, 18	0	0
F	30	D/J	**30, 11, 11**	1	0
F	30	G	**3, 30**	1	0
F	30	C	**11, 11, 11**	1	0
G	30	C	26, **1, 1**	1	0
G	30	D/J	**1, 13**, 2	1	0
H	30	C	**11, 11, deg**[e]	1	0
H	30	D/J	**11, 11, 11**	1	0
I	30	D/J	**12, 12, 12**	1	0
J	30	D/J	32, **3, 4**	1	0
J	30	C	**3, 15, 3**	1	0
J	30	G	21, 29, 29	1	0
K	23	D/J	**5**	1	0
L	23	D/J	**7**	1	0
M	30	D/J	**3, 3,3**	1	0
M	30	C	**4, 4, 4**	1	0
N	30	L	**10, 10, 10**	0	1

[a] C, caecum; D/J, duodenum/jejunum; G, gizzard; L, liver.
[b] Bold, presence of the *netB* gene.
[c] Lesions in the mucosa.
[d] Lesions in the liver.
[e] DNA degraded by endonucleases.