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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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## 1 **Abstract**

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

17

18

19

## 20 **Keywords**

21 *Clostridium perfringens*, necrotic enteritis, *netB*, pulsed-field gel electrophoresis (PFGE),  
22 genotype diversity

23

24

## 25 1. Introduction

26 *C. perfringens* is a common finding in the intestinal tract of healthy chickens. But this  
27 bacterium can also induce production loss and disease, which currently is of great concern in  
28 the poultry meat production. The severity of disease induced by *C. perfringens* varies on a  
29 continuous scale from no clinical symptoms but slightly impaired production and minor  
30 mucosal ulcers and pseudomembranes (subclinical necrotic enteritis) (Kaldhusdal and  
31 Hofshagen, 1992) to severe clinical outbreaks with high daily mortality (clinical necrotic  
32 enteritis) (Al-Sheikhly and Truscott, 1977a, b; Long et al., 1974). Flocks that have been  
33 affected by any degree of NE during the rearing period show increased frequencies of *C.*  
34 *perfringens*-associated liver lesions at slaughter (Lovland and Kaldhusdal, 1999; Randall,  
35 1991). The most severe of such lesions may cause condemnation of not only of the liver but  
36 the whole carcass. Lovland and Kaldhusdal (1999) demonstrated that the level of such  
37 condemnations at slaughter may be a good indicator of NE occurrence during the rearing  
38 period, providing appropriate inspection routines are practised. Cases of subclinical NE and  
39 cases with mild clinical symptoms but normal mortality may be economically even more  
40 important than outbreaks causing increased mortality, because such disease is more prevalent,  
41 usually or often not detected during rearing and therefore not treated, and consequently  
42 allowed to induce significant production losses (Dahiyaa et al., 2006; Kaldhusdal and  
43 Hofshagen, 1992; Kaldhusdal et al., 2001; Stutz and Lawton, 1984).

44 Genetic diversity among *C. perfringens* isolates originating from poultry has previously been  
45 investigated by pulsed-field gel electrophoresis (PFGE) (Chalmers et al., 2008b; Engstrom et  
46 al., 2003; Gholamiandekhordi et al., 2006; Nauerby et al., 2003). Other molecular typing  
47 methods such as Multiple Locus Variable Number Tandem Repeat Analysis (MLVA)  
48 (Chalmers et al., 2008c) and multi-locus sequence typing (MLST) (Chalmers et al., 2008a)  
49 have also been used. The results of these studies are not easily compared due to differences in

50 methodology. Previous PFGE studies have shown a high degree of genetic diversity among  
51 isolates from healthy birds but little genetic diversity among isolates from clinical outbreaks  
52 of NE (Cooper and Songer, 2009; Timbermont et al., 2009). These studies apparently have  
53 not examined genotype diversity among isolates from cases of NE from flocks with normal  
54 mortality.

55 Recent studies indicate that alpha-toxin, which was believed to be the major virulence factor  
56 in NE, is not an essential factor in disease development (Keyburn et al., 2006; Van Immerseel  
57 et al., 2008). Furthermore, a new toxin designated NetB has been detected in *C. perfringens*  
58 strains isolated from birds affected by NE, and this toxin has been demonstrated to be  
59 associated with experimental NE (Keyburn et al., 2008). The prevalence of *netB* in *C.*  
60 *perfringens* isolates was recently shown to be substantially higher in samples from outbreaks  
61 of NE than in samples from outbreak free flocks (Chalmers et al., 2008b; Martin and Smyth,  
62 2008).

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

68

## 69 **2. Materials and methods**

### 70 *2.1. Study flock*

71 The commercial broiler flock (Ross 208 hybrid) was started on May 11, 2004 and  
72 slaughtered on June 11, 2004. The broilers were reared on commercial feed without any  
73 coccidiostats or antibacterial growth promoters, but the chicks were vaccinated with an  
74 anticoccidial vaccine (Paracox 5<sup>®</sup>, Schering-Plough Animal Health) and given a competitive

75 exclusion product (Broilact<sup>®</sup>, Orion Pharma Animal Health) at the hatchery. The broilers were  
76 reared on wood shavings in a rigorously controlled environment. The examined broilers were  
77 started 43 days after shipping of the previous flock to the slaughterhouse. This previous flock  
78 had been given feed supplemented with narasin (Elanco Animal Health) (declared inclusion  
79 rate 70 ppm). Between successive grow-outs, used litter was removed and the broiler house  
80 was cleaned and disinfected.

81

## 82 2.2. *Sampling and cultivation*

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

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

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

99 The flock was slaughtered at 31 days of age; samples were collected from carcasses with CPH  
100 and NE lesions at the slaughterhouse. Specimens from carcasses (gall bladder and gut mucosa  
101 with lesions) were taken with cotton-tipped applicators and transported to the laboratory in  
102 tubes containing Amies medium with charcoal (Copan International, Corona, Italy).  
103 Materials from all specimens were plated on bovine blood agar and incubated anaerobically  
104 overnight at 37 °C. Bacterial colonies were identified as *C. perfringens*, based on colony  
105 morphology and characteristic double-zone haemolysis. From each plate, preferably three  
106 colonies with different appearances were picked for storage and further examination. The  
107 strains were identified as *C. perfringens* type A by applying standard biochemical tests and  
108 multiplex PCR (Johansson et al., 2006).

109

### 110 2.3. Pathology

111 NE in broilers collected alive was recorded if at least one mucosal ulcer or pseudomembrane  
112 (Novoa-Garrido et al., 2006) was detected in the small intestine. *C. perfringens*-associated  
113 hepatic change (CPH) in birds collected alive was recorded if (a) a distinct pattern  
114 characterized by small, pale islets in the parenchyma (cholangiohepatitis) (Randall, 1991) or  
115 (b) focal, subcapsular nodules (Lovland and Kaldhusdal, 1999; Randall, 1991) were detected.  
116 The small intestine and liver of birds found dead and submitted to the laboratory were  
117 examined to record pathological changes. NE was recorded if at least one intestinal  
118 pseudomembrane was detected. The presence of CPH was determined in the same way as in  
119 birds collected alive. At slaughter all carcasses were inspected for liver lesions by the local  
120 Food Safety Authority. Carcasses with liver lesions indicative of CPH were condemned and  
121 recorded in accordance with routine procedures. A previous study has demonstrated that the  
122 procedures used at this slaughterhouse were appropriate for monitoring occurrence of NE



123 (Lovland and Kaldhusdal, 1999). Furthermore, veterinarians at the slaughterhouse examined a  
124 number of carcasses for NE and CPH lesions, in order to verify the condemnation data.

125

#### 126 2.4. PCR and PFGE analysis

127 The four major toxin genes *plc*, *cpb1*, *iap* and *etx* were detected by multiplex PCR as  
128 described by (Johansson et al., 2006). *NetB* was detected by PCR as described by (Keyburn et  
129 al., 2008). The PFGE protocol of (Lukinmaa et al., 2002) was followed in all essentials. DNA  
130 was digested with *SmaI*. Electrophoresis was performed at 6 V/cm with 2.0% AgaroseNA  
131 agar (Amersham Biosciences, Uppsala, Sweden) by using the CHEF-DR II system (Bio-Rad  
132 Laboratories, Richmond, Cal. USA). Running conditions for *SmaI*-digested DNA were 0.5 to  
133 40 s for 20 h. A lambda ladder (size range 0.13 to 194 kb) (Low Range PFG Marker, New  
134 England Biolabs Inc., MA, USA) and *Salmonella* serotype Braenderup (H9812) digested with  
135 *XbaI*, as described in greater detail by Hunter and co-workers (Hunter et al., 2005) were used  
136 as molecular weight standards. The gels were visualized on an UV transilluminator and  
137 Polaroid photographs of the gels were scanned and images in tif file format were imported to  
138 GelCompar II (Applied Maths, Kortrijk, Belgium). Degrees of similarity between strains were  
139 calculated with 1.4% tolerance and 0.5% optimization by applying the band-based Dice  
140 similarity coefficient. Clustering analysis was performed with the unweighted pair group  
141 method (UPGMA), using average linkages. Gels were also analysed by visual interpretation  
142 of the banding patterns. Isolates with one or more differences between banding patterns were  
143 classified as different PFGE genotypes.

144

### 145 3. Results and discussion

146 3.1. Clinical findings, pathology and isolation of *C. perfringens*

147 Accumulated flock mortality at slaughter was normal (3.0 %). Mortality during the last week  
148 of the growth period was slightly higher than expected (0.9 %) with a modest peak on day 26  
149 (0.2 %) and a lower level (mean daily mortality 0.1 %) during the last three days of the grow-  
150 out (Fig. 1). No clinical symptoms were detected in any of the 40 birds collected alive during  
151 the rearing period. *C. perfringens*-associated lesions were found in 20.0 % of these birds.  
152 Seven out of eight randomly sampled birds with NE lesions were detected on day 30, one bird  
153 with NE lesions was found on day 23. These findings indicate that mild NE emerged at  
154 approximately 3 weeks of age and was prevalent (7/10 birds examined) on the day before  
155 slaughter. Our data suggest no clear covariation between NE occurrence and mortality, but an  
156 association with the modest mortality peak on day 26 cannot be excluded. The mortality data  
157 (Fig. 1) do not suggest that the disease was developing into a severe form during the last days  
158 of the grow-out. *C. perfringens* isolates were recovered from all 10 broilers that were  
159 collected alive and examined for bacteria (Table 1, chicken A to J), including four birds  
160 without NE lesions (Table 1, chicken A, B, C and E). Altogether 58 *C. perfringens* isolates  
161 from these 10 birds were successfully characterized.  
162 Seventeen broilers found dead in the house were submitted for laboratory examinations on  
163 days 23 and 30; NE or CPH was detected in eight of these 17 birds. Four of the eight NE/CPH  
164 birds were examined for *C. perfringens*. Isolates were recovered from all four, and a total of  
165 11 *C. perfringens* isolates were collected for further characterization (Table 1; chicken K to  
166 N).  
167 *C. perfringens* was isolated from all four pooled litter samples that were collected during the  
168 rearing period. A total of 11 isolates from these samples were successfully characterized  
169 (Table 2, strains L1 to L12).  
170 The condemnation percentage due to liver lesions at slaughter was 0.40, i.e. substantially  
171 higher than the mean of 49 narasin-treated broiler flocks (0.09%) raised in the same region

172 and slaughtered during roughly the same period of time (unpublished data from a field study  
173 conducted by one of the laboratories participating in the present study). The gall bladder from  
174 each of six condemned chickens were examined for *C. perfringens*, with positive results  
175 (Table 2, strains S1 to S6). Several carcasses with intestinal NE lesions (focal necroses) were  
176 detected, and the jejunum from each of three of these were examined for *C. perfringens*, with  
177 positive results (Table 2, strains S7 to S9).

178 These findings are consistent with previous results, indicating that NE can be prevalent in a  
179 flock free from obvious clinical findings (Kaldhusdal and Hofshagen, 1992; Kaldhusdal et al.,  
180 2001). In flocks affected by mild NE, the condemnation rate due to CPH is often increased  
181 (Lovland and Kaldhusdal, 1999) as was seen in the present flock.

182

### 183 3.2. PFGE analysis

184 A total of 91 isolates were recovered from 23 birds and four pooled litter samples.

185 Degradation of DNA by endonucleases prevented a PFGE analysis of three isolates. A total of  
186 32 PFGE genotypes were detected among the remaining 88 isolates (Fig. 2).

187 We have genotype data from a total of 12 NE birds collected alive (6 birds), found dead (3  
188 birds) or sampled at the slaughterhouse (3 birds). A total of 26 isolates from small intestinal  
189 NE lesions in these 12 birds were examined for PFGE patterns (Tables 1 and 2). Among these  
190 isolates a total of 14 genotypes were recorded. Further, at the individual NE bird level we  
191 examined three isolates from NE lesions from each of 7 individuals, and detected more than  
192 one genotype in 3 (43 %) of them. Also, in 2 of these 3 birds all 3 isolates from their NE  
193 lesions were different. We examined a total of 9 isolates from CPH livers in 7 individuals, and  
194 detected 5 different genotypes among these isolates (Tables 1 and 2). Finally, we examined  
195 isolates from at least two different tissues from a total of six birds (found dead or collected

196 alive) affected by NE, and found that 5 out of 6 (83 %) birds were colonized by more than one  
197 genotype.

198 These data indicate that numerous PFGE genotypes were prevalent in materials collected  
199 from *C. perfringens*-specific organ lesions in this flock suffering from mild NE. Further, even  
200 at the level of the individual lesion, more than one genotype was detected in a substantial  
201 proportion of the samples. These findings suggest that several genotypes proliferated  
202 concurrently in these tissues, although contamination from other segments of the  
203 gastrointestinal tract cannot be excluded as an explanation. Finally, the findings clearly  
204 indicate that most birds affected by NE or CPH were colonized by more than one (up to six)  
205 PFGE genotype.

206 These findings are not in accordance with the hypothesis that all isolates from a flock, or at  
207 least from an individual, suffering from NE, are generally of the same PFGE genotype,  
208 regardless of the animal or the part of the intestine from which the strain is isolated (Cooper  
209 and Songer, 2009; Timbermont et al., 2009). A possible explanation for this discordance may  
210 be that the hypothesis of one single genotype is mainly based on data derived from severe  
211 outbreaks of NE, whereas our data originate from a flock with mild NE. One of the reasons  
212 that a case of mild NE remains mild may be that one single virulent strain does not achieve  
213 complete predominance. Further work is required to clarify this issue.

214

### 215 3.3. Toxin gene analyses

216 Only the alpha-toxin gene (*plc*) was detected by multiplex PCR testing for the four toxin  
217 genes *plc*, *cpb1*, *iap* and *etx*. We examined a total of 34 isolates from NE and CPH lesions  
218 with *netB* PCR. These isolates originated from a total of 18 broilers collected alive, found  
219 dead or slaughtered (Tables 1 and 2). *NetB* was detected in 31 (91%) of these isolates.

220 Further, from 6 of the same birds (all with small intestinal lesions) a total of 23 isolates were

221 collected from habitats (gizzard and caecae) without NE or CPH lesions. *NetB* was detected in  
222 16 (70%) of these isolates. *NetB* was found in isolates from all 18 NE/CPH birds that were  
223 tested, but only in 25% of healthy birds that were tested. *NetB* was not detected in any of the  
224 11 isolates recovered from used litter.

225 Our findings show that presence of *netB* was associated with *C. perfringens*-specific organ  
226 lesions in this flock suffering from mild NE. Further, these results suggest that mild NE (and  
227 absence of severe NE) cannot be explained by the absence of *netB* carrying strains in the herd.

228

#### 229 *3.4. Concluding remarks*

230 Our findings indicate that mild NE in this commercial broiler flock might be associated with  
231 *netB*, but not with a specific genotype of *C. perfringens*. A possible explanation could be that  
232 *netB* is easily transferred between several *C. perfringens* genotypes, thus increasing their  
233 virulence. However, this assumption cannot explain why severe outbreaks of NE apparently  
234 are associated with one single genotype. Other virulence factors may determine if NE turns  
235 into a severe clinical form or not. One such contributing factor could be the ability of some *C.*  
236 *perfringens* strains to inhibit the growth of less pathogenic or non-pathogenic *C. perfringens*  
237 strains (Barbara et al., 2008; Timbermont et al., 2009).

238

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249 **References**

- 250 Al-Sheikhly, F., Truscott, R.B., 1977a, The interaction of *Clostridium perfringens* and its  
251 toxins in the production of necrotic enteritis of chickens. *Avian Dis* 21, 256-263.
- 252 Al-Sheikhly, F., Truscott, R.B., 1977b, The pathology of necrotic enteritis of chickens  
253 following infusion of crude toxins of *Clostridium perfringens* into the duodenum.  
254 *Avian Dis* 21, 241-255.
- 255 Barbara, A.J., Trinh, H.T., Glock, R.D., Glenn Songer, J., 2008, Necrotic enteritis-producing  
256 strains of *Clostridium perfringens* displace non-necrotic enteritis strains from the gut  
257 of chicks. *Vet Microbiol* 126, 377-382.
- 258 Chalmers, G., Bruce, H.L., Hunter, D.B., Parreira, V.R., Kulkarni, R.R., Jiang, Y.F., Prescott,  
259 J.F., Boerlin, P., 2008a, Multilocus sequence typing analysis of *Clostridium*  
260 *perfringens* isolates from necrotic enteritis outbreaks in broiler chicken populations. *J*  
261 *Clin Microbiol* 46, 3957-3964.
- 262 Chalmers, G., Martin, S.W., Hunter, D.B., Prescott, J.F., Weber, L.J., Boerlin, P., 2008b,  
263 Genetic diversity of *Clostridium perfringens* isolated from healthy broiler chickens at  
264 a commercial farm. *Vet Microbiol* 127, 116-127.
- 265 Chalmers, G., Martin, S.W., Prescott, J.F., Boerlin, P., 2008c, Typing of *Clostridium*  
266 *perfringens* by multiple-locus variable number of tandem repeats analysis. *Vet*  
267 *Microbiol* 128, 126-135.
- 268 Cooper, K.K., Songer, J.G., 2009, Necrotic enteritis in chickens: a paradigm of enteric  
269 infection by *Clostridium perfringens* type A. *Anaerobe* 15, 55-60.
- 270 Dahiya, J.P., Wilkie, D.C., Van Kessela, A.G., Drew, M.D., 2006, Potential strategies for  
271 controlling necrotic enteritis in broiler chickens in post-antibiotic era. *Animal Feed*  
272 *Science and Technology* 129, 60-88.
- 273 Engstrom, B.E., Fermer, C., Lindberg, A., Saarinen, E., Baverud, V., Gunnarsson, A., 2003,  
274 Molecular typing of isolates of *Clostridium perfringens* from healthy and diseased  
275 poultry. *Vet Microbiol* 94, 225-235.
- 276 Gholamiandekhordeh, A.R., Ducatelle, R., Heyndrickx, M., Haesebrouck, F., Van Immerseel,  
277 F., 2006, Molecular and phenotypical characterization of *Clostridium perfringens*  
278 isolates from poultry flocks with different disease status. *Vet Microbiol* 113, 143-152.
- 279 Hunter, S.B., Vauterin, P., Lambert-Fair, M.A., Van Duyne, M.S., Kubota, K., Graves, L.,  
280 Wrigley, D., Barrett, T., Ribot, E., 2005, Establishment of a universal size standard  
281 strain for use with the PulseNet standardized pulsed-field gel electrophoresis  
282 protocols: converting the national databases to the new size standard. *J Clin Microbiol*  
283 43, 1045-1050.
- 284 Johansson, A., Aspan, A., Bagge, E., Baverud, V., Engstrom, B.E., Johansson, K.E., 2006,  
285 Genetic diversity of *Clostridium perfringens* type A isolates from animals, food  
286 poisoning outbreaks and sludge. *BMC Microbiol* 6, 47.
- 287 Kaldhusdal, M., Hofshagen, M., 1992, Barley inclusion and avoparcin supplementation in  
288 broiler diets. 2. Clinical, pathological, and bacteriological findings in a mild form of  
289 necrotic enteritis. *Poult Sci* 71, 1145-1153.
- 290 Kaldhusdal, M., Schneitz, C., Hofshagen, M., Skjerve, E., 2001, Reduced incidence of  
291 *Clostridium perfringens*-associated lesions and improved performance in broiler  
292 chickens treated with normal intestinal bacteria from adult fowl. *Avian Dis* 45, 149-  
293 156.
- 294 Keyburn, A.L., Boyce, J.D., Vaz, P., Bannam, T.L., Ford, M.E., Parker, D., Di Rubbo, A.,  
295 Rood, J.I., Moore, R.J., 2008, NetB, a new toxin that is associated with avian necrotic  
296 enteritis caused by *Clostridium perfringens*. *PLoS Pathog* 4, e26.

- 297 Keyburn, A.L., Sheedy, S.A., Ford, M.E., Williamson, M.M., Awad, M.M., Rood, J.I.,  
298 Moore, R.J., 2006, Alpha-toxin of *Clostridium perfringens* is not an essential  
299 virulence factor in necrotic enteritis in chickens. *Infect Immun* 74, 6496-6500.
- 300 Long, J.R., Pettit, J.R., Barnum, D.A., 1974, Necrotic enteritis in broiler chickens. II.  
301 Pathology and proposed pathogenesis. *Can J Comp Med* 38, 467-474.
- 302 Lovland, A., Kaldhusdal, M., 1999, Liver lesions seen at slaughter as an indicator of necrotic  
303 enteritis in broiler flocks. *FEMS Immunol Med Microbiol* 24, 345-351.
- 304 Lukinmaa, S., Takkunen, E., Siitonen, A., 2002, Molecular epidemiology of *Clostridium*  
305 *perfringens* related to food-borne outbreaks of disease in Finland from 1984 to 1999.  
306 *Appl Environ Microbiol* 68, 3744-3749.
- 307 Martin, T.G., Smyth, J.A., 2008, Prevalence of netB among some clinical isolates of  
308 *Clostridium perfringens* from animals in the United States. *Vet Microbiol*.
- 309 Nauerby, B., Pedersen, K., Madsen, M., 2003, Analysis by pulsed-field gel electrophoresis of  
310 the genetic diversity among *Clostridium perfringens* isolates from chickens. *Vet*  
311 *Microbiol* 94, 257-266.
- 312 Novoa-Garrido, M., Larsen, S., Kaldhusdal, M., 2006, Association between gizzard lesions  
313 and increased caecal *Clostridium perfringens* counts in broiler chickens. *Avian Pathol*  
314 35, 367-372.
- 315 Randall, C.J., 1991, Diseases and Disorders of the Domestic Fowl and Turkey, In: Mosby-  
316 Wolfe, 314 London, pp. 1-175.
- 317 Stutz, M.W., Lawton, G.C., 1984, Effects of diet and antimicrobials on growth, feed  
318 efficiency, intestinal *Clostridium perfringens*, and ileal weight of broiler chicks. *Poult*  
319 *Sci* 63, 2036-2042.
- 320 Timbermont, L., Lanckriet, A., Pasmans, F., Haesebrouck, F., Ducatelle, R., Van Immerseel,  
321 F., 2009, Intra-species growth-inhibition by *Clostridium perfringens* is a possible  
322 virulence trait in necrotic enteritis in broilers. *Vet Microbiol* 137, 388-391.
- 323 Van Immerseel, F., Rood, J.I., Moore, R.J., Titball, R.W., 2008, Rethinking our understanding  
324 of the pathogenesis of necrotic enteritis in chickens. *Trends Microbiol*.

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**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. perfringens*-associated hepatic disease

Chicken	Age (days)	Tissue <sup>1</sup>	PFGE genotype <sup>2</sup>	NE <sup>3</sup>	CPH <sup>4</sup>
A	6	G	25, 26, 26	0	0
A	6	C	25, 25, 25	0	0
B	6	C	24, 24, deg <sup>5</sup>	0	0
C	14	G	28, 28, 28	0	0
C	14	C	25, <b>14</b> , 25	0	0
D	23	D/J	16, <b>16</b> , <b>16</b>	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	<b>30</b> , <b>11</b> , <b>11</b>	1	0
F	30	G	<b>3</b> , <b>30</b>	1	0
F	30	C	<b>11</b> , <b>11</b> , <b>11</b>	1	0
G	30	C	26, <b>1</b> , <b>1</b>	1	0
G	30	D/J	<b>1</b> , <b>13</b> , 2	1	0
H	30	C	<b>11</b> , <b>11</b> , deg <sup>5</sup>	1	0
H	30	D/J	<b>11</b> , <b>11</b> , <b>11</b>	1	0
I	30	D/J	<b>12</b> , <b>12</b> , <b>12</b>	1	0
J	30	D/J	32, <b>3</b> , <b>4</b>	1	0
J	30	C	<b>3</b> , <b>15</b> , <b>3</b>	1	0
J	30	G	21, 29, 29	1	0
K	23	D/J	<b>5</b>	1	0
L	23	D/J	<b>7</b>	1	0
M	30	D/J	<b>3</b> , <b>3</b> , <b>3</b>	1	0
M	30	C	<b>4</b> , <b>4</b> , <b>4</b>	1	0
N	30	L	<b>10</b> , <b>10</b> , <b>10</b>	0	1

<sup>1</sup>C, caecum, D/J, duodenum/jejunum, G, gizzard, L, Liver

<sup>2</sup>Bold, presence of the *netB* gene

<sup>3</sup>lesions in the mucosa

<sup>4</sup>lesions in the liver

<sup>5</sup>DNA degraded by endonucleases

**Table 2.** *C. perfringens* isolated from 9 slaughtered broilers (1 isolate per bird) and from litter in the house during rearing

Strain	Sample at slaughter	PFGE genotype <sup>1</sup>
S 1	L <sup>2</sup> , CPH	<b>17</b>
S 2	L, CPH	<b>8</b>
S 3	L, CPH	<b>11</b>
S 4	L, CPH	11 <sup>nt</sup>
S 5	L, CPH	<b>11</b>
S 6	L, CPH	<b>3</b>
S 7	J <sup>2</sup> , focal NE	<b>9</b>
S 8	J, focal NE	<b>6</b>
S 9	J, focal NE	<b>3</b>
Litter sample		
L 1-3	Day 6	22, 27 deg <sup>3</sup>
L 4-6	Day 14	19, 20, 25
L 7-9	Day 23	25, 25, 25
L 10-12	Day 30	3, 3, 23

<sup>1</sup>Bold, presence of *netB*

<sup>2</sup>J, jejunum and L, Liver

<sup>3</sup>DNA degraded by endonucleases

<sup>nt</sup>not tested for the *netB* gene

**Figure captions**

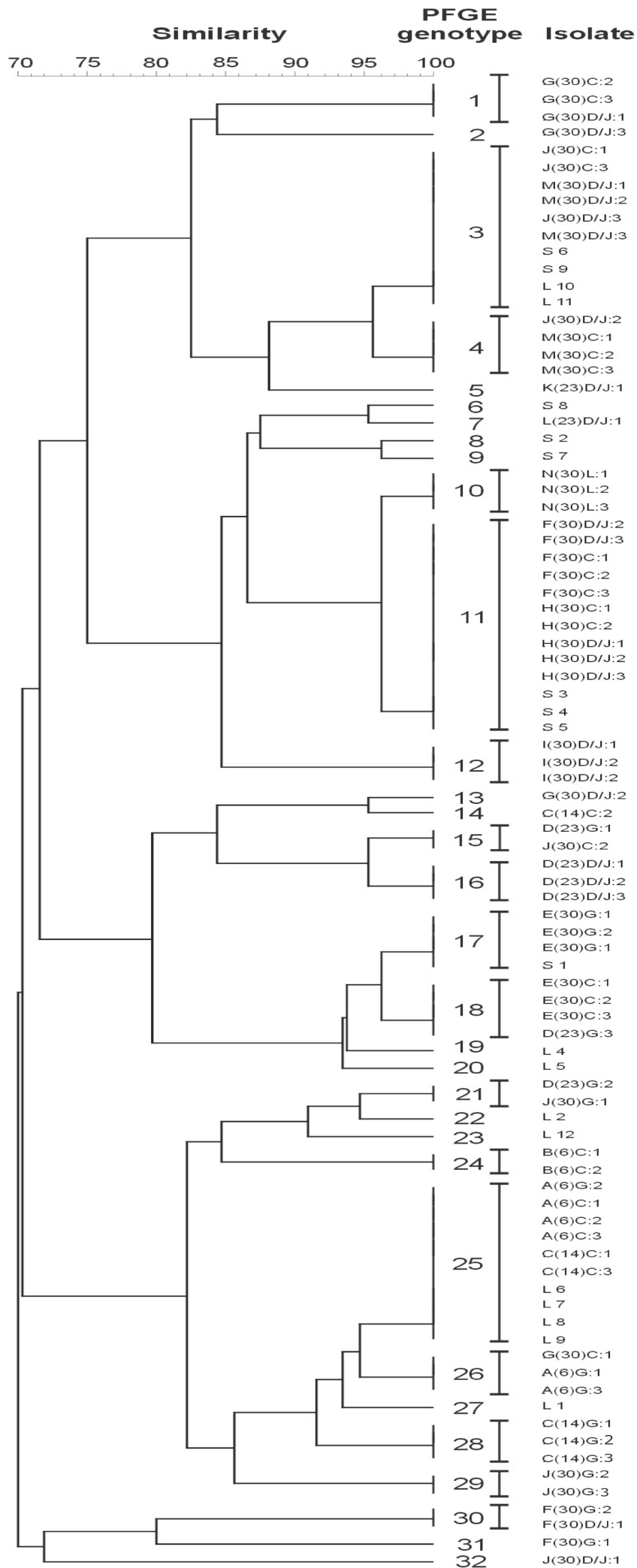
**Figure 1.** Daily mortality rate calculated as percentage of the number of housed day-old chicks (18833 chicks).

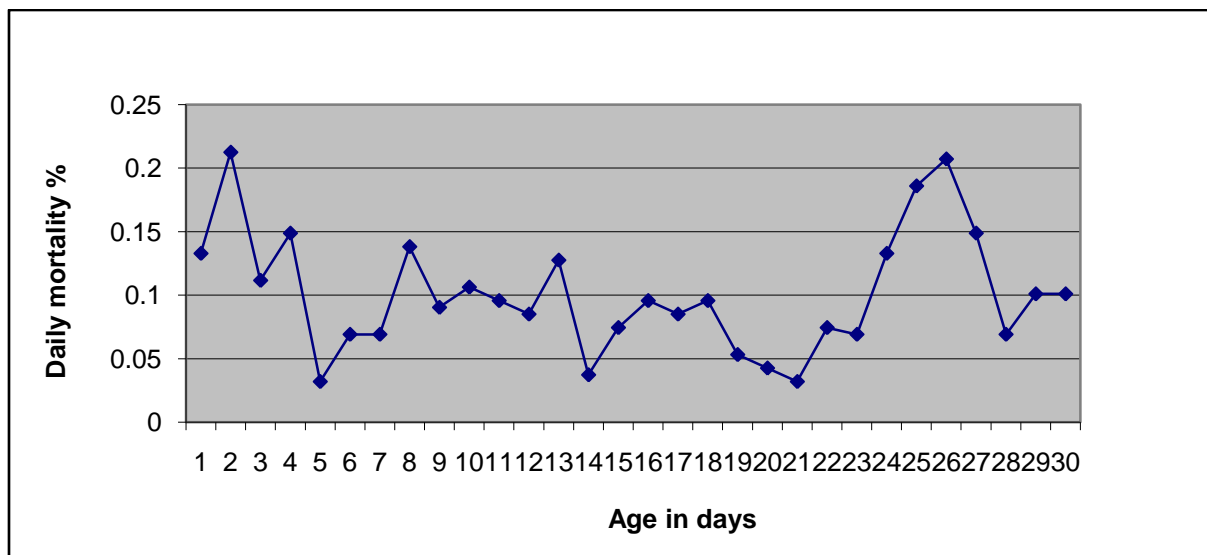
**Figure 2.** Dendrogram based on cluster analysis of *Sma*I-digested DNA from 88 strains of *C. perfringens* separated by PFGE.

Isolate abbreviation: Animal (Age of bird) Tissue:Isolate, 1-3.

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Figure 1





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