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Accepted Manuscript

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PII: S0378-1135(09)00608-7
Reference: VETMIC 4714

To appear in: VETMIC

Received date: 24-4-2009
Revised date: 5-12-2009
Accepted date: 9-12-2009

Please cite this article as: Johansson, A., Aspán, A., Kaldhusdal, M., Engström, B.E., Genetic diversity and prevalence of netB in Clostridium perfringens isolated from a broiler flock affected by mild necrotic enteritis, Veterinary Microbiology (2008), doi:10.1016/j.vetmic.2009.12.017

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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.

Keywords

Clostridium perfringens, necrotic enteritis, netB, pulsed-field gel electrophoresis (PFGE), 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 (Dahiyya 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; Gholamianekordi 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 multi-locus 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 the slaughterhouse. Specimens from carcasses (gall bladder and gut mucosa with lesions) were taken with cotton-tipped applicators and transported to the laboratory in tubes containing Amies medium with charcoal (Copan International, Corona, Italy). Materials from all specimens were plated on bovine blood agar and incubated anaerobically overnight at 37 °C. Bacterial colonies were identified as *C. perfringens*, based on colony morphology and characteristic double-zone haemolysis. From each plate, preferably three colonies with different appearances were picked for storage and further examination. The strains were identified as *C. perfringens* type A by applying standard biochemical tests and multiplex PCR (Johansson et al., 2006).

2.3. Pathology

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

2.4. PCR and PFGE analysis

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

3. Results and discussion

3.1. Clinical findings, pathology and isolation of \textit{C. perfringens}
Accumulated flock mortality at slaughter was normal (3.0%). Mortality during the last week of the growth period was slightly higher than expected (0.9%) with a modest peak on day 26 (0.2%) and a lower level (mean daily mortality 0.1%) during the last three days of the grow-out (Fig. 1). No clinical symptoms were detected in any of the 40 birds collected alive during the rearing period. *C. perfringens*-associated lesions were found in 20.0% of these birds. Seven out of eight randomly sampled birds with NE lesions were detected on day 30, one bird with NE lesions was found on day 23. These findings indicate that mild NE emerged at approximately 3 weeks of age and was prevalent (7/10 birds examined) on the day before slaughter. Our data suggest no clear covariation between NE occurrence and mortality, but an association with the modest mortality peak on day 26 cannot be excluded. The mortality data (Fig. 1) do not suggest that the disease was developing into a severe form during the last days of the grow-out. *C. perfringens* isolates were recovered from all 10 broilers that were collected alive and examined for bacteria (Table 1, chicken A to J), including four birds without NE lesions (Table 1, chicken A, B, C and E). Altogether 58 *C. perfringens* isolates from these 10 birds were successfully characterized. Seventeen broilers found dead in the house were submitted for laboratory examinations on days 23 and 30; NE or CPH was detected in eight of these 17 birds. Four of the eight NE/CPH birds were examined for *C. perfringens*. Isolates were recovered from all four, and a total of 11 *C. perfringens* isolates were collected for further characterization (Table 1; chicken K to N).

*C. perfringens* was isolated from all four pooled litter samples that were collected during the rearing period. A total of 11 isolates from these samples were successfully characterized (Table 2, strains L1 to L12).

The condemnation percentage due to liver lesions at slaughter was 0.40, i.e. substantially higher than the mean of 49 narasin-treated broiler flocks (0.09%) raised in the same region.
and slaughtered during roughly the same period of time (unpublished data from a field study conducted by one of the laboratories participating in the present study). The gall bladder from each of six condemned chickens were examined for *C. perfringens*, with positive results (Table 2, strains S1 to S6). Several carcasses with intestinal NE lesions (focal necroses) were detected, and the jejunum from each of three of these were examined for *C. perfringens*, with positive results (Table 2, strains S7 to S9).

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

3.2. PFGE analysis

A total of 91 isolates were recovered from 23 birds and four pooled litter samples. Degradation of DNA by endonucleases prevented a PFGE analysis of three isolates. A total of 32 PFGE genotypes were detected among the remaining 88 isolates (Fig. 2).

We have genotype data from a total of 12 NE birds collected alive (6 birds), found dead (3 birds) or sampled at the slaughterhouse (3 birds). A total of 26 isolates from small intestinal NE lesions in these 12 birds were examined for PFGE patterns (Tables 1 and 2). Among these isolates a total of 14 genotypes were recorded. Further, at the individual NE bird level we examined three isolates from NE lesions from each of 7 individuals, and detected more than one genotype in 3 (43 %) of them. Also, in 2 of these 3 birds all 3 isolates from their NE lesions were different. We examined a total of 9 isolates from CPH livers in 7 individuals, and detected 5 different genotypes among these isolates (Tables 1 and 2). Finally, we examined isolates from at least two different tissues from a total of six birds (found dead or collected...
alive) affected by NE, and found that 5 out of 6 (83 %) birds were colonized by more than one genotype. These data indicate that numerous PFGE genotypes were prevalent in materials collected from *C. perfringens*-specific organ lesions in this flock suffering from mild NE. Further, even at the level of the individual lesion, more than one genotype was detected in a substantial proportion of the samples. These findings suggest that several genotypes proliferated concurrently in these tissues, although contamination from other segments of the gastrointestinal tract cannot be excluded as an explanation. Finally, the findings clearly indicate that most birds affected by NE or CPH were colonized by more than one (up to six) PFGE genotype. These findings are not in accordance with the hypothesis that all isolates from a flock, or at least from an individual, suffering from NE, are generally of the same PFGE genotype, regardless of the animal or the part of the intestine from which the strain is isolated (Cooper and Songer, 2009; Timbermont et al., 2009). A possible explanation for this discordance may be that the hypothesis of one single genotype is mainly based on data derived from severe outbreaks of NE, whereas our data originate from a flock with mild NE. One of the reasons that a case of mild NE remains mild may be that one single virulent strain does not achieve complete predominance. Further work is required to clarify this issue.

### 3.3. Toxin gene analyses

Only the alpha-toxin gene (*plc*) was detected by multiplex PCR testing for the four toxin genes *plc*, *cpb1*, *iap* and *etx*. We examined a total of 34 isolates from NE and CPH lesions with *netB* PCR. These isolates originated from a total of 18 broilers collected alive, found dead or slaughtered (Tables 1 and 2). *NetB* was detected in 31 (91%) of these isolates. Further, from 6 of the same birds (all with small intestinal lesions) a total of 23 isolates were
collected from habitats (gizzard and caeca) without NE or CPH lesions. NetB was detected in 16 (70%) of these isolates. NetB was found in isolates from all 18 NE/CPH birds that were tested, but only in 25% of healthy birds that were tested. NetB was not detected in any of the 11 isolates recovered from used litter. Our findings show that presence of netB was associated with C. perfringens-specific organ lesions in this flock suffering from mild NE. Further, these results suggest that mild NE (and absence of severe NE) cannot be explained by the absence of netB carrying strains in the herd.

3.4. Concluding remarks

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

Acknowledgements

This study was supported by grants from the Swedish Farmers´ Foundation for Agricultural Research, the Swedish National Veterinary Institute, the Norwegian Centre for Poultry Science and the Norwegian Foundation for Research Levy on Agricultural Products. The authors are grateful to Joann Börjesson for excellent technical assistance. We thank veterinary surgeons Arild Lysaker at Prior Norge and Atle Løvland of the Norwegian Food Safety Authority, for taking samples from the slaughterhouse for bacteriological examination. We
thank Bjarne Bergsjø and Kerstin Nordby, National Veterinary Institute, Oslo, Norway, for isolating *C. perfringens* from broilers.
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Johansson, A., Aspan, A., Bagge, E., Baverud, V., Engstrom, B.E., Johansson, K.E., 2006, Genetic diversity of *Clostridium perfringens* type A isolates from animals, food poisoning outbreaks and sludge. BMC Microbiol 6, 47.


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

<table>
<thead>
<tr>
<th>Chicken</th>
<th>Age (days)</th>
<th>Tissue</th>
<th>PFGE genotype</th>
<th>NE</th>
<th>CPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>G</td>
<td>25, 26, 26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>C</td>
<td>25, 25, 25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>C</td>
<td>24, 24, deg⁵</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>14</td>
<td>G</td>
<td>28, 28, 28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>14</td>
<td>C</td>
<td>25, 14, 25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>23</td>
<td>D/J</td>
<td>16, 16, 16</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>23</td>
<td>G</td>
<td>15, 21, 18</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>30</td>
<td>G</td>
<td>17, 17, 17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>30</td>
<td>C</td>
<td>18, 18, 18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>30</td>
<td>D/J</td>
<td>30, 11, 11</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>30</td>
<td>G</td>
<td>3, 30</td>
<td>1</td>
<td>0</td>
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<tr>
<td>F</td>
<td>30</td>
<td>C</td>
<td>11, 11, 11</td>
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<tr>
<td>G</td>
<td>30</td>
<td>C</td>
<td>26, 1, 1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>G</td>
<td>30</td>
<td>D/J</td>
<td>1, 13, 2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>30</td>
<td>C</td>
<td>11, 11, deg⁵</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>30</td>
<td>D/J</td>
<td>11, 11, 11</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>30</td>
<td>D/J</td>
<td>12, 12, 12</td>
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<td>0</td>
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<tr>
<td>J</td>
<td>30</td>
<td>D/J</td>
<td>32, 3, 4</td>
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<td>0</td>
</tr>
<tr>
<td>J</td>
<td>30</td>
<td>C</td>
<td>3, 15, 3</td>
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<tr>
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<td>G</td>
<td>21, 29, 29</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>K</td>
<td>23</td>
<td>D/J</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>L</td>
<td>23</td>
<td>D/J</td>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>M</td>
<td>30</td>
<td>D/J</td>
<td>3, 3, 3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>M</td>
<td>30</td>
<td>C</td>
<td>4, 4, 4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>N</td>
<td>30</td>
<td>L</td>
<td>10, 10, 10</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

1C, caecum, D/J, duodenum/jejunum, G, gizzard, L, Liver
2Bold, presence of the *netB* gene
3lesions in the mucosa
4lesions in the liver
5DNA degraded by endonucleases
**Table 2.** *C. perfringens* isolated from 9 slaughtered broilers (1 isolate per bird) and from litter in the house during rearing

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sample at slaughter</th>
<th>PFGE genotype(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S 1</td>
<td>L, CPH</td>
<td>17</td>
</tr>
<tr>
<td>S 2</td>
<td>L, CPH</td>
<td>8</td>
</tr>
<tr>
<td>S 3</td>
<td>L, CPH</td>
<td>11</td>
</tr>
<tr>
<td>S 4</td>
<td>L, CPH</td>
<td>11*</td>
</tr>
<tr>
<td>S 5</td>
<td>L, CPH</td>
<td>11</td>
</tr>
<tr>
<td>S 6</td>
<td>L, CPH</td>
<td>3</td>
</tr>
<tr>
<td>S 7</td>
<td>J, focal NE</td>
<td>9</td>
</tr>
<tr>
<td>S 8</td>
<td>J, focal NE</td>
<td>6</td>
</tr>
<tr>
<td>S 9</td>
<td>J, focal NE</td>
<td>3</td>
</tr>
</tbody>
</table>

**Litter sample**

<table>
<thead>
<tr>
<th>Litter sample</th>
<th>Day</th>
<th>22, 27 deg(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L 1-3</td>
<td>Day 6</td>
<td>22, 27 deg(^3)</td>
</tr>
<tr>
<td>L 4-6</td>
<td>Day 14</td>
<td>19, 20, 25</td>
</tr>
<tr>
<td>L 7-9</td>
<td>Day 23</td>
<td>25, 25, 25</td>
</tr>
<tr>
<td>L 10-12</td>
<td>Day 30</td>
<td>3, 3, 23</td>
</tr>
</tbody>
</table>

\(^1\)Bold, presence of netB  
\(^2\)J, jejunum and L, Liver  
\(^3\)DNA degraded by endonucleases  
\(^*\)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.
Figure 2

![Graph showing daily mortality percentage over age in days.](image-url)