Prevalence of *Clostridium perfringens* in commercial turkey and layer flocks*

W. Gad¹, R. Hauck¹, M. Krüger² and H.M. Hafez¹

Dedicated to Prof. Dr. Ulrich Neumann on behalf of his retirement

Introduction

*Clostridium perfringens* is an anaerobic, rod shaped, non-motile bacterium. It is often found in the intestinal tract of chickens, animals, and humans. The presence of *C. perfringens*, especially in higher numbers or in the small intestines is mostly regarded as an indicator of a disturbed balance of the intestinal flora or “dysbacteriosis”. When *C. perfringens* proliferates to high numbers in the small intestine and produces toxins damaging the intestinal lining, it can lead to necrotic enteritis (NE), which is an acute disease accompanied with high economic losses (Shane et al., 1985; Long and Truscott, 1976). Mucosal damage by infection with *Eimeria* spp. (Al-Sheikly and Al-Saieq, 1980; Lanckriet et al., 2010) or diets containing a high amount of indigestible, water-soluble non-starch polysaccharides leading to a high viscosity of the intestinal content (Kaldhusdal and Hophagen, 1992; Craven, 2000; Jia et al., 2009) are important risk factors for NE. NE has been observed in several domestic and wild birds world wide. Recently several reviews were published (Van Immerseel et al., 2004; Williams, 2005; McDevitt et al., 2006; Opengart, 2008; Hafez, 2010). Beside clinically manifested disease, subclinical infections may take place and are mostly accompanied with reduction of performance. *C. perfringens* may also be associated with subclinical cholangiohepatitis and gizzard erosions (Abildgaard et al., 2009; Novoa-Garrido et al., 2006).

Reports from the field indicate that, after the ban of antimicrobial growth promoters in the European Union in 2006 (EC, 2003), infections of poultry with *C. perfringens* increased markedly (Cooper and Songer, 2009; Van Immerseel et al., 2004, van der Sluis, 2010). From the economical point of view the estimated cost of NE to the poultry industry globally is nearly 2 billion US every year (McReynolds et al., 2004).

*C. perfringens* is divided into 5 toxovars A, B, C, D, and E based on the synthesis of four major lethal toxins: alpha (cpa), beta (cpb), epsilon (etx), and iota (iap) (Petit et al., 1999). Along with these four major toxins, some other toxins like enterotoxin (cpe) and beta2 (cpb2) toxin produced by *C. perfringens* are considered as important toxins for enteric diseases (McDonel, 1986; Songer, 1996; Waters et al., 2003, Smedley et al., 2004, McClane et al., 2006). However, it is not clear whether cpe and cpb2 are involved in *C. perfringens*-associated avian enteric diseases (Crespo et al., 2007).

The infections in poultry are mostly caused by *C. perfringens* toxovar A, and to a lesser extent by toxovar C (Kohler et al., 1974, Songer and Meir, 1996; Engstrom et al., 2003). Both toxovar produce cpa while toxovar C strains additionally carry the cpb gene Because *C. perfringens* toxovar A is highly prevalent in the intestines of healthy animals, controversy exists about its real pathogenic role (Smedley et al., 2004; McClane et al., 2006).

Recently, Keyburn et al. (2008) were able to identify a novel toxin (netB) in a *C. perfringens* toxovar A strains isolated from chickens suffering from necrotic enteritis. According to the authors this novel toxin is the first definitive virulence factor to be identified in avian *C. perfringens* strains capable of causing necrotic enteritis.

The aim of the present investigation was to determine the prevalence of *C. perfringens* toxovars in commercial turkey and layer flocks reared in various locations in Germany.

Material and methods

Bacterial strains

*C. perfringens* reference strains ATCC 13124 (Toxovar A), ATCC 3626 (Toxovar B) and ATCC 27324 (Toxovar E) were kindly provided by the Institute of Bacteriology and Epizootic Diseases, Faculty of Veterinary Medicine, Free University Berlin and served as controls for the PCR assays.

Samples

615 pair boot swabs collected from turkey flocks before slaughter on 86 different farms were included in the investigation. 366 swabs originated from tom flocks, 246 from hen flocks. 3 swabs were from flocks whose sex was not reported. In addition 485 samples collected from layer flocks were investigated for the presence of *C. perfringens*. These samples were 329 pair boot swabs, 122 faecal samples and 34 one day old layer chicks. Layer flocks were kept on the floor. All samples were collected from apparently healthy flocks in the frame of the salmonella surveillance program between August 2007 and March 2009.

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* Die Arbeit ist ein Teil der Dissertation von Herrn Wael Gad, die noch nicht abgeschlossen ist.
Bacteriological examination

Boot swabs were first placed in peptone water and vigorously mixed. 10 μl of the suspension were then immediately transferred to Tryptose sulfite cycloserine (TSC) agar (Merck, Darmstadt). Faecal samples were first enriched in reinforced clostridial medium (Oxoid, Wesel, Germany) at 41°C for 24 hours, then 10 μl were spread on TSC agar. TSC plates were incubated anaerobically at 37°C for 36 – 48 hours. Colonies with morphology suggestive of *C. perfringens* were further identified by gram staining and sub cultivation on 5% sheep blood agar supplemented with Neomycin (200 μg/ml) and Polymyxin B (100 μg/ml) and on egg yolk lactose agar. After anaerobic incubation at 40°C for 36 – 48 h, *C. perfringens* was identified by detection of haemolysis, lactose fermentation and lecithinase activity. In doubtful cases, biochemical identification was done using the API 20E kit (BioMérieux, Mercy l’Etoile, France) according to the manufacturer’s instructions.

Molecular identification

DNA was extracted from two to three fresh colonies using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Each isolate was first investigated by a multiplex PCR to detect the major toxin genes, then by a second multiplex PCR for genes encoding the minor toxins cpb2, netb, and cpe. The multiplex PCR was done using the Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany). The used primers are shown in Table 1. Concentrations of primers were 0.04 μM for major toxins and 0.4 μM for minor toxins cpe, cpb2 and netb. The multiplex PCR program consisted of 94°C for 15 min, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, and a final extension step for 5 min at 72°C. PCR-products were separated on a 1.5% agarose gel by electrophoresis in 1× TBE electrophoresis buffer and visualized by ethidium bromide staining and ultraviolet transillumination, together with a 100bp-ladder as molecular weight marker (Figure 1).

Restriction enzyme analysis (REA)

To validate the primer pairs for the detection of cpe and netb, which were designed for this study, 5 μl PCR product were digested in a total volume of 20 μl and 20 units with *Pst I* (cpe) and *HinI* (netb). The resulting fragments were analyzed in a 3% metaphor agarose gel (BioRad Labs, Hercules, CA).

Specificity

To test the specificity, the multiplex PCR assays were performed with DNA of *C. botulinum*, *C. novyi*, *C. sordelli*, *C. septicum*, *C. tetani*, *C. sporogenes*, *C. difficile* and *C. chauvaei* (which were kindly provided by the Institute of Bacteriology and Epizootic Diseases, Faculty of Veterinary Medicine, Free University Berlin). In addition four strains of other bacterial species (*Escherichia coli*, *Salmonella Montevideo*, *Staphylococcus aureus* and *Streptococcus species*) were tested.

Results

Isolation of *C. perfringens* from turkeys

From 59 out of 86 investigated farms 68.6% were tested positive. From most farms, from which several samples were investigated, the swabs yielded positive as well as negative results. *C. perfringens* was isolated from 121 of the 366 samples from toms (33.1%) and 57 samples of the 246 samples from hens (23.2%) were tested positive. From one of the 3 swabs with unknown sex of the birds *C. perfringens* was isolated (Table 2). The difference between toms and hens was significant as tested by Chi square test (P ≤ 0.05).

Isolation of *C. perfringens* from layers

*C. perfringens* was isolated from 134 (27.6%) of the samples from layers. The prevalence of *C. perfringens* in sam-

<p>| Table 1. Primers used for multiplex PCR to detect <em>C. perfringens</em> toxin genes |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Orientation</th>
<th>Sequence</th>
<th>Length of amplicon</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Major toxins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpa</td>
<td>forward</td>
<td>AGTCATCGCTTGGGATGGAA</td>
<td>900 bp*</td>
<td>(BAUMS et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>revers</td>
<td>TTTCTGGGTTGCTCATTTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpb</td>
<td>forward</td>
<td>TCCCCCTTTGAGGGAGGATAAA</td>
<td>611 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>revers</td>
<td>TGAACCTCCTATTTTGTATCCCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>etx</td>
<td>forward</td>
<td>TGGGAACCTCGATACAAAGCA</td>
<td>396 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>revers</td>
<td>TTAACTCATCCATCCAAGTCGACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lap</td>
<td>forward</td>
<td>AAAAAACCATAAAGCTCACACC</td>
<td>293 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>revers</td>
<td>CTGCATAACCTGAGGATGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpe</td>
<td>forward</td>
<td>TGGTTGGATATTAGGGGAACC</td>
<td>752 bp</td>
<td>Designed for this study</td>
</tr>
<tr>
<td></td>
<td>revers</td>
<td>CGAGAAACATATTGTGCCAGCAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>netb</td>
<td>forward</td>
<td>GAAAAATAAATGCGGCGTGA</td>
<td>440 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>revers</td>
<td>CTTGGATTCTTACCACCAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpb2</td>
<td>forward</td>
<td>CAGGCAATTGGGGGAGTTTGA</td>
<td>200 bp</td>
<td>(BAUMS et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>revers</td>
<td>GCAGAACATTGGGGATTTGACCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* basepairs
pled one day old chicks was 11.8%. The prevalence of \( C. \) \( \text{perfringens} \) in birds older than 1 week was between 18.5% in birds between the 1st and 9th week of age. No correlation between age and prevalence was recognizable (Figure 2).

For confirmation of the obtained bacteriological results several isolates were examined using Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) at the Institute of Bacteriology and Mycology, Faculty of veterinary medicine, Leipzig University.

Molecular identification

No DNA of the other pathogens tested showed positive reaction in the PCR with the used primers. In all cases, digestion of PCR products with the restriction endonucleases generated distinct restriction patterns depending on the amplified toxin genes (Figure 3).

143 isolates from turkey flocks and 124 isolates from layer flocks were investigated by the multiplex PCR assays.

Table 2. Prevalence of \( C. \) \( \text{perfringens} \) in commercial turkey flocks

<table>
<thead>
<tr>
<th>Sex</th>
<th>samples</th>
<th>Positive</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>366</td>
<td>121</td>
<td>33.1%</td>
</tr>
<tr>
<td>Female</td>
<td>246</td>
<td>57</td>
<td>23.2%</td>
</tr>
<tr>
<td>Unknown</td>
<td>3</td>
<td>1</td>
<td>33.3%</td>
</tr>
<tr>
<td>Total</td>
<td>615</td>
<td>179</td>
<td>29.1%</td>
</tr>
</tbody>
</table>

Figure 2. Prevalence of \( C. \) \( \text{perfringens} \) in pullet and layer flocks according to age.

Vorkommen von \( C. \) \( \text{perfringens} \) in Jung- und Legehennenherden verschiedener Altersstufen
Figure 3. Restriction enzyme analysis of PCR products
A: cpe product (752 bp) cut with Pst I, expected fragment sizes are 391 bp and 361 bp; Spur 1: 100-bp ladder; Spur 2: strain ATCC 27324.
B: netB product (440 bp) cut with Hinf I, expected fragment sizes are 315 bp and 125 bp; Spur 1: 100-bp Marker; Spur 2: field strain.

Restrikionsenzymanalyse von PCR Produkten
A: cpe Produkt (752 bp) geschnitten mit PstI, die erwarteten Fragmentgrößen sind 391 bp und 361 bp; Spur 1: 100-bp Marker; Spur 2: Stamm ATCC 27324.
B: netB Produkt (440 bp) geschnitten mit Hinf I, die erwarteten Fragmentgrößen sind 315 bp und 125 bp; Spur 1: 100-bp Marker; Spur 2: Feldstamm.

74.1% of isolates from turkeys and 88.7% of isolates from layers belonged to toxovar A. Toxovar D was the second most encountered toxovar with 23.8% of the isolates from turkeys and 7.3% of the isolates from layers. Toxovars C and E were detected very rarely, and no isolate was typed as toxovar B (Table 3).

Minor toxin genes were found in 28.7% of isolates from turkeys and in 32.3% of the isolates from layers. Netb was the most frequently encountered minor toxin. 16.8% of isolates from turkeys and 23.4% of isolates from layers carried its gene. In contrast 12.6% of isolates from turkeys and 8.1% of isolates from layers were tested positive for the cpb2 gene, while 1.4% of isolates from turkeys and 7.3% of isolates from layers carried the cpe gene. Rarely more than one minor toxin was detected in one isolate (Table 4).

Table 3. Results of typing of C. perfringens isolates from turkeys and layers by multiplex PCR to detect major toxin genes

<table>
<thead>
<tr>
<th>Toxovar</th>
<th>Turkeys</th>
<th>Layers</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>106 (74.1%)(^1)</td>
<td>110 (88.7%)</td>
</tr>
<tr>
<td>B</td>
<td>Not isolated</td>
<td>Not isolated</td>
</tr>
<tr>
<td>C</td>
<td>3 (2.1%)</td>
<td>2 (1.6%)</td>
</tr>
<tr>
<td>D</td>
<td>34 (23.8%)</td>
<td>9 (7.3%)</td>
</tr>
<tr>
<td>E</td>
<td>Not isolated</td>
<td>3 (2.4%)</td>
</tr>
<tr>
<td>Total</td>
<td>143 (100%)</td>
<td>124 (100%)</td>
</tr>
</tbody>
</table>

\(^1\) Number of isolates and percentage of all isolates

Table 4. Results of typing of C. perfringens isolates from turkeys and layers by multiplex PCR to detect minor toxin genes

<table>
<thead>
<tr>
<th>Minor toxins</th>
<th>Turkeys</th>
<th>Layers</th>
</tr>
</thead>
<tbody>
<tr>
<td>netB, cpb2, and cpe gene Not detected</td>
<td>2 (1.4%)</td>
<td>2 (1.6%)</td>
</tr>
<tr>
<td>cpe gene only</td>
<td>1 (0.7%)</td>
<td>3 (2.4%)</td>
</tr>
<tr>
<td>netB and cpb2 gene</td>
<td>2 (1.4%)</td>
<td>Not detected</td>
</tr>
<tr>
<td>netB and cpe gene</td>
<td>Not detected</td>
<td>2 (1.6%)</td>
</tr>
<tr>
<td>cpb2 and cpe gene</td>
<td>1 (0.7%)</td>
<td>2 (1.6%)</td>
</tr>
<tr>
<td>netB gene only</td>
<td>22 (15.4%)(^2)</td>
<td>25 (20.2%)</td>
</tr>
<tr>
<td>cpb2 gene only</td>
<td>15 (10.5%)</td>
<td>6 (4.8%)</td>
</tr>
<tr>
<td>cpe gene only</td>
<td>1 (0.7%)</td>
<td>3 (2.4%)</td>
</tr>
<tr>
<td>netB alone or with others</td>
<td>24 (16.8%)</td>
<td>29 (23.4%)</td>
</tr>
<tr>
<td>cpb2 gene alone or with others</td>
<td>18 (12.6%)</td>
<td>10 (8.1%)</td>
</tr>
<tr>
<td>cpe gene alone or with others</td>
<td>2 (1.4%)</td>
<td>9 (7.3%)</td>
</tr>
</tbody>
</table>

\(^1\) Number of isolates and percentage of all isolates

\(^2\) Number of isolates and percentage of all isolates

Discussion

The prevalence of necrotic enteritis is influenced by many factors like rearing, season and age of birds (Craven et al., 2001; Köhler et al., 1977; Long, 1973). In the present investigation the prevalence of C. perfringens in German turkey and layer flocks was determined. Similar previous studies determined the prevalence or incidence of NE by necropsy of dead birds and microscopic lesions (Köhler et al., 1977; Köhler, 2000; Kaldhusdal and Skjerve, 1996) or isolation of the organism from individual birds euthanized for this purpose (Walther, 2009). For the present investigation samples collected for routine Salmonella surveillance of turkey and layer flocks were used. The differences in investigated material and methods make direct comparisons with previous studies very difficult.

The results show, that under current conditions C. perfringens is widespread in the investigated flocks. In turkeys, the prevalence of C. perfringens in male flocks was higher than in female flocks. One reason might be the difference in age, as toms are slaughtered about four weeks later than hens are. On the other hand it is questionable, if this relatively small difference in age can explain the high difference in prevalence, especially since in layers age did not seem to influence the prevalence of C. perfringens. However other differences between toms and hens that might explain the results are not obvious.

To varying degrees compounds used as anticoccidials also have antilisterial effects and their use is even thought to counter negative effects after the ban of AGPs (Eulinger et al., 1998; Martel et al., 2004; Lankriet et al., 2010). Interestingly the prevalence of C. perfringens in layer flocks during productions, which do not receive anticoccidials, was similar to turkeys, which had received anticoccidials up to the 12th week of age.

In the present investigation most of examined isolates were classified as toxovar A, as it was expected. Toxovar D was the second most detected toxovar, and especially prevalent in turkeys. Toxovar C was rarely isolated. This was surprising, since in most studies Toxovar C is more frequently encountered than toxovar D (Köhler et al., 1974; van Immerseel et al., 2004), however similar results have been obtained by Nasr et al. (2007), who found toxovar A
in 70.8% and toxovar D in 12.9% by typing isolates from poultry products.

In this study one or several genes of minor toxins were found in about 30% of investigated isolates. Information about the incidence of minor toxins varies widely. For example ENGSTRÖM et al. (2003) found the cpb2 gene in 9.5% of C. perfringens isolated from birds without enteric disorders, which is similar to our findings. In contrast (CRESPO et al., 2007) found the cpb2 gene in 4 out of 6 isolates from healthy chickens. Similarly the incidence of netb gene was found to be between 8.8% and 60.9% in healthy flocks (MARTIN and SMYTH, 2009; ABILDGAARD et al., 2010). Interestingly MARTIN and SMYTH (2009) also found a strong correlation between the detection of the cpb2 gene and netb gene, which could not be shown here. However, when interpreting the results it has to be kept in mind that the presence of the gene of a toxin does not necessarily mean, that the toxin is produced, as it was shown for netb toxin (ABILDGAARD et al., 2010) or cpb2 (CRESPO et al., 2007).

Our results confirm that the presence of minor toxins like cpb2, or netb in the isolated strains, which where suggested to be the main cause of necrotic enteritis in poultry, in apparently healthy birds indicate that the presence of toxins alone is not enough to develop the lesions but the presence of synergistic factors is required.

Summary

The gram positive anaerobic C. perfringens has been shown to be widespread among poultry farms, especially after the ban of antibiotic growth promoters in the EU in 2006. In the present study we monitored the incidence of C. perfringens in commercial turkey and layer flocks reared in various locations in Germany.

615 boot swabs from turkey flocks and 485 samples from layer flocks were investigated bacteriologically for the presence of C. perfringens and further identified for presence of major and minor toxins using PCR. The samples had been collected from apparently healthy flocks in the frame of the salmonella surveillance program. In 179 (29.1%) boot swabs from turkey flocks and in 134 (27.6%) samples from layers C. perfringens was detected. Toxovar A was the predominant toxovar (74.1% in turkeys and 88.7% in layers), followed by toxovar D (23.8% in turkeys and 7.3% in layers). Minor toxin genes were found in 28.7% of isolates from turkeys and in 32.3% of the isolates from layers.

Key words

Clostridium perfringens, poultry, isolation, Toxin typing, netb toxin

Zusammenfassung

Vorkommen von Clostridium perfringens in Puten- und Legehennenherden

Das gram-positive, anaerobe Bakterium C. perfringens ist, insbesondere nach dem Verbot antibiotischer Leistungs- förderer in der Europäischen Union, in Geflügelbeständen weit verbreitet. In der vorliegenden Arbeit wurde das Vorkommen von C. perfringens in Mastputen- und Legehennenbestände aus verschiedenen Regionen in Deutschland untersucht.


Stichworte

Clostridium perfringens, Geflügel, Isolierung, Toxin Typisierung, NetB Toxin

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