Determination of antibiotic sensitivities of *Clostridium perfringens* isolates from commercial turkeys in Germany *in vitro*

Untersuchungen zur Antibiotikaempfindlichkeit von *Clostridium perfringens* Isolaten aus Putenbeständen in Deutschland *in vitro*

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

**Introduction**

The spore forming anaerobic bacterium *Clostridium perfringens* is considered to be a part of the natural gut flora of animals, including turkeys, but it is also associated with different disease conditions in poultry as necrotic enteritis (Shane et al., 1985; Long and Truscott, 1976), cholangiohepatitis (Lovland and Kalhuisdal, 1999; Onderka et al., 1990; Sasaki et al., 2000; Abildgaard et al., 2009) as well as gizzard erosions (Novoa-Garrido et al., 2006).

Necrotic enteritis has been observed in several domestic and wild birds world wide. Recently several reviews were published (McDevitt et al., 2006; Opengart, 2008; Williams, 2005; Van Immerseel et al., 2004). Besides clinically manifested disease, subclinical infections may take place and are mostly accompanied with reduction of performance.

For decades it is generally known, that supplementation of poultry feed with antibiotic growth promoters (AGPs) improves performance of livestock. The effect of AGP on the gut flora results in a more stable balance of the microbial population, an improvement of digestion and better absorption of nutrients. As consequence this is accompanied with reduced intestinal disorders. However, AGP can also increase the prevalence of drug-resistant bacteria.

Due to concerns about increased prevalence of drug-resistant bacteria, AGPs were banned in the European Union in 2006 (EC, 2003). Reports from poultry farmers and veterinarians in the field indicate increasing problems with enteric problems reaching from "dysbacteriosis", a poorly defined condition characterized by clostridial infections, poor feed conversion, and wet droppings to necrotic enteritis (Hafez, 2010, van der Sluis, 2010; Van Immerseel et al., 2004). This resulted in higher use of antimicrobial drugs to control these conditions (Cooper and Songer, 2009), which again increases the risk of acquired antibiotic resistance.

In this study the minimal inhibitory concentrations (MICs) of some commonly used antimicrobials were determined using 100 *C. perfringens* isolates collected from German turkey flocks between 2008 and 2009, after the ban of AGPs.

**Materials and Methods**

**Bacterial isolates**

One hundred *C. perfringens* field strains were isolated from meat turkey flocks in different regions in Germany between March 2008 and March 2009. Boot swabs were collected in the frame of the salmonella surveillance program from meat turkey flocks before slaughter. The swabs were plated in 225 μl peptone water and vigorously mixed. Then 10 μl of the suspension were immediately transferred on tryptose sulfite cycloserine (TSC) agar (Merck, Darmstadt, Germany) and inoculated anaerobically at 37°C on Colombia blood agar containing Neomycin (200 μg/ml) and Polymixin B (100 μg/ml) and on egg yolk lactose agar for 36 – 48 h at 40°C. Identification as *C. perfringens* was done by detection of haemolysis, lactose fermentation and lecithinase activity. In doubtful cases, the diagnosis was confirmed by biochemical identification using the API 20E kit (Biomerieux, Marcy l’Etoile, France) according to the manufacturer’s instructions. The isolates were identified as *C. perfringens* type A by multiplex-PCR as described by Gao et al. (2010). The isolates were stored at −70°C. Thawed isolates were grown anaerobically at 37°C on Colombia blood agar with 5% sheep blood (Oxoid, Wesel, Germany).

**Antimicrobial susceptibility testing**

MICs were determined by broth dilution procedure using the commercially available broth micro-dilution test plate Avipro® Plate (Lohmann Animal Health, Cuxhaven, Germany). The test comprises 18 antimicrobials from 12 different antimicrobial classes and is based on the documentation of the standard performance criteria issued by the Clinical and Laboratory Standard Institute (CLSI, Wayne, Pennsylvania, USA). The test plates included 2 antimicrobials, namely streptomycin and rifampicin, which are used to differentiate a Salmonella vaccinal strain from field strains. These antibiotics are not included in the results, because only a very high concentration of these two compounds was tested.

The test was done according to the manufacturers instructions. Briefly, *C. perfringens* colonies were suspended in 0.9% NaCl to obtain a McFarland turbidity of 0.5, equal-
ling an estimated concentration of $1 \times 10^8$ colony-forming units (CFU) /ml. 100 μl of this suspension were diluted in Mueller-Hinton II broth to obtain a final concentration of the inoculum of $1 \times 10^6$ CFU/ml. 100 μl of the inoculum were given in each well of the plate, and the plate was incubated for 24 h at 37°C under anaerobic conditions.

The purity of each isolate was checked by plating 10 μl of the inoculum on Colombia blood agar with 5% sheep blood. The density of the inoculum was checked by a serial dilution of the inoculum in sterile 0.9% NaCl solution and plating on Colombia blood agar with 5% sheep blood. The MIC was determined as the lowest concentration of the antimicrobial agent that inhibited visible bacterial growth.

MIC$_{50}$ and MIC$_{90}$ were determined as the minimum concentration of tested antibiotics, at which growth of 50% or 90%, of strains respectively was inhibited.

Results

The results of 16 tested antibiotics as well as MIC$_{50}$ and MIC$_{90}$ are shown in Table 1. Clinical breakpoints to interpret the MIC of C. perfringens from turkey are not available. So different references were used for a tentative classification of the isolates as susceptible, intermediate, or resistant to each antimicrobial (Table 2).

All isolates were susceptible to β-lactam antibiotics penicillin and amoxicillin. Also all isolates were sensitive to the combination of lincomycin and spectinomycin as well as to tylosin. 98% and 83% of the isolates were sensitive to enrofloxacin and oxacillin respectively. Tiamulin, tilmicosin, as well as the combination trimethoprim/sulfamethoxazole showed also high effectiveness in vitro, with 80% and 72% of the isolates being sensitive.

Most of isolates were in the intermediate range for lincomycin (51%), erythromycin (58%), doxycyclin (100%), and tetracycline (100%), but no isolate was resistant. In contrast most or all isolates were resistant against spectinomycin (74%) and neomycin (94%). All isolates were resistant to colistin.

Two strains were resistant against one of the tested antimicrobials, 25 strains against 2 different antimicrobials, 53 strains against 3 different antimicrobials, 18 strains against 4 different antimicrobials and the remaining 2 strains against 5 different antimicrobials.

Discussion

In the present study all isolates showed various degrees of sensitivity to the antimicrobials tested. All isolates were found to be sensitive to the β-lactam antibiotics amoxicillin and penicillin as well as to tylosin. Similar results were reported by several previous investigations on C. perfringens isolated from various farm animals (DUTTA and DEVRIESE, 1980) and from turkey as well as broiler chickens (WATKINS et al., 1997; SILVA et al., 2009; MARTEL et al., 2004).

Also the isolates were highly sensitive to tylosin as reported by DEVRIESE et al. (1993), MARTEL et al. (2004) and GHOLAMIANDEHKORDI et al. (2009). In contrast the strains tested by WATKINS et al. (1997) were highly resistant to tylosin.

Enrofloxacin also showed a high in vitro activity against almost all isolates with only two isolates being intermittent resistant. These results were in agreement with GHOLAMIANDEHKORDI et al. (2009).

Table 1. Minimal inhibitory concentrations (MIC) of 16 antibiotics against 100 C. perfringens isolates from turkeys. The results are shown as number of strains for which the concentration was determined as MIC. Tested concentrations are underlined. The number of isolates whose MIC was above the highest tested concentration of an antibiotic is given in the adjacent cell on the right.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Concentration (μg/ml)</th>
<th>MIC$_{50}$</th>
<th>MIC$_{90}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.125</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Penicillin</td>
<td>86</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lincomycin/Spectinomycin$^1$</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tylosin</td>
<td>88</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>93</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Oxaclin</td>
<td>88</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Tiamulin$^2$</td>
<td>70</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>70</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Trimethoprim/Sulfamethoxazole$^2$</td>
<td>72</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>49</td>
<td>38</td>
<td>13</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>24</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Doxycyclin</td>
<td>77</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>48</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Neomycin</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Colistin</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

$^1$ The tested concentration was 8/32 μg/ml lincomycin/spectinomycin

$^2$ Tested concentrations were 0.5/9.5, 1/19, and 2/38 μg/ml trimethoprim/sulfamethoxazole

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LAMIANDEHKORDI et al. (2009), who investigated strains collected from broiler farms in Belgium. The low frequency of resistance against erythromycin also confirms other study in Belgium and Denmark (GHOLAMIANDEHKORDI et al., 2009; JOHANSSON et al., 2004).

For tilmicosin WATKINS et al. (1997) found MICs at about 2 μg/ml, while in the present study the MIC50 was ≤8 and the MIC90 was 16. Nevertheless most strains were classified as susceptible (72%) with some strains being intermediate (28%).

While in the current study no isolate was classified as resistant against tetracycline, and few strains might have been resistant against lincomycin, many studies reported higher resistance of C. perfringens against these two antibiotics (GHOLAMIANDEHKORDI et al., 2009; JOHANSSON et al., 2004; LEU, 2004; MARTEL et al., 2004; WATKINS et al., 1997).

In contrast the high level of resistance against colistin confirmed findings by BENNO et al. (1988). For the other antibiotics included in the present study comparable results are lacking in literature.

In general, the C. perfringens strains we tested showed a high degree of susceptibility to the antibiotics included in the test. With some antibiotics the percentage of susceptible isolates was even higher than in comparable previous studies. The differences in susceptibility profiles between antimicrobials from different regions may reflect the varying use of antimicrobial drugs in poultry production (WATKINS et al., 1997).

Furthermore laboratory methods used for the detection of resistance vary. Most common are disc diffusion test and determination of minimal inhibitory concentration by broth micro-dilution test. Disc methods are widely used because they are easy to apply and inexpensive, however, there are great variations in the antimicrobial concentrations and the bacterial counts between laboratories. Protocols for commercially available broth micro-dilution test are more standardized. Nevertheless, to correctly monitor the development of the antimicrobial resistance in an area, for comparison between laboratories quality assurance and ring-trials are necessary (JODAS and HAFEZ, 2003).

Finally, it will never be possible to stop the development of resistance completely, but limiting the problem is realistic. This will require a coordinated monitoring system, which is constructed to answer specific questions to give the basis of action and which is not an expensive end in itself (WISE and SOULSBY, 2002).

**Summary**

Minimum inhibitory concentrations of 16 antibiotics for 100 Clostridium perfringens isolates collected between 2008 and 2009 from commercial turkey flocks was determined using a commercially available broth micro-dilution test kit. No isolates were resistant against β-lactam antibiotics (amoxicillin, oxacillin, and penicillin), lincomycin, tylosin, doxycyclin, tetracycline, enrofloxacin, trimethoprim/sulfamethoxazole, lincomycin, and tilmicosin. A low frequency of resistance was detected against erythromycin and tiamulin with 5% and 20% respectively. Spectinomycin, neomycin, and colistin showed the highest incidence of resistance with 74%, 94%, and 100% respectively.

**Key words**

Clostridium perfringens, poultry, resistance, monitoring, MIC

**Zusammenfassung**

Die minimale Hemmkonzentration von 16 Antibiotika für 100 Clostridium perfringens Isolate, die zwischen 2008

**Stichworte**

*Clostridium perfringens*, Geflügel, Resistzenken, Monitoring, MHK

**References**


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