In vitro determination of antibiotic sensitivities of Clostridium perfringens isolates from layer flocks in Germany

In vitro Untersuchungen zur Antibiotikaempfindlichkeit von Clostridium perfringens Isolaten aus Legehennenbeständen in Deutschland

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

Manuscript received 8 August 2011, manuscript accepted 24 September 2011

Introduction

Infections with Clostridium perfringens in poultry can cause several clinical manifestations and lesions including necrotic enteritis, gangrenous dermatitis, cholangihepatitis as well as gizzard erosions. In addition, C. perfringens type A has been shown to cause food poisoning in humans as well as gizzard erosions. In addition, inhibitory concentrations (MICs) of 16 antibiotics of 100

The impact of the ban has been seen on performance parameters like body weight and feed conversion rate. It also led to wetter litter and higher ammonia levels, animal welfare problems like foot pad dermatitis and general health issues of the birds like enteric disorders due to dysbacteriosis and clostridial infections (Van der Sluis, 2010; Hafez, 2011).

In addition, the ban of AGPs indirectly leads to an increase in the use of antimicrobials to control diseases (Cooper and Songer, 2009; Gholamiandehkordi et al., 2009). These factors can increase the risk of acquired antibiotic resistance.

Materials and Methods

C. perfringens isolates

Isolation of C. perfringens field strains from turkeys and layers has been described previously (Gad et al., 2011a). Briefly, 34 paper pads of one day old layer chick’s, 122 faecal samples and 329 boot swabs, which were collected in the frame of the salmonella surveillance program between 2008 and 2010 from layer flocks in different stages of production, were investigated. In addition, samples from internal organs from suspected cases of necrotic enteritis were also included.

Paper pads and boot swabs were placed in 225 ml peptone water and mixed vigorously. Then 10 μl of the suspension was transferred immediately on tryptose sulfite cycloserine (TSC) agar (Merck, Darmstadt, Germany). Swabs from organ samples were directly plated on the same agar. Plates were inoculated under anaerobic conditions at 37°C. Colonies with suggestive morphology were sub-cultivated on 5% sheep blood agar supplemented with 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 as well as Gram staining. For confirmation and determination of the toxin var, isolates were investigated for the presence of major toxin genes as described by Gad et al. (2011a). C. perfringens was isolated from 134 (27.6%) of the samples from layers. In the present investigation 46 isolates were selected for determination of the MICs. These included 2 isolates from paper pads, 9 isolates from faecal droppings, 6 isolates from internal organs and 29 isolates from boot swabs.

References

1 Inst. for Poultry Diseases, Faculty of Veterinary Medicine, Free University Berlin, Germany
2 Inst. of Bacteriology and Mycology, Faculty of Veterinary Medicine, University of Leipzig, Germany

Determination of antibiotic sensitivities

MICs were determined by broth dilution procedure using the commercially available broth microdilution 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 include two 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, equalizing an estimated concentration of $1 \times 10^8$/ml colony forming units (Cfu)/ml. 100 μl of this suspension was 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 was 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 Columbia 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 that growth of 50% or 90% of strains respectively was inhibited.

For quality control as described by CLSI (2007) C. perfringens reference strains ATCC 3124 (type A) and ATCC 27324 (type E), E. coli ATCC 25922, Staphylococcus aureus ATCC 29213 and Bacteroides fragilis (DSMZ 2151) were included in the test. If the ATCC strains exhibited abnormal MIC values for any of the antimicrobials, the results of the test were not used.

Results

Quality control

The broth microdilution method was found to be reproducible within a ± 1 doubling dilution by comparing reference strains used in the study over a series of 10 independent experiments. Variation of the endpoint ± 1 doubling dilution is considered within the limit of error for most MIC methods (CLSI, 2007).

Determination of antibiotic sensitivities

46 isolates were selected and tested in the broth microdilution test. 43 isolates were toxovar A and 3 isolates toxovar D. The results as well as MIC$_{50}$ and MIC$_{90}$ are shown in Table 1 and 2.

Table 1. Minimal inhibitory concentrations (MIC) of 16 antibiotics against 46 C. perfringens isolates from chickens. The results are shown as number of strains for which the concentration was determined as MIC. Tested concentrations are shaded. The number of isolates whose MIC was above the highest tested concentration of an antibiotic is given in the adjacent cell on the right. Breakpoints are indicated by thick vertical lines

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>≤0.125</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>&gt;64</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>39</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>46</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lincomycin/Spectinomycin$^1$</td>
<td>46</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tylosin</td>
<td>44</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>44</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>38</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Tiamulin$^2$</td>
<td>28</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>37</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Trimethoprim/Sulfamethoxazole$^2$</td>
<td>41</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>22</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>44</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>41</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>6</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Neomycin</td>
<td>0</td>
<td>3</td>
<td>43</td>
<td>43</td>
<td>43</td>
<td>43</td>
<td>43</td>
<td>43</td>
<td>43</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>Colistin</td>
<td>0</td>
<td>0</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>46</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
Clinical breakpoints to interpret the MIC of *Clostridium perfringens* in chickens 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 susceptible to the combination of lincomycin and spectinomycin as well as to tylosin and enrofloxacin. 82.6% of the isolates were susceptible to oxacillin. Tiamulin, tilmicosin, neomycin (93.5%) All isolates were resistant to colistin. 87.0% of the isolates were resistant against spectinomycin (87.0%) and neomycin (93.5%). All isolates were resistant to colistin.

Most of isolates were in the intermediate range for lincomycin (52.2%), doxycyclin (100%), and tetracycline (100%), but no isolate was resistant. The MICs of erythromycin covered a broad range with 67.4% of isolating being partially resistant and 17.4% being resistant. In contrast most or all isolates were resistant against spectinomycin (87.0%) and neomycin (93.5%). All isolates were resistant to colistin.

Three strains were resistant against one of the tested antimicrobials, 12 strains against 2 different antimicrobials, 21 strains against 3 different antimicrobials, 21 strains against 4 different antimicrobials and the remaining 4 strains against 5 different antimicrobials.

### Discussion

In a previous study we showed that *C. perfringens* isolates originating from turkey flocks in Germany have various degrees of sensitivity to the tested antimicrobials (Gad et al., 2011b). In the present investigation chicken isolates were also found to be sensitive to the β-lactam antibiotics amoxicillin and penicillin as well as to tylosin. Similar results were reported by several previous examinations of *C. perfringens* isolates from various farm animals (Dutta and Devriese, 1980) as well as from broiler chickens (Watkins et al., 1997; Silva et al., 2009; Leu, 2004; Martel et al., 2004).

There seems to be some geographical and/or regional influence on tylosin resistance, because strains were highly resistant in the USA (Watkins et al., 1997), while European isolates are sensitive as reported by several authors (Devriese et al., 1993; Martel et al., 2004; Gholamiandehkordi et al., 2009; Gad et al., 2011b). Also for tilmicosin Watkins et al. (1997) found MICs at about 2 μg/ml, while in the present study the MIC<sub>50</sub> was ≤8 μg/ml and the MIC<sub>90</sub> was 16 μg/ml. Nevertheless most strains were classified as susceptible (80.4%) with some strains being intermediate (19.6%).

Enrofloxacin also showed a high *in vitro* activity against almost all isolates. These results are in agreement with Gholamiandehkordi et al. (2009), who investigated strains collected from broiler farms in Belgium. In contrast piglet’s isolates collected between 1997 and 2001 in Austria showed variable results (Luger, 2003).

The low frequency of resistance against erythromycin also confirms other reports from Belgium and Denmark (Gholamiandehkordi et al., 2009; Johansson et al., 2004).

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).

Comparing the resistance profiles against tetracycline and doxycyclin between turkey (Gad et al., 2011b) and chicken isolates, MIC<sub>90</sub> in turkey isolates were 4 and 8 μg/ml while in chickens they were ≤2 and 4 μg/ml respectively. This may reflect a higher use of tetracycline compounds in meat turkey flocks than in layers flocks. According to Schetentag et al. (2001) exposure to antimicrobials at concentrations close to or below the MIC for a particular organism is one factor involved in the selection of resistant
bacterial strains. Thus administration of antimicrobials to treat the diseases associated with these organisms may in fact promote resistant strains. This becomes even more significant due to the fact that many of the tetracycline resistance genes are transferable, even between bacterial genera, and conjugative transfer may be induced by subinhibitory concentrations of antimicrobials.

The high level of resistance to colistin confirmed findings obtained by Benno et al. (1988). For the other antibiotics included in the present study comparable results are lacking in literature.

In general, investigation of German C. perfringens isolates from layers we tested showed a similar high degree of susceptibility to the antibiotics included in the test as did the investigation of German isolates from turkeys. This supports the assumption, that differences in susceptibility profiles between antimicrobials from different regions may reflect the varying use of antimicrobial drugs in those regions (WATKINS et al., 1997), while a comparable use of antimicrobial drugs results in comparable susceptibility profiles.

Summary

Minimum inhibitory concentrations of 16 antibiotics for 46 Clostridium perfringens isolates collected between 2008 and 2010 from commercial chicken 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, tetracyclin, erythromycin, and tilmicosin. A low frequency of resistance was detected against erythromycin and tiamulin with 17.4% and 19.6% respectively. However, most of the isolated (67.4%) were partially sensitive to erythromycin. Spectinomycin, neomycin, and colistin showed the highest incidence of resistance with 87.0%, 93.5%, and 100% respectively.

Key words

Laying hen, Clostridium perfringens, antibiotics

Zusammenfassung

In vitro Untersuchungen zur Antibiotikaempfindlichkeit von Clostridium perfringens Isolaten aus Legehennenbeständen in Deutschland

Die minimale Hemmkkonzentration von 16 Antibiotika für 46 Clostridium perfringens Isolate, die zwischen 2008 und 2010 von Legehennenbeständen isoliert wurden, wurde mittels eines kommerziell erhältlichen Mikrodilutions-Test-Kits bestimmt. Kein Isolat war gegen β-lactam Antibiotika (Ampicillin, Oxacillin und Penicillin), sowie gegen Lincopectin, Tylosin, Doxycyclin, Tetracyclin, Enrofloxacin, Trimethoprim/sulfamethoxazole, Lincomycin und Tilmicosin resistent. 17.4% bzw. 19.6% der Isolate waren gegen Erythromycin und Tiamulin resistent. Jedoch waren 67.4% der Isolate partiell empfindlich gegen Erythromycin. Spectinomycin, Neomycin und Colistin wiesen mit 87.0%, 95.5% bzw. 100% den höchsten Anteil resistenter Isolate auf.

Stichworte

Legehenne, Clostridium perfringens, Antibiotika
of Clostridium perfringens-associated hepatitis. Avian Pathology 30, 73-81.


