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The effect of commonly used anticoccidials and antibiotics in a subclinical necrotic enteritis model

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Necrotic enteritis poses an important health risk to broilers. The ionophore anticoccidials lasalocid, salinomycin, maduramicin, narasin and a combination of narasin and nicarbazin were tested in feed for their prophylactic effect on the incidence of necrotic enteritis in a subclinical experimental infection model that uses coccidia as a predisposing factor. In addition, drinking water medication with the antibiotics amoxicillin, tylosin and lincomycin was evaluated as curative treatment in the same experimental model. The minimal inhibitory concentrations (MICs) of all antibiotics and anticoccidials were determined in vitro against 51 Clostridium perfringens strains isolated from broilers. The strains examined appeared uniformly susceptible to lasalocid, maduramicin, narasin, salinomycin, amoxicillin and tylosin, whereas an extended frequency distribution range of MICs for lincomycin was seen, indicating acquired resistance in 36 isolates in the higher range of MICs. Nicarbazin did not inhibit the in vitro growth of the C. perfringens strains even at a concentration of 128 μg/ml. Supplementation of the diet from day 1 onwards with lasalocid, salinomycin, narasin or maduramicin led to a reduction in birds with necrotic enteritis lesions as compared with the non-mediated infected control group. A combination product of narasin and nicarbazin had no significant protective effect. Treatment with amoxicillin, lincomycin and tylosin completely stopped the development of necrotic lesions.

Introduction

Necrotic enteritis is a disease of major economic importance affecting the poultry industry worldwide. Globally, annual costs attributed to necrotic enteritis are estimated to be over US$2 billion due to production losses and medical treatments (Anonymous, 2000).

The causative agents of necrotic enteritis are Clostridium perfringens toxino type A strains that are able to produce the NetB toxin (Engström et al., 2003; Gholamiandekhordi et al., 2006; Keyburn et al., 2006, 2008). The onset of necrotic enteritis is, however, a multifactorial event in which (sub)clinical coccidiosis is believed to be one of the major predisposing factors. Several field cases have been reported in which coccidiosis preceded or coincided with necrotic enteritis, although clinical coccidiosis will not always result in the development of necrotic enteritis (Long, 1973; Broussard et al., 1986; Dhillon et al., 2004; Droual et al., 1994; Hermans & Morgan, 2007). Combined experimental infection with species of Eimeria and C. perfringens leads to higher intestinal lesion scores, increased intestinal numbers of C. perfringens, increased mortality and reduced weight gain compared with infection with C. perfringens alone (Al-Sheikhly & Al-Saieg, 1980; Shane et al., 1985; Kageyama et al., 1987; Baba et al., 1992, 1997; Williams et al., 2003; Gholamiandekhordi et al., 2007; Park et al., 2008).

Until recently, necrotic enteritis was effectively controlled by mixing antimicrobial growth promoters in the broiler feed (Prescott et al., 1978; Elwinger et al., 1992, 1998). Concerns about the potential risk of transmission of antimicrobial resistance, induced in bacterial populations that are carried by domestic animals, towards bacteria infecting humans drove Norway to ban the use of all antimicrobial growth promoters in poultry feed for growth-promoting purposes in 1995. This led to a rise in necrotic enteritis-affected flocks in Norway up to 40% (Kaldhusdal & Lovland, 2000). From 1 January 2006, the use of all antimicrobial growth promoters in poultry feed is also forbidden in the European Union (Regulation (EC) No. 1831/2003).

Since the ban, European farmers rely mostly on curative use of antibiotics such as amoxicillin and tylosin to control necrotic enteritis whenever clinical signs become apparent, but no reports exist on the efficiency of these antibiotics to stop the development of necrotic enteritis during an outbreak (Casewell et al., 2003; Hermans & Morgan, 2007). However, when these curative antibiotics are used without precaution for the treatment of food-producing animals, they may contribute to the development of antimicrobial resistance to important human drugs (Giguère, 2006, Schwarz et al., 2006).

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Previous studies reported that *C. perfringens* strains were susceptible to the antibiotics amoxicillin and tylosin *in vitro*, but that some broiler isolates showed already acquired resistance against lincomycin (Martel et al., 2004). The carboxylic ionophore polyether anticoccidials lasalocid, maduramicin, narasin and salinomycin all possess antibacterial activity and inhibit the growth of *C. perfringens* *in vitro* (Kondo, 1988; Devriese et al., 1993; Martel et al., 2004). These anticoccidials are currently in use in the European Union and therefore, when proven active *in vitro*, could be useful to European farmers in the prevention of necrotic enteritis. In Norway, after the ban of avoparcin, an increase of necrotic enteritis was observed that was believed to be partly levelled out by the introduction of narasin as a feed additive (Grave et al., 2004).

The present study aims to assess the efficacy of ionophore anticoccidials and a selection of commonly used antibiotics to control necrotic enteritis in a well-established *in vivo* model (Gholamiandehkordi et al., 2007; Timbermont et al., 2008).

Materials and Methods

**Strains and vaccines.** Fifty-one *C. perfringens* type A strains belonging to different genotypes, as analysed by pulsed field gel electrophoresis, were included. Thirty-five strains were isolated from broiler chickens in Belgium: 26 strains from clinically healthy broiler chickens and nine strains from broilers suffering from necrotic enteritis (Gholamiandehkordi et al., 2006). Sixteen Danish *C. perfringens* isolates from necrotic enteritis cases were kindly provided by Dr L. Bjerrum (Naurby et al., 2003). *C. perfringens* strain 56, the strain used in the *in vivo* trials, was isolated from the intestine of a broiler chicken with severe necrotic gut lesions. It belongs to toxotype A (no β; or enterotoxin genes) and produces moderate amounts of alpha toxin *in vitro* (Gholamiandehkordi et al., 2006). The strain carries the netB gene and has been used previously to induce necrotic enteritis in an *in vivo* model (Timbermont et al., 2008). Before inoculation of the chickens, the bacteria were cultured for 24 h at 42°C in brain heart infusion broth (Oxoid, Basingstoke, UK) in an anaerobic cabinet. The commercial vaccine Nobilis Gumboro D78 (Intervet, Mechelem, Belgium) and the anticoccidial vaccine Paracox-5™ (Schering-Plough Animal Health, Brus- sels, Belgium), containing live precocious oocysts of *Eimeria acervulina, Eimeria maxima* (two lines), *Eimeria mitis* and *Eimeria tenella*, were used to create predisposing lesions for necrotic enteritis.

**Minimum inhibitory concentrations of anticoccidials and antibiotics.** All antibiotics and anticoccidials were obtained from Alpharma Animal Health (Bridgewater, New Jersey, USA) except for narasin and nicarbazin (Sigma, St Louis, Missouri, USA). Minimal inhibitory concentrations (MICs) of all antibiotics and anticoccidials were tested using the agar dilution method based on the guidelines of the CLSI (2008) (formerly NCCLS). Strains were inoculated onto Columbia agar supplemented with 5% sheep blood (Oxoid) and then incubated in an anaerobic atmosphere for 24 h at 37°C. Cultures were checked for purity. Up to five colonies were then suspended in phosphate-buffered saline at pH 7.2 to a density of 0.5 McFarland, as determined with an ATB 1550 reader. Using a MAST inoculum applicator, a 1/10 dilution of this suspension was inoculated on Mueller Hinton II agar (Oxoid) plates containing serial two-fold dilutions of the antibiotics and anticoccidials. The concentration range tested for each product was 0.03 to 128 μg/ml. The plates were incubated in an anaerobic atmosphere and observed after 24 h for bacterial growth. The MIC was defined as the lowest concentration producing no visible growth. One agar plate without anticoccidials or antibiotics was included to verify growth of all strains tested. Strains used for quality control were *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922.

**Birds and housing.** *In vivo* necrotic enteritis trials were performed using experimental inoculations were performed with Ross 308 broiler chickens that were obtained as 1-day-old chicks from a commercial hatchery. Per trial, all treatment groups were housed in the same room. The birds were reared in cages with a density of 28 birds/m² on wood shavings. All cages were separated by solid walls to prevent contact between birds from different treatment groups. Before the trial, the rooms were decontaminated with Metacetetyl HQ (Clim’o Medic®; Metecta, Belgium) and a commercial anticoccidial disinfectant (OOCIDE; DuPont Animal Health Solutions, Wilmington, Delaware, USA). The chickens were divided in groups of 28 birds. They received *ad libitum* drinking water and feed. A 23 h/1 h light/darkness programme was applied. The bird experiments were carried out according to the recommendations of and following approval of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

**In vivo necrotic enteritis model.** Trials were performed as described previously (Gholamiandehkordi et al., 2007). In short, broilers were fed a wheat/rye-based (43%/7.5%) diet, with soybean meal as protein source. The feed composition was described by Gholamiandehkordi et al. (2007). Gumboro vaccine was given in the drinking water on day 16 in all groups. From day 17 onwards, the same diet was used with the exception that fishmeal (30%) replaced soy bean as the protein source. All groups were orally challenged using a plastic tube (three times a day) with approximately $4 \times 10^8$ colony-forming units of *C. perfringens* bacteria on days 17, 18, 19 and 20. On day 18, all birds were orally inoculated with a 10-fold dose of Paracox-5™. The vaccine was diluted in 1 ml water and given orally with a syringe to each chicken individually. Each chicken received approximately numbers of sporulated oocysts: *E. acervulina* HP, 5000 to 6500; *E. maxima* CP, 2000 to 2300; *E. maxima* MFP, 1000 to 1300; *E. mitis* HP, 10,000 to 13,000; and *E. tenella* HP, 5000 to 6500.

On days 22, 23 and 24, nine birds from each group were euthanized by intravenous T61 (Intervet) injection. Necrotic enteritis lesion scoring and sample collection was performed, as described below.

In the first trial, from day 1 onwards, anticoccidials were mixed in the diet of five out of seven groups according to the recommendations of the suppliers at a concentration of 75 ppm for lasalocid, 70 ppm for salinomycin, 5 ppm for maduramicin, 70 ppm for narasin when used on its own, and 50 ppm for narasin and 50 ppm for nicarbazin when present in the combined product. All anticoccidials were obtained from Alpharma Animal Health except for narasin and the combination product of narasin and nicarbazin (Elanco, Greenfield, Indiana, USA). Analysis of the feed samples was performed by high-performance liquid chromatography in accredited laboratories and confirmed the concentration of the test substances. Two control groups were included: a non-medicated group receiving only the 10-fold dose of the Paracox-5™ vaccine, and a non-medicated group receiving both the 10-fold dose of Paracox-5™ vaccine and the *C. perfringens* infection (positive control).

In the second trial, seven out of nine groups were treated from days 20 to 24 with antibiotics in the drinking water at concentrations of 50, 100 and 150 g/1000 l for amoxicillin, 50 and 150 g/1000 l for lincomycin, and 100 and 200 g/1000 l for tylosin. The same two non-medicated control groups were included as described for Trial 1.

**Macroscopic lesion scoring.** Intestinal lesions in the small intestine (duodenum to ileum) were scored as described by Keyburn et al. (2006). Birds with lesion scores of two (one to five lesions) or more were classified as necrotic enteritis-positive.

**Statistical analysis.** The data were analysed with SPSS Statistics 17.0 software (SPSS Inc., Chicago, Illinois, USA) using the binary logistic regression method to compare the number of necrotic enteritis-positive birds (lesion score $\geq 2$) within the test groups. Bonferroni’s correction for multiple comparisons was applied ($P < 0.05/n$) and significance was determined at $P < 0.007$ for the first trial and at $P < 0.005$ for the second trial.

**Results**

**Minimal inhibitory concentrations of the tested anticoccidials and antibiotics.** The results for the reference strains *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 were within acceptable CLSI quality control limits for...
amoxicillin, lincomycin and tylosin. The MIC values for 51 broiler chicken isolates are presented in Table 1. The MIC of the lincosamide antibiotic lincomycin showed an extended frequency distribution range between 0.25 and 16 mg/ml, indicating acquired resistance in isolates in the higher range of MICs. Acquired resistance was not detected for the other antibiotics and anticoccidials tested. The *C. perfringens* isolates examined were highly susceptible to the ionophore antibiotics lasalocid, narasin, maduramicin and salinomycin. Nicarbazin did not inhibit the *in vitro* growth of the *C. perfringens* isolates even at a concentration of 128 µg/ml.

**In vivo trials.** Birds that died before the infection started were necropsied to determine the cause of death. None of the birds showed necrotic lesions in their intestines. Table 2 summarizes the number of birds having necrotic lesions in the different treatment groups. The intestinal lesions presented as multiple necrotic foci, mostly in the duodenum and jejunum. No necrotic enteritis lesions were observed in the negative control group; that is, the untreated group only inoculated with a 10-fold dose of Paracox-5™. In the positive control group, more than 50% of the birds presented lesions.

Mixing lasalocid, narasin or salinomycin in the feed resulted in a statistically significant decrease in the number of birds having macroscopic necrotic lesions compared with the infected, untreated positive control group (*P* <0.007). The decrease in number of positive birds observed with maduramicin was borderline non-significant (*P* =0.009). When the results for narasin, lasalocid and salinomycin were compared with each other, there was no statistically significant difference in the number of positive birds between these groups. The addition of a combination of narasin (50 ppm) and narcarbazin to the feed was less effective than narasin (70 ppm) alone and did not cause a significant decrease in the number of birds compared with the control group.

In Trial 2, all three antibiotics tested completely stopped the development of necrotic enteritis lesions in the gut, resulting in zero birds with lesions in all medicated groups already at the lowest concentration tested.

**Table 1. MICs of various agents for *S. C. perfringens* strains of broiler chicken origin**

<table>
<thead>
<tr>
<th>Compound</th>
<th>5</th>
<th>0.03 mg/ml</th>
<th>0.06 mg/ml</th>
<th>0.12 mg/ml</th>
<th>0.25 mg/ml</th>
<th>0.5 mg/ml</th>
<th>1 mg/ml</th>
<th>2 mg/ml</th>
<th>4 mg/ml</th>
<th>8 mg/ml</th>
<th>16 mg/ml</th>
<th>32 mg/ml</th>
<th>64 mg/ml</th>
<th>128 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>46</td>
<td>5</td>
<td>17</td>
<td>6</td>
<td>9</td>
<td>3</td>
<td>22</td>
<td>15</td>
<td>11</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Lasalocid</td>
<td>34</td>
<td>17</td>
<td>6</td>
<td>9</td>
<td>3</td>
<td>22</td>
<td>15</td>
<td>11</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>693</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Maduramicin</td>
<td>1</td>
<td>5</td>
<td>17</td>
<td>6</td>
<td>9</td>
<td>3</td>
<td>22</td>
<td>15</td>
<td>11</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Narasin</td>
<td>74</td>
<td>4</td>
<td>17</td>
<td>6</td>
<td>9</td>
<td>3</td>
<td>22</td>
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<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Nicarbazin</td>
<td>51</td>
<td>1</td>
<td>5</td>
<td>17</td>
<td>6</td>
<td>9</td>
<td>3</td>
<td>22</td>
<td>15</td>
<td>11</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Salinomycin</td>
<td>30</td>
<td>21</td>
<td>17</td>
<td>6</td>
<td>9</td>
<td>3</td>
<td>22</td>
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<td>1</td>
<td>1</td>
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<td>1</td>
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<tr>
<td>Tylosin</td>
<td>45</td>
<td>6</td>
<td>17</td>
<td>6</td>
<td>9</td>
<td>3</td>
<td>22</td>
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<td>1</td>
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</tbody>
</table>

**Discussion**

The results obtained in the experimental infection model clearly show a significant decrease in number of broilers with necrotic enteritis gut lesions when lasalocid, salinomycin or narasin were mixed in the feed from day one onwards. All broiler *C. perfringens* isolates tested were uniformly sensitive to these anticoccidials *in vitro*. The results obtained in the *in vivo* necrotic enteritis model confirm earlier reports in which narasin and salinomycin were shown to have beneficial effects on the control of necrotic enteritis in broilers *in vivo* (Elwinger et al., 1992; Bolder et al., 1999; Waldenstedt et al., 1999; Engberg et al., 2000; Vissiennon et al., 2000; Brennan et al., 2001a; Johansen et al., 2007). Narasin significantly reduces the mean necrotic enteritis lesion scores and necrotic enteritis-associated mortality compared with control groups (Vissiennon et al., 2000; Brennan et al., 2001a). Salinomycin has been proven to decrease both the caecal count and prevalence of *C. perfringens* in broilers and reduces the shedding of...
In contrast to a previous report, among the broiler isolates tested, there was no tendency towards a bimodal distribution of MICs of amoxicillin (Martel et al., 2004). The MICs for lincomycin and tylosin were in the same range as those described in 2004 (Martel et al., 2004). No CLSI or EUCAST breakpoints or cut-off values are available for lincomycin (http://www.eucast.org, http://www.clsi.org). Martel et al. (2004) set the wild-type cut-off value for lincomycin, differentiating isolates with normal susceptibility from those with acquired resistance at 1 μg/ml. When applied to our results, 36 out of the 51 isolates tested showed acquired resistance, including the strain 56 used for the in vivo trial, which had a MIC of 4 μg/ml. Nevertheless, in the present study, using strain 56 as challenge, lincomycin treatment successfully stopped the development of necrotic lesions, indicating that the clinical breakpoint of lincomycin against enteric C. perfringens infections in poultry is higher than the wild-type cut-off value proposed by Martel et al. (2004). Although prolonged use of ionophore anticoccidials might induce reduced susceptibility or antibiotic resistance in the intestinal bacterial population, as described for Staphylococcus spp. and Enterococcus spp., these ionophores have a rather limited spectrum and are not used in human medicine (Butaye et al., 2003, Dowling, 2006). Furthermore, no cross-resistance with currently used antibiotics in human medicine is known so there is no danger of transmission of antibiotic resistance selected in animals towards humans (Callaway et al., 2003). In contrast, amoxicillin, lincomycin and macrolides are commonly used in both veterinary and human medicine. Cross-resistance among macrolides, lincosamids and streptogramin group B antibiotics is known to occur (Giguère, 2006; Schwarz et al., 2006). Therefore, the authors recommend to use amoxicillin, lincomycin and tylosin only in acute outbreaks of necrotic enteritis but to avoid recurrent exposure of the intestinal flora to these antibiotics. Both for ionophore anticoccidials and amoxicillin, lincomycin and tylosin, the induction of antibiotic resistance should be monitored regularly.

In conclusion, it was shown in an experimental infection model that ionophore anticoccidials may contribute to the control of necrotic enteritis in broiler.
chickens. Further field trials including a larger number of birds are necessary to verify our results. Amoxicillin, tylosin and lincomycin were shown to be effective in curing necrotic enteritis outbreaks. Acquired resistance was detected only for lincomycin.

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