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► **To cite this version:**

Anouk Lanckriet, Leen Timbermont, Maarten de Gussem, Maja Marien, Dieter Vancraeynest, et al.. The effect of commonly used anticoccidials and antibiotics in a subclinical necrotic enteritis model. Avian Pathology, 2010, 39 (01), pp.63-68. 10.1080/03079450903505771 . hal-00557321

HAL Id: hal-00557321

<https://hal.science/hal-00557321>

Submitted on 19 Jan 2011

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Journal:	<i>Avian Pathology</i>
Manuscript ID:	CAVP-2009-0121.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	26-Oct-2009
Complete List of Authors:	Lanckriet, Anouk; University of Ghent, Pathology, bacteriology and avian diseases Timbermont, Leen; University of Ghent, Pathology, bacteriology and avian diseases De Gussem, Maarten; Alpharma Animal Health Marien, Maja; Alpharma Animal Health Vancraeynest, Dieter; Alpharma Animal Health Haesebrouck, Freddy; University of Ghent, Pathology, bacteriology and avian diseases Ducatelle, Richard; University of Ghent, Pathology, bacteriology and avian diseases Van Immerseel, Filip; University of Ghent, Pathology, bacteriology and avian diseases
Keywords:	Necrotic enteritis, Clostridium perfringens, ionophore anticoccidials, antibiotics

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Cavp-2009-0121.R1

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

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Short title: Anticoccidials and antibiotics

Received 5 May 2009

Abstract

Necrotic enteritis poses an important health risk to broilers. The ionophore anticoccidials lasalocid, salinomycin, maduramicin, narasin and a combination of narasin and nicarbazine were tested in feed for their prophylactic effect on the incidence of necrotic enteritis in a subclinical experimental infection model that uses coccidia as 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 *C. 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 of lincomycin was seen, indicating acquired resistance in thirty-six isolates in the higher range of MICs. Nicarbazine 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 lead to a reduction in birds with necrotic enteritis lesions as compared to the non-medicated infected control group. A combination product of narasin and nicarbazine 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* toxinotype A strains that are able to produce the NetB toxin (Engstrom *et al.*, 2003; Gholamiandekhordi *et al.*, 2006; Keyburn *et al.*, 2006; Keyburn *et al.*, 2008). The onset of NE 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; Droual *et al.*, 1994; Dhillon *et al.*, 2004; 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 to infection with *C. perfringens* alone (Al-Sheikhly & Al-Saieg, 1980; Shane *et al.*, 1985; Kageyama *et al.*, 1987; Baba *et al.*, 1992; Baba *et al.*, 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 (AGPs) in the broiler feed (Prescott *et al.*, 1978; Elwinger *et al.*, 1992; Elwinger *et al.*, 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 AGPs 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 January 1st 2006, the use of all AGPs 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 like 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). 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 vivo*, could be useful to the 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 leveled 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 (Gholamiandekordi *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 analyzed by Pulsed Field Gel Electrophoresis (PFGE), were included. Thirty-five strains were isolated from broiler chickens in Belgium: 26 strains from clinically healthy broiler chickens and 9 strains from broilers suffering from necrotic enteritis (Gholamiandekordi *et al.*, 2006). Sixteen Danish *C. perfringens* isolates from necrotic enteritis cases were kindly provided by Dr. L. Bjerrum (Nauerby *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 toxinotype A (no beta2 or enterotoxin genes) and produces moderate amounts of alpha toxin *in vitro* (Gholamiandekordi *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 (BHI, Oxoid, Basingstoke, England) in an anaerobic cabinet. The commercial vaccine Nobilis Gumboro D78 (Intervet, Mechelen, Belgium) and the anticoccidial vaccine Paracox-5TM (Schering-Plough Animal Health, Brussels, Belgium), containing live precocious oocysts of *Eimeria acervulina* (*E. acervulina*), *E. maxima* (two lines), *E. mitis* and *E. 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 NJ, USA) except for narasin and nicarbazin (Sigma, St. Louis Mo, 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 5 colonies were then suspended in Phosphate Buffered Saline (PBS) 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 ranges tested for each product were 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 using experimental inoculations were performed with Ross 308 broiler chickens that were obtained as one-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/1 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 Metatectyl HQ (Clim'o Medic[®], Metatecta, Belgium) and a commercial anticoccidial disinfectant (OOCIDE, DuPont Animal Health Solutions, Wilmington, US). The chickens were divided in groups of 28 animals. They received ad libitum drinking water and feed. A 23 h/1 h light/darkness program was applied. The animal experiments were carried out according to the recommendations 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 protein source. All groups were orally challenged using a plastic tube (three times a day) with approximately 4×10^8 cfu *C. perfringens* bacteria on days 17, 18, 19 and 20. On day 18, all birds were orally inoculated with a ten-fold dose of Paracox-5TM. The vaccine was diluted in 1 ml of water and given orally with a syringe to each chicken individually. Each chicken got approximate numbers of sporulated oocysts: *E. acervulina* HP, 5000 – 6500; *E. maxima* CP, 2000 – 2300; *E. maxima* MFP, 1000 – 1300; *E. mitis* HP, 10000 – 13000; *E. tenella* HP, 5000 – 6500.

On days 22, 23 and 24, nine animals of each group were euthanized by intravenous T61 (Intervet, Mechelen, Belgium) 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 5 out of 7 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 IN, USA). Analysis of the feed samples was performed by HPLC 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-5TM vaccine and a non-medicated group receiving both the 10-fold dose of Paracox-5 and the *C. perfringens* infection (positive control).

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

Macroscopical lesions scoring. Intestinal lesions in the small intestine (duodenum to ileum) were scored as described by Keyburn *et al.* (2006). Birds with lesion scores of 2 (1 to 5 lesions) or more were classified as necrotic enteritis positive.

Statistical analysis. The data were analyzed with SPSS Statistics 17.0 software (SPSS Inc., Chicago) using the binary logistic regression method to compare the number of necrotic

enteritis positive animals (lesion score ≥ 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 $P < 0.0055$ 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 shown in Table 1. The MIC of the lincosamide antibiotic lincomycin showed an extended frequency distribution range between 0.25 and 16 $\mu\text{g/ml}$, indicating acquired resistance in isolates in the higher range of MICs. Acquired resistance was not detected to 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 $\mu\text{g/ml}$.

***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 their small intestine 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, i.e. 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 to the infected, untreated positive control group ($P < 0.007$). The decrease in number of positive animals obtained 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 number of positive birds between these groups. The addition of a combination of narasin (50 ppm) and nicarbazin to the feed was less effective than narasin (70 ppm) alone and did not cause a significant decrease in the number of animals compared to 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.

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 1 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 lesions scores and necrotic enteritis associated mortality compared to control groups (Vissiennon *et al.*, 2000; Brennan *et al.*, 2001 a). Salinomycin has been proven to decrease both the caecal count and prevalence of *C. perfringens* in broilers and reduces the shedding of *C. perfringens* after experimental infection (Bolder *et al.*, 1999; Engberg *et al.*, 2000; Johansen *et al.*, 2007). This anticoccidial was also able to diminish the severity of the lesions due to necrotic enteritis in an experimental infection model (Elwinger *et al.*, 1998; Engberg *et al.*, 2000; Jackson *et al.*, 2003).

The inhibitory activity of lasalocid or maduramicin against *C. perfringens in vivo* in broilers has not been reported before. The efficiency of lasalocid in reducing the number of birds with lesions was comparable to that of salinomycin and narasin.

In article 11 of the European Council regulation 1831/2003, the European Union states that the use of anticoccidials as feed additives should be evaluated by December 2012 (Regulation, 2003). However, in 2008, the European Commission submitted a report on the use of these substances as feed additives and existing alternatives to the Council and the European Parliament (COM, 2008). In this report, the European Commission clearly recommends to maintain the current legislation and allow the use of anticoccidials, including ionophores as feed additives because of the lack of alternatives and to preserve the economic viability of the poultry industry. Since then, it is unclear whether the European Council will consider the phase-out of anticoccidials as feed-additives and change the regulation for their use or whether the European Council will follow the advice of the European Commission.

Amoxicillin, tylosin and lincomycin were equally effective in abolishing the development of necrotic enteritis after infection with the virulent strain 56, indicating that these antibiotics can be used to treat necrotic enteritis outbreaks. These results confirm previous publications in which tylosin was shown to have beneficial effects on the occurrence of lesions and mortality due to necrotic enteritis (Stutz & Lawton, 1984; Waldenstedt *et al.*, 1999; Vissienon *et al.*, 2000; Brennan *et al.*, 2001b; Collier *et al.*, 2003). Lincomycin is also known to reduce necrotic enteritis associated mortality (Maxey & Page, 1977; Truscott & Al-Sheikhly, 1977; Hamdy *et al.*, 1983). To our knowledge, no reports on field trials using amoxicillin to treat necrotic enteritis in broilers exist

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, thirty-six of the fifty-one 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* sp.

and *Enterococcus* sp., 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 are known so there is no danger of transmission of antibiotic resistance selected in animals towards humans (Callaway *et al.*, 2003). In contrast, amoxicillin, lincosamids and macrolides are commonly used in both veterinary and human medicine. Cross resistance among macrolides, lincosamids and streptogramin groupB antibiotics is known to occur (Giguere, 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 animals, 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 to lincomycin.

Acknowledgements

We would like to thank Renzo Vercammen and Arlette Van de Kerckhove for their skilful technical assistance. This work was supported by the Institute for Science and Technology, Flanders (IWT). Dr. F. Van Immerseel is supported by a Postdoctoral Research Grant of the Research Foundation - Flanders (FWO) and by the Research Fund of Ghent University.

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Table 1. Minimal Inhibitory Concentrations (MIC) of various agents for 51 *Clostridium perfringens* strains of broiler chicken origin

Compound	Number of strains with MIC ($\mu\text{g/ml}$) of												
	≤ 0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	≥ 128
amoxicillin	46 ^a	5	-	-	-	-	-	-	-	-	-	-	-
lasalocid	-	-	34	17	-	-	-	-	-	-	-	-	-
lincomycin	-	-	-	6	9	3	15	11	6	1	-	-	-
maduramicin	-	-	-	-	29	22	-	-	-	-	-	-	-
narasin	-	7	44	-	-	-	-	-	-	-	-	-	-
nicarbazin	-	-	-	-	-	-	-	-	-	-	-	-	51
salinomycin	-	-	30	21	-	-	-	-	-	-	-	-	-
tylosin	-	-	-	-	45	6	-	-	-	-	-	-	-

^a For each antibiotic, the number of isolates sharing the same MIC as strain 56 used in the *in vivo* trial is shown in bold

Table 2. Number of birds with macroscopic necrotic enteritis lesions (lesion score ≥ 2) on each sampling day

Trial	Group	Day 22	Day 23	Day 24	Total
1	lasalocid 75 ppm	2/10	0/9	2/9	4/28 ^a
	salinomycin 70 ppm	1/9	2/9	2/8	5/26 ^a
	maduramicin 5 ppm	3/9	2/9	1/8	6/26
	narasin 70 ppm	0/9	1/9	1/9	2/27 ^a
	narasin 50 ppm + nicarbazin 50 ppm	4/9	4/9	2/9	10/27
	non-medicated, infected control	5/10	7/9	5/9	17/28
	non-medicated, uninfected control	0/9	0/9	0/9	0/27 ^a
2	amoxicillin 50 g/1000 liter	0/9	0/9	0/9	0/27 ^b
	amoxicillin 100g/1000 liter	0/9	0/9	0/9	0/27 ^b
	amoxicillin 150g/1000 liter	0/9	0/9	0/9	0/27 ^b
	lincomycin 50g/1000 liter	0/9	0/9	0/9	0/27 ^b
	lincomycin 150g/1000 liter	0/9	0/9	0/9	0/27 ^b
	tylosin 100g/1000 liter	0/9	0/9	0/9	0/27 ^b
	tylosin 200g/1000 liter	0/9	0/9	0/9	0/27 ^b
	non-medicated, infected control	4/9	5/9	5/9	14/27
	non-medicated, uninfected control	0/8	0/8	0/8	0/24 ^b

^{a,b} Values with superscripts differ significantly ($P < 0.007$ for trial 1 and $P < 0.0055$ for trial 2)

from the non-medicated, infected control in the first (^a) and second (^b) trial.