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Effect of in-feed paromomycin supplementation on antimicrobial resistance of enteric bacteria in turkeys ☆

Isabelle Kempf [a,*], Aurélie Le Roux [a], Agnès Perrin-Guyomard [b], Gwenaëlle Mourand [a], Laetitia Le Devendec [a], Stéphanie Bougeard [a], Pascal Richez [c], Gilles Le Pottier [d], Nicolas Eterradossi [a]

[a] Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES), Université Européenne de Bretagne, Laboratoire de Ploufragan-Plouzané, Zoopole les Croix, F-22440 Ploufragan, France
[b] Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES), Laboratoire de Fougères, La Haute Marche, BP 90203, 35302 Fougères Cedex, France
[c] TransPharm, BP 7, F-34160 Saint Geniès des Mourgues, France
[d] Comité Interprofessionnel de la Dinde Française (CIDEF), 35310 Mordelles, France

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ABSTRACT

Histomoniasis in turkeys can be prevented by administering paromomycin sulfate, an aminoglycoside antimicrobial agent, in feed. The aim of this study was to evaluate the impact of in-feed paromomycin sulfate supplementation on the antimicrobial resistance of intestinal bacteria in turkeys. Twelve flocks of breeder turkeys were administered 100 ppm paromomycin sulfate from hatching to day 120; 12 flocks not supplemented with paromomycin were used as controls. Faecal samples were collected monthly from days 0 to 180. The resistance of *Escherichia coli*, *Enterococcus faecium* and *Staphylococcus aureus* to paramomycin and other antimicrobial agents was compared in paromomycin supplemented (PS) and unsupplemented (PNS) flocks.

E. coli from PS birds had a significantly higher frequency of resistance to paromomycin, neomycin and kanamycin until 1 month after the end of supplementation compared to PNS birds. Resistance to amoxicillin or trimethoprim–sulfamethoxazole was also more frequent in PS turkeys. Resistance was mainly due to the presence of *aph* genes, which could be transmitted by conjugation, sometimes with streptomycin, tetracycline, amoxicillin, trimethoprim or sulfonamide resistance genes. Resistance to kanamycin and streptomycin in *E. faecium* was significantly different in PS and PNS breeders on days 60 and 90. Significantly higher frequencies of resistance to paromomycin, kanamycin, neomycin and tobramycin were observed in *S. aureus* isolates from PS birds. Paromomycin supplementation resulted in resistance to aminoglycosides in bacteria of PS turkeys. Co-selection for resistance to other antimicrobial agents was observed in *E. coli* isolates.

Introduction

Histomoniasis, caused by *Histomonas meleagridis*, is a severe disease of breeder and broiler turkeys and, to a lesser extent, chickens. Infected birds have ulceration of the caeca and necrotic foci in the liver, resulting in mortality. Previously, the disease was controlled with 3,5-dinitrosalicylic acid (5-nitrofurfurylidene) hydrazide (Nifursol), a nitrofuran, but this product was banned in the EU in 2003 (EC regulation 1756/2002) because of residue concerns.

Histomoniasis in turkeys can be prevented by in-feed administration of paromomycin sulfate, an aminoglycoside antimicrobial agent (Lindquist, 1962; Bleyen et al., 2009; Hafez et al., 2010; van der Heijden et al., 2011). Maximum residue limits have been established according to the requirements of EC Council Regulation (EEC) 2377/90. Paromomycin is a 4,5-disubstituted 2-deoxystreptamine structurally similar to neomycin and kanamycin.

Resistance to aminoglycosides is most frequently related to structural modifications by aminoglycoside phosphotransferases (APH), aminoglycoside nucleotidyltransferases (ANT) and aminoglysoside acetyltransferases (AAC). The genes encoding aminoglycoside-modifying enzymes are usually borne on conjugative plasmids, which frequently harbour genes encoding resistance to antimicrobial agents of other families. Supplementation of turkey feed with paromomycin would be expected to result in selection of resistance to aminoglycosides and possibly to other antimicrobial agents in bacteria of the alimentary tract.

Under the authority of the French government, a survey of antimicrobial resistance was implemented before conducting

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* Corresponding author. Tel.: + 33 2 96016281.
E-mail address: isabelle.kempf@anses.fr (I. Kempf).

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large-scale clinical studies under field conditions to evaluate the capacity of paromomycin sulfate to prevent histomoniasis. A field trial was established to compare the antimicrobial resistance of *Escherichia coli*, *Enterococcus faecium* and *Staphylococcus aureus* in the alimentary tract of paromomycin-supplemented (PS) and non-supplemented (PNS) breeder turkeys.

Materials and methods

Experimental protocol for supplementation study

The study included 24 flocks of *Salmonella*-free breeder turkeys (12 PS and 12 PNS, randomly allocated), located in Brittany or Pays de Loire, France (Table 1). Each flock comprised 3300–11,500 birds. For practical reasons, nine farms housed both a PS and a PNS flock, three farms had only one PS flock and three farms had only one PNS flock. On the farms with both PS and PNS flocks, the flocks were bred simultaneously, but in two separate poultry houses, and strict biosecurity measures were taken to avoid cross-contamination.

PS and PNS feed for all the flocks in the study was supplied by a single manufacturer. PS feed was supplemented with 100 mg/kg (100 ppm) paromomycin, i.e. 5 kg Histobloc 2% (containing 2% paromomycin) per tonne of feed; the 2% medicated premix was prepared by Franvet and paromomycin was supplied by Huvepharma. PS birds received paromomycin from the day of hatching (day 0) to day 120. When disease was noted, any additional treatments administered to the flocks were recorded.

Each month, a pool of approximately 15 fresh faecal samples was collected from each flock and sent to the laboratory, beginning on day 0 (before access to supplemented feed) and continuing for 2 months after the end of the treatment period (day 180).

The study was performed in compliance with the principles of Good Clinical Practice as laid down in International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guideline GL9 (June 2000).

Bacteriological analysis

Isolation

On arrival in the laboratory, each faecal sample was labelled for blinded analysis, suspended in buffered peptone water and the suspensions were used to isolate *E. coli*, *E. faecium* and *S. aureus*. *E. coli* was isolated on MacConkey agar plates (Oxoid) and identified using API20E kits (BioMérieux). *E. faecium* were obtained on bile aesculin azide media (BioRad) and identified by PCR (Depardieu et al., 2004). *S. aureus* was enriched in brain heart infusion broth (Becton Dickinson) containing 70 g/L NaCl for 48 h and colonies were isolated on SA Select (BioRad); identification was by PCR (Zhang et al., 2004). As far as possible, one isolate per bacterial species for each flock and each month was stored for further analysis.

Table 1
Description of flocks included in the study.

Farm	Flock number	Paromomycin	Other treatments (days 0–120)	Other treatments (days 120–180)
F1	F1-S	Yes		TTC
	F1-NS	No	AMX, SXT	TTC
F2	F2-S	Yes	TTC, AMX	TTC
	F2-NS	No	CST, TTC	TTC
F3	F3-S	Yes	TTC	
	F3-NS	No	TYL, TTC	
F4	F4-S	Yes	TTC, AMX	
	F4-NS	No	TTC, AMX	
F5	F5-S	Yes		TTC
	F5-NS	No	TTC, CST	TTC
F6	F6-S	Yes		TTC
	F6-NS	No		TTC
F7	F7-S	Yes		
F8	F7-NS	No		
F9	F9-S	Yes	TTC	
F10	F10-NS	No		
F11	F11-NS	No	CST	TTC
F12	F12-S	Yes	TTC, SXT, TYL	
F13	F13-S	Yes		
	F13-NS	No		
F14	F14-S	Yes		
	F14-NS	No		
F15	F15-S	Yes		
	F15-NS	No		

AMX, amoxicillin; CST, colistin; SXT, trimethoprim–sulfonamides; TTC, oxytetracycline, doxycycline or tetracycline; TYL, tylosin.

Susceptibility testing

Antimicrobial susceptibility was determined using the disc diffusion method, with streptomycin (10 IU), neomycin (30 IU), netilmicin (30 μg), gentamicin (15 μg), amikacin (30 μg), kanamycin (30 IU) and tobramycin (10 μg) (BioRad) for *E. coli* and *S. aureus* isolates, and streptomycin (500 μg), gentamicin (500 μg) and kanamycin (1000 μg) for *E. faecium*. Inhibition zone diameters were interpreted according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (CASFM, 2008). Since CASFM does not provide breakpoints for amikacin or netilmicin for *S. aureus*, the resistance breakpoints used in this study for these antimicrobial agents were >15 mm and >19 mm, respectively, according to Bismuth (2006). Minimum inhibitory concentration (MIC) values for paromomycin (Huvepharma) were determined by agar dilution according to CASFM (2008) for all four panels of isolates. *E. coli* isolates with MIC $\geqslant$ 64 mg/L were considered to be resistant to paromomycin. Reference strains (*E. coli* CIP7624 and *S. aureus* CIP7625) obtained from the Institut Pasteur, Paris, France, were used as controls.

Aminoglycoside resistance mechanisms

A panel of 30 *E. coli* isolates with various susceptibility patterns, or isolated from the same farms on different dates, was further characterised to identify aminoglycoside resistance. Aminoglycoside-modifying enzymes *aph* (3′)I and *aph* (3′)II were detected by PCR (Frana et al., 2001; Maynard et al., 2003) using, as controls, *Salmonella enterica* serovar Typhimurium SUO07 kindly provided by Dr Beatriz Guerra, Federal Institute for Risk Assessment, Berlin, Germany, and *E. coli* M155, kindly provided by Dr Josée Harel, University of Montréal, Canada.

Phylogenetic groups of *E. coli* isolates were identified (Clermont et al., 2000) and enterobacterial repetitive intergenic consensus (ERIC)-PCR was used to compare genetic profiles (Versalovic et al., 1991). Patterns of ERIC-PCR fingerprints with $\geqslant$75% similarity were designated as belonging to the same genotype group (Namvar and Warriner, 2006). Conjugation with *E. coli* J5 (rifampicin-resistant) or K12 (sodium azide-resistant) was performed on Mueller Hinton media supplemented with paromomycin (64 mg/L) and rifampicin or sodium azide, respectively (Dheilly et al., 2012), and the susceptibility of transconjugants was analysed by disc diffusion.

Statistical analysis

Fisher's exact test was used to compare the distribution of resistant and non-resistant isolates in PS and PNS flocks for each sampling date. Generalised estimating equations (GEEs) (Liang and Zeger, 1986), including repeated effects, were used. These models were applied for *E. coli* and *E. faecium* by separating two periods of interest, namely the supplementation period (i.e. between 30 and 120 days) and the post-supplementation period (i.e. between 150 and 180 days). The variables to be explained in the GEE models were resistances of *E. coli* and *E. faecium* to the various antimicrobial agents. Only resistances with $\geqslant$10 isolates from treated flocks were modelled. Since resistance is a dichotomous variable, the GEE models were associated with a binomial probability distribution and a logit link function. The time was incorporated into the model as a repeated factor specifying an autoregressive order 1 structure for the working correlation matrix.

Since the flocks belonged to two specific hatcheries, this variable was systematically included as a fixed effect in the model. Since paromomycin supplementation was the topic factor in the analysis, this variable was also systematically included in the model. Moreover, the main additional antimicrobial agents administered (i.e. tetracycline, amoxicillin and colistin) in the month before isolation and their interaction with paromomycin supplementation were also checked and maintained in the GEE model only, in case of significance. The significance of the link between explanatory and outcome variables was provided by the Wald statistic for Type III GEE analysis. GEEs were computed using the geeglm procedure of the R geepack package (Halekoh and Højsgaard, 2006).

Results

Paromomycin supplementation

No side effects attributable to the administration of paromomycin sulfate were observed in breeder turkeys. Other treatments administered to PS and PNS birds included oxytetracycline, amoxicillin, trimethoprim–sulfamethoxazole, tylosin and colistin (Table 1). The number of other treatments administered was similar in both PS and PNS flocks.

Susceptibility of E. coli

On day 0, 23 isolates were inhibited by 2 or 4 mg/L paromomycin. Only one isolate was resistant, with an MIC >64 mg/L (Table 2). According to disc diffusion, 23/24 isolates were susceptible to the