Research Note

Antimicrobial resistance and in vitro biofilm-forming ability of enterococci from intensive and extensive farming broilers

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ABSTRACT Enterococci, major broiler intestinal colonizers, play a recognized role in antimicrobial resistance transmission. Several virulence mechanisms, such as biofilm expression, have been identified. Minimum inhibitory concentrations of vancomycin, enrofloxacin, oxytetracycline, streptomycin, and gentamicin and biofilm production of 34 isolates from intensive and extensive farming system broilers were evaluated. All isolates were susceptible to vancomycin. In extensive-reared broilers (n = 18), resistance to enrofloxacin, oxytetracycline, streptomycin, and gentamicin was high (83.33, 55.56, 100, and 83.33%, respectively). Intensive farming broilers (n = 16) showed a lower resistance level for enrofloxacin and streptomycin and a higher resistance level for oxytetracycline and gentamicin. The relation between antimicrobial susceptibility and farming system was not significant for all drugs tested ($P \geq 0.05$). Enterococci produced biofilm at 24 h (47.0%), 48 h (55.9%), and 72 h (58.8%). Resistance to gentamicin and streptomycin was related to biofilm production at all time points ($P \leq 0.05$), whereas resistance to enrofloxacin was only related to biofilm at 24 h ($P \leq 0.05$; Friedman's test). No relation was found between susceptibility to oxytetracyclin and biofilm formation at any of the 3 time points studied ($P \geq 0.05$). Poultry are colonized by biofilm-producing and antimicrobial-resistant enterococci, independently of the farming system. Results show a relation between resistance to the majority of the drugs tested and biofilm production, which reenforces the importance of these virulence factors in animal and public health.

Key words: *Enterococcus*, antimicrobial resistance, biofilm, broiler, farming system

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INTRODUCTION

Enterococci are saprophytic bacteria that colonize the intestinal tract of mammals and birds, playing a recognized role in antimicrobial resistance transmission (Klein, 2003).

It is well established that antimicrobial exposure selects for resistance, which might spread among commensal and pathogenic bacteria. Antimicrobial drugs are widely used in poultry farming systems as therapeutic agents or growth promoters, or both (Martins da Costa et al., 2006; Poeta et al., 2006). The poultry intestinal tract may act as reservoir of antimicrobial-resistant enterococci that may be directly transferred to humans through the food chain (Poeta et al., 2006) or through contaminated water, fish, and vegetables, given their ability to persist for long periods in the environment (Klein, 2003; Martins da Costa et al., 2006; Kaszanyitzky et al., 2007). Screening for antimicrobial resistance in enterococci isolated from food-producing animals is essential to monitor the risk of antimicrobial resistance gene transfer to humans and to ensure food safety (Khan et al., 2005; Martins da Costa et al., 2006).

Enterococci virulence can be further enhanced by the expression of other virulence traits, such as biofilm formation. These multicellular complexes are characterized by adherent colonies surrounded by an exopolysaccharide matrix that protects bacteria against antimicrobial action and phagocytosis, allowing bacteria to survive in hostile environments (George et al., 2005; Chai et al., 2007). Detection of biofilm-producing strains is important for the selection of control policies to adequately minimize the risk of drug resistance transfer in food animals, with reflexes upon public health.

Because fecal contamination is one of the major sources for enterococci presence in poultry meat, the aim of the present study was to evaluate the antimicrobial resistance profile and the biofilm-forming ability of enterococci isolated from poultry feces in extensive and intensive farming systems.

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MATERIALS AND METHODS

Bacterial Isolates

Thirty-four enterococci isolates from broilers raised in intensive (n = 16) and extensive (n = 18) farming systems were used in this study. These isolates belong to a collection of intestinal commensal bacteria (*Escherichia coli* and *Enterococcus* sp.) from poultry slaughtered for human consumption in 2 Portuguese slaughterhouses. One of the plants exclusively slaughtered poultry produced by intensive farming, characterized by broiler flocks reared in high stocking density (14 to 20 broilers/m^2) until 30 to 40 d of age, whereas the other plant slaughtered broilers from extensive farming, which were reared in open broiler houses at low stocking density (<12 broilers/m^2) until 81 d of age. Bacteria were isolated according to da Costa et al. (2007). Briefly, after an enrichment step in buffered peptone water (CM059, Oxoid, Basingstoke, UK) for 16 h at 37°C, enterococci were isolated on Slanetz & Bartley medium (CM0377, Oxoid). After 24 h at 37°C, presumptive enterococci isolates were selected on the basis of morphology and biochemical traits (catalase testing, esculin hydrolysis) and identified through their biochemical profile (API 20 Strep, BioMérieux, Marcy-l'Etoile, France)

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (**MIC**) of vancomycin (**VAN**; V861987), enrofloxacin (**ENR**; E3369), oxytetracycline (**OXT**; 04636), streptomycin (**STR**; S6501), and gentamicin (**GEN**; G1264) were determined by broth microdilution, following the Clinical and Laboratory Standards Institute guidelines for veterinary susceptibility testing (CLSI, 2008). Antimicrobials were purchased at Sigma (Sintra, Portugal). Antimicrobial dilution range and susceptibility breakpoints considered, based on Clinical and Laboratory Standards Institute document M31-A3 (CLSI, 2008), are described in Table 1. Minimum inhibitory concentration was determined as the lowest dilution inhibiting visible bacterial growth.

All isolates were tested at least twice in separate occasions. Reference strain *Enterococcus faecalis* ATCC 29212 was used for quality control of MIC values (CLSI, 2008).

Biofilm Production

Biofilm production was evaluated in vitro by fluorescent in situ hybridization. The fluorescent in situ hybridization protocol was performed as described elsewhere, with minor modifications (Oliveira et al., 2007). Two 16S rRNA oligonucleotide probes specific for *Enterococcus* spp. were simultaneously used: EFAEC (5′-TTATCCCCCTCTGATGGG-3′; *Escherichia coli* 135–152) (Jansen et al., 2000) and EFAECI (5′-AGCTCCCGGTGGAAAAAGAAG- 3′; *Escherichia coli* 1204–1223) (da Costa et al., 2007). The probes were synthesized by StabVida (Oeiras, Portugal) and labeled with fluorescein in the 5′ end.

Data Analysis

Data analysis was performed using SPSS 15.0 for Windows (SPSS Inc, Chicago, IL). The relation between antimicrobial resistance and biofilm production and between farming system and antimicrobial resistance or biofilm production was evaluated using Friedman's test. The statistical significance of the increase in biofilm expression with time was also evaluated (Wilcoxon matched paired test). For statistical analysis purposes, isolates showing intermediate resistance to the antimicrobial compounds by MIC were considered as resistant.

RESULTS

Isolates from the intensive farming system (n = 16) were identified as *Enterococcus faecalis* (n = 5; 31.5%), *Enterococcus faecium* (n = 10; 62.5%), and *Enterococcus gallinarum* (n = 1; 6.5%). Isolates from the extensive farming system (n = 18) were identified as *E. faecalis* (n = 6; 33.3%), *E. faecium* (n = 11; 61.1%), and *Enterococcus durans* (n = 1; 5.6%).

Minimum inhibitory concentration distribution of enterococci broiler isolates regarding the 5 antimicrobials tested is summarized in Table 2. The MIC values for the reference strain were within the recommended

Table 1. Dilution range and susceptibility breakpoints of vancomycin, enrofloxacin, oxytetracycline, gentamicin, and streptomycin[1]

		Susceptibility breakpoints (μg/mL)		
Item[2]	Dilution range (μg/mL)	Susceptible	Intermediate	Resistant
VAN	0.03 to 64	≤4	8 to 16	≥32
ENR	0.03 to 64	≤0.5	—	≥4
OXT	0.03 to 64	≤4	8	≥16
GEN	0.03 to 64	≤4	8	≥16
STR	0.03 to 64	≤32	—	≥64

[1]Oxytetracycline breakpoints were extrapolated from the class representative tetracycline (CLSI, 2008).
[2]VAN = vancomycin; ENR = enrofloxacin; OXT = oxytetracycline; GEN = gentamicin; STR = streptomycin.

Table 2. Minimum inhibitory concentration distribution and biofilm formation by 34 enterococci isolates from broilers from extensive and intensive farming systems

		Antimicrobial resistance[2]												
		VAN		ENR		OXT		GEN		STR				
		R[3]	S[3]	R	S	R	S	R	S	R	S	Biofilm production		
Farming type[1]	No. of isolates	≥32 μg/mL	≤4 μg/mL	≤4 μg/mL	≤0.5 μg/mL	≥16 μg/mL	≤4 μg/mL	≥64 μg/mL	≤16 μg/mL	≥64 μg/mL	≤32 μg/mL	24 h	48 h	72 h
Extensive														
Enterococcus faecalis	6	0	6	5	1	2	4	6	0	6	0	3	4	4
Enterococcus faecium	11	0	11	10	1	8	3	9	2	11	0	2	3	3
Enterococcus durans	1	0	1	0	1	0	1	0	1	1	0	0	0	1
Enterococcus gallinarum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Subtotal	18	0	18[4]	15	3[4]	10	8[4]	15	3[4]	18	0[4]	5[5]	7[5]	8[6]
Percentage		0	100	83.33	16.67	55.56	44.44	83.33	16.67	100	0	27.78	38.89	44.44
Intensive														
E. faecalis	5	0	5	4	1	5	0	5	0	5	0	4	4	4
E. faecium	10	0	10	5	5	8	2	8	2	8	2	7	8	8
E. durans	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. gallinarum	1	0	1	1	0	1	0	1	0	1	0	0	0	0
Subtotal	16	0	16[4]	10	6[4]	14	2[4]	14	2[4]	14	2[4]	11[5]	12[5]	12[6]
Percentage		0	100	62.50	37.50	87.50	12.50	87.50	12.50	87.50	12.50	68.75	75	75
Total	34	0	34	25	9	24	10	29	5	32	2	16	19	20
Percentage		0	100	73.53	26.47	70.59	29.41	85.29	14.71	94.12	5.88	47.01	55.89	58.82

[1]Extensive farming system = <12 broilers/m^2, reared until 81 d of age; intensive farming system = 14 to 20 broilers/m^2 reared until 30 to 40 d of age.

[2]VAN = vancomycin; ENR = enrofloxacin; OXT = oxytetracycline; GEN = gentamicin; STR = streptomycin.

[3]R = resistant; S = susceptible.

[4]Relation between antimicrobial susceptibility and farming system was not significant for all drugs tested ($P \geq 0.05$).

[5]Significant differences were found between biofilm expression by extensive and intensive isolates at 24 and 48 h ($P \leq 0.05$).

[6]No significant differences were found between biofilm expression by extensive and intensive isolates at 72 h ($P \geq 0.05$).

range for the antimicrobials tested (CLSI, 2008). None of the isolates tested were susceptible to all antimicrobials tested. All isolates were susceptible to VAN. High resistance levels were observed for ENR, OXT, GEN, and STR, and it was observed that all extensive isolates were resistant to STR. The relation between antimicrobial susceptibility and farming system was not significant for all drugs tested ($P \geq 0.05$).

Biofilm production results are also summarized in Table 2. It was observed that almost half of the isolates were able to produce biofilm at 24 h, and this number increased with incubation time. Significant differences were found between biofilm expression by extensive and intensive isolates at 24 and 48 h ($P \leq 0.05$), but no significant differences were found between the isolates from the 2 farming systems at 72 h ($P \geq 0.05$). Within each farming system, the increase in the number of biofilm-positive isolates was not significant for any of the time points analyzed: 24 to 48, 48 to 72, and 24 to 72 h ($P \geq 0.05$).

Biofilm production was related to GEN and STR susceptibility at all time points studied ($P \leq 0.05$). This relation was also significant for susceptibility to ENR and biofilm formation at 24 h ($P \leq 0.05$), becoming nonsignificant at 48 and 72 h ($P \geq 0.05$). No relation was found between susceptibility to OXT and biofilm formation at any of the 3 time points studied ($P \geq 0.05$).

DISCUSSION

All isolates tested were susceptible to VAN, which supports the success of the avoparcin ban by the European Union (Martins da Costa et al., 2006; Kaszanyitzky et al., 2007). Nevertheless, VAN resistance screening should be maintained because some studies report that intermediate levels of resistance to this glycopeptide are still observed (Khan et al., 2005).

Enrofloxacin is one of the quinolones that is licensed for use in broiler production (Martins da Costa et al., 2006), which might contribute to the observed resistance level. Differences in the antimicrobials' mode of action are not likely to account for the high resistance observed to OXT, GEN, and STR because both tetracyclines (OXT) and aminoglycosides (GEN and STR) inhibit bacterial protein synthesis at the ribosomal level. Resistance level found may therefore be caused by the extensive use of broad-spectrum antibiotics for disease prevention in poultry production (Martins da Costa et al., 2006; Kaszanyitzky et al., 2007). The use of tetracycline in veterinary medicine increased after the growth promoter ban (Kaszanyitzky et al., 2007). Streptomycin is also frequently used in veterinary therapy. In contrast, GEN is often administered in combination with cell wall synthesis inhibitors in the treatment of enterococcal infections in humans but is seldom used in veterinary medicine (Kaszanyitzky et al., 2007). Therefore, GEN resistance levels deserve further attention.

No relation was found between antimicrobial resistance levels and farming system, which contradicts the concept that, in broiler flocks reared in high stocking density (intensive farming), the transmission of resistant determinants among bacterial strains is more frequent (Souza et al., 2007). These results alert to the importance of poultry enterococci in the transmission of antimicrobial resistance genes.

Biofilm formation is a recognized bacterial virulence factor that can proceed through several pathways, being regulated by several loci, so its detection may differ according to the time of observation (Oliveira et al., 2007). Approximately one-third of extensive farming enterococci and more than two-thirds of the intensive farming isolates revealed biofilm-forming ability at 24 h. The difference in biofilm expression between both farming systems was significant at 24 and 48 h. However, no significant increase was observed with prolonged incubation times. Overall, these results suggest that intensive farming systems may select for biofilm-producing strains, similarly to what has been described for other virulence determinants (Souza et al., 2007). Nevertheless, it should be noted that future studies regarding biofilm formation under different growth conditions should be performed to confirm this hypothesis.

Biofilm structures are believed to impair the action of antimicrobial compounds (del Pozo and Patel, 2007). In fact, Chai et al. (2007) already established that biofilm formation could be responsible for antimicrobial resistance in *Enterococcus*. In our study, biofilm production was related to resistance to GEN and STR at the 3 time points and to ENR at 24 h. No relation was found between biofilm production and resistance to OXT in any of the time ranges considered. These results suggest that broad-spectrum antibiotics such as OXT may be efficient in the eradication of biofilm producer strains. However, further studies are still required.

To our knowledge, this is the first report of the association between antimicrobial resistance and biofilm formation in poultry enterococci, which reinforces the requirement for surveilling the presence of virulence factors in intestinal microbiota.

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