

Antimicrobial resistance in *Escherichia coli* and *Enterococcus* sp. isolated from swine carcasses at the pre-chill stage

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Abstract

The prevalence of antimicrobial resistant bacteria has been increasingly monitored in animals in order to prevent the spread of these strains through the food supply chain. Particularly, the emergence of vancomycin-resistant *Enterococcus* and Extended-spectrum beta-lactamases (ESBL) producing *Enterobacteriaceae* has been investigated worldwide. In the current study, the frequency of antimicrobial resistance of generic *Escherichia coli* and *Enterococcus* isolated from swine carcasses sampled at the pre-chill stage was assessed. A total of 319 strains of *E. coli* and 240 strains of *Enterococcus* sp. from carcasses sampled in three Brazilian slaughterhouses were tested by the agar diffusion test, according to the guidelines of Clinical and Laboratory Standards Institute. *Escherichia coli* strains showed high frequency of resistance against tetracycline (79.3%), ampicillin (64.3%), sulfonamide and florfenicol (64.9%). Five isolates were resistant to either cefotaxime or ceftazidime, but only one of them displayed a positive result in the confirmatory phenotypic test recommended by CLSI. The five isolates were subjected to PCR for detection of *bla*_{TEM} and *bla*_{PSE} genes and three strains presented amplification for *bla*_{TEM}. The most prevalent resistance profiles found among *Enterococcus* strains were tetracycline (42.5%), erythromycin (26.7%) and high level streptomycin (HLAR, 20.4%). All isolates were susceptible to vancomycin, teicoplanin and ampicillin. Minimum inhibitory concentration (MIC) was determined for resistant and intermediate erythromycin *Enterococcus* strains. The determined MIC ranged from 1 µg/mL to 4 µg/mL for erythromycin intermediate strains, while among the resistant strains it ranged from 6 µg/mL to >256 µg/mL. The results indicate that vancomycin-resistant *Enterococcus* isolates are not prevalent in pig carcasses; however ESBL producing *E. coli* may be present.

Introduction

Monitoring of antimicrobial resistance in pathogenic or commensal bacteria is essential to evaluate the risk of antimicrobial resistance genes spread through the food supply chain. Resistant bacteria present on carcasses that were able to surpass the food processing hurdles, may colonize the consumer's gastrointestinal tract (Apley, 2001). In particular, extended-spectrum β-lactamases (ESBL) resistant bacteria and vancomycin-resistant *Enterococcus* have been investigated worldwide, due to its importance for human therapy. Therefore, the aim of this study was to investigate the frequency of antimicrobial resistance in *Escherichia coli* and *Enterococcus* sp. isolated from pig carcasses sampled at the pre-chill stage.

Material and Methods

Two sampling cycles were conducted in three slaughterhouses located in the state of Santa Catarina, Brazil. A total of 252 pre-chill carcasses were sampled by rubbing individual sterile sponges (Nasco ®) on a 400 cm²-carcass area (Brazil, 2007). After the addition of 40 ml of Buffered Peptone Water (BPW 0.1%) to each sample, aliquots were transferred to Violet Red Bile Agar (Oxoid Brazil Ltda.) and KF *Streptococcus* Agar (Bento, Dickson & Company) for isolation of *E. coli* and *Enterococcus* sp., respectively. After incubation (37°C/48h) typical colonies were identified according to Quinn et al. (2011). The *Enterococcus* genus was confirmed by amplification of *tuf* gene (Ke et al., 1999) and the identification of *E. faecalis* was performed by amplification of *ddl*_{*E. faecalis*} gene (Dutka-Malen et al. 1995).

The isolates were tested for antimicrobial resistance by disk-diffusion test in Müller-Hinton Agar (Oxoid), performed and interpreted according to "Clinical and Laboratory Standards Institute", document M100-S22 and M31-A2 (CLSI, 2008; 2012). *Escherichia coli* strains were tested using disks (Oxoid) of the following antimicrobials: ampicillin (10µg), ceftazidime (30µg), cefotaxime (30µg), gentamicin (10µg), florfenicol (30µg), nalidixic acid (30µg) tetracycline (30µg) and sulfonamide (300µg). Resistant strains to cefotaxime and/or ceftazidime were confirmed phenotypically for ESBL resistance, using disks of cefotaxime and ceftazidime alone and in combination with clavulanic acid (CLSI, 2012). Additionally, genotypic confirmation was conducted by PCR-detection of genes *bla*_{TEM} and *bla*_{PSE} (Sandvang et al. 2002). The *Enterococcus* isolates were tested against: ampicillin (10µg), ciprofloxacin (5µg), chloramphenicol (30µg), erythromycin (15µ), teicoplanin (30µg), tetracycline (30µg), vancomycin (30µg), streptomycin (300µg) and gentamicin (120µg). For isolates displaying erythromycin intermediate and resistant profiles, the minimum inhibitory concentration (MIC) was determined, using the

Etest® (BioMerieux). The frequency of *E. coli* and *Enterococcus* resistant strains among slaughterhouses was compared by chi-square, using the software SPSS 1.8 with a confidence level of 95%.

Results

From 319 tested *E. coli* strains, 12.8% were susceptible to all antimicrobials. The highest frequency of resistance was found against tetracycline (79.3%), sulfonamide, florfenicol (64.9%), and ampicillin (64.3%) (Table 1). Five strains were resistant or intermediate to cefotaxime or ceftazidime (screening test), but only one of them was confirmed in the ESBL phenotypic test. However, three strains were positive in the PCR for *bla*_{TEM} gene. No strain showed amplification for *bla*_{PSE} gene.

All the 240 typical colonies were confirmed by PCR as belonging to *Enterococcus* genus. From them, 217 (90.8%) were identified as *E. faecalis* by PCR. All isolates were susceptible to glycopeptides and ampicillin. The frequency of resistant strains against the tested antimicrobial is depicted in Table 2. Among the seventy-four erythromycin-intermediate strains, two had borderline MIC (4 µg/mL) while the other strains presented MIC between 1 µg/mL and 3 µg/mL. Among the sixty-four resistant strains only six presented MIC between 6 and 64 µg/mL, and 58 presented MIC ≥ 256 µg/mL.

Discussion

Tetracycline resistance was the most frequent in both *E. coli* and *Enterococcus* isolated from all slaughterhouses included in this study. In Brazil, tetracycline resistance is widespread in *Salmonella enterica* isolated from pigs as demonstrated in previous studies (Bessa et al., 2004; Mürmann et al., 2009). Since all the three bacteria species colonize the intestine, they may be exposed to the same selection pressure exerted by the antimicrobial administration. Regarding *E. coli*, those tetracycline-resistant strains presented resistance to sulfonamides and ampicillin as well, suggesting that genes the resistance may be located in mobile elements (Thorsteinsdottir et al., 2010). In our study this association was also observed in most *E. coli* strains tested, although the use of those antimicrobials as growth promoter has been banned since 1998 (Brasil, 2009). However, those antimicrobials are still used for treatment of highly prevalent respiratory and enteric diseases of swine. In this sense, the therapeutic use may have contributed to keep the selective pressure on bacterial population.

The frequency of antimicrobial use influences directly the selection of resistant strains (Wang et al., 2010); therefore variations in the protocol of their administration may justify differences in resistance profiles detected among the slaughterhouses. In Brazil, the pork supply chain is typically organized in a vertical integration system between large companies and small farms. Companies supply medicines and give technical assistance to the farmers and later on transport the market pigs to their own slaughterhouses. The large variation among slaughterhouses in the frequency of resistance against florfenicol and aminoglycosides in *E. coli* and *Enterococcus* strains, respectively, may illustrate the difference in treatment protocols. Specifically the increasing therapeutic use of florfenicol that has occurred in the last years may have contributed for the high percentage of resistant and intermediate strains observed.

Studies conducted in other countries demonstrated ESBL-resistant *E. coli* strains in feces of healthy pigs (Geser et al., 2011), pointing out the risk for carcass contamination. In our study, a low number of ESBL-resistant *E. coli* isolates was observed, indicating that this resistance phenotype is still not highly widespread in the region. Among five resistant strains, one was phenotypically confirmed, and three carried the *bla*_{TEM} gene, demonstrating that they were potentially ESBL producers. In spite of the low frequency, the presence of ESBL-resistant strains in pigs highlights that further studies should be conducted to determine the prevalence and distribution of these strains.

In this study, we included the genus *Enterococcus*, because of its importance as a reservoir of resistance genes against antimicrobials used for treatment of Gram-positive bacteria (Dzidic et al., 2008). In this regard, the reported increase of vancomycin-resistant enterococci in animals have led to the ban of avoparcin administration to pigs in many countries. In Brazil, avoparcin had not been widely administered to pigs up until 1998, when its use was prohibited (Brasil, 1998). Therefore, the absence of resistant strains against glycopeptides was expected. Similar results have been reported in a nationwide monitoring program of poultry carcasses at retail level, where less than 1% of the *Enterococcus* strains were resistant to vancomycin and teicoplanin (ANVISA, 2008).

On the other hand, the high frequency of erythromycin-resistant strains and the high MIC-levels (≥ 256 µg/mL) found is a matter of concern, since *erm* genes confer cross-resistance to the entire macrolide-lincosamides-streptogramins (MLS_B) group, which constitute an important alternative for human treatment (Emanieni et al., 2008). Considering that studies demonstrated that common clones can colonize pigs and humans (Larsen et al., 2011), the widespread of resistant *E. faecalis* strains may constitute a hazard to public health. Recently the use of erythromycin as growth promoter has been banned in Brazil (Brazil, 2012), in order to avoid the selection of resistant strains. Nevertheless, erythromycin-resistance pattern should be kept under monitoring.

Conclusion

The results indicate that vancomycin-resistant *Enterococcus* isolates were not prevalent in pig carcasses; however ESBL producing *E. coli* may be present.

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Table 1 - Frequency of antimicrobial resistance in *Escherichia coli* isolated from pre-chill pig carcasses from three slaughterhouses (A, B, C) of Santa Catarina, Brazil.

	A (n = 102)		B (n = 122)		C (n = 95)		A, B, C (n = 319)		P value
	R (%)	I (%)	R (%)	I (%)	R (%)	I (%)	R (%)	I (%)	
Ampicillin	71.57	1.96	65.57	10.65	54.74	11.58	64.26	8.15	0.023
Cefotaxime	-	0.98	1.64	-	1.05	-	0.94	0.31	0.443
Ceftazime	-	-	1.64	-	-	-	0.62	-	0.197
Florfenicol	87.25	11.76	60.65	29.51	46.31	37.89	64.89	26.33	<0.001
Gentamicin	5.88	2.94	6.55	7.37	9.47	3.16	7.21	4.7	0.382
Nalidixic acid	75.49	0.98	48.36	4.1	50.53	5.26	57.68	3.44	<0.001
Sulfonamide	81.37	-	50.82	0.82	65.26	-	64.89	4.07	<0.001
Tetracycline	93.14	-	69.67	0.82	76.84	-	79.31	0.31	<0.001

Table 2 - Frequency of antimicrobial resistant *Enterococcus* isolated from pre-chill pig carcasses from three slaughterhouses (A, B, C) of Santa Catarina, Brazil.

	A (n = 84)		B (n = 74)		C (n = 82)		A, B, C (n = 240)		P value
	R (%)	I (%)	R (%)	I (%)	R (%)	I (%)	R (%)	I (%)	
Ciprofloxacin	3.6	13.1	17.6	9.5	20.7	8.5	13.8	10.4	0.018
Cloramphenicol	27.4	3.6	2.7	1.4	4.9	2.4	12.1	2.5	<0.001
Erythromycin	41.7	42.9	10.8	20.2	25.6	25.6	26.7	30.8	<0.001
Gentamicin (HLAR)	17.9	-	2.7	-	9.8	-	10.4	-	0.008
Streptomycin (HLAR)	34.5	-	6.8	-	18.3	-	20.4	-	<0.001
Tetracycline	45.2	-	48.6	2.7	34.1	2.4	42.5	1.7	0.209