Antimicrobial Resistance of Enterococcus Isolated from Pre-Chill Swine Carcasses

Thais de Campos¹, Caroline Pissetti¹, Gabriela Orosco Werlang¹, Graciela Volz Lopes¹, Jalusa Deon Kich² & Marisa Cardoso¹

ABSTRACT

Background: As a result of the extensive use of antimicrobials in agriculture, animals have been implicated as possible reservoirs of resistant strains of bacteria. Enterococci are members of the normal flora of the gastrointestinal tract of human and animals. Because of their ubiquity, enterococci have been introduced in programs to evaluate the hazard of transmission of resistant bacterial strains through the food chain. The aim of this study was to assess the antimicrobial resistance profile of Enterococcus isolated from swine carcasses at the pre-chill step of processing.

Material, Methods & Results: Pig carcasses were sampled at three commercial slaughterhouses (A, B and C). On each of two sampling occasions swabs of 100 cm² areas were taken from each ham, back, belly and jowl of a total of 14 pre-chill carcasses. Enterococci were isolated and counted in KF Streptococcus Agar, and typical colonies were confirmed by PCR assay targeting the tuf gene. Enterococcus isolates were tested for susceptibility to nine different antimicrobial agents by agar disc diffusion. From a total of 252 carcasses sampled, 240 (95.2%) presented presumptive colonies of Enterococcus in counts ranging from 0.02 log CFU.cm⁻² to 2.9 log CFU.cm⁻². All isolates were confirmed as belonging to the genus Enterococcus, and the great majority was identified as E. faecalis (218/240; 90.83%). Half (125/240; 52.1%) of the Enterococcus isolates were susceptible to all tested antimicrobials. No resistance to ampicillin, vancomycin or teicoplanin was found. The most frequent resistance was to tetracycline (42.5%), followed by erythromycin (26.7%), high level (HLR) streptomycin (20.4%), ciprofloxacin (13.8%), chloramphenicol (12.1%) and HLR-gentamicin (10.4%). Among the 115 resistant Enterococcus isolates, 55 (47.8%) were multi-resistant, and the distribution of the most common profiles was related to the slaughterhouse from which the isolate originated.

Discussion: Although foodborne enterococci have not yet been clearly involved in direct clinical infection, antimicrobial-resistant isolates from food can be a reservoir of resistance genes. Therefore, the occurrence of resistant isolates to HLR-streptomycin, HLR-gentamicin, tetracycline, erythromycin and ciprofloxacin in swine carcasses may present a risk of spreading enterococci strains that are resistant to treatment. Several drugs of these groups are used in feed for prophylaxis and treatment of respiratory and enteric diseases in pigs and may thus be exerting a high selective pressure in the intestinal microbiota. The sampled slaughterhouses in this study were supplied by production chains that belong to different agribusiness companies. These companies run contracts with farmers which usually specify a common management protocol, including prophylactic and therapeutic administration of antimicrobial drugs. The selective pressure of antimicrobial usage may also explain the significant difference in the frequency of resistance to most tested agents among the slaughterhouses. It was concluded that although Enterococcus isolates from pre-chill swine carcasses presented a low to moderate frequency of resistance against most antimicrobials used in human treatment, isolates displaying resistance to the aminoglycosides and macrolides classes may present a hazard. The multi-resistance patterns were highly associated with the origin of the isolates and may indicate the extent of antimicrobial use on farm.

Keywords: Enterococcus, pork, antimicrobial resistance.
INTRODUCTION

Enterococci are normal components of the flora of the gastrointestinal tract of human and animals and have emerged as important antimicrobial-resistant pathogens causing nosocomial infections in human patients [15]. In particular, enterococci strains resistant to beta-lactams, glycopeptides and aminoglycosides have become a major concern, since these drugs are commonly used in the treatment of human infections [14].

As a result of extensive use of antimicrobials in agriculture, animals have been implicated as possible reservoirs of resistant strains of bacteria. The association between the emergence of glycopeptide-resistant enterococci and the use of avoparcin in poultry and swine production has been cited as an example of the hazard of indiscriminate use of antimicrobials in animals [1]. As a consequence, several antimicrobials formerly administered to livestock have been banned, and monitoring of antimicrobial resistance in bacteria species, such as Escherichia coli and enterococci has been introduced in programs to evaluate the hazard of resistant strain transmission through the food chain [12,16].

In a baseline study of retail chickens, the most frequent resistance in enterococci was to tetracycline and erythromycin [2]. To date, there has been no baseline monitoring of antimicrobial resistance in swine, and to the best of our knowledge no study assessing antimicrobial resistance of enterococci isolated from pig carcasses has been conducted. Thus, the aim of this study was to assess the antimicrobial resistance profile of Enterococcus isolated from swine carcasses at the pre-chill step of processing.

MATERIALS AND METHODS

Sample collection

Samples were collected at three commercial slaughterhouses denoted as A, B and C, which processed between 1500 and 2000 pigs per day. All slaughterhouses were sampled twice. On each sampling occasion a total of 42 carcasses was sampled in a ten-minute interval during one slaughter shift. Immediately before chilling, carcasses were swabbed with sterile sponges (Nasco®) previously moistened with buffered peptone water 1% (BPW 1%)². Each carcass was swabbed over a 100 cm² area from each ham, back, belly and jowl [19]. The four swabs from each carcass were put in a single sterile plastic bag, kept under refrigeration and processed as one sample.

Enterococcus enumeration

Each sample was added to 40 mL of BPW 1%, homogenized (Stomacher)³, and serial diluted to $10^{-4}$ in BPW 1%. Two aliquots (1 mL) from each dilution were transferred to sterile Petri dishes, and melted KF Streptococcus Agar⁴ was added to each plate and mixed. After incubation (48 h at 36 ± 1°C), typical enterococci colonies (red or pink) were counted. The average number of colonies was multiplied by the dilution and divided by 400 to obtain the number of presumptive enterococci colonies on the carcass area (CFU.cm⁻²).

Enterococcus identification

Presumptive Enterococcus colonies were phenotypically identified by biochemistry [13]. Isolates identified as Enterococcus sp. were confirmed by PCR assay targeting the gene tuf, using primers Ent1 (5’-TACTGACAAGACATGCATGATG-3’) and Ent2 (5’-AACTTCGTCACCAACCGGAAC-3’), which results in an amplicon of 112 bp [17]. Enterococcus faecalis was identified by PCR assay targeting the gene ddlE.faecalis using the primers E1 (5’-ATCAAGTACAGTTAGTCTAGTTAGTCT-3’) and E2 (5’-ACGATTCAAAGCTACTG-3’), which results in an amplicon of 941 bp [11]. Genomic DNA for the PCR assays was prepared using NucleoSpin® Tissue Kits⁵. The reference strain Enterococcus faecalis ATCC® 29212 was used as the positive control.

Antimicrobial susceptibility testing

Enterococcus isolates were tested for susceptibility to nine different antimicrobial agents. The agar disc diffusion method was performed and evaluated according to the specifications of the Clinical and Laboratory Standards Institute (CLSI) document M100-S23 [8]. The following discs were used: ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (120 μg), streptomycin (300 μg), teicoplanin (30 μg), tetracycline (30 μg) and vancomycin (30 μg). Multi-resistance was defined as resistance to at least three different classes of antimicrobial agents. Isolates displaying resistance and intermediate resistance to erythromycin and ciprofloxacin were submitted to the minimum inhibitory concentration (MIC) determination, screened by the Etest® or
the agar dilution test following the recommendations of the CLSI document M100-S15, respectively [7]. *Staphylococcus aureus* ATCC® 25923, *Staphylococcus aureus* ATCC 29213® and *Enterococcus faecalis* ATCC® 29212 were used as reference strains for quality control purposes.

**Statistical analysis**

*Enterococcus* counts were transformed to logarithmic counts (log_{10}) and analyzed by ANOVA. The average counts of *Enterococcus* were compared among slaughterhouses by the Tukey-Kramer test. The antimicrobial resistance frequencies of isolates from different slaughterhouses were compared by chi-square test (χ²). A P value of < 0.05 was considered significant. For the purpose of this study, isolates with intermediate susceptibility were categorized as susceptible for statistical analysis. All analyses were performed using the software SAS version 9.2 for Windows.

**RESULTS**

From a total of 252 carcasses sampled, 240 (95.2%) presented presumptive colonies of *Enterococcus* in counts ranging from 0.02 log CFU cm^{-2} to 2.9 log CFU cm^{-2}. The average count was statistically higher (P < 0.05) in slaughterhouse C (2.2 log CFU cm^{-2}) compared to the other two slaughterhouses sampled (0.78 log CFU cm^{-2} and 1.5 log CFU cm^{-2} for A and B, respectively). One presumptive colony was identified from each carcass, and all isolates were confirmed by PCR assay as belonging to the genus *Enterococcus*. The great majority of the *Enterococcus* sp. isolates were identified as *E. faecalis* (218/240; 90.83%).

Half (125/240; 52.1%) of the *Enterococcus* isolates were susceptible to all tested antimicrobial agents. Susceptible isolates accounted for 51.4%; 54.8% and 65.9% of the isolates from slaughterhouses A, B and C, respectively. No resistance to ampicillin, vancomycin and teicoplanin was found. The most frequent resistance was to tetracycline (42.5%), followed by erythromycin (26.7%), high level (HLR) streptomycin (20.4%), ciprofloxacin (13.8%), chloramphenicol (12.1%) and HLR-gentamicin (10.4%). The frequency of isolates resistant to tetracycline was not significantly different among isolates from the three slaughterhouses, while the resistance to the aminoglycosides, erythromycin, chloramphenicol and ciprofloxacin varied significantly according to the origin of the isolates (Figure 1).

Among the *Enterococcus* isolates, 74 (30.8%) presented intermediate resistance to erythromycin and 25 (10.4%) to ciprofloxacin. The MIC presented by resistant and intermediate resistance isolates against these antimicrobials is depicted in Table 1. In all isolates displaying intermediate resistance to ciprofloxacin, the MIC was close to the breakpoint for resistance (1.56 μg.mL^{-1}), while for the resistant isolates MICs ranged from 3.12 μg.mL^{-1} to 50 μg.mL^{-1}. MICs of isolates of intermediate resistance to erythromycin ranged from 1.0 μg.mL^{-1} to 4.0 μg.mL^{-1}, while most resistant isolates (58/64) were inhibited only by the highest antimicrobial concentration tested (256 μg.mL^{-1}).

Among the 115 resistant *Enterococcus* isolates, 55 (47.8%) were multi-resistant, and the distribution of the most common profiles were related to the slaughterhouse from which the isolate originated (Figure 2).

**Table 1.** Number of isolates at minimum inhibitory concentrations (MIC) for ciprofloxacin and erythromycin among *Enterococcus* isolates from swine pre-chill carcasses.

<table>
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<tr>
<th>Antimicrobial</th>
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<th>3.12</th>
<th>4.0</th>
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<tr>
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<tr>
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Vertical lines indicate the breakpoint for resistance [8]. *Result obtained from the disc diffusion test.
DISCUSSION

The genus *Enterococcus* was isolated from 95.2% of the carcasses, similar to frequencies previously reported for pork and chicken [3,5,25]. Enterococci constitute a large proportion of the gastrointestinal flora of animals, and fecal contamination during the slaughter process contributes to their presence on carcasses [14,15]. Therefore, the difference observed in the average counts of *Enterococcus* on carcasses may reflect the variation in processing hygiene among slaughterhouses. It has been demonstrated that despite reductions in average counts of *Enterococcus* during carcass processing, the enterococci population on pork products may increase again after processing, either by recontamination or growth of bacteria that survived the processing [3]. Therefore, the risk of high levels of enterococci contamination may be greater in slaughterhouses where there is a greater residual enterococci population on pre-chill carcasses.

Although foodborne enterococci have not yet been clearly involved in direct clinical infection, antimicrobial-resistant isolates from food can be a reservoir of resistance genes, which may be transferred to bacteria pathogenic to humans [12]. In fact, isolates from *E. faecalis* with similar resistance profiles and belonging to similar genotypes have been found in both human patients and pigs, indicating that this species may play an important role in gene transfer, since it can effectively be transmitted and colonize humans [18].

In southern Brazil, *E. faecalis* is the most prevalent species found in hospital settings, account-
ing for up to 93.6% of the enterococci isolates [4,9]. A high susceptibility to ampicillin (99.5%), teicoplanin (100%) and vancomycin (100%) has been found in the tested E. faecalis human isolates in this region [4], although vancomycin-resistant epidemic strains have recently been reported [24]. In our study, no porcine E. faecalis isolates displayed resistance to ampicillin, teicoplanin or vancomycin. Avoparcin, a vancomycin analogue, has been implicated in the selection of vancomycin-resistant enterococci on-farm [1]. However, it has been rarely administered to animals in Brazil and was ultimately banned [22]. This fact may explain the low level of vancomycin-resistant enterococci reported in foods from this country [2,5,25].

The effective treatment of serious enterococci infection in humans is based on the synergistic action of antimicrobials that act on the cell wall, such as beta-lactams or vancomycin, and aminoglycosides [6]. Therefore, the identification of isolates resistant to HLR-streptomycin (49/218; 22.4%), among which 51% (25/49) were also resistant to HLR-gentamicin, may represent a hazard of spreading enterococci strains that may be resistant to treatment. In particular, the isolates that presented resistance against both streptomycin and gentamicin are of great concern, since they can be considered to be resistant to all the aminoglycoside class [26].

Alternative drugs used in human treatment, such as tetracycline and erythromycin displayed a moderate rate of resistance among the tested isolates (46.3% and 27.5%, respectively). Both drugs have been banned from use to promote animal growth in Brazil, however they are still used for prophylactic and therapeutic purposes [20,21], which may contribute to the selection of resistant enterococci strains in pigs. Among the erythromycin-resistant strains, a remarkably high level of resistance was detected, with 58 of the 64 resistant strains presenting MIC values of 256 μg.mL⁻¹. Resistance to erythromycin is codified by erm genes, which confer resistance and are selected by all drugs in the macrolides-lincosamides-streptogramines group [10]. Several drugs of this group are frequently used in feed for prophylaxis and treatment of respiratory and enteric diseases in pigs and may thus be exerting a high selective pressure on the intestinal microbiota.

The selective pressure of antimicrobial use may also explain the significantly lower frequency of resistance against most tested agents in slaughterhouse B, as well as the concentration of multi-resistant isolates in slaughterhouses A and C. The association of resistance phenotypes and genes, as well as the selection of multi-resistant isolates on-farm, has been reported in other countries [3,23]. The slaughterhouses sampled in this study were supplied by production chains that belong to different agribusiness companies. These companies in turn run contracts with farmers which usually specify a common management protocol, including prophylactic and therapeutic administration of antimicrobial drugs. Therefore, the antimicrobial resistance profile displayed by enterococci may reflect the selective pressure exerted by the extent of antimicrobial use on-farm.

**CONCLUSION**

Enterococcus isolates from pre-chill swine carcasses presented a low to moderate frequency of resistance to most antimicrobials used in human treatment. However, isolates displaying resistance to aminoglycosides and macrolides drugs were present, which may present a hazard. The multi-resistance patterns were highly associated with the origin of isolates, and may indicate the extent of antimicrobial use on-farm.

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**Declaration of Interest.** The authors report no conflicts of interest. The authors alone are responsible for the content of the paper.
REFERENCES


