Prevalence and antimicrobial susceptibility profile of *Enterococcus* spp isolated from frozen chicken carcasses

Prevalência e perfil de susceptibilidade antimicrobiana de *Enterococcus* spp isolados de carcaças de frango congeladas

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RESUMO

No período de setembro de 2004 a junho de 2006, foram avaliadas a prevalência e susceptibilidade antimicrobiana de *Enterococcus* spp. em 360 amostras de carcaças de frangos congeladas, sem tempero, coletadas em estabelecimentos comerciais do Estado de São Paulo, Brasil. *Enterococcus* spp. foi isolado de todas as amostras analisadas e 1.332 cepas foram identificadas. Entre as dez espécies identificadas, houve predominância de *E. faecalis*, *E. gallinarum*, *E. casseliflavus* e *E. faecium*. Todas as cepas de enterococos testadas apresentaram algum nível de resistência aos nove antimicrobianos utilizados no estudo. As porcentagens de resistência antimicrobiana foram: de 89,2% para tetraciclina, 91,4% para quinupristina-dalfopristina, 83,5% para eritromicina, 65% para ciprofloxacina, 55,4% para cloranfenicol, 6,5% para linezolida, 2,3% para vancomicina, 2,3% para teicoplanina e 0,2% para ampicilina. A ocorrência de alto nível de resistência aos aminoglicosídeos (HLR-A) foi detectada em 57,4% dos isolados. As espécies *E. faecalis* e *E. faecium*, consideradas importantes agentes em infecções hospitalares, apresentaram resistência, respectivamente, a oito e sete antibióticos.

Palavras-chave. *Enterococcus*, resistência, antimicrobianos, carcaças de frango.

ABSTRACT

Prevalence and antimicrobial susceptibility of *Enterococcus* spp. were evaluated in 360 frozen unseasoned chicken carcasses samples collected from September 2004 to June 2006 from the retail stores in São Paulo State, Brazil. *Enterococcus* spp. was isolated from all analyzed samples, and 1,332 strains were identified from them. Among the ten identified species, the predominance of *E. faecalis*, *E. gallinarum*, *E. casseliflavus* and *E. faecium* was occurred. All of the Enterococci strains showed some degree of resistance to the nine antimicrobials utilized in the study. The percentages of antimicrobial resistance were: 89.2% for tetracycline, 91.4% for quinupristin-dalfopristin, 83.5% for erythromycin, 65% for ciprofloxacin, 55.4% for chloramphenicol, 6.5% for linezolid, 2.3% for vancomycin, 2.3% for teicoplanin and 0.2% for ampicillin. The occurrence of the high level resistance to amynoglicosides (HLR-A) was detected in 57.4% of the isolates. *E. faecalis* and *E. faecium* species, which are considered as important agents in nosocomial infections, showed resistance to eight and seven antibiotics, respectively.

Keywords. Enterococcus, resistance, antimicrobials, chicken carcasses.

INTRODUCTION

Enterococci comprise a widespread bacterial group and are present in a variety of foods. They normally colonize the intestinal tract of humans and animals, and can be considered as indicators of fecal contamination in food. However, as opportunistic microorganisms, they are also responsible for infections in humans such as endocarditis, infections of the genitourinary tract, meningitis and septicemia. Most infections are caused by *Enterococcus faecalis* and *Enterococcus faecium*, but infections by *Enterococcus gallinarum*, *Enterococcus durans* and *Enterococcus avium* have also been reported[1,2]. Over the last 10 years, enterococci have emerged as major nosocomial pathogens. Approximately 12% of all nosocomial infections in the USA are caused by enterococci[3].

Currently, there is a growing concern as for the increase of acquired antimicrobial resistance in bacteria, which reduce the availability of efficient and indispensible substances for the treatment and prevention of infectious diseases. Many drugs, including some with importance for human medicine, are added to animal feed as growth promoters and with prophylactic purposes. Although this technological alternative allows a better performance of the animals, specially poultry and swine, the use of antibiotics in animal production can select resistant microorganisms that can be transferred to human through ingestion of contaminated foods or via the environment[4].

Enterococci are intrinsically resistant to a number of antimicrobials agents and can acquire resistance to others agents such as aminoglycosides, β-lactams and glycopeptides[5]. In Europe the extensive use of the glycopeptide avoparcin, as feed additive in food farms, has been pointed as the responsible by the emergence of vancomycin resistant *Enterococcus* (VRE) among isolates from human and animals[6]. Avoparcin is a molecule similar to vancomycin that exhibits the same mechanisms of action and resistance. Due to the emergence of cross-resistance to clinically important antibiotics such as vancomycin and teicoplanin, in April, 1997, the use of avoparcin was prohibited in the European Union[7]. In Brazil the use of this drug in animal feed is prohibited since 1998. However, other antimicrobials such as avilamycin, zinc bacitracin, chlorhexidine dichloride, spiramycin, enramycin, flavomycin, lincomycin, colistin sulphate, tilosyn sulphate and virginiamycin are still allowed as additives in animal feed[8].

VRE infections are associated with high morbidity and mortality rates, and excess health care costs[9,10]. Due to emergence of resistant strains, new agents like linezolid, daptomycin, and quinupristin/dalfopristin are also used for therapy of invasive enterococcal infections[11].

In Europe several studies have shown the emergence of vancomycin resistant *Enterococcus* strains in samples of sewage, food and feces of healthy animals[12-14]. In Brazil the first isolation of VRE occurred in 1996[15], and after that many cases have been reported[16-19].

The objective of this work was to evaluate the prevalence of *Enterococcus* spp. in frozen chicken carcasses ready for sale, as well as to identify the species and perform the antimicrobial sensitivity profile of the isolates.

MATERIAL AND METHODS

Samples

A total of 72 samples of frozen unseasoned chicken carcasses were collected from September, 2004 to June, 2006 in retail stores in São Paulo State; each sample were constituted of five units of chicken carcass. A total of 360 units of chicken carcasses from 26 different brands and eight producing states was analyzed (São Paulo, Minas Gerais, Mato Grosso, Paraná, Rio Grande do Sul, Distrito Federal, Santa Catarina and Goiás).

Procedure

The samples were thawed, in a refrigerator, for up to 48 hours. Chicken viscera were removed and each carcass was rinsed in 1% buffered peptone water (BPW; Oxoid, Basingstoke, England). For each 1 g of chicken 1 mL of BPW was added (1:1). After rinsing the whole carcass surface, two 25 mL aliquots of the rinsing water were taken. One of the aliquots was added to 225 mL of Enterococcosel broth (BBL, Beckton Dickinson, Cockeysville, MD, USA), and the other, to 225 mL Enterococcosel (BBL) containing 6 µg/mL vancomycin (Sigma, St Louis, MO, USA). Flasks were incubated at 35 °C for 48 hours. Then, the cultures in the Enterococcosel broths with vancomycin that had turned black were plated on Enterococcosel agar (BBL, Beckton Dickinson, Cockeysville, MD, USA) with vancomycin with the aid of a loop; the cultures from Enterococcosel broths were plated onto the same medium lacking vancomycin. After plates had been incubated for 24 hours at 35 °C, five to ten characteristic colonies were

transferred to Brain Heart Infusion (BHI) Broth (Difco, Detroit, MI, USA). Tubes were again incubated at 35 °C for 18-24 h after which Gram staining and catalase test were performed[20].

Identification of the species and evaluation of antimicrobial resistance

Isolates were identified by conventional biochemicals tests using Gram staining, catalase, reaction on Bile-esculin medium, growth in broth containing 6.5% NaCl, acid formation in carbohydrate (mannitol, sorbitol, sorbose, arabinose, raffinose) broths, hydrolysis of arginine, pyruvate utilization, motility, pigment production, and production of pyrrolidonyl-arylamidase[1].

A multiplex PCR (Polymerase Chain Reaction) assay based on the specific detection of genes encoding D-lanine: D-alanine ligases (*ddl*) was used to confirm the identification of *E. faecalis* and *E. faecium* species. Another multiplex PCR assay based on the specific detection of genes encoding *van*C1 and *van*C2 was used to confirm the identification of *E. gallinarum* and *E. casseliflavus* species[17].

E. faecalis (ATCC 29212), *E. faecium* (*van*A-228), *E. casseliflavus* (NCTC1261) and *E. gallinarum* (NCTC 12359) were utilized as reference strains in the biochemical tests and in the PCR assay.

Antimicrobial susceptibility tests

The criterion for selection of the sampling for determining the antimicrobial susceptibility profile was based on the number of times that each brand was analyzed for the presence of *Enterococcus* spp. The criteria for selection were: 100% of the strains were evaluated when brands were analyzed up to five different times; 50% of the strains when brands were analyzed from six to 10 times; 25% of the strains when brands were analyzed from 11 to 25 times and 15% of those analyzed more than 25 times.

Evaluation of the antimicrobial susceptibility profile was done in 437 strains for vancomycin (Van), teicoplanin (Tei), ampicillin (Amp), ciprofloxacin (Cip), tetracycline (Tet), erythromycin (Ery) and chloramphenicol (Co) and in 245 isolates for linezolid (Lnz) and quinupristin-dalfopristin (Qda). The Minimum inhibitory concentrations (MIC) were determined by broth microdilution according to guidelines of the CLSI[21]. *E. faecalis* (ATCC 29212) was utilized as reference strains to MIC determination.

A total of 437 isolates were tested by agar dilution method to determinate the high-level resistance (HLR) to aminoglicosides using BHI agar plates plus gentamicin (500 µg/ml) and streptomycin (2,000 µg/ml) by CLSI (Clinical and Laboratory Standards Institute) recommendations[21]. For measuring the concentrations of the antibiotics in the BHI agar plates, standard *E. faecalis* ATCC 29212 and ATCC 51299 were utilized as controls for susceptibility and resistance, respectively.

The criteria of susceptibility and resistance adopted for each antimicrobial were those recommended by the CLSI[22].

RESULTS AND DISCUSSION

In the present study, *Enterococcus* spp. was found in 100% of the chicken carcasses analyzed. A total of 1,332 strains were isolated and identified. Similar results have been reported in other studies, confirming the high frequency of enterococci in animal products. Hayes et al.[23] have detected enterococci in 99% of the 981 samples of meat products analyzed. McGowan et al.[24] investigated the prevalence of enterococci in fruits, vegetables and meat products (pork, cow, chicken and turkey) purchased in the retail market; the highest occurrence of this microorganism occurred in meat products (79%), especially chicken and turkey products in which 100% of the samples were positive. In Brazil, among the 120 samples of foods analyzed by Gomes et al.[25], 52% were positive for enterococci, and meat products (60%) and cheeses (83.3%) were those presenting the highest contamination. The high occurrence of enterococci in meat products can be attributed to the natural presence of this microorganism in the gastrointestinal tract of animals and the microorganism's ability to adapt and develop in unfavorable environmental conditions. In addition, the several phases of poultry processing, during slaughter, may contribute for contamination of the carcasses.

As for the different species described for this genus, ten have been identified among the characterized strains (Table 1). However, we observed the predominance of four species: *E. faecalis* (51%), *E. gallinarum* (40%), *E. casseliflavus* (5%) and *E. faecium* (2%), representing about 98% of the total identified strains. Predominance of *E. faecalis* in animal products is in accordance to other studies done in Brazil[26] and in Europe[27,28].

Table 1. Distribution of species of the genus *Enterococcus* in the 360 samples of analyzed chicken carcasses collected from September, 2004 to June, 2006, in retail stores in São Paulo State

Species	N. (%)
E. faecalis	679 (51)
E. gallinarum	532 (40)
E. casseliflavus	69 (5)
E. faecium	24 (2)
E. durans	14 (1)
E. dispar	5 (0.4)
E. hirae	5 (0.4)
E. avium	1 (0.05)
E. columbae	1 (0.05)
E. mundtti	1 (0.05)
Enterococcus spp	1 (0.05)
Total	**1,332 (100)**

Enterococcus, particularly *E. faecalis* and *E. faecium*, showed intrinsic resistance to several antimicrobial drugs, including aminoglycosides, β-lactams and quinolones. In addition, these microorganisms can acquire and transfer genetic elements that confer resistance to other classes of antibiotics, especially glycopeptides such as vancomycin and teicoplanin[5]. All of the enterococci strains tested have shown some level of resistance to the nine antimicrobials utilized in the study, varying from 0.2% (ampicillin) to 89.3% (tetracycline) as show the Table 2. For vancomycin, only 2.3% of the strains were resistant and detected in only two species, *E. faecalis* and *E. faecium*. The intermediate resistance to vancomycin was observed only in the species *E. gallinarum* and *E. casseliflavus*, because this resistance is intrinsic for these two species. For the other drugs we can consider the total resistance by adding the two profiles, intermediate and resistant (I+R) and the strains show 65% resistance to ciprofloxacin, 55.4% to chloramphenicol, 83.5% to erythromycin and 89.3% to tetracycline. However, if we look at each species we observed that *E. faecium* has the lowest resistance profile for these four drugs. To evaluate the profile of resistance to linezolid and quinupristin-dalfopristin, 245 strains were tested as shown in Table 3 and 91.4% of the strains were resistant to quinupristin-dalfopristin.

Among the species, we point out *E. faecalis* which have shown resistance to quinupristin-dalfopristin (97.9%), tetracycline (89.1%), erythromycin (82%), ciprofloxacin (65.2%), chloramphenicol (59.9%), linezolid (5.9%), teicoplanin (0.7%) and vancomycin (0.7%), and *E. faecium* which have shown resistance to tetracycline (93.3%), teicoplanin (53.3%), vancomycin (53.3%), erythromycin (93.3%), quinupristin-dalfopristin (30.8%), ciprofloxacin (20%) and chloramphenicol (20%).

Resistance to vancomycin (VRE) was detected in 10 strains (2.3%) isolated from two units of chicken produced in São Paulo. Although a low resistance rate was observed for this antibiotic, the strains that have shown this characteristic belonged to the *E. faecalis* and *E. faecium* species, which are organisms considered as important agents in hospital infections. Gomes et al.[25] have evaluated the antimicrobial susceptibility of 219 enterococci strains isolated from foods and have detected 3 strains (1.4%) of vancomycin resistant *E. faecium*. VRE *E. faecium* has also been found by Japanese researchers in 9% (2/22) of the chicken carcasses exported by Brazil[29]. In other studies done in Brazil, VRE strains were not detected in samples of chicken and in swabs from chicken cloacae[26,30].

Higher resistance levels were observed for tetracycline, quinupristin-dalfopristin and erythromycin. Resistance to tetracycline was observed among the *E. faecium* (93.3%), *E. gallinarum* (92.5%), *E. faecalis* (89.1%), *E. casseliflavus* (66.7%) and *E. hirae* (50%) isolates. As for erythromycin resistance rates were 84.3% in *E. gallinarum*, 82% in *E. faecalis*, 90.5% in *E. casseliflavus*, 93.3% in *E. faecium*; only one *E. durans* strain has shown resistance to this antibiotic. Although the use of tetracycline as growth promoter is prohibited in Brazil since 1998, this antimicrobial and erythromycin are the drugs most used therapeutically in animal production[31] and may contribute, consequently, for the occurrence of high resistance levels for these antimicrobials.

According to Chopra and Roberts[32], co-resistance can also contribute to high resistance levels to tetracycline and erythromycin since plasmids and/or transposons can simultaneously carry genes that confer resistance to these two antibiotics.

As for quinupristin-dalfopristin, resistance was verified in *E. faecalis* (97.9%), *E. gallinarum* (80.6%), *E. casseliflavus* (85.7%) and *E. faecium* (30.8%). Quinupristin/dalfopristin, which belongs to the streptogramin family, had its use approved by the FDA[33] for treatment of severe infections in humans associated with vancomycin resistant *E. faecium*. According to Manzella[34], resistance to this antimicrobial is not common. *E. faecalis* shows intrinsic resistance to this antibiotic. However, in other species the occurrence of resistance can be related to the use of virginiamycin in animal production as growth promoter[35] since this

Table 2. Susceptibility profile of *Enterococcus* strains isolated from frozen chicken carcasses from September, 2004 to June, 2006 of retail stores in São Paulo State

Species	N. strains evaluated	Ampicillin			Ciprofloxacin			Chloramphenicol			Erythromycin			Teicoplanin			Tetracycline			Vancomycin		
		S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
E. faecalis	267	267	0	0	93	121	53	107	88	72	48	54	165	265	0	2	29	3	235	265	0	2
E. gallinarum	134	133	0	1	43	31	60	64	39	31	21	22	91	134	0	0	10	3	121	0	134	0
E. casseliflavus	21	21	0	0	5	6	10	12	5	4	2	9	10	21	0	0	7	0	14	0	21	0
E. faecium	15	15	0	0	12	0	3	12	1	2	1	9	5	7	0	8	1	0	14	7	0	8
Total n(%)	437	436 (99.8)	0	1 (0.2)	153 (35)	158 (29)	126 (29)	195 (44.6)	133 (30.4)	109 (25)	72 (16.5)	94 (21.5)	271 (62)	427 (97.7)	0	10 (2.3)	47 (10.7)	6 (1.4)	384 (87.9)	272 (62.2)	155 (35.5)	10 (2.3)

Resistance Profile: S= susceptible; I= intermediate; R= resistant; NE= not evaluated

Table 3. The profile of resistance of *Enterococcus* strains to linezolid and quinupristin-dalfopristin

Species	N. strains evaluated	Linezolid			Quinupristin-Dalphopristin		
		S	I	R	S	I	R
E. faecalis	187	176	1	10	4	1	182
E. gallinarum	31	28	2	1	6	8	17
E. casseliflavus	14	12	1	1	2	5	7
E. faecium	13	13	0	0	9	1	3
Total n(%)	245	229 (93.5)	4 (1.6)	12 (4.9)	21 (8.6)	15 (6.1)	209 (85.3)

Resistance Profile: S= susceptible; I= intermediate; R= resistant; NE= not evaluated

antimicrobial shows cross-resistance to quinupristin-dalfopristin[36]. In Brazil the use of virginiamycin as growth promoter in animal feed is allowed in poultry meat production[8]. In the U.S., where virginiamycin is utilized over 20 years in animal production, Hayes et al.[37] verified that 63% of the 127 strains of *E. faecium* isolated from environmental samples in poultry production were resistant to quinupristin-dalfopristin.

Linezolid was the first representative of a new class of antimicrobials called oxazolidinones, utilized in infections caused by multi-resistant Gram positive cocci, among them VRE[38]. Our results alert for the occurrence of resistance to this antibiotic in 5.1% of the strains. According to Scheetz et al.[39], the emergence of linezolid resistant among clinical strains is related to the prolonged use of this drug in the treatment of infections in humans.

The occurrence of High Level Resistance to aminoglycosides (HLR-A) was tested in 437 strains (Table 4). HLR-A (Gentamicin and/or Streptomycin) was detected in 57.4% of the isolates. High resistance levels to gentamicin (HLR-Gn) were observed in *E. gallinarum*, *E. casseliflavus* and *E. faecalis*. High resistance levels to streptomycin (HLR-St) and both gentamicin and streptomycin (HLR-Gn/St) were observed in the species *E. faecalis, E. gallinarum, E. faecium* and *E. casseliflavus.*

Infections by enterococci are frequently treated with a combination of antibiotics, an aminoglycoside (e.g., gentamicin) and an agent that acts on the cell wall, such as penicillin or a glycopeptide. Thus, the percentage (46.5%) of HLR-A strains found in our study is worrisome. Differently from our results, Fracalanzza et al.[26] have detected the occurrence of HLR-A in 10.6% of the strains isolated from chicken samples.

The results of this study revealed that enterococci are common contaminants in chicken purchased in retail stores in São Paulo State, Brazil. Considering that in Brazil the studies on the occurrence of *Enterococcus* in foods are scarce[25,26,40,41] and that the data presented in this study regarding resistance of antibiotics are worrisome, especially for the most prevalent species in human infections (*E. faecalis* and *E. faecium)*, the utilization of new antimicrobials should be done in a very rational

Table 4. Occurrence of High Level Resistance to Amynoglicosides (HLR-A) among *Enterococcus* isolated from frozen chicken carcasses in São Paulo State

Species	Number (%) of isolates that showed HLR-A			
	Number	HLR-Gn	HLR-St	HLR-Gn/St
E. faecalis	267	34 (12.7%)	86 (32.2%)	9 (3.4%)
E. gallinarum	134	62 (46.3%)	32 (23.9%)	13 (9.8%)
E. casseliflavus	21	3 (14.3%)	3 (14.3%)	1 (4.8%)
E. faecium	15	2 (13.3%)	4 (26.7%)	2 (13.3%)
Total	**437**	**101 (23.1%)**	**125 (28.6%)**	**25 (5.7%)**

HLR-Gn: strains with High Level Resistance to gentamicin only;
HLR-St: strains with High Level Resistance to streptomycin only;
HLR-Gn/St: strains with High Level Resistance to gentamicin and streptomycin simultaneously.

way, both in human and animal therapy. Monitoring of antimicrobial resistance is essential since constant surveillance can halt the dissemination of *Enterococcus* clones resistant to several drugs, as well as the emergence of new resistance mechanisms.

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