Antimicrobial Resistance of *Enterococcus* sp. Isolated from the Intestinal Tract of Patients from a University Hospital in Brazil

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This study reports the results about antimicrobial resistance of Enterococcus *spp. isolated from intestinal tract of patients from a university hospital in Brazil. The identification of strains at species level was performed by conventional biochemical tests, API 20 Strep (bioMérieux), and polymerase chain reaction assay. The specie distribution was* E. faecium *(34%), followed by* E. faecalis *(33%),* E. gallinarum *(23.7%),* E. casseliflavus *(5.2%),* E. avium *(1%), and* E. hirae *(1%). Intrinsic resistance to vancomycin characterized by presence of* vanC *genes was found in* E. gallinarum *and* E. casseliflavus. *The high prevalence of* VanC *phenotype enterococci is very important because these species have been reported as causing a wide variety of infections. Vancomycin-resistant* E. faecium *or* E. faecalis *were not found and no one isolate of these species was a β-lactamase producer. Thirteen clinical isolates of enterococci (13.4%) showed multiresistance patterns, which were defined by resistance to three classes of antibiotics plus resistance to at least one aminoglycoside (gentamicin and/or streptomycin). The resistance to several antimicrobials shown by enterococcal strains obtained in this study is of concern because of the decrease in the therapeutic options for treatment of infections caused by enterococci.*

Key words: enterococci - resistance - antimicrobials - Brazil

Enterococci are widespread in nature and are part of the commensal flora of the human gastrointestinal and genitourinary tracts. Enterococci are often implicated in infections of the urinary tract and abdomen or superficial wounds of hospitalized patients, but can also cause bacteremia, endocarditis, perinatal infections, and, occasionally, meningitis or pneumonia. Thus, enterococci are increasing in importance as the cause of hospital-acquired infections. In addition, enterococci colonizing serve as reservoir for antibiotic resistance genes that can be transferred among enterococci and can be acquired by other bacteria (Dukta-Malen et al. 1994, Leclerq 1997).

Several studies have documented that enterococcal infections are most commonly caused by the patients own commensal flora. Colonization may occur long before or immediately before infection, but either way, it plays a major role in the development of nosocomial infection (Montecalvo et al. 1995).

Enterococal antimicrobial resistance to several antimicrobials, particularly resistance to high concentrations of penicillin and gentamicin or to glycopeptides, have hindered the treatment of serious infections caused by enterococci. The synergistic combinations among amynoglycosides and a cell-wall active antibiotic (i.e. amoxicillin or vancomycin) have been implicated in these resistant strains.

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This paper reports a study conducted to determine antimicrobial resistance of *Enterococcus* spp. isolated from the intestinal tract of patients from a university hospital in Brazil.

MATERIALS AND METHODS

Bacterial strains and species identification - From May 2001 to June 2002, 37 stools and 101 rectal swabs were obtained from 125 hospitalized patients in hospital wards where vancomycin and or other antimicrobials are frequently used: Internal Medicine, Infectious Diseases, Kidney Transplant, Bone Marrow Transplant, Emergency Unit, Hematology, and Intensive Therapy Unit at the Hospital das Clínicas, Faculty of Medicine of Ribeirão Preto, University of São Paulo, a university hospital in Brazil, with 551 beds, that admits annually about 34,075 in patients and attends other 524,574 out patients. The patients included in this study were hospitalized at least for five days. All samples were collected randomly and there was no report of any gastrointestinal infection in the patients involved in this study.

The study protocol was approved by the Hospital's Ethical Committee (Process 6539/00).

The samples were plated onto Bile Esculin Azide Agar (Becton, Dickinson & Co.), a selective medium with bile-esculin and sodium azide. Five to six isolated colonies were identified from each plate and only those that presented some difference in minimum inhibitory concentration (MIC) and/or specie identification were considered for each patient.

Isolates recovered from stool or rectal swabs were presumptively identified as enterococci by colonial morphology, Gram's stain, the absence of catalase production, the presence of pyrrolidonylarylamidase by hidrolisis of L-pyrrolidonil-β-naphthylamide (Becton, Dickinson & Co.),

tolerance to 6.5% sodium chloride and ability to grow at 10ºC and 45ºC. Species identification was carried out with a test scheme proposed by Facklam et al. (1999) that is based on carbohydrate fermentation and arginine deamination. Carbohydrate fermentation tests were perfomed in agar containing 1% of the sugar being tested. Arginine deamination was tested with Moeller arginine decarboxylase broth. Yellow pigmentation was observed after over night growth on Müeller Hinton agar (Merck) supplemented with 5% sheep blood and by taking a sweep of the plate with a cotton swab. The pyruvate utilization test was observed with a broth containing 1% pyruvate (Ruoff et al. 1999, MacFaddin 2000). The identification of some species was performed by API 20 Strep (bioMérieux).

Amplification by polymerase chain reaction (PCR) for species identification - PCR was performed to confirm some species identification and to detect vancomycin resistance mediated by *van* genes. Two colonies were obtained from a fresh subculture and were resuspended in 100 µl sterile water (Sigma, St. Louis, US). Two microliters of the suspension were added to each PCR mixture consisting of 23 µl with the following components: 0.625U Taq polymerase (Life Technologies, UK), 0.2 mM each deoxynucleoside triphophate (dNTP), 2.0 mM $MgCl_2$, 1x PCR buffer (20 mMTris, 50 mM KCl), 25 pmol of each primer according to Dukta-Malen et al. (1995) (Table I). After an initial denaturation step at 94ºC for 2 min, the mixture was subjected to 30 cycles under the following conditions: 30 s at 94ºC, 30 s at 54ºC, and 30 s at 72ºC, followed by a final extension step at 72ºC for 10 min. Amplified products were analyzed by 1.5% agarose gel electrophoresis and the size of the amplification products was estimated by comparison with the molecular size standard 123 pb ladder.

Control strains: *E. faecalis* NCTC 775, *E. faecium* NCTC 7171, *E. gallinarum* NCTC 12359, and *E. casseliflavus* NCTC 12361.

Determination of minimum inhibitory concentration - The MIC of the following antimicrobial agents was performed by the agar dilution method according to NCCLS guidelines (2000) with antibiotic dilutions ranging from 0.125 to 256 µg/ml: vancomycin, penicillin, chloramphenicol, tetracycline, erythromycin, and quinupristindalfopristin. Bacterial suspensions equal to a 0.5 McFarland standard were prepared and inoculated onto antibiotic containing medium with Steers replicator to yield a final inoculum of 10^8 cfu/spot. Plates were incubated in ambient air at 35ºC for 24 h. The MIC was defined as the lowest antibiotic concentration which gave a complete absence of growth (NCCLS 2000).

Detection of high level aminoglycoside resistance (HLAR) - The screening of high level aminoglycoside resistance was performed in agar dilution plates prepared with brain heart infusion (BHI - Oxoid, Basingstoke, England) agar with 500 mg gentamicin per ml and 2000 µg streptomycin per ml. The plates were inoculated by spotting 10 µl of a suspension prepared from growth on an 18-24 h agar plate and adjusted to a 0.5 Mc Farland standard, giving a final inoculum of 10^8 cfu/spot. Plates were incubated at 35ºC for 24 h/48 h (NCCLS 2000).

Detection of β-lactamase production - All enterococci isolates were tested for β-lactamase production with nitrocefin (Oxoid) according to the manufacture's instructions. Nitrocefin solution was dropped onto a single colony of an overnight culture. Development of a red color would indicate a positive result.

Control strains: *S. aureus* ATCC 29213 was used as a positive control and *S. aureus* ATCC 25923 as negative control for β-lactamase detection.

RESULTS

Enterococcus spp. were isolated in 67 (53.6%) of the 125 patients included in this study, and 15 of them presented more than one specie of enterococci in the faecal samples.

A total of 97 enterococcal isolates were obtained from 67 patients. The enterococcal isolates were identified to species level and the distribution were *E. faecium* (34%) followed by *E. faecalis* (33%), *E. gallinarum* (23.7%), *E. casseliflavus* (5.2%), *E. durans* (2%), *E. avium* (1%), and *E. hirae* (1%). The scheme proposed by Facklam et al. (1999) did not permit the specie identification in some cases, thus we do need perform the species identification by PCR or API test. No one patient showed only *E. avium* in faecal samples, but this species was isolated in one patient in association with *E. gallinarum*, *E. casseliflavus*, and *E. faecalis*. The association of species most frequently found was *E. faecium*-*E. faecalis* (7.5%) and *E. faecium*-*E. gallinarum* (7.5%). Six patients harbored other association of enterococci species in gastrointestinal tract as indicated in Table II.

The vancomycin resistance genotype in *E. gallinarum*

TABLE I

Sequences of the species-specific primers used in this study

Species	Gene	Product (bp)	Primers (5' – 3')
Enterococcus faecalis	$ddl_{E.\ faecalis}$	941	ATCAAGTACAGTTAGTCTT ACGATTCAAAGCTAACCTG
Enterococcus faecium	$ddl_{E.\ faecium}$	550	GCAAGGCTTCTTAGAGA CATCGTGTAAGCTAACTTC
Enterococcus gallinarum	van*C-1*	822	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT
Enterococcus casseliflavus	van*C 2-3*	439	CTCCTACGATTCTCTTG CGAGCAAGACCTTTAAG

bp: base pairs. All primers used in this study were described by Dukta-Malen et al. (1995)

TABLE II

Distribution of enterococci species in the gastrointestinal tract of the patients studied

Enterococcus species	Patients with enterococci in gastrointestinal tract Nr of patients (%)
E. faecalis	19 (28.3)
E. faecium	16 (23.9)
E. gallinarum	13 (19.4)
E. casseliflavus	1 (1.5)
E. durans	1 (1.5)
E. hirae	1 (1.5)
E. faecalis/ E. faecium	5 (7.5)
E. faecium/ E. gallinarum	5 (7.5)
Enterococcus spp.	6 (8.9) [a]
Total	67 (100)

a: patients colonized by other enterococci association (two or more species).

and *E. casseliflavus* were determined by PCR and all gave positive results for the presence of *vanC-1* and *vanC-2* genes, respectively.

The distribution of antibacterial resistance according to species is presented in Table III. The results show that only *E. gallinarum* and *E. casseliflavus* were resistant to vancomycin. Resistance to penicillin among the *E. faecium* and the *E. faecalis* isolates were 9.1% and 9.4%, respectively. In contrast, resistance to chloramphenicol was 33.3% and 40.6%, erythromycin 66.7% and 75%, tetracycline 42.4% and 56.2%, and to quinupristin-dalfopristin 3% and 12.5%, respectively. *E. faecium* and *E. faecalis*, with high-level gentamicin resistance, were 18.2% and 28.1%, respectively. High-level streptomycin resistance was detected in 21.2% of *E. faecium* and 21.9% of *E. faecalis* isolates. *E. avium* and *E hirae* were susceptible to all drugs tested (Table III).

Although the antibiotic resistance mentioned can exist independently, it can be combined in a single strain that could be a multiresistant strain. In this study the multiresistant strain, defined as that presenting resistance for at least one aminoglycoside (gentamicin and/or streptomycin) plus resistance for another three classes of antibiotics, was verified in 13 isolates of *Enterococcus* spp. (Table IV).

Neither isolate of *E. faecalis* nor *E. faecium* was a β-lactamase producer.

DISCUSSION

This study investigated the species occurrence and antibacterial resistance patterns of enterococci isolated from rectal swabs of hospitalized patients in clinics that have high-risk for vancomycin-resistant enterococci (VRE) colonization in a university hospital in Brazil. The transmission dynamics of VRE and factors contributing to their dissemination are complex. Numerous variables and interactions need to be considered, however gastrointestinal VRE colonization appears to play a major role in the development of VRE infections (Montecalvo et al. 1995).

The isolates obtained in this study were *E. faecium* (34%) followed by *E. faecalis* (33%) and we do not have a clear dominance of *E. faecalis* as expected. According to date published in other Brazilian study (Mondino et al. 2003), *E. faecalis* was the prevalent specie (53.6%) among intestinal strains. A diversity in the species distribution might be obtained when enterococci isolated in different geographical regions are involved. The most frequent enterococcal species isolated from clinical specimens are *E. faecalis* and *E. faecium*, which are responsible for 90% of nosocomial infections (Mutnick et al. 2003), however according to Kühn et al. (2003), these two species represented 78% of the enterococci isolated from intestinal tract of hospitalized patients.

Motile enterococci, including *E. gallinarum* and *E. casseliflavus*, are rarely encountered in human clinical specimens and are primarily found in the gastrointestinal tract in poultry, in foods, and in domestic fowls. Although these species are infrequently isolated from clinical specimens, they have been implicated in a wide variety of invasive infections in humans, especially immunocompromised

TABLE III

Species distribution of enterococci resistant to antimicrobials

Enterococcus species (number tested)	Antimicrobials - Number of strains (%)							
	VAN	PEN	CLO	ERI	TET	QUI/DAL	GEN [a]	STR [a]
E. faecalis (32)	-	3 (9.4)	13 (40.6)	24 (75)	18 (56.2)	4 (12.5)	9 (28.1)	7 (21.9)
E. faecium (33)	-	3 (9.1)	11 (33.3)	22 (66.7)	14 (42.4)	1 (3)	6 (18.2)	7 (21.2)
E. gallinarum (23)	23 (100)	1 (4.3)	3 (13)	8 (34.8)	7 (30.4)	2 (8.7)	3 (13)	1 (4.3)
E. casseliflavus (5)	5 (100)	-	-	2 (40)	1 (20)	-	-	-
E. durans (2)	-	-	2 (100)	1 (50)	-	-	-	-
E. avium (1)	-	-	-	-	-	-	-	-
E. hirae (1)	-	-	-	-	-	-	-	-
Total (97)	28 (28.9)	7 (7.2)	29 (29.9)	57 (58.7)	40 (41.2)	7 (7.2)	18 (8.5)	15 (15.5)

VAN: vancomycin; PEN: penicillin; CLO: chloramphenicol; ERI: erythromycin, TET: tetracycline, QUI/DAL: quinupristin-dalfopristin; GEN: gentamicin; STR: streptomycin. Isolates with MIC ≥ 32 mg/ml for vancomycin and chloramphenicol, ≥ 16 mg/ml for penicillin and tetracycline, ≥ 8 mg/ml for erythromycin, ≥ 4 mg/ml for quinupristin/dalfopristin, > 500 mg/ml for gentamicin, > 2000 mg/ml for streptomycin were considered to be resistant for these agents (NCCLS 2000); *a*: strains were screened for high level aminoglycoside resistance.

TABLE IV
Multiresistance patterns of enterococci strains

Enterococcus strains	MIC (µg/ml)						HLAR	
	VAN	PEN	CLO	ERI	TET	QUI/DAL	GEN	SM
E. faecalis HC 131 S/A	2(S)	16(R)	128(R)	> 256(R)	64(R)	0,5(S)	> 500(R)	< 2000(S)
E. faecium HC 135 S/A	4(S)	4(S)	64(R)	> 256(R)	256(R)	1(S)	> 500(R)	< 2000(S)
E. faecalis HC 135 S/B	4(S)	1(S)	64(R)	> 256(R)	256(R)	1(S)	> 500(R)	< 2000(S)
E. faecium HC 137 S/A	2(S)	1(S)	64(R)	> 256(R)	128(R)	0,5(S)	> 500(R)	< 2000(S)
E. faecalis HC 141 S/A	1(S)	4(S)	64(R)	256(R)	16(R)	0,5(S)	> 500(R)	< 2000(S)
E. faecalis HC 153 S/A	4(S)	1(S)	32(R)	128(R)	32(R)	1(S)	< 500(S)	> 2000(R)
E. faecalis HC 154 S/C	4(S)	0,5(S)	32(R)	128(R)	32(R)	1(S)	< 500(S)	>2000(R)
E. faecium HC 156 S/A	2(S)	16(R)	32(R)	> 256(R)	128(R)	1(S)	< 500(S)	> 2000(R)
E. gallinarum HC 185 S/A	8(S)	2(S)	32(R)	> 256(R)	128(R)	2(S)	> 500(S)	> 2000(R)
E. faecalis HC 186 S/B	1(S)	16(R)	64(R)	8(R)	0,5(S)	1(S)	< 500(S)	> 2000(R)
E. faecalis HC 221 S/A	2(S)	8(S)	64(R)	> 256(R)	0,125(S)	4(R)	> 500(R)	< 2000(S)
E. faecalis BAC 58S/A	2(S)	2(S)	32(R)	> 256(R)	32(R)	0,5(S)	> 500(R)	> 2000(R)
E. faecalis BAC 61 S/C	4(S)	4(S)	64(R)	> 256(R)	32(R)	4(R)	< 500(S)	> 2000(R)

MIC: minimum inhibitory concentration determined by agar dilution method with breakpoints proposed by NCCLS (2000); VAN: vancomycin; CLO: chloramphenicol; ERI: erythromycin; TET: tetracycline; QUI/DAL: quinupristin/dalfopristin; GEN: gentamycin; SM: streptomycin; S: susceptible; R: resistant; HLAR: high level aminoglycoside resistance

or chronically ill patients, and sometimes are nosocomially acquired (Reid et al. 2001, Dargere et al. 2002).

In this study the prevalence of van*C* phenotype enterococci was significantly higher (23.7% of *E. gallinarum* and 5.2% of *E. casseliflavus*) than in several studies that reported intestinal colonization by enterococci van*C*. In 1997, the prevalence of colonization by these species in another hospital in Brazil was 1.2% (Cereda et al. 1997), Canada presented 5% of these species (Toye et al. 1997), Lebanon 1.4% (Zouain & Araj 2001), and Kuwait 4.5% (Udo et al. 2003). The high prevalence obtained in this study is very important because several studies have reported infection or colonization by *E. gallinarum* carrying genes that determine high resistance to vancomycin: *E. gallinarum* van*A* in Belgium (Dukta-Malen et al. 1994), in Italy (Biavasco et al. 2001), in Japan (Takayama et al. 2003), and in Brazil (Camargo et al. 2004). *E. gallinarum* van*B* in Switzerland (Liassine et al. 1998) and Australia (Schooneveldt et al. 2000).

Several hospitals located in São Paulo and some other Brazilian cities reported both outbreaks and isolated cases of VRE infection/colonization (Zanella et al. 1999, 2003, Cereda et al. 2001, 2002, Reis et al. 2001), however the enterococci strains obtained in this study were van susceptible, indicating that vancomycin retains its therapeutic efficacy against *E. faecalis* and *E. faecium* in this hospital.

The prevalence of resistance to penicillin remains low in this hospital, indicating that this antimicrobial agent would be a therapeutic option. On other hand, the absence of vancomycin resistance among enterococci strains studied permits the use of vancomycin in combination with aminoglycoside as an alternative to penicillin to treat allergic patients or against β-lactamase producing strains. However, the high rate of aminoglycoside resistance obtained in this study could not maintain a therapeutic efficacy and the synergistic effect when combined with cell wall active agents such as vancomycin and ampicillin in the treatment of enterococcal infections.

The high rates of resistance to chloramphenicol, erythromycin and tetracycline observed in this study restricts the use of these drugs at this hospital.

Quinupristin-dalfopristin is almost inactive against *E. faecalis*. In contrast, most isolates of *E. faecium* are susceptible to this agent. The mechanism of resistance to dalfopristin in *E. faecalis* is related with an efflux pump that appears to be intrinsic in this species (Eliopoulos 2003). Date obtained in this study are in concordance with those published because 13% of the *E. faecalis* were found to be resistant to quinupristin-dalfopristin. However, among *E. faecium* isolates obtained in this study, 3.8% showed resistance to this agent.

Although the antibiotic resistance mentioned above can exist independently, it can be combined in a single strain resulting in multiresistance. The several multiresistant enterococci strains obtained in this study are a cause of concern due to limitations in clinical use, specially by loss of synergistic combinations which are often needed for treatment of enterococcal infections (Schouten 1999).

In spite of the conditions that could facilitate VRE colonization, we did not find either vancomycin resistant *E. faecalis* or *E. faecium* in this hospital. However, recommendation to prevent the spread of VRE are essential due the resistance to several antimicrobials showed by enterococcal strains obtained in this study.

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