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COMPARISON BETWEEN AUTOMATED SYSTEM AND PCR-BASED METHOD FOR IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY PROFILE OF CLINICAL *Enterococcus* spp

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SUMMARY

Enterococci are increasingly responsible for nosocomial infections worldwide. This study was undertaken to compare the identification and susceptibility profile using an automated MicrosScan system, PCR-based assay and disk diffusion assay of *Enterococcus* spp. We evaluated 30 clinical isolates of *Enterococcus* spp. Isolates were identified by MicrosScan system and PCR-based assay. The detection of antibiotic resistance genes (vancomycin, gentamicin, tetracycline and erythromycin) was also determined by PCR. Antimicrobial susceptibilities to vancomycin (30 µg), gentamicin (120 µg), tetracycline (30 µg) and erythromycin (15 µg) were tested by the automated system and disk diffusion method, and were interpreted according to the criteria recommended in CLSI guidelines. Concerning *Enterococcus* identification the general agreement between data obtained by the PCR method and by the automatic system was 90.0% (27/30). For all isolates of *E. faecium* and *E. faecalis* we observed 100% agreement. Resistance frequencies were higher in *E. faecium* than *E. faecalis*. The resistance rates obtained were higher for erythromycin (86.7%), vancomycin (80.0%), tetracycline (43.35) and gentamicin (33.3%). The correlation between disk diffusion and automation revealed an agreement for the majority of the antibiotics with category agreement rates of > 80%. The PCR-based assay, the *van*(A) gene was detected in 100% of vancomycin resistant enterococci. This assay is simple to conduct and reliable in the identification of clinically relevant enterococci. The data obtained reinforced the need for an improvement of the automated system to identify some enterococci.

KEYWORDS*: Enterococcus;* MicrosScan system; PCR assay.

INTRODUCTION

Enterococci are implicated in a wide diversity of infections and are the third most common pathogen isolated from several infections worldwide[35]. According to a recent epidemiological survey conducted in Brazil, *Enterococcus* spp accounted for 4.5% of all nosocomial bloodstream infections (BSIs), resulting in 49.5% crude mortality[26].

Enterococci infections' greater mortality rates and antibiotic resistance are associated with prolonged hospitalization and increased health-care costs[1,32]. It has recently been reported that inappropriate and delayed antibiotic therapy present an independent risk factor for mortality caused by enterococcal bacteraemia[36]. Besides, the difficulty in treating enterococci infections, particularly with respect to vancomycin resistance isolates, emphasizes the need for safe and therapeutic guidance for rapid identification and effective management.

In this context, the employment of automated systems, that provide rapid identification and susceptibility testing, may lead to a significant reduction of patient morbidity, mortality and cost[3]. However, the identification and susceptibility testing of microorganisms usually takes 24-48 h after initial growth in a routine laboratory. In addition, automated systems may present problems in the identification of members of the genus *Enterococcus* in clinical laboratories[11]. Currently, several studies have compared the direct and standard methods for different automated systems[16,17,20,42].

The employment of polymerase chain reaction (PCR)-based assay in the identification of enterococci and detection of antibiotic resistance genes offered a specific and rapid alternative to standard tests, providing essential information concerning the effective management and appropriate therapy of enterococcal bacteraemia[10,14,21,41].

In this study, we compared for the first time the MicrosScan® system versus PCR-based approach for identification as well as the susceptibility profile of clinical *Enterococcus* sp.

MATERIAL AND METHODS

Isolates: A total of 30 *Enterococcus* clinical isolates were obtained from January 2008 to June 2010, from patients of the University Hospital of State University of Maringá (UEM). The origins of the isolates were

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Table 1
Primers used in this study for identification of *Enterococcus* spp. and detection of different resistance genes by PCR-based method

Gene	Nucleotide sequence (5'- 3')[a]	Ta* (ºC)	amplicon (bp)	References
tuf	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	56	112	21
vanC-1	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	56	822	10
vanC-2, *vanC-3*	CTCCTACGATTCTCTTG CGAGCAAGACCTTTAAG	56	439	
$ddl_{E.faecalis}$	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	56	941	
$ddl_{E.faecium}$	TAGAGACATTGAATATGCC TCGAATGTGCTACAATC	56	550	
vanA	GTAGGCTGCGATATTCAAAGC CGATTCAATTGCGTAGTCCAA	56	231 *E. faecium* 330 *E. faecalis*	2
aac(6')- Ie- aph(2")-Ia	CAGAGCCTTGGGAAGATGAAG CCTCGTGTAATTCATGTTCTGGC	56	348	39
erm(B)	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	56	405	14
tet(L)	GTMGTTGCGCGCTATATTCC GTGAAMGRWAGCCACCTAA	56	696	

Ta (ºC) = temperature of annealing/aM = A or C; R = A or G; W = A or T/ (*) with modification/gene gene *tuf*, *Enterococcus*; *vanC-1*, *E. gallinarum*; v*anC-2*, *vanC-3*, *E.casseliflavus*, *E. flavencens; tet(L)*, tetracycline; *erm(B)*, erythromycin; *aac(6')-aph(2')-Ia*, gentamicin and *vanA*, vancomycin.

urine, blood, orotracheal fluid and rectal swab. The MicrosScan® was used in the identification of enterococci and in susceptibility test by using the standard growth detection algorithms provided by the system.

Isolation of enterococcal DNA, identification and detection of resistance genes by PCR: *Enterococcus* spp. genomic DNA was extracted by the boiling method as described by MARQUES & SUZART[27]. The identification of enterococci species was performed using a polymerase chain reaction (PCR) method. PCR assay was carried out using the following species-specific primers: $ddl_{E.faecalis}$ (*E. faecalis*), $ddl_{E.faecium}$ (*E. faecium*), *vanC-1* (*E. gallinarum*), *vanC-2* (*E. casseliflavus*) and *vanC-3* (*E. flavencens*), and *tuf* for *Enterococcus* sp genus members (Table 1). The detection of resistance genes was conducted by PCR in all isolates of enterococci. The presence of gene *vanA*, *aac(6')-Ie-aph(2")-Ia*, *erm(B)* and *tet(L)*, for vancomycin, gentamicin, erythromycin and tetracycline, respectively (Table 1).

All PCR amplifications were performed in a final volume of 20 µL containing one ρmol of each primer (Forward and Reverse), 0.17 mM dNTPs, 2.5 mM $MgCl_2$, one U of Taq DNA polymerase (Invitrogen), buffer of Taq, and 10 µL template DNA. An initial cycle of denaturation (94 °C for two min), was followed by 30 cycles of denaturation (94 °C for one min), annealing at an appropriate temperature for one min and elongation (72 °C for 10 min). A Thermal Cycler (Techne-Tc3000) was used to carry out the PCR reactions. PCR products were analyzed by gel electrophoresis in 1.5% agarose stained with ethidium bromide (0.5 g.mL-1), observed under UV transillumination and photographed by L-PIX ST (LOCCUS).

Antimicrobial susceptibility testing: Susceptibility testing of four antimicrobial agents (vancomycin, 30 µg; tetracycline, 30 µg; erythromycin, 15 µg; and gentamicin 120 µg) (Laborclin) was performed by the disk diffusion assay on Muller Hinton agar plates. After 18 or 24 h of incubation at 37 °C, inhibition zone diameters around each disc were measured and the diameters of inhibition zones were interpreted according to the criteria recommended by the Clinical and Laboratory Standards Institute, 2011. *Staphlylococccus aureus* 25923 ATCC was used as a control strain. MicrosScan® system was used on the same antimicrobial agents for the antimicrobial susceptibility testing.

RESULTS

In the present study, we firstly evaluated the genetic similarities of the *Enterococcus* isolates using the RAPD-PCR analysis. The fingerprinting revealed no clonal lineage (unrelated strains) among tested isolates (data not shown).

As shown in Table 2, for 27 out of 30 (90%) isolates the identification was concordant between the automated system and the molecular method. All 20 isolates identified as *E. faecium* and seven isolates as *E. faecalis* by automation system were confirmed by PCR assay. Figure 1 illustrated the amplicon size of *Enterococcus* sp. Among the isolates tested, *E. faecium* (76.7%) had a much higher incidence rate followed by *E. faecalis* (23.3%).

The disagreement was observed in the identification of three isolates. The species classified by automation as *E. gallinarum* (isolate 817) and *E. durans/hirae* (isolate 917 and 1000) were all identified as *E. faecium* by the PCR assay.

Table 2
Identification of clinical enterococci isolates by automated systems and molecular method

Strain	origin	Identification	
		automated system	PCR-based assay
802	urine	*E. faecalis*	*E. faecalis*
817	rectal swab	*E. gallinarum*	*E. faecium*
840	blood	*E. faecalis*	*E. faecalis*
848	urine	*E. faecalis*	*E. faecalis*
872	orotracheal fluid	*E. faecalis*	*E. faecalis*
906	urine	*E. faecalis*	*E. faecalis*
917	urine	*E. durans/hirae*	*E. faecium*
924	rectal swab	*E. faecium*	*E. faecium*
925	rectal swab	*E. faecium*	*E. faecium*
928	urine	*E. faecalis*	*E. faecalis*
973	urine	*E. faecium*	*E. faecium*
1000	urine	*E. durans/hirae*	*E. faecium*
1035	rectal swab	*E. faecium*	*E. faecium*
1053	rectal swab	*E. faecium*	*E. faecium*
1062	rectal swab	*E. faecium*	*E. faecium*
1076	rectal swab	*E. faecium*	*E. faecium*
1097	rectal swab	*E. faecium*	*E. faecium*
1112	rectal swab	*E. faecium*	*E. faecium*
1114	rectal swab	*E. faecium*	*E. faecium*
1115	rectal swab	*E. faecium*	*E. faecium*
1125	rectal swab	*E. faecium*	*E. faecium*
1143	rectal swab	*E. faecium*	*E. faecium*
1211	urine	*E. faecalis*	*E. faecalis*
1215	rectal swab	*E. faecium*	*E. faecium*
1227	urine	*E. faecium*	*E. faecium*
1231	urine	*E. faecium*	*E. faecium*
1246	urine	*E. faecium*	*E. faecium*
1280	rectal swab	*E. faecium*	*E. faecium*
1295	rectal swab	*E. faecium*	*E. faecium*
1298	rectal swab	*E. faecium*	*E. faecium*

Antibiotic susceptibility phenotypes and resistance genes profile, detected by PCR, of the enterococcal isolates are shown in Table 3. The presence of resistance genes *erm(B), tet(L), vanA* and *aac(6')-Ie-aph(2")* were 86.7%, 23.3%, 80.0% and 66.7%, respectively. Several isolates harbored resistance genes to more than one antibiotic. Of significance were *tet(L)*$^+$*/erm(B)*$^+$ to *E. faecalis* (42.8%) and *erm(B)*$^+$*/aac(6')-Ie-aph(2")-Ia*$^+$*/vanA*$^+$ to *E. faecium* (69.6%).

The presence of the *vanA* gene was detected in three isolates of *E. faecalis* and twenty-two of *E. faecium*, corresponding to 42.8% and 96.6% of the isolates, respectively. The *van(A)* gene was detected in 100% of vancomycin resistant enterococci (Table 3), however, five isolates harbored the *van(A)* gene and presented vancomycin susceptibility phenotype.

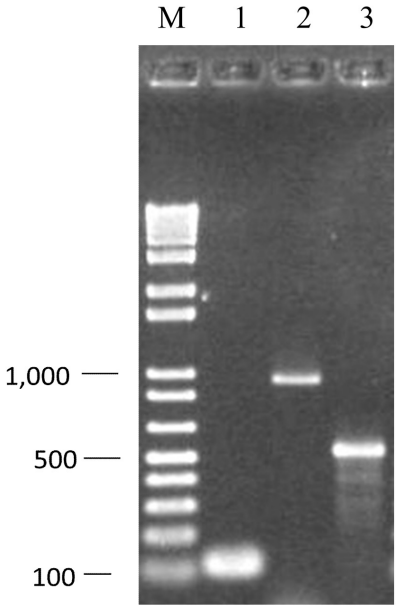

Fig. 1 - Amplification gel pictures characteristic of polymerase chain reaction (PCR) amplification of *Enterococcus* sp gene. Lanes: (1) *Enterococcus* spp. (112 pb), (2) *E. faecalis* (941 pb), (3) *E. faecium* (550 pb). M - Ladder 1kb plus (Invitrogen).

On the other hand, antimicrobial resistance phenotype was detected even in the absence of the respective resistance gene for two isolates to *erm(B),* 10 to *tet(L)* and three to *aac(6')-Ie-aph(2")-Ia* gene.

Additionally, antimicrobial susceptibilities to erythromycin, tetracyclin, vancomycin, and gentamicin were analyzed by disk diffusion. Evaluation revealed excellent agreement for all of the antibiotics with category agreement rates > 80% between automatized method and disk diffusion. Major error rates were for erythromycin, vancomycin and tetracycline with 20.7%, 7% and 16.7% respectively. Minor error rates were found as 12.1% for gentamicin.

Resistance rates obtained by disc diffusion were as follows: 86.7% for erythromycin, 80.0% for vancomycin, 43.35% for tetracycline and 33.3% for gentamicin. Resistance frequencies were higher in *E. faecium* than *E. faecalis*.

DISCUSSION

Enterococci have been implicated in severe human infections as a consequence of associated determinants of virulence and antimicrobial resistance. Accurate identification and rapid analysis of the antibiotic susceptibility pattern of the causative microbial agent leads to earlier targeting of antibiotic therapy and may be lifesaving.

In this study, we describe a comparison between automatic and PCR-based assay for identification of *Enterococcus* spp. Our results showed 90% agreement in the identification of clinically relevant enterococcal species, revealing that the PCR method is reliable and convenient for rapid identification and has potential for use in clinical microbiology laboratories.

Besides, one isolate was identified as *E. gallinarum* and two were

Table 3
PCR presence/absence assays of various antibiotic resistance genes for *Enterococcus* and antibiotic resistant phenotypes by automated systems

Isolates	Genes detected by PCR				Antibiotic resistance phenotype (MIC µg/mL)*			
	em(B)	*tet(L)*	*vanA*	*aac(6')-Ie-aph(2')-Ia*	ERY	TET	VAN	GEN
802	+	+	+	-	> 4R	> 8 R	≤ 2 S	≤ 500 S
817	-	-	+	+	≤ 0,5 S	> 8 R	8 I	≤ 500 S
840	-	-	+	-	>4 R	≤ 4 S	≤ 2 S	≤ 500 S
848	+	+	-	-	2	≤ 4 S	≤ 2 S	≤ 500 S
872	+	+	-	-	> 4 R	> 8 R	≤ 2 S	≤ 500 S
906	+	+	-	-	> 4	> 8 R	≤ 2 S	≤ 500 S
917	+	+	-	-	> 4	> 8 R	≤ 2 S	≤ 500 S
924	+	-	+	+	> 4	≤ 4 S	> 16 R	≤ 500 S
925	+	+	+	+	>4	≤ 4 S	> 16 R	≤ 500 S
928	+	-	+	+	--//--	> 8 R	≤ 2 S	--//--
973	+	-	+	+	> 4	> 8 R	≤ 2 S	--//--
1000	+	-	+	+	> 4	≤ 4 S	> 16 R	> 500 R
1035	+	-	+	+	> 4	> 8 R	> 16 R	> 500 R
1053	+	-	+	+	> 4	> 8 R	> 16 R	≤ 500 S
1062	+	-	+	+	> 4	≤ 4 S	> 16 R	> 500 R
1076	+	-	+	+	> 4	> 8 R	> 16 R	≤ 500 S
1097	-	-	+	+	> 4	> 8 R	> 16 R	≤ 500 S
1112	+	-	+	-	> 4	≤ 4 S	> 16 R	> 500R
1114	+	-	+	+	> 4	≤ 4 S	> 16 R	> 500R
1115	+	-	+	-	> 4	≤ 4 S	> 16 R	> 500 R
1125	+	+	+	+	> 4	≤ 4 S	> 16 R	> 500 R
1143	+	-	+	-	> 4	≤ 4 S	> 16 R	>500 R
1211	-	-	-	-	≤0,5	≤ 4 S	≤ 2 S	≤ 500 S
1215	+	-	+	+	> 4 R	> 8 R	> 16 R	≤ 500 S
1227	+	-	+	+	> 4	≤ 4 S	> 16 R	>500R
1231	+	-	+	+	> 4	> 8 R	> 16 R	≤ 500 S
1246	+	-	+	+	> 4	≤ 4 S	> 16 R	> 500 R
1280	+	-	+	+	> 4 R	≤ 4 S	> 16 R	> 500 R
1295	+	-	+	+	> 4 R	≤ 4 S	> 16 R	> 500 R
1298	+	-	+	+	> 4 R	> 8 R	> 16 R	≤ 500 S

MIC: minimal inhibitory concentration; ERY: erythromycin; TET: tetracycline; VAN: vancomycin; GEN: gentamicin (120 µg/mL); --//--: data not provided; S: sensible; R: resistance; I: intermediate resistance. (*) Result obtained from the automated method.

identified as *E. durans/hirae* by MicrosScan, whereas by PCR-based assay all three isolates were identified as *E. faecium*. Similar discrepancy was described by ROBREDO *et al.*[30], who compared the API20 STREP and colony hybridization for identification of enterococci obtained from several origins. According to these authors, high agreement was obtained for *E. faecalis* identification, however, for eight isolates identified as *E. durans* and *E. casseliflavus* by API20 STREP were identified as *E. faecium* according to the molecular method.

Several studies have found differences between automatic and classical or molecular bacterial identification systems. For instance, concerning Gram positive bacteria, no gram-positive cocci showed concordant identification between the direct and standard methods; other discrepancies consisted of misidentification between various species of coagulase-negative staphylococci[7].

On the other hand, some studies showed the agreement between automatic and classical or molecular bacterial identification systems[16,33]. D'AZEVEDO *et al.*[9] compared the automated Vitek system and standard methods for identification of 80 isolates belonging to different species of *Enterococcus*. The general agreement between results was 83.7%. Among isolates of *E. faecalis* and *E faecium* were observed that the automated system correctly identified 35/40 (87.5%) and 12/14 (85.7%) of the strains, respectively[9].

CEKIN *et al.*[4] demonstrated the consistency of automated systems with the conventional methods. They detected as 97.8% to identification of VRE strains using both methods.

Based on the results presented here and the previous report[39] there is a need for improvement in the automated MicrosScan system to identify enterococci.

In the present study, the genotypic basis of the resistance phenotype found in isolates of *E. faecium* and *E. faecalis* was investigated by PCR based detection of resistance genes. The majority of *Enterococcus* isolates displayed resistance to at least one antibiotic tested. Our results revealed that the *vanA* gene was predominant in *E. faecium* tests since this gene was detected in 100% of vancomycin-resistant isolates, although *Enterococcus* spp. may harbor other genes (*vanB, vanC*-1, *vanC*-2/3 and *vanD*) related to resistance[37].

Antibiotic resistance has played an essential role in the emergence of *E. faecalis* and *E. faecium* as nosocomial pathogens. Vancomycin is an important therapeutic option for the treatment of severe enterococcal infections and resistance to this type of antibiotic is concerning. Identified risk factors for vancomycin-resistant enterococci (VRE) acquisition include a prolonged hospital stay, exposure to intensive care units or residence on transplant oncology wards, prior exposure to antibiotics, and proximity to other patients infected or colonized with VRE[40].

In our study we detected the *tet(L)* gene in 23.3% (7/30) of the isolates, while four and five of these presented resistance to tetracycline in automated and disk diffusion method, respectively. Similar prevalence of *tet(L)* gene (21%) in enterococci was described by STOVCIK *et al.*[35]. In contrast, FRAZZON *et al.*[13] detected the *tet(L)* gene in only 9% of the *Enterococcus* sp isolates. Furthermore, tetracycline resistance phenotype was detected even in the absence of the *tet(L)* gene for 10 isolates. This may be explained by the fact that in enterococci two major groups of tetracycline resistance genes have been identified. One group encoding ribosomal protection proteins include *tet(M)*, *tet(O)* and *tet(S)* genes, and the another one that encodes tetracycline efflux pumps proteins include the *tet(L)* and *tet(K)* genes[18,22,28]. Similarly, erythromycin resistance was detected even in the absence of the *erm(B)*gene. This resistance may be due to the presence of *erm(A* and/or *erm(C)*) genes related to erythromycin resistance phenotype[37].

Gentamicin susceptible phenotype was detected in 36.7% of the isolates. However, 52.6% of these were detected as the *aac(6')-Ie-aph(2")-Ia* gene. Similar results were obtained by POULSEN *et al.*[29].

In our study, the MicrosScan system and disk diffusion method had an agreement of about 80%. GÜLMEZ & HASÇELIK[16] compared the Phoenix system and microdilution method and observed an excellent agreement for all of the antibiotics with category agreement rates of > 97%. In contrast, the API method was considered unreliable in detecting high levels of aminoglycoside resistance among *Enterococcus* strains compared to disc diffusion method[34].

Our data revealed high frequency of *E. faecium* and the occurrence of several multi resistance isolates. Antibiotic resistance appears to have contributed to increasing administration of inadequate antimicrobial therapy for infections, particularly enterococci nosocomial acquired infections, which is associated with greater hospital mortality rates[5,23].

Rapid and reliable identification of these antibiotic resistant organisms is crucial for patient management and infection control measures. Enterococci are intrinsically resistant to many antimicrobial agents, and their ability to acquire resistance to other agents such as aminoglycosides, β-lactams and glycopeptides (vancomycin and teicoplanin) is well known, resulting in invasive human enterococcal infections that are extremely difficult to treat.

The primary objective of the study was to determine whether molecular identification and direct antimicrobial susceptibility testing would provide results comparable to those obtained from an automated system in routine use. This study revealed that the PCR assay and disk diffusion method are in agreement with MicroScan automated system employed for identification and test susceptibility, respectively of clinical *Enterococcus* spp.

RESUMO

Comparação entre o sistema automatizado e PCR na identificação e susceptibilidade de isolados clínicos de *Enterococcus* spp

Os enterococos são cada vez mais responsáveis por infecções hospitalares em todo o mundo. Este estudo foi realizado para comparar a identificação e perfil de suscetibilidade entre o sistema automatizado MicrosScan e a técnica molecular de PCR em espécies de *Enterococcus* spp. Foram avaliados 30 isolados clínicos de *Enterococcus* spp. Os isolados foram identificados pelo sistema MicrosScan® e pela técnica de PCR. A detecção de genes de resistência a antibióticos (vancomicina, gentamicina, tetraciclina e eritromicina) foi determinada por PCR. Suscetibilidades antimicrobianas à vancomicina (30 μg), gentamicina (120 μg), tetraciclina (30 μg) e eritromicina (15 μg), foram testados

pelos métodos automatizados e pelo disco difusão, de acordo com as orientações do CLSI. No que diz respeito à identificação de *Enterococcus* em geral entre os dados obtidos pelo método de PCR e pelo sistema automático foi de 90,0% (27/30). Para todos os isolados de *E. faecium* e *E. faecalis* observamos concordância de 100%. Freqüências de resistência foi maior em *E. faecium* do que em *E. faecalis*. As taxas de resistência obtidas foi maior para eritromicina (86,7%), vancomicina (80,0%), tetraciclina (43,35%) e gentamicina (33,3%). A correlação entre a técnica de disco difusão e automação revelou-se de acordo para maioria dos antibióticos com taxas > 80%. O gene *van*(A) foi detectado em 100% dos *Enterococcus* resistentes á vancomicina. O ensaio baseado em PCR é de simples realização e de confiança para identificação de enterococos clinicamente relevantes. Os dados obtidos reforçam a necessidade de melhoria no sistema automatizado para identificar alguns enterococos.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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