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Original article

ENTEROCOCCUS AND ENTEROCOCCUS-LIKE ORGANISMS RECOVERED IN FAECAL SCREENING FOR VANCOMYCIN-RESISTANCE

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ABSTRACT

Background: Fecal screening of patients for vancomycin-resistant enterococci (VRE) is recommended in an attempt to establish infection control measures. Culture-based methods for VRE detection are widely used, but particular attention should be paid to correct identification of growing isolates. This study is focused on species identification and antimicrobial susceptibility of vancomycin-resistant enterococci and other Gram-positive catalase-negative cocci, recovered from VRE screen cultures.

Materials and methods: A total of 109 immunocompromised patients from University Multiprofile Hospital for Active Treatment "Dr G. Stranski", Pleven were screened for VRE. The determination of cocci was relied on cultural characteristics, manual and automated systems for identification as well as data on antimicrobial sensitivity.

Results: A total of 57 Gram-positive catalase-negative cocci were isolated: 32 VRE, 11 vancomycin-susceptible enterococci and 14 enterococcus-like organisms. Colonization with VanA or VanC enterococci was detected in 29.35% of the patients, with a distinct prevalence of VanC (23.85%) over than VanA (5.50%). Six enterococci were confirmed as *vanA* genotype – 5 *E. faecium* and 1 *E. gallinarum*. All *E. faecium* isolates expressed high-level resistance to vancomycin (MICs ≥256 μg/ml) and low-level resistance to teicoplanin (MICs: 4.0-6.0 μg/ml), whereas a single *E. gallinarum* isolate showed MICs ≥256 μg/ml for both glycopeptides. The isolated VanC enterococci (13 *E. gallinarum* and 13 *E. casseliflavus*) were susceptible to tested antibiotics and possessed low-level resistance to vancomycin (MICs: 4-12 μg/ml). Most of the recovered enterococcus-like organisms were identified as *Leuconostoc* spp.

In conclusion, both species identification and antimicrobial susceptibility pattern have to be taken into account for distinguishing VRE and other Gram-positive catalase-negative cocci, growing in VRE screen cultures.

Keywords: VRE, enterococcus-like organisms, fecal screening,

INTRODUCTION

Members of genus *Enterococcus* are well known pathogens, and their ecological niche is the intestinal tract of humans and animals. The treatment of enterococci is problematic because of their intrinsic, low-level resistance to â-lactams and aminoglycosides, as well as acquired resistance to penicillin/ampicilin, ciprofloxacin and high concentration of aminoglycosides [1].

The appearance and emergence of vancomycin-resistant enterococci (VRE) in the late 1980s additionally restricts the therapeutic options. Nowadays, nine phenotypes of glycopeptide resistance in enterococci have been described [2]. Eight of these are the result of acquired resistance (VanA, VanB, VanD, VanE, VanG, VanL, VanM, VanN) and one type (VanC) is a natural resistant.

It has been found that the fecal colonization with VRE plays an important role in spreading of these microorganisms in the hospitals and routine screening cultures are recommended in an attempt to establish infection control measures [3]. Culture-based methods for VRE detection in fecal samples are widely used in microbiology laboratories, but particular attention should be paid to correct identification of growing isolates, especially to those which produce colonies similar to enterococci.

Many studies worldwide reveal different aspects of determination of VRE isolated in fecal screening of hospitalized patients [3, 4, 5, 6, 7], but the data for Bulgaria are still scarce. These are dated from 2012 when V. Popova et al. [8] studied VRE carriers among dialysis patients. Little is known in our country about the colonization with VRE, predominant glycopeptide resistance types and sensitivity to antimicrobials. This motivates our team to screen hospitalized patients for VRE with attention focused on species identification and antimicrobial susceptibility of enterococcal isolates. Gram-positive catalase-negative nonenterococcal strains were also analyzed.

MATERIALS AND METHODS

Patients and specimens

A total of 109 patients from University Multiprofile Hospital for Active Treatment „Georgi Stranski", Pleven were screened for VRE in the period November 2017 – June 2018. The majority of them were in the Haemodialysis ward (HD) – 97, and 12 were in the Haematology ward (HT). Stools or rectal swabs specimens were collected from the patients.

Selective media for VRE screening

Collected specimens were cultured onto three chro-

mogenic media – Briliiance VRE agar (*Oxoid, UK*), chromID VRE agar (*bioMerieux, France*), HiCrome VRE agar (*HI MEDIA, India*), and also into a bile esculin azide broth with vancomycin – BEAV (*Liofilchelm, Italy*). The antibiotic supplement in the chromogenic media was as defined by the manufacturer (8 μg Vancomycin), whereas BEAV broth was supplemented with 6 μg/ml Vancomycin. Cultures were incubated at 37°C and were examined after 24 and 48 h. BEAV broths with black colour were subcultured onto 5% blood agar plates (BAP) and CPS agar (*bioMerieux, France*).

Preliminary identification

Colonies typical for enterococci and other similar colonies were examined by Gram stain and catalase test. All Gram-positive catalase-negative cocci were tested for pyrrolidonyl arylamidase (PYR), lucine amino peptidase (LAP), the presence of streptococcal D antigen, growth in 6,5% NaCl and bile esculin agar. For presumptive differentiation of enterococci to group level, additional tests were used – production of acid from manitol and sorbose, fermentation of methyl-alpha-d-glucopyranoside (MGP), arginine dihydrolase (ADH), motility and pigmentation.

Species identification

The identification up to species level was performed by RapID STREP Panel (*Oxoid, UK*) and VITEK 2 compact system (*bioMerieux, France*) with version 7.01 software using Gram-positive test panel (GP-67 cards). Species identification of VanA and misidentified VanC enterococci were determined by VITEK MS (*bioMerieux, France*).

Antimicrobial susceptibility testing

The antimicrobial susceptibility was detected by the disk-diffusion method of Bauer-Kirby with standard antibiotic disks (*BD, UK*) and by VITEK 2 compact system (*bioMerieux, France*) with AST P-534 cards. The antimicrobial agents tested were ampicillin (AP), gentamicin (GM), norfloxacin (NOR), ciprofloxacin (CIP), vancomycin (VA), teicoplanin (TEC), daptomycin (DAP), linezolid (LZD) and tigecyclin (TGC). Minimal inhibitory concentrations (MICs) were examined by E-test (*Liofilchem*, *Italy*). The results were interpreted according to the recommendations of the performance standard of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2018. The *vanA* gene status was confirmed by PCR using C1000 Touch™ Thermal Cycler (*BioRad*).

RESULTS

Overall 57 Gram-positive catalase-negative cocci were isolated in fecal screening of patients for VRE. The distribution of cocci according to the species identification and glycopeptide resistance is presented in Table 1. The data reveal a circulation of VRE with VanA (n=6) and VanC (n=26) phenotypes. Vancomycin-susceptible enterococci (VSE) and nonenterococci were also detected.

Table 1. Species distribution of 57 Gram-positive catalase-negative cocci, isolated in fecal screening for VRE

VanA enterococci		**VanC enterococci**		**VSE**		**Other Gram-positive catalase-negative cocci**	
Species	Nr of isolates	Species	Nr of isolates	Species	Nr of isolates	Species	Nr of isolates
E. faecium	**5**	***E. casseliflavus***	**13**	***E. faecium***	**6**	***Leuconostoc* spp.***	**8**
E. gallinarum	**1**	***E. gallinarum***	**13**	***E. faecalis***	**4**	***Pediococcus pentosaceus***	**1**
				E. hirae	**1**	***Lactococcus garvieae***	**2**
						Sreptococcus* spp.*	**3**
Totally	**6**	**Totally**	**26**	**Totally**	**11**	**Totally**	**14**

* *Leuconostoc* spp.: *Leuconostoc psudomesenteroides* (4), *Leuconostoc mesenteroides* (3), *Leuconostoc citreum* (1)
***Sreptococcus* spp.: *Sreptococcus sanguinis* (1), *Sreptococcus mutans* (1), *Sreptococcus gallolyticus ssp. pasteurianus* (1)

Generally, colonization with VanA or VanC enterococci was determined in 32 from 109 patients (29.35%), as VanC (23.85%) being more prevalent than VanA (5.50%). Co-colonization with different VRE was not observed, but combinations of vancomycin-susceptible *E. faecium* and vancomycin-resistant *E. faecium, E. casseliflavus* or *E. gallinarum* were present in three patients.

Detection of VRE with VanA phenotype

Six VanA enterococci were recovered: 5 *E. faecium* and 1 *E. gallinarum*. All vancomycin-resistant *E. faecium* (VR *E. faecium*) yielded α -haemolytic colonies on BAP, violet colonies after subcultivation onto CPS agar and were correctly identified by VITEK 2 compact. The single *E. gallinarum* isolate was α-haemolytic on BAP and also formed violet colonies on CPS agar. It was identified by RapID STREP Panel but misidentified by VITEK 2 compact. The species identification of Van A isolates was confirmed by VITEK MS, and the *vanA* gene was detected by PCR.

The results of antimicrobial susceptibility testing of VanA enterococci are shown in Table 2. All *E. faecium* isolates expressed the same antibiotic pattern: high-level resistance to ampicillin, gentamicin, and susceptibility to linezolid, daptomycin, tigecycline. The data concerning susceptibility to glycopeptides reveal high-level resistance of *E. faecium* isolates to vancomycin (MIC ≥256 μg/ml) and low-level resistance to teicoplanin (MIC: 4.0-6.0 μg/ml), whereas *E. gallinarum* isolates showed high-level resistance to both glycopeptides with MICs ≥256 μg/ml.

Table 2. Antimicrobial susceptibility patterns of enterococci with VanA phenotype

ISOLATE No	Species	MICs (μg/ml)							
		AP	GM	CIP	VA	TEC	LNZ	DAP	TGC
HD 11/17	*E. faecium*	≥256	≥1024	≥32	≥256	6	1.5	1	0.064
HD 16/17	*E. faecium*	≥256	≥1024	≥32	≥256	6	2	1	0.064
HD 36/17	*E. faecium*	≥256	≥1024	≥32	≥256	6	2	1	0.064
HD 47/17	*E. faecium*	≥256	≥1024	≥32	≥256	4	2	1	0.064
HD 75/17	*E. faecium*	≥256	≥1024	≥32	≥256	4	1.5	1	0.064
HD 31/17	*E. gallinarum*	≥256	≥1024	≥32	≥256	≥256	2	2	0.064

Detection of VRE with VanC phenotype

A total of 26 VanC enterococci were isolated: 13 *E. gallinarum* and 13 *E. casseliflavus*. All these were α-haemolytic on BAP and were grown as green colonies on CPS agar.

The data about the species identification and antimicrobial susceptibility of VanC enterococci are listed in Table 3 and Table 4. Among 26 isolates, 17 (65.4%) were correctly determined in terms of species level by RapID STREP Panel and VITEK 2 compact. The remaining 9 strains (34.6%) were with unconformities in the identification, and VITEK 2 compact identified most of them as *E. gallinarum/E. casseliflavus*. Misidentified isolates were motile, so that production of yellow pigment distinguished *E. casseliflavus* from *E. gallinarum* and the definitive species were confirmed by VITEK MS.

Table 3. Species identificatian and antimicrobial susceptibility of *E. gallinarum* isolates with VanC phenotype

ISOLATE No	Identification systems			Diameters of growth inhibition zones (mm)					
	RapID STREP	VITEK 2 compact	VITEK MS	AP 2	GM 30	NOR 10	VA 5	TEC 30	LZD 10
HD 1/17	*E. casseliflavus*	*E. gallinarum*	*E. gallinarum*	16	15	13	8	19	20
HD 5/17	*E. gallinarum*	*E. gallinarum*	—	19	16	11	9	18	24
HD 8/17	*E. casseliflavus*	*E. gallinarum/ E. casseliflavus*	*E. gallinarum*	18	15	12	9	18	24
HD 17/17	*E. gallinarum*	*E. gallinarum*	—	18	14	6	8	18	25
HD 45/17	*E. gallinarum*	*E. gallinarum*	—	16	14	12	9	19	23
HD 59/17	*E. gallinarum*	*E. gallinarum*	—	16	20	18	9	19	21
HD 60/17	*E. gallinarum*	*E. gallinarum*	—	17	14	11	9	18	24
HD 69/17	*E. casseliflavus*	*E. gallinarum/ E. casseliflavus*	*E. gallinarum*	18	16	13	9	18	24
HD 72/17	*E. gallinarum*	*E. gallinarum*	—	18	15	12	9	19	23
HD 76/17	*E. gallinarum*	*E. gallinarum*	—	17	15	12	9	19	23
HD 81/17	*E. gallinarum*	*E. gallinarum*	—	20	18	14	9	19	22
HD 87/17	*E. casseliflavus*	*E. gallinarum/ E. casseliflavus*	*E. gallinarum*	17	16	13	9	19	23
HD 91/17	*E. gallinarum*	*E. gallinarum*	—	17	15	11	9	18	21

Table 4. Species identificatian and antimicrobial susceptibility of *E. casseliflavus* isolates with VanC phenotype

ISOLATE No	IDENTIFICATION SYSTEMS			Diameters of growth inhibition zones (mm)					
	RapID STREP	VITEK 2 compact	VITEK MS	AP 2	GM 30	NOR 10	VA 5	TEC 30	LZD 10
HD 6/17	*E. casseliflavus*	*E. gallinarum/ E. casseliflavus*	*E. casseliflavus*	20	16	15	10	18	24
HD 33/17	*E. gallinarum*	*E. gallinarum/ E. casseliflavus*	*E. casseliflavus*	19	15	14	10	16	21
HD 40/17	*E. casseliflavus*	*E. casseliflavus*	—	18	17	15	9	16	21
HD 41/17	*E. casseliflavus*	*E. casseliflavus*	—	20	17	16	10	19	24
HD 70/17	*E. gallinarum*	*E. gallinarum/ E. casseliflavus*	*E. casseliflavus*	20	17	16	10	19	24
HD 84/17	*E. casseliflavus*	*E. casseliflavus*	—	20	16	16	10	19	23
HD 93/17	*E. gallinarum*	*E. casseliflavus*	*E. casseliflavus*	20	16	16	11	19	24
HD 96/17	*E. casseliflavus*	*E. casseliflavus*	—	16	16	15	10	18	20
HD 99/17	*E. casseliflavus*	*E. casseliflavus*	—	20	19	18	11	19	25
HT 4/18	*E. gallinarum*	*E. gallinarum/ E. casseliflavus*	*E. casseliflavus*	20	19	16	11	19	25
HT 6/18	*E. casseliflavus*	*E. casseliflavus*	—	22	19	17	11	19	20
HT 10/18	*E. casseliflavus*	*E. casseliflavus*	—	22	18	20	11	20	25
HD 27/18	*E. casseliflavus*	*E. casseliflavus*	—	20	16	14	11	16	20

The results of disk-diffusion tests reveal that VanC enterococci were susceptible to ampicillin, gentamicin, teicoplanin, linezolid, and possess reduced susceptibility to vancomycin with a zone of growth inhibition 8-9 mm and 9-11 mm for *E. gallinarum* and *E. casseliflavus*, respectively. For both species, vancomycin MICs varied from 4 to 12 μg/ml, whereas teicoplanin MICs were between 0.5-0.75 μg/ml, which corresponds to VanC phenotype.

Detection of vancomycin susceptible enterococci

Most of isolated VSE belong to the species *E. faecium* and *E. faecalis* (see Table 1). Two enterococcal species, *E. faecalis* and *E. hirae,* were recovered from one patient. VSE appeared as α- or non-haemolytic on BAP and produced typical turquoise colonies on CPS agar. These cocci were tentatively identified by RapID STREP Panel and/or VITEK 2 compact. Two *E. faecalis* isolates were determined by both systems as *E. gallinarum* (HT 1/18) and *E. casseliflavus* (HD 94/17), but they were nonmotile, nonpigmented, MGP-negative and susceptible to vancomycin. Further testing by VITEK MS confirmed *E. faecalis* species. Some variations in susceptibility to antimicrobials were observed among VSE isolates, but all were susceptible to glycopeptides (data not shown).

Detection of Gram-positive catalase-negative cocci other then enterococci

A total of 14 enterococcus-like organisms were detected in the fecal screening for VRE (see Table 1). Most of them (66.28%) belong to genera Leuconostoc (n=8) and Pediococcus (n=1), which are naturally resistant to glycopeptides. Isolates of Lactococcus (n=2) and Sreptococcus (n=3) were also present.

Nonenterococcal species produced colonies similar to those of enterococci, but much smaller, α- or non-haemolytic on BAP and variably coloured on CPS agar – green, pink or colourless. All these were Gram-positive cocci, nonmotile, catalase and PYR-negative, with the exception of both strains *Lactococcus garvieae* (previously named as *Enrerococcus seriolicide*), which were PYR-positive. Most of these cocci didn't produce acid from manitol and sorbose, and expressed variable results regarding LAP, ADH, bile-esculin test, growth in 6,5% NaCl and streptococcal D antigen. The definitive identification of non-enterococci, based on VITEK 2 compact system, was as follows*: Lactococcus garvieae* – 2, *Leuconostoc pseudomesenteroides* – 4, *Leuconostoc mesenteroides* – 3 and single strains of *Leuconostoc citreum*, *Pediococcus pentosaceus*, *Streptococcus sanguinis*, *Streptococcus mutans*, *Streptococcus gallolyticus ssp pasteurianus*. Susceptibility to almost all tested antimicrobials was found in lactococci and streptococci, whereas members of Leuconostoc and Pediococcus were susceptible to ampicillin, gentamicin and linezolid, but resistant to both glycopeptides without a zone of growth inhibition by the disk diffusion method. The values of MIC confirmed high-level resistance to vancomycin (MIC ≥256 μg/ml) and teicoplanin (MIC: 128-256 μg/ml).

DISCUSSION

This study provides information about species identification and antimicrobial susceptibility of intestinal VRE isolated from immunocompromised patients. The data reveal

that culture-based screening for VRE can detect VSE and nonenterococci, too.

In summary, from a total of 57 recovered Gram-positive catalase-negative cocci, 32 were identified as VRE, 11 as VSE and 14 as nonenterococci. Our data, like proportion, are comparable to those of D.F.J. Brown and E. Walpole [4], who reported 128 VRE, 36 VSE and organisms other than enterococci in fecal screening for VRE. According to Gordts et al. [5], out of 135 *enterococcus*-like organisms, 113 (83.8%) were identified as *Leuconostoc* spp. or vancomycin- and teicoplanin-susceptible enterococci. Other trials have also revealed detection of non-VRE in culturing of specimens onto vancomycin selective agars and broths [6, 7].

All studied cocci were recovered from BEAV broth, but 4 VR *E. faecium*, 1 *E. gallinarum* and 1 *E. casseliflavus* isolates were directly growing onto solid chromogenic media. The presumptive identification of VRE culturing on chromogenic media was based on typical coloured colonies as per manufacturer's instructions. In contrast, the recognition of enterococci growing after subcultivation from BEAV broth onto BAP and CPS agar required many supplemental tests as recommended in diagnostic manuals [9, 10]. Gram-positive catalase-negative cocci, which were PYR, LAP and bile esculin positive, possessed streptococcal D antigen and were grown in 6,5% NaCl were determined presumptively as enterococci. Further identification to group level was based on MGP-test, hydrolysis of arginine, production of acid from manitol and sorbose, motility and pigment, but these tests could not fully differentiate the species.

The definitive identification of isolated enterococci was relied on manual and automated systems, but both systems provided some incorrect results. Furthermore, VITEK 2 compact produced results with low discrimination as *E. gallinarum/E. casseliflavus* in about 1/4 of VanC enterococci, but additional tests such as motility and pigment production solved the discrepancies. It is known that the VITEK 2 database cannot reliably differentiate *E. gallinarum* from *E. casseliflavus* and presents most of them as *E. gallinarum/ E. casseliflavus* [11]. According to the publication of M. Abele-Horn et al. [12], VITEK 2 with version 4.01 software had correctly identified 114 of 121 (94.2%) strains to the species level as *E. faecium*, *E. gallinarum* or *E. casseliflavus*. The VITEK 2 system was used for the identification of VRE in routine microbiology work, but this system is less efficient in detecting non-faecalis and non-faecium species [13]. Because of that, isolates with unconformities in the identification were tested with MALDI-TOF (Matrix-assisted Laser Desorption Ionization-Time-of-Flight) technology using VITEK MS (*bioMérieux, France*), which is a highly accurate metod for identification of enterococci [10].

In the present study, cocci other then enterococci were identified to the genus level on the basis of conventional tests (e.g. PYR, LAP, bile-esculin test, grown in 6,5% NaCl, streptococcal D antigen), resistance/susceptibility to vancomycin, and furthermore to the species level by VITEK 2 compact. The commercial diagnostic systems can misidentify unusual Gram-positive catalase-negative cocci such as *Aerococcus*, *Leuconostoc*, etc., mostly at the species level [14, 15]. Conventional phenotypic tests with assistance to genotyping assays (PCR, 16S rDNA sequence analysis, etc.) have been recommended for these purposes [16]. Nowadays, MALDI-TOF MS technology is used successfully for the identification of this group of bacteria [14].

The determination of enterococcal species can somewhat point to the antimicrobial sensitivity, but full susceptibility testing was performed to acquire such data and to determine resistance phenotype. On the other hand, tests for antimicrobial sensitivity can assist in identification [17]. For example, a specific antimicrobial pattern was observed in our *Leuconostoc* spp. – susceptibility to almost all tested antibiotics and high-level resistance to vancomycin and teicoplanin with MICs similar to those already published [18]. Also, overall 26 *E. gallinarum* and *E. casseliflavus* strains were highly sensitive to many antibiotics with the exception of vancomycin – the zone of growth inhibition varied between 8-11 mm and MICs values confirmed low-level vancomycin resistance. These data are related to VanC species. The single unusual *E. gallinarum* strain with high-level resistance to vancomycin and teicoplanin was confirmed as *vanA* genotype.

Interestingly, VR *E. faecium* isolates possessed the same growth characteristics (violet colonies on CPS agar) and multidrug resistant pattern, expressing high-level resistance to vancomycin and low-level resistance to teicoplanin. These results do not fully match to VanA phenotype, which is characterized with high-level resistance to both glycopeptides, but *vanA* gene was detected. Furthermore, identical phenotypic features, concerning colonial morphology and antimicrobial testing, draw attention to the search of genetic relatedness between the isolates. VR *E. faecium* isolates with these specific characteristics have been circulating in our hospital since 2013 [19]. The molecular analysis of our previously recovered VR *E. faecium* (n=60) has determined *vanA* gene and confirmed circulation of a single dominant genotype (95% of isolates) grouped into ST203 (CC17) [20].

Our study has some limitations, concerning the absence of precise genetic techniques for characterization of glycopeptide resistance mechanisms, clonal complexes and allelic profile of strains. Further investigations will be performed in this direction.

In conclusion, both species identification and antimicrobial susceptibility pattern have to be taken into account for distinguishing VRE, but advanced technologies (e.g. MALDI-TOF, nucleic acid sequencing, etc.) are necessarily for definitive identifications at species level and determination of antimicrobial resistance mechanisms.

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