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THE EFFECT OF LINEZOLID AGAINST VANCOMYCIN-RESISTANT ENTEROCOCCI BY VARIOUS METHODS

LİNEZOLİDİN VANKOMİSİNE DİRENÇLİ ENTEROKOK SUŞLARINA ETKİSİNİN ÇEŞİTLİ YÖNTEMLERLE ARAŞTIRILMASI

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ABSTRACT

Objectives: Enterococci are the causative agents of a variety of infections, particularly healthcare-associated infections. Linezolid is an important antibiotic in the treatment of infections caused by vancomycin-resistant enterococci. However, in recent years, an increasing rate of linezolid resistance has been reported in clinical enterococci strains. The aim of this study was to investigate the in vitro efficacy of linezolid against vancomycin-resistant *Enterococcus* (VRE) strains isolated from rectal swab samples of inpatients by disc diffusion, microdilution and E-test methods, and thus to evaluate the efficiency of linezolid against VRE strains by qualitative and quantitative methods.

Material and Methods: Fifty VRE strains were defined as enterococci by conventional methods. The efficiency of linezolid in strains was investigated by disk diffusion, E-test and microdilution methods. Species identification of enterococci strains was done with the GP24 Diagnostics kit.

Results: The identification of fifty enterococcal strains using the conventional methods revealed Gram-positive coccus, catalase-negative, bile growth-esculin hydrolysis positive, salt tolerance test (6.5% NaCl) positive, and L-pyrrolidonyl-β-naphthylamide (PYR) test positive. All strains were found to be susceptible to linezolid in disc diffusion, E-test and microdilution tests. In the microdilution test study, the MIC distrubution, MIC_{50} and MIC_{90} was detected as 1-2, 2 and 2 µg/mL, respectively. The MIC distribution, MIC_{50} and MIC_{90} values of linezolid by E-test were determined as 1-4, 2 and 3 µg/mL, respectively. In the study, 48 (96%) of 50 strains were identified as *Enterococcus casseliflavus* and 2 (4%) were *Enterococcus faecium*.

Conclusion: No linezolid resistance was detected in the study. This suggests that linezolid can be used safely in the treatment of VRE-induced infections. It will be important to conduct continuous and comprehensive studies on this subject and to monitor linezolid resistance surveillance.

Keywords: Vancomycin-resistant enterococci, linezolid, disc diffusion, microdilution, E-test

ÖZ

Amaç: Enterokoklar, özellikle sağlık hizmeti ile ilişkili enfeksiyonlar olmak üzere, çeşitli enfeksiyonların etkenidirler. Linezolid, vankomisine dirençli enterokokların sebep olduğu enfeksiyonların tedavisinde önemli bir antibiyotiktir. Fakat son yıllarda klinik enterokok suşlarında giderek artan oranlarda linezolid direnci rapor edilmektedir. Çalışmada, yatan hastaların rektal sürüntü örneklerinden izole edilen vankomisine dirençli enterokok (VRE) suşlarında linezolidin in vitro etkinliğinin disk difüzyon, E-test ve mikrodilüsyon yöntemleri ile araştırılması ve böylece linezolidin VRE suşlarına etkinliğinin kalitatif ve kantitatif yöntemler ile değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntemler: Elli VRE suşu konvansiyonel yöntemler ile enterokok cinsi olarak tanımlanmıştır. Suşlarda, linezolidin etkinliği disk difüzyon, E-test ve mikrodilüsyon metodları ile araştırılmıştır. Enterokok suşlarının tür tanımlaması GP24 Diagnostics kiti ile yapılmıştır.

Bulgular: Elli enterokok suşunun konvansiyonel yöntemlerle tanımlanmasında Gram-pozitif kok, katalaz negatif, safrada üreme-eskülin hidrolizi pozitif, tuz tolerans testi (%6,5 NaCl) pozitif ve L-pirolidonil-β-naftilamid (PYR) testi pozitif olarak belirlenmiştir. Disk difüzyon, E-test ve mikrodilüsyon testleri ile suşların tümü, linezolide duyarlı bulunmuştur. Mikrodilüsyon test çalışmasında linezolid MİK dağılımı, $MİK_{50}$ ve $MİK_{90}$ değerleri sırasıyla 1-2, 2 ve 2 µg/mL olarak belirlenmiştir. Linezolidin E-test yöntemiyle yapılan MİK araştırması sonucunda MİK dağılımı, $MİK_{50}$ ve $MİK_{90}$ değerleri sırasıyla 1-4, 2 ve 3 µg/mL olarak saptanmıştır. Çalışmada 50 suştan 48'i (%96) *Enterococcus casseliflavus* ve 2 suş (%4) *Enterococcus faecium* olarak tanımlanmıştır.

Sonuç: Çalışmada linezolid direnci saptanmamıştır. Bu da linezolidin VRE kaynaklı enfeksiyonların tedavisinde güvenle kullanılabileceği fikrini vermektedir. Bu konuda devamlı ve kapsamlı çalışmaların yapılması ve linezolid direnç sürveyansının izlenmesi önemli olacaktır.

Anahtar Kelimeler: Vankomisine dirençli enterokoklar, linezolid, disk difüzyon, mikrodilüsyon, E-test

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INTRODUCTION

Enterococci are important bacteria that live commensally in the bowel of humans and many other animals, including invertebrates. They are the first bacteria to colonize newborns and constitute an important part of the healthy adult gut microbiota. The most frequently isolated and clinically important species are *Enterococcus faecalis* and *Enterococcus faecium. Enterococcus casseliflavus* and *Enterococcus gallinarum* are prevalent in the human gut flora and are intrinsically resistant to vancomycin (1).

Although *Enterococci* do not have as wide a range of virulence factors as *Staphylococci* or *Streptococci*, they are important bacteria because they cause life-threatening diseases with antibiotic-resistant strains. There are two general features of virulence. One is the ability to form biofilms by adhering to tissues and the other is the ease with which antibiotic resistance can be developed. Clonal strains adapted to hospital conditions also have superior patient-to-patient transmission abilities (2).

Enterococci are one of the most common types of nosocomial infections. They frequently cause urinary tract infections, and this is usually associated with urinary catheterization or instrumentation. They also cause endocarditis and bacteremia. Pelvic, biliary, intra-abdominal, and wound infections are common. Meningitis may be caused by these bacteria, but only rarely (3).

The first vancomycin-resistant *Enterococcus* (VRE) strain was identified in 1988 in England (4). Since then, it has increasingly spread all over the world. The first antibiotic confirmed for the treatment of VRE infection was quinupristin/dalfopristin. Its use has been largely abandoned due to its effectiveness only against the *E. faecium* strain and its frequent side effects. Linezolid has the advantage that it penetrates well into various tissues (including CFS-Cerebrospinal fluid) and is available in oral form. It is mainly used in the treatment of infections caused by multidrug-resistant bacteria such as VRE, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae* (5). The first linezolid-resistant clinical isolate carrying the *cfr* gene was reported in 2005. It has been reported that the cause of linezolid resistance is related to the overuse of the drug (6).

The goal of this study was to research the efficacy of linezolid on vancomycin-resistant strains of the *Enterococcus* genus isolated from rectal swab samples of patients hospitalized in various clinics of our hospital by disc diffusion, E-test and microdilution methods.

MATERIAL and METHODS

This study was approved by the Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 23.09.2022, No: 17).

In the study, 50 different VRE strains isolated from rectal swab samples taken periodically from patients hospitalized in various wards of Istanbul University's Medical Faculty Hospital between 2022 and 2023 were used. All samples were cultured on Bile-Esculin Agar (BEA) (BD, BBL TM Bile Esculin Agar, USA) supplemented with vancomycin and incubated at 35°C for 24 hours. Cultures hydrolyzing esculin were identified at the *Enterococcus* genus level by conventional methods. Afterwords, the strains were stored in brain-heart infusion broth (Becton Dickonson, USA) storage medium with 20% glycerol and kept at -20°C until the study time (7).

During the study, each strain was seeded on Tryptic Soy Agar (TSA) (Oxoid, United Kingdom) and incubated at 35°C for 18-24 hours. The obtained pure cultures were investigated for growth in bile using the hydrolysis of esculin test, Gram stain, the catalase test, the salt tolerance test, and the L-pyrrolidonyl-β-naphthylamide (PYR) (PYR-Oxoid Biochemical Identification System) test for confirmation of the *Enterococcus* genus. The presence of growth and darkening in the bile-esculin agar medium, the presence of Gram-positive cocci morphology in the microscope examination, the negative catalase test, the in the salt tolerance medium turning from purple to yellow and the PYR test positive strains all indicated the presence of enterococci (7). A commercially available GP24 kit (Diagnostics, Slovak Republic) was used to identify strains at the species level. Identification of the strains was investigated in line with the manufacturer's recommendations and the results were evaluated with the IDmicro software program given. *Enterococcus faecalis* ATCC 29212 standard strain as quality control was studied with the GP24 kit and gave the results of *Enterococcus faecalis* with 100% accuracy in the IDmicro Software program. In addition, the hemolysis, the presence of β-lactamase enzyme, and the existence of high-level aminoglycoside resistance (HLAR) features of the strains were investigated (8).

Disc diffusion test was applied on all strains with vancomycin (30 µg) (Bioanalyse, Ankara, Turkey), teicoplanin (30 µg) (Bioanalyse, Ankara, Turkey) and linezolid (30 µg) (Bioanalyse, Ankara, Turkey) antibiotic discs. In addition, Minimum Inhibition Concentration (MIC) values were investigated for linezolid by both E-test (Bioanalyse, Turkey) and microdilution (Linezolid, Biosynth Carbosynth, United Kingdom) methods (8, 9). The studies were designed in line with the recommendations of the CLSI standard and the results were evaluated as to the same standard criteria. *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Staphylococcus aureus* ATCC 29213 standard strains were used as quality control strains in the studies (8, 10).

RESULTS

The distribution of patients by clinical units and gender is shown in Figure 1. Accordingly, 22 patients (44%) were in pediatrics, 20 (40%) in internal disease, 4 (8%) in anesthesia reanimation, 3 (6%) in general surgery, and 1 (2%) in neurology intensive care unit, respectively. The gender of the patients was determined as 27 (54%) male and 23 (46%) female. The number of patients by age was as follows: 22 (44%) aged 0-10, 1 (2%) aged 11-30, 6 (12%) aged 31-50, 15 (30%) aged 51-70 and 6 (12%) aged 71-90.

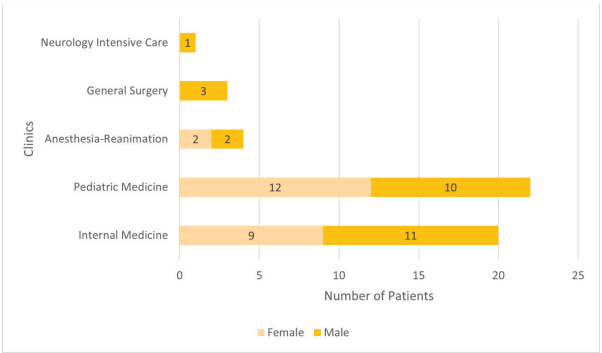

Figure 1: Distribution of 50 VRE strains by gender and clinical units from which they are isolated

In the identification of strains as *Enterococcus* genus by conventional methods, Gram stain, catalase, esculin hydrolysis, growth in bile, growth in 6.5% NaCl and PYR tests were applied. Accordingly, all the strains were catalase-negative, Gram-positive cocci in microscope image, esculin hydrolysis positive, bile growth positive and PYR test positive. All strains were defined as enterococci. The presence of β-laktamase enzyme was also investigated in all strains and it was not detected in any of the strains. All strains were determined to be resistant to vancomycin and teicoplanin (100%) by disc diffusion test. All strains were determined to be sensitive (100%) to linezolid by the same method.

High-level aminoglycoside resistance screening was also performed in strains. While 33 (67%) of 50 VRE strains were found to be HLAR positive, a high level of gentamycin resistance (HLGR) was found in 16 (32%) strains and a high level of streptomycin resistance (HLSR) was found in 1 (2%) strain.

The distribution of linezolid MIC values in the microdilution test study is shown in Figure 2. Accordingly, all strains were found to be susceptible (100%) to linezolid. The MIC distribution, MIC_{50} and MIC_{90} values of linezolid were determined as 1-2, 2 and 2 µg/mL, respectively.

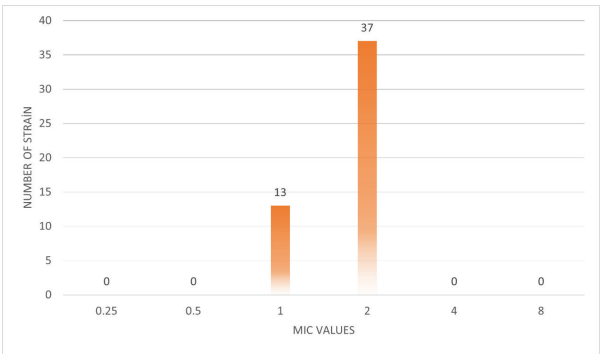

Figure 2: Distribution of linezolid MIC values obtained as a result of microdilution test

As a result of the investigation, the efficacy of linezolid against 50 VRE strains by E-test, the distribution of MIC values, MIC_{50} and MIC_{90} values were determined as 1-4, 2 and 3 µg/mL, respectively. All strains were found susceptible by this method. In addition, the E-test method was used to identify the effectiveness of vancomycin quantitatively, and the distribution of MIC values was determined as MIC distribution: 128 - > 256 µg/mL and $MIC_{50,90}$: > 256, 256 µg/mL, respectively.

Through the identification of *Enterococcus* strains using the GP24 Diagnostics kit, 48 (96%) of 50 VRE strains were determined as *Enterococcus casseliflavus* and 2 (4%) strains as *Enterococcus faecium.* In the study, *Enterococcus faecalis* ATCC 29212 strain was used as quality control strain and identified with 100% accuracy by the diagnostic kit.

DISCUSSION

Healthcare-associated infections are infections that patients encounter while receiving treatment and care for medical or surgical conditions (11). The primary source of healthcare-associated infections is through the contaminated hands of healthcare workers (12). There is growing concern that vancomycin-resistant enterococci are becoming increasingly resistant to the antibiotics used to treat VRE infections and that these antibiotics may be less effective (13).

Because the HLAR does not allow for synergistic treatments, strains with HLAR must be treated with alternative combinations of antibiotics (14). Schouten MA et al. investigated gentamicin resistance of 50 *E. gallinarum* and 21 *E. casseliflavus* strains and determined that 18% of *E. gallinarum* and 18.2% of *E. casseliflavus/flavescens* strains were highly resistant to gentamicin (15). In our study, all strains were screened for HLAR. It was determined in 31 of 48 *(E. casseliflavus)* strains. A high level of gentamicin resistance (HLGR) was determined in 16 strains, and a high level of streptomycin resistance (HLSR) in 1 strain. Additionally, 2 *E. faecium* strains were determined as HLAR.

In a study carried out at Marmara University Pendik Training and Research Hospital in Istanbul in 2021, rectal swab samples collected from patients hospitalized in all units were evaluated by performing VRE scanning. As a result of the study, in which 1710 samples were taken from 771 patients, VRE was detected in 8.1% (137/1710) of all samples. The highest positivity rate was found in intensive care patients (16).

Olearo et al. conducted a study in Germany and reported that the incidence of linezolid and vancomycin-resistant *Enterococcus faecium* (LVRE) is associated with antibiotic consumption. The researchers reported that the use of linezolid could be limited so that it may remain as a treatment alternative in VRE infections (17). According to another study conducted in Germany, the increasing prevalence of linezolid resistance among VRE strains was reported to be less than 1% in 2008, while it was reported to be greater than 9% in 2014 (18).

In a study conducted in Turkey in 2004, linezolid MIC values of 55 VRE strains were investigated using the E-test method. As a result of the study, MIC values of 55 strains were determined

in the range of 0.38-2.0 µg/mL (19). In another study, Aktaş G. et al. investigated linezolid MIC values by microdilution method of 100 VRE strains isolated from rectal swab samples of cases between 2006 and 2007. As a result of the study, linezolid MIC distribution and $MIC_{50,90}$ values were identified as 1-16 µg/mL, 4 µg/mL and 4 µg/mL, respectively, and 2 VRE strains were determined to be resistant to linezolid. MIC values of resistant strains were also investigated with the E-test method and were found to be 8 and 12 µg/mL. These two strains were identified as *E. faecium* (20).

In another study conducted in 2017, 79 *Enterococcus* spp. isolate was found susceptible to linezolid. For *E. faecalis* (69.6%) strains, the linezolid MIC range was found to be 0.25-2 µg/mL, MIC_{50} 0.75 µg/mL and MIC_{90} 1.5 µg/mL. For *E. faecium* (30.4%) strains, the linezolid MIC range was determined as 0.125-2 µg/mL, MIC_{50}: 0.5 µg/mL and MIC_{90}: 1 µg/mL. As a result of the study, attention was drawn to the importance of closely monitoring the changes by monitoring the MIC values of linezolid (21).

Comoglu et al. investigated the linezolid susceptibility of 20 VRE strains in Turkey. The latter study was conducted using disc diffusion and E-test methods. The MIC values of the strains were determined as 0.38-2 µg/mL. As a result of the study, linezolid resistance was not detected, and it was stated that linezolid is an important alternative in the treatment of VRE (22).

In our study, while the $MIC_{50,90}$ values of 50 VRE strains were found to be 2, 2 µg/mL, respectively, by the microdilution method, they were found to be sensitive as 2, 3 µg/mL by the E-test method. When the results of two different quantitative methods (microdilution and E-test) were evaluated, no significant difference was observed in terms of $MIC_{50,90}$ values, and it was determined that all methods, including the disc diffusion method, showed a high degree of parallelism.

A total of 97 enterococcal strains isolated from 67 patients in a university hospital in Brazil (2004) were examined by species identification, and it was determined that 34% of the strains were *E. faecium*, 33% *E. faecalis*, 23.7% *Enterococcus gallinarum* and 5.2% *Enterococcus casseliflavus* (23).

In a study published in 2006, 33 cases of non-faecalis and non-faecium enterococcal bacteremia were examined in a hospital in the USA, and it was determined that 10 of the patients were infected with *E. casseliflavus*, 8 with *E. mundtii*, 7 with *E. avium*, 5 with *E.durans* and 3 with *E. gallinarum*. As a result of the study, it was reported that bacteremia due to non-faecalis and non-faecium enterococci is a nosocomial infection. In addition, the importance of identifying all enterococci at the species level was emphasized in order to initiate appropriate infection control measures (24).

Species prevalence and antibacterial resistance among enterococci isolated in Tehran hospitals in Iran were investigated in 2009. Vancomycin, teicoplanin and linezolid antibiotics susceptibility of 200 enterococcal isolates were tested by disc diffusion and the agar dilution method. As a result of the study, 80% of 200 isolates were identified as *E. faecalis*, 11% as *E. faecium*, 6.5% as *E. casseliflavus*, 2% as *E. gallinarum* and 0.5% as *E. avium*. 2 *E. faecium*, 1 *E. gallinarum* and 1 *E. casseliflavus* strains were found resistant to linezolid. Linezolid MIC values for linezolid vancomycin resistant enterococci (LVRE) strains were between 16 and 32 µg/mL (25).

In a retrospective study conducted in Japan between 2005 and 2014, 410 cases with enterococcal bloodstream infections were studied. *Enterococcus casseliflavus* was detected in 37 (9%) of 410 cases. In the study, it was stated that *E. casseliflavus* was the third factor after *E. faecalis* and *E. faecium* in enterococcal bloodstream infection (26).

In a study conducted in a medical center in Taiwan in 2010 on infections caused by non-faecalis and non-faecium enterococci, 3017 enterococci isolated in blood cultures were examined and the most common species were identified as *E. casseliflavus, E. gallinarum*, *E. avium* and *E. hirae*, respectively. Infections caused by non-faecium non-faecalis enterococci were associated with patients with severely invasive diseases and immunocompromised patients (27).

In a retrospective study carried out in a hospital in the USA in 2015, *E. gallinarum* was found in 29 (60.4%) and *E. casseliflavus* in 19 (39.6%) of 48 patients hospitalized with the diagnosis of non-faecium non-faecalis VRE bloodstream infection (BSI). Generally, treatment with linezolid or daptomycin for vancomycin-resistant *E. casseliflavus* or *E. gallinarum* has been reported to produce better clinical outcomes compared to anti-enterococcal beta-lactam therapy (28).

In a prospective study conducted in India, 371 *Enterococcus* spp. isolates were determined by conventional biochemical tests and the VITEK 2 Compact identification system, and vancomycin resistance was investigated by PCR. As a result of the study, 239 *E. faecalis*, 114 *E. faecium*, 8 *E. avium*, 4 *E. durans*, 4 *E. casseliflavus* and 2 *E. gallinarum* were detected. Vancomycin resistance was detected in 14 *E. faecalis*, 4 *E. faecium*, 4 *E. casseliflavus* and 2 *E. gallinarum* strains, 2 linezolid resistant enterococci and 252 multidrug resistant enterococci (29).

In our study, 50 VRE strains isolated from different clinical care units were identified using the GP24 diagnostics species identification kit. Of the 50 strains, 48 (96%) were determined as *Enterococcus casseliflavus* and 2 strains (4%) were *Enterococcus faecium*. *Enterococcus faecalis* ATCC 29212 standard strain was used as the control strain and was identified as *E. faecalis* with 100% accuracy.

Surveillance follow-up should be performed to prevent healthcare-associated infections and to reduce the risk to patients, employees, and the environment. Infection control programs such as employee health, isolation, training of health personnel, infection prevention policies, and management should be established and implemented. Linezolid is an important antimicrobial agent in the treatment of VRE infections. Unneces-

sary use should be avoided so that resistance does not develop. In our study, the efficacy of linezolid on VRE strains was investigated and no linezolid resistance was found. This suggests that linezolid can safely be used in the treatment of VRE-induced infections. It will be important to carry out continuous and comprehensive studies on this subject and to monitor linezolid resistance surveillance.

Ethics Committee Approval: This study was approved by Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 23.09.2022, No: 17).

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Conflict of Interest: The authors have no conflict of interest to declare.

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