Biofilm and Gelatinase Production, Antibiotic Resistance and Incidence of *Enterococcus faecalis* on Conjunctival Swabs of Diabetic Patients

Diyabetik Hastalardan Alınan Konjonktival Sürüntüde *Enterococcus faecalis* İnsidansı, Biyofilm ve Jelatinaz Üretimi ve Antibiyotik Direnci

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ABSTRACT Objective: *Enterococcus faecalis* is an emerging etiologic agent of hospital infections, with high rates of antibiotic resistance. Enterococci produce biofilms on intraocular lens materials, further highlighting their potential virulence for the eye. **Material and Methods:** Twenty-five patients [15 females (60%) and 10 males (40%)] with type II diabetes mellitus for at least10 years and without any infective or allergic conjunctivitis were included in the study. Both eyes of each patients (total 50 eyes) were analyzed for the presence of enterococci. Presumptive isolates were identified with morphological, cultural and biochemical tests and were confirmed by the VITEK system (BioMerieux). Automated EcoRI Ribotyping was performed with a RiboPrinter® Microbial Characterization System (Dupont Qualicon). Antibiotic susceptibility of the isolates to 10 different antibiotics, which are use as ophthalmic drop was determined by the Kirby-Bauer disc diffusion test. Virulence-related phenotypes (biofilm and gelatinase production) were studied. **Results:** Patients had Type II diabetes mellitus for at least 10 years and the mean value for HbA1c was 8.3±1.61. Seven of 25 patients were using insulin treatment alone, 7 were using oral anti-diabetics alone, 11 were using insulin and oral anti-diabetics together. Among the 26 isolates of enterococci, 24 were defined as *E. faecalis* and 2 as *E. avium*, with biochemical tests. With automated EcoRI Ribotyping, 24 isolates were defined as *E. faecalis* and 2 were defined as *E. faecium*. Resistance to vancomycin was 16.67% among *E. faecalis* isolates. The most effective antimicrobials were moxifloxacin (96.15% of isolates inhibited), gentamycin (92.3% of isolates inhibited) and gatifloxacin (92.3% of isolates inhibited). All *E. faecalis* strains were biofilm producers. While 14 *E. faecalis* (53.85%) isolates had a clear halo around their colonies 2 isolates of *E. faecium* isolates had no halo. **Conclusion:** Antibiotic multi-resistance and strong biofilm production abilities together with a high phenotypic expression of gelatinase are an important equipment of *E. faecalis* to colonize intraocular lens materials.

Key Words: *Enterococcus faecalis*; biofilms; drug resistance, bacterial; ribotyping

ÖZET Amaç: *Enterococcus faecalis* yüksek oranda antibiyotik direnci ile hastane enfeksiyonlarının önemli bir etkenidir. Enterokoklar göz içi lens materyali üzerinde biyofilm oluşturabilir. Bu çalışmada, özellikle diyabetik hastalarda enterokokların göz için potansiyel virülansının gösterilmesi amaçlanmıştır. **Gereç ve Yöntemler:** Çalışmaya en az 10 yıllık Tip II diyabeti bulunan hastalardan 15'i kadın, 10'u erkek toplam 25 hastanın 50 gözü dâhil edilmiştir. Bu hastaların enfektif ve allerjik konjonktivit bulguları olmayan 50 gözü enterokoklar açısından analiz edilmiştir. Elde edilen kökenler morfolojik, kültürel ve biyokimyasal testlere tabii tutulmuş ve VITEK sistemi (BioMerieux) ile doğrulanmıştır. Otomatize EcoRI ribotyping işlemi RiboPrinter® Microbial Characterization System (Dupont Qualicon) ile uygulanmıştır. Kökenlerin antibiyotik dirençleri göz damlası olarak kullanılan 10 farklı antibiyotik için Kirby-Bauer disk difüzyon testi ile değerlendirilmiştir. Virülans ile ilişkili fenotipler (biyofilm oluşturma ve jelatinaz üretimi) incelenmiştir. **Bulgular:** En az 10 yıllık Tip II diyabet hastalarının ortalama HbA1c değerleri 8,3±1,61 bulunmuştur. Yirmi beş hastanın 7'si tek başına insülin tedavisi, 7'si tek başına oral anti-diyabetik, 11'i ise hem insülün hem oral anti-diyabetik kullanmaktadır. Biyokimyasal testlerle, elde edilen 26 enterokok kökeninin 24'ü *E. faecalis*, 2'si *E. avium* olarak tanımlanırken, otomatize EcoRI ribotiplendirmeye göre ise 24'ü *E. faecalis*, 2'si *E. faecium* olarak tanımlanmıştır. *E. faecalis* kökenlerinde vankomisine direnç oranı %16,67 bulunmuştur. En etkili antibiyotikler, moksifloksasin (kökenlerin %96,15'i inhibe olmuştur), gatifloksasin (kökenlerin %92,3'ü inhibe olmuştur) ve gentamisin (kökenlerin %92,3'ü inhibe olmuştur) olarak saptanmıştır. İncelenen tüm *E. faecalis* kökenlerinin biyofilm ürettikleri saptanmıştır. **Sonuç:** Çoklu antibiyotik direnci ve güçlü biyofilm üretme yeteneği, jelatinazın yüksek fenotipik salınımı ile birlikte, bakterinin göz içi lens materyali üzerinde kolonize olmasında çok önemli rol oynamakta ve bu ise göz içi lens uygulanan diyabetik hastalarda potansiyel bir risk oluşturabilmektedir. Antibiyotik tedavisinde bu hususa dikkat edilmesinde yarar vardır.

Anahtar Kelimeler: *Enterococcus faecalis*; biyofilmler; ilaç direnci, bakteriyel; ribotipleme

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Enterococci are ubiquitous Gram-positive bacteria that can be found in soil, food, and water while making up a significant portion of the normal gut flora of humans and animals. *Enterococcus faecalis* is a commensal bacterium inhabiting the hard environment of the human and animal intestines.[1] It is naturally and easily adaptable, quickly finding the way to turn into a true opportunistic pathogen in nosocomial infections. *Enterococcus faecalis* is an emerging etiologic agent of hospital infections, exhibiting high rates of antibiotic resistance. Enterococci are among rare causes of post-operative endophthalmitis, and they are often associated with filtering bleb surgery. However, expressing fewer virulence traits than *B. cereus,* it is more amenable to study. The main toxin expressed by strains of *E. faecalis*, the cytolysin, has been shown to contribute to the severity of infection. Cytolysin is a secreted toxin that can lyse bacteria, erythrocytes, and other mammalian cells. The presence of cytolysin rendered experimental *E. faecalis* endophthalmitis refractory to antibiotic and anti-inflammatory drug treatment.[2,3]

Although microorganisms with high pathogenity were reported as a temporary member of the human ocular flora without colonization, they can be colonized and to be source of serious infections under certain circumstances. Microorganisms present on conjunctival flora gain pathogenity with ocular surgery, immune deficiency or malnutrition and may lead to infection.[4]

Biofilms are bacterial communities attached to a biotic or an abiotic substrate and encased in a matrix that may be composed of carbohydrates, DNA or protein.[5] Enterococci have been associated with biofilms on various kinds of indwelling medical devices, such as intraocular lens materials.[6,7] Organisms within a biofilm are difficult to eradicate by conventional antimicrobial therapy and can cause indolent infections. Biofilm formation may occur rapidly on contact lenses and their cases and hence contribute to the pathogenesis of keratitis. Formation of biofilms is also implicated in delayed post-operative endophthalmitis and crystalline keratopathy. Bacteria within biofilms are 20-1000 times less sensitive to antibiotic than free-living planktonic organisms.[5,8,9]

Here, 26 isolates of enterococci isolated from patients were genotyped by an automated RiboPrinter and analyzed for antimicrobial susceptibility. In addition, biofilm and gelatinase formation was evaluated.

MATERIAL AND METHODS

SUBJECTS

This study included 25 patients [15 female (60%), 10 male (40%); mean age 59.54±6.72 years] with Type II diabetes mellitus for at least10 years and without ocular infection or ocular allergic symptoms. Both eyes of each patient (n=50 eyes) were sampled for microbiologic examination without topical anesthetic drop. Swabs were taken from the conjunctiva using sterile Stuart's swabs, which were placed in Stuart's transport medium for onward transfer to the microbiology laboratory. Oral informed concent was obtained from each patient before conjunctival sampling.

BACTERIAL ISOLATION AND IDENTIFICATION

Swabs were streaked on blood agar, Slanetz and Bartley medium (Oxoid, CM0377A). Cultures were incubated at 37°C to permit bacterial growth for 3 days to ascertain either "growth" or "no growth. Typical colonies (red, maroon or pink) were transferred to tryptone soy agar (Oxoid, CM0131) supplemented with 0.6% yeast extract powder (Oxoid, L0021), were submitted to Gram staining and were tested for catalase production. Gram-positive and catalase negative cocci were tested for growth in De Man-Rogosa-Sharpe (MRS) broth (Oxoid, CM0359) at 10 and 45°C, growth in MRS broth supplemented with 6.5% NaCl, and growth in MRS broth of pH 9.6. The strains were further identified with the Vitek system (BioMerieux). The Vitek identification system is also a carbon source utilization test. The reliability of these systems depends upon the number and diversity of bacteria in the databases.

AUTOMATED RIBOTYPING

For ribotyping, automated RiboPrinterTM Microbial Characterization System (DuPont Qualicon,

Wilmington, DE, USA) was used according to a protocol using EcoRI as restriction enzyme. Pure culture samples were obtained from Brain Heart Infusion (BHI) plates incubated for 24 hours at 37°C using appropriate colony picks. The microbial samples were subsequently analyzed accordign to the recommendations of the producer. The ribotype profiles of the isolates were compared with the reference DuPont identification database DUP2003. Isolates were identified when the corresponding pattern matched one of the patterns of the DuPont Identification Library with a similarity X 0.85. The isolates were automatically grouped in ribogroups by the RiboPrinterTM, based on the similarity of the respective ribotype patterns.

ANTIBIOTIC SUSCEPTIBILITY TESTS

Antibiotic susceptibility tests were performed by the agar diffusion (Kirby–Bauer) method, according to the guidelines of the Clinical and Laboratory Standards Institute.[10,11] Disks containing the following antibacterial agents were used: gatifloxacin (5 μg), cefuroxime (30 μg), ceftazidime (30 μg), vancomycin (5 μg and 30 μg), gentamicin (10 μg), amikacin (30 μg), ciprofloxacin (5 μg), levofloxacin (10 μg), moxifloxacin (5 μg), methicillin (5 U). Plates were incubated at 37°C for 24-48 h.

BIOFILM PRODUCTION

Biofilm measurements were performed by using 30 μl of overnight cultures in trypticase soy broth (TSB) inoculated in 270 μl of TSB supplemented with 1% glucose (TSBG). The optical density of the produced biofilm was measured with a spectrophometer at 570 nm. Measurements were repeated at three different times in triplicate for each strain.[12]

GELATINASE

Gelatinase production was determined as previously described, using trypticase soy agar supplemented with 1.5% skimmed milk; a clear halo around the colonies after 18h at 37°C was considered positive.[13]

RESULTS AND DISCUSSION

Among 25 patients 7 were using insulin treatment alone, another 7 were using oral anti-diabetics alone and 11 were using insulin and oral anti-diabetics together. The mean HbA1c level was 8.3± 1.61. In addition to diabetes mellitus, 17 patients had hypertension, 12 had hypercholesterolemia, two had coronary artery disease, and one had breast cancer.

Among the 50 eyes sampled, 11 yielded 26 Enterococci isolates. All isolates were gram-positive and catalase-negative and non-spore-forming bacteria. All isolates were able to grow at 45°C, at pH 9.6 and in the presence of 6.5% NaCl. Vitek analysis revealed that 24 isolates were *Enterococcus faecalis* (92.31%) and 2 were *Enterococcus avium* (7.69%). *Eco*RI ribotyping confirmed the presumptive classification of the isolates within the enterococci based on the preset identification similarity threshold of 0.86; all the strains were automatically identified. *Eco*RI ribotyping differentiated the isolates into 5 distinct ribotypes. Similarity between those 5 ribogroups ranged from 0.86 to 1.00. The ribogroups belonged to 4 different DUP-IDs (Figure 1). The threshold regarding the measure of similarity was fixed at 0.85%. Figure 1 shows the dendrogram and banding patterns of the isolates and the reference strains based on *EcoRI* ribotypes. EcoRI ribotyping was confirmed as a rapid and reliable method for *E. faecalis.* In all *E. faecalis* strains, the results obtained with the phenotypical identification methods and ribotyping were in good agreement with each other and their identification was considered very reliable. Two *Enterococcus avium* strains were identified as *En-*

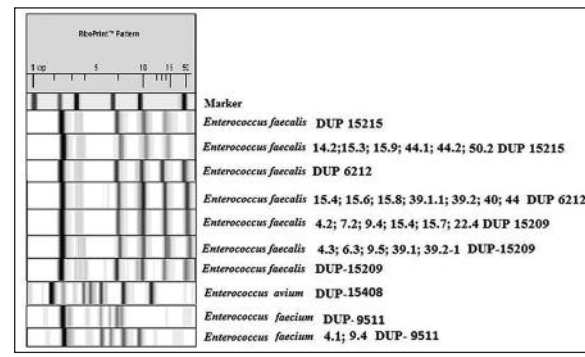

FIGURE 1: Ribotyping profiles of isolates and some standards were shown with DUP numbers.
DUP: DuPont identification database number.

terococcus faecium by automated ribotyping. Automated ribotyping was found to be a reliable and rapid method for generating genetic fingerprints of enterococci. Some heterogeneity in the fingerprints within species was detected. Endophthalmitis caused by *E. faecalis* was identified in 29 eyes of 29 patients.[14]

From 26 isolates, 5 distinct EcoRI ribogroups were identified and various resistance profiles were obtained. Antimicrobial susceptibility of the isolates was investigated using a panel of 10 different drugs and the rate of antibiotic resistance was as follows: Methicillin resistance 65.38%, gentamicin 7.69%, gatifloxacin 7.69%, cefuroxime 65.38%, ceftazidime 57.69%, vancomycin 16.67%, amikacin 15.38%, ciprofloxacin 15.38%, levofloxacin 11.50% moxifloxacin 3.85% (Figure 2). Multiresistance-resistance to at least two antibiotics-was present in 73.08% of the isolates; 11.54% had resistance to two antibiotics, 30.76% (8) to three, 11.54% to four, 11.53% to five, 3.85% to six and 3.85% to seven antibiotics (Table 1). Only four strains out of 26 were susceptible to all tested antibiotics. This was not surprising, because a clone multi-resistant would be expected to prevail and spread. Similarly, epidemic clones of *E. faecalis* responsible for bacteremias in Spanish hospitals were recently reported to be higly resistant to aminoglycosides but not resistant to vancomycin.[15] Low prevalence of vancomycin resistance has also been reported in enterococci in Turkey.[16] Also Scott et al. noticed that their isolates were not resistant to vancomycin.[14] Most vancomycin-resistant enterococci are also associated with high- level resistance to the aminoglycosides and penicillins, rendering synergism irrelevant.[17] Even though resistance to vancomycin was indicated mainly and precociously in the USA reports, European Countries also report that vancomycin resistance is spreading or already widespread, as in the haematology units of the Portugal, where clones resistant to vancomycin are the most prevalent.[18,19] Also in Poland, vancomycin-resistant clones have been recently isolated.[20] Tang et al. reported that community-acquired bleb-related endophtalmitis was caused by vancomycin resistant enterococci.[21] The alarming point about the spreading potential of resistance is that vancomycin resistance genes can be transferred among enterococci and from enterococci to staphylococcus species.[22] In a review of 608 enterococcal isolates, 219 (36%) showed high-level gentamicin resistance.[23] In another study, 53 of 97 nosocomially acquired enterococcal isolates (54.6%) expressed high-level gentamicin resistance.[24] In contrast to the results of our study, another study from Turkey reported high-level resistance to gentamicin by disc diffusion in 22% of the isolates; however, the isolates were not obtained from the conjunctiva. In the same study, moxifloxacin and gatifloxacin resistance among enterococci was low-level Moxifloxacin has been shown to be slightly more active than gatifloxacin against enterococci.[16] This finding indicates that moxifloxacin and gatifloxacin retain their therapeutic efficacy against enterococcal infections.[16]

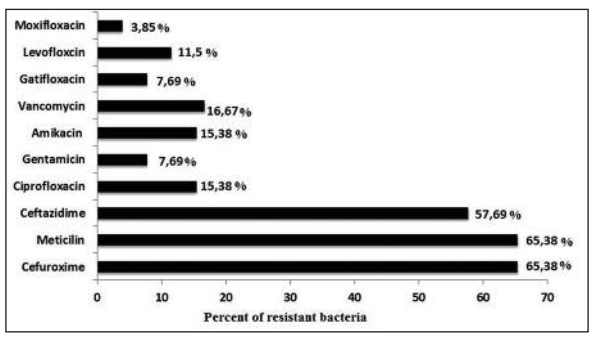

FIGURE 2: Antibiotic susceptibility patterns of *Enterococcus faecalis*.

Enterococci are considered opportunistic pathogens that can cause a variety of infections in patients with severe underlying conditions or are immunocompromized. This relatively high rate of multi-resistant bacteria is comparable to the results of previous studies reporting resistant bacteria as the cause of postoperative endophthalmitis.[25,26] Postoperative endophthalmitis may develop when *E. faecalis* is an element of the ophthalmic flora of diabetic patients.

In the present study, all but 2 *E. faecalis* strains were biofilm producers (Table 2); both nonproducer strains were identified as *E. faecium* with an optic density (OD) below 0.12. Fifteen isolates that showed an OD higher than 0.50 were classified as high producers. Miller et al. reported a case with

TABLE 1: Multidrug resistance pattern among the *Enterococcus* spp.

No. of drug	No of isolates (n=26)	Resistance (%)
≥2	3	11.54
≥3	12	46.15
≥4	3	11.54
≥5	2	7.69
≥6	1	3.85
≥7	1	3.85

diabetes mellitus with recurrent endophthalmitis caused by *E. faecalis* who did not respond to treatment. Resistance to treatment was attributed to the biofilm that developed on the first implantated lens; the case was succesfully treated after the intraocular lens was changed.[27]

While a clear halo around the colonies was observed for 14 isolates of *E. faecalis* (53.85%), 2 isolates of *E. faecium* had no halo. Gelatinase production by *E. faecalis* isolated from inpatients has been reported to be higher than that among community isolates.[28] Gelatinase is able to hydrolyze gelatine and some other bioactive peptides. The gelatinase (GelE) of *E. faecalis* is an extracellular zinc metalloprotease that can hydrolyse gelatin, collagen and casein. Gelatinase influences full virulence in a mouse model of endophthalmitis.[29]

Postoperative endophthalmitis may develop when *E. faecalis* is an element of the ophthalmic flora of diabetic patients. *E. faecalis* has the potential to form biofilm on intraocular lens materials.[6,7] Thus, it may be beneficial to know the bacterial flora of the conjunctiva in diabetic patients underdoing ophthalmic surgery. This provides the opportunity to choose suitable antibiotics for prophylaxis and postoperative use.

TABLE 2: Biofilm and gelatinase production.

Bacteria	Biofilm producer	Gelatinase producer
E. faecalis 14.2	+++	++
E. faecalis 15.3	+++	+++
E. faecalis 15.9	+++	+++
E. faecalis 44.1	+++	+++
E. faecalis 44.2	+++	++
E. faecalis 50.2	+++	+++
E. faecalis 15.4	+++	+++
E. faecalis 15.6	++	+
E. faecalis 15.8	++	+
E. faecalis 39.1.1	+++	++
E. faecalis 39.2	+++	+
E. faecalis 40	++	+
E. faecalis 44	+++	+++
E. faecalis 4.2	+++	+++
E. faecalis 7.2	+++	+++
E. faecalis 9.4	++	+
E. faecalis 15.4	++	+
E. faecalis 15.7	++	+
E. faecalis 22.4	++	+
E. faecalis 4.3	++	+
E. faecalis 6.3	++	+
E. faecalis 9.5	+++	++
E. faecalis 39.1	+++	++
E. faecalis 39.2.1	+++	+++
E. faecium 4.1	-	-
E. faecium 9.4	-	-

-, non-producer (OD 570<0.120); +, weak producer (OD570<0.240); ++, producer (OD 570<0.500);+++, high procducer (OD570> 0.500).

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