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ORIGINAL ARTICLE

Isolation, vancomycin resistance and biofilm production of *Staphylococcus epidermidis* from patients with conjunctivitis, corneal ulcers, and endophthalmitis

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ABSTRACT. The infection frequency associated to bacterial conjunctivitis, corneal ulcers (CU), and endophthalmitis was studied along a five years period. The isolation and identification of microorganisms were performed by culture-based methods and biochemical test respectively. Also, a nested PCR to detect gram-negative and gram-positive bacteria in the clinical samples was assayed. Nested PCR was a more efficient method than culture to detect bacteria in the samples. The most frequently isolated species was *Staphylococcus epidermidis*, a bacterium commonly considered as a human saprophyte. The *S. epidermidis* strains from conjunctivitis, CU, and endophthalmitis exhibited 46, 33.9, and 34.1% of oxacilin-resistance respectively. A total of 28% of intermediate-vancomycin resistance (MIC = 8-16 µg/ml) was observed among *S. epidermidis* strain collection. The UPGMA cluster analysis of the multiresistance profile data of intermediate vancomycin-resistant *S. epidermidis* strains showed a high phenotypic diversity and no relationship between each group and their clinical origin. The biofilm formation capacity was broadly distributed (66%), particularly among intermediate-vancomycin strains (> 75%). In brief, *S. epidermidis* displayed a high diversity of antibiotic resistance profiles and biofilm formation capacity. These phenotypic traits could explain the high isolation frequency of *S. epidermidis* from ocular infections and oblige to review the saprophytic status of these bacteria.

Key words: Conjunctivitis, corneal ulcers, endophthalmitis, *Staphylococcus epidermidis*, vancomycin-resistance.

RESUMEN. La frecuencia de infecciones bacterianas asociadas a conjuntivitis, úlcera corneal (CU) y endooftalmitis se estudió durante un periodo de cinco años. El aislamiento y la identificación de los microorganismos fueron llevados a cabo por métodos basados en el cultivo y en pruebas bioquímicas respectivamente. Asimismo, una PCR anidada se utilizó para detectar bacterias Gram negativas y bacterias Gram positivas en las muestras clínicas. La PCR anidada resultó ser un método más eficiente que el cultivo para detectar bacterias en las muestras. La especie más frecuentemente aislada fue *Staphylococcus epidermidis*, una bacteria comúnmente considerada como un saprófito humano. Las cepas de *S. epidermidis* de conjuntivitis, CU y endooftalmitis mostraron 46, 33.9 y 34.1% de resistencia a oxacilina respectivamente. El 28% de las cepas de *S. epidermidis* resultaron tener una resistencia a vancomicina intermedia (MIC = 8-16 µg/ml). El análisis de agrupamiento UPGMA de los perfiles de multirresistencia de las cepas de *S. epidermidis* con resistencia intermedia a vancomicina mostró una alta diversidad fenotípica y no presentó relación de cada grupo con su origen clínico. La capacidad de formación de la biopelícula estuvo ampliamente distribuida (66%), en particular entre las cepas con resistencia intermedia a la vancomicina (> 75%). En resumen, *S. epidermidis* exhibió una diversidad grande de los perfiles de resistencia a los antibióticos y una ampliamente distribuida capacidad de formación de biopelículas. Estos rasgos fenotípicos pueden explicar la alta frecuencia de aislamiento de *S. epidermidis* a partir de infecciones oculares y obliga a revisar el estatus saprofítico de esta bacteria.

Palabras clave: Conjuntivitis, úlcera corneal, endooftalmitis, *Staphylococcus epidermidis*, resistencia a vancomicina.

INTRODUCTION

Conjunctivitis can be produced by viral or bacterial infection, as well as allergy, trauma, and dietary deficiency. *Staphylococcus epidermidis* and *Staphylococcus aureus* are the most frequently isolated microorganisms from patients with conjunctivitis.[51] Other ocular infectious diseases are bacterial keratitis or corneal ulcers (CU). The spectrum of microorganisms that produce CU is usually influenced by contact lens wear, pre-existing disease, or injury of the cornea.[36] *Staphylococcus*, *Streptococcus*, *Pseudomonas*, and *Serratia* are the most common bacteria cultured from CU.[8] Infectious endophthalmitis, although relatively rare, is the most devastating complication of intraocular surgery. Clinically, two forms can occur after surgery: acute postoperative endophthalmitis, frequently caused by *S. epidermidis*, *S. aureus*, and *Streptococcus* spp.[17] and delayed postoperative endophthalmitis caused

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by *Propionibacterium acnes*,[18] *Actinomyces israelli*, or *Corynebacterium* spp.[44]

S. epidermidis is a saprophyte that is part of the normal mucosa and skin microflora. In recent years, *S. epidermidis* emerged, together with *S. aureus*, as a frequent etiologic agent of infections associated with catheters and other indwelling medical devices. Over the last few years, several studies have been performed to elucidate the structures and pathogenic mechanisms by which staphylococci are able to cause severe and irreducible infections associated with biomaterials.[3] It has recently been shown that *S. aureus* and *S. epidermidis* are capable of biofilm formation,[2,12] an important factor for adherence, antibiotic resistance and protection from host innate defense.[11] Resistance of *S. epidermidis* biofilm to some antibiotics might, in part, be due to a poor antibiotic penetration, nutrient limitation and slow growth, adaptive stress responses, and formation of persister cells. In a biofilm, *S. epidermidis* is protected against attacks from the immune system and antibiotic treatment, making *S. epidermidis* infections difficult to eradicate.[25]

The epidemiology of ocular infections is important because it provides information about the behavior of different microorganisms in the eye. These data help to understand the cause and frequency of infections, as well as to identify resistant strains that can infect the eye. In this work, the frequency of bacteria associated to conjunctivitis, CU, and the endophthalmitis, and particularly the antibiotic resistance and biofilm production of *S. epidermidis* during a 5-years period were analyzed. Besides, a nested PCR procedure to detect gram-negative and gram-positive bacteria from clinical samples was compared with culture assays.

MATERIAL AND METHODS

Patients

This work is a retrospective study in which clinically diagnosed patients with conjunctivitis (n = 3,769), CU (n = 611), and endophthalmitis (n = 167) at the Instituto de Oftalmología "Conde de Valenciana", Mexico City, from 1999 to 2003, were included. CU and conjunctivitis samples were obtained by scraping and swabbing, respectively. The vitreous samples of patients with endophthalmitis were mainly obtained by vitrectomy. Samples of patients with suspicion of fungal, viral, or *Acanthamoeba* infection were excluded.

To compare the efficiency of bacterial detection by nested PCR and culture based methods, a representative random sample of 75 samples from conjunctivitis (n = 35), CU (n = 25), and endophthalmitis (n = 15), from July to December of 2003, was included.

The Medical Ethics and Research Committees of the Instituto de Oftalmología "Conde de Valenciana" of Mexico City, Mexico, approved this study.

Isolation and identification

The clinical samples were inoculated directly on chocolate, blood and mannitol agar plates. The chocolate agar plate was incubated in a 3% CO_2 atmosphere, and all media were incubated at 37°C for 12-48 h. The bacteria were identified by means of the Vitek Jr computerized system (bioMériux, France), using the GPS-101 and V-1305 identification cards for gram-positive bacteria, and GNS-203 and V-1316 for gram-negative bacteria. The resistance to antibiotics was evaluated with BBL™ sensi-disk™ antimicrobial susceptibility test disks (Becton Dickinson, Maryland, USA) and interpreted according to the guidelines established by the National Committee on Clinical Laboratory Standards (NCCLS). The bacteria that presented resistance to five or more antibiotics, detected by disk diffusion assay, were considered as multi-resistant strains.

Determination of vancomycin resistance

Agar and broth dilution methods were used for vancomycin-resistance testing. The procedure was performed according to NCCLS using Mueller-Hinton agar or broth (Becton Dickinson, Sparks, Md.). The definition of a vancomycin-resistance strain was according to NCCLS for which a MIC of ≤ 4 μg/ml is considered vancomicyn-susceptible, while that the MIC is 8 to 16 μg/ml are intermediate-vancomicyn and those for which the MIC is ≥ 32 μg/ml are resistant-vancomycin.

Detection of gram-positive and gram-negative bacteria by nested PCR

The nested PCR was performed according to Carrol *et al.* with the 75 aforementioned clinical samples.[9] Each sample was divided in two parts: a part was used to obtain DNA through the DNeasy Tissue Kit (Qiagen, CA, USA) and the other was used for the routine diagnostic microbiological tests.

Cluster analysis

The antibiotic resistance profile of each vancomycin-resistant *S. epidermidis* strain was recorded as binary data: antibiotic-resistance (1) and antibiotic-nonresistance (0). Pairwise similarities among isolates were calculated from these data using Jaccard´s coefficient.[24] The relationships among resistant isolates were established by cluster and or-

dination analyses performed on the matrix of similarities. Cluster analysis was performed by the unweighted paired group method using arithmetic average (UPGMA).[48] The distortion of the inferred tree was estimated by the cophenetic correlation coefficient (CCCr) with the non-parametric Mantel test and the best-cut test.[30,49] All analyses were made with the NTSYS-PC software, version 2.02j, and PHYLIP version 3.6 software (Phylogeny Inference Package, version 3.6a3; J. Felsenstein, Department of Genomic Sciences, University of Washington, Seattle, WA).

Quantitative determination of biofilm formation

Quantitative biofilm measurement was performed in a microtiter assay as described previously.[10] Biofilm formation positive reaction was considered when A_{490} values obtained were > 0.12. Positive biofilm *Staphylococcus aureus* IOCV17, negative biofilm *Staphylococcus epidermidis* ATCC12228, and six *Staphylococcus epidermdis* strains isolated from healthy subjects were used as controls.

RESULTS

Gram-positive and gram-negative bacteria analysis

A total of 4,547 samples were analyzed during the 5-years of this study; of these, 3,410 samples were positive to culture (75%), in the remainder, 1,137 samples (25%), no bacterial growth was obtained. Basically, the main bacterial positive cultures were obtained from conjunctivitis samples (3,094 of 3,769, 82%) followed in decreasing order by endophthalmitis (103 of 167, 62%), and CU (213 of 611, 35%). The isolated gram-positive bacteria represented 96.8% for conjunctivitis, 91.2% for endophthalmitis, and 75.1% for CU.

The annual infection showed that gram-positive bacteria were more frequently isolated from conjunctivitis, CU, and endophthalmitis cases than gram-negative bacteria (Fig. 1). Regarding CU, 65% of the cultures were without bacterial growth during the five years. In endophthalmitis, the causal agent of most infections in the 1999-2000 period was not detected through culture, but infections by gram-positive bacteria were the most frequently encountered from 2002 to 2003 (Fig. 1).

Bacterial detection by nested PCR

In order to increase the efficiency of bacterial recognition basically in the CU and endophthalmitis samples, which presented a high frequency of negative results by bacterial culture, the nested PCR method was assayed in 75 clinical random samples (Table 1). In conjunctivitis samples, both nested PCR and conventional culture method, were positive to bacteria in 31 of 35 (88.5%) samples analyzed ($p = 1.0$). In CU, nested PCR method detected bacteria in 21 of 25 (84%) samples, whereas the conventional culture method only detected bacteria in 8 of 25 (32%) samples ($p < 0.0003$). Only one gram-negative bacteria was detected by PCR, but none was cultured. In endophthalmitis, 13 of 15 (86%) samples were positive to bacteria by nested PCR whereas only 6 of 15 (40%) samples were positive according to conventional culture ($p <$

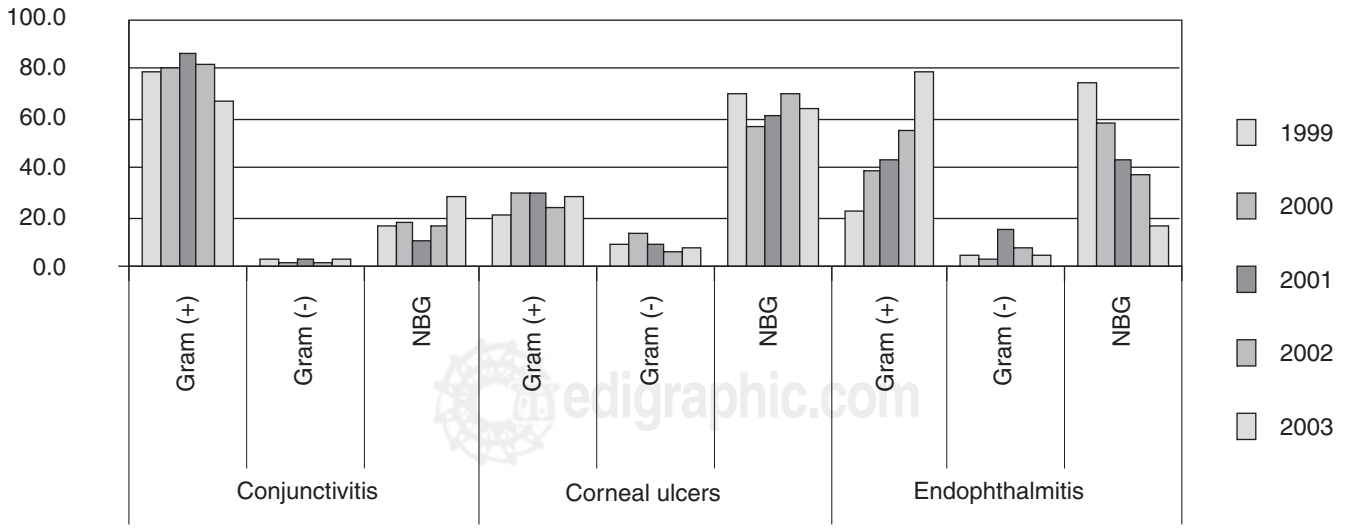

Figure 1. *Bacterial isolation from ocular infections in a five-year period (1999-2003). Distribution of isolated bacteria. No bacterial growth (NBG).*

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0.008). In all cases, the positive samples detected by culture were also detected by nested PCR, but some samples detected by PCR were not cultured. As negative control for PCR, five conjunctiva samples of ophthalmologically healthy subjects were assayed in which no positive PCR reactions were obtained.

Identification of bacteria in eye infections

A total of 65 different bacteria were isolated from samples collected during the 5-years study. Only the most significant data were reported. *S. epidermidis* was the species most frequently isolated from all diseases followed by *S. aureus* strains from CU and endophthalmitis, and *Micrococcus* spp. from conjunctivitis (Table 2A); *Pseudomonas aeruginosa* isolation was relevant from endophthalmitis and CU; and *Proteus mirabilis* and *Escherichia coli* were the most frequently detected in conjunctivitis (Table 2B). Due to the relevance of *S. epidermidis* frequency, posterior analyses were performed with only this species.

Antibiotic resistance among S. epidermidis *strains*

An average of 72.1% of multi-resistance *S. epidermidis* strains were observed among all isolates. Resistance to ceftazidime (in conjunctivitis) and sulfisoxazole (in CU and endophthalmitis) was broadly distributed among *S. epidermidis* strains (Table 3). Oxacilin-resistance was 46%, 33.9%, and 34.1% in strains from conjunctivitis, CU, and endophthalmitis, respectively (Table 3). Vancomycin-resistance of *S. epidermidis* strains, evaluated by the disk diffusion method, was detected in 4.2% and 7.3% from conjunctivitis and endophthalmtis cases. No resistant strains were isolated from CU (Table 3). Besides, a total of 65.6% and 33.3% of the vancomycin-resistant *S. epidermidis* strains from conjunctivitis and endophthalmitis, respectively, exhibited oxacillin-resistance.

Cluster analysis of vancomycin-resistant S. epidermidis *strains*

The cluster analysis of multiresistance profile of vancomycin-resistant *S. epidermidis* strains detected by disk diffusion assay showed a high phenotypic diversity and no relationship of the groups with the origin of the strains (Fig. 2).

Determination of MIC to vancomycin in S. epidermidis *strains*

To verify the disk diffusion data, the vancomycin MIC of the *S. epidermidis* strains was estimated by agar and broth dilution methods. Only 21 of 78 strains (28%) exhibited intermediate-resistance to vancomycin and no resistant strains were isolated (according to NCCLS for which MICs of 8 to 16 μg/ml are considered as intermediate-vancomicyn).

Biofilm formation in S. epidermidis *strains*

The biofilm formation capacity was broadly distributed (66%) in the *S. epidermidis* strains, particularly among intermediate-vancomycin strains (> 75%). No clear association between biofilm formation capacity and multiresistance or vancomycin-intermediate resistance was observed.

DISCUSSION

In this work, bacterial strains isolated from patients with ocular infections collected in a period of 5-years were studied. Gram-positive bacteria were the main cause of conjunctivitis, CU, and endophthalmitis, similarly to previous reports from others countries.[21,27,33,53,54, 56,57] Due to the low efficiency in bacterial detection by culture media obtained in CU and endophthalmitis, 65% and 38% respectively, an alter-

Table 1. Detection of bacteria from conjunctivitis, corneal ulcers, and endophthalmitis by culture and nested PCR.

Samples	Conventional culture medium method			Nested PCR method			Significance[2]
	Gram positive bacteria	Gram negative bacteria	Total positive[1]	Gram positive bacteria	Gram negative bacteria	Total positive[1]	
Conjunctivitis	31/35 (88.5%)	0/35 (0%)	31/35 (88.5%)	31/35 (88.5%)	0/35 (0%)	31/35 (88.5%)	p = 1.0
Corneal ulcers	8/25 (32%)	0/25 (0%)	8/25 (32%)	20/25 (80%)	1/25 (4%)	21/25 (84%)	p < 0.0003
Endophthalmitis	6/15 (40%)	0/15 (0%)	6/15 (40%)	13/15 (86%)	0/15 (0%)	13/15 (86%)	p < 0.008

[1] Positive corresponds to growth in culture or amplified in nested PCR.
[2] Statistical analysis of the data was performed using χ^2 (Chi square) test to compare groups.
The 75 samples were collected during July to December of 2003.

native nested PCR procedure to detect gram-positive and gram-negative bacteria was assayed. Nested PCR significantly increased detection level in CU and endophthalmitis samples from 32 to 84% and 40 to 86% respectively. Other previous reports notified that PCR increases significantly the detection level, specificity and sensitivity of bacteria from keratitis and endophthalmitis samples, as compared to culture-based methods.[4,28,38,52] However, the PCR used in this study only distinguished between gram-positive and gram-negative bacteria, no identification to species can be achieved with this method. To our knowledge, no multiplex PCR has been improved to detect and directly identify the most frequent bacterial pathogens associated to ocular infections; although PCR-RFLP and PCR DGGE mediated detections have been performed.[39,46]

S. epidermidis has been traditionally considered as part of the normal microbiota of skin and mucosa, a saprophyte or an opportunistic pathogen; however, in this study, it was the most frequently isolated bacterium from all ocular infections, similarly to other previous studies.[7,17,51,54] Thus, from our perspective, this species must be considered as a potential primary pathogen in eye, an immunological privileged organ. *S. aureus*,[31] *Haemophilus influenzae*,[40] and *Streptococcus pneumoniae*[32] have also been reported as main bacterial species isolated from conjunctivitis samples. Besides, *S. epidermidis* multiresistant strains frequently cause severe infections in other body locations.[7,21,42,53] The antibiotic multiresistance profile of the potential pathogenic bacteria is a clear selective advantage in an environment with a highly selective pressure driven by several antibiotics used in the clinical treatments. In this work, a high proportion of multiresistant *S. epidermidis* strains were detected. Possibly, the long exposure of this species to antibiotics has promoted the selection of multiresistant strains.

Clinical surveys indicate that 35-65% of clinically important coagulase-negative staphylococci isolates are

Table 2. Bacteria species isolated from ocular infections along the 5 years (1999-2003) study period.

Panel A: Gram-positive bacteria

Bacteria	Conjunctivitis % (n = 2,997)	Endophthalmitis % (n = 94)	CU % (n = 160)
Staphylococcus epidermidis	60.3	38.3	28.1
Staphylococcus aureus	9.4	15.9	11.9
Micrococcus spp.	15.0	7.4	3.1
Corynebacterium spp.	3.9	5.3	8.7
Streptococcus mitis	3.4	7.4	9.4
Staphylococcus hominis	2.2	2.1	2.5
Staphylococcus warneri	2.2	6.4	1.9
Streptococcus oralis	1.1	1.0	5.0
Streptococcus pneumoniae	0.3	1.0	5.0

Panel B: Gram-negative bacteria

Bacteria	Conjunctivitis % (n = 97)	Endophthalmitis % (n = 10)	CU % (n = 53)
Pseudomonas aeruginosa	7.4	20	26.4
Klebsiella pneumoniae	5.3	1	3.7
Morganella morganii	3.2	0	0.0
Serratia marcescens	7.4	20	9.4
Proteus mirabilis	10.6	1	1.9
Escherichia coli	10.6	1	1.9
Enterobacter cloacae	9.6	1	1.9
Haemophilus aegyptus	9.6	0	0.0
Enterobacter sakasakii	0.0	1	0.0
Pseudomonas fluorescens	3.2	1	17.0
Klebsiella oxytoca	6.4	0	9.4
Moraxella spp.	1.1	0	9.4
Acinetobacter calcoaceticus	0.0	0	7.5

Includes only the most representative bacteria.

resistant to methicillin.[47] Strains obtained from ocular infections studied here exhibited a similar oxacillin resistance, a chemically equivalent drug to methicillin. Vancomycin has become the treatment of choice for oxacillin-resistant S. *epidermidis*.[15] Methicillin and vancomycin resistant *S. aureus* strains have increased.[41] However, clinically relevant coagulase-negative staphylococci with a diminished susceptibility or intermediate resistance to vancomycin have been isolated, ranging from 0.6 to 42% of total isolates.[6,16,19,26,45,55] Although, most clinical *S. epidermidis* isolates remain vancomycin sensitive, several vancomycin-intermediate resistant strains have been reported.[1,16,20,29] In our work, a total of 28% of the *S. epidermidis* strains were intermediate-resistance to vancomycin by broth and agar dilution tests. The vancomycin resistance inferred from disk diffusion tests can be difficult to estimate and frequently induces misclassifications,[50] thus, broth or agar dilution tests or E test are the "gold standard" for determining vancomycin susceptibility, although inconsistencies, even among gold standard methods, have been recognized.[14,16]

The collection of *S. epidermidis* strains with a vancomycin resistance were represented in a dendrogram constructed with antibiotic resistance data in order to ex-

Table 3. Antibiotic resistance among *S. epidermidis* strains isolated from ocular infections. The determination of antibiotic resistance was realized with disk diffusion assay.

	Neo (%)	Gen (%)	Tob (%)	Cip (%)	Nor (%)	Ofl (%)	Cep (%)	Cef (%)	Cet (%)	Pol (%)	Tet (%)	Sul (%)	Van (%)	Oxa (%)
Conjunctivitis	19	50.4	63	21	26.6	21.7	5.0	76.4	48.0	52.4	42.7	74.1	4.2	46.0
CU	29	26.8	46	18	12.5	12.5	8.9	62.5	21.4	25.0	33.9	75.0	0.0	33.9
Endophthalmitis	20	46.3	54	20	22.0	17.1	9.7	41.4	7.3	39.0	31.7	63.4	7.3	34.1

Neo, neomycin; Gen, gentamycin; Tob, tobramycin; Cip, ciprofloxacin; Nor, norfloxacin; Ofl, ofloxacin; Cep, cephalothin; Cef, ceftazidime; Cet, ceftriaxone; Pol, polymyxin B; Tet, tetracycline; Sul, sulfisoxazole; Van, vancomycin; Oxa, oxacillin.

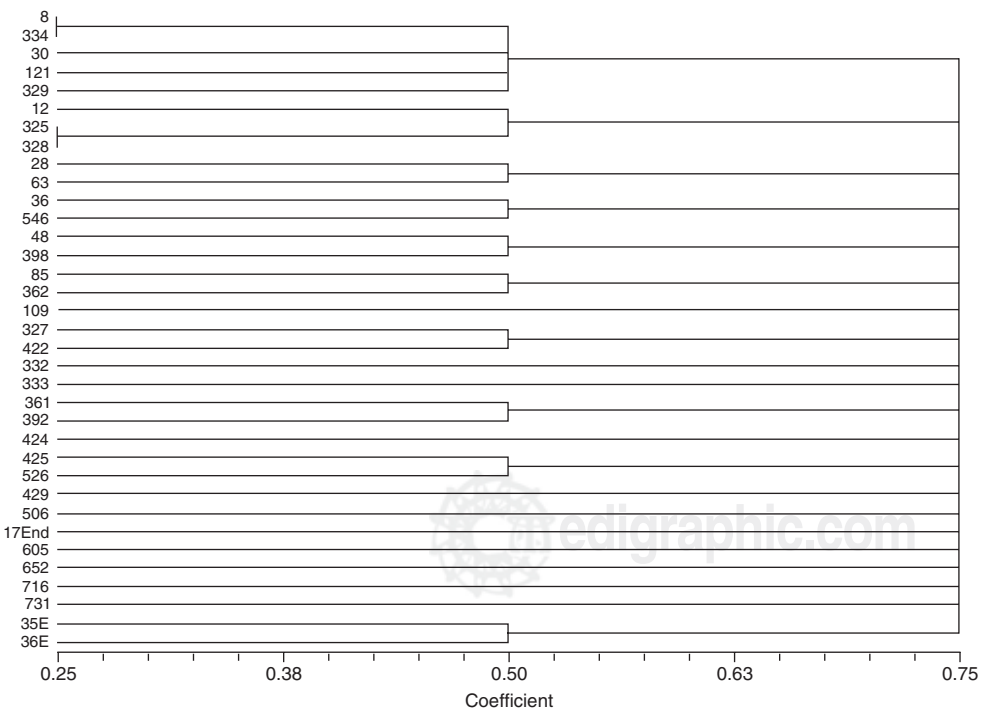

Figure 2. *Dendrogram of* Staphylococcus epidermidis *strains isolated from ocular infections. The dendrograms were generated by the UPGMA method from the multi-resistance data. Cophenetic correlation coefficient = 0.63721; p = 0.0001. This dendrogram was only constructed with multiresistance profile of vancomycin-resistant* S. epidermidis *strains detected by disk diffusion assay.*

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plore the phenotypic diversity. The multiresistance profile cluster analysis of vancomycin- resistant *S. epidermidis* strains showed a high phenotypic diversity and no relationship of the groups with the origin of the strains. This versatility could reflect a broad capacity of the many different strains to colonize and survive in the eye environment and represent a clear potential pathogenic character. More studies to confirm the high variability and eye-specific putative virulence factors are necessary to understand the high prevalence of *S. epidermidis* in eye infections.

Biofilm production by *S. epidermidis* has been considered a putative virulence factor because strains able to form a biofilm are more virulent than biofilm-negative strains.[13] The *ica* operon produces an intercellular adhesin and this adhesin is necessary for cell-to-cell adhesion and biofilm formation.[22] This operon is more prevalent in clinical *S. epidermidis* isolates obtained from catheter-related infections than in isolates from the normal skin and mucosa of healthy individuals.[59] *S. epidermidis* biofilms have been observed in scleral explants of ocular infections, endophthalmitis, and bacterial keratitis.[5,37] Our results show that, regardless of the origin of the strains, the studied *S. epidermidis* strains have a broad biofilm formation capacity.

Additionally, bacteria within the biofilm increases their antibiotic resistance and display a different expression profile that non-associated bacteria.[23,34,43,58] In this work, the capacity to form biofilms was equally distributed among the *S. epidermidis* strains with a vancomycin-intermediate resistance or antibiotic multiresistance. Thus, the multiresistance mechanisms of the studied strains were independent from the capacity to form biofilms, but this population organization can potentiate the resistance effect. The antibiotic resistance of these strains must be confirmed using an artificial biofilm model to compare the effects of antibiotics on cells, as has been previously performed.[35] The genetic and biochemical details of the resistance by biofilms are now beginning to emerge.

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