Antimicrobial Resistance and Production of Biofilms in Clinical Isolates of Coagulase-Negative *Staphylococcus* Strains

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Coagulase-negative *Staphylococcus* (CNS) strains are frequently associated with bacteremia and hospital-acquired infections. 293 CNS strains were isolated from 744 samples from a dialysis center in S. M. de Tucumán, Argentina, from hemocultures, catheters and urine and identified as *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. hominis* and *S. cohnii*. 13 antibiotics were tested for antibacterial resistance. 75% of *S. saprophyticus*, 66% of *S. epidermidis* and 57% of *S. haemolyticus* was resistant to erythromycin and 50% of *S. haemolyticus* was resistant to ciprofloxacin. OXA resistance was found in 43% of *S. haemolyticus*. Presence of PBP 2a in OXA-R strains was confirmed with the modified agglutination assay (MRSA) and presence of the *mecA* gene. 15 strains with intermediate halos for vancomycin and teicoplanin showed a MIC in solid and liquid medium ≤ 1 $\mu\text{g/ml}$ and ≤ 4 $\mu\text{g/ml}$ respectively, which means that they were susceptible. 30% of the CNS strains produced biofilms: 42% of *S. haemolyticus* and 40% of *S. epidermidis*. 76% of the *S. epidermidis* and 59% of the *S. haemolyticus* biofilm-producing strains were isolated from catheters. The disc-diffusion technique together with the agglutination assay proved to be fast and reliable, which is of essential importance in intensive care units. Rapid identification of CNS strains, detection of resistance to methicillin and biofilm production are decisive for a prompt and appropriate antimicrobial therapy and limited use of inappropriate glycopeptides.

Key words coagulase-negative *Staphylococcus*; antimicrobial resistance; biofilm

Coagulase-negative *Staphylococcus* (CNS) strains are frequently associated with bacteremia and hospital-acquired infections, particularly in patients with catheters or other plastic devices. They include a great variety of species such as *S. epidermidis*, *S. hominis*, *S. haemolyticus*, *S. saprophyticus*, *S. simulans* and *S. warneri*, which have shown increasing resistance to antibiotic treatment, mainly to penicillin and synthetic β -lactams represented by oxacillin. Many of these oxacillin-resistant microorganisms (OXA-R) also present resistance to other antimicrobes, thus limiting the use of glycopeptides like vancomycin and teicoplanin. *Staphylococcus* isolations with reduced susceptibility to vancomycin have been described.^{1–3)}

OXA-R CNS strains possess the *mecA* gene, which codes for a modified 78-kDa protein, PBP 2a, which is responsible for the resistance to β -lactams such as dicloxacillin, methicillin, nafcillin and oxacillin. The protein is able to bind to these antibiotics with very low affinity. The presence of the *mecA* gene in *Staphylococcus* strains is a synonym for OXA-R and so the NCCLS (National Committee for Clinical Laboratory Standards) recommends that OXA-R CNS strains isolated from patients with serious infections should be tested for the *mecA* gene or PBP 2a presence.^{4,5)}

Biofilms are microbial communities wrapped in a matrix of exo-polysaccharides produced by the bacteria, which are adhered to an inert surface. This property becomes important in immunocompromised patients, because it produces chronic infections. *S. epidermidis* is thus far the most well known biofilm producer.^{6,7)}

Little is known about the mechanism of resistance (R) to antimicrobial agents in bacterial biofilm producers but apparently it limits antimicrobial diffusion and induces physiological alterations associated with low bacterial growth and the appearance of atypical phenotypes in these cells.^{8–15)}

The objectives of the present paper were to detect mechanisms of antimicrobial resistance and biofilms production in clinical isolates of CNS strains.

MATERIALS AND METHODS

CNS Identification 293 CNS strains were isolated from 744 samples from a dialysis center in S. M. de Tucumán, Argentina: 156 (53%) from urine, 107 (37%) from catheters and 30 (10%) from hemocultures. The bacteria isolated were characterized with conventional biochemical methods: catalase, bacitracin susceptibility and coagulase testing. The scheme proposed by Kloos and Schleifer, 1986, was used, which allowed identification of the CNS strains with the following tests: novobiocin resistance; urea hydrolysis; fermentation of: sucrose, xylose, maltose, trehalose, mannitol, cellobiose and raffinose; phosphatase; nitrate reduction and use of arginine.^{16,17)}

Susceptibility to Antimicrobial Agents Antimicrobial sensitivity was carried out with the disc-diffusion technique in solid medium according to the NCCLS. The following antimicrobial agents were assayed: oxacillin (OXA) 1 μg , vancomycin (VAN) 30 μg , teicoplanin (TEI) 30 μg , gentamicin (GEN) 10 μg , ciprofloxacin (CIP) 5 μg , chloramphenicol (CMP) 30 μg , minocycline (MINO) 30 μg , ofloxacin (OFL) 5 μg , clindamycin (CLIN) 2 μg , erythromycin (ERY) 15 μg , tetracycline (TET) 30 μg , rifampin (RIF) 5 μg , trimethoprim-sulfamethoxazole (TMS) 1.25/23.75 μg .⁵⁾

Phenotypic Assays of the Resistance Mechanism a) Resistance to ERI-CLIN: The ERY-CLIN double disc method was used, which allows phenotypic detection of the inducible methylase (MLS) phenomenon, according to the Hamilton-Miller methodology. This method consists of a flattening of the halo in the CLIN zone due to proximal inhibition pro-

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duced by the ERI disc. This indicates the presence of MLS and resistance to both ERI and CLIN.^{18,19)}

b) Resistance to VAN and TEI: All strains with a halo ≤ 17 mm with VAN and ≤ 13 mm with TEI were screened on Blood-Heart-Infusion (BHI) agar plates with 6 mg/l of both VAN and TEI. The plates were inoculated with 10 μ l of a 0.5 McFarland suspension on the spot and incubated at 35 °C for 24 h.

In accordance with the NCCLS recommendations the MIC for VAN and TEI was assayed using the dilution method in liquid medium and on agar. MICs of ≤ 8 μ g/ml (VAN) and ≤ 16 μ g/ml (TEI) were considered as reduced susceptibility.

c) Resistance to β -Lactams: Of all the CNS strains that produced a halo ≤ 17 mm for OXA using the disc-diffusion method, the PBP 2a protein, a *mecA* gene product, was assayed.

The method consists of an agglutination assay with latex particles coated with monoclonal antibodies against the protein (Slidex MRSA detections). The assay was carried out following the manufacturer's instructions on the commercial kit, except for the following modifications: 3 and 6 loops of 1 μ l were used as inoculum and reading intervals were 3, 6 and 15 min. The assay was carried out with and without induction of oxacillin.^{20–22)}

Genotypic Assays of the Resistance Mechanism Confirmation of oxacillin resistance was carried out by a polymer chain reaction (PCR) through detection of the *mecA* gene.²³⁾

Primers: The following primers were used: M1 and M2 that amplify a 310-bp fragment of the *mecA* gene, which codes for the PBP 2a protein, and DG74 and RW01 that amplify highly conserved regions of the 16S RNA, present in most pathogenic bacteria, obtaining a 370-bp fragment.

DNA Extraction: The template was prepared selecting 5 colonies of (all OXA resistant strains) to be assayed, which were resuspended in 150 μ l of 1% Triton $\times$ 100 in TE buffer $\times$ 1 or sterile bi-distilled water. This suspension was heated at 100 °C for 10 min in a water bath and afterward centrifuged at 10000 rpm for 5 min. Five microliters of the supernatant were taken for further assays.

PRIMER: M1, TGGCTATCGTGTGTCACAATCG

PRIMER: M2, CTGGAACCTGTTGAGCAGAG

PRIMER: DG74, AGGAGGTGATCCAACCGCA

PRIMER: RW01, AACTGGAGGAAGGTGGGGAT

PCR: The mixture selected for PCR consisted of 5 μ l of PCR buffer 10 $\times$ (final concentration: 50 mM KCl, 0.01% gelatin, 10 mM Tris-HCl; pH 8.3), 1.5 mM MgCl₂, 0.1 mM dNTP (dATP, dCTP, dGTP and dTTP), 0.4 pmol/ μ l of each of the specific primers, 2 U of tag polymerase and 5 μ l of the template to get a final reaction volume of 50 μ l. All components were mixed in polystyrene tubes and subjected to amplification temperatures of the nucleic acid in a thermocycler.

DNA from the Joffe reference strain (methicillin resistant *S. aureus* obtained from the Antimicrobial Service of the Malbran Institute, Buenos Aires) was used as positive control of the reaction and sterile bi-distilled water as negative control. The mixture was initially denatured at 94 °C for 5 min and then underwent 29 cycles of: 30 s at 94 °C (denaturing), 30 s at 52 °C (annealing) and 30 s at 72 °C (extension). The reaction was finished with 5 min at 72 °C.²⁴⁾ The amplification products were detected with agarose gel electrophoresis (1% in TAE 1 $\times$ buffer) at 80 V for 30 min.

Biofilm Production Biofilm production was carried out with the micro method according to Pfaller *et al.* using radiation-sterilized, flat-bottomed, polystyrene multiple well plates for cell culture (polystyrene microtiter plate biofilm assay). Two biofilm-producing strains (263 and 1174) and a non-producing strain (ATCC 918) were used as controls.²⁵⁾

Brucella Agar (Difco) was used to maintain the strains at room temperature. They were cultured in BHI broth and incubated at 37 °C for 18 h to initiate the assays. All the cultures were standardized through a standard dilution with BHI (4 °C) characterized by Experimental Transmittance T=30, OD₆₁₀. Growth was stopped at 4 °C by immersion in ice-cold water. From this standard dilution 50 ml of a working suspension with corrected transmittance T=95 (25 μ l of T=30 en 5 ml of refrigerated BHI) was prepared. This dilution was kept at 4 °C, thus assuring cellular quiescence.

Biofilm Production in Multiple Well Assay One hundred microliters of each bacterial suspension and the blank (BHI) were deposited in quadruplicate in each well, maintaining the plate quiet at 37 °C during the incubation. The medium was extracted by inversion and washed twice with PBS buffer (8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 140 mM NaCl and 3 mM KCl; pH 7.2). The contents were emptied by inversion and the plate was dried upside down on absorbent paper. Once dry, when the biofilm showed as a whitish and opaque membrane, it was fixed with methanol for 7 min (Christensen, 1985). The plate was emptied again by inversion and it was dried in the air. When it was dry it was dyed with a watery solution of 0.1% safranin. After 4 min the plate was inverted, washed with water and after inversion it was left to dry.²⁶⁾

Reading was carried out through an ELISA reader at 490 nm. Reading means were analyzed for each strain and they were compared with the blank in order to define the biofilm production. BHI was used as reading blank and the value for determining negative or positive was established calculating the standard deviation of the blank.

RESULTS

Identification: The 293 CNS isolates were identified as: *S. epidermidis* (37%, 32%, 15%), *S. haemolyticus* (13%, 25%, 35%), *S. saprophyticus* (0%, 7%, 20%), *S. hominis* (10%, 5%, 15%), *S. cohnii* (6%, 0%, 28%) from hemocultures, catheters or urine respectively (Fig. 1).

Resistance Patterns: Resistance profiles of the CNS strains against the 13 antibiotics assayed are presented in Table 1. Resistance to ERY was found in 75% of *S. saprophyticus*, 67% of *S. epidermidis* and 57% of *S. haemolyticus* and inducible methylase was found in 50% of *S. saprophyticus*, 17% of *S. epidermidis* and 54% of *S. haemolyticus* (Table 1).

Resistance to TET was observed in 50% of *S. epidermidis* and 43% of *S. haemolyticus* and 50% of the latter was resistant to CIP, whereas *S. cohnii* and *S. epidermidis* showed low resistance: less than 15% of the strains to CIP. *S. saprophyticus* showed high susceptibility to TMS, CIP, GEN, OXA, TET, VAN, TEI, RIF, CMP, OFL and MINO. Of all the strains studied only 15 demonstrated intermediate halos for VAN and TEI, but none of these grew on screening plates and the MIC in solid and liquid medium was less than 1 μ g/ml and less than 4 μ g/ml respectively, thus proving that

Fig. 1. Distribution According to Origin of the 293 Coagulase-Negative *Staphylococcus* (CNS) Strains Isolated from Dialysis Patients

Table 1. Resistance Profile of 293 CNS Strains against 13 Antimicrobial Agents, Using the Disc-Diffusion Technique

Species	Antimicrobial resistance (%)													Inducible methylase MLS
	TMS	CIP	GEN	OXA	CLIN	ERY	TET	VAN	TEI	RIF	CMP	OFL	MINO	
<i>S. epidermidis</i>	17	10	17	16	17	67	50	0	0	11	50	0	8	17
<i>S. haemolyticus</i>	29	50	43	43	50	57	43	0	0	0	36	25	8	54
<i>S. hominis</i>	17	6	17	17	17	37	0	0	0	17	0	17	83	18
<i>S. cohnii</i>	18	15	18	18	27	36	36	0	0	17	0	12	9	17
<i>S. saprophyticus</i>	0	0	0	0	75	75	0	0	0	0	0	0	0	50

TMS (trimethoprim-sulfamethoxazole 1.25/23.75 µg), CIP (ciprofloxacin: 5 µg), GEN (gentamicin: 10 µg), OXA (oxacillin: 1 µg), CLIN (clindamycin: 2 µg), ERY (erythromycin: 15 µg), TET (tetracycline: 30 µg), VAN (vancomycin: 30 µg), TEI (teicoplanin: 30 µg), RIF (rifampin: 5 µg), CMP (chloramphenicol: 30 µg), OFL (ofloxacin: 5 µg), MINO (minocycline: 30 µg), and MLS (inducible methylase).

all the CNS strains assayed were sensitive to these antimicrobes.

Those strains that were resistant to OXA showed a diameter of the bacterial growth inhibition less than 17 mm. With the agar diffusion method OXA resistance was detected in 43% of *S. haemolyticus*, 18% of *S. cohnii* and in 16% of *S. epidermidis* and 17% of *S. hominis*. Presence of PBP 2a was confirmed in all OXA resistant strains with the agglutination assay. It could be observed that with a longer incubation time and higher initial inoculation sensitivity of the method increased as well as the OXA-induced strains.

PCR confirmed the presence of the *mecA* gene in all CNS strains with OXA resistance observed with phenotypic techniques. There was no discrepancy between the disc-diffusion technique and MRSA agglutination, observing excellent sensitivity and specificity.

From all the assays carried out *S. haemolyticus* was the species with the highest resistance profile.

Biofilm Production: 30% of the 293 CNS strains assayed produced biofilms: 42% of *S. haemolyticus*, 40% of *S. epidermidis*, 29% of *S. saprophyticus* and 12% of *S. cohnii*, but none of the *S. hominis* strains. Of all the biofilm-producing strains 76% of *S. epidermidis* and 59% of *S. haemolyticus* were isolated from catheters; the remaining 24% and 41% respectively were from urine samples. No biofilm-producing CNS strain was isolated from hemocultures (Table 2).

The results showed that in *S. haemolyticus* and *S. epidermidis* the biofilm-producing strains presented a high percentage of resistance to two or more antibiotics: 29 strains out of 37 biofilm-producing *S. haemolyticus* (78%), and 20 strains

Table 2. Five Biofilm-Producing Coagulase-Negative *Staphylococcus* (CNS) Species (Comprising 160 out of 293 Isolated Strains) and Their Origin (Uroculture, Catheter or Hemoculture)

Species	Number of strains <i>n</i> =293	Biofilm-producing strains <i>n</i> =88	Origin of biofilm-producing strain		
			Uroculture	Catheter	Hemoculture
<i>S. haemolyticus</i>	88	37 (42%)	15 (41%)	22 (59%)	0
<i>S. epidermidis</i>	73	29 (40%)	7 (24%)	22 (76%)	0
<i>S. cohnii</i>	59	7 (12%)	7 (100%)	0	0
<i>S. saprophyticus</i>	51	15 (29%)	15 (100%)	0	0
<i>S. hominis</i>	22	0	0	0	0

Table 3. Classification of Biofilm-Producing Coagulase-Negative *Staphylococcus* (CNS) Strains and Their Resistance to Two or More Antimicrobial Agents

Species	Biofilm-producing strains <i>n</i> =88	Resistance to 2 or more antimicrobial agents <i>n</i> =88
<i>S. haemolyticus</i>	37	29
<i>S. epidermidis</i>	29	20
<i>S. cohnii</i>	7	15
<i>S. saprophyticus</i>	15	24

out of 29 *S. epidermidis* (69%) showed resistance to two or more antimicrobial. This correlation was not observed in *S. cohnii* and *S. saprophyticus* (Table 3).

S. haemolyticus was the highest biofilm-producing strain

and the species that revealed the highest percentage of resistance to the antibiotics assayed.

DISCUSSION

Although there exist about 20 CNS species only a few of them have been associated with an increase in hospital-acquired infections due to the use of invasive elements. Numerous authors have suggested the need to identify and characterize the species so as to find out the most frequently isolated ones and their association with different degrees of resistance.^{27,28)}

Finelli *et al.* reported in a survey with dialysis patients carried out in 2002 that 40 to 60% of the infections was caused by CNS species.²⁹⁾

The National Reference Laboratory for *Staphylococcus* in the U.S.A., directed by Dr. Peter Petras, reports that the isolated CNS strains from clinical samples from human origin are *S. epidermidis* (30%), *S. hominis* (14%) and *S. haemolyticus* (14%).³⁰⁾

Worthington *et al.*, identified from 56 CNS strains isolated from catheters 54 *S. epidermidis*, 1 *S. lugdunensis* and 1 *S. hominis*. Similarly, De Paulis *et al.*, reported that the isolated CNS strains were: *S. epidermidis* (51%), *S. haemolyticus* (18%), *S. saprophyticus* (16%), *S. lugdunensis* (6%) and *S. warneri* (2%). The results obtained in our study are similar to those of the aforementioned authors coinciding with the fact that the most frequently isolated CNS strain was *S. epidermidis*, followed by *S. haemolyticus* and *S. hominis*.^{31,32)}

Antibiotic susceptibility studies are very important from an epidemiological point of view, because they allow establishing policies for adequate and rational use of antibiotics, thus increasing the quality of attention toward the patient, protecting the lifespan of these drugs and giving economic benefits.

Resistance to antibiotics both in hospitals and the community is an increasing problem in medical treatment. Of particular interest are methicillin resistant (Meth R) *Staphylococci* that carry R genes to other antibiotics such as ERY, TET, streptomycin, TM, GEN and Sulfonamides.³³⁾

Louis *et al.* and Archer, have pointed out the importance to distinguish between *S. aureus* and CNS in clinical samples and their behavior toward antimicrobial agents in order to implement a fast and appropriate antimicrobial treatment.^{16,34)}

Prevalence of methicillin-resistant *Staphylococci* in hospital-acquired infections has increased during the last decade, as well as in community-acquired infections in patients without any clear risk.³⁵⁾

Garrett *et al.* detected and characterized CNS strains with diminished susceptibility to VAN and Sierazki *et al.* detected resistant strains. Failures in the treatment with VAN have been described in Japan and the U.S.A. In a survey in France 23% of 362 CNS strains presented intermediate resistance to TEI; 74% of the resistant strains was identified as *S. epidermidis* and 19% as *S. haemolyticus*.^{36–38)}

In Virginia, 13% of *S. haemolyticus* strains isolated during a period of 10 years showed resistance to VAN and 62% of the isolates had a MIC superior to 6.25 µg/ml.³⁹⁾ Our results demonstrated methicillin resistant CNS strains, but no resistance or diminished susceptibility to glycopeptides was ob-

served.

In Havana, Cuba, González *et al.* detected presence of MLS phenotype in 17% of the strains, 28% of which belonged to *S. aureus* and 5% to a CNS strain.⁴⁰⁾ The results obtained in our study showed that 45% of the CNS isolates presented this phenomenon with a higher incidence in *S. haemolyticus* and *S. saprophyticus*: 54 and 50% respectively.

Several authors have observed the presence of a *mecA* gene in different CNS strains and its correlation with resistance to OXA. However, there have been discrepancies between the genetic results of the presence of the *mecA* gene with phenotypic assays (disc-diffusion) and MIC for resistance to OXA. Recently, the disc-diffusion assay for OXA has been questioned.^{21,34,41)}

The results in our study coincide with the PBP 2a detection with slidex MRSA and detection of the *mecA* gene showing 100% specificity, which agrees with the results by Corso *et al.*²⁰⁾

Although trimethoprim is no drug of choice for *Staphylococci* treatment it is used in combination with sulfamethoxazole to achieve effective treatments in patients infected with multi-resistant *Staphylococci*. Studies carried out by González *et al.* showed resistance of CNS strains to TM. Between 0 and 29% of our strains were resistant to TMS. Our results with ERY, VAN and TEI were similar to those obtained by Barberis *et al.* but resistance to TET and CIP was higher.^{40,45)}

Since *Staphylococci* belong to the normal flora of the skin, interpretation of their isolation is a great challenge to the microbiologist. They must evaluate the significance of their findings through detection of biofilm-producing strains, because there do not exist reliable markers that allow identification of pathogens.⁴²⁾

Staphylococcus is a bacterial pathogen that usually produces biofilms during different infectious processes, which are generally chronic and difficult to treat. As a result, higher doses of antibiotics are needed or sometime a prolonged period of administration to eliminate the planktonic bacteria of exactly the same strain.⁴³⁾

It is well known that a microorganism that is sensitive *in vitro* might be unaffected *in vivo*. In human medicine it has been estimated that 65% of the hospital-acquired infections is associated to a biofilm and that they are 10 to 1000 times more difficult to eliminate with an otherwise successful treatment.^{5,42,44)}

Infection is an important complication of patients undergoing dialysis and 40 to 60% is due to CNS strains. Barberis *et al.* have published about biofilm production in CNS strains associated to human infections and their results are similar to ours.⁴⁵⁾

Rapid identification of CNS strains and detection of resistance to methicillin are essential for a prompt and appropriate antimicrobial therapy and limited use of inappropriate glycopeptides.

Sensitivity and specificity of the PCR technique applied in our study for detection of the *mecA* gene in CNS strains was comparable with phenotypic methods. Consequently, it may be concluded that due to the equipment and supplies needed for PCR, the disc-diffusion technique together with agglutination can be used as a reliable alternative for fast results, especially necessary in intensive care units.

In our study the CNS strains presented a significant association between biofilm production and resistance to a variety of antimicrobial agents, mainly *S. haemolyticus*, which was the species with the highest biofilm production and antimicrobial resistance. It is important to detect biofilm-producing bacteria in order to implement an appropriate antimicrobial therapy at an early stage.⁴⁶⁾

Our results confirm that the CNS strains isolated in the Tucumán area possess high resistance to methicillin and most of them are resistant to two or more antibiotics. Besides, they frequently produce biofilms in infections associated with dialysis patients.

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