Original Article

Survey of molecular determinants in Gram-positive cocci isolated from hospital settings in Argentina

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Abstract

Background: In order to study the resistance mechanisms to aminoglycosides, tetracyclines and erythromycin, we investigated the genetic determinants on 85 *Streptococcus* spp., *Staphylococcus* spp., and *Enterococcus* spp. isolates collected from 46 hospitals of Argentina over a two-year period.

Methodology: The MICs to amikacin, gentamicin, kanamycin, and streptomycin, tetracycline and erythromycin were determined by the standard broth dilution method according to CLSI recommendations. Detection of resistance genes to the antibiotic tested was assessed by the PCR standard technique whereas the clonal relationships of each species was performed by PFGE.

Results: Major heterogeneity was detected in aminoglycoside and erythromycin resistances. Indeed, 37.6% of the isolates harbored the *aac(6')-aph(2'')* genes; 27% harbored the *aph(3')-IIIa* and *ant(6)-Ia* genes along with the *aac(6')-aph(2'')* gene; 7% carried the *ant(4')-Ia* gene; and 71% harbored one or more of the *erm*(A), *erm*(B), *erm*(TR), *mef*(A), *mef*(E) and *msr*(A) genes. The tetracycline resistance was determined by the *tet*(M) gene and was found in 23 isolates that were resistant to this antibiotic. Spreading of *tet*(M) by the Tn*916*-like transposon was not a frequent event since the integrase of this element was detected only in 3 *Streptococcus* spp. isolates. Instead, a 370 bp fragment was detected that corresponded to a region of the *CW459*-like element integrase in 10 of 11 methicillin-resistant *Staphylococcus aureus* and in 3 group G *Streptococcus* isolates, a finding that implies a novel mechanism for tetracycline resistance spreading.

Conclusion: This study demonstrates the wide spreading of resistance mechanisms in our nosocomial cocci population and underscores the importance of continuous and efficient epidemiological surveillance.

Key Words: Gram-positive cocci, multiple resistance, transposon.

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Introduction

The incidence of infections caused by multidrug-resistant Gram-positive organisms has been increasing over the last 20 years as a major clinical problem. Troublesome pathogens in our country include the following: *Staphylococcus aureus* isolates resistant to methicillin, macrolides, lincosamides and aminoglycosides; coagulase-negative *staphylococci* resistant to beta-lactams, aminoglycosides, macrolides, lincosamides and glycopeptides; *Streptococcus pyogenes* and groups B, C, and G *Streptococcus* resistant to macrolides; *Streptococcus pneumoniae* resistant to beta-lactams and macrolides; viridans group streptococci resistant to beta-lactams and aminoglycosides; enterococci resistant to vancomycin, teicoplanin and highly resistant to penicillins and aminoglycosides. The great variability of germs involved requires the performance of a continuous survey of the prevalent microorganisms and their susceptibility.

A number of genes that code for acetyltransferase, phosphotransferase and adenylyltransferase activities are responsible for aminoglycoside resistance. The broadest heterogeneity has been seen in *Enterococcus* spp. where genes such as *aac(6')-aph(2'')*, *ant(9)-I* (or *aad(9)*), *aph(2'')-Ie*, *aph(2'')-Id*, *aac(6')-aph(2'')*-bifunctional, *aph(3')-IIIa*, *ant(4')-Ia*, *aph(2'')-Ib*, *aac(6')-Im*, *aph(2'')-Ib*, *ant(6)-Ia* (or *aadE*), and *aac(6')-Ii* were described (4,10,14,21). In *Staphylococcus*, however, the most commonly described determinants are the following: *aac(6')-aph(2'')*, *aph(3')-IIIa*, *ant(4')-Ia*, *ant(6)-Ia* and *aadA* (1). Of all the genes described, *aac(6')-aph(2')*, *aph(3')-IIIa* and *ant(6)-Ia* have been found to be carried in transposons such as Tn*4001* or Tn*5405* (2,7).

A number of *tet* determinants were described that generate antibiotic efflux or ribosomal protection in Gram-positive cocci such as *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(T), *tet*(U), *tet*(S), *tet* (W) and *tet*38 (19). The *tet*(M) gene is widely spread and is usually found in mobile elements such as the Tn*916*-Tn*1545* family of transposons (16), Tn*5397* (18) and the newly described Tn*2009* and Tn*2010*, (6) where the *tet*(M) gene is cotransferred with macrolide - resistance genes *mef*(A) and *msr*D or with *erm*(B) respectively. Other elements described to carry *tet*(M) were Tn*5801* in *Staphylococcus aureus,* and the conjugative element *CW459* in *Clostridium* spp. (8,18). According to the authors, the nucleotide sequences of Tn*916*, Tn*5801* and *CW459* are related and the encoded integrases of Tn*5801* and *CW459* are identical; however, they are different from that of Tn*916* with an amino acid identity less than 19% (8).

The macrolide-resistance mechanisms described in Gram-positive cocci include target-site modification (*erm*(A), *erm*(B), *erm*(C), *erm*(M), *erm*(TR) and *erm*(Y)), which lead to expression of the MLS_B resistance phenotype, efflux (*mef*(A), *mef*(E), *msr*(A), *msr*(B), *msr*(D), *vga*(A), *vag*(A), *vga*(B), *lsa*, *lin*(A), *lin*(A´) and *mde*A), that differ in the drug specificity and location, and finally, enzyme inactivation that comprises the activity of phosphotranferase (*mph*(C)) or esterase (*ere*(A)) (10,13,20,21,23).

The aim of this study was to establish which genes were involved in the resistance pattern exhibited by 85 Gram-positive cocci isolates of the *Staphylococcus*, *Enterococcus* and *Streptococcus* genera collected from nosocomial patients with invasive diseases over a two-year period. We performed a molecular study that included 46 hospitals from 16 cities of Argentina where we analyzed the possible source of antimicrobial resistance determinants found in these isolates. This study also describes the spreading of tetracycline resistance by transposable elements between staphylococci and group G streptococci.

Materials and Methods

Bacterial strains

Eighty-five bacterial strains, 54 belonging to the genera *Streptococcus* spp: *Streptococcus pyogenes*. (SGA n=11), *Streptococcus agalactiae* (SGB n=10), *Streptococcus dysgalactiae* subsp *equisimilis* group C (SGC n=10) and group G (SGG n=23), 20 *Staphylococcus* spp. [eleven methicillin-resistant *Staphylococcus aureus* (MRSA) and nine coagulase-negative staphylococci (CoNS)] and eleven vancomycin-resistant *Enterococcus faecium* (VRE) were investigated. The isolates were epidemiologically unrelated and collected from different hospitals at distant locations in Argentina. The isolates were obtained over a twenty-month period between 1999 and 2000. The sources of infections ranged from pharingitis to bacteremia (Table 2). Culture conditions. Bacteria were grown on Columbia agar plates (Merck, Darmstadt, Germany) supplemented with 5% v/v of sheep blood and incubated for 24 hours at 37℃ in the presence or absence of a 5% CO2 atmosphere.

Susceptibility test

The MICs to amikacin, gentamicin, kanamycin, streptomycin, tetracycline and erythromycin (Roemmers) were determined by standard broth dilution method according to CLSI recommendations for each genera studied using *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 as controls (5). Briefly, the broth dilution method was performed: 2-fold serial dilutions of the antibiotic were made in Mueller Hinton broth which was inoculated with a standardized number of bacteria and incubated for 18 to 20 hours at 37^0C. The lowest concentration (highest dilution) of antibiotic preventing appearance of turbidity was considered to be the minimal inhibitory concentration (MIC).

PCR amplification

Genomic DNA was extracted with guanidinium thiocyanate method as described by Pitcher *et al*. (14). The presence of *aac(6')-aph(2'')*, *aph(3')-IIIa*, *ant(4')-Ia*, *aadA*, *ant(6)-Ia*, *tet*(M), *tet*(O), *tet*(K), *tet*(L), *mef*(A), *mef*(E), *msr*(A), *erm*(A), *erm*(B), *erm*(C), *erm*(M), *erm*(TR), *qacE*Δ1 (antiseptic resistance) and *sat4* (streptothricin resistance) was determined by the standard Polymerase Chain Reaction (PCR) technique. All PCR amplifications were carried out by denaturation over 10 minutes at 94^0C, 35 amplification cycles of 1 minute at 94^0C, different annealing temperatures for 1 min (Table 1) and a final extension at 72^0C using the primers described in Table 1.

The PCR products were sequenced using the ABI3730XL system (Macrogen, Korea).

Table 1. Primers used in this study.

Gene name or transposon	Primers sequence	Annealing temperature	Product size	GenBank No.
aac(6')-aph (2'')	aacF: 5'-CCAAGAGCAATAAGGGCATA-3' aacR: 5'-CACTATTATAACCACTACCG-3'	55℃	220 bp	M18086
aph(3')-IIIa	aphF: 5'-GCCGATGTGGATTGCGAAAA-3' aphR: 5'-GCTTGATCCCCAGTAAGTCA-3'	55℃	292 bp	AF061336
ant(4')-Ia	ant4F: 5'-GCAAGGACCGACAACATTTC-3' ant4R: 5'-TGGCACAGATGGTCATAACC-3'	55℃	165 bp	CP000253
aadA	aadaF: 5'-TTGCTGGCCGTACATTTG-3' aadaR: 5'-TCATTGGCGTGCCATTC-3'	55℃	266 bp	AF052459
ant(6)-Ia	ant(6)-IaF: 5'-ACTGGCTTAATCAATTTG-3' ant(6)-IaR: 5'-GCCTTTCCGCCACCTCACCG-3'	55℃	597 bp	AF330699
tet(M)	tetmF: 5'-TTATCAACGGTTTATCAGG-3' tetmR: 5'-CGTATATATGCAAGACG-3'	46ºC	397 bp	X90939
tet(O)	tetoF: 5'-AACTTAGGCATTCTGGCTCAC-3' tetoR: 5'-TCCCACTGTTCCATATCGTCA-3'	55℃	515 bp	M18896
tet(K)	tetkF: 5'-TCCTGGAACCATGAGTGT-3' tetkR: 5'-AGATAATCCGCCCATAAC-3'	50ºC	189 bp	S74032
tet(L)	tetlF: 5'-TGAACGTCTCATTACCTG-3' tetlR: 5'-ACGAAAGCCCACCTAAAA-3'	50ºC	993 bp	U17153
erm(A)	ermaF: 5'-TCTAAAAAGCATGTAAAAGAA-3' ermaR: 5'-CTTCGATAGTTTATTAATATTAGT-3'	52℃	645 bp	1
erm(B)	ermBF: 5'-GAAAAGGTACTCAACCAAATA-3' ermBR: 5'-AGTAACGGTACTTAAATTGTTTAC-3'	55℃	639 bp	1
erm(C)	ermCF: 5'-TCAAAACATAATATAGATAAA-3' ermCR: 5'-GCTAATATTGTTTAAATCGTCAAT-3'	46℃	642 bp	1
erm(M)	ermMF: 5'-TCGGCTCAGGAAAAGGG-3' ermMR: 5'-CAAGTTAAGGATGCAGT-3'	50℃	658 bp	M12730
erm(TR)	ermTRF: 5'-TTGGGTCAGGAAAAGGA-3' ermTRR: 5'-GGGTGAAAATATGCTCG-3'	48℃	385 pb	AF002716
mef(A)	mefAF: 5'-CGTAGCATTGGAACAGC-3' mefAR: 5'-TGCCGTAGTACAGCCAT-3'	50℃	316 bp	U70055
mef(E)	mefEF: 5'-CGTAGCATTGGAACAGC-3' mefER: 5'-TCGAAGCCCCCTAATCTT-3'	50℃	513 bp	U83667
msr(A)	msrAF: 5'-CACGTTAGACGGTAGTTT-3' msrAR: 5'-TTCGTTCTTTCCCCACC-3'	50℃	1000 bp	AB016613
Tn*4001*	IS256U + aacF IS256U + aacR	54℃	1,500 bp	M18086
Tn*916*	916F:5'-CGTGGAAACTTGTGGCTA-3' 916R :5'-GATAGGCTTCTTCAACCA-3'	50^{0}C	716bp	U09422
Tn*CW459*	CW459F:5'-CTTGGGATAACCACCACA-3' CW459R: 5'-GTACTTCCTTCCATTCGG-3'	50^{0}C	370bp	AF329848
IS*1181*	IS1181F: 5'-GCCTTCGGCCTGTTATTGT-3' IS1181R :5'-GGCGGCCAGTCCATTATT-3'	54℃	1,200 bp	L43098
IS*1182*	IS1182F:5'-ATGGGCGCGTTTCTTCTC-3' IS1182R :5'-CCATTTGAGAGAGGTGCT-3'	52℃	1,135 bp	L43098
IS*256*	IS256F: 5'-GCGAAGAGATTCAAAGCA-3' IS256R:5'-CCATTCATCATGTAGGTCC-3'	52℃	1,106 bp	M18086
IS*1216*	IS1216F: 5'-GCGGTTAGTGAAGCAGTT-3' IS1216R: 5'-GTGCCTTCTTTTCGGGTT-3'	50℃	270 bp	X69092
IS*256*	IS256U:5'-CGCATCTTCCCCAATCA-3'			M18086
qacEΔ1	QacE deltaF: 5'-GCGAAGTAATCGCAACATCC-3' QacEdeltaR: 5'-AGCCCCATACCTACAAAGCC -3'	55^{0}C	280 bp	U49101
sat4	Sat4U: 5'-ACCCAGCGAACCATTTGA-3' Sat4L: 5'-GTCCTGGGTTTCAAGC-3'	45^{0}C	380 bp	U73026

Transposon detection

The mobile elements Tn*4001* (product size: 1,500 bp), Tn*5405*, the integrases of "Tn*916*-like" and "*CW459*-like" elements and the insertion sequences IS*256*, IS*1181*, IS*1182*, IS*257* and IS*1216* were investigated by PCR. Table 1 lists all

primers that were used, as well as annealing temperatures and PCR product sizes. The protocol included a denaturation step of 5 minutes at 95^0C followed by 30 amplification cycles at different annealing temperatures (Table 1), 1 or 2 minutes at 72^0C, and final extension of 10 minutes at 72^0C. Sequence analysis of the amplification products was performed by automated sequencing.

Genotyping

Genotyping was assessed for each genera with *Sma*I PFGE by a standard protocol using the CHEF DR-III system (Biorad®, Hercules, California). Percent similarity was estimated by the Simple Matching coefficient, and the matrix was clustered by the unweight pair group method (UPMGA). In this study an 80% similarity level was considered, corresponding to differences between 9 to 16 bands. The analysis was performed as previously described (9).

Results

Resistance patterns

From 85 Gram-positive cocci collected, one GGS, (isolate GGS 01), one GCS (isolate GCS 02) and one GBS (isolate GBS 34) exhibited high-level resistance to gentamicin and amikacin (MICs = 1,024 µg/ml). Isolate GGS 01 also showed high level of resistance to streptomycin and kanamycin with MIC values from 1,024µg/ml to 2,048 µg/ml. Also 7 CoNS, 11 MRSA and 11 VRE isolates exhibited resistance to gentamicin, amikacin and kanamycin (MIC=1,024 µg/ml). The remaining isolates were also resistant to streptomycin (MIC=2,048 µg/ml). Nine GGS (GGS 01, 10, 11, 19, 20, 21, 22, 24 and 26) and 2 GCS isolates (GCS 02 and 15) were resistant to tetracycline and minocycline (MICs between 32 and 64 µg/ml). Tetracycline and minocycline resistance was also observed in 11 MRSA and 1 VRE (isolate VRE 09) with similar MIC values as those found in streptococci. Erythromycin resistance was observed in isolate GGS 01 and in 11 GAS with MIC > 4 µg/ml. It was also detected in 11 MRSA, 4 CoNS and in 11 VRE (MIC>8 µg/ml).

Antimicrobial resistance determinants

The aminoglycoside-resistance genes *aac(6')-aph(2'')*, *aph(3')-IIIa* and *ant(6)-Ia* were found in one GGS isolate (GGS 01) as well as in all MRSA, in seven CoNS and in all VRE isolates. In isolates GCS 02 and GBS 34, the *aac(6')-aph(2'')* was the only gene detected whereas in six of the nine CoNS the *ant(4')-Ia* was also detected. The tetracycline-minocycline-resistant isolates (n=23) harbored the *tet*(M) gene. Among erythromycin resistance determinants, *erm*(TR) was found in one GGS isolate (GGS 01), in three GAS (GAS 03, 07 and 16) and in one CoNS (CoNS 03). The sequencing of the amplicon showed 100% homology with sequence AF002716 from *Streptococcus pyogenes*. The *erm*(A) was detected in ten MRSA; *erm*(B) was observed in eleven VRE; and *erm*(M) was found in one CoNS (CoNS 02). We did not observe the presence of *erm*(C) in our population. The *mef*(A) gene was present in nine GAS, in one CoNS (CoNS 03) and in one VRE (VRE 10). The *mef*(E) gene was detected in nine MRSA, and *msr*(A) was found in two CoNS (CoNS 06 and 07).

Transposon detection

In one GGS (GGS 01), in one GCS (GCS 02), in seven CoNS, and in the eleven MRSA isolates, the combination of both *aac* primers with the IS256U primer rendered a 1,500 bp amplification product, whereas in ten of the eleven VRE only the combination aac6R/IS256U rendered a product of the same size. In one VRE isolate (VRE 9) no amplification product was detected with any of the two combinations. Sequence analysis revealed that the *aac(6')-aph(2'')* gene was flanked by two IS*256* in those isolates in which both combinations rendered an amplification product; meanwhile, only one IS*256* downstream of the *aac(6')-aph(2'')* gene was present in VRE isolates (01 to 08, 10 and 11).

Tn*5405* was detected in all the MRSA isolates. Sequence analysis of the amplification products of 3.6 kb (the region between IS*1182* and *ant(6)-Ia)*, 5.7 kb (the region between IS*1182* and *aph3'-IIIa*), 2.2 kb (the region between *ant(6)-Ia* and aph3'-*IIIa*) and 2.5 kb (the region between *aph3'-IIIa* and orfz) revealed that these fragments were identical to portions of the Tn*5405* structure in MRSA. In one GGS (GGS 01) and in the eleven VRE isolates, sequence analysis of the 2.2 kb amplification product demonstrated concomitant presence of *aph(3')-IIIa*, *sat4* and *ant(6)-Ia*.

The integrase of the "Tn*916*-like" element was found in two GGS (GGS 10 and 11) and in one GCS (isolate GCS 15), whereas the integrase of the "*CW459*-like" or "Tn*5801*-like" elements was

detected in three GGS (GGS 21, 22 and 24) and in 10 of the 11 MRSA; both integrases were demonstrated by PCR and blast sequence analysis. Insertion sequences IS*256* and IS*1216* were found in one GGS isolate (GGS 01), one GCS (isolate GCS 02), one GBS (isolate GBS 34) and in the eleven VRE. In *Staphylococcus* spp., IS*256* was detected in seven CoNS and eleven MRSA whereas IS*1182* and IS*1181* and IS*257* were detected only in the MRSA isolates.

Isolates clonal relationships

PFGE analysis of the 23 SGG, six of which were multiresistant isolates, showed twelve band patterns. These 23 isolates were grouped in ten clones or "clusters"; similarity was 80% (I, II, III, IV, V, VI, VII, VIII, IX and X). The number of band differences among the clusters was between seven and ten with three or four bands appearing in the same cluster. The ten SGC were grouped in four patterns, two of which were resistant to tetracycline and the clusters were grouped in four clones at the 80% similarity. The difference of bands among the clusters was between five and ten with four bands appearing in the same cluster (data not shown).

The eleven SGA isolates were resistant to erythromycin and we analysed the spreading of such resistance. Three of them harboured the *erm*(TR) gene (SGA 3, SGA 7 y SGA 16) and nine possessed the *mef*(A) gene (SGA 4, SGA 5, SGA 6, SGA 8, SGA 9, SGA 12, SGA 16, SGA 17 y SGA 18). The PFGE analysis revealed the presence of seven-band patterns among the resistant isolates, which were grouped in seven clones or clusters (A–G). Six different bands were detected among the clones (data not shown).

PFGE analysis of the CoNS isolates revealed the presence of seven-band patterns that were grouped in four clones according to the phylogenetic evaluation (A, B, C and D). The difference in the number of bands among the clones ranged from nine to sixteen, and within the same cluster between one and five. Within the three isolate clone A cluster, the difference between the isolates was the acquisition of various resistance markers. The isolate CoNS 4 did not harbor any of the markers tested (clone A subtype 1), whereas the other two isolates (subtypes 2 and 3) acquired the *mecA*, *aac(6´)-aph(2´´)*, *erm*TR and *mef*(A) genes (Table 2). The acquisition of *mecA* and *aac(6´)-aph(2´´)* differentiated the subtypes 1 from the subtypes 2 in cluster D (isolates CoNS 8 and 9). Among the MRSA isolates, we observed seven PFGE patterns that were grouped in four clones at the 80% similarity. The difference in the number of bands among the clones ranged between eight and twelve bands, and within the same clone between two and five bands. In the MRSA population the difference among the clusters was determined by the presence or absence of macrolides resistance genes *mef*(E) or *erm*A (Table 2). The VRE population showed five PFGE patterns that were grouped in three clusters (A, B and C). The difference among the clusters was of four to six bands and within the same cluster between one and three bands. In this group, one isolate belonging to cluster A (VRE 9) differed from the other members by the acquisition of *tet*(M) gene and one isolate classified by cluster B (VRE 10) harbored the *mef*(A) gene that was not observed in the other isolates.

Table 2. Isolates analyzed in this study.

Isolation	Sample	Antibiogram[a]	Genotypic profile
SGG 01	faringitis	Akn, Gen, Strep, and Kan R, Tet R, Ery R	*aac(6´)-aph(2´´)*, *aph(3´)-IIIa*, *ant(6)-Ia*, *tet*(M), *erm*(TR), *qacEΔ1*
SGC 02	faringitis	Akn R, Gen R, Strep S, Kan S, Tet R, Ery R	*aac(6´)-aph(2´´)*, *tet*(M), *qacEΔ1*
SGA 03	faringitis	Ery R	*erm*(TR)
SGA 04	faringitis	Ery R	*mef*(A)
SGA 05	faringitis	Ery R	*mef*(A)
SGA 06	faringitis	Ery R	*mef*(A)
SGA 07	faringitis	Ery R	*erm*(TR)
SGA 08	faringitis	Ery R	*mef*(A)
SGA 09	faringitis	Ery R	*mef*(A)
SGG 10	faringitis	Tet R	*tet*(M)
SGG 11	faringitis	Tet R	*tet*(M)
SGA 12	faringitis	Ery R	*mef*(A)
SGC 15	faringitis	Tet R	*tet*(M)
SGA 16	faringitis	Ery R	*mef*(A), *erm*(TR)
SGA 17	faringitis	Ery R	*mef*(A)
SGA 18	faringitis	Ery R	*mef*(A)
SGG 19	faringitis	Tet R	*tet*(M)

Isolation	Sample	Antibiogram[a]	Genotypic profile
SGG 20	faringitis	Tet R	*tet*(M)
SGG 21	faringitis	Tet R	*tet*(M)
SGG 22	faringitis	Tet R	*tet*(M)
SGG 24	faringitis	Tet R	*tet*(M)
SGG 26	faringitis	Tet R	*tet*(M)
SGB 34	faringitis	Gen R, Kan R, Akn R	*aac(6´)-aph(2´´)*
CoNS 1	blood	S: Van, Teico, Minoc, Cipro, Tet, Cloran, R: Oxa, Gen, Rif, TMS, Ery, Clin	*aac(6')-aph(2'')*; *ant(4')-Ia*; *qacEΔ1*
CoNS 2	blood	S: Van, Teico, Cip, Tet, R: Oxa, Gen, Rif, TMS, Ery, Clin	*aac(6')-aph(2'')*; *ant(4')-Ia*; *qacEdelta1*; *erm*(M)
CoNS 3	blood	S: Van, Teico, Minoc, Rif , R: Oxa, Gen, TMS, Ery, Clin, Cip, Tet, Clor	*aac(6')-aph(2'')*; *erm*(TR); *mef*(A); *qacEΔ1*; *mecA**
CoNS 4	blood	S: Van, Teico, Minoc, Cip, Tet, Clor, Rif TMS, Oxa, Gen, Ery, Clin	none
CoNS 5	blood	S: Van, Teico, Minoc, Rif, Clor, R: Oxa, Gen, TMS, Ery, Clin, Cip, Tet	*aac(6')-aph(2'')*; *qacEΔ1*; *mecA**
CoNS 6	blood	S: Van, Teico, Minoc, Cip, Tet, Clor, R: Oxa, Gen, Rif,TMS, Ery, Clin	*aac(6')-aph(2'')*; *ant(4')-Ia*; *qacEΔ1*; *msr*; *mecA**
CoNS 7	blood	S: Van, Teico, Minoc, Cip, Clor, Rif, R: Oxa, Gen, Ery,Clin, Tet	*aac(6')-aph(2'')*; *ant(4')-Ia*; *qacEΔ1*; *msr*; *mecA**
CoNS 8	blood	S: Van, R: Teico, Oxa, Gen, Ery, Clin	*aac(6')-aph(2'')*; *ant(4')-Ia*; *mecA**
CoNS 9	blood	susceptible	*ant(4')-Ia*
MRSA 1	Catheter and blood culture	S: Rif, TMS, Van, Teico, R: Oxa, Gen, Ery, Cip, Clin, Tet	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *tet*(M); *mef*(E); *qacEΔ1*; *sat4*; *mecA**
MRSA 2	Bone fistule	S: Van, Teico, R: Oxa, Gen, Ery, Cip, Rif, TMS, Tet	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *tet*(M); *erm*(A); *sat4*; *mecA**; *mef*(E)
MRSA 3	blood	S: Rif, TMS, Van, Teico, R: Oxa, Gen, Ery, Cip, Clin, Tet	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *tet*(M); *erm*(A); *mef*(E); *sat4*; *mecA**
MRSA 4	blood	S: Van, Teico, R: Oxa, Gen, Ery, Cip, Rif, TMS, Clin, Tet	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *tet*(M); *erm*(A); *sat4*; *mecA**
MRSA 5	Bronchoalveolar wash fluid	S: Rif, TMS, Van, Teico, R: Oxa, Gen, Ery, Cip, Clin, Tet	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *tet*(M); *erm*(A); *mef*(E); sat4; mecA*
MRSA 6	Pelvic celulitis	S: Rif, TMS, Van, Teico R: Oxa, Gen, Ery, Cip, Clin, Tet	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *tet*(M); *erm*(A); *mef*(E); *sat4; mecA**
MRSA 7	Conjunctive secretion	S: Rif, TMS, Van, Teico, R: Oxa, Gen, Ery, Cip, Clin, Tet	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *tet*(M); *erm*(A); *mef*(E); *sat4*; *mecA**
MRSA 8	Skin and soft tissue	S: Van, Teico, R: Oxa, Gen, Ery, Cip, Rif, TMS, Clin, Tet	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *tet*(M); *erm*(A); *sat4; mecA**; *mef*(E)
MRSA 9	Thigh celulitis	S: Van, Teico, I: Rifa, R: Oxa, Gen, Ery, Clin, Cip, TMS, Tet	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *tet*(M); *erm*(A); *sat4; mecA**; *mef*(E)
MRSA 10	sputum	S: Rif, TMS, Van, Teico, R: Oxa, Gen, Ery, Cip, Clin, Tet	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *tet*(M); *erm*(A); *mef*(E); *sat4; mecA**
MRSA 11	blood (3:3)	S: Van, Teico, I: Rifa, R: Oxa, Gen, Ery, Clin, Cip, TMS, Tet	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *tet*(M); *erm*(A); *sat4; mecA**
VRE 1 (H1)	Stool	S: Nit, R: Van, Teico, Imip, Mero, Cip,Rif, Gen, Amp, AMS, Ery	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *erm*(B); *sat4*
VRE 2 (H1)	Femoral catheter	Idem E1	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *erm*(B); *sat4*
VRE 3 (H1)	Stool	Idem E1	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *erm*(B); *sat4*
VRE 4 (H1)	Catheter blood culture	Idem E1	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *erm*(B); *sat4*

Isolation	Sample	Antibiogram[a]	Genotypic profile
VRE 5 (H1)	Catheter blood culture	Idem E1	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *erm*(B); *sat4*
VRE 6 (H1)	Colon biopsy	Idem E1	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *erm*(B); *sat4*
VRE 7 (H3)	Rectal swab	Idem E1	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *erm*(B); *sat4*
VRE 8 (H3)	Rectal swab	Idem E1	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *erm*(B); *sat4*
VRE 9 (H3)	Rectal swab	Idem E1+ R: tet	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *tet*(M); *erm*(B); *sat4*
VRE 10 (H3)	Rectal swab	Idem E1	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *mef*(A); *erm*(B); *sat4*
VRE 11 (H6)	Rectal swab	Idem E1	*aac(6')-aph(2'')*; *aph(3')-IIIa*; *ant(6)-Ia*; *erm*(B); *sat4*

a. Akn: amicacin, Gen: gentamicin, Strep: streptomycin, Kan: kanamycin, Tet: tetracycline, Ery: erythromycin, Van: vancomycin, Teico: teicoplanin, Minoc: minocycline, Cipro: ciprofloxacin, Cloran: chloramphenicol, Oxa: oxacillin, Rif: rifampicin, TMS: trimetoprim-sulphametoxazole, Clin: clindamycin

Discussion

The high level of aminoglycoside resistance was due to the presence of several aminoglycoside-modifying enzymes such as *aac(6')-aph(2'')*, *aph(3')-IIIa* and *ant(6)-Ia* harbored in the same strain in our bacterial population. The spreading of *aac(6')-aph(2'')* was due to Tn*4001* present in all resistant isolates in three forms: i) the α-form, which has 2 IS*256* flanking the *aac(6')-aph(2'')* gene in opposite directions, as it was observed in one GGS isolate (GGS 01) and in MRSA; ii) the Tn*4001*-truncated type I-like element, observed in ten of eleven VRE isolates, in which the IS*256* on the right-hand flanking region was absent; or iii) the Tn*4001*-truncated type III-like element observed in one VRE (isolate 09), in which both IS*256* were missing (2). This isolate (VRE 09) was the only one that harbored *tet*(M), a finding that may explain the presence of a truncated Tn*4001*.

The gene cluster *aph(3')-IIIa*, *ant(6)-Ia* and *sat4* (which encodes the resistance to streptothricin) was detected in staphylococci mostly integrated into the Tn*5405* transposable element (7). One hypothesis to explain why these three genes are regularly associated could be the presence of a common promoter (7). The genes *aph(3´)-IIIa*, *ant(6)-Ia* and *sat4* were amplified separately to confirm their presence in the genome of the isolates. As expected, this gene cluster was found inserted in Tn*5405* in all the MRSA isolates investigated in this study, whereas IS*1182*, *orfX*, *orfY* and *orfZ* (elements that are part of Tn*5405*) were not found in either multiresistant GGS isolate 01 or any of the *Enterococcus faecium* isolates investigated. This observation agrees with the results of a report by Werner *et al.* (23), who described that 29.8% of the *Enterococcus faecium* isolates investigated had the 5' end of Tn*5405* deleted, and the cluster *ant(6)-Ia-sat4- aph(3')-IIIa* linked to *erm*(B). In our VRE population, we observed the presence of *erm*(B) and the cluster *ant(6)-Ia-sat4- aph(3')-IIIa*, suggesting a common structure that harbored them. Probably, in a recombination event, Tn*5405* could have lost the IS*1182*, a fact that may explain the finding of the *aph(3')-IIIa*, *sat4* and *ant(6)-Ia* genes together. Six of the eleven VRE isolates and one GGS (isolate 01) were recovered from the same nosocomial setting, suggesting that an interspecies transfer may have occurred among them.

With regard to tetracycline resistance, we found the *tet*(M) gene in tetracycline and minocycline-resistant isolates. From all known conjugative transposons carrying *tet*(M), we designed primers to amplify the integrases of Tn*916* and *CW459* since these are the regions where major differences were found (18). We observed that spreading of *tet*(M) by the *Tn916*-like transposon was not a frequent event since we only detected the integrase of this element in three *Streptococcus* spp. isolates. Instead, we amplified a 370 bp fragment that corresponded to a region of the *CW459*-like element integrase in 10 of 11 MRSA and in 3 GGS isolates, a finding that could suggest a novel mechanism for tetracycline resistance spreading. Ito *et al.* (8) reported similar findings and described a new element called Tn*5801* in MRSA. The Tn*5801* integrase is identical to that of the element *CW459* from *Clostridium* spp., which permits the speculation that the same conjugative transposon harboring *tet*(M) is transmissible between these two genera (8). It can also be hypothesized that the integrase that we detected by PCR might be analogous to the one found in Tn*5801* and that this element was spread into streptococci in a yet undisclosed manner. Further studies are required to determine

which mobile element is responsible for spreading of this transposon in our cocci population and to establish whether there are more strains that harbor this mobile element. The importance of this knowledge is that this putative mobile element seems to be linked to the primary mechanism of *tet*(M) spreading in our country and that we have also observed it in group G streptococci. Different authors have reported the presence of the *tet*(M) gene within a transposable element in *S. pneumoniae*, (6) streptococci group viridans and *Gemella* species being the latter reservoirs of resistance genes that can be transferred to *S .pneumoniae* (3). To our knowledge this is the first study where *tet*(M) is associated with an *intTn* gene in group G streptococci which suggests the presence of elements similar to those of the Tn*916* family in this species.

From the two erythromycin-resistance mechanisms described in the bacterial population under scrutiny, the one mediated by methylases was the most frequently found among Gram-positive cocci (27 isolates), although antibiotic efflux (*mef*(A)) was the most prevalent mechanism in *Streptococcus* spp. (GAS) isolates. We observed that those isolates that harbor an *erm* gene, no matter what type of MLS_B phenotype they expressed, also hold a *tet* gene such as *tet*(M). This finding suggests that these genes could be harbored by a common transposable element such as Tn*2009* (6,19).

The antiseptic resistance was due to *qacE delta1* gene in some isolates (Table 2). The reason for searching this particular gene was the hypothesis that as it is widely spread in Gram-negative bacteria, it could have been transmitted to Gram-positive cocci. In fact Kazama *et al.* (11) found *qacEdelta1* in *Staphylococcus* spp. and *Enterococcus* spp. (11) suggesting an integron-like structure in Gram positive cocci.

From the presence of multiple resistance in our cocci population, we hypothesize the existence of a more complex genetic structure. We searched IS*1216* that flanks the Tn*5385* element described in *Enterococcus faecalis* (17). This element confers resistance to several compounds due to the insertion of different transposons. The IS*1216*-like element was detected in *Streptococcus* spp. and in VRE whereas we did not observe its presence in *Staphylococcus* spp.

In brief, we found a marked heterogeneity in the cocci population under scrutiny since isolates in one genus exhibited the same resistance determinants but belonged in different pulsotype clusters. An exception was the VRE and the CoNS populations, which contained few clones with a highly prevalent one. In conclusion, the appearance of isolates of different genera from different nosocomial settings with the same resistance determinants and transposable elements demonstrates significant horizontal spreading of resistance to aminoglycosides, tetracycline and erythromycin in hospitals in Argentina. These results underscore the importance of planning control strategies in order to limit the spreading of mechanisms of multiple resistance which could have a deleterious impact on human therapy.

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