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ORIGINAL ARTICLE

Macrolide-lincosamide-streptogramin B resistance phenotypes and their associated genotypes in *Staphylococcus aureus* isolates from a tertiary level public hospital of Uruguay

Lorena Pardo[a], Virginia Machado[a], Dianna Cuello[a], Paula Aguerrebere[a], Verónica Seija[b,c], Valeria Braga[a], Gustavo Varela[a,*]

[a] *Bacteriology and Virology Department, Medicine School, Universidad de la República, Montevideo Alfredo Navarro 3051 (south entrance), 11800, Uruguay*
[b] *Bacteriology Laboratory, ''Hospital Pasteur'', Montevideo, Larravide 2458, 11800, Uruguay*
[c] *Laboratory of Public Health, Alfredo Navarro 3051 (north-entrance), Montevideo, Uruguay*

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Abstract This study was undertaken to investigate the resistance phenotypes to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics and their associated genotypes in isolates of *Staphylococcus aureus*. We analyzed one hundred, consecutive, non-duplicate isolates (methicillin-susceptible MSSA, n = 53 and methicillin-resistant MRSA, n = 47) obtained from various clinical samples between July 2012 to December 2013. The resistance profile to MLS_B antibiotics was determined by phenotypic methods and the resistance genes were detected by PCR assays. All of the isolates were subjected to pulsed-field gel electrophoresis (*Sma*I-PFGE). The overall prevalence of resistance to MLS_B antibiotics was 38% and the resistance phenotype distribution was as follows: $cMLS_B$, 22%; $iMLS_B$, 10%; MS_B, 5% and L, 1%. We detected *ermA*, *ermC*, *ermB* and *mrsA/B* genes in these resistant isolates. The single *ermA* gene was commonly observed mainly in those with a $cMLS_B$ R phenotype, whereas the combination *ermA* and *ermC* was more commonly observed in isolates with inducible expression. The patterns of *Sma*I-PFGE suggest a great genetic diversity in both MRSA and MSSA resistant to MLS_B antibiotics. The results demonstrate the local presence of *S. aureus* resistant to MLS_B antibiotics and its most frequently described responsible genes. Some of these isolates, especially those with the $iMLS_B$ phenotype, may be associated with therapeutic failure. Therefore, efforts should be directed to the correct detection of all MLS_B resistant isolates using appropriate laboratory tests. PFGE results reveal a wide spread of resistance genes rather than the circulation of *S. aureus* clones resistant to MLS_B antibiotics.

* Corresponding author.
E-mail addresses: gvarela@higiene.edu.uy, gvarela1961@gmail.com (G. Varela).

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PALABRAS CLAVE
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SmaI-PFGE

Fenotipos de resistencia a macrólidos lincosamidas-estreptograminas B y sus genotipos asociados en *Staphylococcus aureus* aislados en un hospital público de nivel terciario en Uruguay

Resumen Los objetivos de este estudio fueron investigar en *Staphylococcus aureus* la presencia de fenotipos resistentes a los antibióticos macrólidos, lincosamidas y estreptograminas tipo B (MLS_B) y conocer sus genotipos responsables. Analizamos 100 aislamientos consecutivos, no duplicados (53 sensibles a meticilina [MSSA] y 47 resistentes a meticilina [MRSA]), obtenidos entre 2012 y 2013 a partir de diferentes muestras clínicas. El perfil de resistencia a los antibióticos MLS_B fue determinado por métodos fenotípicos y los genes de resistencia se detectaron por PCR. Todos los aislamientos fueron comparados por *Sma*I-PFGE. La prevalencia global de resistencia a los antibióticos MLS_B fue del 38% y la distribución de los fenotipos de resistencia fue la siguiente: $cMLS_B$, 22%; $iMLS_B$, 10%; MS_B, 5%; L, 1%. Se detectaron los genes *ermA, ermC* y *mrsA/B* en los aislamientos resistentes. El gen *ermA* se observó, sobre todo, en aislamientos con fenotipo resistente constitutivo R ($cMLS_B$), mientras que la combinación *ermA* y *ermC* se detectó principalmente en aislamientos con resistencia inducible ($iMLS_B$). Los patrones de *Sma*I-PFGE sugieren una gran diversidad genética en los aislamientos resistentes a los antibióticos MLS_B, tanto MRSA como MSSA. Los resultados demuestran la presencia local de *S. aureus* resistentes a los antibióticos MLS_B y de sus genes responsables más frecuentemente descritos. Estos cultivos, especialmente aquellos con fenotipo resistente $iMLS_B$, pueden asociarse con fallas terapéuticas. Por lo tanto, los esfuerzos deben dirigirse a la correcta detección de todos los cultivos resistentes a MLS_B utilizando pruebas de laboratorio adecuadas. Los resultados de *Sma*I-PFGE sugieren una amplia diseminación de genes de resistencia, más que la circulación de clones resistentes a los antibióticos MLS_B.

Introduction

Staphylococcus aureus is a versatile microorganism that is found in the human microbiota and is also responsible for both hospital- or community-acquired illnesses. The diseases that it causes range from mild lesions that compromise skin and soft tissues to more severe conditions such as pneumonia, septic arthritis, endocarditis, osteomyelitis, and sepsis[12,31,33]. A biological characteristic of *S. aureus* is its ability to acquire exogenous genetic material that encodes several resistance mechanisms for different antibiotics. The mobile staphylococcal chromosomal cassette *mec* (SCC*mec*) that carries the *mecA* gene which encodes PBP2a, which is responsible for methicillin-resistance (MRSA), was likely acquired by horizontal transfer from a related species[8]. Three categories of MRSA are currently recognized, healthcare-associated (HA-MRSA), community-associated (CA-MRSA) and livestock-associated MRSA (LA-MRSA) according to epidemiological criteria, site of infection acquisition (community, hospital or animal contact), associated antibiotic susceptibility profile and genotype, including SCC element type[11,34,35]. *S. aureus* belonging to the three aforementioned categories have been implicated around the world in both sporadic cases and outbreaks of human staphylococcal diseases[4,16,37]. Macrolides, lincosamides, and type B streptogramin (MLS_B) antibiotics can be alternatives for treatment of MRSA infections[36]. However, the increase in macrolide, lincosamide and type B streptogramin resistant isolates complicates the empirical antimicrobial treatment selection in *S. aureus* infections[14]. A fairly common resistance mechanism observed in *S. aureus* is due to the action of efflux pumps, encoded by the *mrsA* and *mrsB* genes responsible for pumping macrolide and streptogramin B antibiotics out of the bacteria (MS_B resistance phenotype). On the other hand, the exclusive resistance to lincosamides (L resistance phenotype) mediated by lincomycin nucleotidyl-transferase enzymes and codified by genes *lnuA-F*, is occasionally observed. Isolates that carry *lnu* genes resist high levels of lincomycin but can be susceptible to clindamycin in the disk induction test[18,23]. In *S. aureus* and other gram-positive bacteria, resistance to MLS_B antibiotics is mainly due to the 23S rRNA modification by adenine-N methyltransferase enzymes. This modification compromises macrolide, lincosamide and type B streptogramin activity. Genes encoding these methylases have been designated as *erm* genes and their expression can be constitutive ($cMLS_B$ resistance phenotype) or inducible ($iMLS_B$ resistance phenotype)[13]. Isolates with the $iMLS_B$ phenotype show in vitro resistance to erythromycin, but can falsely seem to be susceptible to clindamycin. These isolates have a high rate of spontaneous mutation to constitutive resistance phenotypes that can be selected by the usage of clindamycin, resulting in therapeutic failure. This fact highlights the importance of performing a correct laboratory identification using any of the recommended tests[3,6]. In our region, data about the prevalence of resistance to MLS_B antibiotics in isolates of *S. aureus* recovered from adult individuals are scarce. This study aimed to investigate the presence

of the different erythromycin and clindamycin resistance phenotypic profiles and also to determine the responsible genotypes in a set of *S. aureus* isolates recovered in a public, tertiary level hospital of Montevideo city, Uruguay.

Materials and methods

We study 100 consecutive, non-duplicate *S. aureus* isolates obtained from patients admitted at the ''Hospital Pasteur'' (HP) from July 2012 to December2013. *Setting* The HP is a 250-bed tertiary level public and teaching hospital for all medical and surgical specialties. Patients receiving medical care at the HP are adults (>16 years of age) and often come from low-income households.

Bacterial isolates, source, identification and antimicrobial susceptibility testing.

S. aureus isolates were identified using the automated system VITEK 2 version 7.01 and classified as methicillin-resistant (MRSA) or methicillin-susceptible (MSSA) according to the obtained MIC value to oxacillin and the result of the cefoxitin test. The site of infection was taken from the laboratory records. Erythromycin (ERY) and clindamycin (CLI) phenotypes were assessed and interpreted using the D-zone test published by Steward et al.[32] (D phenotype: D-shaped clear zone around the CLI disk near the ERY disk; D+ phenotype: like the previous one but with small colonies growing close to the CLI disk in an otherwise clear zone; HD phenotype: two zones of growth around the CLI disk: one zone is a light growth extending from the CLI disk to the second zone, where the growth is much thicker; R phenotype: growth to the edge of both CLI and ERY disks; MS_B phenotype: a clear zone around the CLI disk; L phenotype: growth only up to the CLI disk; S phenotype: susceptible to both the CLI and ERY disks)[3,2].

The IHSA 23 strain of *S. aureus* was used as a positive control for the D-test. Minimum inhibitory concentration (MIC) to erythromycin, clindamycin, vancomycin, teicoplanin and oxacillin was determined by the agar dilution method according to the Clinical and Laboratory Standards Institute recommendations[6]. The susceptibility patterns to ciprofloxacin, gentamicin, trimethoprim-sulfamethoxazole, tetracycline and rifampicin were taken from the VITEK report. Broth macrodilution assays (BMA) that included combinations of CLI plus ERY were conducted with minor modifications in accordance with the general procedure previously described by Steward et al.[32]. Briefly, the combinations were tested using CLI at final concentrations ranging from 0.5 to 8 μg/ml plus ERY at final concentrations ranging from 0.03 to 0.12 μg/ml. Reading was done after 18–24 h of incubation at 35–37 °C. This procedure was applied to those isolates that showed the $iMLS_B$, $cMLS_B$, MS_B and S phenotypes by disk diffusion tests. *S. aureus* ATCC 29213 (CLI and ERY-susceptible) was included as control. The isolates were stored at −20 °C in skimmed milk for further studies.

Molecular typing and antibiotic resistance gene detection. Bacterial DNA was obtained from isolated colonies using the Wizard genomic DNA preparation kit (Promega. Madison, Wis, USA) adding 20 mg/ml lysostaphin (Sigma Chemical) in the cell lysis step. Detection of the *mecA* gene and SCC*mec* typing was performed in all MRSA isolates by a multiplex PCR assay previously described by Oliveira DC and H. de Lencastre[20]. MRSA isolates that carried the SCC*mec* type IV were arbitrarily taken as community-associated methicillin-resistant *S. aureus* (CA-MRSA) while those that carried the SCC*mec* type I, II, or III were grouped as healthcare associated methicillin-resistant *S. aureus* (HA-MRSA). PCR detection of the *ermA*, *ermB, ermC*, *msrA* and *msrB* genes was performed on all isolates that showed the following resistance phenotypes: $iMLS_B$ (D and D+); $cMLS_B$ (HD and R); and MS_B using standard protocols and previously described primers[15]. *S. aureus* MM122-35 was used as positive control for the *ermA* gene, *S. aureus* MM2 was used as a positive control for the *ermB* gene, and *S. aureus* MM523 for the *ermC* gene. *S. aureus* MM3627 was included as positive control for both *msr A* and *B* genes. All the strains were kindly provided by Genoveva Pensado (Bacteriology and Virology department). Ultrapure RNAse/DNAse free water was used as negative control in all PCR reactions.

Pulsed field gel electrophoresis (PFGE). DNA macrorestriction with the *Sma*I enzyme and further separation of fragments by pulsed field gel electrophoresis (PFGE) were carried out using a previously described protocol[24]. The obtained profiles were analyzed by the unweighted pair group method with arithmetic average (UPGMA) using *BioNumerics* v.7.1. The isolates that showed ≥80% similarity were included into the same pulsogroup (PG), while those that showed ≥95% similarity were considered to be of identical pulsotype (PT)[9]. *S. aureus* subsp. *aureus* (strain NCTC 8325) was included as reference size marker.

Results

Source of analyzed *S. aureus* isolates and resistance phenotypes

We studied 100 isolates of *S. aureus*. Twenty-three of them were recovered from invasive diseases including bacteremia (n = 10; 43.4%), ventilator-associated pneumonia (n = 8; 35%), deep abscesses (n = 4; 17.3%) and acute meningoencephalitis (n = 1; 4.3%). On the other hand, 77 isolates were recovered from skin and soft tissue infections (SSTIs) including superficial abscesses (n = 39; 50%), wounds (n = 22; 28.5%), cellulitis (n = 11; 14.2%) and whitlows (n = 5; 6.4%).

Fifty-three isolates were classified as MSSA and the remaining 47 as MRSA (all of them carried the *mecA* gene); 25 corresponded to CA-MRSA and 22 to HA-MRSA, according to the above described abbreviated criterion. The distribution of the SCC*mec* types in HA-MRSA was as follows: SCC*mec* type I, 5 isolates; SCC*mec* type II, 15 isolates and SCC*mec* type III, 2 isolates. Fifteen percent of the MSSA isolates were resistant to MLS_B antibiotics, whereas this figure increased to 63.8% in the MRSA (CA-MRSA and HA-MRSA) isolates. All *S. aureus* isolates included in this study were susceptible to vancomycin and teicoplanin. Four isolates (4%) were resistant to rifampicin, all of them carrying the SCC*mec* type II. Twenty two out of 47 (46.8%) MRSA showed resistance to ciprofloxacin (HA-MRSA, n = 21; CA-MRSA, n = 1). However, only 4 out of 53 (7.5%) MSSA were resistant to this antibiotic. Eight *S. aureus* isolates (8%) showed resistance to gentamicin, 4 (7.5%) corresponded to MSSA isolates and 4 (8.5%) to MRSA (HA-MRSA, n = 3; CA-MRSA, n = 1). One methicillin-susceptible isolate was resistant

Table 1 Distribution of phenotypes to MLS_B antibiotics in *S. aureus* isolates using the D-zone test.

	MRSA with SCC*mec* type				Subtotal	MSSA n (%)	Total
	I n (%)	II n (%)	III n (%)	IV n (%)			
$iMLS_B$ [a]	0 (0%)	0 (0%)	0 (0%)	5 (5%)	5 (5%)	5 (5%)	10 (10%)
$cMLS_B$ [b]	4 (4%)	14 (14%)	2 (2%)	1 (1%)	21 (21%)	1 (1%)	22 (22%)
MS_B [c]	1 (1%)	1 (1%)	0 (0%)	1 (1%)	3 (3%)	2 (2%)	5 (5%)
L [d]	0 (0%)	0 (0%)	0 (0%)	1 (1%)	1 (1%)	0 (0%)	1 (1%)
S [e]	0 (0%)	0 (0%)	0 (0%)	17 (17%)	17 (17%)	45 (45%)	62 (62%)
Total	5 (5%)	15 (15%)	2 (2%)	25 (25%)	47 (47%)	53 (53%)	100 (100%)

[a] $iMLS_B$: inducible resistance to MLS_B antibiotics.
[b] $cMLS_B$: constitutive resistance to MLS_B antibiotics.
[c] MS_B: resistance to erythromycin.
[d] L: resistance only to clindamycin.
[e] S: erythromycin and clindamycin susceptible.

Table 2 Resistance to MLS_B antibiotics in *S. aureus* isolates.

% of resistance to	MRSA with SCC*mec* IV (n = 25)	MRSA with SCC*mec* I, II, III (n = 22)	MSSA (n = 53)
Macrolides($iMLS_B$, $cMLS_B$ and MS_B resistance phenotypes)	28%	100%	15.1%
Lincosamides($iMLS_B$, $cMLS_B$ and L resistance phenotypes)	28%	90.9%	11.3%

to tetracycline. The only *S. aureus* isolate that showed resistance to trimethoprim-sulfamethoxazole belonged to the HA-MRSA group. Table 1 shows the resistant phenotypes to MLS_B antibiotics found in MRSA and MSSA isolates. Table 2 shows the percentage of resistance to macrolides and lincosamides found in MRSA (HA-MRSA, CA-MRSA) and MSSA isolates. Three out of the 10 isolates with $iMLS_B$ resistance showed the D+ phenotype in the disk-diffusion test. The isolates with the $iMLS_B$ resistance phenotype (n = 10; 5 CA-MRSA and 5 MSSA) showed MICs for CLI $\leq$0.125 μg/ml and those with phenotype S (n = 62; MSSA 45 and CA-MRSA 17) had MICs for CLI $\leq$0.25 μg/ml. In those isolates that exhibited the $cMLS_B$ resistance phenotype (n = 22; HA-MRSA, 21 and MSSA, 1 isolate) MICs for CLI were $\geq$256 μg/ml. As shown in Table 3, the combination of 2 μg/ml of CLI plus 0.12 μg/ml of ERY in the BM assay allowed us to differentiate between isolates displaying $iMLS_B$ or $cMLS_B$ resistance phenotypes and those with the MS_B or S phenotype. Seven out of 10 isolates that showed the $iMLS_B$ resistant phenotype (D, 4 isolates and D+, 3 isolates) grew in the presence of CLY at 8 μg/ml plus ERY 0.12 μg/ml and all the isolates (n = 22) that showed the $cMLS_B$ resistance phenotype (HD and R) grew in the presence of the combination 8 μg/ml of CLI plus ERY 0.12 μg/ml.

Distribution of the resistance genes to MLS_B antibiotics among *S. aureus* isolates

As shown in Table 4, all the isolates that showed the $iMLS_B$ resistance phenotype in the D-test carried the combination of the *ermA* and *ermC* genes. Instead, in those isolates that displayed the $cMLS_B$ resistance phenotype (HD or R) the predominant genotype was *ermA* (alone or in combination with other resistance genes). In those isolates that showed the $cMLS_B$ (HD) phenotype, the combination of the *ermA* and *mrs* genes was seen more frequently (6 out of 8) than in those that displayed the $cMLS_B$ (R) phenotype (2 in 14) (Table 4). The *ermB* gene was found in just one isolate with the cMLSB resistance phenotype. The responsible genotype was not studied in the CA-MRSA isolate that showed the L resistance phenotype.

SmaI-PFGE profiles in MRSA and MSSA isolates. As shown in Figure 1, the *Sma*I-PFGE profile analysis of MRSA isolates revealed a wide variety of PTs (n = 41) and 3 clusters (pulsogroups, PG: A, B and C, with $\geq$80% similarity and more than 2 isolates in each). Cluster A included 6 isolates, one with SCC*mec* IV, one with SCC*mec* III, one with SCC*mec* I and finally 3 isolates that carried the SCC*mec* II element with the cMLSB resistance phenotype that showed 100% identity and were recovered from 3 inpatients located in the same surgery ward. The only isolate with the L resistance phenotype recovered in this study was located in this PG. Cluster B (9 isolates) included 8 CA-MRSA isolates. Three of them (including an isolate carrying the SCC*mec* type II) showed 100% similarity and were visually identical to the main pulsotype found in local CA-MRSA isolates recovered in Uruguay between 2001 and 2002 (PFGE profile A1, type USA1100). Other 3 isolates in this PG showed 100% similarity among them (including the single CA-MRSA isolate with the $cMLS_B$ resistance phenotype recovered in this study) and were also visually similar to the CA-MRSA PFGE profile A1. Cluster C also included CA-MRSA isolates. The 5 CA-MRSA isolates that displayed the $iMLS_B$ resistance phenotype belonged to 5 different PTs (see Fig. 1). In the MSSA isolates, 52 PTs and 3 PGs (A, B and C, $\geq$80% similarity, more than 2 isolates in each) (see Fig. 2) were recognized. Only 2 isolates showed 95% similarity and were located in the PG C. This PG included 7 isolates, 2 of them with the $iMLS_B$ phenotype (see Fig. 2).

Table 3 Results of Broth Macrodilution Assays (BMA).

	Disk diffusion phenotype results				
	iMLS$_B$[a]		cMLS$_B$[b] (HD/R)	MS$_B$[c]	S[d]
	D	D+			
No. of isolates	7	3	22 (8/14)	5	62
No. of isolates that grew with					
CLI 8 μg/ml plus					
ERY 0.12 μg/ml	4	3	22	0	0
ERY 0.06 μg/ml	4	3	22	0	0
ERY 0.03 μg/ml	4	3	22	0	0
CLI 4 μg/ml plus					
ERY 0.12 μg/ml	4	3	22	0	0
ERY 0.06 μg/ml	4	3	22	0	0
ERY 0.03 μg/ml	4	3	22	0	0
CLI 2 μg/ml plus					
ERY 0.12 μg/ml	7	3	22	0	0
ERY 0.06 μ/ml	7	3	22	0	0
ERY 0.03 μg/ml	7	3	22	0	0
CLI 1 μg/ml plus					
ERY 4 μg/ml	7	3	22	0	0
ERY 1 μg/ml	7	3	22	0	0
CLI 0.5 μg/ml plus					
ERY 1 μg/ml	7	3	22	0	0
MIC100 to CLI without ERY	≤0.125 μg/ml		≥250 μg/ml	≤0.125 μg/ml	≤0.250 μg/ml

[a] Inducible resistance to MLS$_B$ antibiotics, D: clear zone around the CLI disk near to the ERY disk; D+: like the previous one but with small colonies growing up to the CLI disk in an otherwise clear zone.
[b] cMLS$_B$ (HD/R) Constitutive resistance to MLS$_B$ antibiotics, HD: two zones of growth appear around the CLI disk, one zone is a light growth extending from the CLI disk to the second zone where the growth is much thicker; R: growth to the edge of both the CLI and ERY disks.
[c] MS$_B$ clear zone around the CLI disk.
[d] S susceptible to both the CLI and ERY disks.

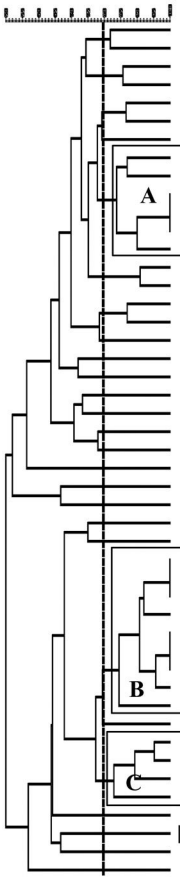
Table 4 Genotypes found in isolates of *S. aureus* resistant to MLS$_B$ antibiotics.

	Resistance phenotype in the D-test assay				
	iMLS$_B$[a] n = 10	cMLS$_B$ (HD)[b] n = 8	cMLS$_B$ (R)[c] n = 14	MS$_B$[d] n = 5	L[e] n = 1
Genotype	*ermA*/10 *ermC*/10 *msrB*/3	*ermA*/1 *ermB*/1 *ermA* + *ermC* + *msrA* + *msrB*/4 *ermA* + *msrA*/2	*ermA*/11 *ermC*/1 *ermA* + *msrA*/1 *ermA* + *msrB*/1	*msrA*/5 *msrB*/5	ND[f]

[a] iMLS$_B$: inducible resistance to MLS$_B$ antibiotics.
[b] cMLS$_B$ (HD): constitutive resistance to MLS$_B$ antibiotics (two zones of growth appear around the CLI disk, one zone is a light growth extending from the CLI disk to the second zone where the growth is much thicker).
[c] cMLS$_B$ (R): constitutive resistance to MLS$_B$ antibiotics (growth to the edge of both the CLI and ERY disks).
[d] MS$_B$: resistance only to erythromycin.
[e] L: growth only up to the CLI disk.
[f] ND, not done, unknown.

Discussion

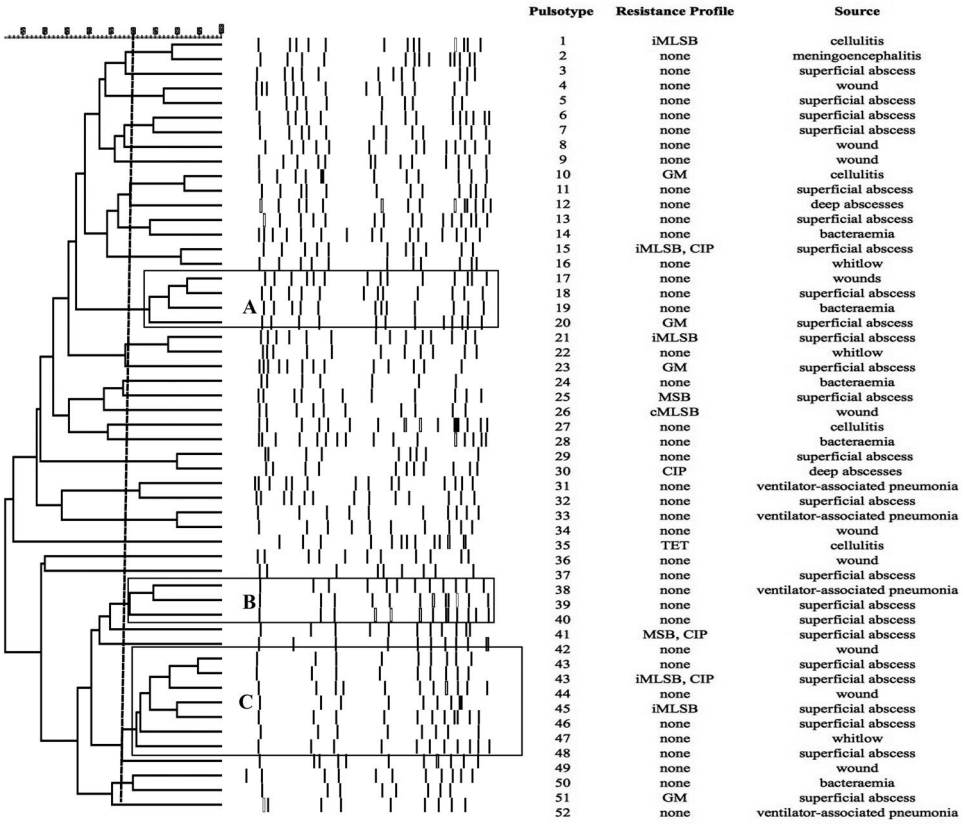
Clindamycin has been used for the treatment of severe staphylococcal diseases, taking into account some of its pharmacological properties. However, resistance can develop in isolates with the iMLS$_B$ phenotype, and spontaneous constitutively resistant mutants have arisen from such isolates during clindamycin therapy, determining therapeutic failures in some patients[10]. This is the first local report about the distribution of resistance phenotypes to



Pulsotype	Associated Resistance Profile	SCC*mec* Type	Source
1	none	IV	deep abscess
2	cMLSB, CIP	III	wound
3	MSB	IV	wound
4	cMLSB, CIP, RIF	II	ventilator-associated pneumonia
5	cMLSB, CIP, GM	I	wound
6	cMLSB, CIP, RIF	II	ventilator-associated pneumonia
7	cMLSB, CIP	II	wound
8	cMLSB, CIP	I	wound
9	cMLSB, CIP, GM	III	superficial abscess
10	cMLSB, CIP	II	ventilator-associated pneumonia
10	cMLSB, CIP	II	bacteraemia
10	cMLSB, CIP	II	cellulitis
11	L/C	IV	superficial abscess
12	cMLSB, CIP, RIF	II	superficial abscess
13	cMLSB, CIP, RIF	II	cellulitis
14	none	IV	wound
15	none	IV	superficial abscess
16	cMLSB, CIP	II	superficial abscess
17	MSB, CIP	II	cellulitis
18	none	IV	wound
19	cMLSB, CIP	II	superficial abscess
20	none	IV	bacteraemia
21	cMLSB, CIP	II	wound
22	none	IV	whitlow
23	cMLSB, CIP, GM, SXT	I	wound
24	cMLSB, CIP	I	bacteraemia
25	cMLSB, CIP	II	cellulitis
26	iMLSB, GM	IV	superficial abscess
27	none	IV	cellulitis
28	cMLSB, CIP	II	bacteraemia
28	none	IV	superficial abscess
28	none	IV	cellulitis
29	none	IV	superficial abscess
30	none	IV	superficial abscess
30	cMLSB, CIP	IV	wound
30	none	IV	whitlow
31	iMLSB	IV	superficial abscess
32	iMLSB	IV	superficial abscess
33	none	IV	bacteraemia
34	none	IV	deep abscesse
35	none	IV	superficial abscess
36	none	IV	superficial abscess
37	iMLSB	IV	cellulitis
38	cMLSB, CIP	II	wound
39	iMLSB	IV	superficial abscess
40	none	IV	ventilator-associated pneumonia
41	MSB	I	wound

Figure 1 Pulsed-field gel electrophoresis (PFGE) dendrogram of methicillin-resistant *S. aureus* isolates showing PTs, PGs, associated profile of resistance, SCC*mec* type and sample source. Dashed line indicates PGs (named as A, B and C, with ≥80% similarity and more than 2 isolates in each). $cMLS_B$, constitutive resistance to macrolide, lincosamide and type B streptogramin; $iMLS_B$, inducible resistance to macrolide, lincosamide and type B streptogramin; MS_B, resistance to macrolide and streptogramin B; L, resistance to lincosamides; CIP, resistance to ciprofloxacin; GM, resistance to gentamicin; RIF, resistance to rifampicin; SXT, resistance to trimethoprim-sulfamethoxazole; None, susceptible to all tested antibiotics.

MLS_B antibiotics and their responsible genes in a set of *S. aureus* isolates recovered from adult inpatients in a public hospital. The overall prevalence of resistance to MLS_B antibiotics was 38%. This figure is similar to that reported by Abbas et al.[1] (40%) in a study conducted in India that included 500 isolates of *S. aureus* recovered in a tertiary hospital and otherwise higher than that also found in India by Prabhu et al. (28.42%)[25]. Similarly to what happened in Serbia and India, in the current study most *S. aureus* isolates (62%) showed susceptibility to both macrolide and lincosamide antibiotics. However, the distribution of the resistance phenotypes was different from that reported by those authors. In Serbia, $iMLS_B$ was the most frequent resistance phenotype detected (31.5–33.7%); instead in India it was MS_B (17%) followed by both $cMLS_B$ and $iMLS_B$ resistance phenotypes (11.6% and 10.8%, respectively)[21,25]. In this study the overall data show that the predominant resistance phenotype was $cMLS_B$ (22%) followed by $iMLS_B$ (10%), MS_B (5%) and L (1%). Similar figures were reported by Sedaghat in the Iranian teaching hospital and also by Paz Pereira et al. in neighboring Brazil[3,28]. The $cMLS_B$ resistance phenotype prevailed among HA-MRSA isolates (SCC*mec* I, II and III) (20/22; 91%), which is in concordance with similar studies carried out in other countries[2,5,29,39]. In this work the prevalence of the $iMLS_B$ resistance phenotype was similar among MRSA (CA-MRSA and HA-MRSA) (5/47; 10.6%) and MSSA (5/53; 9%) isolates. This result is not in concordance with the findings reported by Abbas et al.[1] or Rahbar et al.[26], which showed a higher prevalence of the $iMLS_B$ resistance phenotype in MSSA isolates. Furthermore, in contrast to the findings of Mišic et al.[21] in Serbia, none of the HA-MRSA (SCC*mec* I, II and III) isolates studied here showed the $iMLS_B$ resistance phenotype (Table 1). The prevalence of MSSA isolates with the $iMLS_B$ resistance phenotype found in our study (9%) was lower than that reported by Shoji et al. in Japan (25.4%)[30]. As we will comment below, the results obtained by *Sma*I-PFGE showed great genetic diversity in both MSSA and MRSA isolates with $iMLS_B$ and $cMLS_B$ resistance phenotypes (Figs. 1 and 2). This suggests a broad diffusion of the responsible resistance genes rather than the circulation of a predominant PT. The overall prevalence of the MS_B resistance phenotype was 5%; this figure being lower than that reported by Mišic et al.[21] in Serbia (17.6%) but similar to the results (6%) obtained by Sedaghat et al.[28] in the



Pulsotype	Resistance Profile	Source
1	iMLSB	cellulitis
2	none	meningoencephalitis
3	none	superficial abscess
4	none	wound
5	none	superficial abscess
6	none	superficial abscess
7	none	superficial abscess
8	none	wound
9	none	wound
10	GM	cellulitis
11	none	superficial abscess
12	none	deep abscesses
13	none	superficial abscess
14	none	bacteraemia
15	iMLSB, CIP	superficial abscess
16	none	whitlow
17	none	wounds
18	none	superficial abscess
19	none	bacteraemia
20	GM	superficial abscess
21	iMLSB	superficial abscess
22	none	whitlow
23	GM	superficial abscess
24	none	bacteraemia
25	MSB	superficial abscess
26	cMLSB	wound
27	none	cellulitis
28	none	bacteraemia
29	none	superficial abscess
30	CIP	deep abscesses
31	none	ventilator-associated pneumonia
32	none	superficial abscess
33	none	ventilator-associated pneumonia
34	none	wound
35	TET	cellulitis
36	none	wound
37	none	superficial abscess
38	none	ventilator-associated pneumonia
39	none	superficial abscess
40	none	superficial abscess
41	MSB, CIP	superficial abscess
42	none	wound
43	none	superficial abscess
43	iMLSB, CIP	superficial abscess
44	none	wound
45	iMLSB	superficial abscess
46	none	superficial abscess
47	none	whitlow
48	none	superficial abscess
49	none	wound
50	none	bacteraemia
51	GM	superficial abscess
52	none	ventilator-associated pneumonia

Figure 2 Pulsed-field gel electrophoresis (PFGE) dendrogram of methicillin-susceptible *S. aureus* isolates showing PTs, PGs, associated profile of resistance and sample source. Dashed line indicates PGs (named as A, B and C, with ≥80% similarity and more than 2 isolates in each). $cMLS_B$, constitutive resistance to macrolide, lincosamide and type B streptogramin; $iMLS_B$, inducible resistance to macrolide, lincosamide and type B streptogramin; MS_B, resistance to macrolide and streptogramin B; L, resistance to lincosamides; CIP, resistance to ciprofloxacin; GM, resistance to gentamicin; RIF, resistance to rifampicin; TET, resistance to tetracycline; None, susceptible to all tested antibiotics.

Iranian teaching hospitals[21,28]. The combination of *msrA* and *msrB* genes was detected in all (5) isolates that showed this resistance phenotype. Only one isolate showed the L resistance phenotype; as formerly seen in Argentina, which corresponded to a CA-MRSA isolate carrying SCC*mec* type IV[18]. The occurrence of this resistance type remains low at the HP hospital (1%) as was described previously[17,21,23]. The prevalence of resistance phenotypes to MLS_B antibiotics vary depending on the analyzed country, geographic area and even the type of patient (inpatients vs. outpatients; hospital vs. community origin; children vs. adults; public vs. private institutions; patients or healthcare workers included, among others). This global trend highlights the need for each country, region or hospital to use its own bacterial resistance surveillance program[2,5,26,29]. The obtained results in the BM assay combining 2 μg/ml of CLI plus 0.12 μg/ml of ERY allowed us to differentiate between isolates displaying $iMLS_B$ or $cMLS_B$ phenotypes and those with the MS_B or the S phenotype. However, in contrast with Steward et al.'s findings[32], in this study some *S. aureus* isolates with an $iMLS_B$ resistance phenotype and positive D test were able to grow at higher concentrations of clindamycin. This could be partly explained by the fact that these isolates with the D or D+ phenotype carry the combination of *erm* and *msr* genes, as we discussed further[32]. All analyzed *S. aureus* isolates (n = 100) were susceptible to vancomycin and teicoplanin. However, it is necessary to conduct an active surveillance to early detect the presence of glycopeptide-resistant strains. As expected, most of the *S. aureus* isolates grouped in this study as HA-MRSA (21 of 22) showed ciprofloxacin resistance. Only 4 of 100 isolates were resistant to rifampicin and all of them corresponded to HA-MRSA.

Resistance genes to MLS_B antibiotics and genetic comparison by SmaI-PFGE

The PCR analysis identified the *ermA* gene as the most frequent MLS_B resistance gene followed by *ermC*. The *ermA* gene was detected individually or in combination with other genes such as *ermC*, *msrA* and *msrB* in 30 (79%) out of 38 *S. aureus* resistant to MLS_B antibiotics. In 12 of them, especially in HA-MRSA (11/14), it was individually detected and all these isolates showed a $cMLS_B$ resistance phenotype (R) (see Table 4). This result is in concordance with previous findings obtained in European hospitals[27]. In general, the *ermA* genes are mainly detected in MRSA isolates and are located in transposons related to Tn554, while the *ermC* genes are frequently responsible for resistance in MSSA and are located in plasmids. Based on the *Sma*I PFGE results, we

can rule out the hypothesis of a broad hospital spread of a single pulsotype with a $cMLS_B$ resistance phenotype carrying the *ermA* gene. Only three isolates belonging to cluster A (Fig. 1) showed 100% similarity; they carried the SCC*mec* type II and were recovered from three patients of the same ward. This probably corresponded to a case of limited intrahospital cross contamination. In the two above cited studies, there are no data about the genetic comparison of the included strains; therefore, it cannot be ruled out that they pertained to prevalent clones. In similar studies conducted in Brazil and European countries the *ermC* gene predominated over the *ermA* gene[19,22,37]. As it occurred in Brazil, we merely detected one resistant isolate that carried the *ermB* gene and showed the $cMLS_B$ resistance phenotype[7]. This low prevalence (2.6%) was expected, taking into account that we only included isolates collected from adult patients. This gene is frequently found in *S. aureus* isolates from animal origin and also in pediatric CA-MRSA isolates[7,38]. The PCR analysis identified the *msrA* and *msrB* genes, alone or combined, in 16 isolates belonging to the MSSA, CA-MRSA or HA-MRSA categories with $iMLS_B$, $cMLS_B$ and MS_B resistance phenotypes. An interesting result of this study was the high rate of combination of MLS_B resistance genes. The most commonly detected profile was *ermA* plus *ermC* (n = 14), followed by the combination of *ermA* plus *msrA* (n = 7), many of which also bore the *msrB* gene. All isolates with the $iMLS_B$ resistance phenotype (n = 10) carried both *ermA* and *ermC* genes, and three of them also harbored the *msrB* gene (Table 4). The *Sma*I-PFGE results showed great genetic diversity in both MSSA and MRSA isolates with $iMLS_B$ and $cMLS_B$ resistance phenotypes. They support the wide spread of the resistance genes to MLS_B antibiotics in these studied clinical *S. aureus* isolates rather than the circulation of a predominant PT. This study has two limitations: MRSA isolates were considered to be CA-MRSA or HA-MRSA only according to the SCCmec type that they carried and the use of PCR for *erm* genes does not allow to differentiate between isolates with $iMLS_B$ or $cMLS_B$ resistance phenotypes.

Conclusions

The overall prevalence of resistance to MLS_B antibiotics in this group of clinical *S. aureus* isolates was 38% and the resistance phenotype distribution was as follows: $cMLS_B$, 22%; $iMLS_B$, 10%; MS_B, 5% and L, 1%. In these resistant isolates we detected, *ermA*, *ermC*, *ermB* and *mrsA/B* genes. The single *ermA* gene was commonly found in HA-MRSA (SCC*mec* I, II and III) isolates, mainly in those with a $cMLS_B$ R resistance phenotype; whereas the combination *ermA* and *ermC* was more commonly observed in MSSA or CA-MRSA (SCC*mec* IV) isolates with inducible resistance expression ($iMLS_B$ phenotype). There was a high rate of combination of MLS_B resistance genes and the obtained patterns by *Sma*I-PFGE suggest a great genetic diversity in this group of MRSA and MSSA MLS_B resistant isolates. The obtained results demonstrate the local presence of *S. aureus* resistant to MLS_B antibiotics and its most frequently described responsible genes. These isolates (especially those with the inducible resistance phenotype, iMLSB) may be associated with therapeutic failures; therefore, efforts should be directed toward their correct detection using appropriate laboratory tests, particularly among those recovered from hospitalized individuals suffering from comorbidities or receiving immunosuppressive treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

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