

Antibiotic susceptibility profile and prevalence of *mecA* and *lukS-PV/lukF-PV* genes in *Staphylococcus aureus* isolated from nasal and pharyngeal sources of medical students in Ecuador

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Background: *Staphylococcus aureus* is a common nasal colonizer in 20–30% of the general population. When mucosal and cutaneous barriers are disrupted, *S. aureus* can cause severe infections. While MRSA nasal carriers have an increased risk of infections when compared to non-carriers, prolonged exposure to the hospital environment may cause an increase in carriage of MRSA.

Materials and methods: A survey questionnaire was filled for analyzing risk factors of colonization. Swab isolates were identified as *S. aureus* by traditional microbiological assays. Antibiotic susceptibility profiles were performed following the CLSI standard guidelines. Multiplex PCR was conducted to determine the presence of genes *mecA* and *lukS-PV/lukF-PV*. Chi-squared, univariate, and multivariate logistic regressions were applied to find statistically significant associations between risk factors and the presence of *S. aureus* and MRSA.

Results: One hundred and eighty-six isolates were identified as *S. aureus*. The strains showed high resistance to penicillin, oxacillin, azithromycin, erythromycin, clindamycin (inducible), and tetracycline. The overall prevalence of MRSA in medical students was 45.9% [40.4–51.6] 95% CI. PCR showed a prevalence of *mecA* gene in MRSA isolates of 6.1% while *lukS-PV/lukF-PV* gene was present in 3.2% [1.2–6.9] 95% CI of the *S. aureus* samples. The risk factors frequency of antibiotic intake and repeated visits to hospitals demonstrated statistical significance.

Conclusion: *S. aureus* and MRSA isolates have a high prevalence of colonization, and antibiotic resistance in the population studied. MRSA resistance was not related to the presence of the *mecA* gene. The prevalence of *PVL* genes was low, but it could represent a risk because they are circulating in the community.

Keywords: *mecA*, medical students, MRSA, Panton-Valentine leukocidin, *Staphylococcus aureus*

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Introduction

Staphylococcus aureus is part of the human skin and mucosal microbiota and a common opportunistic pathogen that causes significant infections.¹ *Staphylococcus aureus* is carried in the nasal mucosa in 20–30% of the general population.² However, when mucosal and cutaneous barriers are disrupted, *S. aureus* can produce blood-stream, heart, skin and soft tissues, bones, joints, and device-related infections.^{1–3}

While there are still some strains of *S. aureus* sensitive to more commonly prescribed antibiotics, emerging *S. aureus* isolates have developed antimicrobial resistance. Those resistant to the antibiotic methicillin are termed methicillin-resistant *Staphylococcus aureus* (MRSA)^{3,4} because they have acquired a *mecA* gene, a coding gene of an essential protein to bacterial cell wall synthesis called PBP2', which is found in a mobile genetic element called staphylococcal chromosome cassette *mec* (SSC*mec*).⁴

Prevalence of hospital-associated MRSA (HA-MRSA) in Latin America ranges from 6% to 80%, where Cuba has the lowest percentage and Peru the highest.⁵ Differences in prevalence data between countries may be due to differences between the population studied (from primary, secondary, and tertiary health care facilities origin), microbiology laboratory protocols, and available technologies. Guzman-Blanco et al showed that more than 50% of Latin American countries have more than 50% prevalence of HA-MRSA.⁵ Zurita et al in a study conducted in Ecuador demonstrated that the frequency of MRSA is 46.97%.⁶

MRSA nasal carriers have an increased risk of infections when compared to non-carriers.⁷ However, when the bacterium has virulence factors, such as cytolytic toxin Pantone-Valentine leukocidin (PVL) (a cytotoxin associated with tissue necrosis and leukocyte destruction), the chance of complicated infections increments. Depends on the geographical area or the type of patients, the presence of PVL in *S. aureus* isolates can vary from 3% to 75%,^{8–10} but in Latin America, the presence of these genes in circulating HA-MRSA or community-associated (CA) MRSA has not been fully elucidated.¹¹

Evidence suggests that prolonged exposure to the hospital environment may cause an increase in carriage of MRSA.¹² Work-related factors for MRSA carriage among health care workers include work experience and area of service, employment in areas of high patient MRSA prevalence and close contact with them, high workload, and poor hand hygiene.¹³ Although medical students are not technically health care workers, they have intermittent exposure to hospital environments. Little is known regarding *S. aureus* and MRSA colonization among medical students.^{14–19} Additionally, MRSA surveillance studies carried out in community settings are necessary to better understand the molecular and clinical epidemiology of emerging MRSA isolates.²⁰ Therefore, the purpose of the present study was to determine the antibiotic susceptibility profiles and *S. aureus* and MRSA colonization prevalence

in nose and throat, and its association with risk factors in healthy medical students at the Universidad de Las Américas.

Materials and methods

Population, study design, ethical considerations, and sample collection

Our work was an exploratory and cross-sectional study in a small cohort of medical students from the Universidad de Las Américas (UDLA) in Quito, Ecuador. The study was performed at the Research Laboratories at the UDLA from March to November of 2018. The Ethics Committee for Investigation on Human Beings at Universidad San Francisco de Quito approved the research with the approval code 2018-026E in accordance with the Declaration of Helsinki. All participants signed the previously approved informed consent.

The sample size was calculated using the epidemiological program Epi Info 7.2.2.1, StatCalc based on the number of enrolled students in Medical School in the second period of 2017. The total amount was 1349 students. We used an expected frequency of 46.97% according to the reports of Zurita et al⁶ and an error range of 5%. The final sample size calculated was 298 students, with a reliability level of 95%. The sample was taken from students that signed the informed consent and filled up a survey questionnaire that was designed according to Halablab et al to determine the risk factors of *S. aureus* colonization in healthy carriers.²¹ Nasal and pharyngeal swabs were taken from every participant, cultured in blood agar (Medibac INC S.A, Ecuador), and incubated for 16–18 hrs at 36°C.²²

Phenotypic identification

Isolates that grown with beta-hemolysis in blood agar (Medibac INC S.A, Ecuador) were cultured in mannitol salt agar (BD Difco™, USA) and incubated for 16–18 hrs at 36°C.²² Isolates that were positive for mannitol salt agar were tested by Gram stain, coagulase, and catalase assays. Strains that were mannitol positive, *Staphylococcus* Gram +, catalase positive and coagulase positive were identified as *Staphylococcus aureus* and isolated for future experiments.

Antibiotic susceptibility

Antibiotic susceptibility was performed by the disk diffusion method following the Clinical & Laboratory Standards Institute (CLSI) guidelines.²² Briefly, we took

one or two colonies, previously grown on nutrient agar (BD Difco™, USA), and diluted them in Muller Hinton broth (BD Difco™, USA) until reaching 0.5 McFarland scale. After that, we followed the disc diffusion method according to Kirby-Bauer.²³ The antibiotics used were as follow: penicillin (10U), oxacillin (30 µg), vancomycin (E- Test), gentamicin (10 µg), erythromycin (15 µg), linezolid (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30 µg), rifampicin (5 µg), clindamycin (2 µg), azithromycin (15 µg), mupirocin (100 µg). Clindamycin and erythromycin were used in a D-test to assess inducible clindamycin resistance. The strain *Staphylococcus aureus* ATCC 25923 was used as a control.

DNA extraction and molecular identification of genes *mecA* and *LukS-PV/LukF-PV*

DNA extraction was performed following the Chelex-100 methodology with some modifications.²⁴ Briefly, 6–10 colonies of *S. aureus* were resuspended on 200 µL of Chelex 10% (Sigma-Aldrich, USA) and 5 µL of Proteinase K (Invitrogen, USA). Samples were incubated at 56°C for 60 mins. After that, the samples were vortexed and centrifuged at 8000 g for 2 mins. Another incubation was made at 96°C for 20 mins. Finally, samples were centrifuged at 8000 g for 5 mins, and the supernatant was transferred to a clean tube and stored at –20°C.

Polymerase chain reaction (PCR) was performed with modifications of the protocol reported by Zhang et al.²⁵ The reaction mix for the multiplex PCR contained 1.3 U/µL Platinum Taq DNA polymerase (Invitrogen, USA), 1X PCR buffer (Invitrogen, USA), 2.3 mM MgCl₂ (Invitrogen, USA), 0.4 mM dNTPs (Invitrogen, USA), 10 ng/µL of DNA and 0.6 µM primers Staph 756F and Staph 750 R,²⁶ 0.3 µM of primers Nuc1 and Nuc2,²⁶ 0.6 µM of primers MecA147-F and MecA 147-R²⁷ and 0.5 µM of primers Luk-PV-1 and Luk-PV-2.²⁸ The PCR amplification was performed in a thermocycler Eppendorf, Mastercycler® Gradient (Eppendorf, Germany) with the initial denaturation step at 94°C for 7 min, followed by two cycling steps of 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s and 20 cycles of denaturation at 94°C for 30s, annealing at 63°C for 30 s, and extension at 72°C for 45 s. A final extension was performed at 72°C for 7 mins. PCR products were analyzed by 2% (w/v) agarose gel containing DNA stain SyBr Safe (Invitrogen, USA) in 1X

TBE buffer and electrophoresis was performed at 100 V for 35 min in a Labnet Enduro Gel XL Electrophoresis System (Labnet International, Inc, USA).

The agarose gel was visualized under UV light using a gel documentation system ChemiDoc™ Imaging Systems (BioRad, USA). The length of amplicons was determined by comparing with a 100 bp DNA ladder (Invitrogen, USA). The lengths of amplification of expected products were 756 bp for *16S rRNA* (Stapy756-F and Staph750-R), 279 bp for thermostable nuclease (*nuc*) (Nuc-1 and Nuc-2), 147 bp for *mecA* (*mecA*147-F and *mecA*147-R), and 433 pb for PVL genes *lukS-PV/lukF-PV* (Luk-PV-1 and Luk-PV-2). The strain PV-STAPH (kindly donated by the Hospital del Mar de Barcelona, Spain) was used as a positive control for all the four genes. The strain *S. aureus* ATCC 25923 was used as a control for genes 16S and *nuc*.

Statistical analysis

All results were grouped depending on the outcome of interest: 1) *S. aureus* carriers, 2) *S. aureus* MRSA, 3) Presence of *mecA* gene and 4) Presence of PVL.

Chi-squared, uni, and multivariate logistic regressions were applied to find statistically significant associations between risk factors and the presence of 1) *S. aureus* carriers, 2) *S. aureus* MRSA, 3) Presence of *mecA* gene and 4) Presence of Pantone- Valentine.

For all statistical analyses, *p*-values <0.05 were considered significant. All studies were carried out in R version 3.5.3. and JASP (2018).

The frequency of isolates confirmed as *S. aureus* was determined as sensitive, intermediate resistant, and resistant.

Results

A total of 322 medical students from the first to the eleventh semester participated in the survey. Most of the participants were in the fourth and fifth semesters in the medical curriculum with 36.6% (n=118/322) and 16.1% (n=52/322), respectively. The mean age of the participants was 20.8 years (minimum: 17 years, maximum: 34 years), 46.3% (n=149/322) were males, and 53.7% (n=173/322) were females.

All 322 samples were analyzed using traditional microbiological assays. 17.1% (n=55/322) were isolated from nose, 25.1% (n=81/322) from throat, and 15.5% (n=50/322) from both localizations. *S. aureus* was present in 57.8% [52.2–63.2]95% CI of the samples (n=186/322).

S. aureus antibiotic susceptibility profiles

Using a disc diffusion method, the resistance profiles of the 186 *S. aureus* isolates were determined as follows. The highest percentage of resistance was against penicillin 93.5% (n=174/186), followed by oxacillin with 79.6% (n=148/186), azithromycin with 61.8% (n=115/186), erythromycin with 52.7% (n=98/186), clindamycin (using D-test) with 39.2% (73/186), tetracycline with 19.4% (36/186), ciprofloxacin with 3.2% (n=6/186), gentamicin with 1.1% (n=2/186), and the lowest percentage of resistance was for both trimethoprim/sulfamethoxazole and chloramphenicol with 0.5% (n=1/186). All the 186 isolates were sensitive to vancomycin (using Etest), linezolid (>21 mm), rifampicin (>20 mm), and mupirocin (>1 mm). According to the oxacillin resistance, the overall prevalence of MRSA in medical students was 45.9% [40.4–51.6] 95% CI (n=148/322) (Figure 1).

Molecular analysis of MRSA

Using a multiplex PCR assay, we determined a prevalence of *mecA* gene in MRSA isolates of 6.1% [2.8–11.2]95%

CI (n=9/148). The frequency of *lukS-PV/lukF-PV* genes in *S. aureus* was 3.2% [1.2–6.9]95% CI (n=6/186), from these, 2.7% [0.8–6.2]95% CI (n=5/186) were MRSA. No isolates were found as *lukS-PV/lukF-PV* and *mecA* gene positive at the same time.

Risk factors analysis

Non-statistical association was found for the standard demographic indicators such as age and sex for neither *S. aureus* nor MRSA colonization.

S. aureus colonization

Logistic regression found that *S. aureus* colonization was negatively associated if the students took just one cycle of antibiotic during the 12 months before the sampling ($p=0.039$). *S. aureus* colonization was negatively associated as well with students of the third semester ($p=0.046$).

MRSA colonization

The chi-squared test revealed that MRSA colonization was associated with the intake/not intake of antibiotics due to

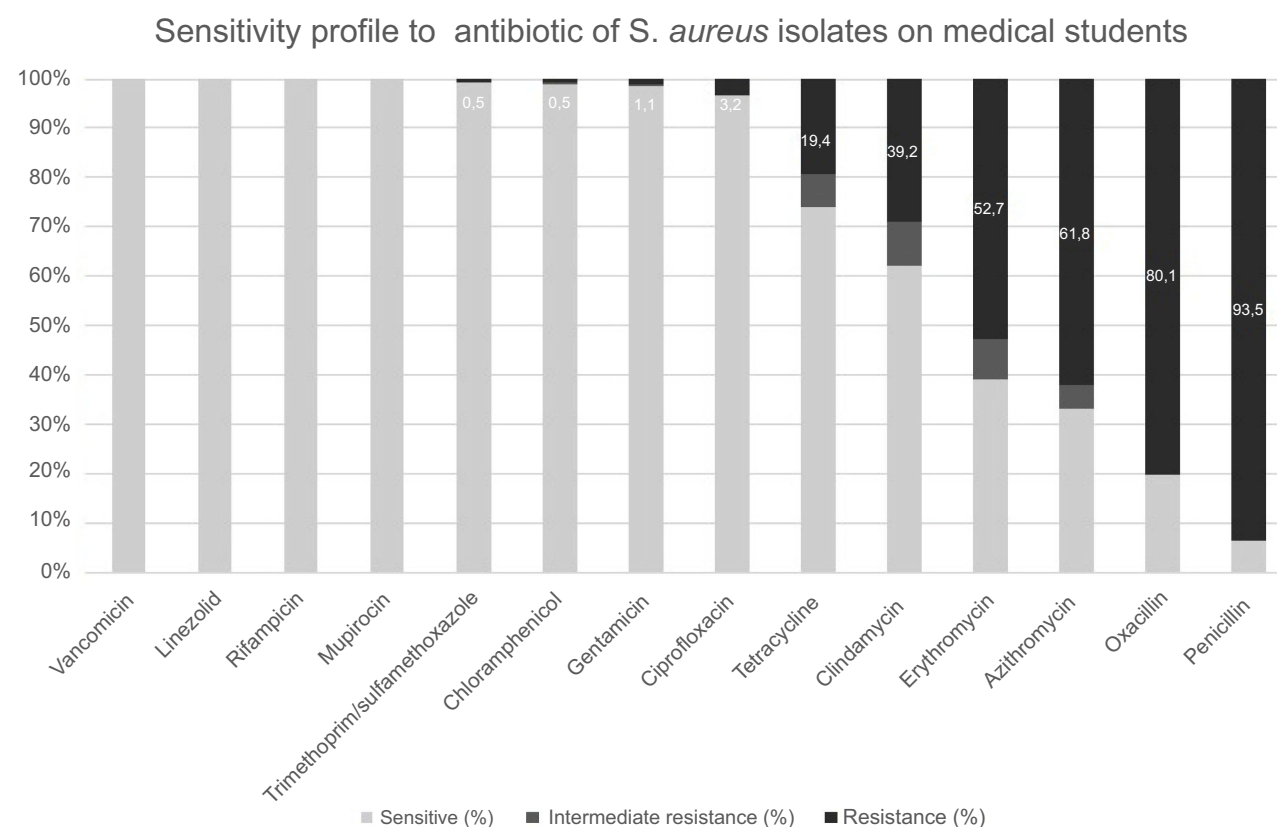


Figure 1 Antibiotic susceptibility profiles of *Staphylococcus aureus* isolated from nasal and pharyngeal swabs from medical students.

Note: Numbers inside the bars indicate resistance.

skin infections ($p=0.038$) or due to another medical condition ($p=0.025$), during the 12 months before taking the sample.

Logistic regression showed a significant negative association of MRSA colonization in students that took antibiotics during skin infections ($p=0.042$), or when they took antibiotics due to other medical conditions ($p=0.026$), or when they took only one cycle of antibiotics ($p=0.029$) during the twelve months before to the sampling.

Related to the level in the medical curriculum and visits to clinics or hospitals during their training, we identified that students who were taking the third semester ($p=0.022$) or those who visited a health unit twice a week ($p=0.035$) were less likely to be colonized by MRSA.

Discussion

To our knowledge, this is the first report that analyses antibiotic susceptibility profiles in *S. aureus* isolates from nose and throat swabs, and its association with risk factors for colonization in healthy medical students in Ecuador.

The overall prevalence of *S. aureus* in our study was 57.8%. Individually, the rate of the nasal carriage was 17.1%, and pharyngeal carriage was 25.4%. Previous studies, based on nasal swabs, have reported similar prevalences in medical students between 15% to 22.1%.^{14,15,17–19} Additionally, other reports have shown high variability in the nasal and pharyngeal carriage of *S. aureus* between populations.^{29–32} When Senn et al combined three sites (nose, groin, and throat), the sensitivity of *S. aureus* detection by culture increased to 96%.³³ In our report, combining two anatomical sites for screening, the overall prevalence reached 57.8%. Although *S. aureus* is most often found in the nose,³⁴ our study has shown that this bacteria colonizes throat (25.4%) more frequently than the nose (17.1%) in medical students at the UDLA. Therefore, it is crucial to carry out investigations for screening samples from different anatomical niches to improve the rate of *S. aureus* detection and understand the real burden of colonization.

Antibiotic susceptibility pattern of *S. aureus* isolates in our study is comparable to other similar studies when were included the most commonly used drugs like penicillin, oxacillin (methicillin), azithromycin, erythromycin, clindamycin, and tetracycline.^{16,19} When we used the D-test, the resistance of *S. aureus* to clindamycin was 39.2% (73/186) instead of 3.2% (n=6/186) (data not shown) when a disc of clindamycin was used alone. It evidences the importance of

including in routine susceptibility test a D-test in all *S. aureus* isolates.³⁵ In contrast with a study conducted in Nepal that found resistances between 20–33.3%, our work reported low rates of resistance against ciprofloxacin, gentamicin, and trimethoprim/sulfamethoxazole¹⁷ maybe because these antibiotics have a low frequency of use in the population studied. In agreement with other studies, all the isolates in our report were sensitive to vancomycin,^{16–18} linezolid,¹⁸ and rifampicin.¹⁸ Similar studies with medical students have not included mupirocin in their analyses.

Interestingly, the frequency of MRSA (using phenotypic methods) reached 79.5% (n=148/186), but only 6.1% (n=9/148) of the MRSA isolates presented the *mecA* gene. Our results contrast sharply with data published in Sudan, which found that 90.2% (111/123) of the isolates were *mecA* positive.³⁶ They suggest that finding of *mecA* gene is the principal evidence for the detection of MRSA isolate. However, we found only 6.1% (n=9/148) isolates *mecA* positives. These data suggest that resistance to oxacillin is due to other genetic alternatives. Patterson et al have identified the emergence of a variant of the *mecA* gene known as the *mecC* gene with a genetic similarity of 70%,³⁷ which could explain the low frequency of the *mecA* gene in this study. Elhassan et al suggest searching for other intrinsic factors that may compete with the *mecA* gene in producing resistance events in regions with a high prevalence of MRSA.³⁶

Additionally, our study identified *lukS-PV/lukF-PV* gene in 3.2% of the samples. The prevalence of PVL-positive MRSA varies considerably from country to country. While in France the prevalence is as lower as 3%,⁹ in the United States reach 98%.⁸ These findings alert to health authorities about the high rate of MRSA circulating in a community and a non-hospital population as would be expected. Based on the chance of person-to-person transmission, we support the implementation of surveillance systems both in hospital and community settings sampling different patient body sites to identify the emergence of new strains, the antibiotic resistance profile, the risk factors associated with infection,³⁸ and the potential contributions of undetected carriers to MRSA transmission.³⁹ Today, the standard MRSA surveillance method is a culture-based strategy, but Ábrók et al developed an approach to minimize time-consuming culturing-based MRSA screening combining MALDI-TOF MS optimized and PBP2' latex agglutination assay.⁴⁰ Therefore, to prevent the MRSA dissemination, rapid and accurate detection of MRSA strains are appropriate measures to understand the

situation of MRSA distribution in the community and health care facilities.

The present report failed to identify a statistical association between age and sex and *S. aureus* and MRSA colonization. We sought to analyze whether the level in the medical curriculum could help prone the students to *S. aureus* and MRSA colonization taking in count that students in higher semesters have longer stays in clinical practice than students in lower semesters. They did not find any significant association between the semester and the acquisition of *S. aureus* among medical students. On the contrary, who was taking the third semester or those who visited a clinic or hospital twice a week were less likely to be colonized by MRSA. In contrast, research conducted in Ethiopia found that more than half of their study participants who were exposed to a university medical center for more than two years were colonized with MRSA.¹⁹ Unexpectedly, our report found that *S. aureus* and MRSA colonization was negatively associated with the use of antibiotics. Fanelly et al recognized that long-term use of antibiotics decreased the prevalence of *S. aureus* colonization by nearly 70%, and a reduced frequency of *S. aureus* colonization was identified with the use of oral and topical antibiotics.⁴¹

The limitations of our study were that we could not distinguish between persistent and intermittent carriers because this was a cross-sectional study. Persistent carriers are individuals that almost always carry one type of *S. aureus* in 20% of the population while 60% of them harbor this bacterium intermittently.⁴² Another factor was that we analyzed only one strain from each participant, so we cannot be able to ensure if participants colonized with *S. aureus* at nose and throat are carriers of the same or different strains. Additionally, only 10% of medical students agreed to participate when they were chosen at random and notified via email. As a result, we invited all 1300 medical students in the same university to increase and achieve the appropriate sample size, and it could have introduced selection bias. Furthermore, information concerning to antibiotics used in the last 12 months, names, doses, and other data could have introduced recall bias in our study.

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Author Contributions

All authors contributed towards data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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