MICROBIOLOGY

Clonal distribution and molecular characterization of *Staphylococcus aureus* isolated strains in Chania and Heraklion, Crete

Eftychios Vittorakis[1,2,3], Mihaela Laura Vica[2], Stanca-Lucia Pandrea[1,4], Amanda Rădulescu[5], Calina Oana Zervaki[3], Evangelos Vittorakis[3], Sofia Maraki[6], Viktoria Eirini Mavromanolaki[6], Michael Ewald Schürger[2], Vlad Sever Neculicioiu[1], Evangelia Papadomanolaki[3], Lia Monica Junie[1]

1) Department of Microbiology, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania

2) Department of Cell and Molecular Biology, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania

3) Agios Georgios General Hospital of Chania, Crete, Greece

4) Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca, Romania

5) University Hospital of Infectious Diseases, Cluj-Napoca, Romania

6) Department of Clinical Microbiology and Microbial Pathogenesis, University Hospital of Heraklion, Crete, Greece

Abstract

Aim. This study investigates the demographic distribution, antibiotic resistance profiles, and molecular characteristics of *Staphylococcus aureus* infections.

Methods. The study was carried out in 141 patients, 60.4% male, in patients from Chania and Heraklion, Crete.

Results. The highest infection prevalence observed in the older adults (≥65 years) age group. The predominant infection types were skin lesions (39.72) and respiratory tract infection (22.7%). Antibiotic resistance testing revealed that 57.44% of strains were *MRSA*, with high resistance to Tetracycline, Ciprofloxacin, Kanamycine Erythromycin and Clindamycin. Molecular analysis showed 19.14% of strains were *Pvl*-positive, highlighting the presence of both *MRSA* and *MSSA* strains with *Pvl* genes.

Conclusions. The study underscores the need for continuous surveillance and targeted infection control strategies to manage the spread of *MRSA*, particularly in vulnerable populations.

Keywords: *Staphylococcus aureus (S. aureus), Plv* (Panton-Valentine leucocidin), *MRSA* (Methicillin-resistant *S. aureus*), Hospital-Associated *MRSA* (HA-*MRSA*), Community-Associated *MRSA* (CA-*MRSA*), *MSSA* (Methicillin-sensitive *S. aureus*), PCR (Polymerase Chain Reaction)

Introduction

Staphylococcus aureus (S. aureus) is a pathogenic bacterium that causes a range of infections in humans. Its ability to cause disease is due to surface components such as capsular polysaccharide, protein A, clumping factor, and fibronectin binding protein. Additionally, it produces extracellular virulence factors including coagulase, hemolysins, enterotoxins, toxic shock syndrome toxin, exfoliatins, and Panton-Valentine leucocidin (*Pvl*) [1,2].

Several genes are linked to the virulence of *Staphylococcus aureus (S. aureus)*. Some genes encode toxins that damages tissue [3].

Hospital-Associated *MRSA* (HA-*MRSA*) and Community-Associated *MRSA (CA-MRSA)* are differentiated based on clinical, epidemiological criteria and on the presence of the *Pvl* gene. HA-*MRSA* is typically found in healthcare settings like hospitals and long-term care facilities, primarily impacting patients with existing health conditions, recent surgeries, or those using medical devices such as catheters. These strains are often resistant to a wide range of antibiotics, complicating treatment.

CA-*MRSA* arises in community settings among healthy individuals,

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Address for correspondence:
Eftychios Vittorakis
vittorakis.eftihis@gmail.com

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frequently presenting as skin and soft tissue infections. CA-*MRSA* strains usually exhibit resistance to fewer antibiotics than HA-*MRSA*. Understanding these differences is essential for effective infection control and treatment approaches [4].

Certain *S. aureus* strains are resistant to methicillin, due to the *mecA* gene, which encodes the penicillin-binding protein (PBP)2a. This resistance is a defining feature of *MRSA* [5-7]. The staphylococcal cassette chromosome *mecA* (*SCCmec*) is a mobile genetic element that enables *S. aureus* to acquire methicillin resistance, making it methicillin-resistant *S.aureus* (*MRSA*). Detection of *mecA gene* through PCR (Polymerase Chain Reaction) is crucial for identifying *MRSA* as further presented in the Molecular Analysis section [8,9].

Determining the roles of staphylococcal virulence factors in invasive infections can be challenging [10,11]. The production of *Pvl* (*Panton-Valentine leucocidin*) is associated with skin and subcutaneous tissue lesions, and severe community-acquired necrotic pneumonia. Research indicates that *Pvl* genes are located on a phage [12-14].

Pvl is a toxin that destroys white blood cells and causes tissue death. It is found in less than 5% of *S. aureus* strains but is present in 93% of strains producing furunculosis and 85% of those associated with severe necrotic hemorrhagic pneumonia. Additionally, *Pvl* genes are detected in strains associated with cellulitis (55%), cutaneous abscess (50%), osteomyelitis (23%), and finger pulp infection (13%). *Pvl* genes are not found in strains producing infections like infective endocarditis, mediastinitis, hospital-acquired pneumonia, urinary tract infections, enterocolitis, and toxic shock syndrome, indicating that *Pvl* is mainly associated with skin or mucosal lesions [15-17].

The purpose of this study is to determine the antibiotic resistance of *S. aureus* strains, aiding in the diagnosis and treatment of patients. Additionally, the study aims to identify genetic determinism of *S. aureus* in 2 areas of Crete. Greece: Chania and Heraklion. [18-20]. All *S. aureus* isolates were tested for the *mecA* gene and for the presence of *Pvl* genes [18-20].

Methods

Patient data collection

Patient data were obtained the General Hospital of Chania and the University Hospital of Heraklion. Information obtained from patient records included gender, age, and the type of care setting, such as outpatient, inpatient, intensive care unit, or surgical ward. Samples collected for the study included abscesses, blood, urine catheter samples, tracheal aspirates, wound ulcers, pus, and joint fluids [12].

Inclusion and exclusion criteria

The study included patients with a positive *S. aureus* culture, with samples collected between January 1, 2020, and March 31, 2022, from the Chania general hospital laboratory and the University Hospital of Heraklion microbiology laboratory. All patients, regardless of age, gender, or care setting, were included. Exclusion criteria were a negative *S. aureus* culture.

Assessment of inflammation

The assessment of inflammation was a critical component of this study, focusing on the inflammatory response triggered by *S. aureus* infections. Patients were observed for clinical indicators of inflammation, including redness, swelling, heat, and pain at the infection site. A clinical scoring system was utilized to quantify the severity of these symptoms, using a scale ranging from 0 to 4, indicating: 0 no symptoms, 1 mild inflammation, 2 moderate, 3 severe, 4 very severe inflammation. This approach allowed the comparison of patients' inflammation severity [3,9,10].

Blood samples were analyzed for inflammatory markers, including C-reactive protein (CRP) (normal upper limit: 0.5 mg/dL), erythrocyte sedimentation rate (ESR) (normal upper limit: 25 mm/hr), and white blood cell (WBC) count (normal range: 4-11 K cells/μL) [12].

Microbial cell culture and analysis

Samples were collected from infection sites: tracheal aspirates, abscesses, blood, urine, and other. Microbial cell cultures were performed to isolate *S. aureus*. A total of 141 *S. aureus* strains were analyzed using the Vitek technique, which identified 81 strains as *MRSA* (methicillin-resistant *S. aureus)*. The remaining 60 strains were identified as methicillin-sensitive *S. aureus (MSSA)* [20].

MDR bacteria are defined as bacteria that are non-susceptible to at least one antimicrobial agent in three or more antimicrobial classes, extensively drug-resistant (XDR), as non-susceptibility to at least one antimicrobial agent in all, but two or fewer antimicrobial classes and pandrug-resistant (PDR) as non-susceptibility to all agents in all antimicrobial classes [21].

Sample analysis - *S. aureus* isolates and antibiotic susceptibility testing

S. aureus was identified using standard methods, and antibiotic susceptibility testing (AST) was conducted using the VITEK 2 system compact bioMerieux, an automated bacteriological analyzer, (for microorganism identification and antimicrobial susceptibility testing) [7,13].

The disk-diffusion method was employed to determine the susceptibility of the strains to a range of antibiotics [1].

The agar dilution method determined the minimum inhibitory concentrations (MICs) of oxacillin. For accuracy, a secondary analysis was performed using the disk-diffusion method with BBL (Becton, Dickinson and Company) disks for Cefoxitin [1,22].

Vancomycin resistance was tested using the E-test method was employed to determine the minimum inhibitory concentration (MIC) for Vancomycin. This method involves placing a plastic strip impregnated with a concentration gradient of Vancomycin on an agar plate inoculated with *S. aureus*. After incubation, the MIC is determined at the point where the elliptical zone of inhibition intersects the strip, including kinetic analysis through multichannel fluorimeter and photometer readings, recording fluorescence, turbidity, and colorimetric signals [5,22].

The susceptibility testing to AB was interpreted according to EUCAST Version 10.0 (2020)**,** with breakpoints established for Glycopeptides and other antibiotics [3].

The Clinical and Laboratory Standards Institute (CLSI) guidelines, as outlined in M100, 30th Edition (2020), were used for interpreting antimicrobial susceptibility testing results**.** These guidelines define the breakpoints used to categorize bacterial strains as susceptible, intermediate, or resistant to specific antibiotics [3,23].

Antibiotic classes tested

β-lactamins (Penems), Glycopeptides: (Vancomycin, Teicoplanin), Aminoglycosides (Kanamycin, Gentamicin), Macrolides (Erythromycin, Clindamycin), Tetracycline, Fluoroquinolones (Ciprofloxacin), Sulfonamides (Sulfamethoxazole-trimethoprim) and Fusidic acid [2,15].

β-lactamase production was tested using nitrocefin disks, and the D-test assessed inducible clindamycin resistance. The D-test is a phenotypic method used to detect inducible clindamycin resistance in *Staphylococcus aureus* [4,16]. To perform the D-test, we inoculate a Mueller-Hinton agar plate with the bacterial suspension, then we placed erythromycin (15 µg) and clindamycin (2 µg) disks about 15-20 mm apart. After incubating at 35° C for 16-18 hours, we checked for a "D" shaped zone of inhibition around the clindamycin disk facing the erythromycin disk. A positive D-test indicates inducible clindamycin resistance, and the isolate should be reported as clindamycin-resistant, even if it initially appeared susceptible. If the zone is circular, the test is negative, indicating no inducible resistance [9,24].

Determination of *S. aureus* genotypes

The genotypes of *S. aureus* isolates were determined at the Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania, using PCR. Staphylococcal cassette chromosome (*SCC*) *mec* types, *Pvl* genes (*lukS* and *lukF*) genes were detected.

DNA isolation for PCR tests

DNA was isolated from 141 *S. aureus* strains and various genes were identified using the PCR technique. DNA extraction was performed using the phenol-chloroform method. A single colony from each *S. aureus* culture was inoculated into brain-heart infusion broth and incubated overnight at 37 °C. The culture was centrifuged, and the pellet was re-suspended in TES buffer with lysostaphin. After incubation and additional processing steps, including the addition of SDS, phenol, chloroform, and RNase, the DNA was ready for PCR reactions [16].

We implemented several measures to avoid cross-contamination between samples and contamination from laboratory personnel. These measures included using pre-aliquoted reagents, wearing gloves, and employing disposable tips with aerosol-resistant filters. Furthermore, the preparation of the amplification reaction mixtures and the analysis of the amplified products were performed in distinct areas with different sets of pipettes. To detect potential contamination, nuclease-free water was used as a negative control in each PCR run instead of a DNA sample.

PCR reaction for the detection of mecA gene

The PCR reaction mixture included 5 µL of 10× reaction buffer, 3 µL of 25 mM MgCl2, 1 µL of 2.5 mM dNTPs (Promega, Wisconsin, USA), 1 µL of *mecA1* primer (20 pmol/µL), 1 µL of *mecA2* primer (20 pmol/µL), 0.2 µL of Taq polymerase (5 U/µL, Promega), 10 µL of DNA, and 28.8 µL of H2O. The primers used for *mecA* detection were:

PCR reactions were carried out using a Mycycler (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of 1 minute at 94 °C, 1 minute at 54 °C, and 1 minute at 72 °C, with a final extension at 72 °C for 7 minutes. The resulting amplicons were separated on a 1% agarose gel (Sigma-Aldrich) in 0.5× Tris Borate EDTA buffer, stained with ethidium bromide (1 µg/mL, Thermo Scientific, Agawam, MA, USA), and visualized under UV light using a GelDoc-XR apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Positive controls included DNA from *MRSA* strain ATCC 33591 (KWIK-STIK, Lansing, MI, USA), and negative controls were DNA from strains ATCC 6538 and ATCC 29213 (Thermo Scientific).

The *GyrA* gene amplification served as a control to verify DNA extraction quality and the absence of PCR inhibitors. The oligonucleotides were synthesized by Eurogentec (Seraing, Belgium) [16,17].

The negative control (nuclease-free water) did not amplify, indicating the absence of contamination, the negative *MRSA* strains ATCC 6538 and ATCC2913 also did not amplify for the *mecA* gene, and the positive control ATCC 33591 strain did amplify, confirming that the primers were correctly designed and confirming the accuracy of the PCR method employed in the study [16].

Characterization of Pvl

All *S. aureus* strains were tested for the presence of *Pvl* using PCR. *S. aureus* ATCC 49775 produced three proteins recognized by antibodies against the S component

of *Pvl* (*luks-PV)* and two recognized by antibodies against the F component (*lufs-PV*). These findings confirmed the presence of two loci encoding *Pvl*, with *luks-PV* and *lukf-PV* coding for the S and F components, respectively. It's noteworthy that the *Pvl* from *S. aureus* ATCC 49775 should not be confused with leucocidin from ATCC 27733, which shares 95% identity with *hlgC* and *hlgB* from gamma-hemolysin.

S. aureus ATCC 49775, initially isolated from an infected area in a patient, was the standard strain for *Pvl* detection. *S. aureus* Newman served as a *hlg*-positive control strain and a negative control for *Pvl* genes, as it lacks *lukS-PV* and *lukF-PV*. Primers for *Pvl* genes were designed based on published sequences (GenBank accession numbers X72700 and AB006796) (Table I).

PCR conditions for *Pvl* detection included initial denaturation at 94 °C for 4 minutes, 30 cycles of 45 seconds at 94 °C, 45 seconds at 56 °C, and 30 seconds at 72 °C, followed by a final extension at 72 °C for 2 minutes. The products were visualized by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. A 433 bp fragment indicated the presence of *Pvl* [12-19].

Table I. Primer sequences**.**

GENE	PRIMER	SEQUENCE (5' TO 3')
mecA	*mecA-1(F)*	5'- TGGCTATCGTGTCA CAATCG-3'
	mecA-2(r)	5'- CTGGAACTTGTTG AGCAGAG-3'
gyrA	*GyrA(F)*	**5'**- AATGAACAAGG TATGACACC-3'
	gyrA(R)	**5'**-TACGCGCTTCAG TATGACACC-3'
Pvl	*luk-PV-1(F)*	5'-TCATTAGGTAAAA TGTCTGGACATGATCCA-3'
	luk-PV-1(R)	**5'**- GCATCAASTGTATTGGATAGC AAAAGC-3'

Statistical analysis

Statistical evaluation was conducted using SPSS 23.0 for Windows, determining descriptive statistics such as mean, median, standard deviation, standard error, and minimum and maximum values.

Results

Demographic representation

In this study, 141 patients were diagnosed with *S. aureus* infection, and their demographic distribution was analyzed. Among these patients, 60.4% were male, and 39.6% were female, indicating a higher prevalence of *S. aureus* infections among males compared to females.

The demographic analysis revealed a diverse age distribution:

The older adults (≥65 years) had the highest number of patients (77). This was followed by adults (25-64 years), which included 48 patients and young adults (20-24 years) with 5 patients. The Adolescents group of 10-19 years had 11 patients (Table II).

These statistics highlight that *S. aureus* infections are more prevalent among older patients, particularly those between 65+ and older.

Table II. Age distribution of patients according to WHO age group criteria**.**

Years	NR-Patients	%
0-19	11	8%
20-24	5	3%
25-64	48	34%
65 and older	77	55%
Total	141	100%

Inflammatory response assessment

The study evaluated inflammation indicators among patients infected with *S. aureus*.

We assessed several laboratory markers of inflammation. High levels of these markers were indicative of an inflammatory response which were consistently elevated across all patient groups:

- **C-reactive protein (CRP)** levels were elevated in 93% of patients, with an average CRP level of 11 mg/dL (normal upper limit: 0.5 mg/dL).
- **The erythrocyte sedimentation rate (ESR)** was elevated in 86% of patients, with an average of 48 mm/hr (normal upper limit: 25 mm/hr).
- **White blood cell (WBC) counts** were elevated in 97% of patients, with an average count of 17 K cells/µL (normal range: 4-11 K cells/µL).

Clinical scores were assigned to quantify the severity of inflammation at the infection site for all 141 patients. The average clinical score across the cohort was 2.5 (range: 1 to 4).

The clinical severity classification (the distribution of scores) among the patients (141) showed that:

Mild infections were observed in 35% of patients (49 patients). These patients generally exhibited less severe symptoms and had lower clinical scores for inflammation (1 and 2), suggesting a less intense inflammatory reaction.

Average infections were found in 55% of patients (78 patients). These showed moderate symptoms, such as fever, and scored between 2 and 3 on the clinical inflammation scale.

Severe infections (fever and/or septic shock and / or other semiology) were identified in 10% of patients (14 patients). These patients were characterized by a severe inflammatory response, with clinical scores 3 and 4.

- Patients with respiratory tract infection and bloodstream infections had the highest scores, of 3.5 (range: 3 to 4).
- Patients with skin lesions generally exhibited

lower scores, of 2.0 (range: 1 to 3).

These results highlight the variability in inflammation severity among different infection types and provide a quantifiable measure of the inflammatory response in the study population.

The severity of the inflammatory response correlated with the type of infection, with more severe responses noted in cases of respiratory tract infection (bronchopneumonia) and bloodstream infections compared to skin lesions. These findings indicate an inflammatory response in a significant portion of the patients with the invasive *S. aureus* infections.

In the following (Table III), we identify the main diseases caused by our strains in patients.

The most common **infection type** was Skin infections identified through pus isolates affecting 56 patients (39.72%). Tracheal aspirate isolates revealed that respiratory tract infection (bronchopneumonia) was the second most prevalent, diagnosed at 32 patients (22.7% of the cases). Urinary tract infections were identified in 17 patients (12.06%). Endocarditis was less common, affecting 9 patients (6.38%), while CNS infections (Meningitis/ Encephalitis) were the least frequent, affecting only 3 patients (2.13%). Blood isolates indicated that bloodstream infections (BSI) accounted for 17.02% of the cases.

BSI present in 24 patients (17.02%), indicate how many patients became critically ill. This condition is known for its potential to cause severe complications, but the specific outcomes for these patients were not tracked in this study.

These data indicate that skin lesions, respiratory tract infection (bronchopneumonia) and BSI are the leading infection types among *S. aureus* patients, as determined by clinical signs, symptoms, and laboratory findings.

For each disease, we also identified the main comorbidities. Each patient may have more than one comorbidity.

The table III shows the frequency of each comorbidity associated with each staphylococcal disease, indicating how often these comorbidities appear in conjunction with the diseases.

The analysis of **past medical histories** among *S. aureus* patients with various infections revealed significant patterns.

Among those with skin infections (39.72%), prevalent comorbidities included cardiovascular system issues (5.67%) psychiatric medical history (4.26%), and neurological conditions (4.96%)

For patients with respiratory tract infection (22.7%), the most common comorbidities were cardiovascular issues (7.09%) and neurological conditions (5.67%)

Patients with urinary tract infections (UTIs) frequently had cardiovascular issues (7.09%) and previous hospitalizations (2.84%).

Patients with heart infections exhibited notable comorbidities, including hematologic conditions (2.13%), pulmonary issues (2.84%), gastrointestinal problems (0.71%), and diabetes mellitus (0.71%). These findings underscore the diverse and complex medical backgrounds of *S. aureus* patients, with cardiovascular and neurological problems, being particularly common across multiple infection types.

Table III. Infection types and associated past medical histories in *S. aureus* patients.

Infection type (Diseases)	%	Patients with past medical history/%
Skin-mucosa lesions	56/39.72%	Cardiovascular system diseases (8/5.67%) Psychiatric diseases (6/4.26%) Neurological conditions diseases (7/4.96%) Diabetes (6/4.26%) Any cancer (6/4.26%) Other past medical history (13/9.22%) Without past medical history (10/7.09%)
Pneumonia	32/22.7%	Cardiovascular issues (10/7.09%) Neurological conditions (8/5.67%) Urinary and reproductive diseases (5/3.55%) Other past medical history (6/4.26%) Without past medical history (3/2.13%)
Urinary tract infection	17/12.06%	Cardiovascular diseases (10/7.09%) Previous hospitalizations (4/2.84%) Other past medical history (3/2.13%)
Heart infections (Endocarditis)	9/6.38%	Hematologic conditions (3/2.13%) Pulmonary issues (4/2.84%) Gastrointestinal problems (1/0.71%), and diabetes mellitus (1/0.71%)
CNS infections	3/2.13%	
Blood stream infections *(Multiple locations, more than one system identification)*	24/17.02%	
Total	141/100%	

Molecular analysis

MecA identification in S. aureus strains (MRSA and MSSA)

Throughout the PCR method, from all *S. aureus* strains 81 strains (57.45%) have the *mecA* gene, being identified as *MRSA* strains and 60 *S. aureus* strains are *mecA* neg, being identified as *MSSA* strains (42.55%). (Table IV).

The *MRSA* strains have been confirmed throughout the phenotypic tests: PBP2a latex test: 78 of them (55.32%) express the protein PBP2a and only three strains (2.13%) has negative result (96.3% concordant results with the *mecA* detection throughout the PCR method).

Table IV. Distribution of *MecA* Gene in *S. aureus* Strains.

S. Aureus	*MecA*	STRAINS
MRSA	Positive	81 (57.45%)
MSSA	Negative	60 (42.55%)
TOTAL		141 (100%)

PLV* (*Panton Valentine leukocidin*) *identification in S. aureus strains

Among the *S. aureus* strains, 19.14% (27 out of 141) were *Pvl*-positive.

The *MRSA* strains were further classified as either Community-Associated *MRSA* (*CA-MRSA*) or Hospital-Associated *MRSA* (*HA-MRSA*) based on clinical, epidemiological criteria and on the presence of the *Pvl* gene.

- *CA-MRSA*: of 81 *MRSA* strains, 18 strains (22.2%) were classified as *CA-MRSA*, based on their *Pvl* gene positivity and association with community-acquired infections.

- *HA-MRSA*: The remaining 63 strains (77.8%) classified as *HA-MRSA*, were isolated from patients with recent hospitalizations or medical procedures and exhibiting resistance to multiple antibiotic classes.

From all *S. aureus* isolated strains (141), the rate of the *Pvl*-positive strains was 19.14% (27/141) and the rate of the *Pvl* (-) strains was 80.85% (114/141) (Table V).

From 114 *PVL*negative *S. aureus,* 63 strains (44.68%) had been *MecA*+ (*MRSA* strains) and 51 strains (36.17%) were *MecA* neg (*MSSA* strains), confirmed through both PCR and (protein PBP2a negative; low MIC values for oxacillin, susceptible to cefoxitin.

Table V. The rate of the *Pvl* gene strains.

***PVL* Gene**	***S. aureus***	**Total**
Pvl positive	*MRSA*: 18 (12.76%) *MSSA*: 9 (6.39%)	27 (19.14%)
Pvl negative	*MRSA*: 63 (44.68%) *MSSA*: 51 (36.17%)	114 (80.85%)
Total		141 (100%)

From 81 (57.44%), ***MRSA* strains** (*mecA* positive), 18 (12.76%) were *Pvl* (+) and 63 (44.68%) *Pvl* (-).

The antibiotic resistance of *MSSA* strains (*mecA* negative strains):

Among 60 (42.55%) ***MSSA*** (*mecA* negative) strains, 9 strains (6.38%) were *Pvl* (+) (CA-*MSSA*), while the remaining 51 strains (36.17%) were *Pvl*-negative (Table VI). All 9 (6.39%) ***MSSA* strains *Pvl* positive**, were sensitive to all AB.

Table VI. The *MRSA* and the *MSSA* strains & the *Pvl* presence (positive) or absence (negative).

***MRSA / MSSA* strains**	***Pvl* presence**
MRSA (81 strains/57.44%)	*-PVL* (+) 18 (12.76%)
	-PVL (-) 63 (46.68%)
MSSA (60 strains/42.55%)	*PVL* (-) 9 (6.38%)
	PVL (-) 51 (36.17%)
Total	141 (100%)

From all 141 strains, **33 *MSSA* strains** (23.4%) were sensitive strains to all tested antibiotics including glycopeptides.

Other 27 ***MSSA*** strains (19.14%) exhibited concomitant resistance to various antibiotic classes (Table VII).

We observe among the *MSSA* strains the existence of 4 different clones, according to their resistance to antibiotics.

In conclusion, we draw attention to the circulation in our geographic area of ***MSSA*** sensitive strains to antibiotics (23.4%) and of ***MSSA*** resistant strains (19.14%) to some antibiotics (Aminoglycosides, Macrolides, Ciprofloxacin) with different resistance phenotypes.-

Table VII. Antibiotic resistance profiles of *MSSA* strains.

1	Macrolides: Erythromycin 6 strains & Erythromicin & clindamycin: 6 strains	12 strains (8.51%)
2	Macrolides (Erythromycin) & Clindamycin	6 strains (4.26%)
3	Aminoglycosides (Kanamycin), Macrolides (Erythromycin ± clindamycin):	6 strains (4.26%)
4	Aminoglycosides (Kanamycin), Macrolides (Erythromycin), Ciprofloxacin	3 strains (2.13%)
	Total	27 strains (19.14%)

Table VIII. Antibiotic resistance profiles of *MRSA* strains: number of resistant strains and corresponding percentages.

	Pvl+	**100%**	***Pvl-***	**100%**
Erythromycin	11/18	61.1%	19/63	30.15%
Clindamycin	11/18	61.1%	27/63	42.3%
Tetracycline	17/18	94.4%	45/63	71.42%
Ciprofloxacin	4/18	22.2%	54/63	85.71%
Kanamycin	8/18	44.4%	44/63	69.84%
Gentamicin	3/18	16.66%	31/63	49.20%
Fusidic acid	2/18	11.1%	3/63	4.76%
Tigecycline	0/18 (12.76%) were	0%	0	0%
Chloramphenicol	1/18	5.5%	-	-
Trimethoprim/sulfamethoxazole	1/18	5.5%	-	-
	18 strains (12.76%)	**100%**	63(44.68%)	

Resistance profiles and the presence of *Pvl* genes in *MRSA* strains

Testing the presence of *mecA* gene and PVL gene, revealed that: *81* S. *aureus* strains were ***MRSA (***57.44%): 63 (44.68%) strains *Pvl* negative (-) and 18 (12.76%) were *Pvl* positive (+).

MRSA (81 strains) 57.44%)	-*Pvl* (+) 18 (12.76%)
	-*Pvl* (-) 63 (44.68%)

Among ***MRSA Pvl (+)*** tested strains, it was observed that 61.1% were resistant to both erythromycin and clindamycin. Testing the strains for tetracycline resistance revealed high levels (94.4%). The study of resistance to aminoglycosides showed that 44.4% of strains were resistant to kanamycin and 16.66% to gentamicin. Moderately increased percentages of antibiotic resistance were found for ciprofloxacin (22.2%). Low levels of antibiotic resistance were observed for Fusidic acid (11.1%), trimethoprim/sulfamethoxazole (5.5%), chloramphenicol (5.5%) and no resistance (0%) to tigecycline (Table VIII).

***MRSA Pvl (-)* strains**: In our study group, 30.15% of strains were resistant to erythromycin and 42.3% were resistant to clindamycin. Additionally, 71.42% strains were resistant to tetracycline, 85.71% to ciprofloxacin. The study of resistance to Aminoglycosides showed that 69.84% of strains were resistant to Kanamycin and 49.20 to Gentamicin. Only 4.76% strains presented resistance to fusidic acid (Table VIII).

All *MRSA* strains were initially found to be sensitive to both Vancomycin and Teicoplanin using standard methods. However, confirmatory testing with the Etest method revealed that 3 strains (3.7%) were intermediate sensitive to Glycopeptides, (*GISA),* to Vancomycin (*VISA*), or resistant to Glycopeptides (*GRSA*) (Vancomycin, Teicoplanin).

It is important to note that no subtyping methods, such as multilocus sequence typing (MLST) or spa typing, were performed in the present study. As a result, we are unable to determine the specific clonal types or subtypes of the strains identified.

Antibiotic resistance profiles among different *S. aureus* subgroups

The antibiotic resistance patterns observed in *MRSA*, *Pvl* positive, and *Pvl* negative strains are summarized in table VIII. This comparison highlights significant differences in resistance across the subgroups. This table provides a comprehensive overview of the resistance patterns, underscoring the importance of subgroup-specific analysis in *S. aureus* infections.

Although the strains carry the *Pvl* gene, is not a correlation between these genes and a specific resistance phenotype, suggesting their independent existence. The research should investigate the biological effects of the *PVL* toxin on human organisms and involve a larger number of strains to confirm the lack of association between the gene presence and antibiotic resistance.

In our study we found high levels of resistance to commonly used antibiotics among *MRSA* strains. These findings are consistent with previously published reports from similar regions and healthcare settings, where resistance to macrolides, aminoglycosides, and quinolones has also been reported at significant levels.

Discussion

The present study aimed to analyze the demographic distribution, antibiotic resistance profiles, and molecular characteristics of *S. aureus* infections in a cohort of 141 patients. Our findings reveal significant insights into the prevalence of *MRSA* and *MSSA* strains, their antibiotic resistance, and the molecular factors influencing their pathogenicity. Comparing these results with other studies provides a broader perspective on the epidemiology and

control of *MRSA* in Europe [18,19].

Demographic distribution and infection types

In our study, 60.4% of the patients diagnosed with *S. aureus* infections were male, while 39.6% were female. The distribution of infections by age group showed that older adults had the highest prevalence (55% of the total cases). Adults aged 25-64 years followed (34%). This distribution suggests that *S. aureus* infections affects particularly older individuals.

The most common **types of infections** identified in this study were skin lesions, which represented 39.72% of all cases. Respiratory tract infection was the second most frequent infection type, accounting for 22.7% of the cases.

These findings are consistent with previous studies, including the EU-funded MOSAR project, which also observed a significant number of *MRSA* infections in older populations, particularly in surgical wards across Europe [18,19,22]. The high prevalence of these infection types underscores the need for targeted prevention strategies in vulnerable populations, particularly older adults and those with comorbidities.

Antibiotic resistance profiles

Our analysis of antibiotic resistance patterns revealed multiple resistance phenotypes among *S. aureus* strains. Notably, 23.4% of *MSSA* strains were sensitive to all the antibiotics tested, indicating that these strains remain susceptible to standard treatments.

However, 19.14% of the ***MSSA*** strains exhibited resistance to several commonly used antibiotic classes, including macrolides, aminoglycosides, and ciprofloxacin. These findings highlight the ongoing challenge of treating *S. aureus* infections, particularly when resistance limits the effectiveness of first-line antibiotics.

To accurately assess Glycopeptide resistance, we employed the E-test method, a reliable technique recommended by guidelines such as those from the CLSI. This method confirmed that while the majority of *Staphylococcus aureus* strains in our study remained sensitive to Glycopeptides, a small percentage of *MRSA* (3.7%) are IS or R to Vancomycin and/or Teicoplanin. While we employed the E-test method to confirm Glycopeptide resistance, the absence of additional confirmatory methods, such as broth microdilution, is a limitation of this study. Future research should incorporate these methods to provide a more thorough validation of Glycopeptide resistance.

Interestingly, the rates of glycopeptide resistance observed in our study are slightly higher than those reported in other parts of Greece. For example, a 2018 study from Crete found 0% resistance to both Vancomycin and Teicoplanin among *MRSA* strains. National surveillance reports from Greece also confirm that *VRSA* cases remain rare. This discrepancy suggests that local factors, such as differences in healthcare settings, antibiotic usage patterns, or patient demographics, may be contributing to the increased glycopeptide resistance observed in our cohort. Further studies are needed to explore the regional variations in glycopeptide resistance across Greece and determine whether the observed increase reflects a broader trend or remains confined to specific clinical environments [25,26]. Our findings suggest that although Glycopeptide resistance is less common in our cohort, it remains a significant concern, especially in healthcare settings where these antibiotics are often reserved for severe infections. Moreover, our results are in line with broader European data, which report high resistance to Erythromycin, Clindamycin, and Ciprofloxacin among *MRSA* isolates [27,28].

In our study the prevalence of *MRSA* strains was 57.44% (81 out of 141 isolated strains).

In Greece, the *MRSA* prevalence remains around 41.9%, comparable to rates observed in southern Europe. These high resistance rates in southern Europe highlight regional differences when compared to northern European countries, where *MRSA* resistance tends to be lower, as reported by the ECDC [23,24,27-30].

Our study found that *MRSA* strains exhibited resistance to erythromycin and clindamycin, which aligns with findings from Greece and Romania, where resistance rates are also significantly high.

However, some regional differences were noted, underscoring the importance of localized surveillance to guide effective treatment strategies.

Molecular characteristics

Our molecular analysis provided further insights into the genetic makeup of the *S. aureus* strains in our study. Specifically, we confirmed the presence of the *mecA* gene in 57.45% of the strains, identifying them as *MRSA*. The presence of the *mecA* gene is a key indicator of methicillin resistance, which complicates treatment options and necessitates the use of alternative antibiotics, such as Glycopeptides. In addition to methicillin resistance, we found that 19.14% (27 out of 141) of the strains were *Pvl* positive. The *Pvl* genes, which are associated with increased virulence, were found in *MRSA* and *MSSA* strains [20,25,29].

The *pvl* gene has been extensively studied in Europe, with findings consistently showing that while it enhances virulence, it does not contribute to antibiotic resistance. This is consistent with findings from other European studies, where *Pvl* genes are predominantly associated with community-acquired *MRSA* clones. The presence of *Pvl*-positive strains is particularly concerning as it suggests a potential for more severe infections, especially in otherwise healthy individuals. The distribution of genetic markers observed in our study suggests potential similarities with clones reported in the EU-funded MOSAR project, such as *EMRSA*-15 (ST22-IVh) and Southern German (ST228-I). These clones have been widely studied across Europe and are known for their ability to spread in hospital settings [20,22]. However, it is important to note that our study

did not include sequencing or detailed subtyping, which would be necessary to confirm the exact clonal identity of the strains. A limitation of this study is the use of PCR for detecting the *mecA* and *pvl* genes, which does not provide the resolution needed to confirm the clonality of the strains. Future studies employing whole-genome sequencing or MLST would provide more comprehensive insights into the genetic relatedness of the strains and their similarity to other clones previously reported. Another limitation of this study is the absence of subtyping techniques like MLST or spa typing, which are necessary for determining the specific clonal relationships between strains. This would allow for a deeper understanding of the spread and epidemiology of antibiotic-resistant strains within clinical settings. Future research should prioritize these methods to identify the clonal distribution of *MRSA* and *MSSA* strains. Therefore, while our findings provide valuable insights into the genetic diversity of *MRSA* and *MSSA* strains in our region, further genetic analysis is required to establish definitive clonal relationships and better understand the spread of these strains [22,24-26,29-31].

In our study, the strains were categorized into two main groups: *Pvl* (+) and *Pvl* (-) strains. *Pvl* (+) strains which accounted for 6.38%% of *MSSA* cases and 12.76%, of *MRSA*, carry the *Pvl* gene, a marker associated with increased virulence and often linked to infections acquired outside of hospital settings (CA-*MRSA*). On the other hand *Pvl* (-) strains, representing 44.68% of *MRSA* cases, were primarily found in hospital environments and exhibited higher resistance to multiple antibiotic classes (HA-*MRSA)*.

This is consistent with findings from other studies conducted in Greece, where HA-*MRSA* constitutes a significant portion of *MRSA* infections. Recent data from Greek hospitals report that HA-*MRSA* rates can range from 20% to 50% of hospital-acquired infections. Additionally, ECDC reports highlight Greece as one of the European countries with a high prevalence of HA-*MRSA* due to factors such as widespread antibiotic use and challenges in infection control practices. These findings emphasize the ongoing need for improved infection control and antibiotic stewardship measures in healthcare settings to mitigate the burden of *MRSA* in hospitals. These findings are consistent with other European studies, highlighting the ongoing challenge of managing *MRSA* in both community and hospital settings [23,25-27,30].

Implications for infection control

The findings from our study underscore the need for robust infection control measures and continuous monitoring to effectively manage the spread of *MRSA* and other antibiotic-resistant strains. The high prevalence of *MRSA*, particularly among older patients and those with chronic conditions such as cardiovascular and neurological disorders, suggests that targeted interventions in these groups could be particularly beneficial. Similar to trends in Romania, where *MRSA* prevalence was reported to be between 33-45% from 2017 to 2022, our study found consistent resistance rates, indicating the persistent challenge *MRSA* poses in southern Europe.This high level of resistance in Romania, which has topped the European list in previous years, is also seen in Greece, with significant resistance levels reported. Focusing on these vulnerable populations may help reduce the overall burden of *MRSA* in healthcare settings. The Glycopeptide resistance detected in our study highlights the importance of judicious antibiotic use, especially in severe infections where these antibiotics are often the last line of therapy. Ensuring that these antibiotics remain effective is crucial, which requires careful management of their use and ongoing surveillance to detect emerging resistance patterns. Comparing our findings with those from other European studies reveals a broader trend of increasing antibiotic resistance and the spread of *MRSA* in hospitals. The project, for example, found that hospitals with lower *MRSA* rates often conducted more intensive surveillance and root cause analyses to understand the factors contributing to *MRSA* transmission. This suggests that enhancing infection prevention and control (IPC) practices could be key to reducing *MRSA* incidence in healthcare settings. Our findings align with these observations, emphasizing the need for collaborative efforts across Europe to monitor and control the spread of resistant strains [24,27-29].

Conclusions

Our results indicate that *MSSA Pvl*-negative strains retain their antibiotic sensitivity, distinguishing them from *MRSA* strains, where resistance to beta-lactam antibiotics is due to the presence of the *mecA* gene. While the presence of the *Pvl* gene in both *MSSA* and *MRSA* strains is linked to increased virulence, it does not correlate with antibiotic resistance to beta-lactams or other antibiotic classes. This finding emphasizes the distinct roles of these genes, with *mecA* conferring resistance and *Pvl* contributing to virulence. This distinction underscores the importance of using PCR methods to trace these strains and explore their role in clinical diseases, which will be further explored in future studies [19,20].

Our data showed resistance to Glycopeptides, including vancomycin and teicoplanin, in a small proportion of strains (3.7%). While this resistance level is lower than in some other studies [23], our findings highlight the importance of continued surveillance, particularly in healthcare settings where these antibiotics are frequently used for severe infections. We did not assess changes in Glycopeptide resistance over time. Our study aligns with national data and highlighting the ongoing challenge of managing antibiotic resistance in clinical settings. Similar to findings in Greece, our strains exhibited high resistance levels to several antibiotics, including Erythromycin, Clindamycin, Tetracycline, and Ciprofloxacin [22].

These resistance patterns underscore the urgent need for continuous surveillance and more targeted antibiotic stewardship.

Our study revealed no resistance to tigecycline rates among *MRSA* strains, a surprising finding given that global data also show much lower rates. In a recent study, the global tigecycline resistance rate was reported to be as low as 0.004%. This highlights the need for further regional investigation.

Future research should aim to investigate the biological effects of toxins encoded by these genes, both in animal models and human systems, to better understand their role in disease. Expanding the scope of these studies to include a larger number of strains will be crucial for confirming the hypothesis that the presence of these genes does not directly associate with antibiotic resistance. Additionally, it is essential to explore the underlying mechanisms of resistance and virulence further to develop more effective treatment strategies [24-26,29-31].

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Institutional review board statement

Ethical approval for this study was obtained from the bioethics committees of the University Hospital of Heraklion (nr. 35/30-12-2020), the General Hospital of Chania (nr. 2819/05-02-2020), and Iuliu Hatieganu UMPh Cluj-Napoca (nr. 4481/01.10.2019).

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