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Antimicrobial Resistance and Detection of Biofilm in *Staphylococcus aureus* Isolates from Casablanca

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MA, MR, TC and KK designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KZ and RAM designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors FM and NR designed the study and wrote the protocol. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The ability of biofilm formation seems to play an essential role in the virulence of *Staphylococcus aureus*. The aims of the present study were to test the sensitivity of the clinical isolates of *Staphylococcus aureus* to antibiotics, detect the ability of these strains to form biofilm and evaluate the correlation between biofilm formation by clinical isolates and the resistance to antibiotics.
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and Biodiversity, Faculty of Sciences and Techniques Mohammedia, University Hassan II Casablanca and laboratory of Bacteriology, Virology and Hygiene, Ibn Rochd University Hospital, Casablanca during October 2015 and January 2016.

Methodology: A total of 117 clinical isolates of staphylococci were collected at the University Hospital Ibn Rochd of Casablanca, Morocco and examined for antimicrobial susceptibility, presence of *mecA* gene and biofilm formation. *Staphylococci* species identification and antibiogram were performed by standard procedures using disk diffusion method. The methicillin resistance was confirmed by PCR using *mecA* specific primers. The biofilm formation assay was realised by the tissue culture plate method (TCP).

Results: Among all strains collected, 74 were identified as *Staphylococcus aureus.* Out of 74 *Staphylococcus aureus,* 22 strains (29.7 %) were found methicillin resistant when tested with cefoxitin disc diffusion method. 20.3 %, 18.9 % and 13.5 % were classified as resistant to kanamicin, ciprofloxacin and erythromycin respectively. All strains were found resistant to penicillin G and sensitive to teicoplanin. All isolates resistant to methicillin by cefoxitin disc diffusion method were confirmed by presence the *mecA* gene by PCR. Of the 74 isolates 16 (21.6 %) were non adherent, 40 (54 %) weakly adherent, 12 (16.2 %) moderately adherent and 6 (8.1 %) strongly adherent.

Conclusion: The results of this study showed that there is a correlation between biofilm formation and resistance to all the antibiotics tested, except to teicoplanin, which was active against the all strains.

Keywords: Staphylococcus aureus; antimicrobial resistance; methicillin-resistant Staphylococcus aureus; biofilm; tissue culture plate method.

1. INTRODUCTION

Staphylococcus aureus is a Gram-positive pathogen that lives as part of the normal microflora on the skin and mucous membranes of humans and animals. If it passes through the epithelial barrier and reaches internal organs, it can cause several diseases, ranging from minor infections such as skin, respiratory, joint and endovascular infections, to severe infections, such as bacteremia, pneumonia, endocarditis, sepsis, and toxic shock syndrome. Approximately 30% of humans are *S. aureus* carriers without symptoms [1]. *Staphylococcus aureus* is also responsible for foodborne intoxications worldwide, caused by the ingestion of food containing staphylococcal heat-stable enterotoxins. The greatest risk of staphylococcal food poisoning is associated with food products contaminated with *S. aureus* after the normal microflora has been destroyed or inhibited [2].

Basic antibiotic for the treatment of *Staphylococcus aureus* infections is generally a derivative of penicillin like oxacillin, cefoxitin or methicillin [3]. During the past two decades, this pathogen has developed resistance to commonly prescribed antimicrobial agents. Today methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of bacterial infection in hospitals [4]. First reported in a British hospital, MRSA clones rapidly spread across international borders. Waves of clonal dissemination with different dominant phage types were reported in the 1960s and were responsible for a large proportion of cases [5,6]. Genetically, MRSA differ from susceptible strains by the presence in their chromosome of a long sequence of DNA (40-60 Kb) named *mec,* and the presence of the *mecA* gene that codes for the formation of penicillin binding protein 2A (PBP 2A). The *mecA* gene (the gene responsible for methicillin resistance) is part of a mobile genetic element found in all MRSA strains. It is part of a genomic island designated as staphylococcal cassette chromosome *mec* (SCC *mec*) [7]. Recently a new *mecA* homolog (*mecC* or $mecA_{LGA251}$, in reference to LGA251 isolates from which it was characterized) was described in a novel staphylococcal cassette chromosome *mec* named type XI. This new *mecA* homolog has been detected in bacteria from dairy cattle in England and humans in England, Scotland, and Denmark [8,9].

Staphylococcus aureus displays a strong capacity to irreversibly attach to the surface of implanted medical devices and forms multilayered communities of bacteria, known as biofilms [10].

Biofilms, surface associated sessile bacterial communities, are formed when planktonic cells colonize a surface, aggregate and grow into multicellular colonies, and embed themselves in an exopolysaccharide [11]. In addition to a large

number of cell surface associated proteins, secreted proteins, Polysaccharide Intercellular Adhesin (PIA) and intracellular adhesin A, D, B and C (*ica*A, *ica*D, *ica*B and ica*C*) which are synthesized by products of the intercellular adhesin A, D, B and C (*ica*A, *ica*D, *ica*B and ica*C*) operon are also required for biofilm formation in *Staphylococci* [10,12-14].

Biofilm formation is a major concern in nosocomial infections because it protects microorganisms from host immune response (opsonophagocytosis) and antibiotics, leading to chronic infection and sepsis [15]. The aims of the present study were to test the sensitivity of the clinical isolates of *Staphylococcus aureus* to antibiotics, detect the ability of these strains to form biofilm and evaluate the correlation between biofilm formation by clinical isolates and the resistance to antibiotics.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of *S. aureus*

A total of 117 strains of *Staphylococcus* spp. were isolated between October 2015 and January 2016 from different sites of infection (blood, pus and other) from patients hospitalized in various services of the University Hospital Ibn Rochd in Casablanca.

The isolates were identified by classic microbiological methods including colony morphology, Gram staining, catalase test, coagulase test, mannitol fermentation and API Staph gallery (bioMérieux, Marcy l'Etoile, France) [16].

2.2 Antibiotic Susceptibility Test

The Antibiotic susceptibility test was done according to Kirby bauer's disc diffusion method on Mueller Hinton (MH) agar using the following antibiotic discs: penicillin G (10 units), gentamicin (10 µg), tobramicin (10 µg), kanamicin (30 µg), ciprofloxacin (5 µg), cefoxitin (30 µg), erythromycin (15 µg), teicoplanin (30µg) and trimethoprim-sulfamethoxazole (25 µg) [17]. The zones of inhibition were interpreted following Clinical and Laboratory Standards Institute (CLSI) guidelines [18].

The methicillin resistance was checked using cefoxitin disc (30 µg) on MH agar following CLSI guidelines and the strains were identified as methicillin resistant *S. aureus* (MRSA) or methicillin sensitive *Staphylococcus aureus* (MSSA).

2.3 MecA Gene Detection and Confirmation of MRSA

This test concerns only the strains which were identified as MRSA using cefoxitin disc (30 µg) on Muller Hinton.

The DNAs of isolates were extracted using the commercial DNA extraction Kit (SIGMA-ALDRICH, USA). PCR assay was performed to detect *mecA* gene, encoding methicillin-resistance gene. For amplification of the *mecA* gene, primers *mecA*-F (5'-GATATCGAGGCCCGTGGATT-3') and *mecA*-R (5'-ACGTCGAACTTGAGCTGTTA-3') were used to produce a 642 bp fragment. The PCR reaction volume was in 25 µl, containing the above-mentioned primers (10 µM each), 100 ng of the extracted DNA, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.5 U of *Taq* DNA polymerase, and buffer 5 mM. The PCR amplification protocol for *mecA* was as follow: an initial 1 min denaturation at 94℃, followed by 35 cycles of 1 min denaturation at 94℃, 1 min annealing at 52℃ and 1 min extension at 72℃, with a final extension at 72℃ for 10 min. PCR products were analyzed by a 1.5% agarose gel electrophoresis with Tris-borate/EDTA in the presence of ethidium bromide 0.5 µg/ml.

2.4 Tissue Culture Plate Method (TCP)

The tissue culture plate method was conducted as previously described [13]. Briefly, the bacterial suspension grown in trypticase soy broth (TSB), supplemented with 1% glucose was diluted for 1:100. 200 µl of this dilution was poured into the wells of sterile flat-bottomed 96-well polystyrene tissue culture plates (200 µl of TSB supplemented with 1% glucose was used as the negative control) and incubated 24 hours at 37℃. All tests were performed in triplicate. Washing was then performed three times for each well with sterile phosphate-buffered saline (PBS; pH 7.2). After that, the fixation step was done by air drying. Subsequently, the adherent biofilm layer was stained by crystal violet for 15 minutes at room temperature. This was followed by the washing steps. Then the plates were air dried and resolubilized with ethanol (95%) for 30 minutes. Optical density (OD) of stained adherent bacteria was determined with an Absorbance Microplate Reader (model EL×800) at wave length of 630 nm. The experiment was

repeated three times separately for each strain and the average values were calculated with standard deviation (SD). Optical density cut-off (ODc) was determined. Formation of biofilm by isolates was analyzed and categorized relying on the absorbance of the crystal violet-stained attached cells (Table1).

Table 1. Interpretation of results

Mean OD values	Biofilm formation
< ODc	Non
ODc < OD < 2 ODc	Weak
2 ODc < OD < 4 ODc	Moderate
> 4 ODc	Strong

ODc = average OD negative control + 3 standard deviation of negative control

2.5 Statistical Analysis

Comparison of rates of resistance to the different antibiotics was performed between strains biofilm producers and strains non biofilm producers. Chi-square tests were done wherever possible. When frequencies of five or less were present, Fisher's exact test was used. One-sided testing was performed. Differences were significant when $P<0.05$. Statistical analyses were performed using SPSS version 17.00.

3. RESULTS

3.1 Isolation and Identification of *S. aureus*

In a total of 117 clinical isolates of *Staphylococcus* spp. isolated from skin surface, blood, catheters, infected devices, bronchial aspiration, burn surface, Protected bronchial sampling, pus etc (Fig. 1), from different services (Medical and Surgical Intensive Care, the National Center of burns, Dermatology, Nephrology, Pediatric, Cardiovascular surgery) (Fig. 2) at the University Hospital Ibn Rochd of Casablanca, 74 strains were identified as *Staphylococcus aureus*.

3.2 Antibiotic Susceptibility Test

Twenty two strains (29.7 %) of *Staphylococcus aureus* were methicillin resistant when tested with cefoxitin disc diffusion method.

The antimicrobial susceptibility patterns of the 74 strains of *Staphylococcus aureus* are presented in Table 2.

3.3 MecA Gene Detection and Confirmation of MRSA

All isolates that were resistant to cefoxitin by disc diffusion method were analyzed by PCR to detect the mec*A* gene to confirming methicillin resistance (Fig. 3).

3.4 Detection of Biofilm Formation

The phenotypic characterization of biofilm formation by all strains of study was realized by the tissue culture plate method (TCP).

Among 74 isolates of *Staphylococcus aureus* studied, 58 (78.4%) were positive for biofilm prodction. 40 (54%) were classified as weakly adherent, 12 (16.2%) as moderately adherent and 6 (8.1%) as strongly adherent. 16 (27.6%) of the 58 positive isolates were obtained from blood culture, 10 (17.2%) from skin surface, 09 (15.5%) from pus, 08 (13.8%) from burn surface, 06 (10.3%) from catheters and 03 (5.2%) from bronchial aspiration and 06 (10.3%) from others sources.

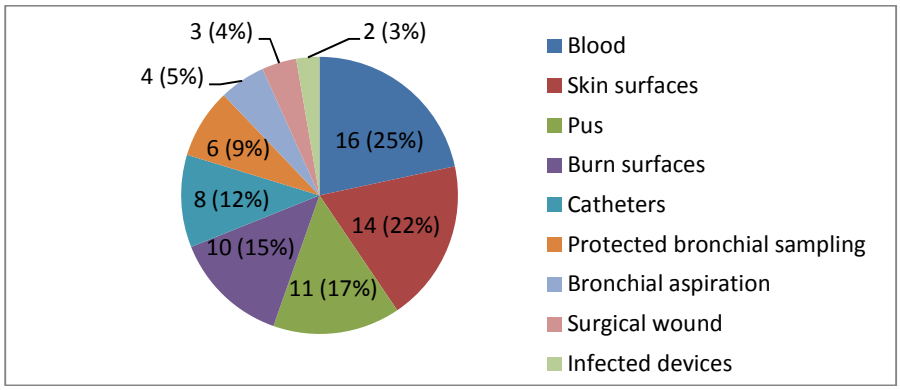

Fig. 1. Distribution of clinical isolates of *Staphylococcus aureus* according to their origins

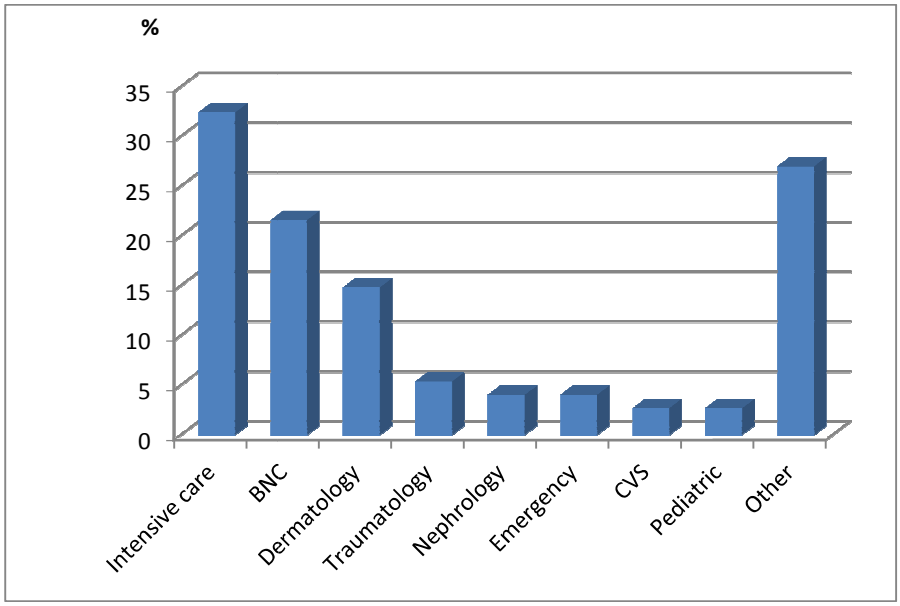

Fig. 2. Distribution of clinical isolates of *Staphylococcus aureus* according to different services of the university hospital Ibn Rochd of Casablanca

BNC: The national center of burns, CVS: Cardiovascular surgery

Table 2. Resistance rate associated to biofilm producers and non biofilm producers

Antibiotics	Resistance rate n (%)		Number of resistant strains	*P*- value
	Biofilm producer n = 58	Non biofilm producer n = 16		
Cefoxitin	21 (95.4)	01 (4.6)	22	< 0.05
Kanamicin	14 (100)	00	14	< 0.05
Tobramicin	13 (100)	00	13	< 0.05
Ciprofloxacin	12 (100)	00	12	< 0.05
Gentamicin	10 (100)	00	10	< 0.05
Erythromycin	09 (90)	01 (10)	10	< 0.05
Trimethoprim-sulfamethoxazole	08 (100)	00	08	< 0.05
Penicillin G	58 (78.4)	16 (21.6)	74	< 0.05
Teicoplanin	00	00	00	-

- : Indeterminate value

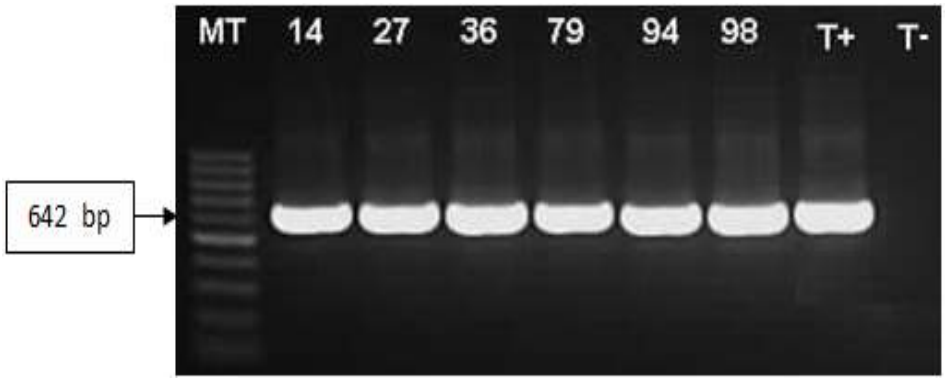

Fig. 3. Result of amplification of the *mecA* gene in 6 strains of *Staphylococcus aureus*

MT: Size marker, T+: Positive control, T-: Negative control, (14, 27, 36, 79, 94 and 98): Strains resistants to cefoxitin (30 µg)

A statistical comparison of rates of antibiotic resistance between the biofilm producers and non biofilm producers showed a significant difference at $p < 0.05$ for all the antibiotics tested, except for teicoplanin, which was 100% active against the two groups of *Staphylococcus aureus*.

4. DISCUSSION

Staphylococcus aureus is one of the bacteria most studied by researchers because of the increase in infections that it causes. In a total of 117 clinical isolates of *Staphylococcus* spp. collected 74 (63.2%) strains were identified as *Staphylococcus aureus*. Similarly, in another study carried out in Tehran between December 2012 and March 2013, *S. aureus* was predominant and was isolated from 65 of 135 (48.14%) samples taken from pus/wounds swabs from skin and soft tissue infections [19].

The majority of tested strains were isolated from the intensive care units 24 (32.4%), the National Center of burns 16 (21.6%) and dermatology unit 11 (14.9%). This result correlates with that of Benouda et al. [20] who found that 41.7% of *Staphylococcus aureus* was isolated in the intensive care units in Morocco in 2009; while the study directed by Gary et al. [21] in the USA in 2005 found that 24% of infections with *Staphylococcus aureus* occur in the national center of burns.

4.1 Susceptibility Pattern of *S. aureus*

Staphylococcus aureus has no natural resistance to antibiotics. The massive use of penicillin G and V has led in 1941 to the emergence of bacteria resistant to these molecules. Currently more than 90% of the strains isolated are resistant to penicillin G [22]. Our study of antibiotic susceptibility showed a high prevalence of β-lactamase production, this mechanism is the most commonly cause of resistance to penicillins (100%); this result is similar to that obtained in another study by Aydin et al. [23], this study included 274 *S. aureus* and 172 coagulase negative staphylococci strains.

Resistance rates against gentamycin, erythromycin and trimethoprim-sulfamethoxazole were determined as 13.5%, 13.5% and 10.8%, respectively. In another study that was carried out in Turkey by Rağbetli et al. [24] a total of 1116 *S. aureus* isolates from various clinics were collected; they found almost the same resistance rates of these antibiotics; they determined resistance to gentamycin at the rate of 13.0%, at 17.7% to erythromycin and at 6.1% to trimethoprim-sulfamethoxazole.

4.2 MRSA

The global emergence of organisms with multiple drug resistances (MDRs), such as methicillin-resistant *Staphylococcus aureus* (MRSA), is an important factor in acute and chronic infections that leads to increase mortality rates and increase healthcare costs. The prevalence rate of MRSA in this study is 29.7% (22 strains). In a Tunisian study conducted by Mastouri et al. [25] between June 2002 and December 2003, 620 strains were isolated from the different pathological samples and 96 (15.5%) were identified MRSA. In another study done in Egypt by Barakat et al. [26] in 161 S. aureus isolated from 513 pus/ wound swabs collected from patients with evidence of surgical site infection over the period July 2013–January 2015, 73 (45.3%) were found to be MRSA. These 22 MRSA isolates were isolated from various services: 9 from patients in the national center of burns, 4 in the intensive care units, 4 in the dermatology unit, 3 in the pediatric service and 2 in the cardiovascular surgery unit. In the study of Benouda et *al*. [21] 15 of 25 MRSA were found in the intensive care units. According to origin, 5 were isolated from skin samples, 5 from burn surfaces, 4 from blood, 4 from catheters, 2 from surgical wounds and 2 from infected devices. While, Skiest et al. [27] have isolated the majority of MRSA (69%) from skin soft tissue.

Vancomycin is commonly used to treat infections caused by MRSA. Nowadays the options for treatment of MRSA infections are considerably limited and vancomycin remained as the last choice for MRSA treatment until recent years [28]. The dramatic increase in use of vancomycin to treat infections caused by methicillin-resistant staphylococci (both coagulase-positive and -negative) preceded the emergence of vancomycin-resistant staphylococci [29].

All strains in this study were susceptible to teicoplanin. This finding is in agreement with other studies that have reported that *Staphylococcus aureus* is almost always susceptible to this antibiotic [30,31].

4.3 Biofilm Production

Biofilms pose a serious problem for public health due to the increased resistance of biofilm-

associated bacteria to antibiotics and host defenses. *Staphylococcus aureus* is ranked among the bacteria that possess great ability to form a biofilm on host tissues and implanted medical devices. This capacity of staphylococcus to form biofilms is one of the major virulence traits underlying persistent and chronic infections [32].

In this study, a tissue culture plate method (TCP method) was selected for assay biofilm formation and to quantify attachment. 74 isolates of *Staphylococcus aureus* were screened for biofilm production by this method.

By TCP method 58 (78.4%) *Staphylococcus aureus* were positive for biofilm production. Our results are correlating well with Gunti et al. [33] and Khan et al. [34] who reported 76 % positivity and 64.9% positivity, respectively by TCP method while Mathur T. et al. [35] and bose et al. [36] reported a less number of biofilm production by *Staphylococcal* species. The distribution of these 58 biofilm producers according to service was as follows:

> 17 from patients in the intensive care units, 14 in the national center of burns, 7 in the dermatology department, 4 in the pediatric service, 3 in the nephrology service, 2 in the cardiovascular surgery unit and 11 in the other services. In the majority of cases (70.7%) strains were isolated from blood, skin samples, burns surfaces and catheters. these results are in accordance with those obtained by Taj et al. [37].

4.4 MRSA and Biofilm Formation

The majority of MRSA strains (95.4%) were positive for biofilm production, and only one isolate was considered to be a non-biofilm-producing strain. These results are similar to studies conducted by several researchers such as Mirzaee et al. [38] and Solmaz et al. [19] which showed that 100% and 97.5% respectively, of the studied strains have an ability to produce a biofilm.

The majority of non-producing biofilm strains are susceptible to all tested antibiotic except penicillin G whereas strains which have resistances are almost all producing biofilm. In this study the comparison of rates of antibiotic resistance between the biofilm producers and non biofilm producers showed a significant difference at $p < 0.05$ for all the antibiotics tested (Table 2).

5. CONCLUSION

Staphylococcus aureus has a great ability to produce biofilms. This characteristic is one of the virulence markers of this pathogen and gives it the ability to resist to antibiotics and to immune defense. Our results indicate that there is a significant association between antibiotics resistance and presence of biofilm in methicillin resistant *Staphylococcus aureus* isolates.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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