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Coagulase negative staphylococci in Anti-Cancer Center, Batna, Algeria: antibiotic resistance pattern, biofilm formation, and detection of *mecA* and *icaAD* genes

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Abstract:

Background: Coagulase-negative staphylococci (CoNS) are normal microbial flora found on the skin and mucous membranes of mammals. Considered for a long time as avirulent commensals, these bacteria are now recognized as opportunistic pathogens by virtue of their high resistance to multiple antibiotics and capacity for biofilm formations, which made them important agents of nosocomial and community-acquired infections. The objectives of this study are to determine the antibiotic resistance pattern and biofilm formation, and to detect *mecA* and *icaAD* genes in clinical CoNS isolates from Batna's Anti-Cancer Center (ACC) in Algeria.
Methods: A total of 66 CoNS were isolated from different samples and identified by API Staph system. *In vitro* antibiotic susceptibility testing (AST) of each isolate to selected antibiotics was determined by the disk diffusion method, and minimum inhibitory concentrations (MICs) of oxacillin and vancomycin were determined by E-test. Biofilm formation was assessed by Tissue Culture Plate (TCP) and Congo Red Agar (CRA) methods. The polymerase chain reaction (PCR) was used to amplify *mecA* gene in 9 oxacillin-resistant and 1 oxacillin-sensitive CoNS, and *icaAD* gene in 9 biofilm forming and 1 non-biofilm forming CoNS. Sequencing of the 16S rDNA of 1 *mecA* and 1 *icaAD* positive isolates was performed by the Sanger method.
Results: Nine species of CoNS were identified, with *Staphylococcus epidermidis* (n=29, 44%) and *Staphylococcus haemolyticus* (n=15, 22.7%) constituting the largest proportion, and isolated mainly from the onco-haematology service unit of the center. The isolates were resistant to penicillin G (98.5%), cefoxitin (80.3%) and oxacillin (72.2%). The TCP method was more sensitive (89.4%) than CRA method (31.8%) in detecting biofilm formation. The *mecA* gene was detected in 66.7% (6/9) of oxacillin resistant CoNS and the *icaAD* gene in 55.6% (5/9) of TCP positive CoNS isolates
Conclusion: *Invitro* resistance to methicillin (oxacillin) and biofilm formation were high among the CoNS isolates in this study, but the association of these with respective carriage of *mecA* and *icaAD* genes was low.

Keywords: Coagulase negative staphylococci, identification, antibiotic resistance, biofilm, PCR

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Staphylocoques à coagulase négative au Centre Anti-Cancer du Batna, Algérie: résistance aux antibiotiques, formation de biofilms et détection des gènes *mecA* et *icaAD*

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Abstrait :

Contexte: Les staphylocoques à coagulase négative (CoNS) sont une flore microbienne normale présente sur la peau et les muqueuses humaines des mammifères. Considérés depuis longtemps comme des commensales avirulentes, ces bactéries sont reconnues comme agents pathogènes opportunistes grâce à leurs multiples propriétés coexistantes de résistance aux antibiotiques et de formation de biofilms qui constituent des agents importants d'infections nosocomiales et communautaires. l'objectif de cette étude est de déterminer la résistance aux antibiotiques, la formation de biofilms et pour rechercher des gènes *mecA* et *icaAD* dans les isolats cliniques de staphylocoques à coagulase négative du Centre Anti-Cancer (AAC) de Batna en Algérie.
Méthodes: au total de 66 des SCN ont été isolés de différents prélèvements et identifiés par galerie API Staph. Le test de sensibilité aux antibiotiques *In vitro* de chaque isolat par rapport aux antibiotiques sélectionnés a été déterminé par la méthode de diffusion sur disque, et les concentrations minimales inhibitrices (MICs) de l'oxacilline et de la vancomycine ont été déterminées par E-test. La formation de biofilm a été évaluée par la méthode de culture de tissu en plaque (TCP) et la méthode de Rouge Congo Agar (CRA). La réaction en chaîne par polymérase (PCR) a été utilisée pour amplifier l'ADN du gène *mecA* dont 9 des SCN résistants à l'oxacilline et 1 sensible à l'oxacilline et le gène *icaAD* dont 9 des SCN formant biofilm et 1 non-formant biofilm. Le séquençage de l'ADNr 16S des isolats positifs, 1 *mecA* et 1 *icaAD* ont été réalisés par la méthode de Sanger.
Résultats: Neuf espèces des SCN ont été identifiées avec *Staphylococcus epidermidis* (n=29, 44%) et *Staphylococcus haemolyticus* (n=15, 22,7%) constituant la plus grande proportion, et isolées principalement de l'unité de service d'onco-hématologie du centre. Les isolats étaient résistants à la pénicilline G (98,5%), à la céfoxitine (80,3%) et à l'oxacilline (72,2%). La méthode TCP était plus sensible (89,4%) que la méthode CRA (31,8%) dans la détection de la formation de biofilm. Le gène *mecA* a été détecté dans 66,7% (6/9) des SCN résistants à l'oxacilline et le gène *icaAD* dans 55,6% (5/9) des isolats positifs des SCN pour CRA.
Conclusion: La résistance à la méthicilline (oxacilline) *in vitro* et la formation de biofilms étaient élevées chez les isolats des SCN de cette étude, mais leur corrélation avec le portage respectif des gènes *mecA* et *icaAD* était faible.

Mots-clés: Staphylocoque à coagulase négative, identification, résistance aux antibiotiques, biofilm, PCR

Introduction:

Coagulase negative staphylococci (CoNS) are normal microbial flora found on the skin and human mucous membranes of mammals (1). These bacteria, which have for a long time adjudged to be avirulent commensals, are currently considered the predominant pathogens (2) and major cause of nosocomial and community-acquired infections (3).

The most common species that cause disease in humans are *Staphylococcus epidermidis* which causes bacteremia in patients with implanted medical devices (such as prostheses and catheters), surgical wound infection, peritonitis in patients with continuous peritoneal dialysis, osteomyelitis, and endophthalmitis; *Staphylococcus haemolyticus* which causes endocarditis, peritonitis, sepsis and infections of the urinary tract, wounds, bones and joints; and *Staphylococcus saprophyticus* which causes urinary tract infections and septicemia. The other important opportunistic pathogenic species include *Staphylococcus hominis*, *Staphylococcus warneri*, *Staphylococcus capitis*, *Staphylococcus simulans*, *Staphylococcus cohnii*, *Staphylococcus xylosus* and *Staphylococcus saccharolyticus* (4).

The treatment of the infections by CoNS is difficult because many clinical isolates exhibit multiple and high resistance to antibiotics, leading to increased inefficiency of a wide range of antibiotics (5). There are several reports in literature showing high resistance to methicillin and other antibiotics among CoNS isolates (6, 7, 8). Methicillin resistance in CoNS is caused by the *mecA* gene that encodes penicillin-binding protein 2a (PBP2a) that has low binding affinity to β-lactams (2, 9**,** 10**,** 11).

The ability to form biofilm is the most important virulence factor in CoNS, which facilitates its adhesion to and colonization of artificial materials (8). Bacteria in biofilms can resist antibiotics at concentrations up to 1000 times higher than those active on the same bacteria in the planktonic state (12). The biofilm consists of layers of cellular clusters integrated in a matrix of extracellular polysaccharide, called polysaccharide intracellular adhesion (PIA). The enzymes implied in the synthesis of PIA are encoded by the *ica* operon including the *icaA*, *icaD*, *icaB* and *icaC* genes (13).

The two common phenotypic methods for detecting biofilm formation, Tissue Culture Plate (TCP) and Congo Red Agar (CRA), were described by Christensen et al., (14) and Freeman et al., (15) respectively. Molecular detection of the *icaAD* locus was initially described by Heilmann, et al., (16) in *S. epidermidis* but few years later, its presence was confirmed in many other species of staphylococci isolated from implant related-infections (12). The objectives of this study are to evaluate antibiotic resistance and biofilm formation in CoNS isolated from patients at the Anti-Cancer Center of Batna, Algeria, and to detect the responsible *mecA* and *icaAD* genes in these isolates.

Materials and method:

Study setting and bacterial isolates

This study was conducted at the Microbiology Laboratory of the Anti-Cancer Center (ACC), Batna, Algeria. Sixty six coagulase negative staphylococci (CoNS) were isolated from clinical samples (urinary probes, urine, blood culture, pus, wound, pleural fluid, cerebrospinal fluid, intravenous catheter, and drain) obtained from hospitalised patients in various service units (onco-haematology, onco-paediatric, intensive care and carcinological surgery) of the center and from outpatients, between 1st of January and 30th of June, 2017. Duplicate samples were excluded.

The samples were cultured on Mannitol Salt (Chapman) agar and incubated at 37°C for 48 hours. All strains were identified by colony morphology, Gram stain reaction, catalase production, coagulase assay, and the API Staph system (bioMérieux, France). The purified isolates were stored at 4°C.

Antibiotic susceptibility testing of isolates

In vitro antibiotic susceptibility testing (AST) was performed by the disk diffusion test on Mueller-Hinton (MH) agar as described by Bauer et al., (17) and the interpretation of the results was done according to Clinical and Laboratory Standards Institute (CLSI) guidelines (18). All isolates were tested with the following antibiotics: penicillin (10 UI), cefoxitin (30 µg), gentamicin (10 µg), amikacin (30 µg), kanamycin (30 µg), erythromycin (15 µg), tetracycline (30 µg), ofloxacin (5 µg), laevofloxacin (5 µg), ciprofloxacin (5 µg), clindamycin (2 µg), chloramphenicol (30 µg), rifampicin (5µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), fusidic acid (10 µg), pristinamycin (15 µg) and teicoplanin (30 µg). The minimum inhibitory concentrations (MICs) of oxacillin and vancomycin for each isolate were determined on MH using E-test method, and results interpreted according to CLSI guidelines (18).

Detection of biofilm formation by Congo Red Agar (CRA) method

The qualitative determination of the biofilm formation was carried out by culturing CoNS isolates on Congo Red Agar as proposed by Freeman et al., (15). The prepared medium was inoculated with CoNS isolates and incubated at 37°C for 24 to 48 hours. Biofilm-forming bacteria appear black with a crystalline lens of dry consistency where the Congo Red dye interacts directly with certain bacterial polysaccharides forming a slime while the non-biofilm forming colonies remained red.

Detection of biofilm formation by Tissue Culture Plate (TCP) method

Quantitative determination of biofilm formation on microplates was evaluated as described by Christensen et al., (14) with some modifications. The microplates used were made of polystyrene with 96 wells on which the bacteria adhere and form biofilm. The isolates were cultivated on nutrient agar for 18-24 hours at 37°C. A colony of each isolate was inoculated into 5 ml Trypticase Soy Broth (TSB) and incubated at 37°C for 24 hours, and the culture was then diluted 1:100 in the TSB (+1% glucose). Each well of the microplate was filled with 200µL of this dilution (three independent cultures for each isolate). A sterile broth of TSB (+1% glucose) was used as a negative control.

The microplates were sealed and incubated for 24 hours at 37°C. Thereafter, the contents of the wells were gently removed and washed four times with sterile physiological water and then dried at 60°C for 30 minutes. The cells adhering to the polystyrene support in each of the wells were stained with 200µl of 1% crystal violet (w/v) and incubated for 30 minutes following which excess crystal violet was removed by 5 successive washes with sterile distilled water and the plates dried at room temperature. The dye incorporated by the adhered cells was solubilized with 200µL of 95% ethanol (v/v).

The amount of dye solubilized was measured by reading the optical density (OD) at 550 nm using Bio-Rad ELISA reader (PR 3100 TSC) (19). The interpretation of the results was performed according to the criteria of Stepanovic et al., (20). The OD of the isolate was obtained by the arithmetic mean of three wells and this value was compared with the mean absorbance of negative control (ODc). The isolates were classified as non-biofilm producer (OD≤ ODc), weak biofilm producer (ODc<OD≤2 ODc), moderate biofilm producer (2ODc<OD≤ 4ODc) and strong biofilm producer (4ODc< OD).

Detection of *mecA* and *icaAD* genes by PCR and 16s rDNA sequencing

The PCR of *mecA* and *icaAD* genes and the sequencing of the 16S rRNA genes were performed in the Laboratory of DIAG-GENE, Angers, France. Nine MRCoNS strains (no 1 to 9) and 1 MSCoNS strain (no 10), were selected for *mecA* gene detection. Similarly, 9 biofilm-forming strains (no 11 to 19) and 1 non-biofilm forming strain (no. 20) were selected from the TCP results for *icaAD* gene detection.

PCR assay

The *mecA* and *icaAD* genes amplifications were performed by PCR as described by

Poulsen et al., (21) and Yazdani et al., (22) respectively, using the following specific primers; *mecA*-F:5'-GGGATCATAGCGTCATTA-TTC-3' and *mecA*-R:5'-AACGATTGTGACACGA-TAGCC-3', *icaAD*-F: 5'-TATTCAATTTACAGTCG-CAC-3' and *icaAD*-R: 5'-GATTCTCTCCCTCTCT GCCA-3'. The DNA amplification was done in a Mycycler thermal cycler (Bio-Rad, USA). The amplification products (10µL) were eletro-phoresed on 1.5% agarose gel followed by staining in an ethidium bromide bath (0.5µg/ml). The amplified DNA products were visualized under UV transillumination.

Sequencing of the 16S rRNA genes

Sequencing of amplified and purified 16S rDNA of strain number 3 (*mecA*+) and strain number 13 (*icaAD*+) was performed as described by Sanger et al., (23), using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and two universal primers (27F and 1492R). The 16S sequences obtained were then compared with those of the GeneBank database using the BLAST programme (24).

Results:

Identification of CoNS isolates

Sixty six CoNS belonging to 9 different species were identified by the conventional methods; *S. epidermidis* 29, *S. haemolyticus* 15, *S. hominis* 8, *S. chromo-genes* 6, *S. xylosus* 4, *S. capitis* 1, *S. sapro-phyticus* 1, *S. cohnii* 1, and *S. simulans* 1 (Table 1).

Table 1: Species distribution of CoNS isolates in Anti-Cancer Center, Batna, Algeria

CoNS isolate	Frequency	Percentage
Staphylococcus epidermidis	29	43.9
Staphylococcus haemolyticus	15	22.7
Staphylococcus hominis	8	12.1
Staphylococcus chromogenes	6	9.1
Staphylococcus xylosus	4	6.1
Staphylococcus capitis	1	1.5
Staphylococcus cohnii	1	1.5
Staphylococcus saprophyticus	1	1.5
Staphylococcus simulans	1	1.5
Total	66	100

Table 2 shows the distribution of the CoNS according to the clinical samples; 28 (42.4%) were from blood cultures (*S. epidermidis* 11, *S. haemolyticus* 8, *S. hominis* 8 and *S. chromogenes* 2); 13 (19.7%) from catheter samples (*S. epidermidis* 8, *S. haemolyticus* 2, *S. chromogenes* 1, *S. xylosus* 1 and *S. capitis* 1); 8 (12.1%) from pus samples (*S. epidermidis* 2, *S. chromogene*s 2, *S. haemolyticus* 1, and *S. xylosus* 3); 6 (9.1%) from urine samples (*S. epidermidis* 3, *S. haemolyticus* 2, and *S. hominis* 1.

Table 2: Specimen distribution of CoNS isolates in Anti-Cancer Center, Batna, Algeria

Samples	Frequency	Percentage
Blood	28	42.4
Vascular catheter	13	19.7
Pus	8	12.1
Urine	6	9.1
CSF	5	7.6
Pleural fluid	3	4.5
Wound	1	1.5
Urinary probe	1	1.5
Drain	1	1.5
Total	66	100

CoNS = coagulase negative staphylococci

The CoNS isolates were found mainly in the onco-haematology service (n=30, 45.5%), out of which 13 were *S. epidermidis* (Table 3).

Table 3: Distribution of CoNS isolates by service unit in Anti-Cancer Center, Batna, Algeria

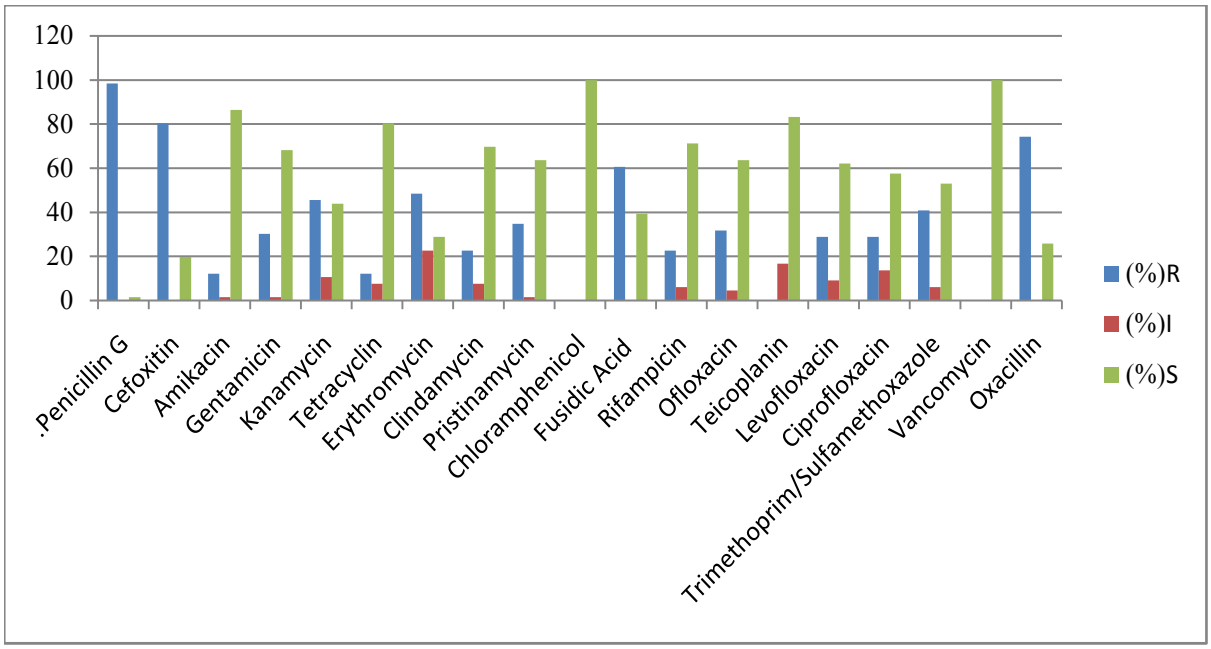
Service unit	No of CoNS	Percentage
Onco-haematology	30	45.5
Carcinological surgery	14	21.2
Onco-paediatric	10	15.2
Intensive care	7	10.6
Outpatient	5	7.6
Total	66	100

CoNS = coagulase negative staphylococci

Antimicrobial susceptibility profiles of CoNS isolates

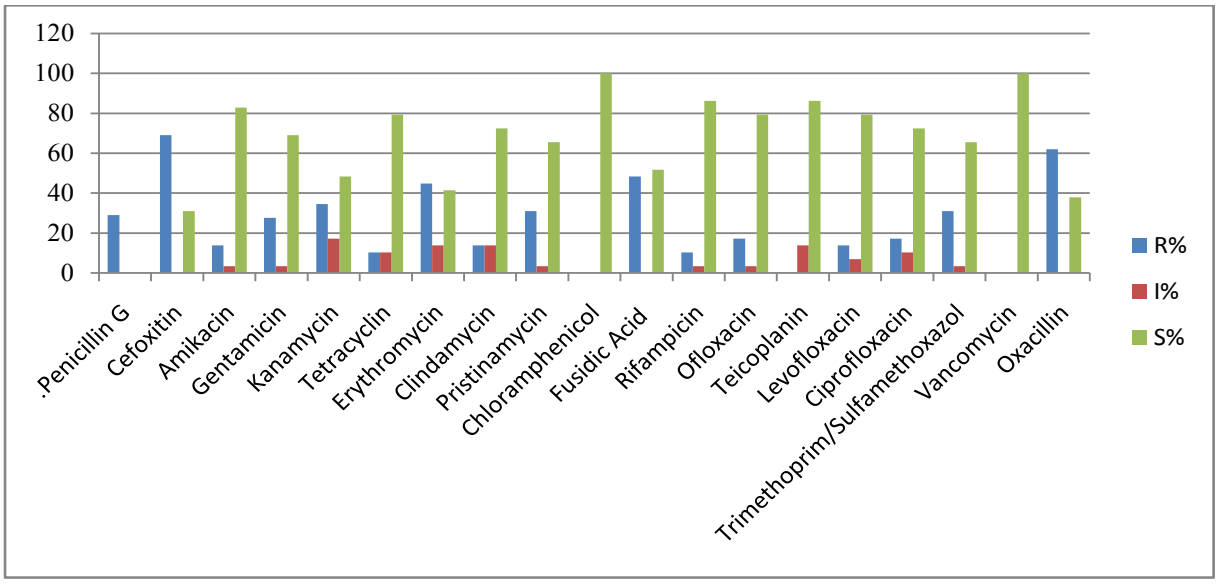
The AST profile of the isolates is detailed in Fig 1. The isolates exhibited high resistance to penicillin G (98.5%), cefoxitin (80.3%), oxacillin (74.2%), fusidic acid (60.6%), but were totally sensitive to chlora-mphenicol and vancomycin (by the E-test). The isolates were also largely sensitive to amikacin (86.4%), teicoplanin (83.3%) and tetracycline (80.3%) but susceptibility was reduced to rifampicin (71.2%), clindamycin (69.7%), gentamicin (68.2%), pristinamycin (63.6%), ofloxacin (63.6%), laevofloxacin (62.1%), erythromycin (50%) and kanamycin (45%).

The most frequent CoNS isolates, *S. epidermidis*, was totally resistant to penicillin G (100%), 69% to cefoxitin, and 60% to oxacillin by the E-test (Fig. 2).



R = resistance; I = intermediate; S = sensitive

Fig 1: Antimicrobial susceptibility profiles of coagulase negative staphylococci isolates



R = resistance; I = intermediate; S = sensitive

Fig 2: Antimicrobial susceptibility profiles of *Staphylococcus epidermidis*

Biofilm formation

Fig. 3 shows the result of biofilm formation by the CRA method with 21 (31.8%) CoNS isolates forming biofilm while 45 (68.2%) were negative. The results of biofilm formation by the TCP method showed that 59 (89.4%) of the 66 isolates formed biofilms with different intensities (Fig 4); 16 (24.2%) were strong biofilm forming, 23 (34.9%) were moderate, 20 (30.3%) were weak, and 7 (10.6%) were non-biofilm forming bacteria.

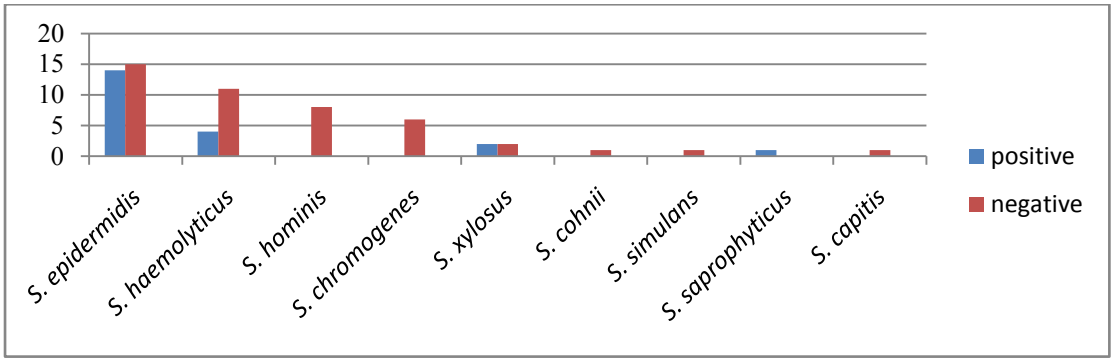


Fig 3: Biofilm formation tested by Congo Red Agar method

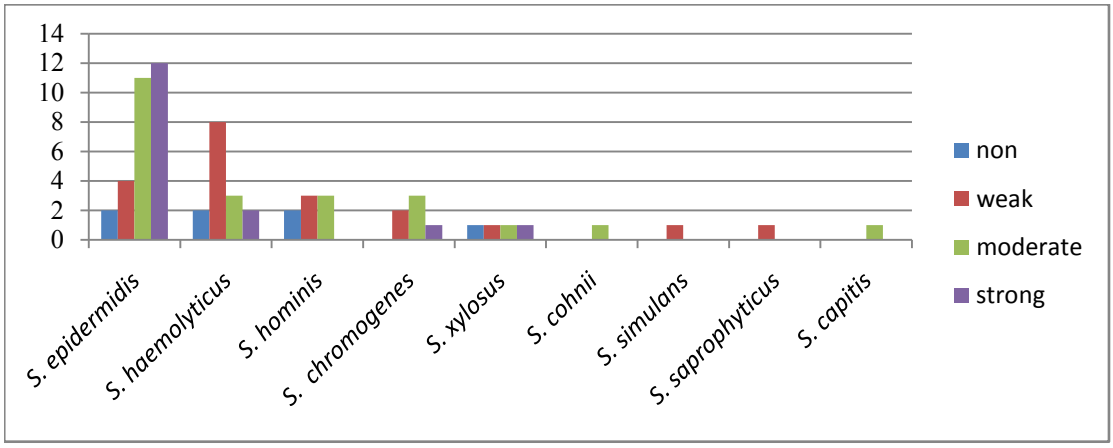
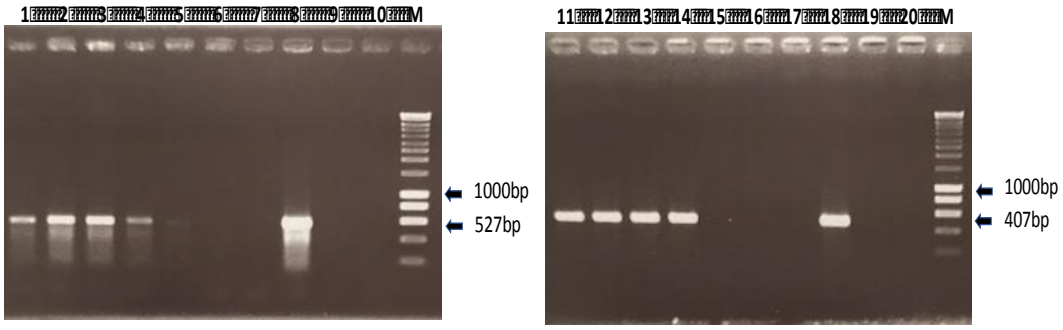


Fig 4: Biofilm formation tested by the Tissue Culture Plate method

Result of *mecA* and *icaAD* genes PCR

Of the 9 CoNS isolates resistant to oxacillin by the AST tested for carriage of *mecA* gene, only 6 amplified for the gene (Fig 5a). Similarly, only 5 of the 9 CoNS isolates positive for biofilm production by the CRA method amplified for the *icaAD* genes (Fig 5b). Sequencing of the 16S rRNA genes of in each isolate positive for *mecA* and *icaAD* shows percentage similarity to respective GenBank strains as shown in Table 4.



(a) *mecA* gene (b) *icaAD* gene

M: molecular size marker (Smart Ladder 200bp, Eurogentech); 1-9: MRCoNS; 10: MSCoNS; 11-19: TCP+ CoNS; TCP- CoNS

Fig 5: Agarose gel electrophoresis of *mecA* and *icaAD* genes

Table 4: Percentage similarity of two CoNS isolates on BLAST analysis

Identification number (N°) isolate	%similarity to strain in GenBanl	Identification
3	100	*Staphylococcus haemolyticus* strain FC2950
13	100	*Staphylococcus epidermidis* strain K121

Discussion:

Our study identified 9 species out of the 66 CoNS characterized, with predominance of *S. epidermidis* (44%), *S. haemolyticus* (22.7%) and *S. hominis* (12.1%). This similar pattern has been reported by Shah et al., (25) who reported 35.1% for *S. epidermidis,* 33.1% for *S. saprophyticus* and 8.8% for *S. haemolyticus,* as well as by many other researchers (1,8,26,27). The CoNS in our study especially *S. epidermidis* were found in onco-haematology service unit mainly from blood cultures which is similar to what Marsik et al., reported in their study (28). CoNS is the most frequently isolated bacteria from blood cultures and is a serious health challenge in developing as well as in many developed countries (4). *S. epidermidis* is the most common species involved in blood stream infections, which is attributed to its ability to colonize central venous catheters and other implanted medical devices (29).

CoNS resistant to multiple antibiotics have become a great challenge in nosocomial infections. In this study, 80.3% of the CoNS isolates were methicillin resistant by the cefoxitin disk and 74.2% by the oxacillin E-test, and resistance to penicillin G was 98.5%. This high resistance rate to both methicillin and penicillin G have been reported by several other researchers (1,6,7,30,31,32). Methicillin resistance is usually associated with *mecA* gene that encodes abnormal penicillin binding protein PBP2a (PBP2a) with low binding affinity for β-lactam antibiotics (5). The *mecA* gene is located on a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*) which can be transferred between and within the staphylococcal species (33). MRSA isolates are usually not reliably detected by phenotypic techniques, especially as the expression of *mecA* gene varies according to strains, some of which may possess heterogeneous resistance, hence the need for genotypic characterization (34).

In our study, no CoNS isolates was resistant to vancomycin by the E-test MIC, which agrees with the reports of Jain et al., (35) and Shrestha et al., (1). Vancomycin and teicoplanin are anti-staphylococcal antibiotics to which resistance is rarely observed. These antibiotics should however be reserved for the treatment of staphylococcal infections resistant to all other antibiotics (32). The CoNS isolates were also largely sensitive to amikacin and tetracycline (>80%) but susceptibility to fluoroquinolones (ofloxacin, ciprofloxacin and laevofloxacin), clindamycin, pristinamycin, erythromycin and trimethoprim/sulfamethoxazole were reduced with resistance rate being over 20% for each of these isolates. Our findings are similar to what others have reported for CoNS clinical isolates (3, 28, 29, 36, 37, 38).

The ability of staphylococci to form biofilms helps the bacterium to resist host immune response and is considered responsible for chronicity as biofilm protects microorganisms from opsono-phagocytosis and antimicrobial agents. In view of the large number of infections caused by biofilm producing bacteria, a reliable method for their diagnosis is necessary. Nasr et al., (39) reported that 50% of CoNS isolated from intravascular blood cultures and catheters were biofilm producers using the CRA method, which is higher than 31.8% reported in our current study. The TCP method has however been reported to be a more sensitive, accurate and reproducible screening method for detecting biofilm production in clinical staphylococci isolates with added advantage of being a quantitative tool for comparing the adherence of different strains (39). Oliveira et al., (26), Soumya et al., (8) and Shrestha et al., (1) reported that 73%, 87.5% and 85% CoNS respectively produce biofilm by the TCP method. This is similar to the high rate of 89.4% reported in our current study.

The ability of CoNS to be resistant to methicillin and produce biofilm is an important factor in infectivity which occurs through expression of *mecA* and *ica* genes respectively. Jain et al., (35) have reported that detection of *mecA* gene by PCR can be a beneficial complement to standard susceptibility test that can allow the identification of intrinsic resistance quickly and efficiently, as most strains carry the classical *mecA* gene. Six of the 9 (66.7%) selected CoNS isolates resistant to methicillin (by oxacillin E-test) in

our study amplified for *mecA* gene. However, other allotypes of *mecA* gene, have been described for subspecies of *S. sciuri* (*mecA1*) and for *S. vitulinus* (*mecA2*) among animal-derived isolates. Another *mecC* allotype, *mecC2*, was recently reported for methicillin resistant *S. saprophyticus* subsp. *saprophyticus* (40). The *icaAD* gene amplified in 5 of the 9 (55.5%) selected CoNS isolates in our study. This rate is higher than the 32% reported by Nasr et al., (39). It has been reported that the *icaAD* gene is not always associated with *in vitro* biofilm formation (39). The biofilm-forming ability of some isolates in the absence of *icaAD* gene highlights the need to investigate the genetic basis of *ica*-independent biofilm formation.

Conclusion:

In vitro resistance to methicillin (oxacillin) and biofilm formation were high among the CoNS isolates in Anti-Cancer Center, Batna, Algeria, but the association of these phenotypes with respective carriage of *mecA* and *icaAD* genes was low. Further studies are needed to investigate the genetic basis of biofilm formation independent of *icaAD* genes.

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