International Journal of Medical Microbiology 307 (2017) 75–82

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Pathogenic features of clinically significant coagulase-negative staphylococci in hospital and community infections in Benin

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ARTICLE INFO

Article history:
Received 18 July 2016
Received in revised form 14 October 2016
Accepted 8 November 2016

Keywords:
Clinically significant coagulase-negative staphylococci
Antibiotic resistance
Hemolytic activity
Enterotoxin
Virulence factors

ABSTRACT

In West Africa, very little consideration has been given to coagulase negative Staphylococci (CNS). Herein, we describe the features contributing to the pathogenicity of 99 clinically-significant independent CNS isolates associated with infections encountered at the National Teaching Hospital Center of Cotonou (Benin). The pathogenic potentials of nosocomial strains were compared with community strains. *S. haemolyticus* (44%), *S. epidermidis* (22%) and *S. hominis* (7%) were the most frequently isolated while bacteremia (66.7%) and urinary tract infections (24.2%) were the most commonly encountered infections. Most strains were resistant to multiple antibiotics, including penicillin (92%), fosfomycin (81%), methicillin (74%) and trimethoprim-sulfamethoxazole (72%). The most frequently isolated species were also the most frequently resistant to methicillin: *S. hominis* (100%), *S. haemolyticus* (93%) and *S. epidermidis* (67%). Screening of toxic functions or toxin presence revealed hemolytic potential in 25% of strains in over 50% of human erythrocytes in 1 h. Twenty-six percent of strains exhibited protease activity with low (5%), moderate (10%) and high activity (11%), while 25% of strains displayed esterase activity. Three percent of strain supernatants were able to lyse 100% of human polymorphonuclear cells after 30 min. Polymerase chain reaction and latex agglutination methods revealed staphylococcal enterotoxin C gene expression in 9% of *S. epidermidis*. A majority of hospital-associated CNS strains (68%) had at least one important virulence feature, compared with only 32% for community-acquired strains. The present investigation confirms that these microorganisms can be virulent, at least in some individual cases, possibly through genetic transfer from *S. aureus*.

1. Introduction

Staphylococci are among the most commonly encountered bacteria in hospital settings and are involved in various infections. *Staphylococcus aureus* is considered as the major species associated with severe staphylococcal infections for which several virulence factors responsible for the symptoms and severity of infections have been identified (Durupt et al., 2007; Baba-Moussa et al., 2008; Fluit et al., 2001). Meanwhile, several studies have emphasized the role of Coagulase-negative staphylococci (CNS) in various infection types (Delaunay et al., 2014; Piette and Verschraegen, 2009). Their growing role as pathogens has been demonstrated in human infections, especially in immunocompromised patients, preterm infants and in patients with implanted devices (Becker et al., 2014).

The most frequent CNS infections associated with humans are primarily nosocomial in nature and are due to *Staphylococcus epidermidis*, *Staphylococcus haemolyticus* isolated from bloodstream infections, peritonitis, prosthetic valve-related endocarditis, and foreign body infections (Ertem et al., 2010; Falcone et al., 2004; Schoenfelder et al., 2010). *S. lugdunensis* and *S. saprophyticus* are involved in arthritis and in urinary tract infections among adolescent females, respectively (Lo et al., 2015; Peel et al., 2015). *S. warneri*, *S. capitis*, *S. saccharolyticus*, *S. caprae*, *S. schleiferi*, *S. hominis* and *S. auricularis* can also be associated with severe infections (Delmas et al., 2008).

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http://dx.doi.org/10.1016/j.ijmm.2016.11.001
1438-4221/

The pathogenicity of CNS and hospital-acquired CNS isolates has been difficult to establish, since the virulence factors of these microorganisms are poorly defined (Becker et al., 2014). Several CNS may carry at least parts of genes encoding exo-enzymes such as proteases, lipases and toxins, which are factors that can impact the severity of the infections (Von Eiff et al., 2002; Pinheiro et al., 2015). *S. epidermidis* produces several adhesion factors, proteins and exo-polymers involved in immune evasion and biofilm production (Otto, 2012). Recently, pathogenicity islands bearing enterotoxin SEC3 and SE1L have been described in *S. epidermidis* (Madhusoodanan et al., 2011). In addition, most CNS display a high antibiotic resistance profile, particularly to methicillin which remains a major challenge for clinicians to find alternative molecules with low grade toxicity (Diekema et al., 2001).

In most African clinical microbiology laboratories, CNS characterization is usually limited to a simple coagulation assay to distinguish *S. aureus* from non-*aureus* isolates. However, because of their involvement in human infections, it now remains essential that clinically significant CNS be identified to the species level in order to establish a correlation between clinical signs and identified bacteria, as well as to identify risks. In Benin and West Africa, the prevalence, pathogenicity and resistance profile of CNS are poorly known because of the lack of such species identification.

The present study was undertaken to characterize clinically significant CNS strains isolated from various samples at the National Teaching Hospital Center, Hubert Koutoukou Maga (NTHC-HKM), in Cotonou, Benin. In this analysis, the variability, resistance profile to antimicrobial agents and virulence factors of isolates were determined and the pathogenic potentials of nosocomial- and community-based CNS were compared.

2. Materials and methods

2.1. Ethical statement

This work was approved by the National Ethics Committee of Benin under protocol number 2015/006. All staphylococcal isolates evaluated in this study were submitted by physicians treating patients for infectious syndromes. Buffy coats from fresh human blood from healthy donors were purchased to the « Etablissement Français du Sang de Strasbourg, France », for which all information remains confidential.

2.2. Study design and clinical relevance assessment of strains

Strains were collected prospectively from March 2015 to January 2016 at the NTHC-HKM, Cotonou, Benin. Clinically significant CNS isolates were retrieved from various specimens from both in- and out-patients. The clinical significance of CNS strains was determined by qualified medical personnel using the clinical records of each patient containing the demographic, clinical and laboratory data. The isolates were considered to be of clinical significance when the following general criteria were fulfilled: 1) strains obtained in bacterial pure culture; 2) presence of infection risk factors (immunosuppressive and/or front door); 3) clinical signs of infection (hyperthermia >38 °C, hypotension, tachycardia, tachypnea); 4) identification of a site of infection (respiratory, digestive, bone and joint, skin and soft tissues) (Mohammad et al., 2014). In the present study, only strains with clinical relevance were considered.

2.3. Identification of CNS

CNS Strains were phenotypically identified by standard microorganism identification methods in the Microbiology Laboratory of the NTHC-HKM. Methods were based on colony morphology, Gram staining, catalase positivity (ID color Catalase; bioMérieux, Marcy l'Etoile, France), non-agglutination of colonies with the Pastorex Staph Plus test (Bio-Rad, Marnes la Coquette, France), and free staphylocoagulase non production with lyophilized rabbit plasma. Identification at the species level was carried out using Api STAPH (bioMérieux, Marcy l'Etoile, France), according to the Manufacturer's instructions, and species identification was further confirmed for species identification with the MALDI-TOF Biotyper™ (Bruker Daltonics) method at the Institute of Bacteriology, University of Strasbourg, France. For two *S. epidermidis* isolates, 16 s rDNA sequencing was achieved (GATC Ltd, Basel, Switzerland) (Ghebremedhin et al., 2008).

2.4. MALDI-TOF mass spectrometry assay for bacterial identification

CNS strains were grown on Columbia agar with 5% (v/v) sheep blood (bioMérieux, Marcy l'Etoile, France) and incubated for 24 h at 37 °C. One colony of each strain was placed in duplicate on a 96-spot polished target plate and allowed to dry at room air. Next, 1 μl of matrix (3 mg/mL alpha-cyano-4 hydroxycinnamic acid in 50% [v/v] acetonitrile–2.5% trifluoroacetic acid [v/v]) was overlaid onto the sample and allowed to dry. The acquisition of protein mass spectra was performed on a Microflex LT™ instrument using the flexControl™ 3.0 software (Bruker Daltonics), with a mass-to-charge ratio (m/z) range of 2–20 kDa. Automated data analysis of raw spectra was performed with MALDI Biotyper™ RTC 3.1.2.0 software (Bruker Daltonics). An identification score of $\geq$2 was considered as high-confidence identification at the species level, whereas scores comprised between 1.7 and 1.99 were considered as intermediate confidence genus-level identification only. Scores of <1.7 were considered as an unacceptable identification, according to the Manufacturer's recommendations, and were excluded from the analysis if any.

2.5. Antibiotic susceptibility and methicillin resistance testing

Antimicrobial susceptibility and methicillin resistance testing of the CNS isolates were performed with the VITEK 2 system (bioMérieux, Marcy l'Etoile, France) according to the recommendations of the Committee for Antimicrobial Susceptibility of the French Society for Microbiology (CA-SFM)-2015. The antibiotics tested were cefoxitin (FOX), benzylpenicillin (BEN), oxacillin (OXA), gentamicin (GEN), kanamycin (K), tobramicin (TM), ofloxacin (OFX), clindamycin (CL), erythromycin (E), lincomycin (L), pristinamycin (PT), linezolid (LIZ), teicoplanin (TEC), vancomycin (VA), tetracycline (TET), fosfomycin (FOS), nitrofurantoin (FT), fusidic acid (FA), rifampicin (RIF) and trimethoprim-sulfamethoxazole (SXT). Methicillin-resistance was assessed by the resistance of the strains with cefoxitin and oxacillin at 37 °C.

2.6. Screening for proteolytic activity

After identification, each strain was first cultured on Mueller Hinton (MH) agar enriched with 5% sheep blood for 18 h at 37 °C after which a clone was selected and inoculated into 100 ml of Brain Heart Infusion (BHI) broth. The suspension was then incubated with shaking at 37 °C for 18 h and centrifuged at 10,000 × g for 10 min. The culture supernatants were collected and filtered with a 0.22 μm filter. Each filtrate was stored at −20 °C until use. Protease activity of the strains was investigated using azocasein (Sigma-Aldrich, France) as substrate (Vandecandelaere et al., 2014). Briefly, 600 μl of buffer (0.2 M Tris HCl, 3 mM $CaCl_2$, pH 8.0), 200 μl of azocasein (25 g/ml) and 200 μl of each strain culture supernatant were incubated at 37 °C. After 3 h, 200 μl of 100% (w/v) trichloroacetic acid (TCA) were added and the new mixture was centrifuged for

5 min at 10,000 × g. One ml of the supernatant was decanted to which 200 µl of 7.5 M NaOH were added. Absorbance was measured at 440 nm to evaluate protease activities with the *S. lugdunensis* AACC51874 strain used as a positive control and brain heart broth without bacterial culture as a negative control. The average OD values of the negative controls were calculated from triplicate assays and subtracted from the mean absorbance values of each strain tested (specific OD). The limit of detection (LD) for this test was defined as 3× the standard deviation of the mean of the negative controls. The strains were classified into four categories according to the classification used by Srdjan et al. (2000): non-proteolytic (OD ≤ LD), weakly proteolytic (LD < OD ≤ 2LD), moderately proteolytic (2LD < OD ≤ 4LD), very proteolytic (OD > 4LD).

2.7. Screening for esterase activity

Nutrient Agar containing 10 g/L yeast extract, 1% (v/v) Tween 80, 0.1 g/L $CaCl_2$, 10 g/L casamino acids and 5 g/L NaCl was used to study esterase activity (Pakshir et al., 2013). The isolates were grown on trypticase soy agar and incubated at 37 °C for 24 h. They were subsequently inoculated by streak on Tween agar plates and incubated at 37 °C for 48 h. The formation of a white precipitate around the streak was taken as an indication of positive esterase activity.

2.8. Screening for hemolytic activity and two bicomponent leukotoxins

Hemolytic activity of strain supernatants on human red blood cells (hRBC) was determined by colorimetry at 414 nm (Pawel et al., 2008). One ml of human red blood cells was centrifuged and washed thrice at 10,000 × *g* for 15 min with 10 ml of phosphate buffered saline (PBS: 10.1 mM Na_2HPO_4; KH_2P0_4 1.8 mM, 2.7 mM KCl, NaCl 140 mM pH 7.3 to 7.4). The suspension was diluted to 50% in PBS and the concentration was adjusted to an absorbance of 8.0 [0.4% (v/v)] at 414 nm. The culture supernatants of the strains were serially diluted with PBS in a 96 well round-bottom plate. One hundred µl of 0.4% red blood cell suspension were added to each well. A negative control (RBC 100 µl + PBS 100 µl) in three wells and a positive control 100 µl PBS +1% (w/v) saponin + (100% RBC) of 3 wells were used. The plate was incubated for 45 min at 37 °C and centrifuged at 3000 × *g* for 3 min. Finally, 100 µl of the supernatant was transferred to a 96-well flat-bottom plate for absorbance reading at 414 nm. The absorbance values at 414 nm of negative controls were calculated and subtracted from the mean absorbance values of each strain tested. The results were expressed as the percentage of 100% hemolysis (Shimuta et al., 2009). In this study, strain supernatants were considered as hemolytic when lysing at least 50% of RBCs at 1:4 dilution. Otherwise, presence of two bicomponent leukotoxins in culture supernatants were checked by using radial gel immunoprecipitation with anti-HlgC, −HlgB, LukS-PV, and −LukF-PV affinity-purified rabbit antibodies as earlier reported (Gravet et al., 1998).

2.9. Cytotoxicity assays

$hPMN_s$ were purified from anonymized buffy coats purchased as previously described (Meyer et al., 2009). The neutrophil cell pellet was resuspended in 25 ml of HBSS buffer and again centrifuged at 1600 × *g* for 6 min. The pellet was mixed in 25 ml of HBSS and allowed to rest for 1 h. Before use, the suspension was centrifuged and the pellet filtered on a 70 µm filter. The cytotoxic activity of the culture supernatant was determined using a commercial kit (CytoTox 96® Cytotoxicity Assay, Promega Corporation, Fitchburg, WI, USA). One hundred microliters of hPMNs adjusted to a concentration of 6×10^5 cells/ml were incubated with 50 µl of culture supernatant for 30 min. The mixture was centrifuged as above and 50 µl of the supernatant was incubated with 50 µl of reagent for 30 min. In the presence of lactate dehydrogenase (LDH), the tetrazolium salt content in the reagent was reduced in a red product, formazan, which was assayed by absorbance measurement at 490 nm. In this assay, the absorbance formed is proportional to the number of lysed cells. Cytotoxicity was represented as the percentage of total LDH release detected by lysing cells with 0.1% (v/v) Triton X-100. To determine the leukotoxic capacity of the isolates, 5 µl of culture supernatant were incubated with 6×10^5 hPMNs/ml. Strains that lysed at least 50% of hPMNs were considered as cytotoxic (Scheifele et al., 1987).

2.10. DNA extraction and amplification for S. aureus *clumping factor*, hlgB, S. epidermidis gseA *and enterotoxin gene detection*

DNA was extracted from CNS strains grown on blood agar plates using the MasterPure™ DNA Purification according to the Manufacturer's recommendations (Epicentre, Le Perray en Yvelines, France). DNA purity was verified using 260/280 ratios; samples with ratios <1.8 were rejected. Presence of genes encoding staphylococcal enterotoxins (se), *sea*, *sec*, *seg* and *seh*, was detected for all strains by Multiplex PCR using published primers (Pinheiro et al., 2015) and methods (Baba-Moussa et al., 2011). International reference strains were included in all reactions as positive [*S. aureus* SCP FRI 722 (*sea* +), SCP FRI 341 (*seb* +), SCP FRI 361 (*sec* +), SCP FRI 137 (*seg* +), SCP FRI 569 (*seh* +)] while negative controls were provided for all reactions by substituting nucleic acid by water. Three other genes, *S. aureus clp* (clumping factor), *S. aureus hlgB* (encoding gamma-hemolysin) and *gseA* encoding the specific *S. epidermidis* glutamyl-endoprotease gene (Ohara-Nemoto et al., 2002), were also investigated by using PCR (see Suppl. Table 1). Amplification products were separated on a 2% (w/v) agarose gel in TEBx0.5 buffer and stained with 0.5 µg/mL ethidium bromide before being analyzed on a UV bench using a GelDoc 2000 (Bio-Rad).

2.11. Detection of enterotoxin production

Extracellular enterotoxin C from strains positive by PCR investigation (*sec*) were identified by the reverse passive latex agglutination (RPLA) method using the SET-RPLA-T900 kit for the detection of SEA, SEB, SEC as described by the manufacturer (Oxoid Diagnostic Reagents). In addition, Western blotting was achieved by using *S. aureus* enterotoxin C affinity-purified rabbit polyclonal antibodies (Attien et al., 2014) and a fluorescein-labeled goat anti-rabbit secondary antibody.

2.12. Statistical analysis

Data are presented as mean ± standard deviation from three independent experiments performed in triplicate. The $\chi 2$ test was carried out with the Minitab 14 (https://www.minitab.com/academic/) for proportions comparison and the level of significance was set at p values < 0.05 for all tests.

3. Results

3.1. CNS isolates and associated infections

A total of 99 clinically significant and independent CNS were collected over a period of ten months in the NTHC-HKM Microbiology laboratory. Amongst these, 62 were hospital-acquired and 37 were community-acquired strains. The strains were from various biological samples including: blood, urine, genital secretion, abscess and intravascular catheter. As shown in Table 1, the most frequently CNS species isolated in this study were *S. haemolyticus*

Table 1
Occurrence of CNS species isolated from various clinical samples.

Species	Source					
	Blood	Urine	Genital secretion	Abscess	Foreign bodies	Total (%)
S. haemolyticus	32	7	5	0	0	44 (44)
S. epidermidis	16	3	0	2	1	22 (22)
S. hominis	7	0	0	0	0	7 (7)
S. saprophyticus	1	6	0	0	0	7(7)
S. cohnii	4	2	0	0	0	6 (6)
S. sciuri	2	0	1	0	0	3 (3)
S. arlettae	1	1	0	0	0	2 (2)
S. capitis	2	0	0	0	0	2(2)
S. warneri	0	2	0	0	0	2 (2)
S. simulans	0	1	0	0	0	1 (1)
S. caprae	0	1	0	0	0	1 (1)
S. lugdunensis	1	0	0	0	0	1(1)
S. kloosii	0	1	0	0	0	1(1)

(44%) followed by *S. epidermidis* (22%), *S. hominis* (7%) and *S. saprophyticus* (7%). Other species such as *S. cohnii* (6%), *S. sciuri* (3%), *S. arlettae* (2%), *S. capitis* (2%), *S. warneri* (2%), *S. simulans* (1%), *S. caprae* (1%), *S. lugdunensis* (1%) and *S. kloosii* (1%) were also identified. Bacteremia (67%) was the most common CNS infection in this study. The majority of sepsis was observed in the neonatal unit [34 (51%)]. Sixty one (92%) of the 66 bacteremia were classified as hospital-acquired bacteremia while only 5 (7%) were community-acquired. *S. haemolyticus* was the leading cause of bloodstream infections [32 (48%)], followed by *S. epidermidis* [16 (24%)] and *S. hominis* [7 (11%)] (Table 1). Urinary tract infections accounted for 24% of all CNS infections, were associated with adult patients (18–78 years) and were community-acquired (Fig. 1). *S. haemolyticus* was also the most frequent causative agent associated with urinary tract infections [7 (29%)] followed by *S. saprophyticus* [6 (25%)] and *S. epidermidis* [3 (13%)]. Genital infections represented 6% of CNS infections, all of which were community-acquired, and 5 of which (83%) were caused by *S. haemolyticus* (Table 1, Fig. 1). Dermatitis [2 (2%)] and foreign body infections [1 (1%) were the other sources of CNS infections (Fig. 1)]. The species most frequently associated with CNS infections at the Cotonou NTHC-HKM were *S. haemolyticus*, *S. epidermidis*, *S. saprophyticus*, S. *hominis* and *S. warneri*.

3.2. Antimicrobial resistance of CNS

Most of the isolated strains were resistant to penicillin (92%) and fosfomycin (81%), followed by oxacillin (74%), trimethoprim-sulfamethoxazole (72%), cefoxitin (74%), kanamycin (65%), gentamicin (54%) and tobramycin (52%). Resistance remained sparse for nitrofurantoin (1%), pristinamycin (6%), teicoplanin (6%) and rifampicin (6%) (Supp. Table 2a,b). *S. haemolyticus*, *S. epidermidis* and *S. hominis* exhibited the highest resistance profile whereas *S. saprophyticus* remained susceptible to most antibiotics tested. The methicillin resistance rate was the highest for *S. hominis* (100%), *S. haemolyticus* (93%) and *S. epidermidis* (67%). Other less frequently isolated species (*S. arlettae*, *S. capitis*, *S. warneri*, *S. simulans*, *S. caprae*, *S. lugdunensis* and *S. kloosii*) showed low resistance to the majority of antibiotics, except for fosfomycin (81%) and benzylpenicillin (70%) (Supp. Table 2a,b). The rate of methicillin-resistant isolates was also found to be particularly higher among newborns (94%). Fig. 2 shows the antimicrobial resistance in methicillin-resistant CNS (MRCNS) and methicillin-susceptible CNS (MSCNS) isolates. MRCNS were significantly more resistant to other antimicrobial agents compared to MSCNS except for fosfomycin and fusidic acid ($p < 0.001$). Finally, a significant association was found between MRCNS and hospital-acquired strains ($p < 0.001$) (Fig. 3).

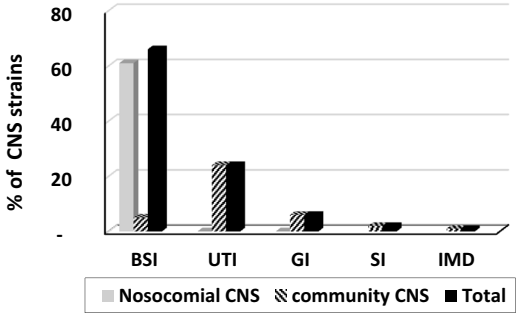

Fig. 1. Distribution of CNS isolates among nosocomial vs. community infections. CNS, Coagulase Negative Staphylococci; BSI, blood stream infections; UTI, urinary tract infections; GI, Genital infections; SI, skin infections; IMD, infections associated with medical devices. Bars represent percentage of strains for each species.

3.3. Extracellular protease and esterase enzyme production

Optical density measurements enabled to rank the 99 strains into four groups: non-proteolytic (OD ≤ 74 mAU; 73 strains), weakly proteolytic (74 mAU < OD ≤ 127 mAU; 5 strains), moderately proteolytic (148 mAU < OD ≤ 295 mAU; 10 strains) and very proteolytic (OD > 295 mAU; 11 strains) (Fig. 4a). Under this classification, 26 of all collected strains had proteolytic activity. Amongst the 17 *S. epidermidis* strains, 7 (41%) were very proteolytic. Only 4 (9%) of the *S. haemolyticus* strains were proteolytic including one very proteolytic. Of the 3 strains of *S. sciuri*, 2 were very proteolytic.

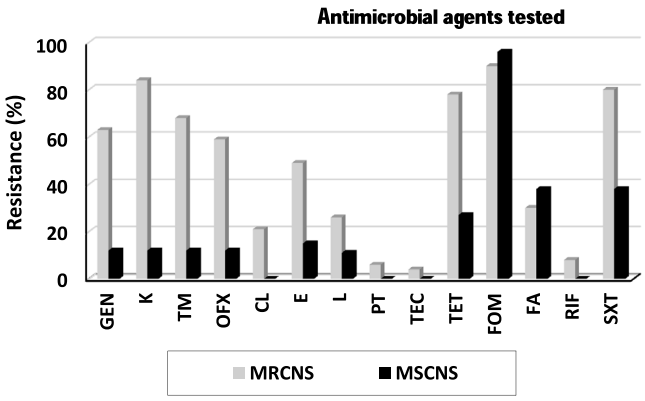

Fig. 2. Antimicrobial resistance patterns in MRCNS and MSCNS isolates. MRCNS; methicillin-resistant coagulase negative staphylococci; MSCNS; methicillin-susceptible coagulase negative staphylococci. GEN, Gentamicin; K, Kanamicin; TM, Tobramicin; OFX, Ofloxacin; CL, Clindamycin; E, Erythromycin; L, Lincomycin; PT, Pristinamycin; TEC, Teicoplanin; TET, Tetracycline; FOM, Fosfomycin; (FA), Fusidic acid; (RIF), Rifampicin; SXT, Trimethoprim-Sulfamethoxazole. Bars represent percentage of strains for each species.

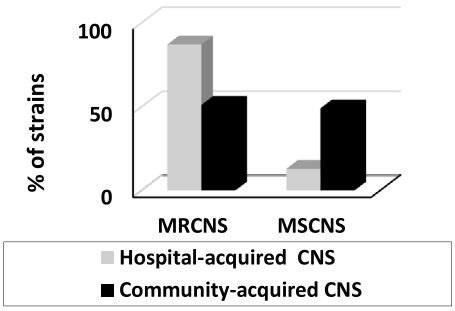

Fig. 3. Distribution of methicillin-resistant and methicillin-sensible CNS according to type of strains. MRCNS; methicillin-resistant coagulase negative staphylococci, MSCNS; methicillin-susceptible coagulase negative staphylococci. Bars represent percentage of strains for each species.

None of the *S. hominis* and *S. haemolyticus* strains produced protease activity. As for the other species, *S. caprae* was very proteolytic.

Esterase activity assayed in agar supplemented with Tween 80 revealed 25 positive strains at 14 to 50% of the control value (Fig. 4b). The highest percentages were found for *S. epidermidis* [n = 11 strains, (50%)] and *S. cohnii* [6 (50%)], followed by *S. haemolyticus* [10 (23%)] and *S. saprophyticus* [1 (14%)]. None of *S. hominis* and *S. sciuri* strains exhibited esterase activity. In the other species, 3 strains (30%) were found to hydrolyze esters.

3.4. Assessment of CNS cytolytic potential on human RBC and PMNs

The distribution of CNS isolates according to their hemolytic activity on human erythrocytes is shown in Fig. 5a. Certain strains produced extracellular compounds that were able to lyse at least 50% of human erythrocytes at 1:4 dilution of the culture supernatants. Of the 99 strains tested, 22 (22%) were hemolytic, with the percentage of hemolysis varying according to species. In the most frequent isolated species, 9 *S. haemolyticus* strains (20%) and 4 *S. epidermidis* strains (18%) were found to be the most hemolytic. High activity (96–73%) was observed for five (56%) of the *S. haemolyticus* positive strains. Only one of the *S. hominis* (14%) and 2 of the *S. cohnii* (33%) isolates were hemolytic. None of the *S. saprophyticus* strains showed any hemolytic activity on HRBCs while five strains (50%) of other species were hemolytic. Except for *S. saprophyticus*, all of the most frequent isolated CNS species were able to induce hemolysis of HRBCs.

Finally, with regard to leukotoxic activity, only 3 strains were considered positive: 1 *S. haemolyticus*, 1 *S. cohnii* and 1 *S. warneri*. All other strains failed to display any leukotoxic activity (Fig. 5b).

3.5. Enterotoxin genes and toxin expression by CNS strains

None of the isolated CNS included in this study carried the enterotoxin *sea*, *seb*, *seg* or *seh* gene. PCR detection for the enterotoxin genes revealed the presence of *sec* genes in two *S. epidermidis* strains (9%) from the 99 staphylococcal strains studied (Fig. 6a). These two isolates (SE90 and SE95) were identified as *S. epidermidis* through mass spectrometry and Biotyper™ with confident scores of 2.07 and 2.09 for isolates 90 and 95, respectively. These isolates were neither positive for the *S. aureus* clumping factor, nor gamma-hemolysins tests, while gamma-hemolysin was previously found in 100% of *S. aureus* isolates (Mahoudeau et al., 1997). However, these two isolates carry the *S. epidermidis gseA* gene, and 16s rDNA amplification confirmed the *S. epidermidis* species identification (Suppl. Fig. 1). Genes were shotgun sequenced through original detecting primers and the corresponding sequences were identical for these two isolates and the already available *S. epidermidis* sequence, whil N-terminal sequence were diverging from *S. aureus* and 9 aminoacid substitutions were noticed inside the polypeptides compared to *S. aureus* again (Suppl. Figs. 2 and 3). Reverse passive latex agglutination (RPLA) assay was performed and confirmed the expression of at least cross-reacting materials by *S. epidermidis* with staphylococcal enterotoxin C (SEC), and further confirmed by Western blotting (Fig. 6a,b). The two enterotoxin C positive *S. epidermidis* originated from blood samples. One of the strains originated from a 2-day-old newborn hospitalized in the neonatal unit for hyperthermia and dyspnea. The second originated from a 12-year-old child hospitalized in a pediatric unit for hyperthermia and digestive disorders. The 2 isolates exhibited distinct antimicrobial susceptibility (tobramicin, gentamicin and erythromycin resistance vs. tobramicin, gentamicin and erythromycin susceptibility) as well as distinct virulence factors (protease+, hemolysin+, esterase- vs. protease+, hemolysin-, esterase+). Amplicon DNA sequencing did not reveal any significant mutation compared with that published by Madhusoodanan et al. (2011).

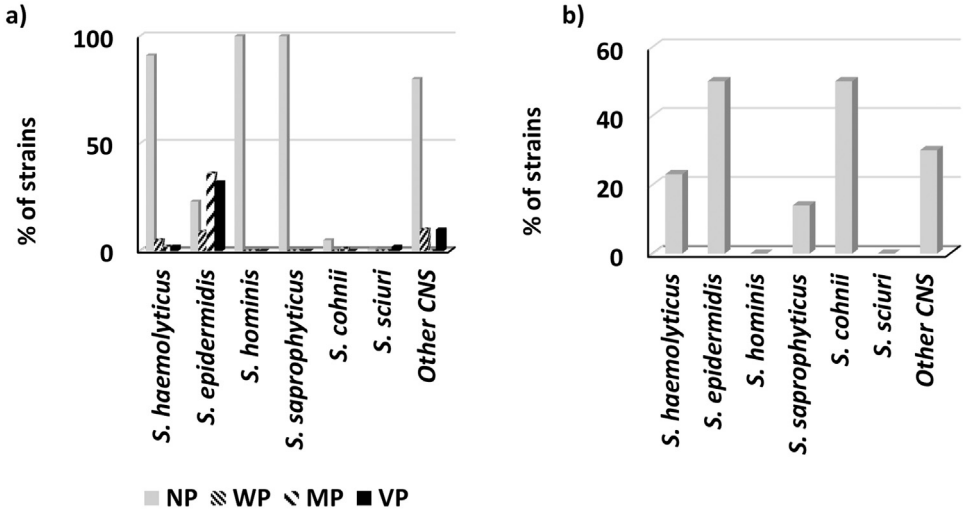

Fig. 4. Extracellular enzyme production by clinical isolates according to species. (a) Proteolytic activity of culture supernatants by CNS isolates. NP; non proteolytic, WP; weakly proteolytic, MP; moderately proteolytic, VP; very proteolytic. Bars represent percentage of proteolytic strains for each species. (b) Esterase production by CNS strains. Bars represent percentage of esterase-producing strains for each species.

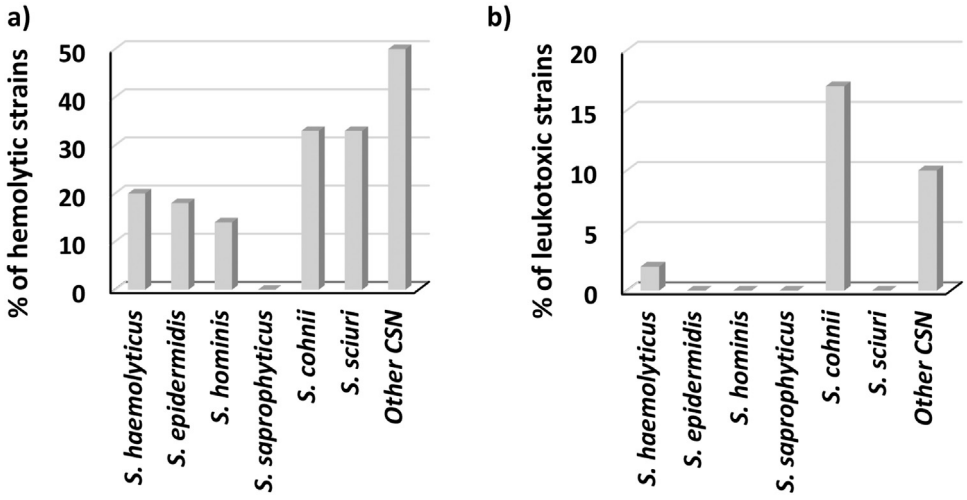

Fig. 5. Cytolytic capacity of CNS isolates on human RBC and PMNs. (a) Hemolytic activity of CNS strains according to species. Bars represent strains percentage of CNS having lysed at least 50% of human erythrocytes up to a quarter dilution of the culture supernatant for each species. (b) Leukotoxic activity of CNS strains according to species. Bars represent strains percentage of CNS having lysed at least 50% of human Polymorphonuclear cells for each species.

3.6. *Virulence factors or functions are more frequently associated with hospital-acquired CNS*

CNS are the major causative agents of nosocomial infections despite the fact that some are widespread skin commensals (Rogers et al., 2009). To explain this phenomenon, we compared the rate of virulence factors between hospital-acquired and community-acquired CNS (Table 2). The results revealed differences between occurrences of protease activity in hospital-associated CNS (65%) compared to community-associated CNS (35%) ($p < 0.001$). With regard to strain toxicity on hRBC and hPMN cells, hemolytic activity was exhibited by 16 (73%) hospital-acquired CNS while 6 (27%) were from community-acquired isolates. Conversely, leukotoxic activity was associated with only community-acquired isolates. The comparison of nosocomial CNS having esterase activity with that of community-acquired isolates demonstrated that the majority of esterase activity was exhibited by nosocomial CNS (64% vs. 36%, $p < 0.001$, Table 2). Two hospital-acquired strains expressed enterotoxigenic capacity (Table 2). Finally, of the 99 CNS screened, 44 (44%) had one or more virulence features, of which 30 (68%) were associated with nosocomial isolates, whereas 14 (32%) stemmed

Table 2
Distribution of virulence features production according to hospital and community-acquired CNS.

Virulence factors	Hospital-acquired CNS	Community-acquired CNS	*p* value
Protease	17 (65%)	9 (35%)	<0.001
Esterase	16 (64%)	9 (36%)	<0.001
Hemolysis	16 (73%)	6 (27%)	<0.001
Leukocytolysis	0 (0%)	3 (100%)	
Enterotoxin	2(100%)	0 (0%)	

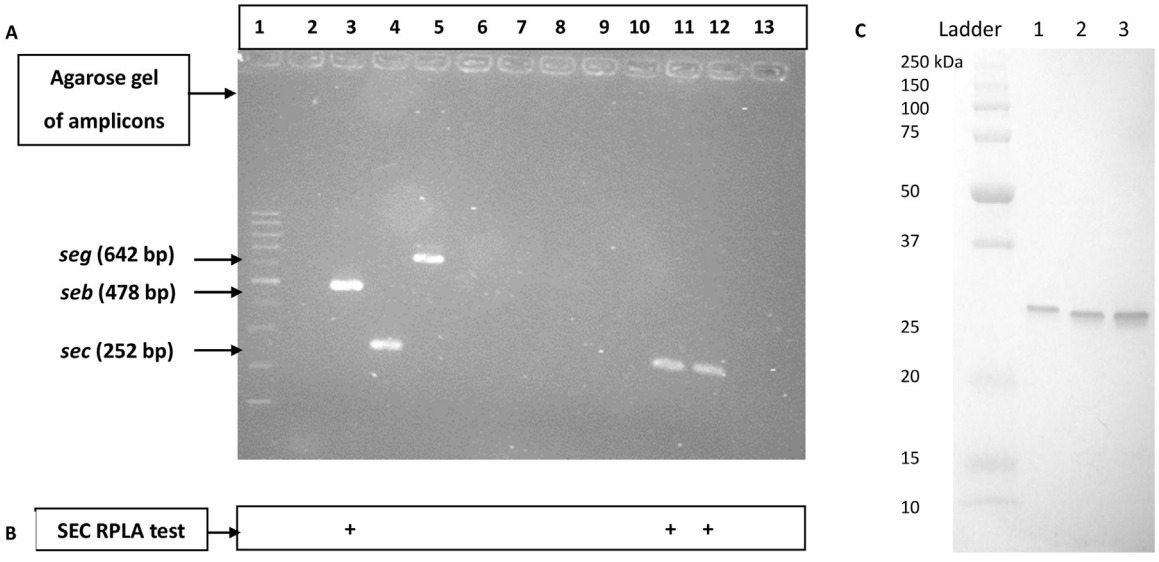

Fig. 6. (A) Agarose gel electrophoresis for the PCR detection of *sec* (252 bp), *seg* (642 bp) and *seb* genes in CNS strains by PCR. Lane 1: 100-bp molecular weight marker, lane 2: negative control, lane 3: *S. aureus* SCP FRI 341 (*seb* +); lane 4: *S. aureus* SCP FRI 361 (*sec* +), lane 5; SCP FRI 137 (*seg* +); lanes 6, 7, 8, 9, 19 and 13: CNS isolates lacking *seb, sec, and seg*; lanes 11 and 12: CNS isolates positive for *sec* gene. (B) Result of reverse passive latex agglutination (RPLA) assay. Lane 4: *S. aureus* SCP FRI 361 positive control for SEC expression. Lanes 11 and 12: CNS isolates positive for SEC expression. (C) Immunoblot of both culture supernatants of *S. epidermidis* isolates using rabbit affinity-purified anti- enterotoxin C antibibodies revealed with a goat anti-rabbit secondary antibody. Lane 1: 5 ng of *S. aureus* purified SEC1; Lanes 2 and 3: 60 ng of culture supernatants from *S. epidermidis* isolates 90 and 95, respectively.

from community-acquired strains, the difference being statistically significant ($p < 0.001$).

4. Discussion

In the present study, *S. haemolyticus* (44%) and *S. epidermidis* (22%) were the most frequent CNS species isolates at the NTHC-HKM in Cotonou, Benin. A study in Nigeria investigating the species distribution from 105 CNS isolates found similar incidences *for S. haemolyticus* and *S. epidermidis* (Azuka and Idahosa, 2013). However, we also detected other species not identified in the Azuka et al. study, including *S. cohnii*, *S. sciuri*, *S. arlettae*, *S. capitis*, *S. warneri*, *S. caprae*, *S. lugdunensis* and *S. kloosii*. The combined use of API STAPH and MALDI-TOF herein enabled us to identify new CNS species and could prove of interest for clinicians as some of these previously neglected species can be highly pathogenic such as *S. lugdunensis* (Argemi et al., 2015).

The results of our antibiotic susceptibility testing revealed multidrug resistance of African CNS as previously described in higher income countries (Dominguez et al., 2002; Mohan et al., 2002). Herein, the highest resistance rates were observed for oxacillin (74%), fosfomycin (81%), trimethoprim-sulfamethoxazole (72%), cefoxitin (74%), kanamycin (65%), gentamicin (54%) and tobramycin (52%). Similar results have also been reported in a study encompassing the United States, Canada, Latin America, Europe, and the Western Pacific region (Diekema et al., 2001). Methicillin resistance (MR) still remains on the rise, especially in hospitalized patients thus complicating their treatment (Monsen et al., 2005). The most frequently isolated species in the present study were also the most resistant to methicillin with rates of 100% for *S. hominis,* 93% for *S. haemolyticus* and 67% for *S. epidermidis*. This suggests that CNS even in Benin could function as a reservoir and a source of antimicrobial resistance genes possibly transferred horizontally from CNS to *S. aureus* (Otto, 2013).

Several studies have described putative virulence factors including exoenzymes and exotoxins that could play a role in their pathogenicity as shown for *S. epidermidis* (Otto, 2012). Herein, as many as 26% of the strains displayed proteolytic activity and 25% were positive for esterase activity. Among the most frequently isolated strains, *S. haemolyticus* and *S. epidermidis* were exoenzymes producers. However, some *S. cohnii* and *S. saprophyticus* isolates also showed esterase activity. The ability of some pathogenic CNS strains to produce extracellular enzymes could allow them to damage host tissues and impair innate immunity, thus promoting their spreading. As an example, the *S. epidermidis* elastase that degrades human IgM, serum albumin, fibronectin and fibrinogen is assumed to be a virulence factor (Otto, 2004). Most previous studies on the hemolytic activity of clinically significant CNS have been based on blood agar containing rabbit blood or sheep blood (Gunn and Davis, 1988), which does not reflect the specific effects of hemolytic strains stemming from humans. In the current study, we quantitatively screened strains for their hemolytic activity on human erythrocytes and obtained a positive reaction for 22% isolates, suggesting the production of extracellular hemolysins, of which *S. haemolyticus* (20%) and *S. epidermidis* (18%) were the most hemolytic.

Staphylococcal enterotoxins (SEs) are superantigens (Casman, 1960; Bergdoll and Surgall, 1959; Bergdoll et al., 1965; Casman et al., 1967; Katsuhiko et al., 2005) that stimulate the immune system to produce an exaggerated inflammatory response, causing cytokine release, clonal expansion and clonal deletion of part of these lymphocytes via apoptosis (Taylor and Llewelyn, 2010; Ortega et al., 2010). In contrast to *S. aureus* that often produces at least one superantigen, superantigen-producing CNS strains remain scarce (Becker et al., 2014). Da Cunha et al. (2007) have reported the presence of *sea*, *seb*, *seg* and *seh* genes in various CNS species isolated from clinical samples obtained from hospitalized newborns as well as a high rate of *sec* genes specifically in *S. epidermidis* from blood (28/50). Nevertheless, the methodology used in this study for staphylococci identification appeared insufficient to confirm that the presence of *sec* genes was not partially due to *S. aureus* misidentification. Expression of these genes was not demonstrated too. While to date pertinent data on the secretion of detectable amounts of enterotoxin or enterotoxin-like products is lacking for CNS isolates, we did found in our study that 2 properly identified *S. epidermidis* strains produced SEC enterotoxin. A recent study in the United States examined 128 CNS (118 *S. epidermidis* and 10 *S. lugdunensis*) isolates from healthy human nares and diseased individuals, and determined that no known enterotoxin gene was present (Christopher et al., 2015). Enterotoxins *sec*3 and *sel*l carried on a pathogenicity island (SePI) were exceptionally described in a *S. epidermidis* strain (FRI909) (Madhusoodanan et al., 2011). All of the aforementioned observations hence indicate a low, but insufficiently determined prevalence of *sec* gene in *S. epidermidis*. In our context, the two strains were isolated from nosocomial bacteremia in pediatric and neonatal units and presented distinct antimicrobial resistance and virulence profiles. These *sec* gene acquisitions are likely the result of independent genetic events that need to be confirmed, since both protein and gene sequences of African strains appeared strictly comparable with the previously reported one in United States of America. Our study also revealed that 68% of virulence features were associated with nosocomial CNS, while only were observed in 32% of the community-acquired strains.

In addition to these data on the variability and antimicrobial susceptibility of CNS strains in Benin (Africa), the present study reveals the presence of virulence features such as protease, esterase, hemolysin and enterotoxins particularly in hospital-associated CNS strains isolated from blood, suggesting that CNS pathogenicity provides a selective advantage for blood colonization in hospitalized patients. Further studies will be conducted on genotyping and biochemical characterization of virulence factors of *S. epidermidis* and *S. haemolyticus*.

Conflict of interest

None.

Acknowledgments

We thank Dr P. Pothier for English reading. We thank research grant from EA7290 (University of Strasbourg) and the Islamic Bank for Development, and the latter also for the doctoral award to Chimène NANOUKON.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2016.11.001.

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