

# Antibiotics Resistance Profile of *Staphylococci* Isolated from Urogenital Infections and Toxins Production of *Staphylococcus aureus* Strains

Haziz Sina<sup>1\*</sup>, Josiane Adjobignon Semassa<sup>1</sup>, Victorien Tamègnon Dougnon<sup>2</sup>, Aurelle A Adjilé<sup>1</sup>, Farid Baba-Moussa<sup>3</sup>, Honoré S. Bankolé<sup>4</sup> and Lamine Baba-Moussa<sup>1</sup>

<sup>1</sup>Laboratoire de Biologie et de Typage Moléculaire en Microbiologie, Faculté des Sciences et Techniques, Université d'Abomey-Calavi, 05 BP. 1604, Cotonou, Benin; <sup>2</sup>Research Laboratory in Applied Biology, Polytechnic School of Abomey-Calavi, University of Abomey-Calavi, Benin; <sup>3</sup>Laboratoire de Microbiologie et des Technologies Alimentaires, Faculté des Sciences et Techniques (FAST), Université d'Abomey-Calavi, 01 BP: 2009 Cotonou, Benin; <sup>4</sup>Laboratoire de Recherche en Biologie Appliquée (LARBA), Ecole Polytechnique d'Abomey-Calavi (EPAC), Université d'Abomey-Calavi, 01 BP. 2009 Cotonou, Bénin

## Corresponding author:

Haziz Sina, Laboratoire de Biologie et de Typage Moléculaire en Microbiologie, Faculté des Sciences et Techniques, Université d'Abomey-Calavi, 05 BP. 1604, Cotonou, Benin, Tel: (+229) 9564450; E-mail: sina\_haziz@yahoo.fr

## Abstract

**Aim:** The aim of this study was to investigate the antibiotic resistance of *staphylococci* and seek toxin production by *Staphylococcus aureus* strains isolated from urogenital infections. **Material and Methods:** The *staphylococci* strains were isolated from urogenital samples collected from hospitalized patients or not. The antibiotic susceptibility was performed by the diffusion method and the search of production of toxin by *S. aureus* was done by radial immunoprecipitation technique. **Results:** Out of the 1904 samples analyzed, 80 *staphylococci* strains were isolated. The major (70%) part of the positive samples were coagulase-negative *staphylococci* composed of *S. saprophyticus* (50.0%), *S. epidermidis* (16.25%), *S. xylosus* (2.5%), and *S. haemolyticus* (1.25%). *S. aureus* was the unique coagulase positive strains. It was observed a multi-resistance of the isolated strains to beta-lactams, aminoglycosides, tetracycline, and co-trimazole. All the *S. haemolyticus* and *S. xylosus* strains were resistant to methicillin. Nitrofurantoin was the most active molecule in all kind of strains. There was no methicillin-resistant *S. aureus* producing Panton-Valentine Leukocidin (PVL) detected but all the *S. aureus* producing PVL were community methicillin-sensitive *S. aureus*. Most of the tested strains produced ETB (83.33%) and ETA (45.33%). **Conclusion:** The presence of multidrug resistance *staphylococci* strains producing toxins indicate an existence of potential reservoir of virulent antibiotics resistance stains in the community.

**Keywords:** Urogenital infections; Antibiotic resistance; *Staphylococcus*; PVL; Exfoliatin; MRSA; Benin

## Introduction

Bacteria are present on our skin, in our mucous membranes and although the vast majority of them are beneficial to us, there are also pathogens. <sup>[1,2]</sup> To control these pathogenic bacteria, antimicrobial agents are often used since 1940 without clearing those bacteria. Indeed, bacteria develop several strategies (natural and/or acquired) to overcome antibiotics effects. <sup>[1]</sup> However, the acquired resistance is the most spread in bacterial and has now become a global phenomenon. <sup>[3]</sup>

The acquired mechanisms of resistance to antibiotics include the antibiotic target modification mechanism especially observed in Gram-positive cocci. <sup>[4,5]</sup> Among these target modification mechanisms, the production of a new penicillin-binding protein (PBP) such as PBP2a or PBP2' with little affinity for beta-lactams and leading to resistance to methicillin is distinguished. <sup>[6,7]</sup> The emergence of resistant *staphylococci* strains to beta-lactams by this mechanism is therefore of concern since the beta-lactam antibiotics are considered very efficacy with few side effects. <sup>[1,8]</sup>

Apart from antibiotic resistance, *Staphylococci*, especially *S.*

*aureus*, also have a high ability to produce virulence factors such as Panton and Valentine Leucocidin (PVL), exfoliatins (ETA and ETB), and so on. PVL is a two-compound toxin (synergistic action of two LukS-PV and LukF-PV compounds) belonging to the family of pore-forming toxins whereas exfoliatins are epidermolytic toxins. <sup>[9]</sup> The prevalence of staphylococcal strains producing PVL is constantly increasing worldwide. <sup>[10]</sup> More worryingly, we have the worldwide emergence of toxinogenic and methicillin-resistant strains of *S. aureus*. <sup>[11]</sup>

*Staphylococci* including coagulase positive (*S. aureus*) and Coagulase negative (*S. saprophyticus*, *S. epidermidis*, etc.) <sup>[12]</sup> are the second class of bacteria isolated from urogenital infections and particularly urinary tract infections, occupy an important place in the nosocomial and community acquired

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infections.<sup>[13]</sup> Indeed, urinary tract infections are a major public health problem and the consequences of untreated or poorly treated urogenital infections causing cystitis, abortion, sterility and even death.<sup>[14]</sup>

In West Africa, staphylococcal infections are widespread and are often associated with urogenital infections<sup>[15]</sup> In Benin, the place of urogenital infections is no less important because apart from Sexually Transmitted Diseases, they represent 2.1% of the main diseases. Studies have been carried out on the antimicrobial resistance of *S. aureus* strains isolated from urinary and/or genital infections and their toxins production. Unfortunately, to date very few studies have taken into account both *S. aureus* and Coagulase negative *Staphylococcus*.<sup>[16]</sup> In order to fill these gaps and to monitor the evolution of bacterial resistance, this study was initiated to study the antibiotic resistance of *staphylococci* isolated from urogenital infections and investigate the production of toxins by *S. aureus*.

## Material and Methods

### Sample collection

Samples of 1904 hospitalized or untreated patients were collected at the National University Hospital of Cotonou (Benin) for four month (from November 2012 to March 2013) various bacteriological screenings in routine. Those samples were composed of urine (1036), sperm (292), urethral (244) and vaginal (332) samples. All the samples were collected following the standard recommendations of each sample.<sup>[17,18]</sup> Once collected, the samples were stored at 4°C until microbial analysis were performed for the research of *Staphylococcus* strains.

### *Staphylococcus* strains identification

Standard microbiological methods for identification of microorganisms were applied<sup>[19]</sup> Briefly, all swabs were inoculated onto mannitol salt agar, incubated at 37°C and inspected. Any suspected colony was subcultured on Mueller-Hinton agar (bioMérieux, Marcy l'Etoile, France) and identified by subsequent Gram staining, catalase test, DNase test, susceptibility to novobiocin test and Slidex Staph Plus (bioMérieux, Marcy l'Etoile, France) and the coagulase test with the rabbit plasma<sup>[20]</sup> Finally, bacterial identification was completed by API Staph (bioMérieux, Marcy l'Etoile, France).

### Antibiotics susceptibility

The susceptibility of the identified *Staphylococcus* strains to 19 antibiotics molecules was determined by the disc diffusion method of Kirby-Bauer on agar Mueller-Hinton (bioMérieux, Marcy l'Etoile, France) as recommended by the Antibiogram Committee of the French Microbiology Society.<sup>[21]</sup> After incubation time (18-24 h) at 37°C, inhibition zone was measured using graduated slide ruler applied in contact with the rear face of the petri dish. For susceptibility to oxacillin, inoculum of 10<sup>7</sup> CFU/ml was prepared and the plate was incubated at 37°C for 24 h on Mueller-Hinton agar + 3% NaCl.

The 19 tested antibiotics provided by Biomérieux (Marcy l'Etoile, France) were: Amoxicillin (AMX 30 µg), Oxacillin

(OXA 5 µg), Penicillin G (PENI G 6 µg), Amoxicillin + clavulanic acid (AMC 20/10 µg), Cefoxitin (FOX 30 µg), Gentamicin (GM 10 UI), Tobramycin (TM 10 µg), Kanamycin (K 30 µg), Netilmycin (NET 30 µg), Erythromycin (ERY 5 µg), Spiramycin (SP 100 µg), Pristinamycin (PT 15 µg), Lincomycin (LIN 10 µg), Ciprofloxacin (CIP 5 µg), Nitrofurantoin (FT 300 µg), Cotrimazol (COT 25 µg), Chloramphenicol (C 30 µg), Tetracycline (TE 30 µg), and Doxycycline (DO 30 UI).

### Toxin production

The capability of the isolated *S. aureus* strains to produce Pantone-Valentine Leukocidin (PVL) and epidermolysins A (ETA) and B (ETB) was performed phenotypically by radial gel immuno-diffusion. Thus, those toxins (PVL, ETA and ETB) were evidenced from culture supernatants after 18-24 h of growth in Yeast Casamino-acid Pyruvate broth medium<sup>[22]</sup> by radial gel immuno-diffusion in 0.6% (wt/vol) agarose with component-specific rabbit polyclonal and affinity-purified antibodies.<sup>[23,24]</sup>

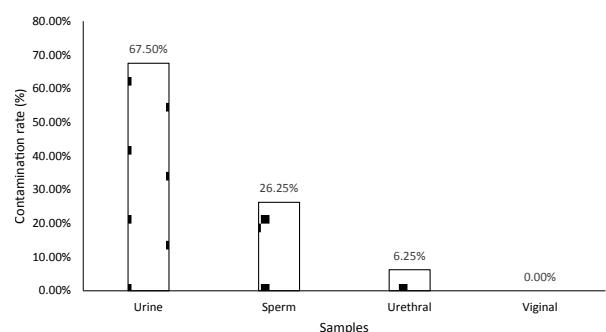
### Statistical analysis

Microsoft Office Excel 2007 spreadsheet was used for statistical data processing. The SPSS (V 16 2007) and Graph Pad Prism 5 software was used to determine whether there is a relationship between the characters being compared. Thus, for comparison tests of positive isolates of each patient group, we used the Student T test, and the Fischer's test for lower number series. P<0.05 was considered statistically significant.

## Results

### Samples contamination

A total of 80 *Staphylococcus* strains were isolated mostly (95%) from non-hospitalized (community) patients sample. Those strains were isolated from urine (67.5%), sperm (26.25%), and urethral samples (6.25%). The vaginal samples were free of *Staphylococcus* strains [Figure 1]. The urine samples containing the *Staphylococcus* strains were at 61% collected from females.



**Figure 1:** Distribution of *Staphylococcal* contamination rate according to samples.

Five different staphylococcal species were isolated at different rate [Table 1] and depend on the samples (P<0.001). Among the isolated staphylococcal strains, only *S. aureus* (50.0%) and *S. saprophyticus* (50.0%) were hospital acquired whereas in the community, all five species were represented. The distribution of *Staphylococcus* species according to the origin of the samples was random [Table 2].

**Table 1: Distribution of *Staphylococcus* strains according to species and collection site.**

Samples	Urethral	Sperm	Urine	Total
<i>Staphylococcal</i> strains	% (n)	% (n)	% (n)	% (n)
<i>S. aureus</i>	80% (04)	4.76% (1)	35.19% (19)	30% (24)
<i>S. epidermidis</i>	20% (01)	23.81% (7)	12.96% (7)	16.25% (13)
<i>S. saprophyticus</i>	00% (00)	61.91% (13)	50.0% (27)	50% (40)
<i>S. haemolyticus</i>	00% (00)	00% (00)	1.85% (1)	1.25% (1)
<i>S. xylosus</i>	00% (00)	9.52% (2)	00% (00)	2.5% (2)
Total	6.25% (5)	26.25% (21)	67.5% (54)	100% (80)

p<0.001

**Table 2: Distribution of *Staphylococci* strains according to the species and sample origin.**

Variables	<i>S. aur</i>	<i>S. epi</i>	<i>S. sap</i>	<i>S. hae</i>	<i>S. xyl</i>	Total
Hospital acquired	50.0% (02)	00% (00)	50.0% (02)	00% (00)	00% (00)	05.0% (04)
Community acquired	28.95% (22)	17.11% (13)	50.0% (38)	01.31% (01)	02.63% (02)	95.0% (76)
Total	30.0% (24)	16.25% (13)	50.0% (40)	01.25% (01)	02.5% (02)	100% (80)

*S. aur*: *S. aureus*; *S. epi*: *S. epidermidis*; *S. sap*: *S. saprophyticus*, *S. hae*: *S. haemolyticus*; *S. xyl*: *S. xylosus*

### Susceptibility to antibiotics

Penicillin G was inactive on all *Staphylococcus* species isolated with a 90% resistance rate for *S. saprophyticus* and 100% for the other four species. The proportion of resistance to methicillin was 54.17% (*S. aureus*), 52.50% (*S. saprophyticus*), 38.46% (*S. epidermidis*) and 100% (*S. haemolyticus*, *S. xylosus*). Amoxicillin and amoxicillin + clavulanic acid were also inactive on the five *Staphylococcus* species but with lower resistance levels for amoxicillin + clavulanic acid.

All the aminoglycoside (gentamicin, kanamycin, tobramycin, and netilmicin) were globally inactive on the five *Staphylococcus* species with at least more than half of the strains of each species resistant to these molecules. *S. epidermidis*, *S. haemolyticus*, and *S. xylosus* were more resistant to aminoglycosides with KTG-phenotypic than *S. saprophyticus* (78.43%).

Macrolides and especially Lincosamides and Streptogramins were more active on the other four species. The lowest resistance levels for pristinamycin were recorded with *S. xylosus* (00%) and *S. epidermidis* (7.7%). Ciprofloxacin was inactive on the

five *Staphylococcus* species with the lowest (47.50%) rate of resistance observed on *S. saprophyticus* and the highest (100%) with *S. xylosus*. Chloramphenicol maintained good activity on *S. haemolyticus* and *S. xylosus* (100%) and *S. epidermidis* (15.39% resistance) and was less active (>40%) on *S. aureus* and *S. saprophyticus*. All *staphylococci* strains were resistant to tetracycline and doxycycline and the most active molecule on all five *Staphylococcus* species was nitrofurantoin [Table 3]. Thus, the resistance levels of the five *Staphylococcus* species depended on the types of antibiotics used (P<0.001).

More than half (54.17%) of *S. aureus* strains were resistant to methicillin. Among the methicillin resistant *S. aureus* (MRSA), 69.23% were isolated from urine, 23.08% isolated from urethral and 07.69% from sperm [Figure 2]. Regarding the origin of MRSA, 92.31% of them were community-acquired.

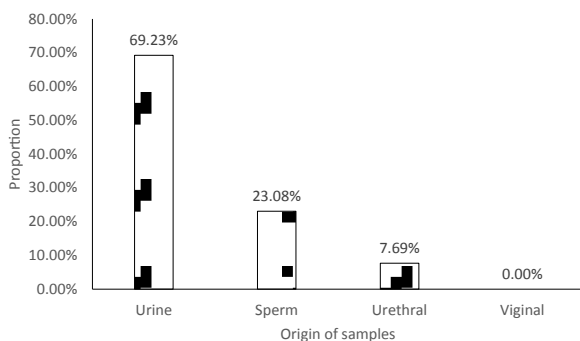
### Toxins productions

In our study, 26.32% of *S. aureus* strains isolated from the urine were LPV-producing; no strain of LPV-producing *S. aureus* was detected in urethral and sperm samples. Concerning the

**Table 3: Resistance profile of *Staphylococcal* strains isolated from urogenital infections.**

Antibiotics	<i>S. aureus</i> (n=24)	<i>S. saprophyticus</i> (n=40)	<i>S. epidermidis</i> (n=13)	<i>S. haemolyticus</i> (n=1)	<i>S. xylosus</i> (n=2)
Penicillin G	100%	90.00%	100%	100%	100%
Oxacillin	58.33%	57.50%	38.46%	100%	100%
Céfoxitine	54.17%	52.50%	38.46%	100%	100%
Amoxicillin	83.33%	70.00%	92.31%	100%	100%
Amoxicillin + clavulanic acid	58.33%	52.50%	53.85%	100%	100%
Gentamycin	54.17%	55.00%	76.9%	100%	100%
Kanamycin	75.00%	55.00%	84.61%	100%	100%
Tobramycin	66.67%	55.00%	76.92%	100%	100%
Netilmicin	54.17%	55.00%	69.23%	100%	100%
Erythromycin	50.00%	60.00%	15.39%	100%	00%
Spiramycin	45.8%	3500%	30.77%	100%	00%
Lincomycin	25.00%	30.00%	15.39%	100%	50.00%
Pristinamycin	20.83%	22.5%	7.7%	100%	00%
Ciprofloxacin	54.17%	47.50%	61.54%	100%	50.00%
Chloramphenicol	50.00%	40.00%	15.39%	00%	00%
Tetracycline	83.33%	82.50%	100%	100%	100%
Doxycycline	87.50%	77.50%	100%	100%	100%
Cotrimazole	50.00%	85.00%	92.31%	100%	100%
Nitrofurantoin	4.17%	00%	00%	00%	00%

enterotoxins, ETB was produced by 83.33% of the *S. aureus* strains whereas about 45.33% produced ETA. Both ETA and ETB were produced by 33.33% of *S. aureus* strains. Regarding the type of sampling, ETA was produced by *S. aureus* isolated from urethral samples (25%) and urinary (52.63%). All the *S. aureus* strains isolated from the urethral and sperm samples produced ETB [Table 4]. The production of toxins by *S. aureus* was not a function of the type of sampling.



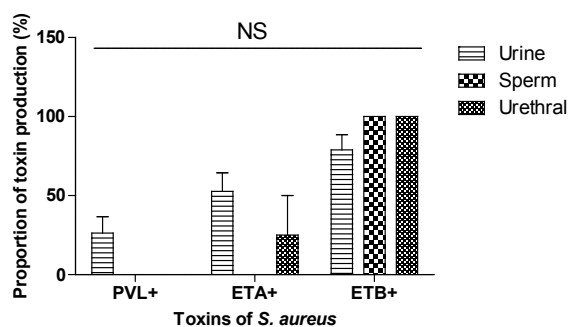
**Figure 2:** Distribution of methicillin resistant *S. aureus* according to the type of sampling.

**Table 4: Frequency of MRSA and MSSA producing LPV, ETA and ETB.**

Variables	MSSA (n=11)	MRSA (n=13)	P (Chi 2)	Significance
PVL+	45.45%	0%	0.006	***
ETA+	54.55%	38.46%	0.431	NS
ETB+	72.73%	38.46%	0.200	NS

NS: Non-Significant; +: Presence, \*\*\* Highly significant.

None of the MRSA strain was LPV-producing whereas 45.45% of MSSAs were LPV-producing. ETA was produced at different level by MSSA (54.55%) strains and of MRSA (38.46%), while ETB was highly produced MSAS (72.73%). The level of *S. aureus* producing PVL was a function of resistance to or not to methicillin [Figure 3].



**Figure 3:** Distribution of *S. aureus* strains producing toxins according to the type of sample.

## Discussion

Among the analyzed vaginal samples, there was no pure or predominant culture of *staphylococci*. Previous studies reported that the main germs involve in the female genital infections are most often *Chlamydiae trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Gardenella vaginalis* and/or yeasts. [25] Staphylococcal strains of urinary origin were predominant (67.50%) and were derived from 61% of female patients, compared with 39% of male patients. Indeed, urine hasn't any antimicrobial property, and can therefore be an excellent culture medium for bacteria growth. In addition, urinary tract

infections (such as cystitis) are five times more common in women than in men because of the clothing habits and also of the proximity of the anal and vulvar regions. [14] The major part of the isolated *staphylococci* was coagulase-negative. This confirms the increasing role of coagulase-negative *staphylococci* (considered commensal) in staphylococcal infections. [16] In urinary infections, the predominant species in our study was *S. saprophyticus* (50%), which is comparable to the results obtained in Morocco. [13] In sperm, the coagulase-negative *staphylococci* were predominantly present with *S. saprophyticus* as the predominant species (61.91%). Thirty percent of the isolated strains were *S. aureus* mostly (35.19%) isolated from the urine. This proportion of *S. aureus* from urogenital origin is not far from the 38% reported in Morocco and Mauritania. [26] Probably due to the samples size and the adaptation of bacteria, the urine contamination rate we observed seems very higher than the 15.2% obtained in Benin by Baba-Moussa et al. [15]

The resistance profile of the *staphylococci* isolated strains varied according to the 19 antibiotics tested in our study [Table 3]. So, considering beta-lactams, penicillin G has been inactive on all *staphylococcus* species. Methicillin resistance level varies from staphylococcal strains to another. Thus, all the *S. haemolyticus* and *S. xylosus* were resistant to that molecule whereas 54.17%, 52.50% and 38.46% of resistance were recorded respectively for *S. aureus*, *S. saprophyticus*, and *S. epidermidis*. Thus, to limit the presence of falsely positive methicillin resistance strains, it is useful to combine oxacillin to cefoxitin or latamoxef. [27] Amoxicillin and amoxicillin + clavulanic acid were also inactive on the five *Staphylococcus* species but with lower resistance levels for amoxicillin + clavulanic acid, and coagulase-negative *staphylococci* are naturally susceptible to beta-lactams. [28] In addition to beta-lactam antibiotics, four other antibiotics used fairly in Benin (ciprofloxacin, tetracycline, cotrimazole and doxycycline) were inactive on all five *Staphylococcus* species. However, *staphylococci* are reported to be naturally sensitive to fluoroquinolones. [29-31] A multi-resistance of *S. aureus* and coagulase-negative *staphylococci* observed in our study is consistent with that reported in other studies in Africa. [28,32] This result could be justified by the fact that in Cotonou, as elsewhere in Africa, several antibiotics molecules are available at affordable prices and are usually delivered without any medical prescription in pharmacies or are found in parallel markets for fake medicines. This sad reality promotes self-medication, over-consumption of antibiotics and consequently increased selection of resistance bacteria.

All the aminoglycosides used in our study were globally inactive on the five staphylococcal species and 83.33% of resistant strains to methicillin were of Kanamycin-Tobramycin-Gentamycin phenotype though the coagulase-negative *staphylococci* strains are reported to be naturally sensitive to aminoglycosides. [29] This high resistance can be explained by the fact that aminoglycosides and gentamicin in particular constitute the antibiotic of choice for the treatment of severe staphylococcal infections and that are increasingly available at very affordable prices. *Macrolides-Lincosamides-Streptogramines* (MLS) were more active on four species (*S. saprophyticus*, *S. epidermidis*, *S.*

*xylosus* and *S. aureus*). The constitutive MLSB phenotype was found in 28.75% of all *staphylococci* resistant to methicillin. Indeed, MLS, because of their high cost are still less accessible for the majority of population and this may explain the good activity of these antibiotics on our strains. The most active molecule on the *staphylococcus* species identified during our study was nitrofurantoin with a resistance rate of 00% for *S. saprophyticus*, *S. epidermidis*, *S. haemolyticus* and 4.17% for *S. aureus*. Our data thus, confirms the efficacy of this antibiotic in the treatment of urinary and/or genital infections as previously reported.<sup>[33]</sup> Thus, patient are strongly invited not to use any molecules for the control of infection if not, the *staphylococcus* involve in the urogenital infection will be more resistant. The reduction of the increase resistance among *Staphylococcus* in particular and globally all the bacterial strains must include all the actors (patient, practitioners, pharmacists and political authorities). The phenomenon is not only a burden for the patient and his family but also for the nation. Then, the populations must be made aware of the dangers resulting from their practices towards to the uses of drugs.

In our study, the rate of 54.17% of methicillin resistant *S. aureus* (MRSA) is close to the 50-57% reported in Senegal,<sup>[34]</sup> but significantly higher than the 1% observed in the Netherlands and Scandinavian countries,<sup>[35]</sup> and the 7.6% in Morocco.<sup>[26]</sup> Thus we can say that the frequency of MRSA varies greatly across countries. This remarkable resistance observed of *S. aureus* to methicillin can be explained by the consumption increasingly important and especially without medical opinion of this antibiotic by the population. This situation is not observed in the developed countries where the acquisition of any antimicrobial drug is certified by a medical prescription. Most (92.31%) of MRSA were isolated from the community. This rate is higher than the overall estimate of 30-40% found by some authors.<sup>[36]</sup> This is not surprising, as over the past two decades, cases of MRSA infections contracted outside the hospital have been regularly reported.<sup>[37]</sup> The high rate of community-acquired MRSA in Benin may be due to the dissemination of hospital-based MRSA outside hospitals through inpatient (or companion) patients through the exchange of these strains within the community or during the home care. There a need to reduce this kind of contamination to reduce dissemination of resistance and pathogenic bacteria in the community. Methicillin resistance of the 13 strains of *S. aureus* was a crossed resistance with all beta-lactams, confirming previous work.<sup>[38,39]</sup> Approximately ninety-two percent (92.31%) of these MRSA displays KTG phenotype. This is normal because more than 90% of MRSA are reported to be resistant to kanamycin, gentamicin and tobramycin.<sup>[28]</sup> In addition, about 40% of our MRSA were constitutive MLSB and 15.38% MLSB inducible. Nitrofurantoin was the only 100% active molecule on both SASM and MRSA and therefore remains an alternative in the treatment of urogenital infections.

Regarding the production of toxins, during our study, 26.32% of *S. aureus* strains all of urinary origin were PVL-producing. This rate is statistically different and slightly higher than the 21.4% found in Benin and France.<sup>[15]</sup> These *S. aureus* producers of PVL in our study were all community-based MSSA. This is

not surprising because MSSAs (clonal complex CC121) LPV + are known to be common in many countries over the world.<sup>[40-42]</sup> Indeed, luk-PV genes can be incorporated into *S. aureus* lines by horizontal transfer, either before or after the acquisition of the *mecA* gene.<sup>[43,44]</sup> In this study, obtaining MSSA LPV + (45.45%) should draw attention to the existence of potential reservoirs of resistance and virulence factors that could lead to the emergence and spread of other MRSA LPV + in Benin. Our study showed that exfoliatins ETB (83.33%) was the most produced by *S. aureus* strains and then ETA (45.33%). This high production of exfoliatins, especially of ETB in urogenital affections, seems exceptional since exfoliatins release *S. aureus* toxins that are associated with well-established specific diseases (generalized exfoliation syndrome, bullous impetigo, etc.).<sup>[45]</sup> The question that may arise is whether exfoliatins are directly associated with urogenital infections or not.

## Conclusion

Urinary-genital and particularly urinary infections are a growing concern in public health. In our study, a high proportion of coagulase-negative *staphylococci* were found. A high proportion of strains of multidrug-resistant *staphylococci* including beta-lactams, aminoglycosides, tetracycline, and cotrimazole were also obtained. This merely reflects the state of progress of the acquired resistance of these germs to antibiotics. Methicillin-resistant *Staphylococcus aureus* was resistant to almost all antibiotics except lincomycin and pristinamycin (for some) and nitrofurantoin (for the whole), confirming the emergence in the community of strains of multi-resistant MRSA. No cases of LPV-producing MRSA (LPV +) strains were detected, but all were community-acquired strains of *Staphylococcus aureus* sensitive to methicillin and LPV-producing, potential reservoirs of MRSA LPV +.

## Conflict of Interest

All authors disclose that there was no conflict of interest.

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