Prevalence of MRSA and Panton-Valentine Leukocidin-Positive strains identified at the trauma service of Tangier Morocco

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Abstract

The epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) in Morocco is poorly documented. The aims of the prospective study were to describe the incidence, microbiologic profiles of patients with both methicillin-sensitive *S.aureus* (MSSA) and MRSA, and to investigate the prevalence of *Staphylococcus aureus* infections carrying Panton-Valentine leukocidin (LPV) genes. From August 2008 to August 2011, eighty *S. aureus* were collected at the trauma service of Tangier's Mohamed V Provincial Hospital. Bacterial identification was based on standard methods and susceptibilities were tested by disk diffusion method. MICs to vancomycin were determined by the broth microdilution. Molecular study (16S RNA, nuc, mecA, and toxin PVL) was determined by multiplex PCR. Multiplex PCR analysis showed that mecA gene was present in 8 (10%) of 80 *S. aureus*. MRSA were predominantly multiantibiotic-resistant hospital-acquired. We noted a high resistance to fluoroquinolones, fusidic acid and tetracycline by 100%, 75%, and 75% respectively. Decreased susceptibility to vancomycin was not detected. 10% of the *S. aureus* isolates carried genes for LPV. Epidemiological data identified that PVL was associated with truly community-acquired disease CA-MSSA ($p<0.001$), younger age ($p<0.05$) and presentation with skin and soft-tissue infections ($p<0.001$). Compared with various countries of the world, our results show a low rate of MRSA and MSSA PVL positive. However, given the genetic plasticity of this species, caution is required and a monitoring system must be set up.

Key words: MRSA; MSSA; LPV; Community-acquired; Hospital-acquired.

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Introduction

The Gram-positive bacterium *Staphylococcus aureus* is the causative agent of human skin and soft tissue infections, invasive disease and bacteremia, in healthcare and community settings [1].The overall burden of staphylococcal diseases caused by antibiotic-resistant *S. aureus*, particularly by methicillin-resistant *S. aureus* strains (MRSA), becomes endemic in most hospitals around the world and accounts for 40–60% of all healthcareassociated *S.aureus* infections [2, 3]. Methicillin resistance is imparted by the 2.1-kb mecA gene, which encodes the 78-kDa penicillin binding protein PBP 2A. PBP2a, as a transpeptidase, facilitates cell wall synthesis and bacterial growth at concentrations of β-lactams inhibitory to native penicillin-binding proteins. Apart from ceftobiprol [4], the presence of PBP2a confers resistance towards all β-lactamsantibiotics.Vancomycin remains the suitable antibiotic for the treatment of MRSA infections. However, dissemination of glycopeptide resistant strains is a concern; multidrug-resistant strains of both glycopeptide-intermediate *S. aureus* (GISA) and glycopeptide-resistant *S. aureus* (GRSA) have been detected, suggesting that the efficacy of antimicrobial agents for systemic infections such as bacteremia, endocarditis, and osteomyelitis may soon be significantly compromised [5]. Although methicillin-resistant *S. aureus* infections were traditionally limited to hospitals, and had been historically associated with longer hospitalization, greater costs in addition to higher mortality and morbidity rates [6], it is worrisome that, in recent years, community-associated cases of methicillin-resistant *S. aureus* infections have been reported with increasing frequency in the community and the so-called community-acquired MRSA (CA-MRSA), which have become a major concern worldwide [7]. CA-MRSA is responsible for a wide array of infections from superficial skin infections to life-threatening diseases. CA-MRSA has also been shown to encode genes for the Panton Valentine Leukocidin [8]. PVL is abicomponent pore-forming cytolytic toxin encoded by the lukF-PV and lukS-PV genes, which are carried by a group of specific bacteriophages. The PVL toxin is a cytotoxin that causes leukocyte destruction and tissue necrosis. It is responsible for many of the clinical symptoms of infection with CA-SA strains such as furunculosis, severe necrotizing pneumonia, necrotic lesions of the skin and soft tissues and also various deep-seated infections such as osteomyelitis [9]. The epidemiology of methicillin-resistant *Staphylococcus* in Morocco is poorly documented. Our present study provides baseline information on antibiotic resistance and epidemiology of S. aureus at the trauma service of Tangier. The aims of the prospective study were to describe the incidence, microbiologic profiles, of patients with both methicillin-sensitive *S.aureus* (MSSA) and

MRSA, and to investigate the prevalence *Staphylococcus aureus* infections carrying Panton-Valentine leukocidin (LPV) genes, as well as to study the characteristics of these strains and the respectively infected patients.

Methodology

Hospital setting

The trauma service described in this report is located in a 250-bed PHC in Tangier, Morocco. The trauma service, with 30-bed consists of one unit and without a separate isolation ward. Patients admitted to the unit include men and women ranged in age from 15 years to 90 years. Ethical approval for the study was granted by the Public Health Minister of the Department of Health Research (ref 319; N°1839).

Collection and identification of *S. aureus* isolates

This prospective study was conducted over a period of three years (August 2008 -August 2011). During this period, all strains were obtained from infected patients at the trauma service. Information on demographic characteristics, clinical presentation, comorbidities, treatment provided, type of specimen and acquisition was collected by physicians in the trauma department. Nosocomial acquisition was defined as a strain firstly isolated from a patient who had been hospitalised for more than 48 hours. Strains were considered to be community-acquired infections CA- if isolates were recovered from patients within 48 h of hospital admission, who were not hospitalized or residents in long-stay care facilities, who had not undergone surgery or dialysis during the previous year, and who did not have permanent indwelling catheters in situ [10].When multiple *S. aureus* strains were isolated from a single patient, only one isolate was included in this study (preferentially that isolated from the most invasive sample). The isolates were cultured in Columbia 5% blood sheep, Cystin Lactose-Electrolyte-Deficient (Cled) and Eosin Methylene Blue (EMB) agar plates; colonies were identified as staphylococci based on colony morphology, gram straining, and positive catalase test. *S. aureus* identification was based on thermostable DNase test agar and free coagulase production (lyophilized rabbit plasma; bioMerieux). *Staphylococcus aureus* ATCC 25923 and ATCC 43300 were used as the control strain.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by the agar dilution method on Mueller-Hinton agar. Antibiogram determined the resistance of isolates to a panel of 14 antimicrobial agents (Oxoid Ltd., Basingstoke, United Kingdom), including: erythromycin (E), lincomycin

(L), pristinamycin (Pri), gentamicin (CN), kanamycin (K), tobramycin (TM), ciprofloxacin (Cip), rifampin (RD), chloramphenicol (C), fusidic acid (AD), tetracycline (TE) and trimethoprim-sulfamethoxazole (SXT). Breakpoints have been interpreted to the criteria of the Committee for Antimicrobial Testing of the French Society of Microbiology CASFM [11]. Methicilin resistance was tested using 30µg cefoxitin (FOX) disk, according to CASFM guidelines. Mueller Hinton Agar was inoculated with 0.5 McFarland preparations of the inoculums and incubated at 37°C for 24 hrs. *S.aureus* with zones of inhibition ≤ 25 mm were interpreted as methicillin resistant, while those with zones of inhibition ≥ 27 mm were interpreted as methicillin susceptible. MICs to vancomycin were determined by the broth microdilution method ranging from 0.06 to 64 mg/l, according to CLSI guidelines [12].

Molecular studies

Genomic DNA was extracted with a standard phenol–chloroform [13]. Presumptive *S. aureus* isolates were confirmed by PCR amplification. Detection of mecA, nuc and 16S rRNA genes was performed by multiplex PCR of the specific genes as reported by Poulsen et al [14]; mecA, a deter-minant of methicillin resistance; nuc, which encodes the *S. aureus* -specific region of the thermonuclease gene; and a genus-specific 16S rRNA sequence used as an internal ampli-fication control for staphylococcal DNA. The expected sizes of the amplified DNA fragments are 527, 255 and 886bp respectively .The sets of primers were previously described [14]. Presence of PVL gene was tested by multiplex PCR as previously described by Lina et al [9]. Oligonucleotide primers were designed according to the published sequences of the PVL genes (GenBank accession numbers X72700 and AB006796. The primer sequences for the PVL genes were as follows: for luk-PV-1, 5′-ATTAGGTAAAATGTCTGGACATGATCCA-3′; luk-PV-2, 5′-GCATCAASTGTATTGGATAGCAAAAGC-3), a genus-specific 16S rRNA sequence as an internal amplification confirm the quality of the extraction and the absence of PCR inhibitors. All these oligonucleotides were synthesized by (Eurofins MWG operon Anzingerstrabe 7a D-85560 Ebersberg). The PCR products were resolved by electrophoresis through 1.5% agarose gels (Promega Corporation 608-274-4330, Madison U.S.A). This, was followed by ethidium bromide staining and analysis

Statistical analyses

Comparisons between proportions were made with Fisher's exact test and the Chi-square test. Differences showing a p value less than 0.05 were considered significant. Statistical analysis was performed on the Statistical Package for the Social Sciences SPSS (2001) [15]

Results

Table 1: Resistance rate associated to MRSA, MSSA, and to hospital-associated and community-associated MRSA

Antibiotic	Number of strains (%)		*P* value	Number of strains (%)		*P* value
	MSSA n=72	MRSA n=8		HA-SA n=57	CA- SA n=23	
Cefoxitin (Fox)	**0(0)**	**8 (100)**	**	**8 (14)**	**0 (0)**	**NS**
Gentamicyn(Cn)	1 (1.38)	1 (12.5)	NS	2 (3.50)	0 (0)	NS
Tobramycin(Tob)	0 (0)	2 (2.5)	NS	2 (3.50)	0 (0)	NS
Kanamycin (K)	3 (4.16)	3 (37.5)	NS	4 (7.02)	2 (8.70)	NS
Pristinamycin (Pri)	0 (0)	0 (0)	NS	0 (0)	0 (0)	NS
Erytromycin (E)	5 (6.94)	5 (62.5)	**	9 (15.79)	1 (4.34)	NS
Lincomycin (L)	0 (0)	2 (25)	**	2 (3.50)	0 (0)	NS
Cotrimoxazol(SXT)	0 (0)	3 (37.5)	**	2 (3.50)	1 (4.34)	NS
Ciprofloxacin (Cip)	20 (27.78)	8 (100)	**	20 (35.08)	8 (34.78)	**
Tetracyclin (Te)	24 (33.33)	6 (75)	NS	21 (36.84)	9 (39.13)	NS
Rifampin (Rd)	0 (0)	0 (0)	NS	0 (0)	0 (0)	NS
Fusidic-acid(AD)	23 (31.94)	6 (75)	NS	22 (38.60)	8 (34.78)	**
Cloranphinicol (C)	8 (11.11)	2 (25)	NS	8 (14.03)	2 (8.70)	NS

NA. not applicable; NS. not significant; CA. Community associated ; HA. hospital-associated ; MRSA. methicillin -resistant *Staphylococcus aureus*; MSSA. methicillin-sensible *Staphylococcus aureus* ; *. p <0.05; **. p <0.001.

Demographic data

During the study period, the trauma service received approximately 2700 patients with an estimated 80 patient/month. The median age of patients was 34 years old (range: <15-90 years). Overall, a male predominance (85%) was found. Longer median length of stay for patients was 24 days.

Bacterial strains

During this period, 288 isolates were collected from skin and soft tissue infections (SSTIs), septic arthritides, osteomyelitis and sepsis. The distribution by groups was *Staphylococcus* 117 strains (40.63%), *Enterobacteriaceae* 73 (25.2%) and other organisms 40 (13.9%). *Staphylococcus aureus* was the most common identifiable cause of infections 80 (27.7%) followed by *Pseudomonas aeruginosa* 34 (11.9%), and *Klebsiella pneumonia* 32 (11.2%). The proportion of Community-Acquired *S. aureus* CA-SA was 23 (28.75%). Interestingly, 18 (78.26%) of strains were collected immediately from surface infections (abscesses, phlegmon, and furunculosis.) and five (21.73%) from deep infections. 57 S. aureus (71.25%) were characteristic of Hospital-Acquired HA-SA and were isolated from 60 percent of purulent wounds, and 40 percent of Bone/joint infections.

Table 2: Distribution of PVL among MSSA and MRSA in community (IC) and in hospital (IH)

Profil of isolates	Total infections(n=80)	CI (n= 23)	HI (n=57)	P value
	Nb (%)	Nb (%)	Nb (%)	
MSSA PVL +	8 (10)	8 (34.78)	0 (0)	**
MSSA PVL -	64 (80)	15 (65.22)	49 (86)	NS
MRSA PVL +	0 (0)	0 (0)	0 (0)	NA
MRSA PVL -	8 (10)	0 (0)	8(14)	NS

NA. not applicable; NS. not significant; PVL. Panton-Valentine Leukocidin; MRSA. methicillin –resistant

Antibiotic susceptibility

In this study, the prevalence of *Staphylococcus aureus* was 80 (27.7%); out of these, 12 (15%) were cefoxitin-resistant; all have been associated with severe nosocomial infections. Of the 12 isolates only eight (10%) were confirmed as *S. aureus* mecA positive by multiplex PCR. The MRSA isolates showed 62.5%, 100%, 37.5%, 75%, and 75% resistance to erythromycin, ciprofloxacin, trimethoprim sulphamethoxazole, fusidic acid, and tetracycline respectively. The MSSA isolates were 6.94%, 27.78%, 31.94% and 33.33% resistant to erythromycin, ciprofloxacin, fusidic acid and tetracycline respectively. All MSSA were susceptible to

trimethoprum sulphamethoxazole. Antimicrobial susceptibility data are given in (Table 1). No high level resistance to vancomycin was observed in either MSSA or MRSA strain.

Comparison between PVL+ and PVL- isolates

To investigate whether PVL strains are associated with invasive disease we compared comorbidities, age, sex, length of hospital stay, and the proportion requiring surgery for patients infected with PVL positive and PVL negative. Among the 80 *S. aureus* strains eight (10%) yielded positive pvl amplification. There was a definite association between detection of the pvl genes and community-acquired infections ($p<0.001$); (Table 2). Patients with PVL positive disease had a shorter hospital stay (median 12 days vs 34 days) ($p<0.001$), and seemed to be younger than those with PVL negative disease (26years old vs 34 years old) ($p<0.05$). PVL positive isolates affected patients with fewer comorbidities ($p <0.05$) (Table 3). Overall, individuals with deep infections were less likely to be infected with a PVL positive strain than were those with skin and soft-tissue disease, ($p<0.001$) (Table 3). Seven out of 18 SSTIs (38.88%) PVL positive cases needed surgery vs four out of 18 (22.22%) PVL negative cases ($p<0.05$).

Table 3. Comparison of the Characteristics of Panton-Valentine Leukocidin PVL positive with PVL negative infections

Variables	*S. aureus* (n=80)		
	SA-PVL+ (n=8)	SA-PVL – (n=72)	*P* value
	Nb (%)	Nb (%)	
Comorbidities	0 (0)	26 (36.11)	*
Demographic traits			
Age. median. Years	26	34	*
Male sexe	7(87)	61(85)	NS
Type and severity of infection SSTIs			
Abscess/furunculosis/ phlegmon	7(87.5)	11(15.27)	**
Deep infections			
Bone/joint infections/ Osteomylit/infection after skin injury/...	1(12.5)	61(84.72)	**
Outcome			
Length of stay, median. days	12	34	**

NOTE. Data are no. (%) of patients or isolates. unless otherwise indicated; SSTIs. skin and soft tissue infections; NS. not significant; *. p <0.05; **. p <0.001;

Discussion

The prevalence rate of MRSA in the investigated period was 10% which is low compared to other studies in Morocco, where 19.3%, 35.4% was reported at university hospitals in Rabat [16] and at Ibn Rochd University Hospital Center in Casablanca [17], respectively. However, several data indicate that MRSA infections vary significantly, even within similar populations, interestingly, the unit and site of isolation of MRSA / specimen type has been found to be associated with prevalence of MRSA [17, 18]. This study was conducted only at the trauma service and might not reflect infection rates throughout the CHR of Tangier. Isolation of *S. aureus* from a single unit may result in underestimating the prevalence of MRSA. The correlation between the presence of the mecA gene and phenotypic resistance to cefoxitin remains less well defined. Four of mecA negative that were phenotypically methicillin resistant were reported. The phenotypic expression of resistance in the absence of a mecA gene could be due to hyperproduction of β-lactamase as described for *S. aureus* [19] or the presence of novel meticillin-resistant *Staphylococcus aureus* (MRSA) clones in human as described by García-Álvarez [20]. Prevalence of MRSA is lower in Morocco than in other European countries such as the UK, Ireland, Portugal and Italy [21]. And it is still lower in comparison with the rates in Algeria, Egypt, and Jordan [22, 23]. Although this prevalence is still low, it is important to note that nosocomial exposure through hand transmission and dissemination among staphylococci via horizontal could raise gradually the rate. MSSA or CNS could be precursors of MRSA [24, 25]. The broth microdilution method does not detect any GISA or GRSA strain. All the srains are susceptible. However, to improve the detection of vancomycin or glycopeptide resistance in *S.aureus*, it is very important to consider the testing method. The Etest and population analyses may offer the best alternative, particularly because not all of these strains will be detected by microbroth dilution methodology [26]. The high resistance reported in the present study, both for MSSA and MRSA isolates to fusidic acid, fluoroquinolones and tetracyclin (Table 1), could be related to the wide use of these antibiotics in unreasonable quantities that led to the rapid emergence of resistance [27]. These antibiotics represent the main antibiotics recommended for staphylococcal in our unit. MRSA isolates displayed multi-drug resistance, and was predominantly nosocomial or health care associated (Table 1). The proportion of CA-MRSA isolates identified (0%) is lower than those reported in many studies [7, 8]. The observation is inconsistent with a study carried out in Portugal where the prevalence of CA-MRSA was lower than 1% [28], although MRSA is a major problem in Portugal and the prevalence of HA-MRSA (49.1%) is among the highest in

Europe [29]. MSSA isolates were susceptible to more antimicrobials than MRSA isolates (Table 1). However, the frequency of MSSA isolates resistance to FA (31.94%) and ciprofloxacin (27.78%) was also clinically relevant. Additionally, few isolates community strains have acquired the Panton-Valentine leukocidin gene, which confers virulence and has been associated with complicated skin and soft tissue infections [7]. PVL is strongly associated with skin and soft-tissue disease ($p<0.001$) and is comparatively less common in strains causing secondary infections after skin injury, or deep-seated infections such as osteomyelitis ($p<0.001$) (Table3), confirming the results of Couppie [29], who detected the toxin in 86% of the *S. aureus* strains responsible for furuncles and only one of 86 *S. aureus* strains isolated from blood and three of 13 cases of osteomyelitis. This is in contrast to data from Senegal [30], which show high Prevalence of pvl genes among *S aureus* myositis and osteomyelitis. The majority of those isolates have the phenotype PVL positive MSSA. In contrast, previous reports revealed that there was a strong association between CA-MRSA infections and the expression of PVL [8]. Thus, PVL was considered as a potential marker for CA-MRSA in many studies [7, 8]. However our results are in agreement with those of other studies published previously in Europe [31], in England, [32] and in Nigeria [33]. Overall, patients with PVL positive infections are likely to be younger ($p<0.05$) and with fewer comorbidities ($p<0.05$). Compared with patients infected with PVL negative strains those with PVL positive SSTI infections report higher rates of surgery (seven out of 18) PVL positive vs four out of 18 PVL negative ($p<0.05$). PVL emerges as a significant predictor of the need for a drainage procedure and surgical interventions, but the higher rates of surgery tend to reduce significantly the length of hospital stays, (median 12 days vs 34 days), ($p<0.001$). This situation contrasts with Europe, where Median hospital stay of 45 days for PVL positive cases vs 13 days for PVL negative cases [34] is reported. The PVL positive MSSA could pose serious problem in the future as potential reservoirs for virulence factors, and could lead to the emergence and spread of PVL positive MRSA clones in Tangier, causing severe infections. The luk-PV genes may be incorporated into *S. aureus* lineages through horizontal transfer, either before or after acquisition of the mecA gene [35].

Conclusion

Phenotypic and Molecular analysis performed in this study reveals diversity between strains of *Staphylococcus* studied isolates. MRSA and MSSA differ in the severity of illnesses they

cause; they have different toxin and antibiotic profiles. All MRSA isolates displayed multi-drug resistance, and were a predominantly nosocomial or healthcare associated. MSSA isolates were susceptible to more antimicrobials than MRSA isolates. However, the frequency of MSSA isolates resistance to FD (31.94) and ciprofloxacin (27.78%) was also clinically relevant. PVL appears to be a possible virulence factor associated with necrotic lesions of skin and subcutaneous tissues and also with community-acquired MSSA. No GISA strain has been detected; however, further study of *S. aureus* and evaluation of optimal practices for screening and detection of glycopeptide nonsusceptibility are clearly needed. In the treatment of the infected patients, it is important to determine the phenotype of resistance to avoid the selection of resistant strains especially for FD, Cip and TE as mentioned before. Lastly, even though the frequency of MRSA is relatively low compared to other regions of the world, adequate measures are necessary to prevent MRSA from becoming endemic in our population. This study presents important clinical and epidemiological information not previously available on *S. aureus* infections in North Morocco. Prospective studies that combine clinical and molecular epidemiology of *S. aureus* infections are warranted.

Conflict of interest

All authors declare to have no conflict of interest.

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