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Fusidic acid-resistant methicillin-sensitive *Staphylococcus aureus* isolates in community of Casablanca (Morocco)

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Fusidic acid-resistant *Staphylococcus aureus* is poorly documented in community acquired infections in Morocco. From 1st January, 2007 - 31 October, 2008, we collected 140 *S. aureus* isolates at Institute Pasteur, Casablanca, 18 of them exhibited resistance to fusidic acid and were negative for gene encoding methicillin resistance (*mecA*). *seh* toxin gene was found in 14 strains (14/18) along with an accessory gene regulator (*agr*) group III, 9 of them were found with two other toxin genes (*sek* plus *seq*). In conclusion, we found a close relationship between the presence of the *seh* gene and the possession of *agr* group III in fusidic acid-resistant methicillin-sensitive *S. aureus* (FAR-MSSA) strains. In addition, our results indicate a relationship between 6 FAR-MSSA strains belonging to the same pulsotype and harbouring *agr* group III with *luk*-PV toxin genes.

Key words: Fusidic acid resistance, *mecA* gene, *seh* toxin gene, *Staphylococcus aureus*, MRSA.

INTRODUCTION

Staphylococcus aureus is both a commensal and an extremely versatile pathogen in humans, causing three superficial lesions such as skin abscesses and wound infections, deep-seated and systemic infections such as osteomyelitis, endocarditis, pneumonia, and bacteremia, and toxic syndromes. It continues to be an important pathogen in both community and hospital-acquired infections (Risson et al., 2007; Ferrara, 2007; Pedersen et al., 2006; Ellington et al., 2003; Novick, 2000; Lina et

al., 1997).

Microbial pathogenicity in this strain is a complex phenomenon involving a number of virulence factors, primarily exotoxins that damage host cells and interfere with immune responses, and cell wall-associated proteins involved in adhesion and protection against host defences. The expression of most of these factors is orchestrated by at least one regulatory locus, the accessory gene regulator (*agr*) (Novick et al., 1993), which encodes a two-component signalling pathway whose activating ligand is a bacterial-density-sensing peptide (autoinducing peptide) also encoded by *agr* (Novick, 2000). A polymorphism in the amino acid sequence of the autoinducing peptide and its corresponding receptor (*AgrC*) has been described. *S.*

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aureus strains can be divided into four major groups (I to IV) on this basis: within a given group, each strain produces a peptide that can activate the *agr* response in the other member strains, whereas the autoinducing peptides produced by the different groups are usually mutually inhibitory (Jarraud et al., 2000; Ji et al., 1997).

It was reported that *S. aureus* strains causing specific syndromes were linked to certain *agr* groups (Obire et al., 2009; Jarraud et al., 2002), and it is believed that strains within a given *S. aureus agr* group are related genetically and share similar biological properties (Novick, 2000).

Fusidic acid is a narrow spectrum antibiotic derived from *Fusidium coccineum*, which has an unusual spectrum of activity that includes *Corynebacteria*, *Nocardia*, Anaerobes, and *Neisseria* species, but is used almost exclusively as an anti-staphylococcal agent (Collignon and Turnidge, 1999; Falagas and Kopterides, 2007; Pan et al., 2008). This antibiotic inhibits bacterial protein synthesis by binding to elongation factor G (EF-G) and preventing its release from the ribosome (Bodley et al., 1969). Although frequencies of resistance to this antibiotic have remained generally low, emerging resistance is a problem that could limit the therapeutic options available for treatment of staphylococcal infections (Norén et al., 2007; Rennie, 2006; Osterlund et al., 2006).

The aim of the present study is to determine the prevalence of fusidic acid-resistant methicillin-susceptible *S. aureus* (FAR-MSSA) isolated from community infections, and to define whether there are links between the antibiotic susceptibility of these strains, their origin and *agr* groups, and to correlate them with their toxic gene profile.

MATERIALS AND METHODS

Bacterial isolates

In this study, community clinical *S. aureus* were collected from the Laboratory of Microbiology, Institute Pasteur of Morocco, and from 14 clinical laboratories located in Casablanca, from the 1st January, 2007 - 31st October, 2008.

Identification of *S. aureus* isolates. Species were identified by colony morphology, gram staining, catalase test, coagulase activity on rabbit plasma (Bio-Mérieux, Marcy l'Etoile, France), and production of clumping factor (Pastorex Plus-Staph, Bio-Rad, Marnes-la-Coquette, France).

Antimicrobial resistance to penicillin G (PG-6 µg), kanamycin (K-30 UI), tobramycin (Tm-10 µg), gentamicin (Gm-15 µg), tetracycline (Te-30 UI), erythromycin (E-15 UI), lincomycin (L-15 µg), pristinamycin (PT-15 µg), chloramphenicol (C-30 µg), pefloxacin (Pef-5 µg), fosfomicine (Fos-5 µg), cefoxitin (Fox-30 µg), fusidic acid (FA-10 µg), rifampicin (RF-30 µg), vancomycin (VA-30 µg) and trimethoprim-sulfamethoxazole (SXT- 1.25/23.75 µg) was determined by the disc (Bio-Rad, Marnes-la-Coquette, France) diffusion method. Fusidic acid (Leo pharma, Denmark) minimum inhibitory concentrations (MICs) were determined by agar dilution technique on Mueller-Hinton agar; the range of dilution used was 0.004 - 128 mg/l. Results were interpreted according to the Committee for Antimicrobial Testing of the French Society of Microbiology guidelines (<http://www.sfm.asso.fr>) (Comité de l'Antibiogramme de la Société Française de Microbiologie, 2007). Strains with MICs higher

than 2 mg/l were taken as being resistant to fusidic acid. *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were used as quality control organisms.

DNA extraction

Fusidic acid-resistant *S. aureus* (FAR-SA) strains were grown in brain heart infusion at 37°C overnight. Their genomic DNA used for polymerase chain reaction (PCR) was extracted by using a standard phenol-chloroform procedure as described by Sambrook et al. (1989). Amplification of *nuc* gene which encodes an extracellular thermostable nuclease of *S. aureus* was used for identification and to confirm the quality of each DNA extract (Brakstad et al., 1992; Kizaki et al., 1994). The *mecA* gene was sought by PCR (Vannuffel et al., 1995) in all FAR-SA. Two reference strains: U2A1593 (methicillin-resistant *S. aureus*) and U2A1594 (methicillin-sensitive *S. aureus*) from the Antibacterial Agents Unit of Pasteur Institute, Paris, France, were used as controls.

Determination of *agr* groups

A multiplex PCR of the *agr* was used to determine the *agr* group (I-IV) (Jarraud et al., 2002). *S. aureus* strains RN6390 (*agr* group I), RN6607 (*agr* group II), RN8465 (*agr* group III), and RN4850 (*agr* group IV) from the National Research Center of Lyon (CNR-Lyon, France) were used as positive controls.

Detection of staphylococcal toxins

All FAR-MSSA isolates were screened for genes encoding staphylococcal enterotoxins A, B, C, D, H, K, L, M, O, P, Q and R (*sea*, *seb*, *sec*, *sed*, *seh*, *sek*, *sel*, *sem*, *seo*, *sep*, *seq* and *ser*), exfoliatin toxins A, B and D (*eta*, *etb* and *etd*), toxic shock syndrome toxin 1 (*tst*), leukocidins (*lukS*-PV, *lukF*-PV, and *lukM*), epidermal cell differentiation inhibitor (*edin* A, B, C) and β-hemolysin (*hly*), as previously described (Jarraud et al., 2002; Tristan et al., 2003).

PFGE typing

Pulsed-field gel electrophoresis (PFGE) of chromosomal DNA of FAR-MSSA was performed using the CHEF DR II System (Bio-Rad, Marnes-la-Coquette, France), as described elsewhere (Maslow et al., 1993). PFGE patterns were compared by physical examination and patterns (pulsotypes) considered being indistinguishable if all bands were shared.

RESULTS

Prevalence and distribution of the FAR-MSSA isolates

Of the 140 isolates studied, 18 (12.9 %) fusidic acid-resistant methicillin-sensitive strains were obtained from different clinical samples, with 9 (50%) being from pus/wound/abscess, 6 (33%) from urinary/genital tract infections and 3 (17%) from Sputum/transtracheal aspirates. Ten of these exhibited a minimum inhibitory concentration (MIC) of 16 µg ml⁻¹ for fusidic acid (Table 1). No FAR *S. aureus* strain with *mecA* gene was detected.

Table 1. MICs of fusidic acid against *S. aureus* isolates (n = 18).

Fusidic acid MIC ($\mu\text{g ml}^{-1}$) ^a	4	8	16
No of isolates resistant to fusidic acid (%)	1 (5.5)	7 (38.9)	10 (55.6)

^a The resistance breakpoint for fusidic acid by the Committee for Antimicrobial Testing of the French Society of Microbiology is $4 \mu\text{g ml}^{-1}$.

Table 2. Distribution of fusidic acid-resistant *S. aureus* strains according to their sources and pattern of resistance.

Source of strains	Number of strains with pattern resistance						Total (%)
	FA	P-FA	RF-FA	P-E-FA	P-K-Te-FA	P-K-Te-E-FA	
Urinary/genital tract infections	2	4					6 (33)
Sputum/transtracheal aspirates			1	1	1		3 (17)
Pus/wound/abscess	3	2			1	3	9 (50)
Total (%)	5 (27.7)	6 (33.3)	1 (5.6)	1 (5.6)	2 (11.1)	3 (16.7)	18 (100)

P = Penicillin G, K = Kanamycin, Te = Tetracycline, RF = Rifampicin, FA = Fusidic acid, E = Erythromycin.

Antimicrobial resistance phenotypes of the FAR-MSSA isolates

We primarily examined the resistance of the FAR-MSSA (n = 18) to antibiotics of different classes and our finding showed that 12 (66.7%), 5 (27.8%), 5 (27.8%), 4 (22.2%) and 1 (5.6%) of the strains were resistant to penicillin G, kanamycin, tetracycline, erythromycin and rifampicin, respectively. Table 2 shows the resistance phenotypes of the respective FAR-MSSA strains. Five strains (27.7%) showed resistance to only fusidic acid. In addition to being resistant to fusidic acid, 6 strains (33.3%) were also resistant to penicillin G, 1 strain (5.6%) was also resistant to rifampicin, 1 strain (5.6%) was also simultaneously resistant to penicillin and erythromycin, 2 strains (11.1%) were also resistant to penicillin, kanamycin tetracycline and 3 strains (16.7%) showed a wide resistance pattern to fusidic acid, penicillin G, kanamycin, tetracycline and erythromycin. Antimicrobial susceptibility revealed that all FAR-MSSA isolates were sensitive to cefoxitin, tobramycin, gentamicin, lincomycin, pristinamycin, chloramphenicol, pefloxacin, fosfomycine, trimethoprim-sulfamethoxazole and vancomycin.

Analysis of *agr* group

As shown in Table 3, the 18 FAR-MSSA isolates were classified according to the four *agr* groups and 14 (77.8%) were found to belong to *agr* group III; 3 were *agr* group I, and only one isolate was *agr* group IV. No strain was found to be *agr* group II.

Analysis of toxin genes

Seventeen (94%) of the 18 FAR-MSSA isolates studied were found to possess one or more virulent genes, with the *seh* gene being the most frequent (78%), and always in combination with at least one other (Table 3). These *seh* genes were only detected in isolates harbouring the *agr* group III. Fourteen of the above 17 strains possessed more than one virulent gene, and they were all of the *agr* group III (Table 3).

Two of the 3 strains with an *agr* group I were found to possess only one virulent gene (the β -hemolysin gene = *hly*); the third possessed no virulent gene. The last strain with an *agr* group IV also possessed a single virulent gene (the *sem* gene) (Table 3). All isolates were found to be negative for genes encoding exfoliative toxins A, B and D, the other staphylococcal enterotoxins B, D, P and R, the epidermal cell differentiation inhibitor genes (*edin* A/B/D) and the toxic shock syndrome toxin gene (*tst*).

Analysis of PFGE typing

PFGE typing produced 12 fingerprints, which were classified as pulsotypes class A to L (Figure. 1). Pulsotype class A was detected in 33.3% (6 isolates) of all FAR-MSSA (Table 3), 5 of these were isolated from pus/wound/abscess, and one from pulmonary aspirates. Pulsotype class B was represented by two strains that were detected from pus or urine and which had two virulent genes: *she* plus *hly*. The remaining 12 isolates belonged to 10 other DNA classes.

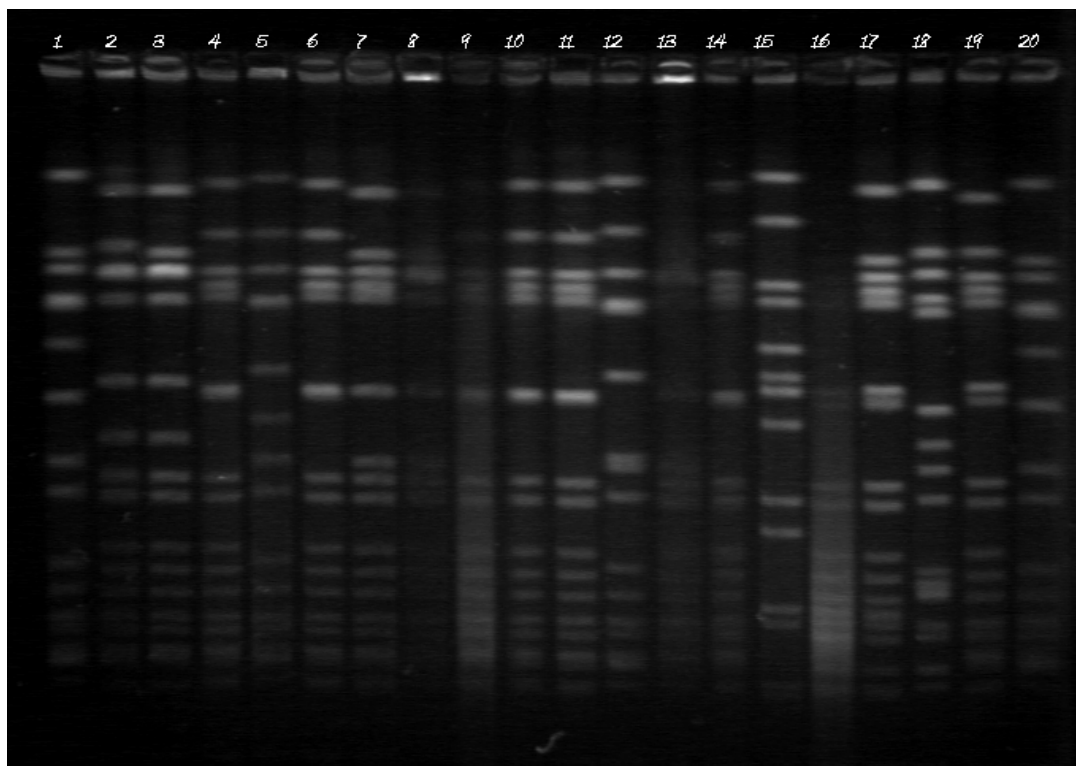


Figure 1. Genomic typing of 18 fusidic acid-resistant methicillin-susceptible *S. aureus*. Lanes 1 and 20: A chromosomal DNA digest of *S. aureus* NCTC 8325 served as the mass standard. Lanes 2 to 19: all 18 strains; Lanes 4, 6, 9, 10, 11, 14: strains class A (predominant class); Lanes 8, 13: strains class B and Lanes 2, 3, 5, 7, 12, 15 - 19: the other strains.

Table 3. Distribution of fusidic acid-resistant methicillin-sensitive *S. aureus* strains according to their agr group, sources, pattern of resistance virulent genes and their pulsotype groups.

Strain	Agr group	Source of samples	Antibiotic resistance phenotype	Positive PCR result for	Pulsotype: letter designation
SA18	3	Pus/wound/abscess	P-K-Te-Fu	<i>seh/seq/sek/pvl</i>	A
SA22	3	Sputum/transtracheal aspirates	Rif-Fu	<i>seh/seq/sek/pvl</i>	A
SA30B	3	Pus/wound/abscess	Fu	<i>seh/seq/sek/pvl/sea</i>	A
SA31B	3	Pus/wound/abscess	P-K-Te-E-Fu	<i>seh/seq/sek/pvl/sea</i>	A
SA32B	3	Pus/wound/abscess	P-K-Te-E-Fu	<i>seh/seq/sek/pvl</i>	A
SA64	3	Pus/wound/abscess	P-K-Te-E-Fu	<i>seh/seq/sek/pvl/seo</i>	A
SA26	3	Urinary/genital tract infections	P-Fu	<i>seh/hlb</i>	B
SA42	3	Pus/wound/abscess	Fu	<i>seh/hlb</i>	B
SA2	3	Urinary/genital tract infections	Fu	<i>seh/sec/sea/sel</i>	C
SA6	3	Urinary/genital tract infections	P-Fu	<i>seh/sea</i>	D
SA24	3	Urinary/genital tract infections	Fu	<i>seh/hlb</i>	E
SA100	3	Sputum/transtracheal aspirates	P-K-Te-Fu	<i>seh/seq/sek</i>	F
SA101B	3	Pus/wound/abscess	Fu	<i>seh/seq/sek/hlb</i>	G
SA102AZ	3	Urinary/genital tract infections	P-Fu	<i>seh/seq/sek/sec/sel</i>	H
SA20B	1	Pus/wound/abscess	P-Fu	<i>hlb</i>	I
SA37	1	Urinary/genital tract infections	P-Fu	<i>hlb</i>	J
SA111	1	Pus/wound/abscess	P-Fu	none	K
SA88	4	Sputum/transtracheal aspirates	P-E-Fu	<i>sem</i>	L

pvl = *luk-PV* genes, P = Penicillin G, K = Kanamycin, Te = Tetracycline, RF = Rifampicin, FA = Fusidic acid, E = Erythromycin

DISCUSSION

Our finding showed a fairly significant resistance to fusidic acid, which was expressed in 18 strains (~13%), this rate of resistance is greater than that reported in Casablanca by Belabbès et al. (2001), which gave only 4.5% rate as well as those reported by other authors, ranging from 0 - 7.7% for both MSSA and community-acquired methicillin-resistant *S. aureus* (CA-MRSA) (Ho et al., 2008; Denton et al., 2008; BenNejma et al., 2008). The present rate however, is about the same as that of Elhamzaoui et al. (2009), who recently reported a resistance rate of 14.21% in a study conducted during the same period (2007 - 2008) on series of MSSA isolated from two hospitals in Rabat, Morocco. This resistance may be the result of the prescription of this antibiotic for outpatient empirical treatment of community-associated skin and soft tissue infections that may be attributable to MRSA, or even use it inappropriately, knowing that *S. aureus* may rapidly develop resistance to this antibiotic when it is used as monotherapy (Darley and MacGowan, 2004). In fact, a causal association between monotherapy and resistance is biologically plausible because of the high spontaneous chromosomal mutation frequency for development of fusidic acid resistance in *S. aureus*, resulting in the emergence of resistant mutants during therapy (O'Neill et al., 2001). For this reason, combination of fusidic acid with other antibiotics is recommended for the treatment of severe and chronic infections but with careful monitoring for the development of resistance.

Some reports have stated that there are clinical trends according to each *agr* group. For example, Yoon et al. (2007) have suggested the relationship between the *agr* group I clones and ear infections; Ji et al. (1997) argued that the presence of the *tst* gene in *S. aureus* is coupled to *agr* group III; while Jarraud et al. (2002) have reported an over representation of *agr* genotype II in *S. aureus* isolates from patients with infective endocarditis. Most exfoliatin-producing strains responsible for staphylococcal scalded skin syndrome (SSSS) belonged to *agr* group IV (Jarraud et al., 2000). On the other hand, the ability of *S. aureus* to cause a variety of diseases in humans and animals may be attributed to its ability to produce a plethora of virulence factors. Thus, we analysed the *agr* group specificity of the different 18 FAR-MSSA strains, and found that 14 of them belong to *agr*-type III, and were all found with at least one virulent gene (*seh*). However, El-Huneidi and co-workers previously reported that the *seh* gene was infrequently detected in clinical isolates of *S. aureus* from Jordan (El-Huneidi et al., 2006). Interestingly, FAR-MSSA that harboured *agr* group III and *seh* gene (14 isolates) were isolated from pus/wound/abscess (50%), urinary/genital tract infections (35.7%) and sputum/transtracheal aspirates (14.3%) and belonged to different clones: the pulsotype A was found in 6 strains, pulsotype B in 2 strains and six other strains were found each with a different pulsotype C to H. We wonder whe-

ther these structures (*agr* group III and *seh* gene) are molecular markers for strains resistant to fusidic acid, whatever their origin, or the existence of any such two structures (*agr* group III and *seh* gene) must coexist with the presence of the other when the strains are resistant to this antibiotic. Moreover, 9 of the 14 strains with *agr* group III and harbouring the *seh* gene were isolated with two other toxin genes: *seq* and *sek* genes. It has been reported that these two genes have been carried by many community-acquired methicillin-resistant *S. aureus* strains (CA-MRSA) such as ST8:USA300, *S. aureus* COL, ST59:USA1000 and ST1:MW2 (Baba et al., 2002) and were detected in all isolates belonging to Brazilian/Hungarian clone, a well-known hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) and having an *agr* group 1 (Deurenberg et al., 2007). As a matter of fact, these toxin genes that encode pyrogenic toxin superantigens were mainly found among *S. aureus* isolated from pus or sputum (8 isolates from 9), suggesting that there might be a relationship between FAR *S. aureus* strains, with an *agr* group III, the simultaneous presence of *seh*, *seq* and *sek* genes and sources of these strains.

Besides the dominant genes mentioned above, we noted that *luk-PV* genes, were detected in 6 FAR-MSSA strains that belonged to the same *agr* group III, and which possess *seh*, *seq*, *sek* genes. Remarkably, these 6 strains were identified with isolates that were identified from pus (5 cases) or sputum (1 case), and they were in most multidrug-resistant and were found to belong to the same clone "pulsotype A". It is known that PVL is a bicomponent exotoxin that causes dermal necrosis and possesses particular cytolytic activity against neutrophils and monocytes (Popovich et al., 2008), and whether caused by MRSA or MSSA, it is associated with suppurative cutaneous disease and necrotizing infections (Gillet et al., 2002; Hidron et al., 2005; Wertheim et al., 2005). Although PVL commonly is found in *S. aureus* isolates responsible for suppurative infections, currently it is found in fewer than 15% of colonizing MRSA isolates in the general community (Gorwitz et al., 2008). It has been postulated that the product of this virulent toxin is the principal factor responsible for the epidemic spread of many CA-MRSA strains (Chambers, 2005), including ST1: MW2 in the Midwest of the United States (Baba et al., 2002), ST30 in Australia (Robinson et al., 2005) and the United States (Pan et al., 2003), and ST80 in Europe (Vandenesch et al., 2003).

It should be noted that the virulence genes studied were significantly more prevalent among the fusidic acid-intermediate or fusidic acid-resistant strains. These virulence genes have detected among our isolates from pus/wound/abscess, and in all strains identified from urine and sputum/transtracheal aspirates. Therefore, the prevalence of virulent genes was found to be higher in fusidic acid-resistant *S. aureus* regardless of their sources. These results are in accordance with those of Van der Mee-Marquet et al. (2004). Thus, resistance to fusidic acid appears to be a pertinent marker of virulent strains.

This resistance marker (fusidic acid-resistance) has been previously identified in community-acquired, non multi-resistant, oxacillin-resistant *S. aureus* strains (Andersen et al., 1999), especially in the PVL-producing strains that are spreading in France (Dufour et al, 2002) and in Germany (Witte et al., 2004).

Conclusion

FAR-MSSA is present in the community of Casablanca (Morocco). Resistance to fusidic acid is a pertinent marker of virulent strains. In our study, the majority of these strains had *agr* type III, and a variety of virulent genes dominated by *seh*, *sek*, *seq* and *luk-PV* genes. It seems that there is relationship between FAR-MSSA strains belonging to the same pulsotype and harbouring *agr* group III with *luk-PV* toxin genes.

So fusidic acid prescription should be limited and if indicated it should be used in combination therapy.

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