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Antimicrobial Susceptibility Patterns of Methicillin-Resistant *Staphylococcus aureus* Isolates Collected from Healthcare and Community Facilities in Libya Show a High Level of Resistance to Fusidic Acid Wareg SE¹², Foster HA^{1*} and Daw MA²

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Abstract

Staphylococcus aureus is an important human pathogen and is implicated in a wide variety of infections in the healthcare and community settings. The organism is often subcategorized as community-associated MRSA (CA-MRSA) or health care-associated MRSA (HA-MRSA). Five hundred and eleven *S. aureus* strains were isolated from clinical specimens submitted to the microbiology laboratories at Tripoli Central Hospital, Tripoli Trauma/ Accident Hospital, Tripoli Medical Centre and Tripoli Burn Hospital between October 2009 and November 2010. MRSA was detected using cefoxitin (30 µg) disc and antibiotic susceptibility pattern was determined using the Kirby and Bauer disc diffusion susceptibility testing method and confirmed for fusidic acid and vancomycin by determination of minimum inhibitory concentration The prevalence of Inpatient Healthcare Associated MRSA (IP-MRSA), outpatient-Healthcare Associated MRSA (OP-MRSA) and community carried MRSA (CC-MRSA) was 43%, 37% and 34% respectively. The isolates of MRSA displayed resistance to fusidic acid and multiple drug resistance (MDR) to 2-9 antibiotics for IP-MRSA, 2-7 antibiotics for OP-MRSA and 2-6 antibiotics for CC-MRSA. The most frequent MDR was resistance to fusidic acid, ciprofloxacin, streptomycin and clindamycin. This study has shown that MRSA is prevalent with similar rates for IP-MRSA, OP-MRSA and CC-MRSA strains. Lack of controls on supply of antibiotics may be responsible for the fusidic acid resistance.

Keywords: Antibiotic resistance; Fusidic acid; Libya; MRSA; *Staphylococcus aureus*; Methicillin resistance

Abbreviations: BSAC: British Society for Antimicrobial Chemotherapy; CA-MRSA: Community acquired MRSA; CC-MRSA: Community Carried MRSA; CLSI: Clinical Laboratory Standards Institute; HA-MRSA: Healthcare Acquired MRSA; IP: Inpatients; IP-MRSA: MRSA Isolated from Inpatients; MIC: Minimum Inhibitory Concentration; Mlsbi: Macrolide, Lincosamide and Group B Streptogramin Resistance; MRSA: Methicillin Resistant *Staphylococcus Aureus*; NB, Nutrient Broth; OP: Outpatients; OP-MRSA: MRSA Isolated From Outpatients

Introduction

Staphylococcus aureus is an important pathogen that causes a wide range of diseases, from mild superficial skin infection to life-threatening diseases such as bacteraemia, pneumonia, infective endocarditis, deep-seated abscess and toxic-shock syndrome [1]. The organism is often subcategorized as community-associated MRSA (CA-MRSA) or health care-associated MRSA (HA-MRSA). Most studies distinguish CA-MRSA from HA-MRSA based on whether infection is present or diagnosed within 72 h of admission [2]). Only a few studies on staphylococcal infections and methicillin-resistant S. aureus (MRSA) in particular in Libya have been reported. Toxic shock syndrome toxin of S. aureus from Tripoli (Libya) was detected by El-Godban et al. [3]. Approx. 75% of strains originating from food were resistant to penicillin but none of the strains were resistant to methicillin or vancomycin. Inducible clindamycin resistance among staphylococci isolated from burn patients in Tripoli, Libya was studied by Zorgani et al. [4] and 65/120 (54%) of S. aureus isolates were MRSA. In a study by Buzaid et al. [5], MRSA was found in 31% of S. aureus isolates examined in a tertiary surgical and trauma hospital in Bengazi, Libya. They also reported the antimicrobial resistance patterns of MRSA to vancomycin, ciprofloxacin, chloramphenicol and erythromycin as 17.7%, 33.9%, 38.7% and 46.8% of cases respectively. A study of 169 clinical samples showed that 32% of S. aureus isolates were MRSA and a further 5% carried the MecA gene but did not express resistance [6]. Screening of 569 doctors and nurses from four main hospitals in Tripoli, Libya for MRSA showed a carriage rate of 19% [7]. This contrasts with an earlier study by Zorgani et al. [8] which showed a carriage rate of 37% in Libyan healthcare workers. These studies suggest that MRSA prevalence was high in many hospitals in Libya. The aim of present study was to identify and verify the extent of the spread of both community and healthcare-associated MRSA infections. Antibiotic resistance was detected using the disc diffusion method. Preliminary results showed a high prevalence of fusidic acid resistance and strains apparently resistant to vancomycin were isolated from outpatients. The resistance was confirmed by determination of minimum inhibitory concentration (MIC).

Materials and Methods

Study area and sampling technique

Inpatient samples (IP; 735) from wound/pus swabs, urine, swabs of catheters and blood culture from patients in the Tripoli Central Hospital, Tripoli Trauma/Accident Hospital, Tripoli Medical Centre and Tripoli Burn Hospital, outpatient samples from surgery out-patient departments (503 wound swabs, skin and soft tissue swabs) were sampled between October 2009 and November 2010. Clinical samples

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were sub-cultured onto Columbia agar base containing 5% sheep blood agar (Oxoid) and incubated at 37°C for 18-24 h. Cultures were screened for colonies typical of *S. aureus* and a total of five-hundred and eleven strains were isolated at random from those samples where *S. aureus* was the main infectious agent. *S. aureus* was similarly isolated from 344 nasal swabs from university students and the general public at random.

Bacterial identification

Clinical samples were streaked to single colonies onto Columbia agar base containing 5% sheep blood agar (Oxoid) and incubated at 37°C for 18-24 h. Colonies consistent with *S. aureus* were streaked to single colonies and identified and confirmed according to their colony morphology, Gram stain, biochemical and microbiological tests including: catalase test using hydrogen peroxide, coagulase test (both slide test and tube test) using human plasma (Liofilchem-Italy), growth in mannitol salt agar (Liofilchem-Italy) and Deoxyribonuclease test (EMD-Germany). *S. aureus* (ATCC 25923) was used as a quality control organism.

Antimicrobial susceptibility testing

S. aureus ATCC25923 and isolates were inoculated into Nutrient Broth (NB; Oxoid) and incubated at 37° C for 18-24 h. The cultures were diluted with fresh NB to give a turbidity equivalent to 0.05 McFarland standard absorbance at 625nm. Susceptibility tests were performed by the disc diffusion method of Bauer et al. [9] as described by the Clinical Laboratory Standard Institute (Anon., 2013) using Mueller-Hinton agar (Difco) supplemented with 20gl-1 NaCl. Antibiotic discs (Oxoid UK) were cefoxitin (FOX) 30 µg, vancomycin (VAN) 30 µg, chloramphenicol (CHL) 30 µg, gentamicin (GEN)10 µg, fusidic acid (FUS)10 µg, erythromycin (ERY) 15 µg, streptomycin (STR) 10 µg, cefotaxime (CTX) 30 µg, clindamycin (CLI) 2 µg, ciprofloxacin (CIP) 5 µg. Zones of inhibition were measured after 18 and 24 h incubation at 35°C. Cefoxitin was used as an indicator of methicillin susceptibility disks and an inhibition zone diameter of \leq 14mm was reported as methicillin resistant, 15-17mm as intermediate and ≥ 18mm was considered as methicillin sensitive. S. aureus ATCC25923 was used in every run. The isolates were reported as sensitive, intermediate and resistant based on the Clinical and Laboratory Standards Institute (CLSI) guidelines [10]. Determination of sensitivity for vancomycin on media containing 6.5% NaCl can give unreliable results [11] and these results were checked by determining the MIC. Interpretative zones of inhibition for fusidic acid are not stated in the CLSI guidelines. Values used (resistant \leq 29 mm and susceptible \geq 30 mm) were as according to the British Society for Antimicrobial Chemotherapy (BSAC) guidelines [12]. Minimum inhibitory concentrations were determined for fusidic acid and vancomycin using dilutions of 128-0.25 mgl-1 in Isosensitest agar (Oxoid). Plates were inoculated with culture suspension (1 μ l) and incubated for 24 h at 35°C. MIC was determined as the lowest concentration that inhibited growth.

Ethical considerations

Ethical permission was obtained from the main management of the hospitals under investigation in Tripoli and the University of Tripoli.

Results

MRSA isolates and antibiotic resistance

A total of 511 *S. aureus* isolates were obtained (Table 1). Twohundred and forty-three isolates were from inpatients (IP; 164 infected wound/pus swabs, 42 urine samples, 16 from swabs of catheters and 12 blood culture). The prevalence of *S. aureus* infection was higher for wound swabs at 42.6% compared to 31-32% for all the other samples. One-hundred and sixty-six strains were isolated from outpatients (OP; 88 from wound swabs, 28 from skin swabs and 50 from soft tissue swabs). The prevalence of *S. aureus* infections in wound swabs was similar to that for inpatients at 32-33%. In addition, 111 community carried (CC) strains were isolated from 344 nasal swabs giving a carriage rate of 32.3%.

The isolates were screened for resistance to cefitoxin as an indicator for methicillin resistance. The prevalence of MRSA was 43%, 37% and 34% in the inpatients (IP-MRSA), outpatients (OP-MRSA) and community carried isolates (CC-MRSA) respectively (Table 1).

The age and sex distribution of the MRSA isolates are shown in Table 2. There was a slightly higher percentage of males than females in the inpatients and outpatients but the reverse was true of the community. Most patients were aged between 30 and 49 but most of the CC-MRSA strains came from those aged below 30, reflecting the number of university students screened.

The MRSA isolates were subjected to antibiotic susceptibility test against 9 antimicrobial agents. The resistance of the IP-MRSA strains is shown in Figure 1a. All of the strains were resistant to fusidic acid. Resistance to vancomycin was 0%, chloramphenicol 31%, gentamicin 37%, erythromycin 48%, streptomycin 56%, cefotaxime 72%, clindamycin 17% and ciprofloxacin 56% (Figure 1a). The resistance pattern of OP-MRSA strains is shown in Figure 1b. Again no strains were susceptible to fusidic acid. Resistance to vancomycin was 8%, chloramphenicol 35%, gentamicin 16%, erythromycin 68%, streptomycin 16%, cefotaxime 16%, clindamycin 58% and ciprofloxacin 10% (Figure 1b). The susceptibility pattern of CC-MRSA

Source	Number sampled	Number of S. aureus isolates	Prevalence of S. aureus (%)	Number of MRSA isolates	% of S. aureus that were MRSA
			Inpatients		
Wound swabs	515	164	31.8	70	42.6
Urine	51	16	31.4	7	43.8
Catheter swabs	132	41	31.1	18	43.9
Blood	37	12	32.4	5	41.6
Inpatient total	735	233	31.7	100	42.9
			Outpatients		
Wound swabs	267	88	32.9	33	37.5
Skin swabs	86	28	32.6	11	35.7
Soft tissue swabs	150	50	33.3	18	36.0
Outpatient total	503	166	33.0	62	37.3
			Community		
Nasal swabs	344	111	32.3	38	34.2

Table 1: Sources and prevalence of isolates of Staphylococcus aureus and MRSA.

Patient sample	IP-MRSA (%)	OP-MRSA (%)	CC-MRSA (%)
Gender Male Female Total	54 (54) 46 (46) 100	34 (55) 28 (45) 62	17 (45) 21 (55) 38
Age breakdown/Y > 30 31-49 > 50 Total	38 (38) 48 (48) 14 (14) 100	20 (32) 33 (53) 9 (15) 62	28 (74) 7 (18) 3 (8) 38

Table 2: Age and gender of patients that MRSA was isolated from IP-MRSA, Inpatient-MRSA; OP-MRSA, Outpatient-MRSA; CC-MRSA, Community carried-MRSA



diffusion assay

(a) Inpatient-MRSA, (b) Outpatient-MRSA (c) Community carried-MRSA ■, resistant; ■, intermediate; ■, sensitive.

VAN: Vancomycin; CHL: Chloramphenicol; GEN: Gentamicin; FUS: Fusidic Acid; ERY:Erythromycin; STR: Streptomycin; CTX: Cefotaxime; CLI: Clindomycin; CIP: Ciprofloxacin

strains (Figure 1c) showed that resistance to fusidic acid was 100%. Resistance to vancomycin was 0%, chloramphenicol 8%, gentamicin 5%, erythromycin 34%, streptomycin 8%, cefotaxime 21%, clindamycin 63% and ciprofloxacin 0% (Figure 1c).

Zone of inhibition of fusidic acid for IP-MRSA, OP-MRSA and CC-MRSA

The zone sizes for fusidic acid were heterogenous (Figure 2). The majority of strains had a zone size in the range 11-15mm for IP-MRSA and CC-MRSA (53/100, 53% and 10/38, 26% respectively) whereas most OP strains had zones of <5 mm (12/62; 20%). A smaller numbers

of strains had zone sizes in the range 26-30 mm which were 2/100 (2%), 0/62 (0%) and 0/38 (0%) respectively for IP-MRSA, OP-MRSA and CC-MRSA respectively (Figure 2).

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Determination of MIC

MIC's were determined for vancomycin and fusidic acid and the results are shown in Table 3. For fusidic acid the breakpoint is 2 mgl-1 and all the strains were resistant with three IP- and 4 OP-MRSA isolates showing a high level of resistance of \geq 64 mgl-1 (Table 3). For vancomycin resistance is defined as an MIC of >16 mgl-1 and no strains were resistant, even in the outpatient isolates in contrast to the results of the disk diffusion assay.

Multi-drug resistance in MRSA isolates

The highest number of resistance phenotypes were shown by the IP-MRSA followed by the OP-MRSA with the CC-MRSA showing the lowest incidence of multiple antibiotic resistance (Table 4). There were no common resistance patterns between the three grouos of isolates. The highest MDR pattern (23%) was observed for two antibiotics with the pattern fusidic acid/streptomycin. The maximum number of antibiotics resisted by one or two isolates was eight antibiotics with MDR pattern fusidic acid/gentamicin/chloramphenicol/ streptomycin/ erythromycin/cefotaxime/clindamycin and ciprofloxacin.

The most frequent MDR pattern (42%) for OP-MRSA strains was observed for five antibiotics. The maximum number of antibiotics resisted by single isolate was six antibiotics with the MDR pattern fusidic acid/streptomycin/erythromycin /gentamicin/ciprofloxacin/ cefotaxime, whereas the highest MDR pattern (34%) for CC-MRSA



Figure 2: Zones of inhibition for fusidic acid for IPHA-MRSA, OPHA-MRSA and CC-MRSA strains by disk diffusion assay

,	Inpatient	(IP); ■,	Outpatient	(OP); ■,	Community	carried	(CC).

	Number of strains					
MIC mg ml ⁻¹ Fusidic a		Fusidic aci	d Vancomycin		ı	
	IP-MRSA	OP-MRSA	CC-MRSA	IP-MRSA	OP-MRSA	CC-MRSA
<2	-	-	-	-	-	-
2	2	14	-	41	4	7
4	18	14	11	46	34	26
8	30	21	24	7	18	5
16	33	7	2	6	6	-
32	14	2	1	-	-	-
64	2	2	-	-	-	-
>64	1	2	-	-	-	-
Total	100	622	38	100	62	38

 Table 3:
 MIC values for vancomycin and fusidic acid for MRSA from inpatients, outpatients and community IP-MRSA, Inpatient-MRSA; OP-MRSA, Outpatient-MRSA; CC-MRSA, Community carried-MRSA

Number of antibiotic	Multi-resistant MRSA strains (number of isolates)					
resistances	Inpatient	Outpatient	Community Carried			
2	Fus/Str (23)	Fus/Ctx (9)	Fus/Cli (13)			
3	Fus/Ery/Ctx (12)	Fus/Ctx/Cli (9)	Fus/Ery/Cli (6)			
4	Fus/Ery/Str/Ctx (12)	Fus/Str/Ctx/Cip (9)	Fus/Ery/Ctx/Cli (13)			
5	Fus/Ery/Str/Ctx/Cip (12)	Fus/Ery/Ctx/Cip/Cli (26)	Fus/Ery/Chl/Ctx/Cli (6)			
6	Fus/Str/Chl/Ctx/Cip/ Gen (12)	Fus/Ery/Gen/Str/Ctx /Cip/ (9)	-			
7	Fus/Ery/Str/Chl/Ctx/ Cip /Gen (17)	-	-			
8	Fus/Ery/Gen/Str/ /Chl/Ctx/Cip/Cli (12)	-	-			

Table 4: Patterns of antibiotic resistance of multi-resistant MRSA isolates Cip: Ciprofloxacin; Chl: Chloramphenicol; Cli: Clindomycin; Ctx: Cefotaxime; Ery: Erythromycin; Fus: Fusidic acid; Gen: Gentamicin; Str: Streptomycin.

strains was observed for four antibiotics with the pattern fusidic acid/ vancomycin/ clindamycin/erythromycin. The maximum number of antibiotics resisted by single isolate or two isolates was five antibiotics with the pattern fusidic acid/ vancomycin/erythromycin/streptomycin/ clindamycin (Table 4).

Discussion

This study has shown the prevalence of MRSA in S. aureus infections in Libya as IP-MRSA, 43% OP-MRSA, 37% and CC-MRSA, 34% respectively. This is rather higher than a similar study of MRSA in a tertiary surgical and Trauma hospital in Bengazi, Libya which showed that that MRSA were 31% of S. aureus isolates [5]. Staphylococcal carriage rate was 32.3% which is slightly higher than in previous studies [11] but may reflect the restricted sample size. MRSA is an increasing important cause of morbidity and can easily spread from hospital to another hospital or community based facility and even spreading from country to another [13]. In this study no vancomycin resistance was found in either IP-MRSA or CC-MRSA strains while 5% of OP-MRSA strains were resistant by disc diffusion assay but the MIC values for these strains indicated sensitivity to vancomycin. This result suggests that vancomycin can be used to treat MRSA infections. Clindamycin resistance represented 17% for IP-MRSA, OP-MRSA, 58% and CC-MRSA, 63% which is higher than in previous studies in Libya [4]. This suggests that clindamycin can be used to treat IPH-MRSA infection but should be used with caution in OP and CC-MRSA infections. A study on detection of inducible clindamycin resistance to) phenotype among MRSA from Libya was reported and of the 128 MRSA isolates collected 24.2% were resistant to clindamycin, 63.2% isolates were resistant to erythromycin and 12 isolates (9.3%) exhibited the MLSBi phenotype [14]. The authors also emphasised that clindamycin could still be used to treat MRSA infection in Libyan hospitals. The findings from the present study suggest that sensitivity to clindamycin should be confirmed. Ciprofloxacin had a resistance of 0% in the CC-MRSA strains which means that it can be used to treat CC-MRSA infections successfully. Gentamicin resistance was 5% in CC-MRSA and 16% for OPH-MRSA strains which indicates that gentamicin could also be used to successfully to treat CC-MRSA and OPH-MRSA infections. These results agree with other studies in Libya by Shebani et al. [15].

There are no established standard international interpretation criteria for zone size for fusidic acid. Early studies on zone size interpretation indicated that the range $\leq 19 \geq 21$ mm as susceptible [16,17]. Interpretive zone diameter breakpoints for *S. aureus* according to the Swedish Reference Group for Antibiotics (SRGA) for fusidic acid using 50 µg disc were sensitive \geq 30 mm and resistant \leq 26 mm [18]. According to these criteria all the MRSA isolates were resistant. The latest BSAC 2013 version guidelines indicate that a zone size of 26-30 mm should be considered as susceptible. In this study BSAC–version 2013 guidelines [11] were used for zone size interpretation for fusidic acid

which is not stated in the CLSI guidelines. Even if interpretation of zone size was based on the previous investigators only 12% of IPHA-MRSA, 1.6% of OPHA-MRSA and 0% of CC-MRSA strains were susceptible which still shows a high rate of fusidic acid resistance. Resistance was confirmed by determination of MIC. The results confirmed the disk diffusion results and showed that most of the resistance was low level and that only 7 of the strains, all from inpatients or outpatients had high level resistance. Such resistance may be due to clonal spread of resistant strains as has been shown in Europe [19] and Taiwan [20]. Fusidic acid has been used widely in Libyan healthcare and community facilities for many years and is still currently used as a topical treatment for skin and soft tissue infection. In particular it is widely used and freely available to the general public and widely used for even minor skin infections. High usage of fusidic acid has been associated with increased levels of resistance elsewhere [21,22].

These results also demonstrated high rates of multi-resistant strains of MRSA in healthcare facilities. If MRSA are not properly identified, clinicians are forced to use different types of antimicrobials where many would have no beneficial effect on the patient and increase the pool of multi-drug resistant organisms such as MRSA. The availability of nearly all antimicrobials on an over-the counter basis in Libyan community settings in particular, the absence of prescription control policies or guidelines further complicates the problem. Further investigation to examine the presence of resistance genetic determinants (i.e., Fus B, Fus C and FusE; [23]) and mutations in Fus A are required. A surveillance study has shown evidence of wider mis-use of antimicrobials in Libyan healthcare and community facilities and will be reported elsewhere.

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