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Antimicrobial resistance profile and molecular characterization of methicillin-resistant staphylococcus isolates in Tripoli Central Hospital, Libya.

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ABSTRACT:

Background: Nosocomial infections caused by methicillin-resistant *Staphylococci* could lead to increased morbidity and mortality, but little is known about the prevalence of infections with these organisms in healthcare facilities and in the community in Tripoli. This study investigated the in vitro susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase negative staphylococci (MRCNS) to antimicrobial agents, and determined the molecular characteristics of MRSA.

Methods: This is a retrospective observational study aiming at determining the prevalence and antibiotic resistance pattern of (MRSA) and (MRCNS) isolated from non-duplicated clinical specimens in Tripoli Central Hospital (TCH) between June 2013 and June 2014. Isolates were identified using standard laboratory procedures. Antimicrobial susceptibility tests were carried out by disk diffusion method and automated systems. DNA of the MRSA isolates was used for PCR to determine the molecular analysis.

Results: 218 isolates of Staphylococci were obtained, 71.6% were coagulase positive staphylococci (CPS) and 28.4% were coagulase negative staphylococci (CNS). 39.7% of CPS were MRSA, while 75.8% of CNS were MRCNS. The rates of hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) among MRSA isolates were 61.3% and 38.7% respectively. A similar trend was detected among MRCNS isolates, where 74.5% were HA-MRCNS and 25.5% were CA-MRCNS. All the MRSA and MRCNS isolates were susceptible (100%) to vancomycin, tigecycline, linezolid, quinupristin/dalfopristin, daptomycin and moxifloxacin. Generally, hospital-acquired strains showed higher resistance rates than community-acquired ones to the most commonly tested non-beta-lactam antibiotics. 35.5% of all

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staphylococcal isolates exhibited *mecA*$^{+}$ gene and 12.9% expressed *mecC*$^{+}$. Meanwhile, 38.7% of MRSA isolates harbored both *mecA* and *mecC*. However, 12.9% of MSSA isolates were negative for both *mecA* and *mecC*. The *mecA* gene was detectable in 59.1% and 40.9 % of HA-MRSA and CA-MRSA isolates respectively.

Conclusion: Hospital-acquired MRSA and MRCNS isolates had higher resistance rates to non-beta lactam antimicrobial drugs than the respective community-acquired isolates. This was shown by early detection of *mecC* gene among MRSA isolates.

Keywords: Antibiotics resistant staphylococci; MRSA; MRCNS; *SCCmec*; *mecA*; *mecC*.

INTRODUCTION

Staphylococcus genus includes some of the major pathogens that are resistant to antibiotics. They colonize the human skin and mucoal surfaces. The disease spectrum caused by *Staphylococcus aureus* ranges from mild to moderate skin and soft tissue infections to life-threatening or fatal systemic infections such as pneumonia and septicaemia [1]. Increasing numbers of MRSA were isolated in community and hospitals settings since the introduction of beta-lactam antibiotics [2]. MRSA has been associated for many years with increased hospital stay and other health care problems. However, in the 1990s, CA-MRSA has appeared with a large number of characteristics different from previously known HA-MRSA. CA-MRSA can spread rapidly among healthy individuals, and outbreaks of CA-MRSA infections have been reported worldwide [1]. MRCNS has recently received more attention as a potential pathogen, specifically for nosocomial infections where a significant increase in the rate of infections is noted and they have recently started to gain resistance to many widely used antibiotics [3]. The resistance was the result of *S. aureus* acquiring the *mecA* gene, which encodes for an altered penicillin-binding protein *(*PBP2a) having a lower affinity for the β-lactam antibiotics; thus allowing the survival of *S. aureus* in the presence of methicillin [4]. *MecA* does not reside on a plasmid but on the chromosome in a large mobile genetic element called *Staphylococcal Cassette Chromosome mec* (SCC*mec*). Seven types of SCC*mec* were identified: types IV, V, VI, and VII code for β-lactam antibiotic resistance, while SCC*mec* types I, II, and III cause resistance to multiple classes of antibiotics, due to additional integrated drug resistance genes [5]. The HA-MRSA and CA-MRSA have been proven to be genetically distinct with respect to the SCC*mec* type; HA-MRSA often carried SCC*mec* types I, II or III, while CA-MRSA often harboured SCC*mec* types IV or V [6]. Although multiple methods of detection of methicillin resistance have been developed, molecular identification of the *mecA* gene is the most reliable reference method of detecting MRSA isolates [7]. A novel *mecA* homologue, *mecA*$_{LGA251}$, has been identified encoded in a new *SCCmec* element designated type XI among human and bovine MRSA isolates in the UK and Denmark [8]. The *mecA*$_{LGA251}$ named *mecC* was 70% identical to *S aureus* *mecA* homologues and was initially detected in 15 *S aureus* isolates from dairy cattle in England [8]. The presence of PBP2a means MRSA is not only resistant to methicillin but also to all β-lactam antibiotics. Few studies have been conducted to investigate the antimicrobial susceptibility or the molecular analysis of the MRSA strains in the healthcare setting in Tripoli [9,10].

METHODS AND MATERIALS

This was a retrospective study conducted from June 2013 to June 2014 in Tripoli Central Hospital (TCH), Tripoli-Libya. 1100 clinical specimens (pus swabs, drains, blood cultures, urine, sputum, vagina swabs, nasal swabs, ear swabs, throat swabs and urethral discharge) were collected from in-patients and out-patients departments.

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Staphylococci isolates were cultured and identified by conventional methods. The data of the patients were obtained from patients' medical record files and laboratory investigation registry. Data of the study subjects included basic demographic profiles, ward admitted, type of specimen, length of hospital stay, clinical notes and details of risk factors associated with the criteria of HA-MRSA infections. MRSA and MRCNS strains were subjected to microbiological and biochemical tests for identification including chromogenic MRSA media (BioMerieux–France), oxacillin screening media supplemented with 4% NaCl and oxacillin (6 µg/ml) (Becton Dickinson BDBBL). MRSA isolates were further classified into HA-MRSA and CA-MRSA according to CDC definition, HA-MRSA was defined as either MRSA isolated from patients of more than 48 hours after hospital admission, or those who had a history of hospitalization, surgery, dialysis, or who had indwelling vascular catheter in place at the time of culture. Patients with none of the above mentioned criteria were classified as having CA-MRSA infection [11].

Antibiogram to all methicillin resistance *Staphylococci* isolates was performed by automated systems BD phoenix and VITEK2 (Bio Merieux, France) using manufacturer criteria. In addition, modified Kirby Bauer disc diffusion method on Mueller-Hinton agar (bioMe´rieux, France) was used for the following antibiotics: linezolid (30µg), quinupristin/dalfopristin (15µg), mupirocin (5µg) according to Clinical and Laboratory Standard Institute (CLSI) guidelines [12] and to the US Food and Drug Administration (FDA) criteria, a tigecycline (15µg) zone diameter of ≥ 19 mm is considered susceptible for *S. aureus* [13]. Both oxacillin (1 µg) and cefoxitin (30 µg) (Oxoid, Basingstoke, UK) were used for detection of resistance to methicillin in accordance to CLSI guidelines to confirm the MRSA and MRCNS [12]. Staphylococcus *aureus* ATCC 25923 standards were used as the control strain.

Molecular analysis of MRSA: DNA isolation and PCR detection:

The MRSA isolates were cultured for 48 hours on tryptic soy broth, and for 24 hours on mannitol salt agar. Single colonies were boiled in 200 µl of diethylpyrocarbonate (DEPC) - treated water and subsequently centrifuged for 5 min at 13000 rpm. In order to confirm previous MRSA phenotypic identification, the isolates were screened by means of specific duplex PCR for *S. aureus* [14] and *SCCmec* carrying the *mecA* and *mecC* genes [15,16]. DNA amplification of each sample was performed in a final volume of 25µl. The mix was: 5µl of DNA, 12.5µl of *accu Prime™ SuperMix* II (Invitrogen), 6.5µl of DEPC Water, 0.5µl of *primers* (50 µM) as in (Table 1).

Table 1: Primers' sequences used for amplification of *SCCmec* genes

Primers names	Genes	Sequences (5'-3')	Predicted amplicon sizes (bp*)	References
***au-F3* (forward)**	*S. aureus*	TCGCTTGCTATGATTGTGG	108	[14]
***au-nucR* (reverse)**		GCCAATGTTCTACCATAGC		
***mecA* (forward)**	*mecA*	AAAATCGATGGTAAAGGTTGGC	533	[15]
***mecA* (reverse)**		AGTTCTGCAGTACCGGATTTGC		
***mecC* (forward)**	*mecC*	GAAAAAAAGGCTTAGAACGCCTC	138	[16]
***mecC* (reverse)**		AAGATCTTTTCCGTTTTCAGC		

**** bp = base pair; mec,= methicillin resistance determinant.***

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The PCR protocol for the detection of *S. aureus*/*mec*A gene was A 95 °C x 1 minute, (94 °C x 1 minute, 55 °C x 1 minute, 72 °C x 1 minute) x 40 cycles, 72 °C x 10 minutes. On the other hand, 95 °Cx 1 minute, (94 °C x 30 seconds, 55 °C x 30 seconds, 72 °C x 30 seconds) x 30 cycles, 72 °C x 10 minutes was used for detection of *mec*C gene. DNA extracted from ATCC 33591 strain was used as positive control and a negative control (DEPC water) was included. PCR products were visualized by electrophoresis on 1.5% agarose gel.

STATISTICAL ANALYSIS

Statistical analysis was computerized using the Statistical Program for Social Sciences (SPSS version 22) that was used for data entry and analysis. Descriptive statistics of the results were presented as frequencies, and percentages. Data were compared using the Chi-square test and Fisher's exact test, if appropriate. $P<0.05$ was considered statistically significant.

RESULTS

During this study, a 218 staphylococcal isolates were obtained out of 1100 clinical samples collected from different hospital wards; 156 out of 218 samples (71.6%) were CPS, while 62 out of 218 samples (28.4%) were CNS. The MRSA isolates constituted 62 samples of 156 of the CPS (39.7%) compared with MRCNS isolates (47/62; 75.8%). The distribution of the isolates according to the classification whether they were hospital or community acquired was as follows: 61.3% HA-MRSA, 38.7% CA-MRSA, 74.5% HA-MRCNS and 25.5% CA-MRCNS (Table 2). The highest percentage of HA-MRSA isolates was detected from surgical ward (30.6%) followed by medical ward and trauma ICU (6.5%) each; whereas the highest percentages of CA-MRSA isolates were obtained from the dermatology 9.7% and traumatic wards 8.1% (Table 2). The highest percentage of HA-MRCNS isolates was obtained from the surgical 31.9% and medical wards 21.3%; whereas the highest rate of CA-MRCNS isolates was in ENT ward (10.6%), and medical ward (6.4%). Most of the MRSA 24.3% and MRCNS isolates 17.4% were demonstrated from pus swabs.

The differences between both hospital acquired and community acquired MRSA or MRCNS were statistically significant with $P<0.05$ (Table 2).

Table 2: Distribution of hospital acquired (HA) and community acquired (CA) MRSA and MRCNS isolates according to hospital wards.

Ward	HA-MRSA=n (%)	CA-MRSA=n (%)	HA-MRCNS=n (%)	CA-MRCNS=n (%)
Medical	4 (6.5)	2 (3.2)	10 (21.3)	3 (6.4)
Surgical	19 (30.6)	3 (4.8)	15 (31.9)	0
ENT	1 (1.6)	3 (4.8)	2 (4.3)	5 (10.6)
Dermatology	3 (4.8)	6 (9.7)	3 (6.4)	2 (4.3)
Orthopaedic	2 (3.2)	3 (4.8)	0	0
Urology	0	1 (1.6)	0	1 (2.1)
Trauma	1 (1.6)	5 (8.1)	5 (10.6)	1 (2.1)
Infectious	3 (4.8)	1 (1.6)	0	0
Surgical ICU	1 (1.6)	0	0	0
Trauma ICU	4 (6.5)	0	0	0
Medical ICU	0	0	0	0
Oncology	0	0	0	0
Total	**38 (61.3%)**	**24 (38.7%)***	**35 (74.5%)**	**12 (25.5%)***

* Statistically significant ($P<0.05$) from the correspondent MRSA. ICU, Intensive care unit

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Vancomycin, tigecycline, linezolid, quinupristin/dalfopristin, daptomycin and moxifloxacin, showed excellent *in-vitro* bactericidal activity with no demonstrable resistance (0%) against all tested isolates (Figures 1 & 2). Low level of resistance rates (0%, 4.8%, and 4.8%) were seen with all MRSA isolates to nitrofurantoin, mupirocin and teicoplanin respectively (Figure 1). On the other hand moderate level of resistance of MRCNS isolates to nitrofurantoin, teicoplanin clindamycin and mupirocin were detected (2.1%, 10.6%, 17.0% and 19.1% respectively- Figure 2). The HA-MRSA and HA-MRCNS strains were more resistant than counterparts CA-MRSA and CA-MRCNS to the most tested non β-lactam antibiotics. However, no significant differences between HA-MRSA and CA-MRSA (Figure 1) or between HA-MRCNS and CA-MRCNS (Figure 2) for all antibiotics were detected.

Figure 1: The resistance profile of the HA-MRSA and CA-MRSA strains to various antibiotics. SXT =Trimethoprim/Sulfamethoxazole, SYN=Qunsopristine/dalphopristine, AMC=Amoxicillin and calvulanic acid.

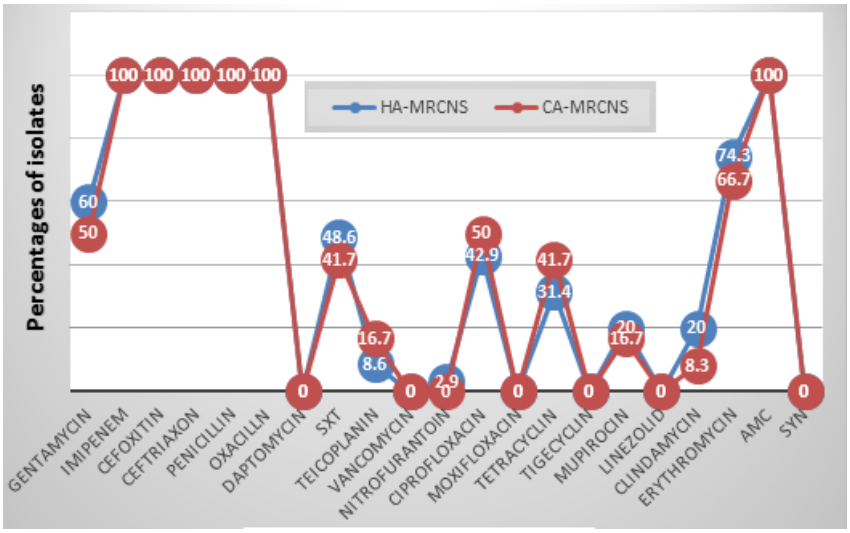

Figure 2: The resistance profile of the HA-MRCNS and CA-MRCNS isolates to various antibiotics.

Citation DOI: 10.21502/limuj.010.02.2017 LIMUJ, Volume 2, PP 74-83, 2017

The MRSA isolates were further analyzed by specific duplex PCR. In addition, the molecular analysis was categorized according to the hospital acquired and the community acquired isolates (Table 3).

Table 3: Distribution of the *SCCmec* genes of MRSA isolates according to hospital acquired and community acquired (HA and CA)

Molecular analysis of *SCCmec genes**	**HA-MRSA**	**CA-MRSA**	**Total**
MRSA - *mecA*	13 (21.0 %)	9 (14.5 %)	22 (35.5 %)
MRSA - *mecC*	4 (6.5 %)	4 (6.5 %)	8 (12.9 %)
MRSA - *mecA/mecC*	16 (25.8 %)	8 (12.9 %)	24 (38.7 %)
MSSA - (*mecA/mecC*) negative	5 (8.1 %)	3 (4.8 %)	8 (12.9 %)
Total	38 (61.3 %)	24 (38.7 %)	62 100 %)

**** SCCmec, Staphylococcal Cassette Chromosome mec; mec, methicillin resistance determinant***

DISCUSSION

In the present study, the prevalence of MRSA among CPS was found to be 39.7% (62/156). Our finding is lower than previously reported in Tripoli (54%) [9], but higher than that reported from Benghazi (31%) [10], and from Egypt (21%) [17]. Meanwhile, a report published from Tunis also revealed nearly similar rate in MRSA (41%) [18]. Our data show that the rate of MRSA among hospital isolates of CPS (61.3%) was much higher than that among community isolates (38.7%). These results are closely similar to those obtained from Saudi Arabia being (69.0%) for HA-MRSA and (31%) for CA-MRSA [19]. CA-MRSA infections were first reported in the early 1990s and then spread all over the world; however, their prevalence varies from one country to another [20]. CA-MRSA is capable of causing a wide range of infections and appears to be predominantly carrying the *mecC* gene [21]. In addition to being found in humans, *mecC* MRSA has also been found in a range of other host species [8]. In this study the rate of MRCNS isolates was 75.8%, which is similar to results in previous reports done in other countries such as Turkey (74.4%), France (71%), and Germany (67.4%) [22]. In the current study, the highest rates of HA-MRSA and HA-MRCNS isolates were obtained from surgical ward (30.6% and 31.9% respectively); whereas the highest rates of CA-MRSA isolates were obtained from dermatology ward (9.7%). A previous study carried out in south India 2013 [23] showed that the highest rates of isolations of methicillin resistant staphylococci were from pus samples, which is in agreement with our finding that most MRSA (24.3%) and MRCNS (17.4%) isolates were obtained from pus swabs, but other investigators reported sputum as the most frequent source of MRSA [24].

In general, we found nearly similar rates of resistance to each tested antibiotic, for both MRSA and MRCNS strains. The MRSA and MRCNS isolates in our study had significantly higher level of resistance ($P < 0.05$) to the antimicrobials tested as compared to MSSA and MSCNS. This finding probably is a reflection of the presence of associated antibiotic-resistance gene clusters among the methicillin resistant isolates. Comparable results were obtained by different

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other authors [3, 25]. The most active agents in *in-vitro* susceptibility tests against MRSA and MRCNS isolates in this study were vancomycin, tigecycline, linezolid, quinupristin/dalfopristin, daptomycin and moxifloxacin with 100% susceptibility reported for each. A similar activity was found for tigecycline, linezolid and quinupristin/dalfopristin with no evidence of resistance (0%) when tested against MRSA and MRCNS isolates [26, 27]. However, Zorgani *et.al*. found that the susceptibility of tigecycline, linezolid and quinupristin/dalfopristin against MRSA isolates were (95.8%, 96.5%, 97.2%) respectively [9]. In our study, both CA-MRSA and HA-MRSA were resistant to β-lactam antibiotics and were susceptible to most non-β-lactam antimicrobial drugs**.** However, the HA-MRSA isolates were more resistant to gentamicin, timethoprim/sulfamethoxazole and tetracycline compared to CA-MRSA isolates but the differences were not statistically significant ($P > 0.05$).

Our data demonstrate a high percentage (35.5%) of *mec*A MRSA isolates, most of these were associated with hospital strains (21.0%) compared to community strains (14.5%). The low percentage of *mecC* MRSA isolates (12.9%) were equally distributed, 4 isolates each, between HA-MRSA and CA-MRSA. This percentage for *mecC* MRSA isolates is high when compared with that of a study from Denmark, being 1.9% in 2010 increasing to 2.8% in 2011 [21]. We found eight (12.9%) isolates that are negative for (*mecA/mecC* genes) despite the fact that they express the MRSA phenotype. Similar observation of discrepancy, but at a lower rate, between phenotypic and genotypic methods in identification of MRSAs was reported in a surveillance study conducted in 2010-2012 by researchers from South Africa [28], where 2/1457 phenotypically characterized MRSA isolates were found to be lacking the *mec*A and *mec*C genes by PCR and in the SCC*mec* typing assay. Also researchers in the United Kingdom [29], reported that 1.1% of 379 *mec*A/*mec*C-negative MRSA isolates, processed at the Cambridge Microbiology and Public Health Laboratory during the period 2006-20012, were resistant to oxacillin but sensitive to cefoxitin. In Ireland, two clone complex 130 (CC130) MRSA isolates from patients in Irish hospitals were phenotypically identified as PBP2a positive but lacked *mecA* by conventional PCR, the isolates were later identified as MSSA using PCR assay [30]. The identification of a *mecA* gene in MRSA that is not detected by *mecA* PCR or other molecular typing method is alarming. Thus, with regard to infection prevention and therapy, isolates harbouring the SCC*mec* element should be treated as MRSA, even though they are identified by PCR to be *mecA/mecC*-negative [28, 29, 30].

Further study may be carried out to characterise those strains by *Staphylococcus aureus* Protein A (spa) typing and whole genome sequencing.

CONCLUSION

HA-MRSA and HA-MRCNS isolates were more common than CA-MRSA and CA-MRCNS isolates. Vancomycin, tigecycline, linezolid, quinupristin/dalfopristin, daptomycin and moxifloxacin are drugs of choice against MRSA and MRCNS isolates with 100% sensitivity. There is a need for continuous monitoring of the antimicrobial susceptibility pattern of MRSA and MRCNS for the selection of appropriate therapy. With regard to infection prevention and therapy, isolates harbouring SCC*mec* element should be treated as MRSA, even they are identified by PCR to be *mecA* gene negative. Further study should be carried out to determine the relation between the community acquired isolates of MRSA and MRCNS and *SCCmec* element *mecC* genes.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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ملخص باللغة العربية

نشاط مقاومة المضادات الحيوية والتحليل الجزيئي للمكورات العنقودية المقاومة للميثيسيلين المعزولة من مستشفى طرابلس المركزى، طرابلس – ليبيا

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الخلفية: عدوى المستشفيات التي تسببها بكتريا المكورات العنقودية المقاومة للميثيسيلين يمكن أن تؤدي إلى زيادة معدلات المرض والوفيات. لا يُعرف إلا القليل عن مدى انتشارها في المستشفيات الليبية. هذه الدراسة اجريت لمعرفة مدى حساسية المكورات العنقودية المقاومة للميثيسيلين المعزولة سريرياً للموبيرسين ولينزوليد وكوينبريستين/دالفوبريستين وتيغسيكلين وبعض المضادات الحيوية الاخرى.

الطرق: تم التعرف على المكورات العنقودية المقاومة للميثيسيلين المعزولة عن العينات السريرية من مستشفى طرابلس المركزي بين يونيو 2013 ويونيو 2014 باستخدام التجارب المخبرية القياسية، وأجريت اختبارات الحساسية المضادة للميكروبات بطريقة نشر القرص والنظم الآلية. كذلك تم استخدام الحمض النووي فى تفاعل البلمرة المتسلسل للعزلات موجبة التخثر لتحديد التحليل الجزيئي لها.

النتائج: تم عزل 218 من المكورات العنقودية حيت كانت إيجابية التخثر 156 (71.6٪) و62 (28.4٪) سالبة التخثر. ومن بين موجبة التخثر كانت نسبة المكورات العنقودية المقاومة للميثيسيلين (39.7%) وكانت نسبة العزلات المرتبطة بمرضى المستشفى (61.3%) والمرتبطة بمرضى العيادات الخارجية (38.7%). وأما عن المكورات العنقودية المقاومة للميثيسيلين سالبة التخثر كانت 62/47 بنسبة (75.8%) وكانت نسبة المرتبطة بمرضى المستشفى (74.5%) والمرتبطة بمرضى العيادات الخارجية (25.5%).

الفانكومايسين ولينزوليد وتيغيسيكلين وكينوبريستين/دالفوبريستين ودابتوميسين ومكسيفلوكساسين الأكثر فعالية حيث أظهرت حساسية (100%) ضد المكورات العنقودية المقاومة للميثيسيلين. عزلات مرضى المستشفى أكثر مقاومة للمضادات الحيوية التي لا تحتوي على حلقة البيتا لاكتام من عزلات مرضى العيادات الخارجية. بالنسبة للتحليل الجزيئ للمكورات العنقودية المقاومة للميثيسيلين موجبة التخثر كانت نسبة جينات *mecC* (12.9 %) ونسبة عالية (35.5 %) من جينات*mecA* وكان انتشارها بمرضى المستشفى (21.0%) ومرضى العيادات الخارجية (14.5%).

الاستنتاج: فانكومايسين، تيغيسكلين، لينزوليد، كينوبريستين/دالفوبريستين، دابتوميسين و موكسيفلوكساسين هي الأدوية المفضلة ضد المكورات العنقودية المقاومة للميثيسيلين موجبة التخثر وسالبة التخثر. الرصد المستمر لنمط حساسية المضادات لهذه الميكروبات لاختيار العلاج المناسب. فيما يتعلق بالوقاية من العدوى والعلاج، العزلات التي تؤوي عنصر *SCCmec* ينبغي أن تعامل على أنها مكورات عنقودية مقاومة للميثيسيلين موجبة التخثر حتى إذا تم التعرف عليها بواسطة تفاعل البلمرة المتسلسل أنها سالبة جينات *mecA*.

الكلمات المفتاحية: مقاومة المضادات الحيوية للمكورات العنقودية، المكورات العنقودية المقاومة للميثيسيلين موجبة التخثر وسالبة التخثر، جينات *mecA* و *mecC* و *SCCmec*.

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