Prevalence of methicillin-resistant *Staphylococcus aureus* and/or intermediate susceptibility to vancomycin isolated from private laboratories in Annaba "Algeria"

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) represents the severe causal agent in nosocomial infections that are becoming increasingly difficult to cure due to their emerging resistance to all current antibiotic classes. The aim of this study is to determine the frequency of MRSA strains and GISA as well as resistance profile to different families of antibiotics. 67 strains of MRSA are isolated from different pathological origins. The isolation and the identification of *S. aureus* strains were based on conventional methods. The resistance to methicillin of these strains was detected by the method of disk diffusion in Mueller-Hinton and a screening by oxacillin (6 µg/ml). Furthermore, a study of the resistance of these strains to different families of antibiotics is done. Decreased sensitivity to glycopeptides of suspected strains was confirmed after determination of the MIC to vancomycin by E-test. The study has shown that 67 strains resistant to methicillin were identified among the 150 strains isolated (44,6%). The MRSA were isolated from pus (71, 6%), urine (17, 9%) and vaginal samples (10,4%). These MRSA strains expressed resistance to different antibiotic families. We identified two MRSA strains (M04 and M47) showing reduced glycopeptides susceptibility. Since the multi-drug resistant MRSA strains are not negligible, a regular supervision is necessary. The "GISA" is an observation phenomenon that should better be defined in terms of detection and prevalence.

Keywords: MRSA, Glycopeptides, GISA, multi-drug resistance, resistance profile

INTRODUCTION

Since the first infected cases with methicillin-resistant *Staphylococcus aureus* (MRSA) reported in 1961 in England, MRSA have been described successively, first in mainland Europe, America and then in the worldwide [1]. In the early eighties, the proportion of MRSA strains on all the strains of *S. aureus* was less than 3%. Ten years later, MRSA strains were a major problem in many hospitals in the United States and Europe, where the proportion of MRSA strains was 40% [2]. MRSA is one of the main pathogens that are associated with serious nosocomial infections because these strains generally show a multi antibiotic resistance which limits the possibility of treatment [3,4]. MRSA has also been spread outside of the hospital environment and it now appears in the community without any identifiable risk factors [5]. Methicillin resistance in *S. aureus* is due to the acquisition of the meca gene, which encodes the low affinity of penicillin-binding protein 2a [6]. This methicillin resistance also causes resistance to all other penicillins and cephalosporins (2). Since 1980, gentamicin resistant strains have appeared and spread rapidly around the world [7]. Then in the 1990s, the frequency of isolation of strains susceptible to gentamicin rose again to become a great majority today (84% according to the data CCLIN Paris-Nord 1998) [7].
Finally, since the late of 90s, strains with intermediate susceptibility to glycopeptides (GISA) appeared and became emerging [8]. This study aims to determine both the frequency of MRSA and GISA strains isolated and characterize their phenotypes of resistance to other antibiotics.

**EXPERIMENTAL SECTION**

**Bacterial strains:**
A total of 150 *S. aureus* were collected from different pathological origins (pus, urine, vaginal sample) of community infections in Annaba which were isolated between 2010 and 2012. Each strain was cultured on agar Chapman and incubated at 36 °C for 18 to 24 hours.

**Identification of the species *S. aureus*:**
The identification of the species *S. aureus* was based on the following microbiological tests: microscopic observation in the fresh state and after Gram staining, the catalase, fermentation of mannitol, coagulation of rabbit plasma test and the API Staph system.

**Antibiotic susceptibility test:**
Antibiotic resistance was determined by the disk diffusion method (Bio-Rad, France) in Mueller-Hinton agar (Bio-Rad) according to the recommendations outlined by the CA-SFM 2013 (Comité de l’Antibiogramme de la Société Française de Microbiologie).

The tested antibiotics were: Penicillin G (PG-6 µg), Oxacillin (Ox-5 µg), Cefoxitin (FOX-30 µg), Gentamicin (GM-15 µg), Tobramycin (TOB-6 µg), Amikacin (AK-30 µg) Kanamycin (K-30U1), Tetracycline (TE-30µg), Lincomycin (MY-15 µg), Erythromycin (E-15 µg), Pristinamycin (PR-15 µg) Chloramphenicol (C-30 µg), Ofloxacin (OFX-5 µg), Fusidic acid (FA-10 µg), Vancomycin (VA-30µg), Teicoplanin (TEC-30 µg), Rifampicin (RA-50 µg), Minocycline (MH-30U1), Fosfomycin (FO-50 µg), Trimethoprim (W-5 µg) and Trimethoprim-sulfamethoxazole (cotrimoxazole) (SXT-1.25 / 23.75 µg).

**Phenotypic detection of methicillin resistance:**

**Method of oxacillin disk diffusion:**
An oxacillin disk (1ug) was applied on a MH agar supplemented with 2% NaCl for the detection of MRSA according to the directives of CLSI 2012[9]. After incubation at 36 °C for 24 hours, strains were considered resistant if the inhibition diameter was ≤10 mm, intermediate if the diameter was 11-12 mm and sensitive for diameters ≥13 mm[10,11].

**Method of Cefoxitin disk diffusion:**
This method was performed by the use of cefoxitin disk (30ug) on MH agar. An inhibition zone ≤ 21 mm reads Methicillin resistance and a diameter ≥ 22 mm indicates sensitivity[11].

**Oxacillin screening Test:**
A 10 µl of bacterial suspension prepared at 0.5 McFarland was inoculated by spot onto Muller-Hinton agar containing 4% NaCl and 6µg/ml oxacillin and incubated at 36 °C for 24 hours. The growth of more than a colony is sufficient to determine methicillin resistance [12,13,14].

**Detection of MLSb resistance:**
To identify the MLSBi phenotype, the D-test was performed. A lawn culture of the isolate which was adjusted to 0.5 Mcfarland’s concentration was made on a Mueller Hinton agar plate and discs of clindamycin (2µg) and erythromycin (15µg) were placed at a distance of 15mm (edge to edge) as per the CLSI recommendations 2009, along with routine antibiotic susceptibility testing.

D Positive (iMLSB Phenotype): Inducible resistance to clindamycin was manifested by flattening or blunting of the clindamycin zone adjacent to the Erythromycin disc, giving a D shape

**Determination of vancomycin MIC by E-test:**
The selection of strains with reduced susceptibility to glycopeptides and confirmation of this reduction were performed according to the recommendations of CA-SFM 2013. After incubation for 24 or 48 hours depending on the composition of the medium, the MIC value corresponded to the intersection of the two ellipses where the
inhibition of growth was completed. The presence of microcolonies within the ellipse must be considered. This phenomenon is mostly observed with heterogeneous resistance to vancomycin. A strain *S. aureus* ATCC 25923, susceptible to glycopeptides was used as a negative control and a laboratory strain of *Enterococcus gallinarum* resistant to glycopeptides was used as a positive control [15,16].

The strain was categorized as sensitive if MIC value was less than or equal to 4 µg/ml, intermediate for MIC values ranging 4< MIC ≤ 8 µg/ml and resistant for MIC values greater than 8 µg/ml

**RESULTS AND DISCUSSION**

Among 150 strains of *S. aureus*: 67 strains (44.6%) were MRSA, forty-eight (71.6%) MRSA were isolated from pus, 12 strains (17.9%) from urine and 7 strains (10.4%) from vaginal specimens.

Among 67 strains of MRSA which were obtained from patients of determined age: 52 (77.6%) were isolated from patients aged between 18 and 65 years, 15 (22.3%) from children aged between 1 year old to 17 years. Most strains were isolated from males patients 48 (71.6%).

The percentage of antibiotic resistance of the 67 MRSA tested are reported in Table 1. (The strains with intermediate susceptibility were considered resistant to antibiotics in the expression of results: I = R).

<table>
<thead>
<tr>
<th>ATB</th>
<th>Resistant MRSA %</th>
<th>sensitive MRSA %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Penicillin</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>22.3</td>
<td>77.6</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>59.7</td>
<td>40.2</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin</td>
<td>34.3</td>
<td>56.6</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>46.2</td>
<td>53.7</td>
</tr>
<tr>
<td>Pristinamycin</td>
<td>4.4</td>
<td>95.5</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>68.6</td>
<td>31.3</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>32.8</td>
<td>67.1</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>74.6</td>
<td>25.3</td>
</tr>
<tr>
<td>Minocycline</td>
<td>83.4</td>
<td>16.6</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>12</td>
<td>88</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>34.3</td>
<td>65.6</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>46.2</td>
<td>53.7</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>46.2</td>
<td>53.7</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole Fusidic acid</td>
<td>65.6</td>
<td>34.3</td>
</tr>
</tbody>
</table>

To characterize more precisely the set of MRSA strains studied, we analyzed their phenotype of resistance to aminoglycosides and macrolides.

The study of resistance phenotypes of our MRSA to aminoglycosides showed three types; involving three inactivating enzymes (Table 2). All MRSA strains were resistant to kanamycin. 23 strains (34.3%) had a phenotype K, due to the production of the enzyme-Aminoglycoside phosphotransferase APH (3')-III. 26 (38.8%) were resistant to kanamycin and tobramycin, KT is the phenotype expressed by the production of the enzyme-Aminoglycosides nucleotidyltransferases ANT (4') (4''), while 16 strains (23.8%) expressed the KTG phenotype and were resistant to the three antibiotics (kanamycin, tobramycin, gentamicin) due to the bifunctional enzyme APH (2'') - Aminoglycosides acetyltransferases AAC (6')[17].

<table>
<thead>
<tr>
<th>Kanamycin</th>
<th>Tobramycin</th>
<th>Gentamicin</th>
<th>Mechanism Inferred</th>
<th>MRSA (%) n=67</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>S</td>
<td>S</td>
<td>Sensitive</td>
<td>0</td>
</tr>
<tr>
<td>R</td>
<td>S</td>
<td>S</td>
<td>APH(3')-III</td>
<td>23 (34.3)</td>
</tr>
<tr>
<td>R</td>
<td>R</td>
<td>S</td>
<td>ANT (4')(4'')</td>
<td>26 (38.8)</td>
</tr>
<tr>
<td>R</td>
<td>R</td>
<td>R</td>
<td>APH(2'')- AAC (6')</td>
<td>16 (23.8)</td>
</tr>
</tbody>
</table>

About macrolides (Table 3), it is noted that 31 strains (46.2%) were resistant to erythromycin. The MLSb phenotype involves cross-resistance to macrolides, lincosamides and streptogramin B by methylation of 23S ribosomal RNA. The phenotype MLSb can be induced by erythromycin in which case an antagonism between erythromycin and
Clindamycin can be observed; it is the case of 27 (40.2%) MRSA strains. Resistance to erythromycin can also be expressed constitutively while also conferring resistance to clindamycin 05 strains (07.4%).

<table>
<thead>
<tr>
<th>Erythromycin</th>
<th>Clindamycin</th>
<th>Pristinamycin</th>
<th>Mechanism Inferred</th>
<th>MRSA (%) n=67</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>S</td>
<td>S</td>
<td>Sensitive</td>
<td>34 (50.7)</td>
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<tr>
<td>R</td>
<td>S</td>
<td>S</td>
<td>MLSB induct</td>
<td>27 (40.2)</td>
</tr>
<tr>
<td>R</td>
<td>R</td>
<td>S</td>
<td>MLSB constit</td>
<td>05 (07.4)</td>
</tr>
</tbody>
</table>

Analysis of the results showed that the most active antibiotic was pristinamycin (4.4% resistance) followed by lincomycin (9%) and chloramphenicol (12%).

According to the criteria of suspicion of reduced sensitivity to glycopeptides which are routinely determined by the agar diffusion method when:
- The diameter of the inhibition zone is <17 mm around the disc of one of the two glycopeptide,
- The diameter of the zone of inhibition around a disc of teicoplanin is lower by at least 3 mm for that of vancomycin
- Some colonies are present in the Zone of inhibition of one of the two glycopeptide

11 strains (16.5%) were suspicious beings GISA only two were confirmed after determination of the MIC of vancomycin. The population analysis showed that these two strains were heterogeneous-VISA (Figure 1).

![Figure1: Determination of vancomycin MIC by E-test](image)

MRSA represents one of the most disturbing and the most consistent aspects in human infections. MRSA is, everywhere in the world, a major cause of nosocomial infections [18] with a recent community outreach [6,19,20]. Treatment of these infections is becoming more difficult due to the emergence of multidrug-resistant strains [16,21].

The rate of MRSA isolated in our study was 44.6%; the prevalence was statistically higher than that of 32.7% obtained by Aouati et al [17] in university hospital Ben Badis Constantine and even higher than in another study at Charles Nicolle hospital in Tunisia (10%) [22] and that reported by Elazhari et al [5] in Casablanca (Morocco) (10%). In Africa the prevalence of MRSA is variable. It was 36% in Benin in 2006 before declining in 2008 with a rate of 14, 5% while in Algeria, the rate of MRSA is increasing with 4.5% in 2002 [23]33, 2% in 2004 [24], 45% in 2006 (25) and 52% in 2009 [26].

In France the rate of MRSA (32%) was observed by «le réseau des microbiologistes Ile de France». The same observation is made by ColBVH «Collège de Bactériologie Virologie Hygiène des Hôpitaux» (30%), the network of Microbiology CCLIN Paris North during the investigation “multi-resistant bacteria” (33%) and ONERBA (35%) [27,28].

In the Algerian hospitals, Amazian et al [29] reported 18.6% a rate of compliance to hygiene rules lack of available devices for hand hygiene and insufficient knowledge of adequate hygiene practices, which could account for the important diffusion of MRSA.

As a result, we can say that the eastern area of Algeria is at a level of some European countries such as: Greece (44%), Italy (38%), Spain (38%), Great Britain (44%) and Ireland (42%) [30]. However, the rate of resistance...
remains lower than that in countries with high prevalence of MRSA such as USA and Senegal which have respective rates of 70% and 72% [31,32].

Other European countries maintain a low prevalence of MRSA, such as Belgium (13%) and Germany (5%) [33], and even below the threshold for Holland, Denmark, Sweden and Finland [30]. This situation is explained by the importance of the commitment of hospitals in substantial programs of anti-MRSA [30,33]. These programs are developed and practiced for a long time; they concern the surveillance of nosocomial infections and their prevention, as better risk management of their occurrence and best control and use of antibiotics.

The rate of MRSA found in our study is influenced by the age of patients, where there is a prevalence of methicillin resistance among adults (77.6%), same results obtained by Garnier et al [34].

A predominance of MRSA in males was observed (71, 6%), this result agrees well with that (59%) found in a study in university hospital Mustapha Bacha in Algiers by Antri et al [35].

Our strains are essentially found in samples of pus (71.6%), however, other investigations have reported that a prevalence of MRSA in urine (61-64%) was much more important than that in blood cultures and pus[15].

The study of antibiotic susceptibility of 67 strains of MRSA determined that the rate of resistance to oxacillin, cefoxitin and penicillin G is of 100%, this rate is the same as that found by Rebiahi and al [26]. Several mechanisms of resistance of Staphylococcus aureus to antibiotics are known, but the resistance, the resistance by change in the molecular target of the β-lactam is the most common. The genetic cause is the production of a penicillin binding protein (PBP) additional, 2a PLP PLP or 2 ', characterized by a low affinity for the β-lactams, in contrast to the four PLP (1 to 4) that are naturally involved in the biosynthesis of peptidoglycan. The gene encoding the PLP 2a is the mecA gene, DNA additional fragment integrated in chromosome of methicillin resistant Staphylococcus aureus[3,16].

Other mechanisms of resistance of resistance much rarer have also been described, including:

- The hyperproduction of penicillinase: the strains called BORSA (borderline SA) do not have mecA gene. Inhibitors of penicillinases restore in vitro the activity of β-lactam antibiotics on these strains;
- Resistance by decreasing the synthesis and / or affinity of a PLP, the strains are referred to MODSA (Modified SA). A modification of the affinity of PLP1 and PLP2 raises the level of MIC, which are not changed in the presence of β-lactamase inhibitor;
- The resistance by production meticillinase or oxacillinase which favored as substrate methicillin and oxacillin [3,36,37].

Other resistances were detected: rifampicin (32.8%), ofloxacin (68.6%), fusidic acid (65.6%).

According to Siegel et al[38], the multidrug-resistant bacteria are defined as micro-organisms resistant to one or more classes of antibiotics. The analysis of resistance profile confirms the multiresistant nature of MRSA to different families of antibiotics [39]. This multidrug resistance is due to the fact that MRSA strains are often resistant to aminoglycosides and macrolides. For aminoglycosides, the rate of MRSA resistant to gentamicin (KTG phenotype) found in our study was 22.3%. This resistance is higher, compared to the situation in Tunisia (18%) and France (10%) [15,28]. This rate is less important compared to results of a study in Abidjan (77.6%) [1]. The resistance to kanamycin and tobramycin is respectively 100% and 59.7%. These rates are higher than those found in Tunisia, where the resistance is respectively 78% and 21% [15].

We note that the rate of resistance to erythromycin is (46.2%), comparable to that observed in a Tunisian study rate (49%) [15]. But it stills lower than that found in the USA (66%) [40]. Furthermore, it should be noted that the low level of resistance to lincomycin (9%) in our study, is significantly lower than that reported by a Tunisian study (21%) [15].

The results show that 95.5% of MRSA strains are sensitive to pristinamycin, a rate comparable with that of 97% found by Leclercq et al [41]. This frequency is also found in a study achieved by (ColBVH) «Collège de Bactériologie Virologie Hygiène des Hôpitaux» of France on strains of S. aureus isolated from blood cultures in 1999. This rate sensitivity has been reported in 1991 by the National Reference Center for Staphylococi (CNRS) [42], suggesting the absence of progression to resistance to this antibiotic. Pristinamycin remains therefore a good alternative for the treatment of MRSA infections.
The decreased susceptibility of *S. aureus* to glycopeptide is a topical problem. Various studies have reported the isolation of strains of *S. aureus* intermediate or resistant to these antibiotics [8,43]. The first description of a strain of MRSA with reduced susceptibility to vancomycin (VISA) was in Japan in 1997 [8]. These MRSA strains are susceptible to vancomycin (MIC 2-4 mg / L) but showed intermediate vancomycin subpopulations (MIC 6-8 mg / L). Subpopulations are present at low frequencies in the range of $10^5 - 10^6$, and can’t be identified with standard susceptibility testing [44].

The glycopeptide resistance in *S. aureus* could be linked to a variety of molecular mechanisms.[45,46,47] These strains have a thickened wall resulting from complex reorganization of peptidoglycan metabolism probably linked to mutations in multiple genes. This reorganization could prevent access of vancomycin to its target [48]. Another hypothesis non-exclusive is the hyperproduction of peptidoglycan precursors acting as decoys for glycopeptides [43,44]. Isolation of strains of *S. aureus* resistant to vancomycin by horizontal transfer of *VanA* operon from *Enterococcus* was recently reported. This high-level resistance to vancomycin is now very limited [44,45]. Now, the great majority of strains are hetero-GISA and the estimated prevalence in the United States is less than 2%, however in Japan, Hiramatsu reported a frequency of 20% [49]. A recent study in the Netherlands showed a prevalence of 7.6% of hetero-GISA [44,50].

In our study 11 strains were suspected to be VISA according to their resistance or susceptibility intermediate to vancomycin and teicoplanin. The confirmation of this reduced susceptibility by E-test vancomycin described two strains. After analyzing the subpopulation, these two strains were heterogeneous VISA (hetero-VISA) where there is a MIC subpopulation between 6 - 8 mg / L for the two strains. A similar result was described in a Tunisian study but with homogeneous GISA [15].

**CONCLUSION**

This study showed that MRSA is a real health problem in our country. The prevalence observed is about 44.6%. We have also the problem of multidrug resistance, where a significant number of our strains showed resistance for aminoglycosides, macrolides and other antibiotics. Regarding the glycopeptides, the study shows that two strains were hetero-VISA. Careful monitoring may be useful to minimize the dissemination of strains with reduced susceptibility to glycopeptides.

**Acknowledgements**

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