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The Phenotypic and Genetic Detection of the Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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Abstract

Methicillin resistant strains of *Staphylococcus aureus* (MRSA) were identified shortly upon the introduction of methicillin into the clinical practice. The *S. aureus* samples were taken from patients hospitalized in General Hospital of Chania "Agios Georgios". The strains were isolated from different pathological products, in the hospital laboratory. All isolates were tested using the cefoxitin disk diffusion, the oxacillin MIC methods and the PBP2' latex agglutination test (bioMérieux), for the production of the Penicillin-Binding Protein 2a (PBP2a or PBP2' protein). *S. aureus* ATCC 29213 was used as a negative control. We followed the detection of the *mecA* gene throughout the PCR method, as the standard "gold" method, in order to identify MRSA strains. Conventional methods for MRSA strains detection were compared with the PCR method. The antibiotic susceptibility testing was performed throughout the Kirby-Bauer disk diffusion method using antibiotic discs from Bioanalyse ltd. The *mecA* gene was found by PCR in 57.5 % of *S. aureus* strains, which allowed defining the isolated strains as MRSA strains. According to the oxacillin MIC values of the studied strains, 25 strains (53.2%) were identified as MRSA, 21 (44.68%) as MSSA and 1*strain (2.13%) as Borderline (BL) MRSA. The *mecA* gene is present in 24 of the MRSA strains with oxacillin MIC ≥ 4 being more common in strains showing the oxacillin MICs ≥ 256 (12/27). Adding the BL strains to the methicillin resistant strains, the rate of the MRSA strains increases to 26 (55.32%), the appropriate values of the MRSA strains percent, as determined by the PCR method (57.45%), which shows a concordance of 96.3% (26/27), between the results obtained by the two tests. Comparing the results obtained using the PCR method; with the oxacillin MICs and the PBP2' latex agglutination test, concordant results were obtained for 89.36% of the strains (42/47) by oxacillin MICs and for 97.87% of the strains (46/47) by PBP2' latex agglutination test. We conclude that the specificity of these methods is 100% for the *mecA* PCR method, 97.87% for the PBP2a latex method and 89.36% for the oxacillin MIC. The comparison of phenotypic methods (the PBP2a latex reaction, oxacillin MICs, the Cefoxitin disk diffusion test with the genotypic methods (the presence of the *mecA* gene), reveals that the PBP2a latex reaction has high sensitivity (97.87%), and can be used as an alternative method to PCR for the MRSA detection, in resource constraint settings.

Keywords: Methicillin-resistant *Staphylococcus aureus* (MRSA); Methicillin-sensitive *Staphylococcus aureus* (MSSA); Oxacillin disk diffusion; Penicillin-Binding Protein 2a (PBP2a); Polymerase chain reaction (PCR); *mecA* gene; Clarithromycin (CLR)

Introduction

Methicillin resistant *Staphylococcus aureus* strains (MRSA) were identified shortly upon the introduction of methicillin into the clinical practice. MRSA should be considered resistant to all penicillins, cephalosporins, β-lactam/β-lactamase inhibitor combinations and also to imipenem [1]. A previous study showed that MRSA had risen up to 33% in hospitals. Rapid detection of MRSA is desirable. Several PCR assays based on the DNA sequence information have been used for MRSA (ORSA) strains detection. Early detection of methicillin resistance (MR) is essential [2]. Several phenotypic methods, PBP2a latex and detection of the *mecA* gene by PCR are recommended [3]. Standardized methods have been used for the detection of resistant strains [4]. Culture based methods require up to 5 days to identify the MRSA strains from patient specimens. However, the phenotypic expression of the methicillin-resistance is usually heterogeneous [5]. In addition, the methicillin-resistance is influenced by the culture conditions such as temperature, pH and NaCl content [6]. These factors complicate the methicillin-resistance detection, especially for the strains with low level resistance. In order to replace these time consuming methods and to avoid the spread of MRSA within

hospitals, molecular methods were developed to identify the MRSA strains. Commercial PCR assays are becoming available for fast MRSA detection and identification of MRSA strains only within 3 hours [7]. Several PCR assays based on the DNA sequence information have been used for the detection of the MRSA strains [8]. Molecular assays that detect the MRSA strains within 2-6 hours have recently been developed for screening specimens. The *mecA* gene detection by PCR methods have high sensitivity and specificity and are independent from the physical and chemical culture conditions and only 24 hours are needed, to carry out a normal PCR method. The detection of the *mecA* gene throughout the PCR method is considered to be the "gold standard" and is replacing the MIC method as reference method, but is not yet generally available, since it is performed in reference laboratories only and is more expensive than conventional tests [9].

Research objectives and assumptions

In the present study, we followed the detection of the *mecA* gene throughout the PCR method, as the standard "gold" method, in order to identify methicillin-resistant *Staphylococcus aureus* strains (MRSA). Conventional methods were compared with the polymerase chain reaction (PCR) method, for the MRSA strains detection.

Materials and Methods

The study took place from January 2020 to April 2020 and included 47 strains of *Staphylococcus aureus* (*S. aureus*) obtained from hospitalized patients in the General Hospital "Agios Georgios" of Chania, Greece. The strains were isolated from patients hospitalized in different hospital wards: the medical, surgical and intensive care wards and other medical units addressing this laboratory. The *S. aureus* strains were isolated from different pathological products: 19 from blood (40.43%), pus (12.76%), 9 from wounds (19.15%), 5 from nasal secretions (10.64%), 5 from ear discharge and conjunctive secretions (10.64%), 3 from catheter tips (6.38%) and from other drain fluids (Figure 1). The strains were isolated from patients aged between 40-60 years (52%), 60-85 years (30%) and 18-40 years (18%) (Figure2).

Strains isolation

The strains isolation was completed on appropriated non-selective, selective and chromogenic culture media. Culture based methods require up to 5 days to identify MRSA from patient specimens.

The identification of the isolated *S. aureus* strains: *S. aureus* strains were identified throughout conventional methods, based on phenotypic properties: morphology, Gram stain, coagulase and catalase test, Staph latex agglutination (Latex beads coated with plasma-detects clumping factor and protein A, *S. aureus* and CNS were differentiated one from the other). The identification of the isolated *S. aureus* strains was made also throughout semiautomatic (Api BioMerieux) and automatic methods (Vitek2 Compact bioMerieux). For a total of 47 strains, the antibiotic susceptibility testing was performed throughout the Kirby-Bauer disk diffusion method using antibiotic discs from Bioanalyse ltd, for AB: β-lactamins, Macrolides (Erythromycin, Clarithromycin), Glycopeptides (Vancomycin, Teicoplanin), Lincosamides (Clindamycin, Lyncomycin) Oxazolidones (Linezolid), Streptogramins: Quinupristin/dalfopristin (Q/D), Pristinamycin, Virginiamycin, Aminoglycosides: (Gentamicin, Tobramycin), Quinolones, Tetracyclines, Cotrimoxazole (Sulphamethoxazol & Thrimetoprim (SXT). Interpretation: Clinical and Laboratory Standards Institute (CLSI) susceptibility documents [10,11].

Rapid detection of the methicillin resistance in the *Staphylococcus aureus* isolates: Molecular detection of *mecA* gene by PCR: All isolates were tested throughout the PCR method for the presence of the *mecA* gene encoding for penicillin-binding protein 2a (PBP2a or *PBP2*' protein). The PCR was done using the standard procedures. The *mecA* gene was amplified as described by Predary, et al. 1992. The DNA amplification was carried out for 30 cycles in 50°C of reaction mixture as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 minute with a final extension at 72°C for 5 minutes. The PCR mixture consists of 0.5 μl of primer. Specific primers for *mecA* gene used were: forward primer (5' AAA TCA GAT GGT AAA GGT TGG C3'), reverse primer (5' AGT TCT GCA GTA CCG GAT TTG C3'). The PCR products (10 ml) were analyzed in 1.5% agarose gel. MSSA standard strain *S. aureus* ATCC 29213 was used as a negative control. MRSA standard strain (ATCC 43300A) was used as a positive control strain. Gel electrophoresis of the PCR amplification using *mecA* gene specific primers, showing the typical patterns of oxacillin (*mecA* gene-533 bp) resistance among the MRSA isolates [12] (Figure 3).

The detection of the Methicillin Resistance in the *Staphylococcus aureus* isolates by phenotypic methods (for detecting the MRSA strains). All isolates were tested, using the cefoxitin disk diffusion and the MIC of oxacillin methods and throughout the PBP2' latex agglutination test (bioMérieux) for the production of the penicillin-binding protein 2a (PBP2a or PBP2' protein) [13].

S. aureus ATCC 29213 was used as a negative control. The Cefoxitin disc diffusion test using 30 μg/ disk Cefoxitin was performed at 23 (48.94%) of the studied strains (47), to assess its value in detecting the MRSA strains and was also compared with other methods: PCR

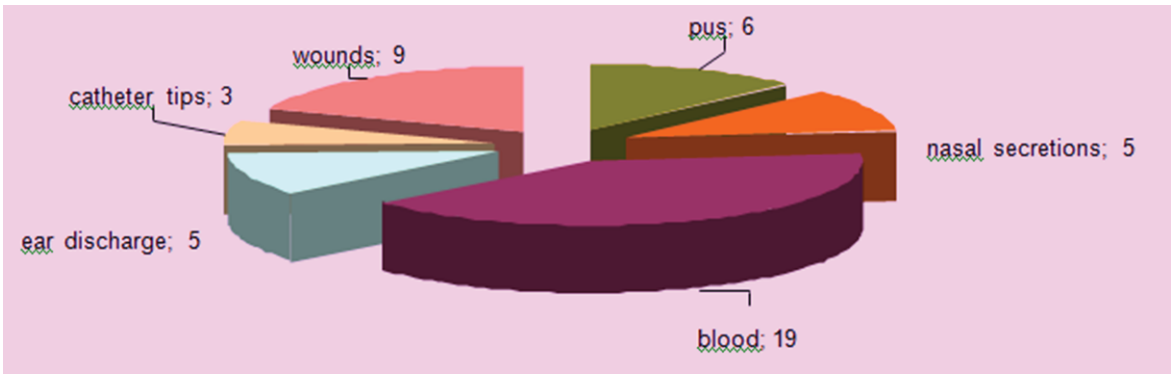

Figure 1: Isolated strains from pathological products.

Figure 2: Age distribution of the patients with staphylococcal infections.

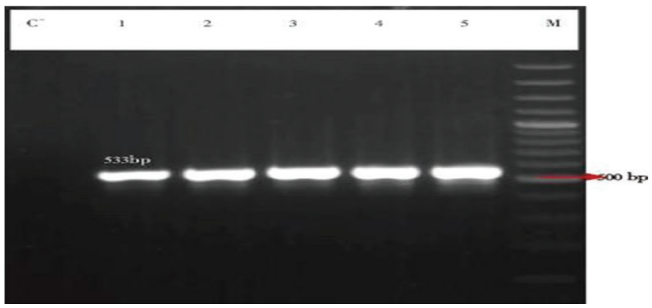

Figure 3: PCR showing the typical patterns of oxacillin resistance (mecA gene +).

Lane 1-5: Lanes: 1 positive control' clinical samples, PCR bands of MRSA isolates mecA gene (533 bp); Lane C: negative control (mecA negative S. aureus); M: molecular size marker (100bp DNA Ladder Plus).

(for *mecA* gene), oxacillin MICs, PBP2a latex reaction). No special medium or incubation temperature is required. The test is less affected by the penicillinase hyper-producers. A 0.5 Mc Farland standard suspension of the isolate was made and a lawn culture that was done on a MHA plate. The plates were incubated at 37°C for 18 h. The inhibition zone diameters were measured. The most recent CLSI supplement (M100-S14) suggests the use of 30 µg cefoxitin discs using a breakpoint of ≤ 21 mm as resistance indicative of *S. aureus* to oxacillin. The isolates were interpreted as sensitive or resistant according to the Clinical and Laboratory Standard Institute-CLSI document M100 S20-2010. The Cefoxitin inhibition zone diameter ≤ 21 was reported as oxacillin resistant *S. aureus* and ≥ 22 mm was considered as oxacillin susceptible/sensitive *S. aureus*. There is no intermediate category with the cefoxitin disk diffusion test. Cefoxitin E-test: selective and differential medium (CHROM agar MRSA) for qualitative direct detection of MRSA was also determined as a possible phenotypic method for the MRSA detection.

The oxacillin MIC for the *S. aureus* strains: For a total of 47 strains, the MIC (minimum inhibitory concentrations) of oxacillin was performed throughout the disk diffusion method [14]. The disks containing 1 µg oxacillin/disk are plotted in Mueller-Hinton agar plates. The isolates were interpreted as sensitive or resistant according to the Clinical and Laboratory Standard Institute-CLSI document M100 S20-2010 Interpretive Criteria (in µg/ml) for the Oxacillin MIC. MIC ≤ 2 µg/ml was considered as Susceptible/sensitive to oxacillin=Methicillin Sensitive S. *aureus* (MSSA), MIC 2-4 µg/ml as Intermediate or Borderline Methicillin-resistant S. *aureus* (BL MRSA) and MIC ≥ 4 µg/ml as Resistant to oxacillin=Methicillin-Resistance *S. aureus* (MRSA). The PBP2a latex agglutination test was compared with the *mecA* PCR ("gold standard") method for the detection of the methicillin resistance in *Staphylococcus aureus* [15].

Results

From the total of *S. aureus* studied strains (n=47), the *mecA* gene was found by PCR in 27 of *S. aureus* strains (57.45 %) and was absent in 20 strains (42.56 %), which allowed defining the isolated strains as methicillin resistant-MRSA strains (57.45%) and methicillin sensitive-MSSA strains (42.56%). *mecA PCR* displayed negative results among 20 (42.56%) *S. aureus* strains (n=47). All the *mecA* negative strains (20 strains) presented negative PBP2a latex reaction, confirming the outcomes obtained by the PCR method and directing the specificity of 100% of the PBP2' latex agglutination test and 100% compliance between the two methods. The PBP2' latex agglutination test detected the PBP2a in 26 (55.32 %) of the *mecA* positive strains. One *mecA* positive strain (2.13%) had negative PBP2a latex reaction, which revealed a 97.87% concordance between the two methods for the methicillin resistance (MR) detection. Taking into account the absence of the PBP2a into the *mecA* positive strains, it is necessary to mention that negative/discordant results are possible, in a low percentage (2.13%), leading to errors in the diagnosis and in the identification of the isolated strains as being MSSA strains. We conclude that PBP2' latex agglutination test has the potential to detect the MRSA strains in a routine microbiology setting; it is more accurate than any susceptibility testing method used alone for the MRSA detection; it approaches the accuracy of PCR (97.87%) and it combines high speed and excellent specificity and sensitivity with limited requirements for special equipment or skilled personnel. It can be recommended as an alternative phenotypic method to identify the MRSA strains and to be successfully used for routine applications in the microbiology laboratory, but it is necessary to confirm the obtained results throughout the detection of the *mecA* gene in the PCR method.

The Oxacillin MIC values of the *S. aureus* strains

Ranged from 0.38-2 µg/ml for 21 strains (44.68%), from 2-4 mg/ml for 1*strain (2.13%) and has been ≥ 4 µg/ml for 25 strains (53.2%). According to the oxacillin MIC values of the studied strains (47), out of isolates, 25 strains (53.2%) were identified as MRSA, 21 strains (44.68%) as MSSA and 1*strain (2.13%) as BL MRSA (Borderline Methicillin-resistant *S. aureus*) (Figure 4). The oxacillin MIC values of the studied strains 25 strains (53,2%) showed high levels of the oxacillin MICs, the most of them being ≥ 64 µg/ml (21 strains): 4 µg/ml (1), 32 µg/ml (1), 64 µg/ml (2), 96 µg/ml (4), 128 µg/ml (3), >256 µg/ml (12).

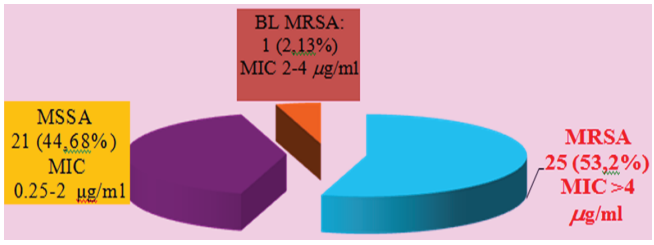

Figure 4: The Oxacillin MICs of isolated strains.

Comparison of the Oxacillin MIC with PCR (the presence of the mec A gene)

Of 27 MRSA (*mecA*+) strains 24 (51.07%) have the oxacillin MIC>4 and 18 of 20 MSSA (*MecA*-) strains (38.3%) have the oxacillin MIC ranged from 0.38-2, which shows a concordance of 89.36% (42/47), between the results obtained by the two tests.

The Oxacillin MIC for the *mecA*+ (MRSA) strains

The *mecA* gene is present in 24 of the MRSA strains with oxacillin MIC ≥ 4 being more common in strains showing the oxacillin MICs ≥ 256 (12/27). Adding the BL strains to the Oxacillin/methicillin resistant strains, the rate of the MRSA strains increases to 26 (55.32%), the appropriate values of the MRSA strains percent, as determined by the *mecA* gene method (57.45%), which shows a concordance of 96.3% (26/27), between the results obtained by the two tests. Discordant results between the oxacillin MICs and the *mecA* gene detection throughout PCR were obtained for 5 MRSA & MSSA strains (10.64%): 2 MSSA (4.25%) *mecA* negative strains had the oxacillin MICs values of 3 μg/ml and 6 μg/ml and 3 MRSA *mecA* positive strains (6.39%) showed low values of oxacillin MICs ≤ 2 μg/ml (0.38-2). Comparing results obtained using PCR for *mecA* gene detection with the oxacillin MICs, concordant results were obtained for 89.36% strains (42/47) and discordant results were obtained for 5 strains (10.64%). The strain with the Oxacillin MICs of 6 μg/ml (strain Nr 1851), is a *mecA* negative strain, sensitive to AB, suggesting the possible correlation between the MIC Oxa breakpoint values and the sensitivity to AB. The strain with the oxacillin MICs of 3 μg/ml (strain Nr 786), is a *mecA* negative strain, sensitive to Cefoxitin (diameter of inhibition area=26), sensitive to AB, β lactamase producing.

The correlation of the β lactamase production of the MSSA strains will be seen later. Of the total *mecA* positive MRSA strains (27), 3 (6.39%) strains showed low levels of Oxacillin MICs ≤ 2 of 0.38 μg/ml (1 strain), 0.75 μg/ml (1 strain), 2 μg/ml (1 strain). These 3 strains are also sensitive (S) to Cefoxitin and can be considered as sensitive strains to Oxacillin (MSSA). All three discordant strains present the *mecA* gene, express the PBP2a, and produce β-lactamase, resulting in their framing as MRSA.

S. aureus clinical isolates that carry the *mecA* gene but appear phenotypically oxacillin susceptible have been increasingly reported recently, by other authors. It has been suggested that such isolates could be classified as a new type of MRSA, designated OS-MRSA, which may be misclassified as MSSA in the daily routine if only susceptibility to antimicrobials is tested. It is generally believed that when treating OS-MRSA infections, we should take precautions, because treatment with beta-lactam antibiotics may result in the emergence of highly resistant MRSA, which is attributable to the presence of the *mecA* gene [16]. From all the strains, 2 *mecA* negative strain (MSSA) had the oxacillin MICs values >4, of 3 μg/ml and 6 μg/ml.

The comparison of results obtained by the Oxacillin MIC with the PBP2a latex and the PCR method (the presence of the *mecA* gene)

Comparing the results obtained throughout PCR for the *mecA* gene detection (100%) in *S. aureus* strains with the Oxacillin MICs and the PBP2' latex agglutination test concordant results were obtained for 89.36% strains (42/47) by the oxacillin MICs and for 97.87% (46/47) by PBP2' latex agglutination test, we conclude that the specificity of these methods is 100% for the *mecA* PCR method, 97.87% for the PBP2a latex method and 89.36% for the oxacillin MIC.

From 47 *Staphylococcus aureus* strains, the Cefoxitin Disk Diffusion test using 30 μg/ disk Cefoxitin was performed at 23 (48.94%) of the studied strains: 16 *mecA* positive strains (34%) and 7 *mecA* negative strains (14,9%) and hadn't been achieved in 24 strains (51.07%): 11 (23.41%) *mecA* positive strains and 13 (27.66%) *mecA* negative strains. There had been obtained the following results: 12 strains (25.5%) from 16 *mecA* positive (34%) strains, were resistant strains to Cefoxitin (inhibition zone diameter ≤ 21 mm) and 11 strains (23.4%): 4 (17.4%) *mecA* positive and 7 *mecA* negative (14.9%) were susceptible strains to Cefoxitin (inhibition zone diameter ≥ 22 mm) (Table 1).

Concordant results with the PCR method were obtained in 82.6% (19 of the 23) of the tested *S. aureus* strains (100%) respectively 12 (52.17%) *mecA* positive strains (MRSA) were R to Cefoxitin and 7 (30.43 %) *mecA* negative strains (MSSA) were sensitive (S) to Cefoxitin. Discordant results with PCR were recorded in 4 (17.4%) *mecA*+ strains (MRSA) S to cefoxitine. All 4 strains present *mecA* gene, express PBP2a, are sensitive to Oxacillin (Oxacillin MICs of 0.38, 0.75, 2); one strain was IS; 3 of the 4 strains produce β-lactamase. The Cefoxitin disk diffusion test which was used typically earlier, but is showing low specificity as we observe also in our results (82.6%) may also lead to identification errors (discordant results in 17.4% of the isolates) leaving some MRSA strains undetected. It may yield false-negative results due to the hyper production of β-lactamases which may lead to the phenotypic expression of the oxacillin resistance. The MRSA & MSSA detection by the Cefoxitin disk diffusion test: out of 23 *S. aureus* tested strains (100%) from witch 16 *mecA* positive strains (69.57%), by the Cefoxitin disk diffusion method, 12 isolates (52.17%) were identified as MRSA and 7 (30.42%) as MSSA.

The MRSA detection by the PCR method (for the presence of *mecA* gene) and by the phenotypic tests

Out of 47 isolates, there were identified throughout phenotypic & molecular methods the following percentage of MRSA: 57.45% by PCR, 55.32% by the PBP2a latex reaction, 53.2% by the Oxacillin MICs test and 52.17% by Cefoxitin disk diffusion. The results of the PBP2a latex reaction method are in concordance with the PCR method for the *mecA* gene (97,87%), the oxacillin MICs test and the Cefoxitin disk diffusion test have a lower concordance (sensitivity) of 89.36% respectively 82.61% with the PCR method for the *mecA* gene. Discordant results between the phenotypic and the genotypic tests reveal possible confusion in the identification, if only classical phenotypic tests (Cefoxitin Disk Diffusion and the Oxacillin MICs) are performed, and show the need to perform the PBP2a latex method and the genotypic tests (the PCR method) to detect the *mec*A gene.

Discussion

During the last decade, MRSA strains have emerged as serious nosocomial pathogens and spread in many regions of world because of its ability to acquiring resistance to antimicrobial chemotherapy [17]. Therefore, rapid recognition of these organisms and detection

Table 1: Cefoxitin Disk Diffusion Test results of using 30 μg/ disk Cefoxitin performed for the studied strains.

S. aureus strains	Cefoxitine R	Cefoxitine S (sensitive)	*S. aureus* tested strains
MRSA mecA positive (+)	12 (25.5%)	4 (8.51%)	16 (34%)
MSSA mecA negative	-	7 (14.9%)	7 (14.9%)
Total	12 (25.5%)	11 (23.4%)	23 (48.94%)

of methicillin resistance are essential for prompting effective therapy, preventing distribution of infection and reducing the risk of patient's mortality [18]. The rate of methicillin-resistant *S. aureus* prevalence determined by Oxacillin disk diffusion method, in other studies [19], was 47.6%; whereas, 45.1% of *S. aureus* isolates were *mecA*-positive in the PCR assay.

The detection of methicillin-resistant *versus* methicillin-susceptible *S. aureus* (MRSA and MSSA) is of significant clinical importance. Detection of methicillin resistance is relatively uncomplicated since it is defined by a single determinant, penicillin-binding protein 2a', which exists in a limited number of genetic variants carried on various Staphylococcal Cassette Chromosomes *mec*. Diagnosis of MRSA and MSSA has evolved significantly over the past decades and there has been a strong shift from culture-based, phenotypic methods toward molecular detection, especially given the close correlation between the presence of the *mec* genes and phenotypic resistance [20].

Epidemiological investigations revealed that the infections produced by MRSA are increasing worldwide [21]. Also, many studies have shown that MRSA isolates are increasing in local settings [22].

The most important challenge in dealing with MRSA bacteria is to reduce the antibiotic choices in empirical therapy or prophylaxis because these isolates are commonly multi-drug resistant [23]. The PBP2a latex agglutination method was found to be more sensitive than the Oxacillin MIC test and the Cefoxitin disk-diffusion method. The PBP2a latex agglutination method is attractive, cost-effective, relatively simple to perform and easy to incorporate in the microbiology diagnosis laboratories. Discrepancies in the MRSA detection by other phenotypic methods have an adverse effect on patient management, thereby highlighting the importance of the accuracy in detection. Hetero-resistance or the presence of susceptible and resistant clones, may lead to diagnosis errors of the conventional susceptibility testing. The development of the molecular methods for the MRSA identification (*mecA* gene detection), emphasized the need for laboratories to reevaluate the role of the conventional phenotypic methods in the MRSA diagnosis. Fast laboratory diagnosis and susceptibility testing is critical in treating, managing and preventing the MRSA infections. The use of the molecular methods for the MRSA detection elude the number of unisolated MRSA and the number of unnecessary pre-emptive isolation days, decrease the MRSA transmission and infection rate, the MRSA-related mortality and are cost saving due to the shorter patient hospital stay. The molecular methods (the PCR techniques) are the main tools used for the identification of pathogenic strains and in the attempt to find the epidemiological relativity of the strains [24].

The presence of MRSA in a hospital is detrimental to patients and to hospital management. Thus, rapid identification of MRSA is needed. MRSA isolates are commonly multi-drug resistant, therefore, antibacterial susceptibility test results should be considered in the treatment MRSA infections [25]. The combined use of the molecular and the conventional techniques provides physicians and health care workers with invaluable information that directly affects the treatment [26]. The number of infections produced by the methicillin-resistant *S. aureus* can be restricted throughout prevention and control measures. Proper surveillance of the medical staff and a rational policy in prescribing antibiotics in these hospitals are therefore mandatory. Controlled trials must determine the potential medical and economic benefit of control strategies using this technology in hospital care units [27].

Conclusions

The comparison of phenotypic methods (the PBP2a latex reaction, oxacillin MICs, the Cefoxitin disk diffusion test with the genotypic methods (presence of the *mecA* gene), reveals that the PBP2a latex reaction has high sensitivity (97.87%), and can be used as an alternative method to PCR for the MRSA detection, in resource constraint settings. Among all the phenotypic methods, the PBP2a latex reaction alone has similar sensitivity and specificity as PCR method.

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