IDENTIFICATION OF THE RESPIRATORY TRACT INFECTION DUE TO METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* BY TAQMAN REAL-TIME PCR

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Abstract

The methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant human pathogenic bacterium that is endemic within hospitals around the world. The identification and inspection of MRSA in clinical samples is quite helpful both in advising individual patients about the required care and in tracking these species. The goal of this study was to present a modern, faster, and more accurate diagnostic technique to operate on the real-time duplex PCR applicable to *S. aureus*/MRSA monitoring in Iraqi patients. For this reason, the *S. aureus*-specific *nuc* gene sequence and the *mecA* gene sequence were checked simultaneously. To estimate the assay efficiency, a set of six target strains, 34 non-target strains, and 296 clinical specimens were used. The findings obtained from the diagnosis of a total of 296 isolates based on phenotypic characteristics and biochemical tests showed that 146 (49.32%) were classified as individuals with respiratory tract infections of *S. aureus* with a total male to female ratio of 1.47, and 142 isolates demonstrated methicillin resistance. 142 MRSA isolates were investigated in the molecular analysis, all MRSA isolates had positive results for the *nuc* gene and 138 isolates were positive for the *mecA* gene. The current real-time PCR assay has 97% sensitivity, 100% specificity, and 98% accuracy.

Running title: Identification of the MRSA by real time PCR

Keywords: Staphylococci, antibiotype, antibiotic resistance genes, real-time PCR

Introduction

The methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain of *S. aureus* that has evolved β -lactam antibiotic resistance. MRSA can be transmitted by physical contact from person to person and, occasionally, by air. The nasopharynx is the major natural habitat of *S. aureus*, so it is found in about all parts of the body [1]. It has been recognized to be a common pathogen that causes a vast array of disease symptoms, including pneumonia, infection of wound, endocarditis, and bacteremia. The β -lactam antibiotic is the drug of choice for treatment. The incidence of methicillin-resistant *Staphylococcus aureus* has gradually increased since methicillin was presented into therapeutic use in 1961, and infections of nosocomial caused by these genotypes are still a global health challenge [2]. Since 1961, successive MRSA disease waves have spread worldwide, to the point that in many countries it is now the most isolated antimicrobial-resistant pathogen. Studies have reported a rise in community-acquired MRSA (CA-MRSA) in patients with or without MRSA infection risk factors over the last few years, which may reflect a shift in epidemiology [3].

There are significant consequences for both the remediation and care of patients infected by *S. aureus* when MRSA strains have been differentiated from several other *S. aureus* strains [4]. *S. aureus* is detected in the clinical laboratory by growth characteristics and identification of catalase and coagulase actions. Resistance to methicillin or oxacillin is routinely measured by traditional *S. aureus* susceptibility tests when disk diffusion tests, agar dilution methods have been used in compliance with the guidelines of the national committee of clinical laboratory standards [5]. However, routine susceptibility test takes time to complete. As a phenotypic manifestation of *in vitro* resistance to methicillin is diverse and most complicated to produce, it is possible to observe false-negative results [6]. Moreover, when commercially obtainable kits are used for monitoring of coagulase, *S. aureus* strains can display a false-negative or non-interpretable result [7].

MRSA strains have been distinguished by a phenotype of beta-lactam resistance; the existence of an extra penicillin-binding protein (PBP) with low-affinity of the subclass PBP2a or hyperproduction of beta-lactamase indicates the key methicillin resistance mechanism. The *mecA* gene encodes PBP2a, which is located within a mobile genetic variable named the staphylococcal cassette chromosome *mec* (SCC*mec*) along with its regulators, *mecR1* and *mecI* [8,9]. An extracellular thermostable nuclease encoded by the *nuc* gene is generated by *S. aureus*, which is one of the most distinctive and effective characteristics that could be used to differentiate *S. aureus* from another *Staphylococcus* spp. This indicates that a particular marker gene is the *nuc* gene and PCR is a useful way of detecting this gene in *S. aureus*. Real-time PCR detection of the *nuc* gene and *mecA* gene

has been characterized as just a quick gold-standard technique to MRSA determination [10].

Reproducibility, speed, and quantitative ability are all advantages of real-time PCR over conventional PCR. Real-time PCR is more sensitive and reproducible, and it can be used in diagnostic routines instead of conventional PCR [11].

A molecular screening test for fast identification of MRSA and a comparison of phenotypic methods with a molecular diagnostic assay was developed and evaluated in this study. The newly designed assay detecting both *nuc* gene that recognized all staphylococci belonged to *S. aureus*, but not other bacteria, and the *mecA* gene using duplex real-time PCR.

Materials and Methods

Collection of specimens

A set consisting of 6 target strains ATCC, 34 non-target strains ATCC (*Staphylococcus epidermidis* (n=10), *Streptococcus agalactiae* (n=9), *Clostridium difficile* (n=8), and *Enterococcus faecalis* (n=7)), and 296 clinical samples were taken from Al-Yarmouk general teaching hospital in Baghdad, over five months, beginning from the first of April to the end of August 2018. Swabs from nares discharge, endotracheal, bronchial aspirates, and sputum were included in the samples. Each patient received only one specimen. Individuals with previously confirmed infection with the MRSA were the preferred chosen. Classical epidemiology data were collected by using standard questionnaires. Information was obtained on name, age, gender, levels of education, country of birth, symptoms, sample type, diagnosis type of test, and present address.

Detection of isolated bacteria

Each clinical sample was inoculated on mannitol salt agar (MSA) medium (Oxoid, England) plates and incubated for 24 hrs at 37 C. The MSA contains 7.5% NaCl, which, other than *Staphylococcus*, is inhibitory to the growth of most bacteria. The medium also contains mannitol and phenol red as pH indicators for the recognition of acid formed by *S. aureus* fermenting mannitol and distinguishes it from *S. epidermidis* non-mannitol fermenting, because mannitol-fermenting *Staphylococcus* create a yellow zone surrounding their growth, and the non-mannitol fermenter does not induce a color change [12].

On a microscope slide, a loop full of a suspected *S. aureus* colony was fixed and then stained with Gram stain to inspect grouping, cell shape, size, and Gram reactivity. Depending on Bergey's manual, *S. aureus* were classified according to the morphological characteristics of culture media and biochemical reactions (Catalase test and Coagulase test). API-staph (Analytic Profile Index System) was followed for further verification. On brain heart infusion agar, bacteria isolates were preserved for a few weeks, the plates were tightly wrapped in parafilm and stored in refrigerator at 4°C [13].

Methicillin-resistant *S. aureus* detection

According to CLSI guidelines [14], the Kirby-Bauer procedure would be used to conduct a disk diffusion test, cefoxitin 30µg disc was used as the alternative methicillin to identify resistance to methicillin in *S. aureus*. All *S. aureus* isolates already recognized by biochemical and morphological screening were grown on Mueller Hinton agar (Himedia, India) plates with turbidity equal to that of 0.5 McFarland tube containing 1.5×10^8 cfu per ml. The inverted plates were aerobically incubated for 18 hours at 37°C and the diameter of the inhibition zone had been determined. The inhibition zone diameter of ≤ 21 mm had been identified as methicillin-resistant and ≥ 22 mm was classified as methicillin-sensitive.

Bacterial DNA extraction

According to the manufacturing instruction of the Wizard Genomic DNA Purification Kit (Promega, USA), the extraction of chromosomal DNA was performed. Three colonies from the 24 hours culture of every strain were transferred into a clean Eppendorf tube and suspend in 480µl 50mM EDTA and 120µl of lysozyme. The mix was centrifuged for 2 minutes at $16,000 \times g$. The cells were lysed with Nuclei Lysis solution and RNase solution. The protein was precipitated by adding protein precipitation solution. The DNA was precipitated and rehydrated. The dsDNA concentration was calculated according to manufacturing instructions by the QuantiFluor dsDNA system with the Quantus Fluorometer (Promega, USA).

TaqMan Real-time PCR

The extracted DNA specimens have been genotyped by TaqMan real-time PCR assays to detected the *S. aureus* species-specific gene, as well as the gene that encoding methicillin antibiotic resistance. The probes fluorescent dyes had been selected to enable genotyping of single-tube PCR, labeled with

different fluorescent reporter dyes (VIC-Yellow and FAM-Green) and non-fluorescent Black Hole Quencher 1 (BHQ1) modified. A pair of PCR primers and a probe attach with 5' reporter and 3' quencher molecules are required for each gene; a probe was known as TaqMan.

Possible target regions were identified using GenBank (<http://www.ncbi.nlm.nih.gov/gene/>), a sequence specificity for *nuc* gene was used (GeneBank Accession Version NC 002952.2) and the *mecA* gene sequence recognition (GeneBank Accession Version NC 021670.1) was utilized to detect methicillin resistance. Primer3web version 4.1.0 (<https://primer3.ut.ee/>) was used for the design of primers and probes, and *in-silico* amplification of PCR (<http://insilico.ehu.es/PCR/index.php?mo=Staphylococcus>) was applied to evaluate the assay *in silico* (Tab. 1).

The lyophilized primers and probe mentioned in Table 1 have been reconstituted with nuclease-free water to make a 100pmol/µl solution. The required quantity of 25µl SmartCycler tubes has been ready for specimens and controls. The real-time PCR was configured and optimized as follows: in a 25µl tube of SmartCycler reaction, 1µl from 5pmol/µl from each one of forward and reverse primers and probes (each labeled with different dyes) for the *nuc* and *mecA* genes, 2µl of DNA was added to 10µl GoTaq Probe qPCR Master Mix (Promega, USA), and 2µl nuclease-free water (Integrated DNA Technologies, USA). All tubes had been centrifuged and placed in the PCR machine (Cepheid SmartCycler, USA). A thermocycler temperature profile was established as follows: 60°C 30secs; 95°C 10mins; 95°C 15secs and 60°C 1min for 40 cycles; 60°C 30secs.

Two controls were prepared for each run to prevent false results: negative control, which is prepared by adding 2 µl of nuclease-free water instead of DNA to the polymerization reaction components, and positive control, indicated by the reference MRSA DNA sample.

TABLE 1 Primers and probes sequence data applied in current research for real-time PCR resistotyping of *S. aureus*

Target gene symbol	Primer and probe sequences 5' → 3'	Nucleotide positions	Aliases	Product size (BasePair)	Source
<i>nuc</i>	Forward primer: GTTGATACACCTGAAACAAAGCA	352-374	SAR0847	156	Current study
	Reverse primer: CGCTAAGCCACGTCCATATTTAT	507-485			
	Probe: VIC-GGTCTCTGAAGCAAGTGCATT-BHQ1	400-419			
<i>mecA</i>	Forward primer: GGAATGCAGAAAGACCAAAGC	406-426	SABB_ RS00325	126	Current study
	Reverse primer: CTTTGGAACGATGCCTATCTCA	531-510			
	Probe: FAM-TGGCCAATACAGGAACAGCA-BHQ1	488-507			

Statistical analysis

The Statistical Analysis System-SAS [15] program had been used to detect the effect of different factors in study parameters. The Chi-square test was used to significantly compare percentages in this study.

Ethical approval

The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the University of Technology-Iraq / Department of Biomedical Engineering- Scientific Committee with resolution no. HT/56.

Results

Isolation and identification of methicillin resistant *S. aureus*

The 296 clinical samples had been screened, 146 (49.32%) were identified as *S. aureus* respiratory tract infection. The data set in **figure 1** exhibited that the number of infected patients was male 87 (59.59%) to female 59 (40.41%), with a total male to female ratio of 1.47 (87/59) with a highly significant difference ($P \leq 0.01$). The findings of this study exhibited that 142 isolates showed resistance to methicillin (oxacillin), 84 (59.15%) in males, and 58 (40.85%) in females with a high difference between percentages ($P \leq 0.01$).

DNA extraction and concentration

Extraction of DNA was performed on all 142 *S. aureus* isolates that were methicillin-resistant, 6 target strains, and 34 non-target strains. The range

of DNA concentrations was (41-56) ng/ μ l. The mean concentration was 47.5 ng/ μ l and 44 ng/ μ l was the median. When detected by real-time PCR, all these different DNA concentrations were adequate for effective DNA amplification.

Molecular identification of *S. aureus* by real time PCR

Extracted DNA specimens from 142 MRSA isolates, 6 target strains, and 34 non-target strains were submitted to real-time PCR molecular diagnosis to identify *S. aureus* identity by amplifying the conserved region of the *nuc* gene and confirming the result obtained from the methicillin resistance test, using designed gene-specific primers and TaqMan probes. This assay was successful for the qualitative detection. Positive results for the *nuc* gene were seen in all isolates of MRSA. For the *mecA* gene, 138 isolates were positive and 4 were negative (**Fig. 2**).

The current real time PCR assay was distinct with 97% sensitivity. Specificity testing gave positive results for all six *S. aureus*/MRSA target strains for *nuc* gene and *mecA* gene, and negative results for both genes when tested the 34 isolates of *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Clostridium difficile*, and *Enterococcus faecalis* (**Tab. 2**).

Discussion

The suspected isolates were able to ferment mannitol on mannitol salt agar and the yellow color was formed because of the generation of acid, Gram staining, and the associated biochemical tests further identified the colonies primarily. Under the microscope, the isolates appeared as Gram-positive coccus-shaped bacteria grouped mainly in clusters.

FIGURE 1 A distribution of MRSA among patients

FIGURE 2 The FAM channel of real-time PCR runs for the three specimens, and two controls (negative control and positive control). The progress of the amplification was shown by the three colored curves, one of which was positive control (red curve) and the other was positive results for the *mecA* gene (MRSA), and the two lines that appeared below the threshold line represent negative results for the *mecA* gene and the negative control

TABLE 2 Real-time PCR test sensitivity, specificity, accuracy, and predictive values were measured in 142 MRSA and 34 control subjects

True positive*	False negative*	True negative*	False positive*	Sensitivity*	Specificity*	Accuracy*	Predictive value of positive result*	Predictive value of negative result*
138	4	34	0	97%	100%	98%	100%	89%

*[16,17]

They were catalase-positive, differentiating them from the species of *Streptococcus*, and coagulase-positive, differentiating *S. aureus* from another *Staphylococcus* spp. Furthermore, a confirmatory test had been conducted using API-staph, a system allowing the suspected isolates to be rapidly identified at the species level. The distribution of MRSA among patients was consistent with previous results reported by Garoy *et al.*; Vijayamohan and Nair [4,18]. *S. aureus* has become resistant to conventional and new antibiotics in recent years and represents a therapeutic challenge, because of this, the observation of the susceptibility pattern helps assess the possible difficulties of effective therapy [19]. The difference between male and female MRSA susceptibility may be due in part to biological differences, socio-economic, and cultural factors that lead to gender differences in access to health care. Hand-hygiene behavior varies by sex, men are less obedient and may be predisposed to higher infection rates. An expression of factors of virulence in *S. aureus* has been inhibited by estrogen hormones in a female.

Further evidence on the impact of gender and risk of infection is needed, the reasons for increased MRSA transmission rates in a male are conditions including the possible role of occupation and contact sports [20]. The higher male incidence is poorly understood and requires further research to identify correlated hazard agents.

The result obtained from the extraction of DNA remained in accordance with the study done by Lara *et al.* [21]. Jaffe *et al.* [22] found that the median concentration of isolated DNA of *S. aureus* was 78.5 ng/ μ l, the difference between the results of this study and the results obtained from the current study may be explained by the use of larger quantities of bacteria in higher concentrations of DNA and kits used in DNA extraction.

Extracted DNA had been amplified by real time amplification and identified using fluorescent reporter dye probes specific for *nuc* gene that detected on the Cy3 (Yellow) channel and *mecA* gene on the FAM (Green) channel (Fig. 2). The assay was constructed to be applicable for the identification of

MRSA. To keep the general criteria of thermal cycling, the primers had been designed for (57.82–59.01) °C annealing temperature. Via the action of 5' nuclease of the Taq DNA polymerase, the probes fluorescent reporters are released. The interruption between the reporter and the quencher of the fluorescence resonance energy transfer causes the fluorescence reporter to raise the signal. Results were accepted as relevant if positive and negative controls of amplification were passed. Finally, the contrast between the post fluorescence of PCR and the reporter's startup basal fluorescence in given samples allows the studied targets to be genotyped. Post amplification manipulations are not mandatory for the real-time PCR diagnostic methods and are available for automated evaluation of the results. The results of the PCR were analyzed with SmartCycler Dx Version 3.0b software that can be graphically evaluated as points on the axis of the VIC/FAM probe. Experimental testing showed that the PCR had a completely reproducible and efficient amplification for the *nuc* and *mecA* genes in one tube. This proposed stable amplification of duplex single-tube without undesirable competitive interactions. *S. aureus* which is phenotypically resistant to methicillin while giving a negative result to *mecA* gene inspection may be referred to as having an alternative mechanism for antibiotic resistance instead of this gene, including the modification of penicillin-binding proteins (PBPs) leading to methicillinase or β -lactamase hyper-production [23,24], this explains why not all β -lactam resistant isolates have revealed positive results for the *mecA* gene detection.

In general, compared to the traditional culture methods, the PCR sensitivity and specificity are higher [25]. Within approximately 3 hours, the entire molecular assay was completed. No contamination at any point during the analysis was observed.

Conclusions

The fast-real time PCR technique identifying MRSA could be provided quick management of the most suitable antibiotic for infections of *S. aureus*. The designed TaqMan probes provide quick and accurate detection of *S. aureus*/MRSA. The technique is rapid, powerful, efficient, laboratory-saving, and reliable for a routine molecular diagnostic based on detection of *S. aureus*/MRSA isolated from clinical samples in a single step.

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Conflict of interest statement

The author declares they have no conflict of interest.

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