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Incidence of Ciprofloxacin-Resistant of Methicillin Resistant *Staphylococcus aureus* isolated from Iraqi patients

Ali A. Mussa*, Harith Jabbar Fahad Al-Mathkhury
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

Abstracts

The resistance of *Staphylococcus aureus* to ciprofloxacin has complicated the problem of treating staphylococcal associated infections in which MRSA is the causative agent since ciprofloxacin was the drug of choice to treat such infections. Our study investigated the incidence of Ciprofloxacin resistant *S. aureus* isolates that were also methicillin resistant among Iraqi patients. The obtained bacterial isolates were tested for Ciprofloxacin resistance using agar dilution method and the sequence of *gyrA* and *parC*. The results revealed that about 8% of the isolated MRSA strains were Ciprofloxacin resistant and the resistance was due to mutation in *gyrA* rather than *parC*.

Keywords: *Staphylococcus aureus*; Ciprofloxacin; MRSA.

انتشار العنقوديات الذهبية المقاومة للمثسلين والسبروفلوكساسين المعزولة من مرضى عراقيين

علي عبد الرضا موسى*، حارث جبار فهد المذخوري
قسم علوم الحياة، كلية العلوم ، جامعة بغداد ، بغداد، العراق

الخلاصة

أن انتشار بكتريا المكورات العنقودية الذهبية المقاومة لمضاد السبروفلوكساسين قد عقد من مشكلة علاج الامراض المصاحبة التي تكون فيها المكورات العنقودية الذهبية المقاومة للميثيسيلين هي المسبب المرضي اذ ان مضاد السبروفلوكساسين كان يستعمل لعلاج تلك الحالات. شملت الدراسة التحري عن انتشار مقاومة بكتريا المكورات العنقودية الذهبية المقاومة لمضاد السبروفلوكساسين بين المرضى العراقيين. تم قياس التركيز المثبط الأدنى لمضاد السبروفلوكساسين للعزلات البكتيرية بطريقة التخفيف بالأغار كما تم تحديد تتابع القواعد النيتروجينية لجيني *gyrA* و *parC*. أظهرت النتائج ان حوالي ٨٪ من العزلات المقاومة للمثيسلين كانت مقاومة للسبروفلوكساسين ايضاً وان المقاومة كانت نتيجة حدوث طفرات في جين ال(*gyrA*) بدلاً من جين ال(*parC*).

Introduction

Staphylococcus aureus is considered to be a major pathogen of increasing importance due to the increase in antibiotic resistance [1]. *S. aureus* has the ability to colonize and infect healthy immune competent as well as hospitalized patients having decreased immunity. Although it normally inhabits the skin and the nasopharynx of the human body, it can cause local infections of the nose, skin, vagina, urethra and gastrointestinal tract [2-4]. The hospital environment can support the acquisition of resistant *S. aureus* strains leading to nosocomial infection [5].

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been associated with hospital-acquired infections since early as 1960s [6]. During the 1990s, the percentage of nosocomial infections in which MRSA was identified to be the causative agent increased considerably, and now MRSA is

*Email: aliredhabiology@gmail.com

considered a principal cause of such infections in the United States [7]. Lately, community acquired MRSA infections have been recognized as emerging pathogens being responsible for extensive morbidity and mortality [8-9]. While there has no agreeable clarification concerning the recent propagation of MRSA, prolonged and extended use of antimicrobial drugs outside the hospital has been proposed to be a major contributor to evolving resistance in the community [10].

Fluoroquinolones are continually being in the lead of the antimicrobial drugs in terms of being prescribed in both the hospital and in the community. Ciprofloxacin, one of the first fluoroquinolones to be used for extensive medical, was originally indicated for its activity against a wide range of pathogens, including MRSA [11].

On the other hand, as early as the beginning of the 1990s, many MRSA clinical isolates were found to be ciprofloxacin resistant [12]. During the second part of the 1990s, the next generation of fluoroquinolones, including levofloxacin, was presented and promised enhanced activity against gram-positive pathogens. Unfortunately surveillance programs confirmed that resistance to several new fluoroquinolones has been also recorded [13].

The fast ascension of fluoroquinolone resistance in *S. aureus* is one of the best modern examples of biological evolution. Two main mechanisms of fluoroquinolone resistance have been described. The first comprises point mutations in *gyrA/gyrB*, which are coding for the subunits of DNA gyrase and topoisomerase IV, respectively [14]. In *S. aureus*, topoisomerase IV is the primary target of ciprofloxacin while DNA gyrase is considered to be a secondary target [15]. The second mechanism includes efflux of fluoroquinolones by NorA that is a membrane-associated protein. This efflux pump is responsible for actively transporting fluoroquinolones along some other structurally unrelated compounds out of the bacterial cell [16].

Upon the aforementioned facts, the present work aimed to investigate the prevalence of MRSA isolates that are resistant to ciprofloxacin in a sample of Iraqi patients.

Materials and methods:

A total of one hundred and sixty specimens were collected over a period between August and December 2016. The collected specimens included anterior nares swabs (n= 20) that were taken from patients and healthcare workers, burn swabs (n= 13), sputum (n= 30), mid-stream urine (n= 85), and blood (n= 12) at Baghdad's medical city (Madinet Al-Teb), Al- Yarmouk teaching Hospital, and Al-Kadhimiyah teaching hospital in Baghdad, Iraq.

All specimens were inoculated onto Mannitol Salt Agar plates (MSA) and incubated at 37°C for 24 hr. Subsequently, the colonies were purified by sub-culturing on BHI agar followed by re-inoculation onto MSA and incubated at 37°C for 24 hr. [17].

Determination of Ciprofloxacin minimal inhibitory concentration (MIC) using agar dilution method

The susceptibility of all of *S. aureus* isolates to Ciprofloxacin was tested by measuring the MIC using agar dilution method [18]. The isolates were interpreted as susceptible, intermediate or resistant to the antibiotic in accordance to CLSI (2016) breakpoints of $\leq$1, 2, or $\geq$4 µg/ml, respectively.

Polymerase chain reaction (PCR)

Bacterial Genomic DNA of all *S. aureus* bacterial isolates was extracted by Presto™ Mini gDNA Bacteria Kit (Geneaid, Thialand) and AccuPower® PCR PreMix was utilized for all of the amplification reactions that was carried out using Gradient master cycler (Eppendorf, Germany).

Staphylococcus aureus-suspected isolates were screened for the presence of *S. aureus 16s rDNA* gene using specific primers SA1 (AATCTTTGTCGGTACACGATATTCTTCACG) and SA2 (CGTAATGAGATTTCAGTAGATAATACAACA), the reaction settings were: Initial denaturation at 92°C for 3 min followed by 30 cycle of 92°C 1 min, 56°C 1 min and 72°C 1 min; following that 3 min at 72°C for final extension [19]. The PCR products were sequenced using Sanger method and after that were aligned with gene sequences from National Center for Biotechnological information (NCBI) (https://www.ncbi.nlm.nih.gov/).

The presence of *mecA* gene encoding for methicillin resistance was detected in all of *S. aureus* isolates using primers MecA1: (GTAGAAATGACTGAACGTCCGATAA) and MecA2: (CCAATTCCACATTGTTTCGGT); The amplification program included initial denaturation at 94°C for 10 min followed by 10 cycle of 94°C 45 sec, 55°C 45 sec and 72°C 75 sec; followed by 25 cycle of 94°C 45 sec, 50°C 45 sec and 72°C 75 sec [20].

Ciprofloxacin-resistant *S. aureus* isolates were designated for the detection of possible mutations in both *parC* and *gyrA* that code for and DNA topoisomerase IV and DNA gyrase subunit A, respectively. The primers used for the amplification of *parC* gene are: ParC F: GTATGCGATGTCTGAACT and ParC: R TTCGGTGTAACGCATTGC for *parC*; whereas those used for the amplification of *gyrA* are: GyrA F: AAATCTGCCCGTGTCGTTGGT and GyrA R GCCATACCTACGGCGATACC. The amplification program involved initial denaturation at 95°C for 2 min followed by 35 cycle of 95°C 30 sec, 55.4°C 60 sec and 72°C 60 sec [21].

The PCR products were sent to Macrogen/ Korea for sequencing using Sanger method and after that were aligned with gene sequences from National Center for Biotechnological information (NCBI) (https://www.ncbi.nlm.nih.gov/) in order to inspect for mutations.

Results and discussion:

Bacterial isolation and identification:

Out of collected specimens, 25 bacterial isolates developed round and yellowish colonies on MSA that would give a primary identification as *S. aureus* isolates. Consequently, these 25 isolates were subjected to several tests (viz. Gram stain, oxidase, catalase, coagulase, and hemolysis) in order to confirm the identification. Results were summarized in Table-1.

Table 1-Biochemical, cultural and microscopic properties of *S. aureus* (n= 25)

Id	Test	Result
1	Mannitol fermentation	Positive with yellow colonies (100%)
2	Gram stain	Gram positive cocci (100%)
3	Catalase	Positive (100%)
4	Oxidase	Negative (100%)
5	Coagulase	Positive (84%)
6	Hemolysis pattern	β (80%)
7	Voges-Proskauer (acetoin)	Positive (100%)

Detection of *Staphylococcus aureus 16SrDNA*

For confirming the identification of *S. aureus* isolates, species specific *16SrDNA* gene was used. The result revealed that all of the 25 isolates identified as *S. aureus* (Figure-1).

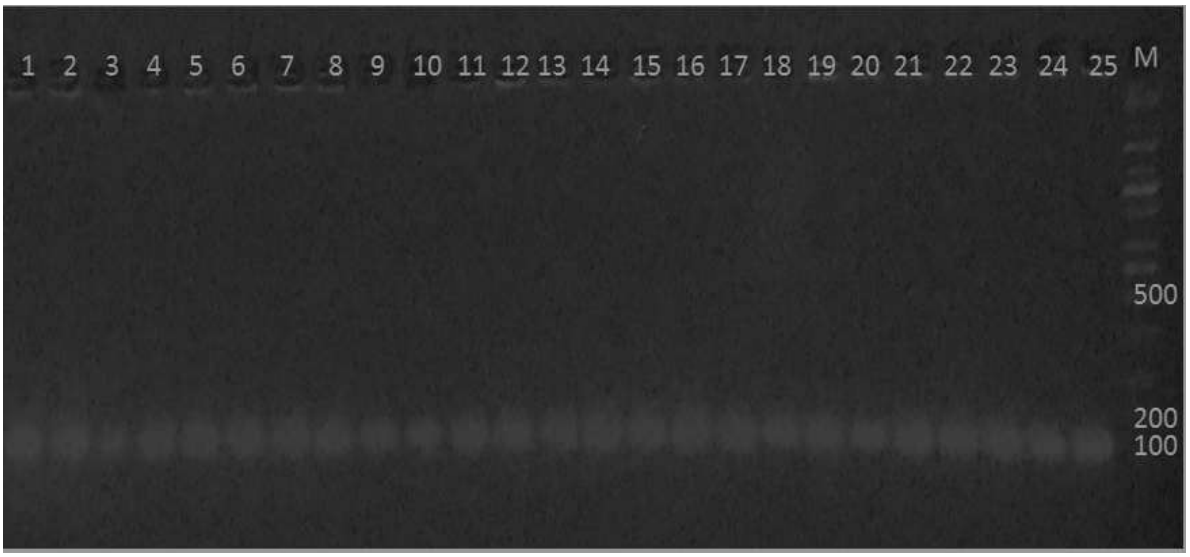

Figure 1- Visualization of *Staphylococcus aureus 16SrDNA* gene by 1.0% agarose gel analysis

The shown bands are representative of PCR products amplified at 108 pb from *S. aureus* isolates (lanes 1 - 25), lane M represents 100 bp DNA ladder.

Additionally, PCR products from isolates were sequenced and compared with standard *16SrDNA* sequence with accession numbers: JN315154, KT369584, MF784283, KX583574, JN084552, MF385269, KF733730, KR265361 and CP012976, the result displayed high similarity level (92- 99%) that further confirms the identification of these isolates as *S. aureus.*

Detection of *mecA* gene in *S. aureus* isolates

All of *S. aureus* isolates (100%) that were included in this study harbored *mecA* as it is depicted in Figure-2. This finding specifies that all of the isolates are methicillin resistant; since there is no *mecA* gene in methicillin-sensitive *S. aureus* (MSSA) strains, detection of this gene in any isolates of *S. aureus* is indicative of MRSA [22].

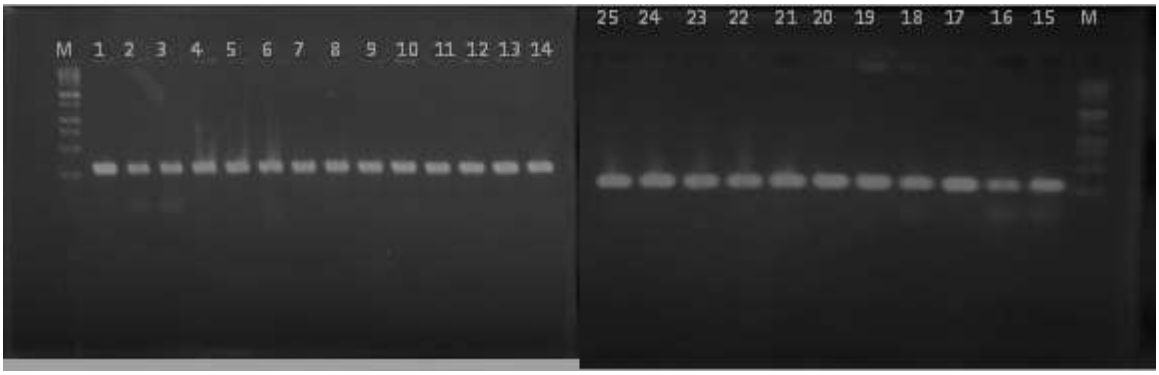

Figure 2-Visualization of *mecA* gene by 1.5% agarose gel analysis. The shown bands are representative of PCR products (310bp) amplified from *S. aureus* isolates (lanes 1 - 25), negative control (lane 26), lane M represents 250 bp DNA ladder

Detection of *gyrA* and *parC* mutations

The sequences of the PCR product was obtained and compared to sequences of *gyrA* and *parC* genes from NCBI, the results shown that 40 and 51 mutations in the forward and reverse strands, respectively, were detected in *gyrA* of the tested isolate; since *gyrA* encode for DNA gyrase. Such mutations would lead to amino acid substitutions, alter the target protein for fluoroquinolone structure and subsequently the fluoroquinolone binding affinity of the enzyme, leading to drug resistance [23]. On the other hand; after comparing the obtained sequence of *parC* from the tested isolate with sequences from NCBI, the result revealed complete similarity and no mutations were recorded. In plain words, resistance to ciprofloxacin in the tested isolates is due to mutation in gyrase rather than topoisomerase IV.

Determination of minimal inhibitory concentration (MIC) using agar diffusion method

Only 8% of *S. aureus* isolates were resistant to Ciprofloxacin (MIC = 4-16 µg/ml); whereas 28% developed intermediate resistance (MIC = 2 µg/ml); and 64% were sensitive to this antibiotic.

The findings of this study agrees reasonably with AL-Marjani *et al*. [24] who had stated that about 16 % of *S. aureus* isolates were resistant to Ciprofloxacin. Moreover, it disagrees greatly with an earlier study achieved by Al-Jebouri and Mdish [25] as they stated that about 40% of *S. aureus* isolates were resistant to Ciprofloxacin.

The increasing resistance of bacteria to Ciprofloxacin could probably be augmented by using it to treat many infections including prostatitis, UTI, endocarditis, gastroenteritis, infections of bones and joints, lower respiratory tract infection and enteric fever, among others, despite the fact that the risk of tendon rupture could increase upon using it. Notably, another factor contributing to the problem is the availability of Ciprofloxacin as oral suspension that is currently flooding the market; even though, it is not licensed by the FDA to treat children with Ciprofloxacin due to the high risk of permanent injury to the musculoskeletal system with the exception of inhalation anthrax and cystic fibrosis [26].

Conclusion

About 8% of isolated MRSA strains are resistant to Ciprofloxacin and this resistance is due mutant *gyrA* rather than *parC.*

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