Original Article

Fluoroquinolone resistant mechanisms in methicillin-resistant *Staphylococcus aureus* clinical isolates in Cairo, Egypt

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Abstract

Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a persistent problem in community and health care settings. Fluoroquinolones are among the drugs of choice used to treat MRSA infections. This study aims to identify different mechanisms of fluoroquinolne resistance in local MRSA random sampling isolates in Cairo, Egypt.

Methodology: A total of 94 clinical isolates of *S. aureus* were collected from two major University hospitals in Cairo. Identification was confirmed by appropriate morphological, cultural, and biochemical tests. The antibiotic susceptibility pattern was determined for all isolates. The possible involvement of efflux pumps in mediating fluoroquinolone resistance as well as changes in the quinolone resistance determining region (QRDR) of *gyrA* and *gyrB* genes were investigated

Results: A total of 45 isolates were found to be MRSA, among which 26 isolates were found to be fluoroquinolone-resistant. The MIC values of the tested fluoroquinolones in the presence of the efflux pump inhibitors omeprazole and piperine were reduced. Measuring the uptake of ciprofloxacin upon the addition of the efflux pump inhibitor omeprazole, an increased level of accumulation was observed. Non-synonymous and silent mutations were detected in the QRDR of *gyrA* and *gyrB* genes.

Conclusions: These results shed light on some of the resistance patterns of MRSA strains isolated from local health care settings in Cairo, Egypt. The resistance of these MRSA towards fluoroquinolones does not depend only on mutation in target genes; other mechanisms of resistance such as the permeability effect, efflux pumps and decreased availability of quinolones at the target site can also be involved.

Key words: Staphylococcus aureus; MRSA; fluoroquinolones; National Cancer Institute; El Damardash Hospital; efflux pumps

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Introduction

The alarming rise in antibiotic resistance among pathogenic bacteria is a persistent issue in antibiotic therapy in health care and community settings. Methicillin was developed in the late 1950's after penicillin failed to control staphylococcal infections [1]. However, by the early 1960's methicillin-resistant (also known as multidrug-resistant) *S. aureus* (MRSA) was isolated in a British hospital; in the following ten years, it became widespread in Europe, Australia and the United States [2,3]. Resistance to antibiotics is often acquired by the horizontal gene transfer from outside sources, although chromosomal mutations and antibiotic selection have also been reported [4]. *S. aureus* developed resistance to methicillin due to acquisition of the *mecA* gene which codes for penicillin binding protein 2A (PBP2A) that has lower affinity for binding β-lactams [5, 6].

Vancomycin is the drug of choice for MRSA isolates. Patients unable to tolerate vancomycin have been treated with minocycline, trimethoprim-sulfamethoxazole, clindamycin, or fluoroquinolones (the subset of quinolone antibiotics in clinical use) [7,8]. Quinolone antibiotics exert their antibacterial effects by inhibiting certain bacterial topoisomerase enzymes, namely DNA gyrase (bacterial topoisomerase II) and topoisomerase IV [9]. Quinolones act by binding to complexes that form between DNA and gyrase or topoisomerase IV. Shortly after binding, the quinolones induce a conformational change in the enzyme. The enzyme breaks the DNA and the quinolone prevents re-ligation of the broken DNA strands. The enzyme is trapped on the DNA, resulting in the formation of a quinolone-enzyme-DNA complex. Quinolone-gyrase-DNA complex formation rapidly inhibits DNA replication [10].

Mutations in gyrase or topoisomerase IV enzymes produce changes that cause resistance to fluoroquinolones by two basic mechanisms, alteration in the interaction of the fluoroquinolones with their target sites and alterations that affect access of the

drug [11]. The mutations responsible for resistance occur in certain regions of each enzyme subunit called the quinolone-resistance-determining-region (QRDR), particularly in the *gyrA* and *gyrB* genes of topoisomerse II, making the enzyme less sensitive to inhibition by fluoroquinolones. For some of the more common QRDR mutations, it appears that the amino acid changes reduce the affinity of the enzyme-DNA complex to fluoroquinolones [12].

A chromosomally encoded multidrug efflux pump, NorA, was identified in *S. aureus* in the 1990's [13]. This pump, a major facilitator superfamily (MFS) member, extrudes quinolone compounds and contributes to low-level quinolone resistance [14]. Together with other resistance mechanisms such as target alterations, the NorA and other efflux pumps can produce high level fluoroquinolone resistance in *S. aureus* [15]. The NorA pump activity could be inhibited by membrane proton pump inhibitors such as omeprazole, and competitive inhibitors such as reserpine [16]. To overcome bacterial resistance, it would be valuable to use inhibitors of resistance mechanisms that are able to potentiate the activity of existing antibiotics. In this approach, the antibiotic is co-administered with an inhibitor that neutralizes the resistance; consequently, the antibiotic is still useful, even in resistant organisms. This strategy can be used when resistance involves antibiotic-inactivating enzymes, and it has been validated with the successful use of β-lactamase inhibitors [17]. A similar approach can be used for target-modifying enzymes [18] and for efflux systems [19].

The goal of this study is to investigate the mechanisms of resistance to fluoroquinolones among MRSA strains isolated from two of the major university hospitals in Cairo and detect any novel mutations in the QRDR of the *gyrA* and *gyrB* genes that would affect fluoroquinolone binding. A large proportion of the population in lower socioeconomic classes relies on public university hospitals spread out across various locations in Cairo for their medical needs. Studying the patterns of antibiotic resistance among patients admitted to these hospitals can be used as a measure for mechanisms behind the spread of resistance.

Methodology

Statement of ethical approval

All experiments involving any samples taken from patients in this study were conducted in accordance with and approval of the ethical committee at Cairo University, Cairo, Egypt. In addition, all patients who contributed any samples (swabs or any other form), provided written informed consent; in case of children, written consent from the guardian or parent was obtained.

Isolation and identification of the strains

A total of 94 clinical isolates were obtained from the National Cancer Institute, Cairo, Egypt and El Damardash Hospital (University hospital of Ain Shames University, Cairo, Egypt). The samples were isolated from the blood of cancer patients and from wound infections, respectively. The isolates were identified according to flow charts of Bergey's Manual of Systemic Bacteriology and confirmed as S. aureus by using API KB004 Histaph identification (HIMEDIA, Mumbai, India) and Dryspot Staphytect Plus Kits (Oxoid, Cambridge, UK). The isolates were confirmed as MRSA by using the PBP2a test, oxacillin resistance screening agar base (ORSAB) (Oxoid, Cambridge, UK) confirmatory media, and antibiotic susceptibility tests against oxacillin (1μg) and methicillin (5 μg) discs. Other antibiotics tested for susceptibility were: amoxicillin (10 μg), amoxycillin/clavulanic (30 μg), ampicillin (10 μg), ampicillin/sulbactam (20 μg), cefepime (30 μg), chloramphenicol (30 μg), sulbactamciprofloxacin (5 μg), gentamicin (120 μg), levofloxacin (5 μg), ofloxacin (5 μg), tetracycline (30 μg), and vancomycin (30 μg). All culture media and antibiotics were from Oxoid, Cambridge, UK). Isolates were given numbers (1 to 94) for identification thought the whole work.

Minimum inhibitory concentration (MIC) determination by micro-dilution method

The test was carried in 96-well plates. The total volume in each well was 200 μl, starting with an antibiotic concentration of 512 μg/ml and applying twofold serial dilutions. Growth in each well was compared with that positive control. MIC was recorded as the lowest concentration that inhibited bacterial division as evidenced by the absence of turbidity in the wells. MIC determination was done in the presence and absence of efflux pump inhibitors omeprazole and piperine. All MIC determinations were done using Clinical and Laboratory Standards Institute guidelines.

Measurement of ciprofloxacin uptake by MRSA in the presence and absence of omeprazole using a fluorometric assay

Ciprofloxacin uptake assay was done according to Giraud et al. [20] with minor modification. Cells were

grown to an absorbance of 0.4 O.D at 600, harvested by centrifugation (5,600 × g for 5 min), washed once with 50 mM sodium phosphate buffer (pH 7.2), suspended in 50 mM sodium phosphate (approximately 40 mg wet weight per ml). Ciprofloxacin was added to a final concentration 10 µg/ml and left for 20 minutes, then centrifuged (5,600 × g for 5 min), washed with a 50 mM sodium phosphate buffer. The supernatant was discarded, the pellet was re-suspended in the same buffer, and 0.5 ml was withdrawn and diluted into 1 ml of the phosphate buffer. The suspension was then divided into two portions, and omeprazole was added to one portion. At appropriate time intervals, 0.5 ml samples were removed and diluted in 1 ml buffer; cells were then pelleted by centrifugation (5,600× g for 1 min) and washed with 1 ml buffer and then treated with 0.1M glycine HCl (PH 3) for 15 hours. The samples were centrifuged (5,600 × g for 5 min) and the fluorescence of the supernatant was determined at 442 nm emission with excitation at 282 nm.

Polymerase chain reaction (PCR)

Primers for gyrA were used to amplify the fragment at positions 2311-2533, while primers for gyrB were used to amplify the fragment at positions 1400-1650. According to Schmitz et al. [21], these regions correspond to QRDR in gyrA and gyrB, respectively. Primers used were gyrA forward primer: 5′- AATGAACAAGGTATGACACC- 3′ (corresponding to nucleotides 2311-2330) and gyrA reverse primer: 5′-TACGCGCTTCAGTATAACGC-3′ (corresponding to nucleotides 2514-2533).

gyrB forward primer: 5′-CAGCGTTAGATGTAGCAAGC- 3′ (corresponding to nucleotides 1400-1419) and gyrB reverse primer: 5′-CCGATTCCTGTACCAAATGC-3′ (corresponding to nucleotides 1631-1650). Reaction was done using the GoTaq DNA polymerase (Promega, Fitchurg, USA) at the following conditions: initial denaturation temperature at 95^{0}C for 10 minutes, followed by 25 amplification cycles of denaturation for 30 seconds at 94^{0}C, annealing for 30 seconds at 50^{0}C, polymerization for 30 seconds at 72^{0}C, and a final extension cycle for 5 minutes at 72^{0}C. The PCR products were purified using the QIA quick purification kit (Qiagen, Hilden, Germany). The molecular size of the PCR product of the two subunits of DNA gyrase was estimated by comparing with the migration of the used DNA marker on agarose gel. The expected PCR products were 222 bp and 250 bp for the gyrA subunit and the gyrB subunit gene fragments, respectively.

DNA sequencing was performed at Clinilab (Cairo, Egypt) using a DNA sequencer (Genetic analyzer, Singapore). The DNA sequences for the gyrA and gyrB genes in Staphylococcus aureus SA74 were retrieved from the NCBI database. Sequences were analyzed using Sequencher software (Gene Codes Inc., Arbor, USA). Multiple sequence alignments were done with the aid of NCBI Blast (URL: http: //blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Isolation and identification

A total of 94 clinical S. aureus isolates were collected from the National Cancer Institute (NCI) and EL Damardash hospital, Ain Shames University, both located in Cairo, Egypt. The 94 staphylococcal isolates were identified morphologically as Gram-positive cocci and confirmed to be *S. aureus* by typical growth on mannitol salt agar medium and conventional biochemical tests (coagulase positive, oxidation/fermentation positive, DNase positive). The findings of those conventional tests were confirmed by the API KB004 Histaph identification system. Isolates were confirmed as MRSA by positive agglutination using the Dryspot Staphytect Plus Kit, PBP2a test positive, blue colonies on ORSAB confirmatory media, and antibiotic resistance to methicillin and oxacillin on susceptibility testing.

Antibiotic susceptibility tests

Among the 94 isolates, 49 (52%) were sensitive to methicillin and oxacillin and 45 (48%) were resistant and identified as MRSA. A total of 26 isolates from the MRSA collection (58%) were resistant to fluoroquinolones (ciprofloxacin and levofloxacin) as well. The antibiotic susceptibility pattern of MRSA isolates showed that none were sensitive to oxacillin, ampicillin and amoxicillin, one was sensitive, and six isolates were intermediate to methicillin. 58% of MRSA isolates were resistant to ofloxacin, 55% were resistant to ampicillin/sulbactam, 100% were resistant to amoxicillin/clavulanic, 94% were resistant to cefepime, 43% were resistant to gentamicin, 67% were resistant to tetracycline, 20% were resistant to chloramphenicol, and 2% were resistant to vancomycin.

Table 1: MICs of MRSA isolates towards ciprofloxacin in presence of omeprazole and piperine

Isolate number	Ciprofloxacin (µg/ml)	Ciprofloxacin/Omeprazole (µg/ml)	Ciprofloxacin/Piperine (µg/ml)
2	64	64	32
6	64	64	32
7	64	32	32
10	64	32	32
13	16	8	4
14	16	4	4
20	128	64	64
36	64	32	32
37	32	8	4
44	64	32	32
50	16	8	8
52	32	32	32
55	64	32	32
57	64	32	32
62	128	64	64
66	64	32	32
67	16	16	8
70	64	32	32
72	16	16	4
73	64	32	32
76	64	32	32
77	128	32	64
79	128	32	32
80	64	32	32
81	64	16	32
83	8	4	4

MICs by microdilution method towards the fluoroquinolnes ciprofloxacin and levofloxacin

The MICs values were determined for fluoroquinolone-resistant MRSA to ciprofloxacin in the presence and the absence of efflux pump inhibitors omeprazole and piperine (Table 1). The 26 fluoroquinolone-resistant MRSA strains were tested; using omeprazole in combination with ciprofloxacin, 21 isolates (81%) were affected as shown by a marked decrease in MIC values, and five MRSA strains were not affected. When ciprofloxacin was combined with piperine, 25 MRSA strains (96%) were affected as shown by a marked decrease in MIC, and one MRSA strain (4%) was not affected. Similarly, the MICs were determined for the 26 MRSA isolates against levofloxacin in the presence and absence of omeprazole and piperine (Table 2). When levofloxacin was combined with omeprazole, six MRSA isolates (23%) showed a decrease in their MIC, and 20 isolates (77%) were not affected. A combination of levofloxacin and piperine resulted in 15 MRSA isolates (58 %) showing a decrease in MICs, while ten isolates (38 %) were not affected, and one isolate (4%) showed an increased MIC.

Measurement of ciprofloxacin uptake by MRSA isolates in presence and absence of omeprazole using a fluorometric assay

For the 12 isolates that showed a decrease in MIC to ciprofloxacin in the presence of the efflux pump inhibitor omeprazole, ciprofloxacin uptake was measured in the presence and absence of omeprazole. Isolates accumulated varying levels of ciprofloxacin. Five isolates (42%) (numbers 7, 10, 20, 73 and 57) showed an increase in ciprofloxacin accumulation in the presence of omeprazole, which explains the reduction in MIC values, while seven others (58%) (numbers 36, 55, 62, 66, 77, 79, 81) showed only a slight increase in accumulation, which indicates that the reduction in MIC is not attributed to increased accumulation, but rather due to other mechanisms such as target site modifications.

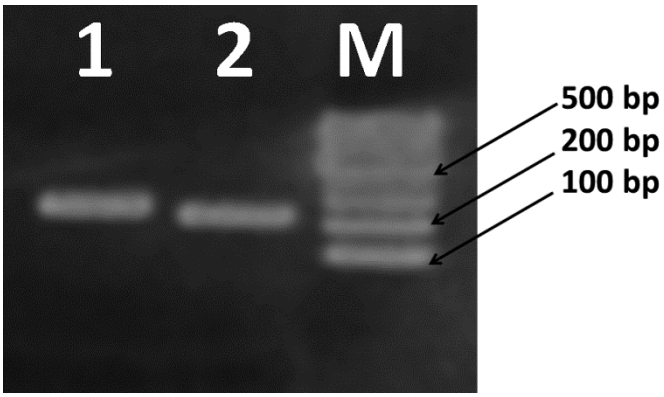
Table 2: MICs of MRSA isolates towards levofloxacin in presence of omeprazole and piperine

Isolate number	Levofloxacin (µg/ml)	Levofloxacin/Omeprazole (µg/ml)	Levofloxacin/Piperine (µg/ml)
2	8	8	4
6	8	8	4
7	8	8	8
10	16	16	8
13	16	16	16
14	4	4	4
20	16	16	8
36	16	16	16
37	16	4	4
44	16	16	16
50	16	16	16
52	8	8	4
55	16	16	8
57	16	8	16
62	16	16	8
66	16	16	8
67	8	4	4
70	8	8	8
72	8	8	4
73	16	16	16
76	32	16	16
77	16	8	8 µ
79	16	16	16
80	16	16	8
81	32	16	16
83	4	4	8

Amplification of QRDR in gyrA and gyrB genes in fluoroquinolone-resistant MRSA

The QRDRs of gyrA and gyrB genes, in the 12 quinolone-resistant MRSA isolates tested for ciprofloxacin uptake, were amplified by PCR. Products of sizes 222 bp and 250 bp were obtained for gyrA and gyrB respectively in the 12 isolates (Figure 1). PCR products were sequenced in three of the twelve isolates to detect possible mutations. Results showed that for gyrA gene, the three isolates (isolates number 73, 77, 79) showed a C2402T mutation, which led to S84I substitution in GyrA. Two isolates (numbers 77, 79) showed a T2409C mutation, which led to silent mutation at I86 in GyrA. One isolate (number 73) showed a T2460G mutation leading to silent mutation at L103 in GyrA. For GyrB, the same three isolates (73, 77, 79) showed a change in nucleotide T1497C; two isolates (numbers 77, 79) showed A1578G mutation leading to silent mutations at A439 and K465 in GyrB, respectively.

Figure 1: Representative PCR products of 1: *gyrA* (222 bp) and 2: *gyrB* (250) genes. M: Marker.



Discussion

Considerable attention has been given to antibiotic resistance found among pathogenic bacteria. Such resistance mechanisms are prevalent in developing countries, particularly among different Gram-negative and Gram-positive bacteria, due to the availability and affordability of antibiotics like ampicillin,

streptomycin, choloramphenicol, tetracycline and trimethoprim/sulphamethoxazole [22]. In addition, resistance to other antibiotics – particularly the new generations of cephalosporins, aminoglycosides and quinolones – has been reported [23-27], along with high rates of extended spectrum β-lactamase and /or Amp-C enzyme production [28]. One explanation for these high resistance rates could be antibiotic usage in the respective institutions. For example, in 1988, Egypt reported that more than 80% of admitted patients were prescribed antibiotics, in many cases without documented proof of infection [29]. Among these patients, more than 30% received repeated courses because of the prolonged periods of disease, though without the appropriate diagnostic tests to determine the exact cause of infection due to the lack of laboratory materials and equipment to perform the tests. Such policies are still in use in public and private hospitals, mainly due to inappropriate prescriptions in the form of wrong drug choice, errors in doses or duration [30], combined with the unavailability of adequate facilities in many public hospitals that can deal with the constantly increasing numbers of patients. In addition, the unrestricted access to antibiotics without a prescription (antibiotics dispensed over the counter) in pharmacies all across Egypt is also a major factor contributing to the problem [31,32].

S. aureus mutations occur first in the *par*C (topoisomerase IV) gene, leading to moderate levels of resistance to fluoroquinolones (MIC: 8 µg/ml) [33]. This is usually followed by a second mutation in the *gyrA* gene. Such *S. aureus* double mutants demonstrate high-level resistance to ciprofloxacin with MIC values ≥ 64 µg/ml. The same result was observed by Decousser *et al*. [34] who stated that mutations in *par*C and *gyrA* are known to raise fluoroquinolones MICs. Schmitz *et al*. [35] also reported that in *S.aureus*, the highest MICs are reached by the strains displaying mutations in the fluoroquinolne targets, DNA gyrase and DNA topoisomerase IV. The number of isolates that showed resistance to ciprofloxacin was similar to the number that showed resistance to levofloxacin. The MIC of ciprofloxacin for 56% of the isolates was 64 µg/ml; for levofloxacin, the MIC for 60% was 16 µg/ml.

Another mechanism involved in quinolone resistance in *S.aureus* is over expression of *nor*A gene. This gene encodes a multidrug efflux protein (NorA) capable of transporting fluoroquinolones outside the bacteria. Studies have shown that a mutant of *S.aureus* with a knockout in the *nor*A gene – coding for the MDR pump – has a substantially increased sensitivity to a large number of antimicrobials, including therapeutically significant compounds [36-38]. As inihibitors of the NorA efflux pump, reserpine, omeprazole, and lansprazole can improve fluoroquinolone activity against strains expressing different levels of NorA [16]. Piperine is a major plant alkaloid within the family Piperaceae and has been reported to enhance the accumulation of ciprofloxacin by *S.aureus* [39]. At concentrations of 12.5 and 25 mg/L, piperine caused a twofold reduction of MIC of ciprofloxacin. The reduction in MIC results of the tested fluoroquinolones in the presence of efflux pump inhibitors omeprazole and piperine showed a much more substantial effect in the majority of the isolates when ciprofloxacin was combined with omeprazole (81% of isolates) and piperine (96% of isolates), compared to levofloxacin when combined with omeprazole (23% of isolates) and piperine (58% of isolates). This might indicate the involvement of an additional mechanism of resistance in the case of levofloxacin. The ciprofloxacin accumulation uptake measurement indicated that only five isolates showed considerable accumulation of ciprofloxacin, indicating the involvement of other mechanism of resistance in the other isolates in addition to the activity of efflux pumps.

Amplification of partial sequences of the QRDRs in *gyrA* and *gyrB* genes in the same 12 MRSA isolates was done in an attempt to assess the association of mutations in *gyrA* and *gyrB* genes with fluoroquinolone resistance. Similar mutation at the same position in GyrA (S84L) was previously reported by Schmitz *et al*.[21]. In addition, three other mutations: E88K, G106D and S112R the same silent mutation at I86; and another silent mutation at F110 were recorded by the same study. Mutations in similar or neighboring positions were also recorded in other studies: S84L, S84A, S85P and E88K in GyrA, and D437N, R458Q in GyrB [40]. Mutations conferring quinolone resistance in the *gyrA* gene of *E. coli* were also confined to that region [14]. The mutations identified in the present study indicate that point mutations at different positions of the QRDR in *gyrA* and *gyrB* sequences lead to decreased affinity between the fluoroquinolones and the enzyme. Several mutations in the *gyrA* and *gyrB* genes were basically silent mutations that led to the presence the same amino acid; however, in general, many fast-growing organisms such as *Escherchia coli* and *Saccharomyces cerevisiae* show codon usage bias, which provides a well-known method for translational control by

changing the rate of incorporating the same tRNA in response to different codons. It is very likely that the silent mutations detected in our MRSA isolates have the same effect, resulting in slower translation rates and low accuracy in an attempt to evade the antibiotic effect.

Conclusion

The observed patterns of resistance in this collection of clinical isolates indicate that different mechanisms of MRSA resistance to fluroquinolones can exist in different isolates or even coexist within the same organism. Such differences in patterns might be a reflection of the source of the isolates and their environment. Due to the diversity of these resistance mechanisms and the constant appearance of new patterns, antibiotic utilization in developing countries should be under strict control and should be monitored to avoid the exhaustion of the antibiotic arsenal that is under intense use.

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