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Identification of DNA gyrase Subunit a Mutations Associated with Ciprofloxacin Resistance in Staphylococcus aureus Isolated from Nasal Infection in Kurdistan-Iran

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Abstract

Fluoroquinolone antibiotics such as ciprofloxacin are useful drugs against infections caused by Staphylococcus aureus and mutations in DNA gyrase which control bacterial DNA topology, can be one of the reason of occurrence resistance to this class of antibiotics. Therefore finding new mutations and study of the quinolone interaction with mutated GyrA can provide important issues for explanation resistance. In this study 5 ciprofloxacin resistance Staphylococcus aureus isolated among 50 collected S.aureus strains. By PCR testing, gyrA genes in resistance strains was amplified and nucleotide sequencing was done. Nucleotide sequences translate to amino acid sequences then by blastp homology between each GyrA mutant and reference GyrA were compared and mutations were recognized, at last molecular docking were done for GyrA protein and ciprofloxacin, based on free energy of binding decide if the mutations are responsible of resistance or not. The results show glutamic acid and threonine adjacent to each other in common positions 21-22, 32-33, 65-66, 84-85, 101-102, 106-107, 128-129 and 138-139 in all 5 strains were inserted . In order to finding association between mutations and ciprofloxacin resistance molecular docking by Molegro Virtual Docker 5.5 was done. Free energy of binding between reference GyrA- ciprofloxacin and mutant GyrA- ciprofloxacin were -92.3477 and -73.1642 respectively. We conclude different mutations can be affected structure of GyrA and make ciprofloxacin resistance. Finding these kinds of mutations are important and preventing them is indispensable.

Keywords: Staphylococcus aureus, disk diffution, Expasy, sequencing

1. Introduction

The introduction of antibiotics for the treatment of infectious diseases was one of the hallmarks in the 20th century medicine. However, shortly after their introduction into the clinical practice, the first bacteria showing antibiotic-resistant were described. Since then the development of new antibiotics have been attend by the constant increase of antibiotic-resistant bacterial strains and different mechanisms used by bacteria to repress the fatal effect of these compounds (Costa et al., 2013). In the last decades concern about bacterial resistance to antibacterial agents because of counter availability, indiscriminate and inappropriate usage of antimicrobial agents has been arised (Neuhauser et al., 2003). Staphylococcus aureus which is a human commensal and well-endowed opportunistic pathogen, can be one of the major delinquent, especially antibiotic-resistant strains (Gao et al., 2015) the wide spread increase in S.aureus antibiotic resistance has significantly restrict treatment agents, especially with the emersion of resistance to antibiotics such as vancomycin and daptomycin (Gao et al., 2013). Fluoroquinolones are DNA-targeting antibiotics and interact with type II topoisomerase, thus augmenting generation of single and double-strand DNA breaks related to stated or collapsed replication forks (Didier et al., 2011). The diversity of fluoroquinolone antibiotics, chiefly ciprofloxacin, made feasible the effective treatment of infectious caused by

S.aureus strains, quickly become resistant to these antibacterial agents (Pourmand et al., 2014). some mechanisms of resistance to quinolones are at the present time identified: mutations that alter the drug targets, mutations that reduce drug accumulation, and plasmids that protect cells from the lethal effects of quinolones (Kaatz & Seo, 1997) fluoroquinolone resistance in S.aureus has been ascribed to mutations arising in the quinolone-resistance determining region (QRDR) of parC, encoding topoisomerase IV, and gyrA, encoding DNA gyraseA. How ever, fluoroquinolone resistance can be mediated by the chromosomally encoded multidrug resistance (MDR) efflux pumps NorA, NorB and NorC which are widely existing in various strains and are recognized based on their ability to confer resistance to quinolones (Kwak et al., 2013).

The primitive target of several fluoroquinolones in S.aureus is thought to be topoisomerase IV (encoded by the grlA and grlB genes), whereas DNA gyrase (encoded by the gyrA and gyrB genes) is considered as the secondry targets (Piddock et al., 2002). Amino acid replacement correlating with fluoroquinolone resistance in GrlA include Ser80->Phe or Tyr, Ala116->Glu or Pro, and Glu84->Lys (Kaatz & Seo, 1997; Agents an chemotherapy, 1992; Ferrero et al., 1994; Ferrero et al., 1995; Yamagishi et al., 1996). With respect to GyrA, Ser84->Leu or Ala, Ser85->Pro and Glu88->Lys mutations are associated with fluoroquinolone resistance (Kaatz & Seo, 1997; Goswitz et al., 1992; Kaatz & Seo, 1995) amino acid replacement in GyrB correlating with fluoroquinolone resistance include Asp437->Asn and Arg458->Glu (Ito et al., 1994; Kaatz & Seo, 1997).

The aim of this study was identifying amino acid replacement in DNA gyrase subunit A (protein GyrA) in staphylococcus aureus isolated from nasal infection in patients reffered to medical centers of Kurdistan province by DNA sequencing method and BLAST instruments, After determining mutations molecular docking were applied to find transformations of ciprofloxacin (ligand) with respect to mutant GyrA (targets) that assemble the two the complex ligand-target.

2. Material and Methods

2.1 Sample Collection and Isolation of Staphylococcus Aureus

50 Staphylococcus aureus strains, collected from patients who suffering from nasal infection and applied to medical centers of Kurdistan province within 3 months period starting from september 2015. Identification of S.aureus were done by gram staining and conventional biochemical tests such as coagulase, novobiocin sensitivity, mannitol fermentation test, DNase, hemolysis, polymyxin sensitivity test.

2.2 Antibiotic Suspetibility Testing

The Kirby-Baur (disk diffusion) method was used to test sensitivity and resistance of antibiotics. The disks which were used included ciprofloxacin (5 μg), vancomycin (30 μg), amikacin (30 μg), oxacillin (10 μg), erythromycin (30 μg), nalidixic acid (30 μg), doxycycline (30 μg), tetracycline (30 μg) and penicillin (10 μg) (Mast Company).

Inoculums were prepared in steril salin solution from grown culture of nutrient agar 0.5 Mc Farland turbidity value was obtained for each bacterial inoculum and by steril cotton swab was incubated on Muller Hinton agar, and then antibiotic disks were placed on plates. The plates were incubated at 37 ℃ for 18-24 hours. After incubation time, inhibition zone diametr were measured and the results were interpreted according to CLSI standard.

2.3 DNA Extraction and Identifying gyrA Gene:

The template DNA were prepared and extracted for PCR amplification through using gram positive bacteria, DNA extraction (Sinaclone Company). And extacted DNAs stored at -20 ℃ untill needed.

Polymerase chain reaction was also carried out for detecting gyrA (885 bp), with specific primer sequences that have been stated in (table 1) and under the conditions stated in (Table 2).

Table 1. Primer sequences used in PCR amplification

Primer	Sequences	size	refrence
gyrA-Fw	5'-GCCACCGTTGTATAAACTGAC-3'	885bp	Kaatz and Seo (1997)
gyrA-Rv	5'-ATACCTACCGCGATACCTGATG-3'		

Table 2. PCR amplicon condition

Initial deanatiration		94 ℃	10min
	deanatiration	94 ℃	30s
30cycle	Annealing	53.1 ℃	30s
	Extention	72 ℃	1min
Final extention		72 ℃	10min

The reaction mixture was prepared in a final volume of 25 µl and the reaction compositions included 1 µl of each of the forward and reverse primers (a total of 2 µl), 8.5 µl of deionized water, 12.5 µl of Master Mix (Sinaclon Company), and 2 µl of the template DNA. The result of the gyrA PCR amplification was determined through loading the PCR product on 1.5% agarose gel staining with 0.5 µg/ml safe stain and analyzed by gel electrophoresis at 100 V voltages for 40 minutes.

2.4 DNA Sequencing

20µl of PCR product of gyrA gene and used primers (forward and reverse), were sent to Bioneer Company for DNA sequencing tests.

2.5 Nucleotide Analysing

Blastn were done for detecting homology between gyrA gene nucleotide sequences in resistance strains and refrence DNA gyrase subunit A (GenBank: AB086041.1) sequence (Table 3).

Table 3. Comparing nucleotid sequencing of gyrA gene in 5 resistant strains with refrence sequence

Gene	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Length	533	529	574	522	17
gyrA-similarity	509/533	444/529	379/574	430/522	15/17
	(95.5%)	(83.9%)	(69.2%)	(82.4%)	(88.2%)
Gaps	20/533	10/529	50/574	16/522	0/17
	(3.8%)	(83.9%)	(8.7%)	(3.1%)	(0.0%)
Score	2269	1808	905	1650	67

2.6 Translate Nucleotide Sequences to Amino Acid Sequences

By Expasy translator server nucleotide sequences of resistance strains and reference strain translate to amino acide sequences, and blastp applied for aligning GyrA amino acid sequences in resistance and refrence strains. The sequence alignments showed how well GyrA query sequence matches with the subject sequence in the database and mutations were found. In the result we obtained five pose (accurate position ond orientation of ligand) that we assumed the first one for analyzing and comparing (Table 4).

Table 4. Comparing GyrA protein sequences in 5 resistant strains with refrence strain

Protein	Sample 1	Sample 2	Sample 3	Sample 4	Sample5
Length	159	171	146	52	4
GyrA-similarity	136/159	146/171	124/146	48/52	3/4
	(84.9%)	(85.4%)	(84.9%)	(92.32%)	(75.0%)
GyrA-Identity	135/196	140/171	119/146	46/52	3/4
	(84.9%)	(81.9%)	(81.5%)	(88.5%)	(75.0%)
Gaps	17/159	18/171	14/146	4/52	0/4
	(10.7%)	(10.5%)	(9.6%)	(7.7%)	(0.0%)
Score	536	565	508	196	18

2.7 Molecular Docking

The interaction study on ciprofloxacin and DNA gyrase A (GyrA) was done by using Molegro Vitual Docker 5.5 software. Docking of ligand and protein repeated 15 times and the average of free energy of binding was cacculated.

$$\Delta G = -RT\ln K_A \qquad K_A = K^{-1}_i = [EI] / [E][I]$$

2.8 Ligand Preparation

3D structure of ciprofloxacin as ligand of DNA gyraseA from zinc database (Zinc numcer: ZINC00020220) were taken.

2.9 Protein Preparation

The X-ray crystal structures of reference GyrA were made by Swissmodel-Expasy server, then common found mutations were inseted to the refrence sequence and again by Swissmodel-Expasy server 3D structure of mutatnt

GyrA were prepared. Molecular docking was done for ligand (ciprofloxacin) and proteins (reference and mutatated GyrA). (Figure 1-A&B)

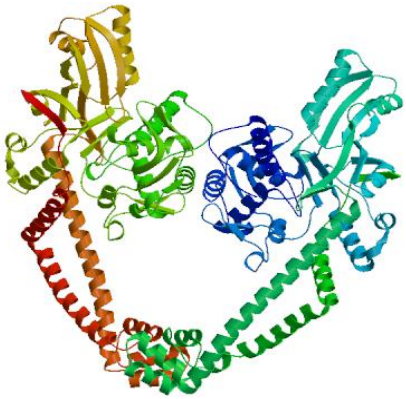

Figure 1-A. Reference GyrA protein structure

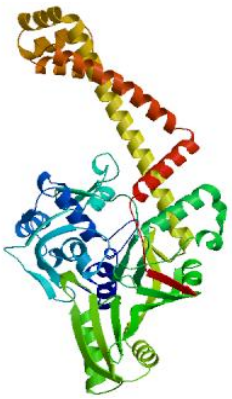

Figure1-B. Mutated GyrA structure

3. Results

50 S.aureus strains were isolated from nasal infection, isolates were collected from women (43%) and men (57%) in the present research. Through disk diffusion method, antibiotic resistance rate were estimated: oxacillin (42%), erythtomycin (46%), deoxycycline (10%), amikacin (20%), tetracycline (24%), penicillin (68%), ciprofloxacin (10%), vancomycin (6%) and nalidixic acid (70%).

After determining sensitivity and resistance rate to used antibiotics, ciprofloxacin resistance S.aureus starins was isolated; among 50 S.aureus 5 starins were resistance to ciprofloxacin. By using PCR testing and specific primers existance of gyrA in all resistant isolates were confirmed (figure 2).

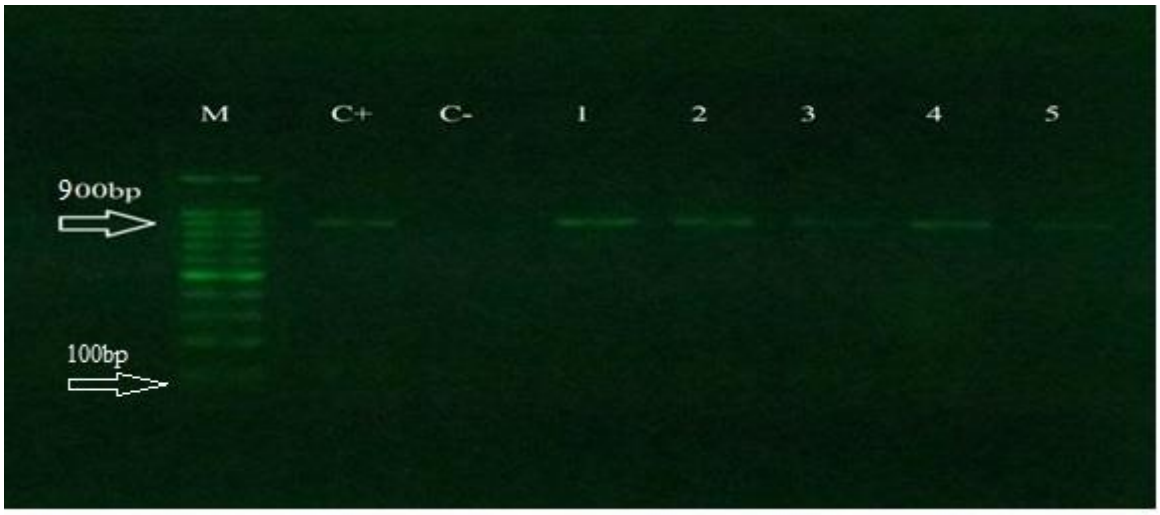

Figure 2. PCR products of gyrA gene run on 1.5% agarose gel (885bp), lane C^+: positive control(Staphylococcus aureus ATCC29213), C^-: negative control (water), lanes 1-5: S.aureus isolated from patients; M: 100 bp size ladder

The results of nucleotide sequencing tests for 5 resistance strains included 10 nucleotide sequencing reading pattern (for each isolates 2 pattren: one for forward strands and one for reverse strands). Based on blastn different nucleotide mutations were seen in each strrains, because of numerous mutations were not stated here but the homology between each isolates and refernce isolate was noted in table 3. then nucleotide sequences translated to amino acid sequences, and by blastp, common amino acid mutations were identified, which Glutamic acid and Threonine were inserted in different adjacent positions:such as in amino acids numbers 21-22, 32-33, 65-66,84-85,

101-102, 106-107, 128-129 and 138-139 in four isolates. Amino acid homology between each isolates and reference isolated was noted in Table 4.

```
+
EMBOSS_001          4 ADLPQSRINERNITSEMETRESFLDYAMETSVIVSRALPDVRDGLKPVHR     53
                      |:|||||||||||||||  |||||||||  ||||:|||||||||||||||
EMBOSS_001          2 AELPQSRINERNITSEM--RESFLDYAM--SVIVARALPDVRDGLKPVHR     47

EMBOSS_001         54 RILYGLNEQGMETTPDKPYKKSARIVGDVMETGKYHPHGDLSIYDAMETV    103
                      ||||||||||  ||||.||||||||||||  ||||||||.|||:||  |
EMBOSS_001         48 RILYGLNEQGM--TPDKSYKKSARIVGDVM--GKYHPHGDSSIYEAM--V     91

EMBOSS_001        104 RMETAQTFSYRYPLVDGQGNFGSMETDGDGAAAMETRYTEAKMETTKITL    153
                      ||  ||.||||||||||||||||  ||||||||  |||||:|  |||||
EMBOSS_001         92 RM--AQDFSYRYPLVDGQGNFGSM--DGDGAAAM--RYTEARM--TKITL    133

EMBOSS_001        154 ELLRDINKDTIDFLDTMETME...     174
                      |||||||||||||:|..:..|
EMBOSS_001        134 ELLRDINKDTIDFIDNYDGNE...     154
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Sequence 1: amino acid sequence alignment of GyrA protein in resistance and refrence strains. The upper strand is related to resistance strain and lower strand is related to refrence amino acid sequence (GenBank: AB086041.1).

Common insetion mutations were showed by red colour. Purple region is QRDR

Molecular docking demonstrates that the free energy of binding (ΔG) for ciprofloxacin- reference GyrA was -92. 3477 and the steric interactions were between Pro 157, Leu338, Leu 35, Asn 340, Val 339 and Asp37 and ciprofloxacin. In the steric interactions of mutant GyrA and ciprofloxacin Val 35, Gly357, Asp41and Arg 52 had been involved, and also the free energy of binding (ΔG) was -73.1642 (Figure 3).

4. Discussion

The aim of this study was identification of amino acid mutations in GyrA protein encoding subunit A of DNA gyrase of Staphylococcus aureus which isolated from nasal infection and studying the influence of identified mutations on the structure of GyrA for interact with ciprofloxacin. Several conventional antibiotics tested on 50 isolates of S.aureus and the results demonestrates that maximum resistance was noted to nalidixic acid (70%), followed by penicillin (68%) and erythromycin (46%) and Maximum susceptibility was noted to vancomycin (6%) followed by ciprofloxacin (10%) and doxycycline (10%). In the antibiotic guidline 2015-2016 mentioned that oxacillin or naficillin and vancomycin for S.aureus suspetible to methicillin and vancomycin for methicillin resistant S.aureus should be Prescripted (Antibiotic Guidelines. (2015-2016)), in our results confirmed that vancomycin can be one of the choices for treatment of infections caused by S.aureus but resistance rate to oxacillin was 42% so this antibiotic can not be usefull in some cases. We based our study on ciprofloxacin resistance which is a quinolone antibiotic, resistance to quinolones has been a problem ever since nalidixic acid was introduced into clinical medicine >40 years ago (Jacoby., 2005). The quinolones operation on DNA gyrase, which relives DNA supercoiling and topoisomerase IV, which seprates concatenated DNA strands. Amino acid changes in critical regions of the enzyme-DNA complex reduce quinolone affinity for its target. Single amino acid mutation are some times sufficient to confer clinical resistance, but for more active fluoroquinolones additional mutations appear necessary (Lowy., 2003).

Amino acid numbers 115-173 (purple color) in S.aureus is Quinolone resistance-determining region (QRDR) (Josep et al., 2005) (Figure 4). We identified four of the common inserted mutations in positions 128-129, 138-139 which placed in QRDR region and increase the possibility of quinolone resistance.

Various methods have been reported to detect point mutations in target genes, including sequence-specific oligonucleotide probe hybridization, sequencing of the target genes, RFLP, radioisotopic or nonradioisotopic SSCP analysis, mismatch amplification mutation assay PCR, and allele-specific PCR in combonation with RFLP (Treatment Recommendations For Adult Inpatients (2015-2016)) which in this sudy sequencing method have been used. The sequncing results for one of the isolates was not efficient but based on nucleotid sequence of other four isolates similar mutations were identified. Although protein blast demonstrated some point mutations in each isolates, but two adjacent insertion mutaions were obvious in all isolates, which Glutamic acid and Threonine were inserted in different positions such as amino acid numbers 21-22, 32-33, 65-66, 84-85, 101-102, 106-107, 128-129, 138-139 and 147-148.

An important point in this study is that, how ever in nearly all reseaches on this topic confirmed that point mutation in position 84 (Ser->Leu) is cause of fluoroquinolone resistance in Staphylococcus aureus, but only in one of our ciprofloxacin resistance isolates this mutation have observed (Sequence1, blue colour) and in other isolates this mutation was not found.

In the study of Wang four types of single point mutations and four types of double mutations were found in gyrA genes of 188 strains that (Ser84-> Leu) were principal and being detected in 137 isolates. point mutations were: Asp73-> Gly, Ser84->Leu, Ilu(silence), Glu88-> Lys, and double mutations were Ser84->Leu and Asp73->Gly, Ser83->Leu and Ser 85-> Pro, Ser84->Leu and Ile(silent), Ser84->Leu and Glu88-> Lys (Wang et al., 1998). In the study of McCurdy gyrA mutations in Staphylococcus aureus 10 point mutations observed that 7 of them were Ser84->Leu and other were Glu88->Lys, S85->P (McCurdy, 2017). In the study of Cheng the results showd 10 point mutation in S84->Leu, four doual mutations in Ser84->Leu with Glu88->Val, one Ser84->Leu with Glu409->Lys, one Ser84->Leu with Ser85->Pro, One Ser 84->Leu with Glu88->Lys (Cheng et al., 2007). The results of Hauschild demonestrate that all amino acid alteration cause fluoroquinolone resistance in gyrA were related to the Ser84->Leu and in one sample Glu88->Asp was observed (Hauschild et al., 2012). In the study of Santos Costa reported that nearly all gyrA mutations related to fluoroquinolone resistance in Staphylococcus aures was Ser84->Leu and in 3 isolates Glu88->Lys was identified (Costa et al., 2013). In the study of Rasha Ser84->Leu, two silent mutations in Ile86 and Leu103, Glu88->Lys, Gly106->Asp, Ser112->Arg in GyrA was reported (Hashem et al., 2013). In our study we could find Ser 84->Leu only in one of the isolates.After indentification new common mutations to understading influence of these mutations on ciprofloxacin resistance, structure of ciprofloxacin and reference and mutated GyrA in pdb format were prepared and molecular docking were done. As we expected free binding energy of reference protein (-92. 3477) was more than mutated protein (-73.1642). Free energy is released by the formation of a number of weak interactions between GyrA and ciprofloxacin, only the correct form of enzyme and ciprofloxacin can participate in most or all the interaction, thus maximal binding energy is released when protein and ligand correctly bind with each other (Berg & Stryer, 1975) so when free binding energy is higher, cmplex is more stable, therefore we can conclude mutations by changing structure of GyrA affected the ineractions and decrease free binding energy and ciprofloxacin can not ineract with GyrA properly so cause resistance (Figure 3).

The observed mutations of resistance in this collection of clinical isolates indicate that different type of mutations can exist in different isolates. Such diffrences can because of the source of isolates and their environments. Due to the diversity of these mutations and emersion of new mutations, antibiotic utilization in all countries should be under strict control and finding ways for preventing antibiotic resistance is an inevitable phenomenon.

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