RESEARCH PAPER | Science | Volume : 4 | Issue : 7 | July 2014 | ISSN - 2249-555X

Comparison Study between Antibiogram and Quorum-Sensing (Qs) Gene Clusters Analysisl for *Staphylococcus Aureus*

KEYWORDS: agr , dendrogram, S. aureus , quorum-sensing (QS).

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ABSTRACT *Thirty-eights S. aureus isolates gathered from differ¬ent hospitals and isolated from various specimens. All isolates under study design and distributed to three groups depending on the type of multidrug resistance as well as dendrogram based on antimicrobial drug sensitivity pattern of 38 non- repetitive staphylococcal isolates and the accessions are clearly divided into two major clusters, A and B. As to the susceptibility to antibiotics, there was no significant variability between test repetitions. The agr specificity group was determined by a PCR method analysis of agr gene polymorphism by specific primers allowed assigning our strains in 1 of 4 major specific agr groups, most of our isolates belonged to agr group I (32%), followed by agr group II (47%), agr group III (16%), and agr group IV (5%). To considering the possible relation between the agr group and the antibiotic resistance in S. aureus isolates, the study showed that isolates with a resistance group A (table 3)table.4) , the dominant genes from (4 isolates agrII) and One isolate each group agrI and agrIII, But not contain agrIV genes. group B the dominant genes from (5 isolates agrII) and (5 isolates agrI) gene and (2 isolates agrIII, 2 isolates agrIV) .the high resistance group C the dominant genes from (9 isolates agrII) and (6 isolates agrI) ,(3 isolates agrIII) But not contain agrIV genes.*

Introduction

Staphylococcus aureus is an extremely versatile human pathogen responsible for a broad range of nosocomial and community-acquired infections due to an impressive array of extracellular and cell-wall-associated virulence determinants that allow it to adhere to surface, invade or avoid the immune system, and cause harmful toxic effects to the host [1]. The coordinated expression of S. aureus virulence factors is regulated by a complex network including the quorum-sensing (QS) system agr and the well characterized virulence gene regulators [2]. The agr (accessory gene regulator) locus consists of two divergent transcription units RNAII and RNAIII driven by two promoters, P2 and P3, respectively. The P2 transcript, RNAII, contains four genes: agrA, agrB, agrC and agrD. The sensor, AgrC, and the response regulator, AgrA, comprise the two component system that responds to autoinducing peptide (AIP). This peptide is encoded by agrD, being post translation ally modified and secreted by AgrB [3]. Under conditions of high autoinducer concentration, e.g., high bacterial density, RNAIII up-regulates the expression of post-exponentially synthesized extracellular virulence factors (toxins, hemolysins, proteases, lipases) and down-regulates the expression of cell-surface-associated proteins [4,5]. Within a given group, each strain produces a peptide that can activate the agr response in the other member strains, whereas the AIPs belonging to different groups are usually mutually inhibitory. The inhibitory activity of these agr groups represents a form of bacterial interference that influences virulence gene expression. Different in vivo studies have shown that agr mutants appear to have diminished virulence in several animal infections models, including intramammary infections, arthritis in mice, and endocarditis in rabbits [6]. Recent studies have shown the existence of a strong association between the agr types and certain of S. aureus diseases [7].. They reported that agr group IV strains were associated with generalized exfoliative syndromes.8 Other studies found that TSS toxin 1-producing isolates belong to agr specificity group III and endocarditis strains mainly belonged to agr groups I and II and also agr group I strains involved in invasive infections, especially bacteremia. [8,9].

The inhibition of staphylococcal pathogenesis could be accomplished without growth inhibition, thus potentially avoiding selective pressures for drug-resistance. The staphylococcal QS system is a cell-density-dependent mechanism for controlling protein expression, including the production of staphylococcal virulence factors such as the α-, β, and δ-hemolysins. It is encoded by the agr locus, which is a quorum-sensing gene cluster of five genes (hld, agrA, agrB, agrC and agrD) [10].

Quantification of δ-toxin produced by S. aureus and found in the culture supernatants allows for the analysis of agr activity at the translational, rather than transcriptional, level. The identification of agr-inhibiting drugs, or staphylococcal QS-inhibitors, has been proposed by several research groups as a potential anti-staphylococcal therapy [11,12].

S. aureus agr is a 3-kb locus showing highly conserved and hypervariable regions. Sequence diversity in the variable region, comprising the last one-third of AgrB, AgrD and the first half of AgrC, has generated the 4 agr specificity groups in S. aureus(I – IV) and it is the target of PCR amplification for defining agr types [13]. Therefore,

The aims of this study wis important to determine agr genotypes and toxin gene profiles of S. aureus recovered from different human sources in Iraq using multiplex PCRs to understand the genetic and pathogenic relatedness, as well as the epidemiology of S. aureus. Finally, it would be interesting to establish a possible relationship between the agr groups and the antibiogram to investigate the contribution of the agr operon to the virulence of S. aureus strains.

Materials and Methods

Bacterial Isolates:

A total of 38 non- repetitive staphylococcal isolates were isolated and identified from 92 clinical samples. The isolates were identified by conventional methods (gram-positive cocci, catalase-positive, mannitol fermenting). coagulase negative staphylococci (CoNS) was based on colony morphology All isolates were stored in brain heart infusion (BHI) broth, to which 15% sterile glycerol was added, at _20 _C. The strains were identified by help of API microtests.

Antibiotic Susceptibility Testing

Susceptibility testing was conducted by disk diffusion according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI)[14] .Antibiotic discs (Becton Dickinson, USA) were placed on Mueller-Hinton agar plates, incubated at 37°C for 24 h, and the diameter of each zone was measured in millimeters. The following antibiotic discs were used:Six

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Antimicrobial disks tested included Oxacilin OX(1µg), Lincomycin L (5µg), Gentamycin CN (10µg), Amikacin AK (30µg), Methecillin ME(µg), Vancomycin VA (10 µg). Staphylococcus aureus ATCC25923 was used for quality control purposes in susceptibility testing.

Biofilm formation

phenotypic method was used for detection of biofilm production of all the staphylococcal isolates; one qualitative (Congo red agar method) (CRA) method According to Freeman et al. [15], the CRA medium was prepared with 37 g/l BHI broth, 50 g/l sucrose, 10 g/l agar, and 0.8 g/l Congo red. Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121 _C for 15 min separately from other medium constituents, and was then added when the agar had cooled to 55 _C. Plates were inoculated and incubated at 37 _C for 24 h. The plates were inspected for the color of the colonies at 24 and 48 h. A positive result was indicated by black colonies whereas nonproducing strains developed red colonies [16]. The Congo red dye directly interacts with certain polysaccharides, forming colored complexes or more likely some metabolic changes of the dye to form a secondary product could play a more important part in the formation of dark colonies [17,18]. For colonies color evaluation, a four-color reference scale was used according to Satorres and Alcara´ z [19]: black and bordeaux almost black were classified as biofilm-producers, while bordeaux and red as non-biofilm-producing strains. This method was performed in triplicate.

Screening for protease activity

Nutrient agar supplemented with 2% casein (Sigma, St. Louis, MO) was used to screen for protease activity. The isolates were subcultured on TSA and incubated at 37°C for 72 h. They were inoculated on casein agar plates and incubated at 37°C for 72 h. The isolates producing opalescent zones around the colony were identified as protease positive [20[.

Haemolysin production

Haemolysis test was done for the detection of haemolysin production by S. aureus. The bacteria were inoculated on 5% human blood agar (group O^+) and incubated overnight at 37 °C. Haemolysin production was detected by the presence of clear lysis zone around the colony and clearing of the medium [21].

Genomic DNA extraction:

Total DNA was extracted from S. aureus grown on mannitol salt agar plates by using the boiling approach as described previously [22]. In brief, for rapid DNA extraction, one to five colonies of each freshly subcultured strain were suspended in 50 µl sterile distilled water and heated at 99°C for 10 min. After centrifugation at 30,000 xg for 1 min, the supernatant was used as a DNA template and stored at -20°C until PCR was performed.

PCR amplification procedure:

Primers obtained from Alpha DNA (USA). Descriptions and sequences of the PCR primers used in this study are displayed in Table 1. Amplification was performed in a thermal cycler (Eppendorf, Germany) according to the methods described by [23].

The characterization of the agr operon in the analyzed S. aureus strains was performed by PCR multiplex using specific primers for the detection of agr groups. The agr locus in S. aureus has been shown to be polymorphic, because it consists from highly conserved and hypervariable regions among S. aureus strains, and can be divided into four distinct genetic groups. The agr specificity groups in the analyzed S. aureus strains were identified by PCR amplification of the hypervariable domain of the agr locus using oligonucleotide primers specific for each of the four major specificity groups. Thus, we used in all reactions a forward primer, pan-agr corresponding to the conserved sequences from the agrB gene, and four reverse primers, each specific for amplification of a single agr group based on agrD or agrC gene nucleotide polymorphism [24].

Table 1: The primer sequences used for amplification of the hypervariable region of agr locus in the analyzed

Primers	Sequence (5′ - 3′)	Amplicon size (bp)
pan forward agr	ATGCACATGGTGCACATGC	
Reverse agrI	GTCACAAGTACTATAAGCT-GCGAT	440bp
Reverse agrII	GTATTACTAATTGAAAAGT-GCCATAGC	572bp
Reverse agrIII	CTGTTGAAAAAGTCAAC-TAAAAGCTC	406 bp
Reverse agrIV	CGATAATGCCGTAATACCCG	588 bp

Gradient PCR amplification procedure:

The reaction was performed in a PCR thermal cycler apparatus, and after several trials, and according to the manufacture's guide. The following program was adopted:-PCR consisted of a preheating at 95°C after this initial denaturation step, the mixture was subjected to 35 amplification cycles as follows:-

Step	Program of gradient PCR for agr gene
1	One cycle for 2 minutes at a temperature of 95°C to initial denaturation DNA template. 35 cycles included:
2	
A	(1) minute at a temperature of 95°C to denaturation DNA template.
B	(1) minute at a temperature of (55°C Gradient Δ 10°C)primer annealing with DNA template.
C	(1) minute at a temperature of 72°Cprimer extension with DNA template.
3	One cycle for (5(minutes at a temperature of 72°C to final extension replicated DNA strand.

Table 2: The conditions used for the amplification of agr gene.

Initial denaturation	No. of cycles	Denaturation in each cycle	Annealing	Primers extension	Final extension
95 °C, 2 min	35	95 °C, 1 min	51.5°C,1 min	72°C, 1min	72 °C, 5 min

The agr specificity groups were identified by the expected product sizes. In the PCR assay, the agr gene was amplified on a Corbett instrument using necessary components provided by Ferment as (DreamTaq™ Green PCR Master Mix kit). The sequence of specific primers used in PCR reactions, and the molecular size of the amplicons are presented in Table 1. The parameters for the amplification cycles used in PCR are presented in Table 2.

PCR products were separated in a 1% agarose gel for 40 min at 100 V, stained with ethidium bromide and detected by UV transillumination. For amplification products whose sizes ranged between 500 and 600 bp PCR we performed two separate tests using DNA samples and primers for agr group II, respectively agr group IV.

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Dendrogram construction and genetic relatedness :
Dendrogram for cluster analysis of all the isolates were subjected to evaluation. The dendrogram was constructed on the basis of the banding pattern produced by ERIC-PCR. A binary table or a haplotype matrix for each strain was constructed by linearly composing presence (1)/absence (0) data derived from analysis of the gel/ antibiogram was subjected for statistical analysis by squared Euclideandistance (SED) (Wards method) using the software.

STATISTICA:
This enabled the plotting of dendrogram showing the level of genetic similarity among the strains.

Results and Discussion
Thirty eight S.aureus isolates gathered from different hospitals were confirmed as S. aureus by standard biochemical tests. Dendrogram analysis of antibiotic used has been able to show a very higher resolving strain differentiation. S. aureus isolated from various specimens and wards is shown in Figure 1:dendrogram (Algoithm paird group .Similarity measure Euclidean) Antibiotic sensitivity pattern of all the isolates.

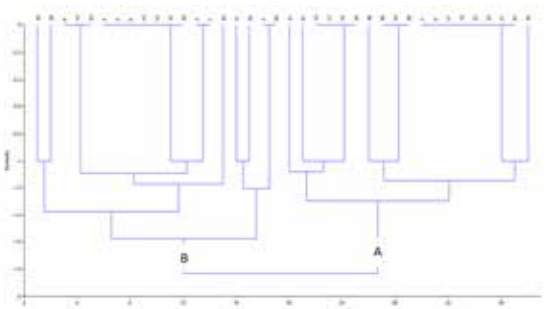

FIG. 1 Dendrogram (Algoithmpaird group .Similarity measure Euclidean) showing the levels of similarity between antibiotic resistance of all isolates.

The dendrogram based on antimicrobial drug sensitivity pattern of 38 non- repetitive staphylococcal isolates represented in Fig 1 and the accessions are clearly divided into two major clusters, A and B. The maximum dissilmiarity distance between the two major clusters A and B was 29 units. The cluster A can be grouped into 2 subclusters A1 and A2 at a linkage distance of 1.7 units. The subcluster A1 is further divided into two subgroups A1.1 and A1.2 at a linkage distance of 1.4 units. The subgroup A1.1 consists 2 units (S35) and (S7,S9,S17,S19,S22,S25, S27,S37) were resistant to (OX,ME,AK,L,VA) and (OX,ME,AK,L,VA,CN) and subgroup A1.2 consists 2 units (S30,S33,S38) and (S36) which were resistant to antibiotic (ME,CN,AK, L,VA) and (CN,AK,L,VA) and sensitive to Oxicillin when compared to subgroup A1.1 (Table3).

Table 3. Multidrug Resistance Patterns of S. aureus Isolates.

NO. of Isolates and percentage	Isolates	Antimicrobial resistance (R) Isolates		Group
1 (2.63)	S35	OX,ME,AK,L,VA	A1-1	A1
8 (21.05)	S7,S9,S17, S19,S22,S25, S27,S37	OX,ME,AK,L,VA,CN		
3 (7.89)	S30,S33,S38	ME,CN,AK,L,VA	A1-2	
1 (2.63)	S36	CN,AK,L,VA		
1 (2.63)	S21	OX,ME,L ,CN		A2
1 (2.63)	S31	OX,CN,L,VA		
4 (10.53)	S10,S12,S18,S24,	OX,ME,L ,CN, VA		
1 (2.63)	S11	CN,L		B
1 (2.63)	S20	L		
2 (5.26)	S2, S26	Sensitive for all Antimicrobial agents		
2 (5.26)	S28,S34	OX ,L		
3 (7.89)	S8,S14,S23,	ME,,L		
7 (18.42)	S4,S5,S6,S13,S15, S16,S29	OX,ME,L		
2 (5.26)	S1,S3	ME,OX		
1 (2.63)	S32	OX,ME,AK,L		

Oxacilin,OX; Gentamycin,CN; Amikacin, AK; Methecillin, ME; Vancomycin,VA; Lincomycin ,L.

All 38 local isolates under study design and distributed to three groups depending on the type of multidrug resistance as well as dendrogram in order to facilitate the distribution process. This table indicates division isolates depending on the number of antibiotics which resisted .six antimicrobial agents (VA, L, AM, CN, OX,ME). As it is shown in table(4) and FIG1 the highest rate of isolates have multidrug resistance was observed with group C isolates, in which this isolates were able to resist (6) antimicrobial agents. While the lowest rate of isolates have multidrug resistance was notice with group A in which the total number of resisted antibiotics were only (0→2).As to the susceptibility to antibiotics, there was no significant variability between test repetitions. The results of Losito et al [25]. were statistically analyzed and clustered in 6 groups, different from the local results, the clustered in to two major group and multiple sub group. An antibiogram-resistogram (AR) typing scheme that can simply and rapidly differentiate Staphylococcus aureus isolates has been devised.(Rossney et al [26] Thus, antibiogram typing is considered to havepoor discriminatory power and is used by microbiologists onlyin the first instance for rapid screening of the similarities between different clinical isolates.[27].

Table.4 The multidrug resistance phenotype in Staph.aureus

Groups	Number of antimicrobail which resisted by isolates	Numbers of the multidrug resistance isolates	Percentage of multidrug resistance
Group A	0 →2	12	32%
Group B	3→5	8	21%
Group C	6	18	47%

S. aureus is a major pathogen in both community and nosocomial infections.[28]. The capacity of this organism to cause a multitude of human diseases, such as endocarditis, osteomyelitis, pneumonia, bacteremia, and TSS, suggests the pathogenesis of S. aureus infections is highly complex. The growth phase is dependent on many cell surface proteins and secreted exotoxins and enzymes, and also the environmental and host signals are contributing to the regulation of virulence factors.[29].

The agr locus is a 2-component regulatory system that responds to host and environmental stimuli and controls the coordinated production of many virulence factors.[29].

The autoinducing peptide pheromone, AgrD, activates the transcription of the agr locus. This leads to the up-regulation of secreted virulence factors genes and the down-regulation of gene-encoding surface proteins.5

In this study, the agr specificity group was determined by a PCR method (Figure 2 and Figure 3 Analysis of agr gene polymorphism by specific primers allowed assigning our strains in 1 of 4 major specific agr groups. As shown in Table 1, most

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of our isolates belonged to agr group I (32%), followed by agr group II (47%), agr group III (16%), and agr group IV (5%).

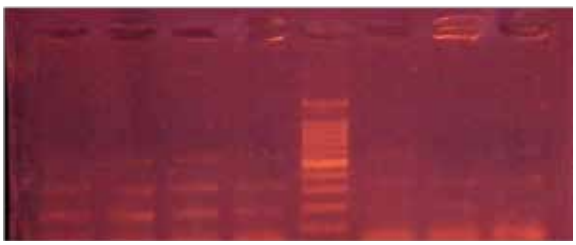

Figure 2_Electrophoretic analysis of PCR products for agr-specific groups. Lane 5: 100 bp size marker. Lane 1 to 8: products from PCR 440 bp for agr I and 588 bp for agrIV, 406 bp for agr III and 572 bp for agr II).

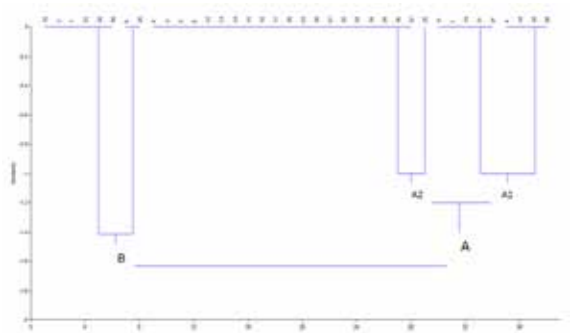

FIG. 3 Dendrogram Algoithm DICE .Similarity measure Euclidean) showing the levels of similarity between Analysis of agr gene of all isolates .

The resulting dendrogram showed TWO major branches A,B represent all positive agr gene, two of which, representing the (isolate) A1(S11,S1,S19,S21,S31) (S4,S18,S24, S38) ,A2(S9,S3,S5,S8,S12,S13,S14,S15,S16,S17,S20,S23,S26,S27,S32,S34,S35,S36,S37,S25) and B (S6,S28) (S10,S2,S7,S22, S29, S30) this isolates from subgroup A1.1 were resistant to (OX,ME,AK,L,VA,CN) and A1.2 were resistant to (ME,CN,AK,L,VA) and have virulence factors Production of (Protease , Hemolysin and Biofilm Production in Congo red).

In this study we assessed the virulence potential of clinical source S. aureus and respect to genomic background and VF profiles, all in relation to antimicrobial resistance. Considering the possible relation between the agr group and the antibiotic resistance in S. aureus isolates, we made disk diffusion agar tests (Table 3). The study showed that isolates with a resistance group A (table 3) table.4) , the dominant genes from(4 isolates agrII) and One isolate each group agrI and agrIII, But not contain agrIVgenes. group B the dominant genes from (5 isolates agrII) and (5 isolates agrI) gene and (2 isolates agrIII, 2 isolates agrIV) .the high resistance group C the dominant genes from (9 isolates agrII) and (6 isolates agrI) ,(3 isolates agrIII) But not contain agrIV genes.

Table.5 : A relationship between antimicrobial resistance and agr genes in

Groups	NO.of isolates contain agrI	NO.of isolates contain agrII	NO.of isolates contain agrIII	NO.of isolates contain agrIV
Group A	1	4	1	0
Group B	5	5	2	2
Group C	6	9	3	0

A relationship between virulence factors Production of (Protease , Hemolysin and Biofilm Production in Congo red) and antimicrobial resistance and agr genes was observed in this studies. The high resistance isolates group C dominant genes agrII and agrI and Production of (Protease , Hemolysin and Biofilm Production in Congo red). But group B moderate Production of (Protease , Hemolysin and Biofilm Production in Congo red) . Group A the dominant genes agrII only. the virulence factors less production of Protease and Biofilm .

Shopsin et al. [24] reported that the distribution of the four agr groups was agr type I, 41.6%; agr type II, 24.7%; agr type III, 33.8%; and agr type IV, 0%; this is somewhat similar to that found in the present study.

This study agree with Layer et al. [30]. The considered variation in agr specificity type has been proposed as a possible influence on population dynamics of S. aureus.

Conclusion :

The results of PCR analysis showed that all analyzed strains, regardless of the source of isolation harbour the agr gene, showing that they have a functional QS system. Also, we have found a specific association between the agr groups distribution with virulence factors (Production of Protease , Hemolysin and Biofilm Production in Congo red) and antimicrobial resistance and, thus, indirectly with certain disease profiles. This report is the first to study the agr locus in Iraq. Our results will be helpful in verifying the characteristics of S. aureus strains in other adjacent countries.

ACKNOWLEDGMENT

Thankful to all staff Collage of science \Department of biology for providing the necessary facilities to carry out the research work.

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