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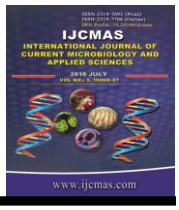

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Prevalence of Accessory Gene Regulator Specificity Groups among Methicillin Resistant *Staphylococcus aureus* Isolated from Iraqi Hospital Patients

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A B S T R A C T

In current study, 140 clinical isolates of *Staphylococcus aureus* were collected from different hospitals in Baghdad and Diyala. 113 of them were diagnosed as Methicillin-resistant *Staphylococcus aureus* (MRSA) by two different methods were used; phenotypic method was done by chromogenic MRSA agar and genotypic method was done by Polymerase Chain Reaction (PCR) technique to detection *mec*A gene. Multiplex PCR technique has been used to investigate accessory gene regulator (*agr*) in 113 MRSA isolates and the results were 84(74.34%) isolates were belonged to *agr* group III, 26(23.01%) isolates were belonged to *agr* group I and 3(2.65%) isolates were untypable , while *agr* groups II and IV were absent.

Introduction

Staphylococcus aureus is a major human pathogen, responsible for a multitude of human infections around the world (Otto, 2012). This diversity is related to a number of virulence factors that are coordinately expressed during different stages of infection by intricate networks of transcriptional regulators and two-component signal transduction systems. The result allows it to adhere to surface, invade or avoid the immune system, and cause harmful toxic effects to the host (Bien *et al.*, 2011).

Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates emerged after the introduction of methicillin by acquisition of the mecA gene, which located on a mobile SCCmec cassette chromosome. This genetic element confers resistance to most currently available beta-lactam antibiotics (Katayama *et al.*, 2000).

The expression of most virulence factors is controlled by global regulators such as accessory gene regulator and staphylococcal accessory regulator A.

The accessory gene regulator (agr) is one of these virulence regulators, being a quorum-sensing system activated by autoinducing peptide (AIP) or the so-called a bacterial

density sensing peptide (Cotar *et al.*, 2012). A polymorphism in the amino acid sequence of the AIP divides *S. aureus* strains into four major groups. Each group is characterized by a specific oligopeptide, in which only the thiolactone structure is conserved (Malone *et al.*, 2007). Agr responsible for the up-regulation in extracellular protein production and the down-regulation of cell wall-associated protein synthesis during the post-exponential growth phase.

The *agr* locus is located on the *S. aureus* chromosome and is considered to be part of the core genome and not in the pathogenicity island (Novick, 1995). It is 3.5kb in size and consists of two divergent transcriptional units, RNAII and RNAIII, whose transcription is driven by the P2 and P3 promoters, respectively (Le and Otto, 2015). The RNAII transcript is an operon of four genes, *agrBDCA*, that encode factors required to synthesize and sense AIP. AgrD is the precursor peptide of AIP and AgrB is an integral membrane endopeptidase essential to biosynthesize AIP. AgrC and AgrA form a two-component pair where AgrC is the membrane histidine kinase and AgrA is a response regulator (Novick, 2003; Li and Tian, 2012).

RNAIII is the major downstream effector of the *agr* system that post transcriptionally regulates expression of virulence factors. The RNAIII operon encodes a δ-haemolysin and is itself a regulatory RNA that plays a key role in the *agr* response (De Kievit and Inglewski, 2000).

Methods and Materials

Isolation of *s. Aureus* from clinical samples

One hundred and forty *S. aureus* isolates were obtained from different hospitals in Baghdad and Diyala governorates. Including, ninety two isolates were collected from Baghdad hospitals; Ibn-El Balady, Baghdad teaching hospital, Al-Numan Hospital and Alemam-Ali hospital during the period between February 2014 to May 2014, while forty eight isolates were collected from Diyala hospitals; Baqubah teaching hospital and Balad-Ruz general hospital during the period between April 2014 to July 2014. They were isolated from different clinical sites including: burn infections, ear infection, throat infection, surgical wound from hospitalized patient, midstream urine from patient suffering urinary tract infections, sputum of patients with respiratory tract infections and blood from patient with septicemia. Each isolate was identified according the morphology, biochemical tests and growth in Chromagar Staph.aureus, the growing colonies that appeared distinctive pink to mauve color diagnosed as *S.aureus.*

Phenotypic detection of MRSA isolates

The colonies were identified as S. aureus by Chromagar Staph.aureus were transferred by sterile loop and directly inoculated on Chromagar MRSA and incubated at 37°C for 24 hr. On this medium, the growing colonies that appeared distinctive pink to mauve color diagnosed as MRSA.

Genotypic detection of MRSA isolates

The prevalence of MRSA isolates was done by using uniplex PCR with specific primer and amplicon size (Table 1) to detecting mecA gene. Genomic DNA of *S.aureus* was extracted according to boiling method described by klingenberg *et al*., (2004). PCR mixture was prepared by adding 12.5µl of GoTaq®Green master Mix (2X) promega, 5µl template DNA, 1.5µl from each forward and reverse primers with final concentration 1 poml /µl, finally volume was completed to 25µl by adding nuclease free water.

Genotypic detection of *agr* groups using PCR technology

The prevalence of agr specific groups among MRSA isolates in this study was done by using multiplex PCR with specific primers and amplicon size (Table 1). Genomic DNA of *S.aureus* was extracted according to boiling method described by klingenberg *et al*., (2004).

PCR mixture was prepared by adding 12.5µl of GoTaq®Green master Mix (2X) promega, 5µl template DNA, 1µl from forward primer and 1µl from each four reverse primers with final concentration 1 poml /µl, finally volume was completed to 25µl by adding nuclease free water.

PCR condition illustrated in table (2) and PCR products were detected in 1 % agarose gel for 1 hr. at 75 V, stained with ethidium bromide and visualized by transilluminator.

Results and Discussion

Isolation and identification of *S.aureus*

In this study, all one hundred and forty clinical isolates produce clear β-hemolysis around their colonies Iin blood agar no growth was appeared in MacConkey agar. In manitol salt agar medium, all isolates caused fermentation of manitole and changed the color of colonies to yellow. Staining and microscopic examination showed that all isolates were gram-positive coccus and arranged in grape shape clearly.

All isolates were catalase positive and oxidase negative. The growing colonies on CHROMagar Staph aureus media appeared as pink to mauve due to the hydrolysis of chromogenic substrates including in media which confirms that they are colonies of *S. aureus* according to manufacture's instruction.

Screening for MRSA

Out of 140 *S. aureus* isolates 113 (80.7%) were MRSA detected by the two methods chromogenic medium and PCR assay, including 65 isolates (77.4%) of 84 isolates collected from Baghdad hospitals, while 48 isolates (85.7%) of 56 isolates collected from Diyala hospitals. Table (3) summarizes the number and source of MRSA isolates which were collected from several hospitals in Baghdad and Diyala.

Phenotypic method

One hundred and forty *S. aureus* isolates cultured on CHROMagar MRAS media in order to diagnosis of methicillin-resistant than other, the growing colonies of 113 (80.7%) *S. aureus* isolates appeared as pink to mauve due to the hydrolysis of chromogenic substrates including in media which indicates they are MRSA, while 27 (19.3%) *S. aureus* isolates did not appear growth which indicates they are MSSA according to manufacture's instruction.

Genotypic method

All *S. aureus* islates were tested for *mecA* gene by PCR. The results revealed that Out of 140 *S. aureus* isolates 113(80.7%) were yielded *mecA* gene by PCR, the figure (1) shows the genetic detection of *mecA* gene to different isolates of the bacteria under study.

Screening for *agr* groups

Hundred and thirteen MRSA isolates from different hospital in Baghdad and Diyala used to study the prevalence *agr* groups in Iraq, it was screened by multiplex PCR technique. The results showed that 74.34% (84 /113) isolates were belonged to *agr* group III, while 23.01% (26 /113) isolates were belonged to *agr* group I and 2.65% (3

/113) isolates were untypable (S17, S41 and S103). *agr* groups II and IV were not detected among all studied strains. Figure (4) shows the positive genetic detection of *agr* group I and III to different isolates of the bacteria under study. The findings of the study also showed that out of 65 MRSA isolates collected from patients in Baghdad were 22(33.85%) isolates belonged to *agr* group I, 41(63.07%) belonged to *agr* group III and 2(3.07%) isolates untypable, while out of 48 MRSA isolates collected from patients in Diyala were 4(8.33%) isolates belonged to *agr* group I, 44(89.58%) belonged to *agr* group III and 1(2.08%) isolate untypable.

The first used this method for classification of *S. aureus* were Dufour and colleagues in 2002, they showed that isolates of this bacterium could be divided into four Groups, I, II, III, IV. The *agr* quorum-sensing and signal transduction system was initially described in *S. aureus* based on the presence four distinct allelic variants have been sequenced.

In this study the predominant *agr* group found in clinical specimens were *agr* group III followed by *agr* group I, the absence of *agr* groups II and IV may be caused that they are uncommon in the region under study or they were present in MSSA isolates. Azimian *et al*. (2012) in Iran, showed that most of MRSA strains belonged to *agr* group I and III while most of MSSA strains belonged to agr groups II and IV, and the strains carrying *agr* group I and *agr* group III were more resistant to oxacillin than *agr* group II and *agr* group IV.

Table.1 Primers and amplified PCR products used in study

Primer name	Primer sequences (5'→ 3')	Product size(bp)	origin	References
*agr*1	F- ATGCACATGGTGCACATGC R- GTCACAAGTACTATAAGCTG CGAT	441	Alpha (Canada)	Gilot *et al.*, 2002
*agr*2	F- ATGCACATGGTGCACATGC R- TATTACTAATTGAAAAGTGCCATAGC	575	Alpha (Canada)	Gilot *et al.*, 2002
*agr*3	F- ATGCACATGGTGCACATGC R- GTAATGTAATAGCTTGTATAATAATA C CCAG	323	Alpha (Canada)	Gilot *et al.*, 2002
*agr*4	F- ATGCACATGGTGCACATGC R- CGATAATGCCGTAATACCCG	659	Alpha (Canada)	Gilot *et al.*, 2002
mecA	F- GTAGAAATGACTGAACGTCCGATAA R- CCAATTCCACATTGTTTCGGTCTAA	310	OligoData (S-Africa)	McClure *et al*.2006

Table.2 PCR condition to genes used in study

Amplified gene	Initial denaturation	No. of cycles	Denaturation	Annealing	Elongation	Final extension
agr	94°C/ 5 min	30	94°C/30 Sec	56°C/1 min	72°C/1 min	72°C/7 min
mecA	95°C/ 5 min	35	94°C/30 Sec	53°C/1 min	72°C/1 min	72°C/7 min

Table.3 distribution of MRSA according to the region under study and source of isolation

Source of isolation	Baghdad hospitals		Diyala hospitals		No. of MRSA	Percentage
	MRSA	MSSA	MRSA	MSSA		
UTI	14	6	14	2	28	24.78%
Burn	20	4	6	2	26	23.01%
Wound	16	3	7	3	23	20.35%
Nasal infection	6	2	11	0	17	15.04%
Ear infection	5	2	5	0	10	8.85%
Blood	3	2	2	0	5	4.42%
Eye infection	0	0	2	1	2	1.77%
Sputum	1	0	1	0	2	1.77%
Total isolates	65	19	48	8	113	100%

Fig.1 Agaros gel electrophoresis (1% agarose,7v/cm2 for 60 min) for mecA gene (310bp amplicon) lane M100bp DNA Ladder, lanes 1-12 represent of bands.

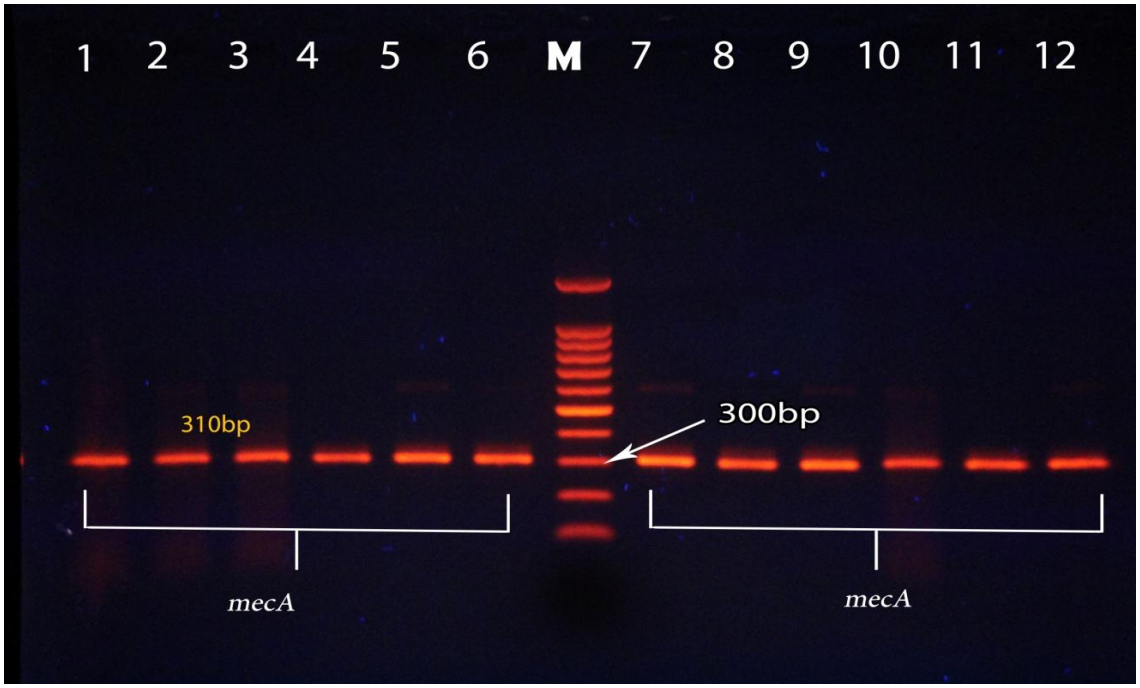
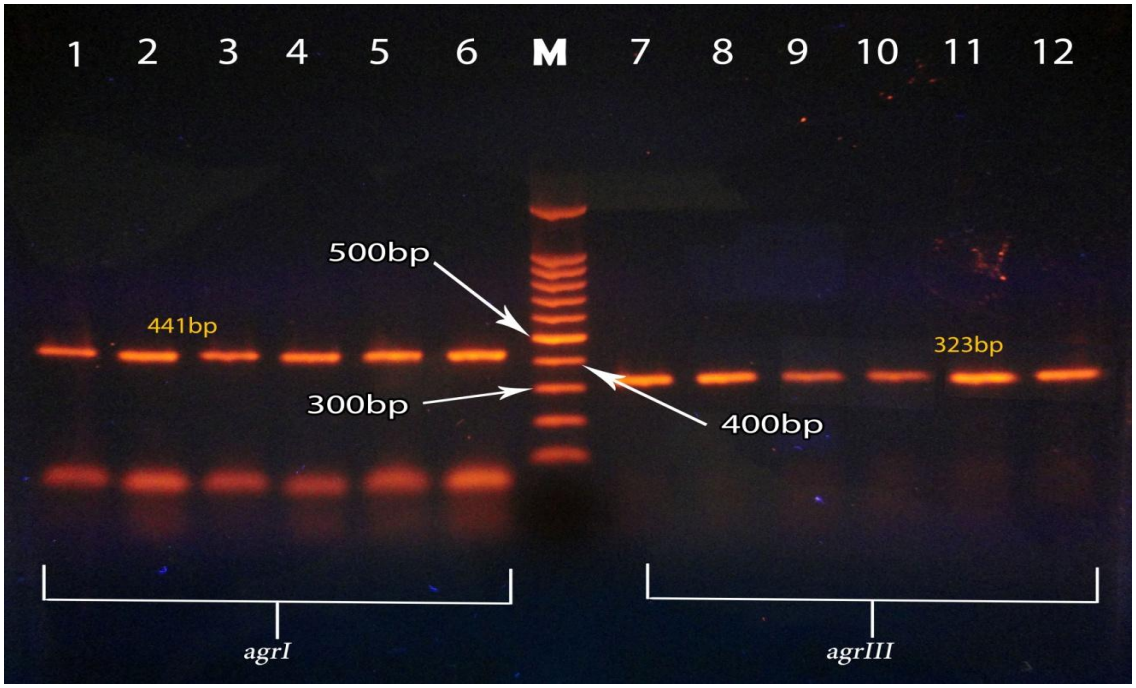


Fig.2 Agaros gel electrophoresis (1% agarose,7v/cm2 for 60 min) for agr I (441bp) and agr III genes (323bp) lane M100bp DNA Ladder, lanes 1-6 represent of agr I bands and lanes 7-12 represent of agr III bands.



There are many studies done around the world to detect prevailing *agr* groups of *S. aureus* in those countries, some of which interested to detects *agr* groups in MRSA. Melake *et al*., (2014), in Egypt showed that most of MRSA strains which Isolated from nasal carriers belonged to *agr* group I followed by *agr* group II and III respectively, while agr groups IV not was not detected. Vaziri *et al*., (2016), in Iran found the predominant *agr* group of MRSA isolated from burn wound of hospitalized patient in Tehran was *agr* group I, while in another study done by Bibalan *et al*., (2016), showed that *agr* group III was predominant in MRSA strains in cases isolated from the clinical sample of patients in Iran and the *agr* group II was predominant in MSSA strains while the *agr* group I was the major *agr* groups in *S. aureus* that were recovered from health care workers and food products. Ayed *et al*., (2006) in Tunis were found *agr* group III were predominant type in MRSA strains that were isolated from patients. Strommenger *et al*., (2004) found that all of the MRSA strains that were isolated from central Europe belonged to *agr* group I, in contrast Dufour *et al*., (2002) showed that *agr* III the vast majority group of MRSA strains in France and around the world. While *agr* II was the predominant group in MRSA isolates collected from multiple centers around the United States (Goerke *et al*., 2005), Paniagua-Contreas *et al*., (2012) agreed with that in a study conducted in Mexico about the strains of MRSA collected from hemodialysis catheters of Mexican patients. Also Gomes *et al*. (2005) suggested that *agr* group II strains were mainly isolated in Japan and North America. The *agr* group IV was absent in many previously reported articles suggests that these strains does not have preference in the competition.

The results of previous studies suggest that the prevalence of predominant *agr*

specificity groups vary from one country to another based on epidemiological and regional factors, in addition to the results affected by sampling biased.

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