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Original Article

Accessory Gene Regulator (*agr*) group polymorphisms in methicillin-resistant *Staphylococcus aureus* and its association with biofilm formation

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the main causes of community- and hospital-acquired infections. The expression of virulence genes in *S. aureus* is arranged by regulators like the accessory gene regulator (*agr*). The present study aims to estimate phenotypic characteristics of *S. aureus* and investigate the occurrence of *agr* genes with their correlation to biofilm formation. In this study, 34 MRSA strains out of 100 *S. aureus* isolates were recovered in a variety of clinical samples. Phenotypic characterization and ability of biofilm formation were assessed. About 8(24%) of isolates were biofilm producers. The percentages of biofilm production among isolates were 3(37.5%), 2(25%), 3(37.5%) as strong, moderate, and weak, respectively. Furthermore, the resistance rates for all antibiotics were higher in biofilm producers and 76% of the isolates were staphyloxanthin producers, around 82% of the strains showed resistance to H_2O_2. Hemolytic activity was detected in 74% of the total isolates. The activity of the protease enzyme was 68%. The lipase enzyme was active in 79% of the tested *S. aureus* isolates. The majority of isolates were established to be *agrI* 84%, followed by *agrII* 53%, *agrIII* 32%, and 30% of the isolates have *agr IV*. Our study indicated that the majority of MRSA isolates were non-biofilm producers and the *agr I* is the most dominant type. Thus, *agr I* is not correlated with biofilm production.

Keywords: *agr* group, Biofilm, Methicillin-resistant, *Staphylococcus aureus*, Staphyloxanthin.

1. Introduction

Staphylococcus aureus is an opportunistic and a commensal pathogen that has been implicated in a variety of illnesses [1], such as infective endocarditis, prosthetic device infections, osteoarticular infections, bacteremia, pneumonia, and infections of the skin and soft tissue [2]. Methicillin-resistant *Staphylococcus aureus* (MRSA), especially community-associated (CA-MRSA) strains outlined a distinct danger because of its hypervirulent nature [3]. *S. aureus* has a large number of virulence determinants that are responsible for its pathogenicity, including cell wall-associated proteins, secreted toxin proteins, and extracellular polysaccharides in addition to other factors which coordinate the infection processes [4]. Quorum-sensing (QS) appears to influence biofilm formation at many of these stages by cell adhesion and proliferation biofilm detachment, *agr* group facilitates the initial attachment of staphylococci to surfaces [5]. Staphylococcal pathogenesis is controlled by quorum sensing, which is one of the most important mechanisms for regulating staphylococcal physiology in the environment [6]. When cells communicate with one another, QS regulates genes based on their local environmental conditions and maintains a stable population density [7]. In *S. aureus*, the accessory gene regulator (*agr*) regulates and controls the expression of virulence genes and it is activated by secreted auto-inducing peptides (AIP)[8]. It is the main and well-characterized operon in the biology of this bacteria, *Agr* controls many genes, counting the utmost of those encoding extracellular and cell-wall-associated protein. The *agr* gene has a length of 3.5 kb and transcribes the P2 and P3 promoters of the two distinct transcriptional units RNAII and RNAIII [9]. The P2 operon encodes 4 proteins (*agrA, agrB, agrC*, and *agrD*) that induce *agr* sensing mechanisms [10]. *AgrB*, an integral membrane endopeptidase, produces and secretes the propeptide encoded by the *agrD* gene. The mature AIP binds to the *agrC* transmembrane receptor. Through autophosphorylation of histidine on the *AgrC* receptor's histidine residue, this binding encodes the standard two-component signaling pathway that leads to trans-phosphorylation of the *agrA* gene product. The agr P2/P3 promoters are assumed to be stimulated by phosphorylated *agrA* [11]. Secreted virulence factors and surface proteins are both regulated by the RNA transcript from the P3 promoter [12] The agr system is one of the main regulatory and control factors in *S. aureus* virulence gene expression. In

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fact, the invasive infection and pathogenicity of *S. aureus* are controlled by genes regulated by the agr operon [13]. In addition, the sequences of the *agrC* and *agrD* genes allow *S. aureus* to be classified into four distinct groupings named *agr* I, II, III, and IV [14]. This study was carried out to investigate the prevalence of MRSA among clinical isolates, considering the specific *agr* groups to which each isolate belonged, as well as their relation to biofilm formation.

2. Materials and methods

1.1. Identification of bacterial isolates

From a total of 100 *S. aureus* isolates from patients admitted to Hawler Teaching Hospital and Central Laboratory in Erbil (34%) were found to be MRSA. Bacterial isolates were collected from different clinical samples such as urine, blood, skin and soft tissue, pus, wound swab, and sputum. Each isolate was cultured on Mannitol salt agar and Blood agar and the plates were incubated at 37°C overnight. Identification of bacterial isolates was performed by Vitek 2.

2.2. Antibiotic susceptibility pattern

Susceptibility testing for different antibiotics was performed following Clinical and Laboratory Standards Institute guidelines (CLSI) on Mueller-Hinton agar. The tested antimicrobial disks were; tetracycline (30µg), rifampicin (5µg), clindamycin (2µg), ampicillin (10µg), vancomycin (30µg), gentamycin (10µg), azithromycin (15µg), amoxicillin/clavulanic acid (30µg), cefotaxime (30µg), ciprofloxacin (5µg), and piperacillin (100µg). Methicillin resistance was confirmed by Oxacillin and Methicillin disk test according to CLSI, 2020 [15]. The *S. aureus* ATCC 25923 strain served as the control.

2.3. Biofilm formation

Biofilm formation was determined by the microplate method (MTP) using tissue culture plates of 96 flat-bottomed wells. In each well, 0.2 ml of a bacterial suspension diluted to 10^5 CFU/ml in TSB was dispensed. After overnight incubation in aerobic conditions at 37 ^{0}C, the contents were discarded to remove planktonic bacteria, and plates were washed twice with phosphate-buffered saline (PBS), pH:7.2. After that, the wells were stained for 30 minutes with 0.1% crystal violet. At a wavelength of 490 nm, the plates were scanned using an ELISA reader (BioTek, ELx800). Sterile TSB was used as a negative control. This experiment was repeated twice, and the values of optical densities (OD) were averaged [16].

2.4. Phenotypic characterization

2.4.1. Staphyloxanthin assay

Simple color change detection of the golden yellow coloration from the quantitative carotenoid assay has also been adapted. Cells were diluted 1:100 in TSB medium and then incubated at 37 ^{0}C for 16 hrs, either with or without flavonoids. Centrifuged for one minute at 16,600x g, 1 ml was then collected and 1 ml phosphate-buffered saline (PBS) was used to wash the sample.

Staphyloxanthin production in cell pellets was photoed. Vortexing cell pellets in 0.2 ml of methanol and heating at 55 ^{0}C for 3 min. to extract carotenoid pigments. Centrifugation at 16,600xg for 10 minutes separated the pigment extraction from the cell debris. Isolates of *S. aureus* were divided into two groups according to their pigment production, as determined by the OD_{450} values [17].

2.4.2. Hydrogen peroxide resistance assay

For the hydrogen peroxide resistance assay, overnight cultures were re-grown in TSB. After that, 0.1 ml of each bacterial suspension was incubated with hydrogen peroxide (H_2O_2), a concentration of 1.5 % (v/v) for 60 minutes shaking at 250 rpm was performed to calculate the bacterial survival rate. A total of three distinct experiments were carried out [18].

2.4.3. Lipase assay

Staphylococcus aureus lipase production was detected on tributrine agar plates [19]. Mueller–Hinton agar containing 1% tributrine emulsified in tween 80 was used to grow the tested strains for three days at 37°C. A positive result was indicated by the presence of a clear zone around the test organism.

2.4.4. Protease assay

Protease enzyme production was examined by spotting 10µl overnight cultures onto milk agar 1 % (w/v) milk to detect casein proteolysis. For the precipitation of undigested casein, following an overnight incubation at 37°C, the agar plates were flushed with an HCl solution that was 1% (v/v) [20].

2.4.5. Hemolysin assay

To test hemolytic activity, 10µl overnight cultures were placed on blood-agar plates and analyzed for hemolysis after incubation 24 hours at 37°C [21].

2.5. Genotypic characterization

2.5.1. DNA Extraction

The genomic DNA extraction kit (ADD BIO Inc., Daejeon, Republic of Korea) was used to extract DNA from the bacterial colony following the manufacturer's instructions. The concentration and purity of DNA were assessed using a Nanodrop-2000 Spectrophotometer (Thermo Fisher Scientific, Inc.).

2.5.2. PCR amplification and agr-typing

Agr polymorphisms amplification was performed in a BioRad Thermocycler (BioRad, Switzerland) with primers listed in Table 1. [22]. The products of amplified samples were analyzed by electrophoresis. The lengths of the PCR products were assessed by comparison with the 100bp DNA ladder (addbio, Korea).

2.6. Statistical analysis

GraphPad Prism (GraphPad Software, San Diego, CA) was used in the current study to analyze the collected data. The data was subjected to a chi-square test. A *P*-value of <0.05 was measured as statistically significant.

3. Results

A total of 34% MRSA and 66% non-MRSA were evaluated out of 100 *S. aureus* isolates, out of 34 isolates, 14 of whom were male and 20 of them were female, 41.2% and 58.8%, correspondingly. In terms of bacterial rate, the following were found in each sample: 17 isolates from urine specimens (50 %), 5 (14.7%) blood, 5 (14.7%) from tissue, 3 (8.8%) pus, 2 (5.9%) wound, and 2 (5.9%) sputa.

Table1. Primer used in the molecular identification of *agr* grouping in this study.

Gene	Primer name	Sequence (5`-3`)	Product size (bp)	Annealing temp.(^{0}C)
*agr*I	Pan Forward	ATG CAC ATG GTG CAC ATG C		
	Reverse *agr*I	GTCACAAGTACTATAAGCTGCGAT	440	61.8
*agr*II	Reverse *agr*II	GTATTACTAATTGAAAAGTG CCATAGC	572	62.2
*agr*III	Reverse *agr*III	CTGTTGAAAAAGTCAACTAAAAGCTC	406	61.6
*agr*IV	Reverse *agr*IV	CGATAATGCCGTAATACCCG	588	58.4

The antimicrobial resistance pattern of MRSA isolates is offered in Figure 1. Within the strains that have been collected, all the MRSA isolates 100% were resistant to ampicillin, amoxicillin methicillin and oxacillin, confirming their methicillin-resistance status. Clindamycin and tetracycline had also the same resistance rate of 82%. Also, 68% of the isolates were resistant to both vancomycin and gentamicin, while 53% of the isolates had similar resistance to cefotaxime and ciprofloxacin.

3.1. Virulence factors

The yellow color staphyloxanthin can be detected in the cell pellets of MRSA. The carotenoid content of the isolates is displayed in Figure 2. The OD_{450} absorbance of the carotenoid ranged from 0.07 to 1.9. Consequently, for non-pigmented isolates, OD_{450} threshold of 0.100 was established (xanthin china 2018). Seventy-six percent of the isolates had OD_{450} more than 0.100 thus they were considered pigmented (26/34). 24% (8/34) of the isolated strains showed no staphyloxanthin production (OD_{450} less than 0,1).

Although *S. aureus* produces different proteases, casein agar plates were used to determine that function, the white zone that was obtained around the colonies after the incubation period served as an indicator of the amount of extracellular protease activity. The activity of the enzyme in isolated *S. aureus* was 68%**.** Staphylococcal lipases randomly hydrolyze the triacylglyceride molecule to glycerol and fatty acid. A positive halo was observed on the Muller-Hinton agar containing Tween 80 after 20 hours. of incubation. This enzyme was active in 79% of the tested MRSA isolated.

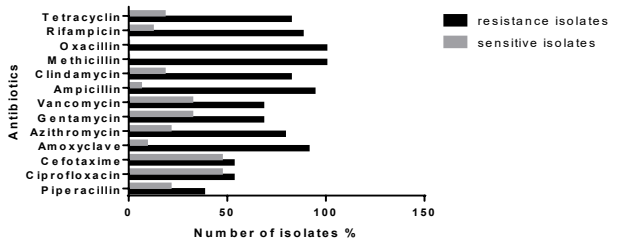

Fig. 1. Antibiotic resistance profiles of the MRSA isolates. The difference is significant (P-value < 0.0001****).

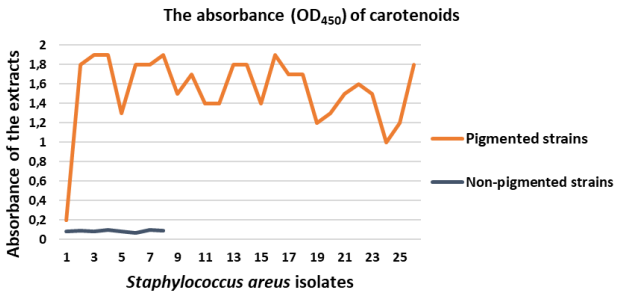

Fig. 2. The absorbance in (OD_{450}) of carotenoids extracted from MRSA. The orange line represents pigmented strains and the blue line represents the non-pigmented strains.

3.2. Biofilm formation

Among the 34 isolates of MRSA, 8(24%) were biofilms producers and the remaining 26(76%) isolates did not produce biofilms under the described experimental conditions. Only three *S. aureus* isolates were strong biofilm producers, two isolates produced an intermediate biofilm, and three isolated *S. aureus* had a weak biofilm Table 2.

3.3. Association of biofilm, antimicrobial resistance, and production of staphyloxanthin

All eight biofilm producers of the tested MRSA were found to be non-susceptible to most of the tested antimicrobial agents except vancomycin and ciprofloxacin. Also, they were a strong staphyloxanthin producer Table 3. The isolates that exhibited biofilm formation ability, express more resistance to antibiotics (46 to 92%), and the ability to staphyloxanthin production (1.2 to 1.8). Staphyloxanthin production ability exhibited lower among strong biofilms compared to moderate and weak biofilm producers.

3.4. *Agr* grouping

Agr specificity groups were recognized by the predictable product sizes. The vast common MRSA isolates across all types were found to be *agr I* 73%, followed by *agr II* 52%, *agr III* 13% and 9% of the isolates belonged to *agr IV* Figure 4.

In terms of the *agr* type from the clinical specimens, type I was found to be more prevalent in pus and wound sources, accounting for 100%; *agr II* was found in all pus specimens; *agr III* was found in 67%; whereas, *agr IV* was found to have a lower prevalence in pus than tissue specimens, accounting for only 33% of MRSA isolates Figure 5.

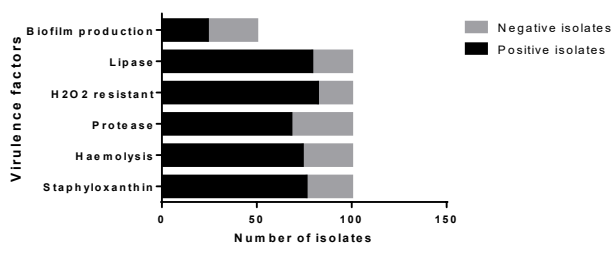

Fig. 3. The percentage of the tested virulence factors of the MRSA isolates. The difference is significant (*P*-value < 0.0002***).

Table 2. Biofilm formation of MR *S. aureus* isolates.

Biofilm Formation	Isolated MRSA			
	Positive 8 (24%)			Negative
Biofilm strength	Weak	Moderate	Strong	
	3(37.5%)	2(25%)	3(37.5%)	26(76%)

Table 3. Association between biofilm and antimicrobial resistance and staphyloxanthin production for MRSA isolates.

Isolates number	Biofilm	Antibiotic resistance rate	Staphyloxanthin
1	strong	92%	1.5
2	strong	92%	1.4
3	strong	77%	1.2
4	moderate	62%	1.7
5	moderate	77%	1.8
6	weak	76%	1.5
7	weak	46%	1.3
8	weak	46%	1.8

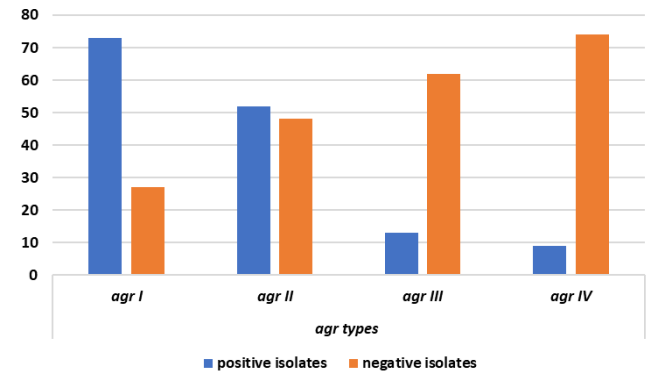

Fig. 4. Percentage of *agr* specificity groups among the MRSA isolates.

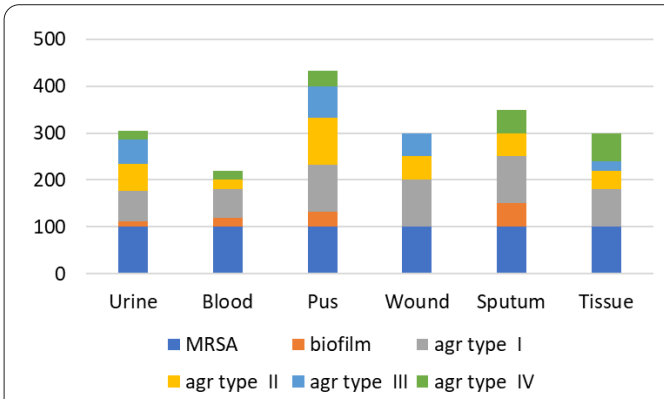

Fig. 5. The distribution of the *agr* types within different sample sources of MRSA isolates. *Agr* groups *agrI, agrII, agrIII,* and *agrIV* are presented as I, II, III, and IV, respectively. The difference is significant (P-value < 0.0001****).

3.5. The correlation between types of *agr* group and biofilm formation

Isolates that were classified as members of the *agrI* group were weak biofilm former (50%). The *agrII*, as opposed generated more strong and moderate biofilm producers with 63%. Besides, *Agr* type III showed a similar rate between biofilm producers and non-biofilm producers and about 38%. Only 13 % of *Agr* type IV isolates produced weak biofilm. Regarding *agr* gene in non-biofilm producers, of the 26 isolates, the *agr* types were identified in 76%. The *agr I* was found in 21 isolates (81%) and *agr type II* in 13 isolates (50%). The agr *III* and *IV* were 38% and 31% respectively Figure 6.

4. Discussion

Methicillin-resistant *S. aureus* has progressively been associated with a wide range of infections worldwide and predictable methods to control are difficult because of their resistance to multiple antimicrobial agents that made this pathogen more headstrong in the hospital situation [23]. There have been few reports regarding molecular characteristics and virulence features of *S. aureus* from our region. This study evaluated the activity of *agr* in MRSA isolates derived from different clinical samples. Also revealed correlations between the *agr* functionality and different virulence factors among the clinical isolates. Under less ideal circumstances, *agr*'s reduced activity promotes the formation of biofilms, which play an advantageous role to the bacteria [24]. The majority of our *S. aureus* isolates were not able to produce biofilm, only 8 (24%) were biofilm producers. In *S. aureus*, most of the virulence genes have been found to be optimally regulated, therefore genes that control pigment production may affect bacteria's ability to cause disease [25]. Researchers hypothesized that the

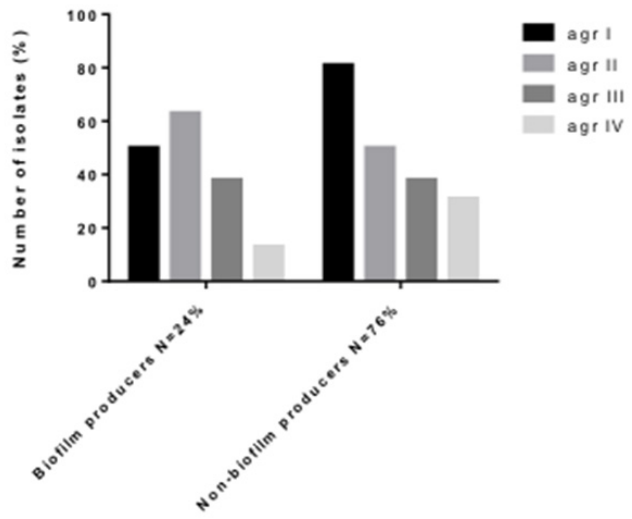

Fig. 6. Biofilm forming pattern of MRSA isolates and *agr* locus distribution. The difference is significant (*P*-value < 0.0052**).

pigment produced by *S. aureus* is not only necessary but also sufficient to stimulate oxidant resistance and blood survival, suggesting an innovative therapeutic target for the treatment [26]. The *agr* group regulates a large number of secreted enzymes. *Agr* regulates proteases, which influence biofilm expansion in vitro by degrading biofilm matrix protein components [27] and *in vivo* by aiding in the breakdown of tissue and host defense mechanisms [28, 29]. Sublethal reactive oxygen species concentrations might play a significant role in *S. aureus* gene dysregulation, Consequently, research demonstrated that S. aureus's in vitro transcriptome responded to sublethal doses of hydrogen peroxide [30]. Other study findings point to a mechanism by which *S. aureus* adjusts to oxidative stress by producing a subpopulation of H_2O_2-resistant with increased catalase production [31]. The two-component signaling systems *Agr* and *Sae* control the expression of hemolysin, this alpha-hemolysin Hla-independent action can lyse human erythrocytes Hla-independent activity [32]. Moreover, 68% of the studied isolates produce protease enzymes as most of them were non-biofilm producers. *S.aureus* can utilize host lipids to change the lipid composition of the bacteria and the expression of virulence factors. While other researchers have recently proven the relevance of lipases [33]. The development of biofilms linked with antimicrobial resistance mechanisms and reduced staphyloxanthin synthesis could enhance MRSA sensitivity to H_2O_2 oxidative stress [34]. Our study displayed that lack of staphyloxanthin enhanced proteolysis and increase hemolysis on blood agar compared to the pigmented strains, the hemolytic activity of the isolated strains was 74%. Furthermore, *Agr* system disrupts biofilm formation by inhibiting adhesion proteins and enhancing the expression of matrix-degrading enzymes such as lipase, nuclease, and protease [35]. Analysis of *agr* gene polymorphism showed that all 34 of the *S. aureus* genotypes could be assigned to one of four major *agr* specificity groups. Based on our findings, we revealed that group I is the most predominant group in the region, followed by other groups such as II, III, and IV. The result in covenant with other countries Iran [36], Pakistan [37], and Netherlands [38] revealed that the most dominant group agr was *agrI* in all sources hospital wards that contain high antibiotic resistance rates. One of the studies proposed that type *II agr* strains were described as strong biofilm producers, whilst *agrI* and *agrIV* tended to be weak to moderate biofilm former, and *agrIII* was mostly moderate biofilm formers [39]. The *agrIII* is prevalent in isolated community-acquired MRSA infections, whereas *agrII* has been over-represented among MRSA strains from hospitals [40, 41]. Several of the previously published articles did not report the presence of *agr IV* [42-44]. Another study also stated a higher incidence of group II strains and a lower occurrence of group I strains among nosocomial infections [43]. Our results also revealed the same results, as the majority of our strains were non-biofilm producers, they belong to *agrI*. Other studies, on the other hand, have revealed an association between the resistance of *S. aureus* isolates and the rates of biofilm production [45, 46]. The results of this study showed that biofilm-producing *S. aureus* had higher levels of antibiotic resistance. According to a study conducted by Cha et al. from a Korean teaching hospital had higher levels of multi-drug resistance among MRSA isolates compared to non MRSA [47]. In another study from Hungary emphasizes that resistant isolates to antibiotics like erythromycin, clindamycin and rifampin were significantly more common among biofilm-producers than non-biofilm producers [48]. Overall, inappropriate and inadequate antibiotic usage may create an environment favorable for the development of more non susceptible pathogens, which may reflect the greater level of resistance in isolated bacteria.

5. Conclusion

In conclusion, the study revealed distribution of MRSA isolates from clinical samples, and *agr I* was the most common *agr*-specific group, followed by *agr II, agr III,* and *agr IV* and isolates belonging to *agrI* group were weak biofilms producers as well as inactivation of *agr* strongly altered virulence determinant regulation and aid in reducing the severity of *S. aureus* infections.

Conflict of Interests

All authors declared no conflict of interest in the manuscript.

Consent for publications

All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate

The study was permitted by the ethics committee at Hawler Medical University College of Pharmacy. Throughout data collection, all patients who attended were requested to obtain verbal consent. Informed agreement from the patients or their families was taken.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Non.

Author's contributions

SO and SH performed research, SH and AG designed, analyzed the data, SO and AG prepared manuscript, SH and AG reviewed then finalized the manuscript. SS and SO collected samples and wrote the method. Final approval of the manuscript was obtained from all of the authors.

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