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## Prevalence of some virulence associated-genes in methicillin resistance *Staphylococcus aureus* isolates from patients infected with septic arthritis and antimicrobial resistance patterns of these isolates.

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This is a Prospective study was done in Al-Najaf Governorate, Iraq during February 2015 to July 2016. Synovial fluid specimens were processed for bacterial culture according to the typical procedures, the prevalence of genes encoding of spa, v8 protease (SspA) and tsst-1 among methicillin resistant *S. aureus* associated with septic arthritis infection was determinants by PCR amplification. Antimicrobial susceptibility test was done using agar disk diffusion method. of the 400 synovial fluid samples, 65(16.25%) showed growth of gram positive and gram negative bacteria while 335(83.75%) of them were affected with aseptic type. The most frequent isolated gram positive bacteria was *S. aureus* 27 (41.53%) and all isolates were resistance to Methicillin. Twenty six (96.29%) strains were found to be spa+, 23(85.19%) strains were found to be v8 protease (SspA)+ and tsst-1 gene were found in 18 isolates (66.66%) . Vancomycin and Rifampicin show high rate of sensitivity compared with other antimicrobial drug.

**Keywords:** Virulence gene; Antimicrobial resistance; MRSA; Synovial fluids.

### INTRODUCTION

Septic arthritis is a rheumatologic emergency. Bacterial replication in the joint and the resulting inflammatory process can lead to rapid local joint destruction, and may be complemented by systemic infection and which can lead to significant morbidity and mortality and incidence of septic arthritis is about 6 cases per 100 000 in the general population and much higher in rheumatoid arthritis patients impending about 70 cases per 100 00 (Sharff et al., 2013; Tarkowski, 2006). *Staphylococcus aureus* is one of the most important gram positive bacteria associated with

different infections such as; abscesses, endocarditis, cellulitis and burns infections, MRSA is a serious problem in orthopaedic (Ryan et al.,1997; Murakami et al .,1991). Virtually half of the patients will progress irreversible joint destruction in spite of optimal antibiotic treatment (Goldenberg,1998) . Haematogenous spread of *S. aureus* to the synovial membrane of joints is the most common reported route of acquiring septic arthritis (Mathews et al.,2010). Infection caused by MRSA is characterized by rapid activated macrophages and fast response to activated T cell that participate destruction of joints (Bremell

et al.,1992) A number of different constituents can produce by *S. aureus* strains that may contribute to virulence and arthritogenicity, including polysaccharides, surface-associated adhesins clumping factor A, exotoxins and exoenzymes (Josefsson et al.,2001). Protein A is an important virulence factor with molecular weight of 42 KD, encoded by *spa* gene and contain repeated part identified as X region; consists of up to 12 units each with a length of 24 nucleotides (Palmqvist et al.,2002; Mitani et al.,2005 ). Protein A inhibition opsonic phagocytosis encouraging apoptosis and inflammatory caused by TNF $\alpha$  (Gomez et al., 2006). The other enzyme is major proteolytic enzymes encoded by the *sspB* gene and located in an operon contiguous to the gene for the serine protease (*sspA*)( Rice et al.,2001). *SspA* gene is secreted in a pro-form, which is proteolytically cleaved to produce a mature and functional enzyme, possibly in an Aur dependent manner, the *SspA* gene is an important in cleave surface proteins including fibrinogen-binding protein and surface protein A (Lindsay and Foster,1999). *Staphylococcus aureus* produced many types of superantigens that bind to T-cell receptors on T-cell and to CD 28 (Kaempfer et al., 2013). TSSI-1 is encoded by *S. aureus* *tsst* gene and it is a 29.1 KDa (Fraser et al., 2002).Responsible for menstruation and non- menstruation cases between general population (McCormick et al., 2003) . And promote the infection in experimental septic arthritis (Abdelnour et al., 1994). In many parts of the world, including Iraq, information concerning the etiologic of virulence factors and antibiotic resistance of bacteria isolated from septic arthritis are rare. Therefore, the aim of this study was to determinate the antimicrobial susceptibility pattern of MRSA isolates from patients infected with septic arthritis and molecular characterization of *spa*, *SspA* and *tsst*-1 genes.

## MATERIALS AND METHODS

Bacterial isolates and data collection:

This is a cross-sectional study; was performed in Al-Najaf Governorate in Iraq, department of Orthopedics and Joints Diseases in Al Najaf General Hospitals and Private Clinics of Orthopedics and Joints diseases during the period from February 2015to July 2016. Four hundred patients from both sexes and different age groups (1-80) years were included in this study. They were attending. Synovial fluid aspirates were taken mostly from affected joints like knee, also from ankle, elbow and shoulder of those patients

by senior Orthopedist and transported to the lab in a cool box as soon as possible for processing, these specimens were manipulated soon following morphological, bacteriological cultures and biochemical required tests. In particular, all identifying isolates were confidence by Vitek $\text{\textcircled{2}}$ -bioMérieux $\text{\textsuperscript{TM}}$ .

Methicillin resistance aureus identification

Methicillin resistance *S.aureus* was identified by cultured onto oxacillin- methicillin plates surface (Gença et al., 2008). All isolates were compared with McFarland tube and streaked onto oxacillin-methicillin plates surface at 37 oC for 24h. The isolates considered methicillin resistance if growth was > one colony.

Antimicrobial susceptibility test

Antimicrobial susceptibility test was done according to Bauer et al., (1966).The resistance and sensitive of bacterial isolates were determined according to CLSI guideline, (2015). The following antimicrobials were used in this study provided from Bioanalyse, Turkey; Penicillin (P,10 $\mu$ g) Vancomycin (VA,30  $\mu$ g) Amoxicillin (AX, 30  $\mu$ g), Gentamicin (CN, 10  $\mu$ g), Amikacin (AK, 10  $\mu$ g), Doxycycline (DO, 30  $\mu$ g), Ciprofloxacin (CIP, 5  $\mu$ g), Cephalexine (CX,30  $\mu$ g ),Cefoxitin (FOX,30  $\mu$ g), Rifampicin,(RA,  $\mu$ g 30).

DNA extraction and Detection of the *spa*, *v8*protease (*SspA*)and *tsst* genes :

virulence determinants of *S. aureus* from patient with septic arthritis infections, emphasizing on the genes encoding *spa* , *SspA* and *tsst* . a suspension with one pure colony into a Luria Bertani medium (LB), which was incubated overnight at 37°C, Then, the inoculum was centrifuged 10 min. at 13000g the supernatant was discarded and the pellet was resuspended, and genomic DNA of *S. aureus* isolates were extracted by using Presto $\text{\textsuperscript{TM}}$  Mini genomic DNA Bacteria Kit Geneaid (Korea) with the modification that 20mg/ml of lysostaphin(Sigma) was added to the cell lysis step .The DNA solution obtained was stored at -20°C and used as target for PCR. DNA amplification was performed in a final volume of 20 $\mu$ l containing taq DNA polymerase1U, dNTP 250 $\mu$ m, tris-HCL (pH 9.0)10mM, ,KCL130 mM, MgCl $_2$  1.5mM and stabilizer and tracking dye. All PCR components were assembled in PCR tube and mixed on ice bag under sterile condition, sequences specific for *spa*, *SspA*, *tsst* genes (Table1) were detected by PCR on thermocycler condition shown in (Table 2) .All PCR products were analyzed by electrophoresis through 1% agarose gels with 0.5 mg/ml of ethidium bromide.

**Table 1: All Primers were used in the current study primer**

primer	Oligo Sequence (3'→5')	Product Size ( bp)	Reference
<i>spaF</i> <i>spa R</i>	GATCTGTAAC TTTAGGTACATTAC ATAGTTCGCCACGACGTC	1200	Afrough et al .,2013
<i>SspA F</i> <i>SspA R</i>	CAACGAATGGTCATTATGCACCCGTA TTTGGTACACCGCCCAATGAA	529	Campbell et al.,2008
<i>tsst F</i> <i>tsst R</i>	ATGGCAGCATCAGCTTGATA TTTCCAATAACCACCCGTTT	350	Johnson et al .,1991

**Table 2: Programs of PCR thermo cycling conditions**

Target gene	Temperature ( c ) / Time				Cycles number	
Initial Denaturation	Cycling condition				Final Extension	
Denaturation	Annealing			Extension		
<i>tsst</i>	94- 2 min.	94- 1 min.	55- 2 min.	27- 1 min.	72- 10 min.	30
<i>spa</i>	94- 3 min.	94- 45 sec.	55- 30 sec.	72- 90 sec.	72- 5 min.	30
<i>SspA</i>	95- 15 min.	95- 1 min.	60 – 1 min.	72 – 1 min.	72 -10 min.	32

The size of the amplified products was estimated by comparison with a DNA ladder (BioNEER) .The image acquisition was obtained through Biometra gel documentation system.

**RESULTS**

A total of 400 synovial fluid diagnosed sample of septic arthritis were included in the study of which 65(16.25%) showed growth of gram positive and gram negative bacteria while 335(83.75%) of them were affected with aseptic type. The most frequent isolated gram positive bacteria was *S. aureus* 27 (41.53%) and all isolates 27(100%) was resistant to Methicillin. The product of PCR adopted allows to identify *spa+* strains by the appearance of an amplified DNA fragment of 1200 bp earlier described (Afrough et al., .,2013) Figure(1) ,The applicant indicating the presence of *spa* gene was detected in 26(96.29%)

of all the 27 staphylococcal clinical isolates Figure (4). The presence of the *SspA* and *tsst* genes was revealed, respectively, by a 350bp and 529 bp, as expected from the respective gene sequence figure(2)and(3) .the presence of these genes was detected in 23(85.19%) and 18(66.66%) among isolates figure(4).

**Antibiotic Susceptibility Profile of MRSA isolates.**

The overall sensitivities of the most commonly tested antimicrobials are shown in Figure 5. MRSA strains showed various degrees of sensitivity to antimicrobials such as Vancomycin (96.29%), Rifampicin (81.48%), Amikacin (77.77%), Ciprofloxacin (70.37%), Gentamycin (70.37%), Doxycycline (66.66%), Cephoxitin (22.22%), Cephalexin and Amoxillin (11.11%). And All MRSA isolates showed complete resistance to Penicillin.

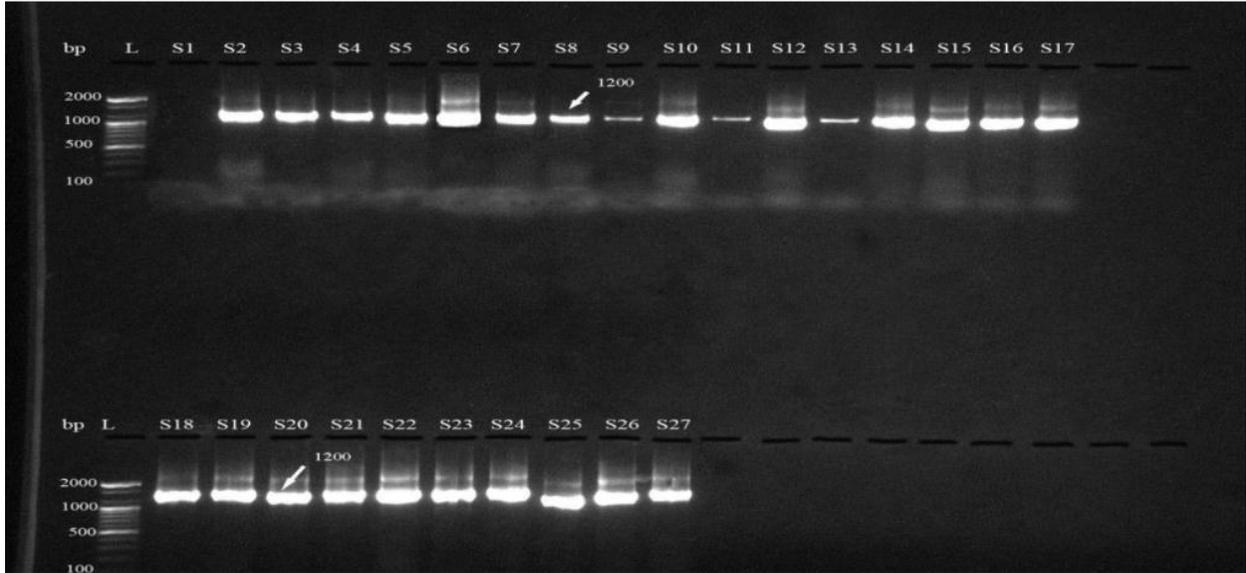


Figure 1: Polymer chain reaction product of *spa* gene was extracted from total DNA of MRSA show positive result at 1200 bp.

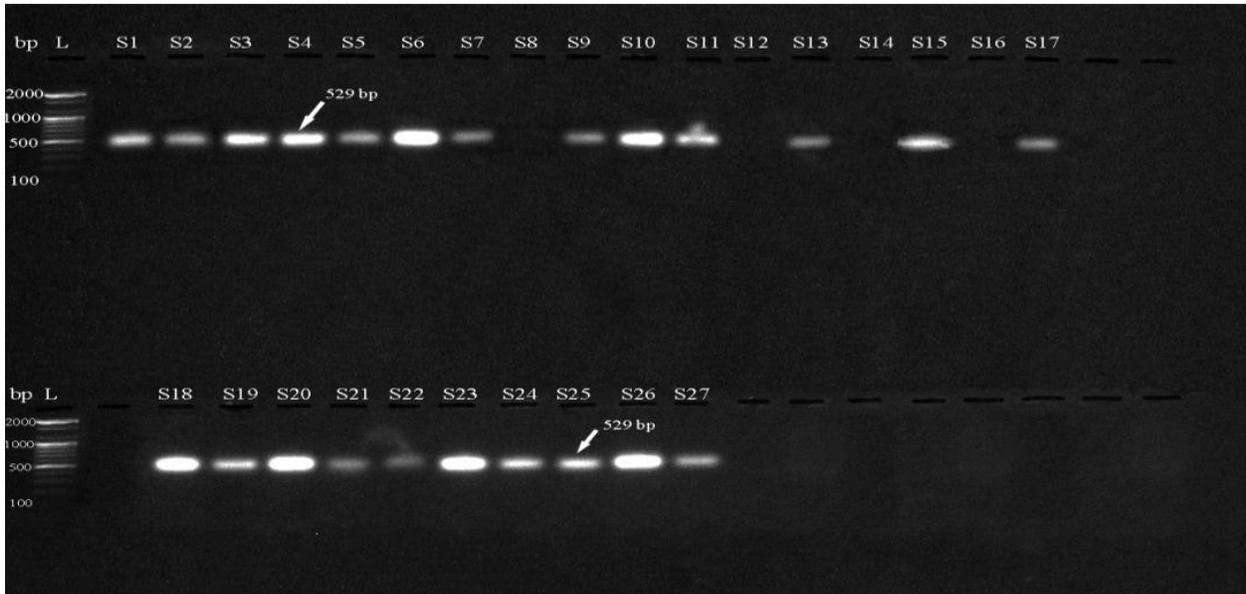


Figure 2: Polymer chain reaction product of *sspA* gene was extracted from total DNA of MRSA show positive result at 529 bp.

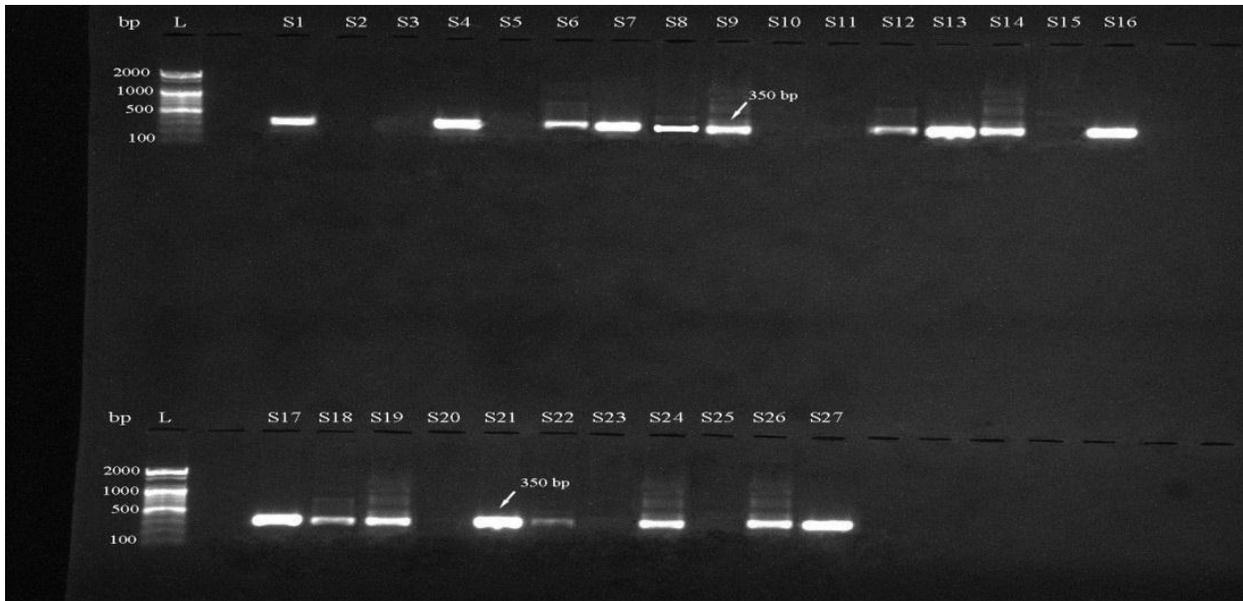


Figure 3: Polymer chain reaction product of *tsst* gene was extracted from total DNA of MRSA show positive result at 350 bp.

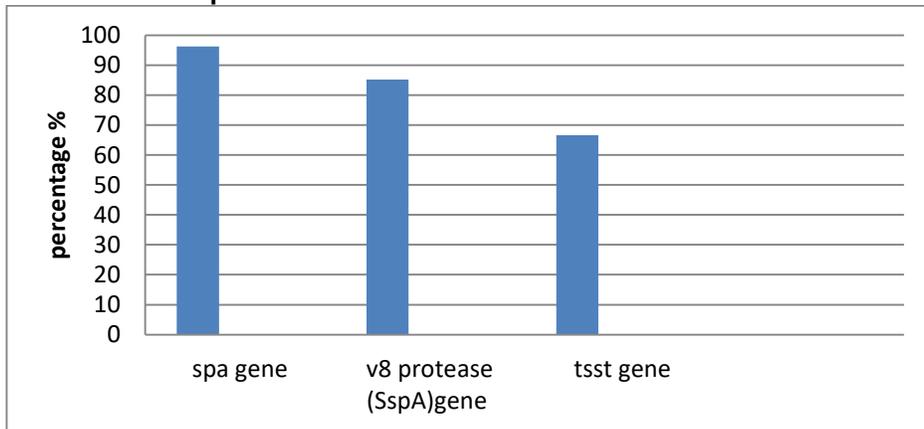


Figure 4: Prevalence of virulence associated-genes in methicillin resistance *Staphylococcus aureus* isolates from patients infected with septic arthritis



**Figure 5: Antimicrobial susceptibility test of 27 MRSA isolates from synovial fluid aspirate.**

## DISCUSSION

Septic arthritis is one of the most dangerous infections caused by *S. aureus*. In this study, 16.25% was as a positive culture, this results are in agreement with previous studies (Kennedy et al.,2015; Brischetto et al.,2016). *Staphylococcus aureus* take the first rank of isolation (41.53%) which is consistent with the epidemiology found in several regions of the world since different rate of *S. aureus* isolation was observed by several reports ( Lin et al.,2015 ;Kalantari et al., ,2007;Chen et al., ,2013). they found that *S. aureus* isolation rate from patient with septic arthritis were 40.9%, 61%, 38% respectively. The results of the present study proved that all *S. aureus* isolates 27 isolates 100% were resistance to Methicillin. Previous studies concluded that the most causative agent of septic arthritis is MRSA (Brischetto et al., ,2016; Mínguez et al.,2015). In this study, *Spa* gene was prevalence in 96.29% of total isolates. This result is similar with some previous studies in different countries such as in Korea according to study by Campbell et al., ,( 2008) which indicated the prevalence of this gene in 100% of total isolates, in Pakistan according to study by Hussian et al.,( 2013) which also concluded that this gene was found in 100% of total isolates. While, in Iran according to study by Shakeri et al., (2010) this gene was prevalence in 97.2% .The high prevalence of this gene in staphylococcal isolates in the current study may be due to the fact that the recent work that implemented protein A (SpA) as a vaccine antigen (Kim et al., 2010). Facilitating loss and destruction

of bone in osteomyelitis and septic arthritis (Widaa et al., 2012) . The prevalence of gene which encoding serine protease enzyme among the *S. aureus* isolates was investigated by PCR assay, the results revealed the abundance of this gene in (85.19%). Abundance of this gene in our study may be due to the fact that *S.aureus* must change its phenotype from adhesive to invasive in order to disseminate within the host (Lowy,1998). The result of present study concerning the prevalence of *SspA* gene was comparable to the results reported by several reports. variations in prevalence of *SspA* recoded in different references , High rate of this gene was recorded by Zdzalik et al., (2012), whom found that 100% of *S. aureus* isolates that recovered from different clinical sources like cystic fibrosis, pneumonia, skin infection, bone infection and urinary tract infection were carried this gene. But the frequencies of other gene encoding other different protease enzymes ranged from 67% to 85% .Another study showed that 95% of *S. aureus* isolates that recovered from skin and soft tissue infections were carried this gene (Campbell et al.,2008).Moreover 92.7% of *S. aureus* isolates that recovered from different clinical sources were carried this gene (Paniagua-Contreras et al.,2012). And 55% of invasive isolates of *S. aureus* were contain this gene (Rasmussen et al.,2013) . These variations in prevalence of gene may be due to the level of SigB-dependent expression of the protease repressor *sarA* that determines the level of protease production in each strain (Karlsson and Arvidson,2002).Or may

be due to different geographical regions, number of isolates that investigation and type of disease. The finding of present study indicated that 18 (66.66%) of *S. aureus* isolates that recovered from synovial fluid have *tsst* gene encoding to toxic shock syndrome toxin, The result of present study concerning the prevalence of *tsst* was comparable to the results reported by several reports which concluded that 68% , 62.16% and 73% of MRSA isolates from different clinical specimens contained the *tsst* genes respectively( Koosha et al.,2016 ;Udo et al.,2016; Campbell et al.,2008 ).Whereas low prevalence of this gene estimated 17% by Rasmussen et al., (2013) in invasive *S. aureus* isolates .However TSST-1 production by *S. aureus* has proved to be an arthritogenic factor and had significantly more interleukin-2 receptor-expressing cells in the inflamed synovium (Abdelnour et al.,1994).

### Antibiotic Susceptibility Profile

Vancomycin appear to be the most effective antibiotics (96.29%), with the lowest levels of resistance in this study . Vancomycin is a glycopeptide which inhibits peptidoglycan synthesis in the bacteria, it is a known drug of choice in the treatment of methicillin resistant *S. aureus* septic arthritis (Sharff et al.,2013).Several studies have been found that MRSA strains that isolated from patient with septic arthritis and joint infection were completely sensitive to vancomycin ( Yadav et al.,2013; Sousa et al.,2010; Motwani et al.,2017). Making it the rational choice for empirical use. Out of 81.48% of MRS isolates were sensitive to Rifampicin . Rifampicin has excellent anti-staphylococcal activity and bioavailability, can eradicate adherent organisms in the stationary phase and can penetrate white blood cells to kill phagocytosed bacteria making it the best antibiotic for bone and joint infection (Darley and MacGowan,2004).A previous done study which reported prevalence of MRSA to be 100% sensitive to Rifampicin in infected knee arthroplasty (Nickinson et al.,2010) .In another studies done in Iran in 2010 and in Jordan in 2015 showed that 89.1% and 85.8% of MRSA strains which isolated from different clinical samples were sensitive to Rifampicin (Japoni et al., 2010;Al-Zoubi et al., 2015) and these observation was in accordance with our result. Out of 77, 77% of MRS isolates were sensitive to Amikacin ,our study is comparable with other studies which found that Amikacin was effective antibiotic with sensitivity of 88%, 78.5% and 71% respectively against *S. aureus* isolated from septic arthritis and

osteomyelitis (Kalantariet al.,2007; Wadekar et al., 2015; Wadekar et al., 2014) . It was found in our study that the organisms isolated from synovial fluid were less sensitive to Gentamycin (70.73%) than Amikacin (77.77%) .Anguita-Alonso et al.,(2005 ) did a study to determine the minimum inhibitory value of 93 staphylococci from patients with PJI and they found that 41% were resistant to gentamicin . A study done in 2018 showed that Resistance profile of the bacterial isolates from pus and body fluid samples was 47.70% for Gentamycin and 30.50% for Amikacin (Perween et al .,2018), these differences may be due to that of aminoglycosides have a slightly different mechanism of resistance due to their different aminoglycoside modifying enzymes chromosomal mutation ( Garneau-Tsodikova and Labby, 2015). In a recent study performed in Tehran, 28.3% of MRSA isolates were resistant to doxycycline (Arianpooret al.,2015) similarly in our study we found that 33.33% of our isolates were resistant to doxycycline. Ciprofloxacin is a quilonone which acts by inhibition of enzymes involved in DNA replication and function .Our data showed that 19 of MRSA isolates was sensitive to ciprofloxacin (70.37%). studies showed that 94.9% ,100% and 79% of *S. aureus* isolates were sensitive to this antibiotic ( Onaolapo et al., 2016; Caraciolo et al.,2012; Anuradha & Nandini,2017.).In Iraq AL-Marjani et al.,(2015) concluded that 37% of *S. aureus* isolates were positive to *gyrA* and *qnrS* genes, characterization of highly ciprofloxacin-resistance in these locally isolates . Thus, *qnrS* and *gyrA* gene may have been spreading in those isolates in Baghdad-Iraq. Only 22.22% and 11.11% of MRSA isolates were found to be sensitive to Cephoxitin and Cephalexin respectively in our in-vitro study. In previous study showed that 76% of MRSA isolates from Orthopaedic Patients were resistance to Cephoxitin (Onaolapo et al.,2015).Current result was not agreement with what was indicated by Campbell et al.,(2008) which included that Susceptibility pattern of bacterial species isolated from children with septic arthritis and osteomyelitis to cephalixin was 78% . High resistant rate of these antibiotics in this study may be due to increase in irrational consumption rate of antibiotics, self-medication and non-compliance with medication. Predictably, the antibiotics more commonly used in the general medical setting, penicillin and amoxillin , the present study concluded higher levels of resistance 100% and 88.88% respectively . Our observation was in accordance with studies that have been showed

higher levels of resistance to penicillin (Kalantari et al.,2007; Nickinson et al.,2010).

### CONCLUSION

This work was performed to evaluate the most common causative organisms of septic arthritis disease and determine the most prevalence genes encoding virulence factor and determination of antimicrobial sensitivity pattern of common isolates. Results of this study showed that Methicillin resistant *Staphylococcus aureus* (MRSA) was the most common important pathogenic bacteria cause septic arthritis ,wide distribution of the *spa* genes among the investigated isolates, indicating that these strains harbouring this virulence determinant may increase the risk for subsequent invasive infection,also the antimicrobial profile of synovial fluids is important so that such life threatening infections can be treated.Vancomycin and Rifampicin show high rate of sensitivity compared with other antimicrobial drug.

### CONFLICT OF INTEREST

There is no any conflict of interest in this work.

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### AUTHOR CONTRIBUTIONS

IAJA designed , performed the experiments and wrote the manuscript.

The author read and approved the final version of this manuscript.

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