Screening the frequency of pannvtale valve leukocidin (pvl) gene isolated from methicillin resistant Staphylococcus aureus isolated from diabetic foot patients in Al-Basrah governorate, south of Iraq

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ABSTRACT
Background: Staphylococcus aureus with methicillin resistant is an important pathogen associated with diabetic foot ulcers (DFUs). The (pvl) gene is considered a marker for MRSA that used widely as acquired in the population, it's responsible for deep dermal infections and soft tissue. The current research aimed to establish the pannvtale valve leukocidin (pvl) frequency of gene between MRSA isolated from infected DFUs individuals in Al-Basrah governorate, south of Iraq.
Methods: One hundred fifty swab sample from diabetic foot ulcers (DFUs) patients who admitted to two local hospitals in Basrah governorate. The S. aureus isolates were identified by criteria of growth on both blood agar and mannitol salt agar, and then it tested for coagulase test. The isolates confirmed as S.aureus by using the vitek® 2 system for identifying the Staphylococcus aureus. MRSA had been confirmed by testing the isolates against oxacillin and cefoxitin disc. The 16S rRNA was used to confirm the identification of S.aureus isolates. The PCR was used to detect the production of pvl genes.
Results: Out of 150 swab samples only 21 isolates which include 18(none) S. aureus, the other 3(14.3%) isolate was identified as Staphylococcus spp. The result of vitek® 2 showed all (18/100%) isolates were identified as S. aureus. On the other hand all (18) S. aureus isolates given the inhibitory resistant criteria for oxacillin disc 1μg and cefoxitin disc 30 μg, these isolates classified as MRSA. Furthermore, the result of amplifying 16S rRNA was given the positive result to identify S. aureus. MRSA was found in all isolates, the 18 (100 %) were pvl positive.
Conclusion: The frequency of pvl gene found in all MRSA isolates in this study. It can be used as community acquired-MRSA marker. The PCR analysis effective in detecting pvl & meca genes.

INTRODUCTION
Diabetes mellitus is a common, chronic disease. Often it causes wounds to recover later, such as diabetic foot ulcers (DFUs). It increasing globally incidence mainly in the elderly (Leung, 2007). The diabetes wound in 25% of cases develop to ulcers infection (Abbot et al., 2005; Laverry et al., 2014). Furthermore the DFUs infections significantly medical, economic issue and social in diabetes patients. If infectious agents are not treated properly, these are related to the amputation of the infected foot (Hartemann-Heurtier et al., 2004). On otherhande the DFUs causes by diverse mixed from Gram- negative and positive bacterial, but most frequently species is S. aureus (Wang et al., 2010; Mendes et al., 2011). Almost the 50% from S. aureus are meticillin-resistant. Several studies found its emergence 15-30% in diabetic wounds (Gadepalli et al., 2006; Wang et al., 2010; Sandhu et al., 2014). Already virulence S. aureus present in DFUs is a major factor that contributes to the based on the severity of a wound, because these factors allow the pathogen to enter and kill the host tissue (Sandhu et al., 2014). The current research aimed to establish the pannvtale valve leukocidin (pvl) frequency of gene between MRSA isolated from infected DFUs individuals in Al-Basrah governorate, south of Iraq.

MATERIAL AND METHODS
Sample collection:
From March-2018 to February-2019, 150 swab samples were collected from diabetic foot ulcer patients who admitted to two local hospitals in Al-Basrah governorate, south of Iraq.

Isolation and Identification of bacteria:
Swab sample was cultivated firstly for 24h. at 37°C in brain heart infusion broth. The broth media that given the positive growth were streaked on both mannitol salt agar and blood agar plates and incubated for 24h. at 37°C. The isolates that given the characteristic of Staphylococcus aureus was selected and checked according to (Harley and Prescott, 2002) for coagulase test. The vitek® 2 system version- 07.01 device was used. The test card of Gram positive bacteria (VITEK® 2 GP 1D-P Reference number 21342, bioMérieux, USA) for identification the S. aureus isolates.

Screening of Staphylococcus aureus methicillin resistant (MRSA):
The oxacillin and cefoxitin diffusion disc methods was used to detected the MRSA. Both tests were preformed according to (Datta et al., 2011; Sharma et al., 2017).
inhibition zone diameter was measured and interpreted according to criteria of CLSI (2007).

Genetic profiling
DNA extraction:
The DNA Presto Mini gDNA Bacteria kit (Geneaid, USA) was used to extract genomic DNA according to the manufacturers specifications.

16S rRNA gene PCR
The extracted DNA was amplified with specific primers with product size 756 bp according to (McClure et al., 2006). The GoTaq promega master mix (promega, USA) was used according to the manufactures specifications. It was performed with: a 1 cycle denaturation step (5 min. at 95°C) and ended with final extension step (5 min. at 72°C). 35( 30s at 95°C; 30s at 55°C; 1 min. at 72°C). Amplified products electrophoresis in 1.5% agarose gel and visualized with ethidium bromide by using UV gel documentation system.

Molecular detection of mecA and pvl genes
Specific primers used for amplification of mecA and pvl gene according to (Pournajaf et al., 2014), and (Lina et al., 1999) respectively.

The reaction of PCR for S. aureus (mecA and pvl)
The GoTaq Promega master mix (Promega, USA) was used in the PCR reaction. It was performed for mecA genes: a 1 cycle denaturation step (3 min at 95°C) and ended with a final extension step (6 min. at 72°C), 30( 1min. at 94°C; 30s at 53°C;1 min. at 72°C), while pvl gene: a 1 cycle denaturation step (1 min at 95°C) and ended with a final extension step (1 min. at 72°C), 35( 30s at 94°C; 30s at 55°C;1 min. at 72°C) with product size 533 bp and 433 respectively. Amplified products electrophoresis in 1.5% agarose gel and visualized with ethidium bromide by using UV gel documentation system.

RESULT
Out of 150 swab samples from March-2018 to February-2019, only 21 isolates which include 18(85.7%) isolates was identified as S. aureus, while the other 3(14.3%) isolate was identified as Staphylococcus spp. The criteria that depended to identify S. aureus included ferment mannitol with changing the colour of the indicator from red to yellow by producing acid when grown on mannitol agar. Also, colonies surrounded by areas of clear β-hemolysis when grown on blood agar and given positive results in a coagulase production test. The vitek® was used to support and confirm the results of the identification tests that used for identification of S. aureus isolates from diabetic foot patients. The result of vitek® showed that all (18) isolates were identified as S. aureus.

Staphylococcus aureus methicillin resistant (MRSA)
The results in the (Figure 1) showed that all (n=18) S. aureus isolates given the inhibitory resistant criteria for oxacillin disc (1µg) and all these isolates classified as MRSA. The results show that isolates with the numbers (3,10,16,18), (2,6,7,11), (4,9,14), (12,15), (5, 13), (1, 8) and (17) gave the inhibition zone less than (9mm, 8mm, 10mm, 7mm, 6mm, 5mm and 4mm) in percentage 22.2%, 22.2%, 16.7%, 11.1%, 11.1%, 11.1% and 5.6% respectively.

Also, all of S. aureus isolates (n=18) were tested against the cefoxitin (30 µg) antibiotic disc to detect the inhibitory resistant criteria and indicated resistance to cefoxitin and the isolates classified as a MRSA. In figure-2 the results showed the isolates with the numbers (7,9,12,13,14,17), (1,4,6,10,7,16), (8), (3,5,11) and (2,15) gave the inhibition zone less than (21mm, 20mm, 18mm, 16mm, and 14mm) with percentage 33.3%, 33.3%, 5.6%, 16.7, and 11.1% respectively.

Figure (1) The percentages of S. aureus isolates that resist for Oxacillin (1 µg) antibiotic disc
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Figure (2) The percentage of the *Staph aureus* isolates that resist for Cefoxitin (30 µg) antibiotic disc

Genetic profiling
16S ribosomal RNA.
The result of amplifying 16S rRNA was showed the all 18 isolates gave the positive result and single band viewed in (750bp) when compared to a typical molecular DNA ladder (2000 bp) (Figures 3).

Molecular detection of mecA and pvl genes
Amplifying mecA gene was showed that all 18 isolates gave the positive result as single band in 533 bp (Figure 4).While the amplifying pvl gene also showed with all 18 isolates and gave the positive result as a single band in 433 bp (Figure 5). All amplified genes were compared with standard molecular DNA ladder (2000 bp).

Figure (3) Gel electrophoresis patterns show PCR amplified products of 16S rRNA. Lane L:(2000 bp DNA ladder) , Lane: (No. 1-9) 16S rRNA band of bacterial isolates.
DISCUSSION

MRSA has emerged as a critical and widespread challenge for diabetic foot ulcer patients (Tentolouris et al., 1999; Game and Jeffcoate, 2004). In both neighborhood and hospital procedures, S. aureus often was the isolated pathogen. S. aureus change antimicrobial pattern sensitivity, particularly in developing countries, it increasingly making antimicrobial agents fewer effective (Bukhari et al., 2004). This study, found the prevalence of S. aureus in diabetic foot ulcers was 18(12%) isolated from 150 diabetic foot ulcers swab samples. This finding is in line with the study conducted in (8.4%) in Bandar Abbas District, Southern Iran (Ahmadishoohi et al., 2020) and (11%) in Malaysia (Goh et al., 2020). However, the prevalence reported in the current study is lower than a study reported (20%) in Brasil (Pontes et al., 2020), (28.8%) in Manchester, UK (Tentolouris et al., 1999), (34%) in Babylon City, Iraq (Al-Allak et al., 2019), (43.8%) in England (Nelson et al., 2018), (51%) in Portugal (Mendes et al., 2011) and (54.7%) in Sulaimani City, Iraq (Qadir et al., 2020). The difference in prevalence may be due to difference in the experimental data obtained, a time taken to research and the procedure used to detect S. aureus.

Also in this study, the prevalence of MRSA was 100% in both antibiotic and molecular detection methods that use to detect MRSA. The prevalence reported in the current study is higher than a study reported in (63.4%) in China (Wang et al., 2010), (29.8%) in USA and (24.5%) in Portugal (Mendes et al., 2011) (Lavery et al., 2014). Excessive and uncontrolled use of antibiotics in Iraq is one of the biggest challenges that lead to the emergence of significant levels of multi-drug resistance. In addition to that, many patients resort to taking many antibiotics without medical advice, especially broad-spectrum antibiotics. Therefore, we suppose that the high percentage of MRSA between isolated of S. aureus is a natural result of the heavy use of broad-spectrum antibiotics. Treating MRSA for any diabetic foot infection is likely to lead to an increase in resistance as well as an increase in the cost burden for health care. Infections with MRSA involving other sites, prior hospitalizations, nursing homes, immunocompromised patients, prior antibiotic treatment (Demling and Waterhouse, 2007; Ding et al., 2012; Lavery et al., 2014).

Panton-Valentine leukocidin is consist of two-component and coded by two co-transcribed genes, lukS-PV and lukF-PV, which exist on a prophage and induce tissue necrosis and leukocyte destruction (Fincl-Barbançon et al., 1993; Prevost et al., 1995). The S. aureus have a variety virulence factors which promote soft tissue and bone infections; however pvl is considered as a one of those virulence characteristics, and it tends to be associated with extreme DFUs (Ambrosch et al., 2011). In our study, the pvl gene present among all S. aureus that isolates from the DFU patients. This finding is in line with the study reported (100%) in France (Naas et al., 2005). The frequency of pvl in S. aureus over the last years given the widespread distribution of pvl-producing MRSA strains (Esmiebadeh et al., 2018).

However, a few studies not shown any relationship between pvl gene and virulence (Campbell et al., 2015; Bae et al., 2009). In these studies, explanation this finding in clinical results may be affected by the efficacy of the administered antibiotic treatment. The pvl gene is use widely as a marker for MRSA acquired in the population, responsible for deep dermal infections and soft tissue (Rieg et al., 2005; Havaei et al., 2010). On the other hand the pvl situation among different isolates from MRSA varies. Statistics from different areas show the growing number of pvl predominance between MRSA isolates. Bhatta et al., (2016) in western Nepal, reported (63.3 %) of pvl prevalence among MRSA also study by D’Souza et al., (2010) in India, reported frequency of 64% positive pvl isolates among MRSA while Rossney et al., (2007) from Ireland show the 76% of pvl-positive for MRSA. In our study show the 100% of MRSA isolates have pvl gene. Therefore, PCR analysis effective of detecting pvl and mecA genes and also being 100% accurate and effective. Besides that, in any microbiology laboratory with PCR capability, this assay is easily suitable for practical clinical use (McClure et al., 2006).

CONCLUSION

The frequency of pvl gene found in all MRSA isolates in this study. It can be used as community acquired-MRSA.

Figure (4) Gel electrophoresis patterns indicate products amplified with PCR of mecA gene. Lane L: (2000 bp DNA ladder), Lane: (No. 1-9) mecA gene bands of S.aureus isolates.

Figure (5) Gel electrophoresis patterns indicate products amplified with PCR of pvl gene. Lane L: (2000 bp DNA ladder), Lane: (No. 1-9) pvl gene band of S.aureus isolates.
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marker. The PCR analysis effective in detecting pvl & mecA genes

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