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Molecular expression of *mecA* gene in *Staphylococcus aureus* isolated from diabetic foot ulcers

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Abstract

Background: The *mecA* gene is a mobile genetic element contains various structures that encoded resistance to non-β-lactam antibiotics.

Aims: Finding the molecular characterization of *mec*A gene in clinical isolates of *S. aureus* obtained from the ulcers of diabetic foot patients with estimating their susceptibility to various antibiotics.

Materials and methods: Swabs of totally 58 diabetic foot ulcers were collected and inoculated in Mannitol salt agar, and then, the isolates of *S. aureus* were confirmed biochemically. Antibiotic susceptibility was performed using the disk diffusion test among an overall 12 types of antibiotics. Molecular PCR assaying was carried out targeting the *mecA* gene.

Results: The findings of traditional isolation and biochemical confirmation revealed the presence of 39.66% positive *S. aureus* isolates. The antibiotic susceptibility testing using the Kirby-Bauer disk diffusion shown that the positive *S. aureus* isolates were significantly sensitive to Nitrofurantoin (86.96%), Oxacillin (82.61%), Ofloxacin (69.57%), and Rifampin (56.52%); but highly resistant to Ampcillin (82.61%), Meropenem (82.61%), Amoxcillin (73.91%), Cephalexin (69.57%), Cefotaxim (60.87%), Ciprofloxacin (60.87%), and Methicillin (60.87%). Targeting the *mecA* gene, molecular testing of 23 positive *S. aureus* isolates by PCR assay detected that 34.78% were positives.

Conclusion: The presence of resistance and virulence genes can affect significantly on the pattern of sensitivity to antibiotics, especially with increasing of antibiotic resistance. Hence, the using of molecular assay in diagnosis of various resistance and virulence genes can support the cure rate.

Keywords: Conventional PCR, Diabetes mellitus, MRSA, Antibiotic susceptibility, Virulence factors, Iraq

Introduction

Staphylococcus aureus strains resistant to methicillin and many other antibiotics are major causes of nosocomial infections worldwide. Resistance to various antibiotic in particular methicillin was determined by the *mecA* gene, which encodes the low-affinity penicillin-binding protein PBP 2A (Ibraheim et al., 2023a, b). The *mecA* gene is part of a 21- to 60-kb staphylococcal chromosome cassette *mec* (SCC*mec*), a mobile genetic element that may also

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contain genetic structures such as Tn*554*, pUB110, and pT181 which encode resistance to non-β-lactam antibiotics (Wielders et al., 2002; Pantosti et al., 2007). Two hypotheses have been raised to explain the evolutionary origin of methicillin-resistant *S. aureus* (MRSA) strains. The single clone hypothesis, based on early analyses of the restriction fragment length polymorphisms obtained for MRSA isolates collected worldwide by using probes for *mecA* and Tn*554*, suggests that *mecA* entered the *S. aureus* population on one occasion and resulted in the formation of a single MRSA clone that has since spread around the world. The second hypothesis, based on the detection of *mecA* in diverse *S. aureus* multilocus enzyme electrophoresis types, proposes that MRSA strains evolved a number of times by means of the horizontal transfer of *mecA* into phylogenetically distinct methicillin-susceptible *S. aureus* (MSSA) precursor strains (Harkins et al., 2017; Lakhundi and Zhang, 2018; Chen et al., 2023). By using DNA microarray technology, *mecA* has been detected in at least five divergent lineages, implying that horizontal *mecA* transfer has played a fundamental role in the evolution of MRSA. The transfer of *mecA* from *S. epidermidis* to *S. aureus* was recently witnessed in vivo, suggesting that *mecA* may transfer more frequently to MSSA (Monecke et al., 2016; Earls et al., 2017).

The prevalence of MRSA in the community is predicted to increase substantially due to the dissemination of a successful SCC*mec* type by horizontal transfer (Steinig et al., 2019). Locally, a study conducted at the Maternity and Children teaching hospital and Al Diwaniya teaching hospital in Iraq, *S. aureus* isolates from different cases were found to be highly resistant to the usually used antibiotics (Al-Saadi and Abd Al-Mayahi, 2021). Another Iraqi study revealed that MRSA strains were resistant to various antibiotics such as azithromycin, methicillin, and ciprofloxacin, but not to ceftaroline (Alwash and Abed Aburesha, 2021). The pathogenicity of MRSA strains relies on several virulence factors; for example, hemolysins which lead to development of diseases and are categorized into three types: alpha (α), beta (β) and gamma (γ). The alpha hemolysin is a toxin produced by the *Hla* gene of *S. aureus* acting as a virulence factor that forms pores in cell membranes, disrupting epithelial barriers and leading to cell lysis and death (Moraveji et al., 2014; Zainulabdeen and Dakl, 2021). Therefore, the current study aims to find the molecular characterization of *mec*A gene in clinical isolates of *S. aureus* obtained from the patients of diabetic foot ulcers, and to estimate the susceptibility of *S. aureus* isolates to various antibiotics.

Materials and methods

Ethical approval

This study was licensed by the Scientific Committee of the College of Veterinary Medicine in University of Wasit (Wasit, Iraq).

Collection of samples and isolation of S. aureus

A total of 58 diabetic foot ulcer patients who attended to private clinics located in different areas in Wasit province (Iraq) were subjected to sampling of ulcer swabs under aseptic conditions. The swabs were kept in tubes containing transport media and inoculated in Mannitol salt agar plates that incubated overnight at 37°C.

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Biochemical testing

The suspected *S. aureus* colonies were tested additionally using catalase, oxidase, and coagulase assays.

Antibacterial susceptibility testing

The method used to detect the antibiotic susceptibility was the disk diffusion test. The culture was incubated on a Muller-Hinton agar plate for 24 hours at 37°C, and the test was performed for all *S. aureus* isolates using the 12 types of antibiotics: Amoxicillin (25µg), Ampicillin (25µg), Azithromycin (15µg), Cefotaxime (10µg), Cephalexin (30µg), Ciprofloxacin (10µg), Meropenem (30µg), Methicillin (10µg), Nitrofurantoin (100µg), Ofloxacin (5µg), Oxacillin (5µg), Rifampin (5µg), and Trimethoprim (10µg) using the Kirby-Bauer disk diffusion (CLIS, 2017).

Molecular analyses

At first, a DNA kit (Geneaid, USA) was used to extract the genomic DNA of all suspected isolates in accordance with the manufacturer's instructions. The PCR MasterMix tubes were prepared for amplification gene primers according to the gene-specific primers (Table 1). The PCR tubes were transferred to a thermal cycler for an amplification reaction (Table 2). PCR products were electrophoresed on 1.5% agarose gel and visualized by ultraviolet (UV) light transilluminator to detect the positive samples at 538bp product size.

Table (1): Primers designed to detect the *mecA* gene in *S. aureus*

Gene	Sequence	Product size	Reference
mecA	F: 5´-CCCAATTTGTCTGCCAGTTT-3´	538bp	Laub et al. (2017)
	R: 5´-ATCTTGGGGTGGTTACAACG-3´		

Table (2): Thermal cycler conditions for amplifying of PCR products

Steps	Temperature (°C)	Time (min)	No. of cycles
Initial denaturation	95	5	1
Denaturation	95	0.5	35
Annealing	56	0.5	
Extension	72	1	
Final extension	72	10	1

Statistical analysis

One-Way Analysis of Variance (ANOVA) and t-test in the GraphPad Prism Software (version 8) were used to statistical analysis of obtained data, and detect significant differences between the obtained values [number (percentage)] at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****), (Gharban, 2022, 2024; Hussen et al., 2024).

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Results

The findings of traditional isolation and biochemical confirmation revealed the presence of 39.66% (23/58) positive *S. aureus* isolates (Figure 1). Targeting the *mecA* gene, molecular testing of 23 positive *S. aureus* isolates by PCR assay detected that 34.78% (total no= 8) were positives (Figure 2).

The antibiotic susceptibility testing using the Kirby-Bauer disk diffusion shown that the positive *S. aureus* isolates were significantly sensitive to Nitrofurantoin (86.96%), Oxacillin (82.61%), Ofloxacin (69.57%), and Rifampin (56.52%); but highly resistant to Ampcillin (82.61%), Meropenem (82.61%), Amoxcillin (73.91%), Cephalexin (69.57%), Cefotaxim (60.87%), Ciprofloxacin (60.87%), and Methicillin (60.87%), (Table 3).

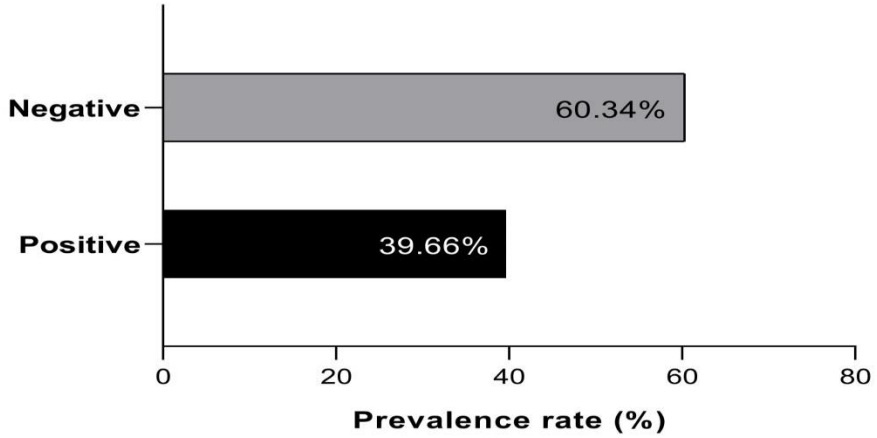

Figure (1): Total prevalence rate (%) of *S. aureus* isolates from ulcers of diabetic foot patients by culture

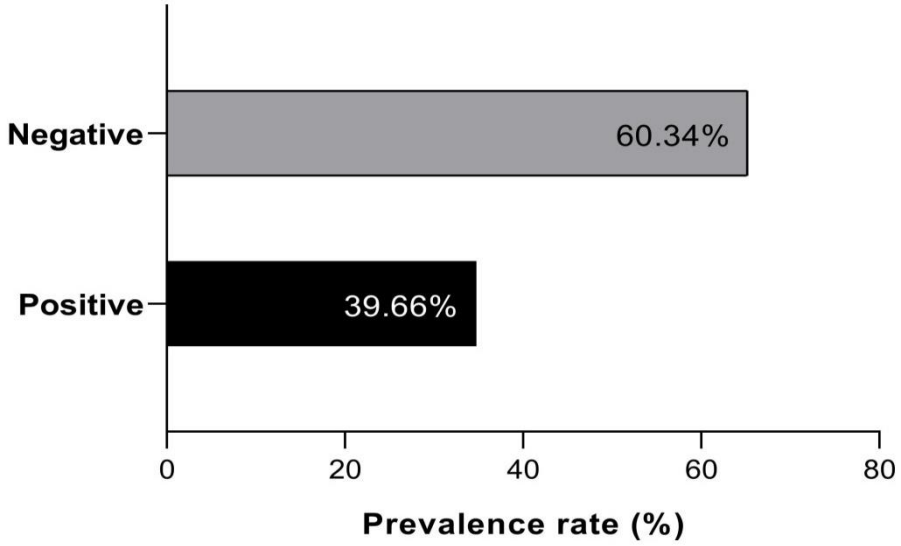

Figure (2): Positive *S. aureus* isolates to *mecA* gene

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Table (3): Antibiotic susceptibility testing of 23 positive *S. aureus* isolates

Antibiotics	**Sensitive No. (%)**	**Intermediate No. (%)**	**Resistance No. (%)**
Amoxcillin	2 (8.7%)	4 (17.39%)	17 (73.91%)
Ampcillin	1 (4.35%)	3 (13.04%)	19 (82.61%)
Azithromycin	9 (39.13%)	8 (34.78%)	6 (26.09%)
Cefotaxim	4 (17.39%)	5 (21.74%)	14 (60.87%)
Cephalexin	5 (21.74%)	2 (8.7%)	16 (69.57%)
Ciprofloxacin	2 (8.7%)	7 (30.43%)	14 (60.87%)
Meropenem	4 (17.39%)	0 (0%)	19 (82.61%)
Methicillin	3 (13.04%)	6 (26.09%)	14 (60.87%)
Nitrofurantoin	20 (86.96%)	1 (4.35%)	2 (8.7%)
Ofloxacin	16 (69.57%)	6 (26.09%)	1 (4.35%)
Oxacillin	19 (82.61%)	3 (13.04%)	1 (4.35%)
Rifampin	13 (56.52%)	5 (21.74%)	5 (21.74%)
Trimethoprim	10 (43.48%)	2 (8.7%)	11 (47.83%)
p-value	0.0001 ****	0.001 ***	0.0001 ****

Discussion

Foot ulcers are common in diabetic patients. Its prevalence varies between 15% and 25% (Singh wt al., 2005). Infection of these ulcers is a frequent (40%-80%) complication representing a major cause of mortality and morbidity (Prompers et al., 2008). It is estimated to be the most common reason of lower-limb amputations (Moulik et al., 2003; Pemayun et al., 2015; Lin et al., 2020). The pathophysiology of diabetic foot infection is quite complex. The prevalence and severity are a consequence of host-related processes (e.g., immunopathy, neuropathy and arteriopathy) and pathogen-related factors (e.g., virulence, antibiotic-resistance and microbial organization) (Dunyach-Remy et al., 2016). *Staphylococcus aureus* is a prevalent pathogen responsible for a wide range of infections, from mild skin conditions to life-threatening systemic diseases (Pal et al., 2023).

The ability of *S. aureus* to cause diabetic foot infection is defined by numerous virulence factors among which secreted toxins play an important role (participation in colonization, persistence, evasion of the immune system and dissemination). These toxins include: the pore-forming toxins, the exfoliatins,the superantigen exotoxins and the epidermal cell differentiation

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inhibitors toxins. These cytolytic toxins can damage membranes of host cells leading to cell lysis. Hemolysins lyse redblood cells, while leukotoxins target white blood cells (Yoong and Torres, 2013; Otto, 2014; Shettigar and Murali, 2020; Ahmad-Mansour et al., 2021). In this study, 39.66% of diabetic foot ulcers were positively infected with *S. aureus* (Singh and Apte, 2014). Many diabetic foot infections are superficial at presentation. However, bacteria can spread to subcutaneous tissues, including tendons, joints, fascia, muscle and bone (Armstrong and Lipsky, 2004; Mendes and Neves, 2012). Diabetic foot infections were classified by their clinical severity, ranging from mild (~35% of cases, depending on site of presentation), through moderate (~30-60%), to severe (~5-25%), (Lipsky et al., 2016). It was proposed simple clinical criteria for classifying the infection of diabetic foot ulcer based on classical signs and symptoms of inflammation (Schaper, 2004; Lipsky et al., 2012a, b). This scheme helps to predict whether hospitalization would be required and the clinical outcome. Moreover, various factors have been suggested as markers of diabetic foot infection when classical signs are not obvious. These include the identification of friable or discolored granulation tissue, necrosis, fetid odor, non-purulent secretions, delay in healing despite otherwise adequate ulcer management and the discovery of unexplained hyperglycemia (Lipsky et al., 2016). Interestingly, neither toxic shock syndrome nor toxinogenic manifestations could be clearly diagnosed in diabetic foot infection (Dunyach-Remy et al., 2016; Rasigade et al., 2016). In Occidental countries, Gram-positive aerobic cocci are the main microorganisms responsible for diabetic foot infection with *S. aureus* the most commonly isolated bacteria, alone or in combination, in superficial or deep infection (David and Daum, 2017). In warmer countries, particularly in Asia and Africa, Gram-negative bacilli are more prevalent, and many cases of deep infections are polymicrobial. Also in this case, *S. aureus* is the main isolated bacteria, present in 30%–60% of cases (Dunyach-Remy et al., 2016; Lipsky et al., 2016).

Molecular findings of the current study found that 34.78% of *S. aureus* isolates were positively having the *mecA* gene. The rise of antibiotic-resistant strains, particularly MRSA, poses a significant challenge to healthcare systems worldwide (Guo et al., 2020). A key factor in the success of this opportunistic pathogen is its ability to adapt and evolve, often through the acquisition of genetic elements that confer antibiotic resistance (Sangappa and Thiagarajan, 2012; Alibayov et al., 2014a, b). One of the most notable examples of this is the *mecA* gene, which is responsible for methicillin resistance in *S. aureus*. The *mecA* gene is a mobile genetic element that encodes a modified penicillin-binding protein, which has a reduced affinity for beta-lactam antibiotics, rendering them ineffective (Ibraheim et al., 2023a, b). The acquisition of the *mecA* gene has facilitated the emergence of community-acquired and healthcare-associated MRSA strains, which are of significant concern due to their ability to spread rapidly and cause severe infections that are challenging to treat (Romero and de Souza, 2021). This result agreed with another study in Iran that showed all MRSA isolates from wound samples harboring *mecA* gene (Alkhafaji et al., 2019); while, another studies in Iran were showed 45.1% and 22.7% of isolates were methicillin resistant and the *mecA* gene was detected in these isolates, respectively (Ghasemian et al., 2015; Pournajaf et al., 2014). In India, Kali et al. (2014) obtained 90.1% positive *S. aureus* isolates to *mecA* gene. Alfatemi et al. (2014) reported that

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the prevalence of MRSA was 42.3%. In Kurdistan showed that 50.4% of isolates were carrying the *mecA* gene (Hussein et al., 2019). In Pakistan, Siddiqui et al. (2018) showed that the prevalence rate of positive *S. aureus* isolates to *mecA* was 35%.

Our findings showed that the positive *S. aureus* isolates were significantly sensitive to Nitrofurantoin (86.96%), Oxacillin (82.61%), Ofloxacin (69.57%), and Rifampin (56.52%); but highly resistant to Ampcillin (82.61%), Meropenem (82.61%), Amoxcillin (73.91%), Cephalexin (69.57%), Cefotaxim (60.87%), Ciprofloxacin (60.87%), and Methicillin (60.87%). In comparison to results of various studies in Iraq, AL-Khozai (2016) showed that *S. aureus* isolates have a high resistance to Amoxicillin (100%), and Ampicillin (100%), and Cefotaxime (72.7%) but not to Meropenem (20.0%). In Erbil, authors have been showed a high resistance (93.42%) to Methicillin and Cephalexin (53.95%), (Rafiq et al., 2017). In another study, *S. aureus* isolates were shown a high resistance level to Methicillin (100%), Ampcillin (98%), Amoxcillin (98%), Cephalexin (95%), Cefotaxim (91.7%), Azithromycin (77.09%) but high low resistance to Meropenem (14.59%), (Refaat et al., 2022). In Iran, Rahimi et al. (2009) showed that 78% of *S. aureus* isolates were resistant to Methicillin, 99% of them were resistant to Ampicillin, and 62% were resistant to Cefotaxime. Momtaz and Hafezi (2014) showed that 62.12% of *S. aureus* isolates were resistant to Azithromycin. The large difference in prevalence rates between different countries may reflect the fact that the infection control policy and other factors in this field differ. With increasing of MRSA colonization rate, there is greater risk in developing drug-resistant wound infections. Therefore, it is necessary to avoid infection as much as possible. Resistance to antibiotics is associated with an increased time of hospitalization, treatment costs and high mortality, including a need for alternative medications.

Conclusion

Diabetic foot ulcers are extremely vulnerable to bacterial infections that can result in lower limb amputations and even death. Though from a clinician's perspective, it is important to differentiate colonization from infection, it might prove cumbersome in diabetic foot ulcers due to the underlying effects of neuropathy and/or ischemia. The polymicrobial community in diabetic foot infection further contributes to synergistic interaction between wound pathogens and induces various virulence traits and modulates host immunity and overall wound deterioration. Prompt recognition of worsening ulcers using predictive molecular markers will hence considerably help in preventing lower limb amputations. Distribution of isolates into different clonal complexes allows comparison between colonizing and infecting strains as well as determining the origin and clonality of the strains infecting wound ulcers. Detection of specific virulence encoding genes along with clonality in different grades will help us in identifying *S. aureus* strains that could cause severe negative wound outcome in diabetic foot infection and also to avoid misuse of antibiotic therapy in uninfected wounds.

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