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Distribution and Molecular Characterization of Staphylococcus aureus Isolates from Outpatient Females with Acute Urinary Tract Infection

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ABSTRACT
Urinary tract infections (UTIs) are common and recurrent among women.Therole of Staphylococcus aureus (S. aureus) as uropathogen is rare and not well documented in young women. Hence, this study was designed for determiningprevalence of S. aureusamong young outpatient womenwith acute UTI and detecting its superantigens' genes, mecA-mediated methicillin resistance, and resistance to some antimicrobials.Out of 137 outpatient women (18-40 years old), only 7 cases (5.1%) were positive forS. aureus (4 from nonpregnant and3 from pregnant women). In addition, six (85.7%) of these isolates were associated with recurrent UTI (RUTI). Five (71.4%) of theseisolates were positive for one or more of classic enterotoxins' genes (sea-see), whereas none of them had tstor epidermolytic toxins (eta, etb, and etd). In order, see (57.1%)was the most prevalent followed by sea(42.8%), and seb(28.5%). Whereas, sec and sedwere not found in any isolate. In three cases these toxins' genes occurred in combination (sea+see; sea+seb+see; and seb+see). mecA genewas found in five isolates (71.4%).Also, 85.7% and 57.1% of the isolates wereresistantto penicillin and cefoxitin, respectively. Whereas, resistance to norfloxacin and trimethoprim was 28.5% each, and ciprofloxacin and gentamicin was 14.2% each. However,nitrofurantoin had complete activity against all of theseisolates.In conclusion, the distribution of uropathogenicS. aureus among pregnant and nonpregnant outpatient females was low but the riskof infection with this bacterium still seriousbecause of their high possession of genesof enterotoxinsand mecA and also their relationship with RUTI.

Keywords: UTI, RUTI, uropathogen, nitrofurantoin, outpatient

INTRODUCTION

Urinary tract infections (UTIs) are common, recurring bacterial infections that have a considerable impact on global healthcare systems[1]. All over the world, UTI is the most predominant infection in females with about 50% to 60% of them experienced at least one UTIDuring their lifetime with around 150 million visits annually[2]. Pregnant women are more likely infected with UTIs with uropathogenicbacteria than nonpregnant women [3]. Gram-negative and Gram-positive bacteria are involved in etiology of UTIs such as Escherichia coli, Klebsiella species, Proteus species, Pseudomonas species, and Staphylococcus species[2].

Staphylococcus aureus appears as coccoid cells arranged as grapelike clusters with positive reaction to Gram-stain, non-motile, non-spore former. It presents on the cutaneous and mucous membranes especially inside the upper respiratory tractas part of the human body normal flora[4]. Like other staphylococci, it produces catalase and reduces nitratebut does nothaveoxidase. Also, it is metabolically facultative anaerobe. Although, it is part of the body flora, it can behave as opportunistic pathogen[5].Different human infections can be caused by this bacterium, ranging from cutaneous and nasal infections to foodpoisoning, bacteremia and UTI. About 30% of humans are nasal carries of S. aureus which has the capacity to resist β-lactam antimicrobialssuch as penicillin and ampicillin. Furthermore, the use of vancomycinhas increased as a result of emergence ofmethicillin resistant S. aureus (MRSA) [6].Versatile sets of structures and products contribute to the pathogenicity of S. aureusincluding virulence genes, production ofenzymes, toxins, adhesin, and cell surface proteins that eases tissuebinding and biofilm formation [7].

Staphylococcus aureus is not a common uropathogen (causing just 0.2-4% of UTIs). Certain populations, such as elderly patients with urinary catheters or those with bacteremia, are vulnerable to infection with S. aureus[8]. In women, UTIs are more common because of their anatomy. Several other factors have also been shown to increase the risk of UTI in women: particularly sexual intercourse and the use of spermicide [9]. During

pregnancy, the microorganisms responsible for UTIs are the same as those responsible for UTIs in nonpregnant patients [10]. However, pregnant women are more likely to be infected with UTIs with uropathogenicbacteria than nonpregnant women [3]. The presence of a UTI has also been shown to increase the riskof preterm labor, preterm birth, pregnancy-induced hypertension, preeclampsia,ammonites and anemia [11]. Increasing S. aureus colonization of pregnant and non-pregnant females, healthy neonates,and hospitalized newborns in the critical care unit, is reported[12]. "The idea of S. aureus predominance in bothnon-pregnant and pregnant women is a complicated one that has yet to be understoodand proven. Many researchers have made various attempts in the past, and the huntis currently ongoing to extract appropriate facts to link the prevalence of S. aureusamong pregnant and non-pregnant women"[13].Hence, this study was suggested to investigate S. aureus participation in UTI-causation in pregnant and non-pregnant outpatient females in terms of spreadratio and the potential risk it poses to both the patients and their fetuses, namely possession of genes encoding superantigens (enterotoxins, toxic shock syndrome toxin-1, and epidermolytic toxins) and mecA-mediatedmethicillin resistance. Both phenotypic and genotypic procedures were followed to achieve these aims.

MATERIALS AND METHODS

- **Urine collection, Processing and Bacterial Culture**

For isolation of bacterial uropathogens, midstream urine samples were obtained from pregnant and non-pregnant women between July 2023 and January 2024, who visited "Al-Hajj Jalal Hospital for Gynecology and Obstetrics in Al-Numaniyah, as well as outpatient clinics in Wasit Province, Iraq". Sterile, screw-capped test tubes were used for collection of urine which was immediately cultured on MacConkey agar, blood agar (BAP), and mannitol salt agar (MSA) plates,subsequently these plates were incubated at 37°C overnight [14]. A count of $\geq 10^5$ CFU/mL, is indicative of bacterial causation of UTI [15].Also, each urine samplewas subjected to general urine examinationseeking microscopically for pus cells, red blood cells, casts, yeast cells, bacteria, and other abnormalities[16].

- **Identification of Staphylococcus aureus**

A. Biochemical Identification

Biochemical tests were performed on all isolates that grew on mannitol salt agar and blood agar. The preliminary identification of S. aureus was based on its growth on mannitol salt agar, Gram stain, and the results of various tests, including catalase, oxidase, and coagulase.

B. Molecular identification

DNA extraction

With minor adjustments, a boiling method described by Mutasher and Fleih (2019) was used for DNA extraction. One mL of sterile 1X TE buffer (pH 8.0) was used for suspending bacteria(3 loopfuls of 24-hr-old bacterial growth on tryptic soy agar)rather than using sterile D.W. After 20 min of heating at 85°C, the bacterial suspension was incubated on ice bath for 10 min. and after that centrifuged for 10 minutes at 10,000 rpm. Thereafter, the DNA-containing supernatant was separated into 100 µL portions and kept at -20ºC until it was required.

Polymerases Chain Reaction (PCR)

Staphylococcus aureus was identified genotypically by PCR detection of species-specific 16S rRNA gene segmentaccording to Martineau et al.,1998 [17] (Table 1).

Table 1: Primers' sequence and amplification conditions for detection of S. aureus[17].

Gene	Primer name	Primer sequence (5'-3')	Product size (bp)	Amplification conditions
16SrRNA	Sa442-1	AATCTTTGTCGGTAC ACGATATTCTTC ACG	108	1. Initial denaturation (94℃/ 5 min). 2. 30cycles of: - Denaturation (94°C/ 30s). - Annealing (55°C/ 30s). - Extension (72°C/30s). 3. Final extension 72°C/ 7 min.
	Sa442-2	CGTAATGAGATTTCA GTAGATAATACAAC A		

In addition, genes encoding S. aureussuperantigens[classic enterotoxins (sea-see); epidermolytic toxins (eta, etb, and etd), and toxic shock syndrome toxin-1 (tst)] and methicillin resistance gene (mecA) were all detected based on PCR protocols (Table 2). Agarose gel (2%) electrophoresis was employed for analysis of PCR products using a 100 bp DNA marker for size comparison.

Table 2: Polymerase chain reaction conditions for detection of S. aureus superantigens and methicillin resistance genes.

Gene	Primer name	Primer sequence (5'-3')	Product size (bp)	Reference and Amplification conditions
sea	GSEAR-1	GGTTATCAATGTGCGGGTGG	102	**Mehrotra et al., 2000 (10):** 1. Initial denaturation (94℃/ 5 min). 2. 35 cycles of: - Denaturation (94°C/ 2 min). - Annealing (57°C/ 2 min). - Extension (72℃/1 min). 3. Final extension 72℃/ 7 min.
	GSEAR-2	CGGCACTTTTTTCTCTTCGG		
seb	GSEBR-1	GTATGGTGGTGTAACTGAGC	164	
	GSEBR-2	CCAAATAGTGACGAGTTAGG		
sec	GSECR-1	AGATGAAGTAGTTGATGTGTATGG	451	
	GSECR-2	CACACTTTTAGAATCAACCG		
sed	GSEDR-1	CCAATAATAGGAGAAAATAAAAG	278	
	GSEDR-2	ATTGGTATTTTTTTTCGTTC		
see	GSEER-1	AGGTTTTTTCACAGGTCATCC	209	
	GSEER-2	CTTTTTTTTCTTCGGTCAATC		
tst	GTSSTR-1	ACCCCTGTTCCCTTATCATC	326	
	GTSSTR-2	TTTTCAGTATTTGTAACGCC		
mecA	GMECAR-1	ACTGCTATCCACCCTCAAAC	163	
	GMECAR-2	CTGGTGAAGTTGTAATCTGG		
eta	ETA1	CTATTTACTGTAGGAGCTAG	741	**Růžičkováet al.,2005 (11):** 1. Initial denaturation (94℃/ 4 min). 2. 25 cycles of: - Denaturation (94°C/ 30s). - Annealing (54°C/ 30s). - Extension (72℃/90s). 3. Final extension 72℃/ 7 min
	ETA2	ATTTATTTGATGCTCTCTAT		
etb	ETB1	ACGGCTATATACATTCAATT	200	
	ETB2	TCCATCGATAATATACCTAA		
etd	ETD1	AACTATCATGTATCAAGG	376	
	ETD2	CAGAATTTCCCGACTCAG		

Antimicrobial Susceptibility Testing

Standard disk diffusion method was employed for antimicrobial resistanceby following CLSI guidelines[18]. Antibiotic discs (Liofilchem, Italy) included: gentamicin(CN: 10 µg), cefoxitin(FOX: 30µg), penicillin(P: 10U),ciprofloxacin(CIP: 30µg), norfloxacin (NOR:10µg), nitrofurantoin(F: 300µg), and trimethoprim(TM: 5µg).

RESULTS AND DISCUSSION

Isolation and Molecular Identification of S.aureus

In this study young female outpatients (18-40 years) clinically diagnosed with acute UTI were enrolled. A total of 318 urine specimens were collected, with 137 (43.0%) testing positive for bacterial growth (≥10 WBCs/µL and bacterial count $\geq 10^5$ CFU/mL). These results align with findings from previous Iraqi studies such as [19] who reported a 35.9% positivity rate in Wasit Province.

These 137 positive urine cultures were distributed as 73 (53.2%) from pregnant and 64 (46.7%) from nonpregnant patients. Furthermore, 54.8% and 45.1% of these cases were RUTI in both pregnant and nonpregnant women, respectively. Biochemically, 81.0% (111/137) of the isolates were suspected to be staphylococci as they grew on MSA, occurred as Gram-positive cocci arranged in irregular clusters and were positive for catalase and negative for oxidase. Also, 29.9% (33/111) of these isolates were coagulase positive.

All of these 111 staphylococcal isolates were surveyed by PCR for S. aureus species(Fig. 1), where only 7 (5.1%)S. aureusisolates were found.Four isolates were obtained from non-pregnant women, while three were from pregnant women. Among these patients, six had RUTI, while one experienced FUTI (nonpregnant). Others [20] reported 14.2% isolation rate of S. aureus from pregnant women. While, in Keffi isolated S. aureus from pregnant and nonpregnant patients at relatively high rates (33.3% and 22.3%, respectively)[13].

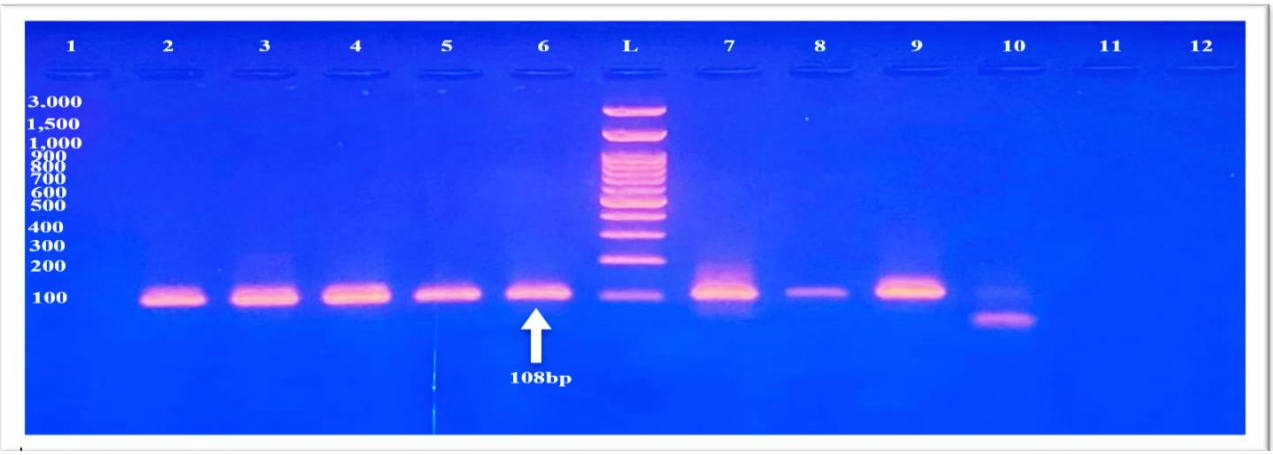

Fig. (1): Detection of PCR product (108bp) for S. aureus-specific 16S rRNA using agarose gel electrophoresis. Lane (L): DNA Ladder (100bp); lanes (2,3,4,5,6,8, and 9): positive results for 16SrRNA; lane (7): positive control; lane (10): negative control; and lanes 11 and 12: unloaded.

Distribution of Virulence factors' genes

Occurrence of genes encoding superantigens of S. aureuswas as in Table 3, where see (57.1%) was the most common among enterotoxins' genes followed by sea (42.8%),and seb(28.5%). However, no isolate contained sec or sed.Furthermore, tst, eta, etb, and etd were not found in any isolate. The link between these enterotoxin genes and UTIs is poorly understood as S. aureus can cause UTIs and may carry enterotoxin genes, UTIs can also be produced by S. aureus strains lacking these genes. The role of enterotoxins in the pathogenesis of S. aureus-associated UTIs requires more research. These toxins may induce the release of specific cytokines, which could suppress the effectiveness of the immune response. This suppression may enable the persistence of S. aureus in the urogenital tract, leading to tissue inflammation or contributing to the chronicity of the infection[21].Absence of other superantigens' genes from among this study included isolates could be related to the study's relatively small number of isolates and the low incidence of these toxins in S. aureus strains from various sources [22]. The low incidence of the tstgene suggests that it may not play an important role in UTI pathogenesis.

Table 3: Occurrence of genes encoding S. aureussuperantigens among isolates from outpatient females with acute UTI.

Superantigen's gene		S. aureus isolates (n=7)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
sea-see		sea see	sea seb see	-	sea	seb see	-	see
tst		-	-	-	-	-	-	-
eta, etb, and etd		-	-	-	-	-	-	-
Patient	Pregnant women	-	-	+	+	-	+	-
	Nonpregnant women	+	+	-	-	+	-	+
Infection	FUTI	-	-	-	-	+	-	-
	RUTI	+	+	+	+	-	+	+

+: present; -: absent; FUTI: first episode UTI; RUTI: recurrent UTI.

mecA-mediated Methicillin Resistance

Phenotypic and genotypic methods were employed to determine mecA-mediated methicillin resistance. All cefoxitin resistant isolates (4/7:57.1%) and one sensitive isolate (1/7: 14.2%) were mecA positive (5/7: 71.4%). Moreover, four (57.1%) of mecA-positive isolates were from RUTI versus only one (14.2%) from FUTI (Table 4).Prevalence of mecAat rate of 73.3%, was reported [23]. Whereas, in reports from Baghdad mecAprevalence

rates ranged from 29.8% to 68.4% [24, 25].Penicillin-binding protein 2a (PBP2a), the product of mecA gene, has a weak interaction with β-lactam antibiotics, rendering S. aureus resistant to methicillin. Therefore, β-lactam antibiotics are no longer effective in treating these patients.This resistance mechanism, mediated by PBP2a, contributes to the enhanced virulence of S. aureus by limiting the efficacy of β-lactam antibiotics [26, 27].

Table 4: Distribution of mecA-mediated methicillin resistance among S. aureus isolates from outpatient females with acute UTI.

Criteria		S. aureus isolates (n=7)						
		1	**2**	**3**	**4**	**5**	**6**	**7**
Cefoxitin resistance		S	R	S	R	R	S	R
mecA gene		+	+	-	+	+	-	+
Patient	Pregnant women	-	-	+	+	-	+	-
	Nonpregnant women	+	+	-	-	+	-	+
Infection	FUTI	-	-	-	-	+	-	-
	RUTI	+	+	+	+	-	+	+

+: present; -: absent; first episode UTI; RUTI: recurrent UTI.

Antibiotic Susceptibility of Staphylococcus aureus

Staphylococcus aureus isolates obtained in this study, exhibited varying levels of susceptibility and resistance to tested antimicrobials (Fig. 2).

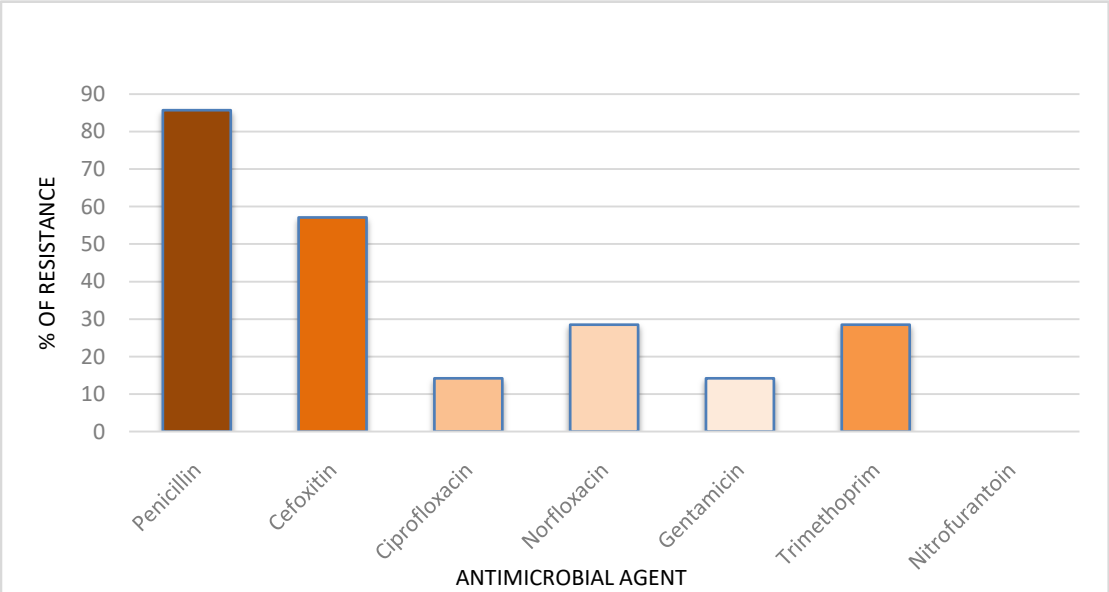

Fig. 2: Antimicrobial resistance of *S. aureus* isolates.

Antibiotic resistance in S. aureus, especially among uropathogenic strains, is a growing global issue. In this research,seven antimicrobials were assessedagainst S. aureus isolates. All ofthese isolates were susceptible to nitrofurantoin. This antimicrobial effectiveness is attributed to its high urine concentration[28]. While, 28.5%were resistant to trimethoprim which works by inhibiting bacterial folic acid synthesis[29]. These findings are similar to those reported by[30], where 1.4% and 33.0%of their isolates were resistant to nitrofurantoin and trimethoprim, respectively.

There was also significant resistance to β-lactam antibiotics, particularly penicillin and cefoxitin, with 85.7% of isolates resistant to penicillin and 57.1% resistant to cefoxitin. This mirrors global patterns of β-lactam resistance, largely driven by the misuse of antibiotics and the rise of β-lactamases such as ESBLs[31]. The excessive use of antibiotics in healthcare and agriculture, alongside the slow development of new antibiotics, exacerbates this issue[32].

Additional resistance was noted against antibiotics like norfloxacin (28.5%). The findings align with[33], who found that 47.5% of isolates showed resistance to norfloxacin, with 14.2% resistance to both gentamicin and ciprofloxacin. Similarly,[34] reported that 13.8% of isolates were resistant to gentamicin (CN) and ciprofloxacin (CIP).Gentamicin targets protein synthesis, while fluoroquinolones like norfloxacin and ciprofloxacin inhibit DNA replication[35]. Though fluoroquinolones are not typically first-line treatments, they remain a part of

empirical therapy for UTIs.The study underscores the importance of considering regional variations in antibiotic resistance patterns to optimize empirical treatment decisions based on local susceptibility data.

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