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Virulence and Resistance Characterization of Staphylococci-associated Urinary Tract Infection in Pregnant Women in Lagos, Nigeria

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Abstract

Background: Urinary Tract Infection (UTI) is the most prevalent bacterial infection in developing countries. UTI is sometimes asymptomatic. The occurrence of UTI in pregnancy can result in complications such as premature or low-birth-weight babies. Escherichia coli is the most common

cause of UTI followed by Staphylococci species. However, E.coli associated UTI has been well studied while there is paucity of data on the virulence and resistance of Staphylococci-associated UTIs in women. This study aimed to characterize the virulence and resistance genes in Staphylococci species isolated from the urine of pregnant women.

Methods: This was a cross-sectional study involving pregnant women attending ante-natal clinic in Lagos State, Nigeria. Clean catch midstream urine specimens were collected from the women and cultured on appropriate agar. Bacteria were identified using both biochemical and molecular methods and evaluated for resistance to antibiotics. Polymerase chain reaction was used to detect the mecA and blaZ resistance genes and the PVL and TSST virulence genes.

Results: Staphylococci species were isolated from 21 (26.25%) of the 80 urine samples of which 7 (29%) were Staphylococcus aureus and 17 (71%) coagulase-negative staphylococcus (CoNS). S. epidermidis and S. saprophyticus were the most commonly isolated CoNS. All the isolates tested were resistant to cotrimoxazole, cloxacillin, erythromycin, ampicillin, amoxicillin-clavulanate, and tetracycline. Both blaZ and the mecA genes were detected in the isolates that were phenotypically susceptible to methicillin and cefoxitin. There was an occurrence of the mecA, blaZ and PVL genes in S. saprophyticus in patients who were co-infected with S. aureus.

Conclusion: UTI caused by Staphylococci is common among pregnant women. There is a need to consider staphylococcal associated UTI screening in both symptomatic and asymptomatic cases of UTI in pregnant women.

Keywords: Urinary Tract Infection, Pregnant Women, Staphylococcus Species, Multi-Drug Resistance

Introduction

Urinary Tract Infection (UTI) is the invasion and subsequent propagation of microorganisms in the urinary tract and it is one of the most prevalent bacterial infections, especially in developing countries, with a high morbidity rate and financial costs (Foxman, 2014; Vasudevan, 2014). UTI is the most common hospital-acquired infection, accounting for up to 35% of nosocomial infections, and it is the second leading cause of bacteraemia in hospitalized patients (Agaba et al., 2017).

UTI is associated with several complications for both the fetus and the mother, including pyelonephritis, preterm birth, low birth weight, and increased perinatal mortality (Ghouri et al., 2019; Vasudevan, 2014). Some women who develop asymptomatic bacteriuria during pregnancy are more likely to have premature or low-birth-weight babies.

The causative agents for UTI are many and differ from location to location and their patterns of susceptibility and resistance often vary. As with *S. aureus*, a high prevalence of coagulase-negative staphylococci (CoNS) causing UTI has also been reported (França et al., 2021; Hashmi et al., 2016; Higashide et al., 2008; Zia Sheikholeslami & Hassanshahi, 2010). The Frequent and many reports about highly resistant staphylococcal-associated UTIs makes it imperative for this study on asymptomatic staphylococci-associated UTI in pregnant women taking into account the pathogenic roles, virulence and resistance genes. This study, therefore, aims to characterize the virulence and resistance genes in *Staphylococci* species isolated from the urine of pregnant women in Lagos, Nigeria.

Materials and Methods

Study design and Area

This was a cross-sectional study carried out in Lagos State, Nigeria. Samples were collected from pregnant women attending the ante-natal clinic of the Lagos University Teaching Hospital (LUTH) in Lagos State, Nigeria. Urine samples of pregnant women with or without symptoms of UTI, at any stage of pregnancy, and having significant bacteriuria were included in this study. Urine samples of pregnant women that had taken antibiotics within the last two weeks before sample collection were excluded from the study.

Ethical Considerations

Ethical approval to carry out this study was obtained from the Research Grants and Experimentation Ethics Committee, College of Medicine, University of Lagos. Participant's consent was obtained before sample collection started. Privacy, confidentiality and personal information of all patients were observed and protected.

Specimen collection, Isolation and Phenotypic Identification of Staphylococcus Species

A total of 80 clean-catch midstream urine specimens were collected into sterile containers. The samples were homogenized, 50 µl of the urine was deposited on the surface of a microscope slide and allowed to dry at ambient temperature. The smears were fixed by passing through the flame of a Bunsen Burner and stained using the gram staining technique. Microscopic examination of the smears was initially done at 20x magnification to ensure that the samples were evenly distributed, and then at 100x magnification. A positive smear, indicative of significant bacteriuria according to the criteria of Washington et al. (1981), was defined as on that had ≥2 per distributed uniformly per oil immersion field after the examination of at least 20 fields (Cardoso et al., 1998; Washington et al., 1981).

The urine samples were cultured onto blood agar and mannitol salt agar (Oxoid, UK), then incubated aerobically for 24 hours at 37°C. Colonies were examined after overnight incubation and sub-cultured to obtain a pure culture. Identification of bacteria was made according to colony morphology, Gram stain and biochemical tests. The isolates were initially identified by gram stain, susceptibility to 0.04U bacitracin (SigmaAldrich), catalase, and tube coagulase tests, and categorized as *S. aureus* and CoNS as described by Cunha et al., 2004. Then other biochemical tests were done to further identify the isolates. These include urease production, nitrate production, resistance to novobiocin, and sugar fermentation tests using the sugars sucrose, trehalose, xylose, a-lactose, fructose, maltose, and mannose (Cunha et al., 2004).

Antibiotic susceptibility patterns

Pure isolates were subjected to antimicrobial susceptibility testing using the modified disk diffusion Kirby-Bauer method as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2020). A freshly prepared broth of 0.5 McFarland concentration of the isolate was cultured inoculated onto Mueller Hinton Agar after which antibiotic discs were applied. The antibiotic discs tested included gentamicin 10µg, ofloxacin 5µg, streptomycin 10µg, erythromycin 5µg, amoxicillin-clavulanate 30µg, cloxacillin 5µg, cotrimoxazole 25µg, tetracycline 25µg, imipenem 10µg, cefoxitin 30µg, methicillin 5µg, and vancomycin 30µg were placed on Müller-Hinton agar and incubated aerobically at 37°C for 24 hours (Oxoid, UK). The zones of inhibition were measured and interpreted as sensitive or resistant using the CLSI criteria.

DNA extraction and Polymerase Chain reaction

DNA was extracted using the boiling – centrifugation method according to Soumet et al., 1994. Polymerase chain reaction (PCR) was carried out to amplify the resistance and virulence gene loci. The *16s rRNA* gene and the virulence genes were detected using a standard single PCR while the resistance genes and the *nuc* genes were detected using duplex PCR (*mecA* and *nuc* combination and *blaZ* and *nuc* combination). PCR was performed in a 20µl reaction mixture containing 1X Master mix (Solis Biodyne) (the master mix contained 1X PCR Buffer, 1.5 mM $MgCl_2$, 200µM of each deoxynucleoside triphosphates, and 2U Taq DNA Polymerase), 20pMol of each primer (BIOMERS, Germany), 2µl of the extracted DNA, and nuclease-free water was used to make up the reaction mixture volume.

Thermal cycling was conducted in a Peltier PTC 200 thermal cycling for an initial denaturation of 95°C for 5 minutes followed by 35 amplification cycles of 30 seconds at 95°C; 45 seconds at the annealing temperature and 1 minute at 72°C, followed by a final extension step of 10 minutes at 72°C. The

primer sequences and the annealing temperatures employed to detect the virulence and resistance genes are presented in Table 1.

The amplification product was separated by electrophoresis on a 1.5% agarose gel at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were stained with ethidium bromide and visualized. 100bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker.

Table 1. Primer sequences, gene locus and Amplicon sizes

Primer	Primer sequence (5′-3′)	Specific gene loci tested	Annealing Temperature (◦C)	Product size (bp)	Reference
16SrRNA-F	5' GTA GGT GGC AAG CGT TAT CC 3'	16S ribosomal ribonucleic acid (*Staphylococcus* genus-specific)	55	228	(Bakheet et al., 2018)
16SrRNA-R	5' CGC ACA TCA GCG TCA G 3'				
nuc-F	5'-GCG ATT GAT GGT GAT ACG GTT-3'	Thermonuclease gene (*S. aureus* specific)	55	279	(Brakstad et al., 1992)
nuc-R	5' AGC CAA GCC TTG ACG AAC TAA AGC 3'				
mecA-F	5' GTG AAG ATA TAC CAA GTG ATT 3'	*mecA* gene	55	147	(Zhang et al., 2005)
mecA-R	5' ATG CGC TAT AGA TTG AAA GGA T 3'				
blaZ-F	5' AAG AGA TTT GCC TAT GCT TC 3'	*blaZ* gene	52	517	(Bakheet et al., 2018)
blaZ-R	5' GCT TGA CCA CTT TTA TCA GC 3'				
Luk-PV-1	5' ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A 3'	Panton Valentine Leucocidin Toxin	55	433	(Lina et al., 1999)
Luk-PV-2	5' GCA TCA AST GTA TTG GAT AGC AAA AGC 3'				
TSST-F	5' GCT TGC GAC AAC TGC TAC AG 3'	Toxic shock syndrome toxin-1	50	559	(Wang et al., 2016)
TSST-R	5' TGG ATT CGT CAT TCA TTC TTA T 3'				

Results

Isolation and identification of *Staphylococcus* species

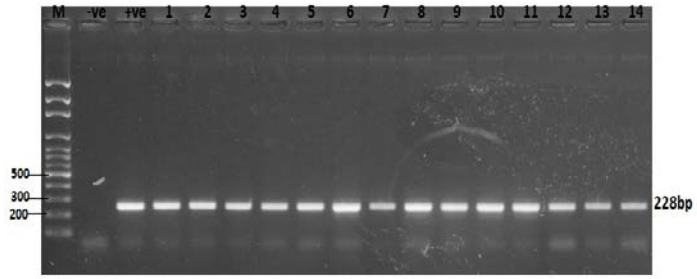
Staphylococcus species were isolated from 21 (26.25%) of the 80 urine samples collected from pregnant women. Of the 21 women, 18 had a single

infection while 3 were infected with a co-infection of *Staphylococcus aureus* and *Staphylococcus saprophyticus,* making a total of 24 Staphylococcus species isolated. These included *S. aureus* 7 (29%) and CoNS 17 (71%). The CoNS were further identified as *Staphylococcus epidermidis* 41.1%, *Staphylococcus saprophyticus* 35.3%, *Staphylococcus haemolyticus* 11.80%, and other staphylococci 11.8%.

Molecular Identification of the Isolates

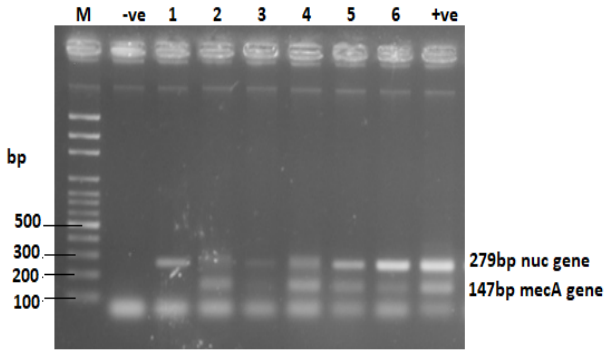
All the 24 isolates showed amplification and were, therefore, confirmed as Staphylococcus species using the Staphylococcus genus-specific 16SrRNA (Figure 1). The *nuc* gene was amplified to further identify the *S. aureus* isolates. Only the 7 isolates presumptively identified as *S. aureus* showed amplification for the nuc gene (Figure 2).

Figure 1. PCR detection of the 16SrRNA (228 bp) gene of Staphylococcus species



Key: M = 100bp DNA Ladder (Solis Biodyne); -ve = negative control; +ve = positive control (NCTC 12493); Lanes 1-14 = Staphylococcal isolates

Figure 2. Multiplex-PCR assay for the detection of *mecA* and *nuc* gene



Key: M = 100bp DNA Ladder (Solis Biodyne); -ve = negative control; +ve = positive control (NCTC 12493); Lane 1 = m*ec*A negative *S. aureus* sample; Lanes 2 - 6 = *mecA* positive S. aureus samples

Antimicrobial Susceptibility Test Result

The result of the antimicrobial susceptibility testing is summarized in Table 2 with all the isolates being multiple resistant to four or more antibiotics.

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All the isolates of both *S. aureus* and CoNS were resistant to cotrimoxazole, cloxacillin, erythromycin, ampicillin, amoxicillin-clavulanate, and tetracycline. All the *S. aureus* isolated were susceptible to vancomycin and imipenem, while cefoxitin, methicillin, vancomycin, and imipenem were the most effective antibiotics in the CoNS with all the CoNS isolates being susceptible to these antibiotics. About four different patterns of resistance were seen in the *S. aureus* isolates with all patterns containing the cotrimoxazole-cloxacillin-erythromycin-ampicillin-Amoxicillin/clavulanate-tetracyclin-chloramphenicol resistant pattern (Table 3). Seven different patterns of resistance were seen in the CoNS isolates with the cotrimoxazole-cloxacillin-erythromycin-ampicillin-amoxicillin/clavulanate-tetracyclin resistance seen in all the seven different patterns (Table 3).

Table 2. Distribution of Antimicrobial Resistance of *Staphylococcus aureus* and Coagulase-Negative Staphylococci from Urinary Tract Infections

Antibiotics	*Staphylococcus aureus* (n =7)		Coagulase Negative Staph (n = 17)	
	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)
Cotrimoxazole	0	100	0	100
Cloxacillin	0	100	0	100
Erythromycin	0	100	0	100
Ampicillin	0	100	0	100
Gentamicin	28.57	71.43	17.65	82.35
Amoxicillin-Clavulanate	0	100	0	100
Streptomycin	28.57	71.43	35.29	64.71
Tetracycline	0	100	0	100
Chloramphenicol	0	100	23.52	76.48
Ofloxacin	57.1	42.9	64.71	35.29
Cefoxitin	85.7	14.3	100	0
Methicillin	85.7	14.3	100	0
Vancomycin	100	0	100	0
Imipenem	100	0	100	0

Table 3. Phenotypic Antimicrobial Resistance Patterns of *Staphylococcus aureus* and CoNS from Urinary Tract Infections

Staphylococcus aureus (n = 7)		
Resistance phenotypes	Number of strains	Number of antibiotic classes
COT, CXC, ERY, AMP, AMC, TET, CHL, GEN	2	6
COT, CXC, ERY, AMP, AMC, TET, CHL, STR	2	6
COT, CXC, ERY, AMP, AMC, TET, CHL, GEN, STR, OFL	2	7
COT, CXC, ERY, AMP, AMC, TET, CHL, GEN, STR, OFL, CFX, MET	1	7
Coagulase Negative Staphylococci (n = 17)		

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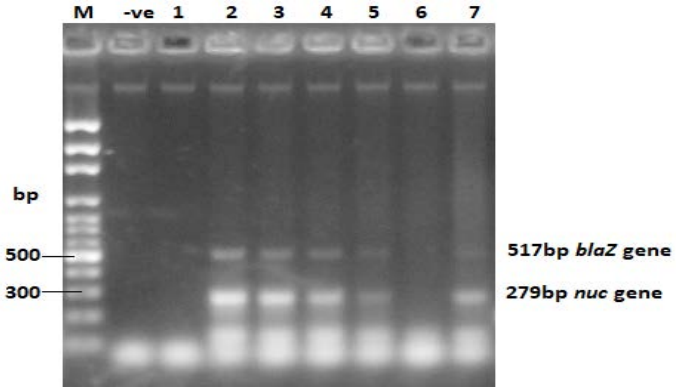
Resistance phenotypes	Number of strains	Number of antibiotic classes
COT, CXC, ERY, AMP, AMC, TET,	2	4
COT, CXC, ERY, AMP, AMC, TET, GEN, STR	1	5
COT, CXC, ERY, AMP, AMC, TET, STR, OFL	1	5
COT, CXC, ERY, AMP, AMC, TET, CHL, GEN,	1	6
COT, CXC, ERY, AMP, AMC, TET, CHL, GEN, STR	7	6
COT, CXC, ERY, AMP, AMC, TET, CHL, GEN, OFL	3	7
COT, CXC, ERY, AMP, AMC, TET, CHL, GEN, STR, OFL	2	7

Key: Cot = Cotrimoxazole,; CXC = Cloxacillin; ERY = Erythromycin; AMP = Ampicillin; GEN = Gentamicin; AMC = Amoxicillin-Claulanate; STR = Streptomycin; TET = Tetracycline; CHL = Chloramphenicol; OFL = Ofloxacin; CFX = Cefoxitin; MET = Methicillin

Detection of the resistant genes mecA and blaZ

mecA and *blaZ* gene detection was carried out on all the isolates irrespective of their phenotypic resistance to penicillin (β-lactam) antibiotics. The *mecA* and *blaZ* amplification products are as presented in Figure 2 and Figure 3. The *mecA* gene was detected in 57.1% (4/7) of the *S. aureus* isolates, even in isolates that were phenotypically susceptible to methicillin and Cefoxitin. Similarly, the *blaZ* gene was also detected in 57.1% (4/7) of the *S. aureus* isolates. The *mecA* gene was also detected in 41.2% (7/17) and the *blaZ* 52.9% (9/14) of the CoNS isolates. The resistant gene patterns of the isolates are presented in Table 4.

Figure 3. Multiplex-PCR assay for the detection of *blaZ* and *nuc* gene



Key: M = 100bp DNA Ladder (Solis Biodyne); -ve = negative control; Lane 1 and Lane 6= *blaZ* negative *CoNS* sample; Lanes 2 – 5 = *blaZ* positive S. aureus samples; Lane 7 = positive control (NCTC 12493)

Table 4. Resistance and Virulence Gene Patterns of Staphylococcus aureus and CoNS from Urinary Tract Infections

Resistance/Virulence Genes	SA (n = 7)	CoNS (n=17)
mecA alone	0	0

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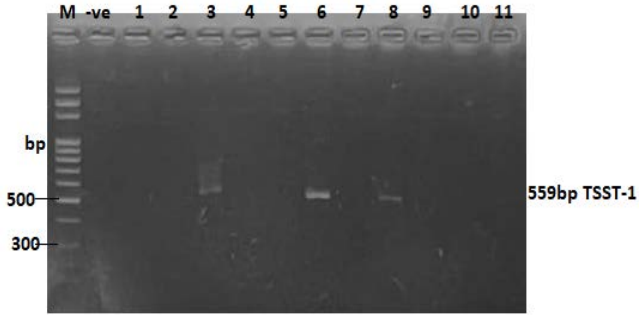
blaZ alone	0	2 (11.8%)
mecA, blaZ	2 (28.6%)	6 (35.3%)
mecA, blaZ, TSST	2 (28.6%)	1 (5.9%)

PVL and TSST Virulent Gene Detection

None of the samples showed amplification for the PVL gene. However, 28.6% (2/7) and 5.9% (1/17) of the *S. aureus* and CoNS isolates respectively showed amplification for the TSST-1 (Figure 4) gene. The virulence and resistance gene patterns of the isolates are presented in Table 4.

Figure 4. Multiplex-PCR assay for the detection of *blaZ* and *nuc* gene



Key: M = 100bp DNA Ladder (Solis Biodyne); -ve = negative control; Lane 1 – 7 = *S. aureus* strains and Lane 8 – 11 = CNS strains

Discussion

UTIs typically manifest as asymptomatic and symptomatic bacteriuria with a prevalence of 15% in pregnant women. Though the treatment of asymptomatic bacteriuria in UTI may not be necessary for healthy adults and non-pregnant women, it is, however, important to screen and treat asymptomatic bacteriuria in pregnant women (Alemu et al., 2012). This is due to complications such as pyelonephritis, preterm birth, low birth weight, and increased perinatal mortality, which UTI can cause in pregnant women. Hence, the treatment of UTI is vital in preventing maternal and neonatal complications (Alemu et al., 2012; Kalinderi et al., 2018).

The majority of pregnant women with bacteriuria were asymptomatic in this study. The prevalence of staphylococci-associated UTI was 26.25%, with the prevalence of *S. aureus* and CoNS being 8.8% and 21.25% of the overall total samples collected respectively, and 29% and 71% of staphylococcal cultures respectively. Though most studies originating from Nigeria on the prevalence of UTI in pregnant women have reported lower rates of CoNS in UTI associated with pregnant women (Okonko et al., 2010; Oladeinde et al., 2015; Simon-Oke et al., 2019), this study reports a higher prevalence of CoNS in staphylococcal associated UTI in pregnant women.

Staphylococcus epidermidis and *Staphylococcus saprophyticus* were the most predominant CoNS isolated from UTIs in pregnant women as seen in this study. This corroborates the study by Hashmi et al. (2016) which also reported *S. saprophyticus* and *S. epidermidis* as the most common CoNS causing staphylococcal associated UTI in women. *S. saprophyticus* has been reported as one of the most common causes of community-acquired urinary tract infections, second only to *Escherichia coli*, and causing about 42% of infections in sexually active women (Hur et al., 2016). The presence of *S. epidermidis* and *S. saprophyticus* in UTIs in pregnant women should not be overlooked. *S. epidermidis*, though considered nosocomial and benign, have been implicated in causing obstetrical complications and infections in neonates (Beksac et al., 2019; Upadhyayula et al., 2012). *S. saprophyticus* has also been implicated as a common culprit in polymicrobial UTIs which are typically seen in the immunocompromised. In this study, subjects that had co-infection of multiple staphylococcal species infections had *S. saprophyticus* as one of the co-infection. This further adds credence to the association of *S. saprophyticus* in polymicrobial UTIs.

All the staphylococcal isolates seen in this study showed resistance to cotrimoxazole, cloxacillin, erythromycin, ampicillin, amoxicillin-clavulanate, and tetracycline, with the most effective antibiotics being vancomycin and imipenem. Apart from being susceptible to cloxacillin and imipenem, the CoNS were also susceptible to vancomycin and imipenem. Hashmi et al. (2016) also reported a 100% susceptibility to Vancomycin in CoNS. In this study, resistance was recorded for almost all the classes of antibiotics apart from the glycopeptides and carbapenems. Resistance of Staphylococcus species to penicillin and the β-lactams is a worldwide problem and the subsequent high levels of resistance to tetracycline, aminoglycosides and to some extent macrolides as seen in this study makes it increasingly difficult for clinicians to make safe antibiotic choices for the treatment of UTIs in pregnancy (Alemu et al., 2012; Mohammad et al., 2002). Easy access and the indiscriminate use of antibiotics such as ampicillin, tetracycline, and co-trimoxazole in Nigeria may influence the increasing resistance seen in these organisms.

Methicillin resistance and the detection of the *mecA* gene in CoNS isolated from UTIs, specifically from *S. Saprophyticus* have been reported (Hashmi et al., 2016). The detection of *mecA* and/or *blaZ* even in isolates that were phenotypically susceptible to methicillin and cefoxitin may imply a further increase in resistance to these antibiotics in the future. All the *mecA* positive isolates were also *blaZ* positive. This can be attributed to the fact that methicillin resistance can also occur as a result of the inactivation of this antibiotic due to an increase in the production of beta-lactamase that is coded by the *blaZ* gene (Soares et al., 2012). Though there are few reports of *mecA*-

mediated resistance in CoNS, this study detected both *mecA* and *blaZ* genes in *S. saprophyticus* and even in *S. epidermidis*. Other studies have also reported the presence of the mecA-mediated resistance in *S. saprophyticus* and *S. epidermidis* (Hanssen & Ericson Sollid, 2007; Hashmi et al., 2016). Both the *mecA* and *blaZ* genes were detected in all the *S. saprophyticus* found in co-infection with *S. aureus*. It is, therefore, possible that the *S. saprophyticus* may have acquired their resistance through horizontal gene transfer from MRSA. This could also imply that *S. saprophyticus* is not only a commensal bacteria but also infectious.

The toxic shock syndrome toxin, which is encoded by the *tst* gene, upsets the cells of the immune system and stimulates the release of cytokines and other non-specific T cell proliferation resulting in toxic shock syndrome which is potentially fatal in humans (Hakimi Alni et al., 2018). In this study, the *tst* gene was detected in two *S. aureus* strains of which one of them was co-infected with *S. Saprophyticus*. This again supports the possibility of horizontal gene transfer from MRSA.

Conclusions

This study shows that both *S. aureus* and CoNS with a prevalence of (26.25%) are uropathogens whose treatment should be considered in pregnancy. The high level of resistance seen in these study means that multi-drug resistance is common in staphylococcal isolates from UTIs, and there is, therefore, a need to consider staphylococcal associated UTI screening in both symptomatic and asymptomatic cases of UTI in pregnant women to alleviate the consequences of asymptomatic UTI and multi-drug resistant staphylococcal infection in pregnancy.

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