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Evaluation of antibiotic resistance pattern in *Staphylococcus saprophyticus* isolated from patients with urinary tract infection using real-time PCR

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ABSTRACT

Staphylococcus saprophyticus is an important agent of urinary tract infections (UTIs), especially among young women. The aim of this study was to identify *S. saprophyticus* as a common cause of urinary tract infections and determine their antibiotic susceptibility. In this study, 51235 clinical samples were collected from therapeutic centers of Mazandaran. *S. saprophyticus* was confirmed by the real-time PCR technique through *rrs* gene and their antibiotic resistance pattern determined by disc diffusion method. Of the 51235 cultivated samples, only 2101 (4.1%) cases had significant bacteriuria. In this study, the prevalence of *S. saprophyticus* was 2.47%. *Escherichia coli* was the most common bacterium isolated from patients with UTIs at the rate of 61.06%. *S. saprophyticus* showed the highest frequency of antibiotic resistance to erythromycin at the rate of 80% and ampicillin and cefotaxime were ranked with the frequency of 55 and 17.5%, respectively. The present study shows that bacterial resistance is a potential problem in Mazandaran province.

1. Introduction

Urinary tract infections caused by antibiotic resistant bacteria are the most common human bacterial infections among all age groups in developing countries and can have serious consequences if timely and appropriately not treated (Oyaert et al., 2018; Stamm & Norrby, 2001). Most urinary tract infections are caused by microorganisms that enter the bladder through the urethra. These infectious agents enter the urinary tract through direct spread via the fecal-perineal-urethral. UTI in women due to the proximity of the anus to the urethra and the shortness of urinary tract is more common than in men (Obirikorang et al., 2012). Across the global, UTI affect about 150 million people every year (Gupta et al., 2001; Moges et al., 2002). UTIs are the second most common infections in children and adults and the most important infections in infants (Yoon et al., 2011). This infection accounts for about 35% of hospital infections in the world (Okonko et al., 2010). The most common bacterial agents of urinary tract infections are bacteria of the Enterobacteriaceae family including *Escherichia coli*, *Klebsiella*, *Enterobacter* and *Proteus*, as well as gram-positive bacteria of *Staphylococcus* spp. (Al-Jiffri et al., 2011). In the past decade, *Staphylococcus* spp. has been identified as one

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of the organisms causing infection in the hospital and today, the resistance of this bacterium to penicillin is increasing due to the production of β-lactamase enzyme. This organism is also resistant to methicillin (Gad et al., 2010). In some reports, negative coagulase staphylococci were also considered as one of the common causes of urinary and nosocomial infections (Keim et al., 2011). Among the *Staphylococcus* spp., *S. saprophyticus* is the most common cause of urinary tract infection, and in some reports, 5-15% of urinary tract infections are caused by this bacterium (Ojo & Anibijuwon, 2010; Sibi et al., 2011). New studies show that antibiotic resistance of bacteria producing urinary tract infections is increasing in the world, and excessive and inappropriate use of antibiotics has increased the number of resistant strains (Eryılmaz et al., 2010; Manikandan et al., 2011). The present study was conducted to determine the antibiotic resistance pattern of *S. saprophyticus* strains isolated from urinary tract infection.

2. Materials and Methods

2.1. Sample collection and initial identification

In this study, 51235 clinical samples from patients with urinary tract infection were collected from therapeutic centers of Mazandaran for 8 months. The age range of the studied population was 25 to 45 years old. Urine samples were cultured in the Blood Agar and EMB Agar Media and then incubated at 37°C for 24 hours. After growing on blood Agar, the colonies were gram stained to determine gram-positive cocci. *S. saprophyticus* was identified by tests of catalase, coagulase, novobiocin and mannitol.

2.2. Identification of Staphylococcus saprophyticus by real time PCR

DNA purification kit (Jena Bioscience, Germany) was used to purify DNA. Real time PCR was used to identify *S. saprophyticus*. Specific primers were designed on the basis of the sequence of 1553 bp of the *S. saprophyticus* subsp. saprophyticus strain ATCC 15305 16S ribosomal RNA gene (GenBank accession no. NR-074999.2). Pick primer and Primer-BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi), from the National center for Biotechnology Information server, were used for primer design and to test their specificity. The specificity of the primers was evaluated using the blast tool against other sequences present in the GenBank database. These primers flank a 172-bp internal fragment of the 16S ribosomal RNA gene gene (Table1).

The final volume of the main mixture was considered to be 25 µl, which included 12.5 µl Sina SYBR Blue HS-qPCR Mix, 2x,1 µl forward primer,1 µl reverse primer, 3 µl DNA Sample and 7.5µl nuclease-free water. The model of Prime-Mid-Size Cycler from Techne Thermocycler Gradient System (ABI Step One Plus Real-Time PCR) was used. The thermal program used for the rrs gene was as a cycle of 95°C for 5 min, 32 repeat cycles of 95°C for 45 seconds, 58°C for one min, 72°C for 45 seconds, and the final cycle of 72°C for 6 min. A melt curve analysis was performed after the last cycle, to investigate the specificity of the amplicon and the presence of reaction artifacts such as primer dimer, using a temperature gradient from 60 to 100°C. *S. saprophyticus* reference strains PTCC No 1440 were used as positive control. The expected size of the amplicons was confirmed by 2% agarose gel containing ethidium bromide and DNA was observed using a UV doc device. The presence of a band with a size of 172 bp was considered as a positive result.

2.3. Determination of antibiotic resistance pattern by disc diffusion method

Bacterial suspension with half- MacFarland turbidity was spread on Muller Hinton Agar medium to check the resistance of *S. saprophytic*us strains to antibiotics. Then impregnated discs to antibiotics of ampicillin, erythromycin and cefotaxime were placed on it. Petri dishes were incubated at 35°C for 18 to 24 hours. Subsequently, the halo diameter of the inhibition of bacterial growth was recorded for any antibiotic based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) as sensitive, semi-sensitive or resistant (Patel, 2015). Accordingly, for ampicillin, the halo diameter is 17≤ mm (sensitive), 14-16 mm (semi-sensitive), ≤13mm (resistant), for erythromycin, 23≤ mm (sensitive), 14-22 mm (semi-sensitive), ≤ 13 mm (resistant), and finally

for cefotaxime, ≥ 23 mm (sensitive), 15-22 mm (semi-sensitive), ≤14 mm (resistant). The resulting data were analyzed by SPSS software version 16.

Table 1. Primers used in this research

gene	Primers	Sequences (5'->3')	Primer length	Tm	GC%	Amplicon size (bp)
rrs	Forward	AGGTAACGGCTTACCAAGG	19	56.74	52.63	172
	Reverse	TACGATCCGAAGACCTTCAT	19	57.91	52.62	

3. Results

Of the total 51235 clinical samples collected, significant bacteriuria was observed only in 2101(4.1%) cases that were identified by biochemical tests up to the genus (Table 2). Out of the total different organisms, *E. coli* was the most isolated organism accounting for 1283 (61.06 %). The second commonest isolate was Klebsiella spp. which accounted for 340 (16.18) followed by *Streptococcus pyogenes* (1.66%), *Staphylococcus aureus* (0.99%), *Enterococcus* spp. (0.66%) and *Pseudomonas* spp. 45 (2.14%).

The results obtained from biochemical tests showed that the prevalence of *S. saprophyticus* was 52 (2/47%) cases.

The existence of the rrs gene (16S rRNA gene) that confirms *S. saprophyticus* in all isolates was identified by the biochemical method. In figure 1, the real time PCR of rrs gene from the isolates of *S. saprophyticus* has been shown indicating the desired gene proliferation.

Table 2. Frequency percentage of identified bacteria in urine samples of patients with UTI

Bacteria family	Genus or Species	Frequency	Frequency percentage
		35	1.66
Streptococaceae	*Streptococcus pyogenes*	52	2.47
	Staphylococcus saprophyticus	21	0.99
Micrococaceae	*Staphylococcus aureus*		
		1283	61.06
	Escherichia coli	340	16.18
	Klebsiella	73	3.47
Enterobacteriaceae	*Citrobacter*	89	4.23
	Enterobacter	68	3.23
	Proteus		
		45	2.14
Pseudomonadaceae	*Pseudomonas*		
		14	0.66
Enterococcaceae	*Enterococcus*		
		81	3.8
Total	Others		
		2101	100

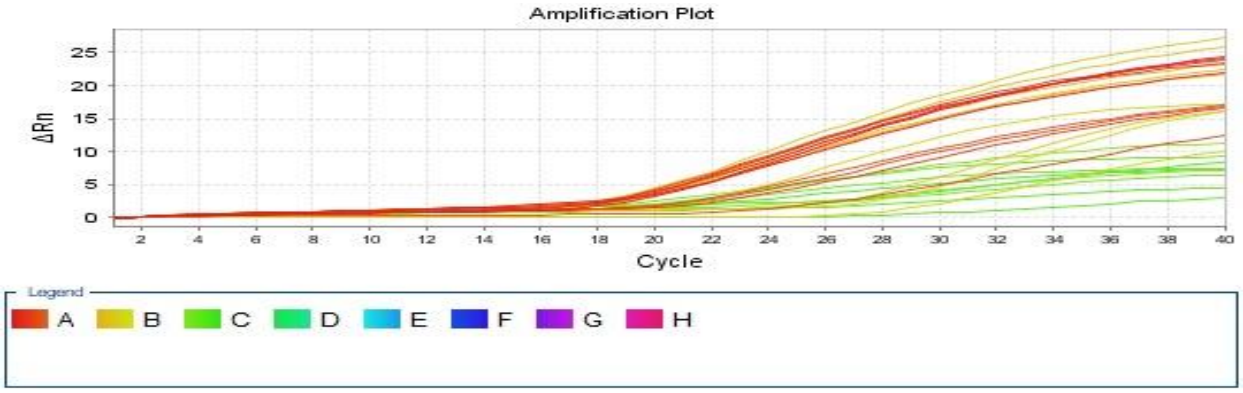

Figure 1. Gene proliferation plot.

The melt curve of products was obtained to confirm the real time PCR product. Figure 2 shows the analysis of the melt curve, which has a single compressed peak, indicating the primers are attached specifically.

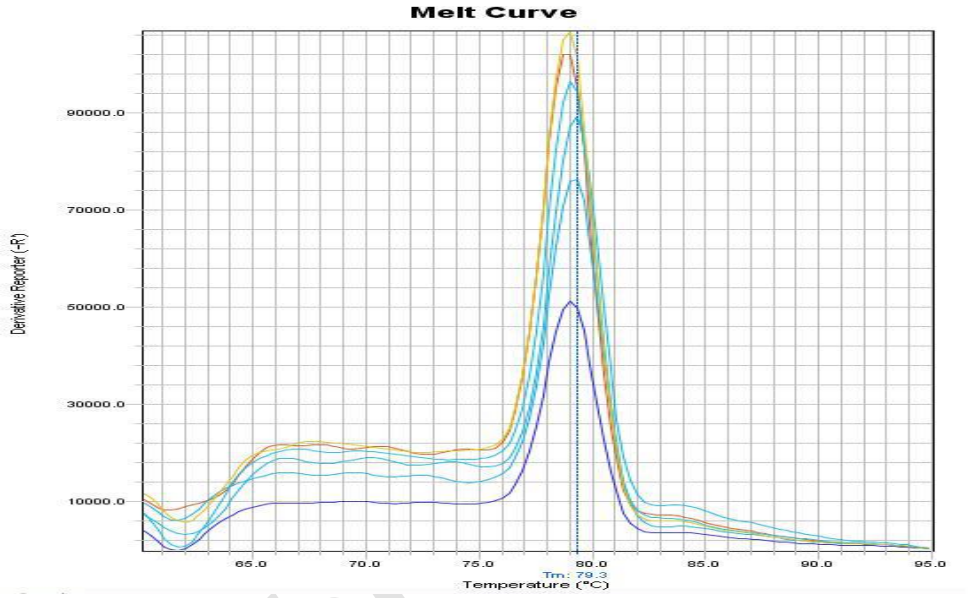

Figure 2. Melt curve.

For further confirmation, gel electrophoresis of PCR products was done with a 2% agarose gel and observed by UV transilluminator after ethidium bromide staining (fig 3).

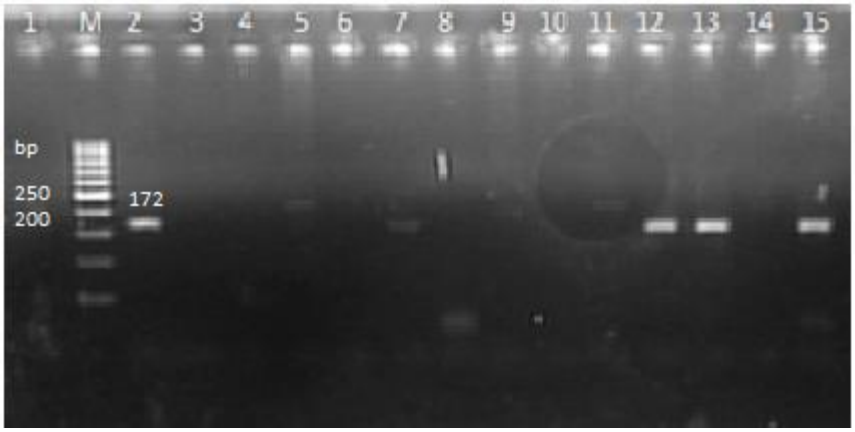

Figure 3. Gel electrophoresis of PCR products using ABI thermocycler device 1: Negative Control, M: DNA Marker (Leader 50 bp), 2,12,13,15: Positive cases (172 bp).

Table 2. Antibiotic resistance pattern of the isolates *of S. saprophyticus* isolated from patients with urinary tract infections

Antibiotic	Resistant (percent)	Semi-sensitive (percent)	Sensitive (percent)
Ampicillin	55%	5%	40%
Erythromycin	80%	12.5%	7.5%
Cefotaxime	17.5%	10%	72.5%

As it can be seen, the highest frequency of isolate resistance to antibiotics was related to erythromycin with 80%, ampicillin and cefotaxime antibiotics were ranked with frequency of 55% and 17.5%, respectively. On the other hand, the highest frequency percentage of sensitivity of isolates to antibiotics is related to cefotaxime with 72.5%.

4. Discussion

Diagnosis of urinary tract infections is based on clinical signs and symptoms and laboratory evidence of the presence of bacteria in the urine (Oyaert et al., 2018). The presence of more than 10^5 colony forming units per mL (cfu/mL) of urine is considered as a significant bacteriuria (Chu & Lowder, 2018). The risk of UTI complications is more common in women, because the disease may be associated with bacteriuria without sign in women (Al-Badr & Al-Shaikh, 2013). Several factors can increase the risk of developing urinary tract infections, including sexual intercourse, poor personal hygiene, diabetes, obesity, vaginal infections and the high use of antibiotics (Hannan et al., 2012). *S. saprophyticus* is a coagulase negative staphylococci which abundantly causes urinary tract infections in the outpatients of young and middle-aged women (Higashide et al., 2008). Effective management of patients with urinary tract infections usually relies on the identification of the types of organisms and the selection of antibiotics effect on them (Beyene & Tsegaye, 2011). In the study performed by Bhatt et al., (Bhatt et al., 2017) out of 17,135 urine samples, a prevalence of 15.9% of positive growth was reported, but in current study, of the total 51235 clinical samples collected, only 2101 (4.1%) samples were detected with microbial culture. Different results indicate that the prevalence of UTI varies in different regions. In study conducted by Nyah-tuku Nzalie et al., (2016), the rate of isolation of the *S. saprophyticus* was 3.6%. In the current study, the detection rate of the bacterium was 2.47 %, which is consistent with the result of the previous study. In this study, the prevalence of *E. coli* (61.06 %), was consistent with the study (60.9%) conducted by Bhatt et al. from Nigeria (Bhatt et al., 2017). In this research, *S. saprophyticus* showed the highest antibiotic resistance rate to erythromycin at the rate of 80% that nearly was consistent with (92.31%) the study carried out by Khoshbakht et al. (2012). In the present study, resistance of *S. saprophyticus* to ampicillin was 55% which is different from the study done by Puneet Bhattet al., with resistance of 95% to ampicillin (Bhatt et al., 2017). In another study conducted by Sajjan et al., (2016) resistance of *S. saprophyticus* to cefotaxime was 46.15% but in the current study, resistance to cefotaxime was 7.5%. The reason for the different results is that, the prevalence of resistance to antibiotics in different areas is different.

Conclusion

Based on the results obtained in this research, Gram-negative and Gram-positive bacteria caused urinary tract infection. According to the results of the antibiotic sensitivity determination test, the prevalence of resistant strains of *S. saprophyticus* is increasing in various antibiotic groups. On the other hand, due to the high consumption of antibiotics for treatment, an accurate and definitive diagnosis should be made to prevent the administration of inappropriate antibiotics in addition to accelerating the treatment. Also, the results of this study showed high levels of resistance of the desired bacterium to studied antibiotics.

Conflict of interest

The authors declare that there is no conflict of interest in this study.

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Refereces

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