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Study the Enterotoxigenixity of *Staphylococcus aureus* Isolated from the Urine Samples of Pediatrics with UTIs

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ABSTRACT

Staphylococcus aureus is an important pathogen associated with urinary tract infections in a variety of hosts including humans. It produces several toxins and virulence factors that contribute to its pathogenic potential such as staphylococcal enterotoxins. This study was conducted to determine enterotoxigenicity of *S. aureus* associated with UTIs in pediatrics patients. One-hundred and seventy two urine samples were collected from pediatrics suffered from UTIs. Samples were cultured immediately and those that were *S. aureus*-positive were analyzed for the presence of *sea*, *seb*,*sec*, *sed* and *see* enterotoxins using PCR. Fifty three out of 172 urine samples were positive for *S. aureus* (30.81%). The prevalence of *S. aureus* in boy and girl patients were 21.25% and 39.13%, respectively (P <0.05). The most commonly detected enterotoxigenic genes in the *S. aureus* isolates of pediatric patients were *sec* (41.50%), *sea* (18.86%), *see* (15.09%) and *sed* (13.20%). There was significant difference between the prevalence of enterotoxigenic genes and sex of pediatric patients (P <0.035). The role of enterotoxin genes in the pathogenesis of UTIs is still unknown. Other newly detected genes may play a role in pathogenesis of diseases. Therefore, further studies should be conducted to demonstrate the role of enterotoxins of *S. aureus* in the cases of UTIs.

Key words: *Staphylococcus aureus*, Enterotoxins, Urinary Tract infections, Pediatric patients, Iran.

INTRODUCTION

Urinary tract infections (UTIs) are among the most common infectious diseases diagnosed especially in pediatric patients[1,2]. UTIs are liable for more than 1.5 million hospitalization and 300,000 cases of severe disease in the United States annually[3].

Among all infectious agents causing UTIs, the *Staphylococcus aureus* (*S. aureus*) is one of those who have recently received considerable attention because its high abilities to resistance against commonly used antibiotics[4, 5]. In despite of such bacteria like *Escherichia coli* (*E. coli*) which has the highest prevalence rate in the cases of UTIs[6], *S. aureus* has a lower prevalence but its high levels of resistance to antimicrobial agents causes many problems in its treatment[7]. Totally, 0.2-1% of the urine samples in developed countries, mainly contains *S. aureus* but the prevalence rate is higher in developing countries[8, 9].

There are documented data concerning the mechanisms by which *S. aureus* induces infections, particularly the role and mode of action of enterotoxigenic genes involved in its pathogenicity[10]. The staphylococcal enterotoxins

(SEs) are a group of low-molecular-mass and single-chain proteins that are similar in composition and biological activity but differ in antigenicity (*sea* to *sej*)[11, 12]. High prevalence of sea gene in the *S. aureus* strains of various types of hospital infections was reported previously[13].

Adwan et al. (2006)[13] reported that more than 50% of the *S. aureus* isolates of the cases of UTIs were positive for various types of enterotoxigenic genes. Unfortunately, there were scarce data about the status of enterotoxigenic genes of *S. aureus* in the cases of UTIs in Iranian pediatrics. Therefore, the present study was carried out to investigate the distribution of enterotoxigenic genes of the *S. aureus* isolated from the urine samples of Iranian pediatric patients suffered from UTIs.

MATERIALS AND METHODS

Samples and *Staphylococcus aureus* identification

From July 2014 to October 2014, a total of 172 urine samples were collected from hospitalized boy (n=80) and girl (n=92) patients of educational hospitals and health centers of Tehran, Iran. The ultrasound technique was used to confirm the presence of UTIs[14]. Urine samples were collected from the midstream using the Suprapubic Aspiration (SPA)[15].

The urine samples were transferred to the Microbiology and Infectious Diseases Research Center in a cooler with ice-packs. All samples were directly cultured into 7% sheep blood agar (Merck, Darmstadt, Germany) and incubated aerobically at 37°C for 48 h. After incubation, suspicious colonies were examined by the use of morphologies compatible with *Staphylococcus* spp. (microscopical morphology, catalase and coagulase production). Studied colonies were cultured on Tryptic Soy Broth (TSB) (Merck, Darmstadt, Germany) and Tryptic Soy Agar (TSA) (Merck, Darmstadt, Germany). After growth, staphylococci were identified on the basis of colony characteristics, Gram staining, pigment production, hemolytic and the following biochemical reactions: catalyses activity, coagulated test (rabbit plasma), Oxidase test, glucose O/F test, resistance to bacitracin (0.04 U), mannitol fermentation on Mannitol Salt Agar (MSA) (Merck, Darmstadt, Germany), urease activity, nitrate reduction, novobiocin resistance, phosphatase, deoxyribonuclease (DNase) test and carbohydrate (xylose, sucrose, trehalose and maltose, fructose, lactose, mannose) fermentation test[16].

DNA extraction and PCR confirmation

Total genomic DNA was extracted from the bacterial colonies. A single colony was inoculated on 5ml of brain heart infusion broth and incubated over night at 37ºC. Then 1.5 ml of a saturated culture was harvested with centrifugation for 5 min. at 14,000 rpm. The cell pellet was resuspended and lysed in 200µl of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, 1% SDS) by vigorous pipetting. To remove most proteins and cell debris, 66 µl of 5M NaCl solution was added and mixed well, and then the viscous mixture was centrifuged at 12,000 rpm for 10min. at 4ºC. After transferring the clear supernatant into a new eppendorf tube, an equal volume of chloroform was added, and the tube was gently inverted at least 50 times when a milky solution was completely formed. Following centrifugation at 14,000 rpm for 5min., the supernatant is then removed to another eppendorf tube and double volume of 100% ethanol was added. The tubes were inverted 5 to 6 times gently, then centrifuged at 10,000rpm for 5minutes. The supernatant was discarded and 1ml of ethanol (70%) was added to the pellet, and tubes centrifuged at 10,000 rpm for 5 minutes. Finally the supernatant discarded and the pellet was dried for 10 min at room temperature, the pellet was resuspended by 100µl H2O. The stock was kept at -20ºC until use. The DNA concentration has been determined by measuring absorbance of the sample at 260 nm using spectrophotometer[17].

Presence of *S. aureus* in each DNA samples was confirmed using the Banada et al. (2012) method[18]. The PCR reaction mix consist of 1 X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 0.001% (w/v) gelatin) with 4 mM MgCl2, 250 mM of each nucleotide (deoxynucleoside triphosphate), 0.5 mM of each primer (forward and reverse), 4 ng of the molecular beacon and 4 U of Jumpstart Taq DNA polymerase (Fermentas, Germany).

PCR amplification for enterotoxigenic genes

The PCR method was used in order to study the distribution of *sea*, *seb*, sec*, sed*, see*, seg*, *seh*, *sei* and *sej* enterotoxins of the *S. aureus* (19-22). Oligonucleotide primers, annealing temperature, PCR programms and size of products is shown in table 1. A programmable thermal cycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) PCR device was used in all PCR reactions. All runs included a negative DNA control consisting of PCR grade water and strains of *S. aureus* ATCC 13565 (*sea*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), FRI 361 (*sed*, *seg*, *sei* and *sej*), ATCC 27664 (*see*) and FRI 137 (*seh*) were used as positive controls.

Statistical analysis

The results were transferred to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA) for analysis. Statistical analysis was performed using SPSS/16.0 software (SPSS Inc., Chicago, IL) for significant relationship between incidences enterotoxigenic genes of *S. aureus* isolated from the boy and girl patients. The chi-square test and Fisher's exact 2-tailed test analysis were performed in this study. Statistical significance was regarded at a P value < 0.05.

Ethical considerations

The present study was accepted by the ethical committees of the educational Hospitals. Written informed consent was obtained from all of the study patients or their parents.

RESULTS

Total distribution of S. aureus in the urine samples of pediatric patients is shown in table 1. Of 172 urine samples studied, 53 samples were found to be positive for *S. aureus* (30.81%). in addition, the total prevalence of *S. aureus* in boy and girls patients of our study were 21.25% and 39.13%, respectively. Significant difference was seen for the prevalence of *S. aureus* between boy and girl patients (P <0.039). Distribution of enterotoxigenic genes in the *S. aureus* isolates of boys and girls is shown in table 3. Results of the gel electrophoresis of PCR amplifications are shown in figure 1-3. We found that girl patients had the highest prevalence of enterotoxigenic genes. There was significant

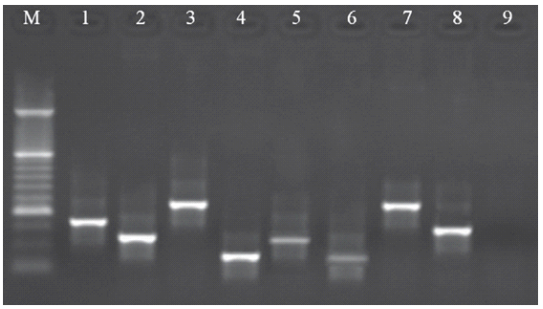

Fig. 1: Results of the gel electrophoresis for identification of enterotoxigenic genes in *S. aureus* strains. M: 100 bp DNA ladder (Fermentas, Germany), Lines 1-4: Positive samples for *sed* (317 bp), *seh* (213 bp), *seb* (478 bp) and *sea* (120 bp), Lines 5-8: Positive controls and Line 9: Negative control

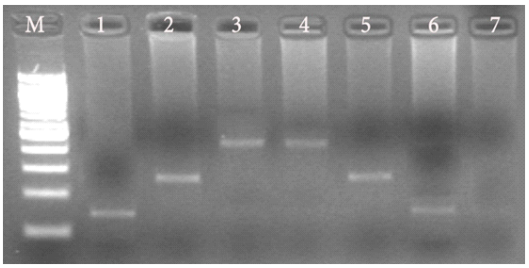

Fig. 2: Results of the gel electrophoresis for identification of enterotoxigenic genes in *S. aureus* strains. M: 100 bp DNA ladder (Fermentas, Germany), Lines 1-3: Positive samples for *sej* (142 bp), *sec* (257 bp) and *sei* (454 bp), Lines 4-6: Positive controls and Line 7: Negative control

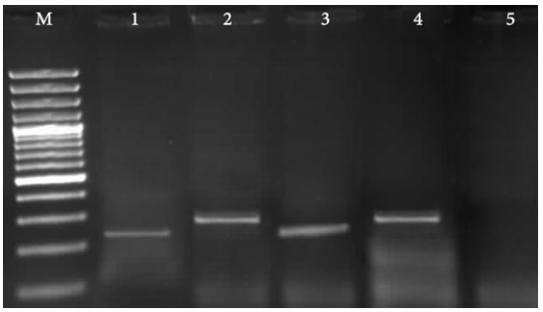

Fig. 3: Results of the gel electrophoresis for identification of enterotoxigenic genes in *S. aureus* strains. M: 100 bp DNA ladder (Fermentas, Germany), Lines 1 and 2: Positive samples for *see* (209 bp) and *seg* (287 bp), Lines 3 and 4: Positive controls and Line 5: Negative control

Table 1. The oligonucleotide primers and the PCR programs used for amplification of enterotoxins of *Staphylococcus aureus* isolates of the urine samples of boy and girl patients

Target gene	Primer sequence (5'-3')	PCR product (bp)	Annealing temperature (°C)	PCR programs	PCR Volume (50µL)
sea	F: TTGGAAACGGTTAAAACGAA R: GAACCTTCCCATCAAAAACA	120	50	1 cycle: 94 °C -5 min. 30 cycle: 94°C -2 min 72°C -1 min 1 cycle: 72°C -5 min	5 µL PCR buffer 10X 1.5 mM $Mgcl_2$ 200 µM dNTP (Fermentas) 0.5 µM of each primers F & R 1.25 U Taq DNA polymerase (Fermentas) 2.5 µL DNA template
seb	F: TCGCATCAAACTGACAAACG R: GCAGGTACTCTATAAGTGCC	478	50		
sec	F: GACATAAAAGCTAGGAATTT R: AAATCGGATTAACATTATCC	257	50		
sed	F: CTAGTTTGGTAATATCTCCT R: TAATGCTATATCTTATAGGG	317	50		
see	F: AGGTTTTTTCACAGGTCATCC R: CTTTTTTTTCTTCGGTCAATC	209	50		
seg	F: AAGTAGACATTTTTGGCGTTCC R: AGAACCATCAAACTCGTATAGC	287	55		
seh	F: GTCTATATGGAGGTACAACACT R: GACCTTTACTTATTTCGCTGTC	213	46.4		
sei	F: GGTGATATTGGTGTAGGTAAC R: ATCCATATTCTTTGCCTTTACCAG	454	50		
sej	F: CATCAGAACTGTTGTTCCGCTAG R: CTGAATTTTACCATCAAAGGTAC	142	50		

Table 2: Distribution of *Staphylococcus aureus* in the urine samples of boy and girl patients

Studied groups of patients	No samples collected	No. positive samples (%)
Boy	80	17 (21.25)
Girl	92	36 (39.13)
Total	172	53 (30.81)

difference between the prevalence of enterotoxigenic genes and sex of pediatric patients (P <0.035). The most commonly detected enterotoxigenic genes in the *S. aureus* isolates of pediatric patients were *sec* (41.50%), *sea* (18.86%), *see* (15.09%) and *sed* (13.20%). There were no positive results for *seg*, *sei* and *sej* enterotoxigenic genes. Statistically significant differences were seen between the prevalence of *sec* and *seh* (P <0.016),

Table 3: Distribution of enterotoxigenic genes of *Staphylococcus aureus* isolates of of the urine samples of boy and girl patients

Studied groups of patients (No. positive)	Distribution of enterotoxigenic genes (%)								
	sea	***seb***	***sec***	***sed***	***see***	***seg***	***seh***	***sei***	***sej***
Boy (17)	3 (17.64)	1 (5.88)	7 (41.17)	2 (11.76)	2 (11.76)	-	-	-	-
Girl (36)	7 (19.44)	3 (8.33)	15 (41.66)	5 (13.88)	6 (16.66)	-	1 (2.77)	-	-
Total (53)	10 (18.86)	4 (7.54)	22 (41.50)	7 (13.20)	8 (15.09)	-	1 (1.88)	-	-

sec and *sed* (P <0.036), *sec* and *seb* (P <0.021) and *sea* and *seb* (P <0.047) enterotoxigenic genes.

DISCUSSION

The results of the present study showed the high prevalence of *S. aureus* and its enterotoxigenic genes in the urine samples of pediatrics suffered from UTIs. Totally, 30.81% of urine samples of our investigation were infected with *S. aureus*. In the other hand, the prevalence of *S. aureus* in the urine samples of boy and girl pediatric patients were 21.25% and 39.13%, respectively.

One possible explanation for the high prevalence of *S. aureus* in the urine samples of pediatrics is the fact that maybe the hospital environment is contaminated and used antimicrobial agents were not efficient for treatment of diseases. A study which has been conducted in Nigeria[23] showed that the total prevalence of *S. aureus in the urine samples of patients suffered from UTIs was* 33.6% which was similar to those of our study. Momtaz and Hafezi (2014)[24] in a cross sectional study which was conducted in Iran showed that 50% of all clinical samples of human hospital's infections were positive for *S. aureus* which was entirely high. In keeping with the high prevalence of *S. aureus* in Iranian hospitals and health care units, increase the growing prevalence of *S. aureus* from other countries have also been reported[25-27].

Possible explanations for the high prevalence of *S. aureus* in this study are the low levels of health care in hospitals, excessive application of urine catheter, lack of sanitary conditions in hospitals, increasing the age of circumcision in boys, improper use of effective drugs and occurrence of antibiotic resistance in *S. aureus*. One possible explanation for the high prevalence of *S. aureus* in girls is that they have relatively short and wide urethra. Also, host factors such as changes in normal vaginal flora may put girls at higher risk for UTIs. Therefore, girls are more prone to get UTIs. Furthermore, management of micturition in girls is very essential. Management faults made by girls or they parents include cleaning perineum forward from the anus to the vulva[28] that can cause urinary tract infection.

Another part of the current study focused on the distribution of enterotoxigenic genes in the *S. aureus* isolates of boys and girls suffered from UTIs. Results showed that *sec* (41.50%), *sea* (18.86%), *see* (15.09%) and *sed* (13.20%) were the most commonly detected enterotoxigenic genes in the *S. aureus* isolates of pediatrics patients. Higher prevalence of *sec* and *sea* enterotoxigenic genes in the *S. aureus* strains of clinical samples has been reported previously[11, 13, 29, 30]. High levels of differences in the prevalence of *S. aureus* and also enterotoxigenic genes which have been seen in the results of various studies are probably due to the differences among the origin of clinical samples, number of test samples, sensitivity of methods, and types of enterotoxins or enterotoxin genes that were detected.

The *S. aureus* strains of UTIs which carried out the enterotoxigenic genes may induce releasing of specific cytokines that may inhibit the efficiency of human immune response by their expressions; this may contribute to the persistence of *S. aureus* in urogenital tract and promote inflammation in these tissues or enhance the chronicity of this disease.

In conclusion, the role of *sea*, *seb*, *sec*, *sed* and *see* genes in the pathogenesis of UTIs is still unknown. However it is possible that UTIs can be caused by *S. aureus* strains at least lack these genes. The current study showed that the *S. aureus* and its enterotoxigenic genes especially *sec*, *sea*, *see* and *sed* had the highest prevalence in pediatrics patients with UTIs. With respect to this condition in Iran, we recommended the initially manage of children affected with a community acquired UTIs with effective drugs to reduce the times of hospitalization.

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