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In silico and Wet Lab Analysis of Two Genes Polymorphism Which Candidate for a Methicillin-resistant *Staphylococcus aureus* Vaccine

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Abstract: Background: *Staphylococcus aureus* is a salient nosocomial infectious agent. The prevalence of antibiotic resistance complicates the treatment of staphylococcal infections. Therefore, the development of an effective vaccine against *S. aureus* is important. *saCOL2291* and *saCOL2581* are potential candidate genes for vaccine development and their sequences are available in GenBank. **Results:** In this study, gene polymorphisms of *saCOL2291* and *saCOL2581* were evaluated. Genomic DNA was extracted from thirty clinical *S. aureus* isolates and target genes were PCR-amplified. All amplicons were sequenced, aligned by using MEGA4 software. Nucleotide polymorphisms were detected that resulted in amino acid sequence changes but these polymorphisms were located in the N-terminal domains while the C-terminal domains of these genes were conserved. At the sametime, all of *saCOL2291* and *saCOL2581* sequences which submitted in GenBank, were aligned withisolatedgenes. All of mutation illustrated both C-terminal of proteins were conserved. Despite the identification of polymorphisms in these genes, both encode candidate proteins for potential use in a universal staphylococcal vaccine. This is the first study for assessing variation in the *saCOL2291* and *saCOL2581* genes.

Key words: *saCOL* gene, *S. aureus*, vaccine, single nucleotide polymorphisms, polymorphism

INTRODUCTION

Staphylococcus aureus is a bacterium that contributes to diseases including endocarditis, pyogenic infections and meningitis. While these diseases can be treated with antibiotics, the bacteria can quickly develop resistance (Shehata, 2008).

A major cause of nosocomial infections is methicillin-resistant *S. aureus* (MRSA) which can be readily transferred between patients in hospitals (Muder *et al*., 1991; Yang *et al*., 2006). Mupirocin is a treatment option with a decreased risk of surgery and carriage. However, Mupirocin resistance was first reported in 1987 in the U.K. (Rahman *et al*., 1987; Yang *et al*., 2006) and has since been reported in other countries (Gales *et al*., 2004; Schmitz *et al*., 1998; Yang *et al*., 2006). Therefore, the prevalence of MRSA is a major concern (Walker *et al*., 2003). *S. aureus* strains that are resistant to penicillin, methicillin and/or vancomycin have been reported by Centers for Disease Control and Prevention (2002). Vancomycin resistance was first reported in Japan (Hiramatsu *et al*., 1997) and its appearance in the U.S in 2002 raised concern (Centers for Disease Control and Prevention, 2004; Chang *et al*., 2003).

Specific antibodies and vaccines can prevent infectious diseases (Josephson *et al*., 2001; McKenney *et al*., 1999). Recently, candidate proteins for a staphylococcal vaccine have been identified that stimulate the immune system and cause an antibody response (Burine *et al*., 2000; Vytvytska *et al*., 2002). Polysaccharides and DNA in vaccines can prevent infection (Foster, 1991). Sixty surface and secretory proteins have been proposed for an immunogenic vaccine (Etz *et al*., 2002).

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The *Sca* family contains nine proteins with conserved C-terminal domains of 40-60% homology (Pourmand and Foster, 2006). In this study, the *Sca* family genes *saCOL2291* and *saCOL2581* which encode the secretory antigens *ScaC* and *ScaD*, respectively, were evaluated for Single Nucleotide Polymorphisms (SNPs). These genes are candidates for a universal staphylococcal vaccine because they are present in most staphylococcal isolates. The purpose of our study was to detect variation in the *saCOL2291* and *saCOL2581* gene sequences and determine whether they are appropriate candidates for a universal staphylococcal vaccine.

MATERIALS AND METHODS

Bacterial strains: Thirty clinical *S. aureus* strains were collected from hospital patients in cities of IRAN from mucus, urine, wounds and CSF. Coagulase, DNase and catalase tests were performed to confirm that the isolates were *S. aureus*.

DNA extraction: All isolates were cultured at 37°C for 16 h in LB broth and genomic DNA was extracted using the Bioneer Kit (South Korea) according to manufacturer's instructions.

Amplification of *saCOL2291* and *saCOL2581* and SNP detection: Molecular biology materials were obtained from Fermentas. Pfu DNA polymerase, a high fidelity enzyme, was used for PCR. Primers for the *saCOL2291* (HM565739) and *saCOL2581* (HQ179767) genes (Table 1) were designed based on the COL strain nucleotide sequences available from NCBI (www.ncbi.nlm.nih.gov/nucleotide). The thermal cycling program consisted of an initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 1 min, 62.7°C for 1 min and 72°C for 1 min and a final extension of 72°C for 5 min. The amplicons were sequenced (Macrogene Company, South Korea) and aligned with the COL strain nucleotide sequence using BLAST (www.ncbi.nlm.nih.gov/Blast). A multiple alignment of the thirty amplicon sequences was created with MEGA4 software. All nucleotide sequences were analyzed with Gene Runner software to deduce the amino acid sequences and multiple alignments were created.

Multiple alignment of submitted *saCOL2291* and *saCOL2581* genes: For assessing of gene polymorphism, all of submitted whole genome sequences of *Staphylococcus* species in Genome project database of NCBI were selected. They were 29 but 23 of them harbor *saCOL2291* gene sequences with following accession numbers: CP002643.1, CP002453.1, FR714927.1, CP001844.2, CP002114.2, CP002120.1, CP001996.1, AM990992.1, FN433596.1, CP001781.1, CP000730.1, AP009324.1, AP009351.1, CP000736.1, CP000703.1, BA000017.4, CP000046.1, CP000253.1, CP000255.1, BX571857.1, BA000018.3, BA000033.2, BX571856.1.

At the same time, 26 *Staphylococcus* species contain *saCOL2581* gene sequence with these accession numbers: CP002643.1, FR714927.1, CP002110.1, CP001844.2, CP002114.2, CP002120.1, CP001996.1, AM990992.1, FN433596.1, CP001781.1, CP000730.1, AP009324.1, AP009351.1, CP000736.1, CP000703.1, BA000017.4, CP000046.1, CP000253.1, CP000255.1, AC074026.14, X97985.1, BX571857.1, BA000018.3, BA000033.2, BX571856.1. Multiple alignment of selected sequences was performed by Mega4 software and all mutations at amino acid level was analyzed. Among 23 selected sequences of *saCOL2291* and 26 *saCOL2581* genes, 2 and 5 sequences,respectively, had nucleotides insertion which interrupt multiple alignment analysis. So, these sequences were omitted and remaining sequences were aligned.

Amplification of the *saCOL2291* and *saCOL2581* genes: The genomic DNA of thirty *S. aureus* clinical isolates was used as a template to PCR-amplify the *saCOL2291* and *saCOL2581* genes. The nucleotide sequences of *saCOL2291* and *saCOL2581* were obtained, aligned and compared with their COL strain counterparts. Alignments of each *saCOL2291* or *saCOL2581* sequence with the appropriate reference sequence revealed average homologies of 98.3 and 97.86%, respectively (results not shown). The C-terminal domains of these genes were conserved. The *ScaC* and *ScaD* amino acid sequences were analyzed to detect silent changes, transitions and transversions. The variation in the samples indicated that the tested strains were unrelated.

RESULTS

Gene polymorphisms in *saCOL2291*: In the *S. aureus* COL strain, the $ssaA_2$ gene (804 bp) of the *saCOL2291* locus encodes the staphylococcal secretory antigen

Table 1: Primer sequences used to amplify the *saCOL2291* and *saCOL2581* genes

Gene	Primer sequence (5′→3′)	Amplicon size (bp)
saCOL2291	Forward: GCGCGCCATATGTCTGAGCAAGATAACTACG GTT	744
	Reverse: GCGCGCCTCGAGGTGAATGAAGTTATAACCAGCAG	
saCOL2581	Forward: GCGCGCCATATGGCAGGACTTGCCACTAT CGC	755
	Reverse: GCGCGCCTCGAGATGAATGAAATTATATGAACCTGC	

$ssaA_2$ (267 aa) with a 1 to 27 aa signal peptide at the N-terminal domain. The mutations listed in Table 2 is based on the multiple alignment of the nucleotide sequences.

An average of 1.6 transition mutations were detected in each sample while an average of 1.3 transversion mutations were detected. Many mutations affected the amino acid sequence; some replaced residues with amino acids of the same family while others altered the native amino acid to one from a different family. Mutations were detected in the N-terminal domains but the C-terminal domains were conserved.

Gene polymorphisms in *saCOL2581*: The *S. aureus* COL strain $ssaA_1$ gene (768 bp) of the *saCOL2581* locus encodes the staphylococcal secretory antigen $ssaA_1$ (255 aa). The signal peptide at the N-terminal domain is composed of residues 1 to 26. Polymorphisms based on the multiple alignments of nucleotides and amino acids are listed in Table 2.

Transitions and transversions were detected at frequencies of 3.4 and 0.7 per sequence, respectively. Transitions were detected most frequently. While several mutations changed residues to an amino acid with similar properties, many produced changes resulting in the replacement of an amino acid with one from a different family.

Study based on bioinformatics method: Multiple alignment of protein sequences was presenting some mutations among sequences which downloaded from GenBank (Fig. 1). These mutations were listed in Table 3.

Table 2: Mutations in the *ScaC* and *ScaD* proteins

Protein	Transition	Transversion	Deletion	Insertion
ScaC	Asn (55)→Asn [9]	Asn (111)→Tyr -[5]	His* [3]	Tyr+Asn#-(61-62)-[6]
	Asn (89)→Asn [6]	Asp (38)→Asn [6]		Ala+Tyr^-(60-61) [6]
ScaD	Pro (130)→Ser [30]	Ala (172)→Val [5]	-	-
	Ala (114)→Val [6]			
	Gly (166)→-Gly [6]			
	Arg (164)→Trp [10]			
	Ala (144)→Val [30]			

Brackets values are the number of isolates with that mutation. Parenthesis values are the amino acid position. *A histidine residue was deleted in 3 isolates but did not affect the reading frame of the amino acid sequence. # Tyrosine and asparagine or ^ alanine and tyrosine amino acids were inserted but did not change the frame. The rates of the analyzed mutations were up to 5 per sequence, excluding deletions and insertions

Table 3: Some mutation of *saCOL2291* and *saCOL2581* genes in NCBI

Protein	Transition	Transversion	Deletion	Insertion
ScaC	Ser (91)→Leu [1]	Tyr (28)→Phe [1]	-	-
	Trp (149)→Arg [1]	Ala (109)→Val [1]	-	-
	Asn (157)→Ser [6]	-	-	-
	Val (172)*→Ala [6]	-	-	-
ScaD	Asn (60)→Ser [3]	Gly (237)→Ser [1]	-	-
	Ser (207)→Asn [1]	Ala (236)→Ser [1]	-	-
	-	Gly (235)→Ser [1]	-	-
	-	Ala (234)*→Ser [1]	-	-

Brackets indicate the number of isolates with that mutation. The numbers in parenthesis indicate the amino acid position. *showed, this mutation is occur the same position as clinical isolates

DISCUSSION

In the 21st century, the rapid acquisition of antimicrobial resistance in *S. aureus* is a major concern. Previously, resistance to methicillin and penicillin was detected; and recently, resistance to linezolid and other antibiotics has increased in frequency (Anderegg *et al*., 2005).

Thus, it is critical to identify resistance mechanisms and design new vaccines to prevent staphylococcal infections. One such vaccine containing conjugated polysaccharide capsule reduced infection in 56% of hemodialysis patients in a 10-month period (Shinefield *et al*., 2002). Another staphylococcal vaccine using the penicillin binding protein 2a (PBP2a) was used against MRSA strains and reduced the number of *S. aureus* cells in kidneys compared with non-immunized patients but the results were not promising (Senna *et al*., 2003).

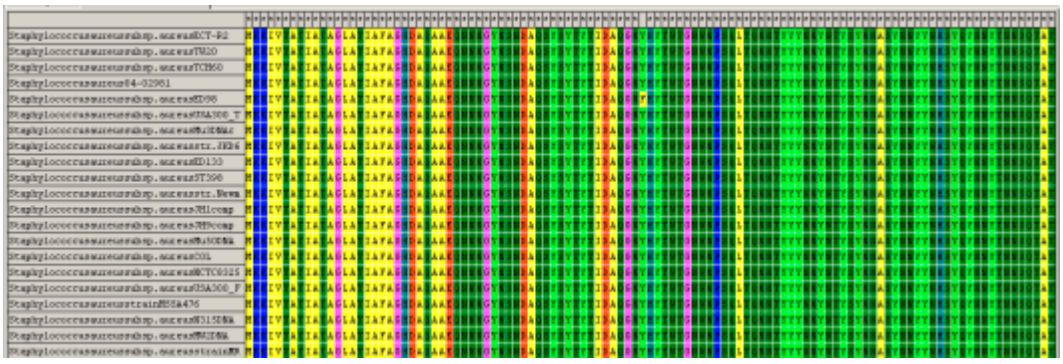

Fig. 1: Multiple protein alignment of ScaC protein, C-terminal domain

The *ScaC* and *ScaD* proteins have been used as immunogens in a mouse model. These proteins induced a humoral immune response and the production of specific antibodies, indicating that these proteins are indeed immunogens (Dryla *et al*., 2005).

Gill *et al*. (2005) sequenced the whole genome of the *S. aureus* COL strain (Gill *et al*., 2005). In our study, the *saCOL2291* and *saCOL2581* genes of the COL strain were used as reference sequences and aligned with the corresponding genes of clinical isolates.

As a result of whole genome sequencing, sixty important surface and secretory proteins were identified as new immunogens in *S. aureus* using bacterial display methods (Etz *et al*., 2002). Pourmand detected *S. aureus* Sca family proteins using bioinformatics methods and designated this family as staphylococcal conserved antigen (Sca). Most of these genes are conserved in the C-terminal domain and are novel vaccine candidates (Foster, 1991; Pourmand and Foster, 2006). We investigated this hypothesis in clinical isolates.

To identify SNPs in the *saCOL2291* and *saCOL2581* genes, Pfu DNA polymerase which has a proofreading function, was used. SNPs were detected in all isolates. Some SNPs altered the amino acid sequences and the resulting functional effects must be evaluated. The C-terminal domains of these genes in the *S. aureus* isolates investigated in this study were conserved and thus are good vaccine candidates.

This study was explained most of submitted whole genome sequences of *Staphylococcus* species, contain *saCOL2291* and *saCOL2581* genes. Moreover, these genes contain point mutation just like *saCOL2291* and *saCOL2581* genes of clinical isolates in our survey. Alignment analysis of submitted sequences clarified conservation in these genes at C and N-terminal domain but the C-terminal domain is more conserve than N-terminal.

The submitted *saCOL2291* and *saCOL2581* genes at GenBank are from different area of the world. In bioinformatics based analysis, all sequences were conserved with some point mutations.

One of the most important properties of a protein candidate for designing an universal vaccine is sequence conservation. So, our data confirmed the conservation of C-terminal domains of these genes and we suggest to using C-terminal domain for designing a new candidate vaccine against methicillin-resistant *S. aureus*.

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