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Characterization of *Staphylococcus aureus* from Sore Throat Patients: Association Among Host Immune Evasion and Toxin Genes

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ABSTRACT

The understanding of bacterial virulence and immune evasion mechanisms provide novel opportunities to adopt preventive measures and design therapeutics against most prevailing resistant and virulent strains. With this background, this study was designed to characterize *Staphylococcus aureus,* the most common pathogen, using conventional microbiological and molecular methods. The agar disc diffusion method was used to evaluate the antibiotic susceptibility. Virulence-associated genes were detected using PCR while association analysis was used to test the likelihood of strains carrying combinations of genes involved in toxin production and/or host immune evasion. Highest resistance was observed against beta-lactam group followed by cephalosporin, lincosamide, tetracycline, macrolides and aminoglycosides. No resistance was observed against vancomycin and linezolid. Among genes involved in host immune evasion, *Staphylococcus* protein A (*spa*) was identified most frequently (81%) and proportions of capsular polysaccharides (*CPs8*), clumping factor A (*clf A)* and intracellular adhesion A (*ica A*) were 78%, 68.5% and 40%, respectively. *ica D* and *CPs5* could not be amplified from any isolate. Toxin genes were present in 43.5% isolates. Among toxin genes, enterotoxins (*SEs*) were most frequently detected followed by enterotoxin (*ETs)* (24.09 %) and toxic shock syndrome toxin (*TSST-1*) (15 %). More than one toxin genes were present in 32.5% isolates. In addition, cluster analysis indicated that host immune evasion and toxin genes were not associated with each other suggesting the presence of diverse lineage specific strains in sore throat patients. However, the intraspecific association was noticed among these genes. *Coa* and *spa* polymorphism and association analysis indicated that *spa* negative isolates possess *Coa* of 1200 and 900bp, whereas *spa* positive isolates contain *Coa* of 650bp and 750bp. The most prevalent genes, *spa*, *CPs8* and *sea* may be considered as molecular targets in designing treatment and control strategies.

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Authors' Contribution
NA and MA designed the study, provided guidance and analyzed the data. AM generated data as a part of her PhD research work and MW helped her in study. IL and NA wrote the article.

Key words
MRSA, Immune evasion genes, Toxin genes, Molecular markers.

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen and frequent part of human microflora that can bring about a multiplicity of self-limiting to life-threatening diseases in both animals and humans particularly in immunocompromised ubjects (Stoke and Ridgway, 1980; Aqib *et al*., 2017). It possesses a remarkable capacity to acquire antibiotic resistance. The resistance to the first antibiotic-penicillin, emerged in 1942 (Rammelkamp and Maxon, 1942). Later on for treatment of infections of penicillin-resistant *S. aureus* strains, semi-synthetic antibiotic methicillin was introduced in 1959 (Jevons, 1961). However, in 1961 there were reports from the United Kingdom that *S. aureus* isolates had acquired resistance to methicillin (MRSA, methicillin-resistant *S. aureus*). From that time resistance to other antibiotics group was increasing and even intermediate resistance to vancomycin has also been reported in Japan (Hinaki *et al*. 2007). Development of multidrug resistance in *S. aureus* is a growing threat to public health.

Molecular characterization and phenotypic methods are important for monitoring the spread and designing therapeutic strategies against bacterial infections (Aman and Adhikari, 2014). Pathogenic potential of *S. aureus* is related to a number of virulence factors that allow it to adhere to host surface, evade the host immune responses and cause harmful toxic effects (Sina *et al*., 2013). The virulence of *S. aureus* is generally considered an upshot of several virulence determinants. Coagulase (*Coa*), nuclease (*nuc*) and factor essential for the expression of methicillin resistance (*femA*) are regarded as a reliable marker for discriminating *S. aureus* from other *Staphylococcus* spp.

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(Kloos and Schleifer, 1985; Rushdy *et al*., 2007). *Coa* causes the coagulation of plasma, *nuc* encodes for DNase production which has species-specific sequences (Brakstad *et al*., 1992; Costaa *et al*., 2012). The *fem A*, a cytoplasmic protein necessary for the expression of methicillin resistance is also involved in the biosynthesis of cell wall. Some of the cell wall associated virulence factors involved in host immune system evasion include *spa*, *CPs*, *clf A* and *ica A*. *spa* encodes surface Ig-binding protein A (42KDa) which functions by capturing IgG molecules through Fc region and preventing phagocytosis of the bacterial cells (Fournier and Philpott, 2005). Capsular polysaccharides (*CPs*) and intracellular adhesion locus A-D (i*ca A-D*) genes are responsible for biofilm formation (Howden *et al*., 2010). They also inhibit phagocytosis and are widely used as vaccine targets for the management of MRSA infection by eliciting a cell surface immune response (Katherin and Lee, 2004; Robbins *et al*., 2004). *clf A* is a gene responsible for secretion of clumping factor, a surface associated adhesion of the *S. aureus*, that improves binding of the bacteria to both soluble and immobilized fibrinogen (Hair *et al*., 2008).

Some *S. aureus* strains produce a variety of *Staphylococcal* enterotoxins (SEs), toxic shock syndrome toxin 1 (TSST-1) and exfoliative toxins (ETs). The TSST-1 and ETS toxins, encoded by *tst* and *ETs*, cause toxic shock syndrome and staphylococcal peeling skin syndrome, respectively (Kim *et al*., 2006). While *SEs* are important in creating a large number of staphylococcal infections including *Staphylococcus* related food poisoning (Mehrotra *et al*., 2000). Balaban and Rasooly (2000) perceived that more than 70% of *S. aureus* strains produced one or more enterotoxins.

The aim of the study was to characterize *S. aureus*, the most common pathogenic strains responsible for sore throat in humans, on the basis of antibiotic resistance, *coa* and *spa* gene polymorphism, host immune evasion and toxin genes. We further tried to relate *coa* and *spa* gene polymorphism and find associations between/among host immune evasion and toxin genes. This information may help to adopt preventive measures and design therapeutics against most prevailing resistant and virulent strains. Few studies have been carried out to determine the association among different genes (Alli *et al*., 2015).

MATERIALS AND METHODS

Sample collection and characterization

Throat samples were collected from patients of sore throat visiting the OPD of tertiary care hospital in Faisalabad with their written consent. All procedures were in compliance with the declaration of Helsinki and the study protocol was approved by the ethical committee of the Zoology Department, University of the Punjab, Lahore. These isolates were identified as *S. aureus* by conventional methods. Briefly, purified colonies were streaked on various media including MacConkey agar, eosin methylene blue agar, blood agar and chocolate agar. Different biochemical tests such as H_2S production, tryptophan deaminase (TDA), catalase, methyl red, citrate utilization, indole, voges Proskauer, denitrification and urease test were performed to characterize bacterial isolates. All isolates were stored in Brain heart infusion broth containing 30% glycerol at -20ºC for further use (Kloos and Schleifer, 1985).

Phenotypic detection of coagulase and thermostable nuclease

Coagulase production was checked by incubating 1ml of rabbit plasma with bacterial suspension. Coagulase activity was considered positive if serum gets clotted after 4-24 h of incubation with bacteria at 37ºC. DNase positive colonies were identified by the clear zone around colony on treatment with 1N HCl on DNase agar (oxoid) carried out by the protocol in Bergey's manual (Kloos and Schleifer, 1985).

Antimicrobial susceptibility testing

The sensitivity of the *S. aureus* isolates against commercially available antibiotics was examined by disk diffusion method following CLSI guideline (Zafar *et al*., 2008). Antibiotics groups include β-lactam, aminoglycosides, cephalosporin, lincosamide, tetracyclines, amphenicols, macrolides, glycopeptides and oxazolidinone.

Genomic DNA extraction

Bacterial DNA was extracted from isolated *S. aureus* (n=200) strains of sore throat patients using Invitrogen DNA mini Spin Column Kit following the manufacturer's instruction (Invitrogen, USA) with slight modification. DNA was extracted from 5 ml of 18-h broth culture. In place of lysozyme 5µl of lysostaphin (1mg/ml) (Sigma) was used and incubated at 37ºC for 30 min. DNA samples were kept at -20°C until further use.

Detection of species-specific gene, immune system escaping gene and toxin gene

The genomic DNA of the isolated *S. aureus* (n=200) strains was examined for the presence of various genes (16S rRNA, species specific, host immune evasion and toxin genes) and antibiotic resistant genes using the primers given in Table I. Genomic DNA from randomly

selected 10 *S. aureus* (n=200) strains was subjected to 16S rRNA gene sequencing for confirmation. Amplification was performed in a 25µl reaction mixture that include 1 µl of template DNA, 12.5 of taq green mater mix, 1 µl of primers both forward and reverse (10 pM/ml) and 10.5 µl of ultrapure water. Genetic marker including *Coa*, *nuc* and *femA*, immune system escaping gene *spa*, *icaA*, *icaD*, *CP5*, *CP8* and *clfA*, antibiotic resistance gene *mecA* and enterotoxin producing genes (*sea-see*), exfoliative toxin (*eta*, *etb*) and *tst* were identified using single or multiplex PCR. Primers, product size and PCR condition are given in Table I. The amplified PCR products (25 µl), along with a 1 kb and 100-bp molecular weight DNA ladder (Fermentas, USA) were subsequently separated by electrophoresis on 1.5% agarose gel (Sigma, USA) stained with 0.5 µg/ml ethidium bromide (Sigma, USA) on horizontal electrophoresis unit at 100 V for 30 min. Electrophoresis was performed at 100 volt for 40 min. DNA bands were visualized and photographed under an ultraviolet transilluminator.

Table I.- List of primers used in this study.

S No	Gene s	Primers	Annealing temperature	Size	Reference
1	*Coa*	5'- CGA GAC CAA GAT TCA ACA AG-3' 5'-AAA GAA AAC CAC TCA CAT CAT CA-3'	58	600-1200	(Toshkova *et al.*, 2001)
2	*spa*	5'-ATC TGG TGG CGT AAC ACC TG-3' 5'-CGC TGC ACC TAA CGC TAA TG-3'	55	1150-1500	(Shakeri *et al.*, 2010)
3	*nuc*	5'-GCG ATT GAT GGT GAT ACG GTT- 3' 5'- AGC CAA GCC TTG ACG AAC TAA AGC- 3'	57.6	279	(Brakstad *et al.*, 1992)
4	*clfA*	5'-GGC TTC AGT GCT TGT AGG- 3' 5' -TTT TCA GGG TCA ATA GC- 3'	55	1329-1309	(Stephan *et al.*, 2000)
5	*ica A*	5'- TCT CTT GCA GGA GCA ATC AA-3' 5'-TCA GGC ACT AAC ATC CAG CA- 3'	53	188	(Lachachi *et al.*, 2014)
6	*ica D*	5'-ATG GTC AAG CCC AGA CAG AG-3' 5'-CGT GTT TTC AAC ATT TAA TGC AA-3'	47	198	(Lachachi *et al*., 2014)
7	*CP5*	5'-ATG ACG ATG AGG ATA GCG-3' 5'-CTC GGA TAA CAC CTG TTG C-3'		880	(Moore and Lindsay, 2001)
8	*CP8*	5'-ATG ACG ATG AGG ATA GCG-3' 5'-CAC CTA ACA TAA GGC AAG-3'	51	1147	(Moore and Lindsay, 2001)
9	*sea*	5'-GGT TAT CAA TGT GCG GGT GG-3' 5'-CGG CAC TTT TTT CTC TTC GG-3'	54	102	(Mehrotra *et al.*, 2000)
10	*seb*	5'-CTA TGG TGG TGT AAC TGA GC-3' 5'-CCA AAT AGT GAC GAG TTA GG-3'	54	164	(Mehrotra *et al.*, 2000)
11	*sec*	5'- AGA TGA AGT AGT TGA TGT GTA TGG-3' 5'-CAC ACT TTT AGA ATC AAC CG-3'	54	451	(Mehrotra *et al.*, 2000)
12	*sed*	5'- CCA ATA ATA GGA GAA AAT AAA AG-3' 5'-ATT GGT ATT TTT TTT CGT TC -3'	54	278	(Mehrotra *et al.*, 2000)
13	*see*	5'-AGG TTT TTT CAC AGG TCA TCC-3' 5'-CTT TTT TTT CTT CGG TCA ATC-3'	54	209	(Mehrotra *et al.*, 2000)
14	*femA*	5'-AAA AAA GCA CAT AAC AAG CG-3' 5'-GAT AAA GAA GAA ACC AGC AG-3'	54	132	(Mehrotra *et al.*, 2000)
15	*mecA*	5'-ACT GCT ATC CAC CCT CAA AC-3' 5'-CTG GTG AAG TTG TTG TAA TCT GG-3'	54	163	(Mehrotra *et al.*, 2000)
16	*eta*	5'-GCA GGT GTT GAT TTA GCA TT-3' 5'-AGA TGT CCC TAT TTT TGC TG-3'	54	93	(Mehrotra *et al.*, 2000)
17	*etb*	5'-ACA AGC AAA AGA ATA CAG CG-3' 5'-GTT TTT GGC TGC TTC TCT TG-3'	54	226	(Mehrotra *et al.*, 2000)
18	*tst*	5'-ACC CCT GTT CCC TTA TCA TC-3' 5'-TTT TCA GTA TTT GTA ACG CC-3'	54	326	(Mehrotra *et al.*, 2000)

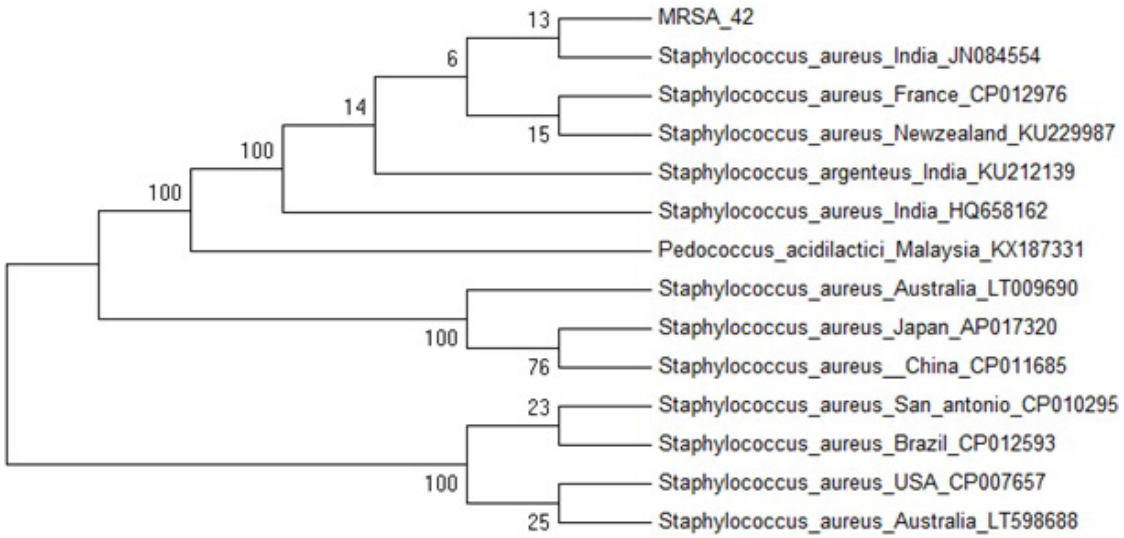

Fig. 1. Phylogenetic tree of 16s rRNA gene sequence of MRSA-42, showing genetic relatedness of different strains with each other.

Statistical analysis

Data were analyzed using SPSS software (version 16.0). The proportion of all genes was calculated and the $\chi 2$ test was used to find an association between and among genes. Furthermore, logistic regression was used to test the likelihood of strains carrying combinations of different genes particularly genes for toxin production and/or host immune evasion. Multivariate statistical analysis (MVSP version 3.2) was used for constructing cluster diagrams.

RESULTS

Phenotypic characterization of S. aureus

Staphylococcus aureus strains isolated from sore throat patients were identified by biochemical and randomly selected 10 strains were confirmed by 16S rRNA gene sequencing. All of the strains (n = 200, one from each patient) were Gram-positive cocci, non-motile, non-spore forming, arranged in grape-like clusters, fermentative and catalase positive. Strains were coagulase positive, β-hemolytic, produced the characteristic golden yellow pigments on nutrient agar and changed the colour of mannitol salt agar from red to yellow. Identification of one selected strain confirmed as *S. aureus* by 16S rRNA gene sequencing is presented in Figure 1.

Antibiotic resistance profiling

Antibiotic resistance profiling of the isolates indicated high resistance (>80%) against β-lactam group followed by cephalosporin (67.5%) The resistance against tetracycline, lincosamide and macrolides was around 25%. Least resistance was observed against aminoglycosides, streptomycin (16%) and gentamycin (6%). However, no resistance was observed for glycopeptides (vancomycin) and oxazolidinones (linezolid) (Table II).

Table II.- Antibiotic resistance pattern among the *S. aureus*.

Chemical group	Antibiotics	Concentration (µg)	Resistance (%)
Beta-lactam	Methicillin	5	88
	Penicillin	10	81
	Oxacillin	1	93
	Ampicillin sulbactam	20	41
Aminoglycosides	Gentamycin	10	16
	Streptomycin	10	9
Cephalosporin	Cefixime	5	67.5
Lincosamide	Clindamycin	2	24.5
Tetracyclines	Tetracycline	30	25
Amphenicols	Chloramphenicol	30	1.5
Macrolides	Erythromycin	15	24.5

Amplification of species specific, host immune evasion and toxin genes

Coa, *nuc* and *fem A* were present in all strains. They were observed with a product size of 600-1200bp, 270bp and 132bp, respectively. Host immune evasion gene were observed in separate PCR reactions, *spa*, *clf A*, *ica A*, *CPs8* and *fem A* were observed as PCR product of 1100-1300bp, 1150bp, 188bp, 1147bp and 132bp, respectively.

The genes of *sea*, *seb*, and *sed* were identified as product size 102bp, 164bp and 278bp, respectively. The genes for enterotoxin, exo-foliative toxin and toxic shock toxin syndrome were amplified by multiplex PCR. The *mec A* and *fem A* were used as an internal control. Primers of *eta* (93bp), *etb* (226bp) *tst* (326bp), *femA* and *mecA* (163bp) were pooled. The amplified products of the multiplex PCR are shown in Figures 2, 3 and 4.

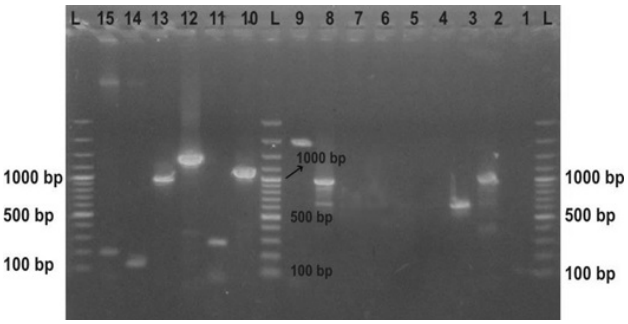

Fig. 2. Electrophoretic profile of *spa* polymorphism product amplified through polymerase chain reaction (PCR). Lane L, molecular marker of 1Kb; Lanes 1, 2 and 3, isolates with 1100bp and 1300bp *spa* gene product and *spa* negative, respectively.

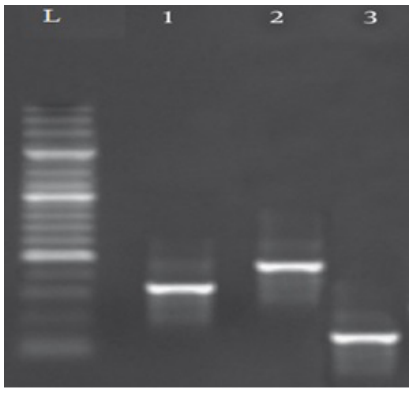

Fig. 3. Electrophoresis profile in 2% agarose gel showing PCR amplification products for the *S. aureus* enterotoxins L., DNA ladder, 1-3 are *sed*, *seb* and *sea.*

Among host immune evasion genes *spa* was the most prevalent gene (81%) followed by *CPs8* (78%), *clf A* (68.5%) and *icaA* (40%), However *icaD* and *CPs5* could not be amplified. Regarding genes responsible for toxins, among all isolates 41.5% (n=83/200) were found to possess at least one genetic marker of toxin production, of which 67.46% (n=56/83) had only one toxin gene whereas 32.53% (n=27/83) had more than one genes. *sea* was most profuse (60.71%, n=34/56) followed by *seb* 12.5 % (n= 7/56) and *sed* 5.35% (n=3/56). Consistently, *sec* and *see* could not be amplified in any isolate. Among ETs, the *eta* was present in 7.14% strains and *etb* in only 1.78%. The frequency of *tst* positive isolates was 12.5% (n = 7/56) (Fig. 5).

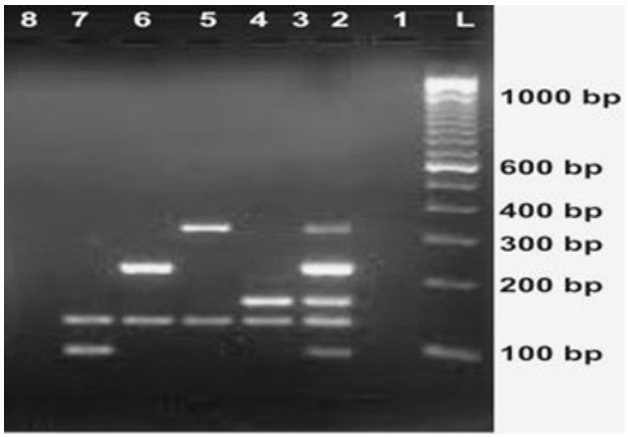

Fig. 4. Electrophoresis profile in 2% agarose gel showing multiplex PCR amplification products for the *S. aureus* exo-foliative toxin tst toxin *mec A* and *fem A* genes. Lane L, DNA marker (100-bp ladder); Lanes 2 to 7, PCR amplicon; Lane 1 and 8, negative control; Lane 2, eta, etb, tst, mecA, and femA; Lane 3, *mecA* plus *femA*; Lane 4, *tst* and *femA*; Lane 5, *eta* and *fem A*; Lane 6, *etb* and *femA*; Lane 7, *eta* and *femA*.

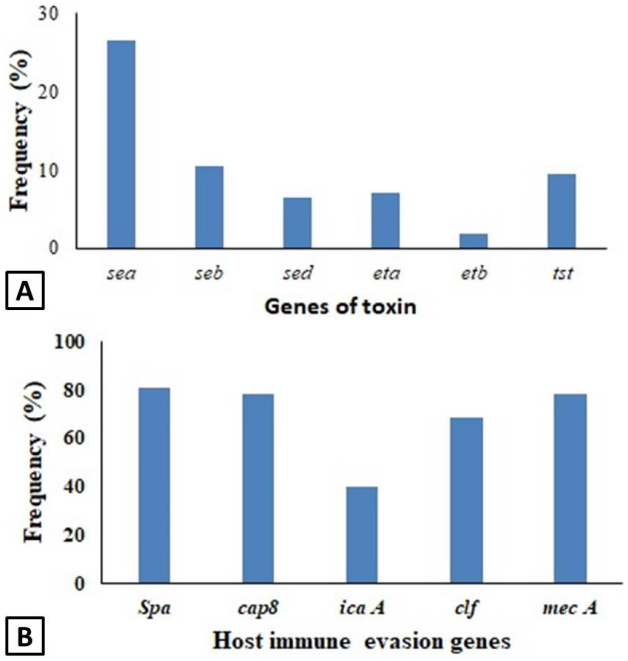

Fig. 5. Frequency of host immune evasion and *mec A* (A) and toxin genes (B) in *S. aureus* isolated from sore throat patients in Punjab.

Table III.- Distribution of different immune evasion and toxin genes in *S. aureus.*

Number of genes	Gene sequence	No. of isolates	Isolates number
1	spa	2	7,135
	CPs8	4	139, 152, 153, 169
	icaA	1	151
	sea	1	170
	mecA	6	28, 34, 132, 166, 198, 17
2	clfA, spa	1	83
	clfA, mec A	1	103
	spa, cap 8	4	74,11,134,144
	spa, ica A	1	75
	spa, mec A	4	2,4,172,194
	CPs8, sea	1	79
	ica A, eta	1	61
	ica A, mec A	2	27, 185
	tst,mec A	1	37
3	clfA, spa, CPs8	4	104,11,148,165
	clfA, spa, mec A	4	32,44,82
	clfA,CPs8, Ica A	1	64
	clfA,CPs8, mec A	1	107
	clfA, icaA, mecA	1	77
	spa, CPs8, icaA	1	98
	spa, CPs8, mecA	9	99, 109, 122, 124, 156, 157, 160, 162, 171
	spa, ica A, mecA	2	16,189
	CPs8,ica A ,mecA	3	140, 193, 147
	CPs8, sea, mecA	1	1
	CPs8, seb, mecA	1	110
	ica A, sea, mecA	1	179
	eta, sea, tst	1	5
4	clfA, spa,CPs8, ica A	3	59,69,164
	clfA, spa, CPs8, sea	2	62,67
	clfA, spa, CPs8, seb	1	43
	clfA, spa, CPs8, mec A	36	10,11,15,20,21,33,41,46,47,50,52,60,68,86,87,88,90,2,106,116,120, 121,123,126,141,146,155,161,167,175,11,16,190,191,195,200
	clfA, spa, CPs8, mecA	1	200
	clfA, spa, tst, sed	2	80, 3
	clfA, spa, icaA, mecA	1	7
	clfA, spa, sea, mecA	1	145
	clfA,CPs8, icaA, sea	1	143
	clfA,CPs8, icaA, mecA	1	136
	clfA, CPs8, sea, mecA	2	76,196
	spa, CPs8, icaA, sed	1	149
	spa, CPs8, icaA, mecA	5	3 ,100,177 ,180,182
	spa, CPs8, sea, mecA	3	66, 125, 184
	CPs8, icaA, sea, mecA	2	154, 199
5	clfA, spa, CPs8, sea, mecA	2	6, 147
	clfA, spa, CPs8, icaA, eta	1	65
	clfA, spa, CPs8, icaA, tst	2	63,118
	clfA, spa, CPs8, icaA, sea	3	51 ,129 ,142

Number of genes	Gene sequence	No. of isolates	Isolates number
5	clfA, spa, CPs8, icaA, mecA	18	13,14,25,30,4,55,56,73,89,91,94,95,114,115,127, 131,183,187
	clfA ,spa, CPs8, eta, mecA	1	176
	clfA, spa, CPs8, test, mecA	1	133
	clfA, spa, CPs8, sea, mecA	6	22,38,45,70,130,192
	clfA, spa, CPs8, seb, mecA	2	36,108
	clfA, spa,CPs8,sed,mecA	1	158
	clfA, spa, icaA, etb, mecA	1	137
	clfA, spa, icaA,tst, mecA	1	18
	clfA, spa, icaA, sea, mecA	1	178
	clfA, spa, icaA, seb, mecA	2	39,54
	clfA, spa, eta, tst, mecA	1	71
	clfA, CPs8, icaA, sed, mecA	1	128
	spa, CPs8, icaA, test, mecA	1	102
	CPs8, eta, sea, seb, mecA	1	117
	CPs8, tst, sea, sed, mecA	1	174
6	clfA, spa, icaA, eta, seb, mecA	1	5
	clfA, spa, CPs8, sed, mecA	1	57
	clfA, spa, CPs8,icaA, eta, mecA	1	81
	clfA, spa, CPs8, icaA, sea, seb	1	26
	clfA, spa, CPs8, icaA, sea, mecA	1	105
	clfA, spa, CPs8, icaA, sea, mecA	6	16,19,49,72,93,150
	clfA, spa, CPs8, icaA, seb, mecA	1	40
	clfA, spa, CPs8, eta, sea, seb	1	9
	clfA, spa, CPs8, eta, sea, mecA	1	24
	clfA, spa, CPs8, tst, sea, mecA	1	29
	clfA, spa, CPs8, tst, sed, mecA	2	23,112
	clfA, spa, CPs8, sea, seb, mecA	2	35,159
7	clfA, spa, CPs8 ,sea, sed, mecA	1	4
	clfA ,spa, CPs8, icaA, eta, sea, seb	1	97
	clfA, spa, CPs8, icaA, tst, sea, mecA	1	12
	spa,CPs8,icaA, eta, sea, seb, mecA	1	188
8	clfA, spa, CPs8, tst, sea, sed, mecA	1	42
	clfA, spa,CPs8, tst, sea, seb, mecA	2	163,101
	clfA, spa, cap, icaA, etb, tst, seb, mecA	1	113
	clfA, spa, CPs8, tst, etb, tst, seb, mecA	1	53
	clfA, spa, CPs8, tst, etb, sea, seb, mecA	1	138
9	clfA, spa, CPs8, icaA, eta, sea, seb, sed, mecA	1	58

Co-occurrence and association of immune evasion, toxin and mecA gene

Isolates were found to have 82 different combinations of immune evasion, toxin and *mecA* gene. The dominant combinations were (*clf A*, *spa*, *Cps8*, *mecA*) and (*clf A*, *spa*, *Cps8*, *ica A*, *mecA*) that were observed in 36/200 and 18/200 isolates respectively. Other combinations are given in Table III. The immune evasion and toxin genes did not show any association while different genes involved in the similar mechanism (immune evasion or toxin production) show association with each other (Table IV).

Polymorphism in Coagulase Coa and spa gene and association in their variates

The coagulase primer pair produced 4 amplicons of 650,750,950 and 1200bp. Among them 124 isolates had only 1 PCR product (650 or 1200), the other 64 had 2 PCR products with size of approximately 650 and 750bp (41 isolates), 600 and 900bp (23 isolates) and other 12 isolates displayed 03 PCR product of 650, 750 and 900 bp, s*pa* amplified with 2 PCR products 1100 (n=119) and 1300 bp (n=46). The s*pa* gene could not be amplified in 35 isolates. *Coa* and *spa* product association analysis indicates that

isolates which do not have s*pa* gene they possess *Coa* of 1200 and 900 bp; whereas, s*pa* 1300 and 1100bp co-occurs with *Coa* of 650 and 750bp (Fig. 6).

Table IV.- Association among host immune evasion and toxin genes.

S No.	Genes function	Genes	Sign.	Odd ratio	Confidence interval
1	Host immune evasion	*Clf A * Spa*	0.000	12.131	5.247-28.044
2		*Clf A * CPs8*	0.000	3.600	1.796- 7.216
3		*Spa* CPs8*	0.000	4.048	1.890- 8.666
4	Toxin production	*tst * sea*	0.000	0.119	0.037-0.383
5		*eta * seb*	0.005	5.062	1.620-15.82
6		*tst * seb*	0.048	0.122	0.015-0.980
7		*tst * sed*	0.001	8.582	2.364-31.160
8		*sea * sed*	0.046	0.286	0.084-0.976

P<0.05 is considered significant.

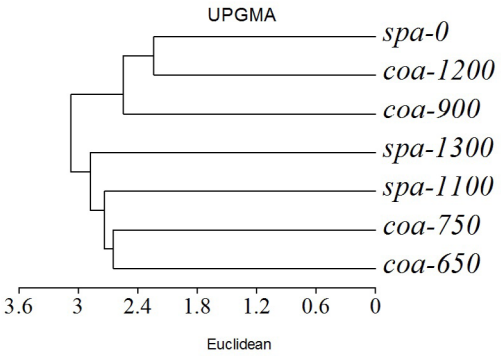

Fig. 6. Association among *Coa* and *spa* genes.

DISCUSSION

S. aureus possesses an extensive repertoire of virulence factors that contribute to its survival and the development of pathologies of variable complexity. The objective for the current study was to characterize *S. aureus* from sore throat on the basis of antibiotic resistance and virulence genes involved in host immune system evasion as well as toxin production and find markers which could be used as a target in control strategies. Identification of strains was validated by biochemical methods followed by PCR-based screening of *Coa*, *nuc* and *femA* since these are considered as gold standard for the rapid identification of the isolates (Strommenger *et al*., 2003). Antibiotic resistance profiling is the first step in characterization and a basic requirement of clinicians for defining the treatment strategies (Rivera and Boucher, 2011). Around 80% isolates were found resistant against β lactam group of antibiotics. However, resistance against other groups were less than 40% and no resistance was observed against vancomycin and linezolid. Studies conducted in different cities of Pakistan including Lahore, Rawalpindi and Karachi reported variable resistance rates against clindamycin and tetracycline (Hannan *et al*., 2009; Shakeri *et al*., 2010, 2014), but methicillin resistance observed in the current study was much higher than previous reports from other cities (Kaleem *et al*., 2010). The drastic increase in methicillin resistance among *S. aureus* strains is alarming, which might be due to irrational use of antibiotic in the treatment of infection and horizontal transfer of resistance gene among bacteria. Resistance pattern of antibiotics revealed that linezolid, vancomycin, chloramphenicol and streptomycin are the drugs of choice. The emergence of vancomycin-resistant enterococcus (VRE) and vancomycin-resistant *S. aureus* have already been reported from some part of the world (Howden *et al*., 2010). Therefore, vancomycin and linezolid may be suggested only under critical and controlled conditions to prevent the emergence of resistance to these antibiotics.

Bacteria adopt different strategies to survive and multiply in the host system. Secretion of staphylococcal protein A, capsule formation, the release of clumping factor and biofilm formation are some of the mechanisms which help microbes to escape the host immune system (Lebeaux *et al*., 2014). The presence of four genes involved in host immune evasion mechanism (*spa*, *Cps8*, *Clf A* and *ica A*) are screened in this study. *spa* transcribes protein A which binds the Fc region of IgG. It acts as antiplatelet, anti-complement as well as anti-phagocytic. In the present study 81% strains were observed to possess *Spa* of two sizes (1100-1300bp) and isolates were categorized into three types a) strains which do not harbor *spa*, b) Strains having *spa* of 1100 bp and c) Strains having *spa* of 1300bp. Kloos and Schleifer (1985), Shakeri *et al*. (2010) and Omar *et al*. (2014) reported the absence of *spa* in their strains. Some workers have related the length of *spa* gene with the ability of adherence and indicated that *spa* > 1000bp is more associated with pathogenicity (Camussone *et al*., 2012).

The encapsulation of *S. aureus* has been regarded as an important trait for the survival of this organism in the host system (Brown *et al*., 2014). It provides protection from phagocytosis. Great variation exists in capsular types strains from animal and human source and information concerning the geographical distribution of capsular serotype has been used for the rational design of a vaccine against *S. aureus* (El-Jakee *et al*., 2010). Therefore, presence of *CPs8* and *CPs5* was investigated. Only *CPs 8* was recorded in 78% isolates pointing towards the

importance of considering this gene in designing control strateges (Anderson *et al*. 2012).

PCR detection of the *Ica* operon may be an effective method to differentiate between virulent and non-virulent strains. Early detection and management of biofilm-forming *Staphylococcus* is considered an essential step towards the prevention and management of nosocomial infections (Higgins *et al*., 2006). The *Ica* operon of *S. aureus* contains *ica* (*A-D*) allows *Staphylococcus* spp. to form slime layers and biofilm. In this study i*ca A* was amplified in 40% isolates while *ica D* could not be amplified. A variable proportion of biofilm forming *S. aureus* (40-87%) has been reported by different authors (Moore and Lindsay, 2001; Grinholc *et al*., 2007).

S aureus expresses many surface proteins of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family, which specifically recognize and bind to the extracellular matrix components of the host. The *clf A* gene encodes an adhesion protein for fibrinogen. It is also important in protecting microbe from phagocytosis during infection (Mayra *et al*., 2013). In this study 68.5% isolates show the presence of this gene. The high proportion of *clf A* containing isolates seems consistent with the fact that these genes play a determinant role in bacterial virulence (Argudín *et al*., 2010).

Production of toxins is another pathogenic characteristic of *S. aureus*. The extracellular toxin with low molecular weight is normally associated with food poisoning and skin related syndromes (Kadariya *et al*., 2014). Several structurally similar but antigenically different staphylococcal enterotoxins (*A-R*) encoded by *sea-ser* are identified by different authors (Vasconcelos *et al*., 2011; Rahimi, 2013). Most important features of *SEs* are resistance to heat, and pepsin digestion and *sea-see* are frequently reported toxins genes (Taj *et al*., 2014). Among *SE* toxins most common was *sea* (84.1%), followed by *seb* (33.3%) and *sed* (20.6%). Taj *et al*. (2014) has also reported sea as a most common toxin in *S. aureus* isolated from Karachi (Peck *et al*., 2009). Other major toxins are *TSST-1* and *ETs*, the causative agent of toxic shock syndrome and staphylococcal peeling skin syndrome, respectively. The frequency of the *eta* in this study was 26.9 % and of *etb* was 4.76%. Peck *et al*. (2009) reported different percentages of *SEs* and *ETs* in Korea and Iran (Kitai *et al*., 2005; Alfatemi *et al*., 2014). Although the properties of *SEs* containing isolates may vary in the different geographical region but the continuous high prevalence of *sea* puts emphasis to adopt the treatment method targeting the *sea* (Kitai *et al*., 2005; Higgins *et al*., 2006; Howden *et al*., 2010). Some isolates (n=26) were found to contain *Ets*, *tst*, and *SEs* depicting a strong virulent potential of these strains.

Coa and *spa* polymorphism are considered important in strain typing. Most prominent PCR product was *Coa* of 650bp which was amplified in 57% isolates. In current study, 05 different variants of *S. aureus* based on *Coa* gene were observed, *i.e.* 650 bp, 650&750 bp, 600&900 bp, 650, 750&900 bp and 1200 bp. Cluster analysis, using MSVP, indicate that *Coa* 900-1200 bp and 900bp can occur independently, while *spa* 1100-1300 bp co-occurs with *Coa* 750bp and 650 bp. Characterization on the basis of *Spa* and *Coa* gene further indicated that they can be collectively used in strain typing.

The association studies among genes provide important information on pathogenicity mechanisms of microbes and guide in designing immunomodulatory therapy (Ahmadrajabi *et al*., 2017). In current study, analysis targeting co-occurrence of genes indicate that immune evasion and toxin genes are not associated. On the other hand, a strong association was found with in different immune evasion and toxin gene groups. It may be due to their presences on different elements which are inherited independently (Cuny *et al*., 2015). The presence of *spa*, *tst* and *SEs* has been reported on SCC element, Pathogenicity Island and plasmid respectively. The widespread nature of toxin and immune evasion genes make them an attractive target for the development of new virulence-targeted therapies. *S. aureus* investigational vaccine group, Pfizer, has already designed vaccine by addressing virulence mechanisms associated with *CP5*, *CP8*, *ClfA* and *MntC* and declared that anti-ClfA antibodies can completely inhibit *S. aureus* binding to human fibrinogen (Andrson *et al*., 2012). Current study highlights the importance of *spa*, *CPs8*, *sea* in *S. aureus* in our area.

CONCLUSION

MRSA is a potential threat to the public health in Pakistan as this bug can disseminate in community causing severe clinical infections. Presence of β-lactam group resistance in our isolates is a cause of concern. However, to the relief, none of our isolates was resistant to glycopeptides (vancomycin) and oxazolidinones (linezolid) suggesting vancomycin and linezolid the drugs of choice in treating MRSA infection. Aassociation analysis revealed that virulence and immune evasion genes are not associated and may be independently utilized as molecular markers for strain typing and designing control strategies. Additionally, high prevalence of *spa*, *CPs8*, *sea* in *S. aureus* make them obvious targets for anti-virulence drugs andinfection control measures.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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