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Virulence Markers in *Staphylococcus aureus* Strains Isolated from Hemodialysis Catheters of Mexican Patients

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* is an increasingly important cause of nosocomial bacterial infections worldwide. *S. aureus* is responsible for several pathologies, including skin infections, endocarditis, meningitis, deep-tissue ulcers, and sepsis. *S. aureus* biofilm formation on catheters and other medical devices is a major post-operative concern, because biofilms are often the source of persistent and difficult to treat bacterial infections. While catheter-related *S. aureus* infections have been reported, the strains responsible for these infections have not been genetically characterized. We genetically characterized *S. aureus* strains isolated from hemodialysis catheters in Mexican patients. The frequency of 35 genes coding for adhesins, toxins, and other virulence-associated products in the 55 isolated *S. aureus* strains was determined using PCR, while real-time PCR was used to examine the level of gene expression. Of the 55 *S. aureus* strains isolated from 109 patients, 45 (81.8%) were determined to be methicillin-resistant. The *icaA*, *rbf*, *sarA*, and *agr* genes are involved in biofilm formation and bacterial dispersion and were detected in 96.3%, 40.0%, 74.5%, and 100% of *S. aureus* strains, respectively, and 70.9% of the strains formed a detectable biofilm. Interestingly, 67.2% of the strains contained the *icaA*, *agr*, *spa*, *clfA*, *sdrC*, *sdrD*, *sdrE*, *seg*, *seh*, and *sei* genes, suggesting that this gene combination is important for successful catheter colonization. The results of this study provide significant insight into the virulence gene make-up of catheter-colonizing *S. aureus* strains, and will assist in developing a more targeted treatment approach for persistent *S. aureus* biofilm contamination of medical devices.

Keywords: *S. aureus*; Hemodialysis; Catheter; Genotyping

1. Introduction

Staphylococcus aureus is a frequent cause of catheter-related infections in patients [1]. The worldwide emergence of methicillin-resistant *S. aureus* (MRSA) is of major concern, as its emergence has dramatically reduced the number of antibiotics available for the prevention and treatment of infections in both hospitals and communities [2]. In 2005 in the United States, invasive MRSA infections occurred in 42.5/1000 dialysis population, a rate exceeding that in the general population by 100-fold [3]. MRSA exhibits a great capacity for biofilm formation on surfaces, making endovascular catheters a favorable nidus for infection. *S. aureus* formation of biofilms requires the synthesis of PNAG (polymeric N-acetylglucosamine), and the enzymes responsible for its synthesis are encoded by the *icaADBC* operon [4]. Additionally, the *rbf* gene promotes biofilm formation by *S. aureus* via repression of *icaR*, a negative regulator of the *icaADBC* operon [5]. The SarA protein, which is encoded by the *sarA* locus, is a positive regulator of PNAG-dependent *S. aureus* biofilm formation [6,7].

The microbial adherence to cells and extracellular matrix is considered as an essential first step in the process of colonization and infection [8]. A well characterized family of staphylococcal surface adhesins, called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) are known to mediate adherence to host extracellular matrix components, such as fibrinogen, fibronectin and collagen [9].

Among the *S. aureus* adhesin-coding genes, *fnbA*, *fnbB*, *spa*, *clfA*, *clfB*, *cna*, *bbp*, *ebps*, *map*/*eap*, *sdrC*, *sdrD* and *sdrE* are found (see **Table 1**). *S. aureus* strains harboring *fnbA* and *fnbB* have been found associated with invasive infections such as endocarditis, septic ar-

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Table 1. Virulence-related genes studied in the *S. aureus* strains [25].

Gene	Protein name	Function
	Adhesins	
clfA	Clumping factor A (ClfA)	Adhesin specific for fibrinogen
clfB	Clumping factor B (ClfB)	Adhesin specific for fibrinogen
fnbpA	Fibronectin-binding protein A (FnBPA)	Adhesin specific for fibronectin
fnbpB	Fibronectin-binding protein B (FnBPB)	Adhesin specific for fibronectin
cna	Collagen adhesin (Cna)	Adhesin specific for collagen
spa	*Staphylococcus* protein A (Spa)	Binds the Fc domain of immunoglobulins and von Willibrand factor
bbp	Sialoprotein-binding protein (Bbp)	Adhesin specific for bone sialoprotein
ebps	Elastin-binding protein of *Staphylococcus aureus* (EbpS)	Adhesin specific for elastin
map/*eap*	MHC class II analog protein or extracellular adhesion protein (Map/Eap)	Major histocompatibility complex class II analogue
sdrC, *sdrD* and *sdrE*	Serine aspartate repeat protein (SdrC, SdrD and SdrE)	Unknown; putative adhesins
	Toxins	
sea, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei* and *sej*	Enterotoxins A, B, C, D, E, G, H, I and J.	Exotoxins with superantigen activity
tst	Toxic shock syndrome toxin 1 (TSST-1)	Exotoxin with superantigen activity
eta and *etb*	Exfoliative toxins A and B	Exotoxin with superantigen activity
pvl	Panton-Valentine leukocidin (PVL)	Bicomponentleukocidin
hlg	Alpha-toxin (Hlg)	Bicomponentleukocidin
	Other genes	
efb	Extracellular fibrinogen-binding protein (Efb)	Extracellular fibrinogen-binding protein
*v*8	Serine protease V8	Serine protease
chp	Chemotaxis inhibitory protein of Staphylococcus *aureus* (CHIP)	Innate immune modulators
arcA	Arginine deiminase pathway	Important role in growth and survival

thritis and osteomyelitis [10]. It has also been shown that protein A (Spa) is an important virulence factor in septic arthritis models and subcutaneous infections in mice [11]. *S. aureus* strains deficient in CNA showed impaired capacity to produce endocarditis [12], osteomyelitis [13] and keratitis [14]. It has been shown that clumping factor A (ClfA) is an important *S. aureus* virulence factor in several experimental infection models, including rat endocarditis [15], mice arthritis [16] and rabbit infective endocarditis [17]. ClfA also inhibits phagocytosis by human polymorphonuclear leucocytes [18].

S. aureus is able to produce a great number of extracellular proteins, including enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and SEJ), Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin 1 (TSST-1), exfoliative toxin (ETA and ETB) and alpha toxin (Hlg), some of which can act as superantigens (**Table 1**).

Other virulence factors are encoded by the genes *chp*, *v*8, *arcA* and *coa* [19]. The expression of most of the virulence factors in *S. aureus* is controlled by the *agr* locus [20].

Despite the fact that severe catheter-related *S. aureus* infections have been reported [21-23], and that these infections have become important causes of morbidity, mortality, and a source of concern in the primary and emergency care context over the past decade [24], the *S. aureus* strains responsible for these infections have not been genetically characterized. Therefore, in order to gain some insight into the combinations of genes present in catheter-colonizing *S. aureus* strains, the presence of 35 virulence genes coding for adhesins, toxins, or other products was determined in 55 *S. aureus* strains isolated from tunneled hemodialysis catheters of Mexican patients with chronic renal failure being treated with hemodialysis. Hence we screened by PCR for the presence of 35 common *S. aureus* virulence-associated genes, including adhesin, toxin, superantigen, and biofilm formation genes. We then used real-time PCR to measure the expression of *icaA*, *rbf*, and *sarA* biofilm formation genes, as well as that of the global virulence gene regulator, *agr*. This study is the first report on the prevalence

of the 35 most common virulence-associated genes in *S. aureus* strains isolated from hemodialysis catheters. These findings will be of significant interest to health professionals and researchers alike, as the discovery of gene sets required for catheter colonization will allow for a more targeted approach to the development of treatment strategies, which would be of great interest to vaccine researchers.

2. Materials and Methods

2.1. Patients

A total of 109 patients presenting with symptoms of infection (e.g., erythema, inflammation, pain, and yellow suppuration) at the insertion site of a Mahurkar-type catheter were selected during September 2009 to May 2010. All of the patients had visited hemodialysis services at four public hospitals located in Estado de Mexico, Mexico [40.4% (n = 44) of the samples were from Hospital General Regional 72 del IMSS; 26.6% (n = 29) from Hospital General Regional N˚ 196 Aragón; 21.1% (n = 23) from ISSEMyM Satélite; and 11.9% (n = 13) of the samples were from Unidad Médica de Atención Ambulatoria (UMAA) N˚ 199]. The patients ranged in age from 17 - 77 years old, with 56% (n = 61) of the patients less than 50 years old. The causes of the chronic renal insufficiency were diabetes mellitus (33% n = 36), glomerulonephritis (17.4%, n = 19), hypertension (10.1%, n = 11), polycystic kidney disease (1.84%, n = 2), renal failure secondary to neurogenic bladder, and congenital malformation, lupus nephritis, or preeclampsia (each with 0.92%, n = 1); the cause was not determined in 34% of the patients (n = 37). The local ethics committee of each hospital approved the study. Inclusion criteria for choosing patients were: persons suffering chronic renal failure, with symptoms of infection and accepting to participate in the study by sign in the informed consent letter. Patients who were under treatment with antibiotics at the moment of sampling or during the last 30 days were excluded. Patients not accepting participate in the study were also excluded.

2.2. Sampling, Bacteria Identification, and PCR Conditions

After obtaining informed consent from each patient, samples were taken from the exterior terminal end of the catheter using sterile cotton swabs. The samples were placed in Stuart transport medium (BD Bioxon, Cuautitlán Izcalli, Edo. de Mexico, Mexico) and taken to the laboratory for further cultivation. For the primary phenotypical species identification of the *S. aureus* strains, tube-coagulase test (Bactident-coagulase, Merck, Darmstadt, Germany) and Api 32 Staph system (BioMerieux, Durham, NC, USA) were applied. This was confirmed by molecular detection of the *S. aureus* specific genes encoding 23S rRNA, thermostable nuclease (*nuc*), clumping factor (*clf*A), coagulase (*coa*) and protein A region X (*spa*), and the *femA* and *femB* genes. **Table 1** shows the virulence-related genes studied in the *S. aureus* strains. Bacterial DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI). One ml of an overnight culture was centrifuged at 13,000 rpm for 2 minutes and the pelleted cells were resuspended in 480 µL of 50 mM EDTA. A mixture of 60 µL of 10 mg/mL lysozyme (Sigma Cat. # L7651) and 60 µL of 10 mg/mL lysostaphin (Sigma Cat. # L7386) were added and the sample was incubated at 37˚C for 60 minutes. Afterwards sample was centrifuged 2 minutes at 13,000 rpm and the supernatant was removed. Six hundred microliters of Nuclei Lysis solution were added in order to resuspend the cells. Sample was incubated at 80˚C for 5 minutes to lyse the cells and then cooled to room temperature. Three microliters of RNase solution were added and the tube was inverted 5 times to mix. Sample was incubated at 37˚C for 60 minutes and then cooled to room temperature; 200 µL of Protein Precipitation Solution were added to the RNase-treated cell lysateand the mixture was vortexed vigorously at high speed for 20 seconds. Sample was incubated on ice for 5 minutes, centrifuged at 13,000 rpm for 3 minutes, and the supernatant containing the DNA was transferred to a clean 1.5 mL microcentrifuge tube containing 600 µL of room temperature isopropanol. The tube was mixed by inversion and centrifuged at 13,000 rpm for 2 minutes. Supernatant was poured off and the tube was drained on absorbent paper. Afterwards, 600 µL of room temperature 70% ethanol were added and the tube was gently inverted several times to wash the DNA pellet. The tube was centrifuged at 13,000 rpm for 2 minutes and the ethanol was aspirated. The tube was drained on absorbent paper and the pellet was allowed to air-dry for 15 minutes. One hundred microliters of DNA rehydration solution were added to the tube and the sample was incubated at 65˚C for 1 hour. DNA was stored at –20˚C until it was used.

PCR amplification of genetic markers was performed in a Corbette Research Thermocycler using PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Piscataway, New Jersey, USA). The amplified products were stained with ethidium bromide after electrophoresis in a 1% or 2% agarose gel. PCR primers, primers and probes for real time PCR and conditions used in PCR and real time PCR assays are listed in **Table 2**. The ATCC 33592 *S. aureus* strain was used as a positive control for PCR amplification of *fnbA*, *fnbB*, *spa*, *clfA*, *clfB*, *cna*, *ebps*, *map*/*eap*, *sdrC*, *sdrD*, *sdrE*, *icaA*, *rbf*, *sarA* and group I *agr* loci. For *bbp*, *eta*, *etb*, *hlg*, *pvl*, *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *tst*, *chp*, *efb*, *V*8, *arcA* and *coa*, the following strains from our collection were used, respec-

Table 2. PCR primers, primers and probes for real time PCR and conditions used in PCR and real time PCR assays.

Gene	Primers and probes descriptions	Sequence (5'-3')	Size of amplified product (bp)	PCR and Real Time PCR conditions cycling
23S rRNA	Forward	ACGGAGTTACAAAGGACGAC	1251	94˚C 5 min; 37 × (94˚C 40 s, 64˚C 60 s, 72˚C 75 s); 72˚C 10 min.
	Reverse	AGC TCAGCCTTAACGAGTAC		
femA	Forward	AGACAAATAGGAGTAATGAT	509	94˚C 5 min; 25 × (94˚C 60 s, 57˚C 60 s, 72˚C 60 s); 72˚C 10 min.
	Reverse	AAATCTAACACTGAGTGATA		
femB	Forward	TTACAGAGTTAACTGTTACC	651	
	Reverse	ATACAAATCCAGCACGCTCT		
nuc	Forward	GCGATTGATGGTGATACGGTT	279	94˚C 5 min; 37 × (94˚C 60 s, 55˚C 30 s, 72˚C 30 s); 72˚C 7 min.
	Reverse	ACGCAAGCCTTGACGAACTAAAGC		
mecA	Forward	GTAGAAATGACTGAACGTCCGATAA	310	94˚C 4 min; 30 × (94˚C 45 s, 50˚C 45 s, 72˚C 60 s); 72˚C 2 min.
	Reverse	CCAATTCCACATTGTTTCGGTCTAA		
fnbA	Forward	GCGGAGATCAAAGACAA	1279	94˚C 5 min; 30 × (94˚C 30 s, 50˚C 30 s, 72˚C 60 s); 72˚C 7 min.
	Reverse	CCATCTATAGCTGTGTGG		
fnbB	Forward	GGAGAAGGA ATTAAGGCG	812	
	Reverse	GCCGTCGCCTTGAGCGT		
spa (X-region)	Forward	CAAGCACCAAAAGAGGAA	Size polymorphisms	94˚C 5 min; 30 × (94˚C 60 s, 60˚C 60 s, 72˚C 60 s); 72˚C 7 min.
	Reverse	CACCAGGTTTAACGACAT		
clfA	Forward	GGCTTCAGTGCTTGTAGG	Size polymorphisms	94˚C 5 min; 35 × (94˚C 60 s, 57˚C 60 s, 72˚C 60 s); 72˚C 7 min.
	Reverse	TTTTCAGGGTCA ATATAAGC		
clfB	Forward	TGGCGGCAAATTTTACAGTGACAGA	404	95˚C 15 min; 32 × (95˚C 60 s, 60˚C 90 s, 72˚C 60 s); 72˚C 10 min.
	Reverse	AGAAATGTTCGCGCCATTTGGTTT		
cna	Forward	TTCACAAGCTTGGTATCAAGAGCATGG	452	
	Reverse	GAGTGCCTTCCCAAACCTTTTGAGC		
bbp	Forward	TCAAAAGAAAAGCCAATGGCAAACG	500	
	Reverse	ACCGTTGGCGTGTAACCTGCTG		
ebpS	Forward	GCAAGTAATAGTGCTTCTGCCGCTTCA	550	
	Reverse	CATTTTCCGGTGAACCTGAACCGTAGT		
map/eap	Forward	GCATGATAGAGGTATCGGGGAACGTG	665	
	Reverse	TCCCTTGATCATTTGCCATTGCTG		
sdrC	Forward	CGCATGGCAGTGAATACTGTTGCAGC	731	
	Reverse	GAAGTATCAGGGGTGAAACTATCCACAAATTG		
sdrD	Forward	CCACTGGAAATAAAGTTGAAGTTTCAACTGCC	467	
	Reverse	CCTGATTTAACTTTGTCATCAACTGTAATTTGTG		
sdrE	Forward	GCAGCAGCGCATGACGGTAAAG	894	
	Reverse	GTCGCCACCGCCAGTGTCATTA		
eta	Forward	CGCTGCGGACATTCCTACATGG	676	
	Reverse	TACATGCCCGCCACTTGCTTGT		
etb	Forward	GAAGCAGCCAAAAACCCATCGAA	419	
	Reverse	TGTTGTCCGCCTTTACCACTGTGAA		

Continued

hlg	Forward	TTGGCTGGGGAGTTGAAGCACA	306	
	Reverse	CGCCTGCCCAGTAGAAGCCATT		
pvl	Forward	TGCCAGACAATGAATTACCCCCATT	894	
	Reverse	TCTGCCATATGGTCCCCAACCA		
sea	Forward	TTGCAGGGAACAGCTTTAGGCAATC	252	
	Reverse	TGGTGTACCACCCGCACATTGA		
seb	Forward	GACATGATGCCTGCACCAGGAGA	355	
	Reverse	AACAAATCGTTAAAAACGGCGACACAG		
sec	Forward	CCCTACGCCAGATGAGTTGCACA	602	
	Reverse	CGCCTGGTGCAGGCATCATATC		
sed	Forward	GAAAGTGAGCAAGTTGGATAGATTGCGGCTAG	830	
	Reverse	CCGCGCTGTATTTTTCCTCCGAGAG		
see	Forward	TGCCCTAACGTTGACAACAAGTCCA	532	
	Reverse	TCCGTGTAAATAATGCCTTGCCTGAA		
seg	Forward	TGCTCAACCCGATCCTAAATTAGACGA	117	
	Reverse	CCTCTTCCTTCAACAGGTGGAGACG		
seh	Forward	CATTCACATCATATGCGAAAGCAGAAG	358	
	Reverse	GCACCAATCACCCTTTCCTGTGC		
sei	Forward	TGGAGGGGCCACTTTATCAGGA	220	
	Reverse	TCCATATTCTTTGCCTTTACCAGTG		
sej	Forward	CTCCCTGACGTTAACACTACTAATAACCC	432	
	Reverse	TATGGTGGAGTAACACTGCATCAAAA		
tst	Forward	AGCCCTGCTTTTACAAAAGGGGAAAA	306	
	Reverse	CCAATAACCACCCGTTTTATCGCTTG		
chp	Forward	AACGGCAGGAATCAGTACACACCATC	479	
	Reverse	GGCAAGTTATGAAATGTCTGCCAAACC		
efb	Forward	CGGTCCAAGAGAAAAGAAACCAGTGAG	303	
	Reverse	TGTGCTTTTCTGTGTGCACTGACAGTATG		
*V*8	Forward	CAACGAATGGTCATTATGCACCCGTA	529	
	Reverse	TTTGGTACACCGCCCCAATGAA		
arcA	Forward	CACGTAACTTGCTAGAACGAG	724	
	Reverse	GAGCCAGAAGTACGCGAG		
icaA	Forward	TCAGACACTTGCTGGCGCAGTC	936	
	Reverse	TCACGATTCTCTCCCTCTCTGCCATT		
rbf	Forward	GAATTCTAGAAAGAGGTAAAGTTATGGC	1200	
	Reverse	CACTCATAAAAGCTTCTTC		95˚C 5 min; 40 × (95˚C 30 s, 60˚C 60 s, 72˚C 30 s); 72˚C 10 min.
sarA	Forward	CAATCACTGTGTCTAATGAA	700	
	Reverse	GTGCCATTAGTGCAAACCTC		
coa	Forward	ATAGAGATGCTGGTACAGG	Size polymorphisms	95˚C 5 min; 30 × (94˚C 60 s, 58˚C 60 s, 72˚C 60 s); 72˚C 10 min.
	Reverse	GCTTCCGATTGTTCGATGC		

Continued

		***Agr* group-specific multiplex PCR**		
agr	PAN	ATGCACATGGTGCACATGC		94˚C 5 min; 26 × (94˚C 30 s, 55˚C 30 s, 72˚C 60 s); 72˚C 10 min.
	agr1	GTCACA AGTACTATA AGCTGCGAT	441	
	agr2	TATTACTAATTGAAA AGTGGCCATAGC	575	
	agr3	GTA ATGTAATAGCTTGTATAATAATACCCAG	323	
	agr4	CGATAATGCCGTAATACCCG	659	
		Real Time PCR		
icaA	Probe	TGGATGTTGGTTCCAGAAACATTGGGAG		95˚C 5 min (Hot Start activation); 40 × (95˚C 5 s, annealing/extension 60˚C 10 s).
	Forward	TGAACCGCTTGCCATGTG		
	Reverse	CACGCGTTGCTTCCAAAGA		
gyrB	Probe	CCGCCACCGCCGAATTTACCACCA		
	Forward	AGTAACGGATAACGGACGTGGTA		
	Reverse	CCAACACCATGTAAACCACCAGAT		
rbf	Forward	TTAGAAGGAATCTTTAA AACCTTATTGAATAA		
	Reverse	TTGTGAATTTTTCTTCTTCGGACA		
sarA	Forward	TTTTTTTACGTTGTTGTGCATTAACA		
	Reverse	CATTTAAACTACAAACAACCACAAGTTG		
agr	Forward	TGAAATTCGTAAGCATGACCCA		
	Reverse	CCATCGCTGCAACTTTGTAGAC		

tively: sa93, sa19, sa55, sa75, sa59, sa9, sa32, sa79, sa66, sa73, sa31, sa33, sa62, sa15, sa63, sa110, sa111, sa43, sa105 and sa35.

The expression of *gyrB*, *icaA*, *rbf*, *sarA* and *agrA* was measured using RT-PCR. To 500 µL of bacterial culture, grown in TSB at 37˚C for 24 h, 1000 µL of RNA Protect Bacteria Reagent (Qiagen, Hilden, Germany) were added. Sample was vortexed for 30 seconds and incubated at room temperature for 5 minutes. Sample was centrifuged at 9400 rpm for 10 minutes and the bacterial cells were resuspended in 200 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing 10 mg/mL lysozyme and 40 mg/mL lysostaphin. Sample was vortexed by 10 seconds and incubated at room temperature for 5 minutes. Afterwards, extraction and purification of total RNA was done using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, including on-column DNase treatment. The concentration and purity of total RNA were analyzed using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Inc.).

To obtain cDNA, a QuantiTec Reverse transcription kit (Qiagen), which includes genomic DNA elimination, was used according to the manufacturer's instructions. The *icaA* probe contained the fluorescent reporter dye FAM covalently linked to the 5'end and BHQ-1 covalently linked to the 3'end as a quencher. The *gyrB* probe contained the fluorescent reporter dye JOE covalently linked to the 5'end and BHQ-1 covalently linked to the 3'end as a quencher. A Rotor Gene Probe PCR Kit (Qiagen, Hilden, Germany) and Rotor Gene SYBR Green PCR Kit (Qiagen) kit were used for the real-time PCR assays.

The sa22, sa103 *S. aureus* clinical strains from our collection, *S. epidermidis* ATCC 35984 and *E. coli* ATCC 11775 were used as negative controls for the real-time PCR assays. *S. aureus* ATCC 33592 was used as the positive control.

2.3. Determination of Biofilm Formation

S. aureus biofilm formation was determined using the microtiter plate assay, as described in [15]. *S. epidermidis* ATCC 35984 and *S. epidermidis* ATCC 12228 were used as positive and negative controls, respectively. In this assay, polystyrene-adhered bacteria were stained with safranin, and the absorbance at 492 nm was recorded. Strains with an A_{492} less than 0.2, between 0.2 and 1.0, or greater than 1.0 were considered biofilm negative, weak biofilm formers, or strong biofilm formers, respectively [26].

2.4. Statistical Analysis

The frequencies of the virulence-related gene combina-

tions among the MRSA (*mecA*+) and MSSA (*mecA*−) *S. aureus* strains were analyzed using Chi-squared tests. A *p* value less than 0.005 was considered significant.

3. Results

Half of the catheter-isolated bacteria (55/109) were identified as *S. aureus* based on PCR amplification of the 23S rRNA, *nuc*, *clf*A, *coa*, *spa*, *femA* and *femB* genes. The remaining isolates (n = 54) were identified as *S. epidermidis*.

The most frequently detected virulence genes in the analyzed strains were i) adhesin-coding genes, including *spa* (100%), *clfA* (100%), *sdrD* (94.5%), *sdrE* (92.7%), *sdrC* (89%) and *ebps* (85.4%); ii) toxin-coding genes, including *hlg* (96.3%), *seg* (94.5%), *sei* (94.5%) and *seh* (78.1%); and iii) genes implicated in biofilm formation, including *icaA* (96.3%), *sarA* (74.5%) and *rbf* (40%) (**Table 3**). The *agr* operon was detected in all *S. aureus* strains; 16 strains harbored the group I *agr* locus, 29 the group II *agr* locus and 10 the group III *agr* locus (**Table 3**). Almost 82% of the strains were *mecA*+. None of the strains harbored the *pvl* or *sej* genes (**Table 3**).

The strains contained combinations of 15 to 25 of the 35 examined virulence genes (**Table 4**). Six strains (11%) carried a combination of 25 genes, with 12 genes coding for adhesins with affinity for fibronectin, fibrinogen and collagen and 5 genes coding for toxins, some of which can act as superantigens (**Tables 1** and **4**). Sixty percent of the strains carried a combination of the *seg*, *seh* and *sei* toxin genes (**Table 4**).

Fifteen strains carried six (*fnbA*, *cna*, *sdrE*, *eta*, *hlg*, *ica*) of the seven virulence genes that have been reported to be significantly more common in invasive *S. aureus* strains [19]. Six of these fifteen strains carried the 25-gene combination (**Table 4**), and the remaining nine strains each harbored a unique combination of virulence genes (data not shown).

3.1. Detection of *IcaA*, *Rbf*, *SarA* and *AgrA* Expression Using Real Time PCR

All of the *S. aureus* strains carrying *icaA* (n = 53), *rfb* (n = 22), *sarA* (n = 41) or *agr* (n = 55) expressed the genes (**Figures 1(a)-(d)**). Constitutive *gyrB* expression was used as a control (data not shown). The Tm was determined for each real time PCR run, when Rotor Gene SYBR Green PCR Kit was used, to differentiate specific amplification from nonspecific amplification (data not shown).

3.2. Determination of Biofilm Formation

Among the *S. aureus* strains studied, 70.9% (n = 39) were able to form a biofilm on polystyrene; 51% (n = 28) were weak biofilm formers, and 20% (n = 11) were strong biofilm formers.

Table 3. Frequency of *mecA* and virulence-related genes in *S. aureus* strains.

Gene	N° of strains positive for the gene (% of total)
Adhesins	
spa	55 (100)
clfA	55 (100)
sdrD	52 (94.5)
sdrE	51 (92.7)
sdrC	49 (89.0)
ebps	47 (85.4)
clfB	45 (81.8)
bbp	43 (78.2)
cna	43 (78.1)
map/eap	35 (63.6)
fnbB	31 (56.3)
fnbA	19 (34.5)
Toxins	
hlg	53 (96.3)
seg	52 (94.5)
sei	52 (94.5)
seh	43 (78.1)
sea	26 (47.2)
eta	23 (42.0)
see	15 (27.2)
tst	14 (25.4)
etb	13 (23.6)
seb	13 (23.6)
sed	6 (10.9)
sec	3 (5.4)
pvl	0 (0.0)
sej	0 (0.0)
Other genes	
icaA	53 (96.3)
ebf	51 (92.7)
*v*8	51 (92.7)
arcA	50 (90.9)
chp	49 (89.0)
mecA	45 (81.8)
sarA	41 (74.5)
rbf	22 (40.0)
***Agr* groups**	
*agr*II	29 (52.7)
*agr*I	16 (29.1)
*agr*III	10 (18.2)

Table 4. Combinations of virulence-related genes in *S. aureus* strains[1].

Virulence gene combinations in the *S. aureus* strains (*n* = 55)	N° of strains (%)	N° of genes per combination (*n* = 35) No. %
Adhesins: *spa*, *clfA*, *clfB*, *bbp*, *ebps*, *sdrC*, *sdrD*, *sdrE.*		
Toxins: *seg*, *seh*, *sei*, *hlg.*	10 (18.2)	20 (57.1)
Other genes: *icaA*, *sarA*, *chp*, *efb*, *v*8, *arcA*, *agr*, *mecA.*		
Adhesins: *spa*, *clfA*, *fnbA*, *fnbB*, *clfB*, *cna*, *bbp*, *ebps*, *map/eap*, *sdrC*, *sdrD*, *sdrE.*		
Toxins: *eta*, *hlg*, *seg*, *seh*, *sei.*	6 (11.0)	25 (71.4)
Other genes: *icaA*, *sarA*, *chp*, *efb*, *v*8, *arcA*, *agr*, *mecA.*		
Adhesins: *spa*, *clfA*, *bbp*, *ebps*, *map/eap*, *sdrC*, *sdrD*, *sdrE.*		
Toxins: *seg*, *seh*, *sei.*	5 (9.0)	17 (48.5)
Other genes: *icaA*, *chp*, *efb*, *v*8, *arcA*, *agr.*		
Adhesins: *spa*, *clfA*, *bbp*, *map/eap*, *sdrC*, *sdrD*, *sdrE.*		
Toxins: *hlg*, *seg*, *seh*, *sei.*	2 (3.6)	17 (48.5)
Other genes: *icaA*, *chp*, *efb*, *v*8, *arcA*, *agr.*		
Adhesins: *spa*, *clfA*, *clfB*, *cna*, *bbp*, *ebps*, *map/eap*, *sdrC*, *sdrE.*		
Toxins: *eta*, *tst*, *sea*, *hlg*, *seg*, *seh*, *sei.*	2 (3.6)	22 (62.8)
Other genes: *icaA*, *chp*, *efb*, *v*8, *agr*, *mecA.*		
Adhesins: *spa*, *clfA*, *cna*, *bbp*, *ebps*, *map/eap*, *sdrE.*		
Toxins: *hlg*, *seg*, *seh*, *sei.*	2 (3.6)	17 (48.5)
Other genes: *icaA*, *sarA*, *v*8, *arcA*, *agr*, *mecA.*		
Adhesins: *spa*, *clfA*, *clfB*, *ebps*, *sdrE.*		
Toxins: *hlg*, *seg*, *seh*, *sei.*	2 (3.6)	15 (42.8)
Other genes: *icaA*, *chp*, *efb*, *v*8, *agr*, *mecA.*		
Adhesins: *spa*, *clfA*, *fnbA*, *fnbB*, *clfB*, *cna*, *sdrD.*		
Toxins: *hlg*, *seg*, *sei.*	2 (3.6)	15 (42.8)
Other genes: *icaA*, *chp*, *arcA*, *agr*, *mecA.*		
Adhesins: *spa*, *clfA*, *sdrC*, *sdrD*, *sdrE.*		
Toxins: *hlg*, *seg*, *sei.*	2 (3.6)	15 (42.8)
Other genes: *icaA*, *sarA*, *chp*, *efb*, *arcA*, *agr*, *mecA.*		
Adhesins: *spa*, *clfA*, *cna*, *sdrC*, *sdrD*, *sdrE.*		
Toxins: *hlg*, *seg*, *seh*, *sei.*	2 (3.6)	18 (51.4)
Other genes: *icaA*, *rbf*, *sarA*, *efb*, *v*8, *arcA*, *agr*, *mecA.*		
Adhesins: *spa*, *clfA*, *fnbB*, *clfB*, *cna*, *ebps*, *sdrC*, *sdrD*, *sdrE.*		
Toxins: *hlg*, *seg*, *seh*, *sei.*	2 (3.6)	21 (60.0)
Other genes: *icaA*, *sarA*, *chp*, *efb*, *v*8, *arcA*, *agr*, *mecA.*		
Adhesins:, *spa*, *clfA*, *fnbB*, *clfB*, *cna*, *bbp*, *ebps*, *sdrC*, *sdrD*, *sdrE.*		
Toxins: *hlg.*	2 (3.6)	18 (51.4)
Other genes: *icaA*, *chp*, *efb*, *v*8, *arcA*, *agr*, *mecA.*		
Other combinations	16 (29.0)	
The most common combination of genes in the strains *icaA*, *agr*, *spa*, *clfA*, *sdrC*, *sdrD*, *sdrE*, *seg*, *seh*, *sei.*	37 (67.2)	10 (28.5)

[1]There were no statistically significant differences in the frequencies of the virulence-related gene combinations among the MRSA (*mecA*+) and MSSA (*mecA*−) strains ($p < 0.005$).

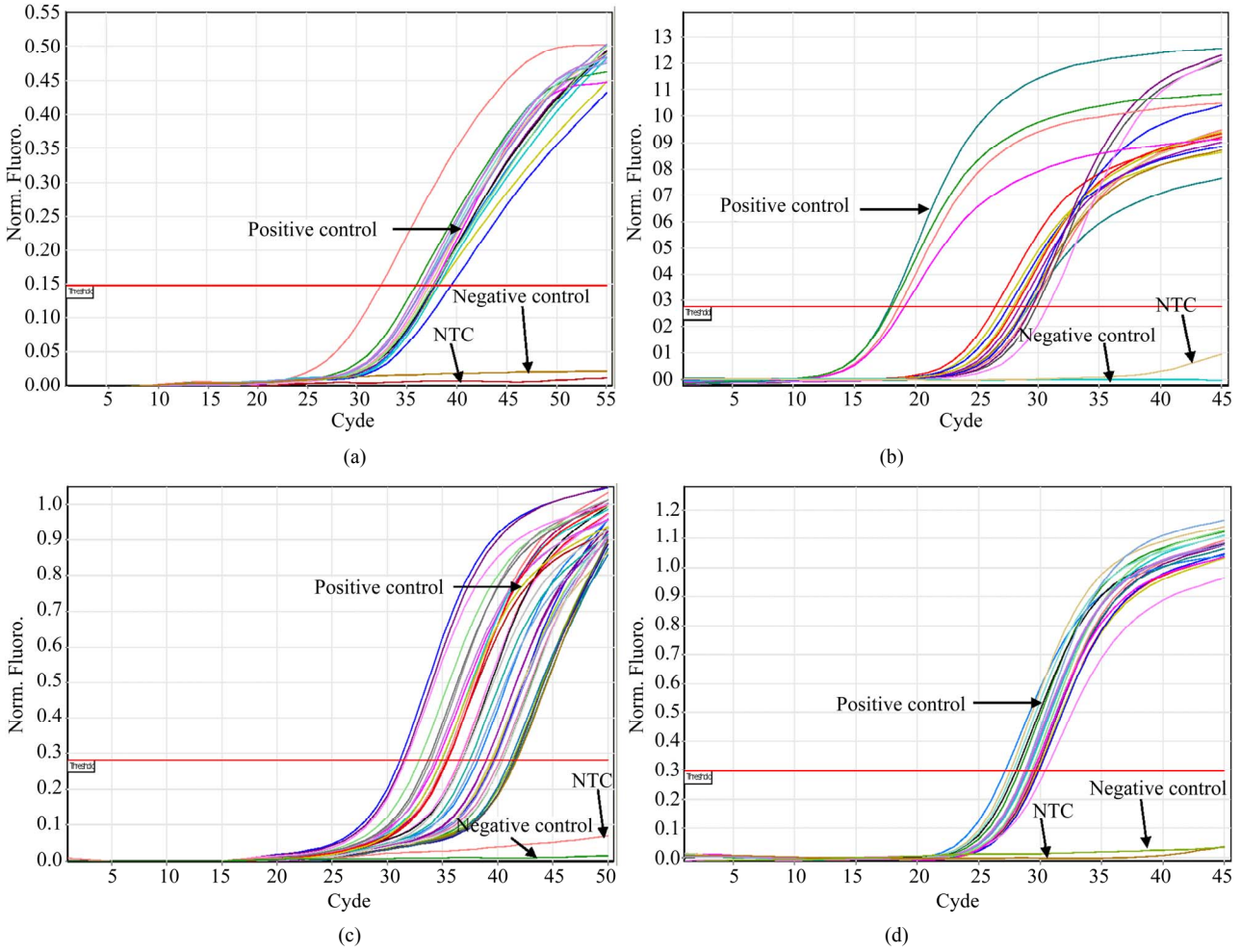

Figure 1. Detection of *icaA* (a), *rbf* (b), *sarA* (c), and *agrA* (d) expression in *S. aureus* strains isolated from catheters using real time PCR. *S. aureus* ATCC 33592 was used as a positive control; the negative controls were *S. epidermidis* ATCC 35984 (a), sa22 (b), sa103 (c) and *E. coli* ATCC 11775 (d). NTC indicates the no DNA control.

4. Discussion

In this study, we found that 55 of 109 catheters in hemodialysis patients harbored *S. aureus*. This high incidence of *S. aureus*-positive catheters may be due to a deficiency in the management of patients within the different hospital settings and/or the high rate of autoinfection by the patients' own indigenous *S. aureus*, which is present in their anterior nares [27].

Despite reports of severe catheter-related *S. aureus* infections [21-23,28], the *S. aureus* strains responsible for these infections have not been genetically characterized. While the genotype of *S. aureus* has been suggested to contribute to the severity of the infection [29], the *S. aureus* virulence factors that are involved in catheter colonization and the subsequent bacteremia are not known. Bacterial adhesins, toxins, and genes involved in bacterial dispersion may be involved in the development of catheter-related infections. Therefore, to gain some insight into the combinations of genes present in catheter-colonizing *S. aureus* strains, the presence of 35 virulence genes coding for adhesins, toxins, or other products was determined in *S. aureus* strains isolated from tunneled hemodialysis catheters. As expected, a large number of strains carried genes for at least five adhesins (maximum of 12, average of 7; **Table 4**), with the combination of *spa*, *clfA*, *sdrC*, *sdrD* and *sdrE* being the most frequently detected combination. The Sdr proteins in *S. aureus* are members of the MSCRAMMs family (Microbial Surface Components-Recognizing Adhesive Matrix Molecules) that are encoded by the tandemly arrayed *sdrC*, *sdrD* and *sdrE* genes [30]. Although the precise role of Sdr adhesins in staphylococcal infection is not known, a strong correlation between the *sdr* genes of *S. aureus* and certain diseases has been reported. There is a significantly increased prevalence of the *sdrE* gene in invasive *S. aureus* strains [31], in *S. aureus* strains responsible for osteomyelitis [32], and in *S. aureus* isolates responsible for bone infections [33].

Among the toxin genes, *hlg* (96.3%), *seg* (92.7%), *sei* (85.4%) and *seh* (78.1%) were the most frequently detected; no *sej*-positive or *pvl*-positive strains were found, and the most frequently observed combination was *seg*, *seh*, and *sei* (74.5%; **Table 4**). The frequency of toxin-encoding genes in the catheter-colonizing strains was higher than the previously reported frequency in a series of 100 MRSA isolates from hospital infections; with *seg* (77%) and *sei* (77%) being the most frequently detected toxin-encoding genes [34]. However, it has been reported that invasive *S. aureus* strains were not more likely than noninvasive strains to carry the *seg*, *seh* and *sei* genes [31].

The virulence markers *ebf*, *v*8, *arcA* and *chp* were identified in most *S. aureus* strains (**Table 2**), with frequencies similar to those reported for *S. aureus* strains isolated from complicated skin and skin-structure infections [35].

All strains carrying genes involved in biofilm formation and bacterial dispersion expressed the genes (**Figure 1**), and 70.9% of the examined strains formed a biofilm. This is an important bacterial virulence property as it has been reported that staphylococci account for more than 60% of all identified pathogens in central venous catheter-related infections [36]. Moreover, in a Center for Disease Control report of 2005, 15% of all reported MRSA infections occurred in dialysis patients [3], and a rate of 0.88 MRSA bacteremias/100 hemodialysis patients/year was reported in England for 2008-2009 [37].

Thirty-seven *S. aureus* isolates (67.2%) had the virulence gene combination of *icA*, *agr*, *spa*, *clfA*, *sdrC*, *sdrD*, *sdrE*, *seg*, *seh* and *sei* (**Table 4**), a combination that was detected in 16 different gene patterns (**Table 4**); 30 of these strains were *mecA*+. This result suggests that this gene combination may be important for successful catheter colonization. We conclude that these *S. aureus* strains containing such array of virulence factors are an element of risk for catheterized Mexican patients, which may acquire bacteremia or other pathologies.

These findings could be used to develop more targeted treatment strategies for *S. aureus* infections, which would be of great interest to vaccine researchers. The discovered gene combination could also be used to quickly identify *S. aureus* strains that are a greater threat to catheterized patients, which would warrant more aggressive treatment in a hospital setting.

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