JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2007, p. 725–729 Vol. 45, No. 3
0095-1137/07/$08.00+0 doi:10.1128/JCM.01572-06

Identification of the Capsular Polysaccharides in *Staphylococcus aureus* Clinical Isolates by PCR and Agglutination Tests▽

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Received 30 July 2006/Returned for modification 23 October 2006/Accepted 17 December 2006

Staphylococcus aureus **is a major cause of nosocomial and community-acquired infections. The predominance of two capsular polysaccharides, types 5 and 8, on the surface of clinical isolates led to the development of a conjugate vaccine (StaphVAX) based on capsular polysaccharides types 5 and 8 conjugated to a carrier protein. We have studied the capsular phenotypes and genotypes of 195 isolates representative of all clinical syndromes that encompassed both hospital and community-acquired infections. These isolates were mainly detected in France between January 2001 and December 2004. In this population, most of clinical isolates (87%) expressed either capsular polysaccharide type 5 (42%) or 8 (45%), whereas 13% were nontypeable by the serotyping method with antibodies specific to capsular polysaccharide type 5 or 8. These 26 nontypeable strains were further serotyped and were demonstrated to express the cell wall surface antigen 336, a polyribitol phosphate *N*-acetylglucosamine, which resembles cell wall teichoic acid. Among methicillin-resistant *Staphylococcus aureus* (MRSA) strains, we found a predominance of serotype 5 for 64% of strains, whereas MSSA isolates were predominantly capsular serotype 8 (60%). All *S. aureus* clinical isolates included in the present study have been investigated by PCR method, demonstrating that all isolates carried either the *cap5* or the *cap8* locus.**

Staphylococcus aureus is a major cause of nosocomial and community-acquired infections. The adaptation of *S. aureus* to the environment has been marked by the acquisition of methicillin-resistant *S. aureus* (MRSA) and the emergence of multiantibiotic resistance. At first, MRSA were described as hospital-acquired MRSA (HA-MRSA), but in recent years community-acquired MRSA (CA-MRSA) strains have been reported worldwide (4). With the emergence of this multiantibiotic resistance, including resistance to vancomycin, the antibiotic of last resort, new strategies are needed to manage staphylococcal infections.

Many virulence factors, including surface-associated adhesins, cytotoxins, superantigens, exoenzymes, and capsular polysaccharides, contribute to the pathogenesis of staphylococcal infections. The capsular polysaccharide or capsule is a cell wall bacterial component that protects bacterium from phagocytic uptake and enhances microbial virulence. Although 11 capsular polysaccharide types are described, only two types, type 5 and type 8, are clinically relevant in that they are predominant among clinical infection isolates of varied geographic origin (1, 10, 12, 16, 19, 20, 23). Although most *S. aureus* clinical infection isolates are capsular type 5 or 8, a capsule-based vaccine, StaphVAX (Nabi Biopharmaceuticals, Rockville, MD), is being developed as a new tool for staphylococcal infection prevention (5, 21, 22). StaphVAX is an *S. aureus* capsular polysaccharide bivalent conjugate vaccine, in which type 5 and type 8 capsular polysaccharides are chemically conjugated to a carrier protein.

Although the majority of *S. aureus* infection isolates are type 5 or 8, the remaining 10 to 20% of clinical isolates, which are nontypeable by serotyping methods, are serotype 336 (1, 10, 23). Type 336 isolates do not express capsule but do express cell surface polysaccharide or the 336 polysaccharide (336PS), which resembles *S. aureus* cell wall teichoic acid (14, 17). Thus, the determination of the 336 serotype prevalence among clinical isolates is important for vaccine development and a *S. aureus* 336-conjugate vaccine is currently under development (Nabi Biopharmaceuticals).

The aim of the present study was to characterize the capsular phenotype of 195 *S. aureus* isolates from diverse clinical diseases mainly from France. Capsular phenotypes were tested by a conventional serotyping method using antibodies specific to type 5 and type 8 capsule. Moreover, the agglutination test with 336 polysaccharide antibodies was included in the study for nontypeable strains by the serotyping method. In parallel, a PCR technique was developed to characterize the *S. aureus* isolates' capsular genotype. Analysis of the data was conducted to determine whether an association between capsular or 336 phenotype and capsular genotype exists.

MATERIALS AND METHODS

Collection of specimens. The French National Reference Center for Staphylococci (Lyon, France) collects more than 1,000 isolates yearly from patients with toxemic and nontoxemic staphylococcal diseases throughout France. For the present study, we selected a subset of 195 *S. aureus* isolates isolated between January 2001 and December 2004 mainly from France (179 of 195). The other isolates were from Japan ($n = 5$), the United States ($n = 2$), Algeria ($n = 2$), Spain ($n = 2$), China ($n = 2$), Romania ($n = 1$), Germany ($n = 1$), and Togo ($n = 1$). Among these strains, 126 were methicillin-sensitive *S. aureus* (MSSA) (65%) and 69 were MRSA (35%). The isolates classified by clinical syndromes

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▽ Published ahead of print on 3 January 2007.

TABLE 1. Distribution of capsular serotype determined by serotyping method among 195 clinical isolates by clinical disease[a]

Clinical disease	No. (%) of isolates												*P*
	MSSA				MRSA				Total				
	No.	Type 5	Type 8	NT	No.	Type 5	Type 8	NT	No.	Type 5	Type 8	NT	
Suppurative infections	113	36 (32)	64 (57)	13 (11)	59	37 (63)	10 (17)	12 (20)	172	73 (42.5)	74 (43)	25 (14.5)	NS
Skin and soft tissues	12	4	6	2	13	9	3	1	25	13	9	3	NS
Muskuloskeletal	12	7	4	1	8	4	1	3	20	11	5	4	NS
Genitourinary tract infection	2	1	1	0	1	0	0	1	3	1	1	1	NS
Non-necrotizing pneumonia	7	1	6	0	7	5	0	2	14	6	6	2	NS
Necrotizing pneumonia	46	8	34	4	6	1	4	1	52	9	38	5	<0.001
Infected endocarditis	3	1	1	1	0	0	0	0	3	1	1	1	NS
Multifocal infection	3	2	1	0	0	0	0	0	3	2	1	0	NS
Bacteremia	28	12	11	5	24	18	2	4	52	30	13	9	0.003
Toxin-associated infections	11	0 (0)	11 (100)	0	8	6 (75)	1 (12.5)	1 (12.5)	19	6 (32)	12 (63)	1 (5)	NS
Bullous impetigo	1	0	1	0	0	0	0	0	1	0	1	0	NS
Toxic shock syndrome	5	0	5	0	1	0	1	0	6	0	6	0	0.023
Scarlet fever	5	0	5	0	2	1	0	1	7	1	5	1	NS
NTED	0	0	0	0	5	5	0	0	5	5	0	0	0.027
Colonization	2	1 (50)	1 (50)	0 (0)	2	1 (50)	1 (50)	0 (0)	4	2 (50)	2 (50)	0 (0)	NS
Total	126	37 (29.3)	76 (60.3)	13 (10.3)	69	44 (64)	12 (17)	13 (19)	195	81 (42)	88 (45)	26 (13)	

[a] NT, nontypeable by serotyping with capsular type 5 and 8 antibodies; non-type 5 and non-type 8. NS, not significant.

were as follows. A total of 172 strains were involved in suppurative or invasive infections (52, necrotizing pneumonia; 52, associated with bacteremia; 25, skin and soft tissue infections; 20, musculoskeletal infections; 14, non-necrotizing pneumonia; 3, infected endocarditis; 3, genitourinary tract infections; 3, multifocal infections), 19 strains were involved in toxin-associated infections (13, scarlet fever or toxic shock syndrome; 5, neonatal toxic shock syndrome-like exanthematous disease [NTED]; 1, bullous impetigo), and 4 strains were isolated from asymptomatic carriers (Table 1). The strains involved in NTED were all isolated in Japan (24).

Identification of *Staphylococcus aureus* isolates. Species were identified by colony and microscopic morphology, coagulase activity on rabbit plasma (bioMérieux, Marcy l'Etoile, France), and production of clumping factor (Slidex Staph Plus; bioMérieux).

Capsular and surface polysaccharide typing. Capsular serotyping was performed for all strains. The strains were grown for 24 h at 37°C on Columbia agar plates containing 2% $MgCl_2$ and 0.5% $CaCl_2$. Several colonies of each strain were suspended in 0.9% saline and tested by slide agglutination with rabbit polyclonal antibodies specific to capsular polysaccharide types 5 and 8 (Nabi Biopharmaceuticals). Nontypeable strains (strains that did not react with antibodies specific to type 5 and type 8 capsular polysaccharide) were further tested with 336 polyclonal antibodies (Nabi Biopharmaceuticals). Sera for 336 serotyping was generated by immunizing New Zealand White rabbits with a 50-μg 336-rEPA conjugate vaccine in Freund adjuvant. Animals were bled, and the sera were stored at −70°C. Slide agglutination was determined to be positive if positive agglutination or clumping was observed in less than 20 s.

DNA extraction. Strains were grown on brain heart infusion agar or in brain heart infusion at 37°C overnight. Genomic DNA was extracted with a standard phenol-chloroform procedure as described elsewhere (3). Amplification of *gyrA* was used to confirm the quality of each DNA extract and the absence of PCR inhibitors.

Detection of capsular genotype by PCR method. Genomic DNA was used as a template for PCR amplification with the primers Cap5 k1 (5′-GTCAAAGATT ATGTGATGCTACTGAG-3′) and Cap5 k2 (5′-ACTTCGAATATAAACTTG AATCAATGTTATACAG-3′) located in *cap5k* for capsular type 5 and the primers Capsule 8 k1 (5′-GCCTTATGTTAGGTGATAAACC-3′) and Capsule 8 k2 (5′-GGAAAAACACTATCATAGCAGG-3′) located in *cap8I* for capsular type 8. Amplification was carried out on a PE-9600 thermocycler (Perkin-Elmer Corp., Norwalk, CT) under the following conditions: an initial 5-min denaturation step at 94°C, followed by 25 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 1 min of extension at 72°C; with a final extension step at 72°C for 5 min. PCR products were analyzed by electrophoresis on ethidium bromide-stained 1.5% agarose gels (Sigma, France). The sizes of the amplicons were 361 bp for capsular type 5 and 173 bp for capsular type 8.

Detection of the *mecA* gene. The *mecA* gene coding for methicillin resistance was detected by PCR as described by Murakami et al. (15).

Identification of *agr* alleles. The *agr* group (*agr-1* to *-4*) was determined by PCR as previously described (11).

Detection of toxin genes. Sequences specific for staphylococcal enterotoxin genes (*sea*), the toxic shock syndrome toxin gene (*tst*), and PVL genes (*lukS*-PV–*lukF*-PV) were detected by PCR as described elsewhere (11).

Statistical analysis. We used the chi-squared test to compare distribution of capsular polysaccharide types 5 and 8 and nontypeable strains among the clinical syndromes using SPSS software version 12.0 (SPSS, Inc., Chicago, IL).

RESULTS

Capsular serotype distribution. When using rabbit polyclonal antibodies specific to capsular polysaccharide types 5 and 8, 81 of the 195 isolates were capsular serotype 5 (T5) (42%), 88 were capsular serotype 8 (T8) (45%), and 26 (13%) were nontypeable (Table 1). The 126 MSSA strains had a capsular serotype 8 in 60.3% (76 of 126) and a capsular serotype 5 in 29.3% (37 of 126). Conversely, the 69 MRSA isolates that belonged to various hospital or community clones had more frequently a T5 for 64% of isolates (44 of 69) than a T8 for 17% of the isolates (12 of 69). The distribution of capsular serotype was classified according to the different *S. aureus* clinical diseases. For suppurative and/or invasive infections, the number of cases for each syndrome was relatively weak, but there was no significant difference between capsular type 5 and 8 for each clinical entity excepted for necrotizing pneumonia, for which 38 isolates were T8 and 9 isolates were T5 ($P < 0.001$) and bacteremia for which 30 isolates were T5 and 13 isolates were T8 ($P = 0.003$). For toxin-associated infections there were a significant difference for toxic shock syndrome, which had all T8 ($P = 0.023$) and for NTED which had all a T5 ($P = 0.027$) (Table 1).

Surface polysaccharide typing. Twenty-six isolates which did not react with T5 or T8 specific antibodies reacted positively with 336 polyclonal antibodies (Table 2); thus, these isolates

TABLE 2. Characteristics of 26 nonserotypeable isolates with antibodies specific for capsular polysaccharide type 5 or 8

Isolate no.	Capsular genotype	336 serotype	Infection characteristic	Clinical disease	*agr* type	Presence (+) or absence (−) of gene(s)			
						mecA	*lukS*-PV and *lukF*-PV	*tst*	*sea*
HT 2003 0151	5	+	Nosocomial	Muskuloskeletal infection	1	+	−	−	+
HT 2004 0020	5	+	Nosocomial	Muskuloskeletal infection	1	+	−	−	+
HT 2004 0230	5	+	Nosocomial	Urinary infection	1	+	−	−	+
HT 2004 0133	5	+	Nosocomial	Non-necrotizing pneumonia	1	+	−	−	+
HT 2004 0291	5	+	Nosocomial	Bacteremia	1	+	−	−	+
HT 2004 0231	5	+	Nosocomial	Bacteremia	1	+	−	−	+
HT 2002 0338	8	+	Community	Necrotizing pneumonia	3	+	+	−	−
HT 2001 0541	8	+	Unknown	Skin and soft tissues infection	3	+	+	+	−
HT 2003 0074	8	+	Unknown	Bacteremia	3	+	+	−	−
HT 2002 0665	8	+	Nosocomial	Muskuloskeletal infection	3	+	−	+	+
HT 2003 0039	8	+	Nosocomial	Bacteremia	3	+	−	−	+
HT 2002 0417	5	+	Unknown	Non-necrotizing pneumonia	2	+	−	+	−
HT 2004 0081	5	+	Unknown	Scarlet fever	1	+	−	−	−
A 98 0642	8	+	Community	Necrotizing pneumonia	3	−	+	−	+
HT 2002 0044	8	+	Community	Necrotizing pneumonia	3	−	+	−	−
HT 2002 0769	8	+	Community	Necrotizing pneumonia	3	−	+	−	−
HT 2001 0522	8	+	Unknown	Skin and soft tissues infection	3	−	+	−	−
HT 2003 0658	8	+	Community	Necrotising pneumonia	1	−	+	−	−
HT 2002 0602	8	+	Community	Muskuloskeletal infection	1	−	+	−	−
HT 2004 0145	8	+	Nosocomial	Bacteremia	1	−	−	−	−
HT 2001 0306	8	+	Community	Infected endocarditis	1	−	−	−	−
HT 2004 0238	8	+	Nosocomial	Bacteremia	1	−	−	−	−
HT 2004 0243	8	+	Nosocomial	Bacteremia	1	−	−	−	−
HT 2004 0112	8	+	Unknown	Bacteremia	2	−	−	−	−
HT 2004 0015	8	+	Unknown	Bacteremia	3	−	−	−	+
HT 2002 0072	8	+	Community	Skin and soft tissue infection	4	−	−	−	−

were defined as having the 336 serotype. These 26 isolates (13 MRSA and 13 MSSA) were involved in various infections: bacteremia ($n = 9$), necrotizing-pneumonia ($n = 5$), musculoskeletal infection ($n = 4$), skin or soft tissue infection ($n = 3$), non-necrotizing pneumonia ($n = 2$), urinary infection ($n = 1$), scarlet fever ($n = 1$), and infected endocarditis ($n = 1$). Six of MRSA strains had an *agr* type 1, harbored the *sea* gene, were associated with hospital-acquired infections, and corresponded to the Lyon clone, which is spreading in France (6). One strain corresponded to the major PVL-positive community-acquired MRSA (CA-MRSA) clone *agr-3* that spreading in Europe (25) and 1 isolate corresponded to a new emerging MRSA clone *tst*-positive *agr-2* responsible for either hospital- or community-acquired infections (3). MSSA isolates agglutinated by 336 antibodies were also associated with a diversity of infections: bacteremia ($n = 5$), necrotizing pneumonia ($n = 4$), skin or soft tissue infection ($n = 2$), infected endocarditis ($n = 1$), and musculoskeletal infection ($n = 1$), and they belonged to the four *agr* groups.

Capsular genotype. A PCR method was developed to detect capsular type of *S. aureus* isolates since serotyping method allowed typing of only 87% of strains (169 of 195). All strains included in the present study have been investigated by PCR method. PCR method allowed genotyping of 100% of strains, and all strains carried either the *cap5* (46% of cases) or *cap8* locus (54% of cases) (Table 3) and demonstrated that the capsular phenotype that was determined by serotyping method was confirmed by PCR. However, all 336 serotype strains ($n = 26$), strains that reacted specifically with 336 antibodies but not with capsular polysaccharide type 5 or 8 antibodies, carried the *cap8* or *cap5* genes (18 and 8 isolates) (Table 2).

Typing French MRSA strains. The present study included 69 MRSA isolates, and 61 of these were from France. The 61 French MRSA isolates belonged to various clones. Twenty-eight MRSA isolates corresponded to the major *sea*-positive *agr-1* MRSA clone spreading in French hospitals (6). Five MRSA isolates were PVL-positive *agr-3* and belonged to the major ST80 CA-MRSA clone spreading all over Europe (25).

TABLE 3. Determination of capsular polysaccharide type 5 or 8 by agglutination tests (serotype) and PCR (genotype) among 195 clinical isolates (126 MSSA and 69 MRSA)

Method	Isolate group	No. (%) of isolates		
		Capsular type 5	Capsular type 8	Nontypeable[c]
Agglutination[a]	MSSA isolates	37 (29.3)	76 (60.3)	13 (6.5)*
	MRSA isolates	44 (64)	12 (17)	13 (6.5)*
	Total isolates	81 (42)	88 (45)	26 (13)*
PCR[b]	MSSA isolates	37 (29.3)	89 (71)	0
	MRSA isolates	52 (75)	17 (25)	0
	Total isolates	89 (46)	106 (54)	0

[a] Serological typing by agglutination with antisera specific to capsular type 5 or type 8.
[b] Detection of capsule genotype with primers located in specific locus cap5 or cap8.
[c] *, Nontypeable by agglutination with antibodies specific for capsular polysaccharide type 5 or 8 but reacted positively with 336 polyclonal antibodies, and all carried the *cap8* or *cap5* genes.

Five others PVL-positive MRSA isolates belonged to various CA-MRSA clones rarely detected in France (ST1, ST5, ST30, ST37, and ST377) described elsewhere (8, 25). Eleven strains corresponded to a new emerging *tst*-positive *agr-2* MRSA clone responsible for either hospital- or community-acquired infections (3). Twelve remaining French MRSA isolates corresponded to minor clones (five strains had *agr* allele type 1, five strains had *agr* allele type 3, and two strains had *agr* allele type 2).

DISCUSSION

This study was designed to characterize the capsular phenotype of 195 *S. aureus* isolates from diverse clinical diseases which encompassed both hospital- and community-acquired infections. These isolates were mainly from France, and 35% were MRSA strains. The majority of these strains were involved in suppurative or invasive infections (172 strains), and among these strains necrotizing-pneumonia was strongly represented (30%) because of the gravity of this pathology (Table 1) (9). However, the present study included tests for surface polysaccharide 336 that were previously unavailable.

In this population most of clinical isolates (87%) expressed either capsular polysaccharide T5 (42%) or T8 (45%), and 13% of the clinical isolates were nontypeable with antibodies specific for capsular polysaccharide types 5 or 8. This distribution of serotype is similar to that observed in previous studies from diverse strain collections representing several geographic regions (1, 7, 10, 16, 23). Na'was et al. studied 254 strains involved in nosocomial infections (238 MSSA and 16 MRSA) and found that 43% were T5, 45% were T8, and 12% were nontypeable (16). Fournier et al. reported a similar distribution (43% were T5, 40% were T8, and 17% were nontypeable) among 406 clinical isolates from six French hospitals (7).

When we considered MRSA strains, 64% were T5, whereas 29.3% of the MSSA strains were T5 (Table 1). These results are in agreement with previous reports noting the predominance of the type 5 serotype among MRSA isolates (7, 16, 19). We also noted that the MSSA strains had a strong association with the type 8 serotype. For suppurative and/or invasive infections, when clinical isolates were classified by clinical syndrome there was a relative equal distribution of the capsular type for each clinical entity except for necrotizing pneumonia, for which we noted a significant predominance of T8, and for bacteremia, for which we noted a significant predominance of T5. These significant differences were not associated with an overrepresentation of clonal MRSA since most of the isolates from necrotizing-pneumonia were MSSA and since there was an approximately equal repartition of MRSA and MSSA isolates from bacteremia. Watts et al. showed that a T5 strain survived preferentially in the bloodstream of infected mice challenged with a mixed inoculum containing T5 and T8 strains (26). Na'was et al. showed in a previous study that capsular type 8 was the prevalent type in MSSA involved in non-necrotizing pneumonia (16). Isolates associated with toxin-associated syndromes, in particular strains involved in NTED, had all a T5. These strains isolated in Japan all corresponded to the New York/Japan clone. Strains involved in TSSS had all a T8, like previous results which showed that toxic shock syndrome toxin 1-positive strains were predominantly T8 (13). Thus, we confirm that capsular polysaccharide T5 and T8 are predominant among clinical isolates.

Although the type 5 and type 8 serotyping method allowed typing of only 87% of the strains (169 of 195), we developed a PCR method to characterize *S. aureus* capsular types at the molecular level. The *cap5* or *cap8* locus was present in all clinical isolates including the 26 strains found to be nontypeable by the serotyping method. Cocchiaro et al. observed the same results and showed that nontypeable strains positive for the *cap5* or *cap8* locus present a lack of capsule expression (2). Three mechanisms can account for the lack of capsule expression by *S. aureus* isolates that carry the *cap5* or *cap8* locus but fail to produce capsular polysaccharide: random point mutations in essential *cap* genes may result in nonfunctional biosynthetic enzymes; mutations in regulatory loci can also result in a capsular polysaccharide-negative phenotype and mutation in promoter can affect capsular polysaccharide production at the transcriptional level (2). Cocchiaro et al. suggest that only four capsular serotypes exist (1, 2, 5, and 8), although some of the older literature suggests that 11 serotypes exist (18, 23). These data are important for vaccine development because although 80 to 90% of clinical isolates produce capsular polysaccharide type 5 or 8, a type 5/type 8 capsular vaccine would be inadequate for 10 to 20% of strains that are nontypeable or noncapsular. These nonencapsulated strains can express the 336 polysaccharide. Antigen 336 has been described as a polyribitol-*N*-acetylglucosamine containing polymer that resembles cell wall teichoic acid (14, 17). In the present study, all nontypeable strains were demonstrated to have the 336 phenotype (Table 2). If we considered our results, while a capsular type 5 and 8 vaccine protects against 87% of *S. aureus* infection isolates, the addition of the 336 antigen to this vaccine should increase the coverage to 100% of all *S. aureus* infection isolates. Thus, a future generation of the vaccine to prevent staphylococcal infection could include antigen 336.

ACKNOWLEDGMENTS

We thank the clinicians and microbiologists who sent us clinical data and isolates. We thank Caroline Bouveyron, Christine Courtier, Christine Gardon, Lys Mayor, Celine Spinelli, and Lauren Tornetta for technical assistance.

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