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Rapid *Staphylococcus aureus agr* Type Determination by a Novel Multiplex Real-Time Quantitative PCR Assay

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The accessory gene regulator (*agr*) is a crucial regulatory component of *Staphylococcus aureus* involved in the control of bacterial virulence factor expression. We developed a real-time multiplex quantitative PCR assay for the rapid determination of *S. aureus agr* type. This assay represents a rapid and affordable alternative to sequence-based strategies for assessing relevant epidemiological information.

Staphylococcus aureus is a major pathogen responsible for both nosocomial and community-acquired infections. The severity of *S. aureus*-associated infections ranges from benign localized skin abscesses to life-threatening diseases, such as arthritis, osteomyelitis, and endocarditis (10). Population analyses based on molecular characterization have proven useful to establish relatedness between clinical isolates responsible for defined diseases. Recently, the presence of genes encoding either the Panton-Valentine leukocidin or other toxins in strains harboring a type IV staphylococcal cassette element were shown to describe community-onset methicillin-resistant strains (8, 15). Association of a particular *agr* type in clinical isolates harboring important virulence factors, such as toxic-shock syndrome toxin (TSST-1) (6) or exfoliatin toxin has already been observed (4). More recently, this association was also suggested for specific diseases such as bullous impetigo, involving strains from *agr* groups II and IV, and TSST-1-mediated diseases, mainly related to *agr* type III isolates (5). Another important study by von Eiff and colleagues reported the prevalence of *agr* type II among strains harboring the bicomponent toxin gene, *lukD-lukE*, isolated from anterior nares or blood (17). Finally, a possible link between specific *agr* types and vancomycin resistance was suggested (16).

The contribution of *agr* to *S. aureus* virulence has been clearly linked to its implication in gene regulation (11, 12) and bacterial interference (6). *S. aureus agr* is a 3-kb locus showing highly conserved and hypervariable regions (11) among *S. aureus* strains. The sequence of this hypervariable segment is the target of PCR amplification (5) for defining *agr* types (11).

The aim of this study was to develop a novel procedure for the rapid typing of *agr* to be used as a high-throughput epidemiological screening assay. Type-specific oligonucleotides targeting the variable moiety of the *agrC* gene encoding the receptor of the autoinducing peptide (11) were selected and validated against reference strains. Finally, *agr*-typed strains were subjected to a recently published genotyping assay (2) that yielded conserved and well-segregated clusters.

Strain collection. Eighty reference *Staphylococcus aureus* strains previously characterized by sequencing (1) were used; they consisted of sets of 20 strains of each *agr* type (types I to IV). In addition, sequenced strains COL, N315, and MW2, belonging to *agr* types I, II, and III, respectively, were simultaneously analyzed during these experiments.

Sequence analysis and primer and probe selection. Sequences of *agr* loci from types I to IV (see Table 1 for accession numbers) were aligned using ClustalW to localize conserved and divergent regions. The designing of type-specific oligonucleotides was performed in variable regions using the software PrimerExpress 2.0 (PE Biosystems, Foster City, CA). Analysis of the *agrC* and *agrD* regions (using SIM; http://www.expasy.org/tools/sim-prot.html) showed similarities ranging from 73 to 93% (considering alignment of *agr* types II and III as well as I and IV, respectively), whereas the conserved moiety showed >93% similarity. Based on these observations, minor groove binder (MGB) probes coupled to dark quenchers were designed to ensure optimal specificity (7) between the different alleles of the *agr* locus.

Bacterial lysis. Genomic DNA was extracted from one colony suspended in 200 μl Tris-EDTA buffer (10 mM Tris, 1 mM EDTA). A total of 100 mg of glass beads (diameter, 100 μm; Schieritz and Hauenstein, Switzerland) was added to the suspension, and bacteria were lysed by vortexing at maximum power for 45 s. The liquid phase was cleared from beads and bacterial debris by centrifugation and diluted 50-fold, and a 5-μl aliquot was used for real-time multiplex PCR assays.

Nucleic acid detection by real-time multiplex PCR and analysis. Each analysis was performed in triplicate; the nucleic acids from the reference strains were simultaneously assayed in each run. Conditions for the amplification on the SDS 7700 (Applied Biosystems) were the following: time 1 (t_1), 2 min at 50°C; t_2, 10 min at 95°C; t_3, 15 s at 95°C; and t_4, 1 min at 60°C (t_3 and t_4 were repeated 30 times). The volume of the PCR mixture (Eurogentec, Seraing, Belgium) was 20 μl and contained all primers and probes (Table 1) at the indicated concentrations. Fluorescent values recorded from cycles 3 to 15

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TABLE 1. Oligonucleotides used in the multiplex quantitative PCR assay

Primer or probe name	Sequence (5′→3′)	Length (bp)	5′ Dye	GenBank accession no.	Concn (nM)
agr type I				AF210055	
F_*agr*	CCAGCTATAATTAGTGGTATTAAGTACAGTAAACT	35			200
R_*agr*	AGGACGCGCTATCAAACATTTT	22			200
P_*agr*[a]	ATAGGAATTTCGACATTATC	20	FAM		100
agr type II				AF001782	
F_*agr*	CAATAGTAACAATTTTAGTGACCATGATCA	30			100
R_*agr*	GCAGGATCAGTAGTGTATTTTCTTAAAGTT	30			100
P_*agr*[a]	TTGCAACAGTAGGTTTGTT	19	TET		50
agr type III				AF001783	
F_*agr*	CATTATAACAATTTCACACAGCGTGTT	27			200
R_*agr*	GCAAGTGCATAAGAAATTGATACATACA	28			200
P_*agr*[a]	ATAGTTCTACCAATCTTTTTGG	22	VIC[b]		100
agr type IV				AF288215	
F_*agr*	GAGTTCTCAAAAAGATTAGCTCATCATATC	30			50
R_*agr*	TAGCTTCATCCGAGTTTATTTGAGAAT	27			50
P_*agr*[a]	TTCTACTGCTTACTTTTTCATTG	23	NED[b]		50

[a] Minor groove binder probes with a nonfluorescent quencher bound to the 3′ end (Applied Biosystems).
[b] Applied Biosystems.

were used to define fluorescent background levels (SDS 1.9 software; Applied Biosystems) using spectral compensation. Cycle thresholds were manually adjusted to 0.1, 0.14, 0.1, and 0.1 for 6-carboxyfluorescein (FAM), 6-carboxy-4,7,2′,7′-tetrachlorofluorescein (TET), NED, and VIC, respectively, to avoid possible cross-talk, mainly between FAM and TET. A reaction was considered positive when fluorescence levels exceeded the detection threshold between cycles 15 and 30.

Rapid genotyping. Rapid genotyping was performed using a recently published variable number of tandem repeats-based (VNTR) method (2), using eight primer pairs and a microcapillary electrophoresis system for the rapid evaluation of the VNTR profile.

This report describes a novel multiplex PCR assay that permits rapid determination of *agr* types based on the discriminatory capacities of short oligonucleotides selected within the

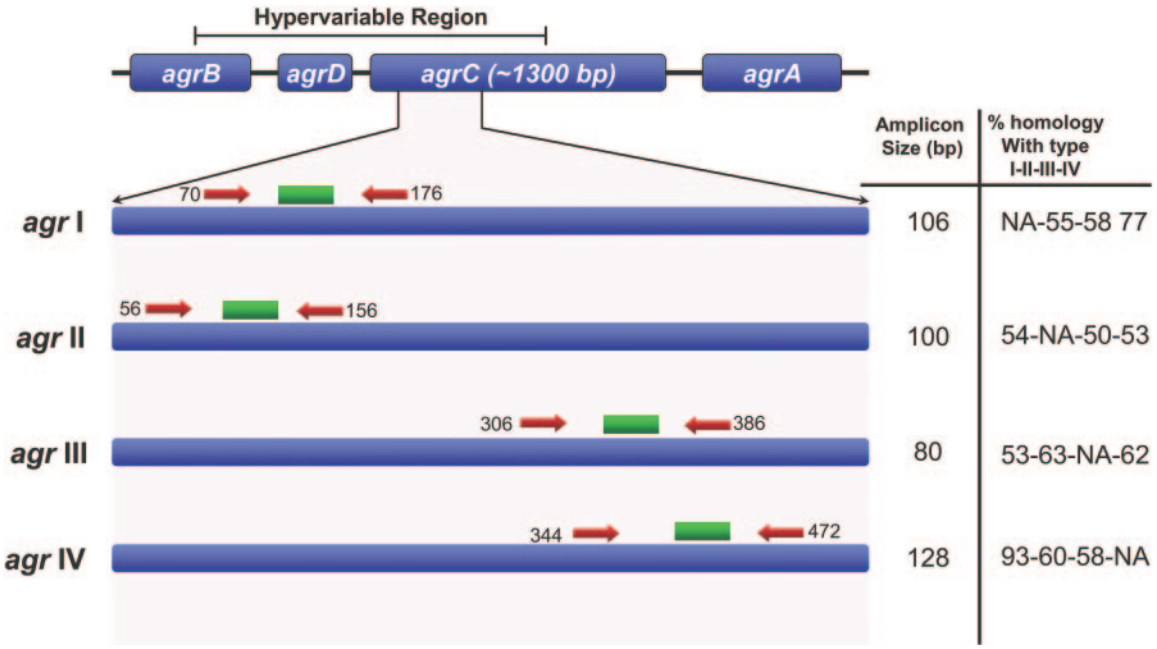

FIG. 1. Schematic representation of the *S. aureus agr* locus that contains variable and conserved regions. Numbers indicate oligonucleotide positions based on the 5′ to 3′ sequence. All primers and probes were selected in the first half of the *agrC* gene, in a region containing enough sequence divergence between the four *agr* groups to allow specificity of the PCR. The exact length of amplicon obtained for each *agr* type and the percentage of homology observed in the PCR-targeted region are also indicated. NA, not applicable.

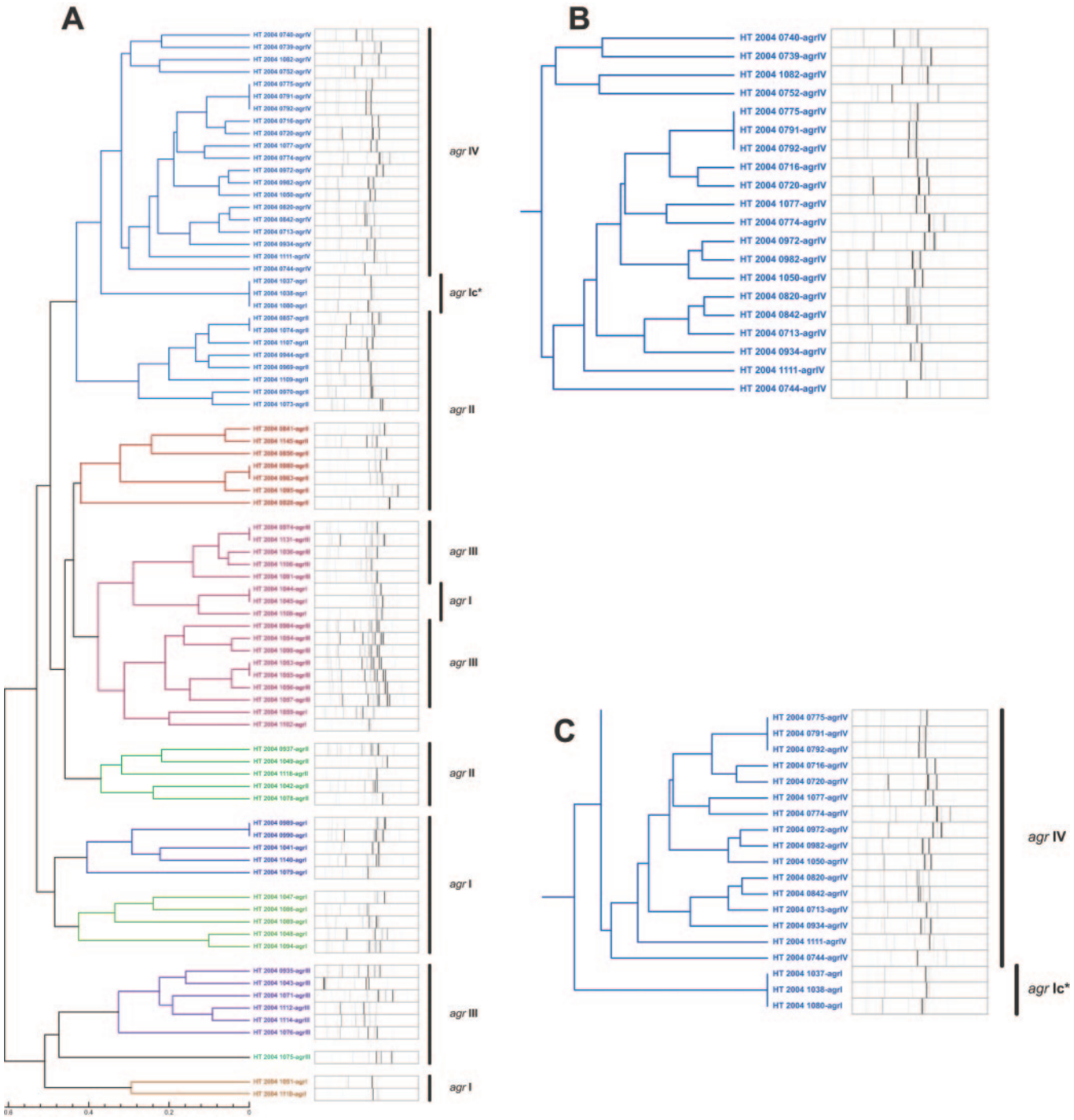

FIG. 2. Clustering tree obtained using high-throughput VNTR assay. (A) General view showing that strains segregated based on their *agr* type in groups of various sizes. (B) Magnification showing a single cluster of *agr* type IV. (C) Small cluster of misclassified *agr* type I coclustered with type IV strains. An asterisk indicates that precise typing was obtained from sequence data (GenBank accession number DQ435772) performed with primers F_agr type I and R_agr type IV (see Table 1).

hypervariable region of the *agr* locus. To date, the only procedure allowing *agr* typing relies on PCR amplification and separation of synthesized products (9) or gene sequencing (5, 14), a time-consuming and labor-intensive strategy. Particular attention was paid to the variable moiety of the *agrC* gene, providing sufficient divergence between each type to allow the selection of type-specific oligonucleotide sequences (Fig. 1). This rapid method was used on a collection of 80 strains originating from the collection of the French National Reference Centre for Staphylococci (this center receives around 1,000 *S. aureus* isolates per year for toxin gene determination) and constituted 20 strains of each type (I to IV). The procedure was

reproducible, specific, and fast, providing results similar to those of conventional sequence-based analysis. All type II to IV strains from the collection were correctly identified by the multiplex PCR assay. However, 18 out of 20 type I strains were confirmed by the multiplex assay, whereas 2 isolates were defined as type IV. Sequencing of the hypervariable region of the *agr* locus revealed that both strains were similar to *agr* type Ic, showing sequence segments of *agr* types I and IV (3) (G. Lina, personal communication). Finally, this new amplification reaction allowed us to rapidly classify *agr* types in approximately 3 h for a reasonable cost of $4.00 to $5.00 per strain, including the four probes and primers and the multiplex PCR enzymatic mixture. This unitary cost represents a drastic reduction compared to that of sequencing using fluorescently labeled nucleotide mixtures.

Rapid genotyping was performed to evaluate a possible link between the *agr* type and the genetic background of the four groups of strains. Figure 2 shows a clear cluster of *agr* group IV strains (Fig. 2B). Other strains are obviously not distributed randomly but constitute large or smaller clusters identified as related groups of strains segregating based on their *agr* type. Figure 2C shows a small group of two strains formerly identified as *agr* type I. Multiplex quantitative PCR reactions classified these strains between isolates from group I and group IV, as previously reported (3, 13). These rare strains harbored elements of variable regions from *agr* I and *agr* IV, and our primers and probe system hybridized with the *agr* IV moiety, thus explaining apparent discrepancies. These discrepancies are, however, rapidly identifiable by molecular assays. Rapid typing results are in concordance with the work of Goerke and colleagues showing that the diversity of type IV isolates is limited and that variation in types I and II is more frequent (3). As a consequence, Fig. 2 clearly shows small clusters of type I and II strains within type III isolates. Strains harboring the *agr* I locus showed the most dispersed pattern, a finding in accordance with the study of Robinson et al., suggesting that this type is probably the ancestor of other *agr* loci (13).

Overall, our data suggest that different *agr* types are distributed in various but limited numbers of genetic backgrounds. This observation is supported by Jarraud and colleagues, who showed a link between toxin patterns and genome contents, as obtained by amplified fragment length polymorphism, suggesting a possible relationship between genomic contents of isolates and specific human infections (5). In this context, our results suggest that a limited number of molecular tests allow the collecting of relevant epidemiological information useful for the characterization of clinical isolates.

In summary, this report describes a rapid, specific, and efficient multiplex PCR allowing the deciphering of important epidemiological information about *S. aureus* clinical isolates. The moderate turnaround time and reagent cost appear compatible with the utilization of this assay in routine laboratories, allowing rapid molecular determination of the *agr* loci of *S. aureus*.

Nucleotide sequence accession number. The nucleotide sequence referred to in the legend of Fig. 2 has been submitted to GenBank under the number DQ435772.

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