

## Molecular enrichment for detection of *S. aureus* in recreational waters

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### ABSTRACT

The identification of rapid methods for the control of recreational water and of aquatic environments with similar characteristics is necessary to provide adequate levels of health safety for users. Molecular techniques have been proposed in recent years as a viable alternative to traditional microbiological methods, as they offer various advantages and are less time consuming than traditional tests. An innovative protocol based on molecular enrichment that allows the identification of low concentrations of *Staphylococcus aureus* in recreational water has been developed. The method is based on the specific amplification of prokaryotic genomic DNA by the usage of universal primers for 23S rDNA; subsequently, a second amplification step is performed with specific real-time polymerase chain reaction (PCR) primers and probe. This approach shows sensitivity levels similar to those observed with microbiological tests, with the additional benefits of the specificity typical of nucleic acids techniques. This methodology is easily applicable also to other microbiological parameters, representing an important milestone in hygiene monitoring by the detection of specific pollution indicators.

**Key words** | molecular enrichment, real-time PCR, recreational water, *Staphylococcus aureus*, 23S rDNA

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### INTRODUCTION

In the last two decades a strong increase in sport activity has been reported in many western countries and a positive trend was also registered in swimming and physical activity in swimming pools (Vandelanotte *et al.* 2010). The increase of users in these recreational environments needs specific procedures for monitoring and maintaining hygiene and water quality (World Health Organization 2006). Microbial contamination is indeed one of the major health risks for swimming pool users and operators, due to the fact that pathogens can find favorable conditions for proliferation and infection. Main sources of this contamination are environment (especially for outdoor facilities) and swimmers. The bathers are largely responsible for the corruption of the water. It has been estimated that each swimmer can release into the pool a significant quantity of urine and sweat, with large amounts of microorganisms (Sato *et al.* 1995; De Laat *et al.* 2011). Moreover, the release of bacteria may be from the peeling of the skin, accidental

fecal release or residual fecal material on swimmers' bodies (World Health Organization 2006; Elmira *et al.* 2007).

The microbiological monitoring of water quality is an essential tool for maintenance of adequate hygienic conditions. This is done by using indicator microorganisms, which are easy to enumerate and are useful to measure the potential presence of hard-to-detect target pathogenic organisms. *Staphylococcus aureus* is an indicator of anthropogenic contamination, and in aquatic environments its presence has been related to the number of bathers; it is also considered a risk indicator for skin, eye and ear diseases (Gabutti *et al.* 2000). Different studies show that human body continuously releases large quantity of coagulase-positive staphylococci, in particular *S. aureus* (Alico & Dragonjac 1986). For these reasons, this microorganism can be suggested as an indicator of overcrowding in swimming pools and of the efficiency of recirculation and disinfection systems (AFSSET 2010).

Procedures for the detection of *Staphylococcus aureus* in water samples are based on the membrane filtration (MF) microbiological method. The past editions of *Standard Methods for the Examination of Water and Wastewater* have included two methods for the enumeration total of staphylococci or *S. aureus* in water samples. Unfortunately, these methods do not respond simultaneously to the requirements of specificity, selectivity and speed of detection. In particular, the simple identification of *S. aureus* by cultural methods and coagulase test can lead to incorrect classification, mainly due to the presence of different species of coagulase-positive staphylococci (cf Blaiotta et al. 2010), but also to the presence of *S. aureus* coagulase negative (Vandenesch et al. 1994). These points are well addressed by molecular approaches and in the last few years several authors proposed interesting protocols applied to the detection of indicator microorganisms in environmental water samples (Noble & Weisberg 2005; Brandi et al. 2007). These techniques are less time consuming and provide highly specific signals due to the fact that they are based on the identification of species-specific nucleotide sequences (Girones et al. 2010). In particular, real-time polymerase chain reaction (PCR) protocols allow the detection and quantization of bacterial species in an accurate and fast mode (Guillemet et al. 2010). A recent study showed that in less than 5 h without the need of culture enrichment, two Gram-negative bacterial species (*Escherichia coli* and *Shigella*) can be detected also at very low concentration (1 cell in 100 ml of water) by real-time PCR performed after a molecular enrichment step based on an isothermal procedure that amplifies genomic DNA (Maheux et al. 2011).

In the present work a different protocol, still built on the principle of a molecular enrichment, but based on an alternative strategy with specificity for prokaryotic DNA sequences, has been utilized in the detection and quantification of *S. aureus* contamination in recreational waters. The new method has been compared with real-time PCR without molecular enrichment and classical microbiological tests. This is the first time that a PCR approach has been suggested for the identification of *S. aureus* in recreational waters.

## MATERIALS AND METHOD

### Bacterial strains, culture and DNA preparation

The *S. aureus* strain used in the study was purchased from the American Type Culture Collection (ATCC). *S. aureus*

was grown at 37 °C in TSB (Tryptic Soy Broth, Oxoid, Germany) for 12 h before DNA extraction (GenElute™ Bacterial Genomic DNA Kit, Sigma Aldrich, USA). DNA from other bacteria species (*E. coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Lactobacillus acidophilus*, *Streptococcus agalactiae*, *Streptococcus salivarius*, *Legionella pneumophila* serogroup 1, *Legionella pneumophila* serogroup 10, *Lactobacillus crispatus*, *Lactobacillus gasseri*) were similarly extracted.

### DNA sequence analysis and design of the universal and specific primers and probe

Sequence alignments of 23S rDNA of several *Eubacteria* were performed by using MEGA software, version 5.0 (Tamura et al. 2011). In particular the following species were analyzed: *Staphylococcus aureus*, *Bacillus cereus*, *Listeria grayi*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Streptococcus parasanguinis*, *Streptococcus downei*, *Lactobacillus crispatus*, *Clostridium difficile*, *Escherichia coli*, *Salmonella enterica*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Legionella longbeachae*. Primer pairs and probes were designed using web-based Primer3 (v. 0.4.0) program and blasted against the other available sequences in GenBank by using BLAST network service. Moreover, gradient PCR of the universal primers was performed to determine the optimum annealing temperature (range between 40 and 68 °C).

### Detection of *S. aureus* in water samples

Several water samples with *S. aureus* titers ranging from 10<sup>1</sup> to 10<sup>3</sup> cfu/ml were prepared for tests. Aliquots of 5 ml were analyzed in duplicate by using both microbiological and molecular methods. The microbiological analysis was conducted by membrane filtration, using nitrocellulose filters (0.45 µm pore size) according to the Standard Methods (APAT-IRSA CNR 2003). The membrane was transferred aseptically in Baird Parker agar (tryptone 10.0 g/l, 'Lab-Lemco' powder 5.0 g/l, yeast extract 1.0 g/l, sodium pyruvate 10.0 g/l, glycine 12.0 g/l, lithium chloride 5.0 g/l, agar 20.0 g/l, pH 6.8 ± 0.2 at 25 °C, completed with 50 ml egg yolk tellurite emulsion equivalent to 3 ml of 3.5% potassium tellurite, Oxoid) and incubated at 37 ± 1 °C for 40 to 48 h. Gray-black shiny convex colonies with narrow white entire margin surrounded by zone of clearing (coagulase reaction) were identified as *S. aureus*. In the molecular analysis, the water aliquots were centrifuged at 4,000 × g for 10 min, bacterial pellet was resuspended in

200 µl of lysis solution buffer for Gram-positive bacteria supplemented with lysozyme (Sigma Aldrich) and transferred in 1.5 ml vial. Glass beads were then added, the pellet was homogenized by pestle and samples were incubated at 37 °C for 30 min. The extraction was then carried out according to the manual of the GenElute™ Bacterial Genomic DNA Kit (Sigma Aldrich). Final elution was performed in 60 µl. Four microliters were analyzed according the procedure described in the following section.

### PCR and real-time conditions

PCR was carried out in 25 µl reaction mixture consisting of 1 × *Taq* master mix (Promega, USA), 1 µM of forward and reverse universal primers (Table 1) and template DNA. Samples were amplified using an Thermal cyclers, Techne® TC-PLUS (VWR, Germany). Thermocycling conditions were as follows: 95 °C for 3 min, then 12 cycles of 95 °C, 30 s, 60 °C, 30 s, 72 °C, 1 min and 30 s, followed by 72 °C for 6 min. Real-time PCR was carried out in 25 µl reaction mixtures consisting of 1 × *TaqMan* master mix (Life Technology, USA), 900 nM of forward and reverse primers specific for *S. aureus* (Table 1), 250 nM of probe, and template DNA. Samples were amplified using an ABI Prism 7,000 sequence detection system (Life Technology). Thermocycling protocol consisted of melting at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min.

**Table 1** | Sequences of the oligonucleotides used in the assay

Oligonucleotide name	Sequence (5'-3')
universal_102_23S	GACCGATAGTGAACCAAGTACC
universal_160_23S	GAGCCGACATCGAGGTGCCA
RT-Sa_23F	CGTTAAGGCTGAGCTGTGAT
RT-Sa_23R	CTTTTCTCGGCAGTGTGAAA
RT-Sa_23Pr	JOE-AGACATTGAGTCTTCGAGTCGTTGA-BHQ1

### Standard curve for real-time quantification

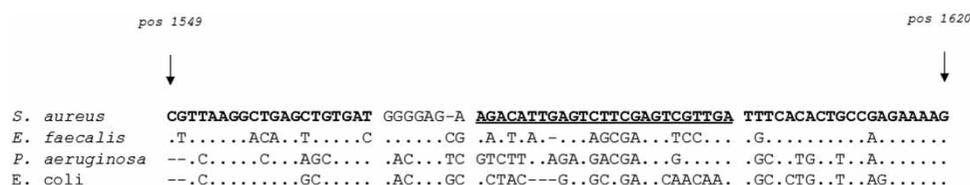
Serial 10-fold dilutions were performed after photometric determination of the DNA concentration of *S. aureus*. Four microliter aliquots of each dilution were used for PCR and real-time PCR (in triplicate) to generate the standard curve.

## RESULTS AND DISCUSSION

DNA sequence analysis of 15 *Eubacteria* allowed the identification of two primers (forward and reverse) annealing on all the genomes considered, generating an amplicon of approximately 1.5 kb (Table 1). In parallel, a set of oligonucleotides for real-time PCR (forward and reverse primers, FAM/quencher probe) was identified annealing specifically on the *S. aureus* sequence (Figure 1). The specificity of these oligonucleotides was also investigated with an alignment of nucleotide sequences of other species of the genus *Staphylococcus* (*S. intermedius*, *S. pseudointermedius*, *S. haemolyticus*, *S. epidermidis*, *S. capitis*): the consensus sequence obtained showed several points of mismatch, in particular in the annealing regions of the forward primer and of the labeled probe, underlining a theoretical specificity of these oligonucleotides for *S. aureus* genome (data not shown).

The universal primers were used in a PCR (molecular enrichment step) of only 12 cycles, in order to avoid to reaching the reaction plateau. In fact, an important consequence of reaching plateau is the increase of nonspecific products (due to mispriming events), and amplification bias that can strongly affect the representativeness of each molecule originally present in the template sample (Jung et al. 2000; Hawkins et al. 2002; Crotty et al. 2007).

Part of the molecular enrichment product (20% of the reaction) was then amplified in the real-time step. The choice to not use all the product in the next step of the assay, is mainly due to possible interferences due to different buffer compositions. The specificity of forward/reverse



**Figure 1** | DNA alignment of partial 23S rDNA of *S. aureus*, *E. faecalis*, *P. aeruginosa*, *E. coli*. Real-time PCR primers are represented in bold while probe is in bold and underlined. Nucleotide positions are referred to *S. aureus* gene for 23S rRNA (GenBank X68425.1).

primers and FAM/quencher probe for *S. aureus* was previously empirically verified on genomes from different bacterial species: a nonspecific signal was detectable at 36  $C_T$  only with *L. gasseri* and *E. faecalis* genomic DNA at a concentration of >100 ng per reaction. The nonspecific amplification can be explained by the elevated concentration of template DNA: in fact, the same genomic samples were negative when amplified at lower concentrations (<10 ng per reaction).

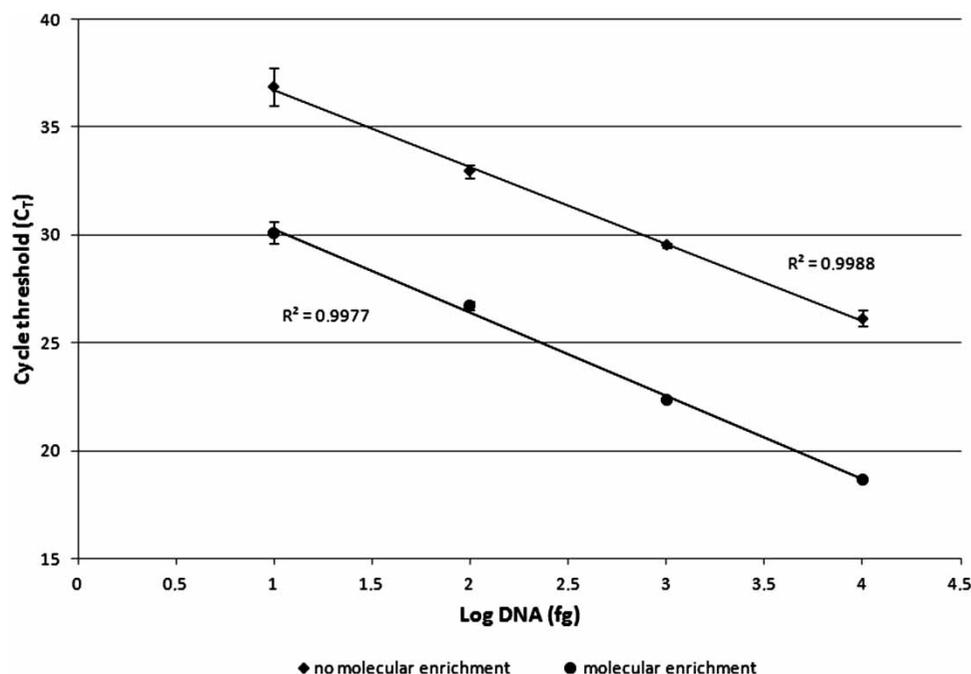
The identification of 23S gene as a target for this approach is based on the consideration that rRNA genes are highly conserved, allowing the design of universal primers annealing on genomes of bacteria belonging to different phyla. At the same time it is possible to identify more variable regions for the discrimination between close species, as well documented for lactic acid bacteria (Mohania et al. 2008).

In order to test sensitivity and linearity limits of the proposed approach, serial 10-fold dilutions of *S. aureus* genomic DNA were analyzed in triplicate, both with and without the molecular enrichment step. The results showed a constant gain of sensitivity in the molecular enrichment protocol, quantified in approximately 7  $C_T$ , for reaction starting from 10 fg–10 pg of template DNA (Figure 2). The two sets of data were characterized by a

correlation coefficient close to 1 ( $R^2 = 0.99$ ). This positive observation sustains the usage of this innovative protocol for the quantization of *S. aureus* in water samples, as observed in other studies utilizing real-time PCR for detection of bacteria (Shannon et al. 2007).

Moreover, the lower limit of detection was of 10 fg of DNA, equivalent to the genomic contents of 2–3 cells of *S. aureus* (Francois et al. 2003). This level of sensitivity is in line with several national laws that require detection of less than 10 cells per liter (cf. Italian Republic 2003).

In order to verify the reliability of the method on environmental samples, six water samples with *S. aureus* titers ranging from  $10^1$  to  $10^5$  cfu/ml were assayed. Each sample was microbiologically tested for the accurate detection of the number of *S. aureus* cells presents in 5 ml. Subsequently another aliquot of 5 ml has been processed for DNA extraction and PCR real-time analysis (with or without molecular enrichment). The results reported in Table 2 confirm the increment of sensitivity after molecular enrichment. In addition, considering the data reported in the plot in Figure 2 as standard curves, it is possible to estimate the number of genomes detected by the amplification reaction: the data obtained are comparable with the microbiological quantification.



**Figure 2** | Real-time PCR of 10-fold dilutions of *S. aureus* genomic DNA. Squares: samples analyzed without molecular enrichment step. Circles: samples analyzed with molecular enrichment step. Error bars represent standard deviation.

**Table 2** | Analysis of six environmental water samples characterized by different titer of *S. aureus*. 'MF': *S. aureus* microbiological titer determined by membrane filtration method. 'No. of genomes': estimated number of genomes present in 4  $\mu$ l of extracted DNA and amplified by PCR. 'C<sub>T</sub>': cycle threshold values with or without molecular enrichment. 'Genomes detected by amplification': extrapolation of the number of genomes detected by real-time PCR after molecular enrichment, considering the data reported in the plot in Figure 2 as standard curves

Sample	MF (cfu/5 ml)	No. of genomes	C <sub>T</sub> (w/o mol. enr.)	C <sub>T</sub> (with mol. enr.)	Genomes detected by amplification
1	$3 \times 10^1$	$2 \times 10^0$	34.33	28.68	5–7
2	$5 \times 10^2$	$3.3 \times 10^1$	29.42	25.65	25–27
3	$7.5 \times 10^3$	$5 \times 10^2$	32.12	25.49	25–27
4	$9 \times 10^5$	$6 \times 10^2$	26.86	21.94	250–500
5	$1.2 \times 10^5$	$8 \times 10^1$	28.70	24.98	50–75
6	$2 \times 10^2$	$1.3 \times 10^1$	33.95	28.87	5–7

## CONCLUSION

In conclusion, the method outlined here can be adopted for the estimation of *S. aureus* cells in water samples, with significantly reduced time to results when compared with standard microbiological tests. The technical approach proposed is not a conclusive validated method but a research output that clearly needs additional developments and comparative tests. The choice of universal oligonucleotides for the molecular enrichment step, is a key element for future expansion of the assay. An appropriate selection of primers/probes combination can lead to the assembling of multiplex real-time reactions that, starting from the same molecularly enriched samples, easily detect more bacterial species. This possible improvement clearly reduces the cost/benefits ratio.

On the other hand, molecular tests maintain a well known limit, due to the fact that they cannot discriminate between cultivable and noncultivable cells (including injured or dead bacteria). In this situation, it is possible to have an overestimation when compared with classical methods. For this reason many concerns have been raised for the application of nucleic acid techniques to drinking water analysis. While further comparison studies will be useful in determining this limit, in the case of *S. aureus* as indicator of overcrowding in swimming pools, the method outlined in this paper could be important, because it is able to correlate the total number of cells with the amount of bathers who access the facility.

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