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# The molecular enrichment approach for the identification of microbiological indicators in recreational waters ☆

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ABSTRACT

The identification of rapid methods for the surveillance of recreational waters and aquatic environments is necessary to provide adequate levels of health safety and quality standards. Molecular techniques have been proposed in recent years as a valuable alternative to traditional microbiological cultural methods, having numerous advantages (speed, specificity and sensibility). We have previously described a method for the detection of *Staphylococcus aureus* in recreational waters by a molecular enrichment approach, based on a two steps PCR performed on 23S rDNA. The approach has been now expanded to other microbiological indicators, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*. This methodological extension to other prokaryotic genomes has required a partial primer re-design and protocol optimization, in order to improve the assay. The usage of native not recombinant Taq-polymerases for detection of *E. coli* eliminated the background probably due to traces of the bacterial genome in many commercially available reagents. The obtained results clearly underline the feasibility of a fast and integrated molecular approach to quantify the standard biological indicators for assessment of safety and quality in recreational waters.



## 1. Introduction

Microbiological monitoring of water quality is an essential tool for the maintenance of adequate hygienic conditions in swimming pools and similar environments. The correct identification of indicator species is a key element for a successful surveillance. A correlation between standard indicators and associated human health risks was recognized in different epidemiological studies [1,2]. According to the World Health Organization guidelines, both environmental and human origin prokaryotic species can be considered as valuable indicators of contamination [3]. In particular *Escherichia coli*, *Enterococcus* spp., *Staphylococcus aureus* and *Pseudomonas aeruginosa* are adopted as microbiological parameters in a large number of local or national laws in Europe [4]. The detection of these bacteria occurs generally with standardized methods, mainly based on microbiological techniques [5]. Conventional microbiological methods, however, have a variety of drawbacks, in primis time consuming procedures [6]. In fact, traditional membrane filtration and most probable number (MPN) methods require 24 to 74 h for enumeration. The importance of reducing analysis time can be critical in outbreaks management, in order to limit dangerous exposure to the community [7]. In addition, for environments subject to rapid condition change, a microbiological datum obtained on a sample collected from a few days does not represent the actual situation, leading to possible wrong considerations on the true health risk [8,9]. The nucleic acid techniques (NATs), based on the identification of species-specific nucleotide sequences, can represent an important methodological improvement. The identification and characterization of microbiota and mfDNA has recently become a key technical approach in many medical fields [10]. NATs can bypass the limit of viable but non-culturable cells (VBNC) that are not visible by classical microbiological tests [11]. However, due to the fact that NATs identify all genomes present in different samples, dedicated sampling and processing are required in order to avoid DNA traces from death microorganisms.

Real-time polymerase chain reaction (PCR) protocols allow the fine discrimination also between closely related genomes, and by the adoption of adequate standard curves it is possible to calculate the bacterial load. Commercially available thermocyclers can simultaneously detect amplifications signals linked to several dyes, enabling multiplex reactions where more than two bacterial genomes can be analyzed. This molecular classification of microorganisms overcomes the problem of some growing media that do not allow the identification at level of species [12,13].

Recently we showed that an indicator of anthropogenic contamination, *S. aureus*, can be detected also at very low concentration by specific real-time PCR performed after a molecular enrichment step based on the amplification of prokaryotic genomic DNA by the usage of universal primers for 23S rDNA [14]. This approach has showed sensitivity levels similar to those observed with microbiological tests, with the additional benefits of the specificity typical of nucleic acid techniques and rapid analysis completion. The methodology is easily applicable also to

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**Table 1**
Sequences of oligonucleotides used in the study.

| Oligonucleotide name | Sequence (5′-3′) |
|---|---|
| universal_102_23S | GACCGATAGTGAACCAGTACC |
| universal_rev_23S | AGGAATTTCGCTACCTTAGGAC |
| RT-Sa_23F | CGTTAAGGCTGAGCTGTGAT |
| RT-Sa_23R | CTTTTCTCGGCAGTGTGAAA |
| RT-Sa_23Pr | JOE-AGACATTGAGTCTTCGAGTCGTTGA-BHQ1 |
| RT-Pa_23 F | TCCAAGTTTAAGGTGGTAGGCTG |
| RT-Pa_23 R | ACCACTTCGTCATCTAAAAGACGAC |
| RT-Pa_23 Pr | FAM-AGGTAAATCCGGGGTTTCAAGGCC-BHQ1 |
| RT-Ef_23 F | TAGCGGAGAAATTCCAAACG |
| RT-Ef_23 R | ACCTCCATCATTCTCAATTCC |
| RT-Ef_23 Pr | JOE-TTGGAGATAGCTGGTTCTCTCCGAAA-BHQ1 |
| RT-Ec_23 F | GTCCCGGTTTAAGCGTGTA |
| RT-Ec_23 R | AGGGCATTTGTTGCTTCAG |
| RT-Ec_23 Pr | FAM-AAATCCGGAAAATCAAGGCTGAG-BHQ1 |

**Table 2**
Specificity analysis of primers and probes used in this assay.

| Template DNA | CT (cycle threshold) | | | |
|---|---|---|---|---|
| | MIX 1 | MIX 2 | MIX 3 | MIX 4 |
| *S. aureus* | 26.15 ± 0.13 | NR | NR | 33 ± 0.19 |
| *E. faecalis* | NR | 17.53 ± 0.23 | NR | 32.39 ± 0. 46 |
| *P. aeruginosa* | NR | NR | 22.66 ± 1.97 | 32.94 ± 0.05 |
| *E. coli* | NR | NR | NR | 24 ± 0.23 |
| Nuclease free water negative control | NR | NR | NR | 33. 10 ± 0.32 |

NR = Not-reactivity; MIX 1 = RT-Sa_23F, RT-Sa_23R, RT-Sa_23PR; MIX 2 = RT-Ef_23F, RT-Ef_23R, RT-Ef_23PR; MIX 3 = RT-Pa_23F, RT-Pa_23R, RT-Pa_23PR; MIX 4 = RT-Ec_23F, RT-Ec_23R, RT-Ec_23PR;

other microbiological parameters, representing an important goal in hygiene monitoring by the detection of specific pollution indicators. In this work, a modified protocol dedicated to the simultaneous detection of *E. coli*, *Enterococcus faecalis*, *P. aeruginosa* is presented, laying the foundation for a possible multiplex real time analysis of the main bacterial indicators in swimming pool and other recreational environments.

## 2. Material and method

### 2.1. Bacterial strains, culture and DNA preparation

The following bacterial strains were used in the study: *E. coli* ATCC 35218, *S. aureus* ATCC 6538, *E. faecalis* ATCC 7080, *P. aeruginosa* ATCC 9027. Liquid growth was performed at 37 °C in TSB (Tryptic Soy Broth, Oxoid, Germany) for 12 h. DNA extraction was conducted with the GenElute™ Bacterial Genomic DNA Kit according to manufacturer's protocol (Sigma Aldrich, St. Louis, USA). DNA from other bacteria species (*Lactobacillus acidophilus*, *Streptococcus agalactiae*, *Streptococcus salivarius*, *Legionella pneumophila* serogroup 1, *L. pneumophila* serogroup 10, *Legionella* spp., *Lactobacillus crispatus*, *Lactobacillus gasseri*) was similarly extracted from clinical and environmental isolates.

### 2.2. Primers and probe design and test

Universal primers were designed as described previous by Valeriani et al. [14]. DNA alignment has been performed with MEGA software, version 5.0.

Universal primers were tested on above mentioned genomic DNA in PCR reaction with Taq master mix (Promega, USA) for 25 cycles: aliquots of each reaction were analyzed by gel electrophoresis (FlashGel DNA Cassette 1.2%, Lonza, Basel, Switzerland). Specific primer pairs and probes for the selected bacteria were designed using web-based Primer3 (v. 0.4.0) program and verified with NCBI/BLAST.

### 2.3. Molecular enrichment PCR and real-time conditions

Molecular enrichment 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 and template DNA (Table 1) on Techne® TC-PLUS thermalcycler (VWR International, LLC, Radnor, USA). Thermocycling conditions were as follows: 95 °C for 3 min, then 12 cycles of 95 °C, 30 s, 60 °C, 30 s, 72 °C, 2 min., followed by 72 °C for 6 min.

Real-time PCR was carried out according Valeriani et al. [14], using forward and reverse primers and probe specific for *E. coli*, *E. faecalis* and *P. aeruginosa* (Table 1).

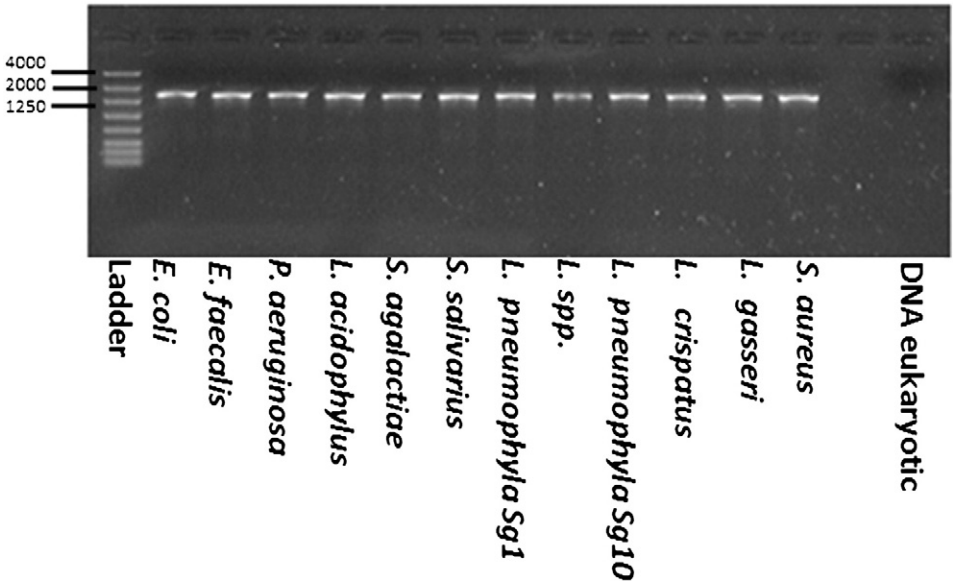


**Fig. 1.** Electrophoresis on agarose gel (1.2%) of the 1.5 kb PCR product generated by universal primers in several bacterial species.