*Analytical Letters*, 38: 1569–1586, 2005
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ISSN 0003-2719 print/1532-236X online
DOI: 10.1081/AL-200065797

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# Development and Comparative Evaluation of Different Screening Methods for Detection of *Staphylococcus aureus*

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**Abstract:** Different ELISA tests to detect and quantify levels of *S. aureus* in broth cultures were developed and compared. In all cases the assays were a modification of a "sandwich" format based on the use of common IgG as well as specific antibodies to bind protein A, an antigen localized in the cellular wall of *S. aureus* and partially extracted by boiling. Initially, human IgG was immobilized on the surface of microtitre plate wells in order to bind, by means of the Fc region, protein A that was present either in standard solutions or broth cultures of *S. aureus* treated by a boiling step.

Received 15 March 2005; accepted 5 April 2005

This work was supported by the Ministry of Health, special programs 1% *(ISPESL Cofin 2003)*, and by the Sixth Framework of European Commission Project "Validation and standardization of PCR-based methods for detection and quantitative risk assessment of foodborne pathogens (FoodPCR 2)."

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The sandwich format was completed using monoclonal (MAb) antibodies specific for protein A. The amount of bound antibody was evaluated using antiglobulins labelled with alkaline phosphatase ($Ab_2$-AP). Reading the absorbance at 405 nm a detection limit (LOD) of 0.6 ng/mL and $2 \times 10^6$ CFU/mL was found for protein A and for *S. aureus*, respectively. In order to improve the performance of the immunoassay, different approaches were pursued: an enzymatic amplification system (Ampli Q); the use of immunomagnetic beads employed both in a colorimetric (ELIMC = Enzyme-Linked Immunomagnetic Colorimetric) and in an electrochemical (ELIME = Enzyme-Linked Immunomagnetic Electrochemistry) assay. Using these systems the detection limit decreased by a factor of about 30-fold for Ampli Q and ELIMC, and about 2000-fold for ELIME formats. In addition, a qualitative polymerase chain reaction (PCR) method, using *nuc* gene primers, was set up and performed in parallel and its various parameters were optimized. This method was able to detect $10^2$ CFU/mL. In terms of minimum detectable concentration of *S. aureus* and total analysis time, the performance of the PCR assay and ELIME, turned out to be comparable.

**Keywords:** *Staphylococcus aureus*, ELISA, ELISA-AmpliQ, ELIMC, ELIME, PCR

## 1. INTRODUCTION

Food poisoning caused by the enterotoxins produced by *Staphylococcus aureus* is a major concern in food hygiene. This microorganism can produce several types (A, B, C, D, E) of enteroxins that cause gastroenteritis (Halpin-Diohnalek, and Marth 1989). Therefore, the presence of this bacterium in food can represent a health hazard when food is stored at temperatures that allow bacterial growth. The *S. aureus* is also an important cause of nosocomial infections (Refsahl and Andersen 1992; Silva et al. 2000).

At present the effective monitoring of this organism is difficult because the standard culture method (ISO 6888) requires almost 2 days to generate results relying as it does on the ability of bacteria to multiply in order to produce visible colonies. Moreover, an additional coagulase test is needed to confirm the identified colonies. For future application of the HACCP system (Van Schothorst and Jongeneel 1994) in which the control of pathogenic microorganisms requires actions at all levels of the transmission chain, there is a clear need for more rapid and efficient methods for detecting of pathogens (Letcher and Rand 1997; Babacan et al. 2002; Rishpon and Invitski 1997; Le et al. 1995; Croci et al. 2001; Perez et al. 1998).

In attempts to develop an alternative to the traditional detection methods some research laboratories have adopted polymerase chain reaction (PCR) technology for use in microbial diagnostics. Several reports based on PCR detection of *S. aureus* are present in the literature (Brakstad et al. 1992; Stuhlmeier and Stuhlmeier 2003; Letertre et al. 2003; Shrestha et al. 2002). Rapidity, low detection limit, good selectivity and sensitivity, along with the potential for automation, are among its important advantages. In spite of this, the use of PCR technology has remained limited to specialized research laboratories, because its high sensitivity could lead to false positive results from even a minute degree of contamination. Well-trained staff, the use of DNA-free reagents, special pipette tips, as well as separate clean pre- and post-PCR areas are required for the application of this method. An additional, potential disadvantage of this technique is the inhibition of the DNA polymerase reaction caused by many substances found in food, as has been reported by some authors (Rijpens et al. 1999).

Taking into consideration all these factors, the use of immunologically based methods remains an efficient and practical alternative for the detection of pathogenic bacteria. In choosing an antigenic marker for *Staphylococcus aureus*, one has to consider a spectrum of virulence factors including cytotoxic hemolysins, leukocidins, enterotoxins, teichoic acid, and protein A (Sompolinsky et al. 1985). Because 99% of *S. aureus* strains have protein A on the cell wall, this protein turns out to be the best marker for the identification of *S. aureus* (Chang and Huang 1995). Several immunoassays based on electrochemical, optical, and piezoelectric detection of *S. aureus* via protein A have been reported in the literature. Although electrochemical assays (Rishpon and Invitski 1997; Jenkins et al. 1991; Mirhabibollahi et al. 1990) are quite sensitive, in most cases the detection procedure for each sample was slow and technically difficult to perform. The fiber-optic sensor (Chang Y. H. et al. 1996) was applied only for the detection of protein A, while the piezoelectric immune-system (Le et al. 1985) was found to not be very sensitive. Moreover, because the crystals are manufactured at high cost they can not be used as disposable transducers; then, during repeated use it is problematical to dissociate the bound analyte from the coated piezoelectric crystal because the antibody-antigen interaction is usually very strong.

Conventional ELISA and ELIMC methods (the latter employs immunomagnetic beads), based on colorimetric detection, have also been reported in the literature for the determination of *S. aureus* and/or *Staphylococcus spp* respectively (Chang T.C. and Huang 1994). The use of these particles to separate and isolate cells of interest in mixed cell populations, via specific ligands attached to the particle surfaces, is described by Safarik and Safarikova (1999) and Safarik et al. (1995).

The immunomagnetic beads have been also coupled with electrochemical detection (ELIME) for rapid and sensitive determination of *Samonella Typhimurium* (Gehring et al. 1996) and *Escherichia coli* O157:H7 (Gehring et al. 1999).

Even though ELISA and ELIMC (for *Staphylococci)* have already been reported in the literature, the objective of this work was to carry out a systematic development and then comparison of different immunological screening methods for detection of *Staphylococcus aureus* using the same antibodies. In fact, a real comparison between the analytical performance of different immunological methods is possible only when the affinity of antibodies used to bind the antigen is the same.

Initially a conventional spectrophotometric assay, with p-nitrophenyl phosphate as substrate for the alkaline phosphatase enzyme label, was used. In order to improve the performance of this assay, three different approaches were pursued. The first one was designed to increase the colorimetric signal generated from the alkaline phosphatase (AP) by use of an enzymatic amplification system (Ampli Q). A second approach was the optimization of an immunoassay called ELIMC (enzyme-linked immunomagnetic colorimetric), which employs tosylactivated immunomagnetic beads (IMBs). A third approach was the development of an immunoassay termed enzyme-linked immunomagnetic electrochemistry (ELIME) in which the beads were localized onto the surface of a magnetized screen printed electrode (SPE).

The lowest detection limit was achieved using the ELIME method, which combines the selectivity of the antibodies with the sensitivity of the electrochemical detection and the possibility of concentrating the immunobeads onto the electrode surface. The enzymatic substrate, 1-naphthyl phosphate, was used and its conversion to an electroactive product (1-naphthol) was measured using differential pulse voltammetry (DPV).

In parallel, a PCR method using *nuc* gene primers was elaborated as an alternative to the immunoassays. The *nuc* gene codes for an extracellular thermonuclease that is produced at a level similar to that of the coagulase (Chesneau et al. 1993). An important modification of the original method was the inclusion of an internal control (IC) in order to identify possible inhibitors of the PCR reaction. This is essential for future applications to real samples, when it will be necessary to avoid false negative results.

## 2. EXPERIMENTAL

### 2.1. Reagents and Materials

Human IgG, protein A, mouse monoclonal antibodies (clone SPA-27, total $\mathrm{IgG} = 7.4\,\mathrm{mg/mL}$) against protein A, p-nitrophenyl phosphate (4-NPP), Tween 20, and bovine serum albumin (BSA) were purchased from Sigma, St. Louis, MO, USA. Antimouse IgG conjugate with alkaline phosphatase ($3\,\mathrm{mg/mL}$) was obtained from Vector Laboratories, Inc. Burlingame, CA, USA; nonfat dry milk, blotting grade, was from Bio-Rad Laboratories, Hercules, CA, USA; the enzymatic amplification system (Ampli Q) was from DAKO Cytomation, Ely, UK. Diethanolamine (DEA) and 1-naphthyl phosphate were purchased from Fluka Chemie, Buchs, Switzerland. Maxisorp$^{\mathrm{TM}}$ surface, 96-well polystyrene microtiter) plates were from Nunk, Roskilde, Denmark; tosylactivated Dynadeads$^{®}$ M-280 ($2 \times 10^9$ beads/mL, about $30\,\mathrm{mg/mL}$), a rotation device (Dynal sample mixer), and a magnetic particle concentrator (Dynal MPC) were from Dynal, Lake Success, NY. Bacterial strains, such as *S. aureus* (ATCC 29213), *S. epidermidis* (ATCC 12228), *S. xylosus* (ATCC 29971), *Streptococcus pyogenes* (ATCC 19615), *Salmonella enteritidis* (ATCC 13076), *Escherichia coli* (ATCC 12900), and all reagents used for culture media were from Oxoid Ltd. Basingstoke, UK. The two primers for *S. aureus* of 21 and 24 bases, 5′-GCGATTGATGGTG ATACGGTT-3′ (staphl) and 5′-AGCCAAGCCTTGACGAACTAAAGC-3′ (staph2) were purchased from M-Medical Genenco, Florence, Italy. The two primers for IC, 5′-GCGATTGATGGTGATACGGTT–CTGTCGTGCCAG CTGGATTA-3′ (staphl-pUC 19) and 5′-AGCCAAGCCTTGACGAACTA AAGC–TGAGCGAGGAAGCGGAAGA-3′ (staph2-pUC19), were also supplied by Medical Genenco. *Taq* DNA polymerase and deoxynucleoside triphosphate were from Applied Biosystems by Roche Molecular Systems, Inc., Branchburg, N.J., pUC19 plasmid (used to construct the internal control) and all other reagents of analytical grade were from Sigma.

### 2.2. Apparatus

For spectrophotometric analysis, a microtiter plate reader (model 550 from Bio-Rad) was used to measure the absorbance at 405 (conventional ELISA) and 490 (ELIMC) nm.

For electrochemical detection, all DPV measurements were performed using a computer-controlled polarographic analyzer, model 433 A (Amel, Milan, Italy). Screen printed electrodes (SPEs) used for DPV measurements were printed with a high performance multipurpose precision screen printer DEK 245 (DEK, Weymouth, UK). Inks were from Acheson Italia (Milan, Italy). The electrodes were printed according to a procedure that had been optimized in the Biosensors Laboratory of the University of Florence (Italy) (Cagnini et al. 1995). The resulting detection strip, which consists of a graphite working electrode, a silver pseudo-reference electrode, and a graphite counter electrode, forms a complete electrochemical cell. The diameter of the working electrode was 0.3 cm, which resulted in an apparent geometric area of 0.07 $\text{cm}^2$.

For PCR amplification, a thermal cycler Model 9700, from Applied Biosystems was used. A spectrophotometer (Biophotometer from Eppendorf AG, Hamburg, Germany) was employed to measure the concentration of the IC DNA.

### 2.3. Preparation of Bacterial Cultures

Pure cultures of *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus xylosus,* and *Streptococcus pyogenes* were grown in 10 mL of Brain Heart Infusion Broth (HBI) at 37°C for 24 h, while pure cultures of *Salmonella* and *E. coli* were grown in 10 mL of Tryptone Soya Broth (TSB) at 37°C for 24 h. The cultures were washed three times, in 0.9% NaCl solution by means of centrifugation (3000 g for 15 min). The microbial suspensions were standardized by turbidimetry (40% transmittance at 540 nm). A parallel count of each strains was done using PCA (Plate Count Agar).

For ELISA assay 10 millilitres of the broth cultures were centrifuged for 15 min at 3000 g (centrifuge PK 121 R, ALC International Srl, Cologno Monzese, Italy). The pellets were resuspended in a final volume of 10 mL with PBS (phosphate buffered saline) and boiled for 10 min; the volume was adjusted to the initial value (i.e., 10 mL). Aliquots of the suspensions were prepared and stored at −20°C for several months. The first immunochemical reaction for all developed immunoassays involved the binding of protein A to the Fc region of human IgG, which occurs with great avidity (Lindmark et al. 1983).

## 2.4. Conventional ELISA

Wells of the microtiter plates were coated with 200 μL of nonspecific human IgG (10 μg/mL) prepared in 0.05 M sodium carbonate buffer pH 9.6. After incubation at 4°C overnight, the wells were blocked [1 h at room temperature (RT)] with 3% dry milk suspension and then standard solutions of the test antigen (200 μL) were incubated 2 h at RT. The antigen was either a solution of protein A or boiled extracts of *S. aureus* cultures. After blocking, each well was incubated with 200 μL of mouse monoclonal antibodies diluted 1:10000 for 1 h at RT. Bound antigen was detected by adding 200 μL of antimouse IgG (1:1000 dilution) conjugated with alkaline phosphatase to each well, for 1 h at RT. Each solution, except the one used for the coating, was prepared in PBS.

Between each step (coating, blocking, human IgG/Ag binding, Ag/MAb binding, labelling with $Ab_2$-AP) a three-cycle washing procedure, using PBS containing 0.05% Tween 20, was adopted. Finally 200 μL of substrate solution (11 mM of 4-NPP in 0.1 M DEA buffer pH $9.8 + 1$ mM $MgCl_2 + 0.15$ M KCl) were added to each well. The enzyme reaction was allowed to proceed for 30 min and the formation of the yellow product of the enzyme reaction was monitored spectrophotometrically at 405 nm. The scheme of the ELISA format is reported in Fig. 1.

## 2.5. ELISA-Ampli Q

Ampli Q is a unique enzyme cycling system designed to amplify the colorimetric signal generated by alkaline phosphatase. The enzymatic reactions involved are the following:

$$\text{NADPH} - \text{AP} \longrightarrow \text{NADH} + \text{P}_i$$

$$\text{NADH} + \text{INT} - \text{DIAPHORASE} \longrightarrow \text{formazan} + \text{NAD}^+$$

$$(\text{INT} = \text{p-iodonitrotetrazolium})$$

$$\text{Ethanol} + \text{NAD}^+ - \text{ADH} \longrightarrow \text{acetaldehyde} + \text{NADH}$$

$$(\text{ADH} = \text{alcohol-dehydrogenase})$$

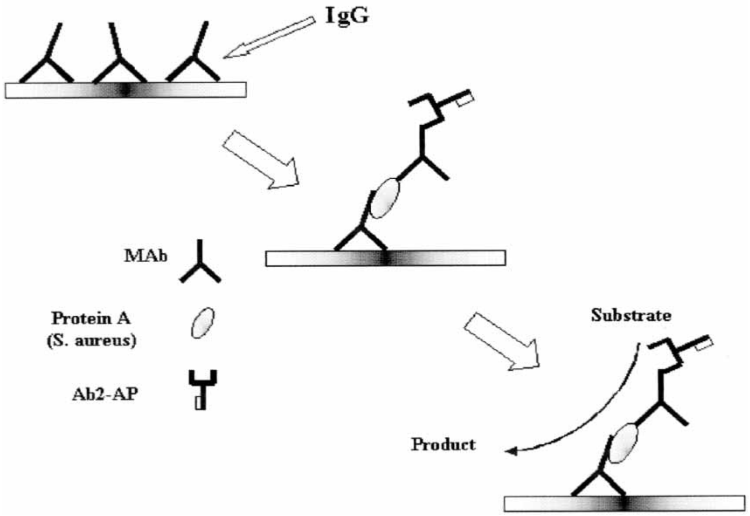

*Figure 1.* Immunoassay scheme.

The two enzymes in the "amplifier" (diaphorase and alcohol dehydrogenase) catalyze the redox cycle, which interconverts NADH and $NAD^+$, and for each turn of the cycle a molecule of colored formazan is generated.

The procedure employed was the same used for the conventional ELISA, but at the end of the immunological chain, 200 μL of the Ampli Q solution was added to each well. After 15 min of incubation the absorbance was read at 490 nm.

## 2.6. ELIMC

This method employs a washing, a coating, and an immunoassay procedure followed by colorimetric detection.

### Washing and Coating Procedures

After resuspension of the tosylactivated Dynabeads M-280, 1 mL ($2 \times 10^9$ beads) was pipetted into 2 mL Eppendorf tube and washed for two times in 1 mL of 0.1 M borate buffer pH 9.5, according with Dynal instructions. Then 200 μL of human IgG (3 mg/mL, prepared in NaCl 150 mM) was added to 1 mL of particles (final IgG concentration of 0.5 mg/mL) and incubate for 20 h at 37°C with slow tilt rotation (using Dynal sample mixer). After incubation, the tube was placed in the magnetic particle concentrator, the supernatant was pipetted off, and the coated beads were washed four times following Dynal instructions.

After washing, the particles were resuspended in 1 mL of PBS pH 7.4 + 0.1% (w/v) BSA and 0.02% $NaN_3$; and the dynabeads were stored (for up to several months) at 4°C.

### Immunoassay Procedure

Dynabeads were then used as a solid phase in immunoassay employing the following steps: (a) the suspension stored at 4°C was shaken and 10 μL transferred into to 2 mL Eppendorf tube (for each *S. aureus* concentration to analyze); (b) the particles were quickly washed three times with 1 mL of PBS pH 7.4 with 0.1% (w/v) BSA; (c) blocked with 1 mL of 3% dry milk suspension for 30 min; (d) 400 μL of *S. aureus* standard solutions (ranging from $10^3$–$10^8$ cells/mL) were added to Dynabeads and incubated for 1 h; (e) 400 μL of MAb (1:50000) were added and incubated for 30 min; (f) 400 μL of $Ab_2$-AP (1:300) were added and incubated for 30 min; (g) the particles were quickly washed three times with 1 mL of PBS pH 7.4 + 0.05% (w/v) Tween 20.

The solutions for steps c, d, e, f were prepared in PBS and the incubation was performed with slow tilt rotation.

Finally, 400 μL of substrate solution (11 mM of 4-NPP in 0.97 M DEA buffer pH 9.8 + 1 mM $MgCl_2$ + 0.15 M KCl) were added to each tube and after 30 min of incubation the colored solution was transferred to a microtiter plate and the absorbance was read at 405 nm.

## 2.7. ELIME

Washing, coating, and immunoassay procedures adopted for the ELIME method, were similar to those employed for ELIMC. Only the immunoreagent concentrations and the detection procedure were changed.

For the coating of the beads, 600 μL of human IgG (3 mg/mL) were added to 0.9 mL of particles (final concentration of IgG was 1.2 mg/mL), while for the immunoassay the best results were obtained using 400 μL of MAb diluted 1:1000 and 400 μL of $Ab_2$-AP diluted 1:100. After incubation (30 min) with $Ab_2$ AP and washing (for three times) with 1 mL of PBS pH 7.4 + 0.05% (w/v) Tween 20, the particles were resuspended in 100 μL of PBS and 10 μL of this suspension was localized (with the aid of a small magnet placed underneath) on the working electrode surface of the SPE. The antibody *S. aureus* complex was revealed by adding 40 μL of the substrate solution [1 mg/mL of 1-naphthyl phosphate in 0.97 M diethanolamine buffer (DEA), pH 9.8, +1 mM $MgCl_2$ + 0.15 M KCl] to cover the three electrode system. After 2 min of incubation, the current response was measured with DPV. All DPV measurements were performed in the potential range 0– +600 mV vs. a silver screen–printed pseudo-reference electrode, with a modulation time (pulse time) of 60 ms, a modulation amplitude (pulse amplitude) of 50 mV and a scan speed of 100 mV/s.

All data points were carried out in triplicate and each value was the average of three determinations.

## 2.8. PCR/Internal Control Construction

The internal control was constructed according to the procedure of A. Abdulmawjood et al. (Abdulmawjood et al. 2002), using the commercial plasmid pUC19 as a template. The primers used to amplify the IC, elaborated using Primer Express 1.5 software, generate a 120-bp product. The length of the amplicon was chosen so as to be sufficiently different from the molecular weights of the possible primer dimers and of the DNA target (*S. aureus*) and, thus, it can be easily identifiable using agarose gel electrophoresis.

One microliter of pUC19 was transferred to a vial containing: 50 μL of a mixture of 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris HC1 (pH 8.3), 200 μM of each deoxynucleoside triphosphate, 1 μM of each primer staphl-pUC19 and staph2-pUC19, and 2.5 U *of Taq* polymerase.

A 30-cycle PCR was carried out using the following conditions: denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 2 min, followed by a final extension incubation at 72°C for 10 min.

Experiments were then conducted to establish the IC concentration so that the amplicon-specific band was always present in the staphylococcus-negative broth cultures and present or absent in the staphylococcus-positive broth cultures, depending on the staphylococcus concentration. For this purpose, 10-fold dilutions of IC (ranging from 80 μg/μL to 80 fg/μL) were tested in the presence of different concentrations of broth cultures of *S. aureus* (10 to $10^8$ CFU/mL).

## 2.9. PCR/DNA Extraction

One milliliter of a broth culture of *S. aureus* ($10^9$ CFU/mL) was centrifuged for 15 min at 14000 g. The pellet was resuspended in a final volume of 100 μL with Dnase-Rnase-free distilled water (Sigma) and boiled for 10 min. The suspension was centrifuged again at 14000 g for 2 min and the supernatant was recovered.

## 2.10. PCR/Coamplification of *S. aureus* Fragment and Internal Control DNA

Serial dilutions ($10^8$–10 CFU/mL) of the supernatant recovered during the DNA extraction were prepared and a five microliter aliquot of each dilution was transferred to a tube containing 50 μL of a mixture of 50 mM KCl, 3 mM $MgCl_2$, 10 mM Tris HCl (pH 8.3), 200 μM of each deoxynucleoside triphosphate, 1 μM of each primer staph1 and staph2, 2.5 U of *Taq* polymerase, and 5 μL of IC (80 fg/μL).

A 30-cycle PCR was carried out using the following conditions: denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 2 min, followed by a final extension incubation at 72°C for 10 min.

The products were separated and visualized by agarose gel electrophoresis (Bio-Rad Laboratories).

## 3. RESULTS AND DISCUSSION

### 3.1. Conventional ELISA and ELISA-Ampli Q

Once the studies to optimize parameters such as concentration of the immobilized IgG, amount of antibodies (MAb and $Ab_2$-AP), blocking agents, and time and temperature of incubation (the best conditions are reported in the experimental section) had been completed, calibration curves for both protein A and *S. aureus* were constructed. These are presented in Fig. 2A and 2B. All experimental data were fit using a "non-linear four-parameter logistic calibration plot" (Fare et al. 1996) as in Eq. (1):

$$f(x) = \frac{a - d}{1 + (x/c)^b} + d \tag{1}$$

in which $a$ and $d$ are the asymptotic maximum and minimum values, $c$ is the value of $x$ at the inflection point, and $b$ is the slope.

The detection limit (LOD), defined as the concentration corresponding to the $f(x)$ value obtained by adding three standard deviations of zero point from the mean of the zero standard measurement (mean value +3 sd), was determined to be 0.6 ng/mL for protein A and $2 \times 10^6$ CFU/mL for *S. aureus.* The value for the sensitivity, calculated as the amount of *S. aureus* needed to produce a 25% increase in the signal, was $9 \times 10^6$ CFU/mL.

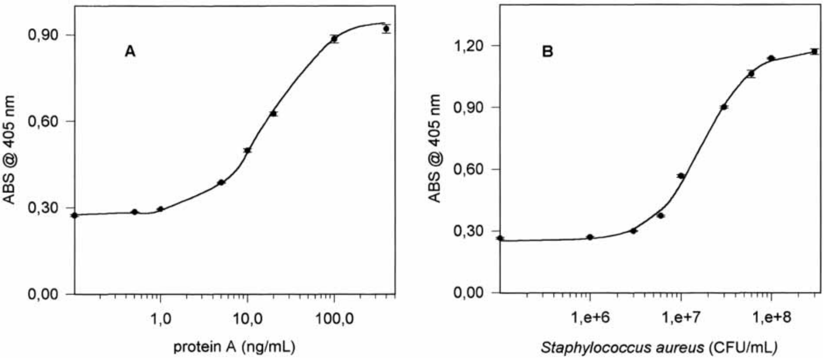

***Figure 2.*** Calibration curves for protein A (A) and *S. aureus* (B) using conventional ELISA.

In addition, a sandwich assay, using rabbit polyclonal antibodies (from Sigma) instead of monoclonal and antirabbit IgG-AP (from Vector) as label, was carried out following a protocol similar to that employed for MAb antibodies. While the best performance was consistently obtained using polyclonal antibodies (LOD $= 0.07\,\text{ng/mL}$ for protein A and $2 \times 10^5\,\text{CFU/mL}$ for *S. aureus*) our experience during this work showed that there was considerable variation in the affinity for protein A between different lots of polyclonal antibody. This prevented a rigorous comparison between these two types of antibodies and also limits the usefulness of polyclonal antibodies for assay development. Therefore, all further experiments were done using monoclonal antibodies that can be produced from a single clone.

Additionally, experiments performed with other bacterial strains of Staphylococcus (*S. epidermidis* and *S. xylosus*) and with *Salmonella* Enteritidis, *E. coli*, and *Streptococcus pyogenes* demonstrated that the antibodies showed good specificity for *S. aureus* and there was no cross-reactivity with the other bacteria tested.

A strategy to enhance the signal generated from the alkaline phosphatase (AP) label, was to incorporate the enzymatic amplification system (Ampli Q) into the protocol.

In Fig. 3 a calibration curve for *S. aureus* is reported. Using this amplification system, both the detection limit and the sensitivity of the assay improved by a actor of about 30. In fact, these analytical parameters were found to be $6 \times 10^4\,\text{CFU/mL}$ and $2 \times 10^5\,\text{CFU/mL}$ respectively.

## 3.2. ELISA Based on Magnetic Beads

The next step was to investigate the improvements that could be achieved using magnetic particles to capture the analyte and to make manipulations more efficient. Tosylactivated immunomagnetic beads (IMBs) were employed to develop two variations of the immunoassays: ELIMC and ELIME.

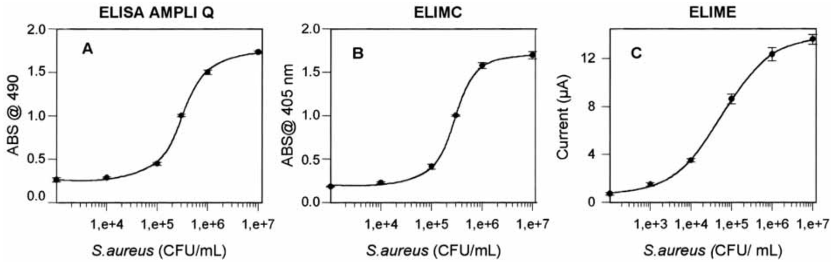

***Figure 3.*** Comparison between calibration curves for *S. aureus* obtained employing ELISA-Ampli Q, ELIMC, and ELIME systems, respectively.

### 3.2.1. ELIMC

In developing a more effective assay with IMBs, numerous parameters such as concentration of the immobilized IgG, antibodies amount (MAb and $Ab_2$-AP), blocking agents, intermediate washing steps, and incubation time also were evaluated. Because different protocols are reported in the literature relative to the use of a blocking phase and of intermediate washing steps to adopt between each step, these two aspects were studied to evaluate their influence on nonspecific adsorption of the biocomponents on the IMBs surface. The results demonstrated (data not shown) the indispensability of the blocking phase and the nonutility of intermediate washings. Omitting the latter, the ELIMC was found to be more rapid than the conventional ELISA. The interaction times between the biocomponents (IgG-protein A/*S. aureus*, protein A/*S. aureus*-MAb, MAb-$Ab_2$) were such that it was possible to carry out the entire protocol in about 3 hours. Moreover, an investigation of the effect of varying the MAb concentration (dilutions from 1:500–1:10000) indicated that, given the fast enzymatic reaction, the absorbance value at 3 min was off scale for the high analyte concentrations. On the basis of these results, a MAb dilution of 1:50000 was ultimately chosen with an enzymatic reaction time of 30 min. The calibration curve for *S. aureus* assayed under these conditions is reported in Fig. 3 B. The detection limit and the sensitivity of the assay are $7 \times 10^4$ and $2 \times 10^5$ CFU/mL, respectively. Thus the performance of ELISA-Ampli Q and ELIMC assays turned out to be comparable.

### 3.2.2. ELIME

In this approach magnetic beads with the immobilized IgG have been successfully used to capture the analyte at the surface of magnetized screen-printed electrodes (SPEs). Parameters such as MAb dilution, concentration of the immobilized IgG, and $Ab_2$-AP dilution were then optimized in order to obtain the maximum electrochemical signal.

Initially, different MAb dilutions (1:500, 1:1000, 1:5000, 1:50000, 1:100000) were tested using the same concentrations of IgG and $Ab_2$-AP employed for ELIMC (0.5 mg/mL and 1:300, respectively) in the presence of a fixed concentration of *S. aureus* ($5 \times 10^5$ CFU/mL). No differences in the electrochemical signal were observed between 1:500 and 1:1000 (Table 1), so a MAb dilution of 1:1000 was then employed for the further experiments.

Various IgG concentrations (0.12 mg/mL, 0.5 mg/mL, 1.2 mg/mL, 2.0 mg/mL), used for the coating of the beads in order to capture the antigen on the surface, were investigated. As reported in Table 1, the maximum electrochemical signal was reached with 1.2 mg/mL of IgG.

Finally three different dilutions of $Ab_2$-AP (1:50, 1:100, and 1:300) were tested at constant MAb dilution (1:1000) and IgG concentration (1.2 mg/mL). Because an insignificant difference between $Ab_2$-AP diluted 1:50 and 1:100 was observed (Table 1), a dilution of 1:100 was chosen.

***Table 1.*** ELIME system: MAb, IgG, and $Ab_2$-AP optimization

| MAb dilution[a] | Current (μA) | RSD% (n = 3) | IgG concentration[b] (ng/mL) | Current (μA) | RSD% (n = 3) | $Ab_2$-AP dilution[c] | Current (μA) | RSD% (n = 3) |
|---|---|---|---|---|---|---|---|---|
| 1:1000000 | 3.8 | 7.4 | 0.12 | 3.1 | 7.9 | 1:300 | 9.7 | 5.7 |
| 1:500000 | 5.7 | 6.8 | 0.5 | 7.9 | 5.9 | 1:100 | 11.5 | 5.4 |
| 1:5000 | 6.8 | 6.4 | 1.2 | 9.5 | 5.7 | 1:50 | 12.0 | 5.5 |
| 1:1000 | 7.8 | 6.0 | 2.0 | 8.6 | 5.8 | | | |
| 1:500 | 7.7 | 5.9 | | | | | | |

[a] Optimization of MAb dilution: experiments performed with immobilized IgG = 0.5 mg/mL and $Ab_2$-AP = 1:300.
[b] Optimization of the IgG concentration: experiments performed with MAb = 1:1000 and Aba-AP 1:300.
[c] Optimization of the $Ab_2$-AP dilution: experiments performed with immobilized IgG = 1.2 mg/mL and MAb = 1:1000.

Also, the effect of two different immunoreagent volumes (400 μL or 4 mL added to each reaction tube) and the boiling time (10–30 min) of the broth cultures of *S. aureus* were evaluated. No significant signal variations were obtained in these experiments (data not shown), so a volume of 400 μL and a boiling time of 10 min were selected as reported in the experimental section.

A calibration curve for *S. aureus*, using the optimized experimental conditions described previously, is shown in Fig. 3C; LOD and sensitivity were $1 \times 10^3$ and $2 \times 10^4$ CFU/mL, respectively.

## 3.3. PCR-Based Detection

The objective of this study was to first develop a PCR method that would meet the requirements for use as an effective screening tool and then to compare its performance with that of the best immunoassay techniques. The *nuc* gene was chosen as a DNA marker for *S. aureus* because its utility and amplification via PCR had been previously demonstrated. The functioning and specificity of the primers that allow the amplification of a 269-bp fragment of the *nuc* gene in *S. aureus* cells have been previously published (Brakstad et al. 1992; Hein et al. 2001).

A first phase of development was to optimize numerous parameters such as $MgCl_2$, primer concentrations and different annealing temperatures. Experimental data show that, using 3 mM of $MgCl_2$, 1 μM of each primer, and an annealing temperature of 60°C, it was possible to detect $10^2$ cells $mL^{-1}$ of *S. aureus.*

A second crucial phase was to incorporate into the procedure an internal control, a fragment of DNA whose replication could be monitored to verify that potential inhibitors present in food samples were not interfering with the enzymatic reactions involved in PCR so as to give a false negative. In fact, the results of the EU-funded research project (FOOD-PCR, www.pcr.dk), indicated the need for inclusion of such an internal amplification control (Hoorfar et al. 2003). The internal control was constructed using the commercial plasmid pUC19 as template.

An issue for the use of the IC is that during the coamplification, the DNA target of *S. aureus* competes with the IC DNA for the primers staph 1 and staph 2. Therefore it was necessary to test different concentrations of IC in the presence of $10^2$ CFU/mL of *S. aureus.* Using the optimized conditions determined earlier, the IC concentration was varied in order to evaluate the most appropriate concentration that allowed for the coamplification of both amplicons, so that the sensitivity of the PCR in the presence of IC remained the same as that for the method performed without the IC. It was determined that 80 fg/μL was a suitable level. At this concentration the 130-bp amplicon of IC was visible in the presence of 10 cells/mL of *S. aureus*, whereas the 269-bp target amplicon of *S. aureus* did not yet show up on the gels. However, both amplicons were visible using $10^2$ CFU/mL of *S. aureus.* The IC amplicon was

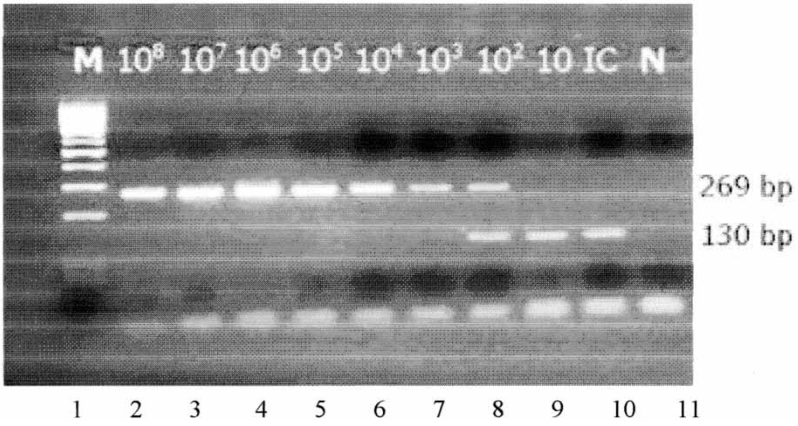

***Figure 4.*** PCR: lane 1 = 100-bp DNA ladder plus marker (M-Medical Genenco), lanes 2, 3, 4, 5, 6, 7 represent the amplified target DNA (269 bp) of *S. aureus* broth cultures = $10^8$, $10^7$ $10^6$, $10^5$, $10^4$, $10^3$ cells $mL^{-1}$; lane 8 represents the amplified target DNA of *S. aureus* = $10^2$ cells $mL^{-1}$ and IC; lane 9 represents the amplified target DNA of *S. aureus* = 10 cells $mL^{-1}$ (not detectable) and the co-amplified internal control (IC = 130 bp); lane 10 represents the amplified IC; and lane 11, the negative control.

absent in the presence of *S. aureus* higher than $10^3$ CFU/mL (Fig. 4). Thus, our adaptation of a PCR method for *S. aureus* allows the detection of a very low cell concentration with a built-in control to guarantee that failure to detect the organism is not due to inhibition of the enzyme reactions involved in amplification.

## 4. CONCLUSIONS

In this paper, results from a comparison of various optimized screening methods for detection of *Staphylococcus aureus* with ELISA-based formats, using alkaline phosphatase as the enzyme label, are reported. Initially a conventional spectrophotometric assay, using p-nitrophenyl phosphate, was optimized; then the introduction of the AmpliQ enzymatic amplification system into the protocol resulted in improved performance in terms of both the detection limit ($6 \times 10^4$ CFU/mL) and sensitivity ($2 \times 10^5$ CFU/mL). The same improvement was achieved by adapting the procedures through the use of immunomagnetic beads with colorimetric detection. However the best results were obtained by using the immunomagnetic beads coupled with a final electrochemical detection. Using this ELIME technique, developed for the first time in our knowledge for the determination of *S. aureus*, a detection limit of $1 \times 10^3$ CFU/mL and a sensitivity of $2 \times 10^4$ CFU/mL were obtained. The overall analysis was performed in about 3 h, thus being extremely rapid compared with the time required for the cultural standard method (i.e., 2–3 days).

Recent experiments, carried out in our laboratory, using lysostaphin, a staphylolytic enzyme that degrades the cell wall of staphylococci, and ELIME have demonstrated a further 10-fold decrease in the detection limit ($\mathrm{LOD} = 10^2\,\mathrm{CFU/mL}$) with respect to the method studied in this work, which includes extraction by a boiling step.

The PCR method that was developed and optimized for comparison with immunoassay approaches allows the detection of $10^2\,\mathrm{CFU/mL}$ in 3 h. Moreover, it was demonstrated that an internal control could be introduced to respond to possible matrix effects that could result in inhibition of the PCR reaction. This is a significant issue for analysis given that false negative results present a risk for the population, whereas false positives merely require a confirmation of the presumptive results by retesting the samples.

On the basis of these studies, both the PCR and ELIME methods have been shown to be rapid and efficient. Thus they represent extremely promising approaches that could facilitate preventive testing of food samples.

While in this work our attention has been focused on the development and comparison among different screening methods for the determination of *S. aureus*, the next experiments will involve a systematic application of PCR and ELIME methods on a large number of food samples, experimentally and naturally contaminated. For confirmation the same samples will be also analyzed with the classical cultural method.

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