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Amperometric Quantification of Total Coliforms and Specific Detection of *Escherichia coli*

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The quantitative determination of total and fecal coliforms, as indicators of fecal pollution, is essential for water quality control. We developed a sensitive, inexpensive amperometric enzyme biosensor based on the electrochemical detection of *β*-galactosidase activity, using *p*-amino-phenyl-*β*-D-galactopyranoside as substrate, for determining the density of coliforms, represented by *Escherichia coli* and *Klebsiella pneumoniae*. The specific detection of *E. coli* was achieved using an antibody-coated electrode that specifically binds the target bacteria. Amperometric detection enabled the determination of 1000 colony-forming units/mL within 60−75 min. Preincubation for 5−6 h further increased the sensitivity more than 100-fold. The present experimental setup allowed the simultaneous analysis of up to eight samples, using disposable screen-printed electrodes.

The quantitative determination of total coliforms, considered as the hallmark of general fecal contamination, is essential for monitoring microbiological water quality. The presence of the fecal coliform, *Escherichia coli*, conveys the potential presence of pathogens originating from humans and warm-blooded animals. Assessing the numbers of both total and fecal coliforms is important because enteric disease contracted from polluted water supplies still constitutes an important public health problem.[1−3] Conventional microbiological plate count counts and other cultivation methods for determining the number of coliforms in drinking water are time-consuming, and the usual incubation period (from 24 to 48 h)[25] is too long when immediate remedial measures must be taken. Furthermore, cultivation-based methods tend to underestimate the number of fecal bacteria because they rapidly lose their colony-forming ability after their release into fresh- or seawaters, while preserving certain metabolic activities and virulence properties.[4−6] The limitations of conventional methods led to the development of rapid methods for determining the densities of total and fecal coliforms, particularly *E. coli*.

The ubiquitous enzyme *β*-D-galactosidase is often used as a general marker for total coliforms, whereas *β*-D-glucuronidase is a specific marker for the major fecal coliform *E. coli*. The differential detection capacity of these enzymes prompted the development of rapid assays for monitoring their activity. The results obtained with such methods have usually shown a good linear correlation with results obtained using traditional methods.[7−11] Moreover, interference from nontarget *β*-D-galactosidase- and *β*-D-glucuronidase-positive bacteria is low and insignificant. The *β*-D-galactosidase activity of noncoliforms is at least 2 log units below that of induced coliforms, affecting the results only when the bacteria are present in high concentrations.[8]

Most rapid enzymatic assays used for total coliform quantitation are based on chemiluminometric or fluorometric procedures. Chemiluminometric methods allow the detection of the very low concentration of 1 coliform/100 mL of water after a 6−9-h propagation phase, followed by a 45-min assay. Permeabilization of the bacteria, which reduces the diffusion barrier for the endogenous enzyme and accelerates the transport of the substrate to the enzyme, is used to enhance the signal.[10] Quite similarly, a fluorometric method detected 1 fecal coliform/100 mL of water within 6 h.[11] The activity of *β*-D-glucuronidase, however, proved to be beyond detection limits in this system. Although the fluorometric method was further improved and optimized,[12] the reliability of these rapid methods for detecting extremely low bacterial concentrations of 1 total coliform or 1 *E. coli* in 100 mL of drinking water is very poor. Under such conditions, the sensitivity-boosting measures that are necessary to achieve the required speed compromise the specificity of the test.[13]

The specific detection of *E. coli* is essential for water quality control because its presence points directly to the presence of enteric disease-causing bacteria.[14] Many rapid detection methods are based on antigen-specific immunoassays. A method described in ref 15 for measuring the number of *E. coli* O157:H7 can detect 5000 bacteria/mL within a short time. The assay involves immu-

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nolabeling, filtration, and the electrochemical detection of alkaline phosphatase activity, which is used as the antibody label. The bacteria are specifically labeled with alkaline phosphatase-conjugated antibodies and captured on a polycarbonate track-etched membrane filter. The filter is placed directly against a glassy carbon electrode, and the signal is detected by square wave voltametry.

Another interesting method[16] involves coupling an immunologic separation, using immunomagnetic beads, with amperometric flow injection analysis detection of viable bacteria. Two different mediators (potassium hexacyanoferrate(III) and 2,6-dichlorophenolindophenol) are used to shuttle electrons from the Krebs cycle of *E. coli* O157 to the Pt working electrode, therefore detecting only viable bacteria. The detection limit of this method is 10^5 cfu/mL, and the assay can be performed in 2 h. An important advantage of this method is that it requires only one antibody, without an enzyme label, therefore accelerating the speed of the assay.

The present communication describes the development of an amperometric biosensor, based on the activity of β-D-galactosidase for quantifying coliforms, represented by *E. coli* and *Klebsiella pneumoniae*. The experimental system allows the simultaneous analysis of eight samples using disposable screen-printed electrodes. The specific detection of *E. coli* was achieved by using an antibody-coated electrode that specifically binds the target bacteria. This system did not require the use of a secondary antibody because detection was based on the activity of the intrinsic β-D-galactosidase enzyme.

EXPERIMENTAL SECTION

Chemicals. *p*-Aminophenyl β-D-galactopyranoside (PAPG), isopropyl β-D-thiogalactopyranoside (IPTG), polymyxin B sulfate, polymyxin B nonapeptide, and lysozyme were purchased from Sigma Chemicals. Toluene was obtained from Frutarom. Glutaraldehyde was from Fluka.

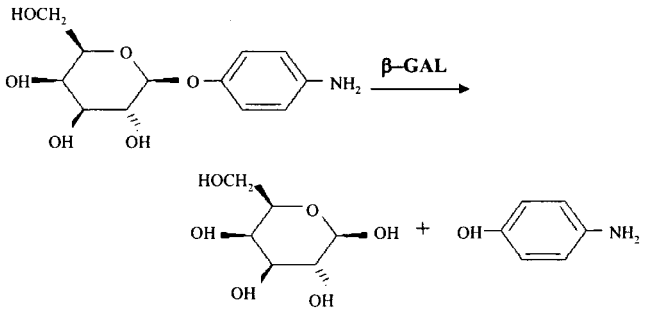
Preparation of Bacterial Cultures. We used three bacterial concentration ranges: high (10^3−10^7 cfu/mL), medium (60 cfu/mL), and very low (∼1 cfu/mL). Two wild-type bacterial strains were used: *E. coli* and *K. pneumoniae*. Both strains were obtained from the strain collection of our laboratory.

For the high concentration, bacterial cultures were grown overnight in Luria broth (LB) medium at 30 °C with aeration by shaking. To begin the experiment, the cultures were inoculated into Erlenmeyer flasks containing 10 mL of LB medium; IPTG was added, to a final concentration of 0.5 mM, to induce β-D-galactosidase activity. The cultures were then grown for 2−3 h at 37 °C with vigorous shaking until they reached the logarithmic phase (∼2×10^8 cfu/mL). The log-phase cultures were then diluted to concentrations of 10^3−10^7 cfu/mL in 1 L of sterile water. The inoculated water was filtered through 0.45-μm filters, which were placed into Erlenmeyer flasks containing 10 mL of LB medium. After the cells were permeabilized, the activity of β-D-galactosidase was electrochemically determined. The number of bacterial colonies was counted on LB agar plates.

For the medium and very low concentrations, an incubation step was required. The log-phase cultures were diluted to final concentrations of 60 and ∼1 cfu/mL in 1 L of sterilized water. The bacterial suspensions were then filtered through 0.45-μmmicron filters, which were placed into Erlenmeyer flasks containing 10 mL of LB medium and 0.5 mM IPTG. The flasks were aerating by shaking at 37 °C, and samples were withdrawn at 1-h intervals for permeabilization and the determination of β-D-galactosidase activity.

Permeabilization, Permeabilization of bacteria was performed in 10 mL of LB medium containing polymyxin B sulfate (10 μg/mL) and lysozyme (25 μg/mL) by vigorous shaking for 45 min at 30 °C.

Amperometric Measurement of Enzymatic Activity. The enzymatic activity was determined electrochemically, using PAPG as substrate.[18,19]



The product of the enzymatic reaction, *p*-aminophenol (PAP), is oxidized at the anode.[18,19] The electrochemical properties of the substrate and the product of β-D-galactosidase have been studied before,[20] and several electroanalytical methods for PAP detection have been reported.[21,22] The electrochemical measurements used here were made with disposable screen-printed electrodes. Graphite ink was used as the counter electrode, and Ag/AgCl ink was used for the reference electrodes. Disposable graphite electrodes of cylindrical form (HB 0.9 mm) were used as the working electrodes. The electrochemical cells were made of polystyrene tubes (volume of 0.3 mL). The electrochemical cells containing the electrodes were placed on a vibrating plate to achieve good mixing.[18,19] The graphite electrode was held at 220 mV versus the reference electrode, using a substrate concentration of 0.8 mg/mL.

Apparatus. We used a PAR VersaStat potentiostat connected to an eight-channel PAR 314 model multiplexer (EG&G Princeton Applied Research) for electrochemical measurements, which was controlled by LabView software. The apparatus allowed the simultaneous measurement of eight samples. The electrical current resulting from the activity of β-D-galactosidase could be visualized simultaneously in all samples in real time on a computer screen. For the low-current measurements, we used a highly

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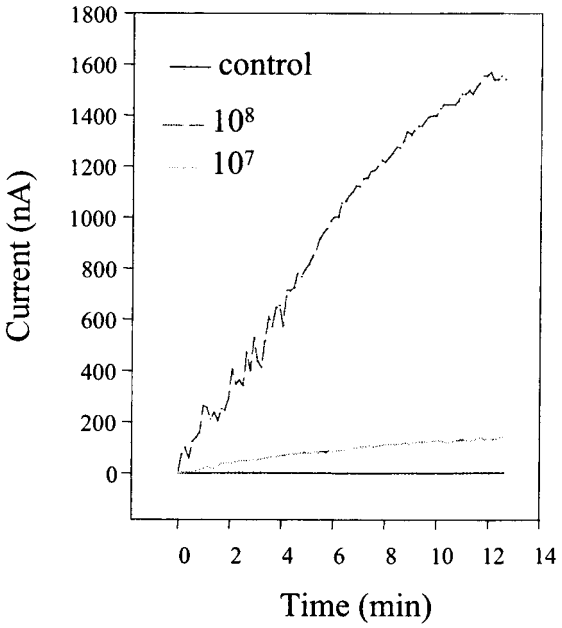

Figure 1. Amperometric response of *E. coli* cultures (10^8, 10^7 cfu/mL), with PAPG as substrate (0.8 mg/mL in LB medium). The control was without bacteria.

sensitive eight-channeled potentiostat (Technion-Israel Institute of Technology).

Specific Detection of *E. coli*. The disposable graphite electrodes of cylindrical form (HB 0.9 mm) were coated with a polyclonal anti-*E. coli* antibody, raised against the bacterial lysate (Dako). The following protocol was used for immobilizing the antibodies on the surface of the electrodes: (1) washing the electrodes for 5 min in ethanol and ultrasonication; (2) immersion overnight in polyethyleneimine (0.5% in water) and then drying for 2 h; (3) immersion for 2 h in glutaraldehyde (2.5% in phosphate buffer, pH 7.5, 0.1 M) and then washing with phosphate buffer; (4) immersion for 1 h in the antibody solution (concentration ~2 mg/mL, as protein) and then washing with water; (5) immersion for 1 h in glycine (0.1 M in phosphate buffer).

Finally, the antibody-coated electrodes were immersed for 30 min in the sample of permeabilized bacteria, with gentle mixing, and then washed and assayed for β-D-galactosidase activity.

Safety Considerations. *E. coli* bacteria were handled according with rules appropriate to biosafety level 1 microorganisms (no special hazard). *K. pneumoniae* bacteria were handled appropriate to rules concerning biosafety level 2 microorganisms (moderate hazard). Biosafety levels are defined by the CDC (Centers for Disease Control USA).

EXPERIMENTAL RESULTS AND DISCUSSION

Amperometric Response of *E. coli* Cultures. We started by incubating a cell culture containing *E. coli* at a concentration of 10^6 cfu/mL for 3 h (to reach the logarithmic phase) with 0.5 mM IPTG. The production of β-galactosidase was measured electrochemically by following the enzymatic activity, using PAPG as a substrate. Samples of the bacteria, appropriately diluted, were placed into electrochemical cells. Figure 1 shows an example of the amperometric response of *E. coli* cultures (10^7 and 10^8 cfu/mL). We used the slope ($\Delta_{current}/\Delta_{time}$) within the linear range (the first 10 min) as the index for β-galactosidase activity.

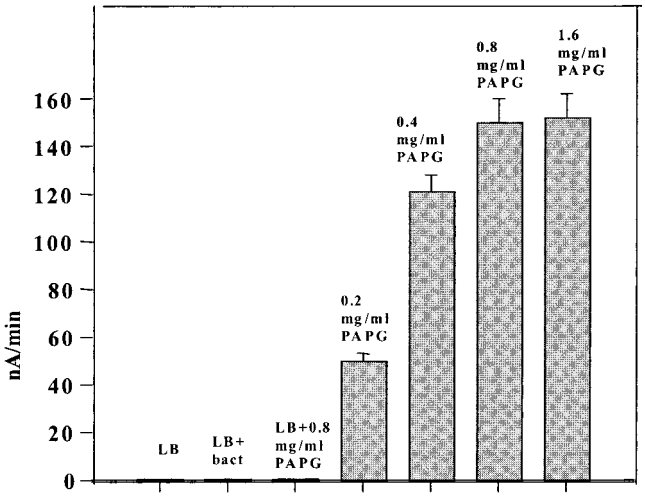

Figure 2. β-D-Galactosidase activity of *E. coli* cultures (~10^8 cfu/mL), with PAPG as substrate (0.2−1.6 mg/mL in LB medium). First column: response with LB medium alone; second column: response with bacteria only (without substrate); third column: response with the substrate alone (without bacteria). Each point represents the mean of three measurements ± standard deviation.

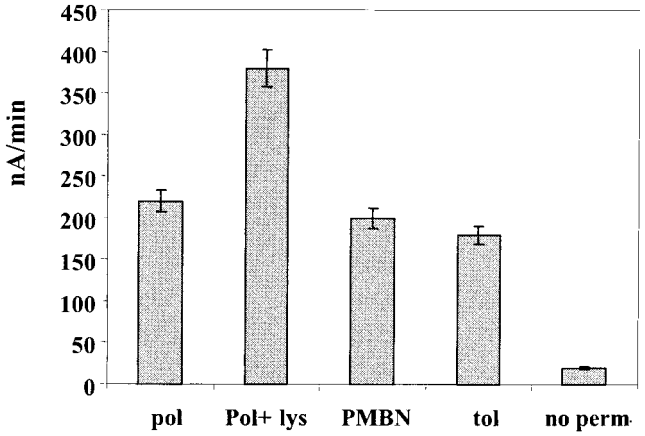

Figure 3. β-D-Galactosidase activity of *E. coli* cultures, using the following membrane permeabilizers: polymyxin B (10 μg/mL), polymyxin B and lysozyme (10 25 μg/mL), and polymyxin B nonapeptide (10 μg/mL) and toluene (5% v/v). One liter of water seeded with ~10^6 cfu/mL was filtered, and the filter was placed in 10 mL of LB medium and permeabilized. Each point represents the mean of three measurements ± standard deviation.

Optimization of the β-D-Galactosidase Monitoring System. To determine the optimal β-D-galactosidase substrate concentration, we monitored the β-D-galactosidase activity of ~10^8 cfu/mL *E. coli* in the presence of various concentrations of PAPG. The results presented in Figure 2 show no electrochemical interference from either LB medium alone, bacteria without the substrate, or the substrate without bacteria. Therefore, any signal obtained could be accredited to the enzymatic activity of the bacteria, without unwanted oxidation reactions. The current signal increased with increasing substrate concentrations, up to 0.8 mg/mL. As the current did not change at a higher substrate concentration (1.6 mg/mL), we selected 0.8 mg/mL PAPG as the optimal substrate concentration.

Permeabilization. We studied the permeabilization efficiency by comparing the resulting enzymatic activity in the electrochemical cells. Permeabilization was accomplished by vigorously shak-

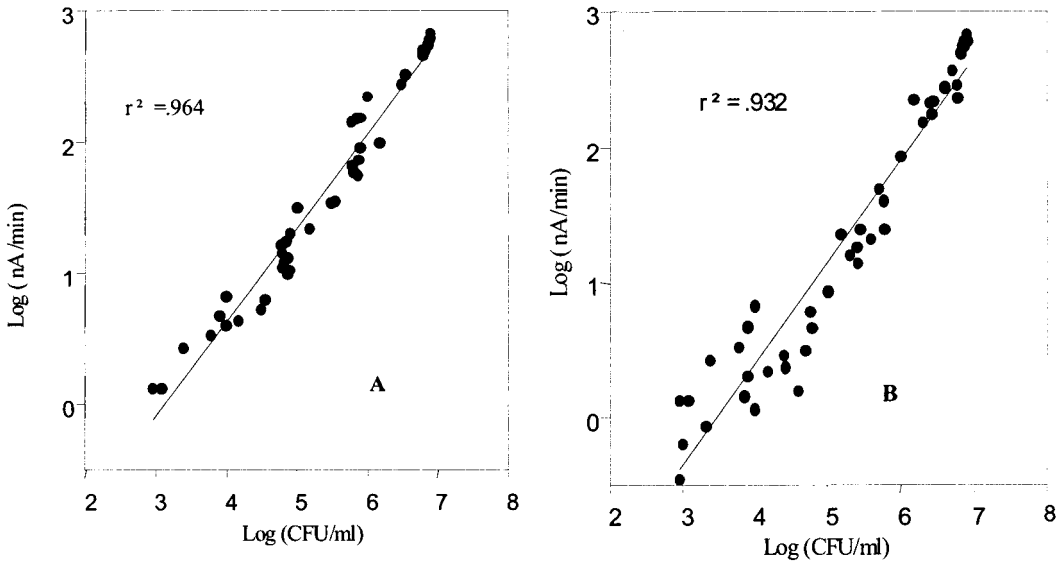

Figure 4. Quantitation of *E. coli* (A) and *K. pneumoniae* (B) after filtering 1 L of water containing bacterial concentrations ranging from ~10^3 to 10^7 cfu/mL. The filter was placed in 10 mL of LB medium and permeabilized.

ing the 0.45-μm filters, containing the previously filtered bacteria, 10 mL of LB medium containing polymyxin B sulfate (10 μg/mL), and lysozyme (25 μg/mL) for 45 min at 30 °C. We checked the permeabilization efficiency of polymyxin B sulfate (10 μg/mL), polymyxin B nonapeptide (10 μg/mL), lysozyme (25 μg/mL), toluene (5% vol/vol), and a combination of polymyxin B sulfate (10 μg/mL) and lysozyme (25 μg/mL), as described in ref 10. The results in Figure 3 show that the highest response was obtained with a combination of polymyxin B and lysozyme. This result agrees with reports in the literature claiming that a combination of polymyxin B with lysozyme, at the concentrations applied here, causes leakage of most of the cellular material.[10] All permeabilizers mentioned above produced a signal that was significantly higher than that of the control without a permeabilizer.

Quantification of Total Coliforms (*E. coli* and *K. pneumoniae*). We used overnight cultures of each type of coliform, inoculated into Erlenmeyer flasks containing LB medium and grown to the logarithmic phase. The bacterial suspensions were inoculated into 1 L of sterilized water, concentrated by filtration, and permeabilized with polymyxin B and lysozyme. The β-D-galactosidase activity was directly determined in electrochemical cells. The results presented in Figure 4 show that a detectable signal could be measured for both coliform types in concentrations ranging between approximately 10^3 and 10^7 cfu/mL.

A total of 45 measurements were made for *E. coli* and 33 for *K. pneumoniae*. A good linear correlation was found between the number of bacteria measured by the plate-counting method and the electrochemical response measured as $-\Delta_{current}/\Delta_{time}$, with a correlation coefficient (R^2) of 0.9641 for *E. coli* and 0.932 for *K. pneumoniae*. The results are better than those reported for fluorometric detection, where correlation coefficients of 0.8 and 0.83 were found.[7] The detection limit for both coliform types was ~10^3 cfu/mL. This sensitivity is comparable with the results obtained with fluorometric and chemiluminometric methods (~10^3 cfu/mL) and better than the sensitivity of the colorimetric method (~10^6 cfu/mL).[10,23] For lower bacterial concentrations, however, further modifications were required.

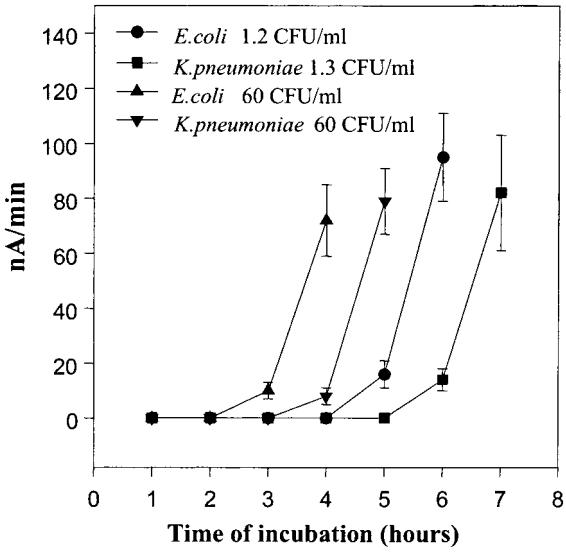

Figure 5. Detection of *E. coli,* at concentrations of 60 and 1.2 cfu/mL, and *K. pneumoniae*, at concentrations of 60 and 1.3 cfu/mL, in 1 L of water after filtration, incubation in LB medium at 37 °C, and permeabilization. Each point represents the mean of three measurements ± standard deviation.

We checked the feasibility of detecting medium concentrations (60 cfu/mL) and very small concentrations of bacteria (~1 cfu/mL) by filtering 1 L of water inoculated with bacteria and incubation in LB medium. At hourly intervals, samples were withdrawn, permeabilized, and analyzed. The results presented in Figure 5 show that *E. coli* at a concentration of 60 cfu/mL could be detected after 3 h of incubation, whereas *K. pneumoniae* at a similar concentration was detectable after 4 h of incubation.

The detection of *E. coli* at the very low concentration of 1.2 cfu/mL after 5 h of incubation and *K. pneumoniae* at a concentration of 1.3 cfu/mL after 6 h of incubation indicates that even very small concentrations of bacteria can be detected within a single

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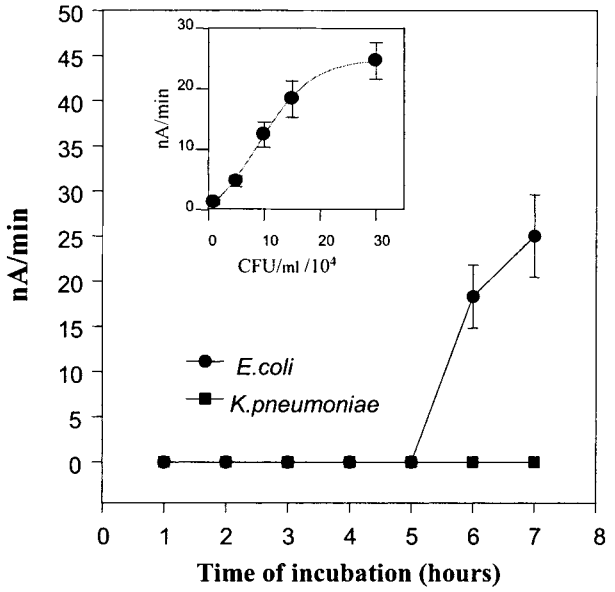

Figure 6. Specific detection by antibody-coated electrodes of 1.2 cfu/mL *E. coli* in 1 L of water after filtration, incubation in LB medium at 37 °C, and permeabilization. Each point represents the mean of three measurements ± standard deviation. Inset: Calibration curve with antibody-coated electrodes after filtration without preincubation.

working day. Although other rapid methods for detecting small concentrations of coliforms, based on fluorescent and chemiluminescent substrates, require similar incubation periods, they are dependent on the availability of an extremely sensitive detection apparatus, such as a CCD camera.[24]

Specific Detection of *E. coli*. The use of immunoassays is one of the presently available approaches to the specific identification of a target protein. We coated the immunosensor with a polyclonal anti-*E. coli* antibody to bind the bacteria to the electrode. The antibody-modified electrodes were immersed in the bacterial suspension (after incubation), washed, and checked for β-D-galactosidase activity. Since this system does not require a secondary labeled antibody, only one washing step was necessary. After an incubation period of 6 h, we could detect *E. coli* at a concentration of 1.2 cfu/mL. *K. pneumoniae*, also β-D-galactosidase positive, was not detected by this method (Figure 6).

The inset in Figure 6 shows that the detection limit of the immunoelectrodes following filtration and without preincubation is 10^4 cfu/mL. Another method not requiring a secondary antibody, based on immunomagnetic separation,[16] has a detection limit of 10^5 cfu/mL after an assay of 2 h.

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It should be emphasized that the signal obtained results only from the β-galactosidase activity and thus the background is actually zero. The detection limit of our electrochemical system is 1 nA/min. Consequently, the detection limit of the bacteria without any preconconetration and preincubation is 10^5 cfu/mL for the general assay and 10^6 cfu/mL for the immunoassay. The filtration step concentrated the bacteria by 100-fold. Preincubation had made the detection of ~1 cfu/mL possible within 5 h and immunospecific detection within 6 h. A comparison of our method with another recently described amperometric system for monitoring β-D-galactosidase activity in *E. coli*[17] revealed that *E. coli* detection is faster in our system. The method described in ref 17 requires 7.3 h for the detection of 4.5 cfu/mL, whereas our method requires a shorter time for a lower bacterial concentration. Furthermore, our system allows the simultaneous analysis of eight samples.

CONCLUSIONS

In this paper we have described a rapid and sensitive electroanalytical technique for the quantification of total coliform bacteria and for the specific detection of *E. coli*. The method uses a sensitive detection of β-D-galactosidase activity, which is considered a good marker for coliforms, with nonsignificant interference from noncoliform bacteria. An important advantage of this amperometric technique is the possibility of specifically detecting the major fecal coliform, *E. coli*, using an antibody-coated electrode that does not require a secondary labeled antibody for detection, thus saving time and lowering costs. Nevertheless, the general limitations of immunoassays, emanating from false positive results, must be considered. It should also be taken into consideration that a second level of selectivity provided by the secondary antibody is also absent. When compared with conventional bacterial plating methods, the method described here is rapid, allowing the detection of very small concentrations of bacteria (~1 cfu/mL) within one working day. The technique is relatively simple and, therefore, does not require the expertise of highly skilled technicians. An important feature of our system is the possibility of the simultaneous processing of up to eight samples and the use of disposable screen-printed electrodes. The validity of this technique with environmental samples is currently under investigation.

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