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ORIGINAL PAPER

Determination of viable *Escherichia coli* using antibody-coated paramagnetic beads with fluorescence detection

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Abstract A rapid and convenient assay system was developed to detect viable *Escherichia coli* in water. The target bacteria were recovered from solution by immunomagnetic separation and incubated in tryptic soy broth with isopropyl-β-D-thiogalactopyranoside, which induces formation of β-galactosidase in viable bacteria. Lysozyme was used to lyse *E. coli* cells and release the β-galactosidase. β-Galactosidase converted 4-methylumbelliferyl-β-D-galactoside to 4-methylumbelliferone (4-MU), which was measured by fluorescence spectrophotometry using excitation and emission wavelengths of 355 and 460 nm, respectively. Calibration graphs of 4-MU fluorescence intensity versus *E. coli* concentration showed a detection range between 8×10^4 and 1.6×10^7 cfu mL^{-1}, with a total analysis time of less than 3 h. The advantage of this method is that it detects viable cells because it is based on the activity of the enzyme intrinsic to live *E. coli.*

Keywords *Escherichia coli* · Immunoassay · Paramagnetic bead · β-Galactosidase · Fluorescence

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Introduction

Escherichia coli is found in the intestinal contents of humans and other warm-blooded animals. Coliforms, fecal coliforms, and *E. coli* have been used as indicators of fecal contamination of water supplies and recreational waters [1]. Only relatively recently has the role of *E. coli* as a pathogen been stressed because of the severity of diseases it causes [2]. Many strains of *E. coli* are non-pathogenic, and only some are involved in food and water-borne diseases [3]. Mostly, outbreaks are associated with contaminated food [4, 5], though in some cases, outbreaks are from contaminated drinking and recreational waters [2, 6, 7].

Standard microbiological methods based on membrane filtration and selective growth on solid media usually take 24–72 h to complete. The long analysis time is a major drawback, and faster methods for detecting *E. coli* in various sample types are being sought. Methods such as the polymerase chain reaction [8–11], flow cytometry [12–14], and immunomagnetic separation assays [14–16] are rapid and sensitive, but instrumentation is benchtop-based in a laboratory. Transferring the sample from field to laboratory can take a relatively long time, but in many cases, a fast response about pollution is needed. Immunoassay, which relies on the specificity of the antigen–antibody reaction, is commonly used to detect pathogens. Immunoassay procedures have been successfully incorporated in microfluidic systems [17, 18], which is an important step in our long-term goal of developing a portable instrument for detecting viable *E. coli* rapidly on-site.

We have reported a bead-based immunoassay for viable *E. coli* that was based on electrochemical detection [19]. The general procedure involved capturing the bacteria on paramagnetic microbeads, rinsing the beads to remove sample components, incubating the captured bacteria to enable viable bacteria to be distinguished from non-viable bacteria based on the production of a particular enzyme, β-galactosidase (β-gal), by the viable bacteria, addition of substrate for β-gal, and electrochemical detection of the enzyme-generated product. Paramagnetic streptavidin-coated microbeads were used as the solid support. Biotinylated antibody specific for *E. coli* was immobilized on the surface of the microbeads to capture bacteria when mixed with a water sample. The beads with captured bacteria were collected by a magnet and rinsed to remove interfering components from the sample. Releasing the beads in a smaller volume served as a preconcentration step as well. Like other bacteria, *E. coli* undergo physiological modifications involving enzyme activities and protein synthesis under different growing conditions [20]. In the presence of lactose, *E. coli* produces β-gal, a catabolic enzyme that cleaves lactose into galactose and glucose for uptake. β-gal is often used as a general marker for *E. coli* and coliforms [21]. Not specific to *E. coli*, β-gal has been found in many microorganisms, animals, and plants, but enzymes from different sources have different properties [22]. The activity of β-gal in bacteria can be induced with isopropyl-β-D-thiogalactopyranoside (IPTG), which induces under gratuitous conditions as it is neither hydrolyzed by β-galactosidase nor used as a carbon source by the organism [21, 23]. After an incubation period to build up the concentration of β-gal, the substrate *p*-aminophenyl β-D-galactopyranoside was added, and the enzyme-generated product *p*-aminophenol was detected electrochemically by oxidation at a gold electrode.

The present study describes a similar bead-based immunoassay for viable *E. coli*, but with fluorescence detection. The general assay procedure was similar to that outlined above for electrochemical detection, but the following enzyme–substrate–product system was used for detection:

The product, 4-methylumbelliferone, fluoresces strongly at 460 nm, with an excitation wavelength of 355 nm. The goal was to develop the assay and compare the two methods of detection, electrochemistry and fluorescence, to determine if either technique offers any significant advantage over the other.

Experimental

Materials

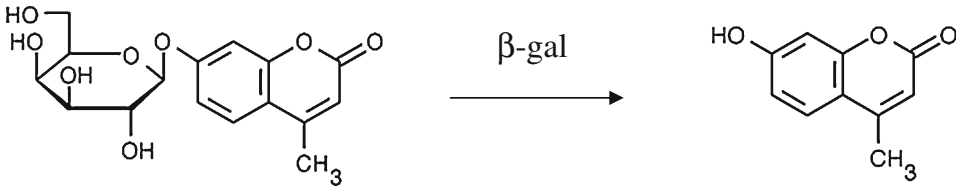
Streptavidin-coated M280 paramagnetic beads (2.8 μm diam.) were from Dynal (Great Neck, NY, USA) as a monodisperse suspension of 6.7×10^8 beads mL^{-1}. Biotin-conjugated goat antibody (Ab) to *E. coli* was from Virostat (Portland, ME, USA). *E. coli* K12 strain was from Refik Saydam National Type Culture Collections (Ankara, Turkey). Sorbitol MacConkey agar (SMAC) and tryptic soy broth (TSB) were from Merck KGaA (Germany). 4-Methylumbelliferyl-β-D-galactoside (MUG), 4-methylumbelliferone (4-MU), dimethylsulfoxide, and IPTG were from Sigma Chemical (St. Louis, MO, USA). Na_2HPO_4 and KH_2PO_4 were used to prepare phosphate-buffered saline (PBS) and were from J.T. Baker (Netherlands).

Instrumentation

Fluorescence measurements were made with a Cary Eclipse Fluorescence Spectrophotometer (Varian, Netherlands) using excitation and emission wavelengths of 355 and 460 nm, respectively, and a quartz microcell (60 μL). The temperature was controlled by Cary Eclipse software and a built-in Peltier system.

Bacteria

E. coli strain K12 was used since it is non-pathogenic. Before each experiment, a colony of *E. coli*, grown on a nutrient agar plate at 37 °C for 24 h, was transferred into TSB medium and incubated at 37 °C for 24 h. After



4-methylumbelliferyl-β-D-galactoside (MUG)

4-methylumbelliferone (4-MU)

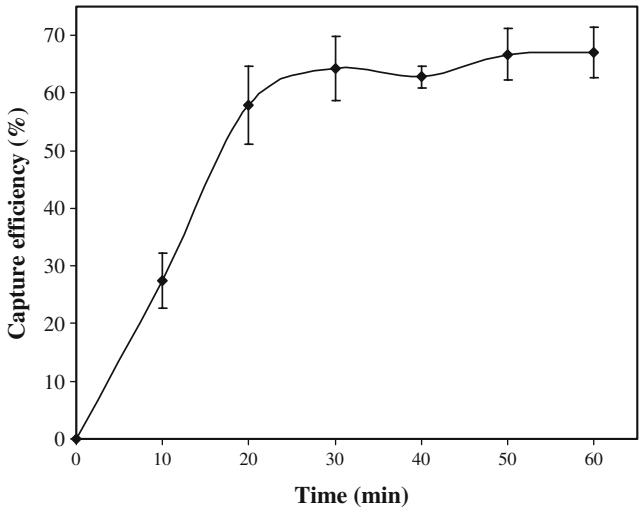


Fig. 1 The effect of reaction time on capturing *E. coli* by antibody-coated paramagnetic beads at room temperature and pH 7.5, 0.1 M PBS

incubation, the culture was kept at 2–4 °C for 12 h. The concentration of bacterial stock solution was determined by the most probable count method. Stock standard was serially diluted, and the lowest dilutions were plated in at least triplicate on SMAC plates and grown for 24 h at 37 °C, then kept at 2–4 °C for 12 h prior to counting colonies.

Experimental procedures

Immunomagnetic separation

Various parameters were optimized sequentially to achieve the best conditions for the assay.

The streptavidin-coated paramagnetic beads (10 μL, 6.7×10^8 beads mL^{-1}) were added to a tube containing biotin-conjugated Ab (10 μL, 0.15 mg mL^{-1}), and the tube was gently shaken on a vortex (Stuart, UK) at room temperature for 10 min. The Ab-coated beads from excess antibody solution were removed magnetically and washed at least three times by re-suspending beads in 50 μL of PBS (pH 7.5, 0.1 M). The beads were then mixed with *E. coli* (250 μL, 3×10^3 cfu mL^{-1}) and incubated at room temperature on the vortex mixer for various times from 0 to 60 min to determine the effect of reaction time on capturing *E. coli.* After incubation, the beads were separated from the supernatant solution, which was kept for further analysis, and then washed three times with PBST (pH 7.5, 0.1 M, 0.05% (*v*/*v*) Tween 20) and three times with PBS.

Both the supernate solution containing uncaptured *E. coli* and the initial concentration *E. coli* solution were plated on SMAC (100 μL per plate) and incubated at 37 °C for 24 h. Then, *E. coli* colonies were counted to determine the percentage of the *E. coli* captured by beads.

Similarly, PBS concentration, pH, and volume of reaction were examined to determine optimal bacteria capture efficiency.

β-Galactosidase conditions and cell lysis

Inducer IPTG (40 μL, 0.5 mM, dissolved in TSB) [18] was added to the bead–*E. coli* complex. The reaction tube was gently mixed and incubated at 37 °C for 2 h.

In order to break the bacteria cell wall and release β-gal enzyme, lysozyme (100 μL, from 0 to 20 mg mL^{-1}) was added to captured *E. coli* (10^6 cfu mL^{-1}) and incubated at 37 °C for 0–45 min.

Fluorescence measurements were done in a 50-μL final volume, consisting of 20 μL PBS-D (0.1 M, 1 mg/mL $MgCl_2$), 10 μL MUG solution, and 20 μL sample. MUG solution was prepared with dimethylsulfoxide and PBS-D buffer. The activity of the released enzyme was determined using calibration graphs of 4-MU fluorescence intensity versus concentration of *E. coli*.

Similarly, the effect of temperature on enzyme activity was investigated between 22 °C and 67 °C (at pH 7.3 and 0.5 mM MUG), and the effect of pH was investigated between 6.5 and 7.7 at 37 °C and 0.5 mM MUG. The effect of MUG concentration on enzyme activity was investigated similarly at 37 °C and pH 7.3 while varying the MUG concentration in the reaction medium between 0.05 to 2 mM.

Construction of the calibration graph

E. coli (from 10^1 to 10^7 cfu mL^{-1}) were captured by paramagnetic beads and lysed after inducing β-gal activity. Sample (20 μL) was added to a quartz microcell containing 20 μL PBS-D (pH 7.3, 0.1 M, 1 mg mL^{-1} $MgCl_2$) and 10 μL MUG (1 mM) at 53 °C. The bacterial cell count was detected by measuring the slope of the increase in intensity of 4-MU, the product of the enzyme reaction.

The colony-forming units per milliliter in each solution was estimated by plating on SMAC, incubating at 37 °C for 24 h, and counting the number of colonies. The average of at least three measurements was taken.

Results and discussion

Immunomagnetic separation

The effects of several conditions in the immunomagnetic separation step of bacteria capture were evaluated.

The effect of reaction time on capturing *E. coli* (250 μl, 3.0×10^3 cfu mL^{-1}) by antibody-coated paramagnetic beads at room temperature (RT) and pH 7.5, 0.1 M PBS is shown in Fig. 1. The percentage of captured bacteria increased

with increasing reaction time up to 30 min and flattened off at approximately 60%. A reaction time of 30 min was used for subsequent experiments.

The effect of the pH of PBS (0.1 M) on the capture efficiency of *E. coli* at RT was evaluated in 1-pH-unit increments over a pH range of 5.0 to 9.0. The best efficiency (approximately 60%) for capturing bacteria (250 μL, 3.0×10^3 cfu mL^{-1}) was observed in a narrow pH range (7.0–8.0) with an optimum at pH 7.5.

The effect of PBS concentration on capturing *E. coli* (250 μL, 3.5×10^3 cfu mL^{1}) at RT and pH 7.5 for 30 min was determined over the range of 0.05–0.40 M. A maximum efficiency (approximately 65%) for capturing bacteria was observed at 0.1 M PBS concentration. One of the most important factors that affect the antibody–antigen interaction is ionic bonds [24]. Thus, the changes in the pH or the ionic strength of the reaction medium can easily affect the binding of the antigen to the antibody.

The effect of immunoreaction volume on capture efficiency of bacteria was examined from 50 to 500 μL (Fig. 2). The same amounts of bacteria (7.5×10^2 cfu) and antibody-coated magnetic beads (6.7×10^6 beads) were used in different volumes, and the capturing was done at RT and pH 7.5 in 0.1 M PBS for 30 min. The capture efficiency exhibited an optimum reaction volume of 250 μL. Above 250 μL, the increasing immunoreaction volume reduces the density of beads and bacteria, which lowers the capture efficiency by reducing the probability of interaction between beads and bacteria in a given time. We attribute the sharp decrease in capture efficiency below 250 μL to steric hindrance of the capture reaction between the beads and the bacteria, which would affect the efficiency negatively. These data were confirmed by measuring the enzyme activity in the captured cells, which exhibited a similar trend.

Our maximum capture efficiency in these optimization experiments was ca. 60%. Similar results of 50% capture efficiency by protein-coated beads were shown by Kieft et al. [8].

To ensure that signal was not caused by nonspecific adsorption of bacteria, a control experiment was done with antibody for a different strain of bacteria, which would not be expected to specifically bind with the K12 strain. Beads were coated with biotinylated antibody against *E. coli* O157:H7 strain, but exposed to K12 strain. The signal observed was at the same level as the usual blank signal when no bacteria were present, which ruled out the capture of bacteria by nonspecific adsorption.

Cell lysis

The effect of lysozyme concentration on releasing β-gal is shown in Fig. 3a. The maximum amount of released enzyme was found at 5 mg mL^{-1} lysozyme concentration. Above this concentration, the amount of released enzyme decreased up to 21%. The effect of reaction time on releasing enzyme was determined (Fig. 3b). The amount of released enzyme increased with increasing incubation time up to 30 min.

There is contradictory information about lysozyme's ability to lyse gram negative bacteria. It was reported that lysozyme does not affect gram negative bacteria without the aid of other factors such as ethylenediaminetetraacetic acid (EDTA) [25]. EDTA decreases β-gal activity and so cannot be used in this assay. According to Wild et al., lysozyme is able to penetrate the outer membrane of the *E. coli* and get into the periplasmic space, but is not able to break the inner membrane [25]. Fortunately, β-gal is located in the periplasm. Higher temperature increases lysozyme penetration and lysozyme's solution prepared in DI water aids cell wall breakage by ionic shock.

Besides lysozyme, we tested other methods for cell lysis. Mechanical breakage methods like French press glass beads are challenging due to the assay design with the paramagnetic beads. We did not try to separate beads from the *E. coli* complex. There are chemical cell wall breakage methods reported, which we tested (polymyxin B, sodium lauryl sulfate, sodium desoxycholate in the presence of toluene, and commercial lysing agents based on detergents), but they were not suitable for the assay due to the negative effect on β-gal activity. In all cases, β-gal activity decreased significantly when compared to the results with lysozyme, and, consequently, the detection limit of *E. coli* increased.

In developing the fluorescence detection method, we found it necessary to rinse the microbeads and their captured bacteria after exposure to the growth solution containing TSB and IPTG because of excessive background

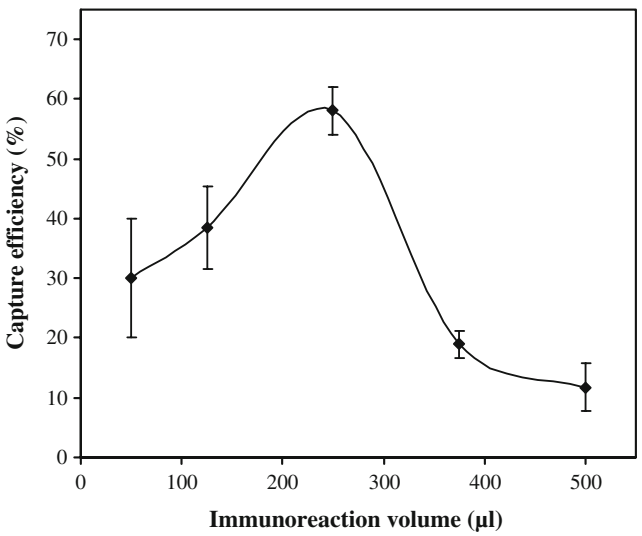


Fig. 2 The effect of immunoreaction volume on capturing *E. coli* by antibody-coated paramagnetic beads at room temperature and pH 7.5 in 0.1 M PBS for 30 min

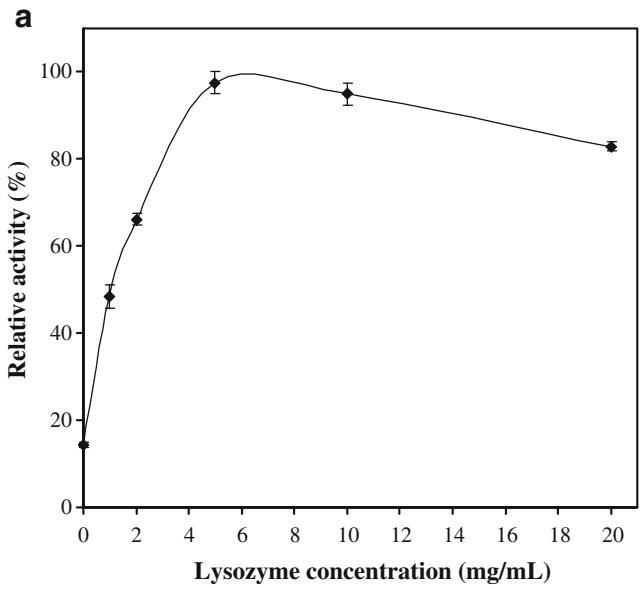


Fig. 3 **a** The effect of lysozyme enzyme concentration on releasing β-galactosidase at 37 °C for 20 min reaction time. **b** The effect of reaction time on releasing β-galactosidase at 37 °C for 5 mg mL^{-1} lysozyme concentration

fluorescence of TSB. The beads and captured bacteria were then lysed with lysozyme to release β-gal, and enzyme substrate 4-MUG was added. Consequently, the fluorescence detection of enzyme-generated 4-MU was done on a solution containing capture beads, lysozyme, lysed bacteria, β-gal, excess MUG, and 4-MU. Two control experiments were done in order to determine if the components of this mixture of lysed cells interfere with fluorescence detection of 4-MU by contributing to background fluorescence, scattering, or quenching fluorescence of 4-MU. First, an assay was carried out the usual way, but without adding

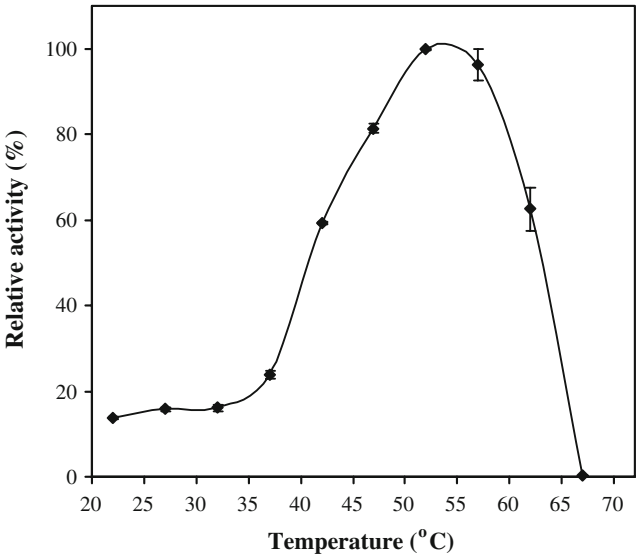


Fig. 4 The effect of temperature on β-galactosidase activity at pH 7.3 and 0.5 mM MUG concentration

IPTG to the incubation solution. Thus, β-gal was not produced in these bacteria samples. The fluorescence signal for these samples was measured and found to be at the same level as a blank signal with no antigen present. In another experiment, the fluorescence response of a known concentration of β-gal (from source *E. coli*) was measured, then enzyme was spiked into the prepared sample, and the difference between both responses was measured. While spiked signals were noisier, thus leading to wider standard deviations, statistically, both responses were the same, meaning that substances in the lysate do not measurably affect fluorescence detection.

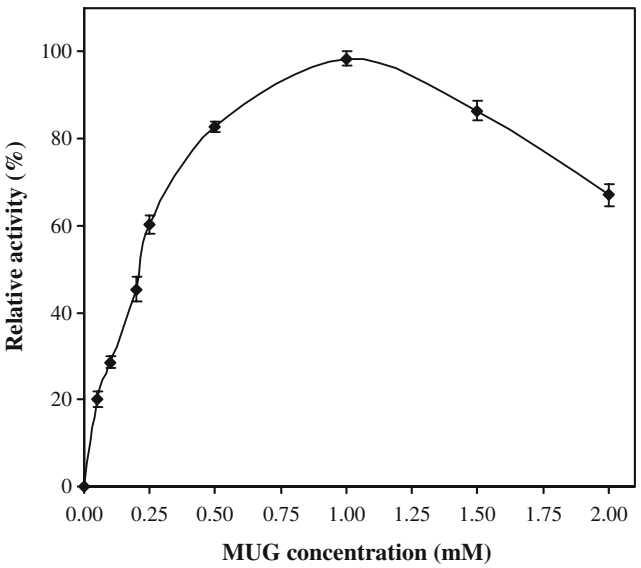


Fig. 5 The effect of MUG concentration on β-galactosidase activity at 37 °C and pH 7.3

Optimization of the reaction parameters of β-galactosidase and its substrate

We monitored the activity between 22 °C and 67 °C to determine the optimal temperature for β-galactosidase activity (Fig. 4). The maximum activity of the enzyme was observed at 53 °C, which is in agreement with previously reported data [26].

The effect of pH on β-galactosidase enzyme activity was determined. The highest enzyme activity was observed at pH 7.25.

The effect of substrate concentration on β-galactosidase activity was examined for various concentrations of MUG (Fig. 5). The enzymatic activity first increased with increasing MUG concentration up to 1.0 mM and then decreased due to substrate inhibition of β-gal [27]. MUG has an advantage over other substrates because it can pass through the bacterial cell wall without cell lysing agents, but is slow [28]. MUG is not an ideal enzyme substrate for our assay because the strongest fluorescence of its product 4-MU is at pH 10.4 (pK_a~7.8), and therefore, at pH 7.3, which is optimum for the enzyme reaction, product fluorescence is significantly lower. Although a fluorinated derivative of MUG (DiFMUG) has a lower pK_a value (~4.9) than MUG and thus an optimal pH closer to the physiological conditions we were looking for [29], DiFMUG has a very high background fluorescence compared to MUG and is expensive.

Fluorescence detection of β-galactosidase activity in *E. coli*

The optimized parameters of the developed assay were applied to detecting *E. coli*. The system was used with stock solutions containing concentrations of *E. coli* between 4×10^1 and 1.6×10^7 cfu mL^{-1}. The activity of the β-galactosidase corresponding to each concentration was determined, and the dose–response curve was generated. The standard curve is sigmoidal, although the high dose tail is not included in the graph, because at the higher concentrations of *E. coli*, clumping and formation of aggregates of beads plus *E. coli* were observed. Those aggregates were problematic to handle during the standard assay procedure and interfered with fluorescence detection by scattering light. The dose–response curve was analyzed by fitting the experimental points to a sigmoidal logistical relationship (Origin 7); a good fit was acquired with correlation coefficient R^2=0.99 (Fig. 6a). The working linear range of the assay was between 8×10^4 and 1.6×10^7 cfu mL^{-1} by fitting to linear least squares relationship (Fig. 6b). It is common to calculate the limit of detection (LOD) as nonspecific signal or blank plus three standard deviations of the nonspecific signal. Since the blank signal is very low, the calculated LOD signal is very low, which we feel is unrealistic to report since the lower asymptote of the curve may be difficult to reproduce and is usually not used [30]. The lower limit of quantitation was set as the lowest concentration standard in the linear range, 8×10^4 cfu mL^{-1}. The total analysis time, which includes capturing the bacteria (30 min), incubating with IPTG (120 min), lysing the cells (30 min), and fluorescence measurement and other activities (20 min), was less than 200 min. If the incubation period were increased, then the detection limit could be lowered. Also, it is possible to improve the number of bacteria captured by using a larger sample volume. However, if the sample volume is increased, then the amount of antibody-coated beads and all other reagents should also be increased, and the cost of the assay will go up as well.

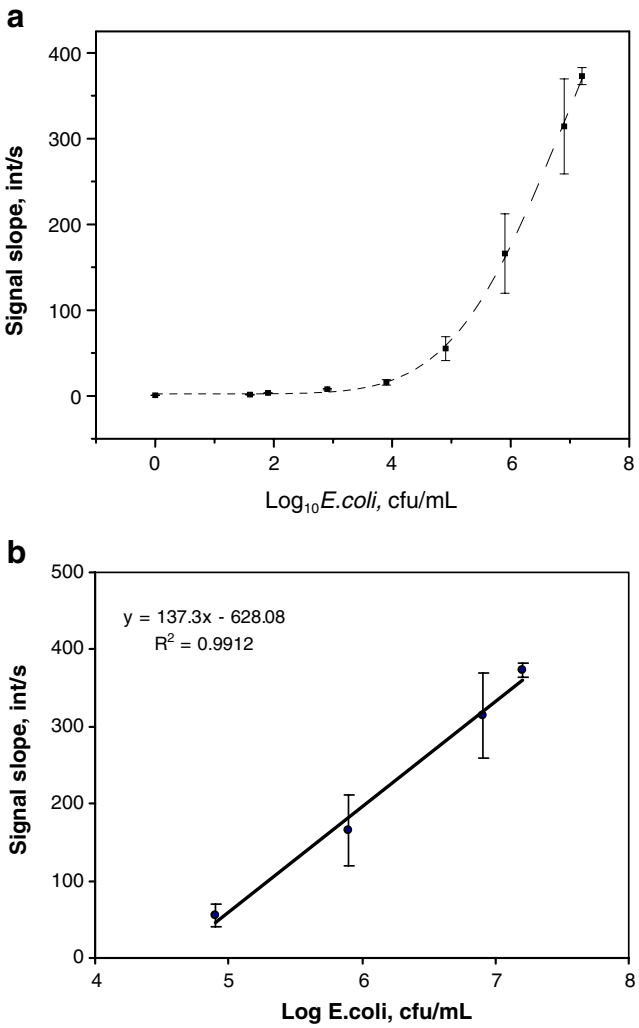


Fig. 6 **a** Response curve for *E. coli.* Each point is the mean of three measurements; error bar represents ±1 SD. The data point of zero represents nonspecific absorption when calibrator is free of *E. coli.* Curve fitted with sigmoidal logistical relationship. **b** Linear region of the dose–response curve, fitted with least square relationship

Conclusions

In this paper, we describe a rapid, sensitive, and convenient fluorimetric assay based on immunomagnetic separation that was designed and tested for detecting *E. coli*. The activity of β-gal enzyme, induced in the *E. coli*, was determined using the fluorogenic enzyme substrate, MUG, which led to the enumeration of the *E. coli*. An important advantage of the technique, based on the activity of the intrinsic enzyme of *E. coli*, is the possibility of detecting viable cells. In many sandwich type immunoanalyses, cells that are dead or are live but not culturable are also detected, thus causing misleading results [31–33]. In the developed method, antigen–antibody interaction was used to separate of *E. coli* from the sample. In most other work, only filtration is used to separate the bacteria from the sample; the filtrate is then incubated in growth medium, and the β-galactosidase activity is measured. Other β-gal positive bacteria may introduce false positive results using this procedure [1, 34, 35], but interference is eliminated if enzymatic activity is measured at 53 °C, because according to Shoichi et al. [26], β-gal activity from other similar organisms is inactivated at this high temperature.

At this point, the assay is designed as a quantitative detection system, but it is possible that the assay could be used as bacteria presence/absence test only, because bacteria may not behave the same way if acquired from different environments. Further studies are required to clarify this question.

Compared to our previously reported electrochemical detection method [19], fluorescence detection shows a slightly better limit of detection. For the electrochemical detection method, we focused on determining the incubation time necessary to detect a few bacteria cells. If incubation was carried out for 2 h as in the fluorescence method, the concentration of bacteria needed to give a signal high enough to be distinguished from the blank signal was above 10^5 cfu/mL. However, a disadvantage of the fluorescence method is that it requires the additional assay step of cell lysis using lysozyme. This step is unnecessary with electrochemical detection because the enzyme substrate used there, *p*-aminophenyl β-D-galactopyranoside, can more rapidly access β-gal in the cell by penetrating the outer wall of the cell and the enzyme product, *p*-aminophenol, can pass back out of the cell for detection at the electrode. The fluorescence method also requires an additional rinsing step to remove the growth solution because TSB fluoresces so strongly. By comparison, the growth solution contained no interference for electrochemical detection. In summary, fluorescence gave a slightly lower limit of detection, but the procedure for electrochemistry had fewer steps.

According to the U.S. Environmental Protection Agency, in 1972, most water-quality standards were set at 1,000 coliforms per 100 mL and the health goal for total coliforms at zero for drinking water [36]. The sensitivity of the system described here is not high enough to detect such low concentrations, but it can be improved with a preconcentration or preincubation step. Although standard microbiological techniques allow the detection of single bacteria, amplification of the signal is required through growth of a single cell into a colony, and this process is time consuming [37].

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