ORIGINAL ARTICLES

Optimization and Evaluation of Rapid Methods for the Assessment of Waterborne *Escherichia coli* in Egypt

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ABSTRACT

Three different methods were developed and evaluated for determination of water borne *E. coli* from two wastewater purification stations and one drinking water station at Cairo, Egypt. The most appropriate parameters used in the enzymatic method were found to be: using the substrate concentration (700 mg/l), temperature of the water bath shaker (44.5º C), pH (7.2), and the time of measurement (30 minutes). Moreover, there was no significant difference between the samples’ enzymatic activity measured with or without addition of the inducer IPTG (isopropyl-β-D-1-thiogalactopyranoside). Multiplex PCR was used to detect *E. coli* that enables its differentiation from biochemically and phylogenetically related bacteria. The target genes have been increased to three genes: *uidA*, *lacZ* and *lacY*. Evaluation of these methods indicated a significant correlation between the microbial count and the enzymatic activity of β-D-galactosidase produced by *E. coli* when using chromogenic (Pearson r = 0.87) or fluorogenic substrate (Pearson r = 0.749). In conclusion, fluorometric method was suitable for detection of low contaminated samples, while colorimetric was suitable for highly contaminated sample, multiplex PCR based on three target genes was suitable for quiet identification of environmental *E. coli* either viable, VBNC (viable but nonculturable cells) or dead cells.

**Key words:** β-D-galactosidase, *Escherichia coli*, evaluation, optimization, waterborne.

Introduction

The presence of pathogenic bacteria threatens public and environmental health that requires safe drinking water. The lack of sensitivity of cultivation-based methods in the detection of stressed bacterial cells leads to contamination-level underestimation and can generate serious problems (Rompré et al., 2002). Consequently, coliforms have the ability to persist in distribution system biofilms creating a high significant problem of ensuring water safety and an issue that requires appropriate solutions and prevention measures to be developed (Murphy et al., 2008).

Conventional microbiological detection of coliform bacteria in drinking water requires 24 to 72 hours to complete. Hence, there is an urgent need to a rapid method to protect the public health, especially in cases of contaminated sources or interrupted treatment (Berg and Fiksdal, 1988). One of the well known rapid methods is the enzymatic reaction that based upon the illustration of β-D-galactosidase (GALase) enzyme activity by using the chromogenic substrate Ortho-nitrophenyl-β-D-galactopyranoside (ONPG) (Bej et al., 1991b) and the fluorogenic one 4-methylumbelliferyl-β-D-galactopyranoside (MUGal) (George et al., 2000).

Besides, Bej et al. (1991b) have reported on the development of PCR gene probe methods for the detection of total coliform bacteria and *E. coli*. The advantages of the PCR system include: its sensitivity (can detect single cells in 100-ml water samples), its specificity for target microorganisms, the speed from the time of sample collection to the completion of analysis, and its ability to simultaneously detect multiple target bacteria (which can include both general indicator species and a series of specific target pathogens).

Interestingly, Lan et al. (2004) suggested considering Shigella strains as pathovars of *E. coli* on the base of the similarity of sequences among housekeeping and plasmid genes of several Shigella and *E. coli* strains. The close relationship between *E. coli* and Shigella species hampers their differentiation. Therefore, it is obvious that many *E. coli* could be called Shigella and vice versa. Alternatively, genes coding for specific virulence markers can be used as target sequences in PCR-based protocols for detection of *E. coli* strains (Ibekwe et al., 2002).

Three target genes were used: *uidA* (coding for β-d-glucuronidase), *lacZ* (coding for β-D-galactosidase) and *lacY* (coding for lactose permease). Products of these genes could be considered as biochemical hallmarks of *E. coli* spp. Indeed, enzymatic products of *lacY* and *lacZ* genes are necessary for lactose fermentation; lactose permease is essential for lactose transportation across the cytoplasmatic membrane and β-D-galactosidase cleaves the disacharide lactose into glucose and galactose (Stoebel, 2005).

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The aim of this study is to optimize and evaluate the different rapid methods of determination of waterborne coliforms to obtain a reliable protocol that is valid to be easily applied in water companies under Egyptian environmental condition.

**Materials And Methods**

**Collection of samples:**

Fifteen *E. coli* isolates were obtained from Zeneen station and Abu Rawash station for wastewater purification, while sixteen *E. coli* were obtained from Embaba station for drinking water purification at Cairo, Egypt. All isolates were identified microscopically and by standard biochemical tests.

**The GALase assay using fluorometric method:**

The GALase assay was performed by using a modification of the method described before (Fiksdal et al., 1994). 100µl of each bacterial concentration (10^1, 10^2, 10^3 and 10^4 cfu ml^{-1}) were placed in a 250-ml flask containing 20 ml of PB (pH 7.2) supplemented with 0.7 gm of MUGal per liter (unless otherwise specified), 0.2 gm of sodium lauryl sulfate per liter, and 0.1% nutrient broth. At least two replicates were analyzed. The flasks were incubated in a shaking water bath at 44.5 ºC (unless otherwise specified). The fluorescence intensities of sample aliquots (2.5 ml of sample and 100 µl of 10 M NaOH) were measured every 5 minutes for 30 minutes with A Perkin Elmer model LS55 fluorometer with excitation at 365 nm and emission at 444.5 nm. Surface viable count was performed to each bacterial concentration using TSA media and growth conditions of (37ºC, 16-18 hours) by spreading method.

**Characterization of optimum parameters for measurement of GALase activity using fluorometric method:**

**The suitable substrate concentration:**

Using 3 groups of environmental isolates (each contains 10 isolates). The substrate (MUGal) was used in 3 different concentrations (0.2, 0.5, and 0.7 gm per liter).The corresponding fluorescence activities were measured.

**The effect of IPTG:**

The isolated pure cultures of nine environmental isolates were grown in 10% TSB supplemented with and without 0.6 gm/L IPTG to achieve GALase induction (Tryland and Fiksdal, 1998). The corresponding fluorescence activities were measured in parallel to determine the effect of the presence of inducer.

**The effect of temperature:**

Using 2 groups of environmental isolates (each has 17 isolates). The flasks were incubated through the time of fluorescence measurement in a shaking water bath at two different incubation temperatures 37ºC (George et al., 2000) and 44.5ºC (Tryland and Fiksdal, 1998).

**The effect of pH of the used PB:**

Using 5 environmental isolates, the fluorescence activities of them were measured using PBs of different pH values (6.5, 7, 7.3, 7.6 and 8).The corresponding results were expressed using the Gaussian relation (George et al., 2000).

**The effect of NaOH on the intensity of the fluorescent product:**

Using 3 groups of environmental isolates (each has 10 isolates), the fluorescence intensities of sample aliquots were measured with and without the addition of 100 µl of 10 M NaOH to the quartz cell (Caruso et al., 2002).

**The effect of time:**

132 different prepared concentrations of 28 tested isolates were used to measure the fluorescence activity through 35 minutes. For each isolate two replicates were analyzed. The mean value of the fluorescence intensity
of all isolates at each time interval (5, 10, 15, 20, 25, 30 and 35 minutes) was calculated and XY relationship was performed between fluorescence values measured and the time by minutes.

**Construction of the standard curve:**

The standard curve represents the relationship between the relative fluorescence values and the MUF concentrations. The 100% of fluorescence intensity of the fluorometer was calibrated using standards of known MUF concentrations from 10 to 10000 nmol l⁻¹ (George et al., 2000).

**Regression analysis of the GALase activity vs. E. coli concentrations:**

The fluorescence values resulted from GALase activity of the tested isolates were calculated per minute and then transformed to MUF concentrations using the equation of the standard curve. The best fitting indicates the relative increase of fluorescence was calculated by least-squares linear regression analysis of the GALase activities of all tested isolates that is represented as (nmole MUF min⁻¹) vs. the log of number of E. coli that is enumerated by surface viable count.

**The GALase assay using colorimetric method:**

Exactly 0.3 ml of bacterial culture and 0.4 ml of a PBS solution (pH 7.2) were added to a microfuge tube. A control tube with 0.3 ml of sterile medium was used. In the hood 100 µl of chloroform and 50 µl of 0.1% sodium lauryl sulphate were added to each tube. The tubes were closed and vortex for 10 seconds, 0.2 ml of ONPG solution was added to each tube, then the caps were closed and the tubes were inverted several times to mix the contents. ONPG is the substrate, so this is time zero for the assay. Incubate at 37ºC and record the times (Miller, 1972).

Whenever a tube develops obvious yellow color, 0.5 ml of 1 M sodium carbonate was added to stop the reaction. All the assay tubes were put in a micro centrifuge and spin for about 5 minute to pellet the CHCl₃ and cell debris. 1.2 ml was transferred from each assay tube to a plastic microcuvette. Distilled water was used as a blank to zero the spectrophotometer. Then the absorbance value of each sample was recorded at 420 nm. For each isolate two replicates were analyzed (Miller, 1972).

**Regression analysis of the standardized sample activity vs. E. coli concentrations of all waterborne pathogens:**

The absorbance at 420 nm is the sum of the absorbance due to o-nitrophenol and light scattering due to the cells that can be determined by measuring the absorbance at 550 nm where o-nitrophenol does not absorb. The light scattering at 420 nm is 1.75× the light scattering at 550 nm, so the absorbance of o-nitrophenol was determined by subtracting 1.75 x OD₅₅₀ nm. Standardized ample activity for each specimen was calculated by division of its absorbance on its incubation time recorded till obtaining the yellow product and sample volume (A₄₂₀/min.ml). The best fitting indicated the relative increase of the enzyme activity was calculated by least-squares linear regression analysis of standardized sample activities of all tested isolates vs. the log of E. coli concentrations that is enumerated by plate counts.

**Statistical analyses:**

Regression models, Gaussian relation and unpaired student t tests were developed using GraphPad Prism 5 (For Windows, © 1992-2007 Graphpad software Inc., V 5.01, USA).

**Multiplex – PCR:**

Total genomic DNA was extracted by the bacterial lysis. The isolated pure cultures were grown in 10% TSB at 37º C for 16-18 hours; the cells were harvested by centrifugation at 6000 rpm for 5 minutes at 4º C in a cooling centrifuge. The broth was washed, and the cell pellets were resuspended in 1 ml a Tris EDTA buffer pH (7.6). This washing procedure was repeated 3 times. The bacterial concentration was adjusted to 10⁸ cfu ml⁻¹ using MacCferland solution (3x10⁸), and then boiled in a water bath for 15 min.

The primers specific for the lacZ and uidA genes were used as described previously (Bej et al., 1991b). A pair of 24-mer primers 5’ATGAAAGCTGCTACAGGAAGGCC3’ and 5’GGTTTATGCAGCAACGAGACGTCA3’ located within the coding region of the lacZ gene of E. coli and a pair of 20- and 21-mer primers 5’-AAAACGGCAAGAAAAAGCAG-3’ and 5’-ACGCCTGGTTACAGTCTTGCG-3’ located within the uidA structural gene of E. coli were used. The sequence
of lacY gene was used as described in (Horakova et al., 2008). The sequences of primers lacY were 5'-ACCAGACCAGCACCAGATAAG-3' and 5'-GCACCTACGATTTTTTGACCA3'.

**Multiplex PCR amplification:**

The reaction mixture consisted of PCR master mix (2X), 0.5 µmol l⁻¹ of each primer and 10 µl of template DNA. PCR reaction was performed in total volume of 25 µl. Conditions of PCR amplification were as follows: initial denaturation at 94º C for 3 minutes and 30 cycles with denaturation at 94º C for 30 seconds, annealing at 58º C for 25 seconds. Extension step was performed at 72º C for 1 minutes and final extension at 72º C for 10 minutes, using MJ research PTC-100 peltier thermal cycler (U.S.A) instrument. A 100-bp DNA ladder was loaded on each gel as a DNA size standard.

**Results:**

**The fluorometric method:**

*The optimum parameters for measurement of GALase activity were found to be:* using 0.7 gm/L of MUGal, The flasks were incubated in a shaking water bath at 44.5ºC using PB of pH 7.2. The fluorescence intensities of sample aliquots (2.5 ml of sample and 100 µl of 10 M NaOH) were measured, every 5 minutes for 30 minutes with fluorometer with excitation at 365 nm and emission at 444.5 nm. The study indicated that the use of IPTG as inducer did not promote the GALase activity significantly (fig 1, 2, 3, 4, 5 and 6)

![Fig. 1](image1.jpg)

**Fig. 1:** Determination of the most appropriate concentration of MUGal leading to maximum GALase activity by linear relationship between log GALase activity and MUGal concentration (E. coli concentration10⁴ cfu ml⁻¹)

![Fig. 2](image2.jpg)

**Fig. 2:** Impact of the two most commonly used temperatures (44.5º C, 37º C) on GALase activity (E. coli concentration10⁷ cfu ml⁻¹) (** means highly significant difference
Fig. 3: Impact of pH of the used buffer on GALase activity measured on 5 different *E. coli* isolates. Data are expressed as a percentage of maximal activity for each sample. The solid line represents the fitting of the experimental data by a Gauss curve (*E. coli* concentration $10^4$ cfu ml$^{-1}$).

Fig. 4: Impact of the alkaline pH shifting of the end fluorescent product on GALase activity (*E. coli* concentration $10^2$ cfu ml$^{-1}$) (***) means extremely significant difference.

Fig. 5: The rate of production of MUF from MUGal by *E. coli* isolates which is expressed as fluorescence over 35 minutes.
Fig. 6: Effect of presence or absence of inducer (IPTG) on GALase activity using two different *E. coli* isolates concentration (10² cfu ml⁻¹)

**Standard curve:**

It was established to convert the fluorescence values to the corresponding MUF concentrations. Fig. 7 showed the linear fitting that was resulted from least squares linear regression analysis of 15 different MUF concentrations and their corresponding fluorescence values, the resulted equation was \( Y = 0.8291X - 0.658 \).

**Linear regression:**

Fig. 8 showed the linear fitting that was resulted from least squares linear regression analysis of the log GALase activities of all tested isolates that was represented as (nmole MUF min⁻¹) vs. the log of *E. coli* concentrations. The resulted equation was \( Y = 0.2129 X + 2.353 \).

**The colorimetric method:**

The results showed that the enzyme activity increased with increasing the bacterial concentration to 10⁸ cfu ml⁻¹ in all the tested isolates. While, decreasing the bacterial concentration to less than 10⁶ cfu ml⁻¹ resulted in diminished or non-detectable enzyme activity. Fig. 9 showed the linear fitting that was resulted from least squares linear regression analysis of the log of standardized sample activity that was represented as (A₄₂₀ min⁻¹ ml⁻¹) vs. the log of *E. coli* concentrations.
Fig. 8: Regression analysis of the log of GALase activity vs. the log of *E. coli* concentration enumerated by plate counts on TSA for *E. coli* isolates (28) $P<0.0001$, Pearson $r = 0.749$, number of X values = 70, slope = 0.2129±0.02282

Fig. 9: Regression analysis of the log of the standardized sample activity vs. the log of *E. coli* concentration enumerated by plate counts on TSA for *E. coli* isolates (31) $P<0.0001$, Pearson $r = 0.87$, number of X values = 86, slope = 0.7403±0.04556

**The results of multiplex PCR:**

Fig. 10 demonstrated the multiplex PCR products of the results of the eight tested isolates with the positive control and the template negative control, using 10 µl of the template DNA. Three distinct bands were easily observed that were corresponding to *uidA* gene (147 bp), *lacZ* gene (264 bp) and *lacY* gene (463 bp).

**Specificity of multiplex PCR:**

Results displayed in Fig. 11 demonstrated the multiplex PCR products of the positive control (*E. coli* K12); *E. coli* isolate 1 in lane 3 that showed three clear bands, which were observed at 463 bp, 264 bp and 147 bp and represented *lacY*, *lacZ*, and *uidA* respectively. *Enterobacter cloacae* in lane 4 and *Citrobacter freundii* in lane 5 showed only two bands, which were observed at 463 bp and 264 bp, represented *lacY* and *lacZ* respectively. *Pseudomonas aeruginosa* in lane 6 and template negative control in lane 7 showed no bands.
Fig. 10: Agarose gel electrophoresis of multiplex PCR products of selected E. coli isolates using lacZ, uidA, and lacY primers (using 10 µl of template DNA)
Lane 1, 100 bp Marker; lane 2, positive control (E. coli K12); lane 3, E. coli isolate 1; lane 4, E. coli isolate 3; lane 5, E. coli isolate 6; lane 6, E. coli isolate 7; lane 7, E. coli isolate 19; lane 8, E. coli isolate 20; lane 9, E. coli isolate 26; lane 10, E. coli isolate 27; lane 11, template negative control; lane 12, 100 bp Marker. The clear bands, which are observed at 463 bp, 264 bp and 147 bp, represent lacY, lacZ, and uidA respectively.

Fig. 11: Agarose gel electrophoresis of multiplex PCR products of selected isolates using lacZ, uidA, and lacY primers (using 10 µl of template DNA)
Lane 1, 100 bp Marker; lane 2, positive control (E. coli K12); lane 3, E. coli isolate 1; lane 4, Enterobacter cloacae; lane 5, Citrobacter freundii; lane 6, Pseudomonas aeruginosa; lane 7, template negative control; lane 8, 100 bp Marker. The clear bands, which are observed at 463 bp, 264 bp and 147 bp represent lacY, lacZ, and uidA respectively.

Discussion:

The assay of GALase activity allows a valuable method for rapid evaluation of the faecal contamination in different water sources since it provides an estimation of the potential activity of cells, which possess GALase, including VBNC forms, which have lost the ability to grow on solid media despite maintaining their enzymatic activity.

The fluorometric method:

The suitable substrate concentration:

George et al. (2000) recorded that routine substrate concentrations were fixed at 500 mg l⁻¹ MUGal while Tryland and Fiksdal (1998) recorded that 200 mg l⁻¹ was enough concentration from which we can conclude that the substrate concentration varied greatly according to the enzyme activity of each environmental isolate. In our study substrate concentrations of 700 mg l⁻¹ MUGal allowed the measurement of enzymatic activities close to the maximum (whether the water was rich in coliforms or not).
The effect of IPTG:

GALase in our study was already induced in the natural water sources and that the fluorometric method can be applied without adding any inducer whatever the abundance of coliforms in the analyzed sample and this was aligned with (George et al., 2000).

The effect of temperature:

Our results show that 44.5º C is considered as the most suitable temperature to measure the GALase activity that were matched with (Caruso et al., 2002). Incubation at 44.5º C offers the advantage of avoiding the problem of interference of contaminating bacteria that active at lower temperature (37º C). Nutrient broth plays a great role in supporting the enzyme activator and enhancing the thermal stability of GALase (Tryland et al., 1998).

The effect of pH of PB:

The most suitable pH to be used in our study ≈ 7.2 at which the maximum enzyme activity was determined in accordance to (George et al., 2000).

Effect of addition of NaOH on the intensity of the fluorescent product:

In the present study addition of NaOH to the quartz cell giving rise to maximal light emission and this result is in accordance with results obtained by George et al. (2000) who recorded that the fluorescence was maximal when the pH of the solution in the quartz cell was greater than 10.

The effect of time:

The time of the fluorometric enzymatic assay varies from protocol to another. George et al. (2000) recorded that the time of the assay is 25 minutes while, Berg and Fiksdal (1988) performed the assay through 30 minutes. Our results show that 30 minutes are sufficient time to accomplish the assay without exposure to the risk of enzyme destabilization.

Regression analysis of the GALase activity vs. E. coli concentrations:

The present study suggests that the determination of the log of GALase hydrolysis has potential as an alternative parameter for the rapid 30 minutes determination of the log of E. coli concentrations using fluorometric methods. Nevertheless, calibration curves have to be preliminarily established for the particular tested environments. The good linear correlation between logarithms of enzyme activities and concentrations of culturable FC bacteria encourages continued use of the rapid enzyme assays for monitoring of sewage pollution in water till coliform concentration of not more than $10^4$ cfu ml$^{-1}$ in agreement with the results explained by (George et al., 2000).

The Colorimetric method:

Van Pouke and Nelis (1995) strongly suggested that the uptake of substrates for GALase, notably ONPG by E. coli, show a relative impermeability or, a partial intracellular retention of the enzymatic cleavage product and it can be substantially improved by pre-treating the cells with permeabilizing agent. In the present study, membrane permeabilization could be achieved by chloroform and sodium lauryl sulfate that enhance activity by improving the transportation of the substrate and/or enzyme across the cell membrane (Berg and Fiksdal, 1988).

The resulted high Pearson r and $P < 0.0001$ indicates the strong correlation between standardized sample activity and the log of E. coli concentrations and that strongly suggests the possibility of using the colorimetric assay as a reliable and rapid method in the quantification of FC.

Detection of E. coli using multiplex PCR:

Early PCR protocols utilized two sets of primers, the first primer sequence (derived from lacZ gene, coded for GALase) served to detect all coliform bacteria, and the second primer sequence (derived from uidA, coded for GLUase) used for detection of E. coli. However, it was found that the primer set of uidA gene could also identify the non-E. coli coliforms (Fricker and Fricker, 1994). In addition, the above-mentioned duplex PCR protocol does not allow distinguishing Salmonella spp. and Shigella spp. from E. coli (Bej et al., 1991a) and (Li
et al., 2004). These facts clearly indicate that duplex PCR protocols are not specific enough to provide a reliable and specific detection of E. coli.

Other investigators utilized four genes (lacZ, uidA, lacY coding for lactose permease), and cyd (coding for cytochrome bd complex)) so as to be able to specifically detect E.coli (Horakova et al., 2008). Horakova et al. (2008) recorded that lactose permease is found in enteric bacteria, including Serratia spp., Salmonella spp., Citrobacter freundii, Klebsiella pneumoniae, and Yersinia pestis (Stoebel, 2005). lacY gene is detected only along with lacZ in strains utilizing lactose (E. coli, Enterobacter cloacae, Citrobacter freundii, Kluyvera ascorbata, Klebsiella pneumoniae subsp. pneumoniae and Raoutella planticola). Interestingly, Shigella spp., the phylogenetically closest relative of E. coli, can be distinguished from E. coli as the PCR product corresponding to lacY gene is absent (Stoebel, 2005). This is in agreement with the findings of Ito et al. (1991) who observed that Shigella flexneri, Shigella boydii and Shigella dysenteriae lack the lacY gene. While, cytochrome bd complex presence in Klebsiella pneumoniae, Klebsiella oxytoca, Raoutella planticola and Raoultella terrigena will be accompanied with the absence of uidA gene and its presence in Shigella sonnei and Shigella flexneri will be accompanied with the absence of lacY. In our study multiplex PCR was performed using only three genes (lacZ, uidA and lacY) to achieve the E. coli detection and these three genes globally can be named as E. coli hallmark genes.

Unequivocal advantage of this approach is that GLUase negative E. coli strains (with undetectable GLUase activity) were identified by PCR amplification targeting the uidA gene (Bej et al., 1991b). That is to say, our multiplex PCR approach seems to be a reliable tool for identification of E. coli - one of the best bacterial indicators of fecal contamination of water and potential pathogen. Application of our multiplex PCR to environmental isolates contributed to the increased reliability and specificity of E. coli detection.

Conclusion:

Under Egyptian conditions, our study has resulted in that there is a great correlation between the microbial count and the enzymatic activity when using chromogenic substrate (Pearson r = 0.87) or fluorogenic one (Pearson r = 0.749) that means the ability of using the enzymatic activity as a method for the quantification of waterborne pathogens. Moreover, the fluorometric methods are suitable to low contaminated samples due to the inner filter effect while; the colorimetric method can be used in the heavily contaminated samples. In addition, multiplex PCR based on three target genes uidA, lacZ, lacY is suitable for identification of environmental E. coli either viable, VBNC or dead cells.

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