African Journal of Biotechnology Vol. 9 (4), pp. 564-572, 25 January, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Development of a competitive PCR assay for the quantification of total *Escherichia coli* DNA in water

Omar Kousar Banu, Barnard Tobias George* and Jagals Paul

Water and Health Research Unit, University of Johannesburg, Republic of South Africa.

Accepted 23 November, 2009

Standard health-related microbial water testing relies on the culturability of *Escherichia coli* (*E. coli) to* estimate their numbers. Competitive PCR (c-PCR) offers the potential to estimate the *E. coli* level of a water source without culturing. The aim was to investigate the use of c-PCR reaction to detect and quantify, without prior enrichment, *Escherichia coli* in water samples. The *E. coli* malate dehydrogenase *Mdh* house-keeping gene was modified and used as an internal control and competitor DNA for the c-PCR. *E. coli* cell concentration equivalents ranging from 20 to 2 x 10^4 cells ml^{-1} could be quantified with the c-PCR. Fifty-three water samples from various sources were tested with the DNA extraction and c-PCR protocol. Due to PCR inhibition *E. coli Mdh* gene copies could only be determined for 20 of the 53 samples (38%). Of the 20 samples tested 15% gave comparable results for competitive PCR and culturable *E. coli* numbers; 55% obtained higher values with competitive PCR and 30% obtained higher values with the culture based experiments. The c-PCR successfully estimated *E. coli* numbers that gave comparable results with the culture based microbiological data obtained.

Key words: Competitive polymerase chain reaction (c-PCR), waters, *Escherichia coli*, internal standard.

INTRODUCTION

Escherichia coli (*E. coli*) is generally regarded as a highly specific bacterial indicator of faecal pollution from humans and warm-blooded animals (World Health Organization (WHO), 2005). Methods for the specific enumeration of *E. coli* from water samples are mostly based on chromogenic and fluorogenic media (Lemarchand et al., 2005). These methods are dependant on the culturability of *E. coli* and therefore would not detect cells in the viable but non-culturable (VBNC) state that may be present in the same water samples (Lleo et al., 2005). VBNC cells are metabolically active but can generally not be cultured with the standard microbiological techniques normally used for health-related microbial water quality testing. Chen et al. (2006) have reported that these cells are able to resume active growth once favourable conditions are restored. The implication of this is that the *E. coli* numbers in a water sample will be underestimated and so will the faecal pollution load in the sample (Lleo et al., 2005). Therefore,a method to estimate the viable and viable but non-culturable *E. coli* in a sample, termed total *E. coli*, is needed.

This can be overcome by direct detection of *E. coli* using molecular techniques. However, the convention is that these methods will also require a pre-culturing step. To avoid culturing, the bacterial cells can be concentrated from the water, followed by DNA extraction and the use of the polymerase chain reaction (PCR) method to amplify selected genes (Kong et al., 2002). However, conventional PCR is qualitative and will only indicate the presence or absence of the target gene and cannot be used to quantify a gene of interest. A modification of PCR, termed competitive PCR (c-PCR), has been developed to detect and estimate bacterial numbers. It has been shown that data from c-PCR testing are comparable to culture-based microbiological data when detecting and estimating bacterial numbers in water samples (Sidhu et al., 1999; Rose et al., 2003). A number of studies have used c-PCR to quantify *E. coli* (Rose et al., 2003; Li and Drake, 2001), *Enterococcus faecalis* (Lleo et al., 2005), *Oxalobacter formigenes* (Sidhu et al.,

*Corresponding author. E-mail: tgbarnard@uj.ac.za. Tel: +2711 5596324. Fax: +2711 5596329.

1999), *Sphingomonas chlorophenolica* (Van Elsas et al., 1998) and *Clostridium proteoclasticum* (Reilly and Attwood, 1998) in environmental samples.

Competitive PCR is based on the co-amplification of the target sequence to be quantified with a known amount of another sequence that shares most of the nucleotide sequence with the target (Van Elsas et al., 1998). These two sequences should ideally be from the same region of DNA, with binding regions for the same primers, in order to amplify each with equal efficiency. The two templates should also differ slightly in size to be distinguishable by agarose gel-electrophoresis (Rose et al., 2003). Using varying concentrations of the template DNA with constant amount of competitor, equivalent band intensities will be observed when the concentration of each is equivalent. The point of equivalence is determined by visual assessment of band intensities or by digital analysis of the gel image (Lim et al., 2001). Thereafter, the log ratios between the genomic DNA (g-DNA) and competitor DNA are applied to construct a standard curve (Lleo et al., 2005) from which the quantities of DNA copies are estimated /extrapolated.

Mathematical models proposed by Connoly et al. (1995), Zachar et al. (1993) and Rose et al. (2003) were adapted for use with competitive PCR for the quantification of products. This mathematical model hypothesizes that the starting amount of the template can be determined if the amount of competitor used in all reactions is kept constant and if the standard curve is linear (Rose et al., 2003). The DNA concentration extrapolated from the standard curve can then be used to calculate the number of copies per microlitre. The gene copy number can then be converted to chromosomal equivalents and then compared to the cell numbers obtained from culture based methods (Li and Drake, 2001).

The aim of this study was to develop a competitive PCR technique for developing countries that might not have access to more technologically advanced PCR methods (e.g. real-time PCR), which could be applied for the screening of total *E. coli* present in untreated waters that people ingest. This was achieved by targeting the *Mdh E. coli* house-keeping gene for quantification of total *E. coli* present in a water sample without prior culturing or enrichment.

METHODOLOGY

Growth and maintenance of bacterial strains

A commensal *E. coli* strain was isolated and confirmed with API 20E. This was cultured on plate count agar (PCA) (Oxoid) and incubated under aerobic conditions at 37ºC for 16 h. *E. coli* Top 10 cells (Whitehead Scientific (PTY) LTD) used for transformational experiments was grown and maintained on nutrient broth (NB) (Oxoid) plates and grown at 37°C for 16 h. Competent *E. coli* Top 10 cells transformed with the vectors were grown on NB plates supplemented with ampicillin (30 $mgml^{-1}$), IPTG (24 $mgml^{-1}$) and X-Gal (20 $mgml^{-1}$) (Fermentas®). Positive *E. coli* transformants were grown in 5 ml NB supplemented with ampicillin (10 $mgml^{-1}$) and grown at 37°C for 16 h with mild agitation at 200 rpm. *E. coli* grown up for genomic DNA isolations were grown in 5 ml NB at 37ºC for 16 h with agitation at 200 rpm.

Polymerase chain reaction (PCR)

PCR reactions were performed in a Biorad Mycycler™ Thermal cycler in a total volume of 20 □l. Each reaction consisted of 10X PCR buffer; 5 µl DNA extract; 2.5 mM dNTP's; 2.5 pmol forward and reverse primers; 0.5 U Hotstar *Taq* polymerase (Qiagen®) and PCR grade water. The reactions were subjected to an initial denaturing step at 95°C for 15 min, followed by 35 cycles denaturing at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 1 min and final elongation at 72°C for 5 min. This procedure was followed for all the experiments except where stated differently.

Gel electrophoresis

DNA was analyzed using a 2.5% (w/v) agarose gel in TAE buffer (40 mmol l^{-1} Tris acetate; 2 mmol l^{-1} EDTA, pH 8.3) with 0.5µgml^{-1} ethidium bromide. Electrophoresis was performed for 1 - 2 h in an electric field strength of 8 V cm^{-1} gel and the DNA visualized with UV light (Gene Genius Bio Imaging system, Vacutec®). This procedure was followed for all the experiments except where stated differently. The relative sizes of the DNA fragments were estimated by comparing their electrophoretic mobility with that of the standards run with the samples on each gel, either a 1 kB or 100 bp markers (Fermentas®).

Construction of the competitor and optimization of competitive PCR (c-PCR)

A 302 bp fragment of the malate dehydrogenase gene was amplified using the Mdh F: 5'-GGT ATG GAT CGT TCC GAC CT-3'and Mdh R: 5' GGC AGA ATG GTA ACA CCA GAG T -3' primer pair (Tarr et al., 2002). The resulting PCR product was subjected to agarose gel electrophoresis and purified using the high pure PCR product purification kit (Roche® Diagnostic Co-operation). Thereafter the product was ligated into the pGEM®T-easy cloning vector (Promega®) using 10 X ligation buffer and 1 U DNA ligase (Fermentas®) in a total volume of 23 µl, the reaction was incubated for 1h at 4ºC. This was transformed into competent *E. coli* Top 10 cells as described by Tang et al. (1994) and plated onto NB plates supplemented with ampicillin, IPTG and X-gal (Fermentas®). Presumptive positive white (*lacZ* gene disrupted) colonies were grown in NB broth with ampicillin and the plasmids isolated using the lysis by boiling method. The insert size was confirmed by restriction enzyme digestion with *Eco*RI, according to the supplier's (Fermentas®) recommendations. Plasmids with the correct insert size after restriction enzyme digestion were subjected to restriction enzyme digestion with *Hpa*I to excise an 84 bp region from the cloned PCR product (Figure 1). The resulting digest was run on a 1% (w/v) agarose gel to separate the 84 bp from the rest of the 3233 bp construct. The 3233 bp construct was purified from the agarose gel using the high pure PCR product purification kit (Roche® Diagnostic Co-operation). This construct was re-ligated as described above, transformed into competent *E. coli* Top 10 cells and plated onto selective plates (As described earlier). White and blue colonies were grown in NB broth supplemented with ampicillin (10 mg ml^{-1}), plasmids were isolated using the lysis by boiling method and the insert size confirmed by restriction enzyme digestion using *Eco*RI and PCR (as described earlier). All confirmations were visualised on 1% (w/v) agarose gels.

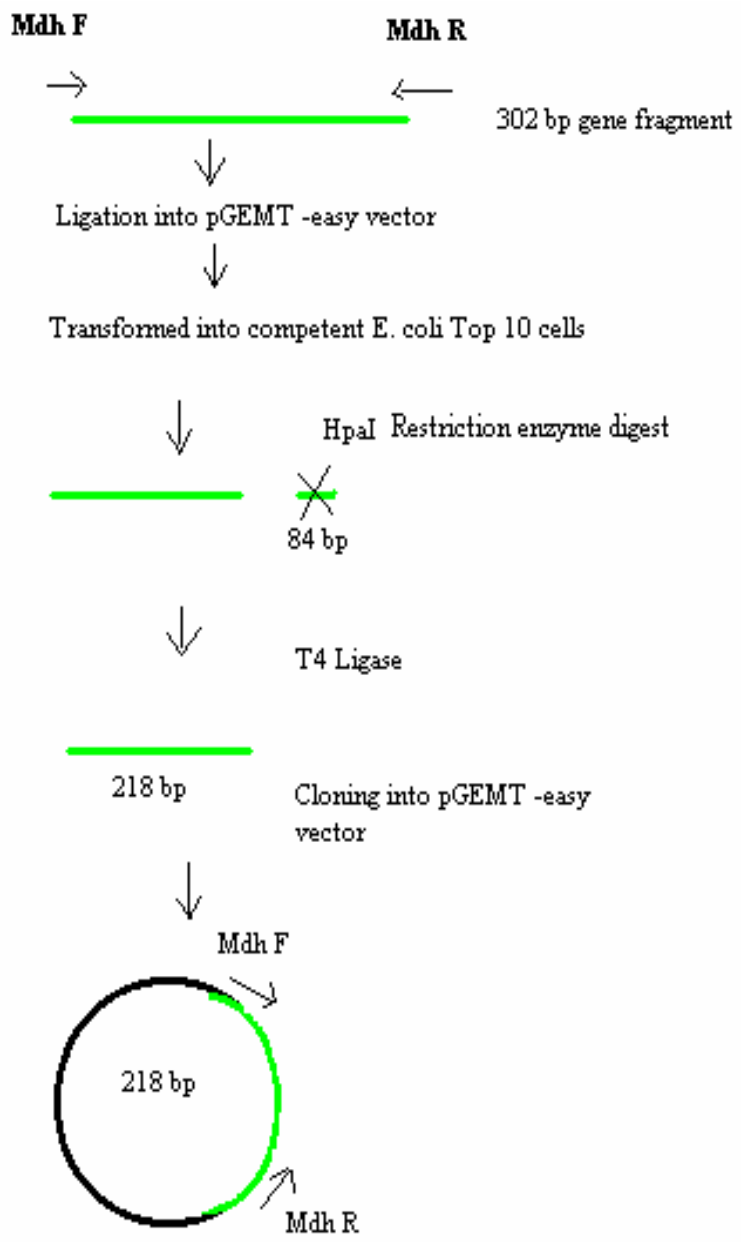

Figure 1. Diagram showing construction of the *Mdh* internal standard.

Optimization of competitive PCR (c-PCR)

The primer pair Mdh F and Mdh R was used to co-amplify the target (Commensal *E. coli* reference strain) and the competitor DNA. Amplifications were performed as described earlier with 5 µl target DNA and 2 µl appropriately diluted internal competitor DNA (pGEMMdh4) added as DNA template. The PCR reaction was used as described earlier. Various combinations of the competitor and genomic DNA concentration were tested to estimate the optimal concentration of the competitor for satisfactory co-amplification of the *Mdh* gene from the genomic DNA and competitor *Mdh* gene.

Construction of standard curve for c-PCR

Standard curves for the c-PCR were constructed using the band intensities of the PCR products from agarose gels using the Biorad® quantification program (Quantity 1). The data were imported to an excel spreadsheet where *E. coli* g-DNA /competitor DNA ratios were calculated and plotted as the log ratios of *E. coli* g-DNA /competitor DNA against the log input of EC g-DNA. Sigma Stat 9.0 was used to calculate the resultant statistical parameters.

Mathematical modelling of c-PCR

The performance of the c-PCR system was determined by the mathematical model that describes and predicts the exponential nature of the PCR reaction as stated by Zachar et al. (1993) and Rose et al. (2003). The formula used for c-PCR was (Rose et al., 2003):

$$\log\left(\frac{Tj}{Cj}\right) = \log To$$

Where , T_j = final amount of the product from genomic DNA template; T_o = initial amount of DNA
C_j = final amount of the product from competitor DNA.

Sampling sites and sample collection

A total of 53 water samples were collected which included 22 wastewater samples from the four points typically found in a wastewater treatment facility (raw, primary, secondary and tertiary), 17 river samples (Gauteng, Kwazulu-Natal and Limpopo provinces) and 14 water samples from household storage containers from rural villages.

Water samples were collected in 11 sampling bottles and kept at 4ºC on route to the laboratory. Samples were analysed within 3 h of collection for bacterial quality using the Colilert® Quanti-Tray/ 2000® (IDEXX).

Microbial analysis of water

Colilert® analysis of the samples was conducted using undiluted (100 ml) as well as various sample dilutions (10, 1 ml of a 10^{-1}, 1 ml of a 10^{-2}). This was mixed with Colilert® media (IDEXX) in sterile 100 ml glass funnels with lids and then sealed in Quanti-tray/2000® containers. Samples required dilution because indicator densities in the samples exceeded the operating range of the test (2400 most probable number (MPN) 100 ml^{-1}). Quanti-trays were incubated for 18 h at 35ºC. After incubation, the Quanti-trays were examined under long wave (366 nm) ultraviolet light and wells that turned both yellow and fluorescent were counted as *E. coli* positive. *E. coli* densities were determined as recommended by the supplier.

DNA extraction from water samples

The water samples were filtered in 100 ml volumes onto polyether sulfone (PES) membranes using standard membrane filtration technique (Standard Methods, 2005; Jagals et al., 2001). DNA was extracted from the trapped bacteria using the silica/guanidium thiocyanate method reported by Boom et al. (1990) as well as adaptations of spin columns reported by Borodina et al. (2003). The adjustments included the addition of 250 µl 100% ethanol to the lysis buffer to enhance the binding of DNA to the celite. The celite containing the bound DNA was loaded onto a DNA binding membrane (Borodina et al., 2003) in the spin columns. DNA was eluted with 50 µl Qiagen elution buffer (Southern Cross Biotechnology®). The extracted DNA was used as a template in all PCR reactions.

Competitive PCR (c-PCR) application

DNA isolated from the water samples was subjected to c-PCR using the protocol stated earlier, where 5 µl of sample DNA extracted was co-amplified with 2 µl of 98000000 $ng\mu l^{-1}$ of pGEMMdh competitor DNA. These PCR's were performed in triplicate to obtain a most probable number (MPN) PCR. The PCR products were analyzed on 2.5% (w/v) agarose gels and the agarose gels analyzed using the Biorad® Quantification system (Quantity 1). Interpolation of the regression equation for a y-value of 0 from the created standard curves (Stated earlier) gave the concentration of the target template in the water sample.

Calculation of DNA concentration

The following calculation was used for the estimation of colony forming units:

For *E. coli* it was accepted that:

1 cell = 1 genome copy = 1 *Mdh* copy (Van der Rest, et al., 2000; Park et al., 1995)…….. (1)

The DNA concentration extrapolated from the standard curve was converted to the number of copies μl^{-1}, using the equation:
Number of *Mdh* copies μl^{-1} = (6.02 x 10^{23} copies mol^{-1}) x (concentration in g l^{-1}) / (MW g mol^{-1})…… (2)

Copies μl^{-1} was then converted to copies per 100 ml^{-1}

But 1 copy of *Mdh* = 1 genome copy = 1 *E. coli* genome equivalent (cell)…… (3)

Therefore 1 copy of *Mdh* = 1 *E. coli* ……..… (4)

Thus x numbers of copies of *Mdh* in 100 ml = x numbers of *E. coli* cells in 100 ml……..…. (5)

RESULTS

Construction of the PCR competitors

The 302 bp fragment was successfully amplified from the *E. coli* malate dehydrogenase gene using the Mdh primer pair (Figure 2). This PCR product was cloned into the pGEMT®-easy vector and the insert size confirmed with *Eco*RI restriction enzyme digestion after the transformation and plasmid isolation experiments. The construct showing the correct insert (pGEMMdh3) was chosen for further experiments (Figures 3a). The selected clone was subjected to restriction enzyme digestion with *Hpa*I to excise a 84 bp segment from the cloned PCR product (Figure 3b). The digested construct was re-ligated with T4 DNA ligase and transformed into *E. coli* Top 10 cells. Plasmid isolated from positive colonies was digested with *Eco*R1 to confirm the insert size and the clone showed that it contained the modified PCR insert cloned into the pGEMT®-easy vector. As expected the estimated insert size was ~218 bp (pGEMMdh4) indicating the successful modification of the *Mdh* PCR product. The construct was used as a template for PCR reactions to confirm the restriction enzyme digestion results. In addition, it confirmed that the primer binding sites were still intact as can be seen in (Figure 2). However, non-specific binding occurred during the PCR and it was decided to amplify and purify the cloned modified insert that was to be used as a competitor during the competitive PCR, Xymoclean Kit (Inqaba Biotechnologies®). This competitor also served as the internal control for the PCRs to determine if there was PCR inhibition.

Optimization of competitive PCR (c-PCR)

Different concentrations of the *Mdh* competitor were

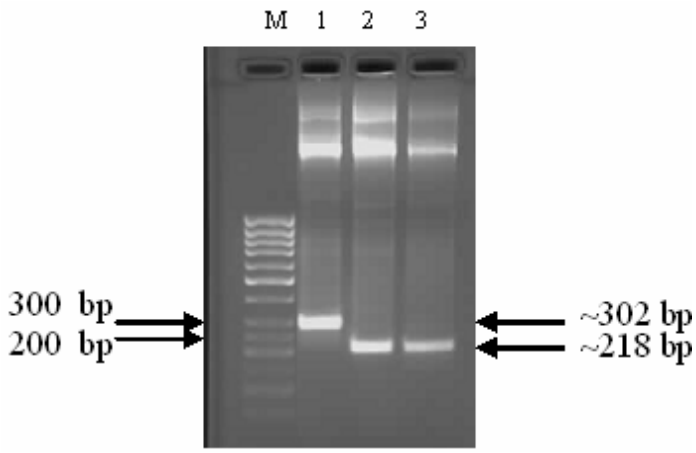

Figure 2. Agarose gel showing the PCR results obtained when pGEMMdh2 and pGEMMdh4 was used as template. Amplification of pGEMMdh3 (lanes 1 and 3) and pGEMMdh4 (lane 2) shows correct amplification of the *Mdh* (~302 bp) and modified *Mdh* (~219 bp) gene products. M indicates the 100 bp Fermentas O' GeneRuler DNA ladder run.

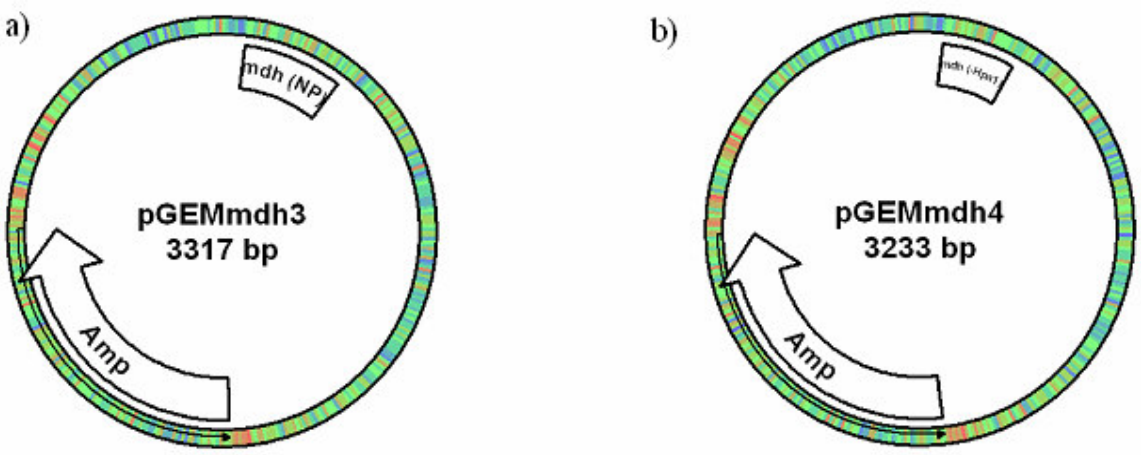

Figure 3. Plasmid map of a) clone pGEMMdh3 for the cloned Mdh PCR product in pGEMT®-easy; b) clone pGEMMdh4 for the cloned and modified *Mdh* PCR product in pGEMT®-easy.

co-amplified with serially diluted *E. coli* genomic DNA (3.73×10^{-2} – $3.73 \times 10^{-5.5}$) until satisfactory co-amplification was obtained. By diluting the EC g-DNA and keeping the competitor constant, the EC g-DNA initially out-competed the competitor for primer binding. This decreased with decreasing [EC g-DNA] concentrations up to a point where equal amplification could be observed. Following this, the competitor started to out-compete the EC g-DNA for binding of the primers and yielded the typical co-amplification profile as seen in Figure 4. The three competitor concentrations that provided the most satisfactory results were 9.75×10^{-6} ng 5 μl^{-1} (referred to as -7 dilution), 9.75×10^{-7} ng 5 μl^{-1} (-8 dilution) and 9.75×10^{-8} ng 5 μl^{-1} (-9 dilution) respectively. The band intensities of the PCR products from the agarose gel images were quantified using the Biorad® quantification program (Quantity 1). The data was then used to construct the standard curves for the various competitor concentrations along with varying concentrations of g-DNA.

The standard curves were constructed as a plot of log ratios of the PCR products against the log of known amounts of *E. coli* g-DNA. From this data the -8 competitor was used, because the gel picture showed that a marked intensity decrease of EC g-DNA against an increasing intensity of c-DNA. The resulting standard curve for the -8 dilution provided the best linear graph between the log ratio of the EC g-DNA and c-DNA amplification versus the log of *E. coli* g-DNA input with a R^2 value of 1 and a slope of 1 (Figure 4). The accuracy of

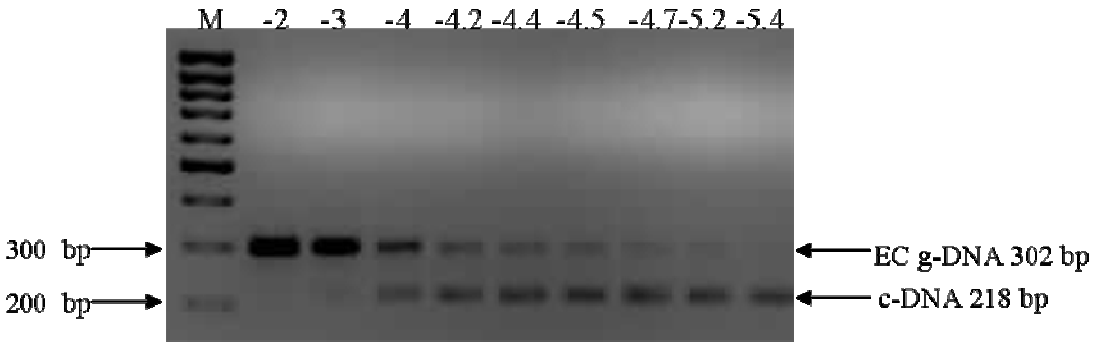

Figure 4. Agarose gels showing the co-amplification of a dilution series of EC g-DNA with constant amounts of competitor –8 pGEMMdh4 dilution.

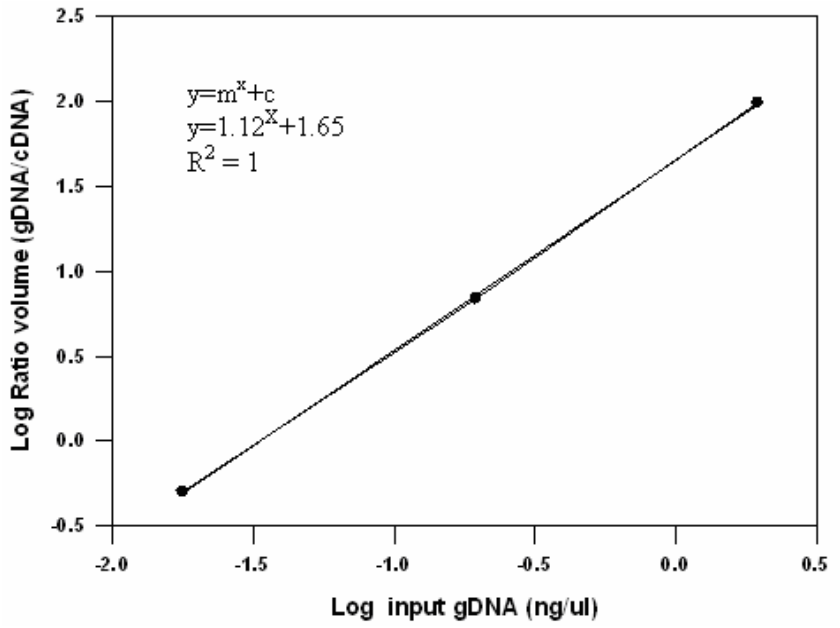

Figure 5. Construction of the standard curve for co-amplification of varying concentrations EC g-DNA with constant amount of competitor for -8 pGEMMdh4 dilution made is indicated above each lane. The dilutions are given as -2 for 10^2 etc.

our c-PCR in practice was comparable to the mathematical requirements predicted by the theoretical model of c-PCR. This means that our c-PCR assay successfully co-amplified serially diluted g-DNA with constant competitor for the construction of a standard curve which was linear with a R^2 value of 1 and slope of 1. This also indicated that the amplification efficiencies and intensities of log ratio of PCR products were equal to the log of g-DNA input. Concentrations of *E. coli* DNA could be calculated from the standard curve between the ranges of 9.75×10^{-3} ng μl^{-1} (equal to 20 *E. coli* cells ml^{-1}) to 10 ng μl^{-1} (equals to 2×10^4 *E. coli* cells ml^{-1}) and could be used to estimate the *E. coli* DNA concentrations for amplification as long as the values for co-amplification fell within this range (Figure 5).

Environmental application

For this part of the project, 53 environmental water samples were analyzed that consisted of 22 wastewater samples, 17 river samples and 14 household water storage containers. Each water sample was analysed for the presence and number of *E. coli*. Bacteria were concentrated from the water samples (100 ml of each) using the PES membrane and g-DNA isolated. The -8 dilution of the *Mdh* competitor served as both the competitor and the internal control (IC) to monitor for the presence of PCR inhibitors. Eighteen of the water samples showed PCR inhibition to the extent that not even the IC was detected. Two adaptations were investigated to remove the PCR inhibition. The first was

Table 1. Comparison between microbiology and molecular biology data on environmental water samples that could be used for c-PCR.

Sample name	Inhibition lifted*	Microbiological cfu 100 ml^{-1}	c-PCR cfu 100 ml^{-1}
Plant 1 raw a	Undiluted	2.36E+07	1.86E+05
Plant 1 raw b	1:5 dilution	1.35E+07	1.71E+06
Plant 1 raw c	Undiluted or BSA	2.36E+06	6.12E+05
Plant 2 raw	1:5 dilution or BSA	2.18E+07	1.60E+04
Plant 1 primary a	Undiluted	1.92E+07	1.09E+06
Plant 1 primary b	Undiluted or BSA	8.16E+06	1.52E+06
Plant 2 primary	Undiluted	7.27E+06	4.78E+05
Plant 1 secondary a	Undiluted or BSA	1.12E+04	3.78E+03
Plant 1 secondary b	Undiluted	9.60E+03	5.22E+04
Plant 2 secondary	Undiluted	3.97E+03	1.01E+03
Plant 1 tertiary a	Undiluted	1.19E+04	1.06E+04
Plant 1 tertiary b	Undiluted or BSA	2.98E+02	5.97E+03
Plant 1 tertiary c	Undiluted or BSA	2.98E+02	5.97E+03
Plant 2 tertiary	Undiluted	<1	7.63E+04
42nd Hill dam	Undiluted or BSA	3.00E+02	3.07E+03
River sample 1	Undiluted	4.20E+01	3.93E+05
River sample 2	Undiluted	3.42E+01	3.48E+03
Container a	Undiluted or BSA	1.27E+05	7.20E+04
Container b	Undiluted	2.00E+03	5.94E+03
Container c	Undiluted	2.67E+02	4.10E+04

*Inhibition was lifted by either adding BSA or diluting the extracted DNA fivefold.

adding bovine serum albumin (BSA) as a PCR facilitator. Of the 18 samples, inhibition was removed for 8 samples (44.4%). The second approach was to dilute the extracted g-DNA in order to dilute the inhibitors present. Of the 18 samples, inhibition was removed for 15 samples (83.3%). For the 3 remaining water samples (0.17%), the inhibition could not be removed either by adding a facilitator or by dilution. The overall results however, demonstrated that by diluting the sample 5–fold, inhibitions for the majority of the water samples inhibition were removed.

Quantifications of the *E. coli* genomic DNA was achieved by co-amplification of the extracted DNA from the water samples with 9.8 x 10^{-7} ng 5 μl^{-1} (-8) Mdh competitor DNA. For this PCR we chose to dilute the water samples instead of adding a BSA facilitator. It was found that BSA greatly enhanced the amplification of the *Mdh* competitor and thus over-competing with sample genomic DNA. A total of 38% (20/53) of the water samples analyzed could be quantified and we were able to detect as low as 3 cells ml^{-1} (Table 1). However, the samples that were diluted 5-fold could not be quantified because the level of target DNA was too low for detection after dilution, allowing for over-competition by the competitor DNA. Therefore comparisons were performed on only 20 samples that gave values for both the culturability (Microbiology) and the c-PCR (Molecular biology) experiments. The data obtained from the c-PCR analysis of the water samples for *E. coli* numbers was comparable with the microbiological data obtained. Three out of 20 samples (15%) tested gave comparable results for c-PCR and culturable *E. coli* numbers; with eleven of the 20 samples (55%) determining higher values with c-PCR and six out of 20 samples (30%) giving higher values with the microbiological experiments in terms of *E. coli* numbers present.

DISCUSSION

Various authors have demonstrated that bacterial cells are capable of entering a viable but non-culturable state (VBNC) where in they cannot be isolated using standard culture based techniques. These cells are however still viable and capable of resuming active growth when favourable conditions are restored (Lleo et al., 1999; Oliver et al., 2005). The aim of this project was to investtigate the use of molecular biology techniques as a supplement and even an alternative to culture based microbiology for the detection and quantification of bacteria using *E. coli* as a model pathogen.

During this study the use of competitive PCR targeting the *E. coli* malate dehydrogenase gene was shown to be a viable option for the detection and quantification of *E. coli* cells in water samples. The pGEMMdh4 competitor was successfully constructed and used as the competitor DNA in the quantitative competitive PCR and as an internal control (IC). Although the competitive PCR could

only be successfully applied to 37% (20 out of 53 samples) of the samples tested, the *E. coli Mdh* gene could be detected in 30 (56.6%) of the remaining samples after the addition of PCR facilitators. No PCR results were detected for the remaining 3 samples due to severe PCR inhibition since the *Mdh* competitor that served as the internal control could not be amplified after diluting the samples 5-fold and/or adding BSA to the PCR mixture. The addition of the *Mdh* competitor to the PCR reaction highlighted an important advantage of c-PCR over conventional PCR. In contrast to an external positive control, an IC is a non-target DNA sequence present in the very same sample tube that is co-amplified simultaneously with the target sequence (Abdulmawjood et al., 2002). In a PCR without an IC, a negative response could either mean that there was no target sequence present in the reaction, or it could mean that the reaction was inhibited because of a malfunctioning thermal cycler, an incorrect PCR mixture, poor DNA polymerase activity or (not least) the presence of inhibitory substances in the sample matrix, revealing any failure of the PCR (Hoorfar et al., 2004).

During this study standard curves were created for the competitive PCR by co-amplifying constant amount of competitor DNA with serially diluted genomic DNA as reported by Zachar et al. (1993) and Rose et al. (2003). The yield of the two products was related to the amount of initial template, which corresponds to the equations stated by Zachar et al. (1993) and Rose et al. (2003). The created standard curve was in the exponential phase with a slope close to 1. This indicated that the two DNA's amplification efficiencies were equal and that the competitor remained constant (Rose et al., 2003). The *Mdh* c-PCR assay allowed for the quantification of the equivalent of 20 to 2 x 10^4 *E. coli* cells ml^{-1} from the extracted DNA for the construction of the standard curve. For the application of c-PCR on the water samples it was possible to detect the equivalent of 3 *E. coli* cells ml^{-1} after extrapolation of the data from the standard curve. Lleo et al. (1999) as well as Lim et al. (2001) were also able to detect 24 to 2 x 10^3 cells ml^{-1} from environmental water samples using a c-PCR assay for *Enterococci* and *E. coli* species.

Although the c-PCR could be used for the quantification of some of the samples, important considerations needs to be kept in mind to do absolute quantification. The first is the DNA extraction efficiency. Several studies have shown the occurrence of variability in DNA recovery efficiency. One such study by Mumy and Findlay (2004) showed that DNA recovery efficiency never exceeded 50% and some of the kits used recovered DNA as low as 2%. These experiments were however done on pure bacterial cultures making it easier to determine the extraction efficiency. It does however emphasize the importance of developing such a system when working with environmental samples to be able to adjust the determined values according to the extraction efficiency.

The second consideration is the degree of PCR inhibition for each sample. Controls need to be put in place to estimate this inhibition and to accurately estimate the amount of DNA present. The IC of the c-PCR allows for this to a certain degree. Because this technique is based on competitive binding of the primers, any condition that will benefit one template is detrimental to the experiments. This problem was encountered during the dilution of the g-DNA to reduce inhibition which allowed for over competing of the competitor DNA for primer binding.

The third consideration is the physiological state of the cells that is detected by the PCR. Methods are needed to distinguish between live, VBNC, dead cells and free DNA in the samples in order to correctly report on the active bacterial fraction that might pose a health risk to consumers. Lleo et al. (1999) has stated that free DNA in environmental water would degrade very rapidly. However, this point was not investigated during this study. However, the results of this study indicated that in some of the water samples, the *E. coli* DNA was quantified using PCR even though *E. coli* bacteria could not be cultured through standard culturing techniques. This demonstrated the value of being able to distinguish between cells that have entered a viable but non-culturable state, culturable cells and dead cells (Oliver et al., 2005; Skelly and Weinstein, 2003; Lleo et al., 2005; Pianetti et al., 2005).

Conclusion

A c-PCR protocol was successfully developed to quantify *E. coli* from environmental waters. The developed protocol is a cheaper alternative that can be used on standard thermo-cycling PCR machines for molecular quantification of *E. coli*. There is however a need to develop and optimise necessary controls before any type of PCR can be used for the quantification of bacteria in environmental samples.

ACKNOWLEDGEMENT

We wish to express our profound gratitude to the University of Johannesburg and National Research Foundation (NRF) for funding this research.

REFERENCES

Abdulmawjood A, Roth S, Bulte M (2002) Two methods for construction of internal amplification controls for the detection of *Escherichia coli* 0157 by polymerase chain reaction. Molecular and cellular probes, 16: 335-339.

Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-Van Dillen PME, Van Der Noordaa J (1990) Rapid and Simple Method for Purification of Nucleic Acids. J. Clin. Microbiol. 28: 495-503.

Borodina TA, Lehrach H, Soldatov AV (2003) DNA purification on homemade silica spin-columns, Analytical Biochem. 321: 135-137.

Chen YC, Higgins MJ, Maas NA, Murthy SN (2006) DNA extraction and *Escherichia coli* quantification of anaerobically digested solids using the competitive touchdown PCR method, Water Res. 40: 3037-3044.

Connoly AR, Cleland LG, Kirkham BW (1995) Mathematical considerations of competitive polymerase chain reaction, J. Immun. Meth. 187: 201-211.

Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P (2004) Practical comsiderations in design of internal amplification controls for diagnostic PCR assays, J. Clin. Microbiol. 42(5): 1863-1868.

Jagals P, Grabow WOK, Griesel M, Jagals C (2001) Evaluation of selected membrane filtration and most probable number methods for the enumeration of faecal coliforms, Escherichia coli and Enterococci in environmental waters, Quantitative Microbiol. 2 (2) June.

Kong RYC, Lee SKY, Law TWF, Law SHW, Wu RSS (2002) Rapid detection of six types of bacterial pathogens in marine waters by multiplex PCR, Water Res. 36: 2802-2812.

Lemarchand K, Berthiaumea F, Maynarda C, Harelb J, Paymentc P, Bayardelled P, Massona L, Brousseau L (2005) Optimization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays. J. Microbiol. Meth. 63: 115–126.

Li W, Drake MA (2001) Development of a Quantitative Competitive PCR Detection and Quantification of *Escherichia coli*, Appl. Environ. Microbiol. 67(7), July, p. 3291-3294.

Lim EL, Tomita AV, Thilly WG, Polz MF (2001) Combination of competitive quantitative PCR and constant denaturant capillary electrophoresis for high resolution detection and enumeration of microbial cells, Appl. Environ. Microbiol. 67(9): 3897-3903.

Lleo MDM, Tafi MC, Signoretto C, Cero CD, Canepari P (1999) Competitive polymerase chai reaction for quantification of non-culturable *Enterococcus faecalis* cells in lake water. FEMS Microbiol. Ecol. 30: 345-353.

Lleo MM, Bonato B, Tafi MC, Signoretto C, Pruzzo C, Canepari P (2005). Molecular vs culture methods for the detection of bacterial faecal indicators in groundwater for human use. Appl. Microbiol. 40: 289–294.

Mumy KL, Findlay H (2004). Convenient determination of DNA extraction efficiency using an external DNA recovery standard and quantitative-competitive PCR, J. Microbiol. Meth. 57: 259-268.

Oliver JD, Dagher M, Linden K (2005). Induction if *Escherichia coli* and *Salmonella typhimirium* into the viable but nonculturable state following chlorination of wastewater, J. Water Health, 03(3): 249-256.

Park SJ, Cotter PA, Gunsalus RP (1995). Regulation of Malate Dehydrogenase (*mdh*) gene expression in *Escherichia coli* in response to oxygen, carbon, and heme availability, J. Bacteriol. 177(22): 6652-6656.

Pianetti A, Falcioni T, Bruscolini F, Sabatini L, Sisti E, Papa S (2005). Determination of the viability of *Aeromonas hydrophila* in different types of water by flow cytometry, and comparison with classical methods, Appl. Environ. Microbiol. 71(12): 7948-7954.

Reilly K, Attwood GT (1998). Detection of *Clostridium proteoclasticum* and closely related strains in the rumen by competitive PCR, Appl. Environ. Microbiol. 64(3): 907-913.

Rose P, Harkin JM, Hickey WJ (2003). Competitive touchdown PCR for estimation of *Escherichia coli* DNA recovery in soil DNA extraction, J. Microbiol. Meth. 52: 29-38.

Sidhu H, Holmes RP, Allison MJ, Peck AB (1999). Direct quantification of the enteric bacterium *Oxalobacter formigenes* in human faecal samples by quantitative competitive- template PCR, 37(5): 1503-1509.

Skelly C, Weinstein P (2003). Pathogen survival trajectories: An eco-environmental approach to the modeling of human Campylobacteriosis ecology, Environ. Health Perspectives, 111(1): 19-28.

Standard Methods. (2005). Standard methods for the examination of water and wastewater, 21st edition, Eaton AD, Clesceri L and Greenberg AE, American Public Health Association, Washington DC, USA.

Tang X, Nakata Y, Li O, Zhang M, Gao H, Fujita A, Sakatsume O, Ohta T, Yokoyama K (1994). The optimisation of preparations of competent cells for the transformation in *E. coli*. Nucleic Acid Res. 22: 2857-2858.

Tarr CL, Large TM, Moeller CL, Lacher DW, Tarr PI, Acheson DW, Whittam TS (2002). Molecular caharacterization of a serotype 0121:H19 clone, a distinct shiga toxin-producing clone of pathogenic *Escherichia coli*, Infect. Immunity, 70(12): 6853-6859.

Van der Rest ME, Frank C, Molenaar D (2000) Functions of the membrane-associated and cytoplasmic Malate Dehydrogenases in the citric acid cycle of *Escherichia coli*, J. Bacteriol. 182(24): 6892-6899.

Van Elsas JD, Rosado AS, Wolters AC, Moore E, Karlson U (1998) Quantitative detection of *Sphingomonas chlorophenolica* in soil via competitive polymerse chain reaction, J. Appl. Microbiol. 85: 463-471.

World Health Organization (WHO). (2005). Guidelines for drinking water quality, 3rd Ed, Geneva.

Zachar V, Thomas RA, Goustin AS (1993). Absolute quantification of target DNA: a simple competitive PCR for efficient analysis of multiple samples, Nucleic Acid Res. 21(8): 2017-2018.