The occurrence of pathogenic *Escherichia coli* in South African wastewater treatment plants as detected by multiplex PCR

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Abstract

The aim of this study was to investigate the use of PCR to detect commensal and diarrhoeagenic *Escherichia coli* concentrated from water samples using membrane filtration. To achieve this, culture-based and PCR-based methods were compared for the detection of *E. coli* in raw sewage and primary, secondary and tertiary effluents from 6 wastewater treatment plants around Johannesburg, Gauteng. *E. coli* was concentrated from the samples using standard filtration techniques with subsequent incubation on *E.* coli/coliform chromogenic media to determine the *E. coli* levels. Bacterial DNA was isolated from bacterial colonies trapped on polyethersulphone membranes after filtration using a celite/guanidium thiocyanate method. A single multiplex PCR (m-PCR) assay was used that targeted the *mdh*, *eaeA, stx1, stx2, st, lt, ial* and *eagg* genes associated with diarrhoeagenic *E. coli*. The *mdh* gene was detected in all of the samples even if no culturable *E. coli* was detected. All the diarrhoeagenic *E. coli* types were detected in one or more of the raw sewage samples from the various plants. EPEC was present in 20% (2/10) of the samples, EHEC in 50% (5/10), ETEC in 80% (8/10), EIEC in 10% (1/10) and EAEC in 90% (9/10) of the samples. In the case of the primary and secondary treatment only ETEC (5/5; 100%) and EAEC (5/5; 100%) were detected in all of the samples. The results demonstrate that molecular techniques such as PCR have the potential to be used for the monitoring of water samples for the presence of pathogenic *E. coli,* without the need to culture the organisms.

Keywords: *E. coli*, multiplex PCR, wastewater treatment plant effluent

Introduction

In South Africa many communities still depend on untreated surface water and ground-water sources for their daily water needs. Water from these sources is often contaminated by faecal pollution from wastewater effluents (Toze, 2004). Wastewater is a matrix consisting of raw sewage and primary, secondary and tertiary treatment effluents (Mara and Horan, 2003). Final effluents are released into receiving waters like dams and rivers. If treatment fails, effluents of poor microbial quality enter public waters (Jagals, 1997).

Microbiological indicators have been used for decades to monitor faecal pollution of water (Grabow, 1996; Standard Methods, 2005) as well as the possible presence of other microbiological pathogens (Medema et al., 2003). *Escherichia coli* is used as an indicator of faecal pollution that originates from human and warm-blooded animals. In the last century, certain *E. coli* strains were identified as a significant cause of gastro-intestinal disease and recognised as highly versatile pathogens (Nataro and Kaper, 1998). At present, 6 groups of *E. coli* patho-types have been identified, of which 5 were selected for this study based on their association with diarrhoeal disease in South Africa. These are classified into 5 categories, namely, entero-pathogenic (EPEC), entero-toxigenic (ETEC), entero-invasive (EIEC), entero-aggregative (EAEC) and entero-haemorrhagic (EHEC) (Ashbolt, 2004; Kaper et al., 2004). *E. coli* strains can thus be either commensal (ComEC; non-diarrhoeagenic) or diarrhoeagenic.

Traditional *E. coli* detection methods are based on chromogenic and fluorogenic media designed to enumerate ComEC with no relationship to the proportion of diarrhoeagenic *E. coli* (DEC). All wastewater treatment plants use culture-based methods based on this principle to monitor the water quality. One complication with culture-based methods is their inability to detect *E. coli* in the viable but non-culturable (VNBC) state (Lleo et al., 2005). VBNC cells are bacterial cells that are metabolically active but cannot be cultured using standard microbiological techniques. These cells are reported to be able to resume active growth when favourable conditions are restored (Chen et al., 2006). It has been reported that pathogenic VBNC cells retain their virulence genes making the water source a potential reservoir of disease (Garcia-Armisen and Servais, 2004).

This inability of traditional culture methods to detect VBNC cells has prompted interest in alternative techniques to monitor the microbiological quality of water (Lleo et al., 2005). Molecular biology techniques, such as the polymerase chain reaction (PCR), have been used to detect DEC types by amplifying specific genes associated with the bacterium's virulence (Kong et al., 2002). To date PCR has mostly been used for the characterisation of isolated *E. coli* cells or the detection of DEC after sample enrichment. DEC genes targeted with PCR include the *eaeA* gene for EPEC (Aranda et al., 2004), the *lt* and *st* enterotoxin genes for ETEC (Pass et al., 2000), *ial* gene for EIEC (Paton and Paton, 1998), the *eagg* gene for EAEC (Kong et al., 2002) and the *eaeA*, *stx1* and *stx2* for the detection of EHEC (Moses et al., 2006).

PCR can, however, not be used for the detection of VBNC cells in water samples if the method is used to characterise bacterial isolates obtained with standard culture methods, since the

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Table 1 Bacterial strains used in molecular characterisation		
Bacterial strain	**Reference nr**	**Genes present**
Escherichia coli (Commensal) [a]		*mdh*
Enterohaemorrhagic (EHEC)	ESCCO 21 [b]	*mdh, stx1*, *stx2* and *eaeA*
Enteroinvasive (EIEC)	ESCCOS ATCC 43893 [b]	*mdh, ipah* and *ial*
Enterotoxigenic (ETEC)	ESCCO 22 [b]	*mdh, lt* and *st*
Enteropathogenic (EPEC)	S-ESCCO 16 Pl [b]	*mdh* and *eaeA*
Enteroaggregative (EAEC)	ESCCO 14 [b]	*mdh* and *eagg*

[a] *Environmental isolate confirmed by API 20E (OMNIMED®) and PCR as commensal E. coli*
[b] *Strains purchased from National Health Laboratory Services (NHLS)*

VBNC bacteria cannot be isolated (Chen et al., 2006). The simplest way to overcome this would be to isolate DNA from bacterial cells concentrated from the water samples, which is then used as template for the PCR, circumventing the need for culturability.

The aim of this study was to investigate the use of PCR to detect commensal and pathogenic *E. coli* concentrated from water samples using membrane filtration. During the study both PCR and culture-based methods were used to detect *E. coli* in raw sewage, and primary, secondary and tertiary effluents from 6 wastewater treatment plants in Gauteng.

Methodology

Growth and maintenance of reference strains

The *E. coli* reference strains (Table 1) were cultured on plate count agar (PCA) (Oxoid) and incubated under aerobic conditions at 37ºC for 16 h. If a liquid culture was required, *E. coli* colonies were inoculated into 5 mℓ nutrient broth and grown overnight at 37°C with mild agitation at 200 r/min.

Sample collection

Samples were collected from 6 wastewater treatment plants in the vicinity of Johannesburg, Gauteng, from August 2006 to December 2007. Thirty-four samples were collected from raw sewage (10), primary treatment effluent (5), secondary treatment effluent (5) and tertiary effluent (14). Water samples were collected in 1 ℓ sampling bottles and kept on ice during transport. Samples were analysed within 3 h of collection.

Microbiological analysis

Microbiological analysis of the samples for *E. coli* was performed using the standard filtration technique (Standard Methods, 2005). Briefly, 100 mℓ of an appropriate dilution of the water sample was filtered in triplicate onto 0.45-µm gridded nitro-cellulose membrane filters (Merck, Germany). The filters were placed onto selective *E. coli*/Coliform Chromogenic Media (Oxoid, UK) and the plates were incubated for 24 h at 37ºC. All pink colonies were counted as total coliforms (glucoronidase positive) and all purple colonies were counted as *E. coli* (β-$_D$-glucoronidase and β-$_D$-galactosidase positive), according to the supplier's instructions. Bacterial counts obtained were expressed as colony forming units in 100 mℓ (cfu/100 mℓ), using the average count of the 3 filters.

Bacterial DNA extraction from wastewater samples

For the DNA extraction, a 100 mℓ sample was filtered onto polyether sulfone (PES) membranes (Microsep (PTY) LTD). DNA was extracted from the trapped bacteria using a modification of the silica/guanidium thiocyanate method reported by Boom et al. (1990) with spin columns prepared as reported by Borodina et al. (2003). The Boom et al. (1990) protocol was followed with 2 exceptions: 250 µℓ 100% (vol/vol) ethanol was added to the lysis buffer, and the celite containing the bound DNA was loaded into the spin columns prior to the washing steps (Borodina et al., 2003). DNA was eluted with 50 µℓ Qiagen elution buffer (Southern Cross Biotechnology®). Controls were included during the DNA extractions and subjected to the same protocol as the samples. The positive control contained 1 mℓ of overnight commensal *E. coli* liquid culture and the negative control contained 1 mℓ of sterile distilled water.

Multiplex polymerase chain reaction (m-PCR)

The oligonucleotide primers used in this study were synthesised by Inqaba Biotec (Pty.) Ltd. as well as Whitehead Scientific (Pty.) Ltd. The sequences, target genes and expected amplification products are listed in Table 2.

All m-PCR reactions were performed in a Biorad Mycycler™ Thermal cycler in a total volume of 20 mℓ. A m-PCR kit (Qiagen®) was used for the m-PCR protocol. Each reaction consisted of 1X Qiagen® PCR multiplex mix (containing HotstartTaq® DNA polymerase, m-PCR buffer and dNTP mix); 2 µℓ of the primer mixture (0.1 mM of *mdh* and *lt* primers Forward (F) and Reverse (R)), 0.2 mM of *ial* and *eagg* primers (F and R), 0.3 mM of *eaeA* and *stx2* primers (F and R), 0.5 mM of *stx1* and *st* primers(F and R) (Table 1), 4 mℓ of sample DNA and 4 mℓ PCR grade water. The reactions were subjected to an initial activation step at 95°C for 15 min, followed by 35 cycles consisting of denaturing at 94°C for 45 s, annealing at 55°C for 45 s, extension at 68°C for 2 min and final elongation at 72°C for 5 min (Omar, 2007). Positive and negative controls for the PCR reaction were also included. The positive control contained a mixture of the 5 pathogenic *E. coli* and commensal *E. coli* DNA. The negative control contained PCR grade water.

Gel electrophoresis

DNA was analysed on a horizontal agarose slab gel (2.5 % (w/v)) with ethidium bromide (0.5 mg/mℓ) in TAE buffer (40 mM Tris acetate; 2 mM EDTA, pH 8.3). Electrophoresis was performed for 1 to 2 h in electric field strength of 80 V; PCR products were visualised with UV light (Syngene, UK). 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 1 kB or 100 bp markers (Fermentas, US).

Table 2
Primers used in the m-PCR reaction

Pathogen	Primer	Sequence(5'-3')	Size (bp)	Reference
E. coli	*Mdh(*F)	GGT ATG GAT CGT TCC GAC CT	300	Tarr et al. (2002)
	Mdh(R)	GGC AGA ATG GTA ACA CCA GAG T		
EIEC	*Ial(F)*	GGTATGATGATGATGAGTGGC	630	Paton and Paton (1998)
	Ial(R)	GGAGGCCAACAATTATTTCC		
EHEC/EPEC	*EaeA*(F)	CTG AAC GGC GAT TAC GCG AA	917	Aranda et al. (2004)
	EaeA(R)	GAC GAT ACG ATC CAG		
EAEC	*Eagg*(F)	AGA CTC TGG CGA AAG ACT GTA TC	194	Kong et al. (2002)
	Eagg(R)	ATG GCT GTC TGT AAT AGA TGA GAA C		
EHEC	*Stx1(F)*	ACA CTG GAT GAT CTC AGT GG	614	Moses et al. (2006)
	Stx1(R)	CTG AAT CCC CCT CCA TTA TG		
	Stx2(F)	CCA TGA CAA CGG ACA GCA GTT	779	Moses et al. (2006)
	Stx2(R)	CCT GTC AAC TGA GCA CTT TG		
ETEC	*LT(F)*	GGC GAC AGA TTA TAC CGT GC	330	Pass et al. (2000)
	LT(R)	CGG TCT CTA TAT TCC CTG TT		
	ST(F)	TTT CCC CTC TTT TAG TCA GTC AAC TG	160	Pass et al. (2000)
	ST(R)	GGC AGG ATT ACA ACA AAG TTC ACA		

F – Forward primer
R – Reverse primer

Table 3
Microbiology and PCR results obtained for the raw sewage, primary treatment, secondary treatment and tertiary effluent samples taken from 6 wastewater treatment plants in the vicinity of Johannesburg

			Microbiology			PCR
Sample		n	Average cfu/100 mℓ	Min cfu/100 mℓ	Max cfu/100 mℓ	Pathogens detected
Plant A	Raw	4	2.22E+07	1.35E+07	2.36E+07	ETEC / EAEC / EHEC / EIEC
	Primary	3	9.95E+06	8.16E+06	1.92E+07	ETEC / EAEC
	Secondary	3	1.96E+05	2.36E+03	1.12E+04	ETEC / EAEC
	Tertiary	3	1.49E+02	0.00E+00	2.98E+02	ETEC / EAEC
Plant B	Raw	1	2.10E+07	-	2.10E+07	ETEC
	Primary	2	9.43E+06	5.99E+06	8.16E+06	ETEC / EAEC
	Secondary	2	8.83E+03	3.97E+03	7.68E+03	ETEC / EAEC
	Tertiary	2	1.63E+02	1.0E+00	6.49E+02	ETEC / EAEC
Plant C	Raw	2	2.34E+07	1.60E+05	4.67E+07	ETEC / EAEC
	Tertiary	2	6.50E+03	0.0E+00	1.30E+04	ETEC / EAEC
Plant D	Raw	2	1.06E+07	3.80E+05	2.09E+07	ETEC / EAEC
	Tertiary	2	7.15E+04	0.0E+00	1.43E+05	ETEC
Plant E	Raw	1	2.0E+07	-	2.0E+07	EPEC / EHEC / ETEC / EAEC
	Tertiary	3	2.67E+00	0.0E+00	7.0E+00	Commensal
Plant F	Tertiary	2	1.69E+04	9.0E+03	2.48E+04	EPEC / EHEC / ETEC / EAEC

Results and discussion

The microbiological data for the enumeration of *E. coli* from the samples is shown in Table 3. The average bacterial counts calculated (min; max) for the various samples were 1.94 x 10^7 cfu/100 mℓ (1.06 x 10^7 cfu/100 mℓ; 2.34 x 10^7 cfu/100 mℓ) for the raw sewage (n=10), 9.69 x 10^6 cfu/100 mℓ (9.43 x 10^6 cfu/100 mℓ; 9.95 x 10^6 cfu/100 mℓ) for the primary treatment effluent (n=5), 1.02 x 10^5 cfu/100 mℓ (8.83 x 10^3 cfu/100 mℓ; 1.96 x 10^5 cfu/100 mℓ) for the secondary treatment effluent (n=5) and 1.59 x 10^4 cfu/100 mℓ (0 cfu/100 mℓ; 7.15 x 10^4 cfu/100 mℓ) for the tertiary effluent (n=14). The breakdown of these counts per treatment plant is also shown in Table 3.

As expected the number of *E. coli* present in the samples declined from raw sewage (starting material) up to the tertiary effluent (final product). Bacterial counts for the tertiary effluents did however cover a broad range from 2.67 x 10^0 cfu/100mℓ up to 7.15 x 10^4 cfu/100mℓ, which provided a range of contexts in which to test PCR as an alternative for the detection and characterisation of *E. coli* strains present.

The m-PCR used for the sample analyses targeted 7 of the virulence genes mostly associated with EHEC (*stx1*, *stx2*, *eaeA*), EPEC (*eaeA*), ETEC (*lt, st*), EIEC (*ial*), EAEC (*eagg*) as well as the *mdh* gene found in both commensal and diarrhoeagenic *E. coli* (Omar, 2007). An example of the complete m-PCR is shown in Fig. 1.

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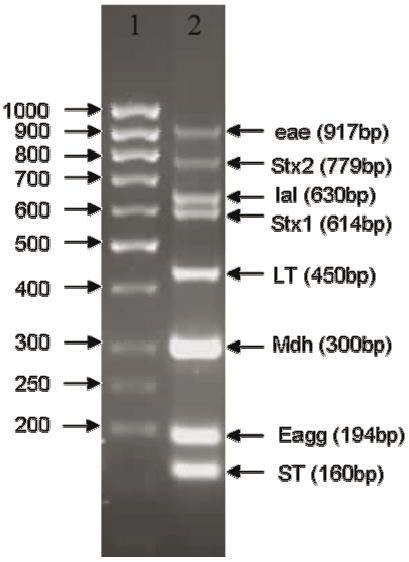

Figure 1
Photograph of an agarose gel showing the PCR products obtained for the E. coli *multiplex PCR (Lane 2). The molecular weight marker is shown in (Lane 1)*

Individual *E. coli* colonies isolated on selective media were not tested using the m-PCR. This was avoided as pathogenic strains would represent only a small proportion of the total *E. coli* community.

The initial PCR results obtained showed PCR inhibition in 21% (7/34) as indicated by the absence of an *mdh* product. Since all the samples had culturable *E. coli* it was expected that at least the *mdh* gene would be detected in the samples. The *mdh* gene could be detected in all 7 samples after the DNA was diluted 5 times, lifting the effect of the PCR inhibitors. The dilution of DNA should always only be considered as a last resort since this can possibly limit the detection of DEC in the sample, especially if the DEC is present in very low numbers. Due to the nature of the samples it was, however, necessary.

The general distribution of the DEC types in the various stages of the wastewater treatment plants is given in Table 4. All the DEC types were detected in 1 or more of the raw sewage samples from the various plants. EPEC was present in 20% (2/10) of the samples, EHEC in 50% (5/10), ETEC in 80% (8/10), EIEC in 10% (1/10) and EAEC in 90% (9/10) of the samples (Table 4). In the case of the primary and secondary treatments only ETEC (5/5; 100%) and EAEC (5/5; 100%) were detected in all of the samples. All the DEC types except for EIEC could be detected in the tertiary effluent samples. The presence of EHEC (2/14; 14.3%) and EPEC (2/14; 14.3%) was however restricted to Plant F (Table 5). The tertiary effluent of Plant F not only showed the presence of high *E. coli* counts but also the presence of EHEC, EPEC, EAEC and ETEC in both samples tested.

In the case of Plant A, ETEC and EAEC could be detected in the raw sewage, primary and secondary treatment effluents as well as the tertiary effluent. The pathogens were, however, not present in all of the samples taken from each point. The same trend was seen with Plant B with the exception that ETEC was detected in all of the samples tested.

Although it was expected that the *mdh* gene would at least be detected in the raw sewage and primary and secondary treatment effluents due to the high number of *E. coli* present, the ability of the PCR method in detecting not only *mdh* but also virulence genes in the tertiary effluents was very encouraging. The *mdh* gene could even be detected in samples with low or no *E. coli* detected using culture-based methods. It should, however, be noted that although DEC were detected with the PCR, especially in the tertiary effluent, no conclusions can be made about the viability of the bacterial cells detected. This is mainly due to the fact that PCR will amplify genes from the DNA of both viable and dead cells.

Conclusion

PCR has the potential to be used for the monitoring of water samples for the presence of pathogenic *E. coli* without the need to culture the organisms. This method can be extended to test for other bacterial pathogens such as *Vibrio cholerae* in water samples if an appropriate PCR protocol is available. For this process to be more efficient more research is needed into the DNA extraction method to ensure that no PCR inhibitors are present after DNA extraction, especially when working with samples such as raw sewage. This will ensure that no DNA dilutions are required that might reduce the chance of detecting bacterial pathogens in the samples.

It is important to consider that although the pathogens were detected with the m-PCR, the genes could have been amplified

Table 4
Summary of the total percentages of pathogenic *E. coli* present at each step of the wastewater treatment process

Samples	HKG	EPEC	EHEC	ETEC	EIEC	EAEC
Raw	100%(10/10)	20%(2/10)	50%(5/10)	80%(8/10)	10%(1/10)	90%(9/10)
Primary treatment	100%(5/5)	0%(0/5)	0%(0/5)	100%(5/5)	0%(0/5)	100%(5/5)
Secondary treatment	100%(5/5)	0%(0/5)	0%(0/5)	100%(5/5)	0%(0/5)	100%(5/5)
Tertiary treatment	78.5%(11/14)	14.3%(2/14)	14.3%(2/14)	78.6%(11/14)	0%(0/14)	57.1%(8/14)

Table 5
Summary of the total percentages of pathogenic *E. coli* present at the various wastewater treatment plants

Samples	HKG	EPEC	EHEC	ETEC	EIEC	EAEC
Plant A	100%(13/13)	7.7%(1/13)	7.7%(1/13)	7.7%(1/13)	7.7%(1/13)	100%(13/13)
Plant B	100%(7/7)	0%(0/7)	0%(0/7)	100%(7/7)	0%(0/7)	85.1%(6/7)
Plant C	100%(4/4)	0%(0/4)	0%(0/4)	75%(3/4)	0%(0/4)	75%(3/4)
Plant D	100%(4/4)	0%(0/4)	0%(0/4)	75%(3/4)	0%(0/4)	50%(2/4)
Plant E	25%(1/4)	25%(1/4)	25%(1/4)	25%(1/4)	25%(1/4)	0%(0/4)
Plant F	100%(2/2)	100%(2/2)	100%(2/2)	100%(2/2)	0%(0/2)	100%(2/2)

from DNA recovered from dead and/or viable cells. Since humans might use the water downstream of the treatment plant, there is a need to better understand what pathogens are released and whether they are still virulent. Olivier et al. (2005) showed that *E. coli* and *Salmonella typhimurium* can enter the viable but non-culturable state if secondary treated wastewater is chlorinated, leading to a drastic underestimation of the actual quality of the water released into the environment.

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