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ORIGINAL ARTICLE

Specific detection of *Escherichia coli* isolated from water samples using polymerase chain reaction targeting four genes: cytochrome bd complex, lactose permease, β-D-glucuronidase, and β-D-galactosidase

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Abstract

Aims: To develop a PCR-based method for reliable detection of *Escherichia coli* that enables its differentiation from biochemically and phylogenetically related bacteria.

Methods and Results: Using multiplex PCR targeting four genes (cytochrome bd complex, lactose permease, β-D-glucuronidase, and β-D-galactosidase) the possibility of specific detection of various control *E. coli* strains was tested. It was found that four PCR fragments of the predicted size were observed only for *E. coli* strains, but not for relatives as close as *Shigella* sp. or other enterobacteria. Not surprisingly, this method enabled us to identify also *E. coli* strains which did not exhibit the β-D-glucuronidase activity. Our multiplex PCR was also successfully used for identification of 95 environmental isolates of *E. coli*.

Conclusions: The developed PCR-based method, in which four genes coding for lactose permease, cytochrome bd complex, β-D-glucuronidase, and β-D-galactosidase, serve as target DNA sequences, allows precise and reliable detection of *E. coli* strains.

Significance and Impact of the study: The suggested approach increases the specificity of detection of *E. coli* since it enables to distinguish *E. coli* from *Shigella* sp. and other relative enterobacteria.

Introduction

Escherichia coli is a common inhabitant of intestinal tract of humans and other warm-blooded animals. *E. coli* can easily enter surface waters in faecal material where it persists for weeks. Therefore, *E. coli* serves as an indicator of faecal contamination of water and its presence in water resources refers to possible occurrence of bacterial enteric pathogens. Moreover, some strains of *E. coli* can cause various intestinal or extraintestinal infections (Nataro and Kaper 1998; Dufour and Till 2004).

Detection of *E. coli* in aquatic environments is thus important for ensuring bacteriological safety of drinking and bathing water and protection of public health. Standard techniques used for detection of *E. coli* from water samples are based on cultivation on selective growth media in combination with conventional biochemical tests (Augoustinos *et al.* 1993; Eckner 1998; Rompré *et al.* 2002). Although the standard methods are still widely used they have many drawbacks such as low specificity (Rompré *et al.* 2002). Indeed, standard methods failed to detect some *E. coli* strains, especially pathogenic serotypes such as *E. coli* O157, which do not exhibit β-glucuronidase activity but carry the *uid*A gene (Doyle and Schoeni 1984; Monday *et al.* 2001).

Progress in methods of molecular biology offered researchers a significant increase in specificity since microorganisms can be identified according to their

unique genetic properties encoded in genomic DNA. Polymerase chain reaction (PCR)-based methods are applied most frequently to detect and identify particular bacterial species. Number of PCR protocols for detection of *E. coli* from environmental samples has been described until now (Bej *et al.* 1991a,b,c; Cebula *et al.* 1995; Juck *et al.* 1996; Franck *et al.* 1998; Frahm and Obst 2003). PCR approaches usually rely upon amplification of one or more target DNA sequences, which are derived from genes coding for enzymes widely expressed among all of the *E. coli* strains (Bej *et al.* 1991a,b,c; Juck *et al.* 1996; Frahm and Obst 2003). Most of the published PCR applications used genes coding for β-D-glucuronidase (*uid*A) and β-D-galactosidase (*lac*Z) as target DNA sequences (Bej *et al.* 1991a,b,c; Fricker and Fricker 1994; Juck *et al.* 1996; Frahm and Obst 2003). This is particularly important since it enables to identify *E. coli* strains that do not exhibit β-glucuronidase activity although they carry the *uid*A gene (e.g., *E. coli* O157) as it was mentioned above (Doyle and Schoeni 1984; Monday *et al.* 2001). On the other hand, *uid*A and *lac*Z genes are not entirely unique to *E. coli* and can be found also in other bacteria, e.g. in *Shigella* species (Manafi *et al.* 1991; Tryland and Fiksdal 1998; Unkmeir and Schmidt 2000; Rompré *et al.* 2002). Molecular analysis of selected genes of enteroinvasive *E. coli* strains and *Shigella* strains revealed very close evolutionary relationship between these species (Lan *et al.* 2001, 2004; Wang *et al.* 2001). Interestingly, Lan *et al.* (2004) recently suggested to consider *Shigella* strains as pathovars of *E. coli* on the base of sequence similarity 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 clinically important *E. coli* strains (Cebula *et al.* 1995; Franck *et al.* 1998; Ibekwe *et al.* 2002). However, none of the described method is universal enough for reliable and unequivocal detection of all possible *E. coli* strains isolated from water samples.

To improve the specificity of PCR-based method for identification of *E. coli* we increased the number of target genes to four: *uid*A, *lacZ*, *lac*Y (coding for lactose permease), and *cyd* (coding for cytochrome bd complex) genes. Products of these genes could be considered as biochemical hallmarks of *E. coli* sp. Indeed, enzymatic products of *lac*Y and *lacZ* genes are necessary for lactose fermentation; lactose permease is essential for lactose transport across cytoplasmatic membrane and β-D-galactosidase cleaves the disacharide lactose into glucose and galactose (Kaback 1990; Ito *et al.* 1991; Stoebel 2005). Lactose fermentation is used for standard *E. coli* identification by cultivation methods (Ito *et al.* 1991; Stoebel 2005). Detection of β-D-glucuronidase enzyme activity (coded by *uid*A gene), which is observed in approximately 94% strains of *E. coli,* is usually used for standard *E. coli* identification by cultivation methods, as well (Rompré *et al.* 2002).

The cytochrome bd complex is one of two respiratory oxidases in *E. coli*, which functions under conditions of very low aeration. (Hill *et al.* 1990; Juty *et al.* 1997). This gene was already successfully applied for detection of *E. coli* (Horakova *et al.* 2006). Tests with DNA isolated from 46 culture collection strains, 110 strains from water samples, and one clinical isolate proved outstanding specificity and reliability of this newly designed multiplex PCR.

Materials and methods

Bacterial strains

In this study, 157 bacterial strains were used; 123 *E. coli* strains and 34 non-*E. coli* strains. 110 isolates of *E. coli* (95 from surface water, 15 from drinking water) were collected from various localities in the Czech Republic during the period 2003–2004. One clinical isolate of *E. coli* (from faeces of diarrhoeic patient was kindly provided by St. Ann's University Hospital, Brno, Czech Republic). Twelve control strains of *E. coli* were obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic, Table 1). Finally, 34 non-*E. coli* bacterial strains, which are biochemically and taxonomically close to *E. coli,* were obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic, Table 1).

Biochemical tests for identification of *E. coli*

Environmental *E. coli* isolates were determined according to the EU Standards ČSN EN ISO 9308-1 (2001) used for drinking water analysis and National Standards TNV 75 7835 (2003) used for surface water analysis.

Identification of *E. coli* according to ČSN EN ISO 9308-1 is based on cultivation on Lactose-TTC agar with Tergitol 7 at 37°C followed by testing of β-D-galactosidase (LAC)-positive colonies for activity of cytochrome c oxidase (OXI) and tryptophanase (TRP). Identification of *E. coli* according to TNV 75 7835 relies on cultivation on m-FC agar at 44°C and assay of β-D-glucuronidase activity (GUR) in colonies of thermotolerant coliform (β-D-galactosidase-positive) bacteria. For simplicity, all the water samples were analyzed by both methods according to ČSN EN ISO 9308-1 and TNV 75 7835. Final confirmations of such identified *E. coli* strains were done by multiple tube fermentation tests (ENTEROtest 24, Pliva-Lachema, Brno, Czech Republic). Control *E. coli* strains

Table 1 Bacterial culture collection (CCM) strains tested

Escherichia coli CCM 2024, *Escherichia coli* CCM 2260, *Escherichia coli* CCM 3773, *Escherichia coli* CCM 3880, *Escherichia coli* CCM 3954, *Escherichia coli* CCM 3988, *Escherichia coli* CCM 4225, *Escherichia coli* CCM 4517, *Escherichia coli* CCM 4723, *Escherichia coli* CCM 4724, *Escherichia coli* CCM 4787, *Escherichia coli* CCM 5172, *Shigella sonnei* CCM 4421, *Shigella flexneri* CCM 4422, *Klebsiella pneumoniae* subsp. *pneumoniae* CCM 2800, *Klebsiella pneumoniae* subsp. *pneumoniae* CCM 4415, *Klebsiella pneumoniae* subsp. *pneumoniae* CCM 5852, *Klebsiella pneumoniae* subsp. *ozaenae* CCM 5792T, *Klebsiella pneumoniae* subsp. *rhinoscleromatis* CCM 5791T, *Klebsiella oxytoca* CCM 2934, *Klebsiella oxytoca* CCM 3565, *Raoutella planticola* CCM 3721T, *Raoutella planticola* CCM 4428, *Raoutella terrigena* CCM 3568T, *Raoutella terrigena* CCM 3570 *Edwardsiella tarda* CCM 2238, *Yersinia enterocolitica* CCM 5671, *Enterobacter cloacae* CCM 1903, *Citrobacter koseri* CCM 2535, *Pseudomonas aeruginosa* CCM 3955, *Kluyvera ascorbata* CCM 3669, *Serratia marcescens* CCM 303, *Rahnella aquatilis* CCM 4086, *Citrobacter freundii* CCM 7187, *Serratia odorifera* CCM 3388, *Pragia fontium* CCM 3716, *Butiauxella agrestis* CCM 4664, *Budvicia aquatica* CCM 3714, *Morganella morganii* subsp. *morganii* CCM 680, *Providencia rettgeri* CCM 4504, *Hafnia alvei* CCM 2636, *Bacillus subtilis* subsp. *spizizenii CCM* 1999, *Proteus* sp. CCM 1799, *Staphylococcus aureus* CCM 3953, *Clostridium perfringens* CCM 4435, *Salmonella enterica* subsp. *enterica* serotype Enteritidis CCM 4420

(obtained from CCM) and one clinical isolate were also analysed in both ways as mentioned above.

Preparation of DNA extracts

Total genomic DNA was extracted by alkaline lysis (Horakova *et al.* 2008). A single bacterial colony grown on Nutrient agar (HiMedia Laboratories, Mumbai, India) was suspended in 1 ml of physiological saline (approximately 10^7 CFU ml^{-1}). The bacterial suspension was pelleted by centrifugation (6000 ***g***, 10 min, at room temperature). The pellet was suspended in 20 μl of 0·1 mol l^{-1} KOH, and then boiled in a water bath for 15 min. After cooling 30 μl of 0·1 mol l^{-1} HEPES (free acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was added to neutralize the lysate.

Primer design

The primers specific for the *lac*Z, *uid*A and *cyd* genes were used as described previously (Bej *et al.* 1991c; Horakova *et al.* 2006). The sequence of *lac*Y gene was obtained from a public database (Entrez, Gene ID: 949083). The sequences of primers *lac*Y were following: EClpma (-1): 5′-ACCAGACCCAGCACCAGATAAG-3′, EClpma (+1): 5′-GCACCTACGATGTTTTTGACCA-3′. Primers were carefully designed using FastPCR software to permit co-amplification in one tube. Primers were synthetized using the PerSeptive Biosystems equipment (Core Laboratory, Department of Functional Genomics and Proteomics, Masaryk University, Brno, Czech Republic). All primers were purified on Sephadex G-25.

Multiplex PCR amplification

The mixture consisted of 1× PCR buffer (10 mmol l^{-1} Tris-HCl pH 8·8, 1·5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl, 0·1% Triton X-100), 1 U of Taq DNA polymerase (Finnzymes, Espoo, Finland), 0·5 μmol l^{-1} of each primer, 200 μmol l^{-1} of each dNTPs (Invitek, Berlin, Germany) and 2·5 μl of template DNA. PCR reaction was performed in total volume of 25 μl. Conditions of PCR amplification were as follows: initial denaturation at 94°C for 90 s, and 30 cycles with denaturation at 94°C for 30 s, annealing at 58°C for 25 s and extension at 72°C for 30 s.

Sensitivity of the assay

Test of sensitivity of the assay was carried out on serially diluted suspension of control strain *E. coli* CCM 3954. 1 ml of bacterial suspension was inoculated on m-FC agar to detect colony counts of *E. coli,* simultaneously, 1 ml of bacterial suspension was used for DNA extraction. Aliquot of extracted DNA was used as template in multiplex PCR reactions.

Detection of PCR products

The amplified products were loaded onto a 1·8% agarose gel containing ethidium bromide (0·25 μg ml^{-1}) and run in 1× TBE buffer (tris-borate buffer) for 1 h at 80 V. PCR fragments were visualized with UV transilluminator. A 100-bp DNA ladder (Fermentas, Burlington, Canada) was loaded on each gel as a DNA size standard.

Results

Biochemical identification of *E. coli*

All the 123 tested *E. coli* strains exhibited properties of *E. coli* according to EU Standards (ČSN EN ISO 9308-1, Table 2). However, nine of them (three control, five surface water isolates and one clinical isolate) did not meet all the criteria for *E. coli* set by the National Standards TNV 75 7835 as they did not exhibit GUR activity (Table 2). Using ENTEROtest 24 (Pliva-Lachema, Brno, Czech Republic), a set of specific biochemical tests for *E. coli* identification, all 123 strains were proved to be *E. coli.* Taken together, not all standard tests based on biochemical properties of bacteria provided reliable identification of *E. coli.*

Table 2 Biochemical characteristics of all *Escherichia coli* strains tested according to National and EU Standards

		EU Standards (ČSN EN ISO 9803-1)			National Standards (TNV 75 7835)		
E. coli strains	Number of tested strains	LAC +	OXI −	TRP +	LAC +	GUR +	ENTERO test 24 *E. coli*
Control (CCM)	12	12	12	12	12	9	12
Surface water	95	95	95	95	95	90	95
Drinking water	15	15	15	15	15	15	15
Clinical	1	1	1	1	1	0	1
Total	123	123	123	123	123	114	123

LAC, β-D-galactosidase activity; OXI, cytochrome c oxidase activity; TRP, tryptophanase activity; GUR, β-D-glucuronidase activity; +, positive reaction; −, negative reaction.

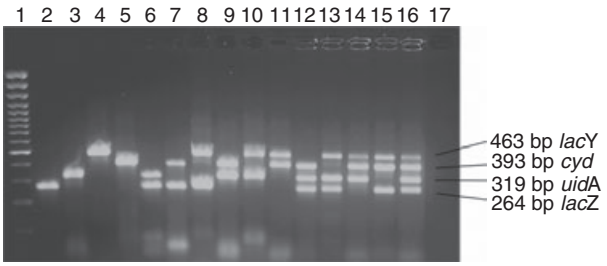

Figure 1 Co-amplification of *lac*Z, *uid*A, *cyd*, and *lac*Y gene fragments using the same template DNA isolated from *E. coli* CCM 3954. Lane 1: DNA size marker (Fermentas); lane 2: *lac*Z; lane 3: *uid*A; lane 4: *lac*Y; lane 5: *cyd*; lane 6: *lac*Z, *uid*A; lane 7: *lac*Z, *cyd*; lane 8: *lac*Z, *lac*Y; lane 9: *uid*A, *cyd*; lane 10: *uid*A, *lac*Y; lane 11: *cyd*, *lac*Y; lane 12: *lac*Z, *uid*A, *cyd*; lane 13: *lac*Z, *uid*A, *lac*Y; lane 14: *uid*A, *cyd*, *lac*Y; lane 15: *lac*Z, *cyd*, *lac*Y; lane 16: *lac*Z, *uid*A, *cyd*, *lacY*; lane 17: negative control.

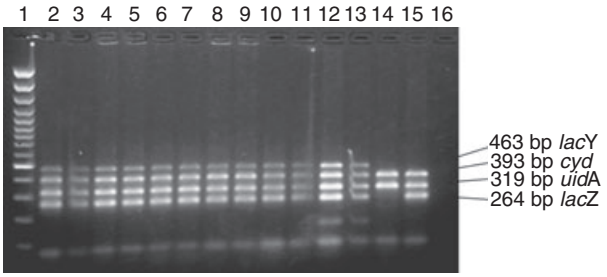

Figure 2 Multiplex amplification of DNA from control *E. coli*, *Shigella flexneri* and *Shigella sonnei* strains using *lac*Z, *uid*A, *cyd*, and *lac*Y primers. Lane 1: DNA size marker (Fermentas); lane 2: *E. coli* CCM 4724; lane 3: *E. coli* CCM 4723; lane 4: *E. coli* CCM 5172; lane 5: *E. coli* CCM 4787; lane 6: *E. coli* CCM 4517; lane 7: *E. coli* CCM 4225; lane 8: *E. coli* CCM 3988; lane 9: *E. coli* CCM 3954; lane 10: *E. coli* CCM 3880; lane 11: *E. coli* CCM 3773; lane 12: *E. coli* CCM 2260; lane 13: *E. coli* CCM 2024; lane 14: *Shigella flexneri* CCM 4422; lane 15: *Shigella sonnei* CCM 4421; lane 16: negative control.

Specificity of multiplex PCR

First, we tested the ability of each primer pair to detect a specific target DNA sequence within *lac*Z, *uid*A, *cyd*, and *lac*Y genes, respectively (Fig. 1). DNA extracted from *E. coli* CCM 3954, one of the fully characterised *E. coli* strains, served as a template. Then co-amplifications using different combination of two, three, and finally all four primer pairs specific for *lac*Z, *uid*A, *cyd*, and *lac*Y genes were tested with the same template DNA (Fig. 1). To further prove the specificity of the multiplex PCR reaction, we tested all the fully characterized bacterial strains (i.e., control strains) obtained from CCM (Table 1). Amplification of PCR mixtures containing template DNA, which were extracted from control *E. coli* strains, resulted in appearance of four fragments of the predicted size (Fig. 2). All four PCR products were also detected for GUR-negative *E. coli* strains CCM 4723, 4724 and 4787 (Fig. 2). Amplification with DNA template extracted from control strains *Citrobacter koseri*, *Shigella flexneri*, *Kluyvera ascorbata*, *Citrobacter freundii*, *Enterobacter cloacae* or *Shigella sonnei,* which are relative to *E. coli,* gave one to three PCR fragments of the predicted size (Table 3 and Fig. 3). *Klebsiella* sp. and *Raoutella* sp. were frequently positive for *cyd* but negative for at least one of other three genes *uid*A, *lac*Z, and *lac*Y (Table 3). Importantly, four fragments corresponding to *lac*Z, *uid*A, *cyd*, and *lac*Y genes were detected only for *E. coli*.

Application of multiplex PCR to environmental and clinical isolates of *E. coli*

In total, 95 isolates of *E. coli* from surface water, 15 from drinking water, and one clinical isolate, were subjected to our multiplex PCR. Each of *E. coli* isolates showed positive amplification of all four DNA fragments derived from *lac*Z, *uid*A, *cyd,* and *lac*Y genes (Table 4). Interestingly, six isolates (five from surface water and one clinical) were GUR-negative using standard biochemical test (Table 2).

These results indicated that our novel multiplex PCR approach based on co-amplification of four target DNA

Table 3 The overview of specific products of multiplex PCR for non-*Escherichia coli* control strains

Control strains	PCR products			
	*lac*Z	*uid*A	*cyd*	*lac*Y
Shigella sonnei CCM 4421	+	+	+	–
Shigella flexneri CCM 4422	–	+	+	–
Enterobacter cloacae CCM 1903	+	–	–	+
Citrobacter koseri CCM 2535	+	–	–	–
Citrobacter freundii CCM 7187	+	–	–	+
Kluyvera ascorbata CCM 3669	+	–	–	+
Klebsiella pneumoniae subsp. *pneumoniae* CCM 5852	+	–	–	+
Klebsiella pneumoniae subsp. *pneumoniae* CCM 2800	–	–	+	–
Klebsiella pneumoniae subsp. *pneumoniae* CCM 4415	–	–	+	–
Klebsiella pneumoniae subsp. *ozaenae* CCM 5792T	–	–	+	–
Klebsiella pneumoniae subsp. *rhinoscleromatis* CCM 5791T	–	–	+	–
Klebsiella oxytoca CCM 2934	–	–	+	–
Klebsiella oxytoca CCM 3565	–	–	+	–
Raoutella planticola CCM 3721T	–	–	+	–
Raoutella planticola CCM 4428	+	–	–	+
Raoutella terrigena CCM 3570	–	–	+	–
Raoutella terrigena CCM 3568T	–	–	+	–

*lac*Z, gene sequence coding for β-D-galactosidase; *uid*A, gene sequence coding for β-d-glucuronidase; *cyd*, gene sequence coding for cytochrome bd complex; *lac*Y, gene sequence coding for lactose permease; +, presence of PCR product; –, absence of PCR product.

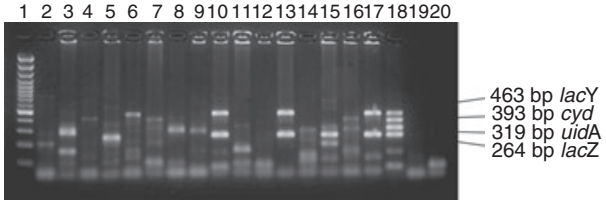

Figure 3 Multiplex amplification of DNA from various control strains using *lac*Z, *uid*A, *cyd*, and *lac*Y primers. Lane 1: DNA size marker (Fermentas); lane 2: *Bacillus subtilis* subsp. *spizizenii* CCM 1999; lane 3: *Hafnia alvei* CCM 2636; lane 4: *Providencia rettgeri* CCM 4504; lane 5: *Morganella morganii* subsp. *morganii* CCM 680; lane 6: *Budvicia aquatica* CCM 3714; lane 7: *Butiauxella agrestis* CCM 4664; lane 8: *Pragia fontium* CCM 3716; lane 9: *Serratia odorifera* CCM 3388; lane 10: *Citrobacter freundii* CCM 7187; lane 11: *Rahnella aquatilis* CCM 4086; lane 12: *Serratia marcescens* CCM 303; lane 13: *Kluyvera ascorbata* CCM 3669; lane 14: *Pseudomonas aeruginosa* CCM 3955; lane 15: *Citrobacter koseri* CCM 2535; lane 16: *Klebsiella pneumoniae* subsp. *pneumoniae* CCM 4415; lane 17: *Enterobacter cloacae* CCM 1903; lane 18: *E. coli* CCM 3954; lane 19: *Yersinia enterocolitica* CCM 5671; lane 20: *Edwardsiella tarda* CCM 2238.

sequences derived from *lac*Z, *uid*A, *cyd*, and *lac*Y genes enabled unmistakable identification of *E. coli*. Moreover, clear differentiation of species with similar biochemical and genetic properties, such as *Shigella* sp. and *E. coli*, was allowed for the first time.

Sensitivity of the assay

Sensitivity of the designed multiplex PCR was estimated to be 10^2 cells when alkaline lysis (not shown) was used for DNA extraction from both types of suspensions, i.e., suspension of control strain *E. coli* CCM 3954. It is necessary to note that detection limit strongly depended on method used for the DNA extraction and varied from 10^1 cells to 10^3 cells for UltraClean microbial DNA isolation kit (MoBio, USA) and freeze-thaw method according to Bej *et al.* (1991b), respectively (Horakova *et al.* 2008).

Table 4 The overview of specific products of multiplex PCR for environmental and clinical isolates of *Escherichia coli*

E. coli isolates	Number of tested isolates	PCR products			
		*lac*Z +	*uid*A +	*cyd* +	*lac*Y +
Surface water	95	95	95	95	95
Drinking water	15	15	15	15	15
Clinical	1	1	1	1	1
Total	111	111	111	111	111

*lac*Z, gene sequence coding for β-D-galactosidase; *uid*A, gene sequence coding for β-d-glucuronidase; *cyd*, gene sequence coding for cytochrome bd complex; *lac*Y, gene sequence coding for lactose permease; +, presence of PCR product; –, absence of PCR product.

Discussion

Although many different conventional cultivation methods for detection of *E. coli* combined with specific biochemical tests are available at present time, this bacterial species is still slightly problematic to detect properly in

complex environmental samples. The PCR-based methods have the potential to overcome the difficulties of standard detection methods.

Early PCR protocols utilized two sets of primers, the first primer set derived from *lac*Z gene sequence served to detect all coliform bacteria, and the second primer set derived from *uid*A gene sequence was used for detection of *E. coli*. Unequivocal advantage of this approach was that *E. coli* strains with undetectable β-D-glucuronidase activity (i.e., GUR-negative) were detectable by PCR amplification targeting the *uid*A gene (Bej *et al.* 1991c). However, it was demonstrated that the primer set derived from the *uid*A gene could also identify the non-*E. coli* coliforms (Fricker and Fricker 1994). In addition, the above mentioned duplex PCR protocol does not allow to distinguish *Salmonella* sp. and *Shigella* sp. from *E. coli* (Bej *et al.* 1991a; Li *et al.* 2004). These facts clearly indicate that so-far reported PCR protocols are not specific enough to provide a reliable detection of *E. coli*.

In order to increase the reliability and specificity of the PCR-based method for detection of *E. coli* we searched for other genes that are typical for these bacteria. We chose two genes, *cyd*, coding for cytochrome bd complex, and *lac*Y, coding for lactose permease, which could serve as *E. coli* hallmark genes.

The cytochrome bd complex (i.e., cytochrome bd quinol oxidase) is one of two respiratory oxidases in *E. coli*. It oxidizes dihydroubiquinol or dihydromenaquinol while reducing dioxygen to water. The bd-type oxidases found in prokaryotes only are induced under conditions of very low aeration, either to generate a proton motive force by reducing O_2 to water or by scavenging O_2 to protect the cell (Hill *et al.* 1990; Juty *et al.* 1997). This gene was already successfully applied for detection of *E. coli* (Horakova *et al.* 2006). However, *cyd* is expressed also in other bacterial species including enteric bacterium *Klebsiella pneumoniae* (Juty *et al.* 1997). This corresponds to our results, which revealed *cyd* gene sequence in *E. coli* and in most of tested *Klebsiella* and *Raoutella* strains (Table 3).

Lactose permease, which is product of the *lac*Y gene, transduces free energy stored in the electrochemical H^+ gradient into a sugar concentration gradient by catalyzing the coupled stoichiometric translocation of galactosides and H^+ (lactose/H^+ symport, reviewed by Kaback 1990). Lactose permease is also found in other enteric bacteria, including *Serratia* sp., *Salmonella* sp., *Citrobacter freundii, Klebsiella pneumoniae,* and *Yersinia pestis* (Stoebel 2005). Our results (Table 3) showed that *lac*Y gene was detected only along with *lac*Z in strains utilizing lactose (*E. coli*, *Enterobacter cloacae, Citrobacter freundii, Kluyvera ascorbata, Klebsiella pneumoniae* subsp. *pneumoniae* and *Raoutella planticola*). Interestingly, *Shigella* sp., the phylogenetically closest relative of *E. coli* (e.g., Stoebel 2005), could be distinguished from *E. coli* as the PCR product corresponding to *lac*Y gene was absent (Fig. 2, Table 3). This is in agreement with the findings of Ito *et al.* (1991), who observed that *Sh. flexneri*, *Sh. boydii* and *Sh. dysenteriae* lack the *lac*Y gene. Interestingly, in *Sh. sonnei* this gene was found to be nonfunctional. In addition, the possession of *lac*Z gene, which is necessary for lactose utilization either, was detected only in *Sh. dysenteriae* and *Sh. sonneii* with high homology to that of *E. coli* (Ito *et al.* 1991). This confirms our results as the specific *lac*Z gene product was found only in *Sh. sonnei* (Fig. 2, Table 3).

In conclusion, our multiplex PCR approach, which includes gene *lac*Y as novel target DNA sequence in addition to *uid*A, *lac*Z and *cyd* genes, seems to be a reliable tool for identification of *E. coli* - one of the best bacterial indicators of faecal contamination of water and potential pathogen. To our knowledge this approach enables for the first time to distinguish *E. coli* from phylogenetically closest relatives such as shigellae. Application of our multiplex PCR to environmental isolates will contribute to the increased reliability of *E. coli* detection due to higher specificity of the method. Moreover, this method allows elimination of false negative results obtained by standard biochemical methods when enzymatic activities of examined genes reached undetectable values. This will be of the highest importance when testing drinking and bathing water.

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