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# Development of a Multiplex PCR (m-PCR) Test for Rapid Identification of Genes Encoding Heat-labile (LTI) and Heat-stable (STI and STII) toxins of Enterotoxigenic *Escherichia coli* (ETEC) with Internal Control of Amplification

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Abstract

A multiplex PCR system was developed for specific identification of genes encoding heat-labile (LTI) and heat-stable (STI and STII) toxins of enterotoxigenic *Escherichia coli* (ETEC) strains. In addition, primers specific for the *E. coli* gene coding for 16S rRNA were used as an internal control of the DNA amplification. The specificity of the method was validated by single PCR tests performed with reference to *E. coli* strains as well as pig-isolated bacteria and 100% correlation was observed. The developed multiplex PCR allowed rapid and specific identification of enterotoxin-positive *E. coli* and may be used as a sensitive and specific method for a direct determination of ETEC and to differentiate them from other *E. coli* isolates.

Key words: ETEC, m-PCR, LTI, STI, STII enterotoxins

## Introduction

Most of *Escherichia coli* strains are harmless commensals in the gut but some of them are important enteric pathogen, causing diarrhoea in humans and animals (Holland, 1990; Nataro and Kaper, 1998). *E. coli* isolates associated with diarrhoea have been classified into six major groups, on the base of their distinct virulence properties: enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), diffusely adherent (DAEC) and enteroaggregative (EAEC) (Nataro and Kaper, 1998). Among them, ETEC is a predominant bacterial agent of diarrhoea in young animals as well as in infants and in adults travelling to developing countries (Nataro and Kaper, 1998; Nagy and Fekete, 1999). Bacteria of this group are defined as *E. coli* which are able to produce at least one enterotoxin: heat-labile I (LTI) heat-stable I (STI) or heat-stable II (STII) (Seriwatana *et al.*, 1988; Celemin *et. al*., 1994; Sears and Kaper, 1996; Nair and Takeda, 1998). LTI and STI have been found in both human and animal ETEC whereas STII toxin is characteristic only for animal *E. coli* (Seriwatana *et al*., 1988; Celemin *et al*., 1994). ETEC have the ability to cause profuse, watery diarrhoea by releasing of either LT or ST enterotoxins or both. Differentiation between ETEC and other pathogenic and non-pathogenic *E. coli* bacteria as well as other enteric isolates requires detection of enterotoxins or their genes and it is essential for proper detection and control of diarrhoeal diseases. Conventional techniques of *E. coli* diagnosis depend on growth in pure cultures and subsequent biochemical identification of relevant bacterial colonies. These procedures are time-consuming and can take up to 3–5 days. Moreover, differentiation of pathogenic ETEC isolates needs additional toxin detection procedures (Ojeniyi *et al*., 1994). One of the approaches used for the toxin determination is polymerase chain reaction (PCR) which is a very sensitive and highly specific molecular biology tool widely used for diagnosis of ETEC. PCR performed with the use two or more primer pairs to amplify two or more target sequences, called multiplex PCR, has been applied for detection and differentiation of LTI, STI or LTI, STII and Shiga toxin genes (Nataro and Kaper, 1998; Tsen and Jian, 1998; Nagy and Fekete, 1999; Osek *et al.* 1999, Osek and Truszczyński, 2000, Osek 2001). However, these

multiplex PCR systems do not allow the simultaneous detection of all enterotoxin genes and *E. coli*-specific 16S rRNA gene, used as an internal control of amplification. Although significant advancement has been made in the molecular identification of ETEC, there is still a need to improve the existing PCR assays for detection of genes encoding all enterotoxins, which are important in pathogenesis of ETEC diarrhoea and specific differentiation of ETEC from non-toxigenic *E. coli* bacteria.

In the present study, a rapid and specific multiplex PCR for the simultaneous detection of the genes encoding LTI, STI, STII and *E. coli*-specific 16S rRNA gene used as an internal control has been described.

## Experimental

### Materials and Methods

**Bacterial strains.** *E. coli* strains (n = 69) were isolated from weaned pigs (four- to six-weeks old) with diarrhoea characterized previously (Osek *et al.*, 1999; Osek, 2000; Osek, 2001). For control purposes the following *E. coli* strains were used: 293 (LTI-STI-STII-positive), 256 (STI-STII-positive), 259 (LTI-STII-positive), 335 (STI-positive), 276 (STII-positive) and C600 (LTI-STI-STII-negative).

**Template DNA.** Bacterial strains were grown on Luria-Bertani (LB) agar at 37°C for 18 h and one individual colony of each *E. coli* isolate was suspended in 50 µl of sterile, DNase, RNase-free deionized water (ICN Biomedicals, Costa Mesa, USA). The suspensions were centrifuged at 13 000 × g for 1 min and the supernatant (5 µl) was subsequently used as a source of DNA template.

**PCR amplifications.** PCR was used for the determination of the following genes encoding the *E. coli* enterotoxins: *eltI* (LTI toxin), *estI* (STI toxin), *estII* (STII toxin) as well as the gene responsible for expression of 16S rRNA of *E. coli*. All PCR primers (synthesized by IDT, Coralville, USA) used in the present study are listed in Table I. The primers flanking the 16S ribosomal RNA gene were included as an internal control to identify the presence of *E. coli* DNA in the PCR samples. Template DNA (5 µl) was added to the PCR mixture consisting of: 5 µl of PCR buffer (10 × concentrated), 5 µl of each deoxynucleotide (dNTPs; final concentration 0.2 mM), 10 µl of 25 mM $MgCl_2$ (final concentration 5 mM), 2.5 µl of 10 µM LT3 and LT4 primers (final concentration 0.5 µM each), 0.5 µl of 10 µM STA1 and STA2 primers (final concentration 0.1 µM each), 1.0 µl of 10 µM STIIB1 and STIIB2 primers (final concentration 0.2 µM each), 0.125 µl of 10 µM 16S-F and 16S-R primers (final concentration 0.025 µM each), 2 U of the *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania) and water to final volume of 50 µl. The PCR program was as follows: after initial DNA denaturation at 94°C for 5 min, 30 cycles (94°C for 1 min, 72°C for 2 min., 55°C for 1 min.) were used. The amplified PCR products (10 µl aliquots) were visualized by gel electrophoresis in 2% agarose gel (Type 1, low EEO, Sigma Chemicals, St. Louis, USA) in Tris-Acetate-EDTA (TAE) buffer at 100 V. The gels were stained with ethidium bromide (5 µl $ml^{-1}$) for 2 min, washed in distilled water, analyzed under UV light (300 nm) and photographed using the Gel Doc 2000 documentation system (Bio-Rad, Hercules, USA). The size of the obtained amplicons was compared to the 100 bp DNA ladder (Fermentas).

Table I
Characteristics of the primers used in the study

| Primer code | Sequence (5'→ 3') | Target gene | Gene product | PCR amplicon (bp) | Reference |
|---|---|---|---|---|---|
| LT3 | TATCCTCTCTATATGCACAG | *eltI* | Heat-labile enterotoxin LTI | 480 | Leong *et al.*, 1985 |
| LT4 | CTGTAGTGGAAGCTGTTATA | | | | |
| STA1 | TCTTTCCCCTCTTTAGTCAG | *estI* | Heat-stable enterotoxin STI | 166 | Ojeniyi *et al.*, 1994 |
| STA2 | ACAGGCCGGATTACAACAAAG | | | | |
| STIIB1 | GCCTATGCATCTACACAATC | *estII* | Heat-stable enterotoxin STII | 278 | Picken *et al.*, 1983 |
| STIIB2 | TGAGAAATGGACAATGTCCG | | | | |
| 16S-F | AGAGTTTGATCATGGCTCAG | 16S rRNA | 16S ribosomal RNA | 798 | Ehresmann *et al.*, 1972 |
| 16S-R | GGACTACCAGGGTATCTAAT | | | | |

## Results and Discussion

The reaction conditions for the multiplex PCR assays were optimized to ensure that all the target gene sequences were satisfactorily amplified. Initially, the same concentrations (0.1 mM) of each set of primers were used but this approach resulted in uneven intensity or even lack of some of the amplified products. To overcome this problem the change in the proportions of the various primers in the reaction mixture was required to increase the concentrations of primers in the case of weak amplicons and to decrease the oligonucleotide concentration in the case of strong PCR bands. Moreover, the multiplex PCR test was

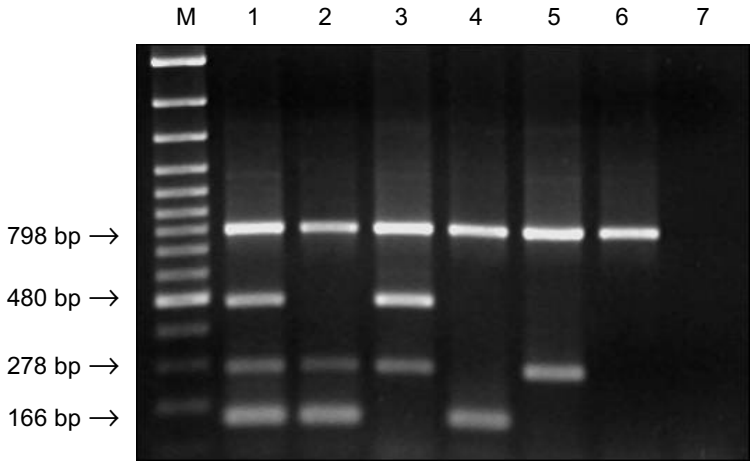


Fig. 1. Simultaneous identification of LTI, STI, and STII enterotoxin genes and *E. coli*-specific gene encoding 16S rRNA using a multiplex PCR system with LT3/LT4, STA1/STA2, STIIB1/STIIB2 and 16S-F/16S-R primers.
Lane M: 100 bp ladder; lane 1: *E. coli* LTI/STI/STII-positive; lane 2: *E. coli* STI/STII-positive; lane 3: *E. coli* LTI/STII-positive; lane 4: *E. coli* STI-positive; lane 5: *E. coli* STII-positive; lane 6: *E. coli* LTI/STI/STII-negative; lane 7: $H_2O$

simplified by the use of DNA templates prepared by a direct suspension of bacterial colonies in water instead of a time-consuming DNA extraction procedure.

The specificity of the multiplex PCR system developed was tested with *E. coli* reference strains listed in the "Materials and Methods" section. Moreover, this test was also validated using *E. coli* isolates (n = 69) originated from weaned pigs with diarrhoea and characterized previously by PCR performed with the primer pairs specific for particular enterotoxin genes analysed (Osek *et al*., 1999; Osek, 2000). It was demonstrated that enterotoxin-positive *E. coli* isolates showed the specific 480 bp (LTI), 166 bp (STI) or 278 bp (STII) amplicons of the enterotoxin genes tested (Fig. 1, lanes 1–5). There was a 100% correlation between the results obtained with the multiplex PCR assay and with PCR analyses separately performed with single primer pairs specific for LTI, STI, and STII enterotoxin genes, respectively.

The presence of the 798 bp amplified product (the 16S rRNA gene) was observed in all bacterial samples tested in the mPCR system indicating that all of them contained *E. coli* DNA. The *E. coli* enterotoxin-negative strain C600 generated only 798 bp amplification product of the 16S rRNA gene (Fig. 1, lane 6).

Table II shows the gene profiles of 69 *E. coli* isolates tested in the present study. All isolates harboured the 16S rRNA gene. The most prevalent ETEC marker detected during this study was the *estII* gene that was found in 40 isolates (the 278 bp PCR amplicon). Among them, 20 isolates harboured the *eltI* gene only, 15 – *estI* (amplified DNA band corresponding to 480 and 166 bp, respectively) and 2 isolates had both of these genes. Moreover, 7 strains carried the *estI* gene and 3 isolates had the *estII* gene as the only marker of ETEC. The remaining 22 isolates possessed none of the ETEC markers that were sought.

Table II
Multiplex PCR results derived from 69 *E. coli* strains isolated from pigs with diarrhoea

| Gene profile | | | | No of strains |
|---|---|---|---|---|
| *16S rRNA* | *LTI* | *STI* | *STII* | |
| + | + | + | + | 2 |
| + | + | + | – | 0 |
| + | + | – | + | 20 |
| + | – | + | + | 15 |
| + | + | – | – | 0 |
| + | – | + | – | 7 |
| + | – | – | + | 3 |
| + | – | – | – | 22 |

In the previous studies of Osek (Osek *et al.* 2000; Osek 2001), the primers specific for the universal stress protein A (UspA) of *E. coli* and enterotoxins genes were used and allowed a simultaneous amplification of the *E. coli*-specific *uspA* and the respective toxins genes. In the present study, the multiplex PCR for the simultaneous detection of the genes encoding LTI, STI, STII and *E. coli* 16S rRNA gene used as an internal control was developed.

The m-PCR described in this study has been shown to be an efficient tool for simultaneous detection of three enterotoxin genes of ETEC strains and it also allows to identify potentially pathogenic (enterotoxin-positive) *E. coli* and to differentiate them from commensally (non-toxigenic) isolates. One of the most important advantages of the multiplex PCR described here is the specific detection of ETEC bacteria without any biochemical identification and subculturing of the isolates. As described earlier (Ehresmann *et al*. 1972), the 798 bp amplification product of 16S ribosomal RNA is presented in *E. coli* bacteria only and therefore, the primers specific for this genetic marker were used in the mPCR developed. This improvement would simplify the detection process and eliminate the requirement for confirmation procedures such as biochemical tests or toxin detection in vitro (Ojeniyi *et al*., 1994).

PCR analysis of all reference *E. coli* strains as well as pig-isolated bacteria yielded consistent results with the PCR test separately performed with single primer pairs specific for particular three enterotoxin genes analysed. These results demonstrate high discriminatory power of the developed m-PCR assay and the potential of the test for rapid and specific identification and toxin profiling of ETEC.

## Literature


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