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Original Contribution

POLYMERASE CHAIN REACTION ASSAY FOR DETECTION OF ENTEROPATHOGENIC *ESCHERICHIA COLI* STRAINS IN MEAT

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ABSTRACT
Enteropathogenic *Escherichia coli* (EPEC) strains are a significant cause of acute and persistent diarrhea in humans, which are transmitted primarily through consumption of contaminated foods. The present study was performed for the identification of EPEC strains from contaminated meat by a PCR technique. A panel of reference *E. coli* strains purchased from NBIMCC; Sofia, Bulgaria was used for the experiments. Using the optimized amplification conditions, PCR with the purified DNA template was amplified for *eae* gene and obtained amplicons were visualized by 2% agarose gel electrophoresis. Our result demonstrated that two strains: E. coli O 111-НБПМКК-4447 and *E. coli* O 26-НБПМКК-7434 have amplified a 482 bp fragment with used primers. In our experimental condition the lowest cell concentration which exhibit well visualized 482 bp amplification band is less then 54 viable *E. coli* cells in 1 ml. The lowest bacterial cell concentration (CFU/g) in the contaminated beef meat samples which was detected in our experiments was 0,7 CFU/g meat. In conclusion the developed PCR method could be used for detection of typical and atypical EPEC in isolated colonies and in food.

Key words: *E. coli*, EPEC, PCR, *eae* gene

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) strains are a significant cause of acute and persistent diarrhea in humans and animals (1, 2). Most of *E. coli* strains are harmless commensals in the gut, but EPEC can cause profuse, watery diarrhea due to its ability to adhere in vitro to tissue culture cells in a pattern called localized adherence (3). The localized adherence phenotype is specific for *E. coli* of enteropathogenic serotypes and is associated with a high molecular weight plasmid present in most EPEC strains. A second phenotype that is recognized as a virulence characteristic of EPEC is the ability to produce a characteristic histopathology known as attaching and effacing (A/E) lesion in the intestines of infected infants and animals (4, 5). These lesions are characterized by the close adherence of bacteria to the enterocyte and by the effacement of the microvili and the disruption of the cytoskeleton of the affected cell. A/E lesions are thus far the best clue as to how EPEC cause diarrhea, with some authors proposing that EPEC produce diarrhea via malabsorption resulting from brush border effacement and other reporting a close correlation between A/E activity and increased calcium and phosphorylation of host proteins potentially leading to intestinal secretion (5).

Enterohaemorrhagic E. coli (EHEC), produces toxins, known as verotoxins or Shiga-like toxins because of their similarity to the toxins produced by *Shigella dysenteriae*. The ability to produce A/E lesions has also been detected

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in strains of Shiga-toxin-producing *E. coli* (STEC) and in strains of other bacterial species. STEC and EPEC that cause characteristic A/E lesions in the intestinal mucosa are also classified as attaching and effacing *E. coli* (AEEC).

Despite the fact that food supply is strictly controlled many cases of foodborne illnesses occur every year. Therefore, the pathogen safety of food has become an important concern of consumers. EPEC and STEC strains as well as many others pathogens are transmitted primarily through consumption of contaminated foods and are a significant cause of acute and persistent illnesses in humans. Since its introduction polymerase chain reaction (PCR) technology has proven to be an invaluable method for the detection of pathogens in food (6).

Jerse and Kaper, 1991 identified in a wild-type EPEC strain a chromosomal locus, *eae* (*E. coli* attacing and effacing), that is necessary for the production of A/E activity on human intestinal tissue culture cells. The *eae* gene consist of a 2817-bp open reading frame capable of encoding a 102-kDa protein. Using immunohistochemistry they identified a 94-kDa membrane protein, called intimin, as the product of the *eae* gene (7). Characterisation of the intimin shows conservative N-terminal region and variability in the last 280 amino acids in the C-terminal region, where protein binding to the enterocytes (8). The 5' regions of *eae* nucleotide sequence are conserved, whereas the 3' regions are heterogeneous (9). Based on this observation it has been developed different PCR methods to detection of AEEC strains using different PCR primers. In this study we report the development of a PCR technique for the identification of *eae* gene in *Escherichia coli* strains and to apply this technique for analysis of contaminated meat and detection of EPEC.

MATERIALS AND METHODS

Bacterial strains, growth conditions and DNA extraction.

A panel of reference *E. coli* strains was used for the experiments. These strains were *E. coli* O 26"H311b"-НБПМКК 4361 (named № 1), *E. coli* O 111-НБПМКК 4447 (named № 2), and *E. coli* O 26-НБПМКК 7434 (named № 3) purchased from National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC; Sofia, Bulgaria). The obtained lyophilized samples were restored in tryptic soy broth (TSB, Merck) and were grown on tryptic soy agar (TSA, Fluka) supplemented with 5 % defibrinated ovine blood at 37ºC for 24h. Single, two or three bacterial colonies grown on the medium were collected in 0.5 ml TBE buffer and the test tubes were incubated for 10min at boiled water bath . Then mixtures were centrifuged at 12 000 rpm for 5min and supernatants were used for DNA template. Isolation of genomic DNA was also performed by bacterial DNA extraction kit (Clonit S.r.l., Italy) according to the manufacture's instructions. The DNA was dissolved in 30 to 50 μL of Tris-EDTA buffer and stored at –20°C. The DNA concentration was determined using the GeneQuant 1300 RNA/DNA calculator.

Sensitivity and specificity of the used PCR

Overnight TSB broth culture of *E. coli* reference strains № 2 was used for 10-fold serial dilutions in Maximum Recovery Diluent (Merck) up to level 10^{-10}. From each dilution 1 ml was transferred in Eppendorf microtube for DNA extraction using thermo method. Real bacterial count in dilutions was estimated by agar spread method using 0,1 ml over double TSA agar plates.

Meat samples and bacterial inoculation

Fresh chilled ground beef meat was minced to 3 mm particle size and divided in 40 g samples, placed in sterilized glass backer. Overnight bacterial broth culture of *E. coli* reference strains № 2 (concentration 10^8 CFU/ml) obtained in TSB after incubation at 37°C for 18 h was used for serial 10 fold dilutions up to level 10^{-7}. Aliquot 0,2 ml of dilutions 10^{-5}, 10^{-6} and 10^{-7} was used for meat sample contamination. The added aliquot of bacterial suspension into the meat sample was mixed very well for equally spreading in the total sample volume. Control samples were inoculated with 0,2 ml of sterile TSB. After inoculation all the samples were placed at 20°C for 30 minutes for better bacterial adaptation and adhesion. Real bacterial count in used dilutions was estimated by agar spread method using 0,1 ml over double TSA plates.

Bacterial DNA extraction from meat samples

From each meat sample 25 g was weight in a plastic bag (Stomacher, 400 ml) and 225 ml TSB were added. Diluted sample (1:10) was gently homogenised in Stomacher (Seward) for 120 sec at 205 rpm^{-1}. Total homogenate was

placed at 37°C for 4 h (pre-incubation) and after that 1 ml was transferred to 1,5 ml eppendorf microtube and used for DNA extraction as described above. Detection of *E. coli* in the samples was carried on by PCR using unspecified DNA template.

***PCR amplification*.**

PCR was performed to detect virulence genes: *eae*; shiga-like toxin1 (*stx1*) and shiga-like toxin 2 (*stx2*) of diarrheagenic *Escherichia coli.* PCR reaction mixture contains 1xPCR buffer, 1.5mM MgCl2; 200 μM each dNTP, 0.5μM each primer; 2.0 U Taq polymerase and 1μl DNA in a volume of 20 μl. The final cycling parameters were as follows: initial incubation step of 3 min at 94°C; 35 cycles: 60 sec at 94°C, 90 sec at 60°C, and 90 sec at 72°C and a final extension step of 7 min at 72 °C completed the reaction.

Based on the previously published reports for the detection of EPEC and STEC virulence genes (10, 11) the following primers were selected:

eae :<F - 5`-TCAATGCAGTTCCGTTATCAGTT-3';
R – 5' -GTAAAGTCCGTTACCCCAACCTG-3'>
stx1 :< F - 5' -CAGTTAATGTGGTGGCGAAGG-3';
R-5' –CACCAGACAATGTAACCGCTG-3'>
stx2 :<F- 5' – ATCCTATTCCCGGGAGTTTACG -3';
R- 5'- GCGTCATCGTATACACAGGAGC -3'>

PCR was performed individually using the above primer pair or as multiplex PCR. The PCR products (10μl) from amplification were separated on 2% agarose gel stained with ethidium bromide (0.5 mg/ml) and photographed using a gel documentation system. PCR amplification was performed in a PCR System (Quanta Biotech QB-96 thermocycler). Primers were supplied by LKB, Austria and components for PCR reactions were supplied by Fermentas, Lithuania.

RESULTS

Three reference *E. coli* strains were used for the experiments. Single, two or three bacterial colonies grown on the medium were collected in 0.5 ml TBE buffer and used for genome DNA isolation. Using the optimized amplification conditions, PCR with the purified DNA template was amplified for *eae* gene with primers given in methods and obtained amplicons were visualized by 2% agarose gel electrophoresis. The representative results from three independent experiments are presented in **Figure 1**. It is obviously those two strains: *E. coli* O 111-НБПМКК 4447 (№ 2), and *E. coli* O 26-НБПМКК 7434 (№ 3) have amplified a 482 bp fragment with used primers. Moreover the range of DNA concentration of sample used for PCR varied from 38.5 ng to 138.5 ng. Our result show also that *E. coli* strain O 26"H311b"-НБПМКК 4361 (№ 1) do not posses *eae* gene.

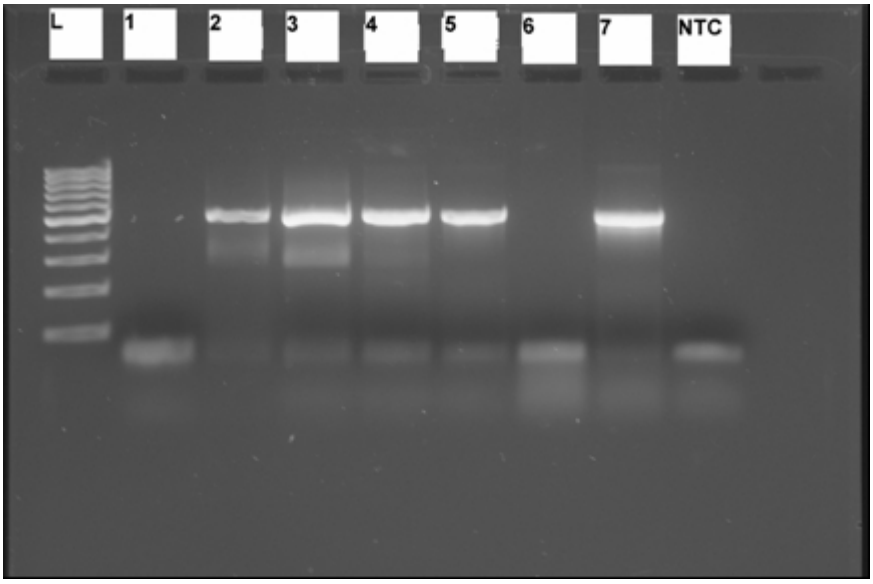

Figure 1. Agarose gel electrophoresis (2,0%) of PCR performed on *E. coli* strains for the detection of EPEC *eae* gene amplification product with 482bp. Line1 and 6: E. coli O 26"H311b"-НБПМКК 4361 (1 and 3 colony); Line 2,3 and 7: *E. coli* O 111-НБПМКК 4447 (1,2 and 3 colony); Line 4 and 5: *E. coli* O 26-НБПМКК 7434 (1 and 3 colony); L- indicate DNA ladder (100bp) and NTC-indicated non template control.

When primers for shiga-like toxin1 (*stx1*) and shiga-like toxin 2 (*stx2*) were used no amplification was observed in all tested *E. coli* strains.

To determine the sensitivity of the used PCR a serial 10-fold dilutions of overnight broth culture of *E. coli* reference strain № 2 were done. The initial cell concentration in the incubated broth was $8,4x10^8$ CFU/ml. Aliquots of 1000 µL from dilutions 10^{-2} to 10^{-10} were used for DNA isolation. Bacterial cell concentration in the used dilutions was decreasing, ranging form $8,4x10^6$ CFU/ml in dilution 10^{-2} to <1 CFU/ml in dilutions 10^{-9} and 10^{-10}. Dilution 10^{-7} contains from 97 to 54 viable *E. coli* cells in 1 ml. In our experimental condition the lowest cell concentration which exhibit well visualized 482 bp amplification band is dilution 10^{-7} (**Figure 2**). From the next dilution (10^{-8}) containing between 50 and 1 CFU/ml the PCR amplification using 1 µl of DNA template results in very fade but also visible shadow in the ethidium bromide stained gel.

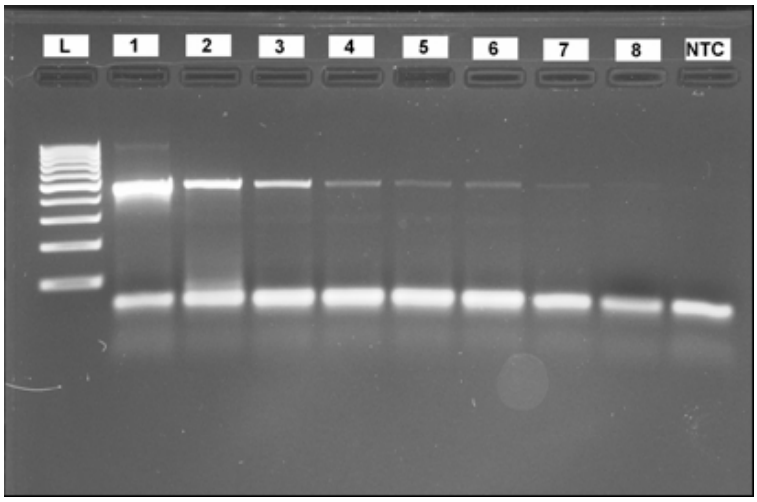

Figure 2. Agarose gel electrophoresis (2,0%) of PCR performed on *E. coli* O 111-НБПМКК 4447 strains serial 10 fold dillution for the detection of EPEC *eae* gene. Line1: Reference *E. coli* strains O 26"H311b"-НБПМКК 4361-positive control; Line 2 to 8: 10 fold dilution of *E. coli* strains O 26"H311b"-НБПМКК 4361 started from $8,4x10^6$ to 8,4 CFU/ml; Line L- DNA ladder (100n) and NTC-indicated non template control.

Bacterial cell concentration (CFU/g) in the contaminated beef meat samples are presented in **Table 1**. All the samples were inoculated with *E. coli* reference strain № 2. Using different broth dilutions we obtain different level of *E. coli* contamination in the samples. The highest cell concentration was 28,3 CFU/g meat by using dilution 10^{-5}. The lowest established contamination level was 0,7 CFU/g meat obtained by using *E. coli* broth dilution 10^{-7}. From each contamination level DNA was isolated after 4h pre-incubation at 37°C and PCR amplification was carried on. The results are shown in **Figure 3**. Well visualized 482 bp amplicon of *eae* gene was obtained from all the samples containing *E.coli*. The lowest initial cell concentration 0,7 CFU/g after pre-incubation resulted in 44 CFU/ml of meat sample homogenate. This very low initial concentration do not reproduce visible PCR product, but after pre-incubation we have increased *E. coli* number which is clear detectable by PCR.

***Table 1**. Concentration of E. coli cells in the used inoculation dose and in the contaminated beef meat samples*

	E.coli cell concentration		
	Level 10^{-5}	Level 10^{-6}	Level 10^{-7}
CFU/0,2 ml inoculation dose	1132	184	26
CFU/g meat after contamination	28.3	4.6	0.7
CFU/g meat after 4h pre-incubation at 37°C	1810	294	44

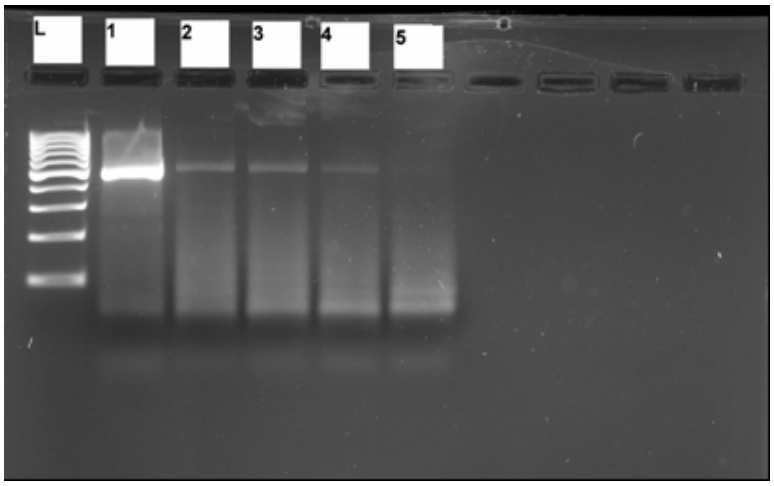

Figure 3. Agarose gel electrophoresis (2,0%) of PCR performed on meat contaminated with *E. coli* O 111-НБПМКК 4447 strains for the detection of EPEC *eae* gene. Line1: Reference *E. coli* strains O 26"H311b"-НБПМКК 4361-(6,0x10^8 CFU/ml); Line 2: meat contaminated with 28,3 CFU/g *E. coli* strains O 26"H311b"-НБПМКК 4361; Line 3: meat contaminated with 4,6 CFU/g *E. coli* strains O 26"H311b"-НБПМКК 4361; Line 4: meat contaminated with 0,7 CFU/g *E. coli* strains O 26"H311b"-НБПМКК 4361; Line 5: non-contaminated meat; Line L- DNA ladder (100n) and NTC-indicated non template control.

DISSCUSION

Escherichia coli (*E. coli*) is a bacterium that is commonly found in the gut of humans and warm-blooded animals. Most *E. coli* strains are commensal and harmless. Some strains however, such as enteropathogenic (EPEC) and enterohaemorrhagic *E. coli* (EHEC), can cause severe foodborne disease. It is transmitted to humans primarily through consumption of contaminated foods, such as raw or undercooked ground meat products and raw milk Strains that cause enteric infections are designated diarrheagenic *E. coli*, a group that includes emergent pathogens with public health relevance worldwide (12). In 1987, the World Health Organization (13) recognized EPEC serotypes of 12 different O serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158). The epidemiological significance of each *E. coli* depends on their genotype. In 1996, EPEC strains were defined as intimin-containing DEC (diarrhoeagenic *E. coli)* isolates that possess the ability to form A/E lesions on intestinal cells and do not possess Shiga toxin (*stx*) genes (14,15). The formation of A/E lesions is governed by a pathogenicity island known as the locus of enterocyte effacement (LEE), which contains the *eae* gene encoding the intimin protein. A wide range of animal species are known to carry STEC and EHEC strains, but ruminants are the most important natural reservoir and excrete these bacteria with their feces (16).

Vidal et al. (17) detected EPEC as the most frequent category (10,6%) of *E. coli* in diarrheic stool samples. *Eae* and *bfp* gene detection primers were included in multiplex PCR. From the same samples eight shigatoxin producing strains were detected. Two of them had the stx_2 gene and six strains were non-O157:H7 and harbored stx_1.

The prevention of infection requires control measures at all stages of the food chain, from agricultural production on the farm to processing, manufacturing and preparation of foods in both commercial establishments and the domestic environment. Despite microbiological identification, PCR provides an equally reliable but much faster diagnostic method. Prevalence of *E. coli* O157:H7 in minced beef products in Ireland showed 2.8% positive samples (18). The number of *E.coli* in 21 of the 43 positive samples ranged from 0.52 – 4.03 log10 cfu g^{-1} while in the remaining 22 positive samples, the pathogen was detectable by enrichment only i.e. 0.52 log10 units. The authors used primers designed for detection *eae*, verotoxin1 and verotoxin2 genes by producing 384, 180 and 255 bp. amplicons, respectively. In a study of beef meat Carney et al. (19) used multiplex PCR in detection of *E.coli* pathogen bacteria. The pathogen was found at a prevalence of 3.0% (4 of 132 samples) on beef carcass samples at concentrations ranging from 0.70 and 1.41 $\log_{10}$ cfu g^{-1}.

The present study was performed for the identification of enteropathogenic *Escherichia coli* strains from contaminated meat by a PCR technique. We used specific primers described by Vidal et al., 2004 for amplification of 482 bp fragment, located in 5' region of *eae* gene. The 5' regions of *eae* genes are conserved, whereas the 3' regions are heterogeneous. This observation has led to the construction of universal PCR primers, which have made it possible to differentiate at present 21 variants of the *eae* gene encoding 21 different intimin types and subtypes (20,21). Our results are in agreement with these observations for PCR detection of *eae* gene fragments in diarrheagenic *Escherichia coli* strains.

The results of this study indicate that it is possible to detect amplification of virulence gene - *eae* from enteropathogenic *E. coli* strains and to apply this technique for analysis of contaminated meat.

The sensitivity of this method had a detection limit between 1 and 50 CFU in liquid substrates like diluted broth or diluted meat homogenate. After 4 hours pre-incubation it was possible to detect meat contamination with initial level of 0,7 CFU/g for fresh meat.

In conclusion the developed PCR method could be used for detection of typical and atypical EPEC in isolated colonies and in food.

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