IDENTIFICATION OF SHIGA TOXIN PRODUCING
ESCHERICHIA COLI O157:H7 IN BEEF SAMPLES STORED
AT DIFFERENT TEMPERATURES USING MULTIPLEX PCR

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Abstract

The multiplex PCR was used to identify the Shiga
toxin-producing Escherichia coli in meat. Raw beef samples
inoculated with E. coli O157:H7 were stored in a cooler for 1
h, 6 h, 12 h, 24 h, 48 h, and 96 h at 2°C and in a freezer for 1 h,
6 h, 12 h, 24 h, 48 h, 72 h, 96 h, 1 week, 2 weeks, 3 weeks, 4
weeks, and 2, 3, 4, 5 or 6 months at -18°C, and after the
storage period were tested with multiplex PCR assay. It was
found that stx gene of E. coli O157:H7 could be properly
identified by the use of the mPCR technique in beef samples
stored up to 96 h at 2°C or 6 months at -18°C.

Key words: beef, storage, E. coli, Shiga toxin, PCR.

Shiga toxin-producing Escherichia coli (STEC) strains, also called Vero toxin-producing E. coli
(VTEC), are an important cause of severe
gastrointestinal diseases in humans, such as bloody
diarrhoea, haemorrhagic colitis (HC), haemolytic
uraemic syndrome (HUS), and thrombotic
thrombocytopenic purpura (TTP) (16). The transmission
of STEC occurs through consumption of contaminated
food, especially undercooked meat, unpasteurised dairy
products, and vegetables or water contaminated by
ruminants, that are the main reservoir of these bacteria.
STEC often belong to O157:H7; however, several other
serogroups have been identified (O26, O111, O113) (1,
2, 8, 10). The pathogenicity of STEC is mainly mediated
by two virulence markers: Shiga toxin (Stx) and the 94
kDa surface protein - intimin, which are involved in the
intimate adhesion of the bacteria to enterocytes (14, 15).
STEC strains may produce two types of Shiga toxin:
Stx1 and Stx2. The detection of STEC generally
involves a colony isolation on selective agar followed by
biochemical and serological testing. These procedures
are time- and labour-consuming and can take up to 3–5
d (11, 13). More rapid detection may be possible by
using polymerase chain reaction (PCR) for the
determination of STEC genotypic virulence markers
(14). On the other hand, they have several limitations
connected with PCR inhibitors present in the reaction
mixture, low DNA polymerase activity, and DNA from
background microflora, which may give false negative
results. Moreover, a long period of the samples storage
may decrease the procedure sensitivity (4, 9). In the
present study, the multiplex PCR was used for the
identification of STEC in meat samples stored at 2°C
and -18°C.

Material and Methods

Raw beef samples (25 g) were inoculated with
reference strain E. coli O157:H7 EDL933 (10^2 cells per
sample). Then, the samples were stored in a cooler (2°C)
for 1 h, 6 h, 12 h, 24 h, 48 h, and 96 h, and in a freezer
(-18°C) for 1 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h, 1 week,
2 weeks, 3 weeks, 4 weeks, and 2, 3, 4, 5 or 6 months.
Afterwards, they were tested by the use of mPCR assay.
The meat was added to 225 ml of TSB broth and
incubated at 37°C for 18 h with shaking (150 rpm). The
cultures were then transferred to TSB with mitomycin C,
incubated at 37°C for 18 h with shaking (150 rpm). The
cultures were then transferred to TSB with mitomycin C,
incubated for another 18 h, and centrifuged twice (5 000
x g and 1 300 x g, respectively). The supernatant was
suspended in 50 µl of sterile DNase- and RNase-free
denatured water (ICN Biomedicals, USA). The
supernatant (5 µl) was used as a source of
DNA template.

The multiplex PCR assay was used to identify
Shiga toxins genes (stx conserved sequence) and, as an
internal control of PCR 16S rRNA E. coli gene (16S
rRNA). Each DNA amplification was performed in the
reaction mixture consisting of DNA template, 1X Taq
buffer, 1 U of Taq DNA polymerase, 200 µM of dNTPs,
MgCl2 (5 mM), nucleotide primers and water to the final
volume of 50 µl. Sequences and characteristics of the
primers used in this study are shown in Table 1.
Table 1
Sequences and characteristics of the primers used in the study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5´→3´)</th>
<th>Target gene</th>
<th>Size of amplicon (bp)</th>
<th>Concentration in mPCR (µM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK1</td>
<td>TTTACGATAGACTTCTCGAC</td>
<td>stx</td>
<td>230</td>
<td>0.5</td>
<td>12</td>
</tr>
<tr>
<td>MK2</td>
<td>CACATATAAATTATTCGCTC</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e16S-a</td>
<td>CCCCCCTGGACGAAGACTGAC</td>
<td>16S rRNA</td>
<td>401</td>
<td>0.05</td>
<td>19</td>
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<tr>
<td>e16S-b</td>
<td>ACCGCTGGCAACAAGGATA</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Multiplex PCRs were run in a thermocycler (PTC-100) under the following conditions: initial DNA denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min. The final extension step was done at 72°C for 5 min. After staining with ethidium bromide (5 µg/mL) for 1 min and washing in distilled water, the gels were photographed under UV light using the Gel Doc 2000 documentation system (Bio-Rad). The sizes of the PCR amplicons were compared to the 100 bp DNA marker (Fermentas).

Results

The multiplex PCR test was used to identify Shiga toxin-producing *E. coli* (STEC) and to differentiate them from other non-STEC isolates. Two pairs of primers were used: *stx*-specific, designated for the detection of all known variants of the *stx* gene and, as an internal control, specific for the *E. coli* 16S rRNA gene. Two respective PCR bands (230 bp and 401 bp for *stx* and 16S rRNA genes, respectively) were visible in the agarose gel when the inoculated beef samples were stored in a cooler for 1 h, 6 h, 12 h, 24 h, 48 h, and 96 h at 2°C (Fig. 1, lanes 1-7). In the meat samples that were not inoculated, the amplicon of 16S rRNA was observed due to the presence of non-STEC *E. coli* strains (Fig. 1, lane 8).

Fig. 1. PCR amplicons obtained in the mPCR test. Lanes 1-7: bacterial cells inoculated into meat (0.4 CFU/g) and stored in a cooler for 1 h, 6 h, 12 h, 24 h, 48 h, and 96 h at 2°C; lane 8: beef contaminated during meat processing with non-STEC; lane 9: H2O; lane 10: *E. coli* strain EDL933 cultured on LB agar; lane M: 100 bp DNA marker.

Fig. 2. PCR amplicons obtained in the mPCR test. Lane 1-7: bacterial cells inoculated into meat (0.4 CFU/g) and stored in freezer for 1 h, 6 h, 12 h, 24 h, 48 h, and 96 h at -18°C; lane 8: beef contaminated during meat processing with non-STEC; lane 9: H2O; lane 10: *E. coli* strain EDL933 cultured on LB agar; lane M: 100 bp DNA marker.
When the beef samples inoculated with STEC and stored in a freezer for 1 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h, 1 week, 2 weeks, 3 weeks, 4 weeks, and 2, 3, 4, 5 or 6 months at -18°C were used in mPCR assay, two bands with molecular masses of 230 bp and 401 bp, respectively, were observed (Figs 2 and 3). When a beef contaminated during meat processing with non-STEC was used as a DNA template in mPCR test, only one PCR amplicon corresponding to the 16S rRNA gene was observed (Fig. 2, lane 8; Fig. 3, lane 10).

Discussion

Raw beef samples inoculated with E. coli O157:H7 were stored in a cooler for 1 h, 6 h, 12 h, 24 h, 48 h, and 96 h at 2°C, and in a freezer for 1 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h, 1 week, 2 weeks, 3 weeks, 4 weeks, and 2, 3, 4, 5 or 6 months at -18°C. After the storage period, the samples were tested with mPCR assay. The positive results were observed when the beef samples were stored up to 96 h in the cooler or 6 months in the freezer. Several reports on influence of sub-zero temperatures on the survival of E. coli O157:H7 were published before. Doyle and Schoeni (6) examined inoculated meat samples, which were fast-frozen at -80°C and then transferred to -20°C. The authors found that the STEC strains could survive for 9 months. Moreover, they found that inoculation level of 6.7x10^3 CFU/g decreased to only 6.2x10^3 CFU/g. These results are opposite to a research of Chapman et al. (4) who froze 91 beef samples containing E. coli O157:H7 to -70°C and after 19-58 months in 20 samples no STEC were observed. Moreover, among the remaining 71 samples tested, only 53 had the same virulence markers before and after the storage. The lost of pathogenic markers was explained by the lack of plasmids stability of either DNA or bacteriophage origin encoding the virulence properties of the examined strains. Other researchers had found that freezing and defrosting of food has an influence on a decrease in the number of bacterial cells: the highest one was noted in the food of plant origin (9). Dykes (7) also noted a decrease in E. coli O157:H7 cells between 0.5 and 2.0 log CFU/g in beef samples stored at -18°C and -35°C for 12 weeks. Moreover, recent studies have described that meat samples should be taken to the laboratory and examined with the use of molecular techniques within 2-8 h (3, 5, 17, 18).

In conclusion, the results of the present study indicate that a beef samples containing E. coli O157:H7 could be stored up to 96 h at 2°C or 6 months at -18°C and the stx and 16S rRNA genes are properly identified by the use of the mPCR technique. This method may be used in routine analysis of these pathogens.

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References

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