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Investigation of Shiga-like toxigenic *Escherichia coli* in meat products by quantitative PCR

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A b s t r a c t: The aim of this study was to investigate and evaluate effectiveness of quantitative PCR (qPCR) in quantification of Shiga-like toxigenic E. coli (STEC) in artificially contaminated deli meat slices compared to plate count technique. Experiment was designed to investigate capability of qPCR using selected pair of primers amplifying rpoB household gene and serial decimal dilution of an overnight suspension of E. coli O157:H7 culture. Subsequently, slices of smoked pork loin were contaminated by respective decimally diluted dose of STEC. DNA was extracted from the samples and qPCR was run in triplicate. Mean Ct values of amplified rpoB gene were compared to each inoculum dose and standard curves were generated. Results clearly showed that the lowest detectable level of STEC in pork loin slices using qPCR was 6.8×10^1 CFU/g. Further optimization of method should be done in order to resolve, if possible, discrepancies at the levels of contamination less than 100 CFU/g.

Key words: *qPCR, E. coli O157, quantification.*

Introduction

Shiga-like toxigenic E. coli have emerged as important food-borne pathogens, causing hemorrhagic colitis, which is sporadically complicated by hemolytic uremic syndrome. Onset of the illness requires a very low dose of STEC, in between 10-700. Standard or validated alternative methods are available and are recommended to be used for the detection and isolation of STEC O157 from food and animals. For the other serotypes, there are no universally accepted and validated methods, but pragmatic approaches have been produced. Pathogenic *E. coli* strains can be divided into Enteropathogenic *E. coli* (EPEC) strains, which "only" cause A/E (attaching-and-effacing) lesions, Shiga-toxin producing *E. coli* (STEC) strains, which possess and express *stx* genes, and Enterohemorrhagic *E. coli* (EHEC), which constitute a subset of STEC as classical EHEC can cause both hemorrhagic colitis (HC; due to AE lesion) and hemolytic uremic syndrome (HUS; due to the Shiga toxins) (*Bunčić,* 2000). Different virulence genes, such as stx_1 and stx_2 and their variants, which encode Shiga toxins, *eae*, which encodes the bacterial outer-membrane protein intimin, *nle*, which encodes translocated substrates of the type III secretion system, *ehx*A, which encodes the EHEC hemolysin, *iha*, which encodes an adherence-associated protein, *esp*P, which encodes the serine protease, and *hly*A,which encodes enterohemolysin, have been targeted to assess the presence of STEC (*Coombes et al.,* 2008; *Paton and Paton*, 1998; *Pradel et al.,* 2008).

Improved methods for the detection and isolation of STEC non-O157 from foods, animals and the environment should be developed and validated. There is no standard protocol for enumeration of STEC O157 or other STEC serotypes in food or environmental samples and such quantitative methods should be developed. Enumeration of STEC is generally not conducted as part of routine monitoring or testing programs, although quantitative data are essential to better understand the human health risks. Recent advances in molecular detection methods combine the traditional detection methods and target serotype specific genes, *stx*, as well as other virulence genes. However, isolation of STEC, and subsequent strain characterization is still needed in order to ensure that the detected genes are present on the same bacteria.

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Microbial cross-contamination at production site is one of the major factors of contamination of foods with STEC. Good hygiene practices at the abattoirs and at processing plants, including monitoring for microbiological indicators (*Enterobacteriaceae* and in generic *E. coli*), is likely to be the most effective method for reducing the public health risks for STEC infection. However, compliance with the hygiene criteria does not necessarily guarantee the absence of STEC at concentrations sufficient to cause human disease. Application of efficient validated HACCP-procedures for production of raw ready-to-eat meat, meat preparations and other foods is important to reduce the public health risks for STEC infection (EFSA 2008).

Generally, the DNA amplification-based techniques are rapid and will give a result within hours. One of the advantages of DNA-based methods is that it is possible to simultaneously investigate cultures for several genes at the same time. However, when testing mixed cultures the detected genes might not originate from the same STEC strain. By using DNA-based methods it is also possible to differentiate between the different *stx* subtypes. Furthermore, several quantitative PCR methods can be used to assist in subculturing of selected enrichment broths with priority given to the highest target concentration since there is a correlation between the number of *stx* gene copies and the success of isolation of STEC from an enrichment broth. DNA based methods have the disadvantage of being unable to distinguish between DNA from viable and non-viable cells, although this may only be important in specific situations.

In the last 10 years, Real-Time PCR systems based on SYBR Green I and TaqMan technologies have increasingly been used for accurate and reliable detection and quantification of various foodborne pathogens (*Rodríguez-Lázaro et al*., 2004a; *Rawsthorne and Phister*, 2006; *Skånseng et al.,* 2006; *Malorny et al.*, 2007), including STEC in contaminated samples along the food production chain. Compared to conventional PCR-based methods, Real-Time PCR technologies involve a lower risk of cross-contamination because the presence of the target sequence(s) in the sample is indicated by an increase in fluorescence signal, and no post-PCR processing of the sample is required (*Rodríguez-Lázaro et al*., 2004c; *Rossmanith et al*., 2006; *Cocolin and Rantsiou*, 2007).

Materials and methods

E. coli O157:H7 ATCC 35150 strain was used for assessing Real Time PCR efficiency, optimization of amplification conditions and inoculation of pork loin slices.

BHI broth (Oxoid, UK) was used for cultivation of strain preceding DNA extraction and for inoculation of dry pork loin slices. MRD (Merck, Germany) was used for preparation of serial dilutions. ChromID O157:H7 agar (Biomerieux, France) was used for quantification of *E. coli* O157:H7 in both overnight (o/n) culture broth and in inoculated samples. The final concentration of cells in the meat product ranged from 10^8 to 10^1 CFU/g.

Extraction of DNA from cultures and inoculated samples

One mL of an o/n culture was centrifuged at 13.000 rpm for 5 min at 4°C and resuspended in 100 µl of PrepMan Ultra reagent (Applied Biosystems, Foster City CA, USA) placed in a 1.5 mL micro centrifuge tube. The samples were heated in boiling water for 10 minutes, allowed to cool to room temperature and centrifuged at 13.000 rpm for 2 min. The supernatant (containing the DNA) was transferred to a clean 1.5 mL micro centrifuge tube. DNA was quantified by using the UV Biophotometer instrument (Eppendorf, Germany) and diluted to a final concentration of 100 ng/µL.

For loin slices, 10 g of sample was diluted in 90 ml of Maximum Recovery Diluent (MRD) in a stomacher bag and homogenized in a stomacher machine (AES Chemunex, France) for 1 min. The debris was left to deposit for about 5 min. One mL of homogenate was transferred to a 1.5 ml sterile tube

Table 1. List of primers used in experiment
Tabela 1. Spisak prajmera koji su korišćeni u ogledu

Primer name/ Ime prajmera	**Sequence (*5′–3′*)**	**Amplified product size/ Veličina amplifikovanog proizvoda**
rpoB-F	GGTAGTGAATTTCGTCAGTTACA	130 bp
rpoB-R	GTATGTCCAATCGAAACCCCT	

and centrifuged at 13.000 rpm for 5 min. The extraction was further carried as described above.

The oligonucleotides used as PCR primers are shown in Table 1. These amplified a region of *rpoB* housekeeping gene encoding RNA polymerase β subunit.

Amplification conditions

Real Time PCR amplification was performed using a Brilliance III SYBR Green Real Time PCR kit (Agilent, USA) in a total volume of 20 µL containing 10 µL of 2× reaction buffer, 1 µL of each primers, 1 µL of template DNA and 7 µL of PCR water to make up the final volume. Amplification was performed using an Agilent MX3005P thermo cycler (Agilent Technologies, USA). Thermal cycling conditions was as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 20 s.

Enumeration

An overnight culture of *E. coli* O157:H7 used to contaminate the sliced pork loin was enumerated on ChromID O157:H7 agar to determine the exact count of colony forming unit (CFU) inoculated in the samples. The signals obtained (threshold cycle, Ct) for the serial dilutions of *E. coli* O157:H7 in MRD and in pork loin were plotted against the $\log_{10}$ CFU/mL or CFU/g to construct the calibration curves. Determination coefficients (R^2) and amplification efficiency (AE) were calculated as described previously by *Higuchi et al.* (1993). Due to plating of 0.1 mL volume of each dilution the samples inoculated by <10 CFU/mL were not detectable.

Results and discussion

When cells were diluted in MRD (Figure 1.), the linearity range was from 8.78 $\log_{10}$ CFU/mL to 1.78 $\log_{10}$ CFU/mL, covering 7 orders of magnitude. The efficiency was 99.25% and the correlation coefficient (R^2) was 0.959.

Due to design of experiment, results of 5 levels of inoculation of sliced pork loins with serially diluted *E. coli* O157:H7 cells are displayed (Figure 2). The efficiency in this case was 101.35% and the correlation coefficient (R^2) was 0.972.

Results showed that it was possible to quantify count of *E. coli* O157:H7 in MRD using standard curve down to the level of less than 100 CFU/mL, more precisely at about 60 CFU/mL. At lower dilution levels (from 1 to 10 CFU/mL) Ct signals couldn't be detected. Moreover, even at the 10-fold higher level, discrepancies of the Ct signals were noticed (data not shown) which could be attributed to the very small amount of initial DNA, non-homogenous distribution of bacteria in samples, or variable amplification efficiency during the first several cycles. Regarding quantification of *E. coli* O157:H7 in sliced smoked pork loin, we determined that

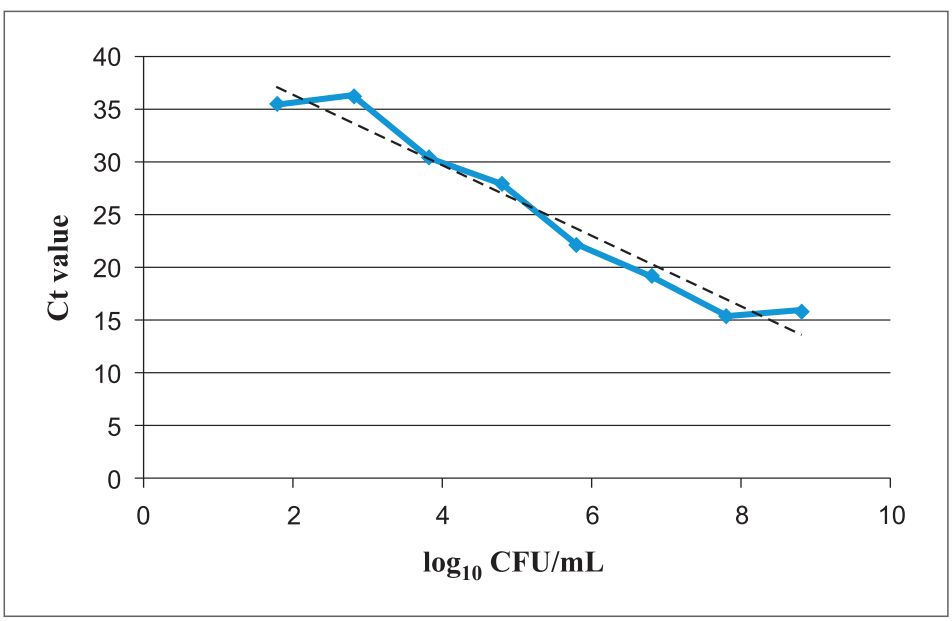

Figure 1. DNA standard curve of E. coli O157:H7; decimal dilutions in MRD
Slika 1. Standardna prava DNK E. coli O157:H7; decimalna razblaženja u MRD

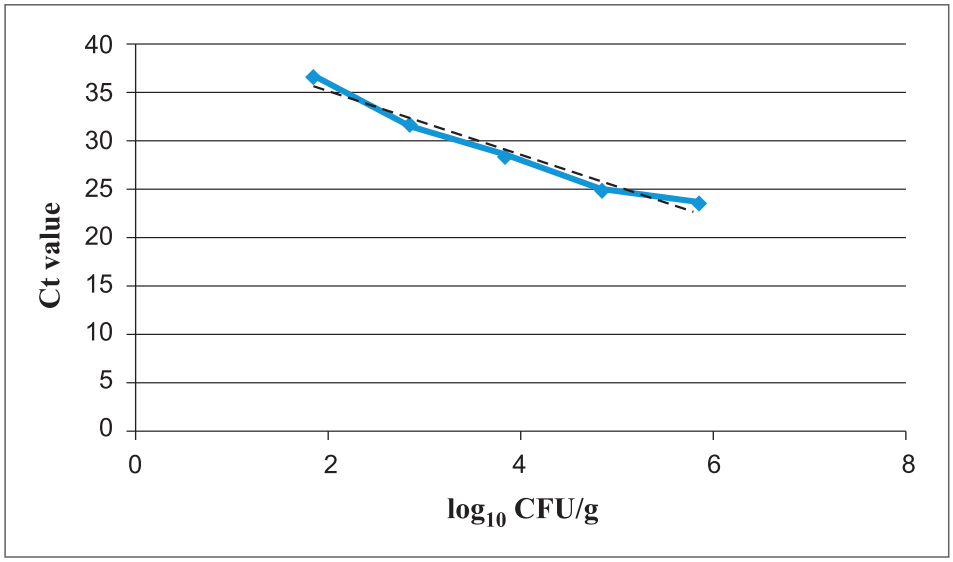

Figure 2. DNA standard curve of E. coli O157:H7 decimal dilutions in slices of smoked pork loin
Slika 2. Standardna prava DNK E. coli O157:H7; decimalna razblaženja u uzorcima svinjske dimljene pečenice

absolute limit of quantification using standard curve was at approximately 70 CFU/g. However, this data should be taken with great care since choice of kit for DNA, possible carryover of impurities or inhibitors, presence of aggregated cells etc., can have a significant effect on quantification results and could lead to misinterpretation.

In our experience which derived from repeated experiments of this type, safety margin (95% CI) in terms of LOQ of *E. coli* O157:H7 in this type of meat product should be in range 100-300 CFU/g.

For the DNA standard curves, the efficiencies were different based on the matrix used, however the R^2 value was always acceptable (≥ 0.930).

Regarding alternative protocols of quantification of food-borne pathogens, *Fukushima et al.* (2007) proposed a buoyant density gradient centrifugation as concentration method for 12 food-borne pathogens. The detection limit of the protocol varied from 10–10^3 CFU/g, presenting favorable applicability for *Salmonella* spp. and *C. jejuni,* for which the detection of 10–10^2 CFU/g in naturally contaminated chicken was obtained in 3 hours.

However, the results in terms of quantification limit obtained here are in agreement with the reports of other authors who developed qPCR protocols to quantify *L. monocytogenes* in meat (*Rodriguez-Lázaro et al*., 2004) and in salmon products (*Rodriguez-Lázaro et al*., 2005).

Conclusion

In this study, we evaluated efficiency of Real-Time PCR quantitative methods against the Plate Count technique for quantification of decimal dilution series of STEC in deli meat matrix. The Real-Time PCR methods showed similar accuracy for quantitative detection of examined samples, but the sensitivity of Plate Count Technique was 1–2 logs lower than the investigated molecular assays. According to the obtained results and with respect to the advantages of the molecular systems, this assay could be considered as a potential alternative to traditional cultural methods used for quantification of food borne pathogens in different foods and culture media matrixes. Care should be taken when calculating counts in the "risky" range of values obtained by using the standard curve, i.e. 100–1000 CFU/g or mL and further studies should be carried out in order to optimize protocol and performance characteristics.

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Ispitivanje Shiga-like toksigenih *Escherichia coli* u proizvodima od mesa pomoću kvantitativnog PCR-a

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R e z i m e : Cilj ovog rada bio je da se ispita, uporedi i oceni efikasnost kvantitativnog PCR-a u kvantifikaciji Shiga-like toksigenih E. coli (STEC) u eksperimentalno kontaminiranim narescima proizvoda od mesa u odnosu na klasičnu tehniku brojanja kolonija. Ogled je osmišljen tako da se primarno ispitaju mogućnosti kvantitativnog PCR-a korišćenjem izabranog para prajmera koji amplifikuje deo rpoB „household" gena u ekstraktima DNK iz serijskih decimalnih razređenja prekonoćne suspenzije kulture E. coli O157:H7. Uzorci svinjske dimljene pečenice kontaminirani su odgovarajućim serijskim razređenjima STEC. Nakon što je ogled ponovljen tri puta, napravljena je standardna prava odnosa srednje Ct vrednosti amplifikacionog signala za gen rpoB i odgovarajućeg razređenja inokuluma. Dobijeni rezultati jasno ukazuju da je limit kvantifikacije STEC u veštački inokulisanoj pečenici koji se može detektovati kvantitativnim PCR-om 68 CFU/g. Neophodna je dalja optimizacija ove metode kako bi se uklonila neslaganja koja se često pojavljuju kod nivoa kontaminacije manjih od 100 CFU/g.

Ključne reči: *qPCR, E. coli O157, kvantifikacija.*

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