Multiplex Real-Time PCR for Detection of Shiga toxin and Intimin genes of pathogenic *Escherichia coli*

Pavlovic, M.; Konrad, R.; Wolf, S.; Lindermayer, M.; Fräßdorf, J.; Busch, U.; Huber, I.

Bavarian Health and Food Safety Authority, Veterinärstr. 2, 85764 Oberschleißheim, Germany

Some *E. coli* strains harbour specific virulence factors which classify them as pathogens. In human they mainly cause infections of the gastrointestinal and urinary tracts and are among the most frequent bacterial causes of diarrhea. Especially in young children, infections with Shiga toxin producing enterohaemorrhagic *E. coli* (EHEC) can lead to the life threatening hemolytic uremic syndrome (HUS). EHEC and enteropathogenic *E. coli* (EPEC) strains can induce attaching and effacing lesions. They harbor the locus of enterocyte effacement (LEE) pathogenicity island with the intimin gene *eae*. Intimin mediates the intimate attachment of bacteria to epithelial cells. The diagnosis of EHEC strains in stool and food is based on detection of Shiga toxin genes and of EPEC strains on the *eae* gene, respectively.

This multiplex real-time PCR with 5´-nuclease probes allows the simultaneous detection of Shiga toxin genes *stx1* and *stx2* as well as the intimin gene *eae* (Pavlovic et al., 2010). As internal amplification control (IAC) plasmid DNA *ntb2* was included (Anderson et al., 2010).

Material and Methods

Extraction of DNA: High Pure Template Preparation Kit, Roche, Mannheim or heat lysis (Bacteria grown on appropriate agar media were suspended in 1.5 mL physiological saline (0.9%). 20 µL were then transferred to 400 µL sterile water and heated at 95°C for 15 min. After centrifugation (14.000 g, 5 min) the supernatant was used for amplification reactions.)

Multiplex real-time PCR: The reaction contained 1x QuantiTect Multiplex real-time (RT)-PCR NoROX Mastermix (Qiagen, Hilden), 400 nM of each primer and probe of the *stx1*-, *stx2*-, *eae*-system and 100 nM of the *ntb2*-primer and probes. 4 µL DNA extract and 25 copies of *ntb2* plasmid DNA were added. The final reaction volume was 20 µL. The PCR program on the LightCycler 480 PCR machine was 15 min, 95°C, 40 cycles of 60 sec/95°C, 60sec/58°C.

Results

Table 1: Characteristics of the multiplex real-time PCR for EHEC/EPEC diagnostic

				Detection limit (genome copies per µL)		Linearity of multiplex ($E = 10^{-1/slope}$)		
Gene	**Length of PCR product**	**Channel**	**Reference**	**Singleplex**	**Multiplex**	**Slope**	**R^2**	**Efficiency of multiplex**
stx1	***87 bp***	***FAM***	***Sharma et al., 1999***	**50**	**50**	**3.37**	**0.99**	**1.97**
stx2	***114 bp***	***TexRed***	***Pavlovic et al., 2010***	**5**	**5**	**3.32**	**1.00**	**1.99**
eae	***107 bp***	***YAK***	***Pavlovic et al., 2010***	**50**	**50**	**3.35**	**0.99**	**1.98**

Inclusivity was tested using **30 EHEC and EPEC** reference strains with gene variants of ***stx1*** (*stx1, stx1c, stx1d*), ***stx2*** (*stx2, stx2c, stx2d, stx2e, stx2f, stx2g*) and ***eae*** (*eae-α1, eae-α2, eae-β1, eae-β2, eae- γ, eae-γ1, eae-γ2,eae-ζ, eae-η, eae-η1, eae-η2, eae-θ, eae-ι, eae-ι 1, eae-κ, eae-ε*). All gene variants were detected as expected.

Exclusivity was tested using 152 strains:

94 were non-EHEC/EPEC *E. coli* pathotypes including 1 ETEC, 2 EIEC, 5 EAEC, 84 clinical *E. coli*-harbouring samples and 2 non-pathogenic reference strains.

58 non-*E. coli* strains including 45 strains of the *Enterobacteriaceae* family and 13 other strains of the gastrointestinal flora.

No strain gave a false-positive signal. In summary **selectivity** was 100%.

Table 2: Comparison of results between the multiplex real-time PCR and the real-time PCR so far used in the routine diagnostics of human stool, animal faeces and food samples for the presence of EHEC and EPEC bacteria.
96.7% showed the same result for the *stx1* gene, 97.3% for the *stx2* gene and 97.1% for the *eae* gene.
The multiplex PCR detected 42 more positive samples than the diagnostic PCR: *stx1*: n=17; *stx2*: n=14; *eae*: n=11
but failed to detect 10 samples positive in the diagnostic PCR (*stx1*: 5; *stx2*: 4, *eae*: 1).

	stx1 (n = 665)		*stx2* (n = 665)		*eae* (n = 405)	
Routine samples	**positive**	**negative**	**positive**	**negative**	**positive**	**negative**
Multiplex	**151**	**514**	**148**	**517**	**125**	**395**
Diagnostic real-time PCR so far used (Reischl et al. 2002)	**139**	**526**	**138**	**527**	**115**	**405**

Literature

Anderson A., Pietsch, K., Zucker, R., Mayr, A., Müller-Hohe, E., Messelhäusser, E., Sing, A., Busch, U., Huber, I. (2010) Validation of a Duplex Real-Time PCR for the Detection of Salmonella spp. in different food products. *Food Anal. Methods*, DOI 10.1007/s12161-010-9142-8.

Pavlovic M., Huber I., Skala H., Konrad R., Schmidt H., Sing A., Busch U. (2010) Development of a Multiplex Real-Time Polymerase Chain Reaction for simultaneous detection of enterohemorrhagic Escherichia coli and enteropathogenic Escherichia coli strains. *Foodborne Pathogens and Disease* 7:801-808.

Reischl U., Youssef M.T., Kilwinski J., Lehn N., Lan Zhang W., Karch H., Strockbine N.A. (2002) Real-time fluorescence PCR assays for detection and characterization of Shiga toxin, intimin, and enterohemolysin genes from Shiga toxin-producing Escherichia coli. *Appl Environ Microbiol* 40:2555-2565.

Sharma V.K., Dean-Nystrom E.A. und Casey T.A. (1999) Semi-automated fluorogenic PCR assays (TaqMan) for rapid detection of *Escherichia coli* O157:H7 and other Shiga toxigenic *E. coli*. *Molecular and Cellular Probes* 13: 291-302.

Kontaktdaten: **Telefon:** 089/31560-886 **E-Mail: regina.konrad**@lgl.bayern.de **Internet:** www.lgl.bayern.de