Detection of Enterotoxigenic *Escherichia coli* in food by Real Time PCR amplification of the *lt*, *sth,* and *stp* genes, encoding the heat-labile and heat-stable enterotoxins

1. Aim and field of application

Enterotoxigenic *Escherichia coli* (ETEC) represent a group of diarrheagenic *E. coli* that is a common cause of infantile and traveller diarrhea in developing countries. However, ETEC are increasingly recognized as an important cause of foodborne illness in industrialized countries, and their prevalence could be underestimated, because they are not detected by standard stool culture methods and need specific assays to be distinguished form the other *E. coli* strains usually present in fecal samples. Moreover, the symptoms of ETEC infection are relatively nonspecific, and ETEC outbreaks may be incorrectly attributed to a viral etiology. In industrialized countries, foodborne outbreaks have been associated with salads, fresh basil used for preparation of pesto sauce, tuna paste. It is therefore important that the procedures allowing the detection of ETEC in foodstuffs are available in public health laboratories.

ETEC strains cause diarrhea through the action of enterotoxins: the heat-labile (LT) and heat-stable (ST) enterotoxins. Some strains may express an LT only, an ST only, or both an LT and an ST. Two variants of ST can be produced by strains isolated from human cases of diarrhea: the human variant (STh) and the porcine variant (STp). Therefore the genes encoding these toxins (*lt*, *sth* and *stp*) represent targets for ETEC identification and detection.

The present procedure describes a molecular methodology to screen food samples for the presence of ETEC by the detection of targets designed on the *lt*, *sth,* and *stp* genes. The same genetic markers are currently used for the detection of ETEC in the feces of patients with diarrhea.

2. Screening of food samples

Enrichment cultures are obtained by adding 25 gr test portions of the food specimen (or 25 ml of liquid) to 225 ml of Buffered Peptone Water (BPW), homogenizing in a peristaltic blender, and incubating at 37 ±1 °C for 18-24 h.

One ml of the enrichment culture is used for DNA extraction. This step is accomplished by any method in use in the laboratory for the extraction and purification of DNA from food enrichment cultures.

The Real Time PCR targeting the *lt*, *sth,* and *stp* genes is performed with the primers and probes reported below.

Enrichment cultures positive for the presence of at least one of the *lt*, *sth,* or *stp* genes are streaked onto suitable solid media (MacConkey agar plates or other media suitable for *E. coli* isolation, such as TBX) for attempting the isolation. This step is accomplished as follows:

- ❑ Pick up to 50 colonies with *E. coli* morphology.
- ❑ Point-inoculate on Nutrient Agar (NA) (single colonies).
- ❑ Test the isolated colonies or pools of 10 colonies by real time PCR for the presence of the gene(s) detected in the screening step.
- ❑ Subculture the positive colonies for further isolation and characterization.

3. Real Time PCR amplification of the enterotoxin genes

This paragraph illustrates the primers and probes sequences and the Real Time PCR conditions for the amplification of the genetic markers of typical ETEC: the plasmid-located genes *lt*, coding for the heat-labile enterotoxin (LT), and *st,* coding for the heat-stable (ST) enterotoxin. The present procedure includes primers and probes targeting both STh and STp coding genes.

The protocol is based on the 5'-nuclease PCR assay. The primers and probes targeting the *lt* and *st* genes have been described by Liu et al. (2013) and their sequences are reported in **Table 1**.

The nature of the Reporter and Quencher is not indicated, as it may depend on the Real Time PCR apparatus available in the laboratory.

Table 1. DNA sequence and characteristics of the primers and probes used for the detection of ETEC.

Target gene	Primer/Probe name	Forward Primer, Reverse Primer and Probe sequences (5'-3')	Amplicon size (bp)	Location within sequence	Sequence accession number
lt	LT F	TTCCCACCGGATCACCAA	62	17201-17218	CP000795.1
	LT R	CAACCTTGTGGTGCATGATGA		17242-17262	
	LT probe	CTTGGAGAGAAGAACCCT		17220- 17237	
st human	ST Fh	GCTAAACCAGYAGRGTCTTCAAAA	147	94199-94222	FN822745.1
	ST Rh	CCCGGTACARGCAGGATTACAACA		94345-94322	
	ST Probeh	TGGTCCTGAAAGCATGAA		94279-94296	
st porcine	ST Fp	TGAATCACTTGACTCTTCAAAA	136	57204-57183	FN649417.1
	ST Rp	GGCAGGATTACAACAAAGTT		57069-57088	
	ST Probep	TGAACAACACATTTTACTGCT		57112-57092	

An internal amplification control (IAC) must be run simultaneously to identify possible inhibition of the test samples.

The amplification conditions will depend on the system used and will refer to the instructions supplied with the instrument and the kit of choice, and should be set up in each laboratory. However, standard reaction conditions together with a two-steps thermal profile applied at EU-RL VTEC are detailed below.

3.1. Real Time PCR conditions:

Master Mix 2X to 1X (usually containing $MgCl_2$ to final concentration of 3mM)

Primer Fwd	500 nM
Primer Rev	500 nM
Probe	200nM
DNA	X (2 μl of DNA purified from 1 ml of culture can be sufficient)
Water	to final volume

The primers and probes have been evaluated at the EU-RL VTEC with a Corbett Rotorgene, by using the following basic two steps thermal profile:

95°C 10 minutes
35-40 cycles of:

- 95°C 15 seconds
- 58°C 60 seconds

4. References

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