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The objective of this study was to develop and validate fast and reliable real-time PCR based methods for detection of VTEC and *Escherichia coli* O157 in raw milk from cows within 20 hours and to use the methods to obtain information about the occurrence of VTEC and *E. coli* O157 in samples taken from bulk milk tanks on Danish farms. An aditional aim was to determine the quantitative levels of *E. coli* in milk samples.

Raw milk from cows may be contaminated with verocytotoxin producing *E. coli* (VTEC) including serogroup O157 (VTEC O157). To overcome this hazard, milk is usually heat treated before it is used for production of dairy products. Despite the risk of diseases many consumers choose to drink unpasteurized milk and eat dairy products made from minimally heat treated milk, e.g. soft cheeses. A safe production of dairy product made from minimally heat treated milk requires that the milk is free for VTEC.

Materials and Methods

Real-time PCR based detection: Twenty-five millilitre milk samples were incubated in 225 ml of tryptic soy broth supplemented with novobiocin at 8 mg/L (TSBn) for 16 hours at 37ºC for non-specific enrichment. DNA was isolated from 1 ml of enrichment culture using the kit MagneSil® KF, Genomic System (Promega Corporation, USA). The purified DNA was analyzed for genes specific for *vtx*1, *vtx*2, *eae*, and *E. coli* O157 (*rfb*E), respectively, by real-time PCR assays based on dual-labeled probes (1). An internal amplification control (IAC) was included to ensure that no false negative PCR reactions was due to the presence of PCR inhibitors in the purified DNA samples. Samples that were *vtx* real-time PCR positive were further analyzed for genes specific for *E. coli* serogroup O26 and O111 following the technical specification issued by CEN (2). Real-time PCR was performed on a Rotor-Gene 3000 thermo cycler (Corbett Research, Australia).

Culture methods for detection of VTEC, *E. coli* O157 and *E. coli* : The milk samples were investigated for *E. coli* O157 using the method described in ISO 16654:2001 (2). The *E. coli* O157 analysis was performed using *E. coli* O157 antibody coated magnetic beads (Dynabeads® anti-*E. coli* O157, Invitrogen) and a BeadRetriver instrument (Invitrogen). Sorbitol MacConkey (SMAC) agar was used as the secondary isolation medium.

VTEC was isolated from *vtx* real-time PCR positive samples by seeding the primary enrichment cultures on TBX (Tryptone Bile Agar with X-Glucuronide) agar and investigate between 10 and 50 colonies by real-time PCR using a pooling strategy, where material from 10 colonies were investigated in one pool. Pure *vtx* positive isolates were verified as *E. coli* using standard morphological and biochemical testing and tested for genes encoding verocytotoxin production (*vtx*1 and *vtx*2) and *eae*. The isolated strains were serotyped by Statens Serum Institut, Copenhagen, Denmark.

Attempts were made to isolate *E. coli* O157 from real-time PCR positive samples. Isolation was performed by seeding the enrichment culture directly on to blood agar plates or by applying the isolation procedure described in ISO 16654:2001. Presumptive *E. coli* O157 was verified using slide agglutination tests, and the pure *E. coli* O157 isolates were tested for genes encoding verocytotoxin production (*vtx*1 and *vtx*2), *eae* and O157 by real-time PCR analysis (1).

Generic *E. coli* was enumerated using Petrifilm™ count plates from 3M™.

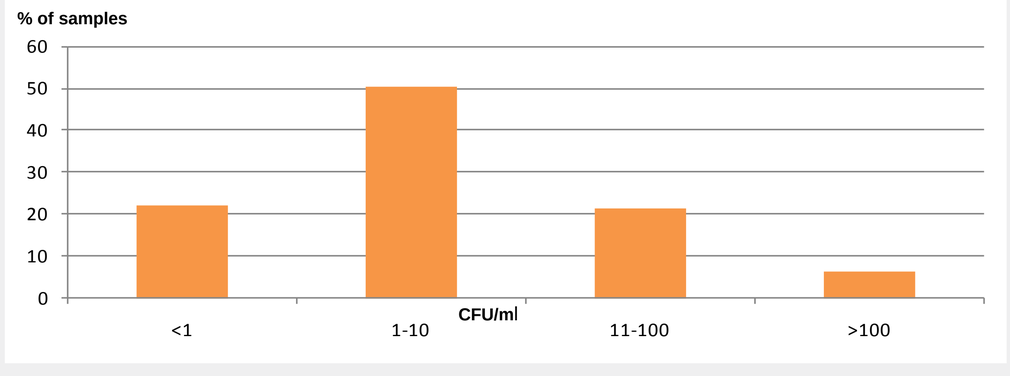
Validation of the real- time PCR detection methods against the reference culture method: A total of 60 milk samples taken from bulk tanks on a Danish dairy were used to validate the real-time PCR based methods. Thirty of the samples were artificially inoculated (spiked) with VTEC O157 at low (~5 CFU) or high (~50 CFU) levels. The 30 spiked samples were made by inoculating 15 samples with 50 CFU of the target organism (high level) and 15 samples with 5 CFU of the target organism (low level). The samples were spiked with 5 different VTEC O157 strains (EDL 933, E32511, two bovine, and one human clinical isolate). Each strain was used to inoculate 2 x 3 milk samples at high and low levels, respectively. The spiking cultures were prepared from overnight TSB cultures (37°C) stored at 4°C for 24 hours by dilution in 0.9 % NaCl and added to 25 ml of milk. The exact inoculation levels were determined by spreading known volumes of the inoculated dilution on blood agar in triplicates. The spiked milk samples were stored at 4°C for 24 hours before being analysed by the reference culture methods and the real-time PCR based methods.

Survey on VTEC and *E. coli* O157 in raw milk from farms: A total of 312 samples were obtained from Danish dairy farms in the period from September to December 2008. The analysis was initiated within 48 hours after sampling. The samples were analyzed with the described real-time PCR methods and further analyzed as described above.

Results and discussion

Generic E. coli:

The level of generic *E. coli* (CFU/ml) in the 312 analyzed samples of raw milk from bulk tanks on Danish farms is shown in the bar chart. Half of the samples were contaminated at the level 1-10 CFU/ml.



Conclusions:

- **The real-time PCR based methods for detection of VTEC and *E. coli* O157 in raw milk from cows were robust and had specificities and sensitivities that were equal to the standard ISO *E. coli* O157 reference method**
- **The real-time PCR based prevalence of VTEC in raw cow's milk from bulk tanks was19.6%**
- **VTEC was isolated from two samples (0.6%)**
- ***E. coli* O157 was isolated from 6.4% of samples but none of these were VTEC**

Validation study:

The results of the validation studies are summarized in the Table. There were full agreement between the results of the *E. coli* O157 real-time based method and the ISO 16654:2001 method; the 30 spiked samples were positive and the 30 non-spiked samples were negative*. Similarly, the *vtx*1 and *vtx*2 real-time PCR assays gave the expected results. The observed Ct values in the spiked samples were consistent and only marginal differences were observed between the Ct values in the samples that were spiked with high and low levels of VTEC O157. The amplifications of the IAC control indicated that the analysed DNA preparations were free of PCR inhibition. These data indicate that the real-time PCR based methods are robust, and that the performance of the methods are satisfactory and equal to the performance of the reference culture method for detection of *E. coli* O157.

Spiking level	No. of samples	Real-time PCR vtx1/vtx2 and O157	Ct values (SD)			O157 ISO culture method
		No. positive	*vtx*1	*vtx*2	O157	No. positive
High ~50 CFU	15	15	19.4 (2.6)	20.1 (2.3)	19.2 (1.8)	15
Low ~5 CFU	15	15	20.8 (2.1)	21.4 (2.5)	20.8 (2.7)	15
Unspiked samples	30	0*				0

***A few of the analyzed non-spiked milk samples generated weak positive PCR signals, all with Ct values of >30**

Raw milk survey:

The PCR assays were used to investigate 312 milk samples from dairy farms for the occurrence of the genes *vtx1, vtx2, eae* and O157; given as percentage of positive samples in the table below:

Gene	vtx	vtx1	vtx2	vtx 1 + vtx2	eae	vtx + eae	O157	vtx + O157
% positive	19.6	5.6	10,6	3.5	32.7	11.9	8.0	1.3
Ct mean		27.9	29.8		22.6		22.8	
SD		5.2	6.2		4.6		5.7	

The *vtx* positive samples were analysed for O26 and O111 specific genes; three samples were O26 positive whereas none of the samples were O111 positive. The obtained Ct values for naturally infected samples were gennerally higher than the Ct values generated in the validation study.

The VTEC isolation protocol was applied on each of the 61 *vtx* positive enrichment broths. A minimum of 10 and up to 50 colonies were tested for *vtx*. The apporach yielded VTEC isolates from two samples; these were *vtx*2 postive, *eae* negative and of serotype O116:H- and O126:H20.

Twenty five (8.0%) of the samples were positive for *E. coli* O157 with the real-time PCR based method. Strains of *E. coli* O157 were isolated from 20 (6.4%) of the samples by investigating subcultures on blood agar. It was not possible to culture verify the remaining five samples when the enrichment broths were investigated in accordance with the ISO protocol for *E. coli* O157 isolation. The PCR and culture positive samples generated an average Ct value of 20.3 (SD = 3.1), whereas the PCR positive but culture negative samples all had Ct values above 31. The 20 isolated *E. coli* O157 strains all tested negative for verocytotoxin encoding genes by PCR analysis. The high prevalence of verocytotoxin negative *E. coli* O157 emphasizes the need for isolation and further characterization of *E. coli* O157 if the purpose of the analysis is to ensure that the samples are free of human pathogenic VTEC O157.

This study shows that real-time PCR assys are efficient for screening of raw cow's milk for VTEC, but also highlights the difficulties in obtaining isolates from PCR screening positive samples. The results indicate that the prevalence of VTEC, including serogroup O157 is low in Danish milk, but genes encoding verocytotoxin and other VTEC associated genes are frequently found. The study also shows that non-pathogenic *E. coli* O157 prevails in raw milk in Denmark.

References:

1. Breum SØ, Boel J. (2010). Int J Food Microbiol. 141, 90-6. 2. Anonymous (2001). Microbiology of food and animal feeding stuffs — horizontal method for the detection of *Escherichia coli* O157. ISO 16654: 2001. 3) Anonymous. Microbiology of food and animal feed -- Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens -- Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) belonging to O157, O111, O26, O103 and O145 serogroups. ISO/PRF TS 13136

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