Year: 2012

Application of a real-time PCR-based system for monitoring of O26, O103, O111, O145, and O157 Shiga toxin-producing Escherichia coli in cattle at slaughter

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Abstract: Kotproben von 573 geschlachteten Rindern im Alter von drei bis 24 Monaten wurden in sieben Schlachthöfen gesammelt. Nach Anreicherung (mTSB mit Novobiocin) wurden die Proben mittels real-time PCR zunächst auf stx untersucht und, falls positiv, mittels serogruppenspezifischer PCR auf die Top-Fünf STEC-Serogruppen O26, O103, O111, O145 und O157 getestet. Von 563 Proben mit auswertbarem Resultat waren 74.1% positiv für die stx-Gene. In diesen konnten die Serogruppen O145, O103, O26, O157 und O111 in 41.9%, 25.9%, 23.9%, 7.8% respektive 0.8% detektiert werden. Aus 95 O26-, 166 O145- und 30 O157-PCR-positiven Proben konnten mittels Kolonienhybridisierung nach immunomagnetischer Anreicherung 17 O26-, 28 O145- und 12 O157-Stämme isoliert werden. Die 17 O26-Stämme waren eae-positiv, aber nur neun davon besassen stx (acht stx1 und einer stx2). Von den 28 O145-Stämmen waren zehn eae-positiv einschliesslich vierer Stämme, die stx1 oder stx2 besassen, während 18 negativ waren für stx und eae. Fünf der 12 O157-Stämme hatten stx2 und eae und wurden identifiziert als STEC O157:H7/H-. Die anderen sieben O157-Stämme waren negativ für stx und eae oder nur eae-positiv. Shiga-toxin-Gene und die Top-Fünf STEC-Serogruppen können bei jungen Rindern bei der Schlachtung in der Schweiz häufig detektiert werden, aber die Isolationsrate von Stämmen ist tief und nur wenige der Stämme zeigen ein Virulenzspektrum von humanpathogenen STEC. Summary Fecal samples were collected from 573 slaughtered cattle aged between three and 24 months in seven abattoirs. After enrichment (mTSB with novobiocin), samples were screened by real-time PCR for first stx and if positive, tested for the top-five STEC serogroups using PCR assays targeting genes specific for serogroups O26, O103, O111, O145, and O157. Of 563 samples with available results, 74.1% tested positive for stx genes. Amongst them, the serogroups O145, O103, O26, O157, and O111 were detected in 41.9%, 25.9%, 23.9%, 7.8%, and 0.8%, respectively. From 95 O26, 166 O145, and 30 O157 PCR-positive samples, 17 O26, 28 O145, and 12 O157 strains were isolated by colony hybridization after immunomagnetic separation. The 17 O26 strains were eae-positive but only nine strains harbored stx (eight possessing stx1 and one stx2). Of the 28 O145 strains ten were eae-positive including four harboring stx1 or stx2, whereas 18 were negative for stx and eae. Five of the 12 O157 strains harbored stx2 and eae, did not ferment sorbitol, and were identified as STEC O157:H7/H-. The other seven O157 strains were negative for stx and eae or positive only for eae. Shiga toxin genes and the top-five STEC serogroups were frequently found in young Swiss cattle at slaughter, but success rates for strain isolation were low and only few strains showed a virulence pattern of human pathogenic STEC.

Other titles: Anwendung eines real-time PCR-basierenden Systems für die Überwachung von O26, O103, O111, O145 und O157 Shiga-toxin-produzierenden Escherichia coli bei Rindern bei Schlachtung

Dissertation
Published Version

Originally published at:
Hofer, Eveline. Application of a real-time PCR-based system for monitoring of O26, O103, O111, O145, and O157 Shiga toxin-producing Escherichia coli in cattle at slaughter. 2012, University of Zurich, Vetsuisse Faculty.

Institute for Food Hygiene and Safety
of the Vetsuisse Faculty, University of Zürich

Director: Prof. Dr. Roger Stephan

Scientific supervision was provided by
PD Dr. Claudio Zweifel

Application of a real-time PCR-based system for monitoring of O26, O103, O111, O145, and O157 Shiga toxin-producing *Escherichia coli* in cattle at slaughter

Inaugural-Dissertation

to be awarded the Doctoral Degree of the
Vetsuisse Faculty, University of Zürich

submitted by

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Prof. Dr. Roger Stephan, Referent

Zürich 2012

Application of a real-time PCR-based system for monitoring of O26, O103, O111, O145, and O157 Shiga toxin-producing *Escherichia coli* in cattle at slaughter

Running head: O26, O103, O111, O145, and O157 STEC in cattle

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Accepted for publication in Zoonoses and Public Health

In fulfillment of the doctoral thesis of Eveline Hofer

Contents

Summary

Fecal samples were collected from 573 slaughtered cattle aged between three and 24 months in seven abattoirs. After enrichment (mTSB with novobiocin), samples were screened by real-time PCR first for *stx* and if positive, tested for the top-five STEC serogroups using PCR assays targeting genes specific for serogroups O26, O103, O111, O145, and O157. Of 563 samples with available results, 74.1% tested positive for *stx* genes. Amongst them, the serogroups O145, O103, O26, O157, and O111 were detected in 41.9%, 25.9%, 23.9%, 7.8%, and 0.8%, respectively. From 95 O26, 166 O145, and 30 O157 PCR-positive samples, 17 O26, 28 O145, and 12 O157 strains were isolated by colony hybridization after immunomagnetic separation. The 17 O26 strains were *eae*-positive but only nine strains harbored *stx* (eight possessing *stx*1 and one *stx*2). Of the 28 O145 strains, ten were *eae*-positive including four harboring *stx*1 or *stx*2, whereas 18 were negative for *stx* and *eae*. Five of the 12 O157 strains harbored *stx*2 and *eae*, did not ferment sorbitol, and were identified as STEC O157:H7/H$^-$. The other seven O157 strains were negative for *stx* and *eae* or positive only for *eae*. Shiga toxin genes and the top-five STEC serogroups were frequently found in young Swiss cattle at slaughter, but success rates for strain isolation were low and only few strains showed a virulence pattern of human pathogenic STEC.

Keywords: Shiga toxin-producing *Escherichia coli*; top-five STEC serogroups; cattle; real-time PCR; strain isolation.

Impacts

- Using a real-time PCR-based system, Shiga toxin genes were frequently found in young Swiss cattle at slaughter (>70%) and the top-five STEC serogroups, especially O26, O103, and O145, were also detected.
- Success rates for strain isolation from bovine fecal samples were low and only a few of the isolated strains showed a virulence pattern of human pathogenic STEC.
- The value of a STEC screening aiming at the top-five serogroups with the described approach in slaughtered cattle remains questionable, especially as long as fast and reliable isolation of strains is not warranted.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are responsible for human gastrointestinal illnesses, including non-bloody or bloody diarrhea (Kaper et al., 2004; Karch et al., 2005). These conditions may be complicated by neurological and renal sequelae, including the life-threatening hemolytic-uremic syndrome (HUS), which is the main cause of acute renal failure in children (Tarr et al., 2005). Most outbreaks and sporadic cases of bloody diarrhea and HUS have thereby been attributed to STEC O157:H7. STEC have been isolated from the intestines of a variety of healthy domestic and wild animals, but ruminants, mainly cattle, are regarded as the principal reservoir of STEC (Caprioli et al., 2005; Karmali et al., 2010). The majority of human infections are correlated with the consumption of fecally contaminated food and water, but transmission by animal-to-person and person-to-person contact has also been reported. STEC are characterized by the production of one or more Shiga toxins: Stx1, Stx2, and variants (Johannes and Römer, 2010). Pathogenic STEC tend to feature Stx2 and intimin mediating attaching and effacing lesions on intestinal epithelial cells (Friedrich et al., 2002; Brooks et al., 2005; Orth et al., 2007).

In the European Union (EU), Directive (EC) 99/2003 on the monitoring of zoonoses and zoonotic agents requires monitoring for STEC along the food chain. In 2009, the European Food Safety Authority (EFSA) published technical specifications for STEC surveys to ensure EU-wide standards (EFSA, 2009). According to a risk-based sampling strategy, these specifications aim at estimating the prevalence in young cattle (and sheep) at slaughter. Monitoring for STEC belonging to serogroups O26, O103, O111, O145, and O157, the so-called top-five serogroups (Beutin, 2006; Johnson et al., 2006), is thereby proposed (EFSA, 2009). The ISO 16654:2001 method is recommended for detection of *Escherichia* (*E.*) *coli* O157:H7, and the use of draft CEN TC275/WG6 standard (submitted to ISO for evaluation) is proposed for detection of serogroups O26, O103, O111, and O145. This standard is based on real-time PCR-based screening followed by a strain isolation step. Primers listed for use in PCR assays (*stx*, O26,

O103, O111, O145, O157) were developed by Perelle and colleagues (Perelle et al., 2003; Perelle et al., 2004; Perelle et al., 2005). Based on the same primers, the closed GeneDisc® multiplex real-time PCR system (Pall GeneDisc Technologies, Bruz, France), which was recently evaluated by Beutin et al. (2009), is commercially available.

The aims of the present study were (i) to screen bovine samples by real-time PCR for Shiga toxin genes and if positive for the top-five STEC serogroups O26, O103, O111, O145, and O157, and (ii) to isolate strains from O26, O145, and O157 PCR-positive samples. Thus, application and challenges of a real-time PCR-based system for monitoring of O26, O103, O111, O145, and O157 STEC in slaughtered cattle were evaluated.

Materials and methods

Abattoirs and sampling

This study was based on investigation carried out within an 11-month period (January to November 2011) in seven abattoirs (A-G). Abattoirs were selected to get a widespread sample distribution throughout Switzerland and the five biggest abattoirs slaughtering cattle were included (A-E). Two smaller abattoirs located in southern Switzerland (F, G) were also included in the study because only few cattle from southern Switzerland were slaughtered in the abattoirs A-E. The present survey was part of a project of the Federal Veterinary Office evaluating the abattoir level as data source for monitoring programs.

Cattle aged between three and 24 months were sampled. Such animals tend to shed STEC more frequently and EFSA recommends investigating cattle within this age spectrum (EFSA, 2009). The minimum number of samples was calculated with the assumption of an infinite population size, a prevalence of 50%, a desired confidence level of 95% and an accuracy of 5%. In total, 573 bovine fecal samples were examined. From animals delivered together to the

abattoir, not more than one animal was sampled. Fecal samples were preferred to hide samples to avoid potential cross contaminations and for practical reasons as a precedent pilot survey showed that they were easier to obtain. Fecal samples were collected from the large intestine using swabs after evisceration. After opening the large intestine and sample collection, swabs were placed in sterile bags and transported to the laboratory chilled.

Screening for Shiga toxin genes and serogroups O26, O103, O111, O145, and O157 by real-time PCR

Upon arrival at the laboratory, samples were enriched in modified tryptic soy broth (mTSB, CMO983, Oxoid AG, Pratteln, Switzerland) with 16 mg/l novobiocin (novobiocin sodium, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Therefore, 20 ml mTSB were added to each of the bags with the swabs, the bags were stomachered and incubated for 12-18 h at 37 °C. From 50 µl of the enrichment broth a lysate was made (lysis tube, Pall GeneDisc Technologies, Bruz, France). All samples were screened by real-time PCR in a first step for *stx* and in a second step (only the *stx* positive samples) for the serogroups O26 (*wzx*), O103 (*wzx*), O111 (*wbd*I), O145 (*ihp*1), and O157 (*rfb*E) with an open LightCycler-based system (LightCycler 2.0, Roche Diagnostics AG, Rotkreuz, Switzerland) or the commercially available, closed GeneDisc® system (Pall GeneDisc Technologies). Primers and probes (Table 1) were the same for both approaches and are in accordance to the requirements of CEN TC275/WG6 standard. The commercially available GeneDisc® system allowed simultaneous detection on multiple targets in 36 reaction microchambers preloaded with the necessary reagents (Beutin et al., 2009), and was performed according the manufacturer's instructions. For the LightCycler system primers and probes were purchased from Microsynth (Microsynth AG, Balgach, Switzerland). Amplification was performed with 20 µl of reaction mixture containing 1 x concentration of QuantiFast™ Multiplex PCR+R Kit (Qiagen AG, Hombrechtikon, Switzerland), 1 µM of each primer, 200 nM

of each probe, and 2 µl of template DNA. Amplification conditions for the LightCycler assays were 5 min at 95 °C followed by 45 consecutive cycles of first 30 s at 95 °C and then 30 s at 60 °C, and a cooling step of 1 min at 40 °C.

Strain isolation

From samples positive for *stx* and serogroups O26, O145, or O157, strain isolation was attempted. Therefore, 1 ml of the first enrichment broth was incubated in 10 ml of brain heart infusion (BHI) broth (CM1135, Oxoid AG) overnight at 37 °C. One milliliter was used for the respective (O26, O145, O157) immunomagnetic separation according to the manufacturer's instructions (Dynabeads® EPEC/VTEC, Invitrogen Corporation, Zug, Switzerland). Diluted aliquots were plated onto sheep blood agar (Difco™ Columbia Blood Agar Base EH, Becton Dickinson AG, Allschwil, Switzerland; 5% sheep blood SB055, Oxoid AG) and incubated overnight at 42 °C. Probes for colony hybridization were prepared by labeling serogroup-specific amplicons (PCR products generated with the same primers that were used for the serogroup-specific PCR assays) with PCR DIG Probe Synthesis Kit (Roche Diagnostics AG). Colony hybridization was performed according to the manufacturer's protocol for hybridization with DIG-labeled probes (Roche Diagnostics AG). Briefly, colonies were transferred to a nylon membrane and lysed following standard methods. After washing, cross-linking, and pre-hybridization in DIG-Easy-Hyb buffer for about 60 min at 42 °C, hybridization with serogroup-specific probes was performed overnight at 42 °C. After washing and blocking, the presence of labeled probe was detected with an alkaline phosphatase-conjugated antibody detection kit and NBT/BCIP stock solution. One to four positive colonies were picked from the sheep blood agar and the respective serogroup was confirmed as described above (LightCycler 2.0). Isolated strains were then examined for the presence of *stx*1, *stx*2, *eae* encoding intimin, and *hly*A

encoding EHEC hemolysin (Schmidt et al., 1995; Møller Nielsen and Thorup Andersen, 2003; Perelle et al., 2004).

Results

Screening for Shiga toxin genes and serogroups O26, O103, O111, O145, and O157 by real-time PCR

Of the 573 fecal samples collected from slaughtered cattle aged between three and 24 months, Shiga toxin genes (*stx*) were detected by real-time PCR in 417 samples. The remaining samples tested negative (n=146) or were inhibited (n=10) in the LightCycler-based system. Thus, of 563 samples with available results, 74.1% tested positive for *stx*. The proportion of positive samples within the abattoirs ranged from 61.4% at abattoir D to 83.7% at abattoir B (Table 2). The non-analyzable results for both *stx*- and serogroup-specific assays were due to inhibition of PCR reactions in the LightCycler-based system, a problem not evident in the GeneDisc® system. Amongst the *stx*-positive samples, serogroups O145, O103, O26, O157, and O111 were detected by real-time PCR in 41.9%, 25.9%, 23.9%, 7.8%, and 0.8%, respectively (Table 3). The proportion of samples positive for serogroups O145, O103, O26, and O157 thereby ranged from 31.1% to 76.0% (abattoir F), 9.0% to 50.6% (abattoir E), 12.5% to 31.2% (abattoir E), and 1.3% to 32.0% (abattoir F), respectively. Only a few samples from two abattoirs (C, E) tested positive for serogroup O111. In 136 samples one serogroup, in 67 samples two serogroups, and in 22 samples three serogroups were simultaneously found. Six samples tested positive for four serogroups (data not shown).

Strain isolation

From 95 O26, 166 O145, and 30 O157 PCR-positive samples, 17 O26, 28 O145, and 12 O157 strains were isolated by colony hybridization after immunomagnetic separation. Thus, success

rates for isolation were 17.9% for O26 strains, 16.9% for O145 strains, and 40.0% for O157 strains. The 17 O26 strains were all *eae*-positive, but only nine of them harbored *stx* genes (Table 4). Eight *stx*-positive strains thereby possessed *stx*1 and one strain harbored *stx*2. In addition, 15 O26 strains, including all *stx*-positive strains, harbored *hly*A. Of the 28 O145 strains, more than half were negative for *stx*1, *stx*2, *eae*, and *hly*A (Table 4). The other ten O145 strains were *eae*-positive but only four of them harbored *stx* genes. Two O145 strains were thereby positive for *stx*1 and two for *stx*2. Furthermore, five of the 12 O157 strains harbored *stx*2 and *eae* and did not ferment sorbitol (Table 4). The other seven O157 strains were negative for *stx* and *eae* (three strains) or tested positive only for *eae* (four strains). Six strains were sorbitol fermenting, whereas one *eae*-positive strain was sorbitol non-fermenting. Of the 12 O157 strains, seven strains, including four of the five *stx*2-positive strains, harbored *hly*A. Moreover, three of the five *stx*2-positive/*eae*-positive O157 strains and the sorbitol non-fermenting, *stx*-negative/*eae*-positive O157 strain agglutinated with anti-H7 antiserum.

Discussion

To evaluate the application of a real-time PCR-based system for monitoring of O26, O103, O111, O145, and O157 STEC in young cattle at slaughter, 573 samples were screened for Shiga toxin genes and for the top-five STEC serogroups. Shiga toxin genes were found in almost three quarters (74%) of the fecal samples with available results. More than one third of the *stx*-positive samples tested negative for the top-five serogroups. Comparisons of our results with literature data on the occurrence of STEC and certain serogroups in cattle are hampered by differences in e.g. the target animal population, the sampling strategy, or the detection procedure, but high *stx* detection rates (up to 71%) have also been reported in other studies (Cerqueira et al., 1999; Pradel et al., 2000; Kobayashi et al., 2001). Statistical analysis (Table 2) showed that *stx* prevalence was significantly influenced by age of slaughtered animals and abattoir. To evaluate

the reasons of the varying serogroup prevalence between the abattoirs, further investigations are required, especially in view of the high O157 and O145 prevalence in one abattoir.

With regard to serogroup distribution, serogroups O26, O103, and O145 were more frequently detected in the present study than O157 and in particular O111. Other studies also frequently reported *E. coli* O26 and O103 in cattle, whereas serogroup O145 was less prevalent (Jenkins et al., 2003; Joris et al., 2011). As expected for Switzerland (Al-Saigh et al., 2004; Zweifel et al., 2005), serogroup O157 was not predominant in our bovine samples. A recent study (Käppeli et al., 2011a) investigating human infections with non-O157 STEC in Switzerland reported that serogroup O26 was most frequently isolated (28.9%) followed by O145 (10.3%), O103 (6.2%), and O121 (6.2%). In contrast to other countries (Elliot et al., 2001; Brooks et al., 2005), serogroup O111 was rarely detected. Besides, it must be considered that STEC of other serogroups as e.g. O91, O113, O121, or O128 play an important role as a cause of human disease in certain regions (Pradel et al., 2000; Beutin et al., 2004; Brooks et al., 2005; Bettelheim, 2007).

To assess the potential pathogenicity of STEC for humans, strain isolation and characterization is of central importance. Simultaneous detection of *stx* and a certain serogroup in a sample by PCR does not necessarily implicate that these properties are featured by the same strain. A reliable conclusion about STEC shedding in cattle or a potential health hazard can therefore not be drawn based on such results. This fact is also emphasized by the finding of two or more of the top-five STEC serogroups in our *stx*-positive samples.

For strain isolation, we selected O26, O145, and O157 PCR-positive samples. These serogroups were selected due to our screening results and their relevance in human disease (CDC, 2007; EFSA/ECDC, 2011; Käppeli et al., 2011a,b). Using colony hybridization after immunomagnetic separation, the isolation rate was less than 18% for O26 and O145 strains and about 40% for O157 strains. A great deal of time and effort was thereby required to isolate the 28

O145, 17 O26, and 12 O157 strains. Data directly comparable to our results are lacking in the literature. Barlow and Mellor (2010) used a similar screening approach but serogroup-specific strain isolation by immunomagnetic separation was restricted to only few samples. Basically, different isolation procedures have been proposed for STEC or *E. coli* of certain serogroups, but fast and reliable strain isolation from animals and food remains a continuous problem (Jenkins et al., 2003; Bettelheim, 2007; Barlow and Mellor, 2010; Fratamico et al., 2011; Verstrate et al., 2012). Noteworthy selective agars for isolation of O26, O103, O111, O145, and sorbitol-fermenting O157 *E. coli* have recently been described (Possé et al., 2008; Joris et al., 2011).

Only a few of the 57 isolated strains showed a virulence pattern of STEC typically associated with human disease. Twenty-one strains (18 of serogroup O145 and three of serogroup O157) were negative for both *stx* and *eae* genes. Moreover, eight O26, six O145, and four O157 strains harbored *eae* but were lacking *stx*. Cattle thereby constitute an important source of serologically and genetically diverse *eae*-harboring *E. coli* (Blanco et al., 2005). Such strains can undergo ephemeral interconversions via loss and gain of Stx-encoding phages, which leads to different pathotypes. This was already shown for *E. coli* O157 and *E. coli* O26 isolated from human (Bielaszewska et al., 2007; Friedrich et al., 2007; Mellmann et al., 2008). Nevertheless, in a recently published study (Stephan et al., 2009) two *stx*-negative/*eae*-positive *E. coli* strains isolated from a cattle and pig could not be converted to STEC using stx_2-phages even though each of the strains had an intact phage integration site. Further investigations are required to elucidate the role of animal *stx*-negative/*eae*-positive *E. coli* belonging to STEC serogroups in their natural source and in human infections.

Shiga toxin genes were only detected in about one third of our isolated strains. The proportion of STEC amongst isolates was 14% for O145 strains, 42% for O157 strains, and 53% for O26 strains. Pearce et al. (2006) also reported that carriage of *stx* in *E. coli* O145 shed by Scottish cattle was rare (4.7%), whereas 49% of *E. coli* O26 isolates possessed *stx*. The

infrequent occurrence of Shiga toxin genes in *E. coli* O145 might partly explain that studies investigating STEC shedding in cattle mainly detected serogroup O145 in low frequencies or not at all (Pradel et al., 2000; Kobayashi et al., 2001; Hornitzky et al., 2002; Blanco et al., 2004b). Amongst our STEC strains that were all *eae*-positive, STEC O26 mainly harbored only *stx*1, STEC O145 either *stx*1 or *stx*2, and STEC O157 only *stx*2. Bovine STEC O26 can harbor *stx*1 and *stx*2, but in various studies *stx*1 predominated (Hornitzky et al., 2002; Blanco et al., 2004b; Pearce et al., 2006; Bardiau et al., 2009). With regard to STEC strains associated with human illnesses in Switzerland (Käppeli et al., 2011a,b), STEC O26 mainly harbored either *stx*1 or *stx*2 and *eae*, STEC O145 mainly possessed *stx*2 and *eae*, and STEC O157 featured *stx*2 alone or in combination with *stx*1 and also harbored *eae*, a pattern typically found in *E. coli* O157:H7 (Blanco et al., 2004a; Karch et al., 2005; Gilmour et al., 2009). STEC O26 and especially STEC O145 isolated from sporadic cases of human ilnesses are highly heterogeneous as evidenced e.g. by their *stx* genotypes, plasmid profiles, or plasmid gene composition (Karch et al., 2005).

In summary, using a real-time PCR-based system, Shiga toxin genes were frequently found in fecal samples from young Swiss cattle at slaughter and the top-five STEC serogroups, especially O26, O103, and O145, were also detected in *stx*-positive samples. Real-time PCR proved to be suitable for screening purposes but it must be considered that this does not result in a bacterial isolate. By taking into consideration only the screening results without strain isolation, a distorted impression on the occurrence of STEC O26, O103, O111, O145, and O157 in the cattle population would have resulted. However, fast and reliable isolation of respective strains poses a major challenge. Success rates for strain isolation from our samples were low and only few of the isolated strains showed a virulence pattern of STEC pathogenic to humans. The value of STEC monitoring aimed at the top-five serogroups with the described approach in slaughtered cattle remains therefore challenging. In particular, methods must be improved to obtain more efficiently isolated strains, which are a prerequisite to assess the pathogenic traits.

Acknowledgments

We thank the staff of the abattoirs for their assistance with the collection of data and Sonja Hartnack from the Section of Epidemiology, Vetsuisse Faculty University of Zurich, for her assistance with statistical analysis. This study was partly funded by the Swiss Federal Veterinary Office.

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Table 1. Primers and probes used in this study

Target gene	Forward primer, reverse primer, and probe sequences (5'-3') *	Amplicon size (bp)	Location within sequence	GenBank accession number	Reference
*stx*1	TTTGTYACTGTSACAGCWGAAGCYTTACG	132	878-906	M16625	Perelle et al., 2004
	CCCCAGTTCARWGTRAGRTCMACRTC		983-1008		
	Probe: CTGGATGATCTCAGTGGGCGTTCTTATGTAA		941-971		
*stx*2	TTTGTYACTGTSACAGCWGAAGCYTTACG	128	785-813	X07865	Perelle et al., 2004
	CCCCAGTTCARWGTRAGRTCMACRTC		785-8132		
	Probe: TCGTCAGGCACTGTCTGAAACTGCTCC		838-864		
wzx	CGCGACGGCAGAGAAAATT	135	5648-5666	AF529080	Perelle et al., 2004
(O26)	AGCAGGCTTTTATATTCTCCAACTTT		5757-5782		
	Probe: CCCCGTTAAATCAATACTATTTCACGAGGTTGA		5692-5724		
wzx	CAAGGTGATTACGAAAATGCATGT	99	4299-4323	AY532664	Perelle et al., 2005
(O103)	GAAAAAAGCACCCCCGTACTTAT		4397-4375		
	Probe: CATAGCCTGTTGTTTTAT		4356-4373		
*wbd*I	CGAGGCAACACATTATATAGTGCTTT	146	3464-3489	AF078736	Perelle et al., 2004
(O111)	TTTTTGAATAGTTATGAACATCTTGTTTAGC		3579-3609		
	Probe: TTGAATCTCCCAGATGATCAACATCGTGAA		3519-3548		
*ihp*1	CGATAATATTTACCCCACCAGTACAG	132	1383-1408	AF531429	Perelle et al., 2003
(O145)	GCCGCCGCAATGCTT		1500-1514		
	Probe: CCGCCATTCAGAATGCACACAATATCG		1472-1498		
*rfb*E	TTTCACACTTATTGGATGGTCTCAA	88	348-372	AF163329	Perelle et al., 2004
(O157)	CGATGAGTTTATCTGCAAGGTGAT		412-435		
	Probe: AGGACCGCAGAGGAAAGAGAGGAATTAAGG		381-410		

* In the sequence Y is (C,T), S is (C,G), W is (A, T), R is (A,G), M is (A,C)

Table 2. Detection of *stx* genes encoding Shiga toxins by real-time PCR in bovine fecal samples obtained from different abattoirs

	No. of examined samples	No. of analyzable samples	No. (%) of *stx*-positive samples*
Abattoir A	102	96	67 (69.8%)
Abattoir B	104	104	87 (83.7%)
Abattoir C	104	103	83 (80.6%)
Abattoir D	104	101	62 (61.4%)
Abattoir E	97	97	77 (79.4%)
Abattoir F	36	36	25 (69.4%)
Abattoir G	26	26	16 (61.5%)
Total	**573**	**563**	**417 (74.1%)**

* Prevalence of *stx* was significantly influenced by age of slaughtered animals ($p<0.0001$) and abattoir ($p=0.024$), whereas the interaction between age and abattoir was not significant ($p=0.082$). Analysis was performed using a logistic regression approach with presence or absence of *stx* as outcome variable and abattoir, age and an interaction between age and abattoir as explanatory variables or fixed effects. Model selection was based on Akaike's information criterion with lower values indicating a better model fit. P-values were derived from likelihood ratio tests.

Table 3. Detection of serogroups O26, O103, O111, O145, and O157 by real-time PCR in 417 *stx*-positive, bovine fecal samples

	Serogroup				
	O26	O103	O111	O145	O157
No. of analyzable samples	397	394	384	396	385
No. (%) of samples with detection of the specific serogroup*	95 (23.9%) [A]	102 (25.9%) [A]	3 (0.8%) [B]	166 (41.9%) [C]	30 (7.8%) [D]

* Values with different letters were significantly different (Chi Square test/Fishers exact test, $P<0.05$)

Table 4. Occurrence of *stx* and *eae* genes among 17 isolated O26 strains, 28 isolated O145 strains, and 12 isolated O157 strains

Serogroup	No. of strains	*stx*1	*stx*2	*eae*	*hly*A
O26	8°	+	-	+	+
	6°	-	-	+	+
	2°	-	-	+	-
	1°	-	+	+	+
O145	16°	-	-	-	-
	6°	-	-	+	+
	2°	+	-	+	+
	2°	-	+	+	+
	2°	-	-	-	+
O157	4*	-	+	+	+
	3°	-	-	-	-
	2°	-	-	+	+
	1*	-	+	+	-
	1*	-	-	+	+
	1°	-	-	+	-

° sorbitol fermenting

* sorbitol non-fermenting

Acknowledgements

I would like to express my gratitude to all those who contributed to this work.

Special thanks go to:

Prof. Roger Stephan, Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zürich, for giving me the opportunity to work on this scientific project, for his optimistic support and help when ever needed.

PD Dr. Claudio Zweifel, Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zürich, for his patience, for his help whenever necessary and for the preparation of the main review.

The whole ILS Team for their assistance and creation of a friendly working atmosphere.

All the other PhD and doctoral thesis students for the good times.

My parents Vreni and Hansueli Hofer-Rohner for their care and appreciation.

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