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Pathogenic potential of *Escherichia coli* O157 and O26 isolated from young Belgian dairy calves by recto-anal mucosal swab culturing

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Running headline:

E. coli O157 and O26 in young dairy calves

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Abstract

Aims: The purpose of this study was to investigate the occurrence of *E. coli* O157 and O26 on Belgian dairy cattle farms, the presence of virulence genes in the confirmed isolates, and the association of *E. coli* O26 presence with calf diarrhea.

Methods and Results: In total, 233 recto-anal mucosal swabs (RAMS) were obtained from healthy and diarrheic dairy calves on three farms, each alternately visited three consecutive times. RAMS were analyzed for presence of *E. coli* O157 and O26, and *stx1*, *stx2* and *eae* virulence genes. Overall, 19% of RAMS tested positive for *E. coli* O157, while 31% tested positive for *E. coli* O26. The majority of isolates possessed both *stx* and *eae*, denoting a high pathogenic potential to humans. While both serogroups persisted at farm level, persistence within the same animal over time appeared to be relatively rare. Interestingly, *E. coli* O26 was already abundantly present at a younger age compared to *E. coli* O157. Calf diarrhea could not be associated with presence of *E. coli* O26.

Conclusions: Young dairy calves are important on-farm reservoirs of potentially pathogenic *E. coli* O157 and O26. A role of *E. coli* O26 in calf diarrhea could not be confirmed.

Significance and Impact of Study: O157 and O26 are responsible for the majority of human STEC infections. Gaining more epidemiological information regarding their occurrence and persistence on cattle farms will contribute to a better understanding of STEC ecology and risk of human transmission.

Key words:

Escherichia coli, O157, O26, dairy cattle, recto-anal mucosal swab, persistence, Belgium

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are important zoonotic pathogens of public health concern, responsible for severe clinical symptoms in humans, such as hemorrhagic colitis (HC) and the possibly life-threatening hemolytic-uremic syndrome (HUS) (Gonzalez and Cerqueira, 2020). Ruminants, mainly cattle, are generally recognized as the primary natural reservoir and excrete these bacteria in their feces without showing clinical signs. Consequently, human infection occurs predominantly through the consumption of foodstuffs contaminated with bovine feces (Persad and LeJeune, 2014).

The most important virulence factor of STEC is the production of Shiga toxins (Stx), encoded by the *stx1* and/or *stx2* genes. Additionally, some STEC strains also possess the *eae* gene, encoding for intimin, involved in the formation of typical attaching-effacing (A/E) lesions (Tozzoli and Scheutz, 2014). These strains have also been referred to as enterohemorrhagic *E. coli* (EHEC). However, the EHEC terminology, based on the opinions from the 1980s that only certain types of STEC were able to cause severe disease in humans, is now considered obsolete by EFSA (Koutsoumanis *et al.*, 2020). Therefore, the STEC nomenclature will be used in this manuscript. Enteropathogenic *E. coli* (EPEC) are characterized by production of A/E lesions, but do not produce Stx and may, or may not, belong to the same serogroups as STEC, such as O26 (Eichhorn *et al.*, 2015).

The most frequent and pathogenic STEC worldwide include O26, O45, O103, O111, O121, O145 and O157 (referred to as the top 7 serogroups) (Fratamico *et al.*, 2017). Additionally, other novel hybrid pathogenic strains can emerge from time to time, like the O104:H11 strain during the German outbreak in 2011 (Bielaszewska *et al.*, 2011) and the O80:H2 outbreaks in France (Cointe *et al.*, 2018) and Switzerland since 2013 (Nüesch-Inderbinen *et al.*, 2018).

Nevertheless, recent data has indicated that O157 and O26 were responsible for more than half of the total number of confirmed human cases (34.5% and 16.6%, respectively) where the serogroup was known (EFSA and ECDC, 2019). This highlights the need for both increased epidemiological surveillance as well as intervention strategies that can effectively prevent the entry of these pathogens into the food chain, since specific therapies to combat or prevent human STEC infections are currently still lacking.

STEC O157 generally colonize the lymphoid follicle-dense mucosa of the recto-anal junction (RAJ) in cattle (Naylor *et al.*, 2003), while STEC O26 colonize the bovine large intestine

(van Diemen *et al.*, 2005). Consequently, sampling the RAJ using recto-anal mucosal swabs (RAMS) can be considered a good indicator for presence of both serogroups, since fecal material also adheres to the swab (Rice *et al.*, 2003). Furthermore, it has also been shown that younger animals are more likely to harbor STEC O157 and O26 than older animals (Cobbold and Desmarchelier, 2000; Mellor *et al.*, 2016; Browne *et al.*, 2018). Moreover, although bovine STEC carriage generally occurs asymptotically in adult cattle, some STEC, including O26, have been implicated in calf diarrhea (Fakih *et al.*, 2017).

Therefore, the objectives of the present study were: (i) to assess recto-anal mucosal swabs (RAMS) for detection of *E. coli* O157 and O26 in young dairy calves on three Belgian farms, (ii) to investigate the presence of *stx1*, *stx2* and *eae* virulence genes in the obtained isolates, (iii) to investigate persistence of *E. coli* O157 and O26 in these young calves at farm level, and (iii) to associate presence of *E. coli* O26 with calf diarrhea.

Materials and Methods

Farm and animal selection

Three Belgian dairy cattle farms (A, B and C), situated in East Flanders and rearing animals belonging to the Holstein-Friesian cattle breed, were included in this study carried out between June 2018 and October 2018. Presence of both *E. coli* O157 and O26 at farm level was previously confirmed by sampling of the pen beddings using disposable, liquid-absorbing overshoes, as described previously (Cobbaut *et al.*, 2008). All three selected farms were alternately visited three times, with a time interval of 4-10 weeks, during the summer season in order to reduce the seasonal effect on the prevalence of *E. coli* O157 and O26. All sampled animals were monitored for clinical signs of diarrhea. The average age of all calves sampled was 2 months, ranging between 1 day to 6 months of age. At the first visit, all animals under the age of 6 months were sampled. Hereafter, all newborn animals were included in the study at each subsequent visit, in addition to the remaining animals < 6 months of age.

RAMS collection

Following tail restraint of the animals, RAMS were collected by inserting a sterile cotton prepping ball (Covidien™, IRL) approximately 2 to 5 cm into the anus, and applying several circular motions. After collection, RAMS were placed in individual stomacher bags (Corning® Gosselin™, DE), appropriately labelled, transported on ice to the laboratory, and processed the following morning after overnight storage at 4°C. In total, 233 RAMS were collected: 74 RAMS were obtained from farm A, 71 RAMS from farm B, and 88 RAMS from farm C.

Isolation of *E. coli* O157 and O26

Upon processing, 25 ml of maximum recovery diluent (MRD, Oxoid, UK) was added to each RAMS and homogenized for 1 min with a stomacher blender (Minimix, Interscience).

For *E. coli* O157 detection, 10 ml thereof was added to an equal volume of double-concentrated modified tryptone soy broth (Oxoid, UK), containing 20 mg/ml of novobiocin (Oxoid, UK) (mTSBn), and subsequently incubated for 6 h at 37°C (Cobbaut *et al.*, 2008). Immuno-magnetic separation (IMS) was performed on 1 ml of enrichment broth, using 20 µl of anti *E. coli* O157 Dynabeads (Invitrogen, US), according to the manufacturer's recommendations. The washed beads were then spread-plated onto sorbitol-MacConkey agar supplemented with cefixime (0.05 mg/l) and potassium tellurite (2.5 mg/l) (CT-SMAC) (Oxoid, UK), and incubated for 24 h at 37°C. Following incubation, up to 4 non-sorbitol fermenting (NSF) colonies per plate

with typical morphology were sub-cultured on CT-SMAC for 24 h at 37°C. Finally, suspect *E. coli* O157 colonies were serologically tested for the O157 antigen with an O157-specific latex agglutination assay (Oxoid, UK).

For *E. coli* O26 detection, 10 ml of MRD suspension was added to an equal volume of double-concentrated brilliant green bile lactose broth (Brila broth, Merck, DE) and subsequently incubated for 20 h at 37°C, followed by a selective acid treatment (Verhaegen *et al.*, 2016). Briefly, 2 ml of the enriched broth was centrifuged at $12,000 \times g$ for 3 min. The pellet was resuspended in acidified (pH = 2) tryptone soy broth (TSB, Oxoid, UK) and incubated on a rotary mixer (Labinco L-28, NL) at room temperature for 30 min. The samples were again centrifuged at $12,000 \times g$ for 3 min, and the pellet was resuspended in 1 ml of sterile phosphate buffered saline (PBS, Oxoid, UK). Finally, 100 μ l was plated onto supplemented CHROMagarTM STEC (CHROMagarTM, FR) and incubated for 24 h at 37°C. Ten suspect mauve colonies from each plate were sub-cultured on plate count agar (PCA, Oxoid, UK) for 24 h at 37°C.

DNA extraction, molecular serotyping and virulotyping

All confirmed *E. coli* O157 isolates were individually boiled in 100 μ l of RNase-free water at 95°C for 17 min, followed by centrifugation ($12,000 \times g$, 2 min), while all 10 suspect *E. coli* O26 isolates, originating from the same sample, were first pooled in 100 μ l of RNase-free water and processed in an identical manner. All supernatants were stored at -20°C until use in PCR.

Individual O157 supernatants and pooled suspect O26 supernatants were then tested for the O157 and O26 serogroup by serogroup-specific PCR, respectively, using previously described primers (Possé *et al.*, 2007). If the pooled colonies were positive for the O26 serogroup, all ten individual colonies were subsequently tested for the O26 serogroup. PCR was performed using a PTC-100TM programmable thermal controller (MJ Research, Inc., US). Briefly, PCR conditions for O-serogroup determination (targeting the O-antigen flippase, *wzx*) were 95°C for 40 s, followed by 30 cycles of 95°C for 30 s, 59°C for 45 s, and 72°C for 45 s. The final cycle was followed by an extension step at 72°C for 3 min. Amplification was performed using 25 μ L of PCR mix containing molecular grade RNase-free water, 5 $\times$ Green GoTaq[®] Flexi Buffer, 10mM dNTPs, 1000 nM of forward and reverse primer, 25 mM MgCl₂, 0.75 U GoTaq[®] DNA polymerase, and 2 μ L of bacterial DNA lysate.

Hereafter, all confirmed O157 and O26 isolates were tested for presence of virulence genes (*stx1*, *stx2* and *eae*) by multiplex polymerase chain reaction (mPCR) using previously described

primers (Possé *et al.*, 2007). Briefly, PCR conditions for detection of virulence genes were 95°C for 3 min, followed by 30 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 30 s. The final cycle was followed by an extension step at 72°C for 8 min. Amplification was performed using 25 µL of PCR mix containing molecular grade RNase free water, 5 × Green GoTaq® Flexi Buffer, 10mM dNTPs, 400 nM of *stx2* primer, 1000 nM of *stx1* and *eae* primer, 25 mM MgCl₂, 1.75 U GoTaq® DNA polymerase, and 1 µL of bacterial DNA lysate.

Finally, PCR products (10 µl) were electrophoresed (EHS3500, Consort, BE) in 1.5 % (w/v) agarose gels in 1 × TAE buffer (0.1 M Tris, 0.1 M acetic acid, and 0.002 M NaEDTA), stained using ethidium bromide (1 µg/ml), and photographed under ultraviolet light (Bio-Rad, Gel Doc™ EZ Imager).

Results

Presence of *E. coli* O157 and O26 in RAMS

In total, 45/233 RAMS (19%) tested positive for presence of *E. coli* O157 (Table 1). Farm A showed the overall highest number of *E. coli* O157 positive RAMS (25/74; 34%), followed by farm C (20/88; 23%). However, all RAMS collected from farm A at the first sampling occasion, and all RAMS collected from farm B throughout the entire study period remained *E. coli* O157 negative. In addition, 72/233 RAMS (31%) tested positive for *E. coli* O26 (Table 1). More than half (37/71; 52%) of the RAMS collected from farm B were *E. coli* O26 positive, while farm A (18/74; 24%) and C (17/88; 19%) showed lower but similar percentages of *E. coli* O26 present. In contrast to *E. coli* O157, all three farms were consistently positive for presence of *E. coli* O26 in RAMS throughout the entire study period. Furthermore, a small proportion of animals (11/233 RAMS, 5%) was also found to be simultaneously positive for both *E. coli* O157 and O26 in RAMS: eight animals on farm A and three animals on farm C.

While it was not our primary objective to investigate persistence of *E. coli* O157 and O26 in individual animals, our study design, nevertheless, resulted in a small cohort of animals (24, 19 and 23 on farm A, B and C, respectively) that were sampled on more than one sampling occasion on each farm (Table 2). On farm A, only one animal was positive for *E. coli* O26 and the same virulence genes on two sampling occasions. On farm B, five animals were found to be positive for *E. coli* O26 on two sampling occasions. However, when comparing the virulence gene profiles of the obtained *E. coli* O26 isolates, only one animal showed a consistent profile of *stx1* and *eae* between the different sampling occasions. The profiles of three other animals shifted from *stx1* and *eae* to *stx2* and *eae*, while the profile of the fifth animal shifted from *stx1* and *eae* to only *eae*, suggesting that different strains belonging to the same serogroup infected the animals. On farm C, three animals were positive for the same *E. coli* serogroup and virulence genes: one animal was *E. coli* O26 positive on the first two sampling occasions, one was *E. coli* O157 positive on the first and last sampling occasion, and one was *E. coli* O157 positive on the last two sampling occasions.

Presence of *stx1*, *stx2* and *eae* virulence genes

A total of 143 *E. coli* O157 colonies from 45 RAMS were isolated and screened for presence of the *stx1*, *stx2* and *eae* genes by mPCR. With the exception of one isolate from farm C that was *stx1*, *stx2* and *eae* positive, all other *E. coli* O157 isolates (142/143) were *stx2* and *eae* positive (Table 3). Additionally, out of the 424 *E. coli* O26 isolates obtained from 72 RAMS, 31

(7%) were only *stx1* or *stx2* positive, 230 (54%) were *stx* and *eae* positive, and 163 (38%) were only *eae* positive (Table 3). In 63 of the 72 RAMS (87.5%), all confirmed *E. coli* O26 isolated from the same sample possessed the same virulence genes. However, in 9 RAMS (12.5%), the O26 isolates differed in one or more virulence genes within the same sample. Furthermore, the virulence gene profiles of the *E. coli* O26 isolates were farm-dependent, with the majority of isolates from farm A being only *eae* positive (71/78, 91%), while the majority of isolates on farm B (155/245; 63%) and C (71/101; 70%) possessed both the *stx* and *eae* genes.

Effect of age on presence of *E. coli* O157 and O26

The influence of animal age on the presence of *E. coli* O157 and O26 is shown in Figure 1. The highest percentage of *E. coli* O157 positive animals were found to be those between 90-120 days of age (36%), while the highest percentage of *E. coli* O26 positive animals were those between 15-30 days of age (44%). In contrast, very few animals <14 days of age were positive for *E. coli* O157 or O26 (2% and 7%, respectively).

Association between presence of *E. coli* O26 and calf diarrhea

Throughout this study, a total of 22 calves exhibited clinical signs of diarrhea. In RAMS from 16 diarrheic calves, no *E. coli* virulence genes and no *E. coli* O26 could be detected. In RAMS from five diarrheic calves, *E. coli* O26 could be detected: three calves with O26 EPEC (*eae* only) and two calves with O26 STEC (*stx1* + *eae*). Additionally, one diarrheic calf was positive for *E. coli* O157 STEC (*stx2* + *eae*).

Discussion

The objectives of this study were to assess RAMS for detection of *E. coli* O26 and O157, to investigate their occurrence on three Belgian dairy cattle farms, and to investigate the presence of virulence genes in the confirmed isolates, since these two *E. coli* serogroups are most commonly associated with human illness. Furthermore, O26 STEC has been implicated as causative agent in calf diarrhea (Mainil and Fairbrother, 2014; Fakih *et al.*, 2017), which still remains a major cause of financial losses for cattle farmers.

Our results confirmed that collecting and culturing RAMS was a suitable method to detect both *E. coli* O157 and O26, as previously reported (Murphy *et al.*, 2016; McCabe *et al.*, 2019). Despite the fact that both serogroups exhibit different tissue tropisms, RAMS allows the simultaneous sampling of the rectal mucosa and fecal material, making it an appropriate method to investigate presence of *E. coli* O157 and O26. The *E. coli* O157 isolation procedure applied in this study is generally considered the gold standard for isolation of this specific serogroup (ISO 16654:2001), and found 19% of all RAMS to be positive for *E. coli* O157. On the other hand, implementation of a post-enrichment acid treatment procedure, originally implemented for improved isolation of STEC from contaminated food samples (Fedio *et al.*, 2012), indicated that 31% of all RAMS was positive for *E. coli* O26. However, while urease-mediated acid resistance has been shown to be associated with major STEC groups, such as O26, diarrheagenic *E. coli* strains of other pathogroups, including EPEC, do not share this association (Friedrich *et al.*, 2005). Therefore, the possibility exists that not all *E. coli* strains with the O26 serogroup were recovered in this study.

In accordance with previous observations (Pearce *et al.*, 2004; Bibbal *et al.*, 2015; Murphy *et al.*, 2016), the present study identified only a small proportion of animals (11/233 RAMS, 5%) that were simultaneously positive for *E. coli* O157 and O26, suggesting competition between different strains to assert intestinal dominance. However, since this study focused only on the two serogroups most implicated in human illness, additional *E. coli* serogroups might also be involved.

An age trend was also observed in this study, with the lowest prevalence of *E. coli* O157 and O26 found in newborn animals (<14 days). Interestingly, peak percentages of *E. coli* O26 positive animals were observed at an earlier age (15-30 days) compared to *E. coli* O157 (90-120 days), suggesting that the former have an advantage over the latter regarding colonization of these animals. Additionally, the number of *E. coli* O157 positive animals gradually increased according

to animal age, while the opposite occurred for *E. coli* O26, which was already abundantly present at a younger age. Factors such as immunity, changes in nutrients, age-related rumen development, the microbiome or differences in virulence factors between both *E. coli* serogroups might play a role, but these were not examined in this study (Magnuson *et al.*, 2000; Menge *et al.*, 2015; Fitzgerald *et al.*, 2019). Nevertheless, our findings demonstrate the wide distribution of both serogroups throughout the entire calf population, suggesting transmission within and between these age groups contributes to *E. coli* O157 and O26 persistence on dairy cattle farms, thus underlining the importance of adequate hygienic practices in veal husbandry and meat processing facilities. Although the majority of human STEC infections are associated with the consumption of fecally contaminated foodstuffs or water, direct contact with infected animals or farm environments has also been reported as a possible route for human STEC transmission (Heuvelink *et al.*, 2002; Schlager *et al.*, 2018). Young dairy calves are often housed at petting zoos in Belgium, where mostly children get the chance to pet and feed these animals. Consequently, they may be exposed to circulating pathogenic *E. coli* strains, with the risk of developing STEC-associated HUS, which is still the leading cause of acute renal failure in children (Harkins *et al.*, 2020). However, the majority of visitors are often ignorant of the possible risk of infection through contact with farm animals, stressing the need to raise public awareness regarding STEC transmission and the importance of proper hand hygiene after contact with animals.

The combination of *stx2* and *eae* is generally a reliable predictor that the STEC strain may potentially cause HC or HUS (FAO and WHO, 2019). All *E. coli* O157 isolates, except one *stx1*, *stx2* and *eae* positive isolate, possessed both *stx2* and *eae*, and can therefore be considered as potentially highly pathogenic strains. This predominant *E. coli* O157 virulence gene profile in cattle has also been described previously (Bibbal *et al.*, 2015; Murphy *et al.*, 2016). Two decades ago, a virulence shift in STEC O26 was observed from *stx1-eae* producing strains to *stx2-eae* producing strains that were more frequently associated with HUS than *stx1*-only strains (Bielaszewska *et al.*, 2013). However, out of the 230 *stx-eae* positive O26 colonies isolated in the present study, 194 (84%) were *stx1* and *eae* positive, and only 32 (14%) were *stx2* and *eae* positive, and 4 (2%) were *stx1*, *stx2* and *eae* positive (Table 3).

Interestingly, our results also demonstrated that the majority of *E. coli* O26 found on farm A was *eae* only positive, while the majority of *E. coli* O26 on farms B and C was positive for both *stx* and *eae*. In other words, this suggests that farm A was dominated by the O26 EPEC pathotype, while farms B and C were dominated by the O26 STEC pathotype. However, interconversion

between EPEC and STEC by loss or gain of Stx-encoding bacteriophages has been described (Bielaszewska *et al.*, 2007), questioning the true molecular identity of these O26 EPEC strains and requiring further characterization.

Occurrence of *E. coli* O157 and O26 within the same animal over time appeared to be relatively transient. Out of the 66 individual animals that were sampled more than once, only 5 animals (8%) were positive for the same *E. coli* serogroup and the same virulence genes on more than one sampling occasion, suggesting repeated exposure and subsequent consecutive infections with different strains over time, rather than persistence of a more dominantly colonizing strain, as previously reported (Rahn *et al.*, 1997).

A few STEC serogroups, including O26, are responsible for diarrhea in young calves (Mainil and Daube, 2005; Moxley and Smith, 2010; Mainil and Fairbrother, 2014). However, the majority of diarrheic calves (16/22; 73%) were negative for both *E. coli* virulence genes and the O26 serogroup. Thus, a correlation between *E. coli* O26 presence and calf diarrhea was absent in this study and we cannot confirm that O26 STEC or EPEC was responsible for calf diarrhea on these farms.

In conclusion, the present study focused on the two most prevalent STEC serogroups in humans, O157 and O26, and demonstrated that they can be frequently found in mostly clinically healthy animals, with an overall higher prevalence of *E. coli* O26 (31%) compared to O157 (19%). While both serogroups persisted at farm level for a period of up to five months, their occurrence within the same animal over time appeared to be relatively transient. Importantly, the majority of the *E. coli* O157 and O26 virulence gene profiles suggest a high pathogenic potential to humans, emphasizing the need for effective control strategies to reduce the risk of human transmission. Additionally, no correlation between presence of *E. coli* O26 and calf diarrhea could be observed. Further studies are currently being undertaken to investigate the occurrence of other conventional and non-conventional O-serogroups isolated from RAMS collected in the present study.

Conflict of interest

There is no conflict of interest to declare.

References

- Bibbal, D., Loukiadis, E., Kérourédan, M., Ferré, F., Dilasser, F., Garam, C.P. de, Cartier, P., Oswald, E., Gay, E., Auvray, F. and Brugère, H. (2015) Prevalence of Carriage of Shiga Toxin-Producing *Escherichia coli* Serotypes O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 among Slaughtered Adult Cattle in France. *Appl Environ Microbiol.* **81**, 1397–1405.
- Bielaszewska, M., Mellmann, A., Bletz, S., Zhang, W., Köck, R., Kossow, A., Prager, R., Fruth, A., Orth-Höller, D., Marejková, M., Morabito, S., Caprioli, A., Piérard, D., Smith, G., Jenkins, C., Čurová, K. and Karch, H. (2013) Enterohemorrhagic *Escherichia coli* O26:H11/H-: A New Virulent Clone Emerges in Europe. *Clin Infect Dis.* **56**, 1373–1381.
- Bielaszewska, M., Mellmann, A., Zhang, W., Köck, R., Fruth, A., Bauwens, A., Peters, G. and Karch, H. (2011) Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. *Lancet Infect Dis.* **11**, 671–676.
- Bielaszewska, M., Prager, R., Köck, R., Mellmann, A., Zhang, W., Tschäpe, H., Tarr, P.I. and Karch, H. (2007) Shiga toxin gene loss and transfer *in vitro* and *in vivo* during enterohemorrhagic *Escherichia coli* O26 infection in humans. *Appl Environ Microbiol.* **73**, 3144–3150.
- Browne, A.S., Midwinter, A.C., Withers, H., Cookson, A.L., Biggs, P.J., Marshall, J.C., Benschop, J., Hathaway, S., Haack, N.A., Akhter, R.N. and French, N.P. (2018) Molecular Epidemiology of Shiga Toxin-Producing *Escherichia coli* (STEC) on New Zealand Dairy Farms: Application of a Culture-Independent Assay and Whole-Genome Sequencing. *Appl Environ Microbiol.* **84**(14):e00481-18.
- Cobbaut, K., Houf, K., Doudah, L., Van Hende, J. and De Zutter, L. (2008) Alternative sampling to establish the *Escherichia coli* O157 status on beef cattle farms. *Vet Microbiol.* **132**, 205–210.
- Cobbold, R. and Desmarchelier, P. (2000) A longitudinal study of Shiga-toxigenic *Escherichia coli* (STEC) prevalence in three Australian dairy herds. *Vet Microbiol.* **71**, 125–137.
- Cointe, A., Birgy, A., Mariani-Kurkdjian, P., Liguori, S., Courroux, C., Blanco, J., Delannoy, S., Fach, P., Loukiadis, E., Bidet, P. and Bonacorsi, S. (2018) Emerging Multidrug-Resistant Hybrid Pathotype Shiga Toxin-Producing *Escherichia coli* O80 and Related Strains of Clonal Complex 165, Europe. *Emerg Infect Dis.* **24**, 2262–2269.

van Diemen, P.M., Dziva, F., Stevens, M.P. and Wallis, T.S. (2005) Identification of Enterohemorrhagic *Escherichia coli* O26:H– Genes Required for Intestinal Colonization in Calves. *Infect Immun.* **73**, 1735–1743.

Eichhorn, I., Heidemanns, K., Semmler, T., Kinnemann, B., Mellmann, A., Harmsen, D., Anjum, M.F., Schmidt, H., Fruth, A., Valentin-Weigand, P., Heesemann, J., Suerbaum, S., Karch, H. and Wieler, L.H. (2015) Highly Virulent Non-O157 Enterohemorrhagic *Escherichia coli* (EHEC) Serotypes Reflect Similar Phylogenetic Lineages, Providing New Insights into the Evolution of EHEC. *Appl Environ Microbiol.* **81**, 7041–7047.

European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC) (2019). The European Union One Health 2018 Zoonoses Report. *EFSA J.* **17**, e05926.

Fakih, I., Thiry, D., Duprez, J.-N., Saulmont, M., Iguchi, A., Piérard, D., Jouant, L., Daube, G., Ogura, Y., Hayashi, T., Taminiau, B. and Mainil, J.G. (2017) Identification of Shiga toxin-producing (STEC) and enteropathogenic (EPEC) *Escherichia coli* in diarrhoeic calves and comparative genomics of O5 bovine and human STEC. *Vet Microbiol*, ECMIS 2015 - *Escherichia coli* and the Mucosal Immune System. **202**, 16–22.

FAO & WHO (2019). Attributing illness caused by Shiga toxin-producing *Escherichia coli* (STEC) to specific foods.

Fedio, W.M., Jinneman, K.C., Yoshitomi, K.J., Zapata, R. and Weagant, S.D. (2012) Efficacy of a post enrichment acid treatment for isolation of *Escherichia coli* O157:H7 from alfalfa sprouts. *Food Microbiol.* **30**, 83–90.

Fitzgerald, S.F., Beckett, A.E., Palarea-Albaladejo, J., McAteer, S., Shaaban, S., Morgan, J., Ahmad, N.I., Young, R., Mabbott, N.A., Morrison, L., Bono, J.L., Gally, D.L. and McNeilly, T.N. (2019) Shiga toxin sub-type 2a increases the efficiency of *Escherichia coli* O157 transmission between animals and restricts epithelial regeneration in bovine enteroids. *PLOS Pathog.* **15**, e1008003.

Fratamico, P.M., Bagi, L.K. and Abdul-Wakeel, A. (2017) Detection and Isolation of the “Top Seven” Shiga Toxin-Producing *Escherichia coli* in Ground Beef: Comparison of RapidFinder Kits to the U.S. Department of Agriculture Microbiology Laboratory Guidebook Method. *J Food Prot.* 829–836.

Friedrich, A.W., Köck, R., Bielaszewska, M., Zhang, W., Karch, H. and Mathys, W. (2005) Distribution of the Urease Gene Cluster among and Urease Activities of Enterohemorrhagic

Escherichia coli O157 Isolates from Humans. *J Clin Microbiol.* **43**, 546–550.

Gonzalez, A.G.M. and Cerqueira, A.M.F. (2020) Shiga toxin-producing *Escherichia coli* in the animal reservoir and food in Brazil. *J Appl Microbiol.* **128**, 1568–1582.

Harkins, V.J., McAllister, D.A. and Reynolds, B.C. (2020) Shiga-Toxin *E. coli* Hemolytic Uremic Syndrome: Review of Management and Long-term Outcome. *Curr Pediatr Rep.* **8**, 16–25.

Heuvelink, A.E., van Heerwaarden, C., Zwartkruis-Nahuis, J.T.M., van Oosterom, R., Edink, K., van Duynhoven, Y.T.H.P. and de Boer, E. (2002) *Escherichia coli* O157 infection associated with a petting zoo. *Epidemiol Infect.* **129**, 295–302.

ISO 16654:2001(en), Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Escherichia coli* O157 (2020). URL <https://www.iso.org/obp/ui/#iso:std:iso:16654:ed-1:v1:en>.

Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bover-Cid, S., Chemaly, M., Davies, R., Cesare, A.D., Herman, L., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Jenkins, C., Pires, S.M., Morabito, S., Niskanen, T., Scheutz, F., Felício, M.T. da S., Messens, W. and Bolton, D. (2020) Pathogenicity assessment of Shiga toxin-producing *Escherichia coli* (STEC) and the public health risk posed by contamination of food with STEC. *EFSA J.* **18**, e05967.

Magnuson, B.A., Davis, M., Hubele, S., Austin, P.R., Kudva, I.T., Williams, C.J., Hunt, C.W. and Hovde, C.J. (2000) Ruminant Gastrointestinal Cell Proliferation and Clearance of *Escherichia coli* O157:H7. *Infect Immun.* **68**, 3808–3814.

Mainil, J. and Fairbrother, J. (2014) Pathogenic *Escherichia coli* in domestic mammals and birds. *Pathog Escherichia Coli Mol Cell Microbiol.* 19–44.

Mainil, J. g. and Daube, G. (2005) Verotoxigenic *Escherichia coli* from animals, humans and foods: who's who? *J Appl Microbiol.* **98**, 1332–1344.

McCabe, E., Burgess, C.M., Lawal, D., Whyte, P. and Duffy, G. (2019) An investigation of shedding and super-shedding of Shiga toxigenic *Escherichia coli* O157 and *E. coli* O26 in cattle presented for slaughter in the Republic of Ireland. *Zoonoses Public Health.* **66**, 83–91.

Mellor, G.E., Fegan, N., Duffy, L.L., McMILLAN, K.E., Jordan, D. and Barlow, R.S. (2016) National Survey of Shiga Toxin-Producing *Escherichia coli* Serotypes O26, O45, O103, O111, O121, O145, and O157 in Australian Beef Cattle Feces. *J Food Prot.* **79**, 1868–1874.

Menge, C., Loos, D., Bridger, P.S., Barth, S.A., Werling, D. and Baljer, G. (2015) Bovine macrophages sense *Escherichia coli* Shiga toxin 1. *Innate Immun.*

Moxley, R.A. and Smith, D.R. (2010) Attaching-effacing *Escherichia coli* Infections in Cattle. *Vet Clin North Am Food Anim Pract*, Emerging, Reemerging, and Persistent Infectious Diseases of Cattle. **26**, 29–56.

Murphy, B.P., McCabe, E., Murphy, M., Buckley, J.F., Crowley, D., Fanning, S. and Duffy, G. (2016) Longitudinal Study of Two Irish Dairy Herds: Low Numbers of Shiga Toxin-Producing *Escherichia coli* O157 and O26 Super-Shedders Identified. *Front Microbiol.* **7**.

Naylor, S.W., Low, J.C., Besser, T.E., Mahajan, A., Gunn, G.J., Pearce, M.C., McKendrick, I.J., Smith, D.G.E. and Gally, D.L. (2003) Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. *Infect Immun.* **71**, 1505–1512.

Nüesch-Inderbinnen, M., Cernela, N., Wüthrich, D., Egli, A. and Stephan, R. (2018) Genetic characterization of Shiga toxin producing *Escherichia coli* belonging to the emerging hybrid pathotype O80:H2 isolated from humans 2010-2017 in Switzerland. *Int J Med Microbiol IJMM.* **308**, 534–538.

Pearce, M.C., Jenkins, C., Vali, L., Smith, A.W., Knight, H.I., Cheasty, T., Smith, H.R., Gunn, G.J., Woolhouse, M.E.J., Amyes, S.G.B. and Frankel, G. (2004) Temporal shedding patterns and virulence factors of *Escherichia coli* serogroups O26, O103, O111, O145, and O157 in a cohort of beef calves and their dams. *Appl Environ Microbiol.* **70**, 1708–1716.

Persad, A.K. and LeJeune, J.T. (2014) Animal Reservoirs of Shiga Toxin-Producing *Escherichia coli*. *Microbiol Spectr.* **2**, EHEC-0027-2014.

Possé, B., De Zutter, L., Heyndrickx, M. and Herman, L. (2007) Metabolic and genetic profiling of clinical O157 and non-O157 Shiga-toxin-producing *Escherichia coli*. *Res Microbiol.* **158**, 591–599.

Rahn, K., Renwick, S.A., Johnson, R.P., Wilson, J.B., Clarke, R.C., Alves, D., McEwen, S., Lior, H. and Spika, J. (1997) Persistence of *Escherichia coli* O157:H7 in dairy cattle and the dairy farm environment. *Epidemiol Infect.* **119**, 251–259.

Rice, D.H., Sheng, H.Q., Wynia, S.A. and Hovde, C.J. (2003) Rectoanal Mucosal Swab Culture Is More Sensitive Than Fecal Culture and Distinguishes *Escherichia coli* O157:H7-Colonized Cattle and Those Transiently Shedding the Same Organism. *J Clin Microbiol.* **41**, 4924–4929.

Schlager, S., Lepuschitz, S., Ruppitsch, W., Ableitner, O., Pietzka, A., Neubauer, S., Stöger, A., Lassnig, H., Mikula, C., Springer, B. and Allerberger, F. (2018) Petting zoos as sources of

Shiga toxin-producing *Escherichia coli* (STEC) infections. *Int J Med Microbiol IJMM*. **308**, 927–932.

Tozzoli, R. and Scheutz, F. (2014) Diarrhoeagenic *Escherichia coli* Infections in Humans. In *Pathogenic Escherichia Coli, Molecular and Cellular Microbiology, 1st Edn.* pp.1–18.

Verhaegen, B., Van Damme, I., Heyndrickx, M., Botteldoorn, N., Elhadidy, M., Verstraete, K., Dierick, K., Denayer, S., De Zutter, L. and De Reu, K. (2016) Evaluation of detection methods for non-O157 Shiga toxin-producing *Escherichia coli* from food. *Int J Food Microbiol*. **219**, 64–70.

Authors contribution statement

FE, DT, BD, MH, JM, LDZ and EC designed the study. FE performed the experiments. MH and LDZ gave technical support. FE, BD, LDZ and EC analyzed the data. FE wrote the manuscript. LDZ, JM and EC revised the manuscript. DT and BD reviewed the manuscript.

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Conflict of interest

There is no conflict of interest to declare.

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Table 1

Isolation of *E. coli* O157 and O26 in RAMS from young dairy calves on three Belgian farms

Table 2

No. of dairy calves per farm testing positive for the same *E. coli* serogroup at multiple sampling points

Table 3

Presence of virulence genes in *E. coli* O157 and O26 isolates

*positive for the same *E. coli* serogroup at more than one sampling point

NS: not sampled

Table 1

Farm	Sampling (date)	No. RAMS collected	<i>E. coli</i> serogroup	
			O157 positive (%)	O26 positive (%)
A	1 (11/06/2018)	16	-	1 (6)
	2 (06/08/2018)	24	8 (33)	10 (42)
	3 (24/09/2018)	34	17 (50)	7 (21)
	Total	74	25 (34)	18 (24)
B	1 (18/06/2018)	23	-	10 (43)
	2 (27/08/2018)	26	-	14 (54)
	3 (24/09/2018)	22	-	13 (59)
	Total	71	-	37 (52)
C	1 (02/07/2018)	26	5 (19)	6 (23)
	2 (20/08/2018)	25	1 (4)	5 (20)
	3 (08/10/2018)	37	14 (38)	6 (16)
	Total	88	20 (23)	17 (19)
Overall		233	45 (19)	72 (31)

Table 2

Farm	No. animals sampled more than once				Serogroup and virulence factors in positive samples		
	Total	Twice	Thrice	Positive*	Sampling 1	Sampling 2	Sampling 3
A	24	18	6	1	NS	O26 (<i>eae</i>)	O26 (<i>eae</i>)
B	19	19	/	5	NS	O26 (<i>eae</i> , <i>stx1</i>)	O26 (<i>eae</i> , <i>stx1</i>)
					NS	O26 (<i>eae</i> , <i>stx1</i>)	O26 (<i>eae</i> , <i>stx2</i>)
					NS	O26 (<i>eae</i> , <i>stx1</i>)	O26 (<i>eae</i> , <i>stx2</i>)
					NS	O26 (<i>eae</i> , <i>stx1</i>)	O26 (<i>eae</i> , <i>stx2</i>)
					NS	O26 (<i>eae</i> , <i>stx1</i>)	O26 (<i>eae</i>)
C	23	10	13	3	O26 (<i>eae</i>)	O26 (<i>eae</i>)	/
					O157 (<i>eae</i> , <i>stx2</i>)	/	O157 (<i>eae</i> , <i>stx2</i>)
					NS	O157 (<i>eae</i> , <i>stx2</i>)	O157 (<i>eae</i> , <i>stx2</i>)

Table 3

Farm	Serogroup	No. of isolates	Virulence genes		
			<i>eae</i>	<i>stx1</i>	<i>stx2</i>
A	O157	90	+	–	+
	O26	71	+	–	–
		2	+	+	–
		2	+	–	+
		3	–	–	+
B	O157	/	/	/	/
	O26	67	+	–	–
		125	+	+	–
		30	+	–	+
		23	–	–	+
C	O157	52	+	–	+
		1	+	+	+
	O26	25	+	–	–
		67	+	+	–
		4	+	+	+

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