Article

Identification of Shigatoxigenic and enteropathogenic *Escherichia coli* serotypes in healthy young dairy calves in Belgium by recto-anal mucosal swabbing

**Audrey Habets 1°, Frederik Engelen 2, Jean-Noël Duprez 1, Brecht Devleesschauwer 3,4, Marc Heyndrickx 5,6, Lieven De Zutter 4, Damien Thiry 1#, Eric Cox 2, Jacques Mainil 1**

1 Bacteriology, Department of Infectious Diseases, Institute for Fundamental and Applied Research in Animals and Health (FARAH) and Faculty of Veterinary Medicine, University of Liège, Quartier Vallée II, Avenue de Cureghem 6, B-4000 Liège, Belgium [damien.thiry@uliege.be](mailto:damien.thiry@uliege.be)

2 Laboratory of Immunology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium [eric.cox@ugent.be](mailto:eric.cox@ugent.be)

3 Department of Epidemiology and Public Health, Sciensano, Rue Juliette Wytsmanstraat 14, B-1050 Brussels, Belgium [brecht.devleesschauwer@sciensano.be](mailto:brecht.devleesschauwer@sciensano.be" \t "_blank)

4 Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium [lieven.dezutter@ugent.be](mailto:lieven.dezutter@ugent.be)

5 Institute for Agricultural and Fisheries Research, Unit Technology and Food, Brusselsesteenweg 370, B-9090 Melle, Belgium [marc.heyndrickx@ilvo.vlaanderen.be](mailto:marc.heyndrickx@ilvo.vlaanderen.be)

6 Department of Pathology, Bacteriology and Poultry Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium [marc.heyndrickx@ugent.be](mailto:marc.heyndrickx@ugent.be)

**°** Current address: Eurogentec SA, Seraing, Belgium

**#**Correspondence: [damien.thiry@uliege.be](mailto:damien.thiry@uliege.be) ; (D.T.)

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**Abstract:** Enterohemorrhagic *Escherichia coli* (EHEC), enteropathogenic *E. coli* (EPEC) and Shigatoxigenic *E. coli* (STEC) are carried by healthy adult cattle and even more frequently, young calves in their intestinal tract, especially at the height of the recto-anal junction. The purpose of the present study was to assess the presence of ten EHEC, EPEC and/or STEC O serotypes (O5, O26, O80, O103, O111, O118, O121, O145, O157, O165) in calves sampled by recto-anal mucosal swabs (RAMS) in three dairy farms in Belgium. A total of 233 RAMS were collected on 3 consecutive times from healthy <6-month-old Holstein-Friesian calves and submitted to a PCR targeting the *eae*, *stx1* and *stx2* genes after non-selective overnight enrichment growth. The 148 RAMS testing positive were streaked on four (semi-)selective agar media: of the 2146 colonies tested, 294 from 69 RAMS were PCR-confirmed as EHEC, EPEC or STEC. The most frequent virulotype was *eae*+ EPEC and the second one was *stx1*+*stx2*+ STEC while the *eae*+*stx1*+ and *eae*+*stx1*+*stx2*+ virulotypes were the most frequent amongst EHEC. The majority of EHEC (73%) tested positive for one of the 5 O serotypes detected (O26, O103, O111, O145, or O157) *vs.* 23% of EPEC and 45% of STEC. Similarly, more RAMS (73%) harbored EHEC isolates positive for those 5 serotypes, than EPEC (53%) or STEC (52%). This survey confirms that (i) healthy young dairy calves are asymptomatic carriers of EHEC and EPEC in Belgium; (ii) the carrier state rates, the virulotypes and the identified O serotypes differ between farms and in time; and (iii) a majority of EPEC belong to still unidentified O serotypes.

**Keywords:** *Escherichia coli*, EPEC, STEC, dairy calves, recto-anal mucosal swab, Belgium

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are important human pathogens, responsible for hemorrhagic colitis (HC), at the origin of their name, and the life-threatening hemolytic-uremic syndrome (HUS). Their most important virulence-associated properties are the Shiga toxins (Stx1 and/or Stx2) encoded by phage-located genes (*stx1* and *stx2*), and the production of the histological Attaching-Effacing (AE) lesion, encoded by genes located on a pathogenicity island (the Locus of Enterocyte Effacement or LEE), including the *eae* gene coding for the intimin adhesin. There exists a broad variety of human EHEC serotypes of whom O26:H11, O103:H2, O111:H-, O121:H19, O145:H-, O157:H7, and O165:H25 (the so-called “gang of seven”) are the most frequent and pathogenic worldwide and O157:H7 and O26:H11 represent the majority of the confirmed human cases in the EU [1-4]. However, not all Stx- and AE-producing *E. coli* cause HC in humans and the acronym EHEC is not appropriate for them. Therefore, the acronym AE-STEC, after Attaching-Effacing Shiga toxin-producing *E. coli* will be used throughout this manuscript, as previously proposed [5].

Human infection most frequently occurs via the consumption of animal or plant-derived foodstuffs contaminated with ruminant, mostly cattle feces. Cattle can indeed be asymptomatic carriers in their intestinal tract, more especially in the colon and at the height of the recto-anal junction [2,6-8]. Furthermore, young calves are more frequently healthy carriers of AE-STEC than older animals [9-11]. In addition, AE-STEC are also responsible for diarrhea in <3-month-old calves. The majority belong to some “gang-of-seven”, like O26:H11 and O111:H-, and to a few other serotypes, like O5:H-, O80:H2 and O118:H16 [12-14].

Besides AE-STEC, enteropathogenic *E. coli* (EPEC) harbor the LEE and the *eae* gene and produce the AE lesion, but no Stx, while STEC sensu stricto produce Stx but no AE lesion. EPEC are responsible for diarrheic diseases in humans and several animals, including young calves. EPEC can belong to host-specific serotypes (like O127 in humans or O15 in rabbits) or to host-non-specific, including “gang-of-seven” serotypes (like O26:H11, O80:H2 and O111:H- in calves). However, the serotypes of the majority of bovine EPEC remain unidentified. Like AE-STEC, EPEC and STEC are also present in asymptomatic cattle [1,12-17].

The purpose of the present study was to assess the presence of the “gang-of-seven” and of 3 other (O5, O80 and O118) (AE-)STEC and EPEC O serotypes in healthy <6-month-old dairy calves of three farms sampled by recto-anal mucosal swabs (RAMS) [18,19], following a serotype non-specific enrichment and isolation procedure [17].

2. Material & Methods

2.1 Farms, Animals and Sampling

A total of 233 RAMS was collected from <6-month-old Holstein-Friesian calves in 3 Belgian dairy farms situated in East Flanders (74 in FarmA, 71 in FarmB, and 88 in FarmC) on 3 consecutive times (65 at Sampling1, 75 at Sampling2, 93 at Sampling3) 2 to 4 weeks apart between June and October 2018 [20]. Eighteen calves in FarmA, 19 calves in FarmB and 11 calves in FarmC were sampled twice while 6 calves in FarmA and 13 calves in FarmC were sampled thrice during the survey. Sterile cotton prepping balls (CovidienTM) were inserted into the anus of the calves and circular motions were applied to sample the entire mucosal surface of the recto-anal junction. Clinical signs (diarrhea), if any were recorded at each visit.Fecal sampling by recto-anal swabbing is considered as non-invasive. Therefore, the calves in this study did not fall into the definition of experimental animals and no ethical approval was required.

2.2 Preliminary Screening

All RAMS were transported on ice to the laboratory of UGent, stored overnight at 4°C and homogenized in 25 ml of maximum recovery diluent (Oxoid, Belgium) by stomaching [20]. At ULiège, the whole procedure to isolate EPEC and (AE-)STEC was slightly adapted from Thiry and collaborators [17].

Briefly, 100 µl of the suspension were added to 5 ml of Lauryl-Sulfate broth (Sigma Aldrich, Germany) and incubated overnight at 37°C. Bacterial DNA was extracted from 1.5 ml of the enrichment broths by the alkaline-boiling method and stored at -20°C. Lysates were tested with a triplex PCR targeting the *eae*, *stx1* and *stx2* genes [21]. Each PCR-positive broth was subsequently streaked onto four (semi-)selective agar plates and incubated overnight at 37°C: McConkey’s (MC), Chromocult Coliform ES (ES) (VWR, Belgium), Chromocult Coliform ES supplemented with 2.5 mg/mL of potassium tellurite (ESTe) (Sigma-Aldrich, Germany) [22], and supplemented CHROMagarTM STEC base (STECB) (I2A, France).

2.3 Identification of Virulotypes and O Serotypes

Up to 5 colonies per agar plate were picked up, inoculated into 200 µl Luria-Bertani (LB) broth in 96-well microtiter plates, and tested by colony hybridization (CH). CH was performed with PCR-derived 32P radioactively-labelled gene probes targeting the *eae*, *stx1* and *stx2* genes to identify EPEC and (AE-)STEC [23]. After overnight incubation at 37°C, 1 µl from each well was transferred onto LB agar using a transfer comb, followed by overnight incubation at 37°C. The colonies were transferred by contact onto Whatman 541 paper filters (VWR, Belgium) and treated to lyse the cells and denature the DNA. All probe-positive colonies were stored at -80°C in LB broth with 40% glycerol till further use.

Probe-positive colonies were subsequently grown overnight on LB agar plates at 37°C. DNA was extracted from a single colony by the alkaline-boiling method and subjected to the triplex PCR for the *eae*, *stx1* and *stx2* genes for confirmation of the virulotypes [21,24]. Isolates with CH/PCR discordant results were retested with the PCR. Finally, confirmed triplex PCR-positive colonies were tested by PCR for the ten O serotypes listed above: the “gang-of-seven”, O5, O80 and O118 [21].

3. Results

3.1 PCR-Positive RAMS

Of the 233 RAMS after overnight enrichment in Lauryl-Sulfate, 148 tested positive with the triplex PCR (Table 1): 49 in FarmA (66%), 47 in FarmB (66%) and 52 in FarmC (59%). The number of PCR-positive RAMS increased with the sampling time (Table 1): at Sampling1, 13 RAMS (20%) were PCR-positive *vs.* 50 RAMS (77%) at Sampling2 and 85 RAMS (91%) at Sampling3. Eleven of the PCR-positive RAMS were sampled from calves with signs of diarrhea. All 148 PCR-positive broths were subsequently streaked on MC, ES, ESTe and STECB agar plates and incubated overnight at 37°C.

3.2 CH- and PCR-Positive Colonies

Of the 2146 colonies studied, 721 were isolated on MC, 711 on ES, 233 on ESTe and 481 on STECB media. Of them, 338 (16%) from 77 RAMS (52%) tested positive with the probes for the *eae*, *stx1* and/or *stx2* genes (Table 1). Of these 338 CH-positive colonies, 294 (87%) from 69 RAMS (90%) were confirmed by the triplex PCR (Table 1). Seven of the 8 RAMS with no confirmed PCR-positive colonies had only one or two CH-positive colonies. Some CH-positive PCR-negative colonies were also observed in 20 other RAMS. CH and PCR results were in agreement for 257 PCR-positive colonies (87%).

In summary, 69 of the 148 PCR-positive RAMS (47%) and of the 233 collected RAMS (30%) harbored PCR-positive colonies. Those percentages in the three farms were: 31% and 20% (FarmA); 55% and 37% (FarmB); 54% and 32% (FarmC), respectively. The same respective percentages according to the sampling time were: 31% and 6% (Sampling1); 42% and 28% (Sampling2); 52% and 47% (Sampling3). However, the percentage of triplex PCR-positive RAMS differently increases with sampling in the 3 farms: in FarmA only at the Sampling3 (6%-4%-38%); in FarmB already at Sampling2 to stabilize at Sampling3 (9%-46%-55%); in Farm C consecutively from Sampling1 to Sampling2 and to Sampling3 (4%-32%-51%).

Seventeen RAMS harbored only one PCR-positive colony, while the remaining 52 RAMS harbored up to 15 PCR-positive colonies. Of the 11 PCR-positive RAMS from diarrheic calves, only one PCR-positive colony was isolated in FarmA at Sampling3.

3.3 Virulotypes of the PCR-Positive Colonies

The 294 PCR-positive colonies from 69 RAMS were identified as either EPEC (131 isolates including the one from one diarrheic calf), or AE-STEC (89 isolates) or STEC (74 isolates) (Table 2). EPEC were identified in 36 of the 69 RAMS with PCR-positive colonies (52%), AE-STEC in 23 RAMS (33%) and STEC in 27 RAMS (39%). Of the 52 RAMS with more than one PCR-positive colony, 4 RAMS from FarmA, 6 RAMS from FarmB and 1 RAMS from FarmC harbored *E. coli* belonging to 2 different pathotypes while 3 RAMS from FarmB and 1 RAMS from FarmC harbored *E. coli* belonging to 3 different pathotypes.

The most frequent virulotype amongst colonies was *eae*+ EPEC and the second one was *stx1+stx2+* STEC. The *eae+stx1+* and *eae+stx1+stx2+* virulotypes were the most frequent amongst AE-STEC, while the *eae+stx2+* AE-STEC and the *stx1+* STEC virulotypes were the less frequent (Table 2).

No calf sampled twice or thrice in FarmA was more than once positive, while 2 calves in FarmB sampled twice were twice positive, 3 calves in FarmC sampled thrice were positive twice (at the 2nd and 3rd samplings), and one calf in FarmC sampled thrice was positive 3 times. The virulotypes were identical (*eae+* and *eae+stx1+stx2+*) in only the 2 calves in FarmB sampled twice.

3.4 O serotypes of the PCR-Positive Colonies

Of the 294 PCR-positive colonies, 136 (46%) tested positive for 5 of the 10 serotypes searched for: O26, O103, O111, O145, or O157 (Table 3). The majority of AE-STEC (73%) tested positive for one of the 5 O serotypes detected *vs.* a minority of EPEC (23%) and almost half of STEC (45%). Similarly, a majority of RAMS harboring AE-STEC isolates (83%) were positive for one of those 5 serotypes *vs.* half of the RAMS harboring EPEC (53%) or STEC (52%) (Table 3). Nevertheless, 65% of the RAMS with EPEC and/or (AE-)STEC harbored isolates not belonging to the “gang-of-seven” serotypes.

The most frequent were the O111 (62 colonies from 18 RAMS) and O145 (43 colonies from 16 RAMS) serotypes, while the O26, O103 and O157 serotypes were the less frequent. In 27 RAMS, all PCR-positive colonies, including the one from the diarrheic calf, tested negative with the PCR for the 10 searched-for serotypes. The identified serotypes were not equally distributed amongst the different virulotypes, but they were all identified only amongst *eae*+ EPEC. Of the 52 RAMS with more than one PCR-positive colony, 34 (65%) harbored EPEC, AE-STEC and/or STEC belonging to different serotypes, including unidentified ones.

The serotype O26 was identified only in FarmB while serotypes O157 and O103 were not detected in FarmB and FarmC, respectively (Table 4). The number of serotypes identified actually increased with sampling time: from 2 (O111 and O145) at Sampling1 to four at Sampling2 (O26, O111, O145 and O157) and all 5 at Sampling3 (O26, O103, O111, O145 and O157). Similarly, the number of RAMS with typeable isolates also increased with sampling time, more especially those positive for the O111 and O145 serotypes (Table 4).

These same 2 serotypes O111 and O145 were identified, along with other serotypes in the 6 calves sampled twice or thrice in FarmB and FarmC. Nevertheless, only one of the 2 calves positive twice in FarmB had the same serotype (O111) identified at both samplings (all isolates were *eae+stx1+stx2+*) and the calf positive thrice in FarmC had the same serotype (O111) identified at Sampling1 and Sampling3, but the virulotypes were not identical (*stx1+stx2+* and *eae+stx1+stx2+*).

3.5 Agar Media and PCR-Positive Colonies

The STECB medium gave the highest rate of PCR-positive colonies: 45% of the tested colonies growing on STECB were PCR-positive *vs.* 3%-5% for the other 3 media. The highest rate of RAMS with PCR-positive colonies was also obtained with the STECB medium (Table 5): 54% *vs.* 8-12% for the other 3 media. Similarly, 73% of the 294 PCR-positive colonies and 84% of the 69 RAMS with PCR-positive colonies were obtained with the STECB medium, *vs.* 2%-13%, and 7%-25% for the other 3 media, respectively. Of the 52 RAMS with more than one PCR-positive colony, 13 were positive on 2 agar media, 4 on 3 agar media and 2 on the 4 agar media. Besides the selective properties of ESTe and STECB, there was no correlation between the agar medium and the identified virulotypes.

Figures, Tables and Schemes

**Table 1. Results of the different tests performed on the 233 Recto-Anal Mucosal Swabs (RAMS) and isolated colonies from <6-month-old calves in 3 farms during the 3 sampling times.**

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Performed tests | RAMS | N° positive RAMS (n=69) | | | | | | | | | |
| Farms | FarmA | | | FarmB | | | FarmC | | | Total |
| Samplings (S) | S1 | S2 | S3 | S1 | S2 | S3 | S1 | S2 | S3 |
| Lauryl sulfate broth triplex PCR | | 8 (50%) 1 | 8 (33%) | 33 (97%) | 4 (17%) | 23 (88%) | 20 (91%) | 1 (4%) | 19 (76%) | 32 (86%) | 148  (64%) |
| Growth on the four agar media 2 | | 8  (70) 3 | 8 (131) | 33 (495) | 4 (42) | 23 (326) | 20 (270) | 1 (14) | 19 (294) | 32 (504) | 148  (2146) |
| Colony triplex hybridization (CH) 4 | | 3 (14) 5 | 3 (4) | 13 (50) | 3 (14) | 13 (59) | 12 (58) | 1 (5) | 8 (34) | 21 (100) | 77  (338) |
| Colony triplex PCR 6 | | 1 (9) 5 | 1 (2) | 13 (42) | 2 (10) | 12 (51) | 12 (52) | 1 (3) | 8 (34) | 19 (91) | 69 (294) |

1 N° PCR-positive RAMS (% of RAMS tested). 2 Only the PCR-positive broths were inoculated onto the 4 agar media. 3 N° RAMS with growing colonies (N° colonies picked up). 4 Only the growing colonies from the PCR-positive broths were tested by CH. 5 N° CH/PCR-positive RAMS (N° positive colonies). 6 Only the CH-positive colonies were tested by PCR.

Table 2. Pathotypes and virulotypes of the triplex PCR-positive colonies according to the farms and sampling time.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Pathotypes | | | EPEC | AE-STEC 1 (= EHEC) | | | STEC | | | PCR-positive colonies | |
| Virulotypes | | | *eae* | *eae,stx1* | *eae,stx2* | *eae,stx1,stx2* | *stx1* | *stx2* | *stx1,stx2* | Per Farm | Per Sampling |
| FARMS | A | S1 2 | 9 (1) 3 | --- | --- | --- | --- | --- | --- | A: 53 (15) | 1: 22 (4) |
| S2 | --- 4 | --- | 2 (1) | --- | --- | --- | --- |
| S3 | 23 (9) 5 | 15 (4) | --- | --- | --- | 2 (2) | 2 (2) |
| B | S1 | 9 (1) | --- | --- | --- | --- | --- | 1 (1) | B: 113 (26) | 2: 87 (21) |
| S2 | 28 (10) | 9 (4) | --- | 1 (1) | --- | 13 (3) | --- |
| S3 | 29 (8) | --- | 1 (1) | 5 (2) | 5 (4) | 12 (3) | --- |
| C | S1 | --- | --- | --- | --- | --- | --- | 3 (1) | C: 128 (28) | 3: 185 (44) |
| S2 | 18 (3) | --- | --- | 1 (1) | --- | --- | 15 (4) |
| S3 | 15 (4) | 9 (2) | 8 (1) | 38 (7) | --- | --- | 21 (8) |
| Total virulotypes | | | 131 (36) | 33 (10) | 11 (3) | 45 (11) | 5 (4) | 27 (8) | 42 (16) | 294 (69) | |
| Total pathotypes | | | 131 (36) | 89 (23) 6 | | | 74 (27) 6 | | |

1 AE-STEC = Attaching-Effacing Shigatoxigenic *Escherichia coli*. 2 S = Sampling. 3 No. *E. coli* isolates (No. RAMS). 4 No positive colony detected. 5 Including one isolate from one diarrheic calf. 6 One RAMS harbored *E. coli* belonging to 2 different virulotypes

Table 3. O somatic serotypes identified amongst triplex PCR-positive colonies.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| O somatic serotypes 1 | EPEC  n=131 (36) 2 | AE-STEC 3 (= EHEC)  n=89 (23) | | | STEC  n=74 (27) | | | Total  n=294 (69) |
| *eae*  n=131 (36) | *eae,stx1*  n=33 (10) | *eae,stx2*  n=11 (3) | *eae,stx1,stx2*  n=45 (11) | *stx1*  n=5 (4) | *stx2*  n=27 (8) | *stx1,stx2*  n=42 (16) |
| O26 | 4 (3) 4 | 5 (2) | 0 | 0 | 0 | 0 | 0 | 9 (4) |
| O103 | 4 (2) | 7 (3) | 0 | 0 | 0 | 0 | 0 | 11 (4) |
| O111 | 5 (4) | 2 (2) | 0 | 38 (8) | 0 | 2 (2) | 15 (6) | 62 (18) |
| O145 | 21 (9) | 6 (2) | 4 (2) | 1 (1) | 0 | 4 (2) | 7 (3) | 43 (16) |
| O157 | 4 (1) | 0 | 2 (1) | 0 | 0 | 0 | 5 (1) | 11 (3) |
| Total | 38 (19) | 20 (8) | 6 (3) | 39 (9) | 0/5 | 6 (4) | 27 (10) | 136 (42) |
| 38 (19) | 65 (19) | | | 33 (14) | | |

1 O5, O80, O118, O121 and O165 serotypes were not identified. 2 No. PCR-positive *Escherichia coli* isolates (No. RAMS with PCR-positive colonies). 3 AE-STEC = Attaching-Effacing Shigatoxigenic *E. coli*. 4 No. serotype-positive *E. coli* isolates (No. RAMS).

Table 4. Distribution of the O somatic serotypes according to the farms and sampling times in the 42 RAMS with typeable colonies The O5, O80, O118, O121 and O165 serotypes were not identified.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sampling / Farm | A | B | C | TOTAL |
| S1 1 | O145 (1) 2 | O111 (1) | O111 (1) | O111 (2), O145 (1) |
| S2 | O157 (1) | O26 (3), O111 (2), O145 (2) | O111 (3), O145 (2) | O26 (3), O111 (5), O145 (4), O157 (1) |
| S3 | O103 (3), O111 (4)  O145 (3) | O26 (1), O103 (1), O145 (4) | O111 (7), O145 (4), O157 (2) | O26 (1), O103 (4), O111 (11), O145 (11), O157 (2) |
| TOTAL | O103 (3), O111 (4), O145 (4), O157 (1) | O26 (4), O103 (1), O111 (3), O145 (6) | O111 (11), O145 (6), O157 (2) | O26 (4), O103 (4), O111 (18), O145 (16), O157 (3) |

1 S = Sampling. 2 O serotype (N° positive RAMS).

Table 5. Pathotypes and virulotypes of the triplex PCR-positive colonies according to the agar media.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Pathotypes | | EPEC | AE-STEC 2 (= EHEC) | | | STEC | | | Total PCR-positive colonies (%) | Total RAMS with PCR-positive colonies (%) |
| Virulotypes | | *eae* | *eae,stx1* | *eae,stx2* | *eae,stx1,stx2* | *stx1* | *stx2* | *stx1,stx2* |
| Agar media 1 | MC | 11 (5) 3 | 2 (1) | 3 (1) | 6 (2) | 2 (2) | 12 (5) | 1 (1) | 37 (5%) | 16 (11%) |
| ES | 12 (5) | 2 (1) | 0 | 0 | 3 (2) | 14 (6) | 3 (3) | 33 (5%) | 17 (12%) |
| ESTe | 5 (4) | 1 (1) | 0 | 1 (1) | 0 | 0 | 0 | 7 (3%) | 5 (8%) |
| STEC | 103 (30) 4 | 28 (10) | 8 (3) | 38 (11) | 0 | 1 (1) | 38 (12) | 216 (45%) | 58 (54%) |
| Total virulotypes | | 131 (36) | 33 (10) | 11 (3) | 45 (11) | 5 (4) | 27 (8) | 42 (16) | 294 (14%) | 69 (45%) |
| Total pathotypes | | 131 (36) | 89 (23) | | | 74 (27) | | |

1 MC = McConkey’s; ES = Chromocult Coliform ES; ESTe = Chromocult Coliform ES supplemented with TeK; STEC = supplemented CHROMagarTM STEC base. 2 AE-STEC = Attaching-Effacing Shigatoxigenic *Escherichia coli*. 3 No. PCR-positive *E. coli* isolates (No. RAMS with PCR-positive *E. coli* isolates). 4 Including one isolate from one diarrheic calf.

4. Discussion

Using RAMS, <6-month-old dairy calves from 3 farms in Belgium are identified as healthy carriers not only of (AE-)STEC serotypes, but also of EPEC. Although the populations are different (dairy calves and beef cattle), as are the regions in Belgium (Flanders and Wallonia), more calves in farms than adult cattle at slaughterhouse harbor one “gang-of-seven” serotype (61% *vs.* 19%) and more “gang-of-seven“ serotypes (O26, O103, O111, O145 and O157 *vs.* O26, O103 and O157) are detected during this study than during the study by Thiry and collaborators [17]. Such a higher prevalence of “gang-of-seven” serotypes in young calves has already been reported during different surveys in Australia and New-Zealand in dairy, beef or veal calves compared to adult animals sampled at slaughterhouses [9,10,25].

Nevertheless, the identified serotypes are not evenly distributed in the three farms at the 3 samplings (Table 4). For instance, the serotype O26 is identified only in FarmB while serotypes O157 and O103 are not detected in FarmB and FarmC, respectively. These 5 serotypes are neither evenly distributed between the pathotypes and virulotypes (Table 3). For instance, the O26 and O103 serotypes are detected only amongst eae+ EPEC and eae+stx1+ AE-STEC. In addition, more RAMS harboring AE-STEC isolates (83%) are positive for one of those 5 serotypes, than STEC (52%) and EPEC (53%) (Table 3). These latter results are similar to those in diarrheic calves, but different from those in healthy cattle in Belgium [13,17].

Conversely, the other 3 serotypes that were looked for in this study, O5, O80 and O118 were not identified, confirming previous findings in Belgium [17,26] and in Europe in general [4] that they are absent, or very rare in asymptomatic calves and adult cattle. This situation is quite different from the situation in young diarrheic calves that can be infected with O5, O118 and O80, in addition to O26 and O111 AE-STEC and EPEC [12-14,16]. Their absence could mean that these O serotypes are present in very low numbers, under the detection level, in healthy calves and adult cattle, or that the sampling was not appropriate: recto-anal junction and large intestinal content instead of small intestine in diarrheic calves.

Of the 69 RAMS (30% of all RAMS) with triplex PCR-positive colonies (Table 1), 36 RAMS (15%) harbor EPEC, 23 RAMS AE-STEC (EHEC) (10%) and 27 RAMS (12%) STEC. The *eae+* EPEC is the most frequent virulotype while the *eae+stx1+stx2+* virulotype is the most frequent amongst AE-STEC and the *stx1+stx2+* amongst STEC. Moreover, 15 RAMS (6%) harbor *E. coli* belonging to more than one virulotype, a situation previously reported in Belgium [27]. These results are to some extent different from those obtained during the study in 2 slaughterhouses in Belgium [17]. For instance, although the percentage of colon samples harboring triplex PCR-positive colonies is similar (25%), more of the colon samples harbor *E. coli* belonging to different virulotypes (15%). Moreover, though the *eae+* EPEC is the most frequently identified virulotype in both surveys, the most frequent AE-STEC and STEC virulotypes are different: *eae+stx1+* and *stx2+*.

Differences in the percentage of RAMS positive for the presence of EPEC, AE-STEC or STEC are observed between farms and between samplings. This percentage is much lower in FarmA (20%) than in Farm B (37%) and in FarmC (32%) and at Sampling1 (6%) than at Sampling2 (28%) and at Sampling3 (47%). The distributions of pathotypes are also different according to the farms and to the samplings (Table 2). A majority of RAMS are positive for EPEC in FarmA and FarmB and for STEC in FarmC while a minority of RAMS are positive for AE-STEC in FarmB and for EPEC in FarmC. Not enough RAMS are positive at Sampling1 for a detailed analysis, but the percentage of EPEC-positive RAMS decreases from Sampling2 to Sampling3 while the percentage of STEC-positive RAMS increases and the percentage AE-STEC-positive remains stable. Several hypotheses may explain for such differences between farms and samplings. Were the STEC and EPEC present in such low numbers in FarmA that testing only 5 colonies per agar medium was not enough? Was Farm A less contaminated with STEC and EPEC? Or were the *stx* genes more unstable *in vivo* or in *vitro* in the *E. coli* isolates of FarmA? Similarly, the reason for the increase of RAMS with PCR-confirmed colonies according to the sampling in the 3 Farms (though at different rates) can only be hypothesized, e.g. by the introduction of new asymptomatic carriers and/or by an increase of the shedding of STEC and EPEC in the feces of young or adult animals following some stress. Spread of one (AE-)STEC or EPEC serotype within a farm has been observed as soon as one calf becomes a super-shedder and the numbers of shedders increase with the number of calves and with their age [11]. Another hypothesis would be the occurrence of EPEC- or AE-STEC-associated diarrhea in some of these young calves. However, this last hypothesis is of a very low probability since only one of the 11 diarrheic calves sampled excrete EPEC, and none (AE-)STEC.

Besides their prevalence, the second purpose of this study was to follow the persistence of the different virulotypes and serotypes in the same calves. The results confirm not only that one negative calf can become a shedder a few weeks later probably after being contaminated by another shedder, but also that EPEC, AE-STEC and STEC belonging to different virulotypes and serotypes can be excreted at different times by one single calf illustrating possible multiple contamination events. These results are similar to those obtained by Rice and collaborators [18] who could differentiate between cattle colonized by and cattle transiently shedding O157:H7 *E. coli* using RAMS. Therefore, more than one sampling over a period of several weeks should be recommended when performing a survey in any farm.

Although less healthy dairy calves in those 3 farms than adult cattle in slaughterhouses in Belgium harbor “non-gang-of-seven” serotypes (65% *vs.* 97%) and although the identification of their actual serotypes will be the purpose of future studies, the question in surveys is the same as previously [17]: “How to isolate and identify them”? Indeed so far, the selective methodologies target only the “gang-of-seven” serotypes. The results of this study were obtained using a first enterobacteria enrichment step followed by growth on four (semi-)selective agar media. MC and ES are selective for enterobacteria and coliforms in general, respectively. ESTe and STECB are selective for tellurite-resistant coliforms, including the majority, if not all “gang-of-seven” and several “non-gang-of-seven” (AE-)STEC and EPEC, in opposition to the majority of non-STEC non-EPEC strains [28,29]. This selective property is reflected by the lower number of RAMS with colonies growing on ESTe and STECB, 63 and 107 out of 148, respectively (Table 5). STECB is the most performant during this study with a similar rate than in the previous study [17]. While MC and ES also have a similar efficiency in either studies, the situation is highly different for ESTe (7% of RAMS *vs.* 53% of colon contents). The reason for this lower efficiency of ESTe in this study is unknown.

The choice between serotype-selective *vs.* a non-selective procedure depends on the actual purpose of the study: studying the distribution and circulation of some “gang-of-seven” serotypes or of all EPEC and (AE-)STEC in one farm or one region? The answer to this question is most important since prevalence and incidence results can differ according to the procedure. For instance, using a serotype-selective procedure on the same RAMS [20], O157 EPEC and (AE-)STEC are detected in FarmA and in FarmC at more sampling times than in this study and O26 EPEC and (AE-)STEC are detected not only in FarmB like in this study, but also in FarmA and in FarmC.

The final question is the actual place of the EPEC belonging to same serotypes as AE-STEC. So far, to our knowledge indeed, no classical genetic method has been able to fully distinguish between true EPEC and AE-STEC having lost the *stx* genes [23,30,31]. Recently however we performed a phylogenetic analysis of ca. 50 AE-STEC and EPEC O80:H2 isolated from humans and calves, based on the Single Nucleotide Polymorphims (SNP) and constructed a Maximum Likehood (ML) tree that could distinguish between true EPEC and AE-STEC suspect of having lost the *stx* genes [14]. This may also represent a possibility of analyzing other serotypes containing both AE-STEC and EPEC [1-4,12,15,19,32,33].

5. Conclusion

The results of this survey confirm (i) that, besides suffering diarrhea from AE-STEC and EPEC [12-16], healthy young calves in farms in Belgium can also be asymptomatic carriers of (AE-)STEC and EPEC at least at the same rate as adult cattle and (ii) that several (AE-)STEC and EPEC belong to several other O serotypes that are not considered in most surveys and must still be identified.

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