

ORIGINAL ARTICLE

Low prevalence of the 'gang of seven' and absence of the O80:H2 serotypes among Shigatoxigenic and enteropathogenic *Escherichia coli* (STEC and EPEC) in intestinal contents of healthy cattle at two slaughterhouses in Belgium in 2014

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Abstract

Aims: The purpose of this survey was to estimate the respective prevalence of the 'gang of seven' and 'non-gang of seven' serotypes of Shigatoxigenic and enteropathogenic *Escherichia coli* and to identify the O80:H2 serotype in 245 intestinal contents collected at two slaughterhouses in Belgium in 2014.

Methods and Results: After overnight enrichment growth, the 69 intestinal contents testing positive with PCR targeting the *eae*, *stx1* and *stx2* genes were inoculated onto four agar media. Of the 2542 colonies picked up, 677 from 59 samples were PCR confirmed. The most frequent virulotypes were *eae*⁺ in 47 (80%) samples, *stx2*⁺ in 20 (34%) samples and *eae*⁺ *stx1*⁺ in 16 (27%) samples. PCR-positive colonies belonged to different virulotypes in 36 samples. No colony was O80-positive, whereas two *eae*⁺ colonies from two samples were O26:H11, 50 *eae*⁺ *stx1*⁺ and *eae*⁺ from eight samples were O103:H2 and two *eae*⁺ *stx1*⁺ *stx2*⁺ colonies from one sample were O157:H7.

Conclusions: The 'non-gang of seven' serotypes are more frequent than the 'gang of seven' serotypes and the O80:H2 serotype was not detected among Shigatoxigenic and enteropathogenic *Escherichia coli* in the intestines of cattle at these two slaughterhouses.

Significance and Impact of the Study: Although the identification protocols of Shigatoxigenic *Escherichia coli* focus on the 'gang of seven' serotypes, several other serotypes can be present with possible importance in public health. Innovative selective identification procedures should be designed.

Introduction

The 'enterohaemorrhagic *Escherichia coli*' (EHEC) pathotype is defined on the basis of one clinical sign that can be observed in humans, that is, haemorrhagic colitis. Their most important virulence-associated properties are

the production of the histological attaching–effacing (AE) lesion and of Shiga toxins (Stx1 and/or Stx2). However, not all AE- and Stx-producing *E. coli* cause haemorrhagic colitis in humans. Therefore, these pathogenic *E. coli* are also members of attaching–effacing *E. coli* (AEEC) and of the Shigatoxigenic *E. coli* (STEC) (Moxley and Smith

2010; Mainil and Fairbrother 2014; Tozzoli and Scheutz 2014). Recently, the acronym AE-STE C was proposed to distinguish them from other AE-negative STE C pathotypes (Saa-STE C , Agg-STE C , etc.) (Piérard *et al.* 2012).

Human AE-STE C belong to hundreds of serotypes of whom the O26:H11, O103:H2, O111:H-, O121:H19, O145:H-, O157:H7 and O165:H25 ('the gang of seven') are the most frequent and pathogenic worldwide (Tozzoli and Scheutz 2014; Beutin and Fach 2015; Stevens and Frankel 2015). Nevertheless, other STE C serotypes can be emerging either occasionally or on the long term, like the O104:H4 Agg-STE C in Germany in 2011 (Navarro-Garcia 2015) and the O80:H2 AE-STE C serotype recently in France (Soysal *et al.* 2016). Human infection frequently occurs via consumption of animal or plant-derived food-stuffs contaminated by faecal material of ruminants, mostly bovines, that can be asymptomatic carriers in their intestinal tract (Beutin and Fach 2015; Persad and Lejeune 2015).

Besides AE-STE C , enteropathogenic *E. coli* (EPEC) also produce the AE lesion, but no Stx toxins. Based on the production of the type 4 fimbriae called bundle-forming pili (BFP), the EPEC pathotype is divided into typical (tEPEC) and atypical (aEPEC): the former produce BFP and have been isolated almost exclusively from humans, while the latter do not produce BFP and are responsible for diarrhoeic diseases in humans and several animals, including young calves. aEPEC can belong to similar serotypes as AE-STE C , like O26:H11, though the serotypes of the majority are still unidentified (Moxley and Smith 2010; Mainil and Fairbrother 2014; Tozzoli and Scheutz 2014). Recently, however, the O80:H2 serotype was identified in 40% of the calf EPEC isolated between 2009 and 2015 from diarrhoeic calves in Belgium. According to recent results of the molecular virulotyping, the calf O80:H2 EPEC are close to human AE-STE C , since they all harbour the ξ (XI) variant of the *eae* gene and the *fliC_{H2}* gene (Thiry *et al.* 2017). But whether cattle can be at the origin of human contamination by aEPEC is still a matter of debate.

Therefore, to assess bovines as a source of contamination of humans and of calves with 'non-gang of seven' STE C and/or EPEC serotypes, respectively, it is important to determine their prevalence in healthy cattle at slaughterhouses. Initially all isolation procedures and methodology focused on O157:H7 AE-STE C . Today other 'gang of seven' AE-STE C , especially belonging to the O26, O103, O111 and O145 somatic serogroups, can also be specifically isolated using different specific and selective methods (Beutin and Fach 2015). But one question remains: How much are those results relevant for the 'non-gang of seven' serotypes, such as O80:H2?

The purpose of this survey at two slaughterhouses in Belgium was therefore: (i) to identify the most frequent

AE-STE C , EPEC and STE C virulotypes in the intestinal contents of healthy cattle; (ii) to estimate the respective prevalence of the 'gang of seven' and 'non-gang of seven', including O80:H2, among the different AE-STE C , EPEC and STE C virulotypes and (iii) to assess a procedure of identification of AE-STE C , EPEC and STE C using colony hybridization and PCR on colonies after growth on four different (semi-) selective agar media.

Materials and methods

Sampling and preliminary screening

Two hundred forty-five samples of intestinal contents (terminal colon) were collected at two slaughterhouses in Belgium in 2014: from 25 culled cows, 4 heifers and 66 bulls in slaughterhouse no. 1 and from 150 bulls at slaughterhouse no. 2. One gram of all samples were incubated overnight at 37°C in 9 ml lauryl sulphate broth (Sigma-Aldrich, Germany) for enterobacteria enrichment, that were tested with a triplex PCR targeting the *eae*, *stx1* and *stx2* genes (Iguchi *et al.* 2012).

Each PCR-positive broth was subsequently streaked onto four agar plates that were incubated overnight at 37°C: McConkey's and Chromocult Coliform ES (VWR, Belgium), Chromocult Coliform ES supplemented with 2.5 mg ml⁻¹ of potassium tellurite (TeK) (Sigma-Aldrich, Germany) (Zadik *et al.* 1993) and supplemented Chromagar STE C base (I2A, France).

Identification of AE-STE C , EPEC and STE C pathotypes and virulotypes

Up to 10 colonies per agar plate were picked up, inoculated into 200 μ l Luria-Bertani (LB) broth in 96-well microtitre plates, incubated overnight at 37°C and 1 μ l from each well was transferred onto LB agar plates using a 'transfer comb' that were incubated overnight once more. The colonies were transferred by contact onto Whatman 541 paper filters (VWR, Belgium) that were treated to lyse the cells and to denature the DNA. The colony hybridization was performed with PCR-derived ³²P radioactively labelled gene probes targeting the *eae*, *stx1* and *stx2* genes, as previously described (Szalo *et al.* 2002; Iguchi *et al.* 2012). 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. The same triplex PCR for the *eae*, *stx1* and *stx2* genes (Iguchi *et al.* 2012) was performed after DNA extraction by the alkaline boiling method (Mainil *et al.* 2011) for confirmation of the virulotypes. Isolates with hybridization/PCR discordant results were retested with the PCR.

Serotyping and genotyping of the AE-STEC, EPEC and STEC pathotypes

Triplex PCR-positive AE-STEC, EPEC and STEC were further tested with one heptaplex PCR for the O26, O103, O111, O121, O145, O157, O165 antigens, and with PCR for the O80 antigen (Iguchi *et al.* 2015). The O antigen PCR-positive isolates were subtyped with appropriate PCR for the *fliC* genes coding for the H flagellar antigens (Gannon *et al.* 1997; Bardiau *et al.* 2009; Thiry *et al.* 2017), for the *eae* gene subtypes (China *et al.* 1999; Blanco *et al.* 2004) and for the *stx1* and *stx2* gene subtypes (Schmidt *et al.* 2000; Scheutz *et al.* 2012). The O26- and O157-positive isolates were genotyped by the IS621 and IS629 fingerprinting respectively (Ooka *et al.* 2009; Mainil *et al.* 2011).

Results

Screening of intestinal samples and isolates

Sixty-nine intestinal contents from 9 culled cows and 60 bulls tested positive with the triplex PCR for the *eae*, *stx1* and/or *stx2* genes after overnight enrichment in lauryl sulphate broth (Table 1). All 69 PCR-positive enrichment broths grew on McConkey's and Chromagar STEC, while 68 gave a positive growth on Chromocult Coliform ES and 53 on TeK Chromocult Coliform ES. Of the total of 2542 coliform colonies picked up, 744 isolated from 62 intestinal contents tested positive with at least one of the three *eae*, *stx1* and *stx2* gene probes (Table 1). Of these 744 probe-positive colonies, 677 (91%) isolated from 59 of the 245 intestinal contents (24%) were confirmed with the triplex PCR targeting the same three genes (Table 1). Hybridization and PCR virulotypes were in agreement for 611 of the 677 PCR-positive colonies (90%).

PCR-positive colonies were identified in 25 of the 59 intestinal contents (42%) after growth on McConkey's

and/or Chromocult Coliform ES, whereas 56 intestinal contents (95%) gave PCR-positive colonies after growth on TeK Chromocult Coliform ES and/or Chromagar STEC. Nevertheless, PCR-positive colonies were identified after growth on each of the four agar media in only seven intestinal contents (12%).

Identification of AE-STEC, EPEC and STEC virulotypes

More than one PCR-positive colony (up to 29) was identified in 57 of these 59 intestinal contents (97%) and they belonged to different virulotypes in 36 intestinal contents (61%). The most frequent virulotypes (Table 2) were *eae+* EPEC in 47 intestinal contents (80%), *stx2+* STEC in 20 intestinal contents (34%) and *eae+* *stx1+* AE-STEC in 16 intestinal contents (27%). These three virulotypes were more frequently identified after growth on TeK Chromocult Coliform ES and/or Chromagar STEC (from 40, 18 and 17 intestinal contents respectively) than after growth on McConkey's and/or Chromocult Coliform ES (from 21, 3 and 2 intestinal contents respectively). The other virulotypes were isolated from 4 to 10 intestinal contents (Table 2) after growth on only TeK Chromocult Coliform ES and/or Chromagar STEC, with one exception.

Identification of AE-STEC, EPEC and STEC serotypes

Although 57 of the 59 PCR-positive intestinal contents (97%) harboured 'non-gang of seven' AE-STEC (22 animals), EPEC (47 animals) and/or STEC (32 animals), none of the 677 PCR-positive colonies tested positive with the O80 serogroup PCR. Conversely, 11 animals harboured 'gang of seven' serotypes (Table 3): O26 *eae+* EPEC (two isolates), O103 *eae+* EPEC (38 isolates) and *eae+* *stx1+* AE-STEC (12 isolates) and O157 *eae+* *stx1+* *stx2+* AE-STEC (two isolates). The serotyping PCR results were confirmed with the O26, O103 and

Table 1 Colony hybridization and PCR results on the intestinal contents and isolated colonies from different healthy cattle at two slaughterhouses in Belgium

Tests	No. positive samples from (No. isolates)			
	Culled cows	Bulls S#1	Bulls S#2	Total
Lauryl sulphate broth triplex PCR	9/25	14/66	46/150	69/245*
Growth on the four agar media†	9 (320)	14 (516)	46 (1706)	69 (2542)
Colony filter triplex hybridization	8 (79)	10 (54)	44 (611)	62 (744)
Colony triplex PCR‡	8 (65)	7 (44)	44 (568)	59 (677)

*Four heifers were also sampled at slaughterhouse no. 1, but were negative at the broth triplex PCR.

†Only the PCR-positive broths were inoculated onto the four agar media.

‡Only the colony hybridization-positive isolates were tested by PCR.

S#1, slaughterhouse no. 1; S#2, slaughterhouse no. 2.

Table 2 Pathotypes and virulotypes of triplex PCR-positive colonies

Pathotypes	Virulotypes	No. positive samples from (No. isolates)			Total virulotypes	Total pathotypes
		Culled cows	Bulls S#1	Bulls S#2		
AE-STEC	<i>eae+</i> <i>stx1+</i>	–	2 (9)	14 (98)	16 (107)	24 (125)
	<i>eae+</i> <i>stx2+</i>	–	1 (1)	3 (4)	4 (5)	
	<i>eae+</i> <i>stx1+</i> <i>stx2+</i>	–	2 (11)	2 (2)	4 (13)	
EPEC	<i>eae+</i>	8 (62)	3 (12)	36 (294)	47 (368)	47 (368)
STEC	<i>stx1+</i>	–	1 (1)	9 (21)	10 (22)	39 (184)
	<i>stx2+</i>	–	2 (8)	18 (108)	20 (116)	
	<i>stx1+</i> <i>stx2+</i>	1 (3)	1 (2)	7 (41)	9 (46)	

S#1, slaughterhouse no. 1; S#2, slaughterhouse no. 2.

Table 3 Identification and typing of the ‘gang of seven’ serotypes

Serotypes (No. isolates)	Virulotypes	<i>eae</i> gene subtypes	<i>stx</i> gene subtypes	Samples of origin		
				Culled cows	Bulls S#1	Bulls S#2
O26:H11 (2)	<i>eae+</i> (2)	<i>eaeβ+</i> (2)	Not relevant	1 (1)*	–	1 (1)
O103:H2 (50)	<i>eae+</i> <i>stx1+</i> (12)	<i>eaeε+</i> (12)	<i>stx1a+</i> (12)	–	–	2 (12)†
	<i>eae+</i> (38)	<i>eaeε+</i> (38)	Not relevant	–	–	7 (38)†
O157:H7 (2)	<i>eae+</i> <i>stx1+</i> <i>stx2+</i> (2)	<i>eaeγ+</i> (2)	<i>stx1a+</i> <i>stx2c+</i> (2)	–	1 (2)	–

*Number of animals.

†One bull harboured both O103:H2 EPEC and AE-STEC.

S#1, slaughterhouse no. 1; S#2, slaughterhouse no. 2.

O157 serogroup uniplex PCR. Those ‘gang of seven’ serogroups were identified along with ‘non-gang of seven’ serogroups in 9 of the 11 positive intestinal contents. Both O26 EPEC harboured the *fliC_{H11}* gene, all 50 O103 EPEC and AE-STEC harboured the *fliC_{H2}* gene and both O157 AE-STEC harboured the *fliC_{H7}* gene (Table 3).

One O26:H11 EPEC and the two O157:H7 AE-STEC were isolated on Chromagar STEC and the second O26:H11 EPEC on TeK Chromocult Coliform ES, while the 38 O103:H2 EPEC were isolated on McConkey’s and Chromocult Coliform ES and the 12 O103:H2 AE-STEC were isolated on TeK Chromocult Coliform ES and Chromagar STEC.

Identification of O26, O103 and O157 AE-STEC and EPEC genotypes

Both O26 EPEC also tested positive with the PCR for the *eaeβ* genes and both O157 AE-STEC with the PCR for the *eaeγ1*, *stx1a* and *stx2c* genes. All 50 O103 EPEC and AE-STEC tested positive with the PCR for the *eaeε* genes and the 12 AE-STEC with the PCR for the *stx1a* gene (Table 3). The two O26:H11 EPEC belonged to two different IS621 fingerprints (6732– and 6733–; Fig. 1), either different from the IS621 type (6733+) of the control human O26:H11 AE-STEC (Mainil *et al.* 2011).

Conversely, both O157:H7 AE-STEC belonged to the same ‘I’ IS629 fingerprint (Fig. 2), like human AE-STEC isolated in Belgium in 2011 and 2014 (Piérard and De Rauw 2016).

Discussion

Today, most studies on the STEC prevalence in humans, bovines and/or foodstuffs are directed towards the isolation of some or all ‘gang of seven’ STEC serotypes (Joris *et al.* 2011, 2013; Beutin and Fach 2015) and neglect dozens of other serotypes. Nevertheless, those rarer serotypes can also cause isolated cases or outbreaks in humans, like the O104:H4 Agg-STEC in Germany in 2011 (Navarro-Garcia 2015), and the since-2015-emerging AE-STEC O80:H2 in France (Soysal *et al.* 2016). Similarly, these procedures also neglect EPEC, although several belong to the same serotypes as STEC (Moxley and Smith 2010; Mainil and Fairbrother 2014; Tozzoli and Scheutz 2014). In the present study, one fourth (24%) of the 245 sampled culled cows, bulls and heifers were positive for the presence of AE-STEC, EPEC and/or STEC (Tables 1 and 2), with 36 (15%) of them harbouring more than one virulotype, as seen in previous studies (Beutin and Fach 2015). Nevertheless, only 11 animals (4.5%) (one culled cow and 10 bulls) harboured ‘gang of seven’ AE-STEC

Figure 1 IS621 right (3') end fingerprints of the two O26:H11 bovine EPEC: isolate 2665 (6733– fingerprint) from the culled cow and isolate 800 (6732– fingerprint) from the bull. The O26:H11 human EH324 AE-STE C (6733+ fingerprint) is the internal control strain (Mainil *et al.* 2011; Piérard and De Rauw 2016).

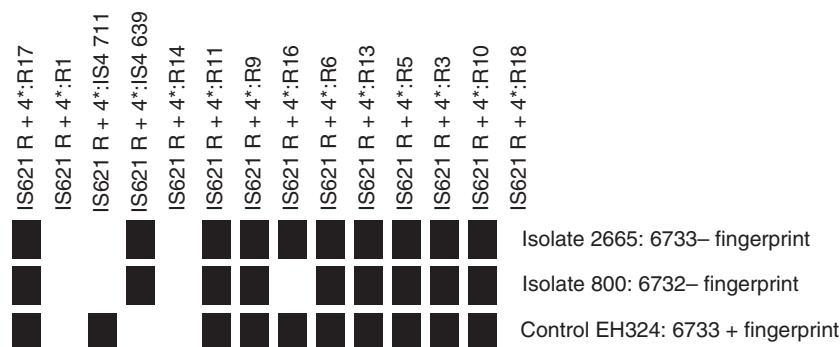
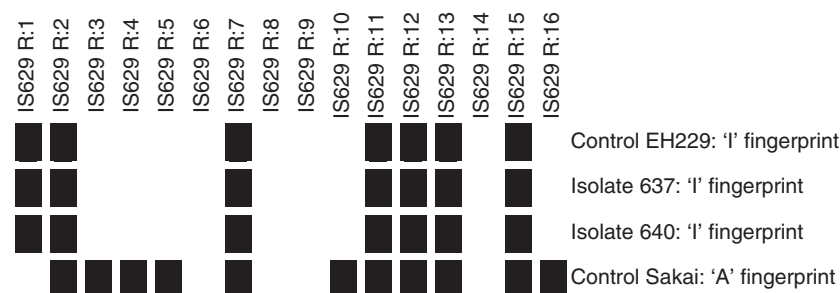


Figure 2 IS629 right (3') end fingerprints of the two O157:H7 AE-STE C: isolates 637 and 640 from one bull. The O157:H7 human EH2429 AE-STE C is the 'I' fingerprint control strain and the O157:H7 human Sakai AE-STE C is the internal control strain (Ooka *et al.* 2009; Piérard and De Rauw 2016).



and/or EPEC serotypes (O26:H11, O103:H2 and O157:H7) (Table 3), with nine of them also harbouring 'non-gang of seven' serotypes.

The bovine O103:H2 and O157:H7 AE-STE C were closely related, if not identical, to some O103:H2 and O157:H7 human STE C isolates of the Belgian NCR collection based on their virulotypes (Table 3). Moreover, the two bovine O157:H7 AE-STE C belong to the same 'I' IS629 fingerprint as human O157:H7 AE-STE C isolated in 2011 and 2014. Therefore, they may indeed represent a potential threat for humans (Piérard and De Rauw 2016). Conversely, the actual status of the O26:H11 and O103:H2 EPEC that could also be related to O26:H11 and O103:H2 human AE-STE C isolates (Table 3) is a matter of debate. Some EPEC can indeed derive from AE-STE C after *in vitro* or *in vivo* loss of the *stx* genes, while others might be a precursor of AE-STE C, and still others unrelated clones (Moxley and Smith 2010; Mainil and Fairbrother 2014; Tozzoli and Scheutz 2014). The IS621 fingerprints (6732– and 6733–) of the two O26:H11 EPEC for instance have not been found previously among Belgian human O26:H11 AE-STE C or EPEC, and only one of them (6733–) was already observed in two American bovine AE-STE C isolated from under 6 months of age healthy cattle (Mainil *et al.* 2011). The completeness of their virulotypes and genotypes (PFGE, MLST and SNP types) should help to understand their actual clonal relationship with corresponding human AE-STE C (Bugarel *et al.* 2011; Iguchi *et al.* 2012).

In contrast, all but two animals (97%) harboured 'non-gang of seven' AE-STE C (22 animals), EPEC (47 animals) and/or STE C (32 animals). The absence of O80 AE-STE C and EPEC among the 'non-gang of seven' isolates may have different explanations: (i) a *geographical bias*—O80:H2 AE-STE C are emerging in humans in France (Soysal *et al.* 2016), not in Belgium. Conversely, the O80:H2 EPEC were isolated from diarrhoeic calves in Wallonia (Thiry *et al.* 2017) where the two slaughterhouses are located; (ii) a *time bias*—The intestinal samples were collected in 2014. Nevertheless, the O80:H2 AE-STE C have been emerging since *c.* 2010 (Soysal *et al.* 2016) and the O80:H2 EPEC were isolated between 2009 and 2014 (Thiry *et al.* 2017); (iii) a *sampling bias*—Bulls represent up to 90% of the sampled animals *vs* 10% of cows. This might be an explanation for O80:H2 EPEC, but is unlikely for O80:H2 AE-STE C. Alternatively, O80 *E. coli* were not present at all on these 245 intestinal contents, since the O80 antigen PCR performed on all enrichment broths gave only negative results (not shown); (iv) an *isolation procedure bias*—Both TeK Chromocult Coliform ES and Chromagar STE C may inhibit the growth of O80:H2 AE-STE C and EPEC that would not be present at a sufficient concentration to be detected on McConkey's and/or Chromocult Coliform ES.

As a first conclusion, the AE-STE C, EPEC and STE C 'non-gang of seven' serotypes are much more frequent than the 'gang of seven' serotypes in the intestines of cattle at these two slaughterhouses in Belgium. Identification

of their actual serotypes will be the purpose of future studies, but the question is already 'how to isolate and identify them'? The results of this study were obtained using a first enterobacteria enrichment step followed by growth on four (semi-) selective agar media: McConkey's and Chromocult Coliform ES are selective for enterobacteria and coliforms in general respectively; TeK Chromocult Coliform ES and Chromagar STEC are selective for Te^{++} resistant coliforms. Therefore, most, if not all, 'gang of seven' STEC and EPEC should selectively grow on the latter two agar media as would several, but not all, STEC and EPEC belonging to other serogroups, at the opposite of the majority of non-STEC non-EPEC strains that are not Te^{++} -resistant (Verhaegen *et al.* 2015). The PCR results confirm this tendency since 56 intestinal contents (95%) gave PCR-positive colonies on TeK Chromocult Coliform ES and/or Chromagar STEC vs 25 intestinal contents (42%) on McConkey's and/or Chromocult Coliform ES. Nevertheless, (i) three intestinal contents gave PCR-positive colonies only on McConkey or Chromocult coliform ES, but at a very low rate; and (ii) some virulotypes were identified only in colonies growing on McConkey's and Chromocult Coliform ES, for example, the O103:H2 EPEC. Those results indicated that different (semi-) selective agar media should be used for screening and isolating the target pathogenic *E. coli*.

The same reasoning can also be applied to colony hybridization vs the PCR, keeping in mind that 90% of the colony hybridization-positive colonies were PCR positive and that their full virulotypes were PCR confirmed for 90% of them. PCR is the method of choice and is of course easier to apply. Nevertheless, the colony hybridization that was applied in medical microbiology in the early 1980s (Moseley *et al.* 1980) can still be helpful as a cheap first-line screening assay when studying several thousands of isolates.

As a general conclusion, future studies should be designed (i) to perform surveys in other slaughterhouses in Belgium to confirm the absence of O80 AE-STEC and EPEC, (ii) to identify the serotypes of the numerous 'non-gang of seven' isolates and (iii) to test different (semi-) selective media to grow and isolate the most threatening serotypes for human health and for young calves, like the O80:H2 AE-STEC and EPEC respectively.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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