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Identification of Shiga toxin-producing (STEC) and enteropathogenic (EPEC) *Escherichia coli* in diarrhoeic calves and comparative genomics of O5 bovine and human STEC

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ABSTRACT

Escherichia coli producing Shiga toxins (Stx) and the attaching-effacing (AE) lesion (AE-STEC) are responsible for (bloody) diarrhoea in humans and calves while the enteropathogenic *E. coli* (EPEC) producing the AE lesion only cause non-bloody diarrhoea in all mammals. The purpose of this study was (i) to identify the pathotypes of enterohaemolysin-producing *E. coli* isolated between 2009 and 2013 on EHLY agar from less than 2 month-old diarrhoeic calves with a triplex PCR targeting the *stx*1, *stx*2, *eae* virulence genes; (ii) to serotype the positive isolates with PCR targeting the genes coding for ten most frequent and pathogenic human and calf STEC O serogroups; and (iii) to compare the MLSTypes and virulotypes of calf and human O5 AE-STEC after Whole Genome Sequencing using two server databases (www.genomicepidemiology.org). Of 233 isolates, 206 were triplex PCR-positive: 119 AE-STEC (58%), 78 EPEC (38%) and 9 STEC (4%); and the *stx1+eae+* AE-STEC (49.5%) were the most frequent. Of them, 120 isolates (84% of AE-STEC, 23% of EPEC, 22% of STEC) tested positive with one O serogroup PCR: 57 for O26 (47.5%), 36 for O111 (30%), 10 for O103 (8%) and 8 for O5 (7%) serogroups. The analysis of the draft sequences of 15 O5 AE-STEC could not identify any difference correlated to the host. As a conclusion, (i) the AE-STEC associated with diarrhoea in young calves still belong to the same serogroups as previously (O5, O26, O111) but the O103 serogroup may be emerging, (ii) the O5 AE-STEC from calves and humans are genetically similar.

1. Introduction

While most *Escherichia* (*E.*) *coli* strains are commensal members of the intestinal microbiota of animals and humans, some strains are important pathogens that cause a wide spectrum of diseases ranging from self-limiting to life-threatening intestinal and extra-intestinal illnesses. Of the many pathogenic *E. coli* strains, those producing the Shiga toxins (Stx) are responsible for the haemolytic–uremic syndrome (HUS) in humans, whereas those producing the histological Attaching-Effacing (AE) lesion are responsible for non-bloody diarrhoea in humans and several animal species (Köhler and Dobrindt, 2011; Mainil and Fairbrother, 2014; Tozzoli and Scheutz, 2014). Strains producing the Stx are named STEC (after Shigatoxigenic *E. coli*) and strains producing the AE lesion are named EPEC (after enteropathogenic *E. coli*), while strains producing both Stx and AE lesion have been named EHEC (after enterohaemorrhagic *E. coli*) on the basis on the clinical syndrome most often observed in humans, i.e. (haemorrhagic) colitis (HC) and (bloody) diarrhoea, followed by the HUS in 10% of the cases (Davis et al., 2015; Kaper and O'Brien, 2015). However some debate exists about the EHEC name because not all of Stx- and AE-positive strains are associated with HC in humans (Tozzoli and Scheutz, 2014; Beutin and Fach, 2015). Therefore two of us

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recently proposed to rename them AE-STEC (Piérard et al., 2012) and this nomenclature will be followed in this manuscript.

AE-STEC are a serious public health hazard in developed countries. The primary source of human infection is foodstuffs contaminated by the faeces of ruminants, especially cattle that can be healthy carriers in their intestinal tract. Numerous studies have been published about the prevalence of the carrier state, the incidence of faecal shedding and the virulotypes of AE-STEC present in asymptomatic cattle (Mekata et al., 2014; Beutin and Fach, 2015; Persad and Lejeune, 2015). Besides the O157:H7 serotype, that is the most frequently responsible for large outbreaks and the most pathogenic to humans, AE-STEC can belong to scores of serotypes (Tozzoli and Scheutz, 2014; Beutin and Fach, 2015). Of these, 5 have been frequently identified worldwide and are most pathogenic for humans together with O157:H7: O26:H11, O103:H2, O111:H-, O121:H19, and O145:H-. In addition to the phage-located *stx1* and *stx2* genes, those AE-STEC can harbour several other genes coding for virulence-associated factors: a set of genes on a pathogenicity island named the Locus of Enterocyte Effacement (LEE) code for a type three secretion system (T3SS), T3-secreted effectors and the outer membrane intimin adhesin (the *eae* gene); different chromosomal non LEE-located genes code for other T3-secreted effectors and for other adhesins or toxins; and still other genes located on a plasmid (pO157 plasmid) code for several virulence markers, including one entero-haemolysin (Ehly) (Mainil and Daube, 2005; Bugarel et al., 2010; Beutin and Fach, 2015; McWilliams and Torres, 2015; Stevens and Frankel, 2015).

Some AE-STEC can be causing diarrhoea in young calves (Mainil and Daube, 2005; Moxley and Smith, 2010; Mainil and Fairbrother, 2014). However, those AE-STEC from young calves receive much less attention today and they are seldom included in comparative studies of isolates from healthy cattle and from humans (Beutin and Fach, 2015; Caprioli et al., 2015). Therefore their actual prevalence, virulotypes and host specificity are largely unknown. According to the latest review by Mainil and Fairbrother (2014), several AE-STEC from diarrhoeic calves belong to some of the most frequent and pathogenic serotypes in humans such as O26:H11 and O111:H-, while others belong to subdominant serotypes that are quite rarely isolated from diseased humans and/or healthy cattle, though some of them such as O5:H- and O118:H16 are listed as emerging AE-STEC serotypes in humans (Beutin and Fach, 2015). Moreover if the genome analyses of several non-O157 AE-STEC have been or are being conducted by different means (Ogura et al., 2009; Lindsey et al., 2014; Sadiq et al., 2015), the genomics of the O5:H- and O118:H16 AE-STEC from diarrhoeic calves has not been.

The aim of this study was twofold (i) to identify the virulotypes and O serogroups of AE-STEC associated with diarrhoea in less than 2 month-old calves in Belgium (Wallonia); (ii) to compare the genomes of bovine and human O5 and O118 AE-STEC identified during this study or already present in our collections.

2. Materials and methods

2.1. E. coli *isolates*

Faecal samples from less than 2 months of age diarrhoeic calves from the south of Belgium (Wallonia) are routinely inoculated onto Enterohemolysin (EHLY) agar plates containing washed sheep blood erythrocytes (Oxoid, Drongen, Ghent, Belgium) at the bacteriological diagnostic laboratory of ARSIA. Between November 2008 and February 2014 a total of 233 Ehly-producing *E. coli* were isolated and stored in 80% glycerol at −70 °C before further studies. Seven O5 AE-STEC from our collections were included in the genome sequencing study: four were isolated from diarrhoeic calves between 1967 and 1993 at the Veterinary Faculty of the

Table 1
Primers and control strains used in this study for the pathotyping (a) and for the serogrouping (b) (adapted from Iguchi et al., 2015).

(a)

PCR	Virulence factor	Primer	Sequence (5′–3′)	Target gene	Amplicon size (bp)
Triplex	Shiga toxin 1	LP30	CAGTTAATGTGGTGGCGAAGG	*stx1*	348
		LP31	CACCAGACAATGTAACCGCTG		
	Shiga toxin 2	LP43	ATCCTATTCCCGGGAGTTTACG	*stx2*	584
		LP44	GCGTCATCGTATACACAGGAGC		
	Intimin adhesin	SK1	CCCGAATTCGGCACAAGCATAAGC	*eae*	881
		SK2	CCCGGATCCGTCTCGCCAGTATTCG		

(b)

PCR	O-genotype	Serogroup	Primer	Sequence (5′–3′)	Target gene	Amplicon size (bp)
Pentaplex 1	Og5	O5	Og5-PCR_F	AGGGCAATCTTCCGTAATGA	*wzy*	566
			Og5-PCR_R	CCTCTTGGGCTATAAACAACC		
	Og26	O26	Og26-PCR_F	GGGGGTGGGTACTATATTGG	*wzx*	241
			Og26-PCR_R	AGCGCCTATTTCAGCAAAGA		
	Og104[a]	O104	Og104-PCR2_F	GCTCGACTTTTTGGTGCGGA	*wzx*	852
			Og104-PCR2_R	TCCTGCTACAGGCACTCCCAA		
	OgC4	O118	OgC4-PCR_F	GTGGGAGTCTGAATCAAGTTGCGA	*wzy*	344
			OgC4-PCR_R	AGCAACCTTACCCAATCCTAAGGG		
	Og145	O145	Og145-PCR_F	TTCGCGCACAGCATGGTTAT	*wzy*	609
			Og145-PCR_R	TACAATGCACCGCAAACAGT		
Pentaplex 2	Og103	O103	Og103-PCR_F	TAAGTACGGGGGTGCTTTTT	*wzx*	716
			Og103-PCR_R	AAGCTCCCGAGCACGTATAA		
	Og111	O111	Og111-PCR_F	CAAGAGTGCTCTGGGCTTCT	*wzx*	451
			Og111-PCR_R	AACGCAAGACAAGGCAAAAC		
	Og121	O121	Og121-PCR_F	CAAATGGGCGTTAATACAGCC	*wzy*	193
			Og121-PCR_R	TTCCACCCATCCAACCTCTAA		
	Og157	O157	Og157-PCR_F	CAGGTGAAGGTGGAATGGTTGTC	*rfbE*	296
			Og157-PCR_R	TTAGAATTGAGACCATCCAATAAG		
	Og165	O165	Og165-PCR_F	GGCGTAAATAAAATATGGGGG	*wzx*	1042
			Og165-PCR_R	GCCCTCTAACAAACGAATTGT		

[a] This O104-specific primer pairs was newly designed in this study.

University of Liège after growth on Gassner agar plates (Merck, Darmstadt, Germany) and were later typed by colony hybridization (Mainil et al., 1993) and three were isolated from humans with (bloody) diarrhoea in 2004 and 2009 at the *Universitair Ziekenhuis* of the *Vrije Universiteit Brussel* after culture on sorbitol-MacConkey (SMAC) and on SMAC with cefixime and tellurite (CT-SMAC) (LabM, Heywood, Lancashire, UK), and typing by PCR (Buvens et al., 2012).

2.2. PCR virulotyping and O segrouping

The virulotyping was performed applying a triplex PCR to detect the *stx1*, *stx2* and *eae* genes. The triplex-PCR positive isolates were assayed with two pentaplex PCRs to detect the O antigen-encoding genes specific to the ten most frequent and pathogenic STEC serogroups in humans and animals (Mekata et al., 2014; Iguchi et al., 2015) (Table 1a and b). Control strains for the triplex and the pentaplex PCRs were included. All *E. coli* isolates and control strains were grown overnight on LB (Luria Bertani) agar at 37 °C. A sample from one colony was re-suspended in one μl of sterile distilled water in 96-well microtitre plates. The PCRs were performed in a total volume of 50 μl after addition of dNTP mixture (Thermo Fisher, Aalst, Belgium), the ThermoPol reaction buffer (New England Biolabs, Bioké, Leiden, The Netherlands), Taq DNA polymerase (New England Biolabs, Bioké, Leiden, The Netherlands) and primers (Eurogentec, Seraing, Belgium). The PCR protocol consisted of an initial denaturation step of one min at 94 °C, followed by 25 cycles of 30 s denaturation at 94 °C, 30 s annealing at 58 °C, one min extension at 72 °C, and a final extension step of 2 min at 72 °C. Amplified fragments from the virulotyping PCR were separated in E-Gel (Invitrogen, Thermo Fisher, Aalst, Belgium) according to the manufacturer's instructions. Amplified fragments from the two serogrouping PCRs were separated by electrophoresis in a 1.5% agarose gels for 30–45 min in TAE buffer (BioRad, Temse, Belgium).

2.3. Whole genome sequencing

Genomic DNA of a total of 12 bovine and 3 human O5 AE-STEC was extracted from a single colony (DNeasy® Blood&Tissue kit, Qiagen, Venlo, Belgium) after overnight growth on LB agar. Genomic libraries were prepared according to the manufacturer's instructions and sequenced on an MiSeq Illumina sequencer using the 2 × 250 v2 reaction chemistry (GIGA, Groupe Interdisciplinaire de Génoprotéomique Appliquée, Liège, Belgium). The raw read sequences (GenBank BioProject PRJNA295953) were assembled into scaffolds using the PLATANUS genome assembler version 1.1.4 (Kajitani et al., 2014) and subjected to an automated annotation pipeline by the Rapid Annotation Sequence Tool (RAST2.0) (Aziz et al., 2008) using as reference the only bovine draft whole genome sequence of an O5:H- AE-STEC isolated from a cow in the USA in 2010 (*E. coli* 970246, GenBank Bioproject PRJNA51087).

2.4. Comparative genomic MLSTyping and virulotyping

Seven housekeeping genes (*adk, fumC, icd, gyrB, mdh, purA, recA*) and different virulence-related genes present in the sequenced isolates were identified using two databases through the Centre of Genomic Epidemiology (CGE) website (www.genomicepidemiology.org): the MLST server database v1.7 (Larsen et al., 2012) and the Virulence Finder server database v1.2 (Joensen et al., 2014). The scaffolds of each isolate were incorporated into these tools as described in CGE, with an identity threshold of 98%. The nucleotide sequences of the concatenated 7 housekeeping and of the *stx1*, *stx2*, and *eae* genes were subsequently extracted and compared using the BioEdit Sequence Editor version7.0 software (Hall, 1999).

In order to study the genetic relationship of the sequenced *E. coli* isolates, the concatenated sequences of the 7 housekeeping genes were aligned with sequences of related genes present in GenBank keeping a query length of 3423 nucleotides. The phylogenetic relationships of all reads were analysed with the MEGA6 software package by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura et al., 2013).

3. Results

3.1. PCR virulotyping and O serogrouping (Tables 2 and 3)

Of the 233 *E. coli* growing on EHLY agar, 206 isolates (88%) tested positive at the triplex PCR for at least one of the three genes: 120 isolates for the *stx1* gene, 24 isolates for the *stx2* gene and 197 isolates for the *eae* gene. AE-STEC were the most frequent with 119 isolates, including 102 *stx1+eae+* isolates, 4 *stx2+eae+* isolates, and 13 *stx1+stx2+eae+* isolates, followed by EPEC (78 isolates), while STEC were rare (9 isolates).

Of these 206 triplex PCR-positive isolates, 120 (58%) tested positive with one of the PCR for the O serogroups. The majority of the AE-STEC (84%) tested positive compared to only 23% of the EPEC and 22% of the STEC. The O26 and O111 serogroups were the most frequently identified: 57 isolates including 42 AE-STEC, and 36 isolates, all but one AE-STEC, respectively.

As for the other 8 serogroups, 10 isolates (9 AE-STEC and 1 EPEC) tested positive with the PCR for the O103 and 8 isolates with the PCR for the O5 serogroup (all 8 were AE-STEC). Finally a total of only 9 isolates tested positive with the PCR for the O121, O145 or O157 serogroups and none with the PCR for the O104, O118 or O165 serogroups.

Since no O118 AE-STEC was isolated, draft genome sequence was obtained from a total of 12 bovine and 3 human O5 AE-STEC isolates. The O serogroup and the pathotypes of the four calf and of the three human O5 AE-STEC from our collections were confirmed by PCR prior to sequencing (Table 4).

3.2. Genome sequencing and MLSTyping

Estimated genome sizes of those 15 bovine and human O5 AE-STEC isolates and of the 970246 strain ranged between 4,881,752 and 5,203,424 bp and the G + C% between 50.4% or 50.6% (Table 4). Based on the genome sequences, all 15 isolates and the 970246 strain were found to harbour not only the *wzy* gene coding for the O5 somatic antigen, but also the *fliC* gene coding for the H9 flagellar antigen (Table 4).

The pairwise alignment of the nucleotide sequences of the *adk*, *fumC*, *icd*, *gyrB*, *mdh*, *purA*, and *recA* genes, which were extracted from the genome sequences, indicated more than 99.8% identity. In a phylogenetic tree constructed on these 7 housekeeping genes (Fig. 1), all 16 O5 *E. coli* isolates from calves, adult bovine and humans grouped in a single cluster whatever the year of isolation.

Table 2
Pathotypes and O serogroups of 206 enterohaemolysin-producing *E. coli* isolated from calves with diarrhoea.

Pathotypes	N° Isolates	Serogroups (N° Isolates)
stx1+	2	O111(1)
stx2+	4	––
stx1 + stx2+	3	O26(1)
stx1 + eae+	102	O5(7), O26(40), O103(9), O111(28), O145(2)
stx2 + eae+	4	O26(2), O145(1), O157(1)
stx1 + stx2 + eae+	13	O5(1), O111(7), O145(1), O157(1)
eae+	78	O26(14), O103(1), O121(2), O145(1)
Total (%)	206 (100%)	120 (58%)

Table 3
Prevalence of the O serogroups according to the pathotype (no O104, O118 or O165 isolate was identified).

Pathotype/Serogroup	O5	O26	O103	O111	O121	O145	O157	Total (%)	NI[a] (%)
STEC (n = 9)	–	1	–	1	–	–	–	2 (22%)	7 (78%)
AE-STEC (n = 119)	8	42	9	35	–	4	2	100 (84%)	19 (16%)
EPEC (n = 78)	–	14	1	–	2	1	–	18 (23%)	60 (77%)
Total (n = 206)	8	57	10	36	2	5	2	120 (58%)	86 (42%)

[a] NI = not identified.

Table 4
Comparison of general and specific genome features of 15 O5:H- AE-STEC isolates from diarrhoeic calves and humans (GenBank BioProject PRJNA295953) and of bovine 970246 strain.

Host	Isolate	Year of isolation	N° of scaffolds (>300 bp)	Total length of scaffolds	Pathotype		Enterohaemolysin		GenBank accession numbers	References
					PCR	Sequencing	EHLY agar	Sequencing		
Calf	20	2009	351	5037936	*stx1* + *eae*+	*stx1a* + *eaeβ*+	+	*ehxA*	LJWY01000000	This study
	60	2010	386	5015475	*stx1* + *eae*+	*stx1a*+ *eaeβ*+	+	*ehxA*	LKCZ01000000	
	86	2010	381	5118737	*stx1* + *eae*+	*stx1a*+ *eaeβ*+	+	*ehxA*	LKDA01000000	
	97	2010	381	4985541	*stx1* + *eae*+	*stx1a*+ *eaeβ*+	+	*ehxA*	LKDB01000000	
	109	2011	236	5203424	*stx1* + *eae*+	*stx1a*+ *eaeβ*+	+	*ehxA*	LKDC01000000	
	174	2012	396	5046409	*stx1* + *eae*+	*stx1a*+ *eaeβ*+	+	*ehxA*	LKDD01000000	
	188	2013	345	4951927	*stx1* + *stx2* + *eae* +	*stx1a* + *stx2a*[a]+ *eaeβ*+	+	*ehxA*	LKDE01000000	
	196	2013	361	4881752	*stx1* + *eae*+	*stx1a*+ *eaeβ*+	+	*ehxA*	LKPK01000000	
	093A	1967	328	5037943	*stx1* + *eae*+	*stx1a*+ *eaeβ*+	ND[d]	–	LKDI01000000	Mainil et al., (1993)
	33645	1984	529	4924512	*stx1* + *eae*+	*stx1a* + *eae*?[b]	ND	*ehxA*	LLEW01000000	
	35402	1986	302	5076698	*stx1* + *eae*+	*stx1a*+ *eaeβ*+	ND	–	LKDH01000000	
	46285	1993	281	4992119	*stx1* + *eae*+	*stx1a*+ *eaeβ*+	ND	*ehxA*	LMZP01000000	
Human	EH1332	2004	300	4950457	*stx1* + *eae*+	*stx1a*+ *eaeβ*+	ND	*ehxA*	LKPL01000000	Piérard, UP[e]
	EH1813	2009	373	5119156	*stx1* + *eae*+	*stx1a*+ *eaeβ*+	ND	*ehxA*	LKDF01000000	Buvens et al., (2012)
	EH1856	2009	332	5051080	*stx1* + *eae*+	*stx1a*+ *eaeβ*+	ND	*ehxA*	LKDG01000000	
Cow	970246	2011	49	5497467	*stx1* + *stx2* + *eae* +	*stx1a* + *stx2d*[c]+ *eaeβ*+	ND	*ehxA*	AEZJ00000000	Aziz et al., (2008)

[a] Previously named *stx2d2*.
[b] Only 76.40% of this *eae* gene sequence was obtained.
[c] Previously named *stx2d1*.
[d] ND = not determined.
[e] UP = unpublished.

The tree also indicated that the O5 AE-STEC grouped together with *E. coli* strains belonging to phylogroups A and B1.

3.3. Genomic virulotyping

Several known virulence genes were detected in the draft genome sequences of O5 isolates by a search using the Virulence Finder server database v1.2 (cut-off: 98% sequence identity). In all PCR-positive Belgian isolates and in the American bovine 970246 strain, the *stx1*, *stx2* and *eae* genes were detected (Table 4). More than 99% of nucleotide sequence homology was observed between those three virulence genes in the 16 O5 AE-STEC strains, excluding the *eae* gene of isolate 33645 due to gaps in its sequence. All *stx1* genes identified were found to belong to the *stx1a* variant, the two *stx2* genes to the *stx2a* and *stx2d* variants, and the 15 fully sequenced *eae* genes to the *eaeβ* variant (Table 4).

In addition to the *stx1*, *stx2* and *eae* genes, several other genes highly homologous (>98%) to known virulence-associated genes were identified in the 15 isolates and the 970246 strain. They include the *ehxA* gene in all phenotypically positive isolates and in 6 of the other isolates (Table 4), genes coding for different LEE-encoded (*tir*, *espA*, *espB*, *espF*) and non-LEE encoded (*cif*, *espJ*, *nleA*, *nleB*, *nleC*) T3S effectors, and genes coding for other adhesins (*iha*, *lpfA*) and toxins (*astA*, *espP*, *subA*). Nevertheless no specific combination of such genes could be correlated to the host of origin or to the year of isolation.

4. Discussion

AE-STEC represent a major issue for public health because of their capability to cause large outbreaks and of the severity of the associated illnesses in humans, HC with (bloody) diarrhoea and HUS with renal failure. The most frequent origin of AE-STEC infections in humans is foodstuffs contaminated by ruminant faeces, mainly cattle that are asymptomatic carriers in their intestines. Scores of AE-STEC O:H serotypes have been associated with HC and/or HUS in humans, but a few ones are also responsible for diarrhoea in young calves, namely O26:H11 and O111:H- and also O5:H- and O118:H16 that are considered as emerging serotypes in humans. In addition, some EPEC belonging mainly to the O26:H11 serotype also isolated from ruminants, including diarrhoeic calves could be AE-STEC having lost their *stx* genes (Bugarel et al., 2011; Moxley and Smith, 2010; Mainil and Fairbrother, 2014; Tozzoli and Scheutz, 2014; Beutin and Fach, 2015; Ferdous et al., 2015). Nevertheless, since most of those data are from the years 1980 and 1990, the purpose of this study was therefore to update the information on AE-STEC and EPEC from diarrhoeic calves.

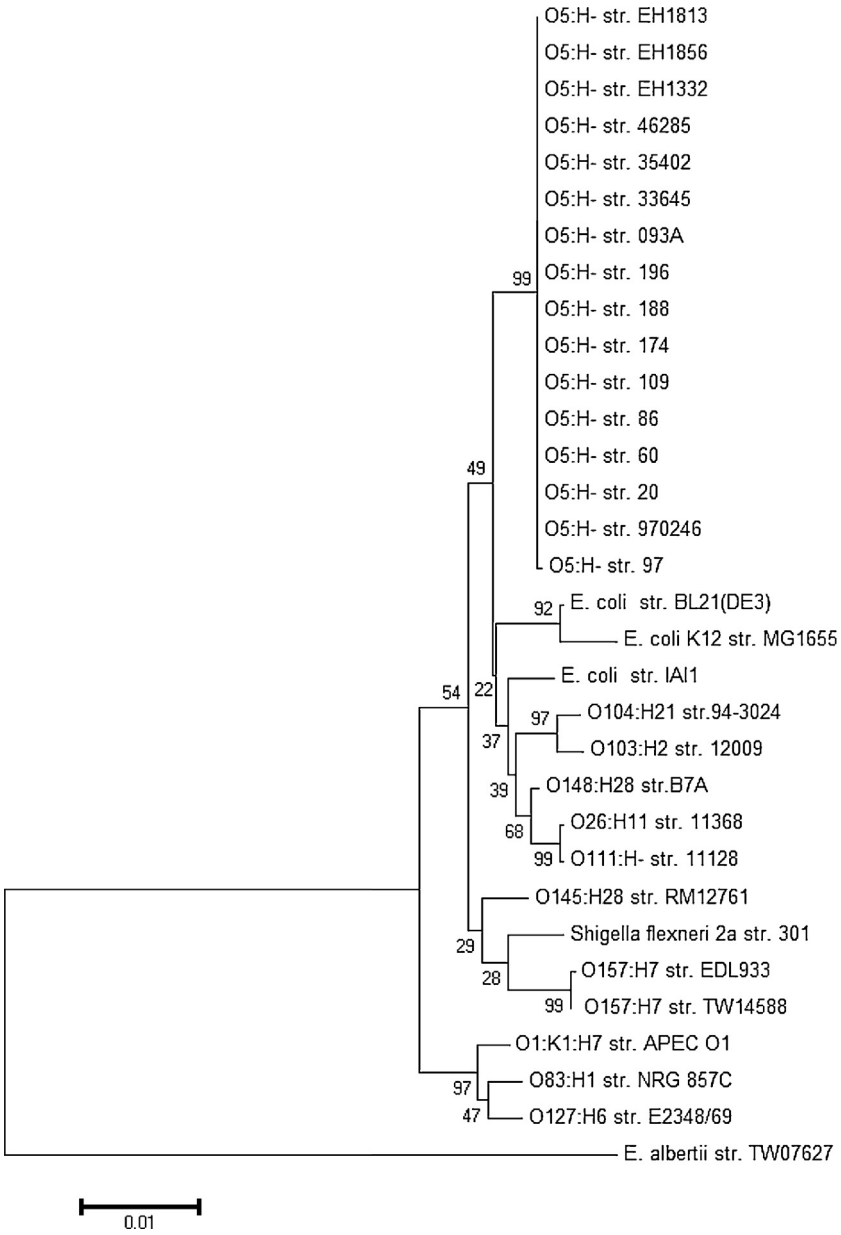

Fig. 1. Phylogenetic tree of concatenated MLST gene alleles (*adk*,*fumC*, *icd*, *gyrB*, *mdh*, *purA*, *recA*), extracted from the O5 genome sequences compared to others strains (based on Lukjancenko et al., 2010).
The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model and 1000 bootstrap replicates (Tamura et al., 2013). The tree with the highest log likelihood (-7091.2200) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories < + G, parameter = 0.0500>). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 32 nucleotide sequences.

All PCR results obtained (Tables 2 and 3) confirm that the majority: (i) of the 233 Ehly-producing *E. coli* isolated from diarrhoeic calves between November 2008 and February 2014 are AE-STEC or EPEC (85%); (ii) of the 119 AE-STEC are positive with the *stx1* PCR (97%) and a minority with the *stx2* PCR (14%); (iii) of the 119 AE-STEC belong to O26 and O111 serogroups (65%) in contrast to a minority of the 78 EPEC (18%) and of the 9 STEC (11%); (iv) of the 18 EPEC that could be serogrouped also belong to the O26 serogroup (78%).

Those results regarding the identity of AE-STEC in diarrhoeic calves therefore highly suggest that the situation has not much changed over the years in Belgium (Mainil et al., 1993) and raise different questions and comments. A first question concerns the role of the Stx toxins in the pathogenicity of AE-STEC-associated diarrhoea in young calves. If everyone agrees that such diarrhoea is related to the production of the AE lesion, just like for EPEC, nobody to our knowledge understands the role, if any, of the Stx toxins, since they seem to have no intestinal activity and are unable to cross the bovine enterocytes, in contrast to what occurs in humans. Another question, with no straight answer either, concerns the high percentage of AE-STEC from young diarrhoeic calves harbouring the *stx1* gene and the low percentage harbouring the *stx2* gene. But this might to some extent reflect the relative high percentage of O serogroups harbouring only this *stx1* gene (Mainil and Daube, 2005; Moxley and Smith, 2010; Mainil and Fairbrother, 2014).

Nevertheless, in contrast to the previous study, those *E. coli* were isolated in a veterinary routine diagnostic laboratory from

faecal materials using the EHLY agar that detects the production of enterohaemolysin (Beutin and Fach, 2015). Now, not all AE-STEC produce an Ehly and Ehly production is no evenly distributed amongst the different AE-STEC serotypes. For instance, all O157:H7 and the great majority of O26:H11 AE-STEC produce an enterohaemolysin while the proportion varies amongst the other serotypes, like O5, O103, O111, O118, O5 and also O103 (Mainil and Daube, 2005; Beutin and Fach, 2015). Therefore we may have over-identified some AE-STEC serogroups, like O26 (although the O26 serogroup was already the most frequent in the past) (Mainil et al., 1993; Moxley and Smith, 2010; Mainil and Fairbrother, 2014), and missed others, like O118 or O165. The use of the EHLY agar may also explain why several O103 AE-STEC were identified, that are not usually considered as responsible for diarrhoea in calves. Like for O5, O26, O111 and O118 AE-STEC (Mainil and Fairbrother, 2014), challenge experiments in calves should be performed to precisely define the role of O103 AE-STEC. Another interesting finding is the high number of Ehly-positive EPEC that were identified. Since Ehly is coded by pEHEC plasmid-located genes, future studies should identify the plasmid profiles of those EPEC and the *ehxA* gene localization (Tozzoli and Scheutz, 2014) and complete their virulotyping to confirm that they are not AE-STEC having lost the *stx* gene(s) (Bugarel et al., 2011; Ferdous et al., 2015).

Future studies must also bring additional information in the following domains: identification of the O serogroups especially of EPEC; role, if any of AE-STEC and EPEC belonging to other serogroups identified like f.i. O103, or so far unidentified in causing diarrhoea in calves; and comparison at the genome level of those calf isolates with isolates from humans and from asymptomatic cattle belonging to the same serogroups, by Pulsed Field Gel Electrophoresis (PFGE), Whole Genome PCR Scanning (WGPS), IS typing, micro-arrays, Multi-Locus Sequence Typing (MLST), and/or Whole Genome Sequencing (WGS) (Ogura et al., 2006; Ooka et al., 2009; Bugarel et al., 2010; Mainil et al., 2011; Sadiq et al., 2015). Such comparison was performed after WGS as described in the materials and methods on 12 calf and 3 human O5 AE-STEC isolated between 1967 and 2013.

From a genomic point of view, the O5 AE-STEC are very stable over time and similar between hosts. Indeed the genetic comparison of the 16 O5 AE-STEC isolates (including the reference strain) detects no relevant difference in the MLSType and in the *stx1*, *stx2*, *eae* gene sequences, either between recent or several year-old isolates or between isolates from calves and humans. Regarding the serotype, the presence of the *wzy* gene coding for the O5 somatic antigen was confirmed, but the *fliC* gene coding for the H9 flagellar antigen was also detected in all isolates. This is surprising because the O5 AE-STEC are usually phenotypically non motile and serologically H antigen-negative (Mainil and Fairbrother, 2014; Tozzoli and Scheutz, 2014). Testing the actual expression of the H9 flagella-encoding genes in different growth media should be performed in future experiments.

The presence of the *stx1a* gene variant in all 16 isolates and of the β variant of the gene encoding the intimin adhesin (*eae* gene) is identical to the report in the literature for O5 AE-STEC isolates from adult cattle (Monaghan et al., 2012; Lukinmaa-Aberg et al., 2013). Conversely, the *stx2* gene was present in only two isolates and was identified to the *stx2a* and *stx2d* variants respectively, according to the Virulence Finder server database v1.2 of the CGE (Joensen et al., 2014). Nevertheless, no particular virulence-associated gene could be associated with the calf isolates in comparison to the human isolates (Table 4). Therefore the ability of such AE-STEC and EPEC to cause diarrhoea in young calves, and not in adults may rely upon another virulence property not identified by the Virulence Finder server database because we used a threshold of 98% sequence identity, or not listed in this server database, or simply still unknown to our knowledge, if any.

5. Conclusion

As a conclusion, the high homology between calf and human isolates suggests the existence of a zoonotic potential and emphasizes the need for additional typing studies and for more systematic inclusion of calf isolates in comparative studies of AE-STEC and EPEC. So far those belonging to the O5 serogroup, that is today considered as an emerging AE-STEC (Beutin and Fach, 2015) are more frequently associated with (bloody) diarrhoea than with HUS, a clinical feature compatible with the presence of the *stx1a* gene in all isolates and the rare presence of any *stx2* gene (Basu and Tumer, 2015). Further studies are also necessary to circularise the genomes in order to complete the whole genome comparison and the identification of all virulence-associated genes and to analyse different O5 AE-STEC isolated in different countries from humans, calves and adult cattle to determine if the genomic homogeneity found over the years is also geographically present (Irshad et al., 2014).

Conflict of interest

The authors have nothing to disclose.

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