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Characterization of two major groups of diarrheagenic *Escherichia coli* O26 strains which are globally spread in human patients and domestic animals of different species

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Abstract

Twenty-three *Escherichia coli* O26 strains from humans, cattle, sheep, pigs and chicken were investigated for virulence markers and for genetic similarity by pulsed field gel electrophoresis and multi locus sequence typing. Two groups of genetically closely related O26 strains were defined. One group is formed by enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *E. coli* strains, which do not ferment rhamnose and dulcitol and most of these carry a plasmid encoding enterohemolysin. The other group consists of rhamnose and dulcitol fermenting EPEC strains, which carry plasmids encoding α-hemolysin. Multiple species of domestic animals were shown to serve as a reservoir for human pathogenic O26 EPEC and EHEC strains.

Keywords: *Escherichia coli* O26; Enteropathogenic; Enterohemorrhagic; Humans; Animals; Genotypes

1. Introduction

Escherichia coli strains of serogroup O26 are known as agents of diarrhea in children since 1951 [1]. This serogroup harbors enteropathogenic *E. coli* (EPEC) strains which cause severe watery diarrhea in infants, as well as enterohemorrhagic *E. coli* (EHEC) types which cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). EPEC and EHEC O26 strains were reported as human pathogens in different regions of the world [2–5]. Apart from EHEC O157:[H7], O26:[H11] is the most frequently isolated EHEC type from patients with HUS and diarrhea in Austria, Germany and Italy [6–8]. EPEC and EHEC O26 strains are also common in healthy and diarrheic animals and were isolated from cattle, pigs, sheep, goats, rabbits and chicken [9–15].

Investigation of EPEC and EHEC O26 strains resulted in the detection of two major phenotypical groups. One group is formed by intimin beta (*eae*-β1) and enterohemolysin (E-*hlyA*) positive O26:[H11] strains which are further subdivided into EHEC strains which produce Shiga-toxins (Stx), and into EPEC strains which are negative for production of Stx and *stx*-genes [16–18]. Motile and non-motile members are found in this group of

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genetically closely related O26:[H11] strains which are negative for fermentation of rhamnose and dulcitol [15,19,20]. The second phenotypical group is constituted of non-motile (NM) O26 strains which are negative for Stx, but produce plasmid encoded α-hemolysin and were isolated from children with diarrhea [21]. Apart from these findings, only little is known about the virulence properties of this group of O26:NM strains and their genetic relationship to the well characterized EHEC and EPEC O26:[H11] strains. In this work, we have compared representative human and animal O26 strains of different phenotypical groups, and from different countries, for their genetic similarity and their virulence attributes. We were interested in the possible animal reservoir for EPEC and EHEC O26 strains which cause diarrhea and HUS in human patients.

2. Materials and methods

2.1. E. coli *strains*

Twenty-three *E. coli* O26:[H11] strains from the laboratory collections of the Robert Koch-Institut in Berlin, Germany and the Laboratory of Medical and Veterinary Microbiology, University of São Paulo, São Paulo, Brazil, were investigated. The strains were selected as representatives for EPEC and EHEC O26 strains for different phenotypical groups as described previously [16,21] and were isolated from humans and animals between 1947 and 2003 (Table 1).

2.2. Examination of phenotypical traits and of virulence genes

Serotyping of *E. coli* O:H antigens and molecular typing of flagellar (*fliC*) genes by PCR was performed as described [7]. The virulence genes were investigated by PCR as listed in Table 2. *E. coli* strains used as controls for virulence genes are listed elsewhere [7,26]. Vero cell toxicity tests and detection of α- and enterohemolytic phenotypes was performed as described [7].

2.3. Pulsed-field gel electrophoresis (PFGE) and calculation of similarity indices

Preparation of genomic DNA for PFGE was performed as described [34]. The samples were digested

Table 1
Properties of *E. coli* O26 strains investigated in this study

Strains	Source	Origin[c]	Grouping[a]		Virulence genes[b]								
			PFGE	MLST	stx	α-hlyA	E-hlyA	katP	espP	paa	iutA	efa1 3′	efa1 5′
CB 9853[d,e]	Cattle	BR, 2003, this work	A	V	–[f]	–	+[g]	+	+	+	+	+	+
CB 9855[d,e]	Cattle	BR, 2003, this work	A	VI	–	–	+	+	+	+	+	+	+
CB 9857[d,e]	Cattle	BR, 2003, this work	A	I	–	–	+	+	+	+	+	+	+
CB 9862[e]	Cattle	BR, 2003, this work	A	I	–	–	+	+	+	–	+	+	+
CB 1025[e]	Human	BR, 1981, [22]	A	I	–	–	+	–	+	+	–	–	+
CB 9410[e]	Human	BR, 2000, this work	A	I	1	–	+	+	+	+	+	+	+
CB 6706[e]	Cattle	D, 1965, [23]	A	IV	–	–	–	–	–	+	+	+	+
CB 7505[e]	Cattle	D, 1998, this work	A	I	1	–	+	+	+	–	+	+	+
CB 8112[e]	Cattle	RA, 1996–2000 [24]	A	VII	1	–	+	+	+	+	+	+	+
CB 9271[e]	Cattle	D, 2002, this work	A	I	1	–	+	+	+	–	+	+	+
DG 223/5[e]	Sheep	D, 1990, [13]	A	I	–	–	+	+	+	+	+	+	+
DG 237/1[e]	Pig	D, 1990, [13]	A	I	–	–	–	+	–	+	+	+	+
DG 501/5[e]	Chicken	D, 1990, [13]	A	I	–	–	+	+	+	+	–	+	+
H19[e]	Human	GB, 1965. [25]	A	VIII	1	–	+	+	+	+	+	+	+
CB 9866[h]	Cattle	BR, 2003, this work	B	III	–	+	–	–	–	+	–	+	+
CB 1027[h]	Human	BR, 1981, [22]	B	IX	–	+	–	–	–	+	–	+	+
CB 1030[h]	Human	BR, 1981, [22]	B	X	–	+	–	–	–	+	–	+	+
DG 11/2[h]	Cattle	D, 1989, [13]	B	II	–	+	–	–	–	+	–	+	+
DG 70/2[h]	Cattle	D, 1989, [13]	B	II	–	+	–	–	–	+	–	+	+
DG 113/5[h]	Sheep	D, 1989, [13]	B	II	–	+	–	–	–	+	–	+	+
C4115[h]	Human	D, 1984, [21]	B	II	–	+	–	–	–	–	–	+	+
KK111/1[h]	Human	D, 1984 [26]	B	II	–	+	–	–	–	+	–	–	–
F41[h]	Human	GB, 1947 [21]	B	XI	–	+	–	–	–	+	–	+	+

[a] Assignment to PFGE and MLST groups.
[b] Virulence genes were investigated by PCR as described. All 23 strains were positive for *cif*, *lpfA*, *irp2* and *fyuA* and negative for *etpD*.
[c] Geographical origin (BR, Brazil, D, Germany, GB, United Kingdom, RA, Argentina) and year of isolation.
[d] All strains were isolated from the same animal.
[e] Rha- Dul-O26:[H11] strains.
[f] –, PCR-negative.
[g] +, PCR-positive.
[h] Rha + Dul +, O26:NM strains.

Table 2
Virulence markers investigated by PCR

Gene	Gene product	Designation and primer sequence (5′–3′)	PCR product (bp)	Ref.
eae-common	Intimin	SK1 – CCCGAATTCGGCACAAGCATAAGC SK2 – CCCGGATCCGTCTCGCCAGTATTCG	863	[18]
eaeβ	Intimin β	SK1 – CCCGAATTCGGCACAAGCATAAGC LP4 – CCCGTGATACCAGTACCAATTACGGTC	2.287	[18]
fliC common	Flagellin	FliC-1 – CAAGTCATTAATAC(A/C)AACAGCC FliC-2 - GAC AT(A/G)TT(A/G)GA(G/A/C)ACTTC(G/C)GT	950–2500	[27]
stx1	Shiga toxin 1 family	KS7 – CCCGGATCCATGAAAAAAACATTATTAATAGC KS8 – CCCGAATTCAGCTATTCTGAGTCAACG	285	[7]
stx2	Shiga toxin 2 family	LP43 – ATCCTATTCCCGGGAGTTTACG LP44 – GCGTCATCGTATACACAGGAGC	584	[7]
α-hlyA	α-Hemolysin	F16 – CAGTCCTCATTACCCAGCAAC B14– ACAGACCCCTTGTCCTGAAC	355	[7]
E-*hlyA*	EHEC- hemolysin	eha1 – GGTGCAGCAGAAAAAGTTGTAG eha4 – TCTCGCCTGATAGTATTTGGTA	1551	[7]
katP	pO157 catalase-peroxidase	wkatB – CTTCCTGTTCTGCTGATTCTTCTGG wkatF – AACTTATTTCTCGCATCATCC	2125	[28]
EspP	pO157 serine protease	espA – AAACAGCAGGCACTTGAACG espB – GGAGTCGTCAGTCAGTAGAT	1830	[28]
etpD	pO157 type II secretion system	D1 – CGTCAGGAGGATGTTCAG D13R – CGACTGCACCTGTTCCTGATTA	1062	[28]
paa	Porcine attaching-effacing	155 f1 – ATGAGGAACATAATGGCAGG 155 r1 – TCTGGTCAGGTCGTCAATAC	350	[29]
iutA	Aerobactin receptor	aero f – GGCTGGACATCATGGGAACTGG aero r – CGTCGGGAACGGGTAGAATCG	280	[30]
EAF	Part of EAF-plasmid	EAF1 – CAGGGTAAAAGAAAGATGATAA EAF25 – TATGGGGACCATGTATTATCA	397	[26]
bfpA	bundle forming pili	EP1 – AATGGTGCTTGCGCTTGCTGC EP2 – GCCGCTTTATCCAACCTGGTA	326	[26]
efa1 3′	EHEC factor adherence	Efa1 3′f – TGCGCACAATTGACTACAGAGGAA Efa1 3′r – ATACGACCATCAGGGGAATCAC	692	[31]
efa1 5′	EHEC factor adherence	Efa1 f – TGGGCAGAACATTTTCACCAGTTC Efa1 r – CTTTCAGGTGGGGAACCATATGGC	725	[31]
cif	Cell cycle inhibiting factor	Cif-int-s – AACAGATGGCAACAGACTGG Cif-int-as – AGTCAATGCTTTATGCGTCAT	383	[18]
lpfA	Long polar fimbriae	lpfA-F – ATGAAGCGTAATATTATAG lpfA-R – TTATTTCTTATATTCGAC	573	[32]
fyuA	Pesticin receptor	fyuA f – GCGACGGGAAGCGATTTA fyuA r – CGCAGTAGGCACGATGTTGTA	780	[33]
irp2	Iron repressible protein	irp2 f – AAGGATTCGCTGTTACCGGAC irp2 r – TCGTCGGGCAGCGTTTCTTCT	280	[33]

with *Xba*I and DNA fragments were resolved in 1% agarose gels in the CHEF-DR-II system (Bio-Rad Laboratories, Munich, Germany). Electrophoresis was performed for 23 h at 14 °C, with a constant voltage of 200 V, using a linear pulse ramp of 5–40 s in running buffer 0.5× TBE, 50 μM thiourea. Evaluation of PFGE profiles for similarity was performed with bionumerics software Dice similarity indices, complete linkage, optimization: 1%, position tolerance 1.3%, as described [34].

2.4. Multi locus sequence typing (MLST)

Seventeen housekeeping genes were investigated by MLST (Table 3). The selection of gene loci, primers

Table 3
Allele frequencies of housekeeping genes in *E. coli* O26:[H11] strains from this study

Allele	*arcA*	*aroE*	*aspC*	*ClpX*	*cstA*	*cyaA*	*dnaG*	*fadD*	*grpE*	*icdA*	*lysP*	*mdh*	*mtlD*	*mutS*	*pgi*	*rpoS*	*uidA*
1	9	23	23	23	23	21	23	22	23	23	21	23	23	23	23	18	23
2	14	–	–	–	–	1	–	1	–	–	2	–	–	–	–	1	–
3	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	1	–
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
Total	2	1	1	1	1	3	1	2	1	1	2	1	1	1	1	6	1

and PCR conditions was performed according to published protocols (STEC Center, Michigan State University, http://www.shigatox.net/cgi-bin/mlst7/index). Single colonies of *E. coli* O26 strains grown on LB-plates were used as target DNA source. PCR products were purified with QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Sequencing reactions were carried out using dye terminator chemistry (Applied Biosystems, Darmstadt, Germany) and separated on an automatic DNA sequencer (ABI PRISM 3100, Genetic Analyzer).

DNA sequences were analysed with BioEdit, version 4.8.10 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), converted into fasta files and loaded into START (*S*equence *T*ype *A*nalysis and *R*ecombinational *T*ests, http://www.medawar.ox.ac.uk/maiden/software.shtml). The dendrograms were compiled with START using the unweighted pair group method with arithmetic mean (UPGMA). This software constructs a phylogenetic tree on the basis of allele numbers. The sequences obtained from the twenty-three O26 strains were compared to those of *E. coli* K-12 strain MG1655 (Accession No. NC000913) and *E. coli* O157:H7 strain EDL933 (Accession No. NC002655).

2.5. Nucleotide sequence Accession Numbers

The nucleotide sequences obtained by sequencing of the PCR products of each allele of all genes have been entered into the EMBL Nucleotide Sequence Database under continuous Accession Numbers from AJ875429 to AJ875455.

3. Results

3.1. Phenotypical properties of E. coli O26 strains

A group of 23 representative *E. coli* O26 strains which were isolated in Europe and South-America between 1947 and 2003 and originated from humans ($n = 8$), cattle ($n = 11$), sheep ($n = 2$), pigs ($n = 1$) and chicken ($n = 1$) were investigated (Table 1). Phenotypically, two groups of strains could be distinguished: One group consisting of 11 motile and three NM strains which did not ferment rhamnose and dulcitol (Rha^-Dul^-); twelve of these strains produced enterohemolysin and five produced Vero toxins. The second group is formed by nine O26:NM strains which fermented rhamnose and dulcitol (Rha^+Dul^+), produced α-hemolysin and did not show toxicity to Vero cells. The motile strains were serotyped as O26:H11 and a *fliC* gene coding for flagellar type H11 was detected in all *E. coli* O26 strains.

3.2. Genetic analysis of virulence markers

The strains were investigated for a set of virulence genes which are associated with EPEC and EHEC groups (Table 1). An intimin beta 1 (*eae*-β1) gene was present in all *E. coli* O26 strains. The two phenotypical groups are characterized by different virulence attributes. Twelve of the 14 Rha^-Dul^-, O26:[H11] strains carried EHEC virulence plasmid associated genes for enterohemolysin (E-*hlyA*), catalase-peroxidase (*katP*), and serine protease (*espP*), as well as the aerobactin receptor gene (*iutA*). The five strains with Vero cytotoxic activity were shown to carry *stx1* genes whereas all other strains were negative for *stx*-genes. In contrast, the nine Rha^+Dul^+ O26:NM strains carried plasmid encoded α-hemolysin genes (α-*hlyA*), but were negative for EHEC virulence plasmid encoded genes as well as for *stx*- and *iutA* genes.

Other virulence markers such as the porcine attaching and effacing associated (*paa*) gene, the EHEC factor for adherence (*efa*), long polar fimbriae (*lpfA*), the cell cycle inhibiting factor (*cif*), and the high pathogenicity-island associated genes *irp2* and *fyuA* were found in most or all O26 strains from both phenotypical groups (Table 1). In contrast, none of the strains were positive for bundle forming pili (*bfpA*) and EPEC adherence factor (EAF) plasmid associated sequences.

According to their virulence attributes the group of Rha^-Dul^-, O26 strains splits into EHEC ($n = 5$) and EPEC ($n = 9$). The group of Rha^+Dul^+, O26 strains is exclusively formed by EPEC (Table 1). The virulence attributes of the O26 strains were not related to their origin, source, and isolation date. In order to investigate the genetic relationship between the *E. coli* O26 strains we have examined them by PFGE and MLST.

3.3. Genotyping by pulsed field gel electrophoresis (PFGE) and by multilocus sequence analysis (MLST)

PFGE typing with *Xba*I resulted in 22 different patterns, only two strains from cattle (DG70/2 and DG11/2) showed identical profiles (Fig. 1). Analysis for similarity revealed two major clusters, called A (fourteen strains with >60% similarity) and B (nine strains with >63% similarity). The clusters corresponded to the major phenotypes, as Cluster A gathered all strains which were Rha^-Dul^-, and Cluster B gathered all Rha^+Dul^+ strains.

MLST was performed with 17 unlinked housekeeping genes (Table 3). Sequences were obtained from all twenty-three O26 strains and could be aligned without gaps. The different alleles of the 17 genes were randomly numbered and eleven MLST profiles (I–XI) were obtained (Table 4). The MLST profiles could be divided into two major clusters 1 and 2, comprising closely related strains. MLST Cluster 1 (nine strains with MLST

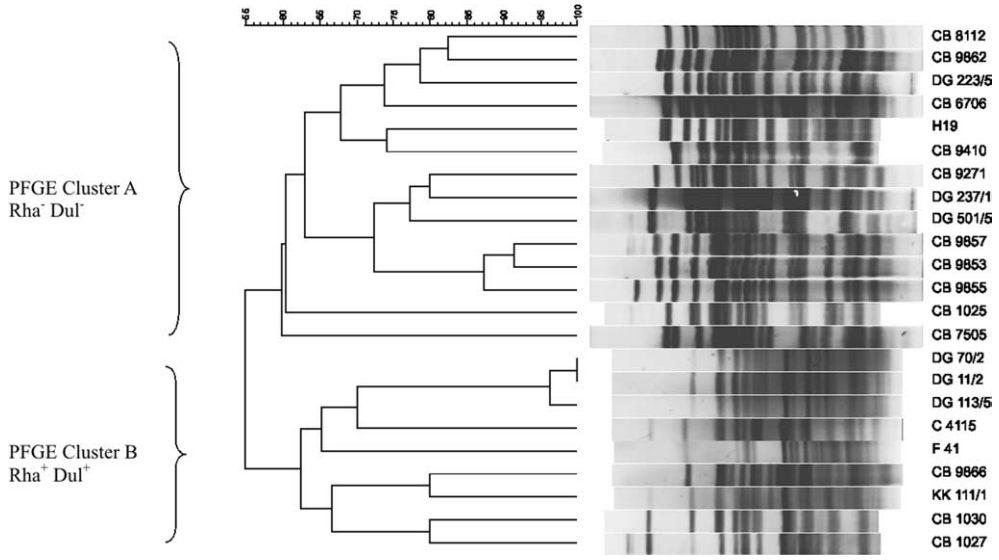

Fig. 1. PFGE profiles and clusters of O26 EPEC and EHEC strains.

Table 4
Allelic profiles associated with *E. coli* O26:[H11] strains and with the reference strains EDL933 (O157) and MG1655 (K-12)

Allelic profiles[a]	Profile designation	Relative frequency
2, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1	I	9
1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1	II	5
1, 1, 1, 1, 1, 2, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1	III	1
2, 1, 1, 1, 1, 1, 1, 1, 1, 2, 1, 1, 1, 1, 1, 1	IV	1
2, 1, 1, 1, 1, 3, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1	V	1
2, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 2, 1	VI	1
2, 1, 1, 1, 1, 1, 1, 2, 1, 1, 1, 1, 1, 1, 1, 1	VII	1
2, 1, 1, 1, 1, 1, 1, 1, 1, 2, 1, 1, 1, 3, 1	VIII	1
1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 4, 1	IX	1
1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 5, 1	X	1
1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 6, 1	XI	1
3, 2, 2, 2, 2, 4, 2, 3, 2, 2, 3, 2, 2, 2, 7, 2	EDL933	1
4, 3, 3, 3, 3, 5, 3, 4, 3, 3, 1, 2, 3, 3, 3, 1, 3	MG1655	1

[a] Order of genes from left to right: *arcA*, *aroE*, *aspC*, *clpX*, *cstA*, *cyaA*, *dnaG*, *fadD*, *grpE*, *icdA*, *lysP*, *mdh*, *mtlD*, *mutS*, *pgi*, *rpoS*, *uidA*.

profiles II, III, IX, X and XI) and Cluster 2 (13 strains with MLST profiles I, IV, V, VI and VII) were closely linked to each other (linkage distance 0.14) and corresponded to PFGE Clusters B and A, respectively, and to the phenotypical groups (Fig. 2). The only exception was made by strain H19 (MLST profile VIII) which was more distant (linkage distance 0.18) from the other O26 strains. However, H19 shared the same *arcA* sequence with all MLST Cluster 2 strains, and the *arcA* gene allele discriminated clearly between the two phenotypical groups of *E. coli* O26 strains (Tables 3 and 4). Two predominating MLST profiles I and II were found which were fully associated with the MLST Clusters 2 and 1, respectively (Fig. 2). All other MLST profiles (III–XI) were associated with single O26 strains. Interestingly, three O26 strains with three different MLST profiles (I, V, VI) originated from a single cow (Table 1). In general, all O26 strains were found to be genetically fully different from strain EDL933 (*E. coli* O157) and far distant from the laboratory *E. coli* K-12 strain MG1655 (Table 4, Fig. 2).

4. Discussion

In this study, we could identify two major genetic groups of diarrheagenic O26 strains. One group is constituted of $Rha^{-}Dul^{-}$ EHEC and EPEC strains which are very similar for their genotypes and their virulence markers, except for Shiga toxins. Larger numbers of O26 strains belonging to this group have been characterized previously in regard to their adhesive properties, some virulence attributes and for their genetic similarity [16,17,20].

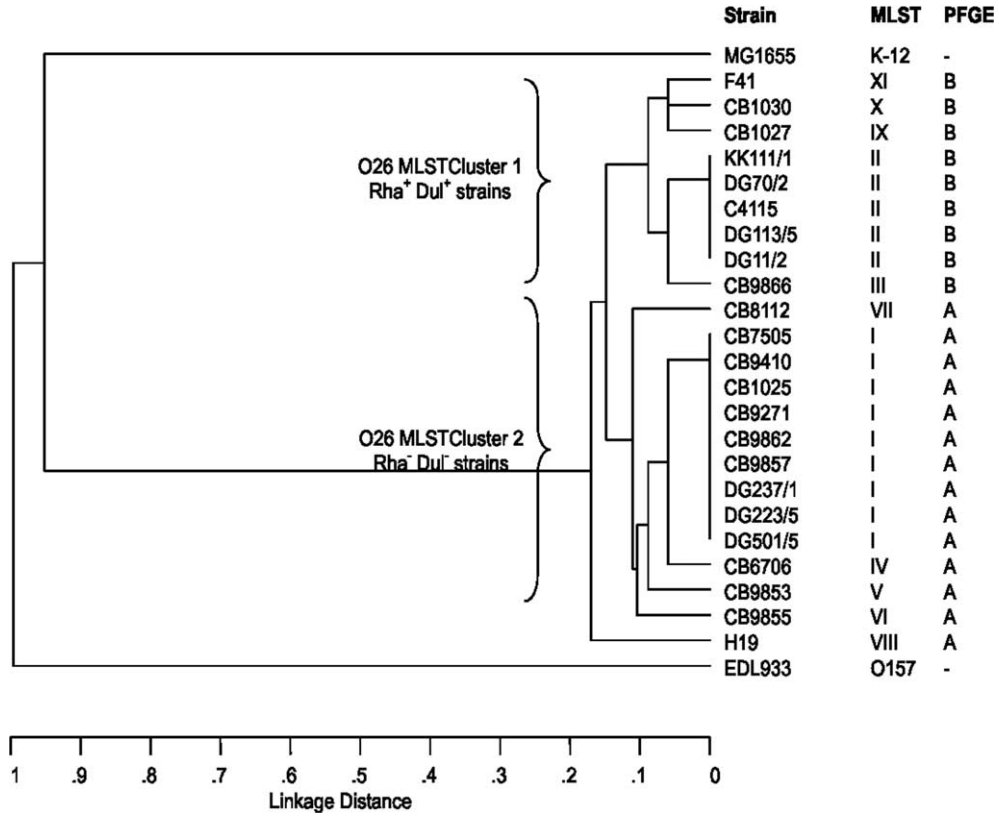

Fig. 2. MLST profiles and clusters of O26 EPEC and EHEC strains. A distance matrix was created on the basis of allele numbers and this was used as basis for the UPGMA dendrogram. Linkage distance are relative distances. Isolates with 17 identical alleles have a distance of 0, and those with no alleles in common have a distance of 1.

The second group of EPEC O26 strains has not been well characterized before and was analyzed more deeply in this study. Members of this group are phenotypically different from classical EPEC and EHEC O26:[H11] strains by lack of motility, production of α-hemolysin, and by their ability to ferment rhamnose and dulcitol. Strains belonging to this group are characterized by maintenance of large transmissible plasmids encoding α-hemolysin [20]. Genetically, all Rha^+Dul^+ O26:NM strains were found to be closely related.

On the basis of MLST data, both groups of O26 strains are similar to each other and far distant from laboratory *E. coli* K-12 and from EHEC O157 strains. Common attributes of both groups of O26 strains are the intimin (*eae*-β1) and flagellar (*fliC*-H11) genes, but also other virulence genes coding for adhesion, cytotoxin and the high pathogenicity island. It appears possible that both groups of O26 strains have evolved from a common O26:H11 (*eae*-β1) ancestor and have split into two lineages of Rha^-Dul^- O26:[H11] EPEC/EHEC, and Rha^+Dul^+ O26:NM EPEC strains.

The evolution of EHEC as a subgroup of Rha^-Dul^- O26:[H11] strains could be explained by uptake of Stx-encoding bacteriophages. Analysis of MLST data and of other virulence markers strains indicate high similarity between O26:[H11] EPEC and EHEC strains and the major MLST profile I is constituted by strains belonging to both pathogroups (Table 1). Furthermore, it cannot be excluded that some of the EPEC strains from this group represent former EHEC strains which have lost their *stx*-genes. Recently published data indicate that EHEC O26:[H11] strains have continued to evolve by uptake of stx_2 genes [17]. In contrast to many other EPEC strains, EPEC O26:[H11] are characterized by possession of the EHEC-virulence plasmid, which a typical attribute of classical EHEC strains [35].

EHEC and EPEC O26:[H11] strains represent one of the most important groups of diarrheagenic *E. coli* worldwide. One reason for the widespread of these pathogens could be the broad animal reservoir which is based on the wide spectrum of animal hosts which can be colonized by EPEC and EHEC O26 strains. Both groups of EPEC and EHEC O26 strains were found to carry genes for multiple colonization factors (*paa*, *lpf*, *efa*) which might enable them to colonize different host species. Animal and human EPEC and EHEC O26 strains were similar for their genotypes and virulence attributes indicating that strains from animals can serve as human pathogens.

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