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First isolation and further characterization of enteropathogenic *Escherichia coli* (EPEC) O157:H45 strains from cattle

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Abstract

Background: Enteropathogenic *Escherichia coli* (EPEC), mainly causing infantile diarrhoea, represents one of at least six different categories of diarrheagenic *E. coli* with corresponding distinct pathogenic schemes. The mechanism of EPEC pathogenesis is based on the ability to introduce the attaching-and-effacing (A/E) lesions and intimate adherence of bacteria to the intestinal epithelium. The role and the epidemiology of non-traditional enteropathogenic *E. coli* serogroup strains are not well established. *E. coli* O157:H45 EPEC strains, however, are described in association with enterocolitis and sporadic diarrhea in human. Moreover, a large outbreak associated with *E. coli* O157:H45 EPEC was reported in Japan in 1998. During a previous study on the prevalence of *E. coli* O157 in healthy cattle in Switzerland, *E. coli* O157:H45 strains originating from 6 fattening cattle and 5 cows were isolated. In this study, phenotypic and genotypic characteristics of these strains are described. Various virulence factors (*stx*, *eae*, *ehxA*, *astA*, EAF plasmid, *bfp*) of different categories of pathogenic *E. coli* were screened by different PCR systems. Moreover, the capability of the strains to adhere to cells was tested on tissue culture cells.

Results: All 11 sorbitol-positive *E. coli* O157:H45 strains tested negative for the Shiga toxin genes (*stx*), but were positive for *eae* and were therefore considered as EPEC. All strains harbored *eae* subtype $\alpha 1$. The gene encoding the heat-stable enterotoxin 1 (EAST1) was found in 10 of the 11 strains. None of the strains, however, carried *ehxA* genes. The capability of the strains to adhere to cells was shown by 10 strains harbouring *bfp* gene by localized adherence pattern on HEp-2 and Caco-2 cells.

Conclusion: This study reports the first isolation of typical O157:H45 EPEC strains from cattle. Furthermore, our findings emphasize the fact that *E. coli* with the O157 antigen are not always STEC but may belong to other pathotypes. Cattle seem also to be a reservoir of O157:H45 EPEC strains, which are described in association with human diseases. Therefore, these strains appear to play a role as food borne pathogens and have to be considered and evaluated in view of food safety aspects.

Background

Enteropathogenic *Escherichia coli* (EPEC), mainly causing infantile diarrhoea, represents one of the at least six different categories of diarrheagenic *E. coli* with corresponding distinct pathogenic schemes [1]. Most of the EPEC strains belong to a series of O antigenic groups known as EPEC O serogroups: O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158.

The main mechanism of EPEC pathogenesis is the ability to introduce attaching-and-effacing (A/E) lesions on intestinal cells characterized by microvillus destruction, intimate adherence of strains to the intestinal epithelium, pedestal formation and aggregation of polarized actin at sites of bacterial attachment [2].

The genetic determinants for the production of A/E lesions are located on the locus of enterocyte effacement (LEE), a pathogenicity island that contains the genes encoding intimin (*eae*), a type III secretion system, a number of secreted proteins (ESP) and the translocated intimin receptor (Tir) [2]. Characterization of *eae* genes revealed the existence of different *eae* variants. At present, 15 ($\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2/\theta$, δ/κ , ϵ , ζ , η , ι , λ , μ , ν , ξ) genetic variants of the *eae* gene have been identified [3-7]. It is discussed that different intimins may be responsible for different host- and tissue cell tropism [8,9]. In epithelial culture cells EPEC strains produce characteristic adherence patterns: (i) localized adherence (LA), where microcolonies attach after 3 hours incubation to one or two small areas on the cells [10,11]; (ii) diffuse adherence (DA), where bacteria cover the cells uniformly [10]; (iii) enteroadherent-aggregative adherence (AA), where the bacteria have a characteristic stacked-brick-like arrangement [12] and (iiii) localized adherence-like adherence (LAL), characterized by formation of less-compact microcolonies or clusters of bacteria than those by LA [13]. Localized adherence is associated with the presence of the large EPEC adherence factor (EAF) plasmid, on which also the cluster of genes encoding bundle-forming pili (BFP) is present [1]. EPEC are classified as typical, when possessing the EAF plasmid; whereas atypical EPEC strains do not possess the EAF plasmid [14,15].

Typical and atypical EPEC strains usually belong to certain serotype clusters, differ in their adherence patterns on cultured epithelial cells (typical: LA; atypical: DA; AA; LAL), are typically found in different hosts (typical EPEC strains have only been recovered from humans), and display difference in their intimin types [15].

The role and the epidemiology of non-traditional enteropathogenic *E. coli* serogroup strains in human diarrhea are not well established. *E. coli* O157:H8 and *E. coli* O157:H45 EPEC strains have been described to be associ-

ated with diarrhea in human [4,16]. Makino et al. [17] reported the first large outbreak with *E. coli* O157:H45 EPEC in Japan in 1998. Nevertheless, these O157 EPEC strains are not well characterized.

This study reports the first isolation of O157:H45 EPEC strains from cattle and phenotypic and genotypic characteristics of the isolated strains are described.

Results and discussion

All 11 sorbitol-positive *E. coli* O157:H45 strains, six strains isolated from fattening cattle and five strains isolated from cows tested negative for Shiga toxin genes (*stx*). The *eae* gene, which is strongly correlated to the attaching-and-effacing lesions, was present in all strains. Therefore, these strains are considered to be EPEC. Our findings emphasize the fact that *E. coli* with the O157 O antigen are not always STEC but may belong to other pathotypes. Further results of strain characterization are shown in Tab. 2.

In all tested strains, LEE was inserted in *selC* and thus these strains could be evolutionarily more akin to the EPEC 1 and EHEC 1 clones than to other clones [18]. Further characterization of the *eae* variants showed in all strains *eae* subtype $\alpha 1$. Oswald et al. [4] testing in their study the distribution of intimin types among different EPEC strains, found the same *eae* subtype in O157:H45 strains isolated from human with diarrhea.

The gene encoding the heat-stable enterotoxin (EAST1), representing an additional determinant in the pathogenesis of *E. coli* diarrhea, was found in ten of the 11 strains. None of the strains, however, carried *ehxA* genes.

Nine strains were positive for the EAF plasmid and 10 strains harbored the *bfpA* gene encoding bundlin, the structural subunit of the bundle-forming pilus expressed by typical EPEC strains. The EAF plasmid negative but *bfpA* positive strain may have a defect in the EAF region that does not interfere with the plasmid's function. All *bfpA* gene positive strains showed a localized adherence (LA) pattern, the phenotypical characteristic associated with the expression of bundle-forming pili and therefore seem to be a typical EPEC. For HEp-2 and Caco-2 cells similar results were obtained (Figure 1, 2). Caco-2 cells instead of HeLa cells were used for adhesion assay and FAS test, because they represent a system close to the human intestinal cells. Furthermore, Vieira et al. [19] have shown that results obtained by Caco-2 cells are comparable to HeLa cells.

10 of the 11 strains showed an A/E property with the fluorescent actin staining (FAS) test (Figure 3).

Table 1: Sequences and predicted lengths of PCR amplification products of oligonucleotide primers used

Target	Primer	Oligonucleotide sequence (5'-3')Sequence	Product size	Reference
stx	VT1	ATTGAGCAAAATAATTTATAT GTG	523 bp	21
	VT2	TGATGATGGCAATTCAGTAT	520 bp	
eae	SK1	CCCGAATTCGCGACAAGCATAAGC	863 bp	22
	SK2	CCCGGATCCGTCTCGCCAGTATTCCG		
eae- α 1	EAE-FB	AAAACCGCGGAGATGACTTC	820 bp	7
	EAE-A	CACTCTTCGCATCTTGAGCT		
eae- α 2	IH2498aF	AGACCTTAGGTACATTAAGTAAGC	517 bp	7
	IH2498aR	TCCTGAGAAGAGGGTAATC		
eae- β 1	EA-B1-F	CGCCACTTAATGCCAGCG	811 bp	7
	EAE-B	CTTGATACACCTGATGACTGT		
eae- β 2	EA-B2-F	CCCGCCACTTAATCGCACGT	807 bp	7
	EAE-B	CTTGATACACCTGATGACTGT		
eae- γ 1	EAE-FB	AAAACCGCGGAGATGACTTC	804 bp	7
	EAE-C1	AGAACGCTGCTCACTAGTGTC		
eae- γ 2/0	EAE-FB	AAAACCGCGGAGATGACTTC	808 bp	7
	EAE-C2	CTGATATTTTATCAGCTTCA		
eae- δ /k	EAE-FB	AAAACCGCGGAGATGACTTC	833 bp	7
	EAE-D	CTTGATACACCCGATGGTAAC		
eae- ϵ	EAE-FB	AAAACCGCGGAGATGACTTC	722 bp	7
	LP5	AGCTCACTCGTAGATGACGGCAAGCG		
eae- ζ	Z1	GGTAAGCCGTTATCTGCC	206 bp	7
	Z2	ATAGCAAGTGGGGTGAAG		
eae- η	EAE-FB	AAAACCGCGGAGATGACTTC	712 bp	7
	LP8	TAGATGACGGTAAGCGAC		
eae- ι	EAE-FB	AAAACCGCGGAGATGACTTC	807 bp	7
	LP7	TTTATCCTGCTCCGTTTGCT		
eae- λ	68.4F	CGGTCAGCCTGTGAAGGGC	466 bp	7
	68.4R	ATAGATGCCTCTTCCGGTATT		
eae- μ	FV373F	CAACGGTAAGTCTCAGACAC	443 bp	7
	FV373R	CATAATAAGCTTTTTGGCCTACC		
eae- ν	IH1229aF	CACAGCTTACAATTGATAACA	311 bp	7
	IH1229aR	CTCACTATAAGTCATACGACT		
eae- ξ	EAE-FB	AAAACCGCGGAGATGACTTC	468 bp	7
	B49R	ACCACCTTTAGCAGTCAATTTG		
ehxA	HlyA1	GGTGCAGCAGAAAAAGTTGTAG	1551 bp	23
	HlyA2	TCTCGCCTGATAGTGTGGTA		
astA	EAST1	CCA TCA ACA CAG TAT ATC CGA	111 bp	24
	EAST2	GGT CGC GAG TGA CGG CTT TGT		
EAF	EAF1	CAG GGT AAA AGA AAG ATG ATA A	397 bp	25
	EAF25	TAT GGG GAC CAT GTA TTA TCA		
bfpA	EPI	AAT GGT GCT TGC GCT TGC TGC	326 bp	26
	EP2	GCC GCT TTA TCC AAC CTG GTA		

According to Kaper [14] most of our O157:H45 strains have to be classified as typical EPEC strains, because they are possessing the EAF plasmid and are showing the localized adherence (LA) pattern. However, they are harboring *astA* genes, which is more common for atypical EPEC strains [15].

Further studies are needed to evaluate the role of such EPEC strains isolated from asymptomatic animals.

Conclusions

Cattle have not only to be considered as important asymptomatic carriers of O157 STEC but can also be a reservoir of O157 EPEC strains, which are described in association with human diseases. These strains appear to play a role as food borne pathogens and have to be considered in view of food safety aspects.

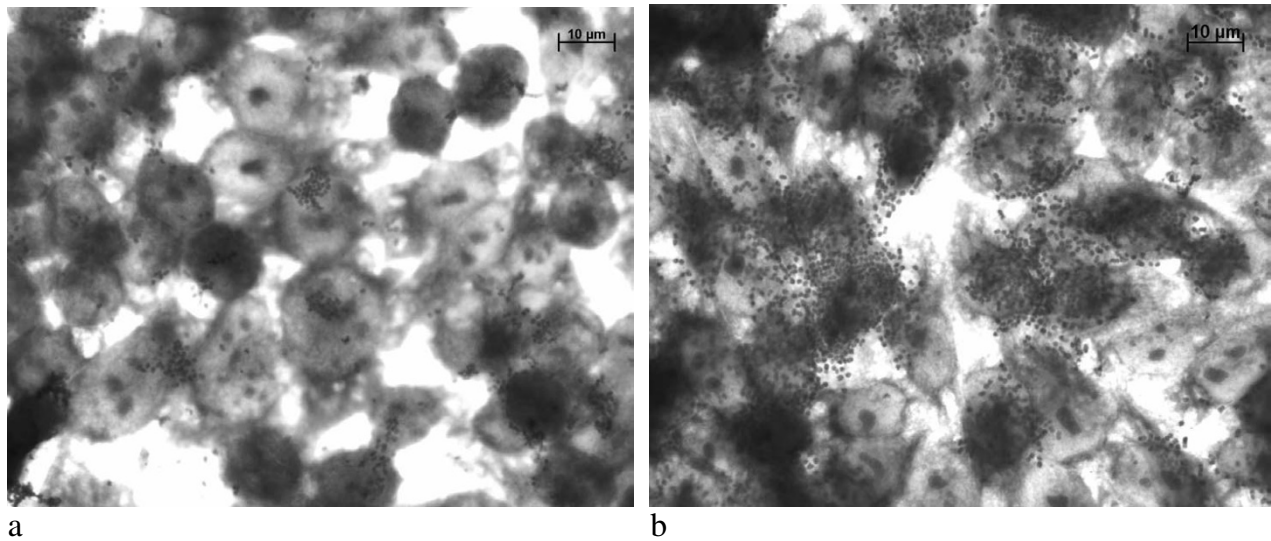


Figure 1
Localized adherence (LA) pattern of the *E. coli* O157:H45 strain I070/03 on HEp-2 cells. (a) 3 h assay, (b) 6 h assay

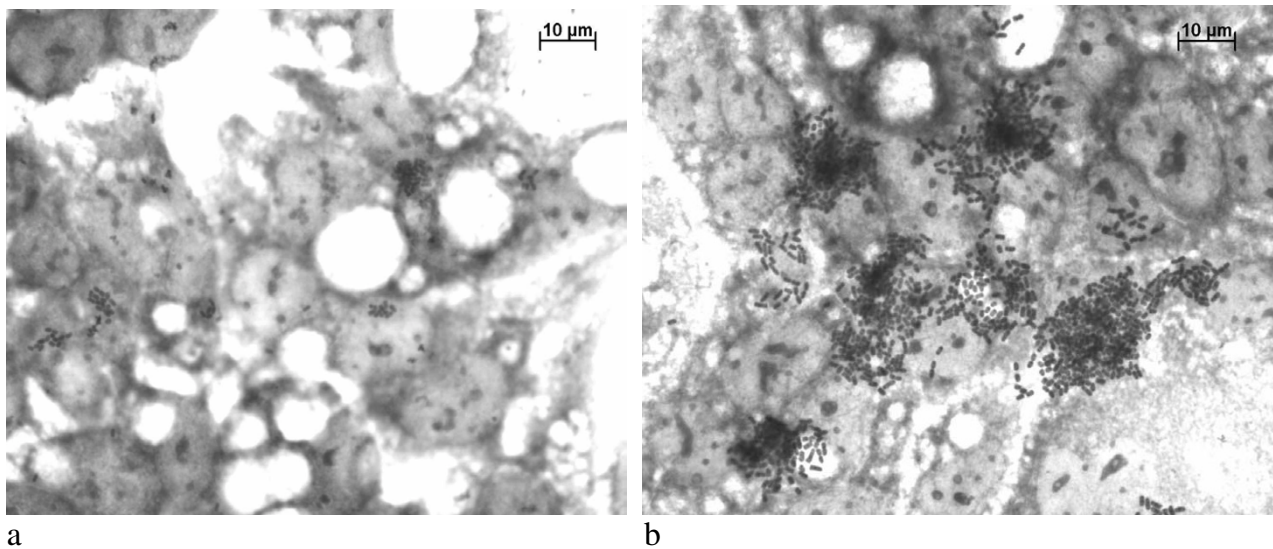


Figure 2
Localized adherence (LA) pattern of the *E. coli* O157:H45 strain I070/03 on Caco-2 cells. (a) 3 h assay, (b) 6 h assay

Methods

Origin of the *E. coli* strains

The strains characterized in this work were isolated during a recent study on prevalence of *E. coli* O157 in healthy cat-

tle in Switzerland [20]. The study was based on investigations that were carried out within the last one and a half years (January 2002 – June 2003) in two EU-approved slaughterhouses. From 2,930 bovine fecal samples (1,183

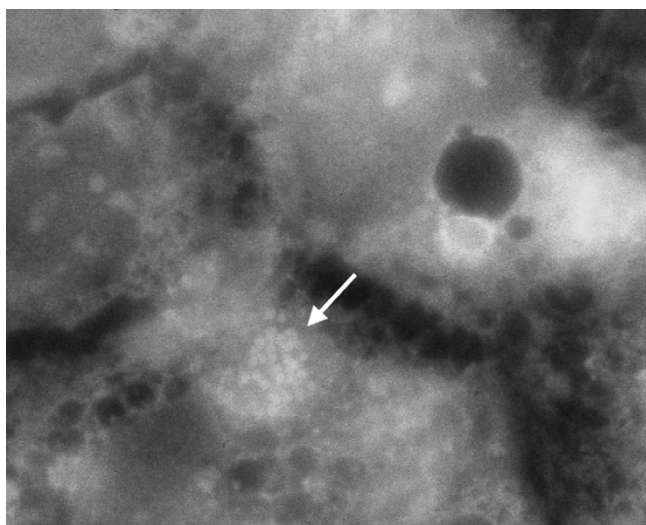


Figure 3
Actin fluorescence micrograph showing *E. coli* O157:H45 strain 1070/03-infected Caco-2 cells

Table 2: Characterization results of 11 sorbitol-fermenting O157:H45 EPEC strains isolated from cattle in Switzerland

No. of strains	LEE in <i>selC</i>	Intimin type	EAF Plasmid	<i>bfp</i>	Adherence pattern	<i>astA</i>	<i>ehx</i>
9	yes	α 1	+	+	LA	+	-
1	yes	α 1	-	+	LA	+	-
1	yes	α 1	-	-	NA	-	-

LA: localized adherence; NA: no adherence

calves, 1,201 fattening cattle and 546 cows), 11 sorbitol-fermenting *E. coli* O157:H45 strains (from 6 fattening cattle and 5 cows) were isolated. The determination of the O and H antigens was performed as previously described using all available O (O1 to O181) and H (H1 to H56) antisera [6].

Genotypic characterization of the O157:H45 strains

PCR for the detection of putative virulence genes were performed in a T3 thermocycler (Biometra, Germany). PCR reagents were purchased from PROMEGA (Madison, Wis.) and primers were synthesized by MICROSYNTH (Balgach, Switzerland). The 50- μ l PCR mixtures normally consisted of 2 μ l of bacterial suspension in 42 μ l of double-distilled water, 5 μ l of 10-fold-concentrated polymerase synthesis buffer containing 2.0 mM MgCl₂, 200 μ M (each) desoxynucleosid triphosphate (dNTP), 30 pmol of each primer and 2.5 U of *Taq* DNA polymerase. The PCR primers, target sequences and product sizes are

listed in Table 1. Differences in the normal PCR mixture and cycling conditions for each PCR were previously described in the cited literature.

To verify whether LEE was inserted downstream of the *selC* locus, PCR reactions that amplify the junctions of this locus with the *E. coli* chromosome were performed according to Sperandio et al. [27]. Primers K255 (5'-GGTTGAGTTCGATTGATCTCTGG-3') and K260 (5'-GAGCGAATATCCGATATCTGGTT-3') were used for the right junction; K296 (5'-CATTCTGAAACAACTGCTC-3') and K295 (5'-CGCCGATTTTCTTAGCCCA-3') for the left junction, and K261 (5'-CCTGCAAATAAACACGGCGCAT-3') and K260 for the intact *selC* gene.

Adhesion assay and FAS test

E. coli colonies were characterized by the pattern of adherence to HEp-2 and Caco-2 cells as described by Karch et al. [28] with minor modifications. Briefly, HEp-2 and Caco-2 cells were grown for 48 h with 5% CO₂ on sterilized coverslips (13 mm diameter; Bibby Sterilin™ Ltd., Stone, England) in 24-well flat-bottom tissue culture plates with low evaporation lid (16 mm diameter; BD Falcon™, Bedford, USA) containing Minimum essential medium (MEM with Earle's salts, 25 mM HEPES, without L-Glutamine; GIBCO™ Invitrogen AG, Basel, Switzerland) supplemented with fetal calf serum (FCS, 10%, Bio Concept™, Allschwil, Switzerland), MEM Non Essential Amino Acids (MEM NEAA (100x) without L-Glutamine, GIBCO™ Invitrogen AG, Basel, Switzerland) and GlutaMAX™I Supplement 200 mM (100x), (GIBCO™ Invitrogen AG, Basel, Switzerland). Bacteria used for the adhesion assay were grown over night in LB-broth Miller (Becton Dickinson, Maryland, USA). Prior to incubation, the confluent cells were washed two times with Dulbecco's phosphate buffered saline (D-PBS (1x) without Calcium and Magnesium, GIBCO™ Invitrogen AG, Basel, Switzerland). *E. coli* diluted 1:100 in 1 ml fresh media (MEM with supplements) was added to the cells and was incubated for 3 h at 37°C and 5% CO₂. After this incubation period, monolayers were washed five times with D-PBS (3 h assay) or monolayers were washed ten times with D-PBS, fresh media (MEM with supplements) was added and an additional incubation period of 3 h proceeded (6h assay). Subsequently, the monolayers were washed five times with D-PBS and the cells were fixed for 10 min with 4 Methanol. After the staining with May-Grünwald-Giemsa (Fluka™, Buchs SG, Switzerland), the coverslips were examined by light microscopy to determine the adherence patterns of the strains.

The following fluorescent actin-staining (FAS) test was performed on Caco-2 cells according to Karch et al. [28] with minor modifications. The cells were fixed with 4% formaldehyde in D-PBS, then washed three times with D-

PBS and incubated for 5 min with 0.1% Triton X-100 (Fluka™, Buchs SG, Switzerland) in D-PBS. After another three washes with D-PBS, the monolayers were incubated with 5 µg/ml of fluorescein isothiocyanate labelled phalloidin (Sigma, Steinheim, Germany) for 30 min. After another three washes with D-PBS, the coverslips were examined by fluorescence microscopy (Leica™).

Authors' contributions

RS isolated the strains and drafted the manuscript, CZ was responsible for further strain characterization, NB performed adhesion assays and FAS tests, MB performed the PCR typing of *eae* genes and JE has done serotyping of the strains. All authors read, commented on and approved of the final manuscript.

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