Molecular analysis of *Escherichia coli* O157 strains isolated from cattle and pigs by the use of PCR and pulsed-field gel electrophoresis methods

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ABSTRACT: Fourteen *Escherichia coli* O157 strains isolated from cattle and pigs in Poland and in Germany were investigated, using PCR, for the genetic markers associated with Shiga toxin-producing *E. coli* (STEC). Only two strains, both of cattle origin, were positive for the *fliC* (H7) gene and could be classified as O157 : H7. Nine isolates had *stx* shiga toxin genes, either *stx*1 (1 strain), *stx*2 (4 isolates) or both (4 strains). The *stx*2-carrying samples were further subtyped by PCR for the *stx*2c, *stx*2d, and *stx*2e toxin variants. It was shown that all but one *stx*2-positive bacteria possessed the *stx*2c Shiga toxin gene type and one *stx*2 STEC isolate had the *stx*2d virulence factor subtype. The *eaeA* (intimin) gene was found in 9 strains (8 isolates from cattle and one strain from pigs); all of them harboured the genetic marker characteristic of the gamma intimin variant. The translocated intimin receptor (*tir*) gene was detected in 7 isolates tested and among them only one *tir*-positive strain was recovered from pigs. The *ehly E. coli* enterohemolysin gene was amplified in all but one strains obtained from cattle and only in one isolate of porcine origin. The genetic relatedness of the analysed *E. coli* O157 strains was examined by restriction fragment length polymorphism (RFLP) of chromosomal DNA digested with *Xba*I. Two distinct but related RFLP pattern clusters were observed: one with 9 strains (8 isolates of bovine origin and one strain obtained from pigs) and the other one comprises the remaining 5 *E. coli* isolates (4 of porcine origin and one strain recovered from cattle). The results suggest that pigs, besides cattle, may be a reservoir of *E. coli* O157 strains potentially pathogenic to humans. Moreover, epidemiologically unrelated isolates of the O157 serogroup, recovered from different animal species, showed a clonal relationship as demonstrated by the RFLP analysis.

Keywords: *E. coli* O157; cattle; pigs; virulence marker genes; RFLP; genetic relatedness

Shiga toxin-producing *Escherichia coli* (STEC), including *E. coli* O157 : H7 and some non-H7 types are an important emerged group of foodborne pathogens (Armstrong *et al.*, 1996; Karch *et al.*, 1999). These bacteria can cause a range of human syndromes, including hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Griffin and Tauxe, 1991; Paton and Paton, 1998). STEC are defined by production of Shiga toxin(s) (Stx1 and Stx2), encoded by the *stx* genes of temperate, lambdoid bacteriophages that remain integrated in the *E. coli* chromosome (O'Brien and Holmes, 1987; Paton and Paton, 1998). STEC may carry either *stx* subtype *stx*1, *stx*2 or both *stx*1 and *stx*2. There is a close association between the production of Stx and the presence of several accessory virulence factors implicated in the pathogenesis of human diseases. One of those virulence markers is the *eaeA* gene, which encodes the intimin protein involved in the intimate attachment of bacteria to

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enterocytes (Jerse *et al.*, 1990). Another secreted protein encoded by the genes of the STEC chromosomal pathogenicity island called LEE (locus of enterocyte effacement) is the translocated intimin receptor Tir (McDanniel *et al.*, 1995; Kenny *et al.*, 1997; Paton *et al.*, 1998). Moreover, the plasmid-located enterohemolysin gene (*ehly*), which has been found in nearly all *E. coli* O157 strains, has been suspected to play a role in pathogenicity of STEC infections (Beutin *et al.*, 1989; Schmidt *et al.*, 1995).

Cattle has been found to be the principle reservoir for *E. coli* O157 strains that are transmitted to humans through foods contaminated with fecal material (Armstrong *et al.*, 1996; Mainil, 1999). STEC of the O157 serogroup have also been isolated from sheep, goats, dogs, poultry, and seagulls (Paton and Paton, 1998; Mainil, 1999). However, there is little information concerning the prevalence and characteristics of these bacteria originating from pigs (Beutin *et al.*, 1993; Chapman *et al.*, 1997; Heuvelink *et al.*, 1999; Nakazawa *et al.*, 1999). Moreover, the *E. coli* O157 recovered from pigs were often different from other animal and human STEC in the absence of *stx* and accessory virulence marker genes (Wray *et al.*, 1993; Wittig *et al.*, 1995).

E. coli strains of the O157 serogroup show a clonal nature and highly sensitive molecular biology-based subtyping methods are needed to compare and to differentiate unrelated strains isolated from different sources (Whittam *et al.*, 1988; Feng *et al.*, 1998). Several methods have been used for genetic analysis of STEC O157 and among them differentiation of chromosomal DNA based on restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) appear to be the most powerful techniques that provide the greatest discrimination and reproducibility (Bohm and Karch, 1992; Meng *et al.*, 1995).

The aim of this study was to analyse, using polymerase chain reaction (PCR), the presence of the STEC-associated virulence genes *stx*1, *stx*2, *eaeA*, *ehly*, and *tir*, in *E. coli* strains of the O157 serogroup isolated from cattle and from pigs. The genetic relationship between these isolates, belonging to the same serogroups and expressing various pathogenic factor genes, was also tested by the RFLP method. These data may provide some new epidemiological information about the clonal nature of *E. coli* O157 isolated from different sources.

MATERIAL AND METHODS

Bacterial strains

Fourteen *E. coli* strains of the O157 serogroup were isolated from cattle (n = 9) and from pigs (n = 5). The strains of cattle origin were recovered from 3–5 months old animals as described previously (Osek *et al.*, 2000). The cattle farms were located in the eastern part of Poland and they were separated by 60–220 km. No animal movement among those farms was noted. The seven Polish *E. coli* O157 isolates (designated with numbers 422, 426, 427, 443, 444, 445, and 448) were recovered from seven animals that represent seven different cattle farms. The additional two *E. coli* strains of cattle origin (numbers 419 and 441) were isolated on two distinct animal farms located in the eastern part of Germany. The five O157 strains recovered from pigs (numbers 263, 272, 275, 297, and 326) were obtained from five weaned pigs with diarrhoea (at the age of 4–6 weeks) from five different geographically separated pig farms in the western part of Poland as described previously (Osek, 1999b).

All bacterial samples were cultured directly on MacConkey's agar (Oxoid, Basinstoke, England) and were identified as *E. coli* using the API20E biochemical system (bioMerieux, Marcy l'Etoile, France). The isolated *E. coli* strains were stored in deep agar tubes at room temperature and they were not subcultured more than twice before the examination.

PCR amplification methods

The following genes were amplified using PCR: *rfb*O157 of *E. coli* LPS O157, *fliC* of the H7 flagellar antigen, *stx*1 and *stx*2 of Shiga toxin 1 and 2, respectively, *eaeA* of STEC intimin, *tir* of the translocated intimin receptor Tir, and *ehly* of *E. coli* enterohemolysin. The characteristics of the DNA primers and PCR conditions were described previously (Cebula *et al.*, 1995; Schmidt *et al.*, 1995; Batchelor *et al.*, 1999; Maurer *et al.*, 1999; Osek, 1999a, 2001; Osek *et al.*, 2000; Wang *et al.*, 2000). The *eaeA* gene subtyping was performed with primer pairs specific for the α, β, γ, δ, and ε variants as described (Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000). The *stx*2d and *stx*2e Shiga toxin variant genes were tested with primers VT2-cm/VT2-f and SLT2vB1/SLT2vB2, respectively (Imberechts

Table 1. PCR primers used in the study

Primer code	Primer sequence (5′→3′)	Target gene	Gene product	PCR amplicon (bp)	Reference
PF8	CGTGATGATGTTGAGTTG	*rfb*O157	LPS O157	420	Maurer *et al.*, 1999
PR8	AGATTGGTTGGCATTACTG				
1806	GCTGCAACGGTAAGTGAT	*fliC*	H7	984	Wang *et al.*, 2000
1807	GGCAGCAAGCGGGTTGGT				
LP30	CAGTTAATGTCGTGGCGAAGG	*stx1*	Shiga toxin 1	348	Cebula *et al.*, 1995
LP31	CACCAGACAATGTAACCGCTG				
LP43	ATCCTATTCCCGGGAGTTTACG	*stx2*	Shiga toxin 2	584	Cebula *et al.*, 1995
LP44	GCGTCATCGTATACACAGGAGC				
GK3	ATGAAGAAGATGTTTATG	*stx2c*	Shiga toxin 2c	260*	Schmidt *et al.*, 1994
GK4	TCAGTCATTATTAAACTG				
VT2-cm	AAGAAGATATTTGTAGCGG	*stx2d*	Shiga toxin 2d	256	Pierard *et al.*, 1998
VT2-f	TAAACTGCACTTCAGCAAAT				
SLT2vB1	ATGAAGAAGATGTTTATAGCG	*stx2e*	Shiga toxin 2e	267	Imberechts *et al.*, 1992
SLT2vB2	TCAGTTAAACTTCACCTGGGC				
Int.Fc	CCGGAATTCGGGATCGATTACCGTCAT	*eaeA*	Intimin (general)	840	Batchelor *et al.*, 1999
Int.Rc	CCCAAGCTTTTATTTATCAGCCTTAATCTC				
Int-d	TACGGATTTTGGGGCAT	*eaeA*-γ	Intimin gamma	544	Adu-Bobie *et al.*, 1998
Int-Ru	TTTATTTGCAGCCCCCCAT				
Tir-F3	CGGGATCCTTAGACGAAACGATGGGATCC	*tir*	Translocated intimin	1 700	Paton *et al.*, 1998
Tir-R3	GGAATTCATGCCTATTGGTAA		receptor		
hlyAF	GCATCATCAAGCGTACGTTCC	*ehlyA*	Enterohemolysin	534	Schmidt *et al.*, 1995
hlyAR	AATGAGCCAAGCTGGTTAAGCT				

*this PCR amplicon was digested with *Hae*III restriction enzyme into two products: 128 bp and 142 bp

et al., 1992; Pierard *et al.*, 1998), whereas the presence of the *stx*2c variant was determined after digestion with *Hae*III (5′-GG↓CC-3′) endonuclease (Fermentas, Vilnius, Lithuania) of the PCR product obtained with the DNA amplification performed with the primer pair GK3 and GK4 according to the protocol of Schmidt *et al.* (1994). All PCR primers used in the study are presented in Table 1.

The PCR amplicons were separated by standard gel electrophoresis in 2% agar gel stained with ethidium bromide solution (5 µg/ml) for 1 min.

RFLP and PFGE

The preparation of genomic bacterial DNA was performed essentially as described previously (Osek, 2000) with one modification for the cell concentration that was 1.25×10^9 bacterial cells per one agarose block. Digestion of the genomic DNA embedded in agarose plugs was carried out using *Xba*I restriction enzyme (5′-T↓CTAGA-3′), (Fermentas), 40 U/agarose block, for 18 h at 37°C in a thermoblock (Eppendorf, Hamburg, Germany).

PFGE was performed with the CHEF DR II electrophoresis system (Bio-Rad, Hercules, CA) in 0.5X Tris-borate-EDTA (TBE) buffer at 14°C in 1% agarose gel (pulsed-field certified; Bio-Rad). The gels were run at pulse ramps from 5 to 40 s for 23 h at constant voltage of 6 V per 1 cm. After electrophoresis, the gels were stained with ethidium bromide solution (0.5 µg/ml) for 30 min, destained with distilled water for 15 min and photographed under UV light using the Gel Doc 2000 documentation system (Bio-Rad). A 50 kb λ PFGE DNA marker (Sigma, St. Louis, MO) was included into each electrophoresis run.

Data analysis

Gel images were scanned and analysed using the GelCompar II software (Applied Maths, Kortrijk, Belgium). Dendrogramme was created with the Dice coefficient and the unweighted pair group method with arithmetic mean analysis (UPGMA).

Table 2. Prevalence of virulence marker genes in *E. coli* O157 strains as tested by PCR

Strain number	Source	Virulence marker gene					
		fliC	*stx*1	*stx*2 (subtype)	*eaeA* (subtype)	*tir*	*ehly*
419	Cattle	–	–	+ (*stx*2c)	+ (γ)	+	+
448	Cattle	–	–	+ (*stx*2c)	+ (γ)	+	+
422	Cattle	–	+	+ (*stx*2c)	+ (γ)	+	+
445	Cattle	–	+	+ (*stx*2c)	+ (γ)	+	+
444	Cattle	–	–	–	–	–	–
426	Cattle	–	+	+ (*stx*2c)	+ (γ)	+	+
443	Cattle	–	+	+ (*stx*2c)	+ (γ)	+	+
427	Cattle	+	+	–	+ (γ)	–	+
441	Cattle	+	–	+ (*stx*2d)	+ (γ)	–	+
272	Pig	–	–	–	–	–	–
263	Pig	–	–	–	–	–	–
297	Pig	–	–	–	–	–	–
275	Pig	–	–	–	–	–	–
326	Pig	–	–	+ (*stx*2c)	+ (γ)	+	+

RESULTS

Fourteen *E. coli* strains of the O157 serogroup (the *rfb*O157 gene-positive) were analysed by PCR for the *stx*1, *stx*2 (including *stx*2c, *stx*2d, and *stx*2e subtypes), *eaeA*, *ehly*, and *tir* virulence marker genes, respectively. Moreover, all isolates were tested for the *fliC* genetic marker responsible for the production of the H7 *E. coli* flagellar protein. It was shown (Table 2) that only two bovine strains, one isolated in Poland and one in Germany, were positive in PCR performed with primers 1806 and 1809 flanking the internal fragment of the *fliC* gene, therefore they could be classified as *E. coli* O157 : H7. The remaining 12 isolates did not generate a PCR amplicon of 948 bp or any other size. Thus, these strains were of the O157 group without the H7 antigen (O157 : H7^{-}).

Further PCR analysis of the *E. coli* O157 isolates was performed for the genetic markers characteristic of STEC, i.e. Shiga toxin 1 and 2 (including the *stx*2 subtypes) and accessory virulence genes. It was found (Table 2) that nine isolates were *stx*-positive, either *stx*1 (one strain), *stx*2 (4 strains) or both *stx*1/*stx*2 (4 isolates), therefore they could be classified as STEC. All but one *stx*2-positive strains had genes for the *stx*2c toxin variant as determined by the presence of the 128 bp and 142 bp *Hae*III-digested PCR products. The last one *stx*2-positive, H7-positive *E. coli* strain generated the 256 bp amplicon in PCR with VT2-cm and VT2-f primers indicating that it has the *stx*2d toxin subtype. Five remaining *E. coli* O157 isolates tested (one strain of bovine-origin and 4 strains recovered from pigs) were negative in all *stx*-associated PCR amplifications.

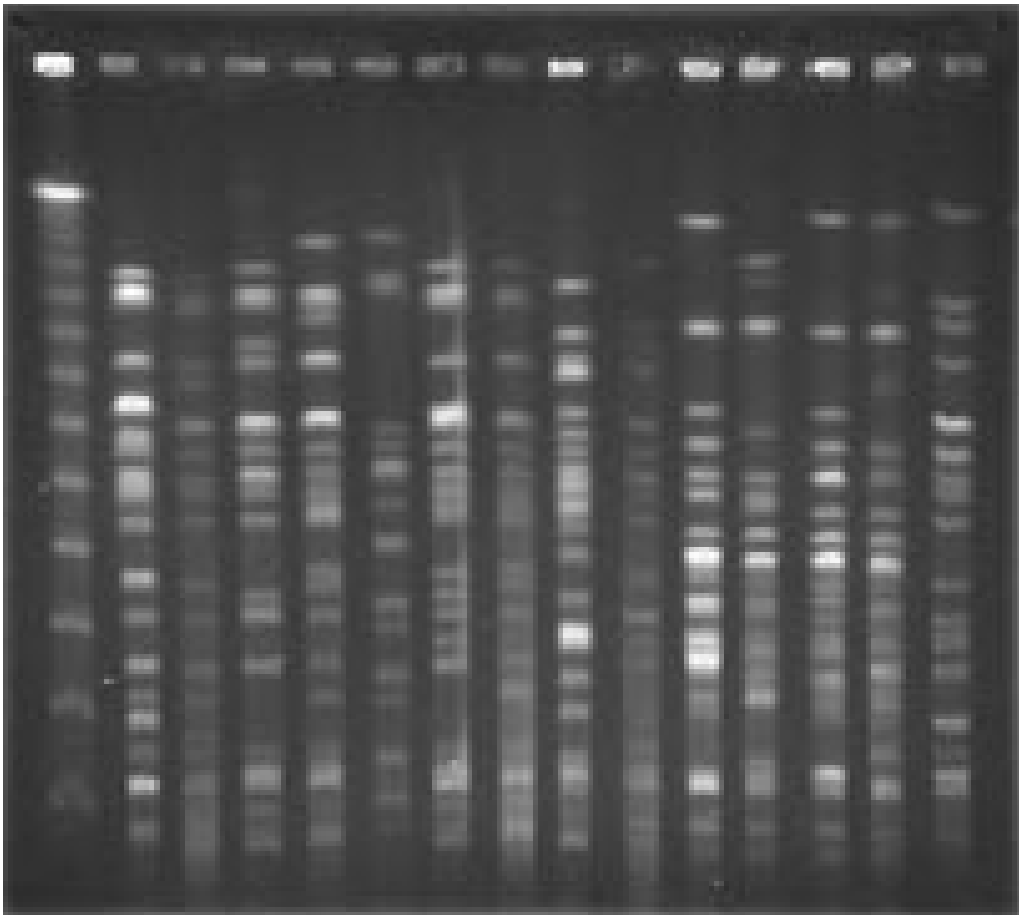

Figure 1. PFGE patterns of *Xba*I-digested genomic DNAs of *E. coli* O157 strains. Lane M – molecular size standard (50 kb λ DNA). In lanes 1 to 9 – the following strains isolated from cattle are shown: lane 1, 419; lane 2, 448; lane 3, 422; lane 4, 445; lane 5, 444; lane 6, 426; lane 7, 443; lane 8, 427, and lane 9, 441. In lanes 10 to 14 – *E. coli* strains isolated from pigs are shown as follows: lane 10, 272; lane 11, 263; lane 12, 297; lane 13, 275, and lane 14, 326

The *eaeA* (intimin) gene was found in 9 strains whereas the remaining 5 O157-positive isolates were *eaeA*-negative as determined by PCR with the Int-Fc and Int-Rc primers (Batchelor *et al.*, 1999). Further analysis of the intimin-positive strains revealed that all of them possessed the genetic marker characteristic of the gamma intimin variant (*eaeA*-γ) as tested with PCR primers SK1 and LP3 (Oswald *et al.*, 2000).

The *tir* gene was found in 7 out of 14 isolates tested; among them only one *tir*-positive strain was recovered from pigs. Interestingly, two *E. coli* O157 : H7 strains of bovine origin, positive for the *eaeA* gene, were negative in the PCR assay with primers Tir-F3 and Tir-R3, for the *tir* determinant (Paton *et al.*, 1998). On the other hand, these two isolates were of the STEC group (*stx*1- or *stx*2-positive) and had the enterohemolysin gene as determined by PCR with primers hlyAF and hlyAR (Schmidt *et al.*, 1995).

PCR performed with primers specific for the *E. coli* enterohemolysin gene revealed that all but one strains of bovine origin generated the 534 bp amplicon characteristic of this virulence marker. On the other hand, only one *E. coli* O157 isolate of porcine origin was positive in this assay, whereas the remaining 4 strains did not possess the *ehly* gene.

PFGE analysis performed with *Xba*I endonuclease, which recognises rarely used nucleotide sequences, revealed that *E. coli* O157 strains tested produced 14 distinct DNA fragment profiles (Figure 1). Each PFGE profile displayed several bands within the 50 and 600 kb range. The PFGE patterns of all but one bovine isolates (strain 444) were closely related to each other. In contrast, the DNA restriction profiles of the four porcine strains (isolates 263, 272, 275, and 297, respectively) differed from the patterns of *E. coli* O157 of bovine origin.

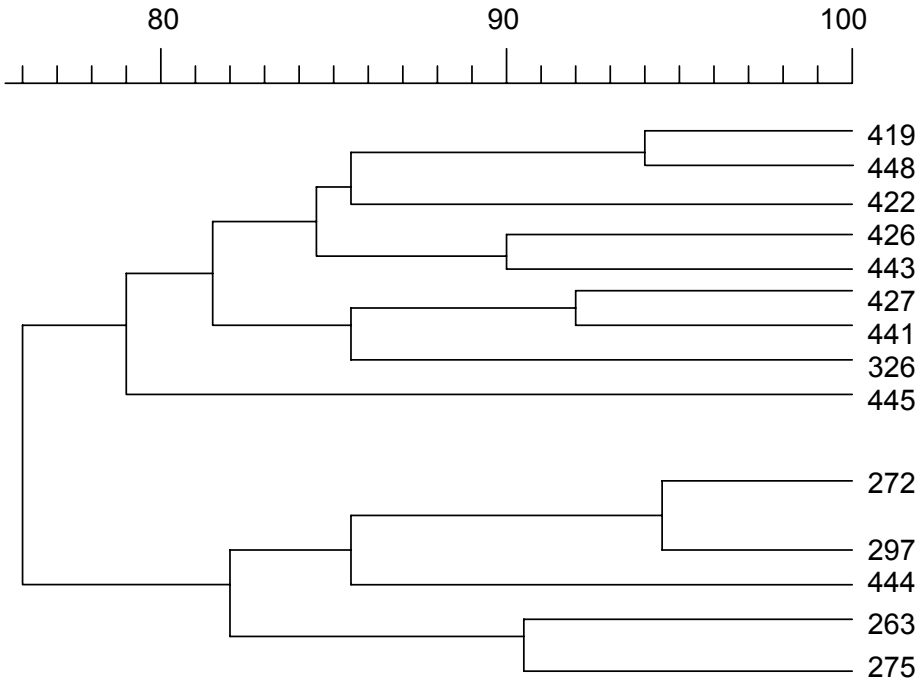

Figure 2. Cluster analysis, derived from PFGE data, on *E. coli* O157 isolates recovered from cattle and pigs. Strains 419, 422, 426, 427, 441, 443, 444, 445, and 448 were isolated from cattle; strains 263, 272, 275, 297, and 326 were of pig-origin. The phenogramme outlining the clonal relationship of the isolates was constructed using the Dice coefficient and UPGMA analysis. The degree of similarity (in percentage) is shown on the scaleFigure 2. Cluster analysis, derived from PFGE data, on *E. coli* O157 isolates recovered from cattle and pigs. Strains 419, 422, 426, 427, 441, 443, 444, 445, and 448 were isolated from cattle; strains 263, 272, 275, 297, and 326 were of pig-origin. The phenogramme outlining the clonal relationship of the isolates was constructed using the Dice coefficient and UPGMA analysis. The degree of similarity (in percentage) is shown on the scale

The cluster analysis of the PFGE profiles performed by the UPGMA method revealed that the tested *E. coli* strains belonged to two genetic groups (Figure 2). One cluster comprised 9 isolates (8 strains of bovine origin and one isolate recovered from pigs) whereas the other group consisted of the remaining five strains, both of pig origin (4 strains) and one isolate recovered from cattle. These two PFGE profiles were lower than 80% by the Dice similarity index. The *E. coli* O157 strains within each RFLP cluster showed a close genetic relationship as demonstrated by the UPGMA analysis. Interestingly, one isolate of porcine origin (strain 326) was much more closely related to 8 bovine strains, whereas the cattle strain 444, which was negative for all virulence marker genes tested, showed a high degree of similarity with porcine *E. coli* O157 isolates (Figure 2).

DISCUSSION

There is little information concerning the isolation and characterisation of *E. coli* O157 strains in Poland (Szych *et al.*, 1998; Osek, 2001). Most of these isolates were recovered from cattle and from children with diarrhoea. Moreover, there is only one report describing the prevalence of *E. coli* O157 of pig origin (Osek, 2002) and there are few papers about such strains from other countries (Beutin *et al.*, 1993; Chapman *et al.*, 1997; Heuvelink *et al.*, 1999; Nakazawa *et al.*, 1999). In the present study, twelve Polish isolates (seven of bovine origin and five recovered from pigs, respectively) and two German strains (both isolated from cattle) were tested for STEC virulence marker genes and for their clonal relationship using PCR and PFGE methods, respectively. The results indicate that most *E. coli* O157 lacked the H7 flagellar protein gene as tested by PCR amplification of the *fliC* operon. Such H7-negative strains have recently been identified in Germany and in the Czech Republic and it was demonstrated that they represent a distinct clone within the *E. coli* O157 serogroup that, however, shares several virulence traits with other STEC of the O157 : H7 serotype (Bielaszewska *et al.*, 1998, 2000; Ammon *et al.*, 1999). As shown in the present study, the PFGE profiles of bacterial DNA digested with *Xba*I endonuclease, analysed with the UPGMA method, revealed a close molecular relationship of the two O157 : $H7^+$ isolates with other O157 but H7-negative strains of bovine origin. All these strains belonged to one genetic branch of the created dendrogramme (Figure 2). As demonstrated by other authors (Bielaszewska *et al.*, 1998; Paton and Paton, 1998; Ammon *et al.*, 1999), O157 : $H7^-$ strains isolated from HC and from HUS were usually *stx*- and *eaeA*-positive, and often possessed the enterohemolysin gene. As shown in the present study, all but one of the bovine isolates were also *stx*-positive, whereas only one out of five *E. coli* O157 of pig origin had the *stx* gene. On the other hand, this *stx*-positive porcine isolate harboured the *eaeA*, *tir*, and *ehly* accessory virulence markers suggesting a potential public health risk.

PCR analysis performed for the *eaeA*, *tir*, and *ehly* accessory virulence marker genes showed that all but one bovine isolates possessed most of these tested factors. Interestingly, two O157 : H7-positive strains were negative for the *tir* gene and another strain did not amplify any of the gene analysed. On the other hand, only one porcine strain had all three accessory virulence markers tested and the remaining 4 isolates were negative for *stx* and other genes as tested by PCR.

All *eaeA*-positive STEC tested in the present study also bore the enterohemolysin gene. As reported by several authors (Beutin *et al.*, 1989; Bielaszewska *et al.*, 1998; Paton and Paton, 1998; Karch *et al.*, 1999), the combination of these two pathogenic marker genes seems to be a more important indicator of pathogenicity of STEC for humans than either factor alone. Therefore, the STEC strains of the O157 serogroup tested can be a potential health risk for man.

Cattle have been implicated as a major reservoir of *E. coli* O157 and there are only few reports describing the isolation and characterisation of this bacteria from pigs (Beutin *et al.*, 1993; Chapman *et al.*, 1997; Heuvelink *et al.*, 1999; Nakazawa *et al.*, 1999). In the present study, five isolates of pig origin were analysed and compared with nine O157 strains isolated from cattle. There were markedly different gene profiles of the strains belonging to these two groups: only one porcine isolate was *stx*-positive, harbouring also the tested accessory virulence marker genes. Moreover, none of the 5 strains of porcine origin was H7-positive as tested by PCR for the *fliC* operon. Clonal analysis of *E. coli* O157 strains also indicated that *stx*-, *eaeA*-, *tir*-, and *ehly*-negative isolates, irrespective to the origin, belonged to one distinct genetic group as analysed by PFGE and UPGMA. Interestingly, one *stx*-negative O157 strain isolated from cattle was much more

closely related to porcine *stx*-negative isolates than to other strains of bovine origin. On the other hand, one *stx*-positive O157 *E. coli* isolated from pigs belonged to the same dendrogramme branch bearing *stx*-producing bovine *E. coli* isolates.

The genetic analysis of all tested *E. coli* O157 isolates revealed that bacteria can possess different virulence associated factors yet be in the same clonal linkages as evaluated by RFLP. This method has widely been used as a molecular subtyping method of STEC strains, including O157, due to its high discriminatory power and good reproducibility (Bohm and Karch, 1992; Meng *et al.*, 1995). In the present study, it was shown that epidemiologically unrelated O157 *E. coli* isolates produced very similar *Xba*I restriction profiles. Moreover, a marked similarity between H7-positive and H7-negative strains further supports the theory that all of them had a common progenitor (Feng *et al.*, 1998). The results also suggest that, besides cattle, pigs may be a reservoir of *E. coli* O157 strains potentially pathogenic to humans although in the present study only one of the five tested porcine isolates possessed *stx* and other STEC-associated virulence marker genes. However, this strain, together with several bovine isolates, represents one clonal group as shown by the PFGE and UPGMA analysis.

The PFGE method used in this study efficiently showed DNA polymorphism and interstrain variation in *E. coli* O157 serogroup isolated from cattle and from pigs. These data indicate that PFGE is a powerful tool to reveal intraserotype specific genetic differences between *E. coli* strains isolated from various animal species.

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