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Genomic fingerprinting of shigatoxin-producing *Escherichia coli* (STEC) strains: comparison of pulsed-field gel electrophoresis (PFGE) and fluorescent amplified-fragment-length polymorphism (FAFLP)

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SUMMARY

For epidemiological studies of shigatoxin-producing *Escherichia coli* (STEC) infections, rapid, reproducible and highly discriminative methods are required. In this study, we examined the performance of the fluorescent amplified-fragment-length polymorphism (FAFLP) technique for epidemiological fingerprinting of STEC isolates and compared it to the acknowledged fingerprinting method pulsed-field gel electrophoresis (PFGE). A total of 88 STEC isolates, including 82 of serotype O157:H7 or O157:H$^-$, were subjected to fingerprinting by both PFGE and FAFLP. The isolates included sporadic and epidemiologically related strains of both animal and human origin from widespread geographical locations. The FAFLP fingerprint patterns confirmed the clonal nature of STEC O157 strains. Among the 82 O157:H7/H$^-$ isolates belonging to 49 distinct groups of epidemiological unrelated isolates, 24 FAFLP profiles and 51 PFGE patterns were obtained. Thus, PFGE had a higher discriminatory power than FAFLP and overall correlated better to available epidemiological data. Consequently, the PFGE technique remains the method of choice in epidemiological investigations of STEC infections.

INTRODUCTION

Shigatoxin-producing *Escherichia coli* (STEC) has in recent years emerged as an important foodborne pathogen and has caused severe outbreaks in Japan, North America and Europe [1–4]. Infection with STEC is associated with a spectrum of symptoms including diarrhoea, haemorrhagic colitis and life-threatening haemolytic uraemic syndrome. Although *E. coli* O157:H7 is the predominant pathogenic STEC in the world, other STEC serotypes are increasingly recognized in many countries [5–7]. The clonal nature of strains within many STEC serotypes especially *E. coli* O157:H7 [8, 9], has made subtyping of these strains dependent on highly discriminatory and reproducible techniques. The ability to differentiate between individual strains of the organism is essential both to trace the spread of an organism and to identify the source of infection and is an important adjunct in epidemiological surveillance. In this context, typing methods allowing easy inter-laboratory comparison are also in demand. Phenotypic typing methods for strain differentiation of STEC O157 strains such as phage typing and verotoxin typing are valuable techniques, which could remove the need for more expensive DNA-based methods. As for the phenotypic methods, the discriminatory power of molecular typing techniques also differs considerably and is in many cases not sufficient to trace the source of an infecting organism in an outbreak situation [10]. Pulsed-field gel electrophoresis is a reliable and highly

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discriminating technique and is now the method of choice for molecular fingerprinting of many bacteria. However, due to the clonal nature of *E. coli* O157:H7 and other STEC serotypes, PFGE may fail to discriminate between epidemiological unrelated isolates [11]. The PFGE patterns may also be difficult to interpret when the profiles do not match exactly [12]. The inability of PFGE to type certain strains of STEC due to degradation of genomic DNA during the PFGE procedure has also been documented [2, 13, 14].

Combinations of techniques are therefore recommended to increase the discriminatory power in epidemiological investigations [11, 15]. Relatively recently, a whole-genome PCR-based DNA fingerprinting method termed amplified-fragment length polymorphism (AFLP) was developed [16]. The AFLP method is based on selective amplification of restriction enzyme digested genomic fragments by primers in which one is radioactively labelled. An approach replacing the radioactively labelled primers with fluorescent labelled primers (FAFLP) together with electrophoresis and fingerprint analysis on automated sequencers offers the ability of high throughputs combined with automatic compilation of FAFLP patterns in a database [17, 18]. This also enables inter-laboratory comparison and exchange of FAFLP fingerprints. The FAFLP assay has therefore been described as a rapid, reproducible and highly discriminating method with universal applicability (reviewed by Savelkoul and colleagues [19]). It could thus be an alternative or supplement to PFGE for fingerprinting of STEC isolates. To investigate the performance of FAFLP on subtyping of STEC isolates, sporadic and epidemiologically related isolates were subjected to FAFLP fingerprinting and the results were compared with those obtained by the acknowledged method of PFGE.

MATERIALS AND METHODS

Strain characteristics

A total of 88 STEC strains, including 26 isolates from human sporadic cases, 40 isolates from outbreaks, 20 isolates from cattle faeces and 2 from pig faeces, were analysed (Table 1). Eighty-two isolates belonged to *E. coli* serogroups O157:H7 or O157:H$^-$. The STEC strains were from diverse geographic locations, including Norway (44 strains), Sweden (12 strains) Finland (6 strains), United States (20 strains) and United Kingdom (6 strains). Presence of *stx* genes (*stx*1 and *stx*2) was ascertained by PCR as described by Brian and colleagues [20].

FAFLP

A modified method of Vos and colleagues [16] was used. Initially, 10 combinations of restriction enzymes (rare cutter + frequent cutter) with corresponding adaptors and primer pairs were evaluated for their ability to subtype STEC strains by FAFLP. The following primer combinations were tested: *Eco*RI +0/*Csp*6I + A (no selective nucleotide (+0) added to the *Eco*RI primer; an additional adenine (+A) was added as a selective nucleotide to the 3′-end of the *Csp*6I primer), *Eco*RI + 0/*Mse*I + C (*Mse*I primer contained additional 3′-cytosine (+C)), *Eco*RI +0/*Mse*I + TA (*Mse*I primer contained additional 3′-thymidine and adenine (+TA)), *Bgl*II + 0/*Csp*6I + A (*Csp*6I primer contained additional 3′-adenine (+A)), *Eco*RI + A/*Mse*I + C (*Eco*RI primer contained additional 3′-adenine (+A)), *Hin*dIII + 0/*Taq*I + C (*Taq*I primer contained additional 3′-cytosine (+C)), *Eco*RI + 0/*Hin*PI + 0, *Eco*RI + 0/*Mse*I + 0, *Xba*I + 0/*Mse*I + 0 and *Bgl*II + 0/*Mfe*I + 0. The choice of the restriction enzymes and primers was mainly based on previous reports describing AFLP/FAFLP fingerprinting of bacteria [16, 21–25]. Restriction enzyme *Csp*6I was purchased from Fermentas AB, Vilnius, Lithuania. Other restriction enzymes were from New England Biolabs, Beverly, MA, USA.

Reproducibility was only tested on restriction enzyme/primer combinations showing the highest potential to discriminate between epidemiological unrelated STEC O157:H7/H$^-$ isolates after the pilot study. These primer combinations included *Eco*RI +0/*Csp*6I + A, *Bgl*II + 0/*Mfe*I + 0, *Eco*RI + 0/*Mse*I + C and *Xba*I + 0/*Mse*I + 0. Lack of reproducibility was experienced with two restriction enzyme/primer combinations (*Eco*RI + 0/*Csp*6I + A; *Bgl*II + 0/*Mfe*I + 0) although the FAFLP procedure was performed under highly standardized conditions. Reproducible fingerprint patterns in experiments performed at different times and including template DNA from different isolations were obtained for the enzyme/primer combinations, *Eco*RI + 0/*Mse*I + C and *Xba*I + 0/*Mse*I + 0. These enzymes/primers were therefore chosen in further FAFLP experiments.

Genomic DNA was isolated by a commercial kit (Easy DNA, Invitrogen BV, Leek, The Netherlands). For restriction enzyme digestion, 500 ng of genomic DNA were incubated at 37 °C for 5 h in a total

Table 1. *Characteristics of STEC isolates and their PFGE and AFLP profiles*

Isolate	Serotype	*stx**	Origin†	Country of isolation	PFGE (*Xba*I) pattern	AFLP (EMc) pattern
1113/93	O157:H$^-$	2	H; S	Norway	P1	A2
IH 53436‡	O157:H7	2	H; S	Finland	P2	A7
I47K§	O157:H7	1+2	C	Sweden	P3	A11
I62K§	O157:H7	1+2	C	Sweden	P3	A11
C21b/95	O157:H$^-$	1+2	C	Norway	P4	A9
1452/99	O157:H7	2	H; O1	Norway	P5	A3
1521/99	O157:H7	2	H; O1	Norway	P5	A3
1721/99	O157:H7	2	H; O1	Norway	P5	A3
1856/99	O157:H7	2	H; O1	Norway	P5	A3
1235/99	O157:H$^-$	1+2	H; S	Norway	P6	A12
1366/99	O157:H7	2	H; S	Norway	P7	A15
174/99	O157:H$^-$	2	H; S	Norway	P8	A8
G5300**	O157:H7	1+2	H; O2	USA	P9	A6
G5301**	O157:H7	1+2	H; O3	USA	P10	A6
1079/96	O157:H7	1+2	H; S	Norway	P11	A6
G5293**	O157:H7	1+2	H; O4	USA	P12	A10
G5294**	O157:H7	1+2	H; O4	USA	P12	A10
G5309**	O157:H7	1+2	H; O5	USA	P13	A6
G5310**	O157:H7	1+2	H; O5	USA	P14	A6
H0616**	O157:H7	1+2	H; O6	USA	P15	A6
H0617**	O157:H7	1+2	H; O6	USA	P15	A6
H0619**	O157:H7	1+2	H; O6	USA	P15	A6
H0620**	O157:H7	1+2	H; O6	USA	P15	A6
G4917**	O157:H7	1+2	H; O7	USA	P16	A6
G4918**	O157:H7	1+2	H; O7	USA	P16	A6
G4920**	O157:H7	1+2	H; O7	USA	P16	A6
G4919**	O157:H7	1+2	H; O7	USA	P17	A6
H0618**	O157:H7	1+2	H; O6	USA	P18	A6
G4921**	O157:H7	1+2	H; O7	USA	P19	A6
1175/96	O157:H7	1+2	H; S	Norway	P20	A6
177/98	O157:H7	2	Pig	Norway	P21	A22
1268/98	O157:H7	2	Pig	Norway	P22	A21
956/98	O157:H7	2	H; S	Norway	P23	A23
1480/96	O157:H7	2	H; O8††	Norway	P24	A17
1720/96	O157:H7	2	H; O8††	Norway	P24	A17
1607/96	O157:H7	2	H; O8††	Norway	P25	A17
610/96	O157:H7	2	H; S	Norway	P26	A7
G5307**	O157:H7	2	H; O9	USA	P27	A18
G5308**	O157:H7	2	H; O9	USA	P27	A18
IH 53425‡	O157:H7	2	H; O10	Finland	P28	A16
IH 53427‡	O157:H7	2	H; O10	Finland	P28	A16
IH 56827‡	O157:H7	2	H; S	Finland	P28	A16
3605§§	O157:H7	2	H; O11	UK	P29	A20
3606§§	O157:H7	2	H; O11	UK	P29	A20
3444§§	O157:H$^-$	1+2	H; O12	UK	P30	A5
3445§§	O157:H$^-$	1+2	H; O12	UK	P30	A5
1406/94	O157:H7	2	H; S	Norway	P31	A16

volume of 40 μl containing 4 U each of two restriction enzymes (*Eco*RI + *Mse*I or *Xba*I + *Mse*I) and 250 ng bovine serum albumin (BSA) in 1 × 'One-Phor-All-Buffer PLUS' (Pharmacia, Uppsala, Sweden). Restriction site specific adaptor oligos were ligated to the digested DNA by adding 10 μl mix containing 5 pmol

Table 1 (*cont.*)

3130/98	O157:H$^-$	1+2	H; O13††	Norway	P32	A3
3190/98	O157:H$^-$	1+2	H; O13††	Norway	P32	A3
1/99	O157:H$^-$	1+2	H; S	Norway	P33	A3
IH 53441‡	O157:H7	2	H; S	Finland	P34	A16
IH 53489‡	O157:H7	2	H; S	Finland	P35	A19
27K§	O157:H7	2	C	Sweden	P36	A24
H83/95	O157:H$^-$	1+2	C/Herd I	Norway	P37	A1
H90/95	O157:H$^-$	1+2	C/Herd I	Norway	P37	A1
H82/95	O157:H$^-$	1+2	C/Herd I	Norway	P38	A1
H88/95	O157:H$^-$	1+2	C/Herd I	Norway	P39	A1
H89/95	O157:H$^-$	1+2	C/Herd I	Norway	P39	A1
3179/92	O157:H$^-$	1+2	H; S	Norway	P40	A3
2K§	O157:H7	2	C	Sweden	P41	A14
204/99	O157:H7	2	C	Norway	P41	A14
205/99	O157:H7	2	C	Norway	P41	A14
8K§	O157:H7	2	C	Sweden	P42	A14
126/97	O157:H7	2	C/Herd II	Sweden	P43	A13
127/97	O157:H7	2	C/Herd II	Sweden	P43	A13
128/97	O157:H7	2	C/Herd II	Sweden	P43	A13
130/97	O157:H7	2	C/Herd III	Sweden	P43	A13
131/97	O157:H7	2	C/Herd III	Sweden	P43	A13
3K§	O157:H7	1+2	C	Sweden	P44	A3
64K§	O157:H7	1+2	C	Sweden	P44	A3
644/98	O157:H$^-$	1+2	H; S	Norway	P45	A3
3108/97	O157:H7	1+2	H; S	Norway	P46	A4
122/98	O157:H$^-$	1+2	H; O14††	Norway	P46	A4
125/98	O157:H$^-$	1+2	H; O14††	Norway	P46	A4
127/98	O157:H$^-$	1+2	H; O14††	Norway	P46	A4
G5295**	O157:H7	2	H; O15	USA	P47	A21
G5296**	O157:H7	2	H; O15	USA	P47	A21
5235§§	O157:H7	2	H; O16	UK	P48	A20
5236§§	O157:H7	2	H; O16	UK	P48	A20
2963/96	O157:H$^-$	1+2	H; S	Norway	P49	A3
1145/96	O157:H7	2	H; S	Norway	P50	A17
1236/96	O157:H7	2	H; S	Norway	P51	A7
2409/96	O113:H21	1+2	H; S	Norway	P52	A25
2945/96	O?:H$^-$	1	H; S	Norway	P53	A26
385/97	O?:H$^-$	1	H; S	Norway	P54	A27
1049/97	O?:H?	2	H; S	Norway	P55	A28
2317/97	O128:H?	2	H; S	Norway	P56	A29
214/98	O?:H?	2	H; S	Norway	P57	A30

* Presence of genes encoding shigatoxin 1 (*stx1*) and/or shigatoxin 2 (*stx2*) is indicated.

† H, human; C, cattle; S, sporadic; O, outbreak. Isolates from the same outbreak are designated with identical numerical value. The outbreaks are numbered consecutively from O1–O16. Sporadic (S) and outbreak (O) associated strains are only indicated for human cases.

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§ Kindly provided by E. Borch, Swedish Meat Research Institute, Kävlinge, Sweden.

** Kindly provided by T. J. Barrett, Centers for Disease Control and Prevention, Atlanta, USA.

†† Family outbreak.

§§ Kindly provided by F. Thomson-Carter, Scottish Reference Laboratory for *Campylobacter* and *E. coli*, Aberdeen, Scotland.

Table 2. *Polymorphisms of AFLP(EMc) profiles and total number of STEC O157 isolates within each profile*

FAFLP profile	No. of isolates	Presence or absence of fragments of the following sizes (bp)*																
		58	62	70	73	82	113	119	135	137	147	274	319	362	381	401	403	434
A1	5	+	−	+	+	+	−	−	−	−	−	−	+	−	+	−	−	−
A2	1	+	−	+	+	+	−	−	−	−	−	−	+	+	+	−	−	−
A3	12	+	−	+	+	+	−	−	−	−	−	−	+	+	−	−	−	−
A4	4	+	−	+	−	+	−	−	−	−	−	−	+	+	−	−	−	−
A5	2	+	−	+	+	−	−	−	−	−	−	−	+	+	−	−	−	−
A6	16	+	−	+	+	+	−	+	−	−	−	−	+	+	−	−	−	−
A7	3	+	−	+	+	+	−	+	−	−	−	−	+	+	+	−	−	−
A8	1	+	−	+	−	+	−	+	−	−	−	+	+	+	−	−	−	−
A9	1	+	−	+	+	+	−	+	−	−	−	+	+	+	−	−	−	−
A10	2	+	−	+	−	+	−	+	+	−	−	−	+	+	−	−	−	−
A11	2	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
A12	1	−	−	+	+	+	−	−	−	−	−	+	−	+	−	−	−	−
A13	5	+	−	+	+	+	−	−	−	−	+	−	+	+	+	−	−	−
A14	4	+	−	+	−	+	−	−	−	−	+	−	+	+	+	−	−	−
A15	1	+	−	+	+	+	−	−	−	−	+	−	+	−	−	−	−	−
A16	5	+	−	+	+	+	−	+	+	−	−	−	+	+	+	−	−	+
A17	4	+	−	+	+	+	−	+	+	−	−	−	+	+	−	−	−	−
A18	2	+	+	+	+	+	−	+	+	−	−	−	+	+	−	−	−	+
A19	1	+	−	+	+	+	−	+	+	−	−	+	+	+	+	−	−	+
A20	4	+	−	+	+	+	−	+	+	−	+	−	+	+	+	−	−	−
A21	3	+	−	+	+	+	−	+	−	−	+	−	+	+	+	−	−	+
A22	1	+	−	+	−	+	−	+	−	−	+	−	+	+	+	−	−	+
A23	1	+	−	+	+	−	+	−	−	−	+	−	+	+	+	+	+	−
A24	1	+	−	+	+	+	−	+	−	−	−	−	+	+	−	−	−	−

* Presence (+) and absence (−) of polymorphic fragments for each profile are indicated.

*Eco*RI adaptors [16] or *Xba*I adaptors [25], 50 pmol *Mse*I adaptors [16], 1 mM ATP, 250 ng BSA and 1 U T4-DNA ligase (New England Biolabs) in 1 × 'One-Phor-All-Buffer PLUS' (Pharmacia). After overnight incubation at 16 °C, 50 μl of TE buffer was added to make the PCR template solution. All PCR amplifications were performed in a total volume of 20 μl containing 2 μl PCR template solution, 10 pmol each of *Xba*I + 0/*Mse*I + 0 or *Eco*RI + 0/*Mse*I + C primers, 2 mM of each dNTP and 1·5 U *Taq* polymerase (Sigma, St. Louis, MO, USA) in 1 × *Taq* buffer. Both *Eco*RI + 0 and *Xba*I + 0 PCR primers [16, 25] were 5′-labelled with the dye FAM (6-carboxyfluorescein).

All PCR reactions were carried out on a Perkin–Elmer GeneAmp PCR system 9700 (PE Biosystem, Foster City, CA, USA). High annealing temperature was used for the first 10 cycles ('touchdown' PCR) to ensure specific primer matches and reduce PCR artifacts [26]. The PCR programme (*Eco*RI + 0/*Mse*I + C primerset) included denaturation at 95 °C for 5 min followed by 10 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 45 s and 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min and finally a 5 min extension step at 72 °C. The same profile was run for the *Xba*I + 0/*Mse*I + 0 primer set except for annealing temperature at 60 °C for the first 10 cycles followed by 56 °C annealing temperature for the remaining 30 cycles. The PCR products were diluted 1:5 and 1 μl was mixed with 12 μl formamide and 0·5 μl Genescan TAMRA-500 (PE Biosystem) as internal standard. After denaturation at 95 °C for 5 min, samples were subjected to capillary electrophoresis on an ABI-310 Genetic Analyzer with POP4-polymer (PE Biosystems).

PFGE

Pelleted cells of 1 ml overnight grown (37 °C) Luria Bertani culture were washed in 1 × TEN buffer (1 M NaCl, 10 mM Tris HCL (pH 8), 10 mM EDTA) and centrifuged. Plugs were prepared by resuspending the cells in 250 μl of EC buffer (1 M NaCl, 10 mM Tris HCl (pH 8), 200 mM EDTA, 0·5% *N*-lauroylsarcosine, 0·2% sodium deoxycholate) adding 350 μl of 1·5% LGT agarose (FMC Bioproducts, Rockland, ME, USA) and 30 μl lysozyme (20 mg/ml) before dis-

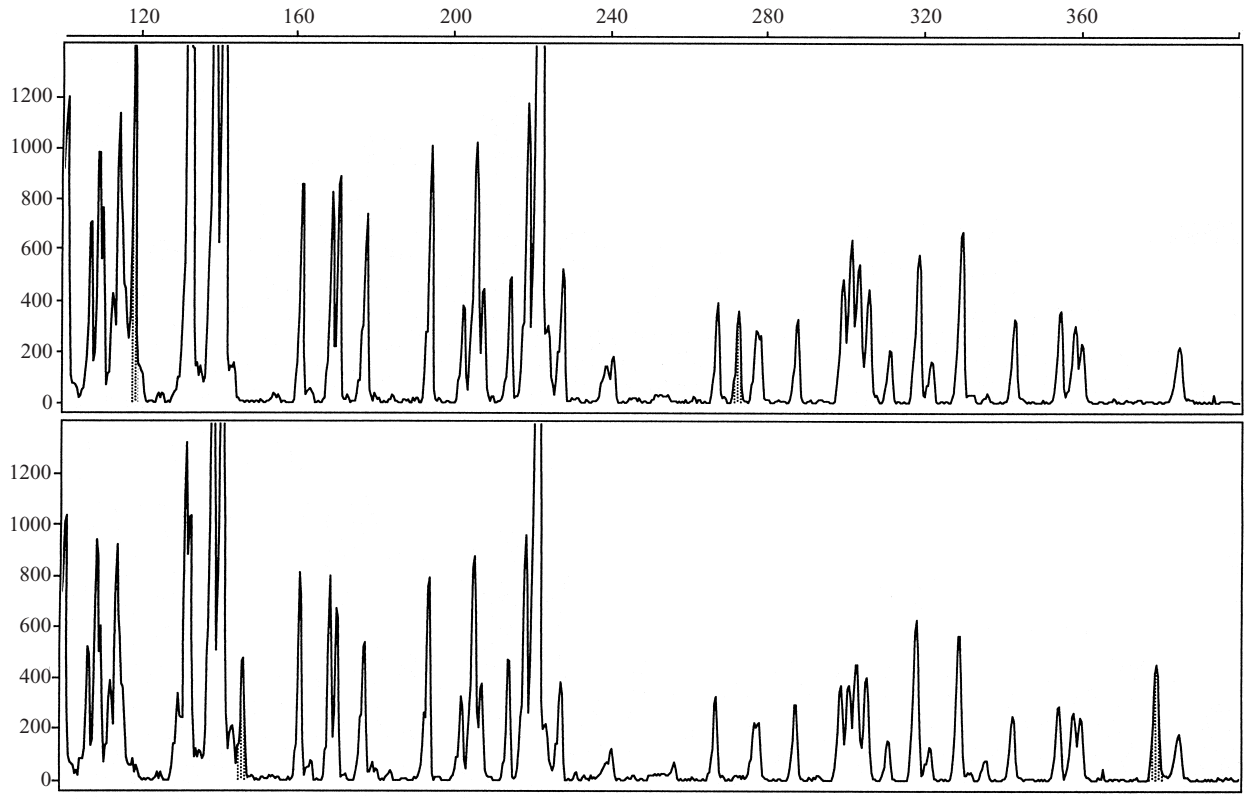


Fig. 1. Electropherograms of two STEC O157 isolates showing examples of polymorphisms (shaded peaks) obtained by FAFLP with the primer combination *Eco*RI + 0/*Mse*I + C. Fragments in the region 100–400 bp are shown. Upper panel: strain C21b/95 (profile A9). Lower panel: Strain 204/99 (profile A14). Fragment sizes (bp) are shown above the electropherograms.

pensing. After solidification, lysis in 5 ml EC buffer containing 0·8 mg/ml lysozyme and digestion with 1 mg/ml proteinase K in 2·5 ml EC buffer under appropriate conditions were performed. The plugs were washed once in 10 ml TE buffer (10 mM Tris, 1 mM EDTA) and incubated for 2 h at 37 °C in 5 ml TE buffer containing 0·2 mM Pefabloc SC (Boehringer–Mannheim, Mannheim, Germany). The plugs were washed twice with TE buffer and digested with 20 U *Xba*I in 100 μl NEB buffer 2 (New England Biolabs) at 37 °C overnight. The DNA fragments were separated by electrophoresis (1 % SeaKem GTG agarose, FMC Bioproducts) in 0·25 × modified TBE buffer (25 mM Tris, 25 mM boric acid, 0·05 mM EDTA) for 22 h at 350 V and 12 °C, with pulse times from 5 s to 40 s using a Beckman GeneLine II apparatus (Beckman, Fullerton, CA, USA).

Data analysis

The FAFLP fingerprints were visually compared by superimposing different strains in the Genescan software (PE Biosystems) and then imported into the computer program GelCompar II (Applied Maths, Kortrijk, Belgium) for further analysis. Scanned images of PFGE gels were stored in tagged image file format and processed with GelCompar II. Similarity between fingerprints was calculated using the Dice coefficient. Band position tolerances of 0·2 and 1·1 % were used for analysis of FAFLP and PFGE patterns, respectively. In data analysis, only fragments in the range 50–480 bp (FAFLP) and 48·5–630·5 kbp (PFGE) were considered. Cluster analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA). Any non-identity in the presence, absence or apparent mobility of bands was considered when assigning FAFLP and PFGE profile numbers. The discriminatory power of FAFLP(EMc) (FAFLP with the primer combination *Eco*RI + 0 and *Mse*I + C) and PFGE typing methods was determined by calculating Simpson's index of diversity (DI) as described by Hunter and Gaston [28].

RESULTS

FAFLP (EMc)

FAFLP with the primer combination *Eco*RI + 0 and *Mse*I + C, termed FAFLP(EMc), detected a total of

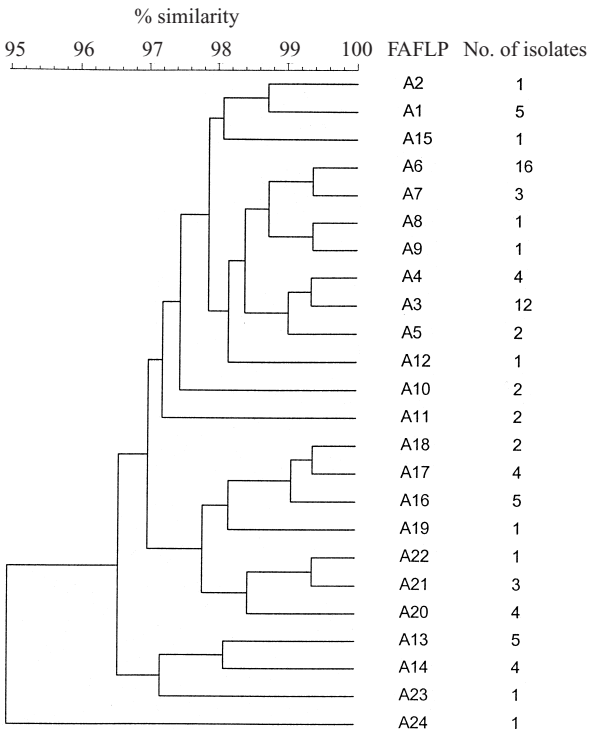


Fig. 2. Dendrogram representing all 24 FAFLP profiles of STEC O157:H7/H$^-$ isolates obtained with the primer combination *Eco*RI + 0/*Mse*I + C. The numbers of isolates representing each profile are indicated.

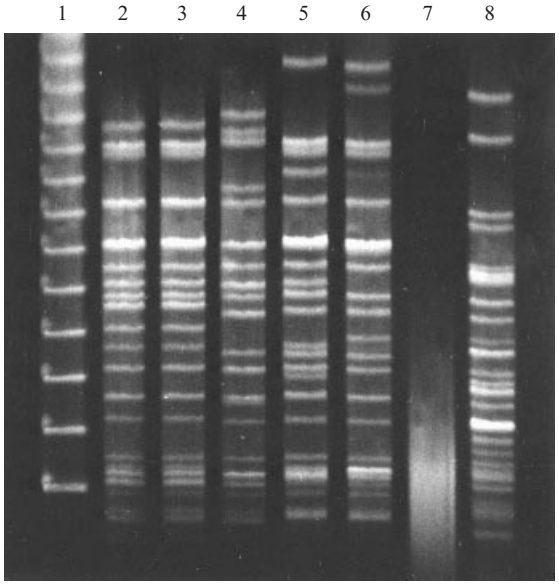


Fig. 3. PFGE patterns of *Xba*I-digested DNA of five O157:H7 STEC isolates (lanes 2–6), one non-typable Stx$^-$ O157:H7 isolate and one non-O157:H7 STEC isolate. Lane 1, bacteriophage lambda ladder (New England Biolabs); lane 2, 1721/99; lane 3, 1856/99; lane 4, 3605; lane 5, G4920; lane 6, G4921; lane 7, non-typable *stx*$^-$ O157:H7 isolate; lane 8, 2409/96.

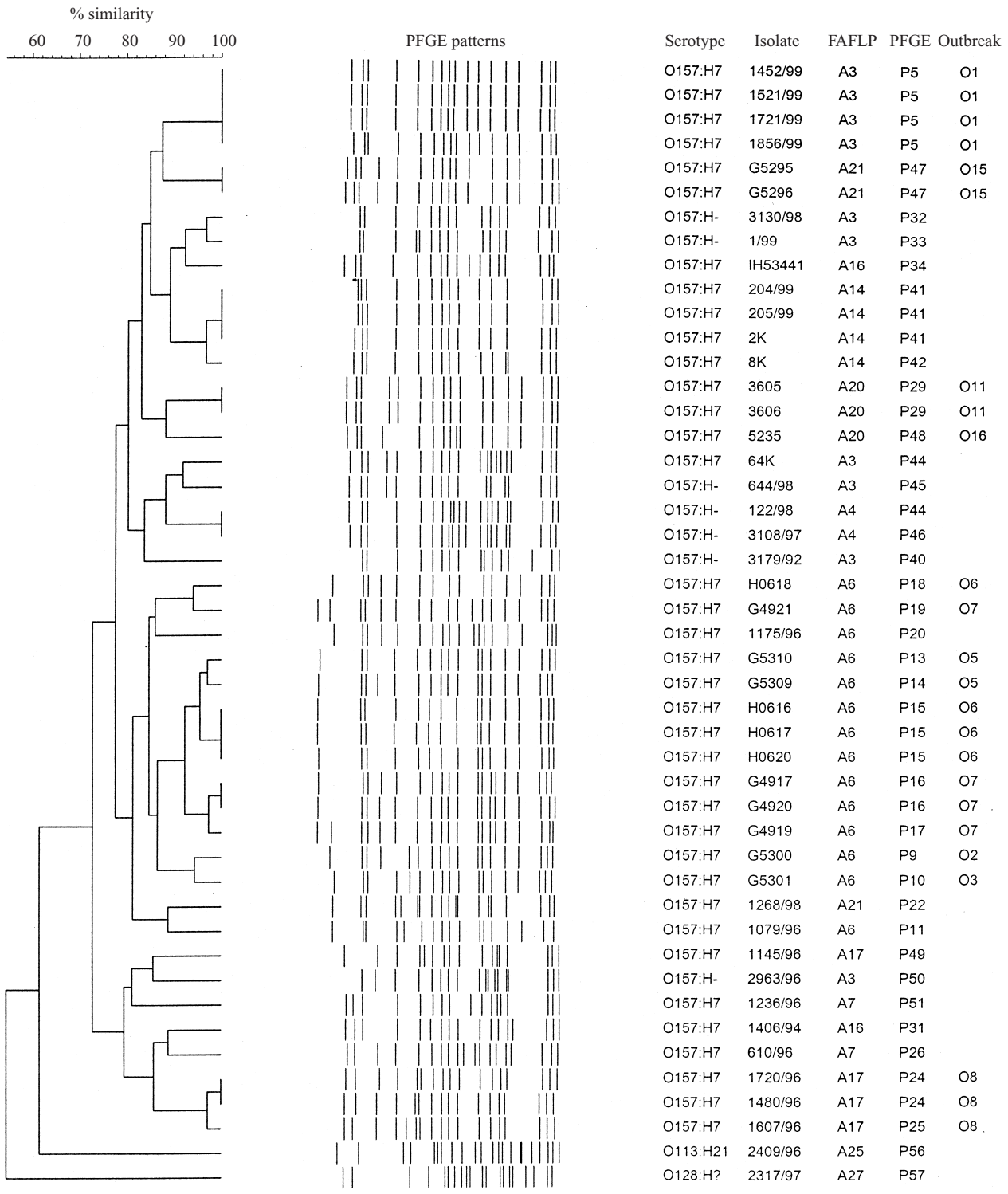
30 profiles among the 88 STEC isolates. We identified 24 different FAFLP(EMc) profiles (A1–A24, Table 1) among the 82 O157:H7/H$^-$ strains. In addition, the six non-O157 STEC isolates generated unique and clearly distinct profiles (A25–A30). Each FAFLP-(EMc) profile consisted of 80–90 fragments between 50–480 bp. The 82 strains of serotype O157:H7/H$^-$ exhibited various combinations of 17 polymorphic fragments (Table 2). Certain other fragments exhibited variation in fluorescence intensity. These fragments were not included in data analysis. FAFLP electropherograms (Fig. 1) were the basis of the dendrogram that illustrates the clustering of the different FAFLP profiles (Fig. 2).

PFGE

A total of 57 different PFGE profiles were detected among the 88 STEC strains. Fifty-one electrophoretic profiles were observed for the 82 O157:H7/H$^-$ isolates (P1–P51, Table 1) while unique, distinct profiles were assigned to the remaining six STEC strains (P52–P57, Table 1). PFGE generated 15–20 fragments for the O157:H7/H$^-$ strains, ranging in size from 50–600 kbp (Fig. 3). Cluster analysis clearly separated the O157:H7/H$^-$ strains from the non-O157:H7/H$^-$ strains (Fig. 3 and Fig. 4, only a subset of the strains are represented).

Comparison of FAFLP and PFGE

The 82 O157:H7/H$^-$ isolates represented a total of 49 epidemiological unassociated incidents (Table 1). Identical FAFLP(EMc) profiles were always obtained for epidemiologically related strains. However, Table 1 also points out that identical FAFLP(EMc) profiles were observed for epidemiological unrelated strains. The dendrogram in Fig. 4 illustrates the ability of PFGE to discriminate between strains which by FAFLP(EMc) were undistinguishable although unlinked epidemiologically. Each of the FAFLP(EMc) profiles A3, A6, A7, A16, A17, A20, A21 included epidemiologically unassociated isolates. Of these, profiles A3 and A6 were the most dominant comprising 12 and 16 isolates, respectively (Table 2). Strains with the A3 profile included the four isolates of the 1999 Norwegian outbreak together with eight epidemiologically unassociated/sporadic strains (Table 1). PFGE generated nine distinct profiles for these strains, in full accordance with the epi-



Serotype	Isolate	FAFLP	PFGE	Outbreak
O157:H7	1452/99	A3	P5	O1
O157:H7	1521/99	A3	P5	O1
O157:H7	1721/99	A3	P5	O1
O157:H7	1856/99	A3	P5	O1
O157:H7	G5295	A21	P47	O15
O157:H7	G5296	A21	P47	O15
O157:H-	3130/98	A3	P32	
O157:H-	1/99	A3	P33	
O157:H7	IH53441	A16	P34	
O157:H7	204/99	A14	P41	
O157:H7	205/99	A14	P41	
O157:H7	2K	A14	P41	
O157:H7	8K	A14	P42	
O157:H7	3605	A20	P29	O11
O157:H7	3606	A20	P29	O11
O157:H7	5235	A20	P48	O16
O157:H7	64K	A3	P44	
O157:H-	644/98	A3	P45	
O157:H-	122/98	A4	P44	
O157:H-	3108/97	A4	P46	
O157:H-	3179/92	A3	P40	
O157:H7	H0618	A6	P18	O6
O157:H7	G4921	A6	P19	O7
O157:H7	1175/96	A6	P20	
O157:H7	G5310	A6	P13	O5
O157:H7	G5309	A6	P14	O5
O157:H7	H0616	A6	P15	O6
O157:H7	H0617	A6	P15	O6
O157:H7	H0620	A6	P15	O6
O157:H7	G4917	A6	P16	O7
O157:H7	G4920	A6	P16	O7
O157:H7	G4919	A6	P17	O7
O157:H7	G5300	A6	P9	O2
O157:H7	G5301	A6	P10	O3
O157:H7	1268/98	A21	P22	
O157:H7	1079/96	A6	P11	
O157:H7	1145/96	A17	P49	
O157:H-	2963/96	A3	P50	
O157:H7	1236/96	A7	P51	
O157:H7	1406/94	A16	P31	
O157:H7	610/96	A7	P26	
O157:H7	1720/96	A17	P24	O8
O157:H7	1480/96	A17	P24	O8
O157:H7	1607/96	A17	P25	O8
O113:H21	2409/96	A25	P56	
O128:H?	2317/97	A27	P57	

Fig. 4. Clustering of 46 selected STEC isolates based on PFGE(*Xba*I) fingerprint patterns. The serotype, isolate no., FAFLP(EMc) and PFGE(*Xba*I) profiles for each isolate are indicated as well as the outbreak no. of outbreak-associated isolates.

demiological data. The strains generating the A6 profile comprised seven epidemiologically unassociated cases. A total of 10 PFGE profiles were discerned for these strains. This demonstrates that in some cases different PFGE profiles were obtained for strains originating from a single outbreak (e.g. outbreaks O5, O6, O7 and O8, Table 1). In most of these cases, the observed PFGE profile differences were small (1–2

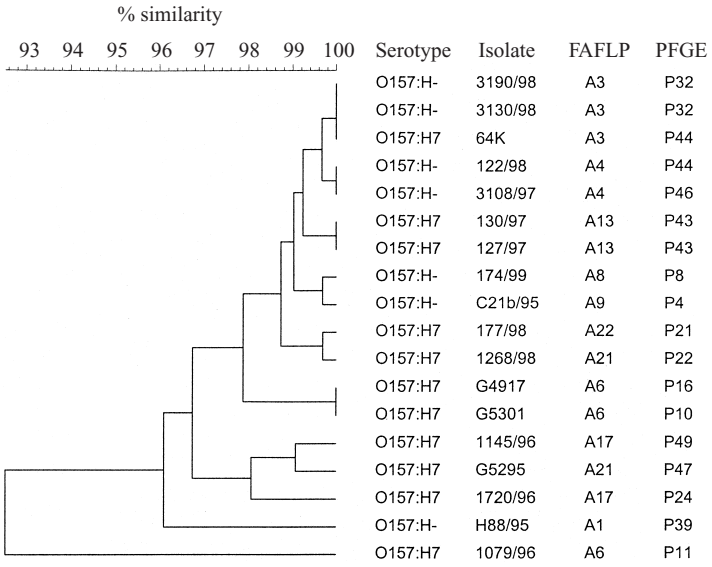


Fig. 5. Dendrogram constructed by combined data analysis of FAFLP patterns obtained by the two separate primer combinations *Eco*RI+0/*Mse*I+C and *Xba*I+0/*Mse*I+0 on 18 STEC O157:H7/H$^-$ isolates representing the 10 FAFLP(EMc) and 15 PFGE(*Xba*I) profiles as indicated. Percentages of similarity are shown on a scale above the dendrogram.

band differences), and the isolates were clustered in close proximity on the dendrogram (Fig. 4). Single-band differences among isolates from epidemiologically associated cases have also been described previously [27]. In this study, an identical PFGE profile was observed for two unlinked sporadic strains, a phenomenon also reported by Böhm and Karch [11] and Barrett and colleagues [15].

To obtain an objective assessment of the ability of FAFLP(EMc) and PFGE to distinguish between the 82 O157:H7/H$^-$ isolates included in the study, the discriminatory power of the AFLP(EMc) and PFGE typing methods was determined by calculating Simpson's index of diversity (DI) [28]. PFGE had a higher discriminatory power (DI = 0·985) than FAFLP(EMc) (DI = 0·925).

To investigate whether a second set of restriction enzymes and primers could increase the discriminatory power of the FAFLP method, we applied the *Xba*I+0/*Mse*I+0 restriction enzyme/primer combination. The *Xba*I is the enzyme of choice for PFGE fingerprinting of *E. coli* O157:H7. We therefore evaluated *Xba*I in combination with *Mse*I for FAFLP fingerprinting (termed FAFLP(XM)) of STEC strains. A total of approximately 60 fragments between 51 and 453 bp were generated. Nineteen fragments were polymorphic. The study included 18 O157:H7/H$^-$ strains, both epidemiologically linked and unlinked isolates, representing 10 FAFLP(EMc) and 15 PFGE profiles (Fig. 5). These isolates were subdivided into six FAFLP(XM) profiles. A dendrogram based on the combined similarity matrixes of both FAFLP(XM) and FAFLP(EMc) showed a total of 13 profiles (Fig. 5). Thus, the combination of two FAFLP procedures increased the discriminatory power of FAFLP although a better resolution was still obtained with PFGE (*Xba*I).

DISCUSSION

Molecular techniques with high discriminatory power are essential to differentiate between bacterial isolates of clonal descent like STEC O157:H7/H$^-$. We have fingerprinted 88 STEC isolates by FAFLP and PFGE, reportedly two of the most discriminatory techniques applied in strain typing [19, 29]. The FAFLP method has the potential to detect more genomic variations than PFGE. While PFGE profiles reflect restriction digests with a rare cutter only, FAFLP genomic profiles are most often generated by both a frequent cutter and a rare cutter. Analysis of FAFLP data on automatic sequencers enables detection of both single nucleotide differences caused by insertions or deletions in amplified fragments, as well as large-scale genomic alterations.

Fingerprinting of various bacterial pathogens has shown AFLP/FAFLP to have comparable or even higher discriminatory power than PFGE [23, 25, 30–32]. In our study, FAFLP often failed to discriminate between epidemiologically unrelated iso-

lates. Seven of the 24 distinct FAFLP(EMc) profiles obtained for the 82 O157:H7/H$^-$ isolates included epidemiologically unlinked strains. The isolates within each of the 17 remaining FAFLP(EMc) profiles were epidemiologically related. A combination of AFLP-(EMc) and AFLP(XM) was also less discriminatory than PFGE(*Xba*I). Limited discriminatory power was observed despite using FAFLP primer combinations intended to provide a large number of fragments for high-resolution analysis of homogenous STEC isolates.

Three other studies have used FAFLP for molecular fingerprinting of STEC O157 isolates. Arnold and coworkers [22] performed FAFLP with primers *Eco*RI +0/*Mse*I+TA (AFLP(EMta)) on 15 STEC O157 isolates. All isolates gave distinct strain-specific profiles. Our pilot study included FAFLP(EMta) on 19 STEC O157:H7/H$^-$ isolates. Less discrimination was obtained compared to the AFLP(EMc) procedure. Iyoda and colleagues [33] performed FAFLP on 46 STEC O157 strains with primers *Eco*RI(+C) and *Mse*I(+C). Although an overall good correlation between FAFLP and PFGE results was obtained, FAFLP was not useful for discriminating between some major PFGE profiles. Both these methods produced fewer fragments than the FAFLP(EMc) assay applied in this study. Zhao and colleagues [34] applied three selected sets of FAFLP primers and obtained a greater genetic resolution for this combined FAFLP compared to PFGE. The high number of both total fragments and polymorphic fragments reported compared to other studies [22, 33, this study] could illustrate the need for method standardization although other factors including diversity within the strain collections and primer sets applied could not be ruled out. The study illustrates the possibility of applying primers with separate dyes. Thus, different FAFLP reactions can be mixed before electrophoresis and run simultaneously to obtain maximum efficiency of combined FAFLP. We focused on primer combinations that gave informative, reproducible results with a large number of bands. This was done to give us a greater chance of discovering genetic differences between STEC strains of clonal descent. Also other studies have shown that the choice of restriction enzymes is crucial for the discriminating power of FAFLP [21, 22]. The optimal combination of restriction enzymes and primers for fingerprinting of STEC O157:H7/H$^-$ isolates may still be untried. Thorough standardization and evaluation must be performed for each set of restriction enzymes applied in the FAFLP method. A rational suggestion is that restriction enzyme combination well suited for FAFLP analysis should be modelled from the whole genome sequence once this information is available [22].

Although several restriction enzymes have been applied for PFGE analysis of *E. coli* O157:H7, the most discriminatory and preferred restriction enzyme is *Xba*I [11, 15, 35]. The 51 PFGE (*Xba*I) profiles obtained for 82 O157:H7/H$^-$ strains were in overall good correlation to the 49 epidemiological cases and confirmed the discriminatory power of PFGE. The dendrogram based on PFGE patterns (Fig. 4) also indicates the tight clustering of O157:H7/H$^-$ strains and that the differences in PFGE profiles between isolates from wide geographic origin and different sources are based on few banding pattern differences. This and other studies [15, 36] have shown that small variations in PFGE patterns could have epidemiological significance, and Tenover's criteria [37] for interpretation of PFGE data may be too strict and not practically applicable for clonal bacterial lineages like *E. coli* O157:H7/H$^-$. Barrett and colleagues suggested that isolates with PFGE profiles that differed by more than one band were probably not related and that isolates differing by a single band by PFGE did not warrant firm conclusions regarding epidemiological relationship [15]. As long as epidemiologically related isolates with minor PFGE pattern differences were grouped in close proximity by the cluster analysis, we assumed such strains to be involved in the outbreak.

We and others [11, 13, 14] have experienced that certain *E. coli* O157:H7/H$^-$ strains (mostly non-Stx producers) are untypable by PFGE, probably due to the presence of DNA degrading nucleases. We successfully performed FAFLP on three *E. coli* O157:H7 strains (non-Stx producers) untypable by PFGE (data not shown). Thus, FAFLP could be an alternative method for typing such strains as also mentioned by Zhao and colleagues [34].

For highly clonal bacterial populations like STEC O157:H7/H$^-$, both PFGE and FAFLP are vulnerable to *in vivo* genotypic changes as the different profiles and clusters are based on very few fragment differences. Therefore, conclusions regarding bacterial fingerprinting should be based on more than a single method [12]. A combination of highly discriminatory methods, e.g. PFGE and FAFLP together with cluster analysis of fingerprint data could be advantageous in epidemiological surveys. Reliable conclusions should

be based on fingerprint data, phenotypic and genotypic properties of the organisms under study as well as on available epidemiological information.

The FAFLP data can easily be stored in electronic libraries. By a proper and standard choice of a set of restriction enzymes and primers, the FAFLP method performed under highly standardized conditions could be quick, reliable and universal fingerprinting method with high throughput that enable inter-laboratory exchange and comparison of fingerprint data. PFGE protocol standardization has also enabled inter-laboratory comparison of PFGE data. However, the PFGE method is laborious and time consuming with a limited sample throughput compared to the FAFLP procedure.

In conclusion, PFGE is still the method of choice for typing STEC O157:H7/H$^-$ strains. Further studies are required to determine the choice of restriction enzymes for optimal discrimination of strains like *E. coli* O157:H7/H by PFGE and FAFLP. Genetic information that could contribute to a rational choice of methods for fingerprinting of various microbial species is needed.

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