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Clonal differentiation of uropathogenic *Escherichia coli* isolates of serotype O6:K5 by fimbrial antigen typing and DNA long-range mapping techniques

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Abstract. *Escherichia coli* isolates of serotype O6:K5 are the most common causative agents of cystitis and pyelonephritis in adults. To answer the question, as to whether strains of this particular serotype represent one special clonal group, out of a collection of 34 serotype O6:K5 isolates [Zingler et al. (1990) Zentralbl. Bakteriol Mikrobiol Hyg [A] 274:372–381] 15 strains were selected and analyzed in detail. The flagellar (H) antigen and the outer membrane protein (OMP) pattern were determined. Further serum resistance properties and the genetic presence and expression of other virulence factors, including hemolysin, aerobactin, P fimbriae, S/F1C fimbriae and type 1 fimbriae was evaluated. In addition the *Xba*I-macrorestriction pattern of ten representative isolates was elaborated and the fimbrial (F) antigen type of the P fimbriae was determined, to obtain the complete O:K:H:F pattern. These analyses could clearly show that the O6:K5 isolates do not represent one clonal group. The *Xba*I-macrorestriction profiles were heterogeneous and marked differences in the hybridization patterns, using virulence-associated gene probes in Southern hybridization of long-range-separated genomic DNA, were observed among the strains. However, some of strains showed similarities in the genomic profiles, arguing for clonal groupings among the O6:K5 isolates. Interstingly the strains grouped together exhibited the same fimbrial F type that many indicate a coincidence of this phenotypic trait with clonality.

Introduction

Escherichia coli is the most common pathogen in urinary tract infections (UTI) (Abraham et al. 1983; Ling et al. 1979). Properties associated with virulence in

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In memoriam of Prof. G. Naumann

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uropathogenic *E. coli* strains include certain O and K antigens (Kaijser et al. 1977; Mabeck et al. 1971; Orskov and Orskov 1985), production of hemolysin (Hacker and Hughes 1985), secretion of aerobactin (Johnson et al. 1988), and resistance to serum bactericidal activity (Lomberg et al. 1984). Furthermore, adherence to uroepithelial cells represents a prerequisite for colonization of host tissues. This initial step of the infection is mediated by fimbrial or non-fimbrial adhesins, distinguishable by their receptor specificities. P fimbriae [also termed Pap-pili (Normark et al. 1983)] recognize the α-D-Gal-(1–4)-β-D-Gal moiety of the P blood group antigen (Källenius et al. 1980; Väisänen-Rhen et al. 1984). The S fimbrial adhesin (Sfa) interacts with sialic acid-(2-3)-β-Gal receptor molecules (Korhonen et al. 1984). Type 1 fimbriae bind to α-D–mannose-containing receptors (Orskov and Orskov 1983). Fimbriae of serotype F1C represent adhesins that bind specifically to cells of the human urinary tract, but do not agglutinate erythrocytes (Van Die et al. 1985; Virkola et al. 1988).

O:K:H serotyping, i.e., characterization by combinations of O, K, flagellar antigen (H), and outer membrane protein patterns (OMP), and electrophoretic typing of alloenzymes (ET) have been used to group bacterial isolates into clones (Orskov and Orskov 1989; Achtmann and Pluschke 1986). Attempts have also been made to correlate the various virulence factors to clonal types (Blum et al. 1990; Ott et al. 1991a).

Serval questions have been addressed in respect to the emergence of clonal grouping among pathogenic *E. coli* exhibiting a common clinical picture. *E. coli* strains of serotype O6 represent one of the most important group of strains found in patients with UTI (Peŗe et al. 1985; Zingler et al. 1990; Kaijser and Jodal 1984; Marlid et al. 1988; Stenquist et al. 1989). Among this serotype preferentially the O6:K5 strains cause cystitis or pyelonephritis. The objective of this study was to analyze this group of isolates in detail, and to answer the question as to whether O6:K5 isolates represent a homogeneous clonal group among uropathogenic *E. coli*. For this purpose molecular tools were applied. Additionally the serotype pattern was enlarged by determining the H antigen and the fimbrial (F) type of P fimbriae of the isolates. Our data provide a clear-cut evidence that strains of serotype O6:K5 are evolutionary heterogeneous; furthermore, the usefulness of the F antigen as clonal marker is discussed.

Materials and methods

Bacterial strains

All the 15 *E. coli* O6:K5 strains (Table 1) investigated were isolated as monocultures in significant concentrations ($> 10^5$ bacteria/ml) from adult UTI patients, suffering from either cystitis for pyelonephritis. The mid-stream urine samples from patients of Rostock University Hospital were all processed within 4 h after sampling and characterized by conventional microbiological techniques. One strain (RZ 533), was obtained from the Department of General Microbiology, University of Helsinki, Finland. The strains were stored on nutrient agar stabs at room temperature and were subcultured twice on nutrient agar plates for further investigations. O, K, and H antigens were determined as described (Zingler et al. 1990). Fimbriae were analyzed serologically by crossed-line-immunoelectrophoresis (CLIE) according to the method of Orskov and Orskov (1983, 1990). *E. coli* test strains for fimbrial antigens were as follows (see Orskov and Orskov 1990): C 1212-77 (O6:K2:H1; $F7_{1,2}$, F1C), C 1388-83 (recombinant rough: H^-; F 13), C 1023-79 (083:K24:H31; F14, F1C), C 1007-79 (025:K5H^-; $F14_{rel.}$) C 1805-79 (075:K5:H^-;

Table 1. Phenotypic and genotypic characteristics of uropathogenic *Escherichia coli* O6:K5 strains

No.	*Strain designation*		H1	OMP	SR	*Aerobactin*		Hemolysin		Adhesins				Fim	*fim*	Fimbrial antigens	
	Current	Orginal				*Aer*	*aer*	Hly	*hly*	P	*pap/ prs*	S	*sfa foc*				
1.	*RZ439*	Z 34	+	1	−	+	+	−	−	+	+	−	+	+	+	F15	F1C
2.	*RZ442*	Z 233	+	1	+	+	+	−	−	+	+	−	+	+	+	F15	F1C
3.	*RZ471*	1202/85	+	1	+	+	+	−	−	+	+	−	+	+	+	F15	F1C
4.	*RZ525*	Ki 81	+	1	+	+	+	−	−	+	+	−	+	+	+	F15	F1C
5.	*RZ498*	W 145	+	1	+	+	+	−	−	+	+	−	−	+	+	F15	−
6.	*RZ495*	9057/85	−	1	+	+	+	−	−	+	+	−	+	+	+	$F15_{rel.}$	F1C
7.	*RZ440*	Z 56	+	1	+	+	+	−	+	+	+	−	+	+	+	F14	F1C
8.	*RZ441*	Z117	+	1	+	+	+	+	+	+	+	−	+	+	+	F14	F1C
9.	*RZ468*	Z 136	+	1	+	+	+	+	+	+	+	−	+	+	+	F14	F1C
10.	*RZ475*	3360/85	+	1	+	+	+	+	+	+	+	−	+	+	+	F14	F1C
11.	*RZ526*	Z 142	+	1	−	+	+	+	+	+	+	−	+	+	+	F14	F1C
12.	*RZ443*	Z 394	−	2	+	+	+	+	+	+	+	−	+	+	+	F13	F1C
13.	*RZ512*	40/86	−	2	+	+	+	+	+	+	+	−	+	+	+	F13	F1C
14.	*RZ513*	62/86	−	2	+	+	+	+	+	+	+	−	+	+	+	F13	F1C
15.	*RZ533*	IH11238	−	7	+	+	+	+	+	+	+	−	+	+	+	$F13_{rel.}$	F1C

SR, serum resistance; H, flagellar antigen; OMP outer membrane protein

F15), C 134-73 (04:K12:H5; F16, F13, F14, F1C) and 20025 (O4:K12:H$^-$; F16, F13, F14, F1C). *E. coli* K-12 strain HB 101 was used as a carrier for recombinant DNA.

Adhesion testing

The presence of adhesins was determined by hemagglutination (HA) assays with plate-grown bacteria using human A, sheep, bovine, and pig erythrocyte suspensions obtained locally with and without 2% mannose as described elsewhere (Orskov and Orskov 1983; Zingler et al. 1990). P-specific adherence was detected by mannose-resistant (MR) HA using P_1 human blood cells and a Gal-Gal-specific latex test (PF-Test) obtained from Orion Diagnostica (Espoo, Finland; De Man et al. 1987). Expression of S fimbrial adhesins was analyzed as described recently (Ott et al. 1991a). Type I fimbriation was identified by mannose-sensitive agglutination of *Saccharomyces cerevisiae* cells as described elsewhere (Orskov and Orskov 1983).

Aerobactin test

Aerobactin production was detected by a cross-feeding bioassay as described (Braun et al. 1983; Ott et al. 1991a).

Hemolysin production

Nutrient agar plates containing 5% sheep blood were used to test for hemolysin production of strains after overnight culture at 37°C (Noegel et al. 19881).

OMP patterns

Outer membrane patterns (OMP) of *E. coli* O6:K5 strains were analyzed by sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE) as described by Achtmann et al. (1986).

Whole bacteria ELISA

An ELISA using whole bacteria as an antigen was carried out as described recently (De Ree et al. 1986). Monoclonal antibodies (mAb) specific for the antigens F14 (20025-F 1) and F13 (21344-F) were provided by K. Jann (Freiburg, FRG). F1C specific mAb (F4C1) was obtained from A. Pere (Helsinki, Finland).

SDS-PAGE

The disintegration of fimbriae and their electrophoretic separation on polyacrylamide gels (separating gel 15%, stacking gel 5%) according to Laemmli (1970) has been described elsewhere (Hacker et al. 1985).

Resistance to bactericidal effect of serum

Sensitivity to the bactericidal effect of 75% pooled normal human serum was tested as described previously (Falkenhagen et al. 1991).

DNA techniques

Plamid DNA isolation, DNA cleavage with restriction enzymes, agarose gel electrophoresis, and elution of DNA fragments from agarose gels were performed according to Sambrook et al. (1989).

DNA probes

The DNA probes used were described in detail recently (Blum et al. 1990; Ott et al. 1991a). Briefly, a 4.4-kb *Hind*III fragment isolated from the plasmid pRHU 845 (Tc^r) was used as P fimbriae-specific probe (Normark et al. 1983), which is also specific for the *prs* gene cluster coding for P-related adhesins (Lund et al. 1988). A 1.8-kb *Cla*I-*Eco*RI fragment of pANN 801-13 (Ap^r) served as a *sfa*-specific DNA (Schmoll et al. 1990). This probe also detects *foc*-specific sequences coding for F 1C fimbriae (Ott et al. 1987, 1988). The 6.0-kb *Pst*I fragment pf plasmid pPKL 4 (Ap^r) was used as a probe for *fim* DNA (Klemm et al. 1986). The *aer*-specific probe was a 7.0-kb *Hind*III-*Eco*RI fragment of plasmid pRG12 (Ap^r) (Gross et al. 1985). The 3.2-kb *Hind*III fragment of plasmid pANN215 (Cm^r) was used as a *hly*-specific probe (Noegel et al. 1981). DNA probes were ^{32}P-labeled using the random priming system (Feinberg and Vogelstein 1983) from Boehringer (Mannheim, FRG).

DNA-dot blot procedure

Colony dot hybridization was performed as described previously (Sambrook et al. 1989). Stringent conditions were used for hybridization and washing.

Pulsed-field gel electrophoresis

Chromosomal DNA was prepared in agar plugs according to Grothues and Tümmler (1987). Agarose blocks were equilibrated in restriction enzyme buffer and the chromosomal DNA embedded in the agarose was digested with *Xba*I. A slice of each insert plug was then sealed into a well of a 4-mm-thick horizontal agarose gel and electrophoresed (CHEF DR II, Bio-Rad Laboratories, Richmond, USA) in 0.5×TRIS-borate-EDTA buffer for 25 h at 14°C and 200 V with the pulse time increasing from 10 to 40 s. Lambda concatemers (Pharmacia, Freiburg, FRG), yeast chromosomes (*S. cerevisiae* WAY 5-4A, Biometra, Göttingen, FRG) and *Hind*III cleaved lambda DNA were used as size markers.

Southern hybridization

Cleaved genomic DNA separated in agarose gels was transferred to nitrocellulose paper as described elsewhere (Southern 1975). Stringent conditions were used for hybridization and washings (Sambrook et al. 1989).

Results

Serotype, OMP and serum resistance

All 15 *E. coli* O6:K5 strains were characterized by H antigen typing, OMP patterns and resistance to bactericidal activity of pooled human serum (Tab. 1). All motile

strains were H1 and 3 different OMP patterns were found. Only two of the O6:K5 isolates were sensitive to the bactericidal activity of serum.

Genetic presence and phenotypic expression of virulence factors

All 15 *E. coli* isolates were analyzed by DNA-DNA dot blots for the presence of different virulence-associated genes. Additionally the phenotypic expression of the corresponding properties was evaluated. The results are summarized in Table 1.

Aerobactin

Aerobactin production was detected in all of the isolates.

Hemolysin

The eight hemolytic isolates were detected by the probe, and one probe-positive isolate (RZ440) was negative for hemolysin production.

P-fimbrial adhesins

All the isolates produced adhesins which mediated binding to Gal-Gal receptor molecules (P-specific binding) and they also caused MRHA of human, sheep, and pig erythrocytes. Five serologically different F antigen types were identified by CLIE: F13, F14, F15 and the related antigens $F13_{rel.}$ and $F15_{rel.}$.

S/F1C-fimbrial adhesins

With one exception, all of the strains reacted in DNA-DNA dot blots using a probe specific for S and F1C gene clusters (Ott et al. 1987, 1988), but none of the isolates expressed S fimbrial adhesin-specific binding properties. F1C fimbriae are expressed by all of the probe-positive isolates, which was demonstrated by CLIE as well as by ELISA with the mAb F4C1.

Type 1 fimbriae

All strains contained *fim*-specific sequences, and expressed type 1 fimbriae.

Combination of virulence factors and the F antigen

P fimbriae, serum resistance, aerobactin production, F1C and type 1 fimbriae were found in nearly all strains of OMP1 which were F15 or $F15_{rel.}$. Strains also of OMP1 expressing the F14 antigen carried the same virulence factors, but additionally produced hemolysin. Isolates expressing F1C and F13 produced the

same virulence factors as the F14 strains but were of OMP2, the strain expressing the $F13_{rel.}$ antigen was of OMP7.

Genomic profiles

For further analysis, genomic DNA of ten representative strains were isolated and cleaved with *Xba*I, a rare cutting enzyme for *E. coli* DNA. Pulsed-field gel electrophoresis (PFGE) was used to elaborate restriction fragment length polymorphisms (RFLP). Figure 1a shows that the strains grouped according the F antigen show identical or very similar restriction fragment patterns. Lanes 1, 2 and 3 contain OMP1 and F15 antigen strains, lanes 4, 5, and 6 represent isolates also of OMP1 but with F14 antigen. Small differences of the pattern indicate that the respective strains are not identical isolates, but belong to the same evolutionary lineage. Strains of OMP2 with F13 (lanes 7 and 9) also display a similar pattern which is quite different from that of the $F13_{rel.}$ strain RZ533 from Finland (lane 10). This strain also had a different OMP pattern (cf. Table 1). Also the $F15_{rel.}$ strain (lane 8) had a restriction pattern different from that of the F15 strains in lanes 1–3.

DNA long-range mapping using virulence-associated DNA probes

Southern hybridization was carried out using *pap, hly, sfa/foc,* and *aer*-specific gene probes (Fig. 1b–e). The $F15/F15_{rel.}$ strains exhibited hybridization in identical *Xba*I fragments using the *pap* (panel c) and *aer* (panel e) gene probes (lanes 1, 2, 3

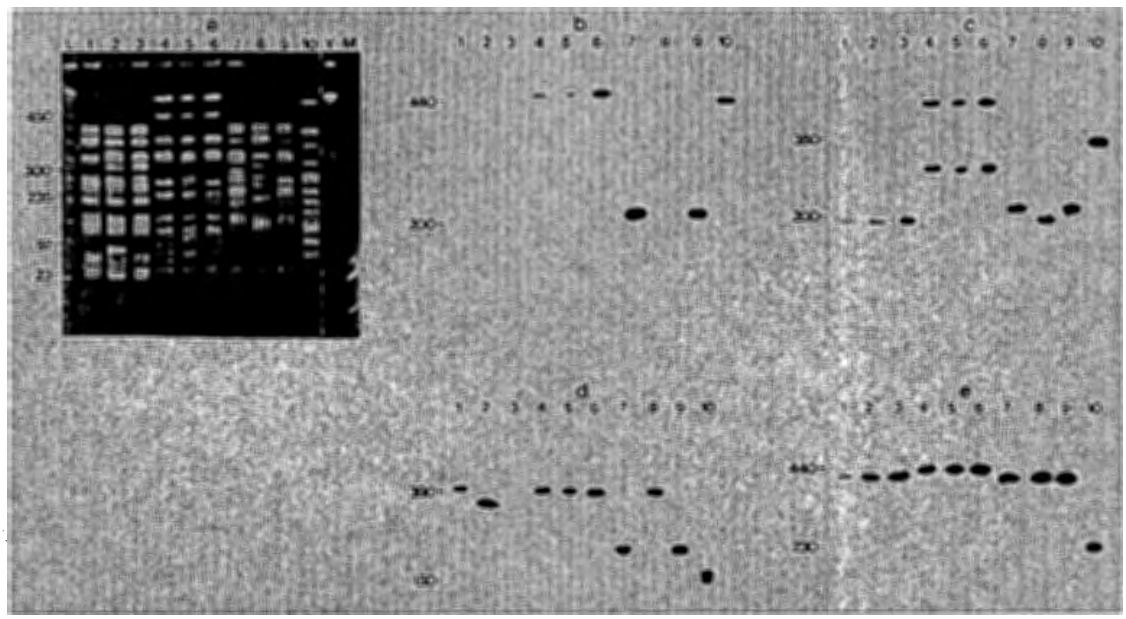

Fig. 1a–e. *Xba*I genomic profile of *E. coli* O6:K5 strains obtained by pulsed-field gel electrophoresis (**a**) and Southern hybridization to *hly* (**b**)-, *pap* (**c**)-, *sfa/foc* (**d**)-, and *aer* (**e**)-specific gene probes. Strains are as follows (cf. Table 1, fimbrial serotype is given in parantheses): *1,* RZ439 (F15): *2,* RZ525 (F15); *3,* RZ498 (F15); *4,* RZ440 (F14); *5,* RZ468 (F14); *6,* RZ526 (F14); *7,* RZ443 (F13); *8,* RZ495 ($F15_{rel.}$); *9,* RZ513 (F13); *10,* RZ533 ($F13_{rel.}$) DNA size standards were lambda concatemers (*L*), yeast chromosomes (*Y*), and *Hind*III-cleaved lambda DNA (*M*)

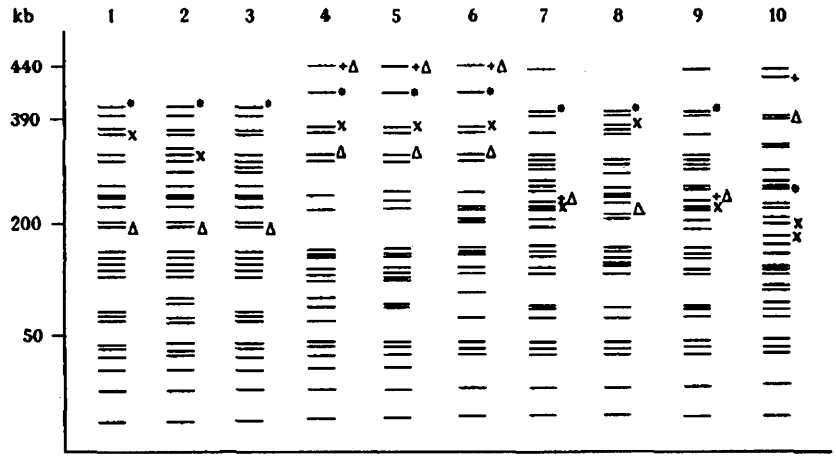

Fig. 2. Interpretation scheme of *Xba*I hybridization patterns of strains analyzed in Fig. 1. The fragments hybridizing to individual gene probes are marked as follows: *, *aer*; +, *hly*; Δ *pap*; ×, *sfa*

and 8), however, had a different pattern with the *sfa/foc* probe (panel d). The F14 strains (lanes 4, 5, 6) showed hybridization to all the four DNA probes in fragments of identical size. Interestingly, using the *pap* probe (panel c) two fragments (440 kb and 300 kb) appeared of which the 440-kb band also hybridized to the *hly* probe, which may indicate that the strains carry two copies of P fimbriae determinants and that one of these copies is located in the vicinity of the *hly*-encoding genes.

Strains expressing the F13 antigen (lanes 7 and 9) were also identical in the hybridization pattern to all of the gene probes used, whereas the $F13_{rel.}$ strains (lane 10) exhibited fragments of different size. This strain exclusively hybridized in two fragments using the *sfa/foc*-specific DNA probe which may argue for the presence of two copies of *foc* determinants. The data obtained by DNA long-range mapping are summarized in Fig. 2.

Discussion

From a collection of 34 *E. coli* serotype O6:K5 strains isolated from patients with UTI (Zingler et al. 1990), 15 isolates were selected for analyzing the clonal relationship among them. This particular group of strains was shown to be most frequently isolated from cases of cystitis or pyelonephritis (Pere et al. 1985; Zingler et al. 1990; Kaijser and Jodal 1984).

Various techniques, including multilocus enzyme electrophoresis (MLEE) (Selander et al. 1987), SDS-PAGE of OMP (Caugant et al. 1986) and serotyping, indicated that most isolates causing upper UTI represent a limited subset of the lineages found among the commensal flora (Selander et al. 1987; Orskov and Orskov 1989); however, little is known about the evolutionary relatedness of strains causing a particular clinical picture of UTI. The analysis of the virulence patterns of uropathogenic *E. coli* added to the classification of isolates, but, as shown here, this is not discriminative enough to disclose the evolutionary divergence of strains of a given O:K serotype. The evaluation of data from recent studies (Blum et al. 1990; Zingler et al. 1992) on UTI strains of serotype O6, and the results obtained in this study show that the strains were characterized by various combinations of well-known virulence factors. However, clonal differentiation

could not be achieved easily for all isolates. Therefore, we analyzed an additional trait, the F type of the widely distributed P fimbriae. As shown here, the complete O:K:H:F serotype was conclusive by concomittantly evaluating the macrorestriction patterns obtained by PFGE analysis. The strains analyzed in this study displayed highly different *Xba*I profiles, arguing against a common evolutionary origin. Our data show that there is a need to base conclusions of evolutionary relationship on precise molecular analyses, rather than simply applying the classical techniques alone. Of high interest should be the coincidence of the F antigen and the similarities in the macrorestriction pattern among the heterogenous strains. The DNA sequences of the major P fimbrial subunits, which determine the various F types are highly heterogeneous (Hacker 1990), which argues against point mutations for the evolvement of dissimilarity. The different clones might have emerged by acquisition of different P fimbriae determinants by horizontal gene transfer. Recent data provided by Plos et al. (1989) and Marklund et al. (1992) support this hypothesis.

DNA long-range mapping revealed some interesting features according the copy number and physical linkage of virulence determinants, as for example in the case of the strains carrying the F14 antigen two copies of P fimbriae gene clusters seem to exist, from which one is in the vicinity of the *hly* determinant. Such linkages of the genes encoding hemolysin production and P fimbriae expression have been described recently (Ott et al. 1991a; Hacker etl al. 1990) in other pathogenic *E. coli* strains. This seems to be a more general phenomenon of the *E. coli* chromosome. Interestingly, the strains with the F15 antigen do not produce hemolysin. The loss of hemolysin determinants by spontaneous deletion events has been reported recently for extraintestinal *E. coli* strains, including UTI isolates (Hacker et al. 1990; Knapp et al. 1986). It might be concluded that also in these strains deletions occurred in the evolutionary lineage.

The PFGE technique has been used recently for epidemiological studies of pathogenic *E. coli* strains (Blum et al. 1990; Ott et al. 1991a; Arbeit et al. 1990; Tschäpe et al. 1992; Böhm and Karch 1992), and other bacteria (Grothues and Tümmler 1987; Ott et al. 1991b; Lück et al. 1991). The strains used in this study which were grouped together show highly related, but not identical macrorestriction patterns. Thus, the application of PFGE is not only helpful for evaluation of clonality, it further enables precise strain identification that cannot be easily achieved by other methods. For the clinical practice this approach should be useful for differentiating between recurrent UTI infections and reinfections with the same strain.

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