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Clonal analysis of *Escherichia coli* serotype O6 strains from urinary tract infections*

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A total of 36 *Escherichia coli* urinary tract isolates (UTI) of serotype O6, with different combinations of capsule (K) and flagellin (H) antigens, were analysed according to the outer membrane pattern (OMP), serum resistance properties, mannose–resistant hemagglutination using various types of erythrocytes, and also for the genetic presence and the expression of P-fimbriae, S fimbriae/F1C fimbriae, Type 1 fimbriae, aerobactin and hemolysin. Twenty selected strains were further analysed by pulsed field gel electrophoresis (PFGE), elaborating genomic profiles by *Xba*I cleavage and subsequent Southern hybridization to virulence-associated DNA probes. It could be shown that O6 UTI isolates represent a highly heterogeneous group of strains according to the occurrence and combination of these traits. Relatedness on the genetic and the phenotypic level was found for some of the strains exhibiting the same O:K:H:F serotype. DNA long-range mapping further indicated some interesting features, according to the copy number and the genomic linkage of virulence genes.

Key words: *E. coli* serotype O6; urinary tract infection; virulence factors; clonal analysis; molecular epidemiology.

Introduction

Bacterial infections of the urinary tract encompass a wide spectrum of clinical syndromes, ranging from asymptomatic bacteriuria to symptomatic cystitis and pyelonephritis. A single bacterial species, *Escherichia coli*, causes more than 80% of these infections, and neither anatomical differences nor defects in host defense mechanisms provide an adequate explanation for the different clinical patterns that have been observed.[1] *Escherichia coli* O6 strains isolated from urinary tract infections often express specific properties that are not prevalent among strains from the commensal fecal flora. These properties include the expression of adhesins mediating attachment to specific receptors on uroepithelial cells, production of hemolysin, serum

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resistance (SR), release of aerobactin and the presence of particular capsular (K) antigens.[2-5]

The ability to adhere to uroepithelial cells is considered to be a crucial virulence factor which allows certain fecal clones of *E. coli* to colonize the urinary tract. Several adhesins of uropathogenic *E. coli* have been analysed by agglutination assays. Type 1 fimbriae bind to α-D-mannose and thereby agglutinate guinea-pig erythrocytes and *Saccharomyces cerevisiae* cells.[6] P-fimbrial adhesins recognize specifically the globo series of glycolipids present on both human erythrocytes bearing the P blood group antigen and uroepithelial cells.[7] The minimal binding receptor of this adhesin type is the disaccharide α-D-galactosyl-(1–4)-β-D-galactopyranose (Gal–Gal). S-fimbrial adhesin binds to sialyl(β 2–3)galactosides moieties, present on human and bovine erythrocytes.[8,9] Fimbriae of serotype F1C are unable to agglutinate erythrocytes, but do seem to interact with cells of the human urinary tract.[10-12]

Recently we analysed *E. coli* serotype O6 strains, originating from various extra-intestinal sources, which were shown to be highly heterogeneous.[13] Several other groups investigated *E. coli* O6 isolates[14-16] according to the relation of serotype and genetic and phenotypic diversity, including the analysis of virulence factors. In the light of previous studies, we attempted to analyse serotype O6 isolates from urinary tract infections, in order to get an insight into the correlation of genetic and phenotypic traits with serotype and type of infection, by combining classical and molecular typing methods.

Results

Serotypes and outer membrane patterns (OMP)

Among the 36 *E. coli* O6 strains in the collection of uropathogenic isolates, 12 of the strains had complete three-antigen serotypes, resulting in four specific serotypes, 13 had a complete serotype for O and K antigens, but were non-motile (H^-), and 11 had incomplete serotypes, i.e. they lacked a capsular antigen (K^-) or were non-typable for the K antigen (K^+). The O6:K:H serotypes, and the results of most of the tests performed on these strains, are presented in Table 1. Six different OMP patterns associated with these strains were obtained. Thirteen of the 36 strains possessed pattern 1; this pattern is typical for strains of serotype O6:K53:H1, O6:K2:H1, and O6:K^-:H1. Most other non-motile strains of our collection, except RZ430 and RZ451, had pattern 2, whereas patterns 3, 4, 5, and 6 were found only in a few isolates.

Serum resistance

Bacteria were also tested for resistance to the bactericidal activity of pooled human serum. 39% of the strains were serum resistant.

Mannose-resistant hemagglutination (MRHA)

Various MRHA types were determined on the basis of the hemagglutination (HA) using erythrocytes from different species (human A_1, pig, bovine, sheep). MRHA of sheep erythrocytes was most common (95%), followed by HA of pig and bovine erythrocytes (47%). Only one-third of our strains showed a MRHA of human erythrocytes.

Genetic presence and phenotypic expression of virulence factors

All of our *E. coli* isolates assigned to different O6:K:H serotypes were analysed by

DNA–DNA dot blots for the presence of different virulence-associated genes. The phenotypic expression of the corresponding properties was also tested and the results are summarized in Table 1.

P-fimbrial adhesins. One-quarter of the isolates produced adhesins, detectable by binding to Gal–Gal receptor molecules (P-specific binding), while 78% hybridized with a *pap* (pili associated with pyelonephritis) gene probe. This gene probe shares homology with the P-related sequences (*prs*).[7] P-fimbrial adhesins are well expressed by strains of serotypes O6:K2:H1/OMP1, O6:K$^-$:H1/OMP1 and O6:K$^-$:H$^-$/OMP4. The *pap/prs* specific gene cluster was also detected on O6:K53:H1, O6:K14:H$^-$, O6:K13:H1 and O6:K$^+$:H1 isolates, but never on O6:K2:H$^-$ strains of OMP type 2. However, none of these isolates mediates P-specific binding and agglutination of sheep erythrocytes indicates the expression of Prs in these strains. Crossed-line immunoelectrophoresis (CLIE) analysis showed that fimbriae of O6:K53:H1 and O6:K13:H1 strains causing MRHA of sheep erythrocytes were strongly related serologically to F12/2. The fimbrial composition of strains with P-receptor binding specificity (Table 1) indicated the existence of antigenic heterogeneity among P fimbriae.

S/F1C-fimbrial adhesins. A total of 33 (92%) of the 36 strains were positive in DNA–DNA dot blots using a gene probe specific for S and F1C fimbriae, but only 47% of the isolates expressed S-specific binding properties. Except for one strain (RZ458; O6:K2:H1/OMP4), all isolates expressing S-specific binding (Sfa) were also positive in CLIE for identification of the main peptide subunit of S fimbriae (SfaA) and spot tests using monoclonal antibody mAbA1 which is specific for the S adhesin (SfaS, see Materials and methods). Fimbriae with S-specific binding activity were found on isolates of serotypes O6:K14:H$^-$, O6:K2:H$^-$, O6:K$^+$:H$^-$ and O6:K$^+$:H31. All these isolates had two fimbrial peptides with apparent molecular weights of 17.2 and 16.2 kDa by SDS–PAGE, but only the O6:K14:H$^-$ and O6:K$^+$:H31/H$^-$ strains of this group had strongly reactive fimbrial antigens of type F12/1. None of the S-fimbriated O6:K2:H$^-$ isolates reacted with any of the P fimbrial antisera used. The presence of *sfa/foc* coding sequences was detected not only among these serotypes but also in P-fimbriated isolates, which were shown to express F1C fimbriae, which are highly related to S-fimbriae at the genetic level.[17,18]

Type 1 fimbriae. As indicated in Table 1 for type 1 fimbrial expression, the phenotype is in total agreement with the genotype in all strains.

Hemolysin. 92% of the strains were hemolytic and generally carried the corresponding genes. Three strains of serotypes O6:K2:H$^-$ and O6:K$^-$:H1 were non-hemolytic and also gave no hybridization signal.

Aerobactin. Aerobactin production was detected in 36% of the isolates, and all of these strains carry the *aer* gene clusters. Aerobactin-positive strains were particularly common among isolates of serotypes O6:K2:H1/OMP1 and O6:K$^-$:H1/OMP1.

Combination of virulence factors as basis for clonal classification. The data in Table 1 show an obvious correlation between virulence patterns and the O6:K:H serotype, and it becomes stronger if the OMPs of the isolates are also taken into account. Among the analysed O6 strains, 16 out of 36 were assigned to membrane patterns 1 or 4 and yielded positive hybridization results with the *pap, fim, aer* probes, with three exceptions in each case, to the *sfa/foc*- and *hly*-specific gene probes.

A total of 17 *E. coli* O6 strains which were non-motile or H31 and had membrane pattern 2 or 6, carried the *sfa/foc, fim* and *hly* gene clusters and expressed the corresponding phenotypic properties. However, these strains were always negative in hybridization tests with the *aer* gene probe. Moreover, we found that *E. coli* strains of serotype O6:K53:H1 were not distinguishable from O6:K13:H1 isolates by all

Table 1 Virulence patterns of UTI *Escherichia coli* O6 strains

					MRHA[c]				Adhesins								Aerobactin		Hemolysin		
No.	Strain	Serotype	OMP[a]	SR[b]	hA	Bv	Sh	Pg	P	*pap/prs*	Sfa	SfaS	SfaA	*sfa/foc*	Fim	*fim*	Aer	*aer*	Hly	*hly*	F-serotype
1.	RZ446	O6:K53:H1	1	+	−	−	+	−	−	+	−	−	−	+	+	+	−	−	+	+	F12/2
2.	RZ448	O6:K53:H1	1	+	−	−	+	−	−	+	−	−	−	+	+	+	−	−	+	+	F12/2
3.	RZ536	O6:K53:H1	1	+	−	−	+	+	−	+	−	−	−	+	+	+	−	−	+	+	F12/2
4.	RZ537	O6:K53:H1	1	−	+	−	+	+	−	+	−	−	−	+	+	+	−	−	+	+	F12/2
5.	RZ422	O6:K14:H⁻	2	+	−	+	+	−	−	+	+	+	+	+	+	+	−	+	+	+	F12/1
6.	RZ423	O6:K14:H⁻	2	+	−	+	+	−	−	+	+	+	+	+	+	+	−	−	+	+	F12/1
7.	RZ461/1	O6:K14:H	2	+	−	+	+	+	−	+	+	+	+	+	+	+	−	−	+	+	F12/1
8.	RZ461/2	O6:K14:H⁻	2	+	−	+	+	+	−	+	+	+	+	+	+	+	−	−	+	+	F12/1
9.	RZ424	O6:K14:H⁻	2	−	−	+	+	−	−	+	+	+	+	+	+	+	−	−	+	+	F12/1
10.	RZ505	O6:H14:H	2	−	−	+	+	+	−	+	+	+	+	+	+	+	−	−	+	+	F12/1
11.	RZ436	O6:K13:H1	3	+	−	−	+	−	−	+	−	−	−	+	+	+	−	−	+	+	F12/2
12.	RZ447	O6:K13:H1	3	−	−	−	+	−	−	+	−	−	−	+	+	+	−	−	+	+	F12/2
13.	RZ454	O6:K2:H⁻	2	−	−	+	+	−	−	−	+	+	+	+	+	+	−	−	+	+	—
14.	RZ460	O6:K2:H⁻	2	−	−	+	+	−	−	−	+	+	+	+	+	+	−	−	+	+	—
15.	RZ463	O6:K2:H⁻	2	−	−	+	+	−	−	−	+	+	+	+	+	+	−	−	+	+	—
16.	RZ484	O6:K2:H⁻	2	−	−	+	+	−	−	−	+	+	+	+	+	+	−	−	+	+	—
17.	RZ502	O6:K2:H⁻	2	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−	+	+	—
18.	RZ462	O6:K2:H	2	−	−	+	+	−	−	−	+	+	+	+	+	+	−	−	+	+	—
19.	RZ458	O6:K2:H1	4	−	+	+	+	+	−	+	+	−	−	+	+	+	+	+	+	+	F1C

20.	RZ485	O6:K2:H1	1	−	+	−	+	+	+	+	−	−	−	+	+	+	+	+	+	+	F7/1; F7/2; F1C
21.	RZ467	O6:K2:H1	1	+	+	−	+	+	+	+	−	−	−	+	+	+	+	+	+	+	F14; F7/1; F1C
22.	RZ470	O6:K2:H1	1	+	+	−	+	+	+	+	−	−	−	+	+	+	+	+	+	+	F7/2
23.	RZ486	O6:K2:H1	1	−	−	−	+	−	−	+	−	−	−	+	+	+	+	+	+	+	F1A; F1B
24.	RZ438	O6:K2:H$^-$	4	−	−	−	−	−	−	+	−	−	−	+	+	+	+	+	−	−	F1C
25.	RZ451	O6:K18/22:H31	2	−	−	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	F12/1
26.	RZ479	O6:K$'$:H$^-$	2	+	−	+	+	−	−	+	+	+	+	+	+	+	−	−	+	+	F12/1
27.	RZ496	O6:K$^+$:H$^-$	2	−	−	+	+	+	−	+	+	+	+	+	+	+	−	−	+	+	F12/1
28.	RZ532	O6:K$'$:H31	6	−	−	+	+	−	−	+	+	+	+	+	+	+	−	−	+	+	F12/1
29.	RZ411	O6:K$^-$:H1	1	−	+	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	F14rel.; F12/1
30.	RZ412	O6:K$^-$:H1	1	+	+	−	+	+	+	+	−	−	−	−	+	+	+	+	+	+	F12/1; F7/1
31.	RZ418	O6:K$^-$:H1	1	−	+	−	+	+	+	+	−	−	−	+	+	+	+	+	+	+	F7/2; F1C
32.	RZ449	O6:K$^-$:H1	1	−	+	−	+	+	+	+	−	−	−	−	+	+	+	+	+	+	F14; F1C
33.	RZ501	O6:K$^-$:H1	4	−	+	−	+	+	+	+	−	−	−	+	+	+	+	+	−	−	F14rel.; F1C
34.	RZ507	O6:K$^-$:H1	1	−	+	−	+	+	+	+	−	−	−	+	+	+	+	+	+	+	F7/2; F1C
35.	RZ504	O6:K$^-$:H1	5	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	+	+	F1C
36.	RZ430	O6:K$^-$:H31	2	+	−	−	+	−	−	−	−	−	−	+	+	+	−	−	+	+	F1C
% Positive:				39	33	47	95	47	25	78	47	44	44	92	100	100	36	36	92	92	

[a] OMP: Outer membrane protein pattern.
[b] SR: Serum resistance.
[c] MRHA: Mannose-resistant hemagglutination, using human A_1 (hA), bovine (Bv), sheep (Sh) and pig (Pg) erythrocytes.

phenotypic and genotypic characteristics, although they differed in their outer membrane protein pattern.

Restriction fragment length polymorphism (RFLP)

For further analysis, genomic DNA of 20 representative strains was isolated and cleaved with *Xba*I, which infrequently cuts *E. coli* DNA. The fragments were separated by PFGE and the restriction fragment patterns were compared (Fig. 1a). The two O6:K53:H1 isolates (Fig. 1a, lanes 3, 4) seem to be identical because they had many fragments in common, whereas the two other strains of this serotype (lanes 1, 2) differed from them and from each other. Similarity of restriction fragment patterns was also found among the O6:K14:H$^-$ (Fig. 1a, lanes 5–9, 20) and the O6:K2:H$^-$ strains (Fig. 1a, lanes 10–13, 19), but the profiles of these groups differed from each other. Sometimes identical profiles (lanes 6, 8 or 12, 13) were found in strains of the same serotype, which were isolated from different patients. In contrast, markedly different *Xba*I patterns were observed among the five O6:K2:H1 strains (Fig. 1a, lanes 14–18); only the three isolates expressing P-specific receptor binding (lanes 14, 15, 17) share some common fragments. After *Sfi*I cleavage, similar results were obtained (data not shown).

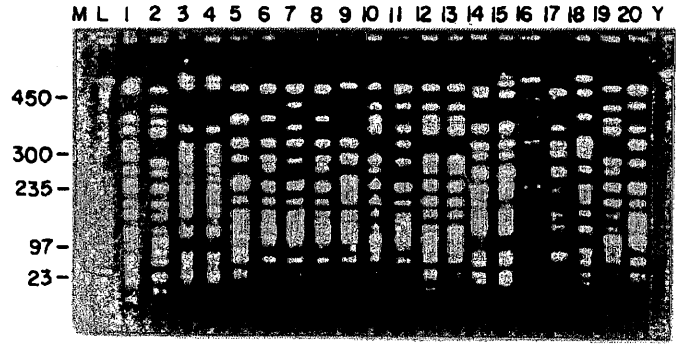
DNA long range mapping using virulence associated gene probes

In order to assign the specific virulence gene clusters to definite *Xba*I fragments, Southern hybridizations were carried out using the same DNA probes as used for the DNA–DNA dot blots (Fig. 1). The results are summarized in Fig. 2. It can be seen that not only the *Xba*I profile, but also the hybridization pattern, is highly heterogeneous between strains of different O6:K:H serotypes. Strains of the same serotype which are related by the *Xba*I pattern also show identical hybridization patterns, with few exceptions. The strains of serotype O6:K2:H1 sharing only few common fragments (lanes 14–18) also displayed some kind of different hybridization patterns. It can be concluded that relatedness of strains also is reflected by the *Xba*I hybridization patterns using virulence associated gene probes.

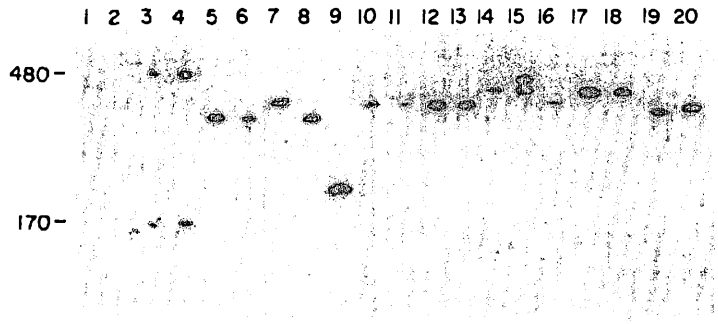
The hybridization data further show that in some isolates two different fragments were detected by one particular gene probe. Especially, all the O6:K14:H$^-$ strains (lanes 5–9, 20) display two fragments using the *pap* gene probe (Fig. 1c) and concomittantly one of these fragments also hybridized to the *hly* probe (Fig. 1b). These data might argue for the presence of two different gene clusters of P fimbriae, from which one is in vicinity to the *hly* determinants. Similar results were obtained for strain RZ467 (O6:K2:H1) (lanes 15, Fig. 1b,c). On the other hand, the O6:K53 strains RZ536 and RZ537 (lanes 3, 4) hybridized in two fragments with *hly* (Fig. 1b) and displayed hybridization with the *pap* probes in only one of these fragments (Fig. 1c), but hybridized with the *sfa* probe (Fig. 1d) to the other *hly* band. In these strains, a linkage of *hly–pap* and *hly–sfa/foc* might exist. Also, these two strains exhibited another band with the *sfa* probe, but distinct from that of the *hly* specific fragments. Two different bands hybridizing to *sfa/foc* were also seen in some other strains (lanes 2, 10, 12, 13, 16, 19), which might reflect two copies of these gene clusters.

Fig. 1 (Opposite). *Xba*I genomic profile of *E. coli* O6 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): (1) RZ446; (2) RZ448; (3) RZ536; (4) RZ537; (5) RZ422; (6) RZ423; (7) RZ461/1; (8) RZ424; (9) RZ505; (10) RZ454; (11) RZ460; (12) RZ484; (13) RZ462; (14) RZ485; (15) RZ467; (16) RZ458; (17) RZ470; (18) RZ486; (19) RZ502; (20) RZ461/2. DNA size standards were lambda concatemers (L), yeast chromosomes (Y) and *Hin*dIII cleaved lambda DNA (M).

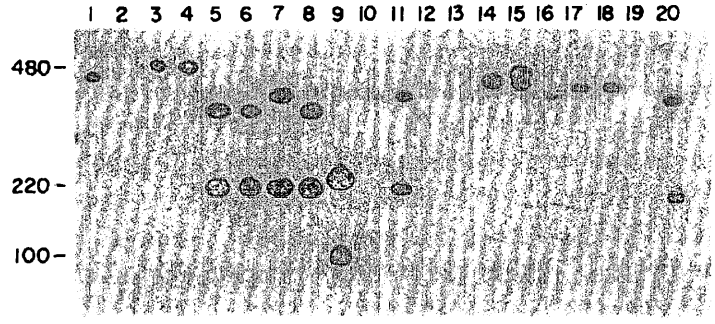
a


M L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 Y
450
300
235
97
23

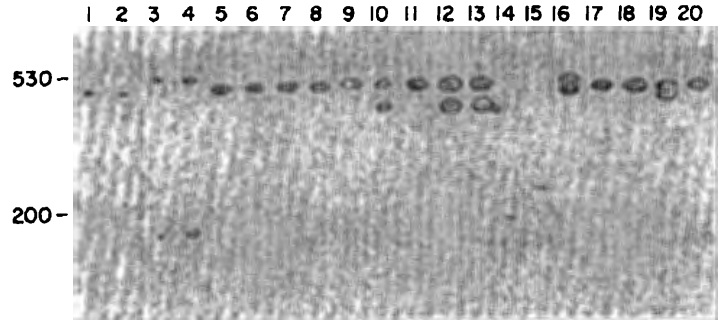
b


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
480
170

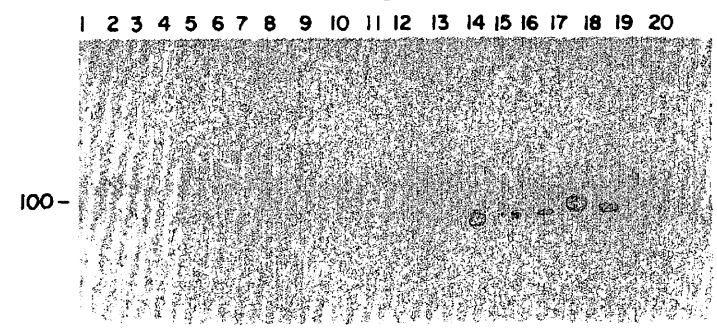
c


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
480
220
100

d


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
530
200

e


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
100

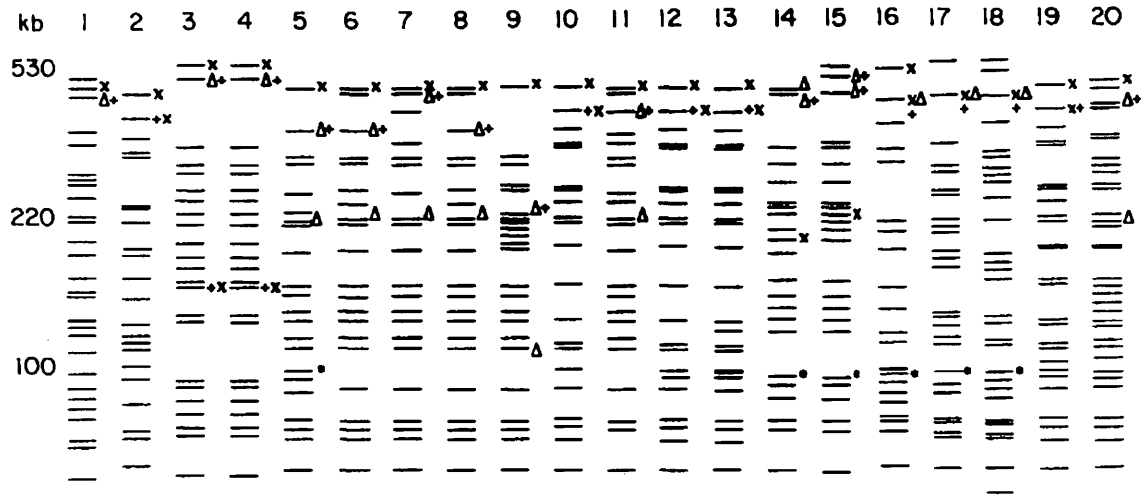


Fig. 2. Interpretation scheme of *Xba*I hybridization patterns of strains analysed in Fig. 1. The fragments hybridizing to individual gene probes are marked as follows: (•) *aer*; (+) *hly*; (△) *pap*; (×) *sfa/foc*.

Discussion

Human uropathogenic strains belong to a limited number of O groups, particularly O1, O2, O4, O6, O7, O16, O18 and O75.[5] One of the most common O antigens among these uropathogenic O groups is *E. coli* O6, which is found in various O6:K:H combinations, whereas the H antigen types are restricted to H1.[3,5] Examination of the O:K:H serotype strains most commonly found among pyelonephritis strains and sometimes among cystitis strains, for the presence of different virulence factors, revealed an association between virulence factors and O:K:H serotype.[2,19] Recent studies have shown that strains of the same serotype display genetic and phenotypic diversity, mainly based on the analysis of electrophoretic types (ET).[14] Also, elaboration of RFLP and the analysis of virulence patterns, especially of *E. coli* O6 strains of extraintestinal source, revealed a highly heterogeneous picture,[3,13,15,16] but a linkage between the K antigen, type of virulence factors and type of infection could be observed.[5,20] O6:K13 strains without P fimbriae are often isolated from cystitis patients, whereas P fimbriated O6:K2 isolates belong to the pyelonephritis-associated clones.[20]

In this report, we present data on the genetic presence and phenotypic expression of fimbrial adhesins, aerobactin and hemolysin among 36 *E. coli* O6 strains with the capsule types K2, K13, K14, K18/22 and K53. Our material also includes strains without capsule antigens and with capsule types which have not been identified (K^+). Molecular epidemiological analysis by either phenotypic or genotypic assays has sought to correlate several virulence factors, for example, adhesins and specific patterns of infection.[20–23] Each of these approaches has intrinsic limitations. Phenotyping may be spurious owing to variations in gene expression, uncertainties as to appropriate receptor-based assays, and the likelihood that most clinical isolates can express several different types of adhesins. On the other hand, colony hybridization studies do not discriminate homologous operons encoding different binding specificities and may be further confounded by other alterations at the nucleotide level. Although a combination of both approaches has been used in recent studies,[13,21–24] the study described here involved a substantially larger number of exactly serotyped *E. coli* O6 isolates than have been analysed previously, more definitive phenotypic assays, and probing with DNA fragments from several virulence factor genes in DNA–DNA dot blots, as well as in Southern hybridization assays after separation of the *Xba*I cleaved genomic DNA by PFGE.

From this study, it can be seen that data primarily elaborated for epidemiological purposes also can be useful for detecting strains with interesting genomic arrangements of virulence factors, such as linkages of virulence determinants. This might build a bridge to the molecular investigation of uropathogenicity. DNA long-range mapping revealed multiple copies of *sfa/foc* gene clusters and the determinants encoding P fimbriae and hemolysins, as well as linkage of *hly* and P fimbriae determinants and *sfa/foc*-sequences respectively, in some of the strains in our collection. Multiple copies and linkage of virulence factors seems to be a general phenomenon, as described recently.[13,16,25–29] The occurrence of such 'virulence clusters' offers the possibility of co-deletions of *hly* and P-specific determinants in certain isolates.[27]

The virulence pattern of strains analysed here was homogeneous among isolates of the same O:K:H serotype. Furthermore, the fimbrial serotypes supported the clonal analysis of our *E. coli* O6 isolates. Our data confirm the recent findings[13,24] that virulence features do reflect close genetic relatedness, however, DNA long-range mapping revealed differences not only among isolates of different serotypes, but also within strains of one serotype. Although, in general, relatedness elaborated by the analysis of virulence factors goes along with similar restriction fragments, the exceptions from the rule should be interpreted carefully in regards to the necessity of molecular tools for clonal analysis. DNA long-range mapping begins to play a major role in epidemiological surveys also, as shown recently, for pathogens other than *E. coli*.[30–34] However, we emphasize that the combination of both classical and molecular analysis is important to get a complete picture for evaluation of clonality.

Materials and methods

Bacterial strains and plasmids. All *E. coli* O6 strains in Table 1 were isolated from patients suffering from either cystitis or pyelonephritis at the Rostock University Hospital. The O6:K:H serotypes have already been described,[3] and the outer membrane patterns of the strains were determined according to Achtman *et al.*[35] Fimbriae were analysed serologically by CLIE as previously described.[36]

Plasmids used as source for DNA-probes were described recently (see below).[13,24] *Escherichia coli* K-12 strain HB101 was used as a recipient strain for recombinant DNA.[37] Cultivation was carried out under antibiotic pressure using the following antibiotics: ampicillin, 50 μg/ml; chloramphenicol, 20 μg/ml; tetracycline, 15 μg/ml.

Media, chemicals and enzymes. Bacteria were grown either in Luria Bertani (LB) broth or on LB-agar. Radiochemicals were purchased from NEN Research, Dreieich, Germany. Antibiotics were a gift from Bayer, Leverkusen, Germany. All other chemicals were obtained from Sigma, München, Germany. Restriction enzymes were purchased from Gibco, Eggenstein, Germany.

Adhesion testing. The presence of adhesins was determined by HA assays using human A_1, sheep, bovine and pig erythrocyte suspensions obtained locally with and without 2% mannose as described elsewhere.[3,36]

P-specific adherence was detected by mannose-resistant HA using P_1 human blood cells and the Gal–Gal specific latex test (PF test) obtained from Orion-Diagnostica (Espoo, Finland).[38,39] S-specific binding was assayed in an HA-test using bovine erythrocytes incubated with neuraminidase or fetuin as described previously.[40] Type 1 fimbriation was identified by mannose-sensitive agglutination of *S. cerevisiae* cells as already described.[6]

Aerobactin test. Aerobactin production was detected by a cross-feeding bioassay[41] as described.[13]

Hemolysin production. Nutrient agar plates and 5% sheep blood were used to test for hemolysin production. Strains producing a clear halo larger than the overlying colony, after overnight culture at 37°C, were defined as hemolysin-positive.

Resistance to bactericidal effect of serum. Sensitivity to the bactericidal effect of 75% pooled normal human serum was tested as described previously.[42]

Spot-test for the reaction of mAbA1 with denaturated fimbriae. A spot-test for the reaction of the mAbA1[43] specific for the S-fimbrial adhesin SfaS was carried out with denaturated fimbriae, as described.[44] Briefly, fimbriae were denaturated by boiling in a buffer containing beta-mercaptoethanol and SDS. Denaturated fimbriae were spotted on nitrocellulose paper and incubated with the mAb.

Immune complexes were detected by incubation with goat anti-mouse peroxidase conjugate (DAKO, Hamburg, Germany) using diaminobenzidine as a substrate.

SDS–PAGE. The disintegration of fimbriae and their electrophoretic separation on polyacrylamide gels (separating gel 15%, stacking gel 5%) according to Laemmli,[45] has been described elsewhere.[40]

DNA-techniques. Chromosomal DNA was isolated as described.[46] Plasmid DNA was isolated by the clear lysate method.[47] DNA fragments were eluted from agarose gels by the freeze-squeeze method.[48] DNA was cleaved with restriction enzymes under appropriate conditions and electrophoresed in 1% agarose gels.[49] The *Hin*dIII fragments of lambda DNA were used as a DNA size marker.

Pulsed field gel electrophoresis (PFGE). Chromosomal DNA was prepared in agar plugs as described by Grothues and Tümmler.[32] Agarose blocks were equilibrated in restriction enzyme buffer, and the chromosomal DNA embedded in the agarose was digested with either *Xba*I or *Sfi*I. A slice of each insert plug was then sealed into a well of 4-mm-thick horizontal agarose gel and electrophoresed (CHEF DR II, Bio-Rad Laboratories, München, Germany) in 0.5×Tris-borate–EDTA buffer[49] for 25 h at 14°C and 200 V, with the pulse times increasing from 10 to 40 s. Lambda concatemers (Pharmacia, Freiburg, Germany), yeast chromosomes (*S. cerevisiae* WAY 5-4A, Biometra, Göttingen, Germany) and *Hin*dIII cleaved lambda DNA were used as size markers.

Gene probes and radioactive labelling. The gene probes used were described recently.[13,24] After isolation from agarose gels,[48] the DNA fragments were radioactively labelled by the method of Feinberg and Vogelstein[50] using a random priming kit purchased from Boehringer, Mannheim, Germany.

The *pap* specific probe was a 4.4 kb *Hin*dIII fragment isolated from the plasmid pRHU845 (Tc^r).[51] It has been shown that this probe is also specific for the *prs* gene cluster coding for P related adhesins.[7] A 1.8 kb *Cla*I-*Eco*RI fragment from the recombinant DNA pANN801-13 (Ap^r) was used as an *sfa/foc* specific DNA probe.[40] This fragment was also subcloned into pBR322, to produce plasmid pANN801-21 (Ap^r). This probe also detects *foc* specific sequences, encoding F1C fimbriae.[17] The specific DNA probe for type 1 fimbriae gene clusters consisted of the 6.0 kb *Pst*I fragment of plasmid pPKL4 (Ap^r).[52] The aerobactin specific probe was a 7.0 kb *Hin*dIII–*Eco*RI fragment of plasmid pRG12 (Ap^r).[53] The 3.2 kb *Hin*dIII fragment representing the insert DNA of plasmid pANN215 (Cm^r) was used as an *hly* specific probe. Plasmid pANN215 is derived from the wild-type plasmid pHly152.[54]

Colony–dot hybridization. The colony–dot hybridization procedure described by Maniatis *et al.*[49] was used for rapid detection of specific virulence factors in the genomes of strains.

Southern hybridization. The transfer of DNA from agarose gels to nitrocellulose paper and the washing and autoradiography of the filters were performed as described earlier.[49,55] The filters were hybridized in 50% formamide for 24 h at 42°C.

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