CHARACTERISATION AND TYPING OF BACTERIA

Characterisation of pathogenic *Yersinia enterocolitica* serogroups by pulsed-field gel electrophoresis of genomic *NotI* restriction fragments

ELISABETH SAKEN, A. ROGGENKAMP, STOJANKA ALEKSIC* and J. HEESEMANNT†

Institute for Hygiene and Microbiology, University of Würzburg, Josef-Schneider-Strasse 2, 79080 Würzburg and

* Hygienisches Institut, Markmannstrasse 129a, 20539 Hamburg, Germany

Summary. Enteropathogenic *Yersinia enterocolitica* is an important cause of human and animal disease. Phenotypic and genotypic characteristics currently used to identify *Y. enterocolitica* are not necessarily sufficient to differentiate pathogenic from non-pathogenic strains or to analyse the epidemiology of yersiniae at a molecular level. To improve the characterisation of *Yersinia* isolates, *NotI* restriction fragment length polymorphisms (RFLPs) of chromosomal DNA of more than 100 clinical, animal and environmental isolates were analysed in pulsed-field gel electrophoresis. Highly conserved RFLP patterns with fragments ranging from 15 to 400 kb were detected within each of 10 *Y. enterocolitica* serogroups tested. Determination of RFLP types makes it possible to discriminate between isolates of different *Y. enterocolitica* serogroups and other *Yersinia* spp. Moreover, *NotI* restriction endonuclease analysis allows even subtyping of strains belonging to a unique serogroup-biotype. Identification of *NotI* fragments hybridising with *inv*- or *ail*-homologous sequences was used as an additional discriminating marker. The results indicate that *NotI* RFLP typing can provide a powerful new tool for the differentiation of clinical *Y. enterocolitica* isolates.

Introduction

Enteropathogenic *Yersinia enterocolitica* has been recognised as an important cause of human and animal disease. Diarrhoea, gastro-enteritis and mesenteric lymphadenitis are the most frequent clinical manifestations of human yersiniosis. Furthermore, septicæmia, with abscesses in the liver and other organs as well as sequelae such as reactive arthritis and erythema nodosum, have been observed.¹ *Y. enterocolitica* has been isolated all over the world, with most cases reported from Japan, North America and northern Europe. Whereas serogroups O3, O5,27 and O9 ('European serogroups') are isolated most frequently in Europe, serogroups O8, O13 and O20 predominate in America.^{2–9} However, during the last decade the incidence of serogroup O3 has been increasing in the USA.^{10,11} *Y. enterocolitica* is found in the environment and in domestic and wild animals and birds, but most strains from environmental sources are avirulent.^{6,7,12}

Four methods have been established for routine subdivision of *Y. enterocolitica*: serotyping, flagellar antigen typing, biotyping and phage typing.^{2,8,9,13,14} Serotyping in combination with biotyping is usually sufficient in most cases to differentiate between patho-

genic and non-pathogenic strains. However, in some instances there are misleading results from serotyping alone because, e.g., O8 antigen is also expressed by non-pathogenic *Y. enterocolitica* O7,8, or O3 antigen can be present on *Y. intermedia* or *Y. frederiksenii*.^{2,8,9,12} Recently, a more direct virulence marker became available with the detection of the 70-kb virulence plasmid pYV which is present in all human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* strains.^{1,4,15–18} However, this virulence plasmid can be rapidly lost in isolates subcultured several times at 37°C or stored for prolonged periods in the laboratory.^{1,4,17}

As more stable virulence-associated genetic markers, the chromosomal loci such as *inv* and *ail*, which encode for invasive phenotypes of *Y. enterocolitica*, and *yst* which encodes for the heat-stable enterotoxin of *Y. enterocolitica*, have been characterised and evaluated as epidemiological tools.^{15,19–22} However, it was impossible to differentiate between human pathogenic and non-pathogenic (environmental) strains of *Y. enterocolitica* with the *inv* probe alone.^{20,21} In contrast, the *ail* probe recognised the majority of human pathogenic serogroups of *Y. enterocolitica*, but not exclusively.²⁰ The *yst* probe was the most reliable virulence marker because it hybridised exclusively to chromosomal DNA of human pathogenic serogroups of *Y. enterocolitica* but not to that of *Y. pseudo-*

tuberculosis or non-pathogenic environmental *Yersinia* spp. (except *Y. kristensenii*).¹⁹

However, none of these methods (biotyping, serotyping or virulence gene typing) is sufficient to permit a finer degree of differentiation in epidemiological investigations.^{2, 8, 9, 13-15} The recent advances in population genetics have provided several new typing methods such as multilocus enzyme electrophoresis (MLEE, defining electrophoretic types by measuring allelic diversity) and pulsed-field gel electrophoresis (PFGE, to resolve genomic macrorestriction fragment length polymorphism) which differentiate isolates indistinguishable by the phenotypic and genotypic procedures mentioned above.²³⁻²⁷ Two recent studies showed that human pathogenic strains of *Y. enterocolitica* from diverse geographical locations could be divided into 13 and 24 electrophoretic types (ET), respectively, by MLEE.^{25, 28} In comparison with small fragment restriction endonuclease analysis of genomic DNA (REAC), the discriminatory power of MLEE typing for *Y. enterocolitica* strains of serogroup O3 was slightly higher (0.752 versus 0.623) than for REAC typing.²⁹ Recently, the impact of PFGE on the analysis of the molecular epidemiology of diverse bacterial pathogens has been demonstrated.^{24, 26} However, only preliminary results of genomic macrorestriction analysis of *Yersinia* spp. have been reported hitherto.³⁰

In a more detailed and extended survey, macrorestriction fragment (MRF) patterns of chromosomal DNA of > 100 clinical isolates of *Y. enterocolitica* of various serogroups were analysed. Moreover, hybridisation results with *inv* and *ail* probes were included to assign these virulence-associated genes to certain MRFs and to differentiate between pathogenic and non-pathogenic strains.

Materials and methods

Bacteria

A total of 109 *Y. enterocolitica* isolates of different serogroups and origin and five *Yersinia* spp. expressing a serogroup O3 cross-reacting antigen was tested (table I). The strains were either isolated from patients with clinical signs of yersiniosis in Hamburg and Würzburg (Germany) and Turku (Finland) from 1980 to 1990, or strains (mostly of human origin) that had been described previously.^{25, 29} A large proportion of these strains has been characterised recently by O- and H-serotyping, phage typing and MLEE.²⁵

Preparation of genomic DNA and restriction endonuclease digests

For PFGE, genomic DNA was prepared as described previously.^{24, 26} Briefly, bacterial cells grown in Luria broth for 16 h at 27°C were harvested into SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.4) and resuspended to an OD of 2.0. This suspension was mixed with an equal volume of melted agarose (GTG

Agarose, Biometra, Göttingen, Germany) 2% w/v and then pipetted into moulds. After solidification at 4°C, blocks were removed from the moulds and sliced into the slot size of the PFGE-agarose gels. After overnight incubation in 20 volumes of a buffer containing 0.5 M EDTA, pH 9.5, N-lauroyl-sarcosine sodium salt 1% w/v and proteinase K 2 mg/ml, the blocks were washed three times with gentle agitation in 15 volumes of TE (10 mM Tris, 5 mM EDTA, pH 7.5) and stored in TE at 4°C. These samples could be stored without detectable degradation of DNA for > 2 years.

For restriction endonuclease digestion of chromosomal DNA, blocks were transferred into 2-ml sterile Eppendorf tubes and equilibrated in 1 ml of *Not*I restriction buffer (Pharmacia, Freiburg, Germany) for 6 h with several changes of buffer. DNA was digested in 0.2 ml of the same buffer containing 10 U of *Not*I for 16 h at 37°C.

PFGE

PFGE was performed with a CHEF system (BioRad, Richmond, CA, USA). Gels containing GTG agarose (Biometra) 1% w/v were run in 0.5 × TBE³² at 8°C at a pulse ramping from 8 to 23 s for 26 h. Lambda concatamers (Pharmacia) and *Hind*III-cleaved lambda DNA were used as size standards.

Generation of digoxigenin-labelled DNA probes

Digoxigenin labelling of the DNA probes specific for the chromosomal genes *inv* and *ail* was performed by direct PCR amplification from the *Y. enterocolitica* O8 strain WA-314 according to the method described by Lion and Haas.³³ Specific primers for *ail* and *inv* were derived from the open reading frame of the published nucleotide sequences of the *Y. enterocolitica* *ail* and *inv* genes.^{22, 24} The primers chosen for *ail* were: Ail-1 (5'-GTCTGTTAATGTGTACGCTGC-3') and Ail-2 (5'-CACCTGGATGCTTGGTGCAG-3'); and for *inv* were: Inv-1 (5'-GAAAACCGGCGAGAGC-GAAGCG-3') and Inv-2 (5'-GGTGTCAAGTCGTCA-CTTTAGA-3'). The expected sizes for the amplified *ail* and *inv* products were 574 bp and 352 bp, respectively. PCR amplification was performed as follows: (i) denaturation of chromosomal DNA for 30 s at 94°C; (ii) annealing of primers for 1 min at 58°C (*ail*) and 57°C (*inv*), respectively; and (iii) extension for 3 min at 72°C. For purification, the PCR products were electrophoresed in a low melting agarose gel (agarose 2% w/v); the labelled products were sliced out and denatured by heat before use as gene probes.

Southern blot and filter hybridisation

Large chromosomal DNA fragments separated by PFGE were transferred to nylon membranes (Zeta probe, BioRad) by vacuum blotting (Vacu gene, Pharmacia) according to the manufacturers' instructions, and fixed at 80°C for 2 h. Pre-hybridisation and hybridisation were at 68°C in 5 × SSC,³² N-lauroyl-sarcosine sodium salt 0.1% w/v, SDS 0.02% w/v and

blocking reagent (Boehringer, Mannheim, Germany) 1% w/v. Hybrid DNA was detected by an enzyme immunoassay with anti-digoxigenin antibodies conjugated to alkaline phosphatase (Boehringer) according to the manufacturer's protocol.

Results

NotI RFLP patterns of diverse *Y. enterocolitica* serogroups

A total of 109 *Y. enterocolitica* strains of different serogroups, isolated in the years 1980–1990, from various sources and countries of origin, were investigated by restriction fragment length polymorphism (RFLP) analysis of genomic DNA. Corresponding to the prevalence of the different serogroups, 54 strains of serogroup O3, 18 of serogroup O9 and 15 of serogroup O5,27 were investigated. Moreover, individual strains of selected *Y. enterocolitica* serogroups and other *Yersinia* spp. (table I) which have been described previously were investigated.^{25, 29}

Y. enterocolitica chromosomal DNA prepared in blocks was digested with several "rare cutting" restriction enzymes (*NotI*, *XbaI*, *SstI* and *NheI*). Cleavage with *NotI* resulted in restriction fragment patterns with good resolution for all *Y. enterocolitica* serogroups tested, whereas cleavage with the other enzymes tested gave too many small fragments (*SstI*, *NheI*), or too many fragments of the same range of size (*XbaI*) (results not shown). *NotI* generated fragments of 15–400 kb (fig. 1) which were suitable for genotyping. Restriction fragments > 100 kb were clearly resolved and were considered for comparison and discrimination of banding patterns. Restriction fragment patterns with few differences in number and size of fragments were classified as the basic (or leading) restriction pattern whereas minor deviations in one to three restriction fragments were considered as subtypes (indicated by small letters) of the leading pattern. This approach revealed that *NotI* RFLP typing clearly differentiated between *Y. enterocolitica* serogroups (table I, fig. 1), i.e., the 10 different *Y. enterocolitica* serogroups tested corresponded to 10 distinct RFLP patterns. Among the "European serogroups", all strains belonging to the same serogroup had a unique RFLP pattern, with few exceptions (see next section, table II, figs 2 and 3). With regard to the number of strains tested, serogroup-specific RFLP types could be defined for the three "European" human pathogenic serogroups: RFLP types of O3, O5,27 and O9 were, therefore, designated as RFLP 3, 5.27 and 9, respectively.

O3 serotyped *Yersinia* strains. Serogroup-specific RFLP patterns were observed among all serogroup O3 strains with but few exceptions. The major group (typical O3 serogroup, biotype 4 or 3) could be subdivided into five subtypes (RFLP 3a–e, fig. 2). Interestingly, two different RFLP subtypes were recog-

nised within one electrophoretic type (ET1 includes RFLP 3a and c, table IV). Two serogroup O3 strains (328/88 and 8018, see table I) which did not correspond to the basic RFLP type 3, were aesculin-positive indicating that they were non-pathogenic *Y. enterocolitica* strains.

O5,27 serotyped *Yersinia* strains. From the rarely isolated *Y. enterocolitica* serogroup O5,27, 15 strains were characterised. All but one strain belonged to a unique and serogroup-specific RFLP (denoted RFLP 5.27). Within this RFLP group, three subgroups could be distinguished (fig. 3), dominated by subgroup 5.27a. The three strains described as ET4 belonged to subgroup 5.27a or 5.27b. One American isolate of *Y. enterocolitica* O5,27 which was *inv*- and *ail*-positive (characteristic of human pathogenic *Y. enterocolitica* strains) exhibited a clearly different pattern from RFLP 5.27, which was also reflected by the size of the *ail*-positive *NotI* restriction fragment (table III).

O9 serotyped *Yersinia* strains. From the second most frequently isolated "European serogroup" O9, 18 strains were characterised; 16 strains showed a similar restriction pattern, denoted RFLP 9, which comprised five subgroups (fig. 3). Four strains that were of ET5 could be divided into three subgroups (RFLP 9a,b and e). Two strains that could not be included in RFLP 9 because of distinct RFLP patterns were *inv*-positive but *ail*-negative (table III). These two strains did not belong to biotype 2 (one was aesculin-positive and one belonged to biogroup 3), which is atypical for *Y. enterocolitica* O9 strains. They were isolated from patients with enteric disease by cold enrichment.

***Y. enterocolitica* strains of biogroup 1B.** Most of the "American serogroups" of *Y. enterocolitica* belong to biogroup 1B. The most frequent isolates are of serogroup O8. All the serogroup O8 strains studied hybridised with *inv* and *ail* probe. Six strains were completely phenotyped and five of them were identical with regard to the phenotypes biotype, phage type and ET, with slight deviations in H-antigen. Surprisingly, only a few strains of this cluster were identical in their RFLP pattern; most strains (including the incompletely phenotyped strains), showed RFLP patterns with major deviations in the number and size of the restriction fragments (fig. 4). Therefore, the RFLP patterns of the 10 serogroup O8 strains were classified as seven main RFLP types and were designated by capital letters (RFLP 8A–F and RFLP 8X) to emphasise putative early evolutionary divergence. The other biotype 1B strains of serogroups O13, O13a, 13b, O20 and O21 showed distinct serogroup-specific RFLP patterns with the greatest difference within the range of restriction fragments < 200 kb. It is interesting to note that the RFLP patterns of all biotype 1B strains showed some similarity within the high-mol. wt area (> 200 kb, fig. 4).

Table 1. Characteristics of strains used in this study

Isolate	Source	Origin	HA	BT	PT	ET	RFLP
<i>Y. enterocolitica</i> O1,2a,3 (n = 5)							
IP197 ^a	Chinchilla	USA	abc	3	II	5	1,2,3a
IP714 ^a	Chinchilla	USA	abc	3	II	5	1,2,3a
IP132 ^a	Chinchilla	Netherlands	abc	3	II	6	1,2,3b
Chinchilla isolates: n = 2		Germany	abc	3	nd	nd	1,2,3a
<i>Y. enterocolitica</i> O2a,2b,3 (n = 2)							
JP1154 ^a	Hare	Belgium	abc	5	XI	7	2,2,3a
JP1142 ^a	Hare	Belgium	abc	5	XI	8	2,2,3b
<i>Y. enterocolitica</i> O3 (n = 54)							
3668 ^a	Man	Finland	abc	4	VIII	1	3a
29C-46 ^a	Man	Norway	abc	4	VIII	1	3c
29C-43a ^a	Man	Norway	abc	4	VIII	1	3c
Y2a ^a	Pig	Norway	ac	4	VIII	1	3c
Y325 ^a	Pig	Norway	abc	4	VIII	1	3c
MCH697 ^a	Man	Canada	abc	4	IXb	1	3c
8265 ^a	Man	France	abc	4	VIII	1	3c
SW13123 ^a	Pig	Japan	ac	4	VIII	1	3c
Y325 ^a	Pig	Norway	abc	4	VIII	1	3c
4147 ^a	Man	Finland	abc	4	VIII	1	3c
M388 ^a	Pig	Japan	—	3	II	1	3d
SW13711 ^a	Pig	Japan	—	3B	II	1	3e
Y764 ^a	Pig	Norway	abc	4	VIII	2	3c
Y-108 ^c	Man	Germany	nd	4	nd	nd	3a
Patient isolates n = 34		Germany/Finland		4	nd	nd	3a
TY5613	Man	Germany	nd	4	nd	nd	3b
Patient isolates n = 4		Germany/Finland		4	nd	nd	3c
328/88	Man	Germany	nd	nd*	nd	nd	NT
<i>Yersinia</i> spp. of serogroup O3 (n = 1)							
8018 ^a	Rodent	Norway	abcd	1A	XI	15	NT
<i>Y. intermedia</i> O3 ^a (n = 1)							
357-36/85	Water	Germany	q	nd	XI	14	NT
<i>Y. frederiksenii</i> O3 ^a (n = 1)							
176-36/90	Water	Germany	p2	nd	XI	16	NT
<i>Y. kristensenii</i> O3 ^a (n = 1)							
Y332	Pig	Norway	—	nd	Xo	18	NT
<i>Y. mollaretii</i> O3 ^a (n = 1)							
87-36/80	Man	Germany	u	6	XI	17	NT
<i>Y. enterocolitica</i> O5,27 (n = 15)							
D113 ^a	Dog	Japan	abc	2	Xz	4	5,27a
PA9436 ^a	Man	Japan	abc	2	Xz	4	5,27a
YE873	Pig	Canada	abc	2	Xz	4	5,27b
H567/90	Man	Germany	abc	3	nd	nd	5,27c
Patient isolates n = 5		various	nd	nd	nd	nd	5,27a
Patient isolates n = 1		Germany	nd	nd	nd	nd	5,27b
Patient isolates n = 1		Germany	nd	nd	nd	nd	5,27c
H735/86	Man	USA	abc	3	nd	nd	NT
Animal isolates n = 2		Germany	abc	3	nd	nd	5,27a
Animal isolates n = 1		Japan	abc	2	nd	nd	5,27a
<i>Y. enterocolitica</i> O5 (n = 1)							
NF-O ^c	Man	Newfoundland	nd	nd	nd	nd	5
<i>Y. enterocolitica</i> O8 (n = 10)							
YE653 ^a	Man	Canada	befiv	1B	Xz	10	8F
A2635 ^a	Man	USA	befi	1B	Xz	10	8B
NY81-68 ^a	Man	USA	befi	1B	Xz	10	8E
WA-314 ^b	Man	USA	befi	1B	nd	nd	8A
WA ^a	Man	USA	befiv	1B	Xz	10	8B
1700 ^a	Man	USA	befi	1B	Xz	10	8C
A2628 ^b	Man	USA	nd	1B	nd	nd	8B
893/87	Man	Italy	befi	1B	nd	nd	8D
900/90	Man	Japan	befi	1B	nd	nd	8D
8081 ^a	Man	USA	befi	1B	Xz	11	8X
<i>Y. enterocolitica</i> O9 (n = 18)							
YE099 ^a	Man	Canada	abv	2	X3	5	9a
W829 ^a	Pig	Belgium	ab	2	X3	5	9b
7Oulua	Man	Finland	abv	2	X3	5	9e
3315a ^a	Man	Netherlands	abv	2	X3	5	9b
Patient isolates n = 5		Germany	nd	2	nd	nd	9a
Patient isolates n = 4		Germany	nd	2	nd	nd	9b
Y-96 ^c	Man	Germany	nd	2	nd	nd	9b
1744/90	Man	Germany	nd	2	nd	nd	9c
PA177	Man	Japan	abc	2	nd	nd	9d
1034/87	Man	Germany	nd	nd*	nd	nd	NT
259/88	Man	Germany	ac	3	nd	nd	NT

Table 1 continues on p. 333

Table I. *Cont.*

Isolate	Source	Origin	HA	BT	PT	ET	RFLP
<i>Y. enterocolitica</i> O13 (n = 2)							
1209-79 ^b	Man	USA	nd	1B	nd	nd	13·1
YE886 ^a	Primate	Canada	abi	1B	Xz	12	13·2
<i>Y. enterocolitica</i> O20 (n = 1)							
1223-75-1 ^b	Man	USA	nd	1B	nd	nd	20
<i>Y. enterocolitica</i> O21 (n = 1)							
YE737 ^a	Man	Canada	befi	1B	Xz	13	21

a, strains as described in ref. 25; b, strains as described in ref. 4; c, strains as described in ref. 31; NT, non-typable; nd, not determined; HA, flagellar antigen (–, non-motile); BT, biotype; PT, phage type; ET, electrophoretic type; RFLP, *NotI* restriction fragment length polymorphism type.

*Aesculin positive.

Fig. 1. *NotI* RFLP of human pathogenic *Y. enterocolitica* serogroups, λ, phage λ concatamers; O3, Y-108; O9, YE099; O5,27, D113; O5, NF-O; O8, WA-314; O13, 1209-79; O20, 1223-75-1; O21, YE737.

O1,2a,3 and O2a,2b,3 serotyped Yersinia strains. Both serogroups expressed the O3 antigen, which is shared with *Y. enterocolitica* serogroup O3 strains. However, the RFLP patterns of these strains were distinct from that of RFLP 3 (fig. 2) and were, therefore, designated according to the serogroup of the strains. Two RFLP subtypes were recognised within each serogroup.

Non-typable strains. The few strains that deviated from the serogroup-specific RFLP type were isolated from asymptomatic carriers or patients with atypical clinical manifestations or were of biotype 1A. These strains were designated "non-typable" (NT) in this study. Five serogroup O3 strains from other *Yersinia*

spp. (table I) were also studied for comparison with the *NotI* RFLP pattern of *Y. enterocolitica* O3 (fig. 2). These RFLP patterns were clearly distinct from RFLP 3.

Identification of NotI restriction fragments hybridising with inv or ail probe

As a further refinement to the genotypic approach, Southern blot hybridisation with *inv* and *ail* probes was used to identify distinct *NotI* restriction fragments. As shown in table III, for all pathogenic *Y. enterocolitica* strains a 90–100 kb *NotI* restriction fragment hybridised with *inv*. *Y. enterocolitica* strains of the same

Table II. Distribution of variants in RFLP of reference strains and clinical isolates

Serogroup	Number of strains	RFLP	Number of strains	Number of variants					NT	
				a	b	c	d	e		
O1,2,3	5	1,2,3	5	4	1	0	0	0		0
O3	54	3	53	36	1	14	1	1		1
O5,27	15	5,27	14	10	2	2	0	0		1
O9	18	9	16	6	7	1	1	1		2
O8	10	8	9	A (1)	B (3)	C (1)	D (2)	E (1)	F (1)	X (1)

Fig. 2. *NotI* RFLP patterns and subtypes (indicated by small letters, e.g., RFLP3a) of *Yersinia* serogroup O3 strains. λ , phage λ concatamers; RFLP 3, 1.2.3 and 2.2.3, *Y. enterocolitica*; RFLP3a, Y-108; RFLP3b, TY 5613/86; RFLP3c, 29C-46; RFLP3d, M388; RFLP3e, SW13711; RFLP1.2.3a, IP197; RFLP1.2.3b, IP132; RFLP2.2.3a, JP1154; RFLP2.2.3b, JP1142; yi, *Y. intermedia*; yf, *Y. frederiksenii*; ym, *Y. mollaretii*; yk, *Y. kristensenii*.

serogroup (excluding serogroup O8) showed a unique *inv*-positive *NotI* restriction band. However, hybridisation with the *ail* probe revealed a significant RFLP among strains of the same serogroup except for serogroup O3 and O9 strains. These strains of "European serogroups" were highly conserved with regard to the *ail*-positive *NotI* restriction fragments.

Comparison of phenotypic and genotypic methods applied to serogroup O3 Yersinia strains

An effective epidemiological analysis requires discriminatory methods that allow differentiation among

isolates of the same species. In table IV, the phenotypes such as biotype, phage type and ET are compared with the *NotI* RFLP types (genotype) of pathogenic (biotype 4, 3A and 3B) *Y. enterocolitica* strains of serogroup O3. Biotype 4 strains could be subdivided into three RFLP types (RFLP 3a, b and c). Strains of the same phage type, H-antigen type of ET were not inter-related with *NotI* RFLP types. Interestingly, the two biotype 3 strains from Japan could be differentiated from those of biotype 4 by RFLP typing, but not by MLEE. Thus, it appears that *NotI* RFLP typing has a higher discriminatory power than MLEE and H-antigen typing.

Fig. 3. *NotI* RFLP patterns and subtypes of *Y. enterocolitica* serogroup O5,27 and O9 strains. λ , phage λ concatamers; RFLP5.27NT¹, H735/86; RFLP5.27a, D113; RFLP5.27b, YE873; RFLP5.27c, H567/90; RFLP9a, YE099; RFLP9b, Y-96; RFLP9c, 1744/90; RFLP9d, PA177; RFLP9e, Oulu; RFLP9NT², 1034/87.

Table III. *NotI* restriction fragments in kb that hybridised with *inv*- and *ail*-specific DNA probes

Strain	RFLP	Number of strains tested	Number of strains <i>inv</i> -positive*	Number of strains tested	Number of strains <i>ail</i> -positive*
<i>Y. enterocolitica</i>					
O1,2a,3	1,2,3a	4	97 (4)	4	60 (4)
	1,2,3b	1	97 (1)	1	40 (1)
	NT	0		0	
O3	3	32	100 (32)	48	40 (48)
	NT	1	110 (1)	1	... (0)
O5,27	5,27a,b	13	95 (13)	13	240 (13)
	5,27c	2	95 (2)	2	300 (2)
	NT	1	95 (1)	1	55 (1)
O8	8	9	80-90 (9)	9	120-150 (9)
	8X	1	90 (1)	1	150 (1)
O9	9	15	100 (15)	15	40 (15)
	NT	2	100 (2)	2	... (0)

*Size of positive *NotI* fragment and number of positive strains.

Discussion

The PFGE technique can be used to separate large fragments of bacterial DNA generated by "rare-cutting" restriction endonucleases. The resulting RFLP pattern can be considered as a characteristic DNA fingerprint of a strain. Strains of the same evolutionary lineage should be encompassed by similar or identical RFLP patterns.^{23, 24, 26, 30} In this study, the potential of genomic RFLP typing for epidemiological

studies of *Y. enterocolitica* infections and for comparison with diverse phenotyping methods including MLEE was evaluated. For this reason, *Y. enterocolitica* strains which had been extensively phenotyped previously (serotyped, biotyped, phage typed, electrophoretically typed) were selected and additional clinical isolates (serotyped and biotyped) were included.^{2-4, 17, 25, 29} By trial and error, it was found that the "rare-cutting" restriction endonuclease *NotI* was well suited to generate resolvable clear and well distri-

Fig. 4. *NotI* RFLP patterns and subtypes of *Y. enterocolitica* biogroup 1B strains. λ , phage λ concatamers; RFLP8.A, WA-314; RFLP8.B, WA; RFLP8.B, A2635; RFLP8.B, A2628; RFLP8.C, 1700; RFLP8.D, 893/87; RFLP8.D, 900/90; RFLP8.E, NY81-68; RFLP8.F, YE653; RFLP8.X, 8081; RFLP13.A, 1209-79; RFLP13.B, YE886; RFLP20, (1223-75-1); RFLP21, YE737.

Table IV. Comparison of *Y. enterocolitica* RFLP 3 with other strain characteristics

ET	BT	PT	RFLP	HA
1 (13)	4 (11)	VIII (9)	3c (8)	abc (6) ac (2)
			3a (1)	abc (1)
		IXb (2)	3c (2)	abc (2)
	3/3B (2)	II (2)	3e (1)	NM (1)
			3d (1)	NM (1)
2 (1)	4 (1)	VIII (1)	3a (1)	abc (1)
nd	4 (1)	nd	3b (1)	nd

nd, not determined; number of strains shown in parentheses.

buted MRFs of chromosomal DNA of diverse *Yersinia* spp. within the range 15–400 kb. *NotI*-generated RFLP patterns of diverse *Yersinia* spp. and also of strains of the same *Y. enterocolitica* serogroup were easily distinguishable. Strains which were incorrectly classified by conventional typing techniques were also discovered by RFLP typing (see table I). These results confirmed those of a preliminary MRF study reported recently³⁰ and encouraged us to focus our interest on the most frequently isolated human pathogenic serogroups of *Y. enterocolitica*.

For several reasons, there is a need for refinement of differentiation techniques to study the molecular epidemiology of yersinia infections. Previously, we and others have shown that restriction endonuclease

analysis of yersinia virulence plasmids (REAP-typing) can hardly differentiate among strains of the same serogroup (except serogroup O8).^{16–18} This prompted several epidemiologists to evaluate restriction enzyme analysis of chromosomal DNA (REAC) of *Yersinia* spp. as a useful genotyping method.^{10, 29} However, as REAC is complicated by the high number of small restriction fragments generated by frequent-cutting endonucleases, this proved to be of low differentiating potential. For instance, *Y. enterocolitica* strains of serogroup O3 but different phage types could not be differentiated by REAC-typing.²⁹ An improvement of the REAC method was achieved when rRNA probes were used for detecting specific RFLPs (ribotyping). There are three relevant reports of the differentiation of *Yersinia* strains by ribotyping.^{10, 28, 35} Picard-Pasquier *et al.* used rRNA probes for *HindIII*- and *EcoRI*-digested chromosomal DNA of yersiniae.²⁸ Surprisingly, they were unable to differentiate between strains of the same biotype. With the restriction endonuclease *NciI* in conjunction with an rRNA probe, two research groups were able to discriminate five and four different ribotypes, respectively, within serogroup O3 strains.^{10, 35} A close inter-relation between ribotypes I and II and phage types IXb and VIII of *Y. enterocolitica* O3, respectively, was demonstrated. However, ribotyping was unable to differentiate between strains of serotype O1,2,3 and O9. Attempts to improve the discriminatory potential by

MLEE also failed because strains of the same O serogroup were also of the same ET and strains of different serogroups could be of the same ET and thus could not be distinguished.²⁵

By RFLP typing with restriction endonuclease *NotI*, the "European serogroups" O3, O9 and O5,27 could be clustered in serogroup-specific RFLP patterns (RFLP 3, RFLP 9 and RFLP 5.27), each consisting of several sub-patterns which could be distinguished by minor deviations of one to three restriction fragments. For instance, the 54 strains of *Y. enterocolitica* of serogroup O3-biotype 4 studied could be subdivided into three RFLP 3 subtypes (designated by small letters, RFLP 3a-c). Most strains of serogroup O3-biotype 4 were distributed among RFLP 3a and c.

Considering serogroup O3-biotype 4 strains, electrophoretic types (all but one strain were ET1), phage types (PT VIII, PT IXb) and H-antigen types did not correspond to distinct RFLP subtypes (tables I and IV).²⁵ However, the majority of the Finnish isolates of REAP type I were RFLP 3c and those of REAP type II were RFLP 3a, respectively (results not shown; P. Toivanen, personal communication).

In contrast to ribotyping, RFLP typing clearly distinguished biogroup 3 *Y. enterocolitica* strains expressing the O3 antigen from those of biogroup 4.^{10, 28, 35} The RFLP patterns of the two Japanese serogroup O3 isolates (RFLP 3d and e) and the five Chinchilla isolates of serogroup O1,2a,3 (RFLP 1.2.3a and b) could be clearly distinguished from serogroup O3-biotype 4 strains by major deviations of the RFLP 3 pattern. Thus, RFLP typing of *Y. enterocolitica* strains expressing the O3 antigen in conjunction with phage typing has a higher discriminatory potential than ribotyping (with *NciI*-RFLP types).

A similar conclusion could be drawn from the O9 and O5,27 strains, that both belong to biogroup 2 but to phage types X3 and Xz, respectively. Fourteen strains of serogroup O5,27 and 16 strains of serogroup O9 were subdivided into three and five RFLP subtypes, respectively. Strikingly, O9 strains were preferentially of RFLP 9a or b and O5,27 strains accumulated as RFLP 5,27a. In contrast, ribotyping of O5,27 strains was less efficient.^{10, 35} The five O5,27 strains which had been ribotyped were all of *NciI*-RFLP type b; however, serogroup O3-biogroup 3 strains also belonged to this ribotype.³⁵ Unfortunately, representative ribotyping results of *Y. enterocolitica* of serogroup O9 are not available for comparison with RFLP types.

RFLP typing of 10 serogroup O8 *Y. enterocolitica* strains of different origin revealed seven RFLP pat-

terns with major differences in many restriction fragments; therefore, these patterns were allocated capital letters, RFLP 8A, B etc. A similar heterogeneity of serogroup O8 strains had been found by REAP typing (six plasmid variants), from ribotyping (four *NciI*-RFLP types) and from REAC typing (10 REAC types of 10 strains).^{18, 25, 29, 35} Surprisingly, serogroup O8 strains appeared homogeneous according to phage type (Xz), biogroup (1B) and ET (ET10).

It is striking that the serogroup O8 strain 8081 could be easily distinguished from the other O8 strains by diverse genotypic techniques. This singularity of strain 8081 is particularly emphasised by the ET11 and the RFLP type 8X. In contrast to the heterogeneity of the RFLP patterns of biogroup 1B strains (including serogroups O8, O13, O20 and O21), these strains share several features: (i) geographical restriction to North America; (ii) high virulence for rodents; (iii) sensitivity to pesticin; (iv) siderophore production; and (v) broad host range.^{1, 4, 7, 36-38} From this aspect, biogroup 1B strains may belong to the same evolutionary lineage. Taking into account the fact that mobile genetic elements (e.g., insertion sequences) render biogroup 1B strains particularly genetically unstable, genetically diverging lineages of one evolutionary clone would be expected. This assumption might explain the relatively low degree of similarity of all RFLP patterns obtained from biogroup 1B strains and it is supported by two recent reports about the genomic instability of *Y. pestis* and *Y. pseudotuberculosis*.^{39, 40} The biogroup 1B strains may benefit from genetical instability because of probable better adaptation to a broad host range and to survival in the environment.⁷

In contrast, the European *Y. enterocolitica* isolates of biogroups 2 and 4 are assumed to be well adapted to pigs (narrow host range) and they are usually not found in the environment (e.g., river water, soil). Probably, these strains have become genetically stable by convergent evolution, e.g., by loss of transposable elements (transposon, IS-element).

In summary, genomic *NotI* RFLP typing permits the distinction of *Y. enterocolitica* of different serogroups and also strains of the same serogroup. Because of the discriminatory potential and practicability of PFGE, *NotI* RFLP typing appears to be more suitable than other genotypic methods (e.g., ribotyping and MLEE) for molecular epidemiological analysis of yersiniosis.

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