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PCR-based characterization of *Yersinia enterocolitica*: comparison with biotyping and serotyping

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SUMMARY

PCR-based DNA fingerprinting was used to characterize 48 clinical isolates of *Yersinia enterocolitica*. The samples were examined by random amplified polymorphic DNA (RAPD-PCR) and inter-repeat PCR (IR-PCR). IR-PCR with two enterobacterial repetitive intergenic consensus primers resulted in patterns which were poorly discriminated; 2 of 11 arbitrary primers (RAPD-PCR) provided sufficient discriminatory power. In comparisons with serotyping and biotyping, RAPD-fingerprinting was the most discriminatory technique and may therefore be a valuable epidemiological tool for the study of *Y. enterocolitica* infections.

INTRODUCTION

Human infections due to *Yersinia enterocolitica* have increased dramatically during the past two decades and are associated with a wide variety of clinical symptoms [1]. Children generally develop enteritis and mesenteric adenitis whereas extraintestinal sequellae such as reactive arthritis occur mainly in adults [2]. Once infected with *Y. enterocolitica*, a patient may become an asymptomatic carrier, but latent infections have been described and may lead to chronic and recurrent illnesses such as chronic ileitis, hepatitis and arthritis [2]. The incidence of *Y. enterocolitica* infections has been estimated at one quarter of that of salmonella infections in The Netherlands. Types most common isolated are O:3, O:9 and, exceptionally, O:8 [3].

A major reservoir for *Y. enterocolitica* serotype O:3 and O:9, are pigs [1, 4]. Animal products, such as raw milk, ice cream, beef and poultry, may also harbour the organism. Environmental sources of *Y. enterocolitica* differ in biotype and serotype of commonly isolated strains from infected patients, except for serotype O:8, of which untreated water has been the source of some outbreaks in the United States. This route of infection may lead to severe outbreaks as has been described in the United States, Canada and Japan [1].

To distinguish different *Y. enterocolitica* strains in epidemiological surveys in human populations, different typing methods are used, including phenotypic characterization such as biotyping [5], serotyping [6, 7], antibiogram typing [8], and phage typing [9]. All share a common theoretical disadvantage since they

depend upon the stability of gene expression *in vitro* [4, 10]. Furthermore, their ability to discriminate between unrelated isolates is generally low.

Recently, several molecular techniques have been described for genotyping, including restriction endonuclease analysis of chromosomal (REAC) and plasmid (REAP) DNA [10, 11] and restriction fragment length polymorphism of rRNA genes (ribotyping) [4, 12]. The advantage of these molecular techniques over phenotypic methods is, that they detect genomic differences which are potentially unique for each individual organism, whereas phenotyping methods depend on the expression of genes [13]. Plasmid analysis of *Y. enterocolitica* (REAP), however, is not useful for epidemiological studies, because pathogenic *Y. enterocolitica* strains only harbour a single plasmid, which is difficult to isolate and easily lost during cultivation [4, 10]. Moreover, little genetic variation is evident in plasmids from different isolates [11]. REAC of *Y. enterocolitica* displays a higher level of discrimination than REAP, but in general very complex fingerprint patterns are found which hamper a reliable comparison [4, 10]. In combination with the use of rRNA gene-probes (ribotyping), fingerprint patterns can be simplified, but the technique is laborious, time-consuming and requires high quality DNA [14].

PCR-based fingerprinting can overcome these problems. Random amplified polymorphic DNA (RAPD)-fingerprinting is based on the random amplification of genomic DNA at low annealing temperatures, with a single primer of arbitrary nucleotide sequence [14] and has been used for the typing of different bacterial species [13]. Inter-repeat-PCR (IR-PCR) on the other hand, amplifies specific genomic regions known to be variable among different pro- and eucaryotic species, as for example *Escherichia coli*, *Salmonella typhimurium* but also *Candida albicans* [15, 16]. Both techniques result in PCR fingerprint patterns which can easily be visualized by agarose gel electrophoresis. Differences between fingerprints arise from the genetic diversity of the chromosomal DNA [13]. Fingerprint patterns are in general simple to interpret and high quality DNA is not required, although the resolution is often higher with purified DNA. Furthermore, PCR-based fingerprinting is a rapid, easy to use, method with high discriminatory power for some species [13]. PCR fingerprinting also discriminates between close relatives among a variety of pro- and eukaryotic species [13]. We investigated the ability of RAPD-PCR and IR-PCR to discriminate between clinical isolates of *Y. enterocolitica* and compared this with the standard biotyping and serotyping results.

METHODS

Yersinia enterocolitica isolates. A total of 48 feacal isolates from 42 patients, with enteritis were studied. All patients lived in the province of Friesland in The Netherlands and most of the patients were epidemiologically unrelated. Two successive isolates were obtained from 6 patients, 5 isolates were obtained from 2 families (2 isolates, from family number 1, and 3 isolates from family number 2). The strains were identified by the API 20E identification system (BioMérieux, Marcy l'Etoile, France) and stored in 50 % glycerol peptone broth at −80 °C until required.

Biotyping. A simplified form of the biotyping scheme described by Wauters and colleagues [5] was used. Only the six most discriminatory tests (lipase, esculine, salicine, indol, xylose and trehalose) were performed.

Table 1. *Primers used in RAPD-PCR and IR-PCR for* Yersinia enterocolitica

Primer	Nucleotide sequence	G+C%	Reference
	Primers IR-PCR		
ERIC1R	5′-ATGTAAGCTCCTGGGGATTCAC-3′	50	15
ERIC2	5′-AAGTAAGTGACTGGGGTGAGCG-3′	55	15
	Primers RAPD-PCR		
D8635	5′-GAGCGGCCAAAGGGAGCAGAC-3′	67	19
1247	5′-AAGAGCCCGT-3′	60	19
1254	5′-CCGCAGCCAA-3′	70	19
1281	5′-AACGCGCAAC-3′	60	19
1283	5′-GCGATCCCCA-3′	70	19
1290	5′-GTGGATGCGA-3′	60	19
RP1-4	5′-TAGGATCAGA-3′	40	23
RP2	5′-AAGGATCAGA-3′	40	23
Soy	5′-AGGTCACTGA-3′	50	24
HLWL85	5′-ACAACTGCTC-3′	50	25
HLWL74	5′-ACGTATCTGC-3′	40	25

Serotyping. Isolates were serotyped by slide agglutination using commercially available O-antisera for O:3 and O:9 (Sanofi Diagnostics Pasteur, Genk, Belgium) and specific rabbit antisera for O:5, 27, O:6, 30 and O:8 [3, 17].

DNA isolation. Isolates were grown overnight on blood agar at 37 °C. A single colony was then suspended in 250 μl STET-buffer (0·1 M-NaCl, 10 mM-Tris-HCl pH 8·0, 0·1 mM-EDTA, 0·5% Triton X-100) and 0·5 mg lysozyme (Sigma, St. Louis, USA). Samples were incubated for 5 min at room temperature, boiled for 45 sec and put on ice for 2 min. Sodium dodecyl sulphate at a final concentration of 1% and 100 μg Proteinase K (Boehringer, Mannheim, Germany) were added to the reaction-mixture and incubated for 2 h at 56 °C. Following phenol-chloroform extraction, 150 μg RNAase (Boehringer) was added to the DNA solution and the mixture was incubated for 1 h at 37 °C. DNA was precipitated with ethanol and the pellet was dissolved in 100 μl distilled water [18]. The concentration was determined using a Pharmacia GeneQuant RNA/DNA Calculator (Pharmacia LKB, Cambridge, UK).

PCR-based fingerprinting. 50 ng of bacterial DNA was used for PCR fingerprinting in a 50 μl reaction volume containing 75 mM-Tris-HCl (pH 9·0), 2·5 mM-$MgCl_2$, 20 mM $(NH_4)_2SO_4$, 0·01% Tween-20, 0·2 mM each dNTP, 50 pmol primer (see Table 1 for primer sequences) and 0·2 U Taq DNA polymerase (Thermoperfectplus DNA polymerase, Integro, Zaandam, The Netherlands). A negative control, consisting of the same reaction mixture but with no template DNA added, was included in each reaction. Amplification was started with an initial denaturation step of 4 min at 94 °C in a Perkin Elmer Cetus 9600 DNA Thermal cycler. This was followed by 40 cycles of denaturation, annealing and extension with 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C for RAPD-PCR for the 10-nucleotide primers. The cycling programme used with primer D8635 was 4 cycles of 94 °C for 5 min, 40 °C for 5 min, 72 °C for 5 min (low stringency amplification), followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min (high stringency amplification), and a final incubation at 72 °C for 10 min [19]. For IR-PCR a cycling programme of 35 cycles, consisting of 1 min at 94 °C, 1 min at 25 °C and 2 min at 72 °C. This was followed by a final extension step at 72 °C for 10 min.

A 20 μl aliquot of each reaction-product was analysed by electrophoresis in 1·5% agarose after which gels were stained with ethidium bromide and photographed using a UV light source. A selection of ten epidemiologically unrelated isolates of *Y. enterocolitica*, representing all biotypes, was initially analysed by RAPD-PCR analysis using 11 arbitrary primers and IR-PCR with two enterobacterial repetitive intergenic consensus (ERIC) primers (Table 1). Only RAPD-PCR primers 1290 and HLWL85 discriminated between the 10 indicator isolates and were therefore used for the genotypic characterization of all 48 isolates. The other primers showed either a very low resolution (i.e. ERIC1, RP1-4 and HLWL74) or patterns too complex (i.e. 1247 and 1254) to interpret [20]. Three repeated DNA isolations from the 10 indicator strains and successive PCR amplification, showed the same fingerprints. In the negative control no amplification was observed with any primer used. Interpretation of the fingerprints was based on visual inspection by three different observers and coded by letters. Closely related genotypes in which only minor-band differences were found were assigned the same letter and accompanied by a prime, for example C′ and C″.

RESULTS

RAPD-PCR using primer 1290 revealed 13 different fingerprints; the HLWL85 primer 14 different fingerprints. The combined patterns from the 2 primers discriminated 22 genotypes. Serotyping and biotyping distinguished 6 serotypes, and 5 biotypes (Table 2) respectively. Together 10 types could be differentiated. The correlation between serotypes O:3, O:9 and genotyping (Table 2) was weak. Most biotypes appeared to have a unique genotype; genotype C/J was associated with biotypes 1B, 2 and 4 and genotype G/J, with biotypes 1B and 2 (Table 2).

Fifteen isolates characterized as biotype 1A belonged to 10 different genotypes (Fig. 1*b*; Table 2). Biotypes 1B and 3 showed 2 different genotypes (Fig. 1*b*, 1*c*; Table 2). The four isolates of biotype 2 were discriminated by 3 different genotypes (Fig. 1*c*; Table 2), 18 isolates of biotype 4/serotype O:3 belonged to 5 different genotypes (Fig. 1*a*; Table 2).

From 3 isolates non-typable by biotyping, belonging to serotypes O:6, 30, O:8 and 0:9 respectively and 5 isolates non-typable by serotyping, belonging to biotypes 1A, 2 and 4 respectively, only 3 isolates showed genotypes which were seen for other isolates. The remaining 5 isolates showed genotypes not previously observed (Fig. 2; Table 2).

The two strains obtained from the members of family number 1 (strain 34 and 35) were isolated with a 3-months interval. They had different bio- and genotypes, and suggests two distinct strains (Table 2). The strains obtained from the family number 2 (strains 40, 41 and 42) had the same serotype, but differed in genotyping. Strains 40 and 41 had been isolated from patient ai 10 days apart and had different genotypes. Strain 42 from patient aj was isolated 2 months after the isolation of strain 41 from patient ai. Strains 40 and 42, had identical bio- and serotypes but differed in genotype. Strain 41 was not biotypable but had the same serotype as strains 40 and 42; the genotype of strain 41 was identical to that of strain 42 (Table 2), suggesting that these isolates were related.

Patients d, h, j, aa and ah each provided two strains after intervals of 1 year (strain 4 and 5), 9 days (strains 9 and 10), 2 months (strains 12 and 13), 3 months

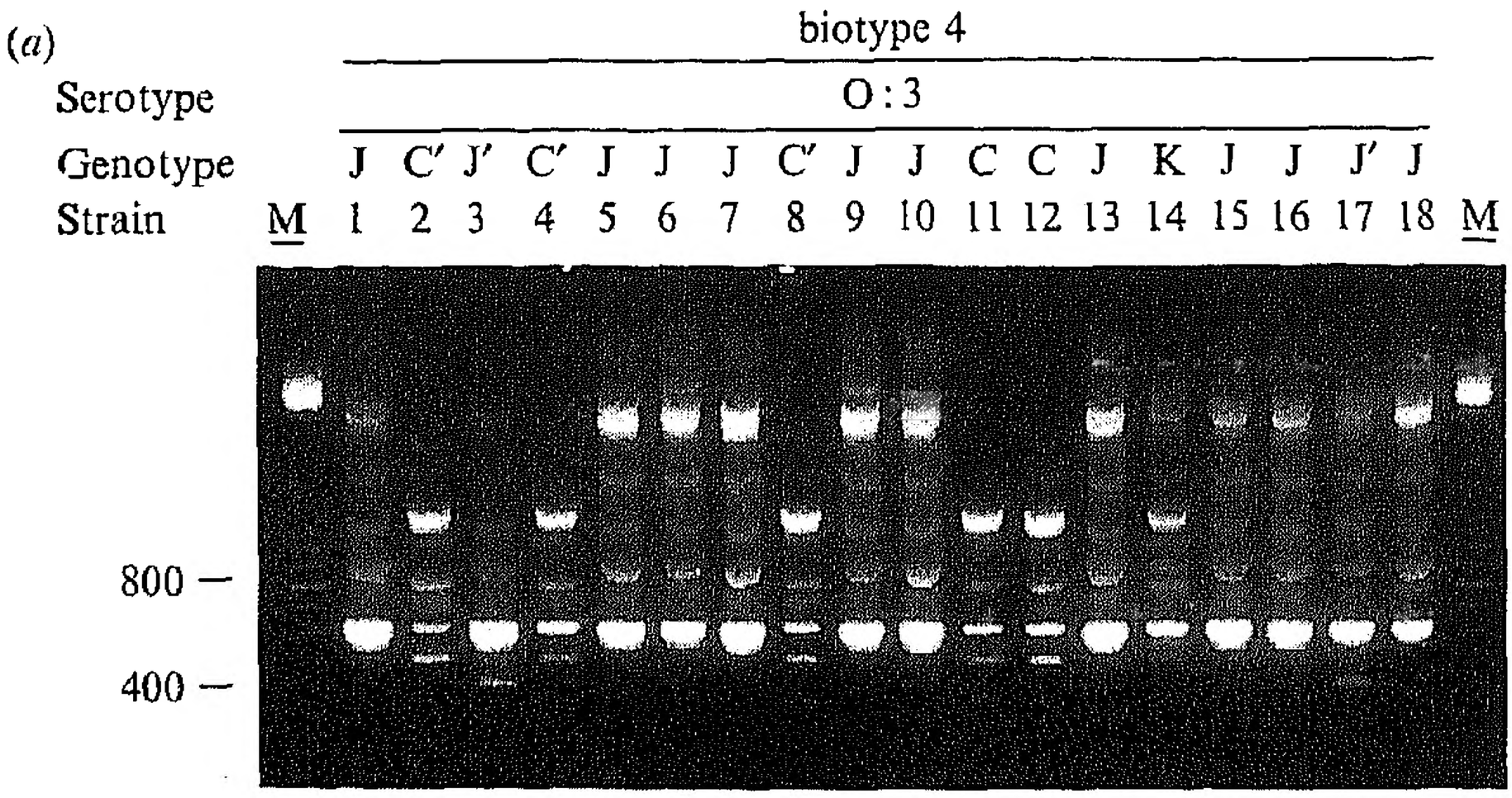
Table 2. *Characterization of* Yersinia enterocolitica *isolates*

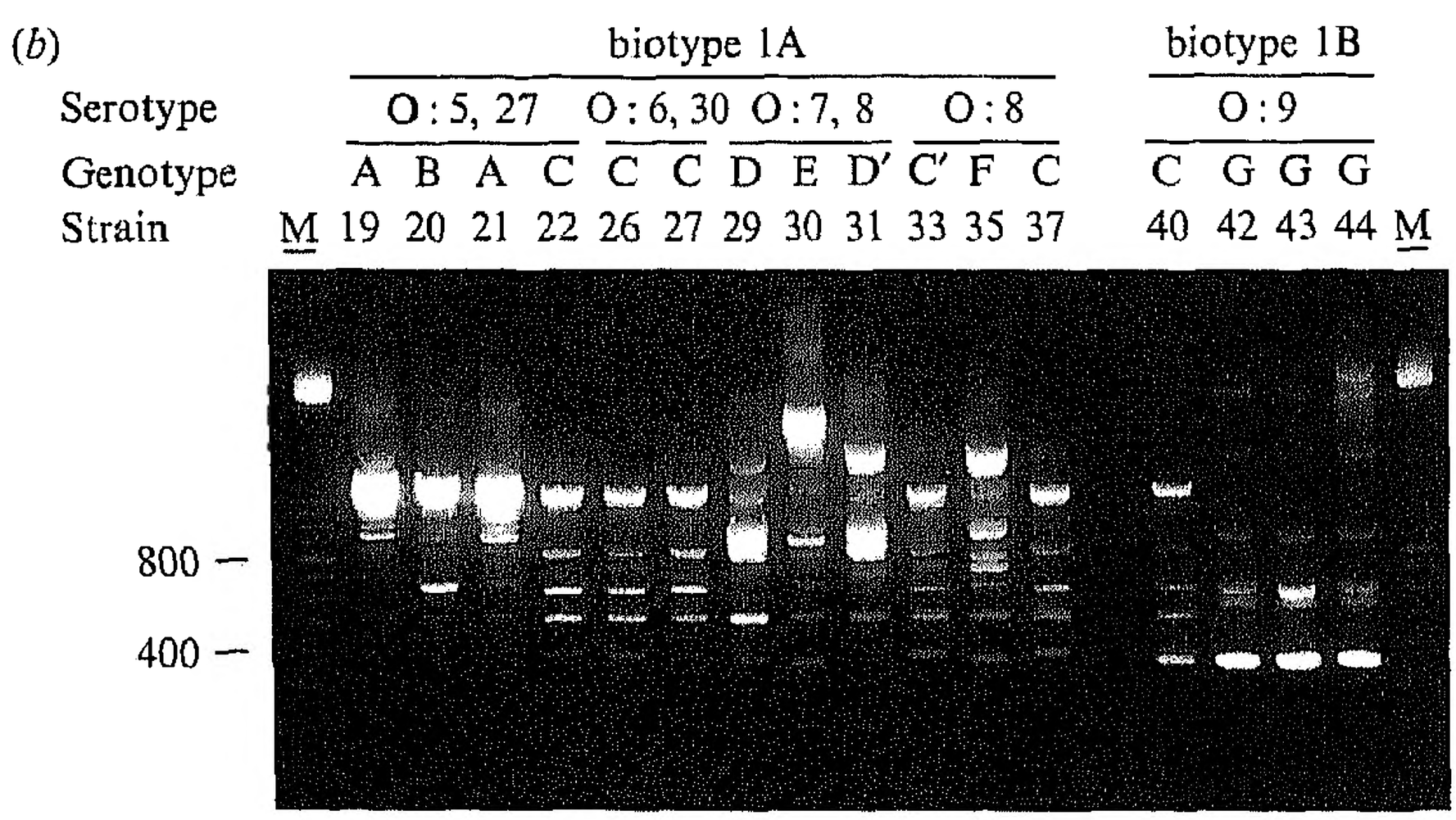
Patient*	Strains	Biotype	Serotype	RAPD† genotype
a	1	4	O:3	J/J
b	2	4	O:3	C′/J
c	3	4	O:3	J′/J
d	4	4	O:3	C′/J
	5	4	O:3	J/J
e	6	4	O:3	J/J
f	7	4	O:3	J/J′
g	8	4	O:3	C′/J
h	9	4	O:3	J/J
	10	4	O:3	J/J
i	11	4	O:3	C/J″
j	12	4	O:3	C/J
	13	4	O:3	J/J
k	14	4	O:3	K/J
l	15	4	O:3	J/J
m	16	4	O:3	J/J
n	17	4	O:3	J′/J
o	18	4	O:3	J/J
p	19	1A	O:5, 27	A/A
q	20	1A	O:5, 27	B/B
r	21	1A	O:5, 27	A/A
s	22	1A	O:5, 27	C/C
t	23	2	O:5, 27	H/J
u	24	2	O:5, 27	H/J
v	25	3	O:6, 30	C″/K
w	26	1A	O:6, 30	C/D
x	27	1A	O:6, 30	C/E
y	28	NT‡	O:6, 30	M/N
z	29	1A	O:7, 8	D/F
aa	30	1A	O:7, 8	E/G
	31	1A	O:7, 8	D′/H
ab	32	3	O:7, 8	I/L
ac	33	1A	O:8	C′/D
ad	34	4	NT	J/J
ae	35	1A	O:8	F/D
af	36	NT	O:8	F′/H
ag	37	1A	O:8	C/I
ah	38	2	O:9	C/J
	39	2	O:9	G/J
ai	40	1B	O:9	C/J
	41	NT	O:9	G/J
aj	42	1B	O:9	G/J
ak	43	1B	O:9	G/J
al	44	1B	O:9	G/J
am	45	1A	NT	L/M
an	46	1A	NT	B/H
ao	47	1A	NT	F/H′
ap	48	2	NT	G/J

* From patients d, h, j, aa, ah and ai two successive isolates were obtained. Patients ad + ae and ai + aj belong to the same family.

† Patterns obtained with primers 1290 and HLWL85, as shown in figures.

‡ NT, not bio- or serotypable.





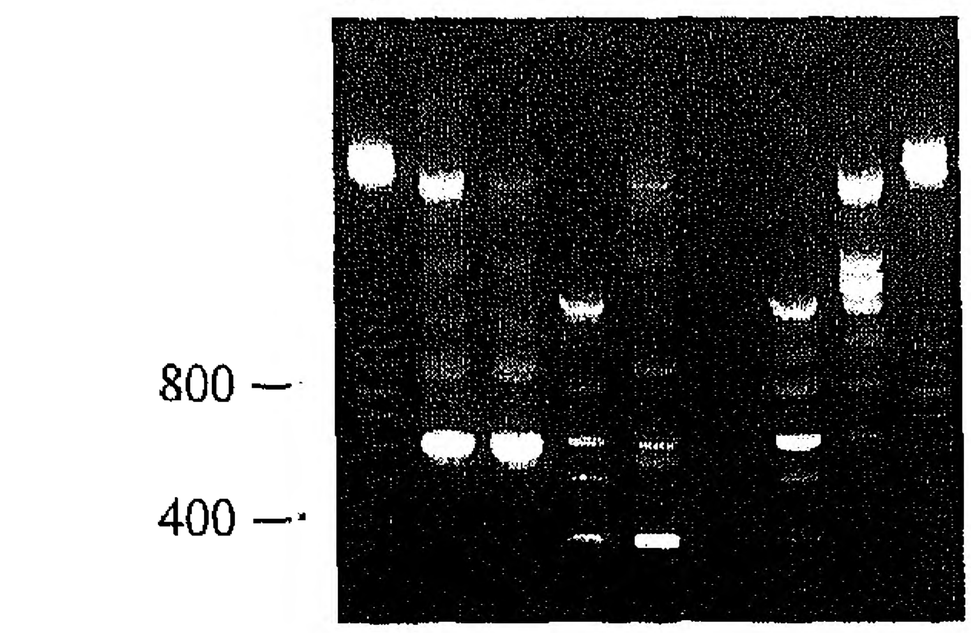


Fig. 1. RAPD analysis of *Y. enterocolitica* clinical isolates. (*a*) Isolates of biotype 4, (*b*) isolates of biotype 1A and 1B, (*c*) isolates of biotype 2 and 3. Genotypes are indicated as obtained using primer 1290. The letters and numbers above the lanes indicate the designated genotypes and isolate numbers respectively (see for details Table 2). M indicates the lane of the size marker (100 bp ladder).

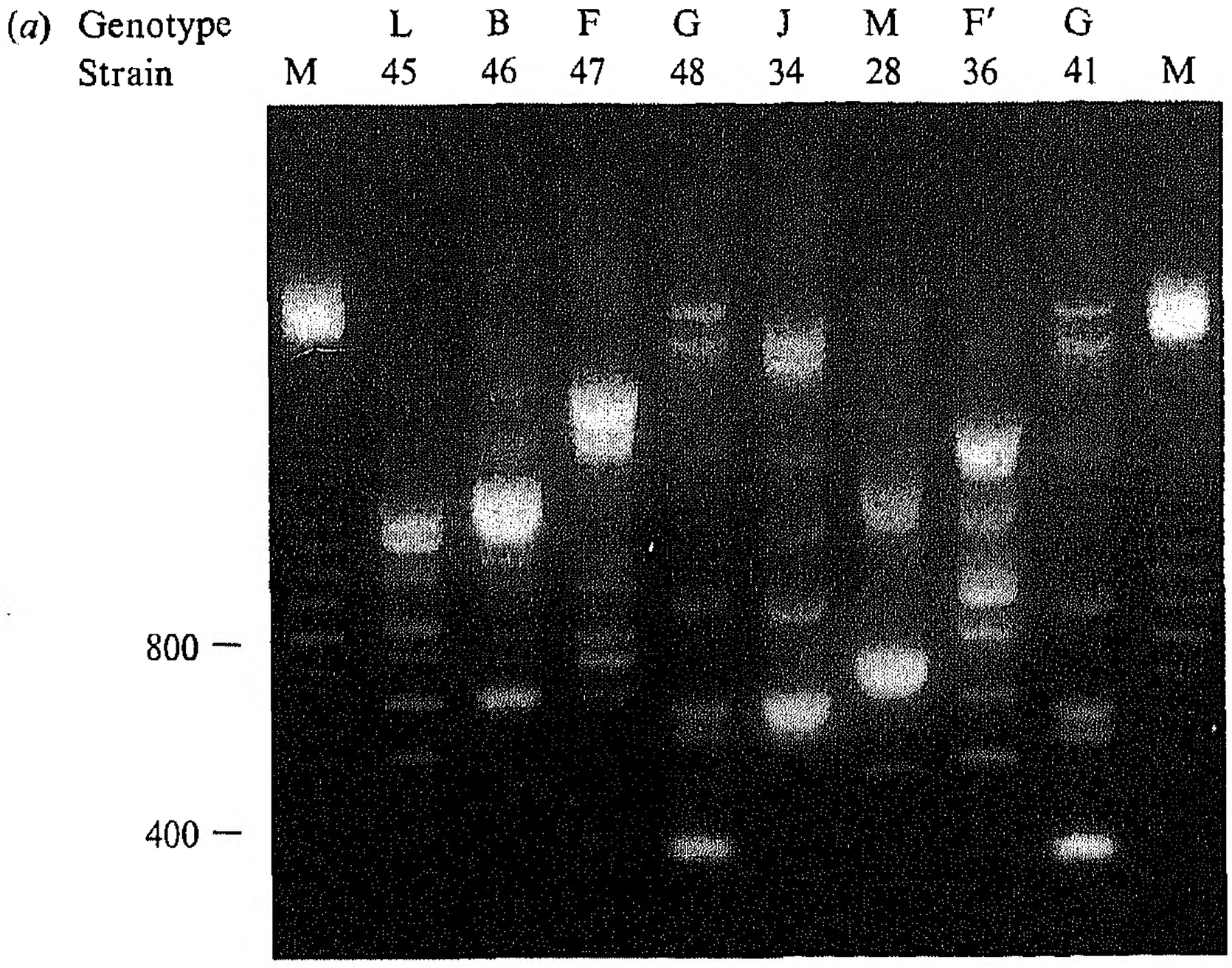


Fig. 2. RAPD analysis of *Y. enterocolitica* clinical isolates which could not be typed by serotyping or biotyping. Genotypes are indicated as obtained using primer 1290 (*a*) and primer HLWL85 (*b*). Other details as in Figure 1.

(strains 30 and 31) and 1 month (strains 38 and 39) respectively. Serotyping and biotyping showed no differences between the types, genotyping showed for all strains different genotypes, with the exception of strains 9 and 10 which had an identical genotype (Table 2).

DISCUSSION

The combination of PCR-fingerprints obtained with the primers 1290 and HLWL85 gave clear and distinct genotypes. Most biotypes were associated with one or more specific genotypes, the genotypes C/J and G/J were found in more than one biotype. The strains isolated from family one, showed beside different genotypes also different bio- and serotypes, which suggests that both members of this family were infected with a different strain of *Y. enterocolitica*. For family two,

all isolates had the same bio- and serotype, but two different genotypes were observed. Similar results were seen with the successive isolates from patients d, j, aa and ah. The finding that strains from one family belonged to different genotypes suggests that the family members were infected with different strains, although they had the same bio- and serotype. It is also possible that either a patient is colonized with several strains and that during colonization, one or other is selected or, minor genetic variation occurs *in vivo* perhaps as a result of the interaction between the host and the bacterium, as has been described for *Legionella* spp. [13]. This may also explain the observation that some successive isolates from single patients show variation. If these differences are a result of *in vivo* variation, then the discriminatory power of PCR-fingerprinting might be too high for epidemiological surveys. Alternatively *Y. enterocolitica* can be acquired by eating contaminated food or by other exogenous sources as surface water [1], leaving open that the feeding habits of patients can play an important role in acquiring *Y. enterocolitica* and equally with this the possibility of colonization by more than one strain and/or successive infection grows. Further work is needed to clarify these possibilities.

One may also conclude from our results that it is possible to recognize unrelated strains within a given serotype or biotype and may therefore yet be helpful in epidemiological studies. Also Makino and colleagues [21] described the genetic characterization of *Y. pseudotuberculosis* by PCR-fingerprinting, and supports the view that PCR-fingerprinting can be useful in epidemiological surveys of *Yersiniae* sp. in general.

PCR-based DNA fingerprinting has been shown to be useful in transmission studies of different infectious agents in human populations [13, 16, 22]. In this study, mainly epidemiologically unrelated *Y. enterocolitica* isolates were examined, but it has been shown that PCR-based DNA fingerprinting can be used to characterize isolates of *Y. enterocolitica*.

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