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Evaluation of pulsed-field gel electrophoresis of genomic restriction fragments in the discrimination of *Yersinia enterocolitica* O:3

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SUMMARY

One hundred and six *Yersinia enterocolitica* serogroup O:3, biotype 4 isolated from human and porcine samples in 1984 and in the years 1993–5 were examined by pulsed-field gel electrophoresis (PFGE). The genomic profiles produced by the enzymes *Not*I and *Xba*I were studied. Sixteen (A–P) and 8 (1–8) different pulsotypes were obtained, respectively. By combining the pulsotypes produced by both *Not*I and *Xba*I 24 different types were distinguished. The two major types, designated as A1 and B1, comprised 36% of all strains tested. The proportions of pulsotypes A1 and B1 were, 35·9 and 25·6%, respectively, among strains isolated in 1984. The corresponding figures among the strains isolated in 1993–5 were 35·8 and 41·8%. Nine pulsotypes were found only in 1984 and nine only in 1993–5. The proportions of the major pulsotypes, A1 and B1, in human isolates were 42·9 and 35·7% and in porcine isolates 22·2 and 36·1% respectively. Six types were found among both human and porcine isolates, 8 only among human strains and 10 only among porcine strains.

INTRODUCTION

Yersinia enterocolitica is one of the three species of the genus *Yersinia* which are unquestionably recognized as human pathogens [1]. The other two species are *Y. pseudotuberculosis* and *Y. pestis*. All three pathogenic *Yersinia* species harbour a 70–75 kb virulence plasmid (pYV) which is considered a prerequisite, but not in itself sufficient for expression of pathogenicity [1–5].

The clinical features of *Y. enterocolitica* infections are diarrhoea, low grade fever and abdominal pain which can be confused with appendicitis. The post-infectious manifestations include reactive arthritis and erythema nodosum that occurs within a few weeks of the acute phase. The former complication is associated with the presence of HLA-B27 histocompability antigen [6–8] and the latter occurs most commonly in women. Neither of these complications is common among children [9]. Transfusion-related *Y. enterocolitica* septicaemia, some of them fatal, have also been reported [10–13].

Y. enterocolitica is a heterogenous group of strains, which are traditionally classified by biotyping into 6 biogroups [14], 5 of which (1B and 2 through 5) are regarded as pathogens, and by serotyping into more than 57 O groups [15]. Only a few of these types have been associated with disease in either humans or animals. Strains that belong to serogroups O:3 (biogroup 4), O:5,27 (biogroups 2 and 3), O:8 (biogroup 1B) and O:9 (biogroup 2) are worldwide most frequently isolated from human samples [16–19]. The most important *Y. enterocolitica* serogroup in many European countries is the serogroup O:3 [19–22]. In Finland about 90% of all isolated *Y. enterocolitica* strains belong to serogroup O:3, the incidence of *Y. enterocolitica* infections reported being 16·6 cases/100000 population/year [23].

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The only known source of these bacteria are pigs. They can frequently be isolated from the tonsils and oral cavity of pigs at the time of slaughter and to a lesser extent from stool samples [24–27]. Epidemiological studies have shown that pigs are a significant reservoir of *Y. enterocolitica* serogroup O:3 for infections in man [17, 28, 29]. New sensitive methods such as DNA–DNA hybridization have revealed a high contamination level of pork products by pathogenic *Y. enterocolitica* [30], which supports the apparent role of pork and pork products as a source of human infections.

Recently, pulsed-field gel electrophoresis (PFGE) combined with the use of restriction of the bacterial genome by endonucleases with rare cutting sites has been demonstrated to be an efficient tool for analysis of the molecular epidemiology of *Y. enterocolitica* [31–34]. The purpose of the present study was to evaluate the efficiency of PFGE to differentiate the most frequently isolated *Y. enterocolitica* bioserogroup 4/O:3. The aim was also to compare the genomic profiles of human and porcine isolates and to produce more information about the transmission of strains. We also studied changed in the genomic profiles and antibiograms that had occurred during the past decade.

MATERIALS AND METHODS

Strains

Strains used in this work were taken randomly from the collections of the Laboratory of Enteric Pathogens, the National Public Health Institute, Helsinki, Finland and the Department of Food Microbiology, the National Veterinary and Food Research Institute, Helsinki, Finland. A total of 106 *Y. enterocolitica* isolates from human stool specimens, swine tongue and tonsils were examined. Of the 39 strains isolated in 1984 and 67 isolated during the years 1993–5, 16 and 20 were of porcine and 23 and 47 of human origin, respectively. All strains were serogrouped by slide agglutination (Denka Seiken, Tokyo, Japan) and biogrouped by the revised biotyping scheme [14]. All strains were bioserogroup 4/O:3.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was studied by the disk diffusion technique on a semisynthetic Iso-Sensitest medium using the zone size criteria standardized for the *Enterobacteriaceae* by the disk manufacturer and on the basis of break-points as used by the Swedish Reference Group for Antibiotics [35]. A range of 14 antimicrobial agents (Rosco, Taastrup, Denmark) was tested: ampicillin (33 μg), amoxycillin/clavulanate (30/15 μg), cefotaxime (30 μg), ceftriaxone (30 μg), chloramphenicol (60 μg), ciprofloxacin (10 μg), impipenem (15 μg), mecillinam (33 μg), nalidixic acid (130 μg), neomycin (120 μg), streptomycin (100 μg), sulphonamide (240 μg), tetracycline (80 μg) and trimethoprim (5·2 μg).

Pulsed-field gel electrophoresis (PFGE)

For PFGE, bacterial cells grown for 20 h in Brain Heart Infusion broth (BHI, 0037-01-6, Difco Laboratories, Detroit, MI, USA) at 26 °C were washed twice with TEN buffer (100 mM Tris-HCl, 150 mM NaCl, 100 mM EDTA, pH 7·5) and then resuspended to 3 ml TEN buffer. Of this suspension 0·5 ml was mixed with an equal volume of low melting point agarose (FMC BioProducts, Rockland, ME, USA) and 20 μl RNAse (500 μl/ml, Boehringer–Mannheim GmbH, Germany) and solidified in moulds on ice. After solidification the agarose plugs were transferred to 3 ml EC buffer (6 mM Tris-HCl, 100 mM EDTA, 1 M NaCl, 0·5% Brij 58, 0·2% Na-deoxycholate, 0·5% lauroyl-sarcosine, all w/v). The buffer was supplemented by 50 μl lyzozyme (20 mg/ml, Boehringer–Mannheim GmbH, Germany). After overnight incubation at 37 °C the EC buffer was replaced with 3 ml of ES buffer (0·5 M EDTA, 1% lauroyl-sarcosine) and 30 μl proteinase K (15·5 mg/ml, Boehringer–Mannheim GmbH, Germany) and then incubated overnight at 60 °C. The buffer supplemented with proteinase K was changed once during the incubation. After 20 h the buffer was removed and the plugs were washed 10 times, for 30–40 min each, in 3–5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA). The plugs were stored in TE buffer at 4 °C until examined.

The strains in this study were not analyzed for a virulence plasmid. Some previous studies have shown that the plasmid does not interfere with chromosomal DNA fragments when using the restriction enzymes *Not*I and *Xba*I. By using *Not*I as restriction enzyme the fragments of the plasmid were detected at 3·5, 25 and 48·5 kb [32]. *Xba*I has been found to reveal three different cutting sites in pYV and produces 5, 12 and 50 kb fragments [36], which were not in the range of

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60–300 kb used for discrimination of strains tested in this study.

For restriction endonuclease digestion the plugs were first cut into one sixth of the whole agarose plug and then equilibrated for 2 h in 100 μl of *Not*I and *Xba*I restriction buffer (Boehringer–Mannheim GmbH, Germany). DNA was digested in 100 μl of the same buffer containing either 10 U of one of the endonucleases *Not*I or *Xba*I for 22 h at 37 °C. PFGE was performed with a Gene Navigator™ system (Pharmacia LKB, Germany). Gels containing 1% (w/v) agarose (Sigma) were run at 200 V in 0·5 × TBE at 8 °C. Switching times from 8 to 23 s were employed for 26 h to fractionate plugs digested by *Not*I and from 5 to 20 s for 22 h to fractionate plugs digested by *Xba*I. Lambda concatamers (BioLabs, Beverly, MA, USA) were used as DNA size standards. The gels were stained with ethidium bromide and visualized under short wave uv-light.

RESULTS

Genomic fingerprints

A total of 106 *Y. enterocolitica* strains of bioserogroup 4/O:3 isolated in 1984 and in 1993–5 from humans and swine were investigated by restriction fragment length polymorphism analysis of DNA. The enzymes used in this study were chosen on the basis of some previous studies [31–33, 36] and preliminary testing, in which both the enzymes *Not*I and *Xba*I were found to be suitable for discriminating *Y. enterocolitica* strains. Various pulse times and run times were also tested in order to obtain the best possible resolution of the fragments. Pulse ramping from 8 to 23 s for 26 h as described earlier [33] was found to be most discriminatory for *Not*I. Pulse ramping from 5 to 20 s for 22 h was chosen for *Xba*I, although pulse times of 10 and 7 s, both for 22 h, were found to be even more discriminatory than the chosen protocol, but were discarded because of strong distortion of the bands (data not shown).

Restriction fragments > 100 kb (12–14 bands) were clearly resolved and were used for comparison of banding patterns and discrimination of strains. The isolates were considered distinguishable when the number of bands was not the same and/or when they were not of the same apparent size. The DNA restriction fragmentation patterns of *Y. enterocolitica* produced by *Not*I fall into 16 PFGE types designated from A to P (Table 1, Fig. 1) and those produced by *Xba*I into 8 PFGE types designated from 1 to 8 (Table 1, Fig. 2).

The pulsotypes designated A and B generated by *Not*I were the main types, their percentages being 39·6 and 40·6%, respectively. The predominance of the pulsotype 1 generated by *Xba*I was obvious in strains isolated both in 1984 and in 1993, the percentage of these types being 84·6 and 91·0%, respectively.

To compare the difference of the DNA patterns of the isolates tested and to assess the possible genetic events causing the difference, the patterns A and 1 were taken as basic patterns produced with *Not*I and *Xba*I, respectively. The patterns B–P (*Not*I) differ from those of the A strains by 2–6 bands and the patterns 2–6 (*Xba*I) from the strain designated 1 by 1–4 bands.

Distribution of combined pulsotypes

A total of 24 different types were recognized by combining the results generated by both *Not*I and *Xba*I. Types A, B, I and O pulsotypes could be further subdivided by *Xba*I into 5, 3, 2 and 2 subtypes, respectively.

Altogether 70 human and 36 porcine isolates were examined by PFGE, which revealed 14 and 16 different pulsotypes, respectively. Of these pulsotypes only 6 (A1, B1, B2, E1 and I1 and P1) were detected in both human and porcine strains (Fig. 3). The other 18 pulsotypes were detected either only among human (8 types) or among porcine (10 types) strains (Fig. 3). There was a distinct difference in the proportions of the major group A1 between the human and porcine strains examined, the proportions being 42·9 and 22·2%, respectively (Fig. 3). The difference was distinct both in strains isolated in 1984 and in 1993–5. No difference in the proportions of the pulsotype B1 was seen. The combined proportion of the major pulsotypes, A1 and B1, was higher among the human strains (78·6%) than the porcine strains (58·3%).

No major change was observed in one of the main pulsotypes, A1, when comparing the proportions of this pulsotype among strains isolated in 1984 and in 1993–5. The total percentages of the group A1 were 35·8% in the two periods (Fig. 4). There was a clear difference in the proportions of the other major group, B1, in 1984 and in 1993–5. The proportions of this pulsotype were 25·6% among the strains isolated in 1984 and 41·8% in 1993–5 (Fig. 4). Altogether six pulsotypes were found both among strains isolated in 1984 and in those isolated in 1993–5. They were A1,

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Table 1. *Distribution of different Not*I *and Xba*I *PFGE types among human and porcine strains in two different periods*

Enzyme type		Year 1984			Years 1993–5			
		Human ($n = 23$)	Porcine ($n = 16$)	Total ($n = 39$)	Human ($n = 47$)	Porcine ($n = 20$)	Total ($n = 67$)	Total ($n = 106$)
*Not*I	A	12	5	17	21	4	25	42
	B	6	7	13	22	8	30	43
	C	0	0	0	0	1	1	1
	D	0	0	0	1	0	1	1
	E	1	0	1	0	1	1	2
	F	1	0	1	1	0	1	1
	G	1	0	1	0	0	0	1
	H	0	0	0	0	1	1	1
	I	1	0	1	1	1	2	3
	J	0	0	0	0	1	1	1
	K	1	0	1	0	0	0	1
	L	0	2	2	0	0	0	2
	M	0	1	1	0	0	0	1
	N	0	1	1	0	0	0	1
	O	0	0	0	0	2	2	2
	P	0	0	0	1	1	2	2
*Xba*I	1	20	13	33	43	18	61	94
	2	1	2	3	2	0	2	5
	3	0	1	1	0	0	0	1
	4	0	0	0	1	0	1	1
	5	1	0	1	0	0	0	1
	6	0	0	0	0	1	1	1
	7	1	0	1	0	0	0	1
	8	0	0	0	1	1	2	2

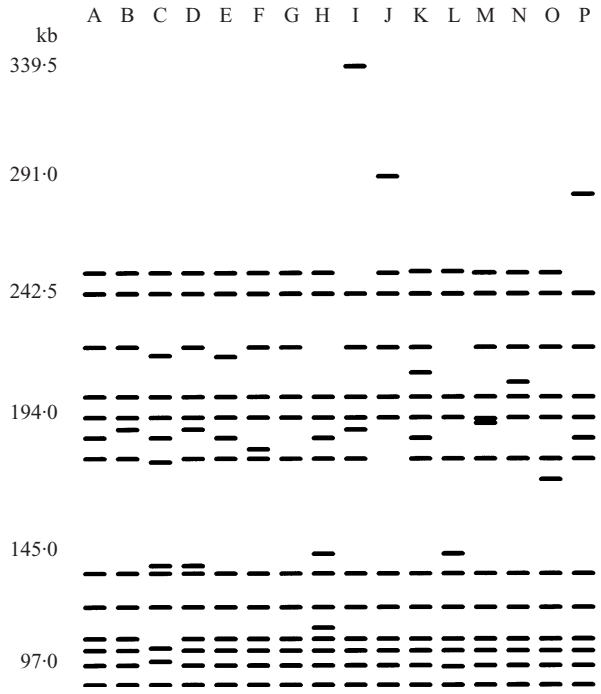

Fig. 1. Diagram of 16 *Not*I PFGE patterns of *Y. enterocolitica* 4/O:3.

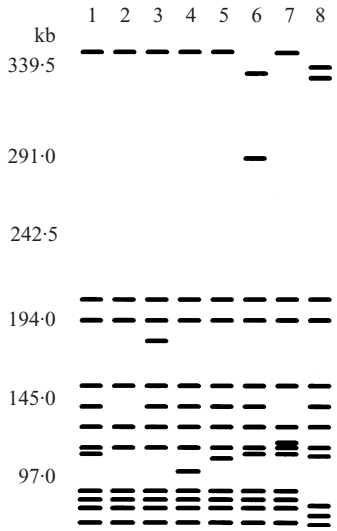

Fig. 2. Diagram of six *Xba*I PFGE patterns of *Y. enterocolitica* 4/O:3.

B1, B2, E1, F1 and I1 (Fig. 4). Eighteen pulsotypes were detected only either among strains isolated in 1984 (9 pulsotypes) or only in 1993–95 (9 pulsotypes).

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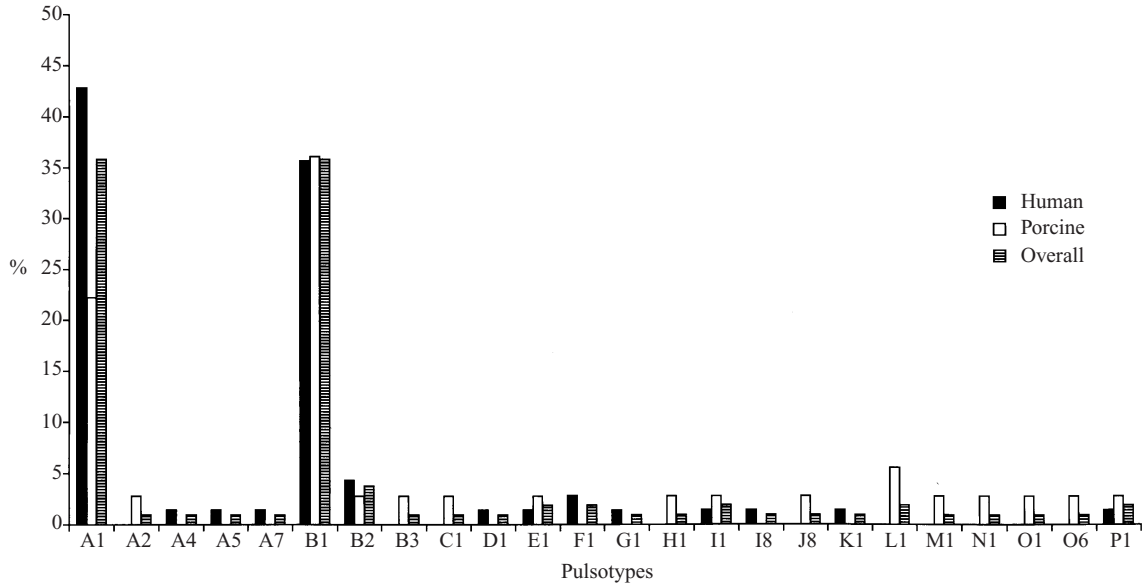

Fig. 3. Distribution of different PFGE types among human and porcine strains.

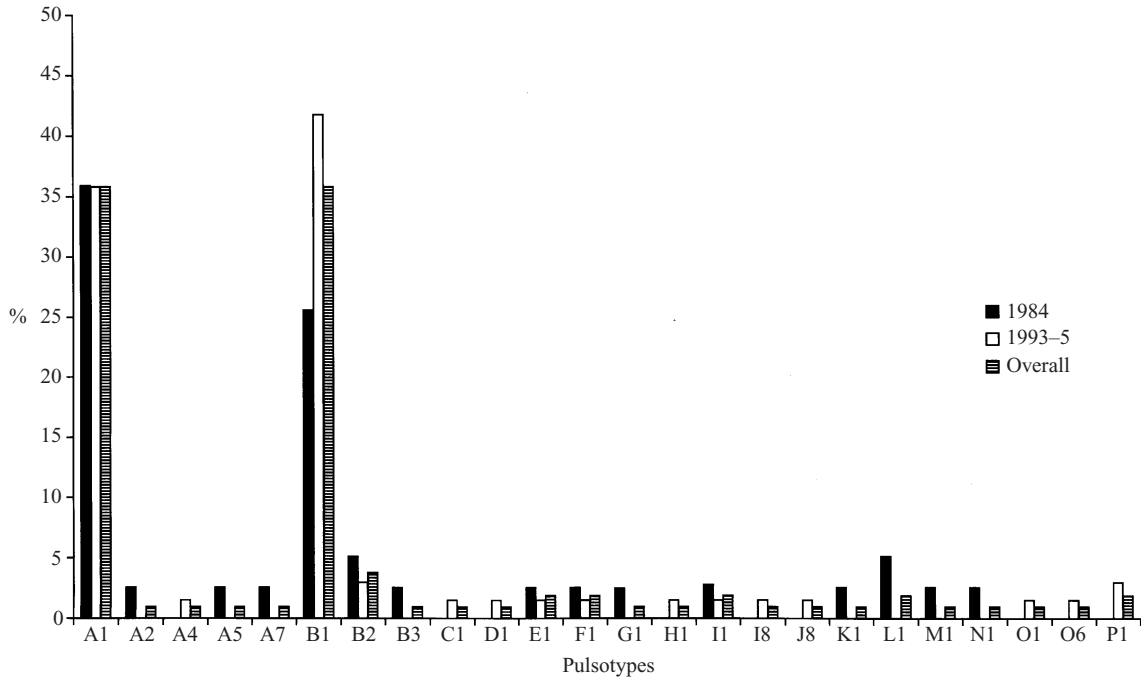

Fig. 4. Distribution of different PFGE types in the years 1984 and 1993–5.

The proportions of these groups were 27·8 and 14·9 %, respectively. Overall, these groups comprised 18·8 % of all the strains tested.

Antibiograms

All strains tested were susceptible to all the antimicrobial agents except to the combination of amoxycillin/clavulanate and ampicillin.

DISCUSSION

Typing of *Y. enterocolitica* strains is usually based on biochemical and serological characteristics. Recently, molecular methods have also been used to enable more precise typing of these strains [20, 31–34, 37–39]. When comparing ribotyping, restriction enzyme analysis of the virulence plasmid (REAP) and PFGE, the latter has been proved to be the most suitable of all these three techniques as an epidemiological tool

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[34]. The pulsotypes of *Y. enterocolitica* has also been proved to be relative stable *in vitro* [32].

In the present study, 106 *Y. enterocolitica* 4/O:3 isolates were examined by PFGE using two restriction enzymes, *Not*I and *Xba*I, in the digestion of DNA of the strains. The material in our study is one of the largest in which PFGE was used in differentiating *Y. enterocolitica* O:3 isolates. This large set of isolates allowed us to evaluate the genomic changes in this *Y. enterocolitica* serogroup that might have occurred during the past decade, as well to compare human and porcine isolates. The combined use of both *Not*I and *Xba*I of these restriction enzymes improves the discriminatory power of PFGE, which is useful in epidemiological surveys and very important when examining possible outbreaks. If the method had relied on pulsotypes generated by a single restriction enzyme, the two major pulsotypes would have accounted for more than 80% (types A and B by *Not*I) or one major type would have represented 88·7% (type 1 by *Xba*I) of all the 106 strains tested. However, care should be taken when estimating whether a strain is part of a possible outbreak, especially if it does belong to either of the major pulsotypes, A1 or B1. The clear predominance of these two major pulsotypes may lead to incorrect conclusions. Also the suitability of the restriction enzymes *Sst*I and *Nhe*I [33] and *Spe*I [32] in PFGE of *Y. enterocolitica* have been tested. They have been found to be unsuitable for discriminating *Y. enterocolitica* by PFGE.

The pulsotype designated as A seems to be the same as the pulsotype 4^{A} in an earlier study [32], in which it was found to comprise as much as half of the 20 *Y. enterocolitica* O:3 strains tested. The pulsotype B differed from the pulsotype A by having one band which is slightly larger. If the pulsotype A is considered as a basic type, the possible genetic events which might have altered the PFGE pattern of A can be evaluated. According to guidelines [40] pulsotypes B, D–H, J–K and N–P can be considered as closely related to the PFGE type A, whereas pulsotypes I, L and M are possibly related and the PFGE type C is different from type of A. When comparing the pulsotypes produced by *Xba*I, pulsotypes 1–7 can be considered as closely related, but pulsotype 8 to be different from all other 7 pulsotypes. Other studies carried out [10, 22, 31, 33] also showed a high degree of similarity within *Y. enterocolitica*. On the basis of the *Xba*I pulsotypes the relationships between the strains could not be established.

The pulsotypes designated A1 and B1 predominated both in strains isolated in 1984 and in those isolated in 1993–5. No major changes in the proportion of pulsotype A1 was observed during this period, but the proportion of the pulsotype B1 increased. Six PFGE patterns were found in strains which were isolated in 1993–5 compared to those isolated in 1984 and six PFGE patterns found in 1984 were not observed in strains from in 1993–5. This finding is in accordance with an earlier study, in which the emergence of new clones was concluded to be rather frequent [32]. Despite the appearance of new clones and the disappearance of others in our material the proportion of the non-A non-B isolates did not appear to change during the past decade, but the A and B isolates maintained their predominance, which can be considered as a rather interesting phenomenon. Although the total number of non-A and non-B strains was rather high in our material, they were too widely distributed to allow any conclusions concerning the possible shift in PFGE patterns.

All the strains of *Y. enterocolitica* were found to be susceptible to all the antimicrobial agents tested except ampicillin and the combination amoxycillin/clavulanate. We did not observe any differences between the human and porcine strains. Our results are in accordance with those of some previous studies with the exception of the combination of amoxycillin and clavunic acid. The majority (73·7%) of *Y. enterocolitica* O:3 strains isolated in Canada were found to be susceptible to a combination of amoxycillin and clavunic acid. Exceptionally high susceptibility has been found to the combination amoxycillin and clavulanate in strains isolated in Australia, New Zealand and Canada. However, strains isolate in Europe, Asia and Brazil were all resistant to this combination [42].

In conclusion, PFGE is an efficient tool for differentiating strains of *Y. enterocolitica* serogroup O:3. However, the great majority of the strains fell into two groups. It is therefore necessary to seek more discriminating enzymes. This may be particularly relevant when testing isolates from putative outbreaks. In addition, the reservoir of the types found in human infections but not in porcine samples needs to be traced.

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