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Molecular typing and virulence markers of *Yersinia enterocolitica* strains from human, animal and food origins isolated between 1968 and 2000 in Brazil

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Molecular typing and virulence markers were used to evaluate the genetic profiles and virulence potential of 106 *Yersinia enterocolitica* strains. Of these strains, 71 were bio-serotype 4/O : 3, isolated from human and animal clinical material, and 35 were of biotype 1A or 2 and of diverse serotypes, isolated from food in Brazil between 1968 and 2000. Drug resistance was also investigated. All the strains were resistant to three or more drugs. The isolates showed a virulence-related phenotype in the aesculin, pyrazinamidase and salicin tests, except for the food isolates, only two of which were positive for these tests. For the other phenotypic virulence determinants (autoagglutination, Ca^{++} dependence and Congo red absorption), the strains showed a diverse behaviour. The *inv*, *ail* and *yst*A genes were detected in all human and animal strains, while all the food isolates were positive for *inv*, and 3 % of them positive for *ail* and *yst*A. The presence of *vir*F was variable in the three groups of strains. The strains were better discriminated by PFGE than by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR). A higher genomic similarity was observed among the 4/O : 3 strains, isolated from human and animal isolates, than among the food strains, with the exception of two food strains possessing the virulence genes and grouped close to the 4/O : 3 strains by ERIC-PCR. Unusually, the results revealed the virulence potential of a bio-serotype 1A/O : 10 strain, suggesting that food contaminated with *Y. enterocolitica* biotype 1A may cause infection. This also suggests that ERIC-PCR may be used as a tool to reveal clues about the virulence potential of *Y. enterocolitica* strains. Furthermore, the results also support the hypothesis that animals may act as reservoirs of *Y. enterocolitica* for human infections in Brazil, an epidemiological aspect that has not been investigated in this country, confirming data from other parts of the world.

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INTRODUCTION

Yersinia enterocolitica is the most prevalent *Yersinia* species connected to disease in humans (Bottone, 1999; Robins-Browne, 2001). It has also frequently been isolated from animals, food and the environment (Fredriksson-Ahomaa *et al.*, 1999; Thoerner *et al.*, 2003; Falcão *et al.*, 2004).

Y. enterocolitica is responsible for gastroenteritis and other syndromes in humans and animals (Bottone, 1999). Human clinical infections with *Y. enterocolitica* most frequently occur after ingestion of food and/or contaminated water (Robins-Browne, 2001; Salyers & Whitt, 2002).

Y. enterocolitica strains have traditionally been classified by a well-established typing system, based on a combination of

Abbreviations: AMK, amikacin; AMP, ampicillin; CEF, cephalothin; CFZ, cephazolin; EGT, ERIC genomic type; ERIC-PCR, enterobacterial repetitive intergenic consensus PCR; FOX, cefoxitin; PGT, pulsed-field genomic type.

biochemical and serological tests that enable the differentiation of the strains into diverse bio-serotypes (Aleksic & Bockemühl, 1999). Also, pathogenic strains are related to some phenotypic characteristics, such as pyrazinamidase production, aesculin hydrolysis, salicin fermentation, calcium dependence, autoagglutination and Congo red absorption (Bottone, 1999). Molecular genetic studies have emphasized the importance of a virulence plasmid (pYV) that encodes various virulence genes, among them *vir*F, which is an important transcriptional regulator of other plasmid genes, as well as the role of chromosomal virulence genes that mediate cell invasion (*inv* and *ail*) and produce a thermostable enterotoxin (*yst*A), among others (Carniel, 1995; Cornelis *et al.*, 1998).

Molecular methods have also been used for typing and in epidemiological studies of *Y. enterocolitica*. The molecular methods most frequently used include restriction analysis of both plasmids (Nesbakken *et al.*, 1987; Kapperund *et al.*, 1990) and chromosomes (Blumberg *et al.*, 1991), randomly amplified polymorphic DNA analysis (Rasmussen *et al.*, 1994), ribotyping (Andersen & Saunders, 1990; Lobato *et al.*, 1998) and PFGE (Najdenski *et al.*, 1994; Saken *et al.*, 1994; Iteman *et al.*, 1996; Fredriksson-Ahomaa *et al.*, 2004). The enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) technique has not been used extensively, and its real potential as a tool for typing and in epidemiological studies of *Y. enterocolitica* has yet to be recognized (Sachdeva & Virdi, 2004; Wojciech *et al.*, 2004).

Y. enterocolitica is not often isolated and studied in Brazil, making it difficult to establish the involvement of this bacterium as a causative agent of human and animal diseases in this country, or to estimate the impact of its presence in food. In particular, to our knowledge, there are no data about the virulence potential of biotype 1A strains isolated from food in Brazil, except for two studies that investigated the production of the heat-stable enterotoxin (ST) by *Yersinia* strains isolated from samples of meat and milk (Tibana *et al.*, 1987; Warnken *et al.*, 1987). Only environmental strains of this biotype have been analysed for the presence of virulence markers (Falcão *et al.*, 2004). In order to answer some of the questions, the aim of the present study was to characterize some phenotypic and genotypic virulence markers of *Y. enterocolitica*, isolated from human patients, animals and foodstuffs in Brazil, and to assess the level of genomic similarity and possible relatedness among these strains by PFGE and ERIC-PCR. All of these data have been brought together to elucidate more thoroughly the epidemiology of *Y. enterocolitica* in Brazil, where its importance as an enteropathogen has been neglected.

METHODS

Bacterial strains. A total of 106 strains of *Y. enterocolitica*, isolated between 1968 and 2000 from human patients (37 strains), animals (34 strains) and food (35 strains), from different regions, states and cities of Brazil, were analysed in this study. They were selected from the collection of the *Yersinia* Reference Laboratory in Brazil (School of Pharmaceutical Sciences, UNESP, Araraquara, São Paulo State). It is important to point out that these strains had already been isolated when received at the Reference Laboratory for further confirmation and typing.

The strains were systematically chosen to represent the range of types of the collection. This was done by selecting the most prevalent bio-serotypes from each of the sources studied, and ensuring that all strains chosen had been isolated from different sources.

The 37 human strains, obtained from different patients, were all isolated from diarrhoeic faeces, the 34 animal strains (from different animals) from pig faeces (24 strains), dog faeces (three strains), rat faeces (one strain), bull faeces (one strain) and marmoset hepatic abscess (five strains). The remaining 35 strains were isolated from various types of food, such as raw and pasteurized milk, pork sausage, pork meat, chicken, bull's liver, ice, beef and lettuce. These 106 strains were isolated by investigators of various institutions located in different cities, states and regions of Brazil: 76 strains from the Southeast Region, comprising São Paulo State (28 strains from the capital, São Paulo, three from Botucatu, four from Campinas, 14 from Araraquara, seven from Ribeirão Preto and one strain from São José do Rio Preto) and Rio de Janeiro State (19 strains from the capital, Rio de Janeiro); 25 strains from the South Region, from Rio Grande do Sul State (21 strains from Porto Alegre) and Paraná State (two strains from Curitiba and two from Londrina); and five strains from the Northeast Region, from Pernambuco State (from the capital, Recife).

Bio-sero-phagetype classification. The isolates were all assigned to biotypes as recommended in Aleksic & Bockemühl (1999). They were serotyped according to Wauters *et al.* (1991) and phage typed according to Nicolle *et al.* (1976).

Drug resistance. All the isolates were tested for drug resistance by disc diffusion, following the National Commitee for Clinical Laboratory Standards (2001) guidelines. The following antimicrobial drugs and quantities were used: ampicillin (AMP),10 μg; cephalothin (CEF), 30 μg; cefotaxime (CTX), 30 μg; cefoxitin (FOX), 30 μg; cephazolin (CFZ), 30 μg; amikacin (AMK), 30 μg; gentamicin (GEN), 10 μg; kanamycin (KAN), 30 μg; streptomycin (STR), 10 μg; tobramycin (TOB), 10 μg; nalidixic acid (NAL), 30 μg; norfloxacin (NOR), 10 μg; ofloxacin (OFX), 5 μg; tetracycline (TET), 30 μg; chloramphenicol (CHL), 30 μg; imipenem (IPM), 10 μg; and sulfamethoxazole-trimethoprim (SXT), 25 μg. All the drugs were bought from the CECON Laboratory (Ribeirão Preto, São Paulo, Brazil).

Phenotypic virulence characteristics. The following tests were performed as described in the cited texts: temperature-dependent autoagglutination, salicin fermentation and aesculin hydrolysis (Farmer *et al.*, 1992), calcium-dependent growth and Congo red absorption (Riley & Toma, 1989), and pyrazinamidase production (Kandolo & Wauters, 1985).

Detection of the genes *inv*, *ail*, *yst*A and *vir*F by PCR. Genomic DNA was extracted as described by Covone *et al.* (1998) and its concentration determined as in Sambrook & Russell (2001). The general PCR procedure was performed by the method given in Saiki *et al.* (1988), using 2·0 U *Taq* DNA polymerase. The primers used and the number of base pairs of the respective products are displayed in Table 1. The PCR reaction conditions used for detection of the various genes were as described in the following: for *inv*, Rasmussen *et al.* (1994); for *ail*, Nakajima *et al.* (1992); for *yst*A, Ibrahim *et al.* (1997); and for *vir*F, Wren & Tabaqchali (1990). The *Y. enterocolitica* strains Ye7660 and Ye197, isolated from diarrhoeic faeces of human origin and food respectively, contain all the tested

Table 1. Primers used to detect the *inv*, *ail*, *yst*A and *vir*F genes in *Y. enterocolitica*

Gene	Primer name	Sequence (5′→3′)	Amplicon size (bp)
inv	YC1	CTG TGG GGA GAG TGG GGA AGT TTG G	570
	YC2	GAA CTG CTT GAA TCC CTG AAA ACC G	
ail	Ail1	ACT CGA TGA TAA CTG GGG AG	170
	Ail2	CCC CCA GTA ATC CAT AAA GG	
*yst*A	Pr2a	A ATG CTG TCT TCA TTT GGA GCA	145
	Pr2c	ATC CCA ATC ACT ACT GAC TTC	
*vir*F	VirF1	TCA TGG CAG AAC AGC AGT CAG	590
	VirF2	ACT CAT CTT ACC ATT AAG AAG	

genes and were used as positive controls. Reactions without DNA as a template or with genomic DNA of *Escherichia coli* DH5-α strain (New England Biolabs) were used as negative controls.

In order to check the reproducibility of the experiments, the PCR reactions were repeated two or three times for some strains.

The PCR products were analysed by agarose gel electrophoresis and visualized by UV light after staining of the gel with ethidium bromide (0·5 μg ml^{-1}).

ERIC-PCR. Genomic DNA was extracted as described as above. One hundred nanograms DNA template was used in each PCR reaction. The PCR reaction mixture also contained 1·25 mM of each deoxyribonucleotide, 5 mM $MgCl_2$, 1·0 U Klentaq1 DNA polymerase (Ab Peptides) and 50 pmol each primer. The primers used were ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′), as described by Versalovic *et al.* (1991). The cycles used in the PCR were: initial cycle of 7 min at 94 °C, 30 cycles of 30 s at 94 °C, 1 min at 52 °C and 8 min at 65 °C, and final cycle of 10 min at 65 °C.

The ERIC-PCR reaction was repeated at least twice for each strain to verify the reproducibility of the experiment. Reactions without the DNA template were used as negative controls.

The ERIC-PCR products were analysed by agarose gel electrophoresis, the gel being stained with ethidium bromide (0·5 μg ml^{-1}) and visualized by UV light.

Data analysis was performed with GelCompar II 2.0 for Windows (Applied Maths). Only the bands between 300 and 5000 bp in size were included in the analysis. The similarity dendrogram was constructed by the unweighted pair group method with arithmetic averages (UPGMA), using the Dice similarity index. A standard molecular weight ladder was included twice on each gel, to allow normalization of images for valid comparisons of fingerprints on different gels. The standard used was the 1 kb Plus DNA Ladder (Invitrogen-Life Technologies).

The ERIC genomic types (EGTs) were considered to be different if any band difference was found in their fragments.

PFGE. The genomic DNAs were prepared in agarose plugs, using a protocol described by Liu *et al.* (1993), with a few modifications. Bacteria were grown overnight in brain heart infusion (BHI) broth (Oxoid) at 28 °C. Each culture was diluted 1 : 50 in 10 ml BHI and reincubated at 28 °C, until reaching OD_{600} 0·6–0·9. The cultures were centrifuged at 2300 ***g*** for 3 min at 4 °C, and the sediment was resuspended in 500 μl cell-suspension buffer (100 mM Tris, pH 7·2, 20 mM NaCl, 100 mM EDTA). The cell suspension was mixed with an equal volume of 2 % low-melting-point agarose (Bio-Rad Laboratories) and cast in DNA-plug moulds. The agarose plugs were treated for 2 h in a lysis solution (100 mM Tris, pH 7·2, 20 mM NaCl, 100 mM EDTA, 1 mg lysozyme ml^{-1}, pH 7·6) at 37 °C and transferred to 5 ml deproteination solution (0·25 M EDTA, pH 9·0, 0·5 % sarcosyl, 5 mg proteinase K ml^{-1}) for 12–16 h at 50 °C. The plugs were washed four times with TE buffer (100 mM Tris, 1 mM EDTA), with 1 mM PMSF added to the second wash. The plugs were stored in 0·5 M EDTA at 4 °C until use.

Before digestion, plugs were washed in TE buffer (100 mM Tris, 1 mM EDTA) for 1 h. They were digested with 40 U *Xba*I (New England Biolabs) overnight. Macrorestriction fragments were resolved by counter-clamped homogeneous electric field electrophoresis in a CHEF-DRIII apparatus (Bio-Rad Laboratories), using an electric field of 6 V cm^{-1} and angle of 120 °C (Guiyoule *et al.*, 1997). Migration of the DNA fragments was achieved in 0·5× Tris/borate/EDTA buffer and 1·0 % agarose maintained at 14 °C. Pulse times were ramped from 1 to 10 s over 29 h.

Restriction fragments were visualized under UV light after the gel was stained with ethidium bromide (0·5 μg ml^{-1})

The genomic profile obtained was analysed as described above for ERIC-PCR. A standard molecular weight ladder was included three times on each gel to allow comparisons of fingerprints on different gels. The molecular weight standard used was Lambda Ladder PFG Marker (New England Biolabs).

The pulsed-field genomic types (PGTs) were assessed as described above for the ERIC-PCR technique.

Discrimination index (DI). The discriminatory power of PFGE and ERIC-PCR was compared by Simpson's diversity index, as presented by Hunter & Gaston (1998).

RESULTS AND DISCUSSION

Bio-sero-phagetyping

The bio-sero-phagetypes and source of origin of the 106 *Y. enterocolitica* strains used in this study are presented in Table 2. All 37 strains isolated from human diarrhoeic faeces were bio-sero-phagetyped as 4/O : 3/VIII and were isolated between 1979 and 2000 in various regions of Brazil, mostly from the Southeast region (31 strains). A similar result was observed for the animal isolates (34 strains), 33 being bio-sero-phagetyped as 4/O : 3/VIII and just one as IXa. These animal strains were isolated from various types of animal clinical material, between 1968 and 1985, all coming from the Southeast (10 strains) and South (24 strains) regions. The 35 strains isolated from diverse types of food were of

Table 2. Bio-sero-phagetypes and source of 106 *Y. enterocolitica* strains isolated from human diarrhoeic faeces (37), animal clinical material (34) and food (35)

No. of strains	Bio-sero-phagetype	(No. of strains) source of isolate		
		Human origin	Animal origin	Food origin
37	4/O : 3/VIII	(37) Diarrhoeic faeces		
33	4/O : 3/VIII		(5) Marmoset hepatic abscess, (3) dog faeces, (24) pig faeces, (1) rat faeces	
1	4/O : 3/IXa		(1) Bull faeces	
1	2/O : 10/Xo			(1) Raw milk
7	1A/O : 5/Xz			(1) Raw milk (5) Pasteurized milk (1) Pork sausage
1	1A/O : 5/Xo			(1) Chicken
5	1A/O : 5,27/Xz			(1) Pasteurized milk, (1) bull's liver, (1) pork sausage, (2) raw milk
1	1A/O : 5,27/Xo			(1) Ice
1	1A/O : 13/Xo			(1) Pasteurized milk
3	1A/O : 13,7/Xz			(2) Meat, (1) lettuce
1	1A/O : 7,8/Xo			(1) Pasteurized milk
1	2/O : 14/Xz			(1) Raw milk
2	1A/O : 6,31/Xz			(1) Pasteurized milk, (1) pork meat
1	2/O : 7,8/Xz			(1) Pasteurized milk
1	1A/O : 10/Xo			(1) Milk
1	1A/O : 10/Xz			(1) Raw milk
1	1A/O : 10,34/Xo			(1) Raw milk
1	1A/O : 4,33/Xz			(1) Chicken
3	1A/O : 14/Xo			(1) Beef, (2) pork sausage
2	1A/O : 8,19/Xz			(2) Raw milk
1	1A/O : 41,42/Xo			(1) Raw milk
1	1A/O : 12,25/Xo			(1) Beef

bio-sero-phagetypes other than 4/O : 3/VIII or IXa, with a prevalence of biotype 1A and serogroups O : 5 and O : 5,27. These food strains were all isolated from the Southeast region between 1980 and 1995. The predominance of strains isolated in the Southeast and South regions of Brazil is probably the result of more efficient medical and laboratory practices than those of other regions of Brazil.

It is interesting to mention that until now the *Yersinia* Reference Laboratory has never received any *Y. enterocolitica* of bio-serotype 4/O : 3 isolated from food. Only strains biotyped as 1A or 2 (of diverse serotypes) have been isolated from foodstuffs in Brazil and received by the Reference Laboratory (Falcão, 1987, 1991; D. P. Falcão and others, unpublished results). However, this does not mean that food cannot function as a vehicle for human infection with *Y. enterocolitica* 4/O : 3 in Brazil. Data from other studies have indicated that the conventional culture methods generally used to detect and isolate pathogenic *Y. enterocolitica* from food are not efficient (Nesbakken *et al.*, 1991; Fredriksson-Ahomaa & Korkeala, 2003; Fredriksson-Ahomaa *et al.*, 2006). Future studies using molecular detection methods, such as DNA hybridization and PCR, in association with conventional methods will be needed to better evaluate this phenomenon in Brazil. In the specific case of the food strains studied here, it is not possible to discuss the procedures used to isolate these strains, as they were isolated by different investigators and sent to the *Yersinia* Reference Laboratory for confirmation and typing only.

Additionally, none of the strains evaluated here can be related to a disease outbreak, as all were isolated from sporadic cases. To our knowledge, no outbreak related to *Y. enterocolitica* has been reported so far in Brazil (Falcão, 1987, 1991; D. P. Falcão and others, unpublished results).

Drug-resistance profile

The tests of drug resistance showed that all the *Y. enterocolitica* strains were resistant to at least AMP, CEF and CFZ. Of the 37 *Y. enterocolitica* strains isolated from humans, only two were resistant to further drugs. Similar results were observed for the 34 *Y. enterocolitica* strains obtained from various animal clinical isolates. Three of these animal strains, besides AMP, CEF and CFZ, were also resistant to AMK. The majority of the 35 strains isolated from food (74·3 %) were resistant to FOX, as well as AMP, CEF and CFZ.

The resistance profile obtained is in agreement with previous studies, in which resistance to AMP and many cephalosporins is frequently observed (Stock & Wiedemann, 1999; Tzelepi *et al.*, 1999; White *et al.*, 2002; Stock & Wiedemann, 2003). In fact, the expression of β-lactamase enzymes A and B has already been associated with *Y. enterocolitica*, *Yersinia intermedia* and *Yersinia frederiksenii* (Stock *et al.*, 1999, 2000; Stock & Wiedemann, 1999, 2003; Tzelepi *et al.*, 1999).

Phenotypic tests related to virulence

The results of the phenotypic tests related to virulence are presented in Table 3. All 37 isolates from humans and 34 isolates from animals showed virulence-related phenotypes with respect to aesculin hydrolysis, salicin fermentation and pyrazinamidase production, with the exception of one human strain (Ye33) that hydrolysed aesculin. In complete contrast, only one food-related strain (Ye197) of bio-serophagetype 2/O : 7,8/Xz, isolated from pasteurized milk, gave similar results.

The phenotypic tests related to the expression of plasmid genes had variable results. A total of 73·0 % (27) of human strains and 41·2 % (14) of animal strains were positive for the tests of autoagglutination at 37 °C, calcium dependence at 37 °C and Congo red absorption. Otherwise, only strain Ye197, among those isolated from food, was positive for these tests.

These results confirm published data, which show a well-established correlation between pathogenic biotype and virulence behaviour in these phenotypic tests (Farmer *et al.*, 1992; Bauab *et al.*, 1995).

We can also conclude that although the plasmid was present in the majority of the strains of human and animal origin, as revealed by the detection of the *vir*F gene (Table 3), in some of these strains, the plasmid genes relating to the expression of autoagglutination, calcium dependence and Congo red absorption were not expressed. Analysing these results, we could speculate that these genes were silenced by a mutation or that the conditions used in the phenotypic assays were not appropriate to induce their expression in some of the strains.

Detection of virulence genes

The incidence of virulence genes detected in the various bio-serotypes is presented in Table 3. The *inv* gene was detected in all the strains studied. The *ail* and *yst*A genes were detected in all the strains isolated from humans and animals, but only in two strains isolated from food. Despite the presence of virulence genes, these two food strains were not implicated as a direct cause of foodborne disease. The *vir*F gene showed a variable incidence, being detected in 94·6 % (35 strains), 61·8 % (21 strains) and 2·9 % (1 strain) of isolates from humans, animals and food, respectively.

These results indicate the virulence potential of the strains isolated from human patients and animals, and reinforce their association with the diseases in the humans and animals from which they were isolated. This was expected, since the bio-serotype 4/O : 3 is the one most frequently isolated from infections caused by *Y. enterocolitica* in Brazil and Europe (Falcão, 1987; Carniel, 2001; Robins-Browne, 2001).

Regarding the food strains, while the *inv* gene was detected in all of them, as expected, since this gene is common to pathogenic and non-pathogenic strains, the *ail* and *yst*A

Table 3. Phenotypic virulence characteristics and PCR detection of virulence genes in 106 *Y. enterocolitica* strains isolated from human diarrhoeic faeces (37), animals (34) and foodstuffs (35)

Phenotypic tests/virulence genes	Number of positive strains (%), bio-serotypes		
	Human origin	Animal origin	Food origin
Expression of plasmid genes			
Autoagglutination at 37 °C	27 (73·0 %), 4/O : 3	14 (41·2 %), 4/O : 3	1 (2·9 %), 2/O : 7,8
Ca^{++} dependence at 37 °C	27 (73·0 %), 4/O : 3	14 (41·2 %), 4/O : 3	1 (2·9 %), 2/O : 7,8
Congo red absorption	27 (73·0 %), 4/O : 3	14 (41·2 %), 4/O : 3	1 (2·9 %), 2/O : 7,8
Other phenotypic tests			
Aesculin hydrolysis	36 (97·3 %), 4/O : 3	34 (100 %), 4/O : 3	1 (2·9 %), 2/O : 7,8
Salicin fermentation	37 (100 %), 4/O : 3	34 (100 %), 4/O : 3	1 (2·9 %), 2/O : 7,8
Pyrazinamidase production	37 (100 %), 4/O : 3	34 (100 %), 4/O : 3	1 (2·9 %), 2/O : 7,8
Presence of virulence genes			
inv	37 (100 %), 4/O : 3	34 (100 %), 4/O : 3	35 (100 %)*
ail	37 (100 %), 4/O : 3	34 (100 %), 4/O : 3	2 (5·7 %), 1A/O : 10, 2/O : 7,8
*yst*A	37 (100 %), 4/O : 3	34 (100 %), 4/O : 3	2 (5·7 %), 1A/O : 10, 2/O : 7,8
*vir*F	35 (94·6 %), 4/O : 3	21 (61·8 %), 4/O : 3	1 (2·9 %), 2/O : 7,8

*The bio-serotypes of the food strains studied were: 2/O : 10; 1A/O : 5; 1A/O : 5,27; 1A/O : 13; 1A/O : 13,7; 1A/O : 7,8; 2/O : 14; 1A/O : 6,31; 2/O : 7,8; 1A/O : 10; 1A/O : 10,34; 1A/O : 4,33; 1A/O : 14; 1A/O : 8,19; 1AO : 41,42; 1A/O : 12,25.

genes were detected in just two strains (5·7 %). One of these two strains, Ye251, was biotyped as 1A, which has been treated as a non-pathogenic biotype. However, the presence of some virulence genes in a strain of this biotype confirms several reports that biotype 1A is not as harmless as was once believed (Grant *et al.*, 1998; Sulakvelidze, 2000; Tennant *et al.*, 2003). In an outbreak of gastroenteritis in Canada described by Ratnam *et al.*, (1982), involving nine hospital patients from the same nursery, the agent was *Y. enterocolitica* biotype 1A, serogroup O : 5. In another outbreak in the UK, described by McIntyre & Nnochiri (1986), the *Y. enterocolitica* strain involved was biotype 1A and serogroup O : 6,30. Tennant *et al.* (2003) have suggested that there are two subgroups of biotype 1A of *Y. enterocolitica*: a group comprising pathogenic strains of clinical origin and another group comprising non-pathogenic strains of environmental origin. Thus, the isolation of *Y. enterocolitica* 1A/O : 10 (Ye251) from food, carrying such important virulence genes as *ail* and *yst*A, is very rare (Grant *et al.*, 1998; Singh & Virdi, 2004), and highly undesirable, as it may represent a hazard to consumers of food contaminated by these bacteria. Although few, there are some published data showing the involvement of *Y. enterocolitica* 1A/O : 10 in human infection (Greenwood & Hooper, 1990; Morris *et al.*, 1991). The other food strain found to carry virulence genes in this study, Ye197, harboured all four genes (*inv*, *ail*, *yst*A and *vir*F). This strain, isolated from pasteurized milk, was bio-sero-phagetyped as 2/O : 7,8/Xz, and showed a virulent profile in all the phenotypic tests used, as mentioned above. According to the literature, biotype 2, associated with serogroups O : 5,27, O : 9 and O : 27, has frequently been related to disease (Robins-Browne, 2001). These results lead us to conclude that this strain, isolated from pasteurized milk, biotyped as 2, showing a virulence phenotype and harbouring some important virulence genes, is a strain with a potential to cause disease. Thus, rigorous control of the time and temperature of pasteurization is necessary to eliminate these micro-organisms. Additionally, our data suggest that serogroup O : 7,8 could be added to the serogroups of *Y. enterocolitica* biotype 2 with potential to cause disease. Despite these observations, these two food strains harbouring virulence genes have not been directly implicated as the cause of any foodborne illness.

ERIC-PCR and PFGE

To examine the genomic similarity among the strains, ERIC-PCR and PFGE were employed.

All 106 *Y. enterocolitica* strains were analysed by the ERIC-PCR technique. Fig. 1(a) shows a representative set of the amplification profiles generated by ERIC-PCR with some of the studied *Y. enterocolitica* strains from all three sources of origin. ERIC-PCR was repeated at least twice for each strain in order to check the reproducibility of the experiments. In all cases, the same pattern of bands was found for each replicate.

ERIC-PCR grouped 84 of the strains (79·2 %) into 10 genomic types (EGT1–EGT10), and 22 strains (20·8 %) showed unique fingerprint profiles (EGT11–EGT32), generating a discriminatory index of 0·908. The 4/O : 3 strains of human and animal origin showed high genomic similarity. These strains were discriminated into seven EGTs, forming two main groups with about 78·0 % similarity. Both groups included isolates of human and animal origin only. The strains isolated from food samples and belonging to biotypes 1A or 2 of various serotypes were more diverse in their fingerprints. It is important to point out that strains Ye197 (2/O : 7,8) and Ye251 (1A/O : 10), the only strains with virulence genes isolated from foods, were grouped closer (almost 70 % similarity) to the strains isolated from human and animal clinical material than to the other food strains (similarity lower than 50 %).

The PFGE technique was performed on 103 *Y. enterocolitica* strains. Three strains isolated from food were omitted from this analysis, as they suffered DNA degradation after repeated pulsed-field runs.

Fig. 1(b) shows representative macrorestriction fragment profiles generated by some of the strains isolated from human patients. By this technique, 53·4 % (55 of the analysed strains) were grouped into 15 PFGE genomic types (PGT1–PGT15), and 46·6 % (48 strains) showed unique fingerprint profiles (PGT16–PGT63), generating a discrimination index of 0·969. Overall, 32 PGTs of *Y. enterocolitica* 4/O : 3 were found, and again, the animal and human isolates proved to be highly similar in their fingerprint profiles, with 84·0 % or more similarity for the majority of the strains (99·0 % of the strains). Some strains isolated from human diarrhoeic faeces were grouped in the same genomic type as strains isolated from animal clinical material, and vice versa.

It may be concluded that PFGE was able to discriminate the strains better than ERIC-PCR. On the other hand, ERIC-PCR grouped the two food strains (Ye197 and Ye251) that carried virulence genes closer to the strains isolated from human patients and animals than to the other food strains. These results suggest that the presence or absence of particular virulence genes affects the band pattern generated by ERIC-PCR. The BLAST search for ERIC sequences on the genome of *Y. enterocolitica* strain 8081 (1B/O : 8) did not reveal any ERIC or homologue sequences flanking virulence genes. Thus, the insertion of virulence genes such as *ail* and *yst*A between ERIC sequences probably cannot explain the differences or similarities in the band patterns of the strains generated by ERIC-PCR. In future experiments, it will be of interest to compare the degree of virulence of the two food strains that carried the virulence genes (Ye197 and Ye251) with that of strains already established as virulent, by using the *in vivo* mouse infection model.

Both ERIC-PCR and PFGE results suggest that animals function as *Y. enterocolitica* reservoirs for human infection, corroborating previous observations from other countries

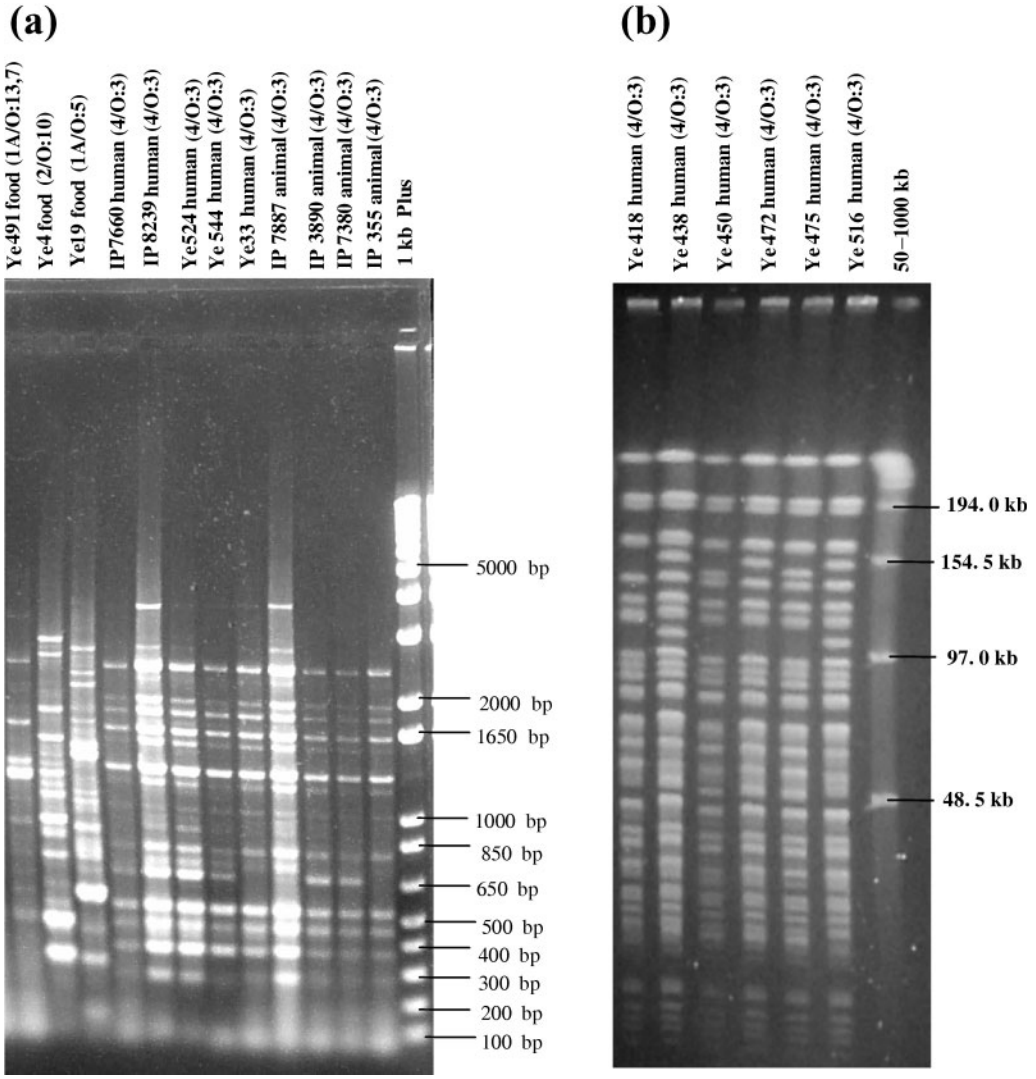

Fig. 1. (a) Representative gel of the band patterns generated by ERIC-PCR; 1·5 % agarose gel. The right-hand lane shows the molecular-weight ladder, 1 kb Plus DNA. (b) PFGE in a 1·0 % agarose gel, after genomic DNA digestion with *Xba*I. The right-hand lane shows the run of a molecular-weight ladder of 50–1000 kb. Both gels show representative strains of *Y. enterocolitica* that are listed above the figure along with their bio-serotype in parentheses.

(Bottone, 1999; Fredriksson-Ahomaa *et al.*, 2004; Lambertz & Danielsson-Tham, 2005) and indicating for the first time that pigs have been a source of *Y. enterocolitica* 4/O : 3 human infections in Brazil. These data also corroborate earlier published work in which a high genetic similarity was demonstrated among *Y. enterocolitica* 4/O : 3 strains (Fredriksson-Ahoma *et al.*, 1999, 2001). On the other hand, the strains isolated from food were mostly from biotype 1A and were more diverse regarding their fingerprints, both among themselves and relative to those isolated from humans and animals, as observed in previous studies (Najdenski *et al.*, 1994; Iteman *et al.*, 1996; Sachdeva & Virdi, 2004).

Recently, Wojciech *et al.* (2004) have described the typing of 35 *Y. enterocolitica* isolates by internal transcribed spacer (ITS) profiling, ERIC- and repetitive extragenic palindromic (REP)-PCR. These authors observed that ERIC-PCR is a suitable technique for this purpose, generating similar results to the other two techniques used, although with a slightly lower discriminatory power. Also, Sachdeva & Virdi (2004) used REP- and ERIC-PCR to assess the genomic heterogeneity among 81 strains of *Y. enterocolitica* biotype 1A isolated from India, Germany, France and the USA. Although the two techniques gave comparable results, ERIC fingerprints discriminated the strains better. To our knowledge, our work is the third that describes the use of ERIC-PCR to discriminate among *Y. enterocolitica* isolates, and the first to compare the results with PFGE, which is considered to be the gold-standard technique for this purpose. Additionally, ERIC-PCR grouped two food isolates with pathogenic characteristics close to the human and

animal clusters, indicating that it may be possible to use it as a tool to group *Y. enterocolitica* strains by their virulence potential.

In conclusion, the group of results obtained in this work affords an improved view of the epidemiology and biology of *Y. enterocolitica* strains isolated from diverse sources, over 32 years, in various regions of Brazil. Furthermore, it reveals the unusual virulence potential of a bio-serotype 1A/O : 10 strain isolated from food, indicating that biotype 1A strains may have been a neglected cause of infection in humans and animals in this country. The absence of pathogenic *Y. enterocolitica* 4/O : 3 strains in food samples in Brazil may be explained by the limited sensitivity of isolation methods. PFGE was able to discriminate the strains better than the ERIC-PCR technique. However, ERIC-PCR discriminated between strains with virulence potential isolated from foodstuffs (Ye197 and Ye251) and those without this virulence potential, indicating that this technique may be used as a virulence-potential screening tool for *Y. enterocolitica*.

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