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Detection of *Yersinia* spp. in meat products by enrichment culture, immunomagnetic separation and nested PCR

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ABSTRACT

The prevalence of *Yersinia enterocolitica* in meat products was assessed by four methods: cold enrichment in trypticase soy broth (A), enrichment in modified Rappaport broth at 25 °C (B), concentration by immunomagnetic separation (C) and *yad*A nested PCR (D). Furthermore, the pathogenic potentials of the isolates were established by phenotypic and genotypic tests, and their genomic relationships were determined by pulsed-field gel electrophoresis (PFGE). A total of 238 samples were collected at retail level in the city of San Luis, Argentina, during the period 2007–2008. The highest *Yersinia* prevalence in meat products was observed by method D (92 positive samples), followed by methods A (13 positive samples) and C (5 positive samples); however, no isolation was obtained by method B. Fourteen *Y. enterocolitica* and 4 *Yersinia intermedia* strains were recovered by culture. All *Y. enterocolitica* 2/O:9 strains gave results related to virulence by phenotypic tests and exhibited the genotype *vir*F$^+$ *myf*A$^+$ *ail*$^+$ *yst*A$^+$. Two biotype 1A strains showed a genotype *vir*F$^-$ *myf*A$^-$ *ail*$^+$ *yst*A$^+$ *yst*B$^+$. The 14 *Y. enterocolitica* strains isolated during this work plus one reference strain were separated into 11 genomic types by PFGE. This genomic heterogeneity of the isolates shows the diversity of *Y. enterocolitica* strains in our region. It is the first time that IMS was used to search *Y. enterocolitica* strains from naturally contaminated meat products.

1. Introduction

Yersinia enterocolitica, an important food-borne enteropathogen, is known to cause a wide variety of clinical manifestations ranging from mild gastroenteritis to invasive syndromes like terminal ileitis and mesenteric lymphadenitis (Bottone, 1999). Even though this species comprises six biotypes and nearly 50 serotypes, the most frequently implicated serotype in human disease worldwide is O:3 with almost all strains belonging to biotype 4. Other biotypes associated with human infection include 1B (serotypes O:8 and O:4), 2 (O:9, O:5,27), 3 (O:5,27, O:1,2,3) and 5 (O:2,3). Isolates belonging to biotype 1A are regarded as avirulent or 'environmental', although they may be opportunistic pathogens (Bottone, 1999).

Pathogenic strains of this bacterial species carry a 72 kb-plasmid (pYV) that encodes various virulence genes including *yad*A whose product is involved in autoagglutination, serum resistance and adhesion. Moreover, chromosomal genes such as *yst*, also known as *yst*A, which encodes a heat-stable enterotoxin (Y-STa), *myf* related to the production of fibrillae (Myf), *vir*F linked to transcriptional activators of the *yop* regulon, and the urease gene complex, may also contribute to virulence traits (Bottone, 1999).

Swine are the main reservoir of pathogenic *Y. enterocolitica* strains, harboring them in tonsils and in the oral cavity (Virtanen et al., 2011). The most common route of transmission of yersiniosis is through contaminated water and foods (Bottone, 1999), and data concerning the incidence of *Y. enterocolitica* and related species in foods are well documented in many countries throughout the world. Thus, this bacterium has been isolated from foods like meat from diverse origins (Bonardi et al., 2010), pasteurized milk (Okwori et al., 2009), and various vegetables (Siddique et al., 2009).

Even though *Y. enterocolitica* 4/O:3 (biotype/serotype) strains have not been isolated from food in our region, *Y. enterocolitica* 2/O:9 strains among other bio-serotypes have been recovered from different kind of foods in San Luis, Argentina (Favier et al., 2005;

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Lucero Estrada et al., 2007; Velázquez et al., 1996). Regarding clinical findings in Argentina, Eiguer et al. (1987) isolated one *Y. enterocolitica* 1A/O5 strain from an asymptomatic patient and another 4/O:3 strain from a child's diarrheic feces. More recently, Paz et al. (2004) reported the isolation of one *Y. enterocolitica* 1A/O:5 strain from a diarrheic patient and Cortes et al. (2010) isolated six *Y. enterocolitica* strains from the diarrheic feces of six children out of 181 patients.

Y. enterocolitica is of particular concern for consumers' safety because it is capable of significant growth in foods stored at refrigeration temperatures without apparent signs of spoilage. However, there are considerable difficulties associated with its detection in foods. The main problem is the small number of pathogenic strains in the samples and the large number of organisms in the background microflora (Fredriksson-Ahomaa and Korkeala, 2003). Additionally, direct isolation is seldom successful so time-consuming enrichment steps, including cold enrichment for up to 3 weeks, are required. Taking into account all these difficulties, it is necessary to find alternative methods for detection of this species in food.

The polymerase chain reaction (PCR) has become an extensively used molecular method for the detection of infectious agents. A number of PCR assays for the detection of *Y. enterocolitica* in food and clinical samples have been developed. Chromosomally-encoded genes like *ail* (Thisted Lambertz et al., 2007), *yst* (Vishnubhatla et al., 2001), or *16S rRNA* (Wolffs et al., 2004) as well as plasmid-encoded genes like *yad*A (Fredriksson-Ahomaa et al., 1999; Lucero Estrada et al., 2007) have been studied.

Another useful technique is the immunomagnetic separation (IMS) which uses small, uniform, paramagnetic particles coated with antibodies specific to bacterial surface antigens. It is known that IMS is effective for the isolation of *Y. enterocolitica* O:3 and O:8 from culture (Kapperud et al., 1993; Ueda et al., 2003), but its utility for the isolation of this bacterium from naturally contaminated food is not well documented yet.

Therefore, the purposes of this work were i) to compare four different methods for determining the prevalence of *Y. enterocolitica* in meat products, ii) to establish the pathogenic potential of the isolated strains by phenotypic and genotypic tests, and iii) to identify their relationships by means of genomic DNA macrorestriction analysis using pulsed-field gel electrophoresis (PFGE).

2. Materials and methods

2.1. Bacterial strains

The reference strain *Y. enterocolitica* W1024 O:9 pYV (+), kindly provided by Dr. Guy Cornelis, Catholic University of Louvain, Belgium, was used in virulence phenotypic assays, PCR and PFGE. Two strains isolated in our laboratory were used to obtain antibodies for immunomagnetic separation: *Y. enterocolitica* 2/O:9 isolated from eggshell (Favier et al., 2005) and *Y. enterocolitica* 3/O:3 isolated from human feces (unpublished data). These strains were kept in Luria broth supplemented with 20% glycerol (LB; Merck Laboratories, Darmstadt, Germany) at −20 °C.

2.2. Combined culture and PCR method

The presence of *Y. enterocolitica* in the food samples was assessed as follow: 25 g of sample were seeded in 225 ml trypticase soy broth (TSB; Merck), homogenized in stomacher for 90 s, and enriched at 25 °C for 18 h. From this TSB culture, i) a 10 ml aliquot was seeded in 90 ml of modified Rappaport broth (MRB) and incubated at 25 °C for 4 days; ii) a 1 ml volume was added to 20 μl of the immunomagnetic coated beads (1.2×10^7 beads/ml), and IMS was performed according to manufacturer's instructions (Dynabeads M-280; Dynal A/S, Oslo, Norway), and iii) another 1 ml aliquot was used for nested PCR analysis. The remaining TSB culture was incubated at 4 °C for 21 days. From this cold enrichment, as well as from IMS and MRB enrichments, platings were carried out on cefsulodin–irgasan–novobiocin agar (CIN, Merck) and Mac Conkey agar (MC; Merck) and incubated at 37 °C for 24 h and 25 °C for 48 h, respectively. The typical "bull's eye" colonies on CIN and the small and creamy colonies on MC were identified by biochemical assays (Bercovier and Morallet, 1984). The final characterization in biotypes and serotypes was performed by Dr. Elisabeth Carniel, National Reference Center of *Yersinia*, Institute Pasteur, Paris, France.

2.3. Food samples

A total of 238 samples was collected at retail level from 23 stores in the city of San Luis, Argentina, during the period 2007–2008, and immediately processed or stored at 4 °C for up to 4 h. The studied samples were: pure pork sausages ($n = 59$), pork and beef sausages ($n = 62$), minced meat ($n = 61$) and chicken carcasses ($n = 56$).

2.4. Immunomagnetic separation (IMS)

2.4.1. Rabbit Y. enterocolitica-specific antisera

Specific O antisera against two local strains, *Y. enterocolitica* 2/O:9 and *Y. enterocolitica* 3/O:3 were prepared by immunizing rabbits with a boiled suspension of bacterial cells. To obtain antigen for immunization, a loopful of each strain was separately seeded in 500 ml of TSB (Merck) and incubated for 24 h at 24 °C. After that, each bacterial suspension was inactivated by autoclaving at 121 °C for 1 h, washed three times with sterile saline, centrifuged at 2000 × g for 10 min in a refrigerated Sigma 3K30 laboratory centrifuge (Sigma, Steinheim, Germany) each time, and finally resuspended in saline at a concentration of 10^6 CFU/ml. Three New Zealand White rabbits, each weighing nearly 3 kg were subcutaneously inoculated with three 0.5 ml doses of each suspension of *Y. enterocolitica* cells, at intervals of fifteen days. One control rabbit received equal doses of saline. Two weeks after the last inoculation, rabbits were bled by heart puncture and sera were separated and stored at −20 °C.

2.4.2. Purification of IgG fraction containing Yersinia-specific antibodies

One milliliter of a pool of rabbit sera was used to obtain immunoglobulin G (IgG) by affinity chromatography using Sepharose CL 4B-protein A (Sigma, Saint Louis, MO, USA) as stationary phase. The elution was performed with 7 ml Tris–HCl 0.1 M pH 8, followed by 7 ml of Tris–HCl 0.01 M. Finally, 2 ml of glycine pH 3 and 2 ml of Tris–HCl 1 M pH 8 were used to wash the column. The eluted liquid was collected in 700 μl/tube fractions and the IgG concentration was determined by reading the optical absorbance at 280 nm (A_{280}), using a spectrophotometer (UV 40 Spectronic, Metrolab S.A., Buenos Aires, Argentina).

2.4.3. Coating immunomagnetic particles

The IgG fraction containing *Yersinia*-specific antibodies was employed to coat monodisperse polystyrene supermagnetic particles 2.8 μm in diameter with covalently linked sheep anti-rabbit IgG (Dynabeads M-280; Dynal A/S) according to the procedure recommended by the supplier. A 50:50 (O:9/O:3) mixture of the two antisera was utilized to coat the beads, at a concentration of 20 μg of IgG/ml immunomagnetic beads.

2.5. DNA extraction method

The Prepman Ultra reagent (Applied Biosystems, Foster City, California, USA) nucleic acid isolation method was carried out according to the manufacturer's instructions.

2.6. PCR

A nested-PCR method targeting the *yad*A gene of *Y. enterocolitica* (Lucero Estrada et al., 2007) was performed in a programmable thermal cycler ("Multigene 1" Thermal cycler, Labnet International Inc., Woodbrige, NJ, USA). Two pairs of primers, Yad 1 (5′-TAA GAT CAG TGT CTC TGC GGC A-3′) and Yad 2 (5′-TAG TTA TTT GCG ATC CCT AGC AC -3′) for the first PCR, and Yad 3 (5′-GCG TTG TTC TCA TCT CCA TAT GC-3′) and Yad 4 (5′-GGC TTT CAT GAC CAA TGG ATA CAC-3′) for the second PCR, were used. The first PCR produced a 747 bp amplicon and the second PCR amplified a DNA sequence of 529 bp. These products were determined by 1% agarose gel electrophoresis comparing them with a 100-bp molecular weight DNA marker (Productos Biológicos, Universidad Nacional de Quilmes, Quilmes, Argentina). The bands were visualized by staining with ethidium bromide (0.5 μg/ml) and photographed with a 6.0 mega pixels digital camera (Sony Cyber-shot; Sony Corporation, New York, USA).

2.7. Virulence phenotypic assays

The following tests were performed as described in the cited texts: temperature-dependent autoagglutination (Laird and Cavanaugh, 1980) and calcium-dependent growth and Congo red absorption (CR-MOX) (Riley and Toma, 1989) in order to differentiate between plasmid-bearing and plasmidless strains; esculin hydrolysis (Farmer et al., 1992) and pyrazinamidase production (Riley and Toma, 1989) to demonstrate other virulence traits.

2.8. Antimicrobial susceptibility

The antimicrobial susceptibility of *Y. enterocolitica* isolates was determined by the disk diffusion method on Mueller Hinton agar (MH; Britania, Buenos Aires, Argentina) performed according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2009). The following antibiotic disks (Britania) were used: ampicillin, 10 μg (AMP); chloramphenicol, 30 μg (CHL); gentamicin, 10 μg (GEN); tetracycline, 30 μg (TET); trimethoprim-sulfamethoxazole, 25 μg (TMS); ciprofloxacin, 5 μg (CIP); nalidixic acid, 30 μg (NAL); cefuroxime, 30 μg (CXM); erythromycin, 15 μg (ERI); fosfomycin, 50 μg (FOS); aztreonam, 30 μg (AZT); and cephalothin, (CEF). Zones of growth inhibition were evaluated according to CLSI standards (2009).

2.9. PCR for virulence genetic markers

Four *Y. enterocolitica* virulence markers: *vir*F, *ail*, *myf*A and *yst*A genes, were assayed by multiplex PCR according to Lucero Estrada et al. (2011). The presence of *yst*B gene in *Y. enterocolitica* B1A strains was investigated by a simple PCR according to Bhagat and Virdi (2007). PCR products were separated in a 2% agarose gel and stained with ethidium bromide.

2.10. Pulsed-field gel electrophoresis (PFGE)

A previously described method for PFGE (Wang et al., 2008) was used with some modifications. Briefly, each bacterial strain was isolated on MH for 48 h at 25 °C and colonies suspended directly in 4 ml of a bacterial suspension buffer (100 mmol/l Tris, 100 mmol/l EDTA, pH 8.0) to an optical absorbance at 610 nm (A_{610}) of 1.00. Two hundred microliters of cell suspension were mixed with equal volume of 1% SeaKem Gold agarose (Cambrex, Rockland, ME, USA). Then, the plugs were treated for 20 h in a lysis solution (50 mmol Tris–EDTA, 1% sodium lauroyl sarcosine, Proteinase K 0.1 mg/ml, pH 8.0) at 37 °C. Before the restriction enzyme digestion, an approximately 1-mm-thick agarose slice was washed four times with TE buffer (10 mmol/l Tris, 1 mmol/l EDTA, pH 8.0) for 30 min at 37 °C. The DNA was restricted with 10 U of *Xba*I (Fermentas, Burlington, Ontario, Canada) for two hours, according to the manufacturer's instructions. DNA fragments were separated by a contour-clamped homogeneous electric field electrophoresis in a CHEF-DRIII apparatus (Bio-Rad, Hercules, CA, USA), using an electric field of 6 V/cm at 14 °C, angle of 120° and switching times of 1.8–20 s over 20 h. Migration of the DNA fragments was achieved in a 1.0% pulsed-field agarose gel (Bio-Rad) submerged in 0.5x TBE buffer (45 mmol/l Tris-borate and 1 mmol/l EDTA). The gels were stained with Gel Red® Acid Gel Stain (Biotum, Hayward, CA, USA) under the conditions suggested by the manufacturer and photographed with a Sony Cyber-shot 6.0 mega pixels camera. The size standard strain was *Salmonella* Braenderup H9812, kindly donated by Dr. Norma Binsztein (National Institute of Infectious Diseases "Dr. Carlos G. Malbrán" INEI-ANLIS, Buenos Aires, Argentina).

2.11. Statistical analysis

All assays were repeated at least three times in independent days. Statistical analysis of the frequency of *Y. enterocolitica* detection/recovery related to the method used and meat product studied was performed using Chi-square test (Analytical Software, Tallahassee FL, USA). Calculations were based on confidence level equal or higher than 95% ($p \leq 0.05$ was considered statistically significant).

The discrimination index (DI) values of PFGE were calculated by Simpson's diversity index, as presented by Hunter and Gaston (1998):

$$DI = 1 - [1/N(N-1)] \sum_{J=1}^{S} nj(nj-1)$$

Where N is the total number of strains, S is the number of profiles obtained, and nj the number of strains belonging to the jth type. Clustering of the patterns obtained by the PFGE was performed using Statistica 6.0 sofware (StatSoft Inc., Tulsa, OK, USA) and the unweighted pair group method with arithmetic average (UPGMA).

3. Results

3.1. Prevalence of Y. enterocolitica in meat products

The presence of *Y. enterocolitica* in naturally contaminated food samples, detected by PCR and culture methods, is shown in Table 1.

Table 1
Yersinia spp. in naturally contaminated food samples detected by PCR and culture methods.

Type of sample	No. of samples	No. (%) of positive results[a]		
		Nested-PCR	IMS	TSB
Pure pork sausage	59	21 (35.59)	3 (5.08)	5 (8.47)
Pork and beef sausages	62	22 (35.48)	2 (3.22)	3 (4.83)
Minced meat	61	33 (54.09)	ND	3 (4.91)
Chicken carcasses	56	16 (28.57)	ND	2 (5.35)
Total	238	92 (38.65)	5 (2.10)	13 (5.46)

[a] IMS: immunomagnetic separation, TSB: trypticase soy broth.

The highest *Yersinia* prevalence in meat products was observed by nested PCR (92 positive samples, 39%), followed by cold enrichment in TSB (13 positive samples, 5%), IMS (5 positive samples, 2%), and enrichment in MRB (0%) ($p \leq 0.05$).

By PCR, *yad*A-positive results were observed from all of the types of examined samples, however, the frequency of *Y. enterocolitica* detection was significantly higher from minced meat samples (33 positive samples among 61 total samples) than from other samples ($p \leq 0.05$).

Table 2 shows the *Yersinia* strains isolated during this work. From a total of 238 samples, 14 *Y. enterocolitica* (5.88%) and 4 *Yersinia intermedia* (1.68%) strains were recovered by culture. By cold enrichment in TSB, a total of 13 *Yersinia* strains were isolated from the samples. Nine strains were characterized as *Y. enterocolitica* belonging to different bio/serotypes and the remaining four strains were characterized as *Y. intermedia*. Three *Y. enterocolitica* 2/O:9 strains were isolated by this technique, two from pure pork sausages and one from pork and beef sausage. No significant differences in the *Yersinia* spp. recovery frequency related to the analyzed food were observed. Using IMS, five *Y. enterocolitica* strains were isolated. Three of them were obtained from pure pork sausages and two from pork and beef sausages. The five isolates corresponded to biotype 2, four strains belonged to O:9 serotype and the other one was autoagglutinable (AA). No *Yersinia* isolates were obtained by MRB method. All *Y. enterocolitica* 2/O:9 strains isolated with TSB were also recovered by IMS and were positive for *yadA* gene by nested PCR.

3.2. Virulence-associated properties of the Y. enterocolitica strains

All *Y. enterocolitica* 2/O:9 strains gave results related to virulence in the four phenotypic tests performed; i.e. all of them exhibited the genotype *vir*F^{+} *myf*A^{+} *ail*$^{+}$ *yst*A^{+}. Regarding the biotype 1A strains isolated by cold TSB enrichment, two serotypes (O:6,30 strain isolated from pork sausage and O:5 strain isolated from pork and beef sausage) were negative for the esculin and pyrazinamidase tests. These strains also showed a genotype *vir*F^{-} *myf*A^{-} *ail*$^{+}$ *yst*A^{+} *yst*B^{+}. Moreover, other two *Y. enterocolitica* 1A strains also were positive for *yst*B gene (Table 3).

3.3. Antimicrobial susceptibility of the Y. enterocolitica strains

All strains were resistant to ampicillin, erythromycin and cephalotin, and only one *Y. enterocolitica* 2/O:9 strain (named CLB197) showed intermediate susceptibility to gentamicin. All strains were susceptible to all the other antimicrobial drugs studied (data no shown).

3.4. PFGE

Fig. 1 shows the restriction pattern and dendrogram obtained by this subtyping method. Among the 15 studied strains, including 14 isolates obtained during this work plus one reference strain, 11 genomic types (GTs) were obtained, generating a DI of 0.962. Two major clusters were observed: cluster A which contained all *Y. enterocolitica* biotype 1A strains of different serotypes, and cluster B which contained all *Y. enterocolitica* biotype 2 strains, including the reference strain.

Most GTs included only one strain (63.64% of all strains) and four GTs (GTA5, GTB1, GTB2 and GTB6) included 2 strains each (36.36%). GTA5 included two *Y. enterocolitica* 1A/O:5 strains isolated from the same sample, one on CIN agar and the other one on MC agar. Six *Y. enterocolitica* 2/O:9 isolates clustered in three GTs (GTB1, GTB2 and GTB6) corresponding to duplicates (CLA and CLB) of three strains. Into each GT the CLA isolate was recovered by IMS and the CLB isolate was recovered by cold enrichment. Strains of GTB1 and GTB2 were isolated from pure pork sausage and the strain included in GTB6 was isolated from pork and beef sausage.

4. Discussion

In this work, the highest *Yersinia* spp. prevalence in the studied samples was observed by using nested PCR, followed by cold enrichment, IMS, and MRB. PCR is considered the most sensitive method for the detection of microorganisms, including *Y. enterocolitica* (Hudson et al., 2008; Thisted Lambertz et al., 2007). Thus, the high nested PCR recovery rates observed in this work must be attributed to the sensitivity of this technique. Since dead or viable but non cultivable cells might be detected by PCR, we used an enrichment step prior to DNA extraction in order to promote the growth of the target organism and to dilute dead bacteria or exogenous DNA present in the sample.

Microbial capture and concentration by IMS method is rapid and simple to perform and this method can allow detection of 1 viable microorganism per 25 g of pre-enriched sample (Kapperud et al., 1993). Although immunomagnetic beads for detecting *Salmonella* spp, *Escherichia coli* O157:H7 and *Listeria monocytogenes* species are commercially available, no commercial kit for *Y. enterocolitica* detection has been developed. This is the first report of IMS being used for assessing the prevalence of *Y. enterocolitica* in naturally contaminated meat products on retail sale. The IMS technique employed in this work was specific for the selected serotypes (O:9 and O:3). Three other studies have used IMS followed by culture for detection of *Y. enterocolitica* in foods and water but in all of them the selectivity and sensitivity of IMS was determined in inoculated

Table 2
Biotypes and serotypes of *Yersinia* spp. recovery from meat products.

Type of sample	*Y. enterocolitica*				*Y. intermedia*			
	Method[a]	Strains number	Biotype	Serotype[b]	Method	Strains number	Biotype	Serotype
Pure pork sausage	IMS	3	2	O:9	TSB	1	2	O:37
	TSB	2	2	O:9	TSB	1	2	NAG
	TSB	1	1A	O:6,30				
Pork and beef sausages	IMS	1	2	O:9				
	TSB	1	2	O:9				
	IMS	1	2	AA				
	TSB	1	1A	O:5				
	TSB	1	1A	NAG				
Minced meat	TSB	1	1A	O:6,30				
	TSB	2	1A	O:5				
Chicken carcasses					TSB	2	2	AA
Total		14				4		

[a] IMS: immunomagnetic separation, TSB: trypticase soy broth.
[b] AA: autoagglutinable, NAG: non-agglutinable.

Table 3
Virulence-associated properties of the *Yersinia enterocolitica* strains.

Biotype/serotype[a]	Local identification	Origin	Phenotypic characteristics[b]				Multiplex PCR				Simple PCR
			Esc	Pyz	AA	CR-MOX	*virF*	*myf*	*ail*	*yst*A	*yst*B
2/O:9	CLA138	pork sausage	–[c]	–	+	+	+	+	+	+	NP[d]
	CLB138	pork sausage	–	–	+	+	+	+	+	+	NP
	CLO216	pork sausage	–	–	+	+	+	+	+	+	NP
	CLA197	pork sausage	–	–	+	+	+	+	+	+	NP
	CLB197	pork sausage	–	–	+	+	+	+	+	+	NP
	CLA170	pork and beef sausage	–	–	+	+	+	+	+	+	NP
	CLB170	pork and beef sausage	–	–	+	+	+	+	+	+	NP
2/AA	CLO162	pork and beef sausage	–	–	+	+	+	+	+	+	NP
1A/O:6,30	CLO225	pork sausage	–	–	–	–	–	–	+	+	+
	CLO121	minced meat	+	+	–	–	–	–	–	–	+
1A/O:5	CLO229	pork and beef sausage	–	–	–	–	–	–	+	+	+
	CLA117	minced meat	+	+	–	–	–	–	–	–	+
	CLB117	minced meat	+	+	–	–	–	–	–	–	–
1A/NAG	CLO036	pork and beef sausage	+	+	–	–	–	–	–	–	–

[a] AA: autoagglutinable, NAG: non-agglutinable.
[b] Esc: esculin hydrolysis; Pyz: pyrazynamidase reaction; AA: autoagglutination; CR-MOX: calcium-dependent growth and Congo red binding.
[c] +/–: positive and negative reactions, respectively.
[d] NP: no performed.

samples only (Kapperud et al., 1993; Ueda et al., 2003; Koujitani et al., 2006).

As no *Y. enterocolitica* strain was isolated from meat products by MRB enrichment at 25 °C, this enrichment method was useless for the isolation of *Y. enterocolitica* from our samples. Other authors have reported the lesser sensitivity of this method compared with cold enrichment for *Y. enterocolitica* recovery from samples hfrom pigs (Laukkanen et al., 2010; Martínez et al., 2009).

In the present work, seven *Y. enterocolitica* 2/O:9, one *Y. enterocolitica* 2/AA, six *Y. enterocolitica* biotype 1A strains belonging to different serotypes, and four *Y. intermedia* of different bioserotype were isolated. Similar recovery rates of *Y. enterocolitica* strains belonging to O:9 and other serotypes from foods of our region were previously obtained using culture methods (Favier et al., 2005; Velázquez et al., 1996). Reports from neighboring countries indicate the recovery of various *Y. enterocolitica* serotypes

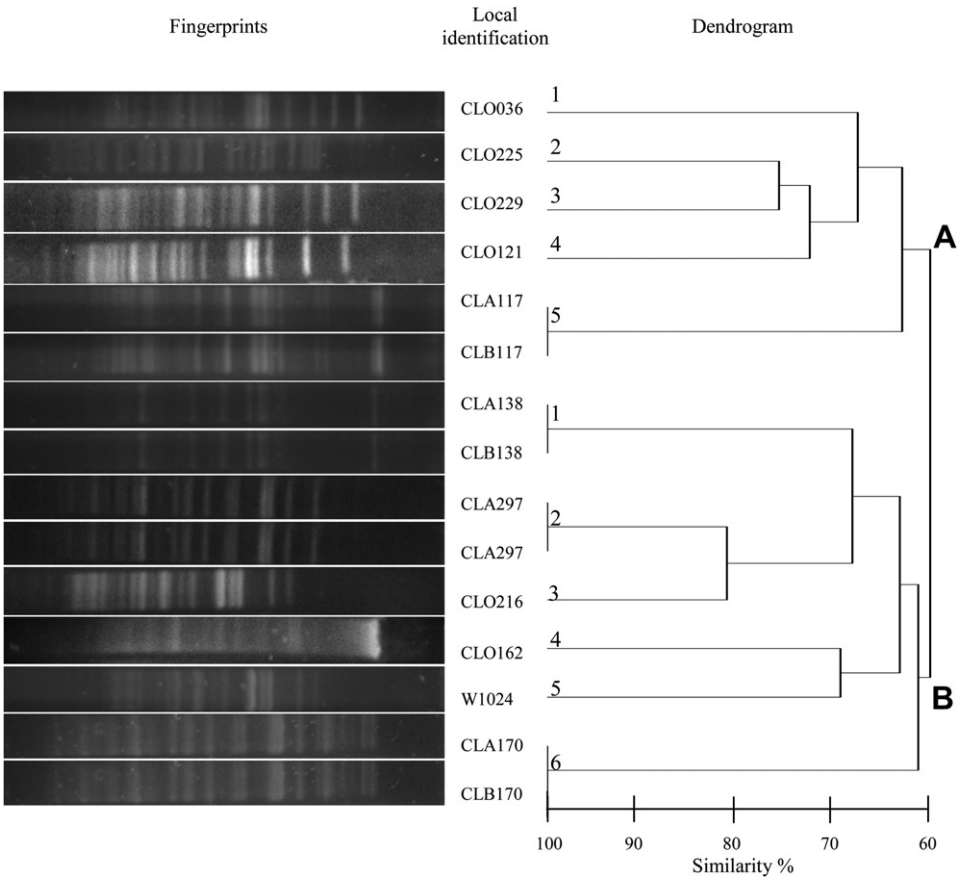

Fig. 1. Fingerprints and dendrogram obtained by PFGE of 14 *Y. enterocolitica* strains isolated in the present work and one reference strain.

from foods and water including *Y. enterocolitica* 2/O:5,27, 1A/O:5, 1A/O:27 and 1A/O:10 strains from water (Falcão et al., 2004) and *Y. enterocolitica* 4/O:3 from pork and goat meat (Borie et al., 1997).

Although the bioserotype most commonly associated with human disease is 4/O:3, biotype 2 associated with serotypes O:5,27, O:9 and O:27, has been also related to illness (Bottone, 1999). The 2/O:9 strains isolated in this work amplified the virulence genes *vir*F, *myf*A, *ail* and *yst*A and were positive in virulence phenotypic tests which indicate their pathogenic potential. However, two *Y. enterocolitica* biotype 1A strains isolated in this study also amplified *ail* and *yst*A genes. Similarly, Falcão et al. (2006) found that strains of this biotype carried *ail*, *yst*A and *vir*F genes that are commonly present in pathogenic strains. Furthermore, it has been proposed that *yst*B gene should be used to identify potentially pathogenic biotype 1A strains because the enterotoxin YstB is a factor of diarrhea caused by this biotype (Kot et al., 2010). In our study, four strains were positive for this gene. Strains of biotype 1A associated with serotypes O:5 and O:6,30 have been associated with gastrointestinal diseases outbreaks in several countries (Greenwood and Hooper, 1990; McIntyre and Nnochiri, 1986; Ratnam et al., 1982). All these data show that biotype 1A strains can be pathogenic, and have lead to increase of efforts to understand the mechanism of virulence of *Y. enterocolitica* biotype 1A strains.

Our antimicrobial susceptibility profiles for *Y. enterocolitica* strains did not significantly differ from those reported by Bonardi et al. (2010), which resistance to AMP, ERI and CEF being shown by all isolates. AMP resistance due to production of β-lactamases by *Y. enterocolitica* is well described in the literature (Bucher et al., 2008); and high rates of resistance to several β- lactam antibiotics and ERI in *Y. enterocolitica* isolated from food animals in Greece have been reported (Kechagia et al., 2007).

Although it was possible to separate *Y. enterocolitica* biotypes into two clonal clusters by PFGE, the clusters were not distinguished by serotype or the source of the isolates. PFGE analysis demonstrated the great heterogeneity among *Y. enterocolitica* strains. Similarly, various clonal groups have been found to be disseminated in South America (Falcão et al., 2006; Favier et al., 2005; Lucero Estrada et al., 2011).

As expected, our research confirmed that nested PCR was more sensitive than culture-based methods for the detection of pathogenic *Y. enterocolitica*, suggesting that culture-based surveys might underestimate their true prevalence. Although cold enrichment was more effective than IMS for the isolation of *Yersinia* spp, IMS was more specific since no *Y. intermedia* or *Y. enterocolitica* biotype 1A strain was isolated by this method. The advantages and disadvantages of culture-based and PCR-based methods complement one another. Therefore, depending on the objectives of the study, it may be valuable to use both approaches to acquire comprehensive results. By means of PFGE it was possible to demonstrate the genomic heterogeneity of the isolates, showing the diversity of *Y. enterocolitica* strains in our region. The pathogenic potential of all *Y. enterocolitica* biotype 2 strains and some biotype 1A strains in this study raises concerns in the field of the human health and highlights the importance of continuing with the detection and characterization of this microorganism from food samples.

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