Food Microbiology 28 (2011) 21–28

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Pulsed field, PCR ribotyping and multiplex PCR analysis of *Yersinia enterocolitica* strains isolated from meat food in San Luis Argentina

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ARTICLE INFO

Article history:
Received 29 April 2009
Received in revised form
19 May 2010
Accepted 23 July 2010
Available online 11 August 2010

Keywords:
Yersinia enterocolitica
Molecular characterization
Multiplex PCR
PFGE
PCR ribotyping

ABSTRACT

The characterization of phenotypic and genotypic virulence markers of *Yersinia enterocolitica* strains belonging to biotypes (B) 1A, 2 and 3, mostly isolated from food in San Luis, Argentina, and the assessment of their genotypic diversity using PFGE and PCR ribotyping, were performed in our laboratory for the first time. Thirty five *Y. enterocolitica* strains, two reference strains and 33 strains isolated in our laboratory were studied. The presence of *virF*, *ail*, *ystA*, and *myfA* genes was investigated by multiplex PCR. The pathogenic potential of B1A strains, the most predominant biotype of *Y. enterocolitica* strains isolated from meat in our region, was investigated by simple PCR. Four B1A strains were positive for *ystB* gene. Four *Y. enterocolitica* 2/O:9 (bio/serotype) and two 3/O:5 strains isolated in our laboratory showed virulence-related results in the phenotypic tests and multiplex PCR. A good correlation between the expression of virulence markers and their corresponding genotypes was observed for most strains. Sixteen genomic types (GT) and 9 different intergenic spacer region (SR) groups were generated by PFGE and PCR ribotyping, respectively. In both cases the *Y. enterocolitica* 2/O:9 strains were separately clustered from 1A and 3/O:5 strains. Meat foods might be vehicles of transmission of pathogenic *Y. enterocolitica* strains in our region.

1. Introduction

Yersinia enterocolitica is considered an important enteric pathogen, causing enterocolitis and other disorders in humans and animals. This bacterial species comprises a large group of phenotypic variants but few of them can cause diseases (Bottone, 1999). It is assumed that the main sources of infection in humans are pigs and pork products and, to a lesser degree, other farm animals (Bottone, 1999).

Virulence in *Y. enterocolitica* results from a complex interplay between a series of chromosomal and plasmid-borne genes (Bottone, 1999; Cornelis et al., 1998). Molecular genetic studies have emphasized the importance of a virulence plasmid (pYV) that encodes various virulence genes, among them *virF*, which is an important transcriptional regulator of other plasmid genes (Cornelis et al., 1998). Moreover, there are several chromosomal virulence genes like those that mediate cell invasion (*inv* and *ail*), produce a thermostable enterotoxin (*yst*), and the fimbrial and adhesin antigen (*myf*) (Carniel, 1995).

Biotype (B) 1A strains of *Y. enterocolitica*, which are generally considered to be avirulent, are highly heterogeneous, and include a large number of serotypes (Bottone, 1997). They occur throughout the world in a wide range of environments and generally lack the common markers associated with virulence of classical invasive *Y. enterocolitica*. Despite these observations, a significant association between *Y. enterocolitica* B1A belonging to different serotypes and diarrhea or other gastrointestinal symptoms in humans has been demonstrated (Butt et al., 1991; Pham et al., 1991). At least three different outbreaks caused by this biotype have been reported (Greenwood and Hooper, 1990; McIntyre and Nnochiri, 1986; Ratnam et al., 1982). Consequently, a thorough study of B1A strains isolated from various sources and belonging to diverse serotypes is imperative.

As a result of their limited discriminatory power, biotyping and serotyping are not useful in studies on heterogeneity and relationship between different isolates of the same species. For epidemiological purposes, such methods are therefore replaced by DNA-based molecular techniques. Pulsed field gel electrophoresis (PFGE) is one of the most used molecular techniques for subtyping

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doi:10.1016/j.fm.2010.07.026

different strains due to its highly discriminative power (Najdenski et al., 1994; Fredriksson-Ahomaa et al., 1999, 2004; Falcão et al., 2006). Another technique is PCR ribotyping which analyzes the sequence divergence of the *rrn* loci. The rRNA genes of this region are highly conserved; in contrast, the flanking sequences and the intergenic spacer region (SR) between the 16S and 23S rRNA genes can show a significant degree of variation in length and sequence within a single species. These differences can be used to discriminate clones and clonal lineages. Although, PCR ribotyping is cheaper, easier to perform and provides fast results, it has not been used extensively and its real potential as a tool for typing in epidemiological studies of *Y. enterocolitica* has not been recognized (Lobato et al., 1998; Wojciech et al., 2004).

In Argentina, relatively few studies have involved the detection of *Y. enterocolitica* in foods and clinical samples; moreover, there is very little information related to its epidemiology. Therefore, it is difficult to establish the role of this bacterium as a causative agent of human disease, or to estimate its impact on food products. In our laboratory, several *Y. enterocolitica* strains belonging to different serotypes have been isolated from different foods of animal origin, such as porcine and bovine tongues, porcine cheese, cooked ham and eggshells; however, they were not associated with food-borne outbreaks (Guzmán et al., 1984; Favier et al., 2005; Velázquez et al., 2005).

The aims of the present study were i) to characterize some phenotypic and genotypic virulence markers of *Y. enterocolitica* strains isolated from food samples in our laboratory and ii) to investigate the level of genetic diversity and possible relationships among these strains by PFGE and PCR ribotyping.

2. Methods

2.1. Bacterial strains and cultivation conditions

A total of 35 strains of *Y. enterocolitica*, 33 local strains and two reference strains were studied. All local strains were isolated during the period 2004–2008 in our laboratory from different meat foods. The reference strains were *Y. enterocolitica* W1024 O:9 pYV (+), provided by Dr. Guy Cornelis, Catholic University of Louvain, Belgium, and *Y. enterocolitica* Ye 099 O:9 pYV (+) and its isogenic plasmidless derivative, kindly provided by Dr. Georg Kapperud, Department of Bacteriology, Oslo, Norway (Table 1). Among the local strains, 25 were B1A (75.75%), six 2/O:9 (18,18%) and two 3/O:5 (6.06%). The bioserotyping of our strains was carried out by Dr. Elisabet Carniel, Pasteur Institute, Paris, France.

One *Yersinia intermedia* 2/O:37 strain, and two strains of *Y. intermedia* B2 autoagglutinable (AA) were used to test the specificity of the PCR developed for *Y. enterocolitica* in this study. These *Y. intermedia* strains were previously isolated in our laboratory.

The *Y. enterocolitica* strains were studied by PFGE and PCR ribotyping. All strains were kept in Luria broth supplemented with 20% glycerol (LB; Merck Laboratories, Darmstad, Germany) at −20 °C.

2.2. Phenotypic virulence characteristics

The following tests were performed as described in the cited text: 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; aesculin hydrolysis (Farmer et al., 1992) and pyrazinamidase production (Kandolo and Wauters, 1985) were used to demonstrate other virulence traits.

2.3. DNA extraction

The *Y. enterocolitica* strains were inoculated onto trypticase soy agar (TSA; Merck) and incubated for 48 h at 25 °C. Three colonies were suspended in one milliliter of trypticase soy broth (TSB; Merck) and the DNA extraction was carried out using the Prepman Ultra reagent (Applied Biosystems, Foster City, California, USA). This nucleic acid isolation method was performed according to the manufacturer's instructions. After the extraction, the DNA concentration was determined reading the OD (UV 40, Metrolab S.A., Buenos Aires, Argentina) at 260 nm, and 10 ng/μl of DNA were used in PCR and PCR ribotyping procedures.

2.4. PCR for virulence markers

Four virulence markers were assayed by multiplex PCR for detection of *virF*, *ail*, *myfA* and *ystA* genes (Table 2). A step-by-step empirical approach was used to determine the optimal annealing temperature, raising the temperature between 55–60 °C with 1 °C increments, and the concentration of primers, $MgCl_2$ and deoxynucleoside triphosphates in the reaction mixture. The final PCR protocol was as follows: 1× PCR buffer (Promega, Madison WI, USA); 1.5 mmol/l $MgCl_2$ (Promega); 1 U of Taq DNA-polymerase (Promega); 200 μmol/l of each dNTP (Promega) and 10 pmol each primer set (Promega). Millipore ultrapure water was added to a final volume of 50 μl. Five μl of DNA were as template. The amplification conditions were: an initial denaturation of 94 °C for 1 min, followed by 30 cycles of 94 °C for 45 s, 57 °C for 45 s and 72 °C for 45 s, with a final extension at 72 °C for 4 min ("Multigene 1" thermal cycler, Labnet International Inc., Woodbrige, NJ, USA).

The presence of *ystB* gene (Table 2) in *Y. enterocolitica* B1A strains was investigated by a simple PCR according to Bhagat and Virdi, (2007). Briefly, each 25 μl PCR reaction mixture contained 1× PCR buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 200 μM of each dNTP (Promega), 25 pmoles of each primer (Invitrogen, California, USA), 2 U Taq DNA-polymerase (Promega) and 2 μL of DNA template. The PCR program used consisted of 10 min at 94 °C of denaturing temperature, followed by 25 cycles of 5 s at 94 °C of denaturing temperature, 30 s at 61 °C of annealing temperature, and 30 s at 72 °C of extension temperature. At the end, a 10-min extension at 72 °C was applied ("Multigene 1" thermal cycler).

The PCR products were separated in a 2% agarose gel and stained with ethidium bromide (0.5 μg/ml). When negative results were observed by multiplex PCR, the analysis was remade. During this new analysis, primer pair(s) that did not generate amplicon(s) was used and the original PCR protocol was assayed (Ibrahim et al., 1997; Hussein et al., 2001; Gierczynski et al., 2002). In order to check the reproducibility of the technique, the PCR assays were repeated three times.

2.5. 16S–23S rDNA spacer region length polymorphism analysis (PCR ribotyping)

It was performed according to Kostman et al. (1992) protocol with few modifications. The primer set used was P1 (5′-TTGTACACACCGCCCGTCA-3′) and P2 (5′-GGTACCTTAGATGTTTCAGTTC-3′) (Invitrogen). Amplifications were performed in a final volume of 25 μl with a reaction mixture containing 1× PCR buffer (Promega); 1.5 mmol/l $MgCl_2$ (Promega); 1 U of Taq DNA-polymerase (Promega); 200 μmol/l of each dNTP (Promega) and 10 pmol of each primer. The DNA was amplified during 30 cycles of PCR consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for

Table 1
Isolates and virulence markers detection using phenotypic tests and two different PCRs.

Collection No.	Serotype	Biotype	Origin	Phenotypic characteristics				Multiplex PCR				Simple PCR
				Aes	Pyz	AA	CR	*virF*	*myfA*	*ail*	*ystA*	*ystB*
W1024	O:9	2	Reference	−	−	+	+	+	+	+	+	NP
Ye 099 (pYV+)	O:9	2	Reference	−	−	+	+	+	+	+	+	NP
Ye 099 (pYV−)	O:9	2	Reference	−	−	−	−	−	+	+	+	NP
3G	O:9	2	pork sausage	−	−	+	+	+	+	+	+	NP
4G	O:9	2	pork sausage	−	−	+	+	+	+	+	+	NP
5G	O:9	2	pork sausage	−	−	+	+	+	+	+	+	NP
170	O:9	2	mix sausage	−	−	+	−	−	+	+	+	NP
6G	O:9	2	minced meat	−	−	+	−	−	+	+	+	NP
7G	O:9	2	minced meat	−	−	+	+	+	+	+	+	NP
1ME	O:5	3	pork sausage	−	−	−	−	−	+	+	+	NP
2LV	O:5	3	hake	−	−	+	+	+	+	+	+	NP
2ME	O:7,8-8-8,19	1A	pork sausage	+	+	−	−	−	−	−	−	−
3M	O:7,8-8-8,19	1A	pork sausage	+	+	−	−	−	−	−	−	−
1C	O:7,8-8-8,19	1A	pork sausage	+	+	−	−	−	−	−	−	+
3C	O:7,8-8-8,19	1A	pork sausage	+	+	−	−	−	−	−	−	−
5C	O:7,8-8-8,19	1A	pork sausage	+	+	−	−	−	−	−	−	−
135	O:7,8-8-8,19	1A	pork sausage	+	+	−	−	−	−	−	−	+
4L	O:7,8-8-8,19	1A	hake	+	+	−	−	−	−	−	−	−
19L	O:7,8-8-8,19	1A	hake	+	+	−	−	−	−	−	−	+
22GA	O:7,8-8-8,19	1A	minced meat	+	+	−	−	−	−	−	−	−
1LV	O:5	1A	chicken	+	+	−	−	−	−	−	−	−
3LV	O:5	1A	chicken	+	+	−	−	−	−	−	−	−
5LV	O:5	1A	chicken	+	+	−	−	−	−	−	−	−
117A	O:5	1A	pork sausage	+	+	−	−	−	−	−	−	−
117B	O:5	1A	pork sausage	+	+	−	−	−	−	−	−	−
16G	O:5	1A	minced meat	+	+	−	−	−	−	−	−	−
1CL	O:5	1A	hake	+	+	−	−	−	−	−	−	−
36	O:41,42-41,43	1A	chicken	+	+	−	−	−	−	−	−	−
231	O:41,42-41,43	1A	chicken	+	+	−	−	−	−	−	−	−
221	O:41,42-41,43	1A	chicken	+	+	−	−	−	−	−	−	−
121	O:6,30	1A	minced meat	+	+	−	−	−	−	+	+	−
12GA	O:5-4,32-4,33	1A	chicken	+	+	−	−	−	−	−	−	−
8LI	O:40	1A	hake	+	+	−	−	−	−	−	−	−
210	NAG	1A	chicken	+	+	−	−	−	−	−	−	−
246	NAG	1A	chicken	+	+	−	−	−	−	−	−	+
17L	NAG	1A	minced meat	+	+	−	−	−	−	−	−	−

+/−, positive and negative reactions, respectively.
Aes, aesculin hydrolysis; Pyz, pyrazynamidase reaction; AA, autoagglutination; CR, congo red absorption.
NP: no performed.
NAG: non-agglutinable.

4 min ("Multigene 1" thermal cycler). The PCR products were separated in a 2% agarose gel and stained with ethidium bromide (0.5 μg/ml).

2.6. Pulsed field gel electrophoresis (PFGE)

The genomic DNA was prepared in agarose plugs using a protocol described by Fredriksson-Ahomaa et al. (1999) with few modifications. Briefly, bacterial cells grown overnight on trypticase soy agar (TSA; Merck) plates were suspended directly into 2 mL of TE buffer (100 mmol/l Tris, 10 mmol/l EDTA, pH 8.0) to a cell concentration corresponding to OD_{600}: 1.35.

Two hundred microliters of cell suspension were mixed with equal volume of 2% low melting point agarose (Low Melt Preparative Grade Agarose, Bio-Rad Laboratories, Hercules, CA, USA) and cast in DNA-plug moulds. The agarose plugs were lysed for 2 h in a lysis solution (2 mol/l Tris, 0.5 mol/l EDTA, 10% sodium lauroyl sarcosine, lysozyme 1 mg/ml, pH 8.0) at 37 °C. Then, each of the plugs was washed four times with TE buffer (10 mmol/l Tris, 1 mmol/l EDTA, pH 8.0) 20 min at 37 °C. Before the restriction enzyme digestion, an approximately 1-mm-thick agarose slice was washed in TE (10 mmol/l Tris, 1 mmol/l EDTA, pH 8.0) for 1 h at 37 °C. After that, the DNA was restricted with 30 U of *Xba*I (Promega) for two hours, according to the manufacturer's instructions. DNA fragments were separated according to Favier et al. (2005) by a counter-clamped homogeneous electric field electrophoresis in a CHEF-DRIII apparatus (Bio-Rad), 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) in 0.5× TBE buffer (45 mmol/l Tris–Borate and 1 mmol/l EDTA). The gels were stained with ethidium bromide and photographed with Sony Cyber-shot 6.0 mega pixels (Sony Corporation) camera. The standard used was *Saccharomyces cerevisiae* chromosomal DNA (Bio-Rad).

Table 2
Primers used for PCRs and amplicons size.

Gene	Sequence (5′–3′)	Amplicon length (bp)	Reference
virF	TCATGGCAGAACAGCAGTCAG ACTCATCTTACCATTAAGAAG	591	Hussein et al., 2001
myfA	CAGATACACCTGCCTTCCATCT CTCGACATATTCCTCAACACGC	272	Gierczynski et al., 2002
ail	ACTCGATGATAACTGGGGAG CCCCCAGTAATCCATAAAGG	170	Hussein et al., 2001
ystA	AATGCTGTCTTCATTTGGAGC ATCCCAATCACTACTGACTTC	145	Ibrahim et al., 1997
ystB	GTACATTAGGCCAAGAGACG GCAACATACCTCACAACACC	146	Bhagat and Virdi, 2007

2.7. Data analysis

The discrimination index (DI) values of PFGE and PCR ribotyping were calculated by Simpson's diversity index, as presented by Hunter and Gaston (1988):

$$DI = 1 - [1/N(N-1)] \sum_{j=1}^{s} n_j(n_j - 1)$$

where N is the total number of strains, S is the number of profiles obtained, and n_j, number of strains belonging to the jth type. Clustering of patterns obtained by the applied techniques was performed using Statistica 6.0 software (StatSoft Inc., USA) and the unweighted pair group method with arithmetic average (UPGMA).

3. Results

3.1. Phenotypic and genotypic virulence characteristics

The results of the phenotypic and genotypic tests related to virulence are presented in Table 1. As expected, the pYV-positive *Y. enterocolitica* reference strains showed virulence-related phenotype with positive results in autoagglutination and Congo red absorption, and negative hydrolysis of pyrazinamide and aesculin. These strains presented the genotype *virF*$^+$ *myfA*$^+$ *ail*$^+$ *yst*$^+$. Among the *Y. enterocolitica* 2/O:9 strains isolated in our laboratory, four strains showed all the phenotypic virulence markers as well as the amplicons of the four virulence genes. The other local *Y. enterocolitica* 2/O:9 strains did not show pinpoint colonies on CR-MOX agar, which was in agreement with the *virF*$^-$ genotype. However, these strains showed unspecific autoagglutination and were positive for *myfA*, *ail* and *ystA* chromosomal genes. One 3/O:5 strain exhibited the genotype *virF*$^+$ *myfA*$^+$ *ail*$^+$ *yst*$^+$, matching results obtained for phenotypic markers; the other one showed the genotype *virF*$^-$ *myfA*$^+$ *ail*$^+$ *yst*$^+$ and consequently it showed negative results for phenotypic markers related with the plasmid presence. Among *Y. enterocolitica* B1A, four strains were positive for *ystB* gene (16%).

Fig. 1 shows the different profiles of virulence genes for *Y. enterocolitica* strains obtained by multiplex PCR. The optimal annealing temperature of the four primers was 57 °C. None of *Y. intermedia* strains produced amplicons with any of these primers, evidencing the specificity of the PCR assay. Phenotypic assays correlated satisfactorily with multiplex PCR results. CR-MOX was a better phenotypic marker of the presence of the virulence plasmid than autoagglutination since none *virF*$^-$ strains showed colonies on CR-MOX agar.

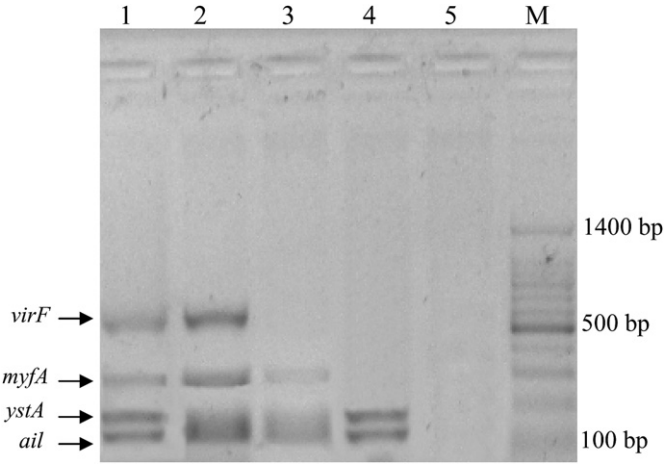

Fig. 1. Multiplex PCR targeting genes encoding virulence-associated properties: *ystA* (145 bp), *ail* (170 bp), *myfA* (272 bp) and *virF* (591 bp). Lane 1: *Y. enterocolitica* W1024 O:9 pYV(+); lane 2: *Y. enterocolitica* 2/O.9 (3G); lane 3: *Y. enterocolitica* 3/O.5 (1ME); lane 4: *Y.enterocolitica* 1A/O:6,30 (121); lane 5: *Y. enterocolitica* 1A/NAG (246); M: 100 bp ladder as the DNA size control.

3.2. PCR ribotyping

Nine SR ribotypes were obtained by this technique (Fig. 2). The SR ribotypes included one to three well-defined amplified DNA fragments ranging in length from approximately 750 to 1400 bp, some stronger than others, but all constant and reproducible under our PCR conditions. Some SR ribotypes showed one or more common fragments (i.e., SR1 and SR2; SR3 and SR4), but none of the fragments was common to all SR ribotypes. Taking into account all the strains, the DI of this technique was calculated as 0.885, but if *Y. enterocolitica* 2/O:9 strains were analyzed separately from the B1A strains, the DIs were 0.638 and 0.820, respectively. The studied strains were divided into two major clusters. Cluster A included all *Y. enterocolitica* B1A and the 3/O:5 strains which were grouped into a different SR; while cluster B contained the 2/O:9 strains.

The *Y. enterocolitica* B1A strains were grouped in SRA2, SRA3, SRA4, SRA5 and SRA6. SRA2, SRA5 and SRA6 were the most frequent SR ribotypes, and included six strains (25%) each one. Among these SR ribotypes, none of them was specific of one serotype. The *Y. enterocolitica* 3/O:5 strains were included in SRA1.

The local *Y. enterocolitica* 2/O:9 strains were included in two SR ribotypes. SRB1 included five strains, three of them isolated from pork sausage and two from minced meat. *Y. enterocolitica* Ye 099 O:9 pYV (+) and pYV (−) as well as one strain isolated in our laboratory from pork and beef sausage were included in SRB2. *Y. enterocolitica* W1024 was the only member of SRB3.

3.3. PFGE

By using PFGE, 30 of the studied strains (85.71%) were grouped into 11 genomic types (GTs) and the remaining five strains (14.28%) showed unique fingerprint profiles, generating a DI of 0.943. By this technique three major clusters were generated: cluster A contained all B1A strains, cluster B included the 3/O:5 strains and cluster C the 2/O:9 strains (Fig. 3). GTA10 was the most common fingerprint; this group included six strains (17.14%). Seven genomic groups (43.75%) included strains of the same serotype (GTA1, GTA4, GTA9, GTB, GTC1, GTC2 and GTC4), and the four remaining (25%) were integrated by strains of different serotypes (GTA5, GTA7, GTA8 and GTA10).

In cluster A, the B1A strains were grouped into ten GTs, DI = 0.900. Three of these groups included strains of the same serotype: GTA2, GTA3 and GTA4.

In relation to the *Y. enterocolitica* 2/O:9 strains, they were separated into five GT groups, with DI = 0.862. The strains that had been isolated in our laboratory from meat products were included in three groups: GTC1 (three strains), GTC2 (two strains) and GTC3 (one strain). The reference strains *Y. enterocolitica* Ye 099 O: 9 and *Y. enterocolitica* W1024, were included in GTC4 and GTC5, respectively.

4. Discussion

In the present work, 33 *Y. enterocolitica* strains were studied by phenotypic and genotypic tests and compared with reference strains. To our knowledge, there are relatively few studies that have investigated the pathogenicity and genetic relationship of *Y. enterocolitica* strains isolated in Argentina, so the real importance

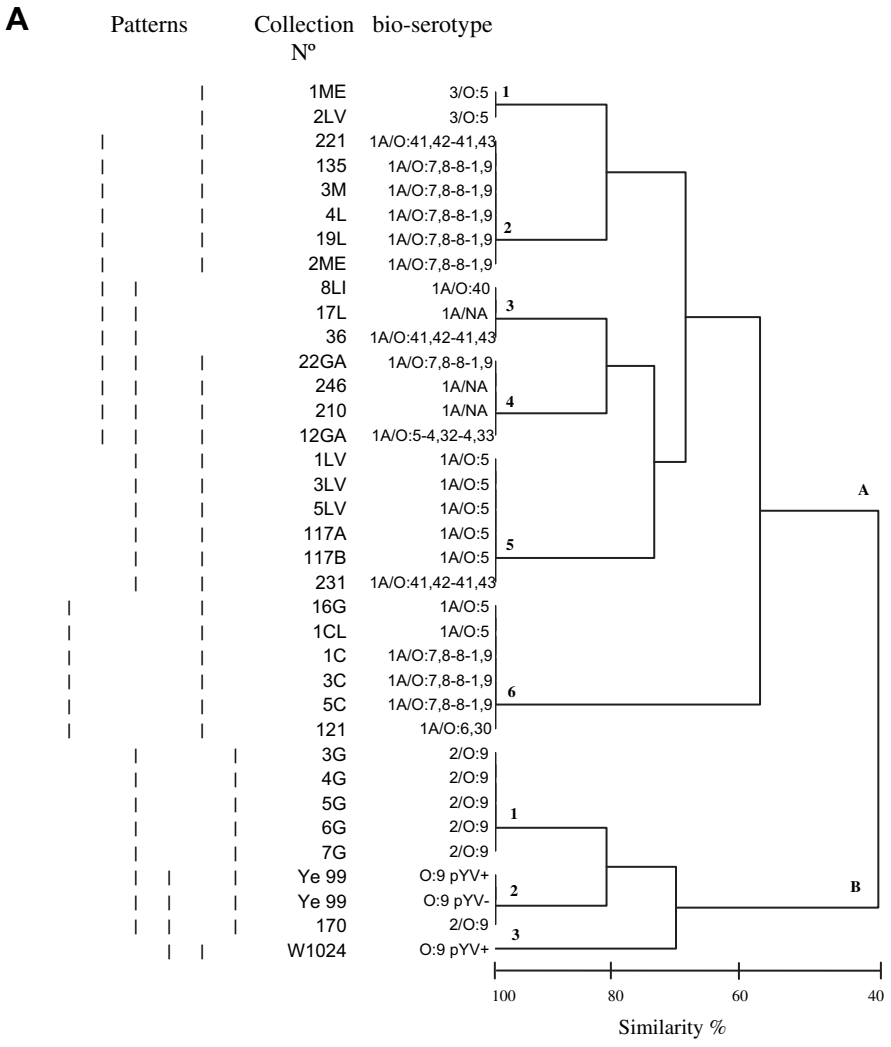

Fig. 2. PCR ribotyping of 33 *Y. enterocolitica* strains isolated in our laboratory and 2 reference strains. A) Patterns and dendrogram, B) representative band patterns: lanes 1–6: SRA, lanes 7–9: SRB.

of this bacterial species is not well-established. Eiguer et al. (1987) recovered one 1A/O:5 (bio/serotype) strain in urine from an asymptomatic woman and one 4/O:3 strain from feces of a child with an acute diarrhea. Furthermore, Paz et al. (2004) reported the isolation of *Y. enterocolitica* 1A/O:5 from a clinical case.

Among the biotypes analyzed in the present study, the biotype 2 associated with serotypes O:5,27, O:9 and O:27 has frequently been related to disease (Bottone, 1997). Although *Y. enterocolitica* 4/O:3 strains is the most common causative agent of yersiniosis in Europe and North America, it has never been isolated in our laboratory. During the period 2005–2009, 13 sporadic *Y. enterocolitica* isolates were recovered from humans in ten different cities in Argentina. These strains corresponded to biotype 4, but they have not been serotyped yet (Drs. R. Terragno and M. Pichel, National Institute for Infectious Diseases "Dr. Carlos G. Malbrán", Buenos Aires, Argentina; personal communication).

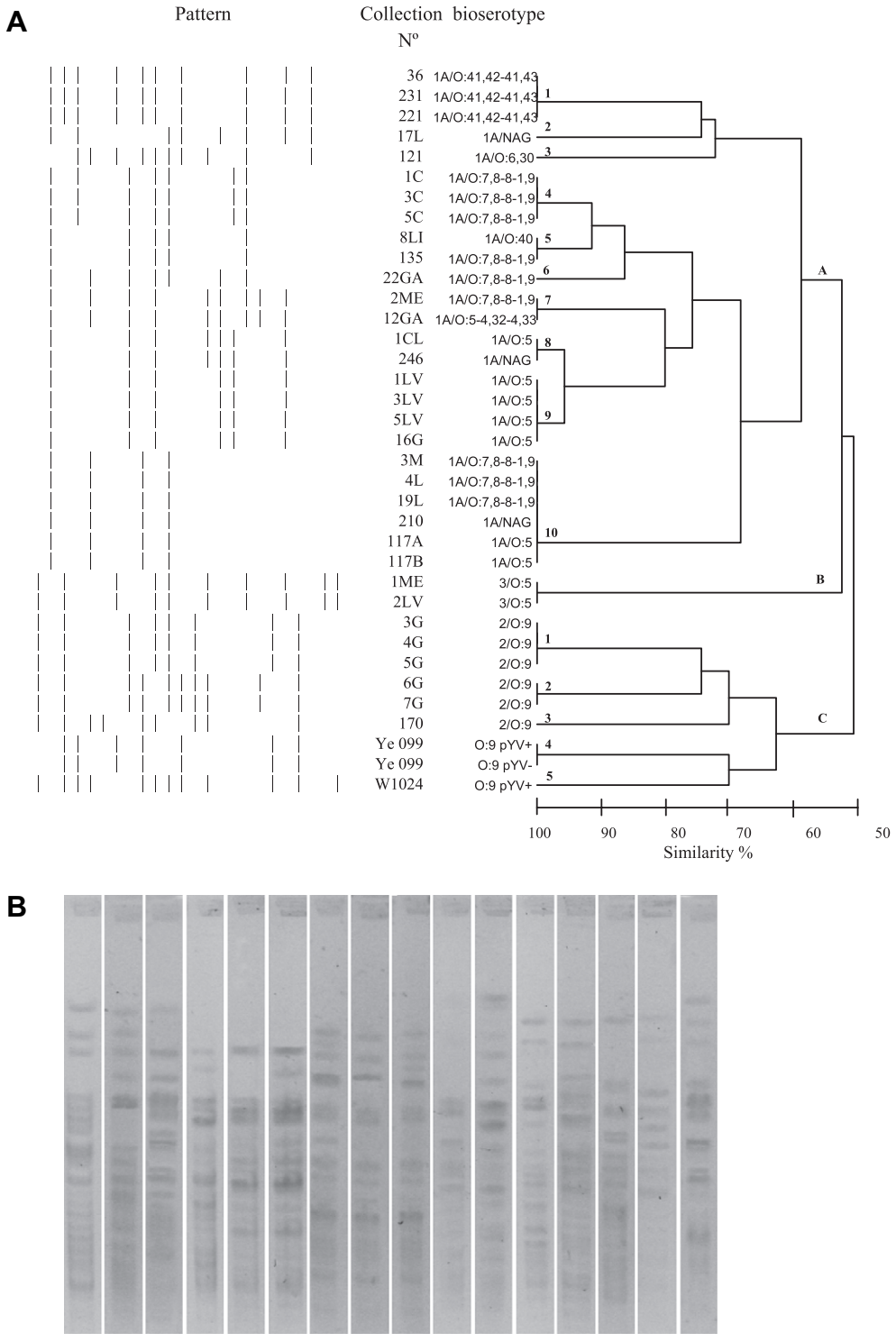

Fig. 3. PFGE of *Y. enterocolitica* strains isolated in our laboratory and the reference strains. A) Schematic patterns and dendrogram, B) representative patterns of each GT: lines 1–10: GTA, line11: GTB, lines 12–16: GTC.

Falcão et al. (2006) found a well-established correlation between pathogenic biotype and virulence behavior in phenotypic tests. Our results show that the *Y. enterocolitica* 2/O:9 strains isolated in our laboratory are potentially pathogenic. All of them were isolated from meat products, three from pork sausage, two from minced meat, and one from pork and beef sausage. Four of these strains showed virulence-related results with respect to chromosomal and plasmid phenotypic tests and amplified *virF*, *myf*, *ail* and *ystA* genes. Conversely, two strains neither amplified *virF* gene nor showed pinpoint colonies on CR-MOX agar, an indicative test of the presence of the virulence plasmid. It could be possible that they were virulent strains since they were positive for chromosomal *myf*, *ail* and *ystA* genes, but that they have lost the plasmid. It has been reported that the virulence plasmid, which

encodes the *virF* gene, could be lost during the bacteriological isolation, storage, or enrichment procedures (Cornelis et al., 1998). Our results are consistent with a previous study, conducted in our laboratory, in which eight *Y. enterocolitica* 2/O:9 strains isolated from chicken eggshells harbored the virulence plasmid and gave virulence-related results with respect to chromosomal and plasmid phenotypes (Favier et al., 2005). From the 13 *Y. enterocolitica* strains isolated by Drs Terragno and Pichel in Argentina, one isolate from human origin and one food-borne isolate (sausage) have been associated to a familiar gastroenteritis outbreak. While the human isolate carried the *ystA* and *ail* genes, the food-borne isolate was negative for these genes (personal communication). Thoerner et al. (2003) reported that 29 of 44 *Y. enterocolitica* biotype 2 strains showed positive virulence phenotypic results and amplified *ail*, *yadA* and *virF* genes. In our work, one *Y. enterocolitica* 3/O:5 strain (1ME) gave positive results for Aes and Pyz virulence tests associated to chromosome and also amplified *ystA*, *myf* and *ail* genes; the other strain (2LV) belonging to this bioserotype gave positive results for every virulence marker tested. These observations suggest that these 3/O:5 strains could have pathogenic potential.

Regarding *Y. enterocolitica* B1A strains, some authors have showed that this biotype is not as harmless as it is believed (Falcão et al., 2006). In Canada, an outbreak of gastroenteritis was described where the causative agent was *Y. enterocolitica* 1A/O:5 (Ratnam et al., 1982); in the United Kingdom another outbreak was described in which the bioserotype involved was 1A/O:6,30 (McIntyre and Nnochiri, 1986). More recently, it was reported that 17 patients on a medical ward in United States were infected with *Y. enterocolitica* 1A/O:6,30; this strain was isolated from the patients and the pasteurized milk supplied to the ward (Greenwood and Hooper, 1990). Kot et al. (2007) have reported a multiplex PCR that amplified *ail* and *ystB* genes for *Y. enterocolitica* B1A. They proposed that the *ystB* gene should be used to identify potentially pathogenic B1A strains because it has been demonstrated that enterotoxin YstB contributed to diarrhea within this biotype (Kot et al., 2007). Moreover, it has been observed that B1A strains of clinical origin showed capacity to penetrate culture epithelial cells, survive within macrophages, colonize the intestinal tract of orally infected mice, and resemble yersiniosis caused by pYV-bearing *Y. enterocolitica* strains of other biotypes in human beings (Tennant et al., 2003). We observed that 16% of B1A strains studied in the present work carried *ystB* gene, showing that it is necessary to pursue further research on the real virulence potential of this biotype.

In the present study, 35 *Y. enterocolitica* strains generated nine different SR groups by PCR ribotyping. All the *Y. enterocolitica* 2/O: 9 strains isolated in our laboratory, except for one, were closely related and went into the same SR group. These strains could be separated from the *Y. enterocolitica* 3/O:5 and B1A strains. Strains of *Y. enterocolitica* B1A could not be separated by serotype, since all SR groups include two or more serotypes. To our knowledge, only three works have used PCR ribotyping to assess the heterogeneity of *Y. enterocolitica* strains, and one of them used it only to characterize human isolates (Lobato et al., 1998). Wojciech et al. (2004) obtained eight different genotypes when comparing 35 strains isolated from human feces, swine and fox. They tested, among others, serotype O:9 and observed that human isolates shared common genotypes with swine isolates. They concluded that *Y. enterocolitica* strains belonging to the same serotype could represent different genotypes and vice versa; they did not test any B1A strain. More recently, Gulati and Virdi (2007) studied *Y. enterocolitica* B1A strains of different origins and serotypes. Their results were similar to ours, although we studied some serotypes that they did not, and we also tried to differentiate them from *Y. enterocolitica* 2/O:9 strains. They could distinguish five SR groups and observed that different serotypes produce identical genotypes.

There is not much information on *Y. enterocolitica* 2/O:9 strains studied by PFGE. The largest work on pathogenic *Y. enterocolitica* is related to bioserotype 4/O:3 (Fredriksson-Ahomaa et al., 2004; Thisted Lambertz and Danielsson-Tham, 2005; Falcão et al., 2006). In our work the 2/O:9 strains showed genomic heterogeneity since the eight studied strains produce 5 GTs. The strains isolated in our laboratory were grouped into three genotypes depending on their origin. These strains were closely related and they were included in separate genogroups from the reference strains. These results were in agreement with Favier et al. (2005), who observed four GTs originated from eight *Y. enterocolitica* 2/O:9 strains isolated from eggshells. Najdenski et al. (1994) reported that 20 strains of biotype 2 were included into 12 genotypes when they performed PFGE with the *Not*I enzyme. On the other hand, Wang et al. (2008) reported a limited heterogeneity in China, since they studied 53 2/O:9 strains and only observed 16 genogroups. In our work, the reference strain *Y. enterocolitica* 099 O:9, with and without the virulence plasmid, was observed in a single genotype. This is in agreement with a previous work reporting that the presence of the virulence plasmid did not interfere with GT patterns (Najdenski et al., 1994).

On the other hand, the 25 *Y. enterocolitica* B1A strains were clustered into ten GTs groups. These data also confirm earlier works where this biotype was more diverse in terms of its genotypes. Najdenski et al. (1994) observed 18 GTs from 20 1A/O:5 strains. Filetici et al. (2000) performed PFGE with *Xba*I enzyme and reported that 5 *Y. enterocolitica* 1A/O:6,30 strains showed 5 different genotypes, and 3 1A/O:5 strains showed 3 genotypes. Falcão et al. (2006) studied 32 B1A strains isolated from different foods by PFGE with the same enzyme, and also observed a great heterogeneity; these strains were grouped into 31 GTs. In China, Wang et al. (2008) reported that 43 *Y. enterocolitica* 1A/O:8 were included into 31 genogroups.

In conclusion, in the present work it is reported that pork meat, pork products and other meat foods obtained in our city harbored *Y. enterocolitica* 2/O:9 and 3/O:5 strains with virulence potential. The application of multiplex PCR was shown to be an efficient tool for the identification of pathogenic *Y. enterocolitica*, separating the pathogenic bioserotypes from non-virulent ones. To our knowledge, it is the first time that a study compares PCR ribotyping with the results obtained with PFGE. Although the PCR ribotyping gave us a lower DI than PFGE, both of them have revealed the high diversity of the food-related *Y. enterocolitica* strains isolated in our region. Our work highlights the role of meat foods as transmission vehicles of potentially pathogenic *Y. enterocolitica* strains in Argentina, with consequent risks for consumer's health.

Acknowledgments

This work was supported by National University of San Luis (project no 8803) and National Council of Science and Technology Research (CONICET). Authors want to thank Biochemist Gabriel Salinas for his technical support.

References

Bhagat, N., Virdi, J.S., 2007. Distribution of virulence-associated genes in *Yersinia enterocolitica* biovar 1A correlates with clonal groups and not source of isolation. FEMS Microbiol. Lett. 266, 177–183.

Bottone, E.J., 1997. *Yersinia enterocolitica*: the charisma continues. Clin. Microbiol. Rev. 10, 257–276.

Bottone, E.J., 1999. *Yersinia enterocolitica*: overview and epidemiologic correlates. Microbes Infect. 1, 323–333.

Butt, H.L., Gordon, D.L., Lee-Archer, T., Moritz, A., Merrell, W.H., 1991. Relationship between clinical and milk isolates of *Yersinia enterocolitica*. Pathology 23, 153–157.

Carniel, E., 1995. Chromosomal virulence factors of *Yersinia*: an update. Contrib. Microbiol. Immunol. 3, 218–224.

Cornelis, G.R., Boland, A., Boyd, A.P., Geuijen, C., Iriarte, M., Neyt, C., Sory, M.-P., Stainier, I., 1998. The virulence plasmid of *Yersinia*, an antihost genome. Microbiol. Mol. Biol. Rev. 62, 1315–1352.

Eiguer, T., Mercado, E.C., Fonchkowsy, B., Foguelman, A., Fernandez, E., Marguet, R., Fratalongo, J.P., Urbano, F.F., Ibáñez, S.B., Trevisan, A.R., 1987. Primer aislamiento y caracterización de *Yersinia enterocolitica* en heces humanas en Argentina. Rev. Argent. Microbiol. 19, 71–76.

Falcão, J.P., Falcão, D.P., Pitondo-Silva, A., Malaspina, A.C., Brocchi, M., 2006. Molecular typing and virulence markers of *Yersinia enterocolitica* strains from human, animal and food origins isolated between 1968 and 2000 in Brazil. J. Med. Microbiol. 55, 1539–1548.

Farmer III, J.J., Carter, G.P., Millar, V.L., Falkow, S., Wachmuth, I.K., 1992. Pyrazinamidase, CR-MOX agar, salicin fermentation, aesculin hydrolysis, and D-xylose fermentation for identifying pathogenic serotypes of *Yersinia enterocolitica*. J. Clin. Microbiol. 30, 2589–2594.

Favier, G.I., Escudero, M.E., de Guzmán, A.M.S., 2005. Genotypic and phenotypic characteristics of *Yersinia enterocolitica* isolated from surface of chicken eggshells obtained in Argentina. J. Food Prot. 68, 94–97.

Filetici, E., Anastacio, M.P., Poursshaban, M., Fantasia, M., 2000. Genotypic and phenotypic characteristics of *Yersinia* spp. isolates from food and man. Food Microbiol. 17, 261–267.

Fredriksson-Ahomaa, M., Hielm, S., Korkeala, H., 1999. High prevalence of yadA positive *Yersinia enterocolitica* in pig tongues and minced meat at retail level in Finland. J. Food Prot. 62, 123–127.

Fredriksson-Ahomaa, M., Koch, U., Klemm, C., Bucher, M., Stolle, A., 2004. Different genotype of *Yersinia enterocolitica* 4/O: 3 strains widely distributed in butcher shops in the Munich area. Int. J. Food Microbiol. 95, 89–94.

Gierczynski, R., Jagielski, M., Rastawicki, W., 2002. Evaluation of usefulness for selected virulence markers for identifying pathogenic *Yersinia enterocolitica* strains. IV. Genes *myfA* and *ureC*. Med. Dosw. Mikrobiol. 54, 347–355.

Greenwood, M.H., Hooper, W.L., 1990. Excretion of *Yersinia* spp. associated with consumption of pasteurized milk. Epidemiol. Infect. 104, 345–350.

Gulati, P.S., Virdi, J.S., 2007. The *rrn* locus and gyrB genotyping confirm the existence of two clonal groups in strains of *Yersinia enterocolitica* subspecies palearctica biovar 1A. Res. Microbiol. 158, 236–243.

Guzmán, A.M.S., Pederiva, N.B., Eiguer, T., Jiménez, D.F., 1984. *Yersinia enterocolitica*: primeros aislamientos a partir de alimentos cárnicos en Argentina. Rev. Argent. Microbiol. 116, 50–56.

Hunter, P., Gaston, M.A., 1988. Numerical index of discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26, 2465–2466.

Hussein, H.M., Fenwick, S.G., Lumsden, J.S., 2001. A rapid and sensitive method for the detection of *Yersinia enterocolitica* strains from clinical samples. Lett. Appl. Microbiol. 33, 445–449.

Ibrahim, A., Liesack, W., Griffiths, M.W., Robins-Browne, R.M., 1997. Development of a highly specific assay for rapid identification of pathogenic strains of *Yersinia enterocolitica* based on PCR amplification of the *Yersinia* heat-stable enterotoxin gene (*yst*). J. Clin. Microbiol. 35, 1636–1638.

Kandolo, K., Wauters, G., 1985. Pyrazinamidase activity in *Yersinia enterocolitica* and related organisms. J. Clin. Microbiol. 21, 980–982.

Kostman, J.R., Edlind, T.D., LiPuma, J.J., Stull, T.L., 1992. Molecular epidemiology of *Pseudomonas cepacia* determined by polymerase chain reaction ribotyping. J. Clin. Microbiol. 30, 2084–2087.

Kot, B., Trafny, E.A., Jakubczak, A., 2007. Application of multiplex PCR for monitoring colonization of pig tonsils by *Yersinia enterocolitica*, including biotype 1A, and *Yersinia pseudotuberculosis*. J. Food Prot. 70, 1110–1115.

Laird, W.J., Cavanaugh, D.C., 1980. Correlation of autoagglutination and virulence of *Yersiniae*. J. Clin. Microbiol. 11, 430–432.

Lobato, M.J., Landeras, E., Gonzales-Hevia, M.A., Mendoza, M.C., 1998. Genetic heterogeneity of clinical strains of *Yersinia enterocolitica* traced by ribotyping and relationships between ribotypes, serotypes and biotypes. J. Clin. Microbiol. 36, 3297–3301.

McIntyre, M., Nnochiri, E., 1986. A case of hospital acquired *Yersinia enterocolitica* gastroenteritis. J. Hosp. Infect. 7, 299–301.

Najdenski, H., Iteman, I., Carniel, E., 1994. Efficient subtyping of pathogenic *Yersinia enterocolitica* strains by pulsed-field gel electrophoresis. J. Clin. Microbiol. 32, 2913–2921.

Paz, M., Muzio, H., Teves, S., Santini, P., 2004. Analysis of a *Yersinia enterocolitica* isolated from human diarrheic feces in Argentina. Rev. Argent. Microbiol. 36, 164–169.

Pham, J.N., Bell, S.M., Lanzarone, J.Y.M., 1991. Biotype and antibiotic sensitivity of 100 clinical isolates of *Yersinia enterocolitica*. J. Antimicrob. Chemother. 28, 13–18.

Ratnam, S., Mercer, E., Picco, B., Parsons, S., Butler, R., 1982. A nosocomial outbreak of diarrhea disease due to *Yersinia enterocolitica* serotype O:5, biotype 1. J. Infect. Dis. 145, 242–247.

Riley, G., Toma, S., 1989. Detection of pathogenic *Yersinia enterocolitica* by using Congo red-magnesium oxalate agar medium. J. Clin. Microbiol. 27, 213–214.

Tennant, S.M., Grant, T.H., Robins-Browne, R.M., 2003. Pathogenicity of *Yersinia enterocolitica* biotype 1A. FEMS Immunol. Med. Microbiol. 38, 127–137.

Thisted Lambertz, S., Danielsson-Tham, M.-L., 2005. Identification and characterization of pathogenic *Yersinia enterocolitica* isolates by PCR and pulsed-field gel electrophoresis. Appl. Environ. Microbiol. 71, 3674–3681.

Thoerner, P., Bin Kingombe, C.I., Bögli-Stuber, K., Bissing-Choisat, B., Wassenaar, T.M., Frey, J., Jemmi, T., 2003. PCR detection of virulence genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and investigation of virulence gene distribution. Appl. Environ. Microbiol. 69, 1810–1816.

Velázquez, L.del C., Barbini, N.B., Escudero, M.E., de Guzmán, A.M., 2005. Resistance of Yersinia enterocolitica, *Escherichia coli* O157:H7 and natural microflora against acidic conditions and freezing-thawing in fresh sausages. Cent. Eur. J. Public. Health 13, 89–95.

Wang, X., Qiu, H., Jin, D., Cui, Z., Kan, B., Xiao, Y., Xu, Y., Xia, S., Wang, H., Yang, J., Wang, X., Hu, W., Xu, J., Jing, H., 2008. O:8 serotype *Yersinia enterocolitica* strains in China. Int. J. Food Microbiol. 125, 259–266.

Wojciech, L., Staroniewicz, Z., Jakubczak, A., Ugorski, M., 2004. Typing of *Yersinia enterocolitica* isolates by ITS profiling, REP- and ERIC-PCR. J. Vet. Med. 51, 238–244.