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ARTICLE

Evaluation of the pathogenic potential, antimicrobial susceptibility, and genomic relations of *Yersinia enterocolitica* strains from food and human origin

Cecilia S.M. Lucero-Estrada, José Miguel Soria, Gabriela Isabel Favier, and María Esther Escudero

Abstract: *Yersinia enterocolitica* is a food-borne pathogen that causes gastroenteritis with occasional postinfection sequels. This study was aimed to determinate the pathogenic potential, antimicrobial susceptibility, and genomic relationships of *Y. enterocolitica* strains of different bioserotypes (B/O) isolated from foods and human samples in San Luis, Argentina. Strains obtained by culture were bioserotyped and characterized by phenotypic and genotypic virulence markers, antimicrobial susceptibility, and pulsed-field gel electrophoresis (PFGE). *Yersinia enterocolitica* was detected in 9.2% of 380 samples, with a distribution of 10.6% (30/284) for food products and 5.2% (5/96) for human samples. Regarding the pathogenic potential, B1A strains of different serotypes were *virF*$^{-}$ *ail*$^{-}$, of which 72.0% (13/18) were *ystB*$^{+}$ with virulence-related phenotypic characteristics. Among B2/O:9 isolates, 75.0% (9/12) exhibited the genotype *virF*$^{+}$ *ail*$^{+}$ *ystB*$^{-}$ along with phenotypic traits associated with virulence; the same genotype was observed in 80.0% (4/5) of B3/O:3 and B3/O:5 strains. By PFGE, it was possible to separate *Y. enterocolitica* biotypes into 4 clonal groups (A to D) with 23 genomic types, generating a discriminatory index of 0.96. All isolates were susceptible to antimicrobials used for clinical treatment. This study highlights the presence of pathogenic bioserotypes and the high genomic diversity of the *Y. enterocolitica* strains isolated in our region.

Key words: Yersinia enterocolitica, foods, human samples, pathogenic potential, PFGE.

Résumé : *Yersinia enterocolitica* est un pathogène d'origine alimentaire qui occasionne des gastro-entérites parfois suivies de séquelles. La présente étude avait pour but de déterminer le potentiel pathogène, la susceptibilité aux antimicrobiens et les liens génomiques de souches de *Y. enterocolitica* de différents biosérotypes (B/O) isolés d'aliments et d'échantillons humains provenant de San Luis en Argentine. Les souches ont été obtenues par culture et caractérisées en vertu de leurs marqueurs phénotypiques et génotypiques de virulence, leur susceptibilité aux antimicrobiens et leur profil de PFGE. On a décelé *Y. entreocolitica* dans 9,2 % des 380 échantillons, à savoir 10,6 % (30/284) des aliments et 5,2 % (5/96) des échantillons humains. Concernant le potentiel pathogène, les souches B1A de différents sérotypes étaient de génotype *virF*$^{-}$ *ail*$^{-}$, desquelles 72,0 % (13/18) étaient dotées de *ystB*$^{+}$ et de caractéristiques phénotypiques liées à la virulence. Parmi les isolats B2/O:9, 75,0 % (9/12) présentaient le génotype *virF*$^{+}$ *ail*$^{+}$ *ystB*$^{-}$ ainsi que des caractères phénotypiques liées à la virulence; on a observé le même génotype chez 80,0 % (4/5) des souches B3/O:3 et B3/O:5. Le PFGE a permis de séparer les biotypes de *Y. enterocolitica* en 4 groupes clonaux (A à D) selon 23 types génomiques et un indice de discrimination de 0,96. Tous les isolats étaient susceptibles aux antimicrobiens d'usage thérapeutique en clinique. Cette étude met en évidence la présence de biosérotypes pathogènes et d'une diversité génomique élevée chez les souches de *Y. enterocolitica* provenant de notre région. [Traduit par la Rédaction]

Mots-clés : Yersinia enterocolitica, aliments, échantillons humains, potentiel pathogène, PFGE.

Introduction

Yersinia enterocolitica is a human food-borne enteropathogen that causes a self-limiting gastroenteritis, extraintestinal manifestations, and postinfectious sequelae such as erythema nodosum and reactive arthritis (Bottone 1999). Pigs are regarded as major reservoirs of *Y. enterocolitica*, and the most common route of transmission of yersiniosis is through raw or inadequately

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cooked foods. The psychrotrophic nature of this bacterium is responsible for its survival in household refrigerators (Bottone 1999). This species includes a heterogeneous group of strains, which are classified into 6 biotypes (B) and more than 57 serotypes (O). Five of the 6 biotypes (1B, 2, 3, 4, 5) carry the 72 kb virulence plasmid (pYV) and several virulence chromosomal genes, and are considered pathogenic to humans and animals (Fàbrega and Vila 2012). However, the B1A strains are generally regarded as avirulent, as they lack the pYV plasmid and major chromosomal virulence genes. Despite this, some B1A strains produce disease symptoms indistinguishable from that caused by known pathogenic biotypes (Bhagat and Virdi 2011).

In Argentina, few studies have been performed to establish the role of this bacterium as a causative agent of human disease or to estimate its impact on foods intended for human consumption. *Yersinia enterocolitica* B1A isolates have been reported from chicken carcasses in Buenos Aires (Floccari et al. 2000), and recently, a *Y. enterocolitica* surveillance on meat foods was carried out in Neuquén, with positive results (Gottardi et al. 2012). In relation to human samples, Eiguer et al. (1987) isolated 1 *Y. enterocolitica* B1A/O:5 strain from an asymptomatic patient and another B4/O:3 strain from a child's diarrheic feces. More recently, Paz et al. (2004) reported the isolation of 1 *Y. enterocolitica* B1A/O:5 strain from a diarrheic patient, and Cortes et al. (2010) isolated 6 *Y. enterocolitica* strains from the diarrheic feces of 6 children out of 181 patients. In San Luis city, *Y. enterocolitica* B2/O:9 strains among other bioserotypes have been isolated from different foods of animal origin, such as porcine, bovine, chicken meat, and eggshells; however, they were not associated with food-borne outbreaks (Favier et al. 2005; Lucero Estrada et al. 2011). The presence of chromosomal and plasmid-encoded virulence markers is related to the potential pathogenicity of *Y. enterocolitica* strains, as has been demonstrated by PCR in isolates from Argentina and Brazil (Lucero Estrada et al. 2012; Paixão et al. 2013). Additionally, the subtyping of this pathogen by molecular methods is especially valuable, since it permits to establish genomic relations between clones. In this way, pulsed-field gel electrophoresis (PFGE) has been widely used to determine strain relatedness, confirm outbreaks, and identify the different sources of isolates (Fredriksson-Ahomaa et al. 2006).

For contributing to the characterization of *Y. enterocolitica* strains from foods and human samples in San Luis, the aims of this study were (*i*) to determine the pathogenic potential of the isolates by phenotypic and genotypic tests, (*ii*) to test the antimicrobial susceptibility, and (*iii*) to establish relationships among the isolates by means of genomic DNA macrorestriction analysis using PFGE.

Materials and methods

Bacterial strains

Yersinia enterocolitica W1024 B2/O:9, provided by G. Cornelis (Belgium), and *Y. enterocolitica* MCH 700 B4/O:3 and *Y. enterocolitica* 29C/43 B4/O:3, both provided by G. Kapperud (Norway), were used as reference strains. Four *Yersinia intermedia*, 2 *Yersinia frederiksenii*, 1 *Salmonella* Enteritidis, and 1 *Salmonella* Typhimurium strains previously isolated in our laboratory were used to test the specificity of the 16S rRNA gene PCR. All strains were maintained in Luria–Bertani broth plus 20% (*v*/*v*) glycerol and stored at –20 °C. For subsequent studies, *Yersinia* and *Salmonella* strains were individually grown in 5 mL of trypticase soy broth (TSB, Britania, Buenos Aires, Argentina) for 18 to 20 h at 22 °C and 37 °C, respectively.

Sample collection

In the 2-year period 2010–2012 a total of 380 samples divided into 2 groups were investigated for the presence of *Y. enterocolitica*:

(*i*) food samples — a total of 284 samples were purchased at random from 7 retail stores and supermarkets located in San Luis city, Argentina. The studied samples were eggshells (n = 72), pork sausages (n = 74), minced meat (n = 68), and chicken carcasses (n = 70). After purchase, each sample was transported in its original container under refrigeration and immediately processed or stored at 4 °C for up to 6 h.

(*ii*) clinical samples — 96 fecal samples from patients with diarrhea were collected from 3 public and private clinical laboratories located in San Luis city, Argentina. The samples were from patients under 10 years old; negative for *Salmonella* spp., *Shigella* spp., and *Escherichia coli* O157 H:7.

Microbiological analysis

Yersinia enterocolitica cultures from food samples were performed according to the FDA *Bacteriological Analytical Manual* (Weagant and Feng 2001). Samples of 25 g were homogenized in a stomacher (IUL Masticator, Germany) for 30 s and incubated in 225 mL of peptone sorbitol bile broth (PSBB; Britania) at 10 °C for 10 days. Eggshells were treated according to Favier et al. (2005) with some modifications. Briefly, entire eggs were individually placed in sterile plastic bags containing 25 mL of TSB (Britania), softly hand shaken for 1 min to facilitate bacterial detachment, and incubated at 25 °C during 24 h. After that, 1 mL of TSB was taken and diluted in 99 mL of PSBB for incubation at 10 °C for 9 days. Isolation from fecal samples was performed according to Z. Haoxuan, W. Jide, Z. Mingjun, S. Yong, and J. Bo (unpublished), with some modifications. In brief, 0.5 g of sample was added into 4.5 mL of 0.85% NaCl. After homogenization, 1 mL was seeded into 9 mL of PSBB and cultured for 10 days at 10 °C. After the enrichment period, 0.1 mL of each sample was spread on MacConkey (Britania) and cefsulodin–irgasan–novobiocin (Merck, Darmstadt, Germany) agars and

Table 1. Primers used for multiplex PCR and amplicon sizes.

Gene	Sequence (5′–3′)	Amplicon length (bp)	Reference
virF	TCATGGCAGAACAGCAGTCAG ACTCATCTTACCATTAAGAAG	591	Hussein et al. 2001
ail	GATGATAACTGGGGAGTAATAGG CGTATGCCATTGACGTCTTAC	238	This study (GenBank No. M29945.1)
ystB	GTACATTAGGCCAAGAGACG GCAACATACCTCACAACACC	146	Bhagat and Virdi 2011

incubated for 48 h at 25 and 37 °C, respectively. Typical colonies were picked and tested by Gram staining and classical biochemical methods (Bottone et al. 2005). Confirmation of biotypes and serotypes was performed by E. Carniel, Reference Center for Yersinia, Pasteur Institute, Paris, France.

Virulence phenotypic tests

The following assays were performed as described previously: autoagglutination at 37 °C (Laird and Cavanaugh 1980), calcium-dependent growth, and Congo red absorption (CR-MOX) to differentiate between plasmid-bearing and plasmidless strains, esculin hydrolysis, and pyrazinamidase production to demonstrate other virulence traits (Farmer et al. 1992; Riley and Toma 1989).

Antimicrobial susceptibility

The antimicrobial susceptibility of *Y. enterocolitica* isolates was determined by the disk diffusion method on Mueller–Hinton agar (Britania) and performed according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2014). The following antibiotic disks (Britania) were used: amikacin (30 μg), ampicillin (10 μg), aztreonam (30 μg), cephalothin (30 μg), cefuroxime (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), colistin (10 μg), erythromycin (15 μg), phosphomycin (50 μg), furazolidone (5 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), neomycin (30 μg), rifampicin (5 μg), tetracycline (30 μg), and trimethoprim–sulfamethoxazole (25 μg, TMS). Zones of growth inhibition were evaluated according to CLSI standards (CLSI 2014). The reference strain *Escherichia coli* ATCC 25922 was used as a control.

DNA extraction

DNA extraction was made using a boiling protocol with minor modifications (Leotta et al. 2005). Briefly, 1 mL of *Y. enterocolitica* suspension was centrifuged at 10 000*g* for 5 min. The pellet was suspended in 150 μL of 1× TE buffer (10 mmol/L Tris (Sigma-Aldrich, St. Louis, Missouri, USA), 1 mmol/L EDTA (Sigma), pH 8.0) supplemented with 1% Triton X-100 (Parafarm, Buenos Aires, Argentina), boiled for 15 min, and then centrifuged at 10 000*g* for 5 min. A 50 μL aliquot of the supernatant containing DNA was transferred to another tube and stored at 4 °C.

Polymerase chain reaction for 16S rRNA gene detection

A 25 μL volume of a reaction mix containing 1× PCR buffer, 0.2 mmol/L dNTPs, 1.5 mmol/L $MgCl_2$, 1 pmol/mL (each) specific primer (forward 5′-GGAATTTAGCAGAGATGCTTTAG-3′ and reverse 5′-GGACTACGACAGACTTTATGTG-3′), 1 U of *Taq* polymerase (PB-L, Quilmes, Argentina), 2 μL of template, and ultrapure water was prepared. The primers were designed in this study using GenBank accession No. NC_015475.1 and OligoCalc software (Kibbe 2007). PCR was performed as follows: 95 °C for 5 min, then 25 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, and a final extension of 72 °C for 3 min in a thermocycler (BioRad, Hercules, California, USA). The products were analyzed by 2% agarose gel electrophoresis in comparison with a 100 bp molecular mass DNA marker (PB-L, Quilmes, Argentina). The bands were visualized by staining with GelRed Acid Gel Stain (Biotium, Hayward, California, USA), 1.5 μL stock solution/40 mL gel, and photographed.

Multiplex PCR for virulence genetic markers

Three virulence genetic markers, *virF*, *ail*, and *ystB*, were assayed by multiplex PCR with primers presented in Table 1. A step-by-step empirical approach was used to establish the optimal annealing temperature, raising the temperature from 55 to 60 °C with 1 °C increments, and to determine the concentration of primers, $MgCl_2$, and deoxynucleoside triphosphates in the reaction mixture. The final PCR protocol was as follows: 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 1 U of *Taq* DNA polymerase, 0.2 mmol/L (each) dNTP, 10 pmol of each primer set, and 2 μL of DNA as template. The amplification conditions were as follows: 95 °C for 3 min, followed by 25 cycles of 95 °C for 45 s, 58 °C for 45 s, and 72 °C for 45 s, with a final extension at 72 °C for 5 min. The products were analyzed and visualized as described previously.

PFGE

All strains were genotyped by PFGE according to the PulseNet protocol (http://www.cdc.gov/pulsenet/PDF/ecoli-shigella-salmonella-pfge-protocol-508c.pdf) with some modifications. Bacterial cells grown overnight on trypticase soy agar (TSA; Britania) plates were suspended directly in 4 mL of the suspension buffer (100 mmol/L Tris, 100 mmol/L EDTA, pH 8.0) to an OD of 1.0 at 600 nm. Two hundred microlitres of bacterial suspension was mixed with an equal volume of 1% SeaKem Gold agarose (Cambrex, Rockland, Maine, USA) and poured into molds to obtain “plugs”. Plugs were treated for 20 h in a lysis solution (50 mmol Tris–EDTA (Sigma); 1% sodium lauroyl sarcosine (Sigma), and 0.1 mg/mL Proteinase K (Fluka Chemie, Buchs, Switzerland); pH 8.0) at 37 °C and then washed 4 times with TE buffer for 30 min at 37 °C. Chromosomal DNA contained in agarose plugs was digested

with 10 U of *Xba*I (Fermentas, Burlington, Ontario, Canada) for 2 h, according to the manufacturer's instructions. Plugs were cut into approximately 1-mm-thick slices, placed in a CHEF-DRIII chamber (BioRad), and PFGE was performed 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 (BioRad) submerged in 0.5× TBE buffer (45 mmol/L Tris–borate and 1 mmol/L EDTA). *Salmonella* Braenderup H9812 strain was used a molecular reference marker. The gels were stained with GelRed® Acid Gel Stain (Biotium) and photographed.

Data analysis

Statistical analysis of the frequency of *Y. enterocolitica* recovery related to the type of sample was performed using a χ^2 test (Analytical Software, Tallahassee, Florida, USA). Calculations were based on a confidence level equal or higher than 95% ($p \leq 0.05$ was considered statistically significant). The levels of relatedness of the isolates were determined through a comprehensive pairwise comparison of restriction fragment sizes, using the Dice coefficient. The discrimination index (DI) values of PFGE were calculated by Simpson's diversity index (Hunter and Gaston 1998). Clustering of the patterns obtained by the PFGE was performed using Statistica 6.0 software (StatSoft Inc., Tulsa, Oklahoma, USA), and the mean values obtained from the Dice coefficients were applied in the unweighted pair group method with arithmetic average (UPGMA).

Results

Distribution of *Y. enterocolitica* in food and clinical samples

Results of distribution of *Y. enterocolitica* from food and clinical samples are summarized in Table 2. A total of 35 (9.2%) of 380 samples were positive for *Y. enterocolitica* isolation. From 10.5% of food samples 30 strains were isolated and from 5.2% human feces samples 5 strains were isolated, with B1A (18 strains) and B2 (12 strains) ($p \leq 0.05$) the most frequently isolated biotypes. Sixteen *Y. enterocolitica* B1A strains were recovered from pork sausage, minced meat, and chicken carcasses (5.6% of total food samples), 2 were recovered from human feces (2.0% of total fecal samples), while 11 *Y. enterocolitica* B2 strains were recovered from eggshell and 1 from pork sausage (4.2% of food). Moreover, 2 *Y. enterocolitica* B3 strains were recovered from pork sausage (0.7% of food) and another 3 strains from clinical samples (3.1% of total fecal samples). The frequency of isolation was significantly higher from eggshell (15.3%) and minced meat (14.7%) than from the other food and human samples ($p \leq 0.05$).

Phenotypic and genotypic characterization of *Y. enterocolitica* isolates

All the strains isolated in this study, including reference strains, amplified a 300 bp region corresponding to the *Y. enterocolitica* 16S rRNA gene. *Yersinia intermedia*, *Y. frederiksenii*, *Salmonella* Enteritidis, and *Salmonella* Typhimurium assayed to test the specificity of the 16S rRNA gene PCR did not show this band (Figs. 1A and 1B).

Table 2. Prevalence of *Yersinia enterocolitica* in food and clinical samples.

Source	No. of analyzed samples	No. of positive samples* (%)	*Y. enterocolitica* biotype/serotype†
Food samples			
Eggshell	72	11a (15.28)	2/O:9 (11)
Pork sausage	74	7b (9.46)	3/O:5 (2)
			2/O:9 (1)
			1A/O:7,8-8-8,19 (1)
			1A/O:4,32-4,33 (1)
			1A/O:7,8-8-8-13-8,19 (1)
			1A/O:5 (1)
Minced meat	68	10a (14.70)	1A/O:5 (3)
			1A/O:7,8-8-8,19 (3)
			1A/O:4,32-4,33 (1)
			1A/O:41,42-41,43 (1)
			1A/O:6,30-6,31 (1)
			1A/NAG‡ (1)
Chicken skin	70	2d (2.86)	1A/O:5 (1)
			1A/O:5-4,34-4,33 (1)
Clinical samples			
Feces	96	5c (5.21)	1A/O:7,8-8-8,19 (1)
			1A/O:5 (1)
			3/O:3 (3)
Total	380	35 (9.21)	

*Values followed by different letters are statistically significantly different ($p < 0.05$).

†Values in parentheses indicate the number of strains belonging to each *Y. enterocolitica* bioserotype per type of sample.

‡NAG, non agglutinable.

The results of the phenotypic and genotypic virulence characteristics of *Y. enterocolitica* strains are shown in Table 3. By multiplex PCR, most B2 (75.0%, 9/12) and B3 (80.0%, 4/5) *Y. enterocolitica* isolates presented the genotype *virF*$^+$ *ail*$^+$ *ystB*$^-$, which was in agreement with the virulence phenotype. Only 4 *Y. enterocolitica*, 3 B2, and 1 B3 isolate showed the genotype *virF*$^-$ *ail*$^+$ *ystB*$^-$ (Fig. 2A; Table 3). All *Y. enterocolitica* B1A strains presented negative results for phenotypic markers related to plasmid presence and positive results for hydrolysis of pyrazinamide and aesculin. Although all B1A strains were negative for *virF* and *ail* genes, most of them (72.0%, 13/18) were positive for the *ystB* gene (Fig. 2B; Table 3).

Antimicrobial susceptibility

All the *Y. enterocolitica* strains isolated in this study were susceptible to amikacin, aztreonam, cefuroxime, ciprofloxacin, chloramphenicol, colistin, phosphomycin, furazolidone, gentamicin, kanamycin, nalidixic acid, neomycin, tetracycline, and TMS, and resistant to ampicillin, cephalothin, erythromycin, and rifampicin (Table 4).

PFGE

Figure 3 shows the DNA restriction patterns and dendrogram obtained by PFGE. Among the 38 studied

Fig. 1. Simple PCR for detection of the 16S rRNA gene in *Yersinia enterocolitica*. (A) Lanes: 1, *Y. intermedia* B1/O:4,32-4,33; 2, *Y. intermedia* B1/O:52; 3, *Y. intermedia* B4/O:17; 4, *Y. intermedia* B4/O:40; 5, *Y. frederiksenii* non agglutinable; 6, standard size marker (100 to 1000 bp); 7, *Y. enterocolitica* W1024 as reference strain; 8, negative control; 9, *Y. frederiksenii* O:16-16,29; 10, *Salmonella* Enteritidis 9,12:g,m:-; 11, standard size marker (100 to 1000 bp); 12, *Salmonella* Typhimurium 4,5,12:I:1,2. (B) Lanes: 1, *Y. enterocolitica* B2/O:9 (W1024); 2, *Y. enterocolitica* B2/O:9 (GFO041); 3, *Y. enterocolitica* B3/O:3 (GFO045); 4, *Y. enterocolitica* B3/O:5 (GFO048); 5, *Y. enterocolitica* B4/O:3 (MCH 700); 6, *Y. enterocolitica* B1A/O:41,42-41,43 (GFO018); 7, negative control; 8, standard size marker (100 to 1000 bp).

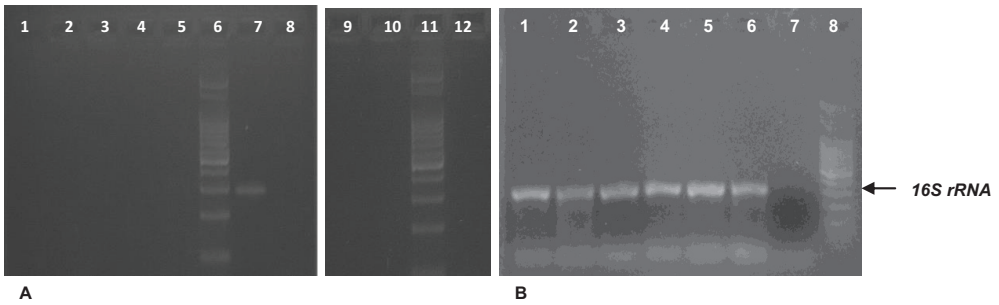
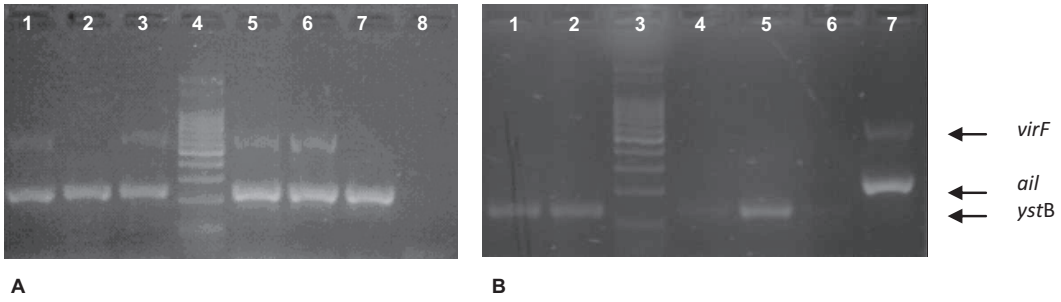


Fig. 2. Multiplex PCR targeting genes encoding virulence-associated properties from *Yersinia enterocolitica*: *ail* (238 bp), *ystB* (145 bp), and *virF* (591 bp). (A) Lanes: 1, *Y. enterocolitica* B2/O:9 (W1024); 2, *Y. enterocolitica* B2/O:9 (GFO041); 3, *Y. enterocolitica* B4/O:3 (MCH 700); 4, standard size marker (100 to 1000 bp); 5, *Y. enterocolitica* B2/O:9 (GFA001); 6, *Y. enterocolitica* B3/O:5 (GFO048); 7; *Y. enterocolitica* B3/O:3 (GFO045); 8, negative control. (B) Lanes: 1, *Y. enterocolitica* B1A/O:7,8-8-8,19 (GFO022); 2, *Y. enterocolitica* B1A/O:41,42-41,43 (GFO018); 3, standard size marker (100 to 1000 bp); 4, *Y. enterocolitica* B1A/O:6,30-6,31 (GFO006); 5, *Y. enterocolitica* B1A/O:5-4,34-4,33 (GFO012); 6, negative control; 7, *Y. enterocolitica* B4/O:3 (MCH 700) as a reference strain.



strains, including 35 isolates obtained in this work plus 3 reference strains, 23 different genomic types (GTs) were obtained, generating a DI of 0.958. Four major clusters were generated: cluster A contained all *Y. enterocolitica* B1A strains grouped into 10 GTs (DI = 0.908), cluster B included local and reference *Y. enterocolitica* B2/O:9 strains separated into 9 GTs (DI = 0.923), cluster C involved only the reference *Y. enterocolitica* B4/O:3 strains, and cluster D contained all *Y. enterocolitica* B3 strains separated into 2 GTs. Seven GTs (30.5%) included strains belonging to the same bioserotype (GTA5, GTA6, GTA7, GTB1, GTB6, GTD1, GTD2), and the remaining 16 GTs (69.5%) comprised a unique strain. GTA5 was the most common pattern band found, with 6 strains (15.7%) included in this group.

The *Y. enterocolitica* GFO017 (B1A/O:7,8-8-8,19) strain isolated from human feces was typed in GTA4 and shared 93% similarity with the GFO010 strain of the same bioserotype, typed in GTA3, and isolated from food. GTA5 included the GFO044 (B1A/O:5) strain isolated from a human sample and 5 strains of the same bioserotype isolated from food samples. In relation to B3/O:3 strains isolated from human samples, they typed together in GTD1 and had 86% similarity with B3/O:5 strains of food origin, included in GTD2.

Discussion

The presence of *Y. enterocolitica* on eggshells (Favier et al. 2005), poultry, pork, and beef samples (Velázquez et al. 1993; Lucero Estrada et al. 2012; Favier et al. 2014) was previously demonstrated in San Luis. These results encouraged further investigations in these kinds of samples and the subsequent characterization of the isolated strains. In this study, 30 *Y. enterocolitica* strains were recovered from 284 food samples after cold enrichment, which represents a prevalence of 10.6%. Biotype 1A combined with different serotypes (16 strains) was the most frequently detected biotype, followed by B2/O:9 (12 strains) and B3/O:5 (2 strains) ($p \leq 0.05$). These results showed a higher recovery rate than those previously obtained in our laboratory: Velázquez et al. (1993) reported 1.1% positive samples from 450 cold foods, which yielded 4 B2/O:9 and 1 B1A/O:5 strain, Favier et al. (2005) isolated 2.3% of *Y. enterocolitica* B2/O:9 from eggshell (8 strains), and Lucero Estrada et al. (2012) recovered

Table 3. Isolates and virulence markers detected using phenotypic tests and multiplex PCR.

				Phenotypic characteristics*				Multiplex PCR†		
Collection No.	Biotype	Serotype	Origin	AA	CR	Pyz	Aes	*virF*	*ail*	*ystB*
W1024	2	O:9	Reference	+	+	–	–	+	+	–
MCH 700	4	O:3	Reference	+	+	–	–	+	+	–
29C/43	4	O:3	Reference	+	+	–	–	+	+	–
GFA001	2	O:9	Egg	+	+	–	–	+	+	–
GFB002	2	O:9	Egg	+	+	–	–	+	+	–
GFC003	2	O:9	Egg	+	+	–	–	+	+	–
GFO111	2	O:9	Egg	+	+	–	–	–	+	–
GFO222	2	O:9	Egg	+	+	–	–	+	+	–
GFO333	2	O:9	Egg	+	+	–	–	+	+	–
GFO444	2	O:9	Egg	+	+	–	–	+	+	–
GFO555	2	O:9	Egg	+	+	–	–	–	+	–
GFG555	2	O:9	Egg	+	+	–	–	+	+	–
GFO055	2	O:9	Egg	+	+	–	–	+	+	–
GFO500	2	O:9	Egg	+	+	–	–	+	+	–
GFO041	2	O:9	Pork sausage	+	+	–	–	–	+	–
GFO045	3	O:3	Clinical sample	+	+	–	–	–	+	–
GFO046	3	O:3	Clinical sample	+	+	–	–	+	+	–
GFO047	3	O:3	Clinical sample	+	+	–	–	+	+	–
GFO048	3	O:5	Pork sausage	+	+	–	–	+	+	–
GFO049	3	O:5	Pork sausage	+	+	–	–	+	+	–
GFO004	1A	7,8-8-8,19	Pork sausage	–	–	+	+	–	–	+
GFO005	1A	4,32-4,33	Pork sausage	–	–	+	+	–	–	+
GFO006	1A	6,30-6,31	Minced meat	–	–	+	+	–	–	+
GFO010	1A	7,8-8-13-8,19	Pork sausage	–	–	+	+	–	–	+
GFO011	1A	NAG‡	Minced meat	–	–	+	+	–	–	–
GFO012	1A	5-4,34-4,33	Chicken skin	–	–	+	+	–	–	+
GFO016	1A	5	Minced meat	–	–	+	+	–	–	–
GFO017	1A	7,8-8-8,19	Clinical sample	–	–	+	+	–	–	+
GFO018	1A	41,42-41,43	Minced meat	–	–	+	+	–	–	+
GFO019	1A	5	Minced meat	–	–	+	+	–	–	+
GFO020	1A	7,8-8-8,19	Minced meat	–	–	+	+	–	–	+
GFO021	1A	7,8-8-8,19	Minced meat	–	–	+	+	–	–	–
GFO022	1A	7,8-8-8,19	Minced meat	–	–	+	+	–	–	+
GFO023	1A	4,32-4,33	Minced meat	–	–	+	+	–	–	+
GFO040	1A	5	Minced meat	–	–	+	+	–	–	+
GFO042	1A	5	Chicken skin	–	–	+	+	–	–	–
GFO043	1A	5	Pork sausage	–	–	+	+	–	–	+
GFO044	1A	5	Clinical sample	–	–	+	+	–	–	–

*+, positive reaction; –, negative reaction. AA, autoagglutination; CR, congo red absorption; Pyz, pyrazynamidase reaction; Aes, aesculin hydrolysis.

†+, positive reaction; –, negative reaction.

‡NAG, non agglutinable.

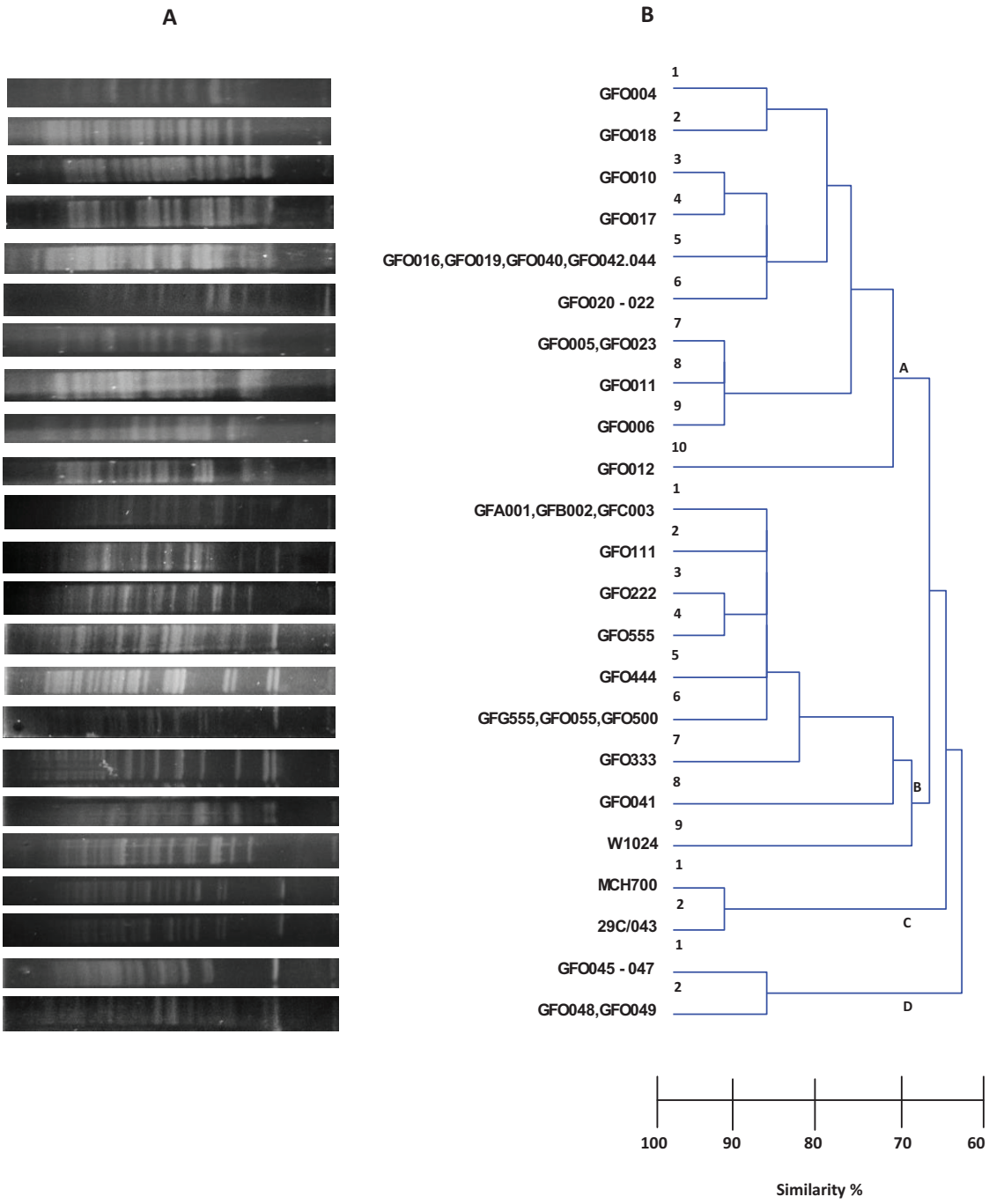
Table 4. Antimicrobial susceptibility of *Yersinia enterocolitica* strains isolated from food and human feces.

Biotype	No. of strains	AKN	AM	AZT	CEF	CXM	CIP	CMP	COL	ERY	FOS	FUR	GEN	K	NAL	NEO	RFA	TET	TMS
2	15	100	0	100	0	100	100	100	100	0	100	100	100	100	100	100	0	100	100
3	5	100	0	100	0	100	100	100	100	0	100	100	100	100	100	100	0	100	100
1A	18	100	0	100	0	100	100	100	100	0	100	100	100	100	100	100	0	100	100
Total	38	100	0	100	0	100	100	100	100	0	100	100	100	100	100	100	0	100	100

Note: AKN, amikacin; AM, ampicillin; AZT, aztreonam; CEF, cephalothin; CXM, cefuroxime; CIP, ciprofloxacin; CMP, chloramphenicol; COL, colistin; ERY, erythromycin; FOS, phosphomycin; FUR, furazolidone; GEN, gentamicin; K, kanamicyn; NAL, nalidixic acid; NEO, neomycin; RFA, rifampicin; TET, tetracycline; and TMS, trimethoprim–sulfamethoxazole.

5.9% of B2/O:9 and B1A strains from meat products (14 strains). Bernardino-Varo et al. (2013) isolated B3 strains in 8.54% of raw cow's milk samples in Mexico City. Furthermore, these authors isolated B1A strains in 70.0%, B2 in 13.2%, and B4 in 8.15% of their samples. Current research in Brazil indicates the recovery of *Y. enterocolitica* B4 and B1A from slaughterhouses, markets, and clinical cases in humans, by culture methods (Paixão et al. 2012, 2013). Even

Fig. 3. Pulsed-field gel electrophoresis (PFGE) patterns of 35 *Yersinia enterocolitica* isolates from food (30) and clinical (5) samples. (A) Representative band patterns of each genotype (GT), and (B) dendrogram. See Table 3 for description of the collection Nos. used in panel B.



though no *Y. enterocolitica* B4/O:3 strain was isolated in the present study, foods might probably act as vehicles for human infection by this bioserotype, in Argentina (Eiguer et al. 1987).

Even though poultry seems not relevant in *Y. enterocolitica* transmission, and eggshells and chicken skin are uncommon foodstuffs, 91.6% of *Y. enterocolitica* B2/O:9 strains were isolated from eggshell and 11.1% of B1A strains from chicken carcasses in this work. The contamination of these products could probably occur by contact with other *Y. enterocolitica*-contaminated animal products on farms and during transportation or handling in retail shops. When outer defense barriers of the eggshell, such as the cuticle (a thin surface membrane of protein nature), are altered or damaged, *Y. enterocolitica* might penetrate into the egg interior (Favier et al. 2009) and be ingested by consumers through raw or unpasteurized egg-based meals. Outbreaks associated with the consumption of *Y. enterocolitica*-contaminated eggs or poultry have not been reported probably because any

Y. enterocolitica remaining on eggshells (Favier et al. 2005) or chicken carcasses is eliminated by washing or cooking.

Regarding clinical isolates, 5 *Y. enterocolitica* strains of this origin were isolated in this work: 2 B1A of different serotypes and 3 B3/O:3. No *Y. enterocolitica* outbreak was reported, and all strains were isolated from sporadic cases. This is the first time that *Y. enterocolitica* B3 has been isolated from human samples in our country, but it was not possible to correlate any human isolate with the probable source of transmission, because we could not analyze household samples or involved foods. To our knowledge, no *Y. enterocolitica* outbreak related to human infection has been reported so far in Argentina. However, strains belonging to B1A/O:5 (Paz et al. 2004), B4 (Sanchez and Gonzalez 2013), and other bioserotypes (Cortes et al. 2010) have been sporadically recovered from human diarrheic feces in this country.

Previously, the 16S rRNA gene has been used for the unambiguous identification of *Y. enterocolitica* within the genus by using PCR (Kechagia et al. 2007). Lantz et al. (1998) developed a pair of primers based on the variable V3 and V9 regions of this gene, which was also used for real-time PCR amplification by Wolffs et al. (2004). Since *Y. intermedia* strains were also detected with these primers (Lantz et al. 1998), we modified them by adding a guanine residue to the 3′ extreme of both primers and by substituting a cytosine with a guanine in the 20th nucleotide of the reverse primer to increase its specificity. So, we demonstrated that these modified primers did not anneal to 16S rRNA of related species and confirmed that all analyzed strains belonged to *Y. enterocolitica*.

We demonstrated the presumptive presence of the pYV virulence plasmid in the majority of the B2 and B3 strains of food and human origin that were positive for the *virF* gene and plasmid-related virulence phenotypic characteristics. However, since this gene was not amplified in 4 strains belonging to these biotypes, we hypothesized that a mutation produced in *virF* would not allow its amplification by PCR, or perhaps, the virulence plasmid was lost during the bacterial isolation, storage, or enrichment procedures (Cornelis et al. 1998). These results were consistent with a previous study conducted in our laboratory in which 4 of 6 *Y. enterocolitica* B2/O:9 and 2 B3/O:5 strains isolated from food samples showed pathogenicity potential in virulence phenotypic tests and amplified *virF*, *myf*, *ail*, and *ystA* genes; however, 2 B2/O:9 strains neither amplified the *virF* gene nor showed pinpoint colonies on CR-MOX agar but exhibited unspecific autoagglutination (Lucero Estrada et al. 2011).

The isolation of B1A strains from human samples with gastrointestinal symptoms (Bhagat and Virdi 2011) and the detection of virulence markers by PCR (Campioni and Falcão 2014) still intrigue many researchers in terms of the pathogenic potential of these strains. In the present work, we observed that although all B1A strains were negative for the *virF* and *ail* genes by PCR in agreement with the phenotypic assays, 72.0% were positive for the *ystB* gene, including one isolated from human feces. This gene has been previously described in *Y. enterocolitica* B1A strains isolated from human samples, foods, and other sources (Wang et al. 2008; Stephan et al. 2013). Also, a study on 259 isolates of *Y. enterocolitica* and related species indicated that Yst-B might be the major contributor to diarrhea produced by this biotype (Singh and Virdi 2004). Besides, there is evidence that infections with B1A strains are more common in individuals that are generally predisposed to infections. So, the pathogenicity of this group might be mainly linked to an attribute of the host that fails to resist the attack of a relatively innocuous agent (Bhagat and Virdi 2011).

Similar to the present study, Favier et al. (2005) showed that all *Y. enterocolitica* strains isolated from the egg surface were susceptible to amikacin, ciprofloxacin, chloramphenicol, and TMS, and resistant to rifampicin, while Lucero Estrada et al. (2012) observed that *Y. enterocolitica* strains isolated from meat products were resistant to ampicillin, erythromycin, and cephalotin, in our region. The intrinsic resistance to β-lactam antibiotics by *Y. enterocolitica* is well detailed in the literature (Bonke et al. 2011). Resistance to many β-lactam antibiotics and erythromycin in *Y. enterocolitica* strains isolated from foods in Italy (Bonardi et al. 2010) and animal foods in Greece (Kechagia et al. 2007) has been detected. Human *Y. enterocolitica* strains belonging to various bioserotypes in Switzerland showed antimicrobial susceptibility results similar to those observed in this study (Fredriksson-Ahomaa et al. 2012).

To subtype *Y. enterocolitica* isolates and establish clonal relationships among them, PFGE was performed, thus it was possible to separate the analyzed isolates into 4 clonal groups (A to D) on the basis of their biotypes. Cluster A comprised the 18 *Y. enterocolitica* B1A strains, which were grouped into 10 GTs. Among these strains, 2 were isolated from human feces, 1 clustered alone (GTA4), and the other 1 clustered together with 5 strains of the same serotype (GTA5) isolated from food. Furthermore, the other strains isolated from foods were separated according to their serotype, but no clustering tendency among GTs related to source or any other identifiable determinant could be demonstrated. These results confirmed earlier works in which this biotype was highly diverse in terms of their genotypes. For instance in San Luis City, Argentina, Lucero Estrada et al. (2011) performed *Xba*I-PFGE and observed that 25 *Y. enterocolitica* B1A strains of diverse serotypes isolated from meat food were clustered in 10 different genotypes. In Brazil, Paixao et al. (2013) studied 22 B1A strains from pork, markets, and slaughterhouses by using *Not*I-PFGE, and also observed a higher heterogeneity, since these strains were grouped into 22 GTs. With this same method, Wang et al. (2008) observed 31 GTs from 43 B1A/O:8 strains isolated from animals and foods in China, and Stephan et al.

(2013) showed a great genetic diversity among all B1A strains isolated from humans in Switzerland.

Cluster B included *Y. enterocolitica* B2/O:9 strains, which showed the highest genomic heterogeneity, since 12 local strains produced 8 GTs by PFGE and only 2 of these genotypes included more than 1 strain, even though all of them were recovered from eggshells. The 3 *virF*$^{-}$ B2/O:9 strains isolated from eggshell and pork sausage clustered separately with each other and separate from the *virF*$^{+}$ B2/O:9 strains. This high genomic heterogeneity is consistent with previous results (Lucero Estrada et al. 2012) and might be attributed to the different locations of the food industries that delivered products to retail shops where samples were purchased, or to cross-contamination during handling and transporting (Favier et al. 2005). Nevertheless, other studies around the world have reported limited genetic diversity of B2/O:9 strains by this technique. Thus, Okwori et al. (2009) in a PFGE analysis using *Not*I, *Apa*I, and *Xho*I enzymes revealed 5 genotypes among 45 *Y. enterocolitica* B2/O:9 strains isolated of human and non-human samples. Meanwhile, Wang et al. (2008) observed 16 PFGE patterns among 53 Chinese isolates of *Y. enterocolitica* B2/O:9.

The 2 reference B4/O:3 strains were grouped in cluster C separate from the local strains. Lastly, the 2 clinical B3/O:3 strains of the present study shared 86% similarity with B3/O:5 strains isolated from pork and were included in cluster D. Taking into account the close genomic relationship between these B3 strains of different serotype and origin, the 100% similarity observed among the 4 food-borne B1A strains and 1 clinical B1A strain in GT5 within cluster A, and the lack of epidemiological data to relate each other, it is possible to suggest that in these cases, sporadic isolations from diverse sources in a determined region during different periods of time could be linked to the spreading of a few *Y. enterocolitica* clones in the environment, animal reservoirs, water, or foods of that area.

In conclusion, the results obtained in this study demonstrated the presence of several pathogenic *Y. enterocolitica* strains in human clinical samples and various foodstuffs of our region. The highest *Y. enterocolitica* recovery was observed in eggshell and minced meat, with B1A in combination with different serotypes and B2/O:9 the predominant bioserotypes. Furthermore, it was possible to show that all isolates were susceptible to antimicrobials used for clinical treatment and to show the presence of several phenotypic and genotypic virulence markers in most of them. PFGE revealed heterogeneous DNA restriction patterns within each bioserotype without discriminating by the source of isolation and demonstrated the high genomic diversity of the *Y. enterocolitica* strains isolated in our region. Regarding B1A strains, our results showed that it is necessary to pursue further research on the true virulence potential of this biotype to be considered as a rapidly emerging group within human pathogens. *Yersinia enterocolitica* isolation from foods and clinical samples in our region emphasizes the need to continue the consumer's education on proper food handling and cooking practices to decrease the risk of transmission of this bacterium.

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