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

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Abstract

Yersinia enterocolitica is a foodborne pathogen that causes gastroenteritis with occasional post-infection 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 PFGE. *Y. enterocolitica* was detected in 9.2% out 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*⁻, being 72.0% (13/18) of them *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 to 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 four 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.

Keywords:

Yersinia enterocolitica; foods; human samples; pathogenic potential; PFGE

Introduction

Yersinia enterocolitica is a human foodborne 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 cooked foods. The psychrotrophic nature of this bacterium is responsible of its survival in household refrigerators (Bottone, 1999). This species includes a heterogeneous group of strains, which are classified into six biotypes (B) and more than 57 serotypes (O). Five of the six biotypes (1B and 2–5) carry the 72 kb virulence plasmid (pYV) and several virulence chromosomal genes, and are considered pathogenic to humans and animals (Fàbrega and Vila 2012). Instead, 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 Viridi 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. *Y. 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 one *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 one *Y. enterocolitica* B1A/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. 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 have 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

Y. enterocolitica W1024 B2/O:9, provided by Dr. G. Cornelis (Belgium); *Y. enterocolitica* MCH 700 B4/O:3 and *Y. enterocolitica* 29C/43 B4/O:3, both provided by Dr. G. Kapperud (Norway), were used as reference strains. Four *Yersinia intermedia*, two *Yersinia frederiksenii*, one *Salmonella* Enteritidis and one *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 two-year period 2010-2012, a total of 380 samples divided into two groups, were investigated for the presence of *Y. enterocolitica*:

i-food samples: a total of 284 samples were purchased at random from seven 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 three 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

Y. 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 stomacher (IUL Masticator, Germany) for 30 s and incubated in 225 ml 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, one milliliter 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 HaoXuan et al. (2006) with some modifications. In brief, 0.5 g of sample was added into 4.5 ml of 0.85% NaCl. After homogenization, one ml was seeded into 9 ml of PSBB and cultured for 10 days at 10°C. After enrichment period, 0.1 ml of each sample was spread on Mac Conkey (MC, Britania) and cefsulodin-irgasan-novobiocin (CIN, Merck, Darmstadt, Germany) agars, and incubated for 48 h at 25°C 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 were performed by Dr. 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) in order 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 (MH; Britania, Buenos Aires, Argentina) 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 (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, one milliliter of *Y. enterocolitica* suspension was centrifuged at 10,000 rpm for 5 min. The pellet was suspended in 150 µl of TE1X buffer [10mM Tris (Sigma Aldrich, St. Louis, MO, USA), 1mM EDTA (Sigma), pH 8.0] added with 1% Triton-X100 (Parafarm, Buenos Aires, Argentina), boiled for 15 min and then, centrifuged at 10,000 rpm for 5 min. A 50 µl aliquot of the supernatant containing DNA was transferred to other tube and stored at 4° C.

Polymerase chain reaction for 16S rRNA gene detection

A 25 µl volume of a reaction mix containing 1X PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 1 pmol/ml of each specific primer (forward 5'- GGAATTTAGCAGAGATGCTTTAG -3' and reverse 5'-GGACTACGACAGACTTTATGTG-3'), 1 U Taq polymerase (PB-L, Quilmes, Argentina), 2 µl template, and ultrapure water was prepared. The primers were designed in this study using GenBank accession number 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, CA, USA). The products were analyzed by 2% agarose gel electrophoresis in comparison with a 100-bp molecular weight DNA marker (PB-L, Quilmes, Argentina). The bands were visualized by staining with GelRed® Acid Gel Stain (Biotium, Hayward, CA, 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 using primers presented in Table 1. A step-by-step empirical approach was used to determine the optimal annealing temperature, raising the temperature from 55 to 60°C with 1°C increments, and the concentration of primers, MgCl₂ and deoxynucleoside triphosphates in the reaction mixture. The final PCR protocol was as follows: 1x PCR buffer, 1.5 mM MgCl₂, 1 U of Taq DNA-polymerase, 0.2 mM of each dNTP, 10 pmol each primer set, and two µl of DNA as template. The amplification conditions were: 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.

Pulsed-field gel electrophoresis (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 mM Tris, 100 mM EDTA, pH 8.0) to

OD_{610nm} 1.0. Two hundred microliters of bacterial suspension were mixed with equal volume of 1% SeaKem Gold agarose (Cambrex, Rockland, ME, USA) and poured in 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 Proteinase K 0.1 mg/ml, Fluka Chemie, Buchs, Switzerland; pH 8.0) at 37°C and then washed four times with TE buffer (10 mM Tris, Sigma; 1 mM EDTA, Sigma; pH 8.0) for 30 min at 37°C. Chromosomal DNA contained in agarose plugs was digested with 10 U *XbaI* (Fermentas, Burlington, Ontario, Canada) for two hours, according to manufacturer’s instructions. Plugs were cut in approximately 1-mm-thick slices, placed in the CHEF-DRIII chamber (Bio-Rad, Hercules, CA, USA), 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 (Bio-Rad) submerged in 0.5 x TBE buffer (45 mmol/l Tris-Borate and 1 mmol/l EDTA). *Salmonella* Braenderup H9812 strain was used as a molecular reference marker. The gels were stained with Gel Red[®] 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 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 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, OK, 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 %) out of 380 samples were positive for *Y. enterocolitica* isolation. Thirty strains were isolated from 10.5% food samples and five from 5.2% human feces being 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, chicken carcasses (5.6 % of total food samples) and two from human feces (2.0 % of total fecal samples), while 11 *Y. enterocolitica* B2 strains were recovered from eggshell and one from pork sausage (4.2% of food). Moreover, two *Y. enterocolitica* B3 strains were recovered from pork sausage (0.7 % of food) and another three 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 *Y. enterocolitica* 16S rRNA gene. *Y. intermedia*, *Y. frederiksenii*, *S. Enteritidis*

and *S. Typhimurium* assayed to test the specificity of the 16S rRNA gene PCR did not show this band (Figure 1A and 1B).

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 four *Y. enterocolitica*, three B2 and one B3 isolates, showed the genotype *virF*⁻ *ail*⁺ *ystB*⁻ (Figure 2A). 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 *ystB* gene (Figure 2B).

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 this method. Among the 38 studied strains, including 35 isolates obtained in this work plus three 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 nine 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 two 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%) were composed by a unique strain. GTA5 was the most common pattern band found, with six strains (15.7%) included into 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% of 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 five 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% of 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, thirty *Y. enterocolitica* strains were recovered from 284 food samples after cold enrichment, which represents a prevalence of 10.6%. The biotype 1A combined with different serotypes (16 strains) was the most frequently detected one, followed by B2/O:9 (12 strains) and B3/O:5 (two 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 four B2/O:9 and one B1A/O:5 strains, Favier et al. (2005) isolated 2.3% of *Y. enterocolitica* B2/O:9 from eggshell (8 strains), and Lucero Estrada et al. (2012) recovered 5.9% of B2/O:9 and B1A strains from meat products (14 strains). Bernardino-Varo et al. (2013) published the isolation of B3 strains in 8.54 % of raw cow's milk in Mexico City. Furthermore, these authors isolated B1A strains in 70.0 %, B2 in 13.2%, and B4 in 8.15% from their samples. Current researches in Brazil indicate 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 though none *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).

Despite poultry seems no 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 . Probably, the contamination of these products could occur by contact with other *Y. enterocolitica*-contaminated animal products on farms and transportation or handling in retail shops. When outer defense barriers of eggshell such as the surface thin membrane of protein nature called cuticle 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, five *Y. enterocolitica* strains of this origin were isolated in this work, two B1A of different serotypes and three 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). As *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 by a guanine in the 20th nucleotide of the reverse primer in order 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 which were positive for *virF* gene and plasmid-related virulence phenotypic characteristics. However, since this gene was not amplified in four 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 four of six *Y. enterocolitica*

B2/O:9 and two B3/O:5 strains isolated from food samples showed pathogenicity potential in virulence phenotypic tests and amplified *virF*, *myf*, *ail* and *ystA* genes; however, two B2/O:9 strains neither amplified *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 Viridi 2011) and the detection of virulence markers by PCR (Campioni and Falcão 2014) still intrigue many researchers on the pathogenic potential of these strains. In the present work, we observed that although all B1A strains were negative for *virF* and *ail* genes by PCR in agreement with the phenotypic assays, 72.0 % were positive for *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 (Stephan et al. 2013; Wang et al. 2008). 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 Viridi 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 Viridi 2011).

Similarly to the present study, Favier et al. (2005) showed that all *Y. enterocolitica* strains isolated from 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 similar antimicrobial susceptibility results 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 four clonal groups (A to D) based in their biotypes. Cluster A comprised the 18 *Y. enterocolitica* B1A strains which were grouped into 10 GTs. Among these strains, two were isolated from human feces, one clustered alone (GTA4) and the other one clustered together with five 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 genomic types related to source or any other identifiable determinant could be demonstrated. These results confirmed earlier works where 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. (2013a) 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. In this way, 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 by performing *Not*I-PFGE.

The cluster B included *Y. enterocolitica* B2/O:9 strains which showed the highest genomic heterogeneity since 12 local strains produced eight GTs by PFGE and only two of these

genotypes included more than one strain, even though all of them were recovered from eggshells. The three *virF*⁻ B2/O:9 strains isolated from eggshell and pork sausage, clustered separately each other and 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 which 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 *NotI*, *ApaI* and *XhoI* enzymes, revealed five 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 two reference B4/O:3 strains were grouped in cluster C separately of the local strains. Lastly, the two 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 four foodborne B1A strains and one 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 being

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 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. *Y. 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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580

Figure captions

Figure 1: Simple PCR for detection of 16S rRNA gene in *Y. enterocolitica*. A) Lane 1: *Y. intermedia* B1/O:4,32-4,33; lane 2: *Y. intermedia* B1/O:52; lane 3: *Y. intermedia* B4/O:17; lane 4: *Y. intermedia* B4/O:40; lane 5: *Y. frederiksenii* no agglutinable; lane 6: standard size (100 bp to 1000 bp); lane 7: reference strain, *Y. enterocolitica* W1024; lane 8: negative control. lane 9: *Y. frederiksenii* O:16-16,29; lane 10: *S. Enteritidis* 9,12:g,m:-; lane 11: standard size (100 bp to 1000 bp); lane 12: *S. Typhimurium* 4,5,12:I:1,2. B) Lane 1: *Y. enterocolitica* W1024 B2/O:9; lane 2: *Y. enterocolitica* B2/O:9 (GFO041); lane 3: *Y. enterocolitica* B3/O:3 (GFO 045); lane 4: *Y. enterocolitica* B3/O:5 (GFO048); lane 5: *Y. enterocolitica* MCH 700 B4/O:3; lane 6: *Y. enterocolitica* B1A/O:41,42-41,43 (GFO018); lane 7: negative control; lane 8: standard size (100 bp to 1000 bp).

Figure 2: Multiplex PCR targeting gene encoding virulence-associated properties from *Y. enterocolitica*: *ail* (238 bp), *ystB* (145 bp) and *virF* (591 bp). A) Lane 1: *Y. enterocolitica* W1024 B2/O:9; lane 2: *Y. enterocolitica* B2/O:9 (GFO041); lane 3: *Y. enterocolitica* MCH 700 B4/O:3; lane 4: standard size (100 bp to 1000 bp); lane 5: *Y. enterocolitica* B2/O:9 (GFA001); lane 6: *Y. enterocolitica* B3/O:5 (GFO048), lane 7: *Y. enterocolitica* B3/O:3 (GFO 045); lane 8: negative control. B) Lane 1: *Y. enterocolitica* B1A/O:7,8-8-8,19 (GFO022); lane 2: *Y. enterocolitica* B1A/O:41,42-41,43 (GFO018); lane 3: standard size (100 bp to 1000 bp); lane 4: *Y. enterocolitica* B1A/O:6,30-6,31 (GFO006); lane 5: *Y. enterocolitica* B1A/O:5-4,32-4,33

603 (GFO012), lane 6: negative control; lane 7: *Y. enterocolitica* MCH 700 B4/O:3 as a reference
604 strain.

605

606 **Figure 3: PFGE patterns of 35 *Y. enterocolitica* isolates from food (30) and clinical (5)**
607 **samples. A) representative band patterns of each genotype (GT), and B) dendrogram.**

Draft

Figure 1

Draft

Figure 2

Figure 3.

Table 1. Primers used for multiplex PCR and amplicon sizes

Gene	Sequence (5'-- 3')	Amplicon length (bp)	Reference
<i>virF</i>	TCATGGCAGAACAGCAGTCAG ACTCATCTTACCATTAAGAAG	591	Hussein et al., 2001.
<i>ail</i>	GATGATAACTGGGGAGTAATAGG CGTATGCCATTGACGTCTTAC	238	This study (GenBank accession number M29945.1)
<i>ystB</i>	GTACATTAGGCCAAGAGACG GCAACATACCTCACAACACC	146	Bhagat and Viridi, 2007.

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

Sample type	Source	N ^o of analyzed samples	N ^o of positive samples (%)	<i>Y. enterocolitica</i> bio/serotype*
Food samples	Eggshell	72	11 ^A (15.28)	2/O:9 (11)
	Pork sausage	74	7 ^B (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	10 ^A (14.70)	1A/O:5 (3) 1A/O: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	2 ^D (2.86)	1A/O:5 (1) 1A/O: 5-4,34-4,33 (1)
Clinical samples	Fecal samples	96	5 ^C (5.21)	1A/O:7,8-8-8,19 (1) 1A/O:5 (1) 3/O:3 (3)
Total		380	35 (9.21)	

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

NAG: non- agglutinable.

^{A,B,C} Values followed by different capital letters indicate statistically significant differences (p<0.05).

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

Collection Number	B*	Serotype†	Origin	Phenotypic characteristics				Multiplex PCR‡		
				AA	CR	Pyz	Aes	<i>virF</i>	<i>ail</i>	<i>yszB</i>
W 1024	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	-	-	+	+	-	-	-

* B: biotype.

† NAG: non-agglutinable.

‡ +/-, positive and negative reactions, respectively. AA: autoagglutination; CR: congo red absorption; Pyz: pyrazinamidase reaction; Aes: aesculin hydrolysis.

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

B	N°	AKN	AM	AZT	CEF	CXM	CIP	CMP	COL	ERY	FOS	FUR	GEN	K	NAL	NEO	RFA	TET	SXT
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

B: biotype, N°: amount of strains
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).