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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Bacterial strains

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

2.2. Combined culture and PCR method

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

2.3. Food samples

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

2.4. Immunomagnetic separation (IMS)

2.4.1. Rabbit Y. enterocolitica-specific antisera

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

2.4.2. Purification of IgG fraction containing Yersinia-specific antibodies

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

2.4.3. Coating immunomagnetic particles

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