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Prevalence, antimicrobial resistance and genetic diversity of Yersinia enterocolitica isolated from retail frozen foods in China

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One sentence summary: This study provides a systematic surveillance of Y. enterocolitica prevalence in frozen foods in China and indicates its high antibiotic resistance.

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ABSTRACT

In this study, our aim was to estimate the extent of Yersinia enterocolitica contamination in frozen foods in China and determine the bioserotype, virulotype, antimicrobial resistance, and enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) genotyping profiles of recovered Y. enterocolitica isolates. Out of 455 samples collected between July 2011 and May 2014, 56 (12.3%) tested positive for Y. enterocolitica. The 70 isolated strains were grouped into five clusters and one singleton based on their ERIC-PCR fingerprint, at a similarity coefficient of 70%. All strains were of biotype 1A, and 35.7% were of bioserotype 1A/O:8. Most strains lacked the virulence genes aii, virF, ystA, and ystC, but harbored ystB, fepD, ymoA, fes and sat. All strains were sensitive to ticarcillin but resistant to two or more antibiotics, and 48.6% of the strains were resistant to four to nine antibiotics. High resistance rates were observed for ampicillin, cephalothin, trimethoprim/sulfamethoxazole, amoxicillin/clavulanic acid, nalidixic acid and chloramphenicol (98.6%, 95.7%, 74.3%, 28.6%, 18.6% and 12.9%, respectively). This study provides a systematic surveillance of Y. enterocolitica prevalence in frozen foods in China and indicates its high antibiotic resistance, which could serve as useful information for the government to control Y. enterocolitica contamination in frozen foods and the use of antibiotics.

Keywords: Yersinia enterocolitica; prevalence; antimicrobial susceptibility; virulence genes; ERIC-PCR; frozen food

INTRODUCTION

Frozen foods, including various types of meats and meat products, have become increasingly popular in China. Compared with fresh foods, frozen foods have a longer shelf-life, can retain nutritional properties more effectively, and are more convenient to use. However, certain pathogenic microorganisms can survive and grow at low temperatures. For example, Yersinia enterocolitica can survive at low temperatures without producing apparent signs of spoilage in refrigerated and frozen foods.

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Yersinia enterocolitica is a major foodborne pathogen that can cause diverse intestinal and extra-intestinal clinical symptoms, including mild gastroenteritis and mesenteric lymphadenitis (Sabina et al. 2011; Drummond et al. 2012; Mikula, Kolodziejczyk and Goldman 2013). Thus, contamination of frozen foods with Y. enterocolitica is a public health concern. Previous studies have characterized Y. enterocolitica bioseotypes and virulence-gene patterns. However, most of these studies have focused on isolates obtained from pigs or a small variety of food products (Bonardi et al. 2010, 2013; Saleh et al. 2012; Faïxão et al. 2013). To date, six biovars (1A, 1B, 2, 3, 4 and 5) and >50 serovars of Y. enterocolitica have been identified. Molecular genetic studies have emphasized the importance of various virulence genes and antibiotic resistance. Virulence genes of Y. enterocolitica include both chromosomally encoded genes (aiu, inv yst) and factors encoded by a 70 kb plasmid called pYV (plasmid Yersinia virulence). The plasmid-encoded virulence factors include the Yersinia adhesin A (yadA) and transcriptional activator (ufrF) genes. Yersinia enterocolitica has been shown to be highly susceptible to most antibiotics except penicillin, ampicillin and first-generation cephalosporin (Bolton, Ivory and McDowell 2013; Bonardi et al. 2013). Conversely, the antibiotic resistance of Y. enterocolitica is mediated by the chromosomal genes blaA and blaB, which encode β-lactamases (Fábrega and Vila 2012), and the prevalence of antimicrobial-resistant Y. enterocolitica strains, including multidrug-resistant Y. enterocolitica strains, has been progressively increasing (Sihvonen et al. 2011; Fábrega and Vila 2012; Bonardi et al. 2014). However, the prevalence of antimicrobial-resistant Y. enterocolitica in frozen foods in China remains poorly characterized.

Based on our previous research on Y. enterocolitica prevalence in certain cities in winter (Hu et al. 2014), we extended the investigated locations from South China to North China, and covered two seasons (winter and summer) for each city selected. Our aims in this study were to investigate the prevalence of Y. enterocolitica in frozen food samples in China, and to assess the microorganism’s pathogenic potential, antimicrobial resistance, and genotypic characteristics.

**MATERIALS AND METHODS**

**Sample collection**

The following 455 frozen food samples were collected between July 2011 and May 2014: 212 frozen chicken-meat samples, 43 frozen duck-meat samples, 8 frozen pork samples, 7 frozen beef samples, 33 frozen sheep-meat samples, 6 frozen ham samples, and 146 frozen pasta samples. The sampling sites were distributed across the 24 provincial capitals of China (see Fig. S1 in the online supplementary material). Each sample was weighed, marked, placed in a separate sterile bag, and immediately transported to the laboratory in an icebox.

**Isolation and identification of Y. enterocolitica**

Yersinia enterocolitica was isolated using a slightly modified selective-enrichment protocol recommended by the National Food Safety Standards of China. Briefly, 25 g of each sample was homogenized in 225 mL of phosphate-buffered saline (PBS) (Huankai, Guangzhou, China) for 30 s in stomacher bags (Huankai). Homogenates were incubated at 26 °C for 48 h, after which a loopful of the PBS-enrichment culture was streaked onto cefsulodin-irgasan-novobiocin (CIN) agar and modified Agar Y (MAY) Yersinia-selective agar (Huankai). The Yersinia-selective agar plates were incubated at 26 °C for 48 h. The typical flat Y. enterocolitica colonies were streaked onto tryptic soy agar (Huankai). Urease activity of the isolates was tested using a urea trace biochemical tube (Huankai) that was incubated at 26 °C for 24 h. Urease-positive isolates were biochemically identified using an API 20E (BioMérieux, Marcy l’Etoile, France).

**Serotyping and biogroup identification**

The Y. enterocolitica isolates were grouped according to biotype by using discriminatory tests (lipase, esculin, salcin, indole, xylene and trehalose) described previously (Thoenner et al. 2003) and were serotyped using the commercial sera agglutinants anti-Y. enterocolitica O:1, O:2, O:3, O:5, O:8 and O:9 (Denka Seiken, Tokyo, Japan).

**Detection of virulence-associated genes**

Conventional PCR assays were performed to detect the presence of 14 virulence-related genes. The primer sequences, PCR conditions and the references are summarized in Table 1.

**Antimicrobial susceptibility test**

Antimicrobial susceptibility was evaluated using the disc-diffusion method for 16 antimicrobial agents, in accordance with the Clinical Laboratory Standards Institute guidelines (CLSI, 2010). These antimicrobials were ampicillin (Amp, 10 μg), amoxycillin/clavulanic acid (2:1) (Amc, 30 μg), cefotaxime (Ctx, 30 μg), ciprofloxacin (Cip, 5 μg), cefalothin (Cf, 30 μg), cefazidime (Caz, 30 μg), chloramphenicol (C, 30 μg), gentamicin (Cn, 10 μg), kanamycin (K, 30 μg), nalidixic acid (Nx, 30 μg), streptomycin (S, 10 μg), sulfonamide (S3, 300 μg), tetracycline (Te, 30 μg), trimethoprim/sulfamethoxazole (Sxt, 1.25/23.75 μg), imipenem (Ipm, 10 μg) and ticarcillin (Tic, 75 μg) (Oxoid, Basingstoke, UK).

**PCR detection of β-lactamase genes**

The primer sequences used for amplifying blaA and blaB have been previously described (Stock, Heisig and Wiedemann 1999; Sharma et al. 2006). The 25 μL PCR mixture contained 1× PeqLab Master-Mix S (Dongsheng Biotech, Guangzhou, China), 200 mmol L⁻¹ of each primer, and 1 μL of the DNA, and blaA and blaB were PCR-amplified using this protocol: initiation at 95 °C for 5 min, followed by 25 consecutive cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s, and a final extension for 10 min at 72 °C.

**Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) fingerprinting**

The primers used for ERIC-PCR were reported previously (Wójciewiech et al. 2004). The PCR mixture (25 μL) contained 1 U of GoTaq® Hot Start Polymerase (Promega, Madison, WI, USA), 0.6 mmol L⁻¹ of each primer, 2.5 mmol L⁻¹ MgCl₂, 0.5 mmol L⁻¹ of each dNTP and 40 ng of template genomic DNA. Amplifications were performed in a DNA thermocycler (Applied Biosystems, Foster City, CA, USA). ERIC-PCR was performed as follows: 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 46 °C and 3 min at 72 °C, and a final extension at 72 °C for 10 min. The products were separated on 2% agarose gels at 80 V for 3 h.
Table 1. Sequences of primers used in this study.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5′–3′)</th>
<th>Amplicon size (bp)</th>
<th>PCR conditions (°C, s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>inv</td>
<td>F: CTGGGGGAGAGTGGGGAAGTTTGG &lt;br&gt; R: GAACTGTGGTGAATCCCTGGAACCC</td>
<td>570</td>
<td>94, 60 55, 60 72, 60</td>
<td>Rasmussen, Rasmussen and Andersen (1994)</td>
</tr>
<tr>
<td>ail</td>
<td>F: ACTCGATGATAACTGGGGAG &lt;br&gt; R: CCCCAGTAATGATCATGAAC</td>
<td>170</td>
<td>94, 60 55, 60 72, 120</td>
<td>Nakajima, Inoue and Mori (1992)</td>
</tr>
<tr>
<td>ystA</td>
<td>F: AATGGTGCTTCTTCTTGGGAGCA &lt;br&gt; R: ATGCCCAATGCTACTGACCC</td>
<td>145</td>
<td>94, 60 55, 60 72, 60</td>
<td>Ibrahim et al. (1997)</td>
</tr>
<tr>
<td>ystB</td>
<td>F: TGTCAAGATTATTCATTCAACT &lt;br&gt; R: GCCGATAATGATCATGACAG</td>
<td>180</td>
<td>94, 60 46, 120</td>
<td>Platt-Samoraj et al. (2006)</td>
</tr>
<tr>
<td>yadA</td>
<td>F: CTTCAAGACTGGTGCGCTGT &lt;br&gt; R: ATGCCTGACTAGAGCGATATCC</td>
<td>849</td>
<td>94, 60 60, 30 72, 90</td>
<td>Thoerner et al. (2003)</td>
</tr>
<tr>
<td>virF</td>
<td>F: TCATGGCAGAAGACAGCCTG &lt;br&gt; R: ACTCATCTTACCATTAAGAAG</td>
<td>591</td>
<td>94, 60 58, 120</td>
<td>Thoerner et al. (2003)</td>
</tr>
<tr>
<td>myfA</td>
<td>F: CAGATACACCTGGCTTCCATCT &lt;br&gt; R: CTCGACATATTCCTCAACACGC</td>
<td>272</td>
<td>94, 60 58, 110 72, 110</td>
<td>Kot and Trafny (2004)</td>
</tr>
<tr>
<td>fepA</td>
<td>F: TAGCAGAAAATACCTTTGAGAT &lt;br&gt; R: TGAAAATACACCACCACCTGAC</td>
<td>438</td>
<td>94, 60 56, 60 72, 60</td>
<td>Schubert, Fischer and Heesemann (1999)</td>
</tr>
<tr>
<td>fepD</td>
<td>F: GTGIATGTCCTTACTTATT &lt;br&gt; R: CGGTCATCCTTTTATTACGG</td>
<td>381</td>
<td>94, 60 56, 60 72, 60</td>
<td>Schubert, Fischer and Heesemann (1999)</td>
</tr>
<tr>
<td>fes</td>
<td>F: CCAGCAAGCCACACGTCAAATTT &lt;br&gt; R: GCCCAACCCACACCAATT</td>
<td>561</td>
<td>94, 60 56, 60 72, 60</td>
<td>Schubert, Fischer and Heesemann (1999)</td>
</tr>
<tr>
<td>tccC</td>
<td>F: GCCGAAGAAAATCGTGGAAGGAGAG &lt;br&gt; R: TTAACGGAATAAGCAGATTTTA</td>
<td>1035</td>
<td>94, 60 51, 110 72, 110</td>
<td>Bhagat and Virdi (2007)</td>
</tr>
<tr>
<td>ymoA</td>
<td>F: GACTTTTTCTCAGGGGGAATAC &lt;br&gt; R: CCTAA CGT TGT GTC TCT</td>
<td>330</td>
<td>94, 60 50, 60 72, 60</td>
<td>Grant, Bennett-Wood and Robins-Browne (1998)</td>
</tr>
<tr>
<td>hreP</td>
<td>F: GCCGGCTATGGTGCTCTGGTG &lt;br&gt; R: CCCGGATGCTACTGCGGCTATC</td>
<td>757</td>
<td>94, 60 60, 60 72, 60</td>
<td>Bhagat and Virdi, (2007)</td>
</tr>
<tr>
<td>sat</td>
<td>F: CGGATGTCGAGGGTTTTTCAG &lt;br&gt; R: GGGATTACGCGCACCAACT</td>
<td>456</td>
<td>94, 60 56, 60 72, 60</td>
<td>Bhagat and Virdi (2007)</td>
</tr>
<tr>
<td>blA</td>
<td>F: GAGATTCAAGGAATGAACGACTCTTCTCG &lt;br&gt; R: TCAGAAATTTGGGGAATCTTAT</td>
<td>896</td>
<td>95, 30 65, 30 72, 90</td>
<td>Stock, Heisig and Wiedemann (1999)</td>
</tr>
<tr>
<td>blB</td>
<td>F: CCACCTTCTTCTATCGGACAAA &lt;br&gt; R: GAAACATATCTCTTGCGCTGGGAAAT</td>
<td>781</td>
<td>95, 30 54, 60 72, 120</td>
<td>Sharma et al. (2006)</td>
</tr>
<tr>
<td>ERIC</td>
<td>ERIC1R: ATCTAACGCTTCTGAGGATTAC &lt;br&gt; ERIC2: AAGTAAAGTGTGAGGGTACCC</td>
<td>–</td>
<td>94, 30 46, 60 72, 180</td>
<td>Wojciech et al. (2004)</td>
</tr>
</tbody>
</table>

*PCR conditions were performed with an initial denaturation step of 94 °C for 5 min, 35 cycles each of denaturation, annealing and extension and an indicated final extension of 10 min at 72 °C.

RESULTS AND DISCUSSION

Prevalence of Y. enterocolitica in frozen food

This is the first systematic study to assess the prevalence of Y. enterocolitica in a broad variety of frozen food samples collected from various main regions of China. Of the 455 frozen food samples examined, 56 (12.3%) were contaminated with Y. enterocolitica, with certain differences being present among the distinct food varieties: Y. enterocolitica was detected in 29/212 (13.7%) frozen chicken meat samples, 6/43 (14%) frozen duck meat samples, 8/33 (24.2%) frozen sheep meat samples and 13/146 (8.9%) frozen pasta samples. By contrast, none of the examined frozen pork, frozen beef and frozen ham samples tested positive for Y. enterocolitica; this result is at odds with the previous suggestion that raw pork products are the main source of Y. enterocolitica (Bonardi et al. 2010; Tan, Ooi and Thong 2014). Furthermore, the prevalence of Y. enterocolitica differed considerably between the main geographic regions of China. Overall, samples from 75% of the investigated sites tested positive for Y. enterocolitica, and the prevalence ranged from 5.3% in Chengdou and Wuhan to 45% in Jinan. The variability among these results might be due to diverse factors, such as season of isolation, and geographical location with regard to distinct temperatures, the system for managing ‘microbial contamination’, and numbers of samples analyzed. These variables can critically affect Y. enterocolitica isolation. For instance, in this study, the majority (60.7%) of Y. enterocolitica-positive samples were collected in wintertime, which agrees with the finding that the isolation rate of Y. enterocolitica is higher in cold climates than in hot climates (Jiang and Kang 2000).
Bioserotypes and virulence genes

Among the six biotypes of Y. enterocolitica, biotype 1A is a ubiquitous biotype isolated from food and includes several serotypes (Tennant, Grant and Robins-Browne 2003), with its most common serotype in China being O:8 (Wang et al. 2008). Here, all Y. enterocolitica isolates were of biotype 1A, and the most prevalent serotype was O:8 (25 strains, 35.7%); the prevalence rates of serotypes O:5, O:9, O:3 and O:1,2 were 14.3%, 12.9%, 7.1% and 2.9%, respectively. Moreover, 19 strains (27.1%) did not belong to any of the tested serovars (simply named 1A/unknown bioserotype). Thus, the primary bioserotype was determined to be 1A/O:8 and our biotype and serotype data agree with those of previous studies (Wang et al. 2008, 2009, 2010).

Biotype 1A strains of Y. enterocolitica have traditionally been considered to be nonpathogenic because they do not harbor the plasmid pYV and chromosomal virulence genes such as ail, myfA, ystA and the ysa locus (Foulquier et al. 2002; Tennant, Grant and Robins-Browne 2003). However, the potential clinical significance of biogroup 1A isolates of Y. enterocolitica is becoming increasingly recognized and warrants increased attention (Batzilia, Heeseman and Rakin 2011; Bhagat and Virdi 2011; Fredriksson-Ahomaa et al. 2012; Bolton, Ivory and McDowell 2013). To assess the pathogenic potential of our isolates, we assayed for the presence of 14 plasmid- and chromosome-encoded virulence genes. Strains that harbor classical virulence genes encoded on chromosomes (imu, ail, ystA) or plasmids (yadA, virF) can generally be considered pathogenic (Zheng et al. 2008). Here, all 70 strains harbored imu and ystB, whereas none harbored ystA, yadA, or virF, which agrees with the results of other studies (Bhagat and Virdi 2007; Saleh et al. 2012; Jamali et al. 2015). Although ail has rarely been detected in biovar 1A strains, all-positive Y. enterocolitica isolates have been occasionally reported in several continents in recent years (Kraushaar et al. 2011; Bonardi et al. 2013); moreover, two ail-positive biotype 1A isolates—one from a vole in Longquan and the other from the feces of a sheep in Wulian—were first reported in China (Liang et al. 2014). In this study, ail was found to be harbored by one bioserotype 1A/O:3 strain isolated from a frozen duck-meat sample from Kunming. However, all 70 Y. enterocolitica strains harbored ymoA, hreP and sat, and most harbored fepD (98.6%) and fes (94.3%), which is consistent with previous reports of these virulence genes being carried by all or most biotype 1A strains (Bhagat and Virdi 2007). Notably, 91.4% of the strains harbored fepA, a level considerably higher than that reported previously (44%) (Bhagat and Virdi 2007). Our findings illustrate the possibility that fepA distribution among Y. enterocolitica isolates varies substantially. The gene tccC is considered to show a geographically diverse distribution among biovar 1A strains (Bhagat and Virdi 2007) and tccC was detected in 15.71% of the isolates in this study. Lastly, the prevalence of myfA (12.86%) was lower than that reported in one previous study (44%) (Bhagat and Virdi 2007).

Collectively, our results revealed that almost all examined isolates lacked the classical virulence genes, and this agrees with previous findings (Saleh et al. 2012) that showed that even if isolates lack some of the classical Y. enterocolitica virulence genes, they might be pathogenic. This could be because the strains possess other recognized virulence-associated genes such as myfA, ymoA and hreP and/or additional unknown virulence genes that could play a key role in diverse pathogeneses (Batzilia, Heeseman and Rakin 2011; Sihvonen et al. 2011). Furthermore, Y. enterocolitica does not have to harbor all virulence genes to cause yersiniosis; for example, published evidence suggests that certain ystB-containing strains could cause pathogenicity (Wang et al. 2009; Kot, Piechota and Jakubczak 2010; Bolton, Ivory and McDowell 2013). Thus, further investigation is required to determine whether the strains isolated in this study are pathogenic.

Antimicrobial susceptibility and β-lactamase gene expression

Yersinia spp. are typically resistant to ampicillin and cephalothin, but susceptible to trimethoprim/sulfamethoxazole. In this study, the results of antibiotic-resistance assays indicated that considerably high percentages of the isolated strains were resistant to ampicillin (98.6%), cephalothin (95.7%) and trimethoprim/sulfamethoxazole (74.3%) (Table 2). The levels of resistance to ampicillin and cephalothin are comparable with those reported widely

Table 2. Results of antimicrobial resistance of Y. enterocolitica isolates in the study.

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Y. enterocolitica (n = 70)</th>
<th>Zone diameters (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.(% of R)</td>
<td>No.(%) of I</td>
</tr>
<tr>
<td>Ampicillin (Amp)</td>
<td>69 (98.6)</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid 2:1 (Amc)</td>
<td>20 (28.6)</td>
<td>4 (5.7)</td>
</tr>
<tr>
<td>Cefotaxime (Ctx)</td>
<td>6 (8.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ciprofloxacin (Cip)</td>
<td>1 (1.4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cefalothin (Cf)</td>
<td>67 (95.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ceftazidime (Caz)</td>
<td>2 (2.9)</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>Chloramphenicol (C)</td>
<td>9 (12.9)</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>Gentamicin (Cn)</td>
<td>3 (4.3)</td>
<td>2 (2.8)</td>
</tr>
<tr>
<td>Kanamicin (K)</td>
<td>1 (1.4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nalidixic acid (Nx)</td>
<td>13 (18.6)</td>
<td>2 (2.8)</td>
</tr>
<tr>
<td>Streptomycin (S)</td>
<td>6 (8.6)</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>Sulphonamide (S3)</td>
<td>2 (2.9)</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td>Tetracycline (Te)</td>
<td>6 (8.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Trimethoprim/ sulfamethoxazole (Sxt)</td>
<td>52 (74.3)</td>
<td>11 (15.7)</td>
</tr>
<tr>
<td>Imipinem (Ipm)</td>
<td>1 (1.4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ticarcillin (Tic)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*R: resistant; I: intermediate resistance; S: susceptibility.
Figure 1. The characterization of Y. enterocolitica isolates from retail frozen foods in China.

### Table: ERIC-PCR serotype, virulence gene profile, and antibiotic profile

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Virulence Gene Profile</th>
<th>Antibiotic Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>pI-schu-1,2</td>
<td>Sensitive</td>
</tr>
<tr>
<td>B</td>
<td>pI-schu-1,2</td>
<td>Sensitive</td>
</tr>
<tr>
<td>C</td>
<td>pI-schu-1,2</td>
<td>Sensitive</td>
</tr>
<tr>
<td>D</td>
<td>pI-schu-1,2</td>
<td>Sensitive</td>
</tr>
<tr>
<td>E</td>
<td>pI-schu-1,2</td>
<td>Sensitive</td>
</tr>
<tr>
<td>F</td>
<td>pI-schu-1,2</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>

*Un: serotype didn’t belong to any kind of test serovars
^VC: virulence genes profiles inr-ystB-smoA-ksp-sat; VC1: virulence genes profiles fePA-fee-feeD
^ace: resistance phenotype A/CC-Sxt

Our data indicate that the development of chloramphenicol-resistant strains (12.9%) might be increasing. Previously, no chloramphenicol-resistant strains were detected in USA, Latvia or Ireland (Bhaduri et al. 2009; Terentjeva and Bazarin 2010; Bolton, Ivory and McDowell 2013), and resistance was found in only 4% of the strains in Czech Republic (Simonova, Borilova and Steinhauserova 2008). The prevalence of amoxicillin/clavulanic acid-resistant strains varies considerably: whereas 0–8% of the Y. enterocolitica strains isolated in Italy were shown to be resistant to amoxicillin/clavulanic acid (Bonardi et al. 2013, 2014), this proportion was 82.8% in the case of isolates from Switzerland (Fredriksson-Ahomaa et al. 2012). Here, 28.6% of the strains showed resistance to amoxicillin/clavulanic acid. Previously, the prevalence of nalidixic acid-resistant Y. enterocolitica strains was reported to range from 0% (in Italy) (Bonardi et al. 2014) to 2.34% (in Switzerland) (Fredriksson-Ahomaa et al. 2012); in this study, the corresponding prevalence was 18.6%. Previous studies indicated that Y. enterocolitica strains were susceptible to the majority of commonly used antimicrobial agents (Dallal et al. 2010; Estrada et al. 2012; Bolton, Ivory and McDowell 2013), but the Y. enterocolitica isolates obtained in this study were only susceptible to ticarcillin. All strains were multidrug-resistant strains: only four strains (5.7%) were resistant to two tested antibiotics, and 66 strains (94.3%) were resistant to $\geq$3 antibiotics, and among these, 32, 21 and 11 isolates were resistant to 3, 4 and 5 antibiotics, respectively. Notably, one strain isolated from a frozen sheep-meat sample from Nanchang was resistant to 7 antibiotics, and...
one strain isolated from a chicken-meat sample from Xiamen was resistant to 9 antibiotics. This could be because no specific policy in animal husbandry governs the use of antibiotics in China, and livestock and poultry farms have abused antibiotics over an extended period and have even included antibiotics with common feed additives, which can potentially explain the presence of multidrug-resistant strains in frozen meats. Moreover, frozen pasta samples commonly include complex raw materials (which typically contain raw meat) and their preparation involves complicated processing and a long supply chain; thus, if drug-resistant strains are present in the raw materials, various strains might gain new resistance genes through cross-contamination from other bacteria during the long supply chain. Thus, the likelihood of the occurrence of drug-resistant and multidrug-resistant strains would be higher here than in the case of food types produced using a single, short supply chain. Given these findings, food regulators must accelerate the regulation of frozen foods, and the government departments related to livestock animal husbandry and fisheries must strengthen the supervision of the use of antibiotics.

ERIC-PCR

Pulsed-field gel electrophoresis (PFGE) has been widely used for subtyping Y. enterocolitica, but PFGE is not useful for distinguishing strains according to serotype or the source of isolates (Silvonen et al. 2011; Estrada et al. 2012; Paixão et al. 2013). ERIC-PCR has also been used in studies on Y. enterocolitica, but the number of studies is low. In this study, ERIC-PCR generated 2–12 amplification bands in the 250–3000 bp size range. At a relative similarity coefficient of 70%, the 70 Y. enterocolitica isolates fell into six major clusters (Fig. 1). Four isolates belonged to Cluster B and showed the same virulence-gene profiles. Three strains belonged to Cluster C and were resistant to gentamicin; a similar antibiotic resistance was not detected in other isolates. Cluster D only included one strain, which was resistant to nine antimicrobials. Four isolates belonged to Cluster E and showed the same antibiotic profiles. Two isolates belonging to Cluster F were obtained from cities in Jinan. Almost all O:8 isolates and almost all isolates harboring tccC and myfA virulence genes belonged to Cluster A, and these were very closely related. These results demonstrated that the ERIC types exhibited high genetic heterogeneity, which agrees with previous studies (Sachdeva and Virdi 2004; Wojciew et al. 2004). Thus, we could not identify a direct relationship among the ERIC types in terms of their origin, isolation site, serovar, virulence profile, or even antibiotic profiles.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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