

ISOLATION, IDENTIFICATION USING PHENOTYPIC AND GENOTYPIC
METHODS AND DETERMINATION OF SOME PATHOGENIC
CHARACTERISTICS OF *YERSINIA* SPP. FROM MEAT AND MILK PRODUCTS

by

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ABSTRACT

ISOLATION, IDENTIFICATION USING PHENOTYPIC AND GENOTYPIC METHODS AND DETERMINATION OF SOME PATHOGENIC CHARACTERISTICS OF *YERSINIA* SPP. FROM MEAT AND MILK PRODUCTS

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The genus *Yersinia* belongs to the family *Enterobacteriaceae* and comprises 11 species, of which *Y. enterocolitica* and *Y. pseudotuberculosis* are well-known enteropathogens in humans and transmitted through contaminated food or water. In the present study, a total of 300 meat and milk products were collected and analyzed to determine the presence of *Yersinia* spp. Out of the 300 samples, 84 (28%) were positive for *Yersinia* spp. Eighteen (6%) out of 84 were identified to be *Y. enterocolitica*. The other *Yersinia* species were *Y. rohdei* 15 (5%), *Y. intermedia* 14 (4.7%), *Y. pseudotuberculosis* 12 (4%), *Y. ruckeri* 12 (4%), *Y. mollaretii* 5 (1.7%) and *Y. bercovieri* 4 (1.3%). Furthermore, 4 (1.3%) atypical *Yersinia* isolates were confirmed as *Yersinia* but could not be identified at *Yersinia* species level. The conventionally identified *Y. enterocolitica* strains were also confirmed by 16S rRNA

amplification and sequencing. The prevalence of *Yersinia* spp. was 40.8% in meat products and 19.4% in milk products. *Yersinia* spp. was most frequently found in meat products at a rate of 51.7% in chicken meat and 30% in minced meat. *Y. enterocolitica* was the most common species in this research that was most frequently found in chicken meat. All *Y. enterocolitica* strains were tested for biotype profiles and phenotypic virulence characteristics including autoagglutination, Congo-red absorption and crystal-violet binding. In this study, as a result of phenotypic virulence tests, it was seen that none of the *Y. enterocolitica* biotyped as 1A had pathogenic potentials. Eighteen *Y. enterocolitica* and the other 66 *Yersinia* strains were genotypically examined for the presence of chromosomal (*ail*, *ystA* and *ystB*) and plasmid-encoded (*yadA* and *virF*) virulence genes. This study demonstrated that neither the *Y. enterocolitica* nor the other *Yersinia* strains harboured the *ail*, *ystA*, *yadA*, and *virF* genes. Only one strain of *Y. intermedia* and two strains of *Y. enterocolitica* had *ystB*. All 84 (100%) *Yersinia* spp. had resistance to clindamycin. Most *Yersinia* spp., namely, 86.9% were resistant to cephalothine, 78.6% to cephazoline, 64.3% to ampicillin and 57.1% to amoxicillin. A multidrug-resistance was observed in 71 (84.5%) of the strains to at least three or more antimicrobial agents. The present study is noteworthy in that it documents prevalence, virulence characteristics and antimicrobial resistance of *Yersinia* spp. and especially *Y. enterocolitica* as foodborne pathogen in meat and milk products. Thus, it is essential that these foods should be closely monitored for sanitation during all the processes from production till consumption and that their quality should be improved.

Keywords: *Yersinia* spp., meat, cheese, raw milk, isolation, antimicrobial resistance, virulence genes.

ÖZET

ET VE SÜT ÜRÜNLERİNDEN *YERSINIA* TÜRLERİNİN İZOLASYONU, FENOTİPİK VE GENOTİPİK YÖNTEMLERLE ADLANDIRILMASI VE BAZI PATOJENİTE ÖZELLİKLERİNİN BELİRLENMESİ

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Yersinia cinsi *Enterobacteriaceae* ailesine dahil olup ve 11 tür içermektedir. Bu cinse ait olan *Y. pseudotuberculosis* ve *Y. enterocolitica* insanlarda iyi bilinen iki enteropatojen olup kontamine gıda ve sular ile taşınırlar. Bu çalışmada, toplam 300 et ve süt ürünü toplanmış ve *Yersinia* türlerinin varlığını belirlemek için analizleri yapılmıştır. 300 örnekten 84'ü (%28) *Yersinia* spp. pozitif olarak bulunmuştur. 84 izolatın 18'i (%6) *Y. enterocolitica* olarak tanımlanmıştır. Tespit edilen diğer *Yersinia* türleri şunlardır: *Y. rohdei* 15 (%5), *Y. intermedia* 14 (%4,7), *Y. pseudotuberculosis* 12 (%4), *Y. ruckeri* 12 (%4), *Y. mollaretii* 5 (%1,7) ve *Y. bercovieri* 4 (%1,3). Ayrıca, 4 (%1,3) atipik *Yersinia* izolatının *Yersinia* cinsine ait olduğu doğrulanmış fakat *Yersinia* tür düzeyinde isimlendirilememiştir. Konvensiyonel olarak tanımlanan *Y. enterocolitica* suşları 16S rRNA amplifikasyonu ve dizi analizi ile de doğrulanmıştır. *Yersinia* türlerinin varlığı, et ürünlerinde % 40,8

ve st rnlerinde %19,4 olarak bulunmuştur. *Yersinia* spp. tavukta %51,7 ve kıymada %30 olmak zere en yaygın olarak et rnlerinde saptanmıştır. Bu araştırmada trler arasında en sık rastlanan trn *Y. enterocolitica* olduđu ve bu trn de en fazla tavukta bulunduđu tespit edilmiştir. Tm *Y. enterocolitica* suşları biyotip profilleri ve otoaglutinasyon, Kongo-kırmızısı bađlama ve kristal viyole bađlama gibi fenotipik virulans zellikleri iin test edilmiştir. 1A olarak biyotiplendirilen *Y. enterocolitica* suşlarının hibiri fenotipik virulans testler sonucunda patojenik potansiyellere sahip bulunmamıştır. 18 *Y. enterocolitica* ve diđer 66 *Yersinia* suşları kromozomal (*ail*, *ystA* and *ystB*) ve plazmit DNA'da (*yadA* and *virF*) kodlanan genlerin varlıđı bakımından genotipik olarak incelenmiştir. Bu alıřma, ne *Y. enterocolitica* ve ne de diđer *Yersinia* suşlarının, *ail*, *ystA*, *yadA*, ve *virF* genlerini tařımadıđını gstermiştir. *Y. intermedia* suşlarının sadece birinin ve *Y. enterocolitica* suşlarının ikisinin *ystB* genine sahip olduđu bulunmuştur. 84 (%100) *Yersinia* suşunun tm klindamisine diren gstermiştir. *Yersinia* spp.'ler iinde en ok diren %86,9 ile sefalotine, %78,6 ile sefazoline, %64,3 ile ampisiline ve %57,1 ile amoksisilline karřı tespit edilmiştir. En az  veya daha fazla antimikrobiyal ajana karřı oklu diren suşların 71(% 84,5)'inde saptanmıştır. Bu alıřma, *Yersinia* spp. ve zellikle gıda kaynaklı patojen olan *Y. enterocolitica*'nın et ve st rnlerindeki varlıđını, virulans zelliklerini ve antimikrobiyal direncini belgelemesi aısından nem arz etmektedir. Dolayısıyla, bu gıdaların retimden tketime kadar tm srelerde sanitasyon aısından sıkıca denetlenmesi ve gıdaların kalitesinin iyileřtirilmesi gereklidir.

Anahtar Kelimeler: *Yersinia* spp., et, peynir, iđ st, izolasyon, antimikrobiyal diren, virulans genler.

To my dear father İsmail ÖZKARDEŞ...

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LIST OF ABBREVIATIONS

Abbreviations	Full Name
Ail	Attachment invasion locus
AMC	Amoxicillin /clavulanic acid
AML	Amoxicillin
AMP	Ampicillin
ANOVA	One-Way Analysis of Variance
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
C	Chloramphenicol
CFU	Colony Forming Unit
CIN	Cefsoludin- Irgasan- Novobiocin
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamicin
CR-MOX	Congo Red-Magnesium Oxalate
DA	Clindamycin
Invasin	Inv
IPM	Imipenem
ISO	International Standard Organization
ITC	Irgasan Ticarcillin Chlorate enrichment broth
K	Kanamycine

KF	Cephalothine
KOH	Potassium Hydroxide
KZ	Cephazoline
MRB	Modified Rappaport broth containing magnesium chloride, malachite green, and carbenicillin
NA	Nalidixic acid
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PMB	Phosphate buffer saline supplemented with mannitol and bile salts
PSB	Peptone Sorbitol Bile broth
pYV	<i>Yersinia</i> virulence plasmid
S	Streptomycine
SSDC	Salmonella-Shigella- deoxycholate calcium chloride
TBE	Tris Borate EDTA
TE	Tetracycline
TOB	Tobramycine
W	Trimethoprim
YadA	<i>Yersinia</i> adhesion A
YstA	<i>Yersinia</i> heat stable toxin A
YstB	<i>Yersinia</i> heat stable toxin B

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1. INTRODUCTION

The genus *Yersinia* belongs to the family *Enterobacteriaceae* includes three well-established pathogens -*Yersinia pseudotuberculosis*, *Yersinia enterocolitica* and *Yersinia pestis*-and several non pathogens. Both pathogenic and non pathogenic species has been isolated from a wide variety of sources in the environment such as water and soil and from healthy humans and various animals such as birds, mammals, and reptiles especially the pig, as well as from foods (Bottone et al., 2005; Carniel et al., 2006). *Y. pseudotuberculosis* is quite common in animals and has been isolated in foods. However, its involvement in foodborne illnesses is confirmed. Infections due to *Y. pseudotuberculosis* and *Y. enterocolitica* result in similar manifestations (Ray, 2004). In humans, *Y. enterocolitica* and *Y. pseudotuberculosis* are well-known foodborne pathogens and are mainly transmitted through ingestion of contaminated pork, milk or water (Bhaduri et al., 1997).

Y. enterocolitica as a foodborne pathogen can cause a broad range of clinical syndromes ranging from mild diarrhea to acute gastroenteritis, enterocolitis, and mesenteric lymphadenitis evoking appendicitis in humans and animals (Bottone, 1997). The association of human illness with the consumption of food contaminated with *Y. enterocolitica* is well documented (Kapperud, 1991; Kwaga et al., 1992; Bhaduri et al., 1997). *Y. enterocolitica* infection has become a significant public health problem. The epidemiology of *Y. enterocolitica* infections is complex and poorly understood. Foodborne yersiniosis was first confirmed in the U.S. in 1976 following an outbreak among a large number of school children from the

consumption of chocolate milk contaminated with *Y. enterocolitica*. *Y. enterocolitica* foodborne outbreaks have occurred in Australia, Finland, Japan, Norway, the United States, and Brazil. Yersiniosis frequently occurs in young children as enterocolitis with fever, diarrhea, and abdominal cramps (Koneman et al., 1997; Bottone et al., 2005; Carniel et al., 2006).

Y. enterocolitica is highly heterogeneous species with worldwide distribution and can be divided into several bioserotypes. Biotyping is a very important part of the identification of *Y. enterocolitica*. This organism is traditionally subdivided into bioserotypes based on the combination of biochemical and serological tests. Currently, *Y. enterocolitica* is represented by six biovars or biotypes including 1A, 1B, 2, 3, 4, and 5. Groups 1B and 2-5 are pathogenic that have the *Yersinia* virulence plasmid (pYV) and certain chromosomal genes (Cornelis et al., 1998). Strains of biogroup 1A that lacked pYV plasmid (68 to 75 kb virulence plasmid) are generally considered to be non pathogenic and widespread in the environment (Schriefer and Petersen, 2011). Biotype 1A strains have frequently been isolated from samples of food and animal origin (Bhagat and Viridi, 2007; Wang et al., 2008). However, biogroup 1A strains may be opportunistic pathogens in patients with underlying disorders (Bottone, 1997). Serotyping of *Y. enterocolitica* provides valuable additional information, but must always be accompanied by biotyping, because serotype by itself is not indicative of human pathogenicity. *Y. enterocolitica* has more than 50 distinct serotypes (on the basis of antigenic variations in cell wall lipopolysaccharide), and few of them are pathogenic. O:8 is the primary infectious serotype in the USA followed by O:3, O:5, 27, O:13a,13b, O:20, O:9, and so forth (Bottone, 1997).

It is very important to confirm the pathogenicity, since *Y. enterocolitica* species comprise both pathogenic and non pathogenic strains. A number of phenotypic characteristics associated with the virulence plasmid have been described. Calcium dependence, measured by growth restriction on magnesium oxalate agar and uptake of Congo red (Riley and Toma, 1989), autoagglutination at 35°C to 37°C (Laird and Cavanaugh, 1980), and uptake of crystal violet (Bhaduri et al., 1987) are the most popular indirect markers. They have been evaluated to predict the pathogenicity of *Y. enterocolitica* based on the presence of virulence plasmid (Frederiksson-Ahooma and Korkeala, 2003a). However, the tests may be unreliable to differentiate the pathogenic and non pathogenic strains due to possible losses of the virulence plasmid (pYV) under various culture conditions (Thoerner et al., 2003).

The pathogenicity of *Y. enterocolitica* also depends primarily on the presence of virulence markers that located on chromosome and on plasmid (Cornelis et al., 1998; Bottone, 1999). Chromosomal virulence genes include *ail*, attachment invasion locus gene, reported to be present only in pathogenic strains (Miller et al., 1990); *ystA*, which encodes a heat-stable enterotoxin (Delor et al., 1990; Ibrahim et al., 1997a); *ystB*, which is responsible for the heat-stable enterotoxin present mainly in biotype 1A strains (Ramamurthy et al., 1997; Singh and Viridi, 2004). The plasmid-encoded virulence factors include *Yersinia* adhesion A (*yadA*), an outer membrane protein involved in autoagglutination, serum resistance, and adhesion (Skurnik and Wolf-Watz, 1989), and the *virF*, which encodes transcriptional activators of the Yop (*Yersinia* outer membrane protein) regulation (Cornelis et al., 1989b; Straley, 1991; Cornelis et al., 1998).

Yersinia species have been isolated from a wide variety of sources in the environment, from animals as well as from foods include contaminated meat and

milk products (Hamama et al., 1992; Frederiksson-Ahomaa et al., 2001; Kechagia et al., 2007) which are the main transmitters of foodborne pathogens. In previous studies, *Y. enterocolitica* has been isolated from meat and meat products (Fukushima, 1985; Logue et al., 1996; Mauro et al., 2008; Güven et al., 2010; Bonardi et al., 2010; Lucero Estrada et al., 2011), raw milk (Schiemann and Toma, 1978; Vidon and Delmas, 1981; Ibrahim and Macrae, 1991) as well as in different types of cheeses (Hamama et al., 1992; Yentür et al., 2008; Özdemir and Arslan, 2011).

Determination of the prevalence of *Yersinia* and especially *Y. enterocolitica* in contaminated food presents difficulties stemming from standard bacteriological methods (Hussein et al., 2001; Frederiksson-Ahomaa and Korkeala, 2003a; Scheriefer and Petersen, 2011). The standard culture methods for the recovery of *Y. enterocolitica* usually involve time-consuming enrichment steps followed by plating on selective media (De Boer, 1992). Several methods that have been recommended for the isolation of *Yersinia* from clinical, food and environmental samples take advantage of their psychrophilic nature. The cold enrichment method (at +4°C) has been widely used for isolation of this organism. At low temperatures, the growth rate of competitive bacteria is slowed sufficiently so the psychrotrophic *Y. enterocolitica* can multiply to numbers necessary for isolation on plating media (Mehlman et al., 1978; Schiemann, 1987). *Yersinia* can alternatively be detected by means of alkali treatment method. This technique is effective in reducing background bacteria in food and it can promote recovery of *Y. enterocolitica* which has a high pH tolerance. The alkali method provides a simple, sensitive, and rapid technique for recovery of *Yersinia* from *Yersinia* contaminated food (Aulisio et al., 1980).

There has been increasing concern on the emergence of multidrug-resistant foodborne pathogens from foods including meat and milk products. The misuse or

overuse of antimicrobials in animal production, veterinary medicine and human therapy is generally associated with an increase in bacterial resistance. Thus, strains that are resistant to antimicrobials can be found in chicken and meat and they can be transmitted to humans (Presi et al., 2009; Soltan-Dallal et al., 2010; Kilonzo-Nthenge et al., 2013). There is also the hazard of therapeutic failure due to the increasing incidence of antimicrobial resistance among foodborne pathogens (Straley et al., 2006).

The objectives of this study were to determine the occurrence of *Yersinia* spp. in meat and milk products, to compare the different isolation methods including the ISO 10273 and the cold enrichment, for the recovery of *Yersinia* from foods which is more difficult than its recovery from other sources and to identify the *Y. enterocolitica* using both the conventional and molecular methods including 16S rRNA gene sequencing. The present study also aimed to investigate the biotypes and phenotypic virulence characteristics (autoagglutination, uptake of Congo red and crystal violet) of *Y. enterocolitica* as well as the presence of chromosomal (*ail*, *ystA* and *ystB*) and plasmid (*yadA* and *virF*) encoded virulence genes and antimicrobial resistance profiles of all *Yersinia* spp. from meat and milk products.

1.1. *Yersinia* spp.

1.1.1. Classification and Taxonomy

Van Loghem in 1944 proposed the separation of the genus *Yersinia* from the genus *Pasteurella*, which became effective in 1974. *Yersinia pseudotuberculosis* was first described by Malassez and Vignal in 1883. *Yersinia enterocolitica* was first described in 1939 by Schleifstein and Coleman who called it *Bacterium enterocoliticum* in 1943 (Carniel et al., 2006). The genus *Yersinia* belongs to the

family *Enterobacteriaceae*. *E. coli* tDNA (the genes coding for transfer RNA) and *Y. pestis* tDNA are 63% related, a value similar to that found for tDNA and *Hafnia alvei* tDNA. All *Yersinia* species express the common enterobacterial antigen. The genus *Yersinia* currently consists of different species. Based on DNA-DNA hybridization studies, all of these species are more closely related to each other than to any other *Enterobacteriaceae* species. The strains originally described as *Y. enterocolitica*-like organisms or atypical *Y. enterocolitica* have been separated into three different species: *Y. intermedia*, *Y. frederiksenii* and *Y. kristensenii* (Bottone et al., 2005). The use of a new approach that combine data obtained by traditional biochemical test and more recent studies including, for example, housekeeping genes sequencing, 16S rRNA gene analysis and DNA-DNA reassociation studies has been permitted to improve taxonomy knowledge (Carniel et al., 2006).

The rRNA gene is the most conserved (least variable) DNA in all cells. Portions of the rDNA sequence from distantly related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria. Thus the comparison of 16s rRNA sequence can show evolutionary relatedness among microorganisms. The 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Janda and Abbott, 2007).

Neubauer et al. (2000b) subdivided *Y. enterocolitica* into two subspecies based on the 16S rRNA sequence and DNA-DNA reassociation values. *Y. enterocolitica* subsp. *enterocolitica* comprises the *Y. enterocolitica* type strain ATCC 9610 and strains from high pathogenic biotype 1B whereas *Y. enterocolitica* subsp. *paleoartica*

comprises the strains from low-pathogenic biotype 2-5 and non pathogenic biotype 1A.

1.1.2. History

DNA/DNA hybridization studies performed in the 1980s demonstrated that *Y. enterocolitica* is the most distantly related pathogenic species of *Yersinia*. *Y. pestis* and *Y. pseudotuberculosis* exhibit such a high degree of DNA relatedness that they should have been included into a single species. However, *Y. pestis* causes plague and is transmitted by flea bites, whereas *Y. pseudotuberculosis* is transmitted by the fecal-oral route and rarely leads to death. The reclassification of *Y. pestis* and *Y. pseudotuberculosis* into a single species has been rejected. *Y. pestis* was found to be a clone that evolved from *Y. pseudotuberculosis* 1500-20.000 years ago. Despite their long existence, the enteropathogenic species *Y. enterocolitica* and *Y. pseudotuberculosis* were not known before the end of the nineteenth century (Carniel et al., 2006). *Y. pseudotuberculosis* was identified in 1883, before *Y. enterocolitica*. This species infects a wider range of animals than *Y. enterocolitica* but its importance in human infections is much lower than that of *Y. enterocolitica* worldwide. Both species are transmitted by the oral route and cause intestinal symptoms such as abdominal pain (especially *Y. pseudotuberculosis*), diarrhea (especially *Y. enterocolitica*), and fever (Carniel et al., 2006).

1.1.3. The genus *Yersinia*

The genus *Yersinia* belongs to the family *Enterobacteriaceae* and is currently composed of 11 species. Three of them are pathogenic for humans and animals: the enteropathogens *Y. pseudotuberculosis* and *Y. enterocolitica* and the plague agent *Y.*

pestis. *Yersinia* are Gram-negative, facultative anaerobic, non-spore forming straight rods or coccobacilli, 0.5 to 0.8 by 1 to 3 µm in size (Bottone et al., 2005).

The optimum growth temperature for *Yersiniae* is 28°C-29°C with the range of 4°C to 42°C. They are often more active biochemically at 25°C than 37°C; for example most of them are motile at 25°C but non-motile at 37°C (Bottone et al., 2005). Phenotypic characteristics are often temperature dependent, and usually more characteristics are expressed by cultures incubated at 25-29°C than at 35-37°C. Placement of the genus *Yersinia* within the *Enterobacteriaceae* family is supported by both biochemical and DNA-DNA relatedness studies (Bottone et al., 2005).

Members of the genus *Yersinia* are psychrotrophic organisms and some strains of *Y. enterocolitica* can grow at temperatures as low as -5°C, although growth is very slow below 0°C. *Y. enterocolitica* survives in frozen food for extended periods and even with stands repeated freezing and thawing (Bergann et al., 1995). On the other hand, *Yersinia* is destroyed quite rapidly by heat; it survives only 18 s at 72°C in milk. Cells are sensitive to pasteurization (Toora et al., 1992).

Yersinia species can grow in a pH range of 4.0–10.0 and tolerate NaCl concentration of up to 5%. *Y. pseudotuberculosis* and *Y. pestis*, however, tolerate a pH range of 5.0–9.6 and NaCl concentration of up to 3.5%. The optimum pH for all species is 7.2–7.4 (Ray, 2004; Bottone et al., 2005; Carniel et al., 2006).

1.1.4. Virulence factors of *Yersinia* spp.

Pathogenic *Yersinia* strains carry several virulence factors encoded on the chromosome and on virulence plasmid (pYV) which are required for adhesion, invasion and colonization of the intestinal epithelial cells and lymph nodes, growth and survival inside the macrophages, and serum resistance (Bhunia, 2008). Chromosomally encoded virulence factors of *Y. enterocolitica* and *Y.*

pseudotuberculosis include *inv* (invasin) and *ail* (attachment invasion locus), which both contribute to invasion of the body. The environmental isolates are shown to be negative for *inv* and *ail* genes and are not associated with human disease (Bhunia, 2008).

In addition, the subgroup of highly pathogenic strains possess a chromosomal entity, the high pathogenicity island (HPI), which is required for their systemic dissemination in the host (Carniel et al., 2006). In *Y. pestis*, an important pathogenicity island termed the *pgm* locus carries the genes for a hemin storage system (*hms*) and a siderophore named yersiniabactin (Ybt). These virulence factors are expressed at 25 °C and / or 37°C. The role of thermostable enterotoxin produced by pathogenic strains of *Y. enterocolitica* is still under discussion, although a clinical association has been suggested (Aleksic and Bockemühl, 1999).

All *Yersinia* species, except *Y. pestis*, are able to use urease to hydrolyze urea to ammonia and carbon dioxide. Urease, a trimeric protein complex requiring nickel ions to be active, is exclusively located in the bacterial cytosol. Three adjacent genes, *ureA*, *ureB* and *ureC*, encode the structural subunits, UreA (11-kDa), UreB (16-18 kDa) and UreC (61-kDa), which together form an inactive form of the enzyme. *Yersinia*'s ureolytic activity is growth phase-dependent and is optimal during the stationary phase, at both 28°C and 37°C (Carniel et al., 2006).

The three pathogenic species share a 70 to 75-kb plasmid carrying a number of genes, whose products may be classified into four main categories: adhesion/invasion (*yadA*; not important in *Y. pestis*), excreted antiphagocytic proteins (YopE, YopH, and YopM in the three species; LcrV in *Y. pestis*; YpkA in *Y. pseudotuberculosis*), processing and excretion-related proteins (Lcr). Loss of the plasmid results in

decreased pathogenicity and inability to cause disseminated disease (Aleksic and Bockemühl, 1999).

1.1.4.1. Chromosomally encoded virulence factors

Although many different virulence factors for surviving and multiplying in host are encoded by pYV, pathogenic *Yersiniae* need chromosomally encoded virulence factors for full virulence (Revell and Miller, 2001).

1.1.4.1.1. Invasin (*inv*)

All strains of *Y. enterocolitica* which carry pYV also produce surface-expressed protein termed invasin. This outer membrane protein was first identified in *Y. pseudotuberculosis* as a 102-kDa protein product of the chromosomal *inv* gene. Despite the difference in size of invasions from *Y. enterocolitica* and *Y. pseudotuberculosis*, the two proteins are functionally highly conserved (Robins-Browne, 2007). Invasin is a 92-kDa outer membrane protein and binds to β 1-integrin receptor and is important virulence factor for the early phase of the intestinal infection. Invasin is encoded by *inv* gene and its expression is high when *Yersinia* was grown in media with a neutral pH at 23°C but low at 37°C (Bhunja, 2008).

Although, DNA sequences homologous to *inv* occur in all *Yersinia* species (except *Y. ruckeri*), this gene is functional only in *Y. pseudotuberculosis* and the classical pathogenic biotypes (1B through 5) of *Y. enterocolitica*, suggesting that invasion plays a key role in virulence (Robins-Browne, 2007). A functional *inv* gene is present in *Y. pseudotuberculosis* and *Y. enterocolitica*, while *Y. pestis* has a disrupted form of *inv*. Invasin is the primary invasion factor of *Y. enterocolitica* in tissue culture invasion models, and is required for efficient translocation of the bacteria across the intestinal epithelium mice. The *inv* expression is regulated by

rovA (regulator of virulence A), which is located on chromosome. The *rovA* was found both in vitro and in vivo (Revell and Miller, 2001).

1.1.4.1.2. Attachment invasion locus (*ail*)

Classical pathogenic strains of *Y. enterocolitica* produce an outer membrane protein, unrelated to invasion which also confers invasive ability on *Escherichia coli*. Ail is a 17-kDa membrane protein and is involved in adhesion and in the serum resistance. The surface protein Ail mediates bacterial attachment to some cultured epithelial cell lines and invasion of others. The *ail* gene occurs only in the classical pathogenic varieties of *Y. enterocolitica* and is expressed optimally in vitro at 37°C (unlike *inv*, which is expressed optimally at 25°C, unless the pH is lowered to 5.5) (Robins-Browne, 2007; Bhunia, 2008).

There are also suggestive data that supports a role for *ail* in virulence of *Y. enterocolitica*. First, *ail* was identified by its ability to confer an attachment and invasion phenotype to *E. coli* K12, suggesting *ail* may be a secondary invasion factor during the infectious process. Secondly, it has been demonstrated that *ail* is required for resistance to killing by human serum. This phenotype may be required for complete virulence in the human but not in the mice, because *Y. enterocolitica* is not killed by mouse serum. Thirdly, the *ail* gene is found in pathogenic serotypes of *Y. enterocolitica*, but is not found in non pathogenic serotypes of *Y. enterocolitica* nor in non pathogenic species of *Yersinia*. Interestingly, the *Y. pseudotuberculosis* *ail* gene does not confer the attachment and invasion phenotype, while it does promote serum resistance (Revell and Miller, 2001).

1.1.4.1.3. *Yersinia* stable toxin (*yst*)

Yersinia stable toxin (*yst*) is structurally and functionally homologous to the heat-stable enterotoxin (ST) of enterotoxigenic *Escherichia coli*, and is encoded by the chromosomal *yst* gene (Delor et al., 1990). In some strains of *Y. intermedia* and *Y. kristensenii*, a gene homologous to *yst* or an inactive *yst* gene has been found and its presence has not always correlated with toxin production (Sulakvelidze, 2000).

The role of enterotoxin in the pathogenesis of *Y. enterocolitica* infection is unclear. When first isolated from clinical material, most strains of *Y. enterocolitica* secrete a heat-stable enterotoxin, known as *yst*. *Y. enterocolitica* has *ystA*, *ystB*, and *ystC* variants. Three subtypes of *yst* exist: *ystA*, *ystB* and *ystC*. The subtypes *ystA* and *ystB* each made of 30 aminoacids, while the *ystC* is much larger and consisted of 53 aminoacids. *Y. enterocolitica* produces an enterotoxin, *yst*, which is heat-stable (100°C for 15 min) and remains active at pH range of 1-11 at 37°C for 4 h and is methanol soluble. The *yst* is involved in diarrhea (Bhunia, 2008).

Despite its similarity to known virulence factors and the fact that the production of *yst* is by and large restricted to the classical pathogenic biotypes of *Y. enterocolitica*, the contribution of *yst* to the pathogenesis of diarrhea associated with yersiniosis is uncertain. Doubts regarding the role of *yst* in virulence stem from the following observations: (i) the toxin is generally not detectable in bacterial cultures incubated at temperatures above 30°C, (ii) the production of *yst* has not been demonstrated in vivo, and (iii) strains of *Y. enterocolitica* that have spontaneously lost the ability to produce *yst* retain full virulence for experimental animals. The availability of diagnostic probes for the genes encoding *ystB* and *ystC* has indicated that biotype 1A *Y. enterocolitica* strains commonly carry the genes for *ystB*, whereas *ystC*, which was originally identified in a serotype O:3 strain of biotype 4, is rare

(Robins-Browne, 2007). The *ystA* is present in pathogenic bioserotypes of *Y. enterocolitica*, *ystB* seems to be associated to biotype 1A strains and *ystC* has been detected rarely (Rammamurthy et al., 1997).

1.1.4.1.4. Iron acquisition and the high-pathogenicity island (HPI)

Iron is an essential micronutrient for almost all bacteria, including *Y. enterocolitica*, and it is readily available in many environments whereas in mammalian tissues it is tightly bound to carrier proteins such as transferrin and lactoferrin. Therefore, iron acquisition is important factor in *Yersinia* pathogenicity. In fact, low-pathogenic *Y. enterocolitica* strains (biotype 2 and biotype 4), usually cause only intestinal symptoms but when iron is available, for example in iron-overloaded patients, these strains are able to cause systemic infections (Robins-Browne, 2007).

Iron acquisition is achieved by siderophore such as yersiniabactin which is chromosome-linked. Under iron starvation, *Yersinia* produces large amounts of iron receptors, *foxA* and *fcuA* on the outer membrane, which bind the siderophores to sequester iron (Bhunia, 2008). Investigation of the relationship of yersiniae to iron has revealed that these bacteria employ a wide array of processes to acquire iron from inorganic or organic sources. The fact that most clinical isolates of *Y. enterocolitica* do not produce siderophores accounts for their reliance on abnormally high concentrations of iron for growth in animals. Interestingly, however, the highly virulent biotype 1B strains, as well as *Y. pestis* and *Y. pseudotuberculosis*, carry genes for the biosynthesis, transport, and regulation of a 482-kDa, catechol-containing siderophore known as yersiniabactin. The ca. 40-kbp *ybt* locus contains high G+C content. These features, which are typical of a pathogenicity island, have led to the *ybt* locus also being known as the *Yersinia* HPI. Interestingly, the *Yersinia*

HPI is also found in a various bacteria, such as *E. coli*, *Klebsiella*, *Citrobacter*, and *Salmonella* (Robins-Browne, 2007).

1.1.4.1.5. Mucooid *Yersinia* factor (*myf*)

Most intestinal pathogens possess distinctive colonization factors on their surfaces which mediate their adherence to the intestinal epithelium. Some strains of *Y. enterocolitica* produce a fimbrial adhesion, named *myf* (for mucooid *Yersinia* fibrillae) because it bestows a mucooid appearance on bacterial colonies which express it. Myf proteins are narrow, flexible fimbriae which resemble CS3, an essential colonization factor of some human strains of enterotoxigenic *E. coli*. The chromosomal region involved in the production of *myf* contains three genes: *myfA* encoding the 21-kDa major subunit, *myfB* and *myfC* encoding an assembly machine involved in the synthesis of many fimbriae. Like *ail* and *yst*, *myf* occurs predominantly in *Y. enterocolitica* strains of the classical pathogenic varieties commonly associated with disease (Robins-Browne, 2007).

1.1.4.2. Plasmid-encoded virulence factors

For full virulence, all *Yersinia* need also a 70-kb plasmid called pYV (for *Yersinia* virulence plasmid) that allow them to survive and multiply in lymphoid tissues of their host. It has been known for decades that pathogenic strains are Ca^{+2} dependent when grown at 37°C and that this property can be lost and that its loss correlates with a loss of virulence. Only later it appeared that virulence and Ca^{+2} dependency are encoded by a 70-kb plasmid (Cornelis et al., 1998).

1.1.4.2.1. *Yersinia* adhesin A protein (*yadA*)

The virulence plasmid (pYV) encodes an outer membrane protein YadA (*Yersinia* adhesin A), a set of secreted proteins called Yops (*Yersinia* outer

membrane proteins), and their secretion apparatus called type III secretion systems. The *yadA* is an important virulence factor and facilitates the bacterial attachment to host cells and protects *Yersinia* from the non specific immune system such as phagocytosis and complement-mediated cell lysis. YadA is a 160-240 kDa protein and appears as fibrillar structure covering the entire bacterial cell surface (Bhunia, 2008). The *yadA* is expressed at 37°C but not at 25°C. Furthermore, *yadA* expression is not affected by pH, salt, or sugar concentration. In *Y. enterocolitica* and *Y. pseudotuberculosis*, it too facilitates pathogen binding to M cells of the intestinal mucosa, signal-induced internalization, and subsequent translocation to the Peyer's patches and mesenteric lymph nodes. But *yadA* is nonfunctional in *Y. pestis* (Scherieffer and Petersen, 2011).

Expression of *yadA* is regulated by two different gene products: *virF* (virulence) and *lcrV* (low calcium response). The *virF* senses the optimal temperature required for protein synthesis, and *lcrV* regulates the *yadA* expression depending on the availability of extracellular calcium concentration (Bhunia, 2008). The function of *yadA* protein in the virulence of *Y. enterocolitica* as follows: serum resistance, surface hydrophobicity, autoagglutination, adhesion to epithelial cells, expression of fibrils on the surface, haemagglutination, binding to intestinal brush border membranes and resistance to killing by polymorphonuclear leukocytes (Bottone, 1997).

1.1.4.2.2. Transcriptional activator (*virF*/*lcrF*)

The mechanism of regulation by temperature involves *virF* and DNA supercoiling. VirF is a pYV-encoded, DNA binding protein. The *virF* plays a central role in the virulence of *Y. enterocolitica* by governing the transcriptional activation of *yadA*, *ylpA*, all of the *yop* genes, and the *virC* operon. Transcription of *virF* itself

is also regulated by temperature (Robins-Browne, 2007). VirF/lcrF is a 30-kDa basic protein that belongs to AraC family of regulators. In *Y. enterocolitica*, the *virF* gene itself is strongly thermoregulated and *virF* is only active at 37°C. The *lcrF* is found in *Y. pestis* and *Y. pseudotuberculosis* (Cornelis et al., 1989a; Carniel et al., 2006).

1.1.4.2.3. Type III secretion system

The genes encoding type III secretion system (TTSS), present in many others Gram negative species, named *Yersinia* secretion apparatus (*ysc*), and the secreted *Yersinia* outer proteins (Yop). The genes are encoded by pYV plasmid and their expression is temperature dependent (37°C). This system is able to inhibit phagocytosis and downregulate the inflammatory response of the host. The TTSS delivers six effector proteins into the host cell cytosol: YopH, YopO, YopT, YopP/J, YopE, and YopM (Bhunia, 2008). TTSS forms a needle structure on the surface of pathogenic *Yersinia* species which interacts with target cells and enables infection of six different Yops which effectively interfere with phagocytosis and other innate host cell responses as well as the adaptive inflammatory cascade, ultimately resulting in target cell apoptosis (Scherieffer and Petersen, 2011).

1.1.4.2.4. *Yersinia* outer proteins (Yops)

Yops are present in the cell surface. Yops are secreted in the presence of very low levels of Ca^{+2} . The expression of Yop is temperature dependent and occurs mostly at 37°C, which is critical during pathogenesis in the host (Bhunia, 2008). The transport of Yops from the bacterial cytoplasm via *ysc* (type III secretory apparatus) into the host cell cytosol via the translocation apparatus is envisaged to occur in one step from bacteria which are closely bound to the host cell. Two of the proteins required for translocation are YopB (42-kDa) and YopD (33-kDa), both interact

directly with host cell membranes YopB suppresses the secretion of macrophage-derived cytokines which are important in inflammatory response against infection. YopB and YopD are encoded by the *lcrGV-sycD-yopBD* operon, which also encodes *lcrV*, *lcrG*, and *sycD*, the chaperone for YopB and YopD (Robins-Browne, 2007). There is no doubt that Yops are produced in vivo because animals and humans infected with virulent *Yersinia* species develop antibodies to these proteins during the course of infection (Robins-Browne, 2007).

1.2. Pathogenic *Yersinia* species

The genus *Yersinia* is currently composed of 11 species. Three of them are pathogenic for humans and animals: the enteropathogens *Y. pseudotuberculosis*, *Y. enterocolitica* and the plague agent *Y. pestis*.

1.2.1. *Yersinia enterocolitica*

1.2.1.1. Habitat of *Y. enterocolitica*

A wide variety of ecological niches may shelter *Y. enterocolitica*. The environment (soil and water), food (milk, retail meats products, vegetables, eggs, cheese, etc.) and a wide range of animals: domestic (cat and dogs), stock farm (pig, rabbit, cow, horse, sheep, etc.) can be contaminated with this bacterium (Carniel et al., 2006). Zoonoses are diseases or infections, which are transmissible from animals to humans. Infections with *Yersinia* species are zoonoses. The subgroups of *Y. enterocolitica* which commonly occur in humans also occur in domestic animals. *Y. enterocolitica* can occupy a broad range of environments and has been isolated from the intestinal tracts of many different mammalian species as well as from birds, frogs, fish, flies, fleas, crabs, and oysters. Foods that may harbor *Y. enterocolitica*

include pork, beef, lamb, poultry, and dairy products, such as milk, cream and ice cream (Robins-Browne, 2007).

Y. enterocolitica is also commonly found in a variety of terrestrial and fresh water ecosystems, including soil, vegetation, lakes, rivers, wells, and streams, and can persist for extended periods in soil, vegetation, streams, lakes, wells, and spring water, particularly at low environmental temperatures (Robins-Browne, 2007).

Most primary pathogenic strains from humans and domestic animals fall within biotype 1B, 2, 3, 4, and 5. Pathogenic *Y. enterocolitica* of biotypes 2 to 5 are mainly isolated from animals (sheep, cattle, goats, and poultry) or food (Carniel et al., 2006). In contrast, *Y. enterocolitica* strains of biotype 1A are commonly obtained from terrestrial and freshwater ecosystems. For this reason, they are often referred to as environmental strains, although some of them may be responsible for intestinal infections (Robins-Browne, 2007). Another line of evidence that is taken to indicate the avirulence of biotype 1A strains is their relatively high prevalence in the environment and healthy animals. Indeed, biotype 1A strains are ubiquitous, inhabiting a wide variety of environmental niches such as soil and various sources of water, including streams, lakes, water wells, and wastewater (Sulakvelidze et al., 1996).

There is a preferential relationship between the bioserotype of the strains and their ecological niches. Bioserotype 4/O:3 strains are the most common pathogenic *Y. enterocolitica* found over the world, followed by 2/O:9 strains. 4/O:3 strains are isolated with a high frequency from pigs; 2/O:9 strains are often found in cows, goats and sheep; the reservoir of strains of bioserotypes 2 or 3/O:5, 27 is not clearly established; strains of the very rare bioserotypes 5/O:2, 3 are isolated from sheep,

hares and goats. Biotype 3 strains are not commonly isolated, and biotype 5 strains are extremely rare (Carniel et al., 2006; Robins-Browne, 2007).

1.2.1.2. Pathogenicity of *Y. enterocolitica*

The pathogenesis of *Y. enterocolitica* depends on ecology, geographic distribution, biochemical and antigenic structures, chromosomal and plasmid-encoded genes (Kechagia et al., 2007).

The urease enzyme is necessary for the survival and pathogenesis of *Y. enterocolitica* and is encoded by the urease gene complex (*ure*) on the chromosome (Ramamurthy et al., 1997). This enzyme catalyzes the hydrolysis of urea to form one molecule of carbon dioxide and two of ammonia, leading to an increase in pH to levels at which the bacteria remain viable. This characteristic makes *Y. enterocolitica* acid tolerance and thereby enhancing survival in acidic environments (Bhunia, 2008).

Lipopolisaccharide (LPS) is an important component of the outer membrane of Gram-negative bacteria and is an endotoxin inducing a strong response from animal immune system. LPS is a large molecule consisting of three parts: lipid A, oligosaccharide core and O-side chain (O-antigen). The lipid A, the hydrophobic part, anchors the LPS into the bacterial outer membrane and it is responsible for the endotoxin activity (Skurnik et al., 1999). O antigen is the outermost domain of LPS molecule thus plays a critical role in the bacterial interaction with the environment (Zhang et al., 1997). *Y. enterocolitica* can be classified as smooth or rough depending on the amount of O side chain polysaccharide attached to the inner core region of the cell wall LPS. Synthesis of O side chain by *Y. enterocolitica* is regulated by temperature, such that colonies are smooth when grown on at temperatures below 30°C but rough at 37°C. The outer core region of the LPS plays a role in maintaining

outer membrane integrity and may contribute to the resistance of *Y. enterocolitica* to bactericidal peptides in host tissues and macrophages. Smooth LPS may also enhance virulence by increasing bacterial hydrophilicity and thus bacteria passage through the mucous secretions which line the intestinal epithelium (Robins-Browne, 2007).

Serotyping of *Y. enterocolitica*, based on LPS surface O antigens flagellar (H) or fimbriae (K) antigens coincides to some extent with biotyping and provides a *Y. enterocolitica* is a heterogeneous species, being divisible into a large number of subgroups, chiefly according to biochemical activity and lipopolysaccharide O antigens. Serotyping provides a useful additional tool to subdivide this species in a way that relates to pathologic significance. At least 76 serotypes based on O antigens and 44 H antigens have been described for *Y. enterocolitica* and *Y. enterocolitica*-like organisms (Carniel et al., 2006; Robins-Browne, 2007). Actually, O antigens are the most used target and antisera for the serogroups O:3, O:5, O:27, O:8 and O:9, which are predominant in human yersiniosis, are commercially available. Same antigens have been found in both pathogenic and non pathogenic strains thus biochemical characterization is also required for correct description (Wauters et al., 1991). Among the pathogenic serogroups, O:3 and O:9 have been commonly associated with *Y. enterocolitica* strains recovered from Europe, Scandinavia, and Canada. Previously in the United States serogroups O:8 and O:5, 27 were the predominating serogroups (Bisset et al., 1990). PCR methods have been developed for the detection and identification of few common specific serotypes, such as O:3 (Weynants et al., 1996; Thisted-Lambertz and Danielsson-Tham, 2005). The biogroups of *Y. enterocolitica* are further subgrouped into serotypes based on variation in the LPS O antigens (Table 1).

Table 1. *Y. enterocolitica* biotypes and serotypes*

<i>Y. enterocolitica</i>					
Avirulent		Low virulence		High virulence	
Biotypes					
1A	2	3	4	5	1B
Serotypes					
O:5	O:9	O:1, 2, 3	O:3	O:2,3	O:8
O:6, 30	O:15,27	O:15,27			O:4
O:7, 8					O:13 a, b
O:18					O:18
					O:20
					O:21

*(Modified from Bottone, 1999).

Biotyping is based on the ability of *Y. enterocolitica* to metabolize selected organic substrates and provides a convenient means to subdivide the species into subtypes of variable clinical and epidemiological significance (Robins-Browne, 2007). *Y. enterocolitica* strains of biotype 1B, 2, 3, 4, and 5 possess' panoply of virulence determinants, including a chromosomally encoded invasins and a ca. 70-kb virulence plasmid. Biotype 1B strains of *Y. enterocolitica* carry two pathogenicity islands, which are associated with enhanced virulence (Robins-Browne, 2007). Biotype 1B yersiniae are usually isolated from patients in the United States and are referred to as "American" strains, although they have also been found in a number of countries in Europe, Africa, Asia, and Australasia (Carniel et al., 2006; Robins-Browne, 2007). Food animals are seldom infected with biotype 1B strains of *Y.*

enterocolitica whose reservoir remains unknown but appears likely to be wild rodents (Robins-Browne, 2007). The most frequent *Y. enterocolitica* biotype obtained from human clinical material worldwide is biotype 4.

Y. enterocolitica biotype 1A is widely present in Europe and, despite has traditionally been designated as non pathogenic, some authors have showed that this biotype is not as harmless as it is believed (Bottone, 1997; Carniel et al., 2006; Robins-Browne, 2007). Biotype 1A strains have also been implicated in nosocomial (Ratnam et al., 1982) and foodborne (Greenwood and Hooper, 1990) outbreaks, and were also isolated from extraintestinal infections (Bisset et al., 1990). Biotype 1A strains of *Y. enterocolitica* are often considered to be non pathogenic primarily because they do not possess the virulence-associated factors of pYV-bearing strains. The biotype 1A strains have been reported to lack both pYV plasmid and most chromosomal virulence genes such as *ail*, *myfA*, *ystA*, *ysa*, TTSS, and HPI, and only occasionally carry *ystA* and *myfA* (Kot et al., 2010). Although the *ail* gene is present in some biotype 1A strains, the *ail* gene alone is an insufficient virulence marker for

detecting the virulence of *Y. enterocolitica* biotype 1A strains (Sihvonen et al., 2011a). Despite the lack of traditional chromosomal-borne and plasmid-borne virulence genes in *Y. enterocolitica* strains of biotype 1A, some biotype 1A strains are isolated frequently from humans with gastrointestinal diseases. The biotype 1A strains isolated from humans and from pigs have been reported to produce *ystB* encoding *Yersinia* heat-stable enterotoxin (Kot et al., 2010). A recent study on 259 isolates of *Y. enterocolitica* and related species; indicated that *ystB* was the major

contributor to diarrhea produced by biotype 1A strains of *Y. enterocolitica* (Singh and Viridi, 2004). Some biotype 1A strains produce symptoms indistinguishable from that produced by isolates belonging to pathogenic biotypes (Burnens et al., 1996).

Most isolates of *Y. enterocolitica* from food or clinical resources have either of two pathogenic properties. First one is the ability to penetrate the intestinal wall, which is thought to be controlled by 70-kb virulence plasmid; that is absent in avirulent strains; second one is the production of heat-stable enterotoxin which is controlled by chromosomal genes (Robins-Browne, 2007; Sabina et al., 2011).

The virulence plasmid of *Y. enterocolitica* plays an important part in the pathogenicity of the organism and encodes several properties that can be detected by in vitro tests as autoagglutination, resistance to killing by normal human serum, and an ability to bind Congo red and crystal violet. Some of these characteristics have been exploited to facilitate the isolation and identification of pYV-bearing culture (Robins-Browne, 2007). The plasmid-linked virulence genes encode *Yersinia* outer membrane proteins (Yops) that are responsible for bacterial adhesion, a type III secretion system (TTSS) to deliver virulence proteins to the host cell cytosol to allow bacterial growth inside macrophages, serum resistance, and septicemia (Bhunia, 2008).

Y. enterocolitica is an invasive enteric pathogen whose virulence determinants have been the subject of intensive investigation, but not all strains of *Y. enterocolitica* are equally virulent (Table 2).

Table 2. Characteristics of pathogenic subgroups of *Y. enterocolitica*

Subgroups	Capacity to invade epithelial cells in vitro	Biotype (s)	Virulence-associated determinants
Classical	High (lower if pYV present)	2, 3, 4, 5	Invasin, Ail, fibrillae (Myf), urease, heat-stable enterotoxin (Yst), LPS, and a virulence plasmid (pYV) encoding YadA, a type III secretory pathways, and translocator and effector proteins
	High (lower if pYV present)	1B	All of the above, together with the HPI, the YSA pathogenicity island, a type II secretory pathway (Yts), and phospholipase A (YplA)
Atypical	Low to moderate	1A	Products of genes homologous to those for insecticidal toxin complex

*(Modified from Robins-Browne, 2007).

1.2.1.3. Clinical manifestations of *Y. enterocolitica* infections

Human clinical infections with *Y. enterocolitica* are usually acquired by ingestions of contaminated food or water. *Y. enterocolitica* enters the gastrointestinal tract after ingestion of contaminated food or water. The median infective dose for humans is not known but is likely to exceed 10^4 colony forming unit (CFU) (Robins-Browne, 2007). The inoculums of *Y. enterocolitica* require to cause intestinal symptoms has been determined to be 10^9 bacteria / mL. Infections with *Y. enterocolitica* typically manifest as nonspecific, self-limiting diarrhea but may give rise to a variety of suppurative and autoimmune complications (Carniel et al., 2006).

Y. enterocolitica is associated with foodborne infections resulting in gastroenteritis, mesenteric lymphadenitis, and septicemia and also causes diarrhea, terminal ileitis, autoimmune arthritis, and abscesses in humans (Bottone et al., 2005; Bhunia, 2008). Other human infections are listed in Table 3 (Bottone et al., 2005).

Table 3. Clinical spectrum of *Y. enterocolitica* infections

Type of infection	Manifestation/population
Gastrointestinal	• Enterocolitis, predominantly in young children; concomitant bacteremia may also be present in infants • Pseudoappendicitis syndrome, acute mesenteric lymphadenitis; terminal ileitis
Septicemia	• Especially in immunosuppressed individuals and those in iron overload or being treated with deferrioxamine • Transfusion related
Metastatic infections	• Focal abscesses: liver; kidney, spleen and lungs
Following septicemia	• Cutaneous manifestations: cellulitis, pyomyositis, pustules and bullous lesions • Pneumonia, cavitary pneumonia • Meningitis; panophthalmitis • Endocarditis, infected mycotic aneurysm • Osteomyelitis
Postinfection sequela	• Arthritis • Myocarditis • Glomerulonephritis • Erythema nodosum
Pharyngitis	

Antimicrobial agents usually active in vitro and clinically against this pathogen also include trimethoprim-sulfamethoxazole, chloramphenicol, newer fluoroquinolones such as ciprofloxacin and ofloxacin, tetracycline hydrochloride, doxycycline, aminoglycosides, imipenem and aztreonam (Bottone, 1997). Recently a study of Sihvonen et al. (2011b) indicated that most of the suspected outbreaks strains were multiresistant to antimicrobial, in particular ampicillin, chloramphenicol, streptomycin and sulfonamide and showed that the last three resistances were carried on a conjugative plasmid. The antimicrobial susceptibility of *Y. enterocolitica* is serogroup-specific. Serogroups O:3 and O:9 present generally a greater panel of antimicrobial resistance to penicillin. Cephalosporinase or penicillinase activities are generally expressed by all isolates due to the chromosomally-mediated production of beta-lactamase but they are sensitive to third generation cephalosporins such as ceftriaxone and cefotaxime (Bottone et al., 2005; Baumgartner et al., 2007; Scheriefer and Petersen, 2011). Two types of chromosomal β -lactamase were described in *Y. enterocolitica*. First is the non-inducible broad spectrum β -lactamase enzyme A, and the inducible cephalosporinase enzyme B. Strains of serotypes O:1, O:2, O:3 and O:9 produced two types of β -lactamase while strains of serotypes O:5, 27 produced only enzyme B. It has been demonstrated that although the genes encoded for both types of β -lactamase are present in all *Y. enterocolitica*, they are not always active (Carniel et al., 2006).

1.2.1.3. Transmission

Transmission of *Y. enterocolitica* occurs by the fecal-oral route. The organisms are ingested with contaminated food. After their journey in the intestinal tract of their host, they are excreted in the feces, which can serve as a contamination of a new host or of the environment. Children below the age of five are much more prone to *Y.*

enterocolitica infections because they have weaker defense mechanisms, immature bacterial flora in their intestines, behaviors favoring bacterial infections, and surveillance of diarrhea is more intensive in infants than in adults. Person-to-person transmission, although scarcely documented, probably represents another mode of circulation of pathogenic *Y. enterocolitica* through infected hands (Carniel et al., 2006). Occasionally *Y. enterocolitica* infection occurs after direct or indirect contact with infected animals. It has been isolated from the intestinal tracts and feces of many animals, including rodents, domestic animals, and other animals and water contaminated by those animals. The pig appears to be the main reservoir for the strains causing infection in humans. Pig feces are a potential mode of direct transmission to farmers. As *Y. enterocolitica* possess the ability to grow under extreme environmental condition, they are well adapted to survival in cooler temperate zones as well as in microaerophilic environments including aquatic environments (Sabina et al., 2011).

1.2.2. *Yersinia pseudotuberculosis*

1.2.2.1. Habitat of *Y. pseudotuberculosis*

Y. pseudotuberculosis is widely distributed among mammals (farm animals, pets and wild animals) and birds in most countries with cold climates. The bacterium is widely spread in the environment (soil, water, vegetables, etc.) contaminated by feces of infected wild animals, and can survive in water for long periods. Serotype O: 1a and 1b strains have been isolated from farm animals, domestic pets, experimental animals, wild mammals, commercially reared fur-bearers, zoological animals.

1.2.2.2. Clinical manifestation of *Y. pseudotuberculosis* infections

Y. pseudotuberculosis has a wide distribution in most countries with cold climates and is recognized as an important causative agent of sporadic and epidemic human enteric diseases (Carniel et al., 2006). *Y. pseudotuberculosis* is a human and animal pathogen responsible for mesenteric lymphadenitis, diarrhea, and septicemia. In humans, the usual presentation includes fever and abdominal pain followed by diarrhea and vomiting. As for *Y. enterocolitica*, *Y. pseudotuberculosis* septicemia often affects patients with a pre-existing condition involving iron overload, and/or liver disease including hemochromatis, sickle cell disease, thalassemia or chronic hemodialysis (Bottone et al., 2005; Carniel et al., 2006).

Y. pseudotuberculosis from clinical isolates is susceptible to most commonly used antibiotics such as ampicillin, cefotaxime, chloramphenicol, cotrimoxazole, nitrofurantoin and the fluoroquinolones. β -lactamase has not been detected in this species. Resistance to tetracyclines has also been reported in some strains isolated from animals (Carniel et al., 2006).

1.2.2.3. Distribution of the subgroups of *Y. pseudotuberculosis*

Y. pseudotuberculosis has 21 different serotypes: O:1a, 1b, 1c, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 (Carniel et al., 2006). The pathogenicity of *Y. pseudotuberculosis* depends on the presence of a 70-kb virulence plasmid termed “pYV”. This plasmid is essential for virulence and its presence differentiates pathogenic from non pathogenic *Yersinia* (pYV virulence plasmid). The pYV is found in almost all serotypes O:1a to 6, 10 and 15 but not in serotypes O:7, 8, 9, 11, 12, 13 and 14. Additionally, a pathogenicity island termed “high-pathogenicity island” and/or chromosomally encoded novel superantigenic toxins designated “*Y. pseudotuberculosis* -derived mitogen” YPMa, YPMb, or YPMc are

found in *Y. pseudotuberculosis* (Superantigens of *Y. pseudotuberculosis*) (Carniel et al., 2006).

1.2.2.4. Transmission

Transmission of *Y. pseudotuberculosis* to humans and animals does not need a vector and occurs by contact with infected animals or by consumption of food (vegetables and meat) or water contaminated by animal reservoirs (Carniel et al., 2006).

1.2.3. *Yersinia pestis*

1.2.3.1. Habitat of *Y. pestis*

Rodents are the natural reservoir of the plague bacteria. The two major environment exploited by *Y. pestis* in nature consist of the flea vector and the mammalian host (Brubaker, 2006). More than 200 mammalian species have been reported to be naturally infected with *Y. pestis*; however, rodents are of principal importance. Highly susceptible hosts include various species of mice, rats, voles, gerbils, ground squirrels, rabbits, and prairie dogs. If such susceptible populations migrate into areas occupied by infected species, this may be followed by dramatic increases in mortality and decimation of the invaders. Their fleas then search for new hosts, including humans, and transmit the disease (Aleksic and Bockemühl, 1999). Three biogroups have been described in relation to the geographical distribution of the organism: (a) biogroup antique produces acid aerobically from glycerol, reduces nitrate to nitrite, does not ferment melibiose, and is found in Central Asia and Central Africa; (b) biogroup medievalis produces acid from both glycerol and melibiose, but does not reduce nitrate to nitrite; (c) biogroup orientalis (synonym: oceanic) does not

produced acid from either glycerol or melibiose but reduces nitrate to nitrite and is distributed worldwide (Bottone et al., 2005).

1.2.3.2. Clinical manifestation of *Y. pestis* infections

Y. pestis is the causative agent of bubonic plague (also known as “the pestilence” or “Black death”) is the most devastating epidemic bacterial disease known to humankind. Human plague can thus be classified into three general syndromes; bubonic plague, primary and secondary septicemic plague, and pneumonic plague (Aleksic and Bockemühl, 1999; Bottone et al., 2005; Brubaker, 2006). The clinical course of plague in animals and humans is similar. Following infection by a flea or animal bite or via otherwise injured skin or mucous membrane, the organisms enter the bloodstream, where they are transported to the nearest lymph node. Ingested by macrophages, the *Y. pestis* organisms survive and proliferate there. Depending on the immunity of the host, bacteria leaking into the bloodstream may cause a secondary septicemia and be transported to various organs. This stage of the disease, septicemic plague, may be followed by meningitis or pneumonia when the organisms parasitize the alveolar macrophages. Patients with plague often develop necrotic lesions in the peripheral blood vessels, led to the name “black death” (Aleksic and Bockemühl, 1999).

1.2.3.4. Transmission

Plague is one of the oldest recorded infectious diseases. Some historical and geographical data suggest the Himalayas to be the most likely birthplace. From there, the disease spread east toward the Middle East and Europe. Transmission in these populations occurs mainly via their fleas but occasionally also by direct contact and

cannibalism. In the flea, the infection is restricted to the alimentary tract, including the salivary glands and stomach (Aleksic and Bockemühl, 1999).

1.3. The nonpathogenic *Yersinia* species

The other *Yersinia* species are *Y. aldovae*, *Y. bercovieri*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. mollaretii*, *Y. rohdei* and *Y. ruckeri*.

1.3.1. *Yersinia aldovae*

Y. aldovae was described in 1984 by Bercovier et al. (1984). The species is composed of isolates previously referred to as *Y. enterocolitica*-like. Two biovars exist-one is typically sucrose negative, and one sucrose positive. *Y. aldovae* has been isolated from aquatic ecosystems-drinking water, river water, fish, and rarely soil. *Y. aldovae* has not been implicated in animal infections and has not been isolated from humans (Bottone et al., 2005; Carniel et al., 2006).

1.3. 2. *Yersinia bercovieri*

Y. bercovieri formerly called *Y. enterocolitica* biogroup 3B. Later it transferred to *Y. enterocolitica* biogroup 6 because of negative Voges-Proskauer reaction. *Y. bercovieri* strains have been isolated from human stool specimens, animals, raw vegetables, soil, and water. There is no evidence of pathogenicity for humans (Bottone et al., 2005).

1.3.3. *Yersinia frederiksenii*

This species is composed of three different genomospecies. One group is positive for β -xylosidase and citrate and the type strain belongs to this group. The other two groups are variable or negative for these tests (Bottone et al., 2005). *Y. frederiksenii* has been isolated primarily from fresh water or sewage, soil, fish, wild

rodents, foods (milk, sandwiches, fruits, and vegetables), domestic animals (cattle, pigs, etc.), and healthy and sick humans (Sulakvelidze, 2000).

1.3.4. *Yersinia intermedia*

Eight biovars have been described based on the fermentation of melibiose, L-rhamnose, α -methyl-D-glucoside, and raffinose, and on the utilization of citrate. *Y. intermedia* has been isolated mainly from fresh water sources, fish, foods, and occasionally from both sick and healthy humans (Bottone et al., 2005). *Y. intermedia* possess a species-specific H antigen and share a few O antigens with *Y. enterocolitica*. Serogroups O:4 and O:17 appear to predominate; however, since most *Y. intermedia* strains are not typeable, the actual predominant serogroup is unclear (Sulakvelidze, 2000).

1.3.5. *Yersinia kristensenii*

Yersinia kristensenii was described to accommodate strains that are trehalose-positive, ornithine decarboxylase-positive and rhamnose-negative. These are mostly adapted to the soil ecosystem. *Y. kristensenii* strains have been isolated from horses, frogs, sheep, wild rodents, earthworms, and monkeys, from human urine, blood, eye, and stool, and from food, water, sewage, and soil in Europe, Japan, Australia, and North America (Bercovier et al., 1980b). Some strains of *Y. kristensenii* produce an enterotoxin (ST) when incubated at 22°C and also 37°C. *Y. kristensenii* may occasionally be associated with human enteritis. *Y. kristensenii* isolates may be lethal to mice pretreated with iron dextrin (Bottone et al., 2005).

1.3.6. *Yersinia mollaretii*

Y. mollaretii is oxidase-negative, fermentative rodsmotile at 25 °C, and, similarly to *Y. enterocolitica* and all other *Y. enterocolitica*-like species, rarely motile

at 37 °C. Some strains of *Y. mollaretii* possess O antigens identical or very similar to those of *Y. enterocolitica* and several other *Y. enterocolitica*-like species (Sulakvelidze, 2000). *Y. mollaretii* formerly called *Y. enterocolitica* biogroup 3A. Later that transferred to *Y. enterocolitica* biogroup 6 because of negative Voges-Proskauer reaction. *Y. mollaretii* strains have been isolated from human stool specimens, meat, raw vegetables, soil, and drinking water, and lack virulence markers. There is no evidence of pathogenicity for humans (Bottone et al., 2005).

1.3.7. *Yersinia rohdei*

Phylogenetically, *Y. rohdei* is most closely related to *Y. frederiksenii*; however, unlike the latter, all *Y. rohdei* strains have been found to be highly interrelated. *Y. rohdei* has been isolated from surface water and feces of dogs and humans, including persons with diarrhea and intestinal cramps. Two biovars of *Y. rohdei* are recognized based on positive (biovar 1) or negative (biovar 2) reactions for both raffinose and melibiose (Sulakvelidze, 2000; Bottone et al., 2005).

1.3.8. *Yersinia ruckeri*

Y. ruckeri is one of the agents responsible for red mouth disease in rainbow trout. The disease can be transmitted experimentally from fish to fish. First isolated in 1959 from a dead muskrat and found in a marshy area at the Bear River Migratory Bird Refuge in northern Utah. Other strains have been isolated from water in the same area (Bottone et al., 2005). The biochemical reactions of *Y. ruckeri* do not fit the general profile of *Yersinia* and some strains may be lysine and gelatin positive (Carniel et al., 2006).

1.4. Food association of *Yersinia* spp.

1.4.1. Milk and dairy products

1.4.1.1. Raw milk

Milk is a good growth medium for many microorganisms because of its high water activity, near neutral pH, and available nutrients. The growth of desirable microorganisms can be adversely affected by several components that are either naturally present or have entered in the milk as contaminants. Normally, raw milk contains $<10^3$ microorganisms /milliliter. In raw milk, microorganisms come from inside the udder, animal body surfaces, feed, air, water, and equipment used for milking and storage (Ray, 2004; Frank, 2007). Pathogens may be present in raw milk due to abnormal udder conditions caused by infections or disease, principally mastitis and contamination during milking caused by faecal material, residues on milk handling equipment, personnel and environmental sources (Hudson et al., 2003). Psychrophilic pathogens such as *Y. enterocolitica* and *Listeria monocytogenes* can multiply in refrigerated raw milk during storage (Ray, 2004).

Y. enterocolitica has been isolated from raw milk in many countries, like Australia, Canada, Czechoslovakia, and USA. There were also a few reports on the isolation of this pathogen from pasteurized milk. It may be due to the malfunction in the pasteurization process leading to inadequate treatment or post process contamination, or it may be due to the contamination of heat-resistant strains of *Y. enterocolitica* (Robins-Browne, 2007; Bhunia, 2008).

A low prevalence (0.6%) was determined in a survey of 352 raw milk samples in Switzerland (Bachman and Spahr, 1994). In samples from Manitoba and Canada *Y. enterocolitica* were detected in 1.6% of 192 farm and 6.3% of 64 dairy raw milk samples (Davidson et al., 1989). The organism has also been isolated from

pasteurised milk associated with yersiniosis (Greenwood and Hooper, 1990). In a survey of 73 pasteurised milk samples from 15 commercial dairies in Scotland *Y. enterocolitica* was isolated from 23% of the samples examined (Bruce et al., 1995).

Careful microbiological monitoring of the milk is required after pasteurization, with a view to determining the efficacy of heat treatment and detecting possible post contamination (Salmeron et al., 2002). Limiting the populations of bacteria primarily involves cleaning, sanitizing, and drying cows' teats and udders before milking, and using cleaned and sanitized equipment (Frank, 2007).

1.4.1.2. Cheeses

Cheeses are made by coagulating the casein in milk with lactic acid produced by lactic acid bacteria and without or with the enzyme rennin, followed by collecting the casein for further processing, which may include ripening. The process was probably accidentally discovered in the Middle East ca. 7000 B.C. from the coagulation of milk stored in a calf stomach by lactic acid produced by lactic acid bacteria (Ray, 2004).

At present, many varieties of cheeses including Cheddar cheese, cottage cheese, Stilton cheese in England, Vacherin Mont d'Or cheese in Switzerland are made from raw or unpasteurized milk worldwide. Several types of cheeses made with pasteurized milk: soft cheeses such as Brie, Camembert, blue-veined cheeses, Mozzarella cheeses, and Mexican-style soft cheeses; hard cheeses such as cheddar, and extra hard grating cheeses such as Parmesan cheeses.

Previous investigations have been performed in several countries on the incidence of *Yersinia* spp. and *Y. enterocolitica* in various cheeses (Hamama et al., 1992; Araujo et al., 2002; Siddik et al., 2007; Yentür et al., 2008; Güven et al., 2010; Özdemir and Arslan, 2011). Survival of pathogenic bacteria in cheese is dependent

on several factors, such as the level of contamination, the competitive microflora, the composition of the cheese and the conditions of manufacture (Bintsis and Papedemas, 2002). The prevalence of *Yersinia* spp. and *Y. enterocolitica* was reported in different type of cheeses at various rates. Hamama et al. (1992) isolated *Yersinia* spp. (7.4%) and *Y. enterocolitica* (2.1%) in cheese samples. In another study, Yücel and Ulusoy (2006) reported that the incidence of *Yersinia* spp. was 14% and the incidence of *Y. enterocolitica* was 35.7% in traditional fresh cheeses. The prevalence of *Y. enterocolitica* in cheese samples was 16% (Yentür et al., 2008) and 4% (Güven et al., 2010).

Cheeses are ready-to-eat food products that do not undergo any further treatment to ensure their safety before consumption. Contamination of cheese with foodborne pathogens may occur at several stages (Kousta et al., 2010). In conclusion, since cheese is a ready-to-eat product, even a low incidence of contamination may pose a risk to consumer health. Therefore, it is essential to ensure high safety standards such as raw milk quality, effective pasteurization process, hygienic production and storage conditions, proper cleaning and sanitation processes in production places (Çolak et al., 2007).

1.5.1. Meat and Meat Products

1.5.1.1. Raw meat and minced meat

Many epidemiological studies have implicated foods of animal origin as major vehicles associated with illnesses caused by *Yersinia* spp. (Khare et al., 1996; Capita et al., 2002; Kechagia et al., 2007; Martinez et al., 2011). Contaminated raw and undercooked meats are particularly important in transmitting this pathogen (Thisted-Lambertz et al., 2008; Soltan-Dallal et al., 2010; Van Damme et al., 2013a).

The muscle tissues of normal healthy cattle presented at slaughter are considered essentially sterile. During slaughter, foodborne pathogens may contaminate carcasses. This contamination arises in a number of ways, including: contact with the hair coat at the time of hide removal, direct contact with rumen and/or intestinal contents during processing, from knives, saws and other equipment, and from the contaminated hands and clothing of personnel handling and processing the carcasses (Troutt and Osburn, 1997).

Following boning, chilled raw meat and ground meat contain microorganisms coming from the carcasses as well as from different equipment used during processing, personnel, air, and water. Chilled meat has mesophiles, such as *Micrococcus*, *Enterococcus*, *Staphylococcus*, *Bacillus*, *Clostridium*, *Lactobacillus*, coliforms, and other *Enterobacteriaceae*. However, because the meats are stored at low temperature (-1 to 5°C), the psychrotrophs constitute major problems. The microbial load of fresh meat varies greatly, with bacteria dominating. Ground meat can have 10^{4-5} microorganisms/gram (Ray, 2004).

The occurrence of *Yersinia* spp. and *Y. enterocolitica* in meat and meat products has been investigated in several countries: in Brazil (Tassinari et al., 1994) Turkey (Siriken, 2004; Güven et al., 2010), Ireland (Logue et al., 1996) South Africa (Nortje et al., 1999), Iran (Soltan-Dallal et al., 2010). Different isolation rates of this pathogen from meat samples were reported in previous studies: Logue et al., (1996) isolated *Yersinia* spp. (89%) and *Y. enterocolitica* (68%) from Irish meat and meat products. Siriken et al. (2004) detected *Yersinia* spp. (32.8%) and *Y. enterocolitica* (27.9%) from ground beef samples. The incidence of *Y. enterocolitica* in the ground beef samples was 15.7% (Nortje et al., 1999). On the other hand, no *Y. enterocolitica* was isolated from minced meat samples in Greece (Mayrhofer et al., 2004).

1.5.1.2. Chicken Meat

Production and consumption of poultry meat and poultry meat products show an upward trend. On the other hand, contamination of poultry meats with bacterial pathogens could be a result of improper handling of poultry meat at the retail and/or consumer level. Storage time and temperature at retail, food service and/or consumer household, including transportation, impacts on the numbers of pathogenic microorganisms present on poultry meat. This includes potential growth (at temperatures $>7^{\circ}\text{C}$) or inactivation (during freezing) of microorganisms. Ultimately, inadequate cooking at the retail outlet or consumer household permits survival of these microbiological hazards (Anonymous, 2005).

High numbers of bacteria are present on the hide, hair, hooves, and feathers of poultry animals, as well as their gastrointestinal tract. They are associated with the microflora of the skin or with species present in faecal material (Nychas et al., 2007). The microflora of poultry is transferred from the primary production sites to production lines, and further, by subsequent contamination. Microflora of crude chicken meat is heterogeneous and originates from slaughtering premises, operators' hands, equipment and outfit, and water and air (Kozacinski et al., 2006; Sırken and Türk, 2013). Chicken meat have consistently been a major source of food poisoning and foodborne diseases, especially *Salmonella*, *Campylobacter* and *Clostridium perfringes*, and possibly also enteropathogenic *Escherichia coli* and *Y. enterocolitica* (Harrigan, 1998). Previous reports in several countries on the incidence of *Yersinia* spp. and *Y. enterocolitica* in chicken samples indicated their presence in these foods. The results of incidence of isolated *Yersinia* spp. and *Y. enterocolitica* are summarized in Table 4 and Table 5, respectively.

Table 4. Literature data on isolation of *Yersinia* spp. from chicken meat

Sample type	Number of samples tested	Country	Incidence (%)	Reference
Chicken products	390	Greece	8	De Boer (1994)
Poultry meat samples	20	Ireland	100	Logue et al. (1996)
Chicken (minced)	40	Mexico	28	Quinones-Ramirez et al. (2000)
Chicken carcass	40	Spain	50	Capita et al. (2002)

Table 5. Literature data on isolation of *Y. enterocolitica* from chicken meat

Sample type	Number of samples tested	Country	Incidence (%)	Reference
Chicken broilers	51	South Africa	15.7	Nortje et al. (1990)
Chicken	615	Japan	3	Fukushima et al. (1997)
Chicken carcass	70	Argentina	4.3	Floccari et al. (2000)
Chicken carcass	40	Spain	50	Capita et al. (2002)
Chicken meat	89	Austria	40	Mayrhofer et al. (2004)
Chicken	40	Italy	0	Mauro et al. (2008)
Chicken	19	Greece	0	Gousia et al. (2011)
Chicken meat	120	Iran	15.8	Mahdavi et al. (2012)

1.6. Isolation, identification and molecular characterization of *Yersinia* spp.

Various methods are available for the detection of *Yersinia enterocolitica* and other *Yersinia* species are ubiquitous in the natural environment, and may be recovered from water, soil, animals, food and clinical samples (Johson, 1998). Recovery of *Y. enterocolitica* from food is more difficult than recovery from human clinical samples. Isolation of *Y. enterocolitica* and *Y. pseudotuberculosis* from diarrhoeal stool samples is usually easier than isolation from other sources, for example, from food where the pathogen is present in low numbers. For example, a faecal sample can contain 10^6 – 10^9 *Yersinia* cells/g in the acute phase of infection (Schriefer and Petersen, 2011).

At present, a variety of reference methods for isolation of *Yersinia* spp. especially *Y. enterocolitica* in foods suggested by different national and international organisations exist including direct plating, various selective enrichments and cold enrichments (Aulisio et al., 1980; ISO 10273, 2003; Frederiksson-Ahomaa et al., 2008). Isolation of *Y. enterocolitica* from food and environmental samples is challenging. The current widely used culturing methods suffer from a lack of sensitivity and are likely to lead to underestimating of the actual prevalence of pathogenic *Yersinia* in foods. Recovery of pathogenic *Y. enterocolitica* is contingent upon a number of factors including: the level of background flora on the product; the amount of background flora coming through enrichment and plating; the level of pathogenic *Y. enterocolitica* present on the sample; the numbers of non pathogenic *Y. enterocolitica* and non pathogenic *Yersinia* spp. present on the product; and loss of virulence factors during enrichment and plating (Johnson, 1998).

Several different enrichment media have been described for the recovery of *Y. enterocolitica* from foods that usually exploit enrichment in one or two non

selective or selective broths such as Peptone Sorbitol Bile (PSB) broth, Irgasan Ticarcillin Potassium Chlorate (ITC), phosphate buffered saline supplemented with mannitol and bile salts (PMB) broth, modified Rappaport broth containing magnesium chloride, malachite green, and carbenicillin (MRB) (Fredriksson-Ahomaa and Korkeala, 2003b; Schrieffer and Petersen, 2011; Van Damme et al., 2013a). In the International Standard Organization method (ISO 10273:2003) mostly used for food samples in Europe, *Y. enterocolitica* is enriched in ITC broth in parallel with PSB broth, and plated on Cefsulodin-Irgasan-Novobiocin (CIN) and Salmonella-Shigella-deoxycholate calcium chloride (SSDC) agars (Van Damme et al., 2010; Martinez et al., 2011; Van Damme et al., 2013a). CIN agar, SSDC agar and MacConkey agar are the most commonly used agars for isolating *Y. enterocolitica* (Fredriksson-Ahomaa and Korkeala, 2003a). CIN agar specifically designed for *Yersinia* spp., inhibits the growth of many other organisms of the family *Enterobacteriaceae* to the advantage of the more slowly growing *Yersinia* species. *Y. enterocolitica* forms distinctive colonies with a deep red centre (bull's eye) with a sharp border surrounded by a translucent zone on CIN agar (Schiemann, 1979).

Cold enrichment at 4°C in buffer or broth for several days to weeks before isolation on a plate has also been used to isolate *Y. enterocolitica* (Pai et al., 1979; Van Noyen et al., 1981; Nordic Committee on Food Analysis, 1996; Fredriksson-Ahomaa and Korkeala, 2003a). Cold enrichment has increased the number of *Y. enterocolitica* recovered from foods in various studies (De Boer, 1992; Logue et al., 1996; Sirken, 2004; Van Damme et al., 2013a). At low temperatures, the growth rate of competitive bacteria is slowed sufficiently so the psychrotrophic *Y. enterocolitica* can multiply to numbers necessary for isolation on plating media (Schiemann, 1987). Among others, cold enrichment in phosphate buffered saline (PBS) or PSB has been

used for *Y. enterocolitica* (Fredriksson-Ahomaa and Korkeala, 2003a). Isolation by cold enrichment seems to be a matter of controversy; it seems that cold enrichment has no advantage over direct plating in acute *Y. enterocolitica* serotype O:3 and O:9 enteritis (Van Noyen et al., 1981), but in some instances it can increase the sensitivity of the detection of pathogenic *Y. enterocolitica* (Pai et al., 1979).

Furthermore, cold enrichment has been shown to increase the recovery of *Y. enterocolitica*-like strains, and *Y. enterocolitica* biotype 1A strains which have questionable pathogenicity for humans or are non pathogens widely distributed in nature (Sulakvelidze, 2000). In a recent study by Sihvonen et al. (2009), 25% of the strains belonging to pathogenic bioserotypes of *Y. enterocolitica* were only detected after cold enrichment of the clinical stool samples. However, cold enrichment also increased the number of isolates representing *Y. enterocolitica* and *Y. enterocolitica* like strains. Selective enrichment yielded a lower recovery of *Y. enterocolitica* and *Y. pseudotuberculosis* among pig samples than cold enrichment (Martinez et al., 2011).

Another culture method employing a potassium hydroxide treatment was used for recovery of *Yersinia* spp. from contaminated food. *Y. enterocolitica* is more tolerant of alkaline conditions than the other gram-negative bacteria, and this property has been used in “alkali treatment” technique for reduction of background bacteria in food enrichments before plating on agar medium (Aulisio et al., 1980).

For many organisms, traditional culture methods for detection and identification of bacteria take approx. 3-5 days or more. Adding polymerase chain reaction (PCR) to a culture method can reduce the analysis process to 1-3 days (Thisted-Lambertz, 2005).

PCR-based methods are used increasingly in food microbiology because they offer a rapid, sensitive and specific detection and identification of pathogenic

microorganisms. PCR is an enzymatic amplification of a specific region of the DNA molecule. The borders of the region must be known because in order to carry out the amplification, two specific oligonucleotides (primers) must be annealed to the DNA molecule, one to each strand of the denatured double helix. This delimits the region that will be amplified. A key enzyme, a thermostable DNA polymerase, carries out the amplification. The reaction cycle consists of the three steps: denaturation of the double-stranded DNA, annealing of primers to the single DNA strands and extension of the primers with the DNA polymerase. The detection of the PCR product is possible through gel electrophoresis, ethidium bromide staining and visual examination under ultraviolet light (Fredriksson-Ahomaa and Korkeala, 2003; Thisted-Lambertz, 2005).

PCR has also been used a sensitive method to detect and identify *Y. enterocolitica* isolates and to easily separate pathogenic from non pathogenic strains within the same species (Wannet et al., 2001; Thisted-Lambertz and Danniellsson-Tham, 2005). *Y. enterocolitica* strain differentiation has relied, until recently, on phenotypic characteristics (biotype, serotype, and phage type). Since these markers exhibit a very low degree of polymorphism, the establishment of an epidemiological link between two strains based on their sharing the same bioserotype is not fully satisfactory. Recent advances in enzymatic and genetic analysis of the plasmid, ribotyping, restriction enzyme digestion of the chromosome, polymerase chain reaction (PCR)-fingerprinting, and pulsed-field gel electrophoresis technique have provided a means to reevaluate the polymorphism of isolates within a given bioserotype (Carniel et al., 2006).

2. MATERIALS AND METHODS

2.1. Sample Collection

A total of 300 meat and milk products including chicken meat (n=60), minced meat (n=60), homemade white cheese (n=60), open white cheese (n=60) and raw milk products (n=60) were purchased from various public bazaars, supermarkets, butchers, and delicatessen shops in Bolu. All food samples in this study were placed in sterile bags and kept on ice during transportation and until they were analyzed in the laboratory.

2.2. Microbiological Analyses

2.2.1. Isolation and Identification of *Yersinia* spp.

Yersinia spp. and *Yersinia enterocolitica* from meat and milk products were isolated in line with the ISO 10273 and the cold enrichment methods (Nordic Committee on Food Analysis, 1996; International Standard Organization, ISO 10273: 2003; Frederiksson-Ahomaa and Korkeala, 2003a). The detection methods used for the isolation of *Yersinia* spp. from meat and milk products are presented in Figure 1.

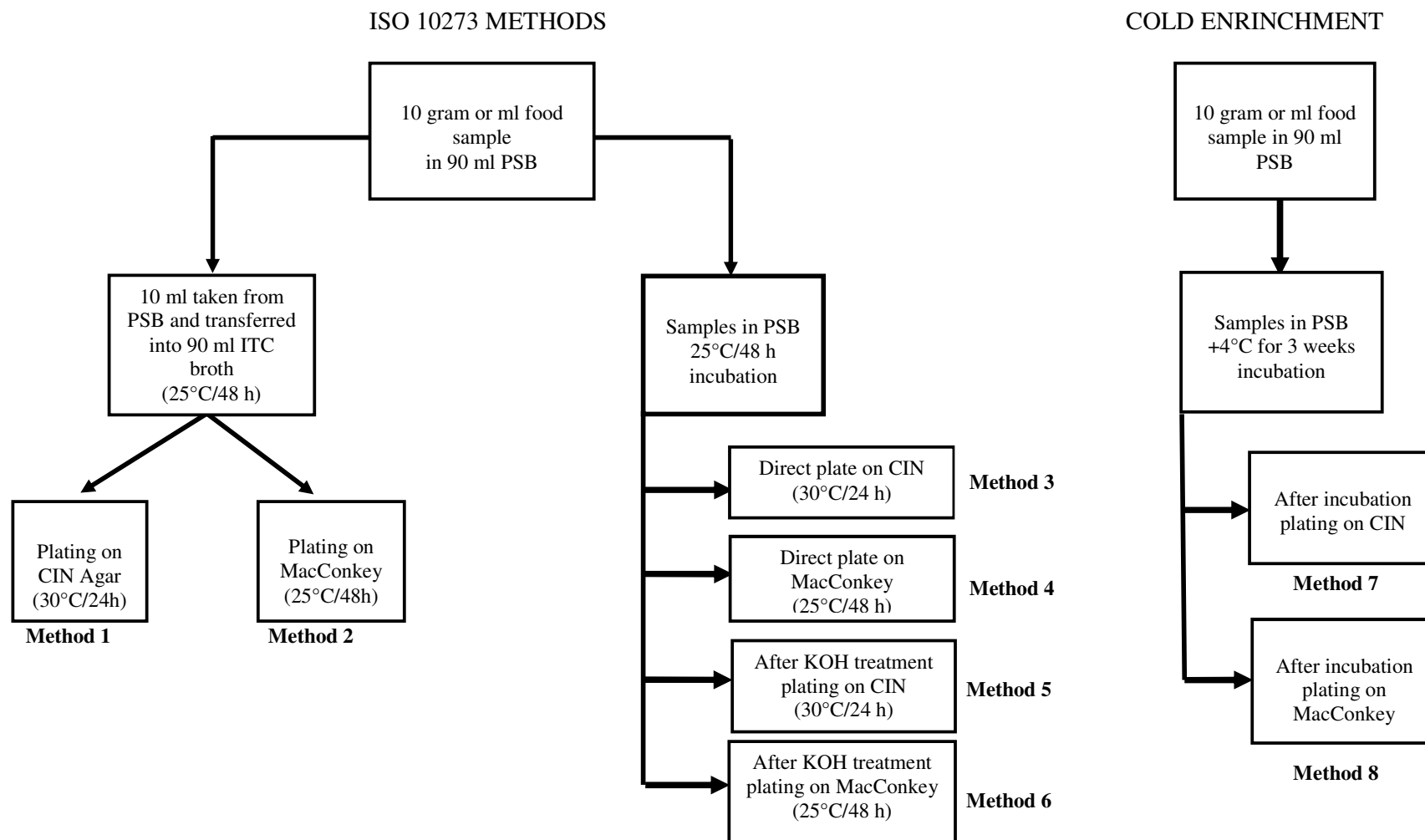


Figure 1. Overview of the different methods for the isolation of *Yersinia* spp. from meat and milk products used in this study.

In this study, five different food samples including chicken meat, minced meat, raw milk, homemade white cheese and open white cheese were analyzed to determine the presence of *Yersinia* spp.

According to the ISO method, a 10 g of homemade white cheese, open white cheese, chicken and minced meat samples and a 10 mL of raw milk were aseptically weighed and added to a 90 mL of sterile Peptone Sorbitol Bile (PSB) broth (Fluka, Steinheim, Germany) and homogenized by stomacher (BagMixer 400; Interscience, Paris, France). From each sample in PSB broth, a 10 mL was taken and inoculated into tubes containing a 90 mL of Irgasan Ticarcillin Chlorate (ITC) enrichment broth base (Fluka) supplemented with ticarcillin (Fluka) and potassium chlorate (Fluka). The samples were enriched in PSB and ITC broth at 25°C for 48 h. Then, a loopful of each samples was streaked onto Cefsoludin-Irgasan-Novobiocin (CIN) agar (*Yersinia* Selective Agar Base supplemented with *Yersinia* Selective Supplement, Oxoid, Basingstoke, UK) and MacConkey agar plates (Merck, Darmstadt, Germany). It is considered that the treatment of enrichment broth with alkali might increase the chances of recovery. Thus, for the alkali treatment, a 0.5 mL of the enriched PSB cultures was transferred to a 4.5 mL of 0.5% potassium hydroxide (KOH) solution and mixed for 20 s as described by Aulisio et al. (1980). Then the alkali treated cultures were streaked onto CIN and MacConkey agar. The plates of CIN agar and MacConkey agar were incubated at 30°C for 24 h and at 25°C for 48 h, respectively.

The cold enrichment method was used in this research to increase the recovery of *Yersinia* species due to the fact that this pathogen has a psychrotrophic feature. In cold enrichment method, a 10 g of meat and cheese samples and a 10 mL of raw milk samples were aseptically added into a 90 mL of PSB broth and homogenized by stomacher. All homogenized samples were incubated at 4°C for up to 3 weeks. After

3 weeks, the cold-enriched samples were streaked on CIN and MacConkey agar plates. CIN agar and MacConkey agar plates were incubated at 30°C for 24 h and at 25°C for 48 h, respectively. After incubation, the CIN agar plates were observed for colonies displaying typical *Yersinia* spp. morphology. Suspected colonies of typical “bulls-eye” appearance were counted as *Yersinia* and subcultured on Nutrient agar (Oxoid). Red or mucoid colonies, flat, colorless, or pale pink colonies on MacConkey agar were selected as suspected *Yersinia* spp. and plated on Nutrient agar. Up to ten representative colonies were selected as presumptive *Yersinia* isolates from both MacConkey agar and CIN agar plates and were transferred to Nutrient agar (Oxoid) for further identification.

Presumptive *Yersinia* spp. colonies were confirmed with following biochemical tests: Gram staining, oxidase and catalase tests, motility at 25°C, 28°C and 37°C, citrate utilization, acid reaction with methyl red, acetoin production at 25°C and 37°C, Kligler’s iron agar reaction, urease, production of each of ornithine decarboxylase and lysine decarboxylase, nitrate reduction, indole and H₂S production, pyrazinamidase test, acid production from glucose, mannitol, trehalose, maltose, rhamnose, sucrose, melibiose, mucate, myo-inositol and raffinose (Holt et al., 1994; Bottone et al., 2005). In all microbiological and molecular experiments, *Y. enterocolitica* ATCC 23715 was used as the reference strain. All biochemical tests were performed twice. Biochemical tests for identification of *Yersinia* species are shown in Table 6. Also, some biochemical reactions at 25-28°C and 37°C for *Yersinia* species are also given in Table 7.

Table 6. Differential characteristics of the species of the genus *Yersinia**

Test ^a	<i>Y. bercovieri</i>	<i>Y. enterocolitica</i>	<i>Y. intermedia</i>	<i>Y. mollaretii</i>	<i>Y. pseudotuberculosis</i>	<i>Y. rohdei</i>	<i>Y. ruckeri</i>
Pathogenic	(+) ^b	+	(+)	(+)	+	(+)	(+)
Nitrate reductase	+	+	+	+	+	+	+
Urease	d	+	+	-	+	d	-
Citrate, Simmons	-	-	-	-	-	+	-
Ornithine decarboxylase	+	+	+	+	-	+	+
Indole	-	V	+	-	-	-	-
H ₂ S production	-	-	-	-	-	-	-
Esculin hydrolysis	-	V	+	-	+	-	-
Methyl red	V	V	+	+	+	+	+
Glucose, acid	+	+	+	+	+	+	+
Sucrose, acid	+	+	+	+	-	+	-
Trehalose, acid	+	V	+	+	+	+	+
Maltose, acid	+	+	+	d	+	-	+
D-Xylose, acid	+	d	+	d	+	d	-
Mannitol, acid	+	+	+	+	+	+	+
Pyrazinamidase	V	V	+	-	-	+	+

* According to Holt et al. (1994) and Bottone et al. (2005). ^aAll *Yersinia* spp. are fermentative; negative for H₂S, oxidase, arginine dihydrolase, phenylalanine deaminase, malonate, dulcitol, DNase (25°C), and pigmentation; all are positive for mannitol and mannose. Motile species have peritrichous flagella, and motility is more pronounced at temperatures less than or equal to 30°C. Except for delayed reactions in some *Y. ruckeri*, all species are negative for gelatin hydrolase and lysine decarboxylase. ^b(+), opportunistic pathogens; V, variable. +, 90-100% of strains are positive; d, 26-75% are positive; -, 0-10% are positive.

Table 7. Reactions of *Yersinia* spp. at 25-28°C and 37°C*

Test	<i>Y. bercovieri</i>	<i>Y. enterocolitica</i>	<i>Y. intermedia</i>	<i>Y. mollaretii</i>	<i>Y. pseudotuberculosis</i>	<i>Y. rohdei</i>	<i>Y. ruckeri</i>
Voges-Proskauer:							
37°C	-	-	-	-	-	-	-
25-28°C	-	+	+	-	-	-	-
Citrate, Simmons:							
37°C	-	-	-	-	-	-	-
25-28°C	-	-	-	-	-	[+]	-
Urease:							
37°C	d	[+]	[+]	[-]	+	d	-
25-28°C	+	+	+	+	+	d	-
Ornithine decarboxylase:							
37°C	[+]	+	+	[+]	-	[-]	+
25-28°C	+	+	+	+	-	[+]	+
Motility:							
37°C	-	-	-	-	-	-	-
25-28°C	+	+	+	+	+	+	[+]
myo-Inositol, acid:							
37°C	-	d	[-]	-	-	-	-
25-28°C	d	d	[+]	d	-	-	-

Table 7. Reactions of *Yersinia* spp. at 25-28°C and 37°C (continued)

Melibiose, acid:							
37°C	-	-	[+]	-	d	d	-
25-28°C	-	-	+	-	+	d	-
Raffinose, acid:							
37°C	-	-	d	-	[-]	d	-
25-28°C	-	-	+	-	[-]	d	-
L-Rhamnose, acid:							
37°C	-	-	+	-	d	-	-
25-28°C	-	-	+	-	+	-	-
Salicin, acid:							
37°C	[-]	[-]	+	[-]	[-]	-	-
25-28°C	-	[-]	+	[-]	d	-	-
D-Xylose, acid:							
37°C	+	d	+	d	+	d	-
25-28°C	+	d	+	+	+	+	-
Mucate, acid:							
37°C	-	-	-	-	-	-	-
25-28°C	+	-	d	+	-	-	-

* According to Holt et al. (1994) and Bottone et al. (2005).

+, 90-100% of strains are positive; d, 26-75% are positive; -, 0-10% are positive;

[+], 76-89% are positive; [-], 11-25% are positive.

2.2.2. Biotyping of *Yersinia enterocolitica*

Yersinia enterocolitica is divided into six biotypes-1A, 1B, 2, 3, 4, and 5- based on biochemical properties. Biotypes of *Y. enterocolitica* show a discrepancy in geographic locations and pathogenic potentials. In the present study, all strains identified as *Y. enterocolitica* were biotyped according to Aleksic and Bockemühl (1999); Schriefer and Petersen (2011) by applying the following tests: lipase production, aesculin hydrolysis, indol production, fermentation of salicin, xylose and trehalose, nitrate reduction, DNase test, and pyrazinamidase activity. Differentiation of biotypes of *Y. enterocolitica* is listed in Table 8.

Table 8. Differentiation of biotypes of *Y. enterocolitica**

Test	Reaction for biotype					
	1A	1B	2	3	4	5
Lipase activity	+	+	-	-	-	-
Salicin	+	-	-	-	-	-
Esculin hydrolysis	+	-	-	-	-	-
Indole production	+	+	(+)	-	-	-
Xylose	+	+	+	+	-	d
Trehalose	+	+	+	+	+	-
Nitrate reduction	+	+	+	+	+	-
DNase	-	-	-	-	+	+
Pyrazinamidase activity	+	-	-	-	-	-

* Aleksic and Bockemühl (1999); Schriefer and Petersen (2011). +, $\geq 90\%$ of strains are positive; d, 11 to 89% of strains are positive; -, $\geq 90\%$ of strains are negative; (+), weakly positive reaction. Tests were performed at 25°C for 48 h.

2.2.3. Phenotypic virulence characteristics of *Yersinia enterocolitica*

The identification of virulent plasmid-bearing strains of *Y. enterocolitica* was carried out according to pathogenicity tests including temperature-dependent autoagglutination (Laird and Cavanaugh, 1980), calcium-dependent growth and Congo red absorption (CR-MOX) (Riley and Toma, 1989) and crystal violet (CV) binding (Bhaduri et al., 1987).

2.2.3.1. Congo Red-Magnesium Oxalate Test

The Congo Red-Magnesium Oxalate (CR-MOX) test provides a simple method to screen for plasmid containing pathogenic *Y. enterocolitica*. Presence of the virulence plasmid was detected with calcium dependency and Congo red absorption on the CR-MOX agar plates (Riley and Toma, 1989). *Y. enterocolitica* strains were first grown on 5% sheep blood agar at 22°C for 18 h. Then, the strains were plated onto CR-MOX agar and incubated for 24 h at 36°C. Positive strains always produced small red colonies, whereas large colorless colonies indicated negative result.

2.2.3.2. Crystal violet binding test

Crystal violet (CV) binding assay may be useful as a rapid screening test to differentiate potentially virulent *Y. enterocolitica* cultures. The ability of strains of *Y. enterocolitica* to bind CV was tested as follows. *Y. enterocolitica* strains were grown separately in Brain Heart Infusion (BHI) broth (Merck) for 18 h at 25°C with shaking. The cells were diluted to a concentration of 10^3 cells per mL and surface plated on BHI agar (Merck). The plates were incubated at 25°C for 30 h. They were gently flooded with 8 mL of CV solution (85µg/mL) for 2 min and decanted. The binding of CV to plasmid-bearing virulent colonies was observed by their dark violet

appearance, while the avirulent plasmidless colonies failed to bind the dye and remained white (Bhaduri, et al., 1987).

2.2.3.3. Autoagglutination test

The positive cultures of *Y. enterocolitica* were inoculated into two tubes containing Methyl Red-Voges-Proskauer broth (MR-VP). The one MR-VP tube incubated at 25°C for 24 h, and the other at 37°C for 24 h. After incubation, the MR-VP tube incubated at the lower temperature should exhibit turbidity from cell growth. The MR-VP tube incubated at 37°C should show agglutination (clumping) of bacteria along walls and/or bottom of tube with clear supernatant fluid. Isolates giving this result are presumptive positive for the virulence plasmid. Any other pattern for autoagglutination at these two temperatures is considered negative (Laird and Cavanaugh, 1980).

2.3. Molecular analyses

In the present study the following molecular analyses were performed: molecular identification of *Y. enterocolitica* based on the 16S rRNA gene sequences and some virulence genes encoding by chromosomal (*ail*, *ystA* and *ystB*) and plasmid (*yadA* and *virF*) were examined using PCR technique. The selected and used primer pairs, primer sequences, and their amplicon size are summarized in Table 9.

2.3.1. DNA extraction

In this research, genomic DNA of all *Yersinia* strains was extracted. All *Y. enterocolitica* and other *Yersinia* strains were grown on Nutrient agar (Oxoid) for 24 h at 28°C and one colony of the culture was inoculated into 5 mL Brain Heart Infusion (BHI) broth was incubated at 28°C for 24 h. DNA extraction was carried out using the commercially available kit (Norgen, Thorold, ON, Canada) by the

manufacturer's instructions. One microliter (10 ng) of DNA was used as a template for each 50 µl PCR reaction. The extracted DNA was stored at -20°C.

2.3.2. Characterization of *Yersinia enterocolitica* using the 16S rRNA amplification and sequencing

The analysis of 16S rRNA sequences is considered as a standard in bacterial classification. The applicability of the 16S rRNA gene sequence analysis for the identification of *Y. enterocolitica* within the genus *Yersinia* is well established (Ibrahim et al., 1993; Ibrahim et al., 1997b; Lantz et al., 1998; Neubauer et al., 2000a; Tan et al., 2014). The identification of *Y. enterocolitica* was performed by using the 16S rRNA gene as described previously (Neubauer et al., 2000a) (Table 9). All amplifications were performed on a XP Thermal Cycler (Bioer Technology Co., Ltd., Japan). PCRs were performed in 50 µl volumes containing 1µl DNA template, 5 µl of 10X PCR buffer (Fermentas, Life Sciences, Canada), 2 µl of 25 mM MgCl₂, 1µl of 10 mM dNTP mix (Fermentas, Life Sciences, Canada), 0.75 µl each of the primers (Biomers, Ulm, Germany) and 0.25 µl of 5 U Taq DNA polymerase (Fermentas, Life Sciences, Canada). The thermal cycling conditions were as follows: 1 cycle denaturation at 94°C for 5 min; 30 cycles of melting at 94°C for 1 min; annealing (depending on the primer pair used) at 55°C for 1:30 min, and elongation at 72°C for 30 s, and a final extension at 72°C for 5 min. After PCR, 5 µl of amplicon was analyzed using electrophoresis in a 1.5% agarose gel. The expected fragment has a size of 330 bp. DNA molecular marker-100 bp Plus DNA Ladder (Fermentas, Life Sciences, Canada) was included as standard. They were visualized with UV transillumination (DNR Minilumi Bio-imaging Systems Ltd., Mahale HaHamisha, Jerusalem, Israel).

In this study, the PCR amplicons of five strains as representatives of food samples (two from chicken meat and one from minced meat, open white cheese and raw milk) were selected for 16S rRNA gene sequencing. The selected PCR products were purified and sequenced using both forward and reverse primer pairs by RefGen Biotechnology (METU Technopolis, Turkey). The results of gene sequences were viewed using BioEdit 7.2.5.0, and compared for similarities with the nucleotide sequences in the National Center for Biotechnology Information (NCBI) library.

2.3.3. Genotypic virulence characteristics of *Yersinia enterocolitica*

In this study, a total of 5 virulence genes on chromosome and plasmid were investigated for all *Yersinia* strains from meat and milk products. Chromosomally encoded virulence genes were *ail*, *ystA* and *ystB* while plasmid-encoded virulence genes contained *yadA* and *virF*. All primers used were designed with Biomers, Ulm, Germany.

Detection of *ail* gene in *Y. enterocolitica* and the other *Yersinia* strains was carried out using conventional PCR as previously described (Bhaduri et al., 1997). The final PCR protocol was as follows: 5 µL of 10X PCR buffer (Fermentas, Life Sciences, Canada); 1.5 mmol / L MgCl₂ (Fermentas, Life Sciences, Canada); 1 U of Taq DNA polymerase (Fermentas, Life Sciences, Canada); 200 µmol / L of each dNTP (Fermentas, Life Sciences, Canada); 10 pmol each primer set (Biomers, Ulm, Germany) and 5 µL of DNA were as DNA template. Molecular grade water was added to a final volume of 50 µL. The amplification conditions were: an initial denaturation of 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57 °C for 30 s and 72°C for 45 s, with a final extension at 72°C for 4 min.

The presence of virulence genes including *ystA*, *ystB*, *yadA* and *virF* genes in *Y. enterocolitica* and the other *Yersinia* strains was investigated according to

Thoerner et al. (2003). PCRs were performed in 50 μ L volumes containing 5 μ L of DNA template, 0.2 mM concentrations of deoxynucleoside triphosphates (Fermentas, Life Sciences, Canada), 5 μ L of 10X PCR buffer (Fermentas, Life Sciences, Canada), 3 mM MgCl₂, 1 μ M concentrations of each forward and reverse primer, 0.5 U Taq DNA polymerase (Fermentas, Life Sciences, Canada). The thermal cycling conditions were as follows: 1 cycle of denaturation at 95°C for 10 min; 25 cycles of melting at 95°C for 15 s annealing at various temperatures depending on the primer pair used (61°C for *ystA* and *ystB*, 60°C for *yadA* and 63°C for *virF*) for 30 s, and elongation at 72 °C for 30 s; and a final extension at 72°C for 10 min. All primers, primer sequences, and their references are given in Table 9. The PCR was performed up to three times with each isolate for all toxin genes. A total of 3 μ L of gel loading dye (Fermentas, Life Science, Canada) was added to 5 μ L of each PCR product, and 8 μ L of the obtained mixture were analyzed in a 2% agarose gel (Merck) using a Tris-borate-EDTA (TBE, Applichem) (1X) supplemented with ethidium bromide (Applichem), then electrophoresis was carried out for 45-50 min at 80 V. DNA molecular marker-100 bp Plus DNA Ladder (Fermentas, Life Science, Canada) was included as standard. They were visualized with UV trans-illumination (DNR Minilumi Bio-imaging Systems Ltd., Mahale HaHamisha, Jerusalem, Israel).

Table 9. Primers used for identification of *Y. enterocolitica* and detection of virulence genes

Target gene	Gene Function	Primer direction	Primer sequence (5'→3')	Amplicon size (bp)	Reference
<i>16S</i> rRNA	Identification	Forward	AAT ACC GCA TAA CGT CTT CG	330	Neubauer et al., 2000a
		Reverse	CTT CTT CTG CGA GTA ACG TC		
<i>ail</i>	Adhesin	Forward	ACT CGA TGA TAA CTG GGG AG	170	Bhaduri et al., 1997
		Reverse	CCC CCA GTA ATC CAT AAA GG		
<i>ystA</i>	Enterotoxin	Forward	ATC GAC ACC AAT AAC CGC TGA G	79	Thoerner et al., 2003
		Reverse	CCA ATC ACT ACT GAC TTC GGC T		
<i>ystB</i>	Enterotoxin	Forward	GTA CAT TAG GCC AAG AGA CG	146	Thoerner et al., 2003
		Reverse	GCA ACA TAC CTC ACA ACA CC		
<i>yadA</i>	Adhesin	Forward	CTT CAG ATA CTG GTG TCG CTG T	849	Thoerner et al., 2003
		Reverse	ATG CCT GAC TAG AGC GAT ATC C		
<i>virF</i>	Transcriptional activator	Forward	GGC AGA ACA GCA GTC AGA CAT A	561	Thoerner et al., 2003
		Reverse	GGT GAG CAT AGA GAA TAC GTC G		

2.4. Antimicrobial Susceptibility Test

Antimicrobial susceptibility patterns of all *Yersinia* strains were performed by the Kirby-Bauer disk diffusion method on Mueller Hinton agar (Oxoid) following the procedures recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006). Sixteen antimicrobial agents were chosen for the study according to their common use: They belonged to the following groups: penicillins (ampicillin, 10 µg; amoxycillin, 25 µg), cephalosporins (cephazolin, 30 µg; cephalothin, 30 µg), beta-lactamase inhibitors (amoxicillin/clavulanic acid, 30 µg), aminoglycosides (gentamicin, 10 µg; kanamycin, 30 µg; tobramycin, 10 µg; streptomycin, 10 µg), tetracyclines (tetracycline, 30 µg), quinolones (nalidixic acid, 30 µg; ciprofloxacin, 5 µg), carbapenems (imipenem, 10 µg), and miscellaneous (chloramphenicol, 30 µg; clindamycin, 2 µg; trimethoprim, 5 µg).

The turbidity of each bacterial suspension in Mueller-Hinton broth (Oxoid) was adjusted to 0.5 McFarland, and then the bacterial suspensions were spread with a sterile cotton swab confluent over the entire surface of Mueller-Hinton agar. The antimicrobial agent disks were placed on the agar surface sufficiently separated so as to avoid overlapping of the inhibition zones. The plates were incubated for 24 h at 30°C. After incubation period, results were recorded by measuring the diameter of the inhibition zones and compared with the Clinical and Laboratory Standards Institute (CLSI, 2006) and were classified as resistant, intermediate and susceptible.

2.5. Statistical Analysis

Analyses were carried out using the statistical software package SigmaPlot version 11.0 (Systat Software, Inc., San Jose California USA). One-way Analysis of Variance (ANOVA) was applied to determine the difference between the isolation

methods. The relationship between the different isolation methods was also tested using a binary correlation (Pearson Product Moment Correlation test). Statistics with a value of $P < 0.05$ were considered significant.

2.6. List of culture media

Peptone Sorbitol Bile (PSB) Broth (Fluka 17192)

Bile salt mixture, 1.5 g/L

Disodium phosphate, 8.23 g/L

Monosodium phosphate, 1.2 g/L

Peptic digest of animal tissue, 5 g/L

Sodium chloride, 5 g/L

Sorbitol, 10 g/L

Irgasan Ticarcillin Chlorate (ITC) enrichment broth..... (Fluka 17156)

Casein enzyme hydrolysate, 10 g/L

Yeast extract, 1 g/L

Magnesium chloride hexahydrate, 60 g/L

Sodium chloride, 5 g/L

Malachite green, 0.01 g/L

Irgasan, 0.001 g/L

Ticarcillin Supplement (Fluka 17778)

Potassium Chlorate Supplement (Fluka 17777)

Cefsulodin Irgasan Novobiocin (CIN) agar (Oxoid CM0653)

Special peptone, 20 g/L

Yeast extract, 2 g/L

Mannitol, 20 g/L

Sodium pyruvate, 2 g/L

Sodium chloride, 2 g /L

Magnesium sulphate, 0.01 g/L

Sodium desoxycholate, 0.5 g/L

Neutral red, 0.03 g/L

Crystal violet, 0.001 g/L

Agar-agar, 12.5 g/L

Yersinia Selective Supplement (Oxoid SR0109)

Cefsulodin, 15 mg/L

Irgasan, 4 mg/L

Novobiocin, 2.5 mg/L

MacConkey agar (Merck 105465)

Peptone from casein, 17 g/L

Peptone from meat, 3 g/L

Sodium chloride, 5 g/L

Lactose, 10 g/L

Bile salt mixture, 1.5 g/L

Neutral red, 0.03 g/L

Crystal violet, 0.001 g/L

Agar-agar, 13.5 g/L

Nutrient Broth (Merck 105443)

Peptone from meat, 5 g/L

Meat extract, 3 g/L

Nutrient Agar (Oxoid, CM0003)

Peptone from meat, 5 g/L

Meat extract, 3 g/L

Agar-agar, 12 g/L

Mueller-Hinton Agar (Oxoid CM0337)

Beef, dehydrated infusion, 300 g/L

Casein hydrolysate, 17.5 g/L

Starch, 1.5 g/L

Agar-agar, 17 g/L

Mueller-Hinton Broth..... (Oxoid CM0405)

Beef, dehydrated infusion, 300 g/L

Casein hydrolysate, 17.5 g/L

Starch, 1.5 g/L

SIMMONS Citrate Agar (Merck 105470)

Ammonium dihydrogen phosphate, 1 g/L

Di-potassium hydrogen phosphate, 1 g/L

Sodium chloride, 5 g/L

Sodium citrate, 2 g/L

Magnesium sulfate, 0.2 g/L

Bromothymol blue, 0.08 g/L

Agar-agar, 13 g/L

SIM Medium (Merck 105470)

Peptone from casein, 20 g/L

Peptone from meat, 6.6 g/L

Ammonium iron (III) citrate, 0.2 g/L

Sodium thiosulfate, 0.2 g/L

Agar-agar, 3 g/L

Urea Broth (Merck 108483)

Yeast extract, 0.1 g/L

Potassium dihydrogen phosphate, 9.5 g/L

Di-sodium hydrogen phosphate, 9.5 g/L

Urea, 20 g/L

Phenol red, 0.01 g/L

Tryptone (Indol) Medium..... (Merck 110859)

Peptone from casein, 10 g/L

Sodium chloride, 5 g/L

MR-VP Broth..... (Merck 105712)

Peptone from meat, 7 g/L

D (+) Glucose, 5 g/L

Phosphate buffer, 5 g/L

Phenol red broth (base) (Oxoid CM0888)

Peptone from casein, 5 g/L

Peptone from meat, 5 g/L

Sodium chloride, 5 g/L

Phenol red, 0.018 g/L

Bile Aesculin Agar (Oxoid CM0888)

Peptone, 8 g/L

Bile salts, 20 g/L

Ferric citrate, 0.5 g/L

Aesculin, 1 g/L

Agar-agar, 15 g/L

Triple Sugar Iron Agar..... (Merck 103915)

Peptone from casein, 10 g/L

Peptone from meat, 10 g/L

Meat extract, 3 g/L

Yeast extract, 3 g/L

Sodium chloride, 5 g/L

Lactose, 10 g/L

Sucrose, 10 g/L

D (+) Glucose, 1 g/L

Ammonium iron (III) citrate, 0.5 g/L

Sodium thiosulfate, 0.5 g/L

Phenol red, 0.024 g/L

Agar-agar, 12 g/L

Tributyryn Agar (Basis) (Merck 101957)

Peptone from casein, 2.5 g/L

Peptone from meat, 2.5 g/L

Yeast extract, 3 g/L

Agar-agar, 12 g/L

Glycerintributyryl (Tributyryn) (Merck 101958)

Decarboxylase Medium Base..... (Difco 287220)

Peptone, 5 g/L

Yeast extract, 3 g/L

Dextrose, 1 g/L

Brom cresol purple, 0.02 g/L

Brain Heart Broth..... (Merck 110493)

Nutrient substrate, 27.5 g/L

D (+) Glucose, 2 g/L

Sodium chloride, 5 g/L

Disodium hydrogen phosphate, 2.5 g/L

Brain Heart (Infusion) Agar..... (Merck 113825)

Nutrient substrate, 27.5 g/L

D (+) Glucose, 2 g/L

Sodium chloride, 5 g/L

Disodium hydrogen phosphate, 2.5 g/L

Agar-agar, 15 g/L

Nitrate Broth..... (Fluka 72548)

Peptone, 5 g/L

Meat extract, 3 g/L

Potassium nitrate, 1 g/L

Congo Red-Magnesium Oxalate (CR-MOX) Agar..... (Merck 105470)

Tryptic soy agar, 40 g/L

Sodium oxalate (0.25 M), 80 mL

Magnesium chloride (0.25M), 80 mL

D-Galactose (%20), 10 mL

Congo red (%1), 5 mL

DNase-Test Agar..... (Merck 110449)

Tryptose, 20 g/L

Sodium chloride, 5 g/L

Deoxyribonucleic acid, 2 g/L

Agar-agar, 15 g/L

2.7. List of chemicals, reagents and dyes

Agar-Agar.....	(Merck 101613)
Sodium Chloride.....	(Merck 106404)
Griess' Reagent for Nitrite	(Merck 109023)
Kovac's Reagent.....	(Merck 109293)
D (+) Glucose-Monohydrate	(Merck 108342)
Sucrose	(Merck 107651)
D (-) Mannitol.....	(Merck 105982)
D (+) Melibiose	Fluka 63630)
Trehalose	(Merck 108353)
D (+) Maltose monohydrate.....	(Roth 8951)
D (+) Xylose	(Merck 108689)
Myo-Inositol.....	(Merck 104728)
Raffinose	(Merck 107419)
L (+)-Rhamnose monohydrate	(Merck 104736)
Salicin	(Sigma S0625)
D (+) Cellobiose	(Sigma C7252)
L-Lysine Monohydrochloride	(Merck 105700)
L-Ornithine Monohydrochloride	(Merck 106906)

2.8. List of antimicrobial agents

Nalidixic acid (30 µg)	(Oxoid CT0031B)
Ciprofloxacin (5 µg)	(Oxoid CT0425B)
Tetracycline (30 µg)	(Oxoid CT0054B)
Streptomycin (10 µg)	(Oxoid CT0047B)
Gentamicin (10 µg)	(Oxoid CT0024B)
Kanamycin (30 µg)	(Oxoid CT0026B)
Amoxycillin (25 µg)	(Oxoid CT0061B)
Amoxycillin/Clavulanic Acid (30 µg)	(Oxoid CT0223B)
Trimethoprim (5 µg)	(Oxoid CT0076B)
Cephazolin (30 µg)	(Oxoid CT0011B)
Cephalotin (30 µg)	(Oxoid CT0010B)
Ampicillin (10 µg)	(Oxoid CT0003B)
Tobramycin (10 µg)	(Oxoid CT0056B)
Imipenem (10 µg)	(Oxoid CT0455B)
Clindamycin (2 µg)	(Oxoid CT0064B)
Chloramphenicol (30 µg)	(Oxoid CT0013B)

3. RESULTS

3.1. Recovery and Prevalence of *Yersinia* spp. in meat and milk products

In this thesis, a total of 300 meat and milk products, 60 chicken meat, 60 minced meat, 60 homemade white cheese, 60 open white cheese, 60 raw milk were tested for the presence of *Yersinia* spp. Out of the 300 food samples analyzed, 84 (28%) were found to be positive for *Yersinia* spp. Of the *Yersinia* positive samples, 18 (6%) isolates were conventionally identified as *Y. enterocolitica*. The other *Yersinia* species were *Y. rohdei* 15 (5%), *Y. intermedia* 14 (4.7%), *Y. pseudotuberculosis* 12 (4%), *Y. ruckeri* 12 (4%), *Y. mollaretii* 5 (1.7%) and *Y. bercovieri* 4 (1.3%). The 4 (1.3%) atypical *Yersinia* strains were also confirmed as *Yersinia* but could not be identified at *Yersinia* species level.

Besides, the distribution of *Yersinia* species isolated from meat and milk products has been summarized in Figure 2.

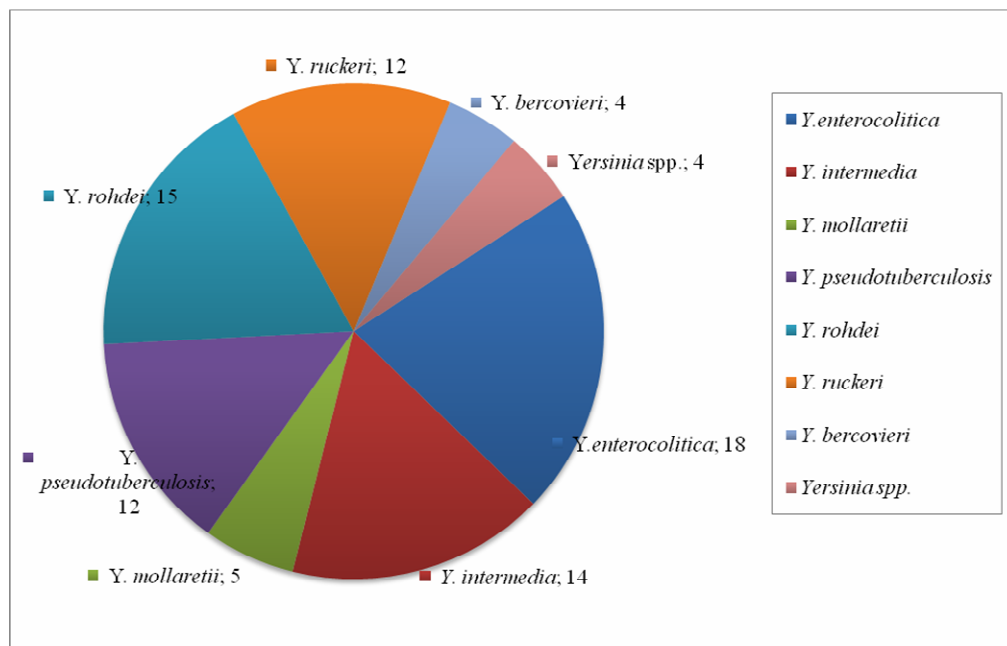


Figure 2. Distribution of 84 *Yersinia* strains isolated from meat and milk products.

Out of the 300 samples, 84 were contaminated with *Yersinia* spp. including 31 chicken meat, 18 minced meat, 15 homemade white cheeses, 13 open white cheese and 7 raw milk samples. The prevalence rates of *Yersinia* spp. were 49 (40.8%) in meat products such as chicken and minced meat and 35 (19.4%) in milk products such as raw milk and cheeses. *Yersinia* spp. were most frequently found in meat products including 51.7% of chicken meat and 30% of minced meat. The incidence rates of *Yersinia* spp. were 25% in homemade white cheese, 21.7% in open white cheese and 11.7% in raw milk. *Y. enterocolitica*, the most commonly isolated *Yersinia* species, was found at the rates of 16.7 %, 5%, 5%, and 3.3% in chicken meat, minced meat, open white cheese, and raw milk, respectively.

Among the isolated *Yersinia* species, *Y. enterocolitica*, *Y. pseudotuberculosis*, *Y. mollaretii* and atypical *Yersinia* spp. were mainly found in chicken meat samples. Besides, homemade white cheese samples in this study did not harbor *Y. enterocolitica* strains. *Y. intermedia*, *Y. rohdei* and *Y. ruckeri* were common in the samples of homemade white cheeses, open white cheeses and minced meat, respectively. The prevalence of all *Yersinia* species isolated with regard to types of meat and milk products is given in Table 10.

Table 10. The prevalence all *Yersinia* species isolated regarding types of meat and milk products

									Atypical	
	Number of	<i>Y.</i>	<i>Y.</i>	<i>Y.</i>	<i>Y.</i>	<i>Y.</i>	<i>Y.</i>	<i>Y.</i>	<i>Yersinia</i>	
	samples	<i>enterocolitica</i>	<i>rohdei</i>	<i>intermedia</i>	<i>pseudotuberculosis</i>	<i>ruckeri</i>	<i>mollaretii</i>	<i>bercovieri</i>	spp.	Total
Chicken meat	60	10 ^a (16.7) ^b	3 (5)	3 (5)	7 (11.7)	2 (3.3)	3 (5)	1 (1.7)	2 (3.3)	31 (51.7)
Minced Meat	60	3 (5)	4 (6.7)	2 (3.3)	1 (1.7)	6 (10)	0 (0)	2 (3.3)	0 (0)	18 (30)
Homemade white cheese	60	0 (0)	1 (1.7)	6 (10)	2 (3.3)	3 (5)	1 (1.7)	1 (1.7)	1 (1.7)	15 (25)
Open White cheese	60	3 (5)	5 (8.3)	2 (3.3)	2 (3.3)	0 (0)	0 (0)	0 (0)	1 (1.7)	13 (21.6)
Raw milk	60	2 (3.3)	2 (3.3)	1 (1.7)	0 (0)	1 (1.7)	1 (1.7)	0 (0)	0 (0)	7 (11.7)

^a, number of strains; ^b, percentage of strains.

In this study, the recovery of *Yersinia* spp. from meat and milk products was performed according to ISO (ISO 10273: 2003) and cold enrichment (Nordic Committee on Food Analysis, 1996) methods. For the application of these methods, several specific culture media and enrichment broths were used (Fig. 1).

Statistical analyses were carried out to compare different isolation methods for the recovery of *Yersinia* spp. from meat and milk products. The results show that at 95% confidence level there is not a statistically significant difference between the isolation methods ($P=0.054$).

In this research, the relationship between the isolation methods was also assessed by using the binary correlation. A statistically significant relationship was observed between Method 3 (enrichment in PSB and plating on CIN) and Method 5 (enrichment in PSB and plating on CIN after KOH-treatment) as well as between Method 3 (enrichment in PSB and plating on CIN) and Method 7 (cold enrichment in PSB and plating on CIN) ($p < 0.05$). Moreover, a high correlation between Method 5 (enrichment in PSB and plating on CIN after KOH-treatment) and Method 7 (cold enrichment in PSB and plating on CIN) was detected ($p < 0.05$).

3.2. Characterization of *Y. enterocolitica* strains based on the 16S rRNA amplification

The identification of *Y. enterocolitica* was performed by using both biochemical tests and the 16S rRNA gene amplification and sequencing. As a result, in this study the conventional and molecular methods were in full agreement with each other. *Y. enterocolitica* isolates were identified at the species level by 16S rRNA homology using PCR with primers as previously described by Neubauer et al. (2000a). After agarose gel electrophoresis, amplified PCR fragments with size about 330 bp from genomic DNAs of *Y. enterocolitica* isolates were observed.

Figure 3 shows PCR amplicons of *Y. enterocolitica* strains isolated from meat and milk products harbored the 16S rRNA gene.

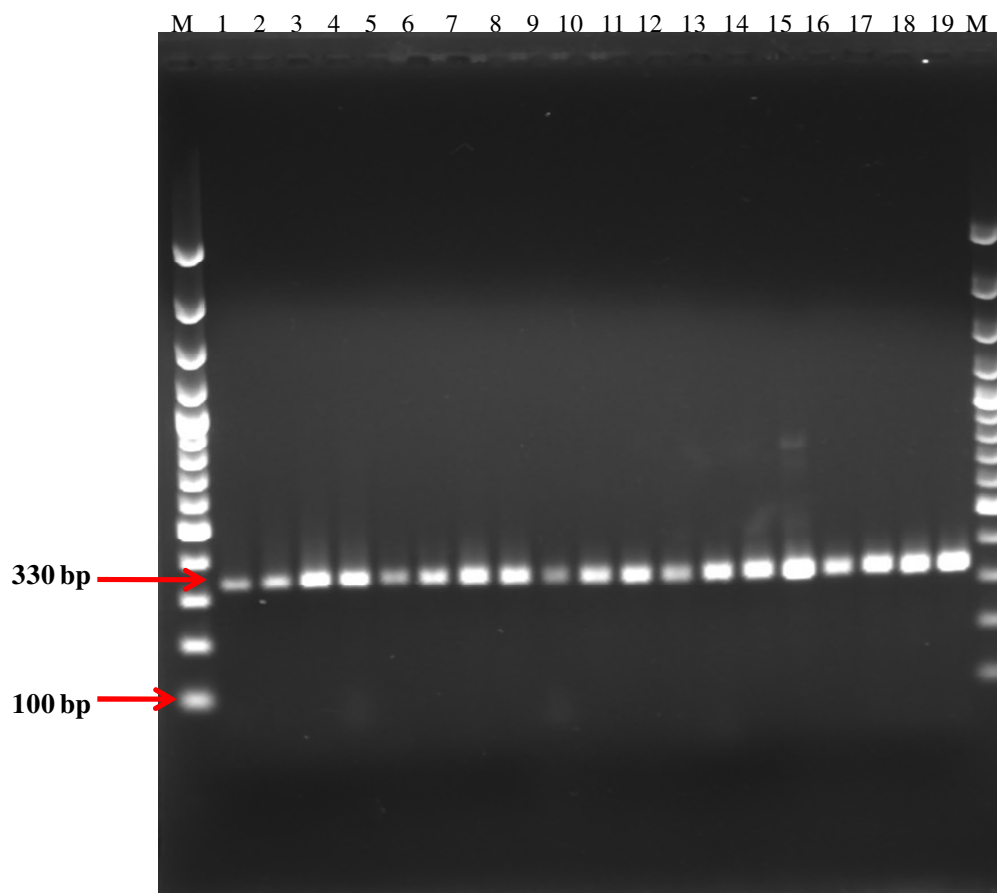


Figure 3. Agarose gel electrophoresis of 16S rRNA gene product amplified with PCR for *Y. enterocolitica* isolates. M, molecular mass marker (GeneRuler 100 bp DNA Ladder Plus; Fermentas). Lane 1 represents PCR products obtained from referential *Y. enterocolitica* ATCC 23715 strain. Lane 2 -19 products of the amplification of 16S rRNA gene fragments from the strains of *Y. enterocolitica* isolated from meat and milk samples under study.

In this research, DNA sequences of the *Y. enterocolitica* 16S rRNA amplicons were checked using the Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov/>) and the selected bacterial strains from meat and milk products showed a 99% similarity to *Yersinia enterocolitica* subsp. *enterocolitica* strain 8081 and *Yersinia enterocolitica* subsp. *paleoartica* strain Y11 in the National Center for Biotechnology Information (NCBI) GenBank. The 16S rRNA gene sequences of *Y. enterocolitica* strains isolated from different food origins including chicken meat, minced meat, open white cheese and raw milk were deposited in the GenBank database. Table 11 represents the molecular identification of *Y. enterocolitica* strains from meat and milk products and their accession numbers based on the 16S rRNA gene sequences.

Table 11. Identification of selected bacterial strains isolated from meat and milk products with partial sequences of 16S rRNA genes

No.	Isolates ^a	Food source	Species ^b	Identity (%)	Accession number ^c
1.	T11/25	Chicken meat	<i>Y. enterocolitica</i> subsp. <i>enterocolitica</i> strain 8081	99	KJ914677
			<i>Y. enterocolitica</i> subsp. <i>palearctica</i> strain Y11	99	
2.	M7/3	Open white cheese	<i>Y. enterocolitica</i> subsp. <i>enterocolitica</i> strain 8081	99	KJ914678
			<i>Y. enterocolitica</i> subsp. <i>palearctica</i> strain Y11	99	
3.	K7/5	Minced meat	<i>Y. enterocolitica</i> subsp. <i>enterocolitica</i> strain 8081	99	KJ914679
			<i>Y. enterocolitica</i> subsp. <i>palearctica</i> strain Y11	99	
4.	S47/3	Raw milk	<i>Y. enterocolitica</i> subsp. <i>enterocolitica</i> strain 8081	99	KJ914680
			<i>Y. enterocolitica</i> subsp. <i>palearctica</i> strain Y11	99	
5.	T53/1	Chicken meat	<i>Y. enterocolitica</i> subsp. <i>enterocolitica</i> strain 8081	99	KJ914681
			<i>Y. enterocolitica</i> subsp. <i>palearctica</i> strain Y11	99	

^a The isolates obtained in this work.

^b Names of the most related strains in the NCBI GenBank.

^c The nucleotide sequences in this study submitted to GenBank databases (<http://www.ncbi.nlm.nih.gov/genbank/>).

3.3. Virulence characteristics of *Y. enterocolitica* and *Yersinia* spp.

Eighteen *Y. enterocolitica* strains from meat and milk samples were biotyped for differentiation of pathogenic and non pathogenic isolates. Some phenotypic virulence tests including autoagglutination, Congo-red absorption in CR-MOX medium and crystal violet binding were performed. In this study, none of the *Y. enterocolitica* strains biotyped as 1A had pathogenic potentials because the phenotypic virulence tests yielded negative results (Table 12).

Genotypic virulence characteristics of all *Y. enterocolitica* and the other 66 *Yersinia* strains were examined by PCR for the presence or absence of chromosomal encoded virulence genes (*ail*, *ystA* and *ystB*) and plasmid encoded genes (*yadA* and *virF*). In relation to our results, none of the *Y. enterocolitica* strains were positive for *ail* and *ystA* genes. However, two of the *Y. enterocolitica* from chicken meat were found to be positive for *ystB* genes (Table 12). All *Y. enterocolitica* strains were found to be negative for plasmid encoded genes including *yadA* and *virF*. The biotype profiles, origins and incidence of phenotypic and genotypic virulence tests of 18 *Y. enterocolitica* strains from meat and milk products are presented in Table 12.

Table 12. Phenotypic and genotypic virulence markers of 18 *Y. enterocolitica* strains

Strains ^a	Food type	Biotype	Phenotypic virulence markers			Genotypic virulence markers					
			AA ^e	CV ^f	CR ^g	16S rRNA	<i>ail</i>	<i>ystA</i>	<i>ystB</i>	<i>yadA</i>	<i>virF</i>
<i>Y. enterocolitica</i> (10)	Chicken meat	1A	- ^b	-	-	+ ^c	-	-	+ (2) ^d	-	-
<i>Y. enterocolitica</i> (3)	Minced meat	1A	-	-	-	+	-	-	-	-	-
<i>Y. enterocolitica</i> (3)	Open white cheese	1A	-	-	-	+	-	-	-	-	-
<i>Y. enterocolitica</i> (2)	Raw milk	1A	-	-	-	+	-	-	-	-	-

^a, number of *Y. enterocolitica* strain; ^b, negative reaction; ^c, presence of the gene; ^d, number of positive strain harbored gene; ^e, autoagglutination; ^f, Congo red absorption; ^g, Crystal violet binding

All the other 66 *Yersinia* species were also tested for *ail*, *ystA* and *ystB* virulence genes located on the chromosome. None of the strains tested had *ail* and *ystA* virulence genes except only one isolate classified as *Y. intermedia* originated from chicken meat which had *ystB* gene. All *Yersinia* strains were found to be negative for *yadA* and *virF* plasmid-encoded genes. Results of virulence genes of the other 66 *Yersinia* strains are given in Table 13.

Table 13. Detection of virulence genes in the other *Yersinia* species from meat and milk products

<i>Yersinia</i> species (66) ^a	Virulence Genes				
	<i>ail</i>	<i>ystA</i>	<i>ystB</i>	<i>yadA</i>	<i>virF</i>
<i>Y. intermedia</i> (14)	- ^b	-	+ ^c (1) ^d	-	-
<i>Y. mollaretii</i> (5)	-	-	-	-	-
<i>Y. pseudotuberculosis</i> (12)	-	-	-	-	-
<i>Y. rohdei</i> (15)	-	-	-	-	-
<i>Y. ruckeri</i> (12)	-	-	-	-	-
<i>Y. bercovieri</i> (4)	-	-	-	-	-
<i>Yersinia</i> spp. (4)	-	-	-	-	-

^a, number of *Yersinia* strain tested; ^b, absence of the gene; ^c, presence of the gene;

^d, number of positive strain harbored the gene.

The use of PCR allowed detecting the 170 bp product of the *ail* gene, 146 bp product characteristic for the *ystB* gene, and 79 bp product of the *ystA* gene for *Y. enterocolitica* and other *Yersinia* strains. The results of PCR were documented by photographs of agarose gels with the presence of appropriate size bands clearly marked; an example of which is shown in Figure 4.

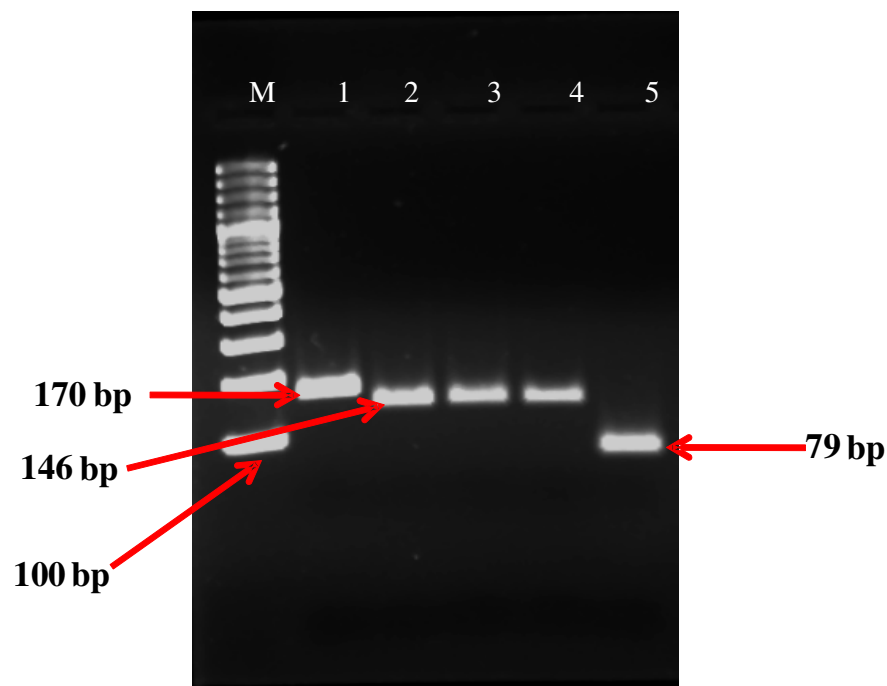


Figure 4. Electrophoretic separation of products of *ail*, *ystA*, and *ystB* genes' amplification from strains of *Y. enterocolitica* and *Y. intermedia* in PCR. M-size marker GeneRuler 100 bp DNA Ladder Plus (Fermentas). Lane 1-positive control for *ail* (170 bp), contains DNA isolated from a reference strain; *Y. enterocolitica* ATCC 23715. Lane 2 and 3-positive for *ystB* (146 bp), contains DNA isolated from *Y. enterocolitica* strains. Lane 4-positive for *ystB*, contains DNA isolated from *Y. intermedia*. Lane 5-positive control for *ystA* (79 bp), contains DNA isolated from a reference strain; *Y. enterocolitica* ATCC 23715.

3.4. Antimicrobial resistance

In this thesis, the antimicrobial susceptibility of the 84 *Yersinia* spp. isolated from meat and milk products to 16 various antimicrobial agents was examined by the standard disk diffusion method.

Results of antimicrobial susceptibility showed that all 84 (100%) *Yersinia* spp. had resistance to clindamycin. Most *Yersinia* strains with a rate of 86.9% were resistant to cephalothine, 78.6% to cephalazoline, 64.3% to ampicillin and 57.1% to amoxicillin. All *Yersinia* strains were susceptible to imipenem and tobramycin. Among all strains, the highest susceptibility was found to ciprofloxacin (98.8%) followed by gentamicin (96.4%), chloramphenicol (91.6%), nalidixic acid (88.1%), kanamycin (86.9%), trimethoprim (85.7%), streptomycin (80.9%), tetracycline (76.2%) and amoxicillin/clavulanic acid (64.3%). Antimicrobial susceptibility rates of 16 antimicrobial agents tested against 84 *Yersinia* strains from meat and milk products are presented in Table 14.

Table 14. Antimicrobial susceptibility rates of 16 antimicrobial agents tested against *Yersinia* strains from meat and milk products (n=84)

Antimicrobial Agents	Conc ^a . (µg/disk)	Number of isolates (%)		
		Resistant	Intermediate	Susceptible
Nalidixic acid	30	4 (4.8)	6 (7.1)	74 (88.1)
Ciprofloxacin	5	0	1 (1.2)	83 (98.8)
Tetracycline	30	4 (4.8)	16 (19)	64 (76.2)
Streptomycine	10	4 (4.8)	12 (14.3)	68 (80.9)
Gentamicin	10	0	3 (3.6)	81 (96.4)
Kanamycine	30	2 (2.4)	9 (10.7)	73 (86.9)
Amoxycillin	25	48 (57.1)	5 (6)	31 (36.9)
Amoxicillin / clavulanic acid	30	17 (20.2)	13 (15.5)	54 (64.3)
Trimethoprim	5	12 (14.3)	0	72 (85.7)
Cephazoline	30	66 (78.6)	7 (8.3)	11 (13.1)
Cephalothine	30	73 (86.9)	5 (6)	6 (7.1)
Ampicillin	10	54 (64.3)	4 (4.8)	26 (30.9)
Tobramycine	10	0	0	84 (100)
Imipenem	10	0	0	84 (100)
Clindamycin	2	84 (100)	0	0
Chloramphenicol	30	2 (2.4)	5 (6)	77 (91.6)
Resistance to ≥ 1 antibiotic		84 (100)		
Multiresistance ^b		71 (84.5)		

n, number of strains tested; ^a Conc, concentration of disk; ^b, Resistance to at least three or more antimicrobial agents.

In this study, all isolated and identified *Yersinia* strains were evaluated from the point of view of antimicrobial resistance of *Yersinia* strains to antimicrobial agents. All strains showed 100% of resistance to only clindamycin. In addition, the highest resistance encountered was 86.9% to cephalothine, 78.6% cephazoline, 64.3% ampicilline, 57.1% amoxycillin. A low percentage of *Yersinia* strains was resistant to amoxicillin/clavulanic acid (20.2%), followed by trimethoprim (14.3%), nalidixic acid (4.8%), tetracycline (4.8%), streptomycin (4.8%), kanamycin (2.4%) and chloramphenicol (2.4%). None of the *Yersinia* strains showed resistance to ciprofloxacin, gentamicin, tobramycin and imipenem. The percentages of antimicrobial resistance of 84 *Yersinia* strains are shown in Figure 5.

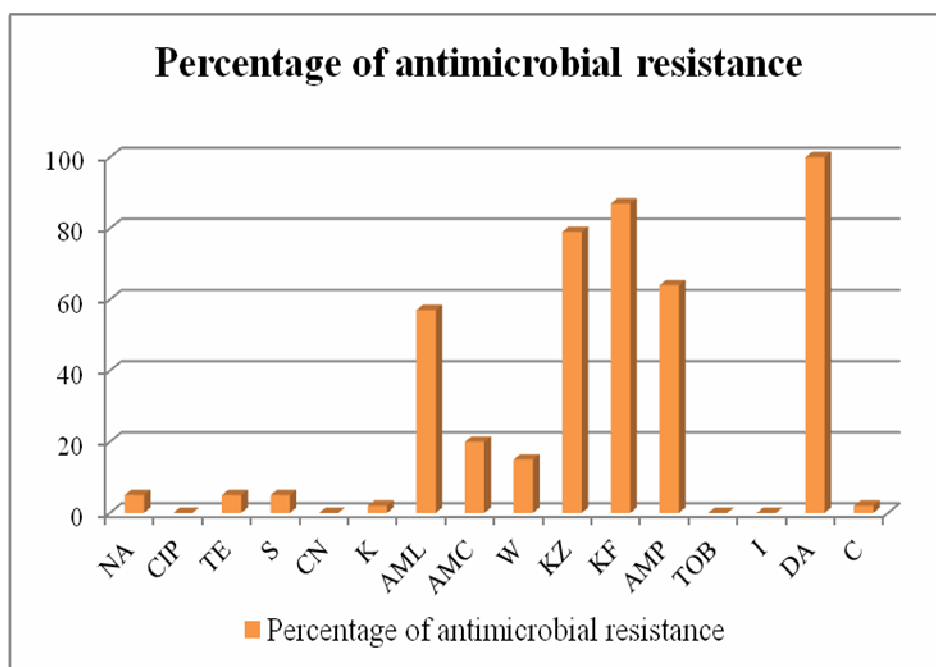


Figure 5. The percentages of antimicrobial resistance of 84 *Yersinia* strains.

AMC, amoxicillin / clavulanic acid; AML, amoxicillin; AMP, ampicillin; C, chloramphenicol; CIP, ciprofloxacin; CN, gentamicin; DA, clindamycin; IPM, imipenem; K, kanamycin; KF, cephalothine; KZ, cephazoline; NA, nalidixic acid; S, streptomycin; TE, tetracycline; TOB, tobramycin; W, trimethoprim.

All *Yersinia* strains were also considered regarding their antimicrobial susceptibilities. More than 75% of the *Yersinia* strains were susceptible to ciprofloxacin, gentamicin, chloramphenicol, nalidixic acid, kanamycin, trimethoprim, streptomycin and tetracycline. All 84 *Yersinia* strains were sensitive to imipenem and clindamycin. Among the *Yersinia* strains, the highest susceptibility was found to ciprofloxacin (98.8%) followed by gentamicin (96.4%), chloramphenicol (91.6%), nalidixic acid (88.1%), kanamycin (86.9%), trimethoprim (85.7%), streptomycin (80.9%), tetracycline (76.2%) and amoxicillin/clavulanic acid (64.3%). The antimicrobial susceptibility patterns obtained from the 84 *Yersinia* strains against 16 antimicrobial agents are given in Figure 6.

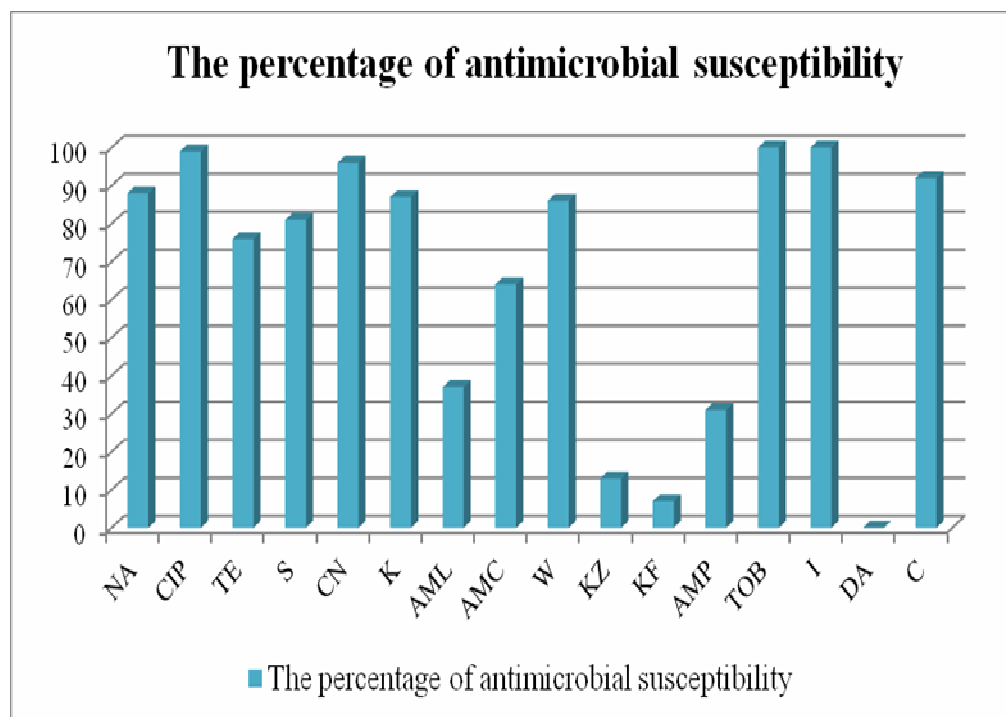


Figure 6. The percentages of antimicrobial susceptibility of 84 *Yersinia* strains.

AMC, amoxicillin / clavulanic acid; AML, amoxicillin; AMP, ampicillin; C, chloramphenicol; CIP, ciprofloxacin; CN, gentamicin; DA, clindamycin; IPM, imipenem; K, kanamycine; KF, cephalothine; KZ, cephazoline; NA, nalidixic acid; S, streptomycine; TE, tetracycline; TOB, tobramycine; W, trimethoprim.

In the present study, a total of 84.5% of the 84 *Yersinia* strains were resistant to three or more antimicrobial agents while 64.3% of them to four or more antimicrobial agents. Among the 84 *Yersinia* strains, 7 were resistant to one antimicrobial agent, 6 to two, 17 to three, 4 to four, 28 to five, 13 to six, 6 to seven, 2 to eight and 1 to nine. Multiple antimicrobial-resistant *Yersinia* spp. and *Y. enterocolitica* in meat and milk products are presented in Table 15.

Table 15. Multiple antimicrobial-resistant *Yersinia* spp. and *Y. enterocolitica* in meat and milk products

Antimicrobial resistance profiles	Number of antimicrobials	Number of strains (%)	
		<i>Yersinia</i> spp. (n=84)	<i>Y. enterocolitica</i> (n=18)
DA, KF, KZ	3	13 (15.5)	6 (33.3)
DA, NA, TE	3	1 (1.2)	0 (0)
DA, TE, W	3	1 (1.2)	0 (0)
AMP, DA, KF	3	2 (2.4)	0 (0)
AML, AMP, DA, KF	4	1 (1.2)	0 (0)
DA, KF, S, TE	4	1 (1.2)	0 (0)
AML, DA, KF, KZ	4	1 (1.2)	0 (0)
AMP, DA, KF, KZ	4	1 (1.2)	0 (0)
DA, KF, KZ, W	4	0 (0)	1 (5.6)
AML, AMP, DA, KF, KZ	5	24 (28.6)	6 (33.3)
AMP, DA, KF, KZ, W	5	3 (3.6)	0 (0)
AMP, DA, K, KF, KZ	5	1 (1.2)	0 (0)
AMC, AML, AMP, DA, KF, KZ	6	10 (11.9)	4 (22.2)
AML, AMP, DA, KF, KZ, S	6	2 (2.4)	0 (0)
AML, AMP, DA, K, KF, KZ	6	1 (1.2)	0 (0)
AMC, AML, AMP, DA, KF, KZ, W	7	4 (4.8)	1 (5.6)
AMC, AML, AMP, DA, KF, KZ, S	7	1 (1.2)	0 (0)
AML, AMP, DA, KF, KZ, TE, W	7	1 (1.2)	0 (0)
AML, AMP, C, DA, KF, KZ, NA, W	8	1 (1.2)	0 (0)
AMC, AML, AMP, C, DA, KZ, NA, W	8	1 (1.2)	0 (0)
AMC, AML, AMP, C, DA, KF, KZ, NA, W	9	1 (1.2)	0 (0)
Total		71 (84.5)	18 (100)

AMC, amoxicillin / clavulanic acid; AML, amoxicillin; AMP, ampicillin; C, chloramphenicol; DA, clindamycin; K, kanamycine; KF, cephalothine; KZ, cephalazolin; NA, nalidixic acid; S, streptomycine; TE, tetracycline; W, trimethoprim.

4. DISCUSSION

The microbiological safety of foods is an increasing public health concern worldwide. Foodborne pathogenic *Yersinia* has been isolated frequently from various foods. It is important to isolate and identify and differentiate foodborne pathogenic *Yersinia* from non pathogenic *Yersinia* strains. Many epidemiological studies have implicated foods as major vehicles associated with illnesses caused by *Yersinia* spp., especially *Y. enterocolitica* (Frederiksson-Ahomaa et al., 1999; Soltan-Dallal et al., 2010; Fukushima et al., 2011).

In the present study, meat and milk products were examined to determine the occurrence of *Yersinia* spp. and *Y. enterocolitica*. The incidence of *Yersinia* spp. in meat and milk products was higher than in the study by Güven et al. (2010) who found 57 (7.6%) *Yersinia* spp. isolates from 750 food samples including ice cream, raw milk, feta cheese, chicken drumsticks and minced meat. In this study, the incidence of *Y. enterocolitica* (6%), was slightly lower than that (7.6%) in the study by Güven et al. (2010). In Brazil, Tassinari et al. (1994) isolated *Yersinia* spp. from 28% of foods including raw vegetables, raw and pasteurized milk as well as meat and meat products.

Yersinia spp. has been isolated from many animals such as cows, chickens, sheep and pigs (Kechagia et al., 2007; Van Damme et al., 2010; Martinez et al., 2011). Therefore, the presence of these microorganisms in raw meat or meat products was not something unexpected. *Yersinia* spp. can be found in environment,

humans and animals as well as food. This pathogen is a common contaminant of meat and meat products because of their ubiquitous nature. Meat and meat products which are the most important link between food producing animals and humans are the major source of foodborne infections. This study showed that the incidence of *Yersinia* spp. was 40.8% and *Y. enterocolitica* 10.8% in meat products including chicken and minced meat. The incidence of *Yersinia* spp. which was 40.8% in meat products in the present study is quite similar to that (40%) reported in Brazil by Tassinari et al. (1994). A lower incidence was observed in Tehran, Iran where 16% of retail chicken and beef samples were found to be positive for *Yersinia* spp. (Soltan-Dallal et al., 2010). On the other hand the incidence of *Y. enterocolitica* in beef meat and chicken samples was as high as 80% in Tehran, Iran (Soltan-Dallal et al., 2010).

In the present study, the incidence of *Yersinia* spp. and *Y. enterocolitica* in minced meat samples was 18 (30%) and 3 (5%), respectively. The prevalence of *Yersinia* spp. and *Y. enterocolitica* was lower in other studies. For instance, Sırıken et al. (2004) isolated *Yersinia* spp. (32.8%) and *Y. enterocolitica* (27.9%) from ground beef samples. Nortje et al. (1999) found that 15.7% of all the ground beef samples contained *Y. enterocolitica*. The incidence of *Y. enterocolitica* in raw beef and poultry was 87.5% and 55%, respectively (Logue et al., 1996). In another study in Belgium, reported by Van Damme et al. (2013a), the prevalence of *Y. enterocolitica* was 4.9% in 82 minced meat samples including pure pork, mixed pork with beef and pork with veal. In the present study, the prevalence of *Yersinia* spp. and *Y. enterocolitica* in minced meat probably resulted from meat-processing equipments, associated surfaces, cross-contamination and inadequate storage temperature. Previously, it has been demonstrated that mincing meat with dirty equipment

significantly increases the level of contamination in minced meats as compared to that in whole carcasses; furthermore, the process of mincing has the potential to introduce these pathogens (Ray, 2004).

Yersinia spp. at a rate of 51.7% and *Y. enterocolitica* at a rate of 16.7% were detected in chicken meat samples in this research. Mayrhofer et al. (2004) isolated *Y. enterocolitica* from various foods including pork, beef, chicken, and turkey meat. In that study, the prevalence rates of *Y. enterocolitica* in beef and chicken meat samples were 31.9% and 44.9%, respectively. The prevalence rate of *Y. enterocolitica* in chicken meat in the study by Mayrhofer et al. (2004) was much higher than that in the current study. Different rates have been reported by several researchers for the prevalence of *Yersinia* spp. and *Y. enterocolitica* in poultry meat. The *Yersinia* spp. were isolated from 40% of the raw meat containing chicken and pork (Elsa Irma et al., 2000). Twenty-six chicken carcasses (65%) harboured *Yersinia* spp. and 22 (55%) contained *Y. enterocolitica* (Capita et al., 2002). Out of the 80 chicken meats, 26 (32.5%) were found to contain *Y. enterocolitica* (Bonardi et al., 2010). Consequently, the level of contamination with *Yersinia* spp. in chicken meat may be influenced by the high numbers of bacteria present on the hide, hair, hooves and feathers of poultry animals as well as in their gastrointestinal tract. Also, cross-contamination, packaging, inadequate cooking and handling can lead to growth of pathogens.

Raw milk contains many types of microorganisms coming from different sources such as inside the udder, animal body surfaces, feed, air, water, and equipment used for milking and storage (Ray, 2004). *Y. enterocolitica* is not normally harbored by milk cows. Therefore, its presence in raw milk must occur through contamination from other sources, which could be human or swine carriers

or contaminated water. Once introduced into a milking system, the organism could easily establish itself if not removed by effective disinfection procedures (Schiemann, 1987). The incidence of *Yersinia* spp. in milk and dairy products (19.4%) in our study was slightly higher than that obtained previously in Brazil by Hamama et al. (1992), who isolated *Yersinia* spp. in 15.4% of milk and dairy samples. On the other hand, the incidence level of *Y. enterocolitica* 2.8% was very close to that (2.7%) found by Güven et al. (2010) and lower than that (6.6%) reported by Hamama et al. (1992).

The occurrence rate of *Yersinia* spp. in 60 raw milk samples was 7 (11.7%) whereas that of *Y. enterocolitica* was 2 (3.3%) in the present study. In previous studies, different incidence rates of *Yersinia* spp. and *Y. enterocolitica* from milk have been reported (Schiemann and Toma, 1978; Vidon and Delmas, 1981; Hamama et al., 1992; Tassinari et al., 1994; Güven et al., 2010). Among the studies on the presence of *Y. enterocolitica* in raw milk, the highest number of *Y. enterocolitica* isolates was reported by Vidon and Delmas (1981) who analyzed 75 raw milk samples in Eastern France with 61 displaying the presence of *Y. enterocolitica* (81%). A higher incidence was observed in Sao Paulo, Brazil, where 45.2% and 19.4% of raw milk samples examined were found to be positive for *Yersinia* spp. and *Y. enterocolitica*, respectively (Tassinari et al., 1994). Hamama et al. (1992) reported that the incidence of *Yersinia* spp. in raw milk was 36.6% and the incidence of *Y. enterocolitica* in raw milk was 30%. The incidence of *Y. enterocolitica* isolated from raw milk (3.3%) is similar to that found by Uraz and Yücel (1998) (3.79%), and lower than those obtained by other authors: 26.7% (Ibrahim and Macrae, 1991) and 7.7% (Ruusunen et al., 2013). On the other hand, the incidence rates as low as 1.6% and 1.3% were reported by Soltan-Dallal et al. (2004) and Güven et al. (2010),

respectively. In general, the presence and incidence rates of *Yersinia* spp. and *Y. enterocolitica* in raw milk could be affected from several factors such as poor milking practices, including dirty, chapped or cracked teats, inadequate cleaning and maintenance of milking equipment, poor personnel hygiene and background microbiota. Besides, inappropriate temperature control and packaging of raw milk after milking can lead to growth of these pathogens.

The occurrence of *Yersinia* spp. and *Y. enterocolitica* in various types of cheese samples has been investigated in many countries (Hamama et al., 1992; Filetici et al., 2000; Araujo et al., 2002; Yentür et al., 2008). In this study, the prevalence rates of *Yersinia* spp. and *Y. enterocolitica* in homemade white cheeses were 25% and 0%, respectively. In an earlier study, *Yersinia* spp. was found to exist in the 24 (12%) out of 200 homemade white cheese samples, and 11(5.5%) out of the 24 *Yersinia* spp. were found to harbor *Y. enterocolitica* (Özdemir and Arslan, 2011). Out of 49 Jben samples, a type of traditional fresh cheese made from raw milk in Morocco, 7 cheese samples (14.3%) were found to be positive for *Yersinia* spp. and 2 (4.1%) were identified as *Y. enterocolitica* (Hamama et al., 1992). In another study, the incidence of *Y. enterocolitica* in traditional fresh cheese was 35.7% (Yücel and Ulusoy, 2006). In the present study, the level of open white cheese contamination with *Yersinia* spp. was 13 (21.7%) and *Y. enterocolitica* was 3 (5 %). However, Araujo et al. (2002) in the city of Rio de Janeiro, Brazil, failed to isolate any *Yersinia* spp. from soft cheese samples collected from three commercial brands. Our data concerning the incidence of *Y. enterocolitica* in open white cheeses (5%) is close to those obtained previously in Turkey by Güven et al. (2010), who isolated *Y. enterocolitica* in 4% of feta cheese samples. In contrast to our result, the incidence rate of *Y. enterocolitica* as high as 16% in 50 cheese samples collected from open markets were reported by Yentür et

al. (2008). In cheeses, the incidence of *Yersinia* spp. and *Y. enterocolitica* may be explained by the nature and sorts of cheeses, eventual contamination from human handlers, storage conditions, environment and water. On the other hand, low pH, salt concentration and starter cultures may inhibit the growth and multiplication of *Yersinia* spp. and *Y. enterocolitica* in cheeses.

The differences between the findings of various authors for the prevalence of *Yersinia* spp. in meat and milk products and those of this study might be due to several factors such as isolation methods, number of analyzed samples, the nature of food and food types, environment, water, the quality on technique used to slaughter animals, contamination during evisceration of the internal organs, temperature, storage conditions, the eventual contamination from human handlers, season, and geographical location. These factors may increase or decrease in the incidence of *Yersinia* spp. in meat and milk samples (Ray, 2004).

In previous studies, many researchers have been used different cultural-based isolation methods for the recovery of *Yersinia* spp. especially *Y. enterocolitica* from various samples. In this research, there is no a statistically significant difference between the isolation methods. By using Fisher's exact test, Bonardi et al. (2014) found that direct plating on CIN agar provided a significantly better performance than each of the other enrichment protocol containing the 2 days enrichment in ITC broth; the 2 days enrichment in PSB broth; the 2 days enrichment in PSB followed by the alkali treatment before plating; the 5 days enrichment in PSB followed by alkali treatment before plating. In a study of Van Damme et al. (2010) reported the 2 days enrichment in PSB and KOH-treatment gave a relatively better performance in isolating the *Y. enterocolitica* from pig tonsil samples. This was followed by the 2 days enrichment in ITC. However, the difference between both methods including 2

days enrichment in PSB and KOH-treatment and 2 days enrichment in ITC was not statistically significant. In another study of Van Damme et al. (2013a) showed that cold enrichment recovered more *Y. enterocolitica* from pig carcass swabs than selective ITC enrichment, but the difference was not significant. Similarly, cold enrichment was more effective than direct plating and selective enrichment for recovery of *Y. enterocolitica* from the slaughter pig tonsils, faeces and carcass surface swabs (Van Damme et al., 2013b).

Y. enterocolitica strains obtained from meat and milk products were biotyped for differentiation of pathogenic and non pathogenic isolates. As a result of biotyping, all of them were found to be biotype 1A which is known as avirulent. Biotype 1A strains have been frequently isolated from food, as in our results (Tassinari et al., 1994; Filetici 2000; Capita et al., 2002; Bonardi et al., 2010; Lucero Estrada et al., 2011). It is important that some extraintestinal infections can be caused by biotype 1A strains that can sometimes act as opportunistic pathogens (Bercovier et al., 1980a; Bottone, 1997).

A number of different biochemical methods have been proposed to predict the virulence of the *Y. enterocolitica* isolates (Laird and Cavanaugh, 1980; Bhaduri et al., 1987; Riley and Toma, 1989; Carniel et al., 2006). In the present study, the strains of *Y. enterocolitica* were tested for some phenotypic virulence characteristics, including autoagglutination, Congo red absorption in CR-MOX medium and crystal violet binding. All *Y. enterocolitica* strains showed negative results for phenotypic virulence tests.

In addition, 18 *Y. enterocolitica* and the other 66 *Yersinia* strains were genotypically examined for the presence of chromosomal (*ail*, *ystA* and *ystB*) and plasmid-encoded (*yadA* and *virF*) virulence genes. None of the *Y. enterocolitica*

strains were positive for the *ail*, *ystA*, *yadA* and *virF* genes. However, two of the *Y. enterocolitica* from chicken meat were found to be positive for the *ystB* genes (Table 12). The other *Yersinia* strains did not contain the *ail*, *ystA*, *yadA* and *virF* genes except one strain identified as *Y. intermedia* from chicken meat which had the *ystB* gene (Table 13).

Several researchers in the past reported the absence or low prevalence of *ystA* in *Y. enterocolitica* biotype 1A strains (Delor et al., 1990; Ramamurthy et al., 1997; Durisin et al., 1997). Thoerner et al. (2003) showed that 43 of the 50 strains of biotype 1A contained *ystB*, while only two strains were positive for *ystA*. On the contrary, in the *Y. enterocolitica* biotype 1A isolates from meat food, the incidence rate of the *ystB* gene was 16%, similar to our results and only one isolate (4%) was positive for the *ail* and *ystA* gene (Lucero Estrada et al., 2011). Bonardi et al. (2010) reported that most biotype 1A strains (84.3%) of *Y. enterocolitica* from chicken meat and pork samples were *ystB* positive. In another research, all *Y. enterocolitica* O:8 strains from foods and animals in China were detected as biotype 1A and none of them carried the virulence genes such as *ail*, *ystA*, *virF* and *yadA* but all of them carried the *ystB* gene (Wang et al., 2008). Similarly, *Y. enterocolitica* 1A strains from foods, animals and humans carried the *ystB* gene and none of them had the *ail*, *ystA*, *yadA* and *virF* genes (Stephan et al., 2013).

All biotype 1A strains from human and porcine were negative for *ail*, *ystA*, *yadA* and *virF* but carried the *ystB* gene (Bolton et al., 2013). The *ystB* gene was the most prevalent enterotoxin in *Y. enterocolitica* biotype 1A strains from different aquatic sources, paediatric patients and swine throat (Singh and Viridi, 2004). The *ystA* gene was found in pathogenic strains and *ystB* was usually restricted to biotype 1A strains (Ramamurthy et al., 1997). All *Y. enterocolitica* strains from clinical

samples were positive for *ail* and *virF* (Hussein et al., 2001). The presence of chromosomal the *ail* gene in pathogenic *Y. enterocolitica* strains has been reported especially in pig (Hussein et al., 2001; Frederiksson-Ahomaa et al., 2006). In another study, the *ystA* and *ail* virulence genes were detected in 132 (77%) and 68 (39.5%) out of 172 *Y. enterocolitica* isolates from different pig production systems (Tadesse et al., 2013).

Antimicrobial agents have been used for different purposes such as growth promotion, prophylaxis, or therapeutics. However, their misuse or overuse in animal production and human therapy has resulted in increased bacterial resistance to many antibiotics (Soltan-Dallal et al., 2010). A variety of research findings related to antimicrobial susceptibility of *Yersinia* spp. and *Y. enterocolitica* in foods have been published (Mayrhofer et al., 2004; Soltan-Dallal et al., 2010; Güven et al., 2010).

In this research, we found that the highest resistance was to cephalothine (86.9%), to cephalazoline (78.6%), to ampicillin (64.3%) and to amoxicillin (57.1%). Besides, all *Yersinia* strains were sensitive to imipenem and tobramycin. The highest susceptibility was found to ciprofloxacin (98.8%) followed by gentamicin (96.4%), chloramphenicol (91.6%), nalidixic acid (88.1%), kanamycin (86.9%), trimethoprim (85.7%), streptomycin (80.9%), tetracycline (76.2%) and amoxicillin/clavulanic acid (64.3%).

Soltan-Dallal et al. (2010) investigated the antimicrobial resistance of *Yersinia* spp. isolated from retail chicken and beef and obtained similar results with a high resistance to cephalothine and high susceptibility to ciprofloxacin. *Y. enterocolitica* isolates from food samples including meat and milk products showed no resistance to ciprofloxacin and imipenem but they all showed resistance to clindamycin, confirming our results (Güven et al., 2010). In contrast to our study, high levels of

resistance to ampicillin 100%, 96% and 75.9% were reported by Bonardi et al. (2010) in meat, by Gousia et al. (2011) in raw and processed meat products and by Soltan-Dallal et al. (2004) in milk samples, respectively. The highest resistance to tetracycline, gentamycin, ampicillin, kanamycin, erythromycin and piperacillin was observed in all of the isolates from raw cow milk while all isolates were sensitive to Co-trimaxazole, chloramphenicol, methicillin (Subha et al., 2009). In previous studies reported by Kwaga and Iversen (1990), all *Y. enterocolitica* and related species from swine and pork products were resistant to clindamycin but sensitive to imipenem, confirming our results. In addition, a high level of resistance to cephalothine and cephazoline among the isolates was detected similar to our results. In a study reported by Bolton et al. (2013), all *Y. enterocolitica* isolates from porcine and human showed resistance to cephalothine, ampicillin and amoxicillin/clavulanic acid and sensitive to ciprofloxacin, gentamicin, chloramphenicol, kanamycine and trimethoprim. In the present study, drug resistance rates of *Yersinia* spp. and *Y. enterocolitica* to at least three or more antimicrobial agents were 84.5% and 100%, respectively. Our results were higher than those reported by Soltan-Dallal et al. (2010).

5. CONCLUSION

Foodborne diseases are an important and growing public health and economic problem in many countries. Bacteria especially are a common cause of foodborne illnesses responsible for high levels of morbidity and mortality in the general population, but particularly of at-risk groups, such as infants and the young, children, the elderly and the immunocompromised. *Yersinia* species can be found in a wide variety of sources including soil, water, healthy humans and various animals. Prevalence of this organism in these sources presents ample opportunities for it to enter the food supply.

The genus *Yersinia* comprises three well-recognized human pathogens -*Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*. Only *Y. enterocolitica* and *Y. pseudotuberculosis* are known as enteropathogens which can cause yersiniosis in humans and animals. They are thought to be significant foodborne pathogens and are important as hygiene indicators in food safety. As a psychrophilic organism, *Yersinia* is able grow at 4 C. Therefore, cold chain food products could offer a protential food safety hazard.

Yersinia species may exist in meat and poultry products. When meat products are inadequately cooked and then left at improper temperatures for too long, this bacterium can cause illness. Meat processing at retail level is likely to contribute to

higher levels of contamination in minced beef as compared to carcasses. The presence of even small numbers of pathogens in carcass meat and edible offal may lead to heavy contamination of minced meat when it is cut into pieces as more microorganisms are added to the surfaces of exposed tissue. During the slaughtering process, bacteria from the oral cavity or intestinal contents may easily contaminate the carcasses and the environment in the slaughterhouse. Good sanitation at all phases of handling, and processing and proper heat treatment are important to control the occurrence of illnesses. Consumption of raw meat or meat cooked at low temperatures should be avoided.

Milk is high in proteins and carbohydrates, which many microorganisms can utilize for growth. The presence of pathogens in milk is likely to arise from contamination by faecal material during the milking process. The detection of pathogens in milk can also indicate a possible contamination of bacteria either from the udder, milk utensil or water supply used. However, milk that has been properly pasteurised and handled correctly is not the cause of significant disease from any of the “traditional” foodborne bacteria. Pasteurisation is a widely accepted process for assuring the safety of milk and milk products. Cheeses generally are made from pasteurized milk worldwide. However, different types of cheeses can make traditionally in rural areas from raw milk or inadequately pasteurized milk. In order to ensure production of safe cheese, the following measures are important: raw milk should be collected and maintained in good hygienic conditions; it should be refrigerated to minimize multiplication of bacteria; it should undergo pasteurization, after pasteurization proper hygienic conditions should be maintained during production of cheeses to prevent contamination.

In this research, a total of 300 food samples, comprise of chicken meat, minced meat, homemade white cheese, open white cheese and raw milk were evaluated for the presence of *Yersinia* spp. and especially *Y. enterocolitica* as a foodborne pathogen. The presence of *Yersinia* spp. in meat and milk products was investigated with regard to different isolation methods including the ISO and cold enrichment. In the application of these methods, several specific culture media and selective enrichment broths were used for increasing the recovery of *Yersinia* spp. from foods. In addition to, in this study, all *Yersinia* strains isolated were tested for determination of genotypic virulence markers and antimicrobial resistance. All of *Y. enterocolitica* strains were also investigated for the biotypes and phenotypic virulence characteristics.

Yersinia spp. was isolated from 84 (28%) out of the 300 samples. The total of 18 (6%) of the 84 contaminated samples were identified as *Y. enterocolitica*. The number and percentage of other *Yersinia* species identified in this study were as follows: *Y. rohdei* 15 (5%), *Y. intermedia* 14 (4.7%), *Y. pseudotuberculosis* 12 (4%), *Y. ruckeri* 12 (4%), *Y. mollaretii* 5 (1.7%) and *Y. bercovieri* 4 (1.3%). Also, 4 (1.3%) atypical *Yersinia* isolates were confirmed as *Yersinia* but could not be identified at the *Yersinia* species level. In the present study, identification results of *Y. enterocolitica* strains by conventional methods were fully consistent with those by molecular methods. All *Y. enterocolitica* strains from meat and milk products belonged to biotype 1A and found to be negative for phenotypic virulence characteristics. In addition, all *Y. enterocolitica* and the other *Yersinia* strains were also found to be negative for *ail*, *ystA*, *yadA* and *virF* virulence genes except only one *Y. intermedia* and two *Y. enterocolitica* strains had *ystB*. Contamination of food with bacteria that are resistant to antimicrobials is a potential public health threat.

Moreover, multidrug-resistance pattern was observed in 84.5% of the *Yersinia* isolates from meat and milk products to at least three or more antimicrobial agents.

The present study is noteworthy in that it documents prevalence, virulence characteristics and antimicrobial resistances of *Yersinia* spp. and especially *Y. enterocolitica* as a foodborne pathogen in meat and milk products. As it is known, biotype 1A strains can occasionally act as opportunistic pathogens. Therefore, their presence in meat and milk products may create a substantial risk to consumer's health. The presence of *Y. enterocolitica* in meat and milk products due to poor hygiene in the increasing number of today's illegal points-of-sale poses a serious health problem. This situation should place on the agenda of those in charge of public health a more careful monitoring and improving of the quality of meat and milk products as well as increase the awareness of consumers regarding risks associated with improper handling of foods.

Furthermore, an important property of *Yersinia* is its ability to multiply at temperatures close to 0°C. Therefore refrigeration cannot be used to control their growth. The presence of *Y. enterocolitica* on foods indicates the importance of monitoring food establishments periodically. Therefore, food handlers and sellers should be educated on the adverse effects of lack of proper personal as well as environmental hygiene and sanitation.

On the other hand, one of the major criteria for producing a safe food is a desirable shelf life which becomes possible as a result of minimization of access of microorganisms to certain foods. This can be achieved by designing proper plants, training personnel, devising equipment that can be sanitized effectively and establishing an efficient cleaning and hygiene procedure.

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