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INVESTIGATION OF YERSINIA ENTEROCOLITICA IN FOOD AND STUDY OF VIRULENCE GENES AND ANTIMICROBIAL RESISTANCE

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ΔΙΕΡΕΥΝΗΣΗ ΤΗΣ ΠΑΡΟΥΣΙΑΣ YERSINIA ENTEROCOLITICA ΣΕ ΤΡΟΦΙΜΑ ΚΑΙ ΜΕΛΕΤΗ ΤΩΝ ΓΟΝΙΔΙΩΝ ΛΟΙΜΟΓΟΝΙΚΟΤΗΤΑΣ ΚΑΙ ΑΝΤΙΒΙΟΑΝΤΟΧΗΣ

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Η Γραμματέας του Τμήματος

ΜΑΡΙΑ ΚΑΠΙΤΟΠΟΥΛΟΥ

Dedicated to my father's Dimitrios A. Petsios loving memory
and my mother Polikseni

Prologue - Acknowledgements

Y. enterocolitica being the most prevalent species of the genus *Yersinia* is the causative agent of one of the five most frequently reported zoonosis in EU countries. Nevertheless, the actual prevalence of pathogenic *Y. enterocolitica* in foods is underestimated due to the complex nature of this bacterium, and existing problems in the detection methods in the foods implicated in its transmission to humans.

In Greece there is limited information about the subtypes, the virulence genes and the antimicrobial resistance of the strains that are contaminating various foods. The present thesis aims to provide data on this field and to highlight the significance of *Y. enterocolitica* in food hygiene and public health.

In order to achieve that aim collaboration of multi-disciplinary people and laboratories was required. The major part of the thesis was conducted in the Department of Microbiology, Faculty of Medicine, School of Health Sciences, University of Ioannina and was supervised by Professor Chrissanthy Papadopoulou, the chief supervisor of my thesis, to whom I am grateful for her persistent interest and continuous support albeit the predicaments and above all her patience until this thesis was completed.

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Thank you,

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CONTENTS

A. GENERAL PART

1 Introduction

In 2016 Yersiniosis was the third most frequently reported zoonosis in EU countries with 6,861 confirmed cases and notification rate 1.82 cases/100,000 population. *Yersinia enterocolitica* was the most prevalent species isolated from human cases and most frequent serotypes were O:3, O:9 and O:8. Pigs are considered to be the major reservoir for *Yersinia*, and pork products are regarded the main source of pathogenic *Y. enterocolitica* infection in humans. Also, *Y. enterocolitica* occurrence has been reported in bovine meat, raw cow's and raw goat's milk and in ready-to-eat (RTE) salads from various EU member states. Positive findings were also reported in other domestic and wild animals (EFSA, 2016; 2017).

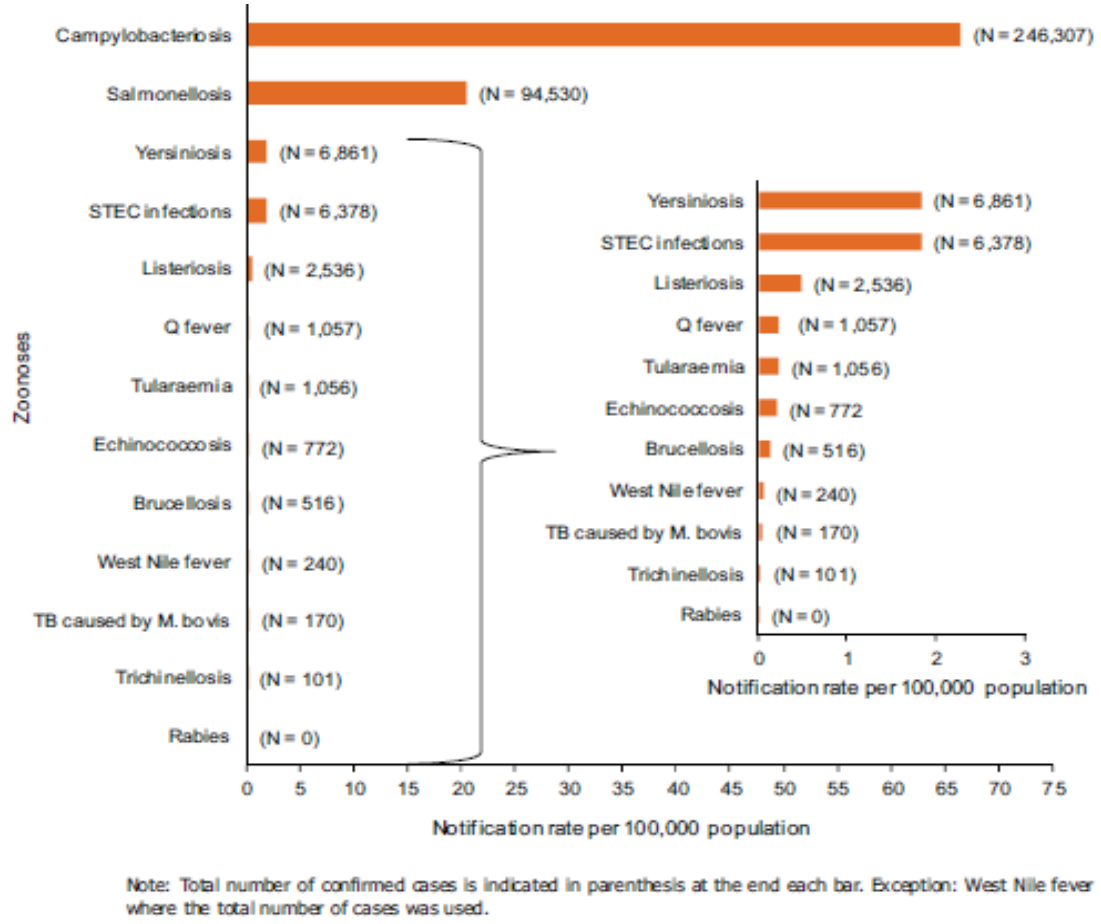

Figure 1. Reported numbers and notification rates of confirmed human zoonoses in the EU, 2016 (EFSA, 2017).

In 2017, the FoodNet identified 489 cases of yersiniosis in USA with incidence rate 1.0 case/100,000 population. Compared with the incidence reported during 2014–2016, the 2017 *Yersinia* incidence was significantly higher (166% increase). The increased incidence is most likely attributed to the increased usage of culture-independent diagnostic tests (CIDTs) employed by clinical laboratories to detect enteric infections, resulting in more accurate incidence estimates for pathogens such as *Yersinia* (CDC, 2018).

Human infections with *Y. enterocolitica* emerge after ingestion of the microorganisms through contaminated food or water or through blood transfusion. Gastrointestinal symptoms range from self-limiting gastroenteritis to acute enteritis (particularly in children), mesenteric lymphadenitis and terminal ileitis mimicking appendicitis (children older than 5 years). Infection with *Y. enterocolitica* can lead to septicemia in immunosuppressed individuals and patients being treated with desferrioxamine or patients with hemochromatosis (Bottone, 1997).

Y. enterocolitica is a Gram-negative bacterium that belongs to the family *Enterobacteriaceae*. It is a psychrophilic microorganism that is characterized by its ability to grow well at refrigeration temperatures, withstand freezing and survive for extended periods of time in frozen food, even after repeated freezing-thawing. The ability to propagate at refrigeration temperature is of outmost importance in food hygiene and safety particularly for foods packaged in vacuum or modified atmosphere (MAP), processes widely used in food preservation aiming for prolonged shelf-life (Feng and Weagant, 1994; Fredriksson-Ahomaa, 2012; Nesbakken, 2006). *Y. enterocolitica* is a highly heterogeneous group of bacteria consisting of different subtypes (biotypes and serotypes). Biotyping and serotyping are both used to determine the strain pathogenicity (Kapperud, 1991), although virulence markers have also been found in non-pathogenic biotype 1A strains (Grant et al., 1998).

2 History

Yersinia pestis, the causative agent of plague is the first reported member of the genus *Yersinia*. It was discovered during the 3rd plague pandemic that originated in China (1855). The finding of the plague causative agent is attributed to the Swiss born French physician Dr. Alexandre Yersin (1863–1943) and to Japanese physician Kitasato Shibasaburo (1852–1931) (Tsiamis et al., 2009).

The two scientistis announced independently, within days of one another, the isolation of a bacillus being responsible for the plague. Although Kitasato was initially accredited with the discovery, it was Yersin who more accurately described the microorganism naming it *Pasteurella pestis* in honor of his mentor, Louis Pasteur. Also, it was Yersin who together with Émile Roux, Albert Calmette and Amédée Borrel at Institute Pasteur in Paris developed the first effective antiserum (1986) that reduced the plague mortality rate from 90% to nearly 7%. Kitasato's failure to descripe correctly his isolate, which he insisted was different from Yersin's organism, is attributed to probable *Streptococcus pneumoniae* contamination (Tsiamis et al., 2009; Tirziu et al., 2011).

The first recorded reference to *Yersinia enterocolitica*, as a gram-negative coccobacillus, was in a publication by McIver and Pike (1934) who worked at the Mary Imogene Basset Hospital in Cooperstown, New York (US). The authors described as "*Flavobacterium pseudomallei* Withmore", a gram-negative coccobacillus isolated from two facial abscesses of a 53-year-old farm worker reporting involvement of the cervical lymph nodes too. As the infection was characterized by sinus tract formation, the researchers initially suspected either actinomycosis, tuberculosis, or glanders. Since, the isolate biochemically resembled the glanders agent (*Burkholderya mallei*) as well as *Pseudomonas pseudomallei*, the two researchers concluded that most likely the isolated microorganism was an atypical form or a variant of some well-known species rather than a new species (Perry and Fetherston, 1997; Tsiamis et al., 2009; Tirziu et al., 2011; Bottone, 1999). In 1939, this isolate was studied again by Schleifstein and Coleman in New York State Department of Health, together with two more isolates from patients with enterocolitis and one isolate from a granulomatous chest lesion of a 13 years old boy. The two researchers described all the isolates as being similar to *Actinobacillus lignieri* and *Yersinia* (*Pasteurella*) *pseudotuberculosis* (Bottone, 1997, 1999; Tirziu et al., 2011). However, since the five isolates were biochemically different from the two latter species

and three of them were from enteric contents, Schleifstein and Coleman in 1943 proposed the name *Bacterium enterocoliticum* forthe yet 'unidentified' microorganism. Retrospectively, based on their biochemical properties, these isolates match to serogroup O:8 *Y. enterocolitica* (Bottone, 1997, 1999).

The microorganism received its current name in 1968, when Sonnenwirth reported the case of a 40 year old teacher who developed *Y. enterocolitica* serogroup O:8 bacteremia, meningitis, and panopthalmitis resulting in eye loss. A few years later in 1974, Keet reported a case of typhoid like *Y. enterocolitica* O:8 septicemia in a hunter by (Bottone, 1999). In 1976, the first major foodborne outbreak of *Y.enterocolitica* serogroup O:8 infection occurred among 222 students and employees of five schools in the area of Holland Patent in upper New York State, who had consumed contaminated chocolate milk (Bottone, 1976).

The interest on the zoonotic potential of *Y.enterocolitica* was initiated after 1955 when Lucas during a series of epizootics among chinchillas and hares in France, isolated strains similar to those isolated in Switzerland (Giourka – Papavasileiou, 1982). In 1960, similar strains were also isolated from healthy pigs. During 1962-1965, epizootics occured in a number of rabbit and chinchilla breeding plants in North America and in Western Europe (Switzerland, Netherlands and Germany), by importing animals from North America (Giourka – Papavasileiou, 1982; Tirziu et al., 2011).

Knapp and Thal in 1963 proved the similarity between the human and animal isolates and in 1964 the species name *Y. enterocolitica* was formally proposed by Frederiksen (Nesbakken, 2006). Furthermore, Mollaret found out the Maclver and Pike's, and Schleifstein and Coleman's american reports which described isolates from human similar to those isolated from animal in Europe (Giourka – Papavasileiou, 1982).

In Greece *Y. enterocotica* was first isolated by Arseni (Arseni et al., 1974) from synovitis pus from a child. Up to date several studies have been published related to the detection and identification of *Yersinia* strains isolated from human faeces (Arseni et al., 1975, 1982, 1986; Maniatis, 1977; Gianneli et al., 1982; Trikka et al., 1986, 1994; Kansouzidou-Kanakoudi et al., 1988; Daneilidis and Kansouzidou-Kanakoudi, 1989; Krokidas et al., 1989; Chrisaki et al., 1995; Kouppari et al., 1994, 1995, Demertzi et al., 1999; Ioannidou et al., 1999; Stamatopoulou et al., 2004; Panagiotaki et al., 2008; Palaiologou et al., 2011; Kiratsa, 2103). There are less studies concerning the prevalence of *Y.enterocotica* in animals in Greece: in tonsil swabs from pigs (Giourka –

Papavasileiou, 1982; Kechagia et al., 2007), in faeces from domestic poultry (Kourti et al., 1984), in rectal swabs from dogs (Kansouzidou et al., 1987), in throat and rectal swabs from slaughtered pigs (Kansouzidou et al., 1995), in rectal swabs from chicken and sheep and throat swabs from cows (Kechagia. et al., 2007). As far as food is concerned there are studies for *Yersinia* incidence in vegetables (Kansouzidou-Kanakoudi and Daneilides, 1994; Xanthopoulos et al., 2010), pasteurized and non pasteurized milk (Kansouzidou-Kanakoudi et al., 1990; Gousia, 2008) and various meat samples (Gousia et al., 2011).

3 Classification

Initially, *Pasteurella pestis* and *Pasteurella pseudotuberculosis* were included in the genus *Pasteurella-Trevisan,* along with *Pasteurella multocida*, *Pasteurella haemolytica and Pasteurella tularensis* in the family of *Parvobacteriaceae*. This classification proved to be unsuccessful due to the larger size of *P. pestis* and *P. pseudotuberculosis in comparison* to other bacteria of this genus and family alike (Tsiamis et al., 2009).

In 1944 J.J. Van Loghem proposed removing of *P. pestis* and *P. rodentium* (pseudotuberculosis) from the *Pasteurella* into a new genus named *Yersinia* in honor of A.J. Yersin (Van Loghem, 1944). Nevertheless, the issue of *Parvobacteriaceae* family stayed unresolved (Tsiamis et al., 2009).

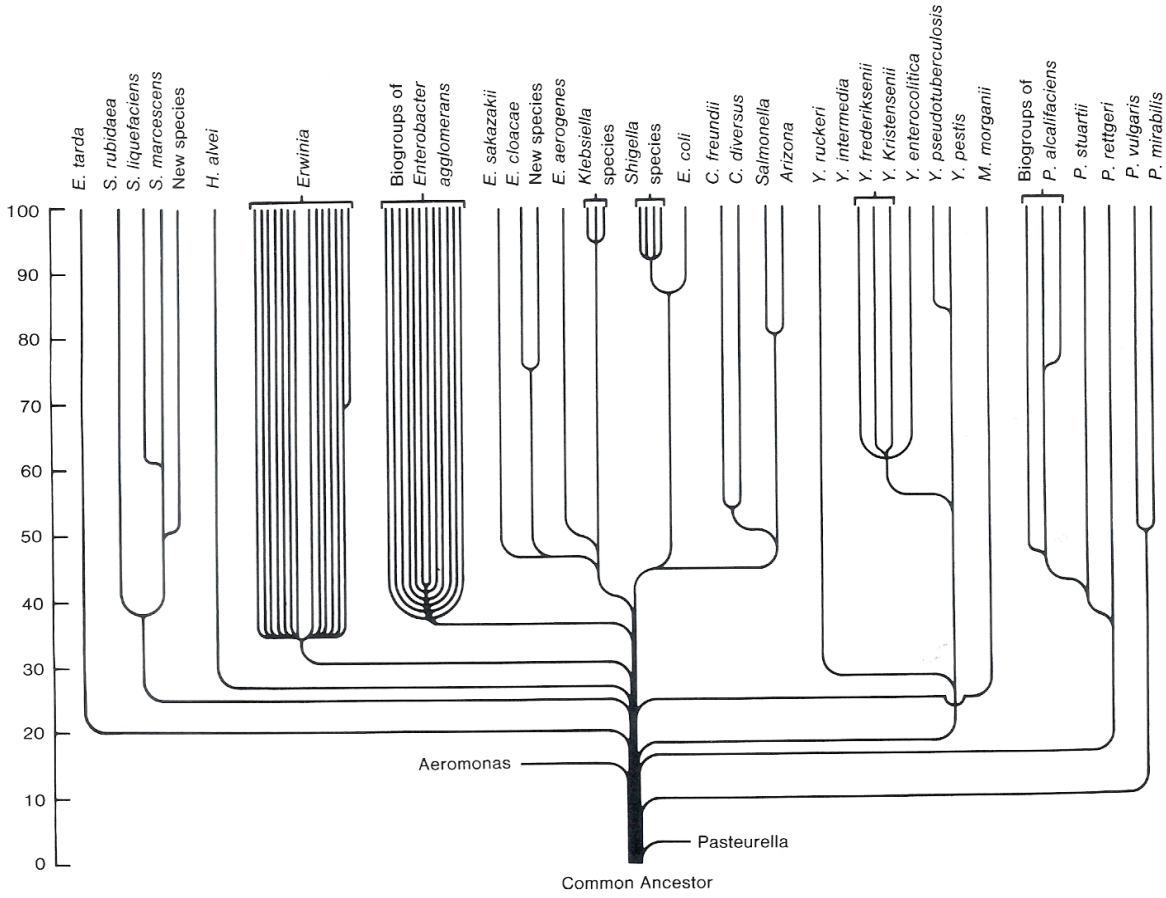

Figure 2. Divergence of *Enterobacteriaceae.* The ordinate is percentage of relatedness. This figure is a simplified attempt to depict relatedness of each species of enterobacteriacae to the rest species. It assumes a common ancestor from which all microorganisms have diverged. The horizontal branches depict the degree of relatedness of the group organisms that have not yet branched (Bercovier and Mollaret, 1984).

Soon there was evidence relating *Yersinia* to the *Enterobacteriaceae.* Since 1958 a general numerical taxonomic study placed *Yersinia* between *Klebsiella* and *Escherichia coli* (Sneath and Cowan, 1958). The allocation of *Yersinia* to the family *Enterobacteriaceae* was further supported by the Danish microbiologist Frederiksen in 1964 (Nesbakken, 2006), who also assigned Schleifstein and Coleman's '*B. enterocoliticum*' to the genus *Yersinia*, changing its name to *Y. enterocolitica*. The following year, Smith and Thal confirmed Frederiksen's assignment of this species to *Yersinia*; however, because some of the strains studied by Frederiksen were indole-positive they proposed the less inclusive name *Yersinia* X until the status of the indole-positive strains could be established (Sulakvelidze, 2000). In 1967, at the First International symposium on *Yersinia* held in Paris, the name of *Bacterium enterocoliticum* was officially changed into *Yersinia enterocolitica* and was classified in the genus *Yersinia* (Tirziu et al., 2011). Five years later, at the Second International symposium on *Yersinia* held in Malmo, it was proposed the genus *Yersinia* to be included in the family *Enterobacteriaceae* (Knapp and Mollaret, 1972).

In 1976 Brenner and colleagues introduced the DNA-DNA hybridization technique in addition to classic biochemical tests for the classification of *Y. enterocolitica* and *Y. pseudotuberculosis* (Brenner et al., 1976), and four years later, they identified three groups of *Y. enterocolitica*-like bacteria thus establishing three new *Yersinia* species: *Y. intermedia, Y. frederiksenii, Y. kristensenii* (Brenner et al., 1980; Ursing et al., 1980; Bercovier et al., 1980c). The DNA hybridization studies revealed that both indole-positive and indole-negative strains of *Y. enterocolitica* were quite closely related (DNA hybridization relatedness of 79 to 100%) to be considered one species (Moore and Brubaker, 1975; Brenner et al., 1976).

Within *Y. enterocolitica* sensu strictu, there was sufficient biochemical heterogeneity to justify the establishment of several biogroups or biotypes. In 1969 Nilehn proposed placing the strains into 'biogroups', according to their biochemical properties, and she identified five biogroups. Later Bercovier proposed two more biogroups 3A and 3B (Bercovier et al., 1978) but Wauters further modified the scheme concluding in six biogroups while omitting 3A and 3B, (Wauters et al., 1987). A year later, Wauters proposed the species designation *Y. mollaretii* and *Y. bercovieri* for isolates originally classified as *Y. enterocolitica* biogroups 3B and 3A, respectively which differed biochemically and antigenically from well-characterized *Y. enterocolitica* biogroups (Wauters et al., 1988a).

Table 1. Biochemical differentiation of *Yersinia* species (Wauters et al., 1988a).

Test	Reactions											
	Yersinia aldovae	*Yersinia bercovieri*	*Y. enterocolitica*		*Y. frederiksenii*	*Y. intermedia*	*Y. kristensenii*	*Y. mollaretii*	*Y. pestis*	*Y. pseudotuberculosis*	*Y. rohdei*	*Y. ruckeri*
			Biovars 1-4	Biovar 5								
Indole production	-	-	d	-	+	+	d	-	-	-	-	-
Voges-Proskauer (25°C)	+	-	+	+[b]	d	+	-	-	-	-	-	-
Citrate (Simmons)	d	-	-	-	d	+	-	-	-	-	+	-
L-ornithine decarboxylase	+	+	+	-	+	+	+	+	-	-	+	+
Mucate, acid	d	+	-	-	d	d	-	+	-	-	-	-
Pyrazinamidase	+	+	d	-	+	+	+[b]	+	-	-[b]	+	ND
Acid from sucrose	-	+	+	d	+	+	-	+	-	-	+	-
Celobiose	-	+	+	+	+	+	+	-	-	+	-	+
L-rhamnose	+	-	-	-	+	+	-	-	-	+	-	-
Melibiose	-	-	-	-	-	+	-	-	d	+	d	-
L-sorbose	-	-	d	d	+	+	+	+	-	-	ND	ND
L-fucose	d	+	d	-	+	d	d	-	ND	-	ND	ND

a + , positive; -, negative; d, different reactions; ND, not determined.

b Some reactions may be delayed or weakly positive.

4 Microbiology of *Y. enterocolitica*

4.1 Morphology

Y. enterocolitica is a small, coccoid-shaped Gram-negative bacterium that resembles the morphology of *Pasteurellaceae* rather than of *Enterobacteriaceae*. Depending on the type of culture medium and incubation temperature pleomorphism can occur and rods and coccobacilli can be observed in Gram stain. The microorganism is smaller in size then other members of the family *Enterobacteriaceae* (0.5-*0.8 µm* in diameter and 1-3 *µm* in length). *Y. enterocolitica* is facultative anaerobic, having both a respiratory and a fermentative type of metabolism, it produces no endospores and has no envelope. Based on biochemical properties and electron microscopy observations Pease (1979) demonstrated that at room temperature some strains of *Y. enterocolitica* can go through a transition to a spheroplast type L-form that can revert back to an irregular shaped structure with intact cell wall (Bercovier and Mollaret, 1984; Bottone, 1999; Mills, 2004; Wanger, 2005).
Yersinia expresses peritrichous flagella at lower temperatures (22-29°C) but it is non flagellated (non motile) at 37°C. Fresh isolates of *Y. enterocolitica* may require a few subcultures to express their motility. Motile cells have 2-15 peritrichous flagella (Bercovier and Mollaret, 1984; Bottone, 1999; Mills, 2004; Wanger, 2005). Bacterial swimming and swarming motility is thought to be regulated by bacterial quorum sensing ability. The bacterium is equipped to maintain biphasic lifestyle, one in the aquatic environment/food system and one in the human host (Bhunia, 2008).

The cell-wall and antigenic composition of *Y. enterocolitica* also resemble those of other members of the family Enterobacteriaceae and sometimes are identical (Bercovier and Mollaret, 1984; Wanger, 2005). There are cell surface structures that play significant role in virulence including outer membrane glycolipids such as lipopolysaccharide (LPS) consisting of a lipid A, enterobacterial common antigen (ECA), an O-specific side chain and several cell surface adhesion proteins which are present only in virulent *Y. enterocolitica* (*Inv*, *YadA* and *Ail*). These structures ensure the correct architecture of the outer membrane, providing adhesive properties as well as resistance to antimicrobial peptides and to host innate immune response mechanisms. The nature of the LPS of *Y. enterocolitica* varies for certain serogroups. The pathogenic serogroups 3, 8, and 9 are composed of galactosamine, glucosamine, 3-deoxyD-mannoctulosonic acid (KDO), 2 heptoses, glucose, and galactose (Wanger, 2005; Białas, 2012).

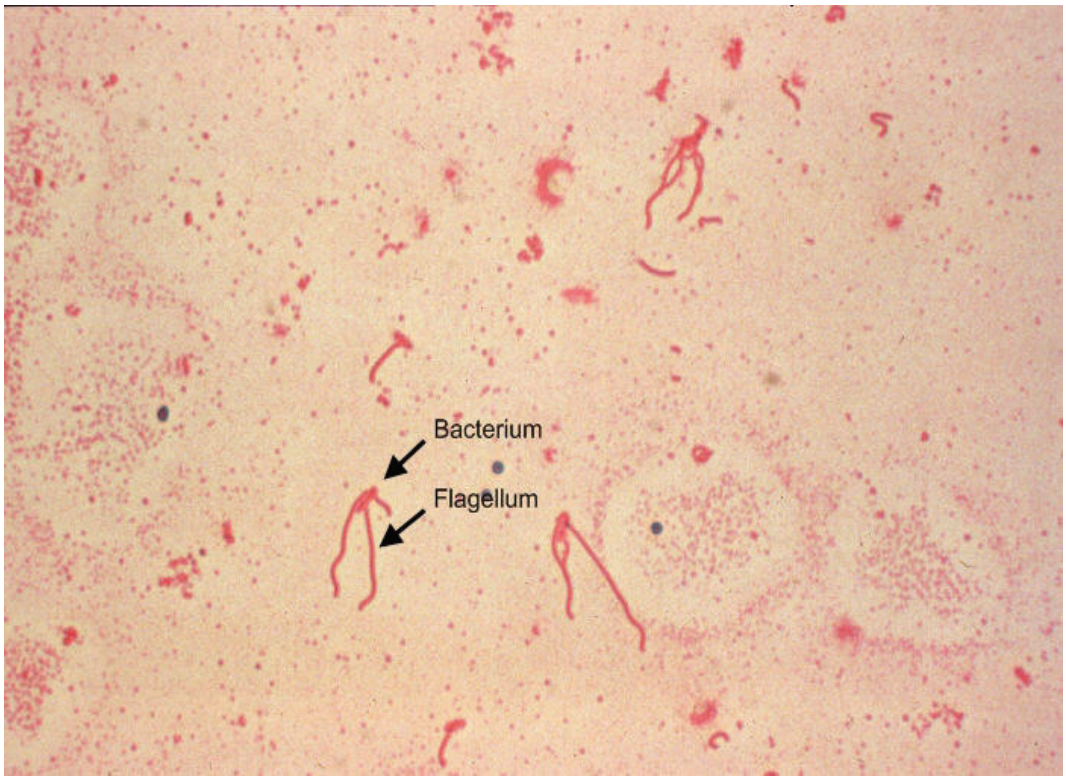

Figure 3. A photomicrograph of *Y.enterocolitica* using Flagella staining technique (CDC).

4.2 Properties

4.2.1 Temperature

Y. enterocolitica is capable to grow at temperatures ranging from 4 to 42°C, with optimum temperature at 28-29°C. The organism has the unusual capability to grow well at refrigeration temperatures (psychrophilic), ability of considerable public health concern for the food industry and food providers (Bercovier and Mollaret, 1984). However, it is reported that its growth may be slowed by the presence of psychrophilic microflora (Feng and Weagant, 1994).

In a rich medium, maximum growth is at the optimum growth temperature (approximately 28 to 30^{o} C) with a doubling time of 34 min. Generation time rises to 40 min when temperature is increased to 40°C, whereas at room temperature it is approximately 1 h. Doubling time increases to 5h at 10°C and at 1°C is approximately 40h. Multiplication can occur between -2 and 0°C, but growth is very slow (Feng and Weagant, 1994; Robins-Browne, 1997; Cosano and Garcia-Gimeno, 2003).

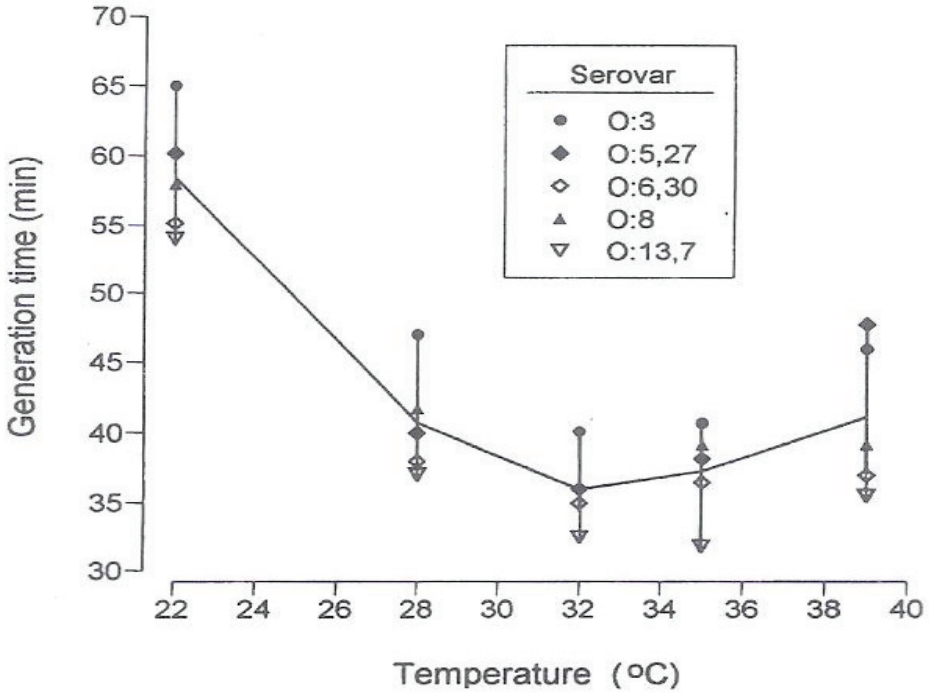

Figure 4. Generation times at different temperatures of *Y, enterocolitica* strains of five different serovars, grown in 4% tryptone, 1% mannitol salt broth, PH 7.6 (Robins-Browne, 1997).

4.2.2 pH

The optimal pH for growth of *Y. enterocolitica* is 7.2 to 7.4 but the organism will grow in pH range 4.6 - 9.0. *Y. enterocolitica* prefers slightly alkaline conditions (Bercovier and Mollaret, 1984). The minimum pH at which growth can occur varies between strains and depends on the acidulent agent used, the environmental temperature, the composition of the medium, and the microorganism growth phase. Acid tolerance depends on the activity of urease, which catabolizes urea to release ammonia, which elevates the cytoplasmic pH (Robins-Browne, 1997; Cosano and Garcia-Gimeno, 2003). *Y enterocolitica* is more tolerant at alkaline conditions than most the rest *Enterobacteriaceae,* and treatment of food enrichments with potassium hydroxide (KON) may be used to selectively reduce the level of background flora (Aulisio et al., 1980).

4.2.3 Heat tolerance

Y. enterocolitica survives for long periods in low temperatures and it is heat-labile. The organism does not survive pasteurization or normal cooking, boiling, baking, and frying temperatures. High-temperature, short-time pasteurization conditions of 71.8°C for 18 s

easily kill *Y. enterocolitica*, as well as heat treatment at 60°C for 1-3 minutes effectively inactivates it in milk and meat products. Exposure of surface-contaminated meat to hot water (80^{o} C) for 10 - 20s reduces bacterial viability by at least 99,9%. (Feng and Weagant, 1994; Robins-Browne, 1997; Nesbakken, 2006). The death rate (90% reduction time) in raw milk is reported to be 0.24 to 0.96 minutes at 62.8^{o}C (Lovett et al., 1982).

The effect of heat exposure depends on growth temperature. The growth temperature at 4–20^{o}C does not influence heat resistance at 54–66^{o}C. However, heat resistance increases fourfold when cells are grown at 37^{o}C. The heat resistance of *Y. enterocolitica* is also influenced by the pH of the heating medium. The pH of maximum heat resistance in citrate phosphate buffer is pH 7 for cells grown at 37^{o}C, but it is pH 5 for those grown at 4^{o}C; in both, the magnitude of the pH effect on heat resistance is constant at all heating temperatures (Cosano and Garcia-Gimeno, 2003).

4.2.4 Irradiation

Y. enterocolitica is readily inactivated by ionizing and UV irradiation (Robins-Browne, 1997). Its ability to survive γ-radiation depends on the food composition and treatment, and the presence of preservatives. *Y. enterocolitica* can be completely eliminated at a dose range of 3–4 kGy since it is more radiosensitive than other common foodborne pathogens such as Salmonella and Listeria. Though irradiation even at 6 kGy fails to prevent survival in raw pork, the bacterium is completely suppressed in cooked ham and salami treated with lower doses of radiation. The reported faster recovery of radiation-injured cells in raw meat than in cured meat with spices or in cooked ham, indicates that *Y. enterocolitica* in irradiated products can be inhibited by using additional processes (Cosano and Garcia-Gimeno, 2003). High CO2 concentration as part of MAP increases the radiation sensitivity of *Y. enterocolitica* suspended in raw ground pork and the pYV (plasmid for *Yersinia* virulence) loss was the greatest when irradiation took place in the presence of air (oxygen) (Bhaduri et al., 2014).

4.2.5 Preservatives and Packaging

The behaviour of *Y. enterocolitica* in response to various preservatives depends on the temperature, the level of the chemical composition, the pH, and the amount of sodium chloride present. At low pH and temperatures, potassium sorbate is very effective against the bacterium. In contrast, sodium phosphite and sodium hypophosphite are inhibitory at

relatively high pH values. *Y. enterocolitica* can survive sodium chloride concentrations of up to 5%. Although it tolerates 5% NaCl in culture media, the addition of this NaCl concentration to foods slows down its growth rate. Delay or growth inhibition can be achieved using other preservatives such as potassium sorbate, sodium hypophosphite, sodium phosphite, sodium nitrate and nitrite (curing salts). Partial resistance to chlorine is observed, particularly when grown under conditions that approximate natural aquatic environments or when co-cultivated with predatory aquatic protozoa. Packaging containing high levels of carbon dioxide is very effective at delaying growth and can cause complete inhibition. (Robins-Browne, 1997; Cosano and Garcia-Gimeno, 2003).

4.2.6 Growth and survival in food

The ability to propagate at refrigeration temperature in vacuum-packed foods with a prolonged shelf-life is of considerable significance in food safety. Refrigeration reduces the multiplication capacity of *Y. enterocolitica* but does not inhibit it completely. Growth at low temperatures depends on other factors, mainly on pH. In neutral pH, good growth is observed, but in a slightly acidic pH (5.4) growth is very little. The organism may survive, can withstand freezing and survive for extended periods in frozen food, even after repeated freezing and thawing. Studies of the ability of organism to survive and grow in artificially contaminated foods under various conditions of storage have shown that this microorganism generally survives better at room temperature and refrigeration temperatures than at intermediate temperatures (Feng and Weagant, 1994; Robins-Browne, 1997; Cosano and Garcia-Gimeno, 2003; Nesbakken, 2006).

According to many reports, the ability of *Y. enterocolitica* to compete with other psychrophilic organisms normally present in foods may be poor (Stern et al., 1980a; Fukushima and Gomyoda, 1986). In contrast, a number of studies have shown that *Y.enterocolitica* is able to multiply in foods kept under chilled storage and might even compete successfully (Hanna et al, 1977; Stern et al, 1980b; Grau, 1981; Gill and Reichel, 1989).

Y. enterocolitica persists longer in cooked foods than in raw foods, probably because of increased availability of nutrients in cooked foods due to absence of other psychrophilic bacteria, including environmental strains of *Y. enterocolitica*, which in unprocessed food may restrict the bacterial growth of the specific foodborne isolates. Presumably, proteins and fats present in the food matrix provide a protective effect for *Yersinia* (Feng and Weagant, 1994; Robins-Browne, 1997). On cooked beef or pork, the number of viable *Y.*

enterocolitica may increase more than 6 log within 10 days at 7°C or within 24 h at 25° C Increased levels of up to 5 log increments in 10–14 days have been recorded in a variety of artificially contaminated cooked foods stored at 4°C. Much slower growth has been reported for raw beef or pork at the afore-mentioned temperatures. Growth at 0–1°C on pork and chicken has been observed and on raw beef kept for 10 days at 0–1°C. Growth is slightly slower in the presence of competitor organisms but at 4°C competes better against other psychrophilic bacteria. Moreover, DFD ("Dark Firm Dry") meat may favour the growth of *Y. enterocolitica,* due to the elevated pH level and primarily the low sugar content, a factor which may result in reduced competition with the lactobacillary flora and the elevated pH (Kapperud, 1991). The bacterium is also able to multiply in vacuum packed meat during refrigerated storage surviving for long periods (Kapperud, 1991; Feng and Weagant, 1994; Robins-Browne, 1997; Cosano and Garcia-Gimeno, 2003).

Proliferation in boiled eggs, boiled fish, pasteurized liquid eggs, pasteurized whole milk, seafoods and tofu (soybean curd) at refrigeration temperature has been reported as well as persistence for extended periods in refrigerated vegetables and cottage cheese (Robins-Browne, 1997). Published studies show that after experimental inoculation into boiled eggs, pasteurized liquid eggs and boiled fish and rapid growth is observed at 4°C growth. In milk, growth at 0–2°C after 20 days has been observed. At 4°C, in milk plate counts reach up to 10^7 CFU ml^{-1} in 7 days, and compete well with the background flora. Generation time at 4°C appears to be strain-dependent, varying between 16 and 26 h in pasteurized milk, whereas growth is preceded by an extended lag time of up to 40 h. (Cosano and Garcia-Gimeno, 2003). In seafoods, such as oysters, raw shrimp, and cooked crabmeat, the proliferation is slower than that in pork or beef. In raw shrimp and cooked crabmeat stored at 5°C, *Yersinia* grew rapidly during the first week but declined in numbers with additional weeks of storage (Peixotto et al., 1979; Kapperud, 1991; Feng and Weagant, 1994; Robins-Browne, 1997; Cosano and Garcia-Gimeno, 2003).

4.3 Culture and isolation

4.3.1 Cultural characteristics and growth requirements

Y. enterocolitica grows in nutrient agar forming small colonies reaching a diameter of 1-1.5 mm after 24 h and 2 or 3 mm after 48 h of incubation at 25-37°C. After 18 h they are translucent, smooth and round with irregular edges, but after 48 h the colony morphology

also changes: the centers become opaque with a yellow color, are elevated while the edges become more regular, producing a "chinese hat" shape. Growth and size of the colonies may be slightly improved by the addition of blood to the medium, although hemolysis is not observed when grown on blood agar (Bercovier and Mollaret, 1984).Growth is moderate in liquid media. While other *Enterobacteriaceae* become turbid in less than 18 h, *Yersinia* yields the same turbidity only after 48 h incubation. Additionally *Yersinia* gives uniform turbidity in nutrient broth and in peptone water (Bercovier and Mollaret, 1984).

Yersinia can grow at 25°C on synthetic mineral-salt media with various carbohydrates as the energy source. When incubated at 37°C, *Yersinia* becomes auxotrophic and supplements are required to promote growth on minimal medium (thiamine, biotin, and other amino acids). Special growth requirements include iron and calcium in low concentration for some virulent strains. The concentration of calcium in the medium and temperature is critical for the expression of virulence factors of *Y. enterocolitica* (Bercovier and Mollaret, 1984; Wanger, 2005).

4.3.2 Enrichments and isolation procedures

The isolation of enteropathogenic *Y. enterocolitica* is demanding. There are several culture methods, including some standardized reference methods, for the isolation of *Y. enterocolitica* from non-human samples, relying on traditional enrichment and selective plating protocols. Due to the heterogeneous nature of *Y. enterocolitica*, no single isolation procedure appears to be optimal for recovery of all pathogenic serotypes or biotypes. Thus, multiple enrichment and plating techniques are utilized. Nevertheless, these methods may be proved insufficient to detect the presence of *Y. enterocolitica*. Factors hindering isolation include the large number of organisms in the background flora and its advancement through enrichment,, especially in food and environmental samples, the presence of non-pathogenic *Yersinia* spp. (including *Y. enterocolitica* strains) in the sample, the small number of pathogenic strains in the samples and loss of the virulence plasmid (pYV) during culture (Mills, 2004; Nesbakken, 2006; Fredriksson-Ahomaa, 2012; Petsios et al., 2016).

The source of *Y. enterocolitica* can markedly affect the methods of isolation. Detection of pathogenic isolates from asymptomatic carriers, food and environmental samples is more difficult than from clinical specimens from infected individuals - patients with acute gastroenteritis or organ abscesses - because in these samples enteropathogenic *Yersinia* are often dominant bacteria and can readily be isolated by direct plating on a variety of

conventional and selective enteric media. On the contrary, because the organism tends to be present in low numbers in a complex microbial flora in asymptomatic carriers, food and environmental samples, direct isolation, even on selective media, is seldom successful. To increase the number of *Yersinia* strains in these samples, cold or selective enrichment in liquid media prior to isolation on solid media is required (Fredriksson-Ahomaa and Korkeala, 2003a). Several different methods available for isolation of *Y. enterocolitica* are presented in Table 2.

Table 2. Methods of isolation of *Y. enterocolitica* most commonly used for clinical, food, and environmental samples (partially adapted by Fredriksson-Ahomaa and Korkeala, 2003a).

Pre-enrichment	Selective/Secondary enrichment	Post-enrichment	Selective agar plate	Serotypes recovered	References
YER at 4°C for 9 days	BOS at 22°C for 5 days		CIN at 30°C for 24 h	O:3, O:8	Schiemann, 1982
PSB at 4°C for 8 days	MRB at 22°C for 4 days		CIN at 30°C for 24 h	O:3, O:8	Schiemann, 1982
PBS at 25°C for 1-3 days			CIN at 30°C for 24 h, MAC at 25°C for 48 h	All	Doyle and Hugdahl, 1983
TSB at 22°C for 1 day or at 2-4°C for 4-7 days	BOS at 22°C for 3-5 days		CIN at 30°C for 24 h	O:3, O:8	Schiemann, 1983
PSB at 4°C for 3-4 weeks			CIN at 30°C for 24 h, MAC at 25°C for 48 h	All	NMKL method No. 117 (EFSA, 2007).
PSB at 22-25ºC for 2-3 days with agitation or 5 days without agitation		±KOH	CIN at 30°C for 24 h	All	ISO 10273:2003
	ITC at 25°C for 2 days			O:3	ISO 10273:2003
PSB at 5±3°C for 5 weeks			direct and weekly plating in CIN at 30°C for 48 h	All	Bonardi et al., 2010
PMB at 4°C for 7 and 14 days	ITC at 25°C for 2 days	±KOH	weekly plating in CIN at 30°C for 48 h	All	Martinez et al., 2011
PMB at 4°C for 7 and 14 days	ITC at 25°C for 2 days	±KOH	direct and weekly plating in CIN at 30°C for 48 h	All	Van Damme et al., 2013a,b

YER, yeast extract-rosebengal broth
PMB, phosphate-buffered saline supplemented with 1% mannitol and 0.15% bile salts

Advantage is taken of the psychrophilic nature of *Yersinia* to outgrow mesophilic organisms such as other *Enterobacteriacea* at low temperatures. Consequently, cold enrichment at 4°C for prolonged periods in different solutions has been used for isolation of *Y. enterocolitica*. Typical cold enrichment methods utilize nonselective media including phosphate-buffered saline (PBS), or PBS supplemented with 1% sorbitol and 0.15% bile salts (PSB) or tryptose soya broth (TSB). This approach allows *Y. enterocolitica* to grow while non psychrophilic bacteria gradually die off. Incubation time depends on temperature; at 4 °C, incubation for 14–21 days is normally recommended, but this can be shortened to 3 days by increasing the temperature to 10 °C. However, major disadvantages

are the presence of non-pathogenic *Y. enterocolitica* and other psychrophilic bacteria, which also multiply during enrichment, and the time consuming incubation period of 14 to 21 days (Fredriksson-Ahomaa and Korkeala, 2003a; Mills, 2004; Petsios et al., 2016).

Selective/secondary enrichment methods boost the selectivity and shorten the incubation time. These incorporate selective enrichment broths with different antimicrobial agents used as selective supplements. Various enrichment media including modified Rappaport medium (MRM), irgasan-ticarcillin-potassium chlorate medium (ITC), bile oxalate sorbose broth (BOS) and modified selenite medium have been developed. These media are highly selective for some strains of *Y. enterocolitica* but are also quite inhibitory for others. The most frequently used media are ITC broth and MRB. Wauters formulated the modified Rappaport broth (MRB), containing magnesium chloride, malachite green, and carbenicillin, in which the sample is incubated at 25°C for 2 to 4 days. Subsequently he developed an enrichment broth derived from the modified Rappaport base, supplemented with irgasan, ticarcillin, and potassium chlorate (ITC) (Wauters et al., 1988b). These are inoculated either direct from sample homogenates or from cold-enriched cultures at ratios of 1 volume inoculum to 100 volumes broth, incubation typically being at 22–25 °C for 2-3 days. Both media have been useful in the recovery of strains of bio-serotype 4/O:3, which is the most common clinical serotype of *Y. enterocolitica* in Europe. Enrichment in BOS, a broth developed by Schiemann is the most effective for the recovery of serotype O:8 strains, the common clinical isolates in North America (Feng and Weagant, 1994; Fredriksson-Ahomaa and Korkeala, 2003a; Mills, 2004; Petsios et al., 2016).

Post-cold and selective enrichment alkali treatment often results in higher isolation rates. Utilizing the resistance of *Y. enterocolitica* to alkaline conditions, treatment of cold enrichments with alkali (potassium hydroxide KOH for some seconds) prior to streaking on to selective agars, has been shown to significantly reduce the number of competing microflora, making selection of *Yersinia* colonies less laborious (Aulisio et al., 1980).

Many different selective agar plating media have been designed for isolation of *Y. enterocolitica* from naturally contaminated samples. Of the traditional enteric media, the most widely used is MacConkey (MAC) agar, in which 1.5 to 2mm translucent, pale nonlactose fermenting colonies are usually seen. Cefsulodin–irgasan–novobiocin (CIN) and *Salmonella–Shigella* deoxycholate calcium chloride (SSDC) agar were developed specifically for the isolation of *Y. enterocolitica*, comprising the two most commonly used media for food samples. CIN agar is the most frequently used agar for naturally

contaminated samples because of the high confirmation rate of presumptive isolates, its high selectivity for faecal specimens and its ability to inhibit the majority of other enteric bacteria (Head et al., 1982). However, differentiation of *Yersinia* from competing organisms, such as *Citrobacter, Enterobacter, Morganella, Pantotea, Providencia, Serratia* and *Stenotrohomonas* can be difficult (Laukkanen et al., 2010; Fondrevez et al., 2010). On CIN agar, characteristic colonies of *Y. enterocolitic*a are small (≤ 1 mm) and smooth with a dark red centre (due to mannitol fermentation) surrounded by a translucent zone (`bull's-eye' colonies). On SSDC they are also small (≤ 1 mm), grey coloured, with an indistinct rim. The use of these media is recommended at 30°C since at 37°C *Yersinia* grows slowly and may be outgrown by other enteric bacteria. Furthermore, the virulence plasmid (pYV) is unstable and may be lost spontaneously during overnight growth at 37°C (Petsios et al., 2016).

Other selective agars, such as CAL medium (Dudley and Shotts, 1979), BABY4 (Bercovier et al., 1984) and virulent *Yersinia enterocolitica* (VYE) (Fukushima, 1987) developed for isolation of *Y. enterocolitica,* are not extensively used and KV202 agar (Jiang et al., 2000)*,* are not extensively used. Three new chromogenic media have been designed to differentiate virulent *Y. enterocolitica:* the *Y. enterocolitica* chromogenic medium (YeCM) (Weagant, 2008), the *Y. enterocolitica* agar-selective chromogenic medium (YECA) (Denis et al., 2011) and the CHROMAgar *Yersinia* (CAY) (Renaud et al., 2013).

Standardized reference methods include the Nordic Committee on Food Analysis (NMKL) method No. 117 "*Yersinia enterocolitica*. Detection in foods" (EFSA, 2007), the Bacteriological Analytical Manual (U.S. FDA) method for the isolation of *Y. enterocolitica* (Weagant and Feng, 2001) and most recently the International Standard Organization method for the detection of presumptive pathogenic *Y.enterocolitica* (ISO 10273:2017) in foods (International Organization for Standardization, 2017).

4.4 Biochemichal identification

Y. enterocolitica do not differ significantly from other *Enterobacteriaceae* in their general metabolism. It produces acid during fermentation of glucose, fructose, galactose, maltose, mannitol, mannose, N-acetylglucosamine and trehalose are fermented too. *Y. enterocolitica* ferments carbohydrates without gas production or may produce a few

Table 3. The main physiological and biochemical characteristics of *Y. enterocolitica* (Bergey's Manual, 1984).

Characteristics	
Indole production	d
Methyl red	+
Voges-Proskauer	-
Citrate, Simmons	-
Hydrogen sulfide on TSI	-
Urea's, Christensen's	[+]
Phenylalaline deaminase	-
Lysine decarboxylase	-
Arginine dihydrolase	-
Ornithine decarboxylase	+
Motility	-
Gelatin liquefaction at 22°C	-
KCN, growth in	-
Malonate utilization	-
D-Glucose, acid production	+
D-Glucose, gas production	-
Lactose	-
Sucrose	+
D-Mannitol	+
Dulcitol	-
Salicin	d
D-Adonitol	-
myo-Inositol	d
D-Sorbitol	+
L-Arabinose	+
Raffinose	-
L-Rhamnose	-
Maltose	d
D-Xylose	d
Trehalose	+
Cellobiose	[+]
α-Methyl-D-Glucoside	-
Esculin hydrolysis	[-]
Melibiose	-
D-Arabitol	d
Mucate	-
Lipase, corn oil	d
Deoxyribonuclease at 25°C	-
$NO_3^- \rightarrow NO_2^-$	+
Oxidase, Kovac's	-
ONPG (β-galactosidase)	+
Yellow pigment	-
D-Mannose	+

Symbols: +, 90-100% of strains are positive; [+] 76-89% of strains are positive; d 26-75% of strains are positive; [-] 11-25% of strains are positive; - 0-10% of strains are positive. The incubation temperature was 36±1°C. Data are calculated for a 48-h incubation period unless otherwise indicated (gelatin liquefaction and deoxyribonuclease).

bubbles after 2 or 3 days at 28°C. Acetoin is produced when incubated at 28°C but not at 37°C. Because the optimum growth temperature of *Yersinia* is 28-29°C, some biochemical activities are often temperature-dependent (cellobiose and raffinose fermentation, ornithine decarboxylase, ONPG (o-nitrophenyl-β-D-galactopyranoside) hydrolysis, indole production, and the Voges-Proskauer reaction) and are more constantly expressed at 28°C rather than at 37°C. It reduces nitrate to nitrite by a type B nitrate reductase. Main physiological and biochemical characteristics and are presented in Table 3. In addition to these characteristics, *Yersinia* is able to attack polypectate in 5-7 days and starch in 3-7 days. It is neither hemolytic nor proteolytic. Lecithinase activity in *Y. enterocolitica* is strain-dependent. *Y. enterocolitica* strains have a lipase that is active on corn oil, but only *Y. enterocolitica* biovar 1 expresses a lipase-esterase that is active on Tween 80 (Bercovier and Mollaret, 1984).

Specific identification of *Y. enterocolitica* requires the use of individual biochemical utilization tests. Sharma et al. (1990) successfully used the API 20E (a commercially available system containing more than 20 biochemical reactions in microtubes inoculated simultaneously for species-level identification of *Y. enterocolitica*, *Y. frederiksenii*. and *Y. kristensenii* using 175 stool isolates collected in Canada. Neubauer et al. (1998) compared the API 20E, API Rapid32 IDE, Micronaut E and polymerase chain reaction (PCR) for identification of ten different species of Yersinia using a set of 118 well-characterized strains. API 20E had the highest sensitivity and specificity, and it was the most cost-effective method for identification. The Vitek enteric pathogens card also correctly identified five isolates of *Y. enterocolitica* (Imperatrice and Nachamkin 1993). Identification of *Y. enterocolitica* can be misinterpreted due to the slow growth of the organism at 37°C. Therefore, biochemical analysis should be performed at temperatures of less than 30°C.

Recently the Fourier transform infrared spectroscopy (FT-IR) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been modified and applied for the identification of *Y. enterocolitica* at the species and subspecies levels. FT-IR is a technique that provides information about the total biochemical composition of the bacterial strain (Petsios et al., 2016).

4.5 Molecular techniques

4.5.1 Molecular detection methods

Molecular techniques have also been reported for direct detection of *Y. enterocolitica* in foods. DNA fragments from the conserved calcium-dependent region of the PYV virulence plasmid were used in colony hybridization analyses of *Y. enterocolitica* in foods (Hill et al., 1983; Jagow et al., 1988). Various genes (16S rRNA genes, virF, ail, yadA, inv and yst) have been used (Robins-Browne et al., 1989; Miliotis et al., 1989; Kapperud et al., 1990; Nkajima et al., 1992; Ibrahim et al., 1992; Feng et al., 1992; Rasmussen et al., 1994; Vishnubhatla et al., 2000; Wannet et al., 2001) but many of the studies reported problems with reduced sensitivity because of the presence of inhibitors in the food matrix or because of the low numbers of organisms present. More recent approaches combining initial enrichment (which dilutes the inhibitors as well as allowing multiplication of *Y. enterocolitica*) and PCR, have been developed to detect low concentrations of *Y. enterocolitica* in food samples with both conventional PCR and real-time PCR, seem to be more successful (Rossen et al., 1992; Bhaduri and Cottrell, 1998; Lantz et al., 1998; Vishnubhatla et al., 2000; Lambertz-Thisted et al., 2000).

However, it is important to note that PCR does not distinguish between viable and non-viable organisms and in a food microbiology diagnostic laboratory there is high risk of false-positives from cross-contamination between samples.

4.5.2 Molecular typing methods

Multiple molecular typing techniques have been used for *Y. enterocolitica*. DNA± DNA hybridization, pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), ribotyping, amplified fragment length polymorphism (AFLP), PCR-ribotyping, randomly amplified polymorphic DNA (RAPD), Repetitive Extragenic Palindrome (REP) and Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences, multiple-locus variable-number tandem repeat analysis (MLVA), multilocus sequence typing (MLST) and whole genome sequencing (WGS). MLVA has been shown to be more discriminatory in *Y. enterocolitica* strains which were identical by other typing schemes including PFGE and is highly applicable in detecting both sporadic and outbreak-related strains. Moreover, MLVA is less time-consuming, less labor-intensive than PFGE and the results are easier to analyse. The better discrimination power of MLVA and WGS for *Y.*

enterocolitica isolates compared to other genotyping methods, and a future cost reduction of these methods will make their use more widespread (Petsios et al., 2016). Additionally, two serotype PCR procedures were developed, targeting the rfbC gene in *Y. enterocolitica* serotype O:3 (Weynants et al., 1996a) and the per gene in *Y. enterocolitica* serotype O:9 (Jacobsen et al., 2005).

4.6 Bio-serotypes

Y. enterocolitica is highly heterogeneous being divided into a large number of subgroups (biotypes and serotypes), chiefly according to biochemical activity and lipopolysaccharide (LPS) O antigens. Biotyping and O-serotyping are both used to type isolates associated with human disease and strong associations between specific biotypes and serotypes are recognized.

4.6.1 Biotypes

Biotyping is based on its ability to metabolise selected organic substrates and provide a convenient means to subdivide this species into subtypes of variable clinical and epidemiological significance. According to Bercovier's biogrouping scheme (Bercovier et al., 1978, 1980c) five biotypes have been established, whereas in the recent and usually applied revised biogrouping scheme (Wauters et al., 1987), there are six biotypes: 1A, 1B, 2, 3, 4 and 5.

Table 4. Biogrouping scheme of *Y. enterocolitica.* Bercovier et al. (1978, 1980c).

	Bercovier et al., 1980c					Bercovier et al., 1978	
Biochemical test	1	2	3	4	5	3A	3B
Lipase	+	-	-	-	-	-	-
Dnase	-	- [(a)]	- [(a)]	+	+	+	+
Indole	+	+	-	-	-	-	-
D-Xylose	+	+	+	-	-	-	-
Sucrose	+	+	+	+	V	+	+
D-Trehalose	+	+	+	+	+	-	+
Nitrate	+	+	+	+	+	-	+
Voges-Proskauer						-	-
Sorbose/Inositol						+	-

Biotypes 1B and 2–5 include strains that are associated with disease in man and animals (biotype 5 has only been isolated from animals and the environment) when biotype 1A is regarded non-pathogenic. Strains of biotype 1A are generally obtained from terrestrial and freshwater ecosystems and are often referred to as environmental strains. However, strains of biotype 1A have constituted a sizeable fraction of strains from patients with gastroenteritis (Burnens et al., 1996; Tennant et al., 2003). Not all isolates of *Y. enterocolitica* obtained from soil, water, or unprocessed foods can be assigned to a biovar. These strains invariably lack the characteristic virulence determinants of the primary pathogenic Yesinia and may represent novel non-pathogenic subtypes or even new *Yersinia* species. Biotype 5 has only been isolated from animals and the environment (Robins-Browne R.M., 1997; Barton, 2002; Fredriksson-Ahomaa, 2007).

The most frequent *Y. enterocolitica* biotype obtained from human clinical material worldwide is 4. Biotype 1B bacteria are usually isolated from patients in the United States and are referred to as American strains, although they have also been identified in a number of countries in Europe, Africa, Asia and Australia. Biovar 1B strains appear to be inherently more virulent than of other pathogenic biotypes and have been identified as the cause of several foodborne outbreaks of yersiniosis in the United States (Robins-Browne R.M., 1997).

Table 5. Revised biogrouping scheme of *Y. enterocolitica.* Wauters et al. (1987).

	Biotypes						
Biochemical test	**1A**	**1B**	**2**	**3**	**4**	**5**	**6**
Lipase (Tween esterase)	+	+	-	-	-	-	-
Esculin	+/-	-	-	-	-	-	-
Salicin (24 h)	+	-	-	-	-	-	-
Indole	+	+	(+)	-	-	-	-
Xylose	+	+	+	+	V	V	+
Trehalose	+	+	+	+	+	-	+
Pyrazinamidase	+	-	-	-	+	-	+
β-D-Glucosidase	+	-				-	-
Voges-Proskauer	+	+	+	+/-[(c)]	+	(+)	-

[a] Some delayed +.
[a] ( ) = Delayed reaction; V = variable reactions.
[c] Biotype of serotype O:3 found in Japan.

4.6.2 Serotypes

Y. enterocolitica isolates are distinguished serologically based on antigenic variation in cell-wall lipopolysaccharide (LPS) O-polysaccharides (O-PS; O-antigen), capsules (K-antigens) and flagellae (H-antigens). The O-antigens are the major antigenic factors responsible for the serological properties. At least 18 flagellar (H) antigens designated by lowercase letters (a,b; b,c; b,c,e,f,k; m, etc.), have been identified, but complete antigenic characterization of isolates by O and H serotyping is rarely attempted (Robins-Browne, 1997; Barton, 2002; Bialas et al., 2012).

Wauters et al. originally devised a scheme of 30 serotypes based on 34 antigenic factors. This was expanded later to add 20 more serotypes, but later many *Y. enterocolitica* like organisms were reclassified into separate species (Weagant and Feng, 2001) (Table 6). Therefore, it was proposed a revised and simplified typing scheme of *Y. enterocolitica* into 18 serotypes containing 20 somatic factors (Aleksic and Bockemuhl, 1984). Moreover, numerous non typeable strains have been recovered, especially from environmental sources (Bottone, 1997).

Table 6. Relationship between O serotype and pathogenicity of *Y. enterocolitica* and related species (Robins-Browne, 1997).

Species	Serotypes[a]
Y.enterocolitica	
Biotype 1A	O:4; O:5; O:6,30; O:6,31; O:6,30; O:7,8; O:7,13; O:10; O:14; O:16; O:21; O:22;
	O:25; O:37; O:41,42; O:46; O:47; O:57; NT
Biotype 1B	**O:4,32; O:8; O:13a,13b;** O:16; **O:18; O:20; O:21;** O:25; O:41,42; NT
Biotype 2	**O:5,27; O:9;** O:27
Biotype 3	**O:1,2,3; O:3; O:5,27**
Biotype 4	**O:3**
Biotype 5	**O:2,3**
Y.bercovieri	O:8; O:10; O:58,16; NT
Y.frederiksenii	O:3; O:16; O:35; O:38; O:44; NT
Y.intermedia	O:17; O:21,46; O:35; O:37; O:40; O:48; O:52; O:55; NT
Y.kristensenii	O:11; O:12,25; O:12,26; O:16; O:16,29; O:28,50; O:46; O:52; O:59; O:61; NT
Y.molaretii	O:3; O:6,30; O:7,13; O:59; O:62,22; NT

a NT, not typeable. Serogroups which include strains considered to be primary pathogens are in boldface.

Although serotyping has been used extensively to study the epidemiology of *Y. enterocolitica*, preparing monospecific antisera to a number of antigenic types is a tedious and technically demanding procedure. Commercially, antisera to only a few serotypes such as O:3, O:5, O:8 and O:9 are available and as a consequence detailed serotyping of *Y. enterocolitica*, for most part, is carried out in reference laboratories. (Barton, 2002; Fredriksson-Ahomaa, 2012).

Since several O-antigens, including O:3, O:8, and O:9 have been recovered from non-pathogenic "avirulent" biogroup 1A *Y. enterocolitica* strains (Chiesa et al., 1993) and different *Yersinia* species, the pathogenic potential of a *Y. enterocolitica* isolate should be based on both serotype and biotype. For instance, serogroup O:8 antigen has been detected among *Y. bercovieri* isolates and serogroup O:3 antigen has been detected among *Y. frederiksenii* and *Y. mollaretii* isolates (Robins-Browne, 1989; Bottone, 1997, 1999; Fredriksson-Ahomaa, 2007). Potential cross-reactions may occur with other members of the *Enterobacteriaceae*, e.g., *Morganella morganii* and *Salmonella* spp. and between serogroup O:9 *Y. enterocolitica* and *Brucella abortus* and *Brucella melitensis,* and between serogroup O:3 and *Rickettsia* spp. (Corbel, 1975; Weynants et al., 1996b; Bottone, 1997; Chenais et al., 2012).

Y. enterocolitica may be separated into approximately 60 serogroups of which only 11 serotypes have been most frequently associated with human infection (Table 7). Of these, the majority of infections on a worldwide basis are caused by serotypes O:3, O:9, O:5,27 with a declining number of O:8 isolations from symptomatic patients. The O:3 serotype is most frequently isolated from humans worldwide, the O:9 serotype is found in Northern Europe and O:8 in United States and Canada, while O:5,27 is more widely distributed. Strains of serotypes O:4,32; O:8, O:13; O:18; O:20 and O:21 are also considered "American" serotypes and along with O:8 are the most virulent compared to other serotypes associated with human diseases, predominantly in the United States and Canada (Feng and Weagant, 1994; Bottone, 1997, 1999; Fredriksson-Ahomaa, 2007).

Table 7. Association of *Y. enterocolitica* with biotype, serotype, and ecologic and geographic distribution (Bottone, 1999; Fredriksson-Ahomaa, 2007).

Associated with human infections	Biotype	Serotype(s)	Ecologic distribution	Geographic distribution
Yes	1B	O:4,32	Man	United States
		O:8	Man, pig, wild rodents	Europe, Japan, North America
		O:13a,13b	Man, monkey	North America
		O:18	Man	United States
		O:20	Man, rat, monkey	United States
		O:21	Man, rat flea	North America
	2	O:5,27	Man, pig, dog, monkey	Australia, Europe, Japan, North America
		O:9	Man, pig, dog, cattle, goat, dog, cat, rat	Australia, Canada, Europe, Japan
	3	O:1,2,3	Chinchilla	Europe, United States
		O:3	Man, pig, dog, Man, pig, dog, rabbit, rat	Japan Korea
		O:5,27	Man, pig, dog, monkey	Australia, Europe, Japan, North America
	4	O:3	Man, pig, dog, cat, rat	Europe, Japan, United States, North America, South Africa
	5	O:2,3	Hare, goat, sheep, rabbit, monkey	Europe, Australia
No[a]	1A	O:5; O:6,30; O:7,8; O:18; O:46, NT	Environment, pig, food, water, animal and human feces	Global

However, this geographical distribution may not be strict, since serotype O:9 has been isolated in the United States (Bisset et al., 1990) and isolations of serotype O:8 also have been reported from Europe and Asia (Ichinohe et al., 1991; Iinuma et al., 1992; Hosaka et al., 1997). In addition, there appears to be a marked increase in incidences of O:3 in clinical infections in the United States (Bottone, 1983; Bisset, 1990). Non-pathogenic strains of serotypes O:5; O:6,30; O:6,31; O:7,8; O:10, O:18; O:46 and non-typable strains are distributed worldwide and are predominantly isolated from the environment, water, feces, and food (Bottone, 1999).

5 Epidemiology

The healthcare sector has to deal with various infections that despite being infrequent, they have considerable impact on health care. *Y. enterocolitica* is a zoonotic bacterial species discovered more than 65 years ago, but prior to the 1960s, it was not considered as a veterinary or human pathogen. However, in the1960s, as *Y. enterocolitica* infections increased it was recognized as causative agent of foodborne gastrointestinal infections. Although the infections of *Y. enterocolitica* have been found to be relatively infrequent; outbreak reports have been evidenced in China, Norway, Finland, Japan, and the United States. Studies highlight that the small household infection clusters might be higher than the known cases.

Y. enterocolitica infections are commonly linked to pigs as they are considered the primary reservoirs, while raw pork products act as significant infection transmitters. *Y. enterocolitica* infections in different countries have different sources e.g in Norway they have been linked to undercooked sausage products, pork and untreated water, whereas in the United States they have been associated with contaminated tofu and pasteurized milk (Ostroff et al., 1994). The Centre for Disease Prevention and Control (CDC) estimates that *Y. enterocolitica* in the US alone causes about 117,000 illnesses, over 6000 hospitalizations and about 35 deaths annually (CDC). *Y. enterocolitica* has attracted health concerns due to the elevated rates of infections in both developed and developing nations. In the following pages the epidemiology and pathogenesis of *Y. enterocolitica* will be described focusing on the sources of infection and routes of transmission.

5.1 Sources of infection

5.1.1 Pigs

Pigs are considered the major reservoir for pathogenic *Y. enterocolitica* involved in human infection. Pigs are asymptomatic carriers of human pathogenic *Y. enterocolitica* which is located in their tonsils and intestines, and can lead to contamination of the carcass during slaughtering. *Y. enterocolitica* of bio-serotype 4/O:3 (the variety most commonly associated with human disease) has been found to be the predominant bio-serotype in asymptomatic pigs (Tauxe et al., 1987; Bottone, 1999; Fosse et al., 2009; Huovinen et al., 2010; EFSA 2012). Serotype O:3 has been almost exclusively isolated with different

degree of frequency from pigs in many European countries, like Denmark (Christensen, 1980; Andersen, 1988; Nielsen and Wegener, 1997), Finland (Fredriksson-Ahomaa et al., 1999, 2000a, 2000b, 2003b; Korte et. al., 2004; Laukkanen et. al., 2010), Norway (Nesbakken et al., 1985, 1988, 2003, 2006b; Skjerve et al., 1998), the Netherlands (De Boer, 1991, 2008), Switzerland (Fredriksson-Ahomaa et al., 2007a), Great Britain (McNally et al., 2004; Milnes et al., 2008; Martinez et al., 2010), Belgium (Wauters, 1979; Wauters et al., 1988b; Martinez et al., 2011; Van Damme et al., 2013; Vanantwerpen et al., 2014), France (Fondrevez et al., 2010, 2014), Germany (Fredriksson-Ahomaa et al., 2001a, 2003b, 2010a; Gurtler et. al., 2005; Bucher et. al., 2008), Estonia, Latvia and Russia (Martinez et al., 2009), Lithuania (Novoslavskij et al.2012, 2013), Poland (Platt-Samoraj et. al., 2006; Kot et al., 2007), Croatia (Zdolec et. al., 2014), Spain (Martinez et al., 2011), Italy (Bonardi et al., 2003, 2007, 2014; Martinez et al., 2011) and Greece (Giourka – Papavasileiou, 1982; Kansouzidou et al., 1995; Kechagia et al., 2007).

Pigs may also be asymptomatic carriers of serovars O:9 and O:5,27. Serotype O:9 is not commonly found among European pigs and has sporadically been isolated in Great Britain, Ireland, Belgium, Switzerland, Germany and Italy (Bonardi et al., 2003, 2007; McNally et al., 2004; Gurtler et al., 2005; Fredriksson-Ahomaa et al., 2007a; Milnes et al., 2008; Martinez et al., 2010, 2011; Bolton et al., 2013). Serotype O:5,27 of *Y. enterocolitica* was the most common bio-serotype isolated among pigs in Great Britain (McNally et al., 2004; Milnes et al., 2008; Martinez et al., 2010), which was reported though in lower prevalence, from Switzerland, Poland, Italy and Greece (Kansouzidou et al., 1995; McNally et al., 2004; Platt-Samoraj et al., 2006; Fredriksson-Ahomaa et al., 2007a; Milnes et al., 2008; Martinez et al., 2010, 2011).

Serovar O:8 biovar 1B, which used to be considered the most common human pathogenic strain of *Y. enterocolitica* in the US and in Western Canada, has rarely been reported in pigs (Nesbakken, 2006). Doyle et al (1981), were the first to isolate. Serovar O:8 from porcine tongues in USA, while in Canada this serotype was isolated from pig intestines (Letellier et al., 1999).

Various serovars have been reported from pigs' isolation worldwide. Serotypes O:3, O:5 and O:9 in Canada (Letellier et al., 1999; Thibodeau et al., 1999; Pilon et. al., 2000; Farzan et al., 2010) and USA (Doyle et al., 1981; Funk et al., 1998; Bhaduri et al., 2005, 2006; Bowman et al., 2007; Wesley et al., 2008), O:5 and O:8 in Argentina (Escudero et al., 1995; Favier et al., 2014), O:3 and O:9 in China (Zheng and Xie, 1996; Wang et al.,

2009; Liang et al., 2012) and Nigeria (Okwori et al., 2009), O:3 in Japan (Fukushima et al., 1983a; 1997), in New Zealand (Hudson et al., 2008), Chile (Borie et al., 1997), Brazil (Falcao et al., 2003; Rusak et al., 2014) and Ivory Coast (Atobla et al., 2012).

Fattening pigs are the most common asymptomatic carriers of human pathogenic strains carrying *Y. enterocolitica* 4/O:3 in their tonsils as documented by surveillance studies at slaughter (Korte et al. 2004; Fredriksson-Ahomaa et al. 2007a; Bucher et al. 2008; Martinez et al. 2009). A close genetic relationship between pig and human *Y. enterocolitica* 4/O:3 strains has been demonstrated by pulsed-field gel electrophoresis (PFGE) (Fredriksson-Ahomaa et al. 2006b). Laukkanen et al. (2009) have demonstrated with PFGE that the *Y. enterocolitica* 4/O:3 strain isolated from pig tonsils at slaughter originates from the farm where the pig was infected during the fattening period. The prevalence of pigs excreting *Y. enterocolitica* into feces is highest at around 12–21 weeks of age, after which the shedding decreases gradually (Laukkannen, 2010; Virtanen et al., 2011). The prevalence of *Y. enterocolitica* in pigs varies among farms, indicating that there are some factors affecting the prevalence of the pathogen within the farms. Factors associated to the within-farm prevalence in pigs have been investigated in several studies indicating both environmental and pig-to-pig transmission (Laukkannen, 2010; Virtanen et al., 2011). Also *Y. enterocolitica* has been isolated from sows but the prevalence is significantly lower compared to young fattening pigs, possibly due to natural resistance and sows may be the main source of infection in piggeries (Virtanen et al., 2011). Enteropathogenic *Y. enterocolitica* strains have occasionally been isolated from pig farm environments particularly from farms with high prevalence of pathogenic *Y. enterocolitica* in pigs (Laukkannen, 2010).

At the age of slaughter, significant numbers of fattening pigs already are asymptomatic carriers of the pathogen in their oral cavity and pharynx (mainly tonsils and tongue), lymph nodes and intestines from where contamination is spread to the carcases during the evisceration process at slaughter, particularly when the head is not removed and handled separately (Borch et al., 1996; Laukkanen et al., 2009, 2010; Virtanen et al., 2011). At slaughter, the prevalence of pathogenic *Y. enterocolitica* in tonsils can be 73 times higher than in the feces suggesting that this tissue is a more important contamination source than intestine (Laukkannen, 2010). The contamination of tonsils is largely linked to the fact that *Y. enterocolitica* feeds on lymphoid tissue (Liang et al., 2012).

5.1.2 Other animals

Y. enterocolitica has also been isolated from domestic and pet animals as well as from wild animals. The organism has been isolated from the feces of cattle (Fukushima et al., 1983b; McNally, et al. 2004; Bonardi et al., 2007; Milnes et al. 2008), sheep and goats (Slee and Button, 1990; Slee and Skilbeck, 1992; McNally, et al. 2004; Lanada et al. 2005; Milnes et al. 2008; Soderqvist et al., 2012; Joutsen et al., 2016), chicken (Kechagia et al., 2007), dogs and cats (Nikolova et al., 1978; Fukushima et al., 1984; Fantasia et al., 1985; Kansouzidou et al., 1987; Fenwick et al., 1994; Fredriksson-Ahomaa et al., 2001b; Murphy et al., 2010; Wang et al., 2010; Stamm et al., 2013), deers (Syczylo et al., 2018), wild boars (Fredriksson-Ahomaa et al., 2011), rodents (Iinuma et al., 1992; Hayashidani et al., 1995; Backhans et al., 2011; Oda et al., 2015; Joutsen et al., 2017) and primates (Iwata et al., 2005; Iwata et al., 2010).

5.1.2.1 Food animals

Positive tests in serological control programs for brucellosis in brucellosis-negative cattle have in some cases proved to be cross-reactions against *Y. enterocolitica* serotype O:9 indicating that cattle can be asymptomatic carriers of this serotype (Bonardi et al., 2007).

In Great Britain *Y. enterocolitica* BT 1A strains have also been isolated from cattle and sheep (McNally et al., 2004). In another British study BT 1A strains were found in 4.5% of the studied cattle, 5% of the sheep and 5% of pigs at slaughter (Milnes *et al.* 2008). In Sweden, nonpathogenic *Y. enterocolitica* biotype 1A has been recovered from sheep (Soderqvist *et al.* 2012). In New Zealand the goats have been recognized as a reservoir of BT 1A (Lanada *et al.* 2005; Arnold *et al.* 2006). Also, 60% of that goat farms found to be positive for *Yersinia,* and the majority of isolates belonged to bio-serotype 5/O:2,3. It has been observed that the prevalence of *Y. enterocolitica* in goats declines as their age increases and similar trend has been noticed in pigs as well. Pathogenic *Y. enterocolitica* was isolated mainly from young goats below one year of age (Lanada, 2005). Enteritis in sheep and goats due to infection with *Y. enterocolitica* biotype 5 (serotypes O:2,3) has been reported in Australia (Slee and Button, 1990). Recently, pathogenic *Y. enterocolitica* serotype O:3 has been detected in hunted wild alpine ibex in Switzerland (Joutsen et al**.,** 2012). In USA, New Zealand, Sweden and Finland strains of very rare bio-serotypes, such as bio-serotype 5/O:2,3, have been isolated from sheep and goats (Slee and Button, 1990;

Lanada *et al.* 2005; Soderqvist et al., 2012; Joutsen et al., 2016). In chicken, *Y. enterocolitica* serotypes O:3 and O:9 have been isolated in Greece (Kechagia et al., 2007).

5.1.2.2 Dogs and cats

Occasionally, pathogenic *Y. enterocolitica* strains, mostly of bio-serotype 4/O:3, have been isolated from dogs and cats (Nikolova et al., 1978; Fukushima et al., 1984; Fantasia et al., 1985; Kansouzidou et al., 1987; Fenwick *et al.*, 1994; Fredriksson-Ahomaa et al., 2001b; Stamm et al., 2013). Dogs excrete this organism in feces for several weeks after infection. Thus, pets can be a source of human infections because of their close contact with people, particularly with young children (Fenwick *et al.*, 1994; Fredriksson-Ahomaa et al., 2001b). PFGE analysis of the pathogenic strains from dogs belonging to farmers showed that they shared the same patterns as strains from diarrhea patients indicating that the strains from domestic dogs have a close correlation with the strains causing human infections (Wang et al., 2010). Murphy et al., (2010) reported the first isolation of biotype 1A strains from canine tonsils thus implicating the oral cavity of dogs as a potential reservoir for this human pathogen.

5.1.2.3 Rats and primates

Wild rodents have been found to be reservoirs for *Y. enterocolitica* 1B/O:8 strains in Japan (Iinuma et al., 1992; Hayashidani et al., 2003; Oda et al., 2015). Another study in Japan yielded similar patterns by restriction enzyme analysis of the virulence plasmid *Y. enterocolitica* O:8 strains among isolates from human patients and wild rodents indicating the important role of wild rodents as a source of human *Y. enterocolitica* O:8 infection (Hayashidani et al., 1995). *Y. enterocolitica* bio-serotype 4/O:3 strains isolated from rodent and pig samples, and compared by PFGE revealed high similarity, which may indicate rodents as vectors transmiting pathogenic *Y. enterocolitica* to pigs, acting as carriers rather than as reservoirs, spreading the bacteria within the farm, especially between different batches of pigs in all-in/all-out systems (Backhans et al., 2011). Joutsen et al. (2017) highlighted the small wild animals as a reservoir of *Yersinia* carrying *ail* gene isolates.

The first report of fatal cases of *Y.enterocolitica* serovar O:8 infection in monkeys has been reported in Japan (Iwata et al., 2005). Two fatal cases of *Y. enterocolitica* bio-serotype 4/O:3 infection in monkeys were reported in Croatia in which cases the ingestion of raw pork was suspected to be the source of the infection (Fredriksson-Ahomaa et al., 2007b).

5.1.3 Food

Y. enterocolitica infection has mainly been linked to ingestion of contaminated foods. It has been isolated from many foods, including beef, pork, minced meat, poultry, turkeys, oysters, fish, crabs, raw and pasteurized milk, powdered milk, chocolate milk, cottage cheese and tofu (cheese-like food made of curdled soybean milk), chow mein dishes (chicken, pork, beef, shrimp or tofu served with fried noodles), eggs, pasteurized liquid eggs, vacuum-packed meat, boiled fish, mushrooms, carrots, lettuce, mixed salad and bean sprouts (especially mung beans, lentils, or edible soybeans).

5.1.3.1 Pork

Pigs from enteropathogenic *Yersinia*-positive farms carry the pathogens to the slaughterhouse in their tonsils and intestines (Laukkanen, 2010). During the slaughter process, the surface of carcasses may become contaminated from feces and tonsils, particularly when the head is not removed and handled separately. Pathogenic *Y. enterocolitica* is frequently isolated from the surface of the organs and carcasses of the slaughtered pigs (Laukkanen, 2010). Meat inspection procedures concerning the head, in particular incision of submaxillary lymph nodes, also appear to represent a cross-contamination risk at slaughtering (Laukkanen-Ninios et al., 2014). Being a psychrophilic microbe, *Y. enterocolitica* is able to multiply along the cold-chain from the slaughterhouse to home refrigerator and thus can be transmitted from slaughterhouses to meat processing plants and then to retail level via contaminated pig carcasses and offal (Fredriksson-Ahomaa *et al.,* 2001b). Cross contamination from offal and pork will occur directly or indirectly via equipment, air and food handlers in slaughterhouses, retail shops and residential kitchens. Pork is likely to be an important vehicle of human infection because they are the only farm animals intended for human consumption that frequently harbour *Y. enterocolitica* 4/O:3, the bio-serotype that is isolated from human yersiniosis cases (Fredriksson-Ahomaa and Korkeala, 2003). The apparent scarcity of *Y. enterocolitica* infection in Moslem countries also supports the potential role of pork as a vehicle of Y. *enterocolitica* transmission to humans (Kapperud et al., 1991).

The observed prevalence of *Y. enterocolitica* in pork meat and products in different countries varies from 0.3% in Germany to 44.4% in New Zealand (Fukushima, 1985; Wauters, et., 1988; Nesbakken et al., 1991; Fukushima et al, 1997; Fredriksson-Ahomaa et

al, 1999, 2000b, 2001a, 2001b; Johannessen et al., 2000; Fredriksson-Ahomaa, 2001; Mayrhofer et al., 2004; Bonardi et al., 2003, 2007, 2010, 2013; Lindbland et al., 2007; Hudson et al., 2008, Gousia et al., 2011; Van Damme et al., 2013, 2015).

Indirect evidence considering pork and pork products indicates that there is an important link between consumption of raw, undercooked, or improperly handled pork product and human *Y. enterocolitica* infections. This positive correlation has been demonstrated in case-control studies in Belgium (Tauxe *et al.,* I987) and Norway (Ostroff et al., 1994). Pork has been implicated in sporadic cases of yersiniosis and has also been identified as the cause of community outbreaks. Seasonal variation has been noted throughout the world with a tendency to occur mostly during winter months in colder climates (Bottone, 1999).

The first Japanese case of *Y enterocolitica* O:8 infection was linked to consumption of imported raw pork (Ichinohe *et al.,* 1991). In the United States, after 1988, several outbreaks of serotype O:3 infections among infants were attributed to chitterlings (pig intestines), in which cases bacteria were transmitted from raw chitterlings to affected children through the hands of food handlers (Lee et al., 1990). A case-control study showed that household preparation of chitterlings was significantly associated with illness (Jones, 2003). In 2006, the first outbreak of *Y. enterocolitica* O:9 in Norway was investigated and involved 11 persons after ingesting traditional pork products consumed during the Christmas holidays (Grahek-Ogden et al., 2007). Another O:3 outbreak due to pork meat consumption occurred in January 2002 on an oil tanker during its return test voyage from Croatia to Trieste and involved 18% of the crew members and workers (Babic-Erceg et al., 2003). A study of *Y. enterocolitica* enteritis in children in Crete revealed that the recorded winter peak is associated with the traditional consumption of homemade pork sausages (Galanakis et al., 2006).

5.1.3.2 Beef and poultry

There are few studies on the detection of *Y. enterocolitica* in beef and poultry. In Japan Fukushima et al (1985) isolated biotype 1 strains from poultry (58,3%) and beef (56,7%) samples. Vishnubhatla et al. (2001), found a high occurrence of *yst*-positive *Y. enterocolitica* in 31% of ground beef samples. In Spain *Y. enterocolitica* was detected in 55% of poultry meat samples and out of 68 isolates 45 belonged to biotype 1A, three to biotype 3 and two of the biotype 3 strains were found to be presumptively virulent according to biochemical tests (Capita et al., 2002). Studies in Brazil have reported isolations of *Y. enterocolitica* from raw

beef (80%) and chicken (60%) (Cosano and Gimeno, 2003). In Austria Mayrhofer et al. (2004) found *Y. enterocolitica* in 31, 9% beef samples three of which belonged to O:3 serotype, while chicken (44, 9%) and turkey (47,6%) meat samples did not share a pathogenic serovar. In Italy 26 (32.5%) of 80 chicken meat samples were contaminated by *Y. enterocolitica* and a total of 32 strains, of which 31 (96.9%) were biotype 1A, and 1 (3.1%) was biotype 2 (O:9). Interestingly a 1A isolate carried the yadA plasmid-borne gene (Bonardi et al., 2010). A study in Iran found *Y. enterocolitica* in 16% and 9,5% in chicken and beef samples respectively (Dallal et al., 2010). In Iran Sharifi-Yazdi et al. (2011) tested 190 samples of poultry meat and 189 samples of beef meat and found *Y. enterocolitica* in 30 (62,5%) samples of poultry and 18 (37,5%) samples of beef meat.

5.1.3.3 Milk and products

Y. enterocolitica has been found in raw milk from cows and goats (Hughes, 1980; Vidon and Delmas, 1981; Moustafa et al., 1982; Greenwood et al., 1990; Kansouzidou-Kanakoudi et al., 1990; Soltan-Dallal et al., 2004; Bonardi et al., 2018), as well as from pasteurized cow milk (Gousia, 2008). Since the microorganisms will not survive pasteurization, contamination of milk by *Yersinia* can be attributed to malfunctioning of the pasteurization process leading to inadequate treatment or post-process contamination (Moustafa et al., 1982; Greenwood et al., 1990). Also, the reduction of psychrophilic bacteria in milk after pasteurization would enable a poor competitor and opportunistic pathogen such as *Y. enterocolitica* to grow better in pasteurized than in raw milk (Bari et al., 2011).

An outbreak at a summer camp in New York, involved 53% of campers and staff members, seven of which required hospitalization while five underwent appendectomies before the disease was identified as yersiniosis. *Y. enterocolitica* O:8 was isolated from humans, a milk suspension made from powdered milk and a milk dispenser (Shayegani et al., 1983). In 1995, another outbreak of *Y. enterocolitica* O:8 infections occurred in Vermont and New Hampshire was associated with the consumption of bottled pasteurized milk from a local dairy, even if the exactly route of contamination was not determined. Milk bottles were likely contaminated by rinsing with untreated well water prior to filling (Ackers et al., 2000). Another culture-confirmed case of infection associated with pasteurized milk was reported in Pennsylvania in July 2011, isolates from both food samples and clinical cases were indistinguishable by pulsed-field gel electrophoresis (Voorhees et al., 2011; Longenberger et al., 2014).

5.1.3.4 Vegetables and fruits

There are reports of yersiniosis human cases due to the ingestion of vegetable and fruit products (fresh salads, fresh juices, smoothies etc) that usually were prepared from fresh produce fertilized with organic material (e.g. farm animals' manure). The risks posed by pathogenic bacteria infestating fresh vegetable and fruit food products have increased in recent years, because of the increased numbers and wider availability of minimally processed vegetable products (wheat germ, bean sprouts, fresh salads) or cold-pressed fruit juices that are not thermally processed. Such products contain almost all the microorganisms existing on the raw materials used for their production and storage under refrigerated conditions is favouring growth of *Yersinia* (Zadernowska et al., 2014). *Y. enterocolitica* has been found in fresh and ready to eat green vegetables (Kansouzidou-Kanakoudi and Daneilides, 1994; Lee et al., 2004; Xanthopoulos et al., 2010; Losio et al., 2015).

Sakai et al. (2005) reported a foodborne outbreak of *Y. enterocolitica* O:8 in Japan where the same PFGE pattern was obtained from all the patient and mixed salad isolates. In March 2011 an investigation was carried out to study a geographically widespread outbreak involving 21 cases of *Y. enterocolitica* O:9 infection with similar profiles produced by MLVA (multiple-locus variable number tandem repeats analysis). In this outbreak the possible source of contamination was bagged salad mix containing "radicchio rosso" imported from Italy and as a result of the investigation, the products of a specific brand were withdrawn from the market (MacDonald et al., 2011).

5.1.3.5 Water and envrironment

Most of the *Y. enterocolitica* isolates recovered from environmental samples, including slaughterhouses, butcher shops, fodder, soil, and water have been non-pathogenic in nature. However, strains of bio-serotype 4/O:3 have occasionally been isolated from slaughterhouses, butcher shops, potable well-water and sewage water (Fredriksson-Ahomaa, 2007). *Y. enterocolitica* can survive for long periods in sterile distilled water, sterile river water and physiological saline. *Y. enterocolitica* can survive better in cold soil and clean surface waters than in warm water or soil. Non-pathogenic *Y. enterocolitica* serotypes survive longer in water and soil than pathogenic serotypes, the latter being rarely isolated from the environment, thus suggesting that the environment is not a significant reservoir for pathogenic strains. Nevertheless contaminated water can serve as a vehicle for pathogenic *Y. enterocolitica* infecting humans (Laukannen, 2010).

In a case-control study on sporadic cases of *Y. enterocolitica* O:3 in Norway between 1988 and 1990, patients were more likely than controls to report drinking untreated water during the 2 weeks preceding the onset of illness (Ostroff et al., 1994). Two *Yersinia* outbreaks have been associated with well water, the one occurring in Pennsylvania (USA) among members of a girl scout troop after eating bean sprouts grown in contaminated well water, the other was a familial outbreak of yersiniosis due to O:3 strain in Canada (Ostroff et al., 1994). Another case-control study in Washington state revealed samples of tofu, water supply and asymptomatic employees to be positive for *Y. enterocolitica* O:8 while inspection of the tofu plant revealed unsanitary conditions, including poor personal hygiene and unsanitary equipment. In Canada, an outbreak of *Y. enterocolitica* O:21, affecting three out of the four members of a family was due to drinking unboiled water obtained directly from the river beside the family's home (Fredriksson-Ahomaa, 2007).

Falcao et al. (2004) recently tested 67 *Y. enterocolitica* strains isolated in Brazil from untreated water for the presence of virulence genes. They found that all 38 strains of serotype O:5,27 possessed *inv*, *ail,* and *yst* genes, suggesting that water may be responsible for human infection with *Y. enterocolitica*. In another study, *Y. enterocolitica* O:8 strains have been isolated from stream water in Japan, which indicate that stream water may be a possible source for human *Y. enterocolitica* O:8 infections (Iwata et al., 2005).

5.2 Routes of trasmission

5.2.1 Foodborne-waterborne transmission

The most common transmission route of pathogenic *Y. enterocolitica* is the fecal–oral via contaminated food and water. More often transmission occurs through the consumption of undercooked or improperly handled cooked pork and other food products (Fredriksson-Ahomaa et al., 2006). Epidemiological investigations have supported the role of pork as a vehicle for *Y. enterocolitica* as well as case-control studies of sporadic cases. Recently application of new subtyping methods has confirmed that edible pig offal is an important source of *Y. enterocolitica* transmission between pigs and humans. Similar genotypes of strains that were isolated from humans and were discovered with MLVA (Virtanen et al., 2013).

As described in previous chapters (5.1.3 and 5.1.3.5) except pork, water and other food products have been involved in yersiniosis outbreaks and case-control studies.

However, pathogenic *Y. enterocolitica* have only infrequently been recovered from food samples. This might be explained by the lack of appropriate selective methodology for isolation of pathogenic strains. Therefore, new conventional and molecular methods have been designed to overcome the challenges regarding detection and subtyping of *Y. enterocolitica* in food (Fredriksson-Ahomaa and Korkeala, 2003; Petsios et al., 2016).

5.2.2 Direct transmission (human to human-animal to human)

Y. enterocolitica rarely causes extraintestinal disease and in such cases, direct inoculation is assumed to be the mode of transmission. A 55 year old patient who worked extensively with pork as a meat cutter and experienced frequent cut wounds on his hands developed axillary abscess due to *Y. enterocolitica* (Kelesidis et al., 2008). In 2009, a 54 year old construction worker reported an axillary abscess due to *Y. enterocolitica* contamination of a finger pustule which was tainted by *Y. enterocolitica* from an environmental source (Menzies, 2010).

Person to person transmission is not common, but Lee et al. (1990) reported *Y. enterocolitica* O:3 infections in infants who were probably exposed to infection by their caretakers who were handlers of pig intestines. This can happen when basic hygiene and hand-washing habits are inadequate or neglected. An outbreak of diarrheal disease as a result of *Y. enterocolitica* bio-serotype 1/O:5 was observed among hospitalized patients indicating a nosocomial outbreak (Sabina et al., 2011). In Japan in July 2006, person-to-person transmission was observed in a familial outbreak of *Y. enterocolitica* bio-serotype 2/O:9 the possible source being an infected carrier suffering from diarrhoea (Moriki et al., 2010).

A direct transmission from pigs to humans was suggested by elevated serum antibody concentrations found among people involved in swine breeding or pork production in Finland; slaughterhouse workers and pig farmers were observed to have elevated antibody levels to *Y. enterocolitica* O:3 twice as frequently as grain- or berry farmers or randomly selected blood donors. Similar differences were also discovered between people involved in swine slaughtering practices and office personnel in Norway (Fredriksson-Ahomaa, 2001).

Feces and intestinal tracts of different animals including rodents (mice and rabbits), domestic animals such as cattle, cats, sheep, pigs, and dogs as well as horses, raccoons, and deer have been incriminated in animal to human infections (Sabina et al., 2011).

Pathogenic *Y. enterocolitica* may be transmitted to humans indirectly from pork and offal via dogs and cats (Fredriksson-Ahomaa et al., 2001b).Transmission of *Y. enterocolitica* 4/O:3 to pets via contaminated pork has been studied using PFGE in Finland. Cats and dogs what were fed raw pork carried in their faeces the same PFGE genotype which was recovered also in raw pork samples. These results show that raw pork should not be given to pets because pathogenic isolates can easily be transmitted from highly contaminated raw pork to pets. Dogs and cats may be an important transmission path of pathogenic *Y. enterocolitica* between pigs and young children (Fredriksson-Ahomaa et al., 2006; Wang et al., 2010).

5.2.3 Blood transfusion-associated transmission

Indirect person-to-person transmission has occurred in several instances by transfusion of contaminated blood products. In 1975, the first incident of transfusion-associated sepsis due to *Y. enterocolitica* was evidenced in Netherlands (Sabina et al., 2011). Over the years, over 60 incidents have been reported globally. Y. enterocolitica has been found in donor blood from healthy individuals or donors who have suffered diarrhea in the past. Such contaminated blood sometimes caused *Yersinia* bacteremia and death of the recipients (Jacobs et al., 1989). Although fatality due to post transfusion bacterial-associated sepsis is rare (Tipple et al., 1990), blood-transfusion associated septicemia due to *Y. enterocolitica* is reported to have high fatality rate. In 2003, a 71-year old patient with refractory anemia underwent fatal septic shock after transfusion of contaminated red blood cells (RBCs), *Y. enterocolitica* bio-serotype 4/O:3 was found in both the transfused RBCs and the patient's blood sample (Sabina et al., 2011). The investigation showed high titers of antibodies against *Y. enterocolitica* in the plasma sample of the donor one month after donation. Although the donor had complained of abdominal discomfort 3.5 months prior to the collection of blood, symptoms of intestinal infections were not been recorded during donation (Leclercq et al., 2005). Furthermore, the potential growth of virulent *Y. enterocolitica* strains in blood units increase the severity of blood transfusion-associated transmission with fatalities occurring in severe cases (Guinet, F.Carniel, & Leclercq, 2011).

6 Pathogenesis

6.1 Virulence factors

The virulence determinants in *Y. enterocolitica* are positioned within the chromosome as well as on a 70 kb virulence plasmid termed pYV (plasmid for *Yersinia* virulence), that is only detectable in virulent strains. Hence, in ensuring the achievement of the temperature gradient linked to infection onset in pathogenesis, *Y. enterocolitica* bacteria utilize both chromosomal and plasmid-associated (pYV+) virulence factors that are temperature-dependent. The presence of pYV enables virulent *Yersinia* strains to survive and multiply in lymphoid tissues of their host (Fredriksson-Ahomaa, 2007).

6.1.1. Chromosome-linked virulence gene products

6.1.1.1 Invasin (inv)

Y. *enterocolitica* invasin protein, is an important factor that plays significant role in facilitating the entry of the bacterium into the epithelial cells (Fabrega and Vila, 2012). This protein facilitates *Y. enterocolitica* to attach and enter the cells of the host (Atkinson, & Williams, 2016). Invasion stimulates internalization into the small intestine epithelial cell by binding to the target host cells identified as β1-integrins, which are available on the surface of the host cell. Upon invasion binding, integrins form clusters that result in the rearrangement of the host cell cytoskeleton, hence the promotion of phagocytosis and eventually internalization of *Y. enterocolitica* microorganism into the epithelial cells (Atkinson, & Williams, 2016). The invasin gene is found in all *Yersinia* spp., however, nonpathogenic strains lack functional *inv* homologous sequences (Fredriksson-Ahomaa, 2007).

Invasin expression in *Y. enterocolitica* is regulated by both temperature and pH. This invasion genetic factor is expressed at 26°C, but peaks in late exponential or early stationary stage with limited expression levels evidenced at 37°C. Although invasin is required for infection at 37°C, it has been found that expression of invasin at 37°C is restored to levels observed at 26°C when the pH was reduced to 5.5 (Atkinson, & Williams, 2016).

6.1.1.2 Attachment invasion locus (ail)

The *ail* locus is chromosomally positioned and codes a 17 kDa surface-associated protein that is regulated thermally through maximum expressions at 37°C (Atkinson, & Williams, 2016). Ail, is greatly associated with virulence due to the fact that it has been

evidenced only among pathogenic *Yersinia* strains (Fredriksson-Ahomaa, 2007). *Ail* plays a part in both adhesion and invasion of specific tissue culture cells and is involved in the survival against the bactericidal effects of serum (Fabrega and Vila, 2012). As with invasin, *Ail*-mediated adhesion to the cells of the host ensures that the delivery of Yops (*Yersinia* outer proteins) is efficient and rapid efficient (Atkinson, & Williams, 2016).

6.1.1.3 Iron acquisition (foxA)

The growth and survival of this pathogen require ferric ion uptake. In *Y. enterocolitica* infections iron supply together with the production of siderophore transport system is critical. Strains of high-pathogenicity biotype 1B/O:8, capture iron by secreting small molecules called siderophores that include *yersinia*-bactin (catechol-type) that is usually encoded by a high pathogenicity island (HIP) (Fredriksson-Ahomaa, 2007).

The other low pathogenic serotypes such as O:3 and O:9 do not produce siderophore but take up ferric ion through the utilization of ectogenic siderophores that include ferrioxamin E and ferrioxamin B (Huang et al., 2010), which are commonly used to treat patients with iron overload (Fredriksson-Ahomaa, 2007). Huang et al. (2010), highlights that a 77 kDa ferrioxamin (*foxA*) receptor on the outer membrane of *Y. enterocolitica* integrates with ferrioxamin to enhance the rapid uptake of ferric ion. The *foxA* is regarded as effective due to its existence in all strains of *Y. enterocolitica.*

6.1.1.4 Yersinia stable toxin (Yst)

Y. enterocolitica produces an enterotoxin, *Yst*, which is heat-stable, structurally and functionally homologous to the heat-stable enterotoxin (ST) of enterotoxigenic *Escherichia coli*, and is encoded by the chromosomal *yst* gene. *Yst* is involved in diarrhea. Three subtypes of *Yst* exist: *Yst-a*, *Yst-b*, and *Yst-c* (Bhunia, 2008). Nevertheless, the contribution of the toxin in a diarrheal disease remains significantly debatable due to issues that relate to the fact that *Yst* is not detectable in diarrheal stool samples during experimental animal models after infection with *Y. enterocolitica* (Fabrega, & Vila, 2012). Furthermore, nonpathogenic strains and strains of related species have been found to produce *Yst* (Fredriksson-Ahomaa, 2007). However, the non-invasive biotype 1A strains causing diarrhea often transport a variant of the *Yst* gene that could possibly be the only virulence factor accounting for the diarrheal illness.

6.1.1.5 Myf fibrialle

Y. enterocolitica synthesizes a fibrillar structure known as *Myf* (mucoid *Yersinia* factor). The chromosomal locus involves three genes: *myfA*, *myfB*, and *myfC*. *Myf* may serve as an intestinal colonization factor for *Y. enterocolitica* (Fredriksson-Ahomaa, 2007). *Myf* appear like CS3, which is an important colonization factor of various human clinical strains of enterotoxigenic *Escherichia coli*. Experiments implemented with *Y. pseudotuberculosis* associate a role in thermoinducible binding and haemagglutination to the myf homolog (the psa locus). However, in *Y. enterocolitica* the *myf* operon is not able to mediate haemagglutination and further experiments regarding its adhesive function and its possible role in pathogenesis have to be performed (Fabrega and Vila, 2012).

6.1.1.6 Pathogenicity islands

Another set of chromosomal associated virulence genes is one that is encoded within the pathogenicity island regularly identified as High-Pathogenicity Island (HPI) and is present in *Y. enterocolitica* biotype 1B only. A significant percentage of the genes positioned on this specific island is involved in the transport, biosynthesis, and regulation of the siderophore *yersinia*-bactin (Fabrega and Vila, 2012). Hence, the HPI could be considered as an iron-capture island. The involved locus is made of 11 genes arranged into four operons (fyuA, irp2, ybtA, and ybtP) that could be grouped into three functional groups: transportation into the bacterial cell (outer membrane receptor and transporters), *Yersinia*-bactin biosynthesis and regulation. Existing evidence highlights that *Yersinia* bactin could up-regulate its own expression together with that of fyuA that is its outer membrane receptor (Fabrega and Vila, 2012).

6.1.2 Plasmid (pVY)-linked virulence gene products

6.1.2.1 Yersinia adhesion protein (yadA)

Yersinia adhesion protein (*YadA*) is a pYV plasmid-encoded protein that facilitates adherence to host epithelial cells, as well as protecting *Yersinia* from the nonspecific immune system such as phagocytosis and complement-mediated cell lysis. *YadA* also promotes bacterial internalization by interacting with the epithelial β1-integrin proteins (Eltahir and Skurnik, 2001; Bhunia, 2008). *YadA* protein forms a fibrillar matrix on the cell surface and is correlated closely with the phenomenon of autoagglutination that occurs after growth in tissue culture medium usually at 37°C (Feng and Weagant, 1994; Fabrega and Vila, 2012).

YadA expression is achieved at 37°C but not at 25°C and is regulated by two different gene products: *VirF* (virulence) and *LcrV* (low calcium response). *VirF* senses the optimal temperature (37°C) required for protein synthesis, while *LcrV* regulates the *yadA* expression depending on the availability of extracellular calcium concentration. Furthermore, *yadA* expression is not affected by pH, salt or sugar concentration (Bhunia, 2008).

6.1.2.2 Yersinia outer membrane proteins (YOPs)

The pYV codes also a set of highly regulated secreted proteins, called *Yops* (*Yersinia* outer proteins) that are responsible for bacterial adhesion and the type III secretion system (TTSS), called *Ysc*. *Yops* protect *Yersinia* from the macrophage by destroying its phagocytic and signaling capacities, and finally, inducing apoptosis. Through TTSS apparatus *Yops* are delivered from extracellularly located *Yersinia* that is in close contact with the eukaryotic cell into the cytosol of the target cell. As with *yadA*, *yop* and *ysc* expression is temperature and calcium regulated, being expressed maximally at 37°C in response to the presence of a low calcium concentration (Fredriksson-Ahomaa, 2007; Bhunia, 2008).

YopB is a major outer membrane protein allowing *Y. enterocolitica* to evade an ensuing immune defense after ingestion which suppresses the production of a macrophage-derived cytokine, tumor necrosis factor alpha (TNF-a), known to play a central role in the regulation of cellular immunity and inflammatory responses to infection (Bottone, 1997). The TTSS delivers six effector proteins into the host cell cytosol: *YopH*, *YopO*, *YopT*, *YopP/J*, *YopE* and *YopM*. These effector proteins affect signaling events, alter actin cytoskeletal structure to induce bacterial entry, phagocytosis, apoptosis, and inflammatory response. The process of phagocytosis involves actin rearrangement to form pseudopods for bacterial internalization (Bhunia, 2008). *YopH* targets a significant group of eukaryotic cell signaling components, RhoA family of small GTPases which regulate actin cytoskeleton formation required for phagocytosis. Also inhibits autophagy following binding of invasin or *YadA* to β1-integrins. *YopO* links with RhoA family proteins enabling the inhibition of phagocytosis through binding and phosphorylating actin, which is utilized by *Y. enterocolitica* as bait for the removal of host regulators that control actin polymerization (Atkinson, & Williams, 2016). *YopT* has also Rho GTPase activity. *YopP/J* blocks inflammation and induce apoptosis in macrophages. *YopE* has cytotoxic action and regulates inflammatory response while *YopM* is a protein with kinase activity with possible role in cellular signaling events (Bhunia, 2008).

6.2 Pathogenic mechanism

Y. enterocolitica after ingestion travels through the stomach to the small intestine and the primary site of infection is terminal ileum and proximal colon. Entrapped in intestinal mucus the bacteria use chromosomally encoded virulence gene products to colonize the intestine until the temperature adaptation to 37°C when the expression of pYV-encoded gene products is initiated (Bhunia, 2008). Bacteria bind to the intestinal mucosa, through M cells using the chromosomal determinants, *inv* and *ail* and plasmid determinant *yadA* and then colonize the Peyer's patches which are part of the gut associated lymphoid tissue (Fredriksson-Ahomaa, 2007). *Inv* binds to β1-integrin receptor located abundantly on the M-cells on the luminal side. Augmenting its role in invasion and spread, the *ail* protein also confers serum resistance to *Y. enterocolitica* (Fredriksson-Ahomaa, 2007; Bhunia, 2008). Binding to the β1-integrin as well as the collagen, fibronectin, and laminin *yadA* also aids in the invasion. From the M-cells engulfed bacteria are released in the basal layer in the lamina propria, multiply within the lymphoid follicle, and cause necrosis and abscess in Peyer's patches. From Peyer's patches, bacteria spread to regional mesenteric lymph nodes causing characteristic lymphadenitis. The three components of the enterotoxin *yst* stimulate the membrane bound guanylate cyclase and increase accumulation and activation of intracellular cyclic guanosine monophosphate (cGMP), followed by an activation of cGMP-dependent protein kinase, which lead to inhibition of Na^+ absorption and stimulation of Cl^- secretion (Bhunia, 2008). Antiphagocytic properties are mainly mediated by *yop* virulon products which enable survival and extracellular multiplication in host lymphoid tissue (Fredriksson-Ahomaa, 2007).

Usually the infection is limited to the intestinal area, but through the mesenteric lymph nodes it can disseminate to liver, spleen and lungs, and survive by resisting phagocytosis by macrophages and polymorphonuclear leukocytes such as neutrophils. The ability to survive and multiply within the lymphoid follicles and other tissue is associated with the presence of virulence plasmids, which are essential for the pathogenesis of *Yersinia*. The delivery of *YOPs* to the macrophages by TTSS blocks phagocytosis and suppresses the immune system thereby ensuring bacterial survival and extracellular multiplication in the host lymphoid tissue (Bottone, 1999; Fredriksson-Ahomaa, 2007; Bhunia, 2008).

6.3 Possible pathogenicity of Y. enterocolitica 1A

Biotype 1A strains have been traditionally considered nonpathogenic due to the lack of virulence-associated factors of pYV-bearing strains. However, the presence of virulence gene *ail* has been documented in these strains (Tennant et al., 2003; Kraushaar et al., 2011). Most of *Y. enterocolitica* biotype 1A strains contain the *ystB* gene, and in experimental conditions, some excrete heat-stable *ystB* enterotoxin at 37°C that is equivalent to those present in the ileum (Singh and Virdi, 2004). Furthermore, these strains have been isolated from patients with gastroenteritis and were reported as the causative agents in outbreaks. Tennant et al. (2003), have suggested that *Y. enterocolitica* biotype 1A may harbor a pathogenic "clinical" subgroup that cannot be readily identified because they lack the well-known virulence determinants of classical pathogenic bio-serotypes. Some strains have been reported of being capable to invade epithelial cells in vitro (Grant et al. 1999) and multiply inside of macrophages, with TNF-a release leading to migration of more macrophages to the site of infection (McNally et al. 2006).

Huovinen et al. (2010) described that the symptoms of the patients with biotype 1A differed from yersiniosis caused by the classic pathogenic bio-serotypes. In addition, the patients with 1A had more protracted gastrointestinal disorders and unspecific complaints suggesting that the original cause may have been other than biotype 1A strains. Nevertheless, there is a possibility of subgroups that can cause a disease within the highly heterogeneous group of *Y. enterocolitica* biotype 1A (Huovinen et al., 2010). Sihvonen et al. (2012) has supported that hypothesis and suggested that in order to understand the virulence mechanisms of these strains more research is needed.

6.4 Clinical diseases

Gastrointestinal infection may manifest itself as an enteritis, enterocolitis, acute mesenteric lymphadentis, and terminal ileitis often mimicking appendicitis ranging from self-limiting enteritis to fatal systematic infection (Table 8). The clinical manifestations of the infection are associated to some extend with the age and physical state of the host, the presence or absence of underlying medical conditions, and the serotype of the invading strain. In children, acute enteritis with fever, inflammatory and occasionally bloody, watery diarrhoea, is the commonest manifestation. Septicaemia may be a concurrent rare

finding in infants with serogroup O:3 infection but may also occur with other serogroups of *Y. enterocolitica*. In young adults, acute terminal ileitis and mesenteric lymphadenitis mimicking appendicitis appear to be a commoner clinical syndrome. Occasionally severe symptoms may develop, such as diffuse ulceration and inflammation of the large and small bowel, peritonitis, toxic megacolon, intestinal perforation, ileocolic intussusception, and mesenteric vein thrombosis leading to intestinal necrosis and cholangitis. Also mild hepatitis and pancreatitis may be symptoms of yersiniosis. *Yersinia* infection may also be asymptomatic and pass unnoticed. Infection with O:8 may include severe symptoms, as extensive ulceration of the gastrointestinal tract and death, whereas symptoms with O:3 and O:9 infections are less destructive. The incubation period ranges for 1 to 11 days. The illness is generally mild and self-limiting persisting for 5–14 d, although diarrhoea may persist several weeks, occasionally even for several months. Excretion of the organism in the stools continues for an average of 4 weeks after cessation of symptoms, which may contribute to intra-familial spread (Bottone, 1999; Fredriksson-Ahomaa, 2007).

Less commonly, *Y. enterocolitica* may spread at extra-intestinal sites with or without bacteremia causing metastatic infection that involves pneumonia, lung abscesses, empyema liver, splenic or renal abscesses, osteomyelitis, septic arthritis, meningitis endocarditis, leading to a mycotic aneurysm (Fredriksson-Ahomaa, 2007). Septicemia caused by *Y. enterocolitica* may occur in immunocompetent, immunosuppressed hosts, and in those with an underlying disease, such as diabetes mellitus and hepatic cirrhosis. More susceptible are the patients in iron overload or those being treated with the iron-chelating agent desferrioxamine (Bottone, 1999; Fredriksson-Ahomaa, 2007). While serogroup O:8 strains produces and secretes its own iron chelator (*Yersinia*-bactin/yersiniophore) which may enhance its virulence, serogroups O:3, O:5,27, and O:9 strains do not produce an iron-sequestering siderophore but rather rely on an exogenous source of an iron chelator such as deferrioxamine. Acquisition of the infecting strain may be via the oral route or associated with blood transfusion (Bottone, 1999).

Infection with *Y. enterocolitica*, especially serogroups O:3 and O:9, often leads to secondary immunologically induced sequelae such as arthritis (most common), erythema nodosum, Reiter's syndrome, glomerulonephritis and myocarditis (Bottone, 1999). Post-infection complications usually develop within 1 week to 1 month after initial infection and may sometimes be the only obvious clinical manifestation of *Yersinia* infection (Fredriksson-Ahomaa, 2007). Eighty percent of the patients experiencing post-*Yersinia*l

reactive arthritis are HLA-B27-positive and predominantly young and middle-aged (Bottone, 1999; Fredriksson-Ahomaa, 2007).The patients may suffer from arthritis, after 1–3 weeks of infection with or without gastroenteritis (Fredriksson-Ahomaa, 2007). Joint symptoms vary from mild arthralgia to severe arthritis. The arthritis is typically an asymmetric oligo- or polyarthritis with sudden onset affecting more often the joints of the lower extremities, such as knee, ankle, and toes, but sometimes also wrist and fingers. Patients with *Yersinia*-triggered arthritis show persistent IgA antibodies against *Yersinia* outer membrane proteins, which is likely the result of chronic stimulation of the gut associated lymphoid tissue by persistent *Yersinia* (Bottone, 1999; Fredriksson-Ahomaa, 2007).

Table 8. Clinical spectrum of *Y. enterocolitica* infections (Bottone, 1999).

Gastrointestinal	Enterocolitis: predominantly in young children; concomitant bacteremia may also be present in infants Pseudoappendicitis syndrome (children older than 5 years; adults) Acute mesenteric lymphadenitis Terminal ileitis
Septicemia	Especially in immunosuppressed individuals and those in iron overload or being treated with deferrioxamine Transfusion related (usually leads to septic shock syndrome)
Metastatic	Focal abscesses: liver, kidney, spleen, lungs Cutaneous manifestations: cellulitis, pyomyositis, pustules and bullous lesions Pneumonia, cavitary pneumonia Meningitis Panophthalmitis Endocarditis, infected mycotic aneurysm Osteomyelitis
Post-infection sequelae	Arthritis (associated with HLA-B-27) Myocarditis Glomerulonephritis Erythema nodosum
Pharyngitis (common after oral ingestion of *Y. enterocolitica*)	

7 Antimicrobial Resistance

The non-prudent use of antimicrobials in veterinary and medical practice selects for resistant bacteria. In animal husbandry, antimicrobials are used for the treatment and prevention of infectious disease and for growth promotion. Although *Y. enterocolitica* infections are usually self-limiting and do not require antimicrobial therapy, antibiotic intervention is required in case of immunocompromised patients or those suffering septicaemia and the current WHO recommendations include tetracycline, chloramphenicol, gentamycin and co-trimoxazole (Bolton et al., 2013).

Y. enterocolitica produces frequently β-lactamases and is usually resistant to β-lactamase-sensitive penicillins, such as ampicillin, cloxacillin, carbenicillin and ticarcillin, the first-generation narrow-spectrum cephalosporins, erythromycin, clidamycin, and vancomycin. However, it is usually sensitive to aminoglycosides (gentamicin, streptomycin, tobramycin, and kanamycin), the third-generation cephalosporins (ceftriaxone, caftazidime, and cefotaxime), co-trimoxazole, tetracyclines, chloramphenicol, fluoroquinolones (ciprofloxacin, norfloxacin, and ofloxacin), trimethoprim–sulfamethoxazole, imipenem, and aztreonam (Fredriksson-Ahomaa, 2007).

Resistance of *Y.enterocolitica* against penicillins (ampicillin, carbenicillin, ticarcillin) and cephalosporins (cephalothin) is primarily due to β-lactamase A (*blaA*). Most *Y.enterocolitica* strains harbor chromosomal genes for two β-lactamases *blaA*, a constitutively expressed Ambler class A penicillinase and *blaB*, an Ambler class C inducible cephalosporinase. The most common mode of microbial resistance to β-lactams is their enzymatic hydrolysis by β-lactamases (Singhal et al., 2018). Over time, β-lactamases have expanded their substrate spectrum with simple mutations in the gene or in the genetic environment of β-lactamases. Modifications in the regulatory regions, mutations in the promoter sequences and integration of insertion sequences containing efficient promoters have frequently been associated with high-level expression of chromosomal β-lactamases (Singhal et al., 2014). A very successful strategy to overcome β-lactamase mediated resistance and restore the efficacy of β-lactams has been to use inhibitors of β-lactamases like clavulanic acid, sulbactam and tazobactam. The β-lactam antibiotic/inhibitor combination, amoxicillin-clavulanate (AMC) is one of the most commonly used antimicrobials, for which an increase in resistance has been noticed in recent years due to the acquisition of point mutations in β-lactamases (Singhal et al., 2018).

Regarding β-lactam susceptibility notable differences are reported among different *Y. enterocolitica* strains bio-serotypes. Recent studies showed that strains of *Y.enterocolitica* bio-serotype 4/O:3 are sensitive to amoxicillin-clavulanate while bio-serotypes 2/O: 5, 27 and 2/O:9 are resistant. Most strains of bio-serotype 4/O:3 are sensitive to amoxicillin/clavulanate and to third-generation cephalosporins, and resistant to ampicillin, carbenicillin, ticarcillin, and cephalothin (Fredriksson-Ahomaa, 2007; Singhal et al., 2014). In comparison, most strains of biotype 2 and biotype 3 are found to be more susceptible to both carbenicillin and ticarcillin (lacking the enzyme A) and resistant to amoxicillin-clavulanic acid (especially bio-serotypes 2/O:5, 27 and 2/O:9) (Fredriksson-Ahomaa, 2007; Singhal et al., 2014). Strains of biotype 1B exhibit high rates of suscebtibility to ampicillin and amoxicillin-clavulanic acid and resistance to carbenicillin, ticarcillin, and cephalothin, whereas strains of biotype 1A have shown to be resistant to amoxicillin-clavulanic acid (Fredriksson-Ahomaa, 2007).

Emerging resistant phenotypes of *Y. enterocolitica* have been recognized and this is attributed to the antimicrobial agents extensively used for therapy, prophylaxis and growth promotion in modern food-animal production. Almost all *Y. enterocolitica* strains from pigs examined in Denmark were resistant to ampicillin but susceptible to streptomycin and sulphonamides. However, a limited number of *Y. enterocolitica* strains were resistant to nalidixic acid and a single isolate was resistant to gentamicin and spectinomycin (Fredriksson-Ahomaa, 2007). Also, clinical isolates resistant to chloramphenicol, streptomycin, sulfonamides, co-trimoxazole, and nalidixic acid have been reported (Fredriksson-Ahomaa, 2007). Sanchez et al. (2003) have reported the emergence of nalidixic acid-resistant clinical *Y. enterocolitica* strains around Madrid in Spain. Similar trend of increase of nalidixic acid resistance in *Y. enterocolitica* O:3 clinical isolates was observed in Zaragoza (Capilla et al., 2003). Fluoroquinolones have been ranked as the second most widely used antimicrobial agent both in Spanish hospitals and the community. This extensive usage, together with the use of antibiotics in animal feed, may explain the increase in the resistance to quinolones in *Y. enterocolitica* clinical isolates. Sanchez et al. (2003) suggest the clonal dissemination of a nalidixic acid susceptible *Y. enterocolitica* strain which has acquired different mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene**,** generating resistance to nalidixic acid and which has probably emerged due to the selective pressure exerted by the overuse of fluoroquinolones.

Another study in Barcelona showed increase of resistance rate up to 90% for streptomycin and sulfonamides, 70% for trimethoprim-sulfamethoxazole, 60% for chloramphenicol, and 5% for nalidixic acid among clinical *Y. enterocolitica* O:3 strains. A study on slaughter swine strains in the United States reported resistance to oxytetracycline and sulfonamides (except trimethoprim–sulfamethoxazole) as well. Oxytetracycline resistance is often plasmid mediated and could be impacted by on-farm antibiotic usage. Tetracyclines and sulphonamides are commonly added to swine feeds for growth promotion and prevention of infectious diseases in swine (Fredriksson-Ahomaa, 2007).

8 Control and Prevention of *Yersinia enterocolitica*

8.1 Methods for controlling spread in pig farms

Virtanen et al. (2012) showed that piglets from certain breeding farms transmit *Y. enterocolitica* strains into a fattening farm and the infection subsequently spreads throughout the whole unit. In order to reduce the prevalence of enteropathogenic *Yersinia* on pig farms, mixing piglets from *Y. enterocolitica*-positive farms with piglets negative for *Y. enterocolitica* should be avoided and prevention methods should be targeted at piglet production units. This principle is in agreement with a previous study by Skjerve et. al (1998). Incoming piglets, possibly infected on their original breeding farm, are a source of infection for other piglets coming from other farms (Virtanen et al., 2012). Taking that into account, the most effective method to prevent *Y. enterocolitica* in fattening pig farms is to get piglets from asserted *Y. enterocolitica* negative breeding farms. The all in/all-out strategy appears to be effective in reducing the on-farm carriage of *Y. enterocolitica* (Laukkanen-Ninios, 2014). Danish experience has shown that strategic removal of infected weaners might lead to *Y. enterocolitica* O:3 negative slaughter animals (Skjerve et. Al, 1998). However, since pathogenic *Y. enterocolitica* is very common in pig production, economically feasible practices to produce enteropathogenic *Yersinia*-free breeding are needed (Laukkanen-Ninios, 2014). The possible risk factors for infection with *Yersinia* spp. during the whole rearing period include the use of a semi-slatted or fully slatted floor in the fattening pig unit, the presence of other pig farms in the area, the number of piglet suppliers and snout contact between pens (Vanantwerpen et al., 2017).

8.2 Methods for controlling spread from slaughterhouse to food

Since the pig is a major source of sporadic human infections with pathogenic bio-serotype 4/O:3 in Europe, control measures have been focused on reducing the contamination of carcasses during slaughter since *Y. enterocolitica* 4/O:3 is commonly found in slaughtered pigs, particularly in tonsils and lymph nodes. In the slaughter process, dehairing, polishing, evisceration, and splitting of the carcass have been identified as process stages that contaminate the carcasses (Laukkanen-Ninios et al., 2014). During evisceration, the tonsils are removed in conjunction with the pluck set

(tongue, oesophagus, trachea, heart, lungs, diaphragm, liver and kidneys) and thus the spread of *Y. enterocolitica* 4/O:3 from the highly contaminated tonsils to the offal is unavoidable especially when they hang together on a hook. Contaminated pig tongues, hearts, livers and kidneys have shown to be an important transmission vehicle of this bacterium from pigs to man (Fredriksson-Ahomaa and Korkeala, 2003). In addition the traditional meat inspection procedures such as submaxillary lymph node excision to detect tuberculosis can spread pathogenic *Y. enterocolitica* on the carcasses. In EU countries, the meat inspection has recently been modified. Palpation and incisions in routine meat inspection were omitted to reduce the contamination of carcasses and other edible parts (Laukkanen-Ninios et al., 2014).

The prevalence of enteropathogenic *Yersinia* can be reduced, but not completely removed, by slaughter hygiene, bagging of the rectum, removal of tonsils and tongue along with the head, and by other carcass decontamination methods. The 'bagging technique', the method of enclosing the anus and rectum of pigs in a plastic bag during slaughter, has been introduced in several slaughterhouses, mainly in Norway, and it has been shown that this effectively reduced carcass contamination by *Y. enterocolitica*. Bagging of the rectum with a plastic bag has been shown to reduce carcass contamination with pathogenic *Y. enterocolitica* due to intestinal carriage significantly (Nesbakken et al., 1994; Laukennen et al., 2010b; Laukkanen-Ninios et al., 2014). Although the effect was significant in the studies, the contamination rate of carcasses after bagging remained relatively high. Head and chest area of the carcass are likely to be contaminated by tonsils. Therefore, prevalence of pathogenic *Y. enterocolitica* 4/O:3 remained relatively high, particularly in chest and head samples, even when bagging of the rectum was applied. The reduction of the *Y. enterocolitica* 4/O:3 prevalence is achieved by removal of the head, with tongue and tonsils prior to evisceration. No effect of the chilling and blast-chilling processes on pathogenic *Y. enterocolitica* on pig carcasses has been reported (Laukkanen-Ninios et al., 2014).

Y. enterocolitica can survive in food and grow at refrigerator temperatures but is easily killed during heating. Since most of the yersiniosis cases are sporadic, good hygienic practices during cooking and preparation when handling pork in domestic and professional kitchens can reduce the risk of food contamination. Cross-contamination can be avoided by separating raw pork from cooked or ready-to-eat foods and also by washing food handlers' hands, cutting boards, and contaminated surfaces. Consumers should avoid

the consumption of raw or undercooked pork, raw milk, or related by products, and washing hands carefully before eating, after contact with animals, and after handling raw meat. After handling raw chitterlings, it is recommended that hands and fingernails be cleaned thoroughly with soap and water before touching children or their toys, bottles, or pacifiers (Cosano and Gimeno, 2003).

B. SPECIAL PART

1 Aims of the study

The objectives of the present study included the following:

1. investigation of the prevalence of pathogenic *Y. enterocolitica* in pork carcasses and fresh vegetables originating from local slaughterhouses and markets of the Epirus region (Northwestern Greece).

2. comparison of different methods for the recovery of human pathogenic *Y. enterocolitica* from pork carcasses.

3. identification of the isolated strains in order to assess:

- the pathogenic nature of the strains (biotype, serotype and virulence properties/markers)
- the presence of genotypic virulence markers in the recovered isolates.
- the antimicrobial susceptibility against antimicrobials routinely used in the medical practice.

4. investigation of potential existence of *Inv* gene sequence pattern between the isolated pathogenic and non-pathogenic strains.

2 Materials and Methods

2.1 Sample collection

2.1.1 Pork carcasses

A total of 145 carcass surface swabs from equal number of pigs at slaughter were gathered during a period of 12 months from August 2013 to July 2014. The samples were collected randomly throughout the year from six slaughterhouses located in the Epirus region. The carcass surface swabs were collected using sponges (18-oz. Whirl-Pak® Speci-Sponge® Environmental Surface Sampling Bag; USA), which were hydrated with 25 ml peptone broth supplemented with 1% sorbitol and 0.15% bile salts (PSB; Biolife Italiana; Italy). All samples were collected after evisceration, but before chilling, wearing sterile gloves while wiping with the sponge to prevent any cross-contamination of the carcasses. From each carcass the swabs were taken from the hind limb and from a lateral area of approximately 100 cm^2 according to the appropriate International Standard Methods protocol (ISO 17604; 2003a). The samples were immediately transported to the laboratory into portable coolers and processed within 4 h after collection.

2.1.2 Vegetables

A total of 144 fresh vegetable samples were collected from April 2013 to March 2014. Samples concluded 22 organic and 22 non-organic curly salads, 37 organic and 37 lettuce salads and 26 ‘ready to eat’ (RTE) salads purchased from local retailer shops and supermarkets. RTE salads contained lettuce (three types), carrot, rocket, cabbage, radicchio, parsley, dill, spring onion and curly salad and their labeled shelf-life twas seven days. The salads were transferred to the laboratory under refrigeration and were analyzed immediately.

2.2 Sample examination

2.2.1 Methods

2.2.1.1. Culture and isolation

For the pork samples each bag with the swab sponge was homogenized in a stomacher (Bag Mixer Interscience; Worthing West Sussex; United Kingdom) for 1 min just before starting the analysis. For the vegetable samples, 25 g of the sample was weighed aseptically and homogenized in 225 ml of peptone, sorbitol and bile salts (PSB) broth for 1 min. All

samples were analyzed for pathogenic *Y. enterocolitica* using (i) direct plating, (ii) selective enrichment and (iii) cold enrichment as described previously (Van Damme et al., 2013a).

(i) For direct plating, 500 µl of PSB homogenate was spread plated onto a cefsulodin–irgasan–novobiocin (CIN) *Yersinia*-selective agar plate in duplicate. For vegetable samples, a CIN agar plate was inoculated with 100 µl of PSB homogenate.

(ii) For selective enrichment, 10 ml of PSB from carcass samples and homogenate from vegetable samples was transferred into 90 ml of irgasan–ticarcillin–potassium chlorate (ITC) broth (Biolife Italiana; Italy) following ISO 10273 (ISO, 2003b). After 2 days enrichment at 25°C, a loopful (10 µl) was streaked onto a CIN agar plate. Additionally, 0.5 ml of the enriched PSB culture was transferred into 4.5 ml of 0.5% KOH in 0.5% NaCl solution for 20s, after which 100 µl was streaked onto a CIN agar plate.

(iii) For cold enrichment, the remaining PSB homogenate was incubated at 4°C for 14 days. On day 7 and 14, a loopful of enriched culture was streaked onto a CIN agar plate. Additionally, after alkali treatment of both 7 and 14-day enrichment (0.5 ml of ITC culture mixed with 4.5 ml 0.5% KOH in 0.5% NaCl solution for 20s), 100 µl was streaked on another CIN agar plate.

All CIN agar plates were incubated at 30°C for 24-48 h and examined each day for `bull's-eye' colonies. From each CIN agar plate, at least one presumptive *Yersinia* colony was selected and streaked onto nutrient agar for further identification. Nutrient plates were incubated at 30 °C for 24 h. The plates were checked for contamination and mixed cultures and in such case the colonies were streaked again on to CIN agar.

2.2.1.2. Detection and identification

Presumptive positive isolates were tested for biochemical characteristics using oxidase test strips (Merck), Christensen's urea agar (LabM) and Kligler Iron Agar (Biolife) which were incubated at 30°C for 24-48 h. Oxidase-negative, urease-positive and lactose-negative isolates were further tested for sucrose and rhamnose fermentation at 25°C for 48 h. Sucrose-positive and rhamnose-negative colonies were picked for species identification using the API 20E microsubstrate system (bioMerieux) incubated at 28°C for 24-48 h. The temperature of 28°C was chosen due to negative Voges-Proskauer reaction of *Y. enterocolitica* at 37°C as recommended from the manufacturer. Isolates were subcultured no more than 3-5 times to prevent plasmid loss due to repeated subculturing and stored at -70 °C in 30% glycerol before biotyping, serotyping, and genotyping.

2.2.1.3. Biotyping and Serotyping

Y. enterocolitica strains were biotyped according to the modified Wauters' scheme (1987). Indole and Voges-Proskauer test results were obtained from API 20E. Pyrazinamidase reaction was tested in tubes. Bacterial mass was streaked on a sloping surface and the tubes were incubated at 30°C for 48 h. After incubation 1ml of ammoniumferrosulfate broth was pipetted in the tube. PYZ positive tubes change the surface of the agar to brownish red. Esculine hydrolysis was tested by streaking bacterial mass on esculine plate as a line and incubation at 30°C for 24 h. Positive reaction was indicated by a black halo around the colonies. Salicine, xylose and trehalose were tested in tubes incubated at 30°C for 24-72 h. Positive tubes turn to yellow. Lipase production (tween 80 or egg yolk agar) was tested by streaking bacterial mass on plates as a line and and incubation at 30°C for 48 h. Positive reaction was indicated by oily, iridescent, pearl-like colony surrounded by outer clearing zone.

Serotyping was performed by slide agglutination with commercially available O antisera for the serogroups O:1,2, O:3, O:5, O:8 and O:9 (Denka Seiken; Japan). Autogglutination was tested with 0.9% NaCl before using antisera. If the strains agglutinated with NaCl, serotyping could not be performed and the isolate had to be inoculated again on nutrient agar. Serotyping was done by dropping a droplet of antisera on a slide and rubbing with a small bacterial mass on the slide. If the bacterial mass formed flakes in the antisera, the reaction was considered positive. The complete biotyping and serotyping tests were performed in the Department of Food Hygiene and Environmental Health at the Faculty of Veterinary Medicine in Helsinki, Finland.

2.2.1.4 Molecular identification and detection of Yersinia virulence genes

The isolated *Y. enterocolitica* strains were tested for confirmatory identification within the genus targeting a 330-bp fragment of the 16S rRNA gene. Additionaly, eight genes were also studied: five chromosome-borne virulence genes (*ail*, *foxA*, *ystA*, *ystB*, *myfA*) and two virulence genes (*yadA*, *virF*) located on the virulence plasmid of the pathogenic *Yersinia* spp. (pYV).

2.2.1.4.1 DNA purification

The template DNA used for the detection of *ail* and *foxA* gene was prepared using QIAamp DNA Mini kit (Qiagen; cat. No. 51304). Kit contents are described in materials

and methods (2.2). Isolation of DNA from bacterial suspension cultures in saline solution was carried as described by the supplier below:

1. Pipet 1 ml of bacterial culture into a 1.5 ml microcentrifuge tube, and centrifuge for 5 min at 5000 x *g* (7500 rpm).
2. Calculate the volume of the pellet or concentrate and add Buffer ATL to a total volume of 180 µl.
3. Add 20 µl proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.
4. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
5. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
6. Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
7. Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.
10. Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the

QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.

12. Repeat step 11.

For long-term storage of DNA eluate in Buffer AE is stored at –30 to –15°C.

2.2.1.4.2 Detection of ail, foxA and inv genes

The PCR reaction mixture and the sequences of primers are summarized in Table 9 and 11. The primers for *inv* were designed using X53368 *Y. enterocolitica* sequence from GenBank. For each amplification reaction, negative controls containing water instead of template DNA were run in parallel. The amplification was performed with denaturation for 10min at 95°C; followed by 25 cycles of 95°C for 15sec; annealing at 57°C (*ail*)/58°C (*foxA*) for 30sec, and primer extension at 72°C for 30sec. The final extension was performed at 72°C for 10min. The specific PCR products 585bp for *ail*, 1532bp for *foxA* and 953bp for *inv* were visualized on 1.5% agarose gels stained with ethidium bromide and photographed under UV-light. The Bench Top DNA 100 bp ladder (Promega) was used as molecular size marker. PCR tests for *ail*, *foxA* and *inv* genes were performed in the Department of Biology, Faculty of Medicine, School of Health Sciences, University of Ioannina, Greece. Purified PCR products of *inv* gene served as template for the sequence reaction, which was performed using an ABI3730xl sequencer in the Department of Cytogenetics and Molecular genetics, Faculty of Medicine, School of Health Sciences, University of Thessaly, Larisa, Greece. *Inv* gene sequences were compared and clustered with BioNumerics version 5.10.

Table 9. Reaction mixture

Components	**Volume**
Buffer 5x	10 µl
MgCl2 25 mM	6 µl
dNTPs 2 mM	5 µl
Forward primers 10 mM	5 µl
Reverse primers 10 mM	5 µl
5U/µl Taq Polymerase (Kapa Biosystems)	0.25 µl
DNA template	5 µl
H_2O	13.75 µl
Total reaction mix volume	50 µl

2.2.1.4.3 Molecular identification and detection of ystA, ystB, virF, yadA and myfA by real-time PCR

DNA was released from bacterial suspension cultures in eppendorf microcentrifuge tubes with TBE buffer solution by heating at 97°C for 10 min and then centrifugation at 12000 rpm for 3 min. The supernatant was placed in new eppendorf tubes and 1 µl of this liquid was added to 19 µl of the mastermix (Table 10).

The PCR reaction mixture and the sequences of primers are summarized in Table 10 and 11. Negative controls containing water instead of template DNA, as well as positive control for each amplification reaction were run in parallel. The amplification was performed with polymerase activation and initial denaturation at 95^{o}C for 3 min; followed by 40 cycles of 95^{o}C for 10sec; annealing at 58^{o}C for 10sec (for all genes), and primer extension at 72^{o}C for 30sec. Melting curve analysis was performed at 55-95^{o}C and increment 0.5^{o}C per 5 s. The fluorescence intensity of SYBR Green and the melting curve analysis were studied using the CFX96 system (Bio-Rad). A threshold cycle (Ct) under 30 and a specific melting temperature (Tm) indicated a positive result. Real-time PCR tests were performed in the Department of Food Hygiene and Environmental Health at the Faculty of Veterinary Medicine in Helsinki, Finland.

Table 10. Reaction mixture for real-time PCR

Components	**Volume**
iTaq™ Universal SYBR®Green supermix (2x)	10 µl (1x)
Forward primers 2 µM	1 µl (0.2 µM)
Reverse primers 2 µM	1 µl (0.2 µM)
DNA template	1 µl
H_2O	7 µl
Total reaction mix volume	20 µl

Table 11. Sequences of primers

Genes	Primer sequence (5'- 3')	Amplicon size (bp)	Reference
16S rRNA	F: AAT ACC GCA TAA CGT CTT CG R: CTT CTT CTG CGA GTA ACG TC	330	Neubauer et al., 2000b
ail	F: GGT TAT TGT ATT AGT ATT GTT R: CAG GTG GGT TTT CAC TAT CTG	585	Huang et al., 2010
ystA	F: ATC GAC ACC AAT AAC CGC TGA G R: CCA ATC ACT ACT GAC TTC GGC T	79	Thoerner et al., 2003
ystB	F: GTA CAT TAG GCC AAG AGA CG R: GCA ACA TAC CTC ACA ACA CC	146	Thoerner et al., 2003
inv	F: ATG GTA GCA CCG TCA CTG TGA CG R: CGT TCA GGC CAA CTG ACC ATG GA	953	In-house
virF	F: TCA TGG CAG AAC AGC AGT CAG R: ACT CAT CTT ACC ATT AAG AAG	590	Wren and Tabaqchali, 1990
yadA	F: TGT TCT CAT CTC CAT ATG C R: TCC TTT CGC TGC TTC AGC A	203	Fukushima et al., 2003
myfA	F: CAG ATA CAC CTG CCT TCC ATC T R: CTC GAC ATA TTC CTC AAC ACG C	272	Bhagat and Virdi, 2007
foxA	F: CTC TGC GGA AGA TAA CTA TG R: ATC CGG GAA TAA ACT TGG CGT A	1532	Huang et al., 2010

2.2.1.5 Resistance to antimicrobial agents

Susceptibility to 15 antimicrobial agents was tested by the Kirby-Bauer disk diffusion method as described by the Clinical Laboratory Standard Institute guidelines (CLSI, 2012). In order to prepare the inoculum of turbidity equivalent to a 0.5 McFarland standard, 4-5 colonies from pure overnight culture were suspended in a 10 ml tube saline solution. With a sterile swab dipped into the inoculum and squeezed against the walls of the tube to remove excess liquid, the inoculum was streaked on the surface of agar petri-dishes to obtain a uniform distribution of the bacterial suspension. The inoculated plates were allowed to stand at room temperature until the moisture in the inoculum spots had been absorbed into the agar. Antimicrobial paper disks were placed and pressed lightly on the surface of the agar with forceps. Disks were placed in distance larger than 24mm between them and no less than 15mm from the petri plate wall. Incubation for 18h at optimal growth temperature of 30^{o}C for *Y. enterocolitica* was used. The commercial antimicrobial susceptibility disks (Oxoid) that were selected are presented in the Table 12.

Table 12. Antimicrobial disks used in the study

Antimicrobial	Abbreviation	Level
Amikacin	AK	30 mg
Amoxicillin/clavulanic acid (2:1)	AMC	30 mg
Ampicillin	AMP	10 mg
Cefixime	CFM	5 mg
Cefotaxime	CTX	30 mg
Cefoxitin	FOX	30 mg
Ceftazidime	CAZ	30 mg
Ceftriaxone	CRO	30 mg
Chloramphenicol	C	30 mg
Ciprofloxacin	CIP	5 mg
Gentamicin	CN	10 mg
Imipenem	IPM	30 mg
Tetracycline	TE	30 mg
Ticarcillin/clavulanic acid (7,5:1)	TIM	85 mg
Trimethoprim/sulfamethoxazole (1.25- 23.75)	SXT	25 mg

Holding the petri plate a few inches above a black background illuminated with reflected light, the diameter of the growth inhibition zones was measured with an electronic caliper and interpreted as resistant or susceptible according to the breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines version 4.0 for *Enterobacteriaceae* or *Escherichia coli*. (EUCAST, 2014) and the breakpoints of CLSI for *Enterobacteriaceae* concerning tetracycline (CLSI, 2013). The control strain was E. coli ATCC 25922. As inhibition zones were considered the zones which were completely free of microbial growth.

2.2.2 Statistical analysis

Confidence intervals of proportions were calculated with Epi tools (http://epitools.ausvet.com.au) using the binomial exact method. Statistical significance of differences of recovery of *Y. enterocolitica* between the isolation methods were evaluated using Fisher's exact test. A confidence level of 95% was applied. Test results were recorded as binary variables (presence/absence of *Y. enterocolitica*) per sample type in Excel spreadsheets.

Results for different isolation methods were recorded as binary variables. Isolation methods were compared only for carcass swabs. Due to the limited number of positive samples isolation methods were not compared for vegetable samples. For each matrix, the relative sensitivity was calculated based on the combined results of all isolation methods (parallel interpretation, a sample is considered positive when tested positive with at least one method). The specificity was assumed to be 100%.

2.3 Materials and reagents

• Yersinia peptone, sorbitol and bile salts (PSB) broth (4022702, Biolife Italiana, Milan, Italy)

Peptone	5.00 g
Sorbitol	10.00 g
Sodium chloride	5.00 g
Disodium hydrogen phosphate	8.23 g
Sodium dihydrogen phosphate	1.20 g
Bile salts n°3	1.50 g

Suspend 31 g in 1000 ml of cold distilled water. Distribute in tubes of flasks and sterilise by autoclaving at 121°C for 15 minutes. Final pH 7.6 ± 0.1 in 25°C.

• Yersinia irgasan, ticarcillin and potassium chlorate (ITC) broth

Yersinia ITC Broth Base (4022652, Biolife Italiana, Milan, Italy)

Tryptone	10.00 g
Yeast extract	10.00 g
Magnesium chloride anhydrous	28.10 g
Sodium chloride	5.00 g
Malachite green	0.01 g

Ticarcillin Irgasan Antimicrobic Supplement (4240060, Biolife Italiana, Milan, Italy)

Ticarcillin	0.25 mg
Irgasan	0.25 mg

Potassium Chlorate Supplement (4240065, Biolife Italiana, Milan, Italy)

Potassium chloride	5% solution 5 ml

Suspend 11 g in 250 ml of cold distilled water. Heat to dissolve completely and sterilise by autoclaving at 121°C for 15 minutes. Cool to approximately 50°C and aseptically add the

contents of one vial of Potassium Chlorate Supplement and one vial of Ticarcillin Irgasan Supplement reconstituted with 5ml of sterile distilled water. Aseptically distribute into sterile tubes or flasks. Final pH 6.9 ± 0.2 in 25°C.

• Potassium hydroxide solution 0.25%

NaCl	0.5 g
Potassium hydroxide (KOH)	0.5 g

Dissolve dry material in 100 ml distilled water, measure 4,5 ml tube and autoclave at 121°C for 15 minutes.

• Yersinia selective cefsulodin, irgasan and novobiocin (CIN) agar

Yersinia selective Agar Base (CM0653, Oxoid Ltd, Hampshire, England)

Special peptone	20.00 g
Yeast extract	2.00 g
Mannitol	20.00 g
Sodium pyruvate	2.00 g
Sodium chloride	1.00 g
Magnesium sulphate	0.01 g
Sodium desoxycholate	0.50 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	12.50 g

Yersinia Selective Supplement (SR0109, Oxoid Ltd, Hampshire, England)

(Vial contents for 500 ml of medium):

Cefsulodin	7.5 mg
Irgasan	2.0 mg
Novobiosin	1.25 mg

Suspend 29 g in 500 ml of distilled water and bring gently to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Allow to cool to approximately 50°C and aseptically add the contents of one vial of Yersinia Selective Supplement SR0109 reconstituted with a 2 ml mixture of 1:1 ethanol and sterilized distilled water. Mix gently and pour into sterile Petri dishes. Final pH 7.4 ± 0.2 in 25°C.

• Nutrient agar (4018102, Biolife Italiana, Milan, Italy)

Peptone	5.00 g
Beef (meat) extract	3.00 g
Agar	15.00 g

Suspend 23 g in 1000 ml of distilled water and bring gently to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Allow to cool to approximately 50°C and mix gently and pour into sterile Petri dishes. Final pH 7.0 ± 0.2 in 25°C.

• Oxidase test strips (113300 Bactident Oxidase, Merck, Darmstadt, Germany)

• Urea agar slant tubes

Urea agar base (LAB130, LabM, Lancashire, United Kingdom)

Peptone	1.00 g
Glucose	2.00 g
Mannitol	20.00 g
Sodium chloride	1.00 g
Disodium phosphate	1.20 g
Potassium dihydrogen phosphate	0.80 g
Phenol red	0.012 g
Agar	12.00 g

40% urea solution

Urea (108487, Merck, Darmstadt, Germany)

Suspend 20 g in 50 ml sterilized water using a sterile filter.

Suspend 2.1 g of urea agar base in 95 ml of distilled water and sterilise in 121°C for 15 minutes. Allow to cool in 47°C, add aseptically 5 ml sterile urea solution 40%. Distribute into sterile bottles and slopes, allow to set in the sloped position.

• Kligler iron agar (4015602, Biolife Italiana, Milan, Italy)

Beef extract	3.00 g
Yeast extract	3.00 g
Peptocomplex	20.00 g
Lactose	10.00 g
Glucose	1.00 g
Ferrous sulphate	0.20 g
Sodium thiosulphate	0.30 g

Sodium chloride	5.00 g
Phenol red	0.024 g
Agar	12.00 g

Suspend 54.5 g in 1000 ml of cold distilled water. Heat to boiling, distribute and autoclave at 121°C for 15 minutes. Pour into tubes to cool in a slanting position to obtain deep butts and short slopes. Final pH 7.4± 0.2 in 25°C.

• Sugar tubes

Sugar base broth

Bacto peptone	10.00 g
NaCl	5.00 g

Suspend 10 g in 1000 ml of distilled water, boil in bath and autoclave in 200 ml bottles in 121°C for 15 minutes.

0.32% phenol red

Phenol red	0,32 g

Rinse with ethanol in a bottle and add 1:1 ethanol-water mixture for total volume 100ml.

10% sugar broth (sucrose, rhamnose, xylose, trehalose, salicine)

Sugar	10.00 g

Suspend 10 g sugar in 100 ml sterilized distilled water using a sterile filter. Salicine needs get warm in water, because it is precipitates.

Final sugar broth

Sugar base broth	94 ml
0.32% bromocresol purple red/bromothymol blue	1 ml
10% sugar broth	5 ml

Mix and measure out in small tubes, about 1ml/tube. Sterility check: incubate in 25°C for 48 hours.

• Bile Esculin agar

Meat extract	3.00 g
Peptone	5.00 g
Bile salts	40.00 g
Iron citrate	0.50 g
Esculin	1.00 g
Agar	15.00 g

Suspend in 1000 ml of distilled water and bring gently to the boil to dissolve completely. Allow to cool to approximately 50°C and mix gently and pour into sterile Petri dishes. Final pH 7.1 ± 0.2 in 25°C.

• Tween 80 agar

Nutrient agar	28.00 g
Tween 80	10 ml

Boil nutrient agar in 1000 ml of distilled water for 30 minutes, add Tween 80 and boil for additional 5 minutes. Set pH 7.4. Autoclave in 121°C for 15 minutes. Allow to cool to approximately 50°C and mix gently and pour into sterile Petri dishes.

• Egg Yolk agar

Yeast extract	5.00 g
Tryptone	5.00 g
Proteose peptone	20.00 g
NaCl	5.00 g
Agar	20.00 g

Suspend in 1000ml of distilled water and bring gently to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Allow to cool to approximately 50°C and aseptically add 80 ml egg yolk emulsion (215329 Difco Bacto Egg Yolk Emulsion 50%). Mix gently and pour into sterile Petri dishes. After solidification dry 2-3 days at ambient temperature or at 35°C for 24 h. Final pH 7.0 ± 0.2 in 25°C.

• PYZ (pyrazinamidase) tubes

Tris-maleate buffer (0.2M, pH 6)

Trizma-maleate buffer $C_4H_{11}NO_3 \cdot C_4H_4O_4$	31.52g
(Sigma, Lot 46H5747)	
H_2O	1000 ml

Dissolve in about 500 ml H_2O. Adjust pH with 1N NaOH or 1N HCl for 6. Add H_2O for final volume of 1000 ml.

PYZ agar

Tryptic soy agar (0369-01-4, Difco)	30.00 g
Yeast extract (0127-01-7, Difco)	3.00 g
Pyrazincarboxamide (P4021, Merck)	1.00 g
Tris-maleate buffer 0,2M pH 6	1000 ml

Autoclave in 121°C for 15 minutes. Cast in long sloping surfaces in normal test tubes, about 5ml/tube.

• Ammoniumferrosulfate broth

$(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$	0.70 g
H_2O	50 ml

Prepared before use, doesn't last storage.

• Mueller-Hinton agar II

Beef extract	2.00 g
Acid digest of casein	17.50 g
Starch	1.50 g
Agar bios special	17.00 g

Suspend 38 g in 500 ml of distilled water. Heat to boiling with frequent agitation and sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C, mix well and pour into sterile Petri plates of 90mm or 140mm with a medium layers of 4mm (25ml of medium per plate of 9cm and 60ml per plate of 14cm). Final pH 7.3 ± 0.1 in 25°C.

• QIAamp DNA Mini kit Catalog no. 51304

Number of preps	50
Collection Tubes (2 ml)	150
Buffer AL	12 ml
Buffer ATL	14 ml
Buffer AW1	19 ml
Buffer AW2	3 ml
Buffer AE	2 x 15 ml
Proteinase K	1.25 ml

• iTaq™ Universal SYBR®Green supermix, 25 ml (5 x 5 ml vials), 2500 x 20 µl reactions #1725124

iTaq™ Universal SYBR® Green Supermix is a 2x concentrated, ready-to-use reaction master mix optimized for dye-based quantitative PCR (qPCR) on any real-time PCR instrument (ROX-independent and ROX-dependent). It contains antibody-mediated hot-start iTaq DNA polymerase, dNTPs, MgCl2, SYBR® Green I Dye, enhancers, stabilizers, and a blend of passive reference dyes (including ROX and fluorescein).

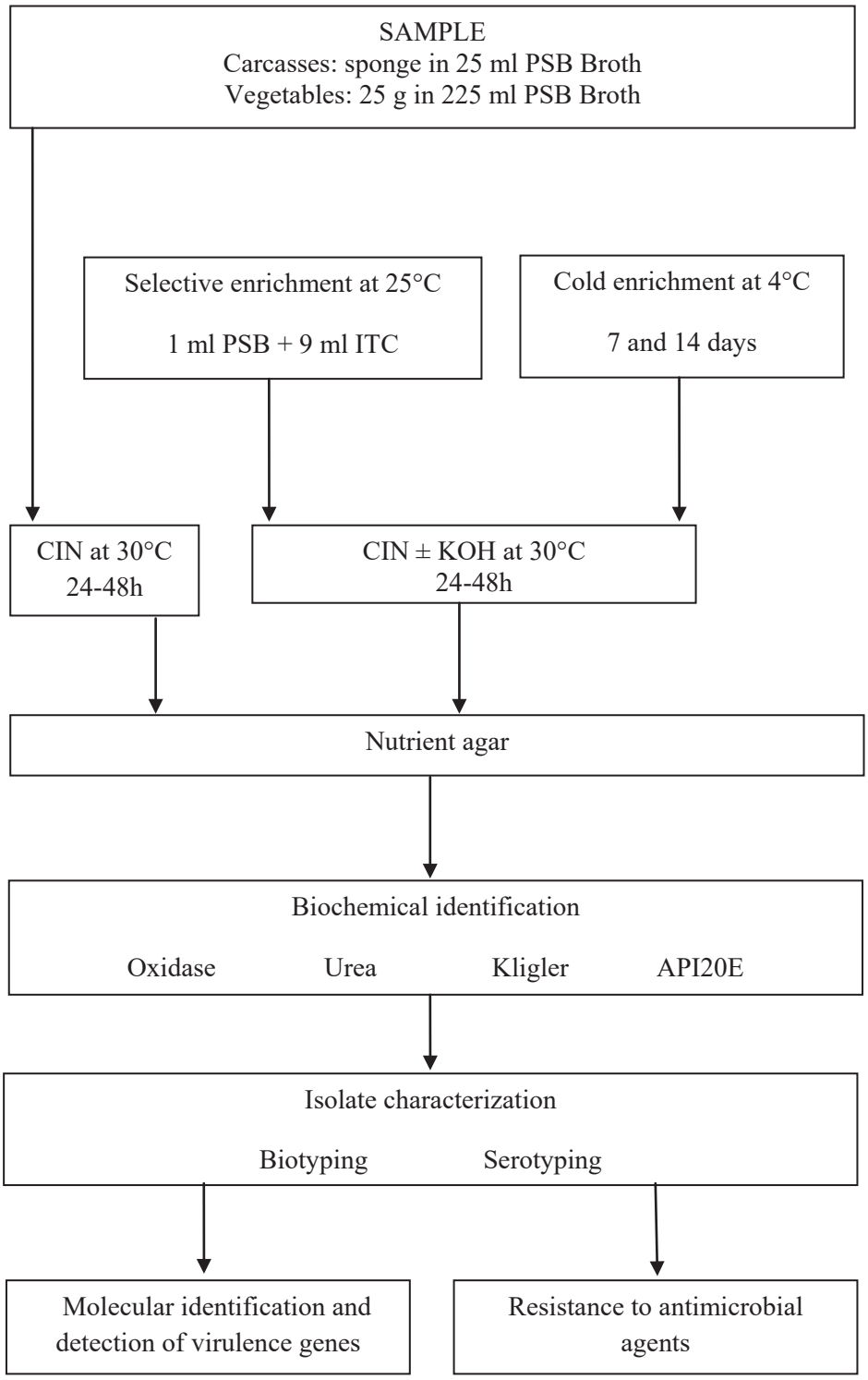

Figure 5. Diagram of procedure

3 Results

3.1 Detection

Y. enterocolitica was found in 15.9% (95% C.I. 10.3% - 22.8%) of the carcass swabs (23/145) and in 2.8% (95% C.I. 0.7% - 7%) of the vegetable samples (4/144). Of the 145 carcass swabs, 15 (10.3%) were identified as human pathogenic bio-serotype 4/O:3 and 9 (6,2%) as bio-serotype 1A. Of the 144 vegetable samples, only one (0.7%) was identified as bio-serotype 4/O:3 (non-organic lettuce) and three (2,1%) as 1A (two mixed salads and one organic lettuce).

A total of 59 strains were isolated and biochemically identified as *Y. enterocolitica*: 54 from the carcass swabs and 5 from the vegetable samples (Table 13). All but one strains were also molecular identified as *Y. enterocolitica* by the detection of the specific of *16S rRNA* gene for the species (Table 15). YE27 strain isolated from a vegetable sample is presumed as *Y. kristensenii* since its appearance in CIN agar plates is often misinterpreted as *Y. enterocolitica*. Use of stereomicroscope with Henry illumination helps the differentiation between the species (Figure 6).

The API 20E profile numbers of these strains, the isolation methods and the strains origin are depicted in Table 13. Interpretation of the API 20E results had some difficulties since most of the profile numbers (as depicted in Table 13) are not matched to those of *Y. enterocolitica* according to API 20E database. This deviation is explained by the fact that the temperature of 28°C instead of 37°C was chosen as recommended from the manufacturer. Voges-Proskauer reaction of *Y. enterocolitica* at 28°C is positive unlike the temperature of 37°C. This led to a different number in the fourth digit of 7-digit profile and finally to a mismatch.

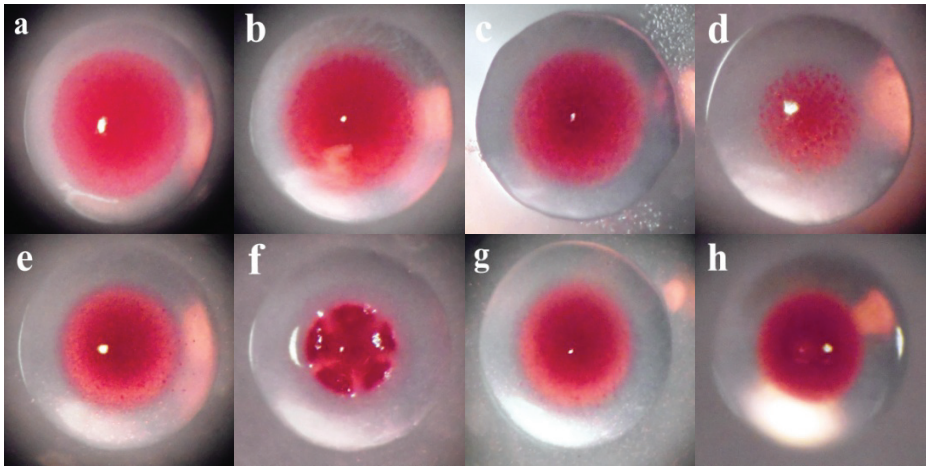

Figure 6. Different strains of *Y. enterocolitica* and one of *Y. kristensenii* on CIN agar through a stereomicroscope. (a-d) *Y. enterocolitica* BT 1A (non-pathogenic biotype). (e-g) *Y. enterocolitica* BT 4 (pathogenic biotype). (h) *Y. kristensenii*.

Table 13. Strains isolated, isolation methods and their origin

Strain codes	API 20E profile numbers	Isolation methods	Origin	Sample number
YE1	*Y.enterocolitica* (0015522)	CE 7d	Lettuce (non organic)	19
YE2	*Y.enterocolitica* (1015520)	CE 7d + KOH	Pork	56
YE3	*Y.enterocolitica* (1015520)	CE 7d + KOH	Pork	56
YE4	*Y.enterocolitica* (0015520)	CE14d + KOH	Pork	56
YE5	*Y.enterocolitica* (1155723)	CE 7d + KOH	Mixed salad	60
YE6	*Y.enterocolitica* (1015522)	CE 14d + KOH	Pork	69
YE7	*Y.enterocolitica* (1015522)	CE 7d + KOH	Pork	70
YE8	*Y.enterocolitica* (1015522)	CE 7d + KOH	Pork	70
YE9	*Y.enterocolitica* (1015522)	CE 14d + KOH	Pork	80
YE10	*Y.enterocolitica* (1015522)	DP	Pork	92
YE11	*Y.enterocolitica* (0015522)	CE 7d	Pork	91
YE12	*Y.enterocolitica* (1015522)	CE 7d + KOH	Pork	91
YE13	*Y.enterocolitica* (1015522)	CE 7d + KOH	Pork	92
YE14	*Y.enterocolitica* (0015522)	CE 7d	Pork	93
YE15	*Y.enterocolitica* (1015522)	CE14d + KOH	Pork	92
YE16	*Y.enterocolitica* (1015522)	DP	Pork	113
YE17	*Y.enterocolitica* (1015522)	DP	Pork	115
YE18	*Y.enterocolitica* (1015522)	SE	Pork	120
YE19	*Y.enterocolitica* (1015522)	CE 7d + KOH	Pork	113
YE20	*Y.enterocolitica* (1015522)	CE 7d	Pork	120
YE21	*Y.enterocolitica* (1015522)	CE 7d + KOH	Pork	120
YE22	*Y.enterocolitica* (1015522)	CE 7d	Pork	136
YE23	*Y.enterocolitica* (1015522)	SE	Pork	120
YE24	*Y.enterocolitica* (1155723)	CE 7d	Lettuce (organic)	126
YE25	*Y.enterocolitica* (1015522)	CE 14d + KOH	Pork	136
YE26	*Y.enterocolitica* (1015522)	CE 14d + KOH	Pork	136
YE27	*Y.enterocolitica* (1054523)	CE 14d + KOH	Mixed salad	151
YE28	*Y.enterocolitica* (1015522)	CE 14d + KOH	Pork	120
YE29	*Y.enterocolitica* (1015522)	CE 14d + KOH	Pork	120
YE30	*Y.enterocolitica* (1015522)	CE 14d + KOH	Pork	120
YE31	*Y.enterocolitica* (1155723)	CE 14d + KOH	Pork	172
YE32	*Y.enterocolitica* (1155723)	CE 14d	Pork	180
YE33	*Y.enterocolitica* (1155723)	CE 14d	Pork	183
YE34	*Y.enterocolitica* (1155723)	DP	Pork	200
YE35	*Y.enterocolitica* (1015522)	CE 7d + KOH	Pork	200
YE36	*Y.enterocolitica* (1155723)	CE 14d	Pork	198
YE37	*Y.enterocolitica* (1155523)	CE14d + KOH	Pork	206
YE38	*Y.enterocolitica* (1155523)	CE14d + KOH	Pork	206
YE39	*Y.enterocolitica* (1155523)	CE14d + KOH	Pork	207
YE40	*Y.enterocolitica* (1155523)	CE 7d	Pork	247

Table 13. Strains isolated, isolation methods and their origin

Strain codes	API 20E profile numbers	Isolation methods	Origin	Sample number
YE41	*Y.enterocolitica* (1155523)	CE14d + KOH	Pork	247
YE42	*Y.enterocolitica* (1015522)	SE + KOH	Pork	262
YE43	*Y.enterocolitica* (1015522)	SE+	Pork	262
YE44	*Y.enterocolitica* (1015522)	CE 7d + KOH	Pork	262
YE45	*Y.enterocolitica* (1155763)	CE14d + KOH	Pork	264
YE46	*Y.enterocolitica* (1155723)	CE 7d	Mixed salad	262
YE47	*Y.enterocolitica* (1015522)	SE+	Pork	262
YE48	*Y.enterocolitica* (1015522)	SE + KOH	Pork	262
YE49	*Y.enterocolitica* (1015522)	CE7d + KOH	Pork	281
YE50	*Y.enterocolitica* (1015522)	SE	Pork	296
YE51	*Y.enterocolitica* (1015522)	SE + KOH	Pork	296
YE52	*Y.enterocolitica* (1015522)	SE + KOH	Pork	296
YE53	*Y.enterocolitica* (1015522)	SE + KOH	Pork	296
YE54	*Y.enterocolitica* (1155523)	CE7d + KOH	Pork	247
YE55	*Y.enterocolitica* (1155523)	CE14d + KOH	Pork	207
YE56	*Y.enterocolitica* (1155523)	CE14d + KOH	Pork	207
YE57	*Y.enterocolitica* (1155523)	CE14d + KOH	Pork	207
YE58	*Y.enterocolitica* (1155523)	CE7d	Pork	247
YE59	*Y.enterocolitica* (1155523)	CE7d + KOH	Pork	247

3.2 Comparison of isolation methods for the recovery of human pathogenic Y. enterocolitica

Pathogenic *Y. enterocolitica* bio-serotype 4/O:3 was detected in total 15 carcass swabs (10.3%) using different isolation methods (Table 14). None of the methods detected all of the 4/O:3 positive samples and none of the samples were detected as positive by all methods.

The relative sensitivity of the combination of cold enrichment for 7 days with or without alkali treatment was higher (73.3%) than others isolation procedures with relative sensitivity. From the 11 positive samples with cold enrichment for 7 days, only two were detected positive with both ± alkali treatment. The combined cold enrichment for 7 days provided a significantly better performance than direct plating (p=0.0268) and selective enrichment (p=0.0092), whereas no significant difference was observed in the recovery rates of cold enrichment for 14 days (p = 0.1394).

Table 14. Number of carcass swabs positive for *Y. enterocolitica* 4/O:3 and relative sensitivities of the different isolation methods.

Isolation method	**Number of positive samples**	**Relative sensitivity**
Direct plating	4	26.7%
Selective enrichment	3	20%
ITC+CIN	2	13.3%
ITC+KOH+CIN	2	13.3%
Cold enrichment 7d	11	73.3%
PSB+CIN	4	26.7%
PSB+KOH+CIN	9	60%
Cold enrichment 14d	6	40%
PSB+CIN	-	-
PSB+KOH+CIN	6	40%
Total	15	100%

3.3 Typing and virulence gene distribution

Bio-serotypes and distribution of virulence genes of *Y. enterocolitica* strains are shown in Tables 15 and 16. Of the 54 *Y. enterocolitica* isolates of the carcass swabs, 37 (68.5%) were identified as human pathogenic belonging to bio-serotype 4/O:3 and the rest 17 isolates belonging to biotype 1A (31.5%) (Table 15). Among these, only two serotypes were identified: 3 isolates as O:5 and one as O: 8, while a high proportion (76.5%) was O-untypeable. The *ail*, *ystA*, *inv*, *virF*, *myfA* and *foxA* genes were detected in 100% of the 4/O:3 isolates, and the *yadA* gene was found in 64.9% (Table 16). All 4/O:3 isolates were negative for the *ystB* gene. The most common virulence-associated gene in 1A isolates was the *inv* (100%), followed by *ystB* (94.1%), and *foxA* (84.4%) genes. All 1A isolates were negative for the other target genes.

Of the 4 *Y. enterocolitica* strains of the vegetable samples which were isolated by cold enrichment for 7 days, only one was identified as 4/O:3 and 3 belonging to biotype 1A/O-untypeable (Table 15). The *ail*, *ystA*, *inv*, *myfA* and *foxA* genes were detected in the 4/O:3 isolate (Table 16). The most common virulence-associated gene in 1A isolates was the *inv* (100%) and *ystB* (100%), followed by *foxA* (66.6%) genes.

As shown in Table 15 there are some strains which are Tween 80 agar negative, although their phenotype is that of biotype 1A. However, they were alternatively tested for lipase production on Egg yolk agar where they showed positive reaction.

Table 15. Biotyping and serotping of *Y. enterocolitica*

Strains	Reactions								Biotype	Serotype
	Lipase (Tween 80)	Esculin	Salicin	Indole	Xylose	Trehalose	Pyrazinamidase	Voges-Proskauer		
YE1	-	-	-	-	-	+	-	+	**4**	**O:3**
YE2	-	-	-	-	-	+	-	+	**4**	**O:3**
YE3	-	-	-	-	-	+	-	+	**4**	**O:3**
YE4	-	-	-	-	-	+	-	+	**4**	**O:3**
YE5	+	+	+	+	+	+	+	+	**1A**	**ND**
YE6	-	-	-	-	+	+	-	+	**4**	**O:3**
YE7	-	-	-	-	-	+	-	+	**4**	**O:3**
YE8	-	-	-	-	-	+	-	+	**4**	**O:3**
YE9	-	-	-	-	-	+	-	+	**4**	**O:3**
YE10	-	-	-	-	-	+	-	+	**4**	**O:3**
YE11	-	-	-	-	-	+	-	+	**4**	**O:3**
YE12	-	-	-	-	-	+	-	+	**4**	**O:3**
YE13	-	-	-	-	-	+	-	+	**4**	**O:3**
YE14	-	-	-	-	-	+	-	+	**4**	**O:3**
YE15	-	-	-	-	-	+	-	+	**4**	**O:3**

Table 15. Biotyping and serotping of *Y. enterocolitica*

Strains	Reactions								Biotype	Serotype
	Lipase (Tween 80)	Esculin	Salicin	Indole	Xylose	Trehalose	Pyrazinamidase	Voges-Proskauer		
YE16	-	-	-	-	-	+	-	+	**4**	**O:3**
YE17	-	-	-	-	-	+	-	+	**4**	**O:3**
YE18	-	-	-	-	-	+	-	+	**4**	**O:3**
YE19	-	-	-	-	-	+	-	+	**4**	**O:3**
YE20	-	-	-	-	-	+	-	+	**4**	**O:3**
YE21	-	-	-	-	-	+	-	+	**4**	**O:3**
YE22	-	-	-	-	-	+	-	+	**4**	**O:3**
YE23	-	-	-	-	-	+	-	+	**4**	**O:3**
YE24	+ [a]	+	+	+	+	+	+	+	**1A**	**ND**
YE25	-	-	-	-	-	+	-	+	**4**	**O:3**
YE26	-	-	-	-	-	+	-	+	**4**	**O:3**
YE28	-	-	-	-	-	+	-	+	**4**	**O:3**
YE29	-	-	-	-	-	+	-	+	**4**	**O:3**
YE30	-	-	-	-	-	+	-	+	**4**	**O:3**
YE31	+	+	+	+	+	+	(+) [b]	+	**1A**	**O:5**

Table 15. Biotyping and serotping of *Y. enterocolitica*

Strains	Reactions								Biotype	Serotype
	Lipase (Tween 80)	Esculin	Salicin	Indole	Xylose	Trehalose	Pyrazinamidase	Voges-Proskauer		
YE32	+	+	+	+	+	+	+	+	**1A**	**ND**
YE33	+	+	+	+	+	+	(+)[b]	+	**1A**	**O:5**
YE34	+	+	+	+	+	+	+	+	**1A**	**O:8**
YE35	-	-	-	-	-	+	-	+	**4**	**O:3**
YE36	+ [a]	+	+	+	+	+	+	+	**1A**	**ND**
YE37	+ [a]	+	+	+	+	+	+	+	**1A**	**ND**
YE38	+ [a]	+	+	+	+	+	+	+	**1A**	**ND**
YE39	+ [a]	+	+	+	+	+	+	+	**1A**	**ND**
YE40	+ [a]	+	+	+	+	+	+	+	**1A**	**ND**
YE41	+ [a]	+	+	+	+	+	(+)[b]	+	**1A**	**ND**
YE42	-	+	+	+	+	+	+	+	**4**	**O:3**
YE43	-	-	-	-	-	+	-	+	**4**	**O:3**
YE44	-	-	-	-	-	+	-	+	**4**	**O:3**
YE45	+	+	+	+	+	+	(+)[b]	+	**1A**	**O:5**
YE46	+	+	+	+	+	+	(+)[b]	+	**1A**	**ND**

Table 15. Biotyping and serotping of *Y. enterocolitica*

Strains	Reactions								Biotype	Serotype
	Lipase (Tween 80)	Esculin	Salicin	Indole	Xylose	Trehalose	Pyrazinamidase	Voges-Proskauer		
YE47	-	-	-	-	-	+	-	+	**4**	**O:3**
YE48	-	-	-	-	-	+	-	+	**4**	**O:3**
YE49	-	-	-	-	-	+	-	+	**4**	**O:3**
YE50	-	-	-	-	-	+	-	+	**4**	**O:3**
YE51	-	-	-	-	-	+	-	+	**4**	**O:3**
YE52	-	-	-	-	-	+	-	+	**4**	**O:3**
YE53	-	-	-	-	-	+	-	+	**4**	**O:3**
YE54	+ [a]	+	+	+	+	+	(+)[b]	+	**1A**	**ND**
YE55	+ [a]	+	+	+	+	+	(+)[b]	+	**1A**	**ND**
YE56	+ [a]	+	+	+	+	+	+	+	**1A**	**ND**
YE57	+ [a]	+	+	+	+	+	(+)[b]	+	**1A**	**ND**
YE58	+ [a]	+	+	+	+	+	(+)[b]	+	**1A**	**ND**
YE59	+ [a]	+	+	+	+	+	(+)[b]	+	**1A**	**ND**

a Tween agar negative, Egg yolk agar positive

b Delayed reaction

ND not determined

Table 16. Virulence gene distribution

Strains	Genes									Bio/sero type
	16sN	Ail	FoxA	ystA	ystB	inv	yadA	virF	myfA	
YE1	+	+	+	+	-	+	-	-	+	**4/O:3**
YE2	+	+	+	+	-	+	-	+	+	**4/O:3**
YE3	+	+	+	+	-	+	-	+	+	**4/O:3**
YE4	+	+	+	+	-	+	-	+	+	**4/O:3**
YE5	+	-	+	-	+	+	-	-	-	**1A/ND**
YE6	+	+	+	+	-	+	+	+	+	**4/O:3**
YE7	+	+	+	+	-	+	+	+	+	**4/O:3**
YE8	+	+	+	+	-	+	+	+	+	**4/O:3**
YE9	+	+	+	+	-	+	-	+	+	**4/O:3**
YE10	+	+	+	+	-	+	-	+	+	**4/O:3**
YE11	+	+	+	+	-	+	+	+	+	**4/O:3**
YE12	+	+	+	+	-	+	+	+	+	**4/O:3**
YE13	+	+	+	+	-	+	+	+	+	**4/O:3**
YE14	+	+	+	+	-	+	+	+	+	**4/O:3**
YE15	+	+	+	+	-	+	+	+	+	**4/O:3**

Table 16. Virulence gene distribution

Strains	Genes									Bio/sero type
	16sN	Ail	FoxA	ystA	ystB	inv	yadA	virF	myfA	
YE16	+	+	+	+	-	+	+	+	+	**4/O:3**
YE17	+	+	+	+	-	+	+	+	+	**4/O:3**
YE18	+	+	+	+	-	+	+	+	+	**4/O:3**
YE19	+	+	+	+	-	+	+	+	+	**4/O:3**
YE20	+	+	+	+	-	+	+	+	+	**4/O:3**
YE21	+	+	+	+	-	+	+	+	+	**4/O:3**
YE22	+	+	+	+	-	+	+	+	+	**4/O:3**
YE23	+	+	+	+	-	+	+	+	+	**4/O:3**
YE24	+	-	-	-	+	+	-	-	-	**1A/ND**
YE25	+	+	+	+	-	+	+	+	+	**4/O:3**
YE26	+	+	+	+	-	+	+	+	+	**4/O:3**
YE27	+	-	-	-	-	+	-	-	-	**-**
YE28	+	+	+	+	-	+	+	+	+	**4/O:3**
YE29	+	+	+	+	-	+	+	+	+	**4/O:3**
YE30	+	+	+	+	-	+	+	+	+	**4/O:3**

Table 16. Virulence gene distribution

Strains	Genes									Bio/sero type
	16sN	Ail	FoxA	ystA	ystB	inv	yadA	virF	myfA	
YE31	+	-	+	-	+	+	-	-	-	**1A/O:5**
YE32	+	-	-	-	+	+	-	-	-	**1A/ND**
YE33	+	-	+	-	+	+	-	-	-	**1A/O:5**
YE34	+	-	+	-	-	+	-	-	-	**1A/O:8**
YE35	+	+	+	+	-	+	+	+	+	**4/O:3**
YE36	+	-	-	-	+	+	-	-	-	**1A/ND**
YE37	+	-	-	-	+	+	-	-	-	**1A/ND**
YE38	+	-	+	-	+	+	-	-	-	**1A/ND**
YE39	+	-	+	-	+	+	-	-	-	**1A/ND**
YE40	+	-	+	-	+	+	-	-	-	**1A/ND**
YE41	+	-	+	-	+	+	-	-	-	**1A/ND**
YE42	+	+	+	+	-	+	-	+	+	**4/O:3**
YE43	+	+	+	+	-	+	-	+	+	**4/O:3**
YE44	+	+	+	+	-	+	-	+	+	**4/O:3**
YE45	+	-	+	-	+	+	-	-	-	**1A/O:5**

Table 16. Virulence gene distribution

Strains	Genes									Bio/sero type
	16sN	Ail	FoxA	ystA	ystB	inv	yadA	virF	myfA	
YE46	+	-	+	-	+	+	-	-	-	**1A/ND**
YE47	+	+	+	+	-	+	-	+	+	**4/O:3**
YE48	+	+	+	+	-	+	+	+	+	**4/O:3**
YE49	+	+	+	+	-	+	+	+	+	**4/O:3**
YE50	+	+	+	+	-	+	-	+	+	**4/O:3**
YE51	+	+	+	+	-	+	-	+	+	**4/O:3**
YE52	+	+	+	+	-	+	-	+	+	**4/O:3**
YE53	+	+	+	+	-	+	-	+	+	**4/O:3**
YE54	+	-	+	-	+	+	-	-	-	**1A/ND**
YE55	+	-	+	-	+	+	-	-	-	**1A/ND**
YE56	+	-	+	-	+	+	-	-	-	**1A/ND**
YE57	+	-	+	-	+	+	-	-	-	**1A/ND**
YE58	+	-	+	-	+	+	-	-	-	**1A/ND**
YE59	+	-	+	-	+	+	-	-	-	**1A/ND**

3.4 Antimicrobial resistance

All 58 *Y. enterocolitica* isolates were susceptible to cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, imipenem, tetracycline and ticarcillin/clavulanic acid. On the contrary all isolates were resistant to ampicillin. Resistances to amoxicillin/clavulanic acid (55.2%), cefoxitin (39.7%), cefixime (18.9%) and chloramphenicol (15.5%) were the most common (Table 17). Among pathogenic 4/O:3 isolates the highest resistance was found to amoxicillin/clavulanic acid (47.4%), while some isolates were resistant to cefoxitin (26.3%) and chloramphenicol (23.7%). A high percentage of the 1A isolates were resistant to amoxicillin/clavulanic acid (70%) and cefoxitin (65%). In addition, 65% of 1A isolates were resistant to both amoxicillin/clavulanic acid and cefoxitin (Chart 1).

There were observed 13 resistance patterns (Table 18). The majority (55.1%) of the isolates was resistant to two (31%) or three (24.1%) antimicrobial agents. Resistance to three or more antimicrobials was observed in 26.6% (27/58) of the isolates. A single 4/O:3 isolate (1%) was resistant to five antimicrobials. No isolate was resistant to more than five antimicrobials. The most common resistance patterns were AmcAmpFox (17.2%), AmcAmp (15.5%) and AmcAmpCfmFox (13.8%).

Table 17. Antimicrobial resistance of strains

Strains	AK	AMC	AMP	C	CAZ	CFM	CIP	CN	CRO	CTX	FOX	IPM	SXT	TE	TIM	Bio/sero type
YE1	S	S	**R**	S	S	S	S	I	S	S	S	S	S	S	S	**4/O:3**
YE2	S	S	**R**	**R**	S	S	S	S	S	S	S	S	S	S	S	**4/O:3**
YE3	S	S	**R**	**R**	S	S	S	I	S	S	S	S	I	S	S	**4/O:3**
YE4	S	S	**R**	**R**	S	S	S	I	S	S	S	S	S	S	S	**4/O:3**
YE5	S	**R**	**R**	S	S	**R**	S	S	S	S	**R**	S	S	S	S	**1A/ND**
YE6	S	S	**R**	**R**	S	S	S	S	S	S	**R**	S	**R**	S	S	**4/O:3**
YE7	S	**R**	**R**	**R**	S	S	S	S	S	S	S	S	**R**	S	S	**4/O:3**
YE8	S	S	**R**	**R**	S	S	S	I	S	S	S	S	**R**	S	S	**4/O:3**
YE9	I	S	**R**	S	S	S	S	I	S	S	**R**	S	S	S	S	**4/O:3**
YE10	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**4/O:3**
YE11	S	**R**	**R**	**R**	S	S	S	I	S	S	S	S	**R**	S	S	**4/O:3**
YE12	I	**R**	**R**	**R**	S	S	S	**R**	S	S	S	S	**R**	S	S	**4/O:3**
YE13	I	S	**R**	S	S	S	S	**R**	S	S	S	S	S	S	S	**4/O:3**
YE14	I	S	**R**	**R**	S	S	S	I	S	S	S	S	**R**	S	S	**4/O:3**
YE15	S	**R**	**R**	S	S	**R**	S	I	S	S	S	S	S	S	S	**4/O:3**

Table 17. Antimicrobial resistance of strains

Strains	AK	AMC	AMP	C	CAZ	CFM	CIP	CN	CRO	CTX	FOX	IPM	SXT	TE	TIM	Bio/sero type
YE16	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**4/O:3**
YE17	S	S	**R**	S	S	S	S	I	S	S	S	S	S	S	S	**4/O:3**
YE18	I	**R**	**R**	S	S	S	S	I	S	S	S	S	S	S	S	**4/O:3**
YE19	S	S	**R**	S	S	S	S	I	S	S	S	S	S	S	S	**4/O:3**
YE20	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**4/O:3**
YE21	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**4/O:3**
YE22	S	**R**	**R**	S	S	S	S	I	S	S	S	S	S	S	S	**4/O:3**
YE23	S	**R**	**R**	S	S	S	S	I	S	S	**R**	S	S	S	S	**4/O:3**
YE24	S	**R**	**R**	S	S	**R**	S	S	S	S	**R**	S	S	S	S	**1A/ND**
YE25	S	S	**R**	S	S	S	S	I	S	S	S	S	S	S	S	**4/O:3**
YE26	S	**R**	**R**	S	S	S	S	I	S	S	S	S	S	S	S	**4/O:3**
YE28	I	S	**R**	S	S	S	S	I	S	S	**R**	S	S	S	S	**4/O :3**
YE29	**R**	S	**R**	S	S	S	S	**R**	S	S	**R**	S	S	S	S	**4/O:3**
YE30	I	S	**R**	S	S	S	S	**R**	S	S	S	S	S	S	S	**4/O:3**
YE31	S	**R**	**R**	S	S	**R**	S	S	S	S	**R**	S	S	S	S	**1A/O:5**

Table 17. Antimicrobial resistance of strains

Strains	AK	AMC	AMP	C	CAZ	CFM	CIP	CN	CRO	CTX	FOX	IPM	SXT	TE	TIM	Bio/sero type
YE32	S	**R**	**R**	S	S	**R**	S	S	S	S	**R**	S	S	S	S	**1A/ND**
YE33	S	**R**	**R**	S	S	**R**	S	S	S	S	**R**	S	S	S	S	**1A/O:5**
YE34	S	**R**	**R**	S	S	S	S	S	S	S	**R**	S	S	S	S	**1A/O:8**
YE35	S	S	**R**	S	S	S	S	S	S	S	**R**	S	S	S	S	**4/O:3**
YE36	S	**R**	**R**	S	S	**R**	S	S	S	S	**R**	S	S	S	S	**1A/ND**
YE37	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**1A/ND**
YE38	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**1A/ND**
YE39	S	S	**R**	S	S	**R**	S	S	S	S	S	S	S	S	S	**1A/ND**
YE40	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**1A/ND**
YE41	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**1A/ND**
YE42	S	**R**	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**4/O:3**
YE43	S	**R**	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**4/O:3**
YE44	S	**R**	**R**	S	S	S	S	S	S	S	**R**	S	S	S	S	**4/O:3**
YE45	S	**R**	**R**	S	S	S	S	S	S	S	**R**	S	S	S	S	**1A/O:5**
YE46	S	**R**	**R**	S	S	**R**	S	S	S	S	**R**	S	S	S	S	**1A/ND**

Table 17. Antimicrobial resistance of strains

Strains	AK	AMC	AMP	C	CAZ	CFM	CIP	CN	CRO	CTX	FOX	IPM	SXT	TE	TIM	Bio/sero type
YE47	S	**R**	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**4/O:3**
YE48	S	**R**	**R**	S	S	**R**	S	S	S	S	**R**	S	S	S	S	**4/O:3**
YE49	S	**R**	**R**	S	S	S	S	S	S	S	**R**	S	S	S	S	**4/O:3**
YE50	S	**R**	**R**	S	S	S	S	S	S	S	**R**	S	S	S	S	**4/O:3**
YE51	S	**R**	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**4/O:3**
YE52	S	**R**	**R**	S	S	**R**	S	S	S	S	S	S	S	S	S	**4/O:3**
YE53	S	**R**	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**4/O:3**
YE54	S	**R**	**R**	S	S	S	S	S	S	S	**R**	S	S	S	S	**1A/ND**
YE55	S	**R**	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**1A/ND**
YE56	S	**R**	**R**	S	S	S	S	S	S	S	**R**	S	S	S	S	**1A/ND**
YE57	S	**R**	**R**	S	S	S	S	S	S	S	**R**	S	S	S	S	**1A/ND**
YE58	S	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	**1A/ND**
YE59	S	**R**	**R**	S	S	S	S	S	S	S	**R**	S	S	S	S	**1A/ND**
Total R %	1.7	55.2	100	15.5	0	18.9	0	6.9	0	0	39.7	0	10.3	0	0	

Chart 1. Antimicrobial resistance of *Y.enterocolitica* strains

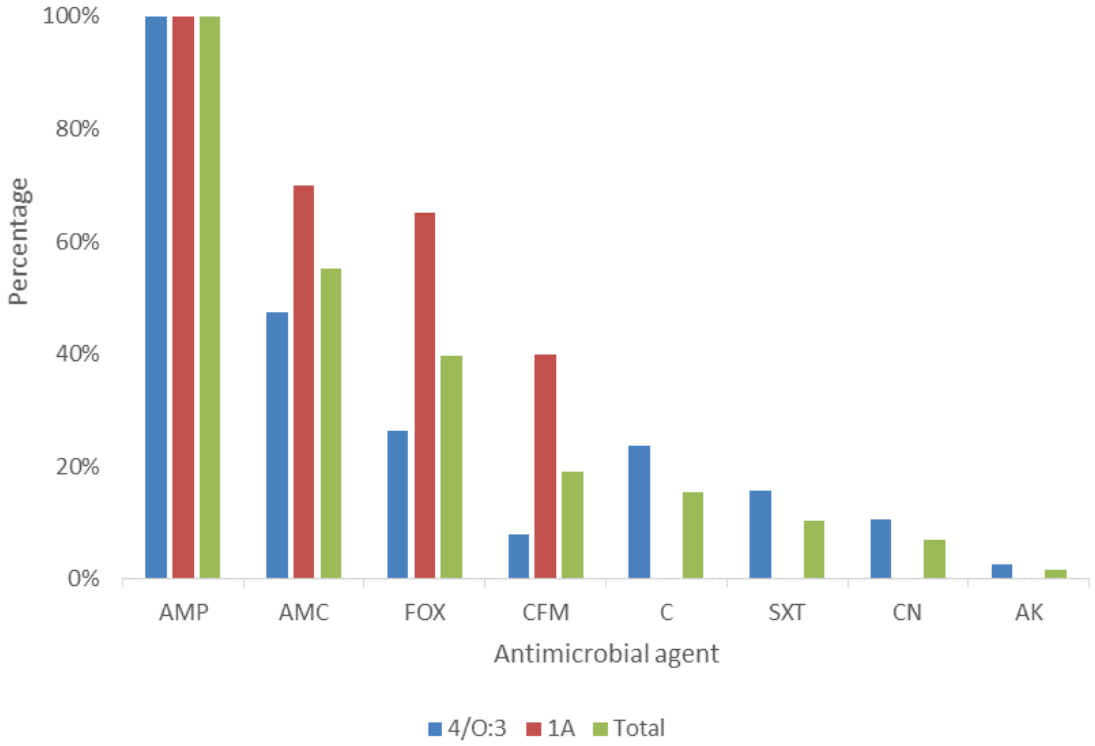

Table 18. Resistance patterns of *Y. enterocolitica* isolates

Bio/ serotype	**N**	**R=2**	**R=3**	**R=4**	**R=5**
1A	20	AmpCfm (1)	AmcAmpFox (6)	AmcAmpCfmFox (7)	
		AmcAmp (1)			
4/O:3	38	AmcAmp (8)	AmcAmpFox (4)	AmcAmpCSxt (2)	AmcAmpCCnSxt (1)
		AmpC (3)	AmcAmpCfm (2)	AkAmpCnFox (1)	
		AmpFox (3)	AmpCSxt (2)	AmpAmcCfmFox (1)	
		AmpCn (2)		AmpCFoxSxt (1)	
Total	**58**	**18 (31%)**	**14 (24.1%)**	**12 (20.6%)**	**1 (1.7%)**

Chart 2. Antimicrobial resistance of *Y.enterocolitica* strains from carcasses

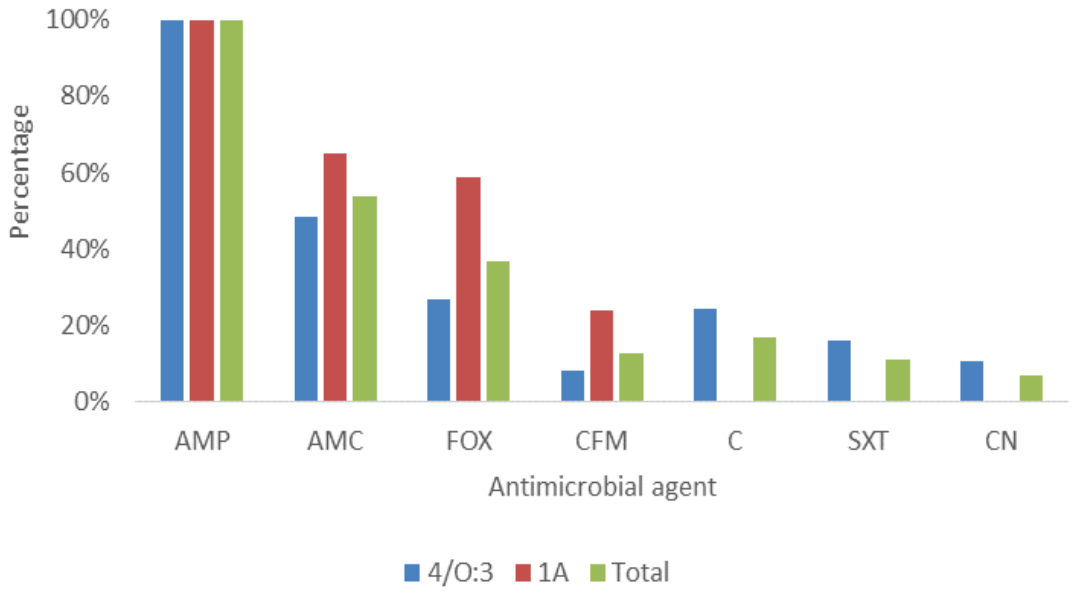

Table 19. Resistance patterns of *Y. enterocolitica* isolates from carcasses

Bio/ serotype	**N**	**R=2**	**R=3**	**R=4**	**R=5**
1A	17	AmcAmp (1)	AmcAmpFox (4)	AmcAmpCfmFox (5)	
4/O:3	37	AmcAmp (8)	AmcAmpFox (4)	AmcAmpCSxt (2)	AmcAmpCCnSxt (1)
		AmpC (3)	AmcAmpCfm (2)	AkAmpCnFox (1)	
		AmpFox (3)	AmpCSxt (2)	AmpAmcCfmFox (1)	
		AmpCn (2)		AmpCFoxSxt (1)	
Total	**54**	**17 (31.5%)**	**12 (22.2%)**	**10 (18.5%)**	**1 (1.9%)**

3.5 Inv gene sequence analysis

Inv gene sequence analysis did not reveal a sequence pattern between the isolated pathogenic *Y. enterocolitica* 4:O3 and non-pathogenic strains 1A since both were in all clusters. Considering the current results the sequence differences in the *inv* gene do not correlate with the biotype.

4 Discussion

Yersiniosis remains the third most commonly reported bacterial food-borne zoonosis in the EU in 2017. There was a significant decreasing trend in yersiniosis cases in the EU between 2008 and 2017, but in the last 5 years (2013–2017) the trend did not show any significant increase or decrease. The total number of reported food-borne yersiniosis outbreaks in the EU varied around 10 during 2013–2017, and outbreak-related cases were below 50 with peaks of more than 100 cases in 2017 and 2014. Almost all cases reported in 2017 were infected domestically or through travel within the EU. Among the 248 travel-associated cases with known information on probable country of infection, 48.0% of the cases represented travel within EU. Spain, Italy and Greece were the most frequently reported travel destinations within the EU (17.4%, 8.0% and 7.5%, respectively) (EFSA, 2018).

Y. enterocolitica has been the most common species reported to be isolated from human cases in Europe with commonest the bio-serotype 4/O:3 (EFSA, 2018), which is isolated from slaughtered pigs, particularly from their tonsils and lymph nodes and these tissues contaminate the carcasses during the slaughtering process (Laukkanen-Ninios et al., 2014). Although the reporting of *Yersinia* occurrence or prevalence in food and animals is not mandatory, member states can report monitoring data on *Yersinia* to the ECDC in accordance with the Zoonoses Directive 2003/99/EC. At present, there is no harmonized surveillance of *Yersinia* in the EU for food or animals and *Yersinia* food and animal monitoring data submitted by the member states to EFSA are collected without harmonized protocol. Thus there are no relevant data provided from Greece since there is no official surveillance system for *Yersinia* infections (EFSA, 2018).

In USA, yersiniosis has been the sixth most commonly reported bacterial food-borne zoonosis during 2018 since the FoodNet identified 465 cases of yersiniosis with incidence rate 0.9 case/100,000 population. The 2018 *Yersinia* incidence was significantly increased (58%) compared with the incidence reported during 2015–2017 (CDC, 2018; 2019). Data about the number of sporadic cases, or potential outbreaks and the *Yersinia* species involved in these incidents are missing.

In New Zealand, the Institute of Environmental Science and Research (ESR) reported 918 cases of yersiniosis during 2017. The 2017 notification rate (19.2 per 100,000) was higher than the 2016 rate (18.3 per 100,000, 858 cases) and the number of notifications for yersiniosis has been steadily increasing since 2010. One outbreak due to *Yersinia* involving

five cases was reported in 2017. *Y. enterocolitica* has been the most common species isolated from human cases with most common the biotype 2 (ESR, 2019).

In Greece, published information on the occurrence of *Y. enterocolitica* in humans shows that the most prevalent bio-serotype is 4/O:3 and the most vulnerable subjects are the β-thalassemia patients. *Y. enterocolitica* has been isolated in 0.3-2.4% from human clinical samples with most common bioserotype 4/O:3 (Daneilides and Kansouzidou-Kanakoudi, 1989; Kansouzidou et al., 1990; Gianneli et al., 1992; Trikka-Grafakou et al., 1994; Kouppari et al., 1995; Demertzi et al., 1999; Stamatopoulou et al., 2004; Panagiotaki et al., 2008; Palaiologou et al., 2011; Kiratsa, 2013). There is no official information reported from National Public Health Organization since yersiniosis is not included in the Mandatory Notification System

During 1984-1988 in the Infectious Diseases Hospital in Thessaloniki, 44 strains of *Y. enterocolitica* were isolated from diarrhea patients, one of which from pus of osteomyelitis in a patient with coexisting diarrhea. The majority of strains (32) were identified as human pathogenic belonging to bio-serotype 4/O:3 (Daneilides and Kansouzidou-Kanakoudi, 1989). Another study in this hospital during the same period reported the isolation of 31 *Yersinia* strains, from 30 out of 3265 children with gastroenteritis 23 strains were identified as 4/O:3 *Y. enterocolitica*. 53% of children were under two years old and three of them were β-thalassemia patients, one of them with concurrent osteomyelitis (Kansouzidou et al., 1990).

Gianneli et al. (1992) in Piraeus Tzaneio Hospital during 1988-1990, detected *Y. enterocolitica* in 2.4% of diarrhea patients most of which were children under 14 years old. During 1986-1993, in Agia Sofia Pediatric Hospital in Athens, 70 *Y. enterocolitica* strains were isolated from 57 children, 34 of which suffered from β-thalassemia aged 2-20 years old. Seven of them had septicemia, five terminal ileitis and septicemia and the rest terminal ileitis mimicking appendicitis. Most strains were of bio-serotype 4/O:3 and two of 2/O:9 (Trikka-Grafakou et al., 1994). At Kiriakou Pediatric Hospital in Athens during 1991-1993, *Y. enterocolitica* was detected in 26 (0.7%) of diarrhea patients of age 2 months-14 years old, five of which suffered from β-thalassemia (Kouppari et al., 1995). Another study in Laiko Hospital in Athens during 1991-1993, detected *Y. enterocolitica* in 1.44% of diarrhea patients (Demertzi et al., 1999).

Stamatopoulou et al. (2005) during 2000-2003 in Xanthi General Hospital isolated a single *Y. enterocolitica* strains among 1046 clinical samples. Similarly Panagiotaki et al.

(2008) detected *Y. enterocolitica* in only 0.1% of clinical samples in Kiriakou Pediatric Hospital in Athens during 2003-2005. In the same hospital prevalence of *Y. enterocolitica* during 2003-2005 varied from 2.8% to 4.1% with the majority (98.1%) of strains identified as 4/O:3 (Palaiologou et al., 2011). Kiratsa (2015) examined 60 strains in Thriasio Hospital in Elefsina during 2003-2005. The strains were isolated from β-thalassemia patients 6 months to 3 years old that suffered from enteritis with/without septicemia. From stool and blood cultures, the 59 strains were identified as 4/O:3 and 1 as 2/O:9.

Concerning the detection of *Y. enterocolitica* in animal tissues and food samples in Greece there is scarce published information. Kansouzidou et al. (1995) found *Y. enterocolitica* in 25% of 376 pig tongue swabs with 10% positive in bio-serotype 4/O:3 and 14.8% in biotypes 1,2. Another study examined 455 tonsil swabs from pigs and detected *Y. enterocolitica* in 15.2% of with predominant serotype O:3 (12.7%) and only 1.5% the non O:3, O:9 serotypes (Kechagia et al., 2007).

As far as food is concerned there are only a few studies for *Yersinia* prevalence in pasteurized and non-pasteurized milk (Kansouzidou-Kanakoudi et al., 1990; Gousia, 2008), various meat samples (Gousia et al., 2011) and vegetables (Kansouzidou-Kanakoudi and Daneilides, 1994; Xanthopoulos et al., 2010). A study on 298 of non-pasteurized and 50 samples of pasteurized cow milk detected 13 *Yersinia* strains only in non-pasteurized milk (4.4%), six of which were *Y. enterocolitica*, the four belonging to bio-serotype 2/ O:5,27 and one to biotype 1 (Kansouzidou-Kanakoudi et al., 1990). In a previous study performed by the Microbiology laboratory (UOI) there were examined 120 of non-pasteurized and 120 samples of pasteurized cow milk, 120 pork meat samples, 80 beef and 80 goat and sheep meat samples, and *Y. enterocolitica* was detected in 20.8% of the pork meat samples and in 1.7% of the pasteurized cow milk (Gousia et al., 2011).

In a study by Kansouzidou-Kanakoudi and Daneilides (1994) on 132 vegetable samples there were found 4 (3%) samples positive to biotype 1 *Y. enterocolitica*. Xanthopoulos et al. (2010) from 26 different RTE salads isolated two strains of *Y. enterocolitica* by both culture-dependent methods and by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole-cell proteins.

To our knowledge in the present study it has been the first time in Greece that a sufficient number of food samples (145 pork carcasses and 144 vegetables) is examined for the presence of pathogenic *Y. enterocolitica* and the isolates are fully investigated (biotyping, serotyping, virulence genes, antimicrobial susceptibility).

4.1 Detection

In this study, 15.9% of the pig carcass swabs were positive for *Y. enterocolitica*. Human pathogenic bio-serotype *Y. enterocolitica* 4/O:3 isolates were detected in 10.3% of carcass swabs, while biotype 1A in 6.2%. These data show that a considerable proportion of pigs slaughtered in Greece carried human pathogenic *Y. enterocolitica* isolates. This study found slightly lower percentage than a previous study executed by our department that had found 20.8% of pork meat samples positive to *Y. enterocolitica* (Gousia et al., 2011) but there was no information for the subtypes of isolates. Prevalence rates of *Y. enterocolitica* originated from pork are classified in Table 20.

In the present doctoral study, the detection rate of *Y. enterocolitica*-positive samples in pork (15.9%) is similar to those reported in Norway, Belgium, Italy and Sweden. In Norway, Nesbakken et al. (1991) detected 4/O:3 *Y. enterocolitica* in 18% of raw pork and Johannessen et al. (2000) found *Y. enterocolitica* positive in 2% of raw pork products by culture method while by PCR in 17% (Johannessen et al., 2000). In Belgium, bio-serotype 4/O:3 was detected in 11.4-15.7% of carcasses (Van Damme et al., 2013a, 2013b). On the contrary, in Italy the predominant biotype in 15.2% of pork products has been found to be 1A (Bonardi et al., 2010). In Sweden, the percentage of 16% in carcasses was detected by PCR (Lindbland et al., 2007). Other studies from Norway, Italy and Germany reported lower rates. In Germany, low rate of 0.3% *Y. enterocolitica* serotype O:3 was detected in carcasses (Gutler et al., 2005). Unlike the above studies, in Italy Bonardi et al. (2003, 2007, 2013) reported low detection rate up to 2.4% in carcasses and in Norway 8.3% of 4/O:3 in carcasses (Nesbakken et al., 2008).

Studies from Austria, Belgium, Lithuania and Finland reported higher prevalence. High rate of pork meat (43.3%) was *Y. enterocolitica* positive and 16.7% was identified as serotype O:3 in Austria (Mayrhofer et al., 2004). In Belgium, Wauters et al. (1988) found positive 25% of ground pork and Van Damme et al. (2015) detected 39.7% of bio-serotype 4/O:3 in carcasses. The same bio-serotype (4/O:3) was found in 25% of carcasses in Lithouania (Novoslavskij et al., 2013). In Finland, detection rates were different depending on the method; 2-12% of minced meat was 4/O:3 positive by culture and 25% by PCR (Fredriksson-Ahomaa et al, 1999, 2001a), 6.3% by culture and 21% by PCR in carcasses (Fredriksson-Ahomaa, 2000b), 26% by culture in carcasses (Laukkanen et al., 2010b) and 22% 4/O:3 positive by PCR in cheek meat samples (Laukkanen-Ninios et al., 2014).

Table 20. Prevalence rates of *Y. enterocolitica* originated from pork

Prevalence	Bio/ serotype	Country	Detection method	Reference
44.4%	-	New Zealand	PCR	Hudson et al., 2008
33.3%			Culture	
43.3%	-	Austria	Culture	Mayrhofer et al., 2004
16.7%	O:3			
39.7%	4/O:3	Belgium	Culture	Van Damme et al., 2015
25%	-	Belgium	Culture	Wauters, et., 1988
25%	4/O:3	Lithuania	Culture	Novoslavskij et al., 2013
26%	-	Finland	culture	Laukkanen et al., 2010b
25%	-	Finland	PCR	Fredriksson-Ahomaa et al, 1999, 2001a
2-12%	4/O:3		culture	
22%	4/O:3	Finland	PCR	Laukkanen-Ninios et al., 2014
21%	-	Finland	PCR	Fredriksson-Ahomaa, 2000b
6.3%			culture	
20.8%	-	Greece	culture	Gousia et al., 2011
18%	4/O:3	Norway	culture	Nesbakken et al., 1991
17%	-	Norway	PCR	Johannessen et al., 2000
2%	O:3		culture	
16%	-	Sweden	PCR	Lindbland et al., 2007
15.9%	-	Greece	culture	Present study
10.3%	4/O:3			
6.2%	1A			
15.2%	1A	Italy	culture	Bonardi et al., 2010
11.4-15.7%	4/O:3	Belgium	culture	Van Damme et al., 2013a, 2013b
8.3%	-	Norway	culture	Nesbakken et al., 2008
3-4.8%	-	Japan	culture	Fukushima, 1985, 1997
2.4%	-	Italy	culture	Bonardi et al., 2003, 2007, 2013
0.3%	O:3	Germany	culture	Gutler et al., 2005

Since higher prevalence of pathogenic *Y. enterocolitica* has been obtained using PCR detection there is probably an underestimation of the true prevalence on carcasses and meats due to the low sensitivity of the culture methods. However, molecular methods should be combined with conventional cultural techniques, since isolation is crucial when studying the epidemiology of the pathogen.

Our findings in regards to predominant bio-serotype 4/O:3 is consistent with the above studies which propose that *Y. enterocolitica* 4/O:3 is the most common bio-serotype isolated in Europe.

Detection rates in Japan and New Zealand are variable. Fukushima et al. (1985, 1997) reported 3-4.8% of pork products *Y. enterocolitica* positive in Japan (Fukushima, 1985, 1997), while in New Zealand Hudson et al. (2008) found *Y. enterocolitica* in 44.4% by PCR and 33.3% by culture in pork meat.

Though the role of vegetables in the epidemiology of human yersiniosis has so far not been investigated, there are studies that have detected *Y. enterocolitica* on fresh vegetables (Delmas and Vidon, 1985; Kansouzidou-Kanakoudi and Daneilides, 1994; Abadias et al., 2008; Cardamone et al., 2015; Losio et al., 2015; Nousiainen et al., 2016; Verbikova et al., 2018) and RTE salads (Lee et al., 2004; Cocolin and Comi, 2005; Abadias et al., 2008; Cardamone et al., 2015; Losio et al., 2015; Verbikova et al., 2018).

In our study *Y. enterocolitica* was detected in 4 out of 144 vegetable samples (2.8%). A single sample (0.7%) was identified as bio-serotype 4/O:3 (non-organic lettuce) and three (2,1%) as 1A/O-untypeable (two mixed salads and one organic lettuce), all isolated by cold enrichment for 7 days. A study by Kansouzidou-Kanakoudi and Daneilides (1994) reported similar results; among 132 vegetable samples examined by cold enrichment for 21 days and there were found 4 (3%) samples positive to biotype 1 *Y. enterocolitica* (lettuce, cucumber, parsley, dill). In another study in Greece, Xanthopoulos et al. (2010) from 26 different RTE salads isolated two strains of *Y. enterocolitica* by both culture-dependent methods and by SDS–PAGE of whole cell proteins. Prevalence rates of *Y. enterocolitica* originated from vegetables are classified in Table 21.

Table 21. Prevalence rates of *Y. enterocolitica* originated from vegetables

Prevalence	Bio/ serotype	Country	Detection method	Reference
33%	-	Finland	culture	Nousiainen et al., 2016
32.3%	-	France	culture	Delmas and Vidon, 1985
11.1%	O:3	Italy	culture and PCR-DGGE	Cocolin and Comi, 2005
7.4%	1A	Czech Republic	culture	Verbikova et al., 2018
3%	1	Greece	culture	Kansouzidou-Kanakoudi and Daneilides, 1994
2.8%	-	Greece	culture	Present study
2.1%	1A			
0.7%	4/O:3			
2.1%	-	Spain	culture	Abadias et al., 2008
0.8%	-	Italy	culture	Cardamone et al., 2015
0.47%	-	Italy	culture	Losio et al., 2015

Similar low detection rates were reported in Spain and Italy. In Spain, Abadias et al. (2008) showed low prevalence of *Y. enterocolitica* of 2.1% (5/236) in RTE salads and fresh. A similarly low prevalence was described by two studies in Italy. Cardamone et al. (2015) and Losio et al. (2015) isolated *Y. enterocolitica* from 0.8% and 0.47% of fresh and

RTE vegetables respectively, using the ISO 10273:2003 reference method. Losio et al. (2015) also examined the samples using "in house" RT-PCR method targeting the virulence *ail* gene none of which was found positive. There is no information for the subtypes of isolates.

Other studies from France, Italy, Finland and Czech Republic reported higher prevalence. In France, Delmas and Vidon (1985) reported that 21 samples (32.3%) were found to be contaminated with *Y. enterocolitica*. Although they found a large variety of environmental serogroups of *Y. enterocolitica*, no human pathogenic serotypes (O:3 and O:9) were isolated. Similarly, a high prevalence of *Yersinia* spp. was observed in Finland where 100 fresh packed leafy green vegetables were examined by cold enrichment for 8 d at 4 °C and alkaline treatment. *Yersinia* spp. were found in 33% of the samples, however, *ail*-positive *Y. enterocolitica* (associated with disease) was isolated only from a single mixed leafy green product containing arugula and spinach (Nousiainen et al., 2016). In Italy, another study by Cocolin and Comi (2005) examined 27 samples of RTE by both culture-dependent and PCR-DGGE (Denaturing Gradient Gel Electrophoresis) analysis and 3 samples (11.1%) were found serotype O:9 positive. A study conducted in Czech Republic found *Y. enterocolitica* positive in 7.4% of fresh and RTE vegetables originated from Czech Republic and other EU countries. All 11 isolates were biotype 1A and one carried the *ail* gene (Verbikova et al., 2018).

In Korea, Lee et al. (2004) reported rate similar to our study by examining 673 RTE vegetable samples by cold enrichment for 10 days at 10°C. Of these samples, 27 (4.0%) were found to be contaminated with *Yersinia* and 18 (2.7%) with *Y. enterocolitica*. In total most strains (77.8%) belonged to biotype 1A and were O-untypeable (61.1%).

4.2 Comparison of isolation methods for the recovery of human pathogenic Y. enterocolitica

Comparison of carcass swab results from different studies is difficult due to differences in study design, pig populations, slaughter age of pigs and slaughter procedures of each country, the areas of the carcass that are swabbed, and culture/molecular methods that are used (Laukkanen et al., 2010b; Van Damme et al., 2013b, 2015; Bonardi et al., 2014; Petsios et al., 2016). In the present study, the hind limb, lateral area was selected for

swabbing. Van Damme et al. (2015) showed the mandibular region as the higher contaminated area of the carcass (29%), followed by the pelvic duct, split surface, and sternum (varying between 7% and 16%). Similarly, Laukkanen et al. (2010b) and Nesbakken (1988) also found the highest number of carcasses contaminated at the chest and head region. Therefore, Van Damme et al. (2015) concluded that inclusion of the head area when analyzing carcasses for the presence of *Y. enterocolitica* results in a considerable increase of the overall carcass prevalence.

In our study, cold enrichment was the most efficient isolation method for the detection of human pathogenic *Y. enterocolitica* 4/O:3. The relative sensitivity of the combination of cold enrichment for 7 days with or without alkali treatment was higher (73.3%) than others isolation procedures providing a significantly better performance than direct plating ($p=0.0268$) and selective enrichment ($p=0.0092$), whereas no significant difference was observed in the recovery rates of cold enrichment for 14 days ($p = 0.1394$). Alkali treatment after cold enrichment for 7 days did not significantly increased the recovery of *Y. enterocolitica* 4/O:3 ($P = 0.1394$).

Similarly, the Van Damme et al. (2013a) study in carcass swabs found cold enrichment more effective than direct plating and selective enrichment for recovery of *Y. enterocolitica* O:3 ($P < 0.001$) with its relative sensitivity in 90.3%. Direct plating and selective enrichment detected 20.8% and 25.9% of positive samples, while on our study 26.7% and 20% respectively. Unlike our findings, cold enrichment for 14 days with alkali treatment was more efficient in isolating pathogenic *Y. enterocolitica* than 7 days without alkali treatment ($P < 0.001$).

In another study Van Damme et al. (2013b) compared PSB enrichment at 25°C, selective enrichment in ITC and cold enrichment in PMB (phosphate-buffered saline supplemented with 1% mannitol and 0.15% bile salts broth). Overall, cold enrichment in PMB was significantly less effective in isolating pathogenic *Y. enterocolitica* 4/O:3 from carcass swabs compared to enrichment in PSB at 25°C ($p = 0.047$), whereas cold enrichment recovered more 4/O:3 positive carcasses than selective ITC enrichment, but the difference was not significant ($p = 0.073$). Similarly to their previous study and unlike to our findings, cold enrichment for 14 days recovered pathogenic *Y. enterocolitica* from significantly more carcass samples than 7 days of cold enrichment - this time when using KOH treatment ($p = 0.0092$).

Recently, cold enrichment procedure using either PSB or CEB (cold enrichment broth) at 4 °C for altogether 22 ± 1 days and plating on CIN with and without KOH treatment has been suggested in the informative Annex D of EN ISO 10273:2017 to be used as an optional procedure (Hallanvuo et al., 2019).

To summarize the data about isolation methods none of the methods was proved efficient to detect all of the 4/O:3 positive samples and none of the samples were detected as positive by all methods. Therefore, when a sample is investigated for the presence of pathogenic *Y. enterocolitica* the use of multiple culture methods is imperative to achieve the maximum efficiency.

4.3 Typing and virulence gene distribution

Virulence gene investigation showed that all 4/O:3 isolates of carcasses carried the *ail*, *ystA*, *inv*, *virF*, *myfA* and *foxA* genes. *YadA* gene was found in 64.9% and none were positive for the *ystB* gene. The most common virulence-associated gene in biotype 1A isolates of carcasses was the *inv* (100%), followed by *ystB* (94.1%), and *foxA* (84.4%) genes. All 1A isolates were negative for the other target genes. Bonardi et al. (2010) from pork meat samples detected 1A isolates positive for *ystB* (90%), *inv* (30%) and *ystA* (66.6%) genes. Another study of Bonardi et al. (2013) in carcass swab samples found a single 4/O:3 isolate which carried *ail*, *ystA*, *inv* and *yadA* genes, while the 1A strains were positive for *inv* (100%) and *ystB* only. Van Damme et al. (2013a) tested isolates from carcass swab samples for the presence of *virF* gene located on the pYV and found it in 80.5% of 4/O:3 strains.

The most common virulence-associated genes in isolates originated from vegetables for biotype 1A were the *inv* (100%) and *ystB* (100%), followed by *foxA* (66.6%) genes, while 4/O:3 isolate carried the *ail*, *foxA*, *ystB* and *myf*. In Lee et al. (2004) study only three out of 18 *Y. enterocolitica* isolates were shown to have a virulence gene. The bio-serotype 3/O:3 strain (crown daisy isolate) had both *yst* and *ail* genes, while 3B/O-untypeable (Chinese cabbage isolate) and 1A/O-untypeable (spinach isolate) contained only the *yst* gene. Interestingly, Verbikova et al. (2018) detected the virulence *ail* gene in a 1A isolate from RTE vegetable, which is generally regarded as non-pathogenic. The presence of virulence gene *ail* in biotype 1A strains has been previously reported (Tennant et al., 2003; Kraushaar et al., 2011).

4.4 Antimicrobial resistance

Studies have reported resistance to ampicillin and first generation cephalosporins due to *Y. enterocolitica* ability to produce β-lactamases. On the other hand, *Y. enterocolitica* is usually susceptible to aminoglycosides, chloramphenicol, tetracyclines, trimethoprim/ sulfamethoxazole, imipenem, the third generation cephalosporins and fluoroquinolones.

All 54 *Y. enterocolitica* isolates originating from carcass swabs were susceptible to cefotaxime, ceftazidime, ceftriaxone (third generation cephalosporins), ciprofloxacin, imipenem, tetracycline and ticarcillin/clavulanic acid. On the contrary all isolates were resistant to ampicillin. Resistances to amoxicillin/clavulanic acid (53.7%), cefoxitin (37%), chloramphenicol (16.7%) and cefixime (13%) were the most common. Among pathogenic 4/O:3 isolates the highest resistance was found to amoxicillin/clavulanic acid (48.6%), while some isolates were resistant to cefoxitin (27%) and chloramphenicol (24.3%). A high proportion of the 1A isolates were resistant to amoxicillin/clavulanic acid (64.7%) and cefoxitin (58.8%).

A study by Gousia et al. (2011) in pork meat isolates found also high resistance rates in ampicillin (96.3%), amoxicillin/clavulanic acid (77.8%) and third generation cephalosporins cefoxitin and cefuroxime (55.6%). There were also isolates resistant to ciprofloxacine, ceftriaxone and imipenem; however, there is no information about the bio-serotypes of these isolates.

In Austria, Mayrhofer et al. (2004) from pork meat detected *Y. enterocolitica* isolates the majority of which were serotype O:3 and were found susceptible to tetracycline, gentamicin, kanamycin, sulfonamids, nalidixic acid, trimethoprim, chloramphenicol and streptomycin. In Italy, 1A isolates also originating from pork meat samples were resistant to cefotaxime, ciprofloxacin, chloramphenicol, nalidixic acid, streptomycin, sulfonamide, tetracycline, trimethoprim, and sulfamethoxazole/trimethoprim (Bonardi et al., 2010). Similar to our study resistance rates were found to ampicillin (85%), and amoxicillin/clavulanic acid (40%) but all isolates were resistant to erythromycin. Bonardi et al. (2013) from finishing pigs isolated *Y. enterocolitica* strains which all were susceptible to ciprofloxacin, ceftazidime, cefotaxime, chloramphenicol, enrofloxacin, gentamicin, kanamicin and neomycin. Resistances to first generation cephalosporin cephalothin (92%), ampicillin (89%) and amoxicillin/clavulanic acid (8%) were the most common. Among pathogenic 4/O:3 isolates, resistance to cefalothin and ampicillin was

both found in 91.3% of the isolates. Resistance to amoxicillin/clavulanic acid was observed in only 10% of the biotype 1A isolates in contrast to higher resistance rates of 1A in our study (64.7%).

The four *Y. enterocolitica* isolates of our study were all susceptible to amikacin, chloramphenicol, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, trimethoprim/sulfamethoxazole tetracycline and ticarcillin/clavulanic acid. On the contrary all isolates were resistant to ampicillin and the three 1A shared the AmcAmpCfmFox resistance pattern. In another study in Greece biotype 1 isolates from vegetable samples were all resistant to ampicillin and cephalothin (Kanakoudi and Daneilides, 1994). Verbikova et al. (2018) found *Y. enterocolitica* 1A isolates similarly resistant to ampicillin (100%), followed by chloramphenicol (22.2%) and tetracycline (5.6%). In Korea, *Y. enterocolitica* isolates were most resistant to cephalothin (100%), followed by ampicillin (94%) and carbenicillin (83%). There are no specific published data regarding the relation of antimicrobial susceptibility and bio-serotype of the isolates, although the majority of these isolates belonged to biotype 1A (Lee et al., 2004).

5 Conclusions

In the present study *Y. enterocolitica* was detected in pork carcasses as well as in vegetables. The majority of the isolated strains belonged to the human pathogenic bio-serotype 4/O:3 that prevails among pig population and based on the existing published data also prevails in clinical strains in Greece. Biotype 1A strains which may have a potential virulence were also isolated. Pigs remain the major reservoir for pathogenic *Y. enterocolitica* 4/O:3 spread among breeding pigs and the consequent contamination of pork carcasses depends on the farming and slaughter practices. Modification of the EU legislation regarding slaughtering methods and meat inspection can decrease the risk of infection with human pathogenic *Y. enterocolitica*. Concerning the involvement of vegetables in the epidemiology of human yersiniosis there is need for further investigation both at local and international level. The observed high resistance rates to broad-spectrum penicillins and second generation cephalosporins of the isolates originating from pork carcasses can be attributed to the non prudent use of antibiotics in the pig farms in Greece.

To achieve more accurate investigation of pathogenic *Y. enterocolitica* in foods intended for human consumption, the use of diverse cultural and molecular methods is essential. The development of an EU directive concerning an ISO method for the detection, isolation and typing of the microorganism is needed for efficient surveillance of yersiniosis and effective implementation of prevention and control strategies. Since *Y. enterocolitica* is an emergent foodborne zoonotic pathogen, a One-health approach based on a multi-disciplinary and interdisciplinary collaboration is an absolute necessity to mitigate the emergence and spread of a new foodborne disease.

6 Abstract

Yersiniosis has been one of the most frequently reported zoonosis in EU countries and *Yersinia enterocolitica* is the most prevalent species isolated from human cases with serotypes O:3, O:9 and O:8 to be the most common. Pigs are considered the major reservoir for *Yersinia*, and pork products are the main source of *Y. enterocolitica* infection in humans. *Y. enterocolitica* has been isolated from bovine meat, raw cow's and raw goat's milk, in ready-to-eat (RTE) salads, as well as from domestic and wild animals from various EU member states.

Y. enterocolitica is a Gram-negative bacterium that belongs to the family *Enterobacteriaceae*. It has the unusual ability among pathogenic enterobacteria to grow well at refrigeration temperatures (psychrophilic). This microrganism can also withstand freezing and survives for extended periods of time in frozen food, even after repeated freezing and thawing. The ability to propagate at refrigeration temperature in vacuum packed or modified atmosphere foods aimed for a prolonged shelf-life is of considerable significance in food hygiene and safety. The large number of organisms in the background microbiota, the presence of non-pathogenic *Yersinia* spp. (including non-pathogenic *Y. enterocolitica* strains) and the low concentration of pathogenic strains, especially in food samples complicate the isolation leading to an underestimated prevalence of pathogenic *Y. enterocolitica* in food. *Y. enterocolitica* is a highly heterogeneous group of bacteria consisting of different subtypes (biotypes and serotypes). Biotyping and serotyping are both used to determine the strain pathogenicity, although virulence markers have also been found in non-pathogenic biotype 1A strains. *Y. enterocolitica* bacteria utilize both chromosomal and plasmid-associated (pYV+) virulence factors that are temperature-dependent. The presence of pYV enables virulent *Yersinia* to survive and multiply in lymphoid tissues of their host.

Pigs from enteropathogenic *Yersinia*-positive farms carry the bacterium in their tonsils and lymph nodes and during the slaughter process the pork carcasses can be contaminated. Fresh vegetables and RTE salads are likely to be contaminated with pathogenic *Yersinia* due to organic fertilizers (manure) used for their production. Storage of vegetables under refrigeration conditions favors growth of *Yersinia*.

Human infections with *Y. enterocolitica* emerge after ingestion of the microorganisms through contaminated food or water or through blood transfusion. Gastrointestinal symptoms range from self-limiting gastroenteritis to acute enteritis (particularly in children), mesenteric

lymphadenitis and terminal ileitis mimicking appendicitis (children older than 5 years). Infection with *Y. enterocolitica* can lead to septicemia in immunosuppressed individuals and patients being treated with desferrioxamine or patients with hemochromatosis.

The objectives of the present study included the following:

1. investigation of the prevalence of pathogenic *Y. enterocolitica* in pork carcasses and fresh vegetables originating from local slaughterhouses and markets of the Epirus region (Northwestern Greece),

2. comparison of different methods for the recovery of human pathogenic *Y. enterocolitica* from pork carcasses,

3. identification of the isolated strains in order to assess the pathogenic nature of the strains (biotype, serotype and virulence properties/markers), the presence of genotypic virulence markers in the recovered isolates and the antimicrobial susceptibility against antimicrobials routinely used in the medical practice,

4. investigation of potential existence of *Inv* gene sequence pattern between the isolated pathogenic and non-pathogenic strains.

A total of 145 carcass surface swabs from equal number of pigs at slaughter and 144 fresh vegetable samples were collected during 12 months period (August 2013-July 2014) from six slaughterhouses located in the Epirus region. Vegetable samples concluded 22 organic and 22 non-organic curly salads, 37 organic and 37 lettuce salads and 26 'ready to eat' (RTE) salads gathered from local retailer shops and supermarkets. RTE salads contained lettuce (three types), carrot, rocket, cabbage, radicchio, parsley, dill, spring onion and curly salad. All samples were analyzed for the detection of *Y. enterocolitica* with different isolation methods; direct plating, selective enrichment and cold enrichment.

Y. enterocolitica strains were biotyped according to the modified Wauters' et al. scheme (1987) and serotyping was performed with commercially available O antisera for the serogroups O:1,2, O:3, O:5, O:8 and O:9. Except for biochemical identification strains were also tested for the unambiguous identification within the genus targeting a 330-bp fragment of the 16S rRNA gene. Presence of virulence genes by PCR were also studied. Susceptibility to antimicrobial agents was tested by the Kirby-Bauer, while inhibition zone results were interpreted according to the breakpoints of the EUCAST guidelines (2014).

Y. enterocolitica was found in 15.9% of the carcass swabs (23/145) and in 2.8% of the vegetable samples (4/144). Of the 145 carcass swabs, 15 (10.3%) were identified as human

pathogenic bio-serotype 4/O:3 and 9 (6,2%) as biotype 1A. Of the 144 vegetable samples, only one (0.7%) was identified as bio-serotype 4/O:3 (non-organic lettuce) and three (2,1%) as 1A (two mixed salads and one organic lettuce).

A total of 59 strains were isolated and biochemically identified as *Y. enterocolitica*: 54 from the carcass swabs and 5 from the vegetable samples. All but one strains were also molecular identified as *Y. enterocolitica* by the detection of the specific of *16S rRNA* gene for the species. YE27 strain isolated from a vegetable sample is presumed as *Y. kristensenii* since its appearance in CIN agar plates is often misinterpreted as *Y. enterocolitica*. Use of stereomicroscope with Henry illumination helps the differentiation between the species.

Pathogenic *Y. enterocolitica* bio-serotype 4/O:3 was detected in total 15 carcass swabs (10.3%) using different isolation methods. None of the methods detected all of the 4/O:3 positive samples and none of the samples were detected as positive by all methods. The relative sensitivity of the combination of cold enrichment for 7 days with or without alkali treatment was higher (73.3%) than others isolation procedures with relative sensitivity. The combined cold enrichment for 7 days provided a significantly better performance than direct plating (p=0.0268) and selective enrichment (p=0.0092), whereas no significant difference was observed in the recovery rates of cold enrichment for 14 days (p = 0.1394).

Of the 54 *Y. enterocolitica* isolates of the carcass swabs, 37 (68.5%) were identified as human pathogenic belonging to bio-serotype 4/O:3 and the rest 17 isolates belonging to non-pathogenic biotype 1A (31.5%). Among these of biotype 1A, only two serotypes were identified: 3 isolates as O:5 and one as O: 8, while a high proportion (76.5%) was O-untypeable. The *ail*, *ystA*, *inv*, *virF*, *myfA* and *foxA* genes were detected in 100% of the 4/O:3 isolates, and the *yadA* gene was found in 64.9%. All 4/O:3 isolates were negative for the *ystB* gene. The most common virulence-associated gene in 1A isolates was the *inv* (100%), followed by *ystB* (94.1%), and *foxA* (84.4%) genes. All 1A isolates were negative for the other target genes.

Of the 4 *Y. enterocolitica* strains of the vegetable samples which were isolated by cold enrichment for 7 days, only one was identified as 4/O:3 and 3 belonging to biotype 1A/O-untypeable. The *ail*, *ystA*, *inv*, *myfA* and *foxA* genes were detected in the 4/O:3 isolate. The most common virulence-associated gene in 1A isolates was the *inv* (100%) and *ystB* (100%), followed by *foxA* (66.6%) genes.

All 58 *Y. enterocolitica* isolates were susceptible to cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, imipenem, tetracycline and ticarcillin/clavulanic acid. On the contrary all isolates were resistant to ampicillin. Resistances to amoxicillin/clavulanic acid (55.2%), cefoxitin (39.7%), cefixime (18.9%) and chloramphenicol (15.5%) were the most common. Among pathogenic 4/O:3 isolates the highest resistance was found to amoxicillin/clavulanic acid (47.4%), while some isolates were resistant to cefoxitin (26.3%) and chloramphenicol (23.7%). A high proportion of the 1A isolates were resistant to amoxicillin/clavulanic acid (70%) and cefoxitin (65%). In addition, 65% of 1A isolates were resistant to both amoxicillin/clavulanic acid and cefoxitin.

Thirteen resistance patterns were observed. The majority (55.1%) of the isolates was resistant to two (31%) or three (24.1%) antimicrobial agents. Resistance to three or more antimicrobials was observed in 26.6% (27/58) of the isolates. A single 4/O:3 isolate (1%) was resistant to five antimicrobials. No isolate was resistant to more than five antimicrobials. The most common resistance patterns were AmcAmpFox (17.2%), AmcAmp (15.5%) and AmcAmpCfmFox (13.8%).

Inv gene sequence analysis did not reveal a sequence pattern between the isolated pathogenic *Y. enterocolitica* 4:O3 and non-pathogenic strains 1A since both were in all clusters. Considering the current results the sequence differences in the *inv* gene do not correlate with biotype.

To our knowledge in the present study it has been the first time in Greece that a sufficient number of food samples (145 pork carcasses and 144 vegetables) is examined for the presence of pathogenic *Y. enterocolitica* and the isolates are fully investigated (biotyping, serotyping, virulence genes, antimicrobial susceptibility).

7 Περίληψη

Εισαγωγή

Η Υερσινίωση είναι μία από τις συχνότερα αναφερόμενες ζωονόσους στις χώρες της Ευρωπαϊκής Ένωσης (ΕΕ) και η *Yersinia enterocolitica* το επικρατέστερο είδος που απομονώνεται από κλινικά περιστατικά με συχνότερους οροτύπους τους O:3, O:9 και O:8. Οι χοίροι θεωρούνται φορείς της *Yersinia* και το χοιρινό κρέας και τα προϊόντα του η κυριότερη πηγή μόλυνσης παθογόνων στελεχών της *Y. enterocolitica* που προκαλούν λοιμώξεις στον άνθρωπο. Η παρουσία της *Y. enterocolitica* έχει αναφερθεί επίσης σε βόειο κρέας, αγελαδινό και γίδινο απαστερίωτο γάλα και σε έτοιμες προς κατανάλωση σαλάτες όπως επίσης και σε κατοικίδια και άγρια ζώα από διάφορες χώρες-μέλη της ΕΕ.

Η *Y. enterocolitica* είναι Gram αρνητικό βακτηρίδιο που ανήκει στην οικογένεια των Εντεροβακτηριοειδών. Είναι ψυχρόφιλος μικροοργανισμός που έχει την ικανότητα να αναπτύσσεται σε θερμοκρασίες ψυγείου, είναι ανθεκτικό σε συνθήκες κατάψυξης και μπορεί να επιβιώσει για παρατεταμένο χρονικό διάστημα σε κατεψυγμένα τρόφιμα, ακόμα και μετά από επαναλαμβανόμενη κατάψυξη – απόψυξη. Η ικανότητα να πολλαπλασιάζεται σε θερμοκρασίες ψύξης σε συσκευασμένα τρόφιμα υπό κενό ή τροποποιημένη ατμόσφαιρα έχει μεγάλη σημασία στην υγιεινή και ασφάλεια των τροφίμων, καθώς και στην βιομηχανία τροφίμων. Ο μεγάλος πληθυσμός της μικροβιακής χλωρίδας που η υπερανάπτυξή τους καλύπτει τις μικροσκοπικές αποικίες της *Yersinia*, η παρουσία μη παθογόνων *Yersinia* spp. (συμπεριλαμβανομένων μη παθογόνων στελεχών *Y. enterocolitica*) και η μικρή συγκέντρωση των παθογόνων στελεχών, ειδικά στα τρόφιμα δυσχεραίνουν την ανίχνευση και απομόνωση του βακτηρίου αυτού υποτιμώντας την πραγματική συχνότητά του στα τρόφιμα.

Το είδος της *Y. enterocolitica* αποτελείται από αρκετά ετερογενή στελέχη και διαφορετικούς υποτύπους (βιοτύπους και οροτύπους). Η βιοτυπία και οροτυπία χρησιμοποιούνται για να καθορίσουν την παθογονικότητα των στελεχών, μολονότι στελέχη του μη παθογόνου βιοτύπου 1A έχουν εντοπιστεί να φέρουν παθογονικούς παράγοντες. Οι παράγοντες αυτοί μπορεί να σχετίζονται με χρωμοσωμικά ή πλασμιδιακά γονίδια (παθογόνο πλασμίδιο pYV - plasmid for *Yersinia* virulence) και η έκφρασή τους ρυθμίζεται από τη θερμοκρασία. Η παρουσία του πλασμιδίου pYV καθιστά τα παθογόνα στελέχη της *Yersinia* ικανά να επιβιώσουν και να πολλαπλασιαστούν στο λεμφικό ιστό του ξενιστή. Τα *inv*, *ail*, *foxA*, *ystA*, *ystB* και *myfA* γονίδια, εντοπίζονται στο χρωμόσωμα, ενώ τα *yadA*, *VirF* και *Yops* στο πλασμίδιο.

Οι χοίροι που προέρχονται από εκτροφές θετικές σε παθογόνα στελέχη *Yersinia* φέρουν το βακτήριο στις αμυγδαλές και στους επιχώριους λεμφαδένες και κατά τη σφαγή μπορεί να επιμολυνθεί ολόκληρο το σφάγιο. Από την άλλη πλευρά, τα φρέσκα λαχανικά και οι έτοιμες προς κατανάλωση σαλάτες είναι πιθανόν να επιμολυνθούν με παθογόνα στελέχη *Yersinia* λόγω της χρήσης οργανικών λιπασμάτων (κοπριά) στην παραγωγή τους. Η συντήρηση των λαχανικών σε συνθήκες ψύξης ευνοεί την ανάπτυξη της *Yersinia*.

Η μετάδοση στον άνθρωπο γίνεται μέσω της κατανάλωσης μολυσμένου τροφίμου ή νερού ή μέσω μετάγγισης αίματος. Τα συμπτώματα της λοίμωξης από *Y. enterocolitica* εξαρτώνται από το στέλεχος (βιο-ορότυπος) που καταναλώθηκε, την ηλικία, την φυσική και ανοσολογική κατάσταση του ξενιστή. Η λοίμωξη στο γαστρεντερικό σύστημα μπορεί να εκδηλωθεί ως εντεροκολίτιδα, οξεία μεσεντέρια λεμφαδενίτιδα και τελική ειλεΐτιδα και συμπτώματα που μπορεί να ομοιάζουν με αυτά της οξείας σκωληκοειδίτιδας κυρίως σε άτομα νεαρής ηλικίας (άνω των 5 ετών). Οι κλινικές εκδηλώσεις ποικίλλουν από αυτοπεριοριζόμενη εντεροκολίτιδα έως συστηματική και θανατηφόρο λοίμωξη. Η σηψαιμία που οφείλεται στη *Y. enterocolitica* μπορεί να συμβεί τόσο σε υγιείς όσο και σε ανοσοκατασταλμένους ή με υποκείμενη νόσο ασθενείς (σακχαρώδης διαβήτης, κίρρωση του ήπατος). Ιδιαίτερα ευπαθή είναι άτομα που λαμβάνουν δεσφεριοξαμίνη και έχουν αυξημένο σίδηρο στο αίμα. Λιγότερο συχνές είναι οι εξωεντερικές λοιμώξεις με ή χωρίς βακτηριαιμία και εκδηλώνονται ως πνευμονία, αποστήματα σε ήπαρ, νεφρούς, σπλήνα και πνεύμονες, οστεομυελίτιδα, αρθρίτιδα, μηνιγγίτιδα, ενδοκαρδίτιδα.

Η *Y. enterocolitica* παράγει δύο τύπους β-λακταμασών. Η β-λακταμάση Α έχει δράση πενικιλλινάσης, ενώ το ένζυμο Β είναι κεφαλοσπορινάση με δυνατότητα επαγωγής. Στα ένζυμα αυτά οφείλεται η αντοχή στις πενικιλλίνες, όπως αμπικιλλίνη, κλοξακιλλίνη, καρβενικιλλίνη, τικαρκιλλίνη και στις κεφαλοσπορίνες πρώτης γενιάς. Επίσης, είναι γενικά ανθεκτική στην ερυθρομυκίνη, κλινδαμυκίνη και βανκομυκίνη. Αντιθέτως, είναι συνήθως ευαίσθητη στις αμινογλυκοσίδες (γενταμικίνη, στρεπτομυκίνη, τομπραμυκίνη, και καναμυκίνη), στις κεφαλοσπορίνες τρίτης γενιάς (κεφτριαξόνη, κεφταζιδίμη, κεφοταξίμη), τετρακυκλίνες, χλωραμφενικόλη, φθοριοκινολόνες (σιπροφλοξασίνη, νορφλοξασίνη και οφλοξασίνη), τριμεθοπρίμη-σουλφαμεθοξαζόλη, ιμιπενέμη και αζτρεονάμη.

Σκοπός

Στους σκοπούς της παρούσας διατριβής περιλαμβάνονται:

1. Η διερεύνηση της παρουσίας *Y. enterocolitica* σε χοιρινά σφάγια και σε φρέσκα λαχανικά. Τα δείγματα συλλέχθηκαν από τα σφαγεία και από διάφορα σημεία πώλησης στην Περιφέρεια της Ηπείρου (ΒΔ Ελλάδα).

2. Η αξιολόγηση της αποτελεσματικότητας των προτεινόμενων διαφόρων μεθόδων απομόνωσης παθογόνων στελεχών της *Y. enterocolitica* που προκαλούν λοιμώξεις στον άνθρωπο

3. Η μελέτη των γονιδίων λοιμογονικότητας και αντοχής σε αντιμικροβιακά των απομονωθέντων στελεχών.

4. Η διερεύνηση της πιθανής παρουσίας μοτίβου αλληλουχίας στο γονίδιο *inv* μεταξύ παθογόνων και μη στελεχών.

Υλικά και μέθοδοι

Συνολικά συλλέχθηκαν 145 επιχρίσματα επιφανείας με χρήση σπόγγου από ισάριθμα χοιρινά σφάγια στη διάρκεια ενός έτους (Αύγουστος 2013 - Ιούλιος 2014) και 144 δείγματα νωπών λαχανικών (Απρίλιος 2013 - Μάρτιος 2014). Στα δείγματα λαχανικών περιλαμβάνονται 22 πράσινες σγουρές σαλάτες βιολογικής καλλιέργειας και 22 συμβατικής καλλιέργειας, 37 σαλάτες μαρούλι βιολογικής και 37 συμβατικής καλλιέργειας και 26 έτοιμες προς κατανάλωση σαλάτες. Τα δείγματα εξετάστηκαν μικροβιολογικά για την ανίχνευση *Y. enterocolitica* με διαφορετικές μεθόδους απομόνωσης: (i) άμεση επίστρωση, (ii) εκλεκτικός εμπλουτισμός και (iii) ψυχρός εμπλουτισμός όπως περιγράφηκε από τους Van Damme και συν. (2013a). Η στατιστική ανάλυση και αξιολόγηση της αποτελεσματικότητας των χρησιμοποιηθέντων μεθόδων έγινε με το τεστ Fisher.

Ο καθορισμός του βιοτύπου των στελεχών έγινε σύμφωνα με το αναθεωρημένο σχήμα των Wauters και συν. (1987) και η ορολογική τυποποίηση με εμπορικά διαθέσιμους αντιορούς για τους οροτύπους O:1,2, O:3, O:5, O:8 και O:9. Εκτός από τη βιοχημική ταυτοποίηση με το σύστημα API 20E, τα στελέχη ταυτοποιήθηκαν και μοριακά ως προς το είδος με την ανίχνευση του *16SrRNA* γονιδίου. Επίσης, με αλυσιδωτή αντίδραση πολυμεράσης μελετήθηκε η παρουσία γονιδίων λοιμογονικότητας. Για τον έλεγχο της

ευαισθησίας των απομονωθέντων στελεχών σε αντιμικροβιακές ουσίες χρησιμοποιήθηκε η μέθοδος της διάχυσης σε άγαρ Bauer-Kirby και ο χαρακτηρισμός του στελέχους με βάση τους ερμηνευτικούς πίνακες που προτείνονται από τον οδηγό EUCAST (2014).

Αποτελέσματα

Στελέχη της *Y. enterocolitica* ανιχνεύθηκαν στο 15.9% των χοιρινών δειγμάτων (23/145) και στο 2.8% των δειγμάτων λαχανικών (4/144). Από τα 145 δείγματα χοιρινών, στα 15 (10.3%) ανιχνεύθηκε ο παθογόνος για τον άνθρωπο βιο-ορότυπος 4/O:3 και στα 9 (6,2%) ο βιότυπος 1A. Από τα 144 δείγματα λαχανικών, μόνο ένα (0.7%) ανιχνεύθηκε θετικό στο βιο-ορότυπο 4/O:3 (μαρούλι συμβατικής καλλιέργειας) και σε 3 (2,1%) βρέθηκε ο βιότυπος 1A (δύο έτοιμες προς κατανάλωση σαλάτες και ένα μαρούλι βιολογικής καλλιέργειας).

Συνολικά απομονώθηκαν 59 στελέχη που ταυτοποιήθηκαν βιοχημικά ως *Y. enterocolitica*: 54 από τα χοιρινά και 5 από τα δείγματα λαχανικών (Πίνακας 13). Όλα εκτός από ένα στέλεχος ταυτοποιήθηκαν και μοριακά ως προς το είδος (Πίνακας 15). Το στέλεχος με κωδικό YE27 που προέρχεται από λαχανικό πιθανολογείται ότι ανήκει στο είδος *Y. kristensenii*, το οποίο ομοιάζει με *Y. enterocolitica* σε τρυβλίο με CIN. Για αυτήν την περίπτωση η παρατήρηση των αποικιών υπό στερεομικροσκόπιο μετά από κατάλληλη εκπαίδευση ενδείκνυται για τη διαφοροποίηση των ειδών (Εικόνα 6).

Η ανίχνευση των θετικών στον παθογόνο βιο-οροτύπο 4/O:3 χοιρινών έγινε με διαφορετικές μεθόδους απομόνωσης (Πίνακας 14). Η σχετική ευαισθησία του ψυχρού εμπλουτισμού για 7 μέρες με ή χωρίς κατεργασία με αλκάλι ήταν μεγαλύτερη (73.3%) από την αντίστοιχη των άλλων μεθόδων. Συγκεκριμένα η μέθοδος ψυχρού εμπλουτισμού για 7 μέρες ήταν στατιστικώς σημαντικά αποτελεσματικότερη από την άμεση επίστρωση σε άγαρ ($p=0.0268$) και τον εκλεκτικό εμπλουτισμό ($p=0.0092$), ενώ δεν παρατηρήθηκε σημαντική διαφορά στην ανίχνευση των θετικών 4/O:3 χοιρινών με τον ψυχρό εμπλουτισμό για 14 μέρες ($p = 0.1394$).

Από το σύνολο των 54 στελεχών προερχομένων από τα χοιρινά, 37 (68.5%) ταυτοποιήθηκαν ως βιο-οροτύπος 4/O:3 και τα υπόλοιπα 17 ως βιότυπος 1A (31.5%). Μεταξύ αυτών, δύο μόνο ορότυποι καθορίστηκαν: σε 3 στελέχη ο O:5 και σε ένα ο O:8, ενώ το μεγαλύτερο ποσοστό (76.5%) δεν ταυτοποιήθηκε με τους διαθέσιμους αντιορούς.

(Πίνακας 15). Τα γονίδια *ail*, *ystA*, *inv*, *virF*, *myfA* and *foxA* ανιχνεύθηκαν σε όλα τα 4/O:3 στελέχη και το *yadA* γονίδιο σε ποσοστό 64.9%, ενώ σε κανένα δεν ανιχνεύθηκε το γονίδιο *ystB*. Τα γονίδια που βρέθηκαν να φέρουν τα 1A στελέχη ήταν τα *inv* (100%), *ystB* (94.1%), και *foxA* (84.4%), ενώ σε κανένα δεν εντοπίστηκαν τα υπόλοιπα γονίδια (Πίνακας 16).

Από τα 4 στελέχη *Y. enterocolitica* των λαχανικών τα οποία απομονώθηκαν με τη μέθοδο ψυχρού εμπλουτισμού για 7 μέρες, ένα μόνο ταυτοποιήθηκε ως 4/O:3 και τρία ως 1A. Τα γονίδια *ail*, *ystA*, *inv*, *myfA* και *foxA* ανιχνεύθηκαν στο στέλεχος 4/O:3. Στα στελέχη βιοτύπου 1A ανιχνεύθηκαν *inv* (100%), *ystB* (100%) και *foxA* (66.6%) (Πίνακες 15 και 16).

Όλα τα 58 στελέχη ήταν ευαίσθητα στην κεφοταξίμη, κεφταζιδίμη, κεφτριαξόνη, σιπροφλοξασίνη, ιμιπενένη, τετρακυκλίνη και τικαρσιλλίνη/κλαβουλανικό οξύ. Αντιθέτως, όλα ήταν ανθεκτικά στην αμπικιλλίνη. Τα μεγαλύτερα ποσοστά αντοχής εμφανίστηκαν στο συνδυασμό αμοξυκιλλίνη-κλαβουλανικό οξύ (55.2%), στην κεφοξιτίνη (39.7%), κεφιξίμη (18.9%) και χλωραμφενικόλη (15.5%). Τα παθογόνα στελέχη 4/O:3 εμφάνισαν μεγαλύτερα ποσοστά αντοχής στο συνδυασμό αμοξυκιλλίνη-κλαβουλανικό οξύ (47.4%), και μερικά στην κεφοξιτίνη (26.3%) και χλωραμφενικόλη (23.7%). Μεγάλο ποσοστό των στελεχών 1A ήταν ανθεκτικό στην αμοξυκιλλίνη-κλαβουλανικό οξύ (70%) και κεφοξιτίνη (65%). Επιπλέον, 65% των 1A ήταν ανθεκτικό και στην αμοξυκιλλίνη-κλαβουλανικό οξύ και στην κεφοξιτίνη (Διάγραμμα 1).

Παρατηρήθηκαν 13 τύποι αντοχής (Πίνακας 18). Η πλειοψηφία των στελεχών ήταν ανθεκτική σε δύο (31%) ή τρία (24.1%) αντιμικροβιακά. Αντοχή σε τρία και πλέον αντιμικροβιακά βρέθηκε στο 26.6% (27/58) των στελεχών, αλλά μόνο ένα (1%) στέλεχος βιο-οροτύπου 4/O:3 ήταν ανθεκτικό σε πέντε αντιμικροβιακά και κανένα σε περισσότερα από πέντε. Οι συχνότεροι τύποι αντοχής ήταν οι AmcAmpFox (17.2%), AmcAmp (15.5%) και AmcAmpCfmFox (13.8%).

Η ανάλυση της αλληλουχίας του γονιδίου *inv* δεν κατέδειξε την ύπαρξη μοτίβου μεταξύ παθογόνων και μη στελεχών. Λαμβάνοντας υπόψιν αυτά τα δεδομένα οι όποιες διαφορές στην αλληλουχία δε σχετίζονται με τον βιότυπο ενός στελέχους.

Συζήτηση

Η Υερσινίωση ήταν η τρίτη συχνότερη αναφερόμενη ζωονόσος στις χώρες της Ευρωπαϊκής Ένωσης (ΕΕ) για το 2017 και η *Yersinia enterocolitica* το επικρατέστερο είδος που απομονώνεται από κλινικά περιστατικά με συχνότερο τον βιο-ορότυπο 4/O:3. Απομονώνεται από σφάγια χοίρων και ειδικά από τις αμυγδαλές και τους επιχώριους λεμφαδένες, ιστοί που επιμολύνουν το σφάγιο κατά τη σφαγή. Μολονότι η δήλωση του επιπολασμού της *Yersinia* σε ζώα και τρόφιμα δεν είναι υποχρεωτική, χώρες-μέλη αναφέρουν δεδομένα σχετικά με τη *Yersinia* στο Ευρωπαϊκό Κέντρο Πρόληψης και Ελέγχου Νόσων. Μέχρι τώρα, δεν υπάρχουν σχετικές για τη *Yersinia* αναφορές από την Ελλάδα καθώς δεν υπάρχει επίσημο σύστημα επιτήρησης για λοιμώξεις από *Yersinia*.

Στην Ελλάδα, οι μέχρι σήμερα δημοσιεύσεις ανθρώπινων υερσινιώσεων αναφέρουν τον βιο-ορότυπο 4/O:3 ως τον πιο επικρατέστερο και πιο ευπαθείς τους ασθενείς με β-μεσογειακή αναιμία. Η *Y. enterocolitica* έχει απομονωθεί σε ποσοστό 0.3-2.4% από κλινικά δείγματα με τους περισσότερους ασθενείς σε ηλικία κάτω των 14 ετών. Επιπλοκές όπως σηψαιμία και οστεομυελίτιδα εμφάνισαν ασθενείς με β-μεσογειακή αναιμία (Δανιηλίδης και Κανσουζίδου-Κανακούδη, 1989; Κανσουζίδου και συν., 1990; Γιαννέλη και συν., 1992; Τρίκκα-Γραφάκου και συν., 1994; Κουππάρη και συν., 1995; Δεμερτζή και συν., 1999; Σταματοπούλου και συν., 2004; Παναγιωτάκη και συν., 2008; Παλαιολόγου και συν., 2011; Κυράτσα, 2013). Δεν υπάρχουν επίσημα στοιχεία από τον Εθνικό Οργανισμό Δημόσιας Υγείας καθώς η Υερσινίωση δε συγκαταλέγεται στα υποχρεωτικώς δηλούμενα νοσήματα.

Όσον αφορά την παρουσία της *Y. enterocolitica* σε ιστούς ζώων και δείγματα τροφίμων η σχετική βιβλιογραφία είναι περιορισμένη. Οι Κανσουζίδου και συν. (1995) ανίχνευσαν τη *Y. enterocolitica* στο 25% από τα 376 επιχρίσματα γλώσσας χοίρου, από τα οποία το 10% θετικό στον βιο-ορότυπο 4/O:3 και 14.8% στους βιοτύπους 1 και 2. Άλλη μελέτη εξέτασε 455 φαρυγγικά επιχρίσματα χοίρων στα οποία ανιχνεύτηκε *Y. enterocolitica* στο 15.2% με κυρίαρχο τον ορότυπο O:3 (12.7%) (Kechagia et al., 2007). Οι μελέτες που αφορούν στα τρόφιμα έγιναν σε δείγματα παστεριωμένου και μη παστεριωμένου αγελαδινού γάλακτος (Κανσουζίδου-Κανακούδη και συν., 1990; Γούσια, 2008), διάφορα δείγματα κρέατος (Gousia et al., 2011) και λαχανικά (Κανσουζίδου-Κανακούδη και Δανιηλίδης, 1994; Xanthopoulos et al., 2010).

Με βάση τα διεθνή επιστημονικά δεδομένα, η παρούσα μελέτη είναι η πρώτη που εξέτασε στην Ελλάδα επαρκή αριθμό δειγμάτων τροφίμων (145 χοιρινών σφάγιων και 144 λαχανικών) για την παρουσία παθογόνων στελεχών *Y. enterocolitica*, τα οποία ταυτοποιήθηκαν πλήρως (βιο-οροτυποποίηση, γονίδια λοιμογονικότητας, αντοχή σε αντιμικροβιακές ουσίες).

Στην παρούσα μελέτη, 15.9% των χοιρινών σφάγιων ήταν θετικά στη *Y. enterocolitica*. Στελέχη του παθογόνου για τον άνθρωπο βιο-οροτύπου 4/O:3 βρέθηκαν στο 10.3% των δειγμάτων, ενώ ο βιότυπος 1A στο 6.2%. Προηγούμενη μελέτη του εργαστηρίου ανίχνευσε θετικά στη δείγματα *Y. enterocolitica* σε μεγαλύτερο ποσοστό (20.8%) σε χοιρινό κρέας, αλλά δεν υπάρχουν δεδομένα σχετικά με τον βιο-ορότυπο των στελεχών (Gousia et al., 2011). Τα ποσοστά *Y. enterocolitica* που απομονώθηκαν από χοιρινά σε διάφορες χώρες συνοψίζονται πίνακα 20.

Τα ποσοστά ανίχνευσης *Y. enterocolitica* της μελέτης μας είναι παρόμοια με αυτά ερευνών από τη Νορβηγία, Βέλγιο, Ιταλία και Σουηδία. Σε άλλες έρευνες από τη Νορβηγία, Ιταλία και Γερμανία αναφέρθηκαν μικρότερα ποσοστά. Αντιθέτως, στην Αυστρία, Βέλγιο, Λιθουανία και Φινλανδία τα ποσοστά ανίχνευσης *Y. enterocolitica* ήταν μεγαλύτερα από αυτά της δικής μας μελέτης. Σε αρκετές από αυτές αποδεικνύεται ότι με τη χρήση μοριακών τεχνικών επιτυγχάνονται μεγαλύτερα ποσοστά ανίχνευσης λόγω της μειωμένης ευαισθησίας των μεθόδων καλλιέργειας. Ωστόσο, η χρήση μοριακών μεθόδων πρέπει να γίνεται παράλληλα με τις συμβατικές μεθόδους καλλιέργειας για την απομόνωση και επιδημιολογική μελέτη ενός στελέχους. Τα αποτελέσματα μας συμφωνούν με τις παραπάνω έρευνες ως προς το συχνότερο βιο-ορότυπο 4/O:3 που απομονώνεται στην Ευρώπη.

Όσον αφορά τα λαχανικά δεν έχει διευρευνηθεί ακόμα ο ρόλος τους στην επιδημιολογία της υερσινίωσης στον άνθρωπο. Τα ποσοστά *Y. enterocolitica* που έχουν απομονωθεί από λαχανικά σε διάφορες χώρες συνοψίζονται στον πίνακα 21.

Στην παρούσα έρευνα, ανιχνεύθηκε *Y. enterocolitica* σε 4 από τα 144 δειγμάτων λαχανικών (2.8%), με τη μέθοδο ψυχρού εμπλουτισμού για 7 μέρες. Μόλις ένα δείγμα (0.7%) ταυτοποιήθηκε θετικό στον βιο-ορότυπο 4/O:3 και 3 (2,1%) στον βιότυπο 1A. Έρευνα των Κανσουζίδου-Κανακούδη και Δανιηλίδη (1994) ανέφερε παρόμοια ευρήματα. Σε 132 δείγματα λαχανικών που εξέτασαν με ψυχρό εμπλουτισμό για 21 ανίχνευσαν 4 (3%) δείγματα θετικά στο βιότυπο 1. Άλλη έρευνα στην Ελλάδα, από τους Xanthopoulos και συν. (2010) απομόνωσε δύο στελέχη από 26 έτοιμες προς κατανάλωση σαλάτες.

Παρόμοια μικρά ποσοστά απομόνωσης αναφέρθηκαν στην Ισπανία και Ιταλία χωρίς να υπάρχουν δεδομένα για τους υποτύπους των στελεχών. Αντιθέτως, σε μελέτες στη Γαλλία και Φινλανδία τα ποσοστά ξεπερνούσαν το 30%, όμως τα στελέχη δε ανήκουν σε παθογόνους βιο-οροτύπους.

Η σύγκριση των αποτελεσμάτων μελετών επιχρισμάτων από τα χοιρινά είναι δύσκολη λόγω διαφορών στο σχεδιασμό τους, το μελετούμενο πληθυσμό ζώων, την ηλικία σφαγής και συνθηκών σφαγής σε κάθε χώρα, την περιοχή δειγματοληψίας και μεθόδων ανίχνευσης (Laukkanen και συν., 2010b; Van Damme και συν., 2013b, 2015; Bonardi και συν., 2014; Petsios και συν., 2016). Στην παρούσα μελέτη επιλέχθηκε η εξωτερική περιοχή του οπισθίου άκρου στο ύψος του μηρού. Οι Van Damme και συν., (2015) απέδειξαν ότι η πιο μολυσμένη περιοχή του σφάγιου είναι αυτή της κάτω γνάθου.

Σε συμφωνία με τη δική μας μελέτη, οι Van Damme και συν. (2013a) σε επιχρίσματα χοιρινών ο ψυχρός εμπλουτισμός αποδείχθηκε πιο αποτελεσματικός σε σχέση με την άμεση επίστρωση και τον εκλεκτικό εμπλουτισμό για την ανίχνευση του παθογόνου οροτύπου O:3 ($P < 0.001$). Σε αντίθεση με τα δικά μας αποτελέσματα, ο ψυχρός εμπλουτισμός για 14 μέρες με επεξεργασία σε αλκάλι ήταν πιο αποτελεσματικός σε σχέση με αυτόν των 7 ημερών χωρίς επεξεργασία σε αλκάλι ($P < 0.001$). Παρομοίως με την προηγούμενη ερευνά τους και σε αντίθεση με τη δική μας, ο ψυχρός εμπλουτισμός για 14 μέρες ανίχνευσε σε σημαντικό βαθμό περισσότερα θετικά σε παθογόνα στελέχη *Y. enterocolitica* δείγματα σφάγιων σε σχέση με τον ψυχρό εμπλουτισμό των 7 ημερών $p = 0.0092$).

Καμία από τις μεθόδους δεν είχε την ικανότητα να ανιχνεύσει όλα τα θετικά στον 4/O:3 δείγματα, αλλά και κανένα δείγμα δε βρέθηκε θετικό με όλους τις μεθόδους. Για το λόγο αυτό, με τη χρήση πολλαπλών μεθόδων καλλιέργειας μπορεί να επιτευχθεί η μέγιστη αποτελεσματικότητα στην ανίχνευση της παθογόνου *Y. enterocolitica*.

Η μελέτη των γονιδίων λοιμογονικότητας κατέδειξε ότι όλα τα στελέχη 4/O:3 από τα σφάγια φέρουν τα γονίδια *ail*, *ystA*, *inv*, *virF*, *myfA* και *foxA* και το *yadA* γονίδιο σε ποσοστό 64.9%, ενώ σε κανένα δεν ανιχνεύθηκε το γονίδιο *ystB*. Τα γονίδια που βρέθηκαν να φέρουν τα 1A στελέχη ήταν τα *inv* (100%), *ystB* (94.1%), και *foxA* (84.4%), ενώ σε κανένα δεν εντοπίστηκαν τα υπόλοιπα γονίδια. Οι Bonardi και συν. (2010) από χοιρινό κρέας ανίχνευσαν στελέχη 1A θετικά στα *ystB* (90%), *inv* (30%) και *ystA* (66.6%) γονίδια. Σε άλλη μελέτη των Bonardi και συν. (2013) σε σφάγια βρήκαν ένα μόνο στέλεχος 4/O:3

που έφερε τα *ail*, *ystA*, *inv* και *yadA* γονίδια, ενώ του βιοτύπου 1A ήταν θετικά στα *inv* (100%) και *ystB* μόνο. Οι Van Damme και συν. (2013a) εντόπισαν το γονίδιο *virF* στο 80.5% των 4/O:3 στελεχών.

Τα γονίδια *ail*, *ystA*, *inv*, *myfA* και *foxA* ανιχνεύθηκαν στο στέλεχος 4/O:3. Στα στελέχη βιοτύπου 1A ανιχνεύθηκαν *inv* (100%), *ystB* (100%) και *foxA* (66.6%). Οι Lee και συν. (2004) εντόπισαν τα γονίδια *yst* και *ail* σε στέλεχος του βιο-οροτύπου 3/O:3, ενώ στελέχη βιοτύπων 3B and 1A έφεραν μόνο το *yst*. Οι Verbikova και συν. (2018) ανίχνευσαν το γονίδιο *ail* σε στέλεχος βιοτύπου 1A το οποίο γενικώς θεωρείται μη παθογόνος.

Έρευνες έχουν αναφέρει αντοχή στην αμπικιλλίνη και σε κεφαλοσπορίνες 1^{ης} γενιάς λόγω της ικανότητας της *Y. enterocolitica* να παράγει β-λακταμάσες. Από την άλλη μεριά, η *Y. enterocolitica* είναι συνήθως ευαίσθητη στις αμινογλυκοσίδες, χλωραμφενικόλη, τετρακυκλίνες, τριμεθοπρίμη/σουλφαμεθοξαζόλη, ιμιπενέμη, κεφαλοσπορίνες 3ης γενιάς και φθοριοκινολόνες.

Όλα τα στελέχη από τα χοιρινά ήταν ανθεκτικά στην αμπικιλλίνη και τα μεγαλύτερα ποσοστά αντοχής ήταν στο συνδυασμό αμοξυκιλλίνη/κλαβουλανικό οξύ (53.7%), κεφοξιτίνη (37%), χλωραμφενικόλη (16.7%) και κεφιξίμη (13%). Τα παθογόνα στελέχη 4/O:3 εμφάνισαν μεγαλύτερα ποσοστά αντοχής στο συνδυασμό αμοξυκιλλίνη/κλαβουλανικό οξύ (48.6%), και μερικά στην κεφοξιτίνη (27%) και χλωραμφενικόλη (24.3%). Επιπλέον, μεγάλο ποσοστό των στελεχών ήταν ανθεκτικό και στην αμοξυκιλλίνη/κλαβουλανικό οξύ (64.7%) και στην κεφοξιτίνη (58.8%). Οι Gousia και συν. (2011) βρήκαν σε στελέχη από χοιρινό κρέας μεγάλα ποσοστά αντοχής στην αμπικιλλίνη (96.3%), αμοξυκιλλίνη/κλαβουλανικό οξύ (77.8%) και στις κεφαλοσπορίνες 3ης γενιάς κεφοξιτίνη και κεφουροξίμη (55.6%). Είχαν επίσης αντοχή στην σιπροφλοξασίνη, κεφτριαξόνη και ιμιπενέμη αλλά δεν υπάρχουν δεδομένα σχετικά με τον βιο-ορότυπο των στελεχών.

Στην Αυστρία, οι Mayrhofer και συν. (2004) από χοιρινό κρέας ανίχνευσαν στελέχη *Y. enterocolitica* στην πλειοψηφία τους οροτύπου O:3 και ευαίσθητα σε τετρακυκλίνη, γενταμυκίνη, καναμυκίνη, σουλφοναμίδες, ναλιξιδικό οξύ, τριμεθοπρίμη, χλωραμφενικόλη και στρεπτομυκίνη. Στην Ιταλία, στελέχη 1A από χοιρινό κρέας ήταν ανθεκτικά στην κεφοταξίμη, σιπροφλοξασίνη, χλωραμφενικόλη, ναλιξιδικό οξύ, στρεπτομυκίνη, σουλφοναμίδες, τετρακυκλίνη, τριμεθοπρίμη και σουλφαμεθοξαζόλη/τριμεθοπρίμη (Bonardi και συν., 2010). Παρόμοια ποσοστά αντοχής με τα δικά μας βρέθηκαν στην αμπικιλλίνη (85%) και αμοξυκιλλίνη/κλαβουλανικό οξύ (40%) αλλά όλα τα στελέχη ήταν

ανθεκτικά στην ερυθρομυκίνη. Οι Bonardi και συν. (2013) απομόνωσαν στελέχη από χοιρινά σφάγια τα οποία ήταν όλα ευαίσθητα στην σιπροφλοξασίνη, κεφταζιδίμη, κεφοταξίμη, χλωραμφενικόλη, ενροφλοξασίνη, γενταμυκίνη, καναμυκίνη και νεομυκίνη. Μεγαλύτερα ποσοστά αντοχής είχαν στην κεφαλοσπορίνη 1^{ης} γενιάς κεφαλοθίνη (92%), στην αμπικιλλίνη (89%) και αμοξυκιλλίνη-κλαβουλανικό οξύ (8%). Η αντοχή στην κεφαλοθίνη και αμπικιλλίνη ήταν στο 91.3% των 4/O:3 παθογόνων στελεχών. Η αντοχή στο συνδυασμό αμοξυκιλλίνη/κλαβουλανικό οξύ παρατηρήθηκε μόλις στο 10% των στελεχών του βιοτύπου 1A σε αντίθεση με τη μεγαλύτερη αντοχή (64.7%) των στελεχών 1A της μελέτης μας.

Τα τέσσερα στελέχη από τα λαχανικά της παρούσας έρευνας ήταν όλα ευαίσθητα στην αμικασίνη, χλωραμφενικόλη, κεφοταξίμη, κεφταζιδίμη, κεφτριαξόνη, σιπροφλοξασίνη, γενταμυκίνη, ιμιπενέμη, τριμεθοπρίμη/σουλφαμεθοξαζόλη, τετρακυκλίνη και τικαρσιλλίνη/ κλαβουλανικό οξύ. Αντιθέτως, ήταν όλα ανθεκτικά στην αμπικιλλίνη και τα τρία είχαν κοινό μοτίβο αντοχής το AmcAmpCfmFox. Σε άλλη μελέτη από Ελλάδα, στελέχη βιοτύπου 1 από λαχανικά εμφάνισαν όλα αντοχή στην αμπικιλλίνη και κεφαλοθίνη (Κανακούδη και Δανιηλίδης, 1994). Οι Verbikova και συν. (2018) απομόνωσαν στελέχη 1A ανθεκτικά στην πενικιλλίνη (100%), χλωραμφενικόλη (22.2%) και τετρακυκλίνη (5.6%). Στην Κορέα, σε στελέχη η πλειοψηφία των οποίων άνηκαν στο βιότυπο 1A τα μεγαλύτερα ποσοστά αντοχής ήταν στην κεφαλοθίνη (100%), αμπικιλλίνη (94%) και καρμπενικιλλίνη (83%) (Lee και συν., 2004).

Συμπεράσματα

Στην παρούσα μελέτη η παρουσία της *Y. enterocolitica* ανιχνεύθηκε τόσο σε χοιρινά σφάγια όσο και σε λαχανικά. Η πλειοψηφία των στελεχών που απομονώθηκαν ανήκουν στον παθογόνο βιο-ορότυπο 4/O:3 που επικρατεί στον πληθυσμό των χοίρων, καθώς και σε κλινικά στελέχη στη χώρα μας. Οι χοίροι είναι κυριότερη πηγή μόλυνσης παθογόνων στελεχών της *Y. enterocolitica* και επιμόλυνση του κρέατος εξαρτάται από τις συνθήκες εκτροφής και σφαγής τους. Τα δεδομένα αυτά δείχνουν ότι σημαντικό ποσοστό των χοιρινών σφάγιων στην Ελλάδα φέρει στελέχη *Y. enterocolitica* του παθογόνου βιο-οροτύπου 4/O:3, σε αντίθεση με τα νωπά λαχανικά στα οποία η παρουσία παθογόνων στελεχών είναι σπάνια. Με τροποποίηση των κανονισμών της ΕΕ σχετικά με τις μεθόδους σφαγής και επιθεώρησης από τον κτηνίατρο μπορεί να μειωθεί ο κίνδυνος επιμόλυνσης.

Όσον αφορά τον ρόλο των λαχανικών στην επιδημιολογίας της υερσινίωσης χρειάζεται περαιτέρω διερεύνηση σε εθνικό και διεθνές επίπεδο. Τα μεγάλα ποσοστά αντοχής στις πενικιλλίνες ευρέως φάσματος και κεφαλοσπορίνες 2ης γενιάς στελεχών από χοιρινά σφάγια αποδίδονται στην αλόγιστηχρήση τους στην εκτροφή χοίρων στην Ελλάδα.

Η χρήση ποικίλων μεθόδων καλλιέργειας και μοριακών τεχνικών είναι επιτακτική για την πληρέστερη διερεύνηση της παρουσίας παθογόνων στελεχών της *Y. enterocolitica* στα τρόφιμα που προορίζονται για ανθρώπινη κατανάλωση. Η θέσπιση οδηγιών από την ΕΕ σχετικών με την αντίστοιχη μέθοδο ISO για την ανίχνευση, απομόνωση και τυποποίηση του βακτηρίου είναι αναγκαία για την αποτελεσματική επιτήρηση της υερσινίωσης και εφαρμογή καταλλήλων στρατηγικών πρόληψης. Στα πλαίσια της Ενιαίας Υγείας η συνεργασία επιστημόνων διαφόρων ειδικοτήτων είναι απαραίτητη για την επιτήρηση της *Y. enterocolitica* ως αίτιο τροφιμογενούς λοίμωξης.

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