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Epidemiology and Comparative Analysis of Yersinia in Ireland

by

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A thesis presented for the
Degree of Doctor of Philosophy

National University of Ireland, Cork

University College Cork
Coláiste na hOllscoile Corcaigh

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Declaration

Submitted thesis is my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

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Abbreviations

aa	amino acid
AAU	Acute Anterio Uveitis
AFLP	Amplified Fragment Length Polymorphism
Ag	Antigen
BD	Becton Dikinson
BOS	Bile Oxalate Sorbose
bp	base pairs
BT	Biotype
CAL	Cellobiose Arginine Lysine agar
CD	Crohn's Disease
CDT	Cytolethal Distending Toxin
CE	Cold Enrichment
CF	Complement Fixation
CFU	Colony Forming Unit
CIN	Cefsulodin Irgazan Novobiocin agar
CR-MOX	Congo Red Magnesium Oxalate agar
CUH	Cork University Hospital
DNA	Desoxyribnucleic acid
ECM	Exra Cellular Matrix
EIA	Enzyme Immuno Assay
ELISA	Enzyme-Linked Immuno Sorbent Assay
EPEC	Enteropathogenic *E. coli*
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction
F	full sequence
F	Forward primer
FDA	Food and Drug Authority
g	gram
GALT	Gut Associated Lymphoid Tissue
HMWP	High Molecular Weight Protein
HPI	High Pathogenicity Island
Ig	Immunoglobulin
IL	Interleukin
ISR	Intragenic Spacer Region
ITC	Irgazan Ticarcillin Chlorate broth
ITC	Insecticidal Toxin complex
kb	kilobase
LB	Luria Bertani
MAC	MacConkey agar
ml	millilitre
MLEE	Multi Locus Enzyme Electrophoresis
MLST	Multi Locus Sequence Typing
MLVA	Multi Locus VNTR Analysis

mM	milliMole
mo	microorganism
MRB	Modified Rappaport Broth
mRNA	messenger Ribonucleic acid
m-YE	modified *Yersinia* Enrichment broth
Myf	Mucoid *Yersinia* factor
ND	Not Determined
NEG	Negative
nt	nucleotide
NT	Not Tested
P	partial sequence
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PMNs	Polymorphonuclear cells
PS	Pig Slurry
PSBB	Peptone Sorbitol Bile Broth
pYV	*Yersinia* virulence plasmid
R	Reverse primer
rDNA	ribosomal Desoxyribonucleic acid
REP-PCR	Repetitive Element Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RPD	Ribosomal Project Database classifier
rRNA	ribosomal Ribonucleic acid
RS	Retail meat Sample
RT	Room Temperature
SNP	Single Nucleotide Polymorphism
SPI	*Salmonella* Pathogenicity Island
SS	*Salmonella-Shigella* agar
SSR	Short-Sequence DNA Repeats
STP	Sewage Treatment Plant
Tc	*Photorhabdus luminescens* insecticidal toxin complex
TSA	Tryptic Soy Agar
TSYB	Tryptone Soy Yeast Broth
TTSS	Type Three Secretion System
U	Unit
UC	Ulcerative Colitis
VNTR	Variable Number Tandem Repeats
VP	Voges-Proskauer
VYE	Virulent *Yersinia enterocolitica* agar
WE	Warm Enrichment
YeCM	*Yersinia* Chromogenic agar
Yen-HPI	*Y. enterocolitica* High Pathogenicity Island
yo	years old

YOP	*Yersinia* Outer Protein
YPM	*Y. pseudotuberculosis* -derived Mitogen
Yps-HPI	*Y. pseudotuberculosis* High Pathogenicity Island
YSE	*Yersinia* Selective Enrichment

Abstract

Yersiniosis is an acute or chronic enteric zoonosis caused by enteropathogenic *Yersinia* species. Although yersiniosis is predominantly associated with gastroenteric forms of infection, extraintestinal forms are often reported from the elderly or patients with predisposing factors.

Yersiniosis is often reported in countries with cold and mild climates (Northern and Central Europe, New Zealand and North of Russian Federation). The Irish Health Protection Surveillance Centre (HPSC) currently records only 3-7 notified cases of yersiniosis per year. At the same time pathogenic *Yersinia enterocolitica* is recovered from pigs (main source of pathogenic *Y. enterocolitica*) at the levels similar to that observed in *Yersinia* endemic countries. Introduction of *Yersinia* selective culture procedures may increase *Yersinia* isolation rates. To establish whether the small number of notifications of human disease was an underestimate due to lack of specific selective culture for *Yersinia* we carried out a prospective culture study of faecal samples from outpatients with diarrhoea, with additional culture of appendix and throat swabs. Higher levels of anti-*Yersinia* seroprevalence than yersiniosis notification rates in endemic countries suggests that most yersiniosis cases are unrecognised by culture. Subsequently, in addition to a prospective culture study of clinical specimens, we carried out serological screening of Irish blood donors and environmental screening of human sewage. Pathogenic *Yersinia* strains were not isolated from 1,189 faeces samples, nor from 297 throat swabs, or 23 appendix swabs. This suggested that current low notification rates in Ireland are not due to the lack of specific *Yersinia* culture procedures. Molecular screening detected a wider variety of *Y. enterocolitica*-specific targets in pig slurry than in human sewage. A serological survey for antibodies against *Yersinia* YOP (Yersinia Outer Proteins) proteins in Irish blood donors found antibodies in 25%, with an age-related trend to increased seropositivity, compatible with the hypothesis that yersiniosis may have been more prevalent in Ireland in the recent past.

Y. enterocolitica is a heterogeneous group of microorganisms that comprises strains with different degree of pathogenicity. Although non-pathogenic *Y. enterocolitica* lack conventional virulence factors, these strains can be isolated from patients with diarrhoea. Insecticidal Toxin Complex (ITC) and Cytolethal Distending Toxins can

potentially contribute to the virulence of non-pathogenic *Y. enterocolitica* in the absence of other virulence factors. We compared distribution of ITC and CDT loci among pathogenic and non-pathogenic *Y. enterocolitica.* Additionally, to demonstrate potential pathogenicity of non-pathogenic *Y. enterocolitica* we compared their virulence towards *Galleria mellonella* larvae (a non-mammalian model of human bacterial infections) with the virulence of highly and mildly pathogenic *Y. enterocolitica* strains. Surprisingly, virulence of pathogenic and non-pathogenic *Y. enterocolitica* in *Galleria mellonella* larvae observed at 37°C did not correlate with their pathogenic potential towards humans.

Comparative phylogenomic analysis detects predicted coding sequences (CDSs) that define host-pathogen interactions and hence providing insights into molecular evolution of bacterial virulence. Comparative phylogenomic analysis of microarray data generated in *Y. enterocolitica* strains isolated in the Great Britain from humans with diarrhoea and domestic animals revealed high genetic heterogeneity of these species. Because of the extensive human, animal and food exchanges between the UK and Ireland the objective of this study was to gain further insight into genetic heterogeneity and relationships among clinical and non-clinical *Y. enterocolitica* strains of various pathogenic potential isolated in Ireland and Great Britain. No evidence of direct transfer of strains between the two countries was found.

Chapter 1 Literature Review

1.1 Epidemiology

The genus *Yersinia* belongs to the family *Enterobacteriaceae* and comprises 18 species [1-7] three of which are pathogenic for humans (*Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*).

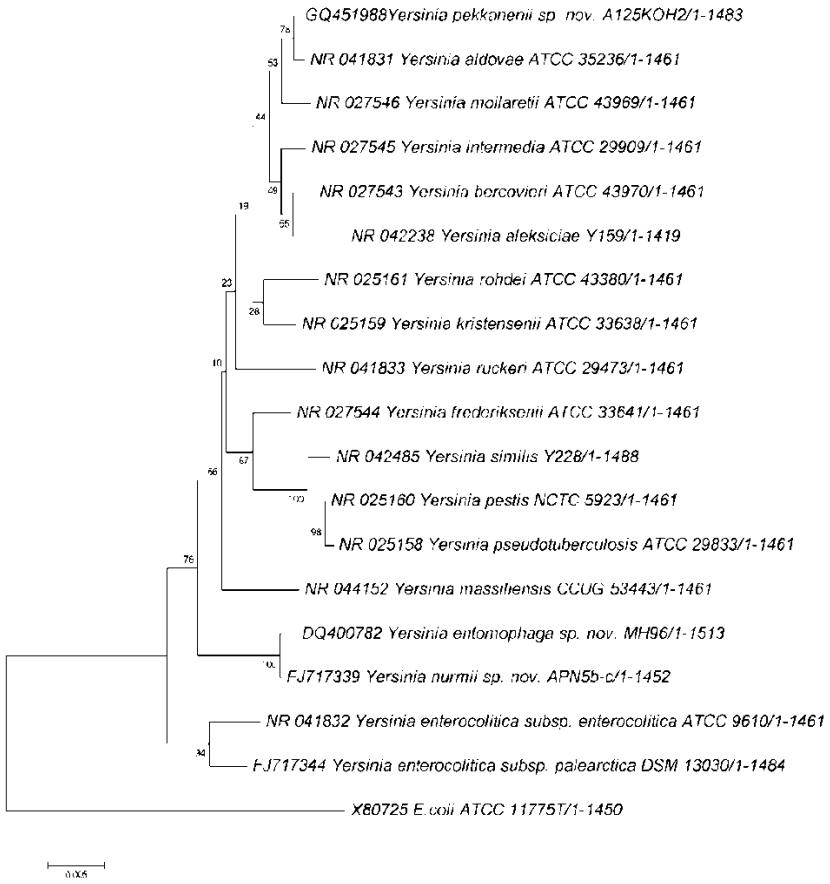


Figure 1.1 Neighbour-joining phylogenetic analysis of the Yersinia taxa based on 16S rDNA sequences

The evolutionary history was inferred using the Neighbor-Joining method [8]. The optimal tree with the sum of branch length = 0.12756774 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [9]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method [10] and are in the units of the number of base differences per site. The analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1403 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [11].

1.1.1 Incidence rates of enteric yersiniosis in humans

Yersiniosis is an acute or chronic enteric zoonosis caused by infection with enteropathogenic *Yersinia* species. The mean annual incidence rate of enteric

yersiniosis notified from 2006-8 inclusive in European Union countries was 2.65/100,000, with the highest incidence rates reported from Northern and Central European countries such as Finland (12.03/100,000), Sweden (6.1/100,000), Germany (5.9/100,000), Czech Republic (5.4/100,000) and Denmark (5.0/100,000). Yersiniosis was generally not reported from Southern European countries [12]. Over the same period, the highest incidences of yersiniosis outside the European Union were reported in the Arkhangelsk region of the Russian Federation (16.5/100,000) (www.epinorth.org) and New Zealand (12.0/100,000) [13]. In Ireland the annual incidence rate of yersiniosis notified during this period was the lowest in Europe, and did not exceed 10 cases per year (≤0.1 per 100,000 population) [12].

Most yersiniosis cases are sporadic [14-17] but outbreaks of *Y. pseudotuberculosis* [18, 19] and *Y. enterocolitica* [20-29] involving food have been reported worldwide (Table 1.1).

Table 1.1 Outbreaks of *Y. pseudotuberculosis* and *Y. enterocolitica* infections

Microorganism	Source	Country	Year	Ref
Y. pseudotuberculosis	ND	Finland	1981-1982*	[30]
Y. pseudotuberculosis	Iceberg lettuce	Finland	1998	[18]
Y. pseudotuberculosis	ND	Finland	2001	[31]
Y. pseudotuberculosis	Grated carrots	Finland	2003	[32]
Y. pseudotuberculosis	Grated carrots	Finland	2006	[19]
Y. enterocolitica	Chocolate milk	USA	1976	[27]
Y. enterocolitica	Food handler	USA	1981	[24]
Y. enterocolitica	ND	Canada	1981	[33]
Y. enterocolitica	Tofu	USA	1981-1982*	[34]
Y. enterocolitica	Pasteurized Milk	USA	1982	[26]
Y. enterocolitica	Pork	USA	1988	[28]
Y. enterocolitica	Pasteurized milk	USA	1995	[20]
Y. enterocolitica	Canteen salad	Japan	2004	[25]
Y. enterocolitica	Pork	Norway	2005-2006**	[21]
Y. enterocolitica	ND	Croatia	2006	[35]
Y. enterocolitica	ND	Japan	2006	[36]
Y. enterocolitica	Salad mix	Norway	2011	[23]

ND - source of the epidemic is not determined
* Late 1981 –:Early 1982
** Late 2005 – Early 2006

1.1.2 Risk factors and transmission of enteropathogenic *Yersinia*

Y. enterocolitica and *Y. pseudotuberculosis* can be isolated from a wide range of wild birds [37-41], rodents [42-46], and domestic animals [45, 47-59] (Table 1.2).

Table 1.2 Isolation of *Yersinia* species from faeces of various animals

Animal	Country	Number of positive specimens		Microorganism	Ref
		Faeces	Tonsils		
Wild Birds	Japan	4.1%	-	*Y. pseudotuberculosis**	[40]
Wild Birds	Japan	0.8%	-	*Y. pseudotuberculosis**	[41]
Wild Birds	Japan	4.8%	-	*Y. enterocolitica**	[39]
Wild Birds	Norway	5.3%	-	*Y. enterocolitica**	[37]
Rodents	Japan	28.9%	-	*Y. enterocolitica**	[46]
Rodents	Sweden	5%**	-	*Y. enterocolitica*	[45]
Rodents	Japan	32.1%	-	*Y. enterocolitica**	[42]
Rodents	USA	12.5%	-	*Y. enterocolitica**	[54]
Goats	Germany	3%	-	*Y. enterocolitica*: 1A -100%	[52]
Goats	China	8.7%	-	*Y. enterocolitica*: 1A – 80% BT2 – 2.5% BT3 – 16.3% BT5 – 1.3%	[53]
Dogs	China	6.7%	-	*Y. enterocolitica*: 1A – 74% BT2 – 6% BT3 – 20%	[53]
Dogs	Japan	19.8%	-	*Y. enterocolitica*: 1A – 79.7% BT2 – 2.9% BT3 – 2.9% BT4 – 14.5%	[49]
Dogs	Japan	6.3%	-	*Y. pseudotuberculosis**	[49]
Dogs	Italy	30.2%	-	*Y. enterocolitica*: 1A – 10.5% BT4 – 78.9%	[47]
		5.3%		*Y. fredriksenii*	
		5.3%		*Y. intermedia*	
Dogs	USA	3.4%	-	*Y. enterocolitica**	[54]

Animal	Country	Number of positive specimens		Microorganism	Ref
		Faeces	Tonsils		
Cattle	USA	6%	-	*Y. enterocolitica**	[54]
Cattle	UK	6.3%	-	*Y. enterocolitica:* 3/O:5, 27- 3.5% 1A – 96.5%	[50]
Cattle	UK	4.5%	-	*Y. enterocolitica*: 1A – 100%	[51]
Cattle	China	5%	-	*Y. enterocolitica*: 1A – 87% BT2 – 4.3% BT3 – 8.7%	[53]
Sheep	UK	10.7%	-	*Y. enterocolitica:* 3/O:5, 27- 38% 1A – 62%	[50]
Sheep	UK	8%	-	*Y. enterocolitica*: 1A – 61% BT3 – 39%	[51]
Pigs	USA	12%	-	*Y. enterocolitica**	[54]
Pigs	Japan	-	24.3%	*Y. enterocolitica**	[55]
Pigs	UK	29.1% 8% 1.1% 1.6%	-	*Y. enterocolitica:* 3/O:5, 27- 22% 3/O:9 – 11% 4/O:3 – 5% 1A – 53.4% *Y. frederiksenii* *Y.rohdei* *Y.intermediae*	[50]
Pigs	UK	10.2%	-	*Y. enterocolitica*: 1A – 50% BT2 – 1% BT3 – 40% BT4 – 9%	[51]
Pigs	China	11.3%	-	*Y. enterocolitica*: BT1A – 83.5% BT2 – 5.8% BT3 – 8.7% BT4 – 1% BT5 – 1%	[53]
Pigs	Germany	16%	62%	*Y. enterocolitica*	[56]

Animal	Country	Number of positive specimens		Microorganism	Ref
		Faeces	Tonsils		
Pigs	Belgium	-	37.4%	*Y. enterocolitica*	[57]
Pigs	USA	22.1% 46.8%**	-	*Y. enterocolitica*	[58]
Pigs	Switzerland	88%**	34%	*Y. enterocolitica*	[59]
Pigs	Sweden	18%**	-	*Y. enterocolitica*	[45]

* Pathogenicity is unknown
** Pathogenic *Yersinia* specific PCR detection

Pulse Field Gel Electrophoresis (PFGE) and Enterobacterial Repeatetive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) DNA profiling of pathogenic *Y. enterocolitica* isolates obtained from symptomatic patients and pigs has demonstrated an epidemiological link between yersiniosis in humans and pigs [60-62]. Consumption of contaminated pig products is a significant risk factor associated with sporadic cases of yersiniosis [16, 63-66] and has been linked to *Y. enterocolitica* gastroenteritis outbreaks among young children in several states of the USA [22, 28, 67] and a recent outbreak of yersiniosis in Norway [21]. Contamination of pig meat with pathogenic *Y. enterocolitica* occurs mainly during lairage and originates from pig tonsils as well as faecal material [68-72].

Contact with domestic animals is also linked to yersiniosis in humans [13, 14, 53, 73]. PFGE profiles of *Y. enterocolitica* strains isolated from pork, pigs, dogs and humans share similar patterns, hence transmission of pathogenic *Yersinia* to pets could also be due to consumption of contaminated pork [53, 73].

It has been suggetsted tha rodents may be possible vectors of pathogenic *Yersinia* transmission to pigs [45, 72] and in some cases directly to humans [41, 42]. Migratory birds [37, 38] as well as flies [74] could also become carriers of *Yersinia* through environmental faecal contamination and consequently transport bacteria over large distances. Drinking untreated water is a risk factor for yersiniosis in humans, contamination of which presumably linked to the prevalence of pathogenic *Yersinia* in animal faeces [13, 16, 65, 75-77].

1.2 Isolation and characterization of *Y. enterocolitica* and *Y. pseudotuberculosis*

Isolation of pathogenic *Yersinia spp.* from stool specimens may require selection for *Yersinia spp.* in the presence of other background microflora. Thus, introduction of *Yersinia* selective procedures for faecal specimens screening may significantly increase *Yersinia* isolation rates [17, 78-80].

1.2.1 *Yersinia* selective enrichment procedures

Proliferation of *Y. enterocolitica* at higher temperatures (32°C) can be inhibited by other faster growing gram-negative bacteria as a result of metabolic crowding [81]. However, lowering the incubation temperature to 15°C equalizes growth rates of *Y. enterocolitica* and other bacteria, and consequently eliminates such antagonism [81]. The mechanism underlying cold enrichment is presumably linked to the greater ability of *Yersinia* to grow at lower temperatures in comparison to other enteric bacteria [82, 83].

Cold enrichment of specimens at refrigerated temperatures (2-8°C) for three weeks is an efficient procedure for isolation of *Yersinia spp.* from food [84, 85] and human stool samples especially when routine culture procedures fail to isolate *Yersinia spp.* [82, 86].

Phosphate Buffered Saline (PBS) is the most frequently used medium for the cold enrichment of clinical [17, 54, 82, 87-92] and non-clinical [50, 54] samples. Cold enrichment in PBS supplemented with 1% sorbitol and 0.15% bile salts gave better results for *Yersinia* isolation than cold enrichment in PBS alone [93] and currently PBS-Sorbitol-Bile Salts broth is recommended by ISO10723:2003 [94] for the detection of pathogenic *Y. enterocolitica* in food.

Cold enrichment may sometimes enhance recovery of pathogenic *Y. enterocolitica* from stool samples obtained from asymptomatic carriers or convalescing patients [87]. However, in most cases non-pathogenic *Yersinia species* are isolated from stool samples after cold enrichment [17, 78, 82, 86-89, 95]. A possible explanation for common isolation of non-pathogenic *Yersinia* biovars is that they produce bacteriocin-like substances that are active against pathogenic *Y. enterocolitica* biotypes [96]. Over 60 percent (61.7%) of clinical *Yersinia* strains were susceptible to the bacteriocins produced by three bacteriocin producing non-pathogenic *Y. enterocolitica* strains [97].

Use of modified *Salmonella* selective Rappaport broth (MRB) (contains increased concentration of magnesium chlorate, decreased concentration of malachite green and

added carbenicillin) increased recovery of pathogenic *Y. enterocolitica* from stool samples [88, 98] and food products [99] in comparison to cold enrichment. MRB was excellent for recovery of *Y. enterocolitica* serotypes O:3 and O:9 but was not efficient for recovery of serotype O:8 [99-101]. Bile Oxalate Sorbose (BOS) broth used as part of two-step enrichment procedure was superior to MRB in the second step of enrichment for the isolation of pathogenic *Y. enterocolitica* O:8 serotype [101]. Further modification of MRB by the addition of irgazan, ticarcillin and potassium chlorate produced a new enrichment broth ITC (Irgazan Ticarcillin potassium Chlorate) with increased selectivity for *Yersinia* [102]. However, isolation of *Y. enterocolitica* serotype O:9 from artificially contaminated minced pork was poor in comparison to recovery of serotype O:3 with ITC [103].

A combination of short enrichment of diarrhoeal stool samples at 16°C in the alkaline (pH 8.4 ± 0.1) *Yersinia* selective broth that contains 2% D-sorbitol, 0.15% bile salts and *Yersinia* selective supplement (Cefsulodin-Irgazan-Novobiocin) and subsequent inoculation onto Cefsulodin Irgazan Novobiocin (CIN) agar (YSE-CIN method) significantly increased isolation of pathogenic *Y. enterocolitica* from diarrhoeal stool samples in comparison to traditional culture methods used for *Yersinia* isolation [79].

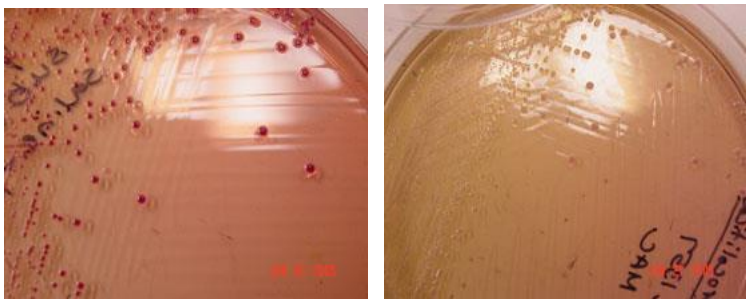
1.2.2 Alkali treatment

Y. enterocolitica and *Y. pseudotuberculosis* survive treatment with dilute alkali (0.5% KOH) which rapidly inactivates other Enterobacteriaceae [104]. Treatment of diarrhoeal stool samples [105], food [104, 106-108] and water [109] with dilute alkali (0.5% KOH) may eliminate competing microflora without prolonged cold enrichment and facilitate better isolation of pathogenic *Yersinia*. However, alkali treatment of experimentally inoculated stool samples did not consistently suppress other enteric microflora [110] and failed to increase the isolation rate of pathogenic *Yersinia* from clinical diarrhoeal stool samples [111]. External factors (peptones, bile salts, pH, bacterial growth phase, and temperature) influence alkalotolerance of *Yersinia* and therefore the reproducibility of alkali treatment [112]. Additionally, variable alkotolerance in different *Y. enterocolitica* [107, 108, 112], and *Y. pseudotuberculosis* [109] strains was detected.

1.2.3 *Yersinia* selective agar medium

Pathogenic *Yersinia species* can be isolated from stool samples using standard enteric media such as MacConkey (MAC) and Salmonella-Shigella (SS) agar [17, 87, 88, 113,

114]. SS agar is more selective for isolation of pathogenic *Y. enterocolitica* than MAC agar [88], however, neither MAC nor SS is very differential for *Yersinia spp* compared to other enterobacteria [114, 115]. In addition, not all *Yersinia species* can grow on SS agar [114, 116]. Cellobiose-Arginine-Lysine (CAL) [117] and Arabinose-Arginine-Lysine (DYS) agars [118] exploited the ability of *Yersinia* to ferment cellobiose and arabinose, and inability to utilize arginine or lysine in comparison with other enteric microorganisms [117, 118]. *Yersinia* resistance to deoxycholate in comparison to other enteric microorganisms was employed in the development of deoxycholate Salmonella-Shigella (d-SS) agar [98] and YM (deoxycholate-citrate-D-mannitol) agar [119].



A. CIN agar B. MAC agar

Figure 1.2 ***Y. enterocolitica*** **colony morphology on CIN and MAC agar plates(adapted from www.fda.gov)**

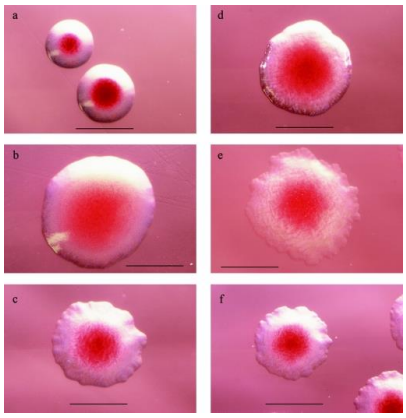


Figure 1.3 ***Yersinia*** **species on CIN agar (adapted from *Hallanvuo et al* [120])**

Differentiating between *Y. enterocolitica*, *Y. bercovieri*, and *Y. mollaretii* on CIN agar (incubation at 30°C, 22 to 24 h) through a stereomicroscope (black bar, 1 mm).
(a) *Y. enterocolitica* 4/O:3 (approximately <1 mm in diameter), typical bull's eye appereance
(b) *Y. enterocolitica* BT 1A (approximately 2 mm in diameter)
(c) *Y. bercovieri* (approximately 1.5 mm in diameter)
(d and e) *Y. mollaretii* (approximately 1.5 mm in diameter); two types exist: mucoid, with a smoother and more convex appearance (d) and a flat, dry, more irregular- and erose-edged colony (e)
(f) *Y. enterocolitica* O:5,27 (pathogenic serotype) (approximately 1 to 1.5 mm in diameter).

Cefsulodin Irgazan Novobiocin (CIN) agar containing sodium deoxycholate and sodium cholate, crystal violet and mannitol with a neutral red indicator was shown to be superior to MAC and SS agar media for selection and differentiation of *Y. enterocolitica* [114, 115] but not *Y. pseudotuberculosis* [55, 121] from other background microflora. Thus, CIN medium is comparable to blood agar for the recovery of *Y. enterocolitica* and the colony morphology of *Y. enterocolitica* on CIN agar is more distinctive when compared with the colony morphology on MAC agar plate (Figure 1.2 and 1.3) [114]. Additionally, CIN medium is also the most effective in the inhibition of other enteric bacteria [114, 115]. It is now the predominant commercially available *Yersinia* selective agar. However, some *Y. pseudotuberculosis* and *Y. entercolitica* biotype 3B strains (now speciated as *Y. bercovieri* [122]) are sensitive to cefsulodin 15mg/L but not 4mg/L [121]. Two formulations of the CIN agar are available commercially: BD™ Diagnostics, USA CIN formulation has cefsulodin concentration of 4mg/L and Oxoid Ltd., UK formulation has cefsulodin concentration of 15mg/L. Another minor weakness of CIN agar is that colony morphology of *Citrobacter freundii, Serratia liquefaciens, Serratia marcescens, Serratia fonticola, Enterobacter agglomerans* and *Morganella morganii* can resemble that of *Yersinia species* [114, 115, 123].

Efforts at producing an improved *Yersinia* selective agar continue with KV202 agar [84], chromogenic *Yersinia* selective agar (YeCM) [124] and VYE agar (virulent *Y. enterocolitica* agar) [125]. YeCM agar media allows differentiation of pathogenic *Y. enterocolitica* from non-pathogenic *Yersinia species,* however, it does not differentiate *Y. pseudotuberculosis* from other enteric microorganisms [124]. VYE, can differentiate pathogenic *Y. enterocolitica* from non-pathogenic *Yersinia* but it is also like YeCM, deficient in recovery of *Y. pseudotuberculosis* [125].

1.2.4 Biochemical identification of enteropathogenic *Yersinia* species

Yersinia species are biochemically more active at 25-30°C than at 35-37°C [126, 127]. Several commercial biochemical test systems for the identification of *Enterobacteriaceae* have been evaluated for their ability to correctly assign a reference [128, 129] and clinical [129-132] panel of enteric *Yersinia species* strains previously identified by conventional biochemical reference methods to genus and species levels (Table 1.3).

Table 1.3 Performance of commercial identification systems

Kit	Manufacturer	Incubation	Genus level assignment	Correct Species assignment	Reference ID methods	Ref
API 20E	*BioMérieux*, France	28°C, 18-24h	ND	93%	Bercovier biochemical tests	[130]
API 20E	*BioMérieux*, France	28°C, 18-24 h	ND	90%	Ewing biochemical tests	[131]
API 20E	*BioMérieux*, France	28°C, 18-24h	91%	79%	ND	[128]
API Rapid ID 32E	*BioMérieux*, France	37°C, 5h	86%	42%	ND	[128]
Vitek GNI Card	*BioMérieux*, France	37°C, 24h	96.3%	57.4%	Bockemühl biochemical tests	[129]
BBL Crystal E/NF	*BD Diagnostics*, USA	37°C, 24h	83.3%	ND*	CDC reference methods	[132]
Micronaut E	*Merlin Diagnostika* GmbH, Germany	37°C, 24h	92%	72%	ND	[128]

*BBL Crystal E/NF identifies *Yersinia species* as *Y. enterocolitica* group (BBL Crystal E/NF package insert)
ND :Not Determined

Incubation of API 20E strips at 28°C resulted in a higher percentage of correct identifications than at 37°C (Table 1.7) [128, 130, 131]. Excluding Voges-Proskauer (VP) reactions from scoring increased the percentage of correct *Yersinia species* identifications with the API20E [128, 130]. A high percentage (70-83%) of *Y. intermedia* strains are misidentified with the API 20E identification system [128, 131] because of slow or unreliable fermentation of rhamnose and melibiose.

API Rapid ID 32E strips incubated at 37°C for 5h could not identify most non-pathogenic *Yersinia* to species level, again because of weak metabolic activity [128]. Sensitivity of Micronaut E system for the identification of *Yersinia* is comparable with the API 20E at genus and species level [128].

1.2.5 Biotyping of *Y. enterocolitica*

Currently six biovars of *Y. enterocolitica* are recognised: 1A, 1B and 2-5. Biotype of *Y. enterocolitica* correlates with the pathogenic potential of the organism: biotype 1A strains are traditionally considered as non-pathogenic, biotype 1B strains (so-called "American strains") as highly pathogenic in animal models and moderately pathogenic strains placed into biotypes 2-5 (Table 1.4) [133].

Table 1.4 Wauters *Y. enterocolitica* biotyping scheme

Tests	Biotypes						
	1A	**1B**	**2**	**3**	**4**	**5**	**6**
Lipase (Tween-esterase)	+	+	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	(+)	-
Indole	+	+	(+)	-	-	-	-
Aesculin	+	-	-	-	-	-	-
Salicin	+	-	-	-	-	-	-
Xylose	+	+	+	+	-	V	+
Trehalose	+	+	+	+	+	-	+
Pyrazinamidase	+	-	-	-	-	-	+
Proline peptidase	V	-	-	-	-	-	+
β-D-Glucosidase	+	-	-	-	-	-	-

(+) :Delayed reaction;
V :Variable reaction

1.2.6 Biotyping of *Y. pseudotuberculosis*

Y. pseudotuberculosis can be divided into 4 biovars by utilization of melibiose, citrate and raffinose (Table 1.5) [134].

Table 1.5 *Y. pseudotuberculosis* biotyping scheme

Tests	Biotypes			
	1	2	3	4
Melibiose	+	-	-	+
Citrate	-	-	+	-
Raffinose	-	-	-	+

Melibiose-fermenting and melibiose non-fermenting *Y. pseudotuberculosis* respectively were shown to be virulent and avirulent, respectively, in intraperitoneally inoculated mice [135] and guinea pigs [136]. However, melibiose non-fermenting *Y. pseudotuberculosis* are able to cause mild infections in humans [137].

1.2.7 Serotyping of *Y. enterocolitica* and *Y. pseudotuberculosis*

Seventy six O- and forty four H-antigens have been described for *Y. enterocolitica* [138]. Several O-antigens (O:3, O:8, O:9 and O:13) typical for pathogenic *Y. enterocolitica* are also shared with non-pathogenic *Y. enterocolitica* and *Y. enterocolitica*-like strains, but H antigens are always species-specific [138, 139].

Based on a combination of 26 O- and 5 H-antigens, the *Y. pseudotuberculosis* antigenic scheme comprises 14 O serogroups of which serogroups O:1 and O:2, and O:4 and O:5 are divided into three (a, b, c) and two (a, b) subgroups respectively [134]. As with *Y. enterocolitica,* O antigens of *Y. pseudotuberculosis* can be shared with other *Enterobacteriaceae* while the H antigens are species-specific [134].

1.3 Molecular detection and characterization of *Yersinia*

1.3.1 Molecular detection of pathogenic *Yersinia* species

Culture methods for pathogenic *Yersinia* have various disadvantages. They have a sensitivity threshold of $\geq 10^3$ CFU/ml [140], cannot select out pathogenic rather than non-pathogenic *Yersinia species* [70, 101], and are particularly poor at detecting *Y. pseudotuberculosis* in diarrhoeal stool samples [121, 141]. Moreover, bacteriocins secreted by non-pathogenic *Yersinia* species may be active against pathogenic *Yersinia* [96, 97, 142, 143] and prevent isolation of pathogenic *Yersinia* species from environmental samples such as soil and sewage [144-146] when they are in fact present.

PCR amplification is more sensitive in detection of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* present in low numbers than bacteriological culture methods (Table 1.6) and subsequently detection rates of pathogenic *Yersinia* are higher after application of PCR amplification in comparison to culture methods (Table 1.7).

Table 1.6 Sensitivity of PCR methods in *Y. enterocolitica* and *Y. pseudotuberculosis* spiked environmental samples

Source	PCR Method	Target gene	Target m.o.	Pre-PCR sample enrichment	Sensitivity	Ref
Environmental water	Simple	ail	YE	-	60 CFU/ml	[145]
Environmental water	Nested	yadA	YE	TSBY, overnight, RT	8 - 17 CFU/100ml	[147]
Environmental water	Nested	yadA	YE	TSBY, 3h, RT	10 – 30 CFU/100ml	[148]
Meat	Nested	yadA	YE	-	10 – 30 CFU/gl	[148]
Meat	Nested	yadA	YE	TSBY, overnight, RT	2 CFU/g	[148]
ITC broth	Fluorogenic – 5' nuclease	ail	YE	-	10 CFU/ml*	[149]
Swine Faces	Fluorogenic – 5' nuclease	ail	YE	ITC, RT, 12h,	≤1 CFU/g	[149]
Ground Pork	Fluorogenic – 5' nuclease	ail	YE	ITC, RT, 12h,	≤1 CFU/g	[149]
Ground Pork	TaqMan	ystA	YE	PSBB, 35°C, 12h	>10 CFU/g	[150]
Carrots	Real-Time PCR	ail	YP	TSBY, 25°C, 18 – 20h	2.8 CFU/g	[151]
Ground Beef	Real-Time PCR	ail	YP	TSBY, 25°C, 18 – 20h	28 CFU/g	[151]
Carrots	Real-Time PCR	ail	YP	TSBY, 25°C, 18 – 20h	28 CFU/g**	[151]
Ground Beef	Real-Time PCR	ail	YP	TSBY, 25°C, 18 – 20h	2.8 CFU/g**	[151]

m.o. :microorganism
YE :*Y. enterocolitica*
YP :*Y. pseudotuberculosis*
RT :Room Temperature
ITC :Irgazan Ticarcilin Chlorate broth
PSBB :Peptone Sorbitol Bile broth
TSBY :Tryptone Soy broth supplementd with 0.6% of yeast extract
* Detection limit in the presence of 10^8 CFU/ml of background microflora
** Detection limit in the presence of pathogenic *Y. enterocolitica*

Table 1.7 Comparison of the yield of bacteriological culture and PCR assays in detection of pathogenic *Y. enterocolitica*

Source	Culture Isolation rates	Culture methods	PCR Detection rates	Target gene	PCR method	Ref
Pig Faeces	22.1%	ITC, 25°C, 48h + CIN	46.8%	YE *ail*	Real-Time-PCR	[58]
Pig Tonsils	25%	ITC, 25°C, 48h + CIN	88%	YE *ail*	Real-Time-PCR	[59]
Human Faeces	6.2%	YSE, 16°C, 48h + CIN	7.23%	YE *ystA*	Real-Time-PCR	[65]
Environmental Water	0%	m-YE, 25°C, 48h + BABY-4	4.6%	*ail*	PCR	[145]

YE :*Y. enterocolitica*
ITC :Irgazan Ticarcilin Chlorate broth
m-YE :modified *Yersinia* enrichment broth
BABY-Y :*Yersinia* selective agar

1.3.2 Molecular identification of *Yersinia* species

Although API 20E is the current biochemical identification system recommended for the identification of *Yersinia* species [128], only *Y. enterocolitica, Y. pseudotuberculosis, Y. kristensenii and Y. fredriksenii/intermedia* identification profiles exist in the API 20E database. Moreover, only 17-30% of non-pathogenic *Y. intermedia* strains were correctly identified with API 20E strips incubated at 28°C [128, 131]. Sequencing of the *Yersinia* 16S rRNA gene and biochemical identification with API 20E in some cases are conflicting (Table 1.8) [3, 4, 120]. Thus, while API 20E strips identified nine *Yersinia* species strains as *Y. enterocolitica* with >90% confidence, 16S rDNA sequencing confirmed only one strain as *Y. enterocolitica* (Table 1.8) [120]. Additionally, 16S rRNA gene sequencing has contributed to the proposal of two new *Yersinia* species – *Y. aleksiciae sp. nov*. [4] and *Y. similis sp. nov*.[3] (Table 1.8), that were identified by API 20E as *Y. kristensenii* [4] and *Y. pseudotuberculosis* [3], respectively.

Table 1.8 Comparison of API20E identification and 16S rDNA sequencing

API 20E Profile	API 20E ID	16S rDNA sequencing	Length of sequence	Ref
1114523	*Y. enterocolitica* (99.7%)	*Y. mollaretii*	P	[120]
1014763	*Y. enterocolitica* (53%)	*Y. rohdei*	P	[120]
1114523	*Y. enterocolitica* (99.7%)	*Y. mollaretii*	P	[120]
0114523	*Y. enterocolitica* (99.9%)	*Y. bercovieri*	P	[120]
1114523	*Y. enterocolitica* (99.7%)	*Y. bercovieri*	P	[120]
1014522	*Y. enterocolitica* (93.9%)	*Y. mollaretii*	P	[120]
1114703	*Y. enterocolitica* (26.2%) *Y. kristensenii* (73.5%)	*Y. bercovieri*	P	[120]
0114523	*Y. enterocolitica* (99.9%)	*Y. bercovieri*	P	[120]
0014523	*Y. enterocolitica* (97.7%)	*Y. bercovieri*	P	[120]
1014522	*Y. enterocolitica* (93.9%)	*Y. mollaretii*	P	[120]
0154723	*Y. enterocolitica* (99.2%)	*Y. enterocolitica*	P	[120]
1014122	*Y. enterocolitica* (13.8%) *Pantoea spp.* (80.8%)	*Y. mollaretii*	P	[120]
1114703	*Y. kristensenii* (73.5%)	*Y. aleksiciae sp. nov.*	F	[4]
NG	*Y. pseudotuberculosis***	*Y. similis sp. nov.*	F	[3]
NG	*Y. pseudotuberculosis***	*Y. similis sp. nov.*	F	[3]
NG	*Y. pseudotuberculosis***	*Y. similis sp. nov.*	F	[3]

**Phenotyped by German *Yersinia* Reference Center, Hamburg [3]
NG :information not given
P :partial sequence
F :Full length sequence

Comparison of 16S rDNA sequencing data for the type strains of eleven *Yersinia* species showed a range in interspecies similarity of 97.4 to 99.9%. *Y. pseudotuberculosis* and *Y. pestis* 16SrDNA sequences showed 99.9% similarity making them virtually indistinguishable [152]. As more 16S rDNA sequences have become available, it has been realised that the resolution of 16S rDNA sequence analysis is sufficient for differentiating distantly related *Yersinia* species, but insufficient to describe relationships of some species. Variability in *gyrB* gene sequences (encoding subunit B of DNA gyrase) is sufficient for discrimination of closely related *Enterobacteriaceae* genomo species [153]. Sequencing of *gyrB* allowed better discrimination of *Y. frederiksenii* [154] and *Y. enterocolitica* biovar 1A [155] in comparison to 16S rDNA sequencing, and has been successfuly combined with 16S rDNA sequences in establishing phylogenetic relationships within the genus *Yersinia* [156]. Sequencing of

the 16S rRNA gene from *Yersinia* strains 50640 and 823 isolated from fresh water in Marseille, France, revealed 1.3% divergence with 16S rRNA gene sequences of *Y. bercovieri,* 1.5% with that of *Y.mollaretii* and 1.6% with that of *Y.frederiksenii.* However, additional sequencing of four housekeeping genes *hsp60, sodA, gyrB and rpoB*, helped assign isolates 50640 and 823 to a novel *Yersinia* species – *Y. massiliensis* [2]. Multi locus sequence typing (MLST) has also been useful in uncovering phylogenetic relationships in *Yersinia* (see below).

Y. enterocolitica strains of American (highly pathogenic biotype 1B) and of European (non-pathogenic bitype 1A and low pathogenic biotypes 2, 3, 4 and 5) origin demonstrate variability of 16S rRNA gene between 451nt and 480nt (Figure 1.4) [157, 158]. Characteristic and distinct 16S rRNA sequences in type strains of American (ATCC 9610 :GenBank/EMBL M59292) and European (Y11 DSMZ13030:GenBank/EMBL U63135) phenotype [158] allowed the description of two *Y. enterocolitica* subspecies. *Y. enterocolitica subsp. enterocolitica* are the highly pathogenic biotype 1B strains, and *Y. enterocolitica subsp. palearctica* are the non-pathogenic and low pathogenic biotypes 1A, 2, 3, 4 and 5 [158].

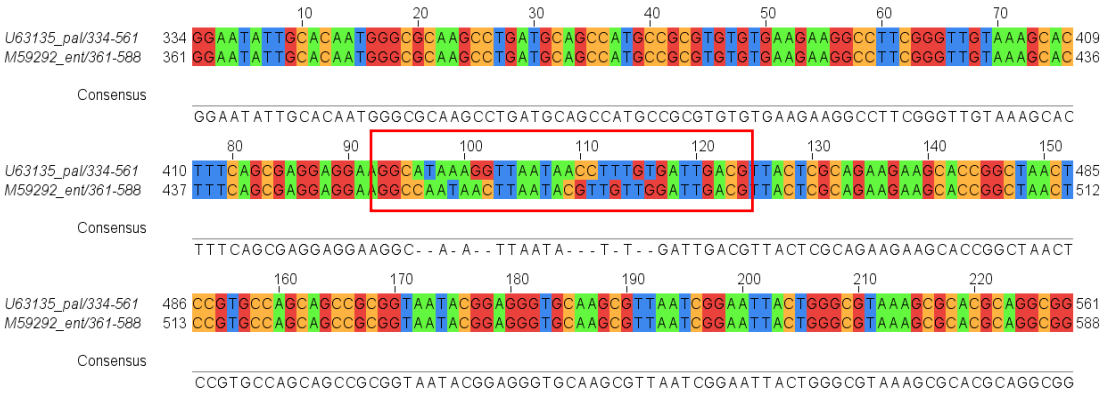


Figure 1.4 BLASTN 2.2.18 alignment results for variable locus of 16S rRNA gene of *Y.enterocolitica subsp. palearctica* (U63135) and *Y.enterocolitica subsp. enterocolitica* (U59292)

1.4 Clinical manifestations of yersiniosis

1.4.1 Gastrointestinal manifestations of yersiniosis

Y. enterocolitica and *Y. pseudotuberculosis* infections mainly involve the gastrointestinal tract [17, 63, 65, 92, 159] and are usually manifest as acute enteritis [92, 160, 161]. Mild or even asymptomatic infections may also occur [17, 162-173]. *Yersinia* gastroenteritis incidence is highest in young children [13, 17, 65, 80, 92, 161]. There is one report of an increased incidence of enteric yersiniosis for adults over 60 years of age compared with adults under the age of 60 [65].

Diarrhoea and abdominal pain are the most common symptoms reported by the patients with *Y. enterocolitica* infections [63, 80, 92]. On the other hand, symptoms of *Y. pseudotuberculosis* enteric infection include abdominal pain without diarrhoea [18, 19, 30, 32, 174-176]. Although diarrhoea due to *Y. enterocolitica* infection is reported in all age groups [63, 65, 80, 92, 161], abdominal pain is more often reported by adolescent and adults than younger children [65, 92, 160]. Other symptoms such as vomiting and fever are reported less frequently [65, 92, 161].

Patients with abdominal pain due to yersiniosis can be diagnosed with appendicitis [92, 177-179], however, the surgically removed appendicitis in yersiniosis often appears to be non-inflamed [90, 178, 180-184]. Abdominal pain due to *Yersinia* infection that can be misdiagnosed as appendicular syndrome (pseudoappendicitis) usually is a result of the inflammation in the mesenteric lymph nodes (mesenteric lymphadenitis) or the terminal end of the small intestine (terminal ileitis) [160, 178, 180-182, 185-188]. Despite this, isolation rates of *Yersinia* from postoperative appendix swabs (2.7% to 10%) [90, 183, 189] are often higher than from faeces (0.2% to 6.2%) [65, 78, 91, 190, 191]. Moreover, molecular evidence with the *Yersinia* specific *yadA* probes has been obtained, hence pointing on the possible involvement of *Y. enterocolitica* and *Y. pseudotuberculosis* in granulomatous appendicitis [186, 187]. However, this form of appendicitis is accountable for only a small percentage (0.38%-0.62%) of acute appendicitis cases world wide [192-194]. In the 1980s serological evidence (agglutinating antibodies) of yersiniosis was seen in 23% of patients presenting with acute abdominal pain in Dublin, Ireland in comparison to only 2% of Irish healthy control subjects [177]. The ratio of *Y. pseudotuberculosis* to *Y. enterocolitica* agglutination seropositivity was 5:1 [177].

Toxic megacolon due to *Y. enterocolitica* has been reported in a 17 year old girl. Culture did not yield pathogenic *Yersinia* species, but diagnosis of yersiniosis was made based

on the presence of an agglutinating *Y. enterocolitica* O:3 antibody titre of 2560 three weeks after the onset of illness. Combined therapy of corticosteroids and antibiotics was successful and in three months *Y. enterocolitica* antibody titre declined to 80 [195]. In Norway, serological evidence of current or recent *Yersinia* infection was detected in 9.4% and 15% of patients with Ulcerative Colitis (UC) and Crohn's Disease (CD), respectively [196]. Moreover, molecular evidence of the pathogenic *Yersinia* was detected in intestinal resections from 31% of patients with CD in a study from the USA [197].

1.4.2 Extraintestinal manifestations of yersiniosis

Extraintestinal infections with *Y. enterocolitica* and *Y. pseudotuberculosis* mainly affect mature adults [65, 159, 198-205] and patients with various underlying conditions such as iron overload [206-218], immunosuppression [219-221], alcoholism [202, 222] and diabetes [210, 223, 224].

Yersinia demonstrate tropism to lymphoid tissue [225-228] and *Yersinia* tonsilar carriage is common in other mammals such as pigs [56, 71] and dogs [49, 229]. While infection with *Y. enterocolitica* and *Y. pseudotuberculosis* mainly involves the gastrointestinal tract with acute enteritis [92, 160, 161] and mesenteric lymphadenitis (mimicking appendicitis) [92, 177-179], in some outbreaks of yersiniosis pharyngitis not diarrhoea was a predominant symptom [198, 199]. Clinical manifestations of *Y. enterocolitica* pharyngitis mimic symptoms of streptococcal throat infection, infectious mononucleosis or viral pharyngitis and can only be diagnosed on throat swab culture [198]. Pneumonia due to *Y. enterocolitica* mainly occurs in patients with weakened immune status due to various predisposing factors and often co-manifested with enteric symptoms of yersiniosis [203, 221, 223, 230].

Multiple liver abscesses due to *Y. enterocolitica* infection are often reported in patients with iron overload and frequently require long term treatment [206-212].

Other extraintestinal manifestations of yersiniosis due to *Y. enterocolitica* include fatal cases of meningitis in patients suffering from alcoholism [202] and thalassemia [217], systemic *Yersinia* infections such as septic arthritis [231-233], osteomyelitis [224] were reported in patients with various predisposing factors. Transient bacteraemia [165, 166, 170, 172, 173] or in some cases even septicaemia [234] was described for individuals without any underlying conditions and previous gastrointestinal yersiniosis.

Renal failure in children due to infection with *Y. pseudotuberculosis* is often reported in Japan [175, 235-238] and Korea [239, 240], but less frequently in Europe [241, 242]. Epidemics of *Y. pseudotuberculosis* systemic infection with clinical presentation similar to scarlet fever caused by *Streptococcus pyogenes* were experienced in the Far-Eastern region of Russia in the 1950s but were not described anywhere else [243-245].

1.4.3 *Yersinia* and blood transfusion

Yersinia is a major cause of fatal blood transfusion septicaemia [246, 247]. Thus, 53.8% of 26 fatal blood transfusion cases reported to the U.S. FDA during 1976 - 1998 were due to *Y. enterocolitica* [246]. The overall worldwide mortality rate of *Y. enterocolitica* post-transfusion sepsis reported during 1975 – 2007 was 54.5% [247].

Most cases of transfusion transmitted *Yersinia* involves blood units older than 14 – 21 days [247-249]. *Yersinia* may be present at undetected levels (<10 CFU/L) in blood donated by asymptomatic donors [173, 247-253], however, long-term storage of such blood units at 4°C enables *Yersinia* multiplication [254, 255]. The risk of post-transfusion *Yersinia* sepsis is substantially decreased when blood components are leucodepleted before their storage [248, 256-259].

It has been difficult to devise a serological screening methodology to exclude blood donations at risk of *Yersinia* contamination because of poor predictivity. Thus, 34% of patients with recent culture-positive *Yersinia* infection were IgA and IgG seronegative against *Yersinia* O:3-LPS Ag and no *Yersinia* was recovered from their blood cultures [260]. Likewise, blood donors whose serum contained IgA antibody against *Y. enterocolitica* O:3 YOPs were not bacteraemic and their blood units did not grow *Yersinia* after long-term storage (35 days) at 4°C [261].

1.4.4 Post infectious complications

Patients with persistent enteric *Yersinia* infection [228, 262, 263] and possessing the histocompatibility gene HLA-B27 [264-267] are at particular risk of developing post-infectious sequelae. Reactive arthritis, erythema nodosum and acute anterior uveitis (AAU) are the most common post infectious complications that may develop after yersiniosis [204, 268-271]. A study undertaken in 1988 in St. James's Hospital, Dublin which involved patients with AAU without any other underlying conditions demonstrated that 24% of them had serological evidence of *Yersinia* infection and additionally were HLA-B27 positive [204].

1.5 Immunology of yersiniosis

1.5.1 Immune response during *Yersinia* infection

During the infective process and recovery, sequential secretion and subsequent disappearance of IgM, IgA and IgG class serum antibodies occurs. While IgM and IgA class antibodies reach peak levels at early stages of infection, IgG immunoglobulins are produced at later stages [272-275]. While young children and immunosuppressed patients show low levels of anti-*Yersinia* antibodies after infection, the serologic response to *Yersinia* gastrointestinal infection among adult and adolescent patients with normally functioning immune systems is profound [276]. Additionally, patients with extraintestinal systemic foci [276] and patients with persistent presence of pathogenic *Yersinia* in Gut Associated Lymphoid Tissue (GALT) experience extended antigenic stimulation [228] leading to a prolonged immune response.

1.5.2 Anti-*Yersinia* seroprevalence

Anti-*Yersinia* antibody seroprevalence among healthy individuals is generally much higher than incidence rates of yersiniosis in the same population (Table 1.10) [76, 162-164].

Normally, declining levels of anti-YOP and anti-LPS IgA immunoglobulins are detected for a few months after yersiniosis [272-275], however, in some patients with reactive arthritis, long term persistence of anti-YOP and anti-LPS IgA class antibodies occurs [274, 277-279]. Anti-LPS IgG class antibodies were found to be persistant for several years in patients after uncomplicated yersiniosis and appendicectomy [76, 179, 280]. Additionally, although the relationship between *Yersinia* infection and thyroid disorders is not clear, a high seroprevalence of anti-YOP and anti-LPS IgG antibodies in patients with thyroid disorders [281-288] and their relatives [289] has been reported. It has been suggested that relatives of patients with thyroid disorders may have a genetic predisposition for persistent *Yersinia* infection [289].

Table 1.9 Anti-*Yersinia* antibody seroprevalence and incidence rates of yersiniosis

Country	Year	Human population	Age Range	Methods	Ag	Ab	Sero Prevalence	Ref	Yersiniosis per 100 000
Norway	1993	Military recruits	19-26	O3 Ag EIA	O-Ag	IgG	7.4%	[76]	6.4[5]*
Finland	1997	Blood donors	18-63 (37)*	O3 and O9 Ag EIA	Whole cell	IgG	10.6%	[162]	14.1*[5]
				Immunoblot	pYV		29.8%		
Germany	1997	Blood donors	20-65 (32)*	O3 and O9 Ag EIA	Whole cell	IgG	24%	[162]	8.7*[5]
Hungary	1999-2000	Blood donors	39.4	Agglutination	O-Ag	IgA	2.6%	[163]	1.4*[5]
				Mikrogen RecomWell	pYV		15.1%		
				Mikrogen RecomLine	pYV		8%		
Greece	2000	Blood donors	49*	Viramed ELISA	pYV	IgG	1.94%	[283]	NR*[6]
Austria	2006	Military recruits	19-58	Mikrogen RecomWell	pYV	IgG	41%	[164]	1.9*[6]
				Mikrogen RecomLine	pYV		29.7%		
Ireland	1987	Healthy individuals	24.2 (m*[2]) 23.3 (f*[3])	Agglutination	O-Ag	NA*[4]	2%	[177]	NA
		Patients with abdominal pain		Agglutination	O-Ag		23%		

* median age; *[2] male (m); *[3] female (f); *[4]NA - Not Available; *[5] Data obtained from EpiNoth (http://www.epinorth.org); [6]* Data obtained from European Centre for Disease Prevention and Control (http://www.ecdc.europa.eu), NR – Not Reported

Agglutinating anti-*Yersinia* antibodies (anti-O-Ag) quickly disappear after the acute phase of infection [276] whilst non-agglutinating antibodies (anti-YOP) have been shown to be more sensitive in the serodiagnosis of persistent, chronic or convalescent forms of yersiniosis [92, 205, 228, 262, 290, 291]. Western blot and ELISA immunoassays (both measuring anti-YOP antibodies) showed high sensitivity (95% both assays) and specificity (95% and 74% respectively) [292]. The highest levels of anti-*Yersinia* YOP seroprevalence in healthy individuals were found with an IgG ELISA assay (Table 1.11) [292].

Table 1.10 Comparison of *Yersinia* antibodies prevalence in the healthy population measured by various techniques (adapted from *Rawlins et al* [292])

Assay	Manufacturer	Antigens used in the assay	Positive sera from healthy individuals		
			IgG	IgA	IgM
CF	"In house "	*Y. pseudotuberculosis* and *Y. enterocolitica* O-antigens	2%*		
Western Blot	Viralab Inc.	YopH (51 kDa), YopM (44 kDa), YopB (41 kDa), LcrV (37 kDa), YopD (35 kDa), YopN (33 kDa), YopE (23 kDa)	6%	2%	2%
ELISA	Mikrogen Diagnostik	YopH (51 kDa), YopM (44 kDa), YopB (41 kDa), LcrV (37 kDa), YopD (35 kDa), YopN (33 kDa), YopE (23 kDa)	18%	10%	4%

* CF :complement fixation, does not identify class of the immunoglobulin

A significant cross reactivity in *Yersinia* ELISA and Western blot YOP assays was observed in a panel of sera known to be positive for *Borrelia burgdorferi*, *Bartonella henselae*, *Brucella*, *Chlamydia pneumoniae* and *Rickettsia rickettsii* antibodies [292]. The highest proportion of cross reactive samples were reported for sera known to contain anti-*Brucella* (4 out of 7) and anti-*Borrelia burgdorferi* (6 out of 11) antibodies in ELISA assays using recombinant YOP antigens [292-294].

1.6 Virulence factors of enteropathogenic *Yersinia* species

1.6.1 Chromosome encoded virulence factors

1.6.1.1 Yersinia resistance to stomach acidity

Gastrointestinal pathogens must withstand stomach acidity before colonising the intestinal mucosa [295, 296]. It is believed that urease production by enteropathogenic *Yersinia* species can assist bacteria to overcome stomach acidity by catalyzing the hydrolysis of urea to form carbon dioxide and ammonia, leading to a pH increase that promotes its survival [297].

1.6.1.2 Invasins

During the next step of the host colonization, enteropathogenic *Yersinia* degrade mucins (glycoproteins of mucus) lining the epithelium of the intestine [298] and penetrate the intestinal barrier by passing through M cells associated with Peyer's patches – lymphoid follicles lining the gastrointestinal tract [299-302]. Invasion of M-cells is mediated by the chromosomally-encoded invasin protein Inv that is exposed on the bacterial cell surface [303]. Inv promotes cell penetration by attaching to ß1 heterodimeric integrin receptors located on the apical end of the cell membrane of the M cell [301, 304, 305].

The Inv protein affinity for lymphoid tissue cells [301, 306, 307] enables dissemination of Inv-producing pathogenic *Yersinia* species via Peyer's patches lining the gastrointestinal tract, while gene knock out of the Inv protein results in delayed systemic spread of orally or intraperitoneally inoculated pathogenic *Y. enterocolitica* [306, 308]. *Y. pseudotuberculosis* Inv mediates transcytosis of exogenous particles across the ileal mucosa via ß1-integrin binding [309]. Gene sequences similar to *inv* are detectable in all *Y. enterocolitica* strains (including non-pathogens) by gene probe or PCR [310, 311]. However, expression of *inv* genes was not detectable as mRNA in non-pathogenic *Y. enterocolitica,* and functional expression of these *inv*-like genes was not detectable when cloned in *E. coli* [310].

Expression of the chromosomal *Yersinia ail* gene in *E. coli* HB101 [312] was able to induce bacterial attachment to, and invasion of, epithelial cells. A similar phenotype was conferred by cloning *ail* in an *inv*- and *ail*-deficient *Y. enterocolitica* 8081c strain [313]. While *ail* is present in pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*

strains associated with disease [314], this locus confers a capability for the attachment and invasion of host cells by *Y. enterocolitica* [312, 313], it does not contribute the same phenotype to *Y. pseudotuberculosis* [315]. However, *ail* does mediate short-term serum (complement) resistance [316] in both *Y. enterocolitica* [313, 315] and *Y. pseudotuberculosis* [315].

1.6.1.3 Enterotoxins

Diarrhoea is usually a result of disturbances in water and electrolyte transport in the small or large intestine that may result in dehydration [317]. Production of enterotoxin resulting in net fluid secretion is an important virulence factor for many enteric pathogens. *Y. enterocolitica* was shown in 1978 to produce an exotoxin detectable in bioassays used for *E. coli* enterotoxins. Rabbits infected with *Y. enterocolitica* W1024 strain that encoded YstA enterotoxin suffered from diarrhoea and weight loss, and most of them died [318]. However, the role of the 71 amino acid YstA [319] in diarrhoeal disease remained controversial, due to its maximum expression in vitro at 30°C rather than at the body temperature of the mammalian host [264], until it was established that *ystA* gene transcription can be induced at 37°C by incubation at osmolarity and pH values normally found in the ileal lumen, thus supporting the idea that *ystA* can be expressed *in vivo* [320]. Gene sequences similar to *ystA* are present in pathogenic *Y. enterocolitica* and *Y. kristensenii* strains but not in *Y. pseudotuberculosis* strains [319].

A homologue of YstA enterotoxin, YstB, was detected in non-pathogenic strains of *Y. enterocolitica* biovar 1A [311, 321-323]. Expression of *ystB in vitro* at 37°C only occurs when the pH of the culture medium is adjusted to 7.5, similar to that found in the ileum [322]. Enterotoxin activity of *Y. enterocolitica* 1A detected in an infant mice assay was not always observed in strains possessing *ystB* gene (Table 1.12) [321, 322].

Table 1.11 Enterotoxin activity of non-pathogenic *Y. enterocolitica* biovar 1A isolates

Source	*ystB*+ve	Enterotoxin Activity*	Ref
Clinical	87.3%	25.3%	[321]
Clinical	100%	77.7%	[322]
Non-clinical	81.3%	40.6%	[321]
Non-clinical	100%	0%	[322]
Non-clinical	96.3%	62.3%	[322]

* Percentage of *Y. enterocolitica* biovar 1A strains exhibiting enterotoxin activity in infant mice model

This discrepancy between the presence of the *ystB* gene and detectable enterotoxin activity could be due to spontaneous mutations in *ystB* in some strains [321, 322]. However, all *Y. enterocolitica* biovar 1A strains containing *ystB* belong to the clonal group A that harbours potentially pathogenic strains that were differentiated from another clonal group based on REP-/ERIC-PCR genotyping [311].

1.6.1.4 Myf functionality

Mucoid *Yersinia* Factor (Myf) of *Y. enterocolitica* is a fibrillar surface antigen expressed at 37°C in acidic conditions. It resembles the CS3 fimbriae of enteropathogenic *E. coli,* and is similar to the *Y. pestis* and *Y. pseudotuberculosis* pH6 antigen encoded by the *psa* locus [324]. Myf is encoded by a 4.4kb locus that comprises three genes: *myfA* (encoding the major subunit), and *myfB* and *mufC* (both encoding the assembly mechanism) [325]. While pH6 antigen is responsible for thermoinducible adhesion to HEp-2 epithelial cells and hemagglutination in *Y. pseudotuberculosis* [315] there is no evidence for a similar Myf role in *Y. enterocolitica* adhesion to the host cells [326, 327]. Some non-pathogenic *Y. enterocolitica* biotype 1A strains possess *myfA* [321].

1.6.1.5 Role of Motility in Yersinia virulence

A connection between motility and *Yersinia* invasiveness has been suggested in various studies because mutations in *flhDC* [328] and *fliA* [328, 329] abolished efficient bacterial invasion of epithelial cells. Additionally, expression of the *inv* gene is regulated by flagellar genes [328, 330]. It was also shown that the flagellar *fliA* gene is responsible for the inverse temperature regulation of plasmid-encoded *Yersinia* virulence genes (*yadA, yomA, yscG, yscM, ISYen1, yopQ,* and *virF*) [331].

The inner and outer-membrane spanning flagella apparatus includes an export system for hook and filament components which can export other proteins. YplA is a phospholipase A that promotes lecithin-dependant haemolysis [332] and contributes to the *Yersinia* pathogenesis by disrupting host cell membranes [333]. The YplA protein is secreted via flagellar type III export system [334]. Expression of class II flagellar genes is sufficient as these encode the flagellum basal body and type III secretion machinery [333]. Mutations in class I flagellar genes abolish YplA secretion as class II flagellar genes expression cannot be activated [333].

1.6.1.6 Iron uptake

Iron is an essential nutrient required for bacterial growth and forms one of the most limiting factors for microbial proliferation in the vertebrate host. Iron uptake by pathogenic *Yersinia* allows systemic dissemination of bacteria in animals and humans with iron overload [215, 335-340]. Highly pathogenic *Y. enterocolitica subsp. enterocolitica* are intrinsically mouse-lethal, but low-pathogenicity *Y. enterocolitica subsp. palearctica* can achieve the same levels of virulence in a mouse in the presence of an exogeneous siderophore or an excess of iron [341]. A variety of systems are present in bacteria for the synthesis of high-affinity iron-binding molecules (siderophores) [342].

Highly pathogenic *Yersinia species* (*Y. pestis, Y. pseudotuberculosis* serotype I and *Y. enterocolitica subsp. enterocolitica*) contain the yersiniabactin locus (*ybt*) [343] which encodes the capacity to synthesize the siderophore yersiniabactin. The chelating activity of yersiniabactin promotes uptake of iron essential for growth in the vertebrate host by pathogenic *Yersinia,* and its presence mediates lethality in mice [344-346]. Yersiniabactin is able to capture iron from host lactoferrin, present in the circulation and in the phagocytic vacuoles of neutrophil leucocytes [347]. Depletion of iron from host lactoferrin reduces the capacity of neutrophils, macrophages and monocytes to produce Reactive Oxygen Species (ROS), presumably by reducing activity of the Haber-Weiss reaction, which generates hydroxyl radicals and superoxide from hydrogen peroxide by Fe (II) catalysis [347]. Therefore yersiniabactin has a dual action, promoting bacterial growth in the host and reducing the effectiveness of the host innate immune response in bacterial killing. The *ybt* locus encodes proteins that are responsible for the biosynthesis

(high molecular weight proteins - HMWP 1 and 2, YbtT, YbtE and YbtS), transport (Psn/FyuA, YbtP and YbtQ) and regulation (YbtA) of the yersiniabactin (Ybt) (Table 1.13) [343, 344, 348]. FyuA contains a region commonly present in TonB-dependent proteins and operates as the outer membrane yersiniabactin receptor [344].

Table 1.12 Yersiniabactin locus genes and their products (adapted from *Carniel* [343])

Gene designation in *Y. pestis* and *Y. pseudotuberculosis*	**Gene designation in *Y. enterocolitica***	**Product**	**Function**
ybtS	*irp9*	YbtS	Ybt biosynthesis
ybtX	*irp8*	YbtX	Unknown
ybtQ	*irp7*	YbtQ	Ybt transport
ybtP	*irp6*	YbtP	Ybt transport
ybtA	*ybtA*	YbtA	Regulator of *ybt* locus expression
irp2	*irp2*	HMWP2	Ybt biosynthesis
irp1	*irp1*	HMWP1	Ybt biosynthesis
ybtU	*irp3*	YbtU	Unknown
ybtT	*irp4*	YbtT	Ybt biosynthesis
ybtE	*irp5*	YbtE	Ybt biosynthesis
psn	*fyuA*	Psn/FyuA	Ybt transport

Similar to the first pathogenicity island described in uropathogenic *E. coli* [349] the *ybt* locus is bounded by an *asn* tRNA with an adjacent integrase gene on one side and a short direct repeat of part of the tRNA gene on the other [350]. Pathogenicity islands widely distributed in pathogenic *Enterobacteriaceae* [351]. In *Yersinia* it has been termed as High Pathogenicity Island (HPI) [350, 352, 353]. Two evolutionary lineages of HPI were identified among highly pathogenic *Yersinia species*: HPI of *Y. pseudotuberculosis/Y. pestis* (Yps HPI) and *Y. enterocolitica* HPI (Yen HPI) [354]. The part of the island that encodes yersiniabactin synthesis, receptor and regulator is highly conserved among *Yersinia species,* while the region downstream of the receptor (*fyuA)* gene is highly variable [350, 354, 355].

In addition to the *ybt* locus, *Y. pestis* also encodes a haemin storage locus (*hms*) [355]. The *hms* locus is not required for *Y. pestis* virulence in mammals [356], but it

mediates biofilm formation [357] and subsequently blockage of the flea foregut, and hence transmission of plague bacilli to mammals [358]. The *hms* locus is also present in *Y. pseudotuberculosis,* however, it does not cause blockage of the flea foregut [359].

Iron uptake systems that are not essential for mammalian virulence have been identified in pathogenic *Yersinia*: *yfe*, *yfu, yin* and a haemin uptake system [360-363]. Other iron uptake systems of unidentified functionality have been detected in *Y. pestis* and *Y. pseudotuberculosis* genomes by bioinformatic analysis (Table 1.14) [364].

Table 1.13 *Y. pestis* and *Y. pseudotuberculosis* iron/hemin uptake systems with unidentified functionality detetected by bioinformatics analysis of their genomes (adapted from *Forman et al* [364])

System	Locus similarities*
Siderophore-dependent systems	
Yersinia non-ribosomal peptide (Ypn)	6 patterns
Ysu	2 patterns
Aerobactin (Iuc; biosynthesis)	2 patterns
ABC iron/siderophore transporters	
Fit	1 pattern
Fiu	2 patterns
Fhu and IutA	2 patterns
FcuA	2 patterns
Non-ABC transporters	
Feo	2 patterns
Efe	2 patterns
Fet	2 patterns
FieF	1 pattern
Hemin transporters	
Hmu	1 pattern
Has	1 pattern

* When relatively few amino acid residues changes and/or small deletions or insertions which maintain the ORF occur, they were classified as one pattern

Non-pathogenic species *Y. intermedia*, *Y. kristensenii*, and *Y. frederiksenii* produce a hydroxamate siderophore, aerobactin, but are avirulent in mice, indicating that this particular siderophore does not contribute to virulence even when these species are

transformed with the virulence plasmid of *Y. enterocolitica* [264].

1.6.1.7 Y. pseudotuberculosis superantigen

Y. pseudotuberculosis strains of Far Eastern origin but not of European origin produce a superantigenic toxin YPM (*Y. pseudotuberculosis*-derived mitogen) [245, 365, 366]. Patients with extraintestinal manifestations of *Y. pseudotuberculosis* infection have higher levels of anti-YPM antibodies than patients with the gastrointestinal symptoms [367, 368].

The role of YPM superantigen in systemic *Y. pseudotuberculosis* illness is mediated by its ability to non-selectively stimulate T-cells resulting in development in acute renal failure, stimulation of PBMC cells leading to the production of IL-12 and subsequent expansion of the skin-homing receptor CLA+ T-cells that manifest as a erythematous skin rash in affected patients [369].

1.6.2 Plasmid encoded virulence factors

Pathogenic strains of *Y. enterocolitica* [370], *Y. pseudotuberculosis* [371] and *Y. pestis* [372] possess a 64- to 75-kb plasmid designated as pYV [373-375]. *Y. pseudotuberculosis* and *Y. pestis* pYV plasmids were shown to be interchangeable [376]. The pYV encodes factors essential for *Yersinia* virulence such as: *Yersinia* Adhesin A (YadA), that mediates binding of bacteria to the host cell membrane [377-379] and serum resistance [380-386], *Yersinia* Outer membrane Proteins (YOPs) that are secreted via the Ysc Type III Secretion System (TTSS) also encoded by pYV, which mediate inhibition of phagocytosis and inflammatory responses by the host [387].

Transcription of pYV genes is thermoregulated and in most cases depends on the presence of Ca^{2+} ions (low calcium response). Thus, *in vitro* transcription of YOPs is observed only in the absence of Ca^{2+} ions and at 37°C (proxy signals for host cell contact), but the secretion of YadA protein is calcium independent although it is still thermoregulated and expressed only at 37°C [388].

1.6.2.1 Function of the adhesin protein YadA

The multiple functionality of YadA is attributed to its multidomain “lollipop-like” structure (Figure 1.5). The following domains and regions of YadA have been

functionally defined: N-terminal leader sequence, head domain, neck domain, stalk domain, linking region, and the C-terminal transmembrane region that consists of four β-strands [389].

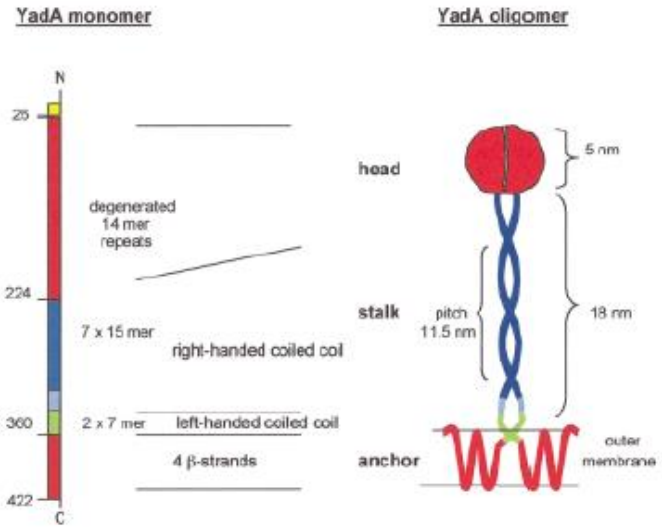


Figure 1.5 YadA protein structure (adapted from *Hoiczyk et al* [389])

The C-terminal outer membrane anchor domain is responsible for stability, oligomerization and anchoring YadA to the bacterial cell surface [389, 390]. YadA-dependent serum resistance [380-383, 386] has been linked to the stalk region but a specific stalk domain associated with serum resistance has not yet been identified [384, 385].

The N-terminal globular domain (head) is responsible for binding extracellular matrix molecules (ECM), adhesion to host cells, and agglutination [389-396]. Inactivation of *yadA* led to a significant reduction in the ability of *Y. enterocolitica* [377, 390, 395, 396], but not *Y. pseudotuberculosis,* to colonise the ileum of oro-gastrically infected mice [377, 397]. The YadA of both *Y. enterocolitica* and *Y. pseudotuberculosis* enables bacterial adhesion to mammalian cells [377-379] and extracellular matrix molecules (ECM) [390, 398-400]. The *Y. enterocolitica* YadA mediates effective adhesion to but not invasion into epithelial cells *in vitro* [401], while *Y. pseudotuberculosis* YadA facilitates both effective bacterial adhesion and invasion of eukaryotic cells, independently of Inv [379, 402]. A unique motif of 31 amino acids within the 53-83 AA region of the *Y. pseudotuberculosis* YadA head

domain (absent from *Y. enterocolitica* YadA), determines the ability to invade eukaryotic cells [391].

1.6.2.2 Ysc-YOP virulon

The *Yersinia* Ysc-YOP virulon comprises the Type III secretion system (TTSS)/Ysc that mediates injection of *Yersinia* outer membrane proteins (YOPs) into mammalian cells [403, 404]. Eight core proteins from the injectisome share a significant similarity with the components of the flagellum (Figure 1.6) [404].

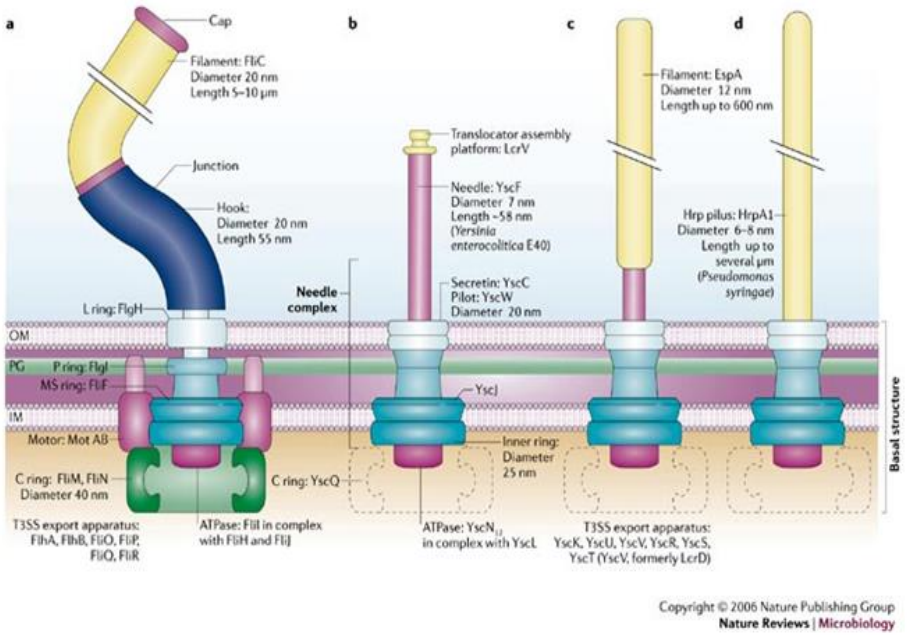


Figure 1.6 Structure of the flagellum and the injectisomes (adapted from *Cornelis* [404])

Schematic representation of the flagellum (**a**), Ysc injectisome (**b**), injectisome from EPECs (**c**) and the injectisome from plant pathogens (**d**). For the injectisomes, the C ring is represented by a dashed line as information on this component is still scarce. IM - inner membrane; OM - outer membrane; PG - peptidoglycan.

Seven families of TTSS are recognised in free-living animal pathogens. The Ysc injectisome of *Yersinia* species is the prototype of one of these families, also seen for example in *Pseudomonas aeruginosa*. The injectisomes first found in *Shigella flexneri* and *Salmonella typhimurium* (encoded on the *Salmonella* SPI-1 pathogenicity island) are members of another family prevalent in animal pathogens. Another family is exemplified by injectisomes found in enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) and *S. typhimurium* (encoded on SPI-2) [404].

Highly pathogenic *Y. enterocolitica subsp. enterocolitica* also encode a TTSS that is distinct from the Ysc/TTSS family and resembles the Mxi-Spa TTSS family of *Shigella species* and *Salmonella typhimurium* [405]. The Ysa/TTSS is encoded by the chromosomal pathogenicity island Ysa-PI that is part of a plasticity zone that also contains other virulence genes [406]. The Ysa/TTSS was shown to be involved in *Yersinia* colonization of gastrointestinal tissues by studies in a mouse model [407].

The Ysc/TTSS system is activated by close contact of pathogenic *Yersinia* with the eukaryotic cell surface [388] (Figure 1.7). After Ysc/TTSS activation a translocation of effector YOPs (YopE, YopH, YopO/YpkA, YopM, YopP/J and YopT) into the cytoplasm of eukaryotic cells occurs [388, 408]. Translocation of effector Yops into eukaryotic cell cytoplasm requires presence of translocator YOPs (YopB, YopD and LcrV) that form pores in the eukaryotic cell membrane [409-414]. The YopN protein forms a link between the Ysc/TTSS needle-like structure YscF and pore, thus, guiding the effector YOPs into eukaryotic cell cytoplasm [415].

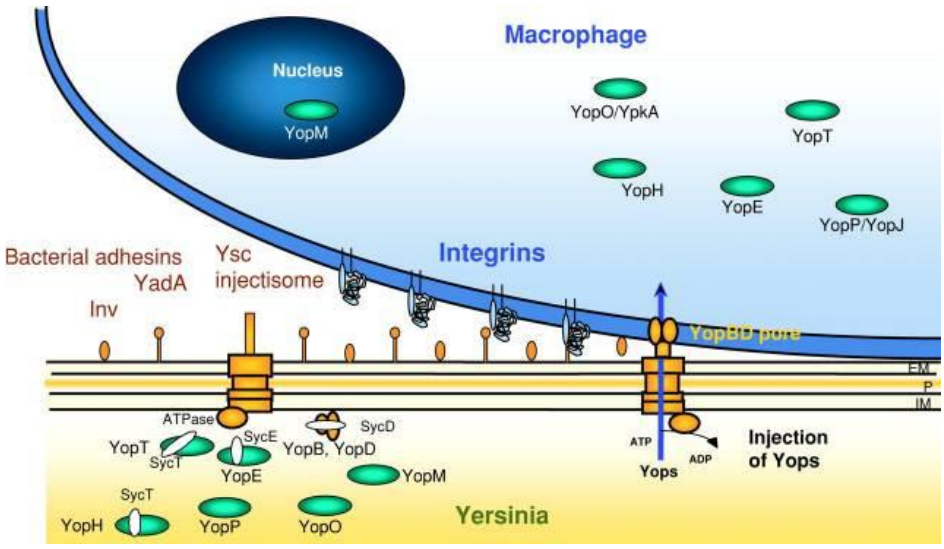


Figure 1.7 Secretion of YOPs by the Ysc injectisome and translocation across the target cell membrane (adapted from *Cornelis* [403])

When *Yersinia* are placed at 37°C in a rich environment, the Ysc injectisome is installed and a stock of Yop proteins is synthesized. During their intrabacterial stage, some Yops are capped with their specific Syc chaperone. Upon contact with a eukaryotic target cell, the adhesins YadA or Inv interact with integrins and the bacterium docks at the cell's surface. Then, the secretion channel opens and Yops are exported. YopB and YopD form a pore in the target cell plasma membrane, and the effector Yops are translocated across this membrane into the eukaryotic cell cytosol. YopM migrates to the nucleus. EM - outer membrane; P - peptidoglycen; IM - plasma membrane.

Effector YOPs suppress phagocytosis [416, 417], modulate cytokine production [418-420] and cause apoptosis of dendritic cells [420] and macrophages [421-424] (Table 1.15). The outcome of this allows *Yersinia* to evade the host immune response and favours persistence and/or systemic dissemination [423, 425].

Table 1.14 Functionality of effector YOPs

YOP	Biological Activity	Results of the biological activity	Ref
YopH	Tyrosine phosphatase that acts on focal adhesion kinase (FAK), $p130^{Cas}$ and NADPH	Disrupts actin cytoskeleton and inhibits phagocytosis	[408, 416, 426, 427]
YopE	GTPase activating protein (GAP) that acts on Rho family GTPases	Disrupts actin cytoskeleton and inhibits phagocytosis and proinflammatory cytokine production	[416, 428, 429]
YopM	Leucin rich protein that binds to protein kinase C-like 2(PRK2) and ribosomal S6 protein kinase 1 (RSK1) in the eukaryotic cell nucleous	Disrupts proinflammatory cytokine production	[430-433]
YopO/YpkA*	Serine/Threonine kinase that binds Rho and Rac-1	Disrupts actin cytoskeleton and inhibits phagocytosis	[434, 435]
YopT	Cystein protease that is cleaving CAAX box from Rho GTPases	Disrupts actin cytoskeleton and inhibits phagocytosis	[416, 428, 436-439]
YopP/YopJ*	Acetyl transferase that disrupts NFkB and MAPK signalling pathways	Disrupts actin cytoskeleton and inhibits phagocytosis by trigerring apoptosis	[424, 440, 441]

* *Y. pseudotuberculosis* effector Yop

1.6.3 Novel virulence factors

Cytolethal Distending Toxins (CDTs) are bacterial heat-labile toxins with an ability to arrest the cell cycle of eukaryotic cells [442-445]. CDTs are produced by a number of bacterial pathogens including: *Campylobacter* species [446-448], *Salmonella enterica* Serovar Typhi [449], *Shigella dysenteriae* [450], *E. coli* [451-454], *Helicobacter* species [455-460], *Actinobacillus actinomycetemcomitans*

[461-463] and *Haemophilus ducreyi* [464, 465]. A putative example was identified by sequence similarity in the genome sequence of the moderately pathogenic *Y. enterocolitica* O:3 strain Y11 [466] and has also been seen in other *Y. enterocolitica* genomes (N. Thomson, personal communication).

CDT is an AB_2 toxin and consists of a hetero-trimeric complex of three proteins: CdtA, CdtB and CdtC [467-470]. CdtA and CdtC subunits are required for delivery of CdtB into host cells. The CdtB subunit resembles type I mammalian DNases and enters the nucleus and binds host DNA, causing double strand breaks [471], leading to arrest of the cell cycle [445, 470, 472-476].

The role of CDT in the pathogenesis of enteropathogenic *Yersinia* is not yet established. Findings of relevance to CDT in *Yersinia* are discussed in Chapter 3 of this thesis, where the Insectidal Toxin Complex is also considered in detail.

1.7 Genotyping of *Yersinia* species

1.7.1 Multilocus Enzyme Electrophoresis (MLEE)

Multi Locus Enzyme Electrophoresis (MLEE) is a molecular technique based on the electrophoretic mobility of the cellular enzymes that can be directly linked to the allelic variation at the corresponding gene [477]. MLEE enabled clustering of *Y. enterocolitica*-like species separately from *Y. enterocolitica* [478, 479]. Furthermore, *Y. enterocolitica* strains were separated in two distinct groups according to their pathogenic potential; moderately pathogenic *Y. enterocolitica subsp. palearctica,* and highly pathogenic *Y. enterocolitica subsp. enterocolitica* [478, 479]. *Y. enterocolitica* strains of biovar 1A were clustered into four separate groups showing high genetic diversity [480]. Also, MLEE typing demonstrated that *Yersinia* strains biochemically identified as *Y. frederiksenii* may be represented by more than one species [479]. This technique provided information about genetic heterogeneity of *Yersinia* species by clearly dividing them to the species and subspecies levels [478-480], however, did not provide clear geographical or source of isolation (host) distinction within clusters [478, 480]. MLEE has largely been replaced by Multi Locus Sequence Typing (MLST).

1.7.2 Multi Locus Sequence Typing (MLST)

Multilocus Sequence Typing (MLST) was developed from MLEE [481]. Indistinguishable electromorphotypes can be encoded by very different nucleotide sequences and MLST typing allows discrimination of more alleles per locus by the amplification of internal fragments of approximately 400-600bp from multiple housekeeping genes that encode fundamental metabolic functions [481, 482]. MLST data can easily be made freely-accessible in curated databases [481] and used for epidemiological, evolutionary and population studies of bacteria regardless of their underlying diversity, population structure or evolution with some caveats given below [481, 482].

The level of discrimination afforded by MLST depends upon factors such as the number and type of genes used, the length of the sequenced gene fragment and the degree of diversity within the sample being characterized. The number of loci is generally minimised to 6-7 to meet the requirements for routine typing [482]. However, for clonal bacterial populations or for evolutionary young (recently diverged) species that have a single clonal lineage, MLST can be poorly discriminatory in some genera when based on the sequencing of 7 core housekeeping genes [483]. Sequencing of 20-30 core housekeeping genes can be useful in such cases but would require an initial genome screening procedure to identify polymorphic loci, an investment in funds and time which is becoming more achievable all the time with next generation sequencing [483, 484]. Variation in housekeeping genes provides basic clonal assignments, whereas sequences from hypervariable loci can be used to "zoom in" on specific clones and tease apart very recent patterns of descent [483, 484].

The intra- and interspecies genetic relationships of 58 strains representing almost all currently known species of the genus *Yersinia* were examined by 4-gene MLST, using sequence data from, *glnA, gyrB, recA and* Y-HSP60 loci in combination and in comparison with 16S RNA gene sequences [485]. MLST and 16S RNA data correlated well in most strains, and agreed with the species assignment based on biochemical tests. *Y. aldovae*, *Y. bercovieri*, *Y. intermedia*, *Y. pestis*, *Y. pseudotuberculosis*, *Y. rohdei*, and *Y. ruckeri* species groupings were genetically more homogeneous than *Y. enterocolitica*, *Y. frederiksenii*, *Y. kristensenii*, and *Y. mollaretii*. In instances where the

16S RNA and MLST data did not agree with each other, MLST data was more consistent with the species' biochemical identification than 16S RNA results [485].

Four bacterial populations were identified by MLST analysis of 417 *Yersinia* strains biochemically identified as *Y. pseudotuberculosis*; these include *Y. pseudotuberculosis sensu stricto, Y. pestis, Y. similis* and new group of *Y. pseudotuberculosis* that is currently undergoing speciation [486].

1.7.3 Repetitive elements PCR

Repetitive element PCR is a method for fingerprinting bacterial genomes by detecting repetitive DNA elements using PCR amplification [487]. Two main sets of repetitive elements are used for typing purposes: small (35bp) Repetitive Extragenomic Palindromic (REP) elements [488], and 126bp Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences [489]. ERIC sequences are major genome components of pathogenic *Yersinia,* accounting for 0.7% and 0.45% of the total DNA of *Y. enterocolitica subsp. enterocolitica* strain 8081 and *Y. pestis* strain CO92 [490], respectively.

Repetitive element PCR clearly distinguish clinical and environmental non-pathogenic biovar 1A *Y. enterocolitica* strains [491]. However, biovar 1A *Y. enterocolitica* strains in general show limited genetic heterogeneity with this method [491]. REP-PCR was shown to be more discriminatory than ERIC-PCR or PCR-ribotyping for genomic profiling of *Y. enterocolitica* strains of various serotypes [492]. In another, study REP-PCR and especially ERIC-PCR provided effective discrimination between various *Yersinia species,* while 16SrDNA sequencing unsurprisingly failed to differentiate *Y. pseudotuberculosis* from *Y. pestis* [152]. *Y. enterocolitica* strains of bioserovar 4/O:3 showed high genomic similarity by ERIC-PCR profiling that could be resolved by Pulse Field Gel Electrophoresis (PFGE) [493].

1.7.4 Ribotyping

Ribotyping is a procedure that analyses the copy number, genomic location and nucleotide sequence variability of the ribosomal operon (*rrn* loci) and was first described by Grimont and Grimont [494]. Ribosomal operons are polycistronic transcriptional units that encode conserved 16S, 23S and 5S rRNA molecules and variable intragenic spacer region (ISR) located between 16S and 23S rDNA.

Conventional ribotyping is based on restriction endonuclease digestion of genomic DNA and subsequent electrophoretic separation followed by Southern blot hybridization to a labelled ribosomal operon probe [495].

Ribotyping provides a clear taxonomic and biotype differentiation of *Yersinia* species [496-499], however, the discrimination index of ribotyping depends on the combination of the restriction endonuclease enzyme used and *Yersinia* strain tested [155, 499-502]. Ribotyping of *Y. enterocolitica* strains of biovars 1-5 with *Hind*III and *Eco*RI restriction enzymes allowed clear differentiation of moderately pathogenic *Y. enterocolitica* biovars 2-5 from *Y. enterocolitica* of biovar 1. Biovar 1 strains could be further separated into highly pathogenic and non-pathogenic clusters [498]. These results were congruent with MLEE typing [478, 479].

Ribotyping of various *Y. pseudotuberculosis* isolates with *EcoR*V and *EcoR*I enzymes allowed subtyping of the strains within a given serotype, that is , the discriminatory power of ribotyping was superior to that of serotyping [503]. In contrast, ribotyping with *EcoR*I gave almost identical ribotypes for all *Y. enterocolitica* O:3 strains tested [496, 498, 501], but DNA digestion with *Nci*I [499] and *Bgl*I-*Hin*dIII [501, 504] was efficient for subtyping of *Y. enterocolitica* O:3 strains. Ribotyping performed with the combination of *Bgl*I-*Nci*I [155] and separate ribotyping with *Pvu*II [500] restriction enzymes allowed the best discrimination of non-pathogenic *Y. enterocolitica* biovar 1A strains.

PCR ribotyping was first described by Kostman [505] and Gurtler [506] and is based on the variability of the PCR amplified internal spacer region (ISR) located between 16S and 23S genes. PCR-ribotyping showed a higher discrimination index in comparison to conventional ribotyping for genotyping of non-pathogenic *Y. enterocolitica* biovar 1A strains [155, 507]. The combination of conventional and PCR-ribotyping significantly increases the discrimination power of the genotyping [504]. However , PCR-ribotyping has a lower discrimination index than PFGE [507].

1.7.5 Pulse Field Gel Electrophoresis (PFGE)

Pulse Field Gel Electrophoresis (PFGE) is based on chromosomal DNA digestion with restriction enzymes and subsequent separation of large fragments on a gel with the assistance of an electric field which is switches periodically between two different

directions with various pulse times.

Single enzyme PFGE with *Not*I [62, 508-510] or *Xba*I [507, 509] clearly differentiates *Y. enterocolitica* according to their bioserotypes into separate PFGE pulsotypes, and also shows genetic homogeneity of *Y. enterocolitica* bioserotype 4/O3 (Table 1.9). *Not*I PFGE pulsotypes for bioserotypes 3/O:3, 3/O:5 and 1A/O:5 are more closely associated with the biotype than serotype [508]. Digestion with more than one restriction enzyme enables fine discrimination of *Yersinia* strains making it a useful epidemiological tool [45, 61, 72, 509, 511-513].

Table 1.15 *Y. enterocolitica* pulsotypes obtained with different restriction enzymes

Microorganism	NotI,	XbaI	NotI+XbaI	NotI+XhoI+ApaI	Ref
Y. enterocolitica* O:3	16	8	24	-	[511]
***Y. enterocolitica* 4/O:3**	15	-	-	30	[514]
***Y. enterocolitica* 4/O:3**	22	-	-	126	[61]

* Biotype information is not given

PFGE is an excellent method for typing biochemically atypical *Yersinia* strains [496, 508, 510] and superior to PCR ribotyping [507], ERIC-PCR typing [493], REAP, REAC and ribotyping [496] for epidemiological tracing of *Y. enterocolitica* strains.

1.7.6 Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) DNA fingerprinting is based on the detection of DNA restriction fragments by random PCR amplification without prior DNA sequence knowledge [515]. Double stranded adaptors containing restriction enzyme sites are ligated to DNA fragments cut with the corresponding frequent or rare-cutting restriction enzymes, and serve as primer binding sites for subsequent amplification of DNA restriction fragments [515].

The AFLP method has been applied to various bioserotypes of *Y. enterocolitica* isolated from a wide range of sources including human, domestic animals and food [516, 517]. AFLP DNA fingerprinting clearly separated pathogenic and non-pathogenic *Y. enterocolitica* biotypes, and allowed further serotype associated subdivision of these strains [516, 517]. There was little correlation between the source of isolation and AFLP clustering. That is, some of the animal and human isolates clustered together and some strains of the same bioserotype clustered

separately [516, 517].

The AFLP technique is helpful in confirmation of *Yersinia* species identification. Thus, *Y. enterocolitica* strains were clearly separated from *Y. enterocolitica*-like species [516, 517].

1.7.7 Multiple Locus Variable number tandem repeat Analysis (MLVA)

ShortSequence DNA Repeats (SSR), also known as variable number tandem repeats (VNTR), undergo frequent variation in the number of individual repeat units through slipped strand mispairing during DNA synthesis [518]. Variation in SSR is detected by PCR amplification with the primers for the regions flanking SSR [518]. VNTR typing, also known as MLVA typing, is widely used for epidemiological typing of human pathogens [519]. MLVA typing is less labour intensive than PFGE typing and does not require large culture volumes [520].

MLVA typing based on six VNTR loci identified in the draft of the *Y. enterocolitica* strain Y11 bioserotype 4/O:3 genome allowed better discrimination of *Y. enterocolitica* bioserovar 4/O:3 strains than PFGE [520, 521] or AFLP [521]. These VNTR loci were also detected in strains of serotypes O:5,27 and O:9 [521].

Although the six VNTR loci selected at the beginning of the study were stable during *in vitro* multiple passages in the laboratory, a comparison of the MLVA genotypes of epidemiologically linked isolates showed that they exhibited a single variation in the V2a locus and apparent hypermutability in V10 locus *in vivo* [521]. Recent examination of mutational events in VNTR loci in *E.coli* O157:H7 showed that the use of hypervariable loci may limit the validity of MLVA for long-term epidemiological investigations [522]. Thus each MLVA scheme used for epidemiological investigations must be validated with a control group of epidemiologically linked strains.

1.7.8 Microarray Genotyping

DNA microarrays allow whole-genome comparisons between a sequenced reference genome and genome of test strains without whole-genome sequencing of the comparator strains [523]. This technique is based on the competitive DNA-DNA hybridization between fluorescently labelled DNA from the test strain and a control strain to the reporter DNA elements on a microarray. After washing of the unbound

DNA, microarrays are scanned by a fluorescence scanner, and the obtained image is analysed by a specific software package.

Two microarray technologies exist – the original Polymerase Chain Reaction product based microarrays (PCR-based) and now oligonucleotide-based DNA microarrays. Both technologies have their advantages and downsides [523]. The advantages of PCR-based microarrays were: flexibility in the design of the microarray; relative ease of production; and its relatively low cost. On the other hand PCR-based microarrays do not allow detection of single nucleotide polymorphism (SNP) and mutant alleles that can be detected by using oligonucleotide-based microarrays. Additionally oligonucleotide-based microarrays reduce cross-hybridization as more specifically designed oligonucleotides are employed and these have now largely replaced PCR product microarrays [523-527].

Microarray technology in combination with phylogenetic analysis is a sensitive and robust method for studying genetic relatedness bacterial populations [528-531].

The genome sequence of the highly pathogenic *Y. enterocolitica subsp. enterocolitica* strain 8081 [532] has allowed whole genome comparison using microarray technology between *Y. enterocolitica* strains of different origin in the same way that *Y. pestis* and *Y. pseudotuberculosis* strains have been analysed [533]. Comparative phylogenomic analysis of microarray data generated in *Y. enterocolitica* strains isolated in the Great Britain from humans with diarrhoea and domestic animals resulted in distribution of strains into the three clades: highly pathogenic, low pathogenic and non-pathogenic clades [531]. Strains isolated from humans and livestock were distributed throughout the clade and did not cluster together by the source host in both low-pathogenic and non-pathogenic clades [531]. Within the low-pathogenicity clade, isolates were partially separated according to the biotype and serotype; the distribution of isolates within non-pathogenic and highly pathogenic clades was more heterogeneous [531].

1.8 Evolution of *Yersinia* species

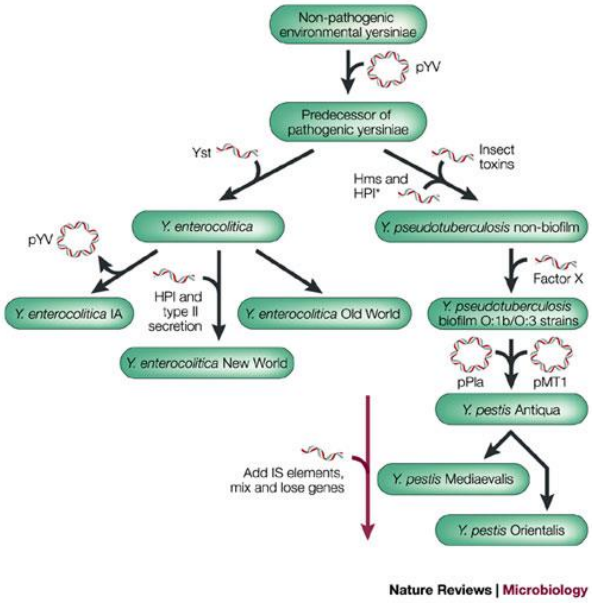


Figure 1.8 Schematic representation of a hypothesis of pathways for pathogenic *Yersinia species* evolution (adapted from *Wren* [534])

Molecular phylogenetics analyses evolutionary relationships among organisms by comparing molecular differences in their DNA and protein sequences [535]. The application of typing methods used in molecular epidemiology can also be extended to studying evolution of bacterial populations [484, 486, 528, 536, 537]. Based on genetic differences in housekeeping genes, the blood-borne pathogen *Y. pestis* was found to be a clone of the gastrointestinal pathogen *Y. pseudotuberculosis*. Using the "molecular clock" hypothesis the former was estimated to have evolved from the latter around 1,500-20,000 years ago (Figure 1.8) [538]. More extensive genomic comparisons using single nucleotide polymorphisms (SNPs) supported this relationship [529] and have allowed detailed phylogeographical studies placing the origin of all current *Y. pestis* strains in China [484]. Ancient DNA studies combined with this detailed SNP data have confirmed *Y. pestis* as the cause of the 14th century Black Death [539-541].

This transition was accompanied by the combined acquisition and loss of a number of genes in the *Y. pestis* genome relative to *Y. pseudotuberculosis* [532, 533, 538, 542, 543]. *Y. pestis* acquired two plasmids: a 100-110 kb pFra plasmid (also called pMT1 [534]) that encodes a phospholipase D homologue Ymt that promotes colonization [544] and survival of *Y. pestis* in the flea midgut [545], and a 9.5 kb pPla plasmid (also termed pPCP1 or pPst [534]), that encodes plasminogen activator Pla and is required for systemic transmission of *Y. pestis* [546]. The pFra plasmid partially resembles the pHCM2 plasmid of *Salmonella typhi* and was probably acquired by the ancestral *Y. pseudotuberculosis* before the emergence of *Y. pestis* [547]. Both *Y. pestis* and *Y. pseudotuberculosis* encode *hms* loci [355, 358], however, the functionality of these loci differ in each microorganism. The *Y. pestis hms* locus mediates blockage of the flea foregut that instigates futile attempts to feed, hence the flea regurgitates infected blood back into the bite site [358]. In contrast, *Y. pseudotuberculosis* infected fleas do not show blockage of the foregut [359].

The virulence plasmid pYV is a consistent feature in pathogenic *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica* and the most parsimonious explanation for this is a single acquisition event as shown in figure 1.8.

In addition to the loss of methionine-salvage and osmoregulated periplasmic glucan (OPG) biosynthetic pathways that are essential for gut colonisation [532, 548] *Y. pestis* has undergone loss or inactivation by mutation of various proteins (YadA, Inv, Urease, flagellar operon) necessary for *Yersinia* gut colonisation or translocation across the intestinal barrier [542]. Although *Y. pestis* has apparently lost the ability to colonise the gastrointestinal tract, it can cause pharyngitis by infection of phayngeal lymphatic tissue [549].

Housekeeping genes and the "molecular clock" in turn suggest *Y. enterocolitica* and *Y. pseudotuberculosis* diverged within the past 200 million years [548]. Surprisingly, although both *Y. enterocolitica* and *Y. pseudotuberculosis* are enteric pathogens occupying a similar ecological niche, comparative genomics suggests *Y. pseudotuberculosis* lost or underwent significant changes in several metabolic pathways (cellulose biosynthesis and tetrathionate respiration pathways, and OPG cluster) presumably essential for gut colonisation [548]. *Y. enterocolitica* has acquired by lateral gene transfer hydrogenase gene clusters (*hyf* and *hyb* loci),

cobalamin (B_{12}) synthesis cluster (*cbi*) and 1,2-propanediol utilisation (*pdu*) pathways that may enhance *Y. enterocolitica* ability to colonise the gastrointestinal tract [548, 550].

Y. enterocolitica species include microorganisms with a variable pathogenic potential (see 1.2.5 Biotyping of *Y. enterocolitica*). Comparative microarray analysis established that mildly and non-pathogenic *Y. enterocolitica subsp. palearctica* strains are closely related by gene content, however, non-pathogenic strains seem more related to highly pathogenic *Y. enterocolitica subsp. enterocolitica* strains than mildly pathogenic strains [531]. Biotype 1A strains generally show a high degree of genomic heterogeneity [516, 517, 531]. AFLP analysis indicated that some non-pathogenic *Y. enterocolitica* 1A of human and animal origin were grouped together with pathogenic biovars [516].

The presence of insecticidal toxin complex genes resembling loci in the insect pathogen *Photorhabdus luminescens* [551-553] in *Y. pestis* [542], *Y. pseudotuberculosis, Y. mollaretii, Y. frederiksenii* [554] and mildly pathogenic [555] and non-pathogenic [556] *Y. enterocolitica subsp. palearctica* isolates suggests ancestral *Yersinia* species were associated with insects or insect pathogens [538, 542]. A baculoviral enhancin gene related to insect parasitism is also present in *Y. pestis* and *Y. pseudotuberculosis*.[533, 542].

It is thought that because of the numerical predominance of invertebrates and similarities of cellular and molecular immune responses of invertebrates and vertebrates, insects may act as a reservoir for emerging human pathogens [557]. Additionally, according to “eco-evo” perspectives on host-pathogen interactions, many bacterial virulence factors were initially evolutionary shaped for interactions with non-human hosts, and only subsequently due to accidental contacts with humans were these factors evolutionary adapted for interactions with humans [558]. Indeed, genomic comparisons of *Y. enterocolitica* and *Ph. luminescens* shows that many of *Yersinia* virulence factors such as Ail, urease, Inv and YplA have apparent homologues in *Ph. luminescens* [559].

1.9 Aims and Objectives

Because of the low incidence rates of yersiniosis in Ireland and the high prevalence of pathogenic *Y. enterocoloitica* in slaughter pigs (*unpublished Murphy et al.*) the aim of this study is to investigate the reasons for such discreapancies. Low notifications of yersiniosis in the presence of high risk might represent a failure to detect pathogenic *Yersinia* in diarrhoeal stool samples or the prevalence of mild self-limiting forms of yersiniosis in the Irish human population. The evidence of active forms of human yersiniosis was pursued through the examination of clinical samples with *Yersinia* selective culture, and substantiation of mild forms of yersiniosis was sought by a serological screening of asymptomatic healthy individuals as well as by molecular and culture screening of human sewage.

Y. enterocolitica is a heterogeneous species that includes pathogenic and non-pathogenic microorganisms. Pathogenic *Y. enterocolitica* biotypes are predominantly gastroenteric pathogens that possess chromosal (required for the initial stages of infection) and pYV plasmid encoded (required for systemic dissemination of *Yersinia*) virulence factors. Non-pathogenic biovar 1A *Y. enterocolitica* do not encode conventional virulence factors. However, their predominance in the collections of *Y. enterocolitica* strains isolated from humans (*gifts of Prof. M. Cormican (University College Galway) and Dr. J. Moore (Queen's University Belfast)*) and retail meat samples (*unpublished Murphy et al.*) incited investigation of their pathogenic potential towards humans. Clinical and non-clinical *Y. enterocolitica* strains of various biovars were screened for the presence of novel virulence factors and their pathogenic potential was compared in a *Galleria mellonella* model.

Because of the extensive human, animal and food exchange between Ireland and United Kindom, investigation of the genetic relationships between clinical and non-clinical *Y. enterocolitica* strains isolated in Ireland and United Kindom is required. Many *Yersinia* genotyping techniques described in section 1.7 are based on knowledge about limited set of genes and often require additional validation for the discrimination of closely related bacterial populations. On the other hand, microarray technology allows comparative genomics without the need for full

genome sequencing for all test microorganisms and was shown to be sensitive and robust. Therefore, genetic relationships between clinical and non-clinical *Y. enterocolitica* strains isolated in Ireland and United Kindom were investiagted by comparative microarray analysis.

Chapter 2 Current evidence for human yersiniosis in Ireland

Ringwood T, Murphy BP, Drummond N, Buckley JF, Coveney AP, Redmond HP, Power JP, Fanning S, Prentice MB: **Current evidence for human yersiniosis in Ireland**. *Eur J Clin Microbiol Infect Dis* 2012. ***(see Appendix)***

2.1 Introduction

Y. enterocolitica and to a lesser extent *Y. pseudotuberculosis* are the leading causes of yersiniosis in Europe and worldwide [12, 13, 560]. Most gastrointestinal *Yersinia* infection cases are sporadic [12, 13, 17, 560] but recent outbreaks of *Y. pseudotuberculosis* [19, 174] and *Y. enterocolitica* [21, 25] infections involving food have been reported. The association of yersiniosis with the consumption of contaminated pork meat is consistent worldwide [14, 16, 22, 61, 66]. Pig tonsils as well as faecal material are the main sources of the pig meat contamination with pathogenic *Y. enterocolitica* [69, 70]. In Ireland tonsillar carriage of pathogenic *Y. enterocolitica* in 31% of pigs at slaughter level has recently been recorded (*unpublished Murphy et al.*), a figure very similar to carriage seen in recent surveys of slaughter pigs in Finland (35%) [561] and Belgium (37.4%) [57] However, the annual incidence of human yersiniosis notified from 2006-8 inclusive in Ireland was the lowest in Europe and did not exceed 10 cases per year (≤0.1 per 100,000 population) [12]. In comparison, in Finland and Belgium, an average of 12.03 and 2.5 yersiniosis cases per 100,000 of population respectively were reported during the same period [12].

In contrast, in the 1980s, a serological survey of agglutinating antibodies reported that 23% of patients with a clinical diagnosis of appendicitis in two hospitals in Dublin had active yersiniosis [177]. Based on the high current prevalence of pathogenic *Y. enterocolitica* in pigs in Ireland, we hypothesized that the current low yersiniosis notification rates might represent a failure to detect *Yersinia* infection in humans, possibly due to the requirement for specific culture methodology to isolate *Yersinia* species from faeces [65, 78].

Isolation of pathogenic *Yersinia* species from stool specimens may require selection for the bacterium in the presence of other background microflora [17, 82, 86]. Cold enrichment of specimens at refrigerator temperatures (2-8°C) for up to three weeks is an effective procedure for isolation of *Yersinia* species from food [84, 85] and human stool samples [82, 86]. Alternatively, alkali shock may rapidly eliminate competing microflora without prolonged cold enrichment for the selective isolation of pathogenic *Yersinia* from stool samples [105] and food [104, 106, 107]. A short

enrichment period in respect of diarrhoeal stool samples in *Yersinia* selective broth at 16°C and subsequent inoculation onto Cefsulodin Irgazan Novobiocin (CIN) agar (YSE-CIN method) significantly increased the isolation of pathogenic *Y. enterocolitica* from diarrhoeal stool samples in comparison to traditional culture methods [79]. Specific enrichment or selective culture for *Yersinia* is not currently routinely practised in most diagnostic laboratories in Ireland.

While infection with *Y. enterocolitica* and *Y. pseudotuberculosis* mainly involves the gastrointestinal tract with acute enteritis [12, 13, 17, 80, 92, 560] and mesenteric lymphadenitis (mimicking appendicitis) [92, 178, 179], in some outbreaks of yersiniosis, pharyngitis and not diarrhoea was a common symptom [198, 199]. Although enteritis is the most common clinical form of yersiniosis [12, 13, 17, 80, 92, 560], isolation rates of *Yersinia* species from postoperative appendix swabs (2.7% to 10%) [90, 183, 189] are often higher than from faeces (0.2% to 6.2%) [65, 78, 91, 190, 191].

Therefore, evidence of *Yersinia* infection in humans in Ireland was sought by carrying out *Yersinia* selective culture of diarrhoeal stool samples with different modes of enrichment, as well as culture screening of throat swabs from patients with pharyngitis and swabs from resected appendix fossae. Furthermore, because subclinical forms of yersiniosis [17, 162-164, 562] do not result in submission of stool samples for culture, a molecular and culture screening of human sewage as well as a serological survey of asymptomatic healthy individuals were carried out.

2.2 Materials and methods

2.2.1 Clinical specimens screening methodology

2.2.1.1 Clinical specimens selection

Yersinia selective culture was carried out on anonymised clinical specimens collected at the Microbiology Department of Cork University Hospital (CUH) during five collection periods (Table 2.3). Diarrhoeal stool samples were obtained from 601 (50.5%) children and 588 (49.5%) adults (≥18 years). Outpatients were prioritised to exclude nosocomial diarrhoea. However, 17.8% of all screened stool samples were acquired from inpatients admitted to the hospital with diarrhoea. Formed stools were excluded from the survey.

All throat swabs from patients with a clinical diagnosis of pharyngitis available during collection period 4 (Table 2.3) and appendix swabs from surgical patients with a clinical diagnosis of appendicitis available during collection period 5 were cultured for the presence of *Yersinia* species. Demographic information was not available for patients with pharyngitis. Appendix swabs were obtained from eleven children and twelve adults (>18 years), for two specimens information about age of patients was not provided. Fifteen (65.2%) surgical patients received pre-operative antibiotic treatment with the amoxicillin/clavulanic acid.

2.2.1.2 Yersinia isolation methodology

During each collection period different isolation procedures were used in progressive order (Table 2.3). Various liquid enrichment procedures were carried out over the course of the study; final incubation was on CIN (LIP, Galway, Ireland) agar plates for three of four of these, and for one period direct plating of faeces onto CIN agar plates was applied. Inoculated agar plates were incubated at 30°C for 18–24 h [127], if no bacterial growth was observed after this period of incubation plates were incubated for an additional 24 hours at 30°C with a maximum incubation time of 48 hours. MacConkey (MAC) (Merck Chemicals) agar plates were used for inoculation of diarrhoeal stool samples following cold enrichment during collection period 1 only.

Cold enrichment (CE) procedure. A loop full (approx. 10 µl) of each stool sample was inoculated into 5 ml of phosphate buffered saline (PBS) (Sigma-Aldrich) and placed into a refrigerator (2-8°C) for cold enrichment (CE) over a 3-week period [127]. CE procedures were used for 375 specimens collected during periods 1 and 2 only (Table 2.3). Stool enrichments were plated onto MAC agar plates [88] during collection period 1 and onto CIN agar plates [91, 563] during collection period 2.

Alkali treatment procedure. Alkali treatment to remove contaminating faecal flora was applied to stool specimens during period 1 only. Prior to alkali treatment PBS suspensions of stool samples (5 ml) were incubated at 30°C for 48 h (warm enrichment, WE) [104, 107]. Three methods of alkali treatment were applied. *Method A (0.25% KOH, 2 min)* [105, 110]*:* equal parts of stool sample after WE and 0.5% KOH solution were mixed for 2 min. *Method B (0.25% KOH, 15 sec):* equal parts of stool sample after WE and 0.5% KOH solution were mixed for 15 sec. *Method C*

(0.5% KOH, 15 sec): equal parts of stool sample after WE and 1.0% KOH solution were mixed for 15 sec. One portion of PBS stool suspensions were alkali treated and inoculated onto MAC agar plates [104, 105, 107], and another portion was plated onto MAC agar plates without prior alkali treatment.

YSE-CIN procedure. During collection periods 4 and 5 all clinical specimens (faeces and swabs) were enriched in *Yersinia* selective (YSE) broth and inoculated onto CIN agar plates. YSE broth was prepared as described [79, 564]: 10 g tryptone (Merck Chemicals), 2.75 g 3-N-morpholino propanesulfonic acid (MOPs) (Sigma-Aldrich), 2.75 g Tris-HCl [pH 8.4] (Sigma-Aldrich), 20 g D-sorbitol (Sigma-Aldrich), 1.5 g Bile salts (Sigma-Aldrich) were dissolved in 1 L of distilled H_2O and autoclaved at 121°C for 15 min. Two vials of *Yersinia* selective supplement (OXOID, UK) containing cefsulodin, irgasan and novobiocin antimicrobials, and 5 ml of sterile 0.8 M $KClO_3$ (potassium chlorate) (Sigma-Aldrich), were added to 983 ml of autoclaved base broth and pH adjusted to pH 8.4.

In period 4, pooled stool samples (5 samples for each 20 ml of enrichment broth) were enriched in YSE broth at 16°C for 48 h [79]. Throat swabs were enriched in YSE broth in pools of 5 for each 20 ml of broth and incubated at 30°C for 96 h.

In period 5 pooled stool samples (10-15 samples for each 35 ml of enrichment broth) were enriched in YSE broth at 2-8°C for 10-12 days. Each appendix swab was enriched in 10 ml of YSE broth and incubated at 2-8°C for period of 3 weeks.

2.2.2 Recovery of *Y. enterocolitica* from experimentally inoculated faecal specimens

Diarrhoeal faeces from 13 individuals known to be negative for enteric pathogens were homogenized (1:1) in sterile PBS solution. Each homogenized faecal specimen was divided into six sterile containers (0.9 ml per container) and spiked with 0.1 ml of six ten-fold serial dilutions of 1.0 $OD_{600\ nm}$ *Y. enterocolitica* IP383 (B2/O:9) to achieve final concentrations in aliquoted spiked stool samples ranging from $1.8x10^6$ to 1.8x10 CFU/ml of faecal homogenate. Thoroughly mixed spiked faeces were processed accordingly to the *Yersinia* selective procedures in Table 2.3 with the exception of YSE-CIN used in periods 4 and 5 for which individual stool samples rather than pooled samples were spiked.

2.2.3 *Yersinia* identification procedures

Biochemical identification of Yersinia. Presumptive *Yersinia* species colonies (small lactose-negative on MAC agar plates [113], and small red *bull-eye* appearance colonies on CIN agar [114]) were further identified with API 20E biochemical galleries (BioMérieux), incubated overnight at 30°C [130, 131]. Isolates identified as *Y. enterocolitica* were biotyped according to the Wauters schema [133] and screened for the presence of *Yersinia* virulence plasmid (pYV) on Congo Red-Magnesium Oxalate (CR-MOX) agar [565].

CR-MOX agar was prepared as described [565]: 40 g of tryptic soy agar (Merck Chemicals) was mixed with 825 ml of distilled water and autoclaved for 15 min at 121°C. The molten medium was cooled to 55°C, and the following solutions were added: 80 ml 0.25 M sodium oxalate (Sigma-Aldrich), 80 ml 0.25 M magnesium chloride (Sigma-Aldrich), 10 ml 20% D-galactose (Sigma-Aldrich), and 5 ml 1% Congo Red (Sigma-Aldrich). The D-galactose solution was sterilized by autoclaving for 10 min at 115°C. All other solutions were autoclaved for 15 min at 121°C. *Yersinia* strains forming small pinpoint bright red colonies on CR-MOX agar after overnight incubation at 37°C were deemed pYV positive. The positive control organism was pYV-positive *Y. enterocolitica* 8081 sourced from D. Portnoy, University of California.

Molecular identification of Yersinia. 16S rDNA from isolates was amplified with the following primers: 16S-27F (5′-*aga gtt tga tcc tgg ctc ag*-3′) [566] and 16S-1435R (5′-*ctt ctt ttg caa ccc act cc*-3′) and the sequences were initially assigned to a taxonomic group with the Ribosomal Database Project Classifier [567] and an RDP Seqmatch search carried out [568, 569].

2.2.4 Human sewage and pig slurry screening for pathogenic *Yersinia*

2.2.4.1 Separation of bacterial cells from the faecal matter

Human sewage samples (500 ml) from five sewage treatment plants (STP) and pig slurry (PS) (500 ml) from six pig farms were collected in county Cork, Ireland. Sewage samples were concentrated by centrifugation at 4300 x g for 5min. For pig slurry the concentration protocol was omitted due to the high concentration of the faecal material. Bacterial cells were separated from concentrated raw sewage and

pig slurry by Nycodenz (Axis Shield) gradient centrifugation as described [570, 571]. Briefly, a 1 ml sample of concentrated sewage was centrifuged with 650 µl of Nycodenz (1.3 g/ml in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA) at 10,000 x g for 6 min at 4°C [571]. 25 ml of neat pig slurry was centrifuged with 9 ml of Nycodenz (1.3 g/ml in 10mM Tris-HCl (pH 8.0) containing 1 mM EDTA) at 10,000 x g for 20 min at 4°C [570]. Separated bacterial cells from Nycodenz first were washed in 5 ml of sterile PBS by centrifugation at 10,000 x g for 10 min and then the washed bacterial pellet was inoculated into 5 ml of YSE broth [79, 564] and incubated with shaking at 200 rpm overnight at 30°C [564]. YSE enrichments were streaked onto CIN agar plates, and 1 ml of each broth was aliquoted for DNA extraction with a Promega Total DNA kit (Promega UK, Southampton). Inoculated CIN agar plates were incubated overnight at 30°C for 18–24h [127]. Presumptive *Yersinia spp.* colonies were identified as described above.

2.2.4.2 Molecular detection of Yersinia contamination

Molecular screening for pathogenic *Yersinia* species in sewage and pig slurry used a modified *yadA* nested PCR protocol [148]. Additionally, the presence of *Y. pseudotuberculosis* specific *ail* and *Y. enterocolitica* specific *ystA* genes were identified using published PCR primers [151, 572] (Table 2.1). PCR master mix for each reaction was prepared with GoTaq Green Polymerase kit (Promega UK, Southampton) according to the manufacturer's recommendations; total volume of the PCR reaction for each protocol was 50 µl. PCR was performed on a PTC-200 Peltier Thermocycler with the following conditions: denaturation at 94°C for 5 min; followed by 29 cycles of each: denaturation at 94°C for 30 sec, annealing depending on Tm of primers ranged from 55°C to 65°C for 30sec, elongation at 72°C for 40 sec, and final elongation at 72°C for 7 min. Amplicons were maintained at 4°C. The first round of *yadA* PCR consisted of 40 cycles, the second round of *yadA* nested PCR consisted of 20 cycles. Because the *yadA* PCR products were of variable size their identity was confirmed by commercial sequencing (GATC, Germany). To confirm the absence of PCR inhibitory substances co-purified from faecal material, environmental DNA samples were spiked with control DNA isolated from *Y. enterocolitica* 8081 in a ratio of 4:1 (Environmental DNA : Control DNA).

Table 2.1 Primers used for environmental screening for *Yersinia* species

Target		Primer Sequence	Product size (bp)	PCR Method	Ref
Y. enterocolitica ystA	F	*5′-aatgctgtcttcatttggagc-3′*	145	Simple	[572]
	R	*5′-atcccaatcactactgacttc-3′*			
Y. pseudotuberculosis ail	F	*5′-cgtctgttaatgtgtatgccgaag-3′*	157	Simple	[151]
	R	*5′-gaacctatcactccccagtcattatt-3′*			
Y. enterocolitica yadA	F	*5′-taagatcagtgtctctgcggca-3′*	747	Nested	[148, 573]
	R	*5′-tagttatttgcgatccctagcac-3′*			
	F	*5′-gcgttgttctcatctccatatgc-3′*	529		
	R	*5′-ggctttcatgaccaatggatacac-3′*			

F :Forward primer
R :Reverse primer

2.2.5 Serological screening

Anonymised blood plasma samples from 200 Irish blood donors were obtained from the Munster Regional Blood Transfusion Centre, St. Finbarr's Hospital, Cork in April 2009 together with 17 blood plasma samples obtained from surgical patients diagnosed with appendicitis, who were admitted to CUH. Blood plasma samples were stored at -20°C for one week before analysing and then transferred for long term storage at -80°C. The nationality of the individual blood donors was not known.

All samples were initially tested for anti-*Yersinia* antibodies with the quantitative *in vitro* recomWell *Yersinia* IgG ELISA (Mikrogen Diagnostik, Germany). The immunoassay was performed and interpreted according to the manufacturer's guidelines. Samples with measured antibody activity levels below 20 U/ml were considered as negative, samples with measured antibody activity levels between 20 and 24 U/ml were assigned as borderline, and samples with the antibody activity levels above 24 U/ml were recorded as positive. Samples with equivocal results were retested. Positive samples in recomWell *Yersinia* IgG ELISA assay were tested with the recomLine *Yersinia* IgG strip immunoassay. This is a qualitative *in vitro* assay that allows specific detection of anti-*Yersinia* antibodies against pYV plasmid encoded virulence factors: YopM, YopH, V-Ag, YopD, YopN and YopE applied to a nitrocellulose membrane strips. Each strip also contains a reaction control, an IgG conjugate control and a cut off control band. All tests were performed and interpreted according to the manufacturer's guidelines. Samples were considered as anti-*Yersinia* IgG positive when the reaction control and IgG conjugate controls were positive for YopD or at least two other bands displayed the same (+) or a stronger (++/+++) intensity (as judged by the un-aided eye) than the cut off control. If no YopD-band (-) or weak YopD-band (+/-) but at least two other bands with the same (+) or a stronger (++/+++) intensity than the cut off control was seen then results were interpreted as borderline.

Blood plasma collected from 17 patients with appendicitis was tested with RecomWell *Yersinia* IgG kit only.

2.2.5.1 Cross reactivity screening

Blood plasma positive for *Yersinia* IgG and gender/age matched anti-*Yersinia* IgG negative blood plasma were screened with a recomBead *Borrelia* IgG assay (Mikrogen Diagnostik, Germany) [574]. This is a semi-quantitative *in vitro* immunoassay based on Luminex xMAP® technology enabling detection of anti-*Borrelia* antibodies against four *Borrelia* species pathogenic for humans: *B. burgdorferi sensu stricto*, *B. garinii, B. afzelii* and *B. spielmanii.* Results were interpreted using specifically designed Excel-based software supplied by Mikrogen Diagnostik (Germany).

2.2.6 Statistical analysis

Linear regression with significance level of 0.05 and χ^2 (Chi-square) were used to model relationships between serological variables in MiniTab® 16 statistical software. P values for spiked cultures were calculated using GraphPad QuickCalcs Web site: http://www.graphpad.com/quickcalcs/Contingency1.cfm (*accessed November 2011*). Power calculations for the sample size of the faeces and throat swab culture studies were carried out as described [575] based on an inability to falsify the null hypothesis that pathogenic *Yersinia* are not responsible for diarrhoea/throat infection in our population, using a binomial model with an assumed population size of >100,000 and an expected true prevalence of pathogenic *Yersinia* causing diarrhoea requiring samples to be sent for culture (threshold of concern) of 0.005, and an expected prevalence of pathogenic *Yersinia* throat carriage of 0.02.

2.3 Results

2.3.1 Clinical specimens screening

Pathogenic *Yersinia* species were not detected in the 1,189 faeces samples, 297 throat swabs and 23 appendix swabs cultured in this study with the procedures summurised in Table 2.3. Three non-pathogenic biovar 1A *Y. enterocolitica* strains (pYV negative on CR-MOX agar and by PCR for *yadA*) were isolated from pooled faeces cold enriched in YSE broth in period 5. Each isolate was cultured from a separate pool: adults (11 samples), children 5 to 9 years old (6 samples) and children 0 to 4 years old (14 samples).

Table 2.2 Clinical and demographic distribution of patients with diarrhoea

Patients age groups	All patients	Inpatients	Blood in stool	Abdominal pain
Children (0-14 yo)	601 (50.5%)	115 (19.1%)	10 (1.7%)	12 (2.0%)
Adolescents (15-17 yo)	4 (0.3%)	0 (0%)	0 (0%)	1 (25%)
Adults (≥18 yo)	584 (49.1%)	97 (16.6%)	16 (2.7%)	15 (2.6%)
Total	1189 (100%)	212 (17.8%)	26 (2.2%)	28 (2.3%)

Table 2.3 Isolation procedures

Period	Duration	Sample Numbers			Isolation Procedures*
		Stool	Throat	Appendix	
1	27th March'07 - 27th April'07	191	-	-	CE, alkali treatment, MAC agar
2	14th January'08 - 12th February'08	184	-	-	CE
3	9th - 18th December'08	97	-	-	Direct plating
4	10th November'09 – 13th April'10	122	297	-	Stool Samples (pools of 5) – YSE broth (16°C, 48 h) Throat swabs (pools of 5) – YSE broth (30°C, 96 h)
5	13th May'11 – 21st June'11	595	-	23	Stool samples (pools of 10-15) – YSE broth (2-8°C, 10-12 days) Appendix swabs – YSE broth (2-8°C, 21 days)
Total		1189	297	23	

* Different liquid enrichment methods employed but all specimens sub cultured on CIN agar, apart from period 1 (MAC agar).

If the true prevalence of *Y. enterocolitica* causing diarrhoea with faecal samples submitted for culture in our population was 0.005 (0.5%), a minimum sample size of 919 would be required to detect the presence of this disease with 99% confidence [575]. This number is exceeded in the current study, even excluding samples processed in period 1 using MacConkey agar, which was inferior in detection of

spiked faeces samples to the culture methods used in periods 2-5 (see below). Similarly, the negative throat swabs exceed the minimal sample size of 228 to exclude 2% pharyngitis caused by culturable *Y. enterocolitica* with 99% power.

2.3.1.1 *Recovery of Y. enterocolitica from experimentally inoculated faecal specimens*

Prolonged cold enrichment in PBS and subsequent inoculation onto CIN agar resulted in the highest recovery rates of *Y. enterocolitica* IP383 (Table 2.4). Culture on MacConkey plates was less likely to result in recovery than other methods ($1.8x10^6$ CFU two-tailed $P < 0.0001$, Fisher's exact test). Inferiority of MAC agar plates was stool-specific (only shown with some stools) and failure to isolate *Yersinia* was because of overgrowth of background enteric microflora.

Table 2.4 Recovery of *Y. enterocolitica* from experimentally inoculated faecal samples

Isolation Procedure	***Y. enterocolitica* IP383 inoculums, CFU/ml of faeces**		
	Period	**$1.8x10^6$**	**$1.8x10^5$**
PBS CE + MAC	1	46.2% (6/13)*	46.2% (6/13)**
PBS CE + CIN	2	100% (13/13)	100% (13/13)
CIN direct plating	3	100% (13/13)	92.3% (12/13)
YSE broth 16°C, 48h	4	92.3% (12/13)	84.6% (11/13)
YSE broth CE	5	100% (13/13)	92.3% (12/13)

* $P < 0.0001$
** $P = 0.006$

2.3.2 Human sewage and pig slurry screening for pathogenic *Yersinia*

Screening of human sewage and pig slurry detected a *Yersinia* specific *yadA* gene fragment in three out of six pig slurry samples and in four out of five human sewage samples (Figure 2.1), *ystA* sequences were detected in two pig slurry samples, but not in human sewage (Table 2.5). Pathogenic *Yersinia* species were not isolated from either pig slurry or human sewage (Table 2.5). Four *Yersinia* isolates were recovered from three (60%) human sewage samples (Table 2.5). These strains did not contain the pYV plasmid as determined by CR-MOX and *yadA* PCR and their 16S rDNA sequences did not match *Yersinia* species pathogenic for humans. Although 16S rDNA is insufficient as sole criterion to separate all *Yersinia* species, *Y. enterocolitica* and *Y. pseudotuberculosis* 16S rDNA sequences are distinct [156, 485] and were not found in these cultures.

Table 2.5 Environmental detection of *Yersinia* species by culture and PCR

Source	Culture Results	RDP Classifier*	RDP Seqmatch*[2]	Environmental PCR on sewage/pig slurry			
				YPT *ail*	*ystA*	*yadA* Simple	*yadA* Nested
PS1	-	NT	NT	-	-	-	-
PS2	-	NT	NT	-	-	-	-
PS3	-	NT	NT	-	+	+	+
PS4	-	NT	NT	-	+	+	+
PS5	-	NT	NT	-	-	-	-
PS6	-	NT	NT	NT	NT	-	+
STP1	-	NT	NT	-	-	-	+
STP2	M1 – API 20E 1105723 *Y. enterocolitica* 93.9%	*Yersinia*	*Y. intermedia* ATCC 29909	NT	NT	-	-
	M3 - API 20E 1014522 *Y. enterocolitica* 1.2%*[3]	*Yersinia*	*Y. kristensenii* ATCC 33638				
STP3	API 20E 1115773 *Enterobacter gergoviae* 83%	*Yersinia*	*Y. frederiksenii* ATCC 33641	NT	NT	-	+
STP4	-	NT	NT	-	-	-	+
STP5	API 20E 1115563 *Y. enterocolitica* 83.5%	*Yersinia*	*Y. aldovae* ATCC 35236	-	-	-	+

PS1 - PS6:Pig slurry samples; **STP1 – STP5**:Human sewage samples; **YPT:***Y. pseudotuberculosis* specific PCR; **"-"**:Negative results; **"+"**:Positive results; **NT**:Not Tested; * 16S rDNA RDP culture assignment; *[2] Type strain with maximal similarity score to culture 16S rDNA with RDP Seqmatch; *[3] Biochemically biotype 3 but lacking pYV and *ystA*

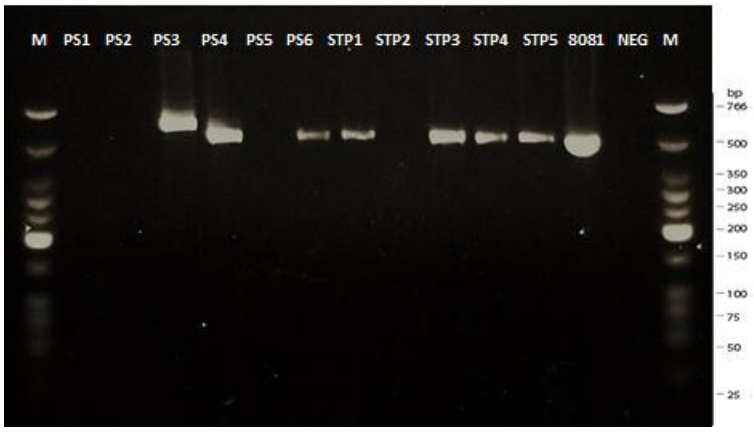


Figure 2.1 *yadA* nested PCR

Expected PCR product 529bp

PS1 - PS6	:Pig slurry samples
STP1 – STP5	:Human sewage samples
8081	:*Y. enterocolitica subsp. enterocolitica* strain 8081
NEG	:Negative control
M	:Low molecular weight marker (NE BioLabs, UK)

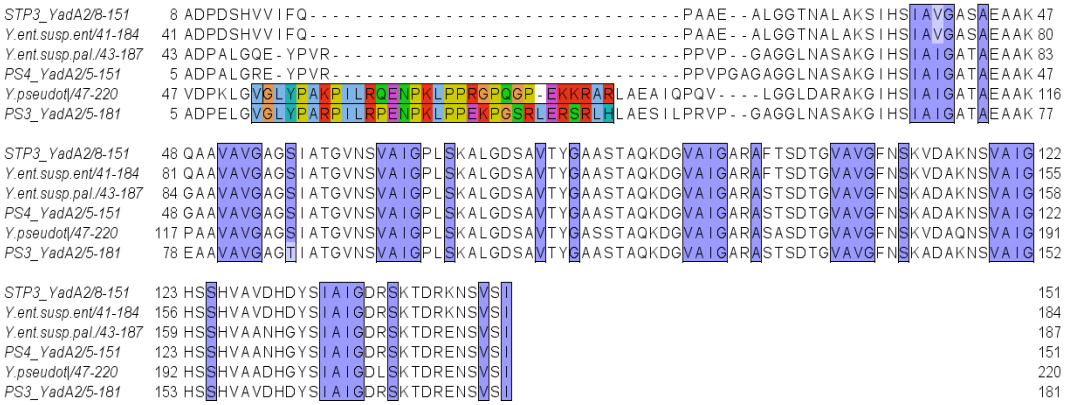


Figure 2.2 ClustalW2 alignment of the *Yersinia spp.* YadA head domain sequences

*In blue boxes YadA head domain degenerate repeat sequences;
** In multicolored boxes N-terminal 32aa insert seen in *Y. pseudotuberculosis* and PS3

Alignment of translated *yadA* PCR fragments from pig slurry and human sewage showed amino acid (aa) repeats characteristic of the *Yersinia* pYV-specific YadA head domain (Figure 2.2, denoted in colour) [389]. The PCR product from PS3 was larger than the expected size (Figure 2.1) and encoded a 32 residue N-terminal insert (Figure 2.2) resembling a spacer domain found in *Y. pseudotuberculosis* YadA [391]. Although the best BLASTX hit for the whole PCR product PS3 was *Y.*

pseudotuberculosis YadA (Table 2.6), molecular phylogenetic analysis of a multiple alignment of the *yadA* fragment nucleotide sequences grouped all PS and STP sequences with *Y. enterocolitica yadA* sequences rather than *Y. pseudotuberculosis* (Figure 2.3). Moreover, a *Y. pseudotuberculosis*-specific *ail* gene fragment [151] was not amplified from the PS3 sample but the *Y. enterocolitica* specific *ystA* [572] was detected (Table 2.5).

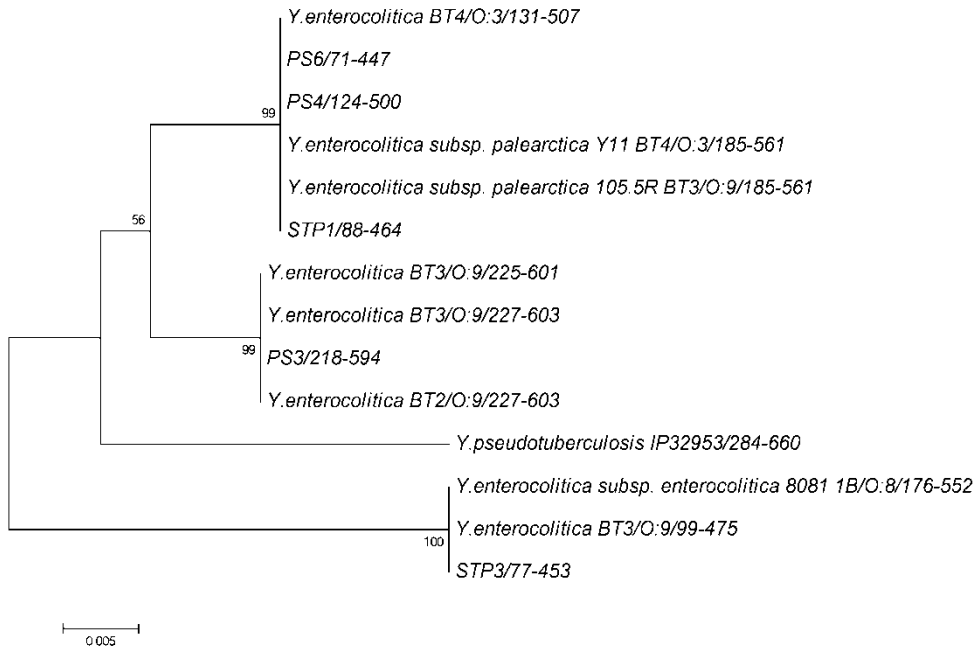


Figure 2.3 Neighbor-Joining tree showing relationship of the *yadA* sequences

The tree was constructed using the Neighbor-Joining method [8]. The optimal tree with the sum of branch length = 0.07758621 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [9]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method [10] and are in the units of the number of amino acid differences per site. The analysis involved 14 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 377 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [11].

Table 2.6 BLASTX *yadA* gene fragments results

Source	AA	BLASTX results					
		Bacterial strain providing best hit	**Database Hit gene/ORF**	**Sequence reference number**	**Max. Identity**	**Query coverage**	**E-value**
PS3	181	*Y. pseudotuberculosis* IP32953	pYV0013	emb CAF253561.1	82%	100%	4e-71
PS4	151	*Y. enterocolitica subsp. palearctica* 105.5R	YadA	gb ADZ44497.1	97%	99%	2e-59
PS6	149	*Y. enterocolitica subsp. palearctica* Y11	Y11_p0221	emb CBY78119.1	100%	99%	9e-73
STP1	154	*Y. enterocolitica subsp. palearctica* Y11	Y11_p0221	emb CBY78119.1	100%	96%	7e-65
STP3	151	*Y. enterocolitica subsp. enterocolitica* 8081	YadA	emb CAL10087.1	100%	97%	3e-77
STP4	152	*Y. enterocolitica subsp. enterocolitica* 8081	YadA	emb CAL10087.1	100%	94%	4e-74
STP5	150	*Y. enterocolitica subsp. enterocolitica* 8081	YadA	emb CAL10087.1	100%	98%	2e-77

AA :length of translated amino acid sequence
PS1 - PS6 :Pig slurry samples
STP1 – STP5 :Human sewage samples

2.3.3 Serology

Anti-Yop *Yersinia* IgG was detected in 57 (28.5%) of Irish blood donors after initial screening with the recomWell (ELISA) assay. The presence of anti-*Yersinia* YopD IgG antibodies was confirmed in 50 of these blood plasma samples (25 % of the total) with the RecomLine strip immunoassay. One sample was borderline positive in the RecomLine assay.

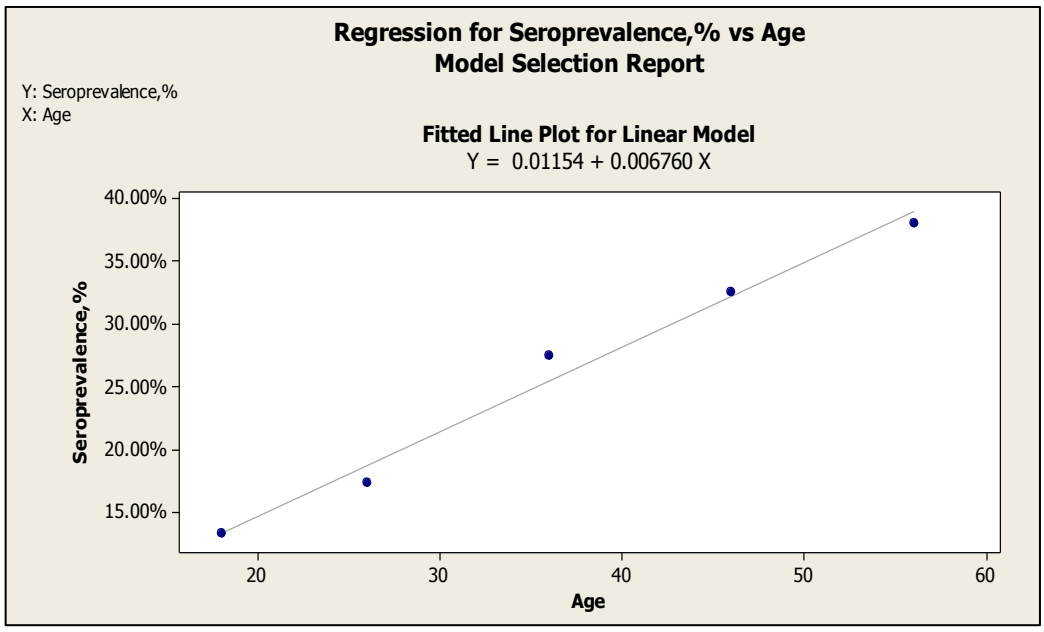


Figure 2.4 Anti-*Yersinia* IgG seroprevalence

A linear regression model analysis (Figure 2.4) showed an age-related increase of anti-*Yersinia* IgG antibodies among healthy individuals: seroprevalence increased from 13.3% in 18- to 25-year old donors to 38.1% in 56-66 year old donors (P-value = 0.001). Anti-YOP *Yersinia* IgG seropositivity showed no significant sex difference.

Cross-reactivity between YopD and *Borrelia burgdorferi* antigens has been reported [292, 293] and the estimated minimum notification rate of Lyme borreliosis in Ireland is 1.67/100,000 [576] and is higher than the notification rates for yersiniosis. However, no anti-*Borrelia* antibodies were detected using the RecomBead Borrelia IgG assay (Mikrogen) in the 50 *Yersinia* antibody-positive specimens or 43 *Yersinia* antibody-negative age and gender matched blood donor plasma controls. No relationship between the presence of anti-*Yersinia* IgG antibody

and appendicitis was observed as 25% of healthy blood donors and 17.6% of patients who underwent appendectomy were anti-*Yersinia* seropositive.

2.4 Discussion

No isolates of pathogenic *Yersinia* were recovered from 1,189 diarrhoeal stool samples processed at CUH, despite the use of standard *Yersinia* selective culture techniques. Three non-pathogenic *Y. enterocolitica* biovar 1A strains were isolated following cold enrichment of diarrhoeal stool samples in YSE broth and subsequent inoculation onto CIN agar plates. *Y. enterocolitica* biovar 1A are frequently isolated from diarrhoeal stool samples [63, 78, 577], but their pathogenicity towards humans remains unclear [556, 578]. Prospective faecal culture studies in other countries where yersiniosis is common reported 1.1% (Finland) [78], 4.1% (Belgium) [89], and 6.2 % (China) [65] of samples positive for pathogenic *Y. enterocolitica*. Tests with spiked samples (Table 2.4) indicated faecal culture methods used in this study (derived from previous published prospective surveys) could detect *Y. enterocolitica* at counts of 10^5-10^8 CFU/g typically found in culture positive stools by real time PCR [65]. Our negative result excluded a true yield of 0.5% or more of diarrhoeal stools containing *Y. enterocolitica* in our study population with 99% power, suggesting *Y. enterocolitica* is a much less common cause of diarrhoea in Ireland than in the above countries, consistent with current notification rates.

Because of the associated presentation of yersiniosis with pharyngitis [198, 199], 297 throat swabs from patients with pharyngitis were also cultured using a sensitive selective enrichment method for *Yersinia* [79], with similarly negative results, excluding a true 2% yield of culturable *Y. enterocolitica* in pharyngitis with 99% power. In contrast pathogenic *Y. enterocolitica* has been cultured from tonsil swabs with CIN agar direct plating from 31% of local slaughtered pigs in Ireland (*unpublished Murphy et al.*), a similar rate to that reported in other countries [57, 561, 579]. These results are further evidence for a low current rate of *Y. enterocolitica* infection in the human population in Ireland, although the positive pig swabs suggest exposure from the local food chain is potentially similar to other countries where isolation rates from humans are higher. Therefore, variations in food preparation methods (including popular raw pork dishes in Germany [80],

Belgium [66] and Norway [16]) may be important determinants in the observed variation in incidence of human yersiniosis between different countries in Europe.

Yersinia selective culture of 23 appendix swabs from surgical patients did not yield any *Yersinia* isolates. The reported isolation rates of *Yersinia* species from postoperative appendix swabs are 2.7% to 10% [90, 183, 189].

Following initial negative faeces cultures from patients with diarrhoea, molecular investigation of different human sewage and pig slurry samples was carried out as a means by which to identify pathogenic *Yersinia* species in a wider sample of human and animal excreta. The PCR targets for pathogenic *Y. enterocolitica* chromosomal target *ystA* and plasmid-located *yadA* were detected in pig slurry and not in human sewage by simple PCR. Nested PCR was required to detect *yadA* in human sewage, consistent with a low level of intestinal excretion of pathogenic *Y. enterocolitica* in humans locally compared with pigs. There was little evidence of *Y. pseudotuberculosis*-specific DNA in either pig slurry or human sewage samples: the *Y. pseudotuberculosis* chromosomal *ail* gene was not detected. pYV-located *yadA* DNA was found in both pig slurry and human sewage samples. Multiple alignment of deduced amino acid sequences suggested these were more likely to represent *Y.enterocolitica yadA* than *Y. pseudotuberculosis yadA.* A single *yadA* sequence amplified from pig slurry encoded a 31 aa N-terminal insert (Figure 2.2) resembling a spacer domain found in *Y. pseudotuberculosis* YadA that is responsible for bacterial uptake by epithelial cells [391], but the remaining sequence of the amplicon more closely resembled *Y. enterocolitica yadA*. The significance of this chimaeric sequence is uncertain.

Negative sewage and pig slurry culture results in the light of molecular detection of pathogenic *Yersinia* could arise from inter-bacterial competition in a complex environment. Bacteriocins secreted by non-pathogenic *Yersinia* species may be active against pathogenic *Yersinia* [96, 97, 142, 143] and may reduce isolation of pathogenic *Yersinia* from environmental samples such as soil and sewage [144-146] while still allowing molecular detection at a low level.

The overall seroprevalence of anti-*Yersinia* YOP IgG antibody in healthy blood donors was 25% and is similar to that recorded in other countries such as Austria

(employing the same detection protocol) [164] and Finland [162], where reported rates of yersiniosis are much higher than in Ireland [12]. Cross-reactivity between anti-*Borrelia* antibodies and *Yersinia* antigens has been reported [292, 293]. However, no blood plasma specimens tested in this study, reacted with *Borrelia* antigens. If the seropositivity in blood donors reflected current or recent infection, we would expect to see a high incidence of transfusion-related *Yersinia* septicaemia in Ireland similar to other countries where yersiniosis is common such as New Zealand [248]. However, there have been no reports of *Yersinia* sepsis following blood transfusion in Ireland, in keeping with the low incidence of yersiniosis. However, it should be noted that all blood components used in Ireland have been leuco-depleted since October 1999 [580], thereby reducing the potential risk of transfusion-related *Yersinia* sepsis [248, 256-259].

There was a significant association between increasing age and the presence of anti-*Yersinia* YOP antibodies, compatible with a cohort effect of increased risk of infection with pathogenic *Yersinia* in Ireland. In Ireland in the 1980s acute anterior uveitis was commonly associated with positive *Yersinia* serology [204], and a serological survey detecting agglutinating antibodies reported that 23% of patients with a clinical diagnosis of appendicitis, and 2% of healthy controls in two hospitals in Dublin had active yersiniosis [177]. The ratio of *Y. pseudotuberculosis* to *Y. enterocolitica* agglutination seropositivity was 5:1 [177], but infections with either species would result in anti-YOP antibodies in the assay used in the current study. Pathogenic *Y. enterocolitica* were present in retail raw and cooked meat samples in Ireland in the mid 1990s [106].

IgG immunoglubulins are secreted at latter stages of an infection cycle, and the presence of anti-Yersinia IgG is commonly used to detect past infections [262]. The duration of detectability of anti-YOP antibodies following *Yersinia* infection is not well documented, but there is evidence for anti-somatic *Yersinia* IgG persistence for several years after occurrence of uncomplicated (untreated) yersiniosis [280], appendicectomy [76], and in relatives of patients with thyroid disorders [289]. *Y. pseudotuberculosis* is notoriously difficult to culture from faeces [121, 141] and was not isolated in the current study or the unpublished pig abbatoir study (*unpublished Murphy et al.*). *Y. pseudotuberculosis* infection has been associated with

contaminated water supplies [75, 581, 582]. In Ireland the quality of drinking water has been recently improved in many areas in response to European legislation [583], and a decreasing level of *Y. pseudotuberculosis* infection since the 1980s could have accompanied this change, contributing to the age-related cohort serology. However, it seems unlikely that over 25% of healthy blood donors have been infected with *Y. pseudotuberculosis* alone, as this organism is generally a rarer cause of yersiniosis than *Y. enterocolitica* even in countries with a high incidence of yersiniosis [12].

The absence of *Y. enterocolitica* as a culturable cause of diarrhoea in this prospective study is compatible with the current low national yersiniosis notification rate and the lack of reports of *Yersinia* associated blood transfusion contamination in Ireland. We suggest the most probable explanation linking absence of culturable *Y. enterocolitica*, with a high seroprevalence of anti-*Yersinia* Yop antibodies in older blood donors, and molecular evidence of the presence of pathogenic *Y. enterocolitica* in human sewage, is a combination of a higher incidence of yersiniosis in Ireland in the past (caused by both *Y. pseudotuberculosis* and *Y. enterocolitica*) and a continuing low incidence of mild self-limited *Y. enterocolitica* infection which does not result in diarrhoea specimens being sent for culture [17, 162-164].

Chapter 3 *Yersinia* insect toxicity

3.1 Introduction

It has been suggested that ancestral *Yersinia* species were associated with insects or insect pathogens [529, 533, 534, 538, 542, 559]. One current *Yersinia* subspecies, *Yersinia pestis*, is a well known insect and mammalian pathogen, which has to infect fleas to be transmitted between mammals. Genome sequences of various *Yersinia* species have demonstrated the presence of several loci found in insect pathogens. For example, baculoviral enhancin has been detected in *Y. pestis* and *Y. pseudotuberculosis* [533, 542] and toxin genes resembling the Insecticidal Toxin Complex (ITC) encoded by the insect pathogen *Ph. luminescens* [551, 552] were found in *Y. pestis* [542], *Y. pseudotuberculosis, Y. mollaretii, Y. frederiksenii* [554] and in mildly pathogenic [555] and non-pathogenic [556] *Y. enterocolitica subsp. palearctica* (but not in the highly pathogenic *Y. enterocolitica subsp. enterocolitica* [532]). There are also bioinformatically identified toxin genes in *Y. enterocolitica* for which activity and targets are at present unidentified, for example apparent cytolethal distending toxin (CDT) genes. In this chapter the toxicity of different ITC and CDT genotypes of *Y. enterocolitica* is investigated in an insect model, *G. mellonella*.

3.1.1 Genetic organisation of *Yersinia* insecticidal toxin complexes

Ph. luminescens is part of the gut flora of entomopathogenic nematodes and an insect pathogen belonging to the family *Enterobacteriaceae* [551, 552]. It was the first bacterium shown to contain ITC genes and the archetypal description of the insecticidal toxin complex operons was established in *Ph. luminescens*. The insecticidal toxin complex (Tc) of *Ph. luminescens* strain W14 comprises four loci (*tca*, *tcb*, *tcc* and *tcd*) [551, 552]. Each locus contains multiple *tca (tcaA, tcaB, tcaC and tcaZ), tcb (tcbA), tcc (tccA, tccB, tccC and tccZ) and tcd* (*tcd*A1, *tcd*B1 and *tcc*2) genes [551, 553]. Based on sequence identity between insecticidal toxin complex genes, they can be grouped into three types: type A - *tcdA*/*tcaAB*/*tcbA*/*tccAB*, type B - *tcdB*/*tcaC* and type C - *tccC* [584]. Consequently, *tca* loci encode type A and type B, *tcd* – type A, B and C and *tcc*- type A and C genes [554].

Yersinia insecticidal toxin complex genes are encoded by tc-PAI^{Ye} genomic islands

and in all cases are inserted into the same location between YE3797 (*tcaR1*) and YE3798 (*tldD*) genes with respect to the chromosome of the *Y. enterocolitica* 8081 strain [554-556] (Figure 3.1). The tc-PAI^{Ye} of pathogenic *Y. enterocolitica* W22703 (2/O:9) [555], *Y. pseudotuberculosis* and *Y. pestis* [553, 554] contains *tca* like operon structures, non-pathogenic *Y. enterocolitica* T83 (1A) have *tcd* like insecticidal toxin complex loci [554, 585]. Thus, type A homology genes in *Y. enterocolitica* W22703, *Y. pseudotuberculosis* and *Y. pestis* split into *tcaA* and *tcaB* genes, unlike in non-pathogenic *Y. enterocolitica* T83 in which the type A gene is encoded as a single continuous ORF (*tcdA* [554, 585] or *tcbA* [556]). The *tcaB* gene in *Y. enterocolitica* W22703 split into separate ORFs (*tcaB1* and *tcaB2*) [555].

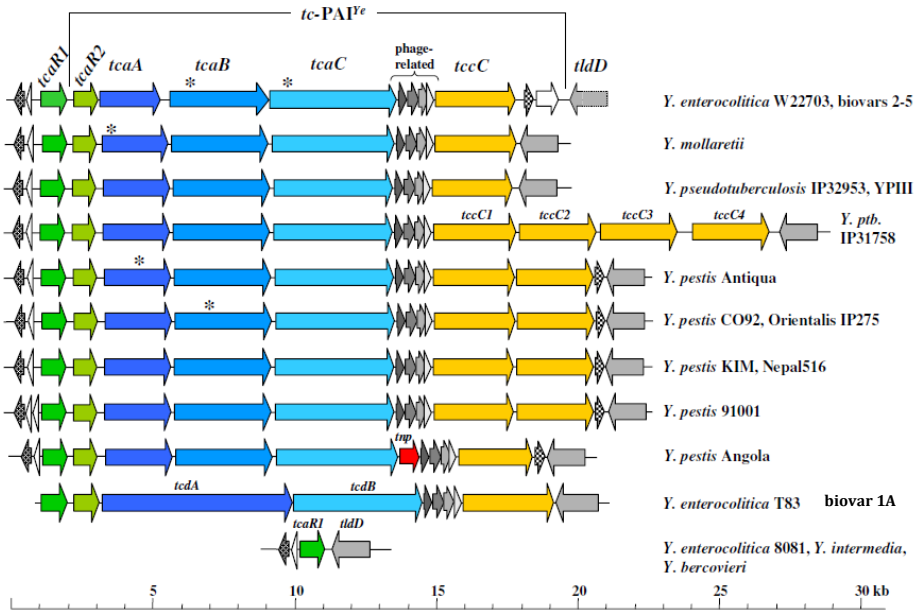


Figure 3.1 *Yersinia* species insecticidal toxin complexes (adapted from *Fuchs et al* [585])

Three homology groups *tcaAB*/*tcdA*, *tcaC*/*tcdB*, and *tccC* are shown. Identically coloured arrows mark homologous genes. *tcaR1* (left) encoding a regulator and *tldD* encoding a putative DNA gyrase modulator (right, checkered) mark the island insertion site common for all *Yersinia* strains that harbour *tc* homologues. A transposase-like gene (*tnp*) is present in the genome of *Y. pestis* Angola (red arrow). The overall gene organisation is similar for all strains harbouring insecticidal determinants, but differences with respect to gene homology, hypothetical ORFs, the presence of transposase-like elements and the number of *tccC* genes are also visible. Gene lengths and intergenic regions are in scale. Asterisks mark frameshifts. With the exception of *tcaC*, all frameshifts result in two ORFs. *tcaB* split into *tcaB1* and *tcaB2* in *Y. enterocolitica* W22703 not shown.*Y. ptb*. - *Y. pseudotuberculosis*

3.1.2 Insect models

Insects have been suggested as a model for studying microbial pathogenicity towards humans [586-589]. *M. sexta* (tobacco horn worm) is a moth of the family *Sphingidae* and *G. mellonella* (Greater Wax Moth) is a moth of the family *Pyralidae*, both belong to order *Lepidoptera* of class *Insecta*. Both *G. mellonella* [590] and *M. sexta* [591] larvae can be kept at 37°C, the optimum temperature for many human pathogens, and constitute a cost-effective non-mammalian model for studying microbial pathogenesis [592, 593]. A *M. sexta* larva is an established model for studying insect pathogenicity of *Ph. luminescens* [551, 552, 594-597]. However, a good correlation between virulence of human pathogens such as *Pseudomonas aeruginosa* [598], *Burkholderia cepacea Complex* [599], *Y. pseudotuberculosis* [600] and *Candida albicans* [601] has been obtained in mammalian and *G. mellonella* infection models. *G. mellonella* larvae also provide an excellent infection model of *Listeria* septic infection that enables clear differentiation of pathogenic and non-pathogenic *Listeria species,* and between virulent and attenuated strains of normally pathogenic *Listeria monocytogenes* [592].

3.1.3 Biological activity of insecticidal toxins

Ph. luminescens insecticidal toxin complex toxin complex (Tc) protein shows clear oral toxicity against *M. sexta* larvae [551, 552, 595-597, 602] and injectable toxicity against *G. mellonella* larvae [603]. There is evidence of differential toxicity of individual proteins and of interaction between different *Ph. luminescens* Tc proteins. Deletion of *tca* or *tcd* loci in *Ph. luminescens* reduced its oral toxicity in *M. sexta* larvae and simultaneous deletion of both loci in a single strain completely abolished oral insecticidal toxicity of *M. sexta* larvae [551]. On the other hand, deletion of either *tcb* or *tcc* loci alone insignificantly reduced mortality of orogastrically infected *M. sexta* [551]. Co-expression of *Ph. luminescens tccC* and *tcdAB* in *E. coli* was required for high levels of oral toxicity to *M. sexta* larvae [596]. Potentiation of TcdA oral toxicity was achieved when TcdB and TccC were expressed in the same bacterial cytoplasm [597]. When the same potentiators were expressed separately in different bacterial cytoplasms, and then mixed with TcdA prior feeding to caterpillars, the toxicity of TcdA was not enhanced. Close examination of co-expressed TcdB and TccC revealed a reduction in TcdB molecular weight suggesting

that TccC may modify TcdB when co-expressed [597]. The same potentiators may increase toxicity of TcaA and TcaB, which unlike TcdA shows little or no activity when fed to caterpillars on its own [597]. The highest biological activity of *Ph. luminescens* W14 insecticidal toxins (TcdA and TcaAB) was achieved in *E.coli* BL21 – a protease deficient strain in comparison with other *E.coli* hosts EC100 and DH5α [602]. Significant levels of biologically active soluble insecticidal toxins were obtained after incubation of bacteria at 15°C for 24 hours, and high levels but biologically inert and insoluble insecticidal toxins were obtained after bacterial incubation at 37°C for 3 hours [602]. Once again, soluble expression of TcaA and TcaB was only achieved when these were simultaneously co-expressed in the same bacterial cytoplasm [602].

To summarise, *Ph. luminescens* type A toxins (TcaAB and TcdA) require potentiation by type B (TcdB) and type C (TccC) proteins for full oral toxicity to *M. sexta* larvae [596, 597]. Moreover, toxins must be co-expressed in the same bacterial cytoplasm [596, 597, 602]. Additionally, insecticidal activity of *Ph. luminescens* Tcs is expression host and temperature-dependent, with maximum biological activity being obtained when insecticidal toxins are expressed in a protease deficient host at 15°C for 24h [602].

In contrast to *Ph. luminescens*, *Y. pseudotuberculosis* toxins TcaC and TccC (TcbB- and TccC-like) are substantially less potent combinations with TcaAB [602]. In fact, some researchers have not found any *M. sexta* oral toxicity of *Y. pestis* or *Y. pseudotuberculosis* TcaAB and TcaC-TccC insecticidal toxin proteins expressed in *E.coli* BL21 [604].

While *Ph. luminescens* TcdA or TcdB-TccC proteins expressed in *E. coli* BL21 are toxic for the rat flea *Xenopsylla cheopis,* neither *Y. pestis* nor *Y. pseudotuberculosis* TcaAB and TcaC-TccC toxins expressed in *E. coli* BL21 mediate acute flea toxicity [359]. In fact, live wild-type *Y. pseudotuberculosis* and isogenic mutants lacking the *tcaAB* locus or the entire *tcaA-tccC* region were equally toxic for *Xenopsylla cheopis* [359]. Moreover, *tcdB-tccC* encoding *Y. enterocolitica* strain CS080 and insecticidal toxins non-encoding *Y. enterocolitica* 8081 were equally toxic to the flea [359].

Also in contrast to *Ph. luminescens* Tcs (TcaAB and TcdBC), *Y. pseudotuberculosis* and *Y. pestis* Tcs (TcaAB and TcaCTccC) expressed in *E. coli* BL21 were not toxic when applied as cell lysates to *Sf9* insect cells [604]. However, *Y. pseudotuberculosis* caused membrane ruffles, actin vacuoles, multinucleation, and actin condensation when applied to human gut cells (Caco-2), and cell lysates of *E. coli* expressing *Y. pseudotuberculosis* insecticidal toxins reproduced this phenotype. Cell lysates of *E. coli*-expressed *Y. pestis* and *Ph. luminescens* Tcs did not affect Caco-2 cells. In contrast, cell lysates of *E. coli*-expressing*Y. pestis* and *Ph. luminescens* Tcs caused actin condensation and nuclear fragmentation in mouse fibroblast NIH3T3 cells which were unaffected by *Y. pseudotuberculosis* Tcs [604]. Transient intracellular expression of *Y. pseudotuberculosis* and *Y. pestis* Tc proteins in these respective cell lines reproduced these phenotypes.

It has been hypothesized that these phenotypes reflect co-option of insecticidal toxins by *Y. pseudotuberculosis* for activity against mammalian intestinal cells to aid enteric pathogenicity, and a similar adaptation by *Y. pestis* for systemic pathogenicity [604]. Interestingly, inactivation of clinical *Y. enterocolitica* biovar 1A insecticidal toxin complex resulted in attenuation of the ability to colonise the intestinal tract of orally inoculated BALB/c mice [556]. Moreover, *tcbA*, *tcaC* and *tccC* toxin proteins of non-pathogenic biovar 1A *Y. enterocolitica* strain T83 [556], and *tcaC, tccC* of *Y. pseudotuberculosis* and *Y. pestis* [604] are preferentially expressed at temperatures of 30°C and 37°C (mammalian body temperature).

However, it should be noted that *tcaA* and *tcaB* of moderately pathogenic *Y. enterocolitica* W22703 are highly expressed at 10°C [555]. No insecticidal activity in *M. sexta* was observed when *Y. enterocolitica* W22703 was grown at 30°C , but cell extracts (not culture supernatants) obtained from grown at 10°C for 48 hours were orally toxic towards *M. sexta* [555]. Cell extracts of highly pathogenic *Y. enterocolitica subsp. enterocolitica* strain 8081v (which does not contain insecticidal toxin complex loci [532]) cultured at 10°C were not orally toxic towards *M. sexta* larvae [555]. Insertional knock out of W22703 *tcaA*, but not *tcaB1* resulted in the loss of oral insecticidal activity against *M. sexta* larvae when cell extracts from bacterial cultures incubated at 10°C were used [555].

However, the presence or absence of an insecticidal toxin complex did not affect the

outcome of the subcutaneous infection of *G. mellonella* at 15°C with different pathogenic *Y. enterocolitica* strains (Table 3.1) [585]. This study did not include biovar IA *Y.enterocolitica* strains.

Table 3.1 Mortality rates of *G. mellonella* larvae incubated at 15°C after subcutaneous infection with *Y. enterocolitica* (adapted from *Fuchs et al* [585])

Microorganism	Strain	ITC presence	*G. mellonella* Mortality rates*		
			5-7.5x10^5 CFU**	5-7.5x10^4 CFU**	Total Dead, % ± SD
***Y. enterocolitica* 2/O:9**	2594	+ve	90% (36/40)	87% (34/39)	90 ± 9% (70/79)
***Y. enterocolitica* 2/O:9**	W22703	+ve	56% (32/57)	36% (20/57)	41 ± 17% (52/114)
***Y. enterocolitica* 2/O:9**	W22703 tca::Tn5lux	*tca* knockout	55% (30/55)	47% (18/380	51 ± 13% (48/93)
Y. mollaretii	NA	+ve	94% (30/32)	75% (24/32)	84 ± 5% (54/62)
***Y. enterocolitica* 1B:O21**	4466	-ve	98% (47/48)	71% (34/48)	88 ± 11% (81/96)

*Results calculated from the table provided in the reference [585]
** CFU of bacteria subcutaneously injected into *G. mellonella* larvae
NA :Not Applicable

3.1.4 Cytolethal distending toxin

Putative Cytolethal Distending Toxins (CDT) have been identified in the genome sequence of pathogenic *Y. enterocolitica* strain Y11 [466] and in the genome sequences of non-pathogenic *Y. enterocolitica* (N.Thomson, personal communication). CDT are able to cause cell cycle arrest and apoptosis in various eukaryotic cells [442-445] including immune cells that may subsequently lead to the inhibition of the adequate immune response towards pathogen [605, 606]. CDT are produced by a number of bacterial pathogens including: *Campylobacter spp.* [446-448], *Salmonella enterica* Serovar *Typhi* [449], *Shigella dysenteriae [450], Escherichia coli* [451-454], *Helicobacter spp.* [455-460], *Actinobacillus actinomycetemcomitans* [461-463], and *Haemophilus ducreyi* [464, 465]. CDT in *Actinobacillus actinomycetemcomitans* [607] and *Haemophilus ducreyi* [608, 609] affect

lymphocytes in a way that may lead to impairment of the immune response and persistent infection. CDT producing *Campylobacter jejuni* [446] and *Shigella dysenteria cdtA*, *cdtB* and *cdtC* cloned in an *E. coli* JM109 strain [606] can cause diarrhoea in a suckling mice model.

3.1.5 Toxicity of *Y. enterocolitica* strains isolated in Ireland for *G. mellonella*

The predominance of non-pathogenic biovar 1A strains in *Y. enterocolitica* isolated from humans and retail meat samples in Ireland prompted investigation of their pathogenic potential in comparison with pathogenic strains in a *Galleria* model. Because the presence of an insecticidal toxin complex in non-pathogenic *Y. enterocolitica* biovar 1A strains has been shown to be linked to an enhanced ability for colonisation of the mammalian gastrointestinal tract [556] we screened for the presence of insecticidal toxin complex genes in clinical and non-clinical *Y. enterocolitica* biovar 1A strains to be studied in the *Galleria* model. Because the insecticidal toxin complex operon differs in mildly pathogenic biovar 2 - 5 *Y. enterocolitica* and biovar 1A strains (Figure 3.1), two sets of PCR assays were required for this. Because the newly demonstrated CDT locus might also play a role in toxicity in the *Galleria* model, we also sought the CDT genotypes among pathogenic and non-pathogenic biovars of *Y. enterocolitica* and *Y. enterocolitica*-like species tested and looked for evidence of expression of CDT toxins in *G. mellonella* larvae incubated at 15 and 37°C.

Thus, to demonstrate potential pathogenicity of pathogenic and supposedly non-pathogenic *Y. enterocolitica* strains isolated in Ireland we compared their virulence towards *G. mellonella* with the virulence of highly and mildly pathogenic *Y. enterocolitica* strains.

3.2 Materials and Methods

3.2.1 *Yersinia* strains

Culture collections of 64 strains of *Yersinia* species were obtained from University College Galway (gift of Professor Martin Cormican) and 25 strains from Queen's University Belfast (gift of Dr. John Moore), identification of strains was confirmed by API 20E *BioMérieux* incubated at 30°C overnight [130, 131]. *Y. enterocolitica* and *Y. enterocolitica* like species from retail meat samples (51), dogs (25) and slaughtered

pigs (287) were obtained from Niall Drummond and Dr. Brenda Murphy in the Veterinary Food Safety Laboratory Cork Council Inniscarra, Co. Cork, Ireland. Reference strains of the main biovars were obtained as shown in Table 3.2.

Table 3.2 *Y. enterocolitica* reference strains

Microorganism	Reference#	Source
Y. enterocolitica 1B/O:8	**8081**	D. Portnoy, University of California
Y. enterocolitica 1A/O:6,30	**IP102**	E. Carniel, Institute Pasteur
Y. enterocolitica 2/O:9	**IP383**	E. Carniel, Institute Pasteur
Y. enterocolitica 2/O:5, 27	**IP885**	E. Carniel, Institute Pasteur
Y. enterocolitica 3/O:5	**IP22228**	E. Carniel, Institute Pasteur
Y. enterocolitica 4/O:3	**IP134**	E. Carniel, Institute Pasteur

All strains were biotyped according to the simplified *Wauter's Biotyping Scheme* (Table 3.3) and serotyped with *MAST Group Yersinia enterocolitica Antisera* that included one poly antisera – O:1,O:2 and four mono antisera – O:3; O:5; O:8; O:9. All isolates were checked for possible autoagglutination in a drop of sterile 0.85% saline solution.

Table 3.3 Simplified Wauters *Y. enterocolitica* biotyping scheme

Tests	Biotypes						
	1A	**1B**	**2**	**3**	**4**	**5**	**6**
Lipase (Tween-esterase)	+	+	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	(+)	-
Indole	+	+	(+)	-	-	-	-
Esculin	+	-	-	-	-	-	-
Salicin	+	-	-	-	-	-	-
Xylose	+	+	+	+	-	V	+
Trehalose	+	+	+	+	+	-	+

(+) :Delayed reaction
V :Variable reaction

3.2.2 DNA isolation

A loopful of overnight bacterial culture grown on tryptic soy agar (TSA) plates at 30°C was gently resuspended with 600µl of Nuclei Lysis solution (Promega Wizard Genomic DNA purification protocol, Promega UK, Southhampton) in 1.5ml tubes. After bacterial culture was fully homogenized in nuclei lysis solution they were incubated at 80°C for 5min, then cooled to room temperature. RNA was removed by adding 3µl of RNAse solution into the cell lysate, inverting tubes 2-5 times to mix and following incubation at 37°C for 60min. Protein precipitation was achieved by adding 200µl of protein precipitation solution into the cooled down to room temperature RNAse-treated cell lysate and following incubation on ice for 5min. Protein was removed from DNA samples by series of washes in isopropanol and in 70% ethanol. DNA samples were rehydrated by adding 100µl of rehydration solution and overnight incubation at 4°C. DNA samples were stored at -80°C. DNA quality was assessed by Nanodrop NT1000.

3.2.3 PCR Protocols

PCR master mix for each reaction was prepared with GoTaq Green Polymerase kit (Promega UK, Southampton) according to the manufacturer's recommendations; total volume of the PCR reaction for each protocol was 50 µl. PCR was performed on a PTC-200 Peltier Thermocycler with the following conditions: denaturation at 94°C for 5 min; followed by 29 cycles of each: denaturation at 94°C for 30 sec, annealing temperature depending on Tm of primers ranged from 52°C to 55°C for 30sec, depending from the expected product size duration of elongation at 72°C lasted from 40sec to 3min, and final elongation at 72°C for 7 - 10min. Amplicons were maintained at 4°C.

All primers (Table 3.4) were designed with *Primer 3* [610] based on publically available reference sequences (Table 3.4). Two sets of primers were designed to detect a putative Cytolethal Distending Toxin (CDT) in *Yersinia* based on the genome sequence of non-pathogenic *Y. enterocolitica subsp. palearctica* (N. Thomson, Welcome Trust Sanger Institute, unpublished). One set of primers was designed to amplify the entire CDT locus (CDT Flanking primers) and another set was designed to amplify targets within CDT genes (CDT Internal primers).

Table 3.4 PCR primers

PCR protocol	Primers	Sequences	Product size, bp	Reference sequence
Insecticidal Toxin Complex of non-pathogenic *Y. enterocolitica*	*tcbA F*	*5′-aacgcttcccagtggtattg-3′*	473	AY647257
	tcbA R	*5′-gagggtagacgcagtcgaag-3′*		
	tcaC F	*5′-atcaatttattggccccaca-3′*	580	
	tcaC R	*5′-aatggtaactggccttgtcg-3*		
	tccC F	*5′-aaaactgtgcgttattccgg-3′*	217	
	tccC R	*5′-tagtccgggtactcctgtgg-3′*		
Insecticidal Toxin Complex PCR of pathogenic *Y. enterocolitica* (W22703-TC)	*tcaC2 F*	*5′-ccgggtatgctttaccaaga-3′*	330	AJ920332
	tcaC2 R	*5′-gattaacgccagatcggaaa-3′*		
	tcaA F	*5′-cacatggttggaaagtcacg-3′*	240	
	tcaA R	*5′-aattgcagcggttcttcact-3′*		
	tcaB2 F	*5′-agcctaaaagagaccgcaca-3′*	192	
	tcaB2 R	*5′-atgctggcgctgtagttctt-3′*		
Flanking Cytolethal Toxin Complex PCR	*CDT-F F*	*5′-aaacttgccagcgatgatct-3′*	1461*	NA
	CDT-F R	*5′-ggcttggttcatggtgactt-3′*	3009**	

PCR protocol	Primers	Sequences	Product size, bp	Reference sequence
Internal Cytolethal Toxin Complex PCR	*CDT-I F*	*5′-atgaaattattgcggccaac-3′*	1461**	NA
	CDT-I-v2 R	*5′-tcgatggttctgttcgactg-3′*		
16S rDNA PCR	*16S F*	*5′-cagccacactggaactgaga-3′*	204	M59292, U63135, X75273, X75275-X75281
	16S R	*5′-gttagccggtgcttcttctg-3′*		

* PCR product expected for CDT negative strains;
** PCR product expected for CDT positive strains;
NA :Not applicable

PCR protocols were validated with the available reference *Y. enterocolitica* strains (Table 3.2). The expected PCR products were detected by gel electrophoresis. All products of partial 16S rRNA gene PCR protocol were sequenced at *GATC Biotech* (Germany, Konstanz).

Insecticidal Toxin Complex of non-pathogenic *Y. enterocolitica* PCR protocol was validated with *Y. enterocolitica* IP102 (positive control) and *Y. enterocolitica* 8081 (negative control), PCR products amplified from DNA of *Y. enterocolitica* IP102 were sequenced at GATC Biotech (Germany, Konstanz*).*

Insecticidal Toxin Complex PCR of pathogenic *Y. enterocolitica* (W22703-TC) PCR protocol was validated with *Y. enterocolitica* IP134, IP383, IP885, IP22228 strains (positive controls). *tcaA, tcaB2* and *tcaC* PCR products amplified from DNA of test strain 5618 (biotype 2) were sequenced at GATC Biotech (Germany, Konstanz).

Flanking and Internal Cytholethal Distending Toxin Complex PCR protocols were validated with *Y. enterocolitica* IP102, IP134, IP383, IP885, IP22228 and 8081 strains. Flanking Cytholethal Distending Toxin Complex PCR products amplified from DNA of all reference strains and some test strains (RS-156 D14, Y/02/02) were sequenced at GATC Biotech (Germany, Konstanz*).* Internal Cytolethal Distending Toxin Complex PCR products amplified from DNA of *Y. enterocolitica* IP102 strain was sequenced at GATC Biotech (Germany, Konstanz).

3.2.4 *G. mellonella* death assay

G. mellonella death assay was performed as described by *Joyce et al* [611].

Selected *Y. enterocolitica* strains were plated on Luria Bertani (LB) agar plates and incubated at 30°C overnight. A loop full of cultured bacteria was inoculated into 5ml of Luria Bertani broth and incubated overnight at 15°C. After overnight incubation in LB broth bacterial inoculums were centrifuged in a Beckman centrifuge using rotor JS-5.3 at 5000 rpm for 5 min at temperature of 4°C. LB supernatant was removed and the bacterial pellet was washed with 1ml of sterile Phosphate Buffered Saline (PBS) and centrifuged as above. After centrifugation the bacterial pellet was re-suspended in 1ml of sterile PBS.

The density of bacterial inoculums in PBS was adjusted to 1.0 OD_{600}. Eight ten-fold serial dilutions of bacterial inoculums were made with PBS. 10µl of each dilution was plated onto LB agar plates for CFU determination by viable counts. 10µl of neat 1.0 OD_{600} suspension and the first two serial dilutions corresponding to approximately $1.8x10^7$, $1.8x10^6$ and $1.8x10^5$ CFU, respectively, were subcutaneously injected into *G. mellonella* larvae that were obtained from Livefood, UK, and stored in the dark until use (not longer than 1 week).

For each bacterial inoculum twenty larvae were injected using a single use sterile BD insulin syringe (cat. N_o. 324884) as described by S. Joyce [611]**.** Larvae (ten per each plate) were placed in sterile petri dishes lined with Whatman paper discs and incubated in parallel at 15°C and 37°C. As a control ten larvae per each temperature regime were also injected with 10 µl of sterile PBS. The numbers of the killed larvae (nonresponsive to the touch) were counted on a daily basis up to 5 days. The assay was repeated three times on different occasions for each *Yersinia* strain.

3.2.5 Bacterial gene expression assay in *G. mellonella*

Bacterial inoculums were prepared as mentioned above for the *G. mellonella* death assay. After injection, larvae were incubated for 4 hours at 37°C and for 24 hours at 15°C [602]. Larvae were then chilled on ice for 30 min and aseptically bled into pre-chilled Rnase-free sterile 1.5 ml tubes (Ambion). Microcentrifuge tubes with 100 µl of haemolymph were kept on ice at all times. To inactivate RNases in larval haemolymph 1 ml of TRI reagent (Sigma) for RNA extraction was added immediately into microcentrifuge tubes with haemolymph and followed by immediate homogenisation. The homogenate was incubated at room temperature for 5 min to allow nucleoprotein complexes to completely dissolve.

To remove insoluble material including DNA from the homogenate it was centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was transferred to a fresh RNase-free microcentrifuge tube. Next, 100 µl of BCP reagent (Sigma) was added and mixed well with the supernatant. Supernatant with BCP reagent (Sigma) was incubated for 15 min at room temperature and then centrifuged at 12,000 g at 4°C for 10 min. The resulting aqueous phase (colourless top layer) was transferred into a fresh RNase-free microcentrifuge tube.

RNA was precipitated by the addition of 500 µl of molecular grade isopropanol (Sigma) followed by incubation at room temperature for 10 min and centrifugation at 12,000 g for 8 min at 4°C. The pellet was washed with 1 ml 75% molecular grade ethanol (Sigma) followed by centrifugation at 7,500 x g for 5 min and then re-suspended in Ambion RNA storage solution and kept at -70°C until needed.

Multiplex single step Reverse-Transcriptase PCR (RT-PCR) for insecticidal toxins was performed with One Step RT-PCR kit (QIAGEN). Master mix was prepared as follows: Qiagen One-Step RT-PCR Buffer 5x -5 µl, Q-Solution 5x - 5 µl, dNTP 10mM - 1µl, of each forward primer 20 pmole/µl - 0.75 µl, of each reverse primer 20 pmole/µl - 0.75 µl, Qiagen RT-PCR enzyme mix – 1 µl, RNase free H_2O – 9.5 µl, RNA template – 0.5 µl. Total RT-PCR reaction volume was 25 µl.

RT-PCR was performed on the *PTC-200 Peltier Thermocycler* with the following conditions: reverse transcription at 50°C for 30 min, initial denaturation at 95°C for 15 min; followed by 39 cycles of each: denaturation at 94°C for 30 sec, annealing at 55°C for 30sec, elongation at 72°C for 1 min; and final elongation at 72°C for 10 min.

As control for the DNA contamination RNA samples were submitted to PCR run with the reverse transcription step (50°, 30 min) omitted.

Primers were designed using *Primer 3* [610] from the reference sequences AJ920332 (*Y. enterocolitica subsp. palearctica* W22703) and AY647257 (*Y. enterocolitica subsp. palearctica* T83) respectively for *tcaC2* and *tcaC* genes and from the conserved regions identified in putative *cdtB2* and *cdtB3*genes detected respectively in non-pathogenic and pathogenic *Y. enterocolitica* strains:

- *cdtB2 Forward* `5′-ttccgagcagaagaggtcat-3′`
- *cdtB2 Reverse* `5′-ctgagtgagtgacgggttca-3′`
- *tcaC Forward* `5′-gcaccagacagcattgaaga-3′`
- *tcaC Reverse* `5′-tgttgctgttctttcgcatc-3′`
- *cdtB3 Forward* `5′-ggatagccggtgttactgga-3′`
- *cdtB3 Reverse* `5′-ttgtcggtctgcatgaggta-3′`
- *tcaC2 Forward* `5′-ccgggtatgctttaccaaga-3′`
- *tcaC2 Reverse* `5′-gattaacgccagatcggaaa-3′`

The expected products of 330bp - tcaC2 gene and 155bp – *cdtB3* of pathogenic *Y. enterocolitica* and 341bp – *tcaC3* and 282bp – *cdtB2* for non-pathogenic *Y. enterocolitica* biovar 1A were detected by gel electrophoresis. PCR protocol was validated with *Y. enterocolitica* IP102, IP134, IP383, IP885, IP22228 and 8081 strains. Amplified PCR products from some of the reference strains (IP102 and IP22228) were sequenced at GATC Biotech (Germany, Konstanz).

3.2.6 Statistical analysis

Statistical comparison was performed using χ^2 test, one-way ANOVA comparison of the means with Tukey's error rate (multiple comparisons) or 2-sample t-test (pairwise comparison) in MiniTab 16 software. Cramér's V^2 coefficient was used to establish the strength of association between two variables by the following criteria: Cramér's V^2 >0.5 – high association, 0.3-0.5 – moderate association, 0.1-0.3 – low association, 0-0.1 – little if any association (http://www.acastat.com/Statbook/chisqassoc.htm). Statistical analysis of the differences in the outcome of *Yersinia* infection in *G. mellonella* larvae between different bacterial strains was applied to pooled data from all experimental replicates for each test strain. Pairwise comparison was applied to establish statistical difference between outcomes of *Yersinia* infection in insect larvae observed at 15°C and 37°C. LD50 for *Yersinia* strains was estimated by the Reed-Muench method where possible [612]. Hierarchical cluster analysis was performed by Dr Ian Jeffery using the Heatplus software package (Bioconductor) in the statistical environment R [613, 614].

3.2.7 Cell culture assay

Cell culture protocols were adapted from *Scott et al* [469] and *Fabris et al* [615].

3.2.7.1 Yersina cell lysates

Bacterial cultures were grown on tryptic soy agar with 0.6% of yeast extract at 37°C and 22°C for 48h. Harvested bacterial cultures were suspended in 10ml of PBS and washed twice. Optical density of bacterial inoculums measured at 600nm was around 3.0 OD. Bacterial inoculums were then sonicated for 1 min at maximum speed of 16,000 x g on ice. To remove cell debris sonicates were centrifuged for 20 min at 3500 rpm at 4°C. Supernatants were further filter sterilized through 0.22µm

impact filters (Millipore) and stored overnight at 4-8°C. Protein concentrations of bacterial cell extracts were measured by NanoDrop.

3.2.7.2 Cell culture

Next day cell lysates (12µl) were inoculated into 120µl (2.4 x10^4 cells/ml) of Chinese Hamster Ovaries (CHO) cell cultures in HAMs' F12 culture medium supplemented with 10% foetal bovine serum, 1% glutamine and penicillin (100 U/ml) and streptomycin (100 µl.ml). Cell cultures were incubated for 120h. Cell cultures were "fed" with 100 µl of fresh HAMs' F12 cell culture medium with all above mentioned supplements after 72h of incubation. At the end of the incubation cycle (120 h) cell cultures were fixed and stained with 0.2% crystal violet. Morphology of CHO cells was examined using an inverted microscope. All experiments were performed in triplicate twice. PBS and *E. coli* strain DHα5 were used as negative controls.

3.3 Results

3.3.1 Identification and biotyping of *Yersinia* strains

Sequencing of the 16S rRNA gene variable locus (204bp) for some *Yersinia* strains that were identified as *Y. enterocolitica* with API 20E strips revealed that these isolates in fact are not *Y. enterocolitica* but other *Yersinia* species (Table 3.5).

Table 3.5 API20E and 16s rDNA identification of *Y. enterocolitica*-like species

Strain Number	Source	API20E Profile	API 20E Identification	16S rRNA
RS-42 D21	Retail meat	1155723	*Y.enterocolitica 99.7%*	*Y. frederiksenii**
29869	Human	1155723	*Y.enterocolitica 99.7%*	*Y. frederiksenii***
58735	Human	1055723	*Y.enterocolitica 94.4%*	*Y.intermedia***
24070	Human	1054723	*Y.enterocolitica 95.4%*	*Y.massiliensis**
26931	Human	1014523	*Y.enterocolitica 96.4% VP+Ind neg*	*Y.bercovieri**
3343	Human	1114563	*Y.enterocolitica 94.5% Melibiose pos*	*Y.rohdei**

VP :Voges-Proskauer reaction
Ind :indole production
* Confirmed by full 16S rDNA sequencing (see protocol in Chapter 2)
** Identified by full 16S rDNA sequencing (see protocol in Chapter 2)

Table 3.6 Biotyping results of *Yersinia* strains

Source	*Y. enterocolitica* biotype				*Y. ent.* like *sp.*	Total
	1A	B2	B3	B4		
Human	72 (80.9%)	3 (3.4%)	3 (3.4%)	4 (4.5%)	7 (7.9%)	89 (100%)
Pig	50 (17.1%)	0	218 (74.7%)	15 (5.1%)	9 (3.1%)	292 (100%)
Retail Meat	36 (70.6%)	0	0	0	15 (29.4%)	51 (100%)
Dog	25 (100%)	0	0	0	0	25 (100%)
Total	183 (40%)	3 (0.7%)	221 (48.4%)	19 (4.2%)	31 (6.8%)	457 (100%)

79 *Yersinia enterocolitica* isolates obtained from UCG and QUB have been serotyped with the *MAST Group Yersinia enterocolitica Antisera* (commercial panel containing common pathogenic serogroups) (Table 3.7). Only pathogenic bioserotypes of *Y. enterocolitica* isolated from slaughter pigs, culled dogs and retail meat samples were serotyped (Dr. B. Murphy and N. Drummond), data summarised in Table 3.8.

Table 3.7 Serotyping of *Y. enterocolitica* strains isolated from humans only

Biotype/Serotype	Number
1A/O:5	17/69 (24.6%)
1A/O:8	11/69 (15.9%)
1A/O:9	1/69 (1.4%)
1A/O:8;O:5	1/69 (1.4%)
1A/O:9;O:5	1/69 (1.4%)
1A/O:9;O:5;O:8	4/69 (5.8%)
1A/NEG	33/69 (47.8%)
1A/Autoagglutination	1/69 (1.4%)
2/O:9	2/3 (66.7%)
2/Autoagglutination	1/3 (33.3%)
3/Autoagglutination	2/3 (66.7%)
3/O:9	1/3 (33.3%)
4/O:3	4/4 (100%)
Total	**79**

Table 3.8 Serotyping results for pathogenic *Y. enterocolitica* isolated from slaughter pigs only

Biotype/Serotype	Number
3/O:5	3/218 (1.4%)
3/O:9	215/218 (98.6%)
4/O:3	10/15 (66.7%)
4/O:9	4/15 (26.7%)
4/NEG	1/15 (6.6%)
Total	233

3.3.2 PCR screening *Y. enterocolitica* for insecticidal toxin complex genes

All pathogenic *Y. enterocolitica* contained insecticidal toxin complex PCR targets resembling those identified in pathogenic *Y. enterocolitica* bioserovar 2/O:9 strain W22703 (EMBL/GenBank Accession number AJ920332) but only 24.8% of non-pathogenic (that is not apparently pathogenic for mammals) *Y. enterocolitica* contained insecticidal toxin complex PCR targets resembling that identified in non-pathogenic *Y. enterocolitica* bioserovar 1A/O:5 strain T83 (EMBL/GenBank Accession number AY647257).

Four types of insecticidal toxin complex toxin operons were detected among *Y. enterocolitica* strains (Table 3.9).

Table 3.9 Insecticidal toxin complex genotypes detected in *Y. enterocolitica* strains

ITC loci type	ITC genes detected
***tcd*-like**	*tcaC*, *tcbA* and *tccC*
***tcbA*-like**	*tcbA*
***tca*-like**	*tcaA*, *tcaB2* and *tcaC2*
***tcaA-tcaC2*-like**	*tcaA* and *tcaC2*

The *tcd* ITC locus, previously reported in non-pathogenic *Y. enterocolitica* [556], and *tcbA* ITC locus were mainly encoded by the non-pathogenic biovar 1A *Y. enterocolitica* strains, while the *tca* and *tcaA-tcaC2* insecticidal toxin complex loci were encoded by mildly pathogenic biovars (Table 3.10). Chi-square test for association indicated that there is a strong relationship between the insecticidal

toxin complex locus encoded by *Yersinia* strains and their biotype (Cramer's V^2 =0.63, P-Value <0.05, Table 3.10). Thus, the *tcd*-like locus was predominantly encoded by biovar 1A *Y. enterocolitica* strains, *tca*-like and *tcaA-tcac2*-like loci were predominantly encoded by mildly pathogenic biovars 2 and 3, and 4, respectively (Table 3.10).

Table 3.10 Biotype dependent distribution of insecticidal toxin complex loci in *Y. enterocolitica* strains isolated from humans, pigs, dogs and retail meat samples

Biotype	Tested strains	ITC +ve strains	ITC type				
			tcd	*tcbA*	*tca*	*tcaA-tcaC2*	*tcaC2*
1A	149	37 (24.8%)	33 (22.1%)	4 (2.7%)	-	-	
2	3	3 (100%)	-	-	3 (100%)	-	
3	64	64 (100%)	-	-	63 (98.4%)	-	1 (1.6%)
4	10	10 (100%)	-	-	-	10 (100%)	
Cramer's V^2 =0.63							
P-value <0.05							

A weak statistical association was detected between insecticidal toxin complex locus type and the source of *Yersinia* isolates (Cramer's V^2 =0.26, P-Value < 0.05, Table 3.11).

Table 3.11 Source dependent distribution of insecticidal toxin complex loci in *Y. enterocolitica* strains isolated from humans, pigs, dogs and retail meat samples

Source	Tested strains	ITC+ve strains	ITC type			
			tcd	*tcbA*	*tca*	*tcaA-tcaC2*
Human	80	22 (27.5%)	12 (15%)	-	6 (7.5%)	4 (5%)
Pig	86	75 (87.2%)	7 (8.1%)	-	61 (73.3%)	4 (4.7%)
Retail meat	35	5 (14.3%)	1 (2.9%)	4 (11.4%)	-	-
Dog	25	13 (52%)	13 (52%)	-	-	-
Cramer's V^2 =0.26						
P-value <0.05						

A moderate statistical association was established between the serotype and the insecticidal toxin complex locus encoded by *Yersinia* strains (Cramer's V^2 =0.38, P-value <0.05, Table 3.12).

Table 3.12 Serotype dependent distribution of insecticidal toxin complex loci in *Y. enterocolitica* strains isolated from humans, pigs, dogs and retail meat samples

Serotype	Tested strains	ITC+ve strains	ITC type				
			tcd	***tcbA***	***tca***	***tcaA-tcaC2***	***tcaC2***
O:3	10	9 (90%)	-	-	-	9 (90%)	
O:5	19	2 (10.6%)	1 (5.3%)	-	1 (5.3%)	-	
O:8	10	1 (10%)	1 (10%)	-	-	-	
O:9	65	65 (100%)	-	-	63 (96.9%)	1 (1.5%)	1 (1.5%)
O:5, O:8	1	0					
O:5, O:9	1	1 (100%)	1 (100%)	-	-	-	
O:5, O:8, O:9	4	0					
Autoagglutinated	4	3 (75%)	-	-	3 (75%)		
Unserotypable*	112	34 (30.4%)	31 (26.8%)	4 (3.6%)	-	-	
Cramer's V^2 =0.38							
P-value <0.05							

* Unserotypable strains represented by biovar 1A recovered from slaughter pigs, culled dogs and retail meat samples for which serotyping was not done and biovar 1A isolated from humans for which serotyping results were negative

3.3.3 Cytolethal distending toxins in *Yersinia* strains

CDT loci encoded by pathogenic and non-pathogenic *Y. enterocolitica* were inserted into a similar location between the genes YE1746 and YE1747 of the *Y. enterocolitica subsp. enterocolitica* 8081 genome (EMBL/GenBank Accession number AM286415). BLASTP analysis of open reading frames (ORFs) of the inserts amplified from pathogenic and non-pathogenic *Y. enterocolitica* strains identified similarity with CdtA and CdtB toxins (Table 3.13, Figure 3.3). Six distinct CDT loci designated in this thesis as A, B, C, D, E and F were identified in 90.5% (239/264) *Y. enterocolitica* and *Y. enterocolitica*-like *species* strains (Table 3.13). CDT loci F encode only CDT locus flanking genes homologous to YE1746 and YE1747 (Table 3.13, Figure 3.3).

Table 3.13 BLASTP hits of open reading frames detected in flanking PCR amplicons of CDT loci

CDT loci (size)	ORF in flanking amplicon	Position (bp) in amplicon	ORF length (amino acids)	First BLASTP GenBank hit	Accession number	Amino acid identity, (%)	ORF designation
A (3kb)	*Orf1*	2-739	245	hypothetical protein YE1746 *Y. enterocolitica subsp. enterocolitica* 8081	CAL11819	233/245 (95%)	YE1746
	Orf2	555-1187	210	Cdt A *Haemophilus ducreyi* 35000HP	AAP95786	41/154 (27%)	CdtA
	Orf3	1180-1626	148	CdtB *Escherichia coli*	EGW63159	26/62 (42%)	CdtB1
	Orf4	1451-2641	396	Cdt B *Campylobacter lari*	BAJ52736	115/316 (36%)	CdtB2
	Orf5	2757-3119	120	hypothetical protein YE1747 *Y. enterocolitica subsp. enterocolitica* 8081	CAL11820	120/120 (100%)	YE1747
B* (4kb)	*Orf1*	3-452	148	Cdt A *Haemophilus ducreyi* 35000HP	AAP95786	38/136 (28%)	CdtA1
	Orf2	551-916	121	Hypothetical protein PUUH_pUUH2392p0167 [Klebsiella pneumoniae]	AET17237	65/92 (71%)	HP**
	Orf3	810-1780	323	Cdt B *Campylobacter lari*	BAJ52736	95/270 (35%)	CdtB2
C (2kb)	*Orf1*	76-612	178	Cdt A *Haemophilus*	AAP95786	40/154 (26%)	CdtA1

CDT loci (size)	ORF in flanking amplicon	Position (bp) in amplicon	ORF length (amino acids)	First BLASTP GenBank hit	Accession number	Amino acid identity, (%)	ORF designation
				ducreyi 35000HP			
	Orf2	605-1600	331	Cdt B *Campylobacter lari*	BAJ52745	111/320 (35%)	CdtB2
	Orf3	1717-2079	120	hypothetical protein YE1747 *Y. enterocolitica subsp. enterocolitica* 8081	CAL11820	120/120 (100%)	YE1747
D (4.5kb)	*Orf1*	1-507	329	hypothetical protein YE1746 *Y. enterocolitica subsp. enterocolitica 8081*	CAL11819	97/100 (97%)	YE1746
	Orf2	392-1441	349	putative transposase for IS1667 *Y. enterocolitica subsp. enterocolitica* 8081	CAL13100	334/339 (99%)	TnIS1667
	Orf3	1587-2306	239	hypothetical protein YE1746 *Y. enterocolitica subsp. enterocolitica 8081*	CAL11819	176/195 (90%)	YE1746
	Orf4	2384-2995	203	Cdt A *Escherichia coli*	BAH78157	56/187 (30%)	CdtA2
	Orf5	2989-3576	195	Cdt B *Campylobacter coli*	ABJ98315	74/192 (39%)	CdtB3

CDT loci (size)	ORF in flanking amplicon	Position (bp) in amplicon	ORF length (amino acids)	First BLASTP GenBank hit	Accession number	Amino acid identity, (%)	ORF designation
	Orf6	3515-3946	143	Cdt B subunit *Helicobacter cinaedi CCUG* 18818	BAM12443	55/126 (44%)	CdtB4
	Orf7	4072-4458	128	hypothetical protein YE1747 *Y. enterocolitica subsp. enterocolitica* 8081	CAL11820	120/128 (94%)	YE1747
E (3kb)	*Orf1*	5-961	318	hypothetical protein YE1746 *Y. enterocolitica subsp. enterocolitica* 8081	CAL11819	270/294 (92%)	YE1746
	Orf2	1039-1650	203	Cdt A *Escherichia coli*	BAH78157	56/187 (30%)	CdtA2
	Orf3	1644-2231	195	Cdt B *Campylobacter coli*	ABJ98315	75/192 (39%)	CdtB3
	Orf4	2170-2601	143	Cdt B subunit *Helicobacter cinaedi* CCUG 18818	BAM12443	55/126 (44%)	CdtB4
	Orf5	2727-3140	137	hypothetical protein YE1747 *Y. enterocolitica subsp. enterocolitica* 8081	CAL11820	121/126 (96%)	YE1747

* Internal locus was only sequenced
** HP :Hypothetical protein

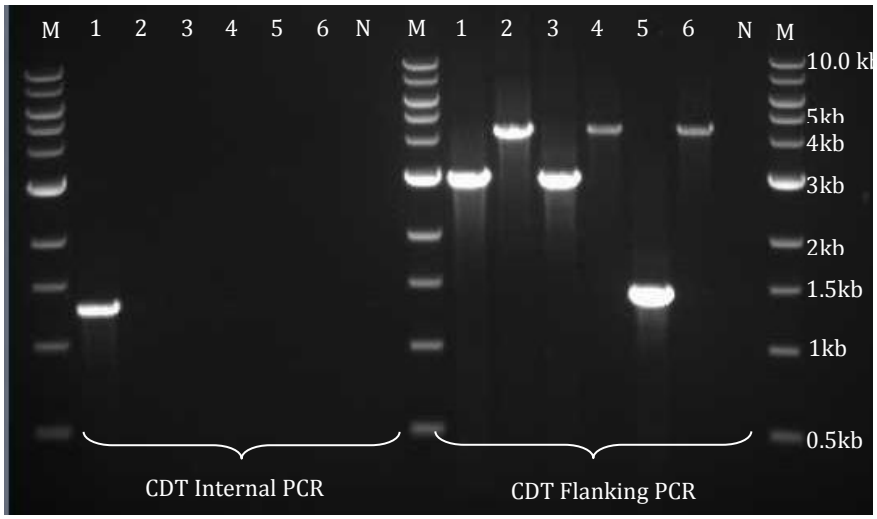


Figure 3.2 Cytolethal Distending Toxin loci amplified from reference *Y. enterocolitica* strains

M :Molecular Marker 1kb (NE BioLabs *Inc.*)
1 :*Y. enterocolitica subsp. palearctica* biovar 1A/O:6,30 strain IP102 (CDT Type A)
2 :*Y. enterocolitica subsp. palearctica* biovar 4/O:3 strain IP 134 (CDT Type D)
3 :*Y. enterocolitica subsp. palearctica* biovar 2/O:9 strain IP383 (CDT Type E.)
4 :*Y. enterocolitica subsp. palearctica* biovar 2/O:5,27 strain IP885 (CDT Type D)
5 :*Y. enterocolitica subsp. enterocolitica* biovar 1B/O:8 strain 8081 (CDT Type F)
6 :*Y. enterocolitica subsp. palearctica* biovar 3/O:5 strain IP 22228 (CDT Type D)
N :Negative control

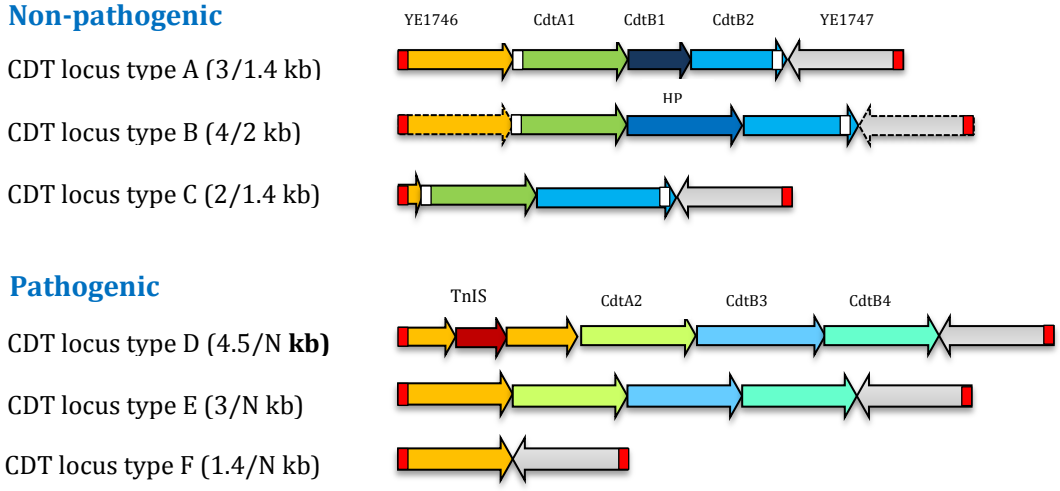


Figure 3.3 Schematic representation of the Cytolethal Distending Toxin loci of *Yersinia* species

All loci were shown by sequencing to be flanked by YE1746 and YE1747 with the exception of; CDT locus B – CDT internal PCR amplicon was sequenced only, the presence of YE1746 and YE1747 is hypothetical based on the non-sequenced CDT flanking PCR amplicon; CDT locus C does not apparently encode YE1746 but a binding site for CDT-Flanking Forward primer only was present. Red boxes indicate binding regions for flanking forward and reverse primers respectively; white boxes indicate binding regions forinternal forward and reverse primers respectively.

3.3.4 Distribution of CDT loci in *Yersinia* strains

The CDT locus was amplified from 264 *Yersinia* strains from our collection and six reference *Y. enterocolitica* strains (Table 3.14).

Table 3.14 CDT loci present in reference *Y. enterocolitica* strains

Yersinia strain	Bioserotype	CDT Type	Flanking PCR Band	Internal PCR Band
Reference strain IP102	1A/O:6, 30	A	3kb	1.4kb
Reference strain IP885	2/O:5, 27	D	4.5kb	NEG
Reference strain IP22228	3/O:5	D	4.5kb	NEG
Reference IP134	4/O:3	D	4.5kb	NEG
Reference strain IP383	2/O:9	E	3kb	NEG
Reference strain 8081	1B/O:8	F	1.4kb	NEG

Only a weak statistical association was established between the source of *Yersinia* strains and the type of CDT locus encoded (Cramér's V^2 =0.24, P-Value <0.05, Table 3.15).

CDT locus A was predominant among non-pathogenic *Y. enterocolitica* biovar 1A (83.3%) and was encoded by isolates recovered from humans, pigs, dogs and porcine retail meat samples (Table 3.16, Figure 3.4). CDT locus B was detected in *Y. enterocolitica* biovar 1A strains isolated from retail meat samples and pigs, and locus C was only detected in *Y. enterocolitica* biovar 1A strains isolated from humans (Table 3.15, Table 3.16, Fig 3.4). CDT loci D and E were most common among pathogenic *Y. enterocolitica* biovars which were mainly from pigs (Table 3.15). Biovar 4 *Y. enterocolitica* mainly encoded loci E (80%), and biovars 2 and 3 – loci D (100% and 90.1% respectively) (Table 3.16, Figure 3.4). *Y. enterocolitica*-like *species* were mostly negative for the CDT toxins with the primers used in this study (Table 3.16, Figure 3.4). There was a strong statistical relationship between the biotype of *Yersinia* and the CDT locus encoded (Cramér's V^2 =0.51, P-value <0.05, Table 3.16).

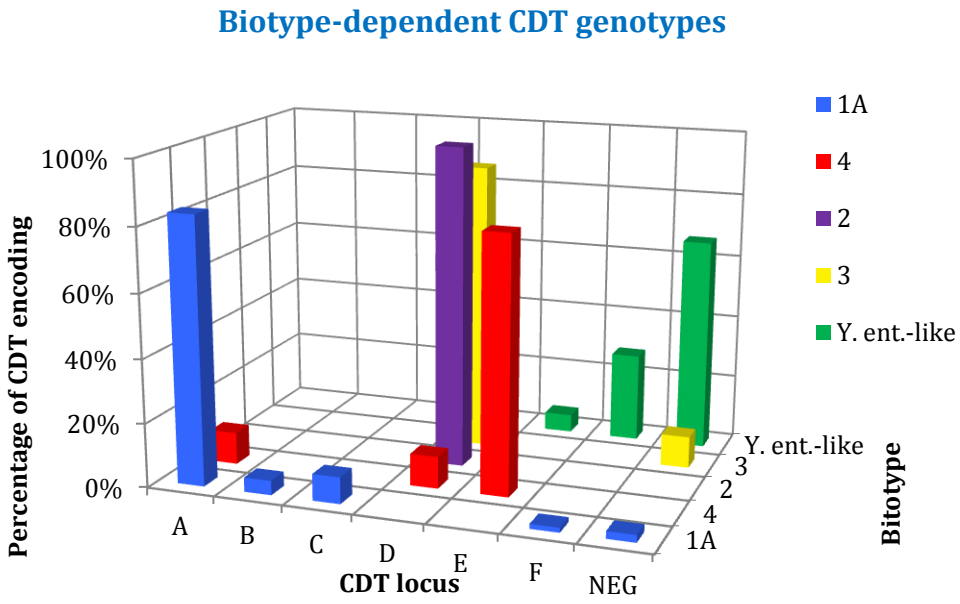
Table 3.15 Source-dependent distribution of CDT loci in *Yersinia* strains isolated from humans, pigs, dogs and retail meat samples

Source	Number of strains	CDT type						
		A	B	C	D	E	F	NEG
Human	86	64 (74.4%)	6 (7.0%)	-	6 (7.0%)	4 (4.7%)	2 (2.3%)	4 (4.7%)
Pig	131	15 (11.5%)	-	6 (4.6%)	88 (67.2%)	4 (3.1%)	2 (1.5%)	16 (12.2%)
Retail meat	33	15 (45.5%)	-	4 (12.1%)	-	-	4 (12.1%)	10 (30.3%)
Dog	14	14 (100%)	-	-	-	-	-	-
Cramér's V^2 =0.24								
P-value <0.05								

Table 3.16 Biotype dependent distribution of CDT loci in *Yersinia* strains isolated from humans, pigs, dogs and retail meat samples

Biotype	Number of strains	CDT type						
		A	B	C	D	E	F	N*
1A	132	110 (83.3%)	6 (4.5%)	11 (8.3%)	-	-	2 (1.5%)	3 (2.3%)
2	3	-	-	-	3 (100%)	-	-	-
3	101	-	-	-	91 (90.1%)	-	-	10 (9.9%)
4	10	1 (10%)	-	-	1 (10%)	8 (80%)	-	-
***Y.enterocolitica*-like**	18	-	-	-	-	1 (5.6%)	5 (27.8%)	12 (66.7%)
Cramér's V^2 =0.51								
P-value <0.05								

* No PCR bands obtained



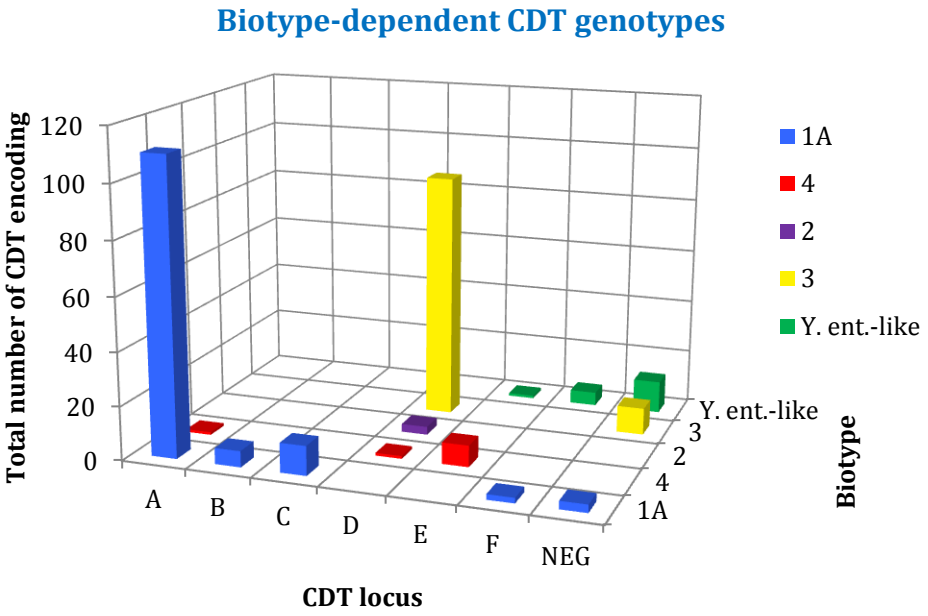


Figure 3.4 Biotype-dependent distribution of CDT loci in *Yersinia* strains isolated from humans, pigs, dogs and retail meat samples

Table 3.17 Serotype dependent distribution of CDT loci in *Yersinia* strains isolated from humans, pigs, dogs and retail meat samples

Serotype	Number of strains	CDT type						
		A	B	C	D	E	F	N**
O:3	9	1 (11.1%)	-	-	-	8 (88.9%)	-	-
O:5	19	17 (89.5%)	-	-	2 (10.5%)	-	-	-
O:8	10	10 (100%)	-	-	-	-	-	-
O:9	101	1 (0.99 %)	-	-	100 (99.01%)	-	-	-
O:5, O:8	1	1 (100%)	-	-	-	-	-	-
O:5, O:9	1	1 (100%)	-	-	-	-	-	-
O:5, O:8, O:9	4	4 (100%)	-	-	-	-	-	-
Auto agglutinated	3	-	-	-	3 (100%)	-	-	-
Unserotypable*	98	76 (77.6%)	11 (11.2%)	6 (6.1%)	-	-	2 (2.0%)	3 (3.1%)
***Y.enterocolitica*-like**	18	-	-	-	-	1/18 (5.6%)	5 (27.8%)	12 (66.7%)
Cramér's V^2=0.36								
P-value <0.05								

* Unserotypable strains represented by biovar 1A recovered from slaughter pigs, culled dogs and retail meat samples for which serotyping was not done and 33 biovar 1A strains isolated from humans for which serotyping results were negative

** Negative, no PCR bands obtained

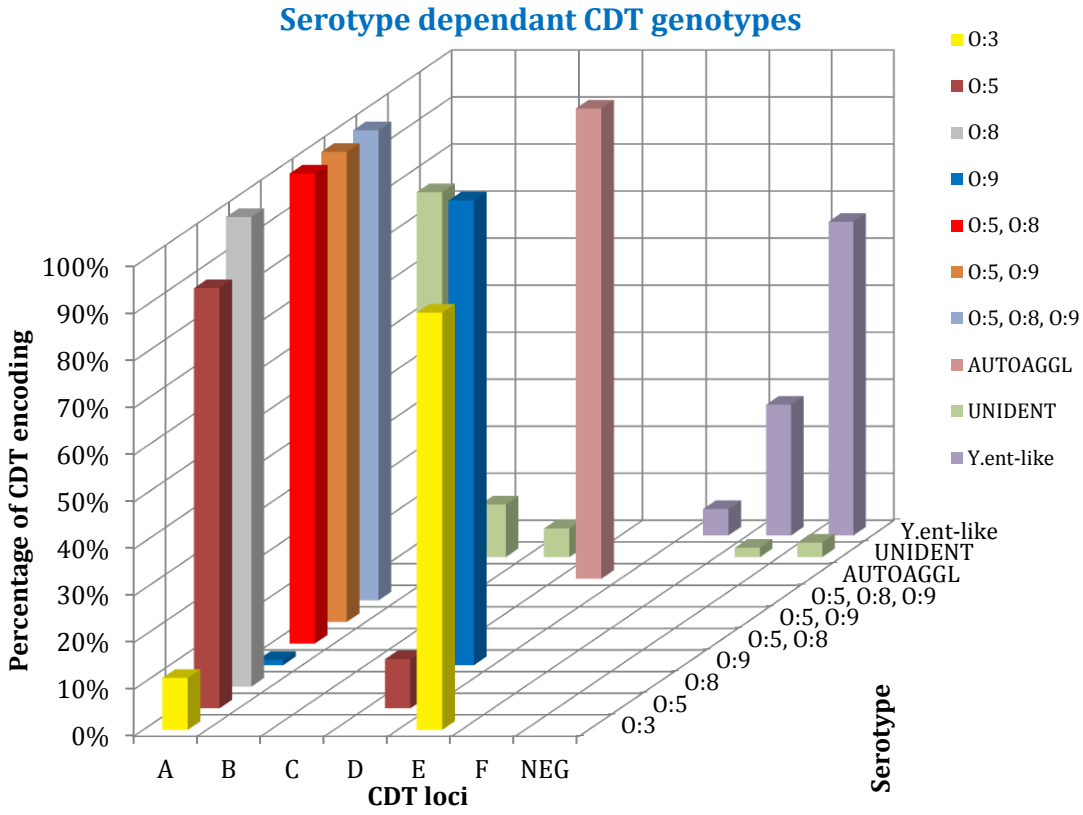


Figure 3.5 Serotype-dependent distribution of CDT loci in *Yersinia* strains isolated from humans, pigs, dogs and retail meat samples

CDT locus A was represented in the majority of *Y. enterocolitica* serotypes, while CDT loci D were mostly encoded by *Y. enterocolitica* serotype O:9 strains (100/105 or 95%) autogglutinating *Y. enterocolitica* strains (3/105, 2.9%) and O:5 strains (2/105,2%); CDT loci E were predominantly encoded by *Y. enterocolitica* serotype O:3 (8/9, 88.9%) (Table 3.17, Figure 3.5). Chi-square test for the association estimated a moderate statistical association between the serotype of *Yersinia* strain and CDT locus type encoded (Cramér's V^2 =0.36, P-Value <0.05, Table 3.17).

3.3.5 Expression of *Yersinia* CDT and insecticidal toxin complexes in *G. mellonella* larvae

Two different multiplex Reverse-Transcriptase PCR protocols were used for pathogenic and non-pathogenic *Yersinia,* respectively, to simultaneously detect expression of CDT and insecticidal toxin complex genes in both groups. The *cdtB2* and *cdtB3* subunits were expressed by non-pathogenic and pathogenic *Y.*

enterocolitica strains, respectively, in the *G. mellonella* infection model equally at both 15°C and 37°C (Table 3.18, Figure 3.6, Figure 3.7). Expression of other Cdt toxins was not examined. *tcaC2* and *tcaC* were also expressed at 15°C and 37°C in both pathogenic and non-pathogenic *Y. enterocolitica* (Table 3.18, Figure 3.6, Figure 3.7). However increased expression of *tcaC* at 15°C was apparent for strains IP102, and 2850 (Figure 3.6), and increased expression of *tcaC2* at 15°C by IP22228 (Figure 3.7). Highly pathogenic *Y. enterocolitica* strain 8081 lacks genes for both insecticidal toxin complex and CDT loci, consequently their expression was not detected by the RT-PCR assay (Table 3.18, Figure 3.7).

Table 3.18 Expression of *cdtB* and *tcaC* of pathogenic and non-pathogenic *Yersinia* in *G. mellonella* larvae

Strain	Bioserotype	CDT type	ITC type	Expression		
				Gene	37°C	15°C
IP102	1A/O:6,30	A	*tcd*-like	*cdtB2*	+	+
				tcaC	+	+
2850	1A/NEG	B	*tcd*-like	*cdtB2*	+	+
				tcaC	+	+
3/C/86 CS D7	1A/O:9	C	NEG	*cdtB2*	+	+
				tcaC	-	-
IP22228	3/O:5	D	*tca*-like	*cdtB3*	+	+
				tcaC2	+	+
Y/00/01(1)	4/O:3	E	*tcaA-tcaC2* like	*cdtB3*	+	+
				tcaC2	+	+
8081	1B/O:8	F (NEG)	NEG	*cdtB3*	-	-
				tcaC2	-	-
5618	2/AUTO	D	*tca*-like	*cdtB3*	+	+
				tcaC2	+	+
RS-42 D21	YF	F	NEG	*cdtB3*	-	-
				tcaC2	-	-

YF :*Y. frederiksenii*

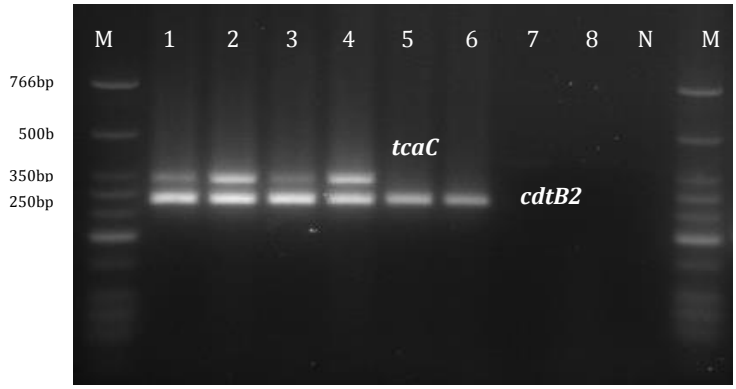


Figure 3.6 RT-PCR for simultaneous expression of non-pathogenic *Y. enterocolitica* biovar 1A *ctdB2* and *tcaC* toxin genes in *G. mellonella* larvae

M :Low Weight Molecular DNA marker (NE BioLabs *Inc.*)
1 :CDT type A, *tcd*-like (IP102) 37°C
2 :CDT type A, *tcd*-like (IP102) 15°C
3 :CDT type B, *tcd*-like (2850) 37°C
4 :CDT type B, *tcd*-like (2850) 15°C
5 :CDT type C, ITC negative (3/C/86 CSD7) 37°C
6 :CDT type C, ITC negative (3/C/86 CSD7) 15°C
7 :PBS 37°C (expression negative control)
8 :PBS 15°C (expression negative control)
N :PCR negative control
tcaC – 341 bp, *cdtB2*-282 bp

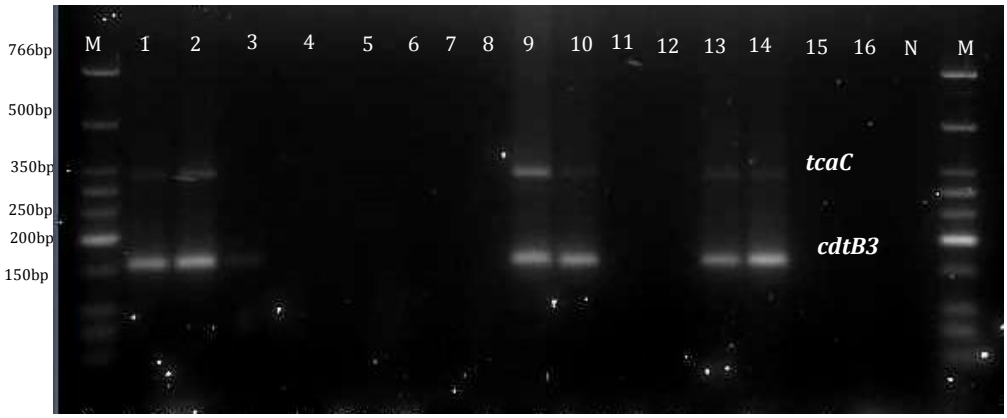


Figure 3.7 RT-PCR for simultaneous expression of *ctdB3* and *tcaC2* toxins of pathogenic biovars of *Y. enterocolitica* in *G. mellonell*a larva

M :Low Weight Molecular DNA marker (NE BioLabs *Inc.*)
1 :CDT type D, *tca*-like (IP22228) 37°C
2 :CDT type D, *tca*-like (IP22228) 15°C
3 :CDT type F, ITC negative (8081) 37°C
4 :CDT type F, ITC negative (8081) 15°C
5 :CDT type negative, ITC negative (3343 *Y. rohdei*) 37°C
6 :CDT type negative, ITC-negative (3343 *Y. rohdei*) 15°C
7 :CDT type negative, ITC negative (26931 *Y. bercovieri*) 37°C
8 :CDT type negative, ITC negative (26931 *Y. bercovieri* 15°C
9 :CDT type D, *tca*-like (5618) 37°C
10 :CDT type D, *tca*-like (5618) 15°C
11 :CDT type F, ITC negative (RS-42 *Y. frederiksenii*) 37°C
12 :CDT type F, ITC negative (RS-42 *Y. frederiksenii*) 15°C
13 :CDT type E, *tcaA-tcaC2*-like (Y/00/01(1)) 37°C
14 :CDT type E, *tcaA-tcaC2*-like (Y/00/01(1)) 15°C
15 :PBS 37°C (expression negative control)
16 :PBS 15°C (expression negative control)
N :PCR negative control
tcaC2 - 330bp, *cdtB3* – 155bp

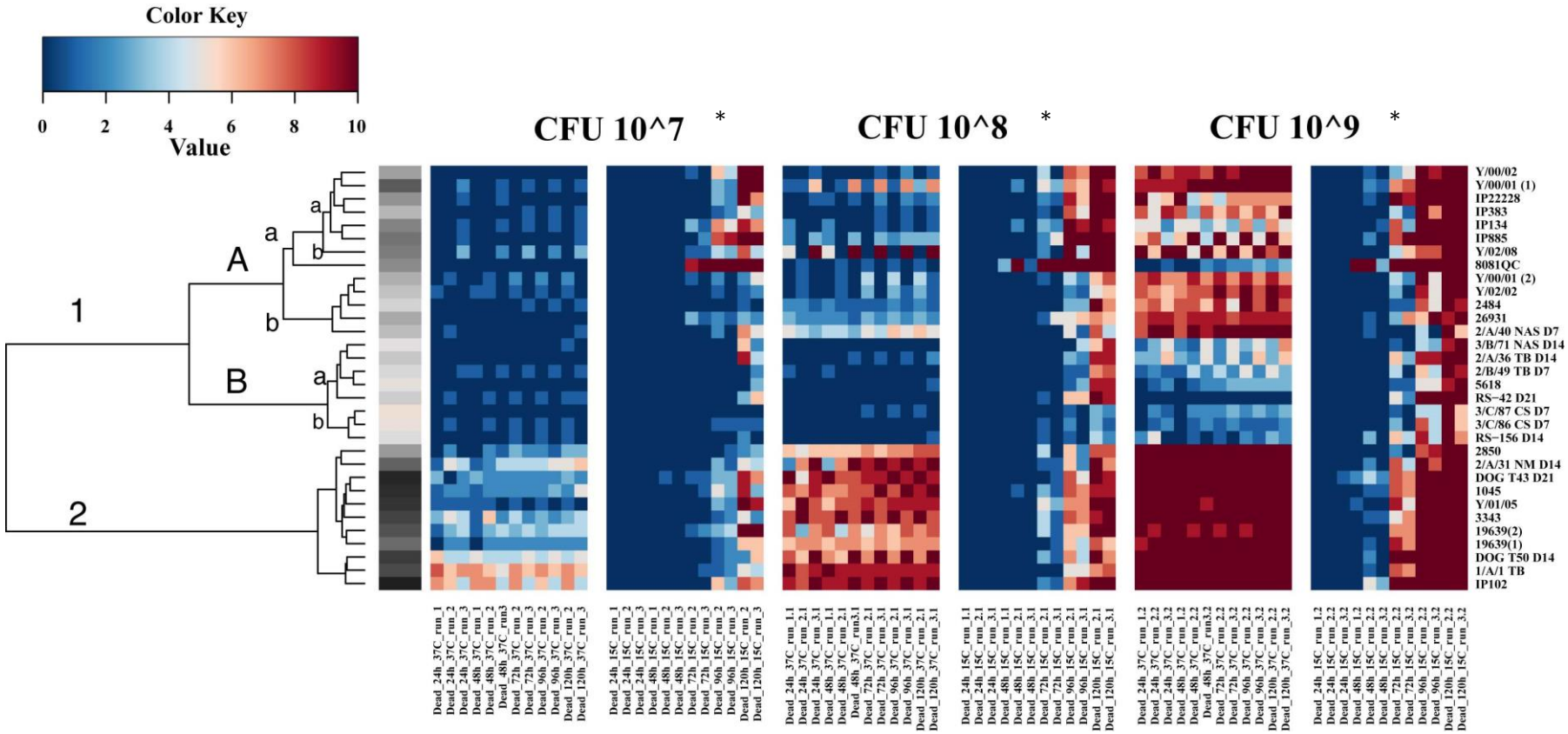


Figure 3.8 Hierarchical clustering of the *G. mellonella* larvae mortality rates incubated at 37°C and 15°C after subcutaneous infection with *Yersinia*

*CFU refer to viable counts per ml of suspensions, of which 10 µl was injected per larva

3.3.6 *Yersinia* infection in *G. mellonella* larvae

Yersinia infection in *G. mellonella* larvae at 15°C and 37°C caused by pathogenic and non-pathogenic strains was accompanied by pigmentation indicating activation of the PPO (ProPhenolOxidase) system causing melanisation [589, 616-619] (data not shown).

Hierarchical clustering of the experimental mortality data obtained after *Yersinia* subcutaneous infection in *G. mellonella* larvae incubated at 15°C and 37°C for 120h grouped strains into two major phenotypic clusters 1 and 2, cluster 1 is further divisible into subclusters 1A and 1B (Figure 3.8). Cluster 2 (comprising only *Y. enterocolitica* strains of Biovar 1A and one *Y. rohdei* strain) is characterised by enhanced killing at 37°C (fast killing) compared to Cluster 1, and subcluster 1B shows even lower mortality at 37°C than cluster 1A. All negative controls for each experiment (10 *G. mellonella* injected with 10µl of the same sterile PBS used for the preparation of the *Yersinia* inocula in experiments) survived after incubation for 120h at 15°C and 37°C.

The chi-square test for association shows that there is a statistically significant strong association between hierarchical mortality cluster type and type of CDT locus encoded by the isolate (Cramér's V^2 =0.65, P-Value < 0.05 , Table 3.19), moderate association between serotype (Cramér's V^2 =0.48, P-Value < 0.05, Table 3.19), source (Cramér's V^2 =0.36, P-Value < 0.05, Table 3.19), ITC locus encoded by microorganism (Cramér's V^2 =0.33, P-Value < 0.05, Table 3.19) and biotype (Cramér's V^2 =0.31, P-Value < 0.05, Table 3.19). A weak association was found for the presence of the pYV and hierarchical mortality cluster type (Cramer's V^2=0.27, P-value < 0.05, Table 3.19).

Overall, subcluster 1A grouped mildly pathogenic *Y. enterocolitica* strains (isolated from humans) of serotype O:3 and O:5, encoding CDT type E and *tcaA-tcaC2*-like insecticidal toxins. Cluster 1B shows over-representation of the mildly pathogenic *Y. enterocolitica* serotype O:9 strains isolated from pigs which encode CDT type D and *tca*-like insecticidal toxins. Cluster 2 mostly includes non-pathogenic *Y. enterocolitica* strains of biovar 1A that encode CDT type A and *tcd*-like insecticidal toxins. All biovar 1A *Y. enterocolitica* strains from cluster 1A encode CDT type B and contain an ITC, and all biovar 1A *Y. enterocolitica* strains from cluster 1B encode CDT type C but are ITC negative. Not all biovar 1A *Y. enterocolitica* strains in cluster 2 are ITC positive but most of them encode CDT type A, except strain 2850 which encodes CDT type B (Table 3.19).

Table 3.19 Outcome of subcutaneous *Yersinia* infection in *G. mellonella* larvae incubated at 37°C and 15°C for up to 120h

Strain	Bioserovar	Source	CDT type	ITC type	pYV	Average Dead Gm larvae ±SD per group of 10*[10]		P-value *[4]	LD_{50}, CFU/ml	
						15°C, 120h	37°C, 120h		15°C, 72h	37°C, 24h
Cluster 1A										
Y/00/02	4/O:3	Human	E	*tcaA-tcaC2*-like	pos	10 ± 0.0	3.5 ± 5.1	<0.001	$2.30x10^7$ (96h) *[5]	$7.94x10^8$
Y/00/01 (1)	4/O:3	Human	E	*tcaA-tcaC2*-like	neg	9.8 ± 0.4	5.2 ± 4.5	<0.001	$3.24x10^8$	$4.27x10^8$
IP22228	3/O:5	Ref**	D	*tca*-like	pos	9.5 ± 1.2	2.8 ± 3.3	<0.001	$6.03x10^8$	$1.05x10^9$
IP383	2/O:9	Ref	E	*tca*-like	pos	8.0 ± 3.2	3 ± 4.1	<0.001	$8.51x10^7$ (96h) *[5]	$9.33x10^8$ (96h) *[7]
IP134	4/O:3	Ref	D	*tcaA-tcaC2*-like	neg	9.2 ± 1.2	2.3 ± 2.9	<0.001	$1.12x10^9$	$1.67x10^9$ (120h) *[7]
IP885	2/O:5,27	Ref	D	*tca*-like	pos	10 ± 0.0	3.8 ± 3.7	<0.001	$3.47x10^8$	$1.07x10^9$
Y/02/08	4/O:3	Human	E	*tcaA-tcaC2*-like	pos	8.7 ± 2.4	5.2 ± 4.8	<0.001	$1.45x10^9$	$1.40x10^8$
8081 QC	1B/O:8	Ref	F	neg	pos	10 ± 0.0	1 ± 1.3	<0.001	$2.19x10^8$ (48h) *[6]	no above 50% *[8]
Y/00/01 (2)	1A/NEG	Human	B	*tcd*-like	NT*[3]	6.8 ± 3.1	3.8 ± 3.5	0.001	$5.01x10^8$ (96h) *[5]	$7.94x10^8$
Y/02/02	1A/NEG	Human	B	*tcd-like*	NT	5.7 ± 3.8	4.0 ± 4.4	0.068	$7.24x10^8$ (96h) *[5]	$8.13x10^8$
2484	3/O:9	Human	D	*tca*-like	neg	6.2 ± 4.5	3.7 ± 4.2	<0.001	$7.08x10^8$ (96h) *[5]	$8.32x10^8$
26931	*Y. bercovieri*	Human	neg	neg	NT	6.2 ± 3.2	3.8 ± 4.2	0.011	$2.10x10^9$	$5.50x10^7$
2/A/40 NAS D7	4/O:3	Pig	E	*tcaA-tcaC2*-like	neg	6.7 ± 2.2	5.3 ± 4.3	0.136	no above 50% at 96h and no below 50% at 120h	$3.02x10^8$

Strain	Bioserovar	Source	CDT type	ITC type	pYV	Average Dead Gm larvae ±SD per group of 10*[10]		P-value *[4]	LD_{50}, CFU/ml	
						15°C, 120h	37°C, 120h		15°C, 72h	37°C, 24h
Cluster 1B										
3/B/71 NAS D14	4/O:9	Pig	D	*tca*-like	pos	7.0 ±3.0	1.8 ± 2.8	<0.001	$2.95x10^7$ (120h) *[5]	$1.57x10^9$ (120h) *[7]
2/A/36 TB D14	3/O:9	Pig	D	*tca*-like	pos	8.5 ± 3.2	1.8 ± 2.4	<0.001	$3.55x10^8$ (96h) *[5]	$1.70x10^9$ (120h) *[7]
2/B/49 TB D7	3/O:9	Pig	D	*tca*-like	pos	6.0 ± 4.0	1.5 ± 2.1	<0.001	$2.10x10^9$ (96h) *[5]	no above 50% *[8]
5618	2/Autoaggl	Human	D	*tca*-like	pos	6.7 ± 4.1	1.2 ± 1.5	<0.001	$1.60x10^9$ (96h) *[5]	no above 50% *[8]
RS-42 D21	*Y. frederiksenii*	Retail meat	F	neg	NT	8.0 ± 2.9	0.3 ± 0.5	<0.001	$1.23x10^8$ (96h) *[5]	no above 50% *[8]
3/C/87 CS D7	1A/Autoaggl	Pig	C	neg	NT	3.5 ± 3.9	1 ± 1.3	<0.001	$5.13x10^8$ (120h) *[5]	no above 50% *[8]
3/C/86 CS D7	1A/O:9	Pig	C	neg	NT	3.7 ± 3.7	0.7 ± 0.8	<0.001	$1.23x10^9$ (96h) *[5]	no above 50% *[8]
RS-156 D14	*Y. frederiksenii*	Retail meat	C	neg	NT	4.2 ± 3.7	1.2 ± 1.2	<0.001	$2.25x10^9$	no above 50% *[8]
Cluster 2										
2850	1A/NEG	Human	B	*tcd*-like	NT	6.8 ± 3.5	7 ± 3.2	0.843	$3.24x10^8$ (96h) *[5]	$1.38x10^8$
2/A/31NM D14	1A/NST*	Pig	A	neg	NT	7.7 ± 2.7	8.3 ± 2.3	0.361	$1.05x10^9$	$6.31x10^7$
DOG T43 D21	1A/NST	Dog	A	neg	NT	9.2 ± 1.2	7.3 ± 3.8	0.008	$8.32x10^8$	$6.46x10^7$
1045	1A/O:8	Human	A	neg	NT	8.3 ± 2.7	7.7 ± 3.4	0.361	$7.76x10^8$	$6.76x10^7$
Y/01/05	1A/Autoggl	Human	A	*tcd*-like	NT	9.8 ± 0.5	6.5 ± 4.7	<0.001	$4.17x10^8$	$7.08x10^7$
3343	*Y. rohdei*	Human	neg	neg	NT	7.7 ± 3.4	7.5 ± 3.2	0.831	$9.55x10^8$	$2.82x10^7$

Strain	Bioserovar	Source	CDT type	ITC type	pYV	Average Dead Gm larvae ±SD per group of 10*[10]		P-value *[4]	LD_{50}, CFU/ml	
						15°C, 120h	37°C, 120h		15°C, 72h	37°C, 24h
19639 (2)	1A/NEG	Human	A	*tcd*-like	NT	10 ± 0.0	7.2 ± 2.7	<0.001	$6.17x10^8$	$8.32x10^7$
19639 (1)	1A/NEG	Human	A	neg	NT	7.7 ± 2.0	6.3 ± 3.6	0.111	$7.76x10^8$	$9.55x10^7$
DOG T50 D14	1A/NST	Dog	A	*tcd*-like	NT	7.5 ± 3.2	7.7 ± 2.7	0.831	$4.57x10^8$	$2.19x10^7$
1/A/1 TB	1A/NST	Pig	A	*tcd*-like	NT	7.3 ±2.5	8.5 ± 1.6	0.116	$8.51x10^8$	$<1.9x10^7$ *[9]
IP102	1A/O:6,30	Ref	A	*tcd*-like	NT	9.2 ± 1.3	8.3 ± 2.4	0.168	$4.47x10^8$	$<1.83x10^7$ *[9]

*NST – not serotyped
** Reference strain
*[3]NT – not tested
*[4] Statistical difference between *G. mellonella* larvae mortality rates observed after their incubation for 120h at 37°C and 15°C
*[5] 50% mortality was not achieved at the specified time (72h)
*[6] LD_{50} was achieved after incubation for 48h
*[7] 50% mortality was not achieved at the specified time (24h)
*[8] Mortality rate of 50% was never achieved
*[9] More than 50% of *G. mellonella* larvae were killed at the lowest CFU/ml injected
*[10] Mean number of dead insect larvae observed for all three different bacterial inocula

One-way ANOVA analysis with Tukey's error of the mortality rates observed for *Yersinia* strains encoding different CDT loci revealed that strains encoding CDT type A locus were the most virulent towards insect larvae at 37°C but not at 15°C (Table 3.20).

Y. enterocolitica strains encoding a *tcd*-like ITC locus were the most virulent towards insect larvae at 37°C, at 15°C all strains encoding different ITC loci were equally pathogenic towards *G. mellonella* (Table 3.21). However, insecticidal activity of *Yersinia* strains encoding *tca*- and *tcaA-tcaC2*-like ITC loci as well as of ITC non-encoding bacteria significantly increased at 15°C (Table 3.21). *Y. enterocolitica* strains encoding a *tcd*-like ITC locus were equally virulent at 15°C and 37°C (Table 3.21).

Presence of the *Yersinia* virulence plasmid pYV did not enhance insecticidal activity of *Yersinia* strains at 37°C, on the contrary pYV encoding strains were the least virulent towards insect larvae at 37°C (Table 3.22). At 15°C virulence of all strains irrespective of pYV presence significantly increased, moreover all strains were equally virulent at 15°C (Table 3.22).

According to Tukey's grouping all *Yersinia* strains were equally virulent at 15°C and 37°C irrespective of their biotype (that reflects pathogenicity) (Table 3.23). While, mildly pathogenic biovar 2-4 and highly pathogenic biovar 1B *Y. enterocolitica* strains demonstrated significantly increased virulence towards *G. mellonella* larvae at 15°C than at 37°C, non-pathogenic biovar 1A *Y. enterocolitica* and *Y. enterocolitica*-like strains were similarly virulent at both 15°C and 37°C (Table 3.23).

Table 3.20 One-way ANOVA comparison of the *G. mellonella* larvae mortality rates caused by *Yersinia* strains encoding different CDT loci

CDT locus	Number of strains	Average Dead Gm larvae ±SD per group of 10, 37°C, 120h*	95% CI**	Tukey's Grouping *3	Average Dead Gm larvae ±SD per group of 10, 15°C, 120h*	95% CI**	Tukey's Grouping *3	P-value *4
A	9	7.5 ± 0.8	6.93, 8.14	A	8.5 ± 1.04	7.72, 9.32	A	< 0.05
B	3	4.9 ± 1.9	0.52, 9.37	B	6.4 ± 0.7	4.77, 8.12	AB	0.306
C	3	0.9 ± 0.3	0.31, 1.58	C	3.8 ± 0.4	2.92, 4.64	B	< 0.05
D	8	2.4 ± 1.0	1.55, 3.20	C	7.9 ± 1.6	6.54, 9.21	A	0.000
E	5	4.4 ± 1.1	3.07, 5.80	B	8.6 ± 1.4	6.92, 10.34	A	< 0.05
F	2	0.7 ± 0.5	-3.57, 4.90	C	9.0 ± 1.4	-3.71, 21.76	A	0.08
N	2	5.7 ± 2.6	-17.63, 28.96	AB	6.9 ± 1.06	-2.61, 16.45	AB	0.642
P-value *5	NA	< 0.05	NA	NA	< 0.05	NA	NA	NA

* Average numbers of dead insect larvae observed for all bacterial inocula
**Confidence Interval
*3 Means that do not share a letter are significantly different ($p < 0.05$), (pairwise differences between means not significant for means with the same letter)
*4 Comparison of mortality rates observed at 15°C against those observed at 37°C
*5 Comparison of mortality rates in different clusters at the same temperature
NA :Not applicable

Table 3.21 One-way ANOVA comparison of the *G. mellonella* larvae mortality rates caused by *Yersinia* strains encoding different ITC loci

ITC locus	Number of strains	Average Dead Gm larvae ±SD per group of 10, 37°C, 120h*	95% CI**	Tukey's Grouping *3	Average Dead Gm larvae ±SD per group of 10, 15°C, 120h*	95% CI**	Tukey's Grouping *3	P-value *4
NEG	11	4.1 ± 3.3	1.86, 6.35	AB	6.9 ± 2.2	5.41, 8.41	A	< 0.05
***tca*-like**	8	2.5 ± 1.01	1.61, 3.30	B	7.7 ± 1.5	6.46, 9.00	A	< 0.05
***tcaA-tcaC2*-like**	5	4.3 ± 1.3	2.65, 5.95	AB	8.9 ± 1.3	7.20, 10.53	A	< 0.05
***tcd*-like**	8	6.6 ± 1.8	5.12, 8.13	A	7.9 ± 1.6	6.57, 9.22	A	0.157
P-value *5	NA	< 0.05	NA	NA	0.252	NA	NA	NA

* Average numbers of dead insect larvae observed for all bacterial inocula
**Confidence Interval
*3 Means that do not share a letter are significantly different ($p < 0.05$), (pairwise differences between means not significant for means with the same letter)
*4 Comparison of mortality rates observed at 15°C against those observed at 37°C
*5 Comparison of mortality rates in different clusters at the same temperature
NA :Not applicable

Table 3.22 One-way ANOVA comparison of the *G. mellonella* larvae mortality rates caused by pYV positive and pYV negative *Yersinia* strains

pYV	Number of strains	Average Dead Gm larvae ±SD per group of 10, 37°C, 120h*	95% CI**	Tukey's Grouping *3	Average Dead Gm larvae ±SD per group of 10, 15°C, 120h*	95% CI**	Tukey's Grouping *3	P-value *4
pYV +ve	10	2.6 ± 1.3	1.61, 3.52	B	8.4 ± 1.5	7.37, 950	A	< 0.05
pYV -ve	4	4.1 ± 1.4	1.88, 6.37	AB	8.0 ± 1.8	5.07, 10.84	A	< 0.05
Non-path	18	5.4 ± 2.9	3.95, 6.85	A	7.2 ± 1.9	6.21, 8.14	A	< 0.05
P-value *5	NA	< 0.05	NA	NA	0.214	NA	NA	NA

* Average numbers of dead insect larvae observed for all bacterial inocula
**Confidence Interval
*3 Means that do not share a letter are significantly different ($p < 0.05$), (pairwise differences between means not significant for means with the same letter)
*4 Comparison of mortality rates observed at 15°C against those observed at 37°C
*5 Comparison of mortality rates in different clusters at the same temperature
NA :Not applicable

Table 3.23 One-way ANOVA comparison of the *G. mellonella* larvae mortality rates caused by *Yersinia* strains of various biotypes

Biotype	Number of strains	Average Dead Gm larvae ±SD per group of 10, 37°C, 120h*	95% CI**	Tukey's Grouping*3	Average Dead Gm larvae ±SD per group of 10, 15°C, 120h*	95% CI**	Tukey's Grouping*3	P-value *4
1A	14	6.02 ± 2.6	4.51, 7.54	A	7.4 ± 2.01	6.21, 8.53	A	0.141
1B	1	1	NA	NA	10*	NA	A	NA
2	3	2.7 ± 1.4	-0.72, 6.06	A	8.2 ± 1.7	4.05, 12.39	A	< 0.05
3	4	2.5 ± 1.0	0.89, 4.03	A	7.5 ± 1.7	4.78, 10.30	A	< 0.05
4	6	3.9 ± 1.6	2.25, 5.53	A	8.6 ± 1.4	7.06, 10.05	A	< 0.05
Non-ent	4	3.2 ± 3.2	-1.93, 8.34	A	6.5 ± 1.8	3.72, 9.28	A	0.147
P-value *5	NA	< 0.05	NA	NA	0.399	NA	NA	NA

* Average numbers of dead insect larvae observed for all bacterial inocula
**Confidence Interval
*3 Means that do not share a letter are significantly different ($p < 0.05$), (pairwise differences between means not significant for means with the same letter)
*4 Comparison of mortality rates observed at 15°C against those observed at 37°C
*5 Comparison of mortality rates in different clusters at the same temperature
NA :Not applicable

The one-way ANOVA comparison with Tukey's error rate of the average numbers of dead larvae observed after incubation at 37°C for 120h revealed that mortality is significantly different across different hierarchical cluster mortality groups (P-value <0.05) (Table 3.24). Comparison of mean dead larvae observed at 15°C for 120h by the same method also found a statistically significant difference in the mortality for the different hierarchical clusters (P-value <0.05) (Table 3.24). For incubation at 37°C the 95% confidence intervals (CI) for means observed for the clusters 1B,1A and 2 do not overlap (Table 3.24,Figure 3.9) and the three clusters show statistically significantly different mortalities at this temperature. For incubation at 15 °C the 95% confidence intervals (CI) for mean death rates observed for clusters 1A and 2 overlap, indicating that they are not significantly different (Table 3.24,Figure 3.9). Likewise, using Tukey's method in ANOVA (95% simultaneous confidence intervals for all pairwise comparisons of the three groups) the difference between average numbers of dead larvae at 15°C achieved by *Yersinia* strains from clusters 1A and 2 is associated with a 95% confidence interval between -1.516 and 1.682, which includes zero. This indicates that *Yersinia* strains from clusters 1A and 2, show indistinguishable virulence towards *G. mellonella* larvae at 15°C. At the same time, *Yersinia* strains from hierarchical cluster 1B are statistically significantly less virulent towards insect larvae at 15°C than microorganisms from clusters 1A and 2 (Table 3.24).

Comparison of *Yersinia* infection outcome observed at 37°C and 15°C between the strains within the cluster revealed that *Yersinia* strains from clusters 1A and 1B cause higher mortality rates at 15°C than at 37°C, with cluster 1B strains causing lower mortality at both 37°C and 15°C than the other groups (Table 3.24, Figure 3.9). *Yersinia* strains from cluster 2 were equally lethal towards *G. mellonella* larvae at 37°C and 15°C (Table 3.24, Figure 3.9). The majority of pathogenic *Y. enterocolitica* strains caused significantly higher mortality rates of insect larvae at 15°C than at 37°C (Table 3.19, Table 3.23).

Table 3.24 One-way ANOVA comparison of the *G. mellonella* larvae mortality rates caused by *Yersinia* strains grouped in specific hierarchical clusters

Cluster	Number of strains	Average Dead Gm larvae ±SD per group of 10, 37°C, 120h*	95% CI**	Tukey's Grouping*[3]	Average Dead Gm larvae ±SD per group of 10, 15°C, 120h*	95% CI**	Tukey's Grouping*[3]	P-value[4]*
1A	13	3.65 ± 1.21	2.92, 4.38	B	8.21 ± 1.69	7.19, 9.23	A	< 0.05
1B	8	1.19 ± 0.53	0.74, 1.63	C	5.94 ± 1.95	4.30, 7,57	B	< 0.05
2	11	7.49 ± 0.72	7.00, 7.97	A	8.29 ± 1.08	7.56, 9.01	A	0.056
P-value *[5]	NA	< 0.05	NA	NA	< 0.05	NA	NA	NA

* Average numbers of dead insect larvae observed for all bacterial inocula
**Confidence Interval
*[3] Means that do not share a letter are significantly different ($p < 0.05$), (pairwise differences between means not significant for means with the same letter)
*[4] Comparison of mortality rates observed at 15°C against those observed at 37°C
*[5] Comparison of mortality rates in different clusters at the same temperature
NA :Not applicable

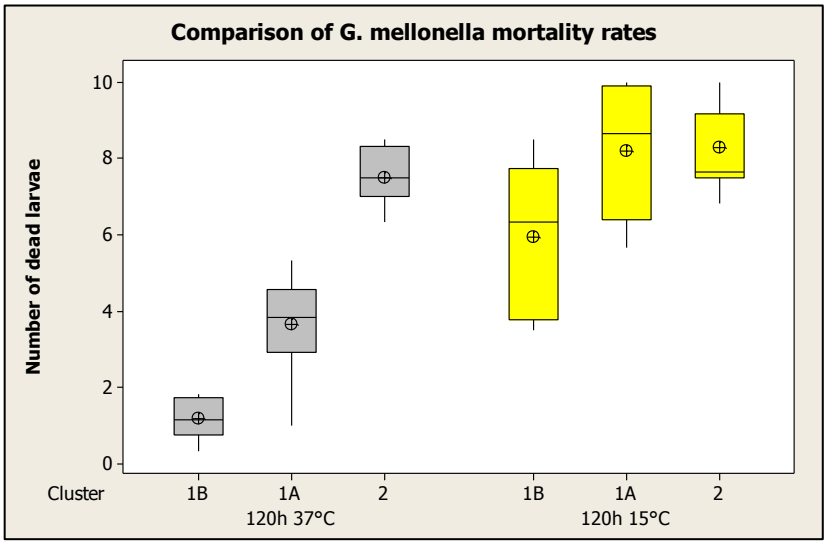


Figure 3.9 Comparison of the *G. mellonella* larvae mortality rates caused by *Yersinia* strains grouped in specific hierarchical clusters analysed by ANOVA in Minitab

⊕ - shows mean death rates; horizontal line indicates median death rates with 95% confidence intervals (box) and standard deviations (whiskers).

In addition to observed mortality rates, an LD_{50} for each *Yersinia* strain was estimated by an approximation of the Reed-Muench method [612] (Figure 3.10) based on at least one of the three inocula reaching mortality rates of insect larvae above 50% on observation after 24h of incubation at 37°C, and after 72h incubation at 15°C (Table 3.19).

1. Estimate proportional distance (PD)

$$PD = \frac{50\% - \text{mortality rate of inoculum below } 50\%}{\text{mortality of higher inoculum above } 50\% - \text{mortality of inoculum below } 50\%}$$

2. Estimate logarithm (log) of the inoculum causing mortality below 50%
3. Estimate sum of PD and log of the inoculum causing mortality below 50%
4. Inverse logarithm of the sum is the LD_{50}

Figure 3.10 Calculation of LD_{50} by Reed-Muench method [612]

Table 3.25 One-way ANOVA comparison of *Yersinia* strain LD_{50} for *G. mellonella* larvae observed at 37°C and 15°C

Cluster	Number of strains*	LD_{50} ± SD, 15°C, 72h	95% CI	Tukey's Grouping *3	Number of strains*	LD_{50} ± SD, 37°C, 24h	95% CI	Tukey's Grouping *3	P-value**
1A	12	$6.8x10^8 \pm 6.1x10^8$	$3.0x10^8$, $1.1x10^9$	A	12	$7.4x10^8 \pm 4.5x10^8$	$4.5x10^8$, $1.0x10^9$	B	**0.8**
1B	8	$1.1x10^9 \pm 9.8x10^8$	$2.9x10^8$, $1.9x10^9$	A	2	$1.6x10^9 \pm 9.2x10^7$	$8.1x10^8$, $2.5x10^9$	A	**0.176**
2	11	$6.8x10^8 \pm 2.4x10^8$	$5.2x10^8$, $8.4x10^8$	A	11	$6.1x10^7 \pm 3.7x10^7$	$3.6x10^7$, $8.6x10^7$	C	**<0.05**
P-value	NA	0.284	NA	NA	NA	< 0.05	NA	NA	NA

*Only strains for which LD_{50} was achieved
** Comparison of LD_{50}, 15°C against LD_{50}, 37°C
*3 Means that do not share a letter are significantly different ($p < 0.05$), (pairwise differences between means not significant for means with the same letter)
NA :Not applicable

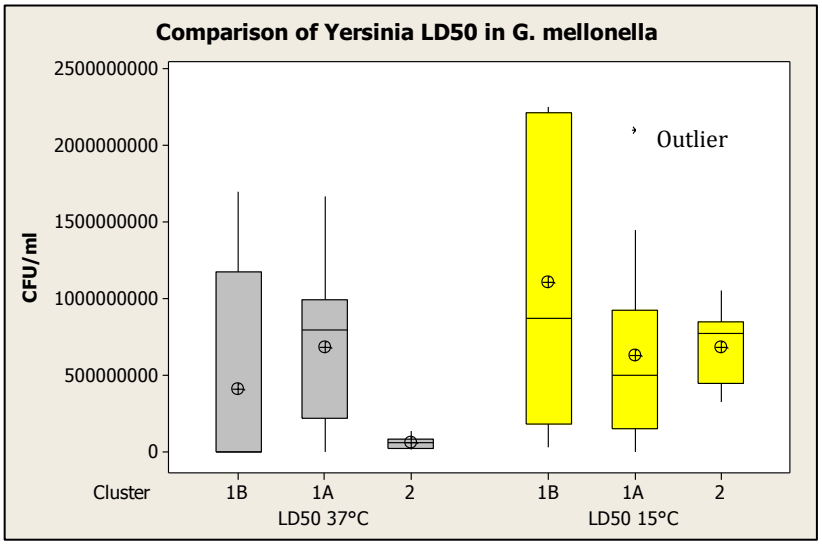


Figure 3.11 Comparison of estimated LD_{50} for Hierarchical Mortality groups observed in *G. mellonella* larvae at 15°C and 37°C analysed by ANOVA in Minitab

⊕- shows mean death rates; horizontal line indicates median death rates with 95% confidence intervals (box) and standard deviations (whiskers).

Yersinia strains from clusters 1A and 1B achieved statistically similar LD_{50} at both 15°C and 37°C (Table 3.25). LD_{50} of *Yersinia* strains from cluster 2 was higher at 15°C than at 37°C of incubation (Table 3.25). The lowest LD_{50} at 37°C was achieved by the non-pathogenic *Y. enterocolitica* strains and *Y. enterocolitica*-like species from cluster 2 in comparison to strains from clusters 1A and 1B, corresponding to the higher death rates shown in Table 3.20 and Figure 3.9. Many *Yersinia* strains from cluster 1B and highly pathogenic *Y. enterocolitica* strain 8081 never achieved 50% mortality rates in insect larvae after incubation at 37°C (Table 3.19).

Although, *Yersinia* strains from all clusters showed statistically similar LD_{50} after incubation of insect larvae at 15°C (Table 3.21), time-dependent difference of insect killing by bacteria was observed (Figure 3.12 and 3.13). A time plot analysis of the same *G. mellonella* larvae mortality rates used to compile the hierarchical cluster analysis revealed distinct time-dependent mortality at 37°C and 15°C (Figure 3.12 and 3.13). Thus, at 37°C all *Yersinia* strains had produced most of their final level of *G. mellonella* larvae mortality by the end of 24 hours (fast killing) and mortality did not change much thereafter during further incubation for 120h (Figure 3.12). However, at 15°C the mortality of infected larvae continuously increased for all strains after the 72h time point (Figure 3.13) (slow killing). Highly pathogenic *Y.*

enterocolitica strain 8081 caused a more rapid form of slow killing at 15°C, with an increase in mortality detectable after 48 hours (Figure 3.13).

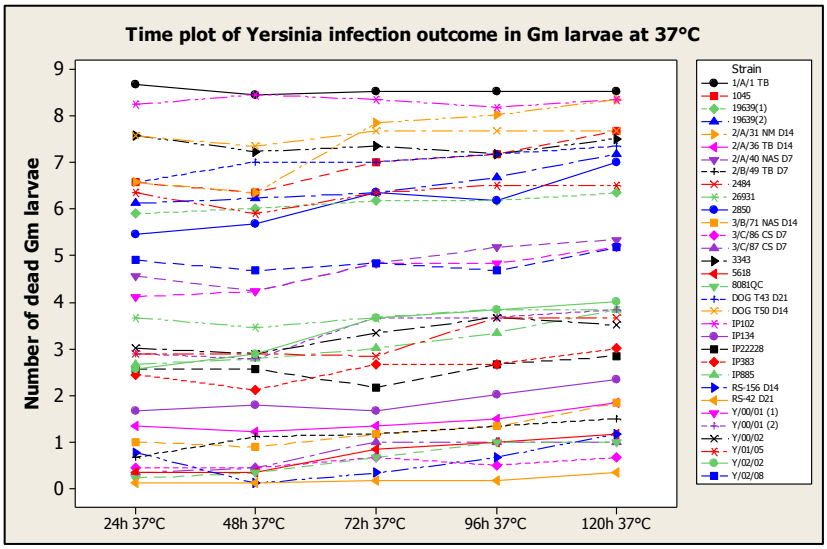


Figure 3.12 Time plot of *Yersinia* infection outcome in *G. mellonella* larvae incubated at 37°C based on mean number of dead larvae observed for all inocula

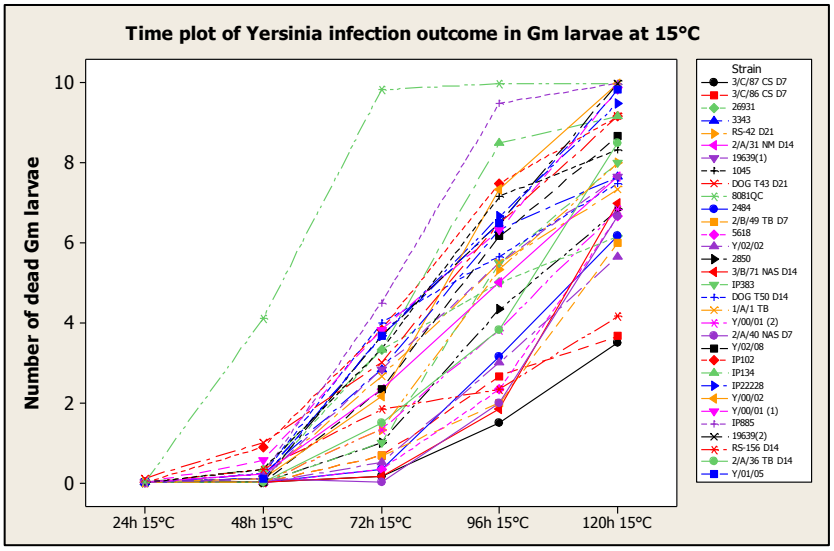
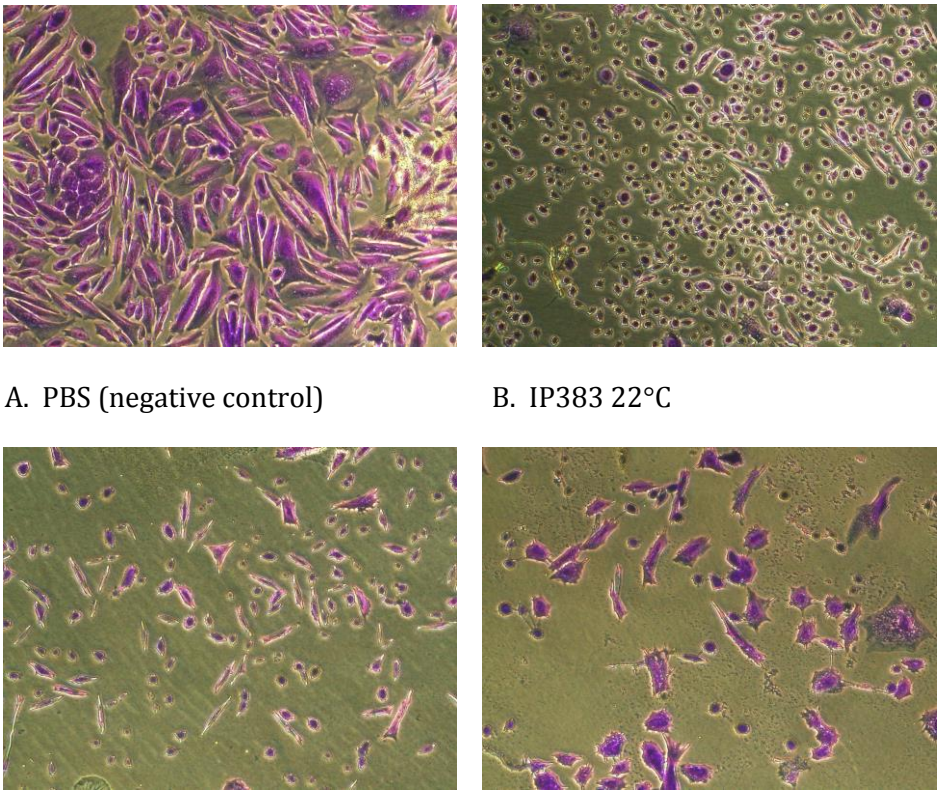


Figure 3.13 Time plot of *Yersinia* infection outcome in *G. mellonella* larvae incubated at 15°C based on mean number of dead larvae observed for all inocula

3.3.7 *Y. enterocolitica* toxicity in CHO cells

While the expression assay demonstrated that CdtB toxins could be expressed at both 15°C and 37°C, eukaryotic cells were more affected by the bacterial cell lysates obtained from *Yersinia* grown at 22°C. However, this could be due to the higher protein concentrations applied. Less protein was detected in the bacterial cell extracts grown at 37°C. Affected CHO cells exhibited characteristic distension (Figure 3.14 (C)) as seen earlier in other studies [469, 615], some CHO cells were dead (round cells considered dead) (Figure3.14 (B), and in some wells there were no confluent growth (Figure 3.14 (C and D)) as seen in negative control wells (PBS) (Figure 3.14 (A)).



A. PBS (negative control) B. IP383 22°C

C. IP102 22°C D. IP134 22°C

Figure 3.14 Effect of *Yersinia* cell lysates in CHO cells

Table 3.26 CDT toxicity of *Y. enterocolitica* cell lysates in CHO cell culture

Strain	Bioserovar	CHO cells Results		Protein conc. µg/µl,		CDT	ITC	pYV	Gm Cluster	Expression		
		37°C	22°C	37°C	22°C					Gene	37°C	15°C
***E.coli* DHα5**	NA	1+	NT	0.36	NT	NT	NT	NT	NT	NT	NT	NT
IP102	1A/O:6,30	1+	4+**	0.24	0.53	A	*tcd*	neg	2	*cdtB2*	+	+
										tcaC	+	+
IP134	4/O:3	2+	3+*	0.19	0.52	D	*tcaA-tcaC2*-like	neg	1A	-	-	-
IP383	2/O:9	3+	4+	0.21	0.4	E	*tca*-like	pos	1A	-	-	-
IP22228	3/O:5	3+	3+	0.16	0.39	D	*tca*-like	pos	1A	*cdtB3*	+	+
										tcaC2	+	+
8081	1B/O:8	2+	3+	0.21	0.36	F	neg	pos	1A	*cdtB3*	-	-
										tcaC2	-	-
2/A/31NMD14	1A/NST	2+	2+	0.22	0.36	A	neg	neg	2	-	-	-
Y/00/01(2)	1A/NEG	2+	3+	0.28	0.42	B	*tcd*	neg	1A	-	-	-
1045	1A/O:8	2+	3+	0.18	0.38	A	neg	neg	2	-	-	-

*Most of the cells are dead
**Some cells distended
NT :Not Tested
NA :Not Applicable

3.4 Discussion

Structural and functional homology between the insect immune response and the vertebrate innate immune response has suggested insects can be a useful model for studying microbial pathogenicity [586-589]. Insect models may be particularly relevant for organisms of the genus *Yersinia* because of the success of *Yersinia pestis* in infecting insects [357, 358, 544, 545], the phylogenetic relationship between *Yersinia* and other genera containing recognised insect pathogens from the family *Enterobacteriaceae* such as *Photorhabdus* [559], and the presence of insecticidal toxins genes in many *Yersinia* species [542, 543, 554-556]. Good correlation of *Y. pseudotuberculosis* virulence has been obtained between mammalian and *G. mellonella* infection models [600].

Previous studies investigating virulence of *Y. enterocolitica* towards *G. mellonella* larvae (Table 3.1, [585]) were confined to mildly pathogenic biovar 2 Serotype O:9 strains and one highly pathogenic biovar 1B strain as well as *Y. enterocolitica*-like strains. Non-pathogenic biovar 1A strains were not studied [585]. Studies were conducted at 15°C only, with a single end point account of death at 5 days [585]. Moreover, it has been shown that classical *Yersinia* mammalian virulence factors such as *yadA, irpI,* and *yopE* do not affect virulence of *Y. enterocolitica* in another non-mammalian model, the nematode *Caenorhabditis elegans*, which requires incubation at less than 25°C [620]. In this study we compared virulence of pathogenic and non-pathogenic *Y. enterocolitica* strains including *Y. enterocolitica*-like microorganisms towards *G. mellonella* larvae at both 15°C and 37°C with daily counts of dead larvae. We found that all *Y. enterocolitica* and *Y. enterocolitica*-like organisms tested showed a consistent temperature-dependant phenotype of fast killing (<24 hours) at 37 °C (Figure 3.12) and a slow (most mortality occurring after 48 hours or longer) killing phenotype at 15°C (Figure 3.13).

Hierarchical clustering of the combined temperature data showed that some biovar 1A strains formed a distinct phenotypic group (Cluster 2, Table 3.19, Figure 3.8) showing enhanced fast killing at 37°C (Table 3.20, Figure 3.9, Figure 3.12). These non-pathogenic *Y. enterocolitica* biovar 1A strains lack recognised conventional

virulence factors seen in pathogenic *Y. enterocolitica* biovars [311, 321, 621] but were more virulent in most cases in *G. mellonella* larvae at 37°C than most of the mildly and highly pathogenic *Y. enterocolitica* strains (Cluster 1, Table 3.19, Figure 3.8). A variety of mammalian pathogens have previously been found to exhibit similarly more rapid *Galleria* killing at higher temperatures, including *Acinetobacter baumanii* [622], the attenuated Live Vaccine Strain (LVS) of *Francisella tularensis* [623], *Bacillus cereus* [624], and the fungal pathogen *Cryptococcus neoformans* [625] (Table 3.27).

Delayed mortality (slow killing) of insect larvae was observed at 15°C and not at 37°C, with continuous increases seen after 72h of incubation (Figure 3.12, Figure 3.13). The slow killing of insect larvae at 15°C eventually accounted for more deaths than the rapid killing at 37°C for both pathogenic and non-pathogenic *Yersinia* strains (Table 3.19, Table 3.23). This phenomenon of enhanced mortality at lower temperatures has not been reported with the other pathogens tested in the *Galleria* model described above, but a similar phenomenon has been observed with *Bacillus weihenstephanensis* [624], and *Fusarium* fungal species [626]. *B. weihenstephanensis* is the proposed name for a group of *B. cereus* strains not so far shown to have mammalian pathogenic properties [624], and *Fusarium* [626] are human opportunist pathogens and plant pathogens prevalent in the environment.

Table 3.27 *G. mellonella* mortality at different temperatures with mammalian pathogens (and some related non-pathogens)

Organism	Inoculum	Final mortality reading (hours)	Final Mortality (%) Incubation 15°C	Final Mortality (%) Incubation 30 °C	Final Mortality (%) Incubation 37 °C	Ref
Acinetobacter baumanii	$3.7x10X10^5$	148	-	<30	>80	[622]
Francisella tularensis **LVS**	$3x10^5$	148	-	50	100	[623]
Listeria innocua **Clip 11262**	$4.2x10^5$	120	-	-	50 (LD_{50})	[611]
Listeria monocytogenes **EGDe**	$3x10^5$	120	-	-	50 (LD_{50})	[611]
Bacillus weihenstephanensis	$5x10^3$ to $1.4x10^4$	120	50-100 (linear regression 80)	-	20-60 (linear regression 40)	[624]
Bacillus cereus	$5x10^3$ to $1.4x10^4$	120	30-100 (linear regression 80)	-	80-100 (linear regression >95)	[624]
Cryptococcus neoformans **strain H99**	$1.5x10^4$	400+	-	80	100	[625]
Fusarium **clinical isolates from humans**	$1x10^5$ (conidia)	100 (30 °C) 190 (37 °C)	-	80-100	0-50	[626]
Fusarium **plant**	$1x10^5$(conidia)	100 (30 °C)	-	>90	25-30	[626]

Organism	Inoculum	Final mortality reading (hours)	Final Mortality (%) Incubation 15°C	Final Mortality (%) Incubation 30 °C	Final Mortality (%) Incubation 37 °C	Ref
pathogen isolates		190 (37 °C)				
***Candida albicans* MEN**	$5x10^5$	72	4°C: 50*	90*	50*	[590]
***Y. pseudotuberculosis* IP32953**	$1x10^6$	24	-	-	47	[600]
***Y. enterocolitica* Cluster 1A mean**	$6.76x10^6$**	120	82	-	37	(this study)
***Y. enterocolitica* Cluster 1B mean**	$6.78x10^6$**	120	59	-	12	(this study)
***Y. enterocolitica* Cluster 2 mean**	$6.78x10^6$**	120	84	-	75	(this study)

* This temperature was applied for 24 hours before inoculation into *Galleria* – all inoculated *Galleria* were incubated at 30 °C. For comparability, studies recording LD50 are given as 50 % mortality

** Mean CFU of three inoculums injected into *G. mellonella* larvae

Differences in *Galleria* mortality at different temperatures and with different *Yersinia* strains could relate to bacterial factors or host (*Galleria*) factors.

Considering bacterial factors, the insecticidal activity of the mildly and highly pathogenic *Y. enterocolitica* strains in Cluster 1 increased at 15°C in comparison to their insecticidal activity towards *G. mellonella* larvae at 37°C (Table 3.24, Figure 3.9). Most non-pathogenic *Yersinia* strains in Cluster 2 were equally virulent towards *G. mellonella* larvae at both 37°C and 15°C (Table 3.24, Figure 3.9). Several different non-pathogenic bacteria (non-pathogenic *E. coli, Bifidobacterium spp*. and *Lactococcus lactis*) are incapable of causing *Galleria* death on inoculation [611]. Lethal effects of bacteria non-pathogenic in mammals in a *Galleria* model have however been reported, but with Gram positive genera, *Listeria* [611] and *B. weihenstephanensis,* mentioned above [624]. *L. innocua* is a species lethal in a *Galleria* model but not in mice or humans (Table 3.4), and *B. weihenstephanensis* [624] (a close relative of *B. cereus*) has a similar phenotype. Interestingly, both of these organisms are psychrotolerant, (psychrotrophic) like *Yersinia* [611, 624].

The majority of mildly pathogenic *Y. enterocolitica* strains in our collection encode a *tca*-like insecticidal toxin complex operon (Table 3.11) similar to that identified in pathogenic *Y. enterocolitica* W22703 strain (2/O:9) [555]. The *tca*-like insecticidal toxin complex operon was previously shown to be repressed at 37°C but increasingly induced as the temperature dropped below 30°C, with maximum expression observed at 10°C in vitro [555]. Therefore, increased levels of virulence towards *G. mellonella* larvae at 15°C demonstrated by mildly pathogenic *Y. enterocolitica* strains could be attributable to expression of the insecticidal toxin complex operon at low temperatures [555]. However, this does not explain the increased insecticidal activity of insecticidal toxin complex non-encoding highly pathogenic *Y. enterocolitica* strain 8081 (1B/O:8) towards insect larvae which was observed in this study at 15°C (Table 3.19, Table 3.23). Moreover, some mildly pathogenic *Y. enterocolitica* strains in our collection encoded a *tcaA-tcaC2*-like insecticidal toxin complex operon that does not contain a *tcaB2* gene detectable by the PCR primers used in this study (Table 3.12). The *tcaB* gene of *Yersinia* species is prone to frameshift mutations [542, 585, 604] that may affect insecticidal toxin complex activity in *Yersinia* at lower temperatures [359, 602, 604]. However, there was no apparent statistical difference in insecticidal activity between *tca*-like and *tcaA-tcaC2*-like-encoding mildly pathogenic *Y. enterocolitica* strains at 15°C (Table 3.21).

Only a small proportion of the biovar 1A *Y. enterocolitica* strains in this study (Table 3.10) encoded a *tcd*-like insecticidal toxin complex operon similar to that previously identified in biovar 1A *Y. enterocolitica* strain T83 [556]. The *tcd*-like insecticidal toxin complex operon is known to be expressed at 30°C and 37°C [556]. At 37°C *tcd*-like insecticidal toxin complex encoding non-pathogenic *Y. enterocolitica* strains were the most virulent towards insect larvae (Table 3.21). However, all of these strains also encoded CDT type A locus (Table 3.19). Although *tcd*-like insecticidal toxin complex encoding *Y. enterocolitica* strains were equally virulent at both 15°C and 37°C (Table 3.21), there was no significant difference between the insecticidal activity at 15°C of biovar 1A *Y. enterocolitica* encoding *tcd*-like insecticidal toxin complex operons and the remaining *Yersinia* strains not encoding a *tcd*-like insecticidal toxin complex operon in this study (Table 3.21).

It has been suggested that *Yersinia* pathogenicity towards *G. mellonella* could be mediated by factors other than the insecticidal toxin complex [559, 585]. Putative Cytolethal Distending Toxins (CDTs) have been identified in the genome sequence of pathogenic *Y. enterocolitica* strain Y11 [466] and in the genome sequences of non-pathogenic *Y. enterocolitica* (N. Thomson, personal communication). CDTs are able to cause cell cycle arrest and apoptosis in various eukaryotic cells [442-445], including immune cells, that may subsequently lead to inhibition of an adequate immune response towards the pathogen [605, 606]. CDTs are AB_2 exotoxins where CdtB is an active subunit and CdtA and CdtC facilitate its entry into eukaryotic cells [445, 468, 627]. It has been proposed that efficient binding of CdtA and CdtC to the target cell influences CDT activity and depends on the amino acid sequence of these CDT-binding subunits as well as presence of specific cell surface biomolecules such as cholesterol, glycoproteins and glycolipids in the target cells [628]. However, no CdtC subunit was identified in the present collection of *Yersinia* strains, suggesting that a different mechanism of CdtB subunit delivery into insect cells must occur. For example, *Salmonella enterica* Serovar *typhi* that does not encode CdtA and CdtC subunits delivers CdtB active subunit via the bacterial internalization pathway [449].

Various cytolethal distending toxin genes were detected by PCR in pathogenic and non-pathogenic *Y. enterocolitica* strains (Table 3.16, Figure 3.4). Expression of *cdtB2* in non-pathogenic and *cdtB3* in mildly pathogenic *Y. enterocolitica* strains was detected at both 15°C and 37°C (Table 3.18, Figure 3.6, Figure 3.7). However, different levels of insecticidal activity were observed for pathogenic and non-pathogenic *Yersinia* strains

at both 15°C and 37°C (Table 3.19). Mildly pathogenic *Y. enterocolitica* strains that encode CDT type E were more virulent towards *G. mellonella* larvae than strains encoding CDT type D at 37°C (Table 3.20). Interestingly, mildly pathogenic *Y. enterocolitica* strains that encode CDT type E also encode *tcaA*-*tcaC2*-like insecticidal toxin complex genes, and CDT type D-encoding strains encode *tca*-like insecticidal toxin complex operons. The *tcaA*-*tcaC2*-like encoding *Yersinia* strains killed more *G. mellonella* larvae at 37°C than *tca*-like encoding strains (Table 3.21). Thus, it is difficult to differentiate between insecticidal toxin complex and CDT genotype contributions to the virulence of mildly pathogenic *Y. enterocolitica* strains towards insect larvae using this data. At 15°C CDT type E and CDT type D-encoding mildly pathogenic strains were not significantly different in insecticidal activity (Table 3.20).

The composition of the CDT loci in non-pathogenic *Y. enterocolitica* strains is also variable. Strains containing CDT type A loci were generally more virulent towards insect larvae than strains encoding CDT type B and type C loci at both 15°C and 37°C (Table 3.20). In general, irrespective of the presence of a recognised insecticidal toxin complex locus, non-pathogenic *Y. enterocolitica* strains that encoded CDT type A (nearly all grouped into hierarchical cluster 2) were more virulent towards insect larvae at 37°C than all other *Y. enterocolitica* (Table 3.19, Table 3.23, Table 3.24, Figure 3.8).

Considering host factors affecting *Yersinia* pathogenesis in insects, the insect immune response operating in *Galleria* has structural and functional homology to the vertebrate immune response [586-589]. Antimicrobial peptides (AMP) are an evolutionary conserved component of the innate immune response and found in many living organisms [629]. AMPs are released at the early stages of the innate immune response and are key defensive molecules in invertebrates [630]. In *G. mellonella* several antimicrobial peptides have been identified with antibacterial and antifungal activity [631-634]. Microbial pathogens may display constitutive and inducible resistance to antimicrobial peptides. *In vitro* detection of antimicrobial activity of purified *Galleria mellonella* (Gm) AMPs demonstrated that gram negative bacteria were mostly resistant to Gm AMPs, only *E. coli* D31 was sensitive to purified Gm cecropin D-like peptide [634]. However, synergetic relationships among different AMPs [635, 636] and between AMPs and lysozyme against gram negative bacteria [637, 638] were described and therefore microbial resistance to purified AMPs observed *in vitro* may not be exhibited *in vivo*.

The ProPhenolOxidase (ProPO) system (also known as the serine proteinase cascade, or melanisation cascade) present in invertebrates such as *Galleria* is a proteolytic process analogous to the alternative complement system of the vertebrate immune response [589, 616]. The process of melanisation is activated by binding soluble pattern recognition receptors to the foreign target surfaces thus initiating the serine protease cascade [589]. The activated serine protease cascade leads to the cleavage of ProPO to PO (PhenolOxidase) that then catalyses production of quinines and subsequent formation of melanin [589]. Melanin is then transported and deposited around the wound or encapsulated foreign material [589, 616, 639]. ProPO is located in haemocytes as a zymogen and released from haemocytes by rupture and death [589, 616] or other mechanisms [639]. All dead insect larvae infected with *Yersinia* strains in this study exhibited melanisation. As was mentioned earlier ProPO cascade resembles the alternative complement cascade in vertebrates [589, 616]. Thus, the insect ProPO has a sequence similar to thiol-ester region of the vertebrate complement proteins C3 and C4 [589, 640]. In the mammalian host, there is evidence that *Yersinia* chromosome-encoded Ail (expressed at 37°C in stationary-phase growth [641]) and pYV-encoded YadA proteins bind the alternative complement cascade regulator Factor H (FH) [385, 386, 395] and the regulator of the classical and lectin complement activation pathways C4b-binding protein (C4BP) [383]. However, low mortality rates seen in this study of *G. mellonella* larvae caused by pathogenic *Y. enterocolitica* strains at 37°C (Table 3.19, Table 3.23, Figure 3.9) suggest that Ail and YadA known to be present in these strains do not facilitate ProPO cascade resistance in this invertebrate host.

The next line of bacterial defence mechanisms against the host innate immune response is the ability to survive or avoid phagocytosis. Although signal transduction of insect phagocytosis is not clearly understood it has been demonstrated that phagocytosis by insect haemocytes stimulates a response similar to the vertebrate respiratory burst pathway involving NADPH oxidase activity [642, 643]. The insect haemolymph contains various cell types, but the most abundant haemocytes of insect haemolymph are plasmocytes and granulocytes that play roles in the insect cellular immune response (phagocytosis) [589]. dSR-C1, TLR-like and integrin receptors similar to receptors on mammalian cells are present on the surface of insect phagocytes (plasmocytes and granulocytes) [644]. Pathogenic *Y. enterocolitica* interacts with mammalian phagocytic cells via binding to β1 integrins by the chromosome-encoded Inv invasion protein [301, 304, 305] and pYV plasmid-encoded YadA protein [645] and subsequent activation of the Ysc/TTSS system [388] that facilitates translocation of

effector YOPs (YopE, YopH, YopO/YpkA, YopM, YopP/J and YopT) into the cytoplasm of eukaryotic cells [388, 408] with consequent suppression of phagocytosis [416, 417]. In vitro *inv* gene expression was observed at 26°C (neutral pH) and 37°C (pH5.5) [646]. Both YadA and YOPs are expressed at 37°C [388]. Therefore, one might anticipate that *Y. enterocolitica* strains which are pathogenic for mammals would show a similar increased capacity to overcome the cellular immune response (phagocytosis) of *G. mellonella* larvae at 37°C. However, in relation to the activity of *Y. enterocolitica* in *Galleria* at 37°C, it is known that pre-incubation of *G. mellonella* larvae at 37°C (and 4°C) results in a stress response including an increase in haemocyte density and antimicrobial peptide synthesis which reduces their mortality in subsequent *Candida albicans* infection compared to when pre-incubated at 30°C (Table 3.4) [590].

Future work to look at the effects of injecting killed *Y. enterocolitica* strains or protein extracts from bacteria would help to show whether a pre-formed toxin was a likely candidate to be responsible for either the rapid (37°C) killing or slow (15°C) killing *Yersinia* insecticidal activity demonstrated in this chapter. *Galleria* toxicity and cytoxicity of culture supernatants grown at different temperatures would also be relevant. In vitro expression analysis (Figure 3.6 and 3.7) suggests that the rapid 37°C killing is unlikely to be due to insecticidal toxin complex activity, because this operon shows less induction at this temperature compared to 15°C (in line with published data [555]). The expression of *cdtB2* and *cdtB3* (i.e. representative component genes for all the CDT loci identified in this chapter) (Figure 3.6 and 3.7) was equally evident at both temperatures *in vitro*, suggesting that CDT toxins are candidates for causing both forms of killing. Preliminary experiments with CHO (Chinese Hamster Ovary) cells indicated that *Y. enterocolitica* cell lysates have toxic effect at both 37°C and 22°C possibly due to the activity of the CDT toxins. However, further experiments are required. The presence of various toxin genes in *Y. enterocolitica* strains which are not recognised mammalian pathogens (e.g. Hierarchical cluster 2, Biovar 1A strains), and their *Galleria*-killing capacity, implies that pathogenicity for insects may be useful for non-mammalian pathogen *Yersinia* strains in environments like soil, and there may be some unrecognized invertebrate host species adapted to temperate environments which provide optimal growth temperatures for these psychrotrophic organisms.

Indeed, it has been suggested that insects may act as a reservoir for emerging human pathogens [557] and ancestral *Yersinia* species were associated with insects or insect pathogens [538, 542]. The *Galleria* pathogenicity of *Y. enterocolitica* strains which are

not pathogenic for mammals strengthens this connection. Understanding of the relationships between bacteria and non-human hosts could give clues to the evolution of human pathogens.

Chapter 4 Comparative genomics

4.1 Introduction

DNA analysis is widely used for investigating bacterial taxonomy, bacterial typing and establishing evolutionary relationships between bacteria [485, 486, 531, 533, 536, 538, 543, 548, 647-650]. Genetic polymorphisms of *Yersinia* strains can be determined by applying DNA analysis techniques that can be grouped into three types (Table 4.1).

Table 4.1 Molecular typing techniques applied for resolving relationships between *Yersinia* strains*

Random whole genome analysis techniques**	Specific gene variation analysis techniques**	Comparative genomics
PFGE	MLEE	Genome sequencing
AFLP	MLST	Microarray genome comparison
RAPD (Random Amplification of Polymorphic DNA)	Ribotyping	
ERIC-PCR	PCR-ribotyping	
REP-PCR	16SrDNA	
MLVA		

*See Chapter 1 for more details
** After Gürtler [651]

Random whole genome analysis and specific gene variation analysis techniques resolve evolutionary and taxonomic relatedness of bacteria based on limited knowledge about their DNA sequences. Comparative genomics facilitates recognition of predicted coding sequences (CDSs) that define host-pathogen interactions and hence provides insights into molecular evolution of bacterial virulence [530-533, 542, 543, 548, 652-654]. Microarray technology allows comparative genomics of test strains against a reference without the need to perform whole genome sequencing on all the test strains.

Microarray technology can be used for genomic typing (genomotyping) – comparative genomic hybridization [655] and for gene expression studies [656]. Comparative microarray genomotyping is based on competitive DNA-DNA hybridization between a mixture of DNA samples from an unsequenced test strain and a sequenced control strain (each labelled with a different fluorescent dye) to the reporter DNA probes designed from the control strain genome or set of genomes bound to a microarray surface [523]. Two microarray technologies exist – the Polymerase Chain Reaction product based microarrays (PCR-based) and oligonucleotide-based DNA microarrays [523, 657]. Although PCR-based microarrays are easy and relatively cheap to produce,

detection of single nucleotide polymorphism (SNP) and mutant alleles is not achievable [657]. On the other hand, oligonucleotide-based microarrays are more expensive but allow detection of single nucleotide polymorphisms (SNP) and mutant alleles [657]. Additionally, oligonucleotide-based microarrays reduce cross-hybridization as they employ more specifically designed oligonucleotides, and these have now largely replaced PCR product microarrays [523-527]. In situ synthesized oligonucleotide arrays (ISO) which can now be made using ink jet printing (Inkjet in situ synthesized (IJISS) Agilent Technologies, USA) are more cost effective as they allow production of the microarrays as required, moreover arrays can be re-used [658]. The IJISS technology also allows greater flexibility in the array design [658].

4.1.1 Analysis of comparative genomic hybridization on microarrays

Comparative genomic hybridization microarray analysis is a multistep procedure that involves initial image acquisition and analysis of the hybridised DNA (spot recognition, normalization); prediction of presence/absence/divergence for each gene in the array, and, finally, phylogenetic analysis [659].

Scanning of the microarray slides – image acquisition – is the first step of the microarray data analysis. The main priority of image acquisition is correct balance of the intensities between two channels corresponding to each fluorescent label (Cy3 and Cy5). Next step is an image analysis that involves spot recognition and normalization. Subtraction of the background intensity from the foreground intensity for each channel gives spot intensity. Spots with low intensity are excluded from further analysis. Intensity ratios between channels Cy3 and Cy5 corresponding to differential hybridisation between the two bacterial strains are then transformed to log-ratios that are used for normalization.

Normalization of microarray data is required to remove systematic errors associated with the microarray procedure which do not reflect underlying biological variation in samples [660]. Numerous microarray data normalization protocols are available [660-666]. They can be grouped into global or intensity-dependent normalization methods. Global normalization methods do not correct for intensity or scale differences and often perform poorly in comparison to intensity-dependent normalization methods [660, 666]. There are two types of intensity-dependent normalization methods – linear and nonlinear, that perform similarly [666].

Many methods (cut-off algorithms) for the prediction of presence/absence/divergence

for each gene represented in the array base their results on log-ratio signals. Log-ratio based cut-off algorithms as applied by the GENCOM [667], GACK [668] and Porwollik [669] methods assume symmetry, normality and linearity of the microarray data [670]. When microarray data do not meet these criteria these algorithms are less able to identify the presence/absence/divergence of the genes correctly [670-672]. Snipen *et al* [673] have proposed a new cut-off algorithm for the prediction of the present/absent/divergent genes based on the supervised learning approach where sequence identities are predicted from bias-corrected array signals in each channel separately. This approach is superior to the log-ratio based cut-off algorithms [673]. The Output of analysis is a binary file scoring gene absence or presence that can be used for estimation of the phylogenetic relationships of the taxa (specimens).

4.1.2 Phylogenetic methods

The most commonly used methods in comparative phylogeny which are applicable to binary character data such as that produced by microarray typing are maximum parsimony, maximum likelihood and Bayesian inference. Maximum parsimony is a method in which different trees are evaluated to find the tree topology which uses the minimum number of changes to explain the observed data [674], it uses non-parametric statistics [675, 676]. Maximum likelihood methods involve scoring different trees and selecting a phylogeny which maximizes the probability of the data D given the parameters of the tree values θ, $\Pr(D|\theta)$ [677] and relies on parametric statistics [678]. Non-parametric or "distribution free" statistical tests do not make numerous or stringent assumptions about the population, on the other hand parametric statistics assume that the data belong to some type of probability distribution [679]. While maximum parsimony is a fast method for reconstructing phylogenetic relationships between closely related taxa, maximum likelihood is useful for the reconstruction of the evolutionary relationships for rapidly evolving taxa or taxa that have been separated for a long time [680]. The Bayesian approach to phylogeny is based on Bayes's theorem in which prior probability of a phylogeny is combined with the likelihood to produce a posterior probability distribution on trees [681]. Bayesian inference attempts to maximise the probability of the tree parameter values given the probability of the observed data, $\Pr(\theta|D)$, known as the posterior probability [677], and uses parametric statistics [680]. Markov Chain Monte Carlo (MCMC) sampling of the trees in proportion to their likelihood during Bayesian analysis is used to produce the most credible tree [682, 683].

Comparative phylogenomic analysis of microarray data obtained from *Y. enterocolitica* strains isolated in Great Britain from humans with diarrhoea and domestic animals [50] using a GACK cut-off algorithm for calling gene presence/absence and Bayesian inference resulted in the distribution of strains into three clades: highly pathogenic, low pathogenic and non-pathogenic clades [531]. Overall the three clades shared only 20.8% of the reference genome set revealing a high genetic heterogeneity of this species [531].

Because of the extensive human, animal and food exchanges between the UK and Ireland (16% of all Irish exports go to the UK and a third of all Irish imports come from the UK) [684], it would be expected that the population structure of *Y. enterocolitica* strains should be similar in the two countries. However, there is currently no data on the gene complement of *Y. enterocolitica* strains isolated in Ireland. The objective of this study is to gain further insight into genetic heterogeneity and relationships among clinical and non-clinical *Y. enterocolitica* strains of varying pathogenic potential isolated in Ireland in comparison with those from Great Britain.

4.2 Materials and Methods

4.2.1 Promega Wizard Genomic DNA purification protocol

A loopful of overnight bacterial culture grown on tryptic soy agar (TSA) plates at 30°C was gently resuspended with 600µl of Nuclei Lysis solution in 1.5ml tubes. After bacterial culture was fully homogenized in nuclei lysis solution they were incubated at 80°C for 5min, then cooled to room temperature. RNA was removed by adding 3µl of RNAse solution into the cell lysate, inverting tubes 2-5 times to mix and following incubation at 37°C for 60min. Protein precipitation was achieved by adding 200µl of protein precipitation solution into the cooled down to room temperature RNAse-treated cell lysate and following incubation on ice for 5min. Protein was removed from DNA samples by series of washes in isopropanol and in 70% ethanol. DNA samples were rehydrated by adding 100µl of rehydration solution and overnight incubation at 4°C. DNA samples were stored at -80°C. DNA quality was assessed by Nanodrop NT1000.

4.2.2 Microarray DNA-DNA hybridization procedure

The *Yersinia* specific microarray YP v3.1.0 contains 9048 predicted CDSs from *Y. enterocolitica subsp. enterocolitica* 8081 and non-8081 *Yersinia* species (*Y.*

enterocolitica subsp. palearctica strains W22703, 647176, R71; *Y. enterocolitica subsp. enterocolitica* strain WA314; *Y. pestis* strains CO92, 1695, KIM; *Y. pseudotuberculosis* strains 3277, 48790, 931474, AH, CN4, Kuratani, M444, M452, M85, Pa3606, R80, YPT1, Yoko), and plasmid encoded genes (pYV, CD1, MT1, PCP1) are also included (http://bugs.sgul.ac.uk/A-BUGS-27; ArrayExpress accession: A-BUGS-27). This microarray was designed and constructed by the Bacterial Microarray Group at St. George's Hospital Medical School, University of London as described by Hinds et. al [685, 686]. Briefly, to avoid cross hybridization, only unique PCR products for each CDS were selected, for optimum hybridization all selected PCR products were in line with a standard set of requirements (length, GC content and Tm) [685]. Microarrays were constructed by robotic spotting of the selected PCR products in duplicate onto UltraGap glass slides (Corning) with a MicroGrid II (BioRobotics) [686].

DNA from test strains (test DNA) and *Y. enterocolitica* 8081 (control DNA), respectively labelled with the fluorescent dyes Cy5-dCTP and Cy3-dCTP, were competitively hybridized onto YP v3.1.0 microarray slides. Labelling and DNA-DNA hybridization procedures were performed according to the protocol devised by Bacterial Microarray Group at St. George's, University of London.

Test and control DNA samples were separately aliquoted into separate 0.2ml sterile eppendorf tubes and then prepared as follows: 2µg of each DNA sample was mixed with 1µl of random primers (3µg/µl, Invitrogen) and then volume of the mixture was increased to 41.5µl with the sterile molecular grade water (Sigma); this mixture was then heated at 95°C for 5min. In the next step test and control DNA samples were labelled with Cy5 and Cy3 dCTPs (GE Healthcare) respectively as follows: 8.5 µl of labelling mixture containing 5µl of 10xREact2 buffer (Invitrogen), 1µl dNTPs (5mM dA/G/TTP, 2mM dCTP, Invitrogen), 1.5µl of respective fluorescent dye Cy3 or Cy5 dCTP (GE Healthcare) and 1µ of Klenow DNA Polymerase (500U; Invitrogen) was mixed with 41.5µl of respective DNA sample, this mixture then was incubated at 37°C in dark for 90 min.

During DNA labelling procedure 50ml of prehybridization mixture (8.75 ml of 20xSSC (Sigma), 250µl of 20% SDS (Sigma), 5 ml of Bovine Serum Albumin (100mg/ml, Sigma) and 36ml of molecular grade water (Sigma) was heated to 65°C in a Coplin jar. Microarray slides were pre-hybridised by their incubation at 65°C for 20min in pre-heated prehybridization solution. After pre-hybridisation slides were washed in 400ml

water and then 400ml isopropanol (Sigma) for 1 min in each wash and then centrifuged at 1,500 rpm for 5 min.

After labelling reactions Cy3 and Cy5 labelled DNA samples were combined in 1.5ml eppendorf tubes and then purified Qiagen MinElute purification kit.

Purified labelled DNA samples (39µl) were mixed with the hybridisation solution containing 12 µl of 20xSSC (Sigma) and 9 µl of 2% SDS (Sigma); this mixture then was heated at 95°C for 2 min. Samples were kept at 70°C in dark until needed.

Hybridisation solution containing labelled DNA samples (total volume 71 µl) was applied on the prehybridized microarray slide covered with two 22x22 mm Lifter Slips (VWR International). Hybridisation was performed in a Hybridisation Chamber II (Corning), submerged in a water bath in the oven at 65°C in the dark overnight (16-20h).

After overnight hybridisation slides were washed for 2min in each of three washes. Wash A (20xSSC – 20ml, 20% SDS – 1ml, distilled H_2O – 379ml) was preheated to 65°C overnight; two washes B (20xSSC – 1.2ml, distilled H_2O – 398.8ml) were kept at room temperature. After washing slides were spanned at 1,500 rpm for 5min.

4.2.3 Microarray data analysis

Microarray DNA-DNA hybridizations were scanned with a dual-laser scanner Affymetrix 428 (MWG Biotech) using various photomultiplier tube (PMT) gain settings. Spot recognition and estimation of the spot fluorescent signal intensity was performed by analysing obtained microarray images with Genedirector v2.7 software package that included Imagene v6.0 software (Biodiscovery) according to BugsBase Imagene settings for array A-BUGS-27 (*Y. enterocolitica* YPv3.1.0).

Between-array normalization and assignment of present and absent genes were performed by Dr. A. Witney (St George's, University of London) using the same methodology for the single colour microarray data obtained during this study (BµG@Sbase (accession number E-BUGS-148 (http://bugs.sgul.ac.uk/E-BUGS-148) and also ArrayExpress (accession number E-BUGS-148),and the raw data from the previous England and Wales study of Howard et al (BµG@Sbase accession number E-BUGS-36 (http://bugs.sgul.ac.uk/E-BUGS-36) and also ArrayExpress (accession number E-BUGS-36), [531]). Single colour data was loaded into R, control spots were removed and then a quantile between-array normalisation was performed using LIMMA [687] with the recommended weak flagging of array spots [673]. Prediction of present and absent

genes was performed as described by Snipen on the single colour normalized data using a published Bias-Corrected S-signal Prediction (BCSP) method in the R statistical environment [673]. Briefly, ranking of present/absent genes was performed according to predicted sequence similarity. The threshold of prediction was 0.7 (70% identity), values above 0.7 were rounded to 1 and correspond to present, values below 0.7 were rounded to 0 and correspond to absent [673]. A binary matrix of presence/absence calls was thereby generated for each gene for each strain for normalized data from microarray experiments E-BUGS-148 and E-BUGS-36.

4.2.4 Comparative phylogenomic analysis

Using the binary matrix data, evolutionary relationships between taxa were estimated by three separate methods. Maximum likelihood using RAxML v7.3.0 [688] and Bayesian inference using MrBayes v3.2.1 [689]. Maximum likelihood phylogenomic reconstruction was carried out by Dr. A. Witney (St George's, University of London) with RAxML v7.3.0 using a GAMMA model of rate heterogeneity [688]. Branch support values were generated by a rapid bootstrap analysis of 1000 replicates. The extended majority-rule consensus tree criterion was used to ensure convergence by 1000 replicates [688]. For Bayesian phylogenetic analysis the binary matrix of presence/absence calls was transformed into NEXUS file format with the Fused Matrix Exporter in Mesquite v2.75 [690]. Bayesian inference was carried out with four-chain MCMC sampling for $1x10^7$ generations with the burn in fraction 0.5 and sample frequency of 1000.

Phylogenetic trees were visualised and formatted with FigTree v1.3.1 software (http://tree.bio.ed.ac.uk/).

4.2.5 Estimation of the genomic content

The binary matrix was used for the estimation of core and specific set of genes encoded by *Y. enterocolitica* strains using the website GeneVenn [691] based on the following list of genes (Table 4.2) as follows. Binary file data was grouped by pathogenicity classification of the strains, and then according to their origin (Great Britain or Ireland) yielding nine data sets: GB only non-pathogenic, GB only highly pathogenic, GB only mildly pathogenic, Irish only non-pathogenic, Irish only highly pathogenic (8081 strain only), Irish only mildly pathogenic, GB and Irish non-pathogenic, GB and Irish highly pathogenic, GB and Irish mildly pathogenic. Any genes absent in any single strain or

multiple strains were manually removed from each data set. Final gene lists for each set were compared in GeneVenn.

Table 4.2 Set of genes used for phylogenomic comparisons

***Yersinia* strain**	**Number of genes**
Y. enterocolitica subsp. enterocolitica strain 8081	4208 (chromosomal) 83 (pYV-encoded)
Y. enterocolitica subsp. enterocolitica strain WA314	4
Y. enterocolitica subsp. palearctica strain 56	1
Y. enterocolitica subsp. palearctica strain 383	2
Y. enterocolitica subsp. palearctica strain 22703	16
Y. enterocolitica subsp. palearctica strain 647176	20
Y. enterocolitica subsp. palearctica strain R71	11
Y. enterocolitica subsp. palearctica strain A12790	10
Y. pseudotuberculosis strain 931474	2
Y. pseudotuberculosis strain AH	5
Y. pseudotuberculosis strain CN4	1
Y. pseudotuberculosis strain M444	4
Y. pseudotuberculosis strain M452	6
Y. pseudotuberculosis strain M85	6
Y. pseudotuberculosis strain Pa3606	2
Y. pseudotuberculosis strain R80	1
Y. pseudotuberculosis strain YPT1	4
Y. pestis strain KIM	32
Total number of genes	**4418**

4.2.6 Samples

Y. enterocolitica strains from patients with yersiniosis were obtained from pre-existing culture collections in Queen's University Belfast (gift of Dr. J. Moore) and University College Galway – isolated during 1997-2003 (gift of Prof. M. Cormican), identification of strains was confirmed by API 20E *BioMérieux* incubated at 30°C overnight [130, 131]. *Y. enterocolitica* strains from retail meat samples and animals were obtained during the contemporaneous abbatoir study of pig carcasses in Ireland carried out in Cork by University College Dublin researchers (Dr. B. Murphy and Prof. S. Fanning) (Table 4.2). All strains were biotyped according to the simplified *Wauter's Biotyping Scheme* (Table 3.3, see Chapter 3) and serotyped with *MAST Group Yersinia enterocolitica Antisera* that

included one poly antisera – O:1,O:2 and four mono antisera – O:3; O:5; O:8; O:9. All isolates were checked for possible autoagglutination in a drop of sterile 0.85% saline solution.

Table 4.3 List of *Y. enterocolitica* strains hybridised (from Ireland unless stated)

Strain Number	BioSerotype	Source	Locality/year
1045	1A/O:8	Human	Galway*
1170	1A/O:8	Human	Galway
1572	1A/O:8	Human	Galway
2850	1A/NST	Human	Galway
19639_1	1A/NST	Human	Galway
19639_2	1A/O:8	Human	Galway
Y0001_2	1A/NST	Human/faeces	Belfast/2000
Y0105	1A/NST	Human/faeces	Belfast/2001
Y0202	1A/NST	Human/faeces	Belfast/2002
2A31NMD14	1A/NST	Pig	Cork/2007
3C86CSD7	1A/O:9	Pig	Cork/2007
3C87CSD7	1A/NST	Pig	Cork/2007
4C115NMD21	1A/NST	Pig	Cork/2008
4C118NMD21	1A/NST	Pig	Cork/2008
6A182TBD21	1A/NST	Pig	Cork/2008
DOGT43D21	1A/NST	Dog	Cork/2008
DOGT50D14	1A/NST	Dog	Cork/2008
RS52D21	1A/NST	Retail	Cork/2007
RS55D21	1A/NST	Retail	Cork/2007
5618	2/O:9	Human	Galway
Y0101	2/O:9	Human/faeces	Belfast/2001
Y0113	2/O:9	Human/faeces	Belfast/2001
1127	3/NST	Human	Galway
1412	3/NST	Human	Galway
2484	3/O:9	Human	Galway
1C23NAS_D7	3/O:5	Pig	Cork/2007

Strain Number	BioSerotype	Source	Locality/year
1C30TB_D7	3/O:9	Pig	Cork/2007
2A36TB_D7	3/O:9	Pig	Cork/2007
2A36TB_D14	3/O:9	Pig	Cork/2007
2B49TB_D7	3/O:9	Pig	Tipperary/2007
2C53TA	3/O:9	Pig	Cork/2007
3C87TB_D7	3/O:9	Pig	Cork/2007
4A92TB_D14	3/O:9	Pig	Cork/2008
5C154NM_D7	3/O:9	Pig	Cork/2008
6A187TB_D21	3/O:9	Pig	Cork/2008
2C_COMMONTABLE_D21	3/O:9	Environment	Cork/2007
3C_COMMONTABLE_D14	3/O:9	Environment	Cork/2007
Y0001_1	4/O:3	Human/faeces	Belfast/2000
Y0002	4/O:3	Human/faeces	Belfast/2000
Y0004	4/O:3	Human/faeces	Belfast/2000
Y0208	4/O:3	Human/faeces	Belfast/2002
2A34TB_D7	4/O:3	Pig	Cork/2007
2A40NAS_D7	4/O:3	Pig	Cork/2007
3A61TB_D21	4/O:3	Pig	Cork/2008
3A70TB_D21	4/O:3	Pig	Cork/2008
3B71NAS_D21	4/O:9	Pig	Tipperary/2007
4C113TB_D7	4/O:3	Pig	Cork/2008
3343	Y. rohdei	Human	Galway
8081	1B/8081	Human	Reference strain (USA)
Total	**49**		

*Exact year of the isolation not available for all strains from Galway

The comparator strains from Great Britain in the historical dataset were isolated from faeces of domestic animals (cattle, sheep, pigs) at slaughter and from patients with yersiniosis during 1999-2000 [50, 531]. A group of highly pathogenic *Y. enterocolitica* biovar 1B strains from the USA was also present in this historical dataset. As in the previous microarray study of strains from Great Britain [531] the reference biovar 1B

Y. enterocolitica 8081 strain which has been sequenced [532] was used as a control in the current experiment (Table 4.2).

4.3 Results

4.3.1 Comparative phylogenomic analysis

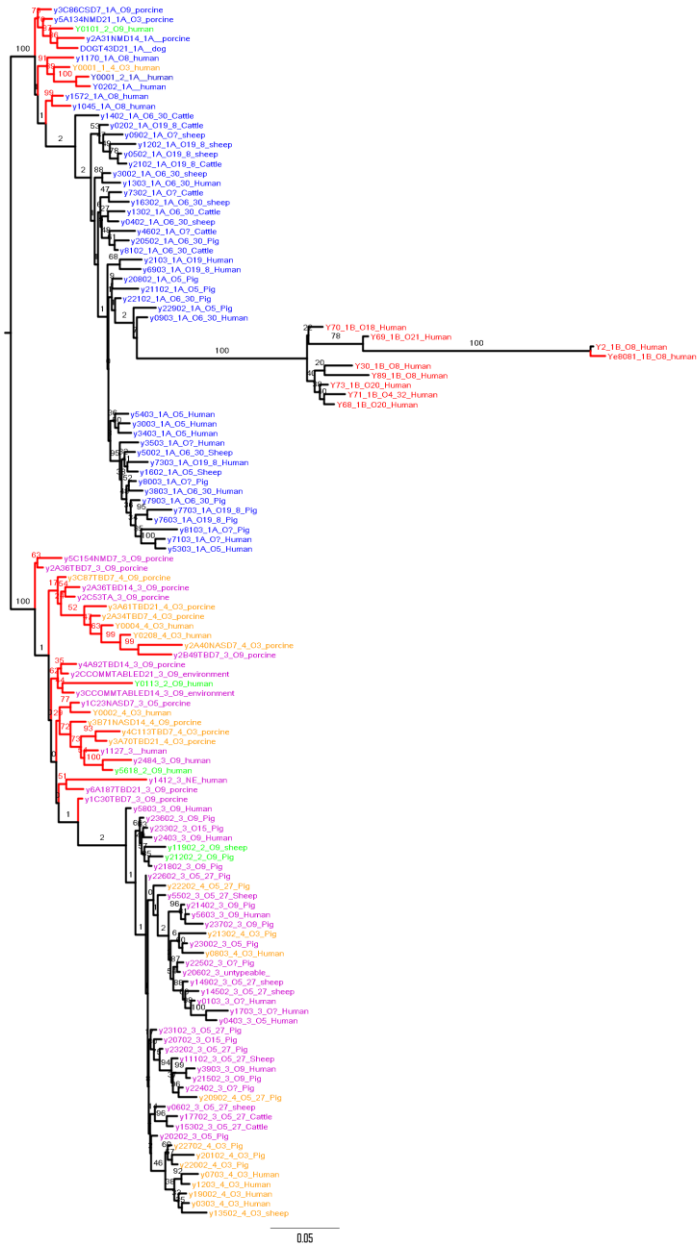


Figure 4.1 RAxML phylogenomic reconstruction

Blue taxa :non-pathogenic biovar 1A strains
Red taxa :highly pathogenic biovar 1B strains
Green taxa :biotype 2 strains
Purple taxa :biotype 3 strains
Orange :biotype 4 strains.
Red branches represent Irish strains

Table 4.4 Rogue strains

Strain	Bioserovar	Source	Country
Y3343	*Y. rohdei*	Human	Ireland
Y2850	1A/NST	Human	Ireland
Y19639_1	1A/NST	Human	Ireland
Y19639_2	1A/O:8	Human	Ireland
Y0105	1A/NST	Human	Ireland
DOGT50D14	1A/NST	Dog	Ireland
RS52D21	1A/NST	Retail pork	Ireland
RS55D21	1A/NST	Retail pork	Ireland
Y3C87CSD7	1A/NST	Pig	Ireland
Y4C115NMD21	1A/NST	Pig	Ireland
Y4C118TBD21	1A/NST	Pig	Ireland
Y6A182TBD21	1A/NST	Pig	Ireland
Y1603	3/O:5	Human	Great Britain
Y4103	1A/O:6,30	Human	Great Britain
Y7403	3/O:9	Human	Great Britain
Y20302	3/O:5,27	Pig	Great Britain
Y20402	4/O:3	Pig	Great Britain
Y23802	3/O:5,27	Pig	Great Britain

RAxML phylogenomic reconstruction grouped all *Y. enterocolitica* strains isolated in Ireland and Great Britain according to their pathogenic potential. Removal of 18 rogue taxa (Table 4.4) was required to improve bootstrap support values of such separation (Figure 4.1). Highly pathogenic *Y. enterocolitica* strains form a subclade within a clade of non-pathogenic biovar 1A strains with the bootstrap support values of 100% (Figure 4.1). Although, there is a clear geographical separation of *Y. enterocolitica* strains isolated in Ireland and Great Britain, this is poorly supported by bootstrap values (below 70%) (Figure 4.1).

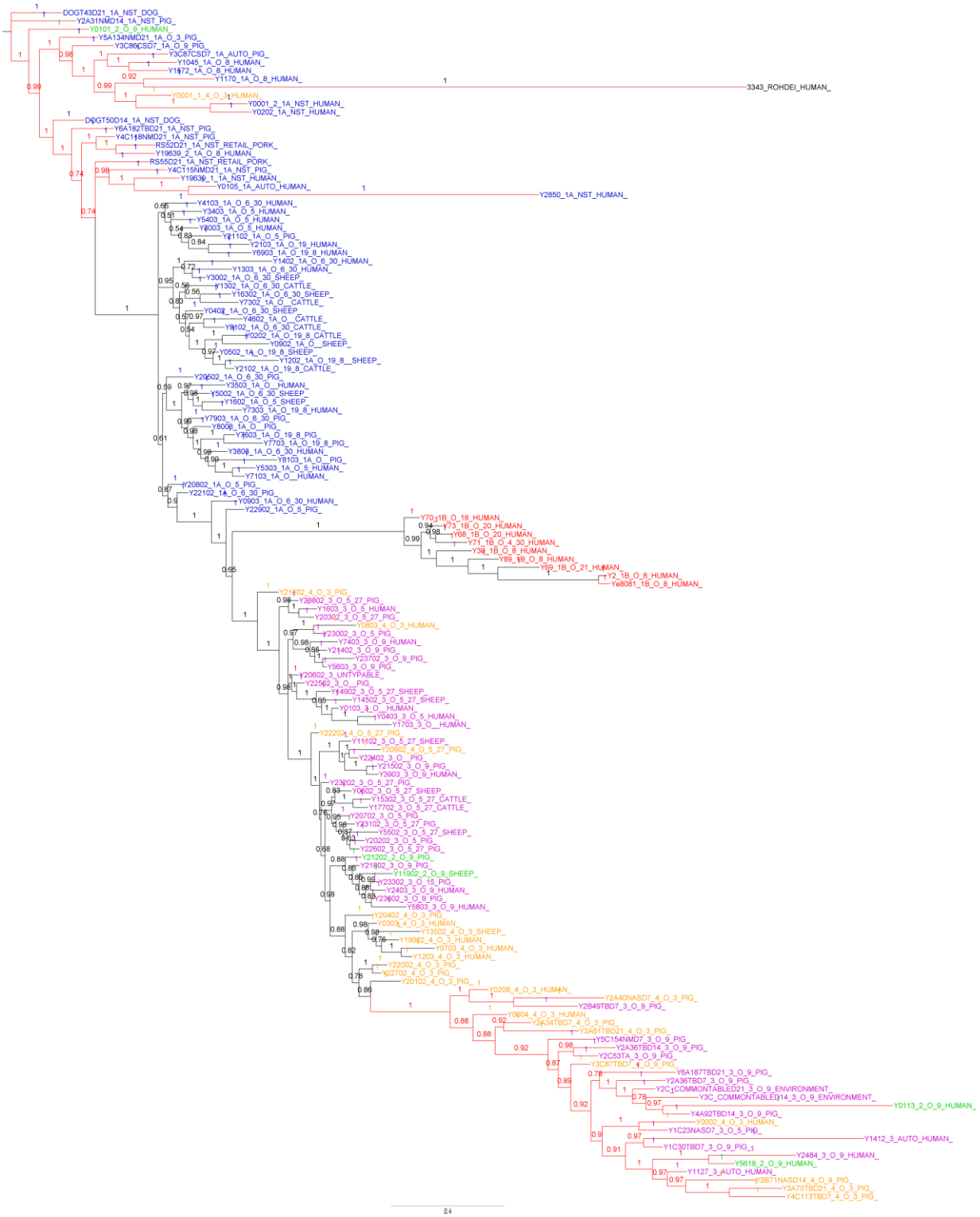


Figure 4.2 MrBayes phylogenomic reconstruction

Blue taxa :non-pathogenic biovar 1A strains
Red taxa :highly pathogenic biovar 1B strains
Green taxa :biotype 2 strains
Purple taxa :biotype 3 strains
Orange :biotype 4 strains
Red branches represent Irish strains

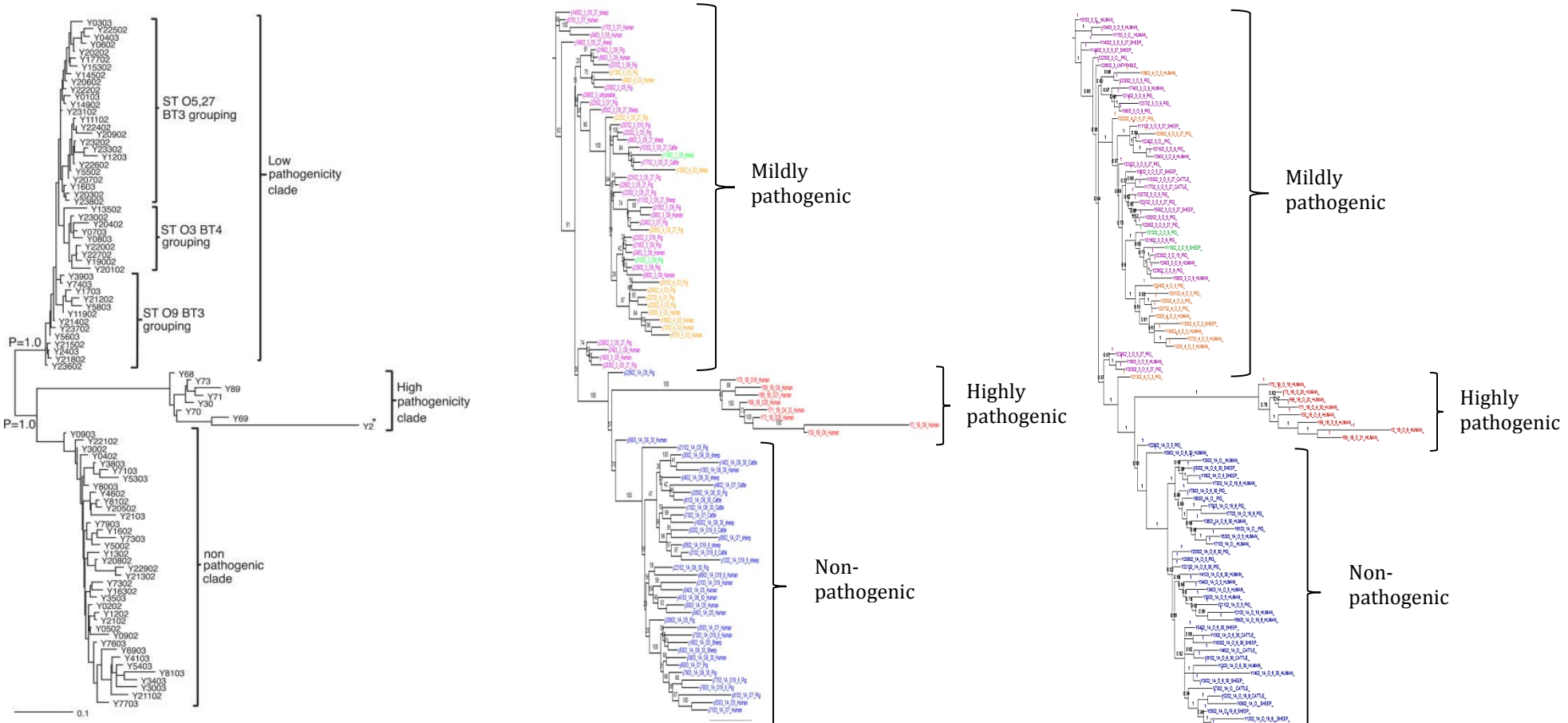
Bayesian inference also grouped all *Y. enterocolitica* strains according to their pathogenic potential, and clearly supported geographic separation of Irish and British strains with the support of branch probabilities above 95%. (Figure 4.2).

RAxML phylogeny of British, Irish and North American (highly pathogenic) suggests that non-pathogenic biovar 1A and mildly pathogenic *Y. enterocolitica subsp. palearctica* evolved separately from a common ancestor (Figure 4.1). RAxML phylogenomic reconstruction aslo shows that highly pathogenic *Y. enterocolitica subsp. enterocolitica* deviated from non-pathogenic biovar 1A *Y. enterocolitica subsp. palearctica* (Figure 4.1). Bayesian inference implies that non-pathogenic biovar 1A *Y. enterocolitica* is a progenitor for both highly and mildly pathogenic *Y. enterocolitica* (Figure 4.2).

In all trees two mildly pathogenic *Y. enterocolitica* strains (Y0101 bioserotype 2/O:9 and Y0001_1 bioserotype 4/O:3) from the Irish collection were clustered together with non-pathogenic biovar 1A strains from the same collection. In addition to the repeatedly confirmed biotyping these two strains encoded a *ystA* gene (see protocol in Chapter 3) characteristic of pathogenic *Y. enterocolitica* strains [572]**.** Y0001_1 bioserotype 4/O:3 also showed a *Galleria* pathogenicity profile commonly seen with mildly pathogenic biovars (Chapter 3). However, the two closest neighbours of Y0001_1 bioserotype 4/O:3 on the Bayesian trees, Y0002_2_1A and Y0202_1A also shared this *Galleria* profile.

Biovar 1B *Y. enterocolitica* strain 8081 used as a control strain in the current microarray experiment has as its closest neighbour *Y. enterocolitica* strain Y2 from the previous dataset that is also *Y. enterocolitica* 8081 [531].

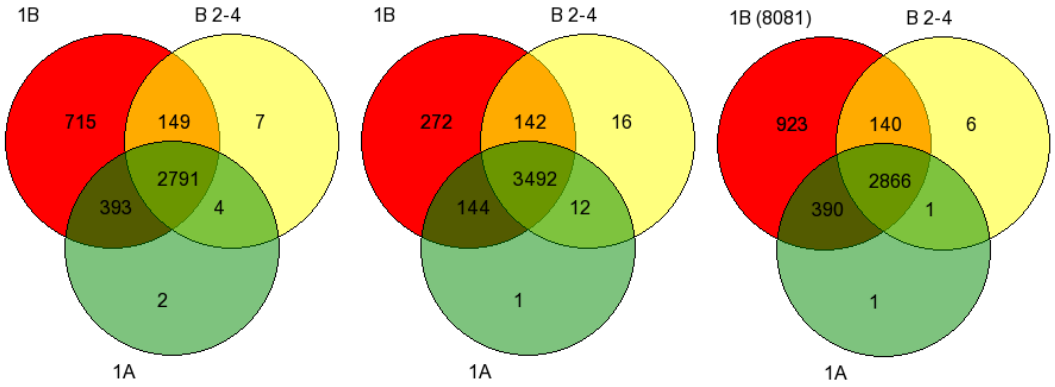
RAxML and Bayesian phylogenomic reconstruction of the re-analysed microarray data for British taxa resulted in the same tree topology as was previously obtained by *Howard et al* [531] (Figure 4.3).



MrBayes, *Howard et al* [531] **RAxML present study** **MrBayes present study**

Figure 4.3 Phylogenomic reconstruction of the re-analysed microarray data for British taxa

4.3.2 Genomic content



A. B. C.

Figure 4.4 Venn Diagram of core genomic content in Irish and British *Y. enterocolitica* strains

1B – Highly pathogenic *Y. enterocolitica* strains: B 2-4 – mildly pathogenic; 1A – non-pathogenic

A. Irish and British *Y. enterocolitica* strains
B. British *Y. enterocolitica* strains only
C. Irish *Y. enterocolitica* strains only

Irrespective of their pathogenicity, Irish and British *Y. enterocolitica* strains share 2791 genes (68.7% of studied genomes (Figure 4.4 (A)). Non-pathogenic *Y. enterocolitica* strains share an additional 393 genes with highly pathogenic strains only (Figure 4.4 (A)). Mildly pathogenic strains share only 149 additional genes exclusively with the highly pathogenic isolates (Figure 4.4 (A)). Mildly and non-pathogenic strains share another 4 genes in addition to the core set of genes (2791 genes) (Figure 4.4 (A)). Highly pathogenic "American" strains encode 715 specific genes that are not found among mildly and non-pathogenic *Y. enterocolitica* strains (Figure 4.4 (A)).

All British *Y. enterocolitica* strains share 3492 (85.6%) genes (Figure 4.4 (B)), and all Irish strains share 2866 (66.2%) genes (Figure 4.4 (C)). British and Irish mildly pathogenic *Y. enterocolitica* strains share respectively 142 and 140 genes with the highly pathogenic strains (Figure 4.4 (B, C)). Irish non-pathogenic biovar 1A *Y. enterocolitica* strains share 390 genes with the highly pathogenic strains (Figure 4.4 (C.)), while British strains shared only 144 genes with the highly pathogenic strains(Figure 4.4 (B)). However, the core gene set for the highly pathogenic strains compared with the Irish strains is larger than for the highly pathogenic strains compared with the GB strains because it comprises *Y. enterocolitica* 8081 alone, rather than a number of highly pathogenic strains as in the case for the GB comparison.

Y. enterocolitica subsp. palearctica (mildly and non-pathogenic *Y. enterocolitica* strains) in this study do not share any other specific genes that are not encoded by highly pathogenic strains, but this is a function of the geneset used for this experiment (8081-based).

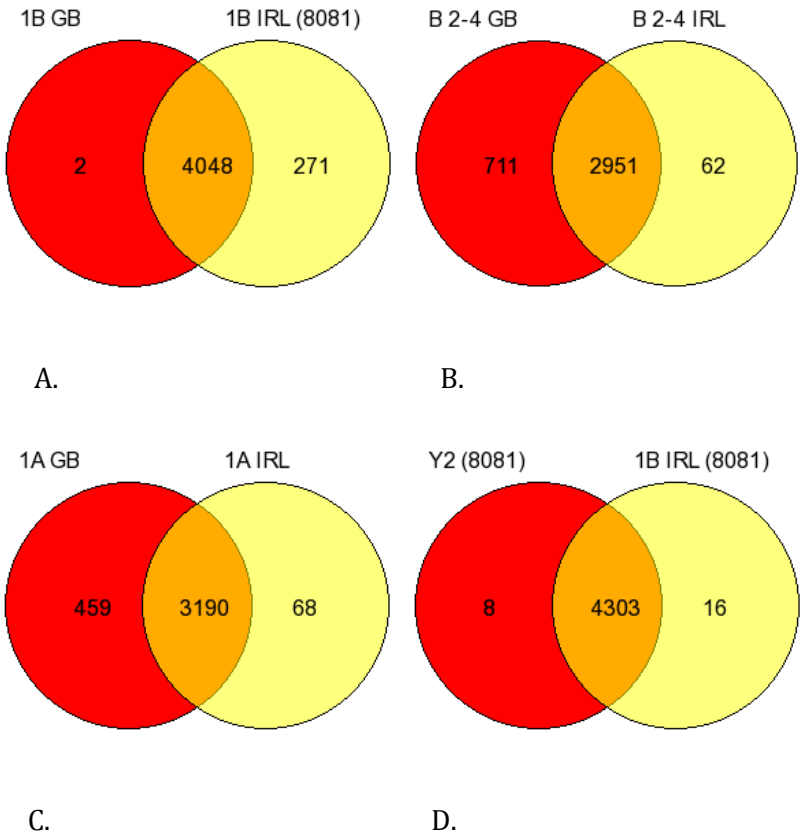


Figure 4.5 Venn Diagram of core genomic content in *Y. enterocolitica* strains

1B – Highly pathogenic *Y. enterocolitica* strains: B 2-4 – mildly pathogenic; 1A – non-pathogenic
GB – British *Y. enterocolitica* strains, IRL – Irish *Y. enterocolitica* strains
A. Highly pathogenic *Y. enterocolitica* strains
B. Mildly pathogenic *Y. enterocolitica* strains
C. Non-pathogenic *Y. enterocolitica* strains
D. Highly pathogenic *Y. enterocolitica* strain 8081 analysed in this study (1B IRL) and previous study by Howard et al [531] (Y2)

Highly pathogenic strains analysed by Howard et al [531] and in this study share 4048 genes, however, Irish strains encode 271 specific genes (Figure 4.5 (A)). Mildly pathogenic strains of GB and Irish origin share 2951 genes, however the British strains encode more unique genes (711) than the Irish (62 specific genes) (Figure 4.5 (B)). British and Irish non-pathogenic biovar 1A *Y. enterocolitica* strains share 3190 genes, additionally British strains encode 459 specific genes and Irish encode only 68 (Figure

4.5 (C)). The same *Y. enterocolitica* strain 8081 hybridized in the current study and as previously published by S. Howard et al. [531] studies share 4303 genes but differ by 24 genes (Figure 4.5 (D)).

4.4 Discussion

Phylogenomic reconstruction of the re-analysed British microarray data resulted in the same phylogenies between British taxa as in the previous study [531] (Figure 4.3) and *Y. enterocolitica* 8081 strain present in both sets of data emerged adjacent in all trees (Figure 4.1 and 4.2), showing reproducibility in data combining hybridisations carried out several years apart.

All *Y. enterocolitica* strains recovered from domestic animals and humans grouped according to their accepted pathogenic potential (mildly pathogenic, highly pathogenic and non-pathogenic clades) (Figure 4.1 and 4.2), consistent with previous studies [531, 692]. Such separation is better supported in MrBayes phylogenetic inference (Figure 4.2). To achieve better reliability of the RAxML phylogenomic reconstruction (Figure 4.1), removal of rogue taxa was required (Table 4.4).

There is no apparent clustering by host (animal or human), or by biotype or serotype within the three groups. Although strains did not cluster according to their geographic origin within each country, *Y. enterocolitica* strains recovered in Ireland were distinct from *Yersinia* strains recovered in Great Britain (Figure 4.1 and 4.2). However, such separation is better supported by Bayesian phylogenetic inference (Figure 4.2). This may indicate a low probability of *Yersinia* exchange between the UK and Ireland.

Both RAxML and Bayesian phylogeny of Irish, British and North American *Y. enterocolitica* strains are in agreement with the previous suggestion that highly pathogenic biovar 1B strains are more closely related to non-pathogenic biovar 1A than to mildly pathogenic *Y. enterocolitica* strains [531, 692], and support the hypothesis that non-pathogenic biovar 1A could be a progenitor of pathogenic *Y. enterocolitica* [692]. According to the genomic content comparison, North American highly pathogenic biovar 1B *Y. enterocolitica* strains are more closely related to Irish and British non-pathogenic biovar 1A strains than to mildly pathogenic strains (Figure 4.4 (A)). It has been previously proposed that highly pathogenic biovar 1B *Y. enterocolitica subsp. enterocolitica* and non-pathogenic biovar 1A *Y. enterocolitica subsp. palearctica* evolved separately from a common ancestor (1A-1B intermediary) due to continental drift [531].

Investigation of gene content revealed that pathogenic and non-pathogenic *Y. enterocolitica* strains share from 2790 to 3492 genes depending on the set of strains (Figure 4.4 (A, B, C)). The figures previously obtained by microarray hybridisation indicated that pathogenic and non-pathogenic *Y. enterocolitica* strains share only 894 genes, that is about 20.8% of the *Yersinia enterocolitica* 8081 genome [531]. However, our estimation of the core genome with the same data indicated that British *Y. enterocolitica* strains share 3492 (79%) CDSs (Figure 4.4 (B)). Such discrepancies could be due to differences in microarray data analysis between two studies. A log-ratio based cut-off algorithm (GACK [668]) was used for calling present and absent CDSs in the previous study [531]. This method assumes symmetry, normality and linearity of microarray data [670] and when these criteria are not met log-ratio based cut-off algorithms are less able to identify the presence/absence/divergence of the genes correctly [670-672] than the algorithm used in the present study [673]. Earlier studies based on whole genome sequencing indicated that genome sequences of eight non-pathogenic species from the genus *Yersinia* share 2497 genes [550], genome sequences of *Y. pestis*, *Y. pseudotuberculosis* and highly pathogenic *Y. enterocolitica subsp. enterocolitica* strain 8081 share 2747 genes [532], and the core gene set of highly pathogenic *Y. enterocolitica subsp. enterocolitica* strain 8081 and mildly pathogenic bioserovar 3/O:9 *Y. enterocolitica subsp. palearctica* strain 105.1R was estimated at 3492 genes [550, 693]. These figures are similar to our estimation of the *Y. enterocolitica* core genome using a different microarray data analysis methodology (single colour microarray data normalization in LIMMA with weak flagging and BCSP cut-off algorithm).

These results confirmed that microarray technology in combination with phylogenetic analysis is a sensitive and robust method for studying genetic relatedness of *Y. enterocolitica*.

Chapter 5 General Discussion and Conclusions

5.1 Literature review

The genus *Yersinia* belongs to the family *Enterobacteriaceae* and comprises 18 species [1-7] three of which are pathogenic for humans (*Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*). Currently six biovars of *Y. enterocolitica* (Table 1.4) [133] and four biovars of *Y. pseudotuberculosis* (Table 1.5) [134] are recognised. In general, the biotype of *Y. enterocolitica* [133] and *Y. pseudotuberculosis* [135-137] correlates with the pathogenic potential of the microorganism.

Pathogenic biovars of *Y. pseudotuberculosis* and *Y. enterocolitica* are predominant causes of an acute or chronic enteric zoonosis known as yersiniosis. However, various extraintestinal manifestations may also occur predominantly in patients with the underlying conditions (described in Chapter 1). Patients with persistent enteric *Yersinia* infection [228, 262, 263] and possessing the histocompatibility gene HLA-B27 [264-267] are at particular risk of developing post-infectious sequelae [204, 268-271]. Transient bacteraemia in healthy blood donors after previous unnoticed yersiniosis [165, 166, 170, 172, 173, 234, 247-253] is a major cause of fatal blood transfusion septicaemia [246, 247].

The highest incidence rates of yersiniosis ranging from 5.0 to 12.03 per 100,000 of population are reported from Northern and Central European countries [12]. In Ireland over the past few years, the annual incidence rate of notified yersiniosis has been the lowest in the European Union, and does not exceed 10 cases per year (≤0.1 per 100,000 population) [12]. Nevertheless, the anti-*Yersinia* antibody seroprevalence among healthy individuals is generally much higher than incidence rates of yersiniosis in the same population (Table 1.10) [76, 162-164].

Most cases of yersiniosis are sporadic [14-17] but outbreaks of *Y. pseudotuberculosis* [18, 19] and *Y. enterocolitica* [20-29] involving food have been reported worldwide (Table 1.1). The main risk factors of yersiniosis include consumption of contaminated pig products [16, 21, 22, 28, 63-67], contact with domestic animals [13, 14, 53, 73] and drinking untreated water [13, 16, 65, 75-77].

5.2 Current evidence for human yersiniosis in Ireland

Despite the high prevalence of pathogenic *Y. enterocolitica* among pigs in Irish slaughter houses it seems these organisms do not enter the food chain at levels that are harmful to the local population as no pathogenic *Yersinia* were isolated from symptomatic patients with diarrhoea, appendicitis or pharyngitis. The number of negative faeces cultures excludes a true yield of 0.5 % or more of diarrhoeal stools containing *Y. enterocolitica* in our study population with 99% power, suggesting *Y. enterocolitica* is indeed a much less common cause of diarrhoea in Ireland than in other European countries. This is consistent with the very low current notification rates of clinical yersiniosis in Ireland. PCR targets for pathogenic *Y. enterocolitica* (chromosomal *ystA* and plasmid-located *yadA*) were detected in pig slurry and not in human sewage by simple PCR. Nested PCR was required to detect *yadA* in human sewage, consistent with a low level of intestinal excretion of pathogenic *Y. enterocolitica* in humans locally compared with pigs.

However, the detection of anti-Yop antibodies in 25% of Irish blood donors suggests occurrence of recent or past *Yersinia* infection in the Irish human population at levels similar to that found in countries where clinical yersiniosis is currently much more common [162, 164]. Anti-*Yersinia* seropositivity in blood donors is a risk factor for transfusion-transmitted *Yersinia* sepsis [173, 248-250, 252, 253, 694], but there have been no reports of *Yersinia* sepsis following blood transfusion in Ireland. There was a significant association between increasing age and the presence of anti-*Yersinia* Yop antibodies, compatible with a cohort effect of increased risk of infection with pathogenic *Yersinia* in Ireland. The most probable explanation linking absence of culturable *Y. enterocolitica*, with a high seroprevalence of anti-*Yersinia* Yop antibodies in older blood donors, and some molecular evidence of the presence of pathogenic *Y. enterocolitica* in human sewage, is a combination of a higher incidence of yersiniosis in Ireland in the past (caused by both *Y. pseudotuberculosis* and *Y. enterocolitica*) and a continuing low incidence of mild self-limited *Y. enterocolitica* infection which does not result in diarrhoea specimens being sent for culture. A much larger prospective study on patients with mild diarrhoea in the community is required to confirm a continuing low incidence of mild self-limiting yersiniosis in Ireland. However, in the absence of a major disease burden, such a study would be difficult to justify. Nevertheless, the presence of pathogenic bioserotypes of *Y. enterocolitica* in the local pig population

means that there is a potential for future food-borne outbreaks of yersiniosis to occur in Ireland.

5.3 *Yersinia* insect toxicity

The prevalence of non-pathogenic *Y. enterocolitica* in isolates from humans (described Chapter 3) and retail meat samples (described Chapter 3) in Ireland prompted investigation of their pathogenic potential.

Invertebrates and vertebrates share structural and functional homology of their immune responses and insects have been suggested as a model for studying microbial pathogenicity [586-589]. Moreover, a good correlation between virulence of *Y. pseudotuberculosis* has been obtained in mammalian and *G. mellonella* infection models [600]. To demonstrate potential pathogenicity of non-pathogenic *Y. enterocolitica* we compared their virulence towards *G. mellonella* larvae with that of highly and mildly pathogenic *Y. enterocolitica* strains. This comparison has not been previously reported.

Virulence of *Y. enterocolitica* in *G. mellonella* larvae observed at 37°C did not correlate with their pathogenic potential towards humans. Irrespective of their pathogenic potential for mammalian hosts, all tested *Yersinia* strains caused fast killing (<24h) of *Galleria* at 37°C and slow killing (≥48h) at 15°C. For most *Yersinia* strains, slow killing at 15°C accounted for the highest mortality rates among insect larvae.

In general, irrespective of the presence of a recognised ITC locus, non-pathogenic *Y. enterocolitica* strains that encoded CDT type A (nearly all grouped into hierarchical cluster 2) were more virulent towards insect larvae at 37°C than all other *Y. enterocolitica* (Table 3.19, Table 3.23, Table 3.24, Figure 3.8). While distinctive CDT complexes are encoded by mildly and non-pathogenic *Y. enterocolitica* strains (see Chapter 3) the expression of *cdtB2* and *cdtB3* (i.e. representative component genes for all the CDT loci identified in chapter 3) (Figure 3.6, 3.7) was equally evident at both temperatures *in vitro*, suggesting that CDT toxins are candidates for causing both forms of insect larvae killing. However, biological activity of these toxins is greatly dependent on their binding affinity to the surfaces of eukaryotic cells [628] and this may explain why other *Yersinia* strains that also encode CDT did not cause the same mortality rates of *G. mellonella* larvae.

High mortality rates of *G. mellonella* larvae at 15°C could be associated with the preferential expression of the insecticidal toxins at low temperatures[555] however the

current study as well as a previous publication [585] did not establish a distinctive link between the presence of ITC genes and subcutaneous toxicity of *Y. enterocolitica* strains in *G. mellonella* larvae. So other factors could be involved. Interestingly, other psychrotrophic bacteria *Bacillus weihenstephanensis* [624] and *Listeria innocua* [611] that do not share recognised pathogenicity factors also cause increased mortality at lower temperatures in a *Galleria* model. Thus, in the absence of recognised shared pathogenicity factors between these different bacteria, enhanced bacterial replication at lower temperatures may be important for this phenotype.

Different physiology of the insect host at 15°C and 37°C could also affect outcome of *Yersinia* infection in *G. mellonella* larvae. Thus, pre-incubation of *G. mellonella* larvae at 37°C (and 4°C) results in a stress response including an increase in haemocyte density and antimicrobial peptide synthesis which reduces their mortality in subsequent *Candida albicans* infection compared to when pre-incubated at 30°C (Table 3.4) [590].

Further studies are required to establish the activity of *Yersinia* CDT toxins against insect cells as well as human epithelial and immune cells. Additionally, knockout of CDT type A loci would be required to confirm whether death of *G. mellonella* larvae is due to its activity. Slow killing of insect larvae at 15°C could be a result of enhanced *Y. enterocolitica* replication at low temperatures. Thus, future experiments are required to establish whether a pre-formed *Yersinia* toxin was responsible for either the rapid (37°C) killing or slow (15°C) killing of *G. mellonella* larvae by *Yersinia.* This could be achieved by injecting *Galleria* with killed *Y. enterocolitica* strains or protein extracts from bacteria grown at different temperatures. Measuring haemocyte density and antimicrobial peptide levels would be required to elucidate the effect of these factors on *Yersinia*-induced *Galleria* mortality.

5.4 Comparative genomics

Genomic microarray proved to be a reliable methodology for studying genomic relationships between *Yersinia.* Comparative microarray analysis of *Y. enterocolitica* strains recovered in Ireland and previously analysed microarray data of strains from Great Britain and North America (highly pathogenic strains only) established that all strains grouped mainly according to their pathogenic potential, not the host. Despite extensive human, animal and food exchanges between the UK and Ireland [684], Irish and British strains clustered separately from each other within the groupings by pathogenic phenotype. Comparison of their genetic content also confirmed that these

strains differ in core genetic content (Figure 4.5). Because these strains were not collected at the same time in the two countries, and possibly because the hybridisations of UK and Irish strains were to different array types (although renormalised and called in the same way), this does not rule out horizontal exchange of *Y. enterocolitica* between the two countries.

Phylogenomic reconstruction agrees with earlier established evolutionary relationships among *Y. enterocolitica* strains [531, 692]. Consequently, phylogeny of Irish, British and North American *Y. enterocolitica* strains implies that *Y. enterocolitica subsp. enterocolitica* evolved separately from *Y. enterocolitica subsp. palearctica* and non-pathogenic *Y. enterocolitica* is a possible progenitor for pathogenic *Y. enterocolitica.*

Twenty two strains that were compared in a *G. mellonella* death assay (Chapter 3) were also used for comparative genomic microarray phylogenomic reconstruction. However, the grouping of these strains by hierarchical clustering of their phenotype in causing *Galleria* mortality at different incubation temperatures and their phylogenetic grouping based on gene content is different. This may indicate that insecticidal pathogenicity factors in *Y. enterocolitica* are phylogenetically widely distributed.

5.5 Concluding remarks

Although, *Yersinia* selective culture results exclude 0.5% prevalence of pathogenic *Yersinia* in diarrhoeal stool samples and 2% prevalence in throat swabs from patients with pharyngitis, high anti-*Yersinia* seroprevalences in Irish blood donors and presence of molecular markers for pathogenic *Yersinia* in human sewage suggest prevalence of mild self-limiting forms of yersiniosis in Ireland that do not result in submission of stool samples for culture.

Virulence of *Y. enterocolitica* in *G. mellonella* larvae observed at 37°C did not correlate with their pathogenic potential towards humans. Non-pathogenic *Y. enterocolitica* strains that encoded CDT type A were the most virulent towards insect larvae at 37°C in comparison to other *Y. enterocolitica* strains. However, at 15°C all *Yersinia* strains were equally highly virulent towards insect larvae possibly due to unobstructed bacterial proliferation in insect host. Future experiments are required to establish CDT activity in mammalian and insect cell cultures and whether a pre-formed *Yersinia* toxin was responsible for either the rapid (37°C) or slow (15°C) killing of *G. mellonella* larvae by *Yersinia*.

Although, comparative microarray analysis of clinical and non-clinical *Y. enterocolitica* strains isolated in Ireland and Great Britain did not show evidence for direct transfer of *Yersinia* between two countries, such exchange can not be excluded as analysed strains are not contemporaneous.

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Publications and Presentations

PUBLICATIONS:

Ringwood T, Murphy BP, Drummond N, Buckley JF, Coveney AP, Redmond HP, Power JP, Fanning S, Prentice MB: **Current evidence for human yersiniosis in Ireland**. *Eur J Clin Microbiol Infect Dis* 2012. ***(attached)***

Ringwood T, Murphy BP, Drummond N, Buckley JF, Fanning S, Prentice MB: **Preliminary survey regarding yersiniosis in Ireland**. *Adv Exp Med Biol* 2012, **954**:59-61.

ORAL PRESENTATIONS:

MOLECULAR DIAGNOSTICS AND SEROPREVALENCE OF *YERSINIA ENTEROCOLITICA* IN THE IRISH HUMAN POPULATION, 11th March, 2011. Workshop: Pork and pork products – veterinary public health protecting consumers and the Irish food industry, RELAY, Cork, Ireland.

SEROPREVALENCE OF ANTI-*YERSINIA* ANTIBODIES IN IRELAND. 16th June, 2010. 2nd Annual Research Conference: Molecules to Medicine, UCC, Cork, Ireland.

POSTER PRESENTATIONS:

THE INTERACTION BETWEEN *YERSINIA ENTEROCOLITICA* CLINICAL AND ENVIRONMENTAL ISOLATES AND *GALLERIA MELLONELLA.* 23-27th October, 2010. 10th *Yersinia* International Symposium, Recife, Brazil.

YERSINIOSIS IN IRELAND. 23-27th October, 2010. 10th *Yersinia* International Symposium, Recife, Brazil.

Appendix