ISOLATION AND CHARACTERIZATION OF *YERSINIA ENTEROCOLITICA* FROM FOOD AND SWINE

TAN LAI KUAN

FACULTY OF SCIENCE
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TAN LAI KUAN

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I/C/Passport No: **851123-14-5636**
Regisration/Matric No.: **SGR100016**
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ABSTRACT

Yersinia enterocolitica is an important foodborne enteric pathogen that causes gastroenteritis. There are limited studies on *Y. enterocolitica* in Malaysia, hence the potential complication due to yersiniosis in the country remains unknown. The objectives of this study were: (i) to determine the prevalence of *Y. enterocolitica* from raw food and pigs in Malaysia; (ii) to characterize the Malaysian *Y. enterocolitica* by using phenotypic and genotypic methods and; (iii) to study the genetic relatedness of Malaysian *Y. enterocolitica* strains from different food sources and pigs in Malaysia; and (iv) to improve the isolation of rate of *Y. enterocolitica* by modifying the composition of Cefsulodin-Irgasan-Novobiocin(CIN) agar.

Between years 2010 to 2011, 106 raw food samples (58 pork products and 48 non-porcine food) and 495 swine specimens (from 165 pigs) were analysed for the presence of *Y. enterocolitica*. The pathogen was isolated in 7/58(12.1%) raw pork products, in which pork (whole meat) had the highest prevalence 5/21(23.8%), followed by liver 1/5(20.0%) and intestine 1/8(12.5%). *Y. enterocolitica* was not isolated from raw non-porcine food. Of 165 pigs, 3(1.8%) were carriers (asymptomatic pigs) for *Y. enterocolitica*. Bioserotyping showed that the isolates were of bioserotypes 3 variant/O:3(n=92), 1B/O:8(n=3), and 1A/O:5(n=3). The 3 variant/O:3 was the most prevalent bioserotype (present in pork products and pigs) and is probably the common bioserotype in Malaysia (warm climate region).

Thirty-two *Y. enterocolitica* isolates were further subtyped by using pulsed-field gel electrophoresis (PFGE) and the antimicrobial profiles and carriage of virulence markers were evaluated. Isolates of three different bioserotypes were distinguished into three clusters (*D* value = 0.87, 90% similarity) by using PFGE. However, isolates were highly clonal within each bioserotype and exhibited minor variation. Of 29

antimicrobials tested, the 1B/O:8 isolates were only resistant to clindamycin and the 1A/O:5, resistant to ampicillin, ticarcillin, amoxicillin, and clindamycin. Majority of the 3 variant/O:3 isolates were resistant to nalidixic acid, clindamycin, ampicillin, ticarcillin, tetracycline and amoxicillin. About 90% were multidrug-resistant(MDR) with multiple antibiotic resistance(MAR) index for isolates of bioserotype 3 variant/O:3 the highest, 0.183, followed by 1A/O:5 and 1B/O:8 with MAR indices at 0.121 and 0.103, respectively. Isolates were examined for the presence of pYV plasmid and 15 virulence genes. Four reproducible virulence genes patterns obverved and each virulotype belonged to a particular bioserotype. The pYV plasmid was only present in the 3 variant/O:3 isolates.

To improve the isolation of *Y. enterocolitica,* the composition of CIN agar was modified. Based on the evaluation on the plating efficiency, detection limit and recovery strength for both CIN and modified CIN media, modified CIN provided a better discrimination of *Y. enterocolitica* from five bacteria exhibiting *Yersinia*-like colonies on CIN than the original CIN while retaining similar detection limit and culture capability for *Y. enterocolitica*.

In conclusion, the occurrence of virulent strains of *Y. enterocolitica* in pigs and raw pork products indicated that pigs are important reservoir of *Y. enterocolitica.* The high incidence of multidrug resistant *Y. enterocolitica* is of public health concern and possibly reflects the abuse of antimicrobial agents in the animal husbandry. The modified CIN might be useful for routine surveillance for *Y. enterocolitica*.

ABSTRAK

Yersinia enterocolitica merupakan patogen enterik bawaan makanan yang menyebabkan gastroenteritis. Kekurangan kajian dalam *Y. enterocolitica* di Malaysia menyebabkan potensi komplikasi yersiniosis di Negara ini tidak jelas diketahui. Tujuan-tujuan kajian ini adalah: (i) mengkaji prevalens *Y. enterocolitica* daripada makan-makanan dan khinzir di Malaysia; (ii) mencirikan *Y. enterocolitica* dengan menggunakan kaedah fenotip dan genotip; (iii) mengkaji hubung-kait genetik bagi strain *Y. enterocolitica* Malaysia yang bersumber daripada makanan and khinzir yang berlainan; dan (iv) membaikan kadar pengasingan *Y. enterocolitica* dengan mengubahsuai komposisi agar Cefsulodin-Irgasan-Novobiosin (CIN)..

Antara tahun 2010 ke 2011, 106 sampel makanan mentah (58 produk khinzir dan 48 makanan bukan khinzir) dan 495 spesimen khinzir (daripada 165 khinzir) telah diperiksa bagi kehadiran *Y. enterocolitica*. *Y. enterocolitica* diasingan daripada 7/58(12.1%) produk khinzir mentah, di mana daging (daging lengkap) memberikan prevalens tertinggi 5/21(23.8%), diikuti dengan hati 1/5(20.0%) dan intestin 1/8(12.5%). Tiada *Y. enterocolitica* terasing daripada makanan bukan khinzir mentah. Daripada 165 khinzir, 3(1.8%) merupakan pembawa (khinzir yang tidak membawa sebarang gejala penyakit) *Y. enterocolitica*. Bioserotip menunjukkan isolat-isolat terdiri daripada bioserotip varian 3/O:3(n=92), 1B/O:8(n=3) dan 1A/O:5(n=3). Bioserotip varian 3/O:3 adalah bioserotip yang paling prevalen (hadir dalam kedua-dua makanan khinzir dan khinzir) dan barangkali merupakan bioserotip yang biasa di rantau ini.

Tiga puluh dua isolat *Y. enterocolitica* dicirikan selanjutnya dengan menggunakan kaedah gel elektroforesis medan-berdeyut (PFGE) dan profil antimikrob dan pembawaan penanda virulens dinilai. Dengan menggunakan PFGE, isolat-isolat daripada tiga bioserotip dibezakan kepada tiga klustur (nilai *D*=0.87, keserupaan 90%).

Akan tetapi, isolat adalah sangat klonal dalam setiap bioserotip dan mempamerkan variasi minor. Daripada 29 antimikrob-antimikrob teruji, isolat 1B/O:8 hanya resistan terhadap ampisilin, tikarsilin, amoxisilin, dan klindamisin. Kebanyakan isolat varian 3/O:3 resistan terhadap asid nalidisik, klindamisin, ampisilin, tikarsilin, tetrasiklin dan amoxisilin. Kira-kira 90% isolat adalah resistan drug kepelbagaian (MDR) dengan indeks resistan kepelbagaian antibiotik (MAR) untuk isolat bioserotip varian 3/O:3 tertinggi, 0.183, diikuti dengan 1A/O:5 dan 1B/O:8 yang mempunyai indeks MAR masing-masing pada 0.121 dan 0.103. Kehadiran plasmid pYV dan 15 gen-gen virulens diperiksa. Terdapat empat rupa susunan yang boleh diulang semula dan setiap virulotip adalah kepunyaan kepada suatu bioserotip. Plasmid pYV hanya hadir dalam isolat varian 3/O:3.

Dalam memperbaiki pengasingan *Y. enterocolitica,* komposisi agar CIN diubahsuai. Berdasarkan penilaian kepada kecekapan pemplatan, had pengesanan dan kekuatan pemulihan untuk kedua-dua agar CIN dan CIN-diubahsuai, CIN-diubahsuai mempunyai keupayaan diskriminasi yang lebih baik berbanding dengan agar CIN dalam membezakan *Y. enterocolitica* daripada lima bakteria bercirian *Yersinia* atas agar CIN dan di samping itu, megekalkan had pengesanan dan keupayaan pengkulturan untuk *Y. enterocolitica* yang sama.

Kesimpulannya, kejadian strain virulen *Y. enterocolitica* dalam khinzir dan makan khinzir mentah menunjukkan khinzir merupakan reservoir penting untuk *Y. enterocolitica*. Kejadian strain MDR *Y. enterocolitica* yang tinggi adalah membimbangan kesihatan awam dan mungkin mencerminkan penyalahgunaan agen antimikrobial dalam industri haiwan. Agar CIN-diubahsuai mungkin berguna dalam pengamatan rutin terhadap *Y. enterocolitica*.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
&	And
~	Approximate
<	Mathematic calculation symbol, lesser than
>	Mathematic calculation symbol, greater than
±	Mathematic calculation symbol, plus or minus
×	Mathematic calculation symbol, times
≤	Mathematic calculation symbol, lesser or equivalent
≥	Mathematic calculation symbol, greater or equivalent
AFLP	Amplified fragment length polymorphism
AMC	Amoxicillin-clavulanic acid, 30 µg
AMK	Amikacin, 30 µg
AMP	Ampicillin, 10 µg
AMX	Amoxicillin, 25 µg
ATCC	American type culture collection
ATM	Aztreonam, 30 µg
bp	base pair
BSA	Bovine serum albumin
ca.	approximately
CAL	Cellociose-arginine-lysine
CAZ	Ceftazidime, 30 µg
CDC	Centre of Disease Control
cfu	Colony Forming Unit
CHL	Chloroamphenicol, 30 µg

CIN	Cefsulodin-irgasan-novobiocin
CIP	Ciprofloxacin, 5 μg
CLB	Cell lysis buffer
CLI	Clindamycin, 2 μg
CLSI	Clinical and Laboratory Standards Institute
CR-BHO	Congo red brain heart infusion agarose
CR-MOX	Congo red magnesium oxalate
CRO	Ceftriaxone, 30 μg
CSB	Cell suspension buffer
CSS	Colistin sulphate, 10 μg
CTM	Cefotaxime, 30 μg
CXM	Cefuroxime, 30 μg
D	Discriminatory Power
D	Delta
ddH_2O	deionized distilled water
DDST	Double-disc synergy test
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleotide-tri-phosphate
DOX	Doxycycline, 30 μg
ENR	Enrofloxacin, 5 μg
ERIC	Enterobacterial Repetitive Intergenic Consensus
ESBL	Extended spectrum β-lactamase
est.	Estimate
g	Unit for gravity
g	Unit of weight in gram

GEN	Gentamicin, 10 µg
h	Hour/hours
H_2S	Hydrogen sulphide gas
HPI	High pathogenicity island
IP	Institut Pasteur, strain collection of the French *Yersinia* Reference Laboratory
IPM	Imipenem, 10 µg
ISO	International Standard Organisation
ITC	Irgasan-ticarcillin-potassium chlorate
KAN	Kanamycin, 30 µg
kb	kilobase pair
KOH	Potassium hydroxide
KV202	*Yersinia*-selective medium
L	Litre/litres
LBA	Luria-Bertani agar
LCI	Lower confidence interval
LOD	Limit of detection
LVX	Levofloxacin, 5 µg
m	Millie
M	Molar
MAC	MacConkey
MAR	Multiple antibiotic resistance
MDR	Multidrug-resistant
mg	Milligram
min	Minute/minutes
MLVA	Multiple-locus variable number tandem repeat analysis

mm	Millimeter
mM	Millie molar
MPN	Most probable number
n	Nano
N	Neomycin, 10 µg
NaCl	Sodium chloride
NAL	Nalidixic acid, 30 µg
NET	Netilmicin, 30 µg
No.	Number
NO_3	Nitrate
℃	Degree Celsius
OD	Optical density
PB	Polymyxin B, 300 µg
PBS	Phosphate buffered saline
PFGE	Pulsed-field gel electrophoresis
psi	Pound per square inch
RAM	1% L-rhamnose and 1% D-arabitol agar
RAPD	Ramdomly amplified polymorphic DNA
REAC	Restriction endonuclease analysis of chromosome
REAP	Restriction endonuclease analysis of plasmid
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
s	Second/seconds
SP	Single enzyme
spp.	Species
SPT	Spectinomycin, 100 µg

SSI	Statens Serum Institut
STR	Streptomycin, 10 µg
TBE	Tris-borated EDTA
TE	Tris-EDTA
TET	Tetracycline, 30 µg
TIC	Ticarcillin, 75 µg
TIM	Trimethoprim-sulphamethoxazole, 25 µg
TMP	Trimethoprim, 5 µg
TTSS	Type III secretion system
U/µl	Unit per micro-litre
UPI	Upper confidence interval
USDA	United States Department of Agriculture
UV	Ultra-violet
V	Volt
VP	Voges-Proskauer
VYE	Virulent *Yersinia enterocolitica*
WHO	World Health Organization
YECA	*Yersinia enterocolitica* agar
YeCM	*Yersinia enterocolitica* chromogenic medium
YSEO	*Yersinia* selective enrichment broth according to Ossmer
β	Beta
µ	Unit of Micro
µg	Microgram
µl	Microliter

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CHAPTER 1

INTRODUCTION

Yersinia enterocolitica is a bacterium which belongs to the *Enterobactericeae* is widely found in natural environments. It is psychrotrophic and has the capability to survive and multiply at low temperature (Annamalai & Venkitanarayanan, 2005; Neuhaus, Francis, Rapposch, Görg, & Scherer, 1999). *Y. enterocolitica* is considered enteropathogenic as it is usually transmitted to through consumption of contaminated food and cause gastrointestinal infection in humans. Typical symptoms include acute enteritis with fever, bloody diarrhoea and pseudo appendicitis, which frequently leads to unnecessary laparotomy in humans (Vlachaki, Tselios, Tsapas, & Klonizakis, 2007). Young children and infants are the most susceptible age group (Rosner, Stark, & Werber, 2010). In most cases, *Y. enterocolitica* infection is self-limiting, and no antimicrobial therapy is needed. However in rare cases like sepsis, antimicrobials may be useful.

Y. enterocolitica is ubiquitous in the nature and is routinely isolated from various animals (swine, cattle, sheep, etc.), food (pork, poultry, ruminant, milk, vegetables, etc.) and environment (Dallal et al., 2010; Fredriksson-Ahomaa & Korkeala, 2003; Fukushima, Hoshina, Itogawa, & Gomyoda, 1997; Novoslavskij et al., 2013; Xanthopoulos, Tzanetakis, & Litopoulou-Tzanetaki, 2010). Among the sources, swine have been implicated as a major reservoir of *Y. enterocolitica* associated with human infections.

Yersiniosis outbreaks that involved ingestion of contaminated food have occurred in several countries such as China, Norway, United States, Japan, and India (Abraham et al., 1997; Ackers et al., 2000; Grahek-Ogden, Schimmer, Cudjoe, Nygard, & Kapperud, 2007; Jones, Buckingham, Bopp, Ribot, & Schaffner, 2003; MacDonald et al., 2012; Sakai et al., 2005; Zheng & Jiang, 2006). In Europe, *Y. enterocolitica* is notified as the fourth most important foodborne enteric pathogen after

campylobacteriosis, salmonellosis and and verotoxigenic *E. coli* (European Food Safety Authority & European Centre for Disease Prevention and Control, 2013).

Studies concerning the incidence and prevalence of *Y. enterocolitica* have seldom been reported in Malaysia. The first case of human yersiniosis in Malaysia was reported by Jegathesan, Paramasivam, Rajagopalan, & Loo (1984) where *Y. enterocolitica* serotype O:3 was isolated from a 34-year-old Indian woman. The only food related prevalence report in Malaysia was from unpublished study of Dzomir (2005), *Y. enterocolitica* (bioserotype 1A/O:52, 53 and 1A/O:41, 42) was isolated from beef burger patty and chicken burger patty. Due to the limited study of this bacterium in Malaysia, the potential complications of yersiniosis in the country remain unknown. Therefore it is interesting to investigate the prevalence of *Y. enterocolitica* in the local food and pigs. It is also interesting to investigate the genetic relatedness and characteristics of the *Y. enterocolitica* strains (phenotypic and genotypic) isolated from various sources in Malaysia.

There are numerous isolation schemes available in isolation and detection of *Y. enterocolitica* and the isolation of *Y. enterocolitica* is considered laborious. Typical isolation method involves selective enrichment, post-enrichment alkaline treatment (0.5 ml enriched broth transferred to 4.5 ml of 0.5% KOH solution and mixed for 20 s) (Aulisio, Mehlman, & Sanders, 1980), selective agar isolation, and a series of characterization tests. In this study, limitations of the current Cefsulodin-Irgasan-Novobiocin (CIN) agar in isolating *Y. enterocolitica* were found. The lack of good isolation medium will thus mask and underestimate the actual incidence of yersiniosis. Efforts in modifying and improving the current CIN agar will therefore improve the isolation rate of *Y. enterocolitica*.

1.1 Objectives of study

The objectives of this research are as follows:

1. To determine the prevalence of *Y. enterocolitica* in food and pigs in Malaysia by using conventional and molecular methods.
2. To characterize the *Y. enterocolitica* isolates in Malaysia by using biotyping, serotyping, pulsed field gel electrophoresis, plasmid profiling, virulotyping and antimicrobials susceptibility test.
3. To study the genetic relatedness of Malaysian *Y. enterocolitica* strains from different food sources and pigs in Malaysia.
4. To modify and improve the composition of the existing Cefsulodin-irgasan-novobiocin (CIN) agar to improve the differentiation of *Y. enterocolitica* from other natural microbiota.

CHAPTER 2

LITERATURE REVIEW

2.1 General background and occurrence of yersiniosis

Yersinia enterocolitica belongs to the genus of *Yersinia* in the *Enterobacteriaceae* family. It is Gram-negative, rod-shaped facultative anaerobes, and psychrotrophic bacterium which is ubiquitous and widely found in the natural environment. *Y. enterocolitica* was first discovered by Schleifstein and Coleman in 1939 (Schleifstein & Coleman, 1939). It is an enteropathogenic as it typically causes gastrointestinal infection in humans. *Y. enterocolitica* is usually transmitted to humans through contaminated food. It is a notifiable disease in Europe (European Food Safety Authority & European Centre for Disease Prevention and Control, 2013). In 2011, the incidence rate of yersiniosis was 1.63 cases per 100,000 population in European Union (European Food Safety Authority & European Centre for Disease Prevention and Control, 2013). In New Zealand, the incidence of yersiniosis is the third most frequently reported disease, 11.5 cases per 100,000 population (Heffernan, 2012). In Malaysia, *Y. enterocolitica* is not routinely isolated as it is not a notifiable disease and therefore, not much is known about its economic importance. The first case of human yersiniosis in Malaysia was reported by Jegathesan, et al. (1984) in which *Y. enterocolitica* serotype O:3 was isolated from a 34-year-old Indian woman.

2.2 Yersiniosis and clinical characteristics

Human yersiniosis occurs when *Y. enterocolitica* enters the gastrointestinal tract after ingestion of contaminated food or water. *Y. enterocolitica* that survive through the barrier of first line of body's defense (stomach acid) will adhere to mucosal cells in the Peyer’s patches (adhesion), invade (invasion) phagocytic cells, extracellular multiplication, and produce a local inflammatory response. The damage to the absorptive epithelial cells results in mal-absorption and fluid loss that characterized as diarrhea (F àbrega & Vila, 2012).

In developed countries, yersiniosis commonly occurs in infants and young children. Approximately 75% of patients with *Y enterocolitica* infection are children aged 5-15 years (Ackers, et al., 2000; Gómez-Duarte, et al., 2010). Yersiniosis usually causes self-limiting diarrhea with symptoms including abdominal pain, fever and diarrhea, sometimes nausea and vomiting, is often indistinguishable from those of acute appendicitis. In some cases, it causes extraintestinal sequelae, septicemia and fatal systematic infection.

2.3 Mode of transmission

2.3.1 Foodborne Transmission

Majority of the incidence of yersiniosis is foodborne transmitted. Human yersiniosis is usually sporadic and the source of infection is unknown. Infection is generally caused by the ingestion of contaminated foods that usually raw or inadequately cooked. Outbreaks of yersiniosis that involved ingestion of contaminated food have occurred in several countries such as China, Norway, United States, Japan, and India (Abraham, et al., 1997; Ackers, et al., 2000; Grahek-Ogden, et al., 2007; Jones, et al., 2003; MacDonald, et al., 2012; Sakai, et al., 2005; Zheng & Jiang, 2006).

2.3.2 Human-to-Human Transmission

Another possible route of transmission is human-human transmission. Human-to-human transmission was reported in a familial outbreak of *Y. enterocolitica* bioserotype 2/O:9 in Japan, where the bacterium is transmitted from a infected carrier to the family members through food and direct human contact (Moriki, et al., 2010). Besides that, another person-to-person *Y. enterocolitica* transmission was reported in an outbreak of diarrheal disease due to *Y. enterocolitica* serotype 0:5, biotype 1 that involved nine hospitalized patients (Ratnam, Mercer, Picco, Parsons, & Butler, 1982).

2.3.3 Animal-to-Human Transmission

Y. enterocolitica infection can occur after the contact with infected or carrier animals. Transmission is possible through direct contact between farm workers and the life stocks (i.e. animal bits or saliva) or indirectly through animal feces or water contaminated by animals. Infected dogs and cats (companion animals or stray pets) can cause human yersiniosis when they are in contact with humans, i.e. through contact with animals' excreta such as saliva and faeces (Fenwick, Madie, & Wilks, 1994; Stamm, Hailer, Depner, Kopp, & Rau, 2013; Wang et al., 2010).

2.3.4 Direct Transmission

Direct transmission is extra-intestinal disease. It is normally transmitted through skin injuries such as cut wound to a person. Many studies showed the infected persons did not show any symptoms of gastrointestinal disease but suffering abscesses (i.e. thigh abscess, axillary abscess, etc) (Gumaste, Boppana, Garcha, & Blair, 2012; Kelesidis, Balba, & Worthington, 2008; Menzies, 2010).

2.3.5 Blood Transfusion-Associated Transmission

Y. enterocolitica that occurs occasionally in blood of a healthy donor (asymptomatic, with diarrhea history) is transmitted to a recipient during blood transfusion. Following a blood transfusion, infected recipients could develop transfusion-associated sepsis or septicemia (Hoelen, Tjan, Schouten, Dujardin, & van Zanten, 2007; Leclercq, et al., 2005). Blood transfusion-associated septicemia is rare, however, the overall fatality rate calculated is about 55% (from the 55 published case reports over the year 1975-2007) (Guinet, Carniel, & Leclercq, 2011).

2.4 Classification and typing of *Y. enterocolitica*

According to Bergey´s Manual of Systematic Bacteriology, the *Y. enterocolitica* belongs to the phylum Proteobacteria, class Gammaproteobacteria, order Enterobacteriales, family *Enterobacteriaceae*, genus *Yersinia*, species *enterocolitica* (Kreig, et al., 1984). Strains of *Y. enterocolitica* are biotyped into six biovars, which include biotypes 1A, 1B, 2, 3, 4, and 5 based on their biochemical reactions (Wauters, Kandolo, & Janssens, 1987), and more than 50 serotypes according to their composition of lipopolysaccharide (LPS) antigens. Strains of *Y. enterocolitica* are further separated into three main pathotypes based on pathogenicity: high pathogenicity biotype 1B; moderate pathogenicity biotype 2, 3, 4, and 5; and no pathogenicity biotype 1A (Bari, Hossain, Isshiki, & Ukuku, 2011; Lamps, Havens, Gilbrech, Dube, & Scott, 2006).

2.5 Geographical distribution of biotypes of *Y. enterocolitica* strains

The geographical distribution of *Y. enterocolitica* is diverse. The bioserotype 1B/O:8 is referred as the American strain, is mainly found in North America followed by Japan but is extremely rare in Europe (Fukushima, Shimizu, & Inatsu, 2011). It can be found in the environment (including water) and responsible for human outbreaks. Biotypes 2, 3, 4, and 5 are referred to as the European strains or non-American strains and are mainly isolated from animals (pig and cattle) and humans and are very seldom reported to be isolated from environment (Fukushima, et al., 2011). Strains of bioserotype 3/O:3 have been frequently reported in many Asian countries like Japan, Taiwan, Korea and China (Fukushima, et al., 1997; Fukushima, et al., 2011; Lee, et al., 2004; Zheng & Xie, 1996). Members of biotype 1A (NP *Y. enterocolitica*) are widely isolated from the environment, animal and also food (Fukushima, et al., 2011; Paixão, et al., 2013).

2.6 Reservoirs of *Y. enterocolitica*

Y. enterocolitica is widely spread in nature and it has been routinely isolated from various natural sources such as animals, foods, and environment (Fredriksson-Ahomaa & Korkeala, 2003; Paixão, et al., 2013). Numerous studies have been carried out in isolating *Y. enterocolitica* from various animals from farms, wildlife, and pet animals. These animals are swine (Liang et al., 2012; Van Damme, et al., 2013), cattle (McNally, et al., 2004), sheep (Chenais, Bagge, Lambertz, & Artursson, 2012; Söderqvist, Boqvist, Wauters, Vågsholm, & Thisted-Lambertz, 2012), goats (Arnold, et al., 2006), rats (Kaneko & Hashimoto, 1981), wild boars (Fredriksson-Ahomaa, Wacheck, Bonke, & Stephan, 2011), dogs (Wang, et al., 2010), cats (Fredriksson-Ahomaa, Korte, & Korkeala, 2001), birds (Niskanen, Waldenstrom, Fredriksson-Ahomaa, Olsen, & Korkeala, 2003) and many other animals. Among them, swine is considered as a major reservoir of *Y. enterocolitica*.

Pigs are often reported to be asymptomatic carriers for strains of bioserotype 4/O:3. The prevalence of this bioserotype in pigs from farms or slaughterhouses in different countries is as follows: Belgium (11.0%) (Van Damme, et al., 2013), Italy (20.9%) (Bonardi, et al., 2013), Finland (56%) (Korte, Fredriksson-Ahomaa, Niskanen, & Korkeala, 2004), Swiss (96%) (Fredriksson-Ahomaa, Stolle, & Stephan, 2007), and southern Germany (60%) (Fredriksson-Ahomaa, Bucher, Hank, Stolle, & Korkeala, 2001). In China, the most prevalent bioserotype in pigs is 3/O:3 (844/850 strains) (Liang, et al., 2012). Other bioserotypes of *Y. enterocolitica* isolated from pigs are 2/O:9, 2/O:5,27, 1B/O:8, and biotype 1A (Fredriksson-Ahomaa, et al., 2007; Liang, et al., 2012; Paixão, et al., 2013).

Y. enterocolitica is often present in the oral cavity of pigs especially tonsils and throat, feces and lymph nodes (Gutler, Alter, Kasimir, Linnebur, & Fehlhaber, 2005; Nesbakken, Eckner, Hřidal, & Rřtterud, 2003; Novoslavskij, et al., 2013; Okwori et al.,

2009). Strains of *Y. enterocolitica* have been frequently isolated in raw pork as a result of cross contamination of the organisms via oral cavity, feces, and intestinal contents during slaughtering, cutting, further processing and distribution of fresh pork and offals (Fredriksson-Ahomaa, Bucher, et al., 2001; Fredriksson-Ahomaa, et al., 2007; Grahek-Ogden, et al., 2007; Ortiz Mart ńez, 2010; Terentjeva & Berzins, 2010). Due to the psychrotrophic behavior of *Y. enterocolitica*, it might survive and further multiply during the storage of the pork meat and other porcine products.

Other vehicles of yersiniosis include ruminant and ruminant products (Fukushima, et al., 1997), poultry (Dallal, et al., 2010), vegetables (Lee, et al., 2004; Xanthopoulos, et al., 2010), milk and dairy products (Ackers, et al., 2000; Harakeh, Saleh, Barbour, & Shaib, 2012; Yucel & Ulusoy, 2006), ready-to-eat food (MacDonald, et al., 2012; Xanthopoulos, et al., 2010) and chitterlings (Lee, et al., 1990).

In Malaysia, there is limited study on *Y. enterocolitica.* The only food related prevalence report in Malaysia was from an unpublished study of Dzomir (2005), *Y. enterocolitica* (bioserotype 1A/O:52, 53 and 1A/O:41, 42) was isolated from beef burger meat and chicken burger meat.

2.7 Isolation and detection methods for *Y. enterocolitica*

2.7.1 Conventional methods for detection of *Y. enterocolitica*

2.7.1.1 Enrichment

There are numerous enrichment schemes available in isolating *Y. enterocolitica* such as the International Standard Organisation method (ISO 10273:2003) (European Food Safety Authority & European Centre for Disease Prevention and Control, 2007), and United States Department of Agriculture (USDA) protocol (Johnson, 1998). These enrichment procedures include direct selective enrichment at higher temperature (normally at ~25 ℃) for 3 to 5 days incubation or cold enrichment (~4 ℃) that takes time up to one-month incubation.

Selective enrichment at higher temperature inhibit the growth of some background microflora (the media contain antimicrobial agents) and at the same time allow the multiplication of *Y. enterocolitica* (in low number) present in samples. Cold enrichment is useful for enrichment of *Y. enterocolitica* as psychrophilic bacteria that able to grow and multiply at 4℃. Cold enrichment in phosphate buffered saline (PBS) or in phosphate buffered saline with sorbitol and bile salts (PSB) has been widely used for clinical, environmental, and food samples (Fredriksson-Ahomaa, et al., 2011; Rahman, Bonny, Stonsaovapak, & Ananchaipattana, 2011). Some researchers claimed that cold enrichment yield better recovery of *Y. enterocolitica* (Fukushima, et al., 2011). However, no single culture protocol which has been described performed equally well for the isolation of *Y. enterocolitica* serotypes from all types of samples. In a recent study, Van Damme, et al. (2013) reported that enrichment in PSB at 25℃ recovered more positive samples than selective enrichment and cold enrichment. Irgasan-ticarcillin-potassium chlorate (ITC) broth is reportedly better in recovering of *Y. enterocolitica* 4/O:3 from pig tonsils than cold enrichment in PSB (Van Damme, Habib, & De Zutter, 2010). *Yersinia* selective enrichment broth according to Ossmer (YSEO) is also reportedly good in isolation of *Y. enterocolitica* (Hudson, et al., 2008; King & Hudson, 2006). Therefore, combination of several enrichment broths should be used concurrently for better isolation rate.

2.7.1.2 Selective or isolation agar for *Y. enterocolitica*

Following the enrichment steps, the enriched samples will be streaked onto selective medium or media for identification of presumptive *Y. enterocolitica.* Many selective agars have been used for isolation of *Y. enterocolitica* from food, clinical, environment and livestock samples. These agars include cellociose-arginine-lysine (CAL) agar (Dudley & Shotts Jr, 1979), Congo red brain heart infusion agarose (CR-BHO) agar (Bhaduri, Turner-Jones, Taylor, & Lachica, 1990), Congo red magnesium oxalate (CR-MOX) agar (Riley & Toma, 1989), Statens Serum Institut (SSI) agar (Blom, Meyer, Gerner-Smidt, Gaarslev, & Espersen, 1999), pectin agar (Bowen & Kominos, 1979), cefsulodin-irgasan-novobiocin (CIN) agar (Schiemann, 1979), *Salmonella-Shigella*-deoxycholate-calcium chloride (SSDC) agar (Wauters, Goossens, Janssens, & Vandepitte, 1988), BABY4 agar (Bercovier, et al., 1984), virulent *Yersinia enterocolitica* (VYE) agar (Fukushima, 1987), *Yersinia*-selective medium (KV202) agar (Jiang, Kang, & Fung, 2000), MacConkey (MAC) agar with Tween 80 (Lee, 1977), DYS agar (Agbonlahor, Odugbemi, & Dosunmu-Ogunbi, 1982), and MAC with 1% L-rhamnose and 1% D-arabitol (RAM) agar (Shehee & Sobsey, 2004).

Among these agars, CIN agar is reportedly to be more specific compared to other conventional selective agars such as SS, MAC, CAL, pectin agars and other lactose-containing media tested (Head, Whitty, & Ratnam, 1982). One of the weaknesses of CIN is that this medium fails to distinguish *Y. enterocolitica* from several other mannitol-fermenting bacterial species such as *Serratia liquefaciens, Enterobacter agglomerans, Aeromonas* spp., *Citrobacter* spp., and other non-pathogenic *Yersinia* spp. as all of them appear as red “bull’s eye” on CIN plates (Head, et al., 1982). Additional biochemical tests such as esculin, phenylalanine deaminase, arginine dihydrolase, hydrogen sulphide, urease, or lysine decarboxylase are needed to further differentiate *Y. enterocolitica* from the others (Weagant & Feng, 2001).

Chromogenic-based media are increasingly popular in recent years for isolation of enterobacteria. To date, two chromogenic media have been developed for the specific detection of virulent *Y. enterocolitica*. These media are named *Yersinia enterocolitica* chromogenic medium (YeCM) (Weagant, 2008) and *Yersinia enterocolitica* agar (YECA) (Denis, Houard, Labbé, Fondrevez, & Salvat, 2011). Both media allow the differentiation of virulent *Y. enterocolitica* from non-virulent *Y. enterocolitica* and other enterobacteria.

2.7.1.3 Identification of *Y. enterocolitica* by using biochemical tests

Presumptive *Y. enterocolitica* isolates from the selective agar plates will be picked and identified by biochemical tests either through conventional tube tests such as: Gram, urease, motility at 25 ℃ and 37 ℃, arginine dihydrolase, lysine decarboxylase, phenylalanine deaminase, H_2S production, indole production, Voges-Proskauer, citrate utilisation, L-ornithine, mucate, pyrazinamidase, sucrose, cellobiose, L-rhamnose, melibiose, L-sorbose, and L-fucose tests or using rapid identification kits such as API 20E, MICRO-ID, Vitek GNI Card, Gene-trak system and BBL Crystal Enteric/Nonfermenter (Archer, Schell, Pennell, & Wick, 1987; European Food Safety Authority & European Centre for Disease Prevention and Control, 2007; Linde, Neubauer, Meyer, Aleksic, & Lehn, 1999; Manafi & Holzhammer, 1994; Sharma, Doyle, Gerbasi, & Jessop, 1990; Varettas, Mukerjee, & Schmidt, 1995).

2.7.2 Polymerase chain reaction (PCR)-based method for detection of *Y. enterocolitica*

The conventional isolation methods for detection of *Y. enterocolitica* normally take approximately 3-5 days for enrichment at higher temperature (~25 ℃) and up to 3-4 weeks for cold enrichment (~4 ℃) to complete the whole sets of isolation procedures in

confirming the identity of isolates. This is laborious and time consuming. PCR-based method can be implemented to shorten the analytical process to 1 - 3 days. Besides that, PCR-based method is a rapid and sensitive technique that can identify isolates and at the same time separates pathogenic and non-pathogenic strains within the same species easily.

For detection of *Y. enterocolitica*, the *Y. enterocolitica* 16S rRNA gene region is used (Wannet, Reessink, Brunings, & Maas, 2001). For the detection of pathogenic *Y. enterocolitica*, different virulence genes are used. These genes are either plasmid- or chromosome-located. Some of the plasmid-located genes are the *virF* gene (Bhaduri & Pickard, 1995; Thoerner, et al., 2003) and *yadA* gene (Lantz, et al., 1998) that responsible for transcriptional activator for many *Yersinia* outer membrane proteins. The chromosome located genes are: the *Yersinia* heat stable enterotoxin gene (*yst*) (Gómez-Duarte, Bai, & Newell, 2009; Thoerner, et al., 2003), the *ail* gene for the attachment invasion locus (Bhaduri & Pickard, 1995; Wannet, et al., 2001); the invasin gene (*inv*); and the *rfbC* gene (Weynants, Jadot, Denoel, Tibor, & Letesson, 1996) located within the *rfb* cluster responsible for the biosynthesis of the O-side chain of *Y. enterocolitica* serotype O:3.

2.8 Characterization

2.8.1 Biotyping and serotyping

Biotyping is essential in the differentiation of pathogenic and non-pathogenic *Y. enterocolitica* strains; whereas serotyping is useful in subgrouping the *Y. enterocolitica* strains within each biotype. According to Wauters, et al. (1987) eight biochemical tests are applied for biotype of *Y. enterocolitica* (Table 2.1) and serotyping is done by using commercial O-antisera.

Table 2.1. Biotyping scheme for *Y. enterocolitica* [a].

Biochemical tests	Biotypes[b]					
	1A	1B[c]	2[c]	3[c]	4[c]	5[c]
Lipase (Tween-esterase)	+	+	-	-	-	-
Esculine/salicin 24h[d]	+, -	-	-	-	-	-
Indole	+	+	(+)[e]	-	-	-
Xylose	+	+	+	+	-	V[f]
Trehalose/NO_3[g]	+	+	+	+	+	-
Pyrazinamidase	+	-	-	-	-	-
β-D-Glucosidase	+	-	-	-	-	-
Voges-Proskauer(VP)	+	+	+	+[h]	+	(+)
DNase	-	-	-	-	+	+

[a] **Modified from Wauters, et al. (1987);** [b] reactions from tests incubated at 25-28 ℃, with the exception of β-D-Glucosidase whichwas incubated at 30 ℃ and salicin which was incubated at 35 ℃. Incubation at other temperatures may result in different results and biotypings; [c] biotype contains pathogenic strains; [d] esculin and salicin reactions for a given strain of *Y. enterocolitica* are nearly always identical so they are listed together in this table; [e] indicates a delayed positive reaction; [f] Indicates variable reactions; [g] trehalose and nitrate reduction reactions for a given strain of *Y. enterocolitica* are nearly always identical so they are listed together in this table; [h] rarely, a serotype O:3 strain may be negative for VP.

2.8.2 Genotyping

There are numerous genotyping methods available in comparing the genetic relatedness of *Y. enterocolitica* strains. These methods include restriction endonuclease analysis of plasmid (REAP), restriction endonuclease analysis of chromosome (REAC) and Southern blotting, ribotyping, ramdomly amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), multiple-locus variable number tandem repeat analysis (MLVA), and DNA sequencing (Fredriksson-Ahomaa, Stolle, & Korkeala, 2006; Virtanen, et al., 2013). Among them, PFGE is the most widely used subtyping method with good discriminatory power and excellent typeability and reproducibility (Fredriksson-Ahomaa, Stolle, Siitonen, & Korkeala, 2006).

PFGE is a technique used for separation of large-sized DNA fragments of the whole bacterial genome (restricted with various rare-cutting restriction enzymes) by applying to a agarose gel with electric field that changes periodically in direction. PFGE is considered the gold standard in bacterial subtyping because it provides highly reproducible restriction profiles as compared to many other genotyping methods. The

most frequently used restriction enzyme in PFGE typing of *Y. enterocolitica* strains is *Not*I, followed by *Xba*I. Paixão, et al. (2013) compared PFGE with single enzyme (SP)-AFLP and Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR, and found that PFGE was the most discriminative technique in subtyping the *Y. enterocolitica* strains. Several studies showed that PFGE allows subtyping of strains that belong to the same or different bioserotype (Fredriksson-Ahomaa, Cernela, Hächler, & Stephan, 2012; Liang, et al., 2012; Lucero Estrada, et al., 2011; Paixão, et al., 2013).

2.8.3 Virulence factors

The virulence of the pathogenic *Y. enterocolitica* biotypes (1B and 2 to 5) depends on the presence of the ~70 kb virulence plasmid (pYV plasmid), Ysc-Yop type III secretion system (TTSS), chromosomal-encoded virulence genes including *ail, myfA, ystA, ysa,* and the high pathogenicity island- (HPI-) associated iron acquisition system (Cornelis, et al., 1998; Revell & Miller, 2001). More than 15 virulence genes have been discovered currently that are associated with the virulence of *Y. enterocolitica* (Table 2.2). In order to develop a full virulence of pathogenic *Y. enterocolitica,* the strains require the expression of the virulence genes that are located in chromosome and pYV plasmid. However, all these virulence genes are not necessarily present and expressed simultaneously in the pathogenic strains (Zheng, Sun, Mao, & Jiang, 2008).

The biotype 1A is considered nonpathogenic primarily due to the loss of virulence pYV plasmid and most of the chromosomal virulence genes such as *ail, myfA, ystA, ysa,* and TTSS, and only occasionally carry *myfA* and *ystA* (Kot, Piechota, & Jakubczak, 2010). Although the biotype 1A strains are nonpathogenic, they are frequently reported to cause gastrointestinal disease in humans (Pham, Bell, & Lanzarone, 1991; I. Singh, Bhatnagar, & Virdi, 2003; Stephan, et al., 2013). The virulence genes such as *ail*, *ystA, ystB, virF* and *yadA* that are normally present in the

pathogenic *Y. enterocolitica* were found in the biotype 1A strains (Paixão, et al., 2013; Sihvonen, Hallanvuo, Haukka, Skurnik, & Siitonen, 2011; Stephan, et al., 2013; H. Zheng, et al., 2008).

2.8.4 Antimicrobial susceptibility profiles

In general, the antimicrobial susceptibility patterns for *Y. enterocolitica* reported by researchers world-wide are different. This may because of the impact of geographical location, local selective pressure and other factors that causes the deviation in the antimicrobial resistance among the strains from different places. However, *Y. enterocolitica* is normally resistant to penicillin, ampicillin and first generation of cephalosporins (Fàbrega & Vila, 2012). In Malaysia, there is limited information on the resistance status of the indigenous strains of *Y. enterocolitica*. In other countries, *Y. enterocolitica* strains isolated from pigs are sensitive to aztreonam, cefotaxim, ciprofloxacin, chloramphenicol, colistin, gentamicin, nalidixic acid and tetracycline, and moderately susceptible to amoxicillin/clavulanic acid. *Y. enterocolitica* strains associated with human infections in Switzerland are sensitive to ceftazidim, ciprofloxacin and gentamicin, and resistant to ampicillin and cefalothin (Fredriksson-Ahomaa, et al., 2012). In China, majority of the *Y. enterocolitica* strains isolated from diarrheal patients are reported susceptible to third-generation cephalosporins, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole, and only small portion is susceptible to the first-generation cephalosporins and penicillins (Zheng, et al., 2008).

Table 2.2. Virulence-associated determinants of pathogenic *Y. enterocolitica.*

Genes	Determinant	Function	References
pYV plasmid			
yadA[c]	YadA, a *Yersinia* outer membrane protein adhesin A	Major adhesion for attachment, being essential for induction of disease and protects the bacterium being killed by neutrophils; involved in autoagglutination, after growth in tissue culture medium at 37 ℃.	(Cornelis, et al., 1998)
virF[c]	Transcriptional regulator	Transcriptional activator controlling the *yop* regulon and responsible for the effect of temperature on the production of the Yops.	(Rouvroit, Sluiters, & Cornelis, 1992)
ysc, *ysa*	Yops, a type III protein secretory apparatus	Resistance to phagocytosis, complement-mediated lysis and allow the proliferate extracellularly in tissues.	(Cornelis, Sluiters, De Rouvroit, & Michiels, 1989)
tccC	Insecticidal toxin-like protease	Virulence expression	(Bhagat & Virdi, 2007)
Chromosome			
inv[c]	Invasin, an outer membrane protein	For efficient translocation of bacteria across the intestinal epithelium	(Miller & Falkow, 1988)
ail[c]	Ail, an outer membrane protein	Contribute to adhesion, invasion, and resistance to complement-mediated lysis	(Miller & Falkow, 1988; Pierson & Falkow, 1993)
ystA[c], *ystB*, *ystC*	Yst, *Yersinia* stable heat-stable enterotoxin	Contribute to the pathogenesis of diarrhea associated with acute yersiniosis	(Huang, Yoshino, Nakao, & Takeda, 1997; Robins-Browne, Still, Miliotis, & Koornhof, 1979; Thoerner, et al., 2003)
myfA[c]	MyfA protein, the major subunit of *Y. enterocolitica* Myf fimbriae	Fimbrial antigen and putative adhesin	(Zacharczuk & Gierczyński, 2010)
fepA, *fepD*	Enterochelin receptor protein and enterochelin ABC transporter, respectively	Enterochelin transport	(Schubert, Fischer, & Heesemann, 1999)
fes	Enterochelin esterase	Release of iron during enterochelin transport	(Schubert, et al., 1999)
ymoA	*Yersinia* modulator	Modulating the expression of virulence functions	(Cornells et al., 1991; Grant, Bennett-Wood, & Robins-Browne, 1998)
hreP	Subtilisin/kexin-like protease/ invasion protein	Bacterial invasion	(Bhagat & Virdi, 2007; Heusipp, Young, & Miller, 2001)
sat	Streptogramin acetyltranferase	Polypeptide antibiotics inhibiting protein	(Bhagat & Virdi, 2007; Seoane & Lobo, 2000)

[c]classical virulence markers

2.8.5 Usage of antimicrobial agents in food-producing animals

It has been well documented that large amounts of drugs are used in food-producing animals, either in feeds or medications in the control, prevention and treatment of disease or promotion of growth (Cromwell, 2002; Rajić, Reid-Smith, Deckert, Dewey, & McEwen, 2006; Rosengren, Gow, Weese, & Waldner, 2010). In Malaysia, the most commonly used antimicrobial agents in animal feeds are procaine penicillin, streptomycin, chlortetracycline, tylosin, oxytetracycline, neomycin, sulphonamides, and spiramycin (Mohamed, Nagaraj, Chua, & Wang, 2000). The application of antimicrobials in food production has successfully suppressed or inhibited the growth of some pathogens and minimised benefits loss to animal industry. However, the indiscriminate use of antimicrobial agents in animals leads to the selection of the antimicrobial resistant bacteria. Several reports indicated that the excessive usage of antimicrobial agents in food-producing farms caused increment in the resistance rates in enteric pathogens and other bacteria (Oliver, Murinda, & Jayarao, 2011; Rajić, et al., 2006; Varga et al., 2009). The release of drug residues and the selection of "superbug" from the food animals to the environment and final consumer (humans) are of a public health concern. Therefore, investigation of the antimicrobial profiles of *Y. enterocolitica* isolated in Malaysia is very important in understanding the current resistant trend in this country.

2.8.6 Treatment and prevention of yersiniosis in humans

According the World Health Organization (WHO) and Centre of Disease Control and Prevention, USA (CDC)'s recommendations, no antimicrobial treatment will be given to normal gasterointestinal infection. Yersiniosis is normally self-limiting, and infected personnel will recover by simple medication and proper rest. Antimicrobials treatment is normally given to patients who are suffering enterocolitis, septicaemia or invasion infection. The recommended antimicrobials given are

tetracyclines, chloramphenicol, trimethoprim-sulphamethaxazole, second and third generation of cephalosporins, ciprofloxacin, fluoroquinolones and aminoglycosides (WHO; CDC). In reducing the emergence of antimicrobial resistant *Y. enterocolitica* strains, the antimicrobial profile of the bacterial strains has to be known in order to avoid blind treatment and guarantee effective treatment in patients.

The prevention of infection with *Y. enterocolitica* depends mainly on good hygiene practices during food processing and food preparation. Poor sanitation and improper cooking or sterilization technique by food handlers are known as the main reason for yersiniosis outbreak. Food handlers should also prevent cross-contamination in the kitchen in using separate cutting boards for meat and other foods. Cutting boards, utensil and other dishes should wash with soap and hot water each time after raw meat preparation. Good hygiene practices also include washing hands thoroughly with soap and water before eating, before and after food preparation and after contact with animals. Consumers should avoid eating raw or undercooked pork or other meat products, consume only pasteurized milk or milk products. For pet's owners, animal faeces are disposed in a sanitary manner.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Media

All the media used for isolation, biochemical tests, culturing and characterization of *Yersinia enterocolitica* are listed in Appendix I.

3.1.2 Chemicals and reagents

Chemicals and reagents used in this study are listed in Appendix II.

3.1.3 Buffers and solutions

Buffers and solutions used in this study are listed in Appendix III.

3.2 Isolation and characterization of *Y. enterocolitica* from raw food samples and swine

3.2.1 Sampling

3.2.1.1 Raw pork products

Between June 2010 to March 2011, 58 raw pork samples were sampled from wet markets at selected states in Peninsular Malaysia (Kuala Lumpur, Perak and Pahang) (Table 3.1). The location of the slaughtering houses from where the raw pork samples came from was unknown because the information was disclosed in the slaughtering house. Background information of the sampling sites, samples and date of sampling is tabulated in Appendix IV. A convenience sampling was performed in choosing the sampling locations. Random selection of participating wet markets was not possible in this observational study as the distribution and number of wet markets selling raw pork and pork products in each state is limited and unknown.

The raw pork samples were further grouped into three categories as raw pork meats (n=25), raw pork internal organs (n=23) and other parts (n=10) (Table 3.2). All

samples in this study were transferred in sterile plastic bags and transported in ice box to the laboratory.

Table 3.1. Locations of wet markets and number of samples collected.

Location	No. of samples collected, n
Kuala Lumpur	**48**
Wet market A	36
Wet market B	12
Perak	**9**
Wet market C	4
Wet market D	5
Pahang	**1**
Wet market G	1
Total samples collected, N	**58**

Table 3.2. Sample types collected from wet markets.

Sample types	No. of samples collected, n
Raw pork meats	**25**
Whole pork	21
Minced pork	3
Raw pork internal organs	**23**
Liver	5
Intestine	8
Heart	5
Kidney	4
Throat	1
Other parts	**10**
Skin	4
Foot	2
Fat tissue	1
Ear	1
Eye tissue	1
Nose	1
Total samples collected, N	**58**

3.2.1.2 Raw non-porcine food

Forty-eight raw non-porcine food were purchased from wet markets (located in Kuala Lumpur, Selangor and Pahang) and examined for the presence of *Y. enterocolitica* (Table 3.3). Background information of the sampling sites, samples and date of sampling are tabulated in Appendix V. A convenience sampling was performed

in choosing the sampling locations. Random selection of participating wet markets was not possible in this observational study as the distribution and number of wet markets in each state was unknown. Food types purchased included raw vegetables (n=19), raw seafood (n=11), raw poultry products (n=9), raw beef (n=6), tofu (n=2), and pasteurised milk (n=1) (Table 3.4).

Table 3.3. Location of wet markets and number of samples collected.

Location	Number of samples collected, n
Kuala Lumpur	***10***
Wet market A	7
Wet market B	3
Selangor	***18***
Wet market E	11
Wet market F	7
Pahang	***20***
Wet market G	20
Total samples collected, N	**48**

Table 3.4. Sample type collected from wet markets.

Food type	No. of samples
Raw beef	***6***
Raw poultry products	***9***
Chicken meat	8
Chicken claw	1
Raw seafood	***11***
Fish	6
Squid	3
Prawn	1
Cockles	1
Raw vegetables	***19***
Leafy vegetables	11
Bitter gourd	3
Cowpea	1
Root	1
Sweet potato	1
Brinjal	1
Lady's finger	1
Raw tofu	***2***
Pasteurised milk	***1***
Total	**48**

3.2.1.3 Pigs (Swab specimens)

The presence of *Y. enterocolitica* from pigs in selected farms was investigated during the period of October 2010 to September 2011. Random selection of participating pig farms was not possible in this observational study as farm access and selections were limited. The sampling schedules were dependent on the availability of the veterinary doctor. A total of nine pig farms located in three states in middle- and north- western part of Peninsular Malaysia (Table 3.5), i.e., Selangor (Farms A, B, C), Perak (Farms D, E, F), and Penang (Farms G, H, I) were enrolled in this study. Pig industry in Malaysia is highly condensed and commercialised in Penang, Perak and Selangor and more than 100 farms are located in these area. Background information of the sampling sites, number of pigs, samples and date of sampling are tabulated in Appendix VI. These three states are important pig-producing states in Malaysia (Department of Veterinary Services, Malaysia, 2011).

A stratified random sampling was performed in categorising the pigs based on the general health condition, i.e. healthy (pigs without prominent disease symptoms) and unhealthy (sick, weak and runt). A total of 165 pigs were selected (Table 3.5; farms A, n=9; B, n=14; C, n=30; D, n=20; E, n=20; F, n=20; G, n=16; H, n=20; I, n=16) and three specimens (nasal, oral and rectal swabs) were collected from each pig and maintained in Cary-Blair transport medium (Oxoid, UK). Age grouping of pig is tabulated in Table 3.6. Pigs with similar age group are fed in the same pens in the farms and the age groups were recorded at the time of the sampling. A veterinary doctor determined the age group determination during the sampling.

Table 3.5. Location of pig farms and number of pigs and samples collected.

Location	Number of pigs	Number of samples
Selangor	***53***	***159***
Farm A, Tanjung Sepat	9	27
Farm B, Tanjung Sepat	14	42
Farm C, Tanjung Sepat	30	90
Perak	***60***	***180***
Farm D, Gopeng	20	60
Farm E, Sungai Siput	20	60
Farm F, Sungai Siput	20	60
Penang	***52***	***156***
Farm G, Sungai Jawi	16	48
Farm H, Kampung Selamat	20	60
Farm I, Kampung Selamat	16	48
Total samples collected, N	**165**	**495**

Table 3.6. Age grouping of pig.

Group	Age
Piglet	< 4 weeks
Weaner	1 –2 months
Grower	2 – 4 months
Finisher	4 – 6 months
Sow	Mother pig

3.2.2 Isolation methods

3.2.2.1 Enrichment methods for raw food samples

3.2.2.1.1 Normal enrichment

Raw food were analysed for the presence of *Y. enterocolitica* by conventional culture methods and post-enrichment PCR screening. Enumeration of *Y. enterocolitica* was performed using a 3×3 most probable number (MPN) method.

Five g of raw food sample was cut into small pieces, added to 45 ml of selective enrichment broth in sterile plastic bag and homogenised manually by hand. Enrichment broths used were phosphate buffered saline (PBS, Sigma, Germany), *Yersinia* selective enrichment broth according to OSSMER (YSEO, Merck, Germany), and irgasan-ticarcillin-potassium chlorate (ITC) broth [ITC broth base (Fluka, Germany) supplemented with ticarcillin supplement (Fluka) and potassium chlorate supplement

(Fluka)]. Food homogenates in ITC and PBS were incubated at 25 ℃ for 2 days and 4 ℃ for 3 weeks, respectively, and food homogenate in YSEO was used for MPN enrichment for food safety enumeration. The enrichment was followed by plating onto selective agars for isolation of presumptive *Y. enterocolitica.*

3.2.2.1.2 MPN enrichment and MPN calculation

After the food particles in YSEO settled down, the fluid was dispensed into a 3 × 3 MPN system consisting of 10 ml of undiluted fluid in each of three 10 ml test tubes (level A), 1 ml of fluid in 9 ml YSEO broth in each three 10 ml test tubes (a 1:10 dilution, level B), and 1 ml of a 1:10 dilution of the fluid in 9 ml YSEO broth in each three 10 ml test tubes (a 1:100 dilution, level C), incubated at 25 ℃ for 18 h (Hudson, et al., 2008). The MPN enrichment was followed by plating onto selective agars for isolation of presumptive *Y. enterocolitica.*

The three digits for each level in the 3 × 3 MPN system were determined based on the post-enrichment PCR screening results (the YSEO enriched tubes). One ml of each post-enrichment tube was retained in a sterile 1.5 ml microfuge tube after its respective incubation period. DNA extraction and PCR screening as described in Section 3.2.4.2 were performed. The MPN/g value was calculated using the Microsoft Excel spreadsheet provided by Institute of Environment Science and Research (ESR), New Zealand (Hudson, et al., 2008). The range over which these nine tubes MPN system operates was between 0.30 MPN/g (lower confidence interval, LCI of 0.07 with one positive at level A) to 44.84 MPN/g (upper confidence interval, UCI of 198.70). The MPN step was not performed for raw non-porcine food and swine specimens. MPN determination was not applicable to swine specimens since the specimens were not categorized as food samples.

3.2.2.2 Enrichment method for swine specimens

Swine specimens were processed with two methods, i.e. (i) direct streaking on selective agar plates and (ii) enrichment in ITC and PBS broths (as described in Section 3.2.2.1.1) followed by streaking on selective agar plates. Direct streaking was used to replace the MPN enrichment method used for raw food samples (swab specimens are not categorized as food). The enrichment was followed by plating onto selective agars for isolation of presumptive *Y. enterocolitica.*

3.2.2.3 Plating on selective media

A loopful of each enriched samples was streaked onto selective agars. Selective agars used were cefsulodin-irgasan-novobiocin (CIN) agar [*Yersinia* Selective Agar Base supplemented with *Yersinia* Selective Supplement (Oxoid, UK)] or modified CIN, and incubated at 25 ℃ for 24-48 h. Modified CIN was made by adding 1% L-arginine (Sigma), 0.8 g/l ferric ammonium citrate (BDH Prolabo, UK), 6.8 g/l sodium thiosulphate (BDH Prolabo), and 2.0 g/l DL-phenylalanine (Sigma) at pH 7.4 ± 0.02 into the CIN agar (Appendix I). In parallel, sample was plated onto CIN agar immediately after alkaline treatment in which 0.5 ml of enriched culture was transferred into 4.5 ml of 0.25% potassium hydroxide (KOH): 0.50% sodium chloride (NaCl) solution (Aulisio, et al., 1980; Hudson, et al., 2008).

3.2.3 Preliminary biochemical tests

At least five typical *Y. enterocolitica* isolates (red bull's eyes) were picked from each plate and tested with four preliminary biochemical tests, i.e. oxidase test, Gram determination, urease and citrate test. All Gram negative, oxidase negative, urease positive and citrate negative isolates were further characterized by using the API 20E identification kit (bio-Mérieux® SA, France). Details of each preliminary biochemical

test and API identification kit are tabulated in Appendix VII and VIII, respectively. The strips were inoculated according to the manufacturer's recommendations with the minor modification of incubation at 28 ℃ (Archer, et al., 1987). The 7-digit API 20E numerical profile obtained was read by using the online APIweb™ identification software.

3.2.4 PCR confirmation

3.2.4.1 Identification of *Y. enterocolitica* isolates

Identity of *Y. enterocolitica* isolates was confirmed by using a duplex PCR targeting *Y. enterocolitica*-specific 16S rRNA and *ail* genes. PCR mixes and the cycling conditions are tabulated in Tables 3.7 and 3.8. PCR amplicons were electrophoresed on 1.5% agarose gel subjected in 0.5× TBE buffer at 100 V for 30 min. The gel was stained in 3× GelRed™ (Biotium, USA) staining solution for 30 min and gel photo was captured using GelDoc™ XR imaging system (Bio-Rad, USA) under UV light. Amplicons of selected PCR products were purified using MEGAquick-spin™ PCR & agarose gel DNA extraction system (iNtRON Biotechnology, Korea) and then submitted to a company (1st BASE) for sequencing. Sequenced genes were analysed using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/).

Table 3.7. Conditions of PCR mixes for duplex PCR targeting *Y. enterocolitica*-specific 16S rRNA and *ail* genes.

Materials	**Stock conc.**	**Working conc.**	**1 × (µl)**
Buffer (×)	5	1	5.000
$MgCl_2$ (mM)	25	3.50	3.500
dNTPs (mM)	10	0.10	0.250
Primers (µM)			
Y. enterocolitica-specific 16S rRNA gene	10	0.08	0.200
ail gene	10	0.16	0.400
Taq polymerase (U/µL)	5	0.50	0.100
ddH_2O	-	-	12.950
DNA templates (ng)	-	~20	2.000
Total			**25.000**

3.2.4.2 Post-enrichment PCR screening from enriched food homogenates

One ml of each enriched samples (YSEO, ITC, and PBS) were retained in a sterile 1.5 ml Eppendorf tube after its respective incubation period. The fluid was centrifuged at 13,400 *g* for 15 min, and the supernatant was discarded. Washing steps for the cell pellet initiated by re-suspension in 1 ml 1 × Tris-EDTA (TE) buffer, and the suspension was centrifuged at 13,400 *g* for 15 min and the supernatant was discarded. The washing step was repeated twice by substituting 1 ml 1×TE buffer with 1 ml sterile ddH_2O water. Finally, the pellet was re-suspended in 100 µl sterile ddH_2O and transferred into a 0.5 ml PCR tubes. The suspension was boiled at 99 ℃ for 5 min, snapped-cold in ice for 10 min and centrifuged at 13,400 *g* for 10 min. The supernatant was used as DNA template. The presence of *Y. enterocolitica* was screened by using the duplex PCR as stated in Section 3.2.4.1.

3.2.5 API 50CH

Isolates that were API 20E identified as *Y. enterocolitica* but negative by using the duplex PCR (as in Section 3.2.4.1) were further identified by using API 50CH identification kit (bio-Mérieux® SA). The kits were inoculated according to the manufacturer's recommendations with the minor modification of incubation at 28 °C. The API 50CH numerical profile obtained was read by using the online APIweb™ identification software.

Table 3.8. Primers sequences and cycling condition of duplex PCR targeting *Y. enterocolitica*-specific 16S rRNA and *ail* genes.

Gene	Primers sequence (5'→3')	Amplicon size (bp)	PCR conditions	References
Y. enterocolitica-specific 16S rRNA	Forward (Y1) – AATACCGCATAACGTCTTCG Reverse (Y2) – CTTCTTCTGCGAGTAACGTC	330	Predenaturation: 94 ℃, 5 mins Denaturation: 94 ℃, 45 s Annealing: 57 ℃, 45 s Extension: 72 ℃, 30 s (30 cycles) Final extension: 72 ℃, 7 mins	(Neubauer, Hensel, Aleksic, & Meyer, 2000)
ail	Forward (A1)– TTAATGTGTACGCTGCGAGTG Reverse (A2) – GGAGTATTCATATGAAGCGTC	430		(Wannet, et al., 2001)

3.2.6 Biotyping of *Y. enterocolitica* isolates

The biogroup of *Y. enterocolitica* was determined by using biochemical tests as described by Wauters, et al. (1987). The biochemical tests included lipase test, esculin hydrolysis, salicin utilisation, indole test, xylose utilisation, trehalose utilisation, nitrate reduction, pyrazinamidase test, β-D-glucosidase test, Voges-Prokauer test, and DNase test. Details of each test are tabulated in Appendix IX. Table 2.1 indicates the biotyping scheme for *Y. enterocolitica.*

3.2.7 Serotyping of *Y. enterocolitica* isolates

Serotyping was determined based on antigenic variations in cell wall lipopolysaccharides (LPS). The serotype of *Y. enterocolitica* was determined by using the O-Antisera "SEIKEN" set purchased (DENKA SEIKEN Co., Ltd, Japan). The kit can determine six *Y. enterocolitica* serotypes (O:1, O:2, O:3, O:5, O:8, and O:9). Results were interpreted according to recommendation of manufacturer.

3.2.8 Further characterization of *Y. enterocolitica* isolates

3.2.8.1 Cultures selection

Cultures selection were based on the results of the basic biochemical tests (Gram, citrate and oxidase tests), API 20E profiles, biogrouping and serotyping. Isolates (with similar sample code) that showed similar results were referred as replicates and only one of them was selected for further characterization. In total, 32 *Y. enterocolitica* isolates (pig, n=20; food n=12) were selected for further characterization in this study.

3.2.8.2 PCR-based virulence gene determination

Crude DNA of the 32 selected *Y. enterocolitica* isolates were prepared by direct cell lysate method. Single well-isolated colony was suspended in 100µl sterile ddH_2O and boiled at 99 ℃ for 5 min and cooled immediately in ice for 10 min. This step was

the heat-shock step that facilitates the released of DNA from cell lyses. Cell lysate was centrifuged at 13,400 rpm for 5 min and the supernatant was used as the DNA template. The presence of 15 virulence genes (*hreP, virF, rfbC, myfA, fes, sat, fepD, inv, ail, ymoA, tccC, yadA, fepA, ystB,* and *ystA*) was detected using five multiplex PCRs. Cycling and PCR mix conditions were re-optimised. *Y. enterocolitica* strains IP102, IP11105, IP383, IP135, IP145, and IP178 were used as the positive controls (kind gifts of Dr. Elisabeth Carniel from Institute Pasteur, the French *Yersinia* Reference laboratory, France).

PCR amplicons were electrophoresed on 2% agarose gel subjected in 0.5× Tris-borated EDTA (TBE) buffer at 100 V for 40 min. The gel was stained in 3× GelRed™ (Biotium) staining solution for 20 min and gel photo was captured using GelDoc™ XR imaging system (Bio-Rad) under UV light. The validity of the amplicons was determined by direct sequencing. Briefly, the PCR products were purified using MEGAquick-spin™ PCR & agarose gel DNA extraction system (iNtRON Biotechnology) and then submitted to a company (1st BASE) for sequencing. Sequenced genes were analysed using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/).

3.2.8.3 Plasmid profiling

3.2.8.3.1 Phenotypic virulence plasmid tests

The presence of pYV virulence plasmid of *Y. enterocolitica* was determined phenotypically by the following tests; temperature-dependent auto-agglutination in MR-VP broth (Oxoid) (Farmer 3rd, Carter, Miller, Falkow, & Wachsmuth, 1992), calcium dependency and Congo red absorption using Congo red magnesium oxalate (CR-MOX) agar (Riley & Toma, 1989) and crystal violet binding (Bhaduri, Conway, & Lachica, 1987). Details of each test are tabulated in Appendix XI.

3.2.8.3.2 PFGE of unrestricted DNA plugs

The same DNA plugs prepared in for PFGE genotyping (Section 3.2.8.5.1) were used. DNA plug slice (3.5 mm wide × 6 mm length) was cut and loaded onto 1% agarose gel (Type 1, Sigma-Aldrich). Genomic DNA (without enzyme restriction) was separated using Chef-Mapper system (Bio-Rad) at a ramping time from 1-12 s over 14 h at 6 V/cm, 15 ℃. Low range PFG marker N0350S (New England Biolabs, USA) was used as DNA standard marker. Bacterial strain IP383 (*Y. enterocolitica* bioserotype 2/O:9) that carried the pYV (~70kb) plasmid was used as the control strain.

3.2.8.3.3 Plasmid DNA extraction

A single bacterial colony of *Y. enterocolitica* was cultured in 10 ml of Luria Bertani (LB) broth, incubated at 28 ℃ for 16-18 h. Cells were harvested by centrifugation at 8000 rpm for 3 min at 4 ℃. Plasmid DNA was extracted according to the recommendation of manufacture using QIAprep® Spin Miniprep kit (QIAGEN, Netherland). Extracted plasmid DNA was electrophoresed on 0.9% agarose gel subjected in 0.5× TBE buffer at 90 V for approximately 10 h. Supercoiled DNA marker set (8-28 kb, Epicentre® Biotechnologies, USA) and supercoiled DNA ladder (2-10 kb, New England Biolabs) were used as plasmid markers.

3.2.8.3.4 Gel staining and imaging

The gels were stained in 3× GelRed™ (Biotium) staining solution for 40 min and gel photos were captured using GelDoc™ XR imaging system (Bio-Rad) under UV light. The plasmid sizes were determined by using Quantity One® 1-D Analysis software.

3.2.8.4 Antimicrobial susceptibility testing

The antimicrobial susceptibility of the 32 selected *Y. enterocolitica* isolates was tested on Mueller-Hinton II agar (BD, USA) with commercial antimicrobial discs (Oxoid, UK) by using the disc diffusion method (Bauer, Kirby, Sherris, & Turck, 1966). Antimicrobials representing 11 different classes were tested (cephalosporins, quinolones/fluoroquinolones, folate pathway inhibitor, aminoglycosides, penicilins, tetracyclines, β-lactam, monobactams, carbapenems, polymixins, phenicol and lincosamide). The antimicrobial agents used were: cefuroxime (CXM, 30 μg), ceftriaxone (CRO, 30 μg), cefotaxime (CTM, 30 μg), ceftazidime (CAZ, 30 μg), nalidixic acid (NAL, 30 μg), ciprofloxacin (CIP, 5 μg), levofloxacin (LVX, 5 μg), trimethoprim (TMP, 5 μg), trimethoprim-sulphamethoxazole (TIM, 25 μg), streptomycin (STR, 10 μg), kanamycin (KAN, 30 μg), amikacin (AMK, 30 μg), gentamicin (GEN, 10 μg), neomycin (N, 10 μg), netilmicin (NET, 30 μg), ampicillin (AMP, 10 μg), ticarcillin (TIC, 75 μg), tetracycline (TET, 30 μg), doxycycline (DOX, 30 μg), amoxicillin-clavulanic acid (AMC, 30 μg), aztreonam (ATM, 30 μg), imipenem (IPM, 10 μg), polymyxin B (PB, 300 μg), chloroamphenicol (CHL, 30 μg), and clindamycin (CLI, 2 μg).

Four other commonly used antimicrobials in pig farms were also included amoxicillin (AMX, 25 μg), colistin sulphate (CSS, 10 μg), enrofloxacin (ENR, 5 μg) and spectinomycin (SPT, 100 μg). All *Y. enterocolitica* isolates were also phenotypically screened for ESBL-production using the modified double-disc synergy test (DDST) (Jarlier, Nicolas, Fournier, & Philippon, 1988). All plates were incubated at 37℃ and results were interpreted (16-18h) according to the Clinical and Laboratory Standards Institute (CLSI) 2012 guidelines (Cockerill, et al., 2012).

Escherichia coli ATCC 25922 was used as a control strain. Multiple antibiotic resistance (MAR) indexing was calculated by referring to Krumperman (1983). Isolates

with MAR index value s a cwere considered originated from high-risk source of contamination (Krumperman, 1983). Multidrug-resistant (MDR) was defined as resistance to om high-risk source of contami

3.2.8.5 Pulsed-field gel electrophoresis (PFGE)

The genetic relatedness of the 32 selected *Y. enterocolitica* isolates was compared by using PFGE.

3.2.8.5.1 DNA plugs preparation

Colonies of an overnight cell culture (incubated at 28 ℃) on Brain Heart Infusion (BHI) agar (Oxoid) were picked and suspended into 2 ml cell suspension buffer (CSB) and cell density was adjusted to 0.8-0.9 by using Dade Microscan turbidity metre (Baxter Diagnostics, Inc., McGraw Park, III). Twenty µl of Proteinase K (20 mg/ml stock, Promega) and 120 µl melted 1% Seakem Gold agarose (maintained at 55-60 ℃; Cambrex Bio Science Rockland, Inc, USA) were added and mixed-gently into 100 µl of the cell suspension. The suspension was dispensed immediately into well of a DNA plug mould and left to solidify at room temperature for 10-15 min. The plugs were lysed in 2 ml cell lysis buffer (CLB) and 10 µl Proteinase K (20 mg/ml). The plugs were incubated in shaking water bath (54 ℃, 175 rpm) for 4 h.

The plugs were washed with two times sterile ddH_2O (15-20 ml, preheated to 50 ℃) followed by six times 1×TE buffer (15-20 ml, preheated to 50 ℃) at room temperature for 15 min with agitation at 100-150 rpm. Plugs were kept in 2 ml 1×TE buffer at 4 ℃ while waiting to be used.

3.2.8.5.2 Restriction digestion of DNA plugs

A slice of the DNA plug (2 mm wide × 8 mm length) was cut and pre-restricted in pre-restriction buffer mixture [containing 1× multicore restriction buffers (Promega) and 0.1 mg/ml Bovine serum albumin (BSA) (Promega)] at 37 ℃ for 10 min. Pre-restriction buffer was removed and restriction master mix [containing 1× multicore restriction buffers (Promega) and 0.1 mg/ml BSA (Promega) and 24 U of *Not*I (Promega) restriction enzyme] was added. Sample was incubated in 37 ℃ overnight.

3.2.8.5.3 DNA standard size marker for PFGE

The PulseNet size standard, *Salmonella enterica* serovar Braenderup H9812 was used as standard size marker. The DNA plug was prepared as described in Section 3.2.8.5.1 and restricted as described in Section 3.2.8.5.2 by replacing the restriction enzyme with *Xba*I.

3.2.8.5.4 Pulse-field electrophoresis condition

Restricted plug slices were loaded onto 1% agarose gel (Type 1, Sigma-Aldrich, Germany). DNA fragments were separated using the Chef-Mapper system (Bio-Rad) at a ramping time from 2–20 s over 25 h at 6V/cm, 14 ℃ (Wang, et. al., 2008). The gel was stained in 3× GelRed™ (Biotium) staining solution for 40 min and gel photo was captured using GelDoc™ XR imaging system (Bio-Rad) under UV light.

3.2.8.5.5 Data analysis

PFGE gels were analyzed. The dendrogram was constructed by using the BioNumerics 6.0 software (Applied Maths, Kortrijk, Belgium). The Dice coefficient and the unweighted pair group method with arithmetic average (UPGMA) were applied, with a band position tolerance of 1.5%. The discriminatory power of PFGE was

calculated based on the Simpson's index of diversity (*D* value) (Hunter & Gaston, 1988).

3.3 Modification and improvement of CIN agar for isolation of *Y. enterocolitica*

3.3.1 Media modification

The CIN agar was modified by adding approximately 1% L-arginine (Sigma), 0.8 g/l ferric ammonium citrate (BDH Prolabo), 6.8 g/l sodium thiosulfate (BDH Prolabo), and 2.0 g/l DL-phenylalanine (Sigma) at pH 7.4 ± 0.02 (Appendix I).

3.3.2 Plating efficiency of CIN and modified CIN

The plating efficiencies of CIN and modified CIN agar were evaluated using the 50 bacterial strains listed in Table 3.9. These include *Y. enterocolitica*, other species of *Yersinia*, other *Enterobacteriaceae*, and selected Gram-negative and -positive bacteria.

Bacterial strains retrieved from glycerol stocks at -20 ℃ were grown overnight on BHI agar (BBL). With the help of the needle inoculators, overnight bacterial cultures were dotted on CIN or modified CIN agar and incubated under aerobic or microaerophilic condition at 25 ℃. Microaerophilic conditions were created by placing the inoculated agar plates in candle jars to facilitate the visualization of H_2S-producing bacteria (Cheesbrough, 2006). The plating efficiency was determined by screening for the presence of colonies with red centre and colourless translucent rim (red bull’s eye) on CIN and modified CIN agar, the expected morphology for *Y. enterocolitica* strains.

Table 3.9. Bacterial strains selected for plating efficiency testing.

Bacterial species	Strain number
Yersinia enterocolitica	
bioserotype 1A/O:6,30	IP102
bioserotype1A/O:5	PC-M16-2
bioserotype 1B/O:8	IP11105, ATCC 9610, YE036c-CY
bioserotype 2/O:9	IP383
bioserotype 3/O:1,2,3	IP135
bioserotype 3 variant /O:3	PC- M1-K1
bioserotype 4/O:3	IP134
bioserotype 5/O:2,3	IP178
Other *Yersinia* spp.	
Y. aldovae	IP6005
Y. bercovieri	IP3443
Y. frederiksenii	IP3842
Y. intermedia	IP955
Y. kristensenii	IP105
Y. mollaretii	IP33766
Y. pseudotuberculosis	IP34476
Other *Enterobacteriaceae*	
Citrobacter,	
freundii, H_2S-producing	YC-K1-3
freundii, non-H_2S-producing	YC-S1-5, YC-T1-1
braakii	YC-T1-K1
koseri	YC-VG2-1
Providencia rettgeri	IC-PP2a-9, IC-PP3a-K1, IC-PP6a-10
Enterobacter cloacae	YC-I1-K1, YC-I1-K2, YC-I1-K3
Pantoae spp.	PI-TSP30a-K1
Serratia,	
odorifera	PC-TSP36b-3
marcescens	YC-M2-11
Morganella morganii	YS-TSP7b-1
Salmonella,	
Paratyphi A	ATCC 9150
Paratyphi B	ATCC 8759
Paratyphi C	ATCC 9068
Typhimurium	ATCC 13311
Typhi	ATCC 6539
enterica	ATCC 10376
Escherichia coli	ATCC25922, O157:H7
Shigella sonnei	ATCC 11060
Proteus penneri	IS-TSP7b-3
Other Gram-Negative Bacteria	
Aeromonas hydrophila	Ae 20
Vibrio spp.	VSP-C12-1210, VS-A29-0810
Pseudomonas aeruginosa	ATCC 9027
Gram-Positive Bacteria	
Enterococcus faecalis	ATCC 29212
Listeria monocytogenes	ATCC 7644
Staphylococcus aureus	ATCC 6538, MRSA 0807-1

The IP strains are a kind gifts of Dr. Elisabeth Carniel from Institute Pasteur, the French Yersinia Reference laboratory, France; ATCC 9610 is a kind gift of Dr. Aziah from Makmal Kesihatan Awam Veterinar, Malaysia; other strains are bacteria collection of Laboratory of Biomedical Science and Molecular Microbiology, UM (Prof. Dr. Thong Kwai Lin).

3.3.3 Limit of detection (LOD) of CIN and modified CIN of *Y. enterocolitica* strains

The LOD was determined using the method recommended by the Microbiological Methods Committee (2011). *Y. enterocolitica* was chosen as representing strain. Overnight *Y. enterocolitica* cultures were serially diluted to a range of 10^8 to 10^1 colony forming unit (cfu)/ml and spread onto CIN and modified CIN agar. The plating efficiency was replicated 6 times with independent cell suspension. The plates were incubated under aerobic condition at 25 ℃. In addition, one set of modified CIN agar was incubated under microaerophilic condition at 25 ℃. The LOD was defined as the lowest concentration of *Y. enterocolitica* with culturable bacteria detectable in at least 50% of the replicates (as low as one colony detectable in each replicate, and at least three out of six positive replicates). Statistical significance was calculated by Student t-test using software R (version 2.12.2). Significant values were for $P<0.05$.

3.3.4 Quantification of *Y. enterocolitica* growth in CIN and modified CIN with that on LBA

The growth of *Y. enterocolitica* on CIN and modified CIN was compared to that on Luria-Bertani agar (LBA) (Oxoid). *Y. enterocolitica* suspension was adjusted to approximately 10^3 cfu/ml and plated on LBA, CIN and modified CIN. The mean cfu/ml (6 replicates) of *Y. enterocolitica* on LBA, CIN and modified CIN was calculated after incubation at 25 ℃ for 48 h. The results were expressed as a percentage of mean cfu/ml on CIN/LBA and modified CIN/LBA at each incubation condition (Savin, Leclercq, & Carniel, 2012).

3.3.5 Limit of detection (LOD) and recovery rate of *Y. enterocolitica* in artificially contaminated raw pork meat on CIN and modified CIN

The effect of the natural microbiota on the recovery of *Y. enterocolitica* from food matrix (with or without stress treatment, kept at -20℃ for three weeks after bacterial spiking) was studied (Microbiological Methods Committee, 2011). An artificially contaminated raw pork meat was used because yersiniosis is frequently associated with the consumption of contaminated pork products. For food matrix without stress treatment, approximately 1.5 kg of freshly purchased minced pork meat was processed immediately following spiking. Cell suspension of *Y. enterocolitica* was serially diluted as previously described and 250 µl of each *Y. enterocolitica* suspension (10-fold higher than the final concentration) was mixed with 25 g of minced meat [homogenised in 24.75 ml PBS (Sigma), final volume = 25 ml, final concentrations of *Y. enterocolitica* were 10^1 to 10^8 cfu/ml]. The meat suspensions were homogenized manually by hand for 30 s and incubated at 25 ℃ for 30 min. An aliquot of each homogenate was then plated onto CIN and modified CIN plates and incubated under aerobic condition at 25 ℃ for 24 - 48 h. The same procedures were repeated for a set of modified CIN agars incubated under microaerophilic condition at 25 ℃ for 24 - 48 h. Six replicates were done for each bacterial concentration. The mean cfu/ml of background microbiota in uninoculated pork meat (25 g, homogenised in 25 ml PBS, 6 replicates) was determined using LBA.

The LOD of *Y. enterocolitica* in the artificially contaminated pork meat was defined as the lowest cfu/ml of culturable *Y. enterocolitica* detectable in ≥50% of the replicates (as low as 1 colony detectable on each replicate, and as least three out of six replicates positive) (Microbiological Methods Committee, 2011). The mean cfu of *Y. enterocolitica* to background microbiota ratio was also determined.

The same spiked minced meat was subjected to stress treatment by keeping the spiked food samples at -20℃ for two weeks after bacterial spiking (to mimic food storage conditions) (Microbiological Methods Committee, 2011). The same plating procedures were performed. Percentage of true *Y. enterocolitica* recovered from the food food (with and without stress treatment) was determined by picking representative colonies from each plate and identified by the duplex PCR targeting *Y. enterocolitica*-specific 16S rRNA and *ail* genes as described in Section 3.2.4.1.

3.3.6 Determination of the recovery of *Y. enterocolitica* from artificial bacterial mixtures

Overnight cultures of *Y. enterocolitica* IP135 and bacteria exhibiting *Yersinia*-like colonies on CIN agar (H_2S-producing *C. freundii, C. braakii, A. hydrophila, P. rettgeri,* and *E. cloacae*) were adjusted to a concentration of 10^4 cfu/ml and mixed together. Six independent mixed bacterial suspensions were made and directly spread on CIN and modified CIN agar, and incubated aerobically at 25 ℃ for 24 - 48 h. Presumptive *Y. enterocolitica* colonies were isolated from each replicate and subjected to PCR confirmation to determine the percentage of true *Y. enterocolitica* on both media. Duplex PCR as stated in Section 3.2.4.1 was used. Statistical significance was calculated by Chi-square (χ^2) test using software R (version 2.12.2). Significant values were for $P<0.05$.

3.3.7 Determination of the recovery rate of *Y. enterocolitica* in naturally contaminated samples

The 52 rectal swabs from swine (from Penang, refer Section 3.2.1.2) were tested for the presence of *Y. enterocolitica* using different enrichment procedures and plating on CIN and modified CIN agars. Briefly, all of the swab specimens were (i) directly

streaked onto the selective medium (Table 3.10, methods 1 and 2), (ii) subjected to cold enrichment (incubation at 4 ℃ for three weeks in PBS) (Table 3.10, methods 3 and 4) before being plated on CIN and modified CIN, and (iii) subjected to cold enrichment and then alkaline treatment before being plated onto the two selective media (Table 3.10, methods 5 and 6). Refer Section 3.2.2 for the details of each isolation method. Presumptive *Y. enterocolitica* colonies were isolated and subjected to PCR confirmation as described in Section 3.2.4.1.

Table 3.10. Summary methods used for the determination of the recovery rate of *Y. enterocolitica* in naturally contaminated samples.

Methods	Medium used
1	Direct streaking onto CIN[a]
2	Direct streaking onto mCIN[b]
3	PBS[c]-CIN
4	PBS-mCIN
5	PBS-KOH[d]-CIN
6	PBS-KOH-mCIN

[a] CIN, Cefsulodin-Irgasan-Novobiocin; [b] mCIN, modified CIN; [c] PBS, phosphate buffered saline, a cold enrichment at 4 ℃ for three weeks; [d] KOH, post-enrichment alkaline treatment.

CHAPTER 4

RESULTS

This study was conducted on 106 raw food samples (58 raw pork products and 48 non-porcine food) and 495 swine specimens (from 165 pigs) to investigate the presence of *Yersinia enterocolitica*. Section 4.1 summarise the prevalence of *Y. enterocolitica* from raw pork products, raw non-porcine food and pigs, respectively. Section 4.2 shows results for the isolation and detection methods for *Y. enterocolitica*. Section 4.3 reports the results for biotyping and serotyping of *Y. enterocolitica* isolates. Results for further characterization of *Y. enterocolitica* isolates by using AST, virulotyping, PFGE and plasmid profiling are in Section 4.4. The last section, Section 4.5, presents results for the evaluation of the modified CIN agar in comparing to CIN. The raw data for sampling date, location, sample type, biochemical tests, PCR results, biotyping and serotyping, virulotyping, antimicrobial profiles and modification and improvement of CIN agar are listed in Appendix IV to XIII.

4.1 Prevalence of *Y. enterocolitica*

4.1.1 Prevalence and MPN/g of *Y. enterocolitica* in raw pork products

The prevalence of *Y. enterocolitica* from raw pork products was low by the cultural method. (Refer Appendix IV for raw data). Out of 58 food tested, seven (12.1%) were contaminated by *Y. enterocolitica*; i.e. raw pork meat (whole meat) 5/21 (23.8%), raw pork liver 1/5 (20.0%) and raw pork intestine 1/8 (12.5%) (Table 4.1.1) Samples involved were M1, M3, M13, M16, YE032, YE036 and YE037 (Appendix IV). All positive samples were from the same hawker stall. Table 4.1.1 summarises the prevalence of *Y. enterocolitica* from raw pork products. Twenty-six isolates of *Y. enterocolitica* (API 20E and PCR confirmed) were isolated from the 7 positive samples (Appendix VIII). Table 4.1.2 shows the summary results of all 26 PCR-confirmed *Y. enterocolitica* isolates.

Post-enrichment PCR detection showed a higher incidence of *Y. enterocolitica*, 35/58 (60.3%) as compared to the cultural methods (raw pork meat n=20, raw pork internal organs, n=14, skin, n=1) (Table 4.1.1). Raw data are in Appendix IV.

MPN/g of *Y. enterocolitica* in raw pork products was determined by using the YSEO enriched tubes and the results are tabulated in Table 4.1.3. The results showed that the concentration of *Y. enterocolitica* in the positive samples ranged from <0.30 MPN/g to >43.84 MPN/g. Although there was no specific requirements for the levels of *Y. enterocolitica* in food under FDA Food Code (Lawley, Curtis, & Davis, 2012), but majority of the positive samples had low MPN/g values (≤18.98 MPN/g), except four samples (>43.84 MPN/g).

Table 4.1.1. Prevalence of *Y. enterocolitica* from raw pork products determined by cultural method and post-enrichment PCR screening.

Food type	No. of sample	No of positives (%)[a]		Isolation rate by strain type[b] (%)	
		Post-enrichment PCR	Culture	Pathogenic	Non-pathogenic
Raw pork meat	**25**	**20 (80.0)**	**5 (20.0)**	**4/5 (80.0)**	**1/5 (20.0)**
Whole meat	21	18 (85.7)	5 (23.8)	4/5 (80.0)	1/5 (20.0)
Minced meat	4	2	0	0	0
Raw pork internal organs	**23**	**14 (60.9)**	**2 (8.7)**	**2/2 (100.0)**	**0**
Liver	5	3 (60.0)	1 (20.0)	1/1 (100.0)	0
Intestine	8	7 (87.5)	1 (12.5)	1/1 (100.0)	0
Heart	5	3 (60.0)	0	0	0
Kidney	4	1 (25.0)	0	0	0
Throat	1	0	0	0	0
Other parts	**10**	**1 (10.0)**	**0**	**0**	**0**
Skin	4	1	0	0	0
Foot	2	0	0	0	0
Fat tissue	1	0	0	0	0
Ear	1	0	0	0	0
Eye tissue	1	0	0	0	0
Nose	1	0	0	0	0
Total	**58**	**35 (60.3)**	**7 (12.1)**	**6/7 (85.7)**	**1/7 (14.3)**

[a] The values are the number of positives detected in either of the enriched samples (YSEO, ITC, and PBS); [b] The isolation rate refers to the number of positive samples determined by cultural methods

Table 4.1.2. Summary results of the 26 PCR confirmed *Y. enterocolitica* isolates isolated from raw pork products.

No.	Isolates Name	Date of isolation	Sample code[a]	Source	API 20E code, % of confirmation[a]		Biotype[b]	Serotype[b]
1	PC-M1-K1	June 2010	M1	Raw pork meat	1014522,	93.9%	3 variant	O:3
2	PC-M1-K2	June 2010	M1	Raw pork meat	1014522,	93.9%	3 variant	O:3
3	PC-M1-K3	June 2010	M1	Raw pork meat	1014522,	93.9%	3 variant	O:3
4	PC-M1-K4	June 2010	M1	Raw pork meat	1014522,	93.9%	3 variant	O:3
5	PC-M1-K5	June 2010	M1	Raw pork meat	1014522,	93.9%	3 variant	O:3
6	PC-M1-K11	June 2010	M1	Raw pork meat	1014522,	93.9%	3 variant	O:3
7	PC-M1-K12	June 2010	M1	Raw pork meat	1014522,	93.9%	3 variant	O:3
8	PC-M1-K13	June 2010	M1	Raw pork meat	1014522,	93.9%	3 variant	O:3
9	PC-M3-6	June 2010	M3	Raw pork meat	1014522,	93.9%	3 variant	O:3
10	PC-M3-K11	June 2010	M3	Raw pork meat	1014522,	93.9%	3 variant	O:3
11	PC-M3-K12	June 2010	M3	Raw pork meat	1014522,	93.9%	3 variant	O:3
12	PC-M13-K13	Oct 2010	M13	Raw pork meat	1014522,	93.9%	3 variant	O:3
13	S18/1-C-O-6a	Jan 2011	YE037	Raw pork intestine	1014523	99.7%	3 variant	O:3
14	S18/1-C-I-10-4-6a	Jan 2011	YE037	Raw pork intestine	1014523	99.7%	3 variant	O:3
15	S18/1-C-O-6b	Jan 2011	YE037	Raw pork intestine	1014523	99.7%	3 variant	O:3
16	S18/1-C-O-5-6b	Jan 2011	YE037	Raw pork intestine	1014523	99.7%	3 variant	O:3
17	S18/1-C-I-4-6b	Jan 2011	YE037	Raw pork intestine	1154523	92.3%	3 variant	O:3
18	S18/1-C-O-6c	Jan 2011	YE037	Raw pork intestine	1014523	99.7%	3 variant	O:3
19	S18/1-C-O-6d	Jan 2011	YE037	Raw pork intestine	1154523	92.3%	3 variant	O:3
20	S18/1-C-O-5-6e	Jan 2011	YE037	Raw pork intestine	1114523	99.7%	3 variant	O:3
21	S18/1-C-O-1a	Jan 2011	YE032	Raw pork liver	1154723,	92.5%	1B	O:8
22	S18/1-C-O-K-5b	Jan 2011	YE036	Raw pork meat	1154523,	92.3%	1B	O:8
23	S18/1-C-O-5c	Jan 2011	YE036	Raw pork meat	1155523,	98.3%	1B	O:8
24	PC-M16-2	Feb 2011	M16	Raw pork meat	1155723,	98.3%	1A	O:5
25	PC-M16-5	Feb 2011	M16	Raw pork meat	1155723,	98.3%	1A	O:5
26	PC-M16-10	Feb 2011	M16	Raw pork meat	1154723,	92.5%	1A	O:5

[a]All samples were from Kuala Lumpur, wet market A, stall 1; [b]Raw data refer Appendix VIII; [b]Raw data refer Appendix IX.

Table 4.1.3. The MPN and MPN/g values (calculated using the results of post-enrichment PCR) and the background information of raw food samples.

Sampling time	Sample code	Food type	Location	Bioserotype	MPN[a], MPN/g	UCI[b]	LCI[c]
June 2010	M1	Raw pork meat	KL[d]	3 v[e]/O:3	0-0-0, <0.30	-[f]	0.07
June 2010	M2	Raw pork meat	KL	neg[g]	0-0-0, <0.30	-	0.07
June 2010	M3	Raw pork meat	KL	3 v/O:3	0-0-0, <0.30	-	0.07
June 2010	I1	Raw pork intestine	KL	neg	0-0-0, <0.30	-	0.07
July 2010	M4	Raw pork meat	KL	neg	0-0-0, <0.30	-	0.07
July 2010	M5	Raw pork meat	KL	neg	0-0-1, 0.30	1.36	0.07
July 2010	M6	Raw pork meat	KL	neg	0-0-0, <0.30	-	0.07
July 2010	I2	Raw pork intestine	KL	neg	0-0-0, <0.30	1.36	0.07
July 2010	I3	Raw pork intestine	KL	neg	1-0-0, 0.36	1.63	0.08
July 2010	D1	Raw pork heart	KL	neg	0-0-0, <0.30	1.36	0.07
July 2010	D2	Raw pork heart	KL	neg	0-0-2, 0.60	2.73	0.13
July 2010	L1	Raw pork liver	KL	neg	2-2-2, 3.12	14.14	0.69
Aug 2010	M9	Raw pork meat	KL	neg	0-0-1, <0.30	1.36	0.07
Aug 2010	S2	Raw pork skin	KL	neg	0-0-0, <0.30	-	0.07
Aug 2010	D4	Raw pork heart	KL	neg	3-3-0, 18.98	86.04	4.19
Sept 2010	M11	Raw pork meat	KL	neg	0-0-0, <0.30	-	0.07
Sept 2010	M12	Raw pork meat	KL	neg	0-3-2, 1.58	7.16	0.35
Sept 2010	M14	Raw pork meat	KL	neg	3-2-0, 7.60	34.44	1.68
Sept 2010	M13	Raw pork meat	KL	3 v/O:3	0-3-3, 1.90	8.60	0.42
Sept 2010	M15	Raw pork meat	KL	neg	0-2-0, 0.62	2.81	0.14
Sept 2010	I5	Raw pork intestine	KL	neg	0-0-1, <0.30	1.36	0.07
Sept 2010	I6	Raw pork intestine	KL	neg	0-0-0, <0.30	-	0.07
Jan 2010	I7	Raw pork intestine	KL	neg	0-3-3, 1.90	8.60	0.42
Jan 2011	M16	Raw pork meat	KL	1A/O:5	3-3-3, >43.84	198.70	-
Jan 2011	M17	Raw pork meat	KL	neg	3-3-3, >43.84	198.70	-
Jan 2011	M18	Raw pork meat	KL	neg	3-3-3, >43.84	198.70	-
Jan 2011	M19	Raw pork meat	KL	neg	3-3-3, >43.84	198.70	-
Jan 2011	M20	Raw pork meat	KL	neg	1-3-3, 2.71	12.29	0.60
Jan 2011	YE032	Raw pork liver	KL	1B/O:8	1-0-0, 0.36	1.63	0.08
Jan 2011	YE036	Raw pork meat	KL	1B/O:8	1-0-0, 0.36	1.63	0.08
Jan 2011	YE037	Raw pork intestine	KL	3 v/O:3	1-0-0, 0.36	1.63	0.08
Jan 2011	S18-3	Raw pork meat	KL	neg	0-0-0, <0.30	-	0.07
Jan 2011	S18-4	Raw pork meat	KL	neg	0-0-0, <0.30	-	0.07
Mar 2011	K3	Raw pork kidney	Taiping	neg	1-1-0, 0.73	3.33	0.16
Mar 2011	L3	Raw pork liver	Taiping	neg	3-1-0, 4.57	20.72	1.01

The MPN/g value was calculated using the Microsoft Excel spreadsheet provided by Institute of Environment Science and Research (ESR), New Zealand (Hudson, et al., 2008). [a] MPN, most probable number; [b] UCI, upper confidence interval; [c] LCI, lower confidence interval; [d] KL, Kuala Lumpur; [e] 3 v, 3 variant; [f]-, value cannot be calculated or identity cannot be determined; [g] neg, sample was negative by using culture method. For the rest samples which were not mentioned in table, *Y. enterocolitica* was negative in both cultural and post-enrichment PCR method in all nine tubes of the 3 × 3 YSEO enrichment tubes.

4.1.2 Prevalence of *Y. enterocolitica* in raw non-porcine food

Overall, no *Y. enterocolitica* was isolated via culture methods from the 48 raw non-porcine food tested (Table 4.1.4). However, the post-enrichment PCR screening indicated *Y. enterocolitica* was present in 20/48 (41.7%) of raw food; i.e. 4/6 (66.7%) raw beef, 5/9 (55.6%) raw poultry products, 5/11 (45.5%) raw seafood, and 6/19 (31.6%) raw vegetables (Table 4.1.4). Raw data for both cultural and PCR methods are tabulated in Appendix V.

Table 4.1.4. Prevalence of *Y. enterocolitica* from raw non-porcine food determined by cultural method and post-enrichment PCR screening.

Food type	No. of samples	No of positives [a] (%)	
		PCR	Cultural
Raw beef	**6**	**4 (66.7)**	**0**
Raw poultry products	**9**	**5 (55.6)**	**0**
Chicken meat	8	5 (62.5)	0
Chicken claw	1	0	0
Raw seafood	**11**	**5 (45.5)**	**0**
Fish	6	4 (66.7)	0
Squid	3	1 (33.3)	0
Prawn	1	0	0
Cockles	1	0	0
Raw vegetables	**19**	**6 (31.6)**	**0**
Leafy vegetables	11	4 (36.4)	0
Bitter gourd	3	1 (33.3)	0
Cowpea	1	0	0
Root	1	0	0
Sweet potato	1	0	0
Brinjal	1	1 (100.0)	0
Lady's finger	1	0	0
Raw tofu	**2**	**0**	**0**
Pasteurised milk	**1**	**0**	**0**
Total	**48**	**20 (41.7)**	**0**

[a] The values are the number of positive detected in either of the enriched samples (YSEO, ITC, and PBS)

4.1.3 Prevalence of *Y. enterocolitica* in live pigs

Based on culture methods, only three out of 165 pigs (1.8%) harboured *Y. enterocolitica* (Tables 4.1.5 and 4.1.6). Raw data are tabulated in Appendix VI. All the positive pigs were healthy grower pigs (asymptomatic) fed in the same pen from Farm I in Penang (Table 4.1.7; Penang's pig: no. 53, 55 and 56). A total of 72 *Y. enterocolitica* isolates were isolated from the three pigs (Table 4.1.7). These isolates were isolated from seven specimens (Table 4.1.8). The involved specimens are PPN53a, PPN55a,

PPN55b, PPN55c, PPN56a, PPN56b, and PPN56c (Appendix VI). Table 4.1.7 shows the summary results of all 72 PCR-confirmed *Y. enterocolitica* isolates.

On the other hand, PCR detected a higher prevalence of *Y. enterocolitica*, in 46 pigs (27.9%), i.e. 11/22 (50.0%) healthy growers, 14/44 (31.8%) unhealthy weaners, 20/68 (29.4%) healthy weaners, and 1/11 (9.1%) unhealthy growers (Tables 4.1.5 and 4.1.6). Raw data for the PCR detection are tabulated in Appendix VI. The PCR results indicated that *Y. enterocolitica* was most frequently found in nasal swabs, 29/165 (17.6%) followed by oral swabs, 25/165 (15.2%) and rectal swabs, 21/165 (12.7%) (Table 4.1.8). Overall, *Y. enterocolitica* was PCR detected in the pigs in three states; i.e. Selangor (n=9), Perak (n=20), and Penang (n=17) (Table 4.1.5).

Table 4.1.5. Prevalence of *Y. enterocolitica* in swine according to each pig farm and state.

States	Farm	n[a]	PCR[b] method	Culture method	Total	
					PCR method (%)	Culture method (%)
Selangor	A	9	3 (33.3)	0 (0)		
	B	14	6 (42.9)	0 (0)	**9 (17.0)**	**0 (0)**
	C	30	0 (0)	0 (0)		
	Total	**53**				
Perak	D	20	5 (25.0)	0 (0)		
	E	20	1 (5.0)	0 (0)	**20 (33.3)**	**0 (0)**
	F	20	14 (70.0)	0 (0)		
	Total	**60**				
Penang	G	16	1 (6.3)	0 (0)		
	H	20	5 (25.0)	0 (0)	**17 (32.7)**	**3 (5.8)**
	I	16	11 (68.8)	3 (18.8)		
	Total	**52**				
Total (%)		**165**	**46 (27.9)**	**3 (1.8)**		

[a]n, number of pigs within each farm; [b]PCR, polymerase chain reaction

Table 4.1.6. Prevalence of *Y. enterocolitica* based on the age and health condition of pigs determined by cultural method and post-enrichment PCR screening.

Health group	n[a]	No of positives samples (%)							
		Selangor (n=53)		Perak (n=60)		Penang (n=52)		Total positive	
		PCR	C[b]	PCR	C	PCR	C	PCR	C
H[c] piglet[d]	4	0	0	-[e]	-	-	-	0	0
UH[f] piglet	4	0	0	-	-	-	-	0	0
H weaner[g]	68	4 (5.9)	0	8 (11.8)	0	8 (11.8)	0	20 (29.4)	0
UH weaner	44	5 (11.3)	0	8 (18.2)	0	1 (2.3)	0	14 (31.8)	0
H grower[h]	22	0	0	3 (13.6)	0	8 (36.4)	3 (13.6)	11 (50.0)	3 (13.6)
UH grower	11	0	0	1 (9.1)	0	-	-	1 (9.1)	0
H finisher[i]	10	0	0	-	-	-	-	0	0
H sow	2	-	-	0	0	-	-	0	0
N[j]	**165**	**9 (5.5)**	**0**	**20 (12.1)**	**0**	**17 (10.3)**	**3 (1.8)**	**46 (27.88)**	**3 (1.8)**

[a] n, number of pigs within each health group; [b] C, cultural method; [c] H, healthy; [d] piglet, < 1 month old; [e] -, sample not collected from this group ; [f] UH, unhealthy; [g] weaner, 1-2 months old; [h] grower, 2-4 months old; [i] finisher, 4-6 months old; [j] N, number of pigs from each state.

Table 4.1.7. Summary results of the 72 PCR-confirmed *Y. enterocolitica* isolates isolated from pigs.

No.	Isolates Name	Pig No.	Source	API 20E code,% of confirmation
1	PCM-PPN53a-1	53	Pig, nasal swab	1114523, 99.7%
2	PCM-PPN53a-2	53	Pig, nasal swab	1114523, 99.7%
3	PCM-PPN53a-3	53	Pig, nasal swab	1014522, 93.9%
4	PCM-PPN53a-4	53	Pig, nasal swab	1114523, 99.7%
5	PCM-PPN53a-K1	53	Pig, nasal swab	1014522, 93.9%
6	PCM-PPN53a-K2	53	Pig, nasal swab	1014522, 93.9%
7	PCM-PPN53a-K3	53	Pig, nasal swab	1114523, 99.7%
8	PCM-PPN53a-K4	53	Pig, nasal swab	1014522, 93.9%
9	DCM-PPN55a-1	55	Pig, nasal swab	1114523, 99.7%
10	DCM-PPN55a-2	55	Pig, nasal swab	1014522, 93.9%
11	DCM-PPN55a-3	55	Pig, nasal swab	1114523, 99.7%
12	DCM-PPN55a-4	55	Pig, nasal swab	1014522, 93.9%
13	PCM-PPN55a-1	55	Pig, nasal swab	1104523, 95.7%
14	PCM-PPN55a-2	55	Pig, nasal swab	1014522, 93.9%
15	PCM-PPN55a-3	55	Pig, nasal swab	1014522, 93.9%
16	PCM-PPN55a-4	55	Pig, nasal swab	1014522, 93.9%
17	PCM-PPN55a-K1	55	Pig, nasal swab	1114523, 99.7%
18	PCM-PPN55a-K2	55	Pig, nasal swab	1014522, 93.9%
19	PCM-PPN55a-K3	55	Pig, nasal swab	1014522, 93.9%
20	PCM-PPN55a-K4	55	Pig, nasal swab	1014522, 93.9%
21	PCM-PPN55b-1	55	Pig, tongue swab	1114522, 99.9%
22	PCM-PPN55b-2	55	Pig, tongue swab	1114523, 99.7%
23	PCM-PPN55b-3	55	Pig, tongue swab	1014522, 93.9%
24	PCM-PPN55b-4	55	Pig, tongue swab	1014522, 93.9%
25	PCM-PPN55b-K1	55	Pig, tongue swab	1014522, 93.9%
26	PCM-PPN55b-K2	55	Pig, tongue swab	1014522, 93.9%
27	PCM-PPN55b-K3	55	Pig, tongue swab	1014522, 93.9%
28	PCM-PPN55b-K4	55	Pig, tongue swab	1014522, 93.9%
29	PCM-PPN55c-1	55	Pig, rectal swab	1114523, 99.7%
30	PCM-PPN55c-2	55	Pig, rectal swab	1014522, 93.9%
31	PCM-PPN55c-3	55	Pig, rectal swab	1014522, 93.9%
32	PCM-PPN55c-4	55	Pig, rectal swab	1014522, 93.9%
33	PCM-PPN55c-K1	55	Pig, rectal swab	1014522, 93.9%
34	PCM-PPN55c-K2	55	Pig, rectal swab	1114523, 99.7%
35	PCM-PPN55c-K3	55	Pig, rectal swab	1014522, 93.9%
36	PCM-PPN55c-K4	55	Pig, rectal swab	1014522, 93.9%
37	PCM-PPN56a-1	56	Pig, nasal swab	1014523, 96.4%
38	PCM-PPN56a-2	56	Pig, nasal swab	1014522, 93.9%
39	PCM-PPN56a-3	56	Pig, nasal swab	1014522, 93.9%
40	PCM-PPN56a-4	56	Pig, nasal swab	1014522, 93.9%
41	PCM-PPN56a-K1	56	Pig, nasal swab	1114523, 99.7%
42	PCM-PPN56a-K2	56	Pig, nasal swab	1114523, 99.7%
43	PCM-PPN56a-K3	56	Pig, nasal swab	1014522, 93.9%
44	PCM-PPN56a-K4	56	Pig, nasal swab	1014522, 93.9%
45	DCM-PPN56b-21	56	Pig, tongue swab	1114522, 99.9%
46	DCM-PPN56b-22	56	Pig, tongue swab	1014522, 93.9%
47	DCM-PPN56b-23	56	Pig, tongue swab	1114523, 99.7%
48	DCM-PPN56b-24	56	Pig, tongue swab	1114523, 99.7%
49	PCM-PPN56b-1	56	Pig, tongue swab	1014522, 93.9%
50	PCM-PPN56b-2	56	Pig, tongue swab	1014522, 93.9%
51	PCM-PPN56b-3	56	Pig, tongue swab	1014522, 93.9%
52	PCM-PPN56b-4	56	Pig, tongue swab	1014522, 93.9%
53	PCM-PPN56b-K1	56	Pig, tongue swab	1014522, 93.9%
54	PCM-PPN56b-K2	56	Pig, tongue swab	1014522, 93.9%
55	PCM-PPN56b-K3	56	Pig, tongue swab	1004522, 94.9%
56	PCM-PPN56b-K4	56	Pig, tongue swab	1014522, 93.9%
57	DCM-PPN56c-21	56	Pig, rectal swab	1114523, 99.7%
58	DCM-PPN56c-22	56	Pig, rectal swab	1114523, 99.7%
59	DCM-PPN56c-23	56	Pig, rectal swab	1014522, 93.9%
60	DCM-PPN56c-24	56	Pig, rectal swab	1014522, 93.9%
61	PCM-PPN56c-1	56	Pig, rectal swab	1114523, 99.7%
62	PCM-PPN56c-2	56	Pig, rectal swab	1014522, 93.9%
63	PCM-PPN56c-3	56	Pig, rectal swab	1014522, 93.9%
64	PCM-PPN56c-4	56	Pig, rectal swab	1014522, 93.9%
65	PCM-PPN56c-5	56	Pig, rectal swab	1014522, 93.9%
66	PCM-PPN56c-6	56	Pig, rectal swab	1014522, 93.9%
67	PCM-PPN56c-7	56	Pig, rectal swab	1114523, 99.7%
68	PCM-PPN56c-8	56	Pig, rectal swab	1014522, 93.9%
69	PCM-PPN56c-K1	56	Pig, rectal swab	1014522, 93.9%
70	PCM-PPN56c-K2	56	Pig, rectal swab	1014522, 93.9%
71	PCM-PPN56c-K3	56	Pig, rectal swab	1014522, 93.9%
72	PCM-PPN56c-K4	56	Pig, rectal swab	1114522, 99.9%

All isolates were bioserotype 3 variant/O:3, isolated on Oct 2011 from Farm I, Penang. Refer Appendix VI and VIII for raw data.

Table 4.1.8. Distribution of the number of positive swab samples of pigs from Selangor, Perak and Penang using post-enrichment PCR screening and cultural methods.

Swab type	n[a]	No of positives samples (%)							
		Selangor (n=53)		Perak (n=60)		Penang (n=52)		Total positive	
		PCR	C[b]	PCR	C	PCR	C	PCR	C
Nasal	165	5 (9.4)	0	14 (23.3)	0	10 (19.2)	3 (5.8)	29 (17.6)	3 (1.2)
Rectal	165	5 (9.4)	0	8 (13.3)	0	8 (15.4)	2 (3.8)	21 (12.7)	2 (1.2)
Oral	165	4 (7.5)	0	13 (21.7)	0	8 (15.4)	2 (3.8)	25 (15.2)	2 (1.2)
N[c]	**495**	**14 (2.8)**	**0**	**35 (7.1)**	**0**	**26 (5.3)**	**7 (1.4)**	**75 (9.9)**	**7 (1.4)**

[a] n, number of samples within each swab type; [b] C, cultural method; [c] N, number of pigs from each state

4.2 Isolation and detection methods for *Y. enterocolitica*

4.2.1 Isolation of *Y. entercolitica*

A total of 4706 presumptive *Y. enterocolitica* isolates were isolated from 106 raw food samples and 495 swine specimens and all presumptive isolates were recovered by using CIN and modified CIN agars. The targeted bacterium, *Y. enterocolitica*, appeared as red bull's eye (red-centred with colourless rim, Figure 4.2.1) and sometimes colourless without red-centred (<0.5mm) on both agars. Table 4.2.1 shows the distribution of the number of presumptive *Y. enterocolitica* isolates according to each sample type.

Table 4.2.1. Number of presumptive *Y. enterocolitica* isolates according to each sample type.

Samples*	**No. of presumptive isolates**
Raw pork products	1267
Raw non-porcine food	496
Swine specimens**	2923
Total	**4706**

*All presumptive *Y. enterocolitica* isolates were picked from CIN agar except swine specimens from Farm I, modified CIN replaced CIN; ** only 52 rectal swabs from Farm I was tested with both CIN and modified CIN media.

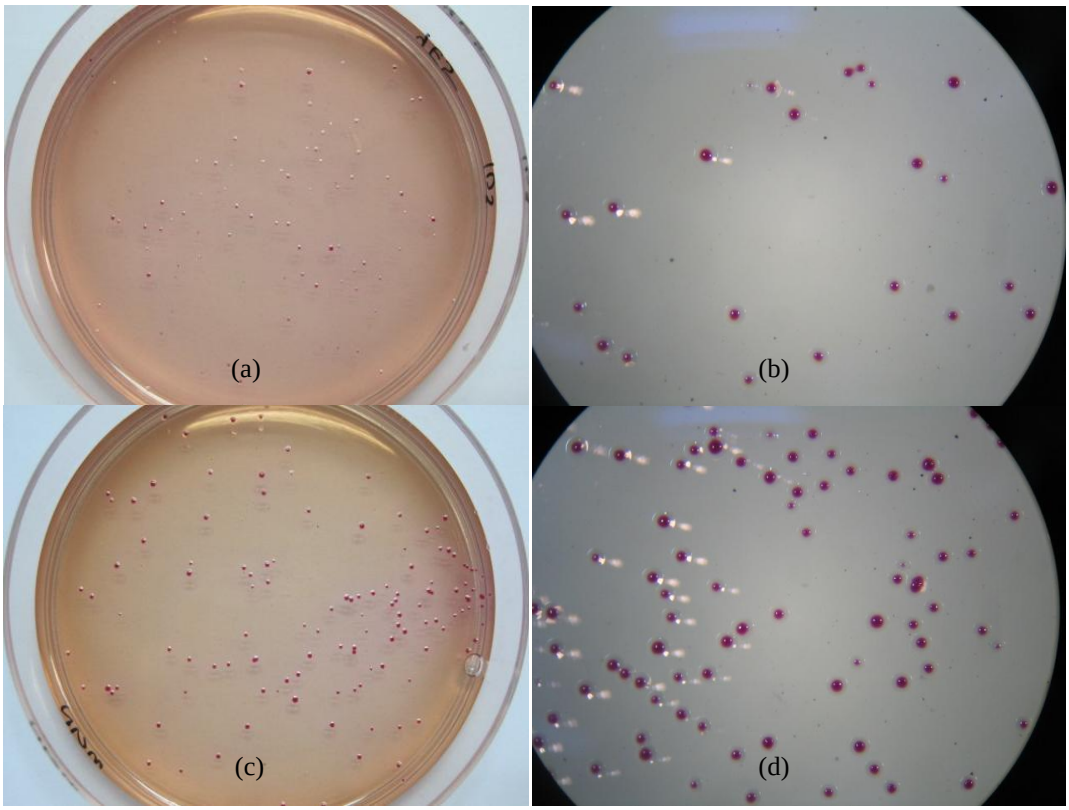


Figure 4.2.1. Colony morphology of *Y. enterocolitica* bioserotype 2/O:9 (IP383) on selective agars- (a) CIN, (b) CIN under 40× light microscope, (c) modified CIN, (d) modified CIN under 40× light microscope.

4.2.2 Biochemical identification for *Y. enterocolitica*

The presumptive *Y. enterocolitica* isolates were then preliminary identified by using biochemical tests such as: oxidase test, Gram determination, urease test and citrate test. Out of 4706 presumptive *Y. enterocolitica* isolates, only 248 (5.3%) were Gram negative, citrate negative, oxidase negative and urease positive. Appendix VII summarises results and representive photos for the preliminary biochemical tests of *Y. enterocolitica*. All 248 presumptive *Y. enterocolitica* isolates that passed the preliminary biochemical tests (Appendix VII) were further identified biochemically by using API 20E identification kit. Only 107 isolates were API 20E identified as *Y. enterocolitica* (92.3% < % of confirmation < 99.9%). Appendix VIII summarises the results of biochemical reactions (API 20E) and raw data of *Y. enterocolitica*. Figure 4.2.2 shows the representative photos of API 20E identified *Y. enterocolitica* isolates.

Nearly 94% (4458/4706) of the presumptive isolates that appeared as red bulls' eye on CIN (~96%, 3977/4153) and modified CIN (~87%, 481/553) were screened out during the preliminary biochemical tests. A part of these isolates were randomly picked and identified as *Providencia rettgeri, Serratia* spp., *Citrobacter freundii, C. braakii, Klebsiella ornithinolytica, Enterobacter cloacae,* and *Pantoea* spp. by using API 20E identification kit (Figure 4.2.3). About 57% (141/248) isolates that passed preliminary biochemical tests were identified as non-*Y. enterocolitica* (*Morganella morganii, Y. frederiksenii*, etc.) by using API 20E identification kit. Figure 4.2.3 shows the representative photos of API 20E identification kit these non-*Y. enterocolitica* bacteria.

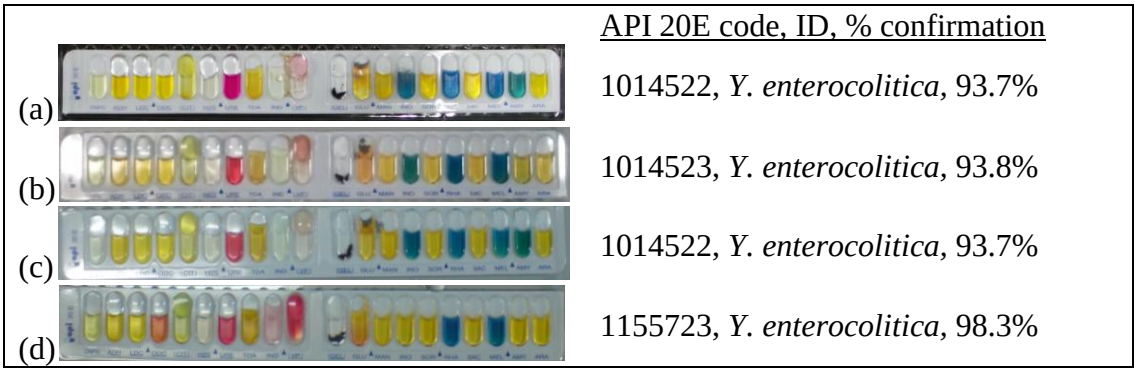


Figure 4.2.2. Representative photos of API 20E identification kit for *Y. enterocolitica* isolates.

(a) PCM-PPN53a-K1; (b) S18/1 C-O-5-6b; (c) PC-M13-K13; (d) PC-M16-2.

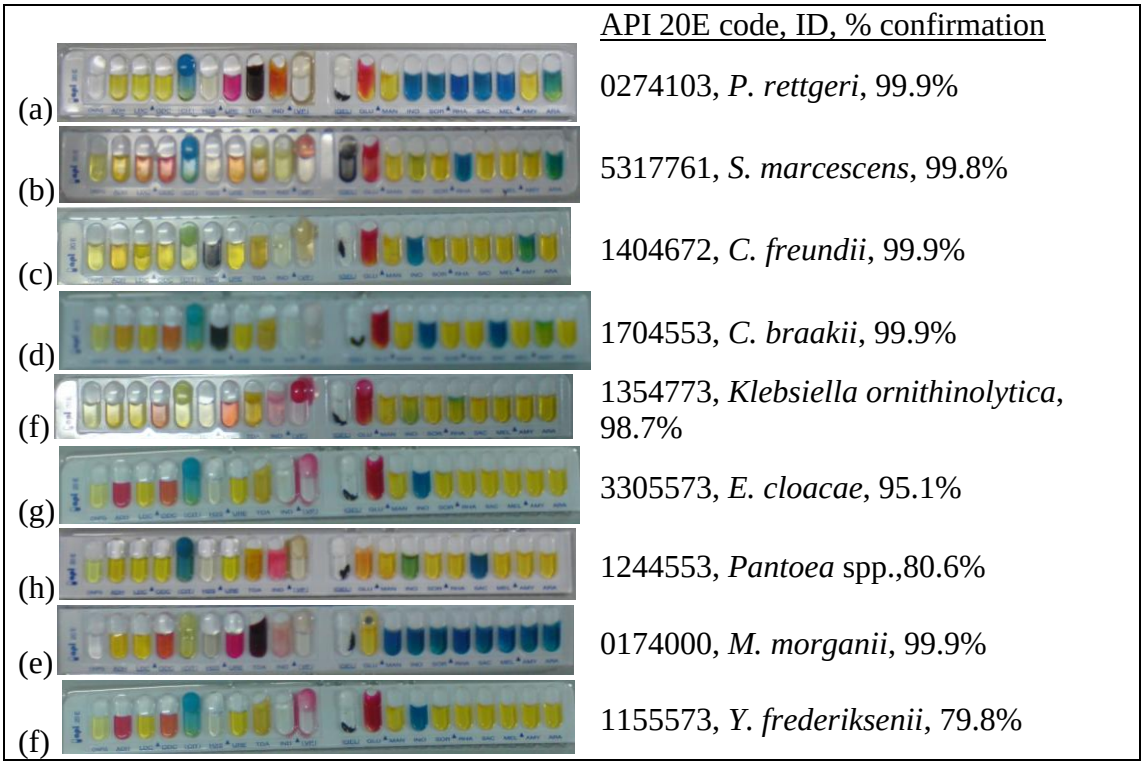


Figure 4.2.3. Representative photos of API 20E identification kit for non-*Y. enterocolitica* bacteria.

(a) *P. rettgeri* (PC-TSP43c-2), (b) *S. marcescens* (YC-M19-2.6), (c) *C. freundii* (YC-I7-2.7), (d) *C. braakii* (YC-T1-K1), (f) *K. ornithinolytica* (PC-M5-K13), (g) *E. cloacae* (YC-I1-K1), (h) *Pantoea* spp. (PC-TSP53b-K1), (e) *M. morganii* (IC-VG2-K1), (f) *Y. frederiksenii* (YC-I1-K1).

4.2.3 Confirmation of API 20E identified *Y. enterocolitica* isolates by PCR and DNA sequencing

API 20E identified *Y. enterocolitica* isolates were further confirmed by using a duplex PCR targeting *Y. enterocolitica*-specific 16S rRNA (Neubauer, et al., 2000) and *ail* (Wannet, et al., 2001) genes. Primers Y1/Y2 and A1/A2 amplified the *Y. enterocolitica*-specific 16S rRNA (330bp) and *ail* (430bp) genes, respectively. The presence of the 330bp amplicon indicates positive for *Y. enterocolitica.* The presence of 430bp amplicon indicates the presence of virulence *ail* gene. Out of the 107 API 20E identified *Y. enterocolitica* isolates, only 98 (91.5%) were PCR confirmed as *Y. enterocolitica* (all positive for *Y. enterocolitica*-specific 16S rRNA gene, Appendix VIII). Five isolates lacked the *ail* gene (refer Appendix VIII). Figure 4.2.4 shows representative gel photo for the duplex PCR using *Y. enterocolitica* isolates. DNA sequences of the 330 bp and 430 bp amplicons (*Y. enterocolitica* 16S rRNA and *ail* genes, respectively) were blast using Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/). The DNA amplicons gave 99% homology for both *Y. enterocolitica* 16S *r*RNA and *ail* genes (refer Appendix X for NCBI blast printscreens).

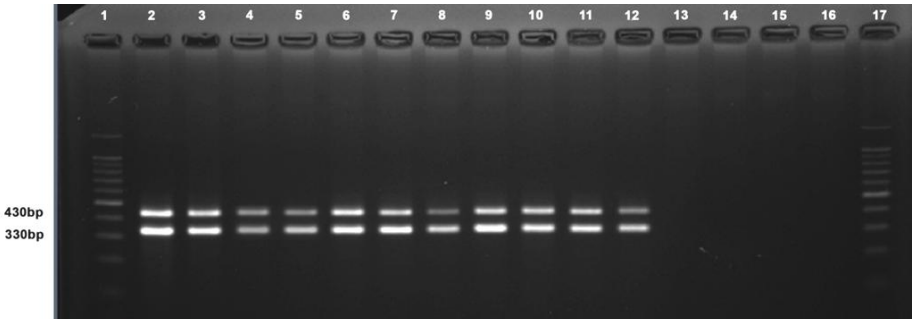


Figure 4.2.4. Representative gel photo for the duplex PCR targeting *Y. enterocolitica*-specific 16S rRNA (330bp) and *ail* (430bp) genes using *Y. enterocolitica* isolates.

Lane 1 and 17, 100bp DNA ladder (Promega); lane 2, positive control/ *Y. enterocolitica* (1992–045); lane 2 to 12, *Y. enterocolitica* (name of isolate starts from left: PC-M1-K1, PC-M1-K2, PC-M1-K3, PC-M1-K4, PC-M1-K5, PC-M1-K11, PC-M1-K12. PC-M1-K13. PC-M3-6, PC-M3-K11, PC-M3-K12); lane 13 to 16, negative control.

4.2.4 Comparison of the recovery power of different isolation methods for *Y. enterocolitica*

Several methods were performed in isolation and detection of *Y. enterocolitica* (three enrichment broths, two selective media, KOH treatment, and also direct plating). Tables 4.2.2 and 4.2.3 summarise the recovery rate of true *Y. enterocolitica* isolates by using different methods. Overall, the recovery rate of true *Y. enterocolitica* isolates was very low by using conventional methods. Only 2.1% (98/4706) presumptive *Y. enterocolitica* isolates (from selective plates) were confirmed as true *Y. enterocolitica*. Among the three enrichment broths, PBS enrichment (cold enrichment) was the best broth in recovery true *Y. enterocolitica* isolates from natural contaminated food, with recovery rate 6.5% as compared to YSEO (1.0%) and ITC (0.1%) (Table 4.2.2). Recovery rate for direct plating method was 1.6%. In comparing the performance of selective plating media, modified CIN was better than CIN, recovery rate for each of them was 13.0% and 0.6%, respectively (Table 4.2.3). Post enrichment KOH treatment increased the recovery rate of both CIN and modified CIN agars in isolating true *Y. enterocolitica,* increased from 0.5% to 1.1% and 7.7% to 31.2%, respectively (Table 4.2.3). Detail results for the modification, comparison and evaluation of CIN and modified CIN are in Section 4.5.

Table 4.2.2. Recovery rate of true *Y. enterocolitica* isolates by using different methods.

Methods	No. of presumptive isolates		No. of true *Y. enterocolitica*[a]		% of recovery		
	CIN	Modified CIN	CIN	Modified CIN	**CIN**	**Modified CIN**	**Total**
Direct plating	744	229	4	12	**0.5**	**5.2**	**1.6**
YSEO	864	-[b]	9	-	**1.0**	**-**	**1.0**
ITC	1531	246	2	0	**0.1**	**0**	**0.1**
PBS	1014	78	11	60	**1.1**	**76.9**	**6.5**
Total	4153	553	26	72	**0.6**	**13.0**	**2.1**

[a]Confirmed by PCR and DNA sequencing; [b]-, method not performed.

Table 4.2.3. Effect of alkaline treatment on the recovery rate of true *Y. enterocolitica* isolates.

Methods	No. of presumptive isolates		No. of true *Y. enterocolitica*[a]		% of recovery		
	Normal plating[a]	Alkaline treatment[b]	Normal plating	Alkaline treatment	**Normal plating**	**Alkaline treatment**	**Total**
CIN	3046	1107	14	12	**0.5**	**1.1**	**0.6**
Modified CIN	428	125	33	39	**7.7**	**31.2**	**13.0**
Total	3474	1232	47	51	**1.4**	**4.1**	**2.1**

[a]Without any post-enrichment treatment; [b]A post enrichment treatment before plating; [c]-, method not performed.

4.2.5 Post enrichment PCR detection for *Y. enterocolitica*

The same duplex PCR (targeting *Y. enterocolitica*-specific 16S rRNA and *ail* genes in Section 3.2.4.1) used for *Y. enterocolitica* isolates confirmation was used. A food sample was counted as PCR positive when either one of the three enriched homogenates (YSEO, ITC and PSB) showed presence of amplicons (*Y. enterocolitica*-specifec 16S rRNA gene, 330bp). Overall, post enrichment PCR detection gave higher prevalence rate as compared to the conventional culture methods (Refer Section 4.1).

Raw data of the post enrichment PCR detection are tabulated in Appendix IV, V and VI. Figures 4.2.5 and 4.2.6 are representative gel photos for the post enrichment PCR detection.

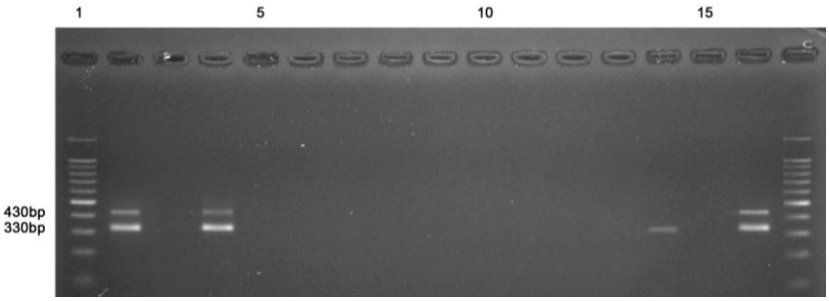


Figure 4.2.5. Representative gel photo for enriched food cultures.

Lane 1 and 17, 100bp DNA ladder (Promega); lane 2 and 16, positive control (1992-045); lane 3, M1 (ITC-enriched); lane 4, M1 (PBS-enriched); lane 5 to 13, M1 (YSEO-enriched MPN tubes); lane14, M2 (PBS-enriched); lane 15, negative control.

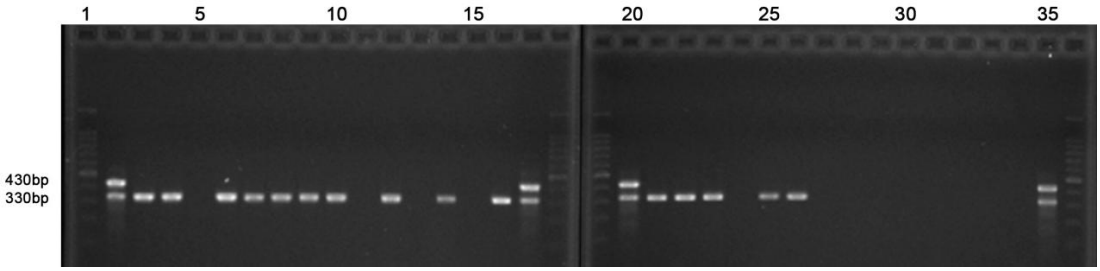


Figure 4.2.6. Representative gel photos for PBS-enriched cultures (Perak's swine specimens).

Lane 1, 18, 19, and 36, 100bp DNA ladder (Promega); lane 2, 17, 20, and 35, positive control (1992-045); lane 3-5, PP49a-c; lane 6-8, PP50a-c; lane 9-11, PP51a-c; lane 12-14, PP52a-c; lane 15, 16 and 21, PP53a-c; lane 22-24, PP54a-c; lane 25-27, PP55a-c; lane 28-30, PP56a-c; lane 31-32, PP57a-b; lane 34, negative control.

4.2.6 API 50CH

The seven isolates that were identified as *Y. enterocolitica* by the API 20E but failed to be amplified by the duplex PCR were further tested with another kit, the API 50CH identification kit (refer Appendix VIII). The results showed that these seven isolates were either *Yersinia intermedia* or *Y. frederiksenii*. Figure 4.2.7 shows representative photo for the API 50CH identification for the tested isolates.

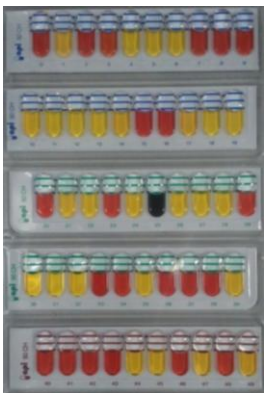


Figure 4.2.7. Representative photo for API 50CH identification kit for *Y. intermedia* (PC-M5-K11).

4.3 Biotyping and serotyping of *Y. enterocolitica* isolates

All 98 PCR-confirmed *Y. enterocolitica* isolates were biotyped by 11 biochemical tests according to Wauters, et al. (1987) and serotyped by using commercial O-antisera "SEIKEN" set purchased (DENKA SEIKEN Co., Ltd, Japan). Results obtained were analyzed by referring to Table 2.1. *Y. enterocolitica* belongs to biotype 1B, 2, 3, 4 and 5 are referred as pathogenic and biotype 1A is non-pathogenic. Tables 4.3.1 and 4.3.2 summarise the results of biochemical and antisera reactions of *Y. enterocolitica*. Raw data and representative photos of each test for all 98 *Y. enterocolitica* isolates are tabulated in Appendix XI. Overall, the *Y. enterocolitica* isolates were bioserotyped as 3 variant/O:3 (n=92), 1B/O:8 (n=3) and 1A/O:5 (n=3).

Table 4.3.1. Summary results for the serotyping of *Y. enterocolitica.*

Serotypes	Antisera					No. of isolates
	O:3	O:5	O:8	O:9	O:1 and O:2	
O:3	+	-	-	-	-	**92**
O:8	-	-	+	-	-	**3**
O:5	-	+	-	-	-	**3**
					Total	**98**

+, positive; -, negative.

Table 4.3.2. Summary results for the biotyping of *Y. enterocolitica.*

Biotypes	Biochemical tests											No. of isolates
	Lipase	Esculine	Salicin	Indole	Xylose	Trehalose	NO_3	Pyrazinamidase	β-D-Glucosidase	VP	DNAse	
3 variant (VP-)	-	-	-	-	+	+	+	-	-	-	-	**92**
1B	+	-	-	+	+	+	+	+	-	-	-	**3**
1A	+	+	+	+	+	+	+	+	+	+	-	**3**
										Total		**98**

+, positive; -, negative.

4.3.1 Bioserotyping of *Y. enterocolitica* isolates from raw pork products

Bioserotyping results revealed that the 26 *Y. enterocolitica* isolates from raw pork products were bioserotyped as 3 variant/O:3 (VP negative variant strain, n=20), 1B/O:8 (n=3), and 1A/O:5 (n=3) (Table 4.1.2 and Appendix IX). Twenty-three isolates were referred as pathogenic (biotypes 3 variant and 1B) and 3 isolates were non-pathogenic (biotype 1A).

4.3.2 Bioserotyping of *Y. enterocolitica* isolates from swine

A total of 72 *Y. enterocolitica* isolates were isolated from the three pigs and all isolates were pathogenic *Y. enterocolitica* bioserotype 3 variant/O:3 (Table 4.1.7).

4.4 Futher characterization of *Y. enterocolitica* isolates

Out of 98 isolates from the previous Sections, 66 were replicate isolates and 32 *Y. enterocolitica* isolates (raw pork products, n=12; pigs, n=20) were selected for further characterization. For each sample, isolates with the same profiles (biochemical tests, API 20E, biotype and serotype) were recorded as replicates and only one isolate was selected for further characterization. More than one isolate (different profiles) might be selected from a sample. Table 4.4.1 summarises the background information of the selected isolates.

Table 4.4.1. Background information of the selected *Y. emterocolitica* isolates.

No.	Name of isolates	Isolation date	Location	Pig No. / sample code	Source	API 20E (code, %)[a]		Biogrouping[b]	Serotyping[b]
1	PCM-PPN53a-1	Sept 2011	Penang, Farm I	53	Pig, nasal swab	1114523,	99.7%	3 variant	O:3
2	PCM-PPN53a-3	Sept 2011	Penang, Farm I	53	Pig, nasal swab	1014522,	93.9%	3 variant	O:3
3	DCM-PPN55a-1	Sept 2011	Penang, Farm I	55	Pig, nasal swab	1114523,	99.7%	3 variant	O:3
4	PCM-PPN55a-1	Sept 2011	Penang, Farm I	55	Pig, nasal swab	1104523,	95.7%	3 variant	O:3
5	PCM-PPN55b-1	Sept 2011	Penang, Farm I	55	Pig, tongue swab	1114522,	99.9%	3 variant	O:3
6	PCM-PPN55b-2	Sept 2011	Penang, Farm I	55	Pig, tongue swab	1114523,	99.7%	3 variant	O:3
7	PCM-PPN55b-4	Sept 2011	Penang, Farm I	55	Pig, tongue swab	1014522,	93.9%	3 variant	O:3
8	PCM-PPN55b-K3	Sept 2011	Penang, Farm I	55	Pig, tongue swab	1014522,	93.9%	3 variant	O:3
9	PCM-PPN55c-1	Sept 2011	Penang, Farm I	55	Pig, rectal swab	1114523,	99.7%	3 variant	O:3
10	PCM-PPN55c-3	Sept 2011	Penang, Farm I	55	Pig, rectal swab	1014522,	93.9%	3 variant	O:3
11	PCM-PPN56a-1	Sept 2011	Penang, Farm I	56	Pig, nasal swab	1014523,	96.4%	3 variant	O:3
12	PCM-PPN56a-4	Sept 2011	Penang, Farm I	56	Pig, nasal swab	1014522,	93.9%	3 variant	O:3
13	PCM-PPN56a-K1	Sept 2011	Penang, Farm I	56	Pig, nasal swab	1114523,	99.7%	3 variant	O:3
14	DCM-PPN56b-21	Sept 2011	Penang, Farm I	56	Pig, tongue swab	1114522,	99.9%	3 variant	O:3
15	DCM-PPN56b-23	Sept 2011	Penang, Farm I	56	Pig, tongue swab	1114523,	99.7%	3 variant	O:3
16	PCM-PPN56b-4	Sept 2011	Penang, Farm I	56	Pig, tongue swab	1014522,	93.9%	3 variant	O:3
17	PCM-PPN56b-K3	Sept 2011	Penang, Farm I	56	Pig, tongue swab	1004522,	94.9%	3 variant	O:3
18	DCM-PPN56c-21	Sept 2011	Penang, Farm I	56	Pig, rectal swab	1114523,	99.7%	3 variant	O:3
19	DCM-PPN56c-23	Sept 2011	Penang, Farm I	56	Pig, rectal swab	1014522,	93.9%	3 variant	O:3
20	PCM-PPN56c-K4	Sept 2011	Penang, Farm I	56	Pig, rectal swab	1114522,	99.9%	3 variant	O:3
21	PC-M1-K1	June 2010	KL, Wet Market A	M1	Raw pork meat	1014522,	93.9%	3 variant	O:3
22	PC-M3-6	June 2010	KL, Wet Market A	M3	Raw pork meat	1014522,	93.9%	3 variant	O:3
23	PC-M13-K13	Sept 2010	KL, Wet Market A	M13	Raw pork meat	1014522,	93.9%	3 variant	O:3
24	S18/1-C-O-6a	Jan 2011	KL, Wet Market A	YE037	Raw pork intestine	1015523,	93.8%	3 variant	O:3
25	S18/1-C-I-4-6b	Jan 2011	KL, Wet Market A	YE037	Raw pork intestine	1155523,	98.3%	3 variant	O:3
26	S18/1-C-O-6d	Jan 2011	KL, Wet Market A	YE037	Raw pork intestine	1154523,	92.3%	3 variant	O:3
27	S18/1-C-O-5-6e	Jan 2011	KL, Wet Market A	YE037	Raw pork intestine	1115523,	92.3%	3 variant	O:3
28	S18/1-C-O-1a	Jan 2011	KL, Wet Market A	YE032	Raw pork liver	1354723,	92.5%	1B	O:8
29	S18/1-C-O-K-5b	Jan 2011	KL, Wet Market A	YE036	Raw pork meat	1354523,	92.3%	1B	O:8
30	S18/1-C-O-5c	Jan 2011	KL, Wet Market A	YE036	Raw pork meat	1355523,	81.3%	1B	O:8
31	PC-M16-2	Jan 2011	KL, Wet Market A	M16	Raw pork meat	1155723,	98.3%	1A	O:5
32	PC-M16-10	Jan 2011	KL, Wet Market A	M16	Raw pork meat	1154723,	92.5%	1A	O:5

Refer Appendix [a]VIII and [b]IX for raw data

4.4.1 Virulotypes of *Y. enterocolitica* isolates

In this study, the cycling and PCR mix conditions were re-optimised for 13 virulence genes into four multiplex PCRs (MP1 to MP4) as the original published conditions were not appropriate. The involved genes are as follow: *hreP, virF, rfbC, myfA, fes, sat, fepD, inv, ail. ymoA, tccC, yadA,* and *fepA.* The optimized cycling and PCR mix conditions are tabulated in Tables 4.4.2 and 4.4.3. Cycling conditions for multiplex MP5 (*ystA* and *ystB* genes) was performed according to recommendations in the original studies. Figure 4.4.1 shows representative agarose gel photo for the multiplexes by using positive control strains.

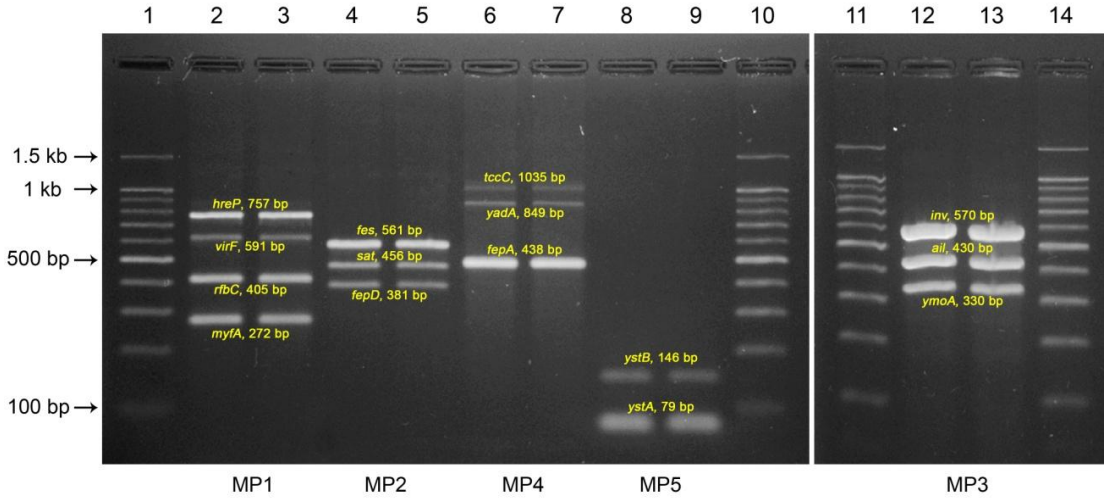


Figure 4.4.1. Representative agarose gel (2%) electrophoresis photo of multiplex MP1 to MP5 by using positive control strains.

Lanes 1, 10, 11 and 14, molecular weight marker (100 bp, Promega, USA); lanes 2 and 3, amplicons of multiplex MP1 (IP383 + IP135; *hreP*, 757 bp; *virF*, 591 bp; *rfbC*, 405 bp; *myfA*, 272 bp); lanes 3 and 4, amplicons of multiplex MP2 (IP135; *fes*, 561 bp; *sat*, 456 bp; *fepD*, 381 bp); lanes 6 and 7, amplicons of multiplex MP4 (IP102 + IP383; *tccC*, 1035 bp; *yadA*, 849 bp; *fepA*, 438 bp); lanes 8 and 9, amplicons of multiplex MP5 (IP102 + IP11105; *ystB*, 146 bp; *ystA*, 79 bp); lanes 12 and 13, amplicons of multiplex MP3 (IP11105 + IP383; *inv*, 570 bp; *ail*, 430 bp; *ymoA*, 330 bp).

Table 4.4.2. Primers sequences and PCR cycling conditions for virulence genes determination of *Y. enterocolitica*.

Multiplex PCR (MP)	Gene	Gene product/ function	Primers sequence (5'→3')	Amplicon size (bp)	PCR conditions (℃, s)[a]			References
					Denaturation	Annealing	Extension	
MP1	*hreP*	subtilisin/kexin-like protease	Forward – GCCGCTATGGTGCCTCTGGTGTG Reverse – CCCGCATTGACTCGCCCGTATC	757	95, 30	55, 60	72, 60	(Bhagat & Virdi, 2007)
	virF	transcriptional activator	Forward – TCATGGCAGAACAGCAGTCAG Reverse – ACTCATCTTACCATTAAGAAG	590				(Bhaduri & Pickard, 1995)
	rfbC	specific detection to YE serotype O:3	Forward – CGCATCTGGGACACTAATTCG Reverse – CCACGAATTCCATCAAACCACC	405				(Weynants, et al., 1996)
	myfA	fimbriae	Forward – CAGATACACCTGCCTTCCATCT Reverse – CTCGACATATTCCTCAACACGC	272				(Kot & Trafny, 2004)
MP2	*fes*	enterochelin esterase	Forward – GCCGGCAGGCACAGCGTAAT Reverse – GGCCAACCCACCCAAAACTT	561	95, 30	55, 60	72, 60	(Schubert, et al., 1999)
	sat	streptogramin acetyltransferase	Forward – CCGATGGTGGGGTTTTCTCAAG Reverse – GGGATTACCGCCGACCACACTA	456				(Bhagat & Virdi, 2007)
	fepD	enterochelin ABC transporter	Forward – GTGTGATTGCCTTACTATTG Reverse – CGGTCATCCTTTTATTACGG	381				(Schubert, et al., 1999)
MP3	*inv*	invasin	Forward – CTGTGGGGAGAGTGGGGAAGTTTGG Reverse – GAACTGCTTGAATCCCTGAAAACCG	570	95, 30	55, 60	72, 60	(Rasmussen, Rasmussen, Andersen, & Olsen, 1994)
	ail	adhesin	Forward – TTAATGTGTACGCTGCGAGTG Reverse – GGAGTATTCATATGAAGCGTC	430				(Wannet, et al., 2001)
	ymoA	yersinia modulator	Forward – GACTTTTCTCAGGGGAATAC Reverse – GCTCAACGTTGTGTGTCT	330				(Grant, et al., 1998)
MP4	*tccC*	insecticidal toxin complex-like protein	Forward – GGGCAAAAAATGCGTGAAGAGAG Reverse – TTTACCGGAATAACGCACAGTTTTA	1035	95, 30	51, 90	72, 90	(Bhagat & Virdi, 2007)
	yadA	auto agglutination, serum resistance, adhesion	Forward – CTTCAGATACTGGTGTCGCTGT Reverse – ATGCCTGACTAGAGCGATATCC	849 759[b]				(Wang, et al., 2008)
	fepA	enterochelin receptor protein	Forward – TACGCCAAAATACCTTACGAT Reverse – TGTAAATACACCCCCACCTGA	438				(Schubert, et al., 1999)
MP5	*ystB*	enterotoxin	Forward – GTACATTAGGCCAAGAGACG Reverse – GCAACATACCTCACAACACC	146	95, 5	60, 20	72, 20	(Thoerner, et al., 2003)
	ystA	enterotoxin	Forward – ATCGACACCAATAACCGCTGAG Reverse – CCAATCACTACTGACTTCGGCT	79				(Thoerner, et al., 2003)

[a] PCRs were performed with a pre-denaturation step of 95 ℃ for 10 min, 30 cycles of denaturation, annealing, extension as indicated above and a final extension of 72 ℃, 10 min
[b] amplicon length in pathogenic *Y. enterocolitica* serotypes O:8

Table 4.4.3. Conditions of PCR mixes of multiplex PCRs for virulence genes determination for *Y. enterocolitica.*

Materials	Stock conc.	Multiplex MP1		Multiplex MP2		Multiplex MP3		Multiplex MP4		Multiplex MP5	
		Working conc.	1 × (µl)	Working conc.	1 × (µl)	Working conc.	1 × (µl)	Working conc.	1 × (µl)	Working conc.	1 × (µl)
Buffer (×)	5	1	5.0	1	5.0	1.5	7.5	1	5.0	1	5.0
$MgCl_2$ (mM)	25	1.5	1.5	1.5	1.5	1.5	1.5	3	1.5	3	3.0
dNTPs (mM)	10	0.2	0.5	0.2	0.5	0.2	0.5	0.2	0.5	0.2	0.5
Primers (µM)	10	*hreP*: 0.2	0.5	*fes*: 0.2	0.5	*inv*: 0.2	0.5	*tccC*: 0.3	0.5	*ystB*: 0.1	0.25
	10	*virF*: 0.2	0.5	*sat*: 0.2	0.5	*ail*: 0.2	0.5	*yadA*: 0.3	0.5	*ystA*: 0.1	0.25
	10	*rfbC*:0.2	0.5	*fepD*: 0.2	0.5	*ymoA*:0.2	0.5	*fepA*: 0.2	0.5	-	-
	10	*myfA*: 0.2	0.5	-	-	-	-	-	-	-	-
Taq polymerase (U/µL)	5	1	0.2	1	0.2	1	0.2	1	0.2	0.5	0.1
ddH_2O	-	-	13.8	-	13.8	-	11.3	-	13.8	-	13.4
DNA templates (ng)	-	~20	2.0	~20	2.0	~20	2.0	~20	2.0	~20	2.0
Total			**25.000**		**25.000**		**25.000**		**25.000**		**25.000**
Positive control strains		**IP383 IP135**		**IP135**		**IP11105 IP383**		**IP102 IP383**		**IP102 IP11105**	

The prevalence of the 15 virulence genes tested are as follows: *hreP* (100%), *sat* (100%), *ymoA* (100%), *myfA* (94%), *inv* (94%), *ystA* (94%), *yadA* (94%), *virF* (84%), *rfbC* (84%), *ail* (84%), *tccC* (84%), *fepA* (34%), *fes* (16%), *fepD* (16%), and *ystB* (6%) (Table 4.4.4). Virulotyping results of the 32 selected *Y. enterocolitica* isolates are tabulated in 4.4.4. Figures 4.4.2 to 4.4.6 show representative agarose gel photos for the PCRs of *Y. enterocolitica* isolates.

Table 4.4.4. Prevalence of virulence genes for 32 selected *Y. enterocolitica* isolates.

No.	Name of isolates	Virulence Genes															Virulotype
		hreP	***virF***	***rfbC***	***myfA***	***fes***	***sat***	***fepD***	***inv***	***ail***	***ymoA***	***ystB***	***ystA***	***tccC***	***yadA***	***fepA***	
1	PCM-PPN53a-1	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
2	PCM-PPN53a-3	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
3	DCM-PPN55a-1	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
4	PCM-PPN55a-1	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
5	PCM-PPN55b-1	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
6	PCM-PPN55b-2	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
7	PCM-PPN55b-4	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
8	PCM-PPN55b-K3	+	+	+	+	-	+	-	+	+	+	-	+	+	+	+	YeVi4
9	PCM-PPN55c-1	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
10	PCM-PPN55c-3	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
11	PCM-PPN56a-1	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
12	PCM-PPN56a-4	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
13	PCM-PPN56a-K1	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
14	DCM-PPN56b-21	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
15	DCM-PPN56b-23	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
16	PCM-PPN56b-4	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
17	PCM-PPN56b-K3	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
18	DCM-PPN56c-21	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
19	DCM-PPN56c-23	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
20	PCM-PPN56c-K4	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
21	PC-M1-K1	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
22	PC-M3-6	+	+	+	+	-	+	-	+	+	+	-	+	+	+	-	YeVi3
23	PC-M13-K13	+	+	+	+	-	+	-	+	+	+	-	+	+	+	+	YeVi4
24	S18/1-C-O-6a	+	+	+	+	-	+	-	+	+	+	-	+	+	+	+	YeVi4
25	S18/1-C-I-4-6b	+	+	+	+	-	+	-	+	+	+	-	+	+	+	+	YeVi4
26	S18/1-C-O-6d	+	+	+	+	-	+	-	+	+	+	-	+	+	+	+	YeVi4
27	S18/1-C-O-5-6e	+	+	+	+	-	+	-	+	+	+	-	+	+	+	+	YeVi4
28	S18/1-C-O-1a	+	-	-	+	+	+	+	+	-	+	-	+	-	+	+	YeVi1
29	S18/1-C-O-K-5b	+	-	-	+	+	+	+	+	-	+	-	+	-	+	+	YeVi1
30	S18/1-C-O-5c	+	-	-	+	+	+	+	+	-	+	-	+	-	+	+	YeVi1
31	PC-M16-2	+	-	-	-	+	+	+	-	-	+	+	-	-	-	+	YeVi2
32	PC-M16-10	+	-	-	-	+	+	+	-	-	+	+	-	-	-	+	YeVi2
No. of isolates		**32**	**27**	**27**	**30**	**5**	**32**	**5**	**30**	**27**	**32**	**2**	**30**	**27**	**30**	**11**	
Prevalence (%)		**100.0**	**84.4**	**84.4**	**93.8**	**15.6**	**100.0**	**15.6**	**93.8**	**84.4**	**100.0**	**6.3**	**93.8**	**84.4**	**93.8**	**34.4**	

+, positive; -, negative.

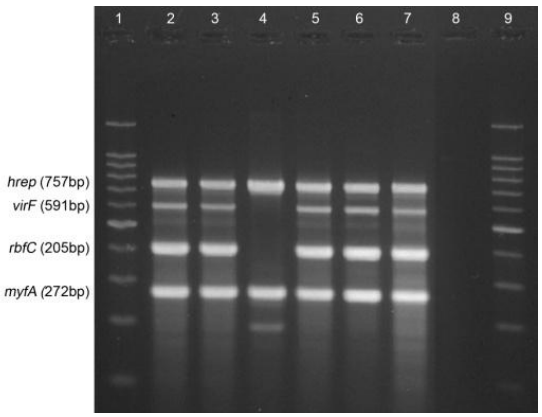


Figure 4.4.2. Representative agarose gel (2%) electrophoresis photo of multiplex MP1.

Lane 1 and 9, DNA marker (100bp, Promega); lane 2 and 7, positive control (IP383 + IP135); lane 3, PC-M1-K1; lane 4, S18/1 C-O-5c; lane 5, S18/1 C-O-6a; lane 6, S18/1 C-O-5-6e; lane 8, negative control.

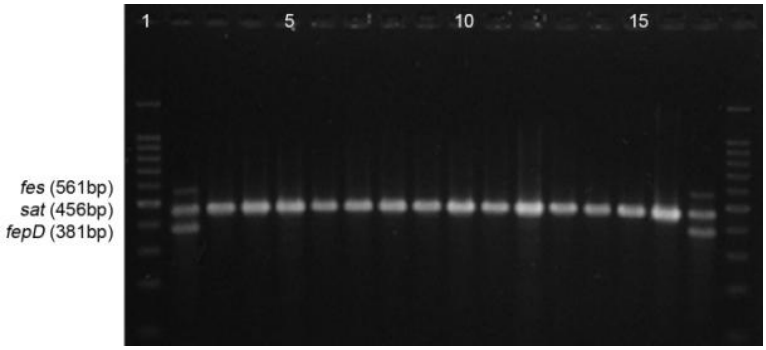


Figure 4.4.3. Representative agarose gel (2%) electrophoresis photo of multiplex MP2.

Lane 1 and 18, DNA marker (100bp, Promega); lane 2 and 17, positive control (IP135); lane 3, PCM-PPN53a-1; lane 4, PCM-PPN53a-3; lane 5, DCM-PPN55a-1; lane 6, PCM-PPN55a-1; lane 7, PCM-PPN55b-1; lane 8, PCM-PPN55b-2; lane 9, PCM-PPN55b-4; lane 10, PCM-PPN55b-K3; lane 11, PCM-PPN55c-1; lane 12, PCM-PPN55c-3; lane 13, PCM-PPN56a-1; lane 14, PCM-PPN56a-4; lane 15, PCM-PPN56a-K1; lane 16, DCM-PPN56b-21.

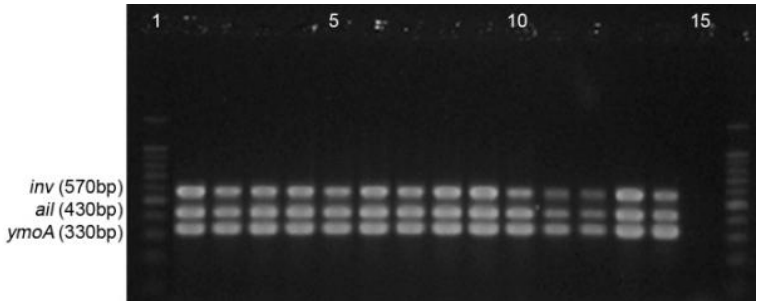


Figure 4.4.4. Representative agarose gel (2%) electrophoresis photo of multiplex MP3.

Lane 1 and 16, DNA marker (100bp, Promega); lane 2 and 14, positive control (IP11105 + IP383); lane 3, PCM-PPN53a-1; lane 4, PCM-PPN53a-3; lane 5, DCM-PPN55a-1; lane 6, PCM-PPN55a-1; lane 7, PCM-PPN55b-1; lane 8, PCM-PPN55b-2; lane 9, PCM-PPN55b-4; lane 10, PCM-PPN55b-K3; lane 11, PCM-PPN55c-1; lane 12, PCM-PPN55c-3; lane 13, PCM-PPN56a-1; lane 15, negative control.

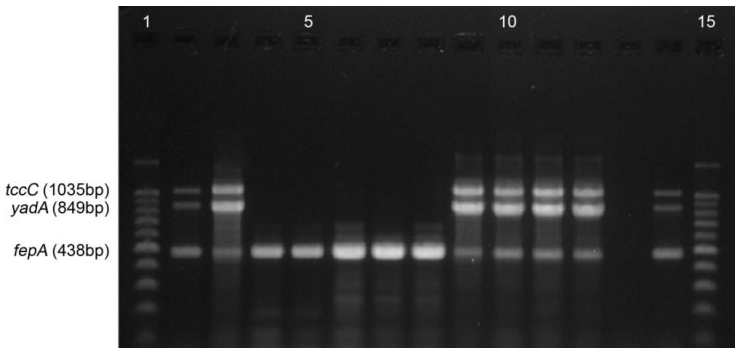


Figure 4.4.5. Representative agarose gel (2%) electrophoresis photo of multiplex MP4.

Lane 1 and 15, DNA marker (100bp, Promega); lane 2 and 14, positive control (IP102 + IP383); lane 3, PC-M13-K13; lane 4, PC-M16-2; lane 5, PC-M16-5; lane 6, PC-M16-10; lane 7, S18/1 C-O-1a; lane 8, S18/1 C-O-K-5b; lane 9, S/18/1 C-O-5c; lane 10, S/18/1 C-O-6a; lane 11, S/18/1 C-I-4-6a; lane 12, S/18/1 C-O-6d; lane 12, S/18/1 C-O-5-6e; lane 14, negative control.

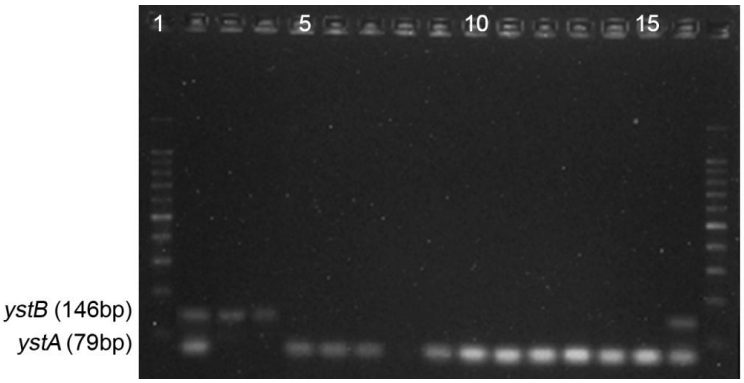


Figure 4.4.6. Representative agarose gel (2%) electrophoresis photo of multiplex MP5.

Lane 1 and 17, DNA marker (100bp, Promega); lane 2 and 16, positive control (IP102 + IP11105); lane 3, PC-M16-2; lane 4, PC-M16-10; lane 5, PC-M1-K1; lane 6, PC-M3-6; lane 7, PC-M13-K13; lane 8, negative control; lane 9, S18/1 C-O-1a; lane 10, S18/1 C-O-K-5b; lane 11, S/18/1 C-O-5c; lane 12, S/18/1 C-O-6a; lane 13, S/18/1 C-I-4-6a; lane 14, S/18/1 C-O-6d; lane 15, S/18/1 C-O-5-6e.

DNA sequences of representative amplicons for all genes were analysed using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/), and results showed high similarity to the sequences of genes in Genebank, with 98-100% homology (Appendix X). There were four reproducible virulence genes patterns (virulotype) and each virulotype associated with a particular bioserotype; i.e. virulotypes **YeVi1** (n=3, bioserotype 1B/O:8), **YeVi2** (n=2, bioserotype 1A/O:5), **YeVi** (n=6, bioserotype 3 variant/O:3), **YeVi4** (n=21, bioserotype 3 variant/O:3). Table 4.4.5 summarises the virulotypes of *Y. enterocolitica.*

Table 4.4.5. Virulotypes of *Y. enterocolitica.*

Virulotype	Virulence genes[a]	*Y. enterocolitica* bioserotype	No. of strains
YeVi1[b]	*hreP, myfA, fes, sat, fepD, inv, ymoA, ystA, yadA, fepA*	1B/O:8	3
YeVi2[c]	*hreP, fes, sat, fepD, ymoA, ystB, fepA*	1A/O:5	2
YeVi3[d]	*hreP, virF, rfbC, myfA, sat, inv, ail, ymoA, ystA, tccC, yadA, fepA*	3 variant/O:3	6
YeVi4[e]	*hreP, virF, rfbC, myfA, sat, inv, ail, ymoA, ystA, tccC, yadA*	3 variant/O:3	21
Total			**32**

[a]The 15 virulence genes tested were *hreP, virF, rfbC, myfA, fes, sat, fepD, inv, ail, ymoA, ystA, ystB,, tccC, yadA,* and *fepA*; [b]The absent genes (PCR detection) were *virF, rfbC, ail, ystB,* and *tccC;* [c]The absent genes (PCR detection) were *virF, rfbC, myfA, inv, ail, ystA, tccC,* and *fepA;* [d]The absent genes (PCR detection) were *fes, fepD, ystB,* and *fepA;* [e]The absent genes (PCR detection) were *fes, fepD,* and *ystB.*

4.4.2 Phenotypic virulence plasmid tests

The presence of virulence plasmid was phenotypically determined by three biochemical tests as stated in Table 4.4.6. CR-MOX test allows visualization of calcium-dependent growth and uptake of Congo red dye (Riley & Toma, 1989). Isolates that contain the pYV virulence plasmid are CR-MOX positive. Crystal violet of the crystal violet binding test binds to the isolates that contain the pYV virulence plasmid (Bhaduri, et al., 1987). Simillarly, isolates that contain the pYV virulence plasmid agglutinate at 37℃ but not 25℃ in the autoagglutination test (Farmer 3rd, et al., 1992). Isolates that lack the virulence plasmid do not agglutinate at either temperature. Representative photo of each tests are in Appendix XI.

Isolates that are pYV-plasmidless are negative in all three biochemical. All *Y. enterocolitica* bioserotype 3 variant/O:3 isolates (n=27) showed positive in all three biochemical tests, indicating they contained the pYV virulence plasmid. Isolates of bioserotypes 1A/O:5 (n=2) and 1B/O:8 (n=3) showed negative in all three tests, indicating did not contain the pYV virulence plasmid.

Table 4.4.6. Results of the auto-agglutination, CR-MOX, and crystal violet binding.

Bioserotype of *Y. enterocolitica* (N=32)	Auto-agglutination	CR-MOX	Crystal violet binding	P/A of pYV plasmid
3 variant/O:3 (n=27)	+	+	+	Present
1B/O:8 (n=3)	-	-	-	Absent
1A/O:5 (n=2)	-	-	-	Absent

+, positive; -, negative.

4.4.3 Plasmid profiles

In this study, the prevalence of plasmids in the 32 selected *Y. enterocolitica* isolates were determined by two ways: (i) PFGE of unrestricted DNA plugs; (ii) electrophoresis of extracted plasmid DNA by using commercial kit. The plasmid sizes were estimated by using Quantity One® 1-D Analysis software (Bio-Rad). Table 4.4.7 shows the plasmid sizes and the plasmid profiles of the 32 selected *Y. enterocolitica* isolates. Gel photos for the PFGE of unrestricted DNA plugs and extracted plasmid DNA are shown in Figures 4.4.7, 4.4.8, 4.4.9, and 4.4.10.

Overall, nine plasmid profiles were observed (Table 4.4.7; YeP1 to YeP9). Twenty-eight isolates (87.5%) carried multiple plasmids ranging from ~2.3 kb to ~102 kb (Table 4.4.7). PFGE of undigested genomic DNA showed the presence of the ~70 kb band indicative of the pYV virulence plasmid, in all isolates of bioserotype 3 variant/O:3 (n=27) (including the control strain, IP383; Figure 4.4.7). In addition, 4-7 small plasmids with size ranging from ~2.3 kb to ~17.7 kb were found (Table 4.4.7). The presence of pYV virulence plasmid (~70 kb) in the 3 variant/O:3 isolates (Table 4.4.7) concurred with the PCR results as both the *yadA* and *virF* genes (pYV plasmid-encoded genes) were present (Tables 4.4.4 and 4.4.5) and the positive phenotypic plasmid detection tests (Table 4.4.6). Two isolates were observed to possess an extra ~102 kb plasmid (Table 4.4.7 and Figure 4.4.7; isolates: PCM-PPN56a-1 and DCM-

PPN55a-1). The two 1B/O:8 and all 1A/O:5 strains did not harbour any plasmid (Table 4.4.7). This is in agreement with the negative results of the phenotypic plasmid detection tests (Tables 4.4.6).

Table 4.4.7. Number of plasmids, plasmid profiles, and plasmid sizes carried by *Y. enterocolitica* isolates.

No.	Name of isolates	Pheno-typic tests	Plasmid Size (kb)*	No. of plasmid	Plasmid profiles
1	PCM-PPN53a-1	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
2	PCM-PPN53a-3	+	70, 25, 8.7, 8.2, 5.6, 5.2	6	YeP1
3	DCM-PPN55a-1	+	102, 65, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	8	YeP7
4	PCM-PPN55a-1	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
5	PCM-PPN55b-1	+	70, 17.7, 7.4, 5.6, 5.2	5	YeP2
6	PCM-PPN55b-2	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
7	PCM-PPN55b-4	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
8	PCM-PPN55b-K3	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
9	PCM-PPN55c-1	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
10	PCM-PPN55c-3	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
11	PCM-PPN56a-1	+	102, 70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	8	YeP8
12	PCM-PPN56a-4	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
13	PCM-PPN56a-K1	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
14	DCM-PPN56b-21	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
15	DCM-PPN56b-23	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
16	PCM-PPN56b-4	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
17	PCM-PPN56b-K3	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
18	DCM-PPN56c-21	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
19	DCM-PPN56c-23	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
20	PCM-PPN56c-K4	+	70, 17.7, 10.9, 8.7, 7.4, 5.6, 5.2	7	YeP3
21	PC-M1-K1	+	70, 14, 12, 8.5, 3.5, 2.6, 2.5, 2.3	8	YeP5
22	PC-M3-6	+	70, 17.7, 8.5, 3.5, 2.6, 2.5, 2.3	7	YeP6
23	PC-M13-K13	+	70, 17.7, 8.5, 3.5, 2.6, 2.5, 2.3	7	YeP6
24	S18/1-C-O-6a	+	70, 17.7, 14.4, 8.5, 3.5, 2.6, 2.5, 2.3	8	YeP4
25	S18/1-C-I-4-6b	+	70, 17.7, 14.4, 8.5, 3.5, 2.6, 2.5, 2.3	8	YeP4
26	S18/1-C-O-6d	+	70, 14, 8.5, 3.5, 2.6, 2.5, 2.3	7	YeP5
27	S18/1-C-O-5-6e	+	70, 14, 8.5, 3.5, 2.6, 2.5, 2.3	7	YeP5
28	S18/1-C-O-1a	-	No plasmid	0	No plasmid
29	S18/1-C-O-K-5b	-	25.3, 12	2	YeP9
30	S18/1-C-O-5c	-	No plasmid	0	No plasmid
31	PC-M16-2	-	No plasmid	0	No plasmid
32	PC-M16-10	-	No plasmid	0	No plasmid

*Estimated plasmid size in kilobases (determined by using Quantity One® 1-D Analysis). Refer Figures 4.4.7, 4.4.8, 4.4.9 and 4.4.10 for gel photos.

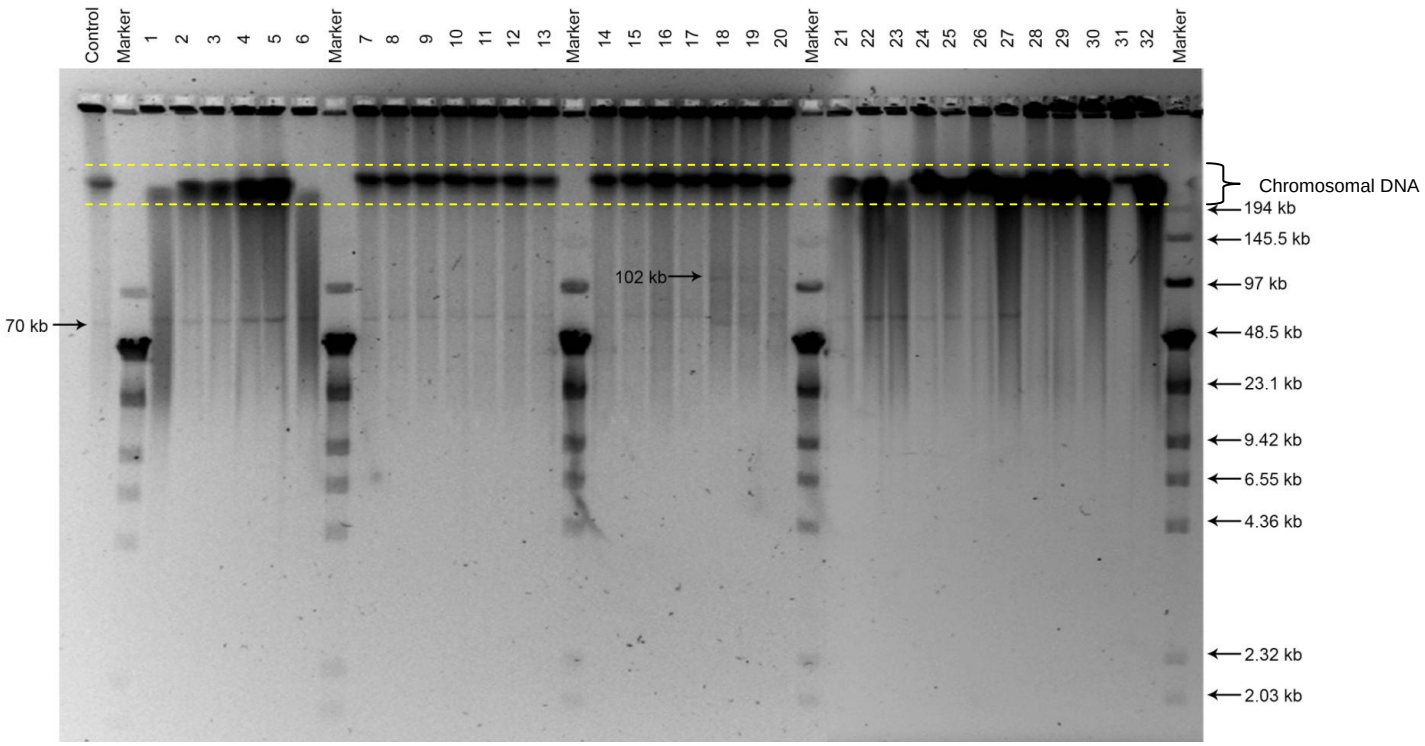


Figure 4.4.7. PFGE gel photos for unrestricted genomic DNA of *Y. enterocolitica* isolates.

Plasmid size was determined by using Quantity One® 1-D Analysis. Lane 1, PCM-PPN53a-3; lane 2, PCM-PPN55c-1; lane 3, PCM-PPN55c-3; lane 4, PCM-PPN56a-4; lane 5, PCM-PPN55a-K1; lane 6, PCM-PPN56b-4; lane 7, PCM-PPN53a-1; lane 8, PCM-PPN55a-1; lane 9, PCM-PPN55b-1; lane 10, PCM-PPN55b-2; lane 11, PCM-PPN55b-4; lane 12, PCM-PPN55b-K3; lane 13, DCM-PPN56b-21; lane 14, DCM-PPN56b-23; lane 15, PCM-PPN56b-K3; lane 16, DCM-PPN56c-21; lane 17, DCM-PPN56c-23; lane 18, PC-M1-K1; lane 19, PC-M3-6; lane 20, PC-M13-K13; lane 21, DCM-PPN55a-1; lane 22, PCM-PPN56a-1; lane 23, PCM-PPN56c-K4; lane 24, S18/1-C-O-6a; lane 25, S18/1-C-I-4-6b; lane 26, S18/1-C-O-6d; lane 27, S18/1-C-O-5-6e; lane 28, S18/1-C-O-1a; lane 29, S18/1-C-O-K-5b; lane 30, S18/1-C-O-5c; lane 31, PC-M16-2; lane 32, PC-M16-10; Control, *Y. enterocolitica* bioserotype 2/O:9 (IP383); Marker , low range PFG marker N0350S (New England Biolabs).

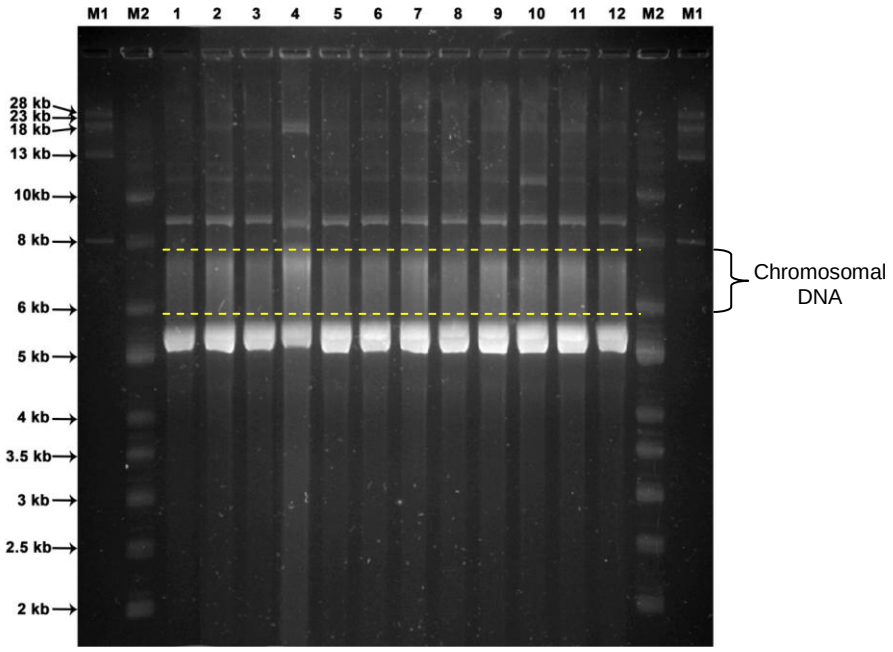


Figure 4.4.8. Gel photo for extracted plasmid DNA.

Plasmid size was determined by using Quantity One® 1-D Analysis. M1, supercoiled DNA marker set (8-28 kb, Epicentre® Biotechnologies, USA); M2, supercoiled DNA ladder (2-10 kb, New England Biolabs, USA); lane 1, PCM-PPN53a-1; lane 2, DCM-PPN55a-1; lane 3, PCM-PPN55a-1; lane 4, PCM-PPN55b-1; lane 5, PCM-PPN55b-2; lane 6, PCM-PPN55b-4; lane 7, PCM-PPN55b-K3; lane 8, PCM-PPN55c-1; lane 9, PCM-PPN55c-3; lane 10, PCM-PPN56a-1; lane 11, PCM-PPN56a-K1; lane 12, PCM-PPN56a-4.

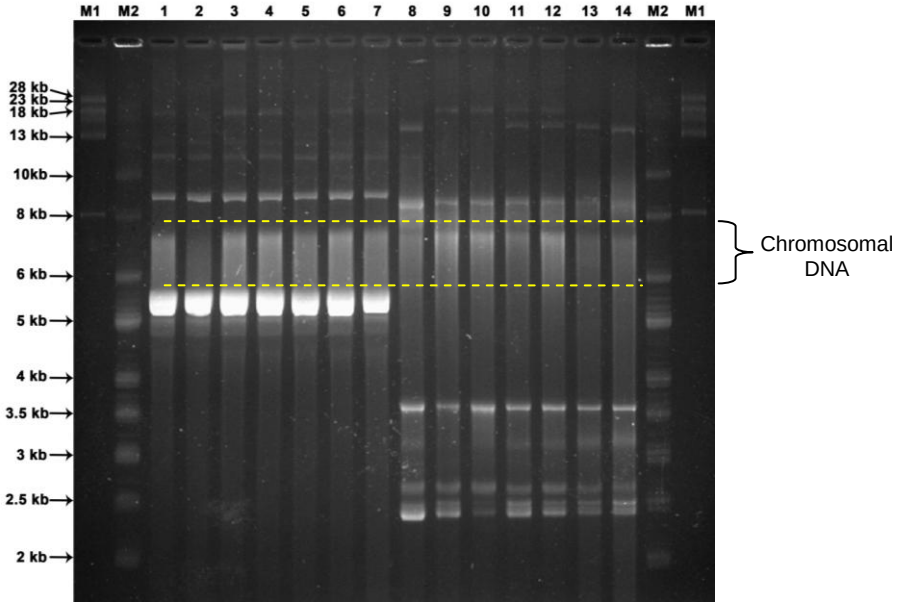


Figure 4.4.9. Gel photo for extracted plasmid DNA.

Plasmid size was determined by using Quantity One® 1-D Analysis. M1, supercoiled DNA marker set (8-28 kb, Epicentre® Biotechnologies, USA); M2, supercoiled DNA ladder (2-10 kb, New England Biolabs, USA); lane 1, DCM-PPN56b-21; lane 2, DCM-PPN56b-23; lane 3, PCM-PPN56b-4; lane 4, PCM-PPN56b-K3; lane 5, DCM-PPN56c-21; lane 6, DCM-PPN56c-23; lane 7, PCM-PPN56c-K4; lane 8, PC-M1-K1; lane 9, PC-M3-6; lane 10, PC-M13-K13; lane 11, S18/1-C-O-6a; lane 12, S18/1-C-I-4-6b; lane 13, S18/1-C-O-6d; lane 14, S18/1-C-O-5-6e.

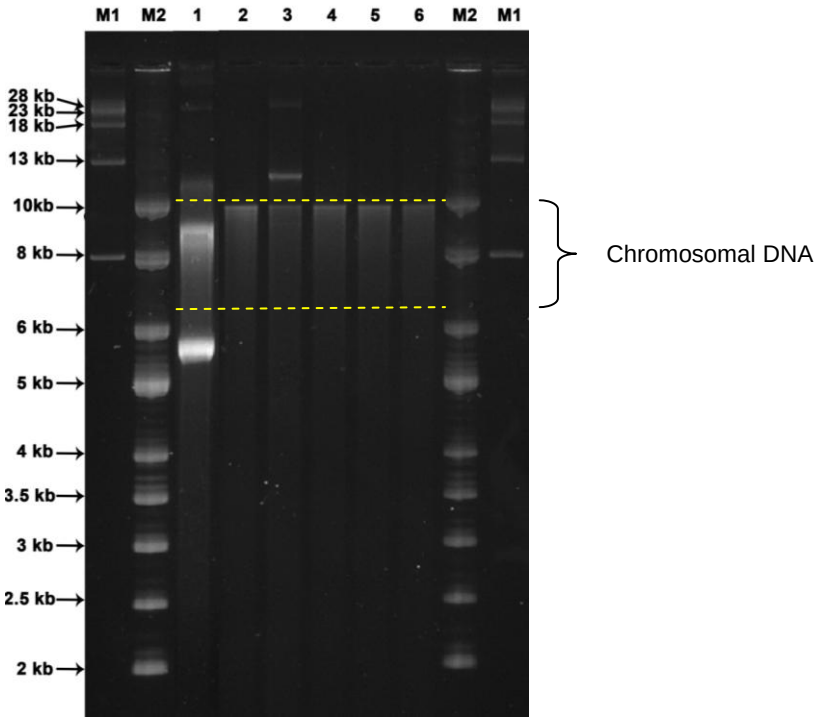


Figure 4.4.10. Gel photo for extracted plasmid DNA.

Plasmid size was determined by using Quantity One® 1-D Analysis.
M1, supercoiled DNA marker set (8-28 kb, Epicentre® Biotechnologies, USA);
M2, supercoiled DNA ladder (2-10 kb, New England Biolabs, USA);
lane 1, PCM-PPN53a-3; lane 2, S18/1-C-O-1a; lane 3, S18/1-C-O-K-5b;
lane 4, S18/1-C-O-5c; lane 5, PC-M16-2; lane 6, PC-M16-10.

4.4.4 Antibiograms of *Y. enterocolitica* isolates

The resistance rates of 32 *Y. enterocolitica* strains against the 29 antimicrobials tested are as follows: CLI (87.5%), AMP (87.5%), AMX (84.4%), TIC (78.1%), NAL (62.5%), TET (62.5%), STR (21.9%), AMC (9.4%), TIM (3.1%). Table 4.4.8 summarises the antimicrobial profiles for each antimicrobials. Detailed antimicrobial susceptibility profiles of the 32 *Y. enterocolitica* isolates are tabulated in Appendix XII.

Overall, 12 resistotypes were observed, with resistotype $NAL^{R}CLI^{R}AMP^{R}TIC^{R}TET^{R}AMX^{R}$ being predominant (Table 4.4.9; n=15, 46.9%). Isolates of different bioserotypes had different resistotypes (Table 4.4.8). All 1B/O:8 isolates (n=3) were resistant to only one antimicrobial, CLI (Table 4.4.8). All 1A/O:5 isolates were resistant to AMP, TIC, AMX, and CLI (Table 4.4.8). Majority of the 3 variant/O:3 isolates were resistant to NAL, AMP, TIC, TET, CLI, and AMX (Table 4.4.8).

About 90% of the *Y. enterocolitica* isolates (n=32) were MDR (resistant to at least three classes of antimicrobials) (Appendix XII). Due to the high resistance observed for penicillin (AMP, 87.5%; TIC, 78.1%), and β-lactam (amoxicillin, 84.4%), phenotypic ESBL-production was tested. However, none of the isolates was an ESBL producer. Isolates of bioserotype 3 variant/O:3 had the highest MAR index 0.183, followed by bioserotype 1A/O:5 and 1B/O:8 with MAR indices at 0.121 and 0.103, respectively (Table 4.4.10). All 32 selected isolates were considered originated from low risk contaminated source (MAR index < 0.2) (Krumperman, 1983).

Table 4.4.8. Antimicrobial profiles (in percentage) of the 32 *Y. enterocolitica* isolates from raw pork products and pigs.

Antimicrobial discs	3 variant/O:3 (n=27)			1B/O:8 (n=3)			1A/O:5 (n=2)			Total (n=32)		
	R	I	S	R	I	S	R	I	S	R	I	S
CLI, 2 µg	85.2	14.8	0	100	0	0	100	0	0	87.5	12.5	0
AMP, 10 µg	96.3	3.7	0	0	0	100	100	0	0	87.5	3.1	9.4
AMX, 25 µg	92.6	7.4	0	0	0	100	100	0	0	84.4	6.3	9.4
TIC, 75 µg	85.2	14.8	0	0	0	100	100	0	0	78.1	12.5	9.4
NAL, 30µg	74.1	0	25.9	0	0	100	0	0	0	62.5	0	37.5
TET, 30µg	74.1	0	44.4	0	0	100	0	0	0	62.5	0	37.5
STR, 10 µg	25.9	14.8	59.3	0	0	100	0	0	0	21.9	12.5	65.6
AMC, 30µg	7.4	14.8	40.7	0	0	100	50	0	50	9.4	12.5	78.1
TIM, 25 µg	3.7	7.4	88.9	0	0	100	0	0	0	3.1	6.3	90.6
CXM, 30µg	0	0	0	0	0	100	0	0	0	0	6.3	93.8
TMP, 5 µg	0	3.7	96.3	0	0	100	0	0	0	0	3.1	96.9
ENR, 5 µg	0	3.7	96.3	0	0	100	0	0	0	0	3.1	96.9
Other antimicrobials[a]	0	0	100	0	0	100	0	0	0	0	0	100

[a]Other antimicrobials are CTM, 30µg; CAZ, 30µg; CRO, 30µg; CIP, 5 µg; LEV, 5 µg; KAN, 30µg; AMK, 30µg; GEN, 10 µg; N, 10 µg; NET, 30µg; DOX, 30µg; ATM, 30µg; IPM, 10 µg; PB, 300 µg; CHL, 30µg; SPT, 100 µg; CSS, 10 µg. R, resistant; I, intermediate; R, resistant.

Table 4.4.9. MAR indices of *Y. enterocolitica* according to each resistotype.

Resistotype	MAR index	No. of isolates (%)
$NAL^{R}CLI^{R}AMP^{R}TIC^{R}TET^{R}AMC^{R}AMX^{R}$	0.241	2 (6.3)
$NAL^{R}CLI^{R}AMP^{R}TIC^{R}TET^{R}AMX^{R}$	0.207	15 (46.9)
$CLI^{R}AMP^{R}TIC^{R}STR^{R}TIM^{R}AMX^{R}$	0.207	1 (3.1)
$CLI^{R}AMP^{R}TIC^{R}STR^{R}AMX^{R}$	0.172	2 (6.3)
$NAL^{R}CLI^{R}AMP^{R}TET^{R}AMX^{R}$	0.172	1 (3.1)
$CLI^{R}AMP^{R}TIC^{R}AMC^{R}AMX^{R}$	0.172	1 (3.1)
$AMP^{R}TIC^{R}STR^{R}AMX^{R}$	0.138	3 (9.4)
$NAL^{R}CLI^{R}AMP^{R}TET^{R}$	0.138	1 (3.1)
$CLI^{R}AMP^{R}TIC^{R}AMX^{R}$	0.138	1 (3.1)
$NAL^{R}CLI^{R}TET^{R}$	0.103	1 (3.1)
$AMP^{R}STR^{R}AMC^{R}$	0.103	1 (3.1)
CLI^{R}	0.034	3 (9.4)
Total		**32 (100)**

^{R}, resistant; NAL, 30 µg; CIP, 5 µg; TIM, 25 µg; STR, 10 µg; AMP, 10 µg; TIC, 75 µg; TET, 30 µg; AMC, 30 µg; CLI, 2 µg.

Table 4.4.10. MAR indices of *Y. enterocolitica* according to each bioserotype.

Y. enterocolitica bioserotype	No. of isolates	No. of antimicrobials resistant to	MAR index
3 variant/O:3	27	143	0.183
1B/O:8	3	9	0.103
1A/O:5	2	7	0.121
Total	**32**	**159**	**0.171**

4.4.5 Genotypes of *Y. enterocolitica* based on PFGE

The genetic relatedness of the 32 *Y. enterocolitica* isolates was determined by PFGE. The *Not*I-digested chromosomal DNA of the 32 *Y. enterocolitica* generated ten reproducible unique patterns (0.74 eproducible unique 32 IM, 25 µg; STR, 10 µg; AMP, 10 µg; TIC, 75 µg; TEbetween 22.3 and 446.7 bp. Gel photos for PFGE are shown in Figures 4.4.11, 4.4.12, 4.4.13, and 4.4.14. The dendrogram based on the profiles obtained by PFGE is shown in Figure 4.4.15.

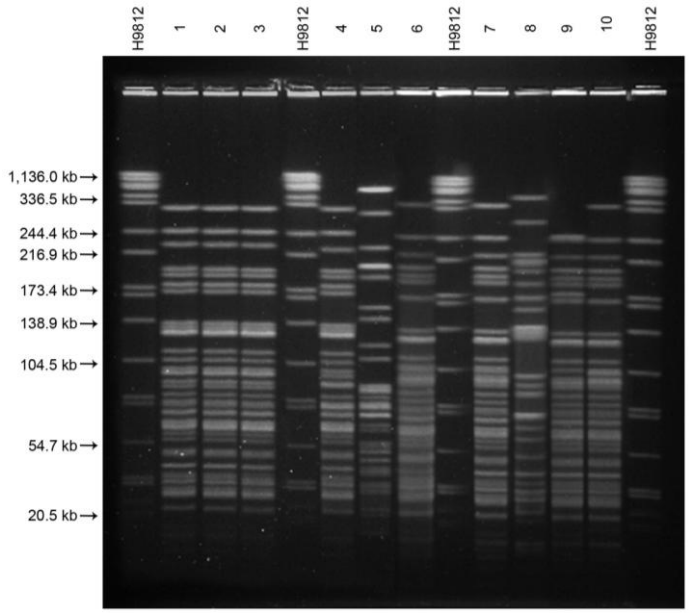


Figure 4.4.11. PFGE (*Not*I-digested DNA plugs) gel photo for *Y. enterocolitica* isolates.

Lane 1, PC-M1-K1; lane 2, PC-M3-6; lane 3, PC-M13-K13; lane 4, S18/6 C-O-6a; lane 5, PC-M16-2; lane 6, DCM-PPN56b-23; lane 7, PCM-PPN56c-K4; lane 8, S18/1 C-O-5c; lane 9, PCM-PPN53a-1; lane 10, PCM-PPN55a-1; H9812, *S. enterica* serotype Braenderup H9812 (ATCC BAA-664) digested with *Xba*I.

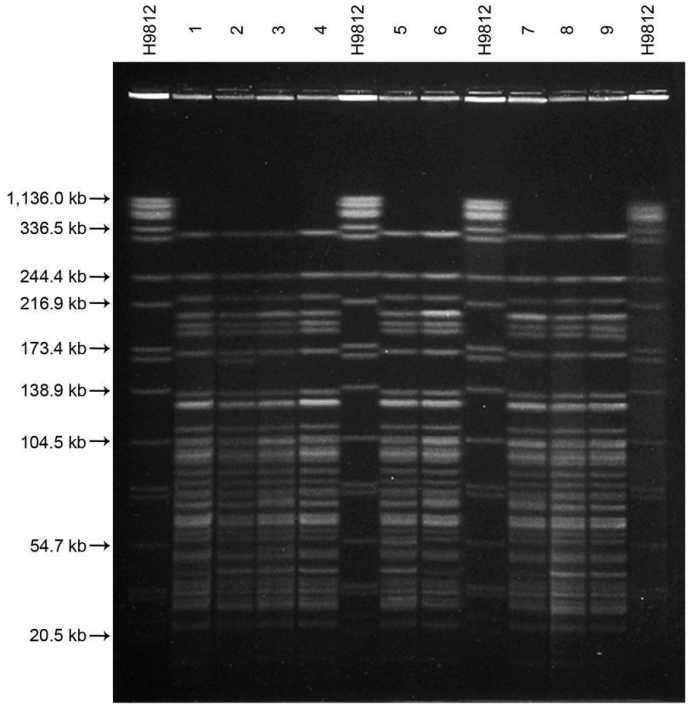


Figure 4.4.12. PFGE (*Not*I-digested DNA plugs) gel photo for *Y. enterocolitica* isolates.

Lane 1, PCM-PPN55b-2; lane 2, PCM-PPN55c-1; lane 3, PCM-PPN55c-3; lane 4, DCM-PPN55a-1; lane 5, PCM-PPN56a-1; lane 6, DCM-PPN56b-21; lane 7, PCM-PPN56b-4; lane 8, PCM-PPN56c-21; lane 9, PCM-PPN55b-K3; H9812, *S. enterica* serotype Braenderup H9812 (ATCC BAA-664) digested with *Xba*I.

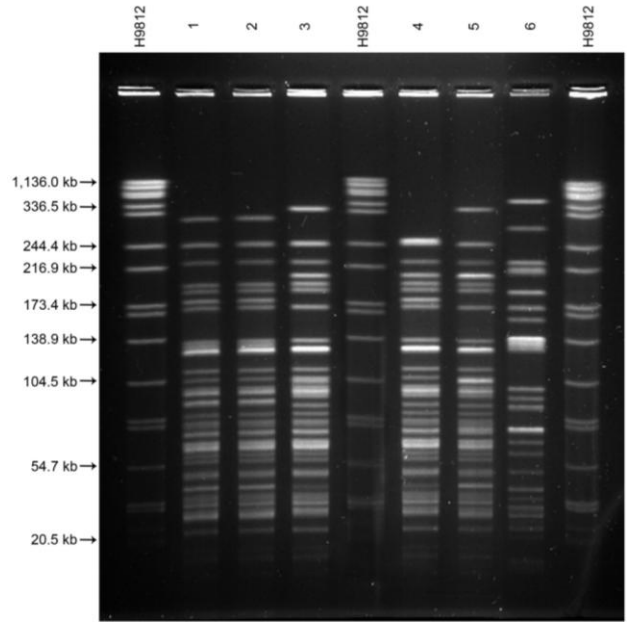


Figure 4.4.13. PFGE (*Not*I-digested DNA plugs) gel photo for *Y. enterocolitica* isolates.

Lane 1, S18/1 C-O-5-6e; lane 2, S18/1 C-O-6d; lane 3, PCM-PPN55b-1; lane 4, PCM-PPN53a-3; lane 5, PCM-PPN55b-4; lane 6, S18/1 C-O-5b; H9812, *S. enterica* serotype Braenderup H9812 (ATCC BAA-664) digested with *Xba*I.

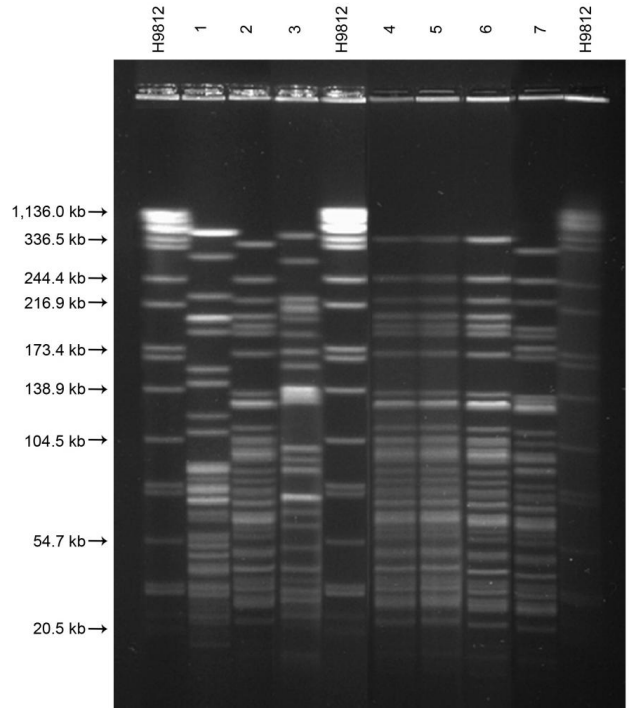
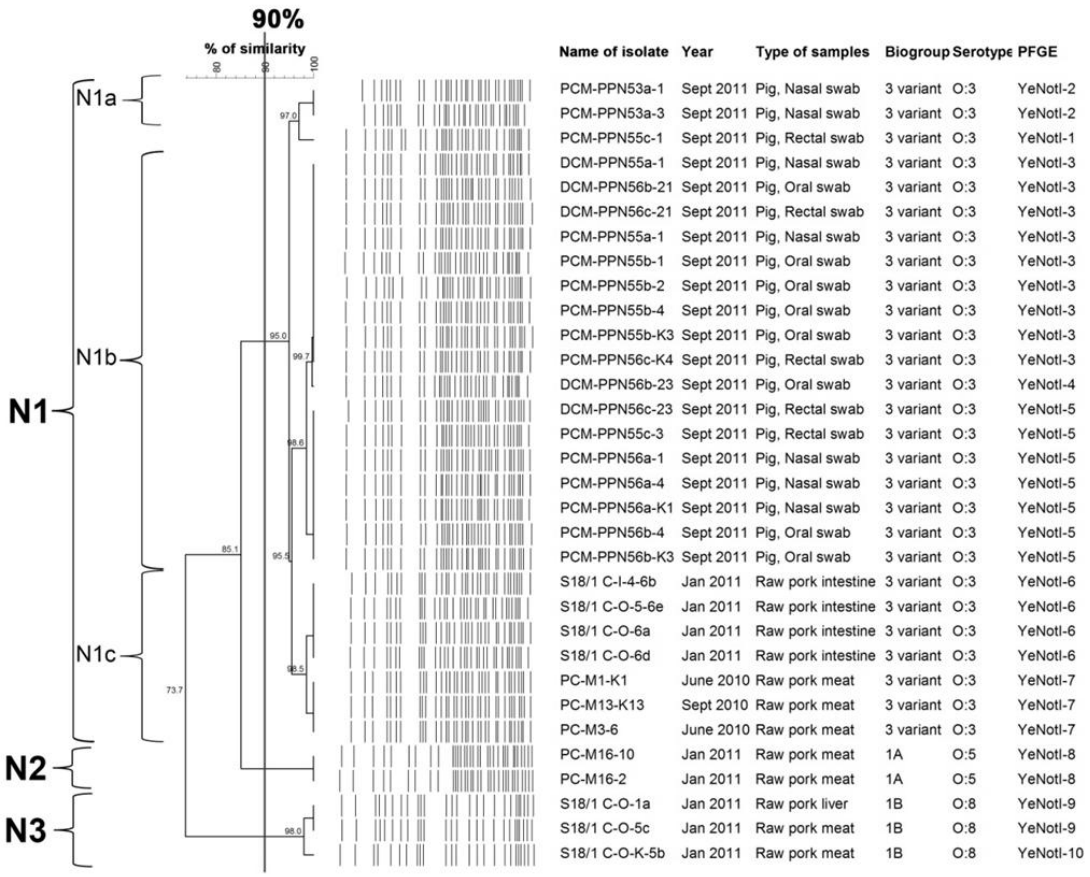


Figure 4.4.14. PFGE (*Not*I-digested DNA plugs) gel photo for *Y. enterocolitica* isolates.

Lane 1, PC-M16-10; lane 2, DCM-PPN56c-23; lane 3, S18/1 C-O-1a; lane 4, PCM-PPN56a-4; lane 5, PCM-PPN56a-K1; lane 6, PCM-PPN56b-K3; lane 7, S18/1 C-I-4-6b; H9812, *S. enterica* serotype Braenderup H9812 (ATCC BAA-664) digested with *Xba*I.



Name of isolate	Year	Type of samples	Biogroup	Serotype	PFGE
PCM-PPN53a-1	Sept 2011	Pig, Nasal swab	3 variant	O:3	YeNotI-2
PCM-PPN53a-3	Sept 2011	Pig, Nasal swab	3 variant	O:3	YeNotI-2
PCM-PPN55c-1	Sept 2011	Pig, Rectal swab	3 variant	O:3	YeNotI-1
DCM-PPN55a-1	Sept 2011	Pig, Nasal swab	3 variant	O:3	YeNotI-3
DCM-PPN56b-21	Sept 2011	Pig, Oral swab	3 variant	O:3	YeNotI-3
DCM-PPN56c-21	Sept 2011	Pig, Rectal swab	3 variant	O:3	YeNotI-3
PCM-PPN55a-1	Sept 2011	Pig, Nasal swab	3 variant	O:3	YeNotI-3
PCM-PPN55b-1	Sept 2011	Pig, Oral swab	3 variant	O:3	YeNotI-3
PCM-PPN55b-2	Sept 2011	Pig, Oral swab	3 variant	O:3	YeNotI-3
PCM-PPN55b-4	Sept 2011	Pig, Oral swab	3 variant	O:3	YeNotI-3
PCM-PPN55b-K3	Sept 2011	Pig, Oral swab	3 variant	O:3	YeNotI-3
PCM-PPN56c-K4	Sept 2011	Pig, Rectal swab	3 variant	O:3	YeNotI-3
DCM-PPN56b-23	Sept 2011	Pig, Oral swab	3 variant	O:3	YeNotI-4
DCM-PPN56c-23	Sept 2011	Pig, Rectal swab	3 variant	O:3	YeNotI-5
PCM-PPN55c-3	Sept 2011	Pig, Rectal swab	3 variant	O:3	YeNotI-5
PCM-PPN56a-1	Sept 2011	Pig, Nasal swab	3 variant	O:3	YeNotI-5
PCM-PPN56a-4	Sept 2011	Pig, Nasal swab	3 variant	O:3	YeNotI-5
PCM-PPN56a-K1	Sept 2011	Pig, Nasal swab	3 variant	O:3	YeNotI-5
PCM-PPN56b-4	Sept 2011	Pig, Oral swab	3 variant	O:3	YeNotI-5
PCM-PPN56b-K3	Sept 2011	Pig, Oral swab	3 variant	O:3	YeNotI-5
S18/1 C-I-4-6b	Jan 2011	Raw pork intestine	3 variant	O:3	YeNotI-6
S18/1 C-O-5-6e	Jan 2011	Raw pork intestine	3 variant	O:3	YeNotI-6
S18/1 C-O-6a	Jan 2011	Raw pork intestine	3 variant	O:3	YeNotI-6
S18/1 C-O-6d	Jan 2011	Raw pork intestine	3 variant	O:3	YeNotI-6
PC-M1-K1	June 2010	Raw pork meat	3 variant	O:3	YeNotI-7
PC-M13-K13	Sept 2010	Raw pork meat	3 variant	O:3	YeNotI-7
PC-M3-6	June 2010	Raw pork meat	3 variant	O:3	YeNotI-7
PC-M16-10	Jan 2011	Raw pork meat	1A	O:5	YeNotI-8
PC-M16-2	Jan 2011	Raw pork meat	1A	O:5	YeNotI-8
S18/1 C-O-1a	Jan 2011	Raw pork liver	1B	O:8	YeNotI-9
S18/1 C-O-5c	Jan 2011	Raw pork meat	1B	O:8	YeNotI-9
S18/1 C-O-K-5b	Jan 2011	Raw pork meat	1B	O:8	YeNotI-10

Figure 4.4.15. Dendrogram of PFGE of *Not*I-digested genomic DNA patterns of *Y. enterocolitica* generated by UPGMA clustering method using Dice coefficient.

PFGE subtyped the isolates into three distinct clusters (N1, N2 and N3), with *D* value of 0.87 based on 90% similarity (Figure 4.4.15). Each cluster contained member of the same bioserotype; i.e. N1, bioserotype 3 variant/O:3; N2, bioserotype 1A/O:5; and N3, bioserotype 1B/O:8.

Cluster N1 was represented by 27 *Y. enterocolitica* isolates (n=7 from food; n=20 from pigs) belonging to bioserotype 3 variant/O:3. Within cluster N1, the isolates were further subgrouped into another three subtypes, N1a, N1b and N1c (Figure 4.4.15). The subtype N1a consisted two indistinguishable isolates isolated from the same nasal swab of the same pig (PFGE profile: YeNotI-2) although they were different by two biochemical tests (API code: 1114523 and 1014522) and plasmid profiles (YeP3 and YeP1) (Figure 4.4.15; Table 4.4.1, pig no. 53 from farm I, Penang). The N1b comprised

of 17 isolates which were isolated from nasal, oral and rectal swabs of two pigs raised in the same pen (Figure 4.4.15, PFGE profile: YeNotI-3, YeNotI-4 and YeNotI-5; Table 4.4.1, pig no. 55 and 56 from farm I, Penang). The N1c comprised of seven *Y. enterocolitica* strains (food origin) with two PFGE patterns (YeNotI-6 and YeNotI-7). The strains were isolated from four different raw pork samples (Table 4.4.1, sample M1, M3 M13 and YE037) purchased at different times from the same hawker stall purchased at different time frame from the same hawker stall (Figure 4.4.15, YeNotI-6 and YeNotI-7).

Within cluster N2, both the bioserotype 1A/O:5 strains were from the same raw pork meat with indistinguishable PFGE pattern (Figure 4.4.15, YeNotI-8), but had different API 20E codes (1155723 and 1154723) and resistant profiles ($CLI^{R}AMP^{R}TIC^{R}AMC^{R}AMX^{R}$/ $CLI^{R}AMP^{R}TIC^{R}AMX^{R}$). The three bioserotype 1B/O:8 strains in cluster N3 were highly similar (F=0.98), with similar antibiogram and virulence genes profile. Of the three strains, two were isolated from raw pork meat and one from raw pork liver, purchased on the same day at the same hawker stall.

4.5 Modification and improvement of CIN agar for isolation of *Y. enterocolitica*

Although CIN agar is widely used in the isolation of *Y. enterocolitica*, however, in this study, plenty of false *Y. enterocolitica* isolates were detected (Refer Section 4.6 for the recovery power of the CIN). Hence, the CIN agar was modified in order to increase the recovery rate of true *Y. enterocolitica* from food and other environmental samples. Results for the evaluation of modified CIN in comparing to CIN are in Sections 4.5.1 to 4.5.5.

4.5.1 Growth characteristics and colony morphology on CIN and modified CIN agar

The capability of bacteria to grow on CIN and modified CIN agar, and the colony morphology of 50 bacterial strains were determined. Both CIN and modified CIN allowed the growth of all *Yersinia* strains tested, except the non-pathogenic strain IP102 (*Y. enterocolitica* bioserotype 1A/O:6,30), which was inhibited on both media. Furthermore, all *Yersinia* colonies displayed the same characteristic, red bull's eye aspect (Table 4.5.1 and Figure 4.5.1, Nb2-17). Therefore, the modifications in the CIN did not alter the growth and colony shape of *Yersinia* strains. The characteristic red bull's eye morphology of *Y. enterocolitica* colonies was observed at ≥30 h incubation on modified CIN instead of 24 h on CIN, indicating that the plates should be read at or after 30 h.

The CIN and modified CIN exhibited the same growth inhibitory effect for various species of *Salmonella, Escherichia coli, Shigella, Proteus, Vibrio, Pseudomonas, Enterococcus, Listeria* and *Staphylococcus* (Table 4.5.1). The two media also gave similar results for non-H_2S-producing *C. freundii, C. koseri, Serriatia odorifera, S. marcescens,* and *Pantoae* spp., which produced colonies with the red bull's eye morphology (Figure 4.5.1 and Table 4.5.1). Hence, the modification of CIN did not improve the differentiation of these bacteria from *Yersinia* spp.

In contrast, the modified CIN but not CIN allowed the differentiation of *Yersinia* spp. from several other *Enterobacteriaceae* and *A. hydrophila*. On the modified CIN, the colonies of *C. braakii* and H_2S-producing *C. freundii* exhibited a black centre (Figure 4.5.1, Nb 18 and 21), the *P. rettgeri* colonies were surrounded by a brown diffusible pigment (Figure 4.5.1, Nb 27-29), the *E. cloacae* colonies were light pink (Figure 4.5.1, Nb 24-26), *Aeromonas hydrophila* appeared as pink colonies surrounded by a brown pigment (Figure 4.5.1, Nb 23), and *M. morganii* appeared as tiny colourless

colonies surrounded by a brown pigment (Figure 4.5.1, Nb 30). The modified CIN is thus more efficient than CIN for the discrimination of *Yersinia* spp. from these *Yersinia*-like bacterial species.

Changing the incubation conditions for the modified CIN had no effect on the colony morphology of the various species tested (Table 4.5.1). However, the formation of a black centre in H_2S-producing colonies could be observed under microaerophilic conditions even when their size was ≤ 1 mm, whereas the black centre appeared when the colonies were larger under aerobic conditions. Colonies that did not turn black at 24 h (aerobic), turned black at 30 h or 48 h when the size of colony grew bigger. Therefore, microaerophilic condition could facilitate better visualization for H_2S-producing bacteria.

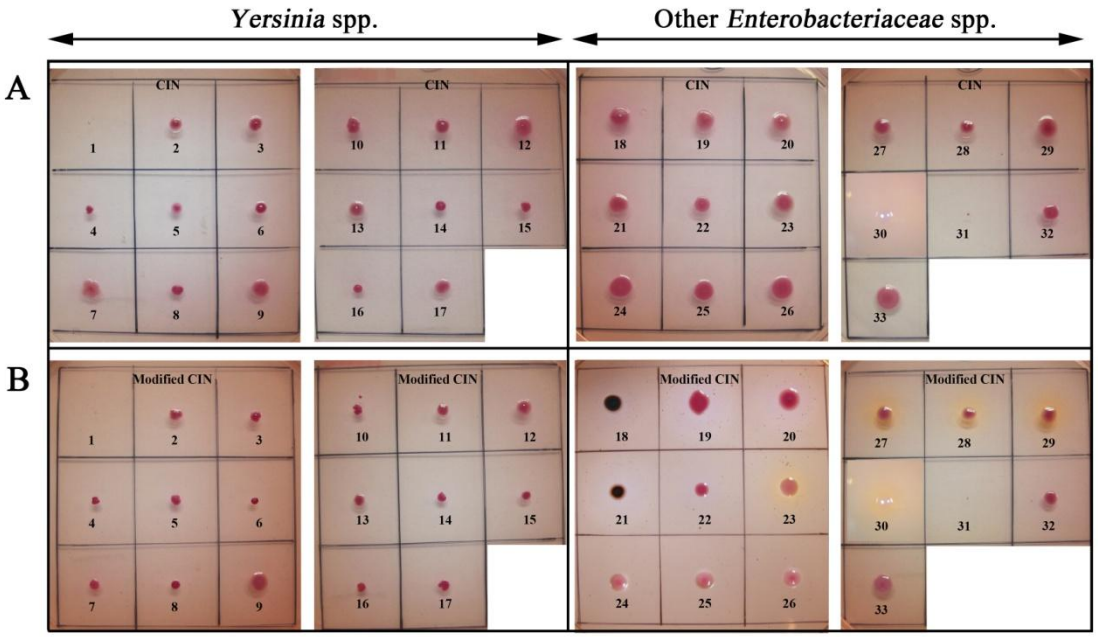


Figure 4.5.1. Bacteria dotted on CIN (A) and modified CIN (B).

1, *Y. enterocolitica* bioserotype 1A/O:6,30 (IP102); 2, *Y. enterocolitica* bioserotype 1B/O:8 (IP11105); 3, *Y. enterocolitica* bioserotype 2/O:9 (IP383); 4, *Y. enterocolitica*, bioserotype 3/O:1,2,3 (IP135); 5, *Y. enterocolitica* bioserotype 4/O:3 (IP134); 6, *Y. enterocolitica* bioserotype 5/O:2,3 (IP178); 7, *Y. enterocolitica* ATCC 9610; 8, *Y. enterocolitica* bioserotype 3 variant/O:3 (PC-M1-K1); 9, *Y. enterocolitica* bioserotype 1A/O:5 (PC-M16-2); 10, *Y. aldovae* (IP6005); 11, *Y. bercovieri* (IP3443); 12, *Y. frederiksenii* (IP3842); 13, *Y. intermedia* (IP955); 14, *Y. kristensenii* (IP105); 15, *Y. mollaretii* (IP33766); 16, *Y. pseudotuberculosis* (IP34476); 17, *Y. enterocolitica* bioserotype 1B/O:8 (YE036c-CY); 18, *C. freundii*, H_2S-producing; 19, 20, *C. freundii*, non-H_2S-producing; 21, *C. braakii*; 22, *C. koseri*; 23, *A. hydrophila*; 24, 25, 26, *E. cloacae*; 27, 28, 29, *P. rettgeri*; 30, *M. morganii*; 31, *Pantoae* spp.; 32, *S. odorifera*; 33, *S. marcescens*.

Table 4.5.1. Comparison of growth and morphology of *Y. enterocolitica* and other bacterial colonies on CIN (aerobic), modified CIN (aerobic) and modified CIN (microaerophilic).

Bacterial species (strain number)	Colony morphology on:		
	CIN (ae[a])	Modified CIN (ae)	Modified CIN (mic[b])
Yersinia enterocolitica			
bioserotype 1A/O:6,30 (IP102)	NG[c]	NG	NG
bioserotype1A/O:5 (PC-M16-2)	Rbe	Rbe	Rbe
bioserotype 1B/O:8 (IP11105, ATCC 9610, YE036c-CY)	Rbe[d]	Rbe	Rbe
bioserotype 2/O:9 (IP383)	Rbe	Rbe	Rbe
bioserotype 3/O:1,2,3 (IP135)	Rbe	Rbe	Rbe
bioserotype 3 variant /O:3 (PC-M1-K1)	Rbe	Rbe	Rbe
bioserotype 4/O:3 (IP134)	Rbe	Rbe	Rbe
bioserotype 5/O:2,3 (IP178)	Rbe	Rbe	Rbe
Other *Yersinia* spp.			
Y. aldovae (IP6005)	Rbe	Rbe	Rbe
Y. bercovieri (IP3443)	Rbe	Rbe	Rbe
Y. frederiksenii (IP3842)	Rbe	Rbe	Rbe
Y. intermedia (IP955)	Rbe	Rbe	Rbe
Y. kristensenii (IP105)	Rbe	Rbe	Rbe
Y. mollaretii (IP33766)	Rbe	Rbe	Rbe
Y. pseudotuberculosis (IP34476)	Rbe	Rbe	Rbe
Other *Enterobacteriaceae*			
Citrobacter,			
freundii, H_2S-producing	Rbe	Rbe + Bc[e]	Rbe + Bc
freundii, non-H_2S-producing	Rbe	Rbe	Rbe
braakii	Rbe	Rbe + Bc	Rbe + Bc
koseri	Rbe	Rbe	Rbe
Providencia rettgeri	Rbe	Rbe + Bp	Rbe + Bp
Enterobacter cloacae	Rbe	P	P
Pantoae spp.	Rbe	Rbe	Rbe
Serratia,			
odorifera	Rbe	Rbe	Rbe
marcescens	Rbe	Rbe	Rbe
Morganella morganii	C[f]	C + Bp	C + Bp
Salmonella,			
Paratyphi A (ATCC 9150)	NG	NG	NG
Paratyphi B (ATCC 8759)	NG	NG	NG
Paratyphi C (ATCC 9068)	NG	NG	NG
Typhimurium (ATCC 13311)	NG	NG	NG
Typhi (ATCC 6539)	NG	NG	NG
enterica (ATCC 10376)	NG	NG	NG
Escherichia coli (ATCC25922 and O157:H7)	NG	NG	NG
Shigella sonnei (ATCC 11060)	NG	NG	NG
Proteus penneri	NG	NG	NG
Other Gram-Negative Bacteria			
Aeromonas hydrophila	Rbe	P[g] + Bp[h]	P + Bp
Vibrio spp.	NG	NG	NG
Pseudomonas aeruginosa (ATCC 9027)	NG	NG	NG
Gram-Positive Bacteria			
Enterococcus faecalis (ATCC 29212)	NG	NG	NG
Listeria monocytogenes (ATCC 7644)	NG	NG	NG
Staphylococcus aureus (ATCC 6538, MRSA 0807-1)	NG	NG	NG

[a] ae, aerobic; [b] mic, microaerophilic; [c] NG, no growth; [d] Rbe, red bull's eye; [e] Bc, black centre; [f] C, colourless; [g] P, pink; [h] Bp, brown diffusible pigment; IP, Institut Pasteur, strain collection of the French *Yersinia* Reference laboratory; ATCC, American Type Culture Collection; Others, strain collection of Laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduate Studies, University of Malaya, Malaysia

4.5.2 Limit of detection (LOD) of CIN and modified CIN agar for *Y. enterocolitica* detection

The capacities of four *Y. enterocolitica* strains, which represented non-pathogenic (PC-M16-2), moderate pathogenic (IP383 and IP135), and highly pathogenic (ATCC 9610) strains, to grow on CIN and modified CIN agar were evaluated. Various cell suspensions (from 10^1 to 10^8 cfu/ml) were spread onto CIN and modified CIN agar, incubated for 24 - 48 h incubation at 25 ℃, and then observed. In addition, one set of modified CIN agar was incubated under microaerophilic condition at 25 ℃ for 24 - 48 h. A score of 100% was recorded for the growth of *Y. enterocolitica* on all six replicates plates; 83% for 5/6 plates and so on. The percentages of plates showing positive for each *Y. enterocolitica* strain are tabulated in Table 4.5.2.

The LOD for all four *Y. enterocolitica* strains was 10 cfu/ml on both CIN and modified CIN under aerobic conditions (Table 4.5.2). Although the LOD for strain ATCC 9610 was slightly higher on the modified CIN under microaerophilic (10^2 cfu/ml) compared with aerobic conditions (10^1 cfu/ml), the difference in sensitivity for all four strains tested under these different conditions was not significant (Student t-test, $P>0.05$). Therefore, microaerophilic conditions did not improve the growth of *Y. enterocolitica* on modified CIN.

Table 4.5.2. Percentage of plates showing positive (seeded with pure cultures of *Y. enterocolitica*) and the limit of detection of *Y. enterocolitica* on CIN and modified CIN.

Dilution of YE[a] seeded on plate (cfu/ml)	Percentage of plates showing positive (%)											
	YE bioserotype 2/O:9 (IP383)			YE bioserotype 3/O:1,2,3 (IP135)			YE bioserotype 1B/O:8 (ATCC 9610)			YE bioserotype 1A/O:5 (PC-M16-2)		
	CIN[b] (ae[c])	mCIN[d] (ae)	mCIN (mic[e])	CIN (ae)	mCIN (ae)	mCIN (mic)	CIN (ae)	mCIN (ae)	mCIN (mic)	CIN (ae)	mCIN (ae)	mCIN (mic)
10^8	100	100	100	100	100	100	100	100	100	100	100	100
10^7	100	100	100	100	100	100	100	100	100	100	100	100
10^6	100	100	100	100	100	100	100	100	100	100	100	100
10^5	100	100	100	100	100	100	100	100	100	100	100	100
10^4	100	100	100	100	100	100	100	83	83	100	100	100
10^3	100	100	100	100	100	100	100	100	67	100	100	100
10^2	83	83	100	100	100	100	100	100	50	100	100	100
10^1	83	83	100	100	100	100	100	67	33	100	100	100
LOD[f] (cfu/ml)	10^1	10^1	10^1	10^1	10^1	10^1	10^1	10^1	10^2	10^1	10^1	10^1

[a] YE, *Yersinia enterocolitica;* [b] CIN, Cefsulodin-Irgasan-Novobiocin; [c] ae, aerobic; [d] mCIN, modified CIN; [e] mic, microaerophilic; [f] LOD, limit of detection; Underlined numbers correspond to the scores of LOD for each *Y. enterocolitica* strain. LOD was defined as the lowest cfu/ml of culturable *Y. enterocolitica* detectable in ica fined as the lowest cfu/ml of c*Y. enterocolitica*

4.5.3 Quantification of *Y. enterocolitica* growth on CIN and modified CIN as compared with LBA

The ability of CIN and modified CIN to support growth of *Y. enterocolitica* as compared with that of LBA was evaluated. The growth of four *Y. enterocolitica* strains (IP383, IP135, ATCC 9610 and PC-M1-K1) on LBA, CIN, and modified CIN was quantified and is presented as percentages of mean cfu/ml on CIN or modified CIN versus LBA. Values between 80% and 120% were considered to indicate growth efficiency on CIN or modified CIN similar to that on LBA (100%) (Savin, Leclercq, & Carniel, 2012). Table 4.5.3 shows the percentages of mean cfu/ml on CIN or modified CIN versus LBA. The cfu counts of IP383, IP135, ATCC 9610 and PC-M16-2 on CIN, modified CIN and LBA are tabulated in Appendix XIII.

As shown in Table 4.5.3, the number of *Y. enterocolitica* colonies recovered on CIN, modified CIN and LBA were similar, whether the bacteria were grown under aerobic or microaerophilic conditions (ranged between 80% and 120%). Therefore the different media and conditions allowed the growth of *Y. enterocolitica* colonies with the same efficiency.

Table 4.5.3. Growth at different incubation conditions of selected *Y. enterocolitica* strains on CIN and modified CIN, as compared with LBA.

Strains	Bio serotype	Percentage of mean cfu/ml (%)			
		Aerobic		Microaero-philic	Aerobic/ Microaero-philic
		CIN[a]/ LBA[b]	mCIN[c] / LBA	mCIN/ LBA	mCIN
PC-M16-2	1A/O:5	110.3	108.9	106.8	102.0
ATCC 9610	1B/O:8	88.5	84.3	84.3	100.0
IP383	2/O:9	109.6	101	94.5	106.9
IP135	3/O:1,2,3	96.7	82.1	82.4	99.6

[a] CIN, Cefsulodin-Irgasan-Novobiocin; [b]LBA, Luria-Bertani agar; [c] mCIN, modified CIN

4.5.4 Limit of detection (LOD) of *Y. enterocolitica* from artificially contaminated raw pork meat

The cfu/ml for IP135 prepared (at dilution 10^8) and microbiota in the meat sample was 2.43×10^8 cfu/ml and 1.00×10^7 cfu/ml, respectively. Therefore, the initial ratio of IP135 to microbiota in the meat sample (IP135:MM) at dilution 10^8 (at 0 h) was 1:0.0412. Logically, the IP135:MM at 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10 were 1:0.412, 1:4.12, 1:41.2, 1:412, 1:4120, 1:41200, and 1:412000, respectively (Table 4.5.4). Raw data of cfu counts are tabulated in Appendix XIII.

The ability of CIN and modified CIN to recover *Y. enterocolitica* from food matrix was determined on raw pork meat (with or without stress treatment, kept at -20 ℃, three weeks after bacterial spiking) mixed with various concentrations (from 10^1 to 10^8 cfu/ml) of *Y. enterocolitica* IP135. A score of 100% was recorded for the growth of *Y. enterocolitica* on all six replicates plates; 83% for 5/6 plates and so on. The percentages of plates showing positive for each *Y. enterocolitica* strain are tabulated in Table 4.5.4. The ratio of LOD to background microbiota was calculated by dividing the mean cfu/ml of LOD to the mean cfu/ml of uninoculated pork meat.

The LOD of IP135 in in both raw pork meat with and without stress treatment was 10^4 cfu/ml on both media incubated under aerobic conditions (Table 4.5.4), indicating that modification of CIN did not impair its capability to recover *Y. enterocolitica* from the studied food matrix. The finding that this LOD was 100-fold higher than that of bacteria grown in pure culture (10 cfu/ml) confirms the impact of the presence of background microbiota on the recovery of *Y. enterocolitica*. At the LOD value of 10^4 cfu/ml, the food homogenates contained a ratio of *Y. enterocolitica* to microbiota of 1:412, indicating that *Y. enterocolitica* colonies cannot be successfully identified in the meat sample if it is below this ratio.

For the food matrix with stress treatment (kept at -20℃ for three weeks after bacterial spiking) and incubated under microaerophilic conditions, the LOD dropped to 10^3 cfu/ml on modified CIN, which suggests that this incubation condition may slightly increase the recovery rate of *Y. enterocolitica* in food since visualization of H_2S-producing bacteria was improved.

Table 4.5.4. Percentage of plates showing positive [seeded with homogenate of raw pork meat spiked with *Y. enterocolitica* bioserotype 3/O:1,2,3 (IP135)] and the limit of detection of IP135 on CIN and modified CIN.

Dilution of IP135 spiked in 25 g of pork meat (cfu/ml)	Initial ratio IP135 : MM[a]	Without stress treatment			With stress treatment at -20℃ for two weeks		
		CIN[b] (ae[c])	mCIN[d] (ae)	mCIN (mic[e])	CIN (ae)	mCIN (ae)	mCIN (mic)
10^8	1:0.0412	100	100	100	100	100	100
10^7	1:0.412	100	100	100	100	100	100
10^6	1:4.12	100	100	100	100	100	100
10^5	1:41.2	100	100	100	100	100	100
10^4	1:412	<u>100</u>	<u>67</u>	<u>67</u>	<u>83</u>	<u>67</u>	83
10^3	1:4120	33	17	33	17	17	<u>50</u>
10^2	1:41200	0	17	0	0	0	0
10^1	1:412000	0	0	0	0	0	0
LOD[f] (cfu/ml), IP135:MM		10^4, 1:412	10^4, 1:412	10^4, 1:412	10^4, 1:412	10^4, 1:412	10^3, 1:4120

[a] MM, microbiota in the meat sample; [b] CIN, Cefsulodin-Irgasan-Novobiocin; [c] ae, aerobic; [d] mCIN, modified CIN; [e] mic, microaerophilic; [f]LOD, limit of detection; Underlined numbers correspond to the LOD scores of LOD for each medium. LOD was defined as the lowest cfu/ml of culturable *Y. enterocolitica* detectable in ≥50% of the replicates

4.5.5 Differentiation of *Y. enterocolitica* colonies from exhibiting *Yersinia*-like morphology on CIN

Suspensions containing similar concentrations (10^4 cfu/ml) of *Y. enterocolitica* IP135 and other enterobacterial species exhibiting *Yersinia*-like colonies on CIN agar such as H_2S-producing *C. freundii*, *C. braakii*, *E. cloacae*, *P. rettgeri*, and *A. hydrophila* were prepared to compare the number of true *Y. enterocolitica* recovered on CIN and modified CIN. Presumptive colonies of *Y. enterocolitica* (appeared as red bull's eye) were picked and analyzed by PCR for confirmation.

Modified CIN gave an excellent differentiation of *Y. enterocolitica* from bacteria exhibiting *Yersinia*-like colonies on CIN agar (H_2S-producing *C. freundii*, *C. braakii*, *E. cloacae*, *P. rettgeri*, and *A. hydrophila*). The percentages of true *Y. enterocolitica* recovered from the bacterial mixtures were 33.3% on CIN and 60.0% on modified CIN (Table 4.5.5), and the difference was significant (χ^2 test, $p<0.05$). Therefore, the use of modified CIN agar enhanced the differentiation of *Yersinia* colonies from those of *Yersinia*-like species.

In artificially contaminated raw pork meat, the percentage of true *Y. enterocolitica* colonies increased from 62.3% on CIN to 72.5% on modified CIN (Table 4.5.5), however this difference was not statistically significant (χ^2 test, $p>0.05$).

Table 4.5.5. Recovery of *Y. enterocolitica* bioserotype 3/O:3 (IP135) from artificially prepared bacterial mixture and from spiked food.

Co-culture bacteria type	Agar	Number of positive isolates		
		True (%)	False (%)	Total
Bacterial mixture[a]	CIN[b]	20 (33.3)	40 (66.7)	60
	Modified CIN	36 (60.0)	24 (40.0)	60
Background microbiota from food	CIN	43 (62.3)	26 (37.7)	69
	Modified CIN	50 (72.5)	19 (27.5)	69

[a] Mixture of bacteria exhibiting *Yersinia*-like colonies on CIN agar (*C. freundii*, *C. braakii*, *E. cloacae*, *P. rettgeri*, *A. hydrophila*)
[b] CIN, Cefsulodin-Irgasan-Novobiocin

4.5.6 Determination of the recovery of *Y. enterocolitica* from naturally contaminated samples

The efficiency of CIN and modified CIN agars for the recovery of *Y. enterocolitica* from the 52 naturally contaminated samples (rectal swabs from swine) was evaluated and compared in three ways: (i) after direct plating on the agars (methods 1 and 2); (ii) after cold enrichment followed by plating on the agars (methods 3 and 4); (iii) after cold enrichment, alkaline treatment, and plating on the agars (methods 5 and 6) (Table 4.5.6). The detection of *Y. enterocolitica* by PCR in post-PBS enrichment

broths was 2/52 (3.8%). Both modified CIN and CIN did not recover any *Y. enterocolitica* from the samples using the direct plating method (methods 1 and 2). After cold enrichment (methods 3 and 4), the modified CIN allowed the recovery of *Y. enterocolitica* from all PCR-positive samples (2/52, 3.8%), while no *Y. enterocolitica* was identified on CIN (Table 4.5.6). Cold enrichment and alkaline treatment followed by plating on modified CIN (methods 5 and 6) also allowed the isolation of *Y. enterocolitica* from all PCR-positive samples (2/52, 3.8%), while no *Y. enterocolitica* was detected on CIN (Table 4.5.6). The results showed that modified CIN resulted in the detection of a larger number of positive samples than CIN for the recovery of *Y. enterocolitica* from naturally contaminated samples.

Table 4.5.6. Recovery rate of *Y. enterocolitica* from the 52 naturally contaminated rectal swabs from swine.

Methods	No. of positive specimens recovered by plating (%)
Method 1 — Direct streaking onto CIN[a]	0 (0)
Method 2 — Direct streaking onto mCIN[b]	0 (0)
Method 3 — PBS[c]-CIN	0 (0)
Method 4 — PBS-mCIN	2 (3.8)
Method 5 — PBS-KOH[d]-CIN	0 (0)
Method 6 — PBS-KOH-mCIN	2 (3.8)

[a] CIN, Cefsulodin-Irgasan-Novobiocin; [b] mCIN, modified CIN; [c] PBS, phosphate buffered saline, a cold enrichment at 4 ℃ for three weeks; [d] KOH, post-enrichment alkaline treatment

CHAPTER 5

DISCUSSION

Yersinia enterocolitica is an important foodborne pathogen that causes human yersiniosis worldwide. The presence of *Y. enterocolitica* in both food and pigs (food animal) has public health implications since the primary transmission route of pathogenic *Y. enterocolitica* to humans is through contaminated food.

5.1 Isolation and detection of *Y. enterocolitica* from food and pigs

The primary goal of the present study is to investigate the occurrence of *Y. enterocolitica* in raw food and also pigs (food animal). Out of the 106 raw food samples (58 pork products and 48 non-porcine food) and 495 swine specimens (from 165 pigs) collected, *Y. enterocolitica* was isolated from seven raw pork products (12.7%) (Table 4.1.1) and seven specimens of three pigs (1.8%) (Tables 4.1.6 and 4.1.7). No correlation was made between the prevalence of *Y. enterocolitica* in the farms and food samples. This was because the location where the raw foods came from was unknown. Three *Y. enterocolitica* bioserotypes were identified, 3 variant/O:3, 1B/O:8 and 1A/O:5. The results confirmed that *Y. enterocolitica* was present in the local raw pork products and pigs.

Interestingly, *Y. enterocolitica* (bioserotype 3 variant/O:3) was isolated from three healthy grower pigs (Table 4.1.6). This indicated that the pigs were carrier for the bacterium. Similar finding is reported in Jos, Nigeria, where *Y. enterocolitica* is isolated from healthy pigs (Okwori, et al., 2009). Pigs appear asymptomatic due to the colonisation of *Y. enterocolitica* in the lymphoid tissue, particularly in tonsils (Horter, Yoon, & Zimmerman, 2003). The colonisation caused the identification of asymptomatic carrier animals difficult in disease control and/or pathogen elimination. These asymptomatic pigs serve as food for humans when they are matured to be sold. Cross-contamination of *Y. enterocolitica* from pigs' oral cavity, intestine and faeces to meat is possible during the slaughtering and dressing

operations through the slaughtering tools and containers (Gill & Jones, 1995; Nesbakken, 1988; Skjerve, Lium, Nielsen, & Nesbakken, 1998). Besides that, cross contamination may happen during food storage. For example, the interior surfaces of household refrigerators (Jackson, Blair, McDowell, Kennedy, & Bolton, 2007) or surfaces of storage containers. *Y. enterocolitica* may be transferred from the contaminated surfaces to other food items, especially the higher risk ready-to-eat foods. Improper food handling, processing and storing practices such as undercooked meats or cross contamination of contaminated meats or surfaces to other food or water are risk factors for yersiniosis in humans.

More than 50 serotypes and 6 biogroups of *Y. enterocolitica* have been identified currently and their geographical distributions are diverse. In Europe, *Y. enterocolitica* particularly bioserotype 4/O:3 has been frequently isolated in humans, pig husbandry and food, followed by the less common bioserotype, 2/O:9 and 2/O:5,27 (European Food Safety Authority & European Centre for Disease Prevention and Control, 2013; Fondrevez, et al., 2010; Fredriksson-Ahomaa, Gerhardt, & Stolle, 2009). In USA, O:8 is the primary infectious serotype, followed by O:5,27, O:13a, 13b, O:20, O:9 (Bottone, 1997; Kwaga, Iversen, & Misra, 1992). *Y. enterocolitica* is frequently isolated in pigs from China in which the bioserotypes isolated are 2/O:9, 4/O:3, 3/O:3, 1A/O:5, 1A/O:8. Among the three bioserotypes identified in this study, *Y. enterocolitica* bioserotypes 3 variant/O:3 was the most common. In the past, the bioserotype 3 variant/O:3 has been reported in imported pork and chicken to Japan and food animals (pigs, rats and rabbits) in China (Fukushima, et al., 1997; Zheng & Xie, 1996). *Y. enterocolitica* bioserotype 3/O:3 is the major bioserotype in pigs, particularly from Jiangxi and Fujian Provinces with warmer climate (Liang, et al., 2012; Wang, et al., 2009). All these reports showed that this particular bioserotype 3/O:3 is frequently isolated from the regions with

warmer climate. Therefore, *Y. enterocolitica* bioserotype 3/O:3 or 3 variant/O:3 strains might be the common strains in warm regions.

Lim & Tay (1992) attempted to isolate *Y. enterocolitica* from over 6000 samples from patients with diarrhoea in Singapore, but no *Y. enterocolitica* was detected. They also suggested that *Y. enterocolitica* had no clinical importance in this region. However, this pathogen is recently isolated from unpacked tofu (Ananchaipattana, et al., 2012a), beef and shrimp samples (Ananchaipattana, et al., 2012b) in Thailand. These recent reports from Thailand indicated that there is a risk of human infection. In Malaysia, yersiniosis is rarely reported. The under-reporting in Malaysia can be due to several possibilities: (i) Malaysians prefer well cooked food to raw or undercooked meats, (ii) *Y. enterocolitica* is not the routine pathogen monitored in the diarrhoeal patients, or (iii) most of the *Y. enterocolitica* cases are self-limiting. Although there is no other official report on yersiniosis in Malaysia since 1984, we should be cognisant that this bacterium could be another potential agent to contribute to the incidence of food poisoning cases in our country since this pathogen was confirmed present in our local food and pigs. Improper food handling and processing may cause cross contamination of this pathogen to humans and therefore affirms a potential risk for the consumers.

5.2 Comparison of conventional cultural and post-enrichment PCR methods in detection of *Y. enterocolitica*

In this study, several methods were performed in the isolation and detection of *Y. enterocolitica*; i.e. post-enrichment PCR detection method and conventional culturing method (initiated by enrichment steps followed by plating onto selective agar plates (with or without KOH treatment, biochemical identification, and finally PCR confirmation of presumptive *Y. enterocolitica* isolates).

In comparing between the conventional cultural and post-enrichment PCR methods in detection of *Y. enterocolitica,* results showed that post-enrichment PCR-based detection method was more sensitive than the conventional cultural method. For raw pork products (Section 4.3), about 60% were PCR detected positive, compared to 12% detected by cultural methods. For raw non-porcine food (Section 4.4), PCR detected *Y. enterocolitica* present in nearly 42% of the food, while no *Y. enterocolitica* was isolated. Besides, *Y. enterocolitica* was PCR detected in nearly 28% of the pigs as compared to 1.8% by using cultural methods (Section 4.5). The results indicated that conventional method might underestimate the real prevalence of this pathogen in the local food and pigs. This result concurred with many other reports that the PCR detection are more sensitive than the cultural method (Bhaduri, Wesley, & Bush, 2005; Johannessen, Kapperud, & Kruse, 2000; Messelhäusser, et al., 2011). For example, Messelhäusser, et al. (2011) reported that 18% of the pork samples analysed is PCR- positive as compared to 10% positive by cultural method. However, these percentages are not necessarily comparable due to the different methods used in the detection of *Y. enterocolitica*. The primers used in the PCR assay are specific, which amplified the targeted gene of *Y. enterocolitica.* In contrast, detection by cultural method is less sensitive as the method is based on the physiology and biochemical activities of bacteria. Besides, DNA templates for PCR assay were prepared from bacterial cells that were concentrated from 1 ml of enriched homogenate, thus increasing the probability in getting more DNA of *Y. enterocolitica.* Moreover, the PCR can detect all kinds of cells in regardless of dead cells or viable including non-culturable cells which may not grow on artificial medium (Parker & Martel, 2002; A. Singh & McFeters, 1987).

5.3 Comparison of the recovery power of different isolation media in isolating *Y. enterocolitica*

In comparing the recovery power of different enrichment and selective media used in the conventional cultural method, PBS enrichment (cold enrichment) was the best broth, with recovery rate 6.5% as compared to YSEO (1.0%), ITC (0.1%), and direct plating (1.6%) (Table 4.2.2). The results concurred with findings of Fukushima, et al. (2011) that reported the PBS (cold enrichment) yields better recovery of *Y. enterocolitica.* Although there are numerous enrichment schemes available in isolating *Y. enterocolitica* such as the ISO 10273:2003 and USDA protocol, however, no single culture protocol which has been described performed equally well for the isolation of *Y. enterocolitica* serotypes from all types of samples. For example, in a recent study, Van Damme, et al. (2013) reported that enrichment in PSB at 25℃ recovers more positive samples than selective enrichment and cold enrichment. Irgasan-ticarcillin-potassium chlorate (ITC) broth is reportedly better in recovering of *Y. enterocolitica* 4/O:3 from pig tonsils than cold enrichment in PSB (Van Damme, et al., 2010). Therefore, combination of several enrichment broths should be used concurrently for better isolation rate.

Following the enrichment steps, the enriched cultures (or samples) were plated (by streaking or/and spread plating methods) onto selective agar. During the initial investigation of *Y. enterocolitica* in this study, particularly for all 106 raw food samples and swine specimens from Selangor and Perak, CIN was the only selective plate used. The recovery power of CIN in isolating true *Y. enterocolitica* was very low (0.6%, Table 4.2.3). Nearly 94% of the presumptive isolates that appeared as red bull's eye on CIN turned out to be *Providencia rettgeri, Serratia* spp., *Citrobacter freundii, C. braakii, Klebsiella ornithinolytica, Enterobacter cloacae,* and *Pantoea* spp. after the preliminary biochemical tests. (Section 4.2.1, Figure 4.2.3). In addition,

about 62% of isolates tht passed the preliminary biochemical tests were then identified as *Morganella morganii, Y. frederiksenii*, etc. by using API 20E identification kit. These bacteria clearly increase the workloads, experimental cost and also create complicates selection of *Y. enterocolitica* colonies during the isolation that may result in false negative findings. In the later stage of isolation of *Y. enterocolitica*, CIN agar was modified in order to increase the differential power of the original CIN in identifying true *Y. enterocolitica.* Discussion for the modification and improvement of CIN agar is in Section 5.6.

5.3 Biochemical tests in identification of presumptive *Y. enterocolitica*

In the identification of presumptive *Y. enterocolitica* isolates, four biochemical tests (preliminary test: Gram, citrate, oxidase, and urease tests) and 20 biochemical tests in the API 20E identification kit were preformed. The preliminary tests were used as the initial screening tests in reducing the amount of non-*Y. enterocolitica* isolates. The results showed the preliminary tests are effective in cutting down the number of non-*Y. enterocolitica* isolates by 94% (Section 4.1.1).

In this study, the incubation temperature of API 20E identification kit was modified to 28 ℃ as recommended in Archer, et al. (1987). In their study, the decrease of incubation temperature from 37 ℃ to 28 ℃ increases the percentage of correct identifications for *Yersinia* spp. from 66 to 93%. By refferring to this recommendation, the accuracy of API 20E in identifying *Y. enterocolitica* in this study was 91.5% (Section 4.1.2). Only seven isolates were misidentified as *Y. enterocolitica* when PCR was carried out. API 50CH results revealed that these isolates were *Y. intermedia* and *Y. frederiksenii.*

5.5 Further characterization of *Y. enterocolitica* isolates

In characterizing the isolates of *Y. enterocolitica*, a total of 32 isolates were selected. During the cultures selection, isolates with similar bioserotypes and API 20E code that originated from a same sample were referred as replicate isolates, and only one isolates was selected for further characterization. The virulotypes, plasmid profiles, antimicrobial susceptibility profiles and the genotypes (PFGE) of *Y. enterocolitica* were determined.

5.5.1 Virulence profiles of *Y. enterocolitica*

The pathogenicity of *Y. enterocolitica* is associated with specific virulence factors that are located in the virulence pYV plasmid (~70kb) and also in the chromosomal DNA (Cornelis, et al., 1998; Revell & Miller, 2001). Among the 15 virulence associated genes tested, the classical virulence genes that are involved in establishing gasterointestinal infection are the *inv, ail, ystA, myfA, virF* and *yadA*, in which, *virF* and *yadA* genes are plasmid-borne (pYV virulence plasmid) (Bottone, 1999). The *ail*, *inv*, *yadA* and *myfA* genes basically contribute to adhesion, invasion and protects the bacterium from being killed by the host defence system (Cornelis, et al., 1998; Miller & Falkow, 1988). The *yst* and *virF* genes are important for the production of *Yersinia* stable heat-stable enterotoxin and Yops, respectively, and cause disease symptoms like diarrhea with fever associated with acute yersiniosis (Fàbrega & Vila, 2012). Other genes are associated with the survival and adaptation in animals or human hosts. All six classical virulence genes (*inv, ail, ystA, myfA, virF* and *yadA*) and in addition *rfbC, hreP, ymoA* and *tccC* and the pYV virulence plasmid were present in all isolates of bioserotypes 3 variant/O:3 (Tables 4.4.4 and 4.4.5). Interestingly, the virulence profiles (inv^+, ail^+, $ystA^+$, $virF^+$, $yadA^+$ and $rfbC^+$) of the bioserotype 3 variant/O:3 isolates in this study were similar to the virulence genes

profiles of clinical 3/O:3 strains reported from South China (Zheng, et al., 2008), indicating that these strains may be potentially virulent to humans.

Isolates of biotype 1B are attributed as highly pathogenic, like other pathogenic strains (biotype 2, 3, 4 and 5) should harbour a highly conserved ~70 kb pYV virulence plasmid and also chromosomal virulence genes (Cornelis, et al., 1998). Surprisingly, the three pathogenic bioserotype 1B/O:8 isolates (isolated from raw pork and liver) in this study did not carry the *ail*, *virF*, *ystA* genes and the pYV virulence plasmid, which normally present in the pathogenic strains (Tables 4.4.5 and 4.4.7). According to the studies by Wang, et al. (2008), the loss of four genes *ail*, *ystA*, *yadA* and *virF* in the O:8 strains did not result in death of their artificially infected mice model experiments. The reason for the loss of plasmid is unknown. The occurrence of plasmid-borne genes (*virF* and *yadA*) in plasmid-less *Y. enterocolitica* has also been reported in other studies by Paixão, et al. (2013) and Zheng, et al. (2008). Although the stability of pYV plasmid is associated with the growth temperature (plasmid loss above 30 ℃) and calcium concentration (Cornelis, et al., 1998), the growth temperature was maintained at <30 ℃ in this study, hence the loss of plasmid due to temperature was minimised.

The non-pathogenic bioserotype 1A/O:5 isolates (n=2) carried the virulence genes *ystB*, *hreP*, *fes*, *sat*, *fepD*, *ymoA*, and *fepA*. The findings concurred with several reports on the presence of these virulence genes in the biotype 1A strains (Bhagat & Virdi, 2007; Paixão, et al., 2013; Sihvonen, et al., 2011; Stephan, et al., 2013; Zheng, et al., 2008). Although the biotype 1A is considered a non-pathogenic strain, it is frequently isolated from diarrheic patients despite the absence of the pYV virulence plasmid and the classical virulence genes (Singh, et al., 2003; Stephan, et al., 2013). Several studies suggested that strains of biotype 1A that possess *ystB*, *hreP*, *sat* and *myfA* genes have a virulence potential of causing infection in humans and animals

(Bhagat & Virdi, 2007; Campioni & Falcão, 2013; Stephan, et al., 2013). In this study, all the bioserotype 1A/O:5 food isolates had similar virulence genes, suggesting their virulence potential.

5.5.2 Antibiograms of *Y. enterocolitica* isolates

The antimicrobial susceptibility patterns for *Y. enterocolitica* reported world-wide are different. This may because of the impact of geographical location, difference in the usage of antimicrobials amongst other factors. In generally the *Y. enterocolitica* is resistant to penicillin, ampicillin and first generation of cephalosporins (Fàbrega & Vila, 2012).

In 2005, majority of *Y. enterocolitica* strains (from meat and meat products) have high percentage of resistance to penicillin, first-generation cephalosporins, and bacitracin, and less than 11% were resistant to TET, NAL, STR and cefaporazone, and 100% susceptible to CHL and GEN (Dzomir, 2005). Similarly, all *Y. enterocolitica* isolates in present study were susceptible to CHL and GEN and resistant to TIC and AMP (penicillin). However, the resistance rates of NAL, TET and STR have increased tremendously over the years. *Y. enterocolitica* with high resistance rates to TET, AMP, or AMX are commonly reported in many countries in recent years from different sources such as marine marketed fishes, pig's carcass and feces, and humans (Akhila, Priya, Murugn, & Thayumanavan, 2013; Bhaduri & Wesley, 2012; Bolton, Ivory, & McDowell, 2013). Nalidixic acid-resistant *Y. enterocolitica* is rarely reported from food and pigs and the reported resistant rate is comparably lower than this study; i.e. 31% in Lebanon (dairy food) (Harakeh, et al., 2012), and 55% in India (marine marketed fish) (Akhila, et al., 2013). High levels of NAL, TET and AMX resistance were also reported in verotoxigenic *Escherichia coli* strains from the same pig farms (Ho, Tan, Ooi, Yeo, & Thong, 2013). The increased

in antimicrobial resistance rates and the emergence of MDR *Y. enterocolitica* in the livestock industry is of public health concern.

Selective pressure due to regular usage of antimicrobials at suboptimal concentrations in the livestock industry is often cited as a cause of antimicrobial resistance (Oliver, et al., 2011; Rajić, et al., 2006; Varga, et al., 2009). In Malaysia, the antimicrobials used in pig farms are for prophylactic (in-feed medication), therapeutic (injectable) purposes and growth promotion purpose. In this study, the antimicrobials used for prophylactic are tetracycline, oxytretracycline, sulphonamide, penicillin, tylosin, lincomycin, florfenicol, and neomycin whereas for enroflaxacin, amoxicillin, gentamycin, florfenicol, ceftiofur, and tylosin are used as therapeutic. The choice of antimicrobials may vary between farms due to the herd health status and farmers' preference. The high resistance in AMX and TET of *Y. eneterocolitica* isolates in this study may be due to the over-usage of antimicrobials in farm disease control purpose. The relevant governmental agencies should regulate the usage of antimicrobials for disease control and prevention in minimizing the breeding and transmission of MDR pathogens that potentially can be transferred to consumers through food or direct contact.

5.5.3 Genotyping of *Y. enterocolitica* by using PFGE

PFGE of the *Not*I restricted genomic DNA is a useful technique in differentiating *Y. enterocolitica* of various biotypes and serotypes (Bonardi, et al., 2013; Fredriksson-Ahomaa, et al., 2007; Liang, et al., 2012; Stephan, et al., 2013). In this study, genomic DNA of the 32 *Y. enterocolitica* isolates was *Not*I-restricted and DNA fragments were separated by PFGE. The *Not*I was the primary restriction enzyme used in this study since the PFGE results yielded discriminating patterns for the *Y. enterocolitica* strains. All isolates of the three different bioserotypes, 3

variant/O:3, 1A/O:5 and 1B/O:8 could be distinguished. Nevertheless, the isolates were highly clonal within each bioserotype and exhibited minor variation. Isolates of bioserotype 3 variant/O:3 were comparably more diverse than the other bioserotype and could be further distinguished according to their geographical origin or sources. Interestingly, pulsotypes YeNotI-7 and YeNotI-9 (Figure 4.5.1) were found consistently in the food strains that were isolated at different times from the same vendor. Cross contamination might have occurred in the slaughtering houses or farms before the raw pork products reach the market (Fredriksson-Ahomaa, Bucher, et al., 2001; Ortiz Martínez, 2010) or during storage from contaminated interior surfaces of refrigerator or surfaces of storage containers (Jackson, et al., 2007). Similarly, *Y. enterocolitica* with indistinguishable PFGE pattern was also observed among specimens originated from different pigs (YeNotI-3 and YeNotI-5, Figure 4.4.15), suggesting transmission of a particular *Y. enterocolitica* clone from a common contaminating source (e.g. feed, water source and breeding environment) among the animals at the farm. It is possible that different subtypes of *Y. enterocolitica* exist within a particular animal host. The phenomenon of co-existing strains in an animal is possibly due to contamination originated from different sources during animal breeding or food processing (Fredriksson-Ahomaa, Korte, et al., 2001; Ortiz Martínez, 2010; Wang, et al., 2010).

Due to the limited research of *Y. enterocolitica* in Malaysia, the PFGE results failed to be compared with other fields in Malaysia. Our PFGE results failed to correlate the raw pork from wet markets with the swine farms because the pulsotypes were different. Pigs and raw pork products may be a potential source of infection for humans in Malaysia and the possible transmissions of *Y. enterocolitica* are directly through pigs to humans via contaminated pork, fecal-oral route, contaminated environment and pets (Fredriksson-Ahomaa, Korte, et al., 2001; Wang, et al., 2010).

5.6 Modification and improvement of CIN agar

In the search for a medium that would allow a better discrimination of *Yersinia* colonies from other bacterial spp. while maintaining the selective properties of CIN, various chemical components have been added to this medium to detect three biochemical activities: phenylalanine deaminase, arginine dihydrolase, and H_2S production. According to Bergey, Breed, Hitchens, & Murray (1948), *C. braakii*, H_2S-producing *C. freundii*, *E. cloacae*, *Providencia rettgeri*, and *Aeromonas hydrophila* can be differentiated from *Y. enterocolitica* based on their biochemical properties in utilizing either of these components.

Ferric ammonium citrate and sodium thiosulphate are substrates for H_2S production, which results in the formation of a black centre on bacterial colonies. Phenylalanine deaminase converts DL-phenylalanine in phenylpyruvate, in which the presence of iron (III) ions and citrate, forms a brown, diffusible pigment in agar around the bacterial colonies. The dihydrolysis of L-arginine produces an alkaline substrate that gives a yellow colour to bacterial colonies. The addition of these substrates did not alter the formation of the red bull's eye feature of *Yersinia* colonies, although the formation of *Y. enterocolitica* characteristic morphology was slightly delayed on modified CIN compared with CIN. Nevertheless, the plates could be read within 48 h (optimal incubation time for *Y. enterocolitica*) in the normal isolation step for *Y. enterocolitica*.

The differentiation of *Yersinia* from other mannitol-fermenting bacteria that exhibit *Yersinia*-like colonies CIN (H_2S-producing *C. freundii*, *C. braakii*, *E. cloacae*, *A. hydrophila*, and *P. rettgeri*) was markedly easier on modified CIN compared with CIN. These bacterial species are naturally present in feces, raw food, and other environmental samples (Bergey, et al., 1948). Because H_2S-producing *C. freundii* and *C. braakii* ferment mannitol and produce H_2S, these appeared as red

bull's eye colonies with a black centre on modified CIN. The capacity of *E. cloacae* and *A. hydrophila* to ferment mannitol and dihydrolyse arginine resulted in the formation of pink colonies. Furthermore, *A. hydrophila* produces phenylalanine deaminase, which generates a diffusible brown pigment around the pink colonies. Similarly, *P. rettgeri* produces a phenylalanine deaminase and therefore appeared as red bull's eye colonies with a diffusible brown pigment. The diffusible brown pigment produced will not mask the appearance of non-phenylalanine deaminase-producing bacteria because this brown pigment has a light colour that is less intense than the dark-brown pigment produced in the VYE agar (Fukushima, 1987). Van Damme, et al. (2013) reported that *Y. enterocolitica* forms small white colonies and not typical red bull's eyes colonies on CIN agar in the presence of an abundant background flora. Similar results were observed in this study when the colony size of *Y. enterocolitica* was $\leq$ 0.5 mm on CIN (Chapter 4.2.1, Figure 4.2.1). The tiny white/colourless *Y. enterocolitica* colonies obtained in this case resembled *M. morganii* on CIN, causing false-negative results during colony selection. A bonus benefit obtained with the modification of CIN was that *M. morganii* appeared as colourless colonies with a diffusible brown pigment on modified CIN due to their capacity to produce phenylalanine deaminase. Based on these differential metabolic properties, it was possible to eliminate a large number of bacterial colonies prior further biochemical testing. The addition of these chemicals in the modified CIN thus reduced the workload and additional costs associated with biochemical testing by decreasing the number of colonies to be tested and enhanced the detection rate by lowering the risk of selecting non-*Yersinia* colonies.

However, the modified CIN still has the limitation of not differentiating pathogenic *Y. enterocolitica* from non-pathogenic *Yersinia* species and from non-H_2S-producing *C. freundii, C. koseri, Pantoea* spp., *S. odorifera*, and *S. marcescens*.

Nevertheless, the combination usage of modified CIN with a chromogenic media, such as CHROMagar *Yersinia* (Renaud, Lecci, Courcol, Simonet, & Gaillot, 2013) and YECA (Denis, et al., 2011), and YeCM (Weagant, 2008), may help eliminate non-pathogenic *Yersinia* without the need to conduct additional biochemical tests (Fondrevez, et al., 2010). This combination usage may also reduce the false-positive results caused by wrong colour interpretations by the user (Denys, Renzi, Koch, & Wissel, 2013).

A better formation of a black centre by H_2S-producing *C. freundii* and *C. braakii* colonies was observed when these were incubated under microaerophilic conditions. The reduction of sulphide to H_2S gas is an anaerobic respiration and normally occurs in the middle of bacterial colonies (Stilinovic & Hrenovic, 2009). Tiny colonies may not provide good anaerobic conditions for the production of H_2S gas, and this phenomenon was observed in the samples (Zone A, Figure 5.1). The formation of a black centre could not be detected when the colonies were small and clumped together. Therefore, a longer incubation time (30 h to 48 h instead of 24 h) was required when the plates were incubated under normal (aerobic) conditions because bacteria grew bigger and fulfilled the anaerobic respiration requirement. Moreover, we observed that the formation of the brown diffusible pigment due to the phenylalanine deaminase reaction was hardly observable when the bacteria grew in clumps (Figure 5.1, zone A) because the brown pigment diffused around the colony. Bacterial clumping may thus be a limitation of modified CIN for the visualisation of these bacteria. However, the clumped bacteria can be re-streaked on modified CIN for further identification.

The addition of chemicals did not inhibit the growth of the *Yersinia* strains tested, indicating that modified CIN has the same capacity as CIN to allow the growth of this species. It has been previously reported that CIN inhibits the growth of

Y. bercovieri (formerly known as *Y. enterocolitica* bioserotype 3B/O:3) and some strains of *Y. pseudotuberculosis* due to the presence of cefsulodin in the medium (Blom, et al., 1999; Fukushima & Gomyoda, 1986; Renaud, et al., 2013; Schiemann, 1979). Because the amount of cefsulodin was not changed in the modified CIN, we expect similar results on this medium.

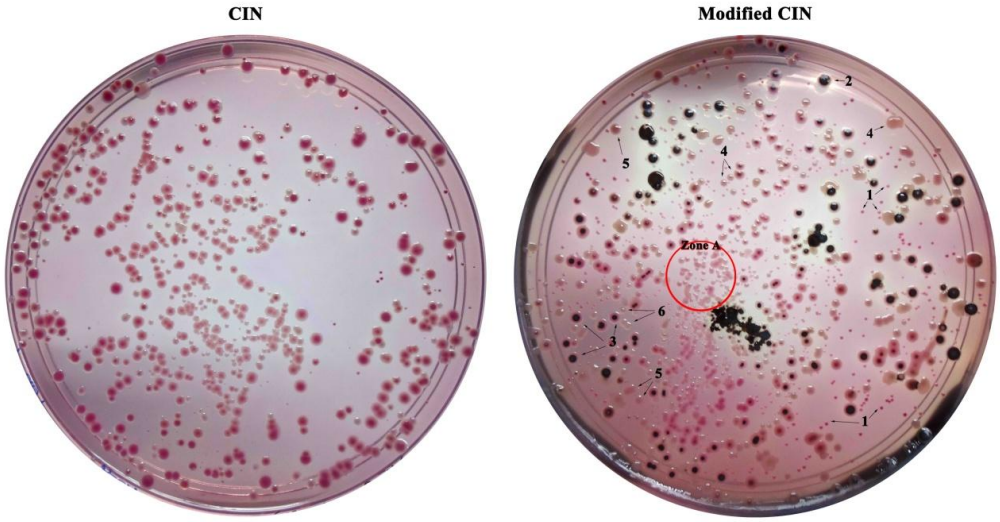


Figure 5.1. Colony morphology on CIN and modified CIN of an artificially prepared bacterial mixture.

1, *Y. enterocolitica* bioserotype 3/O:1,2,3 (IP135); 2, *C. braakii*; 3, H_2S-producing *C. freundii*; 4, *A. hydrophila*; 5, *P. rettgeri*; 6, *E. cloacae*. Zone A was region with heavy bacteria growth (bacteria clumping) and bacteria normally grew tiny due to competition. Limitation of modified CIN in Zone A: (i) formation of a black centre due to H_2S-production could not be detected; (ii) visualization of the brown diffusible pigment produce due to phenylalanine deaminase reaction was hardly seen.

In evaluating the limit of detection (LOD) and influence of background microbiota of both CIN and modified CIN media, *Y. enterocolitica* was chosen as the representating bacterium. The LOD of the modified CIN for the detection of *Y. enterocolitica* (in pure cultures or in artificially contaminated raw pork meat) was comparable to that obtained on CIN. For pure *Y. enterocolitica* cultures, the LOD was 10 cfu/ml (for all four strains tested; IP383, IP135, ATCC 9610 and PC-M16-2) (Table 4.5.2). The LOD of IP135 for artificially contaminated pork meat increased to 10^4 cfu/ml because the presence of natural microbiota interfered with the recovery of

Y. enterocolitica (Table 4.5.4). Bacteria such as *C. freundii, C. amalonaticus, C. diversus, Hafnia alvei, Klebsiella pneumoniae, E. agglomerans, P. rettgeri,* and environmental *Yersinia* species that are naturally present in food may indeed have an inhibitory effect on the growth of *Y. enterocolitica* (Fukushima & Gomyoda, 1986). The antagonistic effect of certain Gram-negative bacteria toward the growth of *Y. enterocolitica* could be due to the limited space for cell multiplication too (Schiemann & Olson, 1984). In a recent study conducted by Savin et al. (2005), CIN was reported to be more efficient (LOD = 3×10^3 cfu/g of faeces) as compared to SSI medium (LOD = 3×10^6 cfu/g faeces) in isolating *Y. enterocolitica*. Therefore, we anticipate the modified CIN would be a useful medium in differentiating *Y. enterocolitica* from contaminated samples such as faeces.

The investigation on the influence of background microbiota was done by three tests: (i) pool of bacterial mixture, (ii) artificially contaminated pork and (iii) naturally contaminated rectal swabs from swine. We observed that the *Y. enterocolitica* colonies were easily distinguished on modified CIN even when surrounded by *Yersinia*-like bacteria and background microbiota, but on CIN, it was much more difficult. The percentage of false-positive *Y. enterocolitica* recovered on CIN (66.7%) from an artificially prepared bacterial mixture can decrease to 40.0% on modified CIN, and the corresponding percentage from artificially contaminated raw pork meat can decrease from 37.7% on CIN to 27.5% on modified CIN (Table 4.5.5). At the same time, the true positive *Y. enterocolitica* isolates increased by nearly 27% and 10% on modified CIN compared to CIN in artificially prepared bacterial mixture and artificially contaminated pork, respectively CIN (Table 4.5.5). The capability of modified CIN in reducing the number of false positive *Y. enterocolitica* isolates, especially in eliminating H_2S-producing *Citrobacter* spp., *M. morganii, P. rettgeri, A. hydrophila, E. cloacae* can shorten the processing time and

reduce the workload and costs associated with biochemical assays that were reported in many studies (Fondrevez, et al., 2010; Head, et al., 1982; Renaud, et al., 2013; Schiemann, 1979; Weagant & Feng, 2001; Zheng & Xie, 1996). Higher recovery rates from the modified CIN compared with that of CIN was further demonstrated using naturally contaminated samples (rectal swabs from swine, Table 4.5.6), indicating the modified CIN improved the recovery of *Y. enterocolitica* even on actual samples. The reason for the failure of CIN in isolating *Y. enterocolitica* from rectal specimens may due to the presence of high amount of *Yersinia*-like bacteria on CIN that caused the visualisation of the *Yersinia* difficult during plate analysis. In this evaluation study, the naturally contaminated samples were only enriched in one enrichment medium, PBS and the results indicated that a cold enrichment could help in recovering a larger number of positive samples than direct streaking method. A post-enrichment alkaline treatment showed no difference for the recovery rates of *Y. enterocolitica* (Table 4.5.6). The modification made on CIN enhanced the differentiating power of CIN while retaining the sensitivity (see results of LOD test) in isolating *Yersinia*. Hence, the modified CIN could also increase the recovery rates compared to CIN when it is used in combination with any other enrichment or isolating media that are reported to be useful in previous studies. For examples the irgarsan-ticarcillin-cholate (ITC)-CIN method (Fondrevez, et al., 2010), followed by streaking on YeCM and enrichment in peptone-sorbitol-bile (PSB) broth for 2 days (Van Damme, et al., 2013) that are reported to be useful in isolation of pathogenic *Y. enterocolitica*.

Therefore, the use of modified CIN may significantly reduce the percentage of false-positive *Yersinia* recovered from a contaminated sample. The higher discriminatory power of the modified CIN compared with that of CIN was further demonstrated in artificially prepared bacteria mixture and naturally contaminated

samples. The results thus demonstrated that the use of modified CIN may be a valuable means to increase the recovery rate of *Y. enterocolitica* from natural samples, which are usually contaminated by multiple types of bacteria.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

This is the first report on the prevalence and characterization of *Yersinia enterocolitica* in pigs and food from Malaysia. *Y. enterocolitica* was isolated from raw pork products and pigs. The prevalence of *Y. enterocolitica* in raw pork products and pigs were 12.1% and 1.8%, respectively. The most common bioserotype isolated was 3 variant/O:3, followed by 1B/O:8 and 1A/O:5. The results showed that healthy grower pig is an important reservoir of *Y. enterocolitica* biotype 3 variant/O:3 harbouring virulence genes. Besides, food such as raw pork meat, liver and intestine might serve as important transmission agents of virulent *Y. enterocolitica* for humans. The virulence genes are not only present in the pathogenic biotypes but also in the non-pathogenic biotype 1A. PFGE subtyping clearly differentiated the *Y. enterocolitica* isolates with different bioserotypes and origin from each other, indicating the isolates are genetically diverse. However, the isolates were highly clonal within each bioserotype. This may due to limitation in the current cultures collection. The increment in the resistance rates of nalidixic acid, tetracycline and streptomycin and the existence of multidrug-resistant *Y. enterocolitica* in food and pigs are of public concern and should be monitored in future surveillance studies. Improper food handling and processing may cause cross contamination of this pathogen to humans. More research is needed in understanding the possible route of transmission of *Y. enterocolitica* in human yersiniosis in Malaysia. The performance of the CIN agar was increased through the modification made in this study. Bacteria such as H_2S-producing *Citrobacter freundii*, *C. braakii*, *Enterobacter cloacae, Aeromonas hydrophila,* and *Providencia rettgeri* which were indistinguishable on CIN agar each now had distinct colony appearances on the modified CIN. In addition, *Morganella morganii* can be differentiated too. The modified CIN may be useful for routine surveillance for the presence of *Y. enterocolitica* in raw pork meat and for those with microbiota such as H_2S-producing *C. freundii, C. braakii, E. cloacae, A. hydrophila, P. rettgeri*, and *M. morganii* as it able to reduce false positive.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications:

Tan, L. K., Ooi, P. T., & Thong, K. L. (2014). Prevalence of *Yersinia enterocolitica* from food and pigs in selected states of Malaysia. *Food Control, 35*(1), 94-100.
(*ISI-Cited Publication*, Q1)

Tan, L. K., Ooi, P. T., Carniel, E. & Thong, K. L. (2014). Improved *Yersinia enterocolitica* differentiation with a modified cefsulodin-irgasan-novobiocin agar. *Plos One* (Accepted on August 3, 2014).
(*ISI-Cited Publication*, Q1)

Tan, L. K., Ooi, P. T., & Thong, K. L. Genetic relatedness and antimicrobial profiling of *Yersinia enterocolitica* strains from pigs and raw food.
(Submitted to *Food Microbiology, ISI-Cited Journal*, Q1)

Proceedings and Conferences:

Tan, L. K., Ooi, P. T., & Thong, K. L. (2013). PCR Detection of Virulence Genes in *Yersinia enterocolitica* from Foods and Pigs. 20th MSMBB Annual Scientific Meeting, Kuala Lumpur, Malaysia (pp. 61-62). Kuala Lumpur, Malaysia: University of Malaya.
(National)

Tan, L. K., Ooi, P. T., & Thong, K. L. (2012). Isolation of *Yersinia enterocolitica* from Various Food in Malaysia. National Postgraduate Seminar, Kuala Lumpur, Malaysia (pp. 33-34). Kuala Lumpur, Malaysia: University of Malaya.
(National)

Tan, L. K., Ooi, P. T., & Thong, K. L. (2012). Improvement of Cefsulodin-Irgasan-Novobiocin Agar for Isolation of *Yersinia enterocolitica* from Food. International Conference on Food Science and Nutrition, Kota Kinabalu, Sabah, Malaysia (pp. 747-749). Kota Kinabalu, Sabah, Malaysia: The Pacific Sutera Hotel.
(International)

Tan, L. K., Ooi, P. T., & Thong, K. L. (2011). Comparison of Different Cultural Protocols in Isolation of *Yersinia enterocolitica*. International Congress of the Malaysian Society for Microbiology, Penang, Malaysia (pp. 654-657). Batu Ferringhi, Penang, Malaysia: Bayview Beach Resort.
(International)

Tan, L. K. & Thong, K. L. (2010). A Comparison of Media and Methods for the Isolation of *Yersinia enterocolitica* from Raw Food Samples in Malaysia. My1Bio Conference, Kuala Lumpur, Malaysia (pp. 91-92). Kuala Lumpur, Malaysia: Berjaya Times Square Hotel.
(National)

APPENDIX I MEDIA

ENRICHMENT BROTHS

1. Phosphate buffered saline (PBS)

PBS (Oxoid, Germany, BR0014)	10 tablets
dH_2O	1 L

The tablets were dissolved well and autoclaved for 10 min at 115 ˚C.

2. *Yersinia* selective enrichment broth according to OSSMER (YSEO)

YSEO (Merck, Germany)	38.7 g
dH_2O	1 L

The suspension was gently brought to boil to dissolve completely. It was then autoclaved at 121˚C for 15min. The solution were kept in dark at the refrigerator temperature (at 4˚C) and used within 7 days.

3. Irgasan-ticarcillin-potassium chlorate (ITC) broth

ITC broth base (Fluka, Germany)	76 g
dH_2O	970 ml
Ticarcillin supplement (Fluka 17778)	1 vial
(Dissolved in 2 ml sterile dH_2O)	
Potassium chlorate supplement (Fluka 17777)	1 vial

Seventy-six gram ITC broth base was suspended in 970 ml dH_2O. Medium was heated to dissolve completely, sterilized by autoclaving at 121 ℃ for 15 minutes, cooled to 45-50 ℃. One vial of each ticarcillin supplement (Fluka 17778) and potassium chlorate supplement (Fluka 17777) added aseptically, and mixed well before dispensing in sterile tubes. The solution were kept in dark at the refrigerator temperature (at 4˚C) and used within 7 days.

DIFFERENTIAL AGARS

1. *Yersinia* Selective Supplement/ CIN agar

CIN agar base (Oxoid, cat no: CM0653)	29 g
dH_2O	500 ml
CIN selective supplement (Oxoid, cat no: SR0109)	1 vial
(Dissolved in ethanol : sterile dH_2O= 1:1)	

Twenty-nine gram of CIN agar base was suspended in 500ml of dH_2O and boiled gently to dissolve completely. Agar was sterilised by autoclaving at 121 ℃ for 15 minutes. Agar was allowed to cool to approximately 50 ℃ and 1 vial of CIN selective supplement (SR0109) was aseptically added into the agar, mixed gently and poured into sterile Petri dishes. The plates were kept in dark at the refrigerator temperature (at 4˚C) and used within 7 days.

2. Modified CIN agar

The following supplements were added into the CIN agar base, pH adjusted to pH 7.4 ± 0.02 before autoclave and CIN selective supplement was added:

a) 1% L-arginine (Sigma, Germany)
b) 0.8 g/l ferric ammonium citrate (BDH Prolabo, UK)
c) 6.8 g/l sodium thiosulfate (BDH Prolabo)
d) 2.0 g/l DL-phenylalanine (Sigma)

NON-SELECTIVE AGARS

1. Brain Heart Infusion (BHI) Agar

BHI agar (Oxoid)	52 g
dH_2O	1 L

The suspension was gently brought to boil to dissolve completely. It was then autoclaved at 121˚C for 15min. It was cooled down to 45-50˚C and was dispensed into Petri dishes. The plates were kept at the refrigerator temperature (at 4˚C).

2. Mueller-Hinton II agar

MH agar (BD)	21.0 g
dH_2O	1 L

The suspension was gently brought to boil to dissolve completely. It was then autoclaved at 121˚C for 15min. It was cooled down to 45-50˚C and about 20-25 ml was dispensed into Petri dishes. The plates were kept at the refrigerator temperature (at 4 ˚C).

MEDIA FOR BIOCHEMICAL TESTS

1. Simmons' citrate agar

Simmons' citrate agar powder (Oxoid)	23.0 g
dH_2O	1 L

Twenty-three g of Simmons' Citrate powder was suspended in 1L of distilled water, boiled to dissolve. It was then autoclaved at 121˚C for 15min. It was cooled down to 45-50˚C and about 20-25 ml was dispensed into Petri dishes. The plates were kept at the refrigerator temperature (at 4 ˚C).

2. Urea agar base

Urea agar base (Oxoid)	2.4 g
dH_2O	95 ml

The suspension was gently brought to boil to dissolve completely. It was then autoclaved at 121˚C for 15min. It was cooled down to 50˚C and 5 ml of filter-sterilized 40% urea solution (Sigma) was mixed well into the warm agar base and immediately dispensed into Petri dishes (20-25 ml). The plates were kept at the refrigerator temperature (at 4 ˚C).

MEDIA FOR BIOGROUPING

1. Lipase test agar (Tween 80 agar)

Peptone (Oxoid)	10 g
NaCl	5 g
$CaCl_2 \cdot H_2O$	0.1 g
Agar	15 g
dH_2O	1 L

The suspension was gently brought to boil to dissolve completely. It was then autoclaved at 121˚C for 15 min. It was cooled down to 45-50˚C and 1% Tween 80 (sterilized by autoclaving at 121℃ for 15 minutes) was added, mixed well and dispensed into Petri dishes. The plates were kept at the refrigerator temperature (at 4˚C).

2. Bile esculine agar

Bile esculine agar (Oxoid)	44.5g
dH_2O	1 L

The suspension was gently brought to boil to dissolve completely. It was then autoclaved at 121˚C for 15 min. It was cooled down to 45-50˚C and was dispensed into Petri dishes. The plates were kept in dark at the refrigerator temperature (at 4˚C).

3. Phenol red agar

Phenol red agar base	30 g
dH_2O	1 L

The suspension was gently brought to boil to dissolve completely. It was then autoclaved at 121˚C for 15 min. . It was cooled down to 45-50˚C and was dispensed into Petri dishes. The plates were kept at the refrigerator temperature (at 4˚C). Phenol red agar was used together with the following carbohydrate discs for carbohydrate fermentation:

a) Salicin carbohydrate disc (Fluka, 92971)
b) Xylose carbohydrate disc (Fluka, 07411)
c) Trehalose carbohydrate disc (Fluka, 92961)

4. Pyrazinamidase agar

Tryptic soy agar (Difco)	30 g
Pyrazine-carboxamide (Sigma)	1 g
0.2 M Tris-maleate buffer (pH 6)	1 L

The suspension was gently brought to boil to dissolve completely. About 5 ml dispensed into (160 × 16 mm) test tubes, autoclaved at 121˚C for 15 min and slanted for cooling. Slants were kept at the refrigerator temperature (at 4˚C).

5. β-D-Glucosidase

0.1 g 4-nitrophenyl-β-D-glucopyranoside (Sigma) was added to 100 ml 0.666 M NaH_2PO_4 (pH 6.0, Sigma), dissolved, and then filter-sterilized. The solution was kept in dark at the refrigerator temperature (at 4˚C).

6. DNase test agar with Toluidine blue

DNase test agar with Toluidine blue (BD)	42 g
dH_2O	1 L

The suspension was gently brought to boil to dissolve completely. It was then autoclaved at 121˚C for 15 min. It was cooled down to 45-50˚C and was dispensed into Petri dishes. The plates were kept at the refrigerator temperature (at 4˚C).

7. MRVP broth (Oxoid)

MRVP broth (Oxoid)	17 g
dH_2O	1 L

The suspension was gently brought to boil to dissolve completely, dispensed into test tubes and then autoclaved at 121˚C for 15 min. The solution was kept at the refrigerator temperature (at 4˚C).

PHENOTYPIC VIRULENCE TESTS

1. MRVP broth (Oxoid) – refer above

2. CR-MOX agar

MOX agar (Fluka)	46.75 g
dH_2O	1 L

The suspension was gently brought to boil to dissolve completely. It was then autoclaved at 121˚C for 15min. It was cooled down to 55˚C and the following solutions were added:

a) 10 ml of 20% D-galactose solution (filter-sterilized by 0.2 µm membrane filter)
b) 5 ml of 1% Congo red solution (sterilized by autoclaving at 121℃ for 15 minutes)

Agar was mixed well and dispensed into Petri dishes. The plates were kept in dark at the refrigerator temperature (at 4˚C).

APPENDIX II CHEMICALS AND REAGENTS

1. API 20 E reagent kit (bio-Mérieux® SA, France)

James reagent, TDA reagent, VP(1+2) reagent, NIT(1+2) reagent, Zn powder

2. Mineral oil (bio-Mérieux® SA, France)

3. 3% Potassium Hydroxide (KOH) solution

KOH pellet	3 g
Sterile ddH_2O	top up to 100 ml

KOH pellet was mixed well and used immediately.

4. 85 µg/ml Crystal violet

Crystal violet	0.085 g
Sterile ddH_2O	100 ml

Reagent powder was mixed well and stored at 28 ℃.

5. α-Naphthol Reagent

α-Naphthol	5 g
Sterile ddH_2O	top up to 100 ml

Reagent powder was mixed well and stored at 4 ℃.

6. 40% KOH solution

KOH pellet	40 g
Sterile ddH_2O	top up to 100 ml

KOH pellet was mixed well and used immediately.

7. 1% iron (II) ammonium sulphate solution

Iron (II) ammonium sulphate (BDH) powder	1 g
Sterile ddH_2O	100 ml

Reagent powder was mixed well and used immediately after preparation.

8. 1.5 % agarose gel

Agarose powder	1.5 g
0.5 × TBE buffer	100 ml

Agarose powder was mixed well and boiled to dissolve. Molten agarose was cooled down to 50-55 ℃ before the gel was casted.

9. 2 % agarose gel

Agarose powder	2 g
0.5 × TBE buffer	100 ml

Agarose powder was mixed well and boiled to dissolve. Molten agarose was cooled down to 50-55 ℃ before the gel was casted.

10. 0.9 % agarose gel

Agarose powder	1.8 g
0.5 × TBE buffer	200 ml

Agarose powder was mixed well and boiled to dissolve. Molten agarose was cooled down to 50-55 ℃ before the gel was casted.

11. 1% Seakem Gold agarose

Seakem Gold agarose powder	1 g
1 × TE buffer	100 ml

Agarose powder was mixed well and boiled to dissolve. Molten agarose was kept in 55 ℃ water bath while waiting for PFGE plug preparation.

12. 1 % PFGE gel

Agarose powder (type 1, Sigma-Aldrich)	1.5 g
0.5 × TBE buffer	100 ml

Agarose powder was mixed well and boiled to dissolve. Molten agarose was cooled down to 50-55 ℃ before the gel was casted.

APPENDIX III BUFFERS AND SOLUTIONS

1. 0.25% potassium hydroxide (KOH): 0.50% sodium chloride (NaCl) solution

NaCl	5 g
KOH	2.5 g
ddH_2O	1 L

The solution was filter-sterilised (0.2 µm filter) or sterilised at 121 ℃ for 15 min. Stored at the refrigerator temperature (at 4˚C) and used within 7 days.

2. 3% KOH solution

KOH pellets	3 g
ddH_2O	100 ml

KOH pellets were dissolved completely and used immediately for String test.

3. 0.85% Saline

NaCl	0.85 g
dH_2O	100 ml

The solution was sterilized by at 121 ℃ for 15 min and stored at 28 ℃.

4. 10× Tris-borated EDTA (TBE), pH 8.3

Trizma base	121.2 g
Orthoboric/Boric acid	61.8 g
EDTA	0.745 g
ddH_2O	1 L

The above ingredients were dissolved in 500 ml of ddH_2O by stirring on the heated stirrer plate. pH was adjusted to 8.3 and top up to 1 L and autoclaved at 121˚C for 15 min.

5. 0.5× Tris-borate EDTA (TBE)

10× TBE	50 ml
dH_2O	950 ml

50 ml of 10× TBE was aliquoted into clean Schott bottle and top up to 1 L by using the dH_2O.

6. 1M Tris, pH 8.0 (Molecular weight = 121.14 g)

Tris	36.342 g
dH_2O	250 ml

36.342 g of Tris powder were dissolved in 250 ml of ddH_2O by stirring on the heated stirrer plate. pH was adjusted to 8.0 and top up to 300 ml and autoclaved at 121˚C; 15 psi for 15 min.

7. 0.5 M EDTA, pH 8.0 (Molecular weight = 372.24 g)

EDTA	55.83 g
dH_2O	250 ml

55.83 g of EDTA powder were dissolved in 250 ml of ddH_2O by stirring on the heated stirrer plate. pH was adjusted to 8.0 and top up to 300 ml and autoclaved at 121˚C; 15 psi for 15 min.

8. Tris-EDTA (TE) buffer (10 mM Tris; 1 mM EDTA; pH 8.0)

Tris, pH 8.0	10 ml
0.5M EDTA, pH 8.0	2 ml

Top up with ddH_2O to 1000 ml, and autoclaved 121˚C; 15 psi for 15 min.

9. Cell Suspension Buffer (100 mM Tris; 100 mM EDTA; pH 8.0)

1M Tris, pH 8.0	10 ml
0.5M EDTA, pH 8.0	20 ml

Top up with ddH_2O to 100 ml. Autoclaved 121˚C; 15 psi for 15 min.

10. 10% Sarcosyl (N-Lauryl-Sarcosine, Sodium Salt) (Molecular weight = 293.39 g)

Sodium N-Lauryl-Sarcosinate solution	10 ml

Top up with ddH_2O to 100 ml. Autoclaved 121˚C; 15 psi for 15 min.

11. Cell Lysis Buffer (50mM Tris; 50mM EDTA; pH 8.0; 1% Sarcosine)

1M Tris, pH 8.0	25 ml
0.5M EDTA, pH 8.0	50 ml
10% Sarcosyl (N-Lauryl-Sarcosine, Sodium Salt)	50 ml

Top up with ddH_2O to 500 ml. Autoclaved 121˚C; 15 psi for 15 min.

12. Proteinase K 20 mg/ml

Proteinase K powder	100 mg
Sterile ddH_2O	5 ml

Solution was mixed well and kept in ice while waiting to be used. The remaining solution was kept at -20 ℃.

APPENDIX IV BACKGROUND INFORMATION OF RAW PORK PRODUCTS

No.	Sampling date	Sample code	Sample	Location	Wet market	Hawker stall	Condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection			
										Overall	YSEO	ITC	PBS
1	7-Jun-10	M1	Raw Pork Meat	Kuala Lumpur	A	stall 1	whole meat	+	+	+	-	n.a.	+
2	7-Jun-10	M2	Raw Pork Meat	Kuala Lumpur	A	stall 1	whole meat	-	-	+	-	n.a.	+
3	7-Jun-10	M3	Raw Pork Meat	Kuala Lumpur	A	stall 1	whole meat	+	+	+	-	n.a.	+
4	14-Jun-10	I1	Raw Pork Intestine	Kuala Lumpur	B	stall 1	minced	-	-	+	-	n.a.	+
5	14-Jun-10	K1	Raw Pork Kidney	Kuala Lumpur	B	stall 1	minced	-	-	-	-	n.a.	-
6	14-Jun-10	S1	Raw Pork Skin	Kuala Lumpur	B	stall 1	minced	-	-	-	-	n.a.	-
7	14-Jun-10	E1	Pig's Ear	Kuala Lumpur	B	stall 1	minced	-	-	-	-	n.a.	-
8	14-Jun-10	T1	Pig's Throat	Kuala Lumpur	B	stall 1	minced	-	-	-	-	n.a.	-
9	14-Jun-10	F1	Pig's fat tissue	Kuala Lumpur	B	stall 1	minced	-	-	-	-	n.a.	-
10	10-Jul-10	M4	Raw Pork Meat	Kuala Lumpur	A	stall 1	whole meat	+	-	+	-	n.a.	+
11	10-Jul-10	M5	Raw Pork Meat	Kuala Lumpur	A	stall 1	minced	-	-	+	+	n.a.	+
12	10-Jul-10	M6	Raw Pork Meat	Kuala Lumpur	A	stall 1	minced	-	-	+	-	n.a.	+
13	10-Jul-10	M7	Raw Pork Meat	Kuala Lumpur	A	stall 1	minced	-	-	-	-	n.a.	-
14	10-Jul-10	I2	Raw Pork Intestine	Kuala Lumpur	A	stall 2	minced	-	-	+	+	n.a.	-
15	10-Jul-10	I3	Raw Pork Intestine	Kuala Lumpur	A	stall 2	minced	-	-	+	-	n.a.	+
16	10-Jul-10	D1	Raw Pork Heart	Kuala Lumpur	A	stall 1	minced	-	-	+	+	n.a.	-
17	10-Jul-10	D2	Raw Pork Heart	Kuala Lumpur	A	stall 1	minced	-	-	+	+	n.a.	+
18	10-Jul-10	L1	Raw Pork liver	Kuala Lumpur	A	stall 1	minced	-	-	+	+	n.a.	-
19	10-Jul-10	P1	Pig's paw	Kuala Lumpur	A	stall 1	whole	-	-	-	-	n.a.	-
20	2-Aug-10	M8	Raw Pork Meat	Kuala Lumpur	B	stall 1	whole	-	-	-	-	-	-
21	2-Aug-10	M9	Raw Pork Meat	Kuala Lumpur	B	stall 1	whole	-	-	+	+	-	-
22	2-Aug-10	M10	Raw Pork Meat	Kuala Lumpur	B	stall 1	whole	-	-	-	-	-	-
23	2-Aug-10	D3	Raw Pork Meat	Kuala Lumpur	B	stall 1	minced	-	-	-	-	-	-
24	2-Aug-10	S2	Raw Pork Skin	Kuala Lumpur	B	stall 1	whole	-	-	+	-	-	+
25	16-Aug-10	D4	Raw Pork heart	Kuala Lumpur	B	stall 1	whole	-	-	+	+	+	-
26	12-Sep-10	L2	Raw Pork Liver	Kuala Lumpur	A	stall6	whole	-	-	-	-	-	-
27	12-Sep-10	I4	Raw Pork Intestine	Kuala Lumpur	A	stall6	whole	-	-	-	-	-	-
28	12-Sep-10	S3	Raw Pork Skin	Kuala Lumpur	A	stall2	whole	-	-	-	-	-	-
29	12-Sep-10	M11	Raw Pork Meat	Kuala Lumpur	A	stall6	whole	-	-	+	+	+	-
30	20-Sep-10	M12	Raw Pork Meat	Kuala Lumpur	A	stall 1	whole	-	-	+	+	-	-
31	20-Sep-10	M13	Raw Pork Meat	Kuala Lumpur	A	stall 1	whole	+	+	+	+	-	-

No.	Sampling date	Sample code	Sample	Location	Wet market	Hawker stall	Condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection			
										Overall	YSEO	ITC	PBS
32	20-Sep-10	M14	Raw Pork Meat	Kuala Lumpur	A	stall 2	whole	-	-	+	+	+	-
33	20-Sep-10	M15	Raw Pork Meat	Kuala Lumpur	A	stall 2	whole	-	-	+	+	+	+
34	20-Sep-10	I5	Raw Pork Intestine	Kuala Lumpur	A	stall6	whole	-	-	+	+	-	+
35	20-Sep-10	I6	Raw Pork Intestine	Kuala Lumpur	A	stall6	whole	-	-	+	-	+	+
36	17-Jan-11	M16	Raw Pork Meat	Kuala Lumpur	A	stall7	whole	+	+	+	+	+	+
37	17-Jan-11	M17	Raw Pork Meat	Kuala Lumpur	A	stall7	whole	-	-	+	+	+	+
38	17-Jan-11	M18	Raw Pork Meat	Kuala Lumpur	A	stall8	whole	-	-	+	+	-	+
39	17-Jan-11	M19	Raw Pork Meat	Kuala Lumpur	A	stall9	whole	-	-	+	+	+	+
40	17-Jan-11	M20	Raw Pork Meat	Kuala Lumpur	A	stall9	whole	+	-	+	+	+	+
41	17-Jan-11	I7	Raw Pork Intestine	Kuala Lumpur	A	stall10	whole	-	-	+	+	+	+
42	15-Mar-11	K2	Raw Pork Kidney	Perak	C	stall 11	whole	-	-	-	-	-	-
43	15-Mar-11	K3	Raw Pork Kidney	Perak	C	stall 11	whole	-	-	+	+	-	-
44	15-Mar-11	S4	Raw Pork Skin	Perak	C	stall 11	whole	-	-	-	-	-	-
45	15-Mar-11	L3	Raw Pork Liver	Perak	C	stall 11	whole	-	-	+	+	-	-
46	15-Mar-11	K4	Pig's Kidney tissue	Perak	D	stall 12	whole	-	-	-	-	-	-
47	15-Mar-11	N1	Pig's nose	Perak	D	stall 12	whole	-	-	-	-	-	-
48	15-Mar-11	EB1	Pig's eye tissue	Perak	D	stall 12	whole	-	-	-	-	-	-
49	15-Mar-11	H1	Raw Pork heart	Perak	D	stall 12	whole	-	-	-	-	-	-
50	15-Mar-11	P2	pig's foot	Perak	D	stall 12	whole	-	-	-	-	-	-
51	27-Sep-10	PO1	Raw Pork Meat	Pahang	G	stall 19	whole	-	-	-	-	-	-
52	18-Jan-11	YE032 (or S18/1-1)	Raw Pork Liver	Kuala Lumpur	A	stall 1	whole	+	+	+	-	+	-
53	18-Jan-11	S18/1-2	Raw Pork liver	Kuala Lumpur	A	stall 1	whole	-	-	-	-	-	-
54	18-Jan-11	S18/1-3	Raw Pork Meat	Kuala Lumpur	A	stall 1	whole	-	-	+	-	-	+
55	18-Jan-11	S18/1-4	Raw Pork Meat	Kuala Lumpur	A	stall 1	whole	-	-	+	-	-	+
56	18-Jan-11	YE036 (or S18/1-5)	Raw Pork Meat	Kuala Lumpur	A	stall 1	whole	+	+	+	+	+	-
57	18-Jan-11	YE037 (or S18/1-6)	Raw Pork Intestine	Kuala Lumpur	A	stall 1	whole	+	+	+	+	-	+
58	18-Jan-11	S18/1-11	Raw Pork Meat	Kuala Lumpur	A	stall 1	whole	-	-	-	-	-	-

APPENDIX V BACKGROUND INFORMATION OF NON-PORCINE FOOD

No.	Sampling date	Sample code	Sample	Location	Wet market	Hawker stall	Condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection			
										Overall	YSEO	ITC	PBS
1	16-Aug-10	VG1	Water-cress	Kuala Lumpur	B	stall 3	Fresh	-	-	+	+	-	+
2	16-Aug-10	VG2	celery cabbage	Kuala Lumpur	B	stall 3	Fresh	-	-	+	+	-	+
3	16-Aug-10	VG3	Lettuce	Kuala Lumpur	B	stall4	Fresh	-	-	+	+	-	-
4	12-Sep-10	VG4	balsam pear	Kuala Lumpur	A	stall5	Fresh, internal content	-	-	-	-	-	-
5	12-Sep-10	VG5	Cowpea	Kuala Lumpur	A	stall4	Fresh	-	-	-	-	-	-
6	12-Sep-10	VR1	Amaranth's root	Kuala Lumpur	A	stall5	Fresh	-	-	-	-	-	-
7	3-Sep-10	S3/9-1	China cabbage	Selangor	E	stall 13	Fresh	-	-	-	-	-	-
8	3-Sep-10	S3/9-2	Chinese broccoli (kai lan)	Selangor	E	stall 13	Slightly spoilt	-	-	-	-	-	-
9	3-Sep-10	S3/9-3	Squid	Selangor	E	stall 14	Fresh	-	-	-	-	-	-
10	3-Sep-10	S3/9-4	Sweet potato	Selangor	E	stall 13	Fresh, dry	-	-	-	-	-	-
11	3-Sep-10	S3/9-5	China cabbage	Selangor	E	stall 13	Fresh	-	-	-	-	-	-
12	3-Sep-10	S3/9-6	China cabbage	Selangor	E	stall 13	Fresh	-	-	-	-	-	-
13	6-Sep-10	S6/9-C1	Chicken	Selangor	F	stall 15	Fresh	-	-	+	-	-	+
14	6-Sep-10	S6/9-C2	Chicken	Selangor	F	stall 15	Fresh	-	-	+	-	-	+
15	6-Sep-10	S6/9-C3	Chicken	Selangor	F	stall 15	Fresh	-	-	+	-	-	+
16	6-Sep-10	S6/9-B1	Beef	Selangor	F	stall 16	Fresh	-	-	-	-	-	-
17	6-Sep-10	S6/9-B2	Beef	Selangor	F	stall 16	Fresh	-	-	-	-	-	-
18	6-Sep-10	S6/9-B3	Beef	Selangor	F	stall 16	Fresh	-	-	+	-	-	+
19	6-Sep-10	S6/9-B4	Beef	Selangor	F	stall 16	Fresh	-	-	+	-	-	+
20	7-Sep-10	S7/9-P1	Prawn	Pahang	G	stall 17	Fresh	-	-	-	-	-	-
21	7-Sep-10	S7/9-F1 or YE015	“Ikan kampong”	Pahang	G	stall 17	Slightly spoilt	+	-	+	-	-	+
22	7-Sep-10	S7/9-F2	“Ikan kampong”	Pahang	G	stall 17	Fresh	-	-	-	-	-	-
23	7-Sep-10	S7/9-F3	“Ikan patin”	Pahang	G	stall 17	Fresh	-	-	-	-	-	-
24	7-Sep-10	S7/9-C1	Chicken	Pahang	G	stall 18	Fresh	-	-	+	-	-	+
25	20-Sep-10	S20/9-V1	Spinach	Selangor	E	stall 13	Spoilt	-	-	-	-	-	-
26	20-Sep-10	S20/9-C1	Chicken (meat)	Selangor	E	stall 21	Fresh	-	-	-	-	-	-
27	20-Sep-10	S20/9-V2	China cabbage	Selangor	E	stall 13	Spoilt	-	-	-	-	-	-
28	20-Sep-10	S20/9-V3	Bitter gourd	Selangor	E	stall 13	Fresh	-	-	-	-	-	-

No.	Sampling date	Sample code	Sample	Location	Wet market	Hawker stall	Condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection			
										Overall	YSEO	ITC	PBS
29	20-Sep-10	S20/9-F4	Fish	Selangor	E	stall 14	Fresh	-	-	+	-	+	-
30	27-Sep-10	S27/9-T1	Tofu	Pahang	G	stall 20	Fresh	-	-	-	-	-	-
31	27-Sep-10	S27/9-S1	Squid	Pahang	G	stall 14	Fresh	-	-	-	-	-	-
32	27-Sep-10	S27/9-V1	Cauliflower	Pahang	G	stall 13	Fresh	-	-	-	-	-	-
33	27-Sep-10	S27/9-C1	Chicken	Pahang	G	stall 18	Fresh	-	-	-	-	-	-
34	27-Sep-10	S27/9-M1	Goat milk (pasteurized)	Pahang	G	mini mart 1	Fresh	-	-	-	-	-	-
35	27-Sep-10	S27/9-T2	Tofu	Pahang	G	stall 20	Fresh	-	-	-	-	-	-
36	27-Sep-10	S27/9-C2	Chicken (feet)	Pahang	G	stall 18	Fresh	-	-	-	-	-	-
37	18-Jan-11	S18/1-7	Cabbage	Kuala Lumpur	A	stall 5	Fresh	-	-	+	-	+	+
38	18-Jan-11	S18/1-8	Bitter Gourd	Kuala Lumpur	A	stall 5	A little mushy and spoilt	-	-	+	+	+	-
39	18-Jan-11	S18/1-9	Brinjal	Kuala Lumpur	A	stall 6	Spoilt, worms observed	-	-	+	+	+	-
40	18-Jan-11	S18/1-10	Lady's Finger	Kuala Lumpur	A	stall 6	Fresh	-	-	-	-	-	-
41	24-Jan-11	S24/1-1	Beef	Pahang	G	stall 22	Fresh	-	-	+	-	-	+
42	24-Jan-11	S24/1-2	Chicken	Pahang	G	stall 18	Fresh	-	-	-	-	-	-
43	24-Jan-11	S24/1-3	Cockles	Pahang	G	stall 14	Fresh	-	-	-	-	-	-
44	24-Jan-11	S24/1-4	"Ikan kampong"	Pahang	G	stall 14	Fresh	-	-	+	-	+	-
45	24-Jan-11	S24/1-5	"Ikan patin"	Pahang	G	stall 14	Fresh	-	-	+	-	+	-
46	24-Jan-11	S24/1-6	Beef	Pahang	G	stall 22	Fresh	-	-	+	-	+	-
47	24-Jan-11	S24/1-7	Squid	Pahang	G	stall 14	Fresh	-	-	+	-	+	-
48	24-Jan-11	S24/1-8	Chicken	Pahang	G	stall 18	Fresh	-	-	+	-	+	-

APPENDIX VI BACKGROUND INFORMATION OF PIG FARMS AND PIG SAMPLES

No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
1	6-Oct-10	Tanjung Sepat, Selangor	A	1	TSP1a	Nasal swab	Weaner	Healthy	-	-	-	-	n.a.
2	6-Oct-10	Tanjung Sepat, Selangor	A	1	TSP1b	Rectal swab	Weaner	Healthy	-	-	-	-	n.a.
3	6-Oct-10	Tanjung Sepat, Selangor	A	1	TSP1c	Tongue swab	Weaner	Healthy	-	-	-	-	n.a.
4	6-Oct-10	Tanjung Sepat, Selangor	A	2	TSP2a	Nasal swab	Weaner	Healthy	-	-	-	-	-
5	6-Oct-10	Tanjung Sepat, Selangor	A	2	TSP2b	Rectal swab	Weaner	Healthy	-	-	+	+	-
6	6-Oct-10	Tanjung Sepat, Selangor	A	2	TSP2c	Palate swab	Weaner	Healthy	-	-	-	-	-
7	6-Oct-10	Tanjung Sepat, Selangor	A	3	TSP3a	Nasal swab	Weaner	Healthy	-	-	-	-	-
8	6-Oct-10	Tanjung Sepat, Selangor	A	3	TSP3b	Rectal swab	Weaner	Healthy	-	-	-	-	-
9	6-Oct-10	Tanjung Sepat, Selangor	A	3	TSP3c	Palate swab	Weaner	Healthy	-	-	-	-	-
10	6-Oct-10	Tanjung Sepat, Selangor	A	4	TSP4a	Nasal swab	Weaner	Healthy	-	-	-	-	n.a.
11	6-Oct-10	Tanjung Sepat, Selangor	A	4	TSP4b	Rectal swab	Weaner	Healthy	-	-	-	-	n.a.
12	6-Oct-10	Tanjung Sepat, Selangor	A	4	TSP4c	Palate swab	Weaner	Healthy	-	-	-	-	n.a.
13	6-Oct-10	Tanjung Sepat, Selangor	A	5	TSP5a	Nasal swab	Weaner	Healthy	-	-	-	-	-
14	6-Oct-10	Tanjung Sepat, Selangor	A	5	TSP5b	Rectal swab	Weaner	Healthy	-	-	-	-	-
15	6-Oct-10	Tanjung Sepat, Selangor	A	5	TSP5c	Tongue swab	Weaner	Healthy	-	-	-	-	-
16	6-Oct-10	Tanjung Sepat, Selangor	A	6	TSP6a	Nasal swab	Weaner	Healthy	-	-	-	-	-
17	6-Oct-10	Tanjung Sepat, Selangor	A	6	TSP6b	Rectal swab	Weaner	Healthy	-	-	-	-	-
18	6-Oct-10	Tanjung Sepat, Selangor	A	6	TSP6c	Palate swab	Weaner	Healthy	-	-	-	-	-
19	6-Oct-10	Tanjung Sepat, Selangor	A	7	TSP7a	Nasal swab	Weaner	Healthy	-	-	+	-	+
20	6-Oct-10	Tanjung Sepat, Selangor	A	7	TSP7b	Rectal swab	Weaner	Healthy	-	-	+	-	+
21	6-Oct-10	Tanjung Sepat, Selangor	A	7	TSP7c	Palate swab	Weaner	Healthy	-	-	+	-	+
22	6-Oct-10	Tanjung Sepat, Selangor	A	8	TSP8a	Nasal swab	Weaner	Unhealthy	-	-	+	+	-
23	6-Oct-10	Tanjung Sepat, Selangor	A	8	TSP8b	Rectal swab	Weaner	Unhealthy	-	-	-	-	-
24	6-Oct-10	Tanjung Sepat, Selangor	A	8	TSP8c	Tonsil swab	Weaner	Unhealthy	-	-	+	-	+
25	6-Oct-10	Tanjung Sepat, Selangor	A	9	TSP9a	Nasal swab	Weaner	Unhealthy	-	-	-	-	n.a.
26	6-Oct-10	Tanjung Sepat, Selangor	A	9	TSP9b	Rectal swab	Weaner	Unhealthy	-	-	-	-	n.a.
27	6-Oct-10	Tanjung Sepat, Selangor	A	9	TSP9c	Tongue swab	Weaner	Unhealthy	-	-	-	-	n.a.
28	20-Oct-10	Tanjung Sepat, Selangor	B	10	TSP10a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
29	20-Oct-10	Tanjung Sepat, Selangor	B	10	TSP10b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
30	20-Oct-10	Tanjung Sepat, Selangor	B	10	TSP10c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
31	20-Oct-10	Tanjung Sepat, Selangor	B	11	TSP11a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
32	20-Oct-10	Tanjung Sepat, Selangor	B	11	TSP11b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
33	20-Oct-10	Tanjung Sepat, Selangor	B	11	TSP11c	rectal swab	Weaner	Unhealthy	-	-	+	+	-
34	20-Oct-10	Tanjung Sepat, Selangor	B	12	TSP12a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
35	20-Oct-10	Tanjung Sepat, Selangor	B	12	TSP12b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
36	20-Oct-10	Tanjung Sepat, Selangor	B	12	TSP12c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
37	20-Oct-10	Tanjung Sepat, Selangor	B	13	TSP13a	nasal swab	Weaner	Healthy	-	-	-	-	-
38	20-Oct-10	Tanjung Sepat, Selangor	B	13	TSP13b	tongue swab	Weaner	Healthy	-	-	-	-	-

No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
39	20-Oct-10	Tanjung Sepat, Selangor	B	13	TSP13c	rectal swab	Weaner	Healthy	-	-	-	-	-
40	20-Oct-10	Tanjung Sepat, Selangor	B	14	TSP14a	nasal swab	Weaner	Healthy	-	-	-	-	-
41	20-Oct-10	Tanjung Sepat, Selangor	B	14	TSP14b	tongue swab	Weaner	Healthy	-	-	+	+	-
42	20-Oct-10	Tanjung Sepat, Selangor	B	14	TSP14c	rectal swab	Weaner	Healthy	-	-	-	-	-
43	20-Oct-10	Tanjung Sepat, Selangor	B	15	TSP15a	nasal swab	Weaner	Healthy	-	-	+	+	-
44	20-Oct-10	Tanjung Sepat, Selangor	B	15	TSP15b	tongue swab	Weaner	Healthy	-	-	-	-	-
45	20-Oct-10	Tanjung Sepat, Selangor	B	15	TSP15c	rectal swab	Weaner	Healthy	-	-	+	+	-
46	20-Oct-10	Tanjung Sepat, Selangor	B	16	TSP16a	nasal swab	Finisher	Healthy	-	-	-	-	-
47	20-Oct-10	Tanjung Sepat, Selangor	B	16	TSP16b	tongue swab	Finisher	Healthy	-	-	-	-	-
48	20-Oct-10	Tanjung Sepat, Selangor	B	16	TSP16c	rectal swab	Finisher	Healthy	-	-	-	-	-
49	20-Oct-10	Tanjung Sepat, Selangor	B	17	TSP17a	nasal swab	Finisher	Healthy	-	-	-	-	-
50	20-Oct-10	Tanjung Sepat, Selangor	B	17	TSP17b	tongue swab	Finisher	Healthy	-	-	-	-	-
51	20-Oct-10	Tanjung Sepat, Selangor	B	17	TSP17c	rectal swab	Finisher	Healthy	-	-	-	-	-
52	20-Oct-10	Tanjung Sepat, Selangor	B	18	TSP18a	nasal swab	Finisher	Healthy	-	-	-	-	-
53	20-Oct-10	Tanjung Sepat, Selangor	B	18	TSP18b	tongue swab	Finisher	Healthy	-	-	-	-	-
54	20-Oct-10	Tanjung Sepat, Selangor	B	18	TSP18c	rectal swab	Finisher	Healthy	-	-	-	-	-
55	20-Oct-10	Tanjung Sepat, Selangor	B	19	TSP19a	nasal swab	Finisher	Healthy	-	-	-	-	-
56	20-Oct-10	Tanjung Sepat, Selangor	B	19	TSP19b	tongue swab	Finisher	Healthy	-	-	-	-	-
57	20-Oct-10	Tanjung Sepat, Selangor	B	19	TSP19c	rectal swab	Finisher	Healthy	-	-	-	-	-
58	20-Oct-10	Tanjung Sepat, Selangor	B	20	TSP20a	nasal swab	Weaner	Healthy	-	-	-	-	-
59	20-Oct-10	Tanjung Sepat, Selangor	B	20	TSP20b	tongue swab	Weaner	Healthy	-	-	+	+	-
60	20-Oct-10	Tanjung Sepat, Selangor	B	20	TSP20c	rectal swab	Weaner	Healthy	-	-	+	-	+
61	20-Oct-10	Tanjung Sepat, Selangor	B	21	TSP21a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
62	20-Oct-10	Tanjung Sepat, Selangor	B	21	TSP21b	tonsil swab	Weaner	Unhealthy	-	-	+	-	+
63	20-Oct-10	Tanjung Sepat, Selangor	B	21	TSP21d	rectal swab	Weaner	Unhealthy	-	-	-	-	-
64	20-Oct-10	Tanjung Sepat, Selangor	B	22	TSP22a	nasal swab	Weaner	Unhealthy	-	-	+	-	-
65	20-Oct-10	Tanjung Sepat, Selangor	B	22	TSP22b	tonsil swab	Weaner	Unhealthy	-	-	-	-	-
66	20-Oct-10	Tanjung Sepat, Selangor	B	22	TSP22d	rectal swab	Weaner	Unhealthy	-	-	-	-	-
67	20-Oct-10	Tanjung Sepat, Selangor	B	23	TSP23a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
68	20-Oct-10	Tanjung Sepat, Selangor	B	23	TSP23b	tonsil swab	Weaner	Unhealthy	-	-	-	-	-
69	20-Oct-10	Tanjung Sepat, Selangor	B	23	TSP23d	rectal swab	Weaner	Unhealthy	-	-	-	-	-
70	21-Mar-11	Tanjung Sepat, Selangor	C	24	TSP24a	nasal swab	Piglet	healthy	-	-	-	-	-
71	21-Mar-11	Tanjung Sepat, Selangor	C	24	TSP24b	tongue swab	Piglet	healthy	-	-	-	-	-
72	21-Mar-11	Tanjung Sepat, Selangor	C	24	TSP24c	rectal swab	Piglet	healthy	-	-	-	-	-
73	21-Mar-11	Tanjung Sepat, Selangor	C	25	TSP25a	nasal swab	Piglet	healthy	-	-	-	-	-
74	21-Mar-11	Tanjung Sepat, Selangor	C	25	TSP25b	tongue swab	Piglet	healthy	-	-	-	-	-
75	21-Mar-11	Tanjung Sepat, Selangor	C	25	TSP25c	rectal swab	Piglet	healthy	-	-	-	-	-
76	21-Mar-11	Tanjung Sepat, Selangor	C	26	TSP26a	nasal swab	Piglet	healthy	-	-	-	-	-
77	21-Mar-11	Tanjung Sepat, Selangor	C	26	TSP26b	tongue swab	Piglet	healthy	-	-	-	-	-
78	21-Mar-11	Tanjung Sepat, Selangor	C	26	TSP26c	rectal swab	Piglet	healthy	-	-	-	-	-

No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
79	21-Mar-11	Tanjung Sepat, Selangor	C	27	TSP27a	nasal swab	Piglet	healthy	-	-	-	-	-
80	21-Mar-11	Tanjung Sepat, Selangor	C	27	TSP27b	tongue swab	Piglet	healthy	-	-	-	-	-
81	21-Mar-11	Tanjung Sepat, Selangor	C	27	TSP27c	rectal swab	Piglet	healthy	-	-	-	-	-
82	21-Mar-11	Tanjung Sepat, Selangor	C	28	TSP28a	nasal swab	Piglet	Unhealthy	-	-	-	-	-
83	21-Mar-11	Tanjung Sepat, Selangor	C	28	TSP28b	tongue swab	Piglet	Unhealthy	-	-	-	-	-
84	21-Mar-11	Tanjung Sepat, Selangor	C	28	TSP28c	rectal swab	Piglet	Unhealthy	-	-	-	-	-
85	21-Mar-11	Tanjung Sepat, Selangor	C	29	TSP29a	nasal swab	Piglet	Unhealthy	-	-	-	-	-
86	21-Mar-11	Tanjung Sepat, Selangor	C	29	TSP29b	tongue swab	Piglet	Unhealthy	-	-	-	-	-
87	21-Mar-11	Tanjung Sepat, Selangor	C	29	TSP29c	rectal swab	Piglet	Unhealthy	-	-	-	-	-
88	21-Mar-11	Tanjung Sepat, Selangor	C	30	TSP30a	nasal swab	Piglet	Unhealthy	-	-	-	-	-
89	21-Mar-11	Tanjung Sepat, Selangor	C	30	TSP30b	tongue swab	Piglet	Unhealthy	-	-	-	-	-
90	21-Mar-11	Tanjung Sepat, Selangor	C	30	TSP30c	rectal swab	Piglet	Unhealthy	-	-	-	-	-
91	21-Mar-11	Tanjung Sepat, Selangor	C	31	TSP31a	nasal swab	Piglet	Unhealthy	-	-	-	-	-
92	21-Mar-11	Tanjung Sepat, Selangor	C	31	TSP31b	tongue swab	Piglet	Unhealthy	-	-	-	-	-
93	21-Mar-11	Tanjung Sepat, Selangor	C	31	TSP31c	rectal swab	Piglet	Unhealthy	-	-	-	-	-
94	21-Mar-11	Tanjung Sepat, Selangor	C	32	TSP32a	nasal swab	Grower	Unhealthy	-	-	-	-	-
95	21-Mar-11	Tanjung Sepat, Selangor	C	32	TSP32b	tongue swab	Grower	Unhealthy	-	-	-	-	-
96	21-Mar-11	Tanjung Sepat, Selangor	C	32	TSP32c	rectal swab	Grower	Unhealthy	-	-	-	-	-
97	21-Mar-11	Tanjung Sepat, Selangor	C	33	TSP33a	nasal swab	Grower	healthy	-	-	-	-	-
98	21-Mar-11	Tanjung Sepat, Selangor	C	33	TSP33b	tongue swab	Grower	healthy	-	-	-	-	-
99	21-Mar-11	Tanjung Sepat, Selangor	C	33	TSP33c	rectal swab	Grower	healthy	-	-	-	-	-
100	21-Mar-11	Tanjung Sepat, Selangor	C	34	TSP34a	nasal swab	Grower	Unhealthy	-	-	-	-	-
101	21-Mar-11	Tanjung Sepat, Selangor	C	34	TSP34b	tongue swab	Grower	Unhealthy	-	-	-	-	-
102	21-Mar-11	Tanjung Sepat, Selangor	C	34	TSP34c	rectal swab	Grower	Unhealthy	-	-	-	-	-
103	21-Mar-11	Tanjung Sepat, Selangor	C	35	TSP35a	nasal swab	Grower	healthy	-	-	-	-	-
104	21-Mar-11	Tanjung Sepat, Selangor	C	35	TSP35b	tongue swab	Grower	healthy	-	-	-	-	-
105	21-Mar-11	Tanjung Sepat, Selangor	C	35	TSP35c	rectal swab	Grower	healthy	-	-	-	-	-
106	21-Mar-11	Tanjung Sepat, Selangor	C	36	TSP36a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
107	21-Mar-11	Tanjung Sepat, Selangor	C	36	TSP36b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
108	21-Mar-11	Tanjung Sepat, Selangor	C	36	TSP36c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
109	21-Mar-11	Tanjung Sepat, Selangor	C	37	TSP37a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
110	21-Mar-11	Tanjung Sepat, Selangor	C	37	TSP37b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
111	21-Mar-11	Tanjung Sepat, Selangor	C	37	TSP37c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
112	21-Mar-11	Tanjung Sepat, Selangor	C	38	TSP38a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
113	21-Mar-11	Tanjung Sepat, Selangor	C	38	TSP38b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
114	21-Mar-11	Tanjung Sepat, Selangor	C	38	TSP38c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
115	21-Mar-11	Tanjung Sepat, Selangor	C	39	TSP39a	nasal swab	Weaner	healthy	-	-	-	-	-
116	21-Mar-11	Tanjung Sepat, Selangor	C	39	TSP39b	tongue swab	Weaner	healthy	-	-	-	-	-
117	21-Mar-11	Tanjung Sepat, Selangor	C	39	TSP39c	rectal swab	Weaner	healthy	-	-	-	-	-
118	21-Mar-11	Tanjung Sepat, Selangor	C	40	TSP40a	nasal swab	Weaner	healthy	-	-	-	-	-

No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
119	21-Mar-11	Tanjung Sepat, Selangor	C	40	TSP40b	tongue swab	Weaner	healthy	-	-	-	-	-
120	21-Mar-11	Tanjung Sepat, Selangor	C	40	TSP40c	rectal swab	Weaner	healthy	-	-	-	-	-
121	21-Mar-11	Tanjung Sepat, Selangor	C	41	TSP41a	nasal swab	Weaner	healthy	-	-	-	-	-
122	21-Mar-11	Tanjung Sepat, Selangor	C	41	TSP41b	tongue swab	Weaner	healthy	-	-	-	-	-
123	21-Mar-11	Tanjung Sepat, Selangor	C	41	TSP41c	rectal swab	Weaner	healthy	-	-	-	-	-
124	21-Mar-11	Tanjung Sepat, Selangor	C	42	TSP42a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
125	21-Mar-11	Tanjung Sepat, Selangor	C	42	TSP42b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
126	21-Mar-11	Tanjung Sepat, Selangor	C	42	TSP42c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
127	21-Mar-11	Tanjung Sepat, Selangor	C	43	TSP43a	nasal swab	Weaner	healthy	-	-	-	-	-
128	21-Mar-11	Tanjung Sepat, Selangor	C	43	TSP43b	tongue swab	Weaner	healthy	-	-	-	-	-
129	21-Mar-11	Tanjung Sepat, Selangor	C	43	TSP43c	rectal swab	Weaner	healthy	-	-	-	-	-
130	21-Mar-11	Tanjung Sepat, Selangor	C	44	TSP44a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
131	21-Mar-11	Tanjung Sepat, Selangor	C	44	TSP44b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
132	21-Mar-11	Tanjung Sepat, Selangor	C	44	TSP44c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
133	21-Mar-11	Tanjung Sepat, Selangor	C	45	TSP45a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
134	21-Mar-11	Tanjung Sepat, Selangor	C	45	TSP45b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
135	21-Mar-11	Tanjung Sepat, Selangor	C	45	TSP45c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
136	21-Mar-11	Tanjung Sepat, Selangor	C	46	TSP46a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
137	21-Mar-11	Tanjung Sepat, Selangor	C	46	TSP46b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
138	21-Mar-11	Tanjung Sepat, Selangor	C	46	TSP46c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
139	21-Mar-11	Tanjung Sepat, Selangor	C	47	TSP47a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
140	21-Mar-11	Tanjung Sepat, Selangor	C	47	TSP47b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
141	21-Mar-11	Tanjung Sepat, Selangor	C	47	TSP47c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
142	21-Mar-11	Tanjung Sepat, Selangor	C	48	TSP48a	nasal swab	Finisher	healthy	-	-	-	-	-
143	21-Mar-11	Tanjung Sepat, Selangor	C	48	TSP48b	tongue swab	Finisher	healthy	-	-	-	-	-
144	21-Mar-11	Tanjung Sepat, Selangor	C	48	TSP48c	rectal swab	Finisher	healthy	-	-	-	-	-
145	21-Mar-11	Tanjung Sepat, Selangor	C	49	TSP49a	nasal swab	Finisher	healthy	-	-	-	-	-
146	21-Mar-11	Tanjung Sepat, Selangor	C	49	TSP49b	tongue swab	Finisher	healthy	-	-	-	-	-
147	21-Mar-11	Tanjung Sepat, Selangor	C	49	TSP49c	rectal swab	Finisher	healthy	-	-	-	-	-
148	21-Mar-11	Tanjung Sepat, Selangor	C	50	TSP50a	nasal swab	Finisher	healthy	-	-	-	-	-
149	21-Mar-11	Tanjung Sepat, Selangor	C	50	TSP50b	tongue swab	Finisher	healthy	-	-	-	-	-
150	21-Mar-11	Tanjung Sepat, Selangor	C	50	TSP50c	rectal swab	Finisher	healthy	-	-	-	-	-
151	21-Mar-11	Tanjung Sepat, Selangor	C	51	TSP51a	nasal swab	Finisher	healthy	-	-	-	-	-
152	21-Mar-11	Tanjung Sepat, Selangor	C	51	TSP51b	tongue swab	Finisher	healthy	-	-	-	-	-
153	21-Mar-11	Tanjung Sepat, Selangor	C	51	TSP51c	rectal swab	Finisher	healthy	-	-	-	-	-
154	21-Mar-11	Tanjung Sepat, Selangor	C	52	TSP52a	nasal swab	Finisher	healthy	-	-	-	-	-
155	21-Mar-11	Tanjung Sepat, Selangor	C	52	TSP52b	tongue swab	Finisher	healthy	-	-	-	-	-
156	21-Mar-11	Tanjung Sepat, Selangor	C	52	TSP52c	rectal swab	Finisher	healthy	-	-	-	-	-
157	21-Mar-11	Tanjung Sepat, Selangor	C	53	TSP53a	nasal swab	Finisher	healthy	-	-	-	-	-
158	21-Mar-11	Tanjung Sepat, Selangor	C	53	TSP53b	tongue swab	Finisher	healthy	-	-	-	-	-

No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
159	21-Mar-11	Tanjung Sepat, Selangor	C	53	TSP53c	rectal swab	Finisher	healthy	-	-	-	-	-
160	29-Jun-11	Gopeng, Perak	D	1	PP1a	nasal swab	Weaner	Healthy	-	-	-	-	-
161	29-Jun-11	Gopeng, Perak	D	1	PP1b	tongue swab	Weaner	Healthy	-	-	-	-	-
162	29-Jun-11	Gopeng, Perak	D	1	PP1c	rectal swab	Weaner	Healthy	-	-	-	-	-
163	29-Jun-11	Gopeng, Perak	D	2	PP2a	nasal swab	Weaner	Healthy	-	-	+	+	-
164	29-Jun-11	Gopeng, Perak	D	2	PP2b	tongue swab	Weaner	Healthy	-	-	-	-	-
165	29-Jun-11	Gopeng, Perak	D	2	PP2c	rectal swab	Weaner	Healthy	-	-	-	-	-
166	29-Jun-11	Gopeng, Perak	D	3	PP3a	nasal swab	Weaner	Healthy	-	-	+	+	-
167	29-Jun-11	Gopeng, Perak	D	3	PP3b	tongue swab	Weaner	Healthy	-	-	-	-	-
168	29-Jun-11	Gopeng, Perak	D	3	PP3c	rectal swab	Weaner	Healthy	-	-	-	-	-
169	29-Jun-11	Gopeng, Perak	D	4	PP4a	nasal swab	Weaner	Healthy	-	-	-	-	-
170	29-Jun-11	Gopeng, Perak	D	4	PP4b	tongue swab	Weaner	Healthy	-	-	-	-	-
171	29-Jun-11	Gopeng, Perak	D	4	PP4c	rectal swab	Weaner	Healthy	-	-	-	-	-
172	29-Jun-11	Gopeng, Perak	D	5	PP5a	nasal swab	Weaner	Healthy	-	-	-	-	-
173	29-Jun-11	Gopeng, Perak	D	5	PP5b	tongue swab	Weaner	Healthy	-	-	-	-	-
174	29-Jun-11	Gopeng, Perak	D	5	PP5c	rectal swab	Weaner	Healthy	-	-	-	-	-
175	29-Jun-11	Gopeng, Perak	D	6	PP6a	nasal swab	Weaner	Healthy	-	-	-	-	-
176	29-Jun-11	Gopeng, Perak	D	6	PP6b	tongue swab	Weaner	Healthy	-	-	-	-	-
177	29-Jun-11	Gopeng, Perak	D	6	PP6c	rectal swab	Weaner	Healthy	-	-	-	-	-
178	29-Jun-11	Gopeng, Perak	D	7	PP7a	nasal swab	Weaner	Healthy	-	-	-	-	-
179	29-Jun-11	Gopeng, Perak	D	7	PP7b	tongue swab	Weaner	Healthy	-	-	-	-	-
180	29-Jun-11	Gopeng, Perak	D	7	PP7c	rectal swab	Weaner	Healthy	-	-	+	+	-
181	29-Jun-11	Gopeng, Perak	D	8	PP8a	nasal swab	Weaner	Healthy	-	-	-	-	-
182	29-Jun-11	Gopeng, Perak	D	8	PP8b	tongue swab	Weaner	Healthy	-	-	-	-	-
183	29-Jun-11	Gopeng, Perak	D	8	PP8c	rectal swab	Weaner	Healthy	-	-	-	-	-
184	29-Jun-11	Gopeng, Perak	D	9	PP9a	nasal swab	Grower	Unhealthy	-	-	-	-	-
185	29-Jun-11	Gopeng, Perak	D	9	PP9b	tongue swab	Grower	Unhealthy	-	-	-	-	-
186	29-Jun-11	Gopeng, Perak	D	9	PP9c	rectal swab	Grower	Unhealthy	-	-	-	-	-
187	29-Jun-11	Gopeng, Perak	D	10	PP10a	nasal swab	Grower	Unhealthy	-	-	-	-	-
188	29-Jun-11	Gopeng, Perak	D	10	PP10b	tongue swab	Grower	Unhealthy	-	-	-	-	-
189	29-Jun-11	Gopeng, Perak	D	10	PP10c	rectal swab	Grower	Unhealthy	-	-	-	-	-
190	29-Jun-11	Gopeng, Perak	D	11	PP11a	nasal swab	Grower	Unhealthy	-	-	-	-	-
191	29-Jun-11	Gopeng, Perak	D	11	PP11b	tongue swab	Grower	Unhealthy	-	-	-	-	-
192	29-Jun-11	Gopeng, Perak	D	11	PP11c	rectal swab	Grower	Unhealthy	-	-	-	-	-
193	29-Jun-11	Gopeng, Perak	D	12	PP12a	nasal swab	Grower	Unhealthy	-	-	-	-	-
194	29-Jun-11	Gopeng, Perak	D	12	PP12b	tongue swab	Grower	Unhealthy	-	-	-	-	-
195	29-Jun-11	Gopeng, Perak	D	12	PP12c	rectal swab	Grower	Unhealthy	-	-	+	+	-
196	29-Jun-11	Gopeng, Perak	D	13	PP13a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
197	29-Jun-11	Gopeng, Perak	D	13	PP13b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
198	29-Jun-11	Gopeng, Perak	D	13	PP13c	rectal swab	Weaner	Unhealthy	-	-	-	-	-

No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
199	29-Jun-11	Gopeng, Perak	D	14	PP14a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
200	29-Jun-11	Gopeng, Perak	D	14	PP14b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
201	29-Jun-11	Gopeng, Perak	D	14	PP14c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
202	29-Jun-11	Gopeng, Perak	D	15	PP15a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
203	29-Jun-11	Gopeng, Perak	D	15	PP15b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
204	29-Jun-11	Gopeng, Perak	D	15	PP15c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
205	29-Jun-11	Gopeng, Perak	D	16	PP16a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
206	29-Jun-11	Gopeng, Perak	D	16	PP16b	tongue swab	Weaner	Unhealthy	-	-	+	+	-
207	29-Jun-11	Gopeng, Perak	D	16	PP16c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
208	29-Jun-11	Gopeng, Perak	D	17	PP17a	nasal swab	Grower	Healthy	-	-	-	-	-
209	29-Jun-11	Gopeng, Perak	D	17	PP17b	tongue swab	Grower	Healthy	-	-	-	-	-
210	29-Jun-11	Gopeng, Perak	D	17	PP17c	rectal swab	Grower	Healthy	-	-	-	-	-
211	29-Jun-11	Gopeng, Perak	D	18	PP18a	nasal swab	Grower	Healthy	-	-	-	-	-
212	29-Jun-11	Gopeng, Perak	D	18	PP18b	tongue swab	Grower	Healthy	-	-	-	-	-
213	29-Jun-11	Gopeng, Perak	D	18	PP18c	rectal swab	Grower	Healthy	-	-	-	-	-
214	29-Jun-11	Gopeng, Perak	D	19	PP19a	nasal swab	Grower	Healthy	-	-	-	-	-
215	29-Jun-11	Gopeng, Perak	D	19	PP19b	tongue swab	Grower	Healthy	-	-	-	-	-
216	29-Jun-11	Gopeng, Perak	D	19	PP19c	rectal swab	Grower	Healthy	-	-	-	-	-
217	29-Jun-11	Gopeng, Perak	D	20	PP20a	nasal swab	Grower	Healthy	-	-	-	-	-
218	29-Jun-11	Gopeng, Perak	D	20	PP20b	tongue swab	Grower	Healthy	-	-	-	-	-
219	29-Jun-11	Gopeng, Perak	D	20	PP20c	rectal swab	Grower	Healthy	-	-	-	-	-
220	29-Jun-11	Sungai Siput, Perak	E	21	PP21a	nasal swab	Weaner	Healthy	-	-	-	-	-
221	29-Jun-11	Sungai Siput, Perak	E	21	PP21b	tongue swab	Weaner	Healthy	-	-	-	-	-
222	29-Jun-11	Sungai Siput, Perak	E	21	PP21c	rectal swab	Weaner	Healthy	-	-	-	-	-
223	29-Jun-11	Sungai Siput, Perak	E	22	PP22a	nasal swab	Weaner	Healthy	-	-	-	-	-
224	29-Jun-11	Sungai Siput, Perak	E	22	PP22b	tongue swab	Weaner	Healthy	-	-	-	-	-
225	29-Jun-11	Sungai Siput, Perak	E	22	PP22c	rectal swab	Weaner	Healthy	-	-	-	-	-
226	29-Jun-11	Sungai Siput, Perak	E	23	PP23a	nasal swab	Weaner	Healthy	-	-	-	-	-
227	29-Jun-11	Sungai Siput, Perak	E	23	PP23b	tongue swab	Weaner	Healthy	-	-	-	-	-
228	29-Jun-11	Sungai Siput, Perak	E	23	PP23c	rectal swab	Weaner	Healthy	-	-	-	-	-
229	29-Jun-11	Sungai Siput, Perak	E	24	PP24a	nasal swab	Weaner	Healthy	-	-	-	-	-
230	29-Jun-11	Sungai Siput, Perak	E	24	PP24b	tongue swab	Weaner	Healthy	-	-	-	-	-
231	29-Jun-11	Sungai Siput, Perak	E	24	PP24c	rectal swab	Weaner	Healthy	-	-	-	-	-
232	29-Jun-11	Sungai Siput, Perak	E	25	PP25a	nasal swab	Weaner	Healthy	-	-	-	-	-
233	29-Jun-11	Sungai Siput, Perak	E	25	PP25b	tongue swab	Weaner	Healthy	-	-	-	-	-
234	29-Jun-11	Sungai Siput, Perak	E	25	PP25c	rectal swab	Weaner	Healthy	-	-	-	-	-
235	29-Jun-11	Sungai Siput, Perak	E	26	PP26a	nasal swab	Weaner	Healthy	-	-	-	-	-
236	29-Jun-11	Sungai Siput, Perak	E	26	PP26b	tongue swab	Weaner	Healthy	-	-	-	-	-
237	29-Jun-11	Sungai Siput, Perak	E	26	PP26c	rectal swab	Weaner	Healthy	-	-	-	-	-
238	29-Jun-11	Sungai Siput, Perak	E	27	PP27a	nasal swab	Weaner	Healthy	-	-	-	-	-

No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
239	29-Jun-11	Sungai Siput, Perak	E	27	PP27b	tongue swab	Weaner	Healthy	-	-	-	-	-
240	29-Jun-11	Sungai Siput, Perak	E	27	PP27c	rectal swab	Weaner	Healthy	-	-	-	-	-
241	29-Jun-11	Sungai Siput, Perak	E	28	PP28a	nasal swab	Weaner	Healthy	-	-	-	-	-
242	29-Jun-11	Sungai Siput, Perak	E	28	PP28b	tongue swab	Weaner	Healthy	-	-	-	-	-
243	29-Jun-11	Sungai Siput, Perak	E	28	PP28c	rectal swab	Weaner	Healthy	-	-	-	-	-
244	29-Jun-11	Sungai Siput, Perak	E	29	PP29a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
245	29-Jun-11	Sungai Siput, Perak	E	29	PP29b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
246	29-Jun-11	Sungai Siput, Perak	E	29	PP29c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
247	29-Jun-11	Sungai Siput, Perak	E	30	PP30a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
248	29-Jun-11	Sungai Siput, Perak	E	30	PP30b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
249	29-Jun-11	Sungai Siput, Perak	E	30	PP30c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
250	29-Jun-11	Sungai Siput, Perak	E	31	PP31a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
251	29-Jun-11	Sungai Siput, Perak	E	31	PP31b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
252	29-Jun-11	Sungai Siput, Perak	E	31	PP31c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
253	29-Jun-11	Sungai Siput, Perak	E	32	PP32a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
254	29-Jun-11	Sungai Siput, Perak	E	32	PP32b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
255	29-Jun-11	Sungai Siput, Perak	E	32	PP32c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
256	29-Jun-11	Sungai Siput, Perak	E	33	PP33a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
257	29-Jun-11	Sungai Siput, Perak	E	33	PP33b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
258	29-Jun-11	Sungai Siput, Perak	E	33	PP33c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
259	29-Jun-11	Sungai Siput, Perak	E	34	PP34a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
260	29-Jun-11	Sungai Siput, Perak	E	34	PP34b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
261	29-Jun-11	Sungai Siput, Perak	E	34	PP34c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
262	29-Jun-11	Sungai Siput, Perak	E	35	PP35a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
263	29-Jun-11	Sungai Siput, Perak	E	35	PP35b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
264	29-Jun-11	Sungai Siput, Perak	E	35	PP35c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
265	29-Jun-11	Sungai Siput, Perak	E	36	PP36a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
266	29-Jun-11	Sungai Siput, Perak	E	36	PP36b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
267	29-Jun-11	Sungai Siput, Perak	E	36	PP36c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
268	29-Jun-11	Sungai Siput, Perak	E	37	PP37a	nasal swab	Grower	Healthy	-	-	-	-	-
269	29-Jun-11	Sungai Siput, Perak	E	37	PP37b	tongue swab	Grower	Healthy	-	-	-	-	-
270	29-Jun-11	Sungai Siput, Perak	E	37	PP37c	rectal swab	Grower	Healthy	-	-	-	-	-
271	29-Jun-11	Sungai Siput, Perak	E	38	PP38a	nasal swab	Grower	Healthy	-	-	-	-	-
272	29-Jun-11	Sungai Siput, Perak	E	38	PP38b	tongue swab	Grower	Healthy	-	-	-	-	-
273	29-Jun-11	Sungai Siput, Perak	E	38	PP38c	rectal swab	Grower	Healthy	-	-	+	-	+
274	29-Jun-11	Sungai Siput, Perak	E	39	PP39a	nasal swab	Grower	Healthy	-	-	-	-	-
275	29-Jun-11	Sungai Siput, Perak	E	39	PP39b	tongue swab	Grower	Healthy	-	-	-	-	-
276	29-Jun-11	Sungai Siput, Perak	E	39	PP39c	rectal swab	Grower	Healthy	-	-	-	-	-
277	29-Jun-11	Sungai Siput, Perak	E	40	PP40a	nasal swab	Grower	Healthy	-	-	-	-	-
278	29-Jun-11	Sungai Siput, Perak	E	40	PP40b	tongue swab	Grower	Healthy	-	-	-	-	-

No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
279	29-Jun-11	Sungai Siput, Perak	E	40	PP40c	rectal swab	Grower	Healthy	-	-	-	-	-
280	30-Jun-11	Sungai Siput, Perak	F	41	PP41a	nasal swab	Weaner	Healthy	-	-	-	-	-
281	30-Jun-11	Sungai Siput, Perak	F	41	PP41b	tongue swab	Weaner	Healthy	-	-	-	-	-
282	30-Jun-11	Sungai Siput, Perak	F	41	PP41c	rectal swab	Weaner	Healthy	-	-	-	-	-
283	30-Jun-11	Sungai Siput, Perak	F	42	PP42a	nasal swab	Weaner	Healthy	-	-	-	-	-
284	30-Jun-11	Sungai Siput, Perak	F	42	PP42b	tongue swab	Weaner	Healthy	-	-	-	-	-
285	30-Jun-11	Sungai Siput, Perak	F	42	PP42c	rectal swab	Weaner	Healthy	-	-	-	-	-
286	30-Jun-11	Sungai Siput, Perak	F	43	PP43a	nasal swab	Weaner	Healthy	-	-	-	-	-
287	30-Jun-11	Sungai Siput, Perak	F	43	PP43b	tongue swab	Weaner	Healthy	-	-	+	+	+
288	30-Jun-11	Sungai Siput, Perak	F	43	PP43c	rectal swab	Weaner	Healthy	-	-	-	-	-
289	30-Jun-11	Sungai Siput, Perak	F	44	PP44a	nasal swab	Weaner	Healthy	-	-	-	-	-
290	30-Jun-11	Sungai Siput, Perak	F	44	PP44b	tongue swab	Weaner	Healthy	-	-	-	-	-
291	30-Jun-11	Sungai Siput, Perak	F	44	PP44c	rectal swab	Weaner	Healthy	-	-	-	-	-
292	30-Jun-11	Sungai Siput, Perak	F	45	PP45a	nasal swab	Weaner	Healthy	-	-	+	+	+
293	30-Jun-11	Sungai Siput, Perak	F	45	PP45b	tongue swab	Weaner	Healthy	-	-	-	-	-
294	30-Jun-11	Sungai Siput, Perak	F	45	PP45c	rectal swab	Weaner	Healthy	-	-	-	-	-
295	30-Jun-11	Sungai Siput, Perak	F	46	PP46a	nasal swab	Weaner	Healthy	-	-	+	+	+
296	30-Jun-11	Sungai Siput, Perak	F	46	PP46b	tongue swab	Weaner	Healthy	-	-	+	+	+
297	30-Jun-11	Sungai Siput, Perak	F	46	PP46c	rectal swab	Weaner	Healthy	-	-	-	-	-
298	30-Jun-11	Sungai Siput, Perak	F	47	PP47a	nasal swab	Weaner	Healthy	-	-	+	+	+
299	30-Jun-11	Sungai Siput, Perak	F	47	PP47b	tongue swab	Weaner	Healthy	-	-	+	+	+
300	30-Jun-11	Sungai Siput, Perak	F	47	PP47c	rectal swab	Weaner	Healthy	-	-	+	+	-
301	30-Jun-11	Sungai Siput, Perak	F	48	PP48a	nasal swab	Weaner	Healthy	-	-	+	-	+
302	30-Jun-11	Sungai Siput, Perak	F	48	PP48b	tongue swab	Weaner	Healthy	-	-	+	+	+
303	30-Jun-11	Sungai Siput, Perak	F	48	PP48c	rectal swab	Weaner	Healthy	-	-	-	-	-
304	30-Jun-11	Sungai Siput, Perak	F	49	PP49a	nasal swab	Weaner	Unhealthy	-	-	+	-	+
305	30-Jun-11	Sungai Siput, Perak	F	49	PP49b	tongue swab	Weaner	Unhealthy	-	-	+	+	+
306	30-Jun-11	Sungai Siput, Perak	F	49	PP49c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
307	30-Jun-11	Sungai Siput, Perak	F	50	PP50a	nasal swab	Weaner	Unhealthy	-	-	+	+	+
308	30-Jun-11	Sungai Siput, Perak	F	50	PP50b	tongue swab	Weaner	Unhealthy	-	-	+	+	+
309	30-Jun-11	Sungai Siput, Perak	F	50	PP50c	rectal swab	Weaner	Unhealthy	-	-	+	+	-
310	30-Jun-11	Sungai Siput, Perak	F	51	PP51a	nasal swab	Weaner	Unhealthy	-	-	+	+	+
311	30-Jun-11	Sungai Siput, Perak	F	51	PP51b	tongue swab	Weaner	Unhealthy	-	-	+	+	+
312	30-Jun-11	Sungai Siput, Perak	F	51	PP51c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
313	30-Jun-11	Sungai Siput, Perak	F	52	PP52a	nasal swab	Weaner	Unhealthy	-	-	+	+	+
314	30-Jun-11	Sungai Siput, Perak	F	52	PP52b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
315	30-Jun-11	Sungai Siput, Perak	F	52	PP52c	rectal swab	Weaner	Unhealthy	-	-	+	-	/
316	30-Jun-11	Sungai Siput, Perak	F	53	PP53a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
317	30-Jun-11	Sungai Siput, Perak	F	53	PP53b	tongue swab	Weaner	Unhealthy	-	-	+	+	+
318	30-Jun-11	Sungai Siput, Perak	F	53	PP53c	rectal swab	Weaner	Unhealthy	-	-	+	+	+

No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
319	30-Jun-11	Sungai Siput, Perak	F	54	PP54a	nasal swab	Weaner	Unhealthy	-	-	+	+	+
320	30-Jun-11	Sungai Siput, Perak	F	54	PP54b	tongue swab	Weaner	Unhealthy	-	-	+	+	+
321	30-Jun-11	Sungai Siput, Perak	F	54	PP54c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
322	30-Jun-11	Sungai Siput, Perak	F	55	PP55a	nasal swab	Weaner	Unhealthy	-	-	+	-	+
323	30-Jun-11	Sungai Siput, Perak	F	55	PP55b	tongue swab	Weaner	Unhealthy	-	-	+	-	+
324	30-Jun-11	Sungai Siput, Perak	F	55	PP55c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
325	30-Jun-11	Sungai Siput, Perak	F	56	PP56a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
326	30-Jun-11	Sungai Siput, Perak	F	56	PP56b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
327	30-Jun-11	Sungai Siput, Perak	F	56	PP56c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
328	30-Jun-11	Sungai Siput, Perak	F	57	PP57a	nasal swab	Sow	Healthy	-	-	-	-	-
329	30-Jun-11	Sungai Siput, Perak	F	57	PP57b	tongue swab	Sow	Healthy	-	-	-	-	-
330	30-Jun-11	Sungai Siput, Perak	F	57	PP57c	rectal swab	Sow	Healthy	-	-	-	-	-
331	30-Jun-11	Sungai Siput, Perak	F	58	PP58a	nasal swab	Sow	Healthy	-	-	-	-	-
332	30-Jun-11	Sungai Siput, Perak	F	58	PP58b	tongue swab	Sow	Healthy	-	-	-	-	-
333	30-Jun-11	Sungai Siput, Perak	F	58	PP58c	rectal swab	Sow	Healthy	-	-	-	-	-
334	30-Jun-11	Sungai Siput, Perak	F	59	PP59a	nasal swab	Finisher	Healthy	-	-	+	-	+
335	30-Jun-11	Sungai Siput, Perak	F	59	PP59b	tongue swab	Finisher	Healthy	-	-	+	-	+
336	30-Jun-11	Sungai Siput, Perak	F	59	PP59c	rectal swab	Finisher	Healthy	-	-	-	-	-
337	30-Jun-11	Sungai Siput, Perak	F	60	PP60a	nasal swab	Finisher	Healthy	-	-	+	+	+
338	30-Jun-11	Sungai Siput, Perak	F	60	PP60b	tongue swab	Finisher	Healthy	-	-	+	-	+
339	30-Jun-11	Sungai Siput, Perak	F	60	PP60c	rectal swab	Finisher	Healthy	-	-	+	-	+
340	24-Sep-11	Sungai Jawi, Penang	G	1	PPN1a	nasal swab	Weaner	Healthy	-	-	-	-	-
341	24-Sep-11	Sungai Jawi, Penang	G	1	PPN1b	tongue swab	Weaner	Healthy	-	-	-	-	-
342	24-Sep-11	Sungai Jawi, Penang	G	1	PPN1c	rectal swab	Weaner	Healthy	-	-	-	-	-
343	24-Sep-11	Sungai Jawi, Penang	G	2	PPN2a	nasal swab	Weaner	Healthy	-	-	-	-	-
344	24-Sep-11	Sungai Jawi, Penang	G	2	PPN2b	tongue swab	Weaner	Healthy	-	-	-	-	-
345	24-Sep-11	Sungai Jawi, Penang	G	2	PPN2c	rectal swab	Weaner	Healthy	-	-	-	-	-
346	24-Sep-11	Sungai Jawi, Penang	G	3	PPN3a	nasal swab	Grower	Healthy	-	-	-	-	-
347	24-Sep-11	Sungai Jawi, Penang	G	3	PPN3b	tongue swab	Grower	Healthy	-	-	-	-	-
348	24-Sep-11	Sungai Jawi, Penang	G	3	PPN3c	rectal swab	Grower	Healthy	-	-	-	-	-
349	24-Sep-11	Sungai Jawi, Penang	G	4	PPN4a	nasal swab	Grower	Healthy	-	-	-	-	-
350	24-Sep-11	Sungai Jawi, Penang	G	4	PPN4b	tongue swab	Grower	Healthy	-	-	-	-	-
351	24-Sep-11	Sungai Jawi, Penang	G	4	PPN4c	rectal swab	Grower	Healthy	-	-	-	-	-
352	24-Sep-11	Sungai Jawi, Penang	G	5	PPN5a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
353	24-Sep-11	Sungai Jawi, Penang	G	5	PPN5b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
354	24-Sep-11	Sungai Jawi, Penang	G	5	PPN5c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
355	24-Sep-11	Sungai Jawi, Penang	G	6	PPN6a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
356	24-Sep-11	Sungai Jawi, Penang	G	6	PPN6b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
357	24-Sep-11	Sungai Jawi, Penang	G	6	PPN6c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
358	24-Sep-11	Sungai Jawi, Penang	G	7	PPN7a	nasal swab	Weaner	Healthy	-	-	-	-	-

No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
359	24-Sep-11	Sungai Jawi, Penang	G	7	PPN7b	tongue swab	Weaner	Healthy	-	-	-	-	-
360	24-Sep-11	Sungai Jawi, Penang	G	7	PPN7c	rectal swab	Weaner	Healthy	-	-	-	-	-
361	24-Sep-11	Sungai Jawi, Penang	G	8	PPN8a	nasal swab	Weaner	Healthy	-	-	-	-	-
362	24-Sep-11	Sungai Jawi, Penang	G	8	PPN8b	tongue swab	Weaner	Healthy	-	-	-	-	-
363	24-Sep-11	Sungai Jawi, Penang	G	8	PPN8c	rectal swab	Weaner	Healthy	-	-	-	-	-
364	24-Sep-11	Sungai Jawi, Penang	G	9	PPN9a	nasal swab	Weaner	Healthy	-	-	-	-	-
365	24-Sep-11	Sungai Jawi, Penang	G	9	PPN9b	tongue swab	Weaner	Healthy	-	-	-	-	-
366	24-Sep-11	Sungai Jawi, Penang	G	9	PPN9c	rectal swab	Weaner	Healthy	-	-	-	-	-
367	24-Sep-11	Sungai Jawi, Penang	G	10	PPN10a	nasal swab	Weaner	Healthy	-	-	-	-	-
368	24-Sep-11	Sungai Jawi, Penang	G	10	PPN10b	tongue swab	Weaner	Healthy	-	-	+	+	-
369	24-Sep-11	Sungai Jawi, Penang	G	10	PPN10c	rectal swab	Weaner	Healthy	-	-	-	-	-
370	24-Sep-11	Sungai Jawi, Penang	G	11	PPN11a	nasal swab	Weaner	Healthy	-	-	-	-	-
371	24-Sep-11	Sungai Jawi, Penang	G	11	PPN11b	tongue swab	Weaner	Healthy	-	-	-	-	-
372	24-Sep-11	Sungai Jawi, Penang	G	11	PPN11c	rectal swab	Weaner	Healthy	-	-	-	-	-
373	24-Sep-11	Sungai Jawi, Penang	G	12	PPN12a	nasal swab	Weaner	Healthy	-	-	-	-	-
374	24-Sep-11	Sungai Jawi, Penang	G	12	PPN12b	tongue swab	Weaner	Healthy	-	-	-	-	-
375	24-Sep-11	Sungai Jawi, Penang	G	12	PPN12c	rectal swab	Weaner	Healthy	-	-	-	-	-
376	24-Sep-11	Sungai Jawi, Penang	G	13	PPN13a	nasal swab	Weaner	Healthy	-	-	-	-	-
377	24-Sep-11	Sungai Jawi, Penang	G	13	PPN13b	tongue swab	Weaner	Healthy	-	-	-	-	-
378	24-Sep-11	Sungai Jawi, Penang	G	13	PPN13c	rectal swab	Weaner	Healthy	-	-	-	-	-
379	24-Sep-11	Sungai Jawi, Penang	G	14	PPN14a	nasal swab	Weaner	Healthy	-	-	-	-	-
380	24-Sep-11	Sungai Jawi, Penang	G	14	PPN14b	tongue swab	Weaner	Healthy	-	-	-	-	-
381	24-Sep-11	Sungai Jawi, Penang	G	14	PPN14c	rectal swab	Weaner	Healthy	-	-	-	-	-
382	24-Sep-11	Sungai Jawi, Penang	G	15	PPN15a	nasal swab	Grower	Healthy	-	-	-	-	-
383	24-Sep-11	Sungai Jawi, Penang	G	15	PPN15b	tongue swab	Grower	Healthy	-	-	-	-	-
384	24-Sep-11	Sungai Jawi, Penang	G	15	PPN15c	rectal swab	Grower	Healthy	-	-	-	-	-
385	24-Sep-11	Sungai Jawi, Penang	G	16	PPN16a	nasal swab	Grower	Healthy	-	-	-	-	-
386	24-Sep-11	Sungai Jawi, Penang	G	16	PPN16b	tongue swab	Grower	Healthy	-	-	-	-	-
387	24-Sep-11	Sungai Jawi, Penang	G	16	PPN16c	rectal swab	Grower	Healthy	-	-	-	-	-
388	24-Sep-11	Kg. Selamat, Penang	H	21	PPN21a	nasal swab	Weaner	Healthy	-	-	-	-	-
389	24-Sep-11	Kg. Selamat, Penang	H	21	PPN21b	tongue swab	Weaner	Healthy	-	-	-	-	-
390	24-Sep-11	Kg. Selamat, Penang	H	21	PPN21c	rectal swab	Weaner	Healthy	-	-	-	-	-
391	24-Sep-11	Kg. Selamat, Penang	H	22	PPN22a	nasal swab	Weaner	Healthy	-	-	-	-	-
392	24-Sep-11	Kg. Selamat, Penang	H	22	PPN22b	tongue swab	Weaner	Healthy	-	-	-	-	-
393	24-Sep-11	Kg. Selamat, Penang	H	22	PPN22c	rectal swab	Weaner	Healthy	-	-	-	-	-
394	24-Sep-11	Kg. Selamat, Penang	H	23	PPN23a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
395	24-Sep-11	Kg. Selamat, Penang	H	23	PPN23b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
396	24-Sep-11	Kg. Selamat, Penang	H	23	PPN23c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
397	24-Sep-11	Kg. Selamat, Penang	H	24	PPN24a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
398	24-Sep-11	Kg. Selamat, Penang	H	24	PPN24b	tongue swab	Weaner	Unhealthy	-	-	-	-	-

No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
399	24-Sep-11	Kg. Selamat, Penang	H	24	PPN24c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
400	24-Sep-11	Kg. Selamat, Penang	H	25	PPN25a	nasal swab	Weaner	Healthy	-	-	-	-	-
401	24-Sep-11	Kg. Selamat, Penang	H	25	PPN25b	tongue swab	Weaner	Healthy	-	-	-	-	-
402	24-Sep-11	Kg. Selamat, Penang	H	25	PPN25c	rectal swab	Weaner	Healthy	-	-	-	-	-
403	24-Sep-11	Kg. Selamat, Penang	H	26	PPN26a	nasal swab	Weaner	Healthy	-	-	-	-	-
404	24-Sep-11	Kg. Selamat, Penang	H	26	PPN26b	tongue swab	Weaner	Healthy	-	-	-	-	-
405	24-Sep-11	Kg. Selamat, Penang	H	26	PPN26c	rectal swab	Weaner	Healthy	-	-	-	-	-
406	24-Sep-11	Kg. Selamat, Penang	H	27	PPN27a	nasal swab	Weaner	Healthy	-	-	-	-	-
407	24-Sep-11	Kg. Selamat, Penang	H	27	PPN27b	tongue swab	Weaner	Healthy	-	-	-	-	-
408	24-Sep-11	Kg. Selamat, Penang	H	27	PPN27c	rectal swab	Weaner	Healthy	-	-	-	-	-
409	24-Sep-11	Kg. Selamat, Penang	H	28	PPN28a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
410	24-Sep-11	Kg. Selamat, Penang	H	28	PPN28b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
411	24-Sep-11	Kg. Selamat, Penang	H	28	PPN28c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
412	24-Sep-11	Kg. Selamat, Penang	H	29	PPN29a	nasal swab	Weaner	Healthy	-	-	+	+	-
413	24-Sep-11	Kg. Selamat, Penang	H	29	PPN29b	tongue swab	Weaner	Healthy	-	-	+	+	-
414	24-Sep-11	Kg. Selamat, Penang	H	29	PPN29c	rectal swab	Weaner	Healthy	-	-	+	+	-
415	24-Sep-11	Kg. Selamat, Penang	H	30	PPN30a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
416	24-Sep-11	Kg. Selamat, Penang	H	30	PPN30b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
417	24-Sep-11	Kg. Selamat, Penang	H	30	PPN30c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
418	24-Sep-11	Kg. Selamat, Penang	H	31	PPN31a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
419	24-Sep-11	Kg. Selamat, Penang	H	31	PPN31b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
420	24-Sep-11	Kg. Selamat, Penang	H	31	PPN31c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
421	24-Sep-11	Kg. Selamat, Penang	H	32	PPN32a	nasal swab	Weaner	Healthy	-	-	-	-	-
422	24-Sep-11	Kg. Selamat, Penang	H	32	PPN32b	tongue swab	Weaner	Healthy	-	-	-	-	-
423	24-Sep-11	Kg. Selamat, Penang	H	32	PPN32c	rectal swab	Weaner	Healthy	-	-	-	-	-
424	24-Sep-11	Kg. Selamat, Penang	H	33	PPN33a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
425	24-Sep-11	Kg. Selamat, Penang	H	33	PPN33b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
426	24-Sep-11	Kg. Selamat, Penang	H	33	PPN33c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
427	24-Sep-11	Kg. Selamat, Penang	H	34	PPN34a	nasal swab	Weaner	Healthy	-	-	-	-	-
428	24-Sep-11	Kg. Selamat, Penang	H	34	PPN34b	tongue swab	Weaner	Healthy	-	-	-	-	-
429	24-Sep-11	Kg. Selamat, Penang	H	34	PPN34c	rectal swab	Weaner	Healthy	-	-	-	-	-
430	24-Sep-11	Kg. Selamat, Penang	H	35	PPN35a	nasal swab	Weaner	Healthy	-	-	-	-	-
431	24-Sep-11	Kg. Selamat, Penang	H	35	PPN35b	tongue swab	Weaner	Healthy	-	-	-	-	-
432	24-Sep-11	Kg. Selamat, Penang	H	35	PPN35c	rectal swab	Weaner	Healthy	-	-	-	-	-
433	24-Sep-11	Kg. Selamat, Penang	H	36	PPN36a	nasal swab	Weaner	Healthy	-	-	-	-	-
434	24-Sep-11	Kg. Selamat, Penang	H	36	PPN36b	tongue swab	Weaner	Healthy	-	-	-	-	-
435	24-Sep-11	Kg. Selamat, Penang	H	36	PPN36c	rectal swab	Weaner	Healthy	-	-	-	-	-
436	24-Sep-11	Kg. Selamat, Penang	H	37	PPN37a	nasal swab	Grower	Healthy	-	-	+	+	-
437	24-Sep-11	Kg. Selamat, Penang	H	37	PPN37b	tongue swab	Grower	Healthy	-	-	-	-	-
438	24-Sep-11	Kg. Selamat, Penang	H	37	PPN37c	rectal swab	Grower	Healthy	-	-	-	-	-

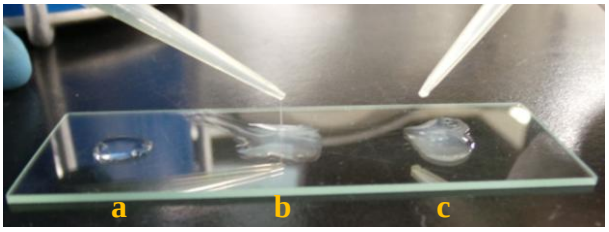
No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
439	24-Sep-11	Kg. Selamat, Penang	H	38	PPN38a	nasal swab	Grower	Healthy	-	-	+	+	-
440	24-Sep-11	Kg. Selamat, Penang	H	38	PPN38b	tongue swab	Grower	Healthy	-	-	-	-	-
441	24-Sep-11	Kg. Selamat, Penang	H	38	PPN38c	rectal swab	Grower	Healthy	-	-	-	-	-
442	24-Sep-11	Kg. Selamat, Penang	H	39	PPN39a	nasal swab	Grower	Healthy	-	-	+	+	-
443	24-Sep-11	Kg. Selamat, Penang	H	39	PPN39b	tongue swab	Grower	Healthy	-	-	+	+	-
444	24-Sep-11	Kg. Selamat, Penang	H	39	PPN39c	rectal swab	Grower	Healthy	-	-	-	-	-
445	24-Sep-11	Kg. Selamat, Penang	H	40	PPN40a	nasal swab	Grower	Healthy	-	-	+	+	-
446	24-Sep-11	Kg. Selamat, Penang	H	40	PPN40b	tongue swab	Grower	Healthy	-	-	+	+	-
447	24-Sep-11	Kg. Selamat, Penang	H	40	PPN40c	rectal swab	Grower	Healthy	-	-	+	+	-
448	24-Sep-11	Kg. Selamat, Penang	I	41	PPN41a	nasal swab	Weaner	Healthy	-	-	-	-	-
449	24-Sep-11	Kg. Selamat, Penang	I	41	PPN41b	tongue swab	Weaner	Healthy	-	-	-	-	-
450	24-Sep-11	Kg. Selamat, Penang	I	41	PPN41c	rectal swab	Weaner	Healthy	-	-	-	-	-
451	24-Sep-11	Kg. Selamat, Penang	I	42	PPN42a	nasal swab	Weaner	Healthy	-	-	-	-	-
452	24-Sep-11	Kg. Selamat, Penang	I	42	PPN42b	tongue swab	Weaner	Healthy	-	-	+	+	-
453	24-Sep-11	Kg. Selamat, Penang	I	42	PPN42c	rectal swab	Weaner	Healthy	-	-	-	-	-
454	24-Sep-11	Kg. Selamat, Penang	I	43	PPN43a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
455	24-Sep-11	Kg. Selamat, Penang	I	43	PPN43b	tongue swab	Weaner	Unhealthy	-	-	+	+	-
456	24-Sep-11	Kg. Selamat, Penang	I	43	PPN43c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
457	24-Sep-11	Kg. Selamat, Penang	I	44	PPN44a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
458	24-Sep-11	Kg. Selamat, Penang	I	44	PPN44b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
459	24-Sep-11	Kg. Selamat, Penang	I	44	PPN44c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
460	24-Sep-11	Kg. Selamat, Penang	I	45	PPN45a	nasal swab	Weaner	Unhealthy	-	-	-	-	-
461	24-Sep-11	Kg. Selamat, Penang	I	45	PPN45b	tongue swab	Weaner	Unhealthy	-	-	-	-	-
462	24-Sep-11	Kg. Selamat, Penang	I	45	PPN45c	rectal swab	Weaner	Unhealthy	-	-	-	-	-
463	24-Sep-11	Kg. Selamat, Penang	I	46	PPN46a	nasal swab	Weaner	Healthy	-	-	-	-	-
464	24-Sep-11	Kg. Selamat, Penang	I	46	PPN46b	tongue swab	Weaner	Healthy	-	-	-	-	-
465	24-Sep-11	Kg. Selamat, Penang	I	46	PPN46c	rectal swab	Weaner	Healthy	-	-	-	-	-
466	24-Sep-11	Kg. Selamat, Penang	I	47	PPN47a	nasal swab	Weaner	Healthy	-	-	+	+	-
467	24-Sep-11	Kg. Selamat, Penang	I	47	PPN47b	tongue swab	Weaner	Healthy	-	-	-	-	-
468	24-Sep-11	Kg. Selamat, Penang	I	47	PPN47c	rectal swab	Weaner	Healthy	-	-	-	-	-
469	24-Sep-11	Kg. Selamat, Penang	I	48	PPN48a	nasal swab	Weaner	Healthy	-	-	-	-	-
470	24-Sep-11	Kg. Selamat, Penang	I	48	PPN48b	tongue swab	Weaner	Healthy	-	-	-	-	-
471	24-Sep-11	Kg. Selamat, Penang	I	48	PPN48c	rectal swab	Weaner	Healthy	-	-	+	+	-
472	24-Sep-11	Kg. Selamat, Penang	I	49	PPN49a	nasal swab	Grower	Unhealthy	-	-	-	-	-
473	24-Sep-11	Kg. Selamat, Penang	I	49	PPN49b	tongue swab	Grower	Unhealthy	-	-	-	-	-
474	24-Sep-11	Kg. Selamat, Penang	I	49	PPN49c	rectal swab	Grower	Unhealthy	-	-	-	-	-
475	24-Sep-11	Kg. Selamat, Penang	I	50	PPN50a	nasal swab	Grower	healthy	-	-	-	-	-
476	24-Sep-11	Kg. Selamat, Penang	I	50	PPN50b	tongue swab	Grower	Healthy	-	-	-	-	-
477	24-Sep-11	Kg. Selamat, Penang	I	50	PPN50c	rectal swab	Grower	Healthy	-	-	+	+	-
478	24-Sep-11	Kg. Selamat, Penang	I	51	PPN51a	nasal swab	Grower	Healthy	-	-	-	-	-

No.	Sampling date	Location	Farm	Animal No.	Sample code	Sample type	Year old	Health condition	Biochemical tests	PCR confirmation	Post enrichment PCR detection		
											Overall	ITC	PBS
479	24-Sep-11	Kg. Selamat, Penang	I	51	PPN51b	tongue swab	Grower	Healthy	-	-	-	-	-
480	24-Sep-11	Kg. Selamat, Penang	I	51	PPN51c	rectal swab	Grower	Healthy	-	-	+	+	-
481	24-Sep-11	Kg. Selamat, Penang	I	52	PPN52a	nasal swab	Grower	Healthy	-	-	+	+	-
482	24-Sep-11	Kg. Selamat, Penang	I	52	PPN52b	tongue swab	Grower	Healthy	-	-	-	-	-
483	24-Sep-11	Kg. Selamat, Penang	I	52	PPN52c	rectal swab	Grower	Healthy	-	-	-	-	-
484	24-Sep-11	Kg. Selamat, Penang	I	53	PPN53a	nasal swab	Grower	Healthy	+	+	+	+	+
485	24-Sep-11	Kg. Selamat, Penang	I	53	PPN53b	tongue swab	Grower	Healthy	-	-	-	-	-
486	24-Sep-11	Kg. Selamat, Penang	I	53	PPN53c	rectal swab	Grower	Healthy	-	-	-	-	-
487	24-Sep-11	Kg. Selamat, Penang	I	54	PPN54a	nasal swab	Grower	Healthy	-	-	-	-	-
488	24-Sep-11	Kg. Selamat, Penang	I	54	PPN54b	tongue swab	Grower	Healthy	-	-	-	-	-
489	24-Sep-11	Kg. Selamat, Penang	I	54	PPN54c	rectal swab	Grower	Healthy	-	-	+	+	-
490	24-Sep-11	Kg. Selamat, Penang	I	55	PPN55a	nasal swab	Grower	Healthy	+	+	+	+	+
491	24-Sep-11	Kg. Selamat, Penang	I	55	PPN55b	tongue swab	Grower	Healthy	+	+	+	+	+
492	24-Sep-11	Kg. Selamat, Penang	I	55	PPN55c	rectal swab	Grower	Healthy	+	+	+	+	+
493	24-Sep-11	Kg. Selamat, Penang	I	56	PPN56a	nasal swab	Grower	Healthy	+	+	+	+	+
494	24-Sep-11	Kg. Selamat, Penang	I	56	PPN56b	tongue swab	Grower	Healthy	+	+	+	+	+
495	24-Sep-11	Kg. Selamat, Penang	I	56	PPN56c	rectal swab	Grower	Healthy	+	+	+	+	+

APPENDIX VII PRELIMINARY BIOCHEMICAL TESTS

1. Gram determination by using KOH string test (3% KOH solution)

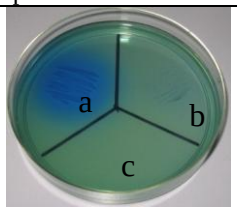
Fresh bacteria culture was picked with sterile pipette tip and suspended in 20 µl 3% KOH. Pipette tip was lifted after 60 s.



Interpretation:
a, uninoculated
b, Gram negative (bacteria string, *Y. enterocolitica* ATCC 9610)
c, Gram positive (no bacteria string, *Listeria monocytogenes* ATCC 7644)

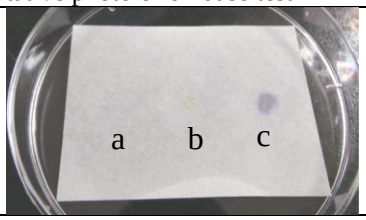
2. Citrate test (Simmon's citrate agar)

A fresh single well-isolated colony was picked from NA and was streaked on the surface of the slant. The tube was incubated at 37±2℃ for 18-24 hours. Colour changes was observed

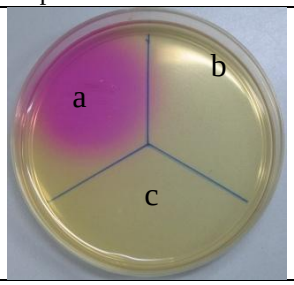
Representative photo of citrate test	Interpretation
	a, citrate positive (agar turned blue, *C. freundii*) **b, citrate negative (agar unchanged, *Y. enterocolitica* ATCC 9610)** c, uninoculated.

3. Oxidase test (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine solution)

A fresh single well-isolated colony was picked from NA by a sterile toothpick and touched onto a piece of filter paper that contained oxidase reagent. Colour changed to purple within 10 sec indicated positive; 10-60 sec indicated delayed positive; and more than 60 sec considered as negative.

Representative photo of oxidase test	Interpretation
	a, uninoculated **b, oxidase negative bacterium (colour unchanged, *Y. enterocolitica* ATCC 9610)** c, oxidase positive bacterium (colony turned blue, *Vibrio cholera*)

4. Urease test

Representative photo of urease test	Interpretation
	a, urease positive (agar turned pink, *Y. enterocolitica* ATCC 9610) b, urease negative (agar unchanged, *E. coli* ATCC 25922) c, uninoculated

APPENDIX VIII API 20E, DUPLEX PCR AND API50CH

1. API 20E identification kit

Fresh culture (18-24 hours) was diluted using sterile 0.85% NaCl solution and 0.5 McFarland was achieved. The cell culture was inoculated into wells of API 20E strip according to the instruction shown in API 20E manual and incubated at 37±2℃ and result was taken within 18-24 hours. After incubation of 37±2℃, the results on the strip were referred to interpretation table for the identification. There were reagents needed to be added before the results were recorded. For examples, TDA test: one drop of TDA reagent was added. Dark brown colour showed positive result; IND test: one drop of JAMES reagent was added. Pink colour indicated positive result; VP test: one drop of α-napthtol was added and followed by one drop of 40% KOH solution. Pink or red colour formation after 10 min showed positive result. Identification software was used and results were coded into the Numerical profile before the database process by computer.

2. Summary of biochemical reactions of *Y. enterocolitica* on API 20E identification kit.

Tests	**Results**		
	Positive	**Negative**	***Y. enterocolitica***
Ortho nitrophenyl-β-galactosidase (ONPG)	Yellow	Colourless	+
Arginine dihydrolase (ADH)	Red – orange	Yellow	-
Lysine decarboxylase (LDC)	Red – orange	Yellow	-
Ornithine decarboxylase (ODC)	Red – orange	Yellow	+/-
Citrate utilization (CIT)	Blue	Light green	-
H_2S production (H_2S)	Black	Colourless	-
Urease production (URE)	Red – orange	Yellow	+
Tryptophane deaminase (TDA)	Brown – red	Yellow	-
Indole production (IND)	Red	Yellow	+/-
Voges-Proskauer (VP)	Light red – red	Colourless	+/-
Gelatinase production (GEL)	Black		-
D-Glucose (GLU)	Yellow	Blue – green	+
D-Mannitol (MAN)	Yellow	Blue – green	+
Inositol (INO)	Yellow	Blue – green	+/-
D-Sorbitol (SOR)	Yellow	Blue – green	+
L-Rhamnose (RHA)	Yellow	Blue – green	-
D-Sacharose (SAC)	Yellow	Blue – green	+
D-Melibiose (MEL)	Yellow	Blue – green	+/-
Amygdalin (AMY)	Yellow	Blue – green	+/-
L-Arabinose (ARA)	Yellow	Blue – green	+
Oxidase (OX)	Violet – blue	Colourless	-
NO_2 production/reduction to N_2 gas ($NO_3 – NO_2$)	Red	Yellow	+

+, positive; -, negative.

3. Raw Data for API 20E, Duplex PCR and API50CH of Y. enterocolitica

No.	Isolates Name	API 20E																							PCR		API 50CH (ID, %)
		code	%	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	YE[a] 16S rRNA	*ail* gene	
1	PCM-PPN53a-1	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
2	PCM-PPN53a-2	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
3	PCM-PPN53a-3	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
4	PCM-PPN53a-4	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
5	PCM-PPN53a-K1	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
6	PCM-PPN53a-K2	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
7	PCM-PPN53a-K3	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
8	PCM-PPN53a-K4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
9	DCM-PPN55a-1	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
10	DCM-PPN55a-2	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
11	DCM-PPN55a-3	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
12	DCM-PPN55a-4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
13	PCM-PPN55a-1	1104523	95.7%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
14	PCM-PPN55a-2	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
15	PCM-PPN55a-3	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
16	PCM-PPN55a-4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
17	PCM-PPN55a-K1	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
18	PCM-PPN55a-K2	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
19	PCM-PPN55a-K3	1014523	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
20	PCM-PPN55a-K4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
21	PCM-PPN55b-1	1114522	99.9%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
22	PCM-PPN55b-2	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
23	PCM-PPN55b-3	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
24	PCM-PPN55b-4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
25	PCM-PPN55b-K1	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
26	PCM-PPN55b-K2	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
27	PCM-PPN55b-K3	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
28	PCM-PPN55b-K4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
29	PCM-PPN55c-1	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
30	PCM-PPN55c-2	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
31	PCM-PPN55c-3	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
32	PCM-PPN55c-4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
33	PCM-PPN55c-K1	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
34	PCM-PPN55c-K2	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
35	PCM-PPN55c-K3	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
36	PCM-PPN55c-K4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
37	PCM-PPN56a-1	1014523	96.4%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-

No.	Isolates Name	API 20E																							PCR		API 50CH (ID, %)
		code	%	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	YE[a] 16S rRNA	*ail* gene	
38	PCM-PPN56a-2	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
39	PCM-PPN56a-3	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
40	PCM-PPN56a-4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
41	PCM-PPN56a-K1	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
42	PCM-PPN56a-K2	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
43	PCM-PPN56a-K3	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
44	PCM-PPN56a-K4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
45	DCM-PPN56b-21	1114522	99.9%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
46	DCM-PPN56b-22	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
47	DCM-PPN56b-23	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
48	DCM-PPN56b-24	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
49	PCM-PPN56b-1	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
50	PCM-PPN56b-2	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
51	PCM-PPN56b-3	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
52	PCM-PPN56b-4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
53	PCM-PPN56b-K1	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
54	PCM-PPN56b-K2	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
55	PCM-PPN56b-K3	1004522	94.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
56	PCM-PPN56b-K4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
57	DCM-PPN56c-21	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
58	DCM-PPN56c-22	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
59	DCM-PPN56c-23	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
60	DCM-PPN56c-24	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
61	PCM-PPN56c-1	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
62	PCM-PPN56c-2	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
63	PCM-PPN56c-3	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
64	PCM-PPN56c-4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
65	PCM-PPN56c-5	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
66	PCM-PPN56c-6	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
67	PCM-PPN56c-7	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
68	PCM-PPN56c-8	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
69	PCM-PPN56c-K1	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
70	PCM-PPN56c-K2	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
71	PCM-PPN56c-K3	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
72	PCM-PPN56c-K4	1114522	99.9%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
73	PC-M1-K1	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
74	PC-M1-K2	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
75	PC-M1-K3	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-

No.	Isolates Name	API 20E																						PCR		API 50CH (ID, %)
		code	%	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	YE[a] 16S rRNA	*ail* gene	
76	PC-M1-K4	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
77	PC-M1-K5	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
78	PC-M1-K11	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
79	PC-M1-K12	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
80	PC-M1-K13	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
81	PC-M3-6	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
82	PC-M3-K11	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
83	PC-M3-K12	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
84	PC-M13-K13	1014522	93.9%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	+	+	-
85	S18/1-C-O-6a	1014523	99.7%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
86	S18/1-C-I-10-4-6a	1014523	99.7%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
87	S18/1-C-O-6b	1014523	99.7%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
88	S18/1-C-O-5-6b	1014523	99.7%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
89	S18/1-C-I-4-6b	1154523	92.3%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
90	S18/1-C-O-6c	1014523	99.7%	+	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
91	S18/1-C-O-6d	1154523	92.3%	+	-	-	+	-	-	+	-	+	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
92	S18/1-C-O-5-6e	1114523	99.7%	+	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	+	-
93	S18/1-C-O-1a	1154723	92.5%	+	-	-	+	-	-	+	-	+	-	-	+	+	+	+	-	+	-	+	+	-	+	-	-
94	S18/1-C-O-K-5b	1154523	92.3%	+	-	-	+	-	-	+	-	+	-	-	+	+	-	+	-	+	-	+	+	-	+	-	-
95	S18/1-C-O-5c	1155523	98.3%	+	-	-	+	-	-	+	-	+	+	-	+	+	-	+	-	+	-	+	+	-	+	-	-
96	PC-M16-2	1155723	98.3%	+	-	-	+	-	-	+	-	+	+	-	+	+	+	+	-	+	-	+	+	-	+	-	-
97	PC-M16-5	1155723	98.3%	+	-	-	+	-	-	+	-	+	+	-	+	+	+	+	-	+	-	+	+	-	+	-	-
98	PC-M16-10	1154723	92.5%	+	-	-	+	-	-	+	-	+	-	-	+	+	+	+	-	+	-	+	+	-	+	-	-
99	YE015f-CPK	1155563	99.4%	+	-	-	+	-	-	+	-	+	+	-	+	+	-	+	-	+	+	+	+	-	-	-	*Y. intermedia*, 99.9%
100	YE015c-CPK	1155563	99.4%	+	-	-	+	-	-	+	-	+	+	-	+	+	-	+	-	+	+	+	+	-	-	-	*Y. intermedia*, 99.9%
101	PC-M5-K11	1155563	99.4%	+	-	-	+	-	-	+	-	+	+	-	+	+	-	+	-	+	+	+	+	-	-	-	*Y. intermedia*, 99.9%
102	PC-M5-K12	1155563	99.4%	+	-	-	+	-	-	+	-	+	+	-	+	+	-	+	-	+	+	+	+	-	-	-	*Y. intermedia*, 99.9%
105	PC-M16-4	1155723	98.3%	+	-	-	+	-	-	+	-	+	+	-	+	+	+	+	-	+	-	+	+	-	-	-	*Y. frederiksenii*, 55.3%
106	PC-M16-6	1155723	98.3%	+	-	-	+	-	-	+	-	+	+	-	+	+	+	+	-	+	-	+	+	-	-	-	*Y. frederiksenii*, 55.3%
107	PC-M20-3	1155563	99.4%	+	-	-	+	-	-	+	-	+	+	-	+	+	-	+	-	+	+	+	+	-	-	-	*Y. intermedia*, 99.9%
																											-
SUMMARY RESULTS				+	-	-	+/-	-	-	+	-	+/-	+/-	-	+	+	+/-	+	-	+	+/-	+/-	+	-	+	+/-	-

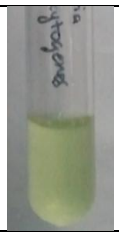
[a]YE, *Y. enterocolitica*

APPENDIX IX BIOROUPING AND SEROTYPING

(A) Biochemical tests of biogrouping

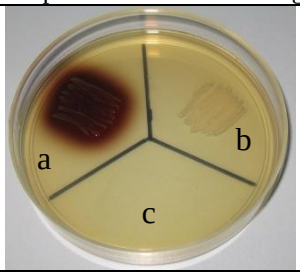
1. β- D-Glucosidase test

Fresh culture (18-24 hours) was diluted using sterile 0.85% NaCl solution and 3 McFarland was achieved. 0.75 ml culture suspension was added into 0.25 ml of β-D-Glucosidase test medium and incubated at 30 ℃ for 16-20 h.

Representative photo of β- D-Glucosidase test			
Representing bacterium	Uninoculated	*Y. enterocolitica* ATCC 9610	*L. monocytogenes* ATCC 7644
Interpretation	Uninoculated	Negative (colourless)	Positive (yellow)

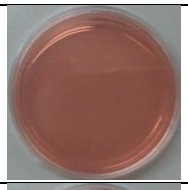
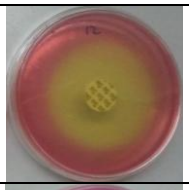
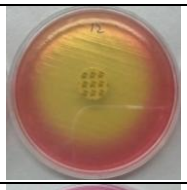
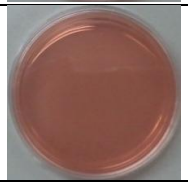
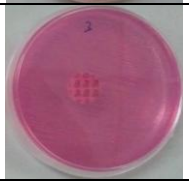
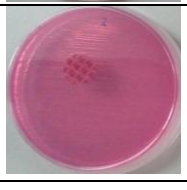
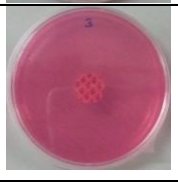
2. Esculine test

Fresh culture (18-24 hours) was streaked onto bile esculine agar. Plates were incubated at 25-28 ℃ for 24 h.

Representative photo of bile esculine agar	Interpretation
	a, positive (brown diffused pigment), *Enterococcus faecalis* ATCC 29212 b, negative (no pigment), *Y. enterocolitica* ATCC 9610 c, uninoculated

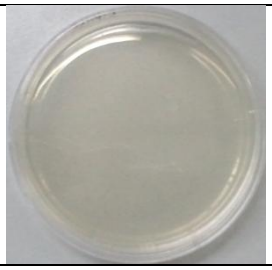
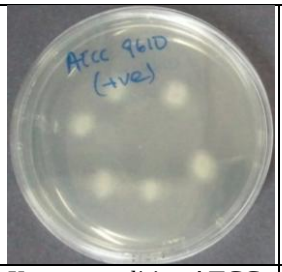
3. Salicin, xylose, trehalose test

Fresh culture (18-24 hours) was diluted using sterile 0.85% NaCl solution and 0.5 McFarland was achieved. Culture was lawned onto phenol red agar and the carbohydrate discs were dispensed onto the surface of the inoculated agar. Plates were incubated at 25-28 ℃ for 24 h.

Interpretation	Phenol red agar (uninoculated)	Phenol red agar with salicin disc	Phenol red agar with xylose disc	Phenol red agar with trehalose disc
Positive				
Negative				

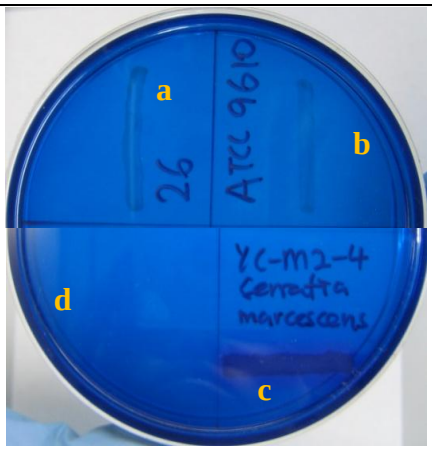
4. Lipase (Tween-esterase) test

Fresh culture (18-24 hours) was streaked onto lipase test agar. Plates were incubated at 25-28 ℃ and the plates were examined after 2 and 5 days.

Representative photo of lipase test agar			
Representing bacterium	Uninoculated	*Y. enterocolitica* ATCC 9610	*Escherichia coli* ATCC 25922
Interpretation	Uninoculated	Positive (opaque halo surrounding streaking)	Negative (no opaque halo observed)

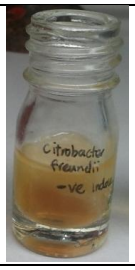
5. DNAse test

Fresh culture (18-24 hours) was streaked onto DNAse test agar. Plates were incubated at 25-28 ℃ for 24 h.

Representative photo of DNase test agar with Toluidine blue	Interpretation
	a, negative (growth without zones), *Y. enterocolitica* bioserotype 3 variant/O:3 (PC-M1-K1) b, negative (growth without zones), *Y. enterocolitica* ATCC 9610 c, positive (growth with rose-pink zone), *Serratia marcescens* (YC-M2-4) d, uninoculated

6. Indole test

Fresh culture (18-24 hours) was stabbed into SIM medium (Oxoid) and the tube were incubated at 25-28 ℃ for 24 h. After incubation, 0.5 ml Indole reagent (Sigma) was added. Immediate development of red colour indicates a positive test.

Representative photo of Voges-Prokauer test			
Representing bacterium	Uninoculated	*Citrobacter freundii* (YC-K1-3)	*E. coli* ATCC 25922
Interpretation	Uninoculated	Negative (yellow)	Positive (red)

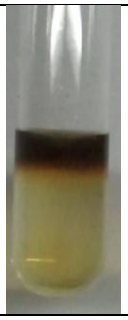
7. Pyrazinamidase test

Fresh culture (18-24 hours) was streaked onto slant's surface. Slant was were incubated at 25-28 ℃ for 48 h. Slant surface was flooded with 1 ml of freshly prepared 1% iron (II) ammonium sulphate solution. Slant was read after 15 min.

Representative photo of pyrazinamidase test			
Representing bacterium	Uninoculated	*Y. enterocolitica* ATCC 9610	*Enterococcus faecalis* ATCC 29212
Interpretation	Uninoculated	Negative (colourless)	Positive (brown color)

8. Voges-Prokauer test

Fresh culture (18-24 hours) was inoculated into MRVP broth and the tubes were incubated at 25-28 °C for 24 h. After incubation, 0.6 ml of α-naphtol reagent was added, mixed gently and then 0.2 ml of 40% KOH reagent was added. Tube was shaken gently for 30 s and allowed to slant to allow maximum oxygen expose for 15 min. Development of red colour indicates a positive test.

Representative photo of Voges-Prokauer test			
Representing bacterium	Uninoculated	*Salmonella enteric* ATCC 10376	*Y. enterocolitica* ATCC 9610
Interpretation	Uninoculated	Negative (dark brown/colour of α-naphtol reagent remained)	Positive (red)

APPENDIX IX RAW DATA FOR BIOTYPING AND SEROTYPING

No.	Isolates Name	Biobhemical tests											Biotype		Antisera					Serotype
		Lipase	Esculine	Salicin	Indole	Xylose	Trehalose	NO_3	Pyrazinamidase	β-D-Glucosidase	VP	DNAse			O:3	O:5	O:8	O:9	O:1 and O:2	
1	PCM-PPN53a-1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
2	PCM-PPN53a-2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
3	PCM-PPN53a-3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
4	PCM-PPN53a-4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
5	PCM-PPN53a-K1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
6	PCM-PPN53a-K2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
7	PCM-PPN53a-K3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
8	PCM-PPN53a-K4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
9	DCM-PPN55a-1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
10	DCM-PPN55a-2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
11	DCM-PPN55a-3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
12	DCM-PPN55a-4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
13	PCM-PPN55a-1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
14	PCM-PPN55a-2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
15	PCM-PPN55a-3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
16	PCM-PPN55a-4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
17	PCM-PPN55a-K1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
18	PCM-PPN55a-K2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
19	PCM-PPN55a-K3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
20	PCM-PPN55a-K4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
21	PCM-PPN55b-1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
22	PCM-PPN55b-2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
23	PCM-PPN55b-3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
24	PCM-PPN55b-4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
25	PCM-PPN55b-K1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
26	PCM-PPN55b-K2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
27	PCM-PPN55b-K3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
28	PCM-PPN55b-K4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
29	PCM-PPN55c-1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
30	PCM-PPN55c-2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
31	PCM-PPN55c-3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
32	PCM-PPN55c-4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
33	PCM-PPN55c-K1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3

No.	Isolates Name	Biobhemical tests											Biotype		Antisera					Serotype
		Lipase	Esculine	Salicin	Indole	Xylose	Trehalose	NO_3	Pyrazinamidase	β-D-Glucosidase	VP	DNAse			O:3	O:5	O:8	O:9	O:1 and O:2	
34	PCM-PPN55c-K2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
35	PCM-PPN55c-K3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
36	PCM-PPN55c-K4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
37	PCM-PPN56a-1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
38	PCM-PPN56a-2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
39	PCM-PPN56a-3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
40	PCM-PPN56a-4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
41	PCM-PPN56a-K1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
42	PCM-PPN56a-K2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
43	PCM-PPN56a-K3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
44	PCM-PPN56a-K4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
45	DCM-PPN56b-21	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
46	DCM-PPN56b-22	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
47	DCM-PPN56b-23	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
48	DCM-PPN56b-24	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
49	PCM-PPN56b-1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
50	PCM-PPN56b-2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
51	PCM-PPN56b-3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
52	PCM-PPN56b-4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
53	PCM-PPN56b-K1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
54	PCM-PPN56b-K2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
55	PCM-PPN56b-K3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
56	PCM-PPN56b-K4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
57	DCM-PPN56c-21	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
58	DCM-PPN56c-22	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
59	DCM-PPN56c-23	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
60	DCM-PPN56c-24	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
61	PCM-PPN56c-1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
62	PCM-PPN56c-2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
63	PCM-PPN56c-3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
64	PCM-PPN56c-4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
65	PCM-PPN56c-5	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
66	PCM-PPN56c-6	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
67	PCM-PPN56c-7	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
68	PCM-PPN56c-8	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3

No.	Isolates Name	Biobhemical tests											Biotype		Antisera					Serotype
		Lipase	Esculine	Salicin	Indole	Xylose	Trehalose	NO_3	Pyrazinamidase	β-D-Glucosidase	VP	DNAse			O:3	O:5	O:8	O:9	O:1 and O:2	
69	PCM-PPN56c-K1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
70	PCM-PPN56c-K2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
71	PCM-PPN56c-K3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
72	PCM-PPN56c-K4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
73	PC-M1-K1	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
74	PC-M1-K2	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
75	PC-M1-K3	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
76	PC-M1-K4	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
77	PC-M1-K5	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
78	PC-M1-K11	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
79	PC-M1-K12	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
80	PC-M1-K13	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
81	PC-M3-6	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
82	PC-M3-K11	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
83	PC-M3-K12	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
84	PC-M13-K13	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
85	S18/1-C-O-6a	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
86	S18/1-C-I-10-4-6a	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
87	S18/1-C-O-6b	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
88	S18/1-C-O-5-6b	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
89	S18/1-C-I-4-6b	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
90	S18/1-C-O-6c	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
91	S18/1-C-O-6d	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
92	S18/1-C-O-5-6e	-	-	-	-	+	+	+	-	-	-	-	3 variant (VP-)		+	-	-	-	-	O:3
93	S18/1-C-O-1a	+	-	-	+	+	+	+	+	-	-	-	1B		-	-	+	-	-	O:8
94	S18/1-C-O-K-5b	+	-	-	+	+	+	+	+	-	-	-	1B		-	-	+	-	-	O:8
95	S18/1-C-O-5c	+	-	-	+	+	+	+	+	-	-	-	1B		-	-	+	-	-	O:8
96	PC-M16-2	+	+	+	+	+	+	+	+	+	+	-	1A		-	+	-	-	-	O:5
97	PC-M16-5	+	+	+	+	+	+	+	+	+	+	-	1A		-	+	-	-	-	O:5
98	PC-M16-10	+	+	+	+	+	+	+	+	+	+	-	1A		-	+	-	-	-	O:5

APPENDIX X NCBI BLAST RESULTS

Strain: PC-M1-K1; target gene: *Yersinia enterocolitica* 16S *r*RNA gene

Yersinia enterocolitica subsp. palearctica partial 16S rRNA gene, strain Y11

Sequence ID: emb|HF558392.1| **Length:** 1480 **Number of Matches:** 1

Range 1: 152 to 421 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
486 bits(263)	3e-134	268/270(99%)	2/270(0%)	Plus/Plus

```
Query  5    CTCACGCCATCGGA-GTGCCCAGATGGGA-TAGCTAGTAGGTGGGGTAATGGCTCACCTA  62
            ||||||||||||||| ||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  152  CTCACGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTA  211

Query  63   GGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTC  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  212  GGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTC  271

Query  123  CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAG  182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  272  CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAG  331

Query  183  CCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGG  242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  332  CCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGG  391

Query  243  CATAAAGGTTAATAACCTTTGTGATTGACG  272
            |||||||||||||||||||||||||||||
Sbjct  392  CATAAAGGTTAATAACCTTTGTGATTGACG  421
```

Strain: PC-M1-K1; target gene: *ail* gene

Yersinia enterocolitica strain SDWL-003 attachment invasion locus protein (ail) gene, complete cds

Sequence ID: gb|JX972143.1| **Length:** 537 **Number of Matches:** 1

Range 1: 101 to 459 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
656 bits(355)	0.0	358/359(99%)	1/359(0%)	Plus/Plus

```
Query  1    CACAAAGCCATGTAAAAG-AAATGGGTATACATTGGATAATGACCCTAAAGGTTTTAACC  59
            |||||||||||||||||  ||||||||||||||||||||||||||||||||||||||||
Sbjct  101  CACAAAGCCATGTAAAAGAAAATGGGTATACATTGGATAATGACCCTAAAGGTTTTAACC  160

Query  60   TGAAGTACCGTTATGAACTCGATGATAACTGGGGAGTAATAGGTTCGTTTGCTTATACCC  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161  TGAAGTACCGTTATGAACTCGATGATAACTGGGGAGTAATAGGTTCGTTTGCTTATACCC  220

Query  120  ATCAGGGATACGATTTCTTCTATGGCAGTAATAAGTTTGGTCACGGTGATCTTGATTACT  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  221  ATCAGGGATACGATTTCTTCTATGGCAGTAATAAGTTTGGTCACGGTGATCTTGATTACT  280

Query  180  ATTCAGTAACAATGGGGCCATCTTTCCGCATTAACGAATATGTTAGCCTTTATGGATTAC  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  281  ATTCAGTAACAATGGGGCCATCTTTCCGCATTAACGAATATGTTAGCCTTTATGGATTAC  340

Query  240  TGGGTGCTGCTCACGGAAAGGTTAAGTCATCTGTATTTGATGGGTCAGTCAGTACAAGTA  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  341  TGGGTGCTGCTCACGGAAAGGTTAAGTCATCTGTATTTGATGGGTCAGTCAGTACAAGTA  400

Query  300  AGACGTCAATGGCATACGGGGCAGGGGTGCAATTCAACCCGCTTCCAAATTTTGTCATT  358
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  401  AGACGTCAATGGCATACGGGGCAGGGGTGCAATTCAACCCGCTTCCAAATTTTGTCATT  459
```

Strain: PC-M1-K1, target gene: *virF* gene

Yersinia enterocolitica subsp. palearctica 105.5R(r) plasmid 105.5R(r)p, complete sequence

Sequence ID: gb|CP002247.1| **Length:** 69704 **Number of Matches:** 1

Range 1: 24983 to 25517 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
989 bits(535)	0.0	535/535(100%)	0/535(0%)	Plus/Plus

```
Query  2      ATATGCTGTTTTTGCGTCGTGGCAGCTATGCTGTTCGATGTGGTACAAAAGAACCCTGCC  61
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  24983  ATATGCTGTTTTTGCGTCGTGGCAGCTATGCTGTTCGATGTGGTACAAAAGAACCCTGCC  25042

Query  62     AATTACTTTGGATTCCATTACCAGGCAGTTTTTTGAGTACTTTTTTACATCGGTTTGGTT  121
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25043  AATTACTTTGGATTCCATTACCAGGCAGTTTTTTGAGTACTTTTTTACATCGGTTTGGTT  25102

Query  122    CTTTGCTTAGTGAAATTAGACGAGACAATGCCACACCCAAGCCATTGTTAATTTTTAATA  181
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25103  CTTTGCTTAGTGAAATTAGACGAGACAATGCCACACCCAAGCCATTGTTAATTTTTAATA  25162

Query  182    TTTCACCAATATTATCACAATCCATTCAAAATCTATGTGCCATATTGGAACGGAGTGATT  241
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25163  TTTCACCAATATTATCACAATCCATTCAAAATCTATGTGCCATATTGGAACGGAGTGATT  25222

Query  242    TTCCGTCAGTATTAACGCAACTGCGTATTGAGGAATTACTGCTTTTGCTTGCCTTTAGCT  301
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25223  TTCCGTCAGTATTAACGCAACTGCGTATTGAGGAATTACTGCTTTTGCTTGCCTTTAGCT  25282

Query  302    CGCAAGGGACTTTATTCCTCTCGGCTCTGCGCCATTTAGGCAACCGCCCAGAAGAACGGT  361
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25283  CGCAAGGGACTTTATTCCTCTCGGCTCTGCGCCATTTAGGCAACCGCCCAGAAGAACGGT  25342

Query  362    TGCAGAAATTTATGGAGGAAAATTATCTACAAGGGTGGAAACTAAGCAAATTTGCGCGAG  421
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25343  TGCAGAAATTTATGGAGGAAAATTATCTACAAGGGTGGAAACTAAGCAAATTTGCGCGAG  25402

Query  422    AATTCGGCATGGGATTAACCACATTCAAAGAACTGTTTGGTACAGTTTATGGCATTTCAC  481
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25403  AATTCGGCATGGGATTAACCACATTCAAAGAACTGTTTGGTACAGTTTATGGCATTTCAC  25462

Query  482    CACGCGCCTGGATAAGCGAGCGACGTATTCTCTATGCTCACCAATTACTTCTTAA  536
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25463  CACGCGCCTGGATAAGCGAGCGACGTATTCTCTATGCTCACCAATTACTTCTTAA  25517
```

Strain: PC-M1-K1; target gene: *rbfC* gene

Yersinia enterocolitica subsp. palearctica Y11
Sequence ID: emb|FR729477.2| Length: 4553420 Number of Matches: 1

Range 1: 1674715 to 1675067 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
652 bits(353)	0.0	353/353(100%)	0/353(0%)	Plus/Plus

Features: dTDP-rhamnosyl transferase RfbF

```
Query  4        AAGCGTCTATACCTTGGTTTTGCTGCAATCATAAAAAAGACGAAAAAGATCATTAACCTA  63
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1674715  AAGCGTCTATACCTTGGTTTTGCTGCAATCATAAAAAAGACGAAAAAGATCATTAACCTA  1674774

Query  64       TATATATGAGCAAGCTTCCATGACCAACTCATTGGTGTACTGGTAACCATATTAATTGTG  123
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1674775  TATATATGAGCAAGCTTCCATGACCAACTCATTGGTGTACTGGTAACCATATTAATTGTG  1674834

Query  124      TTGCGGAAAAATATAATAGTTCCTAAGTGGACTATGTTTAGGAATCTCACGCCATCTTCCC  183
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1674835  TTGCGGAAAAATATAATAGTTCCTAAGTGGACTATGTTTAGGAATCTCACGCCATCTTCCC  1674894

Query  184      ATCCATATCCGAATAACAGAATCCCCAAGAGTATGGTTTAAAAAAGCATCTCCGCATCCA  243
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1674895  ATCCATATCCGAATAACAGAATCCCCAAGAGTATGGTTTAAAAAAGCATCTCCGCATCCA  1674954

Query  244      AATAATTCATACCCACAATTTACCGCCCTAAAACACCATTCTGTATCTACATGATCAATA  303
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1674955  AATAATTCATACCCACAATTTACCGCCCTAAAACACCATTCTGTATCTACATGATCAATA  1675014

Query  304      AAATAATTTTCCTTCATTAAGCCAATATTATGTAACACTTCGGTACGAATTAG  356
                ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1675015  AAATAATTTTCCTTCATTAAGCCAATATTATGTAACACTTCGGTACGAATTAG  1675067
```

Strain: IP135; target gene: *fepD* gene

Yersinia frederiksenii strain C249 ABC transporter (fepD) gene, partial cds
Sequence ID: gb|AY970520.1| Length: 337 Number of Matches: 1

Range 1: 1 to 327 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
564 bits(305)	2e-157	320/327(98%)	1/327(0%)	Plus/Plus

```
Query  11   ATGGGGG-AGATTTAGCCGCCGCAATTGGCGCACGCATTGCCCAAACCCAGTTTTGGGCC  69
            ||||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGGGGGAAGATTTAGCTGCCGCAATTGGCGCACGCATTGCCCAAACCCAGTTTTGGGCC  60

Query  70   GTAGTGACAGTGACCTTGCTATGTGGTGCTGCTACCGCCGCTGTTGGCCCGATCGCCTTT  129
            ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  61   GTAGTGACAGTGACCTTGCTATGTGGTGCAGCTACCGCCGCTGTTGGCCCGATCGCCTTT  120

Query  130  GTCGGCCTGATGGTGCCACATATTGCCCGCTGGATTGTCGGGCCGAATCAATGCTGGATC  189
            ||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  121  GTCGGCCTGATGGTGCCACATATTGCCCGCTGGATTGTCGGGCCGAATCAGTGCTGGATC  180

Query  190  CTTCCTTTCACCTTGGTAATGACTCCCATTTTGCTATTAGTTTCAGATATTGTGGGGCGC  249
            || |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  181  CTCCCTTTCACCTTGGTAATGACCCCCATTTTGCTATTAGTTTCAGATATTGTGGGGCGC  240

Query  250  TTTCTGGTTCCCGGTGAATTACGGGTTTCCATCGTGACGGCGTTCATTGGCGCTCCTTTA  309
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TTTCTGGTTCCGGGTGAATTACGGGTTTCCATCGTGACGGCGTTCATTGGCGCTCCTTTA  300

Query  310  TTGATCTGGCTGGTTCGCCGTAATAAA  336
            ||||||||||||||||||||||||||
Sbjct  301  TTGATCTGGCTGGTTCGCCGTAATAAA  327
```

Strain: IP135; target gene: *hreP* gene

Yersinia enterocolitica subsp. palearctica Y11
Sequence ID: emb|FR729477.2| Length: 4553420 Number of Matches: 1

Range 1: 1440182 to 1440869 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1258 bits(681)	0.0	686/688(99%)	1/688(0%)	Plus/Minus

Features: calcium-dependent protease precursor

```
Query  1        TTTC-TCATAGCCTCCAttttttttCATCTATTTTGTCGCAACTGTCGCGAATGATATCTT  59
                |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1440869  TTTCATCATAGCCTCCATTTTTTTCATCTATTTTGTCGCAACTGTCGCGAATGATATCTT  1440810

Query  60       TGACCTCTTGATAGTTCAGAGTGGGGTTGACTGAAATAATTAAAGCGGCAACACCGGCTG  119
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1440809  TGACCTCTTGATAGTTCAGAGTGGGGTTGACTGAAATAATTAAAGCGGCAACACCGGCTG  1440750

Query  120      CACCGGGACAGGCACTGGAAGTACCACCAAAATTATTAATGTAATTACCAAAAATATCCC  179
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1440749  CACCGGGACAGGCACTGGAAGTACCACCAAAATTATTAATGTAATTACCAAAAATATCCC  1440690

Query  180      CTATTGCAGTGCTGGCCCCTGGATTATCACCATATTCACGACGTAGGTCGGTGGTCCAGA  239
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1440689  CTATTGCAGTGCTGGCCCCTGGATTATCACCATATTCACGACGTAGGTCGGTGGTCCAGA  1440630

Query  240      TGCCCGTTGTTAAAGGCGCTGGGTGCTCGAATACGGGGTCTTCAAAGTCGTTACTGGGAA  299
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1440629  TGCCCGTTGTTAAAGGCGCTGGGTGCTCGAATACGGGGTCTTCAAAGTCGTTACTGGGAA  1440570

Query  300      AAGAGCACCAAAGTGATTTACCGTAATTGCTGTAAACGGAACGGAGACTTCGGTCATTAC  359
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1440569  AAGAGCACCAAAGTGATTTACCGTAATTGCTGTAAACGGAACGGAGACTTCGGTCATTAC  1440510

Query  360      AAGCAGCAACAGCAATAACCGGTTCGTAGCTGGCGTAACCATCTTTTTCGACAGATTCAT  419
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1440509  AAGCAGCAACAGCAATAACCGGTTCGTAGCTGGCGTAACCATCTTTTTCGACAGATTCAT  1440450

Query  420      TGTCGTTACCGGCGGCAAAAAGGACGACACACCCCTTGCCTTTGCGCCCTTTATCAATAG  479
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1440449  TGTCGTTACCGGCGGCAAAAAGGACGACACACCCCTTGCCTTTGCGCCCTTTATCAATAG  1440390

Query  480      CATAATCAATAGCCATACGAGTCAATGCTGGCAGTGCGGCCACAACGTCATGTCGAGGAT  539
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1440389  CATAATCAATAGCCATACGAGTCAATGCTGGCAGTGCGGCCACAACGTCATGTCGAGGAT  1440330

Query  540      CATCTGAGTTAGCCGACCCTTTCTGCCCCCAACTGCAAGAGATAACATCGGCTCCATTAT  599
                |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  1440329  CATCTGAGTTAGCCGACCCTTTCGGCCCCCAACTGCAAGAGATAACATCGGCTCCATTAT  1440270

Query  600      CTGTCGCCCACACGAAAGCGAGAGCTTCGTCTAGTGAACCTAACCCTCCCGCCTGACGTA  659
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1440269  CTGTCGCCCACACGAAAGCGAGAGCTTCGTCTAGTGAACCTAACCCTCCCGCCTGACGTA  1440210

Query  660      TTGGCATAAGATTAGCTTCTGGTGCCAC  687
                ||||||||||||||||||||||||||||
Sbjct  1440209  TTGGCATAAGATTAGCTTCTGGTGCCAC  1440182
```

Strain: IP102; target gene: *fes* gene

Yersinia enterocolitica strain C760 enterochelin esterase (fes) gene, partial cds

Sequence ID: gb|DQ144904.1| Length: 516 Number of Matches: 1

Range 1: 9 to 476 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
859 bits(465)	0.0	467/468(99%)	0/468(0%)	Plus/Plus

```
Query  1    AAACGATCCGTTCAATCACACCGCCCCCCATGCCAGCTATCGCGGAAGGGCACTTTCTGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9    AAACGATCCGTTCAATCACACCGCCCCCCATGCCAGCTATCGCGGAAGGGCACTTTCTGC  68

Query  61   GGTGCATTTAGCTGATGCCATACCTCAGACCGCCTGGCAGCCGATTGATGCAGGCCAGCA  120
            ||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  69   GGTGCATTTAGCTGATGCCATACCTCAGACCGTCTGGCAGCCGATTGATGCAGGCCAGCA  128

Query  121  ACTGCCAACGGATACGCAACGGCTGCAATTGATTACCTGGCATAGCGAATTGCTAGGTAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  129  ACTGCCAACGGATACGCAACGGCTGCAATTGATTACCTGGCATAGCGAATTGCTAGGTAA  188

Query  181  CAGCCGCAATGTGTGGATTTACCACACACACGGCACAGCAGATAACGCCGAGCGGCCATT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189  CAGCCGCAATGTGTGGATTTACCACACACACGGCACAGCAGATAACGCCGAGCGGCCATT  248

Query  241  GGCTATCTTGCTTGATGGGCAGTATTGGGCGACCAGGCAACCGATATTCGGTGTGCTGGA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  249  GGCTATCTTGCTTGATGGGCAGTATTGGGCGACCAGGCAACCGATATTCGGTGTGCTGGA  308

Query  301  TAATGAAACTGATGCCGGACGTTTGCCCGCCAGTGTGTATGTGCTGATCGACATTATTGA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  309  TAATGAAACTGATGCCGGACGTTTGCCCGCCAGTGTGTATGTGCTGATCGACATTATTGA  368

Query  361  CCAACCCCATCGCTCAGTAGAGTTGCCTTGCAATCAGGATTTTTGGCAGGCGCTACAAAC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  369  CCAACCCCATCGCTCAGTAGAGTTGCCTTGCAATCAGGATTTTTGGCAGGCGCTACAAAC  428

Query  421  AGAATTATTGCCACAAGTTGCTGCACTACAACCCTTTACTGACCAAGC  468
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  429  AGAATTATTGCCACAAGTTGCTGCACTACAACCCTTTACTGACCAAGC  476
```

Strain: IP135; target gene: *sat* gene

Yersinia enterocolitica Y56 streptogramin acetyl transferase (sat) gene, complete cds; and PA repeat protein gene, partial cds

Sequence ID: gb|AY143385.1| Length: 1500 Number of Matches: 1

Range 1: 764 to 1165 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
725 bits(392)	0.0	399/402(99%)	1/402(0%)	Plus/Minus

```
Query  3     CACACCGAATCCG-ACATTATTATCGGTGATTATACCTACTACGACGATCCACAAGATTC  61
             ||||| ||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1165  CACACAGAATCCGAACATTATTATCGGTGATTATACCTACTACGACGATCCACAAGATTC  1106

Query  62    TGAAAACTTTGAACGTAACGTGCTTTATCACTACCCCTTTATTGGTGATAAGCTGATTAT  121
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1105  TGAAAACTTTGAACGTAACGTGCTTTATCACTACCCCTTTATTGGTGATAAGCTGATTAT  1046

Query  122   CGGCAAATTCTGTGCATTAGCTCATGGGGTGAAGTTCATTATGAATGGTGCCAACCATAA  181
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1045  CGGCAAATTCTGTGCATTAGCTCATGGGGTGAAGTTCATTATGAATGGTGCCAACCATAA  986

Query  182   AATGTCTGGGTTATCGACTTACCCATTCAATATTTTTGGTAACGGCTGGGAAAGAGTCGC  241
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  985   AATGTCTGGGTTATCGACTTACCCATTCAATATTTTTGGTAACGGTTGGGAAAGAGTCGC  926

Query  242   CCCGTCCAGGGATGAGCTGCCTTATAAAGGCGATACTCATGTCGGAAATGATGTGTGGAT  301
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  925   CCCGTCCAGGGATGAGCTGCCTTATAAAGGCGATACTCATGTCGGAAATGATGTGTGGAT  866

Query  302   TGGCTATGATGTGTTGATTATGCCAGGTGTCACCATTGGCAATGGGGCAATTATTTCATC  361
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  865   TGGCTATGATGTGTTGATTATGCCAGGTGTCACCATTGGCAATGGGGCAATTATTTCATC  806

Query  362   ACGCTCAGTGGTCACGCGCGATGTGCCCGCTTATAGTGTGGT  403
             |||||||||||||||||||||||||||||||||||||||||||
Sbjct  805   ACGCTCAGTGGTCACGCGCGATGTGCCCGCTTATAGTGTGGT  764
```

Strain: IP102; target gene: *fepA* gene

Yersinia enterocolitica strain C842 ferrienterochelin receptor (fepA) gene, partial cds

Sequence ID: gb|DQ144910.1| Length: 427 Number of Matches: 1

Range 1: 22 to 401 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
652 bits(353)	0.0	371/380(98%)	0/380(0%)	Plus/Plus

```
Query  1    CAAACCTCAGTGGGTTATGCCCTTGAGCATTACATCAATGACACCTTTAAATTCCGTCAG  60
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  22   CAAACCTCAGTCGGTTATGCCCTTGAGCATTACATCAATGACACCTTTAAATTCCGTCAG  81

Query  61   AACCTGCGTTACAGCTATAACAAGCAAGACTATAAGTATCTGGTCTTTATGGATTTACTG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  82   AACCTGCGTTACAGCTATAACAAGCAAGACTATAAGTATCTGGTCTTTATGGATTTATTG  141

Query  121  GCTGATAGCCGCACCATGACCCGCCGCCCACAAATTGAGCGCCAGACAACCGCTGAGTTT  180
            || |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  142  GCCGATAGCCGCACCATGACCCGCCGCCCACAAATTGAACGCCAGACAACCGCTGAGTTT  201

Query  181  GCAGTCGATAACCAACTGCAAGCTGATTTCTGGACCGGTCAACTCAACCACACGGTGCTC  240
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||| |||
Sbjct  202  GCAGTCGATAACCAACTACAAGCTGATTTCTGGACCGGTCAACTCAACCACACGGTACTC  261

Query  241  ACCGGATTGGATTACAAACGCACCCGTATTGATAGCCGATTCTATATGGGCACGGTACAG  300
            ||||| || ||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  262  ACCGGGTTAGATTACAAACGCACCCGTATTGATAGCCGATTTTATATGGGCACGGTACAG  321

Query  301  ACAAAATATAATCTGGATTGGGTATCACCGGTTTATGGTCTGAATATCAAAGACAGTGAC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  322  ACAAAATATAATCTGGATTGGGTATCACCGGTTTATGGTCTGAATATCAAAGACAGTGAC  381

Query  361  CTGTCACTGAATAGCAGTGA  380
            ||||||||||||||||||||
Sbjct  382  CTGTCACTGAATAGCAGTGA  401
```

Strain: IP102; target gene: *ystB* gene

Yersinia enterocolitica strain C760 heat stable enterotoxin (ystB) gene, partial cds
Sequence ID: gb|AY966880.1| Length: 122 Number of Matches: 1

Range 1: 11 to 62 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
97.1 bits(52)	6e-18	52/52(100%)	0/52(0%)	Plus/Minus

```
Query  1   GCACGCTTTTCTGTTTATCTCAGCGGCTATTGGTGTCGATAATGTATCATCA  52
           ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62  GCACGCTTTTCTGTTTATCTCAGCGGCTATTGGTGTCGATAATGTATCATCA  11
```

Strain: IP11105; target gene: *inv* gene

Yersinia enterocolitica invA gene for invasin, strain WA-314
Sequence ID: emb|FN594891.1| Length: 3600 Number of Matches: 1

Range 1: 2789 to 3308 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
937 bits(507)	0.0	517/521(99%)	4/521(0%)	Plus/Plus

```
Query  1     GCTGAAGTGG-CATCGGGT-ACTATTGGACTAAAAAGACCAGTACAGATTTTGTCACTAT  58
             |||| ||||| |||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  2789  GCTG-AGTGGCCATCGGGTAACTATTGGACTAAAAAGACCAGTACAGATTTTGTCACTAT  2847

Query  59    GGATATGACCACCGGTGACATACCAACATCTGCGGCTACGGCGTATCCGCTGTGTGCGGA  118
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2848  GGATATGACCACCGGTGACATACCAACATCTGCGGCTACGGCGTATCCGCTGTGTGCGGA  2907

Query  119   GCCGCAATAGTGCTAAATACCAATCTTGCGGCCCAGCAAACTGGCACCTTTAGCGTGACC  178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2908  GCCGCAATAGTGCTAAATACCAATCTTGCGGCCCAGCAAACTGGCACCTTTAGCGTGACC  2967

Query  179   ATCTGGCCCATACAGTGATTGGCCGTGGCGCGTATTCAAAACCGCCAGCGCCTGAGTGTT  238
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2968  ATCTGGCCCATACAGTGATTGGCCGTGGCGCGTATTCAAAACCGCCAGCGCCTGAGTGTT  3027

Query  239   ATGCTCAATATGCTGTTGCAGCAAAAGCCCGTTATGCAGGTTGCCGTAGCGCAGGCCTTC  298
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3028  ATGCTCAATATGCTGTTGCAGCAAAAGCCCGTTATGCAGGTTGCCGTAGCGCAGGCCTTC  3087

Query  299   GGCCAGTTCCAAAATACGCTGCCAGCGCTCAGCTAGCGCAGGAACGTTGCTGTAGGGCGC  358
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3088  GGCCAGTTCCAAAATACGCTGCCAGCGCTCAGCTAGCGCAGGAACGTTGCTGTAGGGCGC  3147

Query  359   TTGAATATTTATGttttttttCGGTGGTGAGCCGGGTCTGGTCCAGATAAGCCAAGGTCGC  418
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3148  TTGAATATTTATGTTTTTTTTCGGTGGTGAGCCGGGTCTGGTCCAGATAAGCCAAGGTCGC  3207

Query  419   CAAAATTGAACTTTTTTGTTCAGTGACGCCTTGCAACACGATACCTTGAATCCGACCGGA  478
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3208  CAAAATTGAACTTTTTTGTTCAGTGACGCCTTGCAACACGATACCTTGAATCCGACCGGA  3267

Query  479   GCACAGCA-GTTGCTGCTCTTGTGCTACTACGGTTTTCAGG  518
             |||||||| ||||||||||||||||||||||||||||||||
Sbjct  3268  GCACAGCAAGTTGCTGCTCTTGTGCTACTACGGTTTTCAGG  3308
```

Strain: IP383; target gene: *yadA* gene

Y.enterocolitica yadA gene for YadA
Sequence ID: emb|X13882.1| Length: 2551 Number of Matches: 3

Range 1: 1072 to 1852 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1432 bits(775)	0.0	779/781(99%)	0/781(0%)	Plus/Plus

```
Query  5     AAAACTCTGTTGCCATTGGACACTCTAGTCACGTTGCGGCAAATCATGGTTATTCAATTG  64
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1072  AAAACTCTGTTGCCATTGGACACTCTAGTCACGTTGCGGCAAATCATGGTTATTCAATTG  1131

Query  65    CAATTGGGGATCGTTCTAAAACTGACCGAGAAAATAGTGTATCCATTGGTCATGAAAGCC  124
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1132  CAATTGGGGATCGTTCTAAAACTGACCGAGAAAATAGTGTATCCATTGGTCATGAAAGCC  1191

Query  125   TTAATCGCCAATTGACACATCTTGCGGCTGGCACTAAAGACACTGATGCAGTGAATGTCG  184
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1192  TTAATCGCCAATTGACACATCTTGCGGCTGGCACTAAAGACACTGATGCAGTGAATGTCG  1251

Query  185   CGCAATTaaagaaagaaattgaaaaaacacaggaaaatacaaataaaaaatCAGCTGAGC  244
             |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  1252  CGCAATTAAAGAAAGAAATTGAAAAAACACAGGAAAATACAAATAAAAGATCAGCTGAGC  1311

Query  245   TGTTAGCAAACGCTAATGCGTATGCAAACAACAAGTCTTCTAGCGTGCTAGGGATCGCAA  304
             |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  1312  TGTTAGCAAACGCTAATGCGTATGCAGACAACAAGTCTTCTAGCGTGCTAGGGATCGCAA  1371

Query  305   ATAACTATACTGATAGTAAAAGTGCTGAAACATTGGAAAATGCGCGTAAAGAGGCTTTTG  364
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1372  ATAACTATACTGATAGTAAAAGTGCTGAAACATTGGAAAATGCGCGTAAAGAGGCTTTTG  1431

Query  365   CTCAGTCTAAAGATGTTTTGAATATGGCAAAAGCACACTCAAATAGTGTTGCTAGAACAA  424
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1432  CTCAGTCTAAAGATGTTTTGAATATGGCAAAAGCACACTCAAATAGTGTTGCTAGAACAA  1491

Query  425   CTTTAGAAACTGCTGAAGAACATGCAAATAGTGTTGCCAGAACAACTTTAGAAACTGCTG  484
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1492  CTTTAGAAACTGCTGAAGAACATGCAAATAGTGTTGCCAGAACAACTTTAGAAACTGCTG  1551

Query  485   AAGAACATGCAAATAAAAAATCAGCTGAGGCGTTAGCAAGCGCTAATGTGTATGCAGACA  544
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1552  AAGAACATGCAAATAAAAAATCAGCTGAGGCGTTAGCAAGCGCTAATGTGTATGCAGACA  1611

Query  545   GCAAGTCTTCTCACACACTAAAAACTGCAAATAGCTATACCGATGTGACTGTAAGTAATT  604
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1612  GCAAGTCTTCTCACACACTAAAAACTGCAAATAGCTATACCGATGTGACTGTAAGTAATT  1671

Query  605   CGACTAAGAAAGCAATCCGTGAATCGAATCAATACACAGATCATAAATTCCGTCAACTTG  664
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1672  CGACTAAGAAAGCAATCCGTGAATCGAATCAATACACAGATCATAAATTCCGTCAACTTG  1731

Query  665   ACAACCGGTTAGATAAACTTGACACACGAGTTGACAAAGGTTTAGCCAGTTCAGCCGCTT  724
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1732  ACAACCGGTTAGATAAACTTGACACACGAGTTGACAAAGGTTTAGCCAGTTCAGCCGCTT  1791

Query  725   TAAACAGCTTGTTCCAGCCATATGGTGTGGGGAAAGTAAACTTTACTGCAGGTGTCGGGG  784
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1792  TAAACAGCTTGTTCCAGCCATATGGTGTGGGGAAAGTAAACTTTACTGCAGGTGTCGGGG  1851

Query  785   G  785
             |
Sbjct  1852  G  1852
```

Strain: IP11105; target gene: *ymoA* gene

Yersinia enterocolitica ymoA gene
Sequence ID: emb|X58058.1| Length: 375 Number of Matches: 1

Range 1: 51 to 330 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
510 bits(276)	2e-141	279/280(99%)	1/280(0%)	Plus/Plus

```
Query  2    AACTTAGGGGA-AGTTTCCCTATATTATTTTTATATTGAGAAGAAAAAACACCATGACAA  60
            ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  51   AACTTAGGGGATAGTTTCCCTATATTATTTTTATATTGAGAAGAAAAAACACCATGACAA  110

Query  61   AAACTGACTACCTGATGCGTTTAAGAAAATGTACGACTATTGATACATTGGAACGTGTAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  111  AAACTGACTACCTGATGCGTTTAAGAAAATGTACGACTATTGATACATTGGAACGTGTAA  170

Query  121  TTGaaaaaaaTAAGTACGAACTTTCTGACGATGAGCTGGAATTGTTTTACTCAGCAGCAG  180
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  171  TTGAAAAAAATAAGTACGAACTTTCTGACGATGAGCTGGAATTGTTTTACTCAGCAGCAG  230

Query  181  ACCACCGCTTAGCTGAACTCACAATGAATAAACTTTATGATAAAATTCCACCTACTGTAT  240
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  231  ACCACCGCTTAGCTGAACTCACAATGAATAAACTTTATGATAAAATTCCACCTACTGTAT  290

Query  241  GGCAACATGTGAAATAGTCTGCTTAACGCTGGTTAAGACA  280
            |||||||||||||||||||||||||||||||||||||||
Sbjct  291  GGCAACATGTGAAATAGTCTGCTTAACGCTGGTTAAGACA  330
```

Strain: PC-M1-K1; target gene: *myfA* gene

Y.enterocolitica MyfA, MyfB, and MyfC genes
Sequence ID: emb|Z21953.1| Length: 4400 Number of Matches: 2

Range 1: 440 to 676 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
425 bits(230)	1e-115	235/237(99%)	2/237(0%)	Plus/Minus

```
Query  3    ATT-ACCTGCTTT-ACCTCGTCGCCAGCAGCAAGAAAGAAAGTCGCTTCCACACGCTCAG  60
            ||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  676  ATTCACCTGCTTTAACCTCGTCGCCAGCAGCAAGAAAGAAAGTCGCTTCCACACGCTCAG  617

Query  61   AACCGTCATCTATCCACCAGTGGTCGTTATCCCACGATAGCTCATTTGTATGTAAGTCAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  616  AACCGTCATCTATCCACCAGTGGTCGTTATCCCACGATAGCTCATTTGTATGTAAGTCAA  557

Query  121  CTCTGGTACCATCTGCAGAAACCATCTGTCCTCCCTCAGATGCACCGGTAGGCCGAACAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  556  CTCTGGTACCATCTGCAGAAACCATCTGTCCTCCCTCAGATGCACCGGTAGGCCGAACAT  497

Query  181  TCCAACCTTTATGTTCCCCTGAGTCCGACATTACCAGATGGAAGGCAGGTGTATCTG  237
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  496  TCCAACCTTTATGTTCCCCTGAGTCCGACATTACCAGATGGAAGGCAGGTGTATCTG  440
```

Strain: IP102; target gene: *tccC* gene

Yersinia enterocolitica strain T83 biotype 1A putative lysR-family transcriptional regulatory proteins, putative insecticidal toxin complex protein (tcbA), putative insecticidal toxin complex protein (tcaC), putative phage-related proteins, putative exported protein, putative insecticidal toxin complex protein (tccC), putative DNA gyrase modulator, and putative carbon-nitrogen hydrolase genes, complete cds
Sequence ID: gb|AY647257.1| Length: 20157Number of Matches: 1

Range 1: 15750 to 16665 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1648 bits(892)	0.0	909/917(99%)	1/917(0%)	Plus/Minus

```
Query  1      TAATTCTAATGAACTGGAGCCGATATTATTATCGAAGTTATAGCGCAGTTGCCCATTCTC  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16665  TAATTCTAATGAACTGGAGCCGATATTATTATCGAAGTTATAGCGCAGTTGCCCATTCTC  16606

Query  61     AATACCTCGTGGTTTGCCTTTTTCCCACAGGAGTACCCGGACTATTGGTCTTCCTGATGT  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16605  AATACCTCGTGGTTTGCCTTTTTCCCACAGGAGTACCCGGACTATTGGTCTTCCTGATGT  16546

Query  121    ACTCAGGGTAATGACCTGAAAATCCTCAGTTAACTTATCGTCATTATGCTGCGTATGCAG  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16545  ACTCAGGGTAATGACCTGAAAATCCTCAGTTAACTTATCGTCATTATGCTGCGTATGCAG  16486

Query  181    TTCTAGCCCTGGCAGGTAAGTGACATTGCTGCGCTGAGTGGTGGTGTTGGTTTGATGAAT  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16485  TTCTAGCCCTGGCAGGTAAGTGACATTGCTGCGCTGAGTGGTGGTGTTGGTTTGATGAAT  16426

Query  241    ACTTTGCTTGATGGCACGTATACCCTCGCTGTCATATAGGTAGTTCTCGCGATCACTCGT  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16425  ACTTTGCTTGATGGCACGTATACCCTCGCTGTCATATAGGTAGTTCTCGCGATCACTCGT  16366

Query  301    ATCATCCTGACGTTCAATCATCGTAACCTGTTGTAACTGATCGTTGACATCCCATTGCAA  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16365  ATCATCCTGACGTTCAATCATCGTAACCTGTTGTAACTGATCGTTGACATCCCATTGCAA  16306

Query  361    TGATTGACCGGGTTGTAATAGCTGCTGGTTACCGGCGGCATCAAACTGGCTACGAATATC  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16305  TGATTGACCGGGTTGTAATAGCTGCTGGTTACCGGCGGCATCAAACTGGCTACGAATATC  16246

Query  421    AGCAGACGTTAAACCATTTCTTTGTTGAATTCCGTGATTACTATTATCGGAGACCGTGAT  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16245  AGCAGACGTTAAACCATTTCTTTGTTGAATTCCGTGATTACTATTATCGGAGACCGTGAT  16186

Query  481    ATGCGTGGTGTATTGGCTGGCACCAATATGCTGGATTTTCAGTAAATTGCCGGCGTTATC  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16185  ATGCGTGGTGTATTGGCTGGCACCAATATGCTGGATTTTCAGTAAATTGCCGGCGTTATC  16126

Query  541    ATAGGTGTAACTCCGGGTGTAATTAATATATTGATGGCTATCAGTTAGTGAGGTTAAAAC  600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16125  ATAGGTGTAACTCCGGGTGTAATTAATATATTGATGGCTATCAGTTAGTGAGGTTAAAAC  16066

Query  601    GGGTAACTGGGCACATTGAGCACTATTGGTATCGGACTCACGGCCTTTGGCTTGGATAAG  660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16065  GGGTAACTGGGCACATTGAGCACTATTGGTATCGGACTCACGGCCTTTGGCTTGGATAAG  16006

Query  661    CTGGTACAGTGCATCGTAGCTGTAGGCGTTTTCCGGTATGACTTTCTGATTGCGATAGAA  720
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16005  CTGGTACAGTGCATCGTAGCTGTAGGCGTTTTCCGGTATGACTTTCTGATTGCGATAGAA  15946

Query  721    GCGAGTCGCTTCTGCATCATTATGGATAGTAAGAATATTCCCAACAGGGTCATAATCGTA  780
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15945  GCGAGTCGCTTCTGCATCATTATGGATAGTAAGAATATTCCCAACAGGGTCATAATCGTA  15886

Query  781    GCGTAAATCCTGTAATAGGGTTGGCCGCTCCTTTAGTACCGAACGGGTGGTTTTAATACC  840
              |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  15885  GCGTAAATCCTGTAATAGGGTTGGCCGCTCCTTTAGTACCGGACGGGTGGTTTTAATACC  15826

Query  841    AAGTAAGCGCTGGGTCTGTGGCTCATAACGGTATTCGGTGACGACACCAATGGCCCTCTC  900
              || |||||||||||||||||||||||||||||||||||||||||||| || | |||||
Sbjct  15825  AATTAAGCGCTGGGTCTGTGGCTCATAACGGTATTCGGTGACGACACCATTGCCACTCTC  15766

Query  901    TTCACTCATTATATTGC  917
              ||||| |||| | ||||
Sbjct  15765  TTCACGCATTTT-TTGC  15750
```

APPENDIX XI PHENOTYPIC VIRULENCE PLASMID TESTS

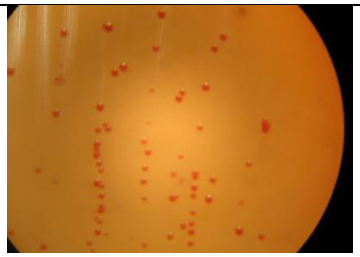
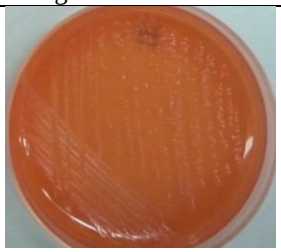
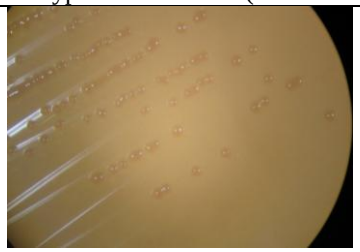
1. Autoagglutination

Cultures were inoculated into two tubes of MR-VP broth (Oxoid). One tube was incubated at 37 ℃ and the other was incubated at 25 ℃. After 18 to 24 h, the tubes were observed for agglutination, with care taken not to shake or disturb the sediment at the bottom and along the sides of the tube. Strains which carried virulence plasmid agglutinated at 37 ℃ but not 25 ℃. Strains that lack the virulence plasmid do not agglutinate at either temperature. Strains that agglutinated at both temperatures were considered "rough".

Representative photo of autoagglutination test using MRVP broth			
Representative bacteria	Uninoculated	*Y. enterocolitica* bioserotype 3 variant/O:3 (PC-M1-K1) at 37 ℃	*Y. enterocolitica* bioserotype 3 variant/O:3 (PC-M1-K1) at 25 ℃
Interpretation	Uninoculated	Clumping	Turbid

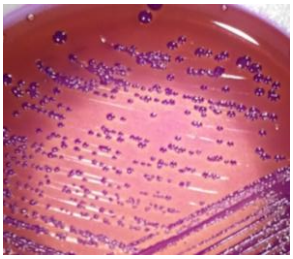
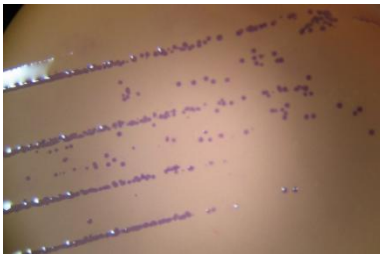
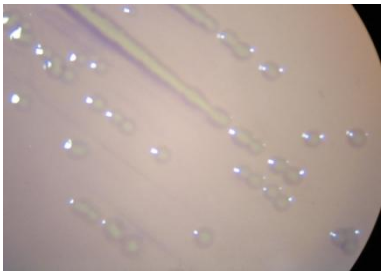
2. Calcium dependency and Congo red binding

CR-MOX agar (Fluka) that allowed visualization of calcium-dependent growth and uptake of Congo red dye on the same plate were used. Fresh *Y. enterocolitica* isolates (16-18 h) were streaked onto CR-MOX agar plates, incubated at 37 ℃, and for 24- 48 h. A predominance tiny red colony is indicative of a positive response for both Congo red binding and calcium dependency.

Representative photo of CR-MOX plate	40× light microscope	Interpretation
	-	Uninoculated
 Representing bacterium: *Y. enterocolitica* bioserotype 3 variant/O:3 (PC-M1-K1)		Positive (red tiny colonies)
 Representing bacterium: *Y. enterocolitica* bioserotype 1A/O:5 (PC-M16-2)		Negative (white colonies)

3. Crystal violet binding

Crystal violet binding on the same CR-MOX agar was performed by flooding the plate with 85 µg/ml crystal violet, allowed to stand for 2 min, and the dye was decanted. Plates were observed with stereo microscope at 40 × magnification. Positive isolates displayed as small and intensely purple colonies.

<table>
<tr><th>Representative photo of CR-MOX plate, flooded with 85 µg/ml crystal violet solution</th><th>Observation under 40× light microscope</th><th>Interpretation</th></tr>
<tr><td>  </td><td>  </td><td rowspan="2">Positive (intensly purple colonies)</td></tr>
<tr><td colspan="2">Representing bacterium: Y. enterocolitica bioserotype 3 variant/O:3 (PC-M1-K1)</td></tr>
<tr><td>  </td><td>  </td><td rowspan="2">Negative (bacteria colonies did not stained by crystal violet)</td></tr>
<tr><td colspan="2">Representing bacterium: Y. enterocolitica bioserotype 1A/O:5 (PC-M16-2)</td></tr>
</table>

APPENDIX XII ANTIMICROBIAL SUSCEPTIBILITY PROFILES OF *Y. enterocolitica* ISOLATES

Antimicrobials \ Name of isolates	PCM-PPN53a-1	PCM-PPN53a-3	DCM-PPN55a-1	PCM-PPN55a-1	PCM-PPN55b-1	PCM-PPN55b-2	PCM-PPN55b-4	PCM-PPN55b-K3	PCM-PPN55c-1	PCM-PPN55c-3	PCM-PPN56a-1	PCM-PPN56a-4	PCM-PPN56a-K1	DCM-PPN56b-21	DCM-PPN56b-23	PCM-PPN56b-4	PCM-PPN56b-K3	DCM-PPN56c-21	DCM-PPN56c-23	PCM-PPN56c-K4	PC-M1-K1	PC-M3-6	PC-M13-K13	S18/1-C-O-6a	S18/1-C-1-4-6b	S18/1-C-O-6d	S18/1-C-O-5-6e	S18/1-C-O-1a	S18/1-C-O-K-5b	S18/1-C-O-5c	PC-M16-2	PC-M16-10	%	S	%	I	%	R
Cefuroxime, 30µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	I	93.8	30	6.3	2	0.0	0
Cefotaxime, 30µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Ceftazidime, 30µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Ceftriaxone, 30µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Nalidixic acid, 30µg	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	37.5	12	0.0	0	62.5	20
Ciprofloxacin, 5 µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Levofloxacin, 5 µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Trimethoprim, 5 µg	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	96.9	31	3.1	1	0.0	0
Trimethoprim-sulphamethoxazole, 25 µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	R	I	S	S	S	S	S	S	S	S	90.6	29	6.3	2	3.1	1
Streptomycin, 10 µg	S	S	S	S	S	I	S	S	S	S	I	S	S	S	S	S	I	S	I	S	R	R	R	R	R	R	S	S	S	S	S	65.6	21	12.5	4	21.9	7
Kanamycin, 30µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Amikacin, 30µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Gentamicin, 10 µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Neomycin, 10 µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Netilmicin, 30µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Ampicillin, 10 µg	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R	R	S	S	S	R	9.4	3	3.1	1	87.5	28
Ticarcilin, 75 µg	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R	R	R	I	R	I	R	R	R	R	R	R	I	S	S	S	R	9.4	3	12.5	4	78.1	25
Tetracycline, 30µg	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	37.5	12	0.0	0	62.5	20
Doxycycline, 30µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Amoxicillin-clavulanic acid, 30µg	I	S	S	S	S	R	S	S	S	S	R	S	I	S	S	S	S	S	S	S	I	S	I	S	S	S	S	S	S	R	S	78.1	25	12.5	4	9.4	3
Aztreonam, 30µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Imipenem, 10 µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Polymyxin B, 300 µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Chloroamphenicol, 30µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Clindamycin, 2 µg	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	I	I	I	R	R	R	R	R	0.0	0	12.5	4	87.5	28
Enrofloxacin, 5 µg	S	S	S	S	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	96.9	31	3.1	1	0.0	0
Amoxicillin, 25 µg	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R	R	S	S	S	R	9.4	3	6.3	2	84.4	27
Spetinomycin, 100 µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Colistin, 10 µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	100.0	32	0.0	0	0.0	0
Total R	6	6	6	6	6	7	6	6	4	6	7	6	6	6	6	6	6	5	6	3	5	6	5	4	4	4	3	1	1	1	5	4						
%	20.7	20.7	20.7	20.7	20.7	24.1	20.7	20.7	13.8	20.7	24.1	20.7	20.7	20.7	20.7	20.7	20.7	17.2	20.7	10.3	17.2	20.7	17.2	13.8	13.8	13.8	10.3	3.4	3.4	3.4	17.2	13.8						
Total I	1	0	1	0	0	1	0	0	2	0	1	0	2	0	0	0	1	1	1	3	2	0	2	1	1	1	2	0	0	0	1	1						
%	3.4	0.0	3.4	0.0	0.0	3.4	0.0	0.0	6.9	0.0	3.4	0.0	6.9	0.0	0.0	0.0	3.4	3.4	3.4	10.3	6.9	0.0	6.9	3.4	3.4	3.4	6.9	0.0	0.0	0.0	3.4	3.4						
Total S	22	23	22	23	23	21	23	23	23	23	21	23	21	23	23	23	22	23	22	23	22	23	22	24	24	24	24	28	28	28	23	24						
%	75.9	79.3	75.9	79.3	79.3	72.4	79.3	79.3	79.3	79.3	72.4	79.3	72.4	79.3	79.3	79.3	75.9	79.3	75.9	79.3	75.9	79.3	75.9	82.8	82.8	82.8	82.8	96.6	96.6	96.6	79.3	82.8						
MAR index	0.207	0.207	0.207	0.207	0.207	0.241	0.207	0.207	0.138	0.207	0.241	0.207	0.207	0.207	0.207	0.207	0.207	0.172	0.207	0.103	0.172	0.207	0.172	0.138	0.138	0.138	0.103	0.034	0.034	0.034	0.172	0.138						

APPENDIX XIII BACTERIAL COUNTS FOR MODIFICATION AND IMPROVEMENT OF CIN AGAR

1. The cfu counts of IP383, IP135, ATCC 9610 and PC-M16-2 on CIN, modified CIN and LBA.

Bacterial strains	Agar plates	Replicate (cfu count)						Average (cfu count)	Dilution factor	cfu/ml[a]
		1	2	3	4	5	6			
IP 383	CIN[b] (ae[c])	197	257	83	102	83	127	142	10^6	1.42×10^8
	mCIN[d] (ae)	120	137	130	130	131	134	130	10^6	1.30×10^8
	mCIN (mic[e])	123	170	73	120	124	122	122	10^6	1.22×10^8
	LBA[f]	148	235	104	116	83	88	129	10^6	1.29×10^8
IP 135	CIN (ae)	81	82	92	132	95	96	96	10^6	9.63×10^7
	mCIN (ae)	59	71	82	97	100	82	82	10^6	8.18×10^7
	mCIN (mic)	76	70	75	99	78	95	82	10^6	8.22×10^7
	LBA	88	96	92	119	90	113	100	10^6	9.97×10^7
ATCC 9610	CIN (ae)	84	137	86	68	71	89	89	10^6	8.92×10^7
	mCIN (ae)	99	85	90	69	63	104	85	10^6	8.50×10^7
	mCIN (mic)	70	85	90	82	88	95	85	10^6	8.50×10^7
	LBA	107	136	133	91	77	61	101	10^6	1.01×10^8
PC-M16-2	CIN (ae)	206	281	233	272	186	259	240	10^6	2.40×10^8
	mCIN (ae)	237	244	228	241	198	271	237	10^6	2.37×10^8
	mCIN (mic)	242	212	209	265	195	268	232	10^6	2.32×10^8
	LBA	217	223	195	265	174	229	217	10^6	2.17×10^8

[a] cfu/ml=cfu/total dilution factor; [b]CIN, Cefsulodin-Irgasan-Novobiocin; [c]ae, aerobic; [d] mCIN, modified CIN; [e] mic, microaerophilic; [f]LBA, Luria-Bertani agar

2. The cfu counts of IP135 and background microflora plated on LBA.

Bacterial strains	Replicate (cfu)						Average (cfu)	Dilution factor	cfu/ml
	1	2	3	4	5	6			
IP135	222	191	235	317	249	246	243	10^6	2.43×10^8
Microbiota in the meat sample	95	105	n.a.	n.a.	n.a.	n.a.	100	10^5	1.00×10^7