Investigation of a framework for epidemiological studies of *Campylobacter jejuni* based on the combination of genetic markers and MALDI-TOF MS typing schemes

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Abstract

Campylobacter jejuni is the most common food and water borne bacterial pathogen responsible for a significant burden to public health worldwide. This is due to the impacts associated with the costs of hospitalization and treatment, public health surveillance and the loss of working hours by those affected by this organism. Bacterial typing helps develop measures to control bacterial infections, and is an important tool in the epidemiological investigation of foodborne infections, by source tracing and revealing the routes of transmission of pathogenic bacteria, particularly foodborne pathogens.

Due to the sporadic nature of *C. jejuni* infections, the gathering of epidemiological data to enable prevention and control is difficult and there is a need for more rapid, cost effective and accurate methods to identify the type of strains associated with *C. jejuni* infections.

This study addressed this problem through the development of a framework for epidemiological investigation of *C. jejuni* infections using a collection of *C. jejuni* strains from environmental and clinical samples collected in Australia.

The framework was developed through the investigation of the suitability of combinations of four sequences based typing schemes and one MALDI-TOF MS based typing scheme.

The applicability of *porA, peb1A, mapA, ceuE* and *cdtA, cdtB* and *cdtC* genes was investigated in tetra, tri and double locus sequence typing schemes for typing of *C. jejuni*. The potential of MALDI-TOF MS for typing of *C. jejuni* strains was explored and assessed. The newly developed sequence based schemes were found to be suitable for local epidemiological investigation of *C. jejuni* infection such as outbreak investigation and zoonotic transfer due to their high discriminatory index and

epidemiological concordance. Incorporation of a small number of genetic loci makes the developed schemes rapid and cheaper alternative to multilocus typing schemes. Simple, rapid and high throughput method was also described for preliminary screening of *C. jejuni* using MALDI-TOF MS. The application of these typing schemes as described in this thesis provides a framework to address the need for more rapid, cost effective and accurate methodological approach to identify the origin of *C. jejuni* infections. The developed schemes should be further evaluated using adequate number of *C. jejuni* strains and in the parallel study using some other relevant typing schemes, *e.g.*, the pulsed field gel electrophoresis.

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Declaration

I, Monir Uddin Ahmed, declare that this thesis is my original work and contains no material that has been accepted for the award of Doctor of Philosophy, or any other degree or diploma, except where due reference is made.

To the best of my knowledge, I declare that this thesis contains no previously published or written materials by any other person except where due acknowledgment is made. Wherever contributions of others were involved every effort has been made to acknowledge the contributions of the respective workers or authors.

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List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
aspA	Aspartase A
bp	Base Pair (S)
BT	Binary Type
^{0}C	Degree Celsius
CDT	Cytolethal Distending Toxin
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
D (SID)	Simpson's Index of Diversity
DNA	Deoxyribonucleic acid
flaA	Flagellin Encoding Gene A
flaB	Flagellin Encoding Gene B
GBS	Guillain-Barre Syndrome
glnA	Glutamine Synthase A gene
gltA	Citrate Synthase gene
*gly*A	Serine Hydroxymethyl transferase gene
HRM	High resolution melting
JlpA	jejuni Lipoprotein A
Kb	Kilo base (S)
LOS	Lipooligoyshaccharides
MALDI-TOF MS	Matirx Assisted laser Desorption/Ionization - Time of Flight Mass Spectrometry
MFS	Miller Fisher Syndrome
Min	Minute

List of Abbreviation

mL	Milliliters
MLST	Multilocus Sequence Typing
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RA	Rheumatic Arthritis
RAPD	Randomly Amplified Polymorphic Deoxyribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
s	Second
SNP	Single Nucleotide Polymorphism
spp.	Species
ST	Sequence Type
SVR	Short Variable Repeat
tkt	Transketolase gene
Tm	Melting Temperature
uncA	ATP Synthase α Subunit
µL	Micro litre (S)

Chapter 1 . Introduction

1.1 The description of scientific problem addressed

C. jejuni is the most common food and water borne bacterial pathogen, which causes gastroenteritis in people of different age groups in developed countries such as Australia and underdeveloped countries worldwide (Parkhill *et al.*, 2000, Wilson *et al.*, 2008). The number of annual infections by *Campylobacter* spp. is estimated between 400 and 500 million cases worldwide (Talukder *et al.*, 2008). *C. jejuni* is primarily a foodborne pathogen (Butzler and Oosterom, 1991, McDowell and McElvaine, 1997, Kothary and Babu, 2001, Dasti *et al.*, 2010). Foods of animal origin, particularly poultry, are the most common risk factor for *C. jejuni* infection (Moore *et al.*, 1996, Mullner *et al.*, 2009b, Blaser, 1997). Milk and water are two other major sources of *C. jejuni* infections (Frost *et al.*, 2002).

The signs and symptoms of *C. jejuni* caused gastroenteritis are fever, abdominal cramps and bloody diarrhoea (Ketley, 1997). Sometimes the self-limiting gastroenteritis is followed by a complicated and long lasting sequelae such as Guillain Barre syndrome, Miller Fisher syndrome and Reactive arthritis (Moore *et al.*, 2005). Most of the *C. jejuni* infections are sporadic, occurring at unpatterned irregular times and localities and thus are disconnected in time and space. Outbreaks or clusters of infection in a distinct population by a single strain or a combination of strains are infrequently detected (Pebody *et al.*, 1997).

In Australia, *C. jejuni* is the most common notifiable food borne bacterial disease, and has a higher rate of *Campylobacter* infection than some other developed countries. For

example, the rates are approximately 8 times higher than in the United States of America (USA) (King and Adams, 2008). However, the real disease burden is substantially higher as these rates are based on notified cases. Being a self-limiting disease, a large number of infections are unreported (Wheeler *et al.*, 1999, Rayner and Scarborough, 2005, Mead *et al.*, 2000, Allos, 2001). Thus campylobacteriosis is a major public health problem in Australia as in other developed countries.

The majority of outbreaks in Australia have been foodborne or suspected foodborne, mostly occurring in settings such as restaurants, aged care centres and school camps. Poultry meats (chicken or duck) have been the major vehicles for the infection, with unpasteurized milk and water also implicated to a lesser extent (Unicomb *et al.*, 2009). Despite the high number of annual notified number of cases of *C. jejuni* in Australia, only a small number of the sources of infections and very few outbreaks are detected (Grills *et al.*, 2010).

The major reasons for this include the sporadic nature of infections making the gathering of epidemiological data resource intensive as well as the need for more rapid, cost effective and accurate methods to identify the type of strains associated with these infections (Grills *et al.*, 2010). The ability to rapidly and accurately detect the types of strains associated with infections, not only assist in conducting accurate and timely epidemiological investigations to identify the association between the disease-outbreak and source, but also enable regulatory authorities to undertake control and preventative measures more effectively.

The large number of infections and the severe sequelae of *C. jejuni* infection affects both the global and the Australian economy as a result of the impacts associated with the costs of hospitalization and treatment, public health surveillance and the loss of working hours by those affected (Grills *et al.*, 2010).

Increasingly outbreak investigations are becoming a major public health activity, hence developing tools, which can help in reducing *C. jejuni* infection by tracing source and route of transmission of *C. jejuni* is an area of intensive research.

Currently there is a greater reliance on subtype-based surveillance networks to detect geographically dispersed outbreaks and to assist in the early detection of contamination sources that otherwise may be difficult to detect. These measures contribute to the identification and addressing of gaps in the food safety system, enabling the prevention of food borne illness (Tauxe, 2006).

Two widely used techniques for developing bacterial typing schemes are DNA sequencing and matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) of bacterial proteins (Ahmed *et al.*, 2012, Eberle and Kiess, 2012, Lartigue, 2013b, Sandrin *et al.*, 2013, Wassenaar and Newell, 2000, Wieser *et al.*, 2012). Though no typing system is optimal for all forms of investigation in regard to cost, rapidity, reliability and discrimination power (Foxman *et al.*, 2005), sequence based methods are preferred for unambiguous data production and ease of data transfer, reproducibility and discriminatory power.

With respect to DNA sequencing based schemes, the two widely used sequences based typing schemes for *C. jejuni* are multilocus sequence typing (MLST) and flagellin (*fla*) gene sequence typing (Wassenaar, 2011, Wassenaar and Newell, 2000).

MLST uses a large number (seven) housekeeping genes and is suitable for global epidemiological studies (Urwin and Maiden, 2003). However, using more stable and less variable housekeeping genes, as stability and variability of genes influence discriminatory power, in large numbers makes MLST costly, laborious and inadequately discriminatory for local epidemiological studies (Dingle *et al.*, 2001, Korczak *et al.*, 2009, Urwin and Maiden, 2003). To use MLST for local epidemiological studies such as an outbreak study, additional markers are needed to supplement the seven loci (Dingle *et al.*, 2008).

Flagellin gene typing uses only one gene, either *fla*A , *fla*B or short variable region (SVR) of the *fla*A gene (Meinersmann *et al.*, 1997, Meinersmann *et al.*, 2005, Mellmann *et al.*, 2004). As a single locus poorly represents the whole bacterial genome, such methods are considered as the least reliable for epidemiological study (De Boer *et al.*, 2000, Olive and Bean, 1999). Moreover, the presence of two copies of the *fla* gene (*flaA* and *flaB*) and possible intragenomic and intergenomic recombination between the two copies undermines the reliability of typing naturally transformation competent *Campylobacter* spp. (Cornelius *et al.*, 2010, Guerry *et al.*, 1991, Harrington *et al.*, 1997). Similar is the case for other single locus typing schemes, clustered regularly interspersed short palindromic repeats (CRISPR) sequence typing and *porA* typing (Jay-Russell *et al.*, 2013, Schouls *et al.*, 2003).

Therefore options for investigating outbreaks of *C. jejuni* in Australia (or for localised infections in other geographical locations) using sequence based typing schemes involve the selection of one of the two extremes. Either multilocus (7 - 10 loci) based MLST typing which is costly, time consuming and labour intensive or single locus *fla* typing which may not provide reliable results. Given these challenges, the development of affordable, reliable and highly discriminatory sequence based typing schemes for epidemiological studies of *C. jejuni* infection is necessary.

Matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) can overcome some of the disadvantages of sequence based typing schemes. Within one to few hours typing results can be obtained, depending on the number of strains analysed, by MALDI-TOF mass spectrometry (Lartigue, 2013a). This technique has been employed for typing of a large number of pathogenic bacteria (Sandrin *et al.*, 2013). However, the applicability of this technique for typing of *C. jejuni* has not been explored.

In order to trace the source and to identify the route of transmission, in both global and local epidemiological studies, potent and well differentiating typing methods are required. In recognition of the need to address the high incidence of *C. jejuni* infections in Australia and the challenges associated with current subtyping methods, the Victorian Department of Health, Australia funded a PhD scholarship aimed at developing more rapid, cost effective and accurate methods of identifying the type of strains associated with *C.jejuni* infections.

This project was the recipient of such a scholarship. In order to address this problem, the project aimed to investigate the development of new typing schemes suitable for routine epidemiological surveillance of *C. jejuni* infection.

1.2 The overall aim the project

The overall aim of the project was to investigate the suitability of combinations of sequence-based and MALDI-TOF MS typing schemes as a framework for epidemiological investigation of *C. jejuni* infections using a collection of *C. jejuni* strains from environmental and clinical samples collected in Australia.

1.3 The specific aims of the Project

(i) Confirm taxonomic affiliation of the environmental and clinical isolates using two complementary molecular techniques such as 16S rRNA gene sequence analysis and matrix assisted laser desorption time of flight (MALDI-TOF) mass spectrometry (MS)

(ii) Comparatively evaluate eleven candidate markers (*cadF, cdtA, cdtB, cdtC, ceuE, flaA, fla SVR, jlpA, mapA, porA* and *peb1A*)

(iii) Design a methodological approach for tetra-, tri- and double-locus sequence – based typing schemes

(iv) Develop a conceptual framework for epidemiological studies of *C. jejuni* as a combination of suitable genetic markers and MALDI-TOF MS typing

1.4 Thesis outline

Sections 1.2 and 1.3 identify the areas of investigation used to define the objectives of the project. Chapter two presents a comprehensive review of literature to introduce the

major concepts relevant to this project. After brief history of *Campylobacter* spp. discovery, general properties of *C. jejuni* are described. Then comprehensive review of existing genotyping schemes is presented along with the guidelines for investigation of new typing schemes. In chapter three, materials and methods used in this project are described along with equipment and software used in analysis of results.

Chapter four outlines confirmation of the identification of collected *C. jejuni* strains by three different methods. In chapter five, selection and combination of markers are assessed based on a preliminary study. In the next chapters, discriminatory power, epidemiological concordance and comparison of different schemes to multilocus sequence typing are presented.

In chapter six, the *cdt* gene cluster was investigated for a sequence based typing scheme for *C. jejuni* using species-specific primers. Chapter seven characterises the tetra locus combination of *porA, peb1A, mapA* and *ceuE* genes for typing of *C. jejuni*. In chapter eight, employability of *porA, peb1A* and *mapA* was investigated as tri-locus typing scheme for *C. jejuni.* In chapter nine, double locus typing scheme based on *porA* and *peb1A* was evaluated in comparison to multilocus sequence typing and *flaSVR* typing. Chapter ten describes the use of MALDI-TOF MS for typing of *C. jejuni* strains using ethanol extracted protein samples. In the last chapter, chapter eleven, the major findings of the research project are summarised and future directions are outlined along with a general discussion.

Chapter 2 . Literature review

2.1 Introduction

Campylobacter jejuni subsp. *jejuni* (referred to as *C. jejuni* throughout this thesis) is a Gram negative, microaerophilic bacterium belonging to the genus *Campylobacter* of the family *Campylobacteriaceae* (Parkhill *et al.*, 2000, Wilson *et al.*, 2008, Veron and Chatelai.R, 1973, Steele and Owen, 1988). It is the major cause of bacterial gastroenteritis in humans in the world, both in developed and developing countries (Friedman *et al.*, 2000). It causes acute diarrhoea in humans and may also cause, though in rare cases, several debilitating sequelae namely Guillain Barre' syndrome (GBS) and Miller Fisher syndrome (MFS), Reactive arthritis (RA) and Reiters syndrome (RS) (Leung *et al.*, 1980, Nachamkin *et al.*, 1998). The number of annual cases of *Campylobacter* spp. infection is estimated at 400 to 500 million worldwide, around 90% of which is by *C. jejuni* (Talukder *et al.*, 2008, Wilson *et al.*, 2008). The most at risk groups both in developed and developing countries are children, the elderly and immunocompromised patients (Banmali *et al.*, 2006, Friedman *et al.*, 2000, Jain *et al.*, 2005). Poultry and livestock are major sources of *C. jejuni* infections (Yamazaki *et al.*, 2009). Most of the infections by *C. jejuni* are sporadic in nature and outbreaks are infrequently detected (Grills *et al.*, 2010, Pebody *et al.*, 1997). Though *C. jejuni* infection is self-limiting and rarely fatal, it causes a huge burden on the national economy from the high rate of infections in terms of medical care, treatment and loss of working hours and productivity (Buzby *et al.*, 1997, Buzby and Roberts, 1997). Hence it is an important pathogen from a public health and economic point of view and substantial effort has been made to better understand the ecology, occurrence and pathogenicity of this global and the most common foodborne pathogen in Australia.

However there is still significant knowledge gaps with respect to the bacterium with genotyping used a basic tool for contributing to these knowledge gaps. In this literature review, the epidemiology, cultural and clinical properties of *C. jejuni* will be discussed along with current genotyping and MALDI-TOF based typing schemes available for *C. jejuni.*

2.2 Historical background and evolution of the genus *Campylobacter*

Campylobacters have been widely known human pathogens since 1970's (Rautelin and Hanninen, 2000). However, German bacteriologist Theodor Escherich reported, first in 1886, that he had observed spiral bacteria in the colons of infants who had died from enteric disease (Escherich, 1886). He could not cultivate these bacteria on solid media, finding these organisms more frequently in stool specimens from infants with enteric disease. Escherick concluded that these bacteria played no role in the aetiology of enteritis (Kist, 1985). In the next few proceeding years, several non-culturable bacteria with similar characteristics were reported but further investigations of those bacteria were still difficult due to lack of suitable selective media. However, two veterinary surgeons, later in 1909, reported the discovery of the unknown *Vibrio*-like organism, frequently isolated from the aborted foetuses of ewes (McFadyean, 1913).

Later in 1919 (Smith and Taylor, 1919), Smith and Taylor isolated a spiral bacterium from aborted bovine foetuses. They observed that this bacterium was identical to the *Vibrio*-like bacterium of McFadyean and Stockman and proposed the name '*Vibrio foetus'* for these organisms (Smith and Taylor, 1919). In 1931, Jones and colleagues linked winter dysentery in calves with a '*Vibrio'* they called '*Vibrio jejuni'*(Jones, 1931). In 1944, a similar organism associated with dysentery in swine was reported

(Doyle, 1944). Thus the role of campylobacters as veterinary pathogens was established. However, the first well-documentation of human campylobacter infection occurred in 1938, which involved a milk-borne outbreak of enteritis. Only 10% of the cases, positively identified by using microscopy only, were grown in liquid blood culture (Levy, 1946).

The genus *Campylobacter* was proposed in 1963, bringing a major change in *Vibrio* taxonomy. Sebald and Véron (Sebald, 1963) proposed the name *Campylobacter* (Greek, campylo: 'curved') for a new genus to separate '*Vibrio*' like bacteria included in this genus and which had low G+C mol % composition and microaerophilic growth requirements. Initially, the genus *Campylobacter* contained two species, '*C. fetus'* and '*C. bubulus'*. Following the development of a filtration technique by Dekeyser and colleagues in 1972 (Dekeyser, 1972), which allowed the first isolation of campylobacters from faeces, the number of species increased dramatically in the following years. The development of a selective medium by Skirrow in 1977 and increased availability and sophistication of equipment and methods for generating microaerophilic environments brought revolution in campylobacter research. The genus *Campylobacter* is still evolving and 28 species were reported with 8 subspecies (as of January, 2014, http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=194). The major species are *C. jejuni, C. coli, C. lari, C concicous, C. upsaliensis, C. ureolyticus, C. helveticus* and *C. fetus.*

2.3 *Campylobacter jejuni*

2.3.1 Morphology

C. jejuni is slender spirally curved rod and motile with rapid corkscrew or darting motion with a single unsheathed polar flagellum at one or both ends (Fields and Swerdlow, 1999). In size, it is 1.5 - 6.0 µm by 0.2 - 0.5 µm. The spiral shape is useful in its identification in a clinical context as few clinically important bacteria are spiral in shape (Levin, 2007).

2.3.2 Growth conditions and metabolism

For growth, *C. jejuni* requires about 10% oxygen, 5% carbon dioxide and 85% nitrogen. It has an optimal temperature range of 42^0C- 45^0C and optimal pH range of 5.8 to 8 but it cannot grow below pH 4.9 and under 30^oC (Kelana and Griffiths, 2003, Levin, 2007). However, the temperature of 37^oC is usually used for growth of *C. jejuni*. Growth occurs based on respiratory metabolism and the bacterium is unable to ferment or oxidize carbohydrates. Energy is obtained from amino acids and intermediates of tricarboxylic acid cycle (Mohammed *et al.*, 2004). This bacterium produces both catalase and superoxide dismutase for the breakdown of H_2O_2 and superoxides but it is highly sensitive to free radicals and superoxides (Grant and Park, 1995, Cabiscol *et al.*, 2000).

2.3.3 Isolation of *C. jejuni*

2.3.3.1 Sources of *C. jejuni*

C. jejuni is isolated mostly from foods and faeces. It can also be isolated from environmental samples (Williams *et al.*, 2012). Foods include raw and under cooked meat of chicken, duck, pig, and cow. Milk and poultry faeces are two other major

sources for *C. jejuni*. Environmental samples such as recreational and potable water and farm soil are investigated for *C. jejuni*. Isolation of *C. jejuni* from these samples is usually performed by direct plating onto selective solid media or enriching and then incubating under microaerophilic conditions.

2.3.3.2 Enrichment media

Preston and Bolton broth are two widely used broths for enrichment of *Campylobacter* spp., particularly *C. jejuni*. Bolton broth is recommended by ISO10272-1: 2006 and Food and Drug Administration (FDA), USA for isolation of *C. jejuni* from a wide variety of samples, such food, faeces and soil (Bi *et al.*, 2013). Preston both is widely used for isolation of *C. jejuni* mainly from foodstuffs (Pearson *et al.*, 2000). However, mExeter was found to be more effective as an enrichment medium for isolation of *C. jejuni* from environmental samples such as farm environment (Williams *et al.*, 2012). Recently developed food borne pathogen enrichment broth has been found as effective as Preston and Bolton broth for enrichment of *C. jejuni* (Hayashi *et al.*, 2013). However, the Preston broth for enrichment and then platting on mCCDA media is the optimal isolation method for *C. jejuni* (Meng *et al.*, 2012).

2.3.3.3 Selective agar media

Two types of selective culture media are used for *C. jejuni* platting: blood based and blood free. Skirrow or Sk medium was developed in 1977 and is one of the most widely used blood based agar medium for *C. jejuni* isolation (Skirrow, 1982). Other blood based agar media for *C. jejuni* include Preston agar, Butzler agar, Karmeli agar and their modified versions (Levin, 2007). Modified Charcoal cefoperazone deoxy cholate agar (mCCDA) is the only blood free agar media widely used for platting *C. jejuni* (Bi *et al.*,

2013). Blood free media have advantages over blood-based media as blood makes the medium expensive, short lived and vulnerable to contamination.

2.4 Clinical manifestations of *C. jejuni* infections

2.4.1 Disease manifestation

Incubation period for *C. jejuni* infection varies from 24 - 72 hours and the infectious dose is as low as 500 bacteria (Kothary and Babu, 2001). Typical signs and symptoms of *C. jejuni* infection include acute, self-limiting gastroenteritis, which is characterized by diarrhoea, fever and abdominal cramps. Diarrhoea is initially watery and may last for more than two weeks in case of traveller's diarrhoea and may also become bloody if persists (Padungton and Kaneene, 2003). Following exposure, *C. jejuni* colonizes in the ileum, jejunum and colon without causing any symptoms. In case of symptomatic infections, the first 1-3 days present prodromal symptoms such as fever, vomiting and headaches which are followed by watery or bloody diarrhoea and abdominal pain (Dasti *et al.*, 2010). People of all ages are infected by *C. jejuni* but children below the age of four and people of the age group 15 - 39 are found more vulnerable to *C. jejuni* infection (Dasti *et al.*, 2010). Males are found more vulnerable to *C. jejuni* infection than female in all age groups (Friedman *et al.*, 2004).

Though campylobacteriosis is mild in nature and self-limiting, serious complication may arise when invasive *C. jejuni* strains translocates and reaches the blood flow (Moore *et al.*, 2005). Invasive disease such as bacteraemia in pregnant women can cause abortion and premature birth (Simor *et al.*, 1986). As maternal bacteraemia caused by *C. jejuni* is associated with fetoplacental involvement, it can cause premature labor, perinatal sepsis and neonatal meningitis (Simor *et al.*, 1990). If the infection occurs

before the third trimester of pregnancy, fetal and neonatal mortality can be as high as 80% (Crushell *et al.*, 2004).

2.4.2 Sequelae of *C. jejuni* infection

Both short term and long-term sequelae follow *C. jejuni* infection. Short-term sequelae include Guillain–Barre´ syndrome (GBS) and Millar Fisher syndrome (MFS). Long-term sequelae include Reactive arthritis (RA) and Reiters syndrome (Leung *et al.*, 1980, Nachamkin *et al.*, 1998).

Guillain–Barre´ syndrome (GBS) is a baleful complication of campylobacter enteritis. It is an acute progressive neuropathy characterized by muscular paralysis, pain, muscular weakness and distal sensory loss. Affected people develop weakness of the limbs and the respiratory muscles and areflexia. GBS is now the leading cause of acute flaccid paralysis in the world after the marked reduction of poliomyelitis and estimated that approximately one case of GBS occurs in every 1000 cases of *C. jejuni* enteritis. Thus *C. jejuni* is the most common antecedent of GBS. From serological and cultural data, it was estimated that 30 to 40% GBS patients had infection with *C. jejuni* between 10 days and 2 weeks before the onset of neurological symptoms. About 20% of GBS patient are left with some disablement, sometimes needing mechanical ventilation and about 2 – 3% of cases result in death with many more occurring in the developing countries of the world (Crushell *et al.*, 2004, Nielsen *et al.*, 2010). Certain types of *C.jejuni* strains are associated with GBS development. Penner's *C. jejuni* serotype HS: 19 were found more frequently present in GBS (67%) than in enteritis (6%) patients. Among O serotype, O: 19 and O: 41 are more frequently found in GBS patient than other types **(**Nachamkin *et al.*, **1998**, Takahashi *et al.*, **2005)**. Respiratory failure,

cardiac arrhythmias and pulmonary embolism resulting from GBS cause death (Peake *et al.*, 2004).

Miller- Fisher syndrome (MFS) is also associated with *C. jejuni* infection. It is considered as a variant of GBS and characterized by ophthalmoplegia, ataxia and areflexia. It is less common than GBS (Crushell *et al.*, 2004). Penner's *C. jejuni* serotype HS:2 was found more frequently in MFS (41%) than in enteritis (14%) patients (Takahashi *et al.*, 2005).

Reactive arthritis (RA) is other long-term sequelae of campylobacteriosis, which is an immune-mediated squeal. It is reactive arthropathy and affects the knee and other peripheral joints. It occurs in between 0.6% and 24% of the patients. Multiple joints can be affected with symptoms of pain and incapacitation, which usually recovers completely within several months (Crushell *et al.*, 2004, Nielsen *et al.*, 2010).

Reiter syndrome (RS), which can be triggered due to infection by a number of bacteria**,** is another long-term sequel of *C. jejuni* infection. It is comprised of the triad of asymmetric arthritis, urethritis and opthalmitis in HLA B-27 positive patients (Crushell *et al.*, 2004).

2.5 Epidemiology of *C. jejuni*

2.5.1 Reservoirs

C. jejuni is ubiquitous in the environment, wild birds and mammals (Cody *et al.*, 2009, Dasti *et al.*, 2010). Campylobacters infectious to human are found in the intestines of

many wild, domestic and farm animals (Moore *et al.*, 2005). Cattle, sheep, poultry (chicken, duck, turkey), and pigs are reservoirs and they harbour highly diverse strains of *Campylobacter* spp. (Colles *et al.*, 2003). They are also found in companion animals such as cats and dogs (Moore *et al.*, 2005). As these animals can excrete *Campylobacter* spp. anywhere, these bacteria can be found throughout the environment including soil, beach sand, sewage, and groundwater (Ogden *et al.*, 2009).

2.5.2 Route of transmission to human

C. jejuni is primarily a food borne pathogen (Butzler and Oosterom, 1991, McDowell and McElvaine, 1997, Kothary and Babu, 2001, Dasti *et al.*, 2010). Foods of animal origin, particularly poultry, are the most common risk factor for *C. jejuni* infection (Moore *et al.*, 1996, Mullner *et al.*, 2009b, Mullner *et al.*, 2009a). Any raw meat may become contaminated with *C. jejuni* and cause infection when eaten undercooked. Raw or undercooked beef, hamburgers, sausages and clams are found to be involved in outbreaks though food borne outbreaks are not frequent (Butzler, 2004). Raw or inadequately treated milk and water have been found as sources of massive outbreaks (Frost *et al.*, 2002). Water activities in contaminated surface water are also potential sources for human infections (Wilson *et al.*, 2008). Direct transmission of *C. jejuni* occurs to personnel involved in poultry industry such as butchers, abattoir workers, poultry processors (Jones and Robinson, 1981). Pets are also sources of such direct transmission (Sopwith *et al.*, 2003). Inter- human transmission is reported for children (Karmali *et al.*, 1984, Shimizu *et al.*, 1986). Neonatal enteritis occurs if the mother is excreting *Campylobacter* spp. Perinatal transmission may occur from asymptomatic mother following passage in the uterus, during passage through the birth canal and first few days of life (Dasti *et al.*, 2010). However, direct infection from man to man is of no

large epidemiological relevance (Friedman *et al.*, 2000). The main route of campylobacter transmission to human was best summarized by Dasti and colleagues (Dasti *et al.*, 2010). The researchers found that humans can be infected mainly from milk/meat, companion animal such as dog, cat, water, chicken and birds and other poultry. They pportrayed the most important routes for human infection by *C. jejuni* in the following figure.

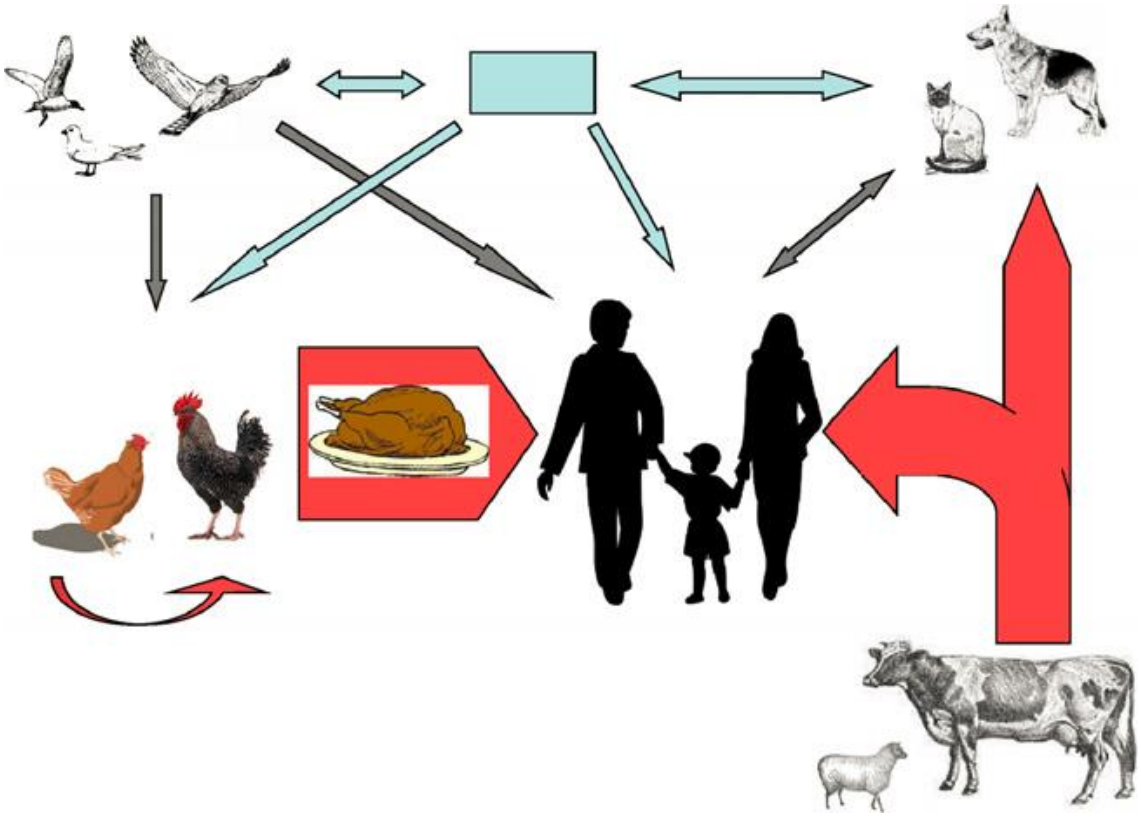

Figure 2.1. The most important routes of human infections by *C. jejuni* (Dasti et al, 2010). [1]

2.5.3 Global scenario of campylobacteriosis

Campylobacteriosis, diseases caused mostly by *C. jejuni*, has been recognized as the most common bacterial gastroenteritis in many countries since the late 1970s (Friedman *et al.*, 2004). It is the major foodborne bacterial enteritis worldwide, both in developed

[1] With permission from Elsevier

and developing countries (Jain, 2008, Senok and Botta, 2009). In the developing countries, there is no national surveillance program for *C. jejuni* though the burden of the disease is substantial. In developing countries, it appears that *Campylobacter* spp. are the most frequently isolated bacteria from infants with diarrhoea (Coker *et al.*, 2002) with infection rates vary between 5% to 20% in children with diarrheal diseases (Jain *et al.*, 2008).

The number of annual cases of campylobacter infection is estimated at 400 to 500 million cases worldwide (Friedman *et al.*, 2000). In the USA and UK, the infection rate was estimated as high as 1% of the population per year. In USA the estimated infection of 2.4 million cases caused 124 deaths per year (Friedman *et al.*, 2004, Jain, 2008). The infection rate of 12.78 per 100,000 populations in 2007 was still above of the national target of 12.30. It was also reported that *Campylobacter* spp. are the most common enteric pathogen in Canada and infection rate was about 29 per 100,000 populations per year (Khan *et al.*, 2009). Similarly in the European countries, *Campylobacter* spp. are the most common cause of reported bacterial gastroenteritis. Campylobacteriosis is also the most frequently reported food-associated infection in Austria. The average incidence for 2007 was calculated as 73.4 per 100,000 populations and the incidence has increased continuously throughout Austria since the start of notification of campylobacteriosis in 1996. In 2007, campylobacteriosis was almost double (6077) of salmonellosis cases (3587) (Feierl and Jelovcan, 2009). Campylobacters were the leading cause of food borne bacterial gastrointestinal illness in Belgium (Sampers *et al.*, 2008). It is the most common food borne enteropathogen in the Nordic countries, Sweden, Norway, Finland and Denmark (Rautelin and Hanninen, 2000). Thus it is a global public health problem including the developed countries.

2.5.4 *C. jejuni* infections in Australia and New Zealand

C. jejuni is the most common notifiable food borne bacterial disease in Australia. According to National Notifiable Diseases Surveillance System (NNDSS) of Australia, the annual infection rate was 102.3 per 100,000 populations in 2012, which is about 8 times higher than that of national target in the USA (12.30 per 100,000 populations). Noteworthy is that these figures show only a proportion of the true incidence as campylobacteriosis is not a notifiable disease in New South Wales (King and Adams, 2008). According to NNDSS, the infection rate in the state of Victoria was 104.3 in 2012 and 98.4 in 2013 per 100,000 populations.

New Zealand is an exceptional country in terms of campylobacteriosis. It has an exceptionally high rate of campylobacter infection. The rate of the infection reached 396/100,000 population in 2003, which was the highest reported rate worldwide. At the same time, the infection rate was 116.5 cases/100,000 population in Australia, 85.4/100,000 in England and Wales, 86.6/100,000 in Scotland and 12.6/100,000 populations in the United States (Baker and Wilson, 2007, Rind and Pearce, 2010). Australia also has a higher rate of campylobacter infection than most other developed countries. However, the real disease burden is substantially higher as the above-mentioned rates are based on notified cases. Being self-limiting disease, a large number of infections are unreported. It was found that only 1 in 7 cases in the United Kingdom (Wheeler *et al.*, 1999) and 1 in 38 in the United States (Mead *et al.*, 1996) were reported. Thus campylobacteriosis is a major public health problem in Australia as in other developed countries.

2.5.5 Outbreak and sporadic *C. jejuni* infection in Australia

Most of the *C. jejuni* infections are sporadic; occurring at unpatterned irregular moments and localities and thus are disconnected in time and space. Outbreaks, cluster of infection in a distinct population by a single strain or a combination of strains, are only infrequently detected (Pebody *et al.*, 1997). From January 2001 and December 2006, there were 33 campylobacter outbreaks reported in Australia, which affected 457 persons (Unicomb *et al.*, 2009). Only 8 campylobacter outbreaks were detected during 2001-2008 (Kirk *et al.*, 2011). These outbreaks most commonly occurred (45%) during the Australian spring (September to November), when notifications of sporadic infections generally peaked. More than eighty precent (82%) of these outbreaks were food borne or suspected foodborne and most of them occurred in commercial settings such as restaurants, aged care centres and school camps. Poultry meats (chicken or duck) were found as the major vehicles for outbreak (Unicomb *et al.*, 2009). Unpasteurized milk and water were also found as vehicles for outbreak (Unicomb *et al.*, 2009). Similar outbreaks were reported in other countries such as England, The Netherlands (Frost *et al.*, 2002, van Duynhoven *et al.*, 2005, Smith *et al.*, 2006). Water borne outbreaks due to contaminated drinking water are common in Nordic countries Sweden, Norway and Finland (Moore *et al.*, 2005). However, the number of identified *C. jejuni* outbreaks is very small in comparison to sporadic infections. In Victoria, only 1-3 outbreaks caused by *C. jejuni* were detected annually from 2002 to 2007. This is despite the number of annual notified number of cases being more than 6000 (Grills *et al.*, 2010). The major reason identified for such small number of outbreak identification is the typing of only a very small fraction of *C. jejuni* strains isolated from apparently sporadically infected gastroenteritis patients. For example, only 12% of campylobacter isolates were subtyped in Victoria in 2007 (Grills *et al.*, 2010). An increased number of

isolates need to be typed for detection of both outbreak and common source of sporadic infections. The ability to rapidly and accurately detect the types of strains associated with outbreaks, not only assists in conducting accurate and timely epidemiological investigations to identify the association between the disease outbreak and source, but also enable regulatory authorities to undertake control and preventative measures more effectively. Increasingly outbreak investigations are becoming a major public health activity, and there is a greater reliance on subtype-based surveillance networks to detect geographically dispersed outbreaks, to assist in the early detection of contamination sources that otherwise may be difficult to detect. This assists in identifying and addressing gaps in the food safety system and thus enable the prevention of food borne illness (Tauxe, 2008).

In order to trace the source and to identify the route of transmission, in both global and local epidemiological studies, potent and well differentiating typing methods are required.

2.6 Control of *C. jejuni* infection in human

Like any other foodborne infections, reduction of the *C. jejuni* infection in human depends on 1) rapid detection of contaminated foods to avoid human consumption of infected foods and 2) detection of sources and route of transmission of the bacterium so that measures can be taken for prevention of food contamination by *C. jejuni* (Sheppard *et al.*, 2009, Velusamy *et al.*, 2010).

To detect *C. jejuni* in foods, cultural, immunological and DNA based methods are being used which are time consuming, laborious and expensive and laboratory confined.

There is a need to develop rapid methods for on-site and real time applications. Biosensors have the desired rapidity with potential in field level application (Ricciardi *et al.*, 2009). Bacterial typing plays crucial role in epidemiological surveillance to trace the source of infection and find out the route of transmission and subsequently develop infection control measures (Li *et al.*, 2009).

2.6.1 Rapid detection of *C. jejuni*

Detection of a contaminating pathogen in food is very important from the public health safety perspective. Rapid detection methods are crucial to evaluate food safety, usefulness of control measures in minimizing pathogens in production and processing environments and biological and ecological properties of pathogenic microorganisms in food environment (Mandrell and Wachtel, 1999). Legislation aimed at promoting food safety exists in developed countries such as USA, Canada, Australia and European Union as failure to detect contamination may be fatal. Failure to detect contamination during food processing can have significant economic impacts on food manufactures, due to issues such as those associated with product recall and consumer confidence if contamination is detected by state or local public health regulatory agencies. Hence methods for rapid detection of food borne pathogens are in high demand from both food processors and regulatory agencies to reduce the incidence of food borne illness, death and economic loss (Lazcka *et al.*, 2007, Taylor *et al.*, 2006).

Cultural identification of *C. jejuni* takes 5 – 7 days for complete identification (Zorman and Mo ina, 2002). The major limitations of immunological methods are that these methods are confined to the laboratory and cannot detect pathogens in real time (Velusamy *et al.*, 2010). As a result, these methods could not be used routinely by food

processors and regulatory agencies in the field level. DNA based methods, particularly PCR based identification, require skilled laboratory personnel and a well-equipped laboratory. The distinction between dead and live pathogen is also not possible by such DNA based methods.

Biosensors can overcome the limitations of immunological and DNA based methods and has the potential for real time pathogen detection. As a result, it is the fastest growing technology for pathogen detection (Lazcka *et al.*, 2007). However, very few biosensors have been described for *C. jejuni*. Those which have been described include the ELISA-enzyme electrode (Che *et al.*, 2001), array biosensor (Sapsford *et al.*, 2004), highly dispersed carbon particles (Chemburu *et al.*, 2005), surface plasmon resonance and flow through immunoassay biosensors (Chemburu *et al.*, 2005). However, none of these biosensors can be used in field level due to high detection limit and failure to miniaturization. Optical biosensors with whisper gallery modes transducer system have the potential to design the label free, miniature and ultra-sensitive biosensors (Vollmer and Arnold, 2008). Such a biosensor for *C. jejuni* detection can be developed using *C. jejuni* specific receptor, such as membrane associated protein A (MAPA) (Stucki *et al.*, 1995) as target bacterial receptor and whisper gallery mode as transducer (Vollmer and Arnold, 2008). Future project can be taken to develop such useful biosensor to complement *C. jejuni* routine identifiocation.

2.6.2 Genotyping methods available for typing *of C. jejuni*

Over the last decades, several typing methods have been developed for *C. jejuni* strain differentiation, with phenotyping methods being routinely used for epidemiological studies of *C. jejuni* (Schouls *et al.*, 2003, Shi *et al.*, 2002). The most widely employed

phenotypic method is the serotyping technique developed by Penner and Hennessy (Shi *et al.*, 2002), which is based on detection of heat-stable (HS) antigens. Other phenotypic methods include biotyping, phage typing, and antibiogram. However, these methods have failed to provide the necessary discrimination power, typeability and reproducibility for effective epidemiological application (Moore and Goldsmith, 2001, Moore *et al.*, 2002). These methods are also considered expensive and labour intensive (Schouls *et al.*, 2003). Molecular genotyping methods have the potential to overcome these difficulties. As a result, a number of molecular genotyping methods have been tested in epidemiological studies of *C. jejuni* (Schouls *et al.*, 2003).

The genotyping methods currently developed for *C. jejuni* typing can be grouped differently based on differentiation criteria. Based on how much of the genome is tested, *C. jejuni* typing methods can be grouped as a) whole genome typing b) multi locus typing and c) single locus typing. This chapter discusses major genotyping methods used for *C. Jejuni* in three groups based on locus used for typing; assess the available genotyping approaches and evaluate their advantages, disadvantages regarding their potential application.

2.6.2.1 Whole genome-based methods

Whole genome based methods investigate polymorphism throughout the genome and thus represent most of the polymorphisms in the strains. There are three methods, pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) and randomly amplified polymorphic DNA (RAPD), that can be employed for comparative evaluation of genomic polymorphism of *C. jejuni* strains.

2.6.2.1.1 Pulsed field gel electrophoresis (PFGE)

PFGE or micro restriction profiling was developed in 1982 by Schwartz and colleagues (Schwartz *et al.*, 1982) to separate large DNA segments, to create genomic maps and calculate the genome size of organisms. Over time, it has evolved as the most commonly used whole genome typing method for its high resolution. Now PFGE is commonly used to study the molecular epidemiology of infectious pathogens, such *as E. coli, Streptococcus* spp., *Staphylococcus* spp., *Neisseria meningitides*, *Vibrio cholerae, Bordetella pertussis,* and *C. jejuni* (Goering, 2010, Ribot *et al.*, 2001). It is generally accepted as one of the most discriminatory genotyping methods available for genotyping of *Campylobacter* spp., particularly *C. jejuni*. However, the sensitivity and discriminatory power of PFGE depends on the organism being subtyped and the restriction enzymes included. *Sal*I, *Kpn*I, *Sac*II and *Bam*HI (On *et al.*, 1998, Gerner-Smidt and Scheutz, 2006, Höök *et al.*, 2005) and *Sma*I (Ge *et al.*, 2006, Höök *et al.*, 2005, Rönner *et al.*, 2005, Wilson *et al.*, 2009a) are commonly used restriction enzymes. The rapid PFGE protocol for *C. jejuni* was based on the standardized PFGE procedure developed by PulseNet (http://www.cdc.gov/ncidod/dbmd/pulsenet/pulsenet.htm), the national network of public health and food regulatory agency laboratories coordinated by the Centers for Disease Control and Prevention (CDC), USA; more details regarding the laboratory protocols can be found in articles by (Tenover *et al.*, 1997, Behringer *et al.*, 2011, De Boer *et al.*, 2000, Oyarzabal *et al.*, 2008, Wilson *et al.*, 2009a).

PFGE is a powerful technique for detection of microevolution in isolates that cannot be distinguished by multilocus sequence typing (MLST) or multilocus enzyme electrophoresis (MLEE) (Sails *et al.*, 2003c, Sails *et al.*, 2003b). This method has been

extensively used in epidemiological studies of *C. jejuni* ranging from the outbreak investigation (Heuvelink *et al.*, 2009, Olsen *et al.*, 2001, Pitkänen *et al.*, 2008), persistence of genotypes in a population (Petersen and Wedderkopp, 2001) or environment (Hakkinen *et al.*, 2007), diversity exploration of sporadic infection isolates (French *et al.*, 2009, Uzunović-Kamberović *et al.*, 2007), dissemination of antibiotic resistant strains (Bae *et al.*, 2007) to the comparison of genotypes within and between hosts (Acke *et al.*, 2010, Parsons *et al.*, 2009, Rozynek *et al.*, 2010), in human (Islam *et al.*, 2009) and in retail meats and broilers (Ge *et al.*, 2006, Oyarzabal *et al.*, 2013).

Though PFGE is considered as the gold standard for microbial epidemiological studies due to its high discriminatory power, however it has several limitations (Sails *et al.*, 2003c). The major limitation is its inapplicability in long term and non-outbreak epidemiological studies due to high sensitivity to small genetic changes (Wassenaar *et al.*, 1998, Sails *et al.*, 2003a). Such limitations were discussed in several studies (De Boer *et al.*, 2002, Barton *et al.*, 2007). Other limitations include non-digestibility of some *C. jejuni* strains' DNA with commonly used restriction enzymes, requirements of pre-treatment of DNA samples with formaldehyde to deactivate DNase activity (Gibson *et al.*, 1994). Poor inter-laboratory comparability is another limitation of PFGE like any other gel-based methods. In spite of all these limitations, PFGE is the most commonly used method to monitor and identify *C. jejuni* outbreaks and source attribution. Recently this method was successfully used for understanding the sources of campylobacteriosis in New Zealand (Gilpin *et al.*, 2013) and detected emerging highly pathogenic strains in the USA (Sahin *et al.*, 2012).

2.6.2.1.2 Amplified fragment length polymorphism (AFLP)

AFLP was developed initially by Vos and colleagues (Vos *et al.*, 1995) for plant genome analysis. Due to its ability to generate typing of any DNA regardless of origin and complexity, it has been successfully adapted for bacterial genomic DNA fingerprinting. AFLP was first evaluated by Duim and colleagues for epidemiological analysis of *C. jejuni* isolates (Duim *et al.*, 1999). Later the method was used to study the persistence of *C. jejuni* clones in the environment and host (Fitzgerald *et al.*, 2001, Manning *et al.*, 2001), genetic relatedness (Alter *et al.*, 2005, Siemer *et al.*, 2004), differentiation among species, subspecies and strain (Fang *et al.*, 2006). It was also used for detection, identification and comparison of disease markers (Duim *et al.*, 2000, Duim *et al.*, 1999, Godschalk *et al.*, 2006) and strain diversity (Coote *et al.*, 2007). As AFLP is based on selective amplification of restriction fragments generated from the whole genomic DNA, this technique is useful for the differentiation of genetically related strains (Janssen *et al.*, 1996). As this method produces multiple bands from the whole genome, there is no under presentation due to point mutations or single locus recombination (Duim *et al.*, 1999).

AFLP is a three-step technique, which involves i) complete digestion of the chromosomal DNA with two restriction enzymes (4 and 6 bp recognition sites) and ii) ligation of oligonucleotide adapters, iii) the selective amplification of restriction fragments, and gel analysis of these amplified fragments (Janssen *et al.*, 1996). The discriminatory power of AFLP is determined by the set of restriction enzymes and primers (Duim *et al.*, 2003, Duim *et al.*, 1999, Janssen *et al.*, 1996).

Whole genome analysis combined with automated data acquisition and analysis led to the development of high resolution AFLP for *C. coli* and *C. jejuni* (Saengthongpinit *et al.*, 2010). This technique does not require prior knowledge of genome sequences as required in PFGE and RAPD-PCR (Vos *et al.*, 1995). However, it needs intact purified double stranded DNA, specialized equipment and software (Duim *et al.*, 1999). Being a gel based method; it shows differences in fingerprints of identical sample and sensitivity depends on the restriction enzymes used. These two factors affect reproducibility of AFLP and limit its routine use (Duim *et al.*, 2001). It's clustering was found not to correlate with that of MLST (Lévesque *et al.*, 2012). The modified version of AFLP, fluorescent amplified length polymorphism (FAFLP) that incorporates fluorescently labelled primers (*Hind*III+A and *Hha*I+A), has overcome this limitation (Desai *et al.*, 2001). This improved AFLP was successfully used for the epidemiological investigation of campylobacters in the food chain (humans, pigs, cattle, poultry, and retail meats) and for the evaluation of the genetic diversity of thermotolerant campylobacters in commercial broiler flocks and broiler farm's environment (Messens *et al.*, 2009). Recently it was used to detect undiagnosed outbreaks in Spain (Pérez-Boto *et al.*, 2013).

2.6.2.1.3 Randomly amplified polymorphic DNA (RAPD)

RAPD is a rapid typing method based on randomly amplified polymorphic DNA. In this method, a single 10-mer primer is used to amplify multiple locations that are randomly distributed throughout the whole genome and produce a spectrum of amplified products characteristic of the template DNA. The amplified fragments are then separated by agarose gel electrophoresis to create a genetic profile for each isolate. As the entire genome becomes accessible to priming and amplification, polymorphisms throughout the genome can be detected (Welsh and McClelland, 1990, Williams *et al.*, 1990).

RAPD method for genotyping of *Campylobacter* spp. was developed by Mazurier and colleagues (Mazurier *et al.*, 1992) using three 10-mer random primer separately. Later various primers and reaction conditions have also been developed for RAPD analysis of *Campylobacter* spp. RAPD was found to be highly discriminatory in investigating the genetic diversity of *C. jejuni* and *C. coli* isolates from different sources (Hilton *et al.*, 1997, Madden *et al.*, 1996, Misawa *et al.*, 2000, Payne *et al.*, 1999). The method is simple, rapid and showed 100% typeability in some studies (Chuma *et al.*, 1997). Another advantage of RAPD is that DNA purification is not necessary (Mazurier *et al.*, 1992). However, RAPD-DNA patterns change with template DNA concentration (Gibson *et al.*, 1994). Poor reproducibility, the inherent limitation of all gel-based methods, outweighs the simplicity, rapidness and cost effectiveness of this method (Fayos *et al.*, 1993, Meunier and Grimont, 1993). Some strains become non typeable due to the variation in DNA extraction and the presence of DNase (Fayos *et al.*, 1993). In spite of these disadvantages, RAPD was used in different epidemiological studies such as genotypic antimicrobial resistance analysis (Giacomelli *et al.*, 2012b), longitudinal study of thermophilic *Campylobacter* spp. for genetic diversity (Giacomelli *et al.*, 2012a).

2.6.2.1.4 DNA Microarrays

DNA Microarray technology is used for large scale genomic interrogation of bacterial pathogens as it is able to provide considerable insights into intra species genetic diversity and microbial evolution (Pearson *et al.*, 2003). Two applications of DNA microarray are complementary DNA (cDNA) expression arrays and comparative genome hybridisation (CGH). Comparative genome hybridisation (CGH) is relevant and used extensively for typing or characterization of *C. jejuni* strains (Taboada *et al.*,

2005). Two different types of CGH approaches are taken based on whether the genome of the strain is characterized by whole genome sequencing or not to study intra-species *C. jejuni* genetic composition. Microarrays for the strains whose genome previously been characterized are constructed from library clones or PCR products generated for each open reading frame (ORF). Shotgun library probes are constructed for those strains that remain uncharacterized by genome sequencing (Poly *et al.*, 2005). Recently this method was used to characterize *C. jejuni* strains from water and wildlife (Stabler *et al.*, 2013) and revealing genetic diversity of *C. jejuni* from commercial turkey (El-Adawy *et al.*, 2013).

2.6.2.2 Multilocus based typing methods

In such cases, multiple loci dispersed throughout the genome come under investigation for genetic polymorphisms. These methods do not reveal the genetic polymorphisms from the whole genome but multiple markers ideally distributed around the genome well represent polymorphisms in the genome and give high discriminatory power.

2.6.2.2.1 Multilocus sequence typing (MLST)

MLST is based on the sequencing of a set of essential or housekeeping genes that are present in all strains of a bacterium. This method was first described by Maiden and colleagues for *Neisseria meningitis* based on sequencing of seven slowly evolving housekeeping genes (Maiden *et al.*, 1998). Since then the method has been applied to many pathogenic and environmental bacteria and eukaryotes (Hannis *et al.*, 2008). For MLST, the housekeeping genes are amplified by PCR and then the sequence is determined in an automated DNA analyser. For each housekeeping gene, the different sequence present within a bacterial species is assigned as a distinct allele and, for each

isolate; the alleles at each of the loci define the sequence type (ST). Allele numbers for new alleles and sequence numbers for new ST are available upon submission of the sequence to the MLST database, http://mlst.zoo.ox.ac.uk/.

Multilocus sequence typing method for *C. jejuni* was developed by Dingle and colleagues based on the sequence of seven housekeeping gene loci, *asp, gln, glt gly, pgm, tkt* and *unc* which code for aspartase A, glutamine synthetase, citrate synthase, serine hydroxymethyl transferase, phosphoglutamase, transketolase and ATP synthase α unit, respectively (Dingle *et al.*, 2001). This method is more suitable for long term and global epidemiological study as it investigates the variation in slowly evolving housekeeping genes (Dingle *et al.*, 2001, Mickan *et al.*, 2007). However, it was successfully used in an outbreak investigation of *C. jejuni* in combination with other typing methods based on antigenic genes such as *flaA* SVR typing or *porA* gene (Dingle *et al.*, 2008, Sails *et al.*, 2003c). The initial MLST method for *C. jejuni* was later supplemented with sequence typing of three more antigenic genes, *flaA*, *flaB* and *porA* to make it suitable for outbreak investigations (Dingle *et al.*, 2008). This supplementation increased the discriminatory power and made the method suitable for both long term and short-term epidemiological studies (Dingle *et al.*, 2008). However amplification and sequencing of nine genes makes the process more laborious, time consuming and costly.

The major advantage of MLST over other genotyping methods such as PFGE, is its very high reproducibility due to minimal variation in DNA sequencing (Enright and Spratt, 1999). Global accessibility of data from the continually expanding database allows electronic portability and inter-laboratory comparison of data without the requirement of

reference isolates, unlike PFGE. As the MLST can be applied directly to clinical material or extracted DNA, the use of live cultures can also be eliminated (Enright and Spratt, 1998, Maiden *et al.*, 1998). However, some disadvantages of MLST make routine use impractical in public health laboratory. These include the analysis time, cost, and the requirement of pure DNA (Hannis *et al.*, 2008). The risk of contamination of samples from post-PCR manipulation of amplicons is also a disadvantage of MLST. However, semi-automation has made the method suitable for use in public health laboratory (Clarke *et al.*, 2001, Sails *et al.*, 2003c).

MLST is a suitable typing method for monitoring of global trends in *C. jejuni* populations, and has been used in a variety of epidemiological studies since its development. Some of such studies include diversity and population structure of *C. jejuni* isolates associated with sporadic cases of gastroenteritis (Colles *et al.*, 2003, Mickan *et al.*, 2007, Wilson *et al.*, 2009b), spatiotemporal homogeneity of *C. jejuni* isolates (Rotariu *et al.*, 2009), comparison of veterinary and human isolates (Manning *et al.*, 2001), sources of *Campylobacter* spp. in broiler flocks during rearing (Bull *et al.*, 2006), source attribution (De Haan *et al.*, 2010, Sproston *et al.*, 2011, Sproston *et al.*, 2010), typing of *C. jejuni* from mediterranean environmental waters (Rodriguez-Martinez *et al.*, 2013), canine (Amar *et al.*, 2013), to study clonal distribution and virulence of *C. jejuni* in blood (Feodoroff *et al.*, 2013). It was also used to study clonal expansion of antimicrobial resistance among *C. jejuni* strains (Wimalarathna *et al.*, 2013, Kittl *et al.*, 2013, Stone *et al.*, 2013) and seasonal origin of *C. jejuni* infections (Strachan *et al.*, 2013) and studying similarities of environmental strains to those from human, poultry and bovines (de Haan *et al.*, 2013).

2.6.2.2.2 Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) based MLST typing of *C. jejuni*

MALDI-TOF-MS, invented in the 1980s by Tanaka in Japan and Karas and Hillenkamp in Germany separately (Tost and Gut, 2006), was initially applied to protein and peptide analysis. Now this method is one of the most powerful alternatives to conventional Sanger or pyro sequencing and a promising genotyping method (Vogel *et al.*, 2010, Vogel *et al.*, 2009). This method determines sequence variation by comparing the mass of target markers. Initially used to study single nucleotide polymorphism in the human genome (Tost and Gut, 2006), MALDI TOF/MS was applied for identification and genotyping of bacterial species such as *Acinetobacter baumannii* (Ecker *et al.*, 2006), *Streptococcus pneumoniae* (Williamson *et al.*, 2008) and *C. jejuni* (Hannis *et al.*, 2008). Hannis *et al*. described MALDI-TOF-MS based genotyping of *C. jejuni* using the housekeeping genes of the conventional MLST method as target markers. A short region (<140 bp) of each housekeeping gene is amplified. Then weighing the mass of the amplicons using high performance mass spectrometry, the base compositions of the amplicons are compared. This method was found rapid, high throughput and comparably discriminatory with MLST (Hannis *et al.*, 2008). Highly skilled personnel and well-equipped laboratories are required for this method. This newly developed method has yet to be evaluated for epidemiological study.

2.6.2.3 Single nucleotide (SNP) profiling

Single nucleotide polymorphisms, the most commonly identified polymorphisms, have often been used as genetic markers for genetic analysis, including genotyping of bacteria (Best *et al.*, 2005, Best *et al.*, 2004). The genotyping method based on SNP has been developed as a rapid and cost effective alternative to cumbersome sequence based

methods such as MLST (Hommais *et al.*, 2005, Robertson *et al.*, 2004). The vast amount of comparative sequence data generated from MLST is used to identify SNPs as targets (Rudi *et al.*, 2006b, Rudi *et al.*, 2006a). MALDI-TOF mass spectrometry, DNA microarray, ligation, electrophoresis, probe hydrolysis, high resolution melting analysis and primer extensions are the methods used to identify SNPs (Merchant-Patel *et al.*, 2008). SNP profiling was first used for identification of *C. jejuni* clonal complex 21 (Best *et al.*, 2004). Later it was used in a PCR platform for characterising a clonal cluster of *C. jejuni* and *C. coli* by SNP profiling. The new scheme was rapid, cost effective and useful for high throughput applications (Robertson *et al.*, 2004). This novel strategy for the use of real-time PCR (RT-PCR) for detection and characterization of *C. jejuni* beyond the species level supplied real-time epidemiological data comparable with MLST results. Resolution optimized SNP was used separately and in combination with binary gene markers to characterize *C. jejuni* by Merchant-Patel and his group in chicken (Price *et al.*, 2006). The single step method allowed a rapid test of epidemiological linkages in *C. jejuni.* In another seven-member SNP genotyping assay, the method efficiently characterized *C. jejuni* isolates in combination with *fla* SVR sequencing (Hannis *et al.*, 2008). However, SNP methods had limited discriminatory power unlike full MLST characterization and cannot be used for studies of *C. jejuni* population structure (Best *et al.*, 2004).

2.6.2.3 Single locus based methods

In such genotyping methods, genetic polymorphism is investigated in only one locus, either by sequencing of the locus or by restriction profiling. These methods are the least representative of the polymorphisms in the strains. *fla* gene is the most studied locus for single locus genotypic methods for *C. jejuni* in which recombination has been shown to

occur (Petersen and Newell, 2001). Other loci studied include LOS gene cluster (Shi *et al.*, 2002). *Campylobacter* spp. are naturally competent to take up and incorporate DNA, and so any genotyping technique based on a single genetic locus may provide inaccurate results for *C. jejuni* (Duim *et al.*, 1999). For this reason, single locus based methods are mostly used in preliminary screening of a large number of strains in epidemiological studies.

2.6.2.3.1 *fla*A - Restriction fragment length polymorphism (*flaA*-RFLP)

flaA –RFLP typing is one of the simplest and most cost effective genotyping methods for the investigation of large number of *Campylobacter* spp. (Petersen and Newell, 2001). This technique is widely used due to its rapidity and simplicity. It involves PCR amplification of the flagellin gene locus and restriction enzyme digestion to generate simple restriction fragment length polymorphic fingerprints. The flagellin locus has two genes, *flaA* and *flaB*, arranged in tandem (Nuijten *et al.*, 1990). The presence of both highly conserved and variable regions in the flagellin locus of *C. jejuni* makes it a suitable genetic marker for restriction fragment length polymorphism (RFLP) of a PCR product (Meinersmann *et al.*, 1997).

A number of *fla* RFLP typing procedures have been developed and have been reviewed previously (Wassenaar and Newell, 2000). These methods vary in the manner of DNA preparation, primer design, annealing temperature, restriction enzyme used and nomenclature (Wassenaar and Newell, 2000). Most of the methods use only *flaA* (Nachamkin *et al.*, 1993, Nachamkin *et al.*, 1996). Both *flaA* and *flaB* were used only by Ayling and colleagues (Ayling *et al.*, 1996) and Harrington and colleagues (Harrington *et al.*, 2003). However, optimal discriminatory power was obtained when

both genes were amplified separately (Petersen and Newell, 2001). This method can be used in combination with other genotyping methods such as MLST if the strains need further subtyping (Djordjevic *et al.*, 2007).

2.6.2.3.2 *fla SVR* sequence typing

C. jejuni genotyping method based on the *flaA* short variable region (SVR) was developed by sequencing 267 bp SVR of the *flaA* gene (Nuijten *et al.*, 1990). This is a simple and useful variation of *flaA*-RFLP method with high sample throughput at reasonable cost (Meinersmann *et al.*, 2005). As *flaA*-SVR sequence typing is a single gene based method, it is not suitable for long term time-location based epidemiological studies. However, the presence of two copies of *fla* gene and possible intragenomic and intergenomic recombination between the two copies undermines the reliability of typing *Campylobacter* spp. (Cornelius *et al.*, 2010, Guerry *et al.*, 1991). Despite this limitation, it is recommended for preliminary screening of large numbers of *C. jejuni* isolates. It was found useful in combination with other typing methods such as MLST to differentiate closely related or outbreak isolates (Dingle *et al.*, 2005, Sails *et al.*, 2003c). The latest report of use of *fla SVR* typing was about typing of C. *jejuni* from market aged broilers and it revealed mutations within the gene in about 25% strains (Singh and Min Kwon, 2013).

2.6.2.3.3 High-resolution melting (HRM) analysis of *flaA* fragment

This is another *flaA* gene based genotyping method for *C. jejuni,* evaluated very recently by Merchant-Patel and group. This is a real time PCR (RT-PCR) based method. In this method, reduction in fluorescence of PCR product stained with double strand specific dye was monitored while the product was heated through its melting

temperature (Merchant-Patel *et al.*, 2010). It can be used for on-site and in preliminary screening of a large number of *C. jejuni* isolates.

2.6.2.3.4 Repetitive Sequence PCR (rep-PCR) analysis

Rep-PCR is a bacterial genome fingerprinting method first described by Versalovic and colleagues (Versalovic *et al.*, 1991) based on PCR amplification and gel electrophoresis of repetitive DNA elements present within bacterial genomes. Two main sets of repetitive elements used in such subtyping are 38 bp repetitive extragenic palindromic (REP) elements and 128 bp enterobacterial repetitive interogenic consensus sequences (ERIC). Another repetitive DNA element, BOX element, is also used for bacterial genotyping. Extracted DNA or whole bacterial cells can be used in this typing, methods that uses whole cells have the advantage of being quicker (Woods *et al.*, 1993). A single primer, a single set of primers or multiple sets of primers can be used for amplification of REP and ERIC elements. Typing can be done by amplifying either REP or ERIC element but combining both elements results in more discriminatory power (Olive and Bean, 1999). The ERIC-PCR method was applied to investigate diversity of *C. jejuni* in humans, poultry and other sources along with PFGE and Penner typing. The method represented genetic relatedness of the strains in relation to their host origin better than PFGE with *Sma*1 (Moser *et al.*, 2002). When the discriminatory power of these three genotyping methods, PFGE, MLST and rep-PCR, were assessed with 63 *C. jejuni* isolates recovered from chickens raised in conventional, organic, and free-range poultry flocks, combined amplification of ERIC, REP and BOX elements (rep REP-PCR) showed greater discriminatory power than PFGE and MLST (Wilson *et al.*, 2009b). On the other hand, ERIC-PCR and BOX-PCR produced the highest number of PCR products and greatest reproducibility (Wilson *et al.*, 2009a). It was found as an effective

screening tool for large epidemiological studies (Patchanee *et al.*, 2012). In a recent studies, it was found more discriminatory than biochemical typing and considered efficient for typing and tracking analyses (Zheng *et al.*, 2013).

2.6.2.3.5 PCR-denaturing gradient gel electrophoresis (PCR-DGGE)

PCR-DGGE analysis distinguishes bacterial isolates based on the sequence differences in a single gene. Hein and colleagues applied this chemical denaturing gradient method to genotype *C. jejuni* using *fla* gene sequence as genotyping marker (Hein *et al.*, 2003). In this method, the variable fragment of 170-210 bp from the 5' end of the *flaA* to the 3' end of the intergenic region that separates the tandemly oriented *flaA* and *flaB* gene of *C. jejuni* was the target marker. A commercially available (Myers *et al.*, 1985b, Myers *et al.*, 1985a) system was used in this method, which was suitable to detect nearly all single-base as well as multiple single-base substitutions in DNA fragments (Farnleitner *et al.*, 2000b, Farnleitner *et al.*, 2000a, Sheffield *et al.*, 1989). This is a rapid method suitable for primary subtyping of *C. jejuni* (Hein *et al.*, 2003). Najdenski and colleagues considered this cultivation independent subtyping method suitable for primary screening of *C. jejuni* and *C. coli* when studied community cecal samples from broilers (Najdenski *et al.*, 2008). This method represents only a very small part of polymorphisms of the whole genome and has poor representation like other single locus method.

2.6.2.3.6 PCR Single strand conformation polymorphism (PCR-SSCP)

SSCP is a culture independent single gene based rapid genotyping method. This method was first developed for detection of polymorphism in human DNA. In SSCP analysis, single-stranded DNA fragments are differentiated according to differences in their electrophoretic mobility due to sequence-dependent conformational changes of the

fragments (Orita *et al.*, 1989). Charvalos and colleagues (Charvalos *et al.*, 1996) applied this method for detecting *gyr*A mutations associated with ciprofloxacin resistance in *C. jejuni*. Later this method was used to investigate *C. jejuni* infection in an immunocompromised patient in association with penner serology, Preston biotyping, phage typing, RFLP, 16S ribotyping and *flaA* RFLP. PCR-SSCP compared well with these other methods (Moore *et al.*, 2001). This simple, rapid and highly discriminatory method is helpful in outbreak characterisation but not suitable for examination of the clonal patterns of *C. jejuni* over a long period of time (Moore *et al.*, 2001). This rapid primary subtyping method was validated by subtyping of 48 clinical *C. jejuni* isolates, 49 *C. jejuni* strains from poultry, 2 strains from ducks and 1 strain from a pheasant. This method can also be applied to *C. coli* as *C. jejuni and C. coli* share flagellin gene types (Hein *et al.*, 2003).

2.6.2.3.7 Ribotyping

Ribotyping has become a well validated and reproducible means of distinguishing between strains in many bacterial genera such as *Pseudomonas, Salmonella, Haemophilus, Mycobacterium, Legionella* and *Campylobacter* (Owen *et al.*, 1993). Foyas and colleagues (Fayos *et al.*, 1992) found the method effective in differentiating strains of *C. jejuni* with 100% typeability. This technique involves electrophoresis of digested whole genome followed by southern blot hybridization with probes specific for rRNA. *Eco*RI is the most frequently used restriction enzyme for digestion (Pavlic and Griffiths, 2009). Other enzymes include *Pst*I, *Hae*III, *Hind*III and *Pvu*III, used alone or either in pairs or in combination (Wassenaar and Newell, 2000). Manual ribotyping is labour intensive, time consuming and an experienced technician is required to maintain a consistent protocol for meaningful results. All these drawbacks make this method

unsuitable for routine surveillance (Pavlic and Griffiths, 2009). To overcome these limitations, Automated Ribotyping (AR) systems have been developed. The RiboPrinter® Microbial Characterization System™ was commercialized by Qualicon (Wilmington, DE) as an automated ribotyping instrument capable of performing 32 assays per day. In the RiboPrinter® system, the instrument conducts cell lysis, nucleic acid digestion, southern blotting and a collection of digitized hybridized DNA patterns (Arvik *et al.*, 2005). This automation has reduced the required time and labour but AR is not a suitable method for routine surveillance for *C. jejuni* as it has shown 98.7% typeability (Fussing *et al.*, 2007) and is much less discriminatory (only 50%) than PFGE (Ge *et al.*, 2006, Schumann and Pukall, 2013). Ribotyping is not recommended as a method of choice for characterisation of *C. jejuni* (Pavlic and Griffiths, 2009) but it can be used in combination with other methods such as MLST or RFLP of *flaA* gene (O'Reilly *et al.*, 2006). It was used in epidemiological study alone or in combination with other methods such as in studying the distribution of predominant *C. jejuni* strain in the slaughterhouse environment (Klein *et al.*, 2007), genotyping diversity in wild bird community and source attribution of sporadic infection (Nielsen *et al.*, 2006).

2.6.2.3.8 Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR) sequence typing

CRISPRs are a subclass of Variable Number of Tandem Repeats (VNTRs), which are the most widely distributed family of repeats within prokaryotic bacterial chromosome (Jansen *et al.*, 2002). The unique characteristic of CRISPRs is the nearly exact direct repeat or palindromic sequences interspersed with similar sized spacer sequences. These repeats conserved within species range from 17-21 bp in length (Jansen *et al.*, 2002). Leader sequence flanking CRISPRs are also conserved within species and span

300-500bp (Jansen *et al.*, 2002). Bacterial strains carrying CRISPR space were found resistant to transmissible genetic elements like phage and plasmids. As a result, CRISPRs spacers are thought to influence bacterial evolution conferring resistance to bacteria and considered as marker for typing bacterial isolates (Mojica *et al.*, 2005).

Schouls and co-workers (Schouls *et al.*, 2003) first used CRISPRs for typing *C. jejuni* and *C. coli* isolates based on both the number of repeats and sequences of spacers. The process involved PCR amplification of the locus, followed by sequencing. The spacer sequence was diverse; 170 spacer sequences were recognized from 137 strains. The resolving power of CRISPR was found comparable to that of AFLP and MLST and combination of CRISPR and MLST had been highly discriminatory in comparison with either method alone (Schouls *et al.*, 2003). The inherent disadvantages of CRISPR typing are those of sequence based methods, high cost and requirement for sequencing equipment.

The evolution of the genotyping schemes for typing of *C. jejuni* over decades can be presented as in the Figure 2.2 (Ahmed *et al.*, 2012). Most of these genotyping methods were first developed for other microorganisms and later tested and applied in epidemiological studies of *C. jejuni*. Genotyping method (Ribotyping) first used for typing of *C. jejuni* was in the late 1980s. Few methods were tested in 1990s. Testing and use of genotyping methods for epidemiological studies of *C. jejuni* increased sharply in 2000s. A large number of genotyping methods were used for typing of *C. jejuni* in this decade. *flaA* gene is the single most used markers (gene) in genotyping of *C. jejuni* either alone or in combination with other markers. Search for new typing methods suitable for outbreak study is still continuing.

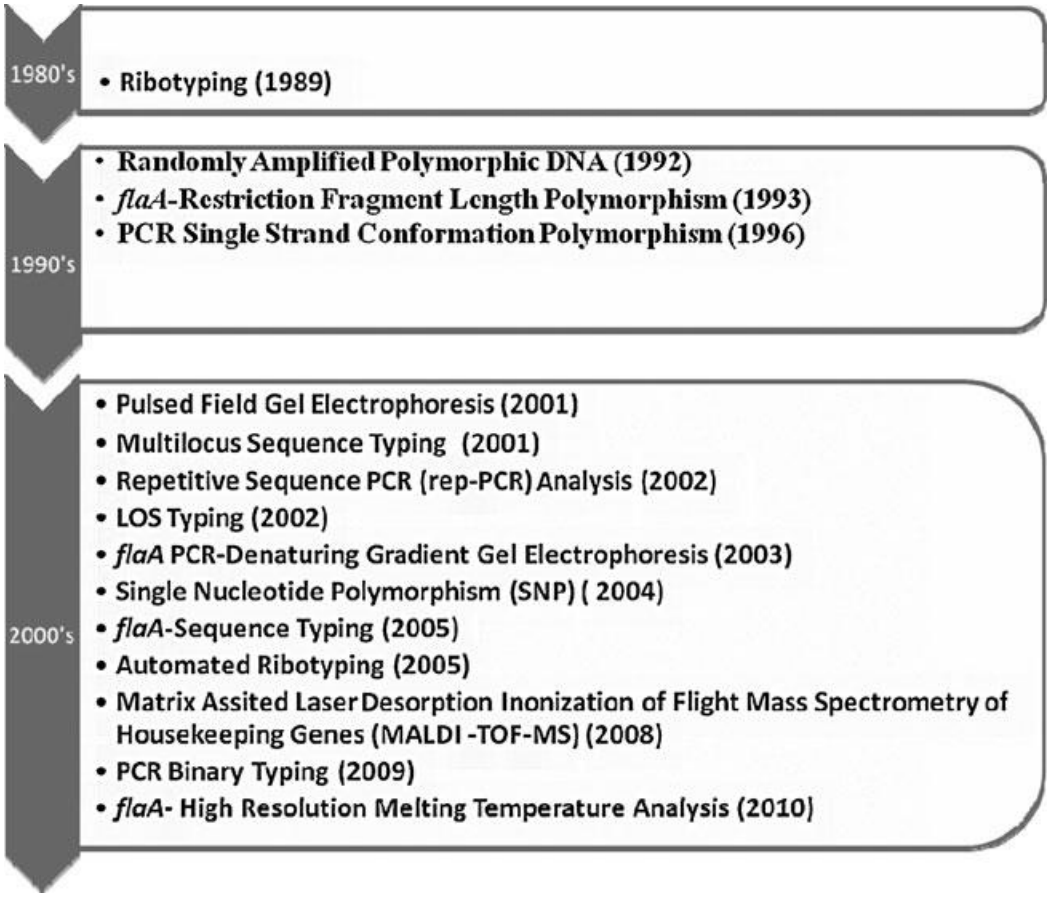

Figure 2.2. Evolution of genotyping methods for *C. jejuni*.[2]

2.7 Investigation of genetic markers for sequence based typing schemes

Three developments have increased the prospect of development of the sequence based typing schemes for pathogenic bacteria. These are: i) improved knowledge of bacterial evolution, genetics and genomics, ii) increasing speed and decreasing cost of nucleotide sequence determination and iii) improved web-based databases and analysis tools (Maiden, 2006).

[2] From self published article

2.7.1 Prerequisites for investigation of new typing scheme

The following are the prerequisites for investigating any typing scheme, particularly for sequence based typing schemes.

2.7.1.1 Marker selection

Selection of marker, genetic loci depend on the purpose of the typing scheme. If the purpose of the typing scheme is to study population genetics, then conserved or slowly evolving genetic loci are selected. Multilocus sequence typing schemes for various bacteria including *C. jejuni* were developed using such conserved genetic loci (Maiden, 2006).

To develop highly discriminatory genotyping scheme, highly variable, that is rapidly evolving, markers are used. Usually antigenic genes, surface protein coding genes, virulence and virulence associated genes are found to be rapidly evolving and hence have been used in developing highly discriminatory genotyping schemes for some pathogenic bacteria such as *L. monocytogenes* (Zhang *et al.*, 2004), trilocus sequence typing of *E. faecalis* (Chowdhury *et al.*, 2009) and double locus sequence typing of *S. aureus* (Kuhn *et al.*, 2007).

2.7.1.2 Strain collection/test population

A diverse and representative collection of strains of the species of bacteria needs to be included to test the suitability of the selected markers for targeted genotyping scheme. However, the number and diversity of collection depended on the purpose of the typing scheme (Maiden, 2006, van Belkum *et al.*, 2007). It should reflect the diversity of the

species as a whole as much as possible or at least in the microbial population to which the typing system will be applied. Thus the test bacterial population should include isolates from all niches that may be subject of future investigation such as particular patient groups based on age, immune status, and geographic origin and from environmental reservoirs, relevant to epidemiology in case of zoonosis and water born infection (van Belkum *et al.*, 2007).

The collection of strains should include epidemiologically unrelated strains, outbreak strains and strains from different sources and reservoirs of the pathogenic bacteria. A few sets of outbreak strains and at least one set of strains from different sources and reservoirs (each set N=5 to 10) should be included in the test population of bacteria (Struelens *et al.*, 1996). Species identity of each strain must be confirmed by biochemical and/or molecular methods.

2.7.1.3 Strain preservation

Preservation of strains over the period of study is important, particularly for stability test of the markers. The strains included in the study should preferably be preserved in 20% glycerol broth at -80^{0}C or freeze dried (van Belkum *et al.*, 2007).

2.7.1.4 Primer design

For sequence based typing schemes, separate sets of primers are suggested to use for amplification and sequencing and the sequencing primers should be nested within the amplified fragments to produce better quality data. The primers should also be such that the same annealing temperature can be used for all the loci in multilocus scheme (Maiden, 2006). This could reduce the time and cost required for the typing scheme.

2.7.1.5 Pilot study

The selected marker or genetic loci should be first tested on a subset of strains out of 100 or more strains collected (Maiden, 2006). This makes the development of new typing scheme efficient by preventing cost of amplification and sequencing all of the selected loci from all of the collected strains.

2.7.2 Evaluation of typing schemes

Typing schemes are evaluated based on their performance (efficacy) and convenience (efficiency) criteria (Struelens *et al.*, 1996, van Belkum *et al.*, 2007).

2.7.2.1 Performance criteria

Major performance criteria are:

1) **Typeability**

   Typeability is the ability of the method to assign a type to all strains tested by it. Mathematically, the typeability is the proportion of strains that are assigned a type by the typing system as follows (Struelens *et al.*, 1996):

   $T = N_t/N$

   Where: N_t is the number of isolates assigned a type and N the number of isolates tested. For a marker to be useful, T should be as close to 1 as possible.

   Ideally it is expected that selected typing marker will be present in all strains irrespective of source and time of isolation of the strain so that a type can be

assigned to each strain. Most of the genotyping schemes can characterise all of the strains within a test population and thus show 100% typeability.

2) Reproducibility

Reproducibility is the ability of the typing scheme to assign the same type to a strain on repeated testing irrespective of time, place or person. Reproducibility is poor for phenotyping methods as phenotypic characteristics of bacteria can change based on growth conditions, sample preparation and reagents used and higher for genotyping methods, particularly for sequence based typing schemes (Struelens *et al.*, 1996).

3) Discriminatory power

Discriminatory power is the ability of the typing scheme to assign two different types to two unrelated random strains from the population of the given bacterial species.

It is expressed as the probability using Simpson's index of diversity (SID or D). The following formula is used to calculate D (Hunter and Gaston, 1988).

$$D = 1 - \frac{1}{N(N-1)} \sum_{J=1}^{S} nj(nj-1)$$

Where N is the total number of strains in the sample population, S is the total number of types described, and nj is the number of strains belonging to the jth type. The index should ideally be 1.00. But in practice, a typing scheme is more or less ideal if it is at least in the order of 0.95. There are some online tools to calculate the discriminatory index of genotyping schemes. Typing schemes that

explore polymorphism at multiple sites of the genome are more likely to have higher discriminatory power than schemes exploring variation at a single site. If any strain appears non-typeable, it should be excluded from the calculation of discriminatory index or grouped together (van Belkum *et al.*, 2007).

4) Epidemiological concordance (E)

E is the ability of the typing method to classify epidemiologically related strains derived from presumably single clone outbreaks into the same clones and can be calculated:

$$E=Ne/N$$

Where Ne is the number of strains assigned to epidemic clones and N the number of strains tested from well-defined outbreaks.

In an evaluation of a new typing method, several sets, e.g. five or more, outbreak related strains (n = 5 or 10 per set) are suggested to be included in the sample of study strains (Struelens *et al.*, 1996). Ideally, E should be equal to 1.

5) Stability

Stability refers to as the stability of the markers used in the typing scheme (ref/s). The traits tested by a typing scheme should remain stable for each strain after its first isolation and subsequent storage and subculturing. In vivo assessment of stability is preferable, though it is not always possible. Typing markers are also considered stable if multiple strains of an epidemic strain

isolated from different patients at different moments appear indistinguishable by typing based on that particular marker (van Belkum *et al.*, 2007).

2.7.2.2 Convenience criteria

Convenience criteria are those criteria, which make the routine use of the typing scheme convenient. These criteria determine the routine use of the typing schemes influencing financial and technical involvement in the scheme (van Belkum *et al.*, 2007, Struelens *et al.*, 1996).

The following criteria are usually considered in the evaluation of convenience of the scheme:

1) **Flexibility or spectrum**

Flexibility refers to the range of species for which the typing method can be used with slight modifications. The more species can be typed using a specific scheme, the more chance the typing scheme will be used in the public health laboratory. Sequence based typing schemes have optimal flexibility as the principle followed; the skills and equipment required are same for different species.

2) **Rapidity**

Rapidity is the time required to get the final typing result from the time of bacterial strains available. In ideal condition, typing result should be available within a day. Such rapidity makes the method more applicable in general medicine. This is an important convenience criterion particularly for outbreak investigation, which requires result within 24-72 hours.

3) Accessibility

Accessibility refers to the usability of the typing schemes influenced by availability of reagents, equipment and technical skill requirement (van Belkum *et al.*, 2007). With an increase of speed and decrease of the cost of sequencing, accessibility of sequence based typing schemes is increasing (Maiden, 2006).

4) Ease of use

Ease of use is the simplicity of the typing scheme technically, workload involvement, possibility of processing large numbers of samples and ease of scoring and interpreting the results.

5) Cost

The cheaper the typing scheme, the more it is used in epidemiological surveillance. However, the cost of the typing scheme depends on a number of factors. These are initial capital to get the principal equipment to its set up, maintenance of equipment, cost of consumable reagents and staffing cost and size and number of markers (locus) under study.

6) Amenability to computerized analysis and incorporation of typing results in electronic databases

These make longitudinal comparisons of a large number of strains possible, both for local and global epidemiological study.

No method possesses all these criteria at a satisfactory level. Each method has some advantages and some disadvantages both in performance criteria and convenience

criteria. Thus no single typing method has yet been found to be universally applicable to enable effective epidemiological application (Olsen *et al.*, 2001). At least two genotyping methods should be combined to accurately answer questions of bacterial lineage and epidemiological questions (Wassenaar *et al.*, 1998). The search for new subtyping methods continues and there is scope for the development of new sequence based method for *C. jejuni.*

2.8 Prospective markers for genotyping schemes for *C. jejuni*

Depending on the purpose of the typing schemes, two different types of genetic markers are selected to develop genotyping schemes. For global epidemiological investigation, usually a typing scheme using more stable genetic markers such as housekeeping genes is used. In global epidemiological studies, multilocus sequence typing schemes have been developed over the decades using housekeeping genes for a wide number of bacteria including *C. jejuni* (Urwin and Maiden, 2003, Maiden, 2006).

For local epidemiological investigation, highly variable markers are used in the typing scheme. Such markers include genes coding antigenic protein, surface protein and virulence factors. Using such markers, highly discriminatory typing schemes have been developed for a number of pathogenic bacteria such as *Staphylococcus aureus* (Kuhn *et al.*, 2007), *Enterococcus faecalis* (Chowdhury *et al.*, 2009), *Bordetella pertussis* (Van Loo *et al.*, 2002) and *Streptococcus pneumoniae* (Enright and Spratt, 1998) for local epidemiological investigation. No such scheme has been reported for *C.jejuni.*

The mechanism of *C. jejuni* pathogenesis includes adhesion, colonization and invasion (Navaneethan and Giannella, 2008, Zilbauer *et al.*, 2008, Van Putten *et al.*, 2009).

Various virulence factors such as adherence, invasive capabilities and toxin production, have been implicated in the multifactor and complex pathogenesis of *C. jejuni* infection (Jain, 2008). Virulence factors involved in adherence of *C. jejuni* include flagella, firbonectin binding outer membrane protein, CadF and surface-exposed lipoprotein, JlpA and major outer membrane protein, *cm*P (Huang *et al.*, 2005). CadF plays a role both in adhesion and invasion (Dasti *et al.*, 2010). The genes encoding these adhesins are well studied. The major toxin produced by *C. jejuni* is cytolethal distending toxin (CDT) (Young and Schauer, 2000). These virulence factors and surface protein coding genes were investigated to develop highly discriminatory typing schemes for local epidemiological investigation of *C. jejuni.*

2.8.1 *Campylobacter* adhesin factor (CadF)

Campylobacter adherence factor (CadF) is an outer membrane protein, which is responsible for adhesion of *Campylobacter* spp. to fibronectin (Fn) of basolateral surface of T84 human colonic cells. It is an important pathogenicity factor for *C. jejuni* and *C. coli* and expressed in every strain. Additionally, *cadF* genes and its products (CadF) are highly conserved among *C. jejuni* and *C. coli*. However, there is a difference in the size of CadF from *C. jejuni* and *C. coli*. CadF protein from *C. coli* strain is larger (by 13 amino acids) than that from *C. jejuni.* This difference can be utilized to discriminate these species in food and clinical samples. This virulence gene was found present in all *C. jejuni* from swine and cattle, human clinical specimens, poultry carcasses and faces in PCR studies. It was also demonstrated that the *cadF* mutants were not able to colonize chicken caecum. Thus CadF protein is an important virulence factor for *C. jejuni* and *cadF* gene can be employed as a genotyping marker in

combination with other virulence genes (Konkel *et al.*, 1999, Krause-Gruszczynska *et al.*, 2007, Talukder *et al.*, 2008, Wieczorek and Osek, 2008, Datta *et al.*, 2003).

2.8.2 Jejuni lipoprotein A (JlpA)

Jejuni lipoprotein (JlpA) is a surface-exposed and a species-specific lipoprotein of *C. jejuni* encoded by *jlpA* gene. It plays an important role in adhesion of the bacterium to HEp-2 epithelial cells (Jin *et al.*, 2001). It was found present in every strain of *C. jejuni* from a wide variety of sources such as humans, poultry, bovine, porcine, ovine, and canine source. Mass spectrometric characterization of the surface-associated 42 KDa lipoprotein JlpA showed strain variation in glycosylation sites of the JlpA protein (Scott *et al.*, 2009). Thus the polymorphic *jlpA* gene encoding the species-specific adhesin is another virulence and antigenic gene suitable candidate for subtyping.

2.8.3 Cytolethal distending toxin (CDT)

Cytolethal distending toxin (CTD) is the most characterized toxin of *Campylobacter* spp. (Yamasaki *et al.*, 2006a). A cluster of three genes, cdtA, cdtB and cdtC, encodes this holotoxin. Interspecies similarities of these genes range from 50-70% but intraspecies similarities are above 99 % when compared among the nucleotide sequences of *cdt* genes in *C. jejuni, C. coli* and *C. fetus* (Asakura *et al.*, 2007). The species specificity and intraspecies polymorphism of *cdt* genes make them suitable candidates for genotyping study. Based on the species specificity of *cdt* genes, multiplex PCR was developed targeting each of three *cdt* genes and it was found 100% specific and sensitive in detecting and identifying *C. jejuni, C. coli* and *C. fetus.* The species-specific primers can be used for amplification of *cdt* genes for genotyping purpose (Asakura *et al.*, 2008a).

2.8.4 Major outer membrane protein, MOMP

The *cmP/porA* gene encodes the surface-exposed major outer membrane protein (MOMP) of *Campylobacter* spp. It exists in every strain of *C. jejuni and C. coli and* functional in the adherence of *C. jejuni* to host cells and the general resistance to many hydrophilic antibiotics. Thus it also plays a role in pathogenesis. Being MOMP, it bears a certain degree of sequence polymorphism and is variable among different strains. These sequence features suggest that the *cmP* gene encoding MOMP can be a potential candidate locus for molecular differentiation of *Campylobacter* spp. It was used as a candidate gene in single- locus typing and was found highly discriminatory (Huang *et al.*, 2005). So it can be used in multilocus sequence typing schemes with other suitable genetic markers. Molecular typing using genes encoding surface and antigenic proteins have been reported in *B Pertussis* and *E. faecalis* (Chowdhury *et al.*, 2009, Van Loo *et al.*, 2002). So *cmP/porA* gene can be combined with other virulence and/ or surface protein genes to develop multilocus sequence typing methods for *C. jejuni.*

2.8.5 The major iron transport protein coding gene, *ceuE*

The enterochelin periplasmic binding protein in *C. jejuni* is encoded by the gene *ceuE*. The encoded protein is a 34.5 - 36.2 KDa lipoprotein component of a binding-protein-dependent transport system for the siderophore enterochelin. Due to crucial role of iron acquisition in bacterial infectivity, this protein is likely to be of importance to virulence. The gene was amplified from *C. jejuni* in the species specific manner for rapid identification and discrimination of *C. jejuni* and *C. coli* (Gonzalez *et al.*, 1997). This ubiquitous gene has been used in fingerprinting of *C. jejuni* strains by resolution optimized binary gene targets (Price *et al.*, 2006). Thus this gene could be considered as a candidate gene for a multilocus typing scheme for *C. jejuni*.

2.8.6 Membrane associated protein-coding gene, *mapA*

The protein encoded by *mapA* gene is a 24 KDa antigenic surface protein and present in all *C. jejuni* strains but not in closely related *C. coli* (Stucki *et al.*, 1995). So amplification of this gene by PCR has been used in numerous studies for identification of *C. jejuni* strains in various types of samples including faeces, foods and colitis and appendicitis patient (De Boer *et al.*, 2013, Tappe *et al.*, 2012, Singh *et al.*, 2011, Lamps *et al.*, 2006, Campbell *et al.*, 2006). Due to its antigenicity and specificity, *mapA* was considered a candidate gene for *C. jejuni* typing.

2.8.7 Cell binding factor coding gene *peb1*A

Peb1A is a major cell binding factor of *C. jejuni* (Pei and Blaser, 1993a). It is also a surface exposed antigenic protein, which plays a role in colonisation and thus in pathogenesis or virulence (Müller *et al.*, 2007a). Mutations in this gene reduce the adherence of the bacterium to epithelial cells and colonization in the intestine (Pei *et al.*, 1998). Peb1A was found as a potential candidate for vaccine against *C. jejuni* infection due to its antigenic properties (Buckley *et al.*, 2010). Due to all these properties, *peb1A* was considered as a candidate gene for typing of *C. jejuni.*

2.9 Matrix assisted laser desorption flight of time mass spectrometry (MALDI-TOF-MS) in bacterial typing

2.9.1 MALDI-TOF MS: applications and advantages

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has revolutionized biological research (Hyytiä-Trees *et al.*, 2007). It is a relatively novel technique. In this technique, co-precipitate of a UV-light absorbing matrix and biomolecules is irradiated by a nanosecond laser pulse which results in order to ionisation of the molecule and generation of gaseous phase. The matrix absorbs most of the laser energy and thus prevents unwanted fragmentation of the biomolecule while ionizing the macromolecule. The ionized biomolecules accelerated in an electric field, to enter the flight tube. Different molecules are separated according to their mass to charge ratio and reach the detector at different times in their flight in this tube. As a result, distinct signal is obtained for each molecule. This distinct signal is used for detection and characterization of biomolecules, such as proteins, peptides, oligosaccharides and oligonucleotides, with molecular masses between 400 and 350,000 Da. It is a highly sensitive method, capable of detection of low (10^{-15} to 10^{-18} moles) quantities of sample with an accuracy of 0.1-0.01%. Short measuring time (few minutes) and negligible sample consumption (less than 1 pmol) together with additional information on microheterogeneity (e.g. glycosylation) and presence of by-products are the advantages of this technique in the case of protein identification. Moreover, hundreds of samples can be analysed on a single instrument with 24 hours (Hannis *et al.*, 2008). In comparison to conventional genotyping methods, MALDI-TOF MS has

rapid turnaround time, low sample volume requirements and modest to negligible reagents or consumable costs (Cherkaoui *et al.*, 2010, Biswas and Rolain, 2013).

Due to these advantages in protein identification, this technique has become a valuable tool for the analysis of microorganisms in clinical, environmental and food microbiology for identification of bacteria at genus, species and subspecies level using whole cells, cell lysates, or crude bacterial extracts (Barbuddhe *et al.*, 2008, Krishnamurthy and Ross, 1996, Saenz *et al.*, 1999).

Its high sensitivity with accuracy has made it an ideal tool for the detection of high- and low-molecular-weight proteins. Thus it has the potential for rapidly distinguishing between pathogenic and non-pathogenic species of bacteria (Krishnamurthy and Ross, 1996).

2.9.2 MALDI-TOF in bacterial typing

MALDI TOF has been used for subtyping of many pathogenic bacteria using extracted protein and intact cells (Sandrin *et al.*, 2013). Extracted protein was used in typing of *L. monocytogenes* (Barbuddhe *et al.*, 2008), *Propinibacterium acnes* (Nagy *et al.*, 2013), mycobacteria (Shitikov *et al.*, 2012), nosocomial outbreak of *Corynebacterium striatum* (Verroken *et al.*, 2013)**,** detection of nosocomial outbreak by *Acinetobacter baumannii* (Mencacci *et al.*, 2013), outbreak study of non-multidrug resistant *S. aureus* (MRSA) in Australia and *Leptospira* strain identification (Rettinger *et al.*, 2012) and study of *Helicobacter pylori* strains (Ilina *et al.*, 2010). Whole cell typing was used in rapid screening of *Salmonella enterica subsp enterica servars* (Dieckmann *et al.*, 2008), rapid typing of *Yersinia pestis* (Ayyadurai *et al.*, 2010), *Yersinia enterocolitica* (Stephan *et*

al., 2011), *Legionella pneumonia* (Fujinami *et al.*, 2011), rapid characterization of *Vibrio* species (Dieckmann *et al.*, 2010), discriminating of environmental strains of *Escherichia coli* (Siegrist *et al.*, 2007), biotyping of multidrug resistant *Klebsiella pneumoniae* (Berrazeg *et al.*, 2013), grouping of *Myxococci* strains (Stackebrandt *et al.*, 2005) and *Burkholderia mallei* and *B. pseudomallei* typing. There is no such report on the typing of *C. jejuni* by MALDI-TOF, which can be used in epidemiological study of *C. jejuni*. In this study, therefore, we examined typing of *C. jejuni* by MALDI-TOF using extracted proteins.

Chapter 3 . Materials and Methods

3.1 Equipment

Table 3.1. Equipment used in the current investigation

Equipment	Purpose	Manufacturer
Micropipettes (0.1-1.0 mL)	Dispensing liquids	Thermo Fisher Scientific, Scoresby, VIC, Australia
Centrifuge	Centrifugation of solutions	Ependorrf, South Pacific, North Ryde, NSW, Australia
Gel Doc XR System, PC and Quantity One 1D software	Capturing images of UV gels	Bio-Rad
Laminar Flow Cabinet	Drying plates, sterile work	Gellman Sciences Australia, Cheltenham, VIC, Australia
Microcentrifuge	Centrifugation of solutions	Ependorrf
Mini-Sub cell GT electrophoresis gel tank	Agarose gel electrophoresis	Bio-Rad
Power Pac Mini power supply	Agarose gel electrophoresis	Bio-Rad
My Cycler™	PCR	Bio-Rad
UV light source	Band visualisation in Agarose gel	Integrated Sciences, Chatswood, NSW, Australia
Water bath	Incubation of cultures, DNA for extraction, media preparation	Labec, Marrickville, NSW, Australia
Vortex mixer	Mixing solutions	Ratek Instruments, North Ryde, NSW, Australia
Microflex LT system	MALDTI-TOF MS analysis	Bruker Daltonic GmbH, Germany

3.2 Chemicals and Buffers

Table 3.2. Commercial kits and solutions

Kit/ Solution	Purpose	Manufacturer
Wizard® Genomic DNA Purification Kit	DNA extraction from *C. jejuni*	Promega, Alexandria,NSW,Australia
MangoMix™	Amplification of DNA	Bioline, London, UK
Wizard® SV Gel and PCR Clean-Up System	PCR product purification	Promega, , Alexandria,NSW,Australia
CampyGen	Microaerophilic environment creation	Oxoid, Hampshire, Uk
Preston selective supplement	Prohibit growth of Unwanted bacteria	Oxoid, Hampshire, Uk
Campylobacter growth Suppliment	Enhance growth of *C. jejuni*	Oxoid, Hampshire, UK
Ladder Mix	Gel electrophoresis	Invitrogen, Mulgrave, VIC, Australia
1kb ladder	Gel electrophoresis	
Ethidium bromide (10 mg/mL)	Gel elctrophoresis	Sigma, Castle Hill, NSW, Australia
Loading dye	Gel electrophoresis	
Ethanol	Protein Extraction	Invitrogen, Mulgrave, VIC, Australia
Formic Acid	Protein extraction	Invitrogen, Mulgrave, VIC, Australia
Acetonitrile	Protein extraction	Invitrogen, Mulgrave, VIC, Australia
Isopropanol	DNA precipitation	Invitrogen, Mulgrave, VIC, Australia

3.3 Software and online tools

The software and online tools used to design primers, alignment and sequence and mass spectra analyses are listed in the Table 3.3

Table 3.3. Software and Online tools used

Name of Software/tool	Purpose	Reference / link
Primer 3	Primer design	(Skaletsky, 2000)/http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm
CLUSTALW	Multiple sequence alignment	http://www.genome.jp/tools/clustalw/
MEGA 5.05	Phylogenetic tree construction	(Tamura *et al.*, 2011)
Discriminatory power calculator	Calculation of Simpson's index of diversity	http://insilico.ehu.es/mini_tools/discriminatory_power/ (Carrico *et al.*, 2006)
Diversity and partition congruence coefficients calculator	Calculation of Adjusted Rand and Adjusted Wallace coefficient	http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool
Biotyper 3.0	MALDI-TOF analysis	Brucker Daltonic, Germany

3.4 Culture media

All culture media used, Preston Agar, Muller Hinton Broth, Nutrient Agar and Broth, were obtained from Oxoid (Hampshire, UK). Media were prepared following manufactures instruction using distilled water and sterilizing by autoclaving at 121°C at 15 p.s.i for 15 minutes. Supplements were added to the media after it had cooled to 50 - 52°C; all media were stored at 4°C.

3.4.1 Antibiotics and supplements

The following antibiotics and supplements from Oxoid (Hampshire, UK) were used in the preparation of media.

3.4.1.1 Antibiotics: Preston Campylobacter selective supplement

The following selective supplements were included in the media for the growth of *C. jejuni*.

Table 3.4. Antibiotics for selective growth of *C. jejuni*

Antibiotic	Quantity*
Polymyxin B	2,500 IU
Rifampicin	5.0 mg
Trimethoprim	5.0 mg
Cycloheximide	50.0 mg

*Vial contents (each vial is sufficient for 500 mL of medium)

Preparations

One vial was reconstituted with 2 mL of pure ethanol and aseptically added the contents to 500ml of sterile basal medium prepared from Nutrient broth No. 2 and 7% v/v lysed horse blood.

3.4.1.2 Growth supplements

Supplement for the enhanced growth of *C. jejuni* for 500 mL of medium of Preston Agar.

Table 3.5. Supplements for enhanced growth of *C. jejuni*

Growth enhancer	Quantity*
Sodium pyruvate	0.125 g
Sodium metabisulphite	0.125 g
Ferrous sulphate	0.125
Sterile water	2 mL

*Vial contents (each vial is sufficient for 500 mL of medium)

Preparation

The content of one vial (2 mL) was added to 500 mL of a sterile nutrient medium cooled to 50-55°C prepared from Nutrient broth No. 2 with 7% lysed defibrinated horse blood. Then it was mixed gently and poured aseptically into sterile petri dishes.

3.4.1.3 Horse blood

7% lysed horse blood was prepared in the laboratory following 3x freeze –thaw described in the guideline of Clinical and Laboratory Standard Institute (CLSI). Briefly, 20 ml defibrinated horse was poured into 50 mL conical plastic tubes labelled with batch no. of the blood. Then it was frozen at -20°C until the next day. Next morning, the blood was thawed at room temperature. These freeze thaw cycle was repeated until the blood has been frozen and thawed three times. Lysis of blood was checked by microscopy using 100× enlargement whether all/almost all erythrocytes are lysed. Then 20 mL sterile Milli Q water was added to plastic tubes containing blood and mixed by inverting the tubes a few times. Sealed tubes were centrifuged at 5000 rpm for 30 minutes and the supernatant was collected carefully into a new tube, leaving the pellet in tube. Properly labelled tube with the batch no. of the blood along with date and initials were stored in a freezer (–20°C) for use in C. *jejuni* media.

3.5 *C. jejuni* strains

The bacterial strains used in this study were obtained from the Enteric Culture collection of Microbiology Diagnostic Unit, Public Health Laboratory, The University of Melbourne. The strains were isolated from different sources, such as human (clinical strains, both sporadic and outbreak strains), chicken, potable water, ovine and canine. The geographical sources of these strains were predominately from the Victoria. In some cases the geographical location of the source of the strain was unknown.

The type strain C. *jejuni* LMG 8841^T (= ATCC 33560^T = CIP 70.2^T) included in this study was obtained from the Belgium Coordinated Collections of Microorganisms/Laboratorium Microbiologie Ghent (BCCM/LMG). *C. jejuni* subsp. *jejuni* 81116 was from culture collection of RMIT University, Melbourne. For outbreak strains, only dead cells in stock media were available. DNA was extracted from such dead cells and hence outbreak strains could not be included MALDI-TOF analysis. Details of the strains used in different studies are presented in the supplementary Table S1.

3.6 Culture conditions for *C. jejuni*

C. jejuni strains were routinely grown on Preston agar (Oxoid, Hampshire, UK) for 36-48 h at 37°C in a microaerobic atmosphere conditions generated by CampyGen gas generation kit (Oxoid, Hampshire, UK).

3.7 Storage of *C. jejuni*

Strains were stored at -80°C in nutrient broth containing 20% (v/v) glycerol (Fisher Scientific, Loughborough, UK). Strains were recovered from stocks when necessary.

3.8 Extraction of genomic DNA

Cells were grown for 36-48 h on Preston agar at culture conditions described above. Then genomic DNA was extracted from a loop full of bacterial colonies suspending in 1 mL peptone water and then using Wizard®Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA) according to manufacturer's instructions. The quality of the DNA was confirmed on 1% agarose gel; and the samples were subsequently stored at -20^{0}C for further use. Genomic DNA from outbreak strains was extracted from stock in glycerol broth, as those strains could not be retrieved, following the same procedures.

3.9 Agarose gel electrophoresis

Both genomic DNA and PCR products were examined and purified on a 1% w/v agarose (Bioline, London, UK) gel in 0.5% Tris-borate EDTA (TBE) buffer. To visualize DNA, 0.5µg/mL ethidium bromide (Fisher, Loughborough, UK) was mixed into the gel whilst still molten. Prior to loading the sample was mixed with 5× loading buffer (Bioline, London, UK). 5-10 µL of sample was run as required. 1kb plus DNA ladder (Bioline, London, UK; 100 bp – 10 kb) was used as a molecular weight marker. The gel was usually run at 100V for 1 h in 1 × TBE (Tris/Borate/EDTA) buffer (0.089 M Tris, 0.089 M borate, and 0.002 M EDTA, pH 8.33) prepared in the laboratory mixing three components proportionately in Milli Q water.

3.10 Primers

Two different sets of primers were used, as recommended (Maiden *et al.*, 1998), for amplification and sequencing of marker genes.

3.01.1 Primers for amplification of selected markers

Primers for amplification of the selected markers *cadF* (Konkel *et al.*, 1997), *mapA* (Stucki *et al.*, 1995), *porA* (Dingle *et al.*, 2008), *peb1A* (Müller *et al.*, 2007a), *ceuE* (Gonzalez *et al.*, 1997) and *jlpA* (Jin *et al.*, 2001) were amplified using previously described primers (Table 3.1). MLST genes were also amplified using previously described primers (Dingle *et al.*, 2001). The details of the primers are described in the table 3.6.

Table 3.6. Primers for amplification of candidate marker genes

Gene	Primer name	Sequence (5'-3')	Amplicons size (bp)	References
cadF	CadFFB CadFRB	TTGAAGGTAATTTAGATATG CTAATACCTAAAGTTGAAAC	400	(Konkel *et al.*, 1997)
cdtA	Cjspau2 Cjspar2	AGGACTTGAACCTACTTTTC CTAATACCTAAAGTTGAAAC	631	(Asakura *et al.*, 2008a)
cdtB	Cjspbu5 Cjspbr6	ATCTTT TAACCT TGC TTT TGC GCAAGCATTAAA ATC GCA GC	714	
cdtC	Cjspcu1 Cjspcr2	TTT AGCCTTTGCAAC TCC TA AAG GGGTAGCAGCTG TTA A	524	
ceuE	JEJ1 JEJ2	CCTGCTACGGTGAAAGTTTTGC GATCTTTTTGTTTTGTGCTGC	793	(Gonzalez *et al.*, 1997)
flaA	flaa664 flaa1494	AATAAAAATGCTGATAAAACAGGTG TACCGAACCAATGTCTGCTCTGATT	855	(Meinersmann *et al.*, 1997)
flaSVR	fla242FU fla625RU	CTATGGATGAGCAATTATAAAAT CAAG(AT)CCTGTTCC(AT)ACTG AAG	425	
jlpA	P4 PR7	GAGAAACATATGAAAAAAGGTATTTTTCTC AACTGCCGCCCATTAACATAGAAAAC	1100	(Jin *et al.*, 2001)
mapA	MAPA-F MAPA-R	CTTGGCTTGAAATTTGCTTG GCTTGGTGCGGATTGTAAA	603	(Stucki *et al.*, 1995)
Peb1A	Peb1AF2 Peb1AR2	GCAGAAGGTAAACTTGAGTCTATT TTATAAACCCCATTTTTTCGCTA	702	(Pei and Blaser, 1993a)
porA	MOMP2 MOMP3	TGA GAA GTT AAG TTT TGG AG AG GAT GGT TTA GTW GGM ACA GG	630	(Dingle *et al.*, 2008)

3.01.2 Primers designed for sequencing selected markers

The primers for sequencing the selected markers were designed in this study as nested primer, that is, the primer set will sequence the region within the amplicons, not the entire amplicons by primer3 online primer design tool (Table 3.2) (Skaletsky, 2000). The details of the designed primers are described in Table 3.7.

Table 3.7. Primers designed for sequencing of selected markers

Genes	Primers	Primers (5′→3′)	Product size (bp)
cadF	cadF_SF cadF_SR	AGCCAAAGAATCACTAAGACG GGATAATCGTTATGCACCAG	304
cdtA	CjspAU2-SF CjspAR2-SR	GGCGGAGTAGTTAAAAACCA ACAAGCAATAGTGCCAACAA	530
cdtB	CjSPBU5-SF CjSPBR6-SR	AGCGGTGGAGTATAGGTTTG CACAGAAAGCAAATGGAGTG	615
cdtC	CjspCU1-SF CjspCR2–SR	ATAGGATCTAGGGTGCAAGG AGCCTTTGCAACTCCTACTG	459
ceuE	ceuE_SF ceuE_SR	CGGTGAAAGTTTTGCCTATT TCCGTGTGTGCCTACTTTTA	626
mapA	mapA_SF mapA_SR	GGTGCGGATTGTAAATTTCT TTTTGAGTGCTTGTGCAACT	497
peb1A	peb1A_SF peb1A_SR	ACGCATCAACTCTTTTAGCA CCGCATTATGCTTTACTTGA	445
porA	porA_SF porA_RF	AGGAAATCTTTACGGTGCTG CAGCTTCTGTTTTTGTTCCA	506

3.10.3 Primers for multilocus sequence typing

Two separate sets of previously described primers were used for amplification and sequencing of 7 genes for multilocus sequence typing (Dingle *et al.*, 2001). The details of the two different sets of primers are described in two different tables. The details of the amplification primers are in the Table 3.8.

Table 3.8. The primers for amplification of MLST markers

Locus	Primer's name	Primer sequence (5'-3')
asp	asp-A9	AGT ACT AAT GAT GCT TAT CC
	asp-A10	ATT TCA TCA ATT TGT TCT TTG C
gln	gln-A1	TAG GAA CTT GGC ATC ATA TTA CC
	gln-A2	TTG GAC GAG CTT CTA CTG GC
glt	glt-A1	GGG CTT GAC TTC TAC AGC TAC TTG
	glt-A2	CCA AAT AAA GTT GTC TTG GAC GG
gly	gly-A1	GAG TTA GAG CGT CAA TGT GAA GG
	gly-A2	AAA CCT CTG GCA GTA AGG GC
pgm	pgm-A7	TAC TAA TAA TAT CTT AGT AGG
	pgm-A8	CAC AAC ATT TTT CAT TTC TTT TTC
tkt	tkt-A3	GCA AAC TCA GGA CAC CCA GG
	tkt-A6	AAA GCA TTG TTA ATG GCT GC
unc	unc-A7	ATG GAC TTA AGA ATA TTA TGG C
	unc-A2	GCT AAG CGG AGA ATA AGG TGG

The primers used for sequencing typing markers are usually nested primers, primers that sequence the region within the amplicons. The details of the primers used for sequencing MLST markers are described in Table 3.9.

Table 3.9. The primers for sequencing MLST genes

Locus	Primer's name	Primer sequence (5'-3')
asp	asp-S3	CCA ACT GCA AGA TGC TGT ACC
	asp-S6	TTA ATT TGC GGT AAT ACC ATC
gln	gln-S3	CAT GCA ATC AAT GAA GAA AC
	gln-S6	TTC CAT AAG CTC ATA TGA AC
glt	glt-S1	GTG GCT ATC CTA TAG AGT GGC
	glt-S6	CCA AAG CGC ACC AAT ACC TG
gly	gly-S3	AGC TAA TCA AGG TGT TTA TGC GG
	gly-S4	AGG TGA TTA TCC GTT CCA TCG C
pgm	pgm-S5	GGT TTT AGA TGT GGC TCA TG
	pgm-S2	CC AGA ATA GCG AAA TAA GG
tkt	tkt-S5	GCT TAG CAG ATA TTT TAA GTG
	tkt-S4	ACT TCT TCA CCC AAA GGT GCG
unc	unc-A7	TGT TGC AAT TGG TCA AAA GC
	unc-A2	TGC CTC ATC TAA ATC ACT AGC

3.10.4 Amplification conditions for marker genes

Selected markers were amplified with oligonucleotide primer pairs described previously. Each 50-ml amplification reaction mixture comprised 25.0 μL of 2x Mango Mix, Red master mix (Bioline, NSW, Australia), 2.0 μL of each primer from a solution of 100 μM/μL, template (genomic DNA) 2.0 μL from 1:10 dilution of extracted DNA, sterile distilled water to make 50.0 μL.

3.10.4.1 *cdt* gene cluster amplification *(cdtA, cdtB and cdtC)*

The amplification reaction conditions for the *cdt* gene cluster was: Initial heating at 95°C for 1 minute, then 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 55^0C and extension for 30s at 72^0C and final extension for 1 min. This is a slight

modification of previously described protocol where the same annealing temperature was used for all three genes instead of 56^{0}C specified for *cdtB* (Asakura *et al.*, 2008b).

3.10.4.2 Amplification conditions for *porA, peb1A, cadF, ceuE, jlpA* and *mapA*

To amplify other markers except *cdt* gene cluster, the protocol was: initial heating at 95°C for 15 min, then 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for the 90 s and then final extension at 72°C for 5 min. This is the protocol for *porA* gene amplification described before (Clark *et al.*, 2007). When tested, it was found that all genes could be amplified using this protocol using previously described primers (Table 3.6).

3.10.4.3 Amplification of *fla SVR*

flaSVR was amplified and sequenced by primers described previously (Meinersmann *et al.*, 1997).

3.10.4.4 Amplification of 16S *rRNA* gene

The 16S rRNA gene sequences were determined using previously described primers and PCR conditions (Lane *et al.*, 1985, Weisburg *et al.*, 1991). The reaction conditions were denaturation at 94°C for 2 min, primer annealing at 50°C for 1 min, and extension at 72°C for 1 min, for 35 cycles.

3.10.5 Purification of PCR products/amplicons

DNA purification was carried out as required. The PCR amplicons were purified using Wizard® SV Gel and PCR Clean-up System (Promega, Madison, Wisconsin, USA). Briefly, the PCR product was transferred to the SV Minicolumn assembly and incubated

for 1 minute at room temperature. Then centrifuged the assembly in a microcentrifuge at 16,000 × g (14,000 rpm) for 1 minute. The liquid in the collection tube was discarded and the SV Minicolumn was returned to the collection tube. Then the column was washed by adding 700 µl of the membrane wash solution, previously diluted with 95% ethanol by centrifugation again for 1 minute at 16,000 × g (14,000 rpm). The liquid in the collection tube was discarded and the wash was repeated with 500 µL of membrane wash solution and centrifuged again for 5 minutes at 16,000 × g. The centrifugation was repeated for one more minute keeping the leading open after discarding the liquid from the collection tube and the SV minicolum was placed in 1.5 µL Ependorrf tube. Then 50 µL nuclease free water was added in the SV column and centrifuged again for 1 minute at 16,000 × g (14,000 rpm). The Microcentrifuge tube contained the eluted DNA and it was stored at -20^{0}C for further use.

3.11 Sequencing

DNA was sequenced on an automated ABI 3700 sequencer using the 'ABI PRISM BigDye Terminator Cycle (Applied Biosystems, Foster City, USA), at the Australian Genomic Research Facility (www. agrf.org.au) following their guidelines. In brief, purified PCR product for each gene was submitted in a volume of 12 µL, which contained 12-18 mg/µL of DNA and (0.8 pmol/µL) of primer. Then DNA labelling (sequencing) reaction was carried out by the BigDye terminator version 3.1 and clean-up capillary separations on the AB 3730*xl*. For each sample sent, the following files were provided:

1) The raw chromatogram trace file

2) A text file of the sequence, as generated by the sequencing instruments

3) A trimmed FASTA formatted text file.

4) A BLAST file (GenBank) of the trimmed FASTA file

3.12 Sequence analysis

Fasta files provided were used for multiple alignments. CLUSTALW from Genome Net Bioinformatics tools (http://www.genome.jp/tools/clustalw/) was used for slow and accurate multiple sequence alignment. An arbitrary allelic number was assigned to sequences having a single nucleotide difference. The evolutionary relationship of the test strains was revealed using MEGA (version 5.05). The GenBank accession numbers of the submitted sequences are listed in the supplementary Table S1.

3.13 Sample preparation for MALDI- TOF MS analysis

3.13.1 Bacterial strains and cultivation

A total of 72 *C. jejuni* strains identified by biochemical tests and collected from the culture collection of the Microbiology Diagnostic Unit, The University of Melbourne was included in this study. The strains were from a variety of sources, which include human patient, chicken, potable and environmental water, bovine, canine and ovine. Strains from the human patient included 4 aged care cluster (C1-C4) and others are sporadic infection strains (C5-C10 and H1-H22). The canine strains (Ca1-Ca3) were isolated from the same canine. Other strains from chicken (10, CH1-CH35), potable water (15, W2- W18), environmental water (3, E2-E9), ovine (9, OV1-OV16), bovine (3, B1-B3) and duck (1, D8) along with the type strain *C. jejuni* LMG 8841^{T} and *C. jejuni* 81116 included in this study were epidemiologically non-related. The strains were isolated during 1993-2012. The bacteria were grown on Preston agar at 37^{o}C for 36-48 hours under microaerophilic conditions.

3.13.2 Sample preparation for MALDI-TOF MS

For MALDI-TOF fingerprinting of bacterial strains, both whole cell and cell extract, are widely used as sample. Cell extract was used as sample for *C. jejuni* typing in this study. Bruker daltonic guideline for sample preparation was followed in the extraction of protein (Ng *et al.*, 2013). In brief, *C. jejuni* cells in full of a 3 mm sterile plastic loop were transferred from the plate to a 1.5 mL ependorrf tube and mixed thoroughly in 300 µL of sterile water by a vortex. A portion of 900 µL of absolute ethanol was added and mixed with the cell suspension by a vortex for 1 min. Then the mixture was centrifuged at 13000 rpm for 2 min and the supernatant was discarded. Subsequently, 60 µL of 70% formic acid was added to the pellet and mixed thoroughly by pipetting and vortex. At the end, 60 µL of acetonitrile was added and mixed well without a vortex. Finally the mixture was centrifuged again at 13000 rpm for 2 min. From this prepared supernatant, only 1 µL was placed onto the spot on the steel plate (96 MSP steel plates, Bruker daltonic, Germany) and allowed to be air-dried at room temperature. The air dried protein solution was overlaid with 1 µL of α–cyano-4- hydroxycinanamic acid (CHCA) matrix solution (saturated solution of-cyno-4-hydroxycinnamic acid in 50% acetonitrile–2.5% trifluoroacetic acid) and allowed to dry before the plate was entered into the machine. All samples were analysed in quadruplicates.

3.13.3 Data acquisition

Samples were analysed using Microflex LT System, the basic MALDI Biotyper™ (Bruker Daltonic GmbH, Germany), equipped with a 60-Hz nitrogen laser was employed, using the Software for FLEX Series 1.3. Spectra were recorded in a linear positive ion detection mode with a mass range from 2,000 to 20,000 Da. Spectrometer settings were set to: Ion Source 1 (IS1) 20 kV; Ion source 2 (IS2) 16.69 kV; Lens

voltage: 7 kV; Pulsed Ion Extraction: 150 ns. Each spectrum was created with the software Flex Control (Version 3.3) in an automatic mode with variable laser power. For each spectrum a total of 240 shots were summed up.

3.13.4 Data analysis

Unknown spectrum identification in the MALDI Biotyper was based on the main spectra (MSPs) in the Biotyper database. When the raw data were loaded into the Biotyper 3.0 software, they were pre-processed in five steps to construct MSPs: mass adjustment, smoothing, baseline subtraction, normalization and peak picking, all with default settings. For identification, the unknowns MSPs were compared with reference spectra stored in the Biotyper 3.0 database. For typing, the unknown MSPs were compared with the created unassigned MSP of the *C. jejuni* LMG 8841^{T}.

Chapter 4 . Identification of *C. jejuni*

Summary

Tentatively identified *C. jejuni* strains were obtained from the Enteric Culture Collection of Microbiology Diagnostic Unit, Public Health Laboratory, The University of Melbourne. For selected strains, their taxonomic identity was further re-confirmed up to the genus and species level using phylogenetic analysis based on 16S rRNA, *mapA* gene sequence similarities and MALDI-TOF MS based identification.

4.1 Introduction

The accurate identification of bacterial isolates at the species level is the prerequisite for strain typing (Struelens *et al.*, 1996). Conventional routine identification of C. *jejuni* is tedious and time consuming as this group of bacteria are slow growing organisms with only few biochemical differential features specific for this species. Hippuricase activity is the one of the commonly used biochemical markers useful for *C. jejuni* differentiation from *C. coli* but this activity is sometimes weak and hippuricase negative *C. jejuni* strains also exist (On *et al.*, 1996, Skirrow and Benjamin, 1980, Totten *et al.*, 1987). Therefore molecular identification is the preferred option and it can be used in combination with conventional methods for reliable verification of taxonomic affiliation of *C. jejuni* strains. Several sequencing-based methods are used for molecular identification of *C. jejuni*. These molecular methods include 16S or 23S rRNA, species-specific *mapA* gene and *cdt* gene cluster sequencing (Stucki *et al.*, 1995, Kabir *et al.*, 2011). Since *C. jejuni, C. coli* and *C. lari* cannot be reliably differentiated by 16S rRNA gene sequence similarities (Gorkiewicz *et al.*, 2003), the *cdt* gene cluster, consisting of 3 genes *cdtA, cdtB* and *cdtC,* was found to be useful for identification of *C. jejuni* (Asakura *et al.*, 2008b, Kabir *et al.*, 2011). It was also shown that *C. jejuni* can be identified reliably by comparative analysis of the *mapA* gene sequence similarities (Stucki *et al.*, 1995, De Boer *et al.*, 2013). In this project, species-specific *mapA* amplification and sequencing was used as a molecular method for confirmation of C. *jejuni* strains. Matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) as recently emerged technique, which is able to overcome the limitations of molecular methods can be also used for rapid identification of *C. jejuni* (Kolínská *et al.*, 2008). Thus in this project, phylogenetic analysis based on 16S rRNA,

mapA gene sequence similarities and MALDI-TOF MS were applied to confirm the taxonomic identity of selected *C. jejuni* strains.

4.1.1 The chapter's aims

The aims of this study were to:

i) Confirm the taxonomic affiliation of the environmental and clinical isolates using 16S rRNA, *mapA* genes sequencing and MALDI-TOF MS;

ii) Compare the performance of the methods used in identification of *C. jejuni.*

4.2 Results and Discussion

4.2.1 *Campylobacter* spp. identification using 16S rRNA gene sequencing

The 16S rRNA gene sequence similarities were analysed for selected, to include strains from different sources, of 43 *C. jejuni* strains. Identification of these *C. jejuni* strains was carried out by comparing 16S rRNA gene sequences with other reference sequences in the GenBank database using the BLAST at the National Centre for Biotechnology Information (NCBI) website (http:// www.ncbi.nlm.nih.gov). For all isolates, 16S rRNA gene sequences were found to exhibit 100% sequence similarities to those of the type strains of *C. jejuni* and *C. coli*. These results reconfirmed that the 16S rRNA gene sequencing is useful for identification up to the genus level and is insufficient for species discrimination. The phylogenetic relationship of *Campylobacter* spp. strains based on 16S rRNA sequences is presented in the Figure 4.1.

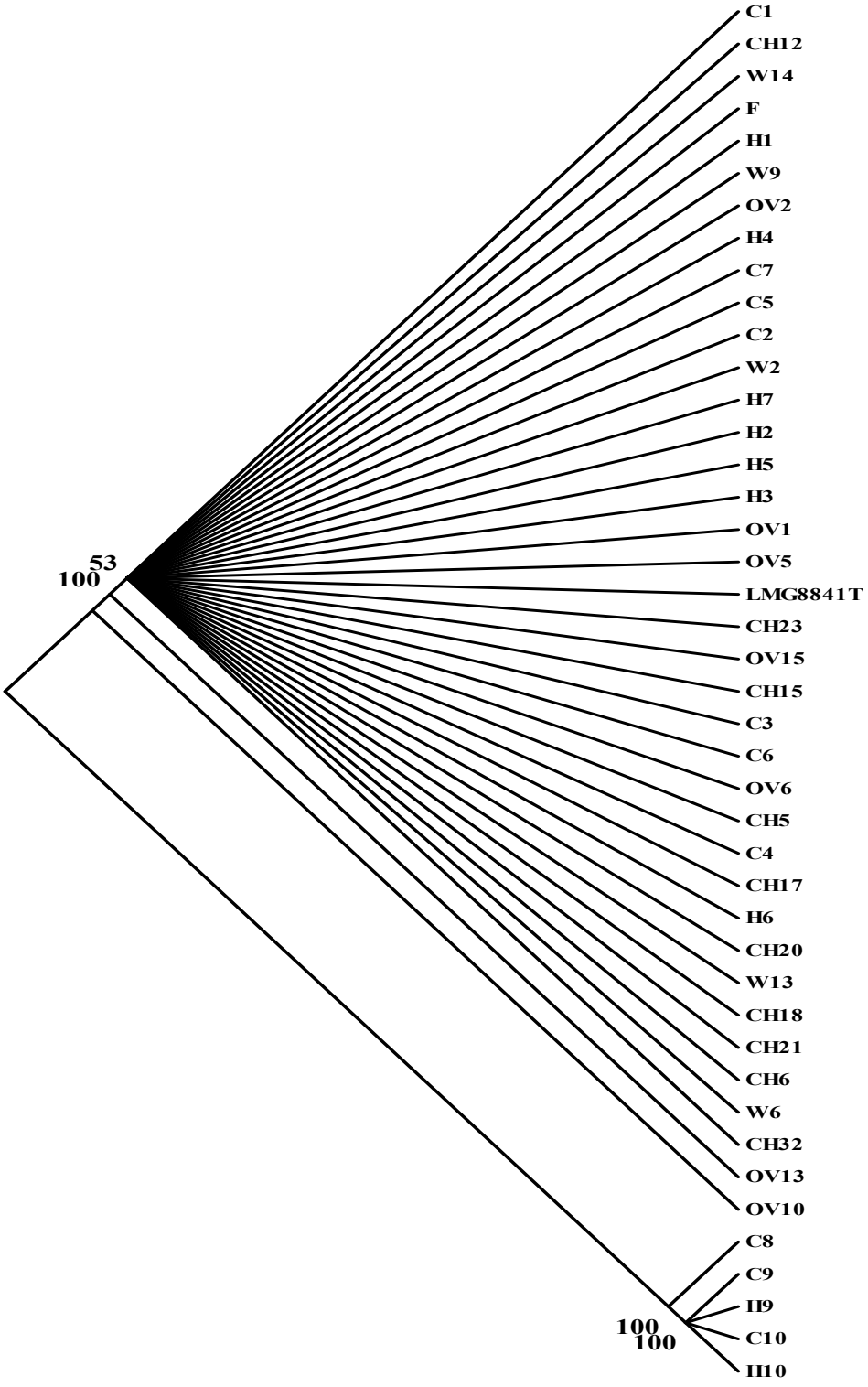

Figure 4.1. Phylogenetic relationships of *C. jejuni* strains based on the analysis of the 16S rRNA gene sequence similarities as inferred from the neighbour joining (N-J) tree with 1000 bootstrap replications using MEGA 5.05.

4.2.2 Identification of *C. jejuni* strains based on the *mapA* gene sequence similarities

The *mapA* gene sequence similarities were analysed for fifty *C. jejuni* strains selected to represent different sources of *C. jejuni* in the evaluation of proposed typing schemes. Identification of the *C. jejuni* strains was carried out by comparing *mapA* gene sequences with other reference sequences in the GenBank database using the BLAST at the National Centre for Biotechnology Information (NCBI) website (http:// www.ncbi.nlm.nih.gov). All isolates were also identified as *C. jejuni* based on $0 \geq 98\%$ sequence similarity with the reference sequence of *mapA* gene of the type strain of *C. jejuni* included in the study. Sequences were aligned and compared with the sequences of *C. jejuni* type strain (LMG8841^{T}) using MEGA 5.05 software (Tamura *et al.*, 2011). The phylogenetic relationship of *C. jejuni* strains based on *mapA* gene sequences is presented in the Figure 4.2.

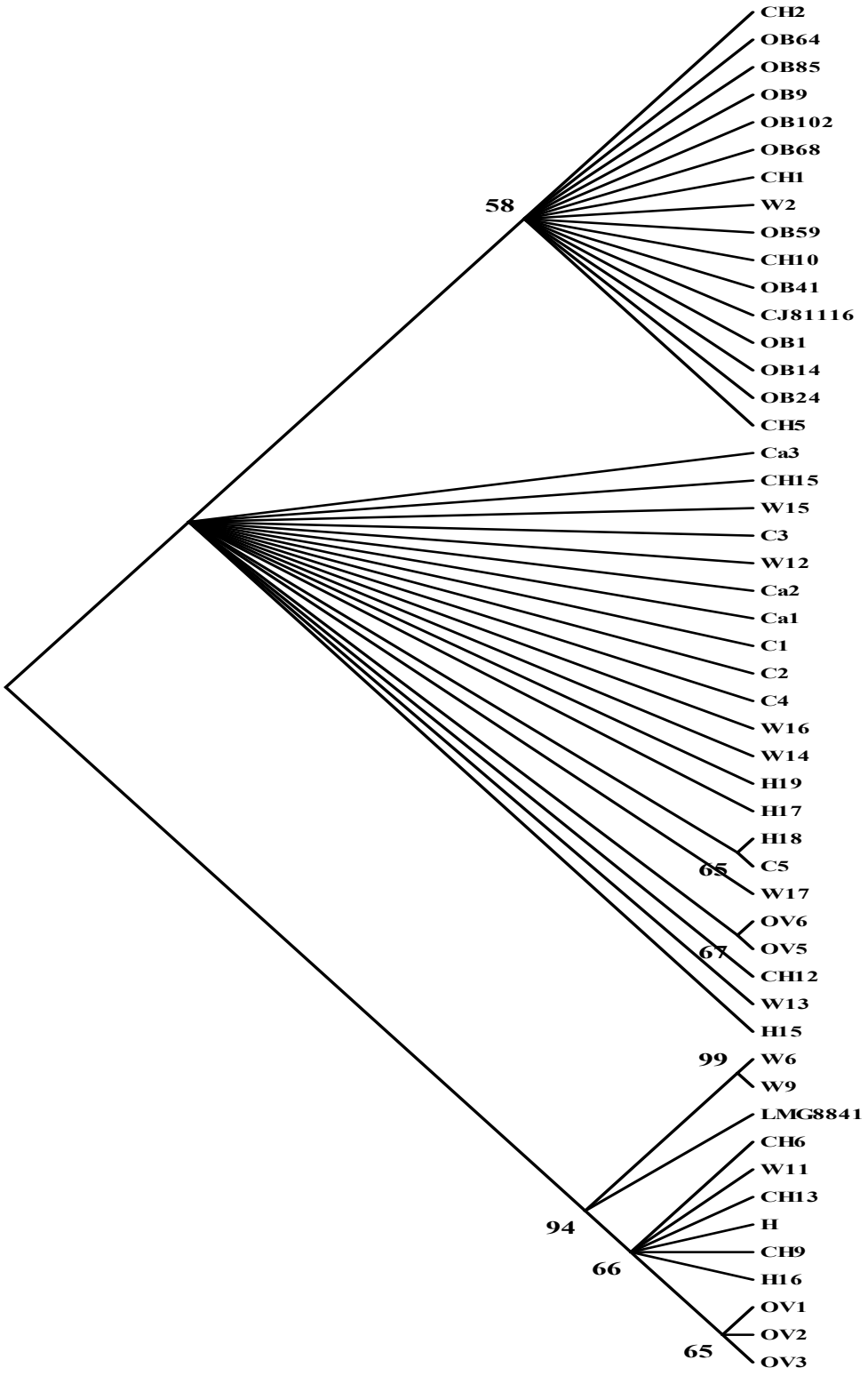

Figure 4.2. Phylogenetic relationships of fifty *C. jejuni* strains based on the analysis of the *map*A gene sequence similarities as inferred from the neighbour joining (N-J) tree and 1000 bootstrap replications using MEGA 5.05.

mapA gene is able to identify *C. jejuni* strains for its species specificity (Stucki *et al.*, 1995). It can also reveal relationships of *C. jejuni* strains as evident from Fig 4.2. All the outbreak strains (OB) are found clustered together. Similar is the case for canine strains (Ca1- Ca3) and other related strains (C1-C4). The strains and their sources identified by 16s rRNA sequencing and *mapA* gene sequencing are presented in Table 4.1.

Table 4.1. *C. jejuni* strains and their sources included in identification studies

Strain ID	Origin/year of isolation/ place
LMG8841^T	Type strain, Belgium
C1	
C2	Human, Aged care cluster, 2005
C3	
C4	
C5	Human, sporadic strain, 2005
C6	
C7	
C8	
C9	
C10	
Ca1	Canine, 2012, identified as genetically indistinguishable by pulsed field gel electrophoresis(PFGE) but different from H; Australian capital territory (ACT)
Ca2	
Ca3	
CH1	Chicken, different firms and retailers; 2002-2004, Melbourne, Australia
CH2	
CH5	
CH6	
CH9	
CH10	
CH12	
CH13	
CH17	
CH18	
CH20	
CH21	
CH23	
CH32	
CJ81116	Reference strain, RMIT culture collection
H (H22)	Human. Sporadic infection strains isolated in 2012

H1	
H2	
H3	
H4	
H5	
H6	
H7	
H9	
H10	
H15	
H16	
H17	
H18	
H19	
OB1	School camp outbreak in 1993, identified as genetically indistinguishable by pulsed field gel electrophoresis(PFGE), only dead cells were available; Gold Coast school camp, Queensland, Australia
OB9	
OB14	
OB24	
OB41	
OB59	
OB64	
OB68	
OB85	
OB102	
OV1	From ovine in Victoria and Tasmania, Australia, 2001-2002
OV2	
OV3	
OV5	
OV6	
OV10	
OV13	
OV15	
W2	Potable water, 2005-2011, Melbourne metropolitan and rural region, Australia
W6	
W9	Environmental, water, 2008-2010; Metropolitan and rural Victoria
W11	
W12	
W13	
W14	
W16	
W17	
W18	
F	Water, 2011, metropolitan Melbourne, Australia

4.2.3 Identifications by MALDI-TOF MS

Seventy-two *C. jejuni* strains were identified using the score value system of Biotyper Real Time Classification (BRT) software. Default cut-off values of BRT were used for identification. Values below 2 stand for probable genus identification or no reliable match with the main spectra (MSP) database (www.bdal.de). Cut off *values* from 2.0 to 2.29 indicate reliable genus identification. Values from 2.3 to 3.0 indicate reliable species identification. The higher is the value, the better is the match. All strains were identified as *C. jejuni* by MALDI-TOF with a log score of ≥ 2.3 or classified as green (Mencacci *et al.*, 2013). The scores of studied strains are shown in the Table 4.1.

Table 4.2. Log score of *C. jejuni* strains by MALDI-TOF MS identification

Strain ID	Score	Strain ID	Score
LMG 8841^T	2.3	B3	2.43
C1	2.312	Ca1	2.43
C2	2.486	Ca2	2.56
C3	2.462	Ca3	2.75
C4	2.425	H1	2.53
C5	2.425	H2	2.35
C6	2.32	H3	2.39
C7	2.396	H4	2.34
C8	2.474	H5	2.33
C9	2.34	H6	2.6
C10	2.41	H7	2.8
F	2.43	H8	2.75
I	2.44	H9	2.7
J	2.56	H10	2.4
K	2.34	H12	2.44
L	2.56	H13	2.46
CH5	2.303	H15	2.32
CH6	2.43	H16	2.47
CH12	2.6	H15	2.32
CH14	2.34	H16	2.47
CH15	2.6	H17	2.56
CH17	2.31	H18	2.59
CH18	2.34	H19	2.57
CH19	2.51	H20	2.62
CH20	2.49	H21	2.72
CH31	2.3	EW2	2.67
CH17	2.31	EW7	2.49
CH18	2.34	EW9	2.45
CH32	2.37	W2	2.43
OV 1	2.39	W6	2.44
OV2	2.45	W9	2.34
OV5	2.36	W11	2.39
OV6	2.34	W12	2.47
OV9	2.323	W13	2.3
OV10	2.42	W14	2.39
OV13	2.5	W15	2.35
OV15	2.41	W16	2.37
OV16	2.34	W17	2.38
B1	2.45	W18	2.34
B2	2.49		

Molecular identification is a multistep process involving amplification, purification of amplified genes and sequencing, which takes 24 hours or more. On the other hand, sample preparation (protein extraction) and sample analysis (spectra taking) by MALDI-TOF MS takes a few minutes to an hour depending on the number of strains. Thus, MALDI-TOF is much faster and high throughput method for identification of *C. jejuni*.

4.3 Conclusion

Thus the results obtained using three independent and complementary techniques clearly indicated that the strains included in this study belong to the genus *Campylobacter* and were identified as *C. jejuni.* MALDI-TOF MS appeared to be fast and reliable and may be used as alternative techniques for *C. jejuni* identification.

Chapter 5 . Selection and evaluation of prospective genes for sequence based typing schemes for *C. jejuni*

Summary

Eleven candidate markers (*cadF, cdtA, cdtB, cdtC, ceuE, flaA, fla SVR, jlpA, mapA, porA* and *peb1A*) were selected based on their function, specificity and probable variability. Amplification and sequencing conditions were optimized for all the selected markers using twenty *C. jejuni* strains, which included clinical strains, and strains isolated from chicken, ovine and water. Based on sequence analysis of those 20 strains, 4 schemes were found being of a satisfactory discriminatory power: *porA, peb1A, mapA* and *ceuE* for a tetra-locus sequence typing scheme, *porA, peb1A* and *mapA* for tri-locus and *porA* and *peb1A* for double-locus sequence typing scheme. Three genes of the *cdt* cluster were also found to be suitable for a separate typing scheme.

5.1 Introduction

A large number of markers are chosen based on the available information regarding the function, variability and their prevalence in the specific bacterial strains (Urwin and Maiden, 2003). New genotyping markers are usually tested on a large number of strains from different sources of the specific pathogen. The recommended number is approximately 100 strains (van Belkum *et al.*, 2007, Struelens *et al.*, 1996). However, before testing the candidate genotyping markers on this large number of strains, the selected markers are recommended to be tested on a subset of the strains to find the suitability of the markers, such as prevalence of the marker in strains originating from different sources, variability of the markers and the right combination of the markers. The recommended subset of strains is usually 10 - 20 strains (Maiden, 2006).

The selected candidate markers for typing schemes for *C. jejuni* were *cadF*, *cdt* gene cluster (*cdtA, cdtB* and *cdtC*), *ceuE*, *flaA, flaSVR, jlpA*, *mapA, peb1A, and porA*. These genes, comprise of several virulence, virulence associated, surface proteins coding and antigenic genes, are highly variable and should be most suitable for highly discriminatory typing. These genes are described in detail in the section 2.8 of the Chapter 2.

Highly discriminatory typing schemes for other pathogenic bacteria such as *Enterococcus. fecalis* (Chowdhury *et al.*, 2009), *Listeria monocytogenes* (Zhang *et al.*, 2004), *S.* aureus (Kuhn *et al.*, 2007) and *Bordetella pertusis* (Chan *et al.*, 2009) were previously developed based on similar highly variable genes. Such markers, however, have not been used for *C. jejuni* typing schemes.

Different pairs of primers were used for amplification and sequencing of each gene. The primers used for amplification of the selected candidate loci were from previous studies (Konkel *et al.*, 2005, Gonzalez *et al.*, 1997, Asakura *et al.*, 2008b, Stucki *et al.*, 1995, Dingle *et al.*, 2008, Jin *et al.*, 2001, Pei and Blaser, 1993a). The primers for sequencing were designed in this study as nested primers using the primer3 online tool (Chapter 3, Table 3.1). The nested primers will amplify region within the amplicons, not full amplicons (Skaletsky, 2000). All the markers were amplified from the subset of strains selected for assessment study and then sequenced. Allele type for each gene was determined by multiple sequence alignment. Then suitable combination of markers for desired typing schemes were chosen based on their combined discriminatory power (Sections 2.7.2.1 and 3.12**)**.

Based on the result of this assessment study, the selected subset of loci was then tested on a larger set of isolates as recommended (Maiden, 2006, Urwin and Maiden, 2003) to develop suitable typing scheme.

5.1.1 The Chapter's aims

The aims were –

i. Comparatively evaluate applicability of *cadF, ceuE, jlpA, mapA, peb1A* and *porA* genes for *C. jejuni* typing schemes;

ii. Investigate the *cdt* gene cluster encoding cytolethal distending toxin for a *C. jejuni* typing scheme**;**

iii. To find combination/s of markers for typing schemes with different number of loci and with the minimum discriminatory power required for outbreak/local epidemiological investigation (≥0.95) to maximum (~1.0).

5.2 Results and Discussion

5.2.1 Selection of virulence genes

To find the best combinations for a series of typing schemes with tetra-, tri- and double-locus, the Simpson's index of diversity (SID) of different combinations was calculated following the formula described previously (section 2.7.2.1.3) and using the online tool (http://insilico.ehu.es/mini_tools/discriminatory_power/). To be suitable for outbreak investigation and other local epidemiological investigation, a typing method should have ≥0.95 as the discriminatory index (Struelens *et al.*, 1996). Based on this criterion, the combinations of candidate markers that showed (SID) values ≥0.95 were considered suitable for further use in this study. It was found that *porA* and *peb1A* as double-locus, *porA, peb1A* and *mapA* as tri-locus and *porA, peb1A, mapA* and *ceuE* as tetra-locus typing scheme showed ≥ 0.95 SID successively increasing with the increase of locus. The combination of markers and respective discriminatory power are summarized in Table 5.2. Though epidemiological concordance is the most important criterion of typing schemes (van Belkum *et al.*, 2007), it is difficult to predict in a pilot study, using a small number of strains. However, it is possible to predict the discriminatory power, another important criterion for typing schemes. Hence the discriminatory power was used to find suitable combinations of markers. Additionally the epidemiological concordance to some extent depends on the discriminatory power of the individual marker and combination of markers. Hence the discriminatory power was selected to find suitable marker combinations, which was calculated from allelic profiles of marker combination for each strain using the formula described by Hunter (Hunter, 1990).

5.2.2 Characterisation of *cdt* gene cluster

From the allelic profiles of *cdtA, cdtB and cdtC* of 20 *C. jejuni* strains included in this study, it was found that *cdt* gene cluster has discriminatory power of SID 0.97 which is satisfactory for local epidemiological studies.

Table 5.1. Combination of candidate markers and their discriminatory power

Name of typing scheme	Locus combination	Simpson's Index of diversity (SID)
Double-locus sequence typing	*porA* and *peb1A*	0.9524
Tri-locus sequence typing	*porA, peb1A and mapA*	0.959
Tetra-locus sequence typing	*porA, peb1A, mapA* and *ceuE*	0.984
cdt gene cluster sequence typing	*cdtA, cdtB* and *cdtC*	0.97

5.3. Conclusion

The combination of the markers suitable for double locus sequence typing was *porA* and *peb1A*, for tri-locus sequence typing were *porA, peb1A and mapA*. The highest discriminatory index of 1.00 was predicted for the tetra locus as a combination of *porA, peb1A, mapA* and *ceuE*. The three genes of the *cdt* gene cluster, *cdtA, cdtB and cdtC*, were also found to show satisfactory discriminatory power which, may be sufficient for local epidemiological study. Therefore, the experimental results for *cdt* gene cluster sequence typing will be presented first followed by a tetra-, tri- and double-locus typing schemes.

Chapter 6 . Investigation of cytolethal distending toxin (*cdt*) gene cluster (*cdt*A, *cdt*B *and cdt*C) for *C. jejuni* typing

Summary

The applicability of the *cdt* gene cluster, which consists of *cdtA, cdtB* and *cdtC*, was investigated using both clinical and environmental strains of *C. jejuni*. Forty five strains included in the study derived from outbreak, sporadic infections and strains isolated from chicken, canine, ovine and water. A total of 1191 bp (197 bp, 620 bp and 374 bp) of the three complete CDSs was analysed for each strain. The 28 unrelated strains were differentiated into 17 types and Simpson's index of diversity was calculated as 0.9656, which is suitable for an outbreak study. Among the 17 related strains, ten outbreak strains were identified as indistinguishable and of the same type. The strains isolated from canine and the aged care centres were also found very closely related. Overall the *cdt* gene cluster sequence typing was in agreement with the epidemiological information confirming the *cdt* gene cluster sequence typing applicability in epidemiological investigation of *C. jejuni* infections.

6.1 Introduction

Cytolethal distending toxin (CDT) is an important virulence factor for *C. jejuni* (Ge *et al.*, 2006, Jain *et al.*, 2009, Jain, 2008). The toxin is an AB-type exotoxin produced also by several other clinically important Gram negative bacterial pathogens such as some other species of the genus *Campylobacter*, *Escherichia coli*, *Shigella* spp., *Helicobacter* spp., *Haemophilus ducreyi* and *Actinobacillus actinomycetemcomitans* (Jinadasa *et al.*, 2011, Yamasaki *et al.*, 2006b). CDT causes cell distension and death by blocking cell division. The toxin has three components, CdtA, CdtB and CdtC encoded by three adjacent genes *cdtA, cdtB* and *cdtC* (Pickett *et al.*, 1996). The CdtA and CdtC subunits are binding protein and help the catalytic cdtB subunit to enter into the susceptible cells. CdtB subunit carries out its cytotoxic activity by its DNase I activity, which causes DNA double strand breaks (Ge *et al.*, 2006, Yamasaki *et al.*, 2006b). All three *cdt* products are required for its functional activity (Asakura *et al.*, 2007). Among the *Campylobacter* spp., *C. jejuni, C. coli* and *C. fetus* produce CDT in a species-specific manner. That is, CDT toxin produced by different *Campylobacter* spp. differ both at the genetic and phenotypic level depending on the species (Asakura *et al.*, 2007, Eyigor *et al.*, 1999, Samosornsuk, 2007). Due to its virulence property and species specificity, the *cdt* gene cluster was considered as a candidate marker group for typing and all three genes of the cluster were investigated for a sequence based typing scheme for C. *jejuni.*

6.2 The Chapter's aims

The aims of this chapter were to-

i) Investigate the typeability of the markers, *cdtA, cdtB* and *cdtC*

ii) Determine the discriminatory power of the *cdt* gene cluster typing scheme

iii) Investigate the epidemiological concordance of the *cdt* gene cluster typing

iv) Investigate the congruence of the *cdt* gene cluster sequence typing scheme to multilocus sequence typing scheme by calculating adjusted Wallace and Rand coefficient

6.3 Results and Discussion

6.3.1 Typeability of *cdt* gene cluster typing

All three *cdt* genes, *cdtA, cdtB* and *cdtC* were amplified simultaneously (using previously described primer mentioned in the table 3.6) and subsequently sequenced using nested primer (Table 3.7) designed in this study from 45 *C. jejuni* strains isolated from different sources, i.e., ten outbreak strains isolated from children in a school camp, four strains isolated from the residents of an aged care facility, 16 clinical strains of sporadic infections, 5 from chicken, 3 from the same canine and 4 from water (Table 6.1). Amplification, sequencing and subsequent allelic profile determination based on multiple sequence alignment of all three *cdt* genes from 45 *C. jejuni* strains from a wide variety of sources indicates high typeability of the selected markers.

Mathematically, typeability of *CDT* cluster typing is as follows:

Tcdt= Tcdt/ T= 45/45=1

The results obtained also confirmed the specificity and sensitivity of the primers used and in agreement with the data reported previously (Asakura *et al.*, 2008a, Kabir *et al.*, 2011). However, it is observed that due to the possible mutations in one of the genes, 100% specificity may not always achievable. Nevertheless, since at least one of three genes can be amplified and sequenced, it is applicable for epidemiological investigation.

Table 6.1. Allelic profile of *cdtA, cdtB* and *cdtC* genes and sequence type for 45 *C. jejuni*

Strains	Epidemiological background of the strains	Allelic profiles			*cdt*
		cdtA	*cdtB*	*cdtC*	Type
LMG 8841^T	Type strain	1	1	1	1
C1	Aged care, 2005	2	2	2	2
C2		2	3	2	3
C3		3	2	2	4
C4		2	3	2	3
C5	Sporadic strain, 2005	3	3	3	5
H	Strain H from a gastroenteritis patient and Ca1-	2	4	3	6
Ca1	Ca3 are from his companion canine, 2012	2	3	3	7
Ca2		2	3	3	7
Ca3		2	3	3	7
CH5	Chicken, different suppliers, 2002 -2010	2	4	3	6
CH6		4	3	3	8
CH10		3	3	3	9
CH12		3	4	3	10
CH13		5	4	3	11
H1	Sporadic strains isolated from gastroenteritis	3	3	3	5
H2	patient, 2011-2012	2	4	3	6
H3		6	3	3	12
H5		2	3	2	3
H6		7	3	3	13
H7		3	3	2	14
H8		5	5	3	15
H9		8	3	3	16
H10		9	6	3	17
H13		3	3	3	5
H15		2	3	2	3
H16		2	3	3	3
H17		3	3	2	14
H18		3	3	3	5
H19		2	3	3	7
H20		3	3	2	14
OB1	School camp outbreak, 1993	3	3	3	5
OB9		3	3	3	5
OB14		3	3	3	5
OB24		3	3	3	5
OB41		3	3	3	5
OB59		3	3	4	5
OB64		3	3	3	5
OB68		3	3	3	5
OB85		3	3	3	5
OB102		3	3	3	5
W2	Potable water, Melbourne metropolitan and rural	3	3	5	18
W6	region, 2005-2008	7	4	5	19
W9		10	5	6	20
W11		5	4	3	11

6.3.2 Markers' resolution and Simpson's index of diversity

The 197 bp fragments from *cdtA*, 620 bp from *cdtB* and 374 bp from *cdtC* were analysed from each of the 45 strains included in this study. There were 10 alleles for *cdtA*, 5 for *cdtB* and 6 for *cdtC* and corresponding discriminatory index were 0.705, 0.474 and 0.492. In total, 1191 bp was analysed from each *C. jejuni* strain, which differentiated 45 strains into 20 allelic profiles (Table 6.1).

However, the final and combined Simpson's index of diversity (described in section 2.7.2.1) was calculated for only 28 epidemiologically unrelated strains as explained in the table 3.6 of the chapter 3. Three groups of related strains, outbreak strains, aged care cluster and canine strains were excluded as diversity index is usually calculated for unrelated strains. The 28 strains were differentiated into 17 allelic profiles and thus calculated Simpson's index of diversity was 0.9656.

6.3.3 Epidemiological concordance

Outbreak strains are expected to be identified as genetically indistinguishable upon application of the typing method suitable for outbreak investigation (Fitzgerald *et al.*, 2001). In this study, the 10 outbreak strains appeared indistinguishable from each other based on the allelic profiles of *cdtA, cdtB* and *cdtC* genes (Table 6.1 and Fig 6.1).

For this group of related strains, the Epidemiological concordance

(E) =1 (Ne/N)= 10/10=1.

In the aged care cluster, strains C1, C2, C3 and C4, isolated from patients living in the same aged care centre, were found to be very closely related. On the other hand, three canine strains isolated were also found genetically indistinguishable by *cdt* gene cluster.

Thus the analysis of the *CDT* cluster typing revealed the epidemiological relationships of three groups of related strains included in the study. These results suggested that *cdt* gene cluster typing showed excellent epidemiological concordance.

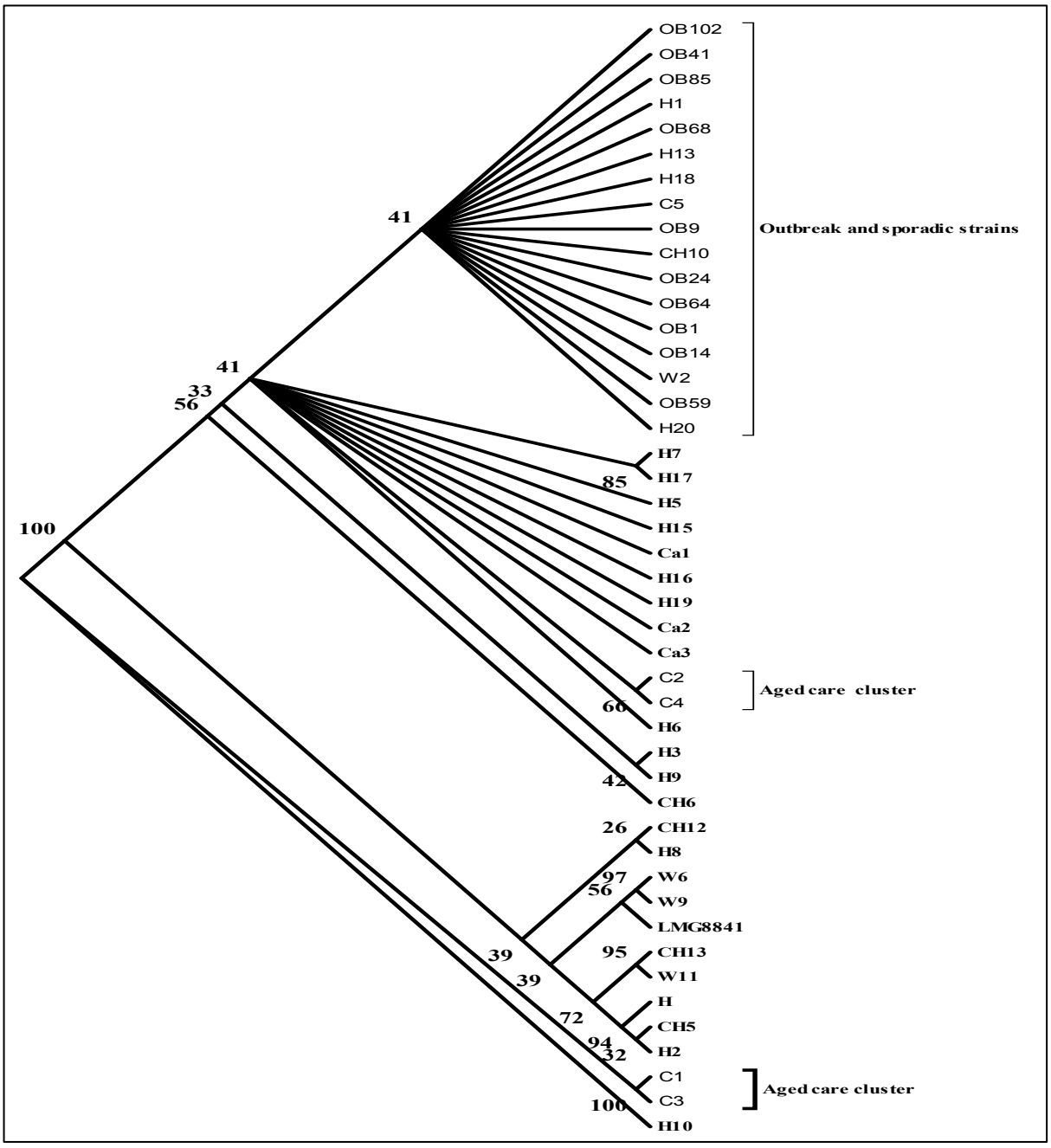

Figure 6.1. Phylogenetic relationships of 45 *C. jejuni* strains based on the concatenated sequence of *cdtA, cdtB* and ***cdtC* genes as established using neighbour joining (N-J) method with 1000 bootstrap replications using MEGA 5.05**

6.3.4 Comparison of *cdt* cluster typing to MLST

The *cdt* cluster typing was compared with MLST using 17 *C. jejuni* strains, which included two groups of related strains, aged care and outbreak strains and 9 non related strains (Table 6.2).

Table 6.2. Allelic profiles of 19 *C. jejuni* strains for MLST and *cdt* cluster typing

Strain	Source and yr. of isolation	MLST Type	*cdt* Type
LMG 8841^T	Type strain	403(10, 27, 16, 19, 10, 5, 7)	1
C1	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C2	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	3
C3	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	4
C4	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	3
C5	Human (sporadic); 2005	2343(2, 4, 5, 2, 10, 1, 5, 2)	5
CH5	Chicken; 2005	45(4, 7, 10, 4, 1, 7, 1)	6
CH6	Chicken; 2005	320(9, 7, 10, 4, 1, 7, 1)	7
OB1	Human (Outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	5
OB9	Human (Outbreak); 1993	48(2, 4, 1, 2, 7, 1,5)	5
OB14	Human (Outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	5
OB24	Human (Outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	5
OB41	Human (Outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	5
W2	Water; 2005	190(2, 1, 5, 3, 43, 3, 5)	8
W6	Water; 2007	800(64, 2, 22, 460, 43, 97, 79)	9
W9	Water; 2008	327(18, 2, 78, 35, 1, 86, 16)	10
W11	Water; 2008	4272(4, 7, 10, 4, 5, 7.)	11

Table 6.3. Comparison of *cdt* cluster typing with MLST using Simpson's index of diversity, adjusted Wallace and adjusted Rand Coefficient

Typing scheme	No. of partition /type	SID (95%CI)	Adjusted Wallace Coefficient (95%CI)		Adjusted Rand Coefficient (95%CI)	
			cdt	MLST	*cdt*	MLST
cdt cluster typing	11	0.882 (0.750-1.000)		0.646 (0.189-1.000)		0.646
MLST	10	0.882 (0.779-0.986)	0.646 (0.457-0.835)			

cdt cluster typing identified 9 singletons against 7 by MLST and *cdt* typing schemes also differentiated among aged care strains (C1-C4). Thus it is found that this three marker typing scheme *cdt* cluster typing is more discriminatory than MLST though the calculated Simpson's index of diversity was found equal for that small number of strains.

To compare the type assignment by *cdt* gene cluster sequencing and MLST, the adjusted Wallace and Rand coefficients (two partition coefficients used to test congruence of typing methods) were also calculated using online tools (Table 3.3). Wallace coefficient (0.646, Table 6.3) showed that the probability that a pair of strains, which were classified as the same type by MLST, were also classified as the same type by *cdt* cluster typing was above 60%. The adjusted Rand index (compares the clustering/grouping of isolates sharing similar characteristics according to a given method) for MLST and *cdt* typing in this study was 0.646, meaning that the congruence

for clustering of *C. jejuni* strains between the two methods is partial and slightly above medium level, which could range from 0 to 1 (Campbell *et al.*, 2011).

6.4 Conclusion

In this chapter, for the first time a typing scheme based on toxin genes is described. With 45 *C. jejuni* strains from a wide variety of sources, it is demonstrated that the *cdt* gene cluster sequence typing scheme is highly discriminatory with high typeability and medium level of congruence to multilocus sequence typing (MLST).

Chapter 7 . Characterisation of a tetra locus sequence typing (TeLST) scheme based on *porA, peb1*A, *mapA* and *ceuE*

Summary

In this chapter, applicability of *porA peb1A, mapA and ceuE* genes was investigated in a tetra locus sequence typing (TeLST) scheme on fifty *C. jejuni* strains isolated from both clinical and environmental sources. Clinical strains included outbreak and sporadic infection strains and environmental strains were from other major sources of *C. jejuni* such as chicken, water, ovine and canine. Four genes *porA, peb1A, mapA* and *ceuE* using in a tetra-locus scheme showed high typeability, extremely high discriminatory power (SID 0.9943) and good epidemiological concordance by identifying outbreak strains and other related strains. The TeLST scheme also showed good congruence to multilocus sequence typing scheme when it was compared using twenty *C. jejuni* strains. It was found that *porA, peb1A*, *mapA* and *ceuE* genes are a tetra locus combination for epidemiological investigation of *C. jejuni*. The scheme can be recommended for epidemiological investigations as a complementary tool along with well established methods such as pulsed field gel electrophoresis or multilocus sequence typing.

7.1 Introduction

Bacterial typing is central to diagnosis and epidemiological surveillance of bacterial infections (Li *et al.*, 2009). Due to the advantages over phenotyping systems in terms of discriminatory power, reproducibility and rapidity, a number of genotyping systems are currently in use for epidemiological surveillance of *C. jejuni* (Wassenaar and Newell, 2000, Ahmed *et al.*, 2012, Eberle and Kiess, 2012), the most common foodborne bacterial pathogen worldwide (Friedman *et al.*, 2004, Senok and Botta, 2009). However, there is no typing system which would be useful for all forms of investigation in regard to cost, rapidity and reliability, and discrimination power (Foxman *et al.*, 2005). Among genotyping methods, sequence based typing methods are preferred for unambiguous data production and inter laboratory transferability (Li *et al.*, 2009). Two commonly used sequences-based typing schemes for *C. jejuni* are flagellin (*fla*) gene sequence typing and multilocus sequence typing (MLST) (Wassenaar and Newell, 2000, Wassenaar, 2011). Flagellin gene typing uses only one gene, *flaA, flaB* or short variable region (SVR) of *flaA* gene (Meinersmann *et al.*, 1997, Meinersmann *et al.*, 2005, Mellmann *et al.*, 2004). Another single locus sequence based typing schemes was described based on *porA or cm*P gene (Huang *et al.*, 2005). Recently this typing scheme was employed for outbreak study and was recommended as complementary to MLST, pulsed field gel electrophoresis (PFGE) and whole genome sequencing (Jay-Russell *et al.*, 2013). As single locus poorly represents the whole bacterial genome, such methods are considered least reliable for epidemiological studies (De Boer *et al.*, 2000, Olive and Bean, 1999). On the other hand, presence of two copies of *fla* gene (*flaA* and *flaB*) and possible intragenomic and intergenomic recombination between the two copies undermines the reliability of typing naturally transformation competent *Campylobacter* spp. (Guerry *et al.*, 1991, Cornelius *et al.*, 2010, Harrington *et al.*, 1997). MLST use

seven housekeeping genes (Olive and Bean, 1999). Using more stable and less variable housekeeping genes in large number makes MLST costly, laborious and inadequately discriminatory for local epidemiological studies (Maiden *et al.*, 1998, Urwin and Maiden, 2003, Dingle *et al.*, 2001, Korczak *et al.*, 2009). Thus it is timely to develop affordable and highly discriminatory sequence based typing scheme for *C. jejuni* as for example recently proposed typing schemes for *E. faecalis*, *S. aureus* and *L. Monocytogenes* which were developed using a small number of highly variable markers such as virulence or a surface protein coding genes (Kuhn *et al.*, 2007, Zhang *et al.*, 2004, Chowdhury *et al.*, 2009).

In search of highly discriminatory typing schemes for *C. jejuni* suitable for the study of multistrain coinfection of the host animal and human patient, in this chapter, a tetra locus sequence typing scheme was investigated with *ceuE*, *porA, peb1A* and *mapA*.

7.2 The chapter's aims

The aims of this chapter were to-

i. Investigate the typeability of the markers *Peoria, peb1A, maps* and *cue*
ii. Determine the discriminatory power of the tetra locus typing scheme
iii. Investigate the epidemiological concordance of the tetra locus typing scheme
iv. Investigate the congruence of the tetra locus typing sequence typing scheme to multilocus sequence typing scheme

7.3 Results and Discussion

7.3.1 Typeability of TeLST typing scheme

porA gene has been considered as a valuable marker in the typing scheme (Cody *et al.*, 2009). This gene was previously included as an additional component in MLST study of *C. jejuni* strains for further differentiation among strains belonging to same types by MLST and found useful in short term epidemiological studies, such as an outbreak investigation (Dingle *et al.*, 2008). The *porA* gene is therefore included in this study to evaluate its performance in combination with other surface associated genes of *C. jejuni*. Peb1A of *C. jejuni* is an antigenic surface associated protein, which plays a role in adherence and host colonization and thus in pathogenesis (Müller *et al.*, 2007b, Del Rocio Leon-Kempis *et al.*, 2006, Pei and Blaser, 1993b). The *mapA* gene encodes a 24 KDa antigenic surface protein in all *C. jejuni* strains in a species specific manner (Stucki *et al.*, 1995). Hence it is widely used in molecular/ PCR based identification of *C. jejuni* strains from different samples (De Boer *et al.*, 2013, Singh *et al.*, 2011). Moreover, the encoded protein is antigenic in nature and such protein coding genes have been used in typing schemes for other bacteria (Chowdhury *et al.*, 2009, Chan *et al.*, 2009). The *ceuE* plays a role in virulence by encoding a protein that facilitates iron acquisition (Gonzalez *et al.*, 1997). Being a species specific and a virulence gene, *ceuE* was considered as a promising marker for typing of *C. jejuni***.**

*por*A*, peb1*A*,map*A and *ceu*E were amplified from the fifty *C. jejuni* strains isolated from human patients, chicken, ovine, water and canine (Table 7.1). Subsequently the amplified genes were sequenced by the nested primers designed in this study (Table

3.2). As all four markers were amplified, sequenced and allelic profiles were determined for all 50 *C. jejuni* strains.

Typeability of tetra locus sequencing typing,

TTe = 50/50=1

Since the collection of fifty *C. jejuni* strains included 5-10 strains from each of the major sources of *C. jejuni*, it can be expected that the scheme will show high typeability when applied for typing *C. jejuni* from different sources and locations.

7.3.2 Marker resolution and Simpson's index of diversity of TeLST

Three hundred fifty nine bp of *porA*, 399 of *peb1A*, 495 bp of *mapA and* 468 bp of *ceuE* coding sequence were sequenced and analysed for the allelic profiles. Thus 1721 bp was analysed for each strain. The number of alleles for *porA, peb1A and mapA* was found to be 18, 9, 7 and 7 respectively in 50 *C. jejuni* strains (Table 7.1). The discriminatory index for individual marker was found 0.8517 for *porA*, 0.858 for *peb1A* 0.751 for *mapA* and 0.5878 for *ceuE*. Thirty-three unrelated strains were differentiated into 31 types and the calculated Simpson's index of diversity was 0.9943. This is a very high discriminatory index, close to the ideal discriminatory index 1.0.

Table 7.1. Allelic profiles of *porA, peb1A, mapA* and *ceuE* and sequence type of 50 *C. jejuni*

Strain ID	Source/origin/isolation year	Allelic profile				Sequence type
		porA	*Peb1A*	*mapA*	*ceuE*	
LMG 8841^T	Type strain	1	1	1	1	1
C1	Aged care facility cluster, 2005	2	2	2	2	2
C2		2	2	2	2	2
C3		2	2	2	2	2
C4		2	2	2	2	2
C5	Sporadic	3	3	3	2	3
Ca1	Ca1- Ca3 are from a companion	3	4	2	2	4
Ca2	canine, 2012	3	4	2	2	4
Ca3		3	4	2	2	4
CH1	Chicken, different farms and	4	5	3	3	5
CH2	retailers, 2002-2004	5	6	3	2	6
CH5		3	5	2	2	7
CH6		6	5	4	4	8
CH9		3	5	4	5	9
CH10		7	2	3	3	10
CH12		3	7	5	5	11
CH13		3	5	4	5	9
CH14		2	4	2	3	12
CH15		3	3	2	2	13
CJ81116	Reference strain	8	8	3	2	14
H	Sporadic strains, 2012	3	5	4	5	9
H15		9	7	6	3	15
H16		10	4	4	4	16
H17		10	4	2	6	17
H18		11	6	3	2	18
H19		10	4	3	6	19
OB1	School camp outbreak, 1993	3	6	3	2	20
OB9		3	6	3	2	20
OB14		3	6	3	3	21
OB24		3	6	3	2	20
OB41		3	6	3	7	22
OB59		3	6	3	2	20
OB64		3	6	3	2	20
OB68		3	6	3	2	20
OB85		3	6	3	2	20
OB102		3	6	3	2	20
OV1	Ovine in Victoria and Tasmania	12	4	7	2	23
OV2	of Australia, 2001-2002	3	5	7	5	24
OV3		3	5	7	5	25
OV5		3	8	3	2	26
OV6		7	5	3	2	27
W2	Potable water, Melbourne	3	2	3	2	28

W6	metropolitan and rural region, 2005-2011	11	3	5	5	29
W9		13	3	5	5	30
W11		14	7	4	2	32
W12		5	7	2	2	33
W13		15	7	6	2	34
W14		16	7	2	2	35
W16		17	4	2	2	36
W17		18	8	2	2	37

7.3.2 Epidemiological concordance (E)

The epidemiological concordance was also found very well for all three related groups of *C. jejuni* strains. TeLST scheme identified 8 out of 10 outbreak strains as genetically indistinguishable (Fig 7.1 and Table 7.1). OB14 and OB59 were differentiated from other 8 outbreak strains. In outbreak set of strains, their epidemiological concordance was found to be: E= 0.8 (E=Ne/N=8/10=0.08)

The three canine strains and four aged care strains were also found genetically indistinguishable (Table 7.1). Thus overall epidemiological concordance of the TeLST can be considered good for all three groups of related *C. jejuni* strains. The scheme has also clustered strains from different sources such as chicken and water strains together.

The differentiation among outbreak strains by TeLST is congruent with its very high discriminatory power (0.9943) and underscores its usefulness in determining whether an outbreak is by single strain or by multiple strains. Multi strain outbreaks or multi strain co-infection of chicken and other animals, even in raw meat and poultry in retail sales are not uncommon for highly genetically variable *C. jejuni (El-Adawy et al., 2013)*. This scheme can be used to study environmental samples**,** whether the chicken flock of a farm or cattle's of a dairy firm carry one genotype of *C. jejuni* or more than one very closely related genotypes.

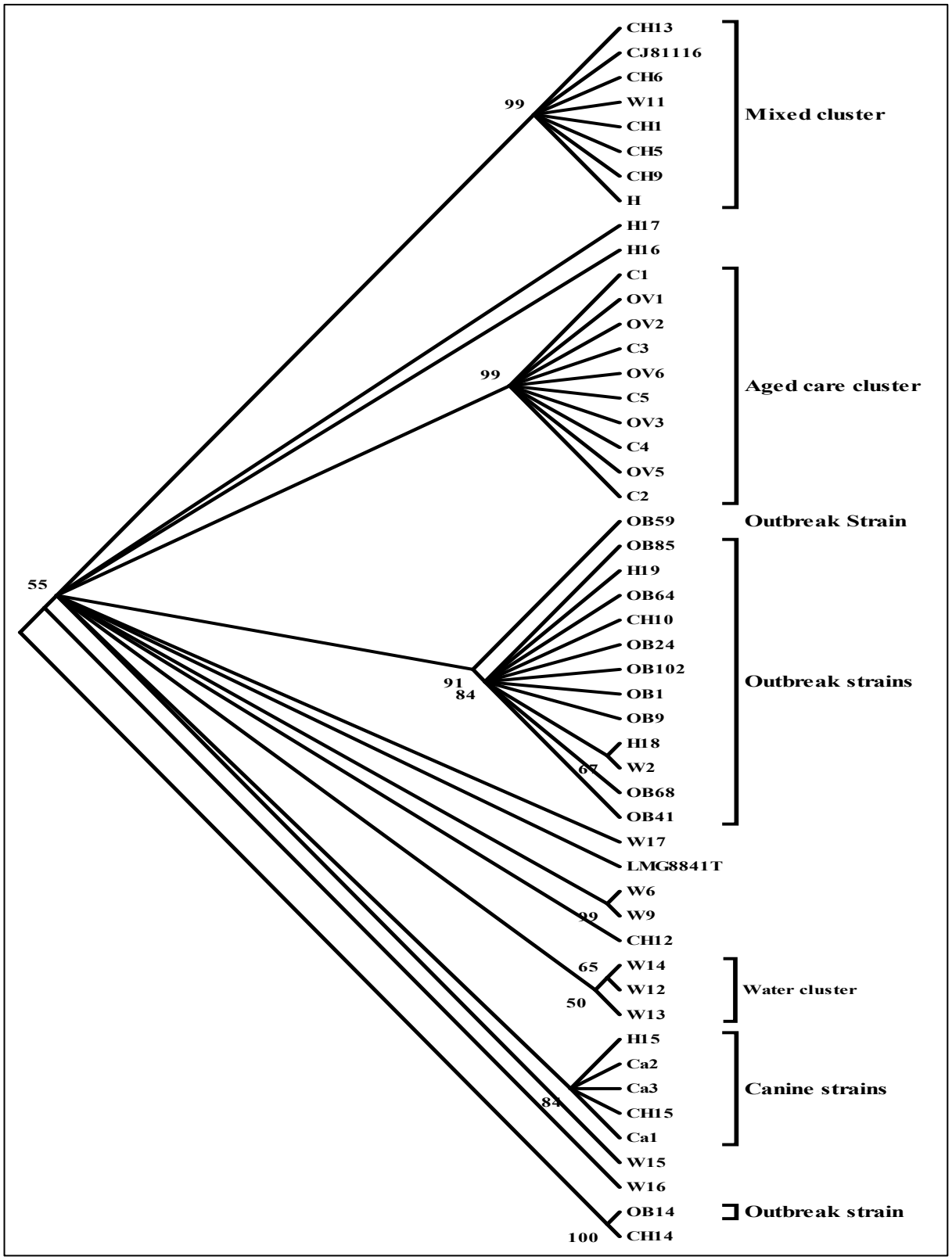

Figure 7.1. Phylogenetic relationship of 50 *C. jejuni* strains based on the concatenated sequence of *porA, peb1A, mapA* and *ceuE* as established using neighbour joining (N-J) method with 1000 bootstrap replications using MEGA 5.05.

7.3.4 Comparative analysis of TeLST to MLST

Twenty *C. jejuni* strains were differentiated into 12 sequence types by MLST and 14 by TeLST (Table 7.2). The TeLST also identified 8 singletons as identified by MLST**,** which are LMG 8841^{T}, C5, CH1, CH5, CH6, CH9, CJ81116 and three strains from water, W2, W6 and W11 (Table 7.2). Doubleton (CH5 and CH9) identified by MLST was differentiated into two different types by TeLST. In this case, all five chicken strains were differentiated into 5 different types (type 4, 5, 6, 7, 8, Table 7.2), which are compatible with its very high discriminatory power. On the other hand, one strain from water, W11 was assigned the same type of some outbreak strains. When calculated for twenty strains, Simpson's index of diversity for TeLST was found higher than that of MLST (Table 7.3).

Table 7.2. Allelic profiles of twenty *C. jejuni* strains according to MLST and TeLST

Strain	Source and year of isolation	MLST Type	TeLST Type
LMG 8841^T	Type strain	403(10, 27, 16, 19, 10, 5, 7)	1
C1	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C2	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C3	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C4	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C5	Human (sporadic); 2005	2343(2, 4, 5, 2, 10, 1, 5, 2)	3
CH1	Chicken; 2002	583(4, 7, 10, 442, 51, 354)	4
CH5	Chicken; 2005	45(4, 7, 10, 4, 1, 7, 1)	5
CH6	Chicken; 2005	320(9, 7, 10, 4, 1, 7, 1)	6
CH9	Chicken; 2008	45(4, 7, 10, 4, 1, 7, 1)	7
CJ81116	Laboratory strain; 2011	267(4, 7, 40, 4, 42, 51, 1)	8
OB1	Human (outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	9
OB9	Human (outbreak); 1993	48(2, 4, 1, 2, 7, 1,5)	9
OB14	Human (outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	10
OB24	Human (outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	9
OB41	Human (outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	11
W2	Water; 2005	190(2, 1, 5, 3, 43, 3, 5)	12
W6	Water; 2007	800(64, 2, 22, 460, 43, 97, 79)	13
W9	Water; 2008	327(18, 2, 78, 35, 1, 86, 16)	9
W11	Water; 2008	4272(4, 7, 10, 4, 5, 7.)	14

The congruence between type assignments by MLST and TeLST was also tested by determination of diversity and partition congruence coefficients, Adjusted Wallace and

Rand coefficient for 20 *C. jejuni* strains from four different groups, outbreak strains, aged care cluster, water and ovine strains were calculated using the online tool (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool). (Table 7.3).

Table 7.3. Comparative characteristics of TeLST and MLST performance criteria

Typing scheme	No. of partition/type	SID (95%CI)	Adjusted Wallace Coefficient (95%CI)		Adjusted Rand Coefficient (95%CI)	
			TeLST	MLST	TeLST	MLST
TeLST	14	0.947 (0.893-1.000)		0.671 (0.436-0.905)		0.484
MLST	12	0.911 (0.833-0.988)	0..379 (0.170-0.588)			

Adjusted Wallace coefficient and the Rand coefficient of TeLST to MLST are considerably lower in comparison to DLST and TLST. Thus, this highly discriminatory typing scheme for *C. jejuni* is least concordant to MLST among this series of sequence based typing schemes. As this scheme uses two *C. jejuni* species specific primer sets for *mapA* (Stucki *et al.*, 1995) and *ceuE* (Gonzalez *et al.*, 1997), separate identification may not be required providing additional advantage over the MLST. Since the scheme employs two times less number of the MLST loci, the cost and labor involved will be also reduced. Most importantly, TeLST exhibited higher discriminatory power than MLST. In some epidemiological studies targeting investigation of a host population in a specific area when closely related strains need to be differentiated, TeLST may be most useful.

7.4 Conclusion

The intragenic sequence of *Peoria*, *peb1A, maps* and *key* genes have been found suitable for a tetra locus sequence typing scheme for *C. jejuni*. The scheme has very high discriminatory power, close to ideal index, 1. The test was found to be able to differentiate among outbreak strains, allowing understanding whether the human patient or host such as chicken, cow and poultry are infected by more than one closely related strains or whether more than one strain of *C. jejuni* involved in the outbreak. The newly developed TeLST scheme with discriminatory power close to 1 can be also used for study co-infection of patient(s) and host.

Chapter 8 . Employment *of porA, peb1*A and *mapA* in a tri-locus sequence typing scheme for *C. jejuni*

Summary

In this chapter, the apapplicability of *porA, peb1A* and *mapA* genes was investigated as a tri locus sequence typing (TLST) scheme based on fifty *C. jejuni* strains derived from outbreak, sporadic infection and sources of *C. jejuni* such as chicken, water, ovine and canine. *porA, peb1A* and *mapA* as a tri-locus scheme showed high typeability, high discriminatory power (SID 0.986) and very good epidemiological concordence in the identification of the outbreak strains. The TLST scheme also showed very good congruence to multilocus sequence typing scheme based on 20 randomly selected *C. jejuni* strains. It is shown that a combination of *porA, peb1A* and *mapA* genes is suitable in a tri-locus typing scheme. The scheme may be applicable for epidemiological investigations as a complementary to other established methods such as pulsed field gel electrophoresis or multilocus sequence.

8.1 Introduction

In search for the typing schemes that will allow accurate differentiation of *C. jejuni* strains, a tri-locus sequence typing scheme is investigated based on *mapA* (membrane associated protein) gene along with *porA* (associated with antibiotic resistance) and *peb1A* (virulence factor by the dual role of adhesin and solute binding protein). Such a combination of markers was found suitable for the trilocus typing scheme in the pilot study. In this study, we report a tri-locus sequence typing scheme for *C. jejuni*.

8.2 The chapter's aims

The aims of this chapter were to -

i. Investigate the typeability of the combination of the selected markers
ii. Determine the discriminatory power of the tri-locus scheme
iii. Investigate the epidemiological concordance of the tri-locus scheme
iv. Investigate the congruence of the tri- locus sequence typing scheme to multilocus sequence typing scheme

8.3 Results and Discussion

8.3.1 Typeability of TLST typing scheme

Each strains was assigned a type based on the allelic profile of *porA, peb1A* and *mapA* gene from 50 *C. jejuni* strains isolated from wide variety of sources, which included strains from human patient, chicken, ovine, water and canine (Table 8.1). Typeability of Tri-locus typing scheme was calculated as follows:

TTL $=Nt/N= 50/50=1$, *Nt* is the number of strains assigned type,

N is the number of strains tested

Typeability of a sequence based typing schemes becomes less than 1.0 if the marker cannot be amplified or sequenced due to mutation in any of primer binding sides or addition /deletion in between the primers. However, in this case, *mapA* along with two other markers *porA* and *peb1A* were amplified, sequenced and allelic profiles were determined from all 50 *C. jejuni* strains from clinical and wide variety of environmental samples. Thus tri-locus typing scheme showed high typeability and can be considered to show similar typeability when applied to a large number of strains in epidemiological studies.

Table 8.1. Allelic profiles of *porA, peb1A* and *mapA* and sequence type of 50 *C. jejuni* isolates

Strain ID	Source/origin/isolation year	Allelic profile			Sequence type
		porA	*Peb1A*	*mapA*	
LMG 8841T	Type strains	1	1	1	1
C1	Aged care cluster, 2005	2	2	2	2
C2		2	2	2	2
C3		2	2	2	2
C4		2	2	2	2
C5	Sporadic	3	3	3	3
Ca1	from a companion canine, 2012	3	4	2	4
Ca2		3	4	2	4
Ca3		3	4	2	4
CH1	Chicken, different farms and retailers, 2002-	4	5	3	5
CH2	2004	5	6	3	6
CH5		3	5	2	7
CH6		6	5	4	8
CH9		3	5	4	9
CH10		7	2	3	10
CH12		3	7	5	11
CH13		3	5	4	9
CH14		2	4	2	10
CH15		3	3	2	11
CJ81116	Reference strain	8	8	3	12
H	Sporadic strains,2012	3	5	4	9
H15		9	7	6	13
H16		10	4	4	14
H17		10	4	2	15
H18		11	6	3	16
H19		10	4	3	17
OB1	School camp outbreak, 1993	3	6	3	18
OB9		3	6	3	18
OB14		3	6	3	18
OB24		3	6	3	18
OB41		3	6	5	19
OB59		3	6	3	18
OB64		3	6	3	18
OB68		3	6	3	18
OB85		3	6	3	18
OB102		3	6	3	18
OV1	Ovine from Victoria and Tasmania of	12	4	7	20
OV2	Australia. 2001-2002	3	5	7	21
OV3		3	5	7	21
OV5		3	8	3	22
OV6		7	5	3	23
W2	Potable water, Melbourne	3	2	3	24
W6	metropolitan and rural region, 2005-2011	11	3	5	25
W9		13	3	5	26
W11		14	7	4	27
W12		5	7	2	28
W13		15	7	6	29
W14		16	7	2	30
W16		17	4	2	31
W17		18	8	6	32

8.3.2 Marker resolution and Simpson's index of diversity of TLST

Three hundred fifty nine bp of *porA*, 399 of *peb1A* and 495 bp of *mapA* coding sequence were sequenced and analysed for the allelic profile. Thus 1253 bp was analysed for each strain. The number of alleles for *porA, peb1A and mapA* was found to be 18, 9 and 7 respectively in 50 *C. jejuni* strains. The discriminatory index for individual marker was found 0.8517 for *porA*, 0.858 for *peb1A* and 0.751 for *mapA*. Thirty three (33) unrelated strains were differentiated into 28 types (Table 6.1) and the calculated Simpson's index of diversity was 0.986. This discriminatory index is high enough to be suitable for outbreak and the local epidemiological study (van Belkum *et al.*, 2007).

8.3.3 Epidemiological concordance

TLST scheme also identified nine out of 10 outbreak strains as of the same type, genetically indistinguishable (except OB41). The diversity index is rising with increased number of markers and the epidemiological concordance was found to be:

E =Ne/N=9/10=0.9, Here Ne= no. of strains found indistinguishable, N= no. of strains outbreak strains tested.

The three strains derived from canine strains and four strains isolated from aged care facility were also found genetically indistinguishable. Thus TLST showed very good epidemiological concordance for all three groups of related *C. jejuni* strains (Table 8.12, Fig. 8.1). Differentiation of 10 outbreak strains into two distinct types indicated that the TLST scheme has the potential to identify multi-strain outbreak which is not uncommon in case of outbreak and infections caused by *C. jejuni* (Devane *et al.*, 2013).

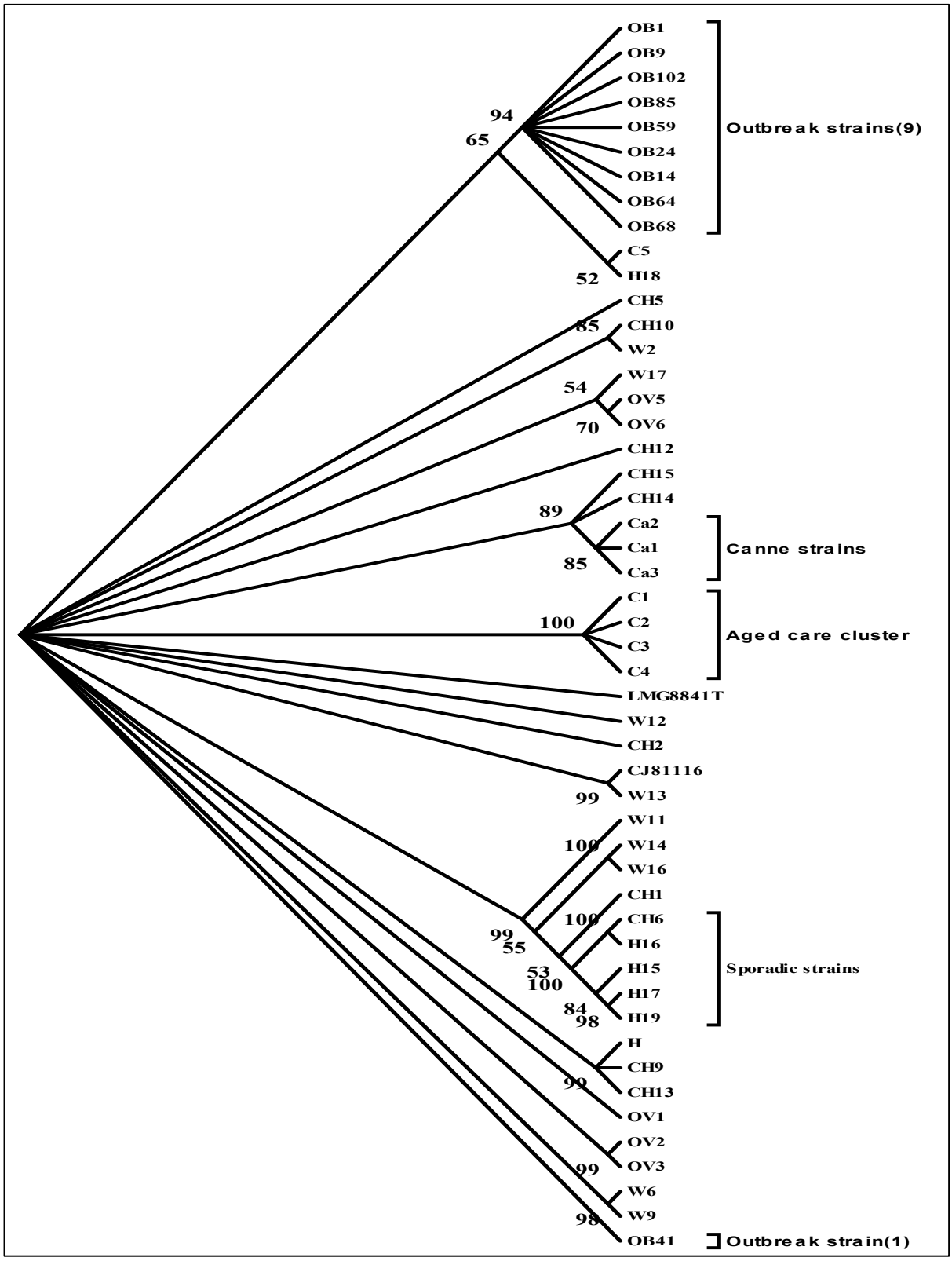

Figure 8.1. Phylogenetic relationship of 50 *C. jejuni* strains based on the concatenated sequence of *porA, peb1A* and *mapA* as established by neighbour joining (N-J) method with 1000 bootstrap replications using MEGA 5.05.

8.3.4 Comparison of TLST to MLST

TLST was compared to MLST using the same group of twenty *C. jejuni* strains at a pilot scale level, which included two groups (aged care facility cluster, C1-C4 and outbreak strains, OB) of related strains and 11 non-related strains (Table 8.2).

Table 8.2. Allelic profiles of 20 *C. jejuni* strains for MLST and TLST

Strain	Source and year of isolation	MLST Type*	TLST Type
LMG 8841[T]	Type strain	403(10, 27, 16, 19, 10, 5, 7)[a]	1
C1	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C2	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C3	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C4	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C5	Human (sporadic); 2005	2343(2, 4, 5, 2, 10, 1, 5, 2)	3
CH1	Chicken; 2002	583(4, 7, 10, 442, 51, 354,)	4
CH5	Chicken; 2005	45(4, 7, 10, 4, 1, 7, 1)	5
CH6	Chicken; 2005	320(9, 7, 10, 4, 1, 7, 1)	6
CH9	Chicken; 2008	45(4, 7, 10, 4, 1, 7, 1)	7
CJ81116	Laboratory strains; 2011	267(4, 7, 40, 4, 42, 51, 1)	8
OB1	Human (Outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	9
OB9	Human (Outbreak); 1993	48(2, 4, 1, 2, 7, 1,5)	9
OB14	Human (Outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	9
OB24	Human (Outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	9
OB41	Human (Outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	10
W2	Water; 2005	190(2, 1, 5, 3, 43, 3, 5)	11
W6	Water; 2007	800(64, 2, 22, 460, 43, 97, 79)	12
W9	Water; 2008	327(18, 2, 78, 35, 1, 86, 16)	13
W11	Water; 2008	4272(4, 7, 10, 4, 5, 7.)	14

*MLST sequence types were assigned in accordance with the database available at http://http://pubmlst.org/campylobacter/. a. Allelic profile of 7 MLST genes as determined from database are shown in the bracket

Twenty *C. jejuni* strains were differentiated into 12 sequence types by MLST and 14 by TLST (Table 8.2). TLST also identified 9 singletons as identified by MLST which were LMG 8841^T, C5, CH1, CH5, CH6, CH9, CJ81116, and all four strains from water, W2, W6, W9 and W11 (Bold in Table 8.2). Doubleton (CH5 and CH9) identified by MLST was differentiated into two different types by TLST. Thus TLST appeared to be highly discriminatory, better than MLST. The two groups of related strains were also identified as two distinct types by both TLST and MLST. TLST and MLST performance parameters are compared in Table 8.3.

Table 8.3. Comparison of DLST and MLST using Simpson's index of diversity (SID), adjusted Wallace (AW) and adjusted Rand (AR) Coefficient for 20 *C. jejuni* strains

Typing scheme	No. of partition/type	SID (95%CI)	Adjusted Wallace Coefficient (95%CI)		Adjusted Rand Coefficient (95%CI)	
			TLST	MLST	TLST	MLST
TLST	14	0.937 (0.871-1.000)		1.0 (1.000-1.000)		0.814
MLST	12	0.911 (0.833-0.988)	0.686 (0.381-0.991)			

It is found that TLST is more discriminatory than MLST. Its ability to predict type assigned by MLST is 100% as evident from adjusted Wallace coefficient (1.0) and probability of clustering of strains is slightly above 80% (0.814 adjusted Rand Coefficient). From these data it can be concluded that TLST scheme is of very good congruence to multilocus sequence typing scheme. Due to reduced numbers of markers this scheme is less laborious and faster than MLST. Since amplification of *mapA* requires *C. jejuni* species specific primers *(Stucki et al., 1995)* the taxonomic identity of newly isolated strains can be simultaneously reconfirmed.

8.4 Conclusion

A combination of *porA*, *peb1A* and *mapA* found to be suitable for a tri-locus *C. jejuni* typing scheme with high discriminatory power of 0.0986 and very good epidemiological concordance and congruence to multilocus sequence typing. Using 1253 bp of *porA, peb1A* and *mapA* genes, the tri-locus sequence typing scheme has shown high discriminatory power to differentiate between non-related strains while showing very good epidemiological concordance for three groups of related strains. It was also possible to differentiate outbreak strain, OB41, from nine other strains. Thus this typing scheme can be useful in identification of multi-strain outbreaks, which is not uncommon in case of *C. jejuni*.

Chapter 9 . Investigation of the *porA* and *peb1A* genes in a double-locus sequence *C. jejuni* typing scheme

Summary

In this Chapter, a DLST scheme for *C. jejuni* based on concatenated partial sequences of *porA* and *peb1A* genes has been investigated. The DLST scheme was validated using fifty clinical and environmental *C. jejuni* strains isolated from human (C5, H, H15-H19), chicken (CH1-CH15), water (W2-W17) and ovine samples (OV1 - OV6). The scheme was found to be highly discriminatory (DI=0.964) and epidemiologically concordant in this study with limited number of *C. jejuni* strains. The DLST showed the discriminatory power above 0.95 and excellent congruence to multilocus sequence typing (MLST) and can be used as a rapid and low cost typing scheme for epidemiological investigation of C. *jejuni*. It is suggested that the DLST scheme is suitable for outbreak strains identification and differentiation of the sporadic infection strains.

9.1 Introduction

The applicability of virulence and surface associated gene *peb1A* (Del Rocio Leon-Kempis *et al.*, 2006, Müller *et al.*, 2007a, Pei and Blaser, 1993a) was investigated in association with *porA* for a double locus sequence typing scheme as this group showed lowest discriminatory power suitable for local epidemiological (≥0.95) study as a double locus scheme.

9.2 The chapter's aims

The major aims of this chapter were to –

i. Investigate the typeability of the markers, *porA* and *peb1A*
ii. Determine the discriminatory power of the double locus scheme
iii. Investigate the epidemiological concordance of the double locus scheme
iv. Investigate the congruence of the double locus sequence typing with multilocus sequence typing scheme.
v. Compare with *fla SVR* sequence typing

9.3 Results and Discussion

Whether any genetic marker or combination of genetic markers is suitable for typing schemes or not depends on its typeability, discriminatory power and epidemiological concordance, hence these properties of the *porA* and *peb1A* were investigated in this study (Struelens *et al.*, 1996).

9.3.1 Typeability of selected markers

To test the typeability, the ability to assign types to each and every strain tested, 48 C. *jejuni* strains from a wide variety of sources such as human, chicken, water, canine and

ovine were included in the study along with *C. jejuni* LMG 8841^T and reference strain *C. jejuni* subsp. *jejuni* 81116 (Table 9.1).

Altogether 50 strains, 5-10 strains from each source following recommended guideline (Struelens *et al.*, 1996), were included to investigate the desired characteristics of *porA* and *peb1A* marker. Among the strains, thirty two *C. Jejuni* strains were epidemiologically unrelated strains isolated from human (C5, H, H15-H19), chicken (CH1 - CH15), water (W2 -W17) and ovine (OV1 - OV6); and seventeen epidemiologically related strains that consisted of three groups, namely, ten *C. jejuni* outbreak strains (OB1 - OB102) from a school camp outbreak in 1993; four strains (C1-C4) from an aged care centre isolated in 2009; three *C. jejuni* strains (Ca1 - Ca3) isolated from the same canine in 2012.

Amplicons for *porA* and *peb1A* were obtained from all fifty strains *C. jejuni* strains stored over a period of 18 years (1993-2012) and isolated from five major sources of *C. jejuni*. Allelic profile of both *porA* and *peb1A* genes from all 50 C. *jejuni* strains were determined by multiple sequence alignment based on nucleotide sequence deference (Table 9.1). Each of the 50 strains was assigned to a type based on the allelic profile of *porA* and *peb1A* gene sequences. So the typeability of the double locus sequence typing schemes was, represented by the formula (Struelens *et al.*, 1996) was found:

$$TD = Nt/N = 50/50 = 1,$$

Here *Nt* is the number of strains assigned a type and N is the number of strains included in the study.

Table 9.1. Allelic profile of *porA* and *peb1A* and sequence type of 50 *C. jejuni* isolates

Strain ID	Source/origin/isolation year	Allelic profile		Sequence type
		porA	*peb1A*	
LMG 8841^T	Type strain	1	1	1
C1	Aged care cluster, 2005	2	2	2
C2		2	2	2
C3		2	2	2
C4		2	2	2
C5	Sporadic	3	3	3
Ca1	From companion canine, 2012	3	4	4
Ca2		3	4	4
Ca3		3	4	4
CH1	Chicken, different farms and retailers,	4	5	5
CH2	2002-2004	5	6	6
CH5		3	5	7
CH6		6	5	8
CH9		3	5	7
CH10		7	2	9
CH12		3	7	10
CH13		3	5	7
CH14		2	4	11
CH15		3	3	3
CJ81116	Reference strain	8	8	12
H	Sporadic strains. 2012	3	5	7
H15		9	7	13
H16		10	4	14
H17		10	4	14
H18		11	6	15
H19		10	4	14
OB1	School camp outbreak, 1993	3	6	16
OB9		3	6	16
OB14		3	6	16
OB24		3	6	16
OB41		3	6	16
OB59		3	6	16
OB64		3	6	16
OB68		3	6	16
OB85		3	6	16
OB102		3	6	16
OV1	Ovine in Victoria and Tasmania of	12	4	17
OV2	Australia, 2001-2002	3	5	7
OV3		3	5	7
OV5		3	8	18
OV6		7	5	19
W2	Potable water, Melbourne metropolitan	3	2	20
W6	and rural region, 2005-2011	11	3	21
W9		13	3	22
W11		14	7	23
W12		5	7	24
W13		15	7	25
W14		16	7	26
W16		17	4	27
W17		18	8	28

Both the markers individually have the expected typeability 1. The typeability of typing schemes can range from 0-1.0 and ideally expected typeability is 1.0 (Struelens *et al.*, 1996). However, if the selected markers cannot be amplified for mutation and subsequently sequenced in case of a sequence based typing or cannot be digested by restriction enzyme due to DNA modifications, then typeability decreases. Usually non typeable strains are grouped together which does not necessarily mean that they are of the same type (van Belkum *et al.*, 2007). In this case, both *porA* and *peb1A* markers were amplified; sequenced and allelic profiles were determined from all 50 *C. jejuni* strains from both clinical and wide variety of environmental samples. As a result, this double locus typing scheme can be expected to show high typeability when applied to a large number of strains in epidemiological studies.

9.3.2 Marker resolution and Simpson's index of diversity of double locus sequence typing of *C. jejuni.*

The number of unique alleles for 359 bp fragment of *porA* was 18 for 50 *C. jejuni* strains. The number of unique alleles for 399 bp fragment of *peb1A* was 8. Thus 758 bp was analysed for each strain. The Simpson's index of diversity for individual marker *porA* and *peb1A* were 0.852 and 0.858 respectively. In combination, *porA and peb1A* differentiated 33 unrelated strains (out of 50) into 25 types and three groups of related strains into three distinct types, aged care group as type 2, canine strains as type 4 and outbreak strains as type 16 (Table 9.1). The Simpson's index of diversity of the double locus typing scheme for 33 unrelated strains was found 0.964 as calculated by the online tool at http://insilico.ehu.es/mini_tools/discriminatory_power/.

9.3.3 Epidemiological concordance (E)

The double locus sequence typing scheme showed good epidemiological concordance (ability of typing method to group bacterial strains conforming available epidemiological information (Struelens *et al.*, 1996) by revealing the relatedness of outbreak strains and other two groups of epidemiologically related strains. All of 10 outbreak strains (OB1-OB102, type 16, Table 9.1) were assigned to the same type as was by PFGE done in 1993 (gel image was not available).

Epidemiological concordance for outbreak strains was calculated as follows:

$$E=Ne/N=10/10=1$$

Here Ne is the number of outbreak strains identified indistinguishable and N is the number of outbreak strains.

DLST also identified three strains (Ca1-Ca3) isolated from the same canine as belonging to the same type (type 4), which is congruent with PFGE result, carried out by the strain provider. Four strains isolated from the patients of the aged care (C1-C4) were also found indistinguishable (type 2, Table 9.1, Fig. 5.1). The analysis of DLST clustering of three epidemiologically related groups of *C. jejuni* strains showed the high epidemiological concordance for outbreak and other local epidemiological investigations. Clustering of four clinical (human) sporadic strains H15, H16, H17 and H19 with the chicken strain CH6 is compatible with the fact that chicken is the major source of infection. Other strains, which are non-related to each other in terms of time and space clustered dispersedly, scattered here and there in the dendrogram. Thus both related and non-related strains clustered in accordance with the epidemiological information.

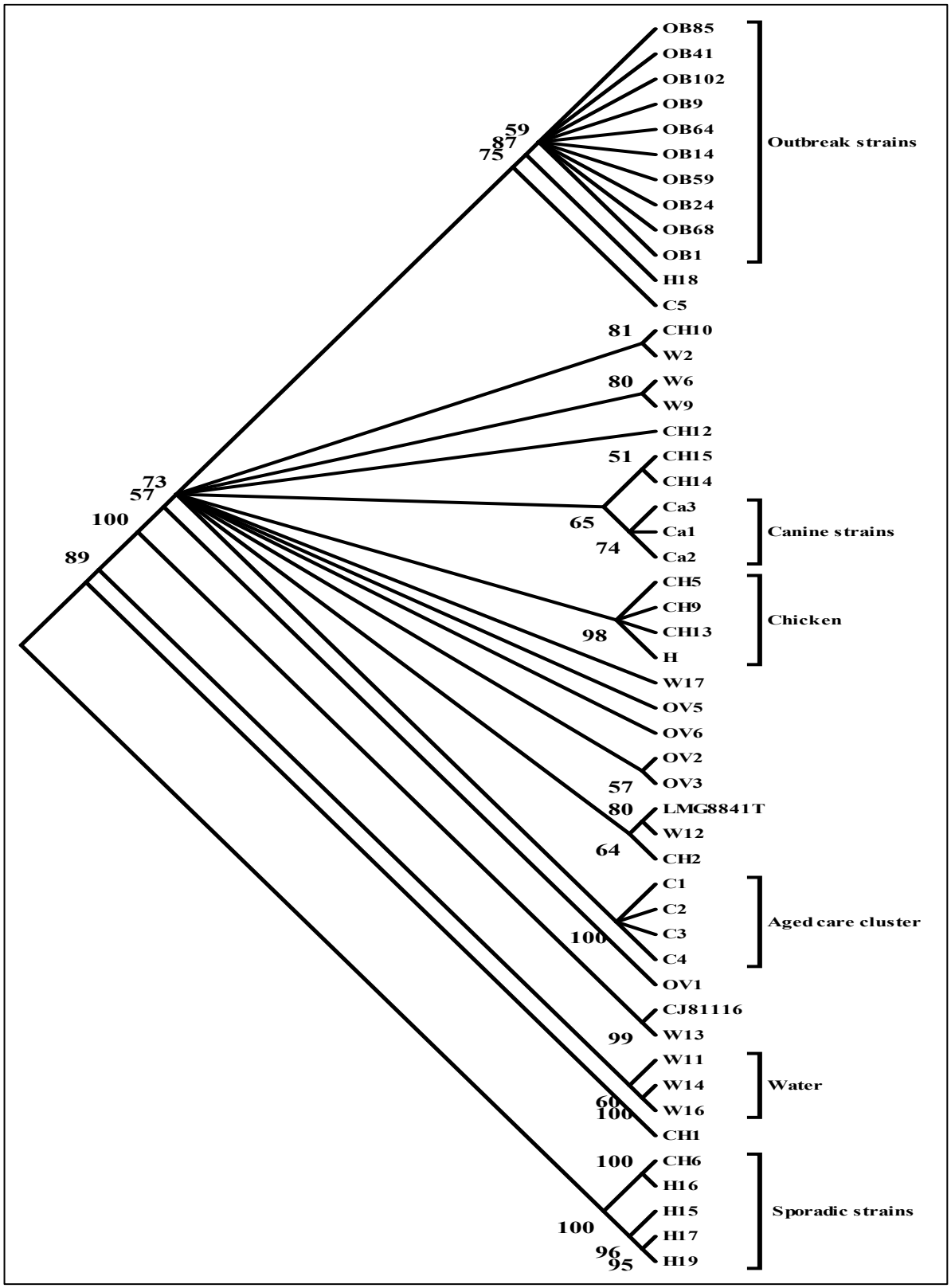

Figure 9.1. Phylogenetic relationships of 50 *C. jejuni* strains based on the analysis of the concatenated sequence of *porA* and *peb1A* as established by neighbour joining (N-J) method with 1000 bootstrap replications using MEGA 5.05.

9.3.4 Congruence comparison to MLST

DLST was compared to multilocus sequence typing (MLST) in a pilot scale level using similar number of strains (20) of the pilot study (Maiden, 2006). A group of twenty representative strains, which included two groups of 9 related strains and 11 non-related strains were included in this comparative study. Five outbreak (OB) strains and four-aged care cluster strains (C1-C4) were related strains. Eleven (11) non-related strains included 4 strains from chicken and another 4 from water along with reference strain *C. jejuni* subsp. *jejuni* 81116 and *C. jejuni* LMG 8841^{T} and sporadic strain C5 (Table 9.2).

Twenty *C. jejuni* strains were differentiated into 12 sequence types by both DLST and MLST (Table 9.2). DLST identified the same nine strains as a singleton (single strains representing a single type). The singletons were *C. jejuni* LMG 8841^{T}, C5, CH1, CH6, CJ81116, and four strains of water, W2, 6, W9 and W11 (Bold in Table 9.2). DLST also identified the same strains, CH5 and CH9, as doubletons (two isolates representing one type) compatibly with MLST. Congruence of DLST to MLST was also found in assigning type of the two groups of related strains, outbreak strains and aged care cluster. Two different types (567 and 48 by MLST; 2 and 16 by DLST) were assigned to these two groups of related strains by both DLST and MLST (Table 9.2). Thus DLST showed excellent congruence to MLST in assigning type and revealing relatedness of C. *jejuni* strains.

Table 9.2. Allelic profiles of 20 *C. jejuni* strains for MLST and DLST

Strain	Source and year of isolation	MLST Type*	DLST type
LMG8841	Type strains	403 (10, 27, 16, 19, 10, 5, 7[a])	1
C1	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C2	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C3	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C4	Human (aged care cluster); 2005	567(1, 3, 6, 4, 3, 3, 1)	2
C5	Human (sporadic); 2005	2343(2, 4, 5, 2, 10, 1, 5, 2)	3
CH1	Chicken; 2002	583(4, 7, 10, 442, 51, 354,)	5
CH5	Chicken; 2005	45(4, 7, 10, 4, 1, 7, 1)	7
CH6	Chicken; 2005	320(9, 7, 10, 4, 1, 7, 1)	8
CH9	Chicken; 2008	45(4, 7, 10, 4, 1, 7, 1)	7
CJ81116	Laboratory strains; 2011	267(4, 7, 40, 4, 42, 51, 1)	12
OB1	Human (outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	16
OB9	Human (outbreak); 1993	48(2, 4, 1, 2, 7, 1,5)	16
OB14	Human (outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	16
OB24	Human (outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	16
OB41	Human (outbreak); 1993	48(2, 4, 1, 2, 7, 1, 5)	16
W2	Water; 2005	190 (2, 1, 5, 3, 43, 3, 5)	20
W6	Water; 2007	800(64, 2, 22, 460, 43, 97, 79)	21
W9	Water; 2008	327(18, 2, 78, 35, 1, 86, 16)	22
W11	Water; 2008	4272(4, 7, 10, 4, 5, 7.)	23

*MLST sequence types were assigned in accordance with the database available at http://http://pubmlst.org/campylobacter/. [Ally's] profile of 7 MLST genes as determined from the database are shown in the bracket

Congruence was also tested statistically by calculating Adjusted Wallace and Adjusted Rand coefficients using the online tool at, http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool (Wallace, 1983, Pinto *et al.*, 2008). The Wallace coefficient calculates the probability that entities grouped in the same cluster under a method will be in the same cluster under another method and vice versa. A high Wallace's coefficient value means that partitions/typing defined by one method could be predicted by another method. In other words, the higher the Wallace coefficient value, the better the congruence between the methods (Carrico *et al.*, 2006).

In bacterial typing, the Wallace coefficient measures the probability score for a pair of strains classified as the same type by two different schemes. The Wallace coefficient of DLST to MLST and MLST to DLST typing scheme was 1.000 (Table 5.3). This means that the chance for two strains to be assigned the same type by both DLST and MLST is 100%. Thus the result of MLST could have been predicted by DLST. On the other hand**,** the adjusted Rand coefficient, which compare the clustering/grouping of isolates sharing similar characteristics according to given methods, for MLST and DLST was also 1.000 (Table 9.3), which means DLST is able to cluster *C. jejuni* strains similar to MLST.

Table 9.3. Comparison of DLST and MLST using Simpson's index of diversity (SID), Adjusted Wallace (AW) and adjusted Rand (AR) Coefficient for 20 *C. jejuni* strains

Typing scheme	No. of partition/type	SID (95%CI)	Adjusted Wallace Coefficient (95%CI)		Adjusted Rand Coefficient (95%CI)	
			DLST	MLST	DLST	MLST
DLST	12	0.911 (0.833-0.988)	1.0	1.0 (1.000-1.000)	1.0	1.0 (1.000-1.000)
MLST	12	0.911 (0.833-0.988)	1.0 (1.000-1.000)	1.0		

Thus the statistical analysis also showed the congruence of double locus sequence typing scheme to multilocus typing was excellent.

9.3.5 Comparison between DLST and *fla SVR* typing

It was possible to compare DLST with *flavour* typing using 24 strains, including outbreak strains as *fla*SVR could not be amplified and sequenced from all strains. DLST differentiated 24 strains into 13 types, whereas *flavour* into 10 types (Table 5.4. DLST scheme identified 8 singletons (LMG8841T, C5, CH6, CJ81116, H15, H18, OV1, OV5 and OV6) where *flaSVR* only 4 singletons (LMG 8841T, C1, CH5, CH9 and H17) (Singletons are in bold ,Table 5.4). DLST to *fla-SVR* adjusted Wallace coefficient and 95% CI was found 0.908 (0.856-0.961) and *fla*-SVR to DLST was 0.788 (0.717-0.860). These data from these small numbers of strains showed that DLST is more discriminatory than *file-SVR* types. However, further study with a large number of strains is needed to ascertain the comparative advantage of these two schemes.

Table 9.4. Comparison between DLST and *fla* SVR

Strains	Allelic profile		DLST type	*fla SVR Type*
	porA	*Peb1A*		
LMG 8841^T	1	1	1	1
C1	2	2	2	2
C4	2	2	2	3
C5	3	3	3	4
CH5	3	4	4	5
CH6	4	4	5	6
CH9	3	4	4	6
CJ81116	5	5	6	7
H15	6	6	7	6
H17	7	7	8	8
H18	8	8	9	9
H19	7	7	8	9
OB9	3	8	10	4
B14	3	8	10	4
OB24	3	8	10	4
OB41	3	8	10	4
OB59	3	8	10	4
OB64	3	8	10	4
OB68	3	8	10	4
OB85	3	8	10	4
OB102	3	8	10	10
OV1	9	7	11	4
OV5	3	5	12	7
OV6	10	4	13	11

The aim of this chapter was to investigate the applicability of *Peoria and peb1A* genes as a double locus typing scheme by determining typeability, discriminatory power and epidemiological concordance for *C. jejuni* typing. High typeability of both the markers was confirmed from assigning types to each of the 50 C. *jejuni* strains from a wide variety of sources. However, *porA* was used as a marker in describing a single locus typing scheme based on partial sequence of the *porA* gene (Huang *et al.*, 2005) and was applied recently for source of outbreak strains in environmental samples (Jay-Russell *et al.*, 2013). In this study, we also found *porA* itself identified the outbreak strains as indistinguishable (Table 9.1, Type 3). On the other hand, *peb1A* also identified the

outbreak strains (OB) indistinguishable separately (Table 9.1**,** Type 6). Similar identification of outbreak strains by *peb1A* can be considered as confirmation of the result of *porA* typing. Thus this identification of outbreak strains by this double locus sequence typing scheme can be considered as more reliable than single locus *porA* typing. On the other hand, the discriminatory power of this proposed double locus sequence typing scheme was also found ≥ 0.95 (SID=0. 964), which is a requirement for a typing scheme to be suitable for outbreak study (van Belkum *et al.*, 2007). This scheme not only identified outbreak strains as indistinguishable but also differentiated them from non-outbreak strains. This is an important factor for a typing scheme to be suitable for outbreak study (Foley *et al.*, 2009).

The relationship of the other two groups of related strains, canine strains and aged care strains were also well depicted complying with epidemiological information and previously performed PFGE result. Thus in this investigation, it was found that *porA* and *peb1A* are suitable markers for a double locus sequence typing scheme for outbreak and local epidemiological investigation. Such double locus typing scheme was found useful for epidemiological investigation of methicillin resistant *S. aureus* (Basset *et al.*, 2010, Basset *et al.*, 2009) .However, the scheme also differentiated non-related isolates well, by differentiating 33 unrelated strains into 25 types. The discriminatory power of DLST appeared to be higher than reported discriminatory power of PFGE (0.93), MLST (0.93 and 0.85) and *flaSVR* (0.92) for *C. jejuni* in different previously reported studies (Sails *et al.*, 2003c, Taboada *et al.*, 2012).

When compared with multilocus sequence typing, though with a limited number of strains, DLST showed excellent congruence to it as both the adjusted Wallace and

adjusted Rand were very high (1.00). That is, DLST can predict the typing pattern of MLST. For example, from DLST we found C1- C4 strains are of same type. Similarly, MLST also identified these four strains as same type (Table 9.1). Outbreak (OB1-OB102) and canine strains (Ca1-Ca3) were also typed similarly by both the schemes. Hence DLST can also be used to screen large population of *C. jejuni* strains to predict relatedness of the strains and then MLST can be used for confirmation on strains if needed.

As DLST is using less than one third of the markers than that of seven loci MLST, the cost of DLST will be less than one third of that of standard MLST. This method will also be rapid in case of purification of amplified products, sequencing and sequence analysis as it requires to carry out these steps for only one third of markers that of standard MLST.

9.4 Conclusion

Using both epidemiologically unrelated and related strains, it was shown that the double locus sequence typing (DLST) scheme based on *porA* and *peb1A* genes has sufficient discriminatory power (0.964) and capability to identify the same type among related strains and to differentiate unrelated/sporadic strains. Thus the DLST scheme was found to be suitable for genotyping of *C. jejuni* as rapid and low cost typing scheme for epidemiological investigations. The DLST scheme should be further compared to other typing schemes using adequate number of *C. jejuni* strains in order to be recommended for practical application.

Chapter 10 . Discrimination and cluster analysis of *C. jejuni* strains isolated from clinical and environmental sources using MALDI-TOF MS

Summary

While MALDI-TOF mass spectrometry has been successfully applied for bacterial identification and typing of many pathogenic bacteria using either whole cell or cell extracts, *C. jejuni* strains have never been subject to similar investigations. The goal of this study was to apply MALDI-TOF MS for discrimination and cluster analysis of *C. jejuni* strains isolated from clinical and environmental sources. Seventy *C. jejuni* strains were included in this study along with the type strain *C. jejuni* LMG 8841^{T} and reference strain *C. jejuni* subsp. *jejuni* 81116. Cell extracts were analysed using Bruker Daltonic Microflex LT System as described in Chapter 3 (3.13). Using standard software package MALDI Biotyper 3.0, three types of analysis were performed: 1) *C. jejuni* strains grouping based on the main spectra dendrogram; 2) subtyping using log score and 3) composite correlation index (CCI). The studied strains were differentiated into 66 types and resulting Simpson's index of diversity was found to be 0.998. Clustering of *C. jejuni* strains were found to be congruent to their epidemiological background, although with some exceptions.

10.1. Introduction

The potential of MALDI-TOF mass spectrometry for identification and subsequently typing of the bacteria such as *Legionella, Salmonella* and *Vibrio* has been recently well documented (Shitikov *et al.*, 2012, Dieckmann and Malorny, 2011, Dieckmann *et al.*, 2010, Dieckmann *et al.*, 2008, Fujinami *et al.*, 2011). MALDI-TOF MS has also been used for outbreak investigation of multidrug resistant *S. aureus* (Schlebusch *et al.*, 2010) and nosocomial outbreak of *Acinetobacter baumannii* (Mencacci *et al.*, 2013). MALDI-TOF MS was shown to have the potential to overcome the shortfalls of phenotyping and genotyping schemes and it has become particularly useful due to the relatively small amounts of bacterial biomass required, simple sample preparation procedure, rapid data acquisition and high throughput allowing the typing results available within an hour after the sample preparation (Sandrin *et al.*, 2013, Fujinami *et al.*, 2011). MALDI-TOF MS analysis of the proteins in the range of 2 - 20 KDa that are dominated by the ribosomal proteins is most useful because it is based on accurate protein spectra and minimally influenced by microbial growth conditions (Wieser *et al.*, 2012, Böhme *et al.*, 2013).

10.2 The chapter's aims

To the best of our knowledge, MALDI-TOF MS so far has been only used for *C. jejuni* identification, not for the typing (Kolínská *et al.*, 2008). In this study, we aimed to explore the applicability of MALDI-TOF MS for *C. jejuni* not only for the identification purposes (presented in Chapter 4) but also for typing, and comparative analysis of the *C. jejuni* clustering on an attempt to reveal the environmental reservoir/s of clinical isolates.

10.3. Results and Discussion

Using the standard software package MALDI Biotyper 3.0, three types of analysis were performed: (i) *C. jejuni* strains grouping based on the main spectra dendrogram; (ii) ssubtyping using log score and (iii) composite correlation index (CCI).

10.3.1 Clustering of *C. jejuni* strains

A score oriented main spectra (MSP) dendrogram was generated based on the *C. jejuni* strains incorporated in the MALDI Biotyper 3.0. The highest scored spectra of all strains were imported in the Biotyper 3.0 software. When the spectra were generated, the raw spectra were loaded in the Biotyper Real time classification software and identification was conducted automatically with default settings. Briefly, main spectra (MSP) series were created, using the action menu, from stored spectra in the reference library as unassigned MSPs. Then using the Biotyper 3.0, all 72 unassigned protein spectra were analysed and presented as dendrogram to examine *C. jejuni* strains clustering (Fig.10.1)

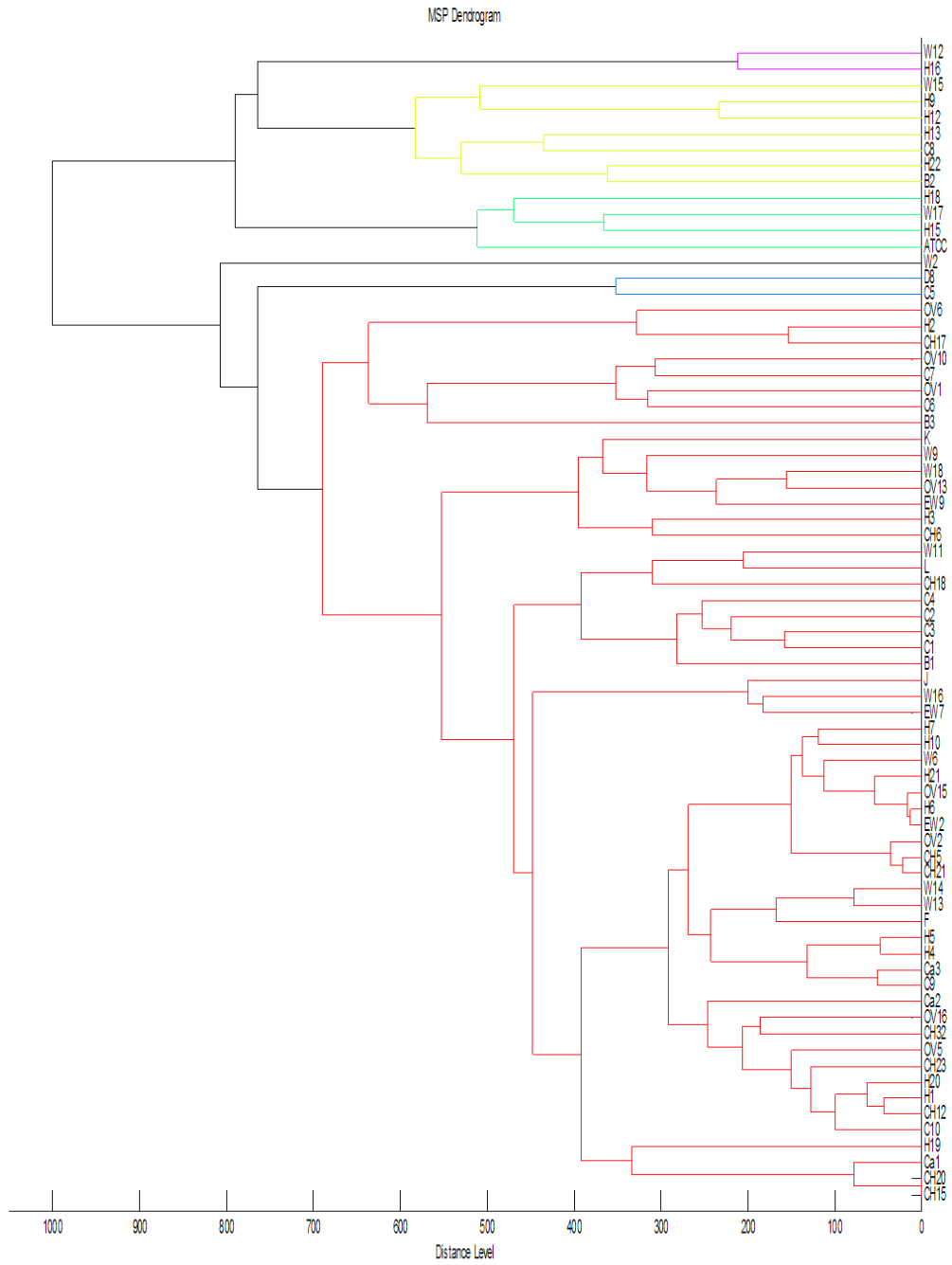

Figure 10.1. MSP dendrogram of 72 *C. jejuni* strains from different sources using Biotyper 3.0. Dendrogram prepared using Mass spectrum profiles (MSPs) of 22 *C. jejuni* strains from different sources to reveal the relatedness of the strains .The dendrogram was created using the Biotyper MSP Dendrogram Creation Standard Method, version 3.0 with default settings. MSP in this context refers to Main Spectrum. Distance is displayed in relative units.

Careful examination of the clustering of the 72 strains revealed that the clusters identified using MALDI-TOF was in accordance with the epidemiological data (Table 9.1). Two distinct clusters according to an arbitrary cut-off at the distance level of 1000 can be distinguished (Fig. 10.1). The top and smaller cluster (Cluster 1) included 8 sporadic infection strains (C8, H9, H12, H13, H15, H16, H18 and H22) from C and H series of clinical strains clustered with 3 strains (W12, W15 and W17) isolated from water, along with the type strain and one bovine strain (B2). The larger cluster (Cluster 2) comprised 67 strains, which were further split into a large number of sub clusters. Four aged care strains (C1-C4), which were isolated from patients of the same aged care centre, formed a subcluster within this biggest cluster.

At the distance level of 500, 10 distinct clusters were identified with 5 double strain clusters. At the distance level 250, 10 clusters further separated 5 double strains clusters. It is noteworthy that sporadic human strains C6 - C10 and H1 - H22 clustered with the strains isolated from different environmental habitats such as water, chicken, ovine, bovine and canine. The strains H9, H12, H13 , H16 and H22 have clustered with potable water; the sporadic infection strain C5 clustered with a strains from duck (D8), the strains, H6 and H21 clustered with strains isolated from environmental water and ovine (EW2 and OV15). *C. jejuni* strains isolated from human (H1 and H20) also clustered with the strains isolated from chicken (CH12)

The sporadic infection strains, C5 - C10 and H1 - H22, did not follow any specific pattern in clustering as it was expected due to their sporadic nature. The three canine strains isolated from the same canine, however, did not cluster together. This is an

indication that the canine might be infected by or carrier of multiple strains, not by a single strain. In contrast, the strains isolated from aged care centre patients (C1 - C4) clustered together with sporadic infection strains and different environmental strains. Overall the analysis of the results suggested that MALDI-TOF analysis allowed to reveal the possible genetic relatedness of *C. jejuni* strains and were in concordance with epidemiological information.

10.3.2 Score based subtyping of *C. jejuni* strains

For subtyping, the spectra of the type strain *C. jejuni* LMG 8841^{T} were used as a reference. The spectra of other 71 strains were scored against the spectra of the type strain. The score of the strains and epidemiological information of the strains are presented in Table 10.1 according to the clustering in the dendrogram (Fig. 10.1). Overlaid spectra of all the strains and mass list of representative strains are presented in the appendix C.

Table 10.1. Subtyping scores against special MSP of type strain *C. jejuni* LMG8841^T (ATCC 33560 ^{T})

C. jejuni Strains	Source, place and year of isolation	Score against LMG 8841^T (ATCC 33560)	*C. jejuni* strains	Source, place and year of isolation	Score against LMG 8841^T (ATCC 33560)
W12	Water, Metropolitan, Vic , 2008	2.123	C2	Human, aged care centre, 2005	2.37
H16	Human, unknown, 2012	2.249	C3	Human, aged care centre, 2005	2.477
W15	Water, Rural Region 1, Vic , 2011	2.092	C1	Human, aged care centre, 2005	2.426
H9	Human, unknown,2011	2.175	B1	Bovine, unknown, 2007	2.459
H12	Human, unknown,2011	2.259	J	Water, VIC metropolitan, 2011	2.275
H13	Human, unknown,2012	2.285	EW7	Env. Water, Rural Region1,VIC, 2008	2.025
C8	Human, unknown, 2005	2.481	H7	Human, unknown, 2011	1.959
H22 (H)	Human, ACT, 2012	2.097	H10	Human, unknown, 2011	2.149
B2	Bovine, unknown, 2007	2.256	W6	Metropolitan, Vic, 2007	2.275
H18	Human, unknown,2012	2.369	H21	Human, unknown, 2012	2.096
W17	Water, Metropolitan, Vic, 2011	2.482	OV15	Ovine, VIC, 2009	2.223
H15	Human, unknown,2012	2.378	H6	Human, unknown,2011	2.147
ATCC	Type strain	3.0	EW2	Rural Region 1, VIC, 2008	2.178
W2	Water, Rural Region 1, Vic , 2005	2.1	OV2	Ovine, Rural region 1,VIC, 2001	2.277
D8	Duck, supplier 6 (VIC), F2010	2.286	CH5	Chicken, Supplier 2, VIC, 2005	2.37
C5	Human, unknown, 2005	2.353	CH21	Chicken, Supplier 5, VIC, 2011	2.164
OV6	Ovine, Tas 1, 2002	2.117	W14	Water, Rural Region 1, VIC, 2011	2.2

H2	Human, unknown,2011	2.167	W13	Water, Rural Region 1, VIC, 2011	2.12
CH17	Chicken, Supplier 4, VIC ,2011	2.211	F	Water, VIC metropolitan, 2011	2.365
OV10	Ovine, Tas1 , 2009	2.114	H5	Human, unknown, 2011	2.302
C7	Human, unknown, 2005	2.277	H4	Human, unknown, 2011	2.055
OV1	Ovine, Vic 1 (rural), 2001	2.11	Ca3	Canine, ACT, 2012	2.095
C6	Human, unknown, 2005	2.098	C9	Human, unknown, 2005	2.274
B3	Bovine, VIC, 2007	2.136	Ca2	Canine, ACT, 2012	2.115
K	Water, VIC metropolitan, 2011	2.139	OV16	Ovine, VIC, 2009	2.233
W9	Water, Rural Region 1, Vic , 2005	2.043	CH32	Chicken, Retailer D, VIC, 2011	2.118
W18	Water, Metropolitan, Vic, 2011	2.124	OV5	Ovine, Unknown, 2002	1.96
OV13	Ovine, Tas 2 ,2009	2.186	CH23	Chicken, Retailer C, VIC, 2011	2.058
EW9	Vic Metropolitan , 2010	2.058	H20	Human, unknown, 2012	2.236
H3	Human, unknown,2011	2.024	H1	Human, unknown, 2011	2.181
CH6	Chicken, Supplier 2, VIC,2005	2.063	CH12	Chicken, Supplier 3, VIC, 2009	2.172
W11	Vic Rural Region 1,2007	2.252	C10	Human, unknown, 2005	2.163
L	Water, VIC metropolitan, 2011	2.276	H19	Human, unknown, 2012	2.241
CH18	Chicken, Supplier 3, VIC, 2011	2.118	Ca1	Canine, ACT, 2012	2.047
C4	Human, aged care centre, 2005	2.401	CH20	Chicken, Supplier 4, VIC, 2011	2.293
			CH15	Chicken, Retailer A, VIC, 2011	2.247

No strains scored 3 against the reference strain (the score of 3, indicative that the strains are indistinguishable, *i.e.*, of absolute identity). Five pairs of strains scored similarly

against the reference strain but clustered differently in the dendrogram (Table 10.1) and therefore according to the MALDI-TOF analysis 71 strains were differentiated into 66 types. There were 61 singletons (one strain representing one type) and 5 doubletons (two strains scored same and consequently can be assigned to same type). Accordingly the calculated Simpson's index of diversity was found to be 0.998 using the online tool (http://insilico.ehu.es/mini_tools/discriminatory_power/index.php). The three canine strains (Ca1 - Ca3), which were identified as genetically indistinguishable by PFGE (unpublished MDU data), were differentiated by MALDI-TOF analysis (Table 10.1) and Fig 10.1) indicating better discriminatory ability of later technique.

10.3.3 Composite correlation index (CCI)

The composite correlation index (CCI) is a statistical analysis of the relatedness of the spectra (Arnold and Reilly, 1998) generated from the strains using Biotyper 3.0. It is one type of the analysis that can be undertaken to assess the relatedness of bacterial strains. CCI values close to 1 indicate a high conformance of spectra. It can also be presented in the colored square diagram. The maximum correlation between any two strains is shown by the colour along the diagonal intersection of the samples (strains) whereas the maximum correlation is self to self-correlation. All inter strain comparisons showed decreasing correlation from the maximal self-relatedness. The closely related the strains are, the similarly they shaded in colour to the maximum. On the other hand, the less related the strains are, the further they are shaded in colour to the maximum. CCI analysis of the spectra of the studied strains demonstrated similar inter-strain relatedness as revealed by the score system (Fig. 10.1 and Table S 2).

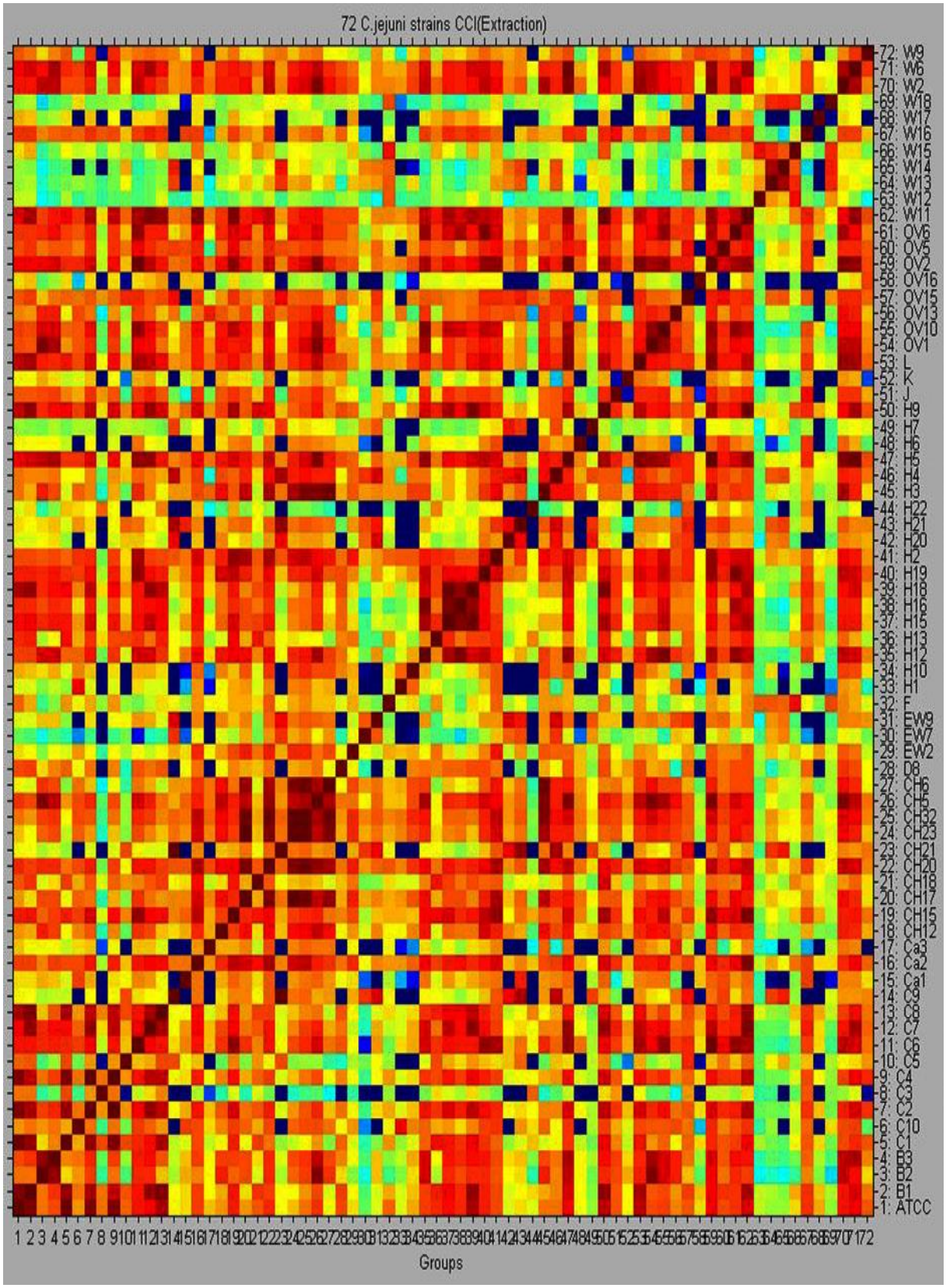

Figure 10.2. Composite correlation index (CCI) of *C. jejuni* strains from different sources. Bruker Biotyper 3.0 was used to create the composite correlation index.

10.4 Conclusion

The present study was performed to investigate the potential of MALDI-TOF MS for discrimination and clustering of *C. jejuni* clinical and environmental strains. Among the three protocols recommended by the MALDI Biotyper system, the ethanol/formic acid extraction procedure was chosen in this study due to its better performance in producing quality spectra (Wang *et al.*, 2012).

In this study, using a large number of strains from diverse *C. jejuni* sources, it was shown that MALDI-TOF MS is capable to discriminate and cluster C. *jejuni* strains revealing their epidemiological relationships. The MALDI-TOF MS allowed accurate differentiation between closely related strains and non-related strains. The discriminatory power was evident from the high discriminatory index (0.998), which appeared to be higher than that of TeLST (0.9943). It can be used as a preliminary typing scheme for screening a large number of strains before confirmation by other molecular methods such as MLST. Further study on the well-defined related strains such as outbreak strains is needed in order to re-confirm MALDI-TOF MS suitability for epidemiological investigation of *C. jejuni* infections.

Chapter 11 . General discussion and conclusions

11.1 Introduction

The preceding chapters described a range of studies undertaken in order to address the overall aim of the project, which was to investigate the suitability of combinations of sequence-based and MALDI-TOF MS typing schemes as a framework for epidemiological investigation of *C. jejuni* infections using a collection of *C. jejuni* strains from environmental and clinical samples collected in Australia.

The specific aims of the project were to:

(i) Confirm taxonomic affiliation of the environmental and clinical isolates using two complementary molecular techniques such as 16S rRNA gene sequence analysis and matrix assisted laser desorption time of flight (MALDI-TOF) mass spectrometry (MS)

(ii) Comparatively evaluate eleven candidate markers (*cad*F, *cdt*A, *cdt*B, *cdt*C, *ceu*E, *fla*A, *fla SVR*, *jlp*A, *map*A, *por*A and *peb1*A)

(iii) Design a methodological approach for tetra-, tri- and double-locus sequence based typing schemes

(iv) Develop a conceptual framework for epidemiological studies of *C. jejuni* as a combination of suitable genetic markers and MALDI-TOF MS typing

This chapter provides an overview of the major findings of this thesis as well as a general discussion, presenting the framework for epidemiological studies, and identifies further scope for research.

11.2 Summary of the findings

11.2.1 Chapter Four

Tentatively identified *C. jejuni* strains were obtained from the Enteric Culture Collection of Microbiology Diagnostic Unit, Public Health Laboratory, The University of Melbourne. For selected strains, their taxonomic identity was further re-confirmed up to the genus and species level using phylogenetic analysis based on 16S rRNA, *mapA* gene sequence similarities and MALDI-TOF MS based identification. Thus the results obtained using three independent and complementary techniques clearly indicated that the strains included in this study belong to the genus *Campylobacter* and were identified as *C. jejuni*. MALDI-TOF MS appeared to be fast and reliable and may be used as alternative techniques for *C. jejuni* identification.

11.2.2 Chapter Five

Eleven candidate markers (*cadF, cdtA, cdtB, cdtC, ceuE, flaA, fla SVR, jlpA, mapA, porA* and *peb1A*) were selected based on their function, specificity and probable variability. Amplification and sequencing conditions were optimized for all the selected markers using twenty *C. jejuni* strains, which included clinical strains, and strains isolated from chicken, ovine and water. Based on sequence analysis of those 20 strains, 4 schemes were found being of a satisfactory discriminatory power: *porA, peb1A, mapA* and *ceuE* for a tetra-locus sequence typing scheme, *porA, peb1A* and *mapA* for tri-locus and *porA* and *peb1A* for double-locus sequence typing scheme. Three genes of the *cdt* cluster were also found to be suitable for a separate typing scheme.

The combination of the markers suitable for double locus sequence typing was *porA* and *peb1A*, for tri-locus sequence typing were *porA, peb1A* and *mapA*. The highest discriminatory index of 1.00 was predicted for the tetra locus as a combination of *porA, peb1A, mapA* and *ceuE*. The three genes of the *cdt* gene cluster, *cdtA, cdtB* and *cdtC*, were also found to show satisfactory discriminatory power, which may be sufficient for local epidemiological study.

11.2.3 Chapter Six

The applicability of the *cdt* gene cluster, which consists of *cdtA, cdtB* and *cdtC*, was investigated using both clinical and environmental strains of *C. jejuni*. Forty five strains included in the study were derived from outbreak and sporadic infections and strains isolated from chicken, canine, ovine and water. A total of 1191 bp (197 bp, 620 bp and 374 bp) of the three complete CDSs was analysed for each strain. The 28 unrelated strains were differentiated into 17 types and calculated Simpson's index of diversity was 0.9656, which is suitable for outbreak study. Among the 17 related strains, ten outbreak strains were identified as indistinguishable and of the same type. The strains isolated from canine and the aged care centres were also found very closely related. Overall the *cdt* gene cluster sequence typing was in agreement with the epidemiological information confirming the *cdt* gene cluster sequence typing applicability in epidemiological investigation of *C. jejuni* infections.

Overall, for the first time a typing scheme based on toxin genes was described. With 45 *C. jejuni* strains from a wide variety of sources, it was demonstrated that the *cdt* gene cluster sequence typing scheme is highly discriminatory with high typeability and medium level of congruence to MLST.

11.2.4 Chapter Seven

The applicability of *porA, peb1A, mapA and ceuE* genes was investigated in a tetra locus sequence typing (TeLST) scheme on fifty *C. jejuni* strains isolated from both clinical and environmental sources. Clinical strains included outbreak and sporadic infection strains and environmental strains were from other major sources of *C. jejuni* such as chicken, water, ovine and canine. Four genes *porA, peb1A, mapA* and *ceuE* using in a tetra-locus scheme showed high typeability, extremely high discriminatory power (SID 0.9943) and good epidemiological concordance by identifying outbreak strains and other related strains. The TeLST scheme also showed good congruence to multilocus sequence typing scheme when it was compared using twenty *C. jejuni* strains. It was found that *porA, peb1A, mapA* and *ceuE* genes are a tetra locus combination for epidemiological investigation of *C. jejuni*. The scheme can be recommended for epidemiological investigations as a complementary tool along with well established methods such as pulsed field gel electrophoresis or multilocus sequence typing.

The intragenic sequence of *porA*, *peb1A, mapA* and *ceuE* genes have been found suitable for a tetra locus sequence typing scheme for *C. jejuni*. The scheme has very high discriminatory power, close to ideal index, 1. TeLST was found to be able to differentiate among outbreak strains allowing understanding whether the human patient or host such as chicken, cow and poultry are infected by more than one closely related strains or whether more than one strains of *C. jejuni* involved in the outbreak. The newly developed TeLST scheme with discriminatory power close to 1 can be also used for study co-infection of patient(s) and host.

11.2.5 Chapter Eight

The applicability of *porA, peb1A* and *mapA* genes was investigated as a tri locus sequence typing (TLST) scheme based on fifty *C. jejuni* strains derived from outbreak, sporadic infection and sources of *C. jejuni* such as chicken, water, ovine and canine. *porA, peb1A* and *mapA* as a tri-locus scheme showed high typeability, high discriminatory power (SID 0.986) and very good epidemiological concordance in the identification of the outbreak strains. The TLST scheme also showed very good congruence to multilocus sequence typing scheme based on 20 randomly selected *C. jejuni* strains. It was shown that a combination of *porA, peb1A* and *mapA* genes is suitable in a tri-locus typing scheme. The scheme may be applicable for epidemiological investigations as a complementary tool to other established methods such as pulsed field gel electrophoresis or multilocus sequence.

11.2.6 Chapter Nine

A DLST scheme for *C. jejuni* based on concatenated partial sequences of *porA* and *peb1A* genes has been investigated. The DLST scheme was validated using fifty clinical and environmental *C. jejuni* strains isolated from human (C5, H, H15-H19), chicken (CH1-CH15), water (W2-W17) and ovine samples (OV1 - OV6). The scheme was found to be highly discriminatory (DI=0.964) and epidemiologically concordant in this study with a limited number of *C. jejuni* strains. The DLST showed the discriminatory power above 0.95 and excellent congruence to multilocus sequence typing (MLST) and can be used as a rapid and low cost typing scheme for epidemiological investigation of *C. jejuni*. It is suggested that the DLST scheme is suitable for outbreak strains identification and differentiation of the sporadic infection strains.

Using both epidemiologically unrelated and related strains, it was shown that the double locus sequence typing (DLST) scheme based on *porA* and *peb1A* genes has sufficient discriminatory power (0.964) and capability to identify the same type among related strains and to differentiate unrelated/sporadic strains. Thus the DLST scheme was found to be suitable for genotyping of *C. jejuni* as rapid and low cost typing scheme for epidemiological investigations. The DLST scheme should be further compared to other typing schemes using adequate number of *C. jejuni* strains in order to be recommended for practical application.

11.2.7 Chapter Ten

The goal of this study was to apply MALDI-TOF MS for discrimination and cluster analysis of *C. jejuni* strains isolated from clinical and environmental sources. Seventy *C. jejuni* strains were included in this study along with the type strain *C. jejuni* LMG 8841^{T} and reference strain *C. jejuni* subsp. *jejuni* 81116. Cell extracts were analysed using Bruker Daltonic Microflex LT System as described in Chapter 3. Using standard software package MALDI Biotyper 3.0, three types of analysis were performed: 1) *C. jejuni* strains grouping based on the main spectra dendrogram; 2) subtyping using log score and 3) composite correlation index (CCI). The studied strains were differentiated into 66 types and resulting Simpson's index of diversity was found to be 0.998. Clustering of *C. jejuni* strains were found to be congruent to their epidemiological background, although with some exceptions.

Using a large number of strains of diverse *C. jejuni* sources, it was shown that MALDI-TOF MS is capable to discriminate and cluster C. *jejuni* strains revealing their epidemiological relationships. The MALDI-TOF MS allowed accurate differentiation

between closely related strains and non-related strains. The discriminatory power was evident from the high discriminatory index (0.998), which appeared to be higher than that of TeLST (0.9943). It can be used as a preliminary typing scheme for screening a large number of strains before confirmation by other molecular methods such as MLST. Further study on the well-defined related strains such as outbreak strains is needed in order to re-confirm MALDI-TOF MS suitability for epidemiological investigation of *C. jejuni* infections

11.3 General discussion

The literature review identified *C. jejuni* as the most common food and water borne bacterial pathogen responsible for a significant burden to public health worldwide. This is due to impacts associated with the costs of hospitalization and treatment, public health surveillance and the loss of working hours by those affected by *C. jejuni* infection (Grills *et al*., 2010). The literature review also identified that due to the sporadic nature of these infections, the gathering of epidemiological data to enable prevention and control is difficult and there is a need for more rapid, cost effective and accurate methods to identify the type of strains associated with *C. jejuni* infections (Grills *et al*., 2010).

This study aimed to address this problem through the development of a framework for epidemiological investigation of *C. jejuni* infections using a collection of *C. jejuni* strains from environmental and clinical samples collected in Australia.

The framework was developed through the investigation of the suitability of combinations of four sequences based typing schemes and one MALDI-TOF MS based

typing scheme. The four sequence based typing schemes included *porA, peb1A, mapA* and *ceuE* for a tetra-locus sequence typing scheme, *porA, peb1A* and *mapA* for tri-locus and *porA* and *peb1A* for double-locus sequence typing scheme and the *cdt* gene cluster typing scheme including *cdtA, cdtB* and *cdtC* genes.

The individual typing schemes were assessed based on their performance criteria, which include typeability, discriminatory power, epidemiological concordance and reproducibility (van Belkum *et al.*, 2007, Struelens *et al.*, 1996). The typing schemes developed in this study have shown satisfactory performance criteria. All schemes have shown high typeability, adequate discriminatory power (≥0. 95) and epidemiological concordance for an outbreak study and other local epidemiological studies. Additionally, sequence based typing schemes are highly reproducible, suggesting that these typing schemes are suitable for use in epidemiological investigations of *C. jejuni*.

The use of a typing scheme in public health laboratories depends also on convenience criteria of the scheme. These include cost, rapidity, throughput and technical difficulty and skill required (van Belkum *et al.*, 2007, Struelens *et al.*, 1996, Foxman *et al.*, 2005). The cost and rapidity of a typing scheme, particularly of a sequence based typing scheme, depend on the number of markers used. The described schemes in this project use only 2 - 4 loci, which is less than one third $\leq (1/3)$ to almost half $\sim (1/2)$ of the numbers of that of the seven loci sequence typing (MLST) scheme. Thus the developed typing schemes are potentially cheaper and more rapid than MLST. Hence from the point of convenience criteria, the described sequence based typing schemes could be schemes of choice for sequence based typing of *C. jejuni* for local epidemiological studies.

11.4 A framework for epidemiological investigation

This project has developed a framework to assist in the local epidemiological investigation of *C. jejuni* infection, which has taken into consideration and meets a range of performance criteria previously described.

The framework involves public health laboratories with sequencing facilities commencing with a double locus sequence typing scheme (Jay-Russell *et al.*, 2013), as this double locus typing scheme showed excellent epidemiological concordance by being able to identify an outbreak and reveal relationships of closely related strains. It also showed excellent congruence to multilocus sequence typing scheme for *C. jejuni*. Thus this rapid and simple method could be the starting point for local epidemiological studies. However, its discriminatory index is 0.96, which means that there are further scopes to differentiate between strains identified as related by DLST.

In order to address this, it is further suggested that for revealing the difference between such closely related strains, additional markers such as *mapA* and *ceuE* can be employed gradually, which raises the discriminatory power to 0.98 and 0.99 respectively. Thus the framework for an epidemiological study could be commenced with DLST and TLST and TeLST can be employed if further differentiation between strains needed. The gradual addition of two markers or schemes with DLST will give equivalent results of extended multilocus typing schemes of *C. jejuni* (Dingle *et al.*, 2008). Thus, these three schemes can work as a complete set of typing schemes for local epidemiological investigation of *C. jejuni* infections.

The *cdt* gene cluster typing is another sequence based typing scheme developed in this project, which has also shown high discriminatory power to identify outbreaks. Many clinical laboratories use the multiplex primers used in this scheme for identification and differentiation of *C. jejuni* from *C. coli* and *C. fetus* (Asakura *et al.*, 2008b). Such laboratories can use this scheme for subsequent typing of *C. jejuni* and outbreak investigation upon sequencing of the amplified *cdtA, cdtB and cdtC* genes using primers designed in this study (Table 3.2).

Alternatively, MALDI-TOF MS is emerging technology which is becoming widely implemented in clinical laboratories mainly for pathogenic bacteria identification. MALDI-TOF MS based scheme described in this project is highly discriminatory, the cost associated per sample strain analysis is very low and given that the turnaround time from bacterial culture availability is few minutes, a large number of samples can be analysed in a day (Wieser *et al.*, 2012, Lartigue, 2013b). Hence this scheme could also be used for the identification of *C. jejuni*-like isolates and as a complementary typing scheme to screen large numbers of *C. jejuni* strains where the facility is available. Due to low cost and short turnaround time, MALDI-TOF is advantageous as a preliminary typing scheme, in preference to a double locus sequence typing scheme for screening large number of *C. jejuni* strains. Using MALDI-TOF MS, results can be obtained within a few minutes to an hour based on the number of strains to be analysed (Lartigue, 2013b).

11.5 Comparative analysis of MALDI-TOF MS with DLST, TLST, TeLST and *cdt* typing

Two groups of epidemiologically related strains were included in the MALDI-TOF MS analysis. These are four aged care strains, C1-C4 and three canine strains isolated from the same canine. The four aged care strains were found to be very closely related but different from each other (Fig 10.1). Similar relationships were found for the aged care strains by *cdt* cluster typing (Fig 6.1 and table 6.1). Other schemes such as DLST, TLST and TeLST revealed the closeness of these four strains but could not differentiate between them (Table 7.1, 8.1, 9.1 and Figs. 7.1, 8.1 and 9.1).

However, three canine strains identified as indistinguishable by all the sequences based typing schemes were differentiated by MALDI-TOF MS. The outbreak strains could not be analysed by MALDI-TOF MS as live cells for those strains were not available. Still, the two studied groups of related strains were easily distinguished confirming that MALDI-TOF MS is more discriminatory than sequence based typing schemes.

The performance and convenience criteria of the developed typing schemes are summarized in the Table 11.1.

Table 11.1. Performance and convenience criteria of the developed schemes.

Typing Scheme	Typeability	Discriminatory power	Epidemiological concordance	Congruence to MLST	Cost	Turnaround time	Throughput
DLST	100%	0.96	Excellent	Excellent	≤1/3 of MSLT	18-36 H	Medium
TLST	100%	0.98	Very good	Very good	≤1/2 of MSLT	18-36 H	Low
TeLST	100%	0.99	good	good	~1/2 of MSLT	18-36 H	Low
cdt cluster typing	100%	0.96	good	good	≤1/2 of MSLT	18-36 H	Low
MALDI-TOF typing	100%	0.99	Good	Good	Very low	10-20 min max	Very high

It is noteworthy that no single typing scheme possesses all the performance and convenience criteria at a sufficient level for epidemiological study. Hence at least two typing schemes are used in epidemiological surveillance of pathogenic bacteria.

After examining all the performance and convenience criteria of the developed genotyping schemes in light of the previously mentioned guidelines in the literature

review, it can be concluded that the proposed sequence based typing schemes are suitable typing schemes for local epidemiological investigation of *C. jejuni*, namely for outbreak investigation. MALDI-TOF MS typing using Biotyper 3.0 can be used for taxonomic purposes and preliminary screening of a large number of environmental isolates. The application of these typing schemes, as described in this project; provide a framework to address the need for more rapid, cost effective and accurate methods to identify the type of strains associated with *C.jejuni* infections.

11.5 Significant contribution of this thesis

1. For the first time, a sequence based typing schemes based on toxin genes and cytolethal distending toxin gene cluster *cdtA, cdtB* and *cdtC* were developed and assessed. It is found that newly developed typing schemes can be employed to identify outbreak strains and differentiate between very closely related strains making the schemes suitable for outbreak investigation and/or local epidemiological investigation.

2. Where high discrimination is required, a tetra locus sequence typing scheme based on the partial sequence of *porA, peb1A, mapA* and *ceuE* can be particularly useful in epidemiological investigations such as ongoing outbreaks in any small set of multi strain infection.

3. Using only two loci, *porA* and *peb1A* as genetic markers, a rapid and highly discriminatory sequence-typing (DLST) scheme having excellent epidemiological concordance for local epidemiological investigation of *C. jejuni* infection and excellent

congruence to multilocus sequence typing for *C. jejuni* is proposed for routine use in public health laboratories

4. A MALDI-TOF MS based typing scheme with simple and rapid analysis has also been proposed and evaluated. This scheme has high potential for routine use in public health laboratory for screening of large number of *C. jejuni* strains.

11.6 Conclusions and future directions

In this PhD thesis, applicability of *porA, peb1A, mapA, ceuE and cdtA, cdtB and cdtC* were investigated in tetra, tri and double locus sequence typing schemes for typing of *C. jejuni*. The potential of MALDI-TOF MS for typing of *C. jejuni* strains was explored and assessed. The newly developed sequence based schemes were found to be suitable for local epidemiological investigation of *C. jejuni* infection such as outbreak investigation and zoonotic transfer due to their high discriminatory index and epidemiological concordance. Incorporation of a small number of genetic loci makes the developed schemes rapid and cheaper alternative to multilocus typing schemes. Simple, rapid and high throughput method was also described for preliminary screening of *C. jejuni* by MALDI-TOF MS. The application of these typing schemes, as described in this project, provide a framework to address the need for more rapid, cost effective and accurate methodological approach to identify with the origin of *C. jejuni* infections. The developed schemes should be further evaluated using adequate number of *C. jejuni* strains and in the parallel study using some other relevant typing schemes, *e.g.*, the pulsed field gel electrophoresis.

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1. Fig 1.1 Reprinted from International Journal of Medical Microbiology, 300(4), Dasti, J.I., Tareen, A.M., Lugert, R., Zautner, A.E. & Groß, U., *Campylobacter jejuni*: A brief overview on pathogenicity-associated factors and disease-mediating mechanisms. 205-11, C(2009) with permission from Elsevier **(**License number 3455281401873, License date Aug 24, 2014)

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Appendix

A. Publication from this thesis

A.1 Review Article (6 citations, www.scopus.com)

Ahmed, M.U., Dunn, L. & Ivanova, E.P. (2012) Evaluation of current molecular approaches for genotyping of *Campylobacter jejuni* strains. Foodborne Pathog Dis, **9**: 375-85.

A.2 Research article

Ahmed, M.U., Dunn, L., Valcanis, M., Hogg, G. & Ivanova, E.P. (2014) Double-locus sequence typing using *porA* and *peb1A* for epidemiological studies of *Campylobacter jejuni*. Foodborne Pathog Dis, **11**: 194-99.

A.3 Conference presentation - Poster

Monir U. Ahmed, Louise Dunn, Mary Valcanis, Geoff Hogg, and Elena P. Ivanova, 2013. Tri-locus sequence typing of *C. jejuni*. Poster presented at the Annual conference of The Australian Society of Microbiology, Jun 07-10, Adelaide, Australia

B.Supplementary Tables

Table S1 *C. jejuni* trains and their origin, year of isolation, places of isolation and genes sequenced and MALDI-TOF MS analysed. 11.2

Strain ID	Origin/year of isolation/ place	Genes sequenced for Sequence based typing							MALDI-TOF MS Ribosomal protein extract analysis
		porA	*Peb1A*	*mapA*	*ceuE*	*cdtA*	*cdtB*	*cdtC*	
LMG8841^T	Type strain, Belgium	KC330942	KC330892	KF754007	KF753957	JX658695	JX658716	JX658737	
B1 B2 B3	Bovine, 2007, Victoria, Australia								
C1	Human, Aged care cluster; 2005	KC330943	KC330893	KF754008	KF753958	JX658696	JX658717	JX658738	
C2		KC330944	KC330894	KF754009	KF753959	JX658697	JX658718	JX658739	
C3		KC330945	KC330895	KF754010	KF753960	JX658698	JX658719	JX658740	
C4		KC330946	KC330896	KF754011	KF753961	JX658699	JX658720	JX658741	
C5	Human, sporadic strain,2005	KC330947	KC330897	KF754012	KF753962	JX658700	JX658721	JX658742	
C6 C7 C8 C9 C10									
Ca1	Canine, 2012 , identified as genetically indistinguishable by pulsed field gel	KC330948	KC330898	KF754013	KF753963	KF754105	KF754081	KF754057	
Ca2		KC330949	KC330899	KF754014	KF753964	KF754106	KF754082	KF754058	
Ca3		KC330950	KC330900	KF754015	KF753965	KF754107	KF754083	KF754059	

	electrophoresis(PFGE) but different from H; Australian capital territory(ACT)								
D8	Duck, 2010, Victoria, Australia								
		KC330951	KC330901	KF754016	KF753966				ND
CH1	Chicken, different								
CH2	firms and retailers;	KC330952	KC330902	KF754017	KF753967				
CH5	2002-2004, Melbourne, Australia	KC330953	KC330903	KF754018	KF753968	KF754108	KF754084	KF754060	
CH6		KC330954	KC330904	KF754019	KF753969	KF754109	KF754085	KF754061	
CH9		KC330955	KC330905	KF754020	KF753970				ND
CH10		KC330956	KC330906	KF754021	KF753971	KF754110	KF754086	KF754062	
CH12		KC330957	KC330907	KF754022	KF753972	KF754111	KF754087	KF754063	
CH13		KC330958	KC330908	KF754023	KF753973	KF754112	KF754088	KF754064	ND
CH14		KC330959	KC330909	KF754024	KF753974				
CH15		KC330960	KC330910	KF754025	KF753975				
CH17									
CH18									
CH20									
CH21									
CH23									
CJ81116	Reference strain,	KC330960	KC330910	KF754025	KF753975				

	RMIT culture collection	1	1	6	6			
H (H22)	Human. Sporadic infection strains isolated in 2012	KC330962	KC330912	KF754027	KF753977		KF754089	KF754066
H1						KF754113	KF754090	KF754067
H2						KF754114	KF754091	KF754068
H3						KF754115	KF754092	KF754069
H4						KF754116	KF754093	KF754070
H5						KF754117	KF754094	KF754071
H6						KF754118	KF754095	KF754072
H7						KF754119	KF754096	KF754073
H8						KF754120	KF754097	KF754074
H9						KF754121	KF754098	KF754075
H10						KF754113	KF754099	KF754076
H11						KF754114	KF754100	KF754066
H15		KC330963	KC330913	KF754028	KF753978	JX658701	JX658722	JX658743
H16		KC330964	KC330914	KF754029	KF753979	JX658702	JX658723	JX658744
H17		KC330965	KC330915	KF754030	KF753980	JX658703	JX658724	JX658745

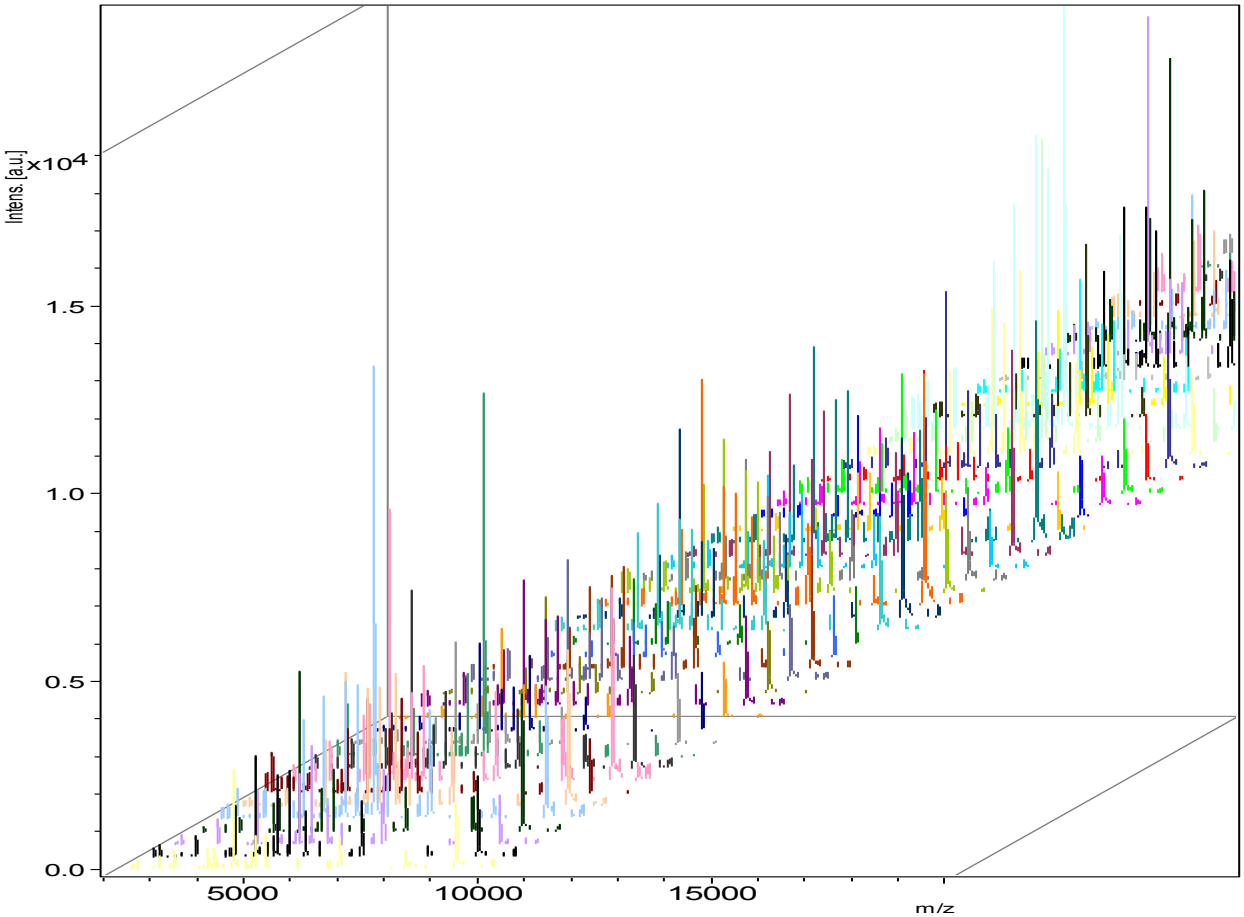
H18		KC330966	KC330916	KF754031	KF753981	JX658704	JX658725	JX658746	
H19		KC330967	KC330917	KF754032	KF753982	JX658705	JX658726	JX658747	
H20						KF754115	KF754101	KF754067	
H21									
OB1	School camp outbreak in 1993, identified as genetically indistinguishable by pulsed field gel electrophoresis(PFGE), only dead cells were available; Gold Coast school camp, Queensland,Australia	KC330968	KC330918	KF754033	KF753983	JX658706	JX658727	JX658748	ND, live cells were not available
OB9		KC330969	KC330919	KF754034	KF753984	JX658707	JX658728	JX658749	
OB14		KC330970	KC330920	KF754035	KF753985	JX658708	JX658729	JX658750	
OB24		KC330971	KC330921	KF754036	KF753986	JX658709	JX658730	JX658751	
OB41		KC330972	KC330922	KF754037	KF753987	JX658710	JX658731	JX658752	
OB59		KC330973	KC330923	KF754038	KF753988	JX658711	JX658732	JX658753	
OB64		KC330974	KC330924	KF754039	KF753989	JX658712	JX658733	JX658754	
OB68		KC330975	KC330925	KF754040	KF753990	JX658713	JX658734	JX658755	
OB85		KC330976	KC330926	KF754041	KF753991	JX658714	JX658735	JX658756	
OB102		KC330977	KC330927	KF754042	KF753992	JX658715	JX658736	JX658757	
OV1	From ovine in Victoria and Tasmania, Australia; 2001-2002	KC330978	KC330928	KF754043	KF753993				
OV2		KC330979	KC330929	KF754044	KF753994				

OV3		KC330980	KC330930	KF754045	KF753995				ND
OV5		KC330981	KC330931	KF754046	KF753996				
OV6		KC330982	KC330932	KF754047	KF753997				
OV10									
OV13									
OV15									
OV16									
		KC330983	KC330933	KF754048	KF753998	KF754122	KF754101	KF754077	
W2	Potable water, 2005-								
W6	2011, Melbourne	KC330984	KC330934	KF754049	KF753999	KF754123	KF754102	KF754078	
	metropolitan and rural								
W9	region, Australia	KC330985	KC330935	KF754050	KF754000	KF754124	KF754103	KF754079	
	Environmental water,								
W11	2008-2010;	KC330986	KC330936	KF754051	KF754001	KF754125	KF754104	KF754080	
	Metropolitan and rural								
W12	Victoria,	KC330987	KC330937	KF754052	KF754002				
W13		KC330988	KC330938	KF754053	KF754003				
W14		KC330989	KC330939	KF754054	KF754004				
W16		KC330990	KC330940	KF754055	KF754005				
W17		KC330991	KC330941	KF754056	KF754006				
W18									
EW2									
EW7									
EW9									

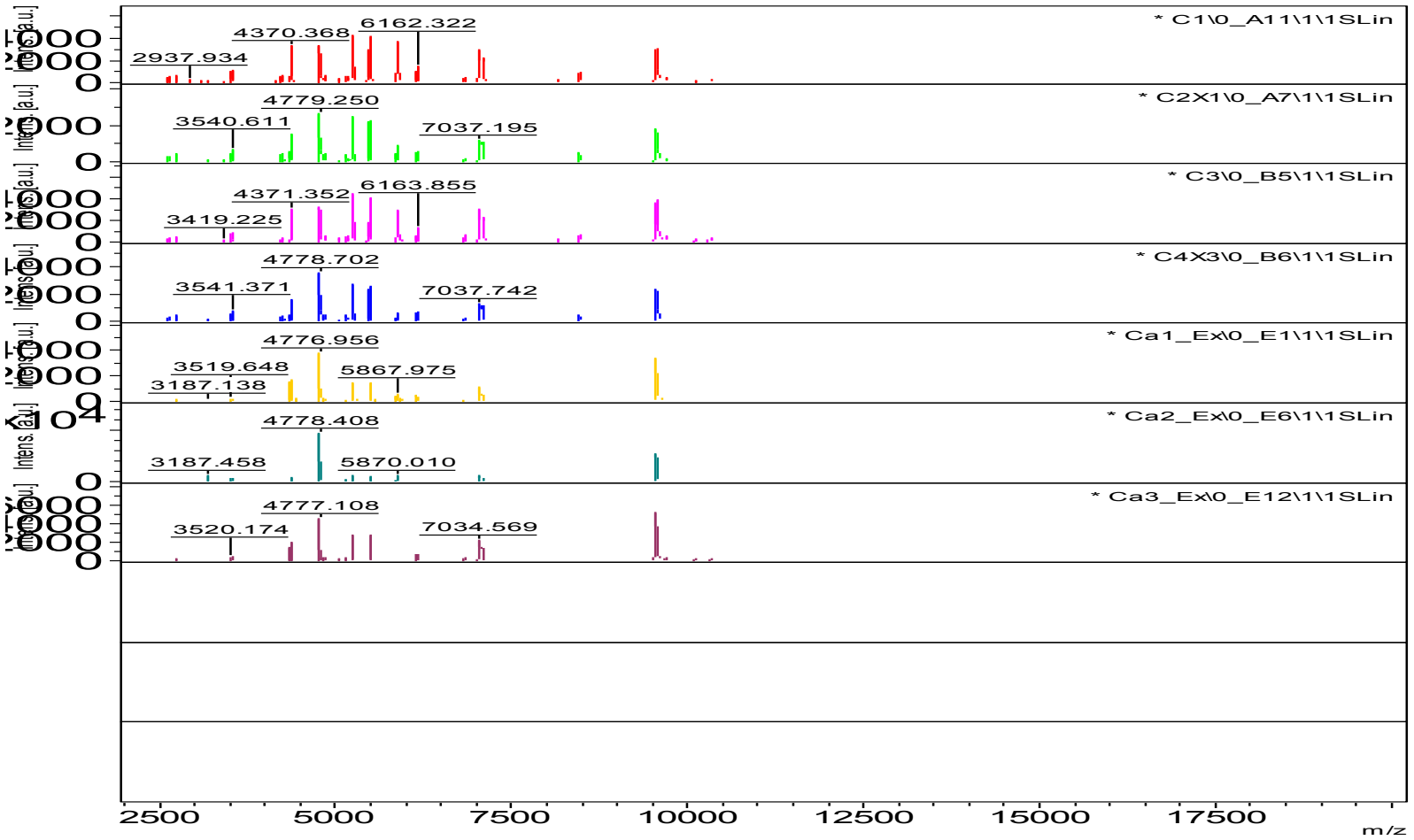
F J K L	Water, 2011, metropolitan Melbourne, Australia	

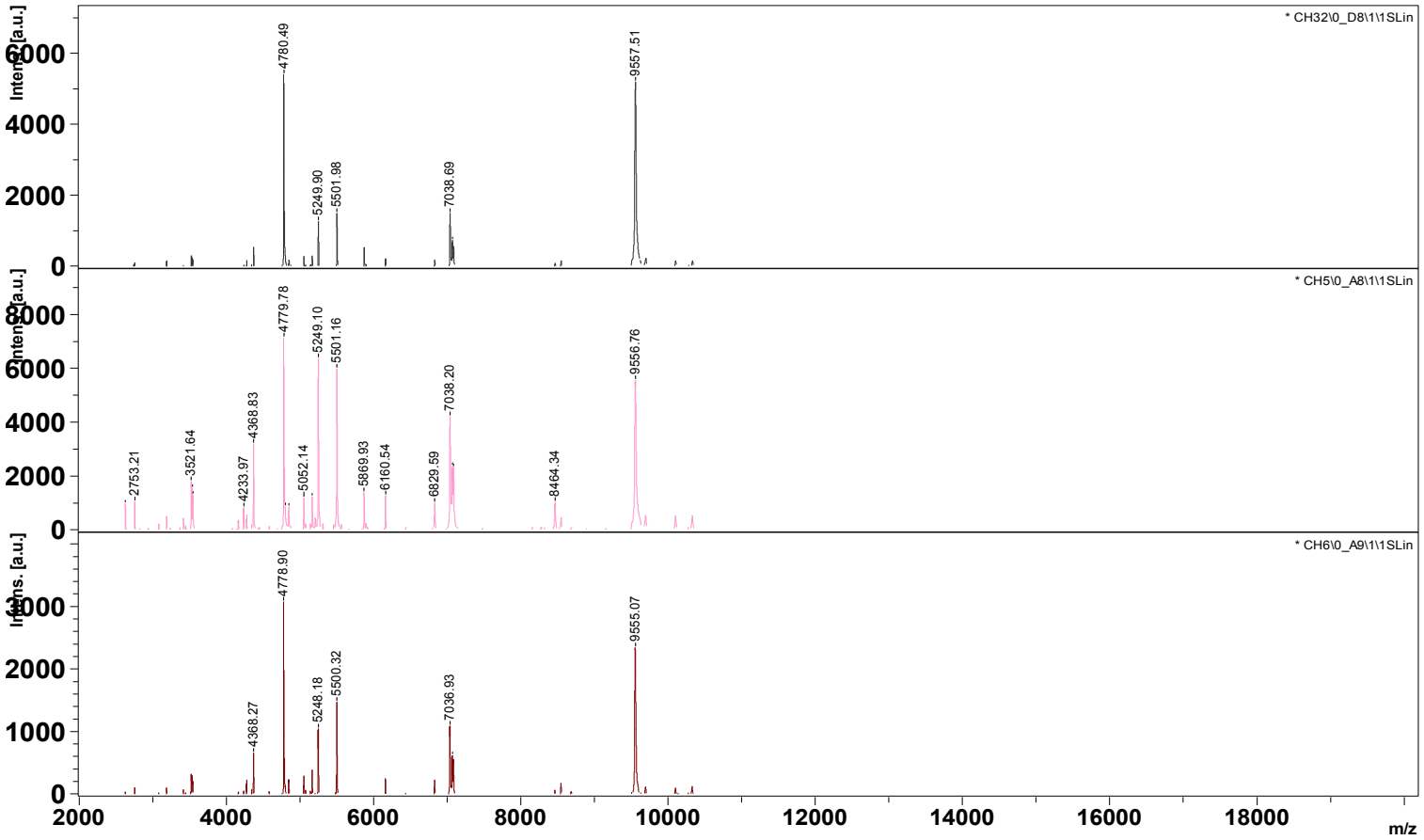
A. Mass spectra of *C. jejuni* strains

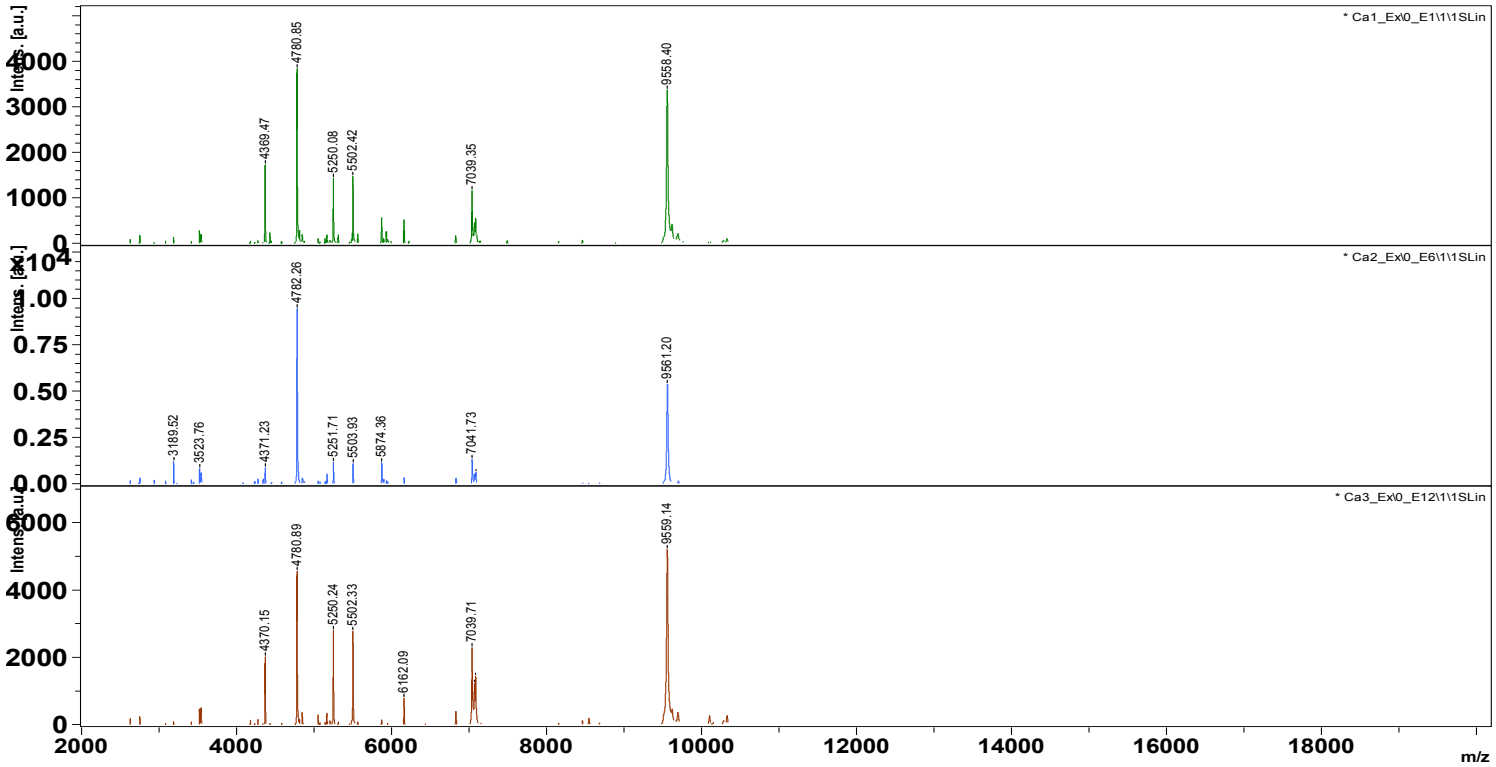
a. Overlaid spectra

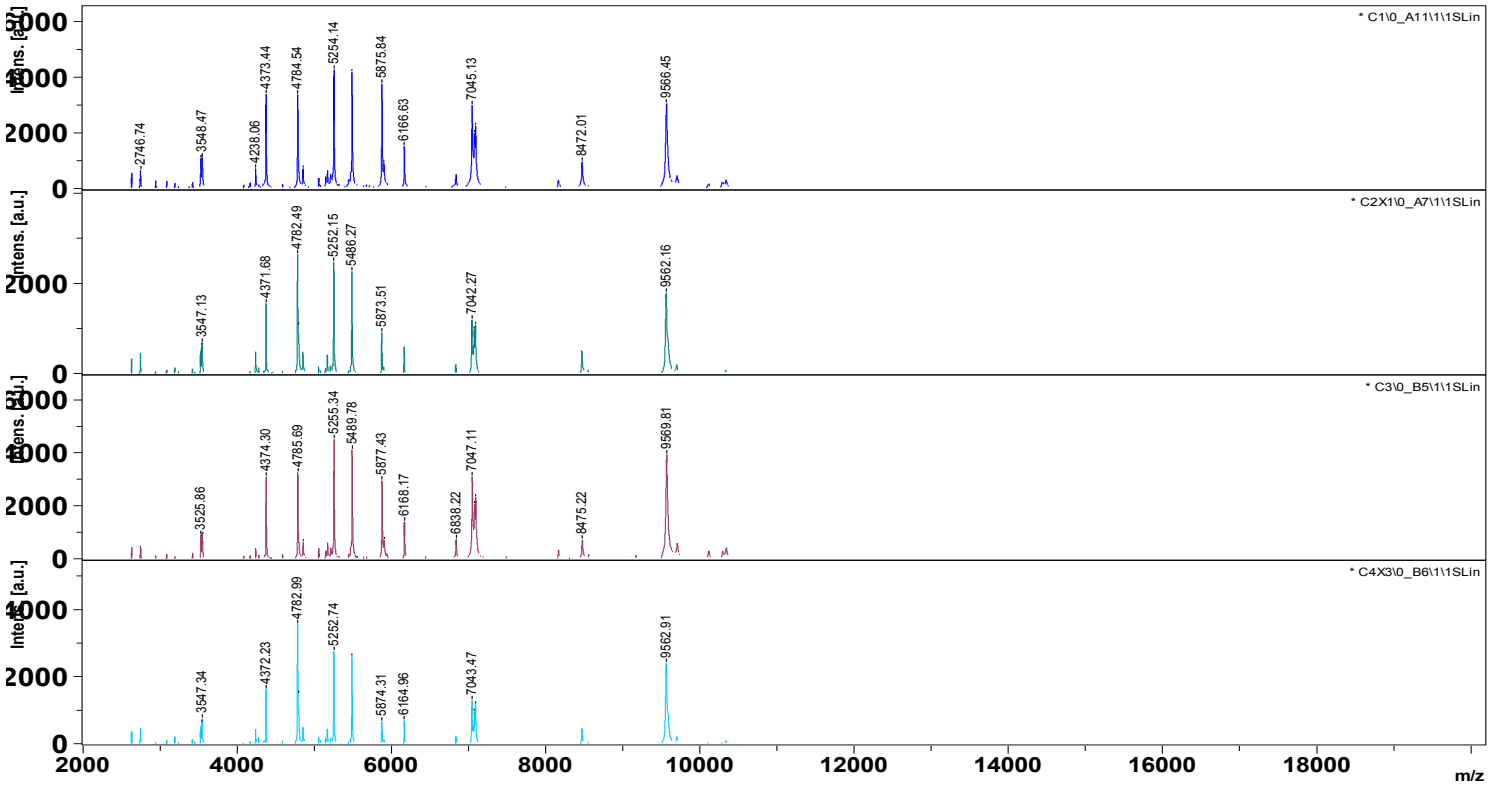


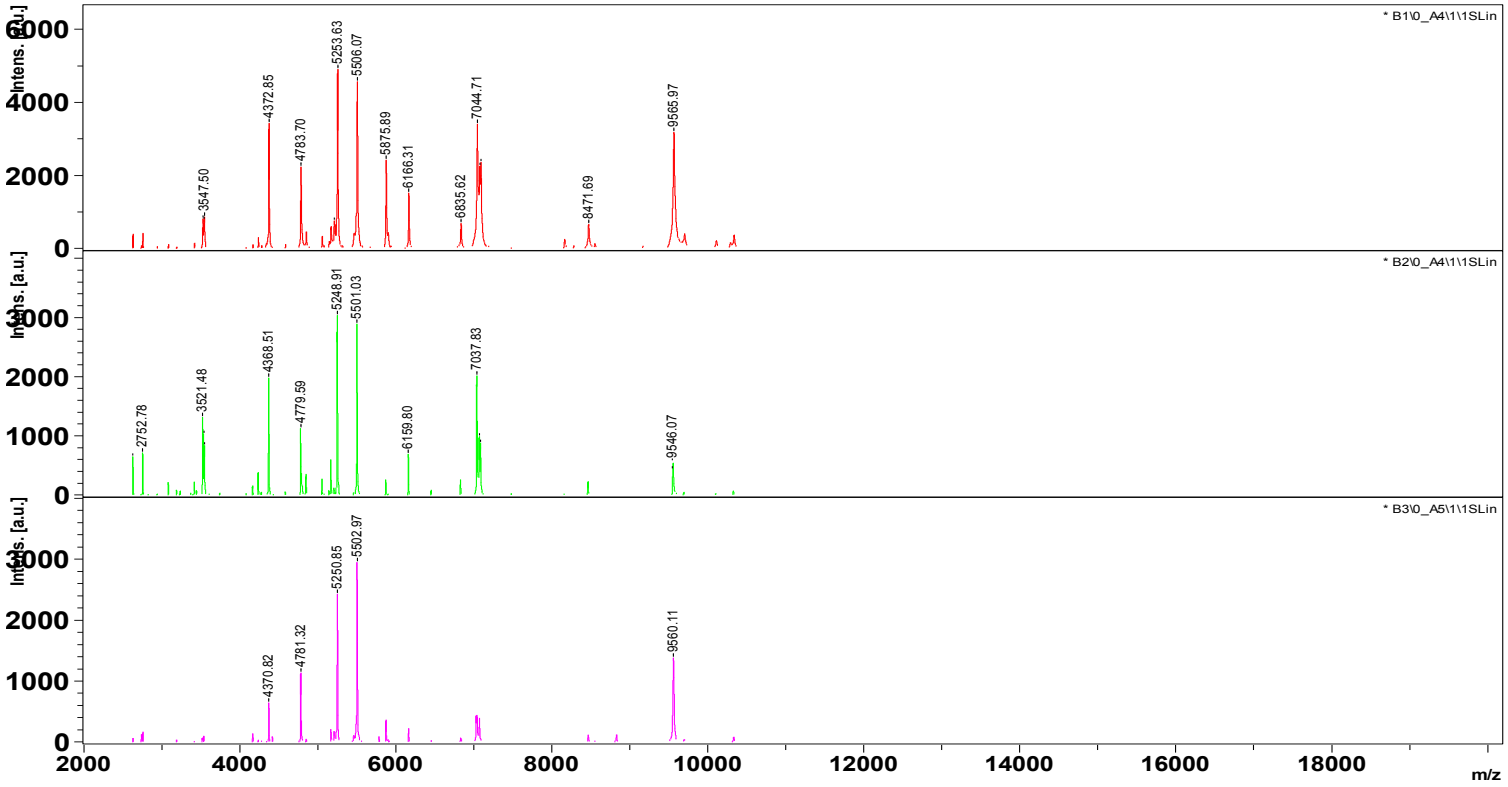
b.Mass list of aged care cluster (C1-C4), chicken (CH), canine(Ca) and bovine(B), water (w) strains.

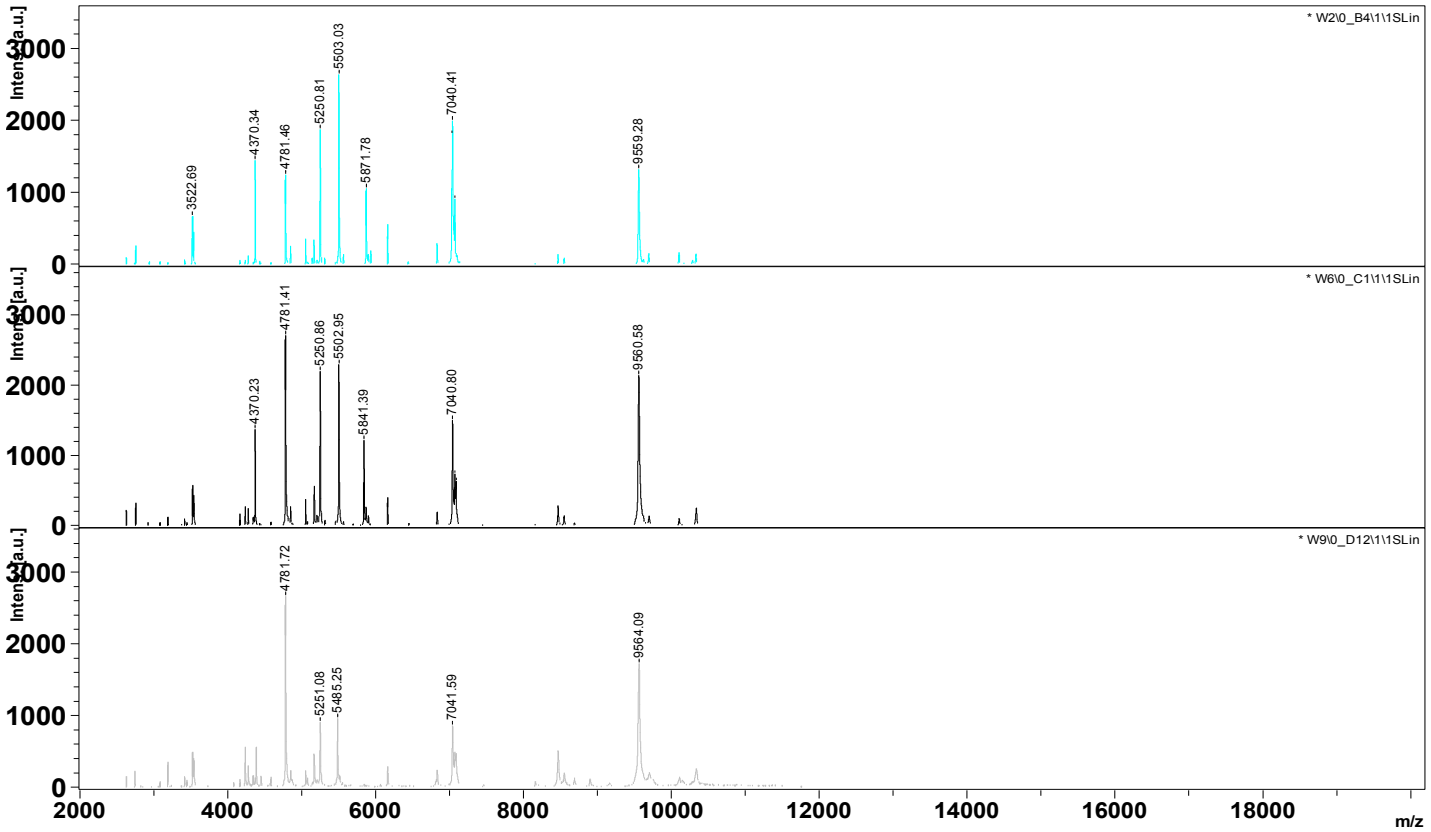



* CH32\0_D8\1\1SLin
Intens. [a.u.]
8000
6000
4000
2000
0
4780.49
9557.51
5249.90
5501.98
7038.69
* CH5\0_A8\1\1SLin
Intens. [a.u.]
8000
6000
4000
2000
0
4779.78
5249.10
5501.16
9556.76
4368.83
7038.20
2753.21
3521.64
4233.97
5052.14
5869.93
6160.54
6829.59
8464.34
* CH6\0_A9\1\1SLin
Intens. [a.u.]
3000
2000
1000
0
4778.90
9555.07
5500.32
5248.18
4368.27
7036.93
2000
4000
6000
8000
10000
12000
14000
16000
18000
m/z


* Ca1_Ex\0_E1\1\1SLin
Intens. [a.u.]
4780.85
9558.40
4369.47
5250.08
5502.42
7039.35
* Ca2_Ex\0_E6\1\1SLin
Intens. [a.u.]
4782.26
9561.20
3189.52
3523.76
4371.23
5251.71
5503.93
5874.36
7041.73
* Ca3_Ex\0_E12\1\1SLin
Intens. [a.u.]
9559.14
4780.89
4370.15
5250.24
5502.33
7039.71
6162.09
m/z


* C1\0_A11\1\1SLin
2746.74
3548.47
4238.06
4373.44
4784.54
5254.14
5875.84
6166.63
7045.13
8472.01
9566.45
* C2X1\0_A7\1\1SLin
3547.13
4371.68
4782.49
5252.15
5486.27
5873.51
7042.27
9562.16
* C3\0_B5\1\1SLin
3525.86
4374.30
4785.69
5255.34
5489.78
5877.43
6168.17
6838.22
7047.11
8475.22
9569.81
* C4X3\0_B6\1\1SLin
3547.34
4372.23
4782.99
5252.74
5874.31
6164.96
7043.47
9562.91
Intens. [a.u.]
m/z


* B1\0_A4\1\1SLin
Intens. [a.u.]
6000
4000
2000
0
3547.50
4372.85
4783.70
5253.63
5506.07
5875.89
6166.31
6835.62
7044.71
8471.69
9565.97
* B2\0_A4\1\1SLin
Intens. [a.u.]
3000
2000
1000
0
2752.78
3521.48
4368.51
4779.59
5248.91
5501.03
6159.80
7037.83
9546.07
* B3\0_A5\1\1SLin
Intens. [a.u.]
3000
2000
1000
0
4370.82
4781.32
5250.85
5502.97
9560.11
2000
4000
6000
8000
10000
12000
14000
16000
18000
m/z


* W2\0_B4\1\1SLin
Intens. [a.u.]
3000
2000
1000
0
3522.69
4370.34
4781.46
5250.81
5503.03
5871.78
7040.41
9559.28
* W6\0_C1\1\1SLin
Intens. [a.u.]
4370.23
4781.41
5250.86
5502.95
5841.39
7040.80
9560.58
* W9\0_D12\1\1SLin
Intens. [a.u.]
4781.72
5251.08
5485.25
7041.59
9564.09
2000
4000
6000
8000
10000
12000
14000
16000
18000
m/z