1.1 INTRODUCTION

Food borne illness is a major international public health concern and estimated to be the cause of 76 million illnesses, 325 000 hospitalizations and 5000 deaths in the developed country of United States annually (Mead *et al*., 1999; Badrie *et al*., 2005). The impact of food borne illnesses on public health has not been well documented in developing countries (King *et al.*, 2000). However, according to the World Health Organization (2005), 90% of the annual deaths from food borne illnesses are children in developing countries.

Food borne pathogens such as *Campylobacter* species, particularly *Campylobacter jejuni* and *Campylobacter coli* are the most commonly identified cause of acute bacterial gastroenteritis (Park and Sanders, 1992; Nachamkin *et al*., 1998; Friedman *et al*., 2000). Food poisoning caused by *Campylobacter* species can be severely debilitating but is rarely life-threatening. Most people who get campylobacteriosis recover completely within 2 to 5 days, although sometimes recovery can take up to 10 days.

Poultry is the most common vehicle for the spread of *Campylobacter*. Some case-control studies indicate that up to 70% of sporadic cases of campylobacteriosis are associated with eating chicken. Surveys by the USDA demonstrated that up to 88% of the broiler chicken carcasses in the USA are contaminated with *Campylobacter* while a recent Consumer Reports study identified *Campylobacter* in 63% of more then 1000 chickens obtained in grocery stores. Other identified food vehicles include unpasteurized milk, undercooked meats, mushrooms, hamburger, cheese, pork, shellfish, and eggs. (Humphrey *et al*., 2000)

Other sources of *Campylobacter*, in addition to food and water, that have been reported include children prior to toilet-training, especially in child care settings and intimate contact with other infected individuals, (Goosens *et al*., 1995). *C. jejuni* is commonly present in the

gastrointestinal tract of healthy cattle, pigs, chickens, turkeys, duck, and geese, and direct animal exposure can lead to infection. Pets that may carry *Campylobacter* include birds, cats, dogs, hamsters, and turtles (Fang *et al*., 1991) The organism is occasionally isolated from streams, lakes and ponds. The outbreaks of campylobacter illness associated with consumption of contaminated vegetables or fruits are uncommon and possible (Centers for Disease Control, 2000)

Campylobacter control is therefore necessary at all key steps of food production to ensure safe products for consumers. This control requires rapid and reliable methods in the isolation, identification and characterization including molecular approaches of *Campylobacter* isolates. Molecular characterization is used to establish the degree of relationship among strains, information that may be useful in epidemiological studies (Shobirin *et al*., 2003). Many molecular typing based on PCR-based techniques have been developed for strain differentiation in *C. jejuni* and other bacteria, these includes enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) (Versalovic *et al*. 1991; Milleman *et al*, 1996), random amplified polymorphic DNA-PCR (RAPD-PCR) (Owen and Hernandez, 1993; Mehmet and Burhan, 2006), PCR ribotyping (Lagatolla *et al*., 1996) and PCR restriction fragment length polymorphism (PCR-RFLP) (Korolik *et al.*, 1995). Bacterial typing in *Campylobacter* using RAPD-PCR and ERIC-PCR have been shown ease to apply and high discriminatory power in strains differentiation (Mehmet and Burhan, 2006; Wilson *et al*., 2009). Bacterial typing is of great value in epidemiology of infectious disease which it helps to recognize the reservoir of infection, sporadic disease setting and recognized virulent clones are present in a set of strains (Eisenstein, 1990).

To better understand the spread of infection within outbreaks, different typing tools are being applied to characterize isolates from individual outbreaks. These include traditional typing

methods such as serological typing or antibiotic resistance patterns. Currently, these techniques are further supported by molecular genetic techniques such as plasmid profiling or DNA fingerprints (Rychlik *et al*., 2000). A number of genotypic methods have been proven useful for species identification, epidemiological typing and determining the genetic relatedness among pathogenic and non-pathogenic bacteria (Son *et al*., 2001).

To date no data have been reported on the outbreak of *C. jejuni* in either poultry or raw vegetables consumption in Malaysia. However, data has shown the prevalence of *C. jejuni* in raw vegetables is rather alarming (Chai *et al.*, 2008). Consequently, the typing of *C. jejuni* is of considerable importance in the surveillance of possible community foodborne outbreaks of campylobacteriosis and as a research tool. In this study we examined the presence of virulence genes and conducted the genotyping variation among raw vegetable isolates of *C. jejuni* using enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) analysis.

1.2 Objectives Of The Study

The objectives of this study are as follows:

1. To detect the virulence genes of *cad*F, *ceu*E, *cdt*a, *cdt*b and *cdt*c genes of *Campylobacter jejuni* isolated from raw vegetables.
2. To characterize the *Campylobacter jejuni* isolates using Enterobacterial Repetitive Intergenic Sequence-Polymerase Chain Reaction (ERIC-PCR) fingerprinting.

2.1 *Campylobacter*

The bacterium *Campylobacter* belongs to the family *Campylobacteraceae*. It is a genus of gram negative bacteria, curved, motile rod shaped usually flagellated commonly found in animal faeces and causes bacterial infections in the body (Ryan *et al*., 2004). The genus *Campylobacter*, meaning 'twisted bacteria' first discovered in 1963 (Nachmankin *et al*., 2008). The organisms have a characteristic spiral and corkscrew appearance and are oxidase-positive (Ryan *et al*., 2004). The first *Campylobacter* genome to be sequenced was *C. jejuni*, in 2000 (Parkhill *et al*., 2000).

Campylobacter species contain two flagellin genes in tandem for motility, *flaA and flaB*. These genes undergo intergenic recombination, further contributing to their virulence. Most of *C. jejuni* strains produce a toxin (cytolethal distending toxin) that hinders the cells from dividing and activating the immune system. This helps the bacteria to evade the immune system and survive for a limited time in the cells (Grant *et al*., 1993).

Campylobacter is grown on specially selective CAMP agar plates at 42 °C, the normal avian body temperature, rather than at 37 °C, the temperature at which most other pathogenic bacteria are grown. Since the colonies are oxidase positive, they will usually only grow in scanty amounts on the plates. Microaerophilic conditions are required for luxurious growth (Ryan *et al*., 2004).

Campylobacter grows best in habitats with an oxygen level lower than 5%, and it is typically found in the intestinal tract of animals. *Campylobacter* colonizes the mucosal surfaces of birds and mammals, using their mobility to survive in the mucous flow and sensitive to salinity, freezing, and pH below five. (Wassenaar *et al*., 2004)

2.2 Campylobacteriosis

Campylobacteriosis is an infection by campylobacter. The common routes of transmission are faecal-oral, person-to-person sexual contact, ingestion of contaminated food or water, and the eating of raw meat. It produces an inflammatory, sometimes bloody, diarrhea, periodontitis or dysentery syndrome, mostly including cramps, fever and pain (Humphrey *et al*., 2007).

One of the effects caused by campylobacteriosis is tissue injury in the gut. The sites of tissue injury include the jejunum, the ileum, and the colon. *C. jejuni* appears to achieve this by invading and destroying the epithelial cells.

Some strains of *C. jejuni* produce a cholera-like enterotoxin, which is important in the watery diarrhea observed in infections. The organism produces diffuse, bloody, edematous, and exudative enteritis. In a small number of cases, the infection may be associated with hemolytic uremic syndrome and thrombotic thrombocytopenic purpura through a poorly understood mechanism (Wikipedia, 2010).

2.3 Pathogenicity of *Camyplobacter*

The pathogenesis of *C. jejuni* infection involves both host- and pathogen-specific factors. The health and age of the host (Tauxe *et al*., 1992) and C. jejuni-specific humoral immunity from previous exposure (Blaser *et al*., 1987) influence clinical outcome after infection. In a volunteer study, *C. jejuni* infection occurred after ingestion of as little as 800 organisms (Black *et al*., 1988). Rates of infection increased with the ingested dose. Rates of illness appeared to increase when inocula were ingested in a suspension buffered to reduce gastric acidity (Black *et al*.,

1988). Many pathogen-specific virulence determinants may contribute to the pathogenesis of *C. jejuni* infection, but none has a proven role. Suspected determinants of pathogenicity include chemotaxis, motility, and flagella, which are required for attachment and colonization of the gut epithelium. Once colonization occurs, other possible virulence determinants are iron acquisition, host cell invasion, toxin production, inflammation and active secretion, and epithelial disruption with leakage of serosal fluid (Ketley *et al*., 1997)

2.4 Sources and Transmission of *Campylobacter*

The common routes of transmission for the disease-causing bacteria are faecal-oral, person-to-person sexual contact, ingestion of contaminated food (generally unpasteurized (raw) milk and undercooked or poorly handled poultry), and waterborne (i.e: through contaminated drinking water). Contact with contaminated poultry, livestock, or household pets, especially cats and dogs, can also cause disease (World Health Organization, 1998).

Exposure to bacteria is often more common during travelling, and therefore campylobacteriosis is a common form of travelers' diarrhea. Most cases of human campylobacteriosis are sporadic. Outbreaks have different epidemiologic characteristics from sporadic infections. Many outbreaks occur during the spring and autumn (Tauxe *et al.*, 1992). Consumption of raw milk was implicated as the source of infection in 30 of the 80 outbreaks of human campylobacteriosis reported to CDC between 1973 and 1992. Outbreaks caused by drinking raw milk often involve farm visits (e.g: school field trips) during the temperate seasons. In contrast, sporadic *Campylobacter* isolates peak during the summer months (Altekruse *et al*., 1999). A series of case-control studies identified some risk factors for sporadic campylobacteriosis, particularly when handling raw poultry (Norkrans *et al*.,1982; Hopkins *et*

al., 1983) and eating undercooked poultry (Kapperud *et al*., 1992; Oosterom *et al*., 1984; Hopkins *et al*., 1984; Harris *et al*., 1986; Deming *et al*., 1987) (Table 2.1). Other risk factors accounting for a smaller proportion of sporadic illnesses include drinking untreated water (Hopkins *et al*., 1984); traveling abroad (Norkrans *et* al., 1982); eating barbequed pork (Oosterom *et al*,.1984) or sausage (Kapperud *et al*., 1992); drinking raw milk (Hopkins *et al*., 1984; Schmid *et al*., 1987) or milk from bird-pecked bottles (Lighton *et al*., 1991); contact with dogs (Kapperud *et al*., 1992) and cats (Hopkins *et al*., 1984; Deming *et al*., 1987), and particularly in contact with juvenile pets or pets with diarrhea (Norkrans *et al*.,1982; Saaed *et al*., 1993). Person-to-person transmission is uncommon (Norkrans *et al*.,1982; Schmid *et al*., 1987). Overlap is reported between serotypes of *C. jejuni* found in humans, poultry, and cattle, indicating that foods of animal origin may play a major role in transmitting *C. jejuni* to humans (Nielsen *et al*., 1997).

In the United States, infants have the highest age-specific *Campylobacter* isolation rate, approximately 14 per 100,000 people per year. As children get older, isolation rates decline to approximately 4 per 100,000 people per year for young adolescents. A notable feature of the epidemiology of human campylobacteriosis is the high isolation rate among young adults, approximately 8 per 100,000 people per year. Among middle-aged and older adults, the isolation rate is < 3 per 100,000 people per year (Tauxe e*t al*.,1992). The peak isolation rate in neonates and infants is attributed in part to susceptibility on first exposure and to the low threshold for seeking medical care for infants. The high rate of infection during early adulthood, which is pronounced among men, is thought to reflect poor food-handling practices in a population that, until recently, relied on others to prepare meals (Tauxe e*t al.*,1992).

2.5 Epidemiology of *Campylobacter*

Awareness of the public health implications of *Campylobacter* infections has evolved over more than a century (Kist *et al*., 1985). In 1996, 46% of laboratory-confirmed cases of bacterial gastroenteritis reported in the Centers for Disease Control and Prevention/U.S. Department of Agriculture/Food and Drug Administration Collaborating Sites Foodborne Disease Active Surveillance Network were caused by *Campylobacter* species. Campylobacteriosis was followed in prevalence by salmonellosis (28%), shigellosis (17%), and *Escherichia coli* O:157 infection (5%). For instance, an estimated 2 million cases of *Campylobacter* enteritis occur annually in the U.S., accounting for 5-7% of cases of gastroenteritis (Altekruse *et al*., 1999).

Furthermore, in the United Kingdom during the year 2000 *Campylobacter jejuni* was involved in 77.3% cases of foodborne illness (Sopwith *et al*. 2008). Fifteen out of every 100,000 people are diagnosed with campylobacteriosis every year, and with many cases going unreported, up to 0.5% of the general population may unknowingly harbour *Campylobacter* in their gut annually (Fang *et al*., 1991).

Developing countries as in Asia, they do not have national surveillance programs for campylobacteriosis, therefore, incidence values in terms of number of cases for a population do not exist. Availability of national surveillance programs in developed countries has facilitated monitoring of sporadic cases as well as outbreaks of human campylobacteriosis (Altekruse *et al*., 1999). Most estimates of incidence in developing countries are from laboratory-based surveillance of pathogens responsible for diarrhea. *Campylobacter* isolation rates in developing countries range from 5 to 20% (Oberhelman *et al*., 2000).

There was no report on the outbreak of *C. jejuni* through poultry or raw vegetables consumption in Malaysia. However, Chai *et al*., (2008) reported the data that showed the prevalence of *C. jejuni* in raw vegetables and *ulam*. The typing of *C. jejuni* is of considerable important in the surveillance of foodborne outbreaks of as research tool. The genotyping variation among raw vegetable isolates of *C. jejuni* using enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) analysis will be expected in this study.

Table 2.1 Epidemiologic studies of laboratory-confirmed cases of sporadic Campylobacteriosis

Number						
Cases	Controls	Date	Population	Location	Foods associated with illness	Animal contacts
52	103	1989-1990	Residents of three counties	Norway	Poultry, sausage	Dogs

218	526	1982-1983	HMO patients	Washington State	Undercooked chicken	Animals with diarrhea
29	42	1990	Residents of Manchester	England	Bottled milk	
45	45	1983-1984	University students	Georgia	Chicken	Cats
53	106	1982-1983	Rural children	Iowa	Raw milk	
40	80	1981	Residents of Denver Ft. Collins	Colorado	Untreated water, raw milk, undercooked chicken	Cats
54	54	1982	Residents of Rotterdam	Netherlands	Chicken, pork, barbequed foods	**NA**
10	15	1982	Residents of Larimer County	Colorado	Preparing chicken	**NA**
55	14	1980	Residents of Göteborg	Sweden	Preparing chicken	Kitten dog with diarrhea

NA= not available

Source: Altekruse, S.F. et al.,(1999). *Campylobacter jejuni*-an emerging foodborne pathogen. *Emerging Infectious Diseases* **5** (1):28-35

2.6 Isolation of *Campylobacter*

Isolation of *Campylobacter* including all species normally were isolated by Chai *et al*. (2008) on modified charcoal cefoperazone deoxycholate agar mCCDA (Merck).

2.6.1 Pre-enrichment media

Food and environmental samples are usually contaminated with relatively low numbers of *Campylobacter*. The incorporation of pre-enrichment procedures into laboratory protocols has been found to increase recovery of *Campylobacter* from most sample types and is generally recommended for analysis of food, water and other environmental samples (Bolton *et al.*, 1984).

Pre-enrichment usually starts with a resuscitation procedure that is included to overcome damage to cells caused by drying, heating, starvation, freezing and/or oxygen radicals. Probably the most widely used resuscitation procedure consists of 4 hours incubation at 37°C (Humphrey, 1989; Bolton, 2000) after which the pre-enrichment broths are transferred to 42°C. It is recommended that resuscitation be limited to 4 hours to prevent overgrowth by contaminants (Goosens and Bultzer, 1992).

Following pre-enrichment, an aliquot of growth from enrichment tubes is sub-cultured to a chosen selective agar that is usually incubated for 48 hours at 42°C to confirm the presence of thermotolerant *Campylobacter* and to yield single colonies for species identification and for sub-species typing (Line *et al*., 2001).

There are many pre-enrichment broths but five in particular seem to be in common use: Preston broth, Exeter broth, Bolton broth, CEB broth (Campylobacter enrichment broth) and Park & Sanders broth (Donnison *et al*., 2003).

2.6.2 Enrichment Media

Enrichment media are liquid or semi-solid agar media that contains additives that selectively allow certain microorganisms to grow and at the same time inhibiting the growth of

other bacteria. Examples of enrichment broths for *Campylobacter* spp. are selenite cystine broth, Campy –Thio broth and *Campylobacter* enrichment broth (Bolton broth). (Patton *et al*., 1981)

While allowing the growth of *Campylobacter* spp., the use of enrichment broth still results in mixed cultures containing other organisms. Therefore, plating to highly selective plating media will allow the development of discrete *Campylobacter* colonies while at the same time inhibiting the growth of other bacteria (Rothenberg *et al*., 1984).

2.6.3 Selective plating media

Selective plating media are solid, selective agar that allows differential growth to varying degrees.

Selective and differential media usually used for campylobacter include blood-free media such as modified charcoal cefoperazone deoxycholate (mCCDA), charcoal based selective medium and semi-solid blood free motility media, and blood containing media such as Skirrow medium (Public Health Laboratory Network Australia, 2000).

Some antimicrobial agents present in selective media may inhibit some *Campylobacter* species. Cephalothin, colistin, polymyxin B, which are present in some selective medium formulations, may inhibit some strains of *C. jejuni* and *C. coli* and are inhibitory to *C. fetus* subsp. fetus, *C. jejuni* subsp. *doylei*, and *C. upsaliensis*. Therefore, the incidence of infection by different *Campylobacter* species maybe variable (Public Health Laboratory Network Australia, 2000)

2.7 Identification of *Campylobacter*

Suspected colonies are subjected to several tests for definitive identification of *Campylobacter*.

2.7.1 Culture Base Methods

Campylobacter colonies may have different appearances depending on the medium. *Campylobacter* species generally produce grey, flat, irregularly spreading colonies (oil drop morphology). As the moisture content decreases, colonies may from round, convex, glistening colonies with little spreading observed (Public Health Laboratory Network Australia, 2000).

Staining with Gram's counterstain (e.g., carbol fuchsin) or a saline wet preparation examination of the colony should be performed, along with an oxidase test. Oxidase positive colonies exhibiting a characteristic microscopic appearance (e.g., "gull wings", curved, S-shaped, or spiral rods that are 0.2 to 0.9 μm wide and 0.5 to 5 μm long) that were isolated from selective media incubated under microaerobic conditions can be reliably reported as *Campylobacter* species. Upon prolonged exposure to air or in old cultures, cells become spherical or coccoid and may be difficult to identify (Public Health Laboratory Network Australia, 2000).

C. jejuni is relatively easy to identify phenotypically; however, hippurate negative strains may occur. *C. coli* are biochemically similar to *C. jejuni* except for hippuricase activity. The use of antibiotic discs (cephalothin and nalidixic acid) for identification is becoming problematic due to fluoroquionlone resistance in *Campylobacter*. Nalidixic acid resistance does not exclude the identification of *C. jejuni* or *C. coli*. Hippurate positive strains should be reported as *C. jejuni* regardless of the nalidixic acid disc results. Detection of a nalidixic acid resistant strain *C. jejuni*

should suggest the strain may be resistant to fluoroquinolone; further susceptibility testing may be warranted (Public Health Laboratory Network Australia, 2000).

2.7.2 Non culture Methods

Commercial assays to assist in identifying *Campylobacter* species at the genus level are available. Two immunological assays (ID Campy [Integrated Diagnostics Baltimore, Md.] and campysides [BBL Microbiology Systems, Cockeysville, Md]) can detect *C. jejuni* and *C.coli* but cannot differentiate between them. A commercial probe assay directed against *Campylobacter* RNA (Accuprobe [Gen-Probe Inc, San Diego, Calif.]) detects *C. jejuni* subspecies *jejuni*, *C. jejuni* subspecies *doylei*, *C. coli* and *C. lari* and is highly sensitive; however, cross hybridisation with *C. hyointestinalis* has been noted for some isolates (Public Health Laboratory Network Australia, 2000).

In-house PCR may be available in some laboratories. A commercial enzyme linked immunosorbent assay for detecting *Campylobacter* antigen directly in stool samples has recently been developed. (Public Health Laboratory Network Australia, 2000).

2.8 Epidemiological typing systems

Phenotype typing systems have been developed to study the epidemiology of *Campylobacter* infections. Serotyping based on O-antigens (i.e., lipopolysaccharide) and serotyping based on heat-labile antigens exist for typing *C. jejuni* and *C. coli*. Biotyping systems also exist (Public Health Laboratory Network Australia, 2000).

2.9 Molecular typing

Conventional methods as described above usually require a few days to complete (Hanes, 2003) and serotyping is complex and difficult even though it is most widely used phenotyping methods (Lim *et* al., 2005).

Molecular epidemiology is a science that focuses on the contribution of potential genetic and environmental risk factors, identified at molecule level, to the etiology distribution and prevention disease within families and across the populations.

There are two major categories of typing that have been used to classify bacteria. There are phenotypic typing and genotypic methods. Phenotypic methods are those that characterized the products of gene expression in order to differentiate strains. Properties such as biochemical profiles, bacteriophage types, antigens present on the cell's surface, and antimicrobial susceptibility profiles all are examples of phenotypic properties that can be determined in the laboratory. Because they involve gene expression, the properties all have a tendency to vary based on changes in growth conditions, growth phase and spontaneous mutation (Fred *et al*., 1997). Meanwhile, genotyping is a molecular approaches that involve direct DNA-based analysis of chromosome, plasmid and insertion sequences or transposons (IS). Genotypic methods are less subject to natural variation, although they can be affected by insertions or deletions of DNA into the chromosome, the gain or loss of extrachromosomal DNA, or random mutations that may create or random mutations that may create or eliminate restriction endonuclease sites (Fred *et* al., 1997). Various molecular typing have been developed; these includes random amplified polymorphic DNA (RAPD or AP-PCR (Arbitrarily primed-PCR), enterobacterial repetitive intergenic consensus (ERIC), antibiotic resistance and plasmid profiling. Genotyping results have the potential of providing more consistent, reproducible data and are applicable to other

species and genera. It also provides information on the clonality among various clinical and environmental isolates, thus permitting and providing greater accuracy in the evaluation of the epidemic spread of pathogens (Eisenstein, 1990).

2.10 Polymerase Chain Reaction

Polymerase chain reaction (PCR) has been used extensively worldwide and has been hailed as 20th century's most important breakthrough in science (Essays on an Information Scientist, 1991). PCR, which is a technique for amplifying a specific region of DNA, has become a powerful and popular tool in microbial identification and also applied in typing of infectious agents.

The PCR technique was originally conceived by Kary Mullis in 1983. Amplification is initiated by thermostable DNA polymerase, defined by a set of two primers, together with four standard deoxyribonucleoside triphosphates. The basic principles of PCR involves 3 steps which are repeated in cycles; denaturation which will separate double stranded DNA into single stranded, annealing which will allow the annealing of the two primers on opposite DNA strands, and extension which will produce complementary copies of the original sequence or polymerization by polymerase-mediated nucleotide (Keith *et al*., 2003)

The ability to amplify a specific DNA sequence *in vitro* using a single automated reaction by PCR has had enormous impact on many areas of biology, including DNA fingerprinting, mapping of the human genome, recovery of sequence information from ancient DNA and molecular taxonomy and evolution. There are many variations of PCR-based techniques such as random amplified polymorphic DNA (RAPD) and enterobacterial repetitive intergenic

consensus sequences (ERIC). RAPD and ERIC were found to be of great discriminatory power (Lim *et al*., 2005). RAPD technique is basically an amplification of genomic DNA in PCR using a single short primer. ERIC meanwhile uses outward-facing primers complementary to each end of the short interspersed repetitive nucleic acid sequence in a PCR. These techniques have been proven successfully useful in epidemiological studies (Lim *et al.*, 2005).

3.0 MATERIAL AND METHODS

3.1 *Campylobacter jejuni* strains

Twenty (n=20) bacterial strains of *C. jejuni* were obtained from the laboratory of Food Science and Biotechnology , University Putra Malaysia, Serdang, Selangor. All isolates were obtained from raw vegetables as described in Table 3.1

Table 3.1: Type of samples for the *Campylobacter* spp. and their location

Strain number	**Sample source**	**Scientific name**	**Location**
CJ1	Kangkung	*Ipomoea aquatica*	Supermarket A, Serdang, Selangor
CJ2	Kangkung	*Ipomoea aquatica*	Supermarket A, Serdang, Selangor
CJ3	Pegaga	*Centella asiatica*	Supermarket A, Serdang, Selangor
CJ4	Pegaga	*Centella asiatica*	Supermarket A, Serdang, Selangor
CJ5	Kangkung	*Ipomoea aquatica*	Supermarket A, Serdang, Selangor
CJ6	Kesum	*Poligonum minus*	Supermarket A, Serdang, Selangor
CJ7	Kacang botol	*Psophocarpus tetragonolobus*	Supermarket B, Putrajaya
CJ8	Kesum	*Poligonum minus*	Supermarket B, Putrajaya
CJ9	Tauge	*Vigna radiata*	Supermarket B, Putrajaya
CJ10	Kesum	*Poligonum minus*	Supermarket B, Putrajaya
CJ11	Kacang botol	*Psophocarpus tetragonolobus*	Supermarket B, Putrajaya
CJ12	Tauge	*Vigna radiata*	Supermarket B, Putrajaya
CJ13	Tauge	*Vigna radiata*	Supermarket B, Putrajaya
CJ14	Tauge	*Vigna radiata*	Supermarket B, Putrajaya
CJ15	Kangkung	*Ipomoea aquatica*	Wet market, Serdang
CJ16	Kacang botol	*Psophocarpus tetragonolobus*	Wet market, Serdang
CJ17	Pegaga	*Centella asiatica*	Wet market, Serdang
CJ18	Pegaga	*Centella asiatica*	Wet market, Serdang
CJ19	Pegaga	*Centella asiatica*	Wet market, Serdang
CJ20	Tauge	*Vigna radiata*	Wet market, Serdang

3.2 Strain maintenance

Bacterial cultures were maintained by means of streaking onto nutrient agar (NA) plates. The cultures were incubated in an anaerobic jar at 42°C and placed in a dry incubator for 48 hours. All strains were stored at 4°C and reinoculated as above to get active culture when it needed.

3.3 Sterilization technique

All plasticware, including microfuge tube, yellow and blue tips, white tips, beakers and polypropylene/polycarbonate centrifuge tubes were autoclaved at 121°C for 30 minutes at 15 psi. Media and solution were autoclaved at 121°C for 20 minutes also 15 psi.

3.4 Solution

3.4.1 Tris-borate EDTA (TBE) buffer (10X concentration)

Tris-borate (TBE) EDTA buffer was used for agarose gel electrophoresis. A 10X stock solution was prepared as follows:

Tris base	890 mM
Boric acid	890 mM
EDTA	20 mM

Distilled water was added to make volume up to 500 ml.

The buffer was subsequently diluted with distilled water to a 1X concentration before use in agarose gel electrophoresis. The pH of the 1X concentration solution was adjusted to 8.3 using 0.1M HCl.

3.5 Medium

3.5.1 Difco ™ Nutrient Agar

The basic growth medium for bacteria, composed of beef extract and peptone. Nutrient agar typically contains (w/v):

- 0.5 % peptone
- 0.3 % beef extract
- 1.5 % agar
- pH adjusted to neutral at 25 °C.

3.5.2 Cyclic adenosine 3',5'-monophosphate (cAMP) agar

Campylobacter is grown on specially selective CAMP agar plates at 42 °C, the normal avian body temperature, rather than at 37°C, the temperature at which most other pathogenic bacteria are grown.

3.6 DNA Extraction

A simple DNA extraction was done involving boiling, freezing and centrifugation (Perera and Murray, 2008). Prior to DNA preparation, the cells were grown onto CAMP agar medium at 42^{o}C for 48 hours in anaerobic jar. The 0.3 ml cells were transferred into 1.5 ml microfuge tube which contained 1.0 ml of sterile distilled water. The cells were vortexed and centrifuged at 12,000 rpm for 1 min. The supernatant was discarded. The pellet was then washed with 1.0 ml sterile distilled water and vortex. Then, it was boiled at 97^{o}C for 10 mins and immediately frozen at

-20^{o}C for 10 mins. The tube was centrifuged at 10,000 rpm for 3 min. The supernatant was used as a template for PCR amplification.

3.7 PCR Detection using *cdtA* gene

The detection of cytolethal distending toxin A (*cdtA*) and PCR condition were optimized using recommendations reported previously (Pickett *et al*., 1996). The detection assay was performed in a 25 µl volume containing 5.0 µl of 5× PCR buffer (100 mmol l^{-1} Tris–HCl, 35 mmol l^{-1} $MgCl_2$, 750 mmol l^{-1} KCl, pH 8.8), 1.0 µl of 200 µmol l^{-1} dNTPs (Promega, Madison, USA), 2.0 µl of 1.5mM stock of $MgCl_2$, 0.5 µl of 400 $nmol^{-1}$ GNW primer (5'-GGAAATTGGATTTGGGGCTATACT-3') and IVH primer (5'-ATCACAAGGATAATGGACAAT-3') 0.3 µl of 2.0 units of *Taq* DNA polymerase (Promega, Madison, USA), 12.20 µl of sterile ultrapure deionized water and 2.5 µl of 100 ng as DNA template. A positive-DNA control was performed by adding 1 µl of DNA from *Campylobacter* spp. cultures from World Health Organization (WHO) in every PCR assay. Amplification was performed in personal Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing for 1 min at 42 °C and polymerization at 72 °C for 40 seconds. Final elongation was at 72 °C for 3 min. Marker size used in this test was also 100 bp from Vivantis.

3.8 PCR Detection using *cdtB* gene

The detection of cytolethal distending toxin B (*cdtB*) and PCR condition were used as described by Pickett *et al.*(1996). The assay was performed in a 25 µl volume containing 5.0 µl of 5 × PCR buffer (100 mmol l^{-1} Tris–HCl, 35 mmol l^{-1} $MgCl_2$, 750 mmol l^{-1} KCl, pH 8.8), 1.0 µl of 200 µmol l^{-1} dNTPs (Promega, Madison, USA), 2.0 µl of 1.5mM stock of $MgCl_2$, 0.5 µl of 400 $nmol^{-1}$ VAT2 (5'-GTTAAAATCCCCTGCTATCAACCA-3') and WMI-R primer (5'-

GTTGGCACTTGGAATTTGCAAGGC-3') 0.3 µl of 2.0 units of *Taq* DNA polymerase (Promega, Madison, USA), 12.20 µl of sterile ultrapure deionized water and 2.5 µl of 100 ng as DNA template. A positive-DNA control was performed by adding 1 µl of DNA from *Campylobacter* spp. cultures from World Health Organization (WHO) in every PCR assay. Amplification was performed in personal Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing for 1 min at 42 °C and polymerization at 72 °C for 40 seconds. Final elongation was at 72 °C for 3 minutes. The amplification products were analysed by electrophoresis in 1.0% agarose in 0.5XTBE 90V for 40 minutes. Gel was stained with ethidium bromide. The amplified fragments are visualized with UV transiluminator (Syngene, USA). The 100 bp DNA ladder (Vivantis, USA) was used as a DNA size marker.

3.9 PCR Detection using *cdtC* gene

The detection of cytolethal distending toxin C (*cdtC*) and PCR condition were optimized using recommendations reported previously (Pickett *et al*., 1996). The detection assay was performed in a 25 µl volume containing 5.0 µl of 5× PCR buffer (100 mmol l^{-1} Tris–HCl, 35 mmol l^{-1} $MgCl_2$, 750 mmol l^{-1} KCl, pH 8.8), 1.0 µl of 200 µmol l^{-1} dNTPs (Promega, Madison, USA), 2.0 µl of 1.5mM stock of $MgCl_2$, 0.5 µl of 400 nmol^{-1} WMI-F primer (5'-TGGATGATAGCAGGGGATTTTAAC-3') and LPF-X primer (5'-TTGCACATAACCAAAAGGAAG-3') 0.3 µl of 2.0 units of *Taq* DNA polymerase (Promega, Madison, USA), 12.20 µl of sterile ultrapure deionized water and 2.5 µl of 100 ng as DNA template. A positive-DNA control was performed by adding 1 µl of DNA from *Campylobacter* spp. cultures from World Health Organization (WHO) in every PCR assay. Amplification was

performed in personal Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing for 1 min at 42 °C and polymerization at 72 °C for 40 seconds. Final elongation was at 72 °C for 3 min. The amplification products were analysed by electrophoresis in 1.0% agarose in 0.5XTBE 90V for 40 minutes. Gel was stained with ethidium bromide. The amplified fragments are visualized with UV transiluminator (Syngene, USA). The 100 bp DNA ladder (Vivantis, USA) was used as a DNA size marker.

3.10 Detection of cad*F* and ceu*E* genes

The detection of cad*F* and ceu*E* genes and PCR condition were used as described by Konkel *et al*. (1999) and Gonzalez *et al*.,(1997). The assay was performed in a 25 µl volume containing 5.0 µl of 5 × PCR buffer (100 mmol l^{-1} Tris–HCl, 35 mmol l^{-1} $MgCl_2$, 750 mmol l^{-1} KCl, pH 8.8), 1.0 µl of 200 µmol l^{-1} dNTPs (Promega, Madison, USA), 2.0 µl of 1.5mM stock of $MgCl_2$, 0.5 µl of 400 $nmol^{-1}$ F2B primer (5'-TGGAGGGTAATTTAGATATG-3') and R1B primer (5'-CTAATACCTAAAGTTGAAAC-3') for cad*F* gene and also JEJ1 primer (5'-CCTGCTCGGTGAAAGTTTTGC-3') and JEJ2 primer (5'- GATCTTTTTGTTTTGTGCTGC-3') for ceu*E* gene. 0.3 µl o f 2.0 units of *Taq* DNA polymerase (Promega, Madison, USA), 12.20 µl of sterile ultrapure deionized water and 2.5 µl of 100 ng as DNA template. A positive-DNA control was performed by adding 1 µl of DNA from *Campylobacter* spp. cultures from World Health Organization (WHO) in every PCR assay. Amplification was performed in personal Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing for 1 min at 42 °C and polymerization at 72 °C for 40 seconds. Final

elongation was at 72 °C for 3 min. The amplification products were analysed by electrophoresis in 1.0% agarose in 0.5XTBE 90V for 40 minutes. Gel was stained with ethidium bromide. The amplified fragments are visualized with UV transiluminator (Syngene, USA). Marker size that used in this test was 100 bp (Vivantis).

3.11 Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR Amplification

The primer used were ERIC1R (5'-CACTTAGGGGTCCTCGAATGTA-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') as described by Versalovic *et al*. (1991). PCR amplification reactions consisted of 25 µl volume containing 10 ng of genomic DNA, 2.5 µl 10x reaction buffer, 0.5 µl of 2 unit *Taq* DNA polymerase, 1.0 µl of 5 µM each of the forward and reverse primers, 2 µl of 1.5 mM $MgCl_2$, and 1 mM each of dNTP. Amplification was done using a Perkin Almer 2400 thermocycler (Perkin-Elmer, Norwalk, USA) as follows: 95^{o}C for 7 min and 35 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 52 ^{o}C for 1 min, and extension at 72 ^{o}C for 5 min. Final elongation step was at 72 ^{o}C for 10 min at the end of 35 cycles. The amplification products were fractionated by electrophoresis through 1.0 % agarose gel in 1X TBE buffer (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA), detected by staining with ethidium bromide and were visualized with UV transilluminator (Syngene, USA). The 100 bp DNA ladder (Promega, USA) was used as a DNA size marker.

3.12 Data Analysis

The banding patterns of individual strains were scored based on the presence or absence of the bands. The banding patterns scored were analyzed using the RAPDistance Package Software (version 1.04) program (Sahilah *et al*., 2010). The scoring was made in the form of binary code with the score '1' indicating presence of band and '0' the absence of band.

The data obtained were recorded and entered in the software CorelDRAW Graphic Suite X3 where a dendogram was produced for further analysis. Clustering was based on the unweighted pair of group average method (UPGMA) and was performed with the RAPDistance software.

4.1 Detection of cdtA gene

Campylobacter spp. is also characterized by the presence of toxin of cytolethal distending toxin (CDT), a toxic activity described by Johnson *et al*. 1998. CDT causes progressive cellular distention and, ultimately death in Chinese hamster ovary (CHO) (Pickett *et al*., 1999).

Figure 4.1 : Detection of *Campylobacter jejuni* isolates using *cdt* A virulence gene among vegetables isolates of *Campylobacter jejuni* electrophoresed on 1.0% agarose gel. Lane M, molecular weight sizes in base pairs (bp) are indicated by numbers on the left; lane 1-20: CJ16, CJ11, CJ12, CJ17, CJ8, CJ19, CJ15, CJ10, CJ7, CJ2, CJ1, CJ3, CJ4, CJ5, CJ6, CJ9, CJ13, CJ14, CJ18, and CJ20; lane C was a positive control

The primer used in this present work (Pickett *et al.* 1996) was used for PCR analysis of twenty (n=20) isolates to detect *cdt*A gene. Figure 4.1 showed the *cdt*A gene detection of *Campylobacter jejuni*. No *cdt*A gene was detected when 20 raw vegetable isolates of *C. jejuni* were examined by PCR amplification using primers that had been mentioned above.

4.2 Detection of cdtB gene

In this study, the primer that has been used for PCR analysis for twenty (n=20) isolates to detect *cdt*B gene is similar to Pickett *et al*. 1999. Figure 4.2 showed the *cdt*B gene detection of *Campylobacter jejuni*. Twelve isolates (12/20) were *cdt*b positive, indicated by formation of band in a range of molecular weight of 495bp. Whereas, eight isolates (8/20) not contained of *cdt*b gene, indicated by no formation of band.

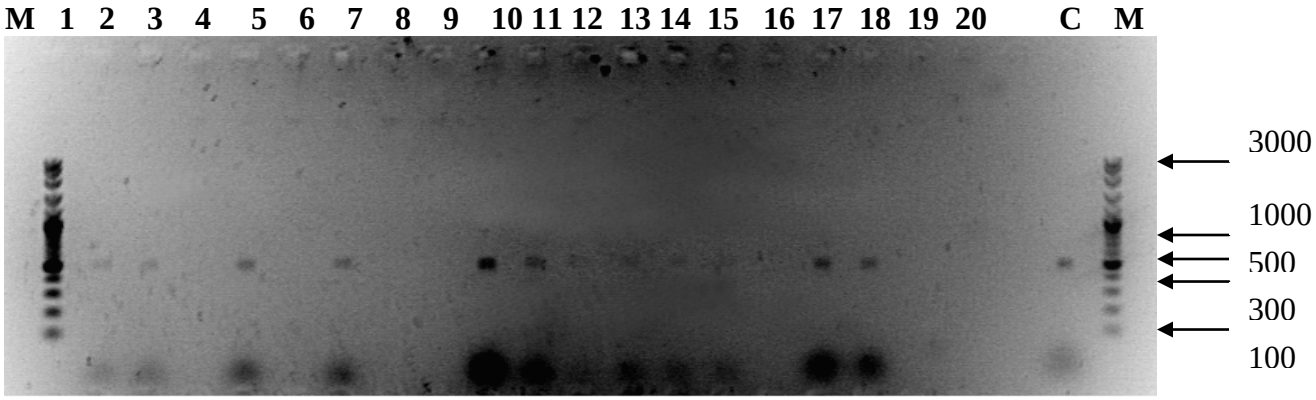


Figure 4.2 : Detection of *Campylobacter jejuni* isolates that used *cdt* B virulence gene among vegetables isolates of *Campylobacter jejuni* electrophoresed on 1.0% agarose gel. Lane M, molecular weight sizes in base pairs (bp) are indicated by numbers on the left; lane 1-20: CJ16, CJ11, CJ12, CJ17, CJ8, CJ19, CJ15, CJ10, CJ7, CJ2, CJ1, CJ3, CJ4, CJ5, CJ6, CJ9, CJ13, CJ14, CJ18, and CJ20; lane C was a positive control

4.3 Detection of cdt*c* gene

Figure 4.3 : Detection of *Campylobacter jejuni* isolates using *cdt* C virulence gene among vegetables isolates of *Campylobacter jejuni* electrophoresed on 1.0% agarose gel. Lane M, molecular weight sizes in base pairs (bp) are indicated by numbers on the left; lane 1-20: CJ16, CJ11, CJ12, CJ17, CJ8, CJ19, CJ15, CJ10, CJ7, CJ2, CJ1, CJ3, CJ4, CJ5, CJ6, CJ9, CJ13, CJ14, CJ18, and CJ20; lane C was a positive control

The detection of cdt*c* gene was conducted as described as Pickett *et al*., (1996). Figure 4.3 showed the *cdt*C gene detection of *Campylobacter jejuni*. Six isolates (6/20) were *cdt*C positive, indicated by formation of band in a range of molecular weight of 555bp. Whereas, fourteen isolates (14/20) were lacked of *cdt*C, indicated by no formation of band.

4.4 Detection of cad*F* and ceu*E* genes by multiplex PCR

In this work the multiplex PCR was conducted as described by Konkel *et al.* (1999) and Gonzalez *et al*. (1997). Two pair of primers (Table 3.2) were used for multiplex PCR analysis of twenty (n=20) isolates to detect cad*F* and ceu*E* gene. Figure 4.4 showed the multiplex PCR profiles of cad*F* and ceu*E* gene detection of *Campylobacter jejuni*. All isolates were cad*F* and ceu*E* positive, indicated by formation of 2 bands in a range of molecular weight of 400bp - 794bp. Table 4.1 summarized the detection of virulence genes in raw vegetables isolates of *C. jejuni*.

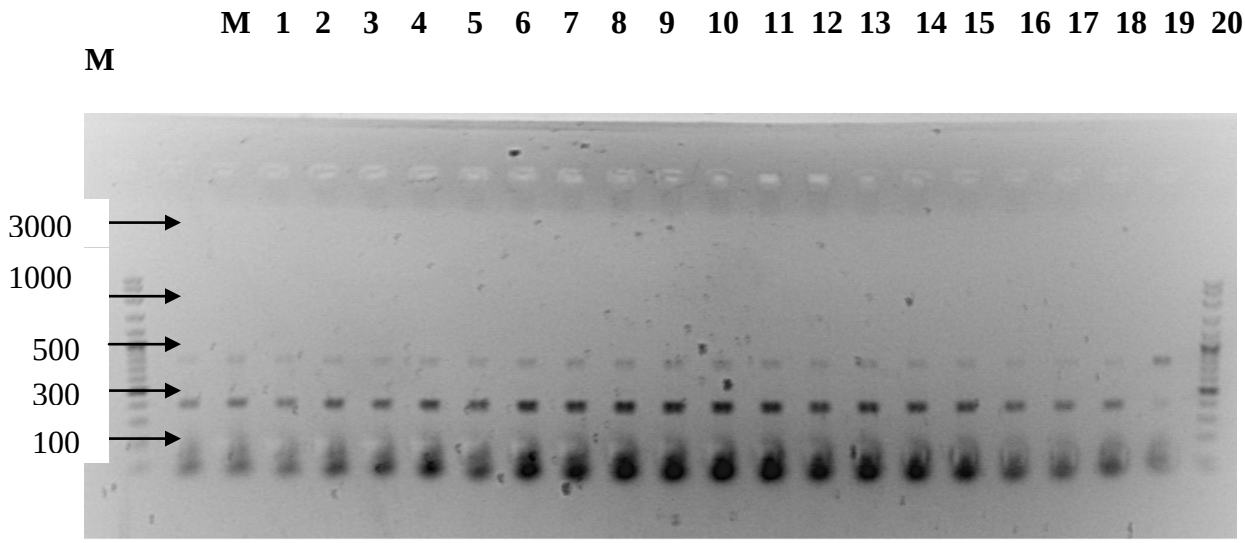


Figure 4.4: Multiplex detection of *cad*F abd *ceu*E virulence gene among vegetables isolates of *Campylobacter jejuni* electrophoresed on 1.0% agarose gel. Lane M, molecular weight sizes in base pairs (bp) are indicated by numbers on the left; lane 1-20: CJ16, CJ11, CJ12, CJ17, CJ8, CJ19, CJ15, CJ10, CJ7, CJ2, CJ1, CJ3, CJ4, CJ5, CJ6, CJ9, CJ13, CJ14, CJ18, and CJ20; lane C was a positive control

Table 4.1: Virulence genes detected in raw vegetables isolates of *Campylobacter jejuni*

Strain number	***cad*F**	***ceu*E**	**Cytolethal distending toxin (CDT)**		
			***cdt*A**	***cdt*B**	***cdt*C**
CJ1	+	+	-	+	-
CJ2	+	+	-	+	-
CJ3	+	+	-	-	-
CJ4	+	+	-	-	+
CJ5	+	+	-	+	-
CJ6	+	+	-	-	+
CJ7	+	+	-	+	-
CJ8	+	+	-	-	-
CJ9	+	+	-	-	+
CJ10	+	+	-	+	+
CJ11	+	+	-	+	-
CJ12	+	+	-	+	-
CJ13	+	+	-	+	-
CJ14	+	+	-	+	-
CJ15	+	+	-	+	-
CJ16	+	+	-	-	+
CJ17	+	+	-	+	+
CJ18	+	+	-	+	-
CJ19	+	+	-	-	-
CJ20	+	+	-	-	-

4.5 Enterobacterial Repetitive Intergenic Sequence

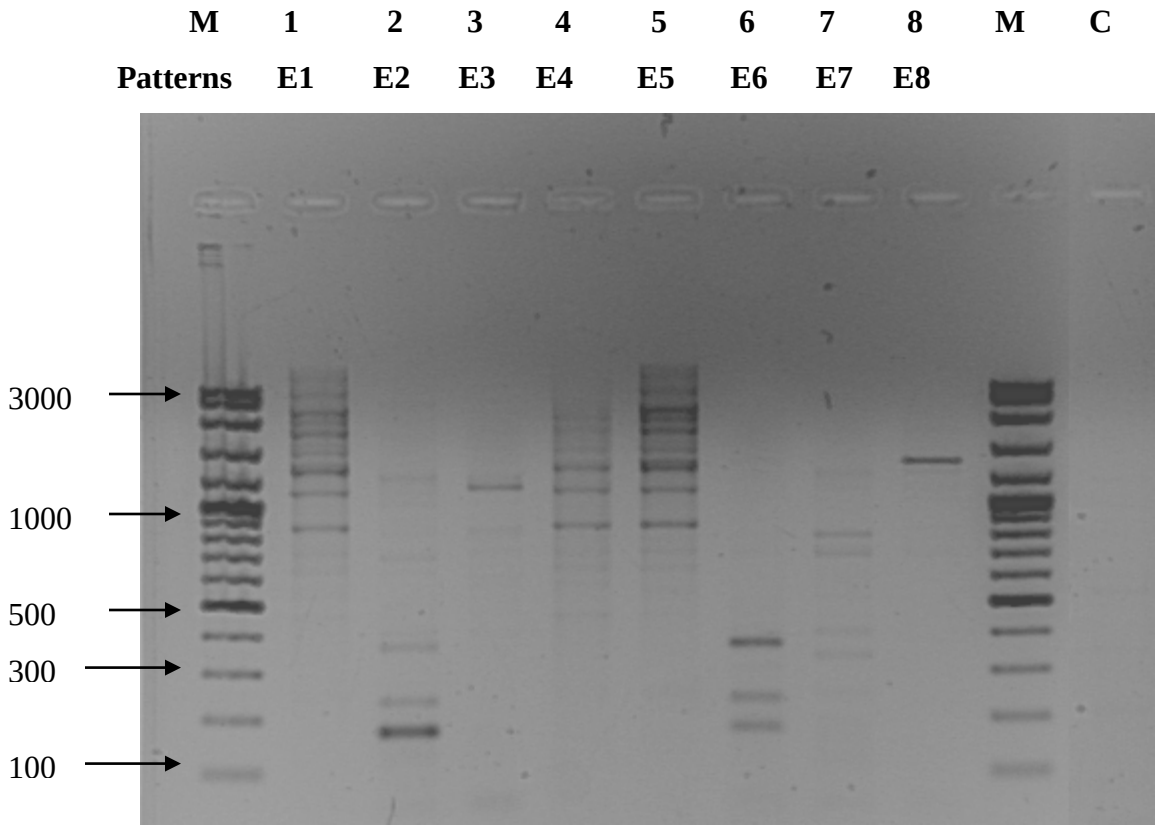


Figure 4.5 : ERIC-PCR fingerprinting (E1 to E8) of *Campylobacter jejuni* isolates electrophoresed on 1.0% agarose gel. Lane M: 100 bp DNA ladder (molecular weight in bp); lane 1-8: isolates number CJ1, CJ2, CJ3, CJ4, CJ5, CJ6, CJ7 and CJ8. Lane C: negative control

Twenty isolates of *Campylobacter jejuni* were used for ERIC-PCR analysis with a pair of primers. ERIC-PCR fingerprinting of *C. jejuni* obtained with primers had been represented by the Figure 4.5 above. The possible number of ERIC-PCR fingerprinting was estimated on the basis of presence in one or more clear bands. Electrophoresis analysis of the amplified products ranging from 0.1 to 3.0 kb in each sample and some of the bands appeared as a faint band in this analysis.

However, there were no band produced with isolates E9 and E11 in lane 9 and lane 11, respectively, (Figure 4.6) with primer that had been used. The number of ERIC bands produced for a given primer ranged from 0 to 14, with molecular sizes ranging from 0.1 to 3.0 kb.

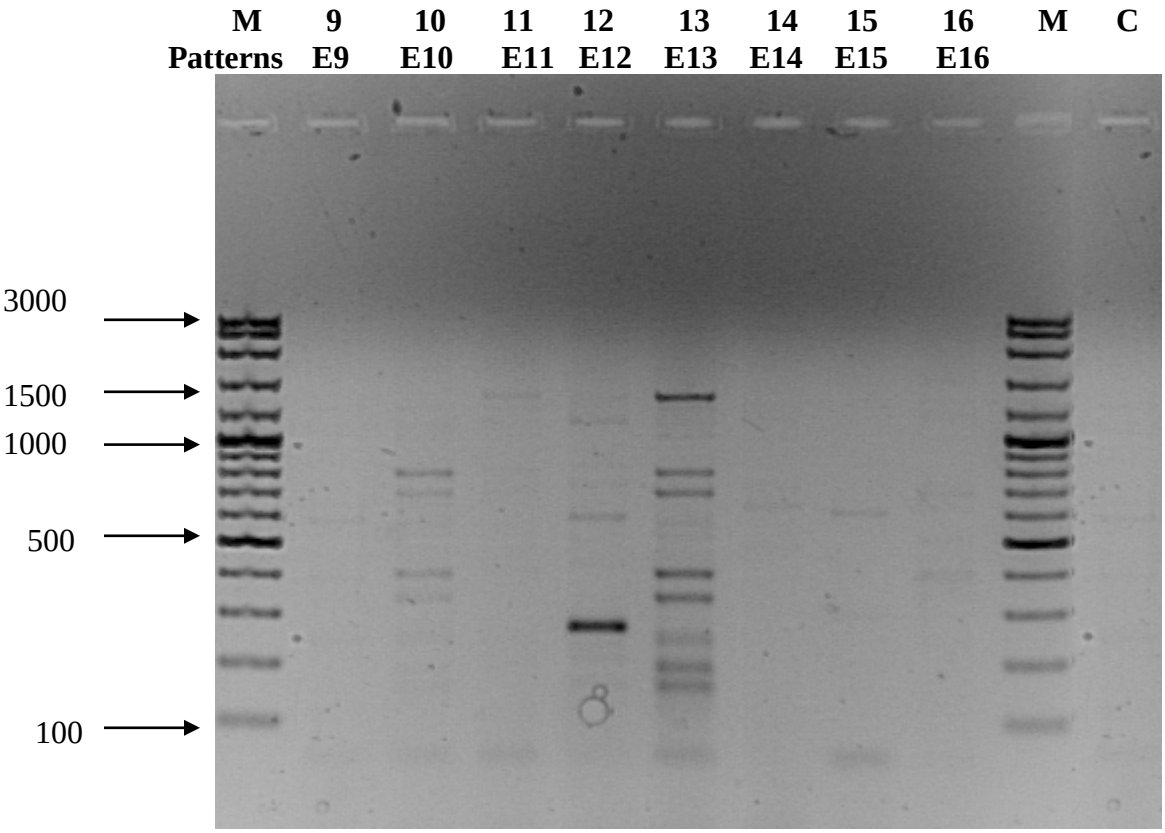


Figure 4.6 : ERIC-PCR fingerprinting (E9 to E16) of *Campylobacter jejuni* isolates electrophoresed on 1.0% agarose gel. Lane M: 100 bp DNA ladder (molecular weight in bp); lane 1-8: isolates number CJ9, CJ10, CJ311, CJ12, CJ13, CJ14, CJ15 and CJ16. Lane C: negative control

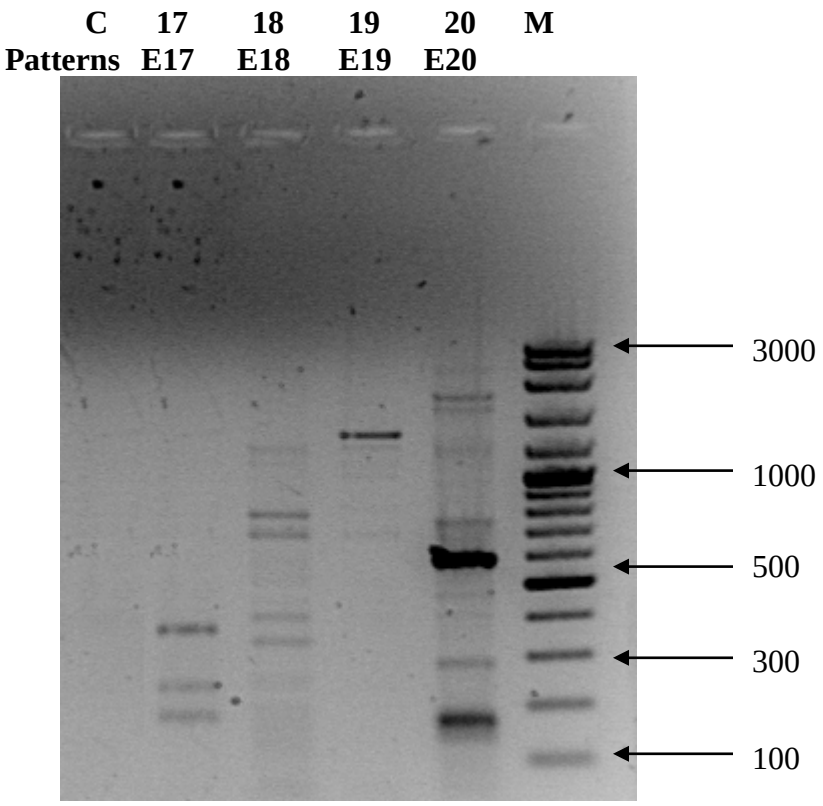


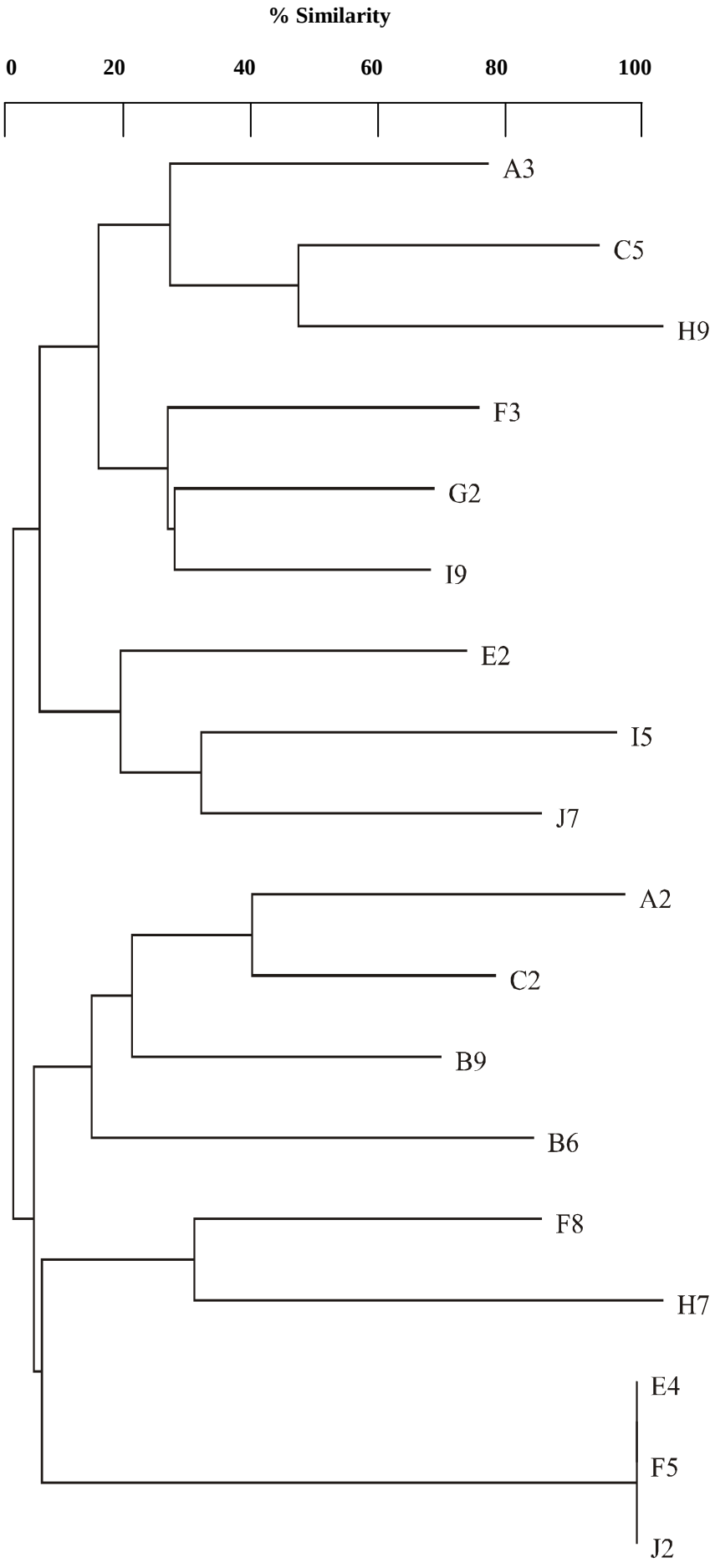
Figure 4.7 : ERIC-PCR fingerprinting (E17 to E20) of *Campylobacter jejuni* isolates electrophoresed on 1.0% agarose gel. Lane M: 100 bp DNA ladder (molecular weight in bp); lane 1-8: isolates number CJ17, CJ18, CJ19 and CJ20. Lane C: negative control

Based on Figure 4.7, the primer that had been used clearly produced ERIC in lane from 17 to 20, with molecular sizes ranging from 0.1 to 3.0 kb.

4.6 Unweighted Pair Group Method with Arithmetic mean (UPGMA)

ERIC-PCR profiles using combination two primers, ERIC1 and ERIC2 was analysed using UPGMA. Figure 4.8 showed the ERIC fingerprinting of *Campylobacter jejuni* of all isolates. Dendogram performed classified twenty *C. jejuni* strains into 2 major clusters. Cluster I and Cluster II. Cluster I, divided into 2 subclusters, subcluster Ia and Ib in which subcluster Ia contained the other 2 subcluster, IaA strains (CJ2, CJ6, CJ16) and IaB strains (CJ9, CJ10, CJ13). Subcluster Ib contained C. jejuni strains CJ7, CJ17, CJ20. It was noted that the subcluster IaA was *C. jejuni* strains isolated from Supermarket A, IaB was *C. jejuni* strains isolated from supermarket B. Whereas, subcluster Ib contained a mixture of *C. jejuni* strains isolated from supermarket B and wet market.

Cluster II divided into two subclusters, subcluster IIa and IIb. The first subcluster, subcluster IIa contained all *C. jejuni* strains isolated from Supermarket A; which are strains CJ1, CJ3, CJ4 and CJ5. Whereas, subcluster IIb contained a mixture of *C. jejuni* strains isolated from Supermarket B which are strains CJ12, CJ8 and CJ11 and wet market which are strains CJ15 and CJ19.


% Similarity
0
20
40
60
80
100
A3
C5
H9
F3
G2
I9
E2
I5
J7
A2
C2
B9
B6
F8
H7
E4
F5
J2

5.1 Discussion

In this study, 20 (n=20) raw vegetable isolates of *Campylobacter jejuni* were examined for their virulence genes and genotypic variation using enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) analysis.

In the detection of *cad*F and *ceu*E genes, all raw vegetable isolates of *C. jejuni* contained both genes. The *cad*F gene is an important virulence factor because it encodes cadF protein involved in adhesion and colonization of the host's intestine (Nuijten *et al*., 1992; Konkel *et al.*, 1999). Whereas, the *ceu*E is important because this gene encodes a binding-protein transport system for the siderophore enterochelin (Park and Richardson, 1995; Gonzales *et al*., 1997). The product of *ceu*E gene is important for pathogenicity because of its involvement in iron acquisition and bacterial infectivity. Similar observation was also reported by Bang *et al.* (2003), where the presence of *cad*F and *ceu*E gene were 100% in Danish pigs and cattles. The *ceu*E gene is highly conserved in *C. jejuni* and *C. coli* neither in pathogenic or non-pathogenic within their respective species (Gonzales *et al*., 1997). This may explain why all raw vegetable isolates of *C. jejuni* were *ceu*E gene positive. The cadF gene was also reported to be common in *C. jejuni* due to its important in adhesion and colonization (Nuijten *et al*., 1992; Konkel *et al*., 1999).

In the detection of cytolethal distending toxin (CDT) of *cdt*A, *cdt*B and *cdt*C genes, none of the *C. jejuni* isolates contained *cdt*A gene. However, for *cdt*B and *cdt*C genes, 12 *C. jejuni* isolates were contained *cdt*B and 6 *C. jejuni* isolates contained *cdt*C gene. The CDT is a toxin produce by *C. jejuni* which can cause progressive cellular distention and ultimately death in Chinese hamster ovary (CHO)(Pickett *et al.*, 1999). In this study, the lacked of *cdt*A gene in all raw vegetables isolates of *C. jejuni*

was inconclusive. In contrast, reported by Bang *et al.* (2003) in which all CDT genes of *cdt*A, cdt*B* and *cdtC* genes were highly detected in *C. jejuni* isolated from Danish pigs and cattles. The presence *cad*F, *ceu*E, *cdt*A, *cdt*B and *cdt*C genes on 20 raw vegetables isolates of *C. jejuni* in this study, were also useful as an aid to identify the *C. jejuni* isolates besides conventional method.

The potential of ERIC-PCR analysis on genome differentiation of 20 *C. jejuni* isolated from raw vegetables has been demonstrated. The ERIC-PCR analysis was highly discriminating in distinguishing the 18 *C. jejuni* strains into 18 distinct ERIC-PCR profiles. Thus, the raw vegetable isolates of *C. jejuni* examined in ERIC-PCR analysis exhibited high level of local geographical genetic variation. This result is in agreement with Wieczorek (2009) who reported the presence of wide heterogeneity within *C. jejuni* isolates from faeces and carcasses.

ERIC-PCR dendrogram was constructed using Unweighted Pair Group Method with Arithmetic mean (UPGMA). Dendogram performed from cluster analysis showed that all the 20 isolates of *C. jejuni* were clustered into two major cluster (I and II) in which each cluster formed the other subcluster (Figure 4.8). Most of the cluster trends were formed according from where the *C. jejuni* were isolated. For example cluster Ia, though *C. jejuni* strains were isolated from supermarket A and wet market assemble in one cluster they were divided into two subcluster (IaA and IaB). While, cluster Ib was a group raw vegetable strains of *C. jejuni* isolated from supermarket B and cluster IIa was a group of raw vegetable strains of *C. jejuni* isolated from supermarket A. However, only cluster IIb was a mixture of *C. jejuni* strains isolated from raw vegetable obtained from supermarket B and wet market which may suggest *C. jejuni* clonal circulate within locations as indicated by 3 strains of *C. jejuni* (CJ8, CJ11 and CJ19).

The results of this study showed exhibited high level of local geographical genetic

variation among the raw vegetable isolates of *C. jejuni* using ERIC-PCR analysis which this technique may be useful in studying the epidemiology of *C. jejuni* isolates within local geographical locations. The detection of virulence genes in raw vegetables isolates of *C. jejuni* is important due to some of the vegetables were eaten by Malaysian as *ulam* (salad) or raw vegetable which may probably can cause campylobacteriosis to consumers.

6.1 CONCLUSION

In the detection of *cad*F and *ceu*E genes, all raw vegetable isolates of *C. jejuni* contained both genes. Whereas in the detection of cytolethal distending toxin (CDT) of *cdt*A, *cdt*B and *cdt*C genes, none of the *C. jejuni* isolates contained *cdt*A gene. However, for *cdt*B and *cdt*C genes, 12 *C. jejuni* isolates contained *cdt*B and 6 *C. jejuni* isolates contained *cdt*C gene.

The potential of ERIC-PCR analysis on genome differentiation of 20 *C. jejuni* isolated from raw vegetables has been demonstrated. The ERIC-PCR analysis was highly discriminating in distinguishing the 18 *C. jejuni* strains into 18 distinct ERIC-PCR profiles. Thus, the raw vegetable isolates of *C. jejuni* examined in ERIC-PCR analysis exhibited high level of local geographical genetic variation.