University of Glasgow

Cytotoxic activity, virulence plasmid profiles and comparative proteomic analysis of *Campylobacter jejuni* strains

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DECLARATION

This thesis is the original work of the author except where otherwise stated.

Abdolmajid Mohammadzadeh

DEDICATION

This thesis is dedicated to my wife, Behnaz, who lived through every moment of my study, and my children, Pouya and Tara, whose arrival brought me more hope and strength.

ACKNOWLEDGMENT

It is with great pleasure that I express my most sincere thanks and best gratitude to my supervisors, Dr. John Coote and Dr. Roger Parton for their continued encouragement, supervision, advice, stimulating discussion, enthusiasm, patience and support throughout my research and during the writing of this thesis.

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Abstract

Human disease due to food-borne pathogens remains a medical problem worldwide. *Campylobacter jejuni* is the most common cause of bacterial food-borne diarrhoeal disease, but the mechanisms by which it causes disease remain unclear. This bacterium is also the most common antecedent to the peripheral neuropathies Guillain-Barré syndrome (GBS) and Miller-Fisher syndrome (MFS).

The main aim of this project was to compare cytotoxicity of isolates of *C. jejuni* towards mammalian cells in culture, to purify any cytotoxic activity and to compare strains by plasmid and proteomic analysis.

Using the API Campy identification kit, 37 of the 39 strains were identified as *C. jejuni jejuni* serotype 1 or 2 with identification (ID) scores > 98.3%; and two strains were identified as *C. jejuni jejuni* 1 with ID scores of 88.3%. GGT (Gamma Glutamyl Transferase) test was the most important test to differentiate between *C. jejuni jejuni* 1 and 2 which showed negative and positive results, respectively.

The cytotoxic activity against Vero and Caco-2 cells of some of the typed strains of *C. jejuni* was tested. Cytotoxic activity was clearly demonstrated using Vero and Caco-2 cells and these activities peaked after incubation of the cell culture with sample for 16h. Comparison of wild-type strains 81-176 and 11168 with mutants of these strains lacking cytolethal distending toxin (CDT) or putative haemolysin or phospholipase toxins indicated that the toxicity observed was not due to any of these proteins, as cytotoxicity was unaffected by their absence. Some isolates (e.g. COL12) showed low cytotoxicity while others, especially *C. jejuni* 81-176, consistently

produced high cytotoxin activity which was heat-labile and lethal to tissue culture cells. Cytotoxicity comparisons of inner and outer membrane preparations with a cytoplasmic fraction of *C. jejuni* strain 81-176 and COL12 indicated that greatest activity was found in the inner membrane fraction. Proteins in the membrane preparation could not be easily fractionated and cell-free extracts of strains with low (COL12) and high (81-176) cytotoxic effects were chosen for further purification and characterisation studies. Although some differences were detected after DEAE-Sepharose fractionation and two-dimensional electrophoretic (2-DE) separation of proteins, no individual proteins could be clearly related to cytotoxicity. However, proteomic analysis did reveal some interesting properties of the *C. jejuni* strains. Of the proteins characterised by mass spectrometry after 2-DE, oxidoreductases were the most frequently identified in the virulent strain 81-176, but not in the type strain 11168.

There was no apparent correlation between toxin production and clinical symptoms in patients from whom the strains were isolated. The study indicated that not all *C. jejuni* strains tested produced cytotoxin (s), but that cytotoxin producers seemed to be predominantly human strains.

The presence of *virB11* and *tetO* genes carried by plasmids was examined by PCR in the 39 *C. jejuni* isolates together with strains 81-176 and NCTC 11168. Detection rates for the *virB11* and *tetO* genes in the clinical isolates were 28.2% and 12.82%, respectively.

Abbreviations

ACMSF	Advisory Committee on the Microbiological Safety of Food
AIFST	Australian Institute of Food Science and Technology
ATP	adenosine triphosphate
BA	blood agar
bp	nucleotide base pair
Caco-2	human colonic carcinoma line
CHO	Chinese hamster ovary
cm	centimetre
dH_2O	distilled water
DNA	deoxyribonucleic acid
ECACC	European Collection of Cell Cultures
EDTA	ethylenediamino tetra acetic acid
Fig.	figure
g	gram
G+C	guanine plus cytosine ratio
h	hour(s)
HaCat	human keratinocyte cell line
HeLa	Human Negroid cervix epitheloid carcinoma cell line
Hep-2	human tumour epithelial cell line
INT 407	human embryonic intestine cell line
IU	international unit(s)
kb	kilobase (1000 base pairs)
kDa	kiloDalton(s)
LB	Luria Bertani
L	Litre
M	molar concentration
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
mg	milligram(s)
$MgSO_4$	magnesium sulphate
min	minute(s)

ml	millilitre(s)
mM	millimolar concentration
MRC-5	human embryonic lung fibroblast cell line
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MWCO	molecular weight cut-off
µ	micro
µg	microgram(s)
µl	microlitre(s)
NaCl	sodium chloride
NaOH	sodium hydroxide
NCTC	National Collection of Type Cultures (UK)
Nmoles	nanomoles
°C	degrees Celsius
p	pico
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pmol	picomole(s)
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second(s)
SLT I	Shiga-like toxin type I (Verotoxin type 1)
SLT II	Shiga-like toxin type II (Verotoxin type 2)
spp.	species
subsp.	sub-species
Taq	*Thermus aquaticus*
TBE	Tris-borate buffer
TE	Tris-EDTA buffer
TEMED	*N,N,N,N*-tetramethylethylenediamine
Tris	Tris-hydroxy-methylamino-methylamine
UV	ultra violet light
V	volt(s)

Vero	African green monkey kidney cell line
VT1	Verotoxin type 1 (Shiga-like toxin type I)
VT2	Verotoxin type 2 (Shiga-like toxin type II)
v/v	volume for volume
w/v	weight for volume
wt	wild ype
%	percent

Chapter one

Introduction

CHAPTER 1: INTRODUCTION

Since the 1970s, *Campylobacter jejuni* and related species have been known to be causative agents of human enterocolitis (Butzler *et al.*, 1973; Dekeyser *et al.*, 1972; Skirrow, 1977). The most common cause of bacterial diarrhoea in many industrialized countries is *C. jejuni* infection, which is consequently responsible for a major public health and economic burden (ACMSF, 1993; Tauxe, 1992), although, the level of public awareness remains limited. Since 1981, in England and Wales, the number of laboratory reports of intestinal infection due to *Campylobacter* spp. has been greater than those due to other enteric pathogens, including the more news-worthy *Salmonella.* The incidence is also rising and this probably reflects both a change in eating patterns, e.g., an increase in the popularity and consumption of chicken, and an increased awareness of *Campylobacter* enteritis by the public health services.

The peak isolation rate of *Campylobacter* spp. in England and Wales is in babies <4 months of age with a second major peak in young adults. Below the age of 45 years, there is an as yet unexplained but consistently higher incidence of campylobacter infection in males than in females; after 45 years the rates become similar (Ketley, 1997). The consumption of undercooked poultry and cross-contamination of other foods with uncooked meat products are leading risk factors for campylobacteriosis in humans. Reinforcing hygienic practices in the food chain, from producer to consumer, is critical in preventing the disease (Fields & Swerdlow, 1999). In spite of campylobacter's importance, effective control of this organism in the food chain and the design of disease prevention strategies are hindered by a poor understanding of the physiology, virulence and genetics of this organism (Parkhill *et al.*, 2000).

1.1 Classification of *C. jejuni*

There has been considerable confusion in the naming of the genus, species and sub-species within this bacterial group of spiral organisms because of the relatively few phenotypic analyses to distinguish them. Some organisms previously known as *Campylobacter* have been placed in the genera *Helicobacter* or *Arcobacter*, since the 9th edition of Bergey's manual (AIFST, 1997).

Bacterial classification prior to 1963 was predominantly based on growth requirements, cell morphology and immunological and biochemical tests (On, 2005). Sebald and Veron (1963) addressed the confused taxonomy of *Vibrio*, and applied Hugh and Leifson's oxidation fermentation test for fermentative metabolism and the G + C ratio in genomic DNA to show that *V. fetus* and *V. bubulus* were notably different from other *Vibrio* species. Therefore a new genus -*Campylobacter*- was proposed to include these taxa. The other microaerobic and/or anaerobic '*Vibrio*' taxa (in addition to '*V. faecalis*' (Firehammer, 1965)) were reclassified as *Campylobacter* spp. in a more comprehensive taxonomic study which used various serological and biochemical tests, and the G + C ratio in DNA (On, 2005). The most dramatic changes in taxonomy of *Campylobacter* took place over the next two decades following the seminal study of Veron and Chatelain (1973).

According to Bergey's Manual of Systematic Bacteriology (Brenner *et al.*, 2005), the families of *Campylobacteraceae* and *Helicobacteraceae* are classified in the order *Campylobacterales*. Three genera, *Campylobacter*, *Arcobacter* and *Sulfurospirillum*, are grouped into the family *Campylobacteraceae*, and the genera *Helicobacter*, *Thiovulum* and *Wolinella* belong to the family *Helicobacteraceae*.

Some characteristic features of the genera *Campylobacter*, *Helicobacter* and *Arcobacter* are shown in Table 1.1.

The genus *Campylobacter* contains 16 species (three may be divided into defined subspecies), six subspecies and several validly named biovars (On, 2005). Distinguishing characteristics are given in Table 1.2 and phylogenetic relationships inferred by comparisons of 16S rRNA gene sequences are shown in Fig 1.1. Although *Campylobacter* includes 16 species, only *C. jejuni* and *C. coli* are of major importance in human medicine. Other species, such as *C. upsaliensis* and *C. lari* (*C. laridis*), have been isolated from patients with diarrhoea, but they are much less common and of unproven pathogenicity.

C. jejuni includes two subspecies (*C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*) which differ substantially in their distribution and to some extent ecology (On, 2005). *C. jejuni* subsp. *jejuni* is often simply referred to as *C. jejuni* and represents the taxon first described by Jones *et al.* (1931) as '*Vibrio jejuni*' from bovine intestine. Since the 1970s, *C. jejuni* has been widely known as the most commonly isolated bacterial cause of human gasteroentritis the world over (Skirrow, 1994). A wide range of other complications including neuropathic disorders and septicaemia may also occur (Skirrow & Blaser, 2000). *C. jejuni* subsp. *jejuni* usually occurs as a commensal in a wide range of animals including chickens, cattle, sheeps, dogs, and ostriches (Skirrow, 1994); its prevalence in food animals make it one of the most important food-borne pathogens today (On, 2005).

C. jejuni subsp. *doylei* (Steele & Owen, 1988) has been found in cases of gastritis, enteritis and septicaemia (Lastovica & Skirrow, 2000). No animal host has been found for this species (On, 2005). Also, this organism fails to grow at 42°C or reduce

Table 1.1: Characteristics for differentiating *Campylobacter, Helicobacter* and *Arcobacter*

Organism	Cell width(μm)	Position of flagella	Flagella sheaths	Nitrate reduction	Growth on 0.5% glycine	Hydrolysis of urea	Aerobic growth	Growth			G+C content (mol%)
								15 °C	30 °C	42 °C	
Campylobacter	0.2-0.5	Polar	Absent	+	V	−	−	−	+	V	30-46
Helicobacter	0.5-1.0	Polar and lateral	Present	V	+	V	−	−	V	V	35-44
Arcobacter	0.2-0.9	Polar	Absent	+	NA	V	+	+	+	−	28-31

+, 90% or more of strains positive; −, 90% or more of strains negative; V, 11%–89% of strains positive; NA, results not available.

Modified from AIFST (1997) and Koneman *et al.* (1994).

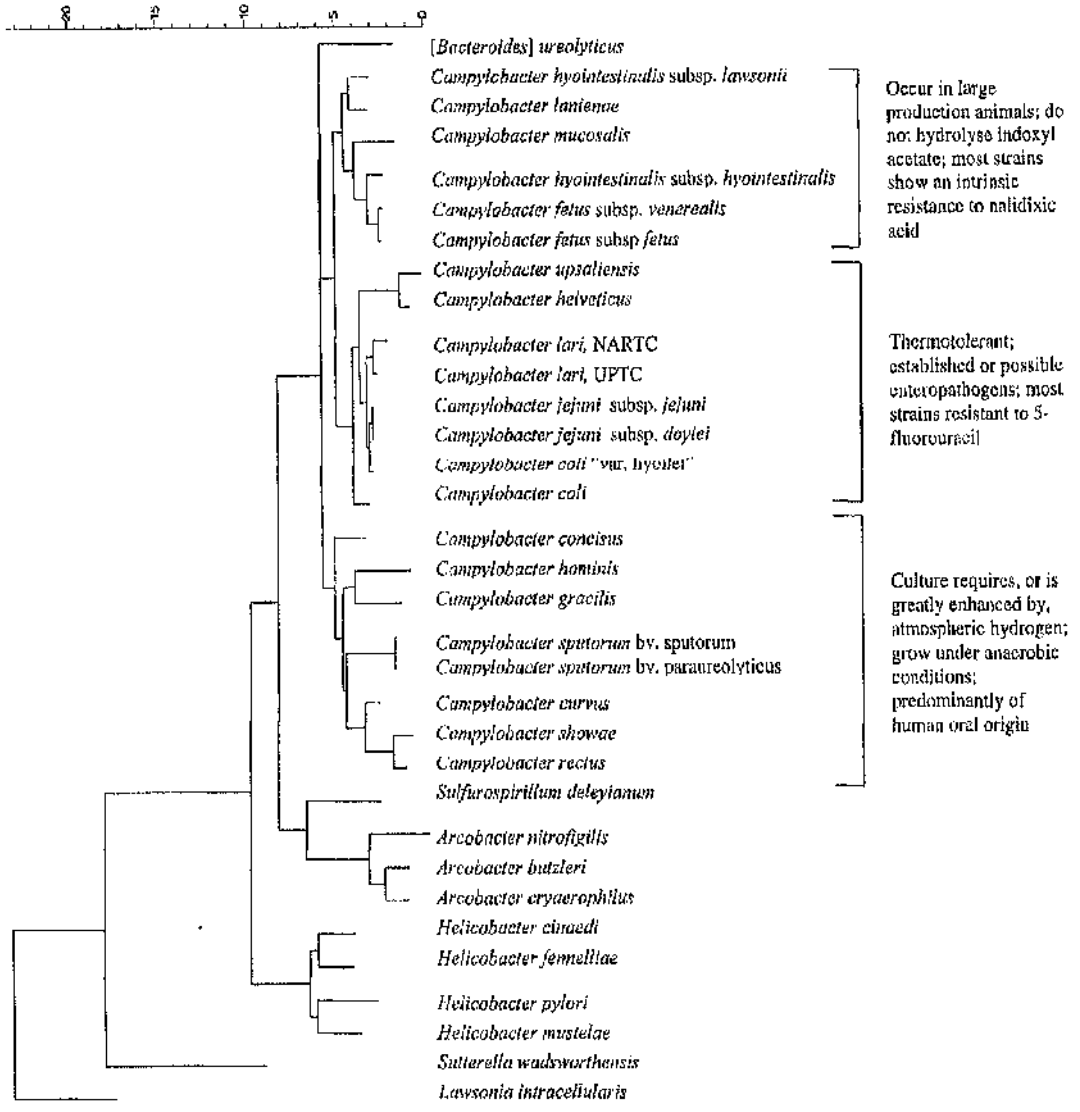
Table 1.2: Phenotypic differential test results for *Campylobacter* spp. and the misclassified *B. ureolyticus*. All values describe the percentage isolates that gave a positive result in the test concerned (On, 2005).

	Oxidase	Catalase	Urease	Indoxyl acetate hydrolysis	Hippurate hydrolysis	Nitrate reduction	Selenite reduction	H_2S/TSI production	Growth at 42 °C	Growth on minimal medium	Nalidixic acid resistance	Growth on 1% glycine medium
C. coli	100	100	0	100	0	100	100	50[2]	100	100	0	94
C. concisus	57	0	0	0	0	14	24	5	76	0	71	24
C. curvus	100	0	0	60	20	100	0	20	60	80	100	100
C. fetus subsp. fetus	100	100	0	0	0	100	94	0	70	71	94	100
C. fetus subsp. *veneralis*	100	93	0	0	0	100	21	0	0	90	69	7
C. gracilis	0	29	0	71	0	86	0	0	71	29	57	100
C. helveticus	100	0	0	100	0	100	0	0	100	0	0	44
C. hominis	100	0	0	0	0	100	0	0	0	0	20	100
C. hyointestinalis subsp. *hyointestinalis*	100	100	0	0	0	100	100	71[2]	100	65	100	100
C. hyointestinalis subsp. *lawsonii*	100	100	0	0	0	100	100	89[3]	100	66	100	22
C. jejuni subsp. *doylei*	100	70	0	100	100	0	0	0	0	0	0	20
C. jejuni subsp. *jejuni*	100	100	0	100	91	100	73	0	100	10	0	91
C. lanienae	100	100	0	0	0	100	100	0	100	0	80	80
C. lari	100	100	64[1]	7	0	100	50	0	100	0	29[2]	100
C. mucosalis	100	0	0	0	0	10	10	100[4]	100	0	80	50
C. rectus	100	20	0	100	0	100	0	0	20	0	80	100
C. showae	50	100	0	50	0	100	0	50[2]	50	50	0	50
C. sputorum	100	Biovar dependent*	Biovar dependent*	0	0	97	45	100[3]	97	NR	82	100
C. upsaliensis	100	0	0	100	0	100	100	0	89	0	0	100
B. ureolyticus	100	20	100	10	0	100	0	0	60	30	0	100

H_2S, hydrogen sulphide; TSI, triple sugar iron agar; *Catalase- and urease-negative strains, b.v. sputorum; catalase-positive strains, bv. Fecalis; urease-positive strains, bv. Paraureolyticus. 1, Urease-positive strains are referred to as the urease-positive thermophilic *Campylobacter* (UPTC) group. 2, weak reaction. 3, Strong reaction in most (87-100%) strains that give a positive test result. 4, Strong reaction in a few (20%) of strains that give a positive test result. NR, test not reproducible.

Figure 1.1: Phylogeny of *Campylobacter* species and related bacteria as inferred by comparison of 16S rRNA gene sequences.

All of the species shown are, or have previously been described as, a *Campylobacter*. The scale bar indicates 0-25% sequence dissimilarity (On, 2005).


[Bacteroides] ureolyticus
Campylobacter hyointestinalis subsp. lawsonii
Campylobacter lanienae
Campylobacter mucosalis
Campylobacter hyointestinalis subsp. hyointestinalis
Campylobacter fetus subsp. venerealis
Campylobacter fetus subsp fetus
Campylobacter upsaliensis
Campylobacter helveticus
Campylobacter lari, NARTC
Campylobacter lari, UPTC
Campylobacter jejuni subsp. jejuni
Campylobacter jejuni subsp. doylei
Campylobacter coli "var. hyoilei"
Campylobacter coli
Campylobacter concisus
Campylobacter hominis
Campylobacter gracilis
Campylobacter sputorum bv. sputorum
Campylobacter sputorum bv. paraureolyticus
Campylobacter curvus
Campylobacter showae
Campylobacter rectus
Sulfurospirillum deleyianum
Arcobacter nitrofigilis
Arcobacter butzleri
Arcobacter cryaerophilus
Helicobacter cinaedi
Helicobacter fennelliae
Helicobacter pylori
Helicobacter mustelae
Sutterella wadsworthensis
Lawsonia intracellularis
Occur in large production animals; do not hydrolyse indoxyl acetate; most strains show an intrinsic resistance to nalidixic acid
Thermotolerant; established or possible enteropathogens; most strains resistant to 5-fluorouracil
Culture requires, or is greatly enhanced by, atmospheric hydrogen; grow under anaerobic conditions; predominantly of human oral origin

nitrate, characteristics that were instrumental in its initial recognition as a distinct subspecies (Steele *et al.*, 1985).

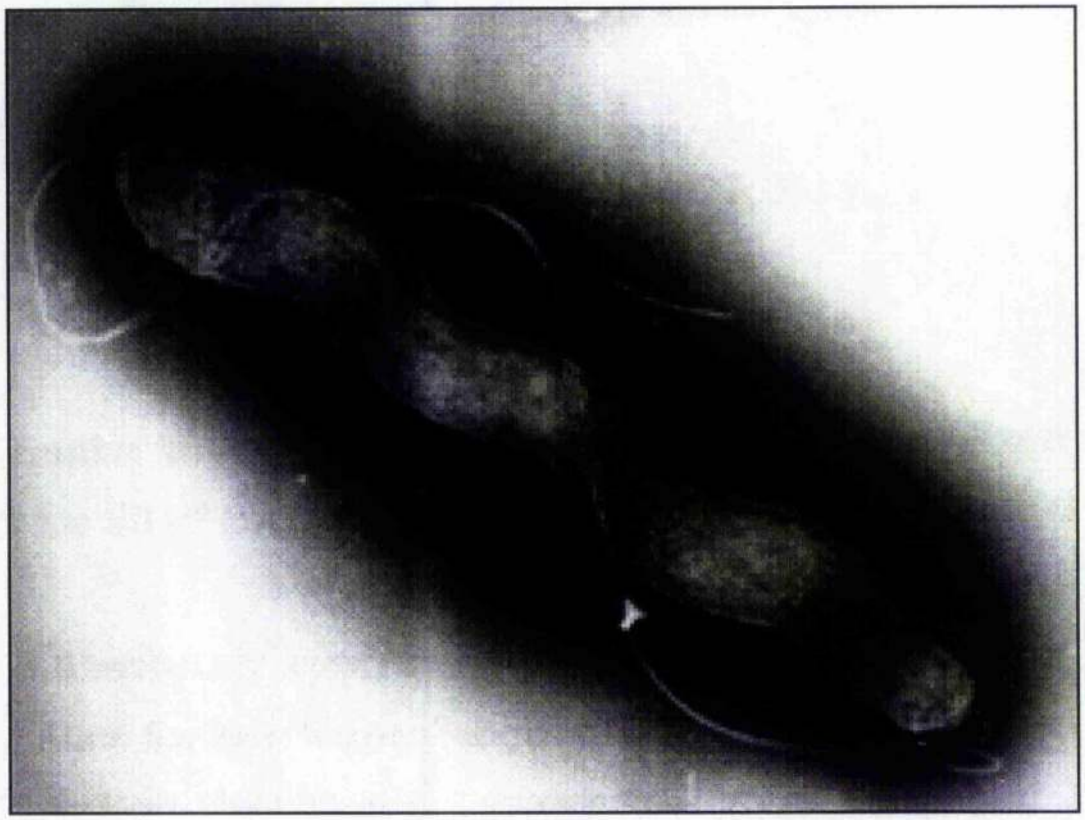
The majority of human infections are due to thermophilic campylobacters, principally *C. jejuni* subsp. *jejuni*, *C. coli* and *C. lari* (Tauxe, 1992). *C. jejuni* accounts for approximately 90% of campylobacter infections, and *C. coli* for most of the remainder (Gillespie *et al.*, 2002). *C. jejuni* and *C. coli* are closely related bacterial species which cause gasteroenteritis worldwide (Dingle *et al.*, 2005). Other species of campylobacteria may also be human pathogens (Edmonds *et al.*, 1987; Fennell *et al.*, 1986; Hsueh *et al.*, 1997; Lerner *et al.*, 1994; Lindblom *et al.*, 1995; Mishu *et al.*, 1992; On *et al.*, 1995; Tee *et al.*, 1988; Vandamme *et al.*, 1992).

1.2 Characterisation of *C. jejuni*

C. jejuni is a Gram-negative, s-shaped rod (0.2-0.8 μm wide and 0.5-5 μm long), non spore-forming, catalase, oxidase and hippurate hydrolysis positive, non saccharolytic (Griffiths & Park, 1990; Ottosson & Stenstrom, 2003; Vandamme, 2000), motile with a characteristic corkscrew-like motion via an unsheated flagellum at one or both poles (Fig. 1.2) and occurs as a commensal in warm-blooded animals, especially poultry (Fields & Swerdlow, 1999; Song *et al.*, 2004). *C. jejuni* may have one or more spirals and two rods forming a short chain often appear in a characteristic S or gull-winged shape. *C. jejuni* is exceptionally sensitive to oxygen and superoxides, yet for growth some oxygen is essential. A strictly microaerophilic environment must be provided for *in vitro* cultivation, because they are microaerophilic (require decreased O_2) and capnophilic (require increased CO_2). 42-43 °C is the best

Figure 1.2: Normal morphological appearance of *C. jejuni*.

Image was taken from: http://www.sanger.ac.uk/Projects/C_jejuni/



temperature for growth (Koneman *et al.*, 1994). It is sensitive to nalidixic acid and resistant to cephalothin. *C. jejuni* is non-fermentative and non-oxidative in its metabolism and derives energy from the consumption of amino acids and four and six carbon Krebs cycle intermediates.

C. jejuni strain 11168 has a circular chromosome of 1,641,481 base pairs (bp) (30.6% G+C) which is predicted to encode 1,654 proteins and 54 stable RNA species (Parkhill et al., 2000). The genome contains two large regions of lower G+C content that encompass CDSs (CoDing Sequences) Cj1135-Cj1148 (25.4%) and Cj1421-Cj1442 (26.5%); these correspond to genes within the lipooligosaccharide (LOS) and extracellular polysaccharide (EP) biosynthesis clusters, respectively. Functional information, including matches to genes of known function or informative hydrophobicity profiles, could be deduced for 77.8% of the 1,654 CDSs, whereas 13.5% CDSs matched genes of unknown function in the database and 8.7% had no database match (Parkhill *et al.*, 2000).

1.3 Clinical aspects

C. jejuni is the most important human pathogen among the campylobacters. This bacterium has worldwide distribution, being recovered from 4% to 35% of faecal specimens of patients with acute diarrhoea. In many practice settings, the rate of recovery of *C. jejuni* exceeds the combined recovery of the enteric pathogens *Salmonella* and *Shigella* species. It is also ubiquitous in domestic animals, house pets and the vast majority of chickens and waterfowl are colonized (Koneman *et al.*, 1994). Ingestion of raw milk, partially cooked poultry, or contaminated water are the usual sources for human infections (Koneman *et al.*, 1994). The incidence of *Campylobacter* infection, as derived from laboratory isolations, is almost certainly a

significant underestimate of the true rate of campylobacteriosis in the general population. This situation reflects under-reporting due to the fact that not all diagnostic laboratories routinely screen and not all physicians request stool cultures for *Campylobacter* spp. (Ketley, 1997).

The clinical spectrum of enteric disease due to *C. jejuni* ranges from a severe inflammatory diarrhoea to generally mild, non-inflammatory, watery diarrhoea (Butzler & Skirrow, 1979; Walker *et al.*, 1986). *C. jejuni* infection that results in inflammatory disease usually begins, in about 50% of patients, with a characteristic acute abdominal pain that is often associates with general malaise and fever, before the symptoms progress to include a profuse diarrhoea that becomes watery. The diarrhoeal stool often contains mucus, fresh blood and an inflammatory exudate with leucocytes (Ketley, 1997). Bacteraemia was observed among a small proportion of patients with the inflammatory condition (Blaser *et al.*, 1986; Mandal, 1984) especially in the early stages of infection. It is probably more common than suspected, but is rarely reported because of infrequent sampling and inappropriate culture conditions. The incubation period prior to the appearance of symptoms usually ranges from 1 to 7 days, although determination of the exact timing and source of infection is often difficult. The acute diarrhoea commonly lasts for 2-3 days but discomfort and abdominal pain persist sometime after the diarrhoea stops. Mucosal changes are usually revealed, ranging from hyperaemia with petechial haemorrhages and oedema to mucosal friability observed by sigmoidoscopy. Inflammation of some areas of the ileum and jejunum with mesenteric adenitis is usually seen in the infection. *C. jejuni* may be isolated from patients for several weeks after the clinical symptoms. Although *Campylobacter* infection can cause a severe illness lasting more than a week, it is usually self-limiting and complications

are uncommon (Skirrow & Blaser, 1992). Diarrhoeal and enteritis syndromes remain the most common manifestation of *Campylobacter* infection, although meningitis, septic arthritis and proctocolitis secondary to *C. jejuni* have been reported (Quinn *et al.*, 1984).

The manifestations of disease are different geographically. In developed countries, in the young adult population the infection is characterised by an inflammatory process (Blaser *et al.*, 1983b; Blaser *et al.*, 1979) and in developing countries, in the first two years of life most cases are likely to present with a profuse watery diarrhoea (Blaser *et al.*, 1983a; Glass *et al.*, 1983). The emergence of antimicrobial-resistance in general and, in particular, of fluoroquinolone-resistant *C. jejuni* infections in the United States and Europe, associated with the use of fluoroquinolones in veterinary medicine, is an important public health concern (Fields & Swerdlow, 1999). Gasteroenteritis by *C. jejuni* is primarily self-limiting and is usually treated by supportive therapy (fluid and electrolyte replacement) (Skirrow & Blaser, 2000). The drug of choice for treating severe clinical infection due to *C. jejuni* is erythromycin (Nachamkin *et al.*, 2000) and the prevelence of resistance to this drug has remained low (Nachamkin *et al.*, 2002).

1.4 Epidemiology

Human disease due to food-borne pathogens remains a medical problem worldwide (Schlundt *et al.*, 2004). Infections caused by ingestion of *C. jejuni* are one of the main sources of severe gasteroentritis in humans and also there is recent evidence that infection by this microorganism may be linked to neurobiological disorders such as Guillain-Barré syndrome (GBS). *C. jejuni* survives in a wide range of environments, including water, milk, and meat products. Under some conditions the

bacteria are exposed to a variety of stresses which they must be able to tolerate in order to permit their transmission to host environments suitable for growth. The association of *Campylobacter* with poultry (e.g. chickens, turkeys, ducks, and geese) as reservoirs has been known for over 30 years (Smith & Muldoon, 1974; Winkenwerder, 1967). Contamination of poultry meat by *C. jejuni* during processing has been reported (Berrang *et al.*, 2001; Chuma *et al.*, 1997; Chuma *et al.*, 1994; Hartnett *et al.*, 2001; Kazwala *et al.*, 1990). In recent years, the number of documented human food-borne outbreaks from raw fruits, vegetables and unpasteurized fruit juices has increased (Buck *et al.*, 2003). *C. jejuni* has been isolated from green onions, potato, mushroom, pepper, lettuce, spinach and parsley (Buck *et al.*, 2003).

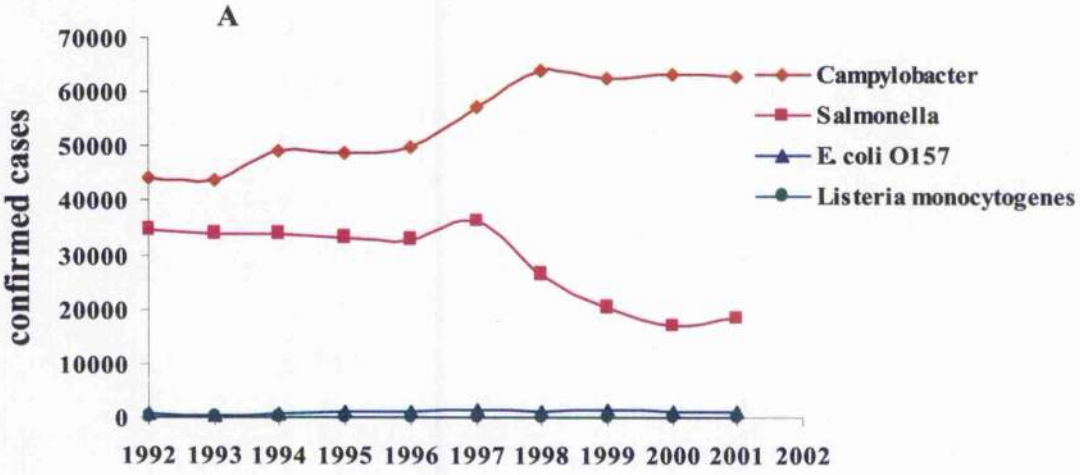
C. jejuni is a food-borne pathogen, thus resistance to physiological stresses in water and food are important for successful transmission and production of infection and, therefore, it is possible that campylobacters can enter a viable non-culturable (VNC) state and this may have a great significance (Jones *et al.*, 1991; Ketley, 1997; Rollins & Colwell, 1986). *C. jejuni* is unable to multiply at temperatures below 30°C, and is sensitive to various environmental stresses, including light, oxygen, heat, drying, high salt concentrations, and low pH values (Park, 2002).

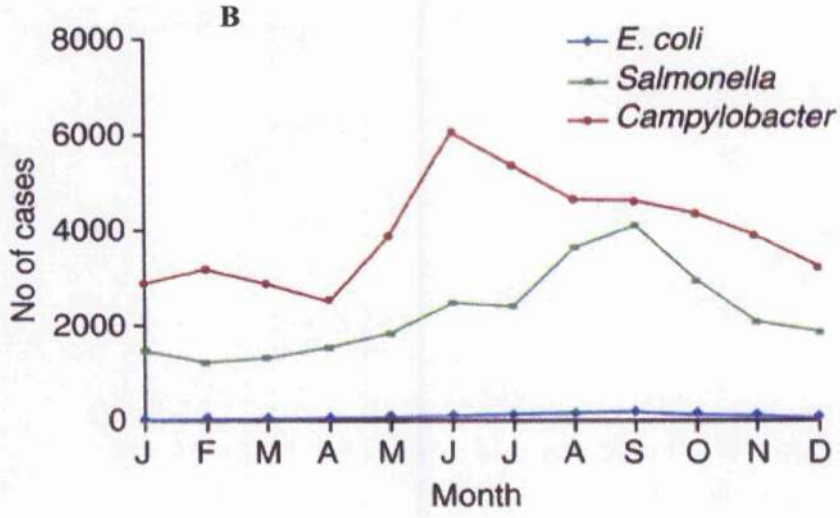
There are more than 60,000 notified cases of campylobacteriosis per annum in the United Kingdom (Fig. 1.3) of which 90% are caused by *C. jejuni*. A peak incidence of disease is seen in children under one year old and in young adults in developed

Figure 1.3:

A- United Kingdom laboratory confirmed cases of gastrointestinal infections between the years 1992-2001

B- Seasonal incidence of confirmed cases of *E. coli*, *Salmonella* and *Campylobacter* in England and Wales (average of 1996-1998) (Jones, 2001)


A
confirmed cases
70000
60000
50000
40000
30000
20000
10000
0
1992 1993 1994 1995 1996 1997 1998 1999 2000 2001 2002
Campylobacter
Salmonella
E. coli O157
Listeria monocytogenes


B
No of cases
8000
6000
4000
2000
0
J F M A M J J A S O N D
Month
E. coli
Salmonella
Campylobacter

countries (Blaser *et al.*, 1983b; MacDonald *et al.*, 1988; Tauxe, 1992). In developing countries, where the organisms are endemic, disease occurs in young children and persistent carriage occurs in adults without symptomatic disease (Calva *et al.*, 1988; Taylor *et al.*, 1988). This indicates that a high level of endemic disease causes the development of specific immune responses and less severe disease (Blaser *et al.*, 1986; Blaser *et al.*, 1985; Glass *et al.*, 1983). Following exposure, although recolonisation may occur, specific serum and secretory antibodies provide strain-specific immunity and protection against disease due to the homologus strain (Black *et al.*, 1985; Black *et al.*, 1988).

1.5 Pathogenesis

Although *C. jejuni* was the first enteropathogen to have its complete genome sequence published (Parkhill *et al.*, 2000), the mechanisms by which it causes disease remain unclear. Several mechanisms may be involved in pathogenesis of infection (Smith, 1996). Possible virulence-associated factors are adherence, invasion, production of cytotoxins (Datta *et al.*, 2003) and motility (Florin & Antillon, 1992; Wassenaar, 1997). To cause gasteroenteritis, *C. jejuni* must adhere to, invade and destroy host cells; thus they must produce adhesion and invasion factors and toxins (Smith, 1996). van Vliet & Ketley (2001) indicated that when colonizing the intestines, campylobacters are predicted to express several virulence factors include motility and chemotaxis, adhesion and invasion, secretion of toxins and lipopolysaccharide (LPS), iron acquisition, oxidative stress defence, and heat shock response which are summerized in Fig. 1.4. Recently, some genes have been

Figure 1.4: The different stages of *Campylobacter* invasion of the intestine.

1, motility

2, chemotaxis

3, oxidative stress defence

4, adhesion

5, invasion

6, toxin production

7, iron acquisition

8, temperature stress response

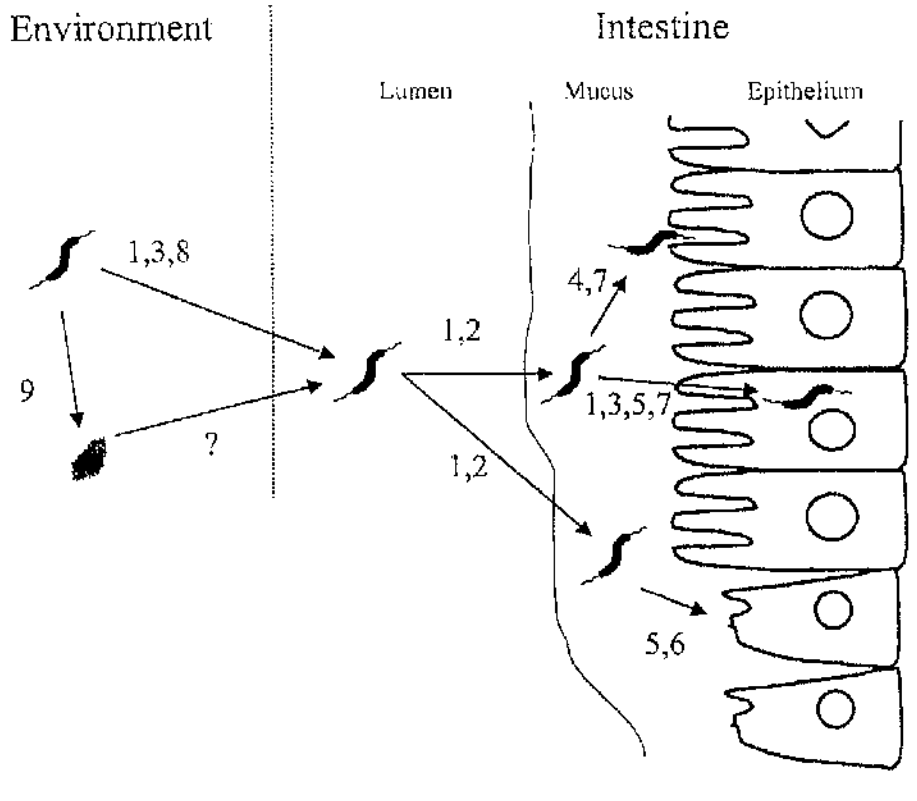
9, coccoid dormant stage

 , viable *Campylobacter* cell

 , coccoid dormant *Campylobacter* cell

 , epithelial cell

Diagram was taken from van Vliet & Ketley (2001).


Environment
Intestine
Lumen
Mucus
Epithelium
1,3,8
9
?
1,2
1,2
4,7
1,3,5,7
5,6

implicated in *C. jejuni* pathogenicity: *flaA* (Nuijten *et al.*, 2000), *dnaJ* (Ziprin *et al.*, 2001), *cadF* (Ziprin *et al.*, 2001), and *racR* (Bras *et al.*, 1999), for the expression of adherence and colonization, *virB11* (Bacon *et al.*, 2000), *pldA* (Ziprin *et al.*, 2001) and *ciaB* (Konkel *et al.*, 1999a; Rivera-Amill *et al.*, 2001) for the expression of invasion, *wlaN* (Linton *et al.*, 2000b) for the expression of Guillain-Barré syndrome and *cdtA*, *cdtB* and *cdtC* as genes responsible for the expression of cytolethal distending toxin (Lara-Tejero & Galan, 2001; Purdy *et al.*, 2000).

1.5.1 Motility and chemotaxis

C. jejuni requires motility and chemotaxis to colonize the intestinal tracts of humans and animals (Black *et al.*, 1988; Pavlovskis *et al.*, 1991) and for invasion *in vitro* (Grant *et al.*, 1993; Wassenaar *et al.*, 1991; Yao *et al.*, 1994). Campylobacters enter the stomach by the host consuming food or water, surviving the acid barrier, and then colonising the distal ileum and colon. This stage needs the production of the flagellum which is the best characterised virulence determinant of *Campylobacter* spp. Experiments with genetically undefined mutants of *C. jejuni* showed that this virulence determinant was required for adhesion and colonisation in a range of animals (Aguero-Rosenfeld *et al.*, 1990; Caldwell *et al.*, 1985; McSweegan & Walker, 1986; Morooka *et al.*, 1985; Newell *et al.*, 1985). The flagellin gene was cloned and characterised in *C. jejuni* and *C. coli* strains (Guerry *et al.*, 1992; Nuijten *et al.*, 1992). The locus consists of two genes: *flaA* and *flaB*, which encode proteins with predicted molecular weights of 59.5 and 59.9 kDa, respectively. It was shown in a genetic study that *flaA* and not *flaB* is essential for colonization (Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1991). Experiments with defined mutants showed that *flaA* was not required for adhesion but was essential for the invasion and transcytosis of

intestinal epithelial cells (Grant *et al.*, 1993; Wassenaar *et al.*, 1991) and the flagellum was necessary for the colonisation of chicks (Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1993). Due to the unusual ability of *C. jejuni* to remain highly motile in mucus, it has been suggested that adhesion to host cells may not actually be necessary as the *C. jejuni* cell is able to remain in the intestine by colonising the mucus blanket overlying the epithelium successfully. *C. jejuni* transcytose through the host cell (Everest *et al.*, 1992; Harvey *et al.*, 1999; Konkel *et al.*, 1992b), to emerge on the basolateral surface, allowing access to the lamina properia (MacCallum *et al.*, 2005a). Influx of neutrophils occurs at the site of bacterial invasion (basolateral surface) through interleukin-8 (IL8) release (Hickey *et al.*, 1999; 2000) from infected cells (enterocytes) by activation of NF-κB (Mellits *et al.*, 2002). Secretion of cytokines and other mediators are involved in this natural immune response leading to an inflammatory response (MacCallum *et al.*, 2005a).

It is possible that the flagella structure may secrete virulence determinants in the absence of another type III secretion system (Parkhill *et al.*, 2000; Rivera-Amill *et al.*, 2001).

1.5.2 Cell adherence and invasion

Humans are infected by *C. jejuni*, like other human enteric bacterial pathogens, by colonisation of the mucosal layer of the intestine followed by adherence and invasion of epithelial cells (Everest *et al.*, 1992; Harvey *et al.*, 1999; Ketley, 1997; Konkel *et al.*, 1992b; Konkel *et al.*, 2001; Kopecko *et al.*, 2001).

Up until recently, few of the determinants involved in pathogenesis were well characterized and some of them were rather controversial (Ketley, 1997). In

volunteer studies, infection has been established by ingestion of as few as 500 bacteria. The first sites to become colonized are the jejunum and ileum, although the infection extends distally to affect the terminal ileum and usually the colon and rectum. It has been suggested that ability to bind to epithelial or to other cells is an essential step to produce disease by *C. jejuni* as well as for a number of other intestinal bacterial pathogens. After colonization of the mucus membrane and adhesion to intestinal cell surfaces, the bacteria affect the normal absorptive capacity of the intestine by damaging epithelial cell function directly or indirectly, directly by cell invasion or the production of toxin(s), and indirectly pursuing the initiation of an inflammatory response. These are possible mechanisms that are not equally exclusive, and any combination may have a role depending on characteristics of the infecting strain and the host condition (Ketley, 1997).

C. jejuni is certainly able to adhere to tissue culture cells without subsequent invasion (Everest *et al.*, 1992), although it can be assumed that adhesion to the epithelial cell surface is essential for subsequent invasion of the cell. Although the production of fimbriae by campylobacters has not been observed, a number of proteins such as outer-membrane proteins (DeMelo & Pechere, 1990; Fauchere *et al.*, 1992; Kervella *et al.*, 1993), LPS (McSweegan & Walker, 1986) and a protein known as PEB1 (Pei & Blaser, 1993) have been suggested as potential bacterial adhesins to bind eukaryotic cells; however, the specific role of these proteins in the binding capacity to host cells of *Campylobacter* is unknown. PEB1 was shown to bind to cells and the gene encoding this potential adhesin has been cloned (Pei & Blaser, 1993). The predicted protein has homology to amino-acid transporter systems of other bacteria. It is not clear whether the cell binding of PEB1 is an artifact or

whether the protein may have the dual function of both amino-acid transport and intestinal cell binding (Ketley, 1995).

The only protein that has been shown to be involved in facilitating the binding of *Campylobacter* to fibronectin is CadF (*Campylobacter* adhesin to fibronectin), which aids in the binding of *C. jejuni* and *C. coli* to intestinal epithelial cells (Konkel *et al.*, 1999b). The involvement of the *cadF* gene product in *Campylobacter* colonization has been shown by *in vivo* colonization using a chicken model (Ziprin *et al.*, 1999).

The importance of host cell invasion in pathogenic mechanisms of campylobacter were strongly suggested by inflammation and occasional bacteraemia (Ketley, 1995). Invasion of host cells in both experimentally-infected infant macaque monkeys (Russell *et al.*, 1993) and in the colon of patients (van Spreeuwel *et al.*, 1985) has been observed.

Caco-2 cells (human colonic carcinoma line) which differentiate to form polarised enterocyte-like microvilliated monolayers, have been used to study adhesion and invasion of *C. jejuni* (Everest *et al.*, 1992). Caco-2 cells have similar properties to colonic enterocytes, as they possess brush borders with microvilli and maintain tight junctions, therefore they exibit the properties of a polarized cell line (Delie & Rubas, 1997). Two clinical groups of patients with either watery (non-inflammatory) diarrhoea or colitis were sources of the *C. jejuni* strains tested (Ketley, 1995). Although the strains adhered to Caco-2 cells, there was a significant correlation between the inability to invade the cells and isolation of the *C. jejuni* strain from patients with non-inflammatory disease. However, some strains classified as "non-inflammatory" were able to invade Caco-2 cells. These strains may have been

isolated from patients suffering from colitis but inflammatory cells were missed or absent in the stool at the time of diagnosis. Alternatively, progress of *Campylobacter* infection is critically influenced by host factors like a strong immune response. The range of symptoms in patients may be to some extent a reflection of strain differences which was suggested by isolation of non-invasive strains from patients with non-inflammatory diarrhoea. In addition, a role for invasion in pathogenesis is supported by a correlation between the ability to invade the cells and to cause colitis (Ketley, 1995).

Caco-2 cells have been used as a model of intestinal epithelium for other studies on the interaction of *C. jejuni* with host cells (Everest *et al.*, 1992; Harvey *et al.*, 1999; Konkel *et al.*, 1992b). MacCallum *et al.* (2005b) suggested that *C. jejuni* inhibits absorption in infected Caco-2 cells. They suggested that, fluid and electrolyte absorption are likely to be compromised, if tight junctional integrity of intestinal epithelium was lost.

Although invasion of intestinal epithelial cells is suggested to play a role in *Campylobacter* virulence, there is a very great range in the *in vitro* invasion level among strains of *C. jejuni* (Bacon *et al.*, 2000; Hu & Kopecko, 1999; Oelschlaeger *et al.*, 1993). *C. jejuni* has been reported to enter host cells *via* a microfilament-dependent process (De Melo *et al.*, 1989; Konkel & Joens, 1989; Konkel *et al.*, 1992a), and *in vivo* observations suggested that cell entry is associated with accumulation of microfilament (Russell *et al.*, 1993). In contrast, other studies (Oelschlaeger *et al.*, 1993; Russell & Blake, 1994) indicated that invasion was not affected by microfilament inhibitors. Campylobacters, after entering the host cells, remain in the cytoplasm within membrane-bound vacuoles, although free *C. jejuni*

bacteria associated with a cytopathic effect were observed *in vivo* within the cytoplasm of cells (Russell *et al.*, 1993). The flagellum is the only *Campylobacter* determinant which is consistently involved in the process of invasion (Grant *et al.*, 1993; Russell & Blake, 1994; Wassenaar *et al.*, 1991).

The ability of *Campylobacter* spp. to translocate across an epithelial cell barrier was observed (Everest *et al.*, 1992; Grant *et al.*, 1993; Konkel *et al.*, 1992b); and such translocation was first observed in salmonellae (Finlay & Falkow, 1990; Finlay *et al.*, 1988). This action has great potential significance for the pathophysiology of campylobacteriosis. Translocated *Campylobacter* were observed below the cell monolayer less than 1h after inoculation of cells above the monolayer (Konkel *et al.*, 1992b) and at least for 6h continued to translocate. Transcytosis (i.e., translocation *via* a cytoplasmic pathway) may be the means to enable campylobacters to cross the cell monolayer following initial invasion of the cell with the exit at the basolateral surface. Nevertheless, these bacteria may also be able to cross the cell monolayer *via* a paracellular path. The passage of campylobacters between cells was indicated by electron microscopic observations (Konkel *et al.*, 1992b; Oelschlaeger *et al.*, 1993), and four different and reproducible phenotypes amongst clinical strains were identified: a. non-invasion; b. invasion; c. invasion with transcytosis; and d. transcytosis without invasion (Everest *et al.*, 1992). It would be predicted that the strains in the group d take a paracellular route between tight junctions.

1.5.3 Cytotoxins

Although tissue invasion by *campylobacter* and inflammatory changes could be responsible for the clinical manifestations of disease, toxins also probably contribute as well to the disease process. Toxin production is, however, an unclear area of

pathogenesis (Ketley, 1997). Lipopolysaccharide (LPS) is a virulence determinant in many species of Gram-negative bacteria and it has been shown to contribute to several aspects of the pathogenesis, including resistance to phagocytic killing, serum resistance and cell toxicity. Sialylation of LPS by enhancing serum resistance was shown to play a role in virulence in some pathogens (e.g., *Haemophilus* and *Neisseria* spp.) (Demarco de Hormaeche *et al.*, 1991; Moxon & Maskell, 1992). The biochemical characterisation of *C. jejuni* LPS has been progressed by several groups (Aspinall *et al.*, 1992a; 1993a; 1993b; 1992b; 1993c; Conard & Galanos, 1990; Mills *et al.*, 1992; Moran *et al.*, 1991) although most of them were concerned with the utilisation of LPS in serotyping. *C. jejuni* LPS can consist of a low mol. wt fraction (similar to the characteristic LPS in *Haemophilus* and *Neisseria* spp.) or it may also contain a high mol. wt fraction (Mills *et al.*, 1992). The role of LPS in the virulence of other Gram-negative bacteria gives a good foundation for the search for such a role in *C. jejuni* (Ketley, 1995). McSweegan & Walker (1986) reported a role for both LPS and flagellin as important adhesins in *C. jejuni*.

The major outer-membrane protein (MOMP) of *C. jejuni* is a porin which is present in 60% of pathogenic strains (Bolla *et al.*, 1995; Huyer, 1986; Kervella *et al.*, 1992). A cytotoxic porin-lipopolysaccharide (LPS) complex was isolated and characterised by Bacon *et al.* (1999). The cytotoxic activity associated with this complex was heat labile at 70 °C and trypsin-resistant and this activity was neutralised by a polyclonal, homologous antiserum which reacted on a Westen blot with a 45-kDa protein (predicted iso-electric point of 4.35), but not by polyclonal antisera raised against a number of other bacterial toxins. Sequencing of the N-terminus of the protein

component of this complex revealed that there was 97% homology with the major outer-membrane porin protein from *C. jejuni* (Bacon *et al.*, 1999).

Campylobacters have been reported to produce various toxic activities including a cholera-like toxin (CLT) and several cytotoxins, including cytotoxins with different patterns of cell specificity, a cytolethal distending toxin (CDT), a shiga-like toxin (SLT) and a haemolysin (Ketley, 1997; Wassenaar, 1997). Parkhill *et al.* (2000) reported that the genome of *C. jejuni* (NCTC 11168) does not contain a cholera-like toxin gene, although genes encoding a cytolethal distending toxin (*cdtA, B* and *C*) are present. A phospholipase (*pldA*), a putative integral membrane protein with a haemolysin domain (Cj0183), and a member of the family of contact-dependent haemolysins found in pathogenic *Brachyspira* (*Serpulina*) and *Mycobacterium* species (Cj0588) were also identified (Parkhill *et al.*, 2000). The cytotoxins may cause enteric cell damage which leads to an inflammatory diarrhoea together with bloody stools and leucocytes in the faeces (Wallace *et al.*, 1997).

Production of enterotoxin by *C. jejuni* was first described by Ruiz-Palacios *et al.* (1983). The evidence for a role and even the production of enterotoxin by campylobacter is not convincing. A toxin that elongated CHO cells like cholera toxin was detected with a GM1-based ELISA, and several groups described fluid accumulation in intestinal loops (McCardell *et al.*, 1986b; Ruiz-Palacios *et al.*, 1985; Ruiz-Palacios *et al.*, 1983; Suzuki *et al.*, 1994). The production of CLT was reported to correlate with the watery diarrhoea observed in developing countries (Guerrant *et al.*, 1992), but CLT-positive strains have been isolated from non-symptomatic carriers (Belbouri & Megraud, 1988; Mathan *et al.*, 1984). There are reports that, under low stringency hybridisation conditions, the *C. jejuni* chromosome contains

nucleotide sequence similarities with cholera toxin and heat-labile toxin (Baig *et al.*, 1986; Calva *et al.*, 1989). In contrast, even with similar strains, several other independent groups could not demonstrate the production of CLT, detect no antibody against it and could not demonstrate any DNA sequence similarity (Konkel, 1992; Mathan *et al.*, 1984; McFarland & Neill, 1992; Perez-Perez *et al.*, 1989; Perez-Perez, 1992; Ruiz-Palacios *et al.*, 1992). It is probable that the nature of toxin production by *campylobacter* spp. is complex and may involve a range of different toxins expressed under various, as yet unknown, conditions.

On the basis of a weighed comparison of the data, the existence of six different cytotoxins was proposed (Wassenaar, 1997): (a) a 70-kDa cytotoxin active on Hela, CHO, and other cells except Vero cells (inactive on Vero cells); (b) a cytotoxin active on Vero and Hela cells; (c) CDT; (d) a cytotoxin neutralized by Stx antitoxin; (e) a cytotoxin(s) displaying haemolytic activity; and (f) a hepatotoxin.

1.5.3.1 70-kDa cytotoxin

A toxic activity against HeLa, MRC-5 and HEp-2 cells but not against Vero or other animal cell lines was described in an early study on the cytotoxicity of bacterial supernatants (Yeen *et al.*, 1983). A molecular weight of more than 30,000 was noted for the cytotoxin, and bacterial culture broth produced diarrhoea in the RITARD (The Removable Intestinal Tie-Adult Rabbit Diarrhoea) model (Pang *et al.*, 1987). The cytotoxin was sensitive to heat, and expression was lost upon bacterial subculturing. Possibly a similar or identical cytotoxin was described by Goossens (1985) as a cytotoxin active on CHO cells which could not be neutralized by Stx (shiga toxins) antitoxin, although the sensitivity for Vero cells was not tested. A cytotoxin that was specific for HeLa and CHO cells and inactive on Vero cells was

detected by Johnson and Lior (1986), and they presumed that this was identical to the cytotoxin described by Goossens (1985). McCardell *et al.* (1986a) described a cytotoxin of 70-kDa that was toxic for CHO cells, although its trypsin resistance and heat stability were in contrast to other reports.

A cytotoxin for HeLa and CHO cells, but not for Vero cells was described by Guerrant *et al.* (1987). This cytotoxin, with 50 to 70-kDa MW estimation, was trypsin- and heat-sensitive and was not neutralized by anti-*Clostridium* or Stx antitoxin (Guerrant *et al.*, 1992; Guerrant *et al.*, 1987). In another study, Mahajan and Rodgers (1990) described a 68-kDa cytotoxin active against CHO and INT407 cells, trypsin sensitive and heat labile, and also they suggested a possible (glyco) protein receptor on chicken embryo fibroblast cells and INT407 cells. Other researchers (Daikoku *et al.*, 1989; Kawaguchi, 1989; Mizuno *et al.*, 1994) described a cytotoxin which was also active on HeLa and CHO cells, trypsin sensitive, heat labile and may be the same 70-kDa toxin, but Vero cell specificity tests or neutralization studies were not carried out to confirm this similarity. It has been reported upon storage (Daikoku *et al.*, 1989; Florin & Antillon, 1992) or subculturing (Daikoku *et al.*, 1989; Pang *et al.*, 1987) of the *Campylobacter* strains, the expression of this cytotoxin was unstable and was lost. The protein was produced intracellularly in the early log phase, and released in the stationary phase (Daikoku *et al.*, 1989; Daikoku *et al.*, 1990; Kawaguchi, 1989).

Briefly, characterisation of this cytotoxin revealed a M.W. of approximately 70 kDa, trypsin sensitivity, heat lability, activity against HeLa, CHO, HEp-2, and INT407 cells and no activity against Vero cells, and also toxic activity could not be

neutralized by anti-*Clostridium* antitoxin or anti-Stx. The mechanism of action (intracellular versus membranolytic) of this cytotoxin is unknown.

1.5.3.2 Vero/HeLa cell cytotoxin

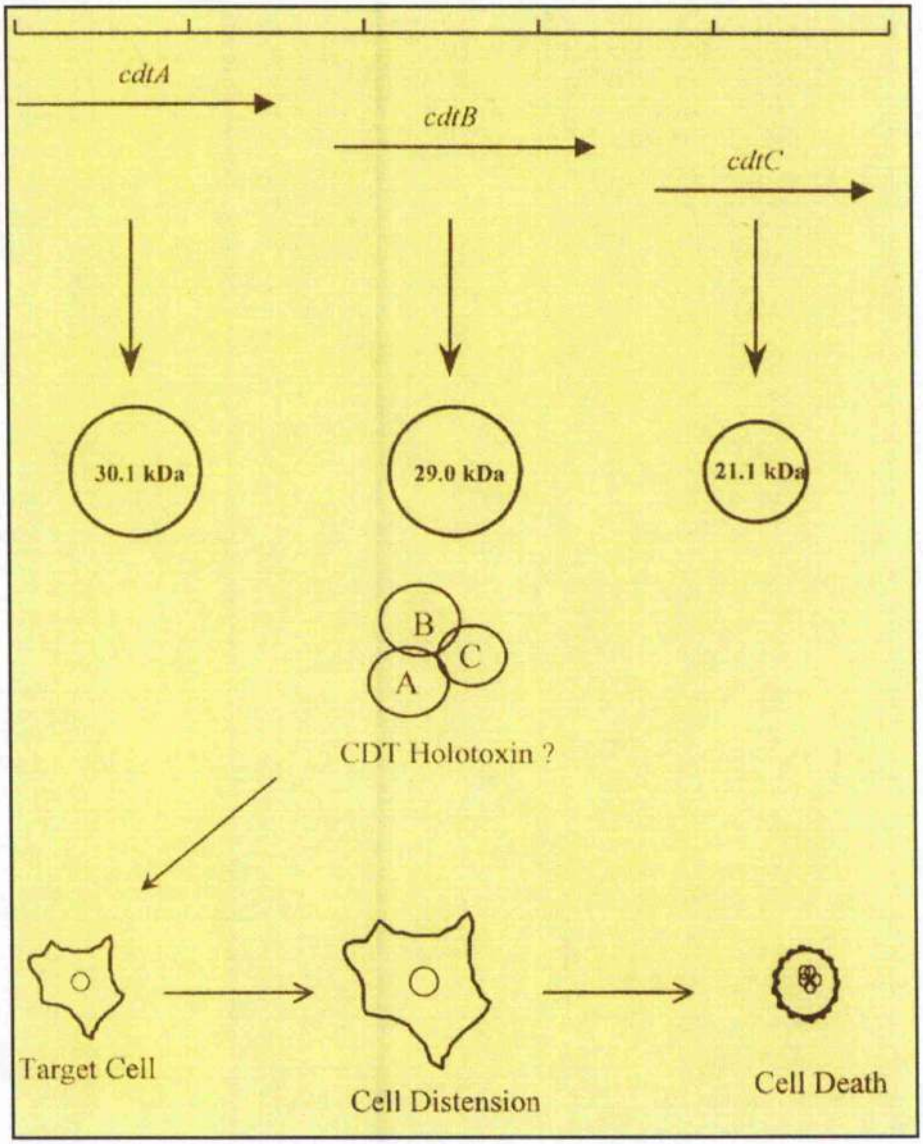
In most reports, Vero cells were found to be more susceptible and sensitive than HeLa cells (Florin & Antillon, 1992; Johnson & Lior, 1984; Johnson & Lior, 1986) against this cytotoxin and, in one study, MRC-5 cells were even more sensitive (Florin & Antillon, 1992). This cytotoxin could not be neutralizaed with anti-*Clostridium* or Stx antitoxin (Johnson & Lior, 1984; Johnson & Lior, 1986). The mechanism of action of this cytotoxin is unknown.

1.5.3.3 Cytolethal distending toxin

Cytolethal distending toxin (CDT) is perhaps the most widely studied cytotoxin and genes with similarity to those encoding *E. coli* CDT were identified in *C. jejuni* (Pickett *et al.*, 1996). CDT causes certain culture cells, including HeLa cells, to become slowly distended and finally to die (Fig. 1.5) (Johnson & Lior, 1987; Johnson & Lior, 1988a; Johnson & Lior, 1988b). CDT was first described by Johnson and Lior (1987) as a distinct and novel toxic entity. They discovered this new activity in certain *E. coli* strains which, during a five-day period, caused Chinese hamster ovary (CHO) cells to become distended. This activity was distinct from those of *E. coli* heat-stable toxin, heat-labile toxin, verotoxin and haemolysin (Johnson & Lior, 1988a; Pickett & Whitehouse, 1999). CDT production by *Campylobacter* spp was first described by Johnson and Lior (1988b) as CLDT. CDT's represent an emerging family that are produced by a number of pathogens, including *E. coli, Campylobacter spp., Shigella dysenteriae, Actinobacillus actinomycetemcomitans, Haemophilus ducreyi* (as a non-diarrhoeagenic pathogen)

Figure 1.5: Genetics and action of CDT

Three adjacent genes, *cdtA, cdtB* and *cdtC*, encode CDT. The arrows underneath the line show the location and length of the genes, and the molecular weight of the translate products. Many sensitive cells become slowly distended and then die by this holotoxin. Diagram was taken from Pickett (2000).


cdtA
cdtB
cdtC
30.1 kDa
29.0 kDa
21.1 kDa
B
C
A
CDT Holotoxin ?
Target Cell
Cell Distension
Cell Death

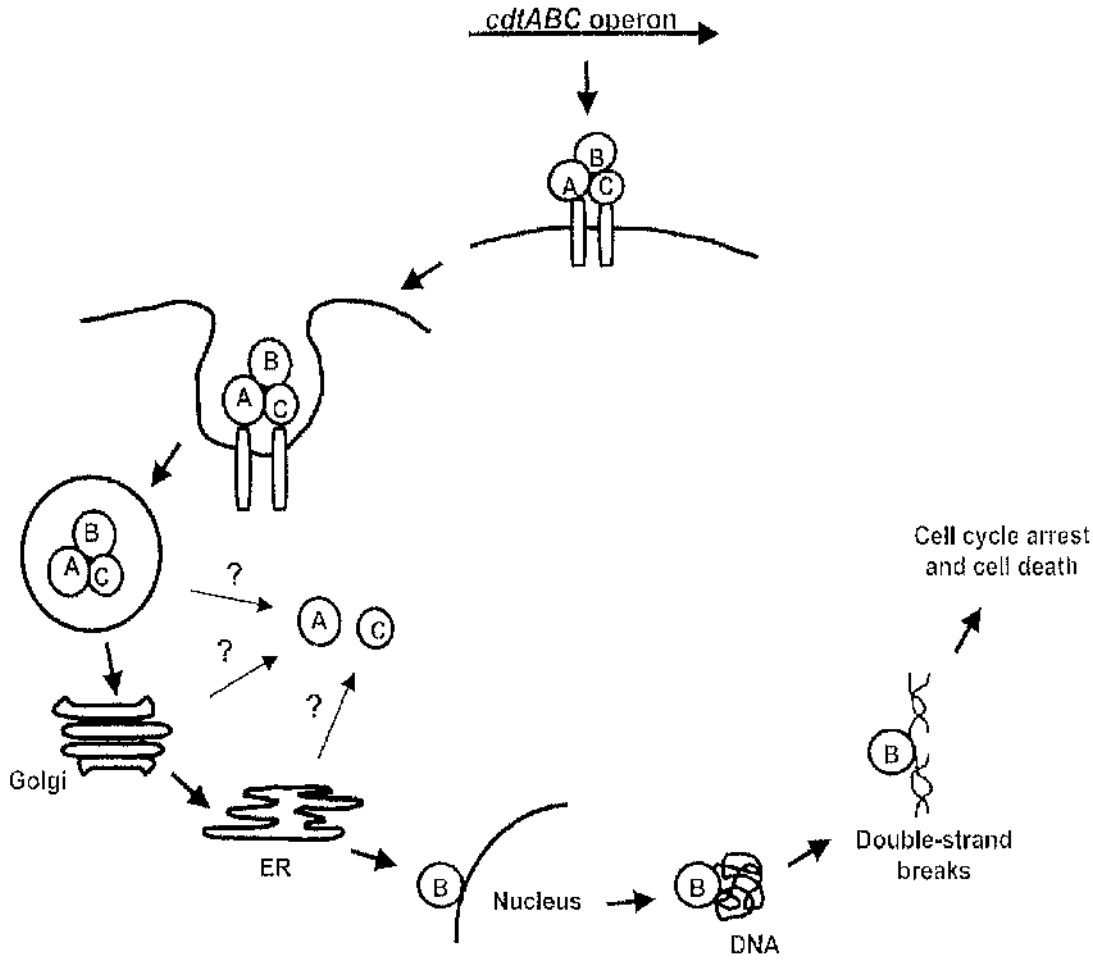
(Cope *et al.*, 1997; Elwell & Dreyfus, 2000) and *Helicobacter* spp. (Young *et al.*, 2000). Eyigor *et al.* (1999a) demonstrated the widespread presence of genes encoding CDT amongst members of the *Campylobacter* genus, showing that CDT may be intimately involved in successful colonisation processes and in the ability of the bacteria to cause disease in man (Purdy *et al.*, 2000). In addition to *C. jejuni*, other diarrhoeagenic bacterial species, including the closely related *C. coli* and *C. fetus* can make CDT (Johnson & Lior, 1988b; Pickett *et al.*, 1996), as well as some *E. coli* strains (Johnson & Lior, 1988a) and *Shigella* spp. (Johnson & Lior, 1987) isolates.

The CDT produced by *Shigella dysenteriae* causes diarrhoeal symptoms in a suckling mouse model (Okuda et al., 1997). CDTs are described as cytotoxins which induce progressive cellular distension and death in a number of cultured and primary cells, including HeLa, Vero, CHO, HEp-2, Caco-2, human keratinocyte cell line (HaCat), Hamster lung (Don) fibroblasts and human T lymphocytes (Elwell & Dreyfus, 2000). NIH 3T3 fibroblasts and mouse Y-1 adrenal cells are apparently not sensitive to CDT (Elwell & Dreyfus, 2000; Pickett & Whitehouse, 1999). Some cell culture types, including HEp-2, CHO (Elwell & Dreyfus, 2000; Pickett & Whitehouse, 1999) and hamster lung (Don) fibroblasts (Pickett & Whitehouse, 1999) rearrange actin in response to CDT, but this does not occur in HeLa cells treated with CDT (Elwell & Dreyfus, 2000).

The *cdt* genes were cloned and sequenced from *E. coli* (Peres *et al.*, 1997; Pickett *et al.*, 1994; Scott & Kaper, 1994), *C. jejuni* (Pickett *et al.*, 1996), *S. dysenteriae* (Okuda *et al.*, 1995), and *H. ducreyi* (Cope *et al.*, 1997). CDT production depends upon the expression of three adjacent genes, *cdtA*, *cdtB*, and *cdtC* (Fig. 1.5 and 1.6),

Figure 1.6: Suggested pattern for uptake, trafficking, and target activity of CDT.

The CDT operon consists of three adjacent genes, *cdtABC*, and the products of these three genes interact to form the CDT holotoxin. Binding of CDT to an unidentified host cell receptor is mediated by CdtA and CdtC, after which the toxin is internalized via receptor-mediated endocytosis (Cortes-Bratti *et al.*, 2001). CDT trafficking, and especially the movement of CdtB, is poorly described, although evidence suggests that the toxin undergoes retrograde transport through the Golgi complex, and that CdtB finally enters the nucleus. In the nucleus, CdtB seems to bind to DNA and causes double-stranded breaks, which lead to trigger the DNA damage checkpoint pathway and finally cell cycle arrest and cell death. The ultimate fate of CdtA and CdtC is unknown as is indicated by the question marks. Diagram was taken from Pickett & Lee (2005).


cdtABC operon
B
A
C
?
?
?
Golgi
ER
Nucleus
DNA
Double-strand breaks
Cell cycle arrest and cell death

and expression of the *cdt* genes in non-toxic *E. coli* strains showed that the three genes were sufficient and required in order to produce CDT (Okuda *et al.*, 1997; Pickett *et al.*, 1994; Pickett *et al.*, 1996). It has been shown that CdtB acts as a nuclease (Elwell & Dreyfus, 2000; Hassane *et al.*, 2001; Lara-Tejero & Galan, 2000), and it has been shown by two groups that CdtB apparently binds to DNA of the host cell and causes double-stranded cuts of the DNA (Fig. 1.6) (Hassane *et al.*, 2003; Li *et al.*, 2002). Recent evidence shows that all three Cdt subunits are present in the CDT holotoxin (Figs. 1.5 and 1.6), in a 1:1:1 stoichiometry (Lara-Tejero & Galan, 2001). About 40% of the sequences of *C. jejuni cdtA* and *cdtC* genes are similar to each other (Pickett & Whitehouse, 1999). CDT is an AB2 toxin composed of CdtB which plays a role as the enzymatically active (A) subunit, and CdtA and CdtC, as the heterodimeric B subunit which is required for the delivery of CdtB into the target cell (Lara-Tejero & Galan, 2001). Lara-Tejero & Galan (2001) reported that toxic activity was not exibited by purified CdtA (29,919.36 Da), CdtB (28,972.97 Da), or CdtC (21,157 Da) when applied to cells individually. However, when CdtA, CdtB, and CdtC were combined, they interacted with one another to form an active tripartite holotoxin. Mao & DiRienzo (2002) reported that the CdtA subunit binds to CHO cells, but they did not observe binding of CdtB and CdtC to these cells. Recently, it has been shown that both CdtA and CdtC bound with specificity to the surface of HeLa cells, but CdtB did not (Fig. 1.6) (Lee *et al.*, 2003). CDT arrests eukaryotic cells in the G2 phase of the cell cycle (Fig. 1.6) and induces interleukin-8 (IL-8) release from intestinal epithelial cells (Hickey *et al.*, 2000; Lara-Tejero & Galan, 2001; Pickett *et al.*, 1996; Whitehouse *et al.*, 1998).

AbuOun *et al.* (2005) challenged one-day old chicks with wild-type and a CDT-negative mutant of *C. jejuni* 81-176 and the same level of colonisation (up to 10^9

CFU per gram of cecal contents) was achieved. This finding suggested that the absence of CDT expression did not affect colonisation potential. In spite of this high level of colonisation, clinical symptoms of disease were not perceived in challenged chicks with either the wild-type or the mutant strain (Abuoun *et al.*, 2005). Also the results showed that CDT was expressed during colonisation of the avian gut (Abuoun *et al.*, 2005). Even though CDT was apparently expressed in the chick, its absence did not interfer with colonisation.

In one study, campylobacters were examined for cytotoxin formation in a CHO cell culture test. The reference strains were *C. jejuni* from persons suffering from diarrhoea, from organs of poultry, *C. jejuni* and *C. fetus* subsp. *fetus* from the gasterointestinal tract of calves and adult cattle. During evaluation, three morphologically different pictures were observed. In the first, there was a formation of rounded or polymorphic, strikingly large and elongated cells and this was associated with reduced growth. These progressive morphological changes corresponded to those described for the Cytolethal Distending Toxin (CDT). A second cytotoxin, that by analogy to CDT was termed Cytolethal Rounding Toxin (CRT), produced a rounding of cells without a change in their size while, at the same time, growth was reduced. A third morphological picture consisted of cell changes characterized by enlarged polymorphic cells as well as by small rounded cells. These cell changes were referred to as the CDT/CRT effect (Schulze *et al.*, 1998). Hanel *et al.*(1998) reported that *C. jejuni* strains were able to produce at least two different cytotoxins, corresponding to CDT and CRT.

1.5.3.4 Shiga-like toxin

Discovery of the Shiga toxins was due to a need to know more about how gram-negative pathogenic bacteria cause disease (Konowalchuk *et al.*, 1977; O'Brien *et al.*, 1992) . These toxins have been proven to be important in the pathogenicity of both *Shigella dysenteriae* type 1 and *E. coli* O157:H7. The term verotoxin (VT) was suggested, due to the observation that *E. coli*-derived toxins were cytotoxic to Vero cells. The terminology has been unified: Stx is from *Shigella dysenteriae* type 1, and Stx1, Stx2, etc. from *E. coli*. The type 1 and 2 toxins from *E. coli* were previously called Shiga-like toxins. The Shiga toxins are responsible for the systemic complications of infections and are not required for the initial colonization of the intestine (Obrig, 1997). Toxins in the Stx family are composed of a holotoxin consisting of one A-subunit (active) responsible for the inhibitory activity and five indentical B-subunits (A1B5) that determine specific binding to eukaryotic cells (Hoey *et al.*, 2003; Jackson, 1990; O'Brien *et al.*, 1992). Stx has been purified from *Shigella dysenteriae* type 1 as a 70 kDa multi-subunit complex comprised of a single 32 kDa A-subunit and 5×7.7 kDa B-subunits (O'Brien *et al.*, 1980; Olsnes *et al.*, 1981). Shiga-like toxins (Stx1, Stx2, Stx2c) have also been purified from *E.coli* and are similar to Shiga toxin (O'Brien *et al.*, 1992). In spite of minor differences between the A-subunit amino acid sequences of the Stx subspecies (Stx1, Stx2, Stx2c, etc.), all exibit identical enzymatic activity. The neutral glycolipid, glycosphingolipid (Gb3) which is present on the cell surface of eukaryotic cells is the receptor for Stx, Stx1, Stx2, and Stx2c (Lingwood, 1993; Lingwood *et al.*, 1987).

The A-subunit inhibits protein synthesis by inactivating ribosomes. Enzymatically a purine base from the 28S rRNA within the 60S ribosomal subunit is removed by the

A-subunit (Endo *et al.*, 1988). Interaction of peptide elongation factors (EF-1 and EF-2) with the ribosome is prevented by the modification of the ribosome and stops protein synthesis at the level of peptide elongation (Obrig, 1994; Obrig *et al.*, 1987).

It has been reported that Stx is cytotoxic to endothelial cells (Obrig *et al.*, 1988). Glomerular vascular capillaries with swollen endothelial cells were revealed by a histopathological test of kidney samples from HUS (haemolytic uraemic syndrome) patients (Koster *et al.*, 1984; Richardson *et al.*, 1988). In all patients diagnosed with HUS and having an infection with *E. coli* (Stx producing), microvascular angiopathy was present. It has been concluded that the putative primary target cells for Stx are endothelial cells (Obrig, 1997). Boyd & Lingwood (1989) demonstrated that human kidney, particularly in young children, contains the Gb3 receptor for Shiga toxin. In another study, endothelial cells isolated from human kidney expressed Gb3 and were very sensitive to Shiga toxin (Obrig *et al.*, 1993). van Setten (1994) demonstrated that murine macrophages directly respond to Stx1 with an increase in production of IL-1 and IL-6.

Due to presence of Gb3 receptor, significant targets for VT are microvascular endothelial cells of the kidney and gastrointestinal tract (Jacewicz *et al.*, 1999; Obrig *et al.*, 1993; Ohmi *et al.*, 1998), which correlate to HUS and haemorrhagic colitis (HC), respectively (Hoey *et al.*, 2003). It has been reported that VT can be detected in HUS patients in the kidney (Chaisri *et al.*, 2001; Uchida *et al.*, 1999), thus considerable levels of this cytotoxin may enter the circulation from the intestinal lumen (Hoey *et al.*, 2003).

Moore *et al.* (1988) detected a toxin activity in 11 of 36 bacterial lysates of *C. jejuni* and *C. coli* strains. This activity cross-reacted with anti-shiga toxin antibodies, but

homology between the stx_1 gene of *E. coli* and the genome of cytotoxic *C. jejuni* strains was not detected (Moore *et al.*, 1988). The putative cell-associated SLT was produced in very low levels equivalent, in fact, to that found in enterotoxigenic *E. coli*, *Vibrio cholerae*, *E. coli* K12 and normal intestinal flora *E. coli* strains (Moore *et al.*, 1988). No SLT gene sequences were found and there was no correlation between production and the clinical background of the strain. Nevertheless, the clinical manifestation, which often involves intestinal tissue damage and an associated inflammatory response, is not inconsistent with the action of cytotoxins (Ketley, 1995).

1.5.3.5 Haemolytic cytotoxins

It is recognized that some *Campylobacter* strains (i.e., *C. jejuni* and *C. coli*) are haemolytic. Haemolysis was described on blood agar plates that were cultivated at 42 °C for 4 days (Arimi *et al.*, 1990). The haemolytic activity was observed only with ageing bacteria, and there is a suggestion that an intracellular component is released when bacteria die and lyse. This suggestion and most other observations describe cell-associated or contact haemolysis.

Alpha haemolysis was rarely observed and its appearance depends on the pH of the medium. The typical green zone around a colony appears within incubation for 48h at 37 °C on rabbit blood agar plates at pH 6.0 to 6.5, which is lower than that of most *Campylobacter* media (Misawa *et al.*, 1995b). Clear zones on blood plates were produced by beta haemolysis, and it was observed with the same strains that produced alpha haemolysis, but only after incubation for 6 days at 37 °C or 3 days at 42 °C. The zone did not spread but remained in close proximity to the colonies (Misawa *et al.*, 1995b).

To perform a contact haemolysin assay, a bacterial suspension was mixed with erythrocytes and was centrifuged to increase the contact between bacteria and cells, so that cell-bound haemolysins could be detected. Haemolytic activity was shown in six of eight *C. jejuni* strains by this assay (Pickett *et al.*, 1992). A more sensitive microplate assay was developed to measure contact haemolysis, which showed moderate titers in some strains and low titers in 94% of *C. jejuni* strains (Tay *et al.*, 1995). Bacterial lysates or culture supernates were always non haemolytic (Tay *et al.*, 1995).

C. jejuni strains were tested for their ability to acquire iron from different iron sources present in humans. The growth of *C. jejuni* strains was stimulated by haemoglobin, haemin, haemoglobin-haptoglobin and haemin-haemopexin in low-iron medium. Lactoferrin, transferrin and ferrin were unable to provide iron to the strains (81-176, C31, and 1376) tested. The haemolytic activity shown to be produced by several *C. jejuni* strains did not appear to be iron regulated (Pickett *et al.*, 1992).

Parkhill *et al.* (2000) identified a member (Cj0588) of the family of contact-dependent haemolysins found in pathogenic *Serpulina* and *Mycobacterium* species, and a putative integral membrane protein with a haemolysin domain (Cj0183), in the genome of *C. jejuni* NCTC 11168.

McCardell *et al.* (1986a) reported secretion of haemolytic activity into the culture medium. A cytotoxin which was toxic to CHO cells and could be neutralized by the anti-haemolysin/cytolysin antitoxin of *V. cholerae*, displayed haemolytic activity. 27 of 60 *C. jejuni* and *C. coli* were positive for the haemolytic phenotype and could be induced in non haemolytic strains by rabbit ligated ileal loop passage (McCardell *et*

al., 1986a). Hossain *et al.* (1993) reported presence of both heat-stable and heat-labile haemolytic activities in culture filtrates of *C. jejuni* isolated from patients with cholera-like and dysenteric diarrhoea, but neutralization with anti-cytolysin of *V. cholerae* was not performed (Hossain *et al.*, 1993). The haemolytic activity of two strains was trypsin sensitive and precipitation by ammonium sulphate indicated its proteinaceous nature. The haemolysins of these two strains differed in sensitivity to temperature, so that one culture filtrate was partially resistant to treatment for 30 min at 100 °C, and the other filtrate was inactivated (Hossain *et al.*, 1993).

More work on haemolytic cytotoxin production by *C. jejuni* is needed. With specific antisera, it could be determined how many different haemolytic cytotoxins exist. Whether haemolytic, membrane-damaging cytotoxins are represented by one or more of the other toxins produced by *Campylobacter* spp. is unknown.

1.5.3.6 Hepatotoxin

Kita *et al.* (1990) reported that some clinical isolates of *C. jejuni* that caused bloody and watery diarrhoea and one strain isolated from an asymptomatic patient could cause hepatitis in mice. A bacterial sonicate fraction was isolated from such a *C. jejuni* strain that caused toxicity to hepatocytes but not to Y1 or CHO cells (Kita *et al.*, 1992; Kita *et al.*, 1990). When the crude sonicate fraction (but not culture filtrates) was applied intravenously to mice, liver lesions similar to the lesions seen after liver colonization by hepatotoxic *C. jejuni* strains were caused (Kita *et al.*, 1992). These reports suggest that some strains have the potential to colonize the liver and express a hepatotoxin to cause hepatitis, although the mechanism of this factor is unknown.

It is probable that the nature of toxin production by *Campylobacter* spp is complex and involves a range of different toxins expressed under a variety of conditions *in vivo*, which are as yet unknown.

1.5.4 Guillain-Barré syndrome (GBS)

Recently, an association between *Campylobacter* infection and GBS has been provided and campylobacteriosis is the most frequent infection associated with this form of neuromuscular paralysis (Fields & Swerdlow, 1999; Parkhill *et al.*, 2000). Unusually, *C. jejuni* has three sets of *neu* genes involved in sialic acid biosynthesis. Sialic acid is an uncommon constituent of bacterial surface structures and, through molecular mimicry, may be important for evasion of host immunity and in post-infection autoimmune disases such as GBS (Parkhill *et al.*, 2000).

Molecular mimicry of gangliosides by lipo-oligosaccharide is in part dependent on the presence of *N*-acetylneuraminic acid of *C. jejuni* (sialic acid) in both structures. Within the lipo-oligosaccharide biosynthetic locus, one group of genes (*neuA*, *neuB* and *neuC*) potentially encode a complete pathway for the biosynthesis of activated *N*-acetylneuraminic acid from *N*-acetylmannosamine, and insertional mutagenesis of the *neuB* gene resulted in a lipo-oligosaccharide lacking *N*-acetylneuraminic acid (Linton *et al.*, 2000a). The biosynthesis of ganglioside-mimicking lipo-oligosaccharide also requires a number of glycosyltransferases, including one or more sialyltransferases. The *C. jejuni* NCTC 11168 genome sequence data was used by Gilbert *et al.* (2000) to clone and sequence the lipo-oligosaccharide biosynthetic locus from a Guillain-Barré syndrome-associated *C. jejuni* strain (OH4384) known to synthesise ganglioside-mimicking lipo-oligosaccharide. Within this locus, they identified three genes encoding sugar transferases, including a sialyltransferase,

involved in biosynthesis of ganglioside-mimicking lipo-oligosaccharide (Gilbert *et al.*, 2000).

1.6 Current knowledge on genetics

Genome sequence data for *C. jejuni* are only available for a single strain, 11168, and this may not be representative of the species as a whole. Several studies have indicated that extensive genetic diversity exists between strains (Dorrell *et al.*, 2001; Leonard *et al.*, 2004; Salama *et al.*, 2000). It would therefore be of considerable interest to have available the genome sequences of a variety of isolates for comparative purposes with regard to the different pathogenic phenotypes exhibited by strains.

In spite of years of research and a complete genome sequence (Parkhill *et al.*, 2000), still there is remarkably little knowledge about the molecular pathogenesis of *C. jejuni*. The genome sequence failed to reveal any specific secretion systems (type III or type IV) that are involved in pathogenesis of many other bacteria (Parkhill *et al.*, 2000). Manifestation of *C. jejuni* disease can range from a mild watery diarrhoea to a dysenteric-like syndrome. This range of differences could be due to either differences in host response to bacteria or to differences in virulence amongst individual strains, or a combination of both. It is obvious that there are great differences amongst *C. jejuni* strains with regard to their ability to promote disease symptoms.

1.6.1 Plasmids

C. jejuni 81-176 is a virulent clinical isolate that has become the predominant model in the study of infection mechanisms of *C. jejuni*. It was originally isolated in 1985 from an outbreak of diarrhoeal disease in children who consumed raw milk during a visit to a dairy farm (Korlath *et al.*, 1985). Strain 81-176 has been chosen for most laboratory work due to the fact that it has been fed to volunteers on two occasions and in each case caused disease (Black *et al.*, 1988). The strain invaded intestinal epithelial cells at much higher levels than most other *C. jejuni* strains (Oelschlaeger *et al.*, 1993) and caused disease in a ferret diarrhoea model but strain NCTC 11168, for which the genome was sequenced, was avirulent (Bacon *et al.*, 2000). Moreover strain NCTC 11168 invaded INT407 cells at < 1% the level of 81-176 (Bacon *et al.*, 2000). Thus, these important and significant differences in virulence phenotypes must have a genetic basis. Dorrell *et al.* (2001) reported that about 5% of the genome of NCTC 11168 is missing in 81-176 using microarray hybridizations. However, the most clear genomic difference between these two strains is the presence of two large plasmids (pVir and pTet) in 81-176.

Until plasmid pVir in strain 81-176 was identified by Bacon *et al.* (2000), there was no evidence for the involvement of plasmids in the virulence of *C. jejuni*. pVir is an ~37.5 kb plasmid which contains components of a type IV secretion system (T4SS) (Bacon *et al.*, 2000; 2002) known to be important for virulence in a number of major bacterial pathogens (Christie, 2001). Bacon *et al.* (2000) suggested that this plasmid is important *in vitro* for invasion and adherence of intestinal epithelial cells in culture.

Tracz *et al.* (2005) reported that although the presence of the pVir virulence plasmid did not correlate with most clinical symptoms (diarrhoea, fever, abdominal pain, and vomiting), patients infected with pVir-positive *C. jejuni* strains were more likely to produce bloody stool than those infected with pVir-negative strains. 53% of patients infected with pVir-positive *C. jejuni* strains had bloody stools, as opposed to 21% of patients infected with pVir-negative *C. jejuni* strains. Also, the presence of pVir in isolates was not associated with the patient's sex, age, antimicrobial therapy, coexisting conditions, or travel (Tracz *et al.*, 2005). *C. jejuni* gasteroentritis with bloody stool indicated that progression of the infection into the tissue of the colon and rectum had occurred (Skirrow & Blaser, 2000). Bacon *et al.* (2000; 2002) reported that pVir was important for the *in vitro* invasion of intestinal epithelial cell lines. The association of pVir in *C. jejuni* infection with bloody diarrhoea in a clinical setting supported the role of this plasmid for *in vivo* epithelial cell invasion and stressed its potential as a marker for the risk of developing a more severe clinical infection (Tracz *et al.*, 2005).

Tetracycline resistance in *C. jejuni* is primarily mediated by plasmids which carry the *tetO* gene (Taylor *et al.*, 1981; 1987). Bacon *et al.* (2000) reported the presence of a 35 kb plasmid (pTet) that encoded tetracycline resistance. Another study reported size of this plasmid 45.2 kb (Batchelor *et al.*, 2004). Tracz *et al.* (2005) reported that the presence of pVir plasmid in *C. jejuni* isolates was associated with the presence of the tetracycline-resistance plasmid (94% of pVir-positive *C. jejuni* isolates were found positive for the presence of the tetracycline-resistance plasmid). However, the results also showed that 66% of *C. jejuni* isolates that contained tetracycline-resistance plasmid did not contain pVir, which demonstrated that plasmid-mediated tetracycline resistance did not occur exclusively with pVir.

1.7 Proteomics

The large and growing application of 2-dimensional electrophoresis (2-DE) is important for "proteome analysis". Proteome analysis is "the analysis of the entire PROTEin complement expressed by a genOME". Proteomics (Wasinger *et al.*, 1995) has become a key technology and also a method of choice for the investigation of expressed gene products. It has applications in the analysis of protein expression changes during cellular processes such as cell differentiation, exposure to adverse environments, host/pathogen interactions and the characterisation and validation of novel therapeutic targets with the investigation of the mode-of-action of drugs (Jungblut *et al.*, 1999). Proteomic techniques have evolved from established protein separation techniques such as 2-DE to include both the rapid identification of polypeptides by mass spectrometry (MS) and the development of bioinformatic techniques developed to correlate enzymatic peptide digest mass fingerprints and peptide fragmentation data with the genome sequence (Parker *et al.*, 1998). The combination of 2-DE along with mass spectrometry makes possible the identification of proteins on 2-DE patterns on a large scale (Shevchenko *et al.*, 1996).

Two-dimensional electrophoresis was first introduced by O'Farrell (1975) and is a widely used and powerful method for the analysis of complex protein mixtures extracted from tissues, cells, or other biological samples. Information on individual proteins such as pI and molecular mass, the number of expressed proteins and their level of expression may be obtained from analysis of gels. 2-DE of proteins allows 10,000 protein species to be separated in one electrophoretic run (Klose & Kobalz, 1995).

Iron homeostasis and the role of the ferric uptake regulator (Fur) in iron acquisition in *C. jejuni* was investigated by a proteomic and transcriptome analysis (Holmes *et al.*, 2005). A proteomic analysis of wild-type and *fur* mutant of *C. jejuni* strain 11168 was done by comparing 2-DE gels of cell protein extracts from bacteria grown in iron-rich and iron-limited conditions. 595 spots on the iron-limited gel and 699 spots on the iron-rich gel (496 protein spots were common to both conditions) were identified by 2-DE gel analysis. The results showed that 30 genes were transcribed at lower levels in the *fur* mutant than in the wild-type strain under iron-rich conditions. Several genes containing perR/Fur box consensus sequences, such as those coding for bacterioferritin (*bfrldps*, Cj1534c), glutamyl-tRNA reductase (*hemA*, Cj0542), thioredoxin reductase (*trxB*, Cj0146c), cytochrome c peroxidase (*ccp*, Cj0358), superoxide dismutase (*sodB*, Cj0169) and the peroxide stress regulator *perR* (Cj0322) might be potentially derepressed in a *fur* mutant (Holmes *et al.*, 2005). The proteomics data supported the microarray data by showing that the major protein spots induced under iron limitation were also components of known iron-uptake systems and components of two iron-regulated ABC transporter systems. In total, 13 different proteins were shown to be regulated at the level of transcription in response to iron stress.

A comparative proteomic analysis was done on three strains (26695, SS1 and J99) of *H. pylori* (a bacterium related to *C. jejuni*) on 2-DE gels (Jungblut *et al.*, 2000). The comparison of the three strains revealed a high genetic variability. Whereas several main spots were found at the same position, many positionally shifted and differentially present or absent spots were observed. 1863 spots were detected on the pattern of *H. pylori* 26695 of which 152 spots were identified by peptide mass fingerprinting using MALDI-MS. The 152 identified proteins spots represented 126

genes which were 8% of the total number of 1590 gene predicted from the genome of *H. pylori* (Jungblut *et al.*, 2000).

Aims of the research

Four major objectives were to be realised: Firstly, development of a reproducible cytotoxicity assay to evaluate and measure cytotoxic activity in different cell fractions of a range of *C. jejuni* strains. Secondly, to screen the isolates of *C. jejuni* from different sources for the presence of virulence plasmids (pVir and pTet) and to seek any correlation of plasmid presence with cytotoxic activity. Thirdly, purification and characterization of cytotoxic activity using column chromatography. Finally, a comparison, by proteomic analysis, of toxin-producing and non-producing *C. jejuni* strains.

Chapter Two

Materials and Methods

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

2.1.1 Bacterial strains

Campylobacter jejuni strains 11168, 11168 *cdtB*, 11168 *cdtA*, 11168 *tlyA*, 11168 *pldA* mutants, 81-176, 81-176 *cdtB* mutant, and clinical isolates strains (COL1-30 and RPH1-10) were used in this study (Table 2.1). *C. jejuni cdtB* mutant strains of 11168 and 81-176 (kindly provided by Dr. D. Purdy, CAMR, Porton Down, UK) were created by insertion of a chloramphenicol resistance (Cm^r) cassette in the *cdtB* gene. 11168 *cdtA*, 11168 *tlyA*, 11168 *pldA* mutants (kindly provided by Dr. P. Everest, Veterinary School, Glasgow University, UK) were created by insertion of a kanamycin resistance (Km^r) cassette in *cdtA*, *tlyA* and *pldA* genes, respectively. *C. jejuni* 81-176 was kindly provided by Dr. D. Newell (Veterinary Laboratory Agency, Weybridge, Addlestone, Surrey, UK), COL1-30 clinical isolates of *C. jejuni* were from Dr. R. Owen, Health Protection Agency (HPA), Colindale, London, UK and RPH1-10 from Dr. F. Bolton, Royal Preston Hospital, Preston, UK. *E. coli* strains E32511A, DH5α and JC3272 were also used in this study as controls. *E. coli* strain E32511A (Verotoxin-producing strain), kindly provided by Dr. G. Willshire, HPA, Colindale, London, UK and *E. coli* DH5α (a non-toxin-producing strain) (obtained from the local culture collection, Division of Infection and Immunity) were used in the cytotoxicity assays as positive and negative controls, respectively. *E. coli* JC3272 strains (one strain containing plasmid pRK 2013 and another one without it) (obtained from the local culture collection, Division of Infection and Immunity) were

Table 2.1 Strains and mutants used in this study

Isolate Number (Strain no.)	Species	Source	Description
81-176	*C. jejuni*	VLA, Weybridge, Surrey	Clinical isolate causing inflammatory diarrhoea in human volunteer studies
81-176*cdtB*	*C. jejuni*	CAMR, Porton Down	Cytolethal distending toxin (CDT) mutant (mutation in B subunit of toxin)
NCTC 11168	*C. jejuni*		Sequenced strain
NCTC 11168*cdtB*	*C. jejuni*	CAMR, Porton Down	CDT mutant (mutation in B subunit of toxin)
NCTC 11168*cdtA*	*C. jejuni*	Vet School, Glasgow	CDT mutant (mutation in A subunit of toxin)
NCTC 11168*pldA*	*C. jejuni*	Vet School, Glasgow	Mutant in contact-dependent phospholipase D
NCTC 11168*tlyA*	*C. jejuni*	Vet School, Glasgow	Mutant in what is characterized as a haemolysin in other pathogenic bacteria but may be a possible methylase or regulator in *C. jejuni*

Table 2.1 (continued)

Isolate Number (Strain no.)	Species	Source	Description
COL1	*C. jejuni*	HPA, Colindale	Human (61 year, male), diarrhoea
COL2	*C. jejuni*	HPA, Colindale	Bovine carcass, abattoir
COL3	*C. jejuni*	HPA, Colindale	Bovine carcass, abattoir
COL4	*C. jejuni*	HPA, Colindale	Chicken caecum
COL5	*C. jejuni*	HPA, Colindale	Chicken carcass, skin
COL6	*C. jejuni*	HPA, Colindale	Human, diarrhoea
COL7	*C. jejuni*	HPA, Colindale	Human faeces
COL8	*C. jejuni*	HPA, Colindale	Human faeces
COL9	*C. jejuni*	HPA, Colindale	Human faeces

Table 2.1 (continued)

Isolate Number (Strain no.)	Species	Source	Description
COL10	*C. jejuni*	HPA, Colindale	Human (Female), persistant diarrhoea
COL11	*C. jejuni*	HPA, Colindale	Human (79 year, female), severe anaemia
COL12	*C. jejuni*	HPA, Colindale	Chicken caecum
COL13	*C. jejuni*	HPA, Colindale	Bovine, farm site
COL14	*C. jejuni*	HPA, Colindale	Human faeces, diarrhoea
COL15	*C. jejuni*	HPA, Colindale	Human, bloody diarrhoea
COL16	*C. jejuni*	HPA, Colindale	Human (31year, female)
COL17	*C. jejuni*	HPA, Colindale	Human (4 year, female)
COL18	*C. jejuni*	HPA, Colindale	Human (22 year, male), diarrhoea

Table 2.1 (continued)

Isolate Number (Strain no.)	Species	Source	Description
COL19	*C. jejuni*	HPA, Colindale	Human, diarrhoea
COL20	*C. jejuni*	HPA, Colindale	Human (54 year, male), diarrhoea
COL21	*C. jejuni*	HPA, Colindale	Bovine (12 week), faeces
COL22	*C. jejuni*	HPA, Colindale	Human (2 year, male), diarrhoea
COL23	*C. jejuni*	HPA, Colindale	Human (36 year, female), diarrhoea
COL24	*C. jejuni*	HPA, Colindale	Human (30 year, male), diarrhoea
COL25	*C. jejuni*	HPA, Colindale	Human (18 year, female), diarrhoea and vomiting
COL26	*C. jejuni*	HPA, Colindale	Human (22 year, female), diarrhoea
COL27	*C. jejuni*	HPA, Colindale	Human (60 year, male), diarrhoea

Table 2.1 (continued)

Isolate Number (Strain no.)	Species	Source	Description
COL28	*C. jejuni*	HPA, Colindale	Ovine carcass, abattoir
COL29	*C. jejuni*	HPA, Colindale	Bovine faeces
COL30	*C. jejuni*	HPA, Colindale	Human (2 year), diarrhoea
RPH1	*C. jejuni*	Royal Preston Hospital	Chicken
RPH2	*C. jejuni*	Royal Preston Hospital	Chicken
RPH3	*C. jejuni*	Royal Preston Hospital	Chicken
RPH4	*C. jejuni*	Royal Preston Hospital	Chicken
RPH5	*C. jejuni*	Royal Preston Hospital	Human
RPH6	*C. jejuni*	Royal Preston Hospital	Human

Table 2.1 (continued)

Isolate Number (Strain no.)	Species	Source	Description
RPH7	*C. jejuni*	Royal Preston Hospital	Chicken
RPH8	*C. jejuni*	Royal Preston Hospital	Human
RPH10	*C. jejuni*	Royal Preston Hospital	Human
WA585	*C. coli*	Glasgow University	Milk
E32511A	*E. coli*	HPA, Colindale	
DH5α	*E. coli*	Glasgow University	
JC3272 (containing pRK 2013)	*E. coli*	Glasgow University	
JC3272	*E. coli*	Glasgow University	

VLA = Veterinary Laboratory Agency
CAMR = The centre for Applied Microbiology and Research
HPA = Health Protection Agency

used in the plasmid purification as a positive and negative control.

2.1.2 Growth of bacterial strains

2.1.2.1 Growth of *C. jejuni* strains

C. jejuni strains were grown on blood agar base (Difco) (Appendix 1) supplemented with 5% (v/v) defibrinated sheep blood (E&O Laboratories Ltd), 0.4% (v/v) Campylobacter selective supplement (Blaser-Wang) (Oxoid) containing: vancomycin 10 μg/ml, polymyxin 2.5 I.U/ml, trimethoprim 5 μg/ml, amphotericin B 2 μg/ml, cephalothin 15 μg/ml, and 0.4% (v/v) Campylobacter growth supplement (Oxoid) containing: sodium pyruvate 0.25 mg/ml, sodium metabisulphite 0.25 mg/ml and ferrous sulphate 0.25 mg/ml. Strains were incubated at 42°C in anaerobic jars under microaerophilic conditions (O_2 5%, CO_2 10% and N_2 85%, BOC Gases).

For growth in liquid medium, a starter culture was prepared from agar by emulsifying several loopfuls of *Campylobacter* growth in 5 ml of Brucella broth (Difco) (Appendix 2) and using this as inoculum for 100 ml of the same medium. The culture was incubated under microaerophilic conditions in an anaerobic jar secured on an orbital shaker running at 100 revolutions per minute (rpm) for 48h at 42 °C. After putting plates or flasks into the jar, first the air of the jar was vacuumed and then the gas mixture above was injected into the jar to make a microaerophilic condition.

When required, antibiotics were added to these media at the following concentrations: tetracycline (20 μg/ml), kanamycin (50 μg/ml), chloramphenicol (20 μg/ml).

2.1.2.2 Growth of *E. coli* strains

E. coli strains were grown overnight at 37 °C on Luria Bertani (LB) agar (Appendix 3), and were inoculated from such plates to LB broth, where necessary. Broths were incubated overnight at 37 °C with shaking at between 150 and 200 rpm.

2.1.3 Long-term storage of *C. jejuni* strains

Several loopfuls of the *Campylobacter* strain were removed from a BA plate and emulsified in 1.5ml of Brucella broth containing 40% (v/v) glycerol (Ridel-deHaën) and aliquots were immediately placed at –70 °C to act as a frozen stock. Cultures were grown from frozen stocks by thawing rapidly at 37 °C and spreading 50µl of the bacterial suspension on a pre-warmed BA plate. Incubation was performed as above.

2.2 Characterisation of the strains

The isolates were identified by standard tests including API CAMPY test, Gram stain, colonial morphology, a selective medium for *Campylobacter*, oxidase and catalase tests.

2.2.1 API CAMPY test

API CAMPY (bioMerieux) is a system for the identification of *Campylobacter* which uses standardized and miniaturized tests with a specially adapted database. API CAMPY test was used to identify the clinical isolate strains. The API CAMPY strip consists of 20 microtubes containing dehydrated substrates, and it is made up of two parts: 1- enzymatic and conventional tests and 2- assimilation or inhibition tests.

2.2.1.1 Enzymatic and conventional tests

The first part of the strip was inoculated with a dense suspension, which rehydrates the substrates. The metabolic end products produced during the incubation period (aerobic conditions) are revealed through colour changes that are either revealed spontaneously or by the addition of reagents.

2.2.1.2 Assimilation or inhibition tests

The second part was inoculated with bacterial cells suspended in a minimal medium and incubated in microaerophilic conditions. *C. jejuni* strains grow if they are capable of utilizing the corresponding substrate or if they are resistant to the antibiotic tests.

After incubation for 24h at 37 °C, the reactions were read visually as positive or negative according to the reading Table (Table 2.2) and identification was obtained by consulting the identification Table and using the identification software.

2.2.1.3 Procedure of API CAMPY test

A well-isolated colony was picked and was subcultured on BA agar. The plate was incubated for 24-48 h at 42 °C in microaerophilic conditions. All the growth from the subculture plate was harvested and suspended in 3 ml of 0.85% NaCl (w/v) in demineralised water (supplied with API kit) (Appendix 4) at a turbidity equivalent to 6 McFarland unit's (supplied with API kit). Approximately 80-100 µl of the bacterial suspension in 0.85% NaCl medium was distributed into the tests URE to PAL of the first strip and test H_2S of the second strip. The cupule of the URE test was overlaid

Table 2.2: API CAMPY reading table

Tests	Reactions	Results	
		Negative	**Positive**
URE	UREase	yellow	orange/red
NIT	Reduction of NITrates	colorless	pink/red
EST	ESTerase	colorless/pale blue	turquoise
HIP	HIPpurate	colorless	violet
GGT	Gamma Glutamyl Transferase	colorless	dark orange
TTC	Triphenyl Tetrazolium Chloride reduction	colorless/pale pink	pink/red
PyrA	Pyrrolidonyl Arylamidase	colorless	orange
ArgA	L-Arginine Arylamidase	colorless	orange
AspA	L-Aspartate Arylamidase	colorless	orange
PAL	ALkaline Phosphatase	colorless	purple
H_2S	Production of H_2S	colorless	black
GLU	GLUcose (Assimilation)	transparent	opaque
SUT	SUccinaTe (Assimilation)	transparent	opaque
NAL	NALidixic acid (Susceptibility)	transparent	opaque
CFZ	CeFaZoline (Susceptibility)	transparent	opaque
ACE	ACEtate (Assimilation)	transparent	opaque
PROP	PROPionate (Assimilation)	transparent	opaque
MLT	MaLaTe (Assimilation)	transparent	opaque
CIT	CItraTe (Assimilation)	transparent	opaque
ERO	ERythrOmycin (Susceptibility)	transparent	opaque

with mineral oil to form a slight convex meniscus. The strip was incubated for 24 hr at 35-37 °C in aerobic conditions.

150 µl of the bacterial suspension was also transferred into an ampoule of AUX medium (7 ml) (supplied with API kit) (Appendix 4), and was mixed well. This new suspension was distributed into the assimilation tubes and cupules, and the strip was incubated for 24-48 h at 42 °C in microacrophilic conditions.

2.2.1.3.4 Reading the strip

After the incubation time, reagents (NIT1, NIT2, NIN and FB) (Appendix 4) were added to the strips according to the manufacturer's instructions. After 10 minutes, the reactions were read by referring to the reading table. If the SUT test (a control for bacterial growth) was positive, all the assimilation tests were read. If it was negative, the second part of the strip was reincubated for a further 24 hr. If the SUT test was negative after 48 h, the other assimilation tests were not considered to provide the necessary information. Identification was obtained using the identification software by manually entering the 7-digital numerical profile via the keyboard.

2.2.2 Oxidase test

The principle of the oxidase test is based on the detection of an intracellular cytochrome oxidase enzyme, which activates the oxidation of reduced cytochrome by molecular oxygen. This in turn acts as an electron acceptor in the terminal stage of the electron transfer systems. Microorganism which produce the oxidase enzyme, in the presence of atmospheric oxygen, cytochrome c, and phenylenediamine oxidase

reagent, oxidize this to the purple coloured indophenol. Ascorbic acid is incorporated in the reagent as a reducing agent to reduce autooxidation and to improve stability.

1-2 drops of reagent (Appendix 5) were dispensed onto a strip of Whatman No. 1 filter paper, and then a colony from *Campylobacter* grown on blood agar was smeared onto the saturated filter paper. A positive test was indicated by the development of a violet to purple colour within 10-30 sec, and a negative test was indicated by delayed reactions or no colour development.

2.2.3 Catalase test

One drop of hydrogen peroxide (Sigma) was placed on a slide and was mixed with a colony of the strain. A positive test was indicated by the production of O_2 gas.

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2} O_2$$

2.3 Fractionation of *C. jejuni* cells

2.3.1 Preparation of bacterial culture supernatant, cell-free extracts and broken cell pellets

Broth cultures (100 ml) were centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was used as bacterial culture supernatant in the assays. The bacterial pellet was resuspended in 1/50 volume of PBS and the bacteria were lysed by

sonication using a cell sonicator (JENCONS Scientific Ltd) (4×30 sec bursts with 30 sec intervals of cooling on ice between each burst). Cell debris and unlysed bacteria (broken cell pellets) were then produced using two different centrifugation speeds; 3,600 ×g for 10 min at 4 °C or 22,000 ×g for 30 min at 4 °C in a Heraeus Biofuge and Sorvall® ultracentrifuge, respectively. The cell-free extracts (supernatants) were divided into aliquots and stored at –70 °C. Broken cell pellets were resuspended to the original lysate volume in PBS and stored at –70 °C.

2.3.2 Preparation of cytoplasmic, inner-membrane protein (IMP) and outer-membrane protein (OMP) fractions of *C. jejuni* strains

A cell-free extract from the low speed centrifugation described above was re-centrifuged at 100,000 ×g for 60 min at 4 °C in a Sorvall ultracentrifuge. The supernatant was used as a cytoplasmic fraction. The pellet was resuspended in envelope buffer (10 mM sodium phosphate, pH 7.2) (Appendix 7) containing sarcosyl 0.5% (w/v) (N-lauroyl-sarcosine) (Sigma) and left for 30 min to solubilise the proteins. After centrifugation at 100,000 ×g for 60 min at 4 °C as described previously, the final supernate contained the sarcosyl-soluble inner membrane proteins and the final pellet contained the sarcosyl-insoluble outer membrane proteins preparations were obtained. The outer membrane fraction was resuspended in 0.5-1 ml of envelope buffer. All preparations were stored as aliquots at –70 °C.

2.4 Protein estimations

Bradford's assay and the modified Lowry method were used to estimate protein concentrations of bacterial culture supernatant, cell-free extract, broken cell pellets, cytoplasmic, IMP and OMP fractions of *C. jejuni*, *C. coli* and *E. coli* strains. After estimation, the protein concentrations of the various fractions were standardised to bring them to equal concentration per ml for use in cytotoxicity assays.

2.4.1 Bradford's assay

The Bio-Rad Protein Assay is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein (Bradford, 1976). This is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs (Reisner *et al.*, 1975; Sedmak & Grossberg, 1977).

A concentration series in duplicate of bovine serum albumin (BSA) (Sigma) as standard was prepared by diluting a stock of BSA (Sigma) (2mg/ml) in PBS. Also serial dilutions of samples were prepared in duplicate. 200 µl of the diluted Dye reagent (1 volume of Dye reagent concentrate diluted with 4 volumes of dH_2O) was added to each sample dilution, and the absorbance value of samples, standard and blank (diluent) were read after 5 min at 595 nm on a spectrophotometer (Anthos). A standard curve was prepared by plotting the average A_{595nm} for each BSA concentration. The absorbance value of samples were then read off against the standard curve to determine the protein concentration.

2.4.2 Modified Lowry method

A sample volume of 1 ml containing test protein diluted in PBS, BSA concentrations (10, 20, 40, 80, 160, 320 and 640 µg/ml) in PBS as a standard and PBS as a blank as two fold serial dilutions in duplicate were prepared. 1 part of reagent B (Appendix 8) was added to 100 parts of reagent A (Appendix 8) to form Reagent C (Appendix 8). 3.0 ml of reagent C was added to the samples, which were incubated at room temperature for 45 min. The samples were then mixed vigorously with 0.3 ml of Folin & Ciocalteu's Phenol reagent (Sigma) (Appendix 8) and were incubated for 45 min at room temperature. Absorbance values of standards, samples and blank (diluent) were read at 660 nm. A standard curve was prepared by plotting the average A_{660nm} for each BSA concentration. The absorbance value of samples were then read off against the standard curve to determine the protein concentration.

2.5 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970) in a vertical gel electrophoresis tank (Bio-Rad). Glass plates were assembled according to the manufacturer's instructions. Resolving gels, containing 7.5, 10, 12.5 or 15% (w/v) acrylamide (Appendix 9.1), were poured between the assembled plates until the gel was approximately 2 cm below the top of the plates and then absolute (100%) ethanol was used as an overlay. The gel was left undisturbed for approximately 30 min at room temperature until set, and then the overlay was poured off. The top of the separating gel was washed with distilled water and dried with the edge of a filter paper. A stacking gel containing 6% (w/v) acrylamide (Appendix 9.2) was poured

directly onto the polymerised separating gel and a comb was placed into the gel solution and allowed to set. The gel was left undisturbed in a vertical position for 30 min at room temperature until the stacking gel was set, and the comb was then carefully removed. The wells were filled with 1× electrode buffer (Appendix 9.4) and the gel was assembled into the electrophoresis tank containing the same buffer. Protein samples (16 µl) were loaded after mixing with 4 µl of 5× sample buffer (Appendix 9.3). The gel was run at 20 mA until the tracker dye entered the separating gel at which point the current was increased to 50 mA. The gel was removed when the tracker dye reached the bottom of the separating gel and stained for protein visualisation. Gels were stained with Coomassie Blue R-250 (Sigma) staining solution (Appendix 9.5) for a minimum of 2 h on a rotating platform and destained by several changes of destaining solution (Appendix 9.6) until the background was decolourised.

2.6 Tissue culture

2.6.1 Growth of mammalian target cells

2.6.1.1 Preparation of Vero cell line

African green monkey kidney (Vero) (ECACC) cells were grown to confluence at 37 °C in 25cm^2 tissue-culture flasks (Iwaki brand) in humidified conditions in an incubator with 5% CO_2 in Modified Eagle Medium (MEM) (Gibco) containing 5% (v/v) foetal calf serum (Labtech), 1% (w/v) penicillin/streptomycin (Gibco), 1% (v/v) fungizone (Gibco) and 1% (w/v) L-glutamine (Gibco). The cells were trypsinized with 3 ml trypsin EDTA solution (Gibco) before harvesting.

2.6.1.2 Preparation of Caco-2 cell line

Human colon adenocarcinoma (Caco-2) (ECACC) cells were grown to confluence at 37 °C as above in MEM culture medium containing 5% (v/v) foetal calf serum, 1% (w/v) penicillin/streptomycin, 1% (v/v) fungizone, 1% (w/v) L-glutamine, 1% (v/v) non-essential amino acid (NEAA) solution (Gibco) and 1% (v/v) insulin-transferrin-selenium (ITS) solution (Gibco) as growth promoters in 80cm^2 tissue-culture flasks (Iwaki brand). The cells were trypsinized with 10 ml trypsin EDTA before harvesting.

2.6.1.3 Preparation of J774A.1 (murine macrophage)

Mouse macrophage-derived (J774A.1) (ECACC) cells were grown to confluence at 37 °C in an incubator with 5% CO_2 and in humidified conditions in phenol red-free Roswell Park Memorial Institute (RPMI 1640) medium (Gibco) containing 5% (v/v) foetal calf serum, 1% (w/v) penicillin/streptomycin, 1% (v/v) fungizone, 1% (w/v) L-glutamine in 25cm^2 tissue-culture flasks. The cells were harvested by banging the flask to release the adhered cells.

2.6.2 Culture and treatment of mammalian cells

After harvesting, the cells were resuspended in the same tissue culture medium as they were grown in to a density of 5×10^4 cells/ml (5×10^5 cells/ml for J774A.1 cells). The trypan blue exclusion test was used to check the cell viability. For this, 50 µl of 0.2% (w/v) trypan blue solution (Appendix 10) was added to 50 µl cell suspension and incubated at room temperature for 5 min. Cells that remained clear and had not

taken up the blue stain were counted as viable cells using an improved Neubauer haemocytometer (Webber, UK).

Resuspended cells were added (as 100 µl for Vero and Caco-2 cells and 50 µl for J774A.1 cells) to the wells of a 96-well flat-bottomed tissue culture plate (Iwaki brand) and maintained at 37 °C. Serial two fold dilutions of fractions of *C. jejuni* (bacterial culture supernatant, cell-free extract, broken cell pellets, cytoplasmic, IMP or OMP preparations) were prepared in MEM or RPMI. After confluent growth of cells (18-24 h), 50 µl of each dilution was added in duplicate to the monolayers and the plates returned to the CO_2/air atmosphere at 37 °C. Cell-free extracts and broken cell pellets of Verotoxin-producing *E. coli* E32511A and a non-toxin producing strain, *E. coli* DH5α, were used as positive and negative controls for the presence of cytotoxin. Tissue culture medium and Triton X-100 (1%) (w/v) (Sigma) were also used as negative and positive controls in the assays to represent 0 and 100% killing, respectively (see formula below).

2.6.3 Cytotoxicity assays

2.6.3.1 MTT dye reduction assay

Cytotoxic activity was determined by the MTT assay as originally described by Mosmann (1983) using the CellTiter 96® non-radioactive cell proliferation assay (Promega) kit.

A tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is used as a quantitative colourimetric assay for mammalian cell survival. The

assay detects living cells, which cleave by dehydrogenase activity in the mitochondria, the MTT tetrazolium ring to produce a dark blue formazan product.

After incubation of the tissue culture cell monolayers in the presence of sample for various times, 15µl of MTT dye solution was added to each well and the plates were incubated at 37 °C for 4 h in a humidified, 5% CO_2 atmosphere. 100 µl of solubilization/stop solution was then added to each well and, after incubation overnight, the absorbance values of the samples were read at 570 nm using a 96 well plate reader (Anthos). The percentage of cell death was calculated using the formula below:

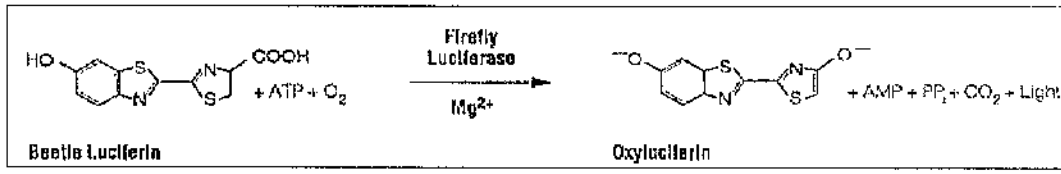
$$\% \text{ Cell Death} = 1 - [(\text{Mean OD}_{\text{test}} - \text{Mean OD}_{\text{+ve control}}) / (\text{Mean OD}_{\text{-ve control}} - \text{Mean OD}_{\text{+ve control}})] \times 100$$

2.6.3.2 ATP-based assay

The Cell Titer-Glo™ luminescent cell viability assay (Promega) was also used to determine cytotoxic activity. This assay determines the number of viable cells in culture based on quantitation of the ATP present, used as an indicator of metabolically-active cells. The amount of ATP is directly proportional to the number of cells present in culture (Crouch *et al.*, 1993). Different cell types have different amounts of ATP, and values reported for the ATP level in cells vary considerably (Andreotti *et al.*, 1995; Beckers *et al.*, 1986; Crouch *et al.*, 1993; Kangas *et al.*, 1984; Stanley, 1986). Factors that affect the ATP content of cells may affect the relationship between cell number and luminescence. Factors that affect the cytoplasmic volume or physiology of cells will also have an effect on ATP content.

One factor known to cause a rapid decrease in ATP content is depletion of oxygen (Crouch *et al.*, 1993).

After incubation of the tissue culture cell monolayers in the presence of sample for various times in 96-well opaque-walled plates, 100 µl of the Cell Titer-Glo™ reagent was added to each well and the contents were mixed for 2 min on an orbital shaker to induce cell lysis. The plate was then incubated at room temperature for 10 min to stabilize luminescence signal. The luminescence was recorded with a luminometer (Anthos Lucy 1).



2.7 Microscopy

Treated (test samples and positive controls) and untreated (negative control) tissue culture cells after different incubation times were examined with an inverted microscope (ZEISS, Axiovert 25) at magnifications of ×20 and ×40. Photographs were taken with a camera (Photometrics) mounted on the microscope.

2.8 Purification of Proteins

2.8.1 DEAE sepharose column chromatography

5 ml DEAE sepharose fast flow (Sigma) was applied to a glass Econo-column (1.5 cm × 20 cm) (BioRad) and washed with 30 ml of water to remove excess ethanol. The column was equilibrated at 4 °C by washing with 25 ml of envelope buffer 10 mM (Tris base, pH 7.2) (binding or starting buffer). A further 50 ml of envelope buffer was applied, followed by 25 ml of envelope buffer. Then 5 ml of low speed centrifugation cell-free extract (crude sample) (3.5 mg total protein) was applied to the column and left for 30 min and the flow-through was collected. The column was washed with 25 ml of envelope buffer and was collected as wash-through no. 1. Then 5 ml of increasing concentrations of NaCl (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 0.9 and 1M) in envelope buffer (pH 7.2) were eluted. Finally, 25 ml of envelope buffer was used to wash the column and was collected as wash-through no. 2. All the procedures were done at 4 °C.

2.8.1.1 Concentration of samples

Centricon (Millipore) centrifugal concentrators with a molecular weight cut-off (MWCO) value of 5,000 kDa were used to concentrate (10 times) some fractions from the DEAE sepharose purification. A 2 ml volume of sample was applied to the Centricon sample reservoir and the tube centrifuged at 2,000 ×g for 20 min at 4 °C to concentrate the sample approximately 10 times as per the manufacturer's instructions.

2.8.2 Fractionation of proteins using Microcon tube

To purify and separate proteins, MicroCon tubes (Millipore) were used. The protein sample was applied sequentially to MicroCon tubes of sizes 100,000, 50,000, 30,000, 10,000, and 5,000 MWCO, respectively. First, 3 ml of sample was applied to a 100,000 MWCO MicroCon tube and was centrifuged at 3,000 ×g for about 15 min until ~50 µl of sample remained in the tube and 2950 µl was filtered. The filtrate was applied to a 50,000 MWCO MicroCon tube and was centrifuged as above until 50 µl of sample remained on the tube and 2900 µl was filtered. This procedure was repeated until the MicroCon 5,000 MWCO tube was reached and 6 fractions were collected.

Bradford's assay was used to measure the protein concentrations of the fractions, and also the fractions were examined by SDS-PAGE.

2.9 Characterisation (identification) of proteins (proteomics)

2.9.1 In-gel digestion after SDS-PAGE (1-dimensional)

The Coomassie-stained gels were destained as normal until the background was clear and bands were visible. Protein bands were then excised for in-gel digestion with trypsin. Usually a small portion from the middle of a gel band is enough rather than the whole band, particularly for stronger bands. This cuts down on the amount of acrylamide in the digest, and improves diffusion of reagents into and peptides out of the gel slice. For this experiment, proteins in the whole gel lane were analysed,

irrespective of mild or strong bands. After the gel pieces were excised, they were cut into several pieces and placed in 1.5 ml eppendorf tube. The gel pieces were then washed for 1 hour in 500 μl of 100 mM ammonium bicarbonate. A volume of 10 μl of 45 mM DTT in 150 μl of 100 mM ammonium bicarbonate sufficient to cover the gel pieces was added, and the proteins were reduced for 30 min at 60 °C. After cooling to room temperature, the DTT solution was replaced with 10 μl of 100 mM iodoacetamide. After 30 min incubation at ambient temperature in the dark with occasional vortexing, the gel pieces were then washed with 500 μl of 50% acetonitrile/ 100 mM ammonium bicarbonate. This step was aided by shaking for 1 h. After discarding the wash, 50 μl of acetonitrile was added to shrink the gel pieces. The liquid phase was removed within 10 min, and the gel pieces were completely dried in a vacuum centrifuge (DNA Speedvac®). The gel pieces were reswollen in 0.2 μg of trypsin (Sigma) in 10 μl of 25 mM ammonium bicarbonate. After 15 min, 20 μl of 25 mM ammonium bicarbonate was added to cover gel pieces and to keep the gel pieces wet. For enzymic cleavage, the sample is then incubated at 37 °C for overnight in flatbed shaker.

After digestion, the remaining supernatant was removed and saved. The remaining peptides were extracted from the gel pieces by incubating in 5% (v/v) formic acid (enough to cover the pieces) for 15 min and then adding the same volume of 100% acetonitrile for a further incubation of 15 min. The extraction was repeated twice and all three fractions were pooled and dried down in a vacuum centrifuge and resuspended in 10 μl of 5% formic acid.

2.9.2 Two-dimensional electrophoresis

2.9.2.1 Sample preparation (sup1 and sup2) for 2-DE gels

C. jejuni colonies were inoculated in 5 ml of Brucella broth, and were incubated for 48 h under microaerophilic conditions at 42 °C. After 48 h, 5 ml of bacterial culture was added to 500 ml of Brucella broth and was incubated as above. After 48h, the OD_{600nm} was measured. Bacterial cultures were centrifuged at 10,000 rpm for 10 min, the supernatant was discarded and the pellets were resuspended in a small volume of Brucella broth medium (10 ml) and shell frozen in liquid nitrogen. They were then freeze dried overnight on a freeze-dryer (E-C, Modulyo).

ProteoPrep™ Universal Extraction Kit (Sigma) was used to prepare material for 2-DE. After freeze drying, 20 mg of lyophilized *C. jejuni* was resuspended in 10 ml of soluble cytoplasmic extraction reagent (S2688, Sigma). The suspension was sonicated on ice using a cell sonicator (JENCONS Scientific Ltd) for 4×30 sec bursts with 30 sec intervals of cooling on ice between each burst to disrupt the cells and break down the DNA. The suspension was centrifuged at 14,000 ×g for 45 min at 4 °C to pellet cell debris and insoluble material. The supernatant was kept on ice. A further 10 ml of soluble cytoplasmic extraction reagent was added to the pellets and sonication and centrifugation were repeated as above. This yielded a supernatant and a pellet. This supernatant and that stored on ice were pooled for a total soluble protein extract of about 20 ml (sup1). sup1 was lyophilized overnight using a freeze-dryer (E-C, Modulyo) and the lyophilized material resuspended in 2 ml of soluble protein resuspension reagent (S 3688, Sigma) and was stored on ice for the next step. The cell pellet was resuspended in 2 ml of cellular and organelle membrane

solubilizing reagent (C0356, Sigma), and then was sonicated as above. The suspension was centrifuged at 14,000 ×g for 45 min at 15 °C to pellet cell debris and the supernatant (sup2) was decanted.

sup1 and sup2 were reduced by adding tributylphosphine (TBP) to each to a final concentration of 5 mM and were incubated for 1 h at room temperature. sup1 and sup2 were then alkylated by adding iodoacetamide to each to a final concentration of 15 mM and were incubated for 1.5 h at room temperature. After the incubations, the reduced and alkylated samples were centrifuged at 20,000 ×g for 5 min at room temperature to remove any insoluble material.

2.9.2.2 TCA precipitation

Due to the low protein concentrations of sup2, precipitation with TCA was performed. TCA was added to 10% (w/v) of the sample volume, and was kept on ice for 30 min. The sample was centrifuged at 13,000 rpm (microcentrifuge) for 30 min, the supernatant was discarded and the pellet was washed several times with acetone before being solubilised.

2.9.2.3 First dimension separation by IEF

Proteins were separated in the first dimension using the IPGPhor® isoelectric focusing system employing immobilised pH gradient (IPG) strips. The required number of strip holders were placed on to the IPGPhor® and 450 µl of sample containing rehydration solution (Appendix 11) was evenly pipetted in the strip holder groove. The 24 cm Immobiline DryStrips® were lowered, gel side down on to the rehydration solution without trapping air bubbles. To minimize evaporation and urea crystallization, this strip was then overlayed with DryStrip Cover Fluid®

(Amersham) before the plastic cover was applied. The proteins were then focused to their isoelectric points according to the programmed conditions in Table 2.3. Rehydration and isoelectric focusing were performed in this integrated system at 20 °C. Following isoelectric focussing, the proteins were reduced and bound to sodium dodecyl sulphate (SDS) by equilibrating each strip for 15 min in 10 ml of SDS equilibration buffer (Appendix 12) containing 100 mg of dithiothreitol (added fresh before use). A second equilibration step in SDS equilibration buffer containing 250 mg of alpha-iodoacetamide (added fresh before use) instead of dithiothreitol was performed in order to prevent protein re-oxidation and to minimise reactions of cysteine residues.

Table 2.3: Isoelectric focusing steps used to focus pH 3-10 nonlinear IPG strips

IPG	Step	Voltage	Duration (hour)
3-10 nonlinear	Rehydration	–	12
	1	500	1
	2	1000	1
	3	8000	6

2.9.2.4 Second dimension separation by SDS-PAGE

After equilibration, the immobilised pH gradient strips were rinsed in electrophoresis buffer before being placed on to 12.5% (w/v) homogeneous polyacrylamide gels. Ettan DALT precast gels® (1 mm thickness × 255 mm width × 196 mm length) cast

onto a plastic support film were used in conjunction with the Ettan DALT*twelve*® system (Amersham). The Ettan DALT precast gel® was inserted into a previously cleaned and dried Ettan DALT precast gel cassette®. The gel side of the precast gel was placed over the glass plate of the gel cassette and the cathodic end (-) of the gel was placed on the cathodic end of the cassette. A roller was used against the plastic support film to press out any bubbles or liquid from between the gel and the glass. Thus the gel is adhered firmly to the glass and resists further movement. The cassette was then closed by pressing the plastic frame and the glass plate tightly together. To load the equilibrated IPG strip, the gel cassettes were placed in an upright position and then the IPG strip was inserted between the glass plates with a spatula or spacer and brought in close contact with the upper edge of the SDS gel. Then the area was sealed with molten 0.5% (w/v) agarose in gel running buffer (supplied with Ettan DALT buffer kit®). After the agarose had set, the loaded gel cassettes were ready to insert into different slots of the Ettan DALT*twelve*® tank.

The Ettan DALT buffer kit® was used to make electrophoresis buffer. Two types of anode and cathode buffer were prepared according to the manufacturers instructions and used for all second dimension separations. The cathode buffer included in the Ettan DALT Buffer kit® was diluted in a separate container to working strength. This was done by adding both bottles of 10x cathode buffer (total volume 250 ml) to 2.25 L of distilled deionised water. The DALT tank was filled to the 7.5 L fill line with distilled deionised water and the entire contents of the bottle of 100x anode buffer (supplied with Ettan DALT Buffer kit®) was added carefully by spreading the tubing elements apart. The pump was switched on to start mixing the buffer with water at a set temperature of 15 °C. The gel cassettes loaded with IPG strips were inserted into different slots of the Ettan DALT*twelve*® tank. Blank cassettes were inserted into any

unoccupied slots. When all 12 slots were occupied, the buffer level was adjusted (by adding distilled deionised water with the help of a pipette) to be slightly below the level of gaskets. The diluted cathode buffer (1×) was then poured into the top of the tank to the fill line. After filling the tank with cathode and anode buffer, electrophoresis were run at 2 w/gel until the bromophenol blue front had completely migrated out of the SDS gel.

2.9.2.5 Visualization of proteins

Gels were stained overnight by incubating in a coomassie staining solution consisting of 0.5 g Coomassie Brilliant Blue G-250 (Sigma), 100 ml glacial acetic acid, 125 ml isopropanol, and ddH_2O up to 1 L. Then the gels were destained by multiple washes in a solution of 10% glacial acetic acid with 20% methanol until background of the gel was clear.

2.9.3 Image analysis

Images of gels were acquired at 300 dots per inch (dpi) using Labscan v3.0 software (Amersham Pharmacia Biotech) on a Umax flatbed scanner (OD maximum 3.4) with integrated transparency adapter and/or Typhoon® scanner 9400 (variable mode imager). The tiff images generated were analysed using ImageMaster® 2D Elite V3.01 software. The authenticity and outline of each spot were validated by visual inspection and edited where necessary. The volume values for each spot were acquired using no-subtraction mode and normalized against the total intensity of the detected spots.

2.9.4 Mass spectrometry

2.9.4.1 Processing of protein for mass spectrometry

A number of spots were selected from different gels with the help of image analysis software and then subjected to the robotic Ettan Spot Handling Workstation® (Amersham Pharmacia Biotech) where the excision of each spot and subsequent digestion with sequencing grade modified porcine trypsin (Promega, Madison, WI, USA), were done automatically.

The spot picking, destaining, digestion, extraction, MALDI sample preparation and spotting on MALDI target slides were carried out robotically in this enclosed, fully-automated, spot-handling workstation. Briefly, each picked gel plug was rinsed by three wash cycles in which 150 μl of 50% methanol/50% water, containing 25 mM ammonium bicarbonate was applied, left to stand for 15 min, and then removed. The plugs were dehydrated in 75% acetonitrile for 20 min, the solution removed, and the trays placed into a heated, air circulating drying module and dried for 10 min. To the dried plugs was added 7 μl of digestion solution (containing 40 ng/μl of sequencing grade trypsin in 25 mM ammonium bicarbonate), and the plate was robotically transferred to the digestion station where it was covered and maintained at 37 °C for 4 h. Eighty microliters of extraction solution (50% acetonitrile containing 0.1% trifluoroacetic acid, TFA) were then added to each well and left to stand for 20 min. The extract solutions were transferred to a clean 96-well plate, and the extraction repeated. The plate containing the combined extracts was then evaporated to dryness. MALDI samples were prepared by reconstituting the dried peptides in 3 μl of 50% acetonitrile containing 0.5% TFA. An aliquot of 0.3 μl was then applied to the clean

MALDI target slide surface and allowed to dry. The spots were redissolved by the addition of 0.3 μl of matrix solution (α-cyano-4-hydroxycinnamic acid saturated solution in 50% acetonitrile containing 0.5% TFA) and finally allowed to dry before MALDI-TOF mass spectrometry. The plate containing the remaining peptides was stored at –20 °C until LC-MS/MS analyses could be performed.

2.9.4.2 MALDI-TOF mass spectrometry

MALDI peptide mass fingerprinting was carried out using the Applied Biosystems Voyager-DE instrument operating in reflectron mode and equipped with a nitrogen laser (337 nm, 3 ns pulse width, 3 Hz repetition rate). Internal calibration was performed using the porcine trypsin autodigestion peaks at *m/z* 842.510 and 2211.1046. Each spectrum corresponded to the sum of 200 acquisitions, in which the threshold signal-to-noise exceeded a set value. Protein identification by peptide mass fingerprinting was performed using the MASCOT® search engine (Matrix Science, London, UK) and database described later in this chapter.

2.9.4.3 Tandem mass spectrometry

Proteins that were not resolved by MALDI-TOF MS were subjected to LC-MS/MS using a capillary HPLC system and a Q-TOF mass spectrometer. For protein identification, the files containing all the MS and MS/MS data obtained during each run were searched against the National Center for Biotechnology Information (NCBInr) protein database (www.ncbi.nlm.nih.gov) using MASCOT®software (www.matrixscience.com).

2.9.5 Database search

Peptide mass fingerprint data from MALDI-TOF and peptide fragmentation data from Q-STAR-MS/MS were used to search genomic and protein databases including NCBInr database (http://www.ncbi.nlm.nih.gov), and Mass spectrometry database (MSDB).

2.9.5.1 The MASCOT® search tool

The MASCOT® search engine (http://www.matrixscience.com) is based on a modified MOWSE computer programme using a probability-based scoring system (Pappin *et al.*, 1993). The probability that the observed match between the experimental data and a protein sequence is random is calculated for each protein sequence in the database. The proteins are classified with increasing probability of being a random match to the experimental data. This probability is based on the size of the database and the frequency of each peptide mass in the database. The search tool guidelines state that if the probability based score is higher than the database's probability (p) then the match is significant within 95% confidence limits. This search engine supports all three types of searches: peptide mass fingerprint, sequence query, and MS/MS ion search. The results are displayed as a histogram showing the distribution of the scores and the 95% confidence limits.

2.9.5.2 The NCBInr database

This database contains the translated protein sequences from the entire collection of annotated DNA sequences kept at GenBank, and also protein sequences in the protein Data Bank (PDB), CDS translations, covering most of the publicly available

data. The NCBI also provide BLAST (Basic Local Alignment Search Tool) programme support for database sequence similarity searching.

2.9.6 Difference gel electrophoresis (DIGE)

The difference gel electrophoresis (DIGE) method allows multiplexing of samples and the use of an internal standard, which is created by using one of the labels for a pooled mixtures of all samples. The method is based upon the specific properties of the three CyDye™ DIGE Fluor minimal dyes which enable multiplexing of up to three separate protein mixtures on the same 2D gel. Cy™2, Cy3 and Cy5 are three spectrally resolvable dyes (CyDye DIGE Fluor minimal dyes) mached for mass and charge. Because gel-to-gel variations are eliminated, this method leads to highly accurate qualitative and quantitative results. Samples for DIGE experiments were submitted to the Functional Genomics Facility (University of Glasgow). Fig. 2.1 shows the workflow of the DIGE system.

2.10 DNA techniques

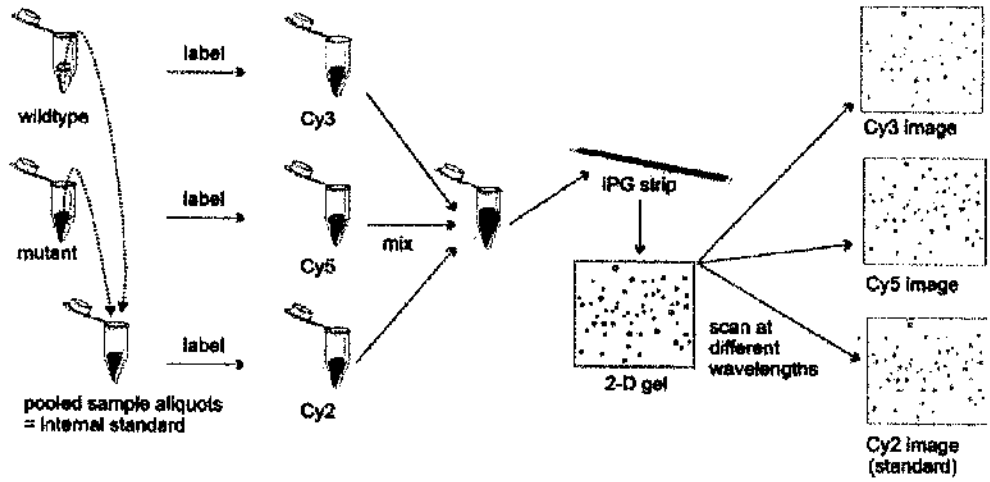
2.10.1 Polymerase chain reaction (PCR) techniques

2.10.1.1 Preparation of boiled extract for PCR (DNA extraction)

Bacterial cell DNA was isolated by the boiling method from cultures grown on blood agar. Several loops of bacterial growth were resuspended in 250 µl of nuclease-free water in microfuge tubes. These were placed in a heating block at 99 °C for 10 min. The tubes were then placed at –20 °C for 5 min to 'snap-freeze' them. Suspensions

Figure 2.1: Schematic workflow of DIGE system.

Diagram was taken from Westermeier & Marouga (2005).


label
wildtype
Cy3
label
mutant
Cy5
mix
label
pooled sample aliquots
= internal standard
Cy2
IPG strip
2-D gel
scan at
different
wavelengths
Cy3 image
Cy5 image
Cy2 image
(standard)

were then centrifuged at 13,000 rpm for 10 min in a Heraeus microfuge after which the supernatant containing DNA was removed and stored at –20 °C.

2.10.1.2 PCR amplification

The sources of the primer sequences and reaction conditions used in this study are listed in Table 2.4. PCR amplifications were performed in a 50 µl volume containing 45 µl of PCR ReddyMix™ master mix (ABgene®), 1 µl (20 pmole) of each primer (Invitrogen) and 3 µl of template.

PCR products were analysed by agarose gel electrophoresis (see section 2.10.3).

Composition of PCR ReddyMix™ master mix:

Taq DNA Polymerase	1.25 units
Tris-HCl (pH 8.8 at 25 °C)	75 mM
$(NH_4)_2SO_4$	20 mM
$MgCl_2$	1.5 mM
Tween® 20	0.01% (v/v)
Each of dATP, dCTP, dGTP and dTTP	0.2 mM

Table 2.4. Primers and PCR programmes used for amplification of various *Campylobacter* genes

Primer pair	Target gene	Primer sequence (5'-3')	Reaction temp. (°C)			Amplicon size (bp)	References
			Denaturation	Annealing	Extension		
CJHF	*hipO*	GTACTGAAAATTTAGTGGCG	94 (1 m) *	55 (1 m)	72 (1 m), 25 cycles	1130	Bang *et al.*, 2001
CJHR		GAGCTTTTAGCAAACCTTCC					
CDTAF	*cdtA*	AGTTTTTATTTTATGTTGTTTTATGAC	94 (1 m)	50 (1 m)	72 (3 m) , 30 cycles	779	This study
CDTAR		CTTGGCGATATAAAGGTTTTGCGG					
CJCF	*cdtB*	GTTAAAATCCCCTGCTATCAACCA	94 (1 m)	42 (2 m)	72 (3 m), 30 cycles	495	Bang *et al.*, 2001
CJCR		GTTGGCACTTGGAATTTGCAAGGC					
VIR BF	*virB11*	GAACAGGAAGTGGAAAAACTAGC	95 (30 s)	50 (30 s)	72 (2 m), 35 cycles	708	Bacon *et al.*, 2000
VIR BR		TTCCGCATTGGGCTATATG					
TET OF	*tetO*	GGCGTTTTGTTTATGTGCG	95 (2 m)	52 (30 s)	72 (1 m), 35 cycles	559	Bacon *et al.*, 2000
TET OR		ATGGACAACCCGACAGAAGC					

*The values in parentheses is the reaction time. m, minute; s, second

2.10.2 Plasmid purification

2.10.2.1 Small-scale isolation of plasmid DNA

The QIAprep® miniprep kit (Qiagen) was used to purify plasmids from *C. jejuni* strains. *E. coli* JC3272 (containing plasmid pRK 2013) and *E. coli* JC3272 were used as positive and negative controls, respectively.

10 ml of bacterial culture was centrifuged at 4,000 rpm for 10 min and the pelleted bacterial cells were resuspended in 250 µl of buffer P1 (resuspension buffer, containing RNase A) and then 250 µl of buffer P2 (lysis buffer) was added and the tube inverted gently 4-6 times to mix (the solution became viscous and slightly clear after inverting). Then 350 µl of buffer N3 was added and the tube gently inverted 4-6 times after which the solution became cloudy. The tube was centrifuged for 10 min at maximum speed (13,000 rpm) in a tabletop microcentrifuge (Heraeus, Biofuge). After centrifugation, a compact white pellet formed and the supernatant was transferred to the QIAprep spin column by decanting or pipetting and this was centrifuged for 30-60 s and the flowthrough was discarded. The QIA prep spin column was washed by adding 0.5 ml of buffer PB (to remove traces of nuclease activity) and then was centrifuged for 30-60 s and the flowthrough was discarded. The column was washed again by adding 0.75 ml of buffer PE (containing ethanol) and was centrifuged for 30-60 s, the flowthrough was discarded and the column was centrifuged for an additional 1 min to remove residual wash buffer. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube and, to elute DNA, 20 µl of buffer EB (10 mM Tris-HCl, pH 8.5) was added to the centre of each QIAprep

column and, after standing for 1 min, was centrifuged for 1 min. The flowthrough was stored at –20 °C.

2.10.2.2 Large-scale isolation of plasmid DNA

QIAGEN® large-construct kit was also used to purify plasmids from *C. jejuni* strains. A single colony from a freshly-streaked selective plate was inoculated as a starter culture into 2-5 ml Brucella broth containing the appropriate selective antibiotic. The medium was incubated for 48 h at 42 °C with vigorous shaking in a flask with a volume at least 4 times greater than the volume of the culture. 0.5-1.0 ml of the starter culture was diluted into 500 ml Brucella broth and was grown at 42 °C for 48 h with vigorous shaking. The bacterial cells were harvested by centrifugation at 6,000 ×g for 15 min at 4 °C. The bacterial pellet was resuspended in 20 ml of buffer P1 (which had Rnase A added, resuspension buffer) (Appendix 15), and then 20 ml of buffer P2 (lysis buffer) (Appendix 15) was added and mixed gently but thoroughly by inverting 4-6 times, and was then incubated at room temperature for 5 min. Then 20 ml of chilled buffer P3 (neutralization buffer) (Appendix 15) was added, mixed immediately by gentle inversion 4-6 times, and was incubated on ice for 10 min, and after that it was centrifuged at 20,000 ×g for 30 min at 4 °C, and the supernatant containing DNA was removed promptly. The lysate was filtered through a folded filter pre-wetted with distilled water. DNA was precipitated at room temperature by adding 0.6 volume (approximately 36 ml) of isopropanol to the cleared lysate, mixed and centrifuged immediately at 15,000 ×g for 30 min at 4 °C, and then the supernatant was decanted carefully. The DNA pellet was washed with 5 ml of 70% (v/v) ethanol at room temperature, and was centrifuged at 15,000 ×g for 15 min, and the supernatant was decanted carefully without disturbing the pellet. The 70%

ethanol removed precipitated salt and replaced isopropanol with the more volatile ethanol, making the DNA easier to redissolve. The tube containing the DNA was placed upside down on a paper towel and the DNA was allowed to air-dry for 2-3 min, any additional liquid visible was removed carefully and the DNA was mixed gently in 9.5 ml buffer EX until the DNA was completely dissolved. Then 200 µl of ATP-dependent exonuclease and 300 µl ATP solution (100mM) were added to the dissolved DNA, mixed gently but thoroughly, and incubation continued in a water bath or heating block at 37 °C for 60 min. During this step genomic DNA and nicked plasmid were digested by exonuclease, and only supercolied DNA remained for further purification. A QIAGEN-tip 500 was equilibrated by applying 10 ml of buffer QBT (equilibration buffer) (Appendix 15), and the column was allowed to empty by gravity flow. 10 ml of buffer QS was added to the DNA sample, and the whole sample was applied to the QIAGEN-tip, and was allowed to enter the resin by gravity flow. The QIAGEN-tip was washed with 2×30 ml of buffer QC (wash buffer) (Appendix 15). DNA was eluted with 15 ml of buffer QF (elution buffer) pre warmed to 65 °C (Appendix 15). DNA was precipitated by adding 10.5 ml (0.7 volume) of isopropanol room-temperature to the eluted DNA, mixed and centrifuged immediately at 15,000 ×g for 30 min at 4 °C, and the supernatant was decanted carefully. The DNA pellet was washed with 5 ml of 70% (v/v) ethanol at room-temperature, was centrifuged at 15,000 ×g for 15 min, and the supernatant was decanted carefully without disturbing the pellet. The pellet was air-dried for 5-10 min, and the DNA was redissolved in a suitable volume of TE buffer, pH 8.0 (Appendix 15).

2.10.2.3 Restriction endonuclease digestion of supercoiled plasmid DNA

*Bgl*II (Promega) enzyme was used to digest the supercoiled plasmid purified by QIAGEN® Large-Construct Kit. A mixture of 5 µl of supercoiled DNA, 1 µl of *Bgl*II enzyme, 5 µl of enzyme buffer and 19 µl of dH_2O were incubated at 37 °C overnight.

2.10.3 Agarose gel electrophoresis

5-20 µl of the sample DNA were mixed with 6× DNA loading buffer (Appendix 13) in a ratio of 5:1 prior to loading into the wells. Molecular weight markers were utilised according to manufacturer's instructions.

Agarose Type II-A (Sigma) was suspended in 0.5× Tris boric EDTA (TBE) (Appendix 14) to the required concentration and heated until the agarose was completely dissolved. The solution was allowed to cool, and ethidium bromide was added to a final concentration of 1.0 µg/ml, after which the agarose was poured into an appropriate gel tray. Upon setting, the gel was immersed in 0.5× TBE in a horizontal gel electrophoresis tank (Bio-Rad). Samples were loaded and a current of 100 mA was passed through the agarose gel using a powerpack (Bio-Rad). Electrophoresis was carried out until the marker dye had migrated an appropriate distance.

A high performance ultraviolet transilluminator (Ultra Violet Products, Cambridge, UK) coupled to an image acquisition system was used to store images of the ethidium bromide-stained DNA electronically using analysis software package (LabWorks). A video graphic printer (model UP-860, Sony) was used to print the images which were edited using Adobe Photoshop Version 4.

Chapter Three

Results

CHAPTER 3: RESULTS

3.1 Growth curve of *C. jejuni*

C. jejuni strain 81-176 was inoculated in Brucella broth and incubated microaerophilically as described in section 2.1.2.1. Every 6h, the absorbance value of the culture was measured at 600 nm and a typical growth curve showed that maximum absorbance (A_{600nm}) was obtained after 48h incubation (Fig.3.1). Thus, cultures of *C. jejuni* for cytotoxicity assays were used after incubation for 48h.

3.2 Characterization of the strains

3.2.1 Growth of *C. jejuni* strains on blood agar containing Blaser-Wang selective supplements

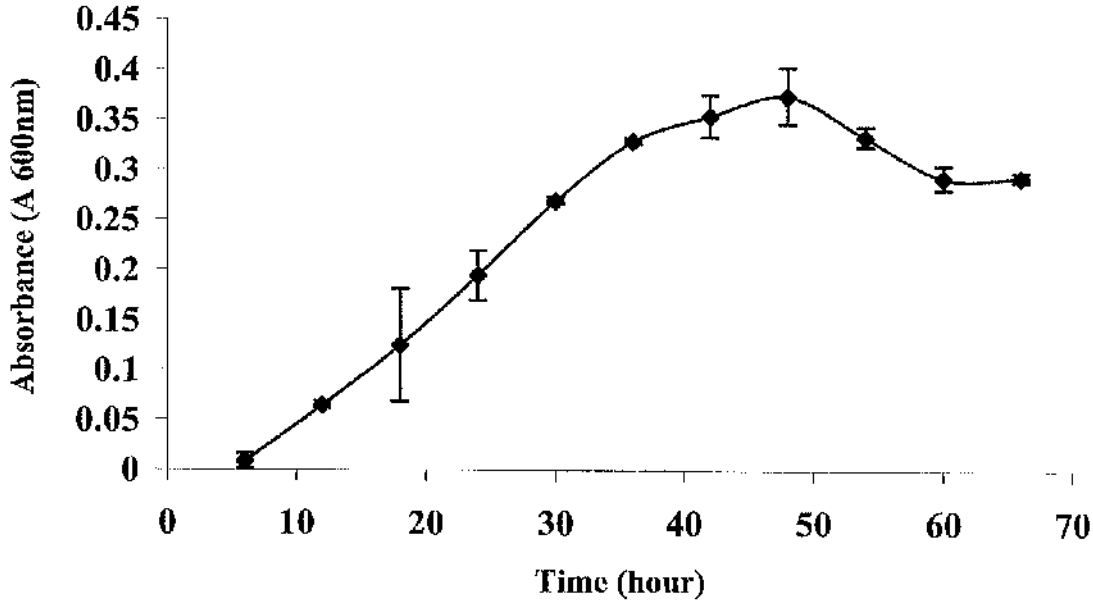
All *C. jejuni* strains that were used in this study grew on Blood agar (BA) containing Blaser-Wang selective supplements and had formed visible colonies after incubation for 36h under microaerophilic condition, indicating that all strains behaved as expected for *C. jejuni*.

3.2.2 API CAMPY identification test

The identity of the 39 clinical isolates was confirmed using the API CAMPY identification kit. Reference strains 11168 (*C. jejuni*) and WA 585 (*C. coli*) were used as positive and negative controls. The results confirmed that all clinical isolates used in further tests were *C. jejuni jejuni*. According to the manufacturer's instructions, it is normal to experience a lack of uniformity between strains in some

Figure 3.1: Growth curve of *C. jejuni* strain 81-176

The organism was grown in Brucella broth medium under microaerophilic conditions in an anaerobic jar secured on an orbital shaker running at 100 rpm at 42 °C. Every 6h, the absorbance value of the culture was measured at 600 nm.


Absorbance (A 600nm)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
70
Time (hour)

test results. Details of API CAMPY test results are shown in Tables 3.1 and 3.2. They show that not all strains produced uniform results, but all were placed as *C. jejuni jejuni*. 37 of the 39 clinical isolates were identified as *C. jejuni jejuni* 1 or 2 with ID score > 98.3%; and two isolates were also identified as *C. jejuni jejuni* 1 with 88.3% ID scores. 19 (48.72%) of the isolates were identified as *C. jejuni jejuni* 1 and 20 (51.28%) of them as *C. jejuni jejuni* 2 (Table 3.2). According to Table 3.1, GGT (Gamma Glutamyl Transferase) test is the most important test to differentiate between *C. jejuni jejuni* 1 and 2 which showed negative and positive results, respectively.

3.2.3 PCR for hippuricase (*hipO*) gene

Differentiation of *C. jejuni* from other *Campylobacter* spp. depends on its ability to hydrolyse hippurate (Wallace *et al.*, 1997) and hippuricase is the product of the *hipO* gene (Hani & Chan, 1995). All *Campylobacter* strains listed in Table 3.1 were tested for the presence of *hipO* gene. *C. jejuni* gave a positive amplimer in all cases, but no PCR product was detected for *C. coli* WA585. Some examples are shown in Fig. 3.2.

3.2.4 Catalase test

The catalase test was used in parallel with API CAMPY test to diagnose the strains. All *Campylobacter* isolates were positive for the catalase test.

3.3 SDS-PAGE analysis of culture supernatants and cell-free extracts

SDS-PAGE showed that less protein was present in culture supernatant than in cell-free extracts (Fig. 3.3). Distortion of the gel was apparent with lanes containing

Table 3.1: API CAMPY test results of *Campylobacter* strains

test / strain	URE	NIT	EST	HIP	GGT	TTC	PyrA	ArgA	AspA	PAL	H_2S	GLU	SUT	NAL	CFZ	ACE	PROP	MLT	CIT	ERO	OXI
11168	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	−	−	+
C. coli	−	+	+	−	−	+	−	+	−	+	−	−	+	−	+	−	+	−	−	−	+
COL1	−	+	+	+	−	+	−	−	−	+	−	−	+	−	+	−	−	+	+	−	+
COL2	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	−	−	+
COL3	−	+	+	+	−	−	+	−	−	+	−	−	+	−	+	−	−	+	−	−	+
COL4	−	+	+	+	−	−	−	−	−	+	−	−	+	−	+	−	−	+	+	−	+
COL5	−	+	+	+	−	−	−	−	−	+	−	−	+	−	+	−	−	+	−	−	+
COL6	−	+	+	+	+	+	+	−	−	+	−	−	+	−	−	−	−	+	+	−	+
COL7	−	+	+	+	−	+	+	−	−	+	−	−	+	−	−	−	−	+	+	−	+
COL8	−	+	+	+	−	+	−	−	−	+	−	−	+	−	+	−	−	+	+	−	+
COL9	−	+	+	+	−	+	−	−	−	+	−	−	+	−	+	−	−	−	+	−	+

Table 3.1: (continued)

test / strain	URE	NIT	EST	HIP	GGT	TTC	PyrA	ArgA	AspA	PAL	H_2S	GLU	SUT	NAL	CFZ	ACE	PROP	MLT	CIT	ERO	OXI
COL10	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	+	−	+
COL11	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	+	−	+
COL12	−	+	+	+	+	+	−	−	−	+	−	−	+	−	+	−	−	+	+	−	+
COL13	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	−	−	+
COL14	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	+	−	+
COL15	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	+	−	+	+	−	+
COL16	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	+	−	+
COL17	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	+	−	+
COL18	−	+	+	+	+	+	+	−	−	+	−	−	+	−	−	−	−	+	−	−	+
COL19	−	+	+	+	+	+	+	+	−	+	−	−	+	−	+	−	−	+	+	−	+
COL20	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	+	−	+

Table 3.1: (continued)

test / strain	URE	NIT	EST	HIP	GGT	TTC	PyrA	ArgA	AspA	PAL	H_2S	GLU	SUT	NAL	CFZ	ACE	PROP	MLT	CIT	ERO	OXI
COL21	–	+	+	+	+	+	+	–	–	+	–	–	+	–	+	–	–	+	+	–	+
COL22	–	+	+	+	–	+	+	–	–	+	–	–	+	–	+	–	–	+	–	–	+
COL23	–	+	+	+	+	+	+	–	–	+	–	–	–	–	+	–	–	+	–	–	+
COL24	–	+	+	+	–	+	+	–	–	+	–	–	+	–	+	–	–	+	–	–	+
COL25	–	+	+	+	–	+	+	–	–	+	–	–	+	–	+	–	–	–	+	–	+
COL26	–	+	+	+	–	+	+	–	–	+	–	–	+	–	–	–	–	+	+	–	+
COL27	–	+	+	+	–	–	+	–	–	+	–	–	+	–	+	–	–	+	+	–	+
COL28	–	–	+	+	–	+	+	–	–	+	–	–	+	–	+	–	–	+	–	–	+
COL29	–	–	+	+	–	–	+	–	–	+	–	–	+	–	–	–	–	+	+	–	+
COL30	–	–	+	+	–	–	+	–	–	+	–	–	+	–	–	–	–	+	+	–	+
RPH1	–	+	+	+	+	+	+	–	–	+	–	–	+	–	+	–	–	–	–	–	+

Table 3.1: (continued)

test / strain	URE	NIT	EST	HIP	GGT	TTC	PyrA	ArgA	AspA	PAL	H_2S	GLU	SUT	NAL	CFZ	ACE	PROP	MLT	CIT	ERO	OXI
RPH2	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	−	−	+
RPH3	−	+	+	+	+	+	−	−	−	+	−	−	+	−	+	−	−	+	−	−	+
RPH4	−	+	+	+	−	+	−	−	−	+	−	−	+	−	+	−	−	+	+	−	+
RPH5	−	+	+	+	−	+	−	−	−	+	−	−	+	−	+	−	−	+	+	−	+
RPH6	−	+	+	+	−	+	+	−	−	+	−	−	+	−	+	−	−	+	−	−	+
RPH7	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	+	−	+
RPH8	−	+	+	+	−	+	+	−	−	+	−	−	+	−	+	−	−	+	+	−	+
RPH10	−	+	+	+	+	+	+	−	−	+	−	−	+	−	+	−	−	+	−	−	+
Total%*	0	92.3	100	100	51.3	84.6	76.9	0	0	100	0	0	97.4	0	84.6	97.4	0	92.3	64.1	0	100

*Percentage of isolates that gave a positive result in the test concerned.

Table 3.2: Results of API CAMPY test

strain	Identified strain (substrain)	Percentage of identification (ID score)	Test against*
COL1	*C. jejuni jejuni* 1	99.4	–
COL2	*C. jejuni jejuni* 2	99.9	PyrA 15%
COL3	*C. jejuni jejuni* 1	98.4	PyrA 10%
COL4	*C. jejuni jejuni* 1	99.3	–
COL5	*C. jejuni jejuni* 1	99	–
COL6	*C. jejuni jejuni* 2	99.7	PyrA 15%, CFZ 93%, CITa 25%
COL7	*C. jejuni jejuni* 1	99.6	PyrA 10%, CFZ 84%,
COL8	*C. jejuni jejuni* 1	99.4	–
COL9	*C. jejuni jejuni* 1	99.4	MLTa 84%
COL10	*C. jejuni jejuni* 2	99.8	PyrA 15%, CITa 25%
COL11	*C. jejuni jejuni* 2	99.8	PyrA 15%, CITa 25%
COL12	*C. jejuni jejuni* 2	99.8	CITa 25%
COL13	*C. jejuni jejuni* 2	99.9	PyrA 15%
COL14	*C. jejuni jejuni* 2	99.8	PyrA 15%, CITa 25%
COL15	*C. jejuni jejuni* 2	99.9	PyrA 15%, CITa 25%
COL16	*C. jejuni jejuni* 2	99.8	PyrA 15%, CITa 25%
COL17	*C. jejuni jejuni* 2	99.8	PyrA 15%, CITa 25%
COL18	*C. jejuni jejuni* 2	99.8	PyrA 15%, CFZ 93%,
COL19	*C. jejuni jejuni* 2	99.9	PyrA 15%, ArgA 9%, CITa 25%
COL20	*C. jejuni jejuni* 2	99.8	PyrA 15%, CITa 25%

Table 3.2: (continued)

strain	Identified strain (substrain)	Percentage of identification (ID score)	Test against
COL21	*C. jejuni jejuni* 2	99.8	PyrA 15%, CITa 25%
COL22	*C. jejuni jejuni* 1	98.7	PyrA 10%
COL23	*C. jejuni jejuni* 2	99.8	PyrA 15%, SUT 96%
COL24	*C. jejuni jejuni* 1	98.7	PyrA 10%
COL25	*C. jejuni jejuni* 1	99.1	PyrA 10%, MLTa 84%
COL26	*C. jejuni jejuni* 1	99.6	PyrA 10%, CFZ 84%
COL27	*C. jejuni jejuni* 1	98.9	PyrA 10%
COL28	*C. jejuni jejuni* 1	98.7	PyrA 10%
COL29	*C. jejuni jejuni* 1	88.3	NIT 98%, PyrA 10%, CFZ 84%
COL30	*C. jejuni jejuni* 1	88.3	NIT 98%, PyrA 10%, CFZ 84%
RPH1	*C. jejuni jejuni* 2	99.9	PyrA 15%, MLTa 84%
RPH2	*C. jejuni jejuni* 2	99.9	PyrA 15%
RPH3	*C. jejuni jejuni* 2	99.8	–
RPH4	*C. jejuni jejuni* 1	99.4	–
RPH5	*C. jejuni jejuni* 1	99.4	–
RPH6	*C. jejuni jejuni* 1	98.7	PyrA 10%
RPH7	*C. jejuni jejuni* 2	99.8	PyrA 15%, CITa 25%
RPH8	*C. jejuni jejuni* 1	99.1	PyrA 10%
RPH10	*C. jejuni jejuni* 2	99.9	PyrA 15%

*Test against, tests not compatible with given identity.

Figure 3.2: PCR to confirm the presence of hippuricase (*hipO*) gene in *C. jejuni* strains.

DNA was extracted from whole bacterial cells and used as a template for PCR using primers for *hipO* gene (see section 2.10.1.2).

M: 1 kb DNA ladder

Lane 1: *C. jejuni* 81-176

Lane 2: *C. jejuni* 81-176 *cdtB* mutant

Lane 3: *C. jejuni* 11168

Lane 4: *C. jejuni* 11168 *cdtB* mutant

Lane 5: *C. jejuni* 11168 *cdtA* mutant

Lane 6: *C. jejuni* COL7

Lane 7: *C. coli* WA 585

hipO gene amplicon: 1130 bp (indicated with the arrow)

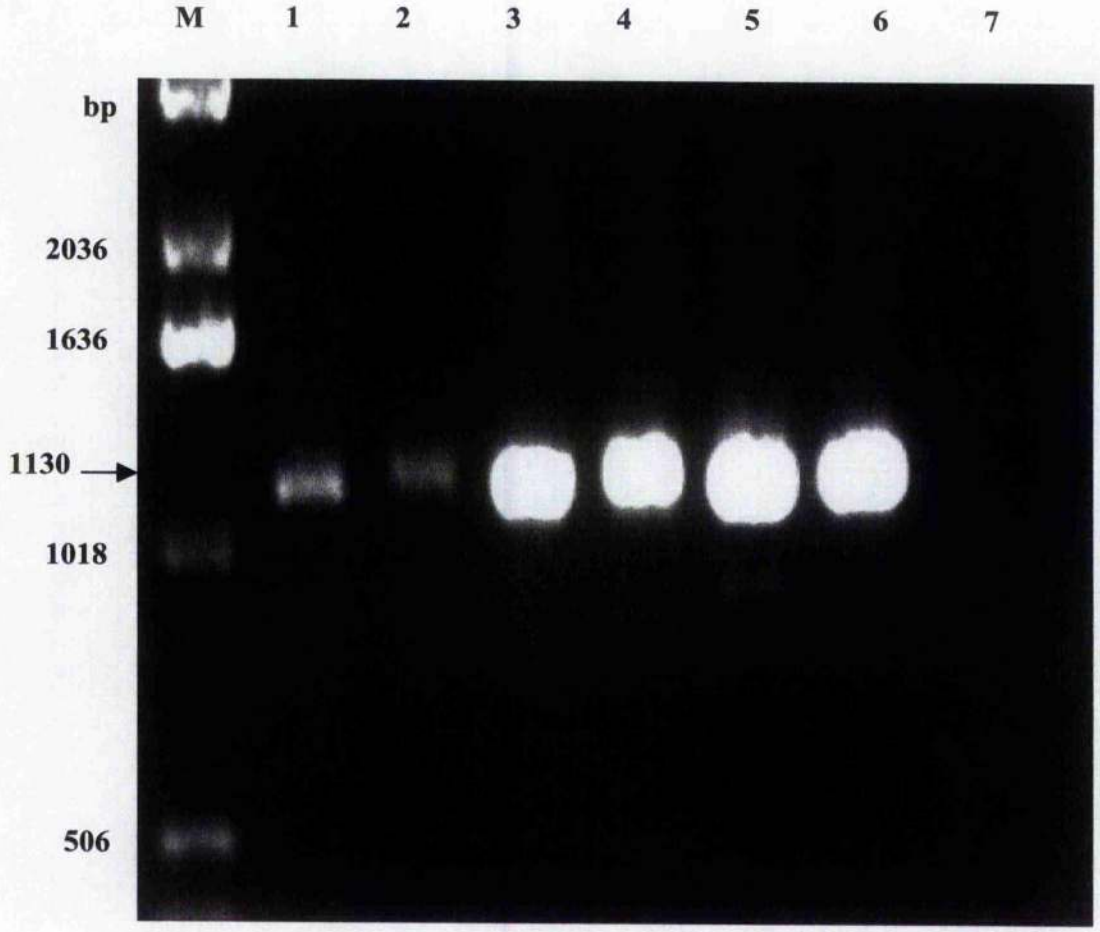

M
1
2
3
4
5
6
7
bp
2036
1636
1130
1018
506

Figure 3.3: SDS-PAGE of cell-free extracts and culture supernatants from *C. jejuni* strains and control strains

Cell-free extracts (with two different centrifugation speeds and times) and culture supernatants of *C. jejuni* clinical isolates (COL1 and COL7) and two *E. coli* strains (E32511 and DH5α) as controls were subjected to electrophoresis on a 10% (w/v) polyacrylamide gel and stained with 0.1% (w/v) Coomassie blue solution (section 2.5).

Lane 1: *C. jejuni* COL1, cell-free extract, after centrifugation at 3,600 ×g/10 min

Lane 2: *C. jejuni* COL1, cell-free extract, after centrifugation at 22,000 ×g/30 min

Lane 3: *C. jejuni* COL7, cell-free extract, after centrifugation at 3,600 ×g/10 min

Lane 4: *C. jejuni* COL7, cell-free extract, after centrifugation at 22,000 ×g/30 min

Lane 5: *C. jejuni* COL1, culture supernatant

Lane 6: *C. jejuni* COL7, culture supernatant

Lane 7: Protein marker (covers a molecular weight range of 10-250 kDa)

Lane 8: *E. coli* E32511, culture supernatant

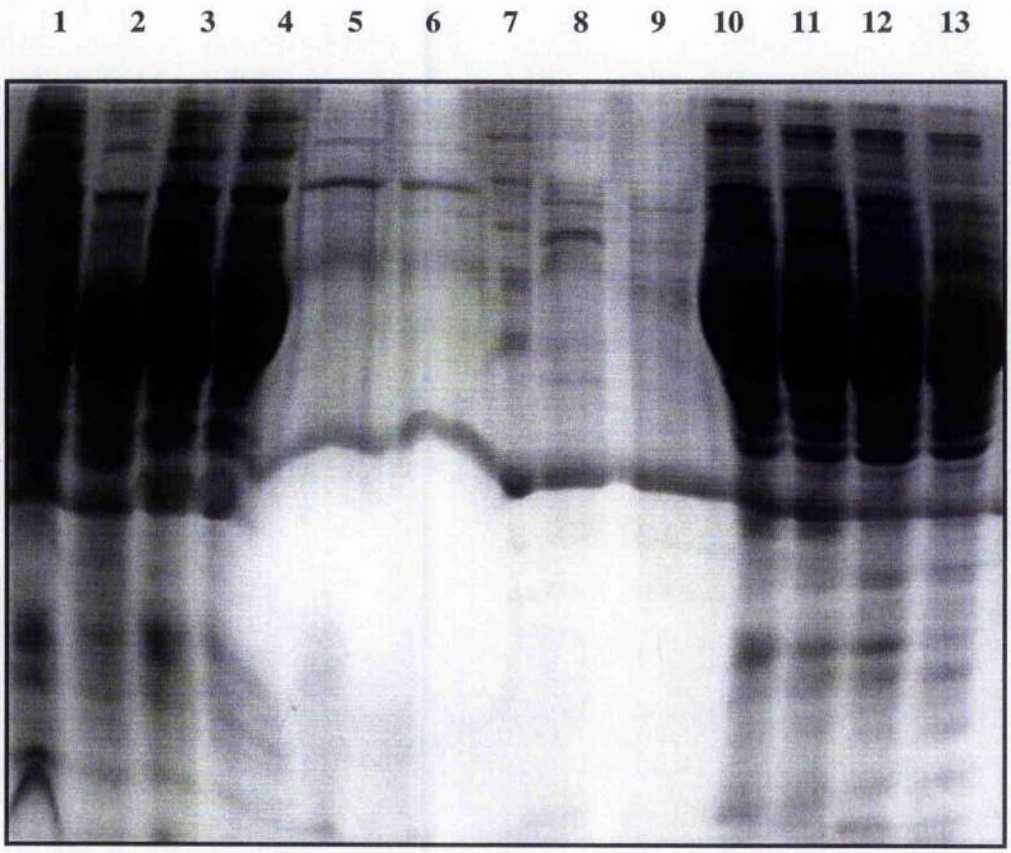
Lane 9: *E. coli* DH5α, culture supernatant

Lane 10: *E. coli* E32511, cell-free extract, after centrifugation at 3,600 ×g/10 min

Lane 11: *E. coli* E32511, cell-free extract, after centrifugation at 22,000 ×g/30 min

Lane 12: *E. coli* DH5α, cell-free extract, after centrifugation at 3,600 ×g/10 min

Lane 13: *E. coli* DH5α, cell-free extract, after centrifugation at 22,000 ×g/30 min


1 2 3 4 5 6 7 8 9 10 11 12 13

culture supernatant, presumely due to the salt content of the growth medium. Differences were apparent between protein bands present in *E. coli* and *C. jejuni* extracts, but no noticeable difference could be seen between high-speed and low-speed centrifugation extracts.

3.4 Cytotoxicity assay

3.4.1 Cytotoxicity assay of *C. jejuni* culture supernatant

After protein estimation, filtered and concentrated culture supernates were tested on Vero cells for cytotoxicity. Some low cytotoxic activity after incubation for 16h was noted, but it was much less than that obtained with cell-free extracts. This result suggests that although some cytotoxin activity may be secreted into the culture medium, it was too little to warrant further investigation.

3.4.2 Cytotoxicity assay of cell-free extracts of *C. jejuni* strains on Vero cells

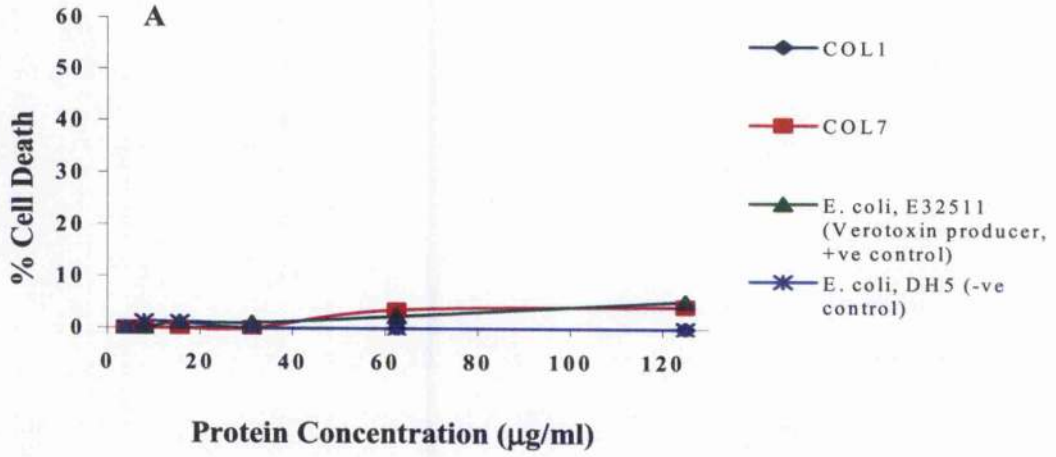
Cell-free extracts obtained after two different centrifugation speeds and time were tested in the cytotoxicity assay. Cell-free extracts from high-speed centrifugation (22,000 ×g) for 30 min and low-speed centrifugation (3,600 ×g) for 10 min of clinical isolates strains COL1 and COL7 were tested for cytotoxicity on Vero cells with different incubation times; 1h, 4h, 8h, 16h, and 24h (Fig. 3.4 to 3.8). Clinical isolates COL1 and COL7 were chosen for cytotoxicity based on previous preliminary data (D. Stewart-Tull, personal communication), which had indicated that COL1 and COL7 had clear cytotoxic activity against Vero cells.

Figure 3.4: Comparison of cytotoxicity of cell-free extracts, obtained after different centrifugation speeds and time on Vero cells after incubation for 1h.

The cytotoxic activities of cell-free extracts of two *C. jejuni* clinical isolates (COL1 and COL7) and two *E. coli* strains (E32511 and DH5α), as positive and negative controls, were determined by MTT assay using two-fold serial dilutions of the extracts.

A. Cytotoxicity of cell-free extracts (low-speed centrifugation) on Vero cells after 1h

B. Cytotoxicity of cell-free extracts (high-speed centrifugation) on Vero cells after 1h


A
% Cell Death
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Protein Concentration (µg/ml)
COL1
COL7
E. coli, E32511 (Verotoxin producer, +ve control)
E. coli, DH5 (-ve control)

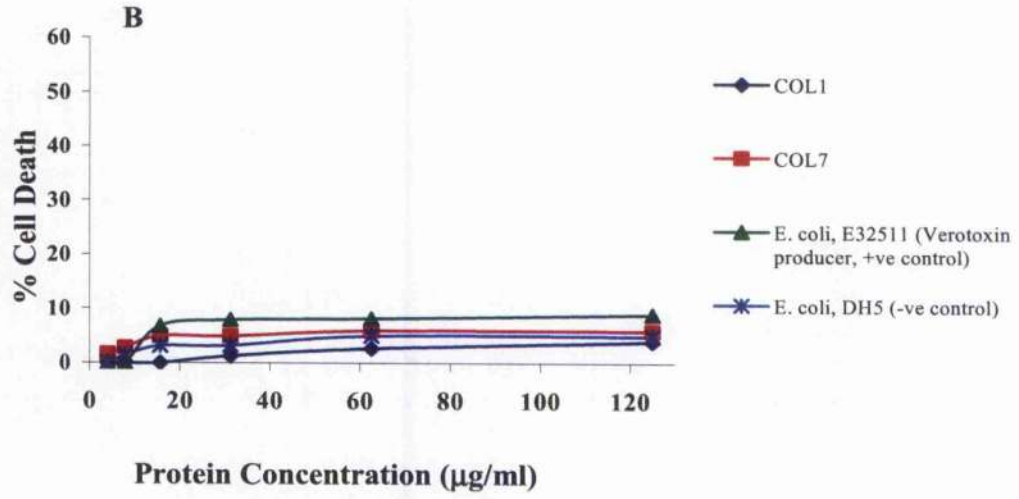
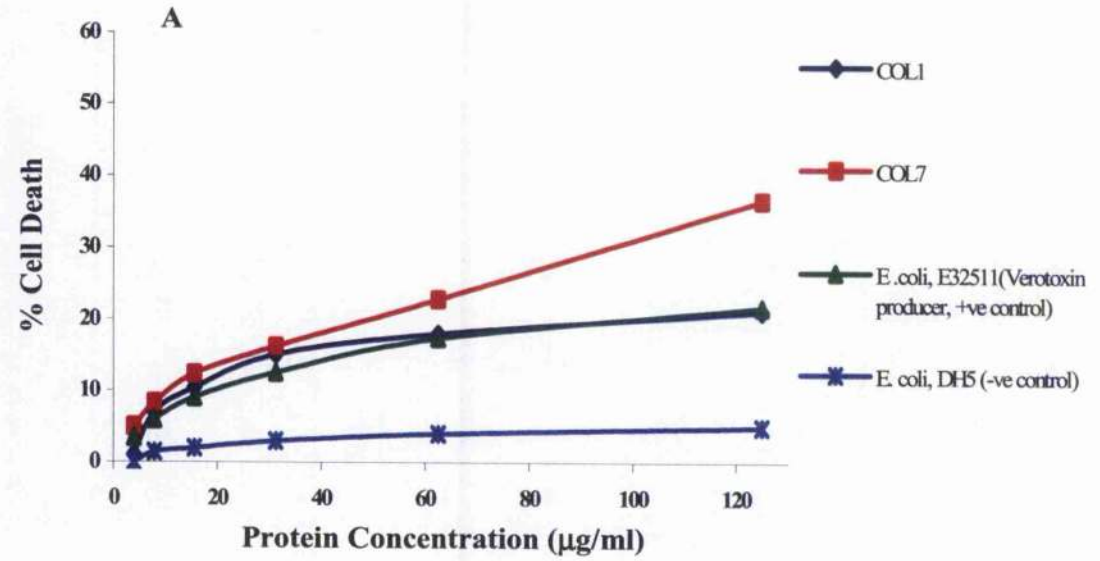

B
% Cell Death
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Protein Concentration (µg/ml)
COL1
COL7
E. coli, E32511 (Verotoxin producer, +ve control)
E. coli, DH5 (-ve control)

Figure 3.5: Comparison of cytotoxicity of cell-free extracts, obtained after different centrifugation speeds and time on Vero cells after incubation for 4h.

The cytotoxic activities of cell-free extracts of two *C. jejuni* clinical isolates (COL1 and COL7) and two *E. coli* strains (E32511 and DH5α), as positive and negative controls, were determined by MTT assay using two-fold serial dilutions of the extracts.

A. Cytotoxicity of cell-free extracts (low-speed centrifugation) on Vero cells after 4h

B. Cytotoxicity of cell-free extracts (high-speed centrifugation) on Vero cells after 4h


A
% Cell Death
Protein Concentration (µg/ml)
COL1
COL7
E.coli, E32511(Verotoxin producer, +ve control)
E. coli, DH5 (-ve control)

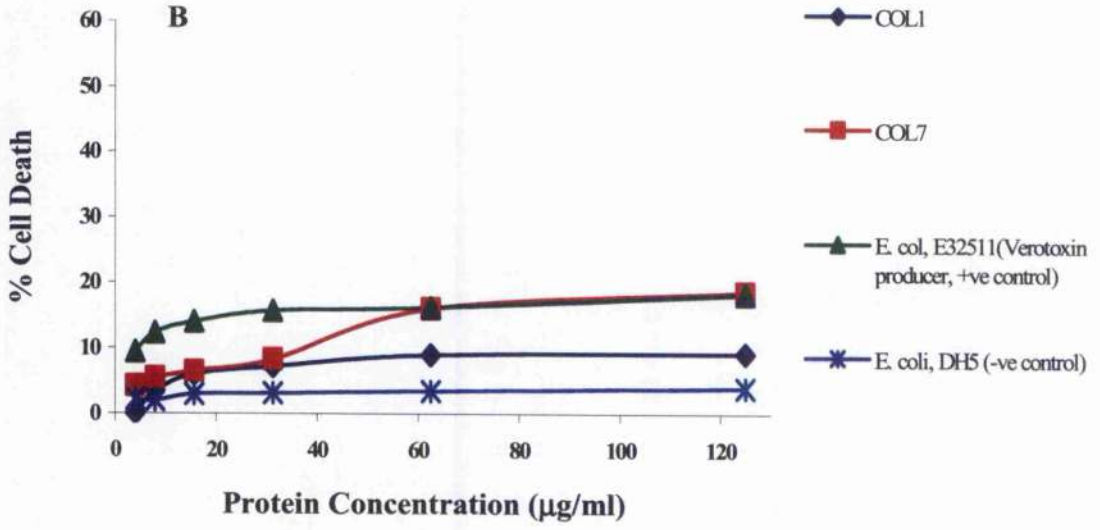
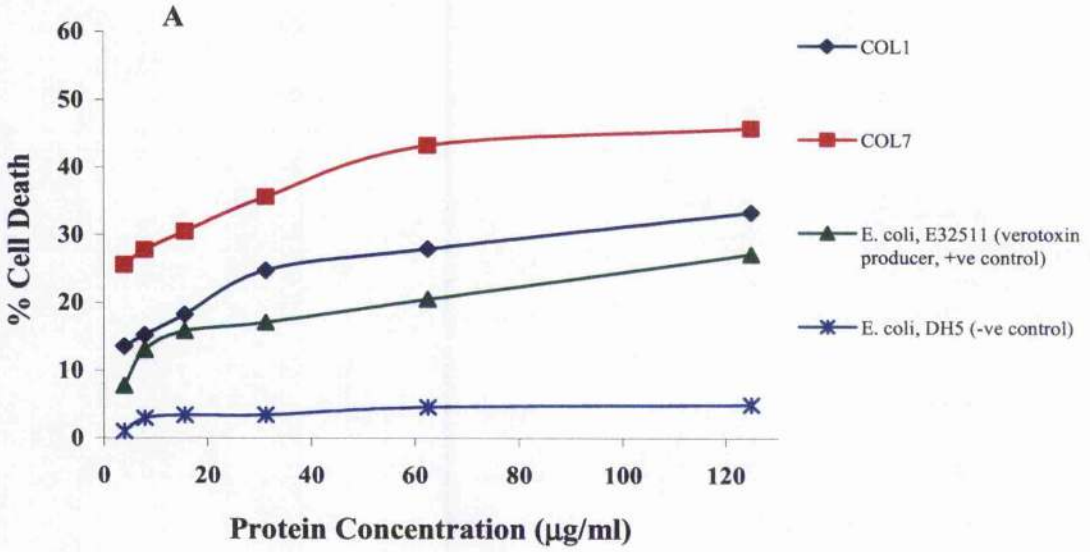

B
% Cell Death
Protein Concentration (µg/ml)
COL1
COL7
E. col, E32511(Verotoxin producer, +ve control)
E. coli, DH5 (-ve control)

Figure 3.6: Comparison of cytotoxicity of cell-free extracts, obtained after different centrifugation speeds and time on Vero cells after incubation for 8h.

The cytotoxic activities of cell-free extracts of two *C. jejuni* clinical isolates (COL1 and COL7) and two *E. coli* strains (E32511 and DH5α), as positive and negative controls, were determined by MTT assay using two-fold serial dilutions of the extracts.

A. Cytotoxicity of cell-free extracts (low-speed centrifugation) on Vero cells after 8h

B. Cytotoxicity of cell-free extracts (high-speed centrifugation) on Vero cells after 8h


A
% Cell Death
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Protein Concentration (µg/ml)
COL1
COL7
E. coli, E32511 (verotoxin producer, +ve control)
E. coli, DH5 (-ve control)

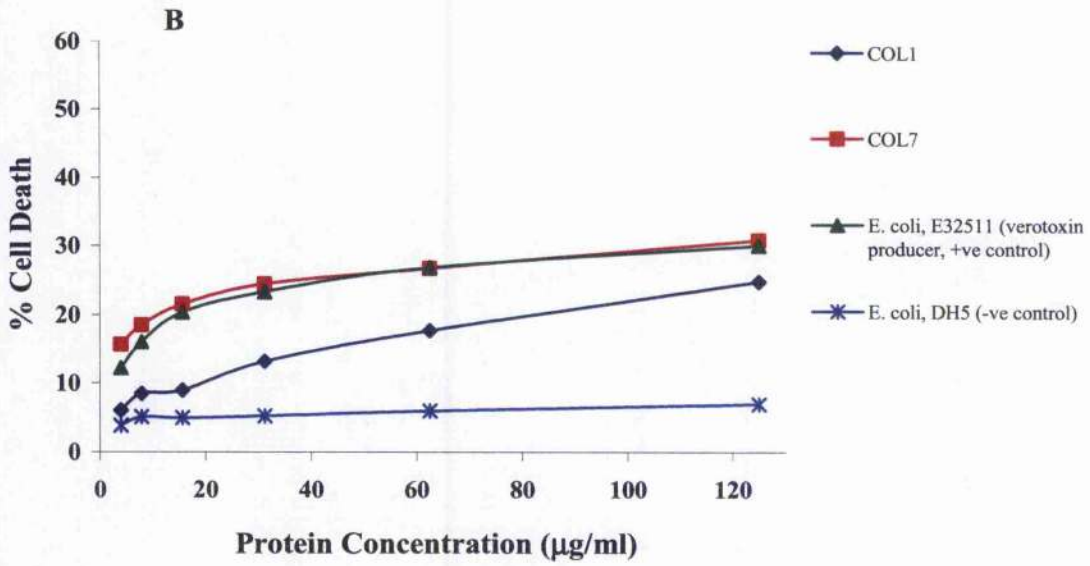
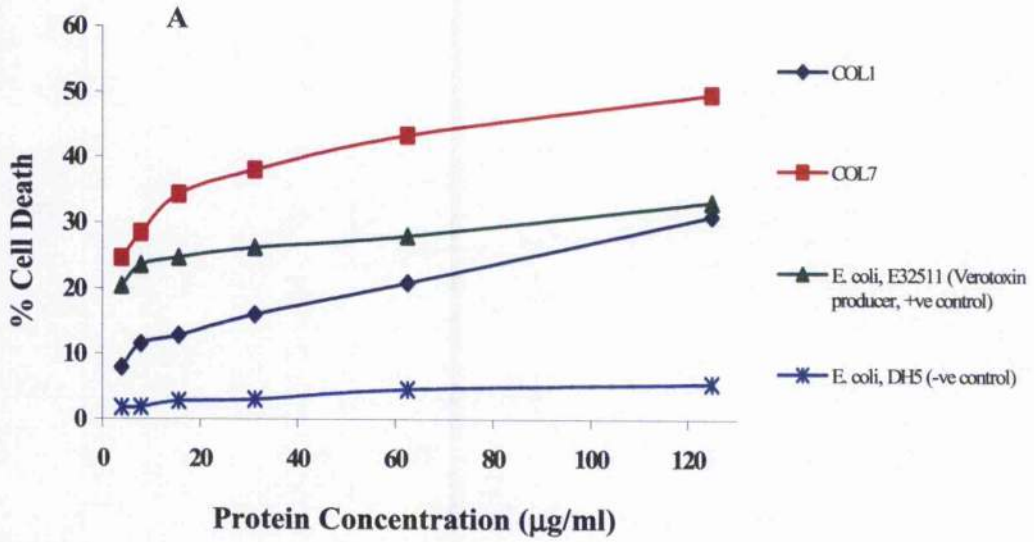

B
% Cell Death
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Protein Concentration (µg/ml)
COL1
COL7
E. coli, E32511 (verotoxin producer, +ve control)
E. coli, DH5 (-ve control)

Figure 3.7: Comparison of cytotoxicity of cell-free extracts, obtained after different centrifugation speeds and time on Vero cells after incubation for 16h.

The cytotoxic activities of cell-free extracts of two *C. jejuni* clinical isolates (COL1 and COL7) and two *E. coli* strains (E32511 and DH5α), as positive and negative controls, were determined by MTT assay using two-fold serial dilutions of the extracts.

A. Cytotoxicity of cell-free extracts (low-speed centrifugation) on Vero cells after 16h

B. Cytotoxicity of cell-free extracts (high-speed centrifugation) on Vero cells after 16h


A
% Cell Death
Protein Concentration (µg/ml)
COL1
COL7
E. coli, E32511 (Verotoxin producer, +ve control)
E. coli, DH5 (-ve control)

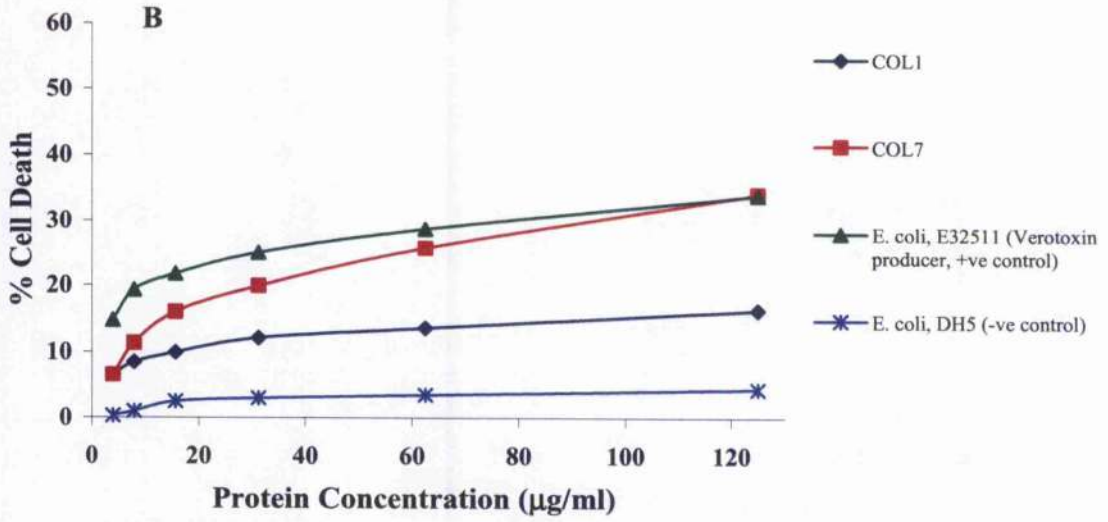
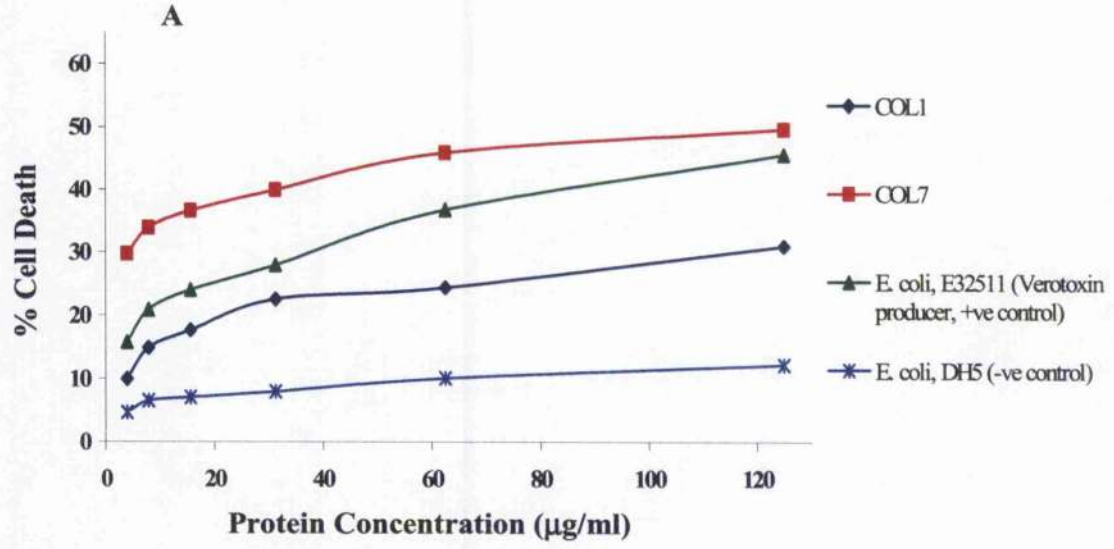

B
% Cell Death
Protein Concentration (µg/ml)
COL1
COL7
E. coli, E32511 (Verotoxin producer, +ve control)
E. coli, DH5 (-ve control)

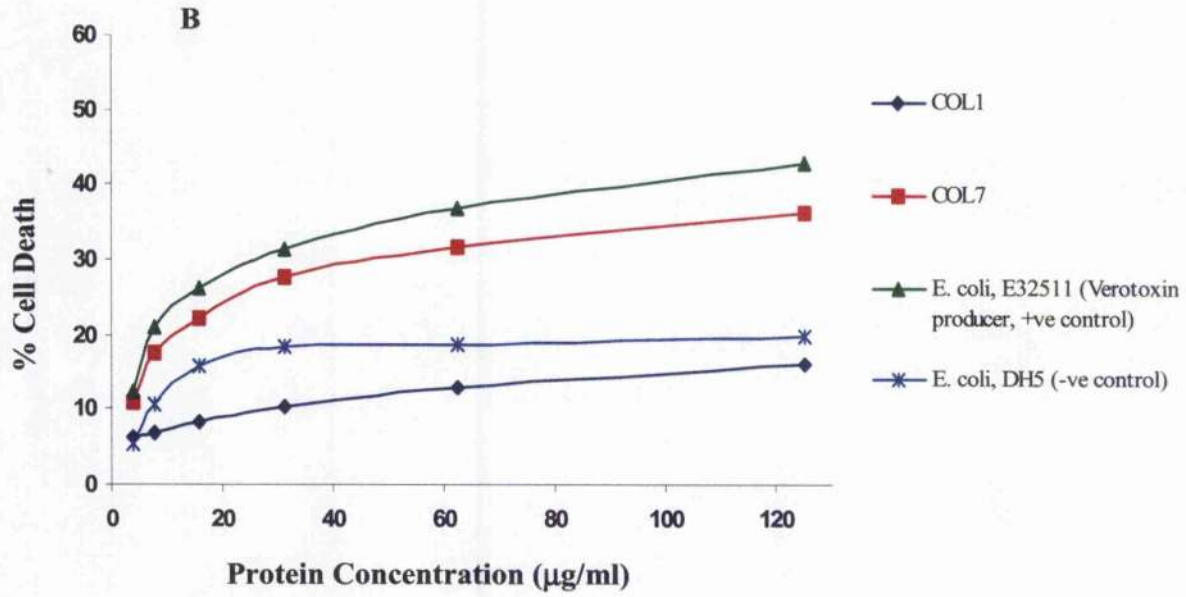
Figure 3.8: Comparison of cytotoxicity of cell-free extracts, obtained after different centrifugation speeds and time on Vero cells after incubation for 24h.

The cytotoxic activities of cell-free extracts of two *C. jejuni* clinical isolates (COL1 and COL7) and two *E. coli* strains (E32511 and DH5α), as positive and negative controls, were determined by MTT assay using two-fold serial dilutions of the extracts.

A. Cytotoxicity of cell-free extracts (low-speed centrifugation) on Vero cells after 24h

B. Cytotoxicity of cell-free extracts (high-speed centrifugation) on Vero cells after 24h


A
% Cell Death
0
10
20
30
40
50
60
0
20
40
60
80
100
120
Protein Concentration (µg/ml)
COL1
COL7
E. coli, E32511 (Verotoxin producer, +ve control)
E. coli, DH5 (-ve control)


B
% Cell Death
0
10
20
30
40
50
60
0
20
40
60
80
100
120
Protein Concentration (µg/ml)
COL1
COL7
E. coli, E32511 (Verotoxin producer, +ve control)
E. coli, DH5 (-ve control)

The results showed that the cytotoxicity was at maximum after 16h incubation and also that the cytotoxicity caused by the cell-free extract from low-speed centrifugation was greater (~50% cell death at the high concentration of COL7 extract) than that of the cell-free extract from high-speed centrifugation (30% cell death at high concentration of COL1 extract). For this reason, the extract prepared by low-speed centrifugation was chosen for subsequent assays. At this time point, *E. coli* DH5α (a non-toxin-producing strain) showed little or no cytotoxicity while the *C. jejuni* strains and *E. coli* E32511 (Verotoxin producer) showed obvious cytotoxicity. The low speed cell extract of COL7 had greater cytotoxin activity than that of *E. coli* E32511 where as the high speed cell extract of this *E. coli* strain had a greater effect.

3.4.3 PCR results to confirm genotype of CDT^+ and CDT^-

A cytolethal distending toxin (CDT) has been described in *C. jejuni* (see section 1.5.2.3). This toxin has been reported to show clear cytotoxic effects only after incubation for about 72h. However, it was important to discount any contribution that CDT might have to the cytotoxicity noted here at 16-24h. For this purpose, cytotoxic activity of cell-free extracts from CDT^- mutants were compared with those from the wild-type *C. jejuni* strains. PCR was performed to confirm the genotypes of the CDT^- mutants in the type strains 81-176 and 11168 using primers for the *cdtB* gene (Fig. 3.9) and for strain 11168 using primers for the *cdtA* gene (Fig. 3.10). The results for *cdtB* gene showed an amplimer of 495 bp from each of the wild-type strains and larger amplimer of 1200 bp from each of the *cdtB* mutant strains (Fig. 3.9). The increased sizes of the amplimers from the mutant strains were consistent

Figure 3.9: Agarose gel electrophoresis of PCR products to confirm the genotype of CDT+ and CDT$^-$ strains of *C. jejuni*

DNA was extracted from whole bacterial cells and used as a template. PCR was performed according to section 2.10.1.2 using primers CJCF and CJCR for *cdtB* gene (Table 2.4).

Lane M: 1 kb DNA Ladder

Lane 1: C. *jejuni* 11168 (CDT$^+$, wild-type)

Lane 2: *C. jejuni* 11168 (CDT$^-$, *cdtB* mutant)

Lane 3: *C. jejuni* 81-176 (CDT$^+$, wild-type)

Lane 4: *C. jejuni* 81-176 (CDT$^-$, *cdtB* mutant)

cdtB gene amplimer: 495bp

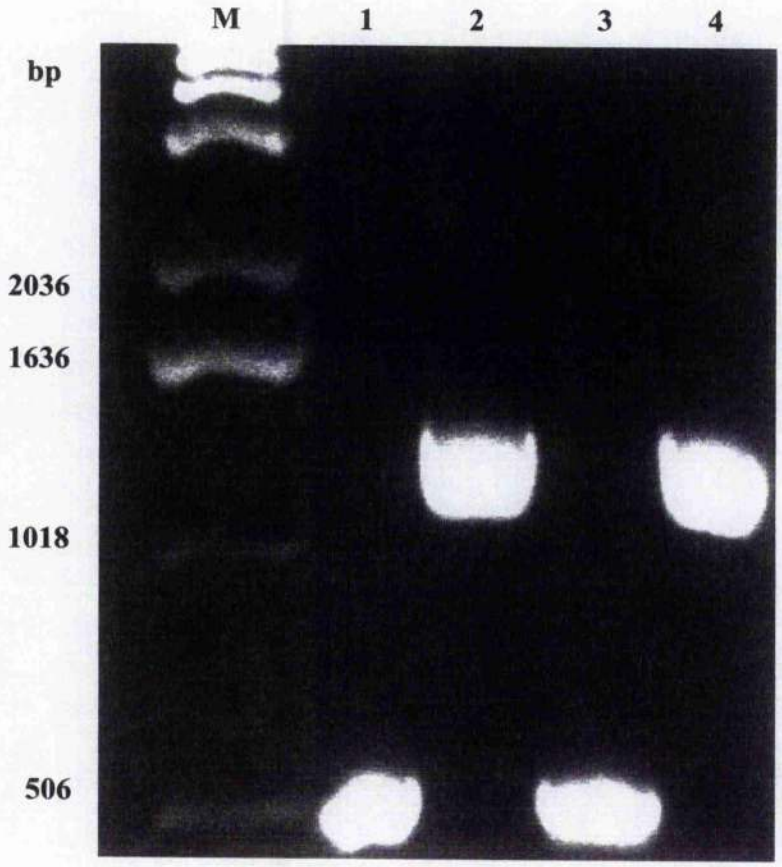

M
1
2
3
4
bp
2036
1636
1018
506

Figure 3.10: Agarose gel electrophoresis of PCR products to confirm the genotype of CDT+ and CDT⁻ strains

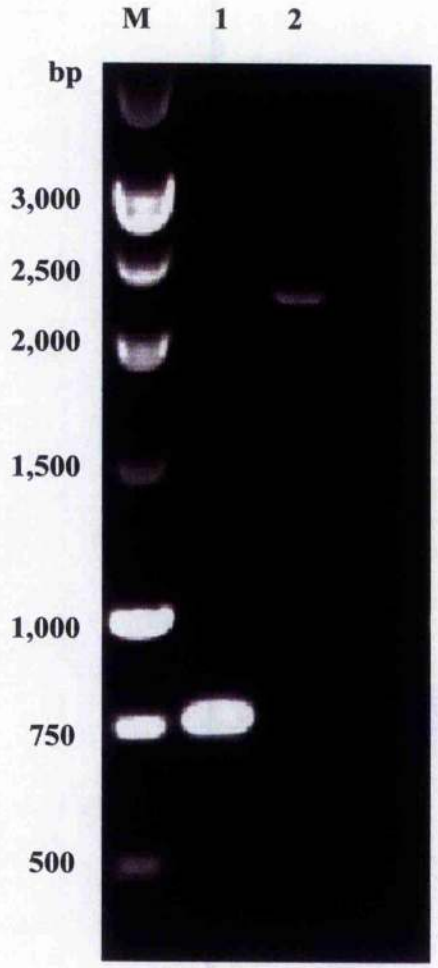
DNA was extracted from whole bacterial cells and used as a template. PCR was performed according to section 2.10.1.2 using primers CDTAF and CDTAR for *cdtA* gene (Table 2.4).

Lane M: 1 kb DNA ladder

Lane 1: *C. jejuni* 11168

Lane 2: *C. jejuni* 11168 (*cdtA* mutant)

cdtA gene amplimer: 779bp


M
1
2
bp
3,000
2,500
2,000
1,500
1,000
750
500

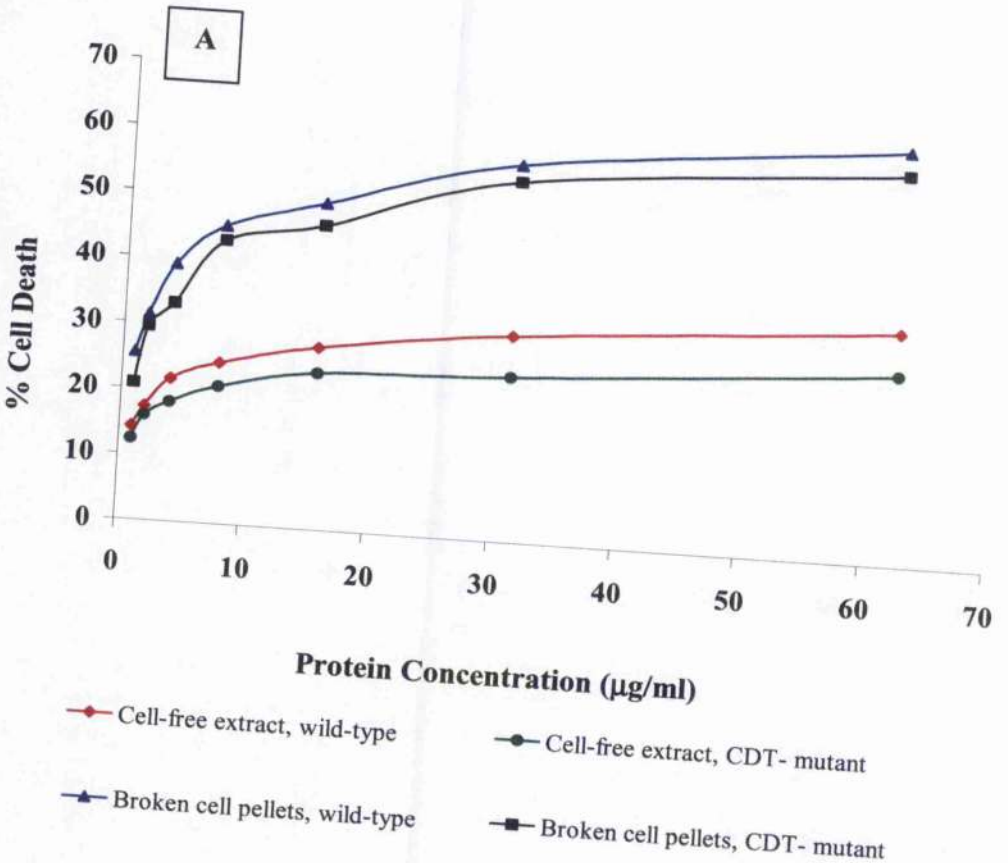
with the incorporation of a 700 bp chloramphenicol-resistance cassette within the *cdtB* gene. A larger amplimer of 2100 bp was obtained from the *cdtA* mutant, consistent with incorporation of a kanamycin resistance cassette (1300 bp) into this gene which produced a 779 bp amplimer from the wild-type strain (Fig. 3.10).

3.4.4 Cytotoxicity assay on Vero cells with wild-type and *cdt* mutant strains

The previous results (section 3.4.2) suggested that the cytotoxic activity may be membrane-associated and therefore retained more in the supernatant (cell-free extract) after low-speed centrifugation. For this reason broken cell pellets were also retained for assay as well as the supernatant cell-free extract after low-speed centrifugation. Cell-free extracts and broken cell-pellets were prepared by low-speed centrifugation of wild-type strain 81-176, the *cdtB* mutant of strain 81-176, wild-type strain 11168, and the *cdtB* and *cdtA* mutants of 11168 and these were tested for cytotoxicity on Vero cells after incubation for 16h and 72h. The longer incubation time was chosen because of the report that the cytotoxic effects of cytolethal distending toxin only become apparent after incubation for 72h. Figs. 3.11 A and B show that the cytotoxicity of cell-free extracts and broken cell pellets of the wild type and CDT$^-$ mutant strains of 81-176 and 11168 after 16h incubation were similar. The cytotoxicity of the broken cell pellets after low-speed centrifugation was noticeably higher than that of the cell-free extracts. After incubation for 16h or 72h, cell-free extracts of 81-176 wild-type and *cdtB* mutant were more active on Vero cells than the extracts from strains 11168 wild-type and *cdtB* mutant (Fig. 3.12). These results indicated that the cell-free extracts and broken cell pellets of the CDT$^-$ mutants still retained cytotoxic activity to approximately similar levels to that of the wild type

Figure 3.11: Cytotoxicity of cell-free extracts and broken cell pellets of *C. jejuni* strains on Vero cells after incubation for 16h.

The cytotoxic activities of cell-free extracts and broken cell pellets from low-speed centrifugation of (A) *C. jejuni* NCTC 11168 wild-type and *cdtB* mutant and (B) 81-176 wild-type and *cdtB* mutant strains were determined by MTT assay in two-fold serial dilutions using Vero cells. Cell death was assessed after incubation for 16h.


A
% Cell Death
0
10
20
30
40
50
60
70
Protein Concentration (µg/ml)
Cell-free extract, wild-type
Cell-free extract, CDT- mutant
Broken cell pellets, wild-type
Broken cell pellets, CDT- mutant

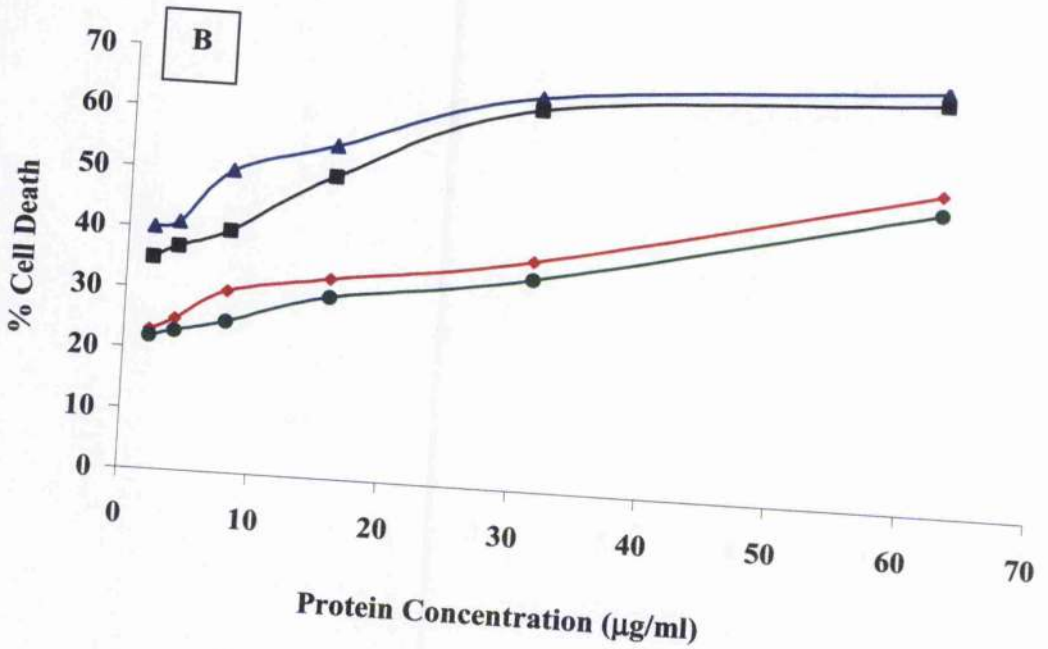
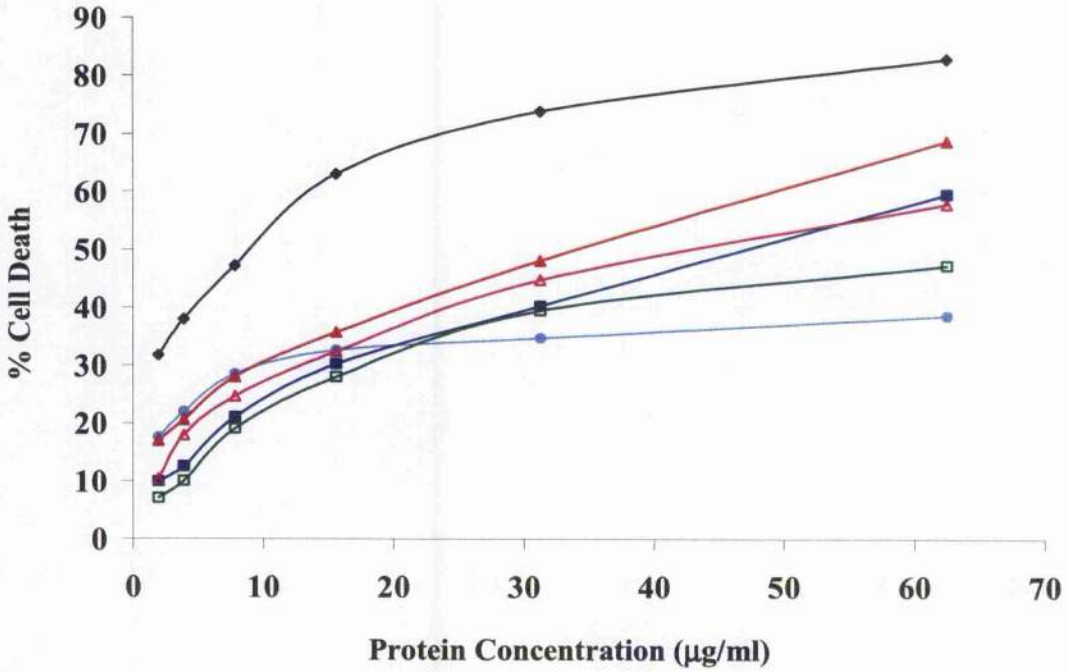

B
% Cell Death
0
10
20
30
40
50
60
70
Protein Concentration (µg/ml)

Figure 3.12: Cytotoxicity of cell-free extracts of *C. jejuni* strains on Vero cells after incubation for 72h.

The cytotoxic activities of cell-free extracts (low-speed centrifugation) of *C. jejuni* 81-176 (wild-type and *cdtB* mutant), 11168 (wild-type and *cdtB* mutant) and two *E. coli* strains (E32511 and DH5α) as positive and negative controls were determined by MTT assay in two-fold serial dilutions using Vero cells. Cell death was assessed after incubation for 72h.


90
80
70
60
50
40
30
20
10
0
% Cell Death
0
10
20
30
40
50
60
70
Protein Concentration (µg/ml)
E. coli E32511 (Verotoxin producer, +ve control)
E. coli DH5 (-ve control)
C. jejuni 81-176
C. jejuni 81-176 cdtB mutant
C. jejuni 11168
C. jejuni 11168 cdtB mutant

strains, indicating that cytotoxicity was not caused by CDT. Also Fig. 3.12 showed that positive control *E. coli* E32511 had greatest effect.

Microscope images of Vero cells after incubation for 72h showed that distension of cells was apparent with extracts from the wild type strain, but not with the CDT$^-$ mutant or control cells (Figs. 3.13 to 3.15).

The trypan blue exclusion dye test was used to differentiate and count the living and dead Vero cells after incubation with the bacterial preparations. The results are expressed as the percentage of dead cells compared to the total number of cells. This test was performed on the Vero cells after incubation for 48, 72 and 96h (Table 3.3). The trypan blue data essentially agreed with the data obtained from the MTT cytotoxicity assay. Broken cell pellets had greater activity than cell-free extracts and *C. jejuni* strain 81-176 exhibited greater activity than strain 11168. The *cdtA* mutant of strain 11168 was included in this analysis and gave similar results to the *cdtB* mutant and the wild-type strain. Morphological changes, including cell rounding, were accompanied by cell death after incubation for 16h.

3.4.5 Use of PCR to identify the presence of *virB11* and *tetO* genes in *C. jejuni* strains

PCR was performed to detect the presence of the *virB11* and *tetO* genes in clinical isolates and type strains of *C. jejuni,* and *cdtA* and *cdtB* mutants. It has been reported that *C. jejuni* strain 81-176 contains two plasmids, each of which is approximately 35 kb in size. pTet carries the *tetO* gene, and four open reading frames have been identified in the second plasmid, pVir, one of which is *virB11*, thought to be involved in the virulence (adhesion and invasion) of *C. jejuni* 81-176 (see section 1.5.1).

Figure 3.13: Untreated Vero cells (control) after incubation for 72h

Note the normal, spindle-shaped appearance of the cells. Photograph was taken at a magnification of ×40.

Figure 3.14: Vero cells treated with cell-free extract (low-speed centrifugation) of *C. jejuni* 11168

Vero cell monolayer incubated with 62.5 μg/ml protein cell-free extract (low-speed centrifugation) of *C. jejuni* 11168 for 72h. The arrows show (A) the grossly distended cells (CDT-treated) and (B) rounded-up dead cells. Photograph was taken at a magnification of ×40.

Figure 3.15: Vero cells treated with cell-free extract (low-speed centrifugation) of *C. jejuni* 11168 *cdtB* mutant

Vero cell monolayer incubated with 62.5 μg/ml protein of cell-free extract (low speed centrifugation) of *C. jejuni* 11168 *cdtB* mutant for 72h. Note that the majority of cells had the normal spindle-shaped appearance but some rounded-up dead cells (arrow) were visible. Photograph was taken at a magnification of ×40.

Table 3.3: Trypan blue exclusion dye test for the effects of *C. jejuni* low-speed cell-free extracts on Vero cells after incubation for 48, 72 and 96h with 60 μg/ml protein.

Trypan blue was added to the cells at the times shown and the percentage of cells taking up the dye was taken as the percentage of non-viable cells.

	Percentage of dead cells after		
Fractions	**48h**	**72h**	**96h**
C. jejuni 81-176, cell-free extract	48	55	58
C. jejuni 81-176, broken cell pellets	49.5	70	75
C. jejuni 81-176 *cdtB* mutant, cell-free extract	35	40	42
C. jejuni 81-176 *cdtB* mutant, broken cell pellets	41.5	56	55
C. jejuni 11168, cell-free extract	22.5	45	47
C. jejuni 11168, broken cell pellets	35	61	58
C. jejuni 11168 *cdtB* mutant, cell-free extract	34.5	37	40
C. jejuni 11168 *cdtB* mutant, broken cell pellets	46	51.5	54
C. jejuni 11168 *cdtA* mutant, cell-free extract	30	34	35
C. jejuni 11168 *cdtA* mutant, broken cell pellets	41.5	49	50
C. jejuni COL7, cell-free extract	24	45.5	46
C. jejuni COL7, broken cell pellets	30	59	60
E. coli E32511, cell-free extract	51.66	60	65
E. coli E32511, broken cell pellets	60	67.5	74
E. coli DH5α, cell-free extract	16	22	21.5
E. coli DH5α, broken cell pellets	18	27	29
Tissue culture medium	4	4	13
Triton-X 100	100	100	100

DNAs extracted (using the boiling method, section 2.10.1.1) from clinical isolates (COL1-30, RPH1-8 and 10), wild-type (81-176 and 11168), *cdt* mutants (81-176 *cdtB*, 11168 *cdtB* and 11168 *cdtA*), *tlyA* mutant (11168 *tlyA*) and *pldA* mutant (11168 *pldA*) were used as templates in the PCR reactions.

A PCR amplimer for *virB11* was obtained for 11 out of 39 (28.2%) of the clinical isolates (Table 3.4). Representative amplimers from some isolates are shown in Fig. 3.16 and 3.17. PCR products showed the presence of the *tetO* gene in 5 out of 39 (12.8%) of the clinical isolates (Table 3.4). Representative amplimers from some isolates are shown in Fig. 3.18 and 3.19. The *tetO* gene was identified in 3 (12%) of 25 human isolates and found in 3 (27.27%) of 11 *virB11*-positive *C. jejuni* clinical isolates compared with 2 (7.14%) of 28 *virB11*-negative isolates. The *virB11* gene was found in 3 (60%) of 5 *tetO*-positive isolates. It should be noted in particular that the strain 81-176 was positive and type strain 11168 was negative for both *virB11* and *tetO*.

C. jejuni strains were grown on blood agar containing tetracycline (20 μg/ml). The results showed that 12.8% of the clinical isolates strains were resistant to this concentration of tetracycline (Table 3.4) which was in agreement with the results obtained by PCR for the presence of the *tetO* gene (*i.* e. all tetracycline-resistant isolates carried the *tetO* gene). Table 3.5 summarises the distribution of the *virB11* and *tetO* genes in isolates from various sources. Results of *virB11* and *tetO* genes in Table 3.5 indicate the similar percentage distribution in human and chicken strains, and there is too few bovine and ovine strains to judge their distribution.

Figure 3.16: PCR results to determine the presence of *virB11* and *tetO* genes in *C. jejuni* 81-176 and 81-176 *cdtB* strains

DNA was extracted from bacterial cells by boiling (see section 2.10.1.1) and used as a template. PCR was performed according to section 2.10.1.2 using primers VIR BF, VIR BR, TET OF and TET OR for *virB11* and *tetO* genes, respectively (Table 2.4).

M: 1 kb DNA ladder

Lane 1: *virB11* in *C. jejuni* 81-176

Lane 2: *virB11* in *C. jejuni* 81-176 *cdtB* mutant

Lane 3: *tetO* in *C. jejuni* 81-176

Lane 4: *tetO* in *C. jejuni* 81-176 *cdtB* mutant

virB11 gene amplicon: 708bp

tetO gene amplicon: 559 bp

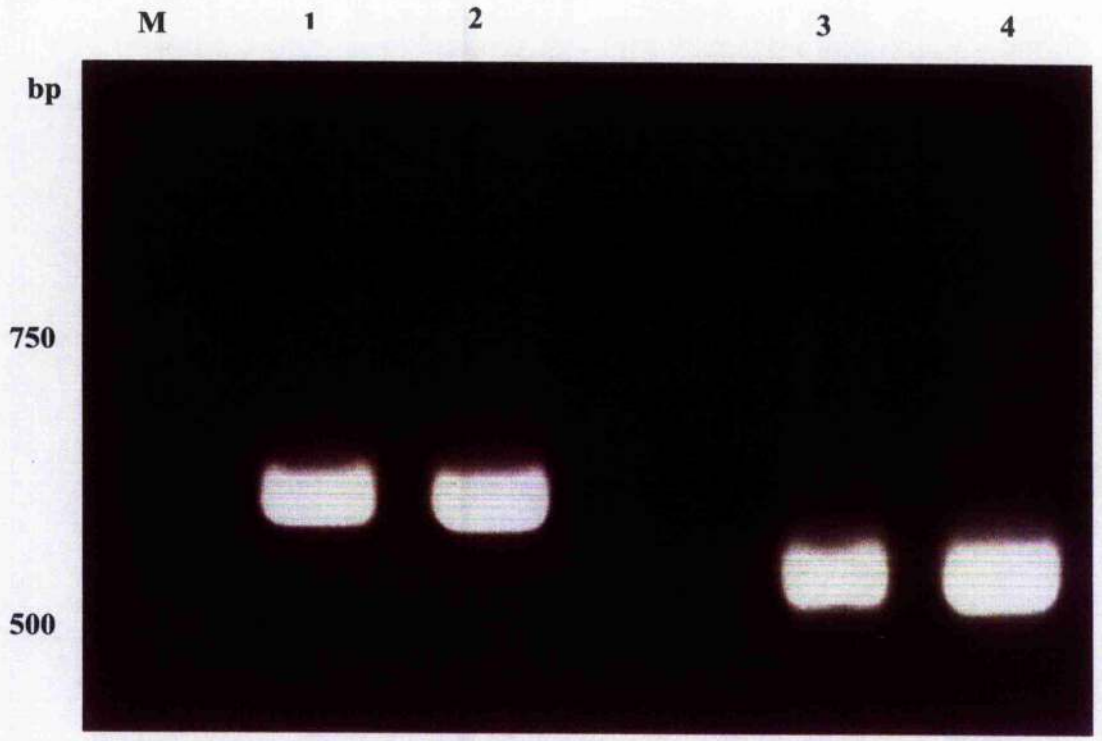

M
1
2
3
4
bp
750
500

Figure 3.17: PCR results to determine the presence of the *virB11* gene in *C. jejuni* strains

DNA was extracted from bacterial cells using boiling (see section 2.10.1.1) and used as a template. PCR was performed according to section 2.10.1.2 using primers VIR BF and VIR BR for *virB11* gene (Table 2.4).

M: 1 kb DNA ladder

Lane 1: *C. jejuni* 81-176 *cdtB* mutant

Lane 2: *C. jejuni* RPH1

Lane 3: *C. jejuni* RPH2

Lane 4: *C. jejuni* RPH3

Lane 5: *C. jejuni* RPH4

Lane 6: *C. jejuni* RPH5

Lane 7: *C. jejuni* RPH6

Lane 8: *C. jejuni* RPH7

Lane 9: *C. jejuni* RPH8

Lane 10: *C. jejuni* RPH10

virB11 gene amplicon: 708bp

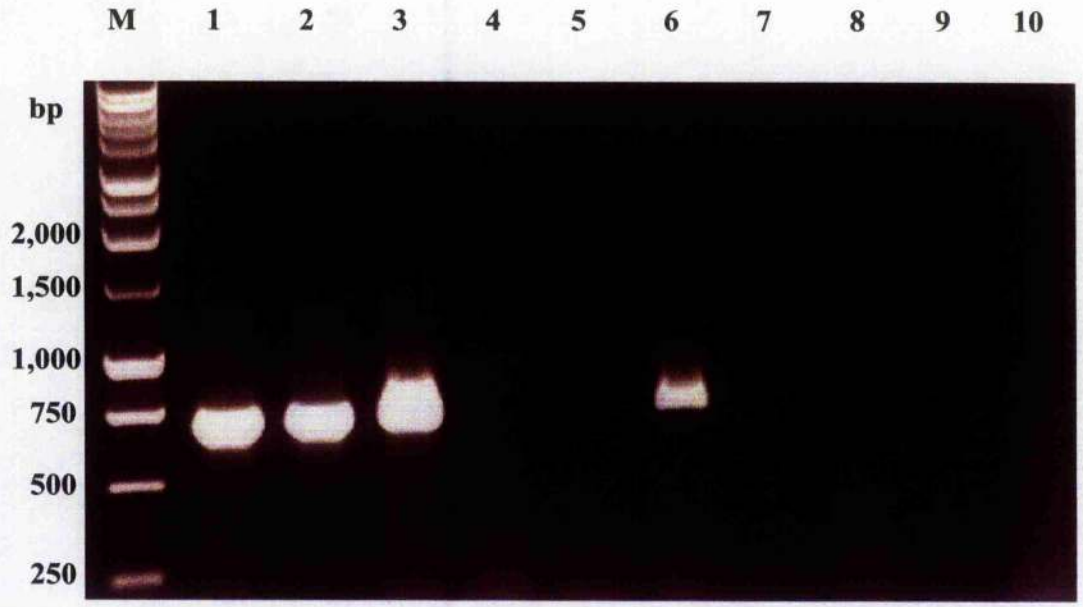

M
1
2
3
4
5
6
7
8
9
10
bp
2,000
1,500
1,000
750
500
250

Figure 3.18: PCR results to determine the presence of the *tetO* gene in *C. jejuni* strains

DNA was extracted from bacterial cells using boiling (see section 2.10.1.1) and used as a template. PCR was performed according to section 2-10-1-2 using primers TET OF and TET OR for *tetO* gene (Table 2.4).

M: 1 kb DNA ladder
Lane 1: *C. jejuni* COL21
Lane 2: *C. jejuni* COL22
Lane 3: *C. jejuni* COL23
Lane 4: *C. jejuni* COL24
Lane 5: *C. jejuni* COL25
Lane 6: *C. jejuni* COL26
Lane 7: *C. jejuni* COL27
Lane 8: *C. jejuni* COL28
Lane 9: *C. jejuni* COL29
Lane 10: *C. jejuni* COL30
Lane 11: *C. jejuni* 81-176

tetO gene amplicon; 559 bp

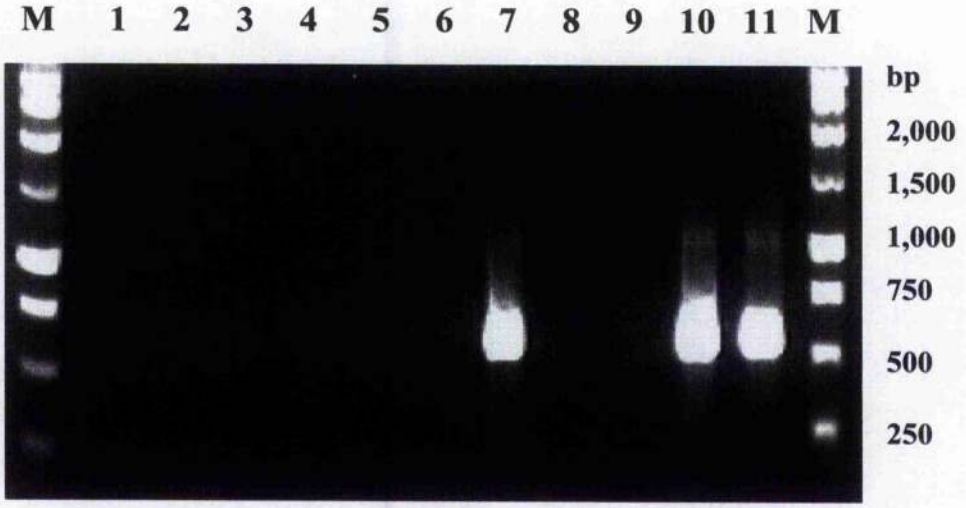

M 1 2 3 4 5 6 7 8 9 10 11 M
bp
2,000
1,500
1,000
750
500
250

Figure 3.19: PCR results to determine the presence of the *tetO* gene in *C. jejuni* strains

DNA was extracted from bacterial cells using boiling (see section 2.10.1.1) and used as a template. PCR was performed according to section 2-10-1-2 using primers TET OF and TET OR for *tetO* gene (Table 2.4).

M: 1 kb DNA ladder
Line 1: *C. jejuni* 81-176 *cdtB* mutant
Line 2: *C. jejuni* RPH1
Line 3: *C. jejuni* RPH2
Line 4: *C. jejuni* RPH3
Line 5: *C. jejuni* RPH4
Line 6: *C. jejuni* RPH5
Line 7: *C. jejuni* RPH6
Line 8: *C. jejuni* RPH7
Line 9: *C. jejuni* RPH8
Line 10: *C. jejuni* RPH10

tetO gene amplicon; 559 bp

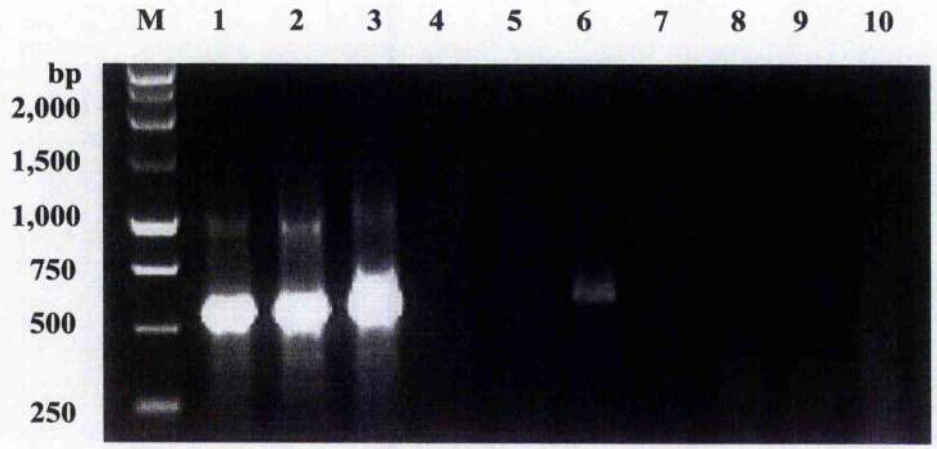

M 1 2 3 4 5 6 7 8 9 10
bp
2,000
1,500
1,000
750
500
250

Table 3.4: Presence of *virB11* gene and tetracycline-resistance locus *tetO* in *C. jejuni* strains

	virB11 (PCR result)	*tetO*	
		PCR result	phenotype*
81-176	+	+	+
81-176 *cdtB* mutant	+	+	+
11168	–	–	–
11168 *cdtB*	–	–	–
11168 *cdtA*	–	–	–
11168 *tlyA*	–	–	–
11168 *pldA*	–	–	–
COL1	–	–	–
COL2	–	–	–
COL3	–	–	–
COL4	–	–	–
COL5	–	–	–
COL6	–	–	–
COL7	–	–	–
COL8	+	–	–
COL9	+	–	–
COL10	+	–	–
COL11	+	–	–
COL12	+	–	–
COL13	+	–	–
COL14	+	–	–
COL15	–	–	–
COL16	–	–	–

Table 3.4: (continued)

	virB11 (PCR result)	*tetO*	
		PCR result	phenotype*
COL17	–	–	–
COL18	–	–	–
COL19	–	–	–
COL20	+	–	–
COL21	–	–	–
COL22	–	–	–
COL23	–	–	–
COL24	–	–	–
COL25	–	–	–
COL26	–	–	–
COL27	–	+	+
COL28	–	–	–
COL29	–	–	–
COL30	–	+	+
RPH1	+	+	+
RPH2	+	+	+
RPH3	–	–	–
RPH4	–	–	–
RPH5	+	+	+
RPH6	–	–	–
RPH7	–	–	–
RPH8	–	–	–
RPH10	–	–	–

* determined on blood agar containing tetracycline (20 µg/ml).

Table 3.5: Presence of *virB11* and *tetO* genes in *C. jejuni* from various sources

Gene	Human ($n = 25$)	Chicken ($n = 8$)	Bovine ($n = 5$)	Ovine ($n = 1$)	Total ($n = 39$)
virB11	7 (28%)	3 (37.5%)	1 (20%)	0	11 (28.2%)
tetO	3 (12%)	2 (25%)	0	0	5 (12.82%)

3.4.6 Plasmid isolation from strain 81-176

It was noted from Table 3.4 that some strains (81-176, RPH1, 2, 5) were positive for both the *virB11* and *tetO* genes which indicated that they might possess the 2 plasmids pVir and pTet. Some strains were positive for *virB11* only (COL8-14, 20) or *tetO* only (COL27, 30) which indicated they may have contained either pVir or pTet, respectively. Attempts were made to purify plasmid DNA from some of these strains, but this was successful only for strain 81-176 when the QIAGEN® Large-Construct Kit was used (see section 2.10.2.2). The lack of success in plasmid isolation may have been due to the low copy number of these large plasmids. A representative gel of plasmid DNA isolated from the 81-176 strain is shown in Fig. 3.20. This shows a high molecular size band which, on digestion with *Bgl*II, produced 11 bands totalling approx. 70 kb. This is consistent with the presence of the two 35 kb pVir and pTet plasmids. It can not be confirmed if digestion continued to completion. However the pattern of DNA fragments very similar to that shown by Bacon *et al.* (2000) who also analysed plasmid DNA from strain 81-176. Analysis of the sequences of pVir (GenBank accession number NC 005012) and pTet (GenBank accession number NC 007141) was carried out using restriction mapping tools at New England Biolabs website. The results were consistent with Fig. 3.20 suggesting the presence of pVir and pTet in strain 81-176.

3.4.7 Cytotoxicity on Vero cells of clinical isolates possessing the *virB11* or *tetO* genes

There was the possibility that one or other of the pVir or pTet plasmids, particularly pVir, coded for the cytotoxic activity identified earlier. In section 3.4.2 only COL1 and COL7 were investigated, but these strains were found to be negative for pVir or

Figure 3.20: Supercoiled and *Bgl*II digestion patterns of plasmids isolated from *C. jejuni* 81-176 *cdtB* mutant

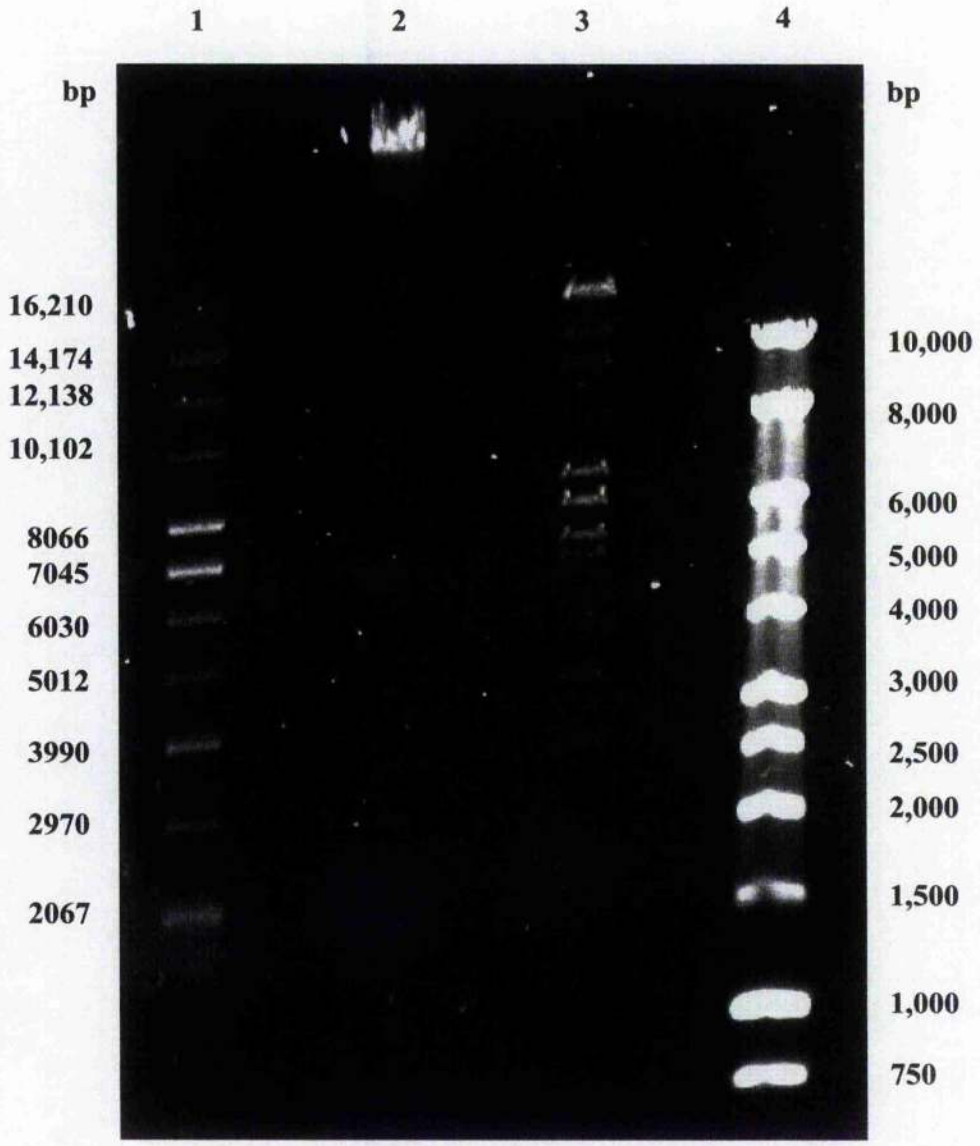
Supercoiled DNA was extracted from whole bacterial cells using QIAGEN® Large-Construct Kit (see section 2.10.2.2) and digested by *Bgl*II enzyme. Both forms were run on 0.4% agarose gel.

Lane 1: Supercoiled DNA ladder (Invitrogen)

Lane 2: *C. jejuni* 81-176 (*cdtB* mutant), supercoiled

Lane 3: *C. jejuni* 81-176 (*cdtB* mutant), *Bgl*II digestion pattern

Lane 4: 1 kb DNA ladder


1
2
3
4
bp
bp
16,210
14,174
12,138
10,102
8066
7045
6030
5012
3990
2970
2067
10,000
8,000
6,000
5,000
4,000
3,000
2,500
2,000
1,500
1,000
750

pTet, although they both possessed cytotoxic activity, particularly COL7. In section 3.4.4, strains 11168 and 81-176 both possessed cytotoxic activity which was particularly strong in strain 81-176 (Fig. 3.12). Strain 81-176 possessed both the pVir or pTet plasmids. Representative clinical isolates were therefore chosen which possessed either pVir (COL12, 20) or pTet (COL27, 30) and their cytotoxic capacities compared with COL1 which possessed neither plasmid. Cell-free extracts of clinical isolates COL1, COL12 (*virB11*+), COL20 (*virB11*+), COL27 (*tetO*+) and COL30 (*tetO*+) were tested for cytotoxicity on Vero cells after incubation for 72h. Extracts from strains COL20, COL1 and COL30 were clearly toxic, particularly COL20 giving >50% cell death at the highest concentration but strains COL12 and COL27 were only slightly more toxic that the *E. coli* DH5α extract (Fig. 3.21). Microscopic images showed that cell distensions were caused by strain COL20 and perhaps COL1 (Figs. 3.22 to 3.24) and these strains showed the greatest cytotoxicity after 72h incubation. Thus, no clear correlation between cytotoxicity and carriage of either the *virB11* and *tetO* genes was apparent from these results.

3.4.8 Tissue culture cell number correlates with luminescent output

The MTT assay for cell cytotoxicity was time-consuming and gave poor results at the higher toxicity level; a toxic activity of 100% was not achieved and maximum cytotoxicity appeared to plateau at 60-70% cell death. For this reason an alternative assay, which used intracellular ATP as a measure of cell viability, was used.

A direct relationship existed between luminescence measured with the CellTiter-Glo™ assay and the number of cells in tissue culture. The results show (Fig. 3.25) that there was a linear relationship (from 0-10,000 cells per well) between the

Figure 3.21: Cytotoxicity of cell-free extracts from low-speed centrifugation of different clinical isolates on Vero cells.

The cytotoxic activities of cell-free extracts (low-speed centrifugation) of *C. jejuni* clinical isolates COL1, COL12 (*virB11*$^+$), COL20 (*virB11*$^+$), COL27 (*tetO*$^+$) and COL30 (*tetO*$^+$) and two *E. coli* strains (E32511 and DH5α) as positive and negative controls were determined by MTT assay. Two-fold serial dilutions of the extracts were applied to Vero cells which were then incubated for 72h.

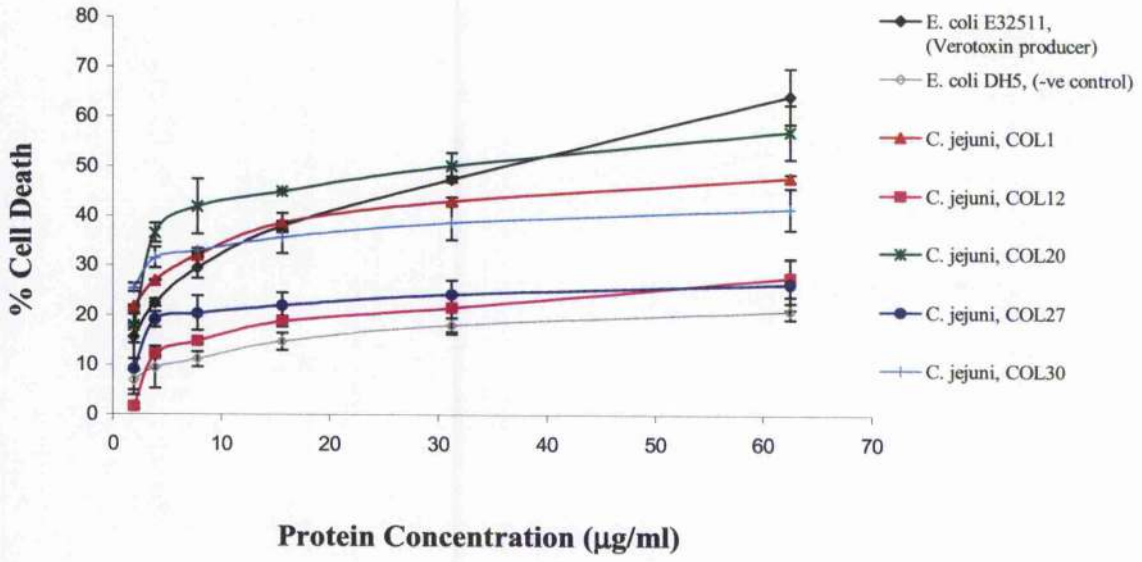

% Cell Death
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Protein Concentration (µg/ml)
E. coli E32511, (Verotoxin producer)
E. coli DH5, (-ve control)
C. jejuni, COL1
C. jejuni, COL12
C. jejuni, COL20
C. jejuni, COL27
C. jejuni, COL30

Figure 3.22:

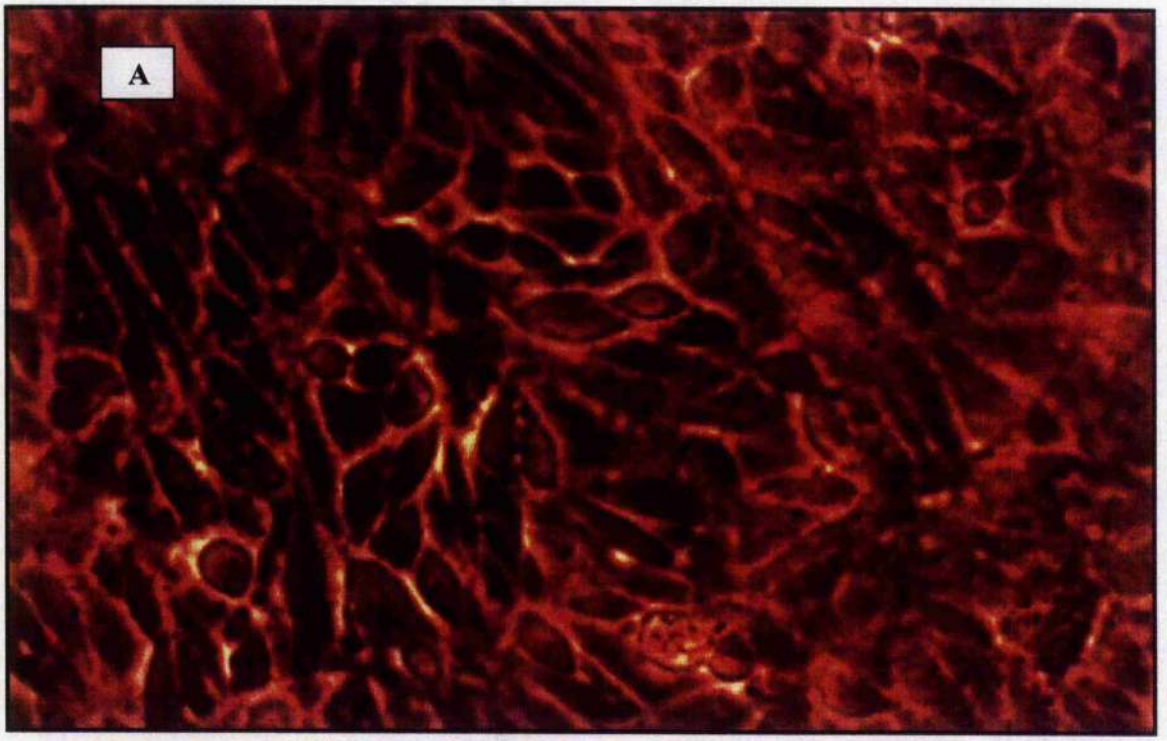
A. Untreated Vero cells after incubation for 72h

Note the normal, spindle-shaped appearance of the cells.

B. Vero cells treated with cell-free extract (low-speed centrifugation) of *C. jejuni* COL1

Vero cell monolayer incubated with 62.5 μg/ml protein cell-free extract (low-speed centrifugation) of *C. jejuni* COL1 for 72h.

Both photographs were taken at a magnification of ×40.


A

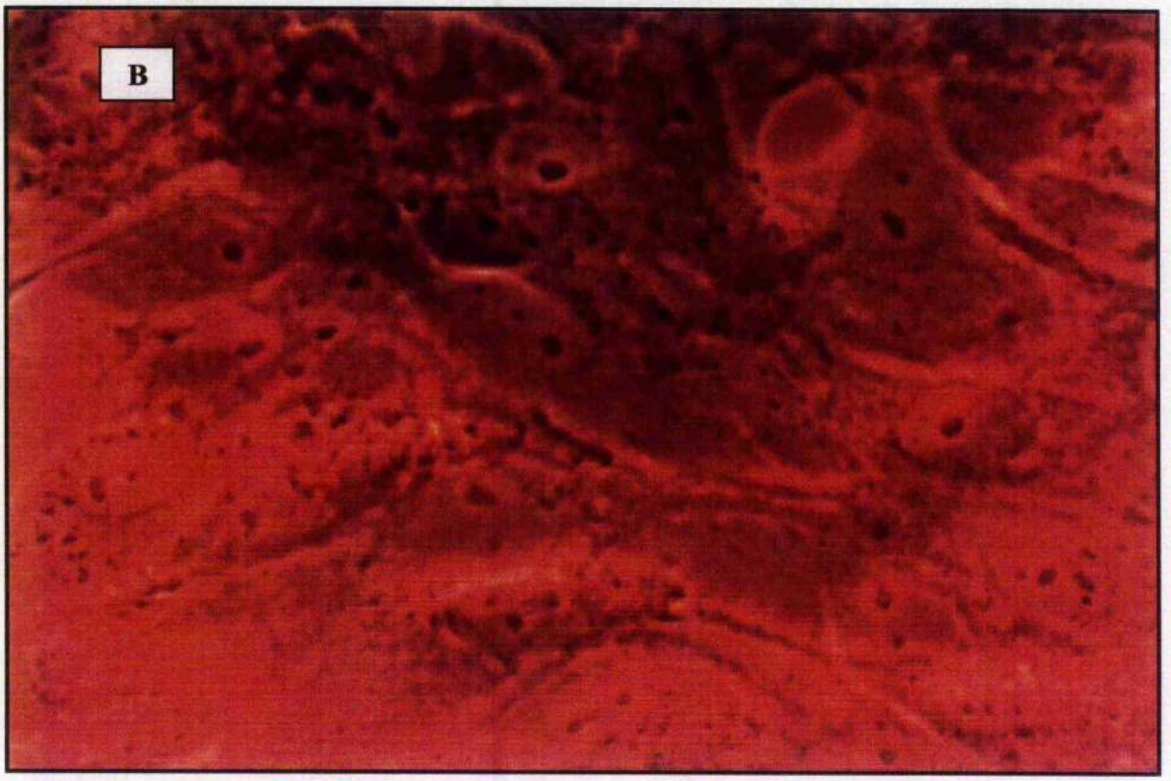

B

Figure 3.23:

A. Vero cells treated with cell-free extract (low-speed centrifugation) of *C. jejuni* COL12

Vero cell monolayer incubated with 62.5 μg /ml protein cell-free extract (low-speed centrifugation) of *C. jejuni* COL12 for 72h.

B. Vero cells treated with cell-free extract (low-speed centrifugation) of *C. jejuni* COL20

Vero cell monolayer incubated with 62.5 μg/ml protein cell-free extract (low-speed centrifugation) of *C. jejuni* COL20 for 72h.

The arrow shows the grossly distended cells (CDT-treated).

Both photographs were taken at a magnification of ×40.

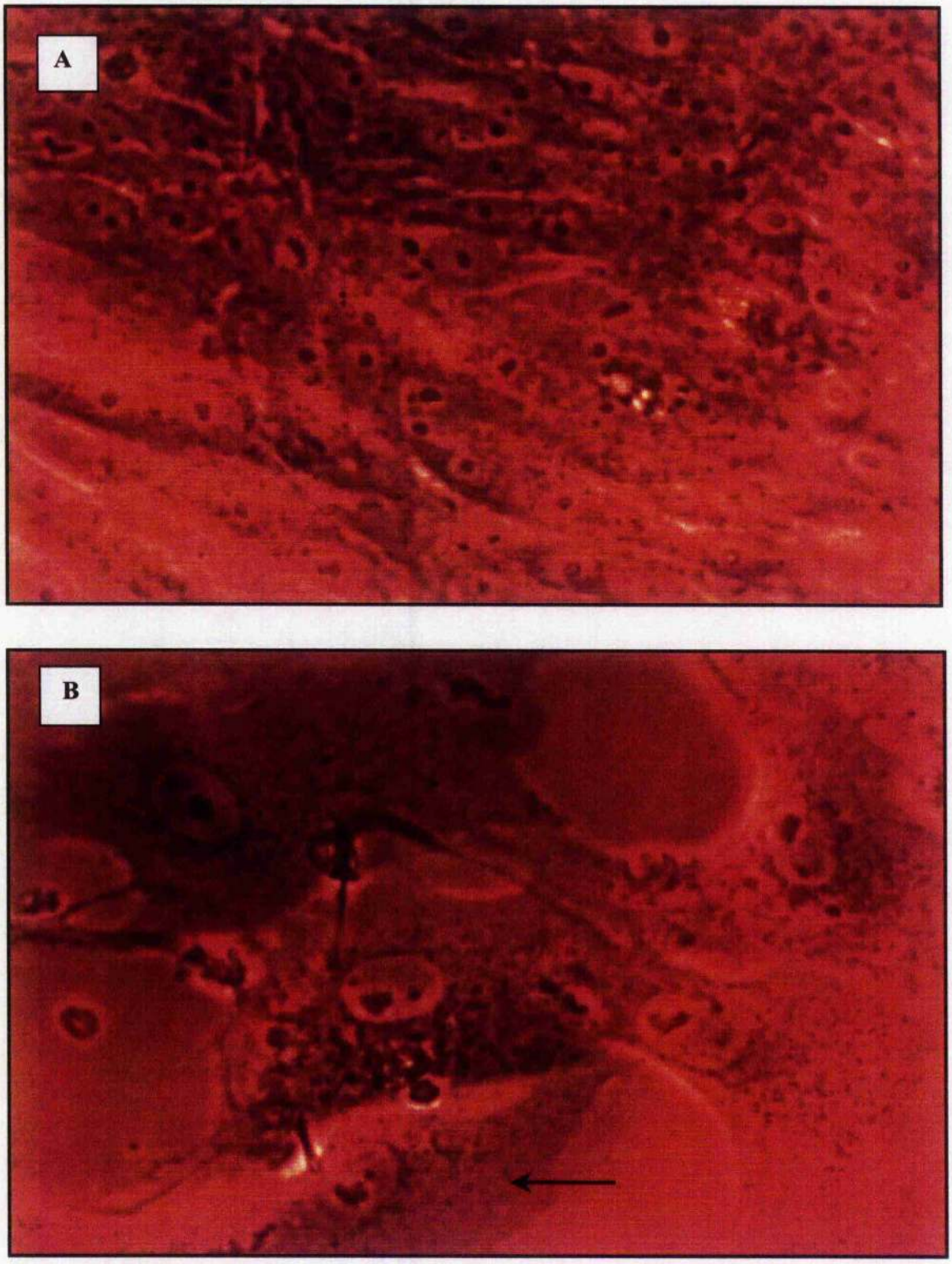

A
B

Figure 3.24:

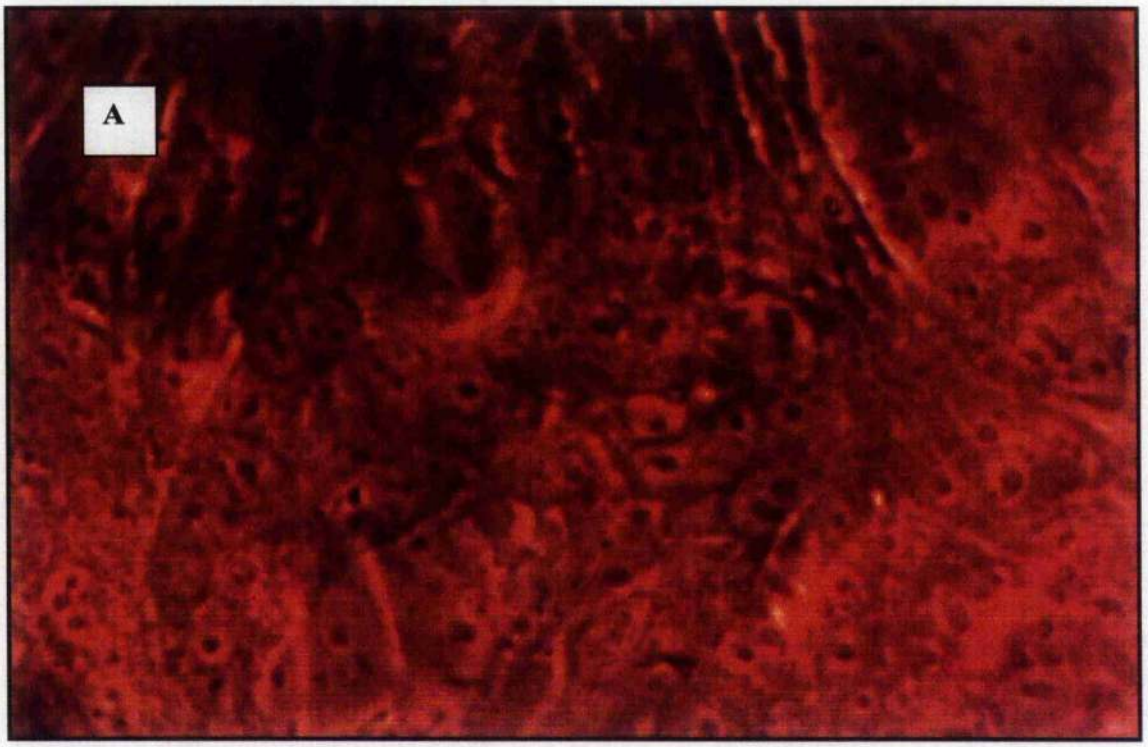
A. Vero cells treated with cell-free extract (low-speed centrifugation) of *C. jejuni* COL27

Vero cell monolayer incubated with 62.5 µg/ml protein cell-free extract (low-speed centrifugation) of *C. jejuni* COL27 for 72h.

B. Vero cells treated with cell-free extract (low-speed centrifugation) of *C. jejuni* COL30

Vero cell monolayer incubated with 62.5 µg/ml protein cell-free extract (low-speed centrifugation) of *C. jejuni* COL30 for 72h.

Both photographs were taken at a magnification of ×40.


A

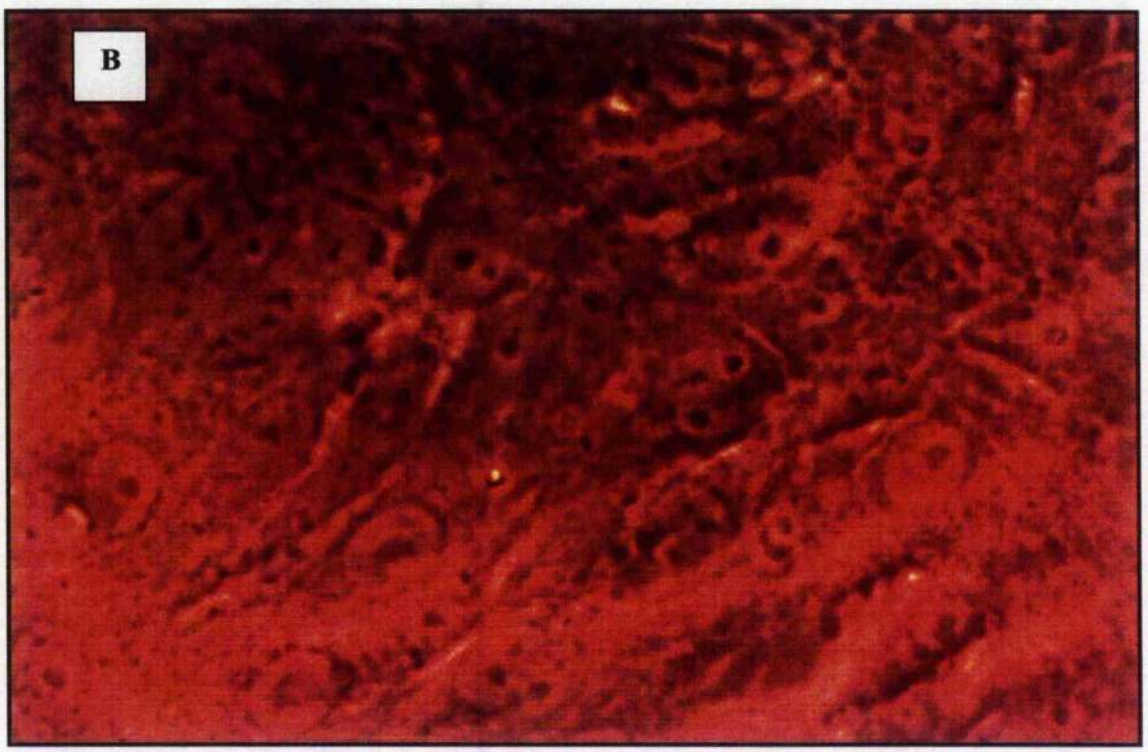
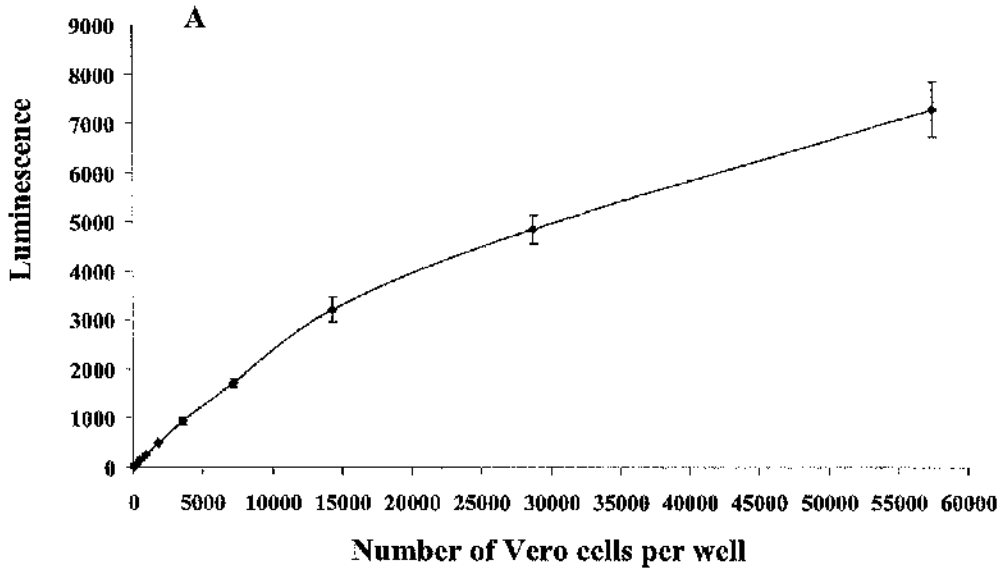
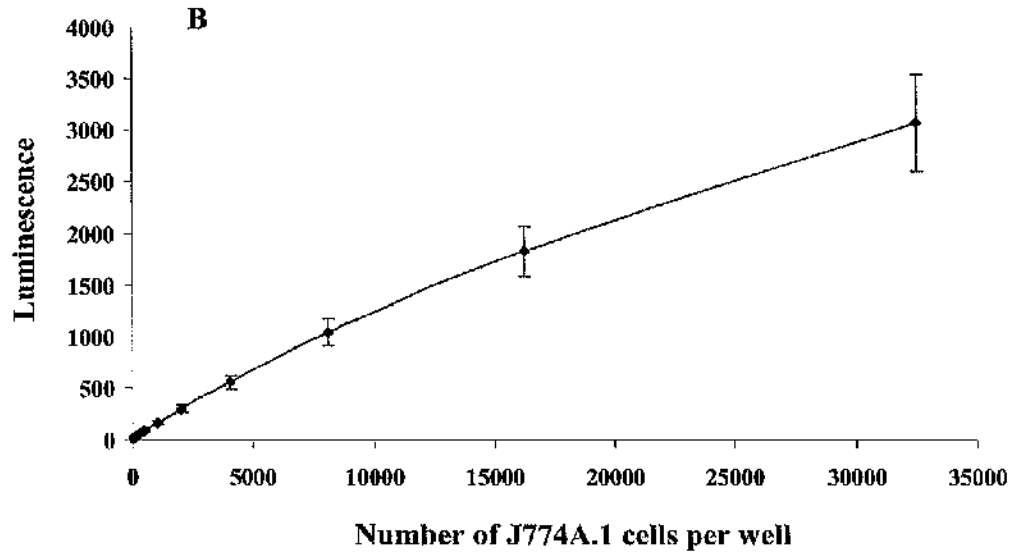

B

Figure 3.25: Correlation of tissue culture cell number with luminescent output (CellTiter-Glo™ Luminescent Cell Viability Assay).

Correlation of Vero (A) and J774A.1 (B) cell number with luminescent output were determined in two-fold serial dilutions using CellTiter-Glo™ Luminescent Cell Viability Assay (ATP-based assay).


A
Luminescence
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0 5000 10000 15000 20000 25000 30000 35000 40000 45000 50000 55000 60000
Number of Vero cells per well


B
Luminescence
4000
3500
3000
2500
2000
1500
1000
500
0
0 5000 10000 15000 20000 25000 30000 35000
Number of J774A.1 cells per well

luminescent signal (a measurement of the ATP content of cells) and the number of tissue culture cells for both Vero cells and the J774A.1 mouse macrophage cell line.

3.4.9 Comparative cytotoxicity of cell-free extracts of strains 11168 (wild type), 11168 *cdtB*, 11168 *tlyA*, and 11168 *pldA* mutants

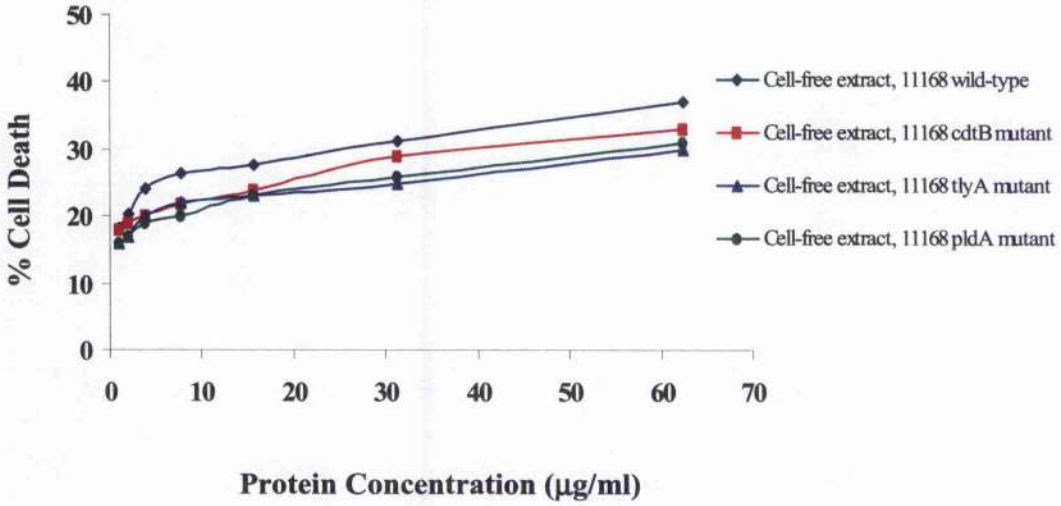
These experiments were done to test the possibility that the putative haemolysin or phospholipase present in *C. jejuni* was contributing to the cytotoxicity of the strains noted here. Cell-free extracts (low speed centrifugation) of *C. jejuni* strains 11168 (wild type), 11168 *cdtB* (CDT$^-$), 11168 *tlyA* (haemolysin$^-$), and 11168 *pldA* (phospholipase$^-$) were incubated with Vero cells for 16h and tested for cytotoxicity using the MTT assay (see sections 2.6.3.1). After incubation of Vero cells for 16h with the cell-free extracts, the cytotoxicity of the four strains were similar (no significant difference) when tested by either assay (Fig.3.26). This suggested that the cytotoxicity measured in the assay was not due to CDT, haemolysin or phospholipase.

3.4.10 Cytotoxicity assay of sup1, sup2 and reagents of the kit used to prepare materials for 2-D electrophoresis

sup1 and sup2 were prepared for use in comparative proteomic analysis of different *C. jejui* strains. Therefore sup1 (cytoplasmic fraction), sup2 (membrane fraction) and reagents (which were used in the kit to prepare material for 2-DE gels) (see section 2.9.2.1) were tested in the cytotoxicity assay on Vero cells using serial dilutions. Reagent 1 (used to prepare sup1) and reagent 2 (used to prepare sup2) were used as controls, but they were toxic for Vero cells. Based on the results with reagent1 and

Figure 3. 26: Cytotoxicity of cell-free extracts of *C. jejuni* NCTC 11168 wild-type, 11168 *cdtB*, *tlyA* and *pldA* mutant strains on Vero cells after incubation for 16h.

The cytotoxic activities of cell-free extracts from low-speed centrifugation of *C. jejuni* NCTC 11168 wild-type, *cdtB*, *tlyA* and *pldA* mutant strains were determined by MTT assay. Two-fold serial dilutions of the extracts were applied to Vero cells which were then incubated for 16h.


50
40
30
20
10
0
% Cell Death
0
10
20
30
40
50
60
70
Protein Concentration (µg/ml)
Cell-free extract, 11168 wild-type
Cell-free extract, 11168 cdtB mutant
Cell-free extract, 11168 tlyA mutant
Cell-free extract, 11168 pldA mutant

reagent 2, the results of cytotoxicity assay of sup1 and sup2 did not show the real cytotoxic activity of cytoplasmic and membrane fractions.

3.4.11 Effect of trypsin on Vero cells

As a prelude to further characterisation of the cytotoxic activity, tests were done to check if the activity was likely to be caused by a protein. One way to do this would be to use trypsin to digest the proteins in a cell-free extract. However, when the cytotoxic activities of two-fold serial dilutions of trypsin (0.25%) were determined using Vero cells, it was shown that trypsin alone was toxic (Fig. 3.27). Thus, trypsin was not used. Instead, the heat stability of the cytotoxicity of the cell-free extract was measured (Fig. 3.28).

3.4.12 Effect of heating on cytotoxic activity of *C. jejuni*

Aliquots of cell-free extract (low-speed centrifugation) of *C. jejuni* 81-176 *cdtB* mutant were heated at different temperatures: 50 °C, 60 °C, 70 °C, 80 °C and 90 °C for 30 min. The *cdtB* mutant strain was chosen to avoid any cytotoxicity due to CDT. The heated cell-free extracts were tested for cytotoxicity on Vero cells. The results showed that heating the extract to 50 °C for 30 min had little effect on its cytotoxicity, but the cytotoxic activity was lost at 60 °C and above (Fig. 3.28). The result indicated that cytotoxic activity was probably due to a protein(s).

3.4.13 Effect of using different temperatures of incubation of *C. jejuni* on cytotoxic activity

C. jejuni 81-176 was grown in Brucella broth at different temperatures (37 °C and 42 °C) for 48h. After growth of the bacteria, cell-free extracts (low speed centrifugation)

Figure 3.27: Effect of trypsin on Vero cells

The cytotoxic activities of two-fold serial dilutions of trypsin (0.25%) were determined on Vero cells after incubation for 16h using the ATP-based assay.

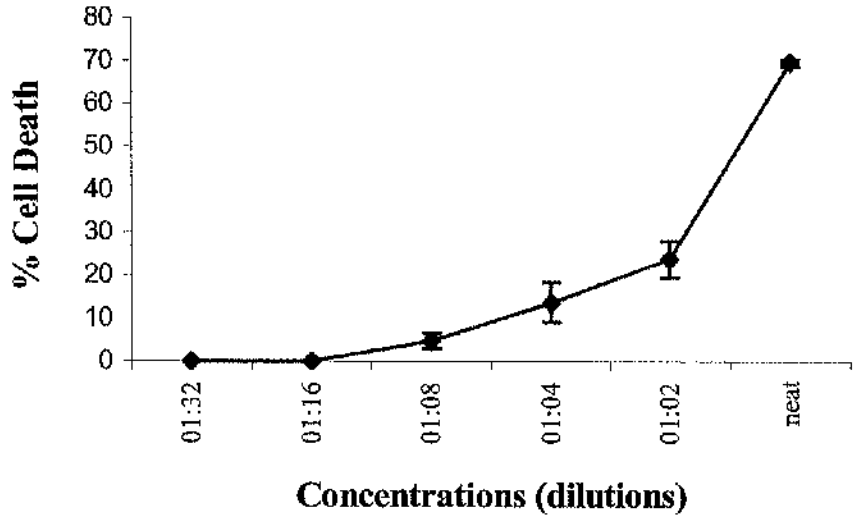
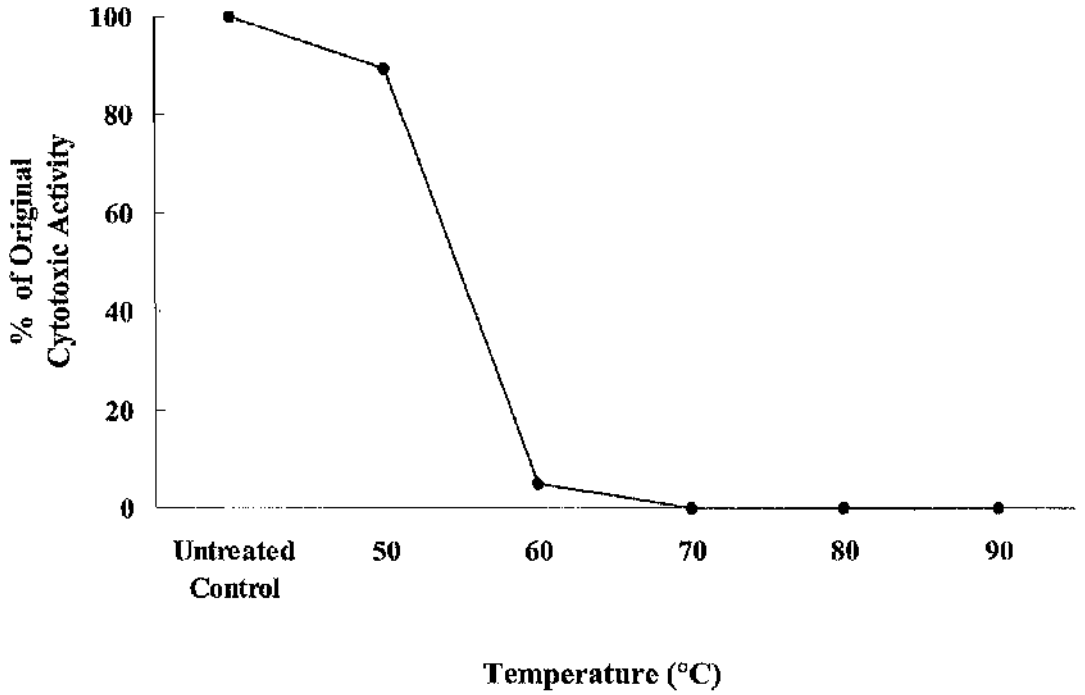

% Cell Death
80
70
60
50
40
30
20
10
0
01:32
01:16
01:08
01:04
01:02
neat
Concentrations (dilutions)

Figure 3.28: Heat stability of cytotoxic activity of a cell-free extract from low speed centrifugation of strain 81-176 *cdtB* using the ATP-based assay.

A cell-free extract (low-speed centrifugation) of *C. jejuni* 81-176 *cdtB* mutant was heated at different temperatures for 30 min. Activities were determined using two fold serial dilutions on Vero cells after incubation for 24h by ATP-based assay.


100
80
60
40
20
0
% of Original Cytotoxic Activity
Untreated Control
50
60
70
80
90
Temperature (°C)

were prepared and used in two different cytotoxicity assays (MTT and ATP-based) (Fig.3.29). Results showed that there was no difference between the two cell-free extracts prepared from cells grown at the two temperatures.

3.4.14 Cytotoxicity of cell-free extracts on Caco-2 cell line

A cell-free extract from low-speed centrifugation of *C. jejuni* 81-176 was tested for cytotoxicity on Caco-2 cells after incubation for 16h (Fig. 3.30). The activity of the strain 81-176 extract on Caco-2 cells was noticeably higher than that of *E. coli* E32511. The activity measured on Caco-2 cells here was similar to the activity of the cell-free extract of 81-176 on Vero cells (Fig.3.11B) (e.g. 40% killing with 40 μg/ml).

3.4.15 Comparison of cytoplasmic, IMP and OMP fractions for cytotoxic activities

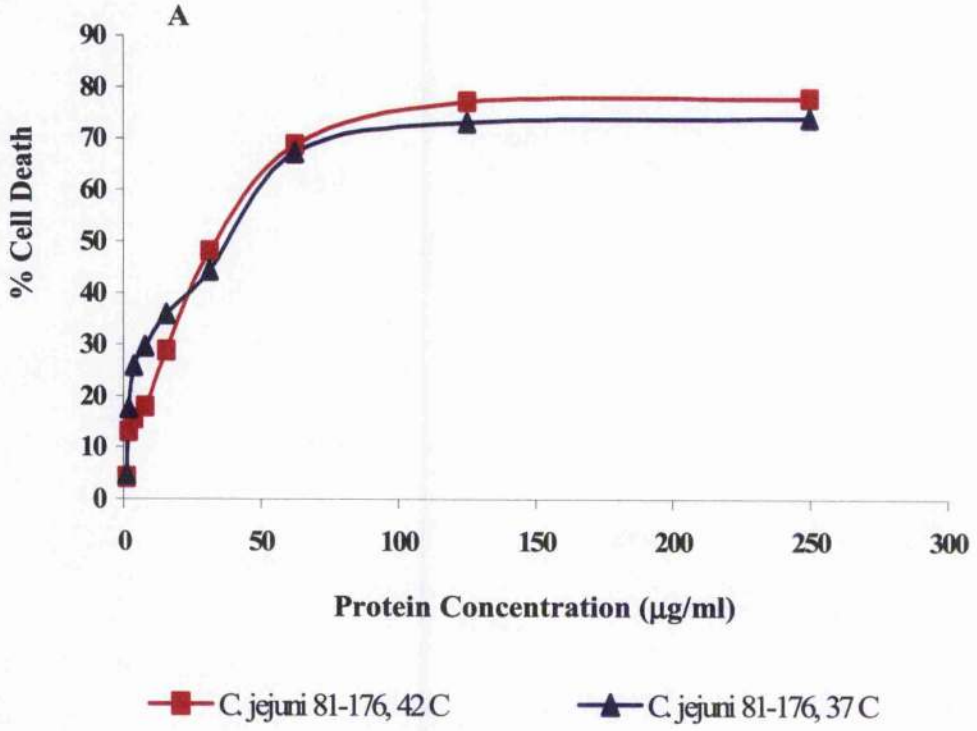
From the previous cytotoxicity tests, strains 81-176 and COL12 were chosen as strongest and weakest strains for cytotoxic activity, respectively. Cytoplasmic, IMP and OMP fractions (see section 2.3.2) of the strains were prepared. Portions of these fractions were analysed by SDS-PAGE (Fig. 3.31). The results showed that more clearly discernible protein bands were found in the OMP and cytoplasmic fractions than in the IMP fraction. Some slight differences in protein bands were visible between strains 81-176 and COL12. This might perhaps be expected as the strains were sourced from widely different locations. The various extracts were incubated with Vero cells for 16h (using confluent Vero cells) and 72h (using non-confluent Vero cells) and were tested for cytotoxicity using the CellTiter-Glo™

Figure 3.29: Effect of incubation temperature of culture medium of *C. jejuni* strain 81-176 on cytotoxicity of cell extract after incubation for 72h with Vero cells.

The cytotoxic activities of cell-free extracts of *C. jejuni* 81-176 (grown in Brucella broth at different temperatures, 37 °C and 42 °C) were determined by two different cytotoxicity assays (MTT and ATP-based) using two-fold serial dilutions of the extracts.

A. MTT assay

B. ATP-based assay


A
% Cell Death
0
10
20
30
40
50
60
70
80
90
0
50
100
150
200
250
300
Protein Concentration (µg/ml)
C. jejuni 81-176, 42 C
C. jejuni 81-176, 37 C

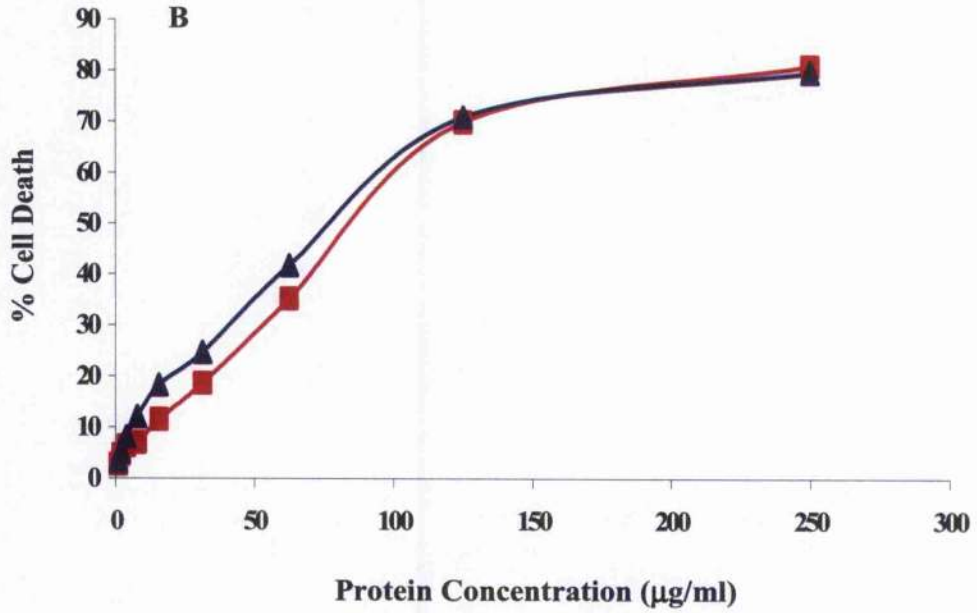

B
% Cell Death
0
10
20
30
40
50
60
70
80
90
0
50
100
150
200
250
300
Protein Concentration (µg/ml)

Figure 3.30: Cytotoxicity of a cell-free extract of *C. jejuni* 81-176 on Caco-2 cells

A cell-free extract from low-speed centrifugation of *C. jejuni* 81-176 was tested for cytotoxicity on Caco-2 cells after incubation for 16h using the MTT assay. *E. coli* E32511 and DH5α strains were used as a positive and negative controls, respectively.

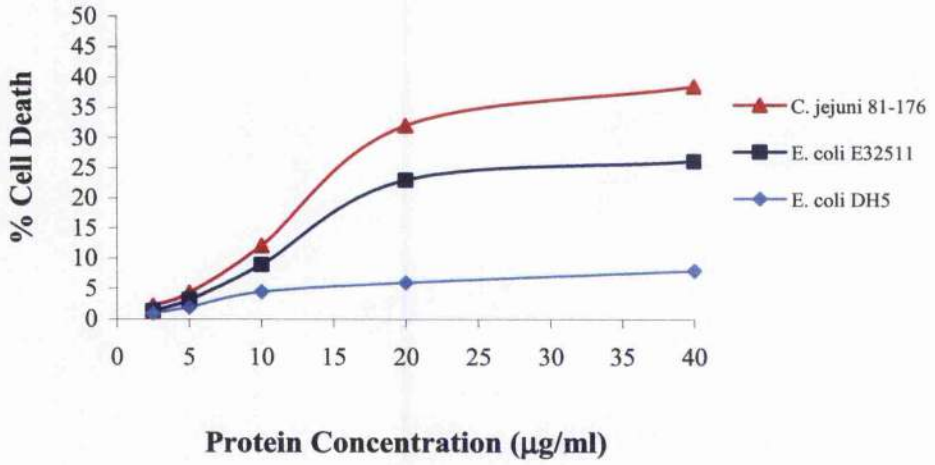

% Cell Death
50
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
Protein Concentration (µg/ml)
C. jejuni 81-176
E. coli E32511
E. coli DH5

Figure 3.31: SDS-PAGE analysis to compare cell fractions of *C. jejuni* 81-176 and COL12.

IMP, OMP and cytoplasmic fractions of *C. jejuni* 81-176 and COL12 (20 µl) were subjected to electrophoresis on a 12.5% (w/v) polyacrylamide gel and stained with 0.1% (w/v) Coomassie blue solution (section 2.5).

M: Protein Marker (molecular weight range as indicated)

Lane 1: IMP fraction from *C. jejuni* 81-176

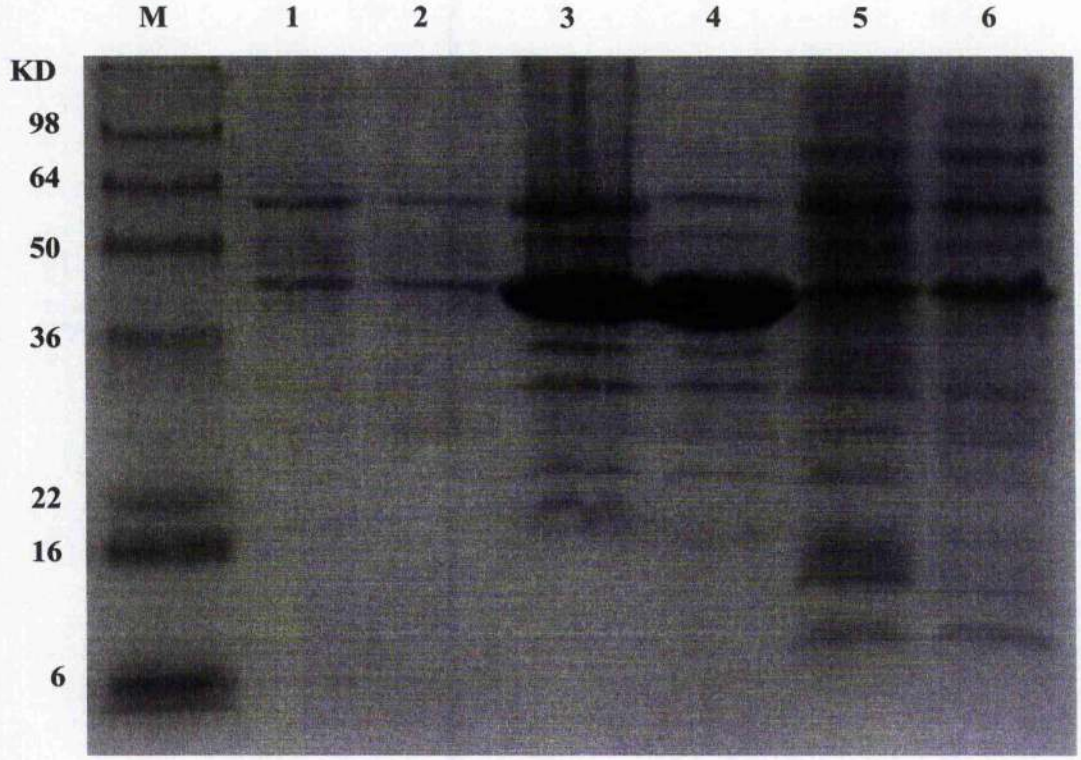
Lane 2: IMP fraction from *C. jejuni* COL12

Lane 3: OMP fraction from *C. jejuni* 81-176

Lane 4: OMP fraction from *C. jejuni* COL12

Lane 5: Cytoplasmic fraction from *C. jejuni* 81-176

Lane 6: Cytoplasmic fraction from *C. jejuni* COL12


M
1
2
3
4
5
6
KD
98
64
50
36
22
16
6

Luminescent Cell Viability assay (Figs. 3.32, 3.33 and 3.34). Summarised data of these figures are shown in Table 3.6. The results showed that all fractions of strain 81-176 had greater activity than those of COL12. Cytotoxic activity of IMP preparations were apparent at quite low protein concentrations, particularly after 72h when >50% killing of cells was achieved by the IMP fraction of 81-176 at 20 μg/ml (Fig. 3.32). OMP fractions were far less active at low protein concentrations with ~50% killing at 250 μg/ml of the OMP fraction of 81-176 (Fig. 3.33) The cytoplasmic fractions also showed little cytotoxicity at low protein concentrations. That of strain 81-176 showed some activity after incubation for 16h, and this activity was noticeably greater after incubation for 72h (approxt. 50% killing at 100 μl/ml), which may have reflected cell death due to the slower, acting CDT toxin.

3.5 Cytotoxin purification

3.5.1 DEAE sepharose column chromatography

3.5.1.1 SDS-PAGE of column chromatography fractions

Purification of a protein fraction with cytotoxic activity was attempted using cell-free extracts. Althought the IMP preparation clearly had greater activity, it was later shown that proteins in this preparation could not be easily fractionated (section 3.5.2). DEAE column chromatography fractions (see section 2.8.1) of cell-free extract of *C. jejuni* strains 81-176 and COL12 were run on SDS-PAGE to find out in which fractions proteins were located. The results showed that some protein bands could be seen in the flowthrough, but most proteins were eluted in fractions

Figure 3.32: Cytotoxicity (ATP-based assay) comparison between IMP fractions of *C. jejuni* 81-176 and COL12.

The cytotoxic activities of IMP fractions from *C. jejuni* 81-176 and COL12 were determined in two-fold serial dilutions on Vero cells using ATP-based assay after incubation for (A) 16h and (B) 72h.

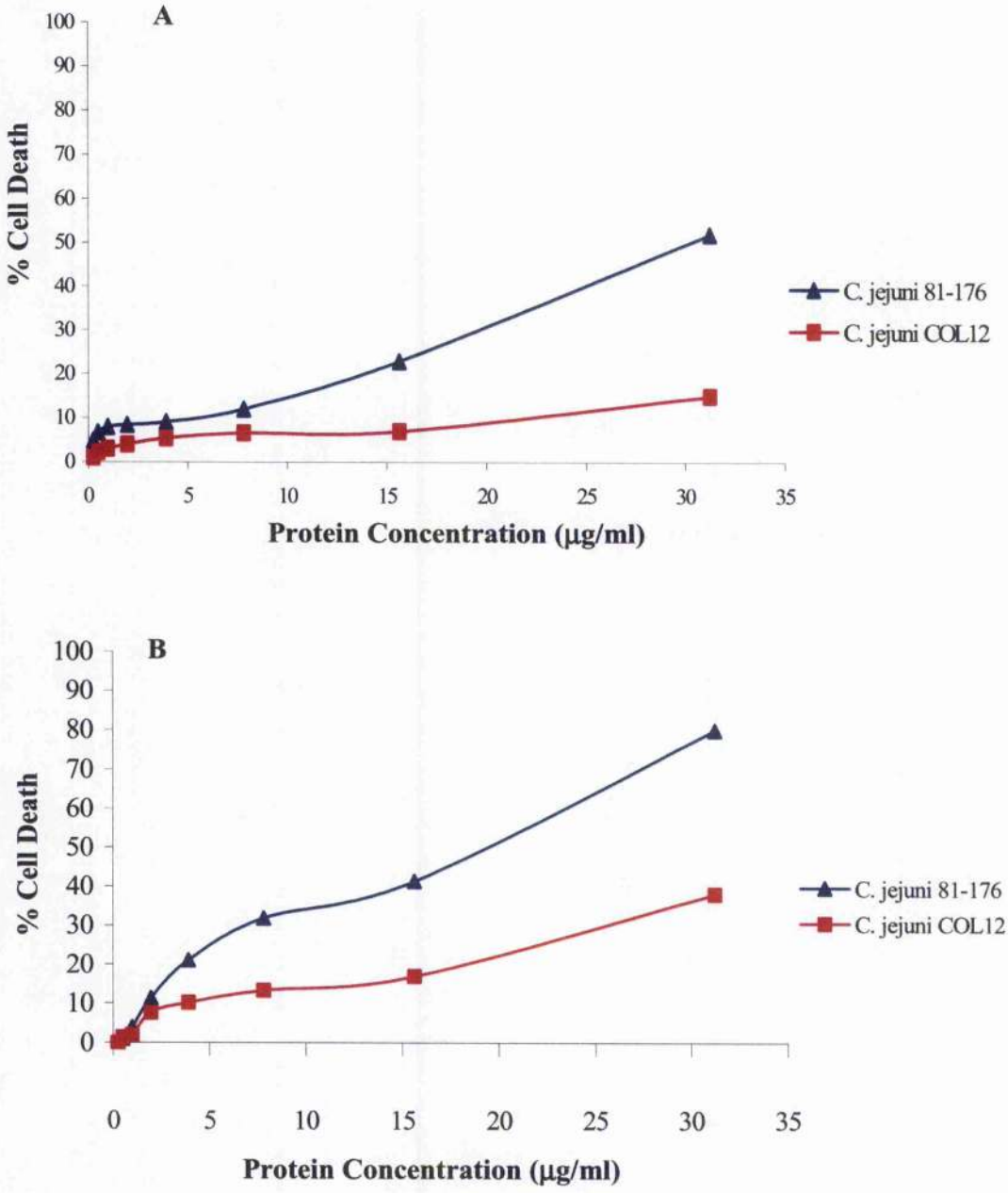
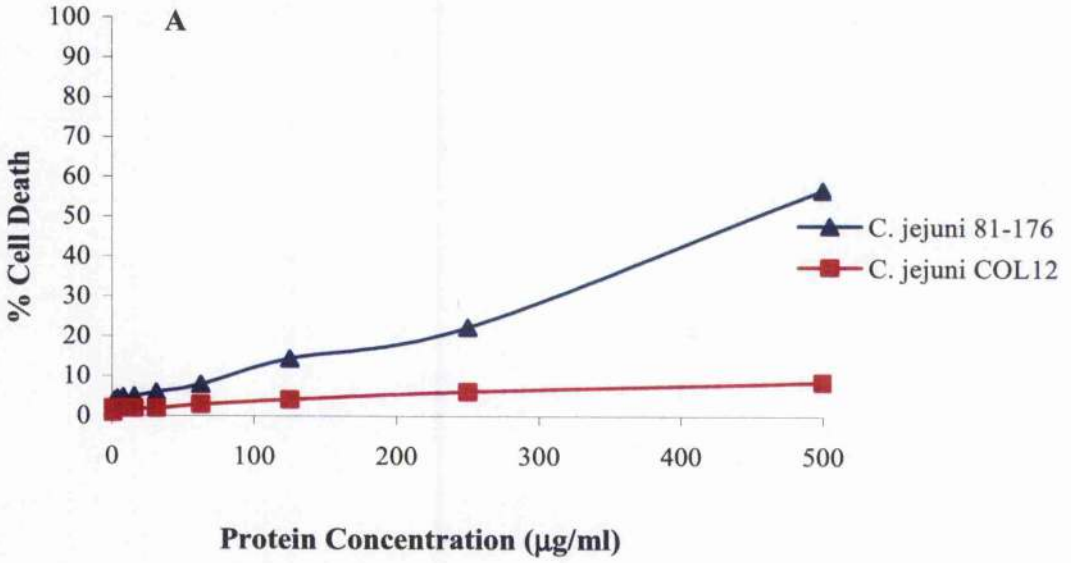

A
% Cell Death
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Protein Concentration (µg/ml)
C. jejuni 81-176
C. jejuni COL12
B
% Cell Death
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Protein Concentration (µg/ml)
C. jejuni 81-176
C. jejuni COL12

Figure 3.33: Cytotoxicity (ATP-based assay) comparison between OMP fractions of *C. jejuni* 81-176 and COL12.

The cytotoxic activities of OMP fractions from *C. jejuni* 81-176 and COL12 were determined in two-fold serial dilutions on Vero cells using ATP-based assay after incubation for (A) 16h and (B) 72h.


A
% Cell Death
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
Protein Concentration (µg/ml)
C. jejuni 81-176
C. jejuni COL12

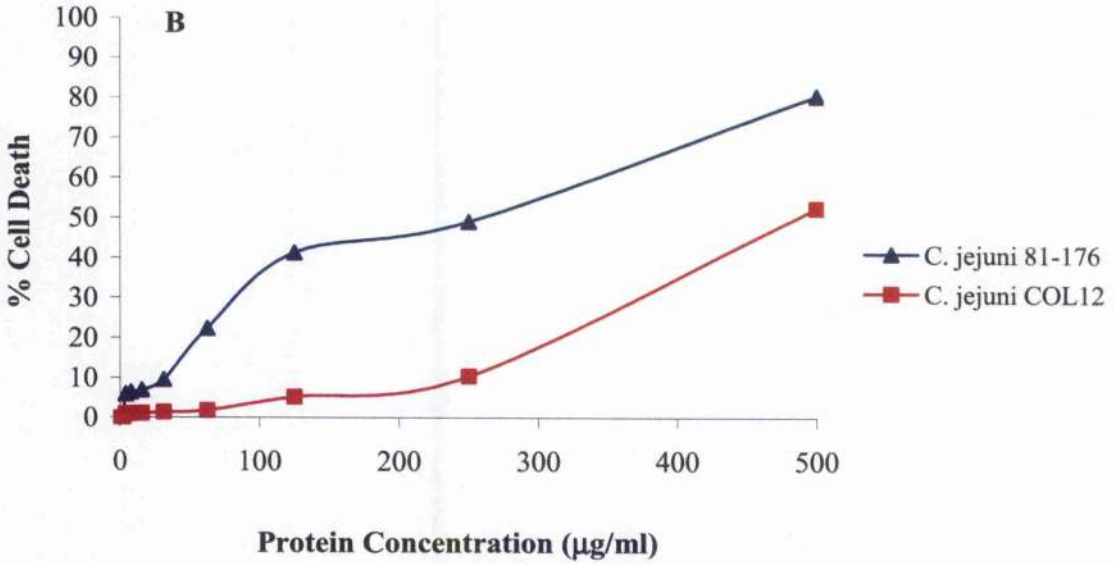
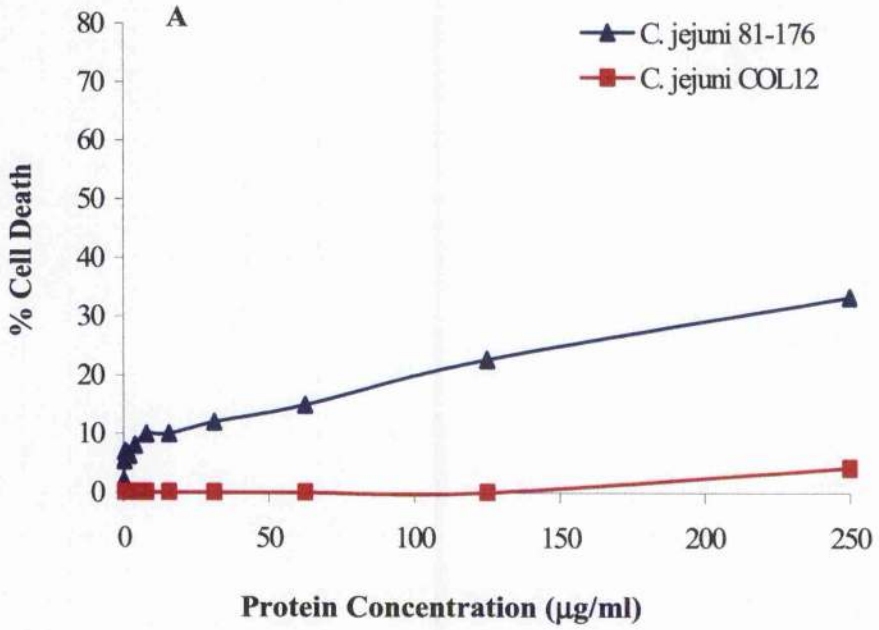

B
% Cell Death
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
Protein Concentration (µg/ml)
C. jejuni 81-176
C. jejuni COL12

Figure 3.34: Cytotoxicity (ATP-based assay) comparison between cytoplasmic fractions of *C. jejuni* 81-176 and COL12.

The cytotoxic activities of cytoplasmic fractions from *C. jejuni* 81-176 and COL12 were determined in two-fold serial dilutions on Vero cells using ATP-based assay after incubation for (A) 16h and (B) 72h.


A
C. jejuni 81-176
C. jejuni COL12
% Cell Death
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
Protein Concentration (µg/ml)

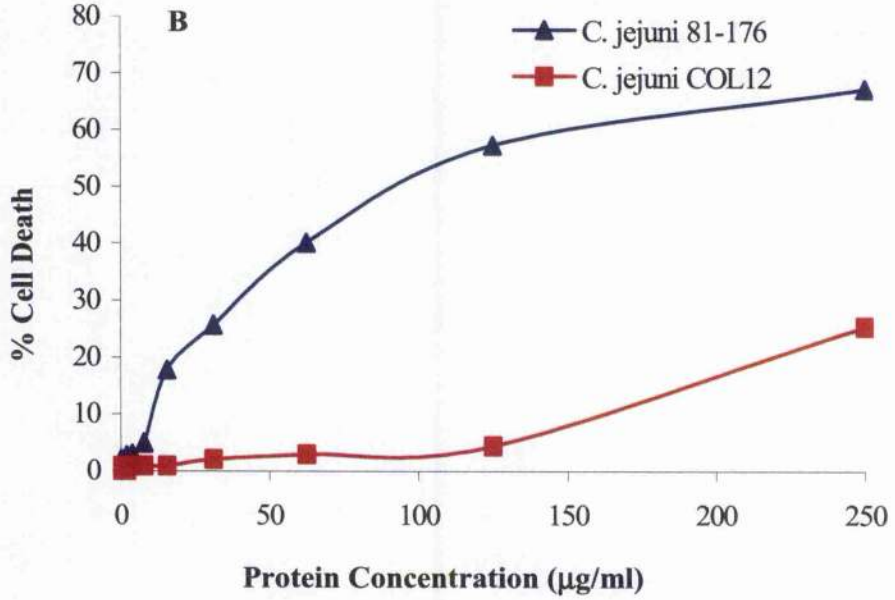

B
C. jejuni 81-176
C. jejuni COL12
% Cell Death
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
Protein Concentration (µg/ml)

Table 3.6: Compiled data from Fig. 3.32 to 3.34

Fractions	Protein Concentration (µg/ml)	%Cell Death after 16h incubation		%Cell Death after 72h incubation	
		81-176	COL12	81-176	COL12
IMP	30	51.91	15.01	80	38.07
OMP	30	6	2	9.65	1.5
Cytoplasmic	30	12	0	25.70	2.15
OMP	250	22.26	6.24	49.19	10.62
Cytoplasmic	250	33.31	4.21	67.21	25.42
OMP	500	56.88	8.63	80.62	52.54

containing 0.1M, 0.2M and 0.3M NaCl for both strains (Fig. 3.35 to 3.38).

3.5.1.2 Concentration of the fractions

A Microcon system with MW cut-off of 5000 Da was used to concentrate (10 times) some of the column chromatography fractions (flow- through, wash through, 0.05M, 0.1M, 0.2M, and 0.3M NaCl) of both 81-176 and COL12 strains. After centrifugation, the supernatant from the Microcon filtration was run on SDS-PAGE (Fig. 3.39). These data showed that little protein was apparent in the concentrated flow-through, wash through and 0.05M NaCl fractions. Many protein bands were, however, visible from the concentrated 0.1M, 0.2M and 0.3M fractions. Some of the bands (no.1, no.2 and no.3) were then identified by Mass spectrometry and the results are shown in Table 3.7. Protein bands 1 and 2 were shown to be the same and were each made up of two identical proteins. Band 3 appeared to contain a number of different proteins. pI values of the identified proteins in bands no.1, no.2 and no.3 (Table 3.7) showed these values in the proteins in band no.1 which eluted first are higher than that in band no.3 which eluted later in DEAE-Sepharose column chromatography (Fig 3.39). This finding confirms the nature of ion-exchange column chromatography.

3.5.1.3 Cytotoxicity of DEAE sepharose column chromatography fractions

Cytotoxicity of fractions of strain 81-176 from DEAE sepharose column chromatography was tested after incubation with Vero cells for 16h. To take account of any toxicity of increased NaCl in the elution buffer, concentrations of 0.05-1M NaCl in buffer were tested in the MTT assay for toxicity (Fig. 3.40). Toxicity was marginal at low NaCl concentrations, but increased significantly at a concentration of 0.4M NaCl and above. According to Figure 3.41 and Fig. 3.42, the most cytotoxic

Figure 3.35: SDS-PAGE analysis of column fractions of cell-free extract of *C. jejuni* 81-176 prepared by DEAE sepharose column chromatography.

Aliquots (20 µl) of column washes and elutions from 0.05 and 0.1M NaCl were subjected to electrophoresis on a 10% (w/v) polyacrylamide gel and stained with 0.1% (w/v) Coomassie blue solution (section 2.5).

M: Protein marker (molecular weight range as indicated)

Lane 1: Crude cell-free extract from 81-176

Lane 2: Flow-through

Lane 3: Wash through (first)

Lane 4: Wash through (second)

Lane 5: First fraction eluted with 0.05 M NaCl

Lane 6: Second fraction eluted with 0.05 M NaCl

Lane 7: First fraction eluted with 0.1 M NaCl

Lane 8: Second fraction eluted with 0.1 M NaCl

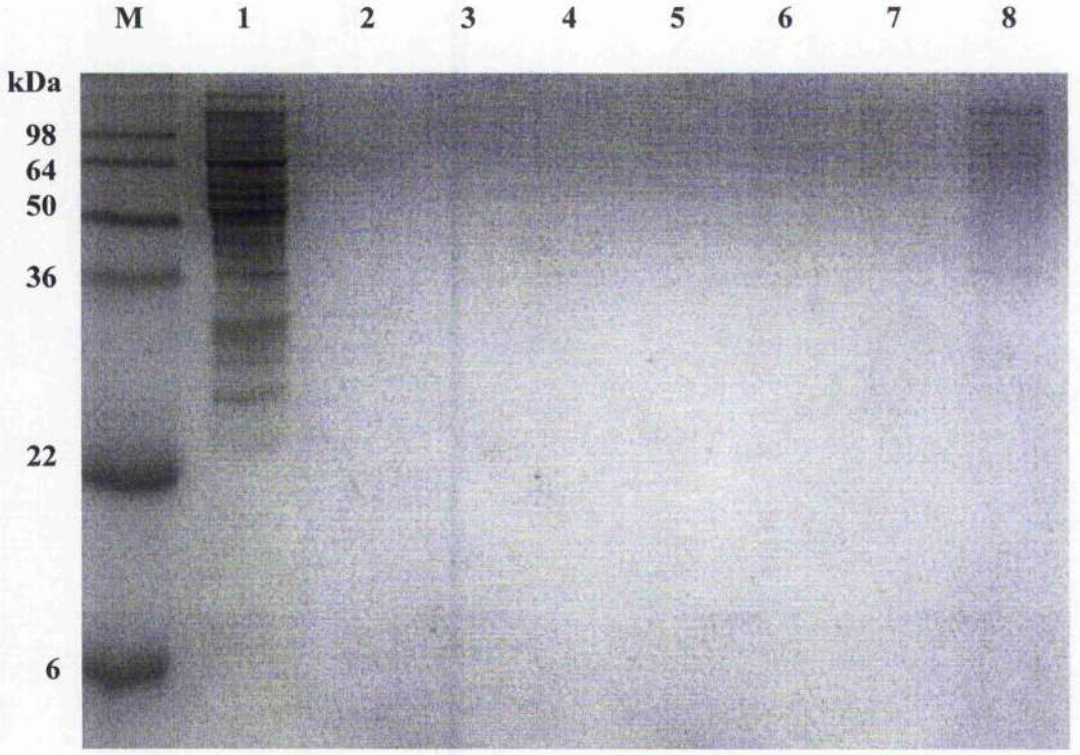

M
1
2
3
4
5
6
7
8
kDa
98
64
50
36
22
6

Figure 3.36: SDS-PAGE analysis of column fractions of cell-free extract of *C. jejuni* 81-176 prepared by DEAE sepharose column chromatography.

Aliquots (20 µl) of elutions up to 0.5 M NaCl were subjected to electrophoresis on a 10% (w/v) polyacrylamide gel and stained with 0.1% (w/v) Coomassie blue solution (section 2.5).

Lane 1: First fraction eluted with 0.2 M NaCl

Lane 2: Second fraction eluted with 0.2 M NaCl

Lane 3: First fraction eluted with 0.3 M NaCl

Lane 4: Second fraction eluted with 0.3 M NaCl

Lane 5: First fraction eluted with 0.4 M NaCl

Lane 6: Second fraction eluted with 0.4 M NaCl

Lane 7: First fraction eluted with 0.5 M NaCl

Lane 8: Second fraction eluted with 0.5 M NaCl

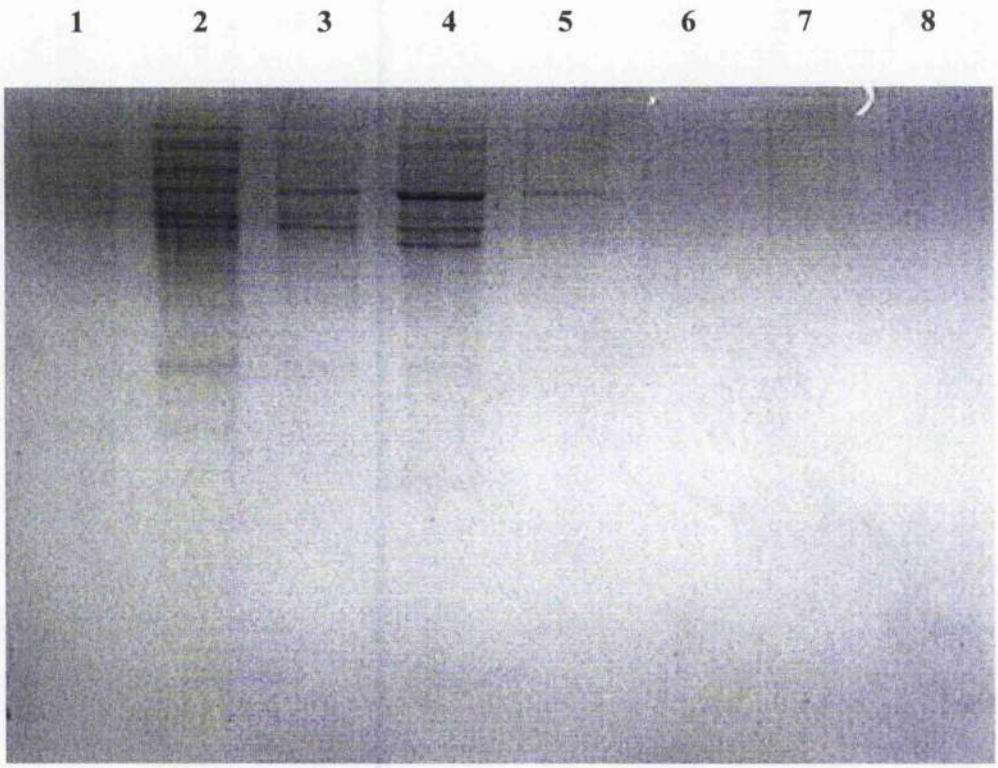

1
2
3
4
5
6
7
8

Figure 3.37: SDS-PAGE analysis of column fractions of cell-free extract of *C. jejuni* COL12 prepared by DEAE sepharose column chromatography.

Aliquots (20 µl) of column washes and elutions from 0.05 and 0.1M NaCl were subjected to electrophoresis on a 10% (w/v) polyacrylamide gel and stained with 0.1% (w/v) Coomassie blue solution (section 2.5).

M: Protein Marker (molecular weight range as indicated)

Lane 1: Crude cell-free extract from *C. jejuni* COL12

Lane 2: Flow- through

Lane 3: Wash through (first)

Lane 4: Wash through (second)

Lane 5: First fraction eluted with 0.05 M NaCl

Lane 6: Second fraction eluted with 0.05 M NaCl

Lane 7: First fraction eluted with 0.1 M NaCl

Lane 8: Second fraction eluted with 0.1 M NaCl

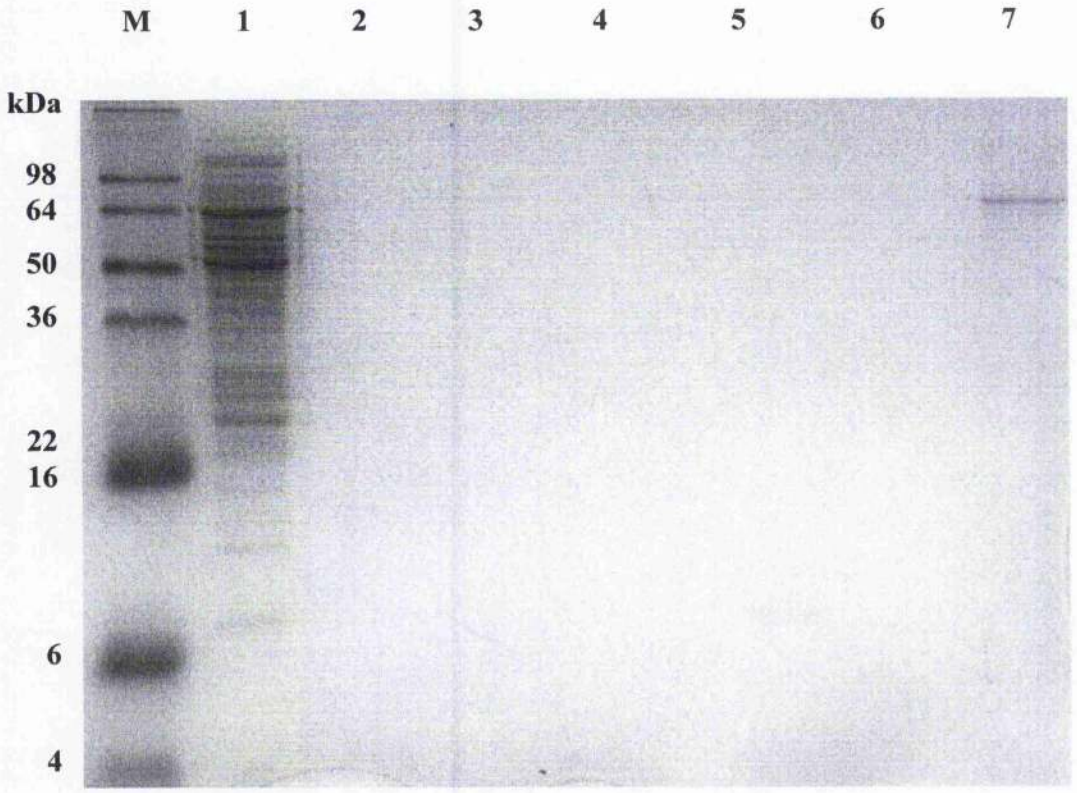

M
1
2
3
4
5
6
7
kDa
98
64
50
36
22
16
6
4

Figure 3.38: SDS-PAGE analysis of column fractions of cell-free extract of *C. jejuni* COL12 prepared by DEAE sepharose column chromatography.

Aliquots (20 µl) of elutions up to 0.5 M NaCl were subjected to electrophoresis on a 10% (w/v) polyacrylamide gel and stained with 0.1% (w/v) Coomassie blue solution (section 2.5).

Lane 1: First fraction eluted with 0.2 M NaCl

Lane 2: Second fraction eluted with 0.2 M NaCl

Lane 3: First fraction eluted with 0.3 M NaCl

Lane 4: Second fraction eluted with 0.3 M NaCl

Lane 5: First fraction eluted with 0.4 M NaCl

Lane 6: Second fraction eluted with 0.4 M NaCl

Lane 7: First fraction eluted with 0.5 M NaCl

Lane 8: Second fraction eluted with 0.5 M NaCl

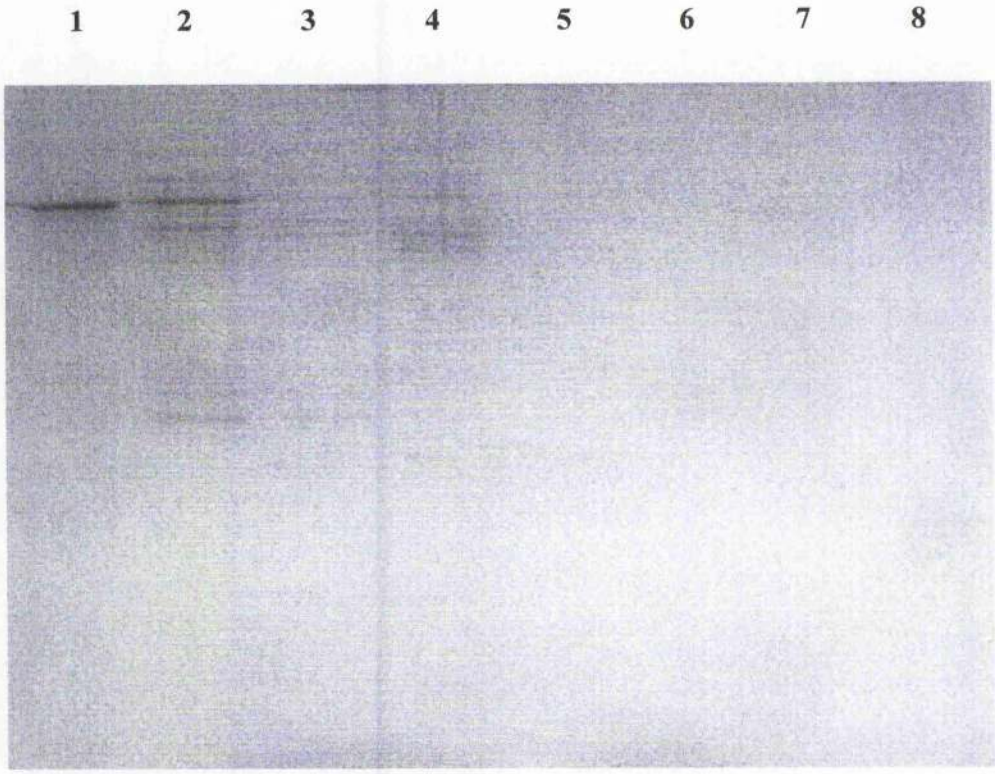

1
2
3
4
5
6
7
8

Figure 3.39: SDS-PAGE analysis of concentrated fractions of *C. jejuni* 81-176 and COL12 prepared by DEAE sepharose column chromatography.

Aliquots (20 µl) were subjected to electrophoresis on a 10% (w/v) polyacrylamide gel and stained with 0.1% (w/v) Coomassie blue solution (section 2.5). Marked bands (1-3) which were not found in strain COL12 were identified by mass spectrometry (Table 3.7).

M: Protein Marker (molecular weight range as indicated)

Lane 1: Flow- through, strain 81-176

Lane 2: Flow- through, strain COL12

Lane 3: Wash through (pooled), strain 81-176

Lane 4: Wash through (pooled), strain COL12

Lane 5: Eluted with 0.05 M NaCl (pooled), strain 81-176

Lane 6: Eluted with 0.05 M NaCl (pooled), strain COL12

Lane 7: Eluted with 0.1 M NaCl (pooled), strain 81-176

Lane 8: Eluted with 0.1 M NaCl (pooled), strain COL12

Lane 9: Eluted with 0.2 M NaCl (pooled), strain 81-176

Lane 10: Eluted with 0.2 M NaCl (pooled), strain COL12

Lane 11: Eluted with 0.3 M NaCl (pooled), strain 81-176

Lane 12: Eluted with 0.3 M NaCl (pooled), strain COL12

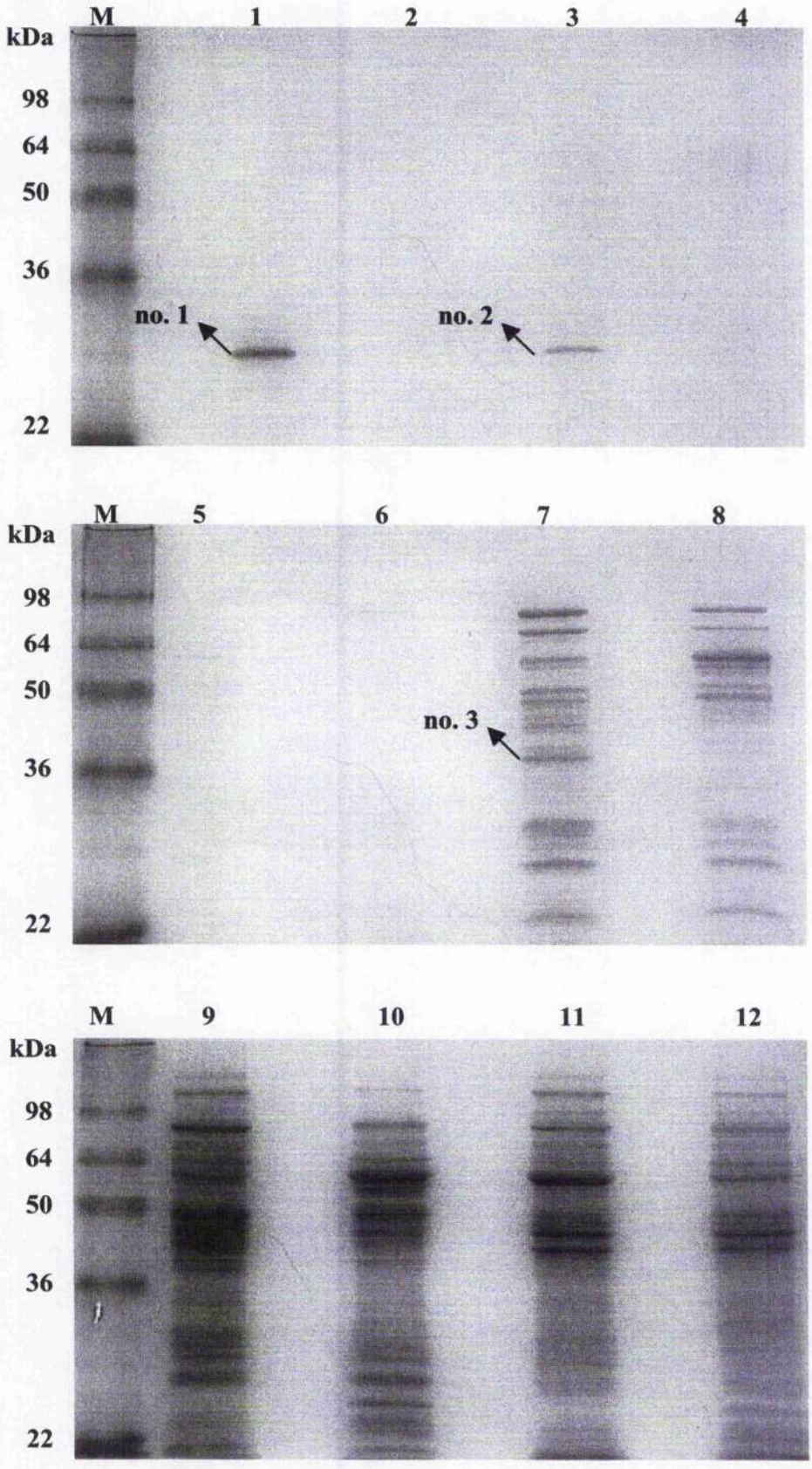

M
1
2
3
4
kDa
98
64
50
36
22
no. 1
no. 2
M
5
6
7
8
kDa
98
64
50
36
22
no. 3
M
9
10
11
12
kDa
98
64
50
36
22

Table 3.7: Identification of proteins from SDS-PAGE of 81-176 using MS.

No. of band on the SDS-PAGE**	Accession number	Locus-tag (Locus)	(Annotation) Protein name	Mr	pI	Matched peptides	Sequence coverage (%)	Mowse Score*
1	gi|6968358	Cj0921c (peb1A)	probable ABC-type amino-acid transporter periplasmic solute-binding protein	28217	8.94	33	64	660
	gi|30721658	Cj0817 (glnH)	GlnH, glutamine-binding periplasmic protein	27658	8.75	10	42	227
2	gi|6968358	Cj0921c (peb1A)	probable ABC-type amino-acid transporter periplasmic solute-binding protein	28217	8.94	14	58	561
	gi|30721658	Cj0817 (glnH)	GlnH, glutamine-binding periplasmic protein	27658	8.75	5	26	105
3	gi|6967567	Cj0069	hypothetical protein Cj0069	39053	5.23	11	39	354
	gi|6967833	Cj0358	Putative cytochrome C551 peroxidase	37051	8.64	8	27	275
	gi|6968027	Cj0559	oxidoreductase	33998	5.73	9	36	218
	gi|6968832	Cj1403c (gapA)	glyceraldehyde 3-phosphate dehydrogenase	36678	6.54	7	21	121
	gi|15559181	Cj1317 (neuB3)	NeuB3, N-acetylneuraminic acid synthetase	38806	5.44	5	11	95
	gi|6968022	Cj0554	hypothetical protein Cj0554	39054	5.97	3	9	63

* Protein identified with MOWSE Score less than 22 were excluded in this table. ** From Fig. 3.39

Figure 3.40: Cytotoxicity of different concentrations of NaCl on Vero cells

The cytotoxic activities of fractions containing the different concentrations of NaCl where proteins were eluted were determined on Vero cells after incubation for 16h using the ATP-based assay.

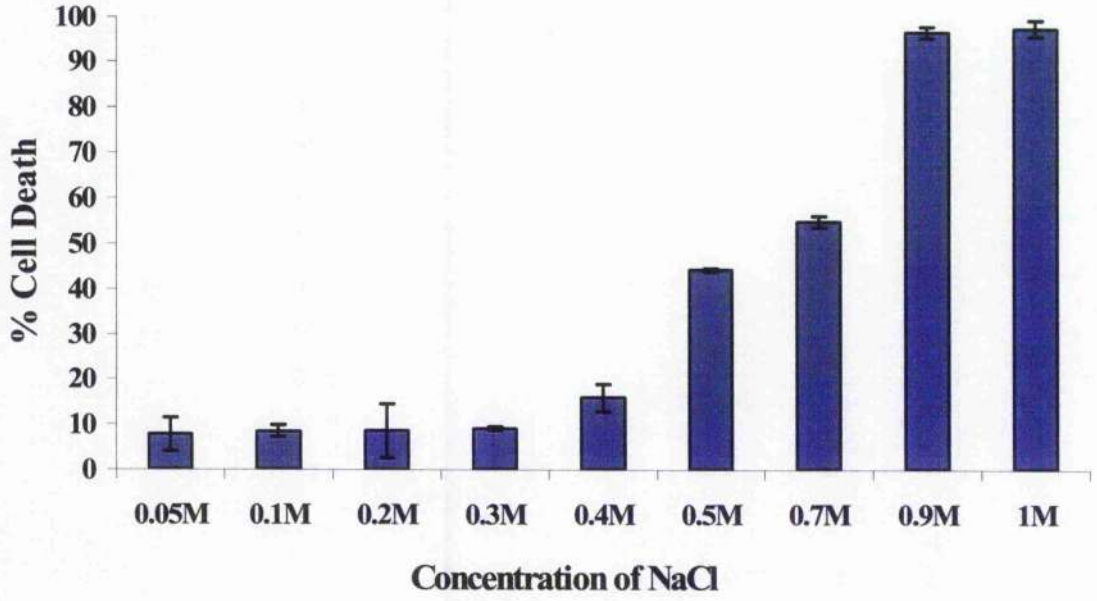

100
90
80
70
60
50
40
30
20
10
0
% Cell Death
0.05M
0.1M
0.2M
0.3M
0.4M
0.5M
0.7M
0.9M
1M
Concentration of NaCl

Figure 3.41: Cytotoxicity on Vero cells of fractions of *C. jejuni* 81-176 cell-free extract prepared by DEAE sepharose column chromatography.

A crude cell-free extract (low speed centrifugation) from *C. jejuni* 81-176 was purified using DEAE sepharose (section 2.8.1). The cytotoxic activities of flow-through (F.T), wash-through (aliquots from the column washes) (W.T), and different elutions were determined in two-fold serial dilutions on Vero cells after incubation for 16h using the ATP-based assay.

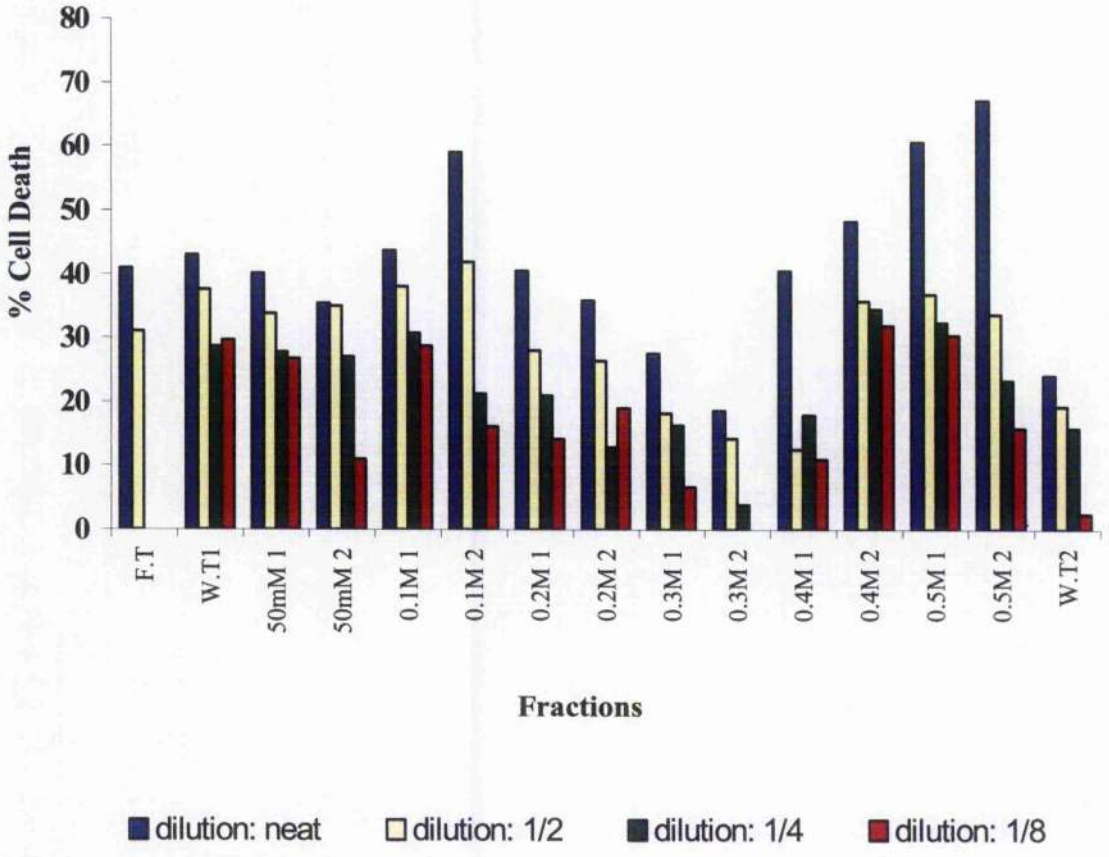
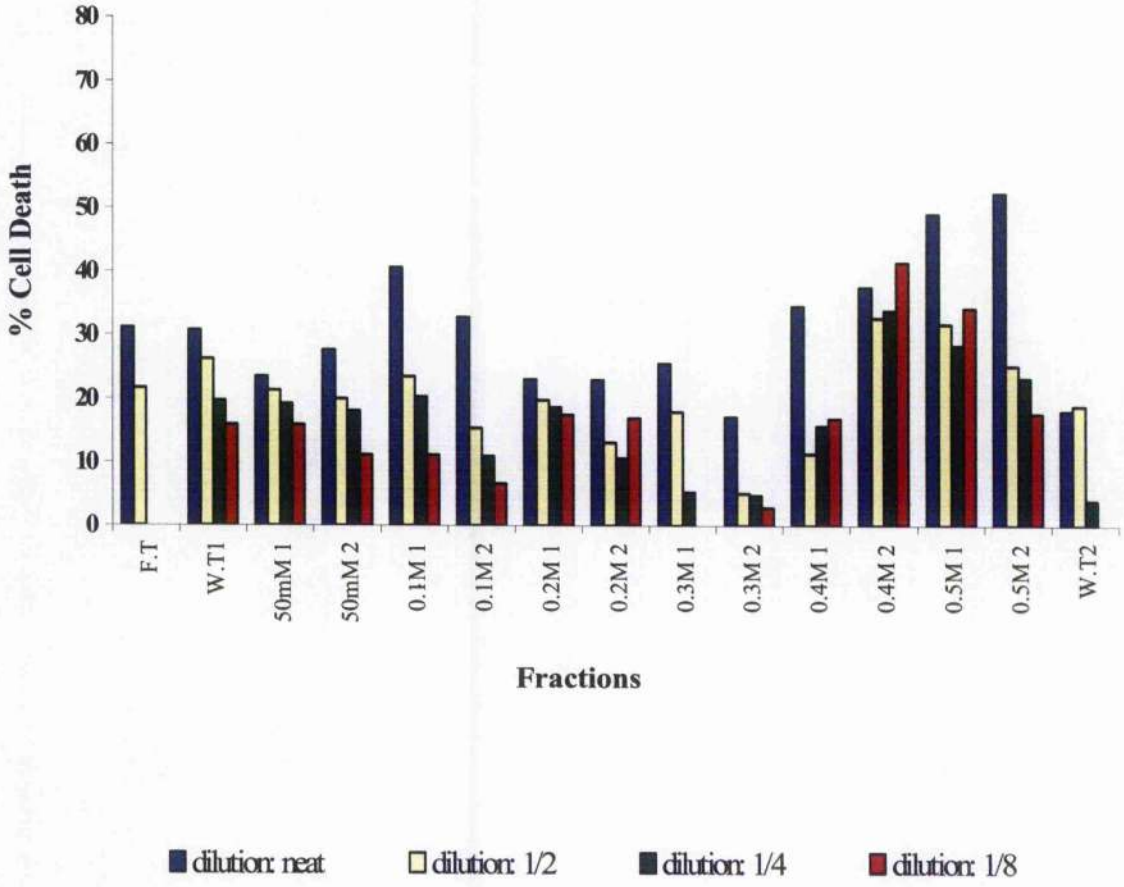

80
70
60
50
40
30
20
10
0
% Cell Death
F.T
W.T1
50mM 1
50mM 2
0.1M 1
0.1M 2
0.2M 1
0.2M 2
0.3M 1
0.3M 2
0.4M 1
0.4M 2
0.5M 1
0.5M 2
W.T2
Fractions
dilution: neat
dilution: 1/2
dilution: 1/4
dilution: 1/8

Figure 3.42: Cytotoxicity on Vero cells of fractions of *C. jejuni* COL12 cell-free extract prepared by DEAE sepharose column chromatography.

A crude cell-free extract (low speed centrifugation) from *C. jejuni* COL12 was purified using DEAE sepharose (section 2.8.1). The cytotoxic activities of flow-through (F.T), wash-through (aliquots from the column washes) (W.T), and different elutions were determined in two-fold serial dilutions on Vero cells after incubation for 16h using the ATP-based assay.


% Cell Death
80
70
60
50
40
30
20
10
0
F.T
W.T1
50mM 1
50mM 2
0.1M 1
0.1M 2
0.2M 1
0.2M 2
0.3M 1
0.3M 2
0.4M 1
0.4M 2
0.5M 1
0.5M 2
W.T2
Fractions
dilution: neat
dilution: 1/2
dilution: 1/4
dilution: 1/8

activities were found in the fractions flow-through, wash-through, 50mM, 0.1M and 0.2M NaCl. From the results of testing 1/8 dilutions of the samples (Fig. 3.41) the most toxicity was detected in wash-through, 50mM, 0.1M and 0.2M NaCl fractions. Considerable activity at this dilution was also detected in 0.4M and 0.5M NaCl fractions, but this was discounted because of the effect of NaCl alone at this concentration. If these experiments were to be repeated, NaCl in the fractions could be removed by using dialysis or MicroCon.

It was hoped that certain fractions from strain 81-176 would show an obviously greater cytotoxicity than the equivalent fractions from COL12 as proteins from each strain eluted from the DEAE-Sepharose in a similar way. At 1/8 dilution there was some indication that fractions at 0.1M NaCl had greater activity in strain 81-176 than COL12, but the flow-through and wash-through fractions also had greater activity. Comparison of the pooled 0.1M NaCl fractions in Fig. 3.39 showed that, aside from the proteins identified in band 3 (Table 3.7) it would be difficult to distinguish clear differences between strains 81-176 and COL12.

3.5.2 SDS-PAGE results of IMP proteins fractionated using Microcon tube

An attempt was made to fractionate the proteins by size from the IMP fraction of 81-176 *cdtB*. It was hoped that if the extract was fractionated, then the proteins present in the fractions might be analysed by column chromatography.

The IMP fraction of 81-176 *cdtB* strain was applied to Microcon filters with different size molecular weight cut offs (MWCO). The Microcon with sizes 100,000, 50,000, 30,000, 10,000 and 5,000 MWCO were used, respectively (Fig. 3.43). Apart from the

Figure 3.43: SDS-PAGE analysis of fractions of IMP from *C. jejuni* 81-176 *cdtB* using microcon

IMP fractions from *C. jejuni* 81-176 *cdtB* were separated using different sizes (MWCO) of microcon filter (section 2.8.2) and subjected in a 20 µl volume, to electrophoresis on a 12.5% (w/v) polyacrylamide gel and stained with 0.1% (w/v) Coomassie blue solution (section 2.5).

M: Protein Marker (molecular weight range as indicated)

Lane 1: IMP Crude

Lane 2: First fraction >100,000 MW

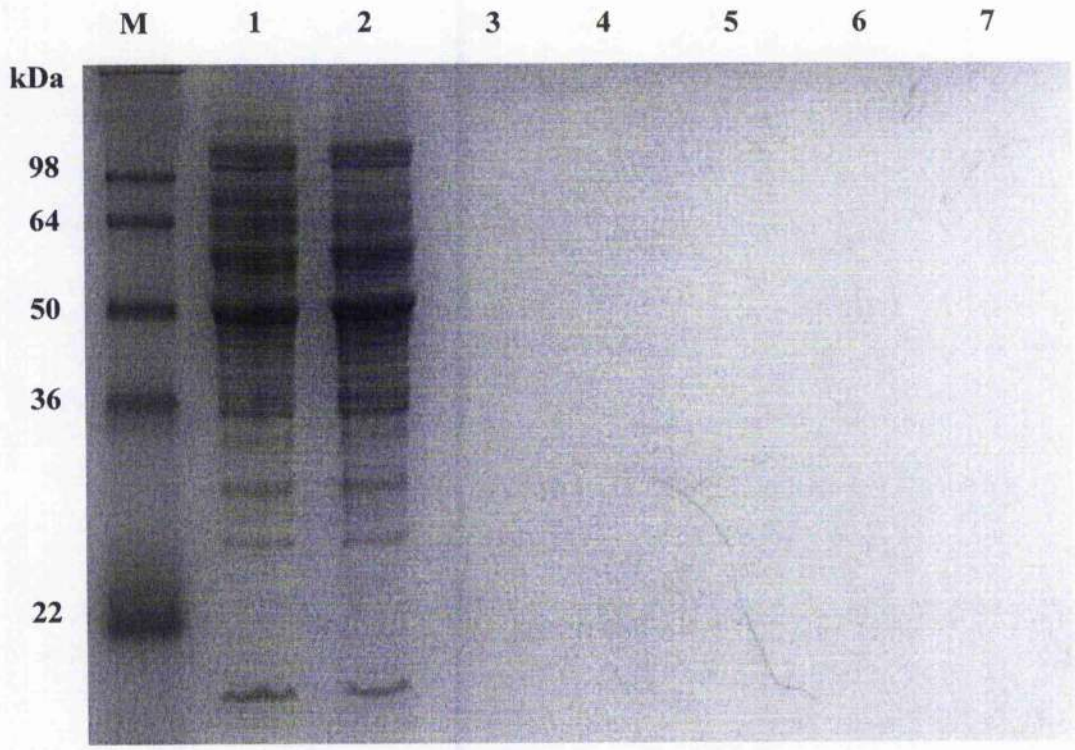
Lane 3: Second fraction 50,000-100,000 MW

Lane 4: Third fraction 30,000-50,000 MW

Lane 5: Fourth fraction 10,000-30,000 MW

Lane 6: Fifth fraction 5,000-10,000 MW

Lane 7: Sixth fraction <5,000 MW


M
1
2
3
4
5
6
7
kDa
98
64
50
36
22

first fraction, no protein bands were visible in the other fractions. It is likely that the starting material for fractionation comprised detergent micelles, each containing a mixture of proteins. When analysed by SDS-PAGE, the proteins would have been freed from the micelles. This would explain the appearance of proteins of modest molecular weight in that fraction held back on the 100 kDa cut-off filter (lane 2, Fig. 3.43).

The results of SDS-PAGE were confirmed by protein assay results which showed that all of the protein content was in the first fraction retained by the filter with a MWCO of 100,000. It was not therefore considered feasible to attempt to purify proteins from the IMP fraction at this stage.

3.6 Identification of proteins (proteomics)

Attempts to identify individual proteins from the DEAE-Sepharose chromatography proved difficult, probably due to insufficient protein in each protein band detected after one-dimensional SDS-PAGE electrophoresis and insufficient separation of the bands into individual proteins. Thus, it was decided to compare protein profiles after 2-DE gel electrophoresis to obtain better resolution of the individual protein components of the cytotoxic extracts. Different strains of *C. jejuni* were chosen, 81-176, 81-176 *cdtB*, 11168 and COL12, which were known to possess different cytotoxic activities. It was assumed that these differences in cytotoxicity arose from differences in the levels of expression of particular proteins. The simplest explanation for the cytotoxicity of the strains was that one produced a protein with

biological activity (e.g. a toxin) and other did not. However, differences could also arise from the levels of protein synthesis. These possibilities were considered when analysing the data from 2-DE gel electrophoresis. Protein profiles from sup1 (cytoplasmic fraction) and sup2 (membrane fraction) extracts of the strains were therefore prepared according to the procedures given in section 2.9.2.1. The cytoplasmic fraction (sup1) was prepared using the ProteoPrep™ Universal Extraction Kit (Sigma) as the supernatant following sonication in the Soluble cytoplasmic extraction reagent (S2688, Sigma). The membrane fraction was prepared after solubilisation in the Cellular and organelle membrane solubilizing reagent (C0356, Sigma).

3.6.1 TCA precipitation of sup2 preparation

Due to the low protein concentration of sup2 in each of the strains, TCA was used to precipitate proteins in an attempt to concentrate the sup2 preparation. After precipitation, the concentrated sup2 was run on SDS-PAGE. As the SDS-PAGE results show, even after precipitation, the concentration of sup2 was still very low (Fig. 3.44). Even after concentration of sup2 there were no obvious protein bands in any strain, but a lot of proteins were seen in sup1 without any concentration. However, the separation was poor, presumbly because of some of the components of the reagents of the kit used in preparation of the extracts.

Figure 3.44: SDS-PAGE analysis of sup1 and sup2 prepared by ProteoPrep™ Universal Extraction Kit

Non-concentrated sup1 and concentrated sup2 (using TCA precipitation method) from *C. jejuni* strains 81-176, 81-176 *cdtB*, 11168, and COL12 (section 2.9.2.1) in a 20 µl volume, were subjected to electrophoresis on a 10% (w/v) polyacrylamide gel and stained with 0.1% (w/v) Coomassie blue solution (section 2.5).

M: Protein Marker (molecular weight range as indicated)

Lane 1: sup1 from *C. jejuni* 81-176

Lane 2: sup2 from *C. jejuni* 81-176

Lane 3: sup1 from *C. jejuni* 81-176 *cdtB* mutant

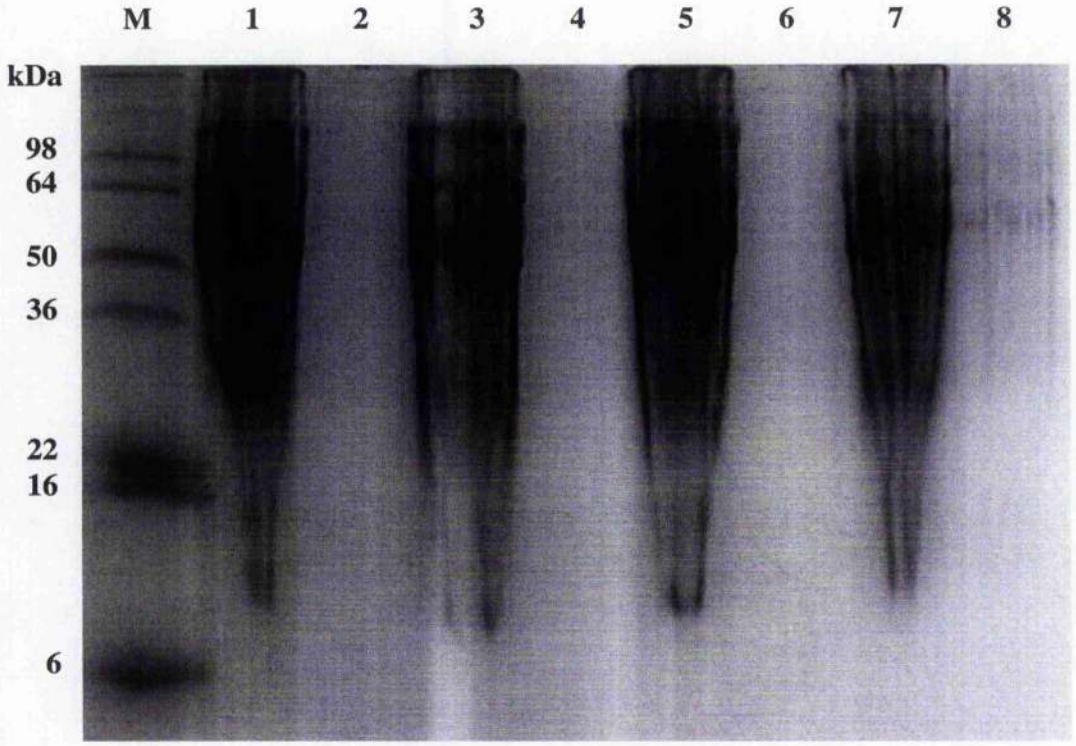
Lane 4: sup2 from *C. jejuni* 81-176 *cdtB* mutant

Lane 5: sup1 from *C. jejuni* 11168

Lane 6: sup2 from *C. jejuni* 11168

Lane 7: sup1 from *C. jejuni* COL12

Lane 8: sup2 from *C. jejuni* COL12


M
1
2
3
4
5
6
7
8
kDa
98
64
50
36
22
16
6

3.6.2 Two-dimensional electrophoresis and characterization of separated proteins

Successful 2-DE gels were obtained for the sup1 fractions of strains 81-176, 81-176 *cdtB* and 11168 (Fig. 3.45, 3.46 and 3.47). Insufficient protein was present on the 2-DE gel for strain COL12 to allow protein identification, but the gel did show good resolution (Fig. 3.48). Nevertheless, the quality of gel was adequate to compare with different strains. Spot numbering on each 2-DE gel was independent and therefore a spot with the same number on different gels need not represent the same proteins. 33 proteins were identified from the sup1 sample of strain 81-176, although no obvious toxin-like proteins were among them (Table 3.8). 22 proteins were identified from strain 81-176 *cdtB* and 24 proteins from strain 11168 (Tables 3.9 and 3.10). A worked example of identification of spot no.19 from 81-176 is shown in Appendices 16, 17 and 18. Proteins identified with a MOWSE score of less than 46 were excluded from Tables 3.8, 3.9 and 3.10. A putative oxidoreductase subunit (gi|15791781 or gi|15791782) appeared in all 3 gels (81-176, 81-176 *cdtB* and 11168). In fact, a striking number of oxidoreductase enzymes and other enzymes associated with central metabolic pathways e.g. phosphoenol pyruvate carboxykinase, citrate synthase and aconitate hydratase were identified. Spot no. 20 in the gel of 11168 was identified as a two-component regulator (Cj0355c) and, while it did not appear in the gels of 81-176 or 81-176 *cdtB*, the spot appeared in the gel of COL12. Comparison by eye of 2-DE gels of 81-176 and COL12 showed that spots no. 16 (Cj0772c, putative periplasmic protein), 17 (Cj1420c, hypothetical protein), 19 (Cj0169, superoxide dismutase, Fe), 23 (Cj0414, putative oxidoreductase subunit), 25 (Cj0779, probable thiol peroxidase), 26 (Cj0779, probable thiol

Figure 3.45: Two-dimensional electrophoresis (2-DE) gels of sup1 from *C. jejuni* 81-176

Sup1 of *C. jejuni* 81-176 (section 2.9.2.1) was focused on a pH3-10 IPG strip. SDS-PAGE was performed on a 12.5% (w/v) gel and stained with Coomassie blue solution. To avoid confusion with spots to be picked for identification, molecular weight and pI standards were omitted from the 2-DE gels. The spots labelled 35 and 36 on this gel, visible on subsequent gels, are non-biological markers used for reference in spot picking.

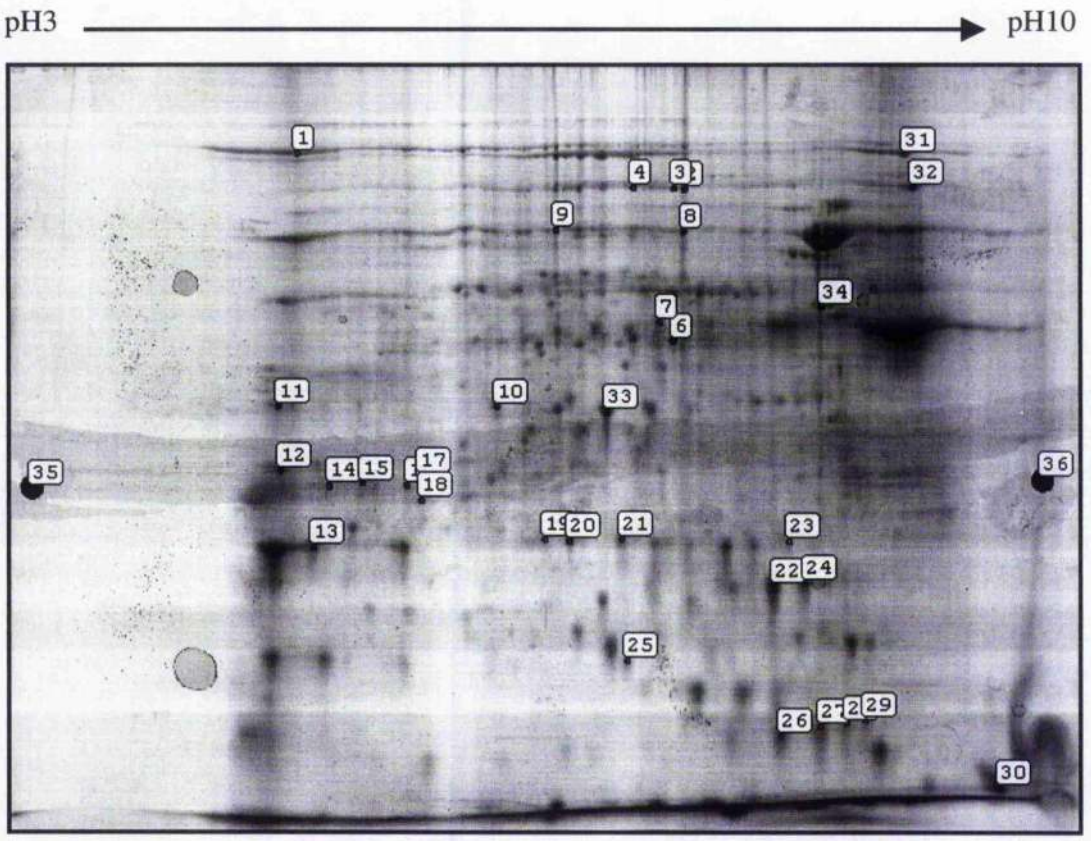

pH3
pH10
1
31
4
3
32
9
8
34
7
6
11
10
33
12
14
15
17
18
35
36
13
19
20
21
23
22
24
25
26
29
30

Table 3.8: Identification of spots on 2-DE gel of *C. jejuni* 81-176

Spot number	Accession number	Locus-tag (Locus)	Protein name (Annotation)	Matched sequences	Sequence coverage (%)	Mowse score*
8	gi|15791782	Cj0415	putative oxidoreductase subunit	12	21	96
9	gi|15791920	Cj0559	oxidoreductase	12	54	103
14	gi|15791895	Cj0534 (sucD)	succinyl-coA synthetase alpha chain	10	42	46
16	gi|15792110	Cj0772c	putative periplasmic protein	5	21	50
17	gi|15792738	Cj1420c	hypothetical protein Cj1420c	5	21	65
19	gi|15791556	Cj0169 (sodB)	superoxide dismutase (Fe)	7	35	59
20	gi|15791556	Cj0169 (sodB)	superoxide dismutase (Fe)	4	22	53
21	gi|15791781	Cj0414	putative oxidoreductase subunit	11	41	125
23	gi|15791781	Cj0414	putative oxidoreductase subunit	9	33	80

Table 3.8 (continued): Identification of spots on 2-DE gel of *C. jejuni* 81-176

Spot number	Accession number	Locus-tag (Locus)	Protein name (Annotation)	Matched sequences	Sequence coverage (%)	Mowse score*
25	gi|15792117	Cj0779 (tpx)	probable thiol peroxidase	8	69	74
26	gi|15792117	Cj0779 (tpx)	probable thiol peroxidase	7	69	99
27	gi|15792117	Cj0779 (tpx)	probable thiol peroxidase	5	52	62
29	gi|15792705	Cj1382c (fldA)	flavodoxin	3	39	59
30	gi|15792173	Cj0835c (acnB)	aconitate hydratase	19	30	142
32	gi|4704601	Cj1478c (cadF)	fibronectin binding protein	13	51	214
33	gi|15791495	Cj0107 (atpD)	ATP synthase F1 sector beta subunit	18	47	252

*Spots no. 35 and 36 were used as markers.

Proteins identified with MOWSE Score less than 46 were excluded from this table.

Figure 3.46: Two-dimensional electrophoresis (2-DE) gels of sup1 from *C. jejuni* 81-176 *cdtB*

Sup1 of *C. jejuni* 81-176 *cdtB* (section 2.9.2.1) was focused on a pH3-10 IPG strip. SDS-PAGE was performed on a 12.5% (w/v) gel and stained with Coomassie blue solution. The spots labelled 23 and 24 are non-biological markers used for reference in spot picking.

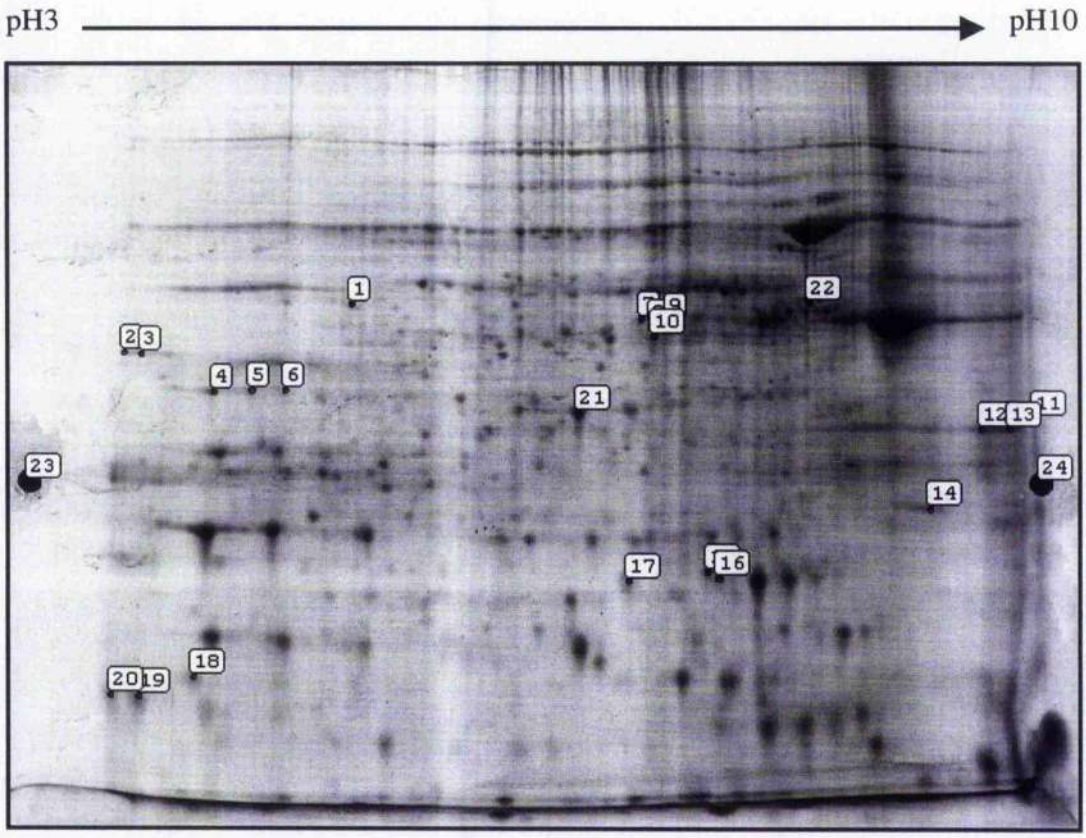

pH3
pH10
1
2
3
4
5
6
7
8
9
10
11
12
13
14
16
17
18
19
20
21
22
23
24

Table 3.9: Identification of spots on 2-DE gel of *C. jejuni* 81-176 *cdtB* mutant

Spot number	Accession number	Locus-tag (Locus)	Protein name	Matched peptides	Sequence coverage (%)	Mowse score*
7	gi|9965857	(cmp)	major outer membrane protein (MOMP)	6	22	78
9	gi|15791894	Cj0533 (sucC)	succinyl-coA synthetase beta chain	10	27	109
15	gi|15791781		putative oxidoreductase subunit	5	20	48
20	gi|4704601	Cj1478c (cadF)	fibronectin binding protein	12	50	188
21	gi|15791495	Cj0107 (atpD)	ATP synthase F1 sector beta subunit	15	35	187
22	gi|15792590	Cj1266c (hydB)	Ni/Fe-hydrogenase large subunit	16	32	153

*Spots no. 23 and 24 were used as markers.

Proteins identified with MOWSE Score less than 46 were excluded from this table.

Figure 3.47: Two-dimensional electrophoresis (2-DE) gels of sup1 from *C. jejuni* 11168

Sup1 of *C. jejuni* 11168 (section 2.9.2.1) was focused on a pH3-10 IPG strip. SDS-PAGE was performed on a 12.5% (w/v) gel and stained with Coomassie blue solution. The spots labelled 33 and 34 are non-biological markers used for reference in spot picking.

pH3 ⟶ pH10

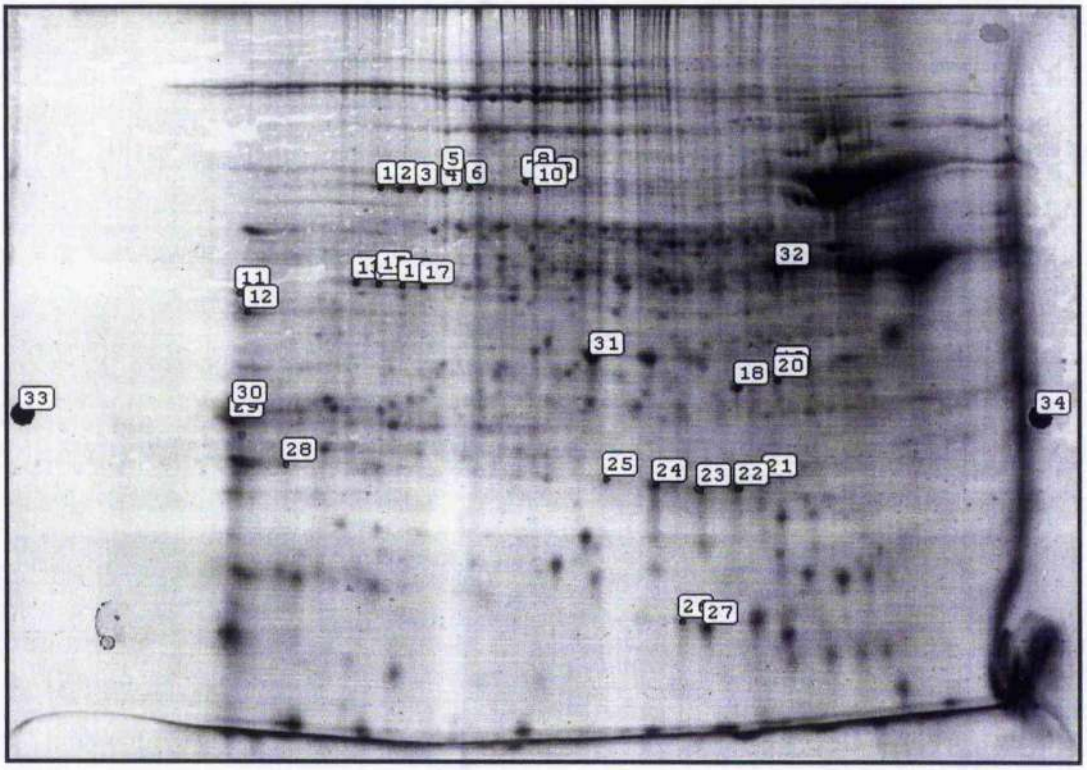


Table 3.10: Identification of spots on 2-DE gel of *C. jejuni* 11168

Spot number	Accession number	Locus-tag (Locus)	Protein name	Matched peptides	Sequence coverage (%)	Mowse score*
1	gi|15792590	Cj1266c (hydB)	Ni/Fe-hydrogenase large subunit	17	32	154
4	gi|15791782	Cj0415	putative oxidoreductase subunit	15	30	110
5	gi|15792590	Cj1266c (hydB)	Ni/Fe-hydrogenase large subunit	11	23	74
6	gi|15792261	Cj0932c (pckA)	phosphoenolpyruvate carboxykinase (ATP)	9	25	76
8	gi|15792261	Cj0932c (pckA)	phosphoenolpyruvate carboxykinase (ATP)	6	16	61
11	gi|15792552	Cj1228c (htrA)	serine protease (protease DO)	8	21	62
13	gi|15792986	Cj1682c (gltA)	citrate synthase	15	39	131

Table 3.10 (continued): Identification of spots on 2-DE gel of *C. jejuni* 11168

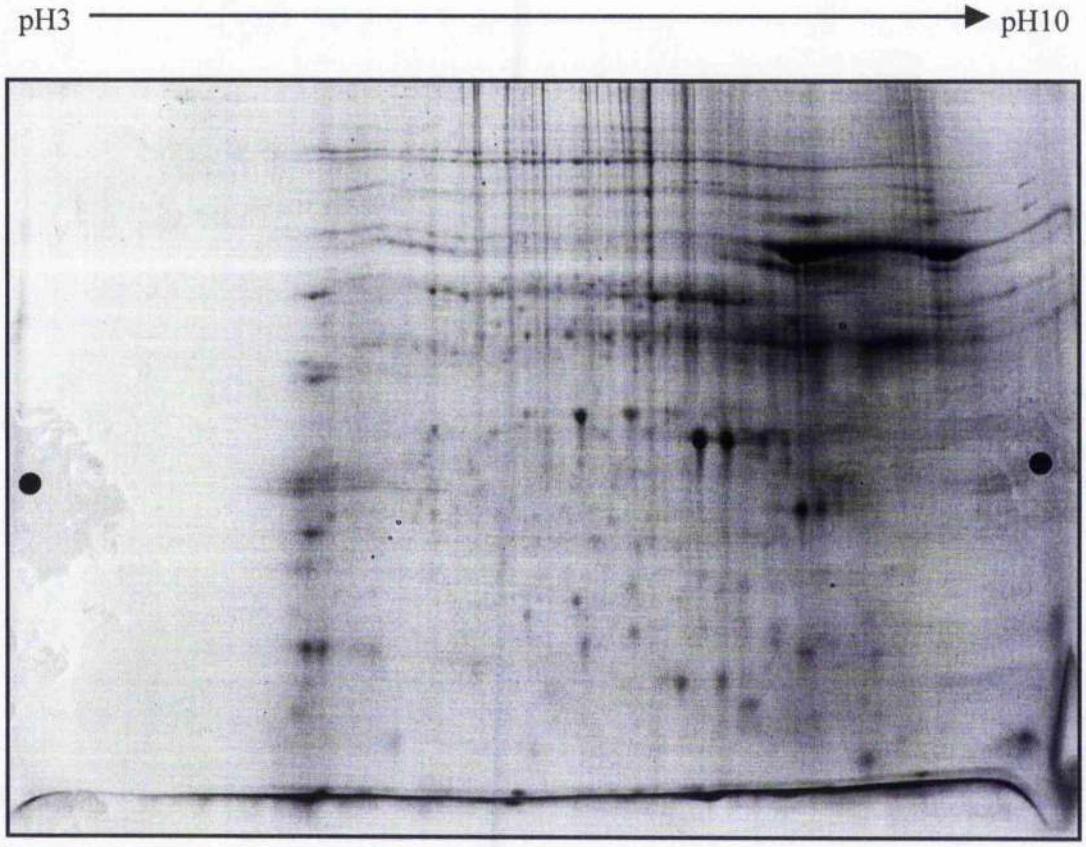
Spot number	Accession number	Locus-tag (Locus)	Protein name	Matched peptides	Sequence coverage (%)	Mowse score*
14	gi|15792986	Cj1682c (gltA)	citrate synthase	7	18	99
16	gi|15791897	Cj0536 (oorA)	OORA subunit of 2-oxoglutarate:acceptor oxidoreductase	14	45	135
20	gi|15791723	Cj0355c	two-component regulator	5	28	51
21	gi|15791723	Cj0355c	two-component regulator	6	26	79
22	gi|28557065	(Rrc)	Rrc	4	26	59
24	gi|15791556	Cj0169 (sodB)	superoxide dismutase (Fe)	4	22	59

*Spots no. 33 and 34 were used as markers.

Proteins identified with MOWSE Score less than 46 were excluded from this table.

Figure 3.48: Two-dimensional electrophoresis (2-DE) gels of sup1 from *C. jejuni* COL12

Sup1 of *C. jejuni* COL12 (section 2.9.2.1) was focused on a pH3-10 IPG strip. SDS-PAGE was performed on a 12.5% (w/v) gel and stained with Coomassie blue solution.


pH3
pH10

peroxidase), 27 (Cj0779, probable thiol peroxidase), 29 (Cj1382c, flavodoxin), and 30 (Cj0835c, aconitate hydratase) which were identified in the gel of strain 81-176, did not appear in the gel of strain COL12. Spots no. 25 (Cj0779, probable thiol peroxidase) in strain 81-176, did not appear in strain 11168. However, multiple spots were identified as probable thiol peroxidase (spots 25, 26 and 27; Fig. 3.45). This observation suggests that probable thiol peroxidase is processed to produce fragments with lower MW than the predicted mature protein. Fibronectin binding protein (Cj1478c, CadF) was identified in strain 81-176, but was not present in strain 11168. Spots no. 32 in strain 81-176 (Fig 3.45) and 20 in 81-176 *cdtB* (Fig. 3.46) both identified as CadF are located in different places on the 2-DE gels and appear to possess different MW. N-liked glycosylation in *C. jejuni* has been reported by Wacker *et al.* (2002). It is speculated that glycosylation of outer membrane protein/peripheral protein can increase MW. Therefore spot no. 32 in Fig 3.45 may be a glycosylated form of CadF and spot no. 20 in Fig. 3.46 may be an unmodified form of the same protein.

Strains 81-176 and 81-176 *cdtB* were chosen to run on 2-DE to check that the 2-DE procedure was reproducible because, apart from the absence of CDT in the *cdtB* strain, it was expected that the profiles of these strains should be similar. Comparison by eye of Figs. 3.45 and 3.46 indicates that this was the case. The most important differences should be CDT subunits (sizes 29.9, 29.0 and 21.2 kDa), which should be present in 81-176 and absent in 81-176 *cdtB*. However, none of the CDT subunits were identified among the 81-176 spots. The reason why 81-176 and 11168 were chosen to run on 2-DE gels was that 11168 was less cytotoxic on Vero cells than 81-176, so it was hoped that there might be some differences in proteins responsible for cytotoxicity when these strains were run on 2-DE gels. This, however, proved to be

too difficult as many differences in spot intensity and position were manifest between the gels. COL12 was chosen because it was less cytotoxic on Vero cells than other strains, and it was also negative for the presence of CDT (according to the microscopic pictures taken after incubation for 72h).

3.6.3 Difference gel electrophoresis (DIGE)

Difference gel electrophoresis (DIGE) analysis was done for the sup1 fractions of strains 81-176 and 81-176 *cdtB* (Fig. 3.49 and 3.50). The results showed that in strain 81-176 *cdtB*, proteins Cpn60 (60 kDa chaperonin) and elongation factor Tu were down regulated 4 and 2-fold compared with 81-176, respectively. Surprisingly, again the absence of CDT subunits in 81-176 *cdtB* was not found by this technique.

Unfortunately, time restraints prevented a DIGE comparison of 81-176 and 11168 or COL12.

Figure 3.49: DiGE (Difference gel electrophoresis) of sup1 of *C. jejuni* 81-176 and 81-176 *cdtB*.

DIGE analysis indicates that the spot arrowed was down regulated ~4-fold in *C. jejuni* 81-176 *cdtB* mutant (right picture), compared with *C. jejuni* 81-176 (left picture). The spot was identified as Cpn60 (60 kDa chaperonin).

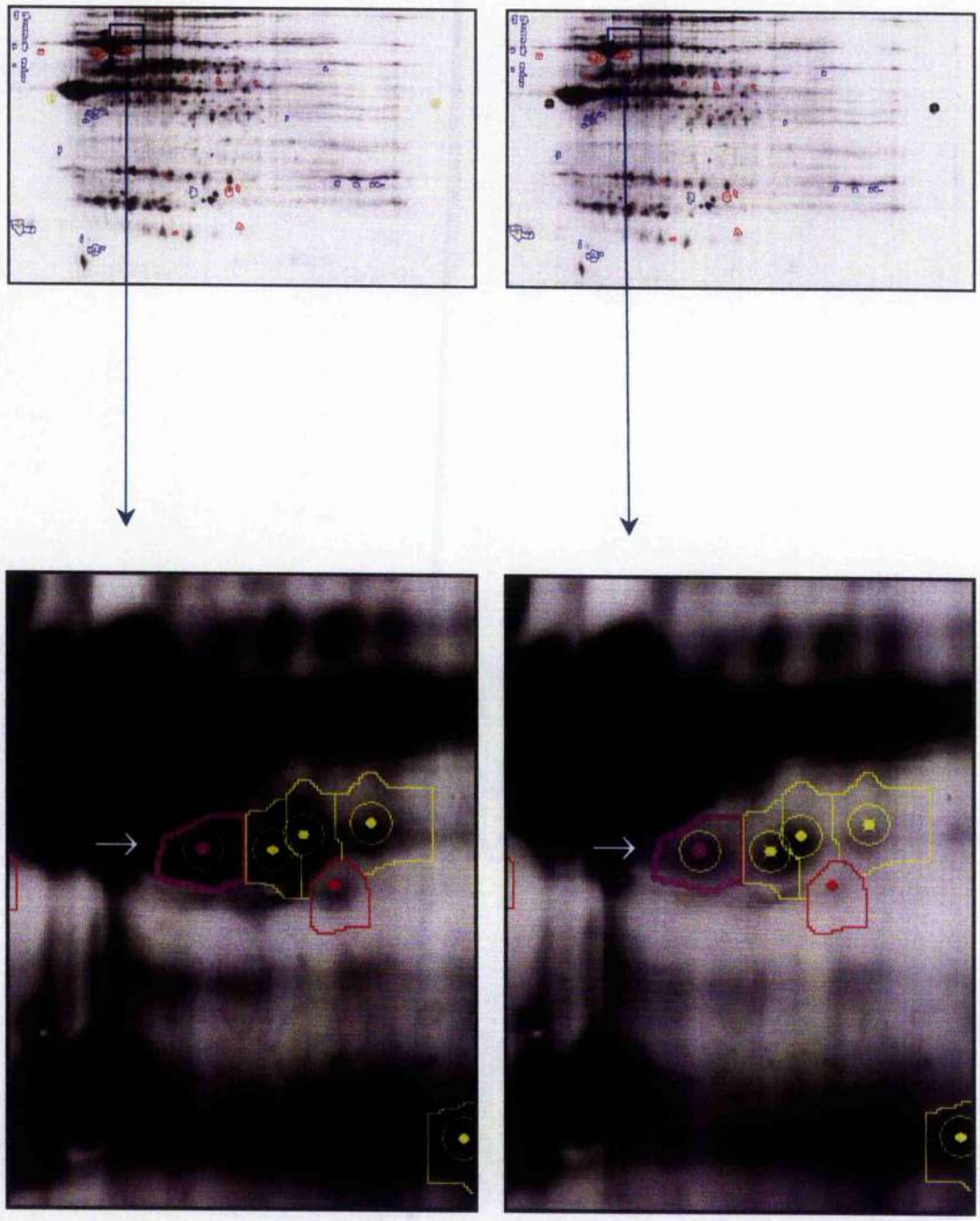
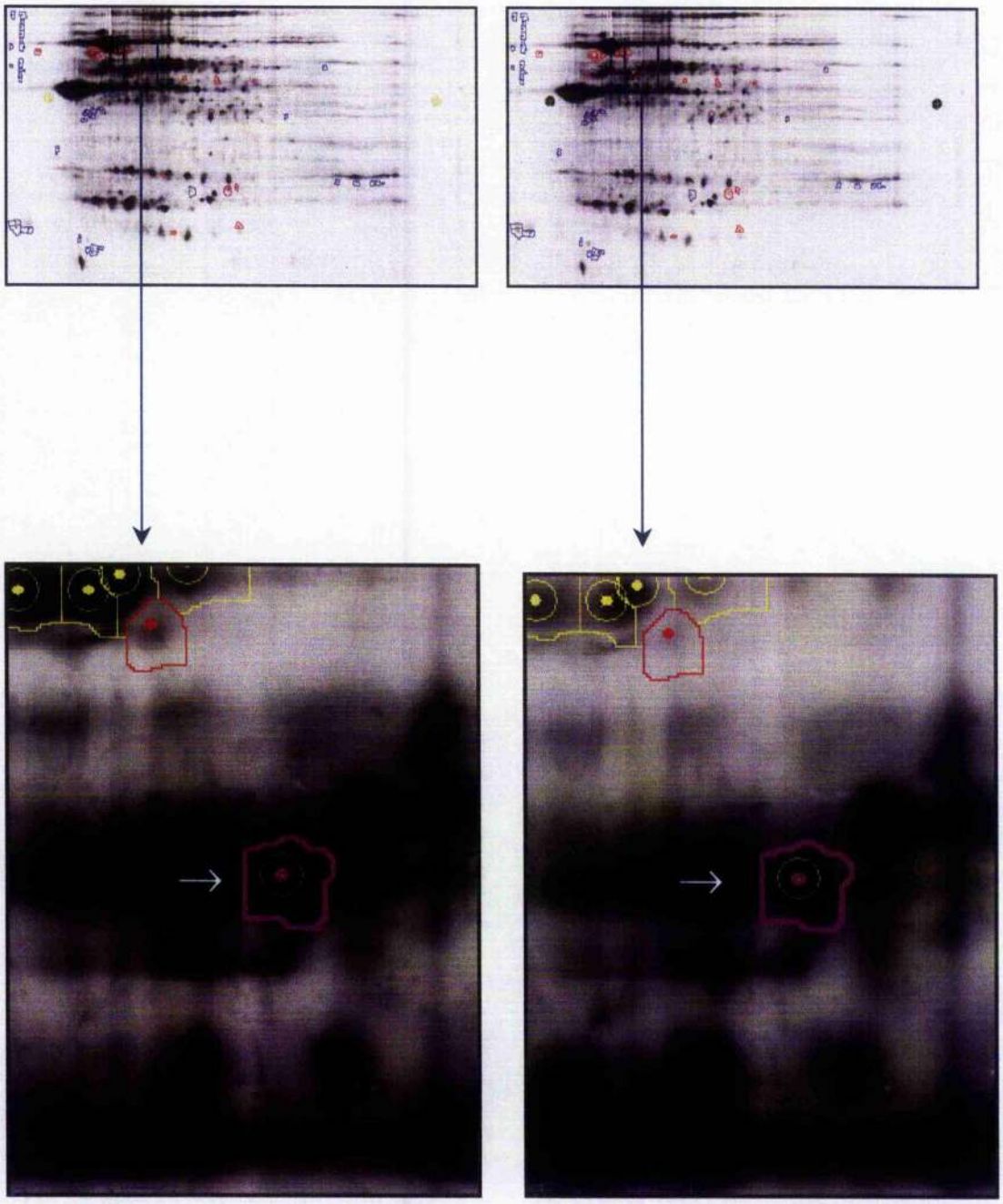


Figure 3.50: DiGE (Difference gel electrophoresis) of sup1 of *C. jejuni* 81-176 and 81-176 *cdtB*.

DIGE analysis indicates that the spot arrowed was down regulated ~2-fold in *C. jejuni* 81-176 *cdtB* mutant (right picture), compared with *C. jejuni* 81-176 (left picture). The spot was identified as elongation factor Tu (Cj0470).



Chapter Four

Discussion

CHAPTER 4: DISCUSSION

4.1 Presence of *hipO* gene in clinical isolates

Diagnostic tests based on the polymerase chain reaction (PCR) are attractive due to their relatively low cost in terms of laboratory manpower, rapid turnaround time, their ease of use, potential to be fully automated (Houng *et al.*, 2001; Linton *et al.*, 1997; Maher *et al.*, 2003; On & Jordan, 2003), high sensitivity and specificity (Dickins *et al.*, 2002; Matsuda *et al.*, 1995; Ragimbeau *et al.*, 1998). A number of conventional PCR assays have been described for the identification and characterization of campylobacters from a spectrum of sample types, including culture (Fermer & Engvall, 1999; Wang *et al.*, 2002), food products (Bang *et al.*, 2002; Burnett *et al.*, 2002; Sails *et al.*, 2003) and stools (Houng *et al.*, 2001; Linton *et al.*, 1997; Maher *et al.*, 2003; On & Jordan, 2003) and using a variety of gene targets e.g. *hipO*, 23S rRNA, *glyA*, *mapA*, and *ceuE* (On & Jordan, 2003).

Harvey (1980) reported that the hippurate hydrolysis test allows clear differentiation between the species *C. jejuni* and *C. coli* and simplifies the biochemical identification of these species. The N-benzoylglycine amidohydrolase (hippuricase), the product of the *hipO* gene (Hani & Chan, 1995), acts to cleave N-benzoylglycine (hippuric acid or hippurate) into the constituent products glycine and benzoic acid. Glycine formation is detected by using a ninhydrin-based system (Harvey, 1980).

In this study, the presence of the *hipO* gene and hippuricase were confirmed in clinical isolates by using a PCR technique and the API CAMPY test, respectively. All *C. jejuni* isolates were tested by PCR for the presence of *hipO* and all were confirmed positive. The *C. coli* strain gave a negative result. This test was therefore

confirmed as accurate and very useful for rapid diagnosis of *C. jejuni*. The PCR technique and API CAMPY test results showed that 100% of the clinical isolates were positive for the presence of the *hipO* gene and hippuricase. This was a greater success rate than that reported by On who gave values of 91% (Table 1.2; On, 2005) (derived from various reports) and 99% (On, 1996) respectively, for hippurate hydrolysis.

4.2 Cytotoxic activities of *C. jejuni* strains

4.2.1 Cytotoxicity of cell-free extracts from *C. jejuni* clinical isolates with different incubation times on Vero cells

Misawa *et al.* (1994) reported that the expression of their cytotoxin was elevated when the *C. jejuni* was grown in Brucella broth and that this medium was excellent for cytotoxin production. In the present study, Brucella broth was used to grow *C. jejuni* for all purposes including cytotoxin production. The absorbance value of the culture at 600 nm after incubation for 48h reached a suitable level to provide enough cells for the cytotoxin assay and thus was in agreement with the finding of Misawa *et al.* (1994).

Vero and HeLa cells have been shown to be extremely sensitive to toxins that inhibit protein synthesis due to the presence of Gb3 sphingoglycolipid receptor on their surfaces (Obrig, 1994). The cytotoxicity towards Vero cells of cell-free extracts produced by low and high centrifugation speeds of *C. jejuni* clinical isolates COL1 and COL7 increased with an increase in incubation time of the extract with the target cells, from 1-24h. This indicated that cytotoxin (s) in the cell-free extract needed

time to affect the Vero cells. Good activity occured after incubation for 16h. Thus, incubation time of 16h was used for other cytotoxicity assays in this study. The cytotoxic activities of cell-free extracts from the low speed centrifugation were always higher than those from the high speed centrifugation at all incubation times (1, 4, 8, 16, and 24h) and with either of the two *C. jejuni* strains (COL1 and COL7). Due to the higher activities in the low speed centrifugation fraction, this fraction was used for further cytotoxicity assays. It suggested that part or all of the cytotoxic activity may be membrane-associated, rather than completely soluble.

Data for the cytotoxicity assay were calculated according to the formula mentioned in the section 2.6.3.1, by comparison of the OD of the test sample with those of negative and positive controls, cells grown in tissue culture medium alone (assumed to be no cell death) or treated with Triton–X 100 (100% killing), respectively. In some graphs, there is no clear dose-response evident e.g. Fig. 3.6 and, even at the lowest doses of sample tested, there was significant cytotoxicity, compared to the controls cells. It would have been of interest to have tested lower concentration of samples for a more accurate comparison of their cytotoxic effects.

4.2.2 Cytotoxicity assay on Vero cells with wild type and *cdt* mutant strains

The frequency of *cdtB* genes among *Campylobacter* isolates obtained from different sources has been reported (Bang *et al.*, 2001; Eyigor *et al.*, 1999a; Eyigor *et al.*, 1999b; Pickett *et al.*, 1996). In general, *C. jejuni* produced much higher CDT in comparison with *C. coli* (Bang *et al.*, 2001; Eyigor *et al.*, 1999a; Eyigor *et al.*, 1999b; Pickett *et al.*, 1996).

Skirrow (1977) originally isolated *C. jejuni* NCTC 11168 from the faeces of a diarrhoeic patient. Results of the effects of cell-free extracts and broken cell pellets of NCTC 11168 wild-type and the *cdtB* mutant on Vero cell after 16h incubation (Fig. 3.11) showed that the cytotoxicity of wild type and *cdtB* mutant were very similar. Microscopic images (Fig. 3.14, 3.15) of both strains after incubation for 72h revealed that distension of Vero cells was apparent with the wild type strain, and not with the CDT^- mutant. This suggested that cytotoxic activity measured after incubation for 16h with wild-type or CDT^- mutant extracts was not due to CDT, and that some other cytotoxin(s) may have been responsible for this activity. Also Fig. 3.11 indicates that cytotoxicity was probably more cell-associated, due to the fact that more activity was found in broken cell pellets. Thus, some cytotoxic activities appear to be present in the broken cell pellet fractions (3.6 and 22K) which are membrane-associated and insoluble proteins.

Lee *et al.* (2000) examined fractions derived from the culture supernatant fluid (CS), sonicated cell supernatant fluid (SCS), pellet wash (PW) and washed cell sonicate (WCS) for cytotoxic activity after incubation for 12 and 24h. They found most activity was located within the bacterial cell (SCS and WCS). Guerrant *et al.* (1987) found cytotoxic activity in sonicated whole bacterial cell preparations. Bacon *et al.* (1999) detected cytotoxic activity in both culture supernatant and supernatants of sonicated *C. jejuni* strain 2483.

It was also found in this study that the cytotoxic effects after incubation for 16h were most prominent in Vero cells grown to a monolayer (confluent) as compared to freshly seeded Vero cells (non-confluent), but for assays that required incubation for

72h, freshly seeded Vero cells produced better results, again indicating that the cytotoxic activities took some time to act.

The cytotoxic activities of *Campylobacter* strains have been classified into those of cytolethal distending toxin (CDT) and non-CDT toxins (Wassenaar, 1997), but due to the confusion concerning *Campylobacter* cytotoxins, comparison of these cytotoxic activities among different studies is difficult (Nadeau *et al.*, 2003). CDT activities have been observed by using sonicated cells or supernatants of *Campylobacter* cultures (Eyigor *et al.*, 1999b; Johnson & Lior, 1988b; Schulze *et al.*, 1998). Non-CDT activities were observed by Nadeau *et al.* (2003) who reported that poultry isolates were generally not toxic for Vero cells or, if so, had weak titres, but four of the five human isolates that were cytotoxic for CHO cells were also cytotoxic for Vero cells. Pickett (2000) reported more than one cytotoxin activity and, since the results obtained for a Vero cell assay by Nadeau *et al.* (2003) were variable among CHO cell-cytotoxic isolates, they could not eliminate the possibility of multiple cytotoxin activities for the tested *Campylobacter* isolates. Thus, there is the possibility that *C. jejuni* strains produce several different cytotoxins.

Previously reported cytotoxicity assays showed that *C. jejuni* 81-176 *cdtB* mutants appeared to retain a low level of toxic activity against HeLa cells or INT407 cells, apparently unrelated to the action of CDT. In contrast, *C. jejuni* 11168 *cdtB* mutants produced no residual toxicity (Purdy *et al.*, 2000). The authors suggested that this weak residual toxicity for HeLa monolayers in *C. jejuni* 81-176 *cdtB* mutants may account for an as yet uncharacterized toxin (s) previously associated by others with campylobacters (Wassenaar, 1997) and suggested that some campylobacters may indeed produce more than one toxin (Purdy *et al.*, 2000).

Lee *et al.* (2000) examined cytotoxicity of sonicated cell supernatant fluid of *C. jejuni* strains against Vero cells by phase-contrast microscopy and reported that wells in which > 30% of the cells showed rounding were considered to be a positive result. Cytotoxicity of cell-free extracts of *C. jejuni* strains examined against Vero cells in the present study never reached 100% cell death even at high protein concentrations, which is keeping with the finding of Lee *et al.* (2000) that maximum dead cells (rounded cells) was 80%.

As different procedures have been used for preparing samples of cytotoxins by different researchers, it is difficult to determine the characteristics of the cytotoxins and it is possible that each (different) assay system for *C. jejuni* cytotoxin might detect a different type of cytotoxin (Misawa *et al.*, 1994).

The activities of Verotoxins (VTs or Shiga-like toxin) are influenced by the presence or absence of its receptor (B-subunits of VT bind to the receptor) and, typically, expression of the Gb3 receptor corresponds with sensitivity to VT (Hoey *et al.*, 2003). Gb3 is present on the cell surface of many eukaryotic cells (Lingwood *et al.*, 1987) such as endothelial cells of kidney (Obrig *et al.*, 1993), bovine colonic epithelial cells (Hoey *et al.*, 2003) and both small and large intestinal mucosa of cattle (Hoey *et al.*, 2002). The Vero cell line was employed for cytotoxicity assays in the present study due to its ease of culture and since these cells have been used extensively by other researchers (Coote & Arain, 1996; Guerrant *et al.*, 1987; Johnson & Lior, 1986; McCardell *et al.*, 1986a; Misawa *et al.*, 1995a; Moore *et al.*, 1988; Yeen *et al.*, 1983). Also, the receptor Gb3 is present on this cell line. Caco-2 cells were used as they are epithelial cells from the human colon and as a result they are related to those cells targeted during natural infection by *C. jejuni*. Different

observations on cytotoxic activities with the different cell lines may be due to the relative amounts of the receptor required for binding of the cytotoxin(s). In this study, cell-free extracts of strain 81-176 showed similar activity on Caco-2 and Vero cells, but identification of a possible receptor requires further study.

4.2.3 The presence of *virB11* and *tetO* genes and relation to cytotoxicity of *C. jejuni* strains

4.2.3.1 Presence of *virB11* and *tetO* genes in *C. jejuni* strains

Bacon *et al.* (2000) reported the absence of *virB11* in strain 11168 which was confirmed here (Table 3.4). They also reported that the *virB11*gene was present in 10.3% of isolates from military personel with diarrhoeal disease. In the present study, the percentage of human clinical *C. jejuni* isolates containing the *virB11* gene was 28%, which is more than that reported above and by others (10.7%) (Datta *et al.*, 2003). The gene was also present in 37.5% of chicken isolates in the present study, which is more than that (9.5%) reported by Datta *et al.* (2003). The detection rate of the *virB11* gene in the present study was 20% for bovine isolates (Table 3.5) while Datta *et al.* (2003) reported 15.4%.

The *tetO* gene was detected only from human (12%) and chicken (25%) sources. The data may reflect the use of tetracycline in treating human infections and also its use in the poultry industry (Avrain *et al.*, 2004). In this study, *tetO* gene was found in all tetracycline-resistant isolates, a result in keeping with the finding of Pratt & Korolik (2005) with Australian *C. jejuni* isolates. However, Tracz *et al.* (2005) reported tetracycline resistance plasmids in only 79% of tetracycline-resistant isolates in Canada. In this study, the prevalence of tetracycline-resistance in human isolates was

12%, compared with 60% in Alberta, Canada (Tracz *et al.*, 2005) which might indicate a contribution to resistance by a means other than expression of the *tetO* gene.

In the present study *tetO* gene was found in 27.3% of *virB11*-positive *C. jejuni* clinical isolates compared with 7.14% of *virB11*-negative isolates. The *virB11* gene was found in 60% of *tetO*-positive isolates. This is in agreement with Tracz *et al.* (2005) who had previously reported that the presence of pVir plasmid was associated with the presence of a tetracycline-resistance plasmid. Apart from strain 81-176, only three strains (RPH1, RPH2 and RPH5) had both *virB11* and *tetO* genes. RPH1 and RPH2 came from chickens and RPH5 was a human isolate.

4.2.3.2 Plasmid purification

Strain 81-176 is one of the best-characterized strains of *C. jejuni* (Black *et al.*, 1988). It was isolated from an outbreak associated with the consumption of unpasteurized milk (Korlath *et al.*, 1985) and has been shown to cause inflammatory diarrhoea in two human volunteer studies as well as disease symptoms in experimental infection models using primates and ferrets (Black *et al.*, 1988). It has been shown that strain 81-176 contains two large (~35 kb) plasmids, pVir and a Tc^R plasmid designated pTet. pVir is involved in invasion of cells by 81-176 (Bacon *et al.*, 2000; 2002), and DNA sequencing of this plasmid revealed the presence of four genes which encode homologues of a type IV secretion system (Bacon *et al.*, 2002). Type IV secretion systems are present in many bacteria and are involved in DNA export, bacterial conjugation, and protein secretion (Christie & Vogel, 2000; Covacci *et al.*, 1999). None of the four genes on the pVir was found to be present in the genome of *C.*

jejuni NCTC 11168 nor in a number of *Campylobacter* reference strains (Bacon *et al.*, 2000).

The presence of pVir genes in a subset of fresh clinical isolates (Bacon *et al.*, 2000) and the absence of this plasmid from other strains of *C. jejuni*, noticeably the type strain, NCTC 11168, suggests that there may be differences in pathogenic mechanisms among strains of *C. jejuni*. In this study, plasmid isolation and subsequent restriction enzyme digestion indicated that strain 81-176 possessed pVir and pTet, in agreement with the positive PCR results obtained for *virB11* and *tetO*.

4.2.3.3 Cytotoxicity assay of isolates of *C. jejuni* possessing the *virB11* and *tetO* genes

Cytotoxicity results on Vero cells using cell-free extracts of *C. jejuni* isolates COL1, COL12, COL20, COL27, and COL30 with different distributions of the *virB11* and *tetO* genes, showed that COL20 was the most active of the strains tested. This strain was positive for the presence of the *virB11* gene (Table 3.4). Cytotoxicity result of strain COL30 which was toxic on Vero cells, but was negative for the presence of CDT showed that the cytotoxicity measured in the assay was not necessarily due to CDT. Some activity of COL20 may have been due to the presence of CDT. All *C. jejuni* strains appear to possess the *cdt* genes (Eyigor *et al.*, 1999a; 1999b; Pickett *et al.*, 1996), but the levels of cytotoxic activities expressed were strain dependent (Bang *et al.*, 2001; Eyigor *et al.*, 1999b). AbuOun *et al.* (2005) observed that the majority of *C. jejuni* strains express detectable CDT activity, while some strains expressed no detectable activity. Indeed, AbuOun *et al.* (2005) reported the isolation of CDT-negative faecal *C. jejuni* strains from patients suffering from enteric disease

which indicated that CDT expression was not essential for the production of human enteric disease.

It has been reported by some authors that there is more non-CDT cytotoxic activity in human isolates than in animal and avian isolates (Akhtar & Huq, 1989; Lee *et al.*, 2000; Prasad *et al.*, 1996; Schulze *et al.*, 1998), although some reported no difference (Lindblom & Kaijser, 1995; Misawa *et al.*, 1995a). Nadeau *et al.* (2003) reported that poultry isolates were generally not toxic for Vero cells or, if so, had weaker titres than against CHO or HeLa cells, but four of the five CHO cell cytotoxic human isolates were also cytotoxic for Vero cells. COL12 was isolated from a chicken and other strains (COL1, COL20, COL27, and COL30) were isolated from human subjects, so low activity of strain COL12 on Vero cells is in keeping with the report of Nadeau *et al.* (2003). The present study indicated that not all *C. jejuni* strains tested produced cytotoxin (s) and that cytotoxin producers seem to be predominantly human strains.

There was no apparent correlation between the clinical findings on the patients from whom the *C. jejuni* isolates were obtained and the presence of cytotoxin produced by the isolates, which is in keeping with the reports from other researchers (Mizuno *et al.*, 1994; Moore *et al.*, 1988). Strain COL1 was isolated from a human with diarrhoeal illness but showed low cytotoxicity compared to COL7 which was from human faeces. This was also confirmed when cytotoxicity results of strains COL1, COL20, COL27, and COL30 were considered (Fig. 3.21). The results were markedly different, although all of the strains were isolated from humans with diarrhoeal illnesses (same manifestation). One explanation for the lack of correlation between the phenotype of the *C. jejuni* isolates and characterisation of disease is that

differences in host immunity factors (Blaser *et al.*, 1986) instead of variability in toxin production among *C. jejuni* isolates may be responsible for the range of clinical manifestations. From observations during outbreaks of food-borne *Campylobacter* enteritis due to a single strain, infected persons manifested different signs and symptoms (Blaser *et al.*, 1987) which supports the above hypothesis. Another possible explanation is the presence of other virulence factors produced by the isolates which were not detected by the assay systems utilized.

4.2.4 Cytotoxicity of cytoplasmic, IMP and OMP fractions of *C. jejuni* 81-176 and COL12

C. jejuni 81-176 was selected for characterization due to its consistency in production of cytotoxin in sufficient quantity to give a good cytotoxic response. As the source of *C. jejuni* 81-176 was a human subject, it would probably have been through several passages. Retaining the ability to produce the cytotoxin suggests that the cytotoxin(s) produced by *C. jejuni* 81-176 is stable, and this stability might suggest that the gene (s) which encode the cytotoxin (s) is (are) located in a stable area of the *C. jejuni* 81-176 genome.

Cytotoxicity of cytoplasmic, IMP and OMP fractions from two *C. jejuni* strains, 81-176 and COL12, on Vero cells showed that the fractions from 81-176 were more active than those from the isolate COL12. As strain 81-176 was isolated from a human (Korlath *et al.*, 1985) and COL12 from chicken caecum (Table 2.1), results of cytotoxic activity of 81-176 and COL12 on Vero cells are in agreement with the report of Nadeau *et al.* (2003). Strain 81-176, a good cytotoxin producer, and COL12, a poor cytotoxin producer, were chosen to use for further experiments

involving protein purification and proteomic analysis. Cytotoxicity of cytoplasmic, IMP and OMP fractions on Vero cells (Figs. 3.32, 3.33 and 3.34; Table 3.6) showed that most of the cytotoxic activity was in the IMP, suggesting that most cytotoxic activity in the cell-free extract is membrane associated.

Results of the analysis of the three fractions of COL12 on Vero cells after incubation for 16h and 72h, indicated that although after 16h, little cytotoxin activity was apparent in the fractions, more activity was noted with increased incubation time (72h) (Figs. 3.32, 3.33 and 3.34). This difference in activities between 16h and 72h may be due to CDT. However as mentioned before, microscopic images indicated that COL12 was negative for the presence of CDT, so it can be speculated that some strains of *C. jejuni* produce both cytotoxin (s) and CDT in low titre.

4.2.5 Comparison of the cytotoxicity assays used

Three cytotoxicity assay systems were utilised to determine which method (MTT, ATP-based and trypan blue exclusion dye assays) would be the best for monitoring cytotoxicity. The trypan blue exclusion assay was the cheapest and quickest to set up but it relied on the operator correctly distinguishing dead cells, making a significant degree of error possible. The MTT dye reduction assay does require a lengthy incubation but the results are easily and accurately read by mechanical means. The Cell Titer-Glo™ Assay (ATP-based assay) is faster than the MTT assay as it requires less prolonged incubation steps. Results obtained in this study indicated that the MTT assay was more sensitive than the ATP-based assay, but suffered from a plateau effect at higher protein concentrations, whereby 100% killing was not

achieved. The reason for this is not clear, but may be related to residual cell death in controls on prolonged incubation.

4.3 Characterization of cytotoxin in this study

Reports to date have shown a confusing and complex picture of *C. jejuni* cytotoxin(s). The varied and different properties of reported cytotoxin (s) could be due partly to variations in the *C. jejuni* strains used in the assays, growth of cultures and the different assay systems used to detect cytotoxins (Lee *et al.*, 2000). As there has been no correlation between different research groups with regard to the cytotoxins detected, therefore it is not yet known whether *C. jejuni* produce more than one type of cytotoxin (Lee *et al.*, 2000).

Apart from CDT, other cytotoxins have been described (Guerrant *et al.*, 1987; Johnson & Lior, 1986; Kawaguchi, 1989; Mahajan & Rodgers, 1989; Mahajan & Rodgers, 1990) and some of them have been partially characterised (Mahajan & Rodgers, 1990), but little is known about the function and structure of these cytotoxins. Yeen *et al.* (1983) first documented a cytotoxin from *C. jejuni* strains that was heat-labile, trypsin-sensitive and induced characteristic rounding of cells. Also a cytotoxic component which was heat-labile at 60 °C, was partially sensitive to trypsin (0.25%) and had a mol.wt > 14 kDa was described by Guerrant *et al.* (1987). This cytotoxic component identified by these researchers could not be neutralised with antisera raised against *E. coli* verotoxins or *C. difficile* toxin B (Guerrant *et al.*, 1987). Moore *et al.* (1988) indicated the presence of a Shiga-like toxin from *C. jejuni* which could be neutralised with monoclonal antibodies directed against the B-subunit of the mature Shiga-toxin, although they also detected a cytotoxin which could not be neutralised by the same monoclonal antibody.

Vero cells, which were used for cytotoxic activity in this study, are sensitive to toxins that inhibit protein synthesis due to the presence of Gb3 sphingoglycolipid on their surface (Obrig, 1994). Thus, the presence or absence of the Gb3 sphingoglycolipid receptor can be used to enable differentiation of toxins that inhibit protein synthesis, like *Shigella dysenteriae* and related *E. coli* Shiga toxins, from other toxins (Obrig, 1994). The rounding of Vero cells by cell-free extracts of *C. jejuni* strains (after incubation for 16h) in the present study indicates the presence of a toxin that inhibits actin filament formation and suggests that the cytotoxin produced (detected) may be more analogous to *Clostridium difficile* toxins A and B than to the *Sh. dysenteriae* and *E. coli* Stx toxins (Lyerly *et al.*, 1988). Morphological cell changes, including cell rounding, were accompanied by cell death after incubation for 16h.

The sensitivity of cell-free extracts of *C. jejuni* 81-176 *cdtB* mutant to heat (60 °C and above for 30 min) indicated that the cytotoxic factor(s) was protein in nature and this is in agreement with the finding of Guerrant *et al.* (1987) where heating the *C. jejuni* C31 filtrates to 60 °C or above for 30 min consistently and progressively abolished the cytotoxicity.

4.4 Proteomics (Protein identification)

One-dimentional SDS-PAGE allowed the identification of several proteins (Table 3.7) which were preferentially present in the cell-free extract of 81-176 compared to that of COL12. It is of interest to note that one of these proteins Cj0559 (an oxidoreductase) was also identified in the 2-DE analysis of 81-176 (Table 3.8). Results of identified proteins from one-dimentional SDS-PAGE by MS showed that

there was more than one protein in the identified bands (Table 3.7), therefore it was decided to do 2-DE to better separate the proteins. It was anticipated that 2-DE analysis would provide additional information on proteins preferentially expressed in strain 81-176, a high toxin producer and a virulent strain.

Two-dimensional electrophoresis (2-DE) is one of the most widely accepted methods for the separation and comparison of complex protein mixtures and has become the main platform for proteomic studies. 2-DE presents greater protein resolution potential than any other current separation technique and has recently been used to map large numbers of proteins from different tissues and organisms, and also to identify changes in protein expression.

The development of an infectious disease in the body involves complex interactions between the microorganism and the host. The important events during infection include entry of the bacteria, invasion and colonization of host tissues, evasion from host immunity and, finally, tissue injury or functional impairment of host tissues. Some microbes, without extensive colonization of host tissues, produce disease by liberating toxins (Abbas & Lichtman, 2003).

In the present study, strains 81-176 (high cytotoxic human strain), 81-176 *cdtB* mutant (cytotoxic but negative for the presence of CDT), 11168 (less cytotoxic strain than strain 81-176 but positive for the presence of CDT) and COL12 (less cytotoxic strain than other strains, also negative for the presence of CDT, isolated from chicken) were chosen to run on 2-DE for a comparative proteomic analysis.

Comparison of 2-DE gels of strains 81-176 and 81-176 *cdtB* showed that the profiles of these strains were similar, as expected, and therefore that the procedure was reproducible.

Comparison of 2-DE gels of *C. jejuni* strains 81-176 and COL12 showed that spots no. 16 (Cj0772c, putative periplasmic protein), 17 (Cj1420c, hypothetical protein), 19 (Cj0169, superoxide dismutase, Fe), 23 (Cj0414, putative oxidoreductase subunit), 25 (Cj0779, probable thiol peroxidase), 26 (Cj0779, probable thiol peroxidase), 27 (Cj0779, probable thiol peroxidase), 29 (Cj1382c, flavodoxin), and 30 (Cj0835c, aconitate hydratase) which were identified in the gel of strain 81-176 did not appear in strain COL12 (Figs 3. 45 and 3.48). Spot no. 25 (Cj0779, probable thiol peroxidase) in strain 81-176 did not appear in the gel of strain 11168. Fibronectin binding protein (Cj1478c) was identified in strains 81-176 and 81-176 *cdtB* mutant, but not in 11168. The results of the 2-DE gels showed that oxidoreductases were amongst the most frequently identified proteins in strains 81-176 compared to NCTC 11168. Some protein differences between 81-176 and 11168 may be due to proteins coded by plasmid pVir or pTet in 81-176 which were not present in 11168. Gaynor *et al.* (2004) noted that the expression of several oxidoreductase enzymes was down-regulated under severe O_2-depletion.

The oxidoreductases identified in the present study may be involved in the ability of *C. jejuni* to adapt to its environment in host body, or in the ability of these bacteria to escape from host defences (immune response). Colonization mechanisms of *C. jejuni* in the intestinal tract are poorly understood. A model of colonization in orally-challenged chickens has enabled some colonization factors to be identified by using defined mutants; these factors include superoxide dismutase (SOD) (Purdy *et al.*,

1999), flagellin (Wassenaar *et al.*, 1993), and GalE (Fry *et al.*, 2000). In addition, signature-tagged transposon mutagenesis has identified 22 different genes involved in colonisation of the chicken gastrointestinal tract by strain 81-176 (Hendrixson & DiRita, 2004).

In an important study, gene expression and animal work data suggested that the ability of *C. jejuni* to shift its metabolism and respiration strategies to accommodate the lower-oxygen microaerobic and largely anaerobic environments encountered in the gut was likely to be very important for *in vivo* survival and colonization (Gaynor *et al.*, 2004). Although to respire, *C. jejuni* requires oxygen as a terminal electron acceptor, it grows poorly if at all in the concentration of oxygen present in air, and so is classified as microaerophilic (Hoffman *et al.*, 1979a; 1979b). However, it has been reported that some campylobacters are able to adapt to an aerobic metabolism and so grow in the presence of air in a humid environment (Jones *et al.*, 1993).

The oxidative stress imposed upon bacteria surviving under aerobic conditions is significantly higher than that survived in microaerophilic conditions. *C. jejuni* can survive in diverse environments including external environments, often aerobic, and *in vivo* environments such as the intestinal tract which will be largely anaerobic. A number of enzymes have been suggested to provide the primary protection against toxicity of oxygen in bacteria, including superoxidase dismutases (SODs), peroxidase, catalases, glutathione reductase, and glutathione synthetase (Farr & Kogoma, 1991). It is suggested that oxidoreductase enzymes have two main protective effects against the toxicity of oxidative reactions for some bacteria:

Firstly, the reaction of an organism to oxygen in its environment depends upon the occurrence and distribution of different enzymes which react with O_2 and various

oxygen radicals that are constantly produced by cells in the presence of O_2. All cells contain enzymes able to react with O_2. The potential for lethal accumulation of superoxide in aerobes and aerotolerant anaerobes is prevented by the enzyme superoxide dismutase (Imlay, 2002). All organisms which can live in the presence of O_2, contain superoxide dismutase (whether or not they use O_2 in their metabolism). Almost all organisms contain catalase which breaks H_2O_2 (Bergamini *et al.*, 2004; Rocha *et al.*, 1996). Even certain aerotolerant bacteria such as the lactic acid bacteria lack catalase, they break H_2O_2 by means of peroxidase enzymes which derive electrons from $NADH_2$ to reduce peroxide to H_2O. Obligate anaerobes bacteria lack superoxide dismutase and catalase and/or peroxidase, and therefore when they are exposed to O_2 undergo lethal oxidations by various oxygen radicals. Recent work has uncovered adaptive strategies by which obligate anaerobes seek to minimize the damage done by hydrogen peroxide and superoxide (Imlay, 2002; Rocha *et al.*, 1996). In enteric bacteria, expression of numerous pathways is induced by exposure to oxidative or nitrosative stress that allow the organism to resist the toxic effects of these compounds during growth in the host. Among food-borne bacterial pathogens, *C. jejuni* and *C. coli* have the unique attribute of being microaerophilic, which require at least 3% oxygen for growth, but 5 to 7% is optimal (Luechtefeld *et al.*, 1982). *C. jejuni* has a single catalase activity, encoded by the *katA* gene, which protects against oxidative stress by changing H_2O_2 to H_2O and O_2 (Grant & Park, 1995) and a superoxide dismutase (SOD) which is important for *C. jejuni* survival and colonisation (Purdy *et al.*, 1999). SOD is a metalloenzyme and has been isolated from many prokaryotic and eukaryotic organisms (Fridovich, 1986). SOD provides the first line of defence against the toxic effects of reactive oxygen derivatives, by conversion of oxygen radicals (O_2^-) to hydrogen peroxide and dioxygen, and as a

result, has a protective role against the effects of oxidative stress. If superoxide radicals are not efficiently scavenged, almost all known biological molecules, including DNA, membrane lipids, and other essential cellular components can be damaged (Carlioz & Touati, 1986; Nakayama, 1992; 1994). *E. coli* expresses three types of superoxide dismutase, whereas in *C. jejuni* and *C. coli*, SOD (Fe) is the only SOD which plays a critical role in defence against oxidative stress, especially when growth has ceased during survival (Purdy *et al.*, 1999). In the present study, the results of 2-DE gels showed the present of a high number of enzymes including oxidoreductases, SOD (Fe), and peroxidases which, together with catalase, were present in all *C. jejuni* isolates (Section 3.2.4) but were particularly prominent in the virulent strain 81-176. They may well be involved in protection against toxicity of oxygen (Tables 3.8-3.10). These results are in keeping with the finding of Gaynor *et al.* (2004) where oxidoreductases were among the most frequently identified proteins in the 2-DE analysis.

Secondly, phagocytes (neutrophil and monocytes), which are the first line of cell-mediated innate immunity, generate reactive oxygen and reactive nitrogen intermediates as an intracellular killing mechanism for phagocytosed microbes. Therefore, the production of these agents is an important host defence mechanism mediated in response to infection by bacterial pathogens. It is not surprising that pathogens have evolved numerous defence strategies to protect themselves against the toxic effects of these agents. The use of specific oxygen radical scavengers and inhibitors, such as superoxide dismutase and catalase, as well as the myeloperoxidase inhibitors, are examples of these evasion strategies (Janssen *et al.*, 2003; Marrack & Kappler, 1994). One of the initial and most crucial responses to infection is the recruitment of leukocytes (primarily neutrophils) from the blood to the site of

infection (Burg & Pillinger, 2001). It has been shown that opsonized *C. jejuni* are phagocytosed by neutrophils and then attacked by the oxidative defence system within the phagosome. Moreover, some *Campylobacter* strains induced the extracellular release of toxic oxygen species which might contribute to the local inflammation due to tissue damage during infection (Walan *et al.*, 1992). However, these authors have found that different clinical isolates showed a great variability in this association and one strain which evoked an intracellular generation of oxygen intermediates also showed the highest sensitivity to killing. Therefore, it is likely that oxidoreductase enzymes, if they contribute to the clinical outcome of infection, may have two different effects. Firstly, in resistance to the extracellular reactive oxygen intermediates (ROI) at the site of colonization and secondly in relation to extracellular release of toxic oxygen species from neutrophils and monocytes. In the current study, the presence of oxidoreductase enzymes was observed more than other proteins in the 81-176 strain. However, it is not still clear if this potential can have an affect on the ability of *C. jejuni* for better colonization of the host or escape from the phagocyte oxidative burst defence. This may require the creation of mutants in the oxidoreductase enzymes and then to assess the colonisation capacities of the mutants in comparison to the parent strain.

In addition, intracellular existence provides bacteria with a niche to shelter from immune surveillance. However, internalized bacteria must be able to survive a variety of reactive oxygen species, particularly in the phagolysosome of professional phagocytes. These products are inactivated by superoxide dismutase and catalase (as bacterial factors) and allow invasive bacteria to persist in host cells and tissues. Experiments to test the effect of oxygen radicals on the survival of *Salmonella typhimurium* have been conducted (De Groote *et al.*, 1997). They found that a *sodC*

mutant of *S. typhimurium* was more susceptible than the wild-type isolate to killing by superoxide and nitric oxide. The role of SOD in *C. jejuni* survival in macrophages has been addressed by assays performed with a *C. jejuni sodB* mutant of 81-176 and the J774A.1 murine macrophage-like cell line. In the survival of the *C. jejuni sodB* mutant in J774A.1 cells, no difference was noted when compared to the *C. jejuni* 81-176 wild-type isolate. However, at 24 h after inoculation, a *C. jejuni katA* mutant (a gene that encodes the enzyme catalase) was not recovered from J774A.1 cells (Day *et al.*, 2000). Moreover, when the respiratory burst or production of nitric oxide was inhibited, the *C. jejuni katA* mutant was recovered. This finding indicates that *C. jejuni* is able to survive intracellularly within mononuclear phagocytes by possession of certain virulence attributes.

The above observations suggest that additional work is required to determine whether the *C. jejuni* oxidoreductase system is able to help in avoiding killing by ROI and the precise role of neutrophils and monocyte/macrophages as an important component of the inflammatory reaction in the development of campylobacteriosis.

Another report (Holmes *et al.*, 2005) showed that there were differences in protein expression between strains of *C. jejuni* grown under different conditions. A proteomic analysis of a wild-type strain and a *fur* (ferric uptake regulator) mutant of *C. jejuni* strain 11168 was done by comparing 2-DE gels of cell protein extracts from bacteria grown in iron-rich and iron-limited conditions (Holmes *et al.*, 2005). The results showed that 30 genes were transcribed at lower levels in the *fur* mutant than in the wild-type strain in iron-rich conditions. In the wild-type strain, the putative iron-transport genes (p19, Cj1658, Cj0177, Cj0178 and *cfrA*) and all of the proposed iron-transport systems for haemin, ferric iron and enterochelin were expressed at

higher levels under iron limitation and, in the *fur* mutant, they were expressed in iron-rich conditions, which suggested that they were regulated by Fur. An ABC transporter encoded by genes Cj1660-1663 was depressed in the *fur* mutant, indicating its role in iron transport. Several genes containing a perR/Fur box consensus sequence, such as those encoding bacterioferritin (*bfrldps*, Cj1534c), glutamyl-tRNA reductase (*hemA*, Cj0542), thioredoxin reductase (*trxB*, Cj0146c), cytochrome c peroxidase (*ccp*, Cj0358), superoxide dismutase (*sodB*, Cj0169) and the peroxide stress regulator *perR* (Cj0322) appeared to be derepressed in *fur* mutants (Holmes et al., 2005).

Taken together, the results suggest that the oxidoreductase proteins may be important among *C. jejuni* strains for adaptation and survival in different growth conditions, particularly growth under stress conditions. The virulence of a bacterium depends on its potential ability for infecting the host and the cytopathic effects that it causes, and also on its strategies for avoiding host defences (Abbas & Lichtman, 2003). *C. jejuni* can use these strategies to benefit its adaptation to new environments. In the current study, alteration of protein profiles, e.g. in binding proteins and oxidoreductases, may be relevant in both of the above strategies. Any change in expression of bacterial binding proteins may influence the adhesion of *C. jejuni* to the host cells, resulting in more or less colonization. 2-DE results (Figs. 3.45 to 3.48; Tables 3.8-3.10) showed that there were differences among strains 81-176, 81-176 *cdtB*, 11168 and COL12 in fibronectin binding protein (Cj1478c, CadF) which was identified as spots no. 32 (Table 3.8) and 20 (Table 3.9) in strains 81-176 and 81-176 *cdtB*, respectively, but did not appear in strains 11168 and COL12. Proteomic results of fractions derived from DEAE sepharose column chromatography of cell-free extracts of strains 81-176 and COL12 on SDS-PAGE (Table 3.7; section 3.5.1.2) showed that

a probable ABC-type amino-acid transporter periplasmic solute-binding protein (PEB1A, MW 28.2 kDa) existed in the column chromatography flow-through fraction of strain 81-176 but was absent in the same fraction from strain COL12. CadF and PEB1 are the best characterized *C. jejuni* adhesins (Konkel *et al.*, 1997; Pei *et al.*, 1998) which may play important roles in mediating *C. jejuni* binding to host cells. It would be of interest to compare the adhesion of strains 81-176 and 11168 or COL12 to mammalian cells *in vitro*. Four outer membrane proteins (omps) with apparent molecular weights of 28, 32, 36 and 42 kDa have been identified which in a ligand-binding assay appeard to play a role in mediating *C. jejuni* binding to host cells (De Melo & Pechere, 1990). Pei *et al.* (1998) suggested that PEB1, a 28 kDa protein, mediates the binding of *C. jejuni* to epithelial cells. PEB1 has homology with membrane proteins from other Gram-negative bacteria that function in amino-acid transporter systems. Konkel *et al.* (1997) cloned and partially characterized a 37 kDa omp termed CadF from *C. jejuni* that mediates the adhesion of *C. jejuni* to fibronectin (Fn), a component of the extracellular matrix. It is not known if the four omps identified by De Melo & Pechere (1990) include PEB1 and CadF. Moreover, our results showed that Peb1A and CadF, which are implicated in the pathogenicity of *C. jejuni*, can be found in strain 81-176 (high cytotoxic strain, isolated from human), while they were absent in strain COL12 (less cytotoxic strain, isolated from chicken).

In conclusion, although one of the aims of the proteomic study was to identify variation in cytotoxins amongst *C. jejuni* isolates, the methods used failed to detect such differences. This may have been due to the method of spot selection for identification since the staining would only reveal the most abundant components of the sample. It is not known exactly how many proteins are to be expected in the

proteome of the *C. jejuni* cytoplasmic fraction. It has been reported that *C. jejuni* NCTC 11168 has a chromosome of 1,641,481 base pairs, which is predicted to encode 1,654 proteins (Parkhill *et al.*, 2000). However, only a proportion of the genes will be expressed in the cytoplasmic fraction, not all will encode proteins and only the reasonably abundant proteins were identified in this study. Of the large number of protein spots which were selected for MALDI identification, only a few of them were identified. Among these spots there was some interesting variation in proteins involved in adhesion and in the way that bacteria can escape from the host immune system, which is likely to have an affect on pathogenesis and clinical outcome. Thus, proteomic analysis by 2-DE in the present study suggests that this approach can be a useful tool for exploring the variation in protein expression profiles among different strains of *C. jejuni* with different clinical outcomes. Overall, the results of this study showed that the use of 2-DE resolution of proteins and identification of differences by proteomic analysis can provide valuable preliminary data for identification of novel proteins or give insight into changes in the protein expression profile in relation to the alteration of bacterial virulence. However, further studies are needed to be performed after this stage to evaluate protein variation by more specific and sensitive methods. In other words, the proteomic analysis with MALDI using 2-DE gels can be used as an initial screening technique and followed by more sensitive and reliable methods such as Western blots and analysis of bands with MS, microarrays and mRNA analysis like that of Holmes *et al.* (2005). Together these techniques can support a better understanding of bacterial pathogenesis and their consistency should magnify the strength of the results.

DIGE (Difference gel electrophoresis) analysis was done for sup1 fractions of strains 81-176 and 81-176*cdtB* mutant (Figs 3.49 and 3.50). The CdtB subunit, the

difference between these two strains, was not detected by this technique which may be due to the possibility that the Cdt subunits are low abundance proteins, but proteins Cpn60 (60 kDa chaperonin) and elogation factor Tu were down regulated 4 and 2-fold in 81-176 *cdtB* compared with parent strain, respectively.

Nalbant *et al.* (2003) reported that upregulation of GroEL-like (60 kDa chaperonin, Cpn60) stress protein was apparent in *cdt* and *ltxA* deletion mutants of *Actinobacillus actinomycetemcomitans*. The authors constructed a series of *Actinobacillus actinomycetemcomitans* deletion mutants Δ*cdtA*, Δ*cdtB*, Δ*cdtC*, Δ*cdtΔBC*, Δ*ltxA* (ltxA is the gene responsible for expression of leukotoxin, Ltx), Δ*ltxA*/Δ*cdtABC*. The ability of the wild type and mutants to induce apoptosis and cell cycle arrest in human peripheral blood mononuclear cells (T-cell) was compared. The results showed that each of the isogenic mutants exibited a reduction in the ability to induce T-cell apoptosis, and in each *cdt* mutant the ability to induce cell cycle block was abolished. A mutant of this strain with simultaneous deletion of *ltxA* and *cdtABC* genes retained ability to induce apoptosis when cell-associated, but not from a supernatant preparation. A neutralisation study showed that addition of *E. coli* anti-GroEL monoclonal antibody significantly diminished the apoptosis of the Δ*ltxA*/Δ*cdtABC* cell associated preparation (Nalbant *et al.*, 2003). Although in our study GroEL was apparently down-regulated in the *cdtB* mutant while in the study of Nalbant *et al.* (2003) it was apparently up-regulated in a *cdt/ltxA* deficient mutant, the apparent association of GroEL synthesis with Cdt production is intriguing and would benefit from further study.

Appendix

Appendix 1: Blood agar base composition (Difco)

Formula per litre:

Bacto Proteose Peptone	15 g
Liver Digest	2.5 g
Bacto Yeast Extract	5 g
Sodium Chloride	5 g
Bacto Agar	12 g

Make up to 1 litre and sterilised by autoclaving at 121 °C for 15 min

Appendix 2: Brucella Broth composition (Difco)

Formula per litre:

Bacto Tryptone	10 g
Bacto Peptamin	10 g
Bacto Dextrose	1 g
Bacto Yeast Extract	2 g
Sodium Chloride	5 g
Sodium Bisulphite	0.1 g

Make up to 1 litre and sterilised by autoclaving at 121 °C for 15 min

Appendix 3: LB agar

Peptone (BDH)	10 g
Yeast extract (BDH)	5 g
Sodium Chloride (BDH)	10 g
Agar	12 g
dH_2O	1000 ml

Appendix 4: Composition of media and reagents of API CAMPY

NaCl 0.85% medium, 3 ml	Sodium chloride	8.5 g
	Demineralized water	1000 ml
AUX Medium, 7ml	Ammonium sulphate	2 g
	Agar	1.5 g
	Mineral base	82.8 mg
	Amino acids	250 mg
	Vitamins and nutritional substances	35.9 mg
	Phosphate buffer 0.04 M, pH 7.1	1000ml
NIT 1 reagent, 5ml	Sulphanilic acid	0.4 g
	Acetic acid	30 g
	H_2O	70 ml
NIT 2 reagent, 5 ml	N,N-dimethyl-1-naphthylamine	0.6 g
	Acetic acid	30 g
	H_2O	70 ml
NIN reagent, 5 ml	Ninhydrin	7 g
	2-methoxyethanol	100 ml
FB reagent, 5 ml	Fast Blue BB(> 0.1 %)	0.35 g
	Sodium lauryl sulphate	7.5 g
	Organic solvents	100 ml

Appendix 5: Composition of the reagent for the oxidase test

N,N,N,N-tetramethyl-1,4-phenylenediamine	10.0 g
Ascorbic Acid	2.0 g
Deionized water	1000 ml

Appendix 6: Phosphate Buffered Saline (PBS)

NaCl	10.0 g
KCl	0.75 g
KH_2PO_4	0.125 g
Na_2HPO_4	1.44 g

Made up to 1 litre in distilled water

Appendix 7: Envelope Buffer (sodium phosphate)

NaH_2PO_4	20 mM
Na_2HPO_4	20 mM

28 ml of first is mixed with 72 ml of second and then 100 ml of dH_2O is added to produce a 10 mM envelope buffer.

Appendix 8: Modified Lowry method reagents

Reagent A:

Na_2CO_3	2.0 g
NaOH	0.4 g
Sodium tartrate	0.16 g
Sodium dodecyl sulphate (SDS)	1.0 g
dH_2O	100 ml

Reagent B:

$CuSO_4$	4 g
dH_2O	100 ml

Reagent C:

Reagent A	100 part
Reagent B	1 part

Folin & Ciocalteu's Phenol:

Folin- Ciocalteu Phenol reagent	1 part
dH_2O	1 part

Appendix 9: SDS-PAGE reagents

Appendix 9.1: Separating gel

	7.5 %	10 %	12.5%	15%
Acrylamide/Bis solution	2.5 ml	3.33 ml	4.16 ml	5 ml
dH_2O	3.8 ml	2.97 ml	2.13 ml	1.3 ml
Tris-HCl (1 M, pH 8)	3.75 ml	3.75 ml	3.75 ml	3.75 ml
10 % (w/v) SDS	100 µl	100 µl	100 µl	100 µl
TEMED	10 µl	10 µl	10 µl	10 µl
10 % (w/v) Ammonium persulphate (APS)	100 µl	100 µl	100 µl	100 µl

Appendix 9.2: Stacking gel (6 %)

Acrylamide/Bis solution	157 µl
dH_2O	714 µl
Tris-HCl (0.5 M, pH 6.8)	300 µl
10 % (w/v) SDS	11.9 µl
TEMED	1.18 µl
10 % (w/v) Ammonium persulphate (APS)	6 µl

Appendix 9.3: Protein sample buffer

Glycerol	5 ml
20% (w/v) SDS	2.5 ml
2-mercaptoethanol	0.5 ml
Tris (0.5M, pH 6.8)	2.5 ml
Bromophenol blue	0.25 % (w/v)

Appendix 9.4: Electrode buffer (10×)

Tris base	30 g
Glycine	144 g
SDS	10 g

Made up to 1 litre in distilled water, pH to 8.3

Appendix 9.5: Coomassie blue stain solution

Methanol	400 ml
Acetic acid	100 ml
Distilled water	500 ml
Coomassie blue	0.1% (1 g)

Appendix 9.6: destain solution for SDS-PAGE

Methanol	400 ml
Acetic acid	100 ml
Distilled water	500 ml

Appendix 10: Trypan blue solution

Trypan blue	0.2 g
NaCl	4.25 g

Each component was dissolved individually in 100 ml dH_2O, then 4 parts of trypan blue solution was mixed with 1 part saline.

Appendix 11: Rehydration solution

Urea	8 M
CHAPS (w/v)	0.5%
DTT (Dithiothreitol) (w/v)	0.2%
IPG Buffer or pharmalyte	0.5%
Bromophenol blue	0.002%

Appendix 12: SDS equilibration buffer (stock solution, store at –20 °C)

Tris-HCl, pH 8.8	10.0 ml
Urea	72.07 g
Glycerol	69 ml
SDS	4.0 g
Bromophenol blue	400 µl of 1% (w/v) solution
Double distilled H_2O	to 200 ml

Appendix 13: 6× DNA loading buffer

Tris	60 mM
EDTA	6 mM
Sucrose	40 % (w/v)
Bromophenol blue	0.25 % (w/v)

Appendix 14: TBE buffer (5×)

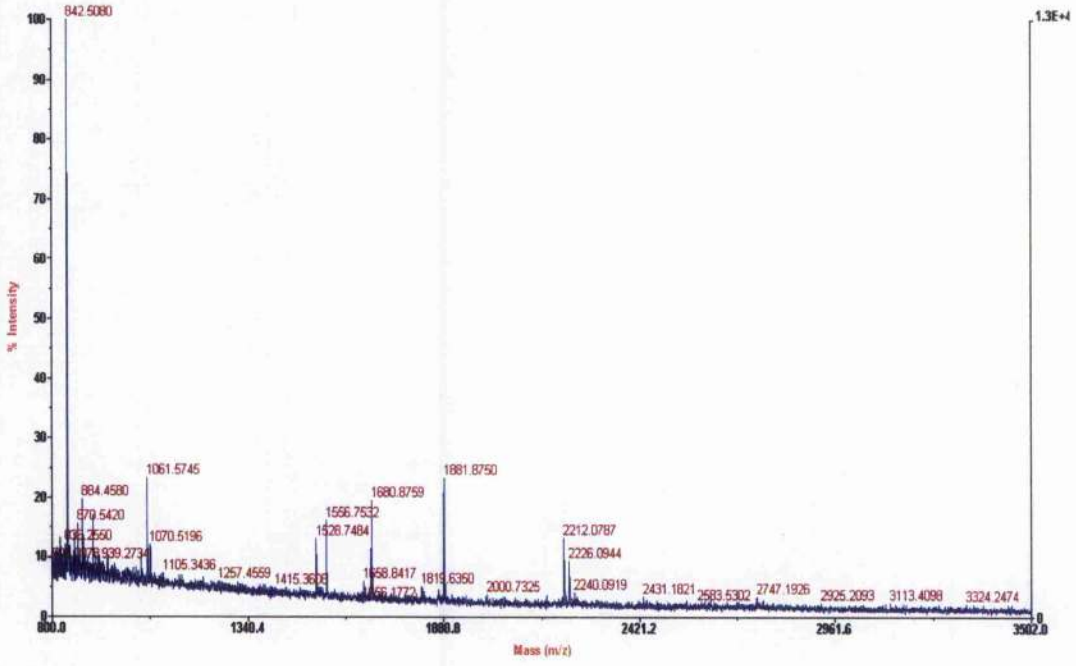
Tris-Base	54 g
Boric Acid	27.5 g
EDTA (0.5 M)	20 ml
dH_2O	980 ml

Appendix 15: Buffers for QIAGEN® Large-Construct Kit

Buffer P1 (Resuspension Buffer)	50 mM Tris.Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A
Buffer P2 (Lysis Buffer)	200 mM NaOH, 1% SDS
Buffer P3 (Neutralization Buffer)	3.0 M potassium acetate, pH 5.5
Buffer QBT (Equilibration Buffer)	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% Triton®, X-100
Buffer QC (Wash Buffer)	1.0 M NaCl; 50 mM MOPS, PH 7.0; 15% isopropanol
Buffer QF (Elution Buffer)	1.25 M NaCl; 50 mM Tris.Cl, pH 8.5; 15% isopropanol
Exonuclease Solvent	20 mM KCl; 20 mM KPO_4, pH 7.5
TE	10 mM Tris.Cl, pH 8.0; 1 mM EDTA

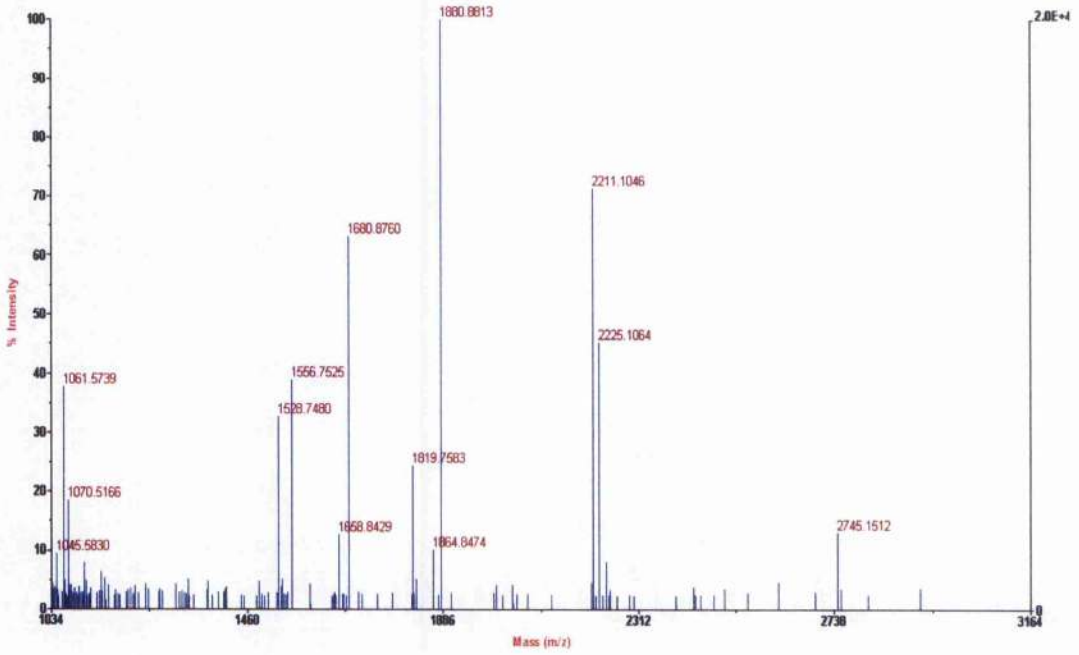
Appendix 16: Maldi mass spectrometry of spot 19 (superoxide dismutase, Fe) from 2-DE gel of sup1 of *C. jejuni* 81-176

A- Raw maldi spectrum Voyager DE pro positive reflectron mode with delayed extraction. 600 transients were accumulated from m/z 800 – 3500 and internal calibration on trypsin autoloysis peaks was performed.



Appendix 16: (continued)

B- Maldi spectrum was deisotoped and truncated to exclude complex regions between m/z 800 and 1000 and Peak detection threshold was set at 5%. Peak masses were entered into the Mascot search engine (Matrix Science Ltd) against the *C. jejuni* database subset of NCBI.



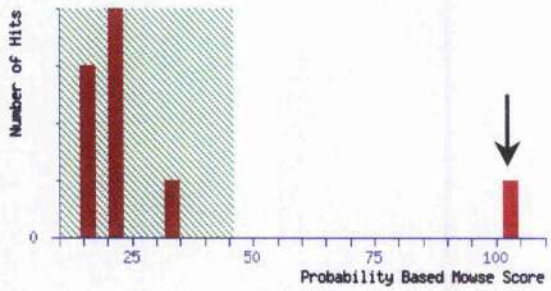
Appendix 17: Mascot pmf search results of spot 19 (superoxide dismutase, Fe) from 2-DE gel of sup1 of *C. jejuni* 81-176

{MATRIX SCIENCE} Mascot Search Results

User : MajidDiGEspot19
Email :
Search title :
Database : NCBInr 20050611 (2503385 sequences; 849188484 residues)
Taxonomy : Campylobacter jejuni (2411 sequences)
Timestamp : 28 Jun 2005 at 15:05:52 GMT
Top Score : 103 for gi|57237176, superoxide dismutase, Fe [Campylobacter jejuni RM1221]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 46 are significant (p<0.05).



Concise Protein Summary Report

Format As: Concise Protein Summary | Help
Significance threshold p< 0.05 | Max. number of hits 20

Re-Search All | Search Unmatched

1. gi|57237176 **Mass:** 25025 **Score:** 103 **Expect:** 1.2e-07 **Queries matched:** 7
superoxide dismutase, Fe [Campylobacter jejuni RM1221]
gi|531402 **Mass:** 25016 **Score:** 78 **Expect:** 4.3e-05 **Queries matched:** 5
superoxide dismutase [Campylobacter jejuni]
gi|57238699 **Mass:** 19575 **Score:** 25 **Expect:** 8.4 **Queries matched:** 2
ribosomal protein L6 [Campylobacter jejuni RM1221]
gi|6967628 **Mass:** 32766 **Score:** 24 **Expect:** 9.6 **Queries matched:** 2
homoserine kinase [Campylobacter jejuni subsp. jejuni NCTC 11168]
gi|6968234 **Mass:** 19426 **Score:** 23 **Expect:** 12 **Queries matched:** 2
hypothetical protein Cj0788 [Campylobacter jejuni subsp. jejuni NCTC 11168]
gi|6968206 **Mass:** 39350 **Score:** 19 **Expect:** 29 **Queries matched:** 2
hypothetical protein Cj0760 [Campylobacter jejuni subsp. jejuni NCTC 11168]

Appendix 18: Superoxide dismutase, Fe (Sod B) amino acid sequence indicating peptide coverage

Matched peptides shown in **Bold Red**

```
  1 MFELRKLPYD TNAFGDFLSA ETFSYHHGKH HNTYVTNLNN LIKDTEFAGK
 51 DLVSIIKTSN GGVFNNAAQV YNHDFYFDCI KPSTGCGCGG SCQSIDANLQ
101 AALEKEFGSL ENFKAEFIKG ATGVFGSGWF WLVYNTKNQK LEFVGTSNAA
151 TPITEDKVPL LVVDVWEHAY YVDHRNARPA YLEKFYAHIN WEFVAKAYEW
201 ALKEGMGSVS FYANELHPVK
```

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
6 - 29	2745.15	2744.14	2744.29	-0.14	1	KLPYDTNAFGDFLSAETFSYHHGK	
30 - 43	1680.88	1679.87	1679.87	-0.01	0	HHNTYVTNLNNLIK	
106 - 114	1070.52	1069.51	1069.51	0.00	0	EFGSLENFK	
106 - 119	1658.84	1657.84	1657.84	0.00	1	EFGSLENFKAEFIK	
176 - 184	1061.57	1060.57	1060.57	0.00	0	NARPAYLEK	
204 - 220	1864.85	1863.84	1863.88	-0.04	0	EGMGSVSFYANELHPVK	
204 - 220	1880.88	1879.87	1879.88	-0.00	0	EGMGSVSFYANELHPVK	Oxidation (M)

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