VIRULENCE

OF

CAMPYLOBACTER JEJUNI

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DEDICATION

I was introduced to the microbial world by the late Professor Anwarul Azim Chowdhury, founder chairman of the Department of Microbiology, University of Dhaka, Bangladesh. I dedicate this thesis to the memory of Professor Chowdhury who not only taught but also inspired myself and many others.

DECLARATION

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

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LIST OF ABBREVIATIONS

A	Absorbance
BA	Brucella Agar
BBA	Brucella Blood Agar (containing 7 % v /v sheep blood)
BA-CR	Brucella Agar containing Congo Red Dye (30 µg ml^{-1})
BATH	Bacterial adherence to hydrocarbon
Bis	N'N'- methylenebisacrylamide
BSA	Bovine Serum Albumin
cm	Centimetre
conc	Concentration
CR	Congo Red
CR^+ Cells	Congo Red dye binding cells
CR^- Cells	Congo Red dye non-binding cells
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzyme-linked Immunosorbent assay
g	Gravity
h r	Hour
ICDDR,B	International Centre for Diarrhoeal Disease Research, Bangladesh
Kb	Kilobase
K dal	Kilo dalton
LPS	Lipopolysaccharide
OMP	Outer Membrane Protein
OPD	ortho-Phenylene Diamine
M dal	Mega dalton
min	Minute
MOMP	Major Outer Membrane Protein
µg	micro gram
µl	micro litre
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
rpm	revolutions per minute
SA	Salt aggregation
SDS	Sodium dodecyl sulphate
spp	Species
TEMED	NNN'N'-tetra-methyl-1,2-diamino-ethane

SUMMARY

The primary aim of this study was to investigate whether the *Campylobacter jejuni* strains causing two clinically distinct form of diarrhoea i.e. cholera-like watery diarrhoea (Group C strains) and dysentery-like mucoid diarrhoea (Group D strains) possess unique sets of virulence characteristics. For this purpose *C. jejuni* strains were assayed for a number of putative virulence factors and the results of the Group C strains were compared with those of Group D strains to assess whether these candidate virulence markers show any significant association with any particular group of strains.

Initially, a comperative study of the enterotoxigenicity of Group C and D of strains was conducted The infant chicken model of Sanyal *et al* (1984a) proved unsatisfactory as convincing evidence of diarrhoea was not obtained. Subsequently, the new-borne chick diarrhoea model of Welkos (1984) was adopted which proved useful for this purpose.

Attention was then turned to determine whether plasmids were involved in the enterotoxin production by *C. jejuni* strains. This aspect of study stemed from the preliminary report by Lee *et al* (1985) that the production of enterotoxin detecteble by Chinese hamster ovary (CHO) cell assay was mediated by a 46.5 Kilo-base transmissible plasmid. No correlation between plasmid profile and enterotoxigenicity was noted in this study.

The *in vitro* assays, GM_1 ganglioside ELISA and CHO cell assay were found to suitable for quantitation of enterotoxin produced by the *C. jejuni* strains and the assays correlated well with each other. Enterotoxigenicity was predominantly associated with the Group C strains. Lines of partial identity were obtained between cholera toxin and *C. jejuni* enterotoxin in immunodiffusion experiments. No hybridization was however noted between Hind III digested chromosomal DNA of 4 enterotoxigenic *C.jejuni* strains and the *E. coli* LT B subunit gene probe.

After successfully demonstrating the enterotoxigenic nature of

C. jejuni strains in the new-born chick model and *in vitro* assays (GM_1 ganglioside ELISA and CHO cell assay), it was decided to explore whether the ligated ileal loop for fluid accumulation could be performed in infant chicks. Reproducible positive results were obtained in 5-day-old chicks, both with whole cells and cell-free culture supernates. This is the first report of accumulation of fluid in the ligated ileal loop of chickens by *C. jejuni.*

C. jejuni strains produced an extracellular haemolysin active against a range of erythrocytes but not against chicken erythrocytes. The haemolytic factor lost its activity partially upon heat-treatment and was trypsin sensitive. The haemolysin which was also cytotoxic towards HeLa cells was predominantly produced by Group D strains.

Adherence and invasion potentials of the *C. jejuni* strains were evaluated in the HeLa cell model by quantitative bacterial counts. The *C. jejuni* strains differentially adhered to and invaded HeLa cells. The Group D strains were significantly more invasive than the Group D strains, but the adherence of the strains belonging to the two groups did not differ significantly. However, the individual strains belonging to the two groups exhibited widely overlapping adherence and invasion potentials. Adherence and invasion of representative strains from both groups were significantly inhibited by L-fucose, cytochalasin B, chicken intestinal mucus and antiserum raised against formalinized bacterial cells. Antiserum against heat-killed bacterial cells had a minimal effect on the adherence and invasion potentials.

Virulence of the representative *C. jejuni* strains belonging to the two groups C and D was assayed in the 11-day-old White Leghorn chicken embryo. The virulence of the strains investigated varied widely; the LD_{50} values was relatively lower in the Group D in comparison to the Group C strains. The gradation of virulence observed in the 11-day-old chick embryo model closely reflected the gradation of invasion and adherence potential of these strains in the HeLa cell model.

The major outer membrane protein (MOMP) of *C.jejuni* isolated and purified to homogeneity as judged by immunodiffusion, SDS-PAGE, and Western

western blotting. Analytical thin-layer isoelectric focusing indicated it to be an acidic protein with pI of ca. 5.1-5.2. This is the first report of isolation of MOMP of *C. jejuni* and determination of its pI. The MOMP could partially inhibit the adherence and subsequent invasion of the *C.jejuni* strains in HeLa cell model, probably by acting as an adhesin.

Cell-surface hydrophobicity of the *C. jejuni* strains was evaluated by salt aggreagation (SA) test and bacterial adherence to hydrocarbon (BATH) test. No significant difference in the cell surface hydrophobicity were noted but in general the Group D strains were relatively more hydrophobic than the Group C strains.

C. jejuni strains were examined for their capacity of binding the dye Congo Red (CR), which has recently been identified as an indicator of virulence in several bacterial species. These parent (CR^+) and variant (CR^-) cells were investigated for cell surface properties and virulence in a variety of assay systems. These included (1) outer membrane protein profile, (2) lipopolysaccharide (LPS) profile, (3) plasmid profile, (4) quantitative binding of the dye CR, (5) cell surface hydrophobicity (SA test and BATH test), (6) adherence and invasion potential in HeLa cell model and (7) lethality in 11-day-old chick embryo model.

No difference was noted among the CR^+ and CR^- variants in terms of plasmid profile and LPS profile. Loss of two protein banda (ca. 63 and 87 K dal) was accompanied by the transition of CR^+ colonial variants to CR^- phase. CR^+ parents bound significantly higher amount of the dye, were relatively more hydrophobic, exhibited relatively higher adherence and invasion potential and was slightly more lethal to the 11-day-old chick embryos.

In conclusion, the Group D strains possessed certain characteristics such as higher invasion potntial in HeLa cell assay, increased lethality in chick embryo model and higher hydrophobicity which are likely to represent them in the cases of dysentery-like, mucoid diarrhoea cases. On the other hand, enterotoxigenicity was the central virulence characteristics of the Group C strains. However, no clear-cut presence or absence of implicated virulence characteristics such as invasion and total absence of enterotoxigenicity in Group D strains and vice-versa for Group C strains, was found.

CR binding may be an indicator of virulence among colonial variants of individual *C. jejuni* strains (as CR^+ colonial variants were more virulent than their CR^- counterpart), but it is probably not a general virulence marker for *C. jejuni* as CR^- variants of certain strains were more virulent than the CR^+ cells of other strains.

Virulence of *C. jejuni* is probably multifactorial and identification of single virulence characteristic may not represent the pahogenic potential of a particular strain. The results of this study have established the importance of working with a range of clinical isolates because they revel a spectrum of virulence. It seems probable that the degree of virulence of a particular strain is influenced by the expression of a number of virulence factors.

1.0 INTRODUCTION

1.1 GENERAL INTRODUCTION

Diarrhoeal diseases caused by a variety of microorganisms constitute one of the greatest sources of morbidity and mortality on a global scale. It is estimated that several billion episodes of diarrhoea are likely to occur, resulting in 5 to 18 million deaths every year (Snyder and Merson, 1982; Guerrant, 1985). Intestinal dysfunction, due to after-effects of diarrhoea e.g. hypernatraemic dehydration together with severe malnutrition have a profound negative effect on the growth of infants and children. This is particularly a problem with the children from developing countries where diarrhoeal disease is endemic (Wanke *et al,* 1987; Molla *et al,* 1987). Diarrhoeal disease also afflicts children and adults in developed countries by causing food-borne epidemics and traveller's diarrhoea (MacDonald and Cohen, 1986; Kean, 1986).

In addition to the classical diarrhoea-causing bacterial pathogens such as *Vibrio cholerae, Salmonella* spp. and *Shigella* spp. a wide array of bacterial, viral and protozoal agents have been recognized as causes of human diarrhoeal disease in the past few decades. Although described early this century as a cause of infectious abortion in cattle and sheep (McFadyean and Stockman, 1909; 1913) (see also Section 1.2), the role of campylobacters as human diarrhoeal pathogens was recognized only recently (Butzler *et al,* 1973; Skirrow, 1977). *Campylobacter jejuni* is now established as a major cause of gastroenteritis all over the world and can occur in sporadic, epidemic and endemic form (Blaser *et al,* 1983c). It is the commonest cause of diarrhoeal disease in many countries (Table 1).

The clinical presentation of the illness (see Section 1.6) due to *C. jejuni* infection is variable, but generally it is self-limiting enteritis (Blaser and Reller 1981; Skirrow, 1984). The incubation period is usually 2-3 days and is manifested by abdominal cramps, varying combinations of diarrhoea, nausea, vomiting and lowgrade fever (Skirrow, 1977; Mandal *et al,* 1984). *C. jejuni* enteritis is not only responsible for a great deal of discomfort and accounts for a considerable loss of time

Table 1: Countries with *Campylobacter jejuni* as the Predominant Enteropathogen.*

Country	References
U.K.	Skirrow (1982; 1987a)
U.S.A.	Riley and Finch (1985)
Sweden	Svedham and Kaijser (1980)
Italy	Crotti *et al* (1988)
Japan	Itoh *et al* (1982)
Mexico	Calva *et al* (1988b)
Nigeria	Olusanya *et al* (1983)
South Africa	Lastovica *et al* (1986)
Gambia	Billingham (1981)
Zaire	De Mol *et al* (1983)
Ruwanda	De Mol and Bosmans (1978)

* These studies represent the diagnosed incidence of *C. jejuni* as a diarrhoeagenic pathogen in a limited number of people in each of the countries listed.

from work and school, it can be severe and is occasionally fatal in infants, the elderly and debilitated patients (Skirrow, 1984; Mandal *et al*, 1984). Moreover, reports are being published with increasing frequency associating *C. jejuni* with a variety of systemic infections (Section 1.6.5). The illness-related cost associated with *Campylobacter* enteritis is also considerable. A recent study in England revealed that a total cost of £31,113 was incurred with 53 cases of human *Campylobacter* enteritis (Sockett and Pearson, 1988). With the estimated 600,000 cases per year in U.K. (Skirrow, 1982), *C. jejuni* infections thus have a substantial economic impact on the health-care services.

Following the discovery of the medical importance of *C. jejuni* and the widespread infections caused by them, it has been the focus of intense scientific interest during the past few years (reviewed by Walker *et al*, 1986). Like many other bacterial pathogens, the pathogenic mechanism(s) of *C. jejuni* enteritis probably involves a multiplicity of virulence factors involving a series of complex interactions between macromolecules of the bacterium and the host cell. Little is known about the putative virulence factors and the pathogenic mechanisms of *C. jejuni* (Walker *et al*, 1986). For these reasons a broad experimental approach was chosen during the course of the present investigation.

Elucidation of the pathogenic mechanisms of the diarrhoeagenic microorganisms will enable us to address better the problem of diarrhoeal disease. Identification and characterization of the virulence factors, which often constitute the antigens of choice for vaccine development may lead the ways by which to improve the control of this major global health problem. The rationale for studies on virulence mechanisms of *C. jejuni* becomes evident when the world-wide features of *C. jejuni* infection, enormous manpower loss associated with it and the huge economic loss in chicken industries are considered.

1.2 HISTORICAL BACKGROUND

Organisms now known as campylobacters were first described in the U.K. by two

veterinary surgeons at the beginning of this century (McFadyean and Stockman 1909; 1913). They observed an unknown bacterium resembling a vibrion in the aborted foetuses during a survey of epizootic abortion in ewes. Smith (1918) reported on the association of similar organisms with bovine abortions in the U.S.A. The following year the pathogen was placed in the genus *Vibrio* because of its resemblance to vibrios and given the specific name *Vibrio fetus* due to its association with abortion in cattle and sheep (Smith and Taylor, 1919)

The microaerophilic nature of campylobacters and their possible role as a causative agent of diarrhoea in calves was first reported by Smith and Orcutt (1927). They also noted that calf diarrhoeal strains were serologically different from previously reported *V. fetus*. Microaerophilic vibrios were linked later to winter dysentery in cattle (Jones and Little, 1931a; 1931b; Jones *et al,* 1931) and swine dysentery (Doyle, 1944). In both cases, the pathogen sufficiently resembled *V. fetus* to be regarded as a closely related group and the calf enteritis strain was named *Vibrio jejuni* (Jones *et al,* 1931) while the swine dysentery organism was named *Vibrio coli* (Doyle, 1944). According to Karamali and Skirrow (1984), the microaerophilic vibrios given the various names *V. fetus, V. jejuni* and *V. coli* by earlier workers were due more to their association with specific diseases in animals rather than to any observed taxonomic differences between them.

After being known for about 35 years as an exclusive veterinary pathogen, *Campylobacter* was first associated with human disease by Levy in 1946. Organisms resembling *V. jejuni* were observed in blood cultures of several victims of a milk-borne outbreak of acute diarrhoea; but the pathogen could not be positively identified as it would not grow on solid medium. Subsequently *V. fetus* was successfully isolated from pregnant women with fever who aborted during illness (Vinzent, 1949)

King (1957) reported that catalase-positive, microaerophilic vibrios could be differentiated by their characteristic growth temperatures *V. fetus* could grow at 25^{o} C and at 37^{o} C but not at 42^{o}; whereas a morphologically indistinguishable group of blood

blood isolates from children with preceding diarrhoea did not grow at 25°C but grew at 37° C, even better at 42° C. She coined the term "related vibrio" to describe these organisms and in a subsequent report (King, 1962) suggested that these organisms were identical to *V. jejuni* (Jones *et al,* 1931) and *V. coli* (Doyle, 1948). As all the children had diarrhoea, she speculated that apart from blood, the pathogen might also have been present in the gut; but attempts to grow them from stools did not succeed because of overgrowing normal faecal flora. She suggested that childhood diarrhoea due to "related vibrios" was possibly more common and emphasized the need to devise methods for culturing them from faeces. The organisms she called "related vibrios" are now *Campylobacter jejuni* and *Campylobacter coli.*

The fundamental differences between *V. fetus* and the true vibrios were pointed out by Sebald and Veron (1963) and a new genus *Campylobacter* (Greek: curved rod) in the family *Spirillaceae* was proposed to include *V. fetus* and related organisms. Following the work of Sebald and Veron (1963), Veron and Chatelain (1973) published the first comprehensive account of the taxonomy of the genus *Campylobacter.* In Bergey's Manual of Systematic Bacteriology, Jones *et al* (1931) and Doyle (1948) are credited with the discovery of *C. jejuni* and *C. coli* which they named as *V. jejuni* and *V. coli* respectively (Smibert, 1984).

Because of the difficulty in isolating *Campylobacter* spp. from diarrhoeal stool by suppressing the normal faecal flora, and the need for a microaerophilic growth environment, their role as a cause of human enteritis went unnoticed for many years after their first description. It is surprising that although *Campylobacter* spp. are now established as a major cause of human diarrhoea, only 12 cases of human infections due to "related vibrios" were known until 1972, all from blood cultures (Middelkamp and Wolf, 1961; Wheeler and Brochers, 1961; White, 1967; Darrell *et al,* 1967). Dekeyser *et al* (1972) made the necessary breakthrough in Belgium and reported the first positive stool culture of "related vibrio". They used a filtration technique for faecal samples (which hold back other faecal organisms but allow *Campylobacter* to

through because of its small size), and antibiotic-supplemented growth medium for selective isolation of "related vibrios". Also in Belgium, Butzler *et al* (1973) isolated *Campylobacter jejuni* from 5% of children with diarrhoea, utilizing the same filtration technique and showing for the first time its importance as a diarrhoea-causing pathogen.

The development of a selective medium for growing these organisms directly from stool without the filtration step by Skirrow (1977) is a milestone in the history of *Campylobacter* research. Isolation and cultivation of *Campylobacter* spp. became much simpler and since then, *C. jejuni* has been isolated with increasing frequency all over the world and is now established as a major cause of human diarrhoea. Recognition of the importance of *C. jejuni* in human enteritis has stimulated prolific research on its pathogenic mechanisms over the past decade.

It is usually considered that the first description of *Campylobacter* in animal and human disease is given by McFadyean and Stockman (1909; 1913) and Levy (1946) respectively. However according to Kist (1985), a number of older predominantly German papers reported the involvement of a spiral bacterium in human diarrhoea. In 1886, 60 years before the publication of Levy's paper Theodor Escherich published a series of papers on the existence of vibrio-like organisms in the intestines and faeces of babies. He named them *Vibrio felinus*. Involvement of similar pathogens in human diarrhoea were reported by several other German authors in subsequent years. Kist (1985) concluded that the organisms described by these German authors were probably *Campylobacter* spp. for several reasons: (1). typical morphology; 2-5 μm in length with 2-6 coils (2). association with enteritis in neonates and infants (3). failure to grow on solid medium despite microscopic detection and (4). the fact that to date, no other bacteria with comparable morphology have been associated with human enteric infection.

1.3 THE GENUS *CAMPYLOBACTER*

The genus *Campylobacter* currently consists of 14 species (Table -2) (Penner,

Table 2: The Genus *Campylobacter*

Species	Reactions and characteristics[b]												
						Growth					Susceptibility[c]		G+C content (mol%)
	Catalase	Nitrate	H_2S (TSI)	Hippurate	Indoxyl acetate	25°C	37°C	42°C	1% Glycine	0.1% TMAO (anaerobic)	Nalidixic acid	Cephalothin	
C. fetus subsp. *fetus*	+	+	−	−	−	+	+	(−)	+	−	R	S	33–34
C. fetus subsp. *venerealis*	+	+	−	−	−	+	+	−	−	−	R	S	33–34
C. hyointestinalis	+	+	+	−	−	(+)	+	+	+	+	R	S	35–36
C. jejuni	+	+	−	+	+	−	+	+	+	−	S	R	30–32
C. coli	+	+	−	−	+	−	+	+	+	−	S	R	31–33
C. laridis	+	+	−	−	−	−	+	+	+	+	R	R	31–33
"*C. upsaliensis*"	(−)	+	−	−	ND	−	+	+	−	−	S	S	35–36
"*C. cinaedi*"	+	+	−	−	(−)	−	+	−	+	−	S	I	37–38
"*C. fennelliae*"	+	−	−	−	+	−	+	−	+	−	S	S	37–38
C. cryaerophila	+	+	−	−	+	+	+	−	−	ND	d	R	29–30
C. nitrofigilis	+	+	ND	−	−	+	+	−	−	ND	S	S	28–29
C. sputorum													
Biovar sputorum	−	+	(+)	−	−	−	+	+	+	d	(S)	S	31–32
Biovar bubulus	−	+	+	−	−	−	+	+	+	+	R	S	31–32
Biovar fecalis	+	+	+	−	−	−	+	+	+	+	R	S	32–33
C. mucosalis	−	+	+	−	−	+	+	+	+	−	R	S	38–39
C. concisus	−	+	+	−	ND	−	+	+	+	−	R	R	38–39
C. pylori	+	d	−	−	−	−	+	+	d		R	S	36–37

[b] +, Positive reaction; -, Negative reaction; ND, Not done; (+), most strains positive but a low percentage negative; (-), most strains negative but some positive or weakly positive; d, different reactions; R, resistant; S, susceptible; I, intermediate zones of inhibition.

[c] Susceptibility to antibiotics determined with 30-μg disks

TAMO, trimethylene N-oxide

From Penner (1988)

1988) and new species are being described with increasing frequency. Campylobacters are generally inert to standard biochemical tests and the number of discriminating characteristics are consequently few. So their classification is dependent upon a few morphological and physiological features and G+C content of DNA (Hebert *et al,* 1984; Karamali and Skirrow, 1984; Smibert, 1984; Roop *et al,* 1984). However, justification of inclusion of all 14 species into a single genus is questioned as they are genetically and metabolically widely diverse (Penner, 1988). Using the phylogenetic comparison of ribosomal RNA method of systematic classification, Romaniuk and Trust (1987) found high interspecies homology in the 16 S ribosomal RNA of *C. jejuni, C. coli, C. fetus, C. laridis* and *C. sputorum.* However, homology data indicate that the spiral bacterium associated with human gastritis, *C. pylori* (formerly *C. pyloridis*) should be considered for reassignment to another genus. Such studies with the other 8 species may conclusively ascertain their status as members of the genus *Campylobacter*

The genus *Campylobacter* was originally included in the family *Spirillaceae* on the basis of general spiral morphology, the presence of a single unsheathed polar flagellum, the inability to accumulate polyhydroxybutyric acid and a 29 to 38 mol % G+C (Veron and Chatelain, 1973; Karamali and Skirrow, 1984). DNA hybridization studies demonstrated that the classification of the genus *Campylobacter* to the species level utilizing the restricted list of phenotypic properties was correct (Belland and Trust, 1982; Benjamin *et al,* 1983; Roop *et al,* 1984). Comparison of partial 16 S rRNA sequence data of *Campylobacter* species with those of representative species from 8 of the 10 currently defined eubacterial group indicate no obvious closeness with any of these phylogenetic groups. Moreover, T1 nucleotide sequence analysis also indicate that the *Campylobacter* species do not belong to any of the currently defined eubacterial group. From these results Romaniuk and Trust (1987) concluded that

the *Campylobacter* spp. constitute a distinct phylogenetic group which has not been described before.

1.4 NATURE OF THE PATHOGEN

C. jejuni is a slender spirally-curved Gram-negative rod, 0.2-0.5 μm wide and 0.5-8.0 μm long. The rods usually have one to several spirals with average wavelength of 1.12 μm and average amplitude of the coil is 0.48 μm. They also appear as S-shaped and gull-winged when two cells form short chains. *C. jejuni* is usually motile with a characteristic rapid darting corkscrew-like motion by means of a single polar unsheathed flagellum at one or both ends of the cell which may be 2-3 times the length of the cell (Smibert, 1984). However, flagellate non-motile and aflagellate non-motile variants have been reported (Newell *et al* 1984; Walker *et al*, 1986).

C. jejuni is microaerophilic; requiring a gas atmosphere of 10% CO_2, 5% O_2 and 85% N_2. Addition of 0.025% (w/w) each of ferrous sulphate, sodium pyruvate and sodium metabisulphite to culture media, increases the aerotolerance of the pathogen by destroying the hydrogen peroxide and superoxide anions produced in the presence of air and thus allow growth at an oxygen concentration of 15-20 %. *C. jejuni* is extremely sensitive to these toxic forms of oxygen although it possesses catalase, oxidase and superoxide dismutase activity (George *et al*, 1978; Hoffman *et al*, 1979a 1979b). It does not grow at 25° C, grows at 37° C and at 45° C but better around 42-43° C.

C. jejuni is chemoorganotrophic in nature with a respiratory type of metabolism; carbohydrates are neither oxidized nor fermented. Energy is obtained from amino acids and tricarboxylic acid cycle intermediates. No acid or neutral products are produced. The growth medium becomes alkaline (pH 8.5-9.0) and coccoidal forms appear when grown for long time or in unfavourable conditions (Moran and Upton,1986; 1987).

1.5 EPIDEMIOLOGY OF *C. JEJUNI* INFECTIONS

The striking increase in the worldwide reported incidences of *C. jejuni* infections

after the discovery of the medical importance of the pathogen (Skirrow, 1977) stimulated several epidemiological investigations in recent years. Such studies have revealed that a broad range of demographic, socioeconomic, clinical and biologic variables are the determinants of the prevalence and transmission of the pathogen, making epidemiological studies difficult. However, it has become apparent from these studies that *C.jejuni* enteritis occurs world-wide and that epidemiologic features vary with the geographic locale and the standard of hygiene enjoyed by the population sampled (Blaser *et al,* 1979a; Blaser *et al,* 1983c; Skirrow, 1984).

1.5.1 Incidence: *C. jejuni* is the commonest cause of acute bacterial enteritis in many countries of the world including several technically advanced countries (Table 1). In many developing countries *C. jejuni* is the third most common diarrhoeagenic pathogen, after enterotoxigenic *E. coli* and Rota virus; although its incidence is much higher than the technically advanced countries where it is the commonest of all enteropathogens (Stoll *et al,* 1982).

In technically advanced countries the rate of subclinical infection leading to asymptomatic excretion is usually much lower than in developing countries. Isolation rate from normal healthy individuals is usually less than 1% in developed countries (Blaser *et al,* 1984c; Skirrow, 1984). Recently, Riordan (1988) reported a much higher (9%) asymptomatic carrier rate in a longitudinal survey of young children attending a day nursery in Manchester. The incidence of *C. jejuni* infection is much higher in developing countries. Molbak *et al* (1988) reported that about 44.9% of the children in an urban slum in Liberia were excretors of *C. jejuni.* Such high carrier rate has also been reported from other countries such as the Central African Republic (Georges-Courbot *et al,* 1987), in a black township of South Africa (Bokkenhheuser *et al,* 1979), India (Mathan *et al,* 1984), Bangladesh (Blaser *et al,* 1980a; Glass *et al,* 1983) and Mexico (Sjogren *et al,* 1989). In developed countries, people of all

age groups are affected as protective immunity is never established significantly (Skirrow, 1987a). But in developing countries illness to infection ratio decreases and the proportion of asymptomatic carriers increases with age as immunity is acquired due to multiple infection at an early age (Glass *et al*, 1983; Blaser *et al*, 1986c; Ani *et al*, 1988; Calva *et al*, 1988a).

1.5.2 Seasonal Distribution: The seasonal distribution of *C. jejuni* infection shows a higher incidence in summer months in many European countries and in North America (Blaser and Reller, 1981; Skirrow, 1984). The reason for this seasonal variation is not known but it is likely to be due to increased concentration of the pathogen in the environment. Poultry have also been implicated as a probable reason; as contamination of retail poultry increases in the summer months (Harris *et al*, 1986a; 1986b) and it is considered to be the most common vehicle of food-borne transmission to man. But this cannot be a generalized phenomenon because the incidence of *C. jejuni* diarrhoea is reported to be higher in the cooler months in many countries such as Bangladesh (Neogi and Shahid, 1987), South Africa (Cameron *et al*, 1982), Hong Kong (McGechie *et al*, 1982) and Israel (Skirrow, 1984).

1.5.3 Influence of Age and Sex: Age specific incidence of *C. jejuni* shows trimodal distribution with peaks at 1-2 years, 14-25 years and about 65 years onwards (Blaser *et al*, 1983c; Skirrow, 1987a). The higher incidence of infection in infants is presumably due to first exposure to *C. jejuni*. In developing countries *C. jejuni* infections follow weaning of infants (Glass *et al*, 1983; Calva *et al*, 1988a). As immunity is acquired by symptomatic infection, probably due to the development of gut mucosal antibody which is most effectively elicited by direct contact with pathogenic bacteria, the children become progressively less susceptible with increasing age (Taylor *et al*, 1988; Calva *et al*, 1988a). Higher incidence of infection in young adults may occur at the time of "second weaning" when they leave their homes of origin

and are first dependent on foods prepared by themselves or other inexperienced cooks (Tauxe *et al,* 1987). But the fact that this particular age group is generally more adventurous and interact more with the environment can also be a possible reason for higher incidence in young adults. The third peak at the elderly age presumably correlates with decreased immunocompetence.

Several studies have shown relatively higher incidence of *C. jejuni* infections among males especially under 30 years of age (Riley and Finch, 1985; Tauxe *et al,* 1987, Skirrow, 1987a; Deming *el al,* 1987). The factors that explain this concentration of *C. jejuni* infection among male is unknown. Deming *et al* (1987) hypothesized that the young male adults may be at greater risk because the cooking practice of male students is less safe than that of female students. However this hypothesis would not explain the higher incidence of infection in male infants (Skirrow, 1984) and according to Sinnecker (1976), the susceptibility or resistance of host populations is influenced not only by environmental factors; the genetic constitution also plays a determinative role.

1.5.4 Reservoirs of Infection: *C. jejuni* has a broad host spectrum; it is found as normal intestinal flora of cattle, sheep, goats, dogs rabbits, monkeys, cats, chickens, ducks, seagulls, pigeons, starlings, sparrows (Smibert, 1984) and a variety of wild birds (Luechtefeld *et al,* 1980, Rogol and Sechter, 1988). All these animal and avian species can thus serve as reservoirs. As campylobacters can survive in water and soil for up to 3 weeks under moderately cold ambient conditions (Blaser *et al,* 1983c) contamination of the environment by these animals may constitute a source of human infections. There are several reports of isolation of the same serovars of *C. jejuni* from infected human beings and from animals from different parts of the world (Luechtefeld *et al,* 1980; Neogi and Shahid, 1987; Rogol and Sechter, 1988). Studies on population genetics of *C. jejuni* strains from human and animal hosts have indicated that (1) *C. jejuni* infection is a zoonosis, (2) human and animal strains do not constitute subpopulations and (3) every animal strain can be

considered as a potential human pathogen (Aeschbacher and Piffaretti, 1989). These studies indicate the possible role of animal reservoirs and the chain of circulation of the pathogen between man and animals.

1.5.5 Routes of Infection: Several potential routes exist by which *C. jejuni* can be transmitted to human beings. Ingestion of bacteria through contaminated food and water followed by dissemination from the gastrointestinal tract is apparently the most common mode of transmission to man (Blaser *et al,* 1983c).

1.5.6 Vehicles of Transmission: Raw milk is a frequent vehicle of transmission of *C. jejuni* infection to human beings (Blaser *et al,* 1979b; Potter *et al,* 1984; Hudson *et al,*1984; Tauxe *et al,* 1987). Birkhead *et al* (1988) reported a case of inadequately pasteurized milk causing an out-break of *C. jejuni* enteritis among school children. Although as low as 500 cells of *C. jejuni* in milk could induce disease in a volunteer study (Robinson, 1981) and the organism could survive in milk for upto 3 weeks when kept at 4^{o} C (Blaser *et al,* 1980b), the epidemic strain could be isolated from the implicated milk only in one case (Hudson *et al,* 1984). The implicated strain was however isolated from the herd (Birkhead *et al,* 1988) and from milk filters (Potter *et al,* 1983; 1984). As *C. jejuni* can be isolated readily from bovine faeces (Svedham and Kaijser, 1981; Hutchinson *et al,* 1985), faecal contamination is considered to be the likely source in milk (Blaser *et al,* 1984a). However bovine mastitis has also been reported as a source of *C. jejuni* in milk (Hudson *et al,*1984). *Campylobacter* enteritis caused by consumption of raw goat milk has also been reported but less frequently (Hutchinson *et al,* 1985; Barrett, 1986).

Water-borne outbreaks of *C. jejuni* enteritis are usually associated with the consumption of untreated water or due to contamination of the water supply system by surface water (Mentzing, 1981; Vogt *et al,* 1982). Sacks *et al* (1986) reported a water-borne outbreak of *C. jejuni* enteritis in which the water supply system

system was unprotected from contact with birds. *C. jejuni* may remain in a viable state in sterile stream water for 33 days at 4° C and for 4 days at 25° C (Blaser *et al,* 1980b); but the infective dose in water is not known. Rollins and Colwell (1986) reported that *C. jejuni* could survive in stream water in a viable form but these cells could not be cultured on sheep blood agar plates. However, they could colonize the intestinal mucosa of experimental animals and resumed normal viability and morphology, indicating their paramount ecological and epidemiological importance. Similar viable but nonculturable states have been described for other pathogens such as *V. cholerae, E. coli* and *Salmonella enteritidis* (Xu *et al,* 1982; Roszak *et al,* 1983). The authors (Rollins and Colwell, 1986) concluded that nonculturability of *C. jejuni* on agar plates cannot be equated with nonviability; which probably explains the inability of isolating the epidemic strains from the suspected vehicle of transmission in numerous outbreaks of *C. jejuni.*

Uncooked or poorly cooked meat and poultry is another major vehicle for human infection. Chicken is considered to be the commonest of all reservoirs of *C. jejuni* and as chicken is a common food all over the world it constitutes the largest potential source (Harris *et al,* 1986a; 1986b). Although the attempts to isolate the organism from the suspected food were not always successful, several studies have shown epidemiological association of undercooked chicken, turkey or other food materials with outbreaks of *C. jejuni* enteritis (Blaser *et al,* 1982; 1983c). Other routes of transmission include contact with animals or their carcasses (occupational and domestic), person to person spread (Skirrow, 1987b), and nosocomial infection (Butzler and Goosens, 1988) but these constitute only a small proportion.

Although the general concept of epidemiology of *C. jejuni* enteritis, which somewhat resembles that of *S. enteritis* is beginning to be recognized, much remains to be known. In particular, the differential epidemiological pattern that exists in technically advanced and developing countries definitely needs to be thoroughly investigated. The routes of transmission, which are probably many and varied, need to be

identified so that effective preventive measures can be formulated and implemented. General control measures such as proper purification of water supplies and pasteurization of milk, public health education emphasizing kitchen hygiene and control of infection in poultry will definitely play a determinative role in the prevention of *C. jejuni* enteritis (Skirrow, 1987b).

1.6 INFECTION IN MAN

The development of specific culture methods for *C. jejuni* has enabled researchers to recognize and investigate the spectrum of enteric and nonenteric infections the pathogen can cause in man. Although *C. jejuni* is primarily an enteropathogen, nonenteric infections were reported long before the enteric ones (see Section 1.2).

1.6.1 Inoculum Size: Considerable variation exists in the relative virulence of the strain and the host susceptibility, both of which determine the inoculum size needed for the establishment of infection. Epidemiological studies indicated that as few as 500 organisms caused disease in a water-borne outbreak (Walker *et al*,1986). The inoculum size of a similar number of organisms in milk, was noted in a human volunteer study (Robinson, 1981). In an extended human volunteer study Black *et al*, (1988) noted that the lowest dose to cause illness was 800 organisms (400 flagellated and 400 nonflagellated) and the attack rate, incubation period and the severity of disease were independent of the size of the inoculum, indicating the critical role of the host factor.

1.6.2 Incubation Period: After exposure, most of the infected individuals become ill within 2-5 days. However, the incubation period may range from 20 hours to 10 days (Butzler and Skirrow, 1979; Itoh *et al*, 1982; Skirrow, 1984). In human volunteer studies the incubation period ranged from 3-6 days (Robinson, 1981, Black *et al*, 1988).

1.6.3 Symptoms: In most cases, the first symptom is abdominal pain but in about one-third, there is a febrile prodromal period characterized by anorexia, malaise, headache, fever, shivering, nausea, vomiting, dizziness and generalized myalgia which may last from a few hours to a few days (Blaser and Reller, 1981; Skirrow, 1984). Usually the patients experiencing the prodromal illness tend to develop a more severe form of illness and attend hospital for treatment in comparison to those starting with diarrhoea (Blaser and Reller, 1981; Skirrow, 1984).

1.6.4 Clinical Disease: *Campylobacter* enteritis is an acute self-limiting diarrhoeal disease with abrupt or gradual onset, which is often clinically indistinguishable from *Salmonella* enteritis or shigellosis (Butzler and Skirrow, 1979; Mandal *et al,* 1984). The diarrhoea may range from a few loose stools to fulminant bloody diarrhoea with abdominal cramps and high fever. Occasionally, the abdominal pain may be so severe as to mimic appendicitis (Mandal *et al,* 1984). Clinical presentation of the disease may be either profuse watery diarrhoea, which is most frequently described in developing countries (Glass *et al,* 1983) to dysentery-like mucoid diarrhoea, which is more common in the technically advanced countries (Skirrow, 1987b) (Section 1.5). Most of the stool samples contain leukocytes, and red cells; frank blood is commonly seen from the second day onward, especially in children (Skirrow, 1984). Human volunteer studies have shown that first the small intestine then the colon is the prominent site of infection (Black *et al,* 1988). The acute diarrhoea lasts generally for about 2-3 days having 10 or more bowel actions per day at the height of the disease (Blaser *et al,* 1979a; Svedhem and Kaijser, 1980). In two outbreaks, the mean duration of illness were recorded to be 4.2 days (Mouton *et al,* 1982) and 5.2 days (Mentzing, 1981). In some cases, the duration of diarrhoea was reported to be as long as 7 days (Mandal *et al,* 1984). The frequency and duration of *C. jejuni* diarrhoea was generally less than in patients with *Salmonella* or *Shigella* infection but the abdominal pain was more severe in *Campylobacter* infected patients

(Price *et al,* 1979; Jewkes *et al,* 1981). The patients generally become completely symptom- free by day 12 and patients remaining ill for any longer is suspected to have developed complications such as ulcerative colitis (Willoughby *et al,* 1979; Skirrow, 1984). When patients start taking solid food after diarrhoea has ceased, a temporary relapse of illness, usually less severe than the original attack, occurs in 16-25% of them (Blaser *et al,* 1979a; Drake *et al,* 1981; Pitkanen *et al,* 1983). The excretion of *Campylobacter* in the stool of the recovered patients decreases exponentially with time with 85% being culture-free by 5 weeks and virtually all by 3 months; The duration of excretion usually did not correlate with the age of the patient, severity of symptoms and duration of disease (Skirrow, 1984).

1.6.4.1 Complications: Although diarrhoea of varying degree is the main symptom of infection in man, various complications may supervene. Colitis, occasionally mimicking the symptoms of Crohn's disease is the most common complication following *C. jejuni* enteritis usually occurring in patients in whom the disease persist for a longer period in comparison to normal enteritis (Lambert *et al,* 1979; Blaser *et al,* 1980c; McKendrick *et al,* 1982). The endoscopic and histological findings in *Campylobacter* colitis resemble those caused by *Shigella, Salmonella,* amoeba and *Clostridium difficile* (Mandal *et al,* 1984). In an autopsy study of diarrhoeal disease deaths in Bangladesh *C. jejuni* was isolated from 16% of the patients and ulcerative disease of the colon and small intestine were noted in the majority of the cases (Butler *et al,* 1987).

The severe abdominal pain (which is reported to be more severe and lasted longer than *Salmonella* enteritis; Jewkes *et al,* 1981) which occurs before the onset of diarrhoea can mimic acute appendicitis and lead to hospitalization and laparotomy; but the appendix is rarely found to be inflamed (Mandal *et al,* 1984). However, the cases of genuine appendicitis due to *C. jejuni* are also reported (Pearson *et al,* 1982, Chan *et al,* 1983; Morlet and Glancy, 1986).

Other complications associated with the gastrointestinal tract, which may or may not be preceded by simple diarrhoea includes proctitis (Quinn *et al,* 1980), inflammatory bowel disease (Newman and Lambert, 1980; Chessin *et al,* 1982); toxic megacolon, haemolytic uraemic syndrome (Chamovitz *et al,* 1983; Shulman and Moel, 1983), severe life-threatening gastrointestinal haemmorhage (Michalak *et al,* 1980) and ulceration of ileal stroma (Meuwissen *et al,* 1981, Skirrow, 1984).

1.6.5 Systemic Infections: Systemic infections caused by *C. jejuni* are reported with increasing frequency and the pathogen *C. jejuni* is being etiologically associated with newer diseases (Table 3) Systemic campylobacteriosis is generally thought to arise from intestinal colonization (Butzler and Skirrow, 1979), and virtually all patients with systemic campylobacteriosis have some sort of predisposition to infection such as immunodeficiency and underlying disease. Prematurity and birth trauma in newborn infants and pregnency has also been occasionally associated with systemic disease (Skirrow, 1984). This probably reflects the opportunistic character of *C. jejuni* in causing various systemic infections.

1.6.5.1 Bacteraemia: Gastrointestinal infection of *C. jejuni* may occasionally lead to bacteraemia (Guerrant *et al,* 1978; Spelman *et al,* 1986) but it is not known whether this is an early consequence of gut infection or whether it may arise sporadically during a period of long term colonization. According to Mandal *et al* (1984), invasion of the blood stream is a part of the pathogenesis in the early stages of the disease and bacteraemia usually goes undetected as blood culture is rarely done in the early stages of the disease. Black *et al* (1988) however failed to detect organisms in the blood after intensive monitoring in human volunteer studies. So the capacity of the *C. jejuni* strains to cause bacteraemia and its significance in clinical disease is still unclear. Various systemic diseases caused by *C. jejuni* are listed in the Table 3.

Table 3 : Systemic Infections Caused by *C. jejuni*

Disease	References
Meningitis	Thomas *et al* (1980); Goossens *et al* (1986)
Septic abortion	Gilbert *et al* (1981)
Female genital tract infection	Lichtenberger and Perlino (1982)
Male genito-urinary infection	Davis and Penfold (1979)
Urinary tract infection	Blaser *et al* (1986b)
Chronic renal failure	Blaser *et al* (1986b)
Acute cholecystitis	Blaser *et al* (1986b)
Pneumonia	Blaser *et al* (1986b)
Ovarian cyst	Blaser *et al* (1986b)
Bronchiolitis	Blaser *et al* (1986b)
Cellulitis and osteitis	Skirrow (1984)
Pancreatitis	Gallagher *et al* (1981)
Hepatitis	Skirrow (1984)
Reactive arthritis	Berden *et al* (1979)
Guillain-Barre syndrome	Kaldor and Speed (1984)
Erythema nodosum	Lambert *et al* (1982); Eastmond and Reid (1982)
Glomerulonephritis	Skirrow (1984)
Miller Fisher syndrome	Roberts *et al* (1987)

1.6.6. TREATMENT

1.6.6.1 Antibiotic therapy: Although erythromycin has been advocated as the drug of choice for uncomplicated *C. jejuni* enteritis based upon *in vitro* studies, several placebo-controlled therapeutic trials with this drug (40 mg kg-1 body weight per day) showed no effect on the clinical course (Anders *et al,* 1982; Robins-Browne *et al,* 1983; Pai *et al,* 1983). But in all these studies the excretion of the pathogen was significantly reduced. However, erythromycin significantly shortened the duration of diarrhoea in a placebo-controlled, double-blind study with children who were ill for only three days using a higher dose of the drug (50 mg kg-1 day-1) (Salazar-Lindo *et al,* 1985). The authors stressed an early therapy with erythromycin in ameliorating the clinical illness and curtailing the excretion of the pathogen. However, a recent study by Williams *et al* (1989) found that although erythromycin was bacteriologically effective (rapidly eliminated *C. jejuni* from stools), it failed to reduce the duration or severity of diarrhoea, abdominal pain and other symptoms. Several recent reports indicated that there is a trend for *C. jejuni* to become resistant to the drug (Altwegg *et al,* 1987; D. N. Taylor *et al,* 1987). *In vivo* development of resistance to this drug during treatment of an AIDS patient has been reported recently (Perlman *et al,* 1988). For the treatment of uncomplicated diarrhoea, public health education and better hygiene might play a far greater role than antibiotic therapy. Moreover, the practicing physician rarely knows the etiological agent causing diarrhoea when first treating the patient; it is usually several days before a suspicious agent is reported. *C. jejuni* strains are sensitive to chloramphenicol and gentamycin and these are used for the treatment of patients with life-threatening infections (Skirrow, 1984).

1.6.6.2 Rehydration Therapy: Profuse watery diarrhoea, which is more commonly seen in developing countries (Glass *et al,* 1983) as a characteristic feature of *C. jejuni* enteritis may cause sufficient dehydration and electrolyte imbalance

especially in infants and children for them to require rehydration therapy. Studies have shown that bacterial and viral diarrhoea can be successfully managed with rehydration therapy (mainly oral) alone without any antibiotic treatment (Molla *et al*, 1987).

1.7 CANDIDATE VIRULENCE MARKERS

The recognition of *C. jejuni* as a major diarrhoeagenic pathogen has stimulated research on the virulence characteristics of the pathogen which enables it to cause a broad spectrum of illness in man and animals (Walker *et al*, 1986). The clinical manifestation of infection with *C. jejuni* may vary from asymptomatic infection to severe diarrhoea leading to ulcerative colitis in some cases (Butzler and Skirrow, 1979; Mandal *et al*, 1984). It can cause various systemic infections even death in the elderly and the debilitated and severe chronic infection in immunodeficient patients (Skirrow, 1984; Perlman *et al*, 1988). The current knowledge of the various putative virulence factors and the parts played by them in *C. jejuni* enteritis is only elementary; our understanding of the systemic disease caused by the pathogen is more fragmentary.

The clinical presentation of the *C. jejuni* enteritis can be categorized into two types; a). dysentery-like bloody, mucoid diarrhoea and b). cholera-like secretory diarrhoea (Klipstein *et al*, 1985) indicating two different pathogenic mechanisms presumably involving two sets of virulence factors. The bloody mucoid diarrhoea which is characteristic feature of *Shigella* spp. infection may be assumed to be caused by strains which are capable of invading intestinal epithelial cells and producing cytotoxin. On the other hand cholera-like diarrhoea may be the manifestation of the infection due to noninvasive, toxigenic strains which are reported to produce an enterotoxin possessing immunobiological similarity with cholera toxin (CT) and *E. coli* heat-labile toxin (LT). Strains causing asymptomatic infection should possess neither of these two sets of virulence characteristics. Such a clear cut correlation between the clinical status of the infected host and the specific pathogenic characteristics of the isolates was

reported in one study (Klipstein *et al,* 1985). Sanyal *et al,* (1984a) however did not observe any correlation between the clinical source of a strain and its pathogenic effects in the infant chicken diarrhoea model. Strains isolated from bloody-mucoid diarrhoea are also reported to produce enterotoxin in addition to being invasive (Ruiz-Palacios *et al,* 1983). Moreover, simultaneous production of enterotoxin and cytotoxin by human clinical strains were also reported (Johnson and Lior, 1984; 1986). Such observations indicate that the virulence characteristics of *C. jejuni* strains cannot be categorized as 'Shigella-like' or 'Cholera-like'; the organism possesses a unique set of virulence markers which may act separately, sequentially or simultaneously to cause disease. To make the investigations on the virulence characteristics of *C. jejuni* more complicated; strains isolated from the asymptomatic carriers have also been reported to produce enterotoxins (Mathan *et al,* 1984). However, it should be considered that the clinical response of the patients following infection is not solely determined by the pathogenic characteristics of the organism, the host immune system also plays a determinative role. In addition, recent studies on the pathogenic mechanisms of diarrhoeagenic pathogens have shown that description of diarrhoea as "Shigella-like" and "Cholera-like" is often confusing as *Shigella* spp. can cause watery diarrhoea (Levine *et al,* 1983) and production of toxic factors other than cholera toxin has been reported in *Vibrio cholerae* (O'Brien *et al,* 1984; Sanyal *et al,* 1984b). Genetically-engineered cholera toxin-negative strains also produced diarrhoea in human volunteers indicating that mere colonization of the gut is often sufficient to induce diarrhoea (Levine *et al,* 1988).

In recent years several virulence markers such as enterotoxin production, cytotoxin production, adherence, invasion, survival within macrophages etc. have been putatively identified in *C. jejuni* strains. The possible involvement of the various surface structures such as outer membrane protein, flagella and lipopolysaccharide (LPS) in the virulence mechanism has also been indicated. However, it should be noted that most of these are preliminary reports yet to be confirmed and extended by others.

The current knowledge on the various candidate virulence markers of *C. jejuni* is reviewed below.

1.7.1 ADHESION: According to the degree of intimacy between bacterial and host mucosal surfaces interaction of pathogenic microorganisms with the host can be

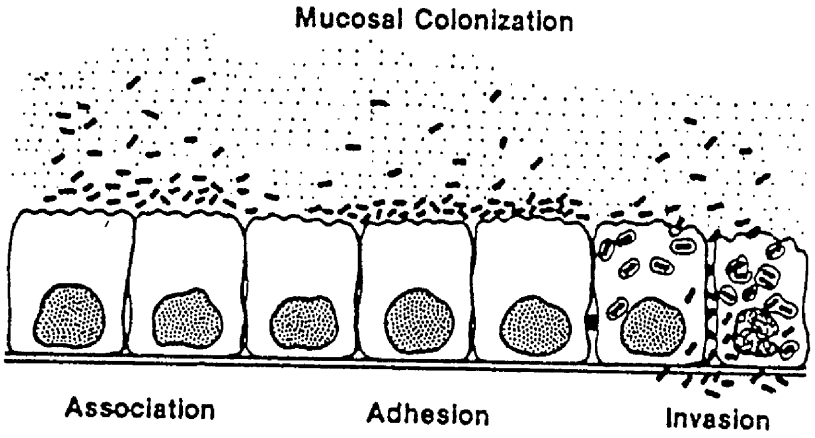


Figure 1: Types of Bacterial-Mucosal Interaction Involved in Bacterial Infection of Mucosal Surfaces.
From Arp (1988)

association, adhesion and invasion (Arp, 1988). Association is weak, reversible attachment or localization of bacteria along a surface. Adhesion describes a relatively stable, irreversible attachment mediated by specialized complementary molecules of the bacterial and mucosal surfaces. Invasion implies the most intimate form of interaction, in which the pathogen penetrates the mucosal barrier to gain entry and establish itself within epithelial cells or adjacent stroma tissue (Arp, 1988).

The capacity to adhere is believed to be an important bacterial determinant related to virulence and is considered as an early event in the complex colonization process of the intestinal mucosa (McNeish *et al*, 1975; Beachey, 1981). Various surface components such as pili, flagella, capsule, lipopolysaccharide, glycocalyx. outer membrane proteins and cell-associated lectins have been implicated as mediators of

interactions of pathogenic microorganisms with host mucosal surfaces (Peterson and Quie, 1981).

Several *in vitro* and *in vivo* studies are reported concerning the adherence process of *C. jejuni* and attempts to identify the contributing cell surface component(s) (Newell *et al*, 1984, McSweegan and Walker, 1986; McSweegan *et al*, 1987). *C. jejuni* can adhere to HeLa cells (Manninen *et al*, 1982; Newell and Pearson, 1984; Fauchere *et al*, 1986). INT 407 cells (Newell and Pearson, 1984; Cinco *et al*, 1984; McSweegan and Walker, 1986) and porcine brush border cells (Naess *et al*, 1983). Differences in the adherence capacity to epithelial cells was noted among *C. jejuni* strains (Manninen *et al*, 1982, Newell and Pearson, 1984; Fauchere *et al*, 1986). Fauchere *et al* (1986) reported the development of an *in vitro* assay to determine the adherence potential of *C. jejuni* strains to HeLa cells which may be used as a rapid and convenient test for estimating the pathogenic potential of a given strain.

1.7.1.1 Proposed Adhesins: The flagella of *C. jejuni* have been implicated to play a contributory part in several studies. Flagellate strains adhered more avidly than their aflagellate variants to epithelial cells. By scanning electron microscopy, Newell and Pearson (1984) showed morphological evidence of close association of flagella of *C. jejuni* with HeLa cells during the process of adherence. Flagellate strains were able to colonize the gut of infant mice more efficiently than the nonflagellate mutants indicating the possible involvement of flagella in this process (Morooka *et al*, 1983). Both flagella and LPS were identified as potential adhesins of *C. jejuni* by McSweegan and Walker (1986). Although purified flagella showed specific binding to INT 407 cells, treatment of *C. jejuni* strains with proteases or glutaraldehyde or mechanical removal of flagella reduced but did not inhibit adherence, indicating flagella were not solely responsible for adherence. Tritiated LPS also specifically bound to INT 407 cells, which was inhibited by prior periodate oxidation. Moreover preincubation of INT 407 cells with LPS inhibited subsequent binding of a *C. jejuni* strain in a concentration dependent manner which showed that LPS could be a second adhesin. Cinco *et al* (1984) also found

that the adherence determinant of *C. jejuni* to INT 407 cell monolayer was heat-stable, presumably LPS. The adherence of LPS of *C. jejuni* to INT 407 cells was fucose-sensitive (McSweegan and Walker, 1986; Cinco *et al*, 1984), whereas the adherence of flagella was fucose-resistant (McSweegan and Walker, 1986). Various other sugars such as glucose, galactose, mannose, N-acetylglucosamine and N-acetylgalactosamine were able to partially inhibit the adherence of *C. jejuni* to epithelial cells in *in vitro* studies (Newell and Pearson, 1984).

McSweegan *et al* (1987) reported that no improvement was observed in the adherence of a *C. jejuni* strain in the presence of lavage fluid absorbed with purified flagella of the same strain. Moreover, monoclonal antibodies against purified flagella failed to inhibit colonization of the infant mouse intestine by homologous strains (Newell, 1986). These studies suggest the involvement of nonflagellar surface components in the adherence and colonization process. A combination of microscopic techniques was used by Lee *et al* (1986) to show that *C. jejuni* associates with the intestinal tissue by mucus colonization and concluded that possession of any specific adhesin is unlikely to be a significant determinant of pathogenicity of *C. jejuni* strains which is yet to be confirmed by other investigators.

The initiation of infections by bacteria that attack the host at mucosal surface is mediated by molecular recognition between ligand molecules or adhesins on the surface of the susceptible host. A series of examples of such specific interactions between bacteria and host surfaces have been reported previously (Beachey, 1981). The identification and characterization of such biomolecules of *C. jejuni* will contribute to the understanding of the process of adherence and its role in the pathogenesis of *C. jejuni* enteritis.

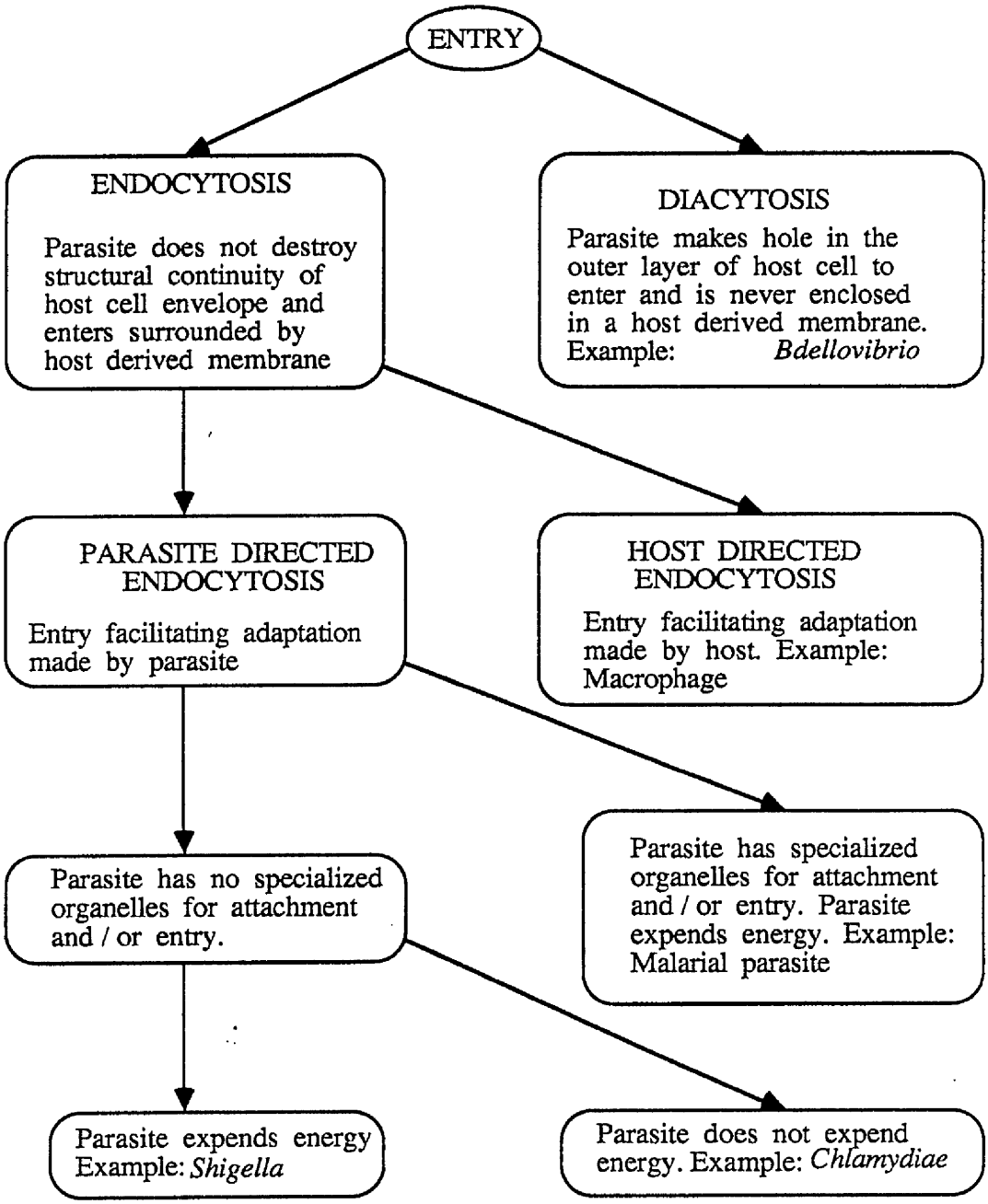
1.7.2 INVASION

The term "invasion" implies forced entry. The transepithelial transport process by which invasion of the host by the pathogenic microorganisms occurs appears to be

partially dependent on microbial factors and is designated as "parasite directed endocytosis" to distinguish it from "host directed endocytosis" by cells such as macrophages that eventually destroy the parasite (McGee *et al,* 1988) (Flow Diagram 1).

The molecular events that lead to invasion of the host cell which is a specialized strategy for virulence of a number of pathogenic microorganisms (Moulder, 1985) is only beginning to be understood. The relative role of both the invading pathogen and the host cells in the process of parasite-directed endocytosis were described by Falkow *et al* (1987) by examining the entrance of bacterial cells into non-professional host cells (cultured epithelial cells) *in vitro.* According to them, the eucaryotic cells offers entry rather than being forced, although the term invasion is used to describe the process. But the critical requirement is that the invading bacterium should possess a structural component on its surface that interacts with the host cell receptor. This fundamental difference between an invasive and noninvasive bacterium enables it to induce its own endocytosis by the epithelial cells. This is exhibited by the rapid and massive entrance of an invasive pathogen such as *S. flexneri* (Sansonetti *et al,* 1986) and *Y. pseudotuberculosis* (Isberg *et al,* 1987) in comparison to their isogenic noninvasive mutants or *E. coli* HB 101. As many as 40 % of the inoculated invasive bacteria can invade an epithelial cell monolayer compared to < 0.01 % with *E. coli* HB 101 (Falkow *et al,* 1987). The presence of a specific receptor on the host cell is exemplified by the fact that invasive bacteria exhibit host cell specificity. Miller and Falkow (1988) cloned two *Y. enterocolitica* chromosomal loci, *inv* and *ail* into *E. coli* HB101. The *inv* locus conferred an invasive phenotype on the *E. coli* allowing uniform high levels of invasion in several tissue culture cell lines. The second locus *ail* showed marked host cell specificity by allowing variable degrees of invasion of HEp-2, HEC1B and CHO cells but no invasion of

Flow Diagram 1: Entry of Pathogens into Host Cells



Adapted from Moulder (1985)

Madin Derby Kidney cells.

The invasive bacteria participate in an active manner during entry into host cells. Inactivation of the invasive organisms by UV irradiation or by mild heat correlated with reduced or no invasion of the epithelial cells. Treatment with antibiotics also resulted in decreased invasiveness demonstrating that metabolically active bacteria are required for entry into host cells (Hale *et al,* 1979; Barrow and Lovell, 1989; Andrade *et al,* 1989). Various compounds which interfere with the uptake of particulate matter by phagocytic cells cytochalasin B, dibutyryl cAMP, cholera toxin, iodoacetate and dinitrophenol also interfered with the entry of invading pathogen into epithelial cells (Hale and Bonventre, 1979; Barrow and Lovell, 1989; Andrade *et al,* 1989). The observations implied that an event analogous to phagocytosis is involved in the internalization of invasive microorganisms by cultured mammalian cells.

The genetic basis of the invasive phenotype is beginning to be understood. In *Shigella* spp. the invasiveness for epithelial cells is determined by a 120-140 M dal virulence plasmid. Strains lacking this plasmid fail to enter HeLa cells and gain this ability when the plasmid concerned is introduced into plasmid-free, avirulent variants (Kopecko *et al,* 1980; Sansonetti *et al,* 1981; 1982; Hale *et al,* 1983). The invasive plasmids differ in size in *Shigella* spp. but they share a high degree of nucleotide sequence homology (Boileau *et al,* 1984). Although the invasion of HeLa cell phenotype is determined by the invasive plasmid, the participation of chromosomal genes is also required for complete expression of virulence i.e. to cause keratoconjunctivitis in guinea-pigs, to invade rabbit ileal mucosa and to induce fluid secretion in the ligated intestinal loops. Sansonetti *et al* (1983) conjugally transferred the invasive plasmid and these three chromosomal loci from *S. flexneri* to *E. coli* K-12, which subsequently expressed all the virulence characteristics of *Shigella.* Hale *et al* (1985) demonstrated that the 140 M dal invasive plasmid of *S. flexneri* encode the

synthesis of several outer membrane proteins in anucleate minicells and postulated that these proteins probably act as functional determinants for the invasive phenotype.

The elegant studies of Falkow and coworkers dissected the invasion process by gene-cloning techniques and showed that a single 3.2 kb chromosomal locus (*inv*) of *Y. pseudotuberculosis* was necessary for its efficient entry into HEp-2 cell monolayer (Isberg and Falkow, 1985). Later, the *inv* gene locus was found to encode a 103 K dal outer membrane protein 'invasin'; the expression of which in *E. coli* HB 101 was sufficient to allow this normally non-invasive bacterium to invade tissue culture cells (Isberg *et al,* 1987). It has been mentioned earlier in this review that in addition to *inv,* another chromosomal locus *ail* of *Y. enterocolitica* was also able to confer invasive phenotype in *E. coli* HB 101. The *ail* gene product was found to be a 15 K dal protein, whose role in the invasion process is yet to be determined (Miller and Falkow, 1988).

Enteroinvasive *E. coli* (EIEC) can also invade cultured epithelial cells like *Shigella* spp; the invasive phenotype is determined by a 140 M dal plasmid (Small *et al,* 1987). Unlike *Yersinia* spp. the genetic regulation of the invasion process in EIEC is complex and at least 70 kb of genetic material scattered in several different locales on the invasive plasmid is required (Falkow *et al,* 1987).

1.7.2.1 Invasiveness of *C. jejuni*: Clinical features of diarrhoea caused by certain strains of *C. jejuni* resemble those of other invasive enteropathogens such as *Shigella* spp. *Salmonella* spp and enteroinvasive *E. coli* (EIEC). These include abdominal pain with bloody-mucoid diarrhoea and the presence of leucocytes in the stool. Bacteraemia and cellular infiltration in biopsy specimens from the colon and small intestine were also noted (Guerrant *et al,* 1978; Blaser *et al,* 1979a; Lambert *et al,* 1979; Blaser and Reller, 1981). These findings led

researchers to investigate the invasive nature of *C. jejuni.*

Although the Sereny test (keratoconjunctivitis in guinea-pigs eyes) (Sereny, 1938), the classical test for determining the invasiveness of enteropathogens, was found to be uniformly negative for *C. jejuni* (Guerrant *et al,* 1978; Manninen *et al,* 1982), cumulative evidence from a variety of *in vitro* and *in vivo* studies indicate that a certain percentage of *C. jejuni* strains possess invasive capability. Van Spreeuwel *et al* (1985) examined colonic biopsy specimens of 22 patients with colitis and positive stool culture for *C. jejuni* using histological, immunological and ultrastructural techniques and observed direct invasion of colonic mucosa by *C. jejuni* in the cytoplasm. Similar findings were also reported by Duffy *et al* (1980) and Blaser *et al* (1980c).

Studies with experimentally infected animals have provided additional evidence for the invasive capacity of *C. jejuni.* Ruiz-Palacios *et al* (1981) observed organisms within the phagocytic cells in the lamina propria of 3-day-old orally infected chicks at 24 hr post-inoculation. Infiltration of gastric epithelial cells of newly-hatched chicks was demonstrated by electron microscopy; intact *C. jejuni* were also observed in the lamina propria both extracellularly and within membrane bound inclusions of lamina cells (Welkos, 1984). In calves, invasion of damaged small intestinal mucosal epithelium by *C. jejuni* was observed in silver-stained sections at day 5 post-inoculation (Al-Mashat and Taylor, 1980a; 1980b). Butzler and Skirrow (1979) demonstrated by electron microscopy the invasion of caecal epithelia of 8-day-old chickens at day 7 post-inoculation by related campylobacters (*C. jejuni / coli*). Invasion of epithelial cells, resulting in damage and destruction of the superficial luminal part of the gut mucosa was also noted by Sanyal *et al* (1984a) in infant chicks infected orally by *C. jejuni.* Electron microscopic observation of irradiated mouse jejunum both *in vitro* and *in vivo* assays revealed definitive evidence of invasion; free organisms in the epithelial cytoplasm not enclosed in vacuoles were seen

(Sosula *et al,* 1988). Intraepithelial invasion was also observed in hamsters (Humphrey *et al,* 1985).

Bacterial translocation is defined as the passage of viable bacteria from the gastrointestinal tract through the lamina propria to the mesenteric lymph nodes and other internal organs (Berg and Garlington, 1979; Berg and Owens, 1979). *C. jejuni* bacteraemia noted by various researchers cannot be solely attributed to translocation because studies with other microorganisms have revealed that the incidence of this process is higher in gnotobiotic animals compared to the conventional animals and is inhibited by normal gut flora (Berg and Garlington, 1979; Berg, 1980). Other factors facilitating translocation includes antibiotic treatment (Berg, 1981), immunosuppressive measures or physical disruption of the mucosal barrier (Wells *et al,* 1988). As none of these factors were operative in the bacteraemia due to *C. jejuni* noted by various researchers (Blaser *et al,* 1984a; Spelman *et al,* 1986) translocation cannot be considered as the only determining factor in the reported cases; involvement of certain properties related to invasiveness may be implicated. Recently, by examining the Peyer's patches in the ligated adult rabbit ileal loops inoculated with live suspensions of *C. jejuni* by electron microscopy at different time intervals, Walker *et al* (1988) provided evidence that the M cells in the Peyer's patches may facilitate transport of the pathogen from the intestine. The authors speculated that the rapid uptake of bacteria could lead to transient bacteraemia by overwhelming the normal clearance mechanism when challenged with large numbers of organisms. However, an invasive enteropathogen such as *S. typhi* (Kohbata *et al,* 1986) and noninvasive cholera vibrios (Owen *et al,* 1986) are also transported by M cells, it is not clear whether this process is associated with the enteroinvasive properties of these organisms.

The clinical significance of transient bacteraemia in the *C. jejuni* enteritis is uncertain. There are several reports of bacteraemia in human beings (Skirrow,

1984; Mandal *et al,* 1984); but most of these are associated with systemic infections with immunocompromised individuals. The bacteraemia occasionally seen in the previously healthy individuals with *C. jejuni* enteritis (Spelman *et al,* 1986) may be related to the invasive capability of this organism. Mandal *et al* (1984) suggested that migration to blood is part of the pathogenesis of the early stages of the disease and pointed out that the reason for the scarcity of reports on human bacteraemia due to *C. jejuni* was: first, the unlikelihood of growing campylobacters by routine blood culture techniques and secondly, the rarity with which blood from patients with enteritis, even when febrile, is sent for culture. Black *et al* (1988) however, failed to detect *C. jejuni* in the blood of human volunteers after intensive surveillance.

1.7.3 OUTER MEMBRANE PROTEINS

The outer membrane protein (OMP) profile of *C. jejuni* consists of 8-10 proteins with a single major band when these are extracted by sarkosyl and visualized by SDS-PAGE (Logan and Trust, 1982; Blaser *et al,* 1983b; Mills and Bradbury, 1984). Another prominent protein band (ca. 62 k dal) represents the flagellin subunit of the *C. jejuni* flagellum (Logan and Trust, 1983; Newell *et al,*1984). The molecular weight and relative concentration of other protein bands were found to vary slightly from strain to strain (Logan and Trust, 1982).

The major outer membrane protein (MOMP) is transmembrane and peptidoglycan associated (Blaser *et al,* 1983b). It is heat-modifiable (Logan and Trust, 1982; Trust and Logan, 1984), and possesses porin activity (Huyer *et al,* 1986) and constitutes about 50-70% of total OMP concentration (Trust and Logan, 1984, Blaser *et al,* 1983b). By two dimensional gel electrophoresis using isoelectric focusing and SDS-PAGE, Dunn *et al* (1987) showed that the MOMP of *C. jejuni* strains consisted of a single isomeric form, whereas the flagella showed considerable charge heterogeneity among strains. The OMP profiles of *C. jejuni* strains were reported to be stable to a variety of growth conditions such as media composition, incubation period and also to the

presence and absence of a tetracycline resistance plasmid. Incubation temperature affected the protein concentration; incubation at 42° C resulted in significantly higher concentrations of OMP compared to incubation at 37° C for the same period. A *C. jejuni* strain had the same OMP profile when compared with the same strain passaged 10 times in laboratory medium. Strains from a common source outbreak had similar OMP profiles (Blaser *et al*, 1983b).

The prediction of Logan and Trust (1982) that the heat-modifiable MOMP of *C. jejuni* could be a porin protein was later proved to be correct (Huyer *et al*, 1986; Page *et al*, 1989). Reconstitution of purified MOMP into lipid bilayer membranes resulted in formation of small channels which preferentially selected for cations. Unlike most other Gram-negative bacteria, *C. jejuni* has only one porin protein and the channels formed are relatively smaller in comparison to other bacteria (Huyer *et al*, 1986). Field *et al* (1986b) reported that the OMP profile of some *C. jejuni* strains changed in response to iron starvation. Three new proteins, ca. 82, 76 and 74 K dal were synthesized during growth in EDDA induced iron-limited medium and one of the 76 K dal or 74 K dal proteins were surface-exposed.

By immunoblotting, radioimmunoprecipitation and immunoautoradiography, several OMP's (15 K dal to 91 K dal) were found to be immunogenic in homologously and heterologously immunized rabbits and infected human beings (Mills and Bradbury, 1984; Blaser *et al*, 1984b; Walker *et al*, 1986). MOMP was found to be most immunogenic and both *C. jejuni* infected and healthy human sera showed an IgA, IgM and IgG response to this protein but infected human sera exhibited a significantly greater response than the control sera (Blaser *et al* 1984b; Blaser *et al*, 1985a). However, flagellin was found to be most immunogenic in other studies (Logan and Trust, 1983; Wenman *et al*, 1985).

An acid glycine extract (pH 2.2) of *C. jejuni* envelopes yields a 31 K dal protein which is absent in the OMP profile prepared by sarkosyl extraction (Logan and Trust, 1983). This protein is surface-exposed, strongly immunogenic and is present

most prominent protein of the outer membrane, flagellin is the immunodominant one as it has the highest ratio of antigenicity to molar representation (Wenman *et al,* 1985). Moreover the flagella of *C. jejuni* were found to possess shared antigens with some unidentified Gram-negative spiral bacteria that colonize the mucus of the rodents (Lee *et al,* 1987). However, no such antigenic cross-reaction was observed with flagellins of *V. cholerae, A. hydrophila, E. coli, S. typhimurium, P. vulgaris,* or *Pseudomonas fluorescens* (Lee *et al,* 1987).

Flagella comprise the major cross-reactive surface antigens in naturally acquired infection in humans and have been proposed as a possible vaccine candidate (Newell, 1983; Wenman *et al,* 1985). The role of flagella in providing protection against colonization upon subsequent challenge has been the subject of several investigations. Intraperitoneal vaccination of dams (mice), before mating, with vaccine prepared by heating *C. jejuni* cells at 62^{o} C resulted in protection of 90 % of the natural and 50 % of the fostered infants when challenged with the homologous strain by the oral route; heat-killed vaccine (boiled at 100^{o}C) did not have any protective effect (Dolby and Newell, 1986; Abimiku and Dolby, 1987). Vaccines given orally were not effective and the degree of protection achieved by intraperitoneal injection varied from strain to strain. Boiled vaccine though non-protective was found to elicit a strong bactericidal antibody response (Abimiku *et al,* 1989). In another study, Abimiku and Dolby (1988) demonstrated the role of heat-labile serotyping (Lior scheme) in providing cross-protection of infant mice. Dams were vaccinated, before mating, with live and heat-killed vaccines and the infants were challenged with homologous and heterologous strains belonging to the Lior typing scheme (Lior *et al,* 1983) in which the flagellum is the determinant antigen (Wenman *et al,* 1985) and the Penner typing scheme (Penner and Hennessy, 1980) which is determined by LPS (Mills *et al,* 1985). In homologous challenge 85 % of the strains were protected and in heterologous challenge (with another strain belonging to the same Lior serotype) 57 % of the mice were protected. On the other hand, none of the infant mice were protected by vaccinated

dams against strains with the matched Penner serotyping antigen (heat-stable antigen) which probably indicate that the LPS of the matched Penner strains may not be as similar as the typing scheme seems to suggest.

1.7.4.1 Possible Involvement in Virulence: The flagella of *C. jejuni* have been identified as probable adhesins for eucaryotic cells and intestinal mucous gel; aflagellate variants exhibited reduced adherence in comparison to the flagellate cells (Newell *et al*, 1984; McSweegan and Walker, 1986). Interestingly, both the research groups observed that flagellate but nonmotile (F^+M^-) variants (flagella were immobilized by KCN treatment) had better adherence capacity than both the flagellate motile (F^+M^+) and aflagellate nonmotile (F^-M^-) variants. To explain this phenomenon McSweegan and Walker (1986) hypothesized that flagella are important in facilitating attachment to epithelial cells, but once the adherence is achieved, motile flagella may impede maximal, long-term adherence. Contradictory evidences also exist in the literature as to the role of flagella in the virulence of *C. jejuni*. No significant difference in the incidence and severity of disease was observed in the removable intestinal tie adult rabbit diarrhoea (RITARD) model challenged with flagellate and aflagellate variants of the same strain (Caldwell *et al*, 1985). Also no difference in lethality was noted among strains with or without flagella and aflagellate variants in lethality in 11-day-old chicken embryo model (Field *et al*, 1986a). It is evident that further studies are needed to establish the role of flagella in the pathogenesis of *C. jejuni* enteritis and it is also important to determine the contribution of the recently described phase variation (Caldwell *et al*, 1985) and antigenic variation which was demonstrated *in vitro* (Harris *et al*, 1987; Logan *et al*, 1987) and also *in vivo* (Logan *et al*, 1989) of *C. jejuni* flagella in the disease process.

1.7.5 LIPOPOLYSACCHARIDE (LPS)

The LPS of *C. jejuni* has been the subject of investigations in which the general nature,

chemistry and biological effects of endotoxin have been investigated. The *C. jejuni* LPS profile as analyzed by SDS-PAGE and silver staining showed the presence of low molecular weight (4.5-5.0 K dal) rough-type LPS consisting of Lipid A, core oligosaccharide and devoid of side-chains (Logan and Trust, 1984; Perez-Perez and Blaser, 1985; Perez-Perez *et al,* 1985). However, some *C. jejuni* strains were found to contain small O side-chains in addition to Lipid A and core oligosaccharide (Naess and Hofstad, 1984a; 1984b). This observation was later confirmed by Preston and Penner (1987), who reported that all *C. jejuni* strains possess low molecular weight LPS readily detected by silver staining and some have high molecular weight LPS containing O side- chains which can be visualized by immunoblotting.

Aqueous phenol extracted LPS was found to contain glucose, galactose, L-glycero-D-manno-heptose and glucosamine and occasionally galactosamine. The fatty acids present were mainly 3-hydroxy-tetetradecanoic acid and n-hexadecanoic acid (Naess and Hofstad, 1982). The Lipid A had a high content of fatty acid relative to neutral sugar (Naess and Hofstad, 1984a) and 2-keto-3-deoxyoctanate (KDO) is present as a high proportion of the total sugar (Perez-Perez *et al,* 1985). Chemical analysis of partially hydrolyzed LPS showed that like enterobacterial LPS, the Lipid A is connected to the polysaccharide part by KDO and various strains showed only minor differences in qualitative sugar composition (Naess and Hofstad, 1984a) .

There are at least 42 serovars of *C. jejuni* based upon heat-stable antigens which were determined by LPS (Penner and Hennessy, 1980; Penner *et al,* 1983; Mills *et al,* 1985). This is surprisingly high for an organism like *C. jejuni* which has a low molecular weight LPS. Other bacteria which have a low molecular weight LPS such as *Bordetella* spp. *Neisseria gonorrhoeae* and *Haemophilus influenzae* confer only a few serovars (Diena *et al,*1978; Inzana, 1983; Peppler, 1984). LPS from different heat-stable serovars acts as a strain-specific antigen (Logan and Trust, 1984; Preston and Penner, 1987). But Perez-Perez *et al* (1985) observed that LPS cross-reacts with antisera raised against different strains. Some degree of intra- and inter-species cross-reactivity was noted by Naess and Hofstad (1985) with heterologous

antisera against LPS. Using immunoblotting technique, Perez-Perez *et al* (1986) showed that the LPS of *Campylobacter* strains share Lipid A antigenic determinants with the core region of LPS of several gram-negative organisms such as *E. coli, V. cholerae, S. typhi, Salmonella minnesota, Pseudomonas aeruginosa* and *C. fetus*. The authors hypothesised that the presence of cross-reactive LPS structure might explain the presence of bactericidal antibodies to *C. jejuni* in normal human serum.

1.7.5.1 Possible Involvement in Virulence: The role of *C. jejuni* LPS in its virulence was investigated by several investigators. Stewart-Tull *et al* (1984) reported that heat-killed *C. jejuni* caused death of 7 day-old-mice in 24 hr and described it as an endotoxin-mediated lethality. In the adult mice however, no deaths were recorded when heat-killed *C. jejuni* or 100 ug of LPS was injected, but symptoms of endotoxaemia (ruffled fur, inactivity, shaking, tearing and hypothermia) was observed (Stanfield *et al*, 1987). The *C. jejuni* LPS exhibit typical properties of endotoxin; it is pyrogenic, antigenic and toxic for mice but its activity was 4 to 8 times lower than that of *E. coli* (Fumarola *et al*, 1985; Naess and Hofstad, 1984b). As with the LPS of other Gram-negative bacteria (Coid, 1976), *C.jejuni* LPS caused resorption of embryos and impaired foetal development in mice when administered on day 13 of pregnancy (O'Sullivan *et al*, 1988a, 1988b). The toxic nature of *C. jejuni* LPS was also investigated by Fumarola *et al* (1985) in an infant mouse model. Bar (1988) observed that in 4-6 weeks old mice, which were depleted of macrophages by injecting silica dust, heat-killed *C. jejuni* was significantly less lethal than an equal number of live cells. Moreover, C3 H/HeJ mice, which are genetically resistant to the action of LPS, showed no increase in resistance to the experimental *Campylobacter* infection (Bar, 1988). The role of *C. jejuni* LPS in mouse deaths thus appears to be complex and probably involves additional factors.

C. jejuni LPS has been implicated as an adhesin for eukaryotic cells (Cinco *et al*, 1984; McSweegan and Walker, 1986). Cinco *et al* (1984) observed that the

and hence has not been thoroughly characterized. The role of these toxins as virulence factors and their interplay with other putative virulence factors controlling adherence and invasion in the pathogenesis of *C. jejuni* enteritis is yet to be determined.

> * Enterotoxins are classified as cytotoxic or cytotonic on the basis of their action on tissue culture cells. Cytotoxic enterotoxins are those enterotoxins which exert cytotoxic effects on the cell monolayer which is characterized by detachment of the cell monolayers from the surface due to cell death; for example: *S. dysenteriae* enterotoxin and *C. perfringens* enterotoxin. Cytotonic enterotoxins are those enterotoxins which can increase intracellular levels of cAMP or induce metabolic changes resulting in morphological changes but with no cellular damage; for example, enterotoxins of *V. cholerae* and enterotoxigenic *E. coli* on the cell monolayer (Keusch and Donta, 1975; Robertson, 1988).

1.7.6.1 Enterotoxin: Ruiz-Palacios *et al* (1983) first reported that some strains of *C. jejuni* produced a heat-labile enterotoxin (CJT) which was immunologically cross-reactive with cholera toxin (CT) and heat-labile enterotoxin of *E. coli* (LT). This toxin (CJT) induced fluid secretion in the ligated illeal loops of rats and caused cytotonic responses in the Chinese hamster ovary (CHO) cells. Later, the production of cytotonic enterotoxin by certain *C. jejuni* strains was reported by various researchers and its properties were investigated.

1.7.6.1.1 Factors Influencing Enterotoxin Production: Various rich growth media such as Brucella broth, Mueller-Hinton broth, brain-heart infusion broth or GC (gonococcus) broth were used for the production of toxin (Ruiz-Palacios *et al,* 1983; McCardell *et al,*1984; Klipstein and Engert, 1984). Biphasic medium using tissue culture medium 199 (M 199) with Mueller-Hinton agar as the solid phase was also used (Johnson and Lior, 1986). The toxin titres cannot be compared directly as different assays were used to quantitate them.

Supplementation of Brucella broth with 0.25 % (w/v) of each of L-asparagine, L-serine and L-cystine (Ruiz-Palacios *et al,* 1983) or addition of vitamin supplement (IsovitaleX, 1 % w/v) to GC broth (Klipstein and Engert, 1984) and to Brucella broth (Johnson and Lior, 1986) enhanced enterotoxin production. This increase in toxin production does not occur with all toxigenic isolates of *C. jejuni*, which may explain why some investigators have not observed this stimulation in CJT production in amino acid supplemented (Shaha *et al,* 1988) or IsovitaleX supplemented medium (Johnson and Lior, 1986). Goossens *et al* (1985a) developed an improved medium for the production of CJT by supplementing Brucella broth with 0.25 % (w/v) L-asparagine, L-serine, L-glutamic acid; 0.05 % (w/v) L-cystine. 0.1 % corn starch, 0.5 % (w/v) yeast extract and 0.48 % (w/v) dextrose; the amount of toxin produced in this medium was twice that produced in the medium used by Ruiz-Palacios *et al* (1983) and Klipstein and Engert (1984). McCardell *et al* (1986a) reported that the production of enterotoxin by *C. jejuni* strains was influenced by the iron concentration of the growth medium. Elevated levels of iron increased the production of the toxin, even some of the toxin-negative strains started producing toxin. On the other hand, some of the toxin producing strains reverted to toxin negativity when grown in iron-depleted medium.

1.7.6.1.2 Treatment of *C. jejuni* cells to Stimulate Release of Toxin: Treatment of the cells with polymyxin B increased the titre of the CJT, indicating that the toxin probably accumulated in the periplasmic space after synthesis, like LT, and is not secreted actively (Ruiz-Palacios *et al,* 1983, Klipstein and Engert, 1984). Johnson and Lior (1986) reported that polymyxin B treatment (2 mg ml^{-1}, 15 min at $37^{o}C$) of washed cell suspension resulted in a 2 to 6-fold increase in the titre of CJT in the CHO cell assay. In contrast, McCardell *et al* (1984) noted that treatment of the cells with polymyxin B or

sonication resulted in a 66 % decrease in the toxin titre and they hypothesized that concomitant release of a protease by these treatments might be responsible for the observed decrease in toxin titre. To date, the presence of such a protease has not been documented; examination of *C. jejuni* strains for IgG, IgM and IgA1 protease yielded negative results (Goossens *et al,* 1985b). Many researchers however, used culture filtrates as the source of toxin without sonication or polymyxin B treatment (Goossens *et al,* 1985; Shaha *et al,* 1988)

1.7.6.1.3 Purification of enterotoxin: There are two reports of partial purification of CJT. Klipstein and Engert (1984) partially purified CJT by utilizing its affinity for the galactose residue of the ganglioside GM_1. This purification procedure resulted in a 2300-fold increase in specific activity as determined by the CHO cell assay and ELISA. The final product was however not homogeneous as judged by SDS-PAGE. McCardell *et al* (1984) reported purification of CJT by anti-CT affinity chromatography. The toxin eluted as a single peak with Tris buffer (pH 10.5) and produced a single band on SDS-PAGE.

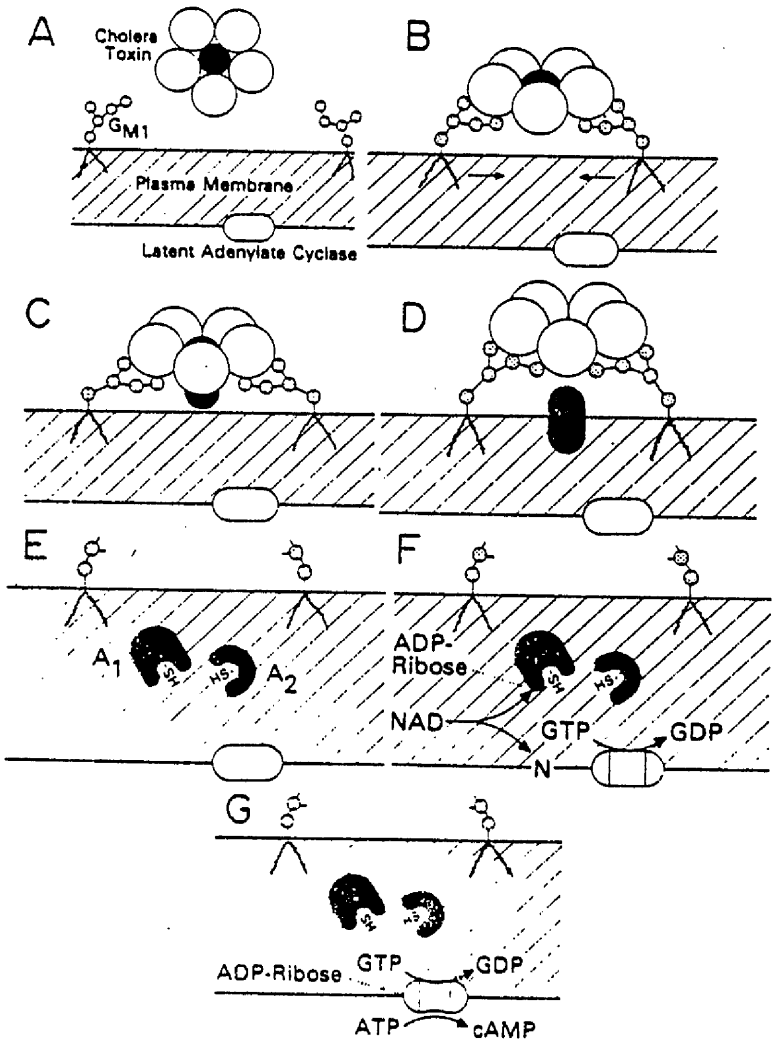
1.7.6.1.4 Physicochemical Properties: Ruiz-Palacios *et al* (1983) suggested that CJT has a molecular weight between $1x10^4$ and $1x10^5$ as judged by the passage of the toxin through ultrafiltration membranes of different molecular weight cut-offs. The CJT purified by McCardell *et al* (1984) was found to have a molecular weight of 60-70 K as determined by SDS-PAGE; but how this single band relates to the possible A5B structure of the CJT is not clear. In pH stability studies, the crude toxin partially lost its activity at pH 4.0 and was completely inactivated at pH 2.0 and 8.0. Storage at 4^{o} C for 1 month or for 1 week at -20^{o} C or -70^{o} C resulted in substantial loss of activity (Ruiz-Palacis *et al,* 1983) possibly due to irreversible aggregation. Conflicting observations were reported with regard to the heat-stability of the CJT which

probably reflected the range of growth conditions and test systems used. It was completely inactivated at 56^{o} C for one hr (Ruiz-Palacios *et al,* 1983) or at 90^{o} C for 10 min (Klipstein and Engert, 1984), although McCardell *et al* (1984) reported that CJT was stable at 100^{o} C for 10 min. Johnson and Lior (1986) reported only a 4-fold decrease in the toxin titre when heated at 70^{o}C for 30 min.

1.7.6.1.5 Biological Properties: The biological properties of CJT is in many respects similar to the cholera toxin (CT) and the heat-labile toxin of *E. coli* (LT). Both CT and LT stimulate adenylate cyclase, resulting in an increase in the intracellular cyclic AMP (Figure 2). In turn, this change results in an accumulation of fluid which is observed in the ligated ileal loops of rabbits and other animals. Concentrated CJT induces fluid accumulation in the ligated ileal loops of rabbits (McCardell *et al,* 1984) and rats (Ruiz-Palacios *et al,* 1983; Klipstein *et al,* 1985; Shaha *et al,* 1988) and evidence is accumulating that CJT may also induce secretory diarrhoea by stimulating the adenylate cyclase system in the intestinal mucosa (Ruiz-Palacios *et al,* 1983; Johnson and Lior, 1986). Like CT and LT, CJT induces a cytotonic response in CHO and Y-1 mouse adrenal cells which can be inhibited by preincubation with GM_1 ganglioside (Ruiz-Palacios *et al,* 1983; McCardell *et al,* 1984). The level of cAMP increased by CJT in CHO cells were comparable to those elevated by CT and LT as determined by radioimmunoassay (Ruiz-Palacios *et al,* 1983). Additionally, CJT could increase permeability in the rabbit skin test like CT and LT (McCardell *et al,* 1984).

Klipstein and Engert (1984) were able to separate the B subunit of CJT by the application of gel filtration in the presence of guanidine to the semipurified preparation of CJT. The B subunit thus obtained was devoid of the holotoxin's cytotonic activity in the CHO cells and secretory activity in the ligated ileal loops of rats was capable of binding to GM_1 ganglioside in the ELISA. These results suggested that CJT may also have a A5B structure like CT and LT, although it

Figure 2: Model of the Mechanism of Action of Cholera Toxin



A. Initial situation

B. Multivalent binding of 𝔹 component of the toxin to accessible oligosaccharide chains of GM_1 ganglioside.

C. Conformational change in the toxin structure that allows hydrophobic regions in 𝔸 component to interect with membrane.

D. Dissociation of toxin components and entry of 𝔸 into fluid lipid bilayer membrane.

E. Penetration of 𝔸 component into membrane and reduction of disulphide bond to generate active $\mathbb{A}_1$ peptide.

F. Cleavage of nicotinamide adenine dinucleotide (NAD) to nicotinamide (N) and ADP- ribose by $\mathbb{A}_1$ peptide of toxin.

G. Transfer of ADP-ribose to adenylate cyclase complex and its activation by inhibition of GTPase

From Fishman (1980).

awaits investigation.

1.7.6.1.6 Immunological Properties: *C. jejuni* enterotoxin (CJT) is a recent addition to the growing list of bacterial protein toxins which are structurally and immunobiologically related to the prototype cholera toxin (CT) (Table 4). Immunological cross-reactivity between CJT with CT and LT has been demonstrated in diverse test systems. Partially purified CJT shows lines of partial identity with CT and LT in gel immunodiffusion tests (McCardell *et al,* 1984, Walker *et al,* 1986). Preincubation of CJT with antisera to CT or LT inhibits cytotonic responses in CHO cells (elongation) and Y-1 mouse adrenal cells (rounding) (Ruiz-Palacios *et al,* 1983; Goossens *et al,* 1985a). Neutralization of CJT rat ileal loop response and elevation of cAMP in CHO cells was demonstrated by cross-reaction with cholera antitoxin (Ruiz-Palacios *et al,* 1983; Klipstein and Engert, 1984; McCardell *et al,* 1984).

Lines of partial identity were also observed between the B subunit of CJT and those of CT and LT (Klipstein and Engert, 1984). Based upon the immunological similarity of CJT with CT and LT, varied forms of ELISA's have been developed (McCardell *et al,* 1984; Klipstein and Engert, 1984: Walker *et al,* 1986). However, ELISA with antisera to the B subunit of LT and CT showed that CJT B subunit was immunologically more closely related to LT B subunit than to CT B subunit (Klipstein and Engert, 1984; 1985). Rats immunized with LT B subunit were significantly protected against challenge with semipurified CJT holotoxin or a viable enterotoxigenic strain of *C. jejuni* (Klipstein *et al,* 1985).

Despite the immunological similarities among CJT, CT and LT, no nucleic acid homology was noted between the chromosome of *C. jejuni* with CT or LT gene probes by several investigators (Olsvik *et al,* 1984; Walker *et al,* 1986). This was not unprecedented as *S. typhimurium* enterotoxin and *Aeromonas hydrophila* enterotoxin do not have nucleic acid homology with CT gene probes although both of these toxins are reported to exhibit immunological cross-reactivity

Table 4: The Family of Cholera Toxin Related Diarrhoeagenic Protein Toxins Produced by Enteric Bacteria.

Toxin	References
Cholera Toxin-1	Finkelstein and Lospalluto (1969)
Cholera Toxin-2	Finkelstein *et al* (1974)
Cholera Toxin-3	Lockman and Kaper (1983)
Cholera Toxin-4	Mekalanos *et al* (1983)
Non 01 *V.cholerae* enterotoxin	Yamamoto *et al* (1983)
V.mimicus enterotoxin	Spira and Fedorka-Cray (1984)
E.coli LT_{h-1} (Strain from human origin)	Geary *et al* (1982)
E.coli LT_{h-2}	Yamamoto and Yakota (1983)
E.coli LT_{p} (Strain from porcine origin)	Clements and Finlkelstein (1979)
Salmonella typhimurium enterotoxin	Finkelstein *et al* (1983)
Aeromonas hydrophila enterotoxin	Potomski *et al* (1987)
Klebsiella pneumoniae enterotoxin	Klipstein and Engert (1977)
Enterobacter cloacae enterotoxin	Klipstein and Engert (1977)
Plesiomonas shigelloides enterotoxin	Gardner *et al* (1987)
Campylobacter jejuni enterotoxin	Ruiz-Palacios *et al* (1983)
Campylobacter coli enterotoxin	Martin *et al* (1989)

with CT (Walker *et al,* 1986). Recently, some nucleotide sequence homology was noted between the genes of LT and CJT in the GM_1 ganglioside binding sites (Calva *et al,* 1988c).

The most important criterion that should be met to implicate an *in vitro* produced bacterial toxin like CJT in the pathogenesis of the disease caused by the bacterium is the demonstration that the toxin is produced *in vivo* during the disease process. Detection of antibody to CJT in the sera of patients with *C. jejuni* diarrhoea has been reported in three independent studies (Ruiz-Palacios *et al,* 1985; Honda *et al,* 1986 and Martin *et al,* 1989). A greater than 4-fold rise in antitoxin antibody was detected in 10 of the children with diarrhoea, whereas none of the asymptomatic carriers seroconverted to the toxin (Ruiz-Palacios *et al,* 1985). A study of the immune response to *C. jejuni* and *C. coli* antigens in children from Bangui, Central African Republic showed that a single *Campylobacter* episode resulted in the development of antitoxic immune response in 53.4 % of the children with asymptomatic carriage and 95 % of the children with diarrhoea (Martin *et al,*1989). Honda *et al* (1986) detected antitoxic antibody in 49 % of the patients with diarrhoea. These studies demonstrate that CJT is probably produced in the gut during the disease process which is consistent with the hypothesis that the cytotonic enterotoxin (CJT) is a virulence factor for *C. jejuni.*

1.7.6.2 Cytotoxin

To account for the bloody-mucoid type diarrhoea which is likely to be associated with a tissue destructive process in the involved segment of the gut caused by certain strains of *C. jejuni,* it was conceptually speculated to be due to the production of cytotoxic factors accompanied by invasion by these strains (Blaser and Reller, 1981; Prescott *et al,* 1981). The *in vitro* production of a cytotoxic factor by *C. jejuni* was first reported from Malasia; Wong *et al* (1983) observed that cell free filtrates of *C. jejuni* contained a heat-stable, trypsin-sensitive

factor which caused a progressive and reproducible cytotoxic effect (cell rounding, loss of adherence and cell death) in the cell lines of human origin (HeLa, MRC-5 and HEp-2). No such cytopathic effect were detected in monkey (Vero, MK2 and PMK) and mouse (L929) cell lines.

After the first report, production of cytotoxin (s) by *C. jejuni* strains were reported from various laboratories. However, in many instances, the reports from many laboratories do not correlate with the number and nature of the toxins. These discrepancies may be due in part to the production of enterotoxin by certain strains thus complicating the bioassay used for cytotoxin and partly because *C. jejuni* may produce low levels of these toxins when cultured in broth by conventional methods. The use of different strains, growth conditions and assay methods may also play a contributory part. As the number of cytotoxins produced by *C. jejuni* strains is uncertain and it is not clear which investigator(s) was reporting which toxin, it appeared to be confusing to review this information on a general basis. Therefore, the cytotoxin(s) of *C. jejuni* are discussed individually as reported by various authors and clinically important considerations are related to the pathogenesis and immunity of the enteritis caused by the pathogen.

Pennie *et al* (1984) observed that treatment of a broth culture of *C. jejuni* with polymyxin B resulted in the release of a cytotoxic factor active against HeLa cells. Concentration of the campylobactors 10-fold prior to polymyxin B treatment led to a 10-fold increase in cytotoxin titre. Antiserum to Shiga toxin failed to neutralize the cytopathic effect.

Although initially observed otherwise (Wong *et al,* 1983), nonhuman cell lines were also found to be susceptible to the action of *C.jejuni* cytotoxin. In a survey of 45 *C. jejuni* strains obtained from stool of children with diarrhoea, Johnson and Lior (1984) reported that the majority of strains produced a heat-labile (70^{o} C for 2 hr) cytotoxin detected in Vero cells either singly or

concomitantly with a heat-stable (70^{o} C) cytotonic toxin detectable in CHO cells. Treatment of the *C. jejuni* cells grown in biphasic medium (tissue culture medium 199 as the liquid phase over Mueller-Hinton agar as the solid phase) with polymyxin B increased the yield of the toxin. The toxin was not neutralized by *C. difficile* antitoxin or *E. coli* vero antitoxin (0157:H7) and no correlation was observed between serovars, biovars and toxigenicity of the strains investigated. In a subsequent study, the same authors (Johnson and Lior, 1986) showed that another cytotoxin was also produced by *Campylobacter* spp. detectable in CHO and HeLa cell lines but inactive in Vero cell assay, in addition to the one described earlier (active against Vero cell, Johnson and Lior, 1984).

CHO cells were used by Goossens *et al* (1985a) to assay cytotoxin produced by *C. jejuni* strains grown in biphasic medium. Antiserum to purified Shiga toxin could not prevent the cytopathic effect indicating no immunological similarity between the two toxins. In accordance with the conceived idea that the production of cytotoxin is a characteristic feature of the *C. jejuni* strains isolated from bloody mucoid type of diarrhoea, none of the strains isolated from watery-type diarrhoea included in this study appeared to produce this toxin (Goossens *et al*,1985a)

In a survey of virulence characteristics of *C. jejuni* strains isolated from patients with watery-type secretory diarrhoea, bloody-mucoid diarrhoea and asymptomatic excretors, Klipstein *et al* (1985) found that a cytotoxin detectable in HeLa and Vero cells was produced only by the strains isolated from bloody mucoid stool. However, in a subsequent report surveying larger numbers of strains, no such clear-cut findings were obtained (Klipstein *et al,* 1986).

C. jejuni strains isolated from 12 patients with inflammatory diarrhoea were found to excrete a cytotoxin when the bacterial cells were treated with polymyxin B which was reactive against HeLa and CHO cell lines but not against Vero and WI-38 cell lines (Guerrant *et al,* 1987). The activity of this toxin was not neutralized by the antisera prepared against Shiga-like toxin 1 and 2 and

the cytotoxins of *C. difficile*, *Aeromonas* spp. and *V. cholerae* non-O1 strains. Heating to 60^{o} C reduced the activity of the toxin and trypsin treatment resulted in 70 % loss of activity. The cytotoxin preparations were devoid of any detectable enterotoxigenic activity as determined by the rabbit ileal loop assay.

Pang *et al* (1987) extended their studies with the previously reported cytotoxin by the same research group (Wong *et al*,1983) by performing further *in vivo* and *in vitro* tests. The cytotoxin was found to have a molecular weight of 30 K ~~dal~~ using Sephadex G-50 gel filtration technique. The culture filtrates from 48 % of the human isolates and 26 % of the faecal filtrates from which cytotoxin-producing *C. jejuni* were subsequently obtained showed cytotoxin titres of 8-32 using ^{51}Cr release assay. In the reversible tie adult rabbit diarrhoea (RITARD) model (Caldwell *et al*, 1983) bacteraemia and severe watery mucus containing diarrhoea resulting in death of rabbits was noted when they were inoculated with the cytotoxin-producing strains. On the other hand, non-cytotoxin producing strains caused less severe diarrhoea in rabbits and none died. As partially purified cytotoxin induced diarrhoea for three days and the toxin was detected in the stool filtrates of the patients with diarrhoea, the authors speculated that this toxin might play a central role in *C. jejuni* enteritis.

Yrios and Balish (1986b) reported that no enhancement in the ability to produce a cytotoxin detectable in the CHO cells was observed with a strain of *C. jejuni* which has undergone a 300-day period of adaptation in the mouse intestinal tract. On the other hand, Pang *et al* (1987) noted a rapid loss of cytotoxin production by clinical faecal isolates of *C. jejuni* upon in vitro passage. They also noted that cytotoxin-producing strains were isolated more frequently from various internal organs in comparison to the strains which produced little or no cytotoxin (Yrios and Balish, 1986a).

The term Cytolethal Distending Toxin (CLDT) was coined by Johnson and Lior (1988) to describe a new cytotoxin produced by *Campylobacter* spp. active

against CHO, Vero, HeLa and HEp-2 cells but negative against Y-1 mouse adrenal cells. The CLDT, which was detected in the culture filtrates of the *Campylobacter* spp. caused progressive cell distension leading to cytotoxicity and continuation of the assay for 96 hr was essential for optimal activity of the toxin. Haemorrhagic responses were observed in rat ligated ileal loops of CLDT but was negative in adult rabbit ligated ileal loops, suckling mouse and rabbit skin test. The CLDT was heat-labile, trypsin sensitive and could only be neutralized by homologous rabbit antitoxin. Out of 718 strains of *C. jejuni*, *C. coli*, *C. laridis*, *C. fetus* subsp. *fetus* and catalase-negative or weakly-positive *Campylobacter* isolated from both human and animal sources and originating from many countries investigated, 41 % of the strains produced this toxin. No correlation was observed between the toxigenicity and the serovar or biovar of these strains.

Although several previous studies indicated that *C. jejuni* toxin was not neutralized by the antisera raised against purified Shiga toxin, Moore *et al* (1988) reported that certain strains of *C. jejuni* produced a cell-associated Shiga-like toxin active against HeLa cells. Cytotoxic activity was considered Shiga-like if neutralised by monoclonal antibody to the B subunit of the Shiga-like toxin 1 (SLT-1) of *E. coli* and rabbit anti-Shiga antitoxin. Under low stringency conditions, no hybridization was observed between a cloned SLT-1 gene and restriction enzyme-digested total DNA from a *C. jejuni* strain producing the toxin. By hybridization under high stringency, genes coding for SLT-1 and Shiga-like toxin-2 (SLT-2) were not found in 30 *C. jejuni* strains isolated from children with diarrhoea in Thailand (Seriwatana *et al,* 1988). These studies indicate that the Shiga-like toxin produced by the *C. jejuni* strains are genetically but not immunologically distinct from SLT-1. As to the role of Shiga-like toxin in the pathogenesis of *Campylobacter* enteritis, the authors speculated that it may not be an important virulence factor because of several reasons. These are (1) some *C. jejuni* strains isolated from asymptomatically infected patients produced

infected patients produced this toxin whereas 53 % of the isolates associated with inflammatory diarrhoea did not produce this toxin, (2) the amount of Shiga-like toxin produced is very low in comparison to other enteropathogens producing such toxins, and (3) no rise in neutralizing titre was observed between acute and convalescent sera of patients with *C. jejuni* infection.

The following question arises considering the rather confusing state of the literature regarding the 'Toxins of *C. jejuni*'. Does *C. jejuni* produce a multiplicity of toxins or a multifunctional toxin ? This problem has to be investigated before it will be possible to ascribe any role to the toxin(s) in the pathogenesis of the *C. jejuni* enteritis.

1.7.7 SERUM RESISTANCE

C. jejuni strains are generally serum-sensitive; both the classical and alternative complement pathways have been implicated in killing (Blaser *et al*, 1985b). *C. jejuni* strains isolated from systemic infections in immunocompromised patients were more sensitive to the bactericidal action of normal human serum than the strains isolated from systemic infections in normal hosts (Blaser *et al*, 1986b). The authors put forward the hypothesis that systemic infection in the compromised hosts reflects the opportunistic nature of the pathogen and the strain should be serum-sensitive. On the other hand, to cause an extraintestinal infection in a normal host, the strain must possess increased virulence which is manifested by increased resistance to bactericidal action of normal human serum. Serum susceptibilty inversely correlated with the carbohydrate or ketodeoxyoctonate (KDO) fraction of the cell weight and the hence was possibly related to the length of the LPS side chain (Blaser *et al*,1986b).

1.7.8 INTERACTIONS WITH PHAGOCYTES

Interactions of *C. jejuni* with phagocytic cells was the subject of several investigations attempting to elucidate the role of nonspecific defense mechanisms in the host's resistance to infection. Kiehlbauch *et al* (1985) reported that *C. jejuni* strains were

rapidly phagocytosed by human and murine macrophages using different microscopic techniques. The ingested bacteria could survive inside the phagocytes for up to seven days as determined by a viable plate count after lysis of the phagocytic cells. As *C. jejuni* cells survived longer inside the macrophages than in the absence of the phagocytic cells, the authors speculated that macrophages may promote the survival of the pathogen as is the case with typical intracellular bacteria such as *S. typhimurium* and *Listeria monocytogenes*. However, Banfi *et al* (1986) reported that *C. jejuni* are not phagocytosed and killed by guinea-pig resident peritoneal macrophages whereas the *C. coli* strains were rapidly ingested and killed, which according to the authors is possibly an indicator of the relatively reduced virulence of the *C. coli* strains in comparison to the *C. jejuni* strains (Karamali and Skirrow, 1984). The authors speculated that the resistance of the *C. jejuni* cells to the action of the phagocytes could possibly be due to the presence of, as in the case of *C. fetus* (McCoy *et al,* 1975), the antiphagocytic protein microcapsule; which was initially also suggested for *C. jejuni* but was later refuted (Newell, 1984). In accordance with the results obtained by Banfi *et al* (1986), Pennie *et al* (1986) studying the interactions of *C. jejuni* strains with human polymorphonuclear leukocytes (PMN) also observed that certain *C. jejuni* strains might show total resistance to phagocytes. In macrophage depleted mice (by injecting silica dust, liquoid or dextran sulphate), a significant increase in the mortality was noted in comparison to the untreated controls (Bar, 1988). He also observed that combined macrophage depletion and decomplementation (by injecting cobra venom factor) did not increase the mortality of the infected mice significantly, in comparison to the macrophage depleted mice, indicating the role of the macrophages in the defense of mice against experimental *Campylobacter* infection (Bar, 1988).

Mice are quite resistant to *C. jejuni* infection even on the first contact (Blaser *et al,* 1983a); pretreatment of the mice such as iron loading (Kazmi *et al,* 1984; Stanfield *et al,* 1987) or preconditioning of the bacteria such as animal passage (Kazmi *et al,* 1984) are needed to establish overt infection. The results of these studies

indicate the possible involvement of a highly effective non-specific defense mechanism, such as the phagocytes may contribute to the resistance of the host. Investigatons are only beginning to explore this aspect of *C. jejuni* infections.

1.8 ANIMAL MODELS

Studies on the pathogenic mechanisms of a microorganism are primarily dependent upon the development of animal models which would reproduce the aspects of human disease under laboratory conditions. As *C. jejuni* is primarily an enteropathogen, the animal models which were adopted for studies on the pathogenesis of other enteropathogens were explored for their suitability for similar studies with *C. jejuni* strains.

1.8.1 CHICKEN MODEL

Ruiz-Palacios *et al* (1981) reported that diarrhoea could be induced in 3-day-old chickens by oral inoculation of *C. jejuni*; as low as 90 organisms caused diarrhoea in 90% of the chicks as determined by consistency of stool and presence of mucus. Localized invasion of the macrophage and epithelial cells were noted by immunofluorescence and electron microscopy. Manninen *et al* (1982) however could not reproduce these observations; no sign of diarrhoea or weight loss was noted in chicks infected with 14 clinical isolates of *C. jejuni*. Sanyal *et al* (1984a) argued that as chickens possess cloaca and pass stools mixed with urine, assessment of diarrhoea in chickens based on consistency and frequency of their excreta as done by Ruiz-Palacios *et al* (1981) could be misleading. They developed a quantitative diarrhoea model in 36-72 hr old chicks based on the measurement of fluid accumulation in the whole gut. Most of the human clinical isolates tested by Sanyal *et al* (1984a) caused watery diarrhoea while only a few caused mucoid diarrhoea. However, the clinical history of the strain (whether from cholera-like watery-type diarrhoea or dysentery-type diarrhoea) did not correlate with the outcome of the type of diarrhoea in chickens. Using newly-hatched chicks (within 12 hr) Welkos (1984) developed a sensitive and reproducible diarrhoea

model. The incubation period in the new-born chicks correlated with the number of organisms introduced orally; the diarrhoea which developed was of the bloody mucoid type and this often led to watery diarrhoea. Cellular infiltration of the gastric mucosa and the intestinal lamina propria were observed by electron microscopy. This new-born chick model for *C. jejuni* enteritis appears to reflect various aspects of natural human infection in comparison to the other chicken models described earlier and surely deserves further consideration.

1.8.2 MOUSE MODEL

The general availability and ease of handling of mice prompted researchers to explore their suitability as experimental models for *C. jejuni* enteritis. Although no overt signs of disease were noted consistently in *C. jejuni* challenged mice, a mucosal lesion, as found in human infections and invasion of intestinal epithelial cells and lamina propria by *C. jejuni* is often seen (Newell, 1984; Walker *et al*, 1986). Intragastric (Field *et al*, 1981) and oral (Stewart-Tull *et al*, 1984, Blaser *et al*, 1983a) administration of the pathogen resulted in colonization of the gut for various lengths of times. Neonatal mice were susceptible to colonization with the greatest number of pathogens being recovered from the caecum and large intestine whereas adult mice were not susceptible to colonization unless antibiotics were given before intragastric administration (Field *et al*, 1984). Other investigators succeeded in colonizing adult mice with *C.jejuni* by using relatively large inocula (Blaser *et al* 1983a) or by performing laparotomies and directly injecting *C. jejuni* into the intestine (Merrell *et al*, 1982). Madge (1980) noted a significantly reduced absorption of D-glucose and D-galactose in young mice challenged with *C. jejuni*; whereas in similarly infected old mice the absorption of these two sugars was unaltered.

Intestinal microflora inhibit the capacity of the *C. jejuni* to colonize the GI tracts of mice (Blaser *et al*, 1983a) and prolonged (up to 10 months) colonization was noted in germ-free mice (BALB/c) (Yrios and Balish, 1986a; 1986b). Antibiotic treatment of the mice was also reported to prolong the duration of colonization (Field *et*

al, 1984). Field *et al* (1981) did not observe dissemination of *C. jejuni* to internal oragans; however in later studies with conventional mice (Blaser *et al,* 1983a), germ free mice (Fauchere *et al,* 1985; Yrios and Balish, 1986b) and monoaxenic mice (Fauchere *et al,* 1985) *C. jejuni* were isolated from mesenteric lymph nodes (MLN), blood and several internal organs. Within 10 min after intraperitoneal (i.p.) inoculation, *C. jejuni* were found in the blood (Blaser *et al,* 1983a; Stanfield *et al,* 1987). As only *C. jejuni,* never the associated strain (*Clostridium perenne*) were isolated from the MLN and the internal organs of monoaxenic mice, Fauchere *et al,* (1985) concluded that *C.jejuni* possessed the specific property to translocate. However, Youssef *et al* (1987) observed no significant difference between the translocation of nine human clinical isolates of *C. jejuni* (from both invasive and non-invasive diarrhoea) and *E. coli* strains with or without various determinants of pathogenicity in adult germ-free mice. So it appears that whether the capacity of the *C. jejuni* strains to translocate to mesenteric lymph nodes and other internal organs is a virulence associated property or not is still uncertain. The increased incidence and the severity of disease (transient diarrhoea, caecal shrinkage, acute inflammatory changes with eosinophilia) in athymic mice in comparison to euthymic mice indicate a role of T cells in the resistance to campylobacteriosis (Yrios and Balish, 1986b); several important aspects of host-pathogen interactions have been revealed by these studies.

As with various other bacterial pathogens, the susceptibility of mice to *C. jejuni* was found to be enhanced by prior or concomitant iron treatment. Kazmi *et al* (1984) developed a diarrhoea model in neonatal BALB/c mice which reflected various features of *C. jejuni* enteritis seen in man. Virulence of the *C. jejuni* strains was enhanced by serial passage in weanling mice and the susceptibility of the infant mice was increased by coinoculating iron dextran or mucin. Intragastric challenge induced severe diarrhoea which was characterized by the discharge of stool containing mucus, occasionally with blood, and reduced weight gain. Diarrhoea usually continued for 8 days and most of the mice recovered thereafter and excreted *C. jejuni* for 2 weeks without

any overt sign of disease. The iron- induced susceptibility of mice to *C. jejuni* infection was also successfully exploited by Stanfield *et al* (1987) to develop a suitable *C. jejuni* diarrhoea model in adult BALB/c mice. The mice were pretreated with iron dextran or ferric chloride immediately before challenge with $1x10^8$-$1x10^9$ live organisms by the intraperitoneal route. Severe diarrhoea was induced within 4 hr and was characterized by unformed stool, containing mucus, blood and faecal leukocytes. Diarrhoea continued for 24 hr and the intestinal contents were positive for 5-7 days. Another characteristic of disease was transient bacteraemia (<24 hr); liver kidney and spleen were positive for *Campylobacter* for 48 hr. Untreated mice developed no diarrhoea and oral inoculation of the pretreated mice only resulted in mild disease. In contrast to the model developed by Kazmi *et al* (1984) this model did not require any in vivo passage of the organisms. These two models are the most sensitive and simplest of all *C. jejuni* diarrhoea model reported.

1.8.3 OTHER MODELS

Among the large animals, the rhesus monkey model of Fitzgeorge *et al* (1981) most closely resembled human disease although the symptoms were mild. Oral inoculation resulted in intermittent diarrhoea, bacteraemia for 2-3 days and prolonged excretion of the organism in the stool. Rechallenged animals resisted reinfection as shown by the lack of clinical disease (no bacteraemia and no excretion of organisms in the stool for only 3-4 days). Recently, Russell *et al* (1989) reported that experimental infection (via a nasogastric tube) of specific-pathogen-free *Macaca nemestrina* monkeys which were fasted and treated with cimetidine resulted in acute diarrhoeal illness, characterized by fluid diarrhoea, bloody stools, and faecal leukocytes. Plasma antibodies to *C. jejuni* group antigen were elevated and rechallenge with the same strain or heterologous strain resulted in a mild diarrhoea with further elevation of specific antibodies. Moreover, no signs of illness were noted in monkeys which had experienced multiple infections with *Campylobacter* spp. Other large animals

that were investigated for such purpose included calves, pigs and dogs (Newell, 1984; Taylor and Al-Mashat, 1984). However, almost all of these animal models did not prove suitable because of the expense and inconvenience of handling. Moreover, none of these models reproduced the overt disease as seen in man, although colonization for various periods accompanied by localized tissue damage in the involved segments of the gut and neutrophil infiltration of the lamina propria were noted.

1.8.4 ILEAL LOOP FLUID ACCUMULATION TEST

Ruiz-Palacios *et al* (1983) first reported accumulation of fluid in the ligated ileal loops of rats inoculated with a human clinical isolate which caused severe diarrhoea. They considered that the failure of previous workers (Guerrant *et al,* 1978; Manninen *et al,* 1982) to demonstrate fluid accumulation by *C. jejuni* in the ileal loop test using various animals could be due to the use of 1) hypotoxigenic strains, or 2) strains which have undergone repeated *in vitro* passages or 3) have been improperly stored or 4) grown under inappropriate conditions. But the strain which evoked fluid accumulation in the rat ileal loop, failed to do so in the rabbit ileal loop test, which is considered to be the prototype of the ileal loop models and is being used extensively for assay of enterotoxigenicity of various enteropathogens. McCardell *et al* (1984) however, noted fluid accumulation in the rabbit ileal loop using a 20-fold concentrated culture supernatant fluid. But later, Shaha *et al* (1988) observed only mild to severe haemorrhage but no fluid accumulation in rabbit ileal loops challenged with live culture or culture filtrate of *C. jejuni* strains isolated from chickens. The insensitivity (or sensitivity) of the rabbit ileal loop to the action of *C. jejuni* enterotoxin has not been investigated any further. The rat model was subsequently used by Klipstein and Engert (1984), Klipstein *et al* (1985) to investigate the enterotoxigenic properties of the *C. jejuni* strains. Shaha *et al* (1988) successfully used the rat ileal loop assay to show that *C. jejuni* strains isolated from chickens also produced enterotoxin; interestingly they also noted that strains which were initially nontoxigenic gave a positive reaction after one or more *in vivo* passages (in the gut), a phenomenon

observed in the case of other enteropathogens such as *V. cholerae* (Sanyal *et al*, 1984b) *Aeromonas hydrophila* (Annapurna and Sanyal, 1977), *Plesiomonas shigelloides* (Sanyal *et al*, 1980) etc.

1.9 PHASE AND ANTIGENIC VARIATION IN *C.JEJUNI*

Many pathogenic microorganisms such as *Salmonella typhimurium*, *Neisseria gonorrhoeae* and *Bordetella pertussis* can undergo phase and antigenic variation, which is considered to have evolved in these pathogens as a means of avoiding the host defence mechanisms. Caldwell *et al* (1985) reported that some *C. jejuni* strains can undergo bidirectional transition or phase variation between flagellated (Fla^+) and nonflagellated (Fla^-) phenotypes *in vitro*. The transition of Fla^+ to Fla^- and of Fla^- to Fla^+ occurred at a rate of $3.1x10^{-3}$ to $5.9x10^{-7}$ and a $4.0x10^{-7}$ to $8.0x10^{-7}$ per cell per generation respectively. No significant difference in characteristics or time course of infection was observed in rabbits in the RITARD model (Caldwell *et al* 1983). With strain A3249, 4 of 5 rabbits challenged with each variant developed mucoid diarrhoea, whereas with strain 81116, 8 of 18 and 5 of 11 rabbits became ill with Fla^- and Fla^+ variants respectively. These results indicate that flagella are probably not involved in virulence at least in this animal model. However, passage through rabbit intestine favored the Fla^+ phenotype. Black *et al* (1988) also noted that only Fla^+ variants were isolated from stool of human volunteers challenged with equal numbers of Fla^- and Fla^+ variants.

Flagella of certain *C. jejuni* and *C. coli* strains were found to undergo antigenic variation: they were capable of producing antigenically distinct flagellin subunits of two different molecular weights, 61,500 (phase 1) and 59,500 (phase 2) (Harris *et al*, 1987). Transition from phase 1 to phase 2 and from phase 2 to phase 1 occurred at a rate of approximately $2.0x10^{-5}$ and $1.2x10^{-6}$ cell per generation respectively. Recently Guerry *et al* (1988) demonstrated that in *C. coli* strain VC 167, the antigenic switch is controlled by a programmed reversible genomic

rearrangement of a 700 base pair region. No such genomic rearrangement was found to be associated with the flagellar phase variation described above. The mechanism of DNA switch in *Campylobacter* and its implication in the virulence mechanisms is yet to be determined.

1.10 GENETIC CONTROL OF VIRULENCE

The presence of plasmids in *C. jejuni* strains were first reported by Austen and Trust (1980). Later, plasmids in varying size and numbers were detected in *C. jejuni* by several workers (Taylor *et al*,1980, 1981; Tenover and Elvrum, 1988). But all attempts to associate plasmid carriage with any of the putative virulence markers were uniformly unsuccessful. Initially, it was reported by Lee *et al* (1985) that enterotoxin production by certain *C. jejuni* strains was linked with the carriage of a 46.5 plasmid, which was later refuted by (Taylor, *et al*, 1987). To date, only resistance to two antibiotics namely tetracycline and kanamycin are the only plasmid mediated phenotypes to be identified in *C. jejuni* (Kotarski *et al*, 1986; Taylor, 1986).

Genetic analysis of the biology of *C. jejuni* and its putative virulence determinants has been difficult due to the absence of standard DNA manipulation methodologies. Systematic approaches to establish standard procedures for gene transfer i.e. conjugation, transformation and transduction were uniformly unsuccessful for *C. jejuni* (Walker *et al*, 1986). A breakthrough in the development of a gene transfer system between *C. jejuni* and other bacteria came from the work of Labigne-Roussel *et al* (1987), who constructed a shuttle cloning vector pIL550 which can be mobilized between *E. coli* and *Campylobacter* spp. The hybrid plasmid was constructed by ligating *C. coli* plasmid pIL 455 to the pBR322 DNA sequences which enabled the plasmid to replicate both in *E. coli* and *Campylobacter.* A kanamycin resistance gene from the *C. coli* plasmid pIP1433 was inserted into the hybrid plasmid to serve as a selectable marker. To ensure the mobilization of the plasmid from *E. coli* to *Campylobacter,* the sequence encoding the origin of transfer (oriT) from a broad host

range plasmid belonging to the incompatibility group P was inserted into the hybrid plasmid. The nonessential regions of the pBR322 were stripped off and polylinker sequences from the tranposon Tn903 were inserted to provide multiple cloning sites. The complete shuttle vector pIL550 containing unique sites for the restriction enzymes *Cla*I, *Pst*I, *Eco*RI, *Sal*I and *Sma*I is thus a useful general vector for cloning *Campylobacter* DNA sequences into *E. coli* and returning them to *Campylobacter* spp. for expression (Labigne-Roussel *et al,* 1987).

Later, the same research group tried to modify the shuttle cloning vector into a shuttle suicide vector carrying Gram-negative (Tn5) or Gram-positive (Tn917) transposible elements to transposon mutagenize the *Campylobacter* genome but were unsuccessful (Labigne-Roussel *et al,* 1988). But in the same communication they reported the development of a method for constructing isogenic mutants of *C. jejuni* by shuttle mutagenesis. This approach involved insertion of a kanamycin resistance gene in a *Campylobacter* DNA fragment encoding the 16S ribosomal RNA (rRNA) gene cloned into the conjugative suicide vector pILL560 in *E. coli* This disrupted, modified 16S rRNA sequence in the suicide vector, when transferred to *C. jejuni*, was integrated (at a frequency of about 10^{-7} transconjugants per cell) into the chromosome. The integration of the modified sequence apparently involved the process of homologous recombination resulting in the simultaneous gene replacement of one of the five 16 S rRNA sequences of *C. jejuni* and loss of the vector. The authors (Labigne-Roussel *et al,* 1988) suggested that the lack of a genetic system for the delivery of transposons for insertional inactivation of genes in *Campylobacter* spp. can be overcome by using the shuttle mutagenesis approach involving *E. coli* as the intermediate host.

Electroporation is the application of high intensity electric fields for short duration to induce transient reversible permeability channels in the biomembranes through which DNA may be introduced. This technique has recently been employed to increase transformation efficiencies of mammalian cells, parasites and yeasts. Miller *et al* (1988) applied the technique of electroporation to transform *C. jejuni* and were able to obtain up to 1.2×10^6 transconjugants per μg of DNA. Several important

features about *C. jejuni* have been revealed in relation to the gene transfer system by the study of Miller *et al* (1988) which probably explains why all the previous attempts to transfer foreign DNA into the organism were uniformly unsuccessful. First, divalent cations (viz. Ca^{2+}, Mg^{2+}) which are used in the conventional transformation protocols drastically reduced the transformation efficiency. Secondly, preincubation of *C. jejuni* cells at 40^{o} C resulted in a 100-fold decrease in transformation efficiency in comparison to those incubated at 22^{o} whereas a heat shock step (40 sec at 40^{o} or 2 sec at 68^{o} C) is critically important in the transformation procedure for other bacteria (Manniatis *et al,* 1982). Thirdly, transformation was only achieved with plate grown cells; broth grown cells were totally ineffective. And most important of all, *C. jejuni* was found to possess a restriction/modification system capable of restricting foreign DNA and modifying endogenous DNA. These studies show that the genetic studies in *C. jejuni* are about to enter an exciting period of active research which will presumably lead to the identification and characterization of the various candidate virulence markers and eventual elucidation of the molecular basis of pathogenicity of this organism.

1.11 CONGO RED BINDING: AN INDICATOR OF VIRULENCE

The ability to bind the dye Congo Red (CR) has been found to be an indicator of virulence in several bacterial species. Agar medium containing CR can serve as a differential medium in which virulent cells absorb and form red colonies (CR^{+}) and avirulent strains and spontaneous avirulent mutants fail to bind the dye and appear colourless (CR^{-}). Payne and Finkelstein (1977) found that CR^{+} variants of *Shigellae, V. cholerae, E. coli* and *Neisseria meningitidis* were more virulent in the chicken embryo model in comparison to the CR^{-} colonial variants. Similarly, the CR-binding phenotype was found to be associated with virulence in *Yersinia enterocolitica* in a mouse model (Prpic *et al,* 1983; 1985) and in *E. coli* causing septicaemic infections in chickens (Berkhoff and Vinal, 1986).

In *S. flexneri,* CR^{-} variants were incapable of successfully invading the

found that in addition to *S. flexneri*, other *Shigella* species also showed CR-binding associated virulence. Spontaneous CR^- colonial variants were negative in the Sereny test (devoid of the property of causing keratoconjunctivitis in the guinea-pig cornea). Parton (1988) reported that loss of the ability to bind CR by *Bordetella pertussis* strains paralleled the loss of all the virulence factors i.e. haemolysin, haemagglutinin(s), pertussis toxin, heat-labile toxin and also the two major cell envelope peptides associated with virulence (X-bands). Moreover, the CR^- variants had reduced lethality in 3-week-old mice. a similar concomitant loss of CR-binding and virulence associated properties was also observed in *Bordetella parapertussis* and *Bordetella bronchiseptica*.

Although CR-binding appears to be a generalized virulence-associated phenomenon in several Gram-negative bacterial species, the molecular basis of this property has not been elucidated. In the fish pathogen *Aeromonas salmonicida*, the dye presumably binds to a cell surface protein layer (protein A), because the ability to bind CR positively correlates with the presence of protein A layer which is an absolute requirement for virulence (Ishiguro *et al*, 1985). Pretreatment of *Shigella flexneri* cells with trypsin resulted in no detectable binding of CR or haemin and approximately 97 % of the prebound CR or haemin was released when cells were treated with trypsin, indicating that these compounds probably bind to the protein components of the cell-surface (Daskaleros and Payne, 1987). Qadri *et al* (1988) investigated isolated cell-surface components such as outer membrane proteins and lipopolysaccharides of *Shigella dysenteriae* type 1 for their ability to bind CR and found that OMP not LPS was capable of binding the dye. No such studies have been done with other bacterial species exhibiting CR binding phenomenon. In some gliding soil bacteria such as *Flexibacter columnais*... (formerly known as *Chondrococcus columnaris*; Johnson and Chilton, 1966) and *Myxococcus xabnthus* (Arnold and Shimkets, 1988), CR binding serves as an important taxonomic indicator and the dye

apparently binds to the extracellular polysaccharide layer.

The biological significance of CR-binding is poorly understood. Payne and Finkelstein (1977) noted that supplementation of iron could restore the virulence of CR^- colonial variants of *V. cholerae, Neisseria meningitidis, E. coli* and *Shigella*, implying that the CR variants are deficient in the capacity of acquiring iron *in vivo*. Earlier studies with *Yersinia pestis* showed that only CR^+ variants had the additional property of absorbing haemin from agar medium (Jackson and Burrows, 1956; Surgalla and Beesley, 1969). Such correlation was later observed in other bacterial species such as *Y. enterocolitica* (Prpic et al, 1983; 1985), *Aeromonas salmonicida* (Kay *et al,* 1985) and *S. flexneri* (Daskaleros and Payne, 1987) suggesting that CR and haemin bind to the same site on the bacterial cell-surface presumably due to their structural similarity. As the pathogenic microorganism must acquire iron from the iron-binding proteins or from cell-free haem of the host to survive and to cause infection (Griffiths *et al,* 1988), the capacity to bind haemin may serve as a mechanism for acquiring iron *in vivo.*

In *S. flexneri,* binding of CR and haemin was found to be independent of the capacity of haemin utilization; as both CR^+ and CR^- colonial variants were capable of utilizing haemin as a sole source of iron. Both the CR^+ and CR^- variants and also a mutant strain deficient in the synthesis of aerobactin were capable of growing in the HeLa cell lysate indicating that the CR or haemin-binding phenotype is probably not required for iron uptake *in vivo.* However, prebinding of *S. flexneri* strains with CR or haemin and the addition of CR or haemin to HeLa cell monolayers resulted in an increased invasiveness for HeLa cells, suggesting that this binding ability was in some way involved in the attachment to the host cells.

Our current knowledge of the genetic control of CR-binding is almost exclusively based upon studies with *Shigella* spp. especially *S. flexneri* A direct correlation has been found between the CR-binding ability and the presence of

large 140 M dal plasmid in *S. flexneri*, *S. boydii* and enteroinvasive *E. coli* (Maurelli *et al*, 1984; Qadri *et al*, 1988). In order to explain the identical plasmid profiles found between CR^+ and CR^- variants of the other two *Shigella* spp. i.e. *S. dysenteriae* and *S. sonnei* it has been suggested that loss of the ability to bind CR may result from the inversion of insertion sequence IS*1* on the plasmid DNA so that although the size of the plasmid remains unchanged, the CR binding phenotype is lost (Qadri *et al*,1988). The CR-binding gene of *S. flexneri* has been cloned (Daskaleros and Payne, 1985; 1986; Sakai *et al*, 1986) and has been found to be highly conserved among *S. flexneri* strains (Daskaleros and Payne, 1986). Use of transposon mutagenesis to obtain isogenic mutants of CR binding having the full complement of the other virulence factor(s) might be useful in assessing the significance of the CR-binding phenomenon in bacterial virulence.

2.0 OBJECTIVES OF THE RESEARCH

OBJECTIVES OF THE RESEARCH

C. jejuni has emerged from obscurity as a major cause of human gastroenteritis in recent years. The pathogenic characteristics of *C. jejuni* have only begun to be investigated. The primary objective of this study was to explore the characteristics of *C. jejuni* which have been been identified as indicators of virulence in other diarrhoea-causing bacteria.

Diarrhoeagenic bacteria usually cause disease either by enterotoxin production (example: *V. cholerae* ; enterotoxigenic *E. coli*) leading to watery diarrhoea or by invasion of intestinal gut mucosa (example: *Shigella* spp. and enteroinvasive *E. coli*), leading to mucoid diarrhoea. But to what extent this generalization applies to *C. jejuni* is unknown, as clinical features of *C. jejuni* enteritis indicates the possible involvement of both of these mechanisms, either singly or concomitantly. So, another aspect of this study was to attempt to make comparative study of *C. jejuni* strains isolated from these two clinically distinct form of diarrhoea to identify the virulence markers which enable them to cause the clinically different forms of diarrhoea. Therefore the various aspects of the initiation and development of *C. jejuni* enteritis such as adherence, colonization, multiplication, invasion, release of toxins etc. and various cell surface properties were also investigated. As the project developed, it became apparent that *C. jejuni* strains vary widely in their virulence characteristics and identification of a single virulence factor may not reflect the pathogenic potential of a particular strain. In addition to be bacterial factors, host factors also play a determinative role in the outcome of clinical disease.

3.0 MATERIALS AND METHODS

3.1 Bacterial Strains

A total of 28 clinical isolates of *C. jejuni* was included in this study. Sixteen strains were isolated at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) (prefix B) and the rest were isolated in Scoland (prefix S). ICDDR,B strains were kindly supplied by Dr. Bradford, A. Kay and Dr. N. Alam. Scottish strains S-1 to S-8 were kindly supplied by Dr. John Gordon, Gartnavel General Hospital, Glasgow; strains S-9, S-10, and S-13 to S-16 were supplied by Dr. Ken Liddle of Law Hospital, Carluke; strains S-11 and S-12 were supplied by Dr. Grace Sweeney of Southern General Hospital, Glasgow. Details of these strains are presented in the Table 5.

Two reference *C. jejuni* strains NCTC 11168 and NCTC 11385 were obtained from the National Collection of Type Cultures, Colindale, London. Strain 11168 was originally isolated from the stool of a patient with enteritis and the strain 11385 was a human gall bladder isolate.

Enteroinvasive *E. coli* (EIEC) 111, which was used as the positive control strain in the adherence and invasion assays in the HeLa cell model was obtained from the ICDDR,B. The negative control strain *E. coli* K-12 HB101 was supplied by Dr. R. M. Brownlie, Department of Microbiology, University of Glasgow.

3.2 Identification of Strains:

The scheme adopted for the isolation of *C. jejuni* strains from diarrhoeal stools and their identification is presented in the Flow Diagram 2. The clinical isolates were identified by Gram-stain, characteristic colonial morphology, motility and biochemical tests.

Table 4: Details of the *C.jejuni* Strains Included in this Study

C.jejuni Strains	Strain Number	Clinical History	Source
S-1*	9	Unknown	Gartnavel Hospital
S-2	10	"	"
S-3	13	"	"
S-4	15	"	"
S-5	21	"	"
S-6	22	"	"
S-7	24	"	"
S-8	786	"	"
S-9	1	Watery Diarrhoea (W.D.)**	Law Hospital
S-10	2	"	"
S-11	1	Mucoid Diarrhoea (M.D.) §§	Southern General Hospital
S-12	2	"	"
S-13	52337	W.D.	Law Hospital
S-14	3	M.D.	"
S-15	4	W.D.	"
S-16	5	"	"
B-7 §	59690	M.D.	ICDDR,B
B-9	TDS-825	W.D.	"
B-10	647000	M.D.	"
B-12	595025	"	"
B-13	597750	"	"
B-14	596900	"	"
B-15	657250	"	"
B-16	595250	"	"
B-17	TDS-163	W.D.	"
B-18	594850	"	"
B-20	TDS-220	M.D.	"
B-23	595300	W.D.	"

* S strains were isolated in Scotland
§ B strains were isolated in Bangladesh
** Strains from Watery diarrhoea cases are designated as Group C strains
§§ Strains from Mucoid diarrhoea cases are designated as Group D strains
¶ International Centre for Diarrhoeal Disease Research, Bangladesh.

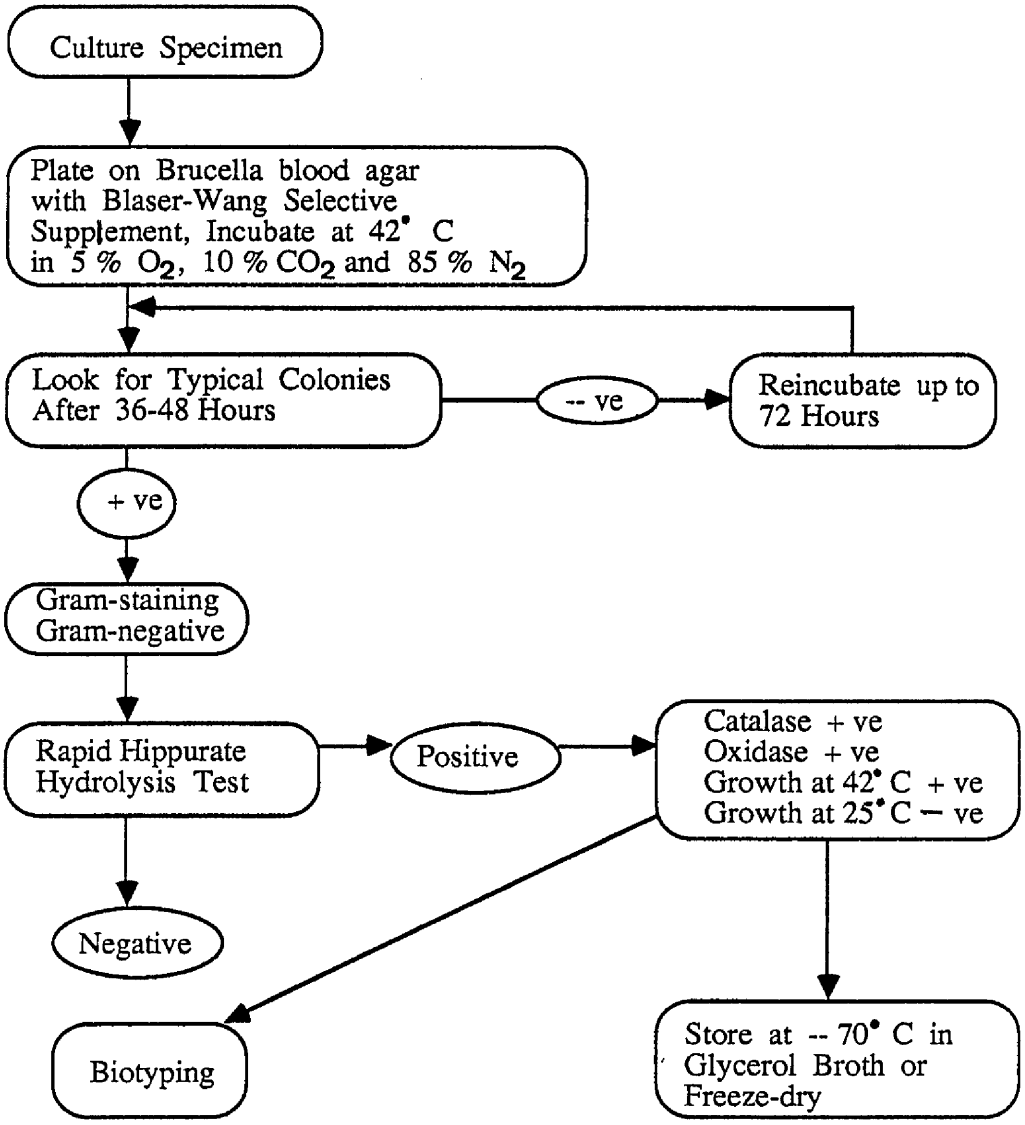
3.2.1 Biochemical Tests for Identification of *C. jejuni*

3.2.1.1 Hippurate hydrolysis test: This test was done as desribed by Lior (1984). A loopful of culture grown on a Brucella blood agar plate for 24 hr was suspended in a tube containing 0.4 ml of 1.0 % (w/v) sodium hippurate in sterile distilled water. After incubation in a 37° C water-bath for 2 hr with frequent mixing, 0.2 ml of freshly prepared ninhydrin solution [3.5% (w/v) in 1:1 mixture of acetone and butanol] was carefully added along the side of the tube. After another 10 min incubation in the 37° C water- bath without mixing, the tube was examined for colour development. A deep purple colour indicated a positive test; whereas a pale purple or no colour was considered negative.

3.2.1.2 Rapid H_2S test: To 97 ml of base medium (BB 2.9 g; Na_2HPO_4 0.118 g; KH2PO4 0.023 g; agar 0.2 g) 1 ml of each [10 % (w/v)] was added in sequence, FeS04.7H20, sodium metabisulphite and sodium pyruvate with mixing after each addition. To 4.0 ml such test medium 3-4 loopful of 24 hr culture from a Brucella blood agar plate was gently suspended (in the upper half of the medium) without mixing. The tube was then incubated in a 37°C water-bath for 2 hr. A positive reaction was indicated by a blackening reaction around the bacterial mass and a negative test showed no such reaction.

3.2.1.3 DNA hydrolysis test: The DNase test agar (Difco Laboratories) plates were prepared by supplementing with 1.35 ml of 0.5% solution of methyl green per 100 ml of the molten medium and the plates were dried well before use. A large loopful of 36 hr culture grown at 37° C on Brucella blood agar was inoculated in a circular area of 1 cm diameter on the DNase test agar plates and the plates were incubated for 5 days at 37° C. A clear, colourless zone surrounding the growth in the blue-green agar was considered positive for DNA hydrolysis.

Flow Diagram 2: Procedure for Identification and Biotyping of *C. jejuni* Strains



Biotyping of *C.jejuni* Strains According to the New Extended Biotyping Scheme of Lior (1984)

Test	*C. jejuni* Biotypes			
	I	II	III	IV
Hippurate Hydrolysis	+	+	+	+
Rapid H_2S Hydrolysis	--	--	+	+
DNA Hydrolysis	--	+	--	+

3.3 Growth Medium

Brucella agar (Appendix 1), supplemented with 7.0 % sheep blood (BBA), was routinely used for growing *C.jejuni* strains on solid medium (Appendix 1). Mueller-Hinton agar plates were used for determination of antibiotic sensitivity. Brucella broth (BB) (Appendix 1) was used for routine growth of the *C.jejuni* strains in liquid medium.

3.4 Growth of *C. jejuni*

Freeze-dried cultures of *C.jejuni* were reconstituted with sterile prewarmed (37° C) Brucella broth and plated on Brucella blood agar plates and incubated in anaerobic jars in a microaerophilic atmosphere (O_2 5%, CO_2 10%, N_2 85%) at 42° C for 24-48 hr. Cultures were grown from frozen stocks (-70° C) by thawing and spreading 100 µl of the bacterial suspension on BBA and incubated as above.

3.4.1 Growth on Plates: Unless otherwise stated Brucella agar (Appendix 1) plates supplemented with 7.0 % (v/v) sheep blood were used for growing *C. jejuni* strains routinely. The plates were incubated in anaerobic jars at 42° C in a microaerophilic atmosphere [O_2 5 %, CO_2 10 %, and N_2 85 % (v/v)] for 24-48 hr. For isolation of *C. jejuni* strains from stool samples, chicken tissues and infected eggs, *Campylobacter* selective plates (BBA plates supplemented with Blaser-Wang selective supplement; Appendix-1) was used.

3.4.2 Growth in liquid medium: Brucella broth (100 ml contained 250 ml , dimpled flasks) were seeded with 5 ml of an 18-20 hr starter culture grown in the same medium. The flasks were incubated in anaerobic jars in microaerophilic conditions on an orbital shaker set at 100 rpm for 24-36 hr.

3.4.3 Harvesting and counting of cells: Cells were harvested from a broth culture by centrifugation (10000xg, 10 min, 4°C), washed once with phosphate buffered saline (PBS, 10mM, pH 7.3), suspended in PBS or sterile Brucella broth as required and spectrophotometrically (Pye Unicam SP 600), adjusted to a suitable optical density at 550 nm. Viable counts were determined on BBA plates according to Miles *et al* (1938).

3.5 Culture Purity Checks: All strains were checked regularly for purity by Gram-stain, growth on selective plates (BBA supplemented with Blaser-Wang selective supplement [Appendix 1]), hippurate hydrolysis, oxidase and catalase tests.

3.6 Determination of Antibiotic Sensitivity

Faintly turbid cell suspensions ($1.0x10^3$-$1.0X10^4$ cells ml^{-1}) of *C. jejuni* strains were made in PBS and 100 µl was uniformly spread on a Mueller-Hinton plate and allowed to dry. An Oxoid Multodisk (Appendix 1) was placed, using sterile forceps, on the surface of the plate, which was incubated as described before (Section 3.4.1). After 36 hr the test strains were determined to be resistant (R) or sensitive (S) to each antibiotic.

3.7 Haemolysin Production by *C. jejuni* strains

3.7.1 Production of Haemolysin: *C. jejuni* strains were grown in Brucella broth supplemented with 1 % (v/v) proteose peptone as described before (Section 3.4.2). Proteose peptone was included, as preliminary studies indicated that this compound enhanced the production of haemolysin. Incubation was continued for 60 hr at 42° C in a microaerophilic gas atmosphere. After incubation, the broth cultures were centrifuged at 12,000xg for 20 min at 4° C and the supernates were passed through a 0.45 µm filter (Millipore, U.K. Ltd., London) and the filtrates were stored at -20° C until used.

filtrates were stored at -20° C until used.

3.7.2 Haemolysin assay: The culture filtrates were serially diluted in PBS (10 mM; pH 7.3) containing BSA at 0.1 % (w/v; PBSA). Freshly collected rabbit erythrocytes (RRBC) were washed three times in PBS and suspended to a concentration of 1.0 % (v/v) in PBSA. The washed RRBC suspension (0.5 ml) was added to the diluted toxin (0.5 ml) and the mixture was incubated at 37° C in a water-bath for 1 hr. After centrifugation at 1,000xg for 5 min, the absorbance of the supernates was read at 540 nm for haemoglobin release. A sample of RRBC suspension was lysed with saponin, diluted with an equal volume of PBSA in order to obtain a 100 % haemolysis end-point at A_{540} nm. One haemolytic unit was defined as the highest dilution of the sample required to produce 50 % haemolysis. Unless otherwise indicated, RRBC in PBSA (1.0 % v/v) was used in all experiments. Erythrocytes from human beings (blood group A, B and O), horse, cows, sheep, rabbits, guinea-pig, rats, mice, and chickens were washed in PBSA as described for RRBC and a 1.0 % (v/v) cell suspension in PBSA was used.

3.7.3 Kinetics of Erythrocyte lysis: Kinetic experiments were done with a Pye Unicam SP8-100 spectrophotometer fitted with a temperature controlled cuvette holder (Pye Unicam, Cambridge, England). Haemolysis was monitored continuously by a reduction in the optical density at 600 nm. Dilutions of the haemolysin from the strain S-11, concentrated by ammonium sulphate precipitation (80 % saturation) (256 HU, 128 HU and 64 HU) were mixed with equal volumes of 1.0 % RRBC in the cuvettes, already temperature-equilibrated in the spectrophotometer and read against a PBSA blank. PBSA mixed with an equal volune of RRBC served as the erythrocyte control. In the case of human blood groups A, B and O erythrocytes, a 0.5 % (v/v) suspension was used.

3.7.4 The effect heat-treatment on the Haemolysin: Serial dilutions of haemolysin in PBSA were heated in a water-bath at different temperatures and for different periods. The tubes were cooled, an equal volume of RRBC suspension was added and the haemolysin titre was determined.

3.7.5 Treatment of *C. jejuni* Haemolysin with Trypsin and Normal Rabbit Serum: Trypsin (Sigma) was added to PBS to give a final concentration of 1 mg ml^{-1} and serial dilutions of haemolysin were prepared. After incubation at 37° C for 1 hr, an equal volume of RRBC suspension was added and the haemolytic titre was determined. To determine the effect normal rabbit serum (NRS) (freshly obtained) the haemolysins from the *C. jejuni* strains S-11 and S-13 were serially diluted in 5 % NRS containing PBSA and the haemolytic titres were determined with RRBC.

3.8 *C. jejuni* Diarrhoea Model in New-born Chicks

Initial experiments of oral inoculation of 3-day-old infant chicks with *C. jejuni* strains to establish the diarrhoea model as described by Sanyal *et al* (1984a) were unsuccessful. However, new-born chicks (< 12 hr of age) were sensitive to oral challenge as reported by Welkos (1984) and were used to evaluate the diarrhoea-causing potential of *C.jejuni* strains.

C. jejuni strains were grown in Brucella broth (BB) for 18 hr as described before (Section 3.4.2). The cells were harvested by centrifugation (7000xg, 10 min, 4° C) washed once in PBS (10.0 mM; pH 7.3) and suspended in sterile BB and spectrophotometrically (Pye Unicam SP 6-550) adjusted to give a cell concentration of $1.0x10^9$ ml^{-1} which was confirmed by plate counts on Brucella agar (Miles *et al,* 1938).

Embryonated eggs from White Leghorn hens (obtained from Marshalls, Edinburgh) were incubated in a humidified rotary incubator at 37° C. The chicks

were removed from the incubator as soon as possible after hatching and were housed in sterile plastic cases in groups of five chicks. The inoculum (100 µl containing ca. $1.0x10^8$ *C. jejuni* cells) was placed in the oral cavity of the chicks with a micropipette; 5 or 10 chicks were inoculated per strain. Control chicks received equal volumes of sterile BB. On day 5 post-inoculation, the chicks were killed under carbon dioxide gas and the abdomen was opened. The cloacal end was clamped with Spencer Wells forceps and the entire gut up to the gizzard was quickly removed and placed in a sterile petri-dish. The intestine was then cut along the length to release the fluid which was measured with a micropipette. Mucoid fluid, when present in some chick guts, was removed with a spatula and the volumes measured. In an initial experiment, 3 chicks were killed off daily from day 3 to 7 post-inoculation and examined for incidence of diarrhoea and fluid accumulation in the gut. The maximum incidence of diarrhoea in the chicks was noted on day 5, which coincided with the mean maximum fluid accumulation in the gut. The incidence of diarrhoea was low before day 5 and declined after day 6 or later. Subsequently, the experiments were terminated on day 5.

3.9 Colonization and Multiplication of *C. jejuni* in Organs and Tissues

The chicks were inoculated as previously described (Section 3.10). From post-inoculation day 1 to 7, five chicks were killed each day under carbon dioxide gas and selected organs and tissues were removed aseptically, weighed and enumerated for *C. jejuni.* The specimens assayed included jejunum, ileum, colon, spleen, liver and heart-blood. These tissues were homogenized in sterile BB with a tissue homogenizer and ten-fold dilutions were made in BB. The viable counts were determined on Brucella blood agar plates supplemented with Blaser-Wang selective supplement (Appendix 1) according to Miles *et al* (1938).

3.10 Determination of Lethality of *C.jejuni* Strains in Chicken Embryos Model

Fertile eggs of White Leghorn hens (Marshall Poultry Co., Edinburgh) were incubated for 11 days at 37° C in a humidified rotary egg incubator. Strains of *C.jejuni* were grown in Brucella broth (BB) for 18 hr as described previously (Section 3.4.2). Cells were harvested by centrifugation (7,000xg, 10 min, 4° C), suspended in sterile BB and spectrophotometrically adjusted to $1.0x10^9$ c.f.u. ml^{-1}, and confirmed by viable plate counts (Miles *et al,* 1938). Serial decimal dilutions were made in BB and embryos were inoculated with 100 µl of each dilution onto the chorioallantoic membrane through a small hole made in the egg shell with a dental drill. Embryos (5 or 6) were inoculated per dilution; control eggs were inoculated with sterile prewarmed (37° C) BB. The eggs were returned to the incubator after inoculation and incubation continued for further 72 hr. The eggs were candled every 24 hr to determine viability. Mean lethal dose (LD_{50}) values were calculated at 72 hr post-inoculation according to the method of Reed and Muench (1938).

3.10.1 Recovery of *C. jejuni* strains from the infected embryos: The shells of the eggs containing dead embryos were removed, and the contents were placed in a sterile petri-dish. The foetus was removed with sterile forceps and scissors, weighed and homogenized in sterile BB with a tissue homogenizer. Ten-fold serial dilutions were made in BB and the viable counts were determined on Brucella blood agar plates supplemented with Blaser-Wang selective supplement (Appendix-1) according to Miles *et al* (1938)

3.11 Ileal Loop Test in Chickens

3.11.1 Preparation of Crude *C.jejuni* enterotoxin (CJT): *C. jejuni* strains were grown in BB as described before (Section 3.4.2) for 24 hr.

Polymyxin B was added to the whole culture at a concentration of 2.0 mg ml^{-1} and the flasks were shaken for a further 15 min. The supernatant fluid was collected by centrifugation (12,000xg, 20 min, 4° C) and sterilized by passage through a 0.22 µm membrane filter (Millipore). Ammonium sulphate (Enzyme grade, BDH) was added slowly to the culture filtrate with gentle stirring at 4° C to a final concentration of 70% (w/v) saturation. After 18 hr at 4° C, the precipitate was collected by centrifugation (16000xg, 20 min, 4°C) and dissolved in 2.5 ml distilled water and dialyzed for 24 hr at 4° C against 3 litres of distilled water with several changes. The dialyzed material was freeze-dried and stored at 4° C. For the ileal loop test, a 2.0 mg ml^{-1} solution of freeze-dried CJT was made in Brucella broth before use.

3.11.2 Cholera toxin: Freeze-dried crude culture filtrate of *Vibrio cholerae* strain B 1307 grown in 2% bacto peptone broth (Craig, 1966), obtained from the National Institute of Health, Bethesda, Maryland, U.S.A. was used as the source of cholera toxin (CT). A 2.0 mg ml^{-1} solution of this toxin was made in 2% (w/v) proteose peptone for the ileal loop test in chickens.

3.11.3 Preparation of *V. cholerae* and *C. jejuni* for Challenge: *V. cholerae* (NCTC 10732 biovar Classical, serovar Inaba) was grown overnight in 2.0 % (w/v) proteose peptone (Oxoid) at 37° C on a shaker set at 100 r.p.m. Two ml of this starter culture was added to 10.0 ml of proteose peptone [2% (w/v)] and incubated for 3 hr as before. This culture [$2.5x10^7$ cells ml^{-1}, Miles *et al* (1938)] was used in the ileal loop assay for fluid accumulation. *C. jejuni* strains were grown in Brucella broth for 18-20 hr as described before. The bacterial cell density was $5.0x10^8$ ml^{-1} (Miles *et al,* 1938).

3.11.4 Operation Procedure: Ileal loop operations in the chicks were done by Dr. D. E. S. Stewart-Tull. Five-day-old White-Leghorn chicks (obtained from

Marshall Chicken Company, Edinburgh) were used for the ileal loop assay to demonstrate fluid accumulation by *C. jejuni*. The chicks were starved of solid food for 24 hr before the experiment. The chicks were anaesthetised by injecting intramascularly 0.1 ml of 1:5 dilution of Valium 20 (Rothe Products Ltd., England) and 0.1 ml of small animal Immobilon (Reckitt & Colman Pharmaceutical Division, Hull, England). The intestine was brought out through a small incision made in the abdomen and the caecal region was isolated by a ligature. Two 4-6 cm long loops were made per gut interspaced by a small blank loop. One of the loops was inoculated with 0.2 ml of broth containing ca. 5.0×10^8 *C. jejuni* ml^{-1}. The control loop received 0.2 ml of sterile Brucella broth (BB). The gut was then replaced and the incision was stitched. The chicks were killed 18 hr post -inoculation and the volume of fluid in the loops was measured and loop ratios (volume of fluid accumulated in ml / length of the loop in cm) were calculated.

3.11.5 Growth of *C. jejuni* Strains in Chicken Intestinal Loops: The excised loop tissues were weighed and homogenized in 10.0 ml of sterile Brucella broth. The tissue homogenate and the corresponding fluid from each loop were serially diluted in BB and quantitatively plated BBA plates suppemented with Blaser-Wang selective supplement. After 24 hr incubation, the viable bacteria in each preparation was determined (Miles *et al,* 1938).

3.12 Immunodiffusion

Double diffusion assays were carried out by the method of Ouchterlony (1949) with 1.0% (w/v) agarose (Sigma) in PBS (pH 7.3). The central well was charged with antisera to cholera toxin (CT) raised in a rabbit (prepared by Dr. D. E. S. Stewart-Tull) and CT and CJT were placed in alternate peripheral wells. After samples were added, the agarose plates were kept at room temperature in a humidified chamber for 24 hr.

microtitre plates were coated with GM_1 ganglioside (10 µg ml^{-1} ; 100 µl per well) overnight at 4° C. The plates were then washed 3 times with wash buffer (PBS-Tween-BSA) with alternate 3 min incubation periods in the same buffer. The test samples, standard antigens and negative controls (uninoculated Brucella broth) were added to quadruplicate wells in 100 µl volumes. After incubation of 4 hr at 37° C, the plates were washed 3 times, as described before. Rabbit antiserum to purified CT diluted 1:1000 was added to each well in 100 µl volumes. The plates were incubated for 2 hr at 37° C and washed 3 times as described before. Horse-radish peroxidase labelled guinea-pig anti-rabbit antisera diluted 1:10,000 in PBS-Tween 20 buffer containing 1.0 % (w/v) BSA was added per well in 100 µl volumes and incubated at 37° C for 2 hr. After 3 washes of the plates, 100 µl of the OPD-H_2O_2 was added per well and incubated for 30 min at room temperaure in the dark. The OPD substrate solution was made up immediately before use and consisted of 34 mg of OPD and 20 µl of H_2O_2 (20 % v/v) in 100 ml of citrate phosphate buffer. The reaction was stopped by adding 50 µl of stop solution per well and the optical density was read at 405 nm with an ELISA reader (Titertek Multiskan, Flow Laboratories). As a negative control Brucella broth persistently gave an optical density reading of <0.1 positive-negative cutoff value was considered to be 0.2 OD reading. A standard curve of purified CT (0-10,000) ng ml^{-1} was prepared and the amount of CT equivalent of CJT was estimated from the standard curve.

3.14 Chinese Hamster Ovary (CHO) Cell Assay for *C. jejuni* Enterotoxin (CJT): The CHO cell assay was done as described by Guerrant *et al* (1974). The CHO cell line was maintained on Eagles minimum essential medium (EMEM), as described for the HeLa cell line (Section 3.21.1). The wells of 96-well flat-bottomed microtitre plates were seeded with 100 µl of CHO cell suspended at a concentration of $2.0x10^4$ cells ml^{-1}. After 24 hr growth at

37° C in an atmosphere of 5% (v/v) CO_2 in air, the growth medium was removed and the cell monolayer was washed once with sterile PBS. The *C. jejuni* enterotoxin preparation used was prepared as described previously (Section 3.11.1). All samples were tested in triplicate in 100 µl working volumes per well. Morphological alteration (elongation) of more than 50% of the cells in a well after 24 hr incubation was considered to be positive. Purified cholera toxin (CT) (Sigma) and uninoculated Brucella broth were used as positive and negative controls respectively.

3.15 Isolation of Outer Membrane Proteins (OMP)

C. jejuni strains were grown in BB (200 ml) and the cells were harvested by centrifugation, as previously described (Section 3.4.2). The cell pellet was washed once with PBS and suspended in PBS and adjusted to an optical density of 1.5 at 550 nm using a Pye Unicam Spectrophotometer. The cell suspension was sonicated (MSE ultrasonicator) for 8-10 30 sec periods with alternate 30 sec rest period in an ice-bath. The cell lysate was treated with 20.0 µg ml^{-1} DNAse (Sigma) for 15 min at 37° C. Intact cells were removed by centrifugation (7,000xg, 10 min, 4° C) and the supernatant fluid containing the cell envelope proteins were collected carefully. The cell envelope preparation was treated with 1.0 % (w/v) Sarkosyl (Sigma) at room temperature for 30 min to solubilize the cytoplasmic proteins according to Filip *et al* (1973). OMP's were collected by centrifugation (100,000xg, 60 min, 4°C), washed once with PBS and suspended in distilled water. The protein concentration was determined (Bradford, 1976) and stored at -20° C.

3.16 Isolation of the Major Outer Membrane Protein (MOMP)

C. jejuni strains were grown in Brucella broth (BB) as described previously (Section 3.4.2) for 36 hr yielding ca. 0.8 gm wet weight of cells from 1000 ml of culture. The cells were harvested by centrifugation (10,000xg, 10 min, 4°C), washed once with 10.0 mM Tris-HCl buffer, pH 7.4, suspended in the same

buffer (10.0 ml) containing 2mM EDTA. Lysozyme was added at a concentration of 1.0 mg ml^{-1} and incubated at room temperature for 30 min. The cell suspension was subjected to 10 cycles of sonication (MSE Ultrasonicator) in an ice-bath for 30 sec each time, with a 30 sec rest period in between. The cell lysate was treated with 20.0 µg of DNase ml^{-1} for 15 min at 37° C and the intact cells were removed by centrifugation (10,000xg, 10 min, 4°C). The cell envelope was sedimented by centrifugation (137,000xg, 60 min, 4°C) and suspended in 20.0 mM Tris-HCl buffer, pH 7.4 containing 10 mM $MgCl_2$ and 2.0 % (v/v) Triton X-100 and incubated at 37° C for 30 min to solubulize the cytoplasmic membrane. The outer membrane material was collected by centrifugation (137,000xg, 60 min, 4° C) suspended in Tris-HCl buffer (10 mM pH 7.4) and adjusted to a protein concentration of 10 mg ml^{-1} (Bradford, 1976). Trypsin was added at a final concentration of 100 µg ml^{-1} and incubated in a 37° C water-bath for 1 hr. As MOMP is the only trypsin-resistant OMP of *C. jejuni*, the other proteins are selectively proteolysed by trypsin. After trypsin treatment, the MOMP preparation was subjected to gel filtration on a Sephadex G-50 column (1.5x30 cm). The column was equilibrated with Tris-HCl buffer pH 7.4 and adjusted to a flow rate of 24 ml hr^{-1}. The sample (2.0 ml) was applied to the column and 3.0 ml fractions were collected which were monitored for absorption at 280 nm. The fractions containing the first protein peak (MOMP) were pooled, freeze-dried, reconstituted in sterile deionized water, and the protein concentration was determined (Bradford, 1976).

3.17 SDS-PAGE of Proteins and LPS

3.17.1 Preparation of OMP Samples for SDS PAGE: The protein concentrations of the OMP's were adjusted to 1.0 mg ml^{-1} mixed with an equal volume of solubilizing buffer (Appendix 2) and boiled at 100° C for 5 min prior to loading 15-20 µl per gel track. To demonstrate the heat-modifiable nature, the OMP

preparations were incubated at 37° C for 30 min after mixing with solubilizing buffer.

3.17.2 Preparation of Whole Cell Samples for SDS-PAGE: Brucella broth (BB) grown cells (Section 3.4.2) were washed once with PBS and suspended in PBS to an optical density of 0.15 at 550 nm. One ml of such a cell suspension was spun in a microfuge, the cell pellet was suspended in 100 µl of solubilizing buffer, boiled at 100° C for 5 min and 15-20 µl was applied per gel track.

3.17.3 SDS PAGE of Proteins: The method used was based on that described by Laemmli (1970) and Ames (1974). Electrophoresis was done on a BRL (Bethesda Research Laboratory) vertical gel system; the thickness of the gel was 0.1 cm. The buffers, stock solutions and the recipe for gel preparation are described in Appendix 2. The separating (lower) and stacking (upper) gels contained 12.5% or 10% (w/v) and 4.5% (w/v) acrylamide respectively. Electrophoresis was done at room temperature at a constant current of 20 mA until the tracking dye had reached the bottom of the gel. The gels were stained with Coomassie blue (Weber and Osborn, 1969) or silver-stained (Oakley *et al,* 1980). Molecular weights of the OMP's were assigned by comparison with the migration of standard proteins: alpha-lactalbumin (14,200), trypsin inhibitor (20,100), trypsinogen ($24x10^3$), carbonic anhydrase ($29x10^3$, glyceraldehyde-3-phosphate dehydrogenase ($36x10^3$), ovalbumin ($43x10^3$), and bovine serum albumin ($66x10^3$).

3.17.4 Silver-Staining for Protein: Silver-staining of SDS-PAGE gels for proteins was done according to the method of Oakley *et al* (1979). The gels were handled with rinsed plastic gloves and were gently shaken on a rotary shaker throughout the process of staining. All the solutions used in the staining of the gels were made up in 100 ml volumes. The gels were prefixed in 10.0 % acetic acid (v/v) and 30.0 % methanol (v/v) for 30 min followed by fixation in a 100 % (v/v)

glutaraldehyde solution (100 ml) for 30 min. After rinsing once with distilled water, the gels were washed overnight with distilled water. Next morning, the gels were washed for 30 min in fresh distilled water and immersed in a solution of dithiothreitol (5.0 μg ml^{-1} for 30 min. The solution was poured off and without rinsing the gels were immersed in 0.1% (w/v) silver nitrate solution for 30 min. The gels were rinsed rapidly once in distilled water and twice in developer solution (50 μl of 37% formaldehyde in 100 ml of 3% (w/v) sodium carbonate). The gels were immersed in a third volume of developer solution and shaken gently until the desired level of staining was achieved. Then 5 ml of 2.3 M citric acid was added to the developer solution and gently shaken for 10 min. Then the gels were washed several times in distilled water and stored in plastic bags.

3.17.5 SDS-PAGE of Lipopolysaccharide (LPS) Proteinase K Digested Whole Cell Lysate of *C. jejuni:* Strains were prepared for SDS PAGE according to Hitchcock and Brown (1983). *C. jejuni* strains were grown in BB for 24 hr and harvested as described previously (Section 3.4.2). Cells were washed once with PBS, pH 7.4 and suspended in PBS to an optical density of 0.5 at 550 nm. A portion (1.5 ml) of such a cell suspension was spun in a microfuge and the cell pellet was suspended in 50 ul of SDS PAGE solubilizing buffer (Appendix 2) and boiled for 10 min at 100° C. Proteinase K solution in PBS was added to the boiled lysate to a final concentration of 1.0 mg ml^{-1} and incubated at 60° C for 60 min.

3.17.6 Silver-Staining for LPS: Throughout the process of staining, the gels were shaken gently on an orbital shaker at room temperature. After overnight fixation in 200 ml of 25% (v/v) isopropanol in 7% (v/v) acetic acid, the gels were subjected to 5 min oxidation in 150 ml of distilled water containing 1.05 gm of periodic acid and 4 ml of 25% (v/v) isopropanol {in 7% (v/v) acetic acid}. The oxidizing solution was prepared immediately before use. The gels were washed 8 times, each time with 200 ml distilled water for 30 min. Silver-staining of the gels was done for 10 min with 150

ml of the staining solution consisting of 0.10 N NaOH (28 ml), ammonia solution (1.0 ml), silver nitrate (5.0 ml, 20% {w/v}) and distilled water 115 ml. The staining solution was made immediately before use and was stirred constantly during preparation. The gels were washed 4 times with 200 ml distilled waster for 10 min each time and immersed in developing solution kept at 25° C. The developing solution was made up of citric acid 12.5 mg and formaldehyde 0.125 ml contained in 250ml distilled water. The developing solution was poured off when the desired level of staining was achieved and the gels were put in a stop-bath [200 ml distilled water containing 7% (v/v) acetic acid]. The gel was finally washed with distilled water and stored in a plastic bag.

3.18 Immunoblotting

Immunoblotting was carried out according to the method of Towbin *et al* (1979) in a Bio-rad "'Transblot' transfer apparatus. The slab gel, nitrocellulose membrane (Hybond-C, Amersham) and filter paper (3 MM, Whatman) were soaked in precooled transfer buffer (Appendix 4), placed inside the cassette and placed in the transfer chamber containing 2.5 litres of precooled transfer buffer with the nitrocellulose membrane facing the cathode. Electrophoretic transfer was accomplished at 220 mA in 3 hr at room temperature using a Bio Rad power pack.

3.18.1 Immunological detection of Protein on Nitrocellulose Membrane

Immediately after transfer the nitrocellulose sheet was immersed in wash buffer (Appendix-4) containing 5.0 % gelatin (Oxoid) and incubated at 42° C for 45 min. The sheet was then transferred to fresh 0.5 % gelatin containing wash buffer in which the appropriate volume of the antiserum (100-200 µl) had been added and incubated for 18 hr at 4° C. The nitrocellulose sheet was washed

with shaking for 4 hr in 8 changes of 100 ml wash buffer at room temperature and immersed in fresh wash buffer containing 1:3,000 dilution of sheep anti-rabbit horseradish peroxidase conjugate (HRP) (Scottish Antibody Production Unit) and incubated for 1 hr and 30 min at 37° C. The nitrocellulose sheet was removed and washed as described above. The substrate, 4-chloro naphthol (Bio Rad) (Appendix 5) was added and gently shaken until the desired level of staining was achieved. The nitrocellulose sheet was finally washed in distilled water, air-dried and stored.

3.19 Electrofocusing

Electrofocusing was done using an LKB Multiphor apparatus mounted on a cooling plate maintained at 4° C by circulating cold water. Ready-made thin layer polyacrylamide gels (LKB, Sweden) with dimensions 245 x 110 x 1 mm, with 5.0 % gel and 2.0 % ampholite concentration with pH range 3.5-9.3 were used. The electrophoresis unit was connected to the power pack (Pharmacia Fine Chemicals, Uppsala, Sweden) via specially designed safety cover and electrophoresis lid. The electric field was applied via two strips of paper soaked in the appropriate anode (1 M NaOH) and cathode (1 M H_3PO_4) buffers. Paraffin oil was spread uniformly over the cooling plate and over the sample application template to place the sample application template and the gel respectively. The gels were prefocused at 10 W for 15 min before putting the sample application strips (10x5 mm) on the gel in appropriate places with the aid of the template. Samples in 10 µl volumes (1.0 mg ml^{-1}) were applied to the strips and electrofocusing was done at 20 W with a 1500 V maximum for 60 min. The minimum electrofocusing time required for protein banding was determined by applying freshly lysed sheep erythrocytes on both sides of the gel and noting the time required for haemoglobin to fuse together from opposite sides of the gel. The run was terminated 40 min after the haemoglobin fusion. Gels were fixed and stained according to Billcliffe and Arbuthnott (1974).

3.19.1 Preparation of Acid Extract of Coomassie blue: Coomassie blue (PAGE Blue 83) 2.5 gm was added to 100 ml of distilled water and stirred vigorously on a magnetic stirrer for 10 min. Then 100 ml of 2.O N H_2SO_4 (warmed to 60° C was added and kept at 60° C for a further 10 min. The solution was filtered through Whatman No.1 filter paper and the pH was adjusted to 5.0 with 10.0 N KOH. Trichloroacetic acid was added to the solution to a final concentration of 12 % (w/v). The resulting solution had a pH of of ca. 1.0 and appeared blue green in colour. The solution was stored in a brown glass bottle and the pH was checked regularly before use. The pH had to be maintained around 1.0 for proper staining.

3.19.2 Staining of the Electrofocusing gel: The gels were stained by immersing in a solution of acid extract of Coomassie blue (prewarmed to 60° C) for 15 min at 60° C. The gels were destained overnight in cold tap water.

3.20 Determination of Cell-Surface Hydrophobicity of *C. jejuni* Strains

3.20.1 Bacterial Adherence to Hydrocarbon (BATH) Test: The cell surface hydrophobicity of the *C. jejuni* strains was determined according to the procedure described by Rosenberg *et al* (1980). Bacterial suspensions from 24 hr cultures in Brucella broth (Section 3.4.2) were washed once with and suspended in phospate urea magnesium (PUM) buffer, pH 7.1 (97 mM K_2HPO_4, 53 mM KH_2PO_4, 30 mM Urea, 0.8 mM $MgSO_4$; Rosenberg *et al,* 1980). The cell suspensions were adjusted to an OD of 1.0 at 550 nm.. To 1.5 ml of bacterial suspension 0.25 or 0.5 ml n-Octane was added and the mixture was incubated at 30° C for 15 min. This mixture was shaken on a vortex mixer (Gallenkamp Spinmix with the setting at 7.0) for 1 min. It was then allowed to stand for 30 min at room temperature for separation of the two phases. The aqeuous layer was removed and its OD at 550 nm was read in a spectrophotometer. The OD 550 value of the aqueous layer of the test mixture was expressed as a

percentage of the OD 550 of the original suspension.

3.20.2 Salt Aggregation (SA) Test: The SAT was carried out as described by (Lindahl *et al* (1981) to determine the cell surface hydrophobicity of the *C.jejuni* strains. Sodium phosphate (2.0 mM, pH 6.8) was used to dilute a solution of 4 M $(NH_4)_2SO_4$ in 2 mM Na-phosphate (pH 6.8). Serial dilutions were made giving $(NH_4)_2SO_4$ concentrations ranging from 4.0 to 0.5M, differing by 0.5 M per dilution. The pH was checked and adjusted when necessary to 6.8 with 1.0 N NH_4OH. *C. jejuni* were grown in Brucella broth for 24 hr as previously described (Section 3.4.2), washed with and suspended in Na phosphate solution (pH 6.8) to $2.5x10^{10}$ ml^{-1}. The cell suspension (25 µl) was mixed with an equal volume of salt solution on a glass slide. The bacteria-salt solution was mixed by rocking gently for 2 min at room temperature and observed for visible agglutination under a table lamp. A reaction causing optimal aggregation that was visible even without the microscope was considered as ++. A reaction giving definite aggregation but visible only with the aid of the microscope was scored +. A reaction giving only a few or no aggregates was considered negative. All readings were compared with the reaction at the highest molarity of salt (for a positive control). The bacterial suspension with 2.0 mM Na phosphate pH 6.8, containing no $(NH_4)_2SO_4$ served as the negative control.

3.21 Adherence to and Invasion of HeLa Cells

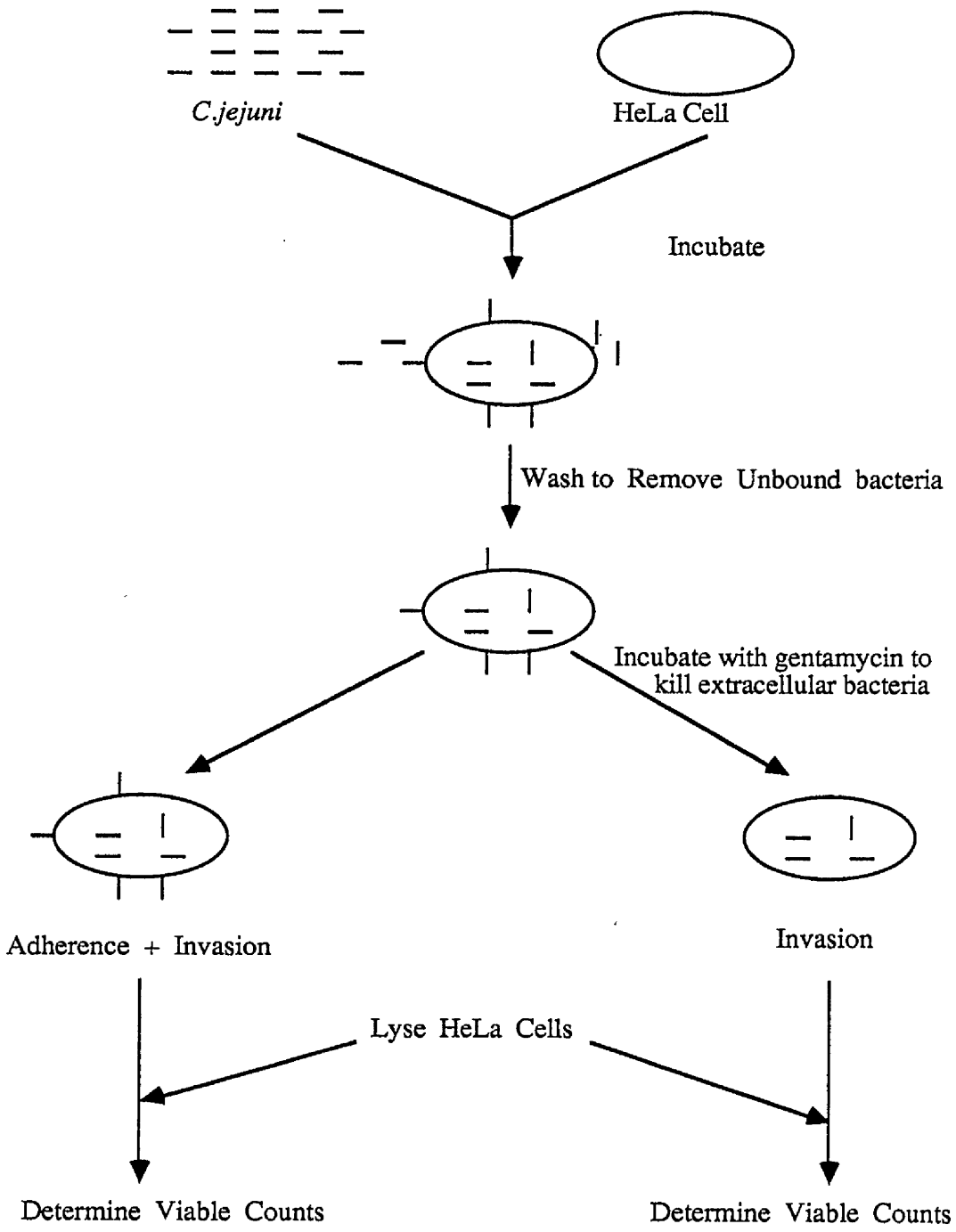
3.21.1 HeLa cell culture: The HeLa cell line (Flow Laboratories) was maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) foetal calf serum 2 mM glutamine, 100 IU penicillin and 100 IU streptomycin ml^{-1} (EMEM-1) (Flow Laboratories). Cells were grown as monolayers in plastic tissue culture bottles in an atmosphere of 5% CO_2 and 95% air. The monolayers were trypsinized once a week and the detached cells were used

to prepare fresh monolayers.

3.21.2 Adherence and Invasion assay: *C. jejuni* strains were grown in Brucella broth for 18 hr as described previously (Section 3.4.2). Cells were harvested, washed once with sterile Dulbecco A PBS (pH 7.3; 10 mM) (Oxoid) and suspended in EMEM without antibiotics and foetal calf serum (EMEM-2) and adjusted to $2.5x10^7$ cells ml^{-1}. Enteroinvasive *E. coli* (positive control) was grown in brain heart infusion broth (10.0 ml) inoculated with 2.0 ml of overnight starter culture grown in the same medium and grown for 2 hr at 37° C. This growth was necessary as actively growing cells were a prerequisite for efficient entry of the organisms into epithelial cells (Small *et al,* 1987).

C. jejuni strains were assessed for their ability to enter HeLa cells according to the procedure described by Isberg and Falkow (1985). HeLa cell monolayers were prepared in 24-well tissue culture plates seeded at a concentration of $2.5x10^5$ cells per well. After 24 hr, the growth medium was removed, the cell monolayer was washed twice with sterile warmed (37° C) PBS and aliquots of $2.5x10^7$ bacteria were added per well, so that the multiplicity of infection was 100 *C. jejuni* : 1 HeLa cell. Infected cultures were incubated at 37° C for 2 hr in an atmosphere of 5% O_2, 10% CO_2, 85% N_2 to allow entry of bacteria in to the HeLa cells. The cell monolayers were then washed 5 times with sterile PBS to remove nonadherent bacteria before adding fresh EMEM-2 containing 100 µg ml^{-1} gentamycin. This antibiotic is unable to enter mammalian cells efficiently (Tronet and Tulkens, 1981; Niesel *et al,* 1985), so the bacteria that enter such cells survive the antibiotic treatment. After 2 hr further incubation at 37° C, the monolayers were washed 5 times with sterile PBS and internalized bacteria were released from the HeLa Cells by lysing them with 0.5 ml of 0.5 % (w/v) sodium deoxycholate in PBS per well.

Flow diagram 3 : Assay of Adherence and Invasion Potential of *C.jejuni*



(Adherence + Invasion) -- (Invasion) = Adherence

After 5 min 0.5 ml of EMEM-2 was added to each well, decimal dilutions were made of the released intracellular bacteria in sterile Brucella broth and viable counts were made on BBA containing Blaser-Wang selective supplement. Results were expressed as follows:

% invasion = the number of bacteria surviving the gentamycin treatment / the number of bacteria added X 100

The total number of cell-associated bacteria (adhered + internalized) was determined in the same way as with the number of internalized becteria with the following exception: after co-incubation of *C. jejuni* with the HeLa cell for 2 hr, the cell monolayer was washed 5 times with PBS, lysed with 0.5% (w/v) sodium deoxycholate as described above and bacterial counts were determined. The proportion of adherent bacteria was calculated as

% adherence = % (adhered + internalized)-% internalized.

In expressing the results, % adherence and % invasion was considered as the index of adherence and invasion potential respectively. Tests were run in quadruplicate and repeated at least twice.

3.21.3 Time Course of Adherence to and Invasion of HeLa Cells by *C. jejuni*

HeLa cell monolayers were inoculated as previously described (section 3.21.2). Adherence and invasion were assayed at 30 min intervals in quadruplicate wells for a 2 hr period.

3.21.4 Factors Influencing Adherence to and Invasion of HeLa Cells by *C.jejuni*

3.21.4.1 Influence of Growth Temperature: *C. jejuni* strain S-11 was grown at 37° C and 42° C (Section 3.4.2) in Brucella broth were suspended in EMEM-2 to the same cell number and used in the adherence and invasion assay as previously

described (Section 3.21.2) .

3.21.4.2 Influence of Growth Phase: Cells from log phase (16 hr) and stationary phase (26 hr) cultures of the *C. jejuni* strain S-11 were compared in terms of their adherence and invasion potential according to the previously described procedure (Section 3.21.2).

3.21.4.3 Influence of Growth Medium: The adherence and invasion potentials of the strain S-11 were determined using cells grown in Brucella broth (for 16-17 hr) (Section 3.4.2) and on Brucella blood agar plates (24 hr) (Section 3.4.1) in parallel experiments. Adherence and invasion assays were done as described above (Section 3.21.2).

3.21.4.4 Influence of Gas atmosphere: After inoculation of the HeLa cell monolayers with *C. jejuni* strain S-11, the tissue culture plates were incubated in microaerophilic gas atmosphere (5% O_2, 10% CO_2, 85% N_2) and under 5 % CO_2-95 % air in parallel assays to determine the influence of gas atmosphere on the adherence and invasion potential of the *C. jejuni* strains.

3.21.4.5 Influence of Assay Temperature: The adherence and invasion potential of the *C. jejuni* strain S-11 were determined at 4^{o} C, 37^{o} C and at 42^{o} C by incubating the tissue culture plates at these temperatures after inoculation with the bacteria to determine the influence of assay temperature on the adherence and invasion potential of the *C. jejuni* strains. Adherence and invasion assays were done as previously described (Section 3.21.2)

3.21.5 Effect of L-fucse on Adherence and Invasion: The influence of L-fucose in the adherence and invasion of HeLa cells by *C. jejuni* strains was

determined by conducting assays in the presence of various monosaccharides. *C. jejuni* inocula were prepared in filter sterilized EMEM-2 containing monosaccharides (50 mM) and incubated at 37° C for 30 min with shaking. Invasion assays were performed as previously described (Section 3.21.2).

3.21.6 Effect of Cytochalasin B: A stock solution of Cytochalasin B (Sigma) (1.0 mg ml^{-1}) made in dimethyl sulfoxide (Sigma) was diluted in EMEM-2 to give a series of concentrations (ranging from 2.0 to 20.0 ug ml^{-1}). These solutions were mixed with equal volumes of EMEM-2 containing $5.0x10^7$ *C. jejuni* cells ml^{-1}. The *C. jejuni* inoculum thus containing $2.5x10^7$ cells ml^{-1} and cytochalasin B at concentrations ranging from 1.0 to 10.0 µg ml^{-1} was used in the invasion assay as previously described (Section 3.21.2).

3.21.7 Effect of Chicken Intestinal Mucus on Adherence and Invasion

3.21.7.1 Isolation of Crude Mucus from Chicken Gut: The chicks were given streptomycin sulphate (5 g $litre^{-1}$) in their drinking water 24 hr before mucus isolation to reduce the bacterial flora of the intestine. The chicken intestinal mucus was isolated according to the method described by Cohen *et al* (1983). The chickens were starved for 24 hr before killing by CO_2. The intestine excluding the jejunum and the caecum was removed and immersed in Dulbecco A PBS. The intestines were cut into approximately 2 cm long pieces and cleared of faeces with swab sticks. The pieces of intestine were transfered to fresh PBS and opened lengthwise with a scalpel. The mucosa (consisting of mucus gel and epithelial cells) was scraped gently with swab sticks and centrifuged (20,000xg, 30 min, 4°) to remove the epithelial cells and any remaining faecal debris. The viscous supernate containing the mucous gel was collected carefully with a pipette, the protein concentration determined (Bradford, 1976), and stored at -20° C until used within 2 weeks.

3.21.7.2 Adherence and Invasion Assay in Presence of Mucus: HeLa cells

monolayers were prepared in 24-well polystyrene tissue culture plates as previous described (Section 3.21.1). Mucus preparation (at a concentration of 250 μg ml^{-1}1 in EMEM-2) was added to the tissue culture wells and the plates were incubated at 37° C for 1 hr before use in the adherence and invasion assay as previously described (Section 3.21.2). In an initial experiment a series of concentrations of mucus (100 to 500 μg ml^{-1} were used and the optimal inhibition of adherence and invasion was found at 250 μg ml^{-1}. Consequently, the mucus preparation was used in this concentration in all subsequent assays. One ml of *C. jejuni* cell suspension in EMEM-2 strains containing $2.5x10^7$cells ml^{-1} was added to each mucus pretreated well. The plates were centrifuged (200xg, 5 min, 4° C; Vesikari *et al,* 1982) prior to incubation at 37°C for 2 hr in a microaerophilic atmosphere. Adherence and invasion assay were done as previously described (Section 3.21.2).

3.21.8 Effect of Antisera on Adherence and Invasion Potential: HeLa cell monolayers were prepared in 24-well tissue culture plates as previously described (Section 3.21.1). *C. jejuni* cells were suspended in EMEM-2 to a concentration of $2.5x10^7$ ml^{-1} in dilutions of either the homologous rabbit antisera or normal rabbit serum (1:25 through 1:400). The tissue culture plates were centrifuged immediately (200xg, 5 min, 4°C; Vesikari, *et al,* 1982) and incubated at 37° C in a microaerophilic atmosphere (5% O_2, 10% CO_2, and 85% N_2) for 2 hr. Adherence and invasion assays were done as previously described (Section 3.21.2)

3.21.9 Effect of Outer Membrane Proteins (OMP) on Adherence and Invasion Potential: Hela cell monolayers were preincubated with different concentrations OMP preparations for 30 min at 37° C. Standard adherence and invasion assays were carried out as previously described (Section 3.21.2).

3.22 Congo Red Binding by *C. jejuni*

3.22.1 Congo Red Binding on Solid Medium: Brucella agar containing 30.0 μg of CR was used as a differential medium for the isolation of CR^+ and CR^- variants. *C. jejuni* strains were streaked on CR plates and incubated at 42° C for 36 hr under microaerophilic atmosphere. CR^+ colonies appeared as dark red whereas CR^- colonies appeared as pale orange among predominantly CR^+ colonies. The colonial variants were subcultured on the same medium, Gram-stained, biochemically tested (hippurate hydrolysis, oxidase test and catalase test) and stored at -70° C in 15.0 % (v/v) glycerol supplemented Brucella broth.

3.22.2 Congo Red Binding in Liquid Medium: The ability of the *C. jejuni* strains to bind the dye in liquid medium was assessed as described by Payne and Finkelstein (1977). Bacterial cells were suspended in PBS (10.0 mM; pH 7.3) containing 20.0 μg ml^{-1} CR and spectrophotometrically (at 550 nm, Pye Unicam SP 6-550) adjusted to the cell concentration of $1x10^9$ ml^{-1}. After incubation at 37° C in a shaking water-bath for 30 min, the cell suspension was spun in a microfuge and the absorbance of the supernate was measured at 500 nm. The concentration of CR remaining was determined by reference to a standard curve.

3.23 Statistical Tests

The Student's t-test (Wardlaw, 1985) was used to evaluate the significance of differences between the experimental and control population means in various experiments. This test was used only if the data had been obtained by random sampling from a normal or approximately normal distribution and the variance of the two populations did not differ significantly when tested using the F-test. The degree of difference between the means of the control and experimental groups were either not significant ($P > 0.05$), marked with NS, or significant at the

levels $P \leq 0.05$, $P \leq 0.01$ or $P \leq 0.001$ which was denoted with *, **, and *** (unless otherwise indicated) respectively.

The χ^2-test was done to determine the proportion of bacterial strains exhibiting a virulence characteristic and the differences were considered as not significant (NS; $P > 0.05$) or significant at the levels $P \leq 0.05$; $P \leq 0.01$ and $P \leq 0.001$ which was denoted with *, **, and *** respectively.

The correlation between the results of various experiments was determined by simple regression analysis (Wardlaw, 1985) and the r values obtained indicated the degree of correlations between different experimental groups.

The statistical analysis was carried out using Statwork™ statistical package on Macintosh Plus (Apple Computer Inc., California) desk-top microcomputer.

3.24 Graphs and Figures

Graphs and Figures were drawn using Cricket Graph Software on Macintosh Plus (Apple Computer Inc., California) desk-top microcomputer.

4.0 RESULTS

4.1 Biotyping of the *C. jejuni* Strains

The results of the biotyping of the *C. jejuni* strains according to the new, extended biotyping scheme of Lior (1984) are presented in Table 6. Out of 20 strains tested, only 3 strains (B-7, B-9 and S-14) were of biovar II, and the rest were of biovar I.

4.2 Production of Enterotoxin by *C. jejuni* Strains

4.2.1 *C. jejuni* Diarrhoea Model in Infant Chicks

Initial experiments with several *C. jejuni* strains (each strain was fed to two chicks) showed that the amount of fluid present in the gut of chicks which were fed with live *C. jejuni* was ≥ 600 µl. On the other hand, sterile Brucella broth fed new-born chicks had ≤ 300 µl of fluid. So according to Sanyal *et al* (1984a), diarrhoea was defined in *C. jejuni* fed chicks as ≥ 500 µl of fluid accumulation in the gut and the strains were evaluated for diarrhoea-causing potential on the basis of this criteria.

In Figure 3, the mean and standard deviation of the volume of fluid present in the gut of chicks challenged with different *C. jejuni* strains (10 or 12 chicks per strain) are presented along with values of the control chicks. Relatively larger volumes of fluids were present in the gut of the chicks fed with the strains S-13 and B-23. The NCTC strain 11385 which was originally isolated from human gall-bladder and the strain S-11 (a Group D strain) were found to be negative in this test. Intermediate amounts of fluid were recovered from the gut of the chicks inoculated with the strains B-7 (a Group D strain) and NCTC 11168, a human enteritis isolate. The volume of fluid accumulated in the gut of all the chicks were significantly higher in comparison to the volume of fluid recovered from the control chicks ($P < 0.001$; unpaired t-test). Although the number of *C. jejuni* strains tested in this model was low, the strains showed good

Table 6: Biotyping of the *C. jejuni* Strains According to the new, Extended Biotyping Scheme of Lior (1984).

	STRAINS *C.JEJUNI*	TEST			BIOTYPE
		HIPPURATE HYDROLYSIS	RAPID H S PRODUCTION	DNA HYDROLYSIS	
GROUP C STRAINS	S-9	+	–	–	I
	S-10	+	–	–	I
	S13	+	–	–	I
	S-15	+	–	–	I
	S-16	+	–	–	I
	B-9	+	–		II
	B-16	+	–	–	I
	S-17	+	–	–	I
	B-18	+	–	–	I
	B-23	+	–	–	I
GROUP D STRAINS	S-11	+	–	–	I
	S-12	+	–	–	I
	S-14	+	–		II
	B-7	+	–		II
	B-10	+	–	–	I
	B-12	+	–	–	I
	B-13	+	–	–	I
	B-14	+	–	–	I
	B-15	+	–	–	I
	B-20	+	–	–	I
		+	+	--	III
		+	+	+	IV

correlation with the *in vitro* assay of enterotoxins, the CHO cell assay (r = 0.7356; Figure 95) and ELISA (r = 0.8406; Figure 96)

4.2.2 GM_1 Ganglioside ELISA and CHO Cell Assay

Twenty human clinical isolates of *C. jejuni* were tested for the production of cholera-like enterotoxin using GM_1 ganglioside ELISA and CHO cell assay. The results of the two assays are presented in the Figures 5 to 8. The ELISA O.D. values were used to determine the concentrations of enterotoxins produced from a standard curve prepared with cholera-toxin as the standard (Figure 4) and were expressed as the ng of toxin per mg of protein. The sensitivity and specificity of the ELISA were 5 ng and 97.5% respectively. The CHO cell titres are expressed as $TCED_{50}$ mg^{-1} of protein i.e. the reciprocal of the highest dilution of toxin causing elongation of 50 % of the cells per mg of protein. The enterotoxin titre determined by these two assays per mg of protein made possible the comparison of the actual amount of toxin produced by these strains on a unified scale.

The cell-free culture filtrates of the *C. jejuni* strains caused morphological changes of CHO cells which were identical to those induced by purified cholera toxin (Sigma) used as the positive control. Preliminary studies with the strain S-13 showed that the morphological changes were maximal at 24 hr although the effect could be convincingly observed at 18 hr. Hence, the morphological changes at 24 hr were recorded in the subsequent studies with the other strains.

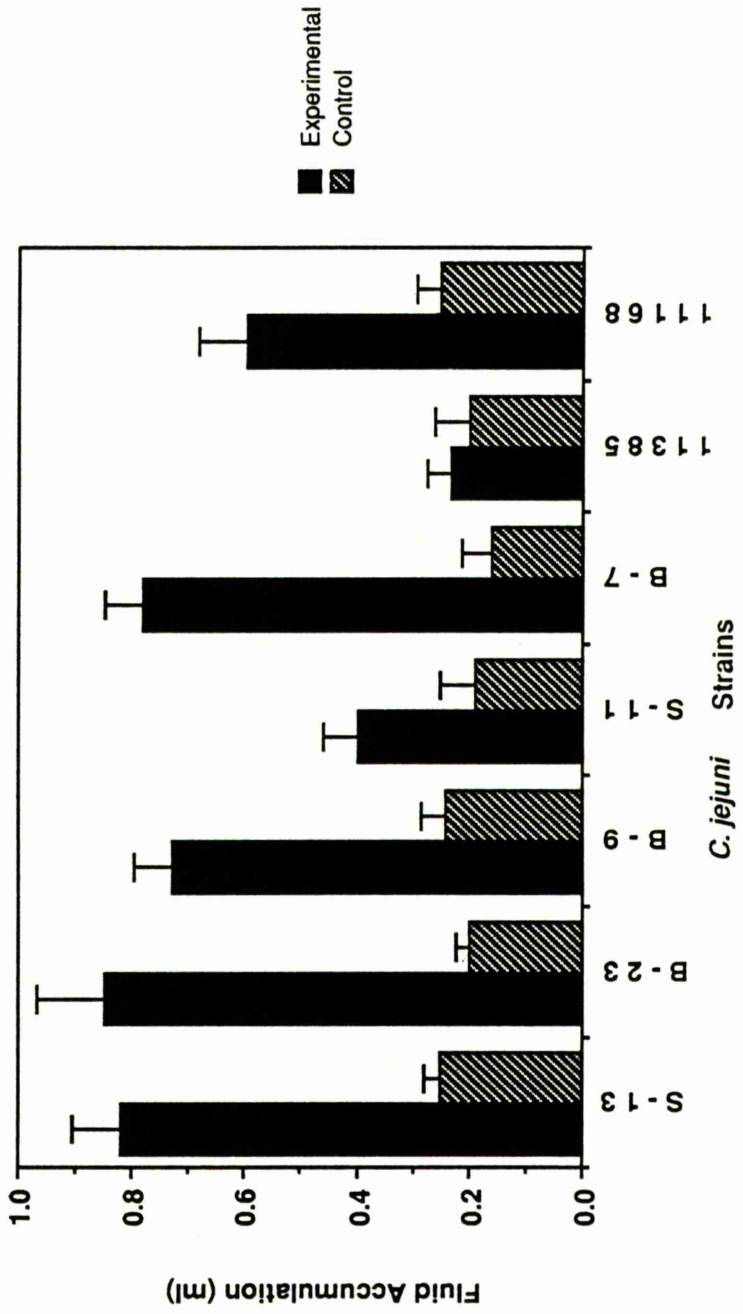
Figures 5-8 show that *C. jejuni* strains produced variable amounts of enterotoxin which ranged from nothing to a high level. Out of the 20 strains tested, only 9 produced detectable amounts of toxin. The production of enterotoxin was significantly associated with the Group C strains ($P < 0.01$; χ^2 test) and the amount of toxin by the group C strains were significantly higher than the Group D strains ($P < 0.05$; unpaired t-test). Seven out of ten Group C strains gave a positive response of variable degree (the range being 12.5-94.78 ng mg^{-1} of protein in ELISA and 3.59-34.39 $TCED_{50}$ mg^{-1} of protein in the CHO cell assay; Figures 5 and 6 respectively).

Figure 3: *C. jejuni* Diarrhoea Model in Infant Chicks

Diarrhoea was induced by oral inoculation of 1.0×10^8 live organisms. The amount of flluid on day 5 post-inoculation per gut of chick was measured. Mean fluid accumulation (+ standard deviation) of 10 to 12 chicks for each *C.jejuni* strain is presented in this figure.

Note that the strain S-11 and 11385 gave a negative response in comparison to the controls in the diarrhoea mode. The amount of fluid induced by rest of the strains was significantly higher in comparison to the control chicks (fed with sterile Brucella broth)

Experimental procedure is described in Section 3.8


Experimental
Control
Fluid Accumulation (ml)
1.0
0.8
0.6
0.4
0.2
0.0
S-13
B-23
B-9
S-11
B-7
11385
11168
C. jejuni Strains

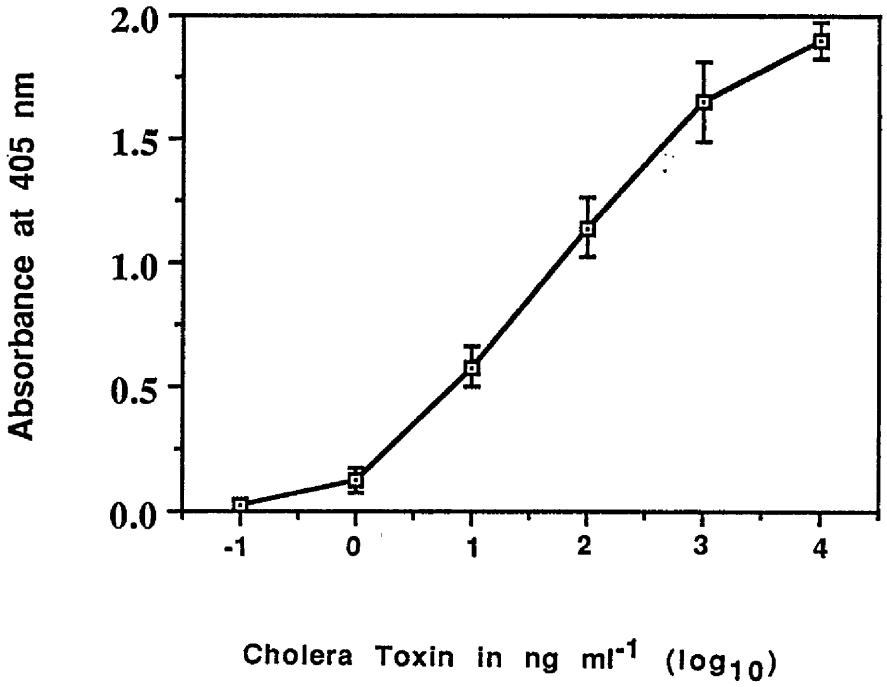


Figure 4: Standard Curve for the Quantitation of *C. jejuni* Enterotoxin

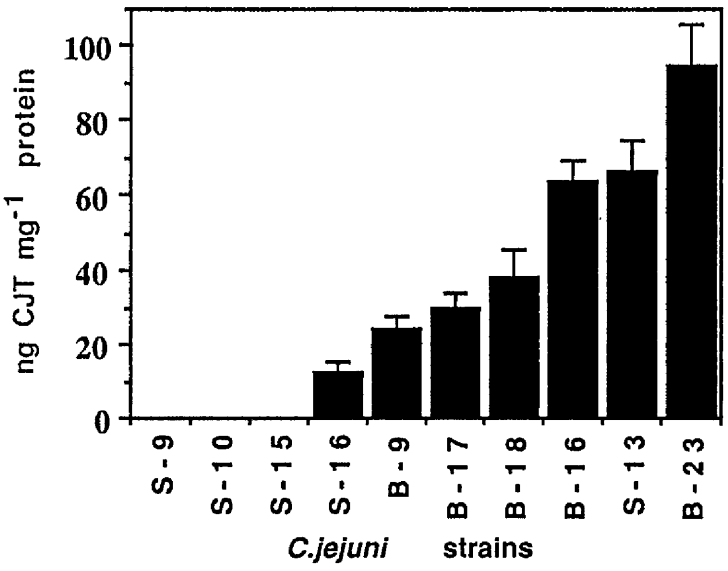
A series of 10-fold dilutions of cholera toxin was used in the GM_1 ganglioside ELISA and absorbances at 405 nm were plotted against the concentration of cholera toxin at each point. Bars represent the standard deviations of two experiments done in triplicate

Figure 5: Quantitation of Enterotoxin Produced by Group C *C.jejuni* Strains by ELISA

The results are presented as mean + standard deviations of two experiments done in triplicate. Experimental procedures are described in Section 3.13.

Figure 6: Quantitation of Enterotoxin Produced by Group C *C.jejuni* Strains by Chinese Hamster Ovary (CHO) Cell Assay

Results are presented as mean + standard deviation of two experiments done in triplicate. Experimental procedures are described in section 3.13.


ng CJT mg-1 protein
100
80
60
40
20
0
S-9
S-10
S-15
S-16
B-9
B-17
B-18
B-16
S-13
B-23
C.jejuni strains

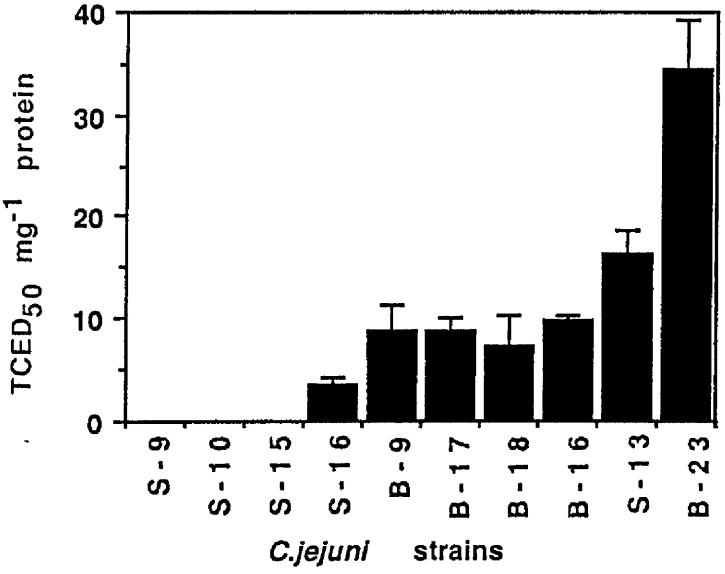
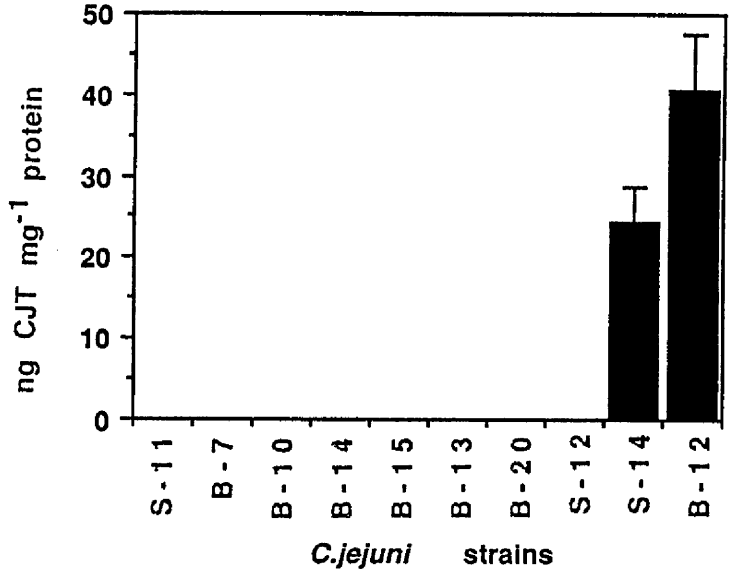

TCED50 mg-1 protein
40
30
20
10
0
S-9
S-10
S-15
S-16
B-9
B-17
B-18
B-16
S-13
B-23
C.jejuni strains

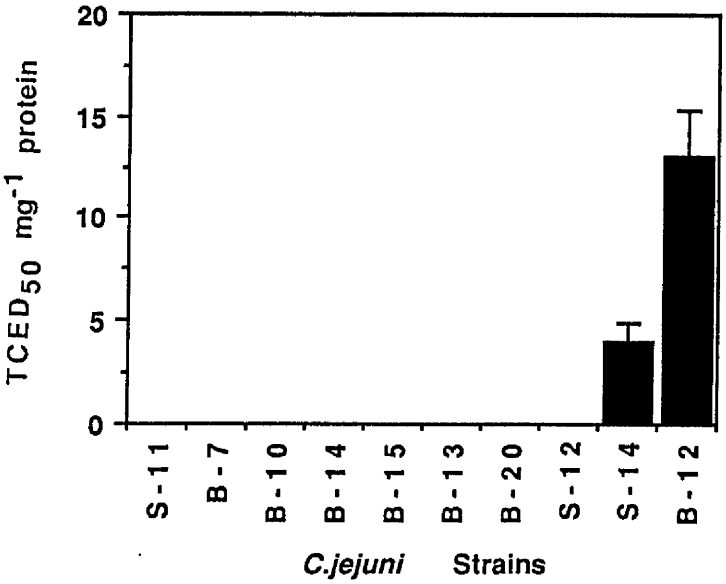
Figure 7: Quantitation of Enterotoxin Produced by Group D *C. jejuni* Strains by ELISA

The results are presented as mean + standard deviations of two experiments done in triplicate. Experimental procedures are described in Section 3.13

Figure 8: Quantitation of Enterotoxin Produced by Group D *C. jejuni* Strains by Chinese Hamster Ovary (CHO) Cell Assay

Results are presented as mean + standard deviation of two experiments done in triplicate. Experimental procedures are described in section 3.13.


50
40
30
20
10
0
ng CJT mg-1 protein
S-11
B-7
B-10
B-14
B-15
B-13
B-20
S-12
S-14
B-12
C.jejuni strains


20
15
10
5
0
TCED50 mg-1 protein
S-11
B-7
B-10
B-14
B-15
B-13
B-20
S-12
S-14
B-12
C.jejuni Strains

On the other hand, only 2 out of 10 Group D strains produced detectable toxin (the range was 24.25-40.60 ng mg^{-1} of protein in ELISA and 3.98-13.12 $TCLD_{50}$ mg^{-1} of protein in the CHO cell assay; Figures 7 and 8 respectively); the production of enterotoxin was not significantly associated with the Group D strains ($P > 0.05$; χ^2 test). The greatest amount of toxin was produced by the strains B-23 and B-9, two Group C strains (Figures 5 and 6).

4.2.3 Effect of Polymyxin B Treatment

Several previous studies suggested that the titre of enterotoxin from *C. jejuni* was increased following the polymyxin B treatment of the cells (Klipstein and Engert; 1984; Johnson and Lior, 1984). Four strains of *C. jejuni,* three from Group C and one from group D, which produced relatively larger amounts of toxin were used to determine the effect of polymyxin B treatment on the enhancement of release of toxin by these strains. A highly significant increase ($P < 0.01$; paired t-test) in the titre of the toxin was observed in all the strains except B-7 which was significant at $P < 0.05$ level (paired t-test) in comparison to the untreated controls as detected by the CHO cell assay (Figure 9). On the other hand, highly significant increase ($P < 0.01$; paired t-test) in toxin titre upon polymxin B treatment of all the strains was noted in ELISA. However, the effect of the polymyxin B on the strains was not uniform; the increase in titre varied from 1.81 to 2.62 fold for the strains B-7 and B-23 respectively, in CHO cell assay. On the other hand, there was from a minimum of 1.72-fold to a maximum of 2.54-fold increase in the CJT titre as determined by the ELISA (Figures 9 and 10).

4.2 4 Immunodiffusion

Immunological relationship between the crude enterotoxins of *C. jejuni* strains (Section 1.7.6.1.6) with purified cholera toxin (CT) were analysed by the Ouchterlony immunodiffusion test using hyperimmune rabbit antiserum against purified CT. Crude

Figure 9 : Effect of Polymyxin B on the Release of Enterotoxin from *C. jejuni* Strains : ELISA

Highly significant ($P < 0.01$) increase in the enterotoxin resulted following Polymyxin B treatment of all the *C. jejuni* strains

Experimental procedures are described in Sections 3.11.1 and 3.13.3

Significance levels (paired t-test) NS = Not Significant

* = $P \leq 0.05$

** = $P \leq 0.01$

*** = $P \leq 0.001$

Figure 10: Effect of Polymyxin B on the Release of Enterotoxin from *C. jejuni* Strains : CHO Cell assay

Highly significant ($P < 0.01$) increase in the enterotoxin resulted following Polymyxin B treatment. of all the strains except the strain B-7, in which the significance level was < 0.05

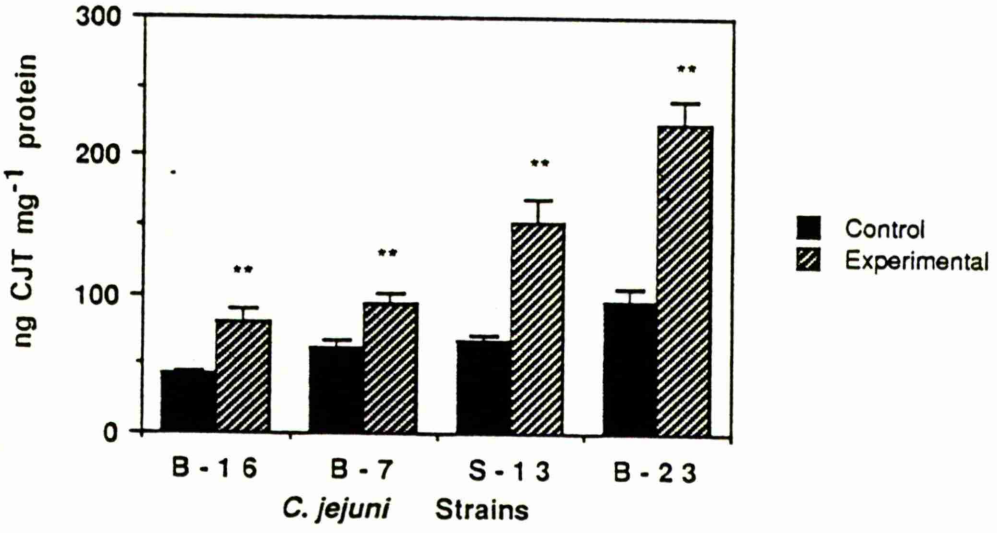
Experimental procedures are described in Sections 3.11.1 and 3.13.3

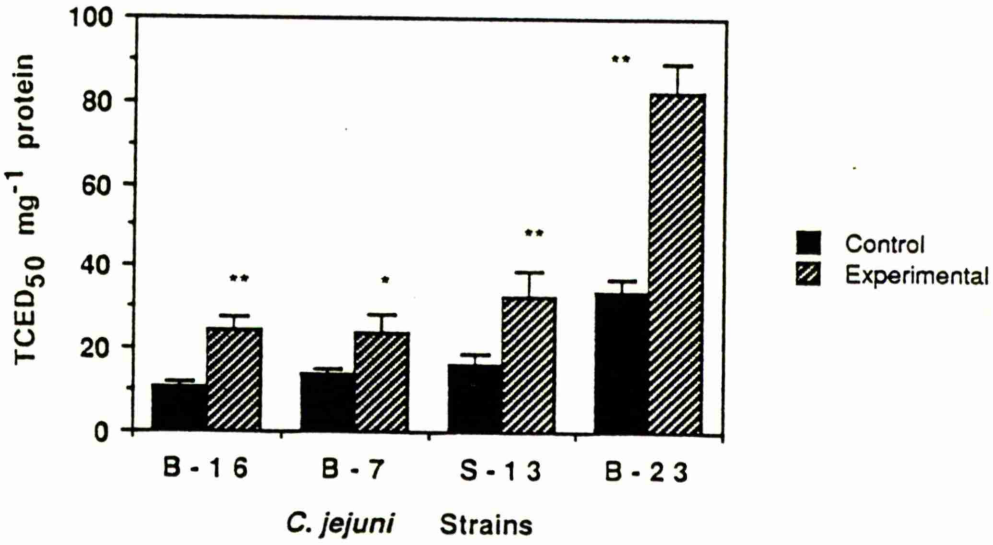
Significance levels (paired t-test) NS = Not Significant

* = $P \leq 0.05$

** = $P \leq 0.01$

*** = $P \leq 0.001$


300
200
100
0
ng CJT mg-1 protein
**
**
**
**
B - 1 6
B - 7
S - 1 3
B - 2 3
C. jejuni Strains
Control
Experimental


100
80
60
40
20
0
TCED50 mg-1 protein
**
*
**
**
B - 1 6
B - 7
S - 1 3
B - 2 3
C. jejuni Strains
Control
Experimental

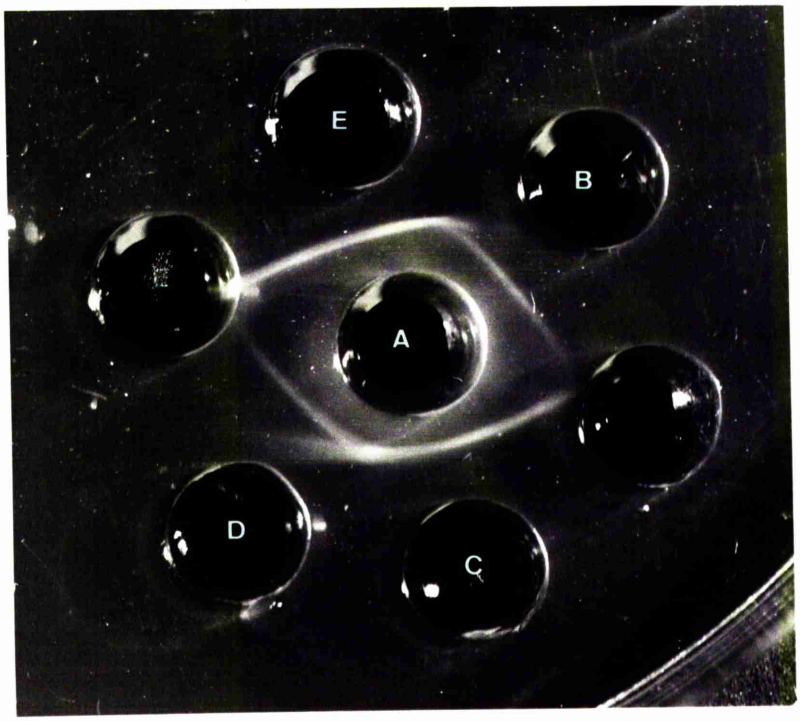


Figure 11: Ouchterlony Immunodiffusion Assay of *C. jejuni* Enterotoxin (CJT) and Cholera Toxin (CT).

Well A: Antisera to CT raised in rabbit

Well B: CJT from strain S-13

Well C: CT from *V. cholerae* strain B-1

Well D: CJT from strain B-23

Well E: CT from *V. cholerae* strain B-1

Note the line of partial identity between CT and CJT. Experimental procedure is described in Section 3.12

C. jejuni enterotoxin reacted with antisera to CT and formed a precipitin line that showed a line of partial identity with purified cholera toxin (CT). Control wells (containing normal saline) showed no such precipitation lines. A typical immunodiffusion experiment showing precipitation lines with spurring between purified CT and *C. jejuni* enterotoxin (CJT) prepared from the strain S-13 is shown in the Figure 11.

4.2.5 Probing of *C. jejuni* Chromosomal DNA with *E. coli* Heat-labile Toxin (LT) Gene Probe

Three *C. jejuni* strains (S-13, B-9 and B-23) which were found to be enterotoxigenic by a variety of *in vitro* (GM_1 ganglioside ELISA and CHO cell assay) and *in vivo* assay (fluid accumulation in the ligated intestinal loop of infant chickens and induction of diarrhoea in new-born chicks) and one strain negative in all these tests (S-11) were examined for DNA sequence homology with *E. coli* LT B subunit gene probe. Chromosomal DNA of the *C. jejuni* strains were isolated according to the method of Bradbury *et al* (1984), digested with the restriction enzyme *Hind*III and Southern blotted to nitrocellulose paper according to Southern (1975). As no hybridization was observed in experiments carried out under high stringency, hybridizations were later performed under low stringent conditions. However, no hybridization was observed even under low stringency between *C. jejuni* chromosomal DNA fragments and *E. coli* LT gene probe. Gene probing experiments were carried out with kind help of Dr. John Coote.

4.2.6 Ileal Loop Test for Fluid Accumulation in Infant Chicks

The capacity of the *C. jejuni* strains to induce fluid accumulation in the ligated ileal loops of 5-day-old chickens was evaluated. *Vibrio cholerae* strain NCTC 10732 and crude cholera toxin (NIH, Bethesda, Maryland) were used as positive controls. Ileal loop experiments were carried out by Dr. D. E. S. Stewart-Tull. The results of two *C. jejuni* strains (S-13 and B-23), their enterotoxin preparations, and positive and negative

Figure 12: Fluid Accumulation in the Ileal Loops of 5-day-old Chicks

The fluid accumulation (ml / cm) is shown as the mean and standard deviation of the group of 4-6 chicks.

Experimental procedure is described in Section 3.11

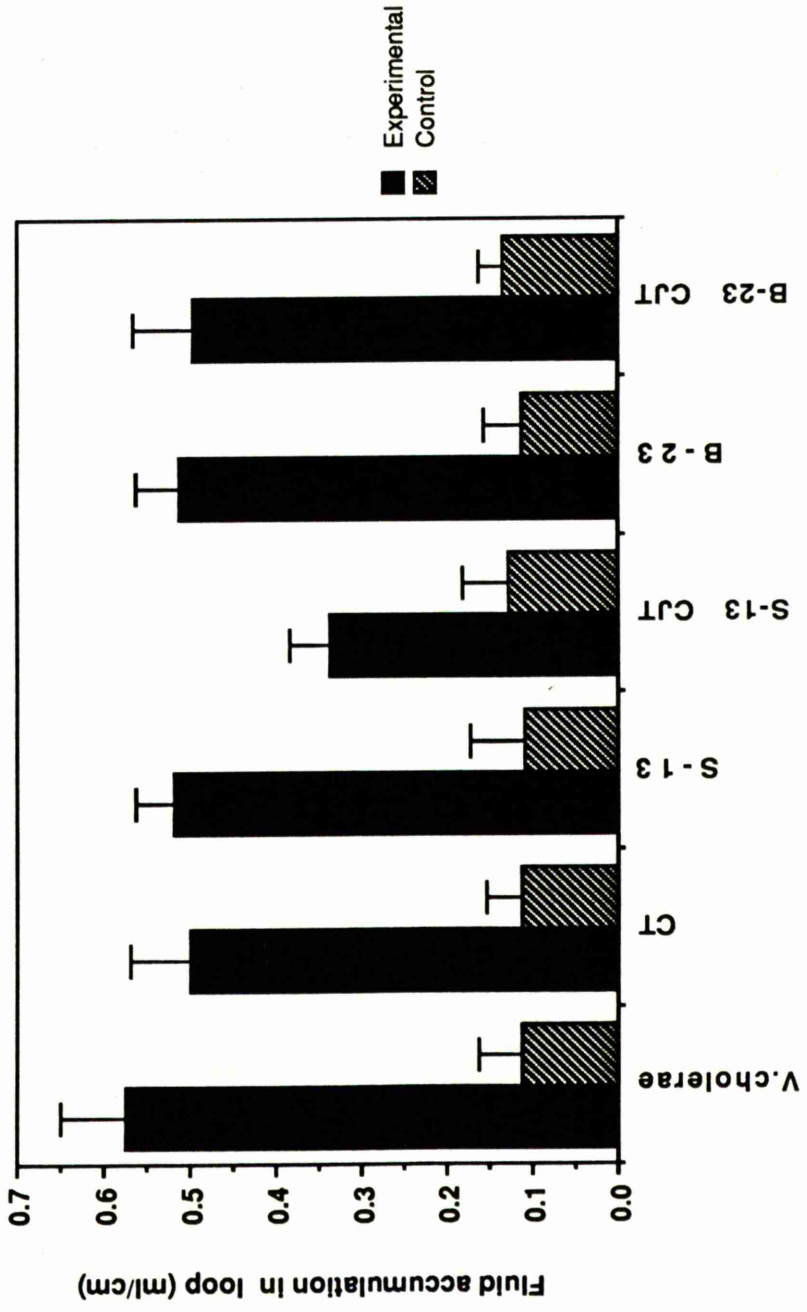

Experimental
Control
Fluid accumulation in loop (ml/cm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
V.cholerae
CT
S-13
S-13 CUT
B-23
B-23 CUT

Figure 13 : Positive Fluid Accumulation Test in 5-day-old Chick Challenged with *C. jejuni* Strain S-13.

E = Experimental loop

C = Control loop

Experimental procedure is described Section 3.11.

Figure 14 : Positive Fluid Accumulation Test in 5-day-old Chick Challenged with *C. jejuni* Strain B-23.

E = Experimental loop

C = Control loop

Experimental procedure is described Section 3.11.

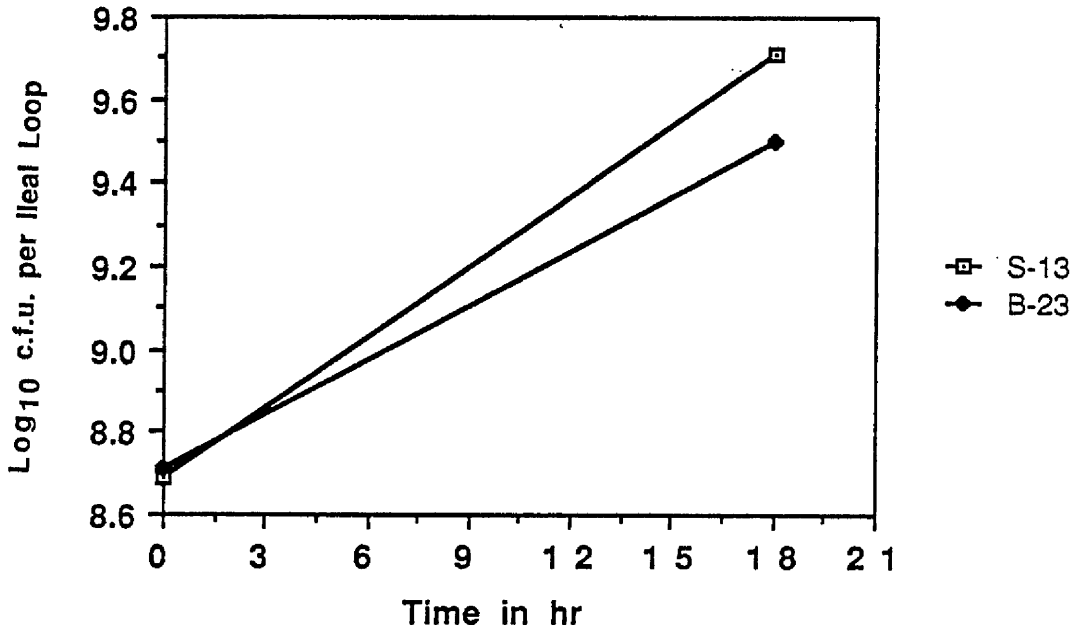


Figure 15: The Differences Between the Growth of the *C. jejuni* Strains S-13 and B-23 in the ileal Loops of the 5-day-old Chicks.

Experimental procedure is described in Section 3.11.5

control samples are presented in the Figure 12 as the mean and standard deviation of the loop ratio (volume / length ratio) values expressed in millilitres of fluid per centimetre of intestine in 6 or 4 chicks. As the loop ratio of the negative control loops was consistently < 0.150 ml cm^{-1}, the chick intestinal loops were considered positive if the volume to length ratio was ≥ 0.30. Negative loops were similar to the normal gut and positive fluid accumulations were characterized by tightly distended loops, filled with turbid fluids. The loop ratios were highly significant ($P < 0.001$; paired t-test) for all the samples (Figure 12) except S-13 CJT which was significant at the level of $P < 0.05$ (paired t-test). Typical positive and negative loops in the 5-day-old chick intestines are exhibited in Figures 13 and 14 for *C. jejuni* strains S-13 and B-23 respectively. Quantitative bacteriology indicated that both the strains S-13 and B-23 efficiently multiplied in the chicken intestinal loops (Figure 15).

4.3 Haemolysin Production by *C. jejuni* Strains

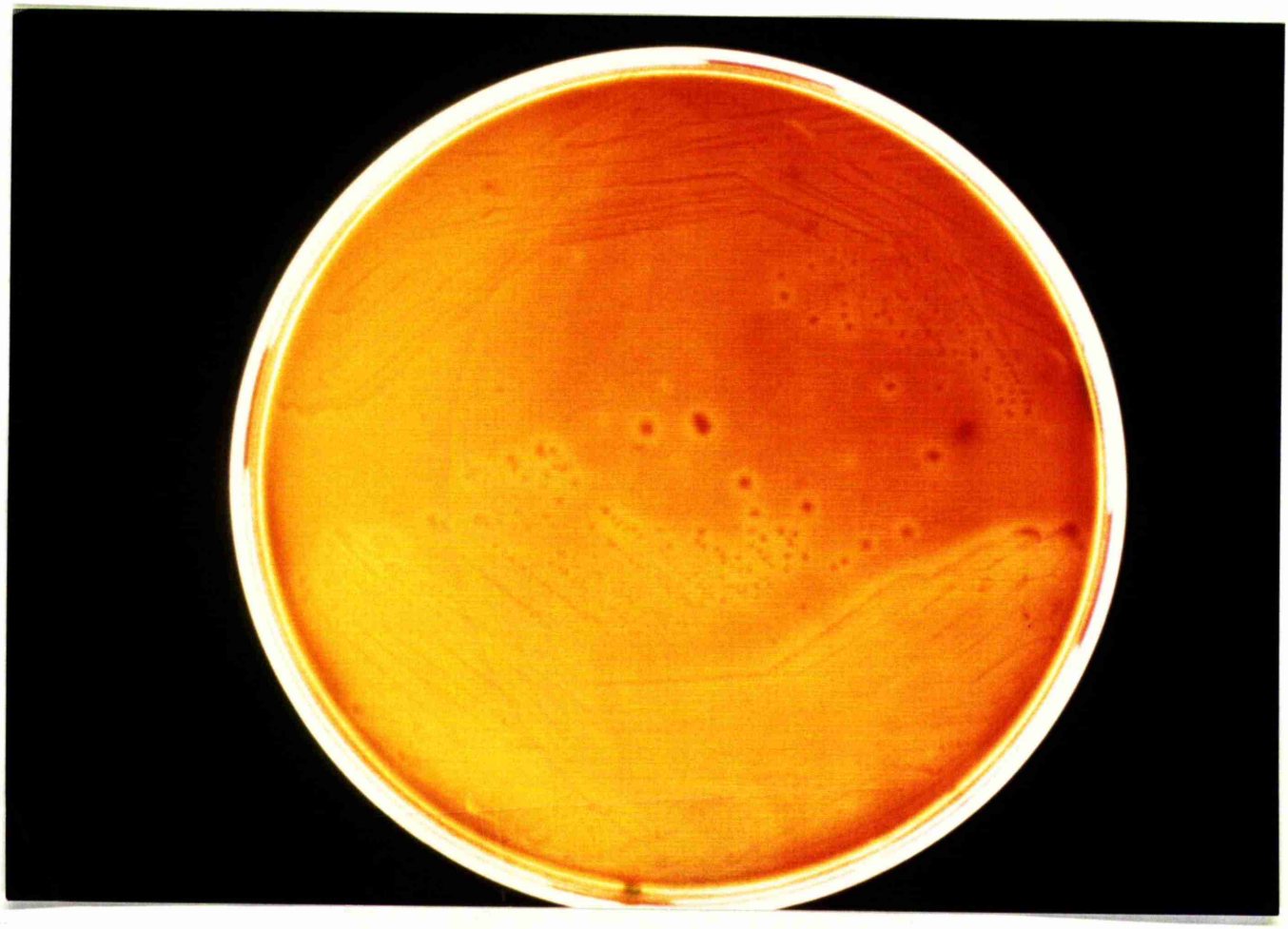
4.3.1 Survey of Strains

A total of 28 clinical *C. jejuni* isolates (Table 5) were examined for the production of a haemolysin active against rabbit erythrocytes; among which 18 (64.28 %) were found to exhibit haemolytic activity in the culture filtrates. The results were expressed as haemolytic units (HU) mg^{-1} and are presented in Table 7. Some of the strains included in this survey could not be assigned to Group C or Group D as the clinical history of these strains was not available (Table 5). The haemolytic activity was predominantly associated with the Group D strains (80 %); contrariwise, only 40 % of the group C strains were haemolytic. The titre of the haemolytic activity ranged from a minimum of 9.09 HU mg^{-1} protein (strain S-16) to 32.1 HU mg^{-1} protein against RRBC (strain S-11) (Table 7).

4.3.2 Haemolytic Spectrum

The cell-free supernates of the *C. jejuni* strains were tested against erythrocytes from 7 different animals, human blood groups 'A', 'B' and 'O' and chicken. Rabbit

S-16



S-11

Figure 16: Photograph Showing Haemolytic Colonies of *C. jejuni* Strains.

Strongly haemolytic strain S-11

Weakly haemolytic strain S-16

The strains were streaked on Brucella blood agar plates and incubated for 36 hr at 42° C under microaerophilic atmosphere (Section 3.4.1).

erythrocytes were the most sensitive to the haemolytic factor of *C. jejuni* and these were lysed by all the positive isolates. The strains showed varied degree of activity against erythrocytes from other animals (Table 7). Hence, further experiments were carried out using rabbit erythrocytes. Two strains S-10 and B-9 showed convincing lysis of erythrocytes on Brucella blood agar plates (containing sheep blood) but failed to show any detectable haemolysis of sheep or rabbit erythrocytes in a tube assay with culture filtrates. Haemolytic factors from two strains S-11 (a Group D strain) and S-13 (a Group C) strain were used for further characterization.

4.3.3 Thermal Inactivation of Haemolysin

The haemolysins of two isolates, S-11 and S-13 were stable for 30 min when incubated at temperatures between 4^{o} C and 55^{o} C prior to assay at 37^{o} C. At higher temperatures a gradual loss of activity was observed and at 60^{o} C ca. 50 % and 75 % of the original activity were lost by S-11 and S-13 haemolysins respectively (Figure 16) Haemolytic activity of the strain S-13 was completely lost upon heating at 80^{o} C. On the other hand, the undiluted haemolytic factor of the strain S-11 retained 6.25 % of the original activity even when heated at 100^{o} C for 10 min (Figure 17).

4.3.4 Proteinaceous Nature of the Haemolysin

The haemolytic factor of S-11 was precipitated with ammonium sulphate {80 % (w/v) saturation} which resulted in a 4-fold increase in titre. The haemolytic activity of undiluted culture filtrates of the strains S-11 and S-13 was completely lost following incubation with trypsin (1.0 mg ml^{-1}) for 30 min at 37^{o} C (Table 8). These results indicate the proteinaceous nature of the haemolysin.

Table 7: Haemolytic Spectrum of the *C.jejuni* Strains

Haemolytic activity of culture filtrates was determined against 1.0 % (v/v) washed erythrocytes from different species.

The results are expressed as haemolytic units (HU) mg^{-1} of protein. One haemolytic unit was defined as the reciprocal of the highest dilution causing lysis of 50 % of erythrocytes.

C. jejuni Strains	Human Blood Group			Horse	Cow	Sheep	Rabbit	Guinea pig	Rat	Mouse	Chicken
	A	B	O								
S-1	9.09	9.09	18.18	4.54	4.54	9.09	18.18	9.09	2.72	1.36	0
S-4	10.10	10.10	20.20	10.10	5.05	5.05	20.20	2.52	5.05	2.52	0
S-5	8.88	8.88	8.88	8.88	4.44	4.44	17.77	4.44	8.88	8.88	0
S-6	6.41	6.41	12.82	6.41	3.20	6.41	12.82	12.82	1.60	1.60	0
S-8	19.90	19.90	19.90	4.95	9.95	9.95	19.90	4.95	2.48	4.95	0
S-11	16.06	16.06	16.06	16.06	8.03	32.12	32.1	16.06	4.01	8.03	2.0
S-13	15.26	15.26	15.26	15.26	7.63	15.26	30.5	15.26	7.63	7.63	1.90
S-14	9.0	9.0	9.0	9.0	4.50	9.0	18.01	9.0	2.25	4.50	0
S-15	8.33	8.33	16.66	8.33	4.16	8.33	16.66	8.33	0	0	0
S-16	2.27	2.27	2.27	0	0	2.27	9.09	0	0	0	0
B-7	31.0	31.0	31.0	15.50	31.0	31.0	31.0	7.75	3.87	7.75	0
B-10	8.69	8.69	8.69	4.34	8.69	8.69	17.39	4.34	4.34	2.17	0
B-11	11.42	11.42	11.42	5.71	5.71	5.71	22.85	5.71	2.85	2.85	0
B-12	12.12	12.12	12.12	6.06	6.06	3.03	24.24	3.03	3.03	3.03	0
B-13	11.11	11.11	11.11	5.55	5.55	5.55	22.22	2.75	2.75	2.75	0
B-16	9.75	9.75	9.75	4.87	4.87	4.87	19.51	2.43	2.43	2.43	0

Titres of C. jejuni haemolysin
against a range of erythrocytes.

C. jejuni Clinical isolate	Haemolysin titres against erythrocytes from: Human group			Horse	Cow	Sheep	Rabbit	Guinea pig	Rat	Mouse	Chicken	C
	A	B	O									
S1	40	80	80	40	80	80	80	80	40	40	20	2(
S4	80	40	80	40	80	80	80	80	40	40	20	2(
S5	40	40	40	40	80	40	160	80	40	40	10	2(
S6	80	80	80	40	40	40	80	80	40	40	10	2(
S7	40	40	40	40	80	40	160	40	80	40	10	2(
S8	40	40	40	40	80	80	160	80	40	40	10	2(
S11	160	160	80	80	160	160	320	160	80	80	20	4(
S12	80	80	80	80	160	80	160	160	80	80	20	2(
S13	160	160	80	80	160	160	320	160	80	80	20	8(
S14	80	80	40	40	80	80	160	80	40	40	20	4(
S15	80	80	40	40	80	80	160	80	80	40	20	2(
S16	80	80	80	80	80	80	80	80	40	40	10	2(
B10	80	80	80	40	80	80	160	80	80	80	20	2(
B11	40	80	40	80	80	80	160	80	40	40	20	2(
B12	40	80	40	40	40	80	80	80	40	40	10	2(
B13	40	80	40	40	40	80	80	40	40	40	10	20
B16	40	80	80	80	160	80	320	80	40	40	20	40
S2,S3,S9,S10*, B9,B14,B15	0	0	0	0	0	0	0	0	0	0	0	0

This strain had a titre of 5 against rabbit erythrocytes after precipitation with 90% aturated $(NH_4)_2SO_4$. All organisms were grown in Brucella broth containing protease pepto 1%). These results were from duplicate experiments.

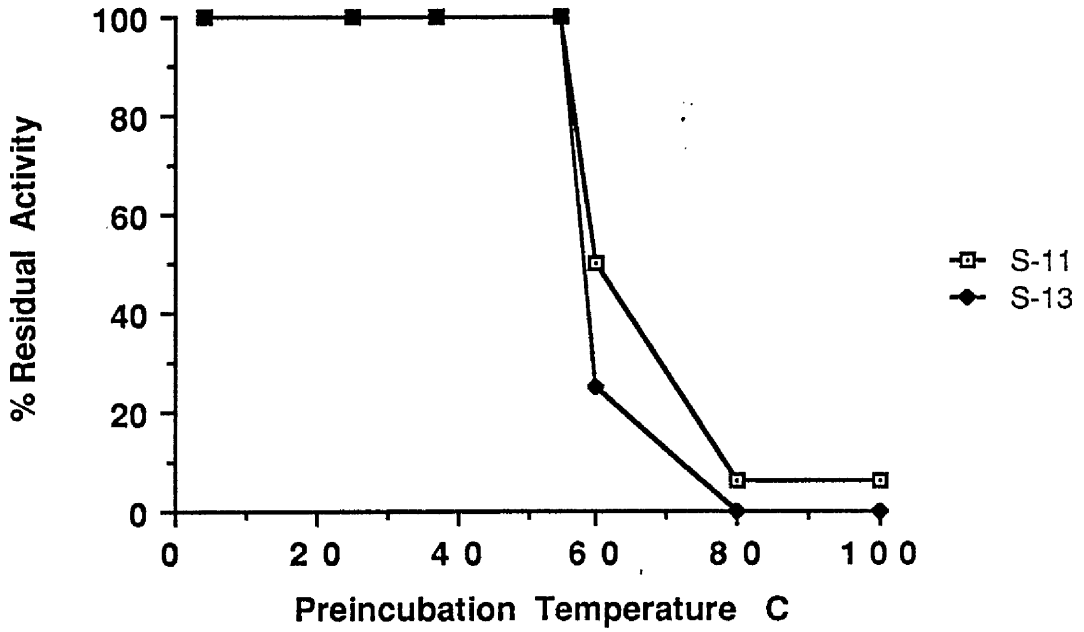


Figure 17: **Effect of Heat on the Activity of the Haemolysin from the *C. jejuni* Strains S-11 and S-13.**

Table 8: Effects of Chemical and Enzymatic Treatments on the Haemolytic Activity of *C. jejuni*.

	Haemolysins from *C. jejuni* Strains	
	S-11	**S-13**
Untreated	28.2	25.91
Trypsin Treatment [a]	0	0
$(NH_4)_2SO_4$ [b] Precipitation	114.49	102.60

Haemolytic activity is expressed as Haemolytic units (HU) mg^{-1} of protein (Section 4.3.1)

a : Incubation with trypsin (1.0 mg ml^{-1}) final concentration at 37° C for 1 hr (Section 4.3.4)

b : $(NH_4)_2SO_4$ used at 80 % saturation (Section 4.3.4)

4.3.5 Effect of Erythrocyte Concentration on Haemolytic Activity

The concentration of erythrocytes in the test system influenced the titre of the haemolysin from the strain S-11. A fixed quantity of haemolysin (1.0 HU) was added to each of different concentrations of rabbit erythrocytes (0.2 % to 4.0 %) and the percentage of cell lysis was determined spectrophotometrically. At lower erythrocyte concentrations (0.2 % to 0.5 %), there was 100 % lysis and the percentage of unlysed erythrocytes increased with the increasing concentration of erythrocytes (Figure 18)

4.3.6 Kinetics of Lysis of Erythrocytes

Kinetic of erythrocyte lysis experiments (Figures 19, 20, 21 and 22) showed that the haemolysin preparation used was very rapid in lysing the rabbit erythrocytes (1.0 % v/v) and erythrocytes of human blood groups 'A', 'B' and 'O' (0.5 % v/v). With 1.0 % rabbit erythrocytes approximately 50 % of the cells were lysed within only 30 sec. Lag phases of different durations, none exceeding 1 min were observed with different erythrocytes especially with haemolysin preparations with low HU. Human blood groups 'A', 'B' and 'O' exhibited a similar lytic pattern but lysis was relatively more rapid with erythrocytes from blood group 'O'. There was very little or no apparent lag phase against erythrocytes (0.5 %) of human blood group 'O' and lysis occurred almost instantaneously.

4.3.7 Isoelectric Focusing and Zymogram Analysis of Haemolysin

Analytical isoelectric focusing experiments with the concentrated culture supernates (adjusted to a protein concentration of 1.0 mg ml^{-1}) of the *C. jejuni* strains S-11 and S-13 were done in polyacrylamide gels. The position of the haemolysin band was located on the polyacrylamide gels by zymogram analysis with an overlay of rabbit erythrocytes (2.0 % v/v) in agarose of one half of the gel and comparing with the Coomassie blue stained other half of the gel in which samples were applied at identical positions (Figures 23 and 24). The pI of the

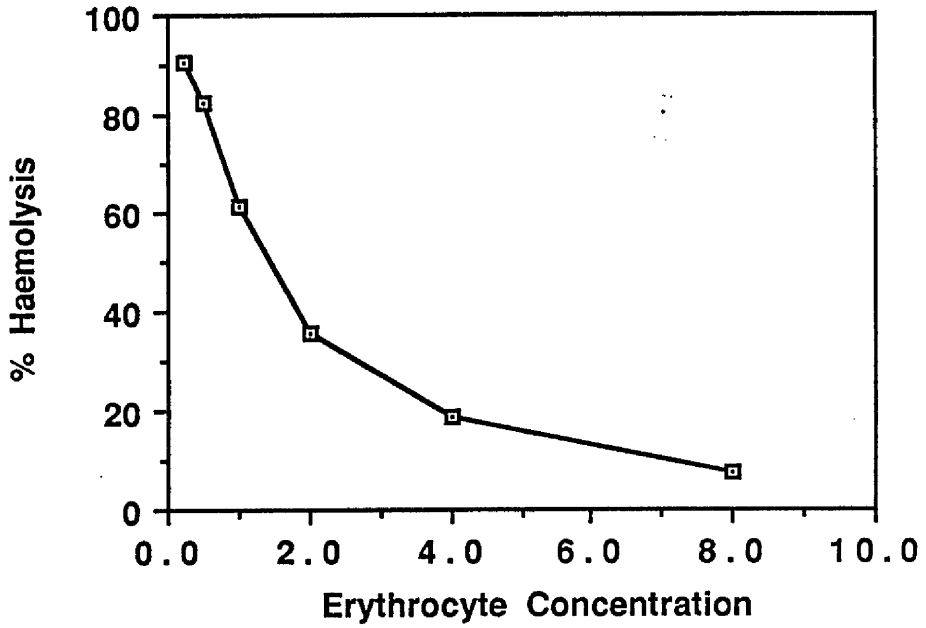


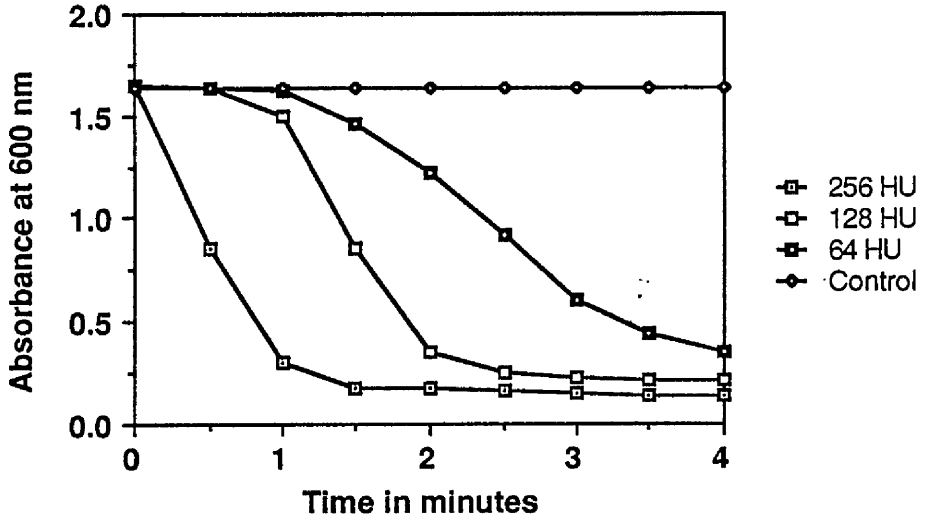
Figure 18: Effect of Different Concentrations of Rabbit Erythrocytes on the Haemolytic Activity

Figure 19: Kinetics of Lysis of Rabbit Erythrocytes by *C. jejuni* Strain S-11 Haemolysin as a Function of Haemolysin Concentration.

Experimental procedure is described in Section 3.7.3.

Figure 20: Kinetics of Lysis of Human Blood Group 'O' Erythrocytes by *C. jejuni* Strain S-11 Haemolysin as a Function of Haemolysin Concentration.

Experimental procedure is described in Section 3.7.3.


2.0
1.5
1.0
0.5
0.0
Absorbance at 600 nm
0
1
2
3
4
Time in minutes
256 HU
128 HU
64 HU
Control

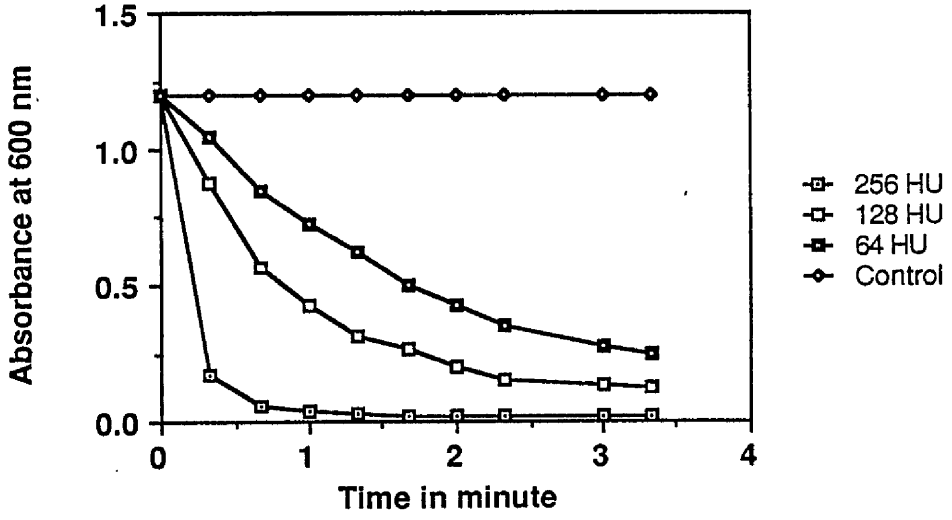
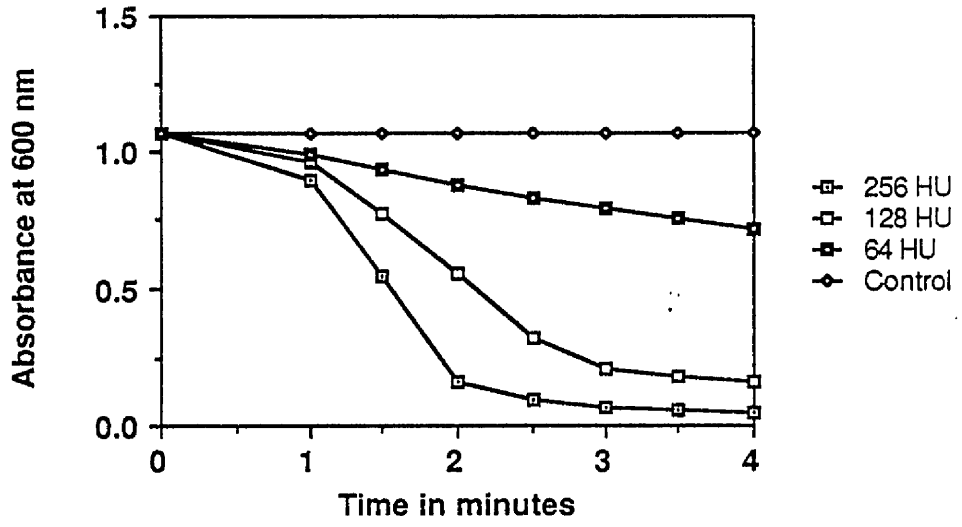

1.5
1.0
0.5
0.0
Absorbance at 600 nm
0
1
2
3
4
Time in minute
256 HU
128 HU
64 HU
Control

Figure 21: Kinetics of Lysis of Human Blood Group 'A' Erythrocytes by *C. jejuni* Strain S-11 Haemolysin as a Function of Haemolysin Concentration.

Experimental procedure is described in Section 3.7.3.

Figure 22: Kinetics of Lysis of Human Blood Group 'B' Erythrocytes by *C. jejuni* Strain S-11 Haemolysin as a Function of Haemolysin Concentration.

Experimental procedure is described in Section 3.7.3.


1.5
1.0
0.5
0.0
Absorbance at 600 nm
0
1
2
3
4
Time in minutes
256 HU
128 HU
64 HU
Control

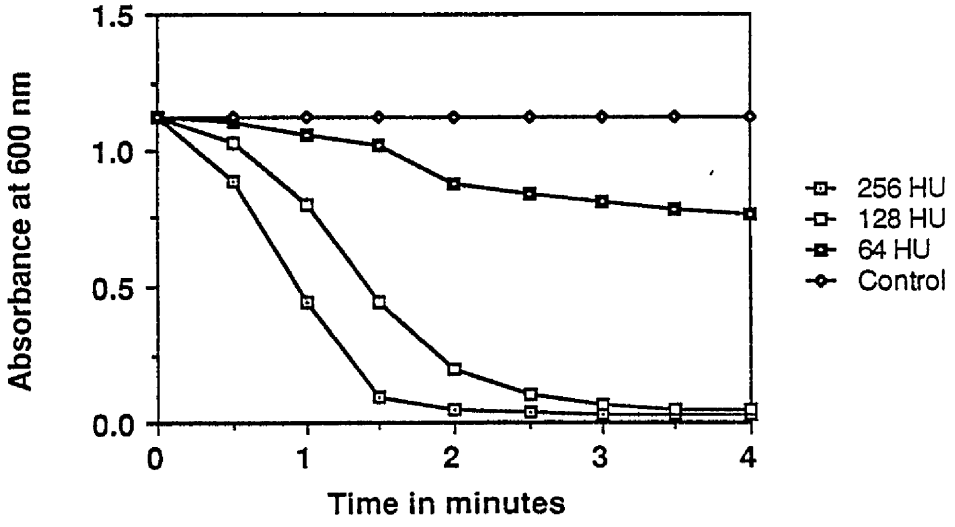

1.5
1.0
0.5
0.0
Absorbance at 600 nm
0
1
2
3
4
Time in minutes
256 HU
128 HU
64 HU
Control

Figure 23 : Analytical thin-layer isoelectric focusing of *C. jejuni* haemolysin.

Distance migrated by the different pI markers from cathode (at the left of the gel) is plotted aginst their pI values.

Lane A : pI markers

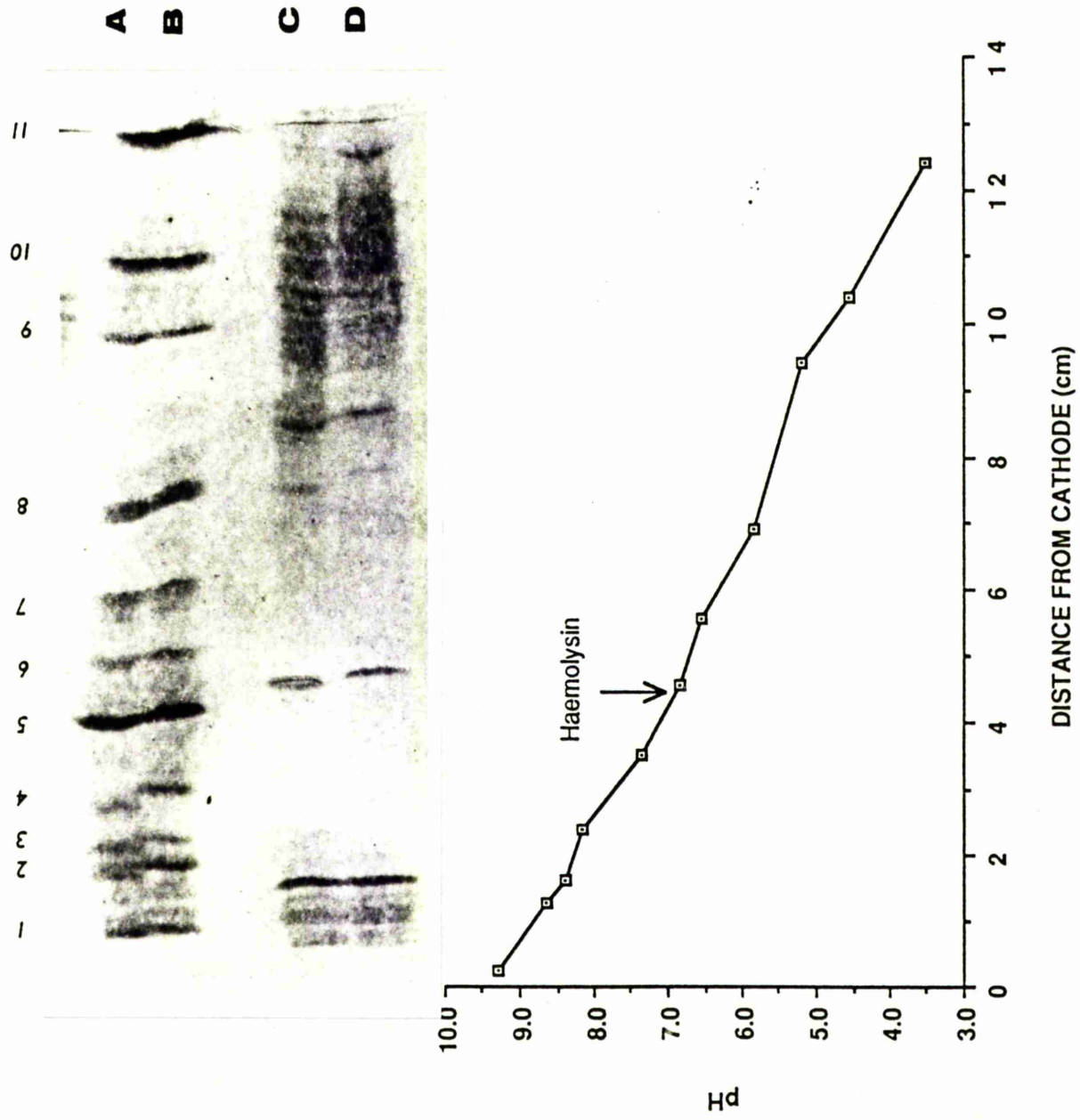
Lane B : pI markers

Lane C : Crude haemolysin from strain S-13 (20 μg)

Lane D : Crude haemolysin from strain S-11 (20 μg)

Name	pI values
1. Trypsinogen	3.50
2. Lentil lectin (basic)	4.55
3. Lentil lectin (middle)	5.20
4. Lentil lectin (acidic)	5.85
5. Horse myoglobin (basic)	6.55
6. Horse myoglobin (acidic)	6.85
7. Human carbonic anhydrase B	7.35
8. Bovine carbonic anhydrase B	8.15
9. ß-Lactoglobulin A	8.45
10. Soyabin trypsin inhibitor	8.65
11. Amyloglucosidase	9.30

Experimental procedures for isoelectric focusing and staining of the gel are described in Section 3.19.


A
B
C
D
11
10
9
8
7
6
5
4
3
2
1
Haemolysin
pH
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
0
2
4
6
8
10
12
14
DISTANCE FROM CATHODE (cm)

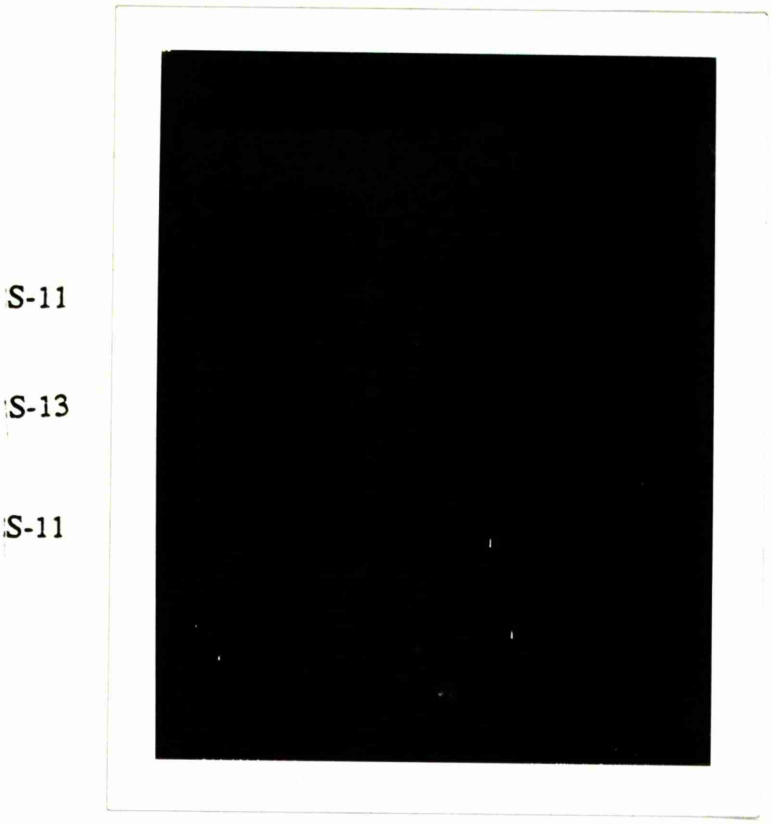


Figure 24: Zymogram Analysis to Determine the Isoelectric Point of the *C. jejuni* Haemolysin

Experimental procedure is described in Section 4.3.7

haemolysins thus determined was ca. 6.7-6.8.

4.3.8 Cytotoxic Effects of Haemolysin in HeLa Cell Monolayer

C. jejuni haemolysin exerted a cytotoxic effect on Hela cell monolayers; this could be reproducibly demonstrated by incubating culture filtrates with Hela cells for 18 hr. Hela cells lost their typical morphology , became round and partially detached from the plastic surface. The titre of the cytotoxic factor was however low as at 1: 8 or 1:16 dilution, the effect was virtually abolished. Cytotoxic effects of the haemolysins from the *C. jejuni* strains S-11 and S-13 on HeLa cells are shown in the Figures 25 and 26. Control HeLa cells (treated with sterile Brucella broth) are shown in Figure 27.

4.4 Cell Surface Hydrophobicity

Bacterial adherence to hydrocarbon (BATH) test (Rosenberg *et al,* 1980) and Salt aggregation (SA) test (Lindahl *et al,* 1983) are two widely used tests for determining cell surface hydrophobicity of microorganisms. In this study BATH and SAT were use to determine hydrophobic properties of the *C. jejuni* strains isolated from patients with cholera-like watery diarrhoea (Group C strains) and dysentery-like mucoid diarrhoea (Group D strains).

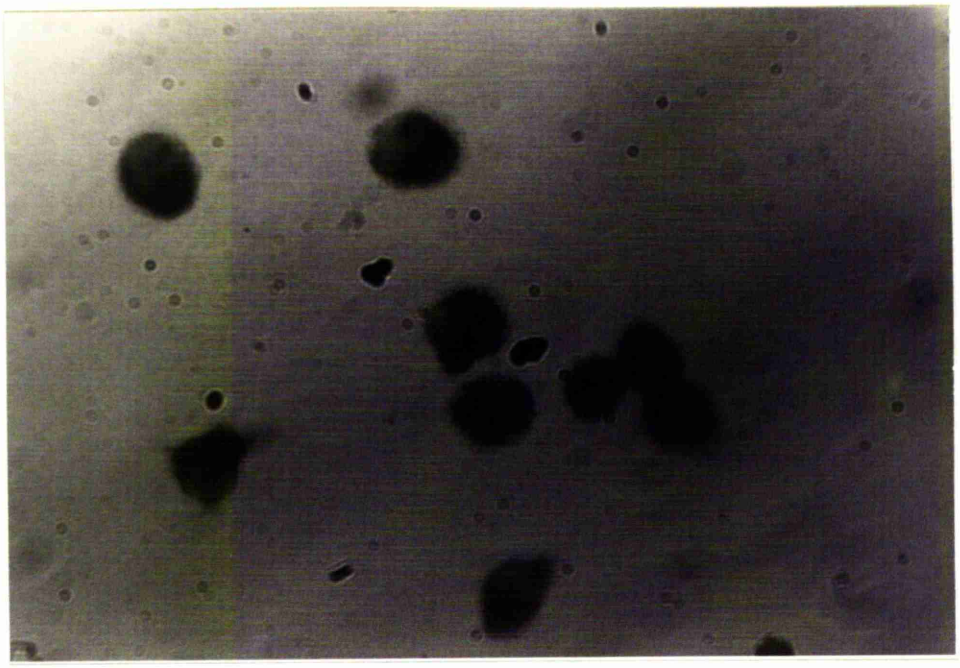
4.4.1 BATH Test: Results obtained from the BATH test with n-Octane as the hydrophobic phase is presented in the Table 23. All the *C. jejuni* strains tested partitioned preferentially into the aqueous phase indicating the relatively less hydrophobic nature of the strains. However, it may be noted that the strains isolated from dysentery-like, bloody mucoid diarrhoea (Group D strains) were relatively more hydrophobic (Mean $\pm$ SD = 22.09 $\pm$ 11.95) in comparison to the strains isolated from cholera-like watery diarrhoea (Group C strains; Mean $\pm$ SD = 13.0 $\pm$ 8.76), which was however not statistically significant ($P > 0.05$; paired t-test). When the proportion of the n-Octane was increased, the strains showing more hydrophobicity were

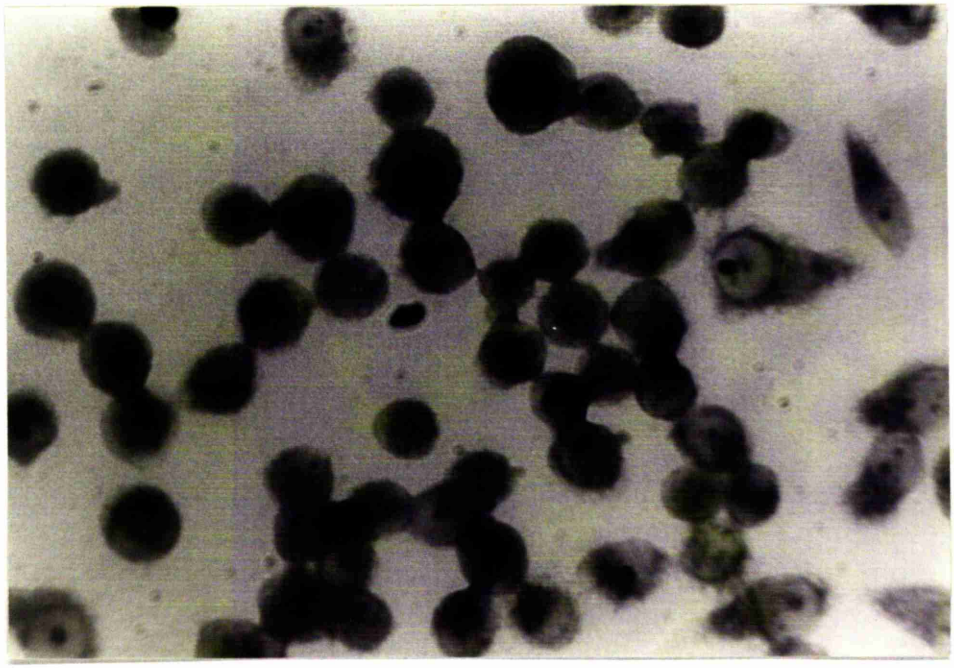
Figure 25: Cytotoxic Effects of *C. jejuni* S-11 Haemolysin on HeLa Cells.

Experimental procedure is described in Section 3.7.6

Figure 26: Cytotoxic Effects of *C. jejuni* S-13 Haemolysin on HeLa Cells

Experimental procedure is described in Section 3.7.6





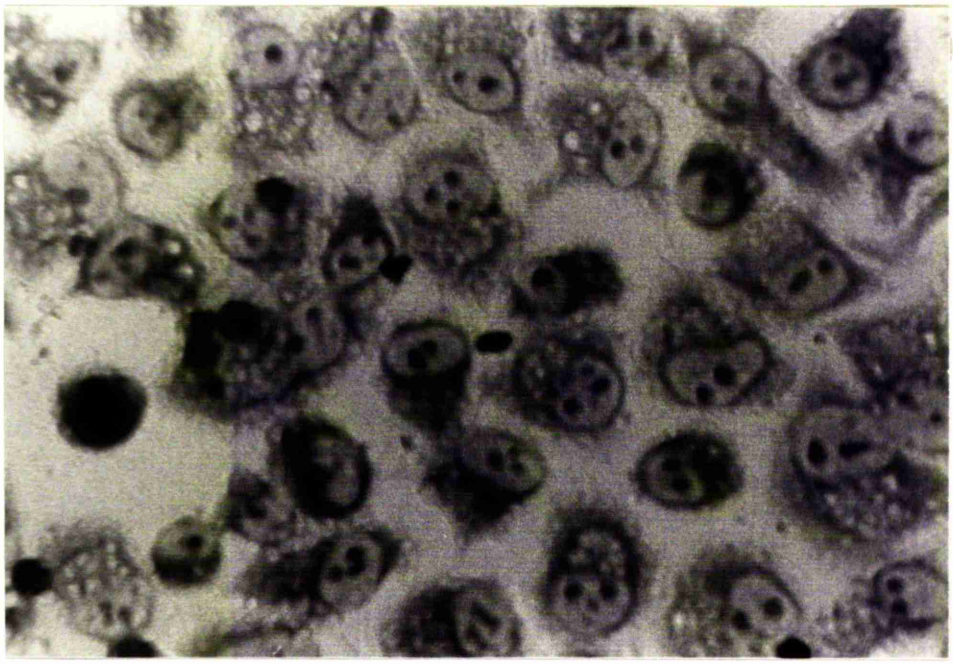


Figure 27: Control HeLa Cells treated with Brucella broth.

Experimental procedure is described in Section 3.7.6

Table 9: Cell-Surface Hydrophobicity of the *C. jejuni* strains: Salt Aggregation (SA) Test and Bacterial Adherence to Hydrocarbon (BATH) Test.

Experimental procedures are described in Sections 3.20.2 and 3.20.1 respectively.

BATH Test values are expressed as mean $\pm$ standard deviation of two experiments done in duplicate. The figures represent the proportion of bacteria partitioned in the hydrocarbon phase.

SA Test values represent the lowest test concentration of $(NH_4)_2SO_4$ required for aggregation. Mean values of two independent experiments are presented.

	C.jejuni Strains	SA Test	BATH Test
GROUP C STRAINS	B-9	3.5	2.46 ± 0.96
	B-16	1.5	15.95 ± 2.13
	B-17	2.5	7.38 ± 1.68
	B-18	2.5	19.19 ± 3.91
	B-23	3.5	3.25 ± 0.92
	S-9	3.0	8.20 ± 2.62
	S-10	1.0	21.30 ± 3.24
	S-13	1.5	30.25 ± 4.36
	S-15	2.5	13.16 ± 3.01
	S-16	2.5	8.94 ± 1.96
GROUP D STRAINS	B-7	1.0	35.02 ± 5.13
	B-10	2.5	19.74 ± 3.26
	B-12	2.5	10.21 ± 2.31
	B-13	2.5	21.33 ± 2.46
	B-14	1.0	37.45 ± 4.81
	B-15	1.5	27.49 ± 3.28
	B-20	2.0	7.79 ± 1.12
	S-11	1.0	38.72 ± 4.92
	S-12	2.0	9.87 ± 1.06
	S-14	3.0	13.36 ± 2.24

increasingly removed from the aqueous phase to the hydrocarbon phase, while the turbidity of the strains showing less hydrophobicity were not significantly affected.

The effect of heat on hydrophobicity was investigated with two strains showing higher hydrophobicity and two strains with lower hydrophobicity, the results are presented in Figures 28 and 29. Heating resulted in the decrease of hydrophobiciity of all the strains tested; strains B-23 and B-9 lost hydrophobicity completely as no decrease in the optical density of the aqueous phase was noted, even with higher (O.5 ml) n-octane.

4.1.2 Salt Aggregation (SA) Test : The SA test values for the Group C and Group D strains are presented in the Table 23. The SA test values for the Group C strains ranged from 1.5-3.5 M (Mean $\pm$ SD = 2.40 $\pm$ 0.84). On the other hand the Group D strains had values ranging from 1.0-3.0 M (Mean $\pm$ SD = 1.90 $\pm$ 0.73) indicating that in this test as well, the Group D strains were relatively more hydrophobic than the Group C strains which was however not statistically significant ($P > 0.05$; paired t-test)

When the results of the two tests that were employed for determining hydrophobicity were compared, both the tests correlated well with each other when the *C. jejuni* strains were tested on a group basis i.e. the group which were more hydrophobic in the BATH test were also more hydrophobic in the SA test and vice-versa. However, when the individual strains were compared the tests did not always correlate (Table 9). For example, strain S-14, which was more hydrophobic than the strain S-12 in the BATH test had higher SA test values in the SAT, indicating it was less hydrophobic than the strain S-12. Moreover, some other strains, for example B-13, B-17, S-15, B-18, S-16, B-10, B-14, and B-12 which had identical SAT values had different BATH test values. These results indicate that the two tests probably measure different characteristics, both of which is related to hydrophobicity.

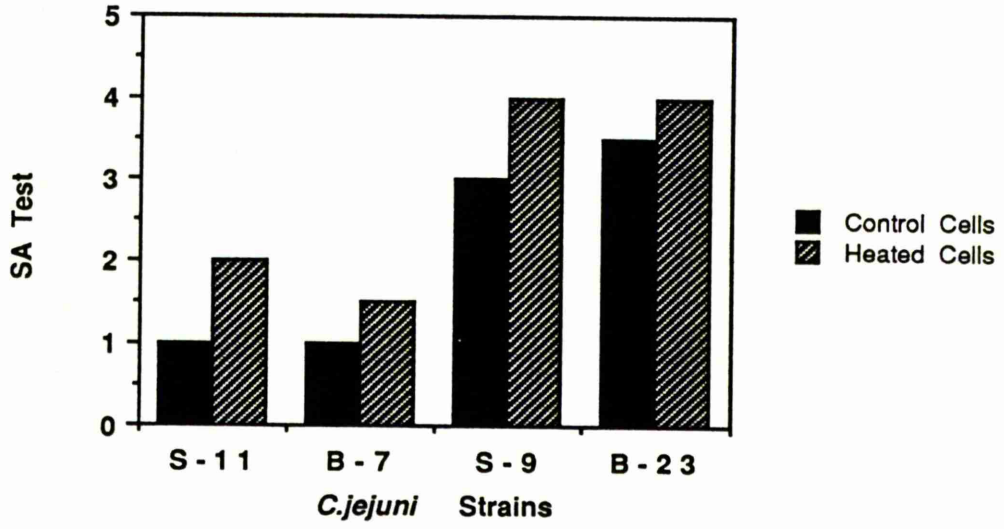
Figure 28: Effect of Heat on the Cell-Surface Hydrophobicity of the *C. jejuni* Strains: SA Test

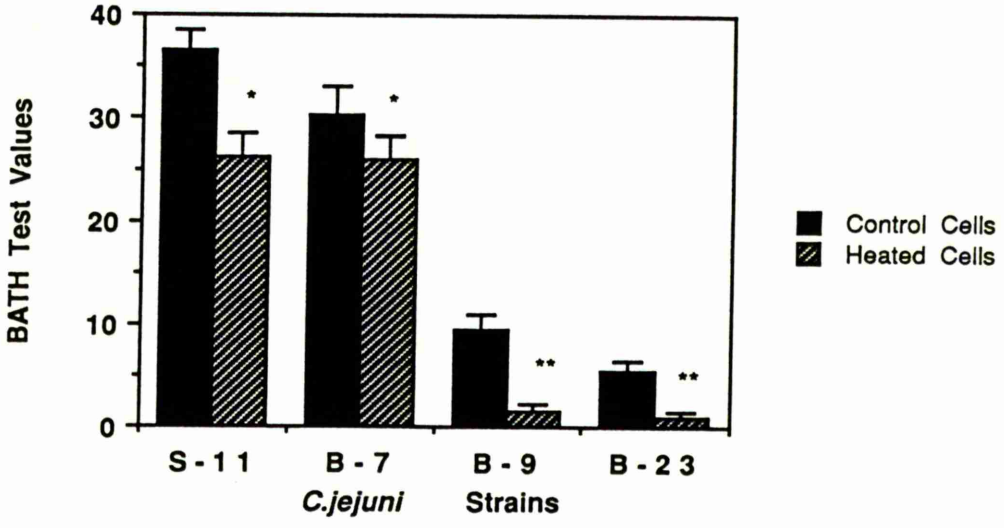
Bacterial suspensions were held at 100° C for 5 min before assay (Section 3.20.2). The results represent the mean of two experiments.

Figure 29: Effect of Heat on the Cell-Surface Hydrophobicity of the *C. jejuni* Strains: BATH Test

Bacterial suspensions were held at 100° C for 5 min before assay (Section 3.20.2). The results represent the mean and standard deviation of two experiments done in duplicate. Differences in hydrophobicity between heated and control cells were evaluated by paired t-test

Significance levels: * = $P \leq 0.05$
** = $P \leq 0.01$


SA Test
5
4
3
2
1
0
S - 1 1
B - 7
S - 9
B - 2 3
C.jejuni Strains
Control Cells
Heated Cells


BATH Test Values
40
30
20
10
0
*
*
**
**
S - 1 1
B - 7
B - 9
B - 2 3
C.jejuni Strains
Control Cells
Heated Cells

4.5 Congo Red Binding by *C. jejuni* Strains

All the strains of *C. jejuni* included in this study (Table 5) were capable of binding the dye Congo Red (CR) when grown on Brucella agar containing 30 μg ml^{-1} (BA-CR) and produced red colonies (CR^+). Some colourless or pale orange colonies (CR^-) were seen in almost all plates amongst the predominantly red (CR^+), which were deficient in the property of dye uptake.

4.5.1 Optimal Dye Uptake Conditions

The concentration of the dye, incubation time and incubation temperature were found to be critical in discriminating between the CR^+ and CR^- colonies. Incubation at 42° C for 36 hours on Brucella agar containing 30 μg ml^{-1} of dye was optimal for distinguishing the CR^+ and CR^- colonial variants. When incubated at 37° C, longer periods of incubation (48-60 hours depending upon the strain) were necessary for maximal dye uptake; whereas incubation at 42° C for the same duration resulted in dye-binding by all colonies and the differences between CR^+ and CR^- became less apparent. The use of a higher dye concentration (50 μg ml^{-1} or more) also made it difficult to distinguish between CR^+ and CR^- colonial variants.

4.5.2 Colonial Morphology of CR^+ and CR^- Variants

All the *C. jejuni* strains when streaked on BA-CR plates gave rise to three types of colonies:- Type 1: compact colonies with a deep red centre and a pale red edge (CR^+); Type 2: compact colonies slightly larger than the Type 1 colonies with pale orange to colourless in appearance (CR^-); Type 3: irregular, diffuse and spreading colonies which spread along the direction of sreaking and were light orange in colour (Figure 30). The type 3 colonies which appeared to be CR^- while growing on BA-CR as they looked pale orange were found to be capable of binding the same amount of dye as the CR^+ colonies in a liquid CR binding assay (Payne and Finkelstein, 1977). The apparent CR^- appearence of the type C colonies were probably due to

Figure 30: Photograph showing the CR^+ and CR^- colonial variants of the *C.jejuni* strain S-11.

Colony type 1: CR^+

Colony type 2: CR^-

Colony type 3: CR^+

The test strain was streaked on a Brucella agar plate containing Congo red dye (30 $\mu g\ ml^{-1}$) and incubated for 36 hr at 42° C under microaerophilic atmosphere. The colony types are described in Section 4.5.2.

their spreading nature although the amount of the dye bound per cell was the same as the type 1 CR^+ colonies. The binding of the dye by the bacterial cells appeared to be rapid and irreversible as once bound (by suspending the *C. jejuni* cells in PBS containing 30 $\mu g\ ml^{-1}$ CR for 3 minutes), the dye could not be released from the cells by repeated vortexing and examination of the supernate for dye release after spinning the mixture in a microfuge.

4.5.3 Stability of the CR^+ and CR^- Cells

Once isolated, the CR binding properties appeared to be fairly stable because upon subculturing only about 5 % of the colonies reverted from CR^+ to CR^- and vice-versa. The typical colonial morphology of Type 1, 2 and 3 was maintained when subcultured on Brucella agar plates without the dye. The CR^+ and CR^- colonial variants were identical in all standard biochemical characteristics such as hippurate hydrolysis, oxidase and catalase test and were stable to the standard storage condition [at -70^{o} C in Brucella broth containing 15-20% (v/v) glycerol].

4.5.4 Comparative Studies with CR^+ and CR^- Colonial Variants

The CR^+ and CR^- colonial variants were compared in terms of various putative virulence factors and cell surface hydrophobicity in an attempt to identify whether any of these properties could be correlated with the observed change of colonial morphology.

4.5.4.1 Comparison of Lipopolysaccharide Profiles: The proteinase K digested whole cell lysate LPS profiles (Hitchkock and Brown, 1983) of the colonial variants were identical, showing the typical rough type at the bottom of the gel upon silver-staining following SDS-PAGE (Figure 32).

4.5.4.2 Comparison of Outer Membrane Protein Profiles: Interestingly, differences with 3 protein bands were seen when the OMP profiles were compared after

isolation by the method of solubilization of the inner membrane by Sarkosyl and separation of the OMP's by SDS-PAGE. The CR^+ variants had two extra bands with apparent molecular weight of 63 K dal and 87 K dal in comparison to the CR^- colonial variants. The CR^- variants on the other hand, had an extra protein band with molecular weight of 16 K dal which was absent with the CR^+ cells (Figure 31). So it appears that the transition of CR^+ colonies to CR^- was accompanied by the loss of two proteins bands and gain of one protein band.

4.5.4.3 Comparison of Plasmid Profile and Drug Resistance: Plasmids were isolated from CR^+ and CR^- colonial variants according to the method described by Takahashi *et al* (1984) and compared by agarose gel electrophoresis. No detectable difference in the plasmid profile of the CR^+ and CR^- variants were noted. The colonial variants also did not show any difference in the susceptibility to a range of antibiotics (Oxoid Multodisc 725E and 1788E).

4.5.4.4 Electron Microscopy : Electron microscopic observation of the CR^+ and CR^- cells after negative-staining revealed that CR^- variants were devoid of flagella, whereas the CR^+ variants possessed typical polar flagella at both ends (Figures 33 and 34). Upto 100 cells (of each variant) were observed in different fields to evaluate the actual distribution of the flagellate and nonflagellate cells among the CR^+ and CR^- morphotypes. Approximately 2-3 % of the CR^+ cells did not have any flagella (depending upon strain) but none of the CR^- cells had any flagella.

4.5.4.5 Motility: The CR^+ and CR^- variants were grown under different growth conditions such as solid medium, in liquid mediem (at different phases of growth), at different temperaturesa (37^o C and 42^o C), in different growth medium (Brucella agar with or without the dye Congo Red) and observed under phase contrast microscopy to investigate the stability of the observed presence of flagella with the CR^+ and the

Figure 31: SDS-PAGE Outer Membrane Protein (OMP) Profile of the CR^+ and CR^- Colonial variants of the *C. jejuni* Strain S-11.

Lane A: CR^+ grown on BA-CR plates (Type-1 colony)

Lane B: CR^- grown on BA-CR plates (Type-3 colony)

Lane C: CR^+ grown on BA-CR plates (Type-2 colony)

Lane D: CR^- grown on BA-CR plates (Type-3 colony)

Lane E: CR^+ grown on BBA plates (Type-1 colony)

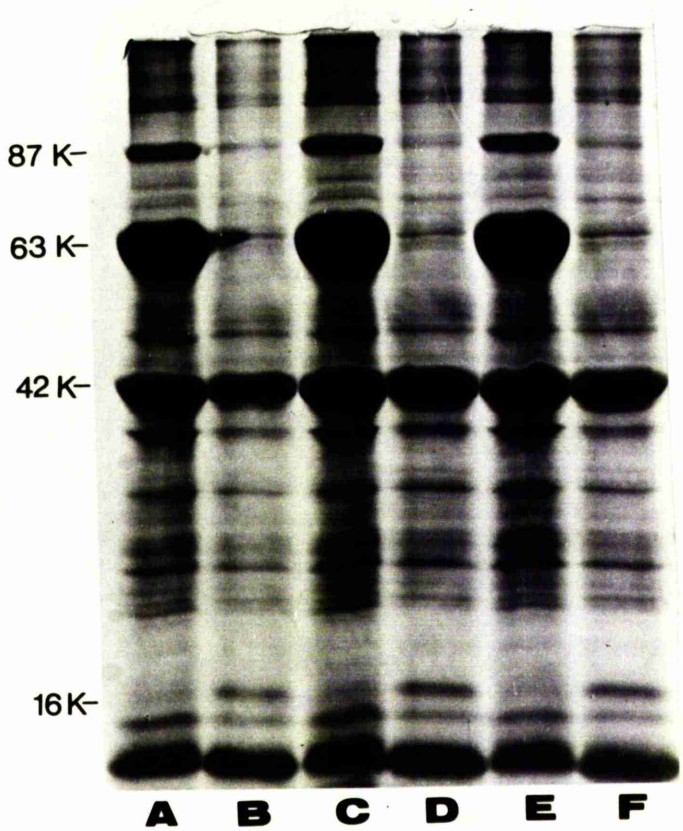
Lane F: CR^- grown on BBA plates (Type-3 colony)

BA-CR = Brucella agar containing Congo Red (30 $\mu g\ ml^{-1}$)

BBA = Brucella blood agar containing sheep blood (7 %, v/v)

Note that transition from CR^+ cells to CR^- cells was accompanied by loss of two protein bands (ca. 87 and 63 K dal) protein bands. CR^- cells had an additional ca. 16 K dal protein which was absent in the CR^+ counterparts. There was no apparent difference in OMP profile between Type I and Type II colonies.

Cathode is at the top of the gel. Methods for isolation of OMP and SDS-PAGE are described on Sections 3.15 and 3.17.3 respectively. The colony types are described in Section 4.5.2.


87 K–
63 K–
42 K–
16 K–
A
B
C
D
E
F

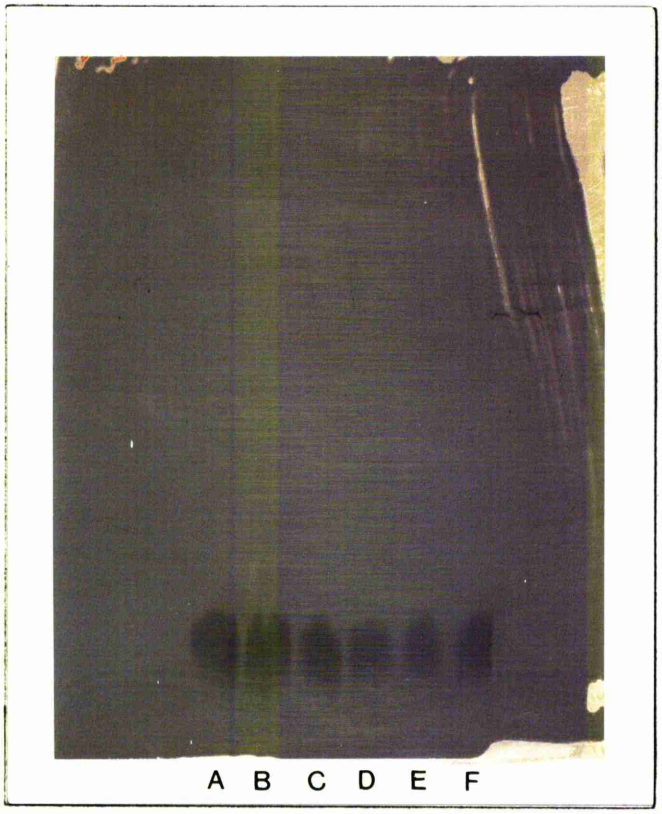


Figure 32: SDS-PAGE Lipopolysaccharide (LPS) Profile of the CR^+ and CR^- Colonial Variants of the *C.jejuni* Strains S-11, S-13 and B-23.

Lane A: S-11 CR^+
Lane B: S-11 CR^-
Lane C: S-13 CR^+
Lane D: S-13 CR^-
Lane E: B-23 CR^+
Lane F: B-23 CR^-

Note that there was no difference in the LPS profile among the CR^+ and CR^- colonial variants of the *C.jejuni* strains.Cathode is at the top of the gel. Methods for preparation of wholecell protenase K digested lysates for LPS and SDS-PAGE is described on Section 3.17.5.

Figure 33: Electron Micrograph of a Negatively Stained CR^+ Cell of *C. jejuni* Strain S-11. Magnification x 80,000

Figure 34: Electron Micrograph of a Negatively Stained CR^- Cell of *C. jejuni* Strain S-11. Magnification x 82,000

absence of flagella with the CR^- cells correlates with the typical *Campylobacter* motility. The CR^+ cells uniformly exhibited typical *Campylobacter* darting motility, whereas the CR^- variants were non-motile.

4.5.4.6 Quantitative Binding of Congo Red: The quantitative CR dye-binding assay revealed that the amount of dye bound by the CR^+ cells of all the strains taken together was significantly higher ($P < 0.001$; paired t-test) than the amount of dye bound by their CR^- colonial variants (Figure 35). The quantitative dye-binding capacity varied from a minimum of 1.27-fold for the strain B-23 to a maximum of 3.8-fold by the strain B-7 (Figure 35). The CR dye-binding capacity did not show any particular association with Group C or Group D strains.

4.5.4.7 Lethality in Chicken Embryo Model: Marked differences in lethality were noted between the paired CR^+ and CR^- variants when compared in the 11-day-old chicken embryo model (Tables 10 to 20). Irrespective of the clinical history of the *C. jejuni* strains (whether Group C or Group D) the CR^+ variants were more virulent than the CR^- counterpart; the LD_{50} values ranged from 10.90 to 120-fold higher for the CR^- variants in comparison to the corresponding CR^+ variant depending upon strain (Table 21). No signifiant differences were observed between the type1and type 3 CR^+ variants when compared in the lethality model using the 11-day-old chicken embryo.

4.5.4.8 Adherence and Invasion of HeLa Cells by CR^+ and CR^- Cells: The results of the comperative adherence and invasion potential of the CR^+ and CR^- variants of the *C. jejuni* strains are presented in Figure 36. The CR^- colonial variants of all the *C. jejuni* strains tested S-11, S-13, B-23 and B-7 exhibited a significantly reduced capacity for adherence to and invasion of HeLa cells.

4.5.4.9 Cell Surface Hydrophobicity: CR^+ and CR^- variants were compared for

Figure 35: Congo Red (CR) Dye Binding of the CR^+ and CR^- Colonial Variants of the *C. jejuni* Strains.

The results presented are the mean and standard deviation of two experiments done in duplicate. Experimental procedure is described in Section 3.22.2.

Significance levels (Paired t-test): NS = Not Significant

* = $P \leq 0.05$

** = $P \leq 0.01$

*** = $P \leq 0.001$

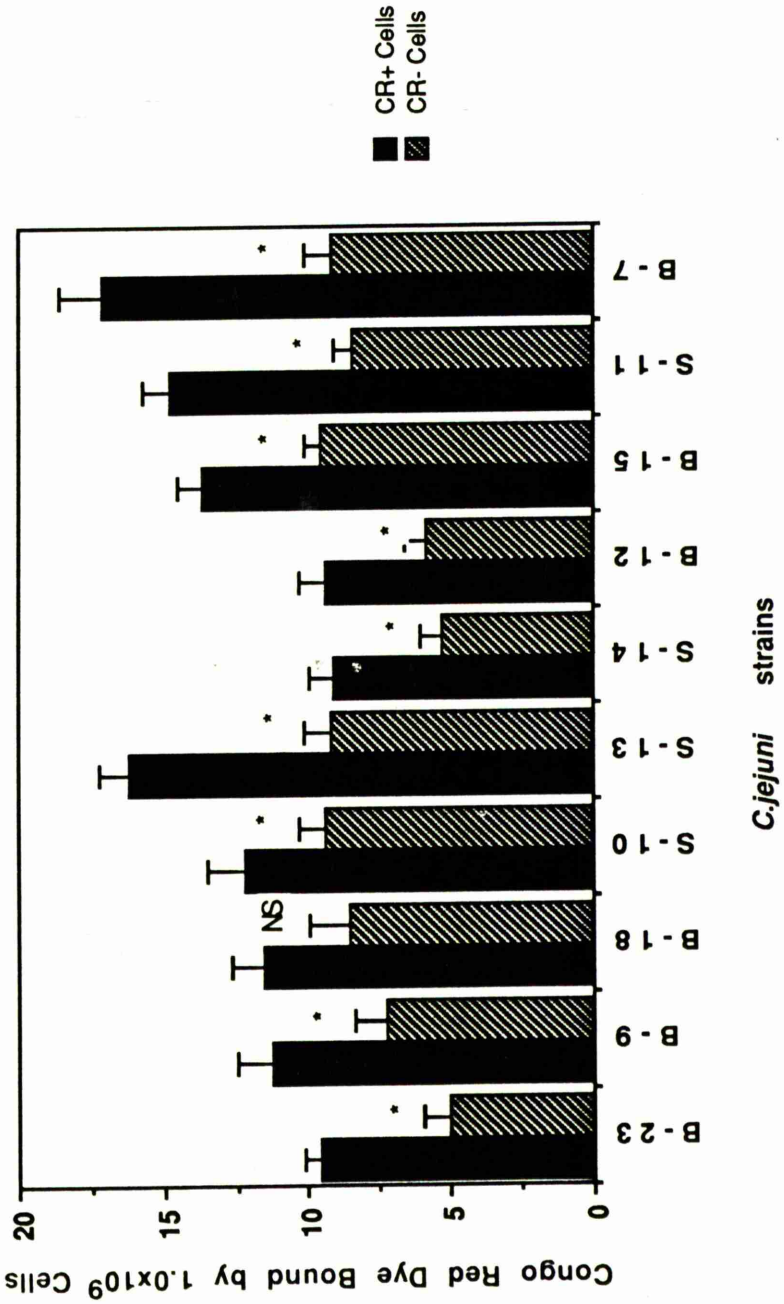

CR+ Cells
CR- Cells
Congo Red Dye Bound by 1.0x10⁹ Cells
0
5
10
15
20
B-23
B-9
B-18
S-10
S-13
S-14
B-12
B-15
S-11
B-7
NS
*
C.jejuni strains

Figure 36: Adherence and Invasion Potential of CR+ and CR- Colonial Variants of the *C. jejuni* Strains in the HeLa Cell Model

A = Adherence potential of CR^+ Cells
B = Adherence potential of CR^- Cells
C = Invasion potential of CR^+ Cells
D = Invasion potential of CR^- Cells

Experimental procedures are described in the Section 3.21.2. The results represent the mean and standard deviation of two experiments done in quadruplicate

Significance levels (Paired t-test): * = $P \leq 0.05$
** = $P \leq 0.01$

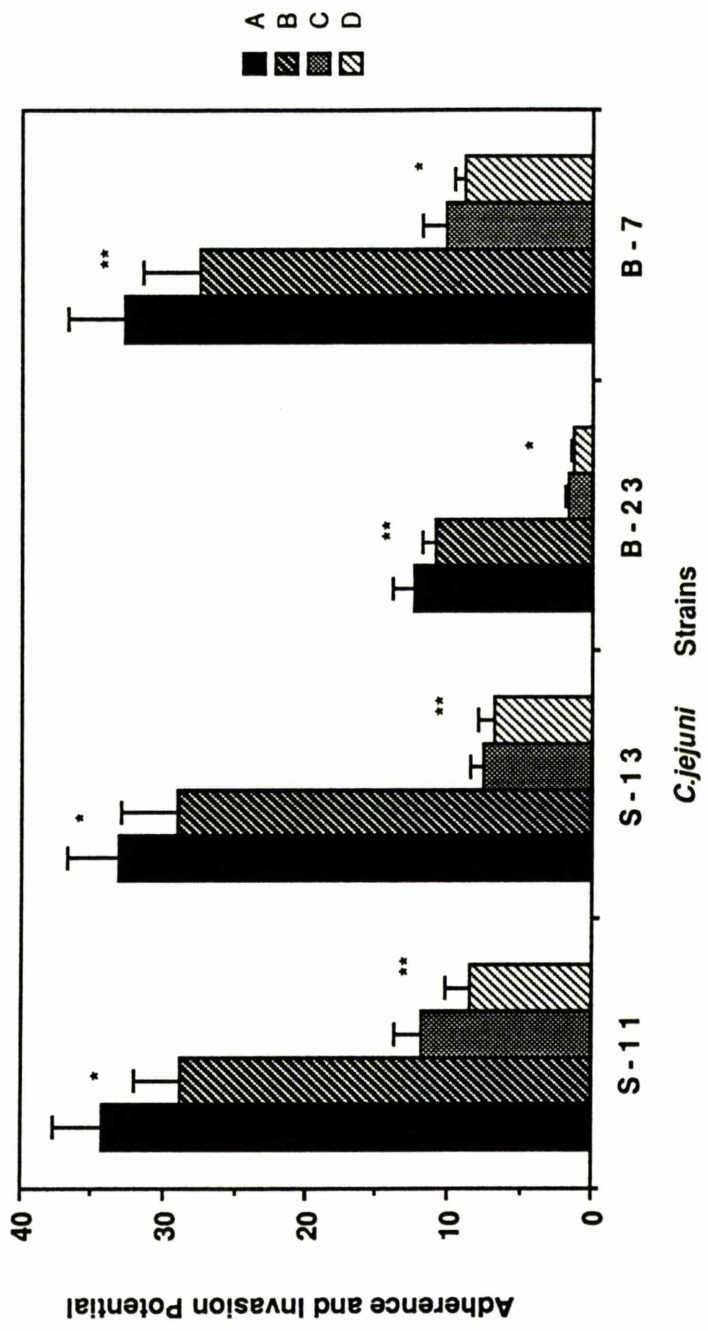

A
B
C
D
Adherence and Invasion Potential
0
10
20
30
40
S-11
S-13
B-23
B-7
C.jejuni Strains
*
**

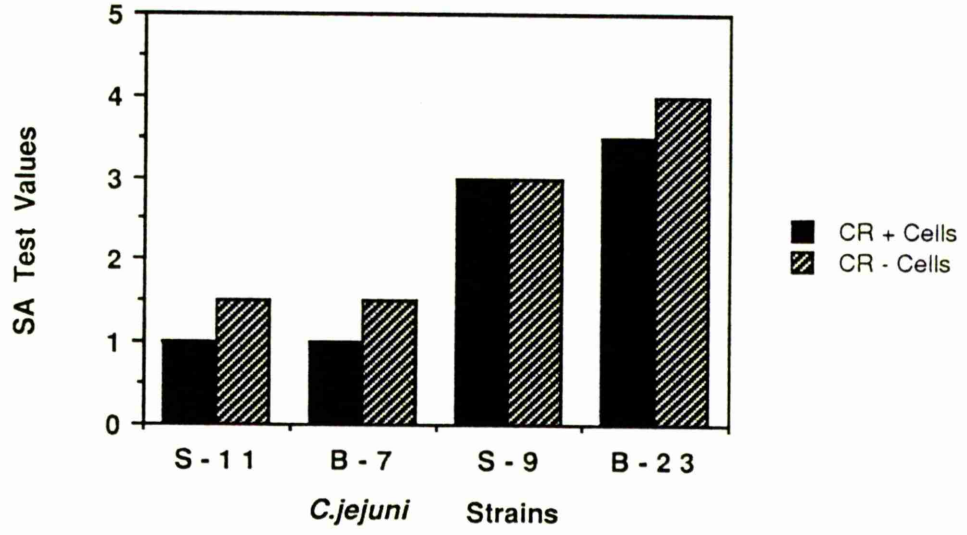
Figure 37: Cell-Surface Hydrophobicity of the CR^+ and CR^- Colonial Variants of the *C. jejuni* Strains: Salt Aggregation (SA) Test.

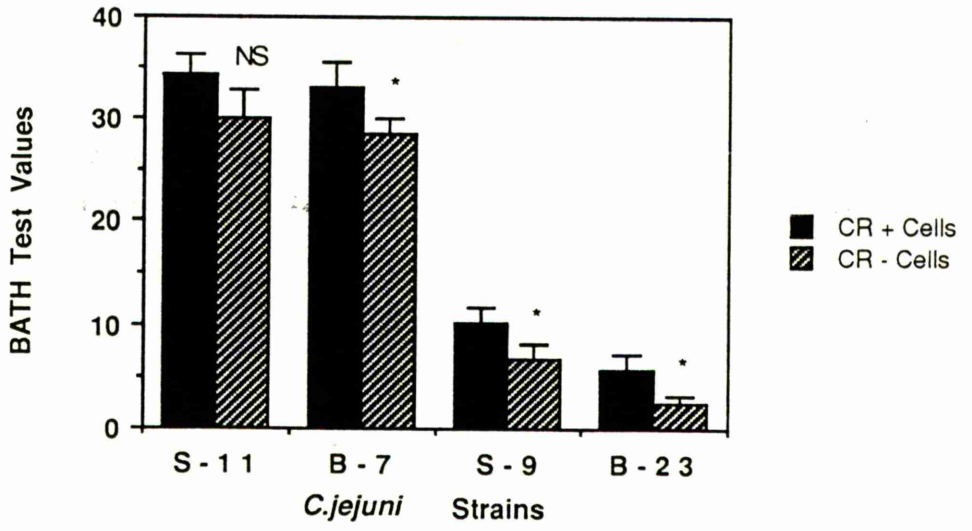
The results represent the mean of three experiments.

Figure 38: Cell-Surface Hydrophobicity of the CR^+ and CR^- Colonial Variants of the *C. jejuni* Strains: Bacterial Adherence to Hydrocarbon (BATH) Test.

The represent the mean and standard deviation of three experiments. Experimental procedure is described in Section 3.20.1

Significance levels (paired t-test) NS = Not Significant
* = $P \leq 0.05$


SA Test Values
5
4
3
2
1
0
S - 1 1
B - 7
S - 9
B - 2 3
C.jejuni Strains
CR + Cells
CR - Cells


BATH Test Values
40
30
20
10
0
NS
*
*
*
S - 1 1
B - 7
S - 9
B - 2 3
C.jejuni Strains
CR + Cells
CR - Cells

cell surface hydrophobicity by bacterial adherence to hydrocarbon (BATH) test (Rosenberg *et al,* 1980) and salt aggregation (SA) test (Lindhal *et al,* 1983). The CR^- colonial variants of strains B-7, S-9 and B-23 exhibited significantly reduced cell surface hydophobicity as determined by BATH test (Figure 38). The cell surface hydrophobicity of the CR^- cells of the strain S-11 was less than CR^+ counterpart but the difference was not statistically significant (Figure 38). The SA test values of the CR^+ cells were either less (strains S-11, B-7 and B-23) or equal (strain S-9) in comparison to the corresponding CR^- variants, indicating either higher or equal hydrophobicity of the CR^+ cells respectively (Figure 37). Although some of the CR^- variants were more hydrophobic than the CR^+ variant of other strains, for paired CR^+: CR^- variants, the CR^+ cells exhibited hydrophobicity either greater than or equal to the CR^- counterparts.

4.6 The Heat-modifiable Major Outer Membrane Protein (MOMP) of *C. jejuni*

Incubation of the outer membrane preparation (Section 3.15) at 37^{o} C and at 100^{o} C with solubilizing buffer (Appendix 2) and subsequent SDS-PAGE revealed that MOMP was the only heat-modifiable OMP which migrated as 30 K dal and 42 K dal protein when denatured in presence of SDS at 37^{o} C and 100^{o} C respectively (Figure 39). It is also apparent from Figure 39 that the MOMP migrated as the low molecular weight form at lower temperature; transition to the high molecular weight form occurred at $\geq 60^{o}$ C.

The isolated MOMP (Section 3.16) of the *C. jejuni* strain S-11 appeared as a single protein band in SDS-PAGE (Figure 40) and showed the same heat-modifiable nature as in the crude OMP preparation (Figure 39). It produced a single precipitin line when reacted with antiserum raised in rabbits against formalinized cells of the *C. jejuni* strain NCTC 11168 (Figure 41). After Western blotting of the MOMP against the same antiserum, a single band was produced

Figure 39: SDS-PAGE Demonstrating the Heat-modifiable Nature of the Major Outer Membrane Protein (OMP) of *C. jejuni.*

Lane	Denaturing Condition
A	37° C; 30 min
B	37° C; 30 min*
C	60° C; 5 min
D	70° C; 5 min
E	80° C; 5 min
F	100° C; 5 min

* Solubilizing buffer without 2-mercaptoethanol

Note that *C.jejuni* MOMP migrates in low molecular weight (30 K dal) form when incubated with SDS containing solubelization bufer. Heating at ≥ 60° C resulted in the transition of the MOMP to the slow migrating (43 K dal) form. MOMP is the only heat-modifiable OMP of *C. jejuni.* Procedures for isolation of OMP and SDS-PAGE are described in Sections 3.15 and 3.17.3 respectively.

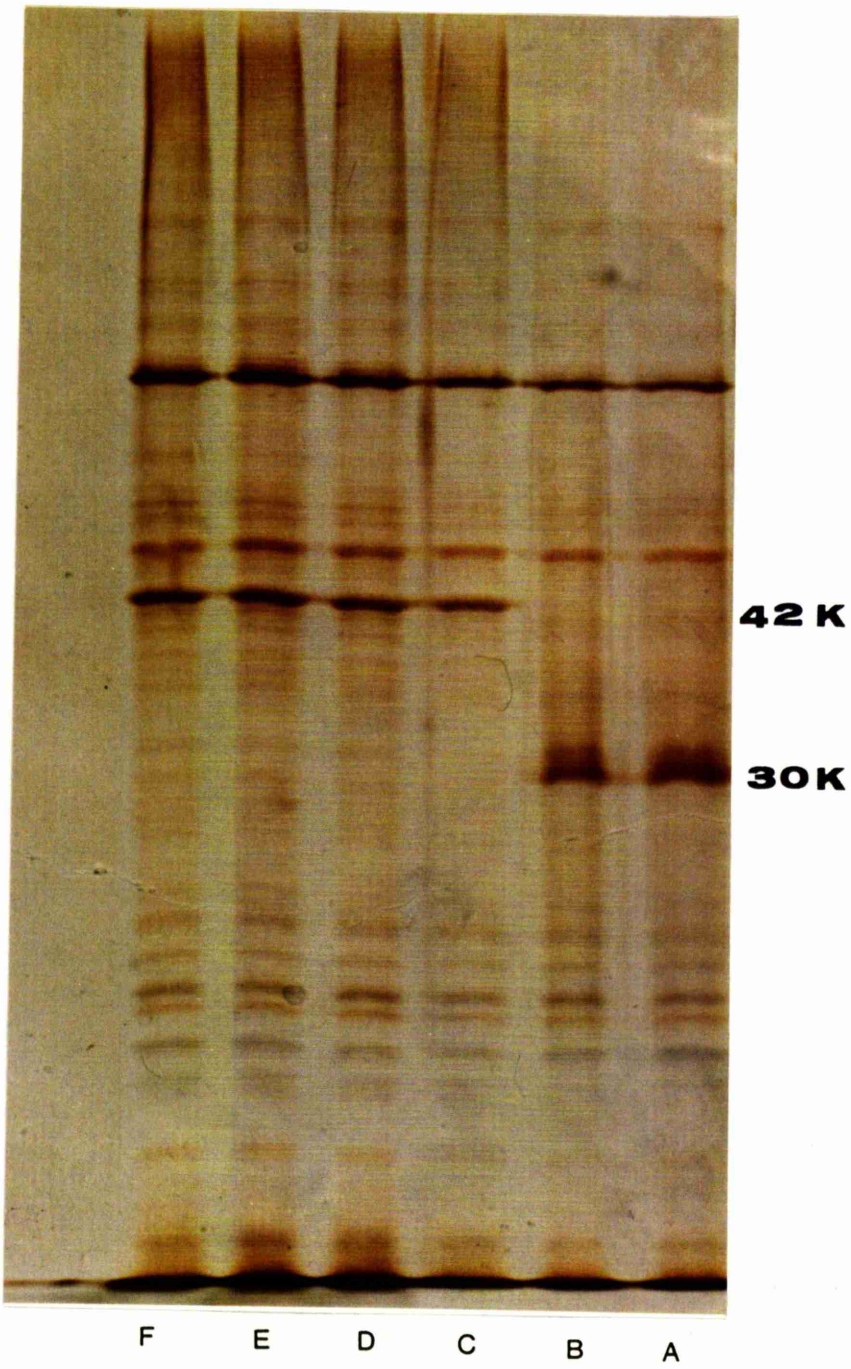

42K
30K
F
E
D
C
B
A

Figure 40:

The Effect of Trypsin Treatment and Denaturation Temperature on the SDS-PAGE of Outer Membrane Protein Preparations of the *C. jejuni* Strain S-11.

Lane	Trypsin treatment	Denaturation Condition
A	None	100° C; 5 min
B	10 min; 37° C	100° C; 5 min
C	10 min; 37° C	37° C; 30 min
D	None	100° C; 5 min
E	30 min; 37° C	100° C; 5 min
F	30 min; 37° C	37° C; 30 min

Trypsin Concentration used: 100 $\mu g\ ml^{-1}$ final concentration. Experimental procedures for SDS-PAGE, OMP isolation are described in Sections 3.16 and 3.17.3 respectively.

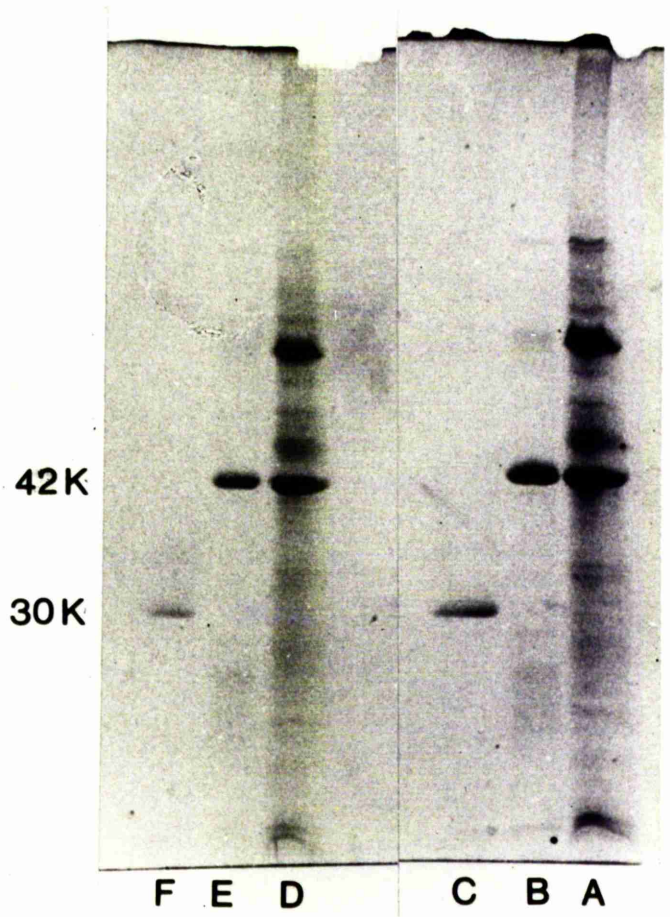

42K
30K
F E D
C B A

Figure 41: Ouchterlony Immunodiffusion Assay of the Major Outer Membrane Protein (MOMP) of *C. jejuni* Strains S-11 and B-23.

Well A: Rabbit antiserum against formalinized bacteria

Well B: MOMP of the strain S-11

Well C: MOMP of the strain B-23

Well D: MOMP of the strain S-11

Well E: MOMP of the strain S-11

Well F: MOMP of the strain B-23

Well G: MOMP of the strain S-11

Figure 42 : Analytical Thin-layer isoelectric focusing of the Major Outer Membrane Protein (MOMP) of *C. jejuni*

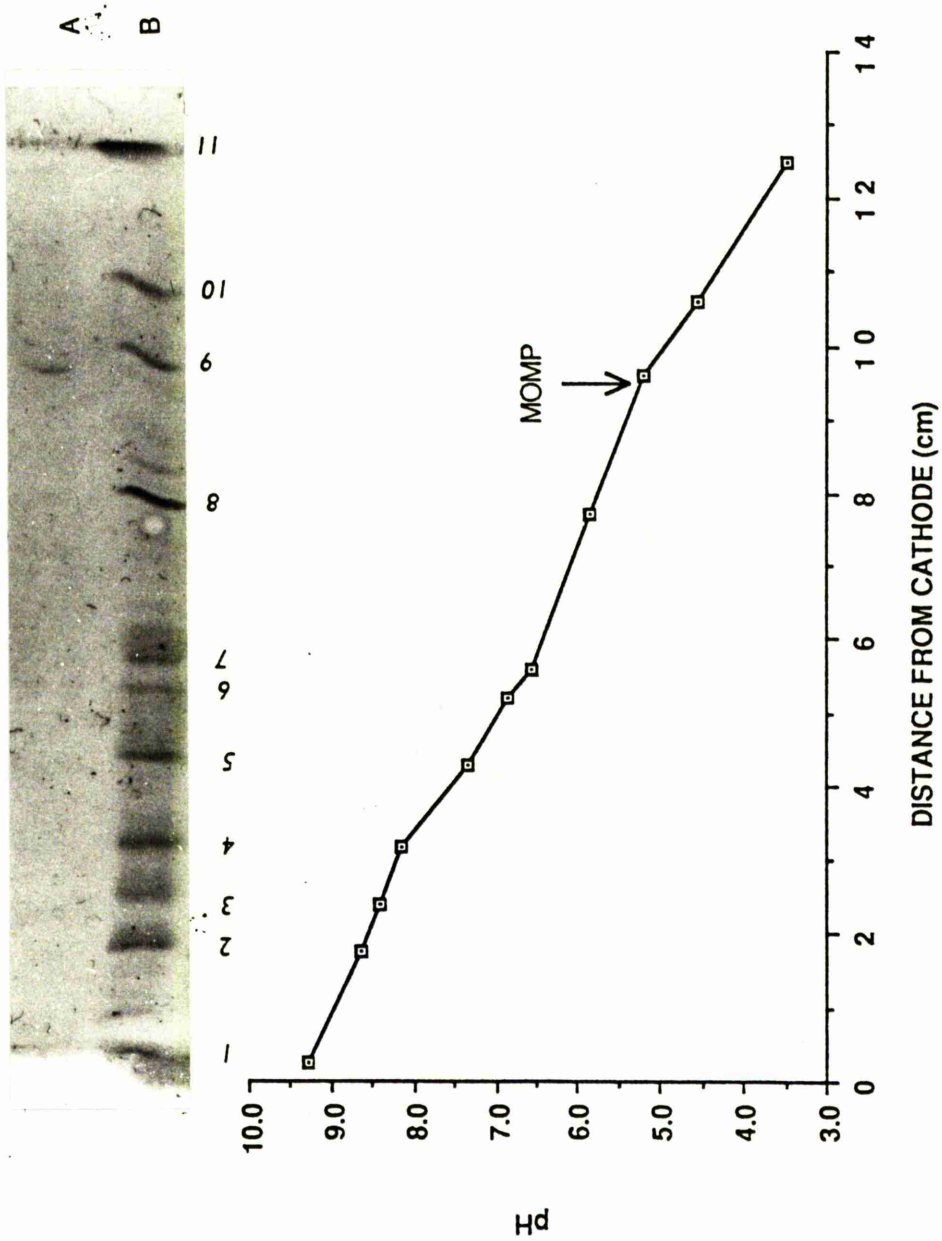
Distance migrated by the different pI markers from cathode (at the left of the gel) is plotted aginst their pI values.

Lane A : MOMP from strain S-11 (10 μg)

Lane B : pI markers

Name	pI values
1. Trypsinogen	3.50
2. Lentil lectin (basic)	4.55
3. Lentil lectin (middle)	5.20
4. Lentil lectin (acidic)	5.85
5. Horse myoglobin (basic)	6.55
6. Horse myoglobin (acidic)	6.85
7. Human carbonic anhydrase B	7.35
8. Bovine carbonic anhydrase B	8.15
9. ß-Lactoglobulin A	8.45
10. Soyabin trypsin inhibitor	8.65
11. Amyloglucosidase	9.30

Experimental procedures for isoelectric focusing and staining of the gel are described in Section 3.19.


A
B
11
10
9
8
7
6
5
4
3
2
1
MOMP
pH
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
0
2
4
6
8
10
12
14
DISTANCE FROM CATHODE (cm)

produced indicating the homogeneity of the MOMP preparation. Analytical thin-layer isoelectric focusing of the MOMP showed that it is an acidic protein with an isoelectric point of ca. 5.0-5.2 (Figure 42). MOMP isolated from the *C. jejuni* strain B-23 showed a complete reaction of identity with strain S-11 MOMP in gel immunodiffusion (Figure 41)

4.7 Colonization and Multiplication of *C. jejuni* strains in New-born Chicks

Two strains of *C. jejuni*, B-23 (a Group C strain) and S-11 (a Group D) strain were investigated for their ability to colonize and multiply in the different portions of the gastrointestinal (GI) tract, spleen and liver and was monitored by determining the number of viable organisms on post-inoculation days 1 to 7. Initial experiments with a graded dose of inoculum (ca. $1.0x10^2$ to $1x10^8$ c.f.u.) revealed that with a lower inoculum (< 10^7 c.f.u) all the chicks were not infected uniformly. This was assessed by a positive faecal culture and isolation of viable organisms by taking swabs from different portions of the gastrointestinal (GI) tract, liver and spleen. The sporadic nature of infection of the GI tract of infant chicks following oral inoculation was not observed with ca. $1.0x10^8$ organisms and large number of viable *C. jejuni* were recovered from the GI tract and other internal organs. Quantitative cultures of organ specimens (jejunum, ileum, colon, heart blood, liver and spleen) for *C. jejuni* after oral inoculation of the strains S-11 and B-23 are shown in Figures 43 to 48. The *C. jejuni* strains B-23 and S-11 quickly colonized the gut of the chicks and the bacterial population progressively increased in number as determined by viable counts on different post-inoculation days. Maximal colonization was usually achieved by day 5 and the counts of bacteria reached the level of ca. $1x10^7$ to 10^8 gm^{-1} gut tissue for ileum and colon (Figures 44 and 45 respectively) whereas relatively lower counts were obtained from the jejunum portion of the gut (Figure 43). The viable counts then fell gradually over the next two days. However, in the jejunum, the bacterial count for the strain B-23 increased until post-inoculation day 3 reaching ca. $1.0x10^{4-5}$

organisms gm^{-1} tissue and was maintained at that level until post-inoculation day 7. In contrast, the bacterial population of the strain S-11 progressively increased until day 6 to the level of $1.0x10^6$ gm^{-1} tissue and then it began to decrease. No significant differences were noted between the strains S-11 and B-23 in their ability to colonize the different parts of the gut.

From the colonization foci in the GI tract, the organisms spread to the spleen, liver and systemic circulation in varying degree. Pronounced differences were noted between the strains in the numbers of viable organisms recovered from blood, spleen and liver. In the blood, the strains B-23 and S-11 were detected at the same level of ca. $3.1x10^1$ ml^{-1} and $3.1x10^2$ ml^{-1} respectively on day 1 post-inoculation. The strain B-23 was rapidly cleared from the blood as revealed by isolation of viable organisms from 3 of the 5 chicks on the postinoculation day 1 but not thereafter. On the other hand, the strain S-11 was cleared more slowly from the blood which remained infected until postinoculation day 5, although the number of viable organisms recovered was lower in comparison to day 1, (Figure 48). However, viable organisms were not consistently recovered from all the chicks. For example, on day 4 postinoculation, 4 out 5 chicks were culture positive and on the postinoculation day 5, heart blood collected from 3 out 5 chicks were positive for *C. jejuni*.

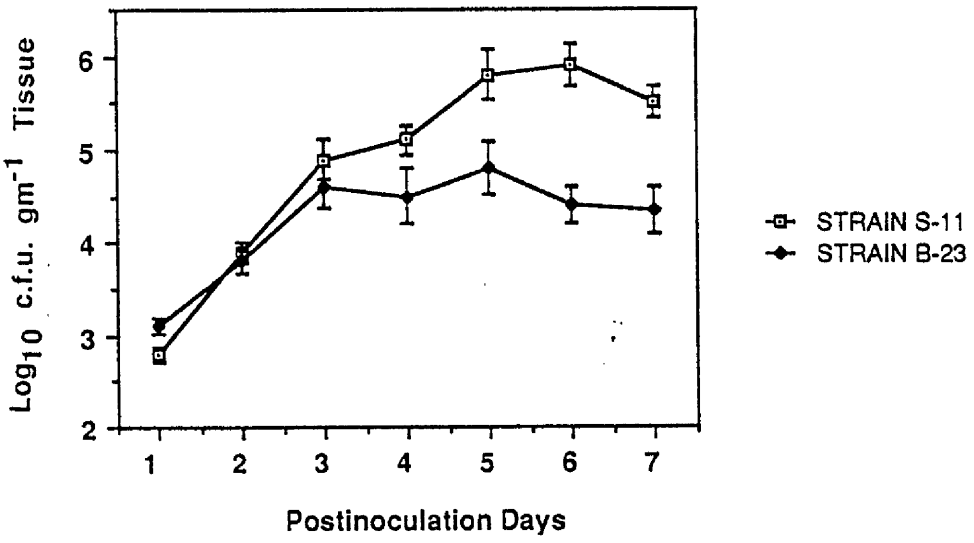
Clearing of the organisms from the blood was accompanied by their accumulation in the liver and spleen. The strain S-11 grew rapidly *in vivo* and reached high counts in test organs and the strain B-23 was maintained in these organs at a fairly low level (Figures 46 and 47). The duration of bacteraemia did not correlate with the number of viable bacteria in the liver and spleen because the chicks carried large numbers of campylobacters in their livers and spleen after the blood became free of organisms. It is apparent from the Figures 43 to 45 that both the strains S-11 and B-23 were almost equally efficient in colonizing the GI tract of the infant chicks. However, marked differences were noted between the strains in the duration of bacteraemia caused

Figure 43 : Recovery of Viable *C. jejuni* Strains S-11 and B-23 from the Jejunum of White Leghorn chicks

The results are presented as Log_{10} c.f.u. gm^{-1} jejunum tissue (Mean $\pm$ standard error of mean) recovered on different post-inoculation days. Experimental procedures are described in Section 3.9.

Figure 44 : Recovery of Viable *C. jejuni* Strains S-11 and B-23 from the Ileum of White Leghorn chicks

The results are presented as Log_{10} c.f.u. gm^{-1} ileum tissue (Mean $\pm$ standard error of mean) recovered on different post-inoculation days. Experimental procedures are described in Section 3.9.


Log_{10} c.f.u. gm^{-1} Tissue
2
3
4
5
6
1
2
3
4
5
6
7
Postinoculation Days
STRAIN S-11
STRAIN B-23

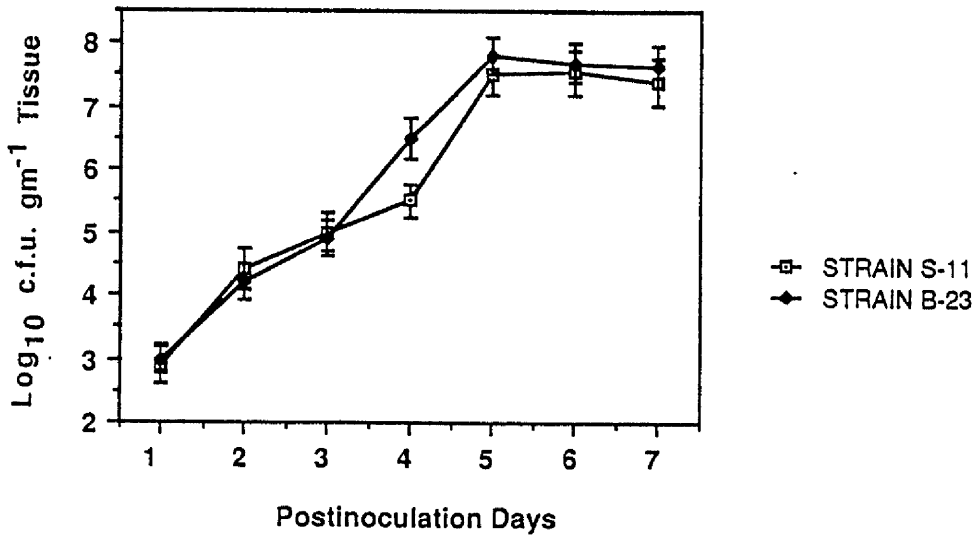
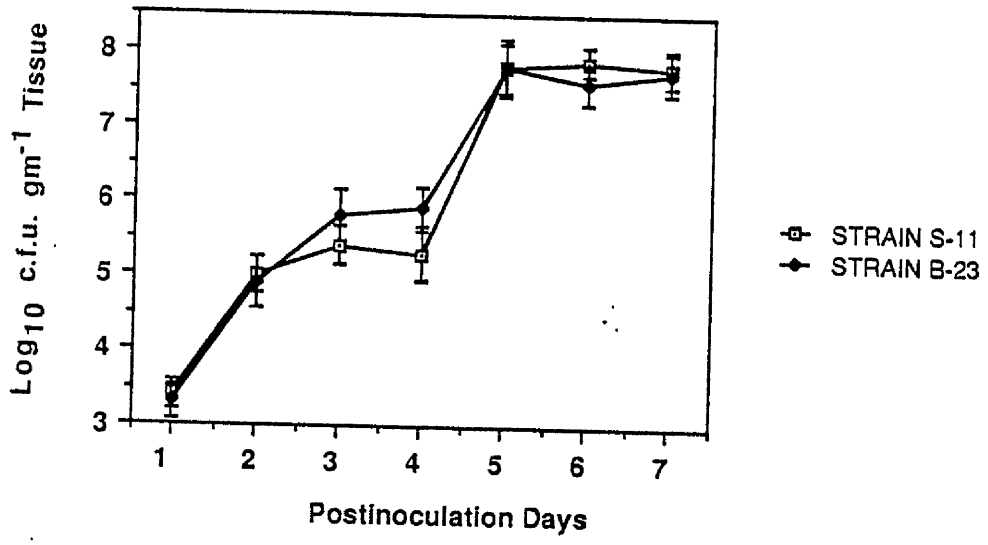

Log_{10} c.f.u. gm^{-1} Tissue
2
3
4
5
6
7
8
1
2
3
4
5
6
7
Postinoculation Days
STRAIN S-11
STRAIN B-23

Figure 45 : Recovery of Viable *C. jejuni* Strains S-11 and B-23 from the Colon of White Leghorn chicks

The results are presented as Log_{10} c.f.u. gm^{-1} colon tissue (Mean ± standard error of mean) recovered on different post-inoculation days. Experimental procedures are described in Section 3.9.

Figure 46 : Recovery of Viable *C. jejuni* Strains S-11 and B-23 from the Spleen of White Leghorn chicks

The results are presented as Log_{10} c.f.u. per spleen tissue (Mean ± standard error of mean) recovered on different post-inoculation days. Experimental procedures are described in Section 3.9.


Log10 c.f.u. gm-1 Tissue
8
7
6
5
4
3
1
2
3
4
5
6
7
Postinoculation Days
STRAIN S-11
STRAIN B-23

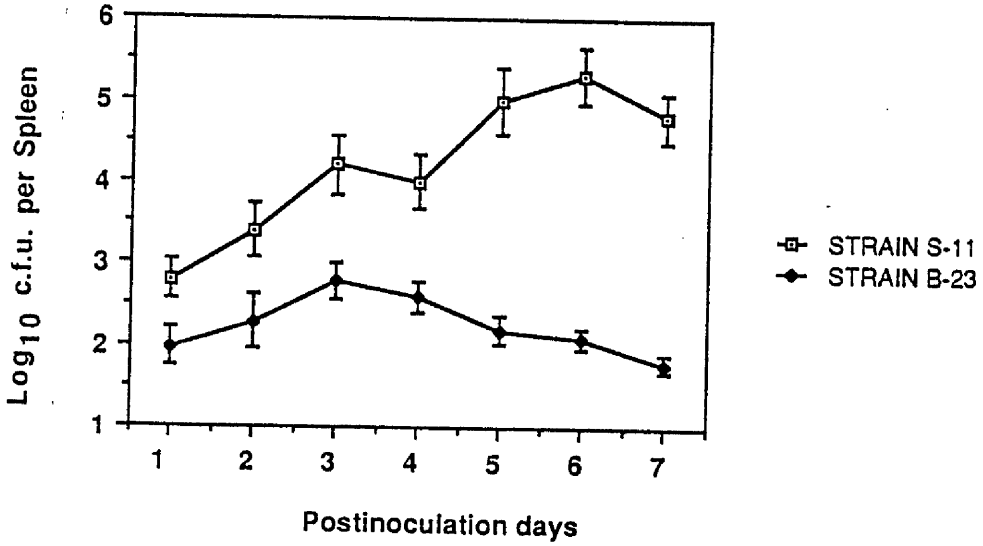
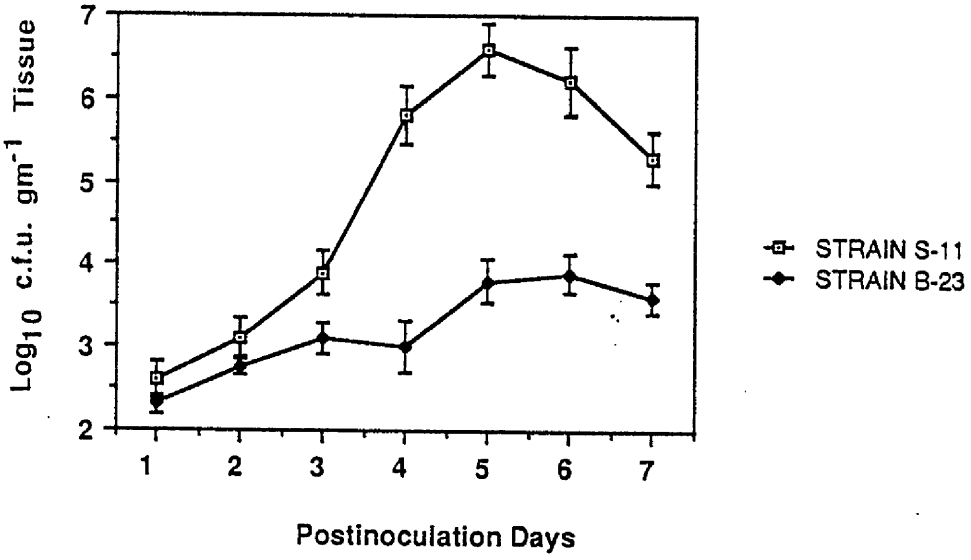

Log10 c.f.u. per Spleen
6
5
4
3
2
1
1
2
3
4
5
6
7
Postinoculation days
STRAIN S-11
STRAIN B-23

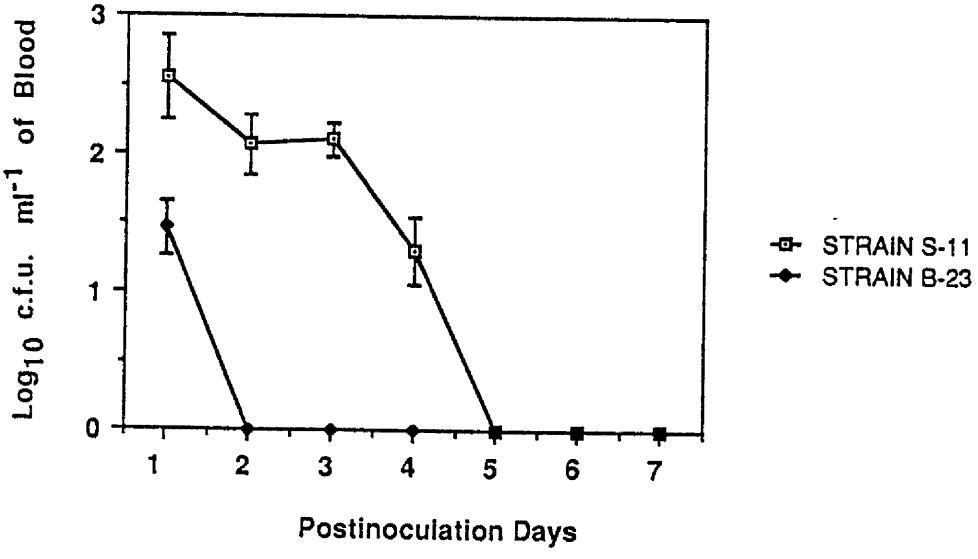
Figure 47 : Recovery of Viable *C. jejuni* Strains S-11 and B-23 from the Liver of White Leghorn chicks

The results are presented as Log_{10} c.f.u. gm^{-1} liver tissue (Mean $\pm$ standard error of mean) recovered on different post-inoculation days. Experimental procedures are described in Section 3.9.

Figure 48 : Recovery of Viable *C. jejuni* Strains S-11 and B-23 from Heart Blood of White Leghorn chicks

The results are presented as Log_{10} c.f.u. ml^{-1} of heart blood (Mean $\pm$ standard error of mean) recovered on different post-inoculation days. Experimental procedures are described in Section 3.9.


Log10 c.f.u. gm-1 Tissue
7
6
5
4
3
2
1
2
3
4
5
6
7
Postinoculation Days
STRAIN S-11
STRAIN B-23


Log10 c.f.u. ml-1 of Blood
3
2
1
0
1
2
3
4
5
6
7
Postinoculation Days
STRAIN S-11
STRAIN B-23

by them and their ability to colonize the spleen and liver of the chicks. In the spleen, the strain S-11 continued to multiply until day 5, then the numbers began to decrease; the maximum number of organisms recovered was ca. $3.1x10^6$ per spleen for the strain S-11. The number of organisms of the strain B-23 continued to increase in the spleen until post-inoculation day 6, but never exceeded the mean value of $8.1x10^3$ gm^{-1} (Figure 46).

A similar difference in the number of viable organisms recovered from the liver was noted between the two strains. The strain S-11 showed a biphasic growth pattern in the liver. Initially, the numbers increased slightly until day 3 and then fell slightly on day 4. The second phase of growth continued until day 6 reaching the number of ca. $1.9x10^5$ gm^{-1} and then it began to fall. On the other hand, the strain B-23 continued to multiply until day 3 then the numbers decreased gradually over the next 4 days of the experiment (Figure 47).

4.8 Determination of Lethality of the *C. jejuni* strains in Chicken Embryo Model

Initially it was decided to use the chicken embryo model to determine the virulence of the *C. jejuni* strains isolated from human clinical sources. Later, the studies on virulence of the *C. jejuni* strains were extended to include the Congo Red (CR) dye binding variants of some of the strains, a phenomenon which is reported to represent a marker of virulence of a variety of pathogenic microorganisms (see Section 1.11). Although, some of the results of the virulence assays of the Congo Red (CR) colonial variants are presented along with those of the strains isolated from different clinical presentations of diarrhoea, they are described in a separate section (Section 4.5).

Eleven-day-old White Leghorn chick embryos were used to compare the virulence of the *C. jejuni* strains isolated from the two clinically distinct forms of enteritis i.e. cholera-like watery diarrhoea (Group C strains) and dysentery-like mucoid diarrhoea (Group D strains). Chorioallantoic inoculation of the embryos was

Table 10 : Daily Mortality of 11-day-old White Leghorn Chicken Embryos After Inoculation with CR^+ and CR^- Colonial Variants of *C. jejuni* Strain S-11 (Experiment 1)

Inoculum and LD_{50} values are expressed as $\log_{10}$. Experimental procedure is described in Section 3.10.

Table 11 : Daily Mortality of 11-day-old White Leghorn Chicken Embryos After Inoculation with CR^+ and CR^- Colonial Variants of *C. jejuni* Strain S-11 (Experiment 2)

Inoculum and LD_{50} values are expressed as $\log_{10}$. Experimental procedure is described in Section 3.10.

STRAIN	INOCULUM	No. DEAD / No. INOCULATED			LD_{50}
		24 hr	48 hr	72 hr	
S-11 CR^+	7.27	1 / 6	3 / 6	6 / 6	3.27
	6.27	1 / 6	2 / 6	6 / 6	
	5.27	0 / 6	2 / 6	6 / 6	
	4.27	0 / 6	0 / 6	4 / 6	
	3.27	0 / 6	0 / 6	3 / 6	
S-11 CR^-	7.39	1 / 6	2 / 6	6 / 6	4.39
	6.39	0 / 6	1 / 6	5 / 6	
	5.39	0 / 6	0 / 6	4 / 6	
	4.39	0 / 6	0 / 6	3 / 6	
	3.39	0 / 6	0 / 6	1 / 6	

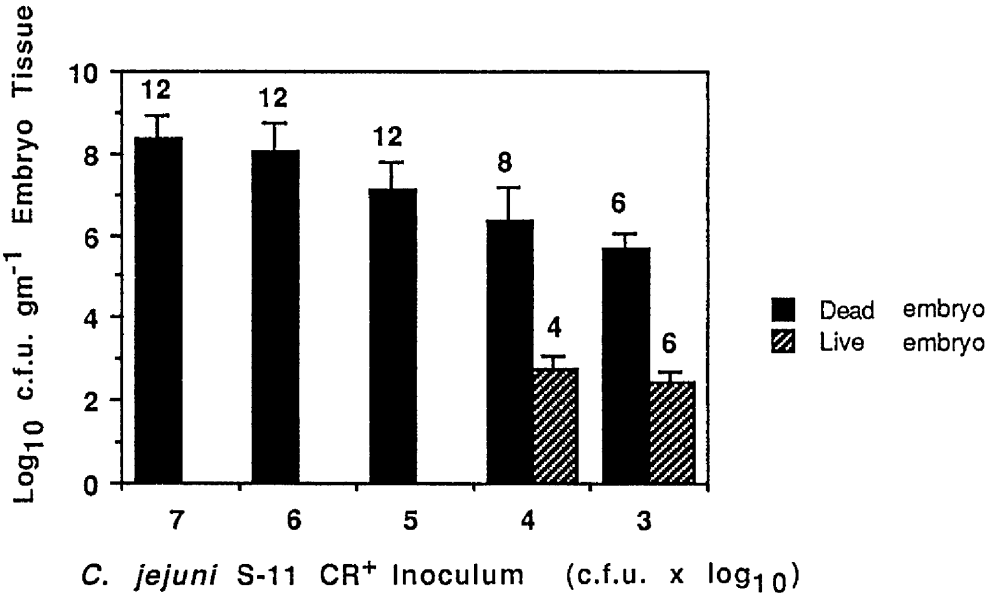
STRAIN	INOCULUM	No. DEAD / No. INOCULATED			LD_{50}
		24 hr	48 hr	72 hr	
S-11 CR^+	7.32	2 / 6	3 / 6	6 / 6	3.32
	6.32	2 / 6	3 / 6	6 / 6	
	5.32	1 / 6	2 / 6	6 / 6	
	4.32	0 / 6	0 / 6	4 / 6	
	3.32	0 / 6	0 / 6	3 / 6	
S-11 CR^-	7.30	1 / 6	3 / 6	6 / 6	4.79
	6.30	0 / 6	2 / 6	5 / 6	
	5.30	0 / 6	1 / 6	4 / 6	
	4.30	0 / 6	0 / 6	2 / 6	
	3.30	0 / 6	0 / 6	1 / 6	

Figure 49 : Recovery of Viable *C.jejuni* Strain S-11 CR+ from 11-day-old White Leghorn Chicken Embryos.

The results are presented as Log_{10} c.f.u. gm^{-1} embryo tissue (Mean and standard error of mean) recovered as a function of the inoculum size (log_{10}). The numbers adjacent to each bar represent the number of embryos averaged. Experimental procedures are described in Section 3.10. See Tables 10 and 11 where LD_{50} has been calculated.

Figure 50 : Recovery of Viable *C.jejuni* Strain S-11 CR- from 11-day-old White Leghorn Chicken Embryos.

The results are presented as Log_{10} c.f.u. gm^{-1} embryo tissue (Mean + standard error of mean) recovered as a function of the inoculum size (log_{10}). The numbers adjacent to each bar represent the number of embryos averaged. Experimental procedures are described in Section 3.10. See Tables 10 and 11 where LD_{50} has been calculated.


Log10 c.f.u. gm-1 Embryo Tissue
10
8
6
4
2
0
12
12
12
8
4
6
6
7
6
5
4
3
C. jejuni S-11 CR+ Inoculum (c.f.u. x log10)
Dead embryo
Live embryo

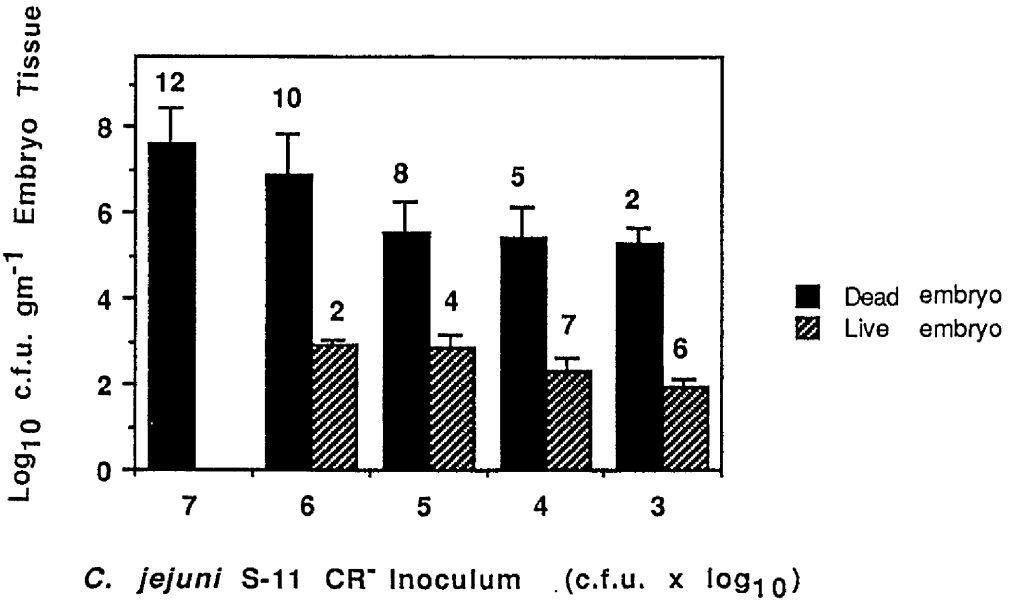

Log10 c.f.u. gm-1 Embryo Tissue
8
6
4
2
0
12
10
2
8
4
5
7
2
6
7
6
5
4
3
C. jejuni S-11 CR- Inoculum (c.f.u. x log10)
Dead embryo
Live embryo

Table 12 : Daily Mortality of 11-day-old White Leghorn Chicken Embryos After Inoculation with CR^+ and CR^- Colonial Variants of *C. jejuni* Strain S-13 (Experiment 1)

Inoculum and LD_{50} values are expressed as $\log_{10}$. Experimental procedure is described in Section 3.10.

Table 13 : Daily Mortality of 11-day-old White Leghorn Chicken Embryos After Inoculation with CR^+ and CR^- Colonial Variants of *C. jejuni* Strain S-13 (Experiment 2)

Inoculum and LD_{50} values are expressed as $\log_{10}$. Experimental procedure is described in Section 3.10.

STRAIN	INOCULUM	No. DEAD / No. INOCULATED			LD_{50}
		24 hr	48 hr	72 hr	
S-13 CR^+	8.41	1 / 6	3 / 6	6 / 6	4.91
	7.41	0 / 6	3 / 6	6 / 6	
	6.41	0 / 6	2 / 6	6 / 6	
	5.41	0 / 6	1 / 6	4 / 6	
	4.41	0 / 6	0 / 6	2 / 6	
S-13 CR^-	8.32	0 / 6	2 / 6	6 / 6	6.32
	7.32	0 / 6	1 / 6	4 / 6	
	6.32	0 / 6	0 / 6	3 / 6	
	5.32	0 / 6	0 / 6	2 / 6	
	4.32	0 / 6	0 / 6	0 / 6	

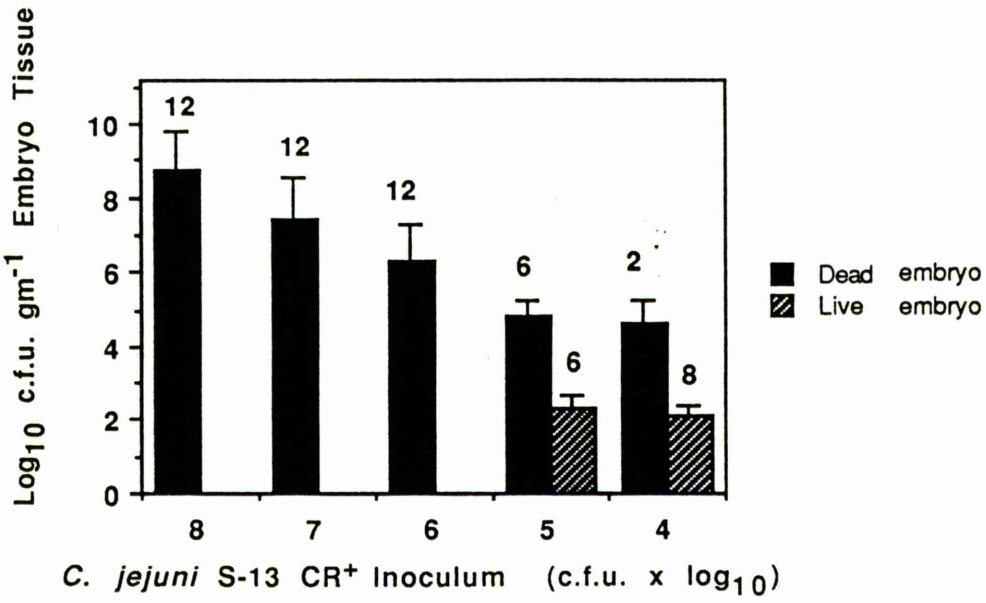
STRAIN	INOCULUM	No. DEAD / No. INOCULATED			LD_{50}
		24 hr	48 hr	72 hr	
S-13 CR^+	8.63	0 / 6	3 / 6	6 / 6	4.63
	7.63	0 / 6	2 / 6	6 / 6	
	6.63	0 / 6	1 / 6	4 / 6	
	5.63	0 / 6	1 / 6	4 / 6	
	4.63	0 / 6	0 / 6	3 / 6	
S-13 CR^-	8.46	0 / 6	2 / 6	6 / 6	6.46
	7.46	0 / 6	1 / 6	4 / 6	
	6.46	0 / 6	1 / 6	3 / 6	
	5.46	0 / 6	0 / 6	1 / 6	
	4.46	0 / 6	0 / 6	0 / 6	

Figure 51 : Recovery of Viable *C.jejuni* Strain S-13 CR$^+$ from 11-day-old White Leghorn Chicken Embryos.

The results are presented as Log_{10} c.f.u. gm^{-1} embryo tissue (Mean and standard error of mean) recovered as a function of the inoculum size (log_{10}). The numbers adjacent to each bar represent the number of embryos averaged. Experimental procedures are described in Section 3.10. See Tables 12 and 13 where LD_{50} has been calculated.

Figure 52 : Recovery of Viable *C. jejuni* Strain S-13 CR$^-$ from 11-day-old White Leghorn Chicken Embryos.

The results are presented as Log_{10} c.f.u. gm^{-1} embryo tissue (Mean and standard error of mean) recovered as a function of the inoculum size (log_{10}). The numbers adjacent to each bar represent the number of embryos averaged. Experimental procedures are described in Section 3.10. See Tables 12 and 13 where LD_{50} has been calculated.


Log10 c.f.u. gm-1 Embryo Tissue
0
2
4
6
8
10
12
12
12
6
2
6
8
8
7
6
5
4
C. jejuni S-13 CR+ Inoculum (c.f.u. x log10)
Dead embryo
Live embryo

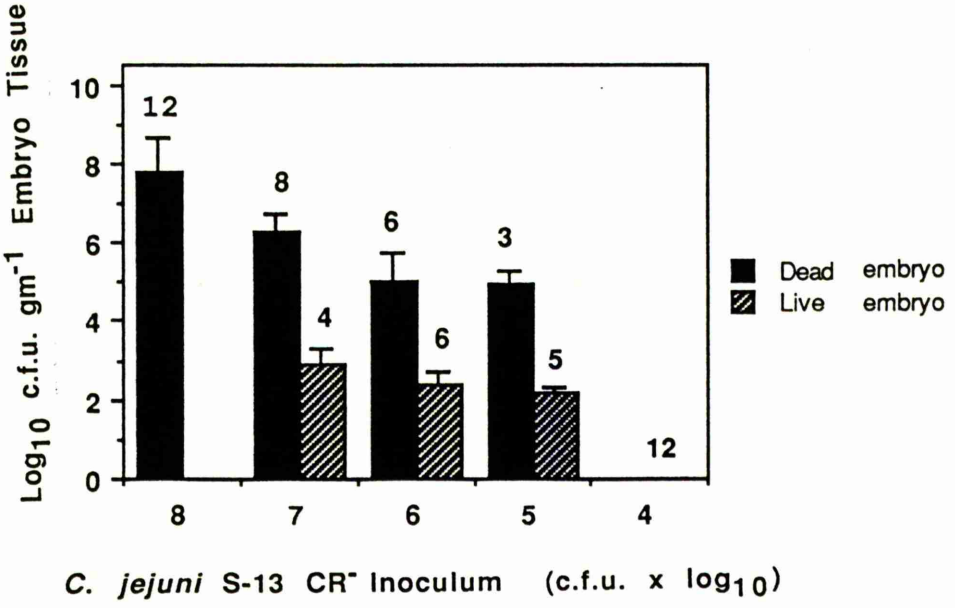

Log10 c.f.u. gm-1 Embryo Tissue
0
2
4
6
8
10
12
8
4
6
6
3
5
12
8
7
6
5
4
C. jejuni S-13 CR- Inoculum (c.f.u. x log10)
Dead embryo
Live embryo

Table 14 : Daily Mortality of 11-day-old White Leghorn Chicken Embryos After Inoculation with CR^+ and CR^- Colonial Variants of *C. jejuni* Strain B-7 (Experiment 1)

Inoculum and LD_{50} values are expressed as $\log_{10}$. Experimental procedure is described in Section 3.10.

Table 15 : Daily Mortality of 11-day-old White Leghorn Chicken Embryos After Inoculation with CR^+ and CR^- Colonial Variants of *C. jejuni* Strain B-7 (Experiment 2)

Inoculum and LD_{50} values are expressed as $\log_{10}$. Experimental procedure is described in Section 3.10.

STRAIN	INOCULUM	No. DEAD / No. INOCULATED			LD_{50}
		24 hr	48 hr	72 hr	
B-7 CR^+	7.34	1 / 6	4 / 6	6 / 6	3.84
	6.34	1 / 6	4 / 6	6 / 6	
	5.34	0 / 6	1 / 6	6 / 6	
	4.34	0 / 6	1 / 6	5 / 6	
	3.34	0 / 6	0 / 6	1 / 6	
B-7 CR^-	7.32	0 / 6	1 / 6	6 / 6	6.32
	6.32	0 / 6	1 / 6	3 / 6	
	5.32	0 / 6	0 / 6	2 / 6	
	4.32	0 / 6	0 / 6	1 / 6	
	3.32	0 / 6	0 / 6	0 / 6	

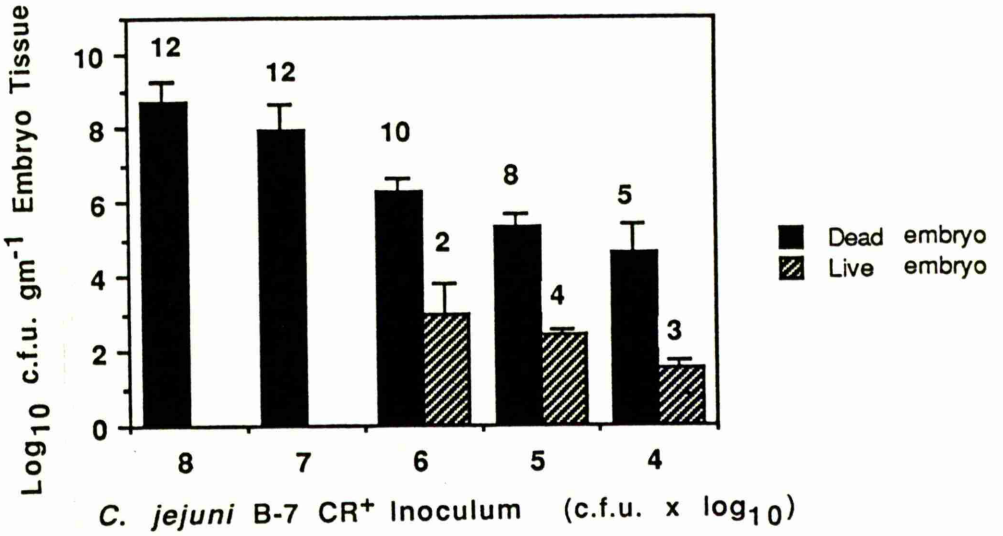
STRAIN	INOCULUM	No. DEAD / No. INOCULATED			LD_{50}
		24 hr	48 hr	72 hr	
B-7 CR^+	7.30	0 / 6	2 / 6	6 / 6	4.54
	6.30	0 / 6	1 / 6	6 / 6	
	5.30	0 / 6	1 / 6	6 / 6	
	4.30	0 / 6	0 / 6	2 / 6	
	3.30	0 / 6	0 / 6	0 / 6	
B-7 CR^-	7.23	0 / 6	1 / 6	6 / 6	6.23
	6.23	0 / 6	0 / 6	3 / 6	
	5.23	0 / 6	0 / 6	2 / 6	
	4.23	0 / 6	0 / 6	2 / 6	
	3.23	0 / 6	0 / 6	0 / 6	

Figure 53 : Recovery of Viable *C.jejuni* Strain B-7 CR+ from 11-day-old White Leghorn Chicken Embryos.

The results are presented as Log_{10} c.f.u. gm^{-1} embryo tissue (Mean and standard error of mean) recovered as a function of the inoculum size (log_{10}). The numbers adjacent to each bar represent the number of embryos averaged. Experimental procedures are described in Section 3.10. See Tables 14 and 15 where LD_{50} has been calculated.

Figure 54 : Recovery of Viable *C. jejuni* Strain B-7 CR- from 11-day-old White Leghorn Chicken Embryos.

The results are presented as Log_{10} c.f.u. gm^{-1} embryo tissue (Mean and standard error of mean) recovered as a function of the inoculum size (log_{10}). The numbers adjacent to each bar represent the number of embryos averaged. Experimental procedures are described in Section 3.10. See Tables 14 and 15 where LD_{50} has been calculated.


Log10 c.f.u. gm-1 Embryo Tissue
10
8
6
4
2
0
12
12
10
2
8
4
5
3
8
7
6
5
4
C. jejuni B-7 CR+ Inoculum (c.f.u. x log10)
Dead embryo
Live embryo

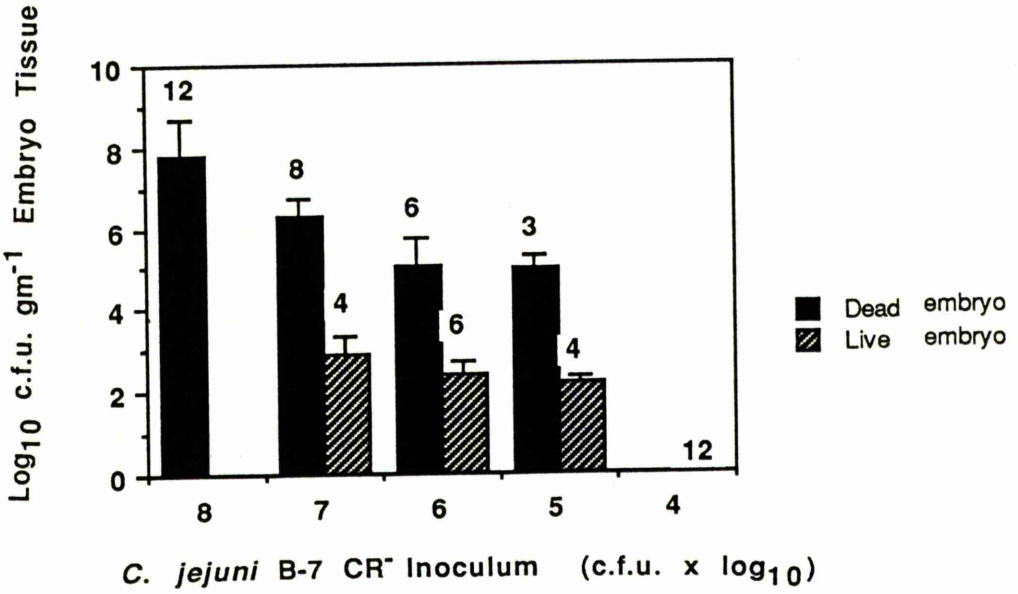

Log10 c.f.u. gm-1 Embryo Tissue
10
8
6
4
2
0
12
8
4
6
6
3
4
12
8
7
6
5
4
C. jejuni B-7 CR- Inoculum (c.f.u. x log10)
Dead embryo
Live embryo

Table 16 : Daily Mortality of 11-day-old White Leghorn Chicken Embryos After Inoculation with CR^+ and CR^- Colonial Variants of *C. jejuni* Strain 11385 (Experiment 1)

Inoculum and LD_{50} values are expressed as $\log_{10}$. Experimental procedure is described in Section 3.10.

Table 17 : Daily Mortality of 11-day-old White Leghorn Chicken Embryos After Inoculation with CR^+ and CR^- Colonial Variants of *C. jejuni* Strain 11385 (Experiment 2)

Inoculum and LD_{50} values are expressed as $\log_{10}$. Experimental procedure is described in Section 3.10.

STRAIN	INOCULUM	No. DEAD / No. INOCULATED			LD_{50}
		24 hr	48 hr	72 hr	
11385 CR+	7.11	0 / 5	1 / 5	5 / 5	3.77
	6.11	0 / 5	0 / 5	5 / 5	
	5.11	0 / 5	0 / 5	4 / 5	
	4.11	0 / 5	0 / 5	3 / 5	
	3.11	0 / 5	0 / 5	1 / 5	
	2.11	0 / 5	0 / 5	1 / 5	
11385 CR-	6.32	0 / 5	0 / 5	3 / 5	5.65
	5.32	0 / 5	0 / 5	2 / 5	
	4.32	0 / 5	0 / 5	1 / 5	
	3.32	0 / 5	0 / 5	0 / 5	
	2.32	0 / 5	0 / 5	0 / 5	
	1.32	0 / 5	0 / 5	0 / 5	

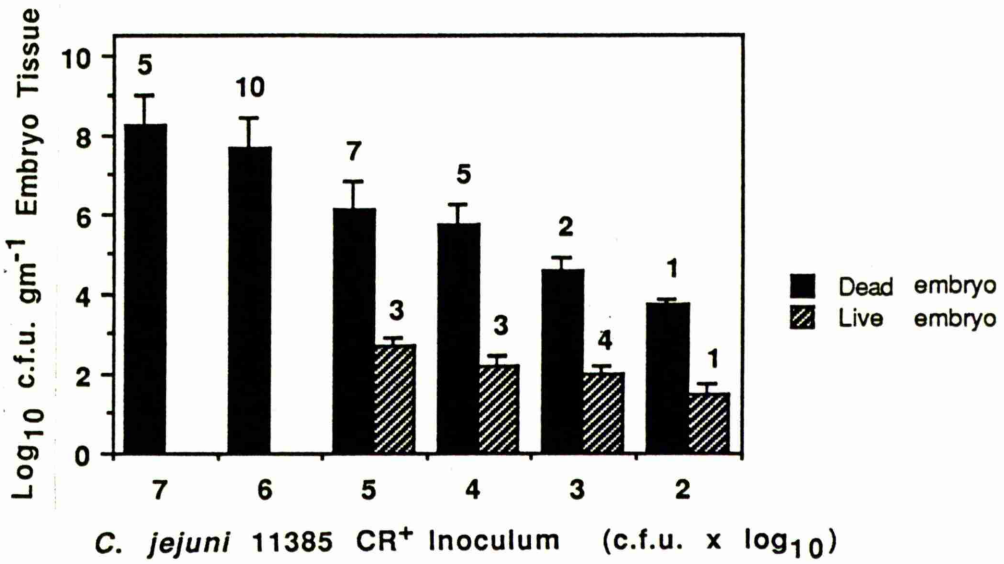
STRAIN	INOCULUM	No. DEAD / No. INOCULATED			LD_{50}
		24 hr	48 hr	72 hr	
11385 CR+	6.04	0 / 5	0 / 5	5 / 5	4.36
	5.04	0 / 5	0 / 5	3 / 5	
	4.04	0 / 5	0 / 5	2 / 5	
	3.04	0 / 5	0 / 5	1 / 5	
	2.04	0 / 5	0 / 5	0 / 5	
	1.04	0 / 5	0 / 5	0 / 5	
11385 CR-	6.07	0 / 5	0 / 5	3 / 5	5.75
	5.07	0 / 5	0 / 5	1 / 5	
	4.07	0 / 5	0 / 5	0 / 5	
	3.07	0 / 5	0 / 5	0 / 5	
	2.07	0 / 5	0 / 5	0 / 5	
	1.07	0 / 5	0 / 5	0 / 5	

Figure 55 : Recovery of Viable *C.jejuni* Strain 11385 CR+ from 11-day-old White Leghorn Chicken Embryos.

The results are presented as Log_{10} c.f.u. gm^{-1} embryo tissue (Mean and standard error of mean) recovered as a function of the inoculum size (log_{10}). The numbers adjacent to each bar represent the number of embryos averaged. Experimental procedures are described in Section 3.10. See Tables 16 and 17 where LD_{50} has been calculated.

Figure 56: Recovery of Viable *C. jejuni* Strain 11385 CR- from 11-day-old White Leghorn Chicken Embryos.

The results are presented as Log_{10} c.f.u. gm^{-1} embryo tissue (Mean and standard error of mean) recovered as a function of the inoculum size (log_{10}). The numbers adjacent to each bar represent the number of embryos averaged. Experimental procedures are described in Section 3.10. See Tables 16 and 17 where LD_{50} has been calculated.


Log10 c.f.u. gm-1 Embryo Tissue
10
8
6
4
2
0
5
10
7
3
5
3
2
4
1
1
7
6
5
4
3
2
C. jejuni 11385 CR+ Inoculum (c.f.u. x log10)
Dead embryo
Live embryo

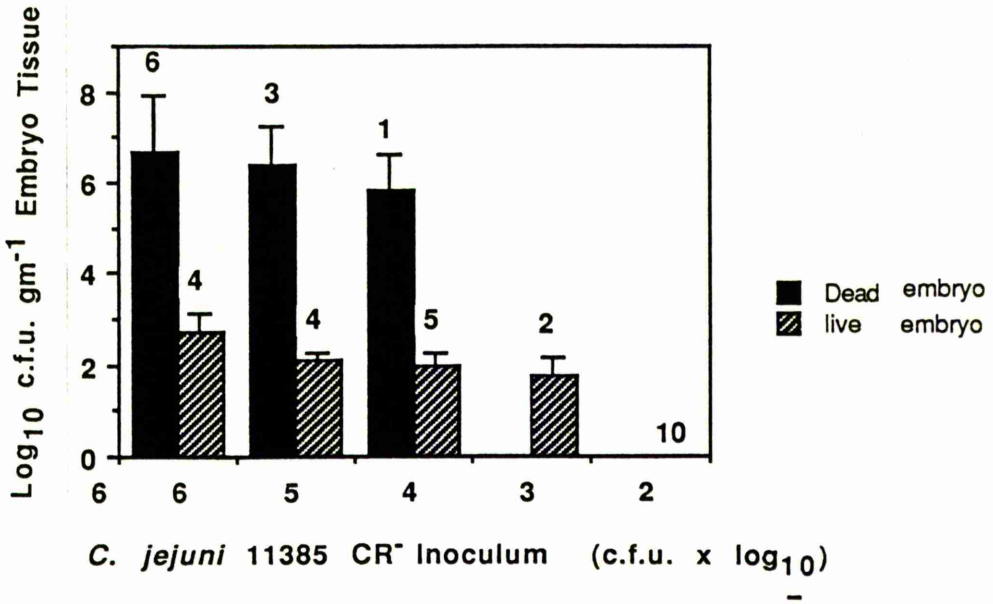

Log10 c.f.u. gm-1 Embryo Tissue
8
6
4
2
0
6
4
3
4
1
5
2
10
6
6
5
4
3
2
C. jejuni 11385 CR- Inoculum (c.f.u. x log10)
Dead embryo
live embryo

Table 18 : Daily Mortality of 11-day-old White Leghorn Chicken Embryos After Inoculation with CR^+ and CR^- Colonial Variants of *C. jejuni* Strain B-23 (Experiment 1)

Inoculum and LD_{50} values are expressed as $\log_{10}$. Experimental procedure is described in Section 3.10.

Table 19 : Daily Mortality of 11-day-old White Leghorn Chicken Embryos After Inoculation with CR^+ and CR^- Colonial Variants of *C. jejuni* Strain B-23 (Experiment 2)

Inoculum and LD_{50} values are expressed as $\log_{10}$. Experimental procedure is described in Section 3.10.

STRAIN	INOCULUM	No. DEAD / No. INOCULATED			LD_{50}
		24 hr	48 hr	72 hr	
B-23 CR^+	7.23	0 / 5	1 / 5	4 / 5	6.72
	6.23	0 / 5	0 / 5	1 / 5	
	5.23	0 / 5	0 / 5	0 / 5	
	4.23	0 / 5	0 / 5	0 / 5	
	3.23	0 / 5	0 / 5	0 / 5	
	2.23	0 / 5	0 / 5	0 / 5	
B-23 CR^-	7.32	0 / 5	0 / 5	0 / 5	>7.32
	6.32	0 / 5	0 / 5	0 / 5	
	5.32	0 / 5	0 / 5	0 / 5	
	4.32	0 / 5	0 / 5	0 / 5	
	3.32	0 / 5	0 / 5	0 / 5	
	2.32	0 / 5	0 / 5	0 / 5	

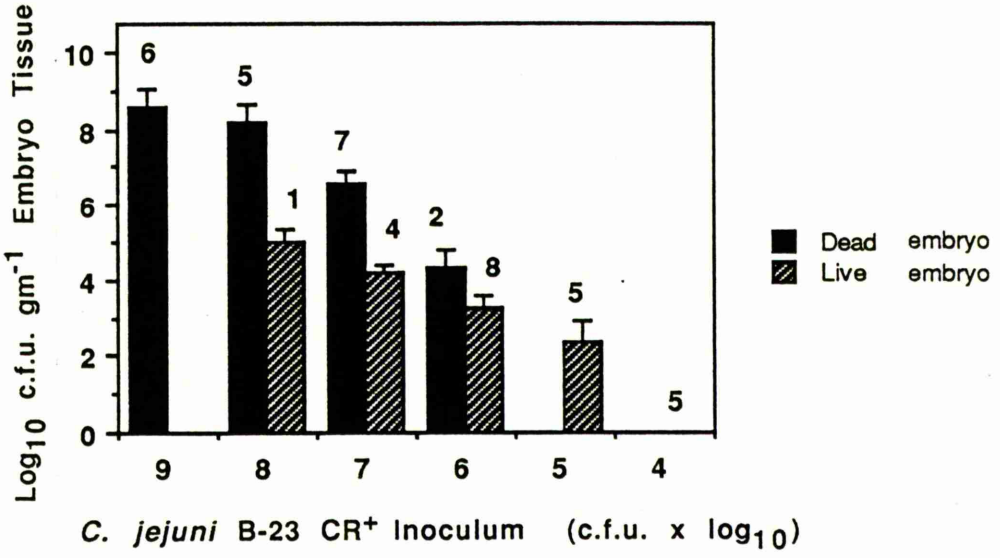
STRAIN	INOCULUM	No. DEAD / No. INOCULATED			LD_{50}
		24 hr	48 hr	72 hr	
B-23 CR^+	9.43	0 / 6	2 / 6	6 / 6	7.43
	8.43	0 / 6	2 / 6	5 / 6	
	7.43	0 / 6	1 / 6	3 / 6	
	6.43	0 / 6	0 / 6	1 / 6	
	5.43	0 / 6	0 / 6	0 / 6	
B-23 CR^-	9.46	0 / 6	2 / 6	5 / 6	8.08
	8.46	0 / 6	1 / 6	4 / 6	
	7.46	0 / 6	1 / 6	2 / 6	
	6.46	0 / 6	0 / 6	0 / 6	
	5.46	0 / 6	0 / 6	0 / 6	

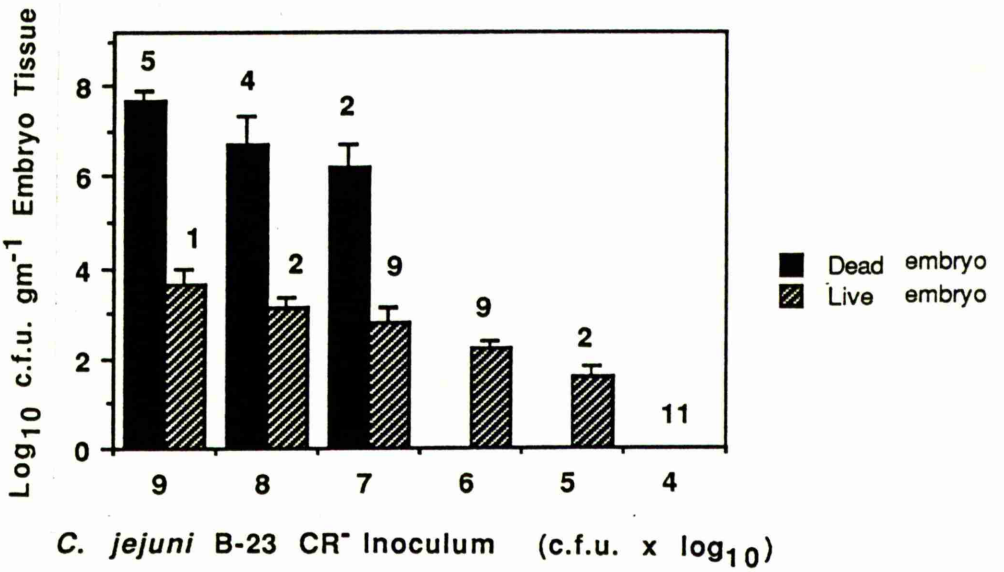
Figure 57 : Recovery of Viable *C.jejuni* Strain B-23 CR+ from 11-day-old White Leghorn Chicken Embryos.

The results are presented as Log_{10} c.f.u. gm^{-1} embryo tissue (Mean + standard error of mean) recovered as a function of the inoculum size (log_{10}). The numbers adjacent to each bar represent the number of embryos averaged. Experimental procedures are described in Section 3.10. See Tables 18 and 19 where LD_{50} has been calculated.

Figure 58 : Recovery of Viable *C.jejuni* Strain B-23 CR- from 11-day-old White Leghorn Chicken Embryos.

The results are presented as Log_{10} c.f.u. gm^{-1} embryo tissue (Mean + standard error of mean) recovered as a function of the inoculum size (log_{10}). The numbers adjacent to each bar represent the number of embryos averaged. Experimental procedures are described in Section 3.10. See Tables 18 and 19 where LD_{50} has been calculated.


Log10 c.f.u. gm-1 Embryo Tissue
10
8
6
4
2
0
6
5
1
7
4
2
8
5
5
9
8
7
6
5
4
C. jejuni B-23 CR+ Inoculum (c.f.u. x log10)
Dead embryo
Live embryo


Log10 c.f.u. gm-1 Embryo Tissue
8
6
4
2
0
5
1
4
2
2
9
9
2
11
9
8
7
6
5
4
C. jejuni B-23 CR- Inoculum (c.f.u. x log10)
Dead embryo
Live embryo

STRAIN	INOCULUM	No. DEAD / No. INOCULATED			LD_{50}
		24 hr	48 hr	72 hr	
B-9 CR^+	8.50	0 / 6	2 / 6	6 / 6	6.50
	7.50	0 / 6	1 / 6	4 / 6	
	6.50	0 / 6	0 / 6	3 / 6	
	5.50	0 / 6	0 / 6	1 / 6	
	4.50	0 / 6	0 / 6	0 / 6	
B-9 CR^-	8.43	0 / 6	1 / 6	4 / 6	7.93
	7.43	0 / 6	0 / 6	2 / 6	
	6.43	0 / 6	0 / 6	0 / 6	
	5.43	0 / 6	0 / 6	0 / 6	
	4.43	0 / 6	0 / 6	0 / 6	

Table 20 : Daily Mortality of 11-day-old White Leghorn Chicken Embryos After Inoculation with CR^+ and CR^- Colonial Variants of *C. jejuni* Strain B-9

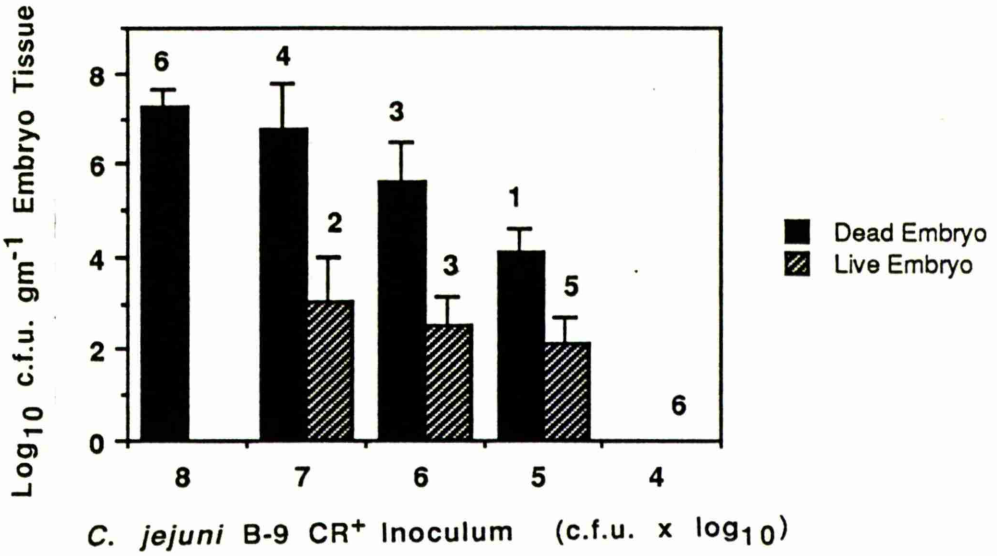
Inoculum and LD_{50} values are expressed as $\log_{10}$. Experimental procedure is described in Section 3.10.

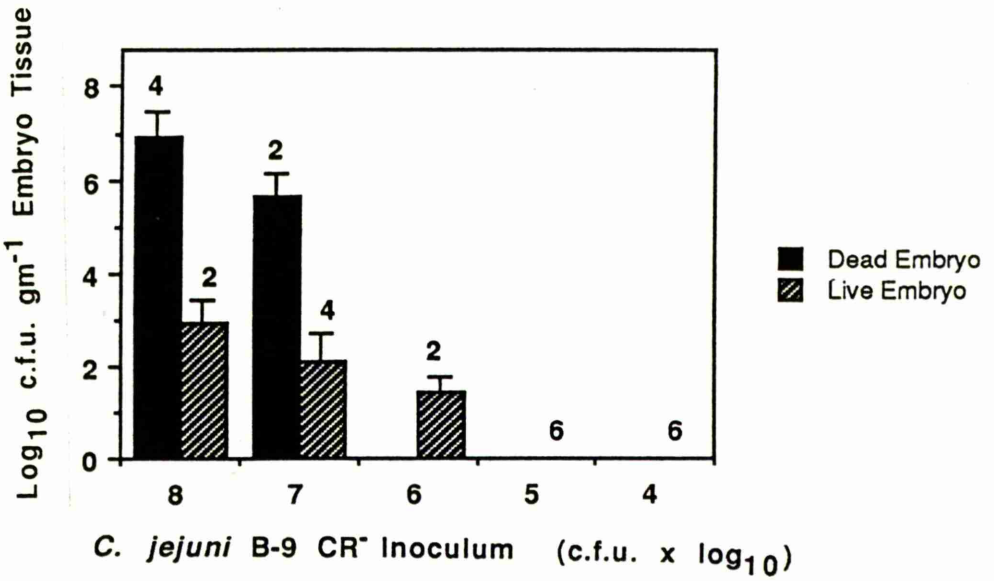
Figure 59 : Recovery of Viable *C.jejuni* Strain B-9 CR^+ from 11-day-old White Leghorn Chicken Embryos.

The results are presented as Log_{10} c.f.u. gm^{-1} embryo tissue (Mean + standard error of mean) recovered as a function of the inoculum size (log_{10}). The numbers adjacent to each bar represent the number of embryos averaged. Experimental procedures are described in Section 3.10. See Table 20 where LD_{50} has been calculated.

Figure 60 : Recovery of Viable *C.jejuni* Strain B-9 CR^- from 11-day-old White Leghorn Chicken Embryos.

The results are presented as Log_{10} c.f.u. gm^{-1} embryo tissue (Mean + standard error of mean) recovered as a function of the inoculum size (log_{10}). The numbers adjacent to each bar represent the number of embryos averaged. Experimental procedures are described in Section 3.10. See Table 20 where LD_{50} has been calculated.


Log10 c.f.u. gm-1 Embryo Tissue
0
2
4
6
8
6
4
2
3
3
1
5
6
8
7
6
5
4
C. jejuni B-9 CR+ Inoculum (c.f.u. x log10)
Dead Embryo
Live Embryo


Log10 c.f.u. gm-1 Embryo Tissue
0
2
4
6
8
4
2
2
4
2
6
6
8
7
6
5
4
C. jejuni B-9 CR- Inoculum (c.f.u. x log10)
Dead Embryo
Live Embryo

STRAIN	LD_{50} (Log 10)			LD_{50} C.F.U.	RELATIVE VIRULENCE CR^+ / CR^-
	EXPT.-1	EXPT.-2	MEAN		
S-11 CR^+	3.27	3.32	3.29	1.9×10^3	20.0
S-11 CR^-	4.39	4.79	4.59	3.8×10^4	
B-7 CR^+	3.84	4.54	4.19	1.5×10^4	120.0
B-7 CR^-	6.32	6.23	6.29	1.8×10^6	
S-13 CR^+	4.92	4.63	4.77	5.8×10^4	41.37
S-13 CR^-	6.32	6.46	6.39	2.4×10^6	
B-9 CR^+	6.50	---	6.50	3.1×10^6	27.41
B-9 CR^-	7.93	---	7.93	8.5×10^7	
B-23 CR^+	6.72	7.43	7.07	1.1×10^7	10.90
B-23 CR^-	>7.32	8.08	8.08	1.2×10^8	
11385 CR^+	3.77	4.36	4.06	1.1×10^4	45.45
11385 CR^-	5.65	4.79	5.70	5.0×10^5	

Table 21: LD_{50} of the CR^+ and CR^- Colonial Variants of the *C. jejuni* Strains in 11-day-old Chicken Embryo Model.

STRAIN B-23 / TEST STRAIN	FOLD INCREASE IN LD50 VALUE
B-23 / S-11	5789
B-23 / 11385	1000
B-23 / B-7	733
B-23 / S-13	189
B-23 / B-12	3.54

Table 22: Relative Virulence of the *C. jejuni* Strains in 11-day-old Chicken Embryo Model.

done with ten-fold doses of viable *C. jejuni*, ranging from ca. $1.0x10^2$ to $1.0x10^9$ (depending upon the strain) and deaths were recorded (by candling the egg) every 24 hr postinoculation up to 72 hr. Some embryos died after 24 hr, however, most of the deaths occurred between 48 and 72 hr. The deaths of the embryos on different post-inoculation days are shown in the Tables 10 to 20. The virulence of the strains was assessed by determining the LD_{50}s according to the method of Reed and Muench (1938) on the basis of the embryo deaths that occured during the 72 hour post-inoculation period (Tables 10 to 20). The strains exhibited marked differences in virulence as determined by their lethality in this model. The LD_{50} values ranged from $1.9x10^3$ to $8.53x10^7$ c.f.u. of *C. jejuni* for the strains S-11 and B-9 respectively; these represent the lowest and the highest values. The group D strains had relatively lower LD_{50} values in comparison to the group C strains (Table 21). The most virulent strain S-11, (a Group-D strain) was 5,789 times more virulent than strain B-23, which is a Group-C strain. The relative virulence of the different strains with reference to strain B-23, a strain of low virulence, is presented in Table 22. The strains also differed in terms of the deaths that occurred at different post-inoculation times. In the case of strain S-11, the deaths started to occur at 24 hr postinoculation and the majority of embryos were dead at 48 hr postinoculation (Tables 10 and 11). On the other hand, most of the deaths that occurred with strain B-23 and B-9 were observed at 72 hour postinoculation (Tables 18 to 20).

The viable *C. jejuni* recovered per gram of embryo tissue for different inocula of the strains are depicted in Figures 49 to 60. All the values represent the pooled results of two separate experiments except for strain B-9, which was tested once. The number of c.f.u.'s recovered per gm embryo tissue exceeded the number of organisms initially inoculated in the case of the strains S-11, B-7, S-13 and 11385, presumably indicating *in vivo* multiplication. On the other hand, the number of c.f.u.'s recovered per gm of embryo tissue for the strains B-23 and B-9 was less than the number inoculated. The strains B-7, S-11, S-13 and 11385 had lower LD_{50} values in

comparison to the strains B-23 and B-9 (Table 21), indicating good correlation between the virulence of the strains and their ability to multiply *in vivo*. It may be noted that most of the deaths occurred only in those embryos in which the number of viable *C. jejuni* recovered reached or exceeded the LD_{50} value of that particular strain. The embryos in which the number of viable *C. jejuni* recovered was less than the LD_{50} value, usually survived (Figures 49 to 60). Occasional deaths, noted in certain embryos challenged with *C. jejuni* less than the LD_{50}, may have been due to the differential susceptibility of the chick embryos as also noted by previous workers (Field *et al*, 1986a)

4.9 Adherence and Invasion Assays in the HeLa Cell Model

C. jejuni strains isolated from two clinically different types of diarrhoea i.e. cholera-like watery diarrhoea (Group C strains) and dysentery-like mucoid diarrhoea (Group D strains) were compared for their relative ability to adhere to and invade HeLa cells by quantitative bacteriology. Enteroinvasive *E. coli* strain 111 and *E. coli* K-12 HB 101 were used as the positive and negative controls respectively. After the 2 hr infection and 2 hr Gentamicin killing period, the number of bacteria recovered after lysis of the HeLa cells relative to the total number of bacteria initially added represented the number of intracellular bacteria. The proportion of intracellular bacteria was considered to reflect the invasion potential of the strain concerned. The mean number of bacteria in the absence of the Gentamicin represented the total number of the extracellular and intracellular bacteria. The number of adherent (extracellular) bacteria was determined indirectly by substracting the intracellular bacterial count from the total number of extracellular and intracellular bacteria (Flow Diagram 3).

4.9.1 Gentamicin Sensitivity

All the strains were tested for sensitivity to the antibiotic Gentamicin to determine

whether they could be used in the adherence and invasion assay in the HeLa cell model, as sensitivity to Gentamicin enabled differentiation of extracellular bacteria from intracellular ones. All the strains were found to be sensitive to the working concentration of Gentamicin (100 μg ml^{-1}) used. The killing of the *C. jejuni* strains S-11 and B-23 by Gentamicin over the 2 hr period is shown in Figure 61. No viable organisms were detected after 2 hr indicating efficient killing of the strains by Gentamicin.

4.9.2 Survey of Strains

In general, Group D strains were more adherent and more invasive in comparison to the group C strains (Fig 66 and Fig 67). The adherence and invasion potentials of the group D strains ranged from 23.70 ± 3.23 to 36.77 ± 4.20 (mean ± SD = 31.05 ± 4.58) and from 4.74±1.07 to 12.30±1.12 (mean 8.78 ± 2.12) respectively. On the other hand, the adherence and invasion potentials of the Group C strains ranged from 11.95 ± 2.44 to 35.5 ± 4.38 (mean ± SD = 24.74 ± 8.63)) and from 1.68±0.31 to 9.51±0.64 (mean ± SD = 5.80 ± 0.36). The invasion potential of the Group D strains was significantly higher than the Group C strains ($P < 0.01$; paired t-test). However, the adherence potential of the two groups of strains did not differ significantly ($P > 0.05$; paired t-test). When the adherence and invasion potentials of the individual strains were considered, it was noticeable that there was considerable overlap among the strains belonging to the two groups. Some of the group C strains were more invasive than some of the Group D strains and vice-versa (Fig. 66 and 67). With most of the strains the adherence and invasion potential positively correlated with each other. For example, a Group D strain S-11, which was most invasive (invasion potential 12.30 ± 1.12) also adhered to HeLa cells in higher numbers (adherence potential 36.77 ± 4.20). On the other hand, a Group C strain B-23, which exhibited the lowest invasion potential (1.68 ± 0.31) was also the least adherent (adherence potential 11.95 ± 2.44) to HeLa cells. However, this was not a generalized phenomenon as some of the strains belonging to

both the Groups C and D did not follow this trend. For example, the strain S-9 with a lower adherence potential (21.5 $\pm$ 3.83) was more invasive (invasion potential 5.74 $\pm$ 1.18) than the strain S-16, which exhibited a higher adherence potential (27.8$\pm$ 3.92) but was relatively less invasive (invasion potential 5.13 $\pm$ 1.02; Figure 66).

The adherence and invasion potential of two NCTC *C. jejuni* strains 11385, 11168; two *C. jejuni* strains isolated from faeces of chickens; the positive control strain enteroinvasive *E. coli* (EIEC) 111 and the negative control strain *E. coli* K-12 HB 101 are presented in the Figure 67. Strain 11385 which was originally isolated from a human gall bladder had a higher adherence and invasion potential than the strain 11168 which was a human stool isolate. The negative control *E. coli* K-12 yielded a negligible number of cells in the presence of Gentamicin (invasion potential 0.002), whereas the positive control strain EIEC 111 gave a high number of c.f.u. (invasion potential 16.75$\pm$3.87) under such conditions (Figure 68).

4.9.3 Reproducibility of the Adherence and Invasion Assay

Table 23 shows the results of adherence and invasion assays of the strain S-11 done in quadruplicate, this experiment is typical of the data produced which were used to determine the adherence and invasion potential of the other *C. jejuni* strains. Analysis of variance (F test) revealed no significant interexperimental variation with most strains. However, the variations noted with certain strains could be due to the fact that the number of bacteria inculated per well varied somewhat in some experiments. This might have resulted in a quantitative difference in the number of bacteria recovered. In addition, the phase (Caldwell *et al,* 1985) and antigenic variation (Harris *et al,* 1987) of *C. jejuni* flagella and the peculiar morphological changes seen in *C. jejuni* strains (Moran and Upton, 1986; 1987) might also have played a contributory role. Routine examination of the strains before each experiment for motility and typical spiral morphology was done to reduce the

possibility of experimental variation due to any morphological changes.

4.9.4 Time Course of Adherence and Invasion of HeLa cell

Two *C. jejuni* strains showing maximum (S-11) and minimum (B-23) adherence and invasion capability were selected for a time course study to ascertain the time and rates of adherence and invasion to HeLa cells. After an initial lag of about 30 min, the adherence and invasion increased progressively with time and became saturated and stabilized around 1.5 hr which was maintained for an additional 30 min (Figure 62 and 63). Therefore, all subsequent assays were run for 2 hr. The degree of adherence and invasion for the strain B-23 was much slower in comparison to the strain S-11 (Figure 62 and 63)

4.9.5 Multiplicity of Infection

The effect of multiplicity of infection (the number of bacteria per HeLa cell) on the adherence and invasion of the strains S-11 and B-23 was investigated in order to standardize the assay. The adherence and invasion capability of both the strains varied with the multiplicity of infection. At low multiplicity (10 *C. jejuni* : 1 HeLa cell), the invasion capacity of the weakly invasive strain B-23 was drastically reduced; in some experiments it was undetectable. On the other hand, with high multiplicity of infection (1000:1), incubation for 2 hr with the highly invasive strain S-11, evidence of a partial cytotoxic effect became apparent as some of the cells became detached and were washed out during the process of washing. Moreover, clumping of the bacterial cells (of strain S-11) was also noted in the EMEM at this high multiplicity of infection. To facilitate comparison of weakly invasive strains e.g. B-23, with strongly invasive strains e.g. S-11, on a common scale, an intermediate multiplicity of infection (100:1) was selected and was found to be suitable for both strains; it allowed the invasion capacity of the B-23 strain to be easily quantitated and no apparent cytotoxic effect was noted with strain S-11.

4.9.6 Intracellular multiplication

As Gentamicin has a very limited capacity to enter eucaryotic cells and hence efficiently kills only extracellular bacteria (Tronet and Tulkens, 1981; Niesel *et al,* 1985), an increase in the number of bacteria with time would reflect intracellular bacterial growth in the assay system employed in this study. In order to determine whether *C. jejuni* strains were capable of multiplication inside the HeLa cells, the number of bacteria protected from Gentamicin killing (intracellular bacteria) at 2, 4, 6, 18, and 24 hr after infection were determined relative to the number of bacteria present after the 2 hr infection period; the result with strain S-11 and the positive control EIEC 111 is shown in Figure 64. There was no change in the number of viable *C. jejuni* over the initial 6 hr of the experiment. At 24 hr, there was a slight decrease in the number of bacteria recovered. On incubation beyond 24 hr, a further decrease in the viable count was observed but at this time there was evidence of cytotoxicity as some of the cells started to round up and become detached; so experiments were not extended beyond 24 hr. On the other hand, the positive control strain EIEC 111 was found to be rapidly multiplying intracellularly as evidenced by the increase in the number of intracellular bacteria recovered (Figure 64). In fact, the kinetics of intracellular growth experiments with EIEC were terminated after 6 hr as the HeLa cell monolayer infected with this bacterium became partially detached presumably due to extensive intracellular multiplication.

4.9.7 Influence of Assay Temperature

The effect of temperature on the adherence and eventual invasion of *C. jejuni* strain S-11 was investigated at three different temperatures; 4^{o}, 37^{o} and 42^{o} C. HeLa cell cultures inoculated with *C. jejuni* strains were incubated at these temperatures for two hr followed by the Gentamicin killing step at 37^{o} C. Incubation at 4^{o} C resulted in a significant decrease in the adherence ($P < 0.05$) and invasion ($P < 0.001$)

capability of the *C. jejuni* strain. The adherence and invasion potenials were found to be slightly but not significantly ($P > 0.05$) lower when the assay was done at 42° in comparison to 37° assay (Figure 65). Hence, all subsequent assays were done at 37° C

4.9.8 Influence of *C. jejuni* Growth Conditions

The effect of growth conditions on *C. jejuni* adherence to and invasion of HeLa cells were investigated by growing the organisms at 37° C and 42° C on both solid mebium (Brucella blood agar) and liquid medium (Brucella broth) (detailed in Sections 3.4.1 and 3.4.2 respectively). Broth grown cells adhered more avidly and invaded more efficiently when compared with the agar grown cells (Figure 65). *C. jejuni* strain S-11 grown at 37° C exhibited a slightly but not significantly decreased adherence and invasion capability than when it was grown at 42° C (Figure 65). The adherence and invasion potential was significantly lower with *C. jejuni* strain S-11 grown on plates at 37° C. With cells grown on plates at 42° C, only the invasion potential was significantly lowered. The adherence potential was slightly but not significantly lowered when the cells were grown at 42° C in Brucella broth. So, all the other strains used in this study were grown in Brucella broth at 42° C.

Cells from the exponential phase of growth (16 hr) were found to be relatively more adherent ($P < 0.05$) and invasive ($P < 0.01$) than the cells from the stationary phase (ca. 26 hr) (Figure 65), so cells from 16 hr cultures were used to infect HeLa cells in subsequent studies.

4.9.9 Influence of Gas Atmosphere

To optimize the assay, the effect of the gas atmosphere in the adherence and invasion process was investigated. After inoculations of the HeLa cell monolayers with *C. jejuni* strains the tissue culture plates were incubated at 37° C in microaerophilic

Table 23: Determination of Adherence and Invasion Potential of the *C. jejuni* strain S-11.

Extracellular Bacteria (c.f.u. ml^{-1})	Adherence Potential	Intracellular Bacteria (c.f.u. ml^{-1})	Invasion Potential
$9.6x10^6$	38.4	$2.4x10^6$	9.6
$7.3x10^6$	29.2	$2.8x10^6$	11.2
$8.3x10^6$	33.2	$3.2x10^6$	12.8
$1.0x10^7$	40.0	$3.7x10^6$	14.8
Mean ± Standard Deviation			
$(8.8 \pm 1.2) x10^6$	35.2 ± 4.94	$(3.0 \pm 0.55) x10^6$	12.1 ± 2.22

The data represent a typical experiment done in quadruplicate.

The procedure for the calculation of the adherence and invasion potentials, which represent the extracellular and intracellular bacteria respectively is detailed in Section 3.21.2 and schemetically illustrated in the Flow Diagram 3.

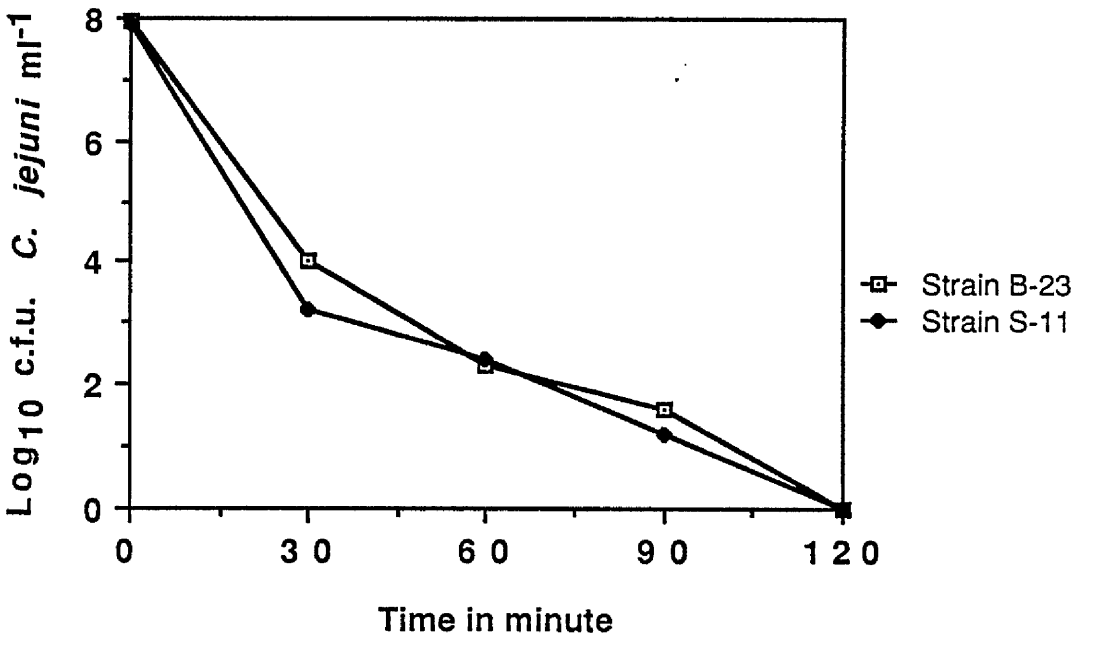


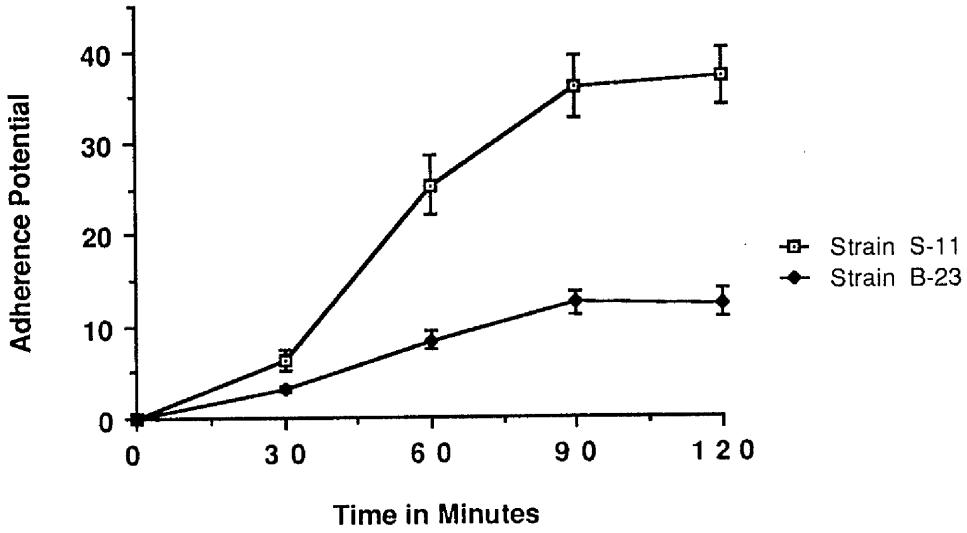
Figure 61: Gentamycin Sensitivity of the *C. jejuni* strains B-23 and S-11

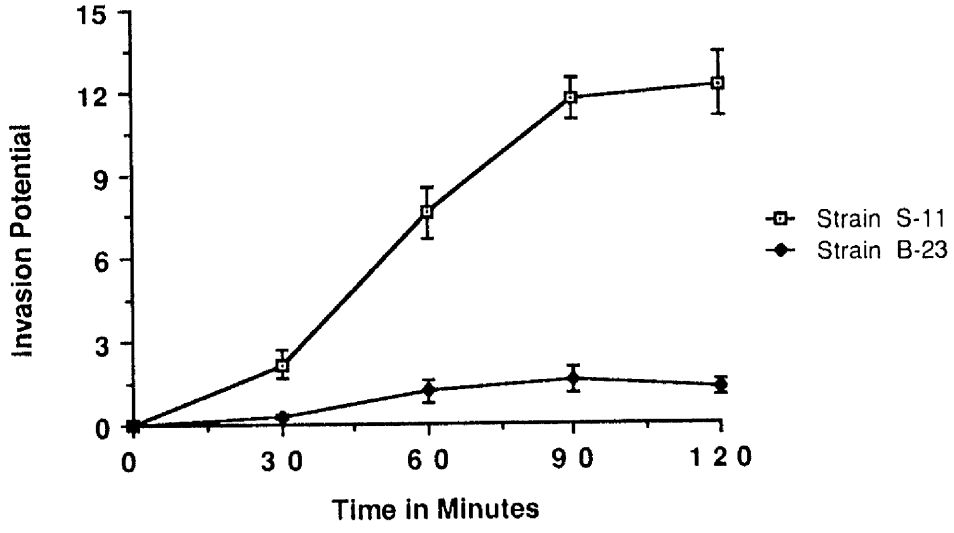
Figure 62: Time Course of Adherence of the *C. jejuni* Strains S-11 and B-23 in HeLa Cell Model.

Results are presented as mean ± SD of two experiments done in quadruplicate. Experimental procedure is described in Section 3.21.3.

Figure 63: Time Course of Invasion of the *C. jejuni* Strains S-11 and B-23 in HeLa Cell Model.

Results are presented as mean ± SD of two experiments done in quadruplicate. Experimental procedure is described in Section 3.21.3.


Adherence Potential
40
30
20
10
0
0
30
60
90
120
Time in Minutes
Strain S-11
Strain B-23


Invasion Potential
15
12
9
6
3
0
0
30
60
90
120
Time in Minutes
Strain S-11
Strain B-23

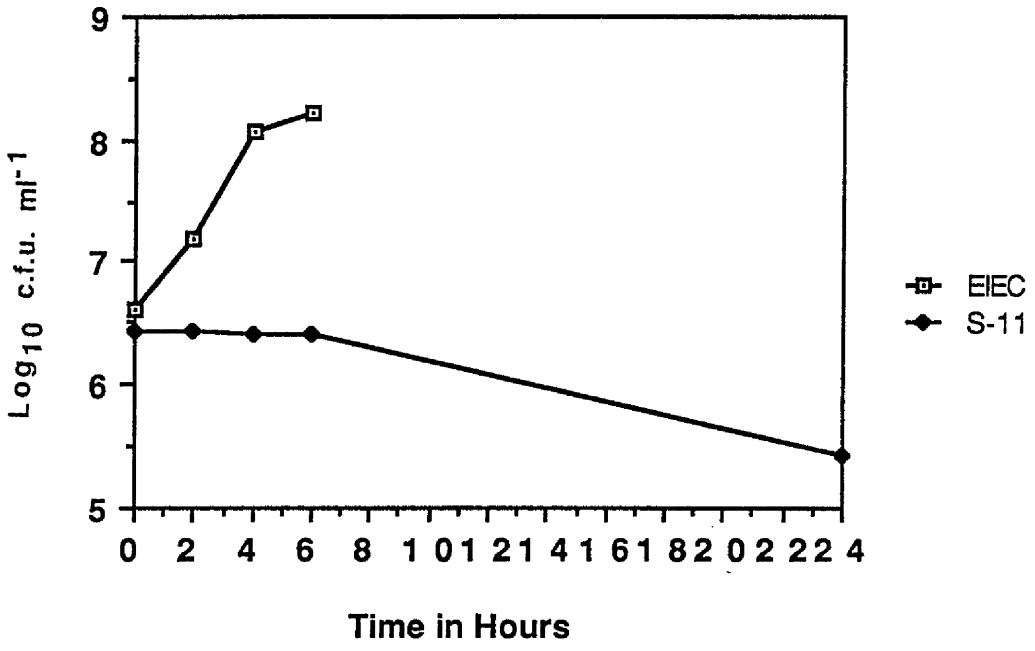


Figure 64: Intracellular Multiplication of the Positive Control Strain EIEC 111 and the *C. jejuni* Strain S-11 in HeLa Cell Model.

Note that experiment with EIEC could not be extended beyond 6 hr as evidences of cytotoxicity became apparent and the cell monolayers became detached presumably due to extensive intracellular multiplication. (See 3.21.2 and 4.9.6).

Figure 65:

Effects of Different Growth Parameters on the Adherence and Invasion Potential of the *C. jejuni* strain S-11

Lanes	Growth Medium	Growth Temperature	Assay Temperature	Incubation Period
A	Brucella Broth	42° C	37° C	16-18 hr
B	"	37° C	"	"
C	Brucella Blood Agar	"	"	24 hr
D	"	42° C	"	"
E	Brucella Broth	42° C	42° C	16-18 hr
F	"	42° C	37° C	26 hr
G	"	"	4° C	16-18 hr
H	"	"	37° C[a]	"

a: Assay was done by incubating *C.jejuni* with HeLa cell monolayer in a gas atmosphere of 5 % CO_2 - 95 % air. The gas atmosphere of all the other lanes (A to G) was microaerophilic (5 % O_2, 10 % CO_2, 85 % N_2)

Adherence and Invasion assays were done as described before (Section 3.21.2)

Significance levels: NS = Not Significant
* = $P \leq 0.05$
** = $P \leq 0.01$
*** = $P \leq 0.001$

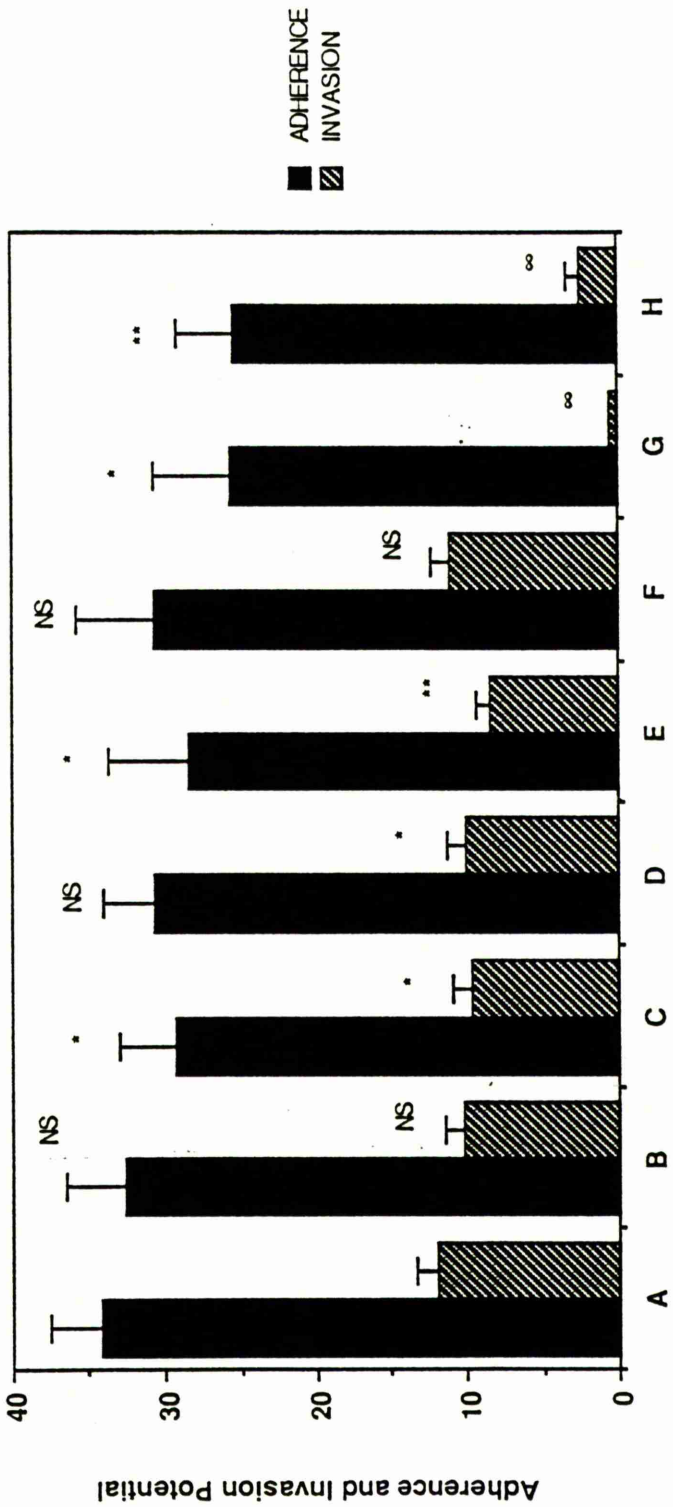

ADHERENCE
INVASION
Adherence and Invasion Potential
40
30
20
10
0
A
B
C
D
E
F
G
H
NS
NS
NS
NS
*
*
*
*
**
**
§
§

Figure 66 : Adherence and Invasion Potential of the Group C *C. jejuni* Strains.

The histograms and the bars represent the mean and standard deviation respectively of two or three experiments done in quadruplicate. Experimental procedure is schematically represented in the Flow Diagram -3 and described in Section 3.21.2.

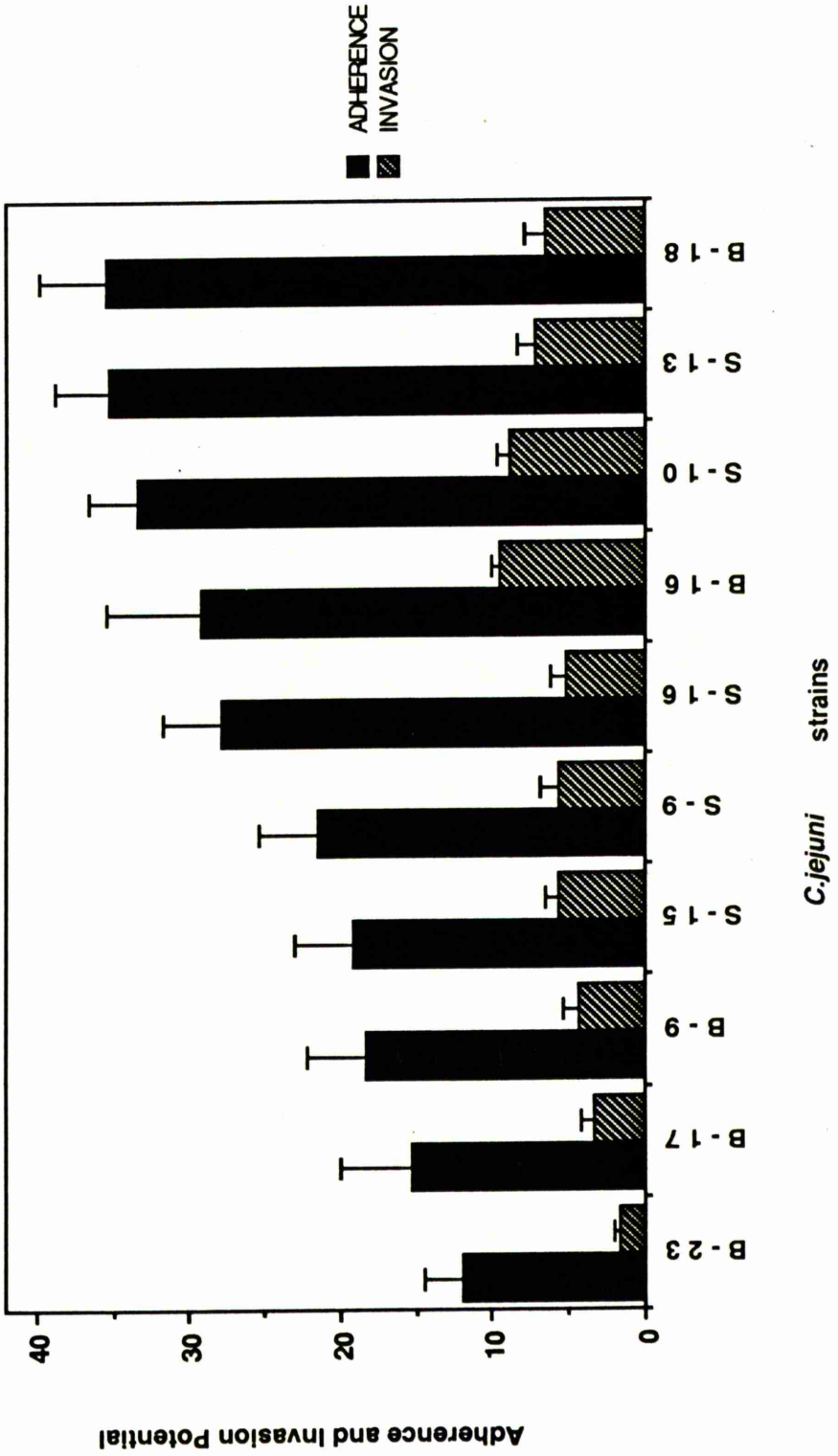

ADHERENCE
INVASION
Adherence and Invasion Potential
0
10
20
30
40
B-23
B-17
B-9
S-15
S-9
S-16
B-16
S-10
S-13
B-18
C.jejuni strains

Figure 67 : Adherence and Invasion Potential of the Group D *C. jejuni* Strains.

The histograms and the bars represent the mean and standard deviation respectively of two or three experiments done in quadruplicate. Experimental procedure is schematically represented in the Flow Diagram -3 and described in Section 3.21.2.

ADHERENCE
INVASION
Adherence and Invasion Potential
0
10
20
30
40
B-12
S-12
B-13
B-20
S-14
B-10
B-15
B-14
B-7
S-11
C.jejuni strains

Figure 68 : Adherence and Invasion Potential of the Control Bacterial Strains.

The histograms and the bars represent the mean and standard deviation respectively of two or three experiments done in quadruplicate. Experimental procedure is schematically represented in the Flow Diagram -3 and described in Section 3.21.2.

ADHERENCE
INVASION
Adherence and Invasion Potential
0
10
20
30
40
11385
11168
CH-1
CH-2
E.coli K-12
EIEC 111
Bacterial Strains

atmosphere (5% O2, 10% CO2, 85% N2) and also in 5% CO2-95% air. The results of the adherence and invasion assays of the strain S-11 assayed under microaerophic gas atmosphere and 5 % CO_2-95 % air are presented in the Figure 65. A significant reduction in adherence ($P < 0.01$) and invasion ($P < 0.001$) potential occurred when incubated in 5% CO2-95% air than in the microaerophilic atmosphere.

4.9.10 Nonspecific Adherence to Tissue Culture Plates

As *C. jejuni* strains were reported to possess the capability of adhering to plastic surfaces (McSweegan and Walker, 1986), control experiments were done to evaluate quantitatively the average number of bacteria that might adhere to the surface of the plates during the assay. Such figures ranged from 0.62 to 1.31% for 5 strains exhibiting strong adherence selected at random (3 from Group D strains; S-11, B-7 and B-14 and 2 from Group C strains; S-13 and B-18). So an arbitrary baseline figure of 2% was adopted and substracted from the percentage of total extracellular and intracellular bacteria recovered for each strain before substracting the percentage of the intracellular bacteria to account for any nonspecific adherence to the tissue culture plates.

4.9.11 Effects of L-fucose

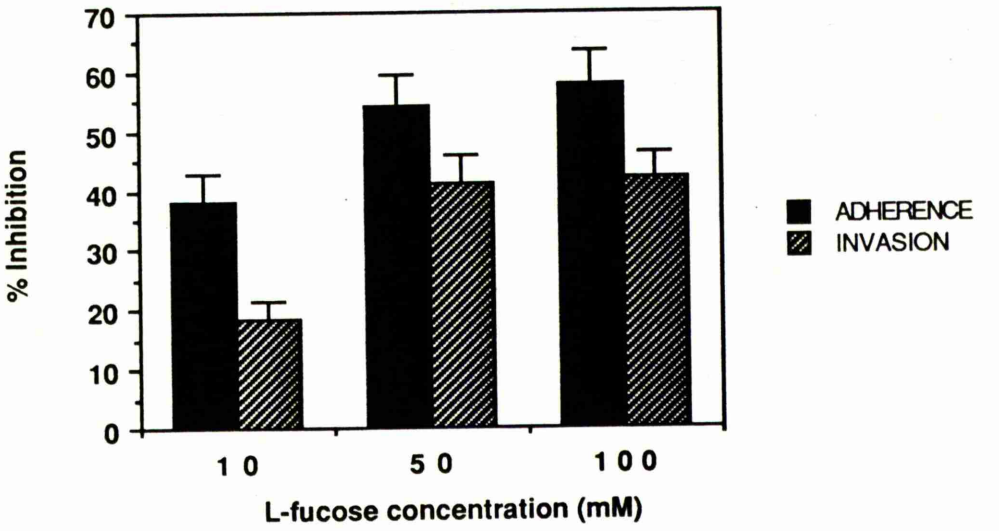
In an attempt to determine the specificity of adherence and subsequent invasion of HeLa cells by *C. jejuni* strains, the assays were done with bacterial cells preincubated with various monosaccharides. Figure 69 shows the dose-dependent adherence curve of the strain S-11 preincubated with L-fucose for 15 min at 37°C. Adherence and invasion were inhibited as a function of L-fucose concentration up to 50 mM; with further increases in the monosaccharide concentraion the effect was not significant. The effect of 50 mM L-fucose on the adherence and invasion potential of the *C. jejuni* strains S-13, B-7 and B-23 is shown in the Figure 70

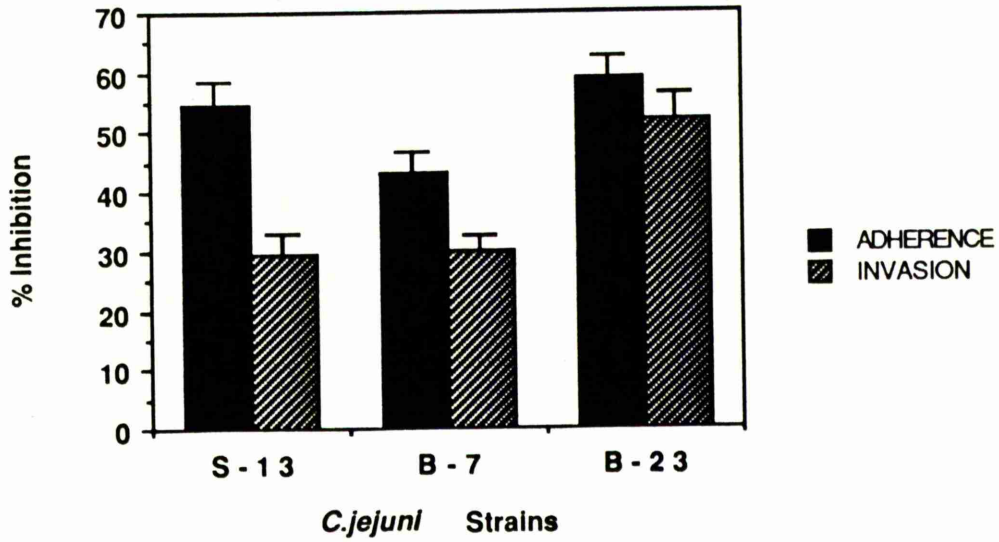
Figure 69: Effect of L-fucose on the Adherence and Invasion Potential of the *C. jejuni* Strain S-11.

Experimental procedure is described in Section 3.21.5. The results are presented as the mean and standard deviation of two experiments done in quadruplicate. Highly significant reduction ($P \leq 0.001$) in adherence and invasion was noted with all the concentrations of L-fucose used.

Figure 70: Effect of L-fucose on the Adherence and Invasion Potential of the *C. jejuni* Strains S-13, B-7 and B-23.

Experimental procedure is described in Section 3.21.5. The results are presented as the mean and standard deviation of two experiments done in quadruplicate. The concentration of L-fucose used was 50 mM. Highly significant reduction ($P \leq 0.001$) in adherence and invasion was noted with all the strains tested.


70
60
50
40
30
20
10
0
% Inhibition
1 0
5 0
1 0 0
L-fucose concentration (mM)
ADHERENCE
INVASION


70
60
50
40
30
20
10
0
% Inhibition
S - 1 3
B - 7
B - 2 3
C.jejuni Strains
ADHERENCE
INVASION

In all cases, the inhibitory effect of L-fucose was more pronounced on invasion than on adherence.

4.9.12 Effect of Antisera

The effect of rabbit antisera against the formalinised and heat killed *C.jejuni* strain NCTC 11168 on the homologous and heterologous *C.jejuni* strains were examined to determine the role of heat-labile and heat-stable surface antigens of *C.jejuni* on the adherence and subsequent invasion of HeLa cells. Figure 71 shows the reduction in adherence and invasion potential of HeLa cells by the strain 11168 by a series of dilutions of the antiserum against formalinised bacteria. Some 80 % reduction in invasion was obtained with 1:25 dilution of the antisera; which decreased to about 19 % with 1:400 dilution. The reduction in adherence potential with 1:25 and 1:400 dilutions of antiserum was approximately 94 % and 68 % respectively (Figure 71). The inhibitory effect of antiserum raised against formalinised bacteria was on the adherence potential was less pronounced in comparison to the invasion process. The inhibitory effect of 1:100 diluted antiserum on the adherence and invasion potential of the *C.jejuni* strains S-11, S-13 and B-23 is shown in the Figure 72. The antiserum had a maximum inhibitory effect on the strain S-13 followed by B-23 and S-11. Interestingly, the inhibitory effect of the antisera was almost the same on the adherence potential of the strains S-13 and B-23 but the effect on the invasion potential was different (62 % and 52 % respectively).

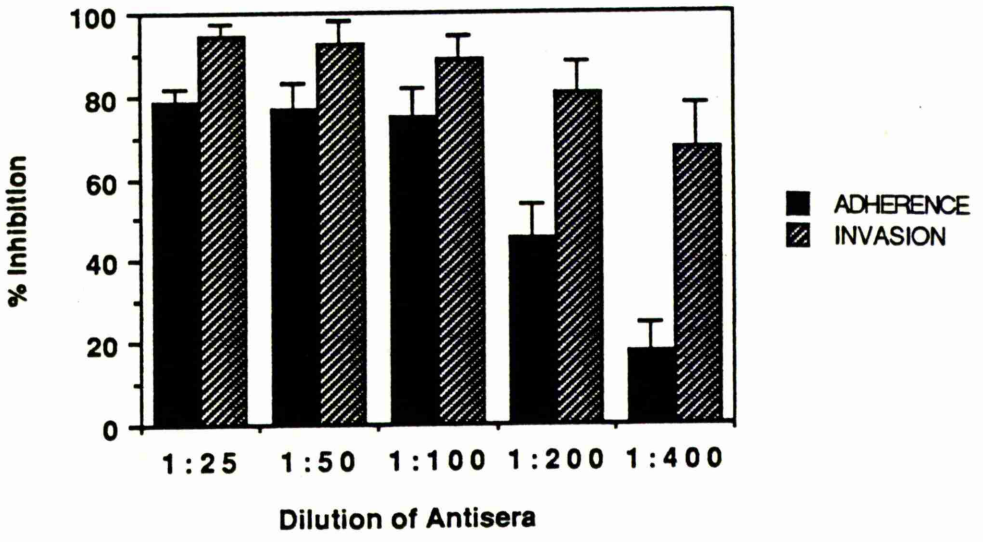
The inhibitory effect of the antiserum against heat-killed *C.jejuni* (1: 25 dilution) on adherence and invasion potential of the strain 11168 were ca. 9.0 % and 7.5 % repectively. As these figures did not differ significantly from the values obtained with normal rabbit serum ca. 6.0 % and 5.5 % respectively, the effect of this antiserum was considered non-specific and was not pursued with other strains.

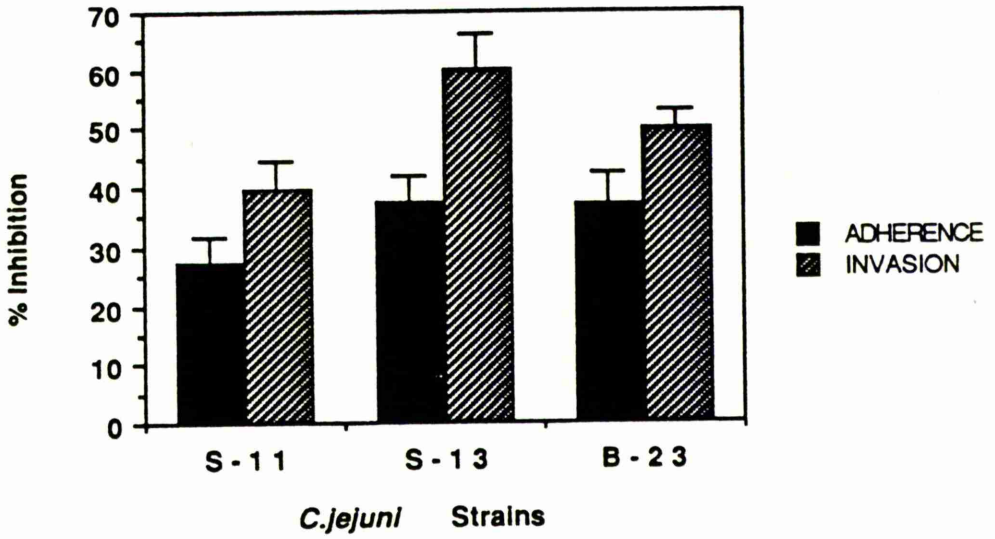
Figure 71: Effect of Antisera on the Adherence and Invasion Potential of the *C.jejuni* Strain 11168

Experimental procedure is described in Section 3.21.8. The results are presented as the mean and standard deviation of two experiments done in quadruplicate. Highly significant reduction ($P \leq 0.001$; paired t-test) in invasion and adherence was noted with all the dilutions of antisera used.

Figure 72: Effect of Antisera on the Adherence and Invasion Potential of the *C. jejuni* Strains S-11, S-13 and B-23.

Experimental procedure is described in Section 3.21.8. The results are presented as the mean and standard deviation of two experiments done in quadruplicate. The dilution of antisera used was 1:100. Highly significant reduction ($P \leq 0.001$; paired t-test) in invasion and adherence was noted with all the strains tested.


100
80
60
40
20
0
% Inhibition
1:25
1:50
1:100
1:200
1:400
Dilution of Antisera
ADHERENCE
INVASION


70
60
50
40
30
20
10
0
% Inhibition
S-11
S-13
B-23
C.jejuni Strains
ADHERENCE
INVASION

4.9.13 Effect of Chicken Intestinal Mucus

Adherence and invasion assays were carried out with HeLa cell monolayers precoated with chicken intestinal mucus to determine the possible effect mucin might have on the efficiency of adherence to and invasion of the *C. jejuni* strains. Pilot experiments with different concentrations of mucus (100 to 500 μg ml^{-1}) showed that the adherence and invasion of HeLa cells by the strain S-11 were inhibited in a concentration dependent manner (Fig 73). With 500 μg ml^{-1}, the inhibitory effect on adherence was slightly higher in comparison to the effect with 250 μg ml^{-1} but the effects on the invasion potential were almost identical. As maximal inhibition (against the control with no mucus) of invasion was obtained with a mucus concentration of 250 μg ml^{-1}, this concentration was used in assays with the other *C. jejuni* strains. The inhibitory effects of mucus on the adherence of the *C. jejuni* strains ranged from a minimum of approximately 37% with strain B-7 to a maximum of 57% with strain B-23 (Figure 74). The inhibitory effect of the mucus on the invasion process was more dramatic; it ranged from a minimum of ca. 35 % with strain B-7 to a maximum of 55 %. The inhibitory effect of mucus was highly significant ($P < 0.001$) for all the strains at all the concentrations of mucus investigated.

4.9.14 Effect of Outer Membrane Proteins

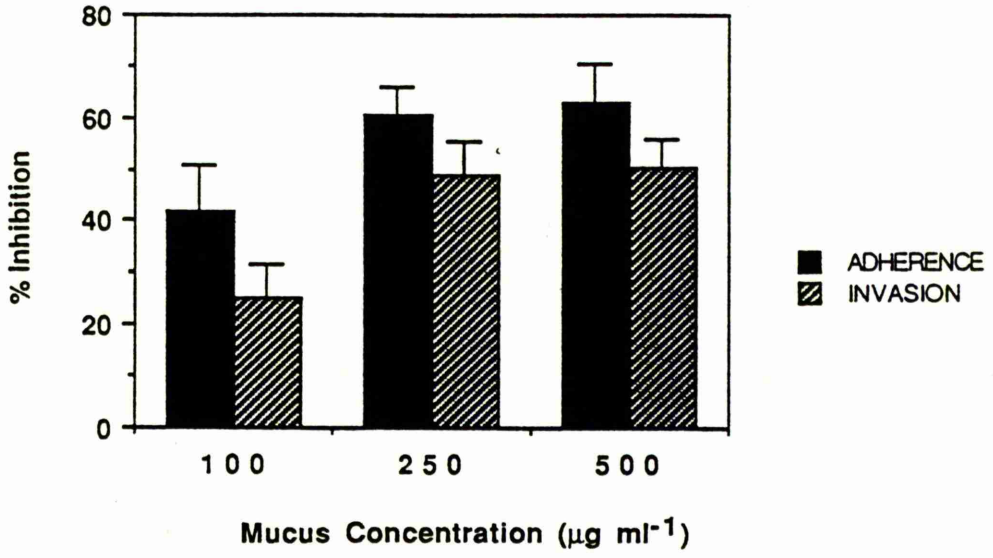
To determine the role played by outer membrane protein (OMP) preparations of the *C. jejuni* strains in the adherence and invasion process, HeLa cell monolayers were incubated with varying concentrations of OMPs prior to carrying out the assays. Figure 75 shows the inhibition of the adherence and invasion process of the *C. jejuni* strain S-11 in HeLa cells as a function of homologous OMP concentration. Virtually no inhibition was observed with OMP concentration below 50 μg ml-1; however with higher concentrations a significant inhibition was noted (Figure 75). To identify the protein component(s) in the OMP preparation exerting the inhibitory

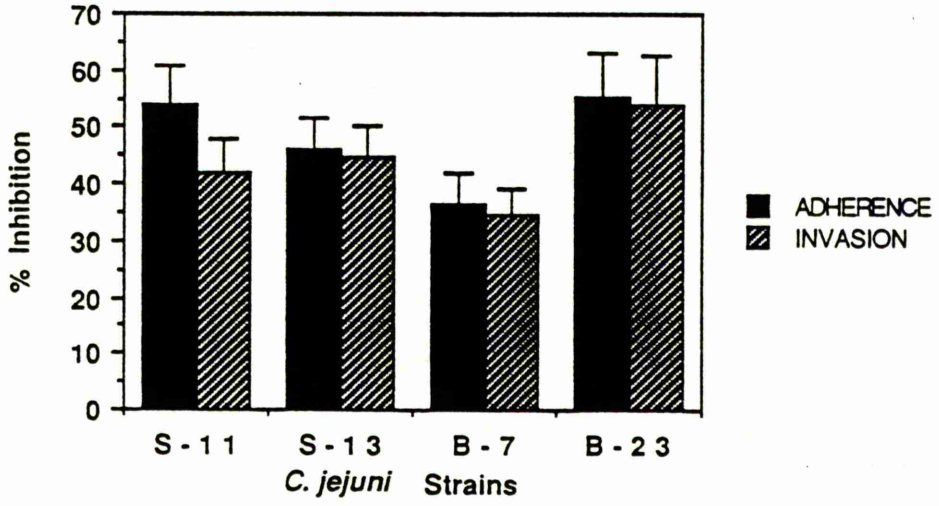
Figure 73: Effect of Chicken Intestinal Mucus on the Adherence and Invasion Potential of the *C. jejuni* Strain S-11.

Experimental procedure is described in Section 3.21.7. The results are presented as the mean and standard deviation of two experiments done in quadruplicate. Highly significant reduction ($P \leq 0.001$; paired t-test) in invasion and adherence was noted with all the mucus concentration used.

Figure 74: Effect of Chicken Intestinal Mucus on the Adherence and Invasion Potential of the *C. jejuni* Strains S-13, B-7 and B-23.

The mucus concentration used was 250 $\mu g\ ml^{-1}$. Experimental procedure is described in Section 3.21.7. The results are presented as the mean and standard deviation of two experiments done in quadruplicate. Highly significant reduction ($P \leq 0.001$; paired t-test) in invasion and adherence was noted with all the strains tested.


80
60
40
20
0
% Inhibition
100
250
500
Mucus Concentration (μg ml-1)
ADHERENCE
INVASION


70
60
50
40
30
20
10
0
% Inhibition
S-11
S-13
B-7
B-23
C. jejuni Strains
ADHERENCE
INVASION

effect on the HeLa cell adherence and invasion process, purified major outer membrane protein (MOMP) of *C.jejuni* (Section 3.16) was also investigated for any inhibitory on HeLa cell assay sytem. The MOMP also exhibited an inhibitory effect in a concentration dependent fashon. The minimum concentration of MOMP which had an inhibitory effect was 10.0 μg ml^{-1}; however the effect on the adherence process was not significant. Higher concentration (50.0 μg ml^{-1}) inhibited adherence and invasion potential significantly (Figure 76).

4.9.15 Effect of Cytochalasin B

The effect of cytochalasin B on the invasion potential of the *C.jejuni* strain S-11 is shown in Figure 77. With 1.0 μg ml^{-1}, the invasion was inhibited by approximately 40 %, in comparison to untreated controls ; and 10.0 μg ml^{-1} completely inhibited invasion. Cytochalasin B had a minimal inhibitory effect on the adherence potential with the concentration range used.

Figure 75: Effect of Outer Membrane Proteins on the Adherence and Invasion Potential of *C. jejuni* Strains S-11 and B-23

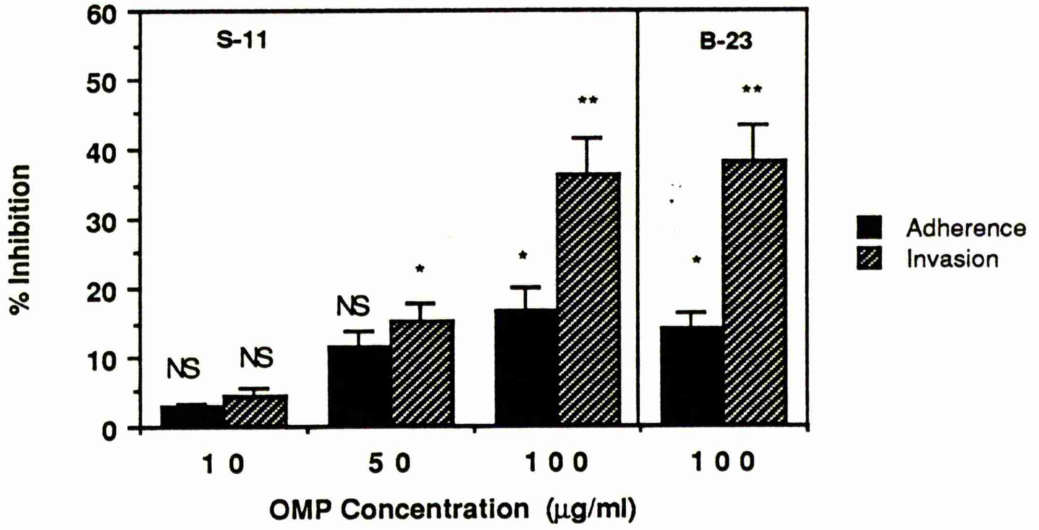
Experimental procedure is described in Section 3.21.9. The results are presented as the mean and standard deviation of two experiments done in triplicate

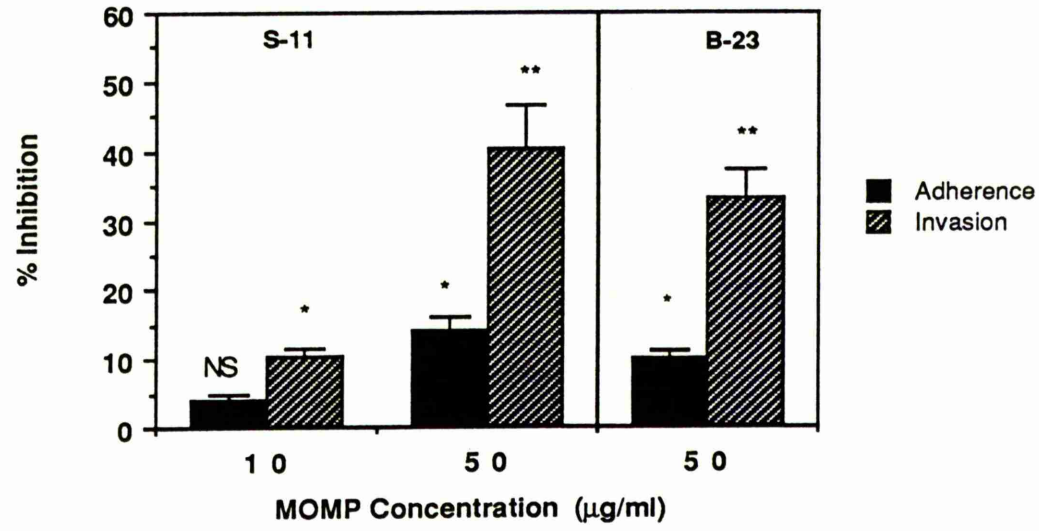
Significance levels (Paired t-test): NS = Not Significant
* = $P \leq 0.05$
** = $P \leq 0.01$
*** = $P \leq 0.001$

Figure 76: Effect of the Major Outer Membrane Protein (MOMP) on the Adherence and Invasion Potential of *C. jejuni* Strains S-11 and B-23

Experimental procedure is described in Section 3.21.9. The results are presented as the mean and standard deviation of two experiments done in triplicate.

Significance levels (Paired t-test): NS = Not Significant
* = $P \leq 0.05$
** = $P \leq 0.01$
*** = $P \leq 0.001$


60
50
40
30
20
10
0
% Inhibition
S-11
B-23
NS
NS
NS
*
*
*
**
**
1 0
5 0
1 0 0
1 0 0
OMP Concentration (µg/ml)
Adherence
Invasion


60
50
40
30
20
10
0
% Inhibition
S-11
B-23
NS
*
*
*
**
**
1 0
5 0
5 0
MOMP Concentration (µg/ml)
Adherence
Invasion

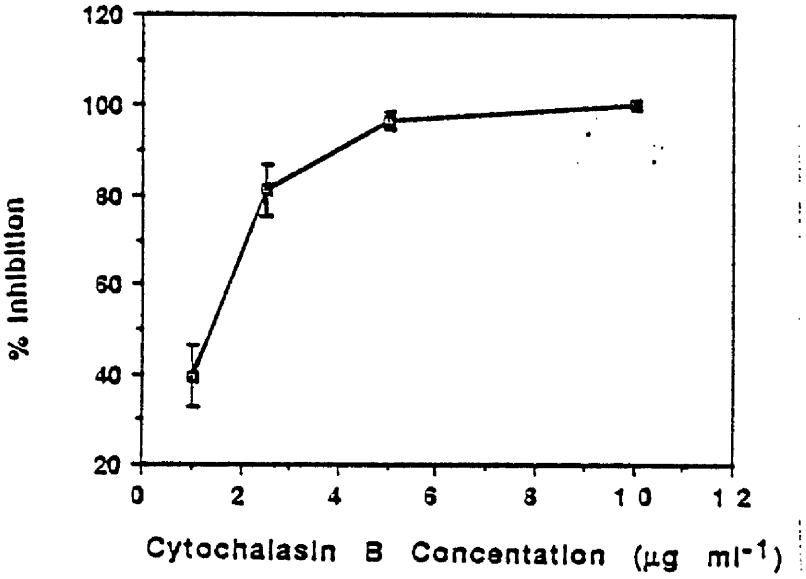


Figure 77: Effect of Cytochalasin B on the Invasion Potential of the *C. jejuni* Strain S-11

Experimental procedure is described in Section 3.21.6. The results are presented as the mean ± SD of two experiments done in quadruplicate.

Figure 78: Correlation Between Adherence and Invasion Potential of the *C. jejuni* Strains

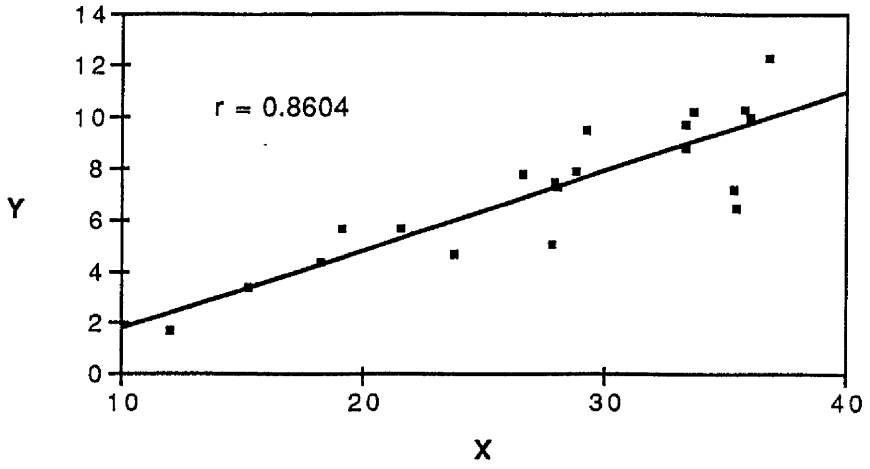
X = Adherence potential
Y = Invasion potential

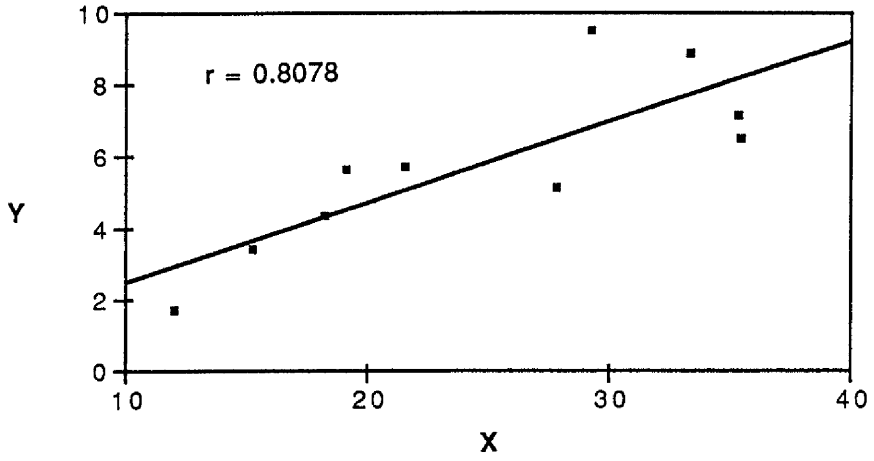
Figure 79: Correlation Between Adherence and Invasion Potential of the Group C *C. jejuni* Strains

X = Adherence potential
Y = Invasion potential

Figure 80: Correlation Between Adherence and Invasion Potential of the Group D *C. jejuni* Strains

X = Adherence potential
Y = Invasion potential


r = 0.8604
Y
X
0
2
4
6
8
10
12
14
10
20
30
40


r = 0.8078
Y
X
0
2
4
6
8
10
10
20
30
40

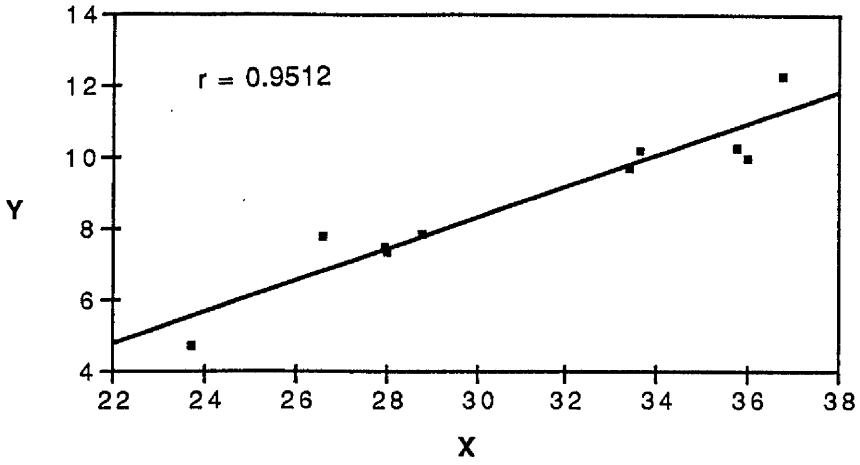

r = 0.9512
Y
X
4
6
8
10
12
14
22
24
26
28
30
32
34
36
38

Figure 81: Correlation Between Lethality in Chicken Embryo Model and Adherence to HeLa Cells

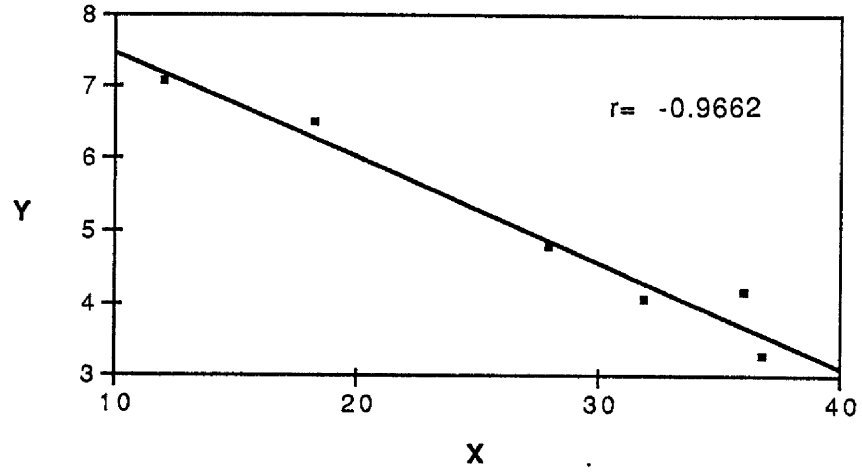
X = Adherence potential
Y = Lethality in chicken embryo model (LD_{50} values)

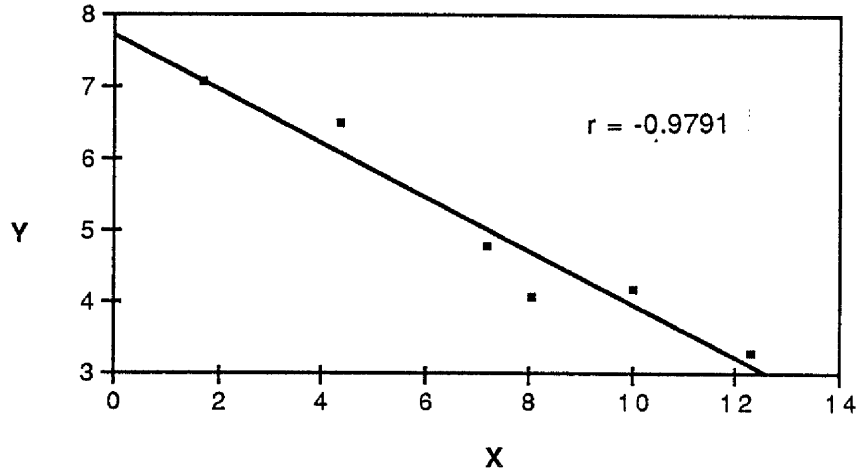
Figure 82: Correlation Between Lethality in Chicken Embryo Model and Invasion of HeLa Cells

X = Invasion potential
Y = Lethality in chicken embryo model (LD_{50} values)

Figure 83: Correlation Between Salt Aggregation (SA) Test and Bacterial Adherence to Hydrocarbon (BATH) Test

X = BATH Test values
Y = SA Test values


r= -0.9662
Y
X
8
7
6
5
4
3
10
20
30
40


r = -0.9791
Y
X
8
7
6
5
4
3
0
2
4
6
8
10
12
14

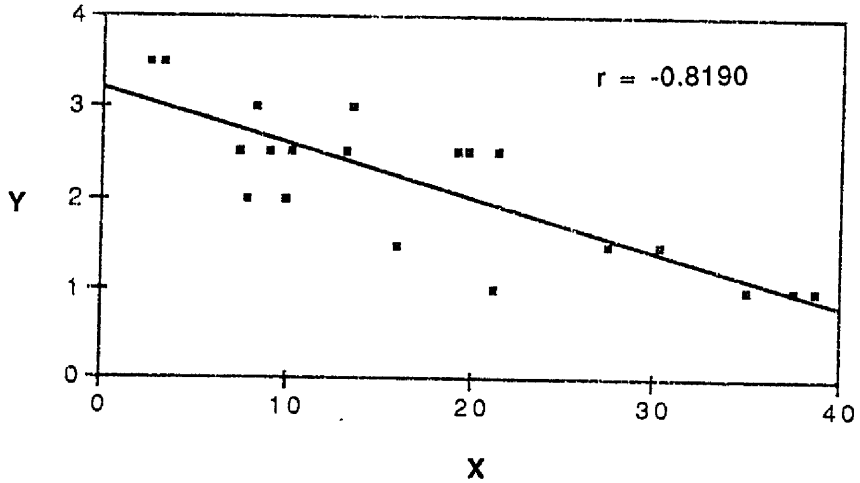

r = -0.8190
Y
X
4
3
2
1
0
0
10
20
30
40

Figure 84: **Correlation Between Adherence to HeLa Cells and Cell Surface Hydrophobicity: SA Test**

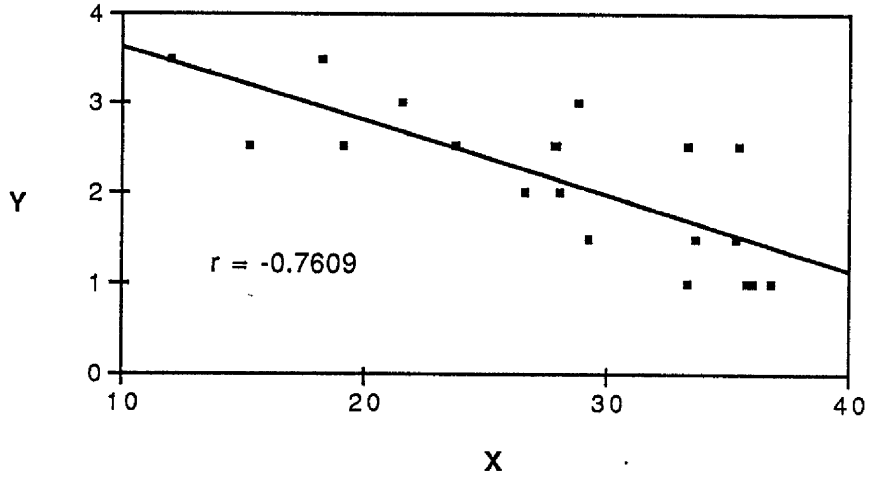
X = Adherence potential
Y = SA Test values

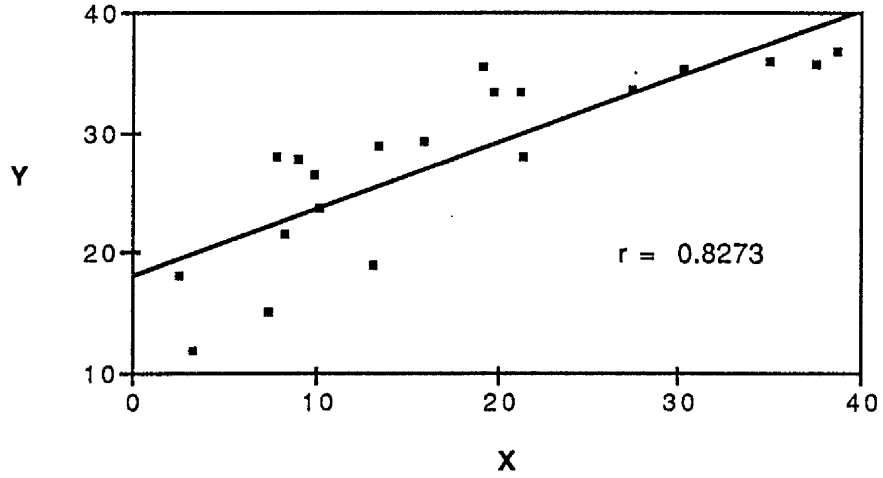
Figure 85: **Correlation Between Adherence to HeLa Cells and Cell Surface Hydrophobicity: SA Test**

X = BATH Test values
Y = Adherence potential

Figure 86: **Correlation Between Invasion of HeLa Cells and Cell Surface Hydrophobicity: SA Test**

X = Adherence potential
Y = SA Test values


Y
4
3
2
1
0
r = -0.7609
10
20
30
40
X


Y
40
30
20
10
r = 0.8273
0
10
20
30
40
X

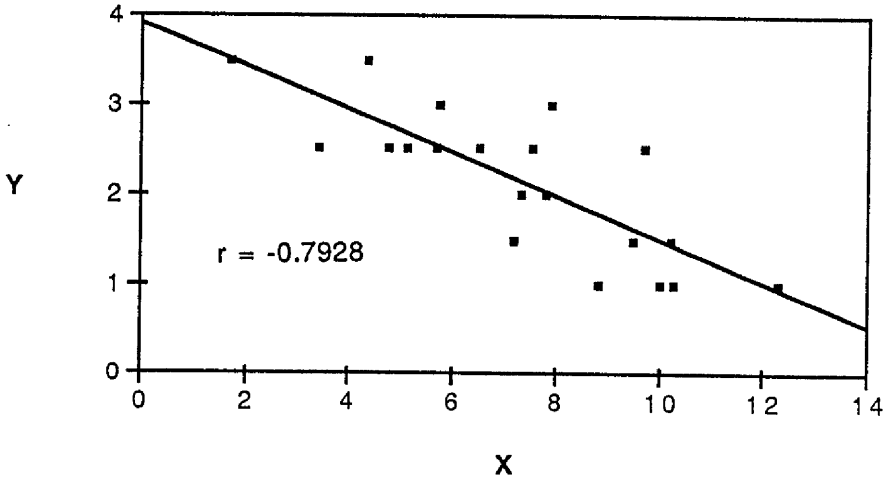

Y
4
3
2
1
0
r = -0.7928
0
2
4
6
8
10
12
14
X

Figure 87: Correlation Between Invasion of HeLa Cells and Cell Surface Hydrophobicity: BATH Test

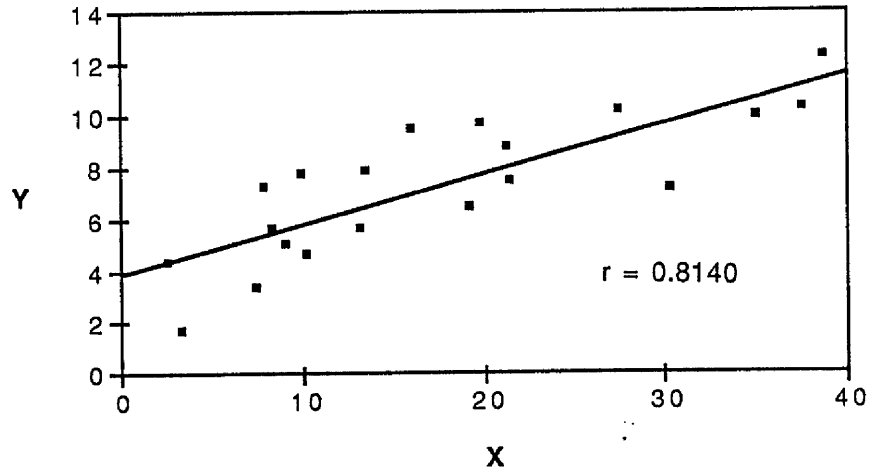
X = BATH Test values
Y = Invasion potential

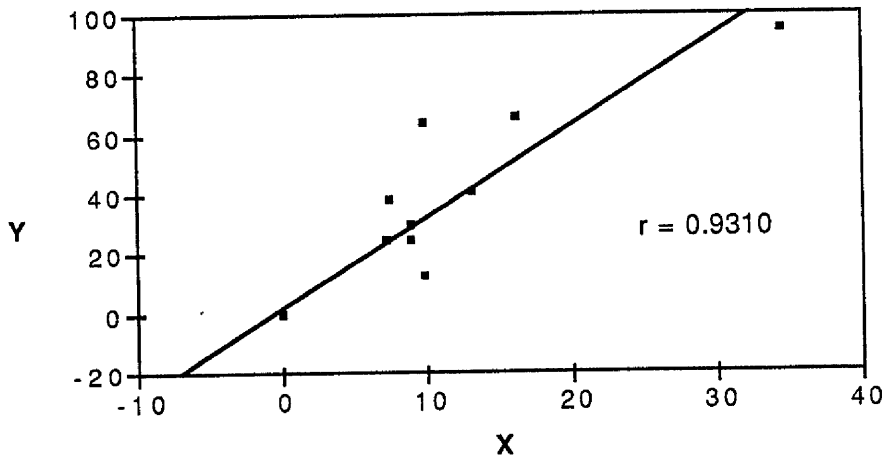
Figure 88: Correlation Between CHO Cell Assay and ELISA for Quantitation of *C. jejuni* Enterotoxin

$X = TCED_{50}\ mg^{-1}$ protein
$Y = ng\ of\ CJT^{a}\ mg^{-1}$ protein
a = *C.jejuni* enterotoxin

Figure 89: Correlation Between Adherence to HeLa Cells and Enterotoxin Production: CHO Cell Assay

$X = TCED_{50}\ mg^{-1}$ protein
Y = Adherence potential


14
12
10
8
6
4
2
0
Y
r = 0.8140
0
10
20
30
40
X


100
80
60
40
20
0
-20
Y
r = 0.9310
-10
0
10
20
30
40
X

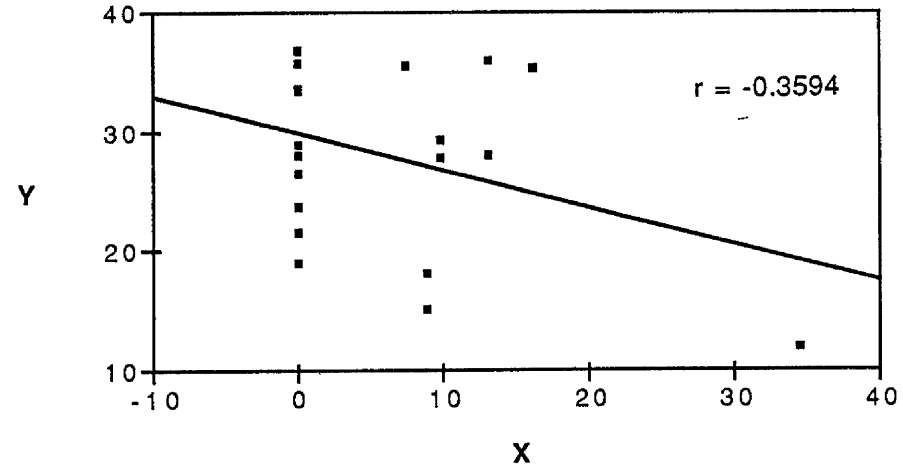

40
30
20
10
Y
r = -0.3594
-10
0
10
20
30
40
X

Figure 90: Correlation Between Adherence to HeLa Cells and Enterotoxin Production: ELISA

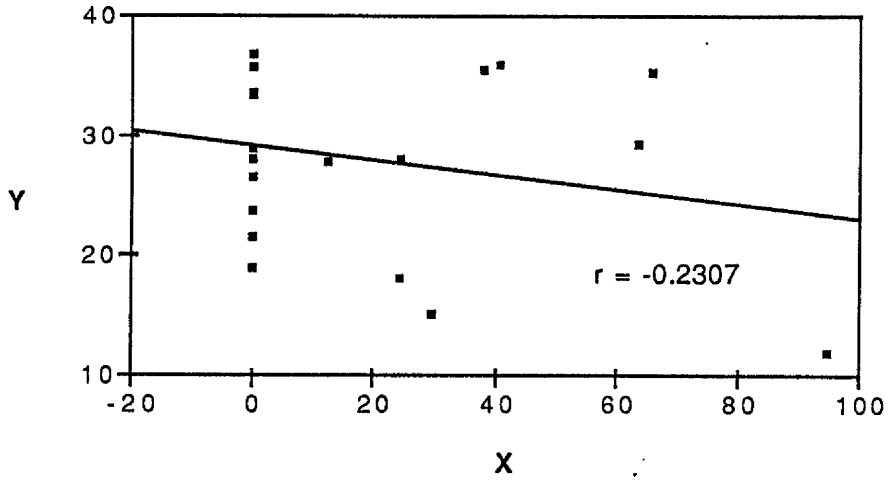
X = ng of CJT ^{a} mg^{-1} protein
Y = Adherence potential
a = *C.jejuni* enterotoxin

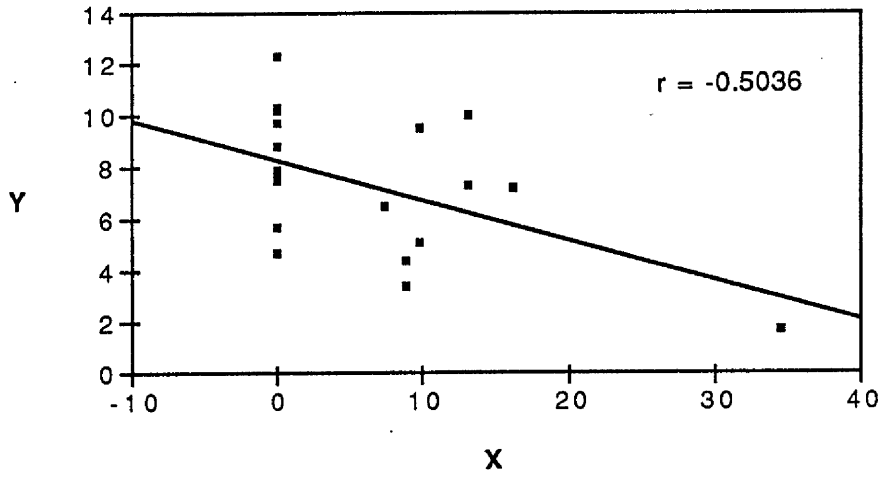
Figure 91: Correlation Between Invasion of HeLa Cells and Enterotoxin Production: CHO Cell Assay

X = $TCED_{50}$ mg^{-1} protein
Y = Invasion potential

Figure 92: Correlation Between Invasion of HeLa Cells and Enterotoxin Production: ELISA

X = ng of CJT ^{a} mg^{-1} protein
Y = Adherence potential
a = *C.jejuni* enterotoxin


40
30
20
10
Y
r = -0.2307
-20
0
20
40
60
80
100
X


14
12
10
8
6
4
2
0
Y
r = -0.5036
-10
0
10
20
30
40
X

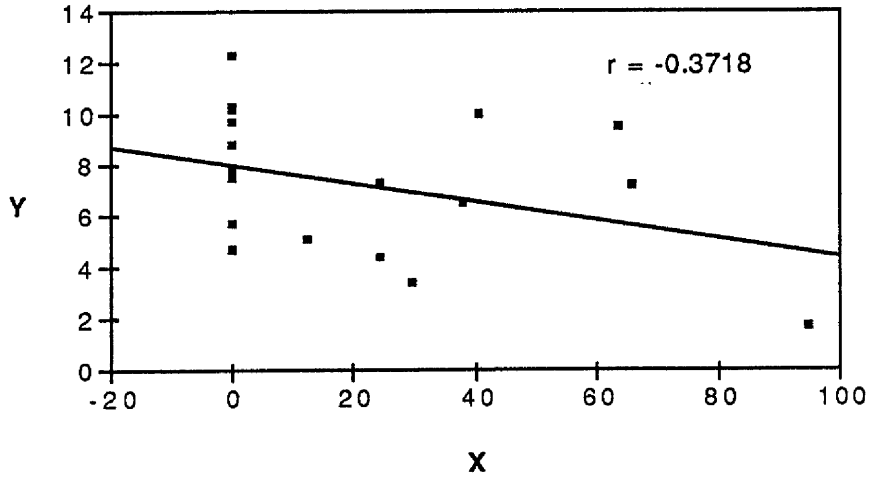

14
12
10
8
6
4
2
0
Y
r = -0.3718
-20
0
20
40
60
80
100
X

Figure 93: Correlation Between Lethality in Chicken Embryo Model and Enterotoxin Production: CHO Cell Assay

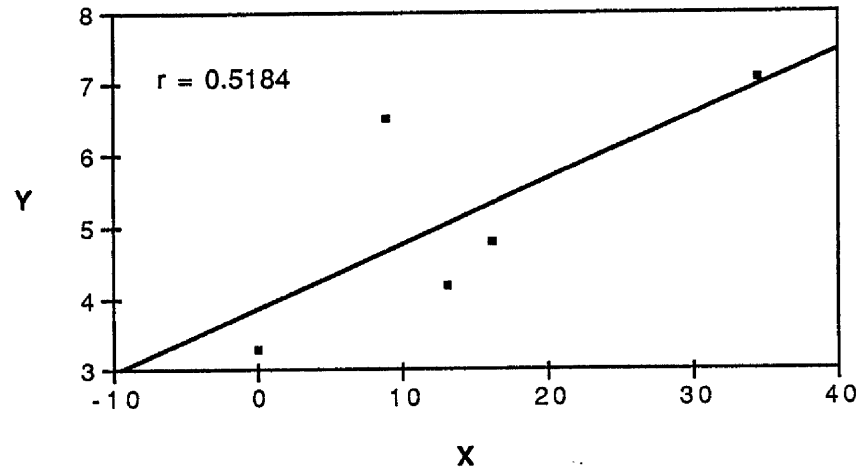
X = $TCED_{50}$ mg^{-1} protein
Y = Lethality in chicken embryo model (LD_{50} values)

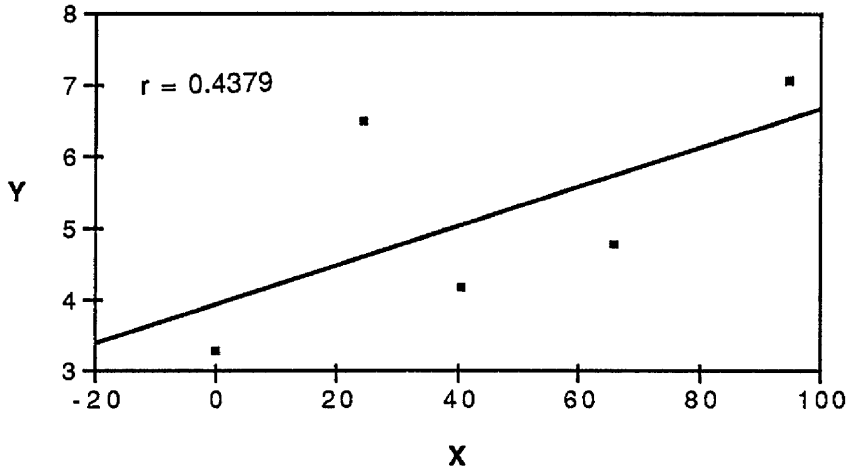
Figure 94: Correlation Between Lethality in Chicken Embryo Model and Enterotoxin Production: ELISA

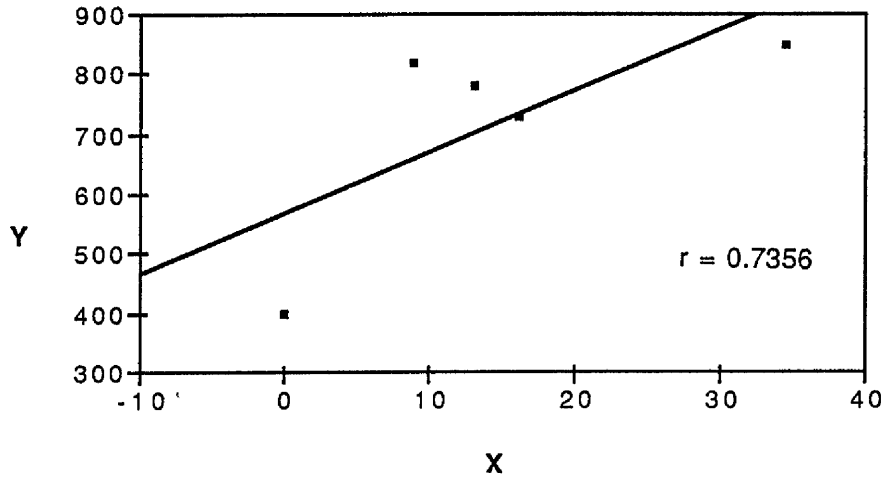
X = ng of CJT [a] mg^{-1} protein
Y = Lethality in chicken embryo model (LD_{50} values)
a = *C. jejuni* enterotoxin

Figure 95: Correlation Between Enterotoxin Production in Infant Chicken Diarrhoea Model and CHO Cell Assay

X = $TCED_{50}$ mg^{-1} protein
Y = Fluid accumulated (µl) per chick gut


r = 0.5184
Y
X
8
7
6
5
4
3
-10
0
10
20
30
40


r = 0.4379
Y
X
8
7
6
5
4
3
-20
0
20
40
60
80
100


r = 0.7356
Y
X
900
800
700
600
500
400
300
-10
0
10
20
30
40

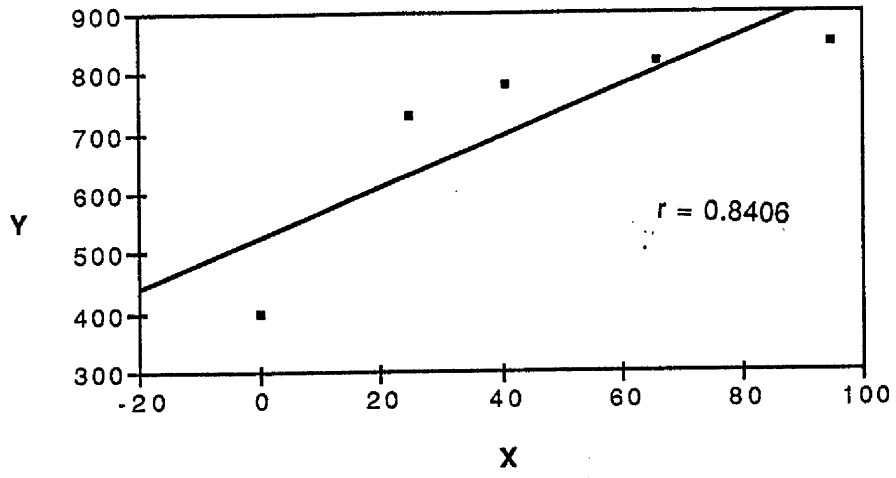


Figure 96: Correlation Between Enterotoxin Production in Infant Chicken Diarrhoea Model and ELISA

X = ng of CJT [a] mg^{-1} protein
Y = Fluid accumulated (µl) per chick gut
a = *C. jejuni* enterotoxin

5.0 DISCUSSION

C. jejuni is a pathogen which reveals a broad clinical spectrum ranging from a few loose stools, to cholera-like watery diarrhoea to dysentery-like mucoid diarrhoea (Blaser and Reller, 1981; Skirrow, 1984). Studies on the putative virulence factors and pathogenic mechanisms of *C. jejuni* were directed towards superimposing its pathogenic characteristics on those of *V. cholerae* to account for the secretory diarrhoea and also on the pathogenic characteristics of *Salmonella-Shigella* to account for the mucoid diarrhoea. A variety of putative virulence associated properties such as enterotoxin, cytotoxin(s), adherence and invasion have been identified in certain clinical *C. jejuni* strains (Walker *et al,* 1986) which have led to the questions concerning the correlation of these properties to the clinical spectrum seen in patients.

Klipstein *et al* (1985) found that virulence characteristics associated with the *C. jejuni* strains correlated with the clinical history of the patients from whom the strains were isolated. The strains isolated from cholera-like diarrhoea cases were toxigenic and failed to show invasive characteristics and the strains from dysentery-like diarrhoea cases were invasive but not toxigenic. Moreover, the production of a cytotoxin which is a characteristic of invasive enteropathogen was predominantly associated with the strains isolated from dysentery-like diarrhoea cases. On the other hand, the strains isolated from asymptomatic carriers were devoid of all these virulence-associated characteristics. The work of Klipstein *et al* (1985) prompted an investigation to determine whether other putative virulence characteristics would show such a definitive association with the type of clinical diarrhoea caused by the pathogen. So a broad research approach was undertaken in the present study, primarily directed towards correlating the various putative virulence markers of *C. jejuni* to the clinical history of the strain i.e. whether one or more putative virulence factors enable the pathogen to cause either of the two clinically distinct forms of diarrhoea. The sequential involvement of the various pathogenic events, such as adherence to the intestinal mucosa, colonisation and multiplication, invasion, elaboration of toxin(s), bacteraemia

etc. were investigated so that their effects on the development of clinical disease could be evaluated.

5.1 The Grouping of Human Clinical Isolates Used in This Study

Based upon the clinical features of the *C. jejuni* enteritis, the strains included in this study were broadly categorized into two groups: those caused cholera-like, watery diarrhoea (Group C) and the other group caused dysentery like, mucoid diarrhoea (Group D) (Table 5).

5.2 Enterotoxin Production

Although the production of 'enterotoxin' and 'cytotoxin' was reported by various workers, the information regarding these virulence-associated factors are fragmentary or indirect as neither of them has been purified and characterized. In this study, the apparent enterotoxic nature of the crude cell-free or cell-associated material of *C. jejuni* was analysed by *in vitro* assays; the CHO cell assay and GM_1 ganglioside ELISA, and by *in vivo* assays such as induction of diarrhoea in new-born chicks and fluid accumulation in the intestinal loops of 5-day-old chicks. The cytotoxic nature was investigated by haemolytic activity against rabbit erythrocytes and cytotoxicity towards HeLa cell monolayers.

5.2.1 *C. jejuni* Diarrhoea Model in Infant Chicks

To assess the enterotoxigenicity of the *C. jejuni* strains, the 3-day-old infant chicken diarrhoea model of Sanyal *et al* (1984a) was initially adopted. But none of the freshly isolated strains from patients with watery-type secretory diarrhoea, and which had been subjected to minimal *in vitro* passage, caused any signs of diarrhoea according to the criteria set by Sanyal *et al* (1984a). Subsequently, the new-born chick (Wilkos, 1984) was used instead of 3-day-old chicks. Reproducible diarrhoea was induced by the *C. jejuni* strains in the new-

born chicks; the volume of fluid recovered per gut of chicks (ca. 0.7-0.8 ml) was comparable to that obtained by Sanyal *et al* (1984a). On the other hand the control chicks had < 0.3 ml of fluid per gut. Quantitative analysis of the fluid accumulation caused by the different strains of *C. jejuni* (Figure 3) showed that Group C strains caused accumulation of larger volumes of fluid than the Group D strains; however, one Group D strain, B-7, also caused substantial fluid accumulation. The clinical relevance of this observation is discussed later along with results of quantitation of *C. jejuni* enterotoxin in the GM_1 ganglioside ELISA and CHO cell assay.

The apparent insusceptibility of the 3-day-old White Leghorn chicks to oral inoculation of *C. jejuni* strains is open to several interpretations. White Leghorn chicks may be relatively less susceptible to the induction of diarrhoea by *C. jejuni.* Sanyal *et al* (1984a) reported that diarrhoea could not be induced regularly in 36 to 72-hr-old White Leghorn chicks and so they changed to the Starbro strain which gave reproducible results. In this study, it was found that White Leghorn chicks of < 12 hr of age are susceptible to oral challenge with *C. jejuni,* leading to diarrhoea, but 3-day-old birds were refractory to infection. Similar insusceptibility of the 3-day-old chicks (broiler chicks) to oral inoculation of $1x10^6$-$1X10^9$ *C. jejuni* was also reported by Manninen *et al* (1982). Ruiz-Palacios *et al* (1981) reported that diarrhoea could be induced in 3-day-old chicks by oral inoculation of 90 c.f.u. of *C. jejuni* and as few as 9 bacteria caused diarrhoea in 20 % of the infected chicks.

The induction of diarrhoea in 3-day-old chicks by inoculating only 9 *C. jejuni* groups the pathogen with *Shigella* spp. which also have such a low infective dose (ca. 10 organisms; Hale and Formal, 1988). However, this is not in agreement with all the other studies which have shown that higher doses are required for colonization and even larger inocula were required for the induction of diarrhoea (Welkos *et al,* 1984; Sanyal *et al,* 1984a; Beery *et al,* 1988). In this study the minimum infective dose was not determined, but initial

experiments with graded inocula (using 2 chicks per inoculum) showed that ca. 1.0×10^8 organisms consistently induced diarrhoea by all of the strains tested. Consequently, a fixed inoculum of 1.0×10^8 c.f.u. was used which reproducibly induced diarrhoea in the newly hatched (< 12-hr-old) chicks. No mortalities of the chicks were observed in this study, unlike that of Ruiz-Palacios *et al* (1981) who reported 32 % mortality of the chicks following the development of diarrhoea. In this regard the results obtained in this study agree with those of Welkos (1984), Sanyal *et al* (1984a) and Beery *et al* (1988) who also did not observe any mortality among the infected chicks.

The results obtained in this study gives rise to the question "Why are newly hatched chicks susceptible to *C. jejuni* infection but older chicks are not ?" Several explanations can be put forward. Age-related development of resistance in chickens to *C. jejuni* has been reported previously. Butzler and Skirrow (1979) failed to induce diarrhoea in 8-day-old chicks, however, the strain of the bird used was not mentioned. Similar resistance to *C. jejuni* infection in adult chickens (unknown strain) was also observed by Ruiz-Palacios *et al* (1981). Young chicks lack a fully developed normal gut flora which may have a protective effect against infections due to enteric pathogens (Abraham and Bishop, 1966: Turnbull and Richmond, 1978; Berg and Garlington, 1979). In fact in several studies, the normal intestinal flora was found to exert an antagonism against oral infection (Freter, 1962; Bohnhoff *et al*, 1964; Savage, 1972).

Apart from this direct protective effect of the normal flora, several indirect factors may also be involved in the increased resistance of the older chicks. Comparative studies of germ-free animals with conventional animals have revealed that the normal flora also influences the morphology and physiology of both the gastrointestinal tract and the associated lymphoid tissue such as structure of the lamina propria, the life-cycle of the epithelial cells of the

mucosa (Ruitenberg *et al,* 1971) and most importantly, intestinal motility (Abraham and Bishop, 1966). Although the newly hatched chicks used in this study were not germ-free, it is probable that their near gnotobiotic condition of these chicks (Popiel and Turnbull, 1984) may be a primary factor in the easy induction of enteritis.

Another important point is that the gizzard mucosa of newly hatched chicks has a pH of 5.1-6.2, which falls to pH 3.3-4.5 upon administration of food and water (Shaffer *et al,* 1964). In this study, the chicks were starved of food and water after hatching until they were inoculated, which might have contributed towards their increased susceptibility as *C. jejuni* is labile to killing at an acidic pH (Blaser *et al,* 1980b). Thus the resistance of the 3-day-old White Leghorn chicks observed in this study and also by Sanyal *et al* (1984a) may be due to the development of a competitive normal gut flora and the low pH of the gizzard mucosa, as the chicks were allowed normal food and water before inoculation.

5.2.2 Immunological Relationship Between Cholera Toxin and *C. jejuni* Enterotoxin

The line of partial identity between the cholera toxin (CT) and *C. jejuni* enterotoxin (CJT) (Figure 11) indicates that CJT possesses both shared and distinct antigenic determinants with CT. Klipstein and Engert (1984) also noted similar partial identity between the B subunits of CJT (CJT-B) with those of CT (CT-B) and *E. coli* heat-labile enterotoxin (LT-B). The CJT-B was isolated by the application of a dissociation technique which involved gel filtration in the presence of guanidine from a semi-purified preparation of CJT. Less spurring was observed between the CJT-B and LT-B than between CJT-B and CT-B which indicated more immunological similarity between CJT-B and LT-B than between CJT-B and CT-B. Unavailability of commercial LT prevented the inclusion of LT

in the immunodiffusion experiments in this study; so the relative similarity between the CJT with LT and CT could not be determined. But the line of spurring indicate close immunological similarity with CT. CT from different strains show immunological differences (Finkelstein *et al,* 1987) and charge heterogeneity between CT molecules has also been reported (Kabir, 1986). These factors might play contributory roles in the degree of relatedness observed between CT and other protein molecule such as CJT.

5.2.3 GM_1 Ganglioside ELISA and CHO Cell Assay

A GM_1 ganglioside ELISA and CHO cell assay were used to determine the enterotoxigenicity of the *C. jejuni* strains. Both the assays gave reproducible results and closely correlated with each other (r= 0.9310; Figure 88). Analysis of the toxigenicity profile of the *C. jejuni* strains shows that the enterotoxin is differentially produced by the strains of *C.jejuni*, which can be detected by GM_1 ganglioside ELISA and the CHO cell assay (Figures 5-8). Moreover, the enterotoxin also produced a line of partial identity with cholera-toxin (CT) (Figure 11). These results confirm previous reports that certain *C. jejuni* strains produce an enterotoxin which is immunologically similar to cholera toxin (Ruiz-Palacios *et al,* 1983; Klipstein and Engert, 1984; Klipstein *et al,* 1985). The observed immunological similarity between the *C. jejuni* enterotoxin with CT led to the study to determine whether such a similarity existed at the genetic level. The only report that was available on this aspect, when this study was initiated was that of Olsvik *et al* (1984) who did not observe any hybridization between *C. jejuni* chromosomal DNA (the strains tested included two strains which were reported to produce enterotoxin by Ruiz-Palacios *et al,* 1983) and the gene probes for A and B subunits of CT and LT. In this study, the chromosomal DNA of 4 strains (3 enterotoxigenic and one non-enterotoxigenic as determined by ELISA and the CHO cell assay) were probed with *E. coli* LT gene probe, but no hybridization was observed even at low stringency which permitted hybridization if there was as low as 80 % homology. Similar results were

also obtained by other workers (Baig *et al*, 1986; Walker *et al*, 1986). Recently Calva *et al* (1988c) reported that homology was noted between the genes encoding LT and chromosomal DNA of *C. jejuni* at the GM_1 ganglioside binding region.

Lee *et al* (1985) in a preliminary abstract reported that the production of enterotoxin by a strain of *C. jejuni* was mediated by a 46.5 Kb plasmid which also encoded for tetracycline resistance. This plasmid could be transferred to non-toxigenic *C. jejuni* by conjugation rendering it toxigenic; the plasmid was not however transmissible to *E. coli.* This report prompted the screening of the freshly isolated human clinical strains of *C. jejuni* for plasmids, but no correlation was observed between the plasmid profile and enterotoxigenicity of the strains as determined by ELISA and CHO cell assay. For example, B-7, which contained 4 plasmids produced approximately the same quantity of enterotoxin as the strain B-18 (Figures 5 and 6) which did not contain any plasmid. And the strain S-13 producing relatively larger quantities of toxin harboured a small (10.2 Kb plasmid). The report of plasmid-mediated enterotoxin production in *C. jejuni* strains was later refuted by Taylor *et al* (1987).

To explain the two clinically distinct forms of diarrhoea caused by *C. jejuni*, two pathogenic mechanisms have been suggested: a) elaboration of an enterotoxin to account for the cholera-like secretory diarrhoea and b) epithelial cell invasion to account for the dysentery-like mucoid diarrhoea. However, involvement of these proposed virulence mechanisms to the outcome of overt disease are circumstantial as direct correlation between the clinical history of the strain and the demonstration of the implicated virulence-associated properties (i.e. production of cholera-like enterotoxin or possession of invasive capability) have not been demonstrated convincingly.

If it is assumed that virulence characteristics of *C. jejuni* strictly correlate with the clinical diarrhoea caused by the pathogens, as seems to be implied by Klipstein *et al* (1985), then isolation of toxigenic *C. jejuni* from

carriers (Mathan *et al,* 1984) and induction of both watery and mucoid diarrhoea by non-toxigenic strains of *C. jejuni* in human volunteer studies (Black *et al,* 1988) needs to be explained.

Infection of human beings by enteric pathogens is not always accompanied by overt disease. Presence of enterotoxigenic bacteria in the intestine does not ubiquitously induce diarrhoea; enterotoxigenic *E. coli* and *V. cholerae* are frequently found in healthy individuals (Merson *et al,* 1980), this has also been reporetd for *C. jejuni* (Mathan *et al,* 1984; Belbouri and Megraud, 1988). A number of host and environmental factors can influence the individual response to an enteric pathogen; namely

(a). Gastric acidity: gastric acid is the first non-specific defence mechanism encountered by enteric pathogens. There is an inverse relationship between basal levels of gastric acid and severity of diarrhoea caused by *V. cholerae* (Nalin *et al,* 1978) and neither clinical cholera nor asymptomatic infection was observed in fasting, chlorhydric individuals in volunteer studies (Cash *et al,* 1974; Levine *et al,* 1981). As *C. jejuni* is susceptible to an acidic pH (Blaser *et al,* 1983b), ingestion of the pathogen may result in subclinical infection and isolation of toxigenic bacteria from stools.

(b). Desensitization of the adenylate cyclase system: in order to explain the prevalence of toxigenic enteropathogens in asymptomatic carriers in endemic areas (Pickering *et al,* 1977; Merson *et al,* 1980), it has been suggested that the development of intestinal resistance to the toxin possibly plays a role and has been proved in mouse (Lonnroth *et al,* 1984; Lange *et al,* 1984) by repeated administration perorally of cholera toxin and *E. coli* heat-labile toxin.

(c).Prostaglandins: on the basis of their capacity to mimic the effects produced by enterotoxins, prostaglandins have been implicated to influence the difference in sensitivity to enterotoxins in human beings (Kanto *et al,* 1974).

(d). Bile acids: bile acids have been reported to have a potentiating effect

on cholera toxin and *E. coli* heat-labile toxin-induced diarrhoea in the ligated intestinal loops of mice (Lange and Lonnroth, 1982) and the authors suggested that determination of bile acid concentrations in the stools from subclinical and convalescent cases may provide information on the sensitivity for enterotoxic diarrhoea.

(e). Blood group antigens: a relationship between predisposition to cholera and the 'O' blood group antigens was noted by Glass *et al* (1985) in an epidemiological survey in Bangladesh.

(f). Genetic control of sensitivity: histocompatibility genes have been shown to regulate the extent of pathogenesis caused by *Yersinia enterocolitica, Mycobacterium bovis* BCG and *V. cholerae.* The sensitivity to cholera toxin induced diarrhoea in the mouse was found to be linked to the *H-2* complex and it was speculated that a similar linkage between diarrhoeal diseases of enterotoxin etiology and the HLA genes in human beings may be demonstrated (Richardson and Kuhn, 1986).

(g). Microecology of the intestinal tract: certain normal gut flora, especially lactobacilli, which are indigenous to the proximal small intestine are inhibitory to enteric pathogens (Johnson and Calia, 1979; Foster *et al,* 1980; Hentges, 1986). Trophozoites (cysts) of intestinal parasites *Giardia muris* and *Giardia lamblia* were found to bind significant amounts of cholera toxin as determined by GM_1 ganglioside ELISA; fluid accumulation in response to orally administered cholera toxin in mice which were infected with high doses of parasite was significantly reduced (Ljungstrom *et al,* 1985). The authors speculated that trophozoites may bind and thus reduce the concentration of toxin available for binding to the receptors resulting in decreased secretion. Giardiasis, both in symptomatic and asymptomatic form occurs in high frequency in the countries in which other enteropathogens are also prevalent. It may be speculated that giardiasis may also play a determinative role in the high carrier rate of *C. jejuni* and other enteropathogens in endemic areas (Merson *et al,* 1980; Mathan

et al, 1984; Molbak *et al,* 1988).

(h). Immune status of the host: the immune status of the host also determines the outcome of infections caused by enteric organisms. Infection to illness ratio decreases with age in endemic areas presumably due to the development of gut immunity resulting from multiple infections at an early age (Glass *et al,* 1983; Calva *et al,* 1988a). Blaser *et al* (1987) reported that even during common source outbreaks of *C. jejuni,* the clinical manifestations of infection varied from transient asymptomatic carriage to severe colitis, indicating the role of host factors in addition to the bacterial factors.

Although most of the studies referred to above dealt with cholera toxin and *E. coli* heat-labile toxin (LT), these observations can be extrapolated to *C. jejuni* diarrhoea, as immnological and genetic similarity of *C. jejuni* enterotoxin (CJT) with CT has been reported (Klipstein and Engert, 1984; Calva *et al,* 1988c). The development of clinical disease or asymptomatic carrier state following ingestion of *C. jejuni* is a complex process, which is probably influenced by some or all of the above mentioned factors and also by factors which may yet to be discovered. So considering the various probable influencing parameters, the isolation of toxigenic *C.jejuni* from healthy individuals (Mathan *et al,* 1984) is not inherently difficult to explain.

It has been stated earlier that nontoxigenic *C. jejuni* strains caused full-blown disease in human volunteers (Black *et al,* 1988), which questions the significance of enterotoxin in the disease process. Three independent studies have demonstrated that *C. jejuni* enterotoxin (CJT) is probably produced *in vivo* as specific antibodies to CJT in serum were detected by ELISA (Ruiz-Palacios *et al,* 1985; Honda *et al,* 1986; Martin *et al,* 1989). Failure to demonstrate production of enterotoxin by Black *et al* (1988) does not mean that it is not produced *in vivo,* as animal passage-induced production of enterotoxin has been demonstrated in several enterotoxigenic bacteria including *C. jejuni* (Sanyal *et al,* 1984b; Shaha

et al, 1988). Attempts should have been made to detect antibodies to CJT in the serum to eliminate categorically the possibility that enterotoxin was not produced *in vivo* and hence did not play any role in the disease process in the human volunteer study of Black *et al* (1988).

Analysis of toxigenicity profiles of the *C. jejuni* hospital isolates (Group C and Group D strains) shows that no clear correlation existed between the production of either toxin and the clinical symptoms of the patients. Among the Group C strains, which clinically resembled *V. cholerae* and enterotoxigenic *E. coli,* 30 % did not produce any detectable toxin. Failure to demonstrate a toxin (or any other implicated virulence factor) in fresh clinical isolates is not an uncommon occurrence which has been reported for *V. cholerae* (Dutta *et al,* 1963; Finkelstein, 1973), *Aeromonas hydrophila* (Turnbull *et al,* 1984) and also for *C. jejuni* (Johnson and Lior, 1986). Additionally, 40 % of these strains also produced a cytotoxic haemolysin. There are reports on the production of both enterotoxin and cytotoxin by the same strain of *C. jejuni* (Johnson and Lior 1984; 1986; Daikoku *et al,* 1989). The production of a cytotoxin in addition to an enterotoxin is not unprecedented among diarrhoea-causing pathogens other than *C. jejuni.* For example, certain strains of *A. hydrophila* produce both enterotoxin and cytotoxin (Ljungh and Wadstrom, 1986). Other enteropathogens producing two such toxins includes *E. coli* (Konowalchuk *et al,* 1977) and *S. typhimurium* (Baloda *et al,* 1983). Recently, the production of toxins other than the classical cholera toxin has been reported for *V. cholerae* strains (O'Brien *et al,* 1984; Sanyal *et al,* 1984b).

Between the two groups (C and D) of *C. jejuni* the production of cytotoxin was predominantly associated with the Group D (60 % versus 40 % in Group C strains). But 20% of the Group D strains also produced the enterotoxin, indicating that two toxins were not specifically associated with the clinical history of the strains. In general, the production of enterotoxin was predominantly (70%) associated with the Group C strains and the cytotoxin was produced by majority of the Group D strains (60 %).

In line with several epidemiological studies, that biovar I is the most frequently encountered biovar of *C. jejuni* both in human clinical cases and also among strains isolated from the environment and food samples (Lior, 1984; Fricker and Park, 1989), 85 % of the strains investigated in this study were found to belong to biovar I (Table 6). A statistically significant association was noted between biovar I and enterotoxin production in a recent study (Fricker and Park, 1989). As the number of strains investigated was small, an association between biovars and the toxigenicity profile could not be assessed properly in this study.

The results obtained in this study are not in total agreement with those observed by Klipstein *et al* (1985) who reported that all the strains isolated from cholera-like diarrhoea cases produced enterotoxin and all the strains from dysentery-like diarrhoea cases were toxin negative. However, the same research group did not find such a clear-cut relation between the toxin profile and the clinical history of the *C. jejuni* strains in a later study with a larger number of strains (Klipstein *et al,* 1986). The apparent lack of correlation between the toxin profile and the disease characteristics may be due to the various factors discussed above. However, it should be considered that toxin production is only one aspect of a presumably multifactorial virulence mechanism of *C. jejuni.*

5.2.4 Chicken Intestinal Loop Test for Determining the Enterotoxigenicity of *C. jejuni* Strains

The Ileal loop fluid accumulation test has been and is being used as the major test to determine the enterotoxigenicity of diarrhoeagenic microorganisms (Bergdoll, 1988). De and Chatterje (1953) first developed the ileal loop test in rabbits for *V. cholerae.* Since then, a number of different animals have been investigated for their suitability in the study of the enterotoxigenic nature of a variety of enteropathogens. An ileal loop test in rats was reported to respond consistently to *C. jejuni* enterotoxin (Ruiz-Palacios *et al,* 1983; Klipstein *et al,* 1985; Shaha *et al,* 1988). But attempts to use rabbits were unsuccessful

(Ruiz-Palacios *et al,* 1983; Shaha *et al,* 1988) although the reason is not immediately clear. McCardell *et al* (1984) however, reported that 20-fold concentrated culture filtrate induced a positive response in the rabbit ileal loop.

In this study following the observation that *C. jejuni* strains induced diarrhoea in the newly-hatched chicks, experiments were initiated to determine whether an ileal loop test could be done in infant chicks which would provide a simple and economical assay for determining the enterotoxigenicity of the *C. jejuni* strains. The results generated from the ileal loop test in infant chicks (Figures 12, 13 and 14) indicate that a new reproducible test has been developed for evaluating the diarrhoeagenic potential of the *C. jejuni* strains. Counts of viable bacteria from the loops revealed that there was extensive multiplication of the bacteria in the gut (Figure 15). The mechanisms by which a positive reaction occurs in ligated intestinal loops differ depending on the particular pathogenic bacterial species. With *V. cholerae,* the positive reaction is caused by toxin (Burrows and Musteikis, 1961), whereas bacterial invasion into the mucosa of the small intestine caused a positive reaction with *Shigella* strains (Arm *et al,* 1965; Sakaguchi *et al,* 1986). As a positive response was noted with crude culture filtrates (Table 12), *C. jejuni* enterotoxin may be considered as the primary determinant of fluid accumulation in the chicken intestinal loops.

New-born Chicks have not been used before for ileal loop test with *C. jejuni.* In fact there is only one report of use of the chickens for the ileal loop test, that of Pal *et al* (1968) for *V. cholerae.* In that study adult (8-16 weeks old) chicken were used for the construction of ileal loops, and results comparable to those obtained with the rabbit ileal loop assay were obtained. In view of the fact that rabbits, which are the most commonly used animal in ileal loop assays, are not sensitive to *C. jejuni* enterotoxin (Ruiz-Palacios *et al,* 1983; Shaha *et al,* 1988), the infant chick ileal loop model may prove to be a useful alternative to the rat model which is the only one available at present.

5.3 Production of Haemolysin by *C. jejuni* Strains

It has been shown in this study that certain clinical isolates of *C. jejuni* produce a cell-free haemolytic factor which can be easily demonstrated by its rapid lytic activity against rabbit erythrocytes. The haemolytic factor was also active against human blood groups 'A', 'B' and 'O'; erythrocytes from other animals (Table 7). Moreover, the haemolysin also exhibited cytotoxic activity against HeLa cells (Figures 25 and 26). Although the the production of enterotoxin and cytotoxin was reported previously, the haemolytic activity of the *C. jejuni* strains received little attention. In fact when this postgraduate study was initiated in 1985, there was no report of the production of haemolysin by *C. jejuni* strains. The investigation of haemolysin production by *C. jejuni* strains stemmed from the fact that occasionally narrow zones of lysis were observed around *C. jejuni* colonies on Brucella blood agar plates (containing 7% sheep blood), especially when the plates were incubated for longer than normal (48-60 hr). *C. jejuni* culture filtrates (48-60 hr) were tested for their lytic activity against 1.0 % washed sheep blood and rapid lysis of the erythrocytes was observed.

The sensitivity of the different erythrocytes to the action of the haemolysin was rabbit > human blood groups 'A', 'B' and 'O' > chicken. In fact chicken erythrocytes did not show convincing haemolysis even with undiluted toxin and hence were considered to be insensitive. The underlying reason for this differential activity of the haemolytic activity against different types of erythrocytes is not known. Bernheimer *et al* (1975) reported that erythrocytes from different animals differ in their phosphatidyl choline content, and the activity of the haemolysin from *A. hydrophila,* 'aerolysin' was directly proportional to the phosphatidyl choline content of the erythrocytes. Whether the activity of the haemolytic factor from *C. jejuni* is related to some structural component of

the erythrocyte membranes is not known. The low haemolytic activity of approximately equal titre against erythrocytes from different animals probably indicates that the *C. jejuni* haemolytic factor does not interact with any specific phospholipid residues in the red-cell membrane. Avian erythrocytes are nucleated whereas mammalian erythrocytes are anucleated. Whether this is a determining factor in regard to the reduced sensitivity of the chicken erythrocyte is unknown. It may be mentioned that haemolysin from different enteropathogenic microorganisms such as *V. cholerae* biovar El Tor (Yamamoto *et al,* 1986), *V. parahaemolyticus* (Zen-Yoji *et al,* 1971), *A. hydrophila* (Bernheimer *et al,* 1975) and *Bacillus cereus* (Turnbull, 1986) also lyse a wide range of erythrocytes.

The proteinaceous nature of the haemolytic factor was demonstrated by the fact that it was sensitive to the action of trypsin. Incubation at 37° C for 1 hr with a trypsin concentration of 1.0 mg ml^{-1} completely abolished the activity of the haemolysin (Table 8). Additionally, the haemolytic factor showed no loss of activity when heated up to ca. 55° C, but rapidly lost activity at higher temperatures (Figure 17), providing further evidence of its protenaceous nature. But resistance to heat does not necessarily imply that the test material is not a protein as the thermostable direct haemolysin of *V. parahaemolyticus* (a protein toxin) is stable to heating at 100° C for 10 min (Takeda *et al,* 1975). A four-fold increase in the haemolytic titre following ammonium sulphate precipitation (80 %) (Table 8) also provided additional evidence that the *C. jejuni* haemolytic factor was a protein molecule.

As the haemolysin was inactivated at temperatures around 60° C or higher, it was decided to investigate whether it exhibits the Arrhenius effect (inactivation when heated around 60° C, but no loss of activity when heated at 100° C; Arbuthnott, 1970). No such effect was noted for the *C. jejuni* haemolytic factor, which is a characteristic phenomenon of *V. parahaemolyticus* haemolysin (Miwatani *et al,* 1972; Takeda *et al,* 1975).

The fact that the haemolysin is not produced during the logarithmic phase of growth indicates that it is probably not a primary metabolite of *C. jejunii.* In contrast, the haemolysin of other pathogenic microorganism such as *A. hydrophila* (Bernheimer and Avigad, 1974), *Vibrio mimicus* (Hossain *et al,* 1988), and *Vibrio vulnificus* (Tison and Kelly, 1984) are produced during logarithmic phase of growth. In this regard *C. jejuni* resembles the fish pathogen *V. angularum* (Munn, 1978) and *V. cholerae* biovar classical and El Tor (Richardson *et al,* 1986) which produce haemolysin when the bacterial growth enters the stationary phase. Another possibility is that it is an intracellular protein, released in the stationary phase due to autolysis or that the haemolytic factor is secreted in an inactive form earlier, but activated later by some other factor released in the stationary phase.

C. jejuni haemolysin probably acts by a two-step process similar to haemolysins from various other microorganisms. These steps are 1), a temperature independent haemolysin-binding step followed by 2), a temperature-dependent lysis of the target cells. This conclusion was drawn from the experiment in which no haemolysis was observed at 4° C but when the same tubes were incubated at 37° C, rapid lysis occurred. Incubation of haemolysin with 1 % RRBC at 4° C resulted in binding of ca. 75 % of the toxin as determined by the titration of the supernate removed from the incubation mixture of RRBC and haemolysin at 4° C, and subsequently tested against RRBC at 37° C.

With an isoelectric point (pI) of ca. 6.8, it appears that the haemolysin is a neutral molecule. The apparent pI was determined by comparing the band of haemolysis obtained by zymogram analysis with an overlay of 2 % washed rabbit erythrocytes in agarose on one half of the identically run analytical thin-layer isoelectric focusing, with the other identical half being stained by Coomassie blue (Figures 23 and 24).

The kinetics of erythrocyte lysis revealed that the rate of lysis was

different for erythrocytes against which the haemolysin exhibited identical titre when incubated at 37° C for 30 min. For example, the titre of the haemolysin S-11 was identical against erythrocytes of human blood group 'O', 'A' and 'B', but when the kinetics of lysis experiments were performed, it became evident that the rate of lysis was blood group 'O' > 'A' > 'B' (Figures 20, 21 and 22). Moreover, comparison of the rate of lysis of each type of erythrocyte by three different concentrations of haemolysin indicates that the rate of lysis was directly proportional to the concentration of haemolysin. Human erythrocytes of different groups differ in the blood group sugars. Whether the presence or absence of different sugar moieties is related to the preferential binding resulting in the different rates of lysis is not known. The continuous monitoring of the haemolytic reaction by spectrophotometry was found to be especially suited to the studies on the kinetics of the lysis of RBC as continuous monitoring of haemolytic reaction was possible (with the help of built-in chart recorder). A decrease in percent haemolysis was observed when the target cell / haemolysin ratio was increased (Figure 18). These observations indicate that probably a multi-hit lytic process (more than one molecule of haemolysin is required to lyse a single erythrocyte) is involved in the lysis of rabbit erythrocytes by *C. jejuni* haemolysin. The multi-hit lytic process has been found to be operative in the cases of many haemolysins such as streptolysin O and *Clostridium perfringens* theta toxin (Inoue *et al,* 1976).

McCardell *et al* (1986) reported the production by *C. jejuni* of two distinct protein toxins which were cytotoxic to CHO cells. Cytotoxin-1 had a molecular weight of 70 K dal after purification by fast protein liquid chromatography (FPLC) and was insensitive to heat and trypsin. This toxin was inactivated by normal rabbit serum but could haemagglutinate rabbit erythrocytes. On the other hand, cytotoxin-2 was found to be heat-labile, sensitive to trypsin and was not inactivated by normal rabbit serum. In contrast to the cytotoxin-1 which was haemagglutinating, cytotoxin-2 was haemolytic towards rabbit

erythrocytes. Cytotoxin-1 (haemagglutinin) was produced by all of the 60 strains of *C. jejuni / coli* tested and cytotoxin-1 (haemolysin) was produced by 45 % of the strains. In the present study the various properties exhibited by the haemolysin such as lytic activity against rabbit erythrocytes, sensitivity to heat, trypsin and normal rabbit serum are similar to those reported by McCardell *et al* (1986). However, as the haemolysin preparation that was worked with here was a crude preparation, it exhibited most of the properties that were noted for both the separated toxins of McCardell *et al* (1986) taken together. But in contrast to McCardell *et al* (1986) no haemagglutinin activity was noted in the haemolysin preparation; probably the haemagglutinin activity was overshadowed by the haemolytic property. These observations indicate that the crude haemolysin preparation that was tested here probably contained both the toxins described by McCardell *et al* (1986). The present study has extended the work of McCardell *et al* (1986) and has confirmed experimentally the presence of a haemolysin. But it should be mentioned that two toxins were reported by McCardell *et al* (1986) in a brief letter in The Lancet, with no experimental detail, so it was not possible to compare various aspects in elaboration.

Since the plasmid profile of the *C. jejuni* strains included in this study did not correlate with the haemolytic profiles, it was assumed that the gene(s) for the production of haemolysin is probably located on the chromosome. McCardell *et al* (1986) noted that a non-haemolytic strain of *C. jejuni* when injected into rabbit ileal loop and isolated again from the blood 3 hours later was haemolytic indicating the production of haemolysin (cytotoxin-2) might be an inducible virulence factor; this observation is yet to be confirmed.

The role played by the haemolysin in the pathogenesis of *C. jejuni* enteritis is only speculative at present. Haemolysins are produced by a variety of Gram-positive and Gram-negative pathogenic organisms including diarrhoea-causing pathogens such as *V.cholerae* biovar El Tor (Honda and Finkelstein, 1979) *V. cholerae* biovar classical (Richardson *et al,* 1986), *V. parahaemolyticus* (Takeda,

1986), *V. mimicus* (Hossain *et al,* 1988), *A. hydrophila* (Ljungh and Wadstrom, 1986). But only in the cases of *V. parahaemolyticus* and *A. hydrophila,* is the relationship between pathogenicity and haemolysin production is strong and constant. However, haemolysins from these two pathogens are not primarily responsible for their diarrhoeagenic activity (Chakraborty *et al,* 1984; Takeda *et al,* 1986). McCardell *et al* (1986) speculated that the cytotoxin-1 (haemagglutinin) of *C. jejuni* may be involved in the attachment of the organism to the mammalian cells, like haemagglutinin factors of other pathogenic microorganism and the cytotoxin-2 (haemolysin) might be involved in obtaining iron while growing *in vivo*. In this study it was found that the capacity to produce haemolysin was directly related to the lethality of the strains in 11-day-old chicken embryos. Haemolytic strains had lower LD_{50} values, while the strains with higher LD_{50} values were nonhaemolytic (this aspect is discussed in Section 5.8). These observations lead to the speculation that the haemolysin of *C. jejuni* may be involved in releasing the iron of haemoglobin, by lysing the erythrocytes, available to the pathogen in the severe iron-restricted conditions *in vivo* (Griffiths *et al,* 1988).

The low specific activity (Table 7) indicates that the haemolytic factor is not produced in significant quantities. Some prelimanry experiments were done to purify the haemolysin. Precipitation with ammonium sulphate (80 % saturation) resulted in a 4-fold increase in titre. But when this material was subjected to Sephadex G-75 gel filtration, a very large peak trailing from the void volume showing minimal activity was generated. After freeze-drying, the sample was found to have reduced specific activity in comparison to the starting material. In view of the original formulation of this research work and the realisation that there was a need to optimise the laboratory conditions for the maximal production of the haemolysin before systematic purification could be attempted, it was decided to leave this topic of research at this stage.

There are many unanswered questions about the origin, production, and activity of the haemolytic factor produced by *C. jejuni.* These preliminary results obtained with the crude culture filtrate of *C. jejuni* strains may not be a true reflection of the pure haemolysin molecule, as the various properties such as heat-stability, erythrocyte spectrum and sensitivity to various chemical agents may be different for a purified toxin and crude preparation possibly due to the presence of some other factor(s) in the latter (Kothary and Kreger, 1985). Further studies are needed to establish conditions for its maximal production so that it can be purified and investigated for its role in pathogenicity of *C. jejuni.*

5.4 Cell-Surface Hydrophobicity

Cell-surface hydrophobicity is currently regarded as an important factor in mediating bacterial adherence to a wide variety of surfaces (Ofek and Beachy, 1980; Rosenberg and Kjellberg, 1986). Since the involvement of hydrophobic interactions are implicated in the adherence of a variety of pathogenic microorganisms to eucaryotic cells (Smyth *et al,* 1978; Sherman *et al,* 1985), the cell-surface hydrophobicity of the *C. jejuni* strains were investigated. It was intended to determine whether the strains causing the two types of diarrhoea could be differentiated by their cell-surface hydrophobicity and if so whether this property showed any correlation with the various other putative virulence factors of the *C. jejuni* strains. Two different approaches namely, bacterial adherence to hydrocarbon (BATH) test (Rosenberg *et al,* 1980) and Salt aggregation (SA) test (Lindhal *et al,* 1981) were undertaken to determine whether a specific hydrophobic pattern could be defined for these two group of strains.

Although the adhesive properties of the *C. jejuni* strains in relation to pathogenicity have been investigated in a variety of systems (see Section 1.7.1), the hydrophobic properties of the cell-surface which might provide valuable insights into the nature of the adhesion process have received little attention. McSweegan and Walker (1986) tested a *C. jejuni* strain with an undefined clinical history for

hydrophobicity using Octyl-sepharose chromatography and found it was less hydrophobic than *E.coli* K-12. The only elaborate investigation on the cell-surface hydrophobicity of *C. jejuni* strains is the recent study of Walan and Kihlstrom (1988), who used hydrophobic interaction chromatography and aqueous two-phase partitioning techniques. They found that the strains were heterogeneous in terms of cell-surface hydrophobicity and the techniques employed showed good but not perfect correlation; however, they did not attempt to correlate the hydrophobic property with the clinical history of the patient from whom the strains were isolated. As the techniques used in this study (SA test and BATH test) were different from those used by Walan and Kihlstrom (1988) no direct comparison can be made between these two investigations. However, it is apparent from the results of both of these studies that *C. jejuni* strains vary widely in cell surface hydrophobicity which can be quantitated by these methods. The two tests for determining hydrophobicity of the test strains did not always correlate with each other (Table 9) indicating that two different criteria are probably evaluated by these two tests. In fact the SA test reflects the overall surface hydrophobicity; on the other hand the BATH test is more sensitive and can detect even localized areas of hydrophobic residues (Jacobson *et al,* 1989).

Although definite conclusions regarding the cell-surface properties cannot be made from studying only 20 strains, the results obtained in this study indicate that like many other enteropathogens the relatively less hydrophobic nature of the *C. jejuni* strains in comparison to some Gram-positive pathogens such as *Staphylococcus aureus* (Reifsteck *et al,* 1987). A similar conclusion was also reached by McSweegan and Walker (1986) investigating a single *C. jejuni* strain by hydrophobic interaction chromatography. Qadri *et al* (1988) also found that cell-surface hydrophobicity of the *Shigella* spp. measured by the SA test was relatively low in comparison to Gram-positive bacteria.

The most interesting aspect that was observed in this study is that the group D strains were relatively, although not significantly, more hydrophobic than the group C strains. As *C. jejuni* strains have not been investigated before by the techniques used

in this study and surface hydrophobicity has not been correlated with the origin of strains, the clinical significance of these results cannot be compared with any other study. However, the increased surface hydrophobicity of the group D strains correlated well with the other virulence- associated properties investigated in this study such as 1). adherence and invasion of HeLa cells and 2). lethality in the 11-day-old chicken embryo model, indicating that it can also be a candidate virulence marker for *C. jejuni.* An increased hydrophobicity of the strains isolated from dysentery-like mucoid diarrhoea, indicate that hydrophobic forces might be operative in close association of the *C. jejuni* with the HeLa cells leading to increased adherence and invasion. Cell-surface hydrophobicity has been correlated with virulence in several pathogenic microorganisms. In *E. coli, Salmonella* spp. gonococci and *S. flexneri,* the capacity to adhere to host tissue in experimental animals was found to be related to increased cell surface hydrophobicity of the organisms (Magnusson *et al,* 1980; Seltmann *et al,* 1986). Qadri *et al* (1988) reported that the cell-surface hydrophobicity correlated closely with the relative virulence of the different species of *Shigella*. The gradual reduction of cell-surface hydrophobicity in the order of *S. dysenteriae, S. flexneri, S. sonnei* and *S. boydii* was in general agreement with the virulence of *Shigella* spp. and the severity of the disease caused by these pathogens. Recently, Pal and Hale (1989) showed that the adherence and subsequent invasion of HeLa cells is closely associated with the cell-surface hydrophobicity of the *S. flexneri* strains. Adherent strains were more hydrophobic than the non-adherent strains as determined by hydrophobic interaction chromatography and pretreatment of bacterial cells before the assay indicated the involvement of plasmid-coded outer membrane proteins. In *Bordetella pertussis* the virulent phase (X-mode) cells were hydrophobic whereas the avirulent phase (C-mode variants) were hydrophilic (Robinson *et al,* 1985; Fish *et al,* 1987). Cell surface hydrophobicity has also been found to contribute towards virulence in the fish pathogen *Aeromonas salmonicida* (Trust *et al,*

1983; Ishiguro *et al*, 1985).

Although the group D strains appeared to be relatively more hydrophobic than the group C strains on a general basis, there were many which did not fit to this pattern when the individual strains were compared (Table 9). For example, a group C strain S-13, was more hydrophobic than most of the group D strains. Cell-surface hydrophobicity measured by the BATH test revealed a better correlation with HeLa cell adherence and invasion potential than the SA test. However, it may be noted that certain strains with higher cell-surface hydrophobicity had lower adherence and invasion potential in HeLa cell model. These results indicate that hydrophobicity alone is probably not the only attribute that determines the interaction of the *C. jejuni* strains with HeLa cells. Schiemann *et al* (1987) also reached similar conclusion studying the cell-surface hydrophobicity and association with epithelial cells of *Yersinia* spp. Although both the BATH test and SA test are widely used techniques for determining cell-surface hydrophobicity, the results obtained in this study indicate that the BATH test probably more accurately reflects this property than the SA test when equated with the other putative virulence-associated properties investigated.

The surface structure(s) involved in determining the relative surface hydrophobicity of *C. jejuni* is sill unrevealed. Several studies with other microorganisms indicate that epithelial cell association is associated with cell surface hydrophobicity which is in turn determined by fimbriae on the bacterial cells (Smyth *et al*, 1978; Faris *et al*, 1982). In this study electron microscopic observation of negatively-stained *C. jejuni* cells revealed no pili on the bacterial cell-surface (Figure 33 and 34); so the involvement of fimbriae in the case of *C. jejuni* can be ruled out. Other outer membrane structures such as lipopolysaccharides, lipoproteins, phospholipids and outer membrane proteins have also been associated with hydrophobicity (Schiemann and Swanz, 1985). Various other surface structures have been implicated as the determinant of cell-surface hydrophobicity in different microorganisms; for example, protein A and other surface proteins in *S. aureus*

(Reifsteck *et al,* 1987) lipoteichoic acid complexed with protein M in group A *streptococci* (Ofek *et al,* 1983), and the surface protein layer A in the fish pathogen *A. salmonicida* (Trust *et al,* 1980). The surface structure(s) involved structures in determining cell-surface hydrophobicity of the *C. jejuni* strains is yet to be determined. The clinical, significance of cell-surface hydrophobicity of the *C. jejuni* strains can only be speculative at this moment; hydrophobic forces might bring the bacterial cells in close association with the intestinal epithelial cells, rendering them more efficient in withstanding the expulsive process of peristalsis leading to increased colonisation and invasion which precipitates the clinical disease.

The relevance of hydrophobic cell-surface properties was demonstrated in a number of biologic adherence processes, including adherence to phagocytes (van Oss, 1978) and host tissue (Jann *et al,* 1981). A correlation between increased cell-surface hydrophobicity and resistance to phagocytosis by rabbit peritoneal neutrophils was reported with *S. typhimurium* (Stendahl *et al,* 1973). As *C. jejuni* can survive inside the macrophages for up to 7 day (Kiehlbauch *et al,* 1985), a mechanism for adhering to phagocytes has important implication toward the understanding of *C. jejuni* interactions with phagocytes.

5.5 Congo Red (CR) Dye Binding by *C. jejuni* Strains

Like many other pathogenic microorganisms, *C. jejuni* strains also exhibited the Congo red (CR) dye binding property; parent and variants can be differentiated by their ability to take up the dye. As the CR binding phenomenon is usually associated with physical and biological differences between the CR^+ and CR^- cells, whether the resultant physical and biological difference (if any) between the CR^+ and CR^- *C. jejuni* cells could be exploited to study their virulence was considered. Hence, in view of the possibility that the CR binding might generate variants deficient in some virulence-associated properties, attempts were made to characterize and optimize the phenomenon. Isolation of CR variants made a comparative study of virulence and cell surface properties of the *C. jejuni* strains possible. CR^+ and CR^- variants were compared in terms of (a). CR

binding capacity, qualitatively and quantitatively, (b). Virulence in the 11 day old chicken embryo model, (a). ability to adhere and invade HeLa cells, (d). outer membrane protein (OMP) profile, (e). lipopolysaccharide (LPS) profile, (f). plasmid profile and (g). cell surface hydrophobicity.

A variety of tests was employed by previous workers to assess the relative virulence of the CR^+ and CR^- variants as discussed in the section 1.11. In this study an *in vivo* (virulence in 11-day-old chicken embryo model) and an *in vitro* test (adherence to and invasion of HeLa cells) were employed for comparison of the relative virulence of the CR^+ and CR^- variants. Irrespective of the clinical source of the strains, the CR^+ variants had LD_{50} values between 11 and 120-fold less than the corresponding CR^- cells (Table 21). As the CR binding property of the *C. jejuni* strains has not been investigated before, the results obtained in this study cannot be compared directly with any previous reports. However, the difference in the LD_{50} values obtained with the *C. jejuni* strains (11 to 120-fold) compares favourably with those obtained with *V. cholerae* (100 fold) but not with *S. flexneri* (5000 fold) (Payne and Finkelstein, 1977).

In the adherence and invasion assay in HeLa cells, a significant reduction in the adherence and invasion potential of the CR^- variants was noted in comparison to the CR^+ variants (Figure 36). The inability of the CR^- variants of *S. flexneri* to invade HeLa cells was always paralleled with the loss of the 140 M dal virulence plasmid (Maurelli *et al*, 1984). However, analysis of plasmid profile of the CR^+ and CR^- variants of the *C. jejuni* strains in the present study indicated no involvement of plasmids in the CR binding phenomenon.

Comparative studies with CR^+ and CR^- variants in several other microorganisms showed that the transition to the CR^- phase from the parent CR^+ phase was associated with a loss of various cell-surface structures and properties. In *Aeromonas salmonicida*, the CR^- variants were devoid of the protein A layer (Ishiguro *et al*, 1985; Kay *et al*, 1985) which is reported to be the determinant of

virulence (Kay *et al*, 1981) and cell-surface hydrophobicity (Trust *et al*, 1983). However, the results obtained with the CR uptake phenomenon in *Bordetella* spp. was the most dramatic. The CR^- variants were devoid of the whole range of virulence factors such as haemolysin, heat-labile toxin, pertussis toxin, haemagglutinin(s) and virulence-associated envelope polypeptides (X-bands) (Parton, 1988). *S. flexneri* 2a grown in the presence of CR is reported to produce three outer membrane proteins (43, 58 and 63 K dal) which reacted with human convalescent-phase sera in immunoblots (Sankaran *et al*, 1989). Indirect immunofluorescence studies with 43 K dal protein specific antiserum showed an increase in the levels of the 43 K dal protein in *S. flexneri* which had invaded epithelial cells, indicating that this CR regulated OMP is involved in virulence of the pathogen. Stugard *et al* (1989) reported that a 101 kilodalton heme-binding protein was associated with CR binding and virulence of *S. flexneri* and enteroinvasive *E. coli*. The CR^+ and CR^- variants of the *C. jejuni* strains differed in terms of three outer membrane proteins (Figure 31). The transition from CR^+ to CR^- phase was accompanied by the loss two proteins (ca. 87 and 63 K dal) and gain of a 16 K dal protein. The 63 k dal band presumably represents the flagellin molecule of *C. jejuni* and the ca. 87 K dal protein is considered to represent the hook protein (Newell *et al*, 1984).

The importance of flagella in the virulence of the *C. jejuni* strains was addressed in several investigations. They have tentatively been identified as an adhesin for eucaryotic cells and intestinal mucus, and also been found to enhance the colonization of the gut in comparative studies with nonmotile strains (Morooka *et al*, 1985; Newell *et al*, 1985a). But the role of motility in the adherence and colonization process is still confusing. McSweegan and Walker (1986) reported that flagellate, non-motile (F^+M^-) adhered better than the flagellate, motile (F^+M^+) and aflagellate, non-motile (F^-M^-) mutants and suggested that only intact flagella were necessary for adherence, and motility could impede-long term adherence. Morooka *et al* (1985) however, stressed the importance of motility by showing that flagellate, motile (F^+M^+) strains were

better colonizers of the mice intestine than flagellate, non-motile (F^+M^-) and aflagellate, non-motile (F^-M^-). However, Monoclonal antibodies directed against the flagella of the *C. jejuni* failed to inhibit motility and colonization of the infant mice (Newell, 1986) and Field *et al* (1986a) did not observe any significant difference between the flagellate and aflagellate strains in lethality in the 11-day-old chicken embryo model. These results thus question the role of flagella in the virulence of *C. jejuni.*

In the present study, CR^+ variants of all the strains investigated exhibited relatively higher virulence than the corresponding CR^- variants in the 11-day-old chicken embryo model. As the CR^+ and the CR^+ variants differed in terms of a 16 K dal protein band (and a 87 K dal protein band) in addition to the 62 K dal protein representing flagella, the CR binding phenomenon cannot be directly equated the presence or absence of flagella, and hence cannot be compared directly with the results of Field *et al* (1986a). However, as all the CR^- variants were devoid of flagella, and as the flagella were considered to be involved in the virulence of *C. jejuni* (Newell and Pearson,1984; McSweegan and Walker, 1986) and other microorganisms such as *V. cholerae* (Attridge and Rowley, 1981), it may be considered to play a role in the virulence in the chick embryo model.

In addition to the adhesin-receptor mechanism for interaction of pathogenic microorganism to the host cell, bacterial cell-surface hydrophobicity is now considered to play a secondary nonspecific role in interacting with the host cell-surface involving hydrophobic forces. Comparative studies with the cell surface properties of *Shigella* spp. have shown that the CR^+ variants were relatively more hydrophobic than the corresponding CR^- variants (Qadri *et al,* 1988). Various other virulence-associated cell-surface properties such as the possession of protein A layer in *Aeromonas salmonicida* (Trust *et al,* 1983; Kay *et al,* 1985), and the presence of X-bands in *Bordetella pertussis* (Robinson *et al,* 1985; Fish *et al,* 1987; Parton, 1988) have been correlated with the binding of Congo red dye and cell surface hydrophobicity; both of which characteristics were found to be indicators of

virulence in these pathogens. As CR^+ cells were relatively more hydrophobic in comparison to the CR^- cells, and hydrophobicity generally correlates with increased adherence, CR-binding property may also be considered as a candidate virulence marker of *C. jejuni.*

In this study, the CR-binding acpacity correlated with several virulence associated characteristics of *C. jejuni. C. jejuni* strains which bound Congo red in higher quantity, and were relatively more hydrophobic, were more virulent in 11-day-old chicken embryos mode. In addition CR^+ cells were exhibited increased adherence and invasion capacity than the CR^- cells. However, these characteristics did not correlate with the enterotoxigenicity of these strains. Both the production of enterotoxin and invasiveness are the proposed virulence mechanism of *C. jejuni* (Klipstein *et al,* 1985). Congo red dye binding and the cell-surface hydrophobicity correlated with the invasiveness but not with enterotoxigenicity of the *C. jejuni* strains. Congo red binding capacity as an indicator of virulence in *C. jejuni* has not been investigated before. And although the cell-surface hydrophobicity of the *C. jejuni* has been studied no attempts were made to correlate this putative indicator of virulence with the clinical characteristics of the strains. In this study it has been found that CR binding and cell surface hydrophobicity correlate with some of the virulence characteristics of the *C. jejuni* strains. It would be interesting to evaluate the clinical significance of these *in vitro* findings.

5.6 The heat-modifiable MOMP of *C. jejuni*

Due to heat-induced changes in protein-SDS interactions resulting in altered electrophoretic mobility, certain outer membrane proteins of Gram-negative bacteria are termed as heat-modifiable because of their migration behaviour upon SDS-PAGE (Parton and Wardlaw, 1975; Nakamura and Mizushima, 1976; Russell, 1976; Benz, 1985). The heat-modifiable nature of the MOMP observed in this

study (that it migrates as a 42 K dal protein when solubilized at 100° C and as a 30 K dal protein when solubilized at 37° C) is in agreement with those observed by Huyer *et al* (1986) and Page *et al* (1989). The apparent molecular weight of the heat-modifiable proteins may either increase or decrease upon heating in presence of SDS. Proteins which exhibit an increase in apparent molecular weight upon heating usually have a high content of ß-sheet structure, which determines the extent of SDS binding. Heat-modifiable proteins which show a decrease in apparent molecular weight upon heating are often porin proteins; which are usually trimeric and heating the trimers in SDS causes them to dissociate and migrate as lower molecular weight monomers in SDS-PAGE (Benz, 1985). *C. jejuni* MOMP is unusual in this regard; although it is a porin protein (Huyer *et al,* 1986; Page *et al,* 1989) it showed an apparent increase in molecular weight upon heating in presence of SDS. The might be due to the monomeric nature of the porin MOMP and because of the fact that some porins also possesses significant ß-sheet structure (Benz, 1985). The presence or absence of the reducing agent ß-mercaptoethanol in the solubilizing buffer did not affect the migration of the MOMP or other OMPs of *C. jejuni* (Figure 39), presumably indicating that these proteins did not possess extensive disulphide bonding. Logan and Trust (1982) also observed no change in protein profile when ß-mercaptoethanol was omitted from the solubilization solution.

Although there are reports in which the heat-modifiable nature of the MOMP was indicated (Logan and Trust, 1982; Huyer *et al* 1986; Page and Taylor, 1988), it has not been characterized in terms of its sensitivity to trypsin and isoelectric point. Huyer *et al* (1986) and Page *et al* (1988) isolated MOMP and examined for its porin activity. But it is evident from the SDS-PAGE gels presented in these papers that the MOMP was not purified to homogeneity as some additional minor bands were also visible. In this study the isolation of MOMP by the method of Huyer *et al* (1986) was slightly modified and

extended by including the trypsin treatment step (Nurminen, 1978) to selectively eliminate the minor bands. As MOMP is the only trypsin resistant protein in the OMP preparation of *C. jejuni,* the procedure adopted here thus resulted in an OMP preparation which was homogeneous in SDS-PAGE, Western blotting, immunodiffusion, and isoelectric focusing (Figures 40, 41 and 42). Nurminen (1978) used trypsin treatment to selectively isolate certain OMPs of *Salmonella typhimurium.* He described the process as very mild since the isolated proteins retained all the biological activities. The MOMP of *C. jejuni* isolated by trypsin treatment was also found to be effective in partially reducing the adherence and invasion potential of homologous and heterologous *C. jejuni* strains.

From the IEF experiment (Figure 42) it appears that the MOMP is an acidic protein with a pI of ca. 5.0-5.2 This is the first report of the determination of the pI of a MOMP of a *C. jejuni* strain. Dunn *et al* (1987) characterized the OMPs of *C. jejuni* by two-dimensional gel electrophoresis involving isoelectric focusing and SDS-PAGE. Although the pI of MOMP was not determined, they tentatively identified the location of it (as crude membrane preparations were used) on the acidic portion of the gel. It may be mentioned in this connection that flagellin of *C. jejuni* is also an acidic protein and, depending upon serovars, varies in pI from 4.7 to 5.7 (Nachamkin and Yang, 1988). The presence of acidic MOMP and flagella is in agreement with Walan and Kihlstrom, (1988) that *C. jejuni* cells possess a negatively-charged surface as determined by ion-exchange chromatography with DEAE-Sepharose.

Seroepidemiological studies have indicated that MOMP is immunogenic and induces antibodies, which are persistant (Walker *et al,* 1986). Although the flagella of *C. jejuni* have been characterized to considerable deapth in recent years (Section 1.7.4), the MOMP has received little attention. The simple method for obtaining highly purified MOMP may make possible the biophysical and immunological characterization of this important structural component of *C. jejuni.*

5.7 Colonization and Multiplication of *C. jejuni* Strains in Tissues and Organs of Infant Chicks

Two strains of *C. jejuni* were investigated for their capacity to colonize the different portions of the gut and various internal organs of newly hatched chicks (within 12 hr of age). These two strains B-23 and S-11 were selected because they were representative of Group C and Group D respectively. Strain S-11, isolated from bloody, mucoid diarrhoeal stool was one of the most adherent and invasive strains (as judged by *in vitro* HeLa cell model and *in vivo* 11 day old chicken embryo model) and produced no detectable enterotoxins. Moreover, it produced a cytotoxic haemolysin which showed lytic activity against a spectrum of erythrocytes from different animals and was also cytotoxic towards HeLa cells. On the other hand, the strain B-23 which was isolated from a watery diarrhoeal stool produced a cholera-like enterotoxin. Moreover, it exhibited the least evidence of adherence and invasion in the HeLa cell model and did not produce the cytotoxic haemolysin unlike the strain S-11. In addition the strain B-23 was least lethal in the 11-day-old chick embryo model. So the presence and absence of these virulence associated properties made these strains ideal representatives for a comparative study of the virulence of the strains isolated from cholera-like (Group C) and dysentery-like (Group D) enteritis cases of *C.jejuni* (Table 23). It was anticipated that comparison of high and low virulent strains might provide information on how these strains differed in colonizing the different segments of the gut and causing systemic infection, which might be indicative of the differences relevant to pathogenesis.

Interestingly, the strains S-11 and B-23 did not differ significantly in colonizing the different parts of the GI tract (Figures 43 to 45). Both the strains were able to establish themselves rapidly and virtually in equal degree. As the largest concentration of viable organisms was found in the colon, this organ may be considered the principal site of colonization. Smaller numbers were found in the ileum and jejunum. The counts from the different portions of the gut

Table 24: Candidate Virulence Markers Associated with the Representative Strains from Dysentery-like and Cholera-like Diarrhoea Cases (S-11 and S-13 respectively)

CANDIDATE VIRULENCE MARKER	STRAIN S-11	STRAIN B-23
1. HeLa cell adherence	High	Low
2. HeLa cell invasion	High	Low
3. Lethality to chicken embryo	High	Low
4. Growth in the GI tract of chicks	High	High
5. Growth in the internal organs of Chicks	High	Low
6. Production of cytotoxic haemolysin	+	–
7. Enterotoxin Production		
a. GM_1 ganglioside ELISA	–	+
b. CHO cell assay	–	+
c. Fluid accumulation in the gut of new-born chicks	–	+
d. Ligated ileal loop assay in 5-day-old chicks	–	+
e. Immunodiffusion with cholera toxin	–	+
8. Cell-surface hydrophobicity		
a. Adherence to hydrocarbon	High	Low
b. Salt aggregation test	High	Low
9. Uptake of the dye Congo Red	High	Low

demonstrate that the behaviour of these organisms in the chicken is not greatly different from that in other animals such as mice of different ages (Field *et al*, 1981; Blaser *et al*, 1983a; Newell *et al*, 1984). Previous studies with chicks also demonstrated preferential colonization of *C. jejuni* in the lower region of the GI tract (Sanyal, *et al*, 1984a; Welkos, 1984). Recently, Beery *et al* (1988) reported that *C. jejuni* strains colonized infant chickens after oral inoculation and quantitative bacteriology showed preferential localization in the large gut and cecum.

Among the internal organs, liver and spleen were selected in the present study because Benjamin *et al* (1986) reported that as many as 80 % of the orally-inoculated *Salmonella enteritidis* were localized in these organs in mice. Quantitative bacteriology of liver and spleen revealed a differential colonization pattern by the two strains of *C. jejuni*; S-11 being more efficient than the B-23, which may be considered as an indicator of relative virulence of these two strains to cause systemic invasion and *in vivo* growth. Figures 46 and 47 indicated that infection occurred simultaneously in the spleen and liver and these two organs remained culture positive during the 7 days of the experiment. Sanyal *et al* (1984a) also isolated viable *C. jejuni* from the liver and spleen of 3-day-old chicks following oral inoculation, but this was done only by taking smears from sections of these two organs and no quantitative analysis was made.

Although the quantitative cultures of organ specimens for *C. jejuni* were not extended beyond day 7 post-inoculation, it is possible that the organisms may survive in these organs for longer periods, as Kita *et al* (1986) showed that a human faecal strain could survive in the livers of orally inoculated mice for up to 5 months post-inoculation. Persistence in the liver was associated with focal infiltrative necrosis in the liver parenchyma and altered liver function tests. However, the results obtained in this study are at variance with those of Beery *et al* (1988) who reported that viable *C. jejuni* organisms were not recovered from the liver and only occasionally from the spleen. Use of different strains and

older chicks (8-day-old) in comparison to those used in this study (ca. 12-hr-old) may account for this discrepancy.

The resistance of the chicks to infection increases rapidly with age and it is probable that the almost gnotobiotic condition of the freshly hatched chick (Popiel and Turnbull 1985) is a primary factor in the ease with which the enteritis can be induced. However, studies with *C. jejuni* (Blaser *et al,* 1983a) and other enteropathogens such as *S. enteritidis* (Collins, 1970) and *Y. enterocolitica* (Carter and Collins, 1974) showed that these pathogens were isolated in large numbers from spleen and liver. Thirty to 45 min after oral inoculation of 1-day-old chicks with *S. enteritidis,* blood, liver and lung became culture positive (Turnbull and Richmond, 1978). Interestingly, ca. 1.0×10^5 c.f.u. gm^{-1} of *C. jejuni* were recovered from liver 10 minutes after intragastric inoculation of adult mice with 1.0×10^8 organisms (Blaser *et al,* 1983a).

A marked difference was noted between the *C. jejuni* strains B-23 and S-11 in their ability to cause bacteraemia. The transient bacteraemia noted with the strain B-23 could be the result of the initial influx of the organisms overwhelming the normal clearing mechanism after inoculation of a large number of organisms. But the strain S-11 was detected in the blood until day 5 post-inoculation indicating that it had certain properties which enabled it to survive longer in the blood. The duration of bacteraemia caused by these two strains also correlated with their persistence and multiplication in the spleen and liver; strain S-11 was recovered in larger numbers than the strain B-23 from these organs, as mentioned earlier. Bacteraemia in chicks following oral inoculation of *C. jejuni* was reported by other researchers. Sanyal *et al* (1984a) found that organisms could be isolated from heart blood of 3-day-old chicks until day 3 post-inoculation, but not thereafter. Beery *et al* (1988) also occasionally detected *C. jejuni* in the blood of 8-day-old chicks. The relatively longer bacteraemia caused by the strain S-11 in this study (Figure 48) compared with

that observed by Sanyal *et al* (1984a), could be an attribute of the strains; the strain S-11 may be more virulent than those investigated by Sanyal *et al* (1984a). As discussed earlier, the immunological immaturity of the younger chicks may be another determining factor. Duration of bacteraemia did not correlate with the persistance of the organism in the liver and spleen as large numbers of organisms could be isolated from these organs after the blood has been cleared of *C. jejuni.*

In all these studies dealing with colonization of chicks with *C. jejuni* (this study, Butzler and Skirrow, 1979; Ruiz-Palacios *et al,* 1981; Sanyal *et al,* 1984a; Beery *et al,* 1988), bacteraemia of varying duration up to day 5 post-inoculation was noted. On the other hand, bacteraemia is rarely seen in other animal models and occasional transient bacteraemia is rapidly cleared (Field *et al,* 1981; Fitzgeorge *et al,* 1981; Al-Mashat and Taylor, 1980b; Field *et al,* 1984). The relatively higher body temperature of chickens (41-42^{o} C) in comparison to other laboratory animals such as rats (37-37.5^{o} C) was suggested as one of the probable reasons for the ease with which chickens are colonized by *C. jejuni* (Kakoyiannis *et al* 1985). As the optimum temperature for growth of *C. jejuni* is 42^{o} C, this phenomenon may also be considered as one of the reasons for longer bacteraemia in chicks than other animals.

5.8 Virulence (lethality) in the Chicken Embryo Model

The 11-day-old chicken embryos inoculated into the chorioallantoic cavity was used as a model for studying the virulence of the *C. jejuni* strains. Although it may be argued that chick embryos do not represent a relevant assay system for studying the pathogenesis of the *C. jejuni* enteritis, two independent studies showed that it may be used to determine the relative virulence of the *C. jejuni* strains (Field *et al,*1986a; Davison and Solomon, 1980). Moreover, the chicken embryo model has been used extensively in studies on virulence of other pathogenic microorganisms such as *E. coli*

(Minshew *et al*, 1978), *V. cholerae* (Finkelstein and Ramm, 1962; Gardner *et al*, 1963), *Neisseria gonorrhoeae* (Buchanan and Gotschlich, 1973) and virulence in this model correlated with virulence for man. In this study, clear-cut differences in lethality were observed among *C. jejuni* strains which paralleled those demonstrated in other assay systems employed.

It was clear from the LD_{50} values and the number of viable c.f.u's recovered per gm embryo tissue that the *C. jejuni* strains could be divided into two groups. In the first group were the strains S-11, B-7, S-13 and 11385 which had low LD_{50} values (Tables 10 to 17) and probably were capable of *in vivo* multiplication as evidenced by the higher number of c.f.u's recovered per gm embryo tissue (Figure 49 to 56) in comparison to the number of c.f.u's present in the inoculum. The other group (strain B-23 and B-9) had relatively higher LD_{50} values (Tables 18 to 21) and probably were incapable of *in vivo* multiplication as shown by the recovery of the lower number of viable *C. jejuni* per gm embryo tissue than the number inoculated (Figurse 57 to 60).

The number of infected surviving embryos appeared to be inversely related to the inoculum size and also to the virulence of the test strains. Viable *C. jejuni* were recovered from these infected but surviving embryos; but the counts were lower in comparison to the dead embryos (Figures 49 to 60); these embryos also might have died if incubation had been continued beyond the 72 hr period. Finkelstein and Ramm (1962) also noted that a proportion of embryos remained viable despite considerable multiplication of *V. cholerae*. In the case of some embryos, no viable bacteria were recovered in plate counts, especially when the inoculum contained a relatively lower number of c.f.u's. This indicates that the embryos were capable of clearing the organism from the system; the mechanism(s) involved are not known. Although the chicken embryos do not have complement and antibodies until shortly after hatching (Beveridge and Burnet, 1946), they have phagocytic capability (Karthigzsu and Jenkin, 1963; Mizejewski and Ramm, 1969) which may be a factor in eliminating the pathogen. In addition, circulating leukocytes

might also play an additional role, although their numbers seems to be small (Romanoff, 1960). The fact that some embryos were more efficient in clearing the bacteria than others inoculated with the same number bacteria probably indicates heterogeneity among chick embryos in susceptibility to *C. jejuni.* Similar variation among embryos was also observed by investigators working with other pathogens (Buchanan and Gotschlich, 1973).

The interesting point is that the chicken embryo model could differentiate between *C. jejuni* strains with varying degrees of virulence and accurately reflected the gradation of virulence of the same strains noted in the *in vitro* adherence and invasion assay in HeLa cells. The results obtained with the chicken embryo model provides additional supporting evidence for the initial idea that, although all the strains included in this study were from human enteritis cases, there was a gradation of virulence among them. Unless a strain with fully expressed virulence properties is investigated, it may appear avirulent despite being freshly isolated.

Much uncertainty exists about the putative virulence factors of *C. jejuni* and their stability. It is generally agreed that *in vitro* passage of a clinical strain results in a gradual decrease in virulence, and Pang *et al* (1987) found this to be true for *C. jejuni.* On the other hand, Black *et al* (1988) reported that in human volunteer studies a human clinical strain, which was passaged several times on laboratory medium induced a more severe disease than that in the patient from whom it had been originally isolated.

The reason(s) for the graded differences in lethality of the *C. jejuni* strains investigated in the 11-day-old chicken embryo is not known. The possible involvement of the various surface structures such as flagella and outer membrane protein (OMP) can be ruled out as all the strains were identical in these characteristics. The LPS profiles of the strains investigated were also identical in SDS-PAGE. But recently Preston and Penner (1987) reported that *C. jejuni* strains exhibiting a rough LPS profile in SDS-PAGE may occasionally possess O-side chains which can only be

demonstrated by immunoblotting with homologous antisera. Along with other characteristics of the pathogenic microorganisms, the possession of a unique O-antigenic structure has been implicated with the invasion capability of the enteric microorganisms (Mroczenski-Wildey *et al,* 1989). As the LPS profile of the *C. jejuni* strains was investigated only by SDS-PAGE in this study, the possible presence of long, smooth-type LPS and hence their possible involvement in the chicken embryo lethality exhibited by the different strains cannot be ruled out. In fact, possession of such LPS by the virulent *C. jejuni* strains was speculated by Field *et al,* (1986a) in the chick embryo model and by Stewart-Tull *et al* (1984) in the infant mouse model.

Differences in lethality among *C. jejuni* strains however correlated well with the production of a cytotoxic haemolysin by them. Strains with lower LD_{50} values (S-11, B-7, S-13) produced this toxin, while strain B-23 and B-9 which had relatively higher LD_{50} values (Table 21) did not. The only exception in this regard was the strain 11385 which did not produce the cytotoxic haemolysin although having relatively low LD_{50} value. Several independent studies published since the commencement of this study documented that the production of cytotoxic factors correlated with *C. jejuni* virulence. Yrios and Balish (1986b) observed that a cytotoxin-producing *C. jejuni* strain persisted longer in different internal organs of orally-infected germ-free mice than the cytotoxin non-producing strain. Recently, Pang *et al* (1987) reported that cytotoxin-producing strains induced a more severe diarrhoea in rabbits, occasionally resulting in deaths; whereas cytotoxin non-producing strains caused less severe diarrhoea and none of the rabbits died. In both of these studies the production of cytotoxin was suggested as a virulence-associated property. The results obtained in this study appear to be compatible with this interpretation. However, the differences in virulence noted in the 11-day-old chicken embryo model were not associated with the enterotoxic activity of the strains investigated.

Recently, Mahajan and Rodgers (1989) reported that both whole cells and cell-free culture filtrates of a single strain of *C. jejuni* caused death of chick embryos.

As they failed to detect any viable organisms in the embryonic tissue, the authors suggested a toxin etiology and speculated that invasion may not play an important role in the *C. jejuni* disease process. However, these results are in total disagreement with those obtained in this study and that of Field *et al* (1986a) where large numbers of viable organisms were recovered from the embryonic tissue indicating likely multiplication of the *C. jejuni* strains *in vivo*. Considering the fact that a). the age of the embryos used by Mahajan and Rodgers (1989) was less than is usually used for similar studies b). the resistance of the embryos increased with age c). even a non-invasive pathogen like *V. cholerae* was reported to multiply and persist in 13-day-old embryos (Gardner *et al,* 1963), the total absence of *C. jejuni* is evidently incompatible with the published studies. Another point that should be considered is that Mahajan and Rodgers (1989) found samples of filtrate originally containing 5.0×10^7 c.f.u. ml^{-1} retained toxicity (which was destroyed by heating at 60^o and was trypsin-sensitive, indicating it was a protein) even when diluted to 1:10,000. This is also much higher a titre for any toxin of *C. jejuni* reported so far; the toxins of this organism are generally produced in low titre. For example, a cytotoxin produced by *C. jejuni* had a maximum titre of 1:32 in CHO cell assay (Guerrant *et al,* 1987). Other studies (Johnson and Lior, 1986; 1988) also reported similar low titres of toxins of *C. jejuni.* The production of low levels of toxin has been pointed out as a problem in the purification and characterization of the toxins of *C. jejuni.*

The difference in age of the embryos used in this study and that of Field *et al* (1986a) with those used by Mahajan and Rodgers (1989) was also considerable. Eleven-day-old embryos were used here and by Field *et al* (1986a) and LD_{50} values were determined at 72 hr post-inoculation, whereas Mahajan and Rodgers (1989) first inoculated the embryos and then incubated for 10 days when inoculated in the allantoic cavity or the chorioallantoic membrane, and for 6 days when inoculated in the amniotic sacks and yolk sacks before LD_{50} determination. Chicken embryos of age 8-days or less have relatively underdeveloped resistance

mechanisms and are insensitive to the differences in virulence noted in older embryos. Gardner *et al* (1963) found that in 8-day-old embryos, strains of *V. cholerae* which were virulent and avirulent for older embryos, were equally lethal and suggested that relatively older embryos should be used so that the embryos achieve sufficient resistance to the pathogen to enable virulence of differences to be determined. So it appears that the reason(s) of virulence of the *C. jejuni* strains in the chick embryo model is not yet clear and needs further investigations.

5.9 Assay of Adherence and Invasion Potential of *C. jejuni* Strains in HeLa Cells

Adherence and subsequent invasion is probably the essential virulence characteristic of *C. jejuni* strains causing the dysentery-like mucoid diarrhoea. Adherence is also considered as a prerequisite for efficient delivery of the toxin into the target cells in the pathogenic mechanism of enteropathogens causing toxin-mediated enteritis (Gianella, 1981). Colonization factor negative mutants of enterotoxigenic *E. coli* were unable to cause diarrhoea in human volunteers although they retained the ability to produce enterotoxin (Satterwhite *et al*, 1978). So it appears that whatever the pathogenic mechanism of the *C. jejuni* enteritis e.g invasion as in *Shigella* and enteroinvasive *E. coli* or enterotoxin production as in *V. cholerae* and enterotoxigenic *E. coli*, adherence is probably a critical primary step in the pathogenesis of *C. jejuni* enteritis.

Although the rationale for the use of mammalian cells, such as HeLa, as a model for the microvilli containing columnar epithelial cells of the intestine which would reflect the essential characteristics of the adherence and invasion process of *C. jejuni* that occur *in vivo* is arguable, several investigations have indicated a close correlation between *in vitro* interaction with the tissue culture cell line and the virulence activity *in vivo* in different enteropathogens. For

example, Giannella *et al* (1973, 1975) demonstrated that *S. typhimurium* strains which were unable to invade epithelial cells failed to induce disease in animals and to cause characteristic pathogenesis in rabbit ileal loops. Similarly, invasion of epithelial cells by *Shigella* spp. closely correlated with their pathogenic potential in animal models (Sansonetti *et al,* 1981; 1982). Moreover, the mammalian cell monolayer represents a uniform and convenient experimental system in which various experimental parameters can be easily controlled and varied. Recent studies with such systems have led to the discovery of many interesting aspects of adherence and invasion at the molecular level; for example, the identification of 'adhesin' and 'invasin', the protein molecules that mediate such processes in *Yersinia* spp. (Isberg and Falkow, 1985; Isberg *et al,* 1987; Miller and Falkow, 1988). In this study HeLa cells were used to assess the adherence and invasion potential of the *C. jejuni* strains and also to determine whether these characteristics correlated with the other *in vitro* and *in vivo* virulence assays. It was also anticipated that if the basic features of the interaction of *C. jejuni* strains with the HeLa cells were established, this information might be useful in applying the techniques of molecular biology to identify such vital biomolecules as in *Yersinia* spp.

Attempts have been made in recent years to understand the mechanism(s) of adherence and to identify the surface component(s) that mediate such interactions (Walker *et al,* 1986). Although adherence of *C. jejuni* strains to cultured mammalian cells have been investigated and occasionally subsequent invasion has also been noted (Manninen *et al,* 1982; Naess *et al,* 1983; Newell *et al,* 1985b; Fauchere *et al,* 1986), no quantitative evaluation of the invasion potential of *C. jejuni* strains was made.

In this study, the assay used was straightforward and was modelled on that of Isberg and Falkow (1985) to investigate the invasion of HEp-2 cell monolayers by *Yersinia pseudotuberculosis.* Adherence and subsequent invasion of

the *C. jejuni* strains were investigated in the HeLa cell model and were quantitated in terms of the number of adherent viable bacteria (adherence) and internalized (invasion) relative to the total number of bacteria present in the inoculum. The strategy of the HeLa cell adherence and invasion assay is that after co-incubation of *C. jejuni* strains with the HeLa cell monolayer, the adherent but non-internalized bacteria are selectively killed by an aminoglycoside antibiotic (such as Gentamicin) which has very limited ability to diffuse into the epithelial cells (Niesel *et al*, 1985). The portion of bacteria surviving the action of Gentamicin is interpreted as having invaded the host cells and is determined by viable count of the bacteria after lysing the HeLa cells (Flow Diagram 3). Incubation of bacteria with tissue culture cells, followed by the use of an aminoglycoside antibiotic such as Gentamicin or kanamycin to kill extracellular, but not intracellular bacteria and recovery of the intracellular bacteria by lysing the cells, is now widely used method for the study of invasion of eucaryotic cells by Gram-positive (Nath, 1989) and Gram-negative pathogenic microorganisms (Bhogale *et al*, 1983; Isberg and Falkow, 1985; Miller and Falkow, 1988). This method was chosen because it is a highly sensitive analysis (as few as 100 c.f.u. can be determined) and is probably more revealing as the invasion potential of a pathogen is determined on the basis of viable bacteria recovered after lysis of the host cell. Other methods employ various physical, chemical, biological and immunological techniques (Labrec *et al*, 1964; Kihlstrom, 1977; Hale and Bonventre, 1979; Newell *et al*, 1985b) in which the viability of internalized bacteria is not considered and is often operationally difficult to determine whether a given bacterium is simply adhering to the epithelial surface or is internalized. The fact that large numbers of bacteria were recovered in this study after lysis of the HeLa cells, following an invasion experiment which involved incubation in growth medium containing Gentamicin for 2 hr can only be explained by an intracellular localization of bacteria since, in control experiments under similar conditions at the same concentration of antibiotic caused ca. 1.0×10^6 fold decrease

in the viable count of *C. jejuni* in 90 min (Figure 61). A major advantage of this method over microscopic methods to establish the proportion of invaded bacteria is that extracellular adhering bacteria do not interfere.

Initially, it was intended to work out the experimental parameters to develop an optimized, accurate and reproducible assay system to evaluate the adherence and invasion potential of *C. jejuni* strains in a HeLa cell model. The various parameters investigated included (1). multiplicity of infection i.e. the ratio of bacterial cells to the HeLa cell, (2). assay temperature, (3). time course of infection, (4). gaseous atmosphere during the infection period, (5). growth temperature of the *C. jejuni* strains, and (6) growth phase of the *C. jejuni* strains. In the process of developing the experimental system, significant differences were noted among various experimental parameters on the adherence and invasion potential of the *C.jejuni* strains included in this study in the HeLa cell assay.

5.9.1 Multiplicity of Infection

Multiplicity of infection (MOI) is a critical requirement for the development of an accurate and reproducible assay for adherence and invasion potential of a pathogen. This is particularly important if the invasion potential is analysed by enumeration of the viable bacteria liberated after lysis of the host cell. Although this critical requirement was usually overlooked previously, it is now analysed and maintained at a level to maximize the invasion process in recent studies with other enteric organisms (Isberg and Falkow, 1985; Sansonetti *et al,* 1986; LeChevallier *et al,* 1987). Unfortunately, most of the studies reported so far dealing with adherence and invasion of the *C. jejuni* did not attempt to maintain MOI at a constant level hence it is difficult to assess whether the adherence and invasion potential of the *C. jejuni* strains were in fact accurately analysed. The use of a constant MOI is particularly important if a number of strains are

compared for their relative adherence and invasion potentials.

In this study the MOI was maintained at the reasonable level of 100 organisms : 1 HeLa cell which enabled the determination of adherence and invasion potential of all the *C. jejuni* strains on a unified scale. Thus the virulence-associated differences between strains, such as S-11 and B-23, which differed widely in virulence, could be compared directly. This particular value of MOI was determined after a series of experiments with graded numbers of organisms of a highly invasive strain S-11 and a weakly invasive strain B-23 in parallel experiments. The MOI used is comparable with those used for other bacterial pathogens investigating for adherence and invasion. For example Isberg and Falkow (1985) used a MOI of 100 for *Y. pseudotuberculosis* in a HEp-2 cell model. For *S. flexneri* a MOI ranging between 61-135 was used in the HeLa cell model (Sansonetti *et al*, 1986) MOI of ca. 200 was used by LeChevallier (1987) for *Y. enterocolitica* in the HeLa cell model and Lenin *et al* (1986) found a MOI of 500 suitable for assay of adherence of *Bordetella pertussis* to mouse fibroblast cells. In studies dealing with the adherence and invasion properties of *C. jejuni* the importance of MOI was not recognized and for discussion purposes, I have calculated the approximate values. A MOI of approximately 100 was used by Newell *et al* (1985b) for adherence and invasion studies in a HeLa cell model. Fauchere *et al* (1986) used a bacterial inoculum of $1.0x10^7$ to $1.0x10^8$ *C. jejuni* per $1.0x10^5$ HeLa cells which yields a MOI of 100-1000. Other studies dealing with adherence and invasion of *C. jejuni* in an epithelial cell model used a relatively high MOI of 10,000 (Cinco *et al*, 1984; McSweegan and Walker, 1986; de Melo and Pechere, 1988). Use of different *C. jejuni* strains, different hosts cell and different assay procedures may justify the use of such high MOIs. But in this study it was found that the use of MOI 1000 for certain *C. jejuni* strains resulted in bacterial aggregation; often the monolayer became partially detached and was washed off during the

process of removing unbound bacteria. This made interpretation the results difficult and prevented comparison with other strains. Use of high MOI may also result in reduced invasion efficiency as has been reported by Small *et al* (1987) for *Y. enterocolitica* in the HEp-2 cells.

5.9.2 Influence of Growth Parameters

The phenotypic expression of bacterial determinants that mediate interaction with host tissue is influenced by the growth conditions (Gaastra and de Graaf, 1982; Guerina *et al*, 1983). Microorganisms from different phases of growth are reported to differ in various phenotypic markers including their capacity to invade tissue culture cells. For example, enteroinvasive *E. coli* had to be grown for 2 hr at 37° C immediately before the invasion assay because bacteria undergoing logarithmic growth entered HEp-2 cells more efficiently than bacteria in the stationary phase of growth (Small *et al*, 1987).

A comparison of the efficiency of invasion of HeLa cell monolayers by *C. jejuni* strain S-11 revealed that bacteria from 16 hr cultures (logarithmic phase of growth) were more efficient than 26 hr cultures (stationary phase). Interestingly, the effect of use of 26 hr culture on invasion potential was more pronounced than on the adherence potential of 16 hr cultures (Figure 65). These results probably indicate that actively metabolizing *C. jejuni* cells are required for the efficient invasion of HeLa cells. On the other hand, this may not be critical for adherence. The relatively higher invasion potential noted in the case of growth at 42° C in comparison to 37° can also be explained on the assumption that the metabolic state of *C. jejuni* influences its invasion potential. The two parameters, viz. growth temperature and the phase of growth, did not affect the adherence potential to the same extent as the invasion potential (Figure 65). This is in contrast to certain well-known bacterial adhesins such as K88 and K99 adhesins of enterotoxigenic *E. coli* (ETEC) which are expressed at 37° C but not in significant amounts at 18° C (Gaastra and de Graaf, 1982; Issacson,

1983). The influence of phase of growth is also well-documented; for example, the K99 adhesive fimbriae of (ETEC) are expressed maximally in the logarithmic phase of growth of shaken culture (Issacson, 1980). On the other hand, the Type 1 fimbriae of *S. typhimurium* and *E. coli* were produced when grown in static broth cultures beyond the logarithmic growth phase, but were only poorly expressed if grown on solid media (Old and Duguid, 1970).

5.9.3 Use of Microaerophilic Gas Atmosphere

The composition of the gas atmosphere during the incubation period of the *C. jejuni* cells with the HeLa cells was found to have a profound influence on the adherence potential of the *C. jejuni* strains. Adherence and invasion were expressed at the maximal level when a microaerophilic gas atmosphere (5 % O_2, 10 % CO_2 and 85 % N_2) was used, and a highly significant reduction in these virulence-associated properties especially on invasion was observed when the standard tissue culture gas atmosphere (95 % air and 5 % CO_2) was used during the infection period (Figure 65). All other studies dealing with the adherence and invasion potential of the *C. jejuni* strains (Cinco *et al,* 1984; Newell *et al,* 1985b; McSweegan and Walker, 1986; De Melo and Pechere, 1988) overlooked this critical requirement for the maximal expression of these virulence-associated properties.

C. jejuni is microaerophilic in nature and normal atmospheric levels of oxygen are inhibitory to growth (Bowdre *et al,* 1976; Karamali *et al,* 1981). *C. jejuni* rapidly loses its viability and typical spiral morphology upon exposure to oxygen and transforms into coccal forms (Moran and Uptan, 1986; 1987). This observation indicates that metabolically active *C. jejuni* cells are required for invasion of HeLa cells. Later, Bukholm and Kapperud (1987) reported that invasion of HEp-2 cells by *C. jejuni* strains occurred only in the

microaerophilic atmosphere and the invasiveness could be selectively blocked by increasing the O_2 tension. But it should be mentioned here that they also observed that *C. jejuni* by themselves were non-invasive to HEp-2 cells, even when incubated in a microaerophilic atmosphere; co-infection with enteroinvasive *E. coli, Shigella* and *Salmonella* was found to exert an effect which rendered the *C. jejuni* capable of invasion HEp-2 cells. In this regard, *C. jejuni* resembles other enteropathogens such as *S. flexneri* where the organisms must be viable and metabolically active to be able to invade mammalian cells (Hale and Bonventre, 1979). *Yersinia* spp. killed by heat, UV irradiation, formalin or glutaraldehyde were non-invasive for tissue culture cells. Inhibitors of protein and RNA synthesis also significantly reduced the invasiveness of *Yersinia* spp. into HeLa cells (LeChevallier *et al,* 1987). The results obtained in this study agree with the general consensus that invasive pathogens do not act as inert particles but participate in an active fashion in the invasion of cultured mammalian cells; some metabolic activity is required on the part of the bacteria to induce 'parasite directed endocytosis'.

5.9.4 Intracellular multiplication

Once it was established that the *C. jejuni* strains could reproducibly invade HeLa cell monolayers, experiments were carried out to investigate how long the internalized bacteria survived within the HeLa cells and whether the bacteria were capable of intracellular multiplication. Although the interaction of *C. jejuni* strains with cultured mammalian cells has been studied previously (Manninen *et al,* 1982; Newell *et al,* 1985; Fauchere *et al,* 1986), this particular aspect was not investigated. In this study, any increase in the c.f.u. of the bacteria over the time of the study would reflect intracellular growth, as the extracellular bacteria were selectively killed by the antibiotic gentamicin. There was no increase in the number of viable bacteria at 2, 4, 6 and 24 hr time

points indicating that there was no intracellular multiplication of *C. jejuni.* The decrease in the viable bacteria at 24 hr time point (Figure 63) could be due to transition from spiral to coccal form (Kiehlbauch *et al,* 1985), followed by digestion by the lysosomes as has been observed by De Melo *et al* (1989) in HEp-2 cell model.

Some invasive enteropathogens are capable of efficient intracellular multiplication following invasion of epithelial cells e.g. enteroinvasive *E. coli* (EIEC) (Small *et al,* 1987), *S. flexneri* (Sansonetti *et al,* 1986) and *Salmonella typhi* (Mroczenski-Wildey, 1989). While others are either incapable or possess poor ability of intracellular growth such as *Y. pseudotuberculosis* (Falkow *et al,* 1987) *Y. enterocolitica* (Small *et al,* 1987) and *S. typhimurium* (Sansonetti *et al,* 1986; Small *et al,* 1987). The capacity of intracellular multiplication has been correlated with the rapid and efficient lysis of the limiting eucaryotic cell membrane resulting in death of the eucaryotic cells (Sansonetti *et al,* 1986; Falkow *et al,* 1987). From the results obtained in this study it may be inferred that *C. jejuni* belong to the latter group of enteropathogens which can penetrate but are incapable of intracellular multiplication. The positive control strain EIEC 111, on the other hand multiplied efficiently as evidenced by ca. 50-fold increase in c.f.u. over the 6 hr study period (Figure 64). In fact, the experiment could not be extended beyond 6 hr due to observed cytotoxicity on the HeLa cell monolayer presumably because of this extensive multiplication. It may be mentioned in this connection that Falkow *et al* (1987) observed that the growth of *E. coli* HB101 (*eic*) (*E. coli* carrying the invasive capability of EIEC) in HEp-2 cells were comparable and in some instances even surpassed that of broth-grown cells.

The clinical significance of the intracellular survival of *C. jejuni* in HeLa cells is an intriguing question with important implications concerning the pathogenicity of the organism. According to Falkow *et al* (1987) the capacity of the enteropathogens to invade and survive is central to the 'pathogenic

personality' of these organisms. This pathogenic strategy is extraordinarily beneficial for the bacteria, which are safely sequestered from the antimicrobial substances present on the mucosal surface as well as from antibody, phagocytes, and some antibiotics. The ability to multiply intracellularly is critically important for *Shigella* spp. and EIEC since the epithelial cell is the primary site of multiplication during infection. Kiehlbauch *et al* (1985) reported that *C. jejuni* can survive for up to 7 days in a peritoneal macrophage cell line derived from BALB/c mice, J77 4G8; resident BALB/c peritoneal macrophages and human peripheral blood monocytes. The fact that *C. jejuni* survived better inside monocytes or macrophages than in control preparations without phagocytes led the authors to imply that the mononuclear phagocytes may provide nutrients or other favourable conditions or both for increased intracellular survival which may contribute to the pathogenicity of the organism.

5.9.5 Inhibition of Adherence and Invasion of HeLa Cells by L-fucose

Studies with a variety of microorganisms strongly indicated that sugar-binding proteins (adhesins) similar to plant lectins are present on the bacterial cell-surface which may mediate attachment of the pathogen to carbohydrate-containing molecules of the eucaryotic cell membrane or glycocalyx sugar residues on the eucaryotic cell surfaces (Lark, 1986). A sugar capable of inhibiting adherence of microorganisms to epithelial cells is generally considered to be an essential component of a mammalian cell receptors to which a bacterial adhesin binds and adhesion inhibition studies involving a battery of carbohydrates is extensively used for identification of putative receptor molecules. Such studies revealed that D-mannose was one of the epithelial cell receptor for *E. coli* (Duguid *et al,* 1979; Mirelman *et al,* 1980) and L-fucose for *V. cholerae* (Jones and Freter, 1976).

Recently, *C. jejuni* strains were investigated for the presence of such

adhesins specific for sugar residues on the mammalian cell-surface using INT 407 cells (Cinco *et al,* 1984; McSweegan and Walker, 1986). In both of these studies, L-fucose significantly inhibited adherence of the *C. jejuni* strains to the INT 407 cells indicating the presence of L-fucose specific adhesin on the bacterial cell-surface. In this study, initially it was decided to investigate whether the L-fucose mediated inhibition of adherence observed with INT 407 cells could be extended to the HeLa cell system and if so, how it might interfere with the invasion of these cells by *C. jejuni* strains, in addition to the adherence process reported by previous workers (Cinco *et al,* 1984; McSweegan and Walker, 1986) with INT 407 cell line.

A significant reduction in adherence and also in the invasion potential of the *C. jejuni* strains in the presence of L-fucose was observed in the HeLa cell model (Figures 69 and 70) indicating that L-fucose residues may constitute at least part of the adhesin receptor for *C. jejuni* on HeLa cells. McSweegan and Walker (1986) found that in addition to L-fucose, D-mannose can also partially inhibit adherence of *C. jejuni* to INT 407 cells. The presence of multiple sugar specificity was found with other bacterial pathogens such as *S. flexneri* (Izhar *et al,* 1982) and *Vibrio cholerae.* In this respect *C. jejuni* exhibited similar characteristics to *V. cholerae* which also possesses a similar adhesin specificity for D-mannose and L-fucose to rabbit intestinal brush border membranes (Jones and Freter, 1976).

It is generally agreed that bacteria must adhere to the mucosal surface of the host cells in order to colonize host tissues to exert their pathogenic effect. This is supported by extensive data showing a positive correlation between the ability of bacteria to adhere to the cell surface and the expression of virulence (Ofek and Beachy, 1980). As adherence is mediated by specific interaction between the adhesin on the bacterial surface and the sugar-containing receptor on the host cell-surface, the sugar-binding capability of pathogenic microorganisms can be implicated in virulence especially in experimental urinary tract infections and gastrointestinal infections in animals. Hirschberger *et al* (1977) reported that intestinal colonization of rabbits

was specifically blocked by D-mannose but not by other sugars. More importantly the incidence of urinary tract infection in experimental animals was found to be markedly decreased by sugars that specifically inhibit the bacterial cell surface lectins (Aronson *et al,* 1979; Fader and Davis, 1980) and by antibodies to the bacterial lectins (Silverblatt and Cohen, 1979; Silverblatt *et al,* 1982; Abraham *et al,* 1985) or to the lectin receptor (Abraham *et al,* 1985). In this study, the virulence of the *C. jejuni* strains (adherence and invasion potential) was significantly reduced by the L-fucose in an *in vitro* model (HeLa cell). The effect of L-fucose on the colonizing capability of the gastrointestinal mucosa of experimental animals has not yet been carried out. Such studies directed towards identification of effective inhibitors of adherence and subsequent invasion *in vivo* may help in the design of therapeutic agents that may be useful in preventing infection.

5.9.6 Inhibition of HeLa Cell Adherence and Invasion by Specific *C. jejuni* Antiserum

Antisera raised against whole cells and / or different surface structures have been used to probe the nature of the cell-surface components responsible for epithelial cell adherence and invasion in different studies. Infection of corneal cells in cell culture by *Shigella* spp. was decreased by immune sera (Ogawa *et al,* 1967) and Bhogale *et al* (1983) found that antisera against live cells of *S. flexneri* inhibited invasion of HeLa cells by the homologous strain by approximately 90 %. Rabbit immune serum and antibody in bovine colostrum has been reported to inhibit invasion of HeLa cell by a calf strain of *E. coli* (Okabe *et al,* 1983). Similar studies have been performed with other bacterial pathogens such as *Y. enterocolitica* (Okamoto *et al,* 1980) and reduction of adherence to epithelial cells was noted. Recently, Ewanowich *et al* (1989) reported approximately 95 % inhibition of invasion of HeLa cells by *Bordetella pertussis* antisera. Generally antibodies decrease the adherence and subsequent invasion of epithelial cells and the slight increase in invasion of HeLa cells by *S. flexneri* in the presence of immune serum observed by Hale and Bonventre

(1979) was attributed later to the use of a relatively larger inoculum in comparison to the titre of the test antiserum (Bhogale *et al*, 1983).

In this study, antiserum raised against formalinized bacteria (*C. jejuni* strain NCTC 11168) significantly reduced the adherence and invasion of HeLa cells by the homologous and heterologous strains (Figures 70 and 71). The antiserum raised against the heat-killed cells exhibited only a slight inhibitory effect which was not significantly different from the control normal rabbit serum used, indicating the probable involvement of the heat-labile surface component(s) (presumably proteinaceous in nature) of *C. jejuni* in the interaction with cultured epithelial cells *in vitro*. The importance of heat-labile surface components in adherence and invasion has been demonstrated for several bacterial pathogens. As specific antiflagella antiserum was not used in this study it remains uncertain whether the flagella or outer membrane proteins were involved. Sherman and Soni (1988) found that antisera against OMPs, but not flagella, could effectively inhibit the adherence of vero-cytotoxin producing *E. coli* of serotype 0157 to human epithelial cells in tissue culture. With antisera against live cells and the same serum absorbed with boiled cells Bhogale *et al* (1983) indicated that heat-labile surface components were important in the adherence and invasion of HeLa cell by *S. flexneri*. Immunization of guinea pigs and rabbits with outer membrane proteins (OMP) of *S. flexneri* but not with heat-killed cells resulted in protection of the animals in the Sereny test, which indicated the importance of heat-labile surface antigens in the protection of invasion in an *in vivo* system (Adams *et al*, 1980). The slight reduction of invasion by the antiserum raised against the heat-killed organisms observed in this study may be due to steric hindrance as speculated by Bhogale *et al* (1983) in explaining the partial reduction of the invasion of HeLa cell by *S. flexneri* with antisera raised against heat-killed cells.

Different explanations have been proposed to explain the observed inhibition of the association of pathogenic microorganisms with epithelial cells by antisera. According to Ogawa *et al* (1967), the inhibitory effect of the antisera on

epithelial cell invasion by microorganisms may be partially due to the agglutination of the bacteria by the antisera. Another attractive theory is that the interaction of the bacterial cell-surface and epithelial cell is mediated by recognition of specific receptor molecule on the epithelial cell-surface by a specific ligand molecule on the bacteria and the effect of antibody may be assumed to be due to neutralization of it (Beachy, 1981). Antibodies were also reported to alter the physicochemical properties of the bacteria (Stendahl *et al*, 1974) which might influence the interaction with epithelial cells and hence the inability of the antibody-coated bacteria to invade epithelial cells would not reflect any type of specific neutralization. In this study, as the antisera raised against the heat-stable surface components of *C. jejuni* virtually failed to inhibit invasion, the possibility of such a nonspecific inhibitory effect could be ruled out. The significant reduction observed with the antisera raised against formalinized bacteria (presumably against the heat-labile protein components of the cell surface) provides further evidence that antibody inhibition involved a specific neutralization of a heat-labile surface component of *C. jejuni*.

5.9.7 Inhibitory Effect of Chicken Intestinal Mucus

The mucous layer covering the intestinal epithelium is a substantial structure consisting of a heterogeneous mixture of proteins and very large molecular weight glycoproteins called mucins secreted from the epithelial goblet cell (Clamp, 1977; Rozee *et al*, 1982). It represents an important defence system in the gastrointestinal tract (Forstner *et al*, 1986). As with other enteropathogens *C. jejuni* must penetrate the mucous blanket to reach the underlying epithelial cells where they can invade the cells (in the case of strains causing dysentery-like diarrhoea) or adhere to the cells and release the toxin inside (in the case of the strains causing cholera-like secretory diarrhoea). So whatever the pathogenic mechanism of the *C. jejuni* diarrhoea, interaction with the mucus presumably plays an important initial role.

In the case of *V. cholerae*, the intestinal mucous blanket constitutes a

physical barrier to the pathogens and the motile strains guided by chemotactic stimuli can penetrate the mucus to adhere to the intestinal epithelium (Freter, 1988). Leitch (1988) reported that cholera toxin (CT) induced mucus secretion and thickening of the mocus blanket in rabbit ileum *in vivo* and speculated that it may represent an attempt by the host to protect itself against infection by the cholera vibrios. Since CT has been found to enhance the colonization potential of the *V. cholerae* strains in the rabbit model (Pierce *et al*, 1985), binding of the CT by mucin leading to its inhibition may represent another aspect of the protective capacity of mucus against infection by *V. cholerae.* Moreover, trapping of the bacteria within the mucus gel facilitates their removal from the intestinal tract by peristalsis (Magnusson and Stjernstrom, 1982).

C. jejuni strains were reported to adhere to rabbit intestinal mucus immobilized on polystyrene plates and isolated LPS was also able to bind to the mucus (McSweegan and Walker, 1986). These results and the report by Dinari *et al* (1986) that guinea-pig intestinal mucus could inhibit the invasion of HeLa cells by *S. flexneri* led to experiments which investigated whether mucus gel would interfere with the adherence and invasion of HeLa cells by *C. jejuni* strains. Chicken intestinal mucus was selected as the test mucus assuming that as chickens are generally colonized by *C. jejuni* (Hood *et al*, 1988), chicken intestinal mucus might play a role in preventing the overt disease in chickens. It was found that an overlay of chicken intestinal mucus inhibited adherence and subsequent invasion in a concentration-dependent manner (Figure 73) and highly significant ($P < 0.001$) reduction in adherence and invasion potential was noted with all the strains tested in this study (Figure 74) with mucus concentration of 250 $\mu g\ ml^{-1}$. McSweegan *et al* (1987) reported that rabbit small intestine mucus impeded the attachment of *C. jejuni* strains to a monolayer of INT 407 cells and mucus from rabbits previously colonized with *C. jejuni* completely inhibited adherence to underlying cells. The results obtained in this study were confirmed by those obtained by McSweegan *et al* (1987) and also in agreement with the general consensus that intestinal mucus acts as a barrier to adherence and invasion of epithelial cells *in vitro.*

Recently, De Mol and Pechere (1988) reported that invasion of HEp-2 cells by *C. jejuni* strains was enhanced by bovine submaxillary mucin (BSM). This invasion enhancing effect of the BSM was strictly concentration-dependent: a concentration of 100 μg ml^{-1} caused maximal enhancement which decreased dramatically when the concentration of BSM was either increased or decreased. The invasion-enhancing effect of mucin was strain dependent, but all the strains that were positive in this test exhibited the critical requirement of 100 μg ml^{-1} mucin for maximal activity. The results obtained in this study and subsequently those of McSweegan *et al* (1987) (that mucus impeded interaction of *C. jejuni* strains with the underlying cell monolayer) are in total disagreement with those obtained by De Mol and Pechere (1988) (that mucus enhanced interaction of *C. jejuni* with underlying cell monolayer). In fact, the authors themselves (De Mol and Pechere, 1988) described their results as 'new' and 'somewhat unexpected' but did not explain or speculate about the reasons for this apparent discrepancy. The use of different strains, cell lines and source of mucin can be put forward as possible explanations. But another point that should be given appropriate consideration is that the interaction of *C. jejuni* strains with HEp-2 cell monolayer is somewhat peculiar. Bukholm and Kapperud (1987) reported that *C. jejuni* strains by themselves were incapable of invasion of HEp-2 cells (and also A-549 cell line) but coinfection with enteroinvasive *Salmonella, Shigella* and *E. coli* and also with a non-invasive *E. coli* exerted an effect which rendered *C. jejuni* strains capable of intracellular location in epithelial cells. Sterile culture filtrate from cell cultures inoculated with *S. typhimurium* or *S. typhimurium* plus campylobacters were not capable of inducing *C. jejuni* internalization and internalization of coinfectants was not essential as many cells contained only campylobacters. Whereas there is ample documention on the invasion of epithelial cell lines, particularly of HeLa cells, by *C. jejuni* strains without the requirement of an invasive coinfectant (Newell *et al,* 1985b; Fauchere *et al,* 1986; Manninen *et al,* 1982). Examples of host cell specificity by invasive enteropathogens exists in the literature. Miller and Falkow

(1988) reported that cloning of two *Y. enterocolitica* chromosomal loci *inv* and *ail* in *E. coli* HB101 conferred an invasive phenotype on this innocuous organism. The cloned *ail* locus allowed invasion to a variable degree of some cell lines (HEp-2, HEC1B and CHO cells) but no invasion of Madin-Darby canine kidney cells), while the *inv* locus permitted a uniformly high level of invasion in these cell lines. Whether this unusual mode of interaction of *C. jejuni* with HEp-2 cells could in some way be related to the reported invasion enhancement by BSM is not apparent at present.

In an interesting series of investigations, Cohen *et al* (1983, 1985) reported that the ability of a human faecal isolate of *E. coli* to adhere to mouse intestinal mucus immobilized in polystyrene wells *in vitro* correlated with the ability to colonize the mouse intestine. They also succeeded in identifying a glycoprotein as the receptor for the *E. coli* strain in the mouse mucus gel. In this study, it was observed that chicken intestinal mucus reduced the interaction of *C. jejuni* strains with HeLa cells . Whether the chicken intestinal mucus contained a specific receptor for *C. jejuni* analogous to that has been found for *E. coli* in mouse mucus (Cohen *et al*, 1985) is unknown. It is not known whether this observed reduced interaction of the *C. jejuni* strains due to the presence of the mucus blanket with the underlying HeLa cell monolayer is due to the presence of specific receptor or alternatively because it presents a physical barrier. It would be interesting to know whether such receptors are present for *C. jejuni* in the mucus preparation from other animals including man.

The fact that chicken intestinal mucus reduced the adherence and invasion of HeLa cells by the *C. jejuni* strains can also be discussed from another point of view. Dinari *et al* (1986) reported that colonic mucus from guinea-pigs but not from monkeys inhibited the invasion of HeLa cell monolayer by *S. flexneri* strains. As monkeys are more susceptible to the *Shigella* infection than guinea-pigs, the authors speculated that monkey mucus possibly lacks invasion-inhibition properties which are not present in guinea-pig mucus. A similar hypothesis can also be put forward to

explain the high carriage of *C. jejuni* strains in the chicken gut. As non-immune chicken intestinal mucus partially inhibited invasion of HeLa cells by *C. jejuni*, it may be assumed that chicken intestinal mucus does possess an invasion inhibiting factor which might be absent in susceptible host of *C. jejuni* such as man. These are definitely interesting research aspects which might lead to the discovery of many unknown facts about the host-pathogen interaction in *C. jejuni* infection.

5.9.8 MOMP as a Putative Adhesin for *C. jejuni*

Although flagella and LPS have been implicated as probable adhesins of *C. jejuni* for eucaryotic cells (McSweegan and Walker, 1986), the role of OMPs in such processes was not investigated. Having developed a mild procedure for isolation of the MOMP in highly purified form, it was decided to investigate whether this protein acted as an adhesin in the adherence and invasion process of *C. jejuni* strains in the HeLa cell model. Preincubation of HeLa cells with OMP and also with MOMP significantly reduced adherence and invasion of HeLa cells by a homologous strain and a heterologous strain of *C. jejuni* (Figure 75 and 76) indicating that the MOMP molecule might act as an adhesin. But as this protein is present on the cell-surface of all the *C. jejuni* strains, it raises question of how this protein might be involved in the differential adherence and invasion capability of different strains observed in this study. Studies with monoclonal antibodies against the outer membrane proteins of *V. cholerae* showed that the conformation of particular surface antigens may vary among strains (Sciorino *et al*, 1985). Similarly, it may be assumed that the MOMP of *C. jejuni* may have a different conformation in different strains resulting in differential adherence and invasion potentials. However, MOMP only partially inhibited the adherence and invasion of the *C. jejuni* strains indicating the involvement of other undefined cell-surface components, which may be differentially expressed in different strains. For example, the flagella of *C. jejuni* can undergo antigenic variation (Harris *et al*, 1987; Logan *et al*, 1989) which might affect the role (if any) played by

flagella in the process of adherence and subsequent invasion. Moreover, the LPS of certain *C. jejuni* strains may exist in structurally variable forms (Penner *et al,* 1987) which might also differentially interfere with the adherence process. So the adherence of the *C. jejuni* strains to epithelial cells may be considered as a multi-component process, contributory role played by different participating surface components may vary among different strains. This is the first report that the MOMP of *C. jejuni* can act as an adhesin for *C. jejuni.* The result obtained in this study that the MOMP of the *C. jejuni* strains may bind to eucaryotic cells resulting in subsequent reduction of adherence and invasion of *C. jejuni* has been supported in part by a recent report of Moser and Hellmann (1989). They found that in addition to LPS and flagella, trypsin-resistant surface proteins of some *C. jejuni* bound to isolated murine small intestinal cells and to a membrane fraction of the isolated brush borders which was determined by ELISA. Flagella and LPS which were implicated as adhesins of *C. jejuni* in previous studies (Cinco *et al,* 1984; McSweegan and Walker, 1986) were found to mediate only 10-33 % and 10 % respectively of the total binding of different strains. However, binding of the surface proteins of certain *C. jejuni* strains was hardly influenced by trypsin treatment while the binding of some strains was reduced by 45-85 % after trypsin treatment. In this study, it was found that MOMP is the only trypsin-resistant protein (Figure 40), so it may be assumed that this surface protein was involved in the binding of the trypsin-insensitive surface protein components of *C. jejuni* in the study of Moser and Hellman (1989).

OMPs have been found to contribute to pathogenesis and immunity in several pathogenic microorganisms. Monoclonal antibodies directed against an *Haemophilus influenzae* type b outer membrane protein was protective against experimental *Haemophilus* disease (Hansen *et al,* 1982). Outer membrane proteins are considered to be involved in the intestinal adhesion and subsequent

colonization of enteric microorganism. Sengupta *et al* (1989) found that antiserum raised against OMP of *V. cholerae,* which was absorbed with LPS inhibited the adhesion of homologous and heterologous strains of *V. cholerae* to rabbit intestinal tissue sections. In addition, passive protection against challenge with *V. cholerae* in the rabbit intestinal loop by antiserum to OMP was also noted. Prophylactic significance of non-LPS components of *V. cholerae* 01 was also noted by Sharma *et al* (1987) in the infant mouse model. Sherman and Soni (1988) found that outer membrane proteins of vero cytotoxin-producing *E. coli* of serotype 0 157: H7 inhibited adherence of the pathogen to human epithelial cells in tissue culture in a concentration dependent manner. These studies clearly indicate the importance of OMP in the disease process.

5.9.9 Inhibition of Invasion by Cytochalasin B

Cytochalasin B is a fungal secondary metabolite (produced by *Helminthosporium dematioideum*) which can suppress the phagocytic activity of polymorphonuclear cells and macrophages by disruption of actin polymers of the subplasmalemmal microfilaments (Allinson *et al,* 1971; Davies *et al,* 1973). Several studies have indicated that this compound has a similar effect on the nonprofessional phagocytes such as HeLa cells and inhibit internalization of a variety of microorganisms by such cells (Kihlstrom and Nilsson, 1977; Hale *et al,* 1979; Bukholm, 1984; Barrow and Lovell, 1989). Pathogenic microorganisms enter mammalian cells either by penetration or by phagocytosis (Flow diagram 1). During penetration the microorganism participates in an active fashion and the metabolic state of the infected cell is not important e.g. penetration of HeLa cells by *Toxoplasma gondii* (Lycke *et al,* 1975). Whereas in the process of phagocytosis is an energy dependent process which may be inhibited by the treatment of the phagocytic cells with the inhibitors of glycolysis such as iodoacetate (Jones, 1975). The metabolic state of the bacterial cell has also been

found to be critically important in the internalization process by nonprofessional phagocytes as heat-inactivation, ultraviolet irradiation and treatment with various inhibitors of metabolism affected this process (Hale and Bonventre, 1979; LeChevallier *et al,* 1987). Surface properties of the bacteria were also important as mutants deficient in various surface components such as flagella and LPS (Mroczenski-Wildey *et al,* 1989) exhibited a reduced capability to interact with mammalian cells. And the term 'receptor mediated endocytosis' has been coined to describe the internalization of microorganisms by nonprofessional phagocytes in a process similar to true phagocytosis.

Use of Cytochalasin B has enabled researchers to determine whether the entrance of the pathogen into the mammalian cells occurs by penetration or by a receptor mediated endocytosis. As there was no report on the effect of cytochalasin B on the invasion process of mammalian cells by *C. jejuni,* this experiment was initiated to determine in the HeLa cell model whether internalization of the *C. jejuni* occurs by penetration or by receptor mediated endocytosis as has been found with other enteropathogens such as *Shigella* (Hale *et al,* 1979), *Salmonella* and *Y. enterocolitica* (Bukholm, 1984). In this study, Cytochalasin B at concentrations 0-10.0 μg ml^{-1} were used to see whether the association of *C. jejuni* strains with HeLa cells was inhibited by this compound. The fact that at a concentration of >5.0 μg ml^{-1} invasion was inhibited completely but large numbers of bacteria were found to be adhered to the HeLa cells (which was not significantly different from the untreated controls) indicate that this compound specifically interferes with the process of invasion, leaving adherence unaffected. Suppression of HeLa cell invasion by cytochalasin B shows that for invasion by *C. jejuni* to occur, activation of the cellular microfilament is essential as has been found for other enteropathogens.

Previous researchers have used various time periods of incubation of the host cells with cytochalasin B for optimal effect of the drug on the invasiveness of the microorganisms. Such studies have revealed that the invasiveness of *S.*

flexneri, *S. typhimurium* and *Y. enterocolitica* are time and dose dependent. A concentration of the drug 0-20.0 μg ml^{-1} was found to be suitable for most bacteria. Some investigators used short pulses (15 min for enteropathogenic *E. coli* for HEp-2 cells; Andrade *et al*, 1989; immediately before inoculation of bacteria for *S. typhi* in HeLa cells; Mroczenski-Wildey *et al*, 1989) while others used longer incubation periods; 2 hr for *Salmonella* spp. and *Shigella* spp. in Vero cells, Barrow and Lovell, 1989; 12-18 hr *Shigella flexneri*, *S. typhimurium* and *Y. enterocolitica*, Bukholm, 1984. In this study, the drug and bacteria were added concomitantly to the HeLa cell according to Hale *et al* (1979) for *S. flexneri* and was found to be effective in preventing invasion. However, the preincubation period involved and the concentration required is not only determined by the bacterial factor, growth phase and degree confluence of the mammalian cells are also important (Bukholm, 1984).

The results obtained show that HeLa cells have the ability to internalize viable *C. jejuni* by a process which can be inhibited by cytochalasin B (Figure 77), but the number of adherent bacteria was not significantly affected. Similar results on the effect of cytochalasin B on the adherence has been found for other bacteria such as enteropathogenic *E. coli* (Andrade *et al*, 1989), *S. flexneri*, *S. typhimurium* (Barrow and Lovell, 1989) and *Y. enterocolitica* (Schiemann *et al*, 1987) and occasionally even an increase in adherence was also noted (Kihlstrom and Nilsson, 1977). Cytochalasin B alters the expression of ionized sialic acid residues resulting in reduced charge density of the mammalian cell surface and the occasional increased adherence may be due to the reduced electrostatic repulsive forces between the negatively-charged bacteria and the cytochalasin B treated mammalian cells (Kihlstrom and Nilsson, 1977).

Although the full significance of the findings of this study require further evaluation, the fact that the invasion of HeLa cells by *C. jejuni* can be inhibited by cytochalasin B implies that this pathogen involves a mechanism

which is partially similar to those adopted by other invasive enteropathogens.

5.9.10 Survey of Strains

All the strains included in this study exhibited to some degree the capacity to adhere and to invade the HeLa cell monolayer (Figures 66 and 67). This was not entirely unlikely as the strains were all from clinical sources (Table 5). However, there was a considerable degree of variability among strains in terms of their adherence and invasion potential. In general, the Group D strains (strains from dysentery-like, mucoid diarrhoea cases) were more invasive and adhered more avidly than the Group C strains (from cholera-like, watery diarrhoea cases). There was some degree of overlap among strains belonging to the two groups in their adherence and invasion potentials. Manninen *et al* (1982) also observed variable degree of adherence and invasion capacity of 14 *C. jejuni* strains isolated from human beings and other animals. The results obtained in the present study are in agreement with those obtained by Fauchere *et al* (1986) who reported that clinical *C. jejuni* strains differentially adhered to HeLa cells. However, they also reported that a proportion of the strains failed to adhere sufficiently to be positively scored in their assay. But when the invasive potential of a strain considered to be negative by Fauchere *et al* (1986) is expressed as in this study, it appeared to be positive (Appendix 5). It should also be noted that the assay technique used by Fauchere *et al* (1986) i.e. counting of adhered bacteria by light microscopy is different from that used in this study.

As none of the previous reports investigating the adherence and invasion potential of the *C. jejuni* strains employed the quantitation of c.f.u. method after lysis of the epithelial cells, the results obtained in this study cannot be compared directly with those of others (Cinco *et al,* 1984; Newell *et al,* 1985b; McSweegan and Walker *et al,* 1986; Fauchere *et al,* 1986). As high as 12.3

% of the inoculated bacteria of strain S-11, (a Group D strain with the highest invasion potential; Figure 67) were able to invade the HeLa cells whereas only 1.68 % of the strain B-23 (a Group C strain with the lowest invasion potential, Figure 66) inoculum were internalized. Similar studies with other bacteria have shown that 26.8 % of *Y. enterocolitica* in HEP-2 cells (Miller and Falkow, 1988), 50.0 % of *Y. pseudotuberculosis* in HEp-2 cells (Isberg *et al*, 1987), 18.3 % of *S. flexneri* in HeLa cells (Bhogale *et al*, 1983), 16.0 % of enteroinvasive *E. coli* in HEp-2 cells (Small *et al*, 1987) and 50.0 % of group G streptococci in HeLa cells (Nath, 1989) were internalized. The adherence potential of the *C. jejuni* strains (range 11.00 for strain B-23 to 36.00 for the strain S-11) also compares favourably with those of other enteropathogens. Chart *et al* (1988) observed that enteropathogenic *E. coli* (EPEC) adhered to HEp-2 cells in numbers of at least 50 bacteria per eucaryotic cell. Percent of Y. enterocolitica inoculum which became cell-associated varied from 67 % with HEp-2 cells to 51 % with CHO cells (Miller and Falkow, 1988).

With immunofluorescence techniques with or without fixation of the HeLa cells in acetone (to determine both intracellular and surface-associated bacteria or only surface-associated bacteria, respectively), Newell *et al* (1985b) demonstrated that approximately 3.1-3.5 % of the inoculated *C. jejuni* (from human clinical cases) were internalized. This is somewhat low in comparison to the values obtained in this study (1.2-12.2). Use of different techniques and strains of *C. jejuni* may probably explain this discrepancy. Another point of considerable importance is that in this study the microaerophilic gas atmosphere (5 % O_2, 10 % CO_2, 85 % N_2) was used during the infection period of HeLa cells by *C. jejuni* strains, whereas 5 % CO_2 and 95 % air was used by Newell *et al* (1985b). Microaerophilic gas atmosphere was found to be critically important for the expression of maximal invasion potential of the *C. jejuni* strains which has been discussed earlier (Section 5.9.3). The result of this study however compares favourably with those obtained by Bukholm and Kapperud

(1987) in which the infection of the HEp-2 cells by *C. jejuni* strains were carried out in a microaerophilic gas atmosphere. Microscopic examination of the infected cells revealed that the number of intracellular bacteria per Hela cell varied from 3.2 to 17.0 depending upon the strain. But it should be mentioned that *C. jejuni* strains by themselves were not invasive, coinfection with enteroinvasive *E. coli, Salmonella* and *Shigella* were necessary to induce invasion (Bukholm and Kapperud, 1987). Another point of definite relevance is that several authors (Gemski and Formal, 1975; Brodeur *et al,* 1977) have cautioned that light microscopy assays for detecting invasion of mammalian cells may not distinguish between intracellular and adherent extracellular bacteria which was used by Newell *et al* (1985b) and Bukholm and Kapperud (1987) for evaluating the invasion potential of the *C. jejuni* strains.

When the adherence and invasion potential of the strains investigated are related to the clinical history of the respective strains, it appears that the results obtained in this study are not in complete agreement with those obtained by Klipstein *et al* (1985) who reported that invasiveness was only exhibited by the strains obtained from dysentery-like mucoid diarrhoea cases, whereas the strains from cholera-like secretory diarrhoea cases and from asymptomatic carriers were totally non-invasive. The techniques used by them for determining invasiveness were ELISA (based upon the presence of a common antigen among invasive strains) and a direct immunofluorescence examination of the histological sections obtained from rat ileal loops. This discrepancy can only be explained by the use of different strains and techniques. Several recent studies have indicated that strains from healthy laying hens, asymptomatic carriers, even environmental isolates possess different putative virulence-associated properties including the capacity to invade epithelial cells and the production of enterotoxin (Newell *et al,* 1985b; Belbouri and Megraud, 1988; Lindblom *et al,* 1989; Fricker and Park, 1989). No correlation was observed between the clinical symptoms and the

serovars of the strains based on either heat-stable (Penner and Hennessy, 1980) or heat-labile (Lior *et al*, 1983) of the *C. jejuni* indicating no involvement of any serogroup surface antigen specifically contributing to the virulence of the pathogen (Kaijser and Sjogren, 1985). No significant correlation was also noted between enterotoxin production and serogroups of *C. jejuni* (Lindblom *et al*, 1989). So as there was no correlation between clinical history of the strains and their serogroup (Kaijser and Sjogren, 1985) and again no correlation between serogroups of the strains and enterotoxin production (Lindblom *et al*, 1989), it may be indirectly concluded that there was apparently no correlation between the clinical history of the strain and enterotoxin production. So in view of the results obtained in this study, and the large numbers of reported investigations, it appears that it is premature to categorize the pathogenic *C. jejuni* strains strictly as enterotoxigenic and enteroinvasive and to consider the strains from asymptomatic carriers as nonpathogenic as described by Klipstein *et al* (1985) who investigated only a few strains.

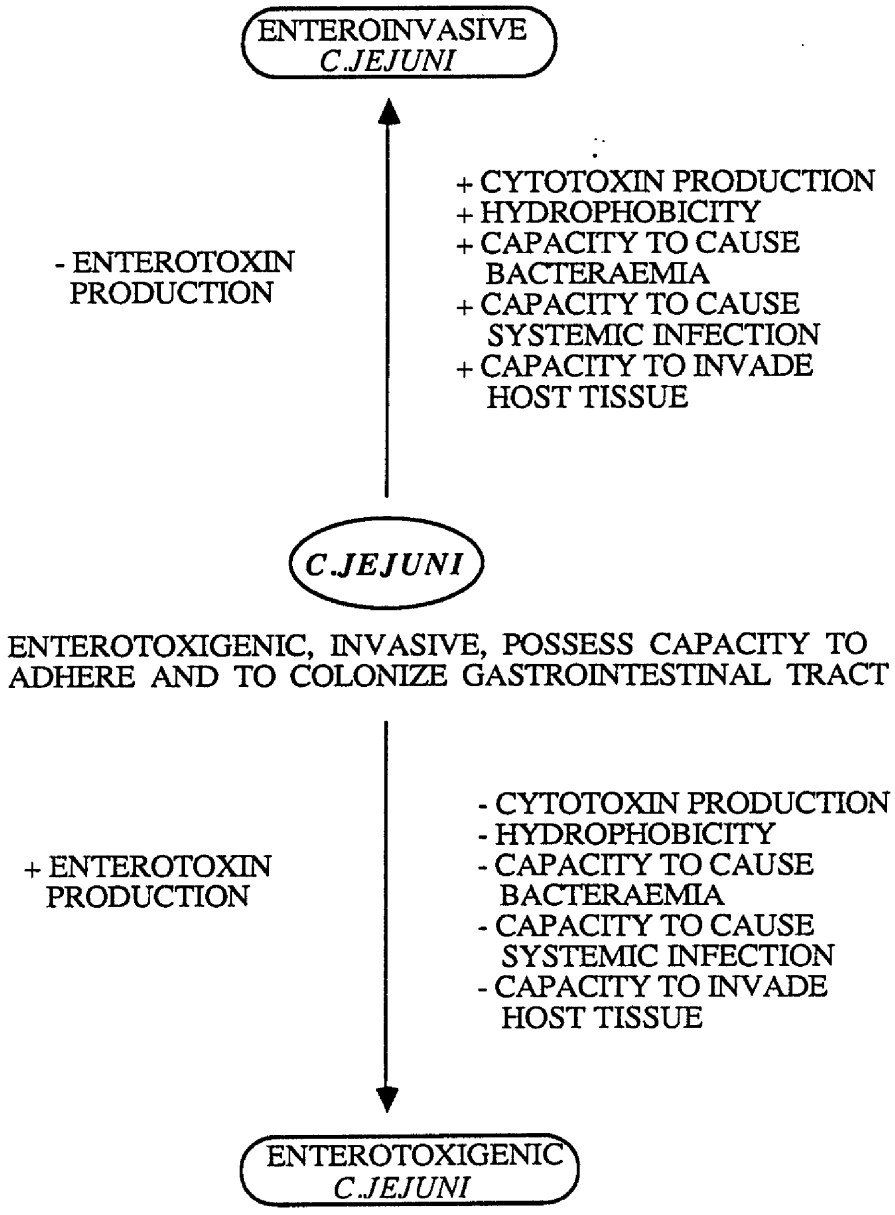
General Discussion

The results presented in this thesis indicate that the virulence of *C. jejuni* is probably multifactorial as is the case with many other pathogenic microorganisms (Smith, 1988; Finlay and Falkow, 1989). In comparison to other diarrhoeagenic microorganisms, the pathogenic mechanism of *C. jejuni* appears to be more complex. It is apparent from the results obtained in this study that adherence, invasion and enterotoxin production are essential virulence characteristics of the *C. jejuni* strains. The presence of a haemolysin has been demonstrated but this seems to be at most an ancillary virulence characteristic.

Unlike *V. cholerae* and enterotoxigenic *E. coli* (ETEC) in which the production of enterotoxin plays the vital role in pathogenesis or *Shigella* spp. and enteroinvasive *E. coli* (EIEC) in which the ability to invade eucaryotic cells is the central virulence property, both these characteristics may be associated with virulence in different strains of *C. jejuni.* But some of these virulence characteristics are probably expressed to a lesser degree in *C. jejuni,* for example, severe watery diarrhoea frequently observed with the cases of *V. cholerae* and ETEC infection is rarely seen in *C. jejuni* infections, and invasion of the enterocytes in some human cases is less dramatic than found with *Shigella* spp. or EIEC. In this regard, *C. jejuni* resembles more closely *Salmonella* spp., in which both the production of a cholera-like enterotoxin and invasion of the epithelial cells has been described to be associated with virulence. A fully virulent *C. jejuni* is thus both enterotoxigenic and invasive; however, strains devoid of invasiveness or enterotoxin production may also be virulent as they were able to cause overt disease in man and positively scored in variety of *in vivo* and *in vitro* assays of virulence-associated properties (Flow Diagram 4). The interplay of the various virulence-associated properties in the outcome of infection and clinical presentation of disease is not clear at present.

Early in this study, the presence of a soluble haemolysin was clearly

Flow Diagram 4: Virulence of *C. jejuni*



demonstrated. This showed a broad species spectrum of activity, although the haemolytic titres were comparatively low in relation to the known values for organisms where a cytolysin is a principal virulence factor. The order of erythrocyte sensitivity was rabbit > human > chicken. It is interesting that the chicken which has nucleated erythrocytes showed little evidence of haemolysis. This might be a possible explanation for the near commensal relationship between *C. jejuni* and the adult chicken.

Although the conclusion above was that the haemolysin was a minor virulence characteristic, it must not be forgotten that the cultural conditions available for *C. jejuni* are undoubtedly ill-defined and probably suboptimal for the efficient production of haemolysin. There is a need to extend the haemolysin study in order to purify and characterize the protein involved. It was possible to show by ammonium sulphate precipitation of the culture supernate that concentration of a protein could be achieved. However, it was considered that the time involved to establish optimum conditions for production of the haemolysin ruled out further investigation within the framework of this thesis.

The haemolysin was also shown to have cytotoxic activity against HeLa cells, this might indicate a more important role in the virulence mechanism but further work would require the use of different cell lines and a purified haemolysin preparation.

There was clear evidence from the results with HeLa cell monolayers that virulence for the chick embryo was associated with the capacity to adhere to and invade the cells. When all the clinical isolates (i.e. Group C and Group D strains) were considered, the correlation coefficient for adherence : invasion was, $r = 0.8604$ (Figure 78). For Group C strains, the r value was 0.8078 (Figure 79) but for Group D strains it was 0.9512 (Figure 80). This adds weight to the division of the strains into dysentery-like bloody, mucoid diarrhoea causing strains (Group D) since it is established that these characteristics (adherene and invasion) are

essential for *Shigella* spp. to cause infection.

The relationship between these characteristics was even more pronounced when the correlation coefficient between chick embryo lethality versus adherence to HeLa cells was considered, r = -0.9662 (Figure 81). Similarly, with invasion of HeLa cells the value was -0.9791 (Figure 82). The 11-day-old chick embryo model was excellent for the determination of the relative virulence of the clinical isolates of *C. jejuni*. The LD_{50} values of the strains tested accurately reflected the relative virulence of these strains determined by other assays. A high degree of correlation between the LD_{50} values of the *C.jejuni* strains in the 11-day-old chick embryos and the HeLa cell adherence (r = -0.9662) and invasion (r = -0.9791) indicate that these *in vitro* assays may be considered as an indicator of virulence in addition to the *in vivo* test. This close correlation indicate that the more convenient HeLa cell assay may be used to determine the relative virulence of the *C. jejuni* strains instead of the time-consuming chick embryo model and reduce the numbers of experimental animals used.

In this study, cell-surface hydrophobicity of the *C. jejuni* strains showed a lower correlation coefficient with adherence to HeLa cell, r = -0.7609 for SA test (Figure 84) and r = 0.8273 for the BATH test (Figure 85). The r values between invasion of HeLa cells and hydrophobicity were -0.7928 and 0.8140 for SA test and BATH test (Figures 86 and Figure 87) respectively.

No correlation was observed, however, between the enterotoxigenicity of the *C. jejuni* strains (determined by ELISA and CHO cell assay) with any of the other tests such as HeLa cell adherence (r = -0.3594 for adherence : CHO cell assay; Figure 89 and r = -0.2307 for adherence : ELISA, Figure 90) and invasion (r = -0.5036 for invasion : CHO cell assay, Figure 91 and r = -0.3718 for invasion : ELISA, Figure 92) assay; virulence test in the 11-day-old chick embryo model (r = 0.5184 for CHO cell assay, Figure 93 and r = 0.4379 for ELISA, Figure 94) or the cell surface hydrophobicity (determined by SA test and

BATH test) of the *C. jejuni* strains. This is not entirely unexpected as toxin is a bacterial product which usually acts independently of the bacterial cells *in vitro* and the above mentioned virulence characteristics are associated with bacterial cell surface properties. The correlation coefficient between fluid accumulation in the new-born chick diarrhoea model and enterotoxin production by *C. jejuni* strains as determined by CHO cell assay and ELISA were r = 0.7356 (Figure 95) and r = 0.8406 (Figure 96) respectively indicating good correlation of the *in vivo* test with the *in vitro* tests.

The production of an enterotoxin by an invasive enteropathogen raises the question of its significance and at what stage of infection the enterotoxin might exert its deleterious effect on the intestinal mucosa. As the enterotoxin increases the intracellular cAMP concentration (Ruiz-Palacios *et al,* 1983), it can also be questioned whether the toxin alters the cAMP levels before or after epithelial cell invasion. In this regard, invasion of epithelial cells by the bacteria may provide a very efficient mechanism of delivery of the small amount of enterotoxin produced by the *C. jejuni* strains to the intestinal epithelial cells resulting in substantial alteration in the water and electrolyte balance during intestinal infection.

It is clear that certain *C. jejuni* strains do produce an enterotoxin which is immunologically similar to cholera toxin (Figure 11) but in much smaller quantities. The evidence appears to indicate, as with *E. coli* LT, a poor mechanism of secretion. Furthermore, more convenient procedures are required to culture *C. jejuni* in the laboratory so that detailed investigations of the enterotoxin may be pursued.

Many bacterial factors contribute to the likeihood that a host-pathogen interaction will result in disease. There are also multitude of host factors which contribute to determination of outcome of an infection (Mims, 1987). So the virulence of the *C. jejuni* strains should be considered as the product of many

interacting variables involving the bacterium and the host. Although several tests were employed in this study to detect the putative virulence factors of *C. jejuni*, it cannot be assumed that these tests are sufficient to evaluate all the characteristics associated with the pathogenic personality of the *C. jejuni*. Virulence characteristics of enteric bacteria can be extremely diverse (Rowe, 1979) and the possibility of yet unrecognized virulence properties in a bacterium like *C. jejuni* which is a versatile pathogen capable of causing two clinically distinct forms of enteritis and in addition various systemic diseases (Table 3) cannot be ruled out.

A major feature of this study has been the investigation of a number of virulence determinants with fresh clinical isolates and not with old laboratory cultures. This has allowed the investigation of isolates from mild to severe cases of diarrhoea or enteritis. As far as is known this is the first time that such a wide variety of tests have been used to assess the virulence of *C. jejuni*.

Recently methods for gene transfer from *C. jejuni* to other bacteria and vice-versa has been described (Lebigne-Roussel *et al*, 1987). Transposon mutagenesis and electroporation techniques have also been established for *C. jejuni* (Lebigne-Roussel *et al*, 1988; Martin *et al*, 1988). Application of these techniques will enable researchers to address at the molecular level, the contribution of each virulence determinant in the causation of disease.

Conclusions

* There seems to be little doubt that a process of adherence and invasion is of prime importance in the establishment of a *C. jejuni* infection. A considerable amount of further work is required to characterize the mechanisms of adhesion and invasion.

* The virulence-associated properties of the *C. jejuni* strains do not always correlate with the clinical history of the strains i.e. production of enterotoxin or invasion of host cells may not be the sole virulence-associated property of the strains which were originally isolated from watery diarrhoea and mucoid diarrhoea cases respectively.

* All clinical isolates are not equally virulent. A gradation of virulence was observed among the strains in all the virulence assays employed which probably explains the presence or absence of various virulence-associated properties noted by different researchers investigating only few strains.

* Investigation of a single virulence property may not reflect the full pathogenic potential of the *C. jejuni* strains. Performance of a series of virulence-associated tests are necessary to establish the 'pathogenic personality' of the *C. jejuni* strains.

* The overall results of this study leads to the general conclusion that none of the virulence determinants studied can be considered as an absolute criterion of pathogenicity. As mentioned before, the virulence of *C. jejuni* is multifactorial, based on a scale of virulence depending on the numbers of factors expressed by the particular strain.

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7.0 APPENDICES

Appendix 1 Growth Media and Buffers

Brucella Broth:

Ingradients	per 1000 ml
Pancreatic digest of casein	10.0 g
Peptic digest of animal tissue USP	10.0 g
Dextrose	1.0 g
Yeast extract	2.0 g
Sodium chloride	5.0 g
Sodium bisulphite	0.1 g

Agar 1.5 % was added to the Brucella broth to prepare Brucella agar

Blaser-Wang Selective Supplement

Vancomycin	5 mg per vial
Polymyxin B	1,250 IU
Trimethoprim	2.5 mg
Amphotericin B	1.0 mg
Cephalothin	7.5 mg

The contents of each vial were rehydrated by aseptically adding 2.0 ml of sterile distilled water and mixing gently to avoid frothing. The antibiotic solution was then added at a concentration of 0.4 ml per 100 ml of Brucella broth containing 1.5 % (w/v) agar.

Phosphate Buffered saline (Dulbecco A)

Ingradients	per 100 ml
NaCl	0.80 g
KCl	0.02 g
Na_2HPO_4	0.15 g

KH_2PO_4	0.02 g

Phosphate-buffered saline tablets (Oxoid) were used. Each tablet was dissolved in 100 ml of distilled water and the solution was autoclaved at 121° C for 15 min at 15 p.s.i. The pH of the subsequent solution was 7.3

Appendix 2: Buffers and Solutions for SDS-PAGE

Buffers and Solutions	Per 100 ml
Acrylamide-Bisacrylamide solution	
Acrylamide	30.0 g
Bis-acrylamide	0.8 g
Upper Buffer	
Tris	6.08 g
SDS	0.4 g
Lower Gel Buffer (pH 8.9)*	
Tris	18.1 g
SDS	0.4 g
Upper Gel Buffer (pH 6.8)*	
Tris	30.0 g
SDS	0.4 g

* pH was adjusted using concentrated HCl

Running Buffer (pH 8.3)

Tris	0.303 g
Glycine	1.44 g
SDS	0.10 g

Temed (undiluted stock)

Ammonium persulphate solution*	10.0 g

* Freshly made up in small volumes (0.5 ml) before use

Solubilising Buffer for Protein

Glycerol	10.0 ml
ß-Mercaptoethanol	5.0 ml
SDA	3.0 g
Bromophenol blue	0.01 g
Tris Buffer (pH 6.8)*	to make 100 ml

* a 1:4 dilutionof the upper Buffer.

Staining Solution

Coomassie Blue R 250	0.25 g
50 % (v/v) methanol	90.0 ml
Glacial acetic acid	10.0 ml

Destaining Solution

Methanol	5.0 ml
Glacial acetic acid	7.5 ml
Distilled water	87.5 ml

Solubilising Buffer for LPS (Hitchcock and Brown, 1983)

Glycerol	10.0	ml
2-mercaptoethanol	5.0	ml
SDS	2.0	g
Sucrose	20.0	g
Bromophenol blue	0.01	g

Slab Gel Preparations

A. Lower (Separating) Gel

	12.5 %	10.0 %
Lower Gel Buffer	10.0 ml	10.0 ml
Distilled water	13.4 ml	16.6 ml
Acrylamide-bisacrylamide solution	16.6 ml	13.4 ml
After degassing for 15 min the followings were added		
Ammonium persulphate solution	200.0 µl	200.0 µl
Temed	20.0 µl	20.0 µl

B. Upper (Stacking) Gel (4.5 %)

Upper Gel Buffer	2.5	ml
Distilled water	6.0	ml
Acrylamide-bisacrylamide solution	1.5	ml
After degassing for 10 min the following were added		
Ammonium persulphate solution	30.0	µl
Temed	20.0	µl

Appendix 3 Buffers for Protein Immunoblotting

Transfer buffer*

Tris	0.303 g
Glycine	1.44 g

* Buffer made up in 20 % (v/v) methanol

Wash buffer (pH 7.5)

Tris	0.242 g
NaCl	2.92 g
Tween 20	0.1 % (v/v)

Substrate Buffer (pH 7.5)

Tris	0.242 g
NaCl	2.92 g

H_2O_2 (60 µl) was added to the above buffer followed by 4-chloro napthol (60 mg, Biorad) which was dissolved in 20 ml of methanol and added to 100 ml of the above buffer immediately before use.

Appendix 4: ELISA Buffers AND Solutions

Buffers and Solutions	per 100 ml

A. Coating Buffer (Carbonate buffer, 0.05 M; pH 9.6)

Sodium carbonate (anhydrous)	0.159 g
Sodium hydrogen carbonate	0.293 g

B. Washing / Incubation Buffer (PBS-Tween; pH 7.4)

NaCl	0.8 g
KH_2PO_4	0.02 g
$Na_2HPO_4.12H_2O$	0.28 g
KCl	0.02 g
Tween-20	0.05 % (v/v)

C. Citrate Phosphate Buffer (0.15 M; pH 5.0)

Solution X (Citric acid)	2.10 g
Solution Y ($Na_2HPO_4.2H_2O$)	3.56 g

49 ml solution X + 51 ml solution B = Citrate Phosphate buffer pH 5.0

D. Susbtrate Solution*

O-Phenylenediamine (OPD)	0.034 g
H_2O_2 (20 % v/v)	20 µl

* Made up in fresh in citrate-phospate buffer each time immediately before use. Once ready it was kept in the dark.

Appendix 5 : Calculation of Invasion potential of *C.jejuni* using the data of Fauchere *et al,* (1986).

Number of HeLa cells	$1.0x10^5$
Number of *C.jejuni*	$1.0x10^7$
AI	2.1

(AI = Number of bacteria adhered per HeLa cell)

So, $2.1X10^5$ *C.jejuni* adhered.

It is mentioned in the paper that as much as 100 % of the adhered bacteria can be internalized. So, it may be considered that $2.1X10^5$ had invaded the HeLa cell monolayer; which means that 2.1 % of the original inoculum has invaded. So the invasion potential of this strain (which was scored to be negative by Fauchere *et al,* 1986 to be nonadherent and nonpathogenic) is 2.1, a value which is higher than that of the the strain B-23; (adherence potential 1.63). So it appears although 2.1 % of the inoculated bacteria ($2.5X10^5$ cells out of $1X10^7$ had invaded the HeLa cells it was scored negative. This calculation thus shows that the quantitative bacteriological method used in this study was more sensitive than the microscopic method used by Fauchere *et al,* 1986.

Appendix-6

Antibiotic	Concentration (µg / disk)
Multodisk 725 E	
Ampicillin	2
Carbenacillin	5
Cephaloridine	10
Erythromycin	10
Kanamycin	5
Penicillin G	1.5
Streptomycin	10
Tetracycline	10
Multodisk 1788 E	
Ampicillin	25
Colistin Sulphate	10
Compound Sulphonamides	300
Nalidixic Acid	30
Nitrofurantoin	200
Streptomycin	25
Sulfamethoxazole / Trimethoprim	25
Tetracycline	50