THE ASSOCIATION BETWEEN *CAMPYLOBACTER JEJUNI* INFECTION AND GUILLAIN BARRÉ SYNDROME

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ABSTRACT

Infection with *Campylobacter jejuni*, a common enteric pathogen, may be one of the major causes of Guillain Barré Syndrome (GBS), a neurological disease of humans. Clinically the disease is characterized by acute progressive paralysis, and sometimes death. Antibodies produced in rabbits against different *Campylobacter jejuni* strains, commonly incriminated in Guillain Barré Syndrome (GBS) were tested in two experimental systems to quantify their effects on peripheral nerve function and neurons. The effect of these antiserum antibodies on peripheral nerve function was determined (*in vivo*) in Sprague-Dawley rats using electrophysiological measurements of the femoral nerve prior to injection of antiserum (Day 0) and on Days 2, 7, and 14 after injection. A two-way Analysis of Variance (ANOVA) indicated a significantly ($p<.05$) higher threshold Compound Action Potential (TCAP) on Days 2 and 7 in treatments vs controls. These electrophysiological changes were not present 14 days post innoculation. The results indicated that serum antibodies against certain strains of *C. jejuni* affect peripheral nerve function and react immunologically in particular neural cell lines. To test the effects of these antibodies on neurons, 7 cultured human neural cell lines were evaluated for reactivity with 7 different anti-*C. jejuni* sera by indirect immunofluorescence. Positive reactions against antibodies tested were noted in 2 of the 7 (29%) cell lines, both reflecting different positive cell proportions (5% and 75%) for each cell line. These effects were most evident with antibodies against the Penner O:19 serotype indicating only certain serotypes may have potential involvement in GBS. To determine the prevalence of GBS and possible sources of organisms associated with GBS in Maritime Canada and to assess whether certain strains of *C. jejuni* are significantly associated with GBS, a retrospective study of GBS patients and suitable controls was completed. Differences in 10 patients and matched control groups were analysed. The reported prevalence over the 2 year study period was 1.1 per 100,000. Household controls were limited as most patients lived alone (70%). The average age of diagnosed females was 44.5 years compared to 45.3 for male, however a higher number of males (60%) were diagnosed with the disease, compared to females (40%). No differences were noted in occupation. Seasonality trends were not noted; however, diagnosis occurred 4 times in duplicate (meaning twice in the same month) compared to two single cases (different months). McNemar's Exact test determined that the only significant risk factor ($p<.05$) was exposure to untreated water. The ODDS RATIO (OR) for exposure to untreated water was 8.0, indicating that the odds of exposure to untreated water was 8 times higher in cases than controls. All stool samples collected were negative for *C. jejuni*, and a two-way ANOVA showed no significant differences in antibody titres against *C. jejuni* by ELISA in patients versus control groups. Overall, adverse nerve function effects were most evident with antibodies against the Penner O:19 serotype indicating potential involvement in GBS. Isolation of *C.jejuni* through serolgy and culture are unreliable, post-diagnosis of GBS. Due to insufficient patient data this study was not successful in identifying statistically significant exposure vectors related to GBS. Factors of interest, and in particular consumption of untreated water, indicated a possible relationship in the development of GBS. in the Maritime Region of Canada.

DEDICATION

To my husband, Daniel
our son, James
and our daughter, Madison.

For your support, patience and love.

And for my parents,
Joan and Harold Tobin.

For everything.

Thank - you.

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- Dr. Paul Hoffmann, Dalhousie University, Halifax, NS, and
- Dr. Pierre Talbot, Institute Armand Frappier, Quebec.

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TABLE OF CONTENTS

TITLE i

CONDITION OF USE ii

PERMISSION TO USE POSTGRADUATE THESIS iii

CERTIFICATION OF THESIS WORK iv

ABSTRACT v

DEDICATION vi

ACKNOWLEDGMENTS vii

TABLE OF CONTENTS viii

LIST OF FIGURES xi

LIST OF TABLES xii

LIST OF ABBREVIATIONS xiii

1.0 GENERAL INTRODUCTION 1
- 1.1 Genus *Campylobacter* 1
  - 1.1.1 History and Characterization 1
  - 1.1.2 Epidemiology of *Campylobacter* Infections 4
    - 1.1.2.1 Clinical Aspects 4
    - 1.1.2.2 Ecology, Transmission and Potential Risk Factors 5
    - 1.1.2.3 Seasonal Variation and Distribution 5
  - 1.1.3 *Campylobacter jejuni* 6
    - 1.1.3.1 Descriptive Epidemiology of *C. jejuni* 6
    - 1.1.3.2 Clinical Features 7
    - 1.1.3.3 Diagnosis / Treatment 7
- 1.2 Guillain Barré Syndrome 8
  - 1.2.1 Introduction 8
  - 1.2.2 Clinical Description / Characteristics 8
  - 1.2.3 Diagnosis and Treatment of GBS 11
  - 1.2.4 Epidemiology 12
    - 1.2.4.1 Incidence, Seasonality and Risk Factors 12
    - 1.2.4.2 Other Potential Risk Factors 13

1.3 Guillain Barré Syndrome and *Campylobacter jejuni* 14
1.3.1 Reported Associations 14
1.3.2 Explanations for the association between *C. jejuni* and GBS 16
1.3.3 Natural and Experimental Animal Models of Guillain Barré Syndrome 18
1.3.3.1 Introduction 18
1.3.3.2 Natural Animal Models 21
1.3.3.3 Experimental Animal Models 21
1.3.3.4 Experimental Animal Models Involving *C. jejuni* 25
1.4 Objectives of the Study 26

2.0 THE EFFECTS OF ANTIBODIES AGAINST *CAMPYLOBACTER JEJUNI* ON PERIPHERAL NERVE FUNCTION AND HUMAN NEURAL CELL LINES IN EXPERIMENTAL MODELS. 27
2.1 ABSTRACT 27
2.2 INTRODUCTION 28
2.3 MATERIALS AND METHODS 30
2.3.1 Bacterial Isolates and Antigen Preparation 30
2.3.1.1 Whole Cell Preparation for Immunization 30
2.3.1.2 LPS Preparation for Immunization 31
2.3.2 Immunization Schedule for Production of Anti-Sera in Rabbits (Whole Cell and LPS) 31
2.3.3 Experimental Models 34
2.3.3.1 Experimental Groups 34
2.3.3.2 Electrophysiological Assessment 34
2.3.3.3 Western Blotting 35
2.3.3.4 Human Neural Cell Lines 37
2.4 STATISTICAL ANALYSIS 38
2.4.1 Peripheral Nerve Function 38
2.4.2 Human Neural Cell Lines 39
2.5 RESULTS 39
2.5.1 Peripheral Nerve Function 39
2.5.2 Western Blotting 41
2.5.3 Neural Cell Lines 48
2.6 DISCUSSION 48
2.6.1 Peripheral Nerve Function 48
2.6.2 Human Neural Cell Lines 56

3.0 IDENTIFICATION OF POSSIBLE RISK FACTORS OF GUILLAIN BARRÉ SYNDROME IN THE MARITIME REGION 59
3.1 ABSTRACT 59
3.2 INTRODUCTION 61

3.3 EPIDEMIOLOGICAL STUDY 64
3.3.1 Study Design 64
3.3.2 Study Population 66
3.3.3 Materials and Methods 66
3.3.3.1 Epidemiological Survey 67
3.3.3.2 Isolation and Identification of Bacteria 67
3.3.3.3 Serological Testing 67
3.4. STATISTICAL ANALYSIS 69
3.4.1 Epidemiological Survey 69
3.4.2 Serological Testing 70
3.5 RESULTS 71
3.5.1 Epidemiological Survey 71
3.5.2 Isolation and Identification of Bacteria 74
3.5.3 Serological Testing 74
3.6 DISCUSSION 78
3.6.1 Epidemiological Survey 78
3.6.2 Isolation and Identification of Bacteria 82
3.6.3 Serological Testing 83

4.0 GENERAL DISCUSSION 86

REFERENCES 91

APPENDIX A: Electrophysiological Recording Equipment. Stimuli were recorded with a P55, A.C. amplified signal and sent to an oscilloscope (below) which in turn measures currents (BK Precision - Model 2522 - A) that are displayed on the grid screen (marked X). Note the faint green line (marked ▲) registered on the grid representing the baseline. 104

APPENDIX B: Maximum response of stimulated rat nerve. A represents stimulus artifact, B = A + B fibers, C + B or C fibers and D represents latency 105

APPENDIX C: Epidemiological Survey: Survey Template 106

APPENDIX D: Epidemiological Survey: Clinical Assessment Template ... 111

LIST OF FIGURES

Figure 1.1: Cultured and microscopic forms of *Campylobacter jejuni* 3

Figure 1.2: Interrelationship among the forms of Guillain Barré Syndrome (adapted from Nachamkin et al., 1998). 10

Figure 2.1: Effects of rabbit antisera to *Campylobacter jejuni*, normal rabbit sera and saline on Mean threshold CAP (TCAP) of femoral nerve function in rats 42

Figure 2.2: Effects of rabbit antisera to *Campylobacter jejuni*, normal rabbit sera and saline on mean conduction velocity (CV) of femoral nerve in rats 43

Figure 2.3: Effects of rabbit antisera to *Campylobacter jejuni*, normal rabbit sera and saline on mean maximum amplitude of CAP of femoral nerve in rats 44

Figure 2.4: Effects of rabbit antisera to *Campylobacter jejuni*, normal rabbit sera and saline on mean maximum stimulus required to reach CAP (MSCAP) of femoral nerve in rats 45

Figure 2.5: Effects of rabbit antisera to *Campylobacter jejuni* antibodies, normal rabbit sera and saline on mean latency of femoral nerve in rats. 46

Figure 2.6: Western Blot of sera from *C. jejuni* immunized rabbits with rat nerve. Lanes 1, 3, 5, 7 and 9 are normal rabbit antisera (pre-immune). Lanes 2, 4, 6, and 8 are rabbit antisera to Penner O:19 (heat-treated), Lior 7 & 11 (combination of heat treated and formalinized), GBS 4382/4384 H, and a negative control (no serum added), respectively. The numbered column to the right of Lane 10 are molecular weight markers. 47

Figure 2.7: Photo micrograph of immunofluorescence of rabbit anti-*C. jejuni*/ sera on cultured neural cell lines against Penner O:19 F with reactivity in 75% of the cells of the SK-N-SH neural cell. The fluorescent yellow area in the green circles above indicates some of the areas of reactivity. . 51

Figure 2.8: Photo micro graph of immunofluorescence of rabbit anti-*C. jejuni* sera on cultured neural cell lines against Lior 7:11- F with reactivity in 5% of the MO3.13 Oligo cell line. The fluorescent yellow area in the green circles above indicates some of the areas of reactivity. 52

Figure 2.9: Photo micrograph of negative controls on cultured neural cell lines. 53

Figure 2.10: Photo micrograph displaying immunofluorescence in the astro marker rabbit (anti-GFAP) at 1/250 dilutions in the U-373MG/250 (astrocyte) cell lines. The fluorescent yellow and green areas in the green circles represent some of the areas of reactivity. 54

LIST OF TABLES

Table 1.1: ***Campylobacter species*** **- (human and non-human sources) 2**

Table 2.1: **Agglutination titres of rabbit antisera raised against strains of *Campylobacter jejuni.* 33**

Table 2.2 **Scale for quantitative grading of binding of *Campylobacter jejuni*-specific antibodies to neural cell lines. 40**

Table 2.3: **Immunofluorescence reactivity of rabbit anti-*Campylobacter jejuni* antisera with cultured neural cell lines. 49**

Table 3.1: **Count data for cases and non-cases (hospital-based controls) of GBS with statistical results for p-value from McNemar's Exact test with Exact ODDS Ratio and confidence intervals. 73**

Table 3.2: **Two-Way ANOVA based on ELISA optical density values at 1/100 serum dilution seven *C. jejuni* antigens preparations. Results of F-Test and presentation of corresponding p-values, all based the 1/100 dilution. 76**

Table 3.3: **Dichotomous values for positive and negative samples as determined by ELISA using end-point titre values for each antigen tested. Each column represents the number of positives (P)or negatives (N) in each of the five groups listed, including positive and negative controls. 77**

LIST OF ABBREVIATIONS

CNS	**- central nervous system**
CHP	**- coonhound paralysis**
CSF	**- cerebral spinal fluid**
d	**- Days**
EAE	**- experimental allergic encephalomyelitis**
EAN	**- experimental allergic neuritis**
ELISA	**- enzyme linked immunosorbant assay**
F	**- formalinized**
FBS	**- fetal bovine serum**
g	**- Gram(s)**
GBS	**- Guillain Barré Syndrome**
H	**- heat treated**
HLA	**- Human Leukocyte Antigen**
i.e.	**- Id est; that is**
IgA	**- immunoglobulin A**
IgG	**- immunoglobulin G**
IgM	**- immunoglobulin M**
IVIG	**- intravenous immunoglobulin therapy**
L	**- Litre(s)**
LCDC	**- Laboratory Center for Disease Control**
LPS	**- lipopolysaccharide**
MCAP	**- maximum amplitude of CAP**
MFS	**- Miller Fisher Syndrome**
mg	**- Milligram(s)**
min	**- Minutes**
ml	**- Milliliter(s)**
mm	**- millimeter(s)**
MSCAP	**- maximum stimulus required to reach TCAP**
O_2	**- Oxygen**
P / p	**- P-value**
PBS	**- phosphate buffered saline**
pH	**- p(otential of) H(ydrogen)**
SEM	**- Standard error of the mean**
St. Dev.	**- Standard deviation**
T	**- Temperature**
TCAP	**- threshold for CAP**
CAP	**- compound action potential**
v/v	**- volume per volume**
w/v	**- weight per volume**
X^2	**- Chi-square**
°C	**- Degrees of Celsius**
µg	**- microgram(s)**
µl	**- microlitre(s)**

1.0 GENERAL INTRODUCTION

1.1 Genus *Campylobacter*

1.1.1 History and Characterization

The identification of *Campylobacter* likely occurred more than 100 years ago in Germany, when in 1886 the renowned bacteriologist, Theadore Escherich, observed a non-culturable, spiral shaped micro-organism, closely resembling the *Campylobacter* of today, which he named *Vibrio felinus* (reported in Lior, 1996). Although the initial observation was made from faeces of both children and kittens with diarrhea, subsequently similar organisms described as *Vibrio fetus* and *Vibrio jejuni* were considered mainly as animal pathogens. In 1963 these organisms were assigned to a new genus, *Campylobacter.* The requirement for a micro-aerophilic environment for growth of *Campylobacters* have eluded their isolation by standard microbiological techniques. However, in 1973, Butzler and colleagues in Belgium reported isolation of related vibrios from the stool of diarrheal patients using a filtration technique. Skirrow, in 1977, using a simpler isolation technique based on the use of antibiotics in blood agar media, substantiated Butzler's findings that this organism, *C. jejuni*, was indeed a common cause of human diarrhea (Lior, 1996).

By the middle of the 1980's , the genus *Campylobacter* contained species with many diverse characteristics which led to the restructuring of the genus. In the early 1990's Vandamme *et al.* proposed that the family *Campylobacteraceae* should include three genera as follows (Vandamme *et al.*, 1991):

1. *Campylobacter* - 19 species and subspecies
2. *Arcobacter* - 4 species

3. *Helicobacter* - 16 species

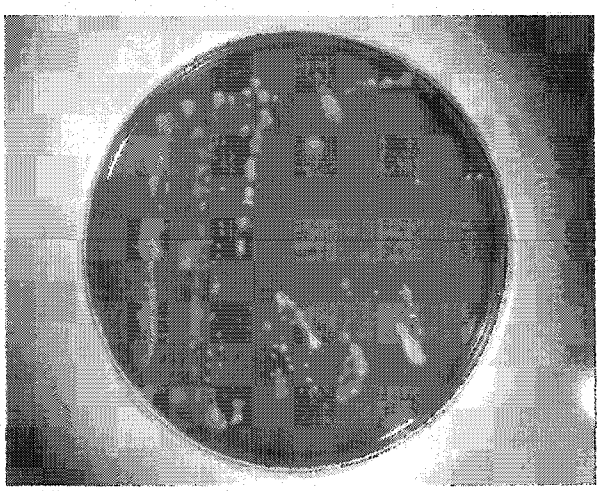
Campylobacters are gram-negative, slender, spirally curved bacterial rods (See Figure 1.1) which require a micro-aerobic atmosphere for growth. Most *Campylobacters* associated with human enteritis grow at 37°C and 42°C. Today *Campylobacters* are recognized globally as the major aetiologic agents in human diarrheal disease. The nineteen species and subspecies presently identified in the genus *Campylobacter* that have been isolated from humans and/or animals are provided in Table 1.1 below: (Lior, 1996).

Table 1.1: *Campylobacter* species - (human and non-human sources)

C. coli	*C. mucosalis*
C. concisus	*C. rectus*
C. curvus	*C. sputorum biovar bubulus*
C. fetus ssp. *fetus*	*C. sputorum biovar fecalis*
C. fetus ssp. *venerealis*	*C. sputorum biovar sputorum*
C. hyointestinalis	*C. upsaliensis*
C. jejuni* ssp. *jejuni	*'C. Helveticus'*
C. jejuni ssp. *doylei*	*'C. Showae'*
C. lari	*'C. Hyoilei'*
	'C. Gracillis'

Among these, *C. jejuni* ssp. *jejuni* (*C. jejuni*) is most commonly isolated from diarrheal disease in humans and animals. *C. jejuni* spp. *doylei*, *C. coli*. and *C. lari* are occasionally involved in human diarrhea as the enteropathogenic *Campylobacters*; hereafter referred to as *Campylobacters*.

A.



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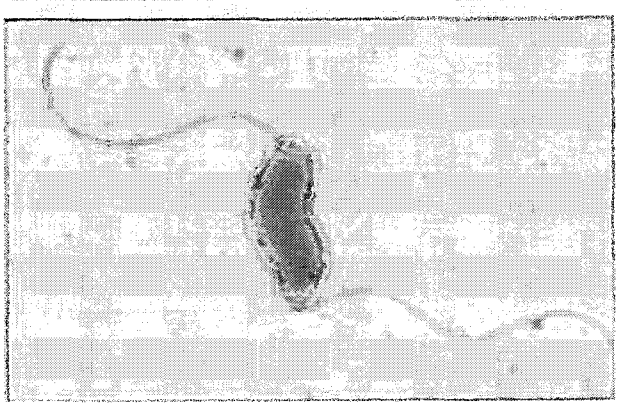

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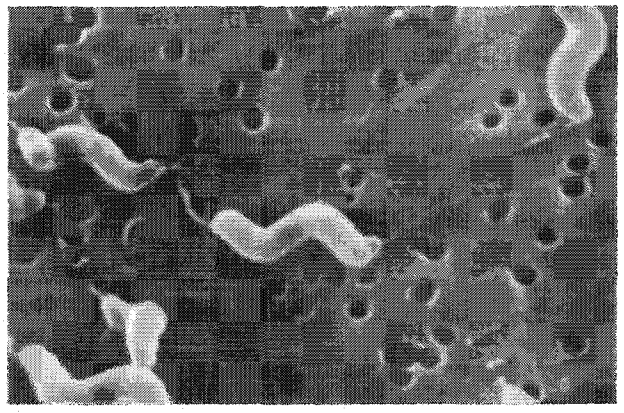


Figure 1.1: **Cultured and microscopic forms of *Campylobacter jejuni*. A. *C. jejuni* on blood agar observed macroscopically (Lior, 1996). Shown are a combination of single colonies and areas of heavy growth. B. *Campylobacter jejuni* as viewed by transmission electron microscopy (Lior, 1996). Shown is a singular *C. jejuni* organism. C. Scanning electron microscopic view of *Campylobacter jejuni* (Nachamkin, 1997). Several single *C. jejuni* organisms, showing spiral shape and flagellar system.**

1.1.2 Epidemiology of *Campylobacter* Infections

1.1.2.1 Clinical Aspects

Two forms of infection have been reported for *Campylobacter*:

1. a disseminated form; and
2. a localized form, mostly enteritis affecting all sexes and ages.

Campylobacters have been isolated from cases of bacteraemia and appendicitis and implicated in various human diseases including: diarrhea, meningitis, peritonitis, salpingitis and septic abortion (Mishu and Blazer, 1993). One of the most serious sequelae of infection with *Campylobacter* is Guillain Barré Syndrome (GBS) (Mishu and Blazer, 1993).

Because of limitations with culture techniques, serological studies combined with cultures and clinical histories are useful in identifying patients likely to have had *Campylobacter* infections (Nachamkin, 1997). The immune response to *Campylobacter* infections is similar to that of other infectious diseases. Serum IgM and IgG levels rise in response to infection and remain elevated for 3 to 4 weeks before declining to baseline levels; serum IgA levels rise only during the first few weeks of infections and fall rapidly (Blazer *et al.*, 1984; Mascart-Lemone *et al.*, 1987 and Kaldor *et al.*, 1983. Enzyme Linked Immunosorbant Assay (ELISA) appears to be the most commonly used method for measuring serum antibodies (Blazer *et al.*, 1984; Herbrink *et al.*, 1988; Nachamkin *et al.*, 1989 and Mishu *et al.*, 1993); other methods include immunoblot assay (Speed *et al.*, 1987 and Nachamkin *et al.*, 1985), diffusion in-gel ELISA (Svedhem *et al.*, 1983) , agglutination tests and complement fixation (Jones *et al.*, 1981).

1.1.2.2 Ecology, Transmission and Potential Risk Factors

Campylobacter enteritis is a zoonosis. Contact, primarily through the faecal-oral route, with poultry, cattle, raw milk, surface water and pets is responsible for most *Campylobacter* infections in the industrialized world (Skirrow, 1987). Specific reports of transmission of the disease include farm workers (likely a result of handling cattle and chickens), occupational exposure to monkeys and household outbreaks due to caged birds. The most common cause of sporadic outbreaks and infections is the consumption of undercooked chicken; however poultry may also be an important means of transmission of infection to humans via cross-contamination (Lior, 1996). Other sources of infections have been reported, specifically in meats. Lior (1996) stated that the isolation of *Campylobacter* has been reported in about 5% of retail meats in the USA and from 2 - 5 % of ground beef and beef flank in Canada. In a two year study in a defined geographical area of the UK, *Campylobacters* were found in 47% of offal, 23% of beef, 18% of pork samples and 15% of lamb samples. In addition, *Campylobacters* have been associated with very large milk and water-borne outbreaks in several countries (Skirrow, 1987).

1.1.2.3 Seasonal Variation and Distribution

In temperate Europe and North America, reports of infections with *Campylobacters* occur predominantly during the warmer months (Skirrow, 1987). Isolations are more frequent in the wet season than the dry season in countries with constant mean temperatures throughout the year. Tauxe (1992) reported that isolations increased in England in the beginning of the second quarter and last until September. In Sweden more isolations are

made in July and August, with another increase noted in January, probably due to travel patterns (Tauxe, 1992).

Canada follows the pattern seen in temperate countries, with the seasonal distribution peaking in late June and lasting through August and September (Lior, 1996). Slight increases in infection of *Campylobacters* are recorded following holidays such as Thanksgiving and Christmas, probably as a result of increased consumption of poultry and turkey (Lior, 1996).

1.1.3 *Campylobacter jejuni*

Campylobacter jejuni is recognized as the most important species of *Campylobacter* associated with diarrheal illnesses in humans. Infection is common throughout the world; however, clinical and epidemiological features differ in developed and developing countries.

1.1.3.1 Descriptive Epidemiology of *C. jejuni*

Diarrhea in humans caused by *Campylobacter jejuni* has only been recognized for the last 3 decades or so, although it was isolated from the jejunum of calves with diarrhea as early as 1931 (Rees *et al.* 1993). *Campylobacter jejuni* may contaminate both surface water and soil due to its widespread reservoir in the animal kingdom. Transmission to humans resulting in endemic infections may occur by ingestion of contaminated foods, untreated water, direct contact with infected animals and less commonly, infected humans and travel (Blaser, 1997). Outbreaks are usually due to ingestion of undercooked poultry, contaminated water and unpasteurised milk (Blaser *et al.*, 1983).

Diarrhea in humans due to *C. jejuni* is quite common and this bacterium has surpassed *Salmonella* as the most common agent of diarrheal disease since the late 1980's (Lior, 1996). However, many hospital laboratories do not routinely culture for this organism and thus infection with *C. jejuni* is considered to be substantially under-reported. The annual incidence of infection is estimated to be 1000 per 100,000 persons (Blazer, 1997). Persons of any age can be infected with *C. jejuni.* In developed countries, the highest age specific attack rates are in children under 1 year old, and a second lower peak of broader range occurs between the ages of 15 and 30 years of age (Tauxe *et al.*, 1988).

Campylobacter jejuni has been isolated from cases of bacteraemia, appendicitis and more recently was associated with reactive arthritis, Reiter's Syndrome and GBS , the latter being one of the most serious sequela of infection related to *C. jejuni* (Blazer *et al.*, 1993 and Ropper *et al.*, 1988).

1.1.3.2 Clinical Features

The usual incubation period after ingestion of *C. jejuni* is 24 - 72 hours; however incubation periods of $\geq$1 week or longer have been reported (Skirrow and Blaser, 1995). Non-specific symptoms include headache, myalgia, chills and fever and can last more than 24 hours. The major clinical manifestation is acute diarrhea, accompanied by severe abdominal cramping and fever.

1.1.3.3 Diagnosis / Treatment

Diagnosis of *C. jejuni* infection is usually based on culture of the organism and serum

antibodies. Mild infections do not require specific antimicrobial treatment; however treatment is often introduced for immunocompromised patients or for persons with increasingly severe symptoms. Erythromycin is currently the treatment of choice; alternates include fluoroquinolones and tetracyclines (Blaser, 1997).

1.2 Guillain Barré Syndrome

1.2.1 Introduction

In the post-polio era, Guillain Barré Syndrome is the most common cause of acute neuromuscular paralysis in both adults and children in North America, including Canada (Rees *et al.*, 1993). If full credit were given to those clinicians who first recognized and described this paralytic disorder, the proper name would be Landry-Guillain-Barré-Strohl Syndrome (Steinberg 1995). The precise etiology of this condition remains unknown, and there is no specific cure. Descriptive accounts of GBS, including history, clinical and pathological aspects are given by Hughes (1990) and Ropper *et al.* (1991). Subsequently, Hughes and Rees (1997) summarized the clinical and epidemiological features of the condition.

1.2.2 Clinical Description / Characteristics

GBS is a human illness, in which the body's immune system attacks the nervous system. GBS is an acute, inflammatory demyelinating disease of peripheral nerves, characterized by rapid ascending paralysis that can lead to respiratory muscle compromise and death. The ascending paralysis is due to conduction block with segmental demyelination

of nerves, macrophage and lymphocytic infiltration of the nerves, and elevated protein in the cerebrospinal fluid (Constantinescu *et al.* 1998).

Motor, sensory, and autonomic nerves supplying the limbs are always affected; but respiratory muscles and facial, bulbar, and ocular motor nerves may be involved (Hughes and Rees, 1997). GBS often follows nonspecific respiratory illness or other primary events including viral or bacterial infections (Ropper *et al.* 1991).

Normally the disease, which is often termed post-infective polyneuropathy, starts after a gastrointestinal or respiratory infection, and the symptoms increase in intensity until the muscles become immobile resulting in near total paralysis. It can become a medical emergency because of the potential to interfere with breathing and death.

GBS was previously considered a single homogeneous clinical entity; currently the syndrome has been divided into several electrophysiological and pathologic patterns suggesting that there are different immune targets of an autoimmune response in the peripheral nervous system (Ho *et al.*, 1997). Griffin *et al.* (1996) proposed a physiological and pathological classification (See Figure 1.2) of GBS patterns (Nachamkin *et al.*, 1998). According to Griffin *et al.* (1996), the most frequently encountered pattern of GBS in Europe and North America is acute demyelinating polyneuropathy (AIDP) which is characterized by an immune-mediated attack on myelin with varying degrees of lymphocytic infiltration (Asbury *et al.*, 1969; Peterman *et al.*, 1959 and Prineas, 1972).

The AIDP form of GBS may be accompanied by demyelination with severe cases characterized by axonal degeneration. Two patterns have been noted in the axonal form of the disease. The first pattern, originally called the axonal GBS form, has been recently

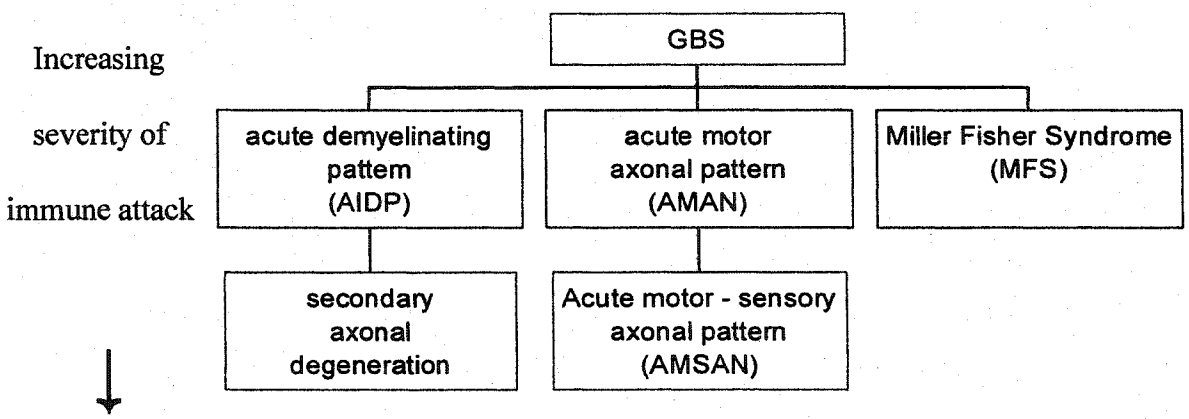


Figure 1.2: Interrelationship among the forms of Guillain Barré Syndrome (adapted from Nachamkin *et al.*, 1998).

termed acute motor-sensory axonal neuropathy (AMSAN) and is usually severe involving both motor and sensory fibres. The second pattern, limited to pure motor involvement, is termed acute motor axonal neuropathy (AMAN) and is more benign. Another related disorder, Miller Fisher syndrome (MFS) is an infrequent variation of GBS, characterized by oculomotor weakness, ataxia and areflexia (van der Meche *et al.* 1997).

1.2.3 Diagnosis and Treatment of GBS

The criteria for diagnosis of this idiopathic neuropathy were described by Asbury *et al.*, 1978, 1981, 1990 and Cornblath, 1990. These include more-or-less symmetrical paresis and a loss of myotatic reflexes (van der Meche *et al.* 1997). The symptoms may be caused by inflammatory demyelination, axonal degeneration, or both (Hughes and Rees, 1997).

Inflammatory neuropathies such as GBS have been suspected to have an autoimmune basis, and are characterized by demyelination of nerves and axonal degeneration. Plasmapheresis and intravenous immunoglobulin therapy (IVIG), the latter being the recently preferred treatment, are often used to reduce the destructive effects by the host immune system (Rees *et al.*, 1993).

The major reduction of mortality in GBS reflects advances in supportive care of critically ill patients. However, increased understanding of the immunologic basis of this disease over the past 15 years has allowed change in the treatment of the disease. Plasmapheresis was the first therapy shown to be effective in speeding up the course of recovery. In this procedure the patient's blood is removed and centrifuged to separate the cellular and plasma components and cellular components are diluted with artificial

plasminate before being reinfused into the patient. The effect is presumably due to removal of inciting circulating factors such as antibodies (Guillain Barré Study Group, 1985). Another proven effective therapy is to administer intravenous immunoglobulin (IVIG) in GBS. One proposed mechanism of action of infused immunoglobulin is it's contents of anti-idio-typic antibodies that inactivate the disease-specific antibodies and production of more antibodies by auto-reactive B cells (van der Meche, 1992).

1.2.4 Epidemiology

1.2.4.1 Incidence, Seasonality and Risk Factors

Presently, GBS is the most common cause of acute neuromuscular paralysis in both adults and children in North America, yet its precise cause and pathogenesis are unknown. From 5 to 15% of GBS patients die during acute illness, and among those who survive, ~15% have residual neurological problems (Allos and Blaser, 1994). In the United States GBS has an annual incidence of 1.7 per 100,000 people (Mishu *et al.*, 1993). Published figures for incidence of GBS in Canada are unavailable; however in 1999, Guillain-Barré Syndrome caused the death of 7 females and 15 males (Statistics Canada, 2001). The median annual incidence of clinically defined GBS in the developed world is 1.3 cases (range 0.4 - 4.0 cases) per 100,000 (Black *et al.*, 1988).

Recently, seasonal variation has been introduced as annual summer peaks have occurred in China, Mexico, Spain and Korea (Lior, 1996). Outbreaks of GBS are rare. In 1978, during an outbreak of gastroenteritis affecting more than 5000 people caused by contaminated water, 16 persons developed GBS 8 to 24 days after the onset of diarrhea

(Khoury, 1978).

GBS, as clinically defined, is slightly more common in males than in females and more common in whites than in blacks (Mishu and Blaser, 1993). The incidence of disease as it relates to age suggests bimodal distribution, with the AIDP form of GBS affecting the older population in Western countries and the AMAN form tending to affect primarily children and young adults (Kaplan *et al.*, 1985).

1.2.4.2 Other Potential Risk Factors

A few studies have retrospectively investigated the relationship between occupation and GBS. Statistically there is no reported confirmation of associations between occupation and the development of GBS. However, there have been trends established in those occupations with exposure to risk factors, such as *Campylobacter* infection, that appear preferentially to precede GBS (Ropper *et al.*, 1991).

Evidence of familial GBS has not been proven and thus there is no evidence that GBS is communicable. Attempts to identify an association between Human Leukocyte Antigen (HLA), often seen in auto-immune diseases, have been negative (Hughes, 1990).

Outbreaks of GBS associated with immunizations are still questionable. However in a swine flu vaccination program (N/J -76 strain), two clusters of GBS and several individual case reports were generated from 45 million treated patients. Although not proven statistically, the five fold increase in adult GBS observed was associated with the vaccine utilized in this case (Ropper *et al.*, 1991). No other influenza vaccines have been implicated in GBS; however there was an increased incidence of GBS in areas with polio vaccination

programs (Kaplan *et al.*, 1985).

1.3 Guillain Barré Syndrome and *Campylobacter jejuni*

GBS is characterized as a "postinfectious" disease with two-thirds of patients reporting antecedent illnesses (Blaser, 1997). Often the precise infection is not clear from the medical history and has resolved prior to the development of neuropathic symptoms. Bacterial and viral cultures are usually negative, and serological tests lack specificity and sensitivity. Respiratory infections are the most frequently reported antecedent infection, followed by gastrointestinal infection, of which *C. jejuni* is the most frequently identified cause (Winer *et al.*, 1988 and Arnason and Soliven, 1993).

1.3.1 Reported Associations

Evidence has recently accumulated to support an association between GBS and infection with the gastrointestinal bacterial pathogen *C. jejuni*. The first reports of an association between *Campylobacter jejuni* and GBS were published in 1984 (Kaldor and Speed, 1984). Evidence of preceding *C. jejuni* infection was found in 38% of 56 GBS patients, in a retrospective study conducted by Kaldor and Speed (1984) in Australia. Subsequently, there have been many reports from different parts of the world documenting strong association between infection with *C. jejuni* and GBS. In a study conducted from 1990 to 1996, 44% of 205 Japanese GBS patients had serological evidence of recent *C. jejuni* infection, compared with 1% in healthy controls (Saida *et al.* 1997). *Campylobacter jejuni*-associated GBS, specifically Miller Fisher Syndrome was seen in 26% of patients in a

prospective case-control study in the United Kingdom (Rees *et al.* 1995). Axonal degeneration, slow recovery, and severe residual disability were more common in patients with *C. jejuni* infection. Cases of acute motor axonal neuropathy (AMAN) and acute motor and sensory neuropathy (ASMAN) associated with *C. jejuni* infection are increasingly recognized, especially in China (Powell and Myers 1996).

Besides serological evidence of *C. jejuni* infection, investigators have also succeeded in isolating *C. jejuni* from the stools of patients with GBS at the onset of neurologic symptoms (Allos 1997). Hariharan *et al.* (1996) found three (38%) of 8 GBS patients in India culture positive for *C. jejuni*; the rate was within the range of culture-positive GBS cases reported elsewhere (Allos, 1997). Two of the three culture positive GBS patients in the above study (Hariharan *et al.*,1996) had a history of antecedent diarrhea. A positive *C. jejuni* stool culture and a history of enteritis preceding GBS are significant supporting evidence, although serologic evidence only may be nonspecific, especially in older patients (Ropper *et al.*, 1991). Evidence of previous *C. jejuni* infection in GBS patients has been reported in several areas, including the United States, Japan and the United Kingdom (Gruenwald *et al.*, 1991; Mishu *et al.*, 1993; Kuroki *et al.*, 1993 and Rees *et al.*, 1995).

Campylobacter jejuni strains are heterogeneous with regard to their heat stable and heat labile antigens for serotyping. The Penner typing method utilizes heat stable lipopolysaccharide (LPS) antigens, and the Lior method uses thermolabile protein antigens. The serotypes Penner 19, Lior 7 and Lior 11 are most commonly associated with GBS (Fujimoto *et al.*, 1992, Rees *et al.*, 1993 and Hartlung *et al.*, 1995), although no predominance of any particular serotype was found in a British study (Rees *et al.*, 1995).

The lipopolysaccharide of *C. jejuni* serotype Penner O:19 has striking homology with peripheral nerve antigens such as gangliosides GM1, and cross react in immunological assays (Aspinall *et al.*, 1994 and MacDonald *et al.*, 1994).

Some recent findings suggest that cases of GBS associated with *C. jejuni* infection are more serious and prolonged than those without infection or due to other causes (Rees *et al.*, 1995). Routine testing of stools and blood sera from GBS patients may have prognostic value. Detection of *C. jejuni*-induced GBS may help determine specific treatment, decreasing the requirement for special care and long-term treatment.

1.3.2 Explanations for the association between *C. jejuni* and GBS

Apparently, certain strains of *C. jejuni* share structural components with human nerves, and immune responses initially mounted against *C. jejuni* are misdirected to peripheral nerves, resulting in the clinical signs of GBS. In this respect some serotypes of *C. jejuni* may be more pathogenic than others. Another possibility is that infection with a certain strain of *C. jejuni* is more strongly associated with GBS than infection with a different strain, or possibly the type of antibodies produced in response to *C. jejuni* infection, or that perhaps GBS patients produce more antibodies against nervous tissue.

Griffin and Ho (1993) reported that the lipopolysaccharide (LPS) of some *C. jejuni* strains expresses an unidentified carbohydrate epitope shared with nerves. A possible association between anti-ganglioside antibodies and *C. jejuni*- associated GBS was suggested in several studies (Yuki *et al.*, 1990, Walsh *et al.*, 1991 and Gregson *et al.*, 1993). Possibly, in some particularly sensitized individuals, exposure to GM1 or cross-reacting antigens may

trigger an immune response to GM1 (Nobile-Orazio *et al.* 1994). Parenteral treatment with gangliosides, for certain neurological diseases has resulted in many instances in an acute, mostly axonal, motor neuropathy clinically presenting as GBS (Nobile-Orazio *et al.* 1994). Human anti-GM1 antibodies cross react with LPS from certain strains of *C. jejuni* (Wirguin *et al.,* 1994). Isolates of *C. jejuni* from GBS cases may belong to several serotypes, although Penner serotype O:19 is the most common one among Japanese patients (Saida *et al.,* 1979). Molecular mimicry between the GM1 ganglioside and the LPS from *C. jejuni*, belonging to Penner serotype 19 has been demonstrated (Yuki, 1997). Although in the United States ~75% of isolates of *C. jejuni* from diarrhea cases do not have GM1-like epitopes, all GBS-associated isolates do possess GM1 or other ganglioside-like epitopes in the core region of LPS (Nachamkin *et al.*, 1999). GBS cases with antecedent *C. jejuni* infection are characterized by poorer recovery and axonal damage, with or without elevated serum antibodies to gangliosides GM1 and GD1b (Yuki *et al.,* 1990, Vriesendorp *et al.,* 1993). Sera from patients with GBS frequently have anti-GM1 antibodies (Oomes *et al.*, 1995; Yuki *et al.*, 1995). Oomes *et al.,* (1995) demonstrated with IgG ELISA that pre-incubation with certain *C. jejuni* and *C. coli* serotypes of GBS patient sera with elevated levels of anti-GM1 antibodies reduced anti-GM1 activity in the samples, thereby providing some evidence for molecular mimicry in the pathogenesis of GBS.

Rees *et al.* (1993) hypothesized that induction of neuropathy following *C. jejuni* infection might be mediated by an enterotoxin which is secreted by some strains, and which binds to ganglioside GM1 on nerves. The ganglioside GM1 enterotoxin complex might then alter recognition of self antigens and trigger production of antibodies directed against axonal antigens. Production of cholera-related enterotoxins occurs in some strains of *C. jejuni*, and

this group of toxins can be detected by immunoblot and ELISA (Hariharan and Panigrahi, 1990 and Hariharan *et al.*, 1986).

1.3.3 Natural and Experimental Animal Models of Guillain Barré Syndrome

1.3.3.1 Introduction

Disease conditions resembling GBS, characterized by inflammatory polyradiculoneuropathy sporadically occurs in animals. In addition to elucidating the mechanisms involved in GBS, both natural and experimental animal models have helped develop experimental approaches to treat autoimmune neuritis, although the immune mechanisms involved are not fully understood. Many small animal neuropathies may be similar or even identical to their human counterparts (Duncan , 1980).

1.3.3.2 Natural Animal Models

Northington *et al.* (1981) and Northington and Brown (1982) noted similarities between idiopathic acute canine polyradiculoneuropathy (ACP) and GBS: rapid progression, (predominantly motor neuropathy), slow nerve conduction velocities, and primary demyelination. Onset of disease is similar in ACP and GBS as acute onset is less than 21 days to the most severe effect. ACP can be confirmed by electromyography, indicating denervation of influenced segments, and by nerve conduction velocity measurements, revealing slow conduction in affected segments (Northington *et al.*, 1981, Northington and Brown 1982). Similar to GBS, pathological changes include degeneration of myelin sheaths, leukocytic infiltration and varying degrees of axonal degeneration (Cummings and Haas,

1972; Collins, 1994 and Cuddon, 1998). The progression of both diseases can lead to respiratory failure, and notwithstanding recovery of up to several weeks, disease can cause permanent damage or death (Northington *et al.* and 1981, High 1996). Relapse occurs in a low percentage of cases in ACP, similar to GBS (Northington *et al.*, 1981, Northington and Brown, 1982). The acute axonal form of GBS has close resemblance to ACP as noted in a study on 12 dogs by Cuddon (1998). Electrophysiologic assessment suggested that ACP represents a peripheral motor axonopathy, with demyelination and axonal involvement occurring in ventral nerve roots. Electromyographic changes occurred in 100% of affected dogs. Two cases of ACP with predominantly axonal degeneration were also reported earlier by Griffiths *et al.* (1983). The underlying cause of the neuropathy in dogs diagnosed with ACP is unclear; however suggestions coincide with those presented for GBS. An infectious agent or an antigen may trigger an autoimmune process, damaging Schwann cells and manifesting as a demyelinating neuritis (Hawe, 1979) .

More specifically, an ACP resembling GBS in humans is Coonhound paralysis (CHP). Similar to GBS, CHP has been identified as a polyradiculoneuritis with initial symptoms being weakness and hyporeflexia in the hind limbs, and progressive rapid paralysis resulting in flaccid symmetric quadriplegia (Cummings and Haas 1967, 1972 and Cummings *et al.*, 1982). The onset of GBS and CHP are similar in that they occur 7 to14 days post infectious symptoms (Kingma and Catcott, 1954). Complete absence of spinal reflexes, facial weakness and laboured respiration are also similarities between GBS and CHP and occur at the peak of the illnesses. Similarities in electromyographic findings in CHP include fibrillations, positive sharp waves and other evidence of denervation. Similar

to GBS, deaths occur due to respiratory failure in CHP and both are characterized by motor conduction delay and CSF albuminocytologic dissociation in affected dogs; however axon damage occurs more consistently in CHP than in GBS (Cummings *et al.*, 1982). Although CHP can occur following raccoon bites, it does occur in dogs that have no history of such exposure (Hawe 79, Northington *et al.,* 1981, Northington and Brown 1982, and Cuddon, 1998). Cases of ACP have also occurred in dogs following rabies vaccination (Collins, 1994), but no infectious agent was isolated.

Pepose *et al.* (1981) observed the similarities of Marek's disease (a demyelinating peripheral neuropathy in chickens) and GBS. Marek's disease is initiated by a viral infection in neuronal supporting cells, and subsequently demyelination occurs via a specific immune response to viral-induced antigens on these cells (Stevens *et al,.*1981). The pathogenesis of GBS in humans and Marek's disease are similar with regard to the autoimmune response to myelin and peripheral nerves and their demyelination with invasion of nerve fibres with lymphocytes and macrophages. Despite this similarity, GBS has shown no evidence of preceding viral infection.

Disease conditions resembling GBS also occur in other animal species. Monkeys and chimpanzees have paralytic illnesses resembling human GBS (Schultz, 1987, Alford and Satterfield, 1995). In the chimpanzee, Alford & Satterfield (1995) reported acute onset of ascending, symmetrical, monophasic flaccid paralysis, with albuminocytologic dissociation in CSF. Muscle atrophy was evident after one month, and several months were required to regain full mobility. The cause was undetermined; however the animal was re-vaccinated for rabies prior to the onset of disease. Goats and horses have also been affected with disease

conditions similar to GBS. Cauda equina neuritis of horses is an uncommon condition in which chronic polyneuritis affects the sacral and coccygeal nerves, and occasionally cranial nerves (Martens *et al.*, 1970, Greenwood *et al.*, 1973, Manning and Gosser, 1973 and Kadlubowski &Ingram, 1981). Characteristics of the disease that share similarities with those in GBS include; paralysis of the extremities, bladder and rectum, eyelids and lower lip (Martens *et al.*, 1970). Upper respiratory disease involving *streptococci* has been implicated in this condition (Martens *et al.*, 1970). Further description by Greenwood *et al.* (1973) indicated swelling of the nerve bundles of the cauda equina and proliferated perineurium. The observation that individual bundles involved had reached varying stages of inflammation, ranging from concurrent demyelination and progressing to degeneration and cellular infiltration was noted as similarities to GBS. Of most interest, was the similarity of this condition to experimental autoimmune neuritis. Circulating antibodies to a neuritogenic myelin protein P2 have been found in horses affected with cauda equina neuritis (Kadlubowski and Ingram, 1981). MacLachlan *et al.* (1982) reported advanced ataxia in a goat, substantiated histologically as polyradiculoneuritis similar to GBS. In this case, segmental demyelination, mononuclear inflammatory cell infiltration and Schwann cell proliferation were seen in spinal nerve roots and in the peripheral nerves of the fore and hind limbs.

1.3.3.3 Experimental Animal Models

Coonhound paralysis was experimentally reproduced for the first time by Holmes *et al.* (1979) in one of two coonhounds injected with 1.0 ml of a pool of raccoon saliva. The

onset of paralysis of hind limbs occurred 9 days later, and progressed rapidly to involve the forelimbs, neck and tail. The course of the disease was comparable to GBS. By 48 h the dog was tetraplegic and areflexic. Respirations weakened at 72 h and the animal was placed on a respirator. Reduced ulnar conduction was recorded on day 5. The dog could stand after 6 weeks, and was able to walk short distances by 8 weeks.

Stevens *et al.* (1981) observed that although coonhound paralysis resembles GBS, the unknown cause and rather limited evidence of experimental transmission of this condition limits its usefulness as a model. They suggested Marek's disease of chickens as a better natural model for GBS, at least in cases where a latent initial viral infection is involved.

Experimental autoimmune ("allergic") neuritis (EAN), an animal model of GBS, was first described by Walksman and Adams (1955, 1956) who produced the condition in rabbits, guinea pigs and mice by injecting peripheral nerve emulsified in complete Freund's adjuvant. Later, others (Levine and Wenk, 1963) used rats successfully. Smith *et al.* (1979) found that Lewis rats were more susceptible to EAN, compared to other strains of rats, rabbits and guinea pigs. Soon, the Lewis rat model of EAN became a widely accepted animal model for GBS (Saida *et al.*, 1981). Clinically, EAN is an acute disease appearing two weeks following immunization and consisting of ataxia and ascending limb weakness (Steiner and Abramsky, 1985). In the Lewis rat model developed by Smith *et al.* (1979), the animals affected with EAN showed weakness of all extremities, leg splaying, inability to hold head upright, facial weakness with loss of blinking, and laboured, shallow respirations. Hind leg dragging and unusually flaccid and weak forelimbs were seen in some animals. Nerve roots of rats injected with bovine root myelin showed EAN lesions characterized by infiltration of

lymphocytes and other mononuclear cells, and demyelination in the areas of inflammation. EAN was produced by injection of myelin from the peripheral nervous system of rat, rabbit, cattle and human.

Experimental autoimmune neuritis has been developed in monkeys and dogs. A "hyperacute" form of EAN could be produced in primates by the addition of pertussis vaccine to the standard neuritogenic dose of Freund's adjuvant and peripheral nerve homogenate (Behan *et al.*, 1971). In rhesus monkeys sensitized with rabbit sciatic nerve myelin, clinical signs also involved cranial nerves (Wisniewski *et al.*, 1974). A canine model of EAN produced by intradermal inoculation of canine nerve tissue, was similar to CHP/ACP in lesions and clinical signs except for the more acute nature of EAN (Holmes and deLahunta, 1974). Recently, Constantinescu *et al.* (1998) developed a novel murine model using SJL/J strains of mice, which will be useful because of the availability of transgenic and knockout mice for numerous immunologically relevant genes.

EAN can be induced by active immunization with peripheral nerve myelin, with single myelin components, or with adoptive transfer of autoimmune T cells (AT-EAN) reactive with myelin proteins such as P2 or P0 (Hartung *et al.*, 1988 and Archelos *et al.*, 1994). Hartung *et al.* (1988) in their review on the immune mechanisms of GBS, compared and discussed the possible mechanisms involved in GBS with the experimental models. In human GBS cases, in addition to demyelination, axonal degeneration can occur, depending on the severity of demyelination, although in some cases these may occur independently. Hartung *et al.* (1988) noted that a mild and entirely demyelinating form of actively induced EAN can be induced by immunization of rats with small doses of myelin, and a more severe

variety with extensive demyelination and significant axonal damage in animals via a large dose of the immunogen. In AT-EAN, the injection of myelin protein P2-specific lymphocytes into Lewis rats produces fulminant neuritis, characterized electrophysiologically by conduction failure resembling acute nerve transection and morphologically by axonal degeneration and prominent endoneurial edema (high cell dose). Transfer of a smaller number of line cells produces a milder condition with later onset of signs of conduction slowing and, histologically, signs of predominant demyelination (Hartung *et al.,* 1988).

The role of humoral factors in the pathogenesis of GBS has been studied experimentally with variable results. Intraneural injection of experimental allergic neuritis serum resulted in demyelination in normal recipient animals (Saida *et al.*, 1978 and Sumner *et al.* 1982a). Antiserum against galactocerebroside, a major component of peripheral nerve myelin, can induce demyelination on intraneural injection (Saida *et al.*, 1979). Later, intra neural injection of serum from GBS patients into rat sciatic nerves caused acute demyelination, although this activity was also seen to a lesser extent with a few non-GBS sera (Saida *et al.*, 1982). Acute conduction block and demyelinating activity of GBS patient sera following subperineurial injection into rat sciatic nerves was demonstrated by others as well (Sumner *et al.*, 1982 b and Harrison *et al.,* 1984). Conduction block was maximal at 5 days after injection, and demyelination appeared to evolve both by vesicular disruption and by macrophage mediated myelin stripping (Sumner *et al.*, 1982 b). These results suggested that demyelinating factor in the serum may be important in the pathogenesis of GBS. Attempts to produce an animal model by systemic transfer of serum were not generally successful. An attempt to determine if acute canine polyradiculoneuritis is immune-based

was made by Griffiths *et al.* (1983) who injected serum from a canine case into rats. However, there was no evidence of demyelination. This early work was a step towards developing an animal model for ACP, and indirectly for GBS. Recently, intraperitoneal injection of serum from GBS patients into mice produced GBS-like signs, and changes in peripheral nerve function (van den Berg *et al.*, 1994 and Notermans *et al.*, 1992).

Bronsnan *et al.* (1988) noticed the differences in disease pattern according to age groups of animals in the Lewis rat EAN model. Older animals exhibited severe acute illness which was monophasic over the period of observation, whereas younger animals developed less severe and frequently relapsing illness, unlike GBS in humans. Powell and Myers (1996) indicated the sharply contrasting patterns of histological injury across species in the EAN models. For instance, the primary demyelination is usually obliterated by intense axonal injury in the guinea pig model of EAN.

1.3.3.4 Experimental Animal Models Involving *C. jejuni*

There is a need for experimental models to identify strains of *C. jejuni* that can cause damage to peripheral nerves via various immune mechanisms. Vriesendorp (1997) indicated that the immune mechanisms by which infection with *C. jejuni* can create peripheral injury, targeted primarily to axons, should be studied. In this regard, Li *et al.* (1996) demonstrated development of paralysis in chickens experimentally infected with GBS-causing *C. jejuni* strain. Paralysis developed 5 to 18 d post-infection, and sciatic nerves of some birds exhibited evidence of degeneration, including demyelination. Published evidence of *C. jejuni*- induced animal models are totally lacking after the work carried out by Li *et al.*, 1996.

1.4 Objectives of the Study

The goal of this study was to understand the relationship between infection with *Campylobacter jejuni* and Guillain Barré Syndrome. The specific objectives of this research are:

1. to determine if *C. jejuni* strains involved in GBS share antigen(s) with nerve components and alter peripheral nerve function (*in vivo*) and react with human neural cells (*in vitro*); and
2. to determine if infection with *C. jejuni* is significantly associated with GBS in the Maritimes and to identify possible sources of infection.

2.0 THE EFFECTS OF ANTIBODIES AGAINST *CAMPYLOBACTER JEJUNI* ON PERIPHERAL NERVE FUNCTION (*IN VIVO*) AND HUMAN NEURAL CELLS (*IN VITRO*).

2.1 ABSTRACT

Antibodies specific for *Campylobacter jejuni* strains, commonly incriminated in Guillain Barré Syndrome (GBS), were tested *in vivo* to determine effects on peripheral nerve function and *in vitro* for their interaction with cultured neurons. Rabbit antisera to different strains of *C. jejuni* were injected into male Sprague-Dawley rats (Charles River Laboratories) and electrophysiological measurements of the femoral nerve were made prior to serum injection (Day 0), and on Days 2, 7, and 14 after injection. A two-way Analysis of Variance (ANOVA) showed interaction between treatment and day, and contrast testing within days showed significance ($p<.05$) for Penner O:19 H on Day 2 and for GBS 4382/ 4384 H and Penner O:19 H on Day 7 specifically for Threshold Compound Action Potential (TCAP). To test reactivity of serum antibodies with neurons, 7 cultured human neural cell lines were evaluated using sera against different *C. jejuni* strains by immunofluorescence. Positive reactions with rabbit antibodies were noted in 2 of the 7 cell lines. The serum antibodies from rabbits immunized with serotype Penner O:19 F reacted with 75% of the cells of the SK-N-SH neuron cell line. Serum antibodies from rabbits immunized with Lior 7:11 F gave a 5% reaction in the MO3.13 Oligo cell line. Serum antibodies against certain strains of *C. jejuni* affect peripheral nerve function and react immunologically *in vitro* with certain neural cell lines. These effects were most evident with antibodies against the Penner O:19 serotype, indicating only certain serotypes may be involved in GBS.

2.2 INTRODUCTION

Certain strains of *C. jejuni* may share common structure with nerves, and an immune response initially mounted against *C. jejuni* may be misdirected to peripheral nerves, resulting in the clinical signs of GBS (Allos and Blaser, 1994). In this respect, certain serotypes of *C. jejuni* may be more pathogenic than others. The serotypes Penner O:19, Lior 7 and Lior 11 are most commonly associated with GBS (Fujimoto *et al.*, 1992, Rees *et al.*, 1993 and Hartlung *et al.*, 1995), although predominance of any particular serotype was not observed in a recent British study (Rees *et al.*, 1995). The lipopolysaccharide (LPS) of *C. jejuni* serotype Penner O:19 has striking homology with peripheral nerve antigens such as gangliosides GM1, and cross reacts in immunological assays (Aspinall *et al.*, 1994 and MacDonald *et al.*, 1994).

The Lewis rat model of experimental autoimmune 'allergic" neuritis (EAN) is a widely accepted animal model for GBS; however only in the effector phase (Saida *et al.*, 1981). Thus, the absence of a reproducible specific animal model limits our understanding of *C. jejuni* - primary axonal GBS. There have been reported similarities in several natural and experimental animal models. Van den Berg *et al.* (1994) and Notermans *et al.* (1992) found botulism-like signs, including insufficient respiration and resulting in a wasp-like waist, in mice injected with serum from GBS patients, and suggested that this model used for studying botulism toxin, may also be valuable in GBS research. Saida *et al.*, (1982), produced GBS-like signs and changes in peripheral nerve function by systemic administration of GBS patient serum via intraperitoneal route into mice (van den Berg *et al.*, 1994 and Notemans *et al.*, 1992). A disease model involving a species for which many

investigative tools are available would be useful to further analyse the immune mechanisms by which infection with *C. jejuni*, in particular, can create peripheral nerve injury targeted to axons or myelin (Vriesendorp, 1997).

A proposed mechanism for *C. jejuni*-triggered GBS is antigenic mimicry and the ability of the immune response to induce endoneural inflammation, some of which is directed toward the Schwann cell (Koski, 1997). This multipotential glial cell, vital for the normal physiologic function of peripheral nerves, has been proposed to be capable of contributing to the inflammatory process through the production of cytokines, acute-phase reactants, and growth factors. In GBS both complement activation and antibody production occur. Detailed pathology studies have shown early complement deposition on the Schwann cell membrane in demyelinating GBS, while complement is deposited in the adaxonal membrane in axonal GBS. (Hafer-Macko, 1996a and Hafer-Macko, 1996b). The frequent association of GBS and *C. jejuni* infection has indicated antibodies with specificity for anti-gangliosides which bind to the axon at the node of Ranvier and at the motor endplate (Illa *et al.*,1995). These anti-ganglioside antibodies apparently are generated in response to *C. jejuni.* This hypothesis that antibodies against *C. jejuni* specifically are responsible for neural damage has not been adequately tested. Furthermore, neural cell line testing on neural lines originating from the central nervous system has not been tested. Although GBS is well documented as a peripheral nerve disease, there may be CNS involvement.

Therefore, the current study examined the effects of antiserum from rabbits immunized with strains of *C. jejuni* on peripheral nerve function in rats and in cultured human neural cells of CNS origin. Specifically, our objective was to determine if antibodies

against specific strains of *Campylobacter* produced electrophysiological change in rat nerve, indicating peripheral nerve dysfunction, and if these antibodies could react with human neural cell lines, suggesting potential damage.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial Isolates and Antigen Preparation

Cultures of *C. jejuni* associated with GBS were received from the National Laboratory for Enteric Pathogens at the Laboratory Centre for Disease Control (LCDC), Bureau of Microbiology in Ottawa and were identified as follows: GBS 4382, GBS 4384, Lior 7, Lior 11, and Penner O:19.

2.3.1.1 Whole Cell Preparation for Immunization

The above strains were individually cultured on Preston's (without antibiotic) plates (Oxoid Laboratories) at 37°C for 48 h in a microaerophilic atmosphere. Cultures were suspended in PBS and adjusted to an optical density of MacFarland's #3 (Remel) and were either heat treated (H) at 62°C for 45 min or formalinized (F) with 0.2% formalinized PBS (pH 7.2) to produce 10 whole cell preparations. Treated preparations showed no growth when cultured. Optical densities were standardized to 1 using an LKB Biochrom: Ultropec II Spectometer at 540 nm wavelength and all suspensions were washed 3 X with PBS and resuspended in preparation for immunization. Serotype classifications were pooled and contained equal quantities, by volume, for the Lior and GBS strains. For immunization, antigen preparations were pooled as follows:

1. Penner O:19 H
2. Penner O:19 F
3. Lior 7 and 11 H
4. Lior 7 and 11 F
5. GBS 4382 and 4384 H
6. GBS 4382 and 4384 F

2.3.1.2 LPS Preparation for Immunization

For LPS extraction, assistance was received from Dr. Paul Hoffman, Department of Microbiology and Immunology, School of Medicine, Dalhousie University, Halifax. *Campylobacter jejuni* strains GBS 4382, GBS 4384 and Penner O:19 were incubated overnight in 1 litre of *Brucella* broth under microaerophilic conditions in a CO_2 incubator (5% CO_2, 7% O_2) and then for up to 48 h on an orbital shaker at room temperature. Bacteria were harvested (8000 x g for 10 min) in normal sterile saline (0.85%). Crude LPS was extracted by the hot phenol-water technique (45%) (Moran *et al.*, 1994, Moran *et al.*, 1992 and Mills *et al.*, 1985). After 73 h of dialysis against distilled water (3 changes), crude LPS was lyophilized and purified by enzyme treatment with DNase, RNase and by proteinase K. After overnight dialysis against water, LPS was pelleted at 100,000 x g for 20 h, re-dissolved in distilled water and lyophilized.

2.3.2 Immunization Schedule for Production of Anti-Sera in Rabbits (Whole Cell and LPS)

After pre-immune bleeding, 6 New Zealand White female rabbits (Charles River

Laboratories) weighing 2 - 2.5 kg were injected subcutaneously in the neck with equal portions of increasing amounts (0.25, 0.5, 1.0, 1.5 and 2.0 ml) of whole cell antigen (see 2.3.1 Bacterial Isolates and Antigen Preparation). One week after the final immunization, blood was collected, serum obtained and titrated. Fifty to 60 ml blood was collected from each rabbit, including controls (not immunized), using cardiac puncture under terminal barbituate anaesthetic. Samples were left at room temperature (18°C) overnight and then centrifuged (Beckman Model TJ - 6) at 200 x g for 30 minutes and serum collected. Antibody levels were tested by titration using the bacterial plate agglutination technique. The highest dilution of antiserum showing agglutination was taken as the titre (See Table 2.1). The highlighted (bold print) titers represent those used in the experiments in this study, chosen due to their response levels. The initial dilution of the antiserum was 1:100 and the absence of agglutination at this dilution was considered a negative reaction. Serum samples were separated, dispensed in 1.5 ml aliquots, and frozen at -80°C.

Lyophilized LPS of the 3 preparations (See section 2.3.1.2.: LPS Preparation for Immunization) were utilized in the following manner. Penner O:19 was prepared as a single unit and GBS 4382/4384 were combined. Both were suspended into a saline stock alum (Imject ®ALUM-77161, Sigma) solution in preparation for immunization. The doses were based on the amount of purified LPS. Following a pre-immune bleeding, rabbits were inoculated intramuscularly with LPS prepared antigens 4 X over a 1 month period to administer a total of 99 and 104 μg of LPS for Penner O:19 and GBS 4382/4384, respectively. The LPS portion of the study showed no response using ELISA through a standard solid phase enzyme immunoassay indicating that antibodies were not raised against

Table 2.1: Agglutination titres of rabbit antisera raised against strains of *Campylobacter jejuni.*

	Whole Cell Antigen: Strain Identification of *C. jejuni*				
Immunized against:	GBS4382	GBS4384	Penner O:19	Lior 7	Lior 11
Penner O:19 H	1/800	**1/800**	1/800	>1/6400	>1/6400
Penner O:19 F	1/400	1/400	1/200	1/400	1/400
Lior 7 and 11 Combo H	1/400	1/400	1/400	**>1/6400**	>1/6400
Lior 7 and 11 Combo F	1/1600	1/800	>1/6400	>1/1600	**>1/6400**
GBS 4382 and 4384 H	**>1/6400**	1/3200	>1/6400	>1/6400	>1/6400
GBS 4382 and 4384 F	1/800	1/1600	1/400	1/200	>1/6400
Control	<1/100	<1/100	<1/100	<1/100	<1/100

these strains.

2.3.3 Experimental Models

2.3.3.1 Experimental Groups

Among the 6 rabbits immunized with antigen preparations, the three that gave high titers (Table 2.1) were selected for experiments in rats to determine effects on peripheral nerve function. The preparations used for injection against rats were as follows:

1 Antiserum against GBS 4382/ 4384 (heat treated only)

2 Antiserum against Lior 7 & 11 (combination of heat treated and formalinized)

3 Antiserum against Penner O:19 (heat treated)

4 Saline Control

5 Pre-immune Serum Control

A total of 48 male Sprague-Dawley rats (Charles River Laboratories) were divided into 3 groups for a triplicate experiment on the 5 preparations. Within each group, the effects of three antibodies (those listed above) were tested for their effect on peripheral nerve function. A saline control and a pre-immunized control was used in each group. Within each group, rats were tested on Days 2, 7 and 14. On Day 0, the day of inoculation, control rats (no treatment, n=3) were included to provide baseline measurements.

2.3.3.2 Electrophysiological Assessment

Electrophysiological assessments of peripheral nerve function were completed in

the neurophysiology laboratory of Dr. Tarek Saleh, Atlantic Veterinary College. Once anaesthetized, the femoral nerve was isolated from the gastrocnemius muscle on the dorsal surface of the rat's left hind limb. A stainless steel, bi-polar stimulating electrode was placed on the proximal end and platinum recording electrode placed on the distal end. The distance between the recording and stimulating electrodes were measured for use in conduction velocity calculations. Both electrodes were fixed in place with dental impression material. The nerve was stimulated at increasing intensities and the peak amplitude of the Compound Action Potential (CAP) was measured and used to construct a stimulus intensity - response curve. The intensity was fixed at approximately 1.5 mA while the duration of the pulse was varied, in order to construct an intensity duration curve for each animal. At the end of the experiment, animals were euthanised with an overdose with somnitol (65 mg/kg). A Grass (Model S88) Stimulator (See Appendix A) was connected to the stainless steel stimulating electrode via a stimulus isolation unit (Grass Instruments, Wareick, Rhode Island). The evoked response was amplified and measured on an oscilloscope (BK Precision - Model 2522 - A). The amplitude and conduction of the compound action potential were measured for all nerve preparations. Recordings included the TCAP (threshold compound action potential), the maximum stimulus to reach TCAP or MSCAP, the maximal amplitude of CAP or MCAP and latency and conduction velocity.

2.3.3.3 Western Blotting

Western Blot was conducted on normal (untreated) rat nerve to compare immune and pre-immune rabbit antisera. Peripheral nerves were collected from 6 normal rats, and

homogenized and resuspended in sample buffer (1 ml of 0.5 M-Tris-HCL, pH 6.8; 0.8 ml of glycerol; 1.6 ml of 10% sodium dodecyl sulphate (SDS); 0.4 ml of dH_20) and heated to 100°C for 10 min. Rabbit antisera specific for 3 strains of *C. jejuni* and pre-immune control sera described earlier were used.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970). A stacking gel with 4% acrylamide was layered on to a 12% separating gel in a BioRad mini Protean II gel apparatus (BioRad, Canada). Five and 10 µl / lane of the molecular weight marker and the normal rat nerve, respectively were loaded and *C. jejuni* antisera at 4 µl / lane and 150 V was applied to the gel for 1 h at room temperature (18°C). Protein transfer was conducted according to Towbin *et al.* (1979). Gels were equilibrated in transfer buffer (25 mM Tris; 192 mM glycine; 20% methanol; pH 8.3) for 30 min before transfer of proteins to nitrocellulose with a BioRad blotting system running for 1 h at 100 V in room temperature.

The nitrocellulose blots were incubated overnight at 4°C in TRIS Buffer (TTBS, 20 mM Tris, 500 mM NaCl, 0.005% Tween-20) containing 3% BSA to block unbound sites and eliminate non-specific antibody binding. Blots were washed after each subsequent step 3 X for 10 min with TTBS on an orbital shaker. Blots were then incubated for 1 h at 37°C with rabbit anti-sera diluted 1:20 in antibody buffer (TTBS, 1% BSA). The secondary antibody solution was horseradish peroxidase anti-rabbit IgG in TTBS + 1% BSA . Finally the substrate (4 chloronapthal (TBS + hydrogen peroxidase)) was added and incubated at 37°C until color had developed, ~5-15 min. The reaction was stopped by washing the blots in distilled water (dH_20). Blots were left overnight in dH_20 in a 4°C refrigerator and dried the

following day.

2.3.3.4 Human Neural Cell Lines

Neural cell testing was completed at the Laboratory of Neuroimmunovirology, Institute Armand-Frapier, Laval, Quebec in collaboration with Dr. P.J. Talbot. An indirect immunofluorescence technique was used to determine if antibodies in sera of rabbits immunized with *C. jejuni* could bind to various neural cells. All tests were run in duplicate. Seven cultured human neural cell lines (MO3.13-Oligo, H4 -Neuron, SK-N-SH-Astrocyte, U-87MG-Astrocyte, U-373MG Microglia, CHME - Astrocyte and GL-15-Astrocyte) and the L132 cell line (control) was used (as described in Arbour et al., 1999a and 1999b). Primary antibody consisted of the rabbit antisera and positive and negative controls.

Cell monolayers were washed with PBS, dissociated with 0.25% (w/v) trypsin (Canadian Life Technologies), resuspended in DMEM (Canadian Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS), then centrifuged for 7 min at 340 x g in a Beckman GS-6R centrifuge.

Cells were resuspended in DMEM containing 10% (v/v) FBS and 25 µl of the suspension (final concentration at 5 X 10^5 cells/mL) was dispensed into the well of a 12-unit glass slide (ICN Biomedical). The slides were incubated in a humidified chamber incubator at 37°C and 5% (v/v) CO_2. Slides were then washed once with PBS and fixed in 100% acetone for 20 min at -20°C. Slides were stored at -20°C.

For immunofluorescence, the primary rabbit antibody was added at the given dilutions and incubated in a humidified chamber for 1 h at 37°C. This was followed by three

washes with PBS. Then, fluorescein-conjugated goat anti-rabbit IgG (Fab'_2 fragment) secondary antibody (1/1000 dilution) was added. Cells were incubated for 30 min at 37°C. Three additional washes in PBS were performed, followed by one in distilled water. Evan's blue (0.003%) w/v was used in the last wash as counterstain and incubated for 15 min. Finally, the slides were mounted in glycerol: PBS (9:1). Slides were observed and immunofluorescence determined with a Leica epifluorescence microscope.

2.4 STATISTICAL ANALYSIS

2.4.1 Peripheral Nerve Function

Data and measurements recorded during the experiment were summarized. Direct readings were used to calculate CAP, TCAP, MSCAP, MCAP, latency and conduction velocity; mean values were utilized in the statistical analysis to compare treatments and controls. Effect by day, and by treatment was tested for each variable.

A two-way Analysis of Variance (ANOVA) was performed on log transformed (natural logarithm) data to test the null hypothesis that treatments and controls had no effect or were equal regarding day and treatment. When significant interaction between treatment and day was observed, interaction plots displayed possible differences of treatments within days, and contrast testing within days by treatment was used to detect specific differences in treatments for each of the parameters measured.

All statistical analyses were carried out using the program MINITAB ™ Inc. Differences were considered significant at the α=0.05 level of probability.

2.4.2 Human Neural Cell Lines

For the classification of immunofluorescence, the legend for the interpretation of results is defined in Table 2.2. The codes presented in Table 2.2 are regularly utilized in the laboratory at the Institute Armand Frappier to determine reactivity levels (provided courtesy of Dr. Pierre Talbot). Samples were determined to be in one of three classes: definite negative, weak positive and definite positive. Those identified as definite positives were assigned percentages that reflected the proportion of cells that were affected.

2.5 RESULTS

2.5.1 Peripheral Nerve Function

There was a significant effect ($p<.05$) of rabbit antiserum against certain strains of *C. jejuni* on aspects of peripheral nerve function suggesting that some serotypes may have similar antigenic determinants shared by peripheral nerves. In addition to the statistical representation of results, trends were also noted and compared with those expected in the case of peripheral nerve dysfunction, by observing Main Effects graphically. The control rats ($n=3$) at Day 0, the day of inoculation, gave similar resutls to saline and pre-immune control (not included in graphical results).

Rats injected intraperitoneally with *C. jejuni*-specific antibodies showed changes in electrophysiological properties of the femoral nerve. A two-way Analysis of Variance (ANOVA) showed significant ($P<.045$) interaction between treatment and day, and contrast testing within days showed significance ($p<.05$) for Treatment 3 (Penner O:19 H) on Day

Table 2.2 **Scale for quantitative grading of binding of *Campylobacter jejuni*-specific antibodies to neural cell lines.**

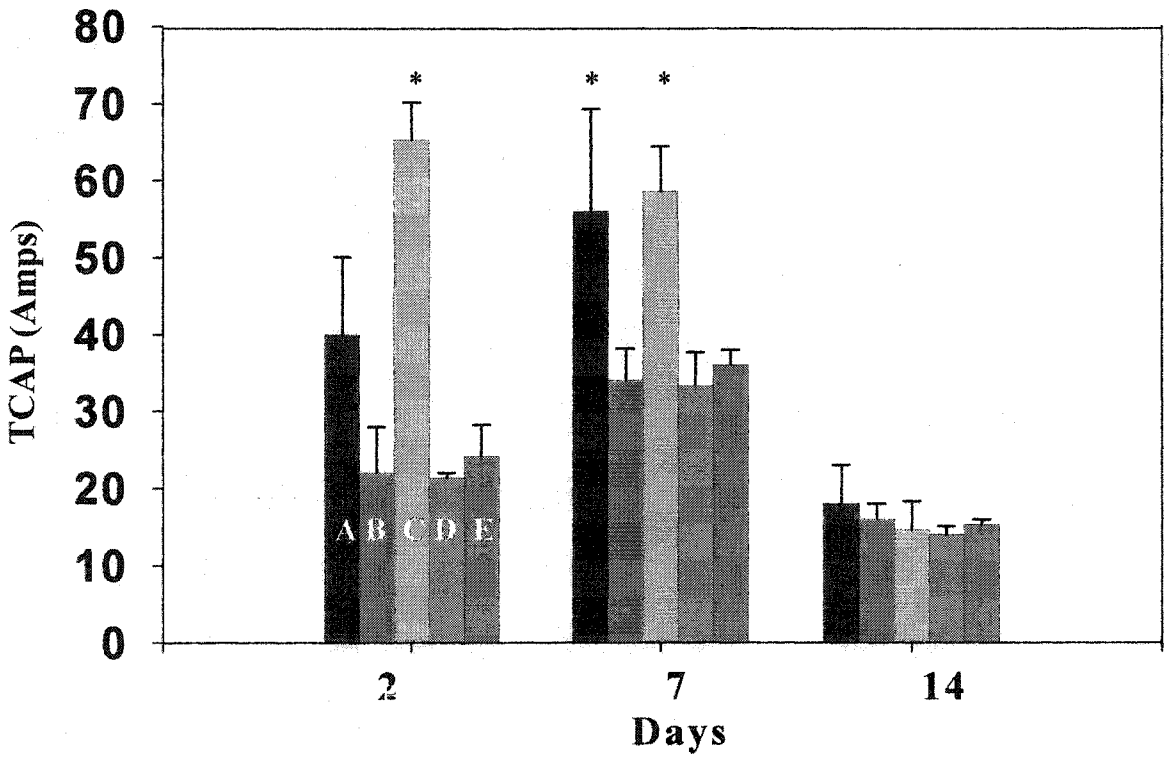
Code	Description of Fluorescence
0	Definite Negative (no Fluorescence)
	From 1 to 3: possible weak positives with non-specific background
1	Fluorescence was hardly visible
2	10 - 50% of non-specific fluorescence
3	50-100% of non-specific fluorescence
	Positive: definite specific fluorescence was detected
4	cut-off level for positivity: 1+
5	2+
6	3+
7	4+

2 and for Treatments 1 (GBS 4382/4384 H) and 3 (Penner O:19 H) on Day 7, specifically for Threshold CAP (Figure 2.1). There were no significant differences or trends by day between treatment and control groups for conduction velocity (Figure 2.2), Maximal Amplitude of CAP (Figure 2.3), MSCAP (Figure 2.4), or latency (Figure 2.5). Clinical signs of peripheral nerve damage was not noted in any of the animals during the experiment.

2.5.2 Western Blotting

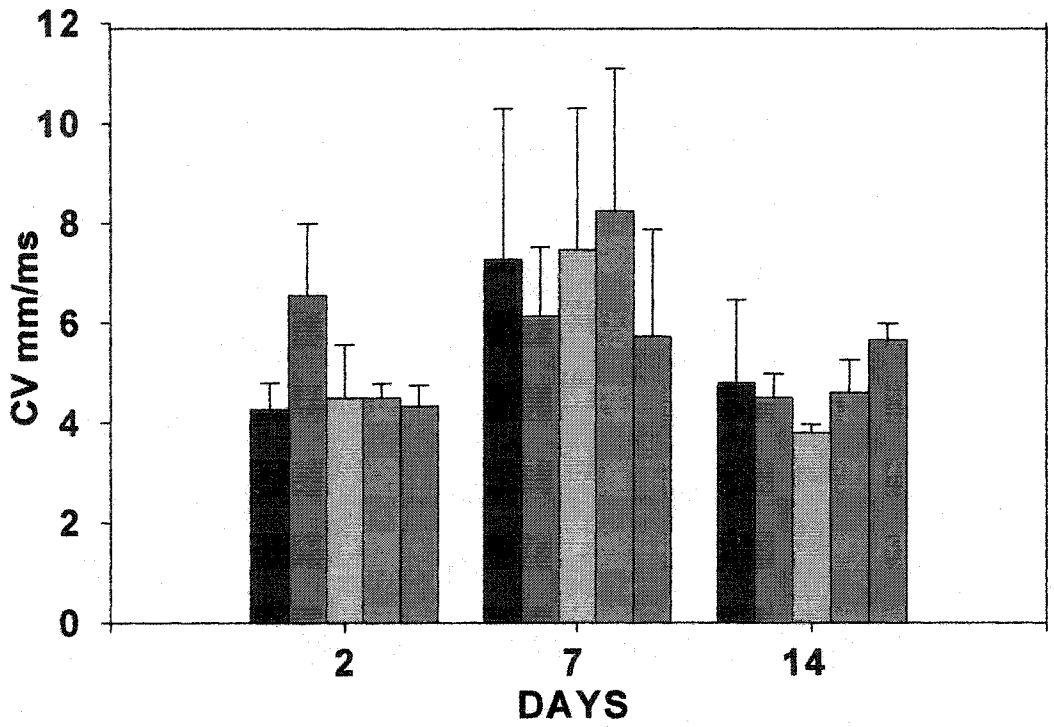
To identify if serum from *C. jejuni* immunized rabbits reacted with rat nerve components, Western blotting was performed. Figure 2.6 shows bands revealed by this technique. This reaction is represented by three notable bands (color coded circles) as presented in Figure 2.6. Band 1 falls between markers 38 and 51 kD, ~ 45 kD and was apparent in rabbit antisera to Penner O:19 H (Lane 4) and GBS 2964 HF (Lane 8) ; both marked with red circles). A second notable band was observed with antisera to strains Lior 7:11 HF (Lane 6) and GBS 4382/4384 H (Lane 8); marked with green circles) at ~62 kD. A third band of distinction was noted in antisera to the Penner O:19 H (faintly visible; Lane 4) and GBS 4383/4384 H (marked with blue circles; Lane 8) strains.

These findings suggest that there is a reaction between rat nerve and *C. jejuni* which may correlate to the results observed in the *in vivo* study where changes were noted in femoral nerve function of rats. Thus, rabbits immunized with *C. jejuni* have antibodies in their serum that are reacting to rat nerves.



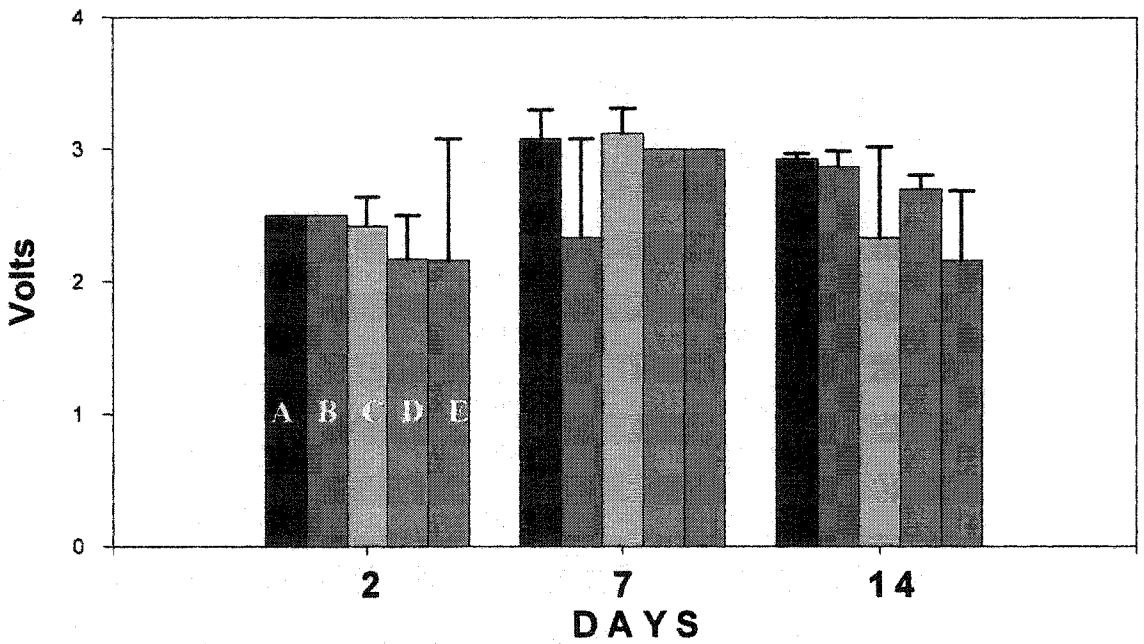
Legend:
A = Treatment 1: GBS 4382 / 4384: H
B = Treatment 2: Lior 7 and 11: H F
C = Treatment 3: Penner O:19: H
D = Treatment 4: Saline Control
E = Treatment 5: Pre-immune Control

Figure 2.1: **Effects of rabbit antisera to *Campylobacter jejuni*, normal rabbit sera and saline on Mean threshold CAP (TCAP) of femoral nerve function in rats. * represents significant differences at $p<.05$, relative to both controls, as determined by statistical analysis. T : Bars correspond to Standard Error of the Mean (SEM).**



Legend:
A = Treatment 1: GBS 4382 / 4384: H
B = Treatment 2: Lior 7 and 11: H F
C = Treatment 3: Penner O:19: H
D = Treatment 4: Saline Control
E = Treatment 5: Pre-immune Control

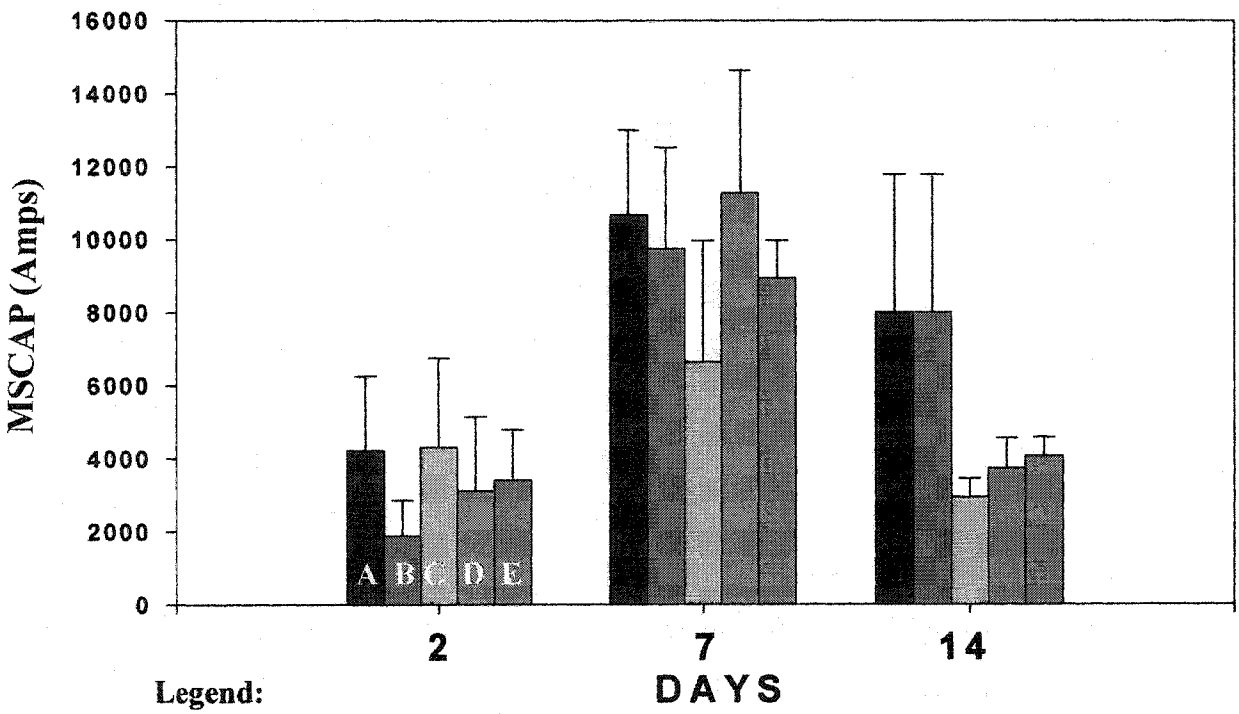
Figure 2.2: Effects of rabbit antisera to *Campylobacter jejuni*, normal rabbit sera and saline on mean conduction velocity (CV) of femoral nerve in rats. T : Bars correspond to Standard Error of the Mean (SEM).



Legend:

A = Treatment 1: GBS 4382 / 4384: H
B = Treatment 2: Lior 7 and 11: H F
C = Treatment 3: Penner O:19: H
D = Treatment 4: Saline Control
E = Treatment 5: Pre-immune Control

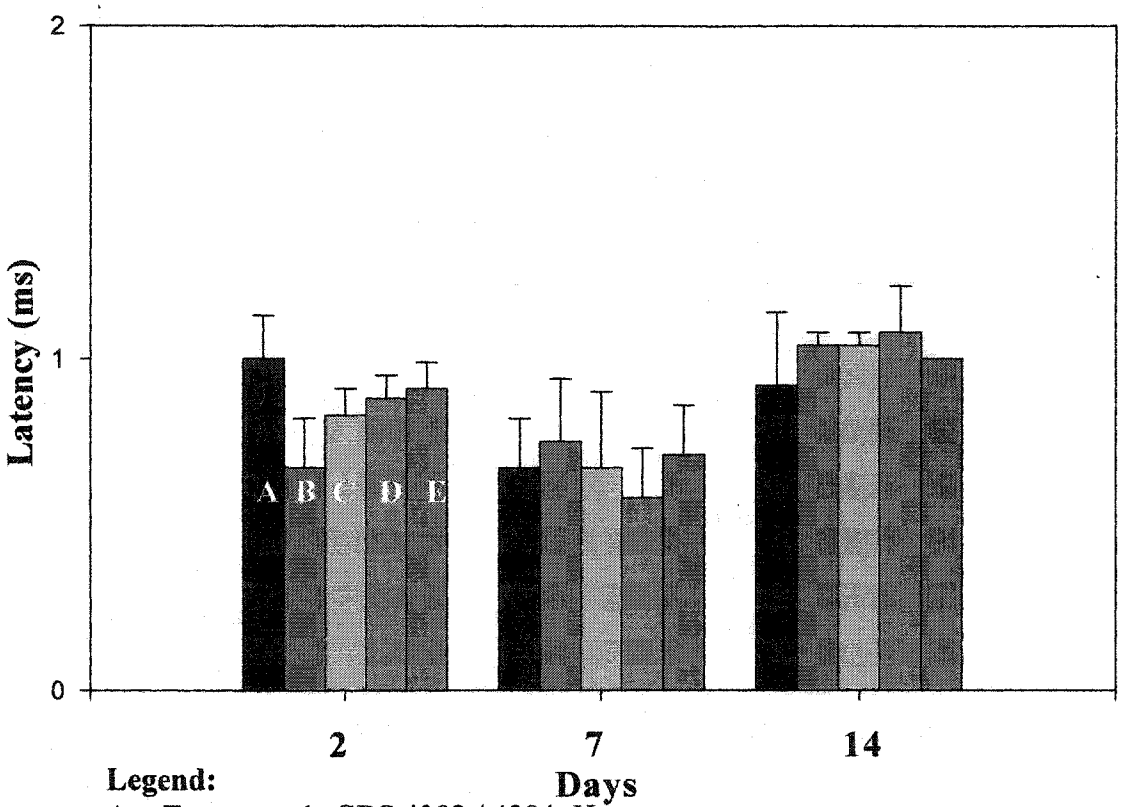
Figure 2.3: Effects of rabbit antisera to *Campylobacter jejuni*, normal rabbit sera and saline on mean maximum amplitude of CAP of femoral nerve in rats. T : Bars correspond to Standard Error of the Mean (SEM).



Legend:

A = Treatment 1: GBS 4382 / 4384: H
B = Treatment 2: Lior 7 and 11: H F
C = Treatment 3: Penner O:19: H
D = Treatment 4: Saline Control
E = Treatment 5: Pre-immune Control

Figure 2.4: Effects of rabbit antisera to *Campylobacter jejuni*, normal rabbit sera and saline on mean maximum stimulus required to reach CAP (MSCAP) of femoral nerve in rats. T : Bars correspond to Standard Error of the Mean (SEM).



Legend:

A = Treatment 1: GBS 4382 / 4384: H
B = Treatment 2: Lior 7 and 11: H F
C = Treatment 3: Penner O:19: H
D = Treatment 4: Saline Control
E = Treatment 5: Pre-immune Control

Figure 2.5: Effects of rabbit antisera to *Campylobacter jejuni* antibodies, normal rabbit sera and saline on mean latency of femoral nerve in rats T : Bars correspond to Standard Error of the Mean (SEM).

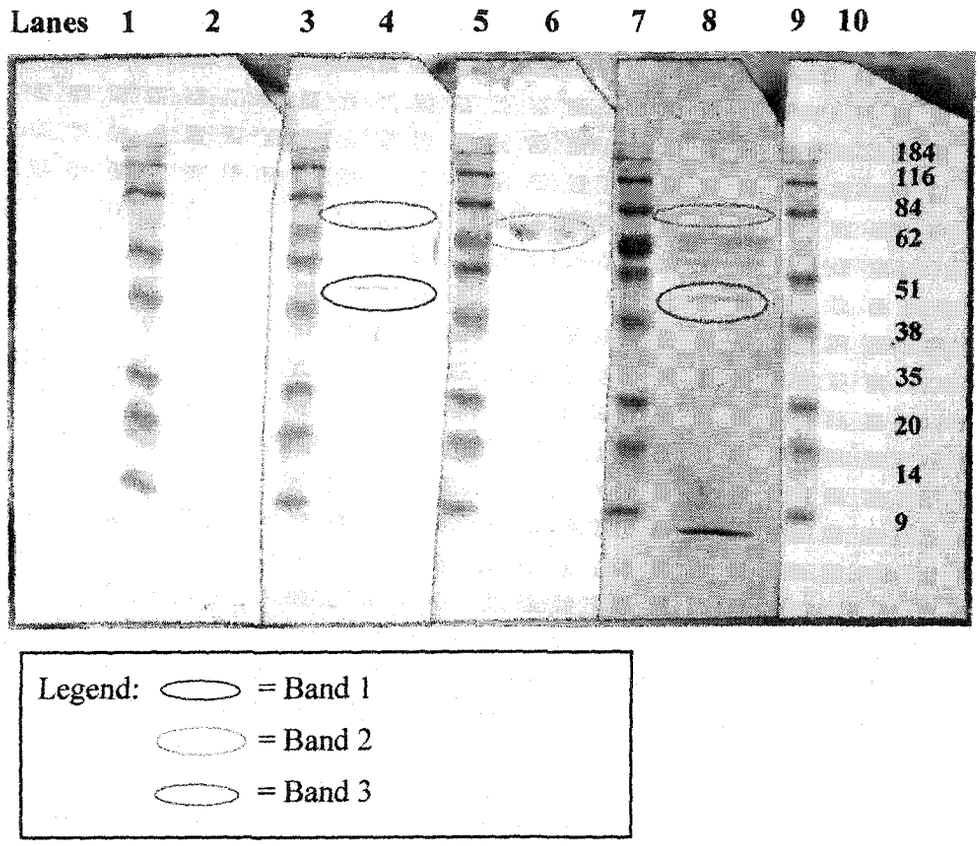


Figure 2.6: **Western Blot of sera from *C. jejuni* immunized rabbits with rat nerve. Lanes 1, 3, 5, 7 and 9 are normal rabbit antisera (pre-immune). Lanes 2, 4, 6, and 8 are rabbit antisera to Penner O:19 (heat-treated), Lior 7 & 11 (combination of heat treated and formalinized), GBS 4382/4384 H, and a negative control (no serum added), respectively. The numbered column to the right of Lane 10 are molecular weight markers.**

2.5.3 Neural Cell Lines

Immunofluorescence on neural cell lines are presented in Table 2.3. Two of the seven neural cell lines reacted in varying degrees with the rabbit antisera. Antibodies from rabbits immunized with serotype Penner O:19 F reacted with 75% of the cells of the SK-N-SH neuron cell line (See Figure 2.7), while serum from rabbits immunized with Lior 7:11 F reacted with only 5% of the MO3.13 Oligo cell line (see Figure 2.8). All other sera including the negative controls were considered negative (see Figure 2.9). Normal rabbit serum gave non-specific fluorescence (at dilution 1/250) in both the MO3.13 Oligo and U-373MG Astrocyte cell lines. The astromarker (rabbit anti-GFAP) displayed 1 weak positive on the SK-N-SH (neuron) at 1/500 dilution and 2 weak positives at dilutions of 1/250 and 1/500 on the U-373MG (Astrocyte) cell lines. Strong positives were observed in the astro marker (rabbit anti-GFAP) at both dilutions (1/250 and 1/500) on the U-373MG (astrocyte), CHME-5 (Microglia), and the Gl-15 (Astrocyte) cell lines (see Figure 2.10). The normal rabbit and rabbit anti-GFAP samples used as negative controls and an astro marker, respectively, displayed the expected results.

2.6 DISCUSSION

2.6.1 Peripheral Nerve Function

Electrophysiological measurement of femoral nerve function of rats indicated an altered function after intraperitoneal injection with antibodies against certain strains of *C. jejuni* of human origin and known to have been associated with GBS. A significant affect by day of *the C. jejuni* specific antibodies against the effect on peripheral nerve function in

Table 2.3: Immunofluorescence reactivity of rabbit anti-*Campylobacter jejuni* antisera with cultured neural cell lines.

Samples of Rabbit Sera	Dilution	MO3.13 Oligo	H4 Neuron	SK-N-SH Neuron	U-87MG Astrocyte	U-373MG Microglia	CHME - 5 Astrocyte	GL-15 Astrocyte
2962: Penner O:19- F	20	0	0	**4 (75%)****	0	0	0	0
	50	0	0	0	0	0	1	0
	titer	≤20	≤20	**20**	≤20	≤20	≤20	≤20
2963: Lior 7: 11 Combo -F	20	**4(5%)****	0	0	0	0	0	0
	50	0	0	0	0	0	0	0
	titer	**20**	≤20	**20**	≤20	≤20	≤20	≤20
2964:4383/84 Combo - H	20	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0
	titer	≤20	≤20	≤20	≤20	≤20	≤20	≤20
2965: Penner O:19 - H	20	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0
	titer	≤20	≤20	≤20	≤20	≤20	≤20	≤20

** degree of reactivity (% of reactivity)

Table 2.3: **Immunofluorescence reactivity of rabbit anti-*Campylobacter jejuni* antisera with cultured neural cell lines (continued).**

Samples of Rabbit Sera	Dilution	MO3.13 Oligo	H4 Neuron	SK-N-SH Neuron	U-87MG Astrocyte	U-373MG Microglia	CHME - 5 Astrocyte	GL-15 Astrocyte
2967: Lior 7: 11 Combo -F	20	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0
	titer	≤20	≤20	≤20	≤20	≤20	≤20	≤20
2968: Lior 7:11 Combo -F	50	0	0	0	0	0	0	0
	titer	≤20	≤20	≤20	≤20	≤20	≤20	≤20
2969 (Experiment Control)	20	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0
	titer	≤20		≤20	≤20	≤20	≤20	≤20
Normal Rabbit (Lab Reference)	20	0	0	0	0	0	**1**	0
	50	0	0	0	0	0	0	0
	titer	≤500	≤500	≤500	≤500	≤500	≤500	≤500
Rab. Anti GFAP (astro marker)	20	0	0	**2**	**1**	**6**	**4**	**5**
	50	0	0	0	**1**	**6**	**4**	**4**
	titer	≤250	≤250		≤250	〉500	≥500	≥500

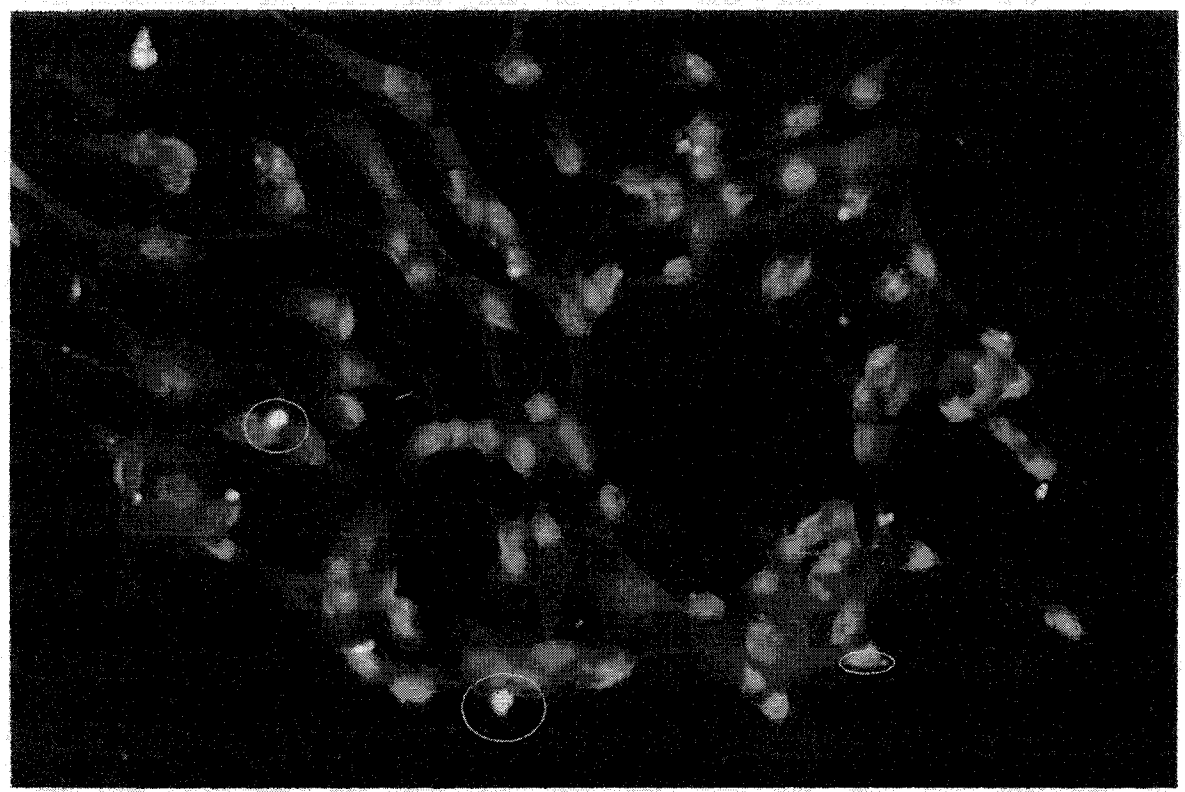


Figure 2.7: **Photo micrograph of immunofluorescence of rabbit anti-*C. jejuni* sera on cultured neural cell lines against Penner O:19-F with reactivity in 75% of the cells of the SK-N-SH neural cell. The fluorescent yellow area in the green circles above indicates some of the areas of reactivity.**

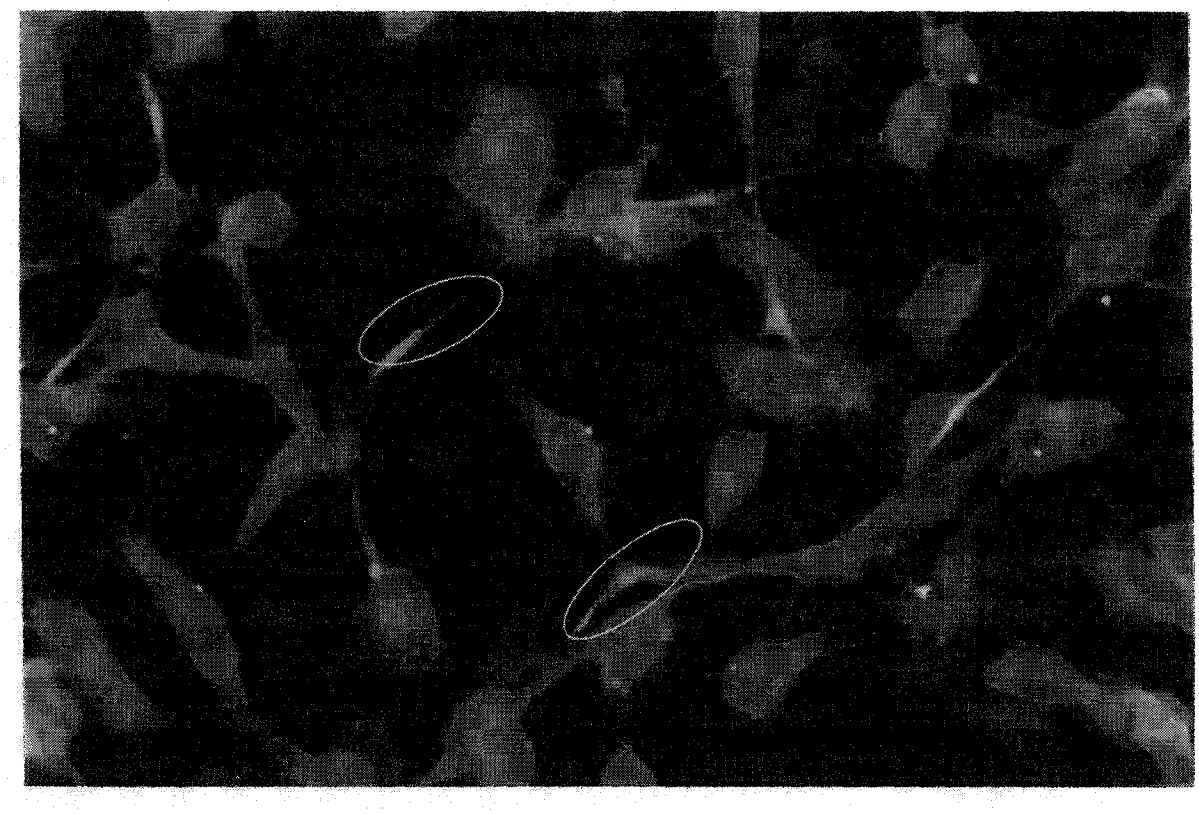


Figure 2.8: **Photo micrograph of immunofluorescence of rabbit anti-*C. jejuni* sera on cultured neural cell lines against Lior 7:11 F with reactivity in 5% of the MO3.13 Oligo cell line. The fluorescent yellow area in the green circles above indicates some of the areas of reactivity.**

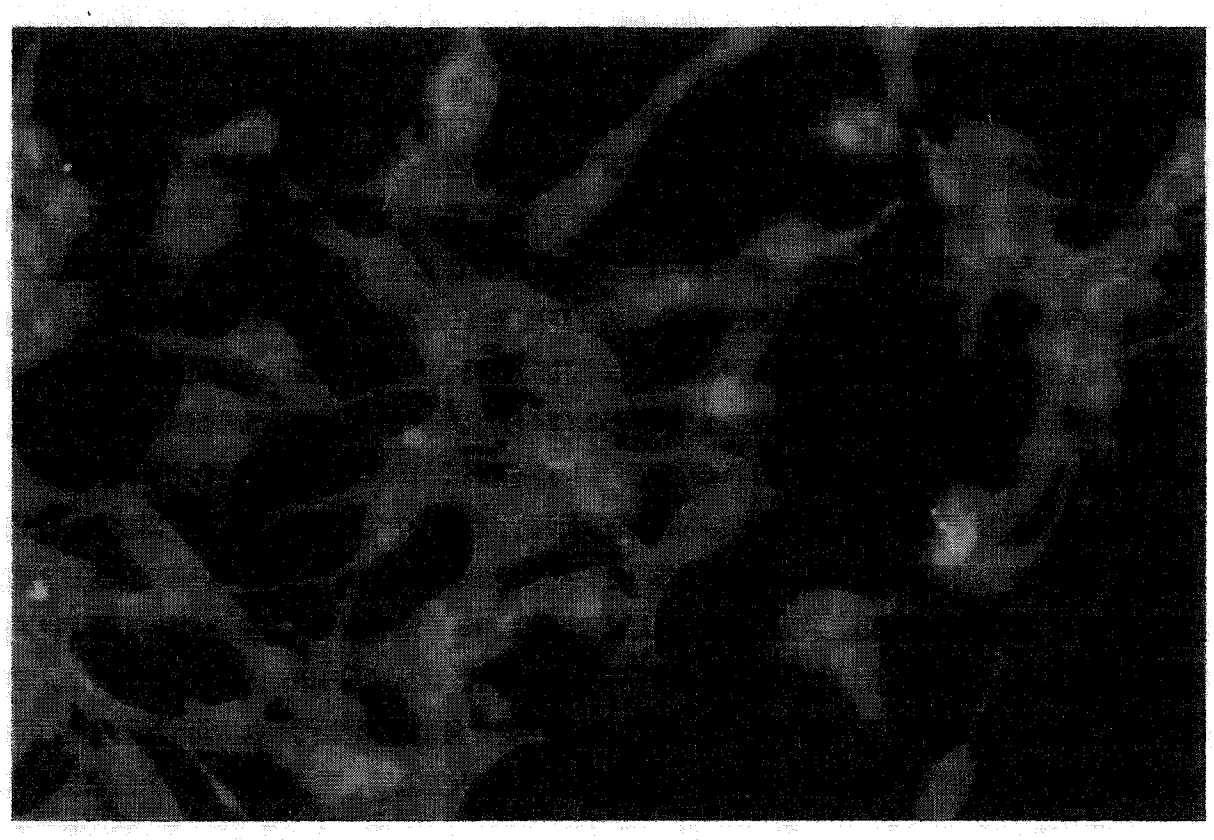


Figure 2.9: Photo micrograph of negative controls on cultured neural cell lines.

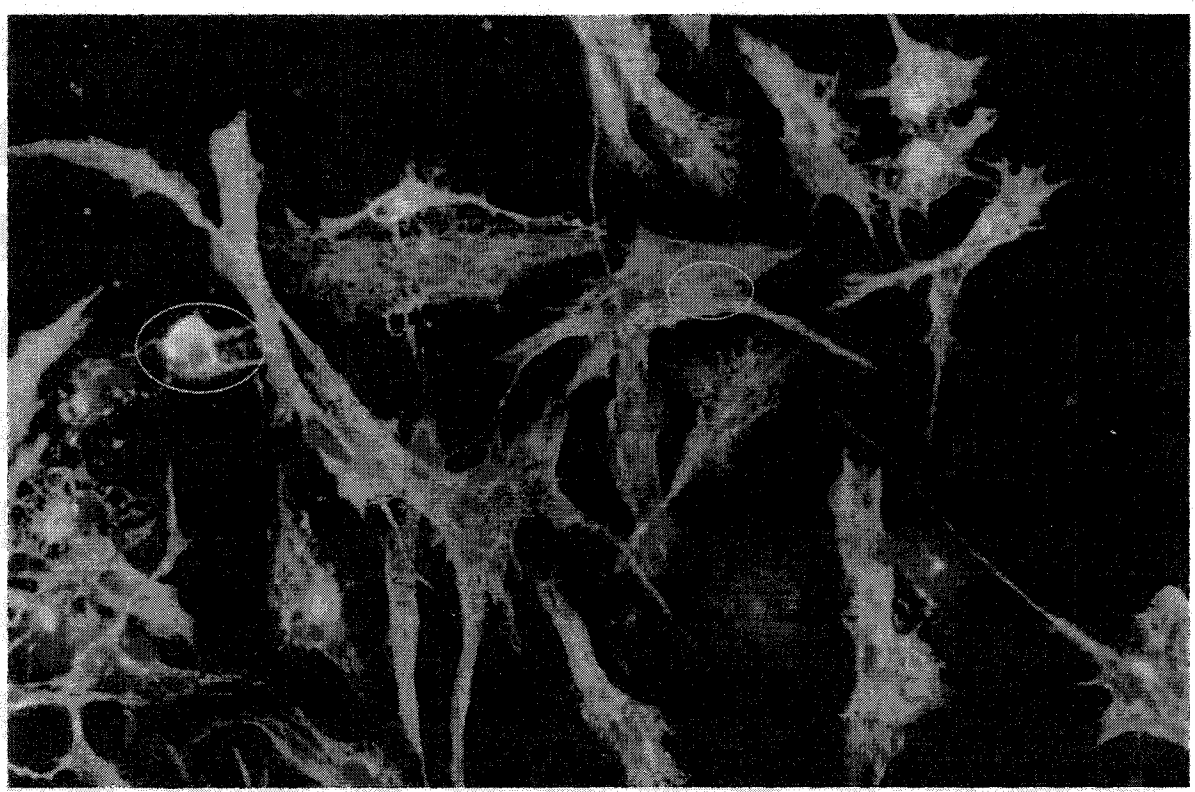


Figure 2.10: Photo micrograph displaying immunofluorescence in the astro marker rabbit (anti-GFAP) at dilutions 1/250 in the U-373MG (astrocyte) cell lines. The fluorescent yellow and green areas in the green circles represent some of the areas of reactivity.

rats, as expected, was observed. For example; the incubation period observed in other animals such as that in dogs (Homes et al., 1979) shows signs of conduction problems at 5 days. In the current study, maximal changes were observed on Day 7 and returned to normal on Day 14. Significance ($p<.05$) for Treatment 3 (Penner O:19 H) on Day 2 and for Treatments 1 (GBS 4382/ 4384 H) and 3 (Penner O:19 H) on Day 7 was noted specifically for Threshold CAP (TCAP), and indicated an increase in electrical current required to generate a nerve response, and thus indicating damage. Although significance was obtained, one must consider that multiple outcomes ($n = 5$) were studied. In the contrast comparisons, only the Day 2 significance would hold if a strict significance level ($p<.05$) was used

These results correlated with the results of the Western blot, where the treatment groups showing reactivity were GBS 4382/ 4384 H, Penner O:19 H and Lior 7 /11 H and F. These results suggest that animals immunized with *C. jejuni* of GBS 4382/ 4384 H, Penner O:19 H and Lior 7 /11 H and F have antibodies in their serum that are reacting to rat nerves. The noted increase in electrical current required to generate a nerve response in this study were similar to the studies of Fujimoto *et al.*, (1992), Rees *et al.*, (1993) and Hartlung *et al.*, (1995) for the serotypes Penner O:19 and Lior 7 and Lior 11. However, this study determined the same response with serum produced with the GBS 4382 / 4384 combination.

The observation that rats did not exhibit signs of clinical nerve dysfunction in the strains showing significant differences between treatments and controls may be due to the amount of treatment sera administered or delivery intervals. Van den Berg *et al.* (1994) suggested that if serum had been injected into mice for more that two consecutive days, more severe signs and prolonged disease could have ensued. Regarding the specificity of

whole cell components, and which ones are responsible for actions on the peripheral nerve function, we cannot comment as the LPS portion of the experiment was forfeited due to difficulties experienced in raising antibodies in rabbits.

2.6.2 Human Neural Cell Lines

There have been several unconfirmed suggestions for the basis of nerve dysfunction in *C. jejuni*- triggered GBS. Koski (1997) advanced the concept of antigenic mimicry and the ability of the immune response to induce endoneural inflammation directed toward the Schwann cell. Berciano *et al.*, 1999 recognized that the pathological basis of nerve inexcitability in Guillain-Barré syndrome has not been conclusively demonstrated and suggested that both primary demyelination and axonal degeneration, secondary to inflammation, are responsible. The frequent association of GBS and *C. jejuni* has been attributed to the specificity of anti-ganglioside antibodies able to bind to the axon at the node of Ranvier and at the motor endplate (Illa *et al.*,1995). These anti-ganglioside antibodies apparently are part of the antibody response to *C. jejuni*. However, the hypothesis that serum antibodies against *C. jejuni* are specifically responsible for neural damage has not been adequately tested.

The results of this experiment indicated reactivity of post- immune sera with whole cell antigen, from different strains of *C. jejuni*, toward human neural cell lines, indicating potential for neural damage. Specifically, reactivity was noted in lines representing neuronal and oligodendroglial cells, both majority cells in the central nervous system.

Neurons are organized in the human body with the vast majority existing entirely in

the brain and spinal cord, which is known as the Central Nervous System (CNS). The remainder form the bridge between the CNS and the tissues, and are designated the Peripheral Nervous System (PNS). The reaction of neurons of the central nervous system was not expected. Specifically, serum antibodies against Penner O:19 F displaying the 75% reaction on neural cells is consistent regarding the incriminated neurological effect of Penner O:19 serotype in Guillain Barré Syndrome. Lior 7:11 has been implicated in the pathogenesis of GBS. The reaction of antibodies to Lior 7:11 F with a neural cell line of oligodendroglia, at such a low percentage (5%) may be explained by the findings of Benn *et al.* (2001). Benn summarized that sensitivity of both Schwann cells and oligodendrocytes to laboratory agents during experimentation, such as those used in the procedures outlined in this experiment, may result in false positives. Nonetheless, considering that oligodendroglia are responsible for the production of myelin for neurons in the CNS, there may well be site specific binding to the contents of the whole cell antigen that cause the dysfunction of this glial cell. Thus, one could consider the role that reactive oxygen and nitrogen species play in demyelination, occurs in the inflammatory demyelinating disorders such as Guillain-Barré syndrome. According to Smith *et al.* (1999) the concentrations of reactive oxygen and nitrogen species (e.g. superoxide, nitric oxide and peroxynitrite) can increase dramatically under conditions such as inflammation overwhelming the antioxidant defenses within lesions. Oligodendrocytes are more sensitive to oxidative and nitrative stress *in vitro* than are astrocytes and microglia (both negative for reactivity to rabbit sera in this study), seemingly due to a diminished capacity for antioxidant defense, and the presence of raised risk factors, including high iron content. If we relate this to an *in vivo* model then,

oxidative and nitrative stress might cause selective oligodendrocyte death resulting in demyelination. In addition, the reactive species may also damage the myelin sheath, promoting its attack by macrophages. Smith *et al.* (1999) reports that evidence for the existence of oxidative and nitrative stress within inflammatory demyelinating lesions includes the presence of both lipid and protein peroxides, and nitrotyrosine (a marker for peroxynitrite formation).

A negative reaction for all serum antibodies against the H4 (neuron) cell line was expected. The H4 cell line is from a neuroglioma (ganglioglioma) that consists of a mixture of cells of neuronal and glial origins. Lack of reaction with CHME -5 (Microglia) may be attributed to the fact that this line was of fetal origin, while the H4 (neuron) was from an adult and both originated from CNS cells.

The results presented here introduce a question of whether CNS cells are involved in GBS and further testing should be completed.

3.0 IDENTIFICATION OF POSSIBLE RISK FACTORS OF GUILLAIN BARRÉ SYNDROME IN THE MARITIME REGION THROUGH EPIDEMIOLOGICAL STUDY

3.1 ABSTRACT

A retrospective case-control study of GBS patients and suitable controls was completed to identify possible risk factors associated with GBS in the Maritime Region and to assess whether exposure to certain strains of *C. jejuni* are significantly associated with GBS. Several pre-disposing factors determined by epidemiological survey, antibody titres to different strains of *C. jejuni* and the culture of stool samples were used to compare differences in 10 patients and matched control groups. All patients were from the province of Nova Scotia and the reported prevalence over the 2 year study period was 1.1 per 100,000. Household controls were limited as most patients lived alone (70%), and thus were not utilized in certain analyses and comparisons. The average age of affected females was 44.5 years compared to 45.3 for males, however a higher number of males (60%) were diagnosed with the disease, compared to females (40%). No differences were noted in occupation and no patients were farm residents. Seasonal trends were not noted; however, diagnosis occurred 4 times in duplicate (meaning twice in the same month) compared to two single cases (different months). Mean onset of disease prior to diagnosis was indeterminant as symptoms were noted by individuals at different intervals ranging from months to years. McNemar's test determined that the only significant risk factor ($p<.05$) was exposure to untreated water. An ODDS RATIO for exposure to untreated water indicated that ODDS of being exposed to untreated water was 8 X higher in cases than in controls. All stool samples collected were negative for *Campylobacter*. A two-way ANOVA showed no significant

differences in antibody titres against *C. jejuni* by Enzyme Linked Immunosorbant Assay (ELISA) in patients versus control groups. Isolation of the causative agent of GBS through serolgy and culture are unreliable, post-diagnosis. Due to insufficient patient data this study was not successful in identifying statistically significant exposure vectors related to GBS. Factors of interest, and in particular consumption of untreated water, indicated a possible relationship in the development of GBS. in the Maritime Region of Canada.

3.2 INTRODUCTION

Guillain Barré Syndrome has been diagnosed in the Maritime region with approximately 30 cases per annum being admitted and referred to the Queen Elizabeth II Health Sciences Centre (QEHSC) in Halifax. Patients are from all three Maritime provinces; Nova Scotia (including Cape Breton), Prince Edward Island, and New Brunswick (Personal Communications, Joanne McCormick and Dr. T. Benstead, Queen Elizabeth II Hospital, Halifax, NS). Although stool samples are collected as a routine procedure at this institution, patient medical and exposure histories, biotyping in the case of positive stools samples or serotyping does not occur. Similarly, many hospital laboratories do not routinely culture specifically for *C. jejuni* and thus infection is considered to be substantially under-reported at an annual incidence of 1000 per 100,000 persons (Blazer, 1997). In the past two decades evidence has accumulated to support an association between GBS and infection with *Campylobacter jejuni.* When evident, this association more commonly results in a more serious and prolonged form of GBS than those without infection with *Campylobacter* or disease due to other causes (Rees *et al.*, 1995).

In the industrialized world, exposure to *Campylobacter* occurs primarily through the faecal-oral contact with pets and to a lesser extent by poultry and cattle (Skirrow, 1987). Specific reports of transmission of the disease also include occupational exposure. *Campylobacter jejuni* may contaminate both water and soil due to its widespread reservoir in animal populations. Transmission to humans considered to result in endemic infections occurs by ingestion of contaminated foods, untreated water, direct contact with infected animals and less commonly, direct human to human contact (Blaser, 1997). Recognized

outbreaks are usually due to ingestion of undercooked poultry, contaminated water and unpasteurized milk (Blaser *et al.*, 1983) .

Diagnosis of *C. jejuni* infection is usually based on isolation of the organism from faeces and presence of serum antibodies. GBS patients can have both cultural and serological evidence of recent *C. jejuni* infection (Saida *et al.* 1997). The number of samples needed for optimal yield of *Campylobacter* species is not firmly established; however several studies suggest multiple samples have higher yield (Nachamkin, 1997). Kuroki *et al.* (1993) reported that of 14 GBS patients with *Campylobacter* positive stool samples, 57% were detected with 1 sample, 93% were detected with 2 samples, and all were detected with 3 samples.

The immune response to *Campylobacter* infections is similar to that of other infectious diseases. Serum IgM and IgG levels rise in response to infections and remain elevated for 3 to 4 weeks before declining to baseline levels. Serum IgA levels rise only during the first few weeks of infections and fall rapidly (Blazer *et al.*, 1984; Mascart-Lemone *et al.*, 1987 and Kaldor *et al.*, 1983. The ELISA technique appears to be the most commonly used method for measuring serum antibodies (Blazer *et al.*, 1984; Herbrink *et al.*, 1988; Nachamkin *et al.*, 1989 and Mishu *et al.*, 1993).

Seasonal trends have been noted and in the temperate climates of countries such as those prevalent in Europe and North America; infections with *Campylobacter* are reported during the warmer months (Skirrow, 1987). Isolations are more frequent in the wet season than the dry season in countries with constant mean temperatures throughout the year. Canada follows the pattern seen in temperate countries, with the seasonal distribution

peaking in late June lasting through August and September (Lior, 1996) with a noted slight increase in *Campylobacter* infection following holidays, when there is an increase in the consumption of poultry and turkey (Skirrow, 1987). Seasonal variation has been reported as a risk factor in the development of GBS as annual summer peaks have occurred in China, Mexico, Spain and Korea (Lior, 1996).

The most serious outcome of infection with *Campylobacter jejuni* (*C. jejuni*) is its association with Guillain Barré Syndrome (GBS). GBS is characterized as a "postinfectious" disease with two thirds of patients reporting antecedent illnesses (Blaser, 1997), with respiratory infections being those most frequently reported (Winer *et al.*, 1988 and Arnason *et al.*, 1993). Often the precise infection is not clear from the medical history and has resolved when neuropathic symptoms develop.

The *C. jejuni* serotypes Penner O:19, Lior 7 and Lior 11 are most commonly associated with GBS (Fujimoto *et al.*, 1992, Rees *et al.*, 1993 and Hartlung *et al.*, 1995), although predominance of any particular serotype was not found in a recent British study (Rees *et al.*, 1995). The isolation of *Campylobacter* from patients with GBS will depend upon the methods used, patient therapy for previous illnesses and the duration of excretion of the organism following the onset of diarrheal illness (Nachamkin, 1997).

Outbreaks of GBS are rare. GBS is slightly more common in males than in females (Mishu and Blaser, 1993). The incidence of the disease as it relates to age suggests a bimodal distribution, with the acute demyelinating pattern (AIDP) form of GBS affecting the older population in the Western countries and the acute motor axonal pattern (AMAN) form tending to affect primarily children and young adults (Kaplan *et al.*, 1985). Few studies

have retrospectively investigated the relationship between occupation and GBS, and statistically there has not been confirmation of associations between the two. However, trends have been established in those occupations with exposure of risk factors, such as *Campylobacter* infection, that appear preferentially to precede GBS (Ropper *et al.*, 1991). Familial cases of GBS do not occur. Outbreaks of GBS associated with immunizations are debatable; however there is evidence suggestive of an increased incidence of GBS in the case of the swine flu inoculation (Ropper *et al.*, 1991) and polio vaccination programs (Kaplan *et al.*, 1985). Due to limitations with culture techniques, serological studies in combination with stool cultures and epidemiological information, such as clinical histories are useful in identifying patients likely to have had *Campylobacter* infections (Nachamkin, 1997).

The purpose of the current study was to determine if infection with *C. jejuni* is significantly associated with GBS in the Maritime region and to identify any other possible risk factors, i.e. those that may be associated with the increased risk of *C. jejuni* exposure. Specifically, a case-control study was implemented to determine differences in the prevalence of risk factors in patient-matched control groups and testing of these groups, both culturally and serologically, were used to establish evidence of preceding *C. jejuni* infection and to determine if infection with *C. jejuni* results in more prolonged and serious GBS than those without.

3.3 EPIDEMIOLOGICAL STUDY

3.3.1 Study Design

Well suited to the evaluation of rare diseases such as GBS (1.3 per 100,000 in the developed countries), case-control studies allow for the evaluation of a wide range of potential etiologic exposures that might relate to a specific disease including interrelationships that may exist among those factors. Aside from being less costly and time consuming than other analytic epidemiologic studies, case-control studies offer a number of advantages for evaluating the association between exposure and disease (Hennekens and Buring, 1987). The strengths of case-control studies include the ability to study rare diseases effectively, their usefulness in early stages of knowledge about an exposure-disease relationship, and their ability to investigate multiple exposures simultaneously. The main disadvantages include difficulty in establishing the temporal relationship between exposure and disease and the increased susceptibility to bias.

This study was a case-control design whereby cases were identified by standard practice of the Queen Elizabeth II Hospital in Halifax, Nova Scotia. Appropriate controls were selected based on their similarities to cases with respect to age and sex with the exception that the controls did not have the disease of interest, namely Guillain Barré Syndrome. Prevalence of risk factors in the case group and the control groups were determined by utilizing the results of epidemiological surveys and *C. jejuni* antibody titres and stool cultures of patient and control groups. Hospital-based controls were age and sex matched individuals admitted to the same institution, within 24 hours of diagnosis, for reasons other than GBS. Household controls included in the study were those individuals living in the same household in the previous 4 months and having regular contact with the GBS patient, particularly in terms of sharing meals. As controls were selected within 24

hours of diagnosis the study design was that of incidence density sampling.

Additional information regarding the clinical assessment of the patients was gathered to report severity of disease, onset of symptoms, duration of the illness and treatments. Results from these data were used to determine if GBS was more prolonged or serious in those with *C. jejuni* infection. Blinding was utilized, regarding the investigator; data were collected and made available by Dr. J.M. Haldane, Director, Department of Pathology and Laboratory Medicine, Dr. Tim Benstead, Clinical Neurologist and Nurse Joanne McCormick at the QEHSC. Criteria for inclusion for patients considered in the study was based on being diagnosed with GBS according to the Hughes Scale of disability currently being used at the QEHSC by the neurologists (Hughes and Rees, 1997). Qualification of neurological deficits/clinical signs for all GBS patients were performed similar to the system used by Rees et al. (1995). After a diagnosis of GBS was confirmed, patients and matching controls were interviewed and tested.

3.3.2 Study Population

In the Maritimes region, the Queen Elizabeth II Health Sciences Centre in Halifax is the largest recipient of GBS patients with referrals from Nova Scotia, Prince Edward Island and, New Brunswick (Personal Communications, Joanne McCormick and Dr. T. Benstead, Queen Elizabeth II Hospital, Halifax, NS). In this study there were 10 GBS patients, 10 matched hospital-based controls and 3 household controls studied during a period of 24 months (1997 to 99).

3.3.3 Materials and Methods

3.3.3.1 Epidemiological Survey

Following an extensive review of the literature on the properties of *Campylobacter* infection and the reported associations of *Campylobacter* and GBS , a survey was developed based on reported risk factors for disease. A case-control was chosen because of its ability to investigate multiple etiologic factors simultaneously. For all cases and controls, information was collected through survey on identification, history, medical and pre-exposure to vectors of transmission, with particular emphasis on risk factors associated with both *Campylobacter* infection and GBS. (See Appendix C: Survey Template).

Specific to patients only, a template (Appendix D: Clinical Assessment Template) was developed and completed by the attending physician, describing the clinical assessment of each patient and used to record onset and duration of disease, severity of illness according to the Hughes Scales of Disability, and treatments.

3.3.3.2 Isolation and Identification of Bacteria

Stool samples were collected immediately, or within 24 hours of a patient being diagnosed with GBS. Faecal samples from matching controls were obtained as soon after as possible. All samples were cultured in duplicate and identified for *Campylobacter jejuni* by standard methods at the QEHSC in Halifax which are similar to those outlined by Rees *et al.*, (1995). Positive *Campylobacter* cultures underwent further serotyping and biotyping at the National Laboratory for Enteric Pathogens (NLEP), Ottawa, Ontario.

3.3.3.3 Serological Testing

Strains of *Campylobacter* incriminated in the development of GBS were obtained from the National Laboratory for Enteric Pathogens at the Laboratory Centre for Disease Control (LCDC), Bureau of Microbiology (Johnson and Woodworth, 1999) in Ottawa and were designated as follows: GBS 4382, GBS 4384, Lior 2, Lior 7, Lior 10, Lior 11, and Penner O:19. These strains were individually cultured on Preston's (without antibiotic) plates (Oxoid Laboratories) at 37°C for 48 h in a microaerophilic atmosphere. Each suspension was adjusted to an optical density of MacFarlands #3 (Remel, 1996). Each bacterial suspension was heat treated at 62°C for 45 min or formalinized with 0.2% PBS (pH 7.2) to produce 7 whole cell preparations and stored at 4°C. There was no growth when plated on blood agar. Optical densities were standardized to 1 using an LKB Biochrom: Ultropec II Spectrometer (540 mm) wavelength.

The ELISA was performed as follows: a ninety-six well Immuno-Plate Max 150 RP-F96 (Sigma BioSciences) was coated with 100 µl each of the whole cell antigen preparations that had been diluted at a ratio of 1:10 in carbonate coating buffer (pH 9.6). After incubation overnight at room temperature (24°C), the plates were frozen and stored at -20°C. On the day of testing, plates were removed from the freezer and allowed to thaw for 1 h and then washed 3 X with phosphate buffered saline containing 0.05% Tween 20 (PBS-T), pH 7.4. Similar washes were performed after each of the subsequent steps. Plates were blocked for 1 h at room temperature with 100 µl/well of 3% bovine serum albumin (BSA) diluted in PBS-T. Patients sera were diluted to 1:50 and 50 µl was added to the first well of each row of a microtitre plate containing 50 µl of PBS-T fetal bovine serum (FBS). The sera were serially diluted with the last well receiving no serum. The plates were incubated at 37°C for

1 h and then washed. A pooled conjugate (secondary antibody) containing Goat-anti-human IgA, IgG and IgM was diluted 1:4000 (Sigma BioSciences) and 100 µl of the diluted conjugate was added, followed by incubation for 1 hour at 37°C, then washed.. Finally 100 µl of the substrate (0.1M, Citric acid, 0.2M Na_2HPO_4, 0.22 g/l of 2,2'azino-di[3-ethyl-benzthiazoline-6-sulfonic acid] (ATBS) (Sigma-Aldrich Canada, Oakville, Ontario) and 0.1% of hydrogen peroxide (11.5 µl per plate v/v) (Fisher Scientific Ltd, Napean, Ontario.) was dispensed into each well and the plate was incubated for 45 min at 37°C. The resulting color reaction was quantified using a Spectra Max 340 plate reader (Molecular Devices, Sunnydale, CA) at a dual wavelength of 490 lambda with a reference wavelength of 405 lambda.

3.4. STATISTICAL ANALYSIS

3.4.1 Epidemiological Survey

McNemar's Exact test of independence ($p<.05$) was used to test the null hypothesis that risk factors observed were not equal to those due to chance for each predisposing factor (Zarr, 1999). Rejection of the null hypothesis indicated that there was a statistically significant association between the particular risk factor tested and Guillain Barré Syndrome. The strength of the association was assessed using the ODDS RATIO (Fletcher *et al.*, 1996), with a value greater than 1 indicating a positive association and a value of less than 1 indicating an inverse association. An ODDS RATIO of 1.0 indicated that the incidence rates of disease in the exposed and nonexposed groups were identical and thus that there is no association between the exposure and the disease in the data collected. Where non-

significance was found, these results were utilized to establish trends.

Data collected for the clinical assessment of patients were utilized and analysed in subsequent sections, namely those regarding serological and stool culture testing, to determine if there was an association between infection with *Campylobacter*.

Any risk factors significantly more prevalent in the case groups than the control groups were considered to be associated with the disease of interest at $p<.05$. Where there was no statistical significance, data were presented descriptively and trends noted regarding the consequences of Guillain Barré Syndrome.

3.4.2 Serological Testing

To complete the analysis of samples and to compare positive and negative sera, the following data was considered:

A. optical density readings at the initial dilution of 1:100 and,

B. final titres (dilution at which the corrected optical density readings reached the arbitrarily chosen cut-off point of 0.1).

Both analyses were carried out separately for 10 pairs and involved 5 treatment groups as follows:

1. Patient
2. Household Control
3. Hospital Based Control
4. Positive Control
5. Negative Control

For optical density readings at 1:100, a two-way ANOVA (analysis of variance) was used to test the null hypothesis that matched pairs were not significantly different from each other regarding optical density reading with 7 different reference strains of *C. jejuni* , as tested by ELISA. If accepted, then no pair effect was present, and a one-way ANOVA was used to test the null hypothesis that there were differences between treatment groups. Fisher's pairwise comparison was utilized ($p<.05$) to determine specific differences in patients and control groups regarding *C. jejuni* antibody levels to all antigens. All statistical analysis was performed using MINITAB^{TM}Inc.

To categorize samples as positive and negative, the titres of sera (dilution at which the corrected optical density (OD) readings reached the arbitrarily chosen cut-off point of 0.1) were compared. If the OD corrected value was 2 X the standard error of the mean of the negative reference serum, then serum samples were considered positive. To ensure validity of the positive reference serum the OD corrected value should be greater than 1.0 and the negative reference serum should have an OD corrected value of less than .015.

3.5 RESULTS

3.5.1 Epidemiological Survey

Patient numbers were considered small (n=10), and unlikely sufficient to establish statistical significance at $p<.05$. All patients originated from the province of Nova Scotia indicating an annual incidence of 1.1/100,000. Household controls were not used for comparisons as most of the 10 patients diagnosed lived alone (70%).

The average age of females was 44.5 years compared to 45.3 for male, however a

higher number of males (60%) were diagnosed with the disease, compared to females (40%). These differences were clearly insignificant. No differences were noted in occupation, as no patients or controls had the same occupation, and no patients were farm residents. Seasonality trends were not noted; however, diagnosis occurred 4 times in duplicate (meaning twice in the same month) compared to two single cases (different months). Mean onset of disease prior to diagnosis was indeterminant as symptoms were noted by individuals at different intervals ranging from months to years.

Chi-square test for matched-pair data was performed as pair analyses is more powerful. McNemar's test of independence ($p<.05$) was used to test the null hypothesis that risk factors observed were not equal to those due to chance for each predisposing factor. Count data and statistical results for paired data are displayed in Table 3.1. McNemar's test determined that the only significant risk factor ($p<.05$) was exposure to untreated water. An ODDS RATIO for exposure to untreated water indicated that exposure to untreated water was 8 X more likely to occur in patients versus controls. Calculations are displayed in Appendix E. Stratification of the significant risk factor (untreated water) failed because of insufficient numbers, and no multivariable analysis were possible given the size of the data set.

Ignoring statistical significance, ODDS ratios (OR) were calculated to determine if trends existed for preceding illnesses and exposure vectors reported in patients. Regarding medical history a notable difference between the hospital based controls and patients regarding preceding infections were those of upper respiratory tract infections (OR = 4.0) followed by myalgia and fever (OR=2.0), suggesting a possible positive association with

Table 3.1: Count data for cases and non-cases (hospital-based controls) of GBS with statistical results for p-value from McNemar's Exact test with Exact ODDS Ratio and confidence intervals.

	Actual Numbers by Pairs		Calculated		
Factor	Cases +/-	non-cases (HB) +/-	p-value	OR	95% C.I.
Medical History:					
Upper Respiratory Tract Infection	37775	37686	0.38	4	(0.40, 197)
Diarrheal Illness	37716	37716	1	1	(0.07, 13.8)
Myalgia	37659	37629	1	2	(0.10, 118)
Fever	37745	37686	0.69	2	(0.29, 22.1)
Pre-Exposure Vectors:					
Cattle	37745	37686	0.73	1.7	(0.32, 10.7)
Pets	37834	37745	0.45	2.5	(0.41, 26.3)
Sewage	37659	37629	1	2	(0.10, 118)
Poultry	37686	37686	1	1	(0.13, 7.5)
Surface Water	37716	37659	0.69	2	(0.29, 22.1)
Birds	37659	37659	n/a	n/a	n/a
Unpasteurized Milk	37630	37630	n/a	n/a	n/a
Raw Eggs	37775	37745	1	2	(0.10, 118)
Untreated Water	37834	37629	0.04	8	(1.07, 355)
Other Humans	37630	37659	0.5	0	(0.0, 5.3)
Allergies	37745	37686	0.69	2	(0.29, 22.1)

GBS, although there is no statistical significance. The ODDS Ratio for diarrheal illnesses for cases and non-cases was 1 indicating no difference in odds of exposure for cases. Surgical history was not considered as hospital based controls would be considered a bias for this comparison.

Regarding general exposure to preceding factors for those preceding factors deemed statistically insignificant, the ODDS RATIO for exposure to pets ranked highest (OR = 2.5) followed by sewage, surface water, raw eggs and allergies (OR = 2) and cattle (OR = 1.7). As all ODD RATIOS for the aforesaid factors were greater than 1, this suggests that these risk factors may have a positive association with GBS.

All OR's calculated for unpasteurized milk, exposure to birds and consumption of poultry were equal to 1 or not calculated (n/a), and thus indicated no evidence of any association between the factors of interest and GBS. Contact with others humans through symptomatic individuals appeared to be protective regarding the association with GBS at a value of zero, although once again this could not be confirmed statistically..

3.5.2 Isolation and Identification of Bacteria

Stool samples from all patients, household controls and hospital based controls were negative for *Campylobacter* species. Therefore, testing to determine serotype and biotype was not performed.

3.5.3 Serological Testing

A two-way ANOVA (analysis of variance) accepted the null hypothesis and thus

indicated that matched pairs were not significantly different from each other regarding patient antibody titres to each of 7 different reference strains of *C. jejuni*, as tested by ELISA. (See Table 3.2 for F -tests and p-values). With no pair effect being reported a 1-way ANOVA ($p<.05$) was used to test the null hypothesis that there were no differences between treatment groups regarding antibody titre for each antigen.(See Table 3.2 for F -tests and p-values).

As expected, the null hypothesis was rejected and significant differences were reported at $p<.05$ in each of the antigens tested. Fisher's pairwise comparison was utilized to indicate specific differences in these treatments groups; however only significant differences were noted between the treatment groups and the control group (positive and negative). Antigen Lior 7 and Antigen Lior 11 exhibited values closer to significance that any of the others for patients versus control groups.

Final titres considered in determining positive and negative serum samples resulted in the following (See Table 3.3). All positive and negative controls were detected. For antigens GBS 4384 and Penner O:19 there were no positive serum samples detected. For the antigen GBS 4382, 20% of samples were positive in both patients and household controls and no positives were seen in hospital based controls. For antigen Lior 2, 20% of the serum samples were positive for both patients and household controls and 10% positive hospital based controls. Serum samples tested against antigen Lior 7 resulted in 20% positives in sera for patients, 10% for household controls and 20% in for hospital based controls. Antigen Lior 12 resulted in highest rates of positive serum samples at 30% for patients, 20% for household controls and 10% for hospital based controls. For antigen Lior 11, the highest

Table 3.2: **Two-Way ANOVA based on ELISA optical density values at 1/100 serum dilution seven *C. jejuni* antigens preparations. Results of F-Test and presentation of corresponding p-values, all based at 1/100 dilution.**

Calculated Variables						
	Factor Pair		Factor Group		Estimates (SE)	
Antigen Tested	F-Test	p-value	F-Test	p-value	Cases and Hospital Controls *	Household, Positive and Negative Controls **
Lior 2	0.37	0.93	20	0.0	.05	.08
Lior 12	0.61	0.77	10	0.0	.05	.09
Lior 7	1.13	0.4	18	0.0	.04	.08
Lior 11	1.04	0.45	10	0.0	.05	.09
Penner O:19	0.61	0.77	30	0.0	.03	.06
GBS 4384	0.33	0.95	10	0.0	.05	.10
GBS 4382	0.57	0.8	10	0.0	.06	.11

Note:
* All SE calculations are the same as n=10 for all groups.
** All SE calculations are the same as n=3 for all groups.

Table 3.3: **Dichotomous values for positive and negative samples as determined by ELISA using end-point titre values for each antigen tested.** Each column represents the number of positives (P) or negatives (N) in each of the five groups listed, including positive and negative controls.

	Number of Positives (P) and Negatives (N)													
	Antigens Tested													
	Lior 2		Lior 12		Lior 7		Lior 11		Penner O:19		GBS 4382		GBS 4384	
	P	N	P	N	P	N	P	N	P	N	P	N	P	N
Group Status														
Positive Control	3	0	3	0	3	0	3	0	3	0	3	0	3	0
Negative Control	0	3	0	3	0	3	0	3	0	3	0	3	0	3
Patients	2	8	3	7	2	8	2	8	0	10	2	8	0	10
Household	2	1	2	1	1	2	1	2	0	3	2	8	0	3
Hospital-based	1	9	2	8	5	5	0	10	0	10	0	10	0	10

positive results were noted for hospital based controls at 50%, and the patient and household controls groups for this antigen were 20% and 10%, consecutively. Validity of the positive reference serum was confirmed as the OD corrected value was greater than 1.0 and the negative reference serum OD corrected value was less than .015. McNemar's Test accepted the null hypothesis that that there were no significant differences between positive and negative samples ($p<.05$) between patient and hospital-based control groups.

3.6 DISCUSSION

3.6.1 Epidemiological Survey

The number of patients diagnosed with GBS was less than the projected 30 cases per annum. Fewer cases than anticipated were noted early in the study and the collection period was extended from 12 to 24 months to acquire additional patients. Although this extension yielded extra data, the final number of cases was still well below those originally expected. Thus, the ability to detect statistically significant differences was greatly inhibited by low sample size.

Household controls only yielded 3 samples, indicating that there may be some confounding factor to consider regarding patients that live alone and develop GBS. A consideration of interest, depicted in the comments section of the survey, may relate to poor personal hygiene. However this factor was not considered in the hospital controls and thus no conclusions can be drawn.

The number of males and females diagnosed was in agreement with that of Mishu and Blaser (1993) with a higher number of males (60%) being diagnosed with the disease,

compared to females (40%); however once again one must re-consider the validity of this statement as sample size of the study was not sufficient. No differences were noted in occupation and no patients were farm residents, unlike trends established in those occupations increased exposure of risk factors, that appear preferentially to precede GBS as reported by Ropper *et al.*, 1991.

During the summer period of study there was a drought, and the winter season was characterized by colder temperatures which both presented conditions considered unfavorable for the development of both *Campylobacter* and GBS. Although all patients originating from the province of Nova Scotia may have been totally due to chance, one should consider that New Brunswick and Prince Edward Island recorded worse drought conditions than Nova Scotia during the collection period. Seasonal trends were not noted, suggesting that weather conditions such as drought may be a determining factor or the number of samples collected were too low to detect this difference. Duplicate (meaning twice in the same month) diagnosis of GBS patients occurring 4 times as compared to two single (different months) cases may indicate that in a year where additional cases were diagnosed, a trend may be developing regarding seasonality. Speculation on the cause of this concurred with Lior (1996) in that isolations are more frequent in the wet seasons than the dry season in countries with constant mean temperatures throughout the year.

McNemar's test of independence ($p<.05$) was used to test the null hypothesis that risk factors observed were not equal to those due to chance for each predisposing factor. McNemar's test determined that the only significant risk factor ($p=.0455$) was exposure to untreated water. An ODDS RATIO for exposure to untreated water was 8.0, indicating that

the ODDS of exposure is 8 X more likely in patients versus controls. Alternatively, one could present the ODDS RATIO as those exposed to untreated water were 8 X more likely to develop GBS than controls.

Stratification of the significant risk factor (untreated water) failed because of insufficient numbers, and an alternative multivariate analysis for exposure to untreated water was not completed due to the study design. According to Hennekens and Buring (1987), when the outcome of interest is a binary variable, it is possible in matched pair data to use a specialized type of multiple regression called conditional logistic regression analysis. This is a powerful tool for estimating the magnitude of the association between an exposure an outcome after adjusting simultaneously for a number of potential confounding factors. These coefficients can be directly converted to an odds ratio that provides an estimate of the relative risk that is adjusted for confounding. However, given the size of the data set, no multi-variable analyses were performed.

Although not statistically significant, ODDS RATIOS (OR) were calculated to determine if trends existed for noted preceding illnesses and exposure vectors reported in patients (See Table 3.1). Regarding medical history, a notable difference between the hospital based controls and patients regarding preceding infections were those of upper respiratory tract infections (OR = 4.0) followed by myalgia and fever (OR=2.0). This agrees with reports that respiratory infections are the most frequently reported antecedent infection (Winer *et al.*, 1988 and Arnason *et al.*, 1993) and that myalgia and fever are two of the reported non-specific symptoms associated with *Campylobacter jejuni* infection.

The ODDS RATIO for diarrheal illnesses for cases and non-cases was 1 indicating

no association to exposure for cases. However, if we consider that the comparison groups are hospital based controls, then one could justify this result by assuming that these types of controls are likely to report diarrheal illnesses. In the same regard, surgical history was not considered as hospital based controls would be considered a bias for this comparison.

Regarding general exposure for those preceding factors deemed statistically insignificant, exposure to pets ranked highest (OR = 2.5) followed by sewage, surface water, raw eggs, allergies (OR = 2.0) and cattle (OR = 2.0) and were in agreement with the those factors noted by Blaser (1997). Once again, should patient number have been sufficient these results may have indicated significance.

ODDS RATIO's could not be calculated (n/a) for unpasteurized milk and exposure to birds due to equivalent numbers by pairs in the cases and non-cases. Thus, in these exposure factors and for the risk factor consumption of poultry (OR = 1.0), there was no evidence of an association with GBS, unlike that presented in the literature. Consider the following: consumption of unpasteurized milk is uncommon in the developed countries and poultry is prepared according to guidelines for the prevention of food-borne illnesses. Although contact with other humans through symptomatic individuals (OR = 0) appeared to be protective regarding the association with GBS, symptomatic individuals are not an easily recognized group as symptoms are not always visible, especially in the early stages of the disease.

Considering that studies of both *Campylobacter* and GBS have suggested consumption of untreated water as a predisposing factor, the findings of this study seem appropriate. However, this was the only significant factor identified of others more

commonly reported, such as surface water and pets, for example, and may relate to the inability of the small data set to detect other significant differences.

When presenting the results obtained in this study, one must be cautious. Under random conditions and at a level of 95% confidence, we are willing to accept a Type 1 error of at least 5%. Considering this, our results could be totally accounted for based on this principle alone. The patients sample size in this study was not indicative of true significance, and thus the results presented should be considered as preliminary findings only.

3.6.2 Isolation and Identification of Bacteria

There are several factors that can explain the failure to isolate *Campylobacter* in faeces of GBS patients. In this study, negative results were obtained for both patients and control groups. The number of samples needed for optimal yield of *Campylobacter* species, in general, has not been firmly established. The isolation of *Campylobacter* from patients with GBS will depend upon isolation techniques, duration of excretion of organisms and therapy in the patient (Nachamkin, 1997). Several studies suggest that multiple samples give greater isolation of *Campylobacter* infection (Nachamkin, 1997) and specifically Kuroki *et al.* (1993) reported that of 14 GBS patients with *Campylobacter* positive stool samples, 93% were detected with 2 samples. Considering that in the current study sampling was duplicated, one would expect that if *Campylobacter* was present there would be recovery unless detection is dependent on the severity of infection. In control patients, prior treatment with agents which affect isolation of *Campylobacter* likely contributed to the failure to obtain positive cultures.

Furthermore, as noted by Rees *et al.* (1995), the acute infection is often resolved before symptoms of GBS develop, and with a mean onset of symptoms of 13 days, perhaps the bacteria were no longer present at the time of diagnosis and testing. Several investigators have succeeded in isolating *C. jejuni* from the stools of patients with GBS at the onset of neurologic symptoms (Allos 1997), and if this sampling had been similar, there may have been isolation of the organism.

Finally, limitations with culture techniques have been reported, and bacterial and viral cultures are usually negative in Guillain Barré patients (Nachamkin, 1997), most likely for those reasons given in previous discussions. Thus, this study was designed to include serological and epidemiological information as to identify patients likely to have had *Campylobacter* infections. These, combined with the small sample population may explain the results obtained.

3.6.3 Serological Testing

The results of ELISA by both types of analysis indicated that there were no significant differences in serum antibody levels of *Campylobacter jejuni*. Although positive sera were detected, there is no evidence that the antibodies to strains tested were involved in the pathogenesis of GBS. This is not in agreement with the literature and thus one must consider the following. Serum IgG and IgM levels in response to infections remain elevated for 3 to 4 weeks before declining to baseline levels. Serum IgA levels rise only during the first few weeks of infections and fall rapidly (Blazer *et al.*, 1984; Mascart-Lemone *et al.*, 1987 and Kaldor *et al.*, 1983. Most of the titres appeared to be due to IgG antibodies when

tested separately. This suggests that these titres may not reflect a very recent exposure. Mean time of onset of the disease in relation to the first symptoms reported was indeterminant as symptoms reported were ongoing in some case for months and even years before the disease was diagnosed. Thus, antibody levels had declined by the time the disease was diagnosed and testing occurred. Also, it should be considered that infection at undetectable levels does not preclude that *Campylobacter* is present and involved in the onset or severity of GBS as the pathogenesis of the bacteria is unknown at this time.

Thus, in going forward it would be useful to know when titres developed, reached maximum or declined. As a follow-up to this study it would also be advantageous to differentiate between acute and convalescent patients in order to monitor the titers of antibodies, in particular IgG antibodies.

Testing and analysis methods used for serum samples would appear not to be determining factor in the results reported as positive and negative controls were detected at 100% accuracy in both. End point titration to determine dichotomous values (positive or negative) may have skewed results as data were censored and results may have misrepresented the actual ELISA values. Therefore, analysis of the 1:100 adjusted OD value was chosen for ability to detect larger differences. Thus, if significance or otherwise was obtained using this method, we can say with confidence that the results were accurate.

The results of this study did not show a significant association between infection with *C. jejuni* strains of Penner 19, Lior 7 and Lior 11 and GBS as found earlier (Fujimoto *et al.*, 1992, Rees *et al.*, 1993 and Hartlung *et al.*, 1995). The number of samples collected over the designated period of time would most likely not be sufficient to generate a data set with

significance.

One should consider that this study was comparative and the results obtained were based on matched pairs, unlike most other studies reporting significant differences in patients and controls regarding serum antibody levels to certain strains of *C. jejuni*. In a standard case control study, the investigator compares a group of controls with a group of cases. As a group, the controls are supposed to be similar to the cases with the exception being the absence of the disease of interest. Additionally, in this study individual cases are matched with individual controls based on gender and age. Displaying and analysing data from matched case-control studies on an ordinary contingency table obscures matching of data. Matching makes the experiment stronger, especially when dealing with a rare disease, as it may ensure an adequate number of cases and controls for each of the subgroups making it possible to efficiently evaluate the association between exposure and disease. Additionally, matching removes any possible confounding variables. The design of this study considered matching for the mentioned reasons. Although matching was appropriate in this study, results would have been more reliable had the sample size been larger.

4.0 GENERAL DISCUSSION

Guillain Barré Syndrome (GBS) of humans is an acute demyelinating polyneuropathy characterized by an immune mediated damage to peripheral nerve myelin. The trigger for this immune attack is unknown; however GBS is frequently preceded by an acute infection. Infection with *Campylobacter jejuni* has emerged as one of the most common antecedent events associated with GBS. *Campylobacter jejuni* infections are common in Canada and the United States, affecting approximately 1% of the population each year (Tauxe, 1992). Approximately 1 in 2,000 *C. jejuni* infections may be complicated by GBS and because *C. jejuni* infections occur far more commonly than GBS, host (Rees *et al.*, 1995) or strain (Kuroki et al., 1993 and Fujimoto *et al.*, 1992) characteristics may determine which infected persons develop GBS and as well there may be differences in individual atypical variation in immune response. The increasing awareness of the importance of *C. jejuni* infection in triggering GBS is an example of how well-described diseases have emerged as sequelae of acute infectious illnesses. This study attempted to characterize the nature of this association in Maritimes region of Canada and the effect of antibodies to certain strains of *Campylobacter* on peripheral nerve function.

In rats injected intraperitonially with rabbit antiserum to *C. jejuni* of human origin, electrophysiological measurements indicated that the femoral nerve function was affected by the presence of these antibodies. Reactivity of the antibodies with nerves was also suggested by Western blots which showed antibodies against GBS 4382/ 4384 H, Penner O:19 H and Lior 7 /11 H F reacted with nerve tissue antigen. The positive responses to these serotypes agree with the observations of other workers regarding the involvement of

these strains in GBS. Additionally this study determined the same response with serum produced against GBS 4382 / 4384.

These same rabbit antisera also reacted immunologically *in vitro* with cultured human neural cell lines suggesting the potential for disruption of function. The specific cause of this potential disruption on function is not apparent from the results of this experiment. Specific reactivity was noted in lines representing neural and oligodendroglia cells. Furthermore, antibodies for Penner O:19 F showed greatest reactivity and this strain has consistently been implicated in GBS. The reaction of the Lior 7:11 F antibodies to the line representing the oligodendroglia, at a low percentage (5%) may represent sensitivity of oligodendrocytes to laboratory reagents during experimentation as per the findings of Benn *et al.* (2001). The results presented here introduce a question of whether CNS cells are involved in GBS and thus, further testing should be completed to determine if there is CNS involvement in GBS.

For the epidemiological study, the number of patients confirmed with GBS was considered less than normal and not expected to yield significance. Despite this, the appropriate analysis was completed and significance and trends were noted. McNemar's Exact test was utilized because of its ability to account for confounding and the use of matched-pair data. McNemar's test determined that the only significant risk factor ($p=.0455$) was exposure to untreated water. The ODDS RATIO for this risk factor indicated that exposure was 8 times more likely to occur in patient versus control groups. Considering that both *Campylobacter* and GBS have previously reported exposure to untreated water as a predisposing factor, this is fitting. However, the fact that it was the only significant factor

identified of others more commonly reported, such as surface water and pets, for example, may relate to the inability of the small data set to detect other significant differences. This is a common problem with rare disease studies, however and the findings cannot be dismissed as there may be other factors of equal importance that have not been identified in this study.

There are several factors which could explain the failure to isolate *Campylobacter* from stool samples. These include the number of samples needed for optimal yield of *Campylobacter* species, methods used, patient's therapy for previous illnesses and the duration of excretion of the organism following the onset of diarrheal illness or other symptoms (Nachamkin, 1997). Considering that samples were done in duplicate for the purposes of this study, one could then attribute the negative results to indeterminent mean onset of symptoms in diagnosed patients, and thus the bacteria was no longer present at the time of diagnosis or testing. However, of greater importance is the need for testing criteria regarding the detection of *C. jejuni* related GBS, if there is a concrete association between the two. Otherwise, preventative measures and treatment may be ineffective.

The results of ELISA by both types of analyses indicated that there were no significant differences in patient and control groups with respect to serum antibodies to some strains of *Campylobacter jejuni*. Although positive samples were detected, based on the results of this study there was no evidence to suggest that the presence of antibodies to antigens tested was correlated with GBS.

One should consider that this study was comparative and the results obtained were based on matched pairs, unlike most other studies reporting significant differences in

patients and controls regarding serum antibody levels to certain strains of *C jejuni*. In a standard case control study, the investigator compares a group of controls with a group of cases. As a group, the controls are supposed to be similar to the cases with the exception being the absence of the disease of interest. Additionally, in this study individual cases are matched with individual controls based on gender and age.

In this type of study antecedent factors can only be *associated* with the risk of an outcome and causation can not be determined. The study's reliability depends on credibility of client recall and/or of records. Beyond the limitations of such a study one can try and limit bias. This study attempted to do so by selecting appropriate controls and methods of analysis. Matching was introduced to ensure valid comparisons. Variables associated with both exposure and outcome, which occur with outcome regardless of exposure, were considered in an effort to reduce confounders. However, sample size was not appropriate in exercising these factors mentioned.

Case-control studies, such as that used in this study, provide several advantages for evaluating the association between exposure and disease and are particularly efficient in terms of time and cost (Hennekens and Buring, 1987). Well suited to the evaluation of rare diseases, such as GBS, they allow for the evaluation of a wide range of potential etiologic exposures that might relate to a specific disease as well as the interrelationships among these factors. They offer a solution to diseases with long latency periods whereby investigators can retrospectively identify antecedent infections rather than waiting a number of years for a disease to develop. Participants are selected on the basis of the disease which usually allows investigators to identify adequate numbers of diseased and control groups, however

in the case of this study the limiting factor was time. It is possible to determine what samples size should be taken, however this only occurs if we start with a given level of precision. Precision can be increased by reducing the size of the standard error. The size of the standard error is based on the size of the sample. However in a situation where no data exists on the incidence, prevalence of the disease or the suspected cause, it is difficult to determine adequate sample size.

This study was useful in studying the effect of variables on the process of disease associated with GBS and in identifying risk factors thereof. Although we cannot conclusively say that there are certain variables that may or may not have an effect upon disease process, we can suggest that there are trends and tendencies towards risk factors, such as untreated water, in GBS in the Maritimes region of Canada. Furthermore, patterns of GBS noted over the two year period of study have been reported and may be useful to subsequent research.

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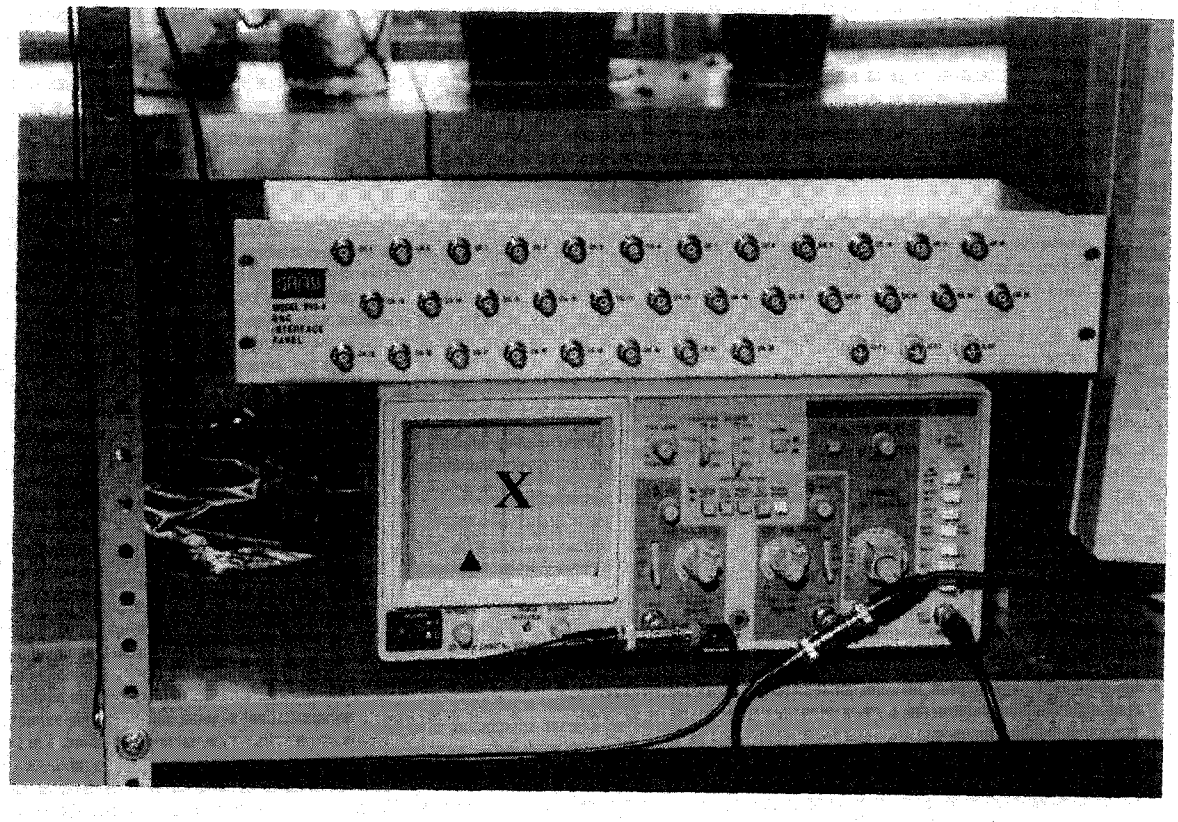
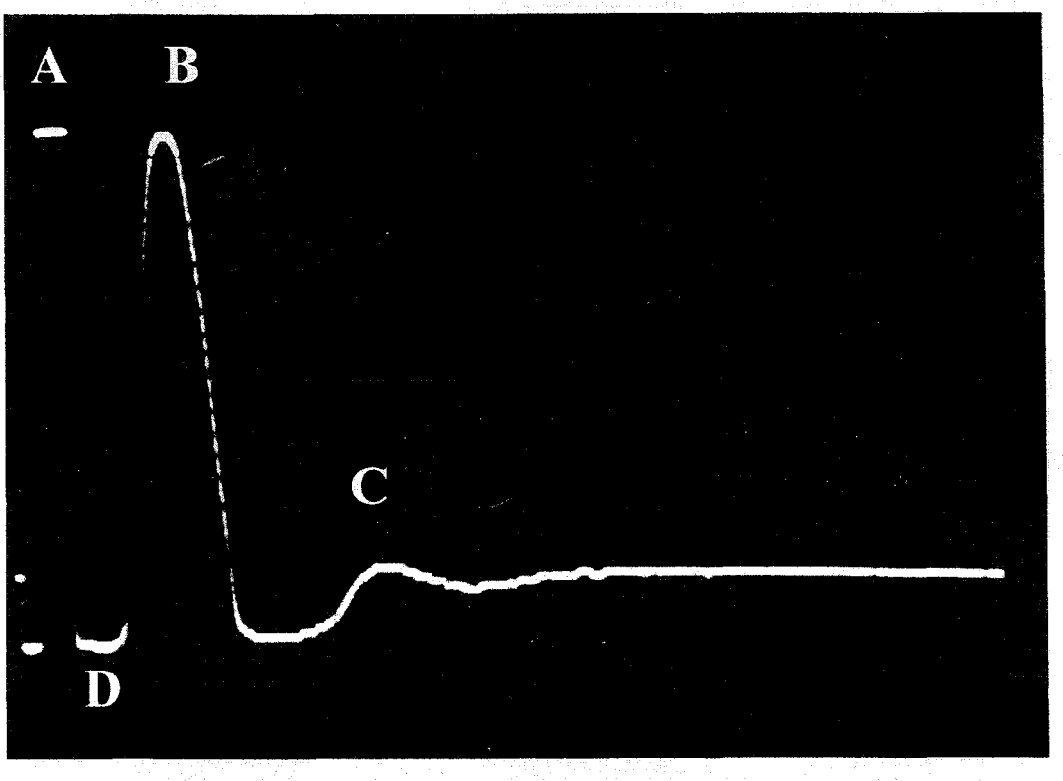
APPENDIX A: **Electrophysiological Recording Equipment. Stimuli were recorded with a P55, A.C. amplified signal and sent to an oscilloscope (below) which in turn measures currents (BK Precision - Model 2522 - A) that are displayed on the grid screen (marked X). Note the faint green line (marked ▲) registered on the grid representing the baseline.**



APPENDIX B: **Maximal response of stimulated rat nerve. A represents the stimulus artifact, B = A + B fibers, C = B or C fibers and D represents latency.**



APPENDIX C: Epidemiological Survey: Survey Template

Guillain Barre Syndrome Survey **Date:** _____, 199___
Personal Identification #:_____
Hospital Unit #:

Part B:
1. Occupation/Hobbies/Lifestyle

a. Which category best describes your occupation?

1. Hospital Worker
2. Farmer
3. Fisherman
4. Office-based
5. Industrial Worker
6. Food-Related Industry
7. Other: Please Specify:

_____
_____
_____

b. Travel within Last 6 months has been:

Specify destinations and dates in space provided.

national	
Destination	Date
international	
Destination	Date
provincial	
Destination	Date

1. Occupation/Hobbies/Lifestyle continued:

c. List hobbies that you have participated in over the past year.

1. _____
2. _____
3. _____
4. _____
5. _____
6. _____
7. _____
8. _____
9. _____
10. _____

Others: _____

d. Have you moved in the past year? ____Yes ____ No

If yes, please specify your previous address or addresses below:

_____
_____
_____
_____
_____
_____
_____
_____

e. Do you live or work on a farm?

1. Yes
2. No

f. Do your eating habits include any of the following?

1. Vegetarian Diet
2. Special Diet (Please Specify)

_____
_____
_____

2. Previous Medical History (1 year):
a. Have you experienced any of the following?
If the answer is Yes, please complete the Table.

			Was this reported to or diagnosed by a physician? If yes, please specify?			Did this occur during travel? If yes, please specify?			Date
	Yes	No	Yes	Physician	No	Yes	Location	No	
1. Upper Respiratory Tract Infection (URTI)									
2. Diarrheal Illnesses:									
Cramps									
Blood in Stool									
Watery Diahrea									
WBC Count in Stool									
3. Myalgia									
4. Fever									
5. Previous GI or Neurological Diseases: (Use Space Provided)									
6. Allergies: (Use Space Provided)									

2. Previous Medical History (1 year) continued:

b. Medication History

Prescription	Non- Prescription	Date

c. Surgical History

Type of Surgery	Date

APPENDIX C (continued)

3. Possible Vectors:

a. Contact with or Exposure to:

____Cattle ____Poultry
____Pets ____Surface Water
____Sewage ____Common Birds
____Symptomatic Individuals

b. Consumption of:

____Red or Bloody Meat
____Raw Milk
____Untreated water (surface, well, cottage, lakes, estuaries)
____Raw or Soft Cooked Eggs

Water source:

At Home:___ well ____city ____Bottled ____other (estuaries, etc.)

At Work:___ well ____city ____Bottled ____other (estuaries, etc.)

4. This Section to be Completed for Household Controls Only

Relation to GBS Patient: ____Immediate Family ____Spouse
____Other: (Specify):_____

5. This Section to be completed by Hospital Based Controls Only

Reason for Admission: _____
_____

Comments:

_____
_____
_____
_____
_____
_____
_____
_____

APPENDIX D: Epidemiological Survey: Clinical Assessment Template

Clinical Assessment
Guillain Barre Syndrome Project
Patient Identification #:_____
Date: _____, 19___ **Attending Neurologist(s):**_____
Admission Date: _____, 19

1. Symptoms: _____

History of Onset of Symptoms: (Include dates)

Symptom(s):	Date:

Disability Scale (at maximum disability)
Hughes Scale: ___1 ___2 ___3 ___4 ___5 ___6

Duration of Maximum Disability: _____
Amount of time on ventilator: _____
Comments: _____

2. Lab Data:
EMG _____abnormal _____ normal
_____axonal _____ demyelinating
Blood Type: _____
HLA typing: _____
CSF: 1. Date:_____ Results:_____
2. Date:_____ Results:_____
_____
Cell count _____ normal _____ elevated
Protein _____ normal _____ elevated

Comments: _____

3. History of Treatment:

Treatment		Date
IVIG	Plasmapheresis	