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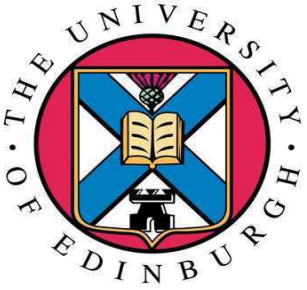
Roles of *Clostridium difficile* cell wall and flagellar proteins in pathogenicity and innate immunity



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CONTENTS

DEDICATION

I dedicate this thesis to those who are browsing this thesis:

My late parents, they are my roots, I have grown up under their supervision, they supplicated Allah to enhance me and my words cannot express the appreciation for them but Allah will

My wife, Samar, for your prayers, love and supporting me for so long has never been ignored.

My children, Mohammad, Rawan, Majed, Mazin, Moaied, Majd and Mulham, they are my Allah gifts. Whose are make my life joyful.

ABSTRACT

The number of cases of *Clostridium difficile* infection (CDI) has been increasing globally. CDI is the main cause of nosocomial diarrhoea, which may be life-threatening in complicated cases, and also costs the health care societies millions of pounds annually. The predominant types and their resistance to antibiotics have been changing and one of the major selective pressures which causes this is antimicrobial use. Although much is known about the role of the toxins in pathogenesis of CDI, the role of immunogenic cell wall components is unclear. They may play a role in colonisation and pathology and a study of these could clarify the infection process. It is therefore important to study the immune responses against these bacterial wall components from different strains and their effects on stimulation of leukocytes to produce cytokines and chemokines.

This study was divided into four parts:

1. An epidemiological study to determine frequencies of the predominant types of *C. difficile*, thus 140 *C. difficile* isolates from surgical patients and their environment during 2009 were investigated to define their PCR ribotype. This utilised capillary sequencing gel electrophoresis for their analysis.
2. The determination of antimicrobial susceptibility to six antibiotics (ampicillin, erythromycin, tetracycline, metronidazole, moxifloxacin and vancomycin) was assessed and MIC determination by agar dilutions.
3. Investigation of host immunity to molecules with conserved molecular patterns. Surface-layer proteins (SLPs), lipocarbohydrate (LC) and flagellar proteins were

separated and purified from five ribotypes of *C. difficile* (001, 002, 027, 078 and106) predominant in Scotland.

a) The immune responses to these molecules were assessed by ELISA by exposing serum of patients and healthy donors and measuring specific IgG levels.
b) Innate immunity was investigated by distinguishing responses of a macrophage cell line (THP1) to the above molecules. Induction of interleukins (IL)-1β, IL-6, IL-8, IL-10 and IL-12 interleukins and TNF-α was detected by ELISA.

In this study 15 different ribotypes were identified. The most frequent were 001, 020, 106 ribotypes (52.8%, 7.4% and 5.7%), respectively, while 13 isolates could not be assigned a ribotype. However, all isolates were sensitive to vancomycin, metronidazole and moxifloxacin, but 74.28% of isolates were resistant to erythromycin.

The IgG level against bacterial antigens (SLPs, LC and flagella proteins) in donors' serum showed almost normal distribution to all antigens from the different ribotypes and the sensitivity of the assays was increased by raising the concentration of antigens. Levels to SLPs were generally the highest, but the flagellar protein exceeded the SLPs of the 027 ribotype. The donors, controls, patients and carrier sera gave similar results.

The greatest induction of interleukins was obtained using 50µg of antigen with the THP-1 cells activated with 50ng of PMA. The highest induction of all antigens was

for IL-10. The highest values for the control LPS was with IL-12. But the best effect for SLPs of 027 was for IL-10 (109.1ng/ml), while the weakest for TNF for SLPs of 027 (4.7ng/ml). In general the IL-1β, IL-6, IL-8 and TNF concentrations ranged from 4.7-60ng/ml for all antigens and in contrast IL-12 and IL-10 average ranged 11-109.1ng/ml.

To conclude, the prevalence of *C. difficile* and their antibiotic susceptibility are constantly changing. IgG antibodies to SLPs and flagellar proteins from the hypervirulent ribotype 027 were highest in the community and hospitalized individuals. The molecules of conserved molecular patterns are immunogenic with various levels of response in the monocytic THP1 cells. SLPs were best in inducing interleukins. Flagellar proteins from 027 ribotypes accompanied SLPs in IL-10 induction levels. Consequently SLPs and flagellar proteins from 027 ribotypes appeared the best immunogenic bacterial molecules.

ACKNOWLEDGMENTS

Grateful thanks to my supervisor, Professor Ian R. Poxton, for his guidance, support and understanding during the last four years; thanks also to Dr Garry Blakely, my second supervisor.

Many thanks to Dr Surekha Reddy who consented the study participants, obtained clinical samples. Many, many thanks to all my friends, past and present, in MPRL and MC group. As well Mr. Adel from Glasgow University for offering THP-1 cell line.

Also I wish to appreciate Dr. Derek Fairley from Belfast Health and Social Care Trust for his kind gift of *C. difficile* standard strains library.

DECLARATION

The author performed all the investigations and procedures presented in this thesis, unless otherwise stated.

ABBREVIATIONS

AMPs Antimicrobial peptides

APS Ammonium persulphate

BA Blood Agar medium

BAM Brucella Agar Medium

BHI Brain-Heart Infusion

bp Base Pair

BSA Bovine Serum Albumen

BSAC British Society for Antimicrobial Chemotherapy

Caco-2 Heterogeneous Human Epithelial Colorectal Adenocarcinoma Cells

CCEY Cefoxitin-cycloserine-egg yolk agar

CDI *Clostridium difficile* Infection

CDAD *C. difficile* associated diarrhoea

CGE Capillary Gel Electrophoresis

CLSI Clinical Laboratory Standards Institute

CWP Cell Wall Protein

Cyt Cytoplasm

CV Coefficient of Variation

DC Dendritic Cells

DSS Dextran Sulphate Sodium

ECACC European Collection of Animal Cell Cultures

ELISA Enzyme-Linked Immunosorbent Assay

ESGCD European Study Group for *C. difficile*

FCS Fetal Calf Serum

FDA Food and Drug Administration

FliC Flagellar protein C

FliD Flagellar protein D

Fbp Fibronectin Protein

GALT Gut-Associated Lymphoid Tissue

HPS Health Protection Scotland

HMW High Molecular Weight

IBD Inflammatory Bowel Disease

IDSA Infectious Diseases Society of America

IgA Immunoglobulin A

IgG Immunoglobulin G

IL Interleukins

IVIG Intravenous Immunoglobulin

kDa Kilo Dalton

LC Lipocarbohydrate

LMW Low Molecular Weight

LPS Lipopolysaccharide

LTA Lipoteichoic Acid

MIC Minimum Inhibition Concentration

MRSA Meticillin Resistant *Staphylococcus Aureus*

NFW Nuclease Free Water

PAGE Polyacrylamide Gel Electrophoresis

PaLoc Pathogenicity Locus

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reaction

PG Peptidoglycan

pIgAR Polymeric Immunoglobulin A Receptor

PMA Phorbol Myristate Acetate (a potent tumour promoter often employed in biomedical research to activate the signal transduction enzyme protein kinase)

PMC Pseudomembranous Colitis

PPY Protease Peptone Yeast Extract Medium

PRR Pattern Recognition Receptors

PSG Penicillin Streptomycin Glutamine

R^2 Statistical value was estimated to indicate the coefficient and effectiveness of results

rRNA Ribosomal Ribonucleic Acid

SDS Sodium Lauryl Sulphate

SEM Standard Error of the Mean

SHEA Society for Health-care Epidemiology of America

SLPs Surface Layer Proteins

SXGC conserved motif in *C. difficile* genome

TcdA Toxin A

TcdB Toxin B

TEMED N, N, N, N- Tetramethylethylenediamine

TGF-β Transforming Growth Factor

THP-1 Human Acute Monocytic Leukaemic Cells from human male one year old

TJC Tight Junctional Complex

TLRs Toll-Like Receptors

TNF Tumour Necrosis Factor

UV Ultra Violet

WGH Western General Hospital, Edinburgh

CHAPTER I INTRODUCTION

1.1 *Clostridium difficile* epidemiology:

The gut's microbiota is critically important as it spreads to varying degrees in the four gastrointestinal areas: stomach, jejunum, ileum and colon. Furthermore, the normal gut microorganisms are useful in food processing, assisting intestinal blood vessels, stimulating progress of the gut-associated lymphoid tissue (GALT), and contributing to organising bowel immunity.

Clostridia are rare in the stomach and jejunum, yet thrive in the colon (Albright and Albright, 2009). While the presence of *C. difficile* in the large intestinal microbiota is commensal in most neonates as well as is in about 3% of healthy adults, colonisation increases up to 40% in the hospitalised elderly patients or debilitated host; *C. difficile* omnipresence is natural. Moreover, it is the main cause of diarrhoea in long-stay hospitalised elderly patients, no doubt due to their consumption of broad-spectrum antibiotics. Susceptibility increases in compromised patients. However, the severity of symptoms ranges from mild to severe colitis with pseudomembranes and toxic megacolon. It may be life-threatening (Sydnor and Perl, 2011).

The first time *C. difficile* was described as being the main factor of antimicrobial-associated diarrhoea and pseudomembranous colitis (PMC) was in 1978 (Larson HE et al 1978). This was the start of many experts studying pathogenicity, epidemiology, risk factors, antibiotic susceptibility, as well as prevention of this infection.

C. difficile may be isolated from soil, fresh, salt and tap water, domestic animals and all foods including meat, vegetables and animal foods (Al Saif and Brazier, 1996). Also, hospital environments could be contaminated by *C. difficile* spores in lavatories, sluice rooms, beds and floors (McCoubrey *et al*., 2003). Furthermore, health-care workers are able to be a source of spores, and long hospitalisation could raise the rates of non-symptomatic *C. difficile* colonisation by 20% more than in a community. Therefore, long hospitalisation, risk conditions and oral consumption of food or drinks contaminated by *C. difficile* vegetative cells or spores, assist the colonisation of *C. difficile* in the bowel (Rupnik *et al*., 2009).

The fight to prevent nosocomial infection-associated diarrhoea has soared in the healthcare societies during the last decades, as it costs various governments billions of dollars. The United States spends about $3.2 billion yearly on the investigation, hospitalisation, and treatment of *C. difficile*-associated diarrhoea (CDAD) (O'Brien, 2007); the United Kingdom spent about £200 million on CDAD in 2005 (Blondeau, 2009). Therefore, studying the epidemiology and pathogenicity provides an overview of epidemics and should help to reduce health care costs.

In 2002, the European Study Group for *C. difficile* (ESGCD) studied the CDI rates in 212 European hospitals, and indicated that the mean incidence of CDI in European countries was 11 cases per 10,000 admissions. However, in 2005, there was progress in the health care service; the statistics indicated the prevalence being 0.13 to 7.1 cases per 10,000 patients per day. Although between Oct. 2003 and Jun. 2004, the largest outbreak of hypervirulent epidemic ribotype 027 in the United Kingdom was

present in Stoke Mandeville Hospital in Buckinghamshire. During this epidemic, nineteen patients died, and was followed by another outbreak in the period of Oct. 2004 to Jun. 2005 by an equal mortality rate and the same type of *C. difficile*. The causes referred to the poorness in the health care facilities and the poor restriction of antibiotic use (Freeman *et al*., 2010). The incidence rates of *C. difficile* infection (CDI) change significantly depending on the diagnosing facility, antibiotic consumption system, the application of infection control, and overall hygiene (HPS 2011).

Bauer *et al*. (2011) studied the incidence of CDI in Europe and the geographical allocation of *C. difficile* ribotypes in 34 European countries. They collected data from 106 laboratories to demonstrate the distribution of *C. difficile* in November 2008, and showed the 014 PCR ribotypes as being the commonest in European investigation centres and 001 in 13 countries. However, the hypervirulent ribotype 027 was ninth, and in regards to pervasiveness and multi-country surveillance. It is important to detect and manage CDI and to show variations between different countries (Table1.1).

Country	Number of toxin positive cases/number of patients tested and percentage of positive cases	Number of patients tested per 10 000 patient-days	Number of typed isolates in country	Commonest ribotype
Finland	52/351 (15%)	141	19	106
U.K	164/1695 (10%)	115	43	106
Ireland	38/493 (8%)	94	18	106
Denmark	28/330 (8%)	74	16	023
Sweden	69/430 (16%)	74	28	014 & 020
Germany	93/602 (15%)	72	22	001

Table1.1: The Data show the most common PCR-ribotypes per European country and number of patients tested per day as well as percentage of positive cases (taken from Bauer *et al*., 2011).

C. difficile strains have produce toxins: enterotoxin (A) TcdA and cytotoxin (B) TcdB, which are responsible for the diarrhoea (Starr, 2005). Subsequently, the severity of CDI symptoms is related to the production of these toxins, and some strains also produce binary toxin (CDT) (Akerlund *et al*., 2006; Bouvet and Opoff, 2008).

The amino acid sequences for toxins A and B are similar for about two thirds of their structure. The enterotoxin and cytotoxin are effective in *C. difficile* pathogenicity; and the action of these toxins includes binding to several receptors on the epithelial cell surfaces and membranes with enzymatic activity on cytoskeleton formation, as well as the discharging of several pro-inflammatory cytokines from the colonic cells, macrophages and mast cells (Rupnik *et al*., 2009).

The hypervirulent ribotype 027, which produces a high level of toxins A and B, appears to have the highest severity and mortality rate (Dupuy *et al*., 2008).

Fortunately, there is a suggestion that insufficient toxin receptors on the epithelial cells in infants could prevent the host from symptomatic colonisation and other factors associated with the resistance of virulent *C. difficile* colonisation. This is because the epithelial toxin binding doesn't happen all over the intestine of neonates (Kaslow and Shiver, 2011).

Furthermore, the hypervirulent ribotype 027 is not the predominant strain in Scotland; the dominant strains change from time to time, and place to place depending on several factors. It has recently been studied in Scotland that the most frequent strains are 001 ribotype and 106 ribotype (HPS reports 2011; Freeman *et al*., 2010).

However, there is still a compromised community risk; all populations are threatened by CDI as a result of changes in epidemiology and the antibiotic susceptibility of new strains of *C. difficile* (Kuntz *et al*., 2011).

During the last decade, the distribution of *C. difficile* has altered in Scotland. Taori *et al*., (2010) investigated the period 1979-2004 for the predominant ribotypes of *C. difficile* in southern Scotland and the changes in antibiotic susceptibility, The study indicated that the most frequent ribotypes were 002 and the incidence during the study period for 001 ribotype increased eight times which reflected the highest antibiotic resistance pattern, while 012 ribotype decrease from 8.7 to 2%. 95.5% of the total isolates were resistant to clindamycin but there is no resistant isolates for vancomycin in their study. However about 18% of isolates are resistant to two antibiotics and fewer than 8% were resistant to three or more antibiotics.

Also, the Wiuff *et al*., (2011) study indicates that ribotypes 106 and 001 have been the dominant strains in Scotland for the years 2008 and 2009. It also shows that more than 93% of *C. difficile* dominant ribotype isolates show resistance to clindamycin, erythromycin, levofloxacin, moxifloxacin and cefotaxime, as they can be declared as

multidrug-resistant strains. In addition, low doses of antibiotics amplifying the ability of resistant strains are the main cause of change in *C. difficile* epidemiology and antibiotic susceptibility, as suggested. Thus, one of the effective ways to avoid hypervirulent strains is through control of antibiotics, which requires a prospective epidemiological study, and antimicrobial susceptibility (Shears *et al*., 2010).

An uncontrolled CDI outbreak is caused by several factors; these factors are related to the patient conditions, hospital facilities, health care systems, microbe ribotypes, and the microbiota disturbance (Goorhuis *et al*., 2011).

The host association factors are divided into two groups: host health conditions, such as haematological malignancy, inflammatory bowel disease, and geriatric, or other compromisation factors (Harbatrth *et al*., 2001). The second group concerns the medical process and treatments, such as nasogastric intubations, acid suppression, proton-pump inhibitor, H-2 receptor antagonist, corticosteroids, long-term hospitalisation, and high exposure to broad-spectrum antibiotics, such as clindamycin and third generation cephalosporin, as well the oral antibiotic consumption contributing to disturbing the gut microbiota, (Starr *et al*., 2003).

The exposure of *C. difficile* to ampicillin and clindamycin may stimulate some binding factors, such as SLPs, Fbp68 and Cwp66, and enzymatic activity, such as cysteine proteases Cwp84, which corrupts bowel epithelium (Dene`ve *et al*., 2008).

Additionally, prophylaxis from low doses of first generation cephalosporins or the use of new generation fluoroquinolones are associated with the lack of control of a CDI outbreak and resistance of the hypervirulent ribotype 027 (Pepin *et al*., 2005).

However, there are some antibiotics that have a low risk, such as aminoglycosides and tigecyline; the latter is a member of the glycylcycline antibiotics and which have a wide spectrum effect on anaerobic bacteria, which causes the toxin production of *C. difficile* to be dormant (Baines *et al*., 2006).

Rupnik *et al.* (2009) claim that the controlled use of third generation, broad-spectrum antibiotics could reduces the CDI infection up to 90%. As well the application of infection control regulations, such as hand washing, patient isolation, using disposable equipment, striving for the surveillance of CDI, and regular teaching programmes for healthcare employees, is recommended by Starr (2005).

Additional to the antibiotics-used factors associated with the CDI, some viral infections also contribute. Wilcox and Fawley (2007) noticed that the CDI rate was higher in the wards infected by norovirus gastroenteritis. There were also factors affecting colonisation, such as gene expression stimulated by some intestinal stressed circumstances, like hyperosmolarity (Dene`ve *et al*., 2008).

Moreover, the weakness of the infection control system in health care organisations as well as the poor diagnostic tools for the control of CDI outbreaks assist the epidemic (Morrison *et al*., 2011). Furthermore, some aspects of *C. difficile* ribotypes,

such as toxin production and genes encoding multi-resistance, have an effect on the virulence of CDI and distribution of types. At the same time, some of the previous factors are related to community-acquired infections and others to the nosocomial infection (Goorhuis *et al.*, 2011).

Patients who are suspected as having CDI were the persons developing diarrhoea in the community or the hospital following consumption of antibiotics. These were accompanied by any debilitating condition or any health risk factors, such as when the liquid stool is sometimes bloody, combined with general signs, like fever, the loss of appetite, nausea and abdominal pain, as well as a rising white blood cell count in about 40% of CDI patients and a decrease in blood albumin in more than two thirds of infected persons (Knoop *et al.*, 1993).

The symptom of vomiting and a typical serial gap in the outbreaks distinguish antibiotic-associated diarrhoea by *C. difficile* from that caused by norovirus and other bacterial infections, as well laxative abuses or other inflammatory gut illnesses. The incubation period of CDI is usually two to ten weeks after the start of antibiotic consumption, to when symptoms appear (Cheng *et al*., 2011).

1.2 *C. difficile* Typing:

Most members of the genus *Clostridium* are motile, and there are variations in oxygen tolerance, but *C. difficile* is typical of the genus being Gram-positive, rod-

shaped, with subterminal spores and are strictly anaerobic and produce exotoxins, which are the main reasons for pathogenicity.

The categorisation of pandemic *C. difficile* isolates depends on accurate typing methods and of detecting the relatedness between *C. difficile* strains in the outbreaks. There have been many techniques developed for typing and these included the detection of phenotypic markers or a genotypic markers, which has become a universal technique to detect epidemics, as it is quick, easy, and suitably discriminative (Indra *et al*., 2008; Bidet *et al*., 2000).

One of the genotypic techniques is the polymerase chain reaction (PCR) ribotyping, which is based on the presence of a number of alleles of the rRNA operon, with variation in the length of the intergenic spacer region. This involves the amplification of the targeted fragments of rRNA between the 16S and 23S genes during several rounds of amplification in a thermocycler to create easily detected amounts of rRNA by using the two primers in the16S and 23S flanking regions (O'Neill *et al* 1995; Bidet *et al*., 2000; Singh *et al*., 2006). Moreover, Indra *et al*. (2008) claim that there are more than 250 ribotypes; the minority of them are human pathogens.

Analysis of PCR ribotype is traditionally based on electrophoresis in agarose gel and has been confirmed as being a useful tool for the epidemiological analysis of *C. difficile* outbreaks in European countries as it is easy to use and is low-cost, comparatively. However, the main defect in this assay is the weakness of resolution of fragment size analysis and difficulty in comparing patterns between labs.

On the other hand, Indra *et al*., (2008) assert that fluorescence dye labelling on the 16S primer for amplification of fragments and they developed the technique which they named capillary gel electrophoresis ribotyping, which is abbreviated as "sr". This new technique was developed to decrease considerably the time required for this test if a capillary sequencer was available in the laboratory. Furthermore, web-based software permitted sharing the data between analysis centres in order to conquer the problems related to contrast typing of *C. difficile* isolates, such as standardised chemicals, methods and tools.

The patterns used in the PCR capillary gel electrophoresis ribotyping are the different sizes of inter-gene spacer regions of the rRNA gene of the microorganisms, and the extent of these areas in *C. difficile* ranges from 233-680 bp. This feature makes PCR more sensitive, as it is able to distinguish *C. difficile* subgroups. For example, the capillary gel electrophoresis ribotyping differentiates seven subgroups from the 014 ribotype, as studied by Indra *et al*., (2008).

1.3 *C. difficile* pathogenicity:

Bacterial pathogenicity is mainly related to the presence of gene-encoded virulence factors. However, most of the CDI in a human takes place during or after antibiotic consumption; also, compromised patients, like the elderly, are able to be colonised by *C. difficile*, and it is possible to isolate *C. difficile* from neonatal faeces as these are carriers without symptoms (Poxton *et al*., 2001).

Adult patients colonised by *C. difficile* were able to be converted to infections when the gut microbiota was disturbed by medication or there were changes in health conditions (Hogenauer *et al*., 1988). Therefore, the colonisation of the bowel starts by orally consuming *C. difficile* vegetative cells or more likely spores, followed by a disturbance of colonic microbiota by medication or host predisposing factors, which makes the colonic epithelial cells susceptible to being attacked by infectious agents, with varying severity of infection (Rupnik *et al*., 2009; Fagan *et al*., 2009).

Then, the vegetative *C. difficile* cells adhere to the colonic epithelial cells by different factors, such as hydrolytic enzymes, putative capsules, and surface-layer proteins (SLPs) and flagella proteins (Tasteyre *et al*., 2001; Dingle *et al*., 2011). However, most of the effective factors in the pathogenicity are toxins A and B, which degrade the actin cytoskeleton of the human intestinal cells and kill epithelial cells. These diminish the gut barrier function and consequently cause fluid to accumulate. Furthermore, these factors stimulate the host immune system; inflammation presents and produces a pseudomembrane. Finally, the diarrhoea, colitis and toxic megacolon are the result and are often life-threatening (Rupnik *et al*., 2009; Kelly and Kyne, 2011). The infection procedure is summarised in Figure 1.1.

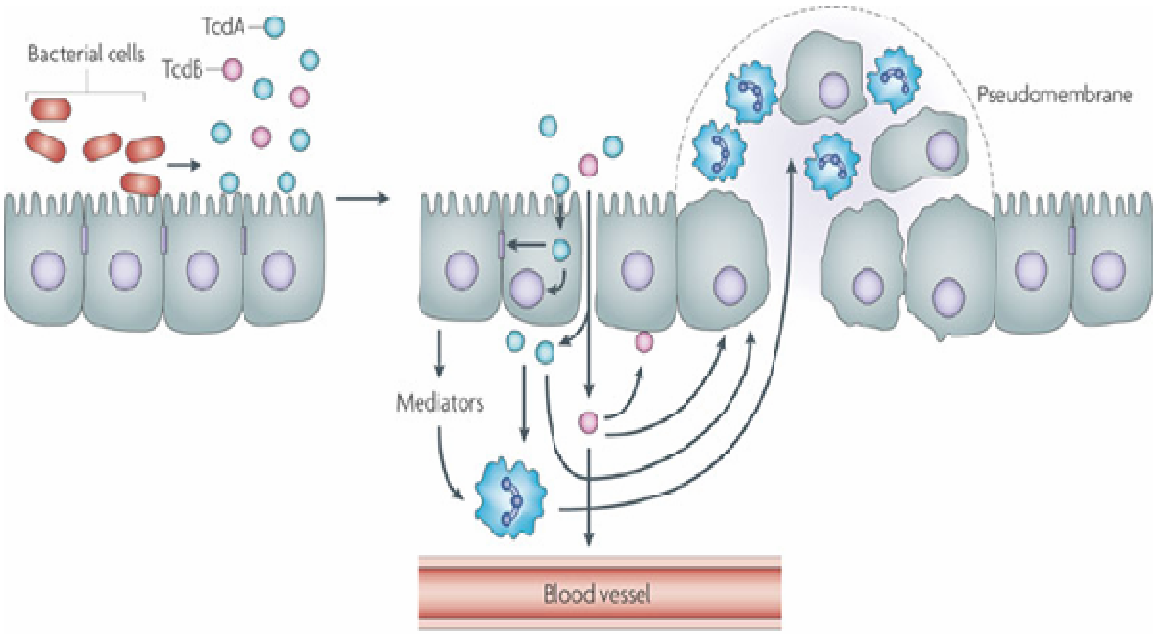

Figure 1.1: The diagram designed by Rupnik *et al*., (2009) reviews and illustrates the infection procedure. TcdA= Toxin A, and TcdB= Toxin B. Blue cells = neutrophils

There are three toxins produced by *C. difficile* - Toxin A, Toxin B and binary toxin. The first two toxins are coded on a pathogenicity locus, as in figure 1.3a. Toxin A (molecular weight 308 kDa and encoded by *tcdA*) is sometimes referred to as the enterotoxin according to the effect of gathering fluid in the intestinal loop model by raising exchange fluids in the epithelial cells, inhibiting built-up proteins, enhancing the making of prostaglandins as well as leukotriene, and breaking through the intestinal border with necrosis of the mucus membrane. Subsequently, blood plasma is secreted into the intestine. Toxin B (molecular weight 269 kDa and encoded by *tcdB*) has a cytotoxic action on cell lines and is often referred to as the cytotoxin reviewed by Rupnik *et al*. (2009).

As well both toxins are shared in two thirds of amino acid sequencing; they have binding activity to the epithelial cell wall and membrane. Also, there is an enzymatic achievement on the cytoskeleton binding layer as well as on macrophage and mast cells (Cohen *et al*., 2010).

Both of these toxins are single protein chains and have three functional areas; a binding domain on the C-terminal by β- solenoid structure, cysteine protease activity in the central part, which separates the glucosyl transferase domain (N-terminal) (figures1.2, 1.3) (Rupnik *et al*., 2009).

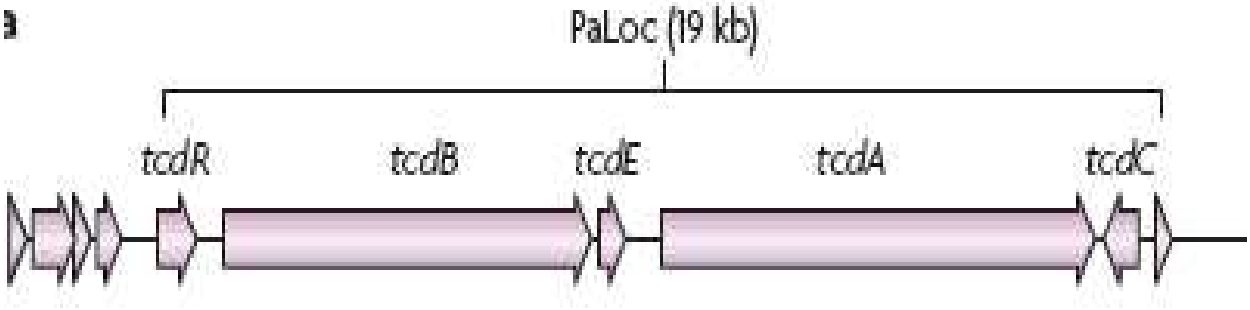

Figure 1.2: The diagram shows the genes encoding TcdA and TcdB. In total there are five genes (*tcdR, tcdB, tcdE, tcdA and tcdC*) and are located on the pathogenicity locus (PaLoc) (Taken from Rupnik *et al*., 2009).

Moreover, the enzymatic cleavage action is the common activity in both toxins, which are cytotoxic for cell lines *in vitro*, while the effectiveness of toxin B is higher by 100-1000 times more than toxin A. After damaging the intestinal epithelial cells, this is followed by the formation pf pseudomembranes as a result of degrading the

actin cytoskeleton, then stimulating the host immune system to produce tumour necrotic factor-α (TNF-α) and interleukins (ILs) (Darkoh *et al*., 2011).

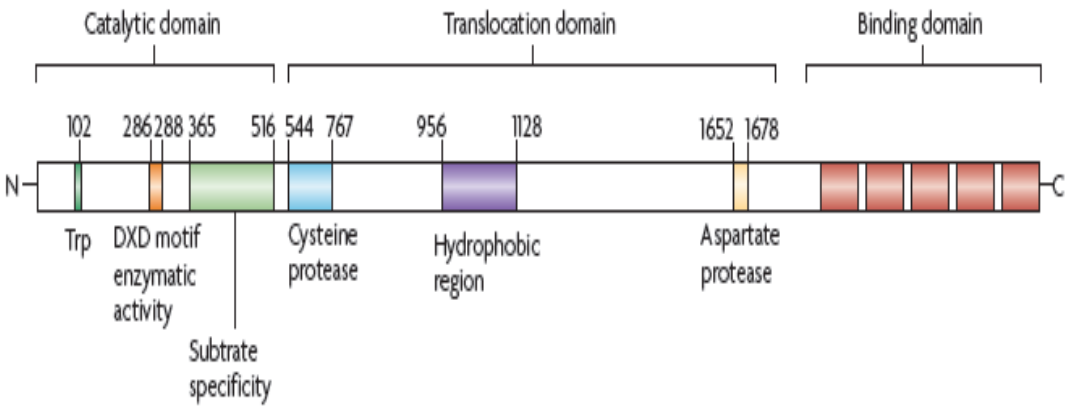

Figure1.3: The structure and functions of toxins A and B, where the catalytic and binding domains are peripheral and the translocation domain is central and contains the hydrophobic region. The enzymatic activity and substrate specificity are on the N-terminal (reviewed by Rupnik *et al*., 2009).

The C- terminal of toxin A, which binds with host cells, is composed of a five part recurring structure consisting of glycopeptides. This part may stimulate the infection by an enterotoxin effect, while toxin B (tcdB) consists of 18 cell wall-binding parts, and has cytotoxic action. These features are used in a standard diagnosis of CDI are cytotoxicity and toxigenic assays (Crobach *et al*., 2009; Cohen *et al*., 2010).

The binary toxin is produced by some strains. However, there is no good evidence for its function in the colonisation of human bowel epithelial cells or for causing cell damage during infection (Bouvet and Opoff, 2008). The binary toxin or CDT is encoded on another part of the chromosome (CdtLoc) and includes three genes (*cdtR*,

cdtA and *cdtB*), the two unlinked proteins are *CdtB* and *CdtA*, but *CdtB* is a binding component while *CdtA* has the enzymatic function (Rupnik *et al*., 2009).

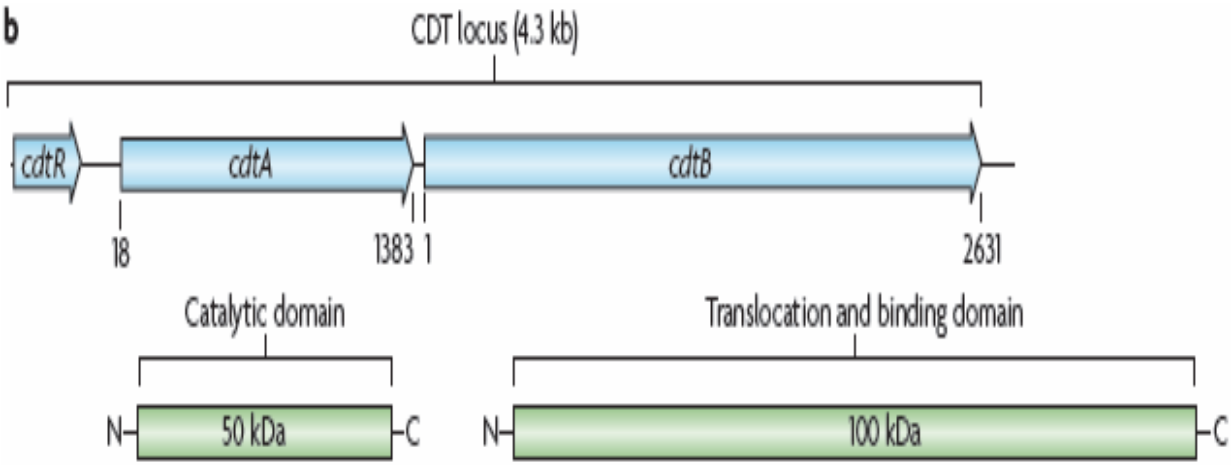

Figure 1.4: This diagram illustrates the structure and functions of the binary toxin (Taken from Rupnik *et al*., 2009).

Additional to toxin A and B, binary toxin is suggested as being a virulence factors in *C. difficile*. It is known as actin-specific ADP-ribosyltransferase toxin or CDT. The CDT was investigated by western blotting, while *TcdA* and *TcdB* genes for toxins A and B were examined by PCR. To date, the role of the CDT in *C. difficile* pathogenicity has not been confirmed in human CDI, but it is known to induce microtubule formation in tissue culture cells and may aid adhesion of *C. difficile* cells in *vivo* (Schwan *et al*., 2009).

Although TcdB and TcdA are predominant in the pathogenicity of *C. difficile*, other factors also support these toxins in the infectious process. The competition

phenomenum between bowel microbiota indicates the contribution of intestinal colonisation by non-toxic *C. difficile,* and that other commensal microbiota protect the host bowel from CDI as a symbiotic relationship; therefore, disturbances of microbiota by the antibiotic use enhance *C. difficile* (Sambol *et al*., 2002).

Dingle *et al*. (2011) suggest that the surface layer proteins (SLPs) have a role in the adherence step in the infection process, since the cell wall proteins (CWPs) fasten SLPs on the external cell surfaces of *C. difficile*, and SWPs 66 and SWPs 84 are concerned with adhering to epithelial cells and may damage outer cell matrices. Furthermore, the flagellar proteins of *C. difficile* (FliC, FliD) and the basic structure of flagella are a part of the connection process to or penetration of the mucus layer in the epithelial cells.

The capsule is suggested as being a virulence factor in bacterial infection and an anti-phagocytic part by Tasteyre *et al*. (2000).

1.4 The relationship between intestinal defence system and microbiota:

The intestine is one of the highest sites of microbial colonisation in the human body, and the relationship between microorganisms and the gut indicate efficiency of the host defence, which is characterised by differentiating between pathogenic and non-pathogenic microbes; some of these microorganisms are commensal and the other pathogens are opportunistic (Schiffrin and Donnet-Hughes, 2011).

The function and structure of the intestine allows the bowel to control the microbiota, where digesting food, removing undigested food and pathogenic agents, are some of intestinal functions. Moreover, regulating the mucus membrane, intercellular linkage, epithelial cells and immune responses are other functions (Kim and Ho, 2010).

There are four types of luminal epithelial cells: absorptive enterocytes, goblet cells, Paneth cells and enteroendocrine cells. The goblet cell range from 4% to 16% in a colon and depend on the rising microbial count. Goblet cells secrete some components of mucus, such as mucins, peptides, and binding proteins. The mucins have roles in human innate and adaptive immunity - for instance, the effect on binding with pathogens, adhesion, growth factors, and cytokines (Dharmani *et al*., 2009).

The major cells are enterocytes, which are absorptive and also responsible for producing cytokines and chemokines in order to control immune cells in the subordinate tissue; the Paneth cells create antimicrobial peptides, and enteroendocrine cells generate peptide hormones. Furthermore, the lymphoid cells activate indigenous particles which are provided to immune cells in the lymph nodes. Although the ability of the cells involved in innate immunity to identify antigens of pathogens in the bowel depends on imperfect feature receptors, which cannot specify the antigens when exposed, they can generally distinguish pathogen-associated molecular patterns and are useful in the organising the adaptive immune response (Schiffrin and Donnet-Hughes, 2011).

The innate immune system has many actions, such as a chemical barrier, like acidity, in the stomach and the intestinal folds with movements, such as physical action; also, pepsin, trypsin and other enzymes, bile and microbiota competition have a role in inhibiting infectious agents, and phagocytes and antibodies attack foreign bodies (Janeway *et al*., 2001).

One of the first lines of protection from foreign bodies in the host bowel is epithelial cells, which sense the pathogens and unusual growth of indigenous microorganisms and promote the immune system. The mucus layer protects the epithelial cells from physical and chemical injury and prevents the cells from direct contact with endogenous antigens.

Immunoglobulin A (IgA), which is bound to the mucus layer and which prevents the attack of pathogens in the gut lumen by neutralising bacterial lipoproteins, whilst also combining with many antimicrobial peptides, which are secreted by Paneth cells that have a function in preventing the intestine lumen from microbial attack. These compounds have an influence on defences such as lysozyme and cathelicidins (Maldonado and McCormick, 2011).

As the main role in the gut homeostasis is influenced by mucosal antibodies, Benckert *et al*. (2011) suggested that the disproportion in the IgA and IgG production by B cells in the intestine may be accompanied by bowel diseases. However, they found that a quarter of intestinal IgA and IgG were polyreactive. Mainly, specific detection of the pathogenic and commensal microbes as well self-antigens and 100%

of the bowel antibodies were somatically modified, which clarified that colonic homeostasis is the result of specific immune B cell responses in the intestine; this provides essential recognition of uncontrolled defence reactions present in inflammatory gut diseases.

Therefore, many microorganisms have modified their invasion methods to avoid these barriers, such as *C. difficile* toxins, which weaken the stability of a tight junctional complex (TJC), such as claudins and occludins, which prevent bacterial adherence to epithelial cells and which control epithelial cell permeability (Nusrat *et al*., 2001).

Identification of pathogens and molecular structures related to microorganisms, like flagellin, peptidoglycan, and lipopolysaccharide (LPS), also formylated peptides in lumen cells, is done by receptors, such as the extracellular Toll-like receptors (TLRs) and the intracellular Nod-like receptors. These receptors induce IL-1, IL-6 and tumour necrosis factor alpha (TNF-α) as part of the innate immune system. In addition, there are huge families of cytosolic pattern recognition receptors, which also have subfamilies that depend on N-terminus patterns. All of these receptors are involved in surveillance of the extracellular, endolysosomal and cytoplasm signs in the infection and damaged epithelium layer (Kawai and Akira, 2006).

Inflammation of innate immunity can be stimulated by antigens, which enhance the adaptive immune reaction by activating the antigens and which achieve the desired stage via interaction with pattern recognition receptors (PRR). These molecules pass

the cell membrane into endosomal compartments inside the cell, and through a series of reactions, cytokines and chemokines are produced. Therefore, innate immunity has the “decision” on protection and bowel stability. For instance, in the steady/healthy bowel, the symbiotic microbiota enhance epithelial cells in order to produce homeostatic cytokines, such as TGF-β, to suppress the dendritic cells (DC), which are required to create a primary antibody, while stimulating the pro-inflammatory cytokine production through epithelial cells to stimulate the DC in the infection (Schiffrin and Donnet-Hughes, 2011).

In addition to the competition of symbiotic microorganisms to the pathogens in the bowel, the microbiota are involved in gut homeostasis, the endorsement of intestinal lymphoid tissues and the amplifying production of intestinal villi also accompanies the life cycle reduction of epithelial cells. It also affects epithelial cell permeability, intestinal mucous layer composition, thickness, compactness as well as mucin formation (Maldonado-Contreras and McCormick, 2011). Moreover, the creation of some antimicrobial peptides by Paneth cells was considerably increased by the presence of bowel microbiota, as opposed to gut-free microorganisms (Karlsson *et al*., 2008).

Consequently, the intestinal innate immune system is a primary defence, which maintains a stable bowel microbiota and symbiotic microorganisms, which assist intestinal immunity, as well as presenting antigens to the adaptive immune system. Additionally, elucidating the pathogen-associated molecular pattern effects will clarify the pathogenicity and will control the infection. Furthermore, figure 1.5,

which was designed by Maldonado-Contreras and McCormick (2011), expresses the role of innate mucosal immunity by intestinal epithelial cell performances.

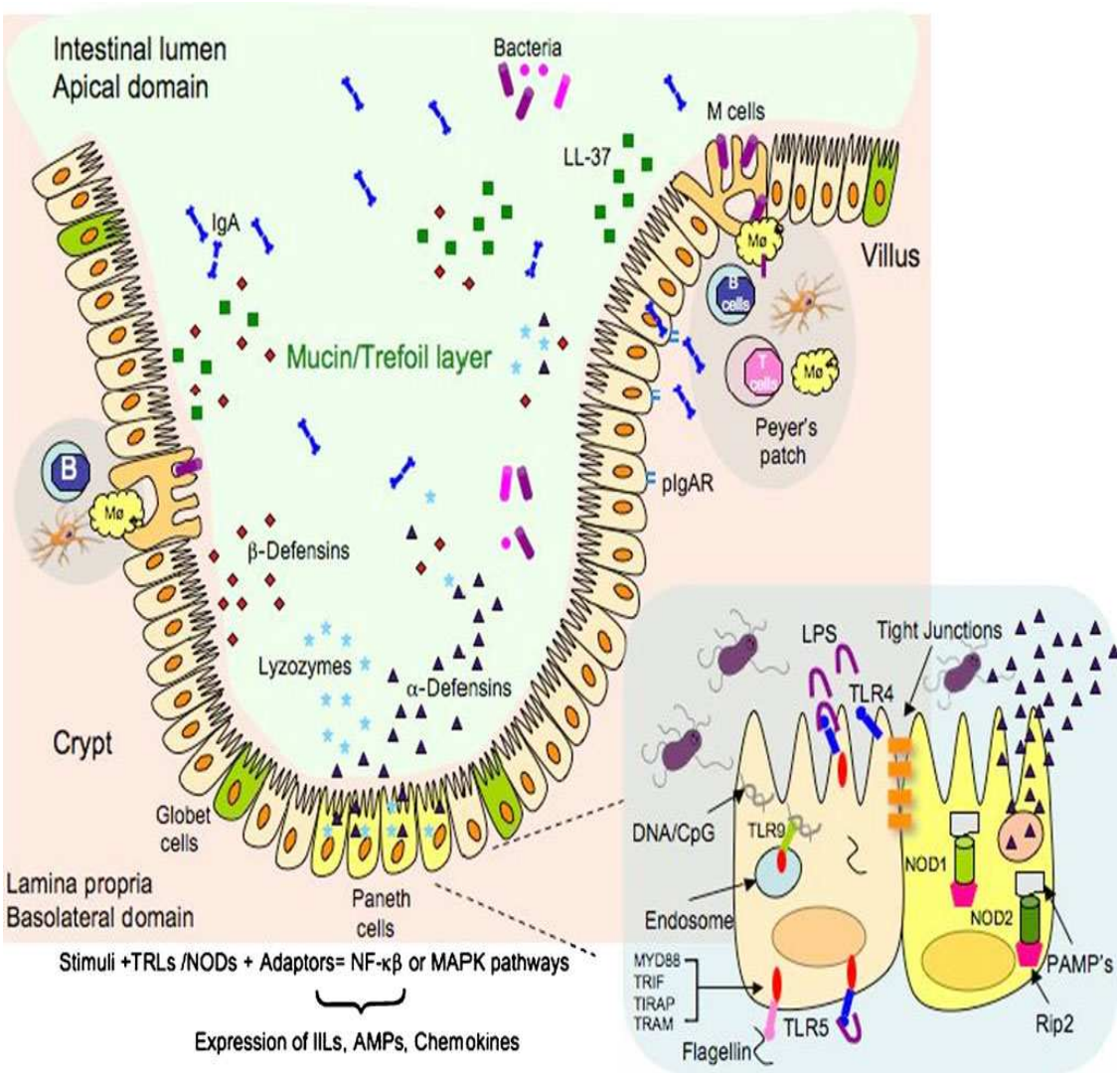

Figure1.5: The intestinal epithelial cell properties that regulate innate mucosal immunity. The abbreviations are: antimicrobial peptides (AMPs), interleukins (IILs), immunoglobulin A (IgA), polymeric immunoglobulin A receptor (pIgAR), human cathelicidin (LL-37), lipopolysaccharide (LPS), M cells microfold cells, macrophage (Mø), mitogen-activated protein kinases (MAPK), adaptor proteins MYD88, TRIF, TIRAP, TRAM, Rip2, nuclear factor kappa-B (NF-κB), nucleotide-binding Oligomerization domain (NODN), pathogen-associated molecular patterns (PAMPs), toll-like receptors (TLR) (Reproduced from Maldonado-Contreras and McCormick, 2011).

1.5 *C. difficile* cell wall structure:

The cell wall structure of *C. difficile* consists of a cytoplasmic membrane from inside the peptidoglycan polymer layer, which is coated by the cell wall proteins and the lipoteichoic acid (lipocarbohydrate antigen LC) included in peptidoglycan (La Riva *et al*., 2011).

Fagan *et al*. (2009) diagrammed the *C. difficile* cell wall and exemplified two molecular weights of surface layer proteins (SLPs), high molecular weight (HMW) and low molecular weight (LMW), as well as other small cell wall protein components (Figure1.6). These cell wall proteins may have roles in bacterial adhesion to epithelial cells (Calabi *et al*., 2002).

The other clostridial wall proteins (CWPs), which are enclosed to the three cell-binding motifs, are in small amounts and have an encoded gene called *cwp*; most of them are supposed to have a role in adhesion, such as Cwp66, which is a phase variable protein, CwpV, Cwp84 and Cwp2, which is a cysteine protease (La Riva *et al*., 2011).

Additional to CWPs, *C. difficile* genomes determine a number of putative surface proteins which have been distinguished at the molecular level, for example, *fbp68* encoding a fibronectin binding protein (Fagan *et al*., 2011).

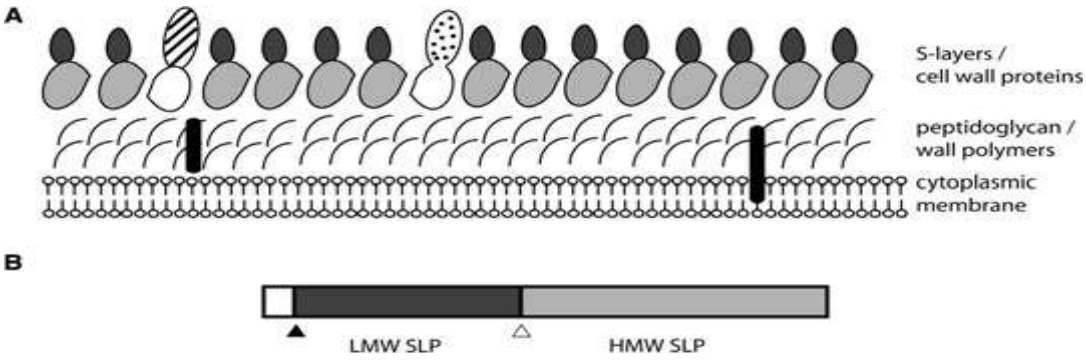

Figure 1.6: Model of the cell wall of *C. difficile*. The diagram illustrates two SLP peptidoglycan layers: the HMW SLP (light grey) and the LMW SLP (dark grey). The white structure indicates other small cell wall proteins (taken from Fagan *et al*., 2009).

La Riva *et al*. (2011) simplified the role of some CWPs in the structure of a cell wall. Firstly, the preprotein production includes a signal peptide. The second step is removing Cwp84 and Cwp13 preproteins, and by autocatalysis activity in Cwp13, the activity is not cleared in Cwp84 when combining Cwp13 to structure the enzymes, which is included in the S-layer. Next, high and low mol. wt. SLPs is formed after the SlpA primer form cleavage by active Cwp84. Finally, the Cwp13 identified the mis-folded proteins and arranged its attachment in the growth medium of the bacterial cell wall (figure 1.7).

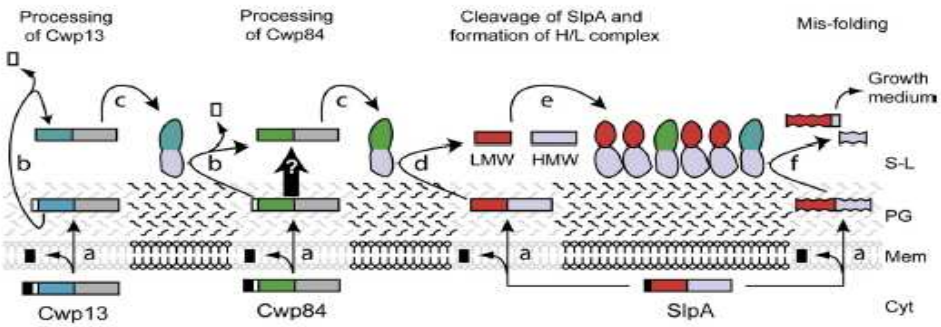

Figure 1.7**:** Represent the processing and activities of Cwp84 and Cwp13 proteins. (S-L, S-layer; PG, peptidoglycan; Mem, membrane; Cyt, cytoplasm). Taken from La Riva *et al* (2011).

The second layer in the clostridial cell wall is peptidoglycan PG, which is located between the cytoplasm membrane and CWPs and is 80 nanometres thick; the teichoic acids are part of it. Despite poor knowledge of PG structure and biosynthesis in *C. difficile*, it seems to contain a high amount of glycan, which is more than 90% N-deacetylated; the non-acetylated amino sugars offer resistance to lysozyme. Also, the PG biosynthesis in *C. difficile* was suppressed by some antibiotics, which may be a hypothetical key in the infection control by antibiotics (Peltier *et al*., 2011; Fagan *et al*., 2011).

The Peltier *et al*., (2011) study of composition and structure of *C. difficile* PG show the homologous proteins in *C. difficile* genome contain ldt_{cd1} (*CD2963*), ldt_{cd2} (*CD2713*) and *CD3007*. It is supposed that these proteins contain a transmembrane area which has catalytic activity with the S*X*GC conserved motif in different levels and the other protein in *C. difficile* genome which has no hydrophobic areas that could act as binding membrane but contain a putative peptidoglycan anchor area consisting of three CWB-2 modules or two SH3 modules. The sequence of preserved motifs of *C. difficile* peptidoglycan is presented in (figure 1.8).

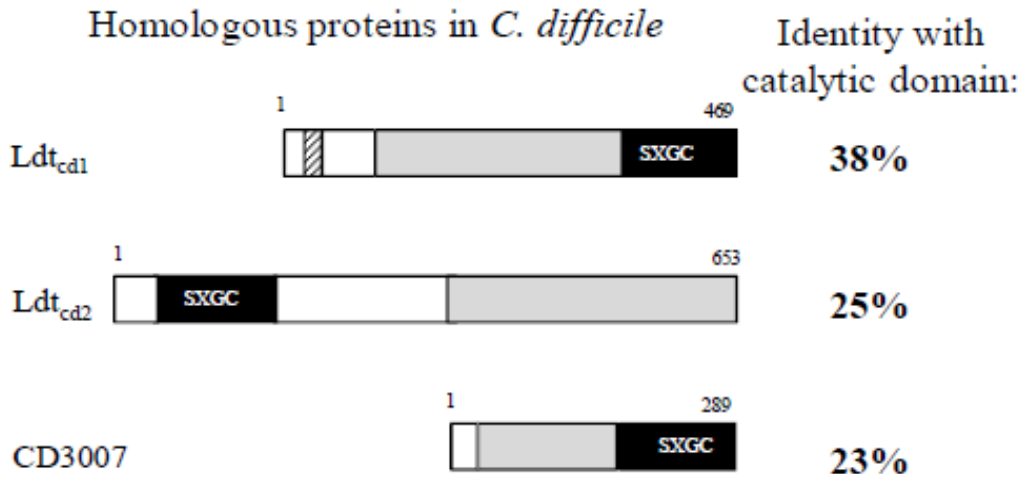

Figure 1.8: The diagram shows the chain of preserved motifs, and L, as well as the D-transpeptidase domain composition of *C. difficile* peptidoglycan and the identity of the catalytic area. The grey terminal is the strap area and the black is the catalytic part; both are supposed peptidoglycan. The Ldt_{cd1} that encodes a protein of 469 residues and Ldt_{cd2} (*CD2713*) and *CD3007* display significant sequence identity with the *Ldtfm* catalytic domain, also all of them have no hydrophobic regions (Peltier *et al*., 2011).

1.6 *C. difficile* antigens:

The pathogenicity of *C. difficile* is related to many components, such as toxins A, B and binary. Also, cell wall-, flagellar- and surface-associated proteins are suspected to be involved in the infection. These compounds are mainly induced in an immunological reaction between host and microorganisms (Sanchez-Hurtado *et al.*, 2008; Fagan *et al*., 2011; Spigaglia *et al*., 2011). Although the majority of *C. difficile* isolates from patients produce one or more toxins, some previous studies claim that the *C. difficile* virulence is related only to Toxin B. However, recently, many researchers have suggested that the infection is associated to two toxins (Permpoonpattana *et al*., 2011).

The cell wall and flagella proteins are a part of the outer surface of bacterial cells, which have direct contact with epithelial cells. These proteins consist of SLPs and CWPs. Up to now, the predominant proteins in the *C. difficile* cell wall are the surface layer proteins, which are composed of two parts of high and low molecular weight surface layer proteins (HMW and LMW) SLPs (Fagan *et al.*, 2011; Spigaglia *et al*., 2011).

The C-terminal proteins are the base of HMW SLPs, which are responsible for mediating attachment to the underlying cell wall by putative cell wall binding motives (Pfam), and the LMW SLP is the immunogenic region which contains the N-terminal proteins (Spigaglia *et al*., 2011).

In fact, it is recognised by specific antibodies from sera of patients infected by *C. difficile*, and the specific immunoglobulin for some of these proteins is present in CDI cases. However, the different versions of *CwpV* may assist *C. difficile* to escape from our innate defences. Moreover, a *C. difficile* congregation in the bowel that is encouraged by *CwpV* might affect the colonisation; the *CwpV* role in infection needs more investigation (Spigaglia *et al*., 2011; Reynolds *et al*., 2011).

The hypotheses of SLP roles in bacterial adhesion, which is suggested by Calabi *et al.* (2002) according to their in-vitro and in-vivo adhesive observations, as well as the immune response investigation to SLPs by Sanchez-Hurtado *et al.* (2008) and Wright

et al. (2008) recommended that the SLPs have a role in bowel colonisation and are a helper factor in *C. difficile* infection and antigenic active proteins.

The *C. difficile* flagellar proteins FliC and FliD and the surface-associated protein Cwp66 and Cwp84 roles in the infection were revealed by Pechine *et al.* (2005), when they detected immune responses to these proteins after CDI.

1.7 The immune response to *C. difficile*:

1.7.1 General view of immunity:

The host differentiates between self and non-self entities by different methods. The immune system is divided into two parts, the innate and adaptive immunity. Innate, or natural immunity is the first line of defence as well as it being characterised by being fixed in the genome, non-clonal, with immediate activation and recognising conserved molecular patterns, like lipopolysaccharide (LPS), lipoteichoic acid (LTA), mannans, and glycans. Also, it responds to co-stimulatory molecules, such as cytokines (IL-1β, IL-6) and chemokines (IL-8). The effector cells consist of macrophages, polymorphonuclear leukocytes, and mast cells, which were created by innate immune detection. While one of the effective natural intestinal barriers is microbiota, normal colon movements and the acidity in the gastrointestinal tract protect the bowel from *C. difficile* colonisation. These phenomena led us to think about a probiotic strategy with non-toxic CD, which prevents hamsters from CDI (Poxton *et al*., 2001; Janeway and Medzhitov, 2002; Sambol *et al*., 2002).

The gut immune system is activated when neutrophils are stimulated by IL-1, IL8, TNF-α and leukotriene B4, which are produced by stimulating resident cells by the antigens. The neutrophils migrate from blood vessels to colonic mucosa in a massive way. The specific antagonistic peptide controls this inflammation and this peptide is present in gut tissue (Poxton *et al*., 2001).

The innate immune mechanisms detect and destroy infectious agents without a prolonged period for induction; it also discriminates between host cells and pathogen cell surface. However, adaptive immunity needs more stages to remove infectious material, beginning with transporting antigens to lymphocytes then a recognition phase after the clonal expansion and differentiation to effector cells. Finally, it removes the infectious agents, but is characterised as specific with memory to control reinfection (Janeway *et al*., 2001).

The adaptive immune system is encoded by clonal genes, is concerned with the details of molecular structure, and specifically identifies proteins, carbohydrates, lipids, nucleic acids and whole pathogens. The two parts of the immune system use the same effecter cells (Janeway and Medzhitov, 2002). As the gut mucosal tissue is the main mediator for pathogenic access for the host, the adaptive immune cells secrete huge quantities of immunoglobulin (Ig) and the majority is an IgA antibody, which is one of the frontline defences to protect the epithelia. It passes between cells, translocating onto mucosal cells and is a part of the homeostasis in the bowel. Also, IgA in humans has two subclasses: IgA1 and IgA2. The IgA2 is more resistant to

bacterial proteases and is present in overloaded bacterial growth areas. A second immunoglobulin detected in the lumen is IgE, and is involved in hypersensitivity reactions (Cerutti *et al*., 2011).

Depending on the structure, cytokines are divided into three families: 1) Hematopoietin comprises growth hormones and some interleukins, such as IL-6; 2) Tumour necrosis factor TNF-α, and 3) Chemokines which are a division of cytokines. All cytokines have roles in both innate and adaptive immunity, as the cytokines are produced when the bacterial products stimulate macrophage (Janeway *et al*., 2001).

C. difficile antigens can be divided into two groups - toxins and non-toxins. The toxins includes toxin A, B and binary, while non-toxin antigen includes SLPs, FliC, FliD, and surface-associated proteins, such as Cwp66 and Cwp84 (Sanchez-Hurtado *et al*., 2008; Kelly & Kyne 2011).

Some studies on the inhibition of the severe reactions in colon epithelial cells caused by *C. difficile* toxins A and B show protection to epithelial injuries. Many studies indicate that more than 50% of a population have obvious levels of IgG and IgA antibodies to *C. difficile* toxin A and toxin B. Also, vigorous anti-toxic immune responses are present in the toxinogenic *C. difficile* carrier without symptoms, as opposed to symptomatic patients (Kelly & Kyne, 2011).

1.7.2 Functional role of surface layer proteins (SLPs):

The SLPs have two components: high molecular weight (HMW) and low molecular weight (LMW). These proteins are created after a translational split of a precursor. SLPs are present in all *C. difficile* strains and the cell wall may have a role in adhesion to epithelial cells. Also, it is an immunodominant antigen by disturbing the cytokine equilibrium in human monocytes, and it stimulates monocytes to produce IL-10 and IL-12. Thus, the hypothesis of SLP effects on irritation of intestinal lining bowels is expected. So, Bianco *et al*., (2011) proposed that SLPs have similar immunomodulatory action. However, there is no variation between SLPs from hypervirulent and epidemic strains.

McCoubrey and Poxton (2001) suggested that the SLPs, which show dissimilarities in isolated antigens, and their molecular weights, have a role in immune reactions and bacterial virulence. Immunoblotting of SLPs can be used to distinguish between isolates.

The LMW SLPs are suspected as being an immunodominant antigen in *C. difficile* isolates by Spigaglia *et al.*, (2011), as they notice that three specific peptides from the two exposed surface regions out of ten regions recognised PCR sequencing for LMW SLP between 027 ribotype and 001 ribotype. The three peptides predicted by human and rabbit anti SLPs for ribotype 027 and against SLPs from ribotype 001 in human sera only, which is supposed to be present in pathogenic bacteria. Consequently, it is significantly interesting to study the proposition of SLP effectiveness on epithelial cell activation and pathogenicity (Bianco *et al*., 2011).

Fibronectin binding proteins (Fbps) and the gene is *FbpA*

Various cell surface receptors are responsible for the adhesion of pathogenic agents; these include fibronectin, fibrinogen, collagen and vitronectin. The SLPs were linked to collagen, thrombospondin and vitronectin, whereas fibronectin-binding proteins Fbps were suspected to join to fibronectin or laminin. Fibronectin is a glycoprotein present in the body fluids and in the extracellular component of human tissue; the Fbp in *C. difficile* is Fbp68, which has a weight of 68 kDa (Hennequin *et al*., 2003).

1.7.3 Functional role of Flagellin:

Although *C. difficile* toxin activity is the most important virulence mechanism in CDI, there is a hypothesis that flagella are involved pathogenicity features, such as virulence, adherence, invasiveness and colonisation, are suggested by Tasteyre *et al*. (2000).

The flagellum structure shows a whip shape and it is responsible for bacterial movement. It consists of a single component polymer called flagellin. It is composed of about 500 amino acids polymerised to make up the flagellum, which is fixed to the cell wall by a base and hook. In addition, the flagella genes consist of three classes: transcriptional regulatory proteins (FliC/FliD) class, basal body and hook proteins (FliA/FlgM) (activator and repressor) class, and the third class is genes encoding the hook filament adaptors, cap, motor, chemosensory system, and the flagellin protein that polymerises to form the flagellar filaments (Aderem *et al*., 2009).

Tasteyre *et al*. (2001) confirmed the function of encoded genes FliC (*fliC* 39-kDa) and encoded genes FliD (*fliD* 56-kDa) and flagellar proteins in the adherence to the luminal cells *in vitro*; they provide colonisation, as they recognised the flagellar *C. difficile* strains had adhered to mouse caecum tissue ten times higher than non-flagellar strains.

The function of the flagellin polypeptide is suspected to encourage the essential immune response by stimulating the Toll-like receptors as well as inducing several cytokines, thus enhancing the adaptive immunity. The activation of the immune system demolishes the foreign body and prevents infection in the gastrointestinal system (Aderem *et al*., 2009).

1.7.4 Functional role of Lipocarbohydrate LC:

The majority of Gram-positive bacteria cell membranes consist of peptidoglycan-attached carbohydrate-based polymers into their cell casing, such as cell-wall glycopolymers and these polymers are involved in the protectiveness of bacterial cells, gut epithelial cell adhesion, irritation and have immunogenicity activity (Weidenmaier and Peschel, 2008).

C. difficile cell envelope consists of a cytoplasmic membrane, SLPs and between them, the peptidoglycan, which includes lipoteichoic acid (LTA) (lipocarbohydrate LC antigen) (Sharp and Poxton 1985; La Riva *et al*., 2011), and the antigenic activity of *C. difficile* LC, which was extracted by aqueous phenol and EDTA, was investigated by Sanchez-Hurtado *et al*., (2008) to IgG and IgM responses, which

shows a high level to both immunoglobulins. However, they did not notice significant differences in the antibody levels between the control and the study case groups.

Mohamadzadeh *et al*. (2011) deny that the lipoteichoic acid enhances the dendritic cells (DCs) to produce proinflammatory cytokines (e.g., IL-12) that are causative of ulcerative colitis and Crohn's disease in humans, and that removing the phosphoglycerol transferase gene, which plays a key role in the lipoteichoic acid biosynthesis in *Lactobacillus acidophilus*, one of the normal gut components, down-regulates IL-12 levels and considerably alleviates induced dextran sulphate sodium (DSS) in mice; thus gene changes of normal gut bacterial flora, such as *Lactobacillus acidophilus*, may propose alternative therapy if immune responses are regulated.

1.7.5 Functional role of Capsule:

Davies and Borriello (1990) proposed that toxigenic isolates of *C. difficile* may have other potential factors, such as capsules, flagella and hydrolytic enzymes, and that the anti-phagocyte activity *in vivo* may be affected by capsules, as its location is in the bacterial cells. However, little more has been reported on capsules in *C. difficile* and it is uncertain whether they exist.

1.8 *C. difficile* antibiotic susceptibility:

Treatment CDI with antibiotic not include asymptomatic carriers as well oral antibiotic consumption disturbs the gut microbiota and enhances *C. difficile* to

colonise the bowel as well as to invade the epithelial cells in order to establish the infection complications, and it can take at least twenty days or more up to months for the human bowel to recover homeostasis (Bartlett *et al*., 1992; Poxton, 2010; Kee, 2012).

However, some antibiotics suppressed the *C. difficile* growth and the pathogenesis ability. The relationship between antibiotics and *C. difficile* is not clear with a wide range of effects, even to anaerobes. For example clindamycin enhances *C. difficile* to colonise the host gut, while moxifloxacin does not. On the other hand, ampicillin does not have a broad spectrum or weak activity to anaerobic bacteria, thus distinguishing it as an associated antibiotic to CDI (Dene`ve *et al*., 2008; Pituch *et al*., 2011).

Thus, discontinuing the present antibiotic in use is essential in the CDI medication or at least should be replaced by safer antibiotics to avoid diarrhoea which may cure 25% of cases within 4-5 days, except for the cases cannot stop antibiotics, which brings about dehydration. Therefore, substitute electrolyte is required (Kee, 2012).

The cheapest treatment choice is oral metronidazole, as opposed to the expensive vancomycin, (and it is essential for MRSA treatment). Furthermore, the normal intestinal peristaltic movement is essential to clean the gut from the pathogens, and anti-peristaltic agents often used to treat diarrhoea are not recommended for CDI, as it can interrupt the improvement also the exchange resins should avoid when treat CDI as it may combine with antibiotics (Poxton, 2010; Kee, 2012).

The standard treatment of metronidazole or vancomycin is 10 days, which supports reinstating the microbiota and stops chances of recurring, as suggested by (Bartlett, 1992). Even though vancomycin is expensive, it is suitable for the incompatible patients to metronidazole; the other disadvantages of metronidazole are decreasing the susceptibility to neurotoxic complications and may replace vancomycin by teicoplanin (Beloosesky *et al*., 2000; Brazier *et al*., 2001; Cheng *et al*., 2011).

Recently a narrow-spectrum macrocyclic antimicrobial fidaxomicin become as novel strong competitor to vancomycin as there is no variation of clinical cure rate between both antibiotics. Moreover fidaxomicin is more durable resolution of CDI and may be has lesser disturbances effect on bowel microbiota during treatment as well in the CDI infection by 027 ribotype, the record rate of recurrent infection is less, which is make fidaxomicin so far the safer antibiotics to treat the CDI as well fidaxomicin approved for adults from food and drug administration (FDA) as vancomycin (Poxton, 2010; Louie *et al*., 2011; Kee, 2012).

1.9 Alternative treatments for CDI:

Many non-antibiotic methods to control CDI have been approach such as passive and active immunization also probiotics are being investigated. Intravenous immunoglobulin (IVIG) as treatment for CDI has not been successful so far, but recently some studies investigated intravenous injection of monoclonal antibodies against toxin A and toxin B and these have shown acceptable results (Lowy *et al*.,

2010). A newly developed vaccine by Sanofi Pasteur which is based on toxoid has been granted a fast-track development from the FDA in 2011 (Kee, 2012).

A considerably effective probiotic organism in CDI treatment was *Saccharomyces boulardii* and has also been used to avoid or decrease diarrhoea when using antibiotics. Other studies used *Lactobacillus rhamnosus* or a combination including bifidobacteria. However, some of these may cause bloodstream infections, and due the shortage of information especially for compromised patients, the Society for Healthcare Epidemiology of America (SHEA) and Infectious Diseases Society of America (IDSA) guidelines do not recommend probiotics to prevent primary CDI (Kee, 2012).

1.10 CDI managing procedures:

The most important precautions to avoid spread of *C. difficile* by visitors include recommending the use of gloves and gowns as well as hand washing with antimicrobial soap after patient contact (figure1.9) and to avoid alcohol-based hand sanitizers. Medical staff should provide a private commode for CDI patients if isolation rooms are not available and they should avoid reusable equipment which could be a source of *C. difficile* spores. Also, strict supervision should be made to restrict antibiotic use especially for cephalosporins, broad spectrum penicillin and clindamycin except for surgery prophylaxis advice. Cleaning areas where CDI patients are present should be done with sporicidal agents and chlorine (hypochlorite solution/bleach) is recommended (Cohen, *et al* 2010; Kee, 2012).

Figure1.9: Picture of hand washing steps from http://www.cvh.on.ca/patient-safety/

To put it briefly, *C. difficile* is one of the superbugs which is terrifying the healthcare organisations. It colonises the human gut causing a range of symptoms from relatively mild watery diarrhoea to life-threatening pseudomembranous colitis and Fulminant colitis and toxic megacolon. Its spores are present in the community and the hospital environments, also it is resistant to many antibiotics (but not to those used for treatment) and the frequencies of their ribotypes and the antibiotic susceptibility are variable.

As the first step of infection is likely to be adhesion, the binding of the *C. difficile* outer cell wall components with the receptors of epithelial cells represents the adhesion stage. As well there are various bacterial outer cell wall compounds with

different immunogenic effect which are suspected to have different roles on the host immune response. Thus, studying the immune responses to these *C. difficile* cell wall structures and flagellar proteins by estimating the immunoglobulin level in the serum of symptomatic and non symptomatic patients as well as normal healthy donors may clarify the distribution of the immune response against bacterial antigens in the different stages of infection.

As well, the effects of bacterial antigen on the innate and adaptive immunity by exposing the antigens to the monocytic cells such as the THP-1 cell line to estimate the production of interleukins to induce the pathogenicity. This may allow the follow up of patients, to investigate CDI, to protect patients and to control the infection.

On the other hand, predicting the changing of *C. difficile* ribotypes distribution and antibiotic susceptibility can help to avoid outbreaks and helps with the health care cost and makes life safer particularly for compromised patients.

1.11 Aims of the thesis:

There is wide debate about the activity of the cell wall and flagellar proteins, such as their effectiveness in adhesion, gut colonisation and the immunogenic activity. It was, therefore, reasonable to study these features to understand the *Clostridium difficile* epidemiology, pathogenicity and the host immune response. Thus, the thesis contains five research topics:

- To identify *C. difficile* isolates from surgical patients and their environment by capillary gel electrophoresis to determine the distribution of *C. difficile* PCR ribotypes by this new technique and to distinguish the predominant ribotypes in the surgical unit, as well as to compare the results to agarose gel electrophoresis.

- To estimate the antibiotic susceptibility in the strains isolated above to six antibiotics (ampicillin, erythromycin, tetracycline, metronidazole, moxifloxacin and vancomycin) and to detect the MICs for these six antibiotics against the *C. difficile* isolates.

- To separate the cell protein SLPs, lipocarbohydrate (LC) and flagellar proteins from the six most frequent strains in the South Eastern area of Scotland, in order to determine the hypothesis of immunogenicity of these *C. difficile* proteins.

- To evaluate the antibody (IgG) levels by ELISA in the healthy donor and hospital-admitted elderly patients from South Eastern area of Scotland to *C. difficile* SLPs, LC and flagellar proteins from the six most common ribotypes (001, 002, 0012, 0027, 078, 106) as well as highly immunogenic LPS from *E. coli* as a control.. Hospital admitted patients were classified as symptomatic cases, asymptomatic patients (carriers) and non-colonized patients as controls, and this depends on the stages of *C. difficile* colonization.

- To determine the affects of different antigens on induction of chemokines and cytokines from the THP-1 cell line: IL-1β, IL-6, IL-8, IL-10, IL-12 and TNFα.

CHAPTER II MATERIALS AND METHODS

The chemicals used in this study were prepared by international standard methods, unless mentioned otherwise. The distilled water (Millipore, Billerica, MA, USA) used in all water base chemicals solution, except in PCR preparation where Nuclease-free water (NFW), was used. Phosphate buffered saline (PBS) was prepared in all research by dissolving ten tablets of PBS (Oxoid, Basingstoke, UK) in one litre of pyrogen-free water and autoclaved before use. All other autoclavable chemicals were autoclaved prior use.

2.1 Samples investigated in the study:

Blood, stool and environmental samples were used in the study analysis.

2.1.1 Samples and ethics:

The Local Research Ethics Committee approved all clinical sampling. Stool and blood samples were collected from surgical ward patients and their environment was also sampled – at the Western General Hospital (WGH), the Royal Victoria Hospital in Edinburgh. Serum samples were collected from the Blood Donor Centre Edinburgh.

2.1.2 *C. difficile* isolates:

The 140 *C. difficile* isolates used in this study were isolated from stools from different patients and their environments in surgical wards of the WGH. These were collected by Dr Surekha Reddy, one of MPRL group members. Control strains were

from the MPRL group culture collection.. All strains were stored in cooked meat broth and subcultured on blood agar or CCEY medium before picked for colonies for any proposes of the research.

2.1.3 Blood samples:

Blood samples consisted of 230 healthy donor samples and the patients samples included 20 samples from symptomatic patients, 20 samples from carriers as well 26 control samples from the collection of MPRL group which are collected during 2006.

2.1.3 Culture media:

The following media used in the investigation were for different purposes: to store bacteria or determine colony characters and sometimes for distinguishing *C. difficile*. However the enrichment growth to produce the biomass and that for determination of MICs required special broth media.

2.1.3.1 Blood Agar medium (BA):

Blood agar medium used for bacterial growth and the ingredient are Proteose peptone 15g/L, Liver digest 2.5 g/L, yeast extract 5g/L, NaCl 5g/L and agar 12g/L , also pH adjusted for 7.4 at 25°C. Horse blood was added to a final concentration of 5% of medium.

2.1.3.2 Cefoxitin-cycloserine-egg yolk agar (CCEY):

This contained Proteose peptone No 2 (Difco) 40g, Na_2HPO_4 5g, KH_2PO_4 5g, NaCl 2g, fructose 6g, $MgSO_4$.$7H_2$ O 0,2g, Neutral red (1% neutral red in ethanol) 3ml and

Agar (Bacteriological agar, Oxoid L11) 20g, after autoclaving added sterile Egg yolk (Oxoid SR047C) 50ml, cefoxitin (1.6g/l) 5ml and D-cycloserine (50g/l) 5ml were added.

The ingredients were dissolved in 1L distilled water and autoclaved at 121°C/15 minutes then cooled to 50°C. The agar was dispensed in 15 to 17 ml amounts into 9cm Petri-dishes and allows setting. To dry the surfaces of plates they were kept at 37°C for about 1 hour, and then stored at 4°C (George *et al*., 1979).

2.1.3.3 Protease Peptone Yeast Extract Medium (PPY medium):

The PPY medium was used for an anaerobic growth of *C. difficile* strains. The ingredients consist of: Protease peptone (Oxoid) 20g; Yeast extracts (Oxoid) 10g, NaCl 5g; cysteine HCl (3.75% w/v solution) 20ml; sodium carbonate (2% w/v solution) 20ml; haemin (250µg/ml)/ menadione (50µg/ml) were added to 850ml distilled water. The pH was adjusted 7.1 and made up to 1 litre with distilled water. It was autoclaved at 121°C for 15min then stored at 4°C, or dispensed in 4ml in bijoux or 15ml to universals tube and autoclave at 121°C/15 minutes with caps loose. The caps were tighten caps when still hot, immediately on removal from the autoclave, and stored at 4°C. (Reference: Practical Medical Microbiology, 14th edition, p507 - 511).

2.1.3.4 Cooked meat broth:

Cooked meat media used to store the *C. difficile* at room temperature. The contents of the medium were Proteose peptone (Oxoid L85) 20g, Yeast extract (Oxoid L21) 5g, Trypticase (BBL 11921) 5g, NaCl 5g, cysteine HCl (3.75% aqueous solution) 20ml (or 750mg dry), Na_2CO_3 (2% aqueous solution) 20ml (or 400mg dry) and 20ml Haemin + menadione solution.

The ingredients were dissolved in 940 ml distilled water then the pH adjusted to 7.1 and the volume completed to 1L. It was dispensed 4ml in bijoux and added 0.5cm dry cooked meat particles or 15ml plus 1cm dry cooked meat particles to universals, or as required, and autoclaved at 121°C/15 minutes after that recapping whilst still hot and store at 4°C. It was pre-reduced at or 37°C for 24h in the anaerobic cabinet before use. (Practical Medical Microbiology, 14th edition, p507 -511)

2.1.4 Chemicals:

The chemical required in epidemiological study include Gram stain and ribotyping reagents.

2.1.4.1 Gram stain reagents:

0.5% w/v aqueous methyl violet, 2% iodine in 0.1M NaOH, acetone and 0.1% aqueous basic fuchsin were used in Gram stain.

2.1.4.2 Chemicals for ribotyping:

The ribotyping methodology followed that of O'Neill *et al*., (1996) with the modification of Indra *et al*., (2008) for capillary gel electrophoresis (CGE).

Utilizing the oligonucleotide primers complementary to the 3' end of the 16S rRNA gene in the positions 1445-1466 and the 5' end of the 23S rRNA gene in the position 1-20 which was labelled by florescent dye carboxy-fluorescein (FAM), the variable length intergenic spacer region was amplified. Primers were obtain from MWG and the sequences of the primers were:

16S 5’ CTG GGG TGA AGT CGT AAC AAG G3'
23S 5’ GCG CCC TTT GTA GCT TGA CC3'

The master mix for 100 samples contained: nuclease-free water 251µl, bovine serum albumen (BSA 2mg/ml) 100 µl, PCR buffer (Promega flexi X5) 200 µl, dNTP mix (2mM) 100 µl, $MgCl_2$ (25mM) 140 µl, primer 16S (100µM) 2 µl from MWG, primer 23S (100µM) 2 µl from MWG and TAQ (5u/µl) 5µl fromPromega.

Diluted samples contained HiDi formamide 995 µl and LIZ-600 size markers 5 µl. Three PCR tubes per plate were required for capillary gel electrophoresis. The final volume of the PCR reaction for each sample was 8 µl + 2 µl DNA extraction.

Firstly, denaturation was done at 95 °C for 5min, and then 25 cycles in 1min at 95 °C for the denaturation of the mixture were carried out. And then annealing at 55°C for 1min was processed, followed by extension at 72°C for 90 sec. The last step was elongation for 5min at 72°C.

The amplification product (1 µl) was diluted ten times in Nuclease-free water 9µl, then mixed by pipette, 1µl transferred to another PCR-microplate tube which contained 9µl of diluent sample and covered by aluminium foil. The PCR-microplate was then centrifuged at 112g for 2min (the diluted amplified product could be stored at 4°C until reading by sequencer). The patterns were identified by contrast with control ribotypes from the library collection.

2.1.5 Identification methods:

C. difficile was identified by colony morphology characteristics and appearance under long-wave UV light. Microscopy was used to confirm bacterial cell shape by Gram-staining reaction and motility was checked in 24-48h old cultures following strict anaerobic growth.

2.1.5.1 Colony characters:

To identify *C. difficile* colonies morphology was used on CCEY agar or blood agar, and exposure to long-wave UV light to reveal the characteristic yellowish-green fluorescence.

2.1.5.2 Gram stains Procedure:

The bacterial film was prepared and heat-fixed. The film was flooded with methyl violet and left for one minute. After a brief rinse in tap water, it was flooded with iodine and left for 1 minute. After rinsing with tap water, it was decolourised briefly

(for 2 - 3 seconds) in acetone and then promptly washed well in tap water. The last stain was basic fuchsin and was left for one minute. Finally, it was rinse briefly in tap water and blotted dry. Gram positive and Gram negative bacterial strains were used as control.

2.1.5.3 Motility investigation:

Hanging-drop techniques was used to differentiate the bacterial motility and investigated by dark field background microscope.

2.1.6 Anaerobic work station:

The anaerobic workstation (MK3, DW Scientific, Shipley, Yorkshire) was used to provide anaerobic condition via hydrogen 10%, nitrogen gas 80% and carbon dioxide 10%.

2.1.7 Ribotyping techniques:

The principle of PCR ribotyping for *C. difficile* is rooted in the intergenic spacer sections of 16S-23S ribosomal RNA gene which is extremely heterogeneous with many alleles also the size of the space between alleles vary between different strains.

Multiple copies of the different sized spacer regions of the *C. difficile* genome were amplified, by the Polymerase Chain Reaction (PCR) (Stubbs, 1999). The products can be differentiated by their banding patterns on agarose gels, or, as here, one of the primers (5`) was labelled with FAM to allow the products to be analysed by capillary gel electrophoresis, and a DNA sequence reader. Final analysis was done by Gene

Marker software V 1.95 and the patterns were matched by eye to the ribotype library developed by this technique.

2.1.7.1 Specimen preparation and DNA extraction:

A few colonies from fresh cultures of *C. difficile* isolates and controls grown an anaerobically for <48 hrs at 37°C on blood agar were collected. They were emulsified in 100µl of Chelex suspension (0.05g/1ml NFW) (Chelex 100 resin Bio-Rad) in Eppendorf tube, then heated for 10-15 minutes in a boiling water bath or heat-block to lyse the bacteria.

The suspensions were next centrifuged for 10min for 14000g, and then 40 µl of the supernatant was transferred to another sterile empty tube by sterile tip. The DNA extraction was used immediately or stored at - 80°C until required.

2.1.7.2 Amplification:

The *C. difficile* ribotype mastermix preparation was made as in table 1. The mastermix was stored at -20°C until required in 400µl aliquots and protected from light. This was stable at least one year.

Components	Stock concentration	×100 samples
Nucleases-free water (NFW)	------	251 µl
Bovine serum albumin (BSA)	2mg/ml	100 µl
PCR buffer (Promega flex)	×5	200 µl
dNTP mix	2mM	100 µl
$MgCl_2$	25mM	140 µl
Primer 16S FAM (MWG)	100µM	2 µl
Primer 23 3S (MWG)	100µM	2 µl
TAQ (GoTaq)	5U/ µl	5 µl

Table 1: Mastermix content for ribotyping-PCR

Sample diluents buffer consisted of:

Components	×100 samples
HiDi formamide	995µl
LIZ-600 size markers	5 µl

Samples diluents buffer were mixed well and frozen at -20°C in 500 µl aliquots and protected from light. They remained stable for one year but were discarded after thawing.

PCR amplification protocol:

The previous prepared *C. difficile* ribotype mastermix was thawed from -20°C storage in 37°C water bath and placed in ice immediately as well as avoiding direct light. An 8 µl volume of mastermix was distributed to a suitable number of PCR tubes on ice, and cover by dome caps. A 2 µl volume of specimen DNA extraction was added, as well relevant controls to the wells. The PCR tubes were place in the thermocycler.

Thermocycler CDIFF protocol was:

Temperature	Time	Cycles
95 °C	5min	1×
95 °C	60sec	25×
55 °C	60sec	
72 °C	90sec	
72 °C	5min	1×

The PCR product after amplification could be storage at -20°C before processing by fluorescent fragment analysis and should be kept in the dark.

2.1.7.3 Fluorescent fragment analysis:

Samples for fluorescent fragment analysis were prepared as follows:
A 9 µl volume of NFW per sample was dispensed into a 96-well PCR plate (A), and 9 µl of sample dilution buffer per sample was dispensed into plate B and discarded of any amount of sample buffer not used.

1 µl of each PCR product was dispensed into the wells in plate A and mixed thoroughly to make 1/10 dilution of PCR products in water, and 1 µl of each diluted sample was transferred into the matching well in plate B which contained HiDi formamide, so the final dilution will be 1/100.

The plate was covered carefully using adhesive foil seal and mixed carefully by the vortex mixer. Finally the sample sheet was completed for the capillary sequencing process in the GenePool laboratory in the Ashworth Building at the King's Buildings, University of Edinburgh.

2.1.7.4 Reading of capillary electrophoresis sequences results:

The software GeneMarker V, 1.95 from AFLEP/ Genotyping used to distinguish the ribotypes.

2.2 Antibiotic susceptibility:

Minimum Inhibitory Concentration (MIC) determination followed the agar plate diffusion CLSI (2007) methods.

2.2.1 Bacterial isolate:

A panel of 140 *C. difficile* isolates from surgical patients and their environment at the Western General Hospital in Edinburgh UK, and the control strains from MPRL group at the Centre for Infectious Disease at the University of Edinburgh.

Five separate colonies (diameter~1mm) of *C. difficile* isolates were selected from the growth on supplemented Brucella horse blood agar which had been incubated in anaerobic conditions for 24-48 hrs. The colonies were suspended in brain-heart infusion (BHI) broth and the turbidity adjusted to an O.D. 0.5 at 625nm (BSAC methods for antimicrobial susceptibility testing V 10.2 May 2011).

2.2.2 Antibiotics:

The NCCLS methods was followed for antimicrobial susceptibility testing of anaerobic bacteria approved standard – seventh edition (M11-A5 Vol.21 No.2) Hecht *et al* (2007). In the MIC technique for this study, the direct colony suspension method was chosen, and six antibiotics were used (ampicillin, erythromycin, tetracycline, metronidazole, moxifloxacin and vancomycin).

For each concentration, 1ml horse blood + 2ml antimicrobial agent solution were added to the plate and 17 ml cooled Supplemented Brucella Agar, poured into it and

mixed gently and left covered to cool at room temperature. Plates were used within 72 hours. A multi-point inculcator was used to inoculate the strains.

Pouring Agar dilution:

Serial dilution of antibiotics were prepare as in Table 2:

Steps	Concentration	Source	Volume	Diluents	Conc. µg/ml	Log2	Final Conc.(g/l)
	5120 µg / ML	Stock	___	___	5120	9	512
1	512	Stock	2ml	2ml	2560	8	256
2	512	Stock	1ml	3ml	1280	7	128
3	512	Stock	1ml	7ml	640	6	64
4	640	Stock	2ml	5ml	320	5	32
5	640	Stock	1ml	3ml	160	4	16
6	640	Stock	1ml	7ml	80	3	8
7	80	Stock	2ml	2ml	40	2	4
8	80	Stock	1ml	3ml	20	1	2
9	80	Stock	1ml	7ml	10	0	1
10	10	Stock	2ml	2ml	5	-1	0.5
11	10	Stock	1ml	3ml	2.5	-2	0.25
12	10	Stock	1ml	7ml	1.25	-3	0.125

Table2: Serial dilution of the antibiotics

2.2.3 Media used in MICs:

2.2.3.1 Brain Heart Infusion broth (BHI: Oxoid):

The BHI broth used to prepare the *C. difficile* suspension in the MIC determination, contained brain infusion 12.5g/L, beef hearts infusion 5g/L, glucose 20g/L, NaCl 5g/L and sodium phosphate 2.5g/L.

2.2.3.2 Brucella Agar Medium (B.A.)(Sigma):

Brucella Agar Medium used in MIC determination for *C. difficile* after adding horse blood (5%), the components were casein 19g/L, peptic digest 10g/L, yeast extract 2g/L, dextrose 1g/L, NaCl 5g/L, hemin 0.1g/L, vitamin K1 0.01g/L and agar 15g/L.

2.2.4 Chemicals:

2.2.4.1 Chemicals for MIC:

The MIC for *C. difficile* isolates requires Brucella agar with horse blood, and the antibiotics with solvent. Also the brain heart infusion broth used for bacterial suspension and sterile PBS to dilute the bacterial suspension for the optical density evaluation by spectrophotometer.

Six antibiotics were used in this study: ampicillin, erythromycin, tetracycline, moxifloxacin, metronidazole and vancomycin by different dilutions table 3.

Antibiotic	ampicillin	erythromycin	tetracycline	moxifloxacin	metronidazole	vancomycin
Potency	≥845 µg per mg	≥850 µg/mg	993µg/mg	~1,000µg/mg	≥900 µg / mg	~1,000 µg/mg
Stock solvent	NFW	Ethanol 100%	NFW	NFW	0.1% acetic acid	NFW

Table 3: The potency and stock solvent used in antibiotic preparations.
NFW = nuclease-free water.

2.2.5 MICs procedure:

Colonies of strains of *C. difficile* from BA plates grown for 48 h were inoculated into tubes of BHI broth and incubated anaerobically for 24 hrs. The optical density of the

bacterial suspensions was read in a spectrophotometer at a wave length of 600 nm and the turbidity adjusted to 0.5 O.D. by addition of sterile PBS. Using a 36 well tile for the multi-inculcator the suspensions of different strains were inoculated onto agar plates contains different antibiotic concentration.

The plates were incubated anaerobically, with one plate without inoculation as control and one plate aerobically as control for facultative anaerobic bacterial contamination. After 24 h the antibiotic susceptibility was checked.

2.3 Extraction of *C. difficile* antigens:

2.3.1 Chemicals:

2.3.1.1 Chemicals for S-layer proteins Extraction:

Cell washing buffer: 0.05 M sodium phosphate buffer pH 7.4, containing 0.15 M sodium chloride.

1.482g $NaH_2PO_4.2H_2O$ and 5.749g Na_2HPO_4 were dissolved in about 900ml distilled water and pH checked that it was 7.4. 8.77g NaCl was added, and when dissolved, made volume up to 1000ml with distilled water.

5M guanidine HCl:

9.553g guanidine hydrochloride was dissolved in about 10ml distilled water and then made up to a total of 20ml with distilled water.

Dialysis buffer: 6.25mM Tris/ HCl buffer, pH 6.8.

757mg Tris (hydroxymethyl) methylamine was dissolved in about 950ml distilled water. The pH was adjusted to 6.8 using 1M HCl and then made up to a total of 1000ml with distilled water.

PPY Medium: Medium was mentioned previously in (2.1.3.3).

Micro-dialysis membrane: Using a 10000 mwco membrane (Medicell international Ltd).

2.3.1.2 Chemicals for Flagella protein Extraction:

Phosphate buffered saline (PBS), Protease Peptone Yeast Extract Medium PPY Medium and Micro-dialyser 10 000 mwco membrane.

2.3.1.3 Chemicals for Lipocarbohydrate (lipoteichoic acid: LC) extraction:

Phosphate buffered saline (PBS), PPY broth, and chloroform/methanol solution (2:1) to delipidated the sample. Aqueous phenol solution 80% (w/w) and acetic acid/ acetate buffer (pH 5.0) containing 0.02 M $MgCl_2$ and 100µg each RNase and DNase (Sigma) and toluene, as well Micro-dialyser a 10000 mwco membrane using.

2.3.1.4 Chemicals for Polyacrylamide gel electrophoresis (PAGE):
PAGE used to determine the purity of proteins.

40% w/v aqueous acrylamide/methylene bisacrylamide solution:

Add 100g acrylamide (BDH Electran) to 2.7g methylene bisacrylamide (BDH Electran) completed by nuclease-free water to 250ml.

Freshly prepared 15g/l w/v ammonium persulphate solution (APS):

0.05% w/v aqueous bromophenol blue solution:

Water saturated butan-2-ol

About 60 ml butan-2-ol was added to a 100ml screw-capped bottle with 10ml nuclease-free water and shaken thoroughly after about 1 min and was checked to see if the solution was biphasic. If not, more water was added and mixed and rechecked until the solution was biphasic.

Sample buffer (Single strength): 0.0625 M Tris/HCl; pH 6.8, 2% SDS, 10% glycerol, 1% 2-mercaptoethanol and 0.001% bromophenol blue.

0.757g Tris (hydroxymethyl) methylamine (BDH analar) was dissolved in 70ml water and adjusted to pH 6.8 with 1 M HCl then add 2g sodium lauryl sulphate (SDS) (BDH especially pure).

12.6g glycerol (BDH analar) was accurately weighed directly into a 100ml volumetric flask and the bromophenol blue added. The Tris/HCl/SDS solution was transferred to the volumetric flask and washed in with several small volumes of water. In a fume cupboard, add 2ml mercaptoethanol (0.05% aqueous solution) (BDH water soluble) and made up to 100ml with water. It was mixed thoroughly and then 5 - 6ml volumes transferred to disposable bijoux wrapped in foil

Sample buffer (Double strength) 0.125 M Tris/HCl pH 6.8, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, and 0.002% bromophenol blue:

1.514g Tris (hydroxymethyl) methylamine (BDH analar) was dissolved in 60ml water and adjusted to pH 6.8 with 1 M HCl. 4g Sodium lauryl sulphate (SDS) (BDH especially pure) was added 25.2g glycerol (BDH analar) (20% v/v glycerol) was accurately weighed directly into a 100ml volumetric flask and 2ml bromophenol blue (BDH) added. The Tris/HCl/SDS solution was transferred to a volumetric flask and washed in with several small volumes of water.

In a fume cupboard, the 2-mercaptoethanol was added and made up to 100ml with water. It was mixed thoroughly and then 5 - 6ml volumes transferred to disposable bijoux wrapped in foil

Electrode buffer: (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3):

6.057g Tris (hydroxymethyl) methylamine (BDH analar) and 28.827g glycine (BDH chromatographically homogeneous) ware dissolved in1800ml water and the pH check to be 8.3. Minor adjustments can be made using 1 M NaOH (but not HCl). 2g sodium lauryl sulphate (SDS) (BDH especially pure) was added and made up to 2000ml.

Separating gel buffer (Double strength) (0.75 M Tris /HCl pH 8.8, 0.2% SDS):

90.855g Tris (hydroxymethyl) methylamine (BDH analar) was dissolved in 800ml water and adjusted, initially with 5 M, and then 1 M HCl to pH 8.8. 2g sodium lauryl

sulphate (SDS) (BDH especially pure) was added and made up to 1000 ml with water.

Stacking gel buffer (double strength) (0.25 M Tris /HCl pH 6.8, 0.2% SDS):

15.142g Tris (hydroxymethyl) methylamine (BDH analar) was dissolved in about 400ml water and adjusted, initially with 5 M, and then 1 M HCl to pH 6.8. 1g sodium lauryl sulphate (SDS) (BDH especially pure) was added and made up to 500ml with water.

TEMED: N, N, N, N- Tetramethylethylenediamine from (Sigma)

2.3.2 Extraction methods for SLPs:

2.3.2.1 *C. difficile* growth:

20ml of PPY medium tube was inculcated and incubated anaerobically overnight at 37°C. The purity was then checked with a microscope.

2.3.2.2 Cell wall separation:

The cells were harvested and washed twice in cell washing buffer (10ml per wash) by centrifugation at 4800g for 15 mins, then the supernatants were discarded. Next the cells were resuspended in 1.5ml of 5M guanidine HCl in a 3ml tube, and mixed in an RBC mixer for two hours. After that, the cells were removed using an ultracentrifuge at 16,000g for two minutes, the supernatants collected and

centrifuged again at 16,000g for two mins. The cell free supernatants were carefully transferred to a clean tube.

2.3.2.3 Dialysis:

The supernatant was dialysed by a 10,000 MWCO membrane, against a 1 litre dialysis buffer at 4°C overnight. Protein was used to concentrate the solution, freeze-drying it if necessary and storing at -20°C.

2.3.3.4 Purification of SLPs:

To check the purification poly-acrylamide gel, electrophoresis was used (PAGE) as described in Section 2.3.6.

2.3.4 Extraction methods for flagellar proteins:

2.3.4.1 *C. difficile* growth:

The *C. difficile* strains were grown anaerobically overnight in 1 litre of PPY medium.

2.3.4.2 Separate of flagellar proteins:

The bacterial suspension was centrifuged at 13,000g for 10 mins at 4°C and supernatants were discarded. The bacterial sediment was resuspended in 20ml of PBS and a vortex for 2 mins in a blender at high speed. It was then centrifuged at 12,000g for 10 mins, or 5,000g for 30 mins at 4°C. The supernatant was collected

and centrifuged at 12,000g for 10 mins at 4°C. Again, the supernatant was collected and centrifuged at 25,000g for 1 hour at 4°C. The sediment (flagellar proteins) was collected and resuspended in 1ml of PBS.

2.3.5 Extraction methods for Lipocarbohydrate (LC):

This method is based on Poxton and Cartmill's (1982) and Sharp and Poxton's (1986) procedure.

2.3.5.1 *C. difficile* growth:

Six litres of PPY broth were inculcated by the *C. difficile* strain and incubated anaerobically overnight at 37°C.

2.3.5.2 Separate cell wall for LC:

Harvesting of the cells was done by ultracentrifuge at 10,000g for 10 mins at 4°C. Then the cells were resuspended in 10ml of PBS and washed twice by centrifugation for two mins at 10,000g and 4°C. The PBS cell suspension was sonicated in ice by MSE Soniprobe for three mins at 10µm. To remove the cell walls and unbroken cells, the suspension was ultracentrifuged at 4000g for 10 mins at 4°C, followed by 35,000g for 30mins at 4°C. The supernatant was freeze-dried overnight in a weighed container, then delipidated twice with 200ml of chloroform/methanol (2:1) solution overnight, and filtered through Whatman No. 1 paper and allowed to dry. The dry delipidated cell wall contents and membrane fraction were resuspended in distilled

water to 10% (w/v) and mixed with an equal volume of 80% (w/w) phenol solution. The mixture was stirred at room temperature for half an hour then centrifuged at 2500g for 20 mins at 4°C.

2.3.5.3 Dialysis:

The supernatant was dialysed by a 10,000 MWCO membrane against tap water overnight to remove the phenol.

The dialysed material was then mixed with an equal volume of 0.2 M acetic acid/acetate buffer (pH 5.0), containing 0.02M Mg Cl_2 and approximately 100µg each of RNase and DNase (Sigma), and incubated overnight at 37°C with the surface covered by toluene.

2.3.5.4 Purification of LC and storage:

The phenol extraction was repeated to remove nucleases, then dialysis repeated overnight against tap water of the upper layer. Next it was freeze-dried overnight in a weighed container, so the crude membrane antigen could be collected and stored at room temperature.

2.3.6 Polyacrylamide gel electrophoresis (PAGE):

PAGE is used to define *C. difficile* proteins by electrophoresis as they have different molecular weights.

2.3.6.1 Sample Preparation:

The samples and double-strength sample buffer were thawed before working, and then equal volumes of sample were mixed with buffer (15µl of each) in a sealed eppendorf tube. Next it was boiled in a heat-block or boiling water bath.

2.3.6.2 Gel Preparation:

The slide and cover were properly cleaned and dried before use, then the slide was fixed with the cover in the cassette. The separating and stacking gels were prepared as described in Table 4.

The separating gel was injected into the space between the slide and cover just 0.5ml lower than the edge. The separating gel was gently covered with 50µl of water saturated butan-2-ol and left for 5-10 mins to polymerise. Then the rest of the water saturated butan-2-ol was removed. A sufficient quantity of stacking gel was added and the comb was fixed to make wells in the stacking gel, before leaving it for polymerisation.

Ingredient	Separating gel 12%	Stacking gel 4%
Acrylamide solution (40%)	3ml	0.5ml
Separating gel buffer (DS)	2.5ml	----------
Stacking gel buffer (DS)	----------	1.25ml
10% SDS	100µl	50µl
Distilled water	4.35ml	3.2ml
10% APS	100µl	50µl
TEMED	10µl	5µl

Table 4: The contents of the separating and stacking gels are enough to prepare two slides.

2.3.6.3 Gel Running:

PAGE electrode buffer was poured into the tank (Bio-Rad) and the slide containing the gel was inserted (note that the surface of the buffer should cover the slide gel). The slide wells face the back (to the middle of the chamber), and the comb was removed and checked to ensure there were no bubbles. 10µl of prepared samples were dispensed and marker ladders were demonstrated in the gel wells using narrow tips and the sample sequence mentioned. (Note that bubbles should be avoided when placing the samples). The tank was covered properly and the power supply connected. The voltage was adjusted to 80 volts and the time to two hours. Finally, notice was paid to when the dye reached the lower edge of the slide.

2.3.7 Blue Stain for PAGE:

The blue stain was used to determine the purification of S-layers proteins, flagellar proteins and lipocarbohydrate. Alcohol and acetic acid were used as fixatives before the gel was stained.

2.4 Immunoassay:

2.4.1 Sandwich enzyme-linked immunosorbent assay ELISA procedure:

This procedure included seven steps, described as follows:

Coating step:

96 microtiter plate wells were coated by investigated antigens with a 5µg/ml concentration in coating buffer, and control and blank wells were coated by antigen diluents, then the plates were sealed and incubated at 4^{o}C overnight.

Washing step:

The following day the wells were aspirated and washed four times with wash buffer (0.05% Tween20 in sterile PBS) by adding 400µl/well. They were then inverted against clean paper towels and the plates tapped to remove any remaining wash buffer.

Blocking step:

The wells were blocked using 200µl of blocking buffer (1% Bovine serum albumin, BSA (Sigma) in sterile PBS) per well, then wrapped in aluminium foil and incubated for two hours at 37^{o}C on a shaker. The wells were then aspirated and the washing step repeated as previously.

Binding step:

Diluted antigen samples of 1:200 in PBS-ST were added to duplicate "test" wells and a single antigen control well. Additionally, a well was diluted using the standard control serum and added to the appropriate "test" as well as the serum control wells. A serum buffer was added to the blank and antigen control wells, then the plate was wrapped in aluminium foil and incubated for 90 mins at 37^{o}C on the shaker. It was then aspirated and the washing step repeated.

Conjugating step:

The conjugate (Rabbit anti-horse IgG alkaline phosphatase) 1 in 10,000 in PBS-TF was prepared and added to all wells, then incubated for 90 mins at 37^{o}C on the shaker. It was then aspirated and the washing step repeated.

Developing colour step:

The new preparation of alkaline phosphatase substrate is essentially composed of Sigma 104-105 phosphatase tablets, (p-nitrophenyl phosphate 5mg/ml) in a substrate solvent. Two tablets in 10 ml per plate are required, and this solution was added to all wells. Then the plate was incubated for one hour at room temperature without cover or until the colour intensity of the control was considered sufficient.

Well content	Antigen	Antigen diluents	Serum	Serum diluents	Conjugate	Substrate
Blank (B)	-	+	-	+	+	+
Antigen control(Ag)	+	-	-	+	+	+
Serum control (S)	-	+	+	-	+	+
Test (T)	+	-	+	-	+	+

Table 5: Summary of ELISA steps.

Evaluation optical density step:

The absorbance measurement of optical density for wells was applied by an auto-micro plate reader (Anthos, 2001) at 405nm (reference 620nm). All values were counted as the means of duplicate measurements and compared to the standard curve.

2.4.2 Enzyme linked Immunosorbent Assay (ELISA) chemicals:

The ELISA sandwich technique was required for at least five solutions, detailed as follows:

2.4.2.1 Antigen diluents:

0.05 M sodium carbonate buffer pH 9.6, 0.02% w/v sodium azide:

In preparation a 0.05 M sodium carbonate buffer (0.848g $Na_{2,}$ CO_3 and 1.428g $NaHCO_3$) was dissolved in 500ml of distilled water and checked to ensure the pH was 9.6. 100mg sodium azide was then added to the buffer. (Note that this should be dispensed in 100ml aliquots and stored at 4°C.)

2.4.2.2 Antiserum and conjugate diluents:

0.05 M sodium phosphate buffer at pH 7.4, 0.85% w/v sodium chloride, 0.05% w/v Tween 20 and 0.02% w/v sodium azide

1.482g $NaH_2PO_4.2H_2O$ and 5.749g Na_2HPO_4 were dissolved in about 950ml of distilled water and checked to ensure the pH was 7.4. Then 8.5g NaCl, 0.5ml Tween 20 and 200mg sodium azide were added (or 2ml of a 10% w/v solution of NaN_3), and the final volume was made up to 1000ml with distilled water.

2.4.2.3 Blocking solution:

0.05 M sodium phosphate buffer at pH 7.4, 0.85% w/v sodium chloride, 0.05% w/v Tween 20, 0.02% w/v sodium azide and 2% teleostean gelatine.

2g of teleostean gelatin was added to 100ml of antiserum diluents and stored at 4C.

2.4.2.4 Substrate solvent (for alkaline phosphatase): 0.05 M sodium carbonate buffer at pH 9.8 and 1.0 mM magnesium chloride.

Preparation of 0.2 M carbonate/bicarbonate buffer stock solutions

1.167g Na_2 CO_3 and 1.176g $NaHCO_3$ were dissolved in 500ml of distilled water and checked to ensure it was pH 9.8. 102mg $MgCl_2$ $_6$ H_2O was added.

2.4.2.5 ELISA wash buffer (single-strength wash buffer):

A preparation was made of 2000ml of a 10 X concentrated solution without the sodium azide; this was dispensed in 380ml aliquots and sterilised by autoclaving at 15psi/15 mins and stored at 4°C. Note that sodium azide must not be autoclaved as it may react with copper or lead components inside autoclaves, creating explosive metal azide.

10 X concentrated solution:

Oxoid phosphate buffered saline tablets (BR14a), 144g NaCl and 3.6g KCl were dissolved in about 1800ml of distilled water. 10ml Tween 20 was added and the volume made up to 1900 with distilled water. The aliquots of 380ml were then autoclaved at 15psi/15 mins and stored at 4°C. (Note that the pH of the 10X solution was 7.0)

2.4.3 Estimated IgG level in donors and patients by ELISA:

The detection of cytokines was followed by the ELISA technique, as described previously in Section 2.4.1.

2.4.4 Detection interleukins and TNF-α by ELISA:

The detection of cytokines was followed by the ELISA technique, as described previously in Section 2.4.1.

2.5 THP-1 cell line:

THP-1 is a human acute monocytic leukaemia cell line separated from a human male at one year old (Tsuchiya *et al*., 1980).

2.5.1 Source of cell line:

The source of the THP-1 cell line is the European Collection of Animal Cell Cultures (ECACC, Salisbury, U.K.) ECACC No: 88081201, and was a kind gift from Mr Adel Alkadidi from Glasgow University.

2.5.2 Essential chemicals:

2.5.2.1 Chemicals for Cell Line:

- Human acute monocytic leukemic cell line (THP-1).

- RPMI-1640 medium (Sigma), Fetal Calf Serum (FCS) (Sigma), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES (Gibco) and Penicillin Streptomycin PS (Gibco).
- Glutamine 200mM (Sigma). The freezing medium for THP-1 cell line storage contained 3ml of sterile glycerol (Sigma), 7ml of FCS and Phorbol myristate acetate (PMA).

2.5.3 Cell line media:

2.5.3.1 RPMI 1640 medium (Sigma) used to grow the THP-1 cell line.

2.5.3.2 4-(-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco) as supplement to the RPMI 1640 medium for enrichments cell line.

2.5.3.3 Fetal Calf Serum (FCS) from Oxoid was used as a supplement to the RPMI 1640 medium for enrichments.

2.5.3.4 (PSG) from Sigma:

Penicillin and streptomycin were used to prevent the cell line from contamination. Supplements of 200nM Glutamine were also added.

2.5.3.5 Phorbol myristate acetate (PMA):

PMA was added to RPMI 1640 medium to enhance the adhesion of bacterial cell wall proteins to the THP-1 cell line.

2.5.3.6 Freezing medium for THP-1:

The ingredients contain 100ml of a sterile nutrient broth (Oxoid CM1), filter-sterilised heat inactivated (at 56°C for 30 minutes) FCS 10ml and sterile 20% aqueous glucose solution. The components were aseptically mixed and dispensed in a 2ml sterile tube, then incubated aerobically at 37°C for 24 hours. The tube cap was loosened then the mixture was anaerobically incubated for 48 hours to detect contamination. Next the cap was tightened again and the mixture stored at -20°C. (Reference: Practical Medical Microbiology, 14th edition, pp.126-127).

2.5.4 Cell line growth:

2.5.4.1 Preparing the cell line:

Precautions:

Wearing lab-coat and gloves are essential in tissue culture room, the reagents and media were immersed in warmed water bath for 20min and the Class II cabinet was cleaned by ethanol 70% and wiped by paper towel. The incubator was disinfected weekly with TrigenII virucidal disinfectant concentrate from MEDI CHEM UK (diluted 5ml/1000ml distilled watter), distilled water, ethanol 70% and Biocidal spray

2.5.4.2 Activating the THP1 cell line:

Frozen cells were transferred to a 25ml falcon tube and 5ml warmed RPMI 1624 medium was added, then centifuged for 5min at 1000 g. The supernatant was then discarded in a discarding bottle. Next, 5ml of the medium was added to the pellet and

the vial was gently shaken until the pellet detached. The cell suspension was transferred to a little flask and 5ml of medium added. Finally the flask was incubated for 24-48 hours at 37°C in a cleaned incubator, and then the medium was changed after its colour changed to orange/yellow.

2.5.4.3 Changing the medium:

The medium was changed approximately every two days, depending on the colour developed about the cell line. The cell line was removed to the falcon tube and centrifuged for 5 min at 100g, after which the supernatant was discarded in the discarding bottle, and around 10ml of fresh warmed medium added to the cell pellet in the small flask, or 20ml to the large flask.

2.5.4.4 THP-1 cell line procedure:

To start with, the cell line was sub-cultured in 30ml of RPMI 1624 medium (Sigma) and 6ml of FCS with 3ml 100mM autoclaved 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and (HEPES) (Gibco) (RPMI/20%FCS/HEPES), then incubated for 24-48 hours at 37°C.

For routine growth, the cell line was sub-cultured in a mixture of RPMI/10%FCS/PS+G, which was prepared previously by adding 50ml of FCS, 5ml of penicillin streptomycin and 15ml of 200mM glutamine (Sigma) to 500ml of RPMI medium.

To discriminate cell growth a RPMI/PS+G mixture was added, which was arranged previously by adding 5ml of PSG to 500ml of RPMI medium. The cells were then stimulated by adding 2ml of 10ng (PMA) (Sigma) to 20ml of the cell suspension.

The THP-1 cells were preserved by being grown in combination with RPMI. 10%/FCS/PSG, which was prepared by adding 50ml of FCS and 5ml of PS+G to 500ml of RPMI medium, and incubated for 24 hours at 37°C. The cell suspension was then transferred to a falcon tube and centrifuged for 5 mins at 100g. Next the THP-1 cells were added to 10ml of freezing medium containing 3ml of sterile glycerol (Sigma), and dispensed in 1ml tubes then stored in liquid nitrogen.

2.5.4.5 Preparation of THP-1 cell line for bioassay:

A confluence of cells (16×10^5 cells/ml) in RPMI/10%FCS was required.
Untreated cells: 100µl of cells were added to each well of 96 well tissue culture plates and incubated at 37°C in 5% CO_2 overnight.

Pre-treated cells: 2ml (10ng/ml & 50ng/ml) of PMA was added to 20ml of cell suspension. Then 100µl of this suspension was distributed to each well of 96 well tissue culture plates and incubated for 24 hours at 37°C in 5% CO2. The medium was gently removed after centrifugation (100g for 5 mins) and replaced by serial dilution of SLPs, LC or flagellin.

2.5.4.6 THP-1 cells viability:
Estimation of cell viability was carried out by spinning one volume of cells at 100g for 5 mins, then mixing the same volume of trypan blue dye with the cell suspension,

and immediately observing the cells under a microscope. The equation of viable cells = (No. of unstained cells / total No. of cells) × 100. The dual chamber haemocytometer slide was used to count the cells.

2.6 Expose HTP-1 cell line to *C. difficile* antigens (SLPs, Flagellin and LC):

2.6.1 Preparation of SLPs, LC and flagellin to expose THP-1 cell line:

Six serial dilutions of antigens in RPMI/10%HS medium, from different strains (1=1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml) were used by thawing 10µl of stock (1mg/ml) and adding it to 990µl of medium, to achieve 10 µg/ml. 30µl was then added to 270µl of medium to reach 1µg/ml (=1000ng/ml), which constituted the starter dilution.

In order to get 200ng/ml, 20µl of 10µg/ml was added to 980µl of the medium. To make 2000ng/ml (2µg/ml), 50µl of 10µg/ml was added to 250µl of medium. This constitutes the starter concentration of 2000ng, 200ng, 20ng, 0.2ng and 0.02ng. The same dilution was used for positive control and 2 wells for negative control.

2.6.2 PMA (50ng/ml and 10ng/ml) cell line media preparation:

PMA 50ng/ml = (0.5ml P.S. + 49.5ml RPMI +2.5µl PMA).

PMA 10ng/ml = (1ml P.S. + 99ml RPMI + 1µl PMA).

2.6.3 Exposure of THP-1 to *C. difficile* antigens:

First the seeded wells of the microplate THP-1 cell line were prepared as described in Sections 2.5.4.6 and 2.6.2. The viability of the cells was checked as described in Section 2.5.4.7, then marked wells as antigens dilution will be used. Next, 100µl

diluted antigens were added to the marked wells as described in Section 2.6.1. Some wells were left without inculcation and blank as controls. Cell line microplate was inculcated and incubated at 37°C, 5% CO_2 for 8 hours for interleukins and 4 hours for TNF-α. Finally, supernatants were collected to evaluate the cytokines level by ELISA.

2.6.4 Preparation of primary, standard and secondary antibodies to detect cytokines:

Antibodies were supplied from eBioscience and PeproTech companies as shown in Table 6 (with kind help from Dr. Prerna Vohra). The standard curve was achieved using four dilutions (8ng/ml, 16ng/ml, 32ng/ml and 64ng/ml) to evaluate the cytokines concentration.

Antibody / Cytokine	Primary antibody and concentration	Standard cytokine and starting concentration	Secondary antibody and concentration
Interleukin -1β	Affinity Purified anti-human IL-1β (eBioscience, 14-7018;clone CRM56) 2µg/ml	Recombinant Human IL-1β (PeproTech,200-01B)32ng/ml	Biotin anti-human Il-1β(eBioscience,13-7016;cloneCRM57) 0.25µg/ml
Interleukin -6	Affinity Purified anti-human IL-6 (eBioscience, 14-7069;clone MQ2-13A5) 2µg/ml	Recombinant Human IL-6 (PeproTech,200-06)32ng/ml	Biotin anti-human Il-6 (eBioscience,13-7068;cloneMQ2-39C3) 1µg/ml
Interleukin -8	Affinity Purified anti-human IL-8 (PeproTech,500-M08;clone MQ2-13A5) 3µg/ml	Recombinant Human IL-8 (eBioscience, 14-8089)32ng/ml	Biotin anti-human Il-8(PeproTech,500-P2Bt) 0.5µg/ml
Interleukin -10	Affinity Purified anti-human IL-10 (eBioscience, 14-7108;cloneJES3-9D7) 2µg/ml	Recombinant Human IL-10 (PeproTech,200-10)32ng/ml	Biotin anti-human Il-10 (eBioscience,13-7109;cloneJES3-12G8) 1µg/ml
Interleukin -12	Affinity Purified anti-human IL-12 (eBioscience, 14-7128;cloneB-T21)2µg/ml	Recombinant Human IL-12 (PeproTech,200-12)32ng/ml	Biotin anti-human Il-12 (eBioscience,13-7129;cloneC8.6) 1µg/ml
TNF-α	Affinity Purified anti-human TNF-α (eBioscience, 14-7348;cloneMAb1) 2µg/ml	Recombinant Human TNF-α (PeproTech, 300-01A)32ng/ml	Biotin antihuman TNF-α (eBioscience,13-7349;cloneMAb11)2µg/ml

Table 6: Preparation of primary antibodies, standard cytokines and secondary antibodies with their concentration is presented.

2.7 Statistical analysis:

In this study Excel and Minitab were used for statistical analysis. The data were checked for normality and then contrasted with each other. An unpaired t-test was used for normal distribution data and R^2 was performed in the linear relationship between the two groups.

CHAPTER III DISTRIBIUTION AND DISSEMINATION OF *C. difficile* RIBOTYPES

3.1 Introduction:

This chapter is an epidemiological study for the *C. difficile* infection (CDI) in the surgical department of Western General Hospital, Edinburgh. Samples collected from the patients and their environments have been used, along with the control isolated from the MPRL group collection have been used.

C. difficile is recognised as the primary cause of infectious nosocomial diarrhoea in Europe, including the United Kingdom. The first known large UK outbreak of the hypervirulent, epidemic ribotype 027 happened in a Buckinghamshire hospital between October 2003 and June 2004, where 19 people died. This was followed by another outbreak between October 2004 and June 2005, with the same cause and a similar mortality level; a lack of patient care, infection control and isolation facilities cause the disaster (Freeman *et al.*, 2010).

As described in Chapter I, *C. difficile* spreads in many environments as it is spore-forming and heat-resistant, as well as not being affected by many antiseptic detergents such as hand soap or alcohol, and having resistance to many antibiotics. Furthermore, it the spread is facilitated by the incidence of diarrhoea caused by and associated with the antibiotics used for treatment (Lin *et al*., 2011).

Hospitals are an important reservoir of *C. difficile* spores; the spores have been isolated from hospital equipment such as linen, furniture surfaces and lavatories (McCoubrey *et al*., 2003). It is present in about 40% of geriatric patients (Sydnor and

Perl, 2011), and the spores are the primary cause of infection in hospitals (Lin *et al*., 2011).

Categorisation of the endemic *C. difficile* strains requires the application of a good typing scheme. Previously, many different techniques have been used for typing *C. difficile* during the course of outbreaks in hospitals. These include genotypic and phenotypic tests, the latter being serotyping, bacteriophage typing, whole cell protein profiles, ^{35}S methionine-labelled protein patterns, antimicrobial resistance patterns and immunoblotting (Knoop *et al*., 1993). However, these techniques may have disadvantages, such as poor reproducibility, weakness with regards to discrimination and the need for special equipment not available in most laboratories (O'Neill *et al*., 1996). The development of these methods has made it easier to identify microorganisms and follow cases up, as well as predicting outbreaks.

PCR-ribotyping based on gel is one of the easiest and most discriminating techniques for typing strains, but it is difficult to compare patterns from different places as the technique uses agarose gel images (Figure 3.4). However, Indra *et al*. (2011) modified the PCR method by labelling the 16S primer with carboxy-fluorescein (FAM) dye, and the results are analysed by a sequencer to make the PCR more accurate at identifying ribotypes (Figure 3.3).

The aim of the study was to isolate *C. difficile* strains from hospitalised patients' samples and samples taken from hospital environments, as well to identify strains and use ribotyping to verify the distribution and dissemination of *C. difficile* ribotypes.

Comparisons are also made between capillary sequencing PCR and metaphor gel PCR.

3.1.1 Isolation of *C. difficile* strains:

To date *C. difficile* has been isolated in many countries around the world. North American and European countries faced a significant challenge in the last decade, with CDAD threatening the lives of geriatric patients and other compromised individuals like pregnant women (Balassiano *et al*., 2012). Nowadays, warnings are sounding in some areas of South America, Asia and Australia as CDI has begun spreading through these countries (Cheng *et al*., 2011). It may be that the remaining countries which have not recorded CDI do not have healthcare protocols that include, facilities to isolate and identify *C. difficile.*

027/BI/NAP-1 was the main cause of CDAD in North America, and has been thought to be the cause of epidemics in Europe as well as Asian countries, such as Japan and Korea (Balassiano *et al*., 2012). However, Mutlu *et al*. (2007) reported that the most frequent ribotype is 001, whereas Taori *et al*. (2010) recounted that the predominant ribotype in Scotland is 002. Recently the Health Protection Scotland (HPS) Network in its second quarter report documented that 106 and 001 ribotypes had decreased compared to the previous years, and 027 was now uncommon. This has lead to the hypothesis that the predominant strains were varying from place to place and time to time.

3.1.2 Ribotype identification by Capillary Sequences PCR and the contrast with gel PCR:

Recently, the identification of etiologic agent techniques has been developed, and this gives microbiologists new equipment for molecular biology typing methods which assist in the identification of microorganisms. Polymerase Chain Reaction (PCR) ribotyping is used to type many organisms because it is fast and simple (O'Neill *et al.*, 1996; Bidet et *al.*, 2000), but its analysis by agarose gel electrophoresis made it difficult to reconcile the patterns from different laboratories.

The PCR method of classification of *C. difficile* depends on the analysis of polymorphisms by specific oligonucleotide primers complementary to the 3' end of 16s primer corresponding to base 1445-1466 of the 16S ribosomal RNA gene and the 5' end of the 23s primer corresponding to base 1-20 of the 23S ribosomal RNA gene.

Indra *et al*. (2011) modified the work of Stubbs *et al*. (1990) on the capillary sequencing method by labelling the 16s primer with florescent dye and 23s without labelling and analysing the PCR fragment with capillary gel electrophoresis, and this has provided a new angle on typing. The modified method achieved reliable peak patterns, dispelling the disadvantage of inter-laboratory comparisons of typing results, and significantly reducing the time and manpower required for PCR. In addition this technique can differentiate the fragment length 2b, which is better than 1.5b, as the electrophoresis requires disconnected fragments (150-650b) to accurately identify the size of a fragment permitted to distinguish seven subgroups for the *C. difficile* 014 ribotype.

3.2 Material and methods:

C. difficile strains were isolated and identified, beginning with an anti-toxin test (ELISA) for stool samples which was completed by Dr. Surekha Reddy and cultured in cooked meat broth. A subculture on blood agar and/or CCEY media was then carried out for morphology characters, as well as an ultraviolet test, a gram stain, motility investigation and DNA extraction.

3.2.1 Bacterial isolates:

Patients' stool and environment samples were collected by Dr. Surekha Reddy from the surgical unit at the Western General Hospital, Edinburgh; there was no interaction between the researchers and the patients selected for this study, as this was a blind study.

A total of 140 *C. difficile* isolates were collected from patients' stools (n=89), and from their close hospital environment: floors, furniture and hospital equipment (n=51) during 2009. Pure cultures were stored in cooked meat broth.

3.2.2 Visual investigation:

- Ultraviolet tests were set up by exposing the bacterial growth to UV light and observing the yellowish shine of the colony.

- Gram stain was made for all strains, followed the procedure of Practical Medical Microbiology (1989, 14th edition, p. 41-42). Also confirmed the Gram positive roads and peripheral spores.

- Motility was investigated using a hanging drop test, prepared by emulsifying a bacterial colony in normal saline, dispensing a drop on coverslip and turning it upside-down on the slide with a well; this was then investigated by a phase contrast microscope.

3.2.3 DNA extraction:

C. difficile isolates were subcultured on horse blood agar plates and incubated for 24 hours at 37°C in an anaerobic work station (Mark 3, Don Whitley Scientific, Shipley, Yorkshire). Five bacterial colonies were then emulsified and extracted, as described in Section 2.1.7.2, following the method promoted by O'Neill *et al*., (1996).

3.2.4 PCR Ribotyping:

PCR ribotyping was originally described by O'Neill *et al*. (1996) and adapted for analysis by the capillary gel electrophoresis method, according to Fawley *et al*. (2008). The 16S primer was labelled with carboxy-fluorescein (FAM) fluorescent dye at the 5' end, as described by Indra *et al*. (2011), in order to identify and quantify the PCR product peaks by the sequencer (16S= P5 5'-GCG CCC TTT GTA GCT TGA CC-3'/ 23S= P3 5'-CTG GGG TGA AGT CGT AAC AAGG-3') (from Integrated DNA Technology, or IDT).

The amplification procedure involved initial denaturation at 95°C for 5 mins, then 25 cycles of amplification with denaturation at 95°C for 60 secs, annealing at 55°C for 60 secs, an additional 90 secs at 72°C, and finally 5 mins at 72°C. The PCR products were stored at -70°C if necessary before dilution.

The amplified DNA was diluted 1:10 by nuclease-free distilled water, then diluted 1:10 by sample diluents (HiDi formamide 995µl + LIZ-600 size Marker 5µl). This product was stored at 4°C until being sequenced by a 3730 DNA analyser at the GenePool Group, Edinburgh University.

The fifty standard strains used in this study were from the MPRL collection and Dr Derek Farley's collection from Belfast Health and Social Care Trust, and were as follows: 001, 002, 003, 005, 009, 011, 012, 014, 015, 017, 020, 023, 026, 027, 029, 032, 035, 038, 039, 046, 053, 054, 056, 057, 059, 064, 069, 070, 072, 075, 078, 081, 087, 092, 095, 103, 104, 106, 111, 117, 119, 120, 125, 126, 131, 137, 185, 186, 200 and 210. They were originally typed by conventional PCR with agarose gels, and confirmed by capillary gel electrophoresis.

3.3 Results:

This study investigated 140 *C. difficile* particular isolates from hospitalised surgical patients (n=89) and their environment (n=51) in the Western General Hospital, Edinburgh. These isolates were stored in a bijoux tube containing cooked meat broth and subcultured on blood agar into characterised colonies, examined for yellowish shine under UV light and subjected to a gram stain and DNA extraction for PCR.

Image A reveals that the *C. difficile* character colonies on blood agar ware not bright, but were glossy, stumpy and curved, had irregular edges which were sometimes broad, were non-haemolytic and had a diameter ranging between 1 and 3mm; this was also the case on the CCEY agar. The growth had a characteristic penetrative smell.

The Gram stain image (Figure 3.2) presented a blue colour for *C. difficile* rods, and the peripheral spores were not stained.

Figure3.1: presented *C. difficile* colony on CCEY and blood agar.

Figure 3.2: Gram stains of *C. difficile* cells and identification of peripheral spores.

The *C. difficile* colony was identified by Capillary Sequencing Electrophoresis, which identifies strains by the patterns produced (size and location of the peak) by the PCR products (Figure 3.3).

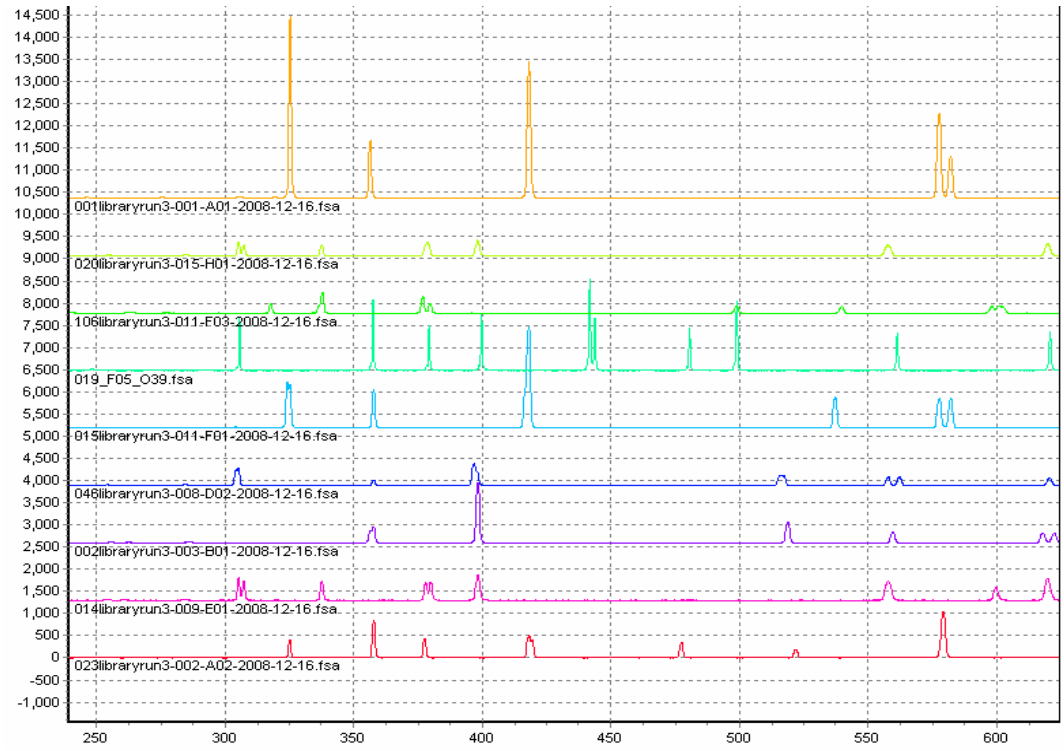

Figure 3.3: The Electrophorogram displays fluorescent signal intensities from Capillary Sequencing PCR by fluorescent labelled primers for 9 *C. difficile* standard isolates (001, 020, 106, 039, 015, 046, 002, 014 and 023). The y-axis show the signal intensities in Relative Fluorescent Units and x-axis for basepair sizes of fragments.The figure shows overlay view to compare easily several sample traces at once.

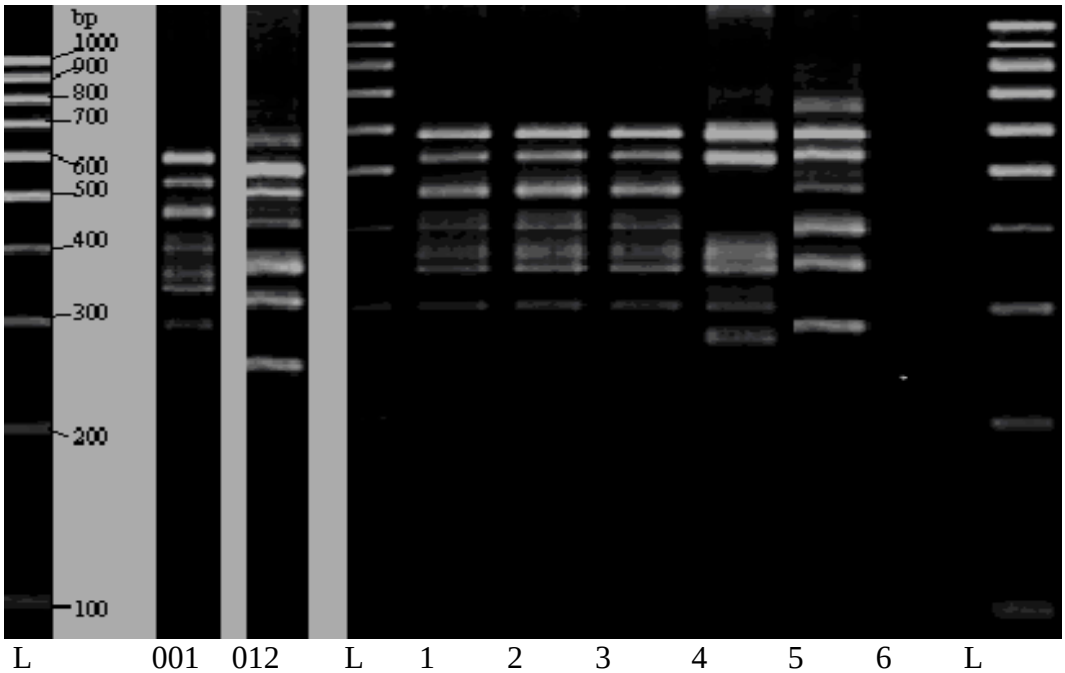

Figure 3.4: Images of PCR ribotyping by agarose gel electrophoresis. There are two standards: ribotype 001 and 012 with examples of 001 (tracks 1-3); 012 (track 5) and an unidentified type (track 4). L = size ladder (100-1000bp).

Analysis showed that 15 different PCR ribotypes were detected, with just four of the most frequent ribotypes representing about 70% of the study isolates. Overall, the epidemic strain ribotype 001 was the most frequent isolate, with a total of 74 isolates, about two thirds of which came from stool samples and 24 from the environment. These were followed by ribotype 020 (10 isolates), divided equally between patient and stool. Ribotype 106 was next with eight isolates (three from patients and five from the environment), then ribotype 039 with six isolates (two from patients and four from the environment). The results are summarised in Figure 3.5.

	Environment	Stool	Total	Percentage
Total	51	89	140	100
001	24	50	74	52.8
020	5	5	10	7.4
106	3	5	8	5.7
039	4	2	6	4.3
015	1	3	4	2.8
046	2	2	4	2.3
002	0	4	4	2.3
014	2	1	3	2.1
005	0	3	3	2.1
023	1	2	3	2.1
012	1	1	2	1.4
032	1	1	2	1.4
081	1	1	2	1.4
003	0	1	1	0.7
064	0	1	1	0.7
Unknown	4	9	13	9.3

Table 3.1: Frequencies of *C. difficile* ribotypes

Notably, the hypervirulent ribotype 027 was absent in this study. Ribotype 002, which was found to be the most common ribotype by Taori *et al*. (2010), was also

uncommon, with only four isolates in this study which originated only from stools; 005, 003 and 064 ribotypes were also isolated from stools only.

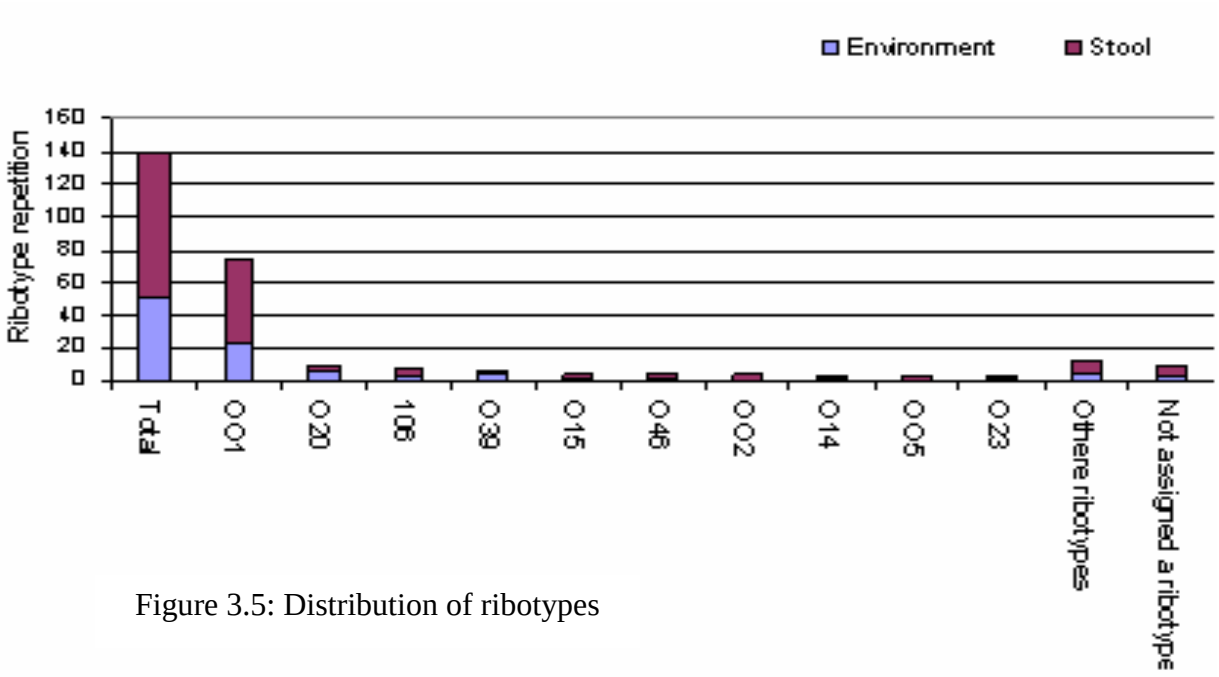

Figure 3.5: Distribution of ribotypes

The agarose gel technique was used with some isolates to compare the results to CGE and the variation can be observed between the agarose gel (Figure 3.4) and CGE (Figure 3.3). The noticeable difficulty when differentiating between the bands and comparing the ribotypes is removed.

3.4 Discussion:

The world at the beginning of twenty-first century has been challenged by CDI caused by *C. difficile*, especially in the elderly and compromised individuals such as the hospitalised community. Many strains have been isolated from infected persons. These strains varied in their ferocity depending on the virulence factors of the

etiologic agent. The prevalence of ribotypes varies from time to time and place to place due to unidentified reasons. However, this study indicates the view of the hospitalised endemic *C. difficile* ribotype, which reflects the general picture of *C. difficile* epidemiology.

The epidemiological study attempts to predict the future, through analysis of past and present accident data. It also highlights the hospital load of the infectious diseases. In this study, out of 140 isolates 15 ribotypes were identified, and the epidemic is of ribotype 001. These results indicate the changing prevalence, as the same ribotype featured in the Mutlu *et al*. (2007) study, and ribotype 002 was present in Taori *et al*.'s (2010) results. The HPS records in 2011 also indicated that ribotypes 106 and 001 are the most prevalent in Scotland, while ribotype 027, which was the most hypervirulent strain, is retreating except for in some cities in Scotland and various countries in Europe (Kuijper *et al*., 2008). Moreover, the *C. difficile* Ribotyping Network (CDRN) indicated that the frequency of ribotype 027 had decreased in the 2008-2009 report in England compared to 2007-2008 (Freeman *et al*., 2010).

In this surveillance, 89 *C. difficile* isolates were collected from patients' stool samples along with 51 isolates from different types of hospital environments. The *C. difficile* ribotype 001 dominates in this study, with 74 isolates, followed by 10 isolates of ribotype 020 and eight of ribotype 106. The other ribotypes were negligible. It is apparent that the source of *C. difficile* may have had some effect on increasing the repetition of the non-hyper-virulent ribotypes.

The epidemic strain ribotype 001 had soared in this study by about 53%, a third of which instances were related to hospital environments, while eight isolates of the epidemic ribotype 106 appeared in the search, five of which were from stool samples and three from the hospital environments. while 001 ribotype represented by 19% in the Wiuff *et al*. (2011) study.

Some studies have suggested that other ribotypes exceed the frequencies of 027 ribotype, such as 078 in some European countries (e.g. the Netherlands) in 2007, compared to 2005, in which period 027 ribotype decreased from 27% to 1% and the prevalence of 078 ribotype jumped from 3% to 13% (Rupnik *et al*., 2008).

However, in previous studies in Scotland, Taori *et al*. (2010) indicated that ribotype 002 was the most frequent strain, although only four isolates in this study were from stools. However, Solomon *et al*. (2011) suggested that ribotype 027 is the most prevalent ribotype (18.8%), followed by 001 (15.8%) in their study in the Republic of Ireland.

On the other side of the world, in Taiwan, Lin *et al*. (2011) investigated 130 isolates presented during the period 2001-2009 that NAP1 027 ribotype did not expose. Additionally, Huang *et al*. (2010) stated that the predominant epidemic ribotype in Fudan University Hospital, Huashan, China, was 017, followed by 001, between December 2008 and May 2009.

Coia (2009) recorded that the Scottish *Clostridium difficile* Reference Service results for characterization by PCR ribotyping testing of 530 isolates in the period November 2007-August 2009 indicated that 75% of isolates related to 106, 001 and 027 ribotypes, and hypothesized that these results were one of the natural reasons to create the new endemics ribotype of the *C. difficile* infection.

There is a lack of knowledge about the frequency rate of CDI by ward specialties in Scotland. However, Smyth *et al*. (2008) studied the infections incidence in hospital wards, and indicated that the prevalence of *C. difficile* was 0.69% in the general surgical word and 1.09% for MRSA in four countries studied (England, Wales, Northern Ireland and the Republic of Ireland) during February 2006 to May 2006. However, this study failed to mention the ribotypes necessary to identify the source of infection.

Accurate identification tools used in searches have assisted microbiologists to correctly define diagnoses. Nowadays, however, the PCR ribotyping has become the standard typing method for *C. difficile* in Europe, despite there being no international unified type strain library (Aspevall *et al*., 2006); this practice can be used with other molecular typing techniques to divide strains into subgroups.

Although the agarose gel electrophoresis PCR technique is cheap and easy to apply, there can be poor resolution of fragment size analysis compared with CGE, which is the reason for this study. Indra *et al*. (2011) indicated that using CGE by labelling only the primer 16s by FAM dye to distinguish ribotypes is a more reliable and more

sensitive method, saves time and makes it easier to compare patterns between laboratories. The other benefit of labelling only the 16s primer is the avoidance of the double peaks which is necessary in routine work.

The lack of standard ribotype collection can lead to different nomenclatures, but this should be avoided and known standards shared. The website http://webribo.ages.at (accessed Sept 2012). offers automatic analysis and comparison of the *Clostridium difficile* capillary-sequencer-based PCR-ribotyping-data, and simplifies laboratory PCR-ribotyping methods. Thus, the continuous investigation of stool and environmental samples to detect and analyse *C. difficile* is required to predict outbreaks. The provision of accurate tools is also essential in proper laboratories.

A weakness in this study was that the ribotype distribution may have been influenced by the sample collection as the samples came from a single unit in one hospital.

To conclude, the predominant *C. difficile* ribotype in the South Eastern of Scotland was 001ribotype, and the predominant ribotype changed time to time and place to another. The spores were presented in hospital environmental as a reservoir to spread the microorganism. Proper laboratory investigations support prediction of CDI outbreaks.

CHAPTER IV ANTIBIOTIC SUSCEPTIBILITY

4.1 Introduction :

The substantial global increase in life-threatening *C. difficile* infections (CDI) has become a major problem for healthcare organisations. *C. difficile* infection is a well-known cause of diarrhoea in geriatric patients and is associated with broad-spectrum antibiotic use (Dallal *et al*., 2002).

The *C. difficile* spore is considered to be the main source of CDI. These spores are resistant to heat and normal antiseptic detergents. *C. difficile* is also drought-tolerant and may be difficult to remove from the surfaces of instruments. It has been isolated from different environments, such as lavatories and the surfaces of furniture in hospitals (Rupnik *et al*., 2009; Lin *et al*., 2011). Al-Saif and Brazier (1996) state that the *C. difficile* isolated from cats' tails, pets, soil and water is indicative of the distribution of *C. difficile* throughout communities and in healthcare buildings.

Communities in developed countries are exposed to many predisposing factors which may lead to CDI infections, such as the uncontrolled use of antimicrobial treatment and the increasing use by elderly individuals of new risky processes such as radiotherapy and chemotherapy to treat malignancies and tumours (Coia, 2009). In Scotland, CDI cases underwent an approximately tenfold increase between 1988 and 2000 (Lee *et al*., 2001), reaching a peak in 2007 but it is now declining (HPS Network, 2011).

Changes in the predominant types and their resistance to antibiotics have also occurred. Antimicrobial use is one of the most significant selective pressures that affects the change in the incidence of the different strains that cause *C. difficile* infection (CDI). Access to up-to-date information on patterns of resistance to antibiotics is therefore important (Taori *et al*., 2010).

4.1.1 Virulence factors of *C. difficile*:

The virulence of *C. difficile* depends on its ability to produce toxins. Strains are likely to be selected by their resistance to antibiotics. Antimicrobial resistance patterns were related to the *tcdC* regulatory locus gens and consequent hyper production of toxins A, B and binary toxin (CDT) (McDonald *et al*., 2005). Lin *et al*., (2011) acknowledge that hyper-virulency is related to one or more of the genes *tcdA*, *tcdB* and *cdtC*.

The other factor that assists CDI is disruption of the bowel microbiota by broad spectrum antibiotics that a *C. difficile*-colonised colon can tolerate. Impaired individuals, for example pregnant women or patients with compromised conditions, are more vulnerable to CDI (Balassiano *et al*., 2012). In addition to bacterial cell wall components, such as SLPs or flagellar proteins, hydrolytic enzymes and putative capsules also have a role in infection (Dingle *et al*., 2011).

Coia, (2009) and Wiuff *et al*., (2011) suggest that in the case of microbial virulent and pandemic strains, antimicrobial resistant strains could lead to the formation of endemic subtypes such as the 106 and 001 ribotypes of *C. difficile*. The Scottish

Clostridium difficile Reference Service identified 530 ribotyped clinical isolates of *C. difficile* and investigated their antimicrobial susceptibility between November 2007 and August 2009. They were found 90% of isolates were resistant to clindamycin and fluoroquinolones, all isolates were susceptible to vancomycin and metronidazole.

In the past, clindamycin was the first antibiotic to be associated with CDI. Widespread use of penicillin was then found to be a predisposing factor for CDI. More recently, the third generation of cephalosporins and fluoroquinolones have been implicated as risk factors for CDI (Wiuff *et al*., 2011).

4.1.2 *C. difficile* antimicrobial therapy:

The Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America (SHEA/IDSA) guidelines recommend oral metronidazole for mild and moderate cases of CDI, or oral vancomycin for severe cases following unsuccessful antimicrobial treatment. However, the European Society of Clinical Microbiology and Infectious Diseases treatment guidance document for CDI advises the use of teicoplanin, a glycopeptide, and vancomycin as supplementary oral therapy. Both American and European guidance favours oral treatment (Musgrave *et al*., 2011).

Louie *et al*., (2011) advocate the use of the narrow spectrum macrocyclic antibiotic fidaxomicin as it is considered safe, there is a high level of active drug in the bowel, a low possibility of developing resistant strains and limited effects on intestinal microbiota. Moreover, it is associated with only a minor rate of recurrent CDI.

Alternative therapies include bismuth subsalicylate, rifamycin, nitazoxanide, tigecycline, linezolid and ramoplanin, as well as many non-antibiotic forms of treatment such as immunological methods, faecal transplantation and surgical procedures (Musgrave *et al*., 2011).

The study aimed to investigate minimum inhibition concentration (MICs) and antibiotic resistance patterns for *C. difficile* isolates from surgical patients and associated environmental samples. The relationship between the sources of isolates and antibiotic susceptibility was also examined.

4.2 Materials and methods:

Colleagues from the surgical wards at the Western General Hospital in Edinburgh provided 89 stool samples during 2009 and purified *C. difficile* isolates were obtained from these samples. Also 51 pure cultures were obtained from the environment of these patients. Pure cultures were stored in cooked meat broth for further investigation.

This study followed guidelines set by the Clinical and Laboratory Standards Institute (CLSI), officially the National Committee for Clinical Laboratory Standards (NCCLS). MIC reference agar dilution methods (NCCLS 2004). Performance Standards for Antimicrobial Susceptibility Testing 2007) were used.

The isolates of standardised McFarland inocula were streaked onto pre-reduced Brucella agar plates supplemented with vitamin K1, haemin and 5% (v/v) horse blood (BD Biosciences, Oxford, UK).

The control strains used in each batch in this study were *C. difficile* ribotypes 001, 002, 012, 027, 078, and 106 from the MPRL collection. The agar dilution method was used to estimate the MICs for the six antibiotics (ampicillin, erythromycin, tetracycline, metronidazole, moxifloxacin and vancomycin).

The break points in this analysis were erythromycin ≥ 8 µg/ml, tetracycline ≥ 16 µg/ml, metronidazole ≥ 32 µg/mL, moxifloxacin ≥ 8µg /ml and vancomycin ≥ 32 µg/ml, (following Taori *et al*., 2010) and ampicillin ≥ 32 µg/ml (from Taori *et al*., 2010 and Lin *et al*., 2011).

4.3 Results:

The MICs were assessed by agar diffusion as described in Section 4.2. The image in Figure 4.1 shows the Brucella agar medium with horse blood, including antibiotic dilution and the growth of *C. difficile* isolates.

4.3.1 Sensitivity to antibiotics:

The susceptibility of 140 *C. difficile* isolates to the six antibiotics used in this study by determination of MICs is summarised in Table 4.1 and Figures 4.2 and 4.3. 100%

of isolates were sensitive to moxifloxacin, metronidazole and vancomycin. These results are generally compatible with Wiuff *et al*., (2011). However, whilst 100% of the environmental isolates were sensitive to tetracycline, more than 85% of isolates were sensitive to ampicillin and around 15% were resistant to erythromycin (Table 4.1).

Antibiotic	Vancomycin		Erythromycin		Tetracycline		Ampicillin		Metronidazole		Moxifloxacin	
Source of isolates	envi	stool	envi	stool	envi	stool	envi	stool	envi	stool	envi	stool
Number of resistance isolates	—	—	42	70	—	13	7	14	—	—	—	–
Number of sensitive isolates	51	89	9	19	51	76	44	75	41	89	51	89

Table 4.1: Antibiotic susceptibility to all *C. difficile* isolates

Figure 4.1: Image of Brucella agar with blood for MICs determination for *C. difficile* isolates.

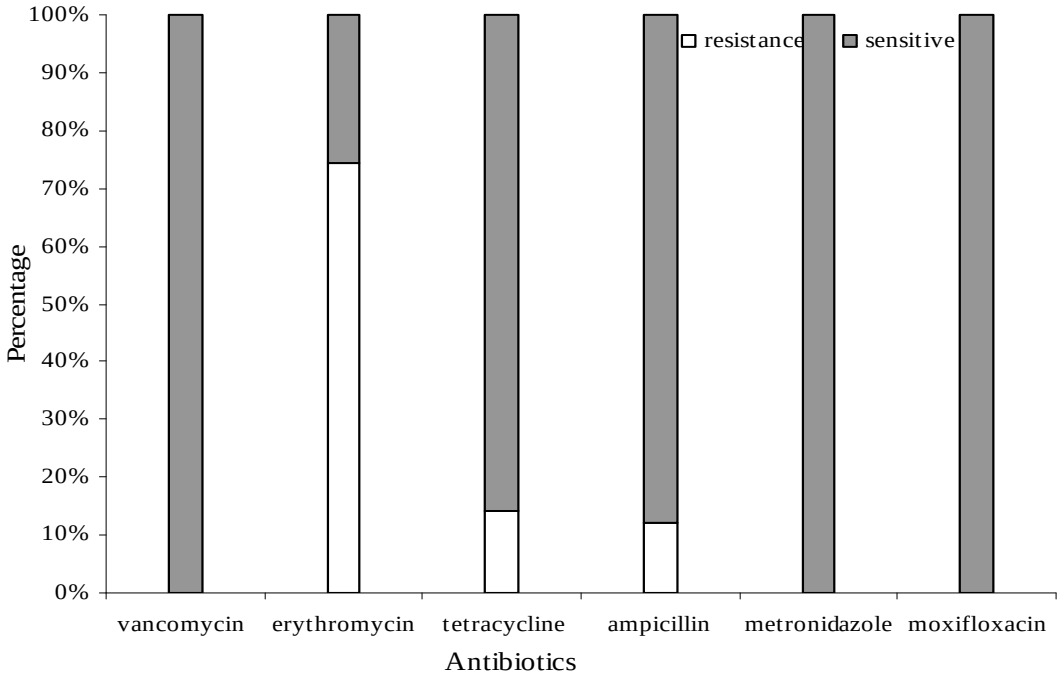

Figure 4.2: Percentage of antibiotics susceptibility of *C. difficile* isolates

Figure 4.2 shows antibiotic susceptibility for *C. difficile* isolates from different sources. The chart shows that 74% of isolates exhibited resistance to erythromycin, whilst 100% of isolates were sensitive to vancomycin, metronidazole and moxifloxacin. More than 12% of isolates were resistant to tetracycline and ampicillin.

4.1 Dominant ribotypes and MIC:

The most frequent ribotype in this study was the epidemic ribotype 001. This strain illustrates susceptibility to vancomycin, moxifloxacin and metronidazole. There was no substantial variation between the 001 ribotype and the other ribotypes and no difference between isolates from stool and hospital environmental isolates for ribotype 001.

Antibiotic	MIC 50 µg/ml	MIC90 µg/ml	Break point µg/ml
Ampicillin	20	160	64
Erythromycin	160	160	8
Tetracycline	5	80	16
Metronidazole	5	5	32
Moxifloxacin	5	5	8
Vancomycin	5	10	16

Table 4.2: MIC 50 & 90 for *C. difficile* ribotype 001

Table 4.2 shows the MIC50 and MIC90 for ribotype 001, which indicates that 50% of isolates were inhibited by 20 µg/ml of ampicillin and by 160 µg/ml of erythromycin. For tetracycline, metronidazole, moxifloxacin and vancomycin, the corresponding figure was 5 µg/ml. The same results were observed in MIC90 for metronidazole and moxifloxacin. Ampicillin and erythromycin inhibit ribotype 001 at concentrations of 160µg/ml for MIC90; the figure for tetracycline was 80µg/ml.

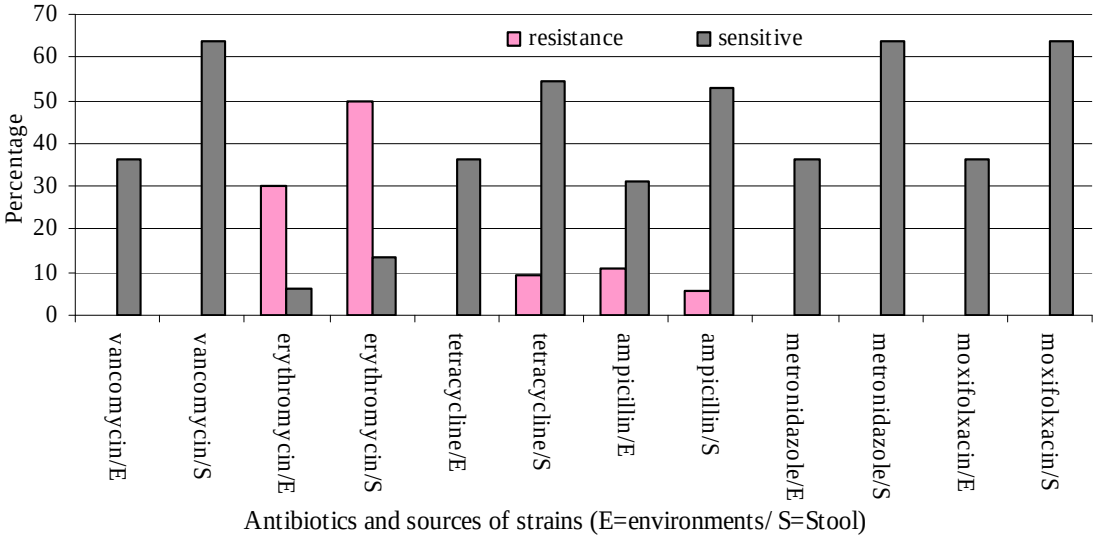

Figure4.3: Antibiotics susceptability for *C. difficile* 001 ribotype

Figure 4.3: The histogram shows that all *C. difficile* isolates are suscepitible to vancomycin, metronidazole and moxifloxacin, but some strains from the environment show resistance to erythromycin and tetracycline, while the stool strains show the highest resistant percentage to eryrthomicin.

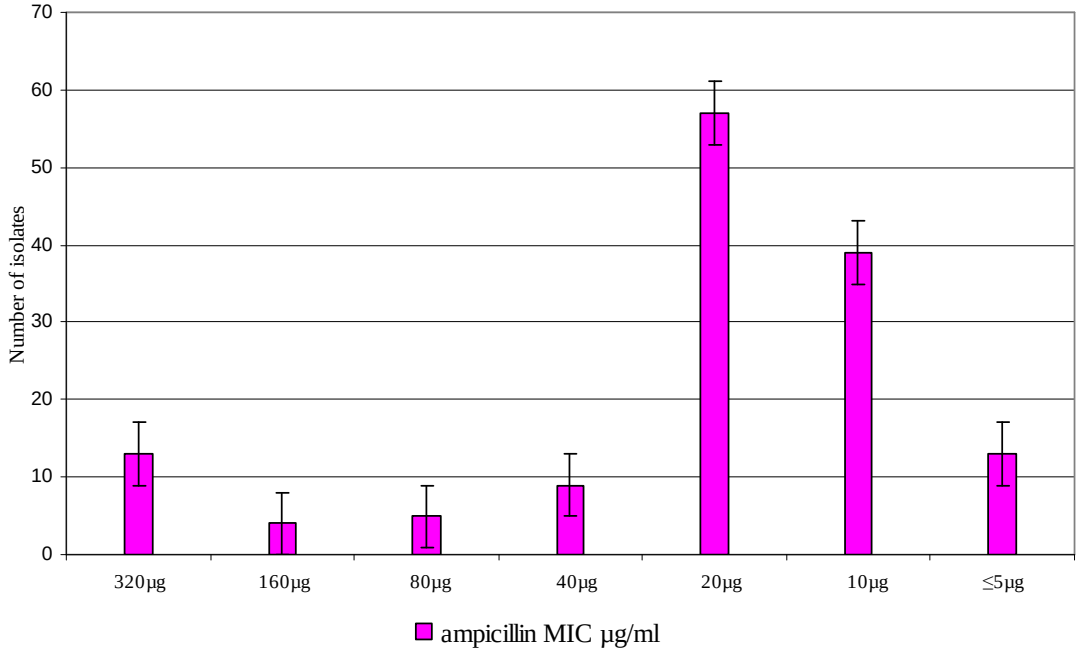

Figure 4.4 This shows an almost normal distribution of ampicillin MICs values against ribotype 001 and a concentration of 20µg was the most effective, the Y error bars by 1 standard deviation and fixed value 4 with 5%.

Through the data from this study we can see that there were similarities in antibiotic susceptibilty between isolates from the hospital enviroments and the stool isolates, which suggests that the sources of *C. difficile* isolates were the same, and the hospital enviroment contaminated patients isolates.

4.4 Discussion:

This study examined the susceptibility of 140 *C. difficile* isolates and found all isolates to be susceptible to vancomycin, metronidazole and moxifloxacin. The HPS 2011 report found vancomycin and metronidazole to be compatible for all ribotypes, but 100% of ribotype 106 and 69% of ribotype 001 isolates were resistant to

moxifloxacin. Ribotype 020 had 28 isolates that showed 100% sensitivity in the HPS reports.

Furthermore, the frequency of resistance by ribotype 001 isolates to erythromycin was 74%. Similarly, HPS reports show a figure of 71%, whilst Mutlu *et al*., (2007) found that 95.4% of ribotype 001 isolates were resistant to three or more antibiotics. Ribotype 001 is often related to multi-antibiotic resistant strains, which may partially explain its predominance in some studies (Taori *et al*., 2010). Furthermore, 100% of ribotype 106 isolates are resistant to erythromycin, but the ribotype 020 isolates were sensitive 100%.

In this study, 7% of isolates were found to be resistant to tetracycline, whilst Barbut *et al*. (2007) indicated that for toxigenic strains 9.2% of isolates are resistant to tetracycline.

No strain was resistant to all six antibiotics used in this study. Mutlu *et al*., (2007) found one isolate of ribotype 001 to be resistant to the five antibiotics studied. As incidence rates of CDI in Scotland have decreased (HPS reports 2011), multidrug resistance is not the only virulence factor in the *C. difficile* endemic. A number of other virulence factors, such as inducers of cytokines, binary toxins, surface layer proteins and sporulation, have been suggested as areas in which further research is needed (Taori *et al*., 2010).

Table 2 shows that 50% of isolates were inhibited by 20 µg/ml of ampicillin and 160 µg/ml of erythromycin compared to 32 µg/ml in 2005 results obtained by Taori *et al.*, (2010), whilst tetracycline, metronidazole, moxifloxacin and vancomycin were inhibited by 5µg/ml. The same results were obtained in MIC90 for metronidazole and moxifloxacin. During the period 1979-2004, the MIC50 (Taori *et al.*, 2010) study obtained the same results for metronidazole, whilst the figure for tetracycline was 1µg/ml, for moxifloxacin 2µg/ml, and 1µg/ml for vancomycin. In addition, ampicillin and erythromycin inhibit ribotype 012 at 160µg/ml for MIC90 and 80µg/ml for tetracycline. Lin *et al.*, (2011) found the value for MIC90 for ampicillin to be 4µg/ml.

Rupnik *et al.*, 2009 and Lin *et al.*, 2011 indicate that the *C. difficle* spores were able to contaminated hospital's surfaces furnture, toilets and linen. The inferences of similarity in antibiotic susceptibility of stool and environmental isolates lead to suggesting that the *C. difficile* spores spread in hospital environments.

The change in the pervasiveness of *C. difficile* and its susceptibility to antimicrobials could be due to numerous factors. Ongoing studies on the change in *C. difficile* ribotypes in hospital environments and their ability to induce cytokines, binary toxins, surface layer proteins and sporulation are required, which will have implications for healthcare workers and hospital environments acting as bacterial reservoirs.

CHAPTER V IMMUNE RESPONSES TO *C. difficile* ANTIGENS IN HEALTHY PEOPLE IN THE COMMUNITY AND IN HOSPITALISED INDIVIDUALS

5.1 Introduction:

A huge range of microorganisms inhabit the human bowel. The host mucosal immune system has the ability to differentiate between pathogenic and commensal organisms. The regular sampling of antigenic molecules from normal microbiota may be used to enhance specific IgA responses from mesenteric lymph nodes against symbiotic microorganisms. The IgA is then secreted to the mucosal layer in the colon to protect the intestine from microbial assault (Kinnebrew and Pamer, 2012).

Viscidi *et al*., (1983) hypothesised that the production of antibodies is stimulated during childhood and continues into older age irrespective whether the body is exposed to the same pathogens or to alternative species.

The host immune response to *C. difficile* immunogenic molecules has been the subject of extensive research over the past decade. Kyne *et al*., (2000) found no significant difference between IgG and IgA levels in serum from CDI patients. Drudy *et al*., (2004) found no variation in the concentrations of IgG, IgM and IgA against S-layer proteins of *C. difficile* in serum from patients, carriers and controls.

Sanchez-Hurtado *et al*., (2008) examined the response of antibodies to different *C. difficile* antigens in symptomatic CDI patients, carriers and controls (non-colonised individuals). No noteworthy variation in IgM levels with all antigens between the three groups was found, while anti-lipocarbohydrate IgG was higher in controls.

Antibodies for other antigens were higher in symptomatic patients and carriers than in the controls.

This study aimed to separate the *C. difficile* cell wall antigen SLPs, lipocarbohydrate (LC) and flagellar proteins from the five most frequent strains encountered in South Eastern Scotland. The objective was to test the hypothesis of immunogenicity of these *C. difficile* proteins by estimating IgG levels against these antigens in the community of Edinburgh and in hospitalised individuals and to determine the hyper immunogenic antigen. The hypothesis of repeated infection due to a lack of creation of antibodies was also examined.

5.2 Materials and methods:

5.2.1 Bacterial growth:

Four of most frequent *C. difficile* ribotypes in South Eastern Scotland (001, 002, 078 and 106) along with ribotype 027, which is very uncommon locally, were grown anaerobically in PPY broth and used to extract SLPs, LC and flagellar proteins.

5.2.2 Extraction of SLPs, LC and flagellar proteins:

The guanidine HCl method was used to extract SLPs from the five *C. difficile* ribotypes as described in Section 2.3.2.2. The extraction methods for flagellar proteins followed the procedure outlined in Section 2.3.4. Extraction methods for lipocarbohydrate (LC) were based on Poxton and Cartmill (1982) and Sharp and Poxton (1986) as described in Section 2.3.5.

5.2.3 PAGE preparations:

Polyacrylamide gel electrophoresis was prepared as described in Section 2.3.6 to confirm the purification of proteins.

5.2.4 Detection of IgG antibodies by ELISA:

Detection of IgG antibodies was performed by ELISA. The procedure followed is described in Section 2.4.1.

5.2.5 Serum samples:

Samples were obtained from the MPRL collection originally obtained under ethical approval from the SE Scotland Blood Transfusion Service in Edinburgh (healthy blood donors) and patients in the Royal Victoria Hospital in Edinburgh: the latter were also used in the study by Sanchez-Hurtado *et al* (2008).

The samples used in this study were divided into four groups:

i. Donor samples were collected from healthy adult individuals from blood donors (230 samples).
ii. Controls were individuals who were culture-negative and toxin A/B negative (26 samples) from the hospital community.
iii. Symptomatic samples were collected from patients with clinically diagnosed CDI by positive investigation for toxin A/B, stools and/or positive identification for PMC on colonoscopy (20 samples).
iv. Carrier samples were collected from people who were culture positive for toxin A/B but asymptomatic (20 samples).

5.3 Results:

5.3.1. Separation and purification of SLPs, LC and flagellar proteins:

Separation of SLPs from different ribotypes presented two layers: HMW about 45 kDa and LMW around 35 kDa of SLPs as shown in the images in Figures 5.I. Flagellar proteins ranged between 40-55 kDa in PAGE as well as lipocarbohydrate LC.

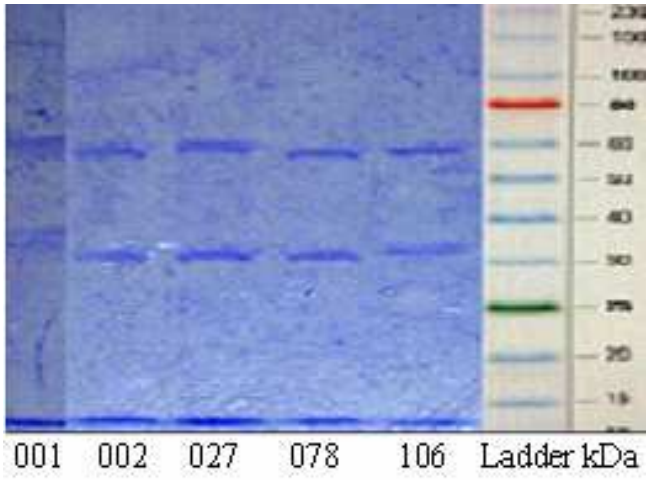

Figure 5.I: Demonstrates the two SLPs bands for ribotypes 001, 002, 027, 078 and 106 comparing to the ladder next to 35 and 45 kDa on PAGE.

5.3.2 Anti-cell wall markers and flagellar proteins IgG levels in the community:

The three antigens of *C. difficile* from every isolate of the five ribotypes were exposed to the donor serum. Measurements of the level of IgG antibodies against antigens were evaluated by ELISA, with the values then divided into groups to demonstrate the distribution. Charts of the distribution of IgG against SLPs, LC and flagellar proteins in donors' serum showed almost normal distribution for all antigens as shown in the Figures 5.IIa, 5.IIb, 5.IIc, 5.IId, 5.IIe, 5.IIIa, 5.IIIb, 5.IIIc, 5.IIId, 5.IIIe, 5.Iva, 5.IVb, 5.IVc, 5.IVd and 5.IVe.

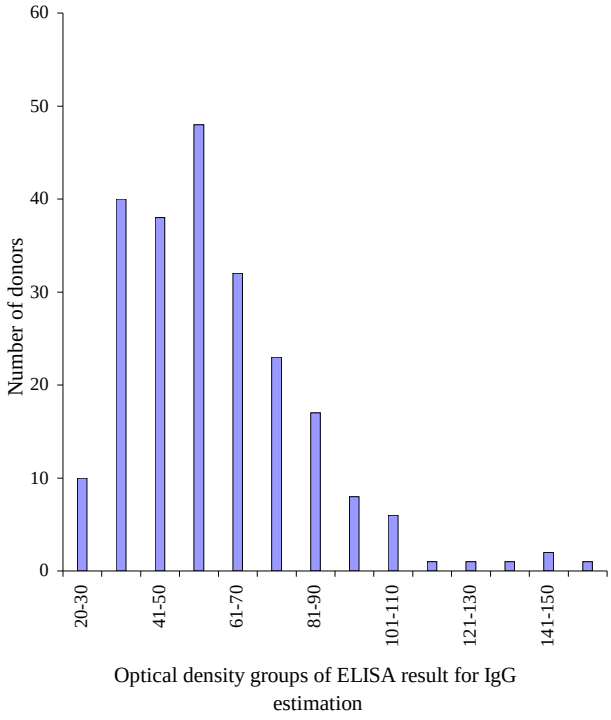

Figure 5.IIa: anti-001 SLPs IgG % in standard serum

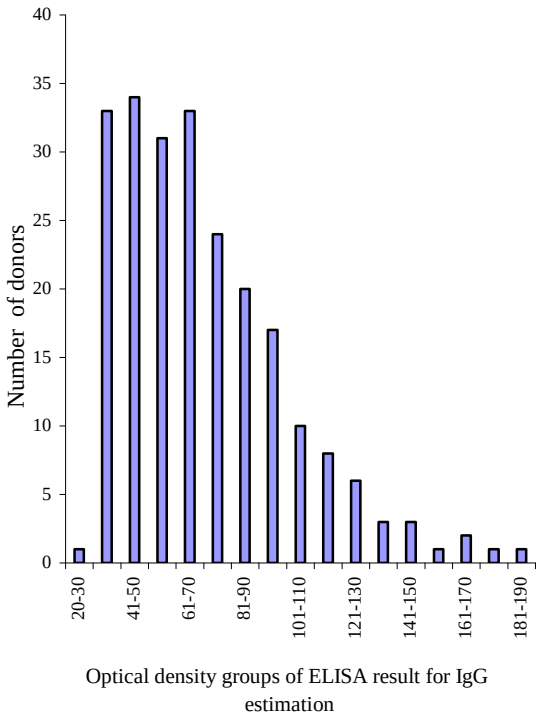

Figure 5.IIb: anti-002 SLPs IgG % in standard serum

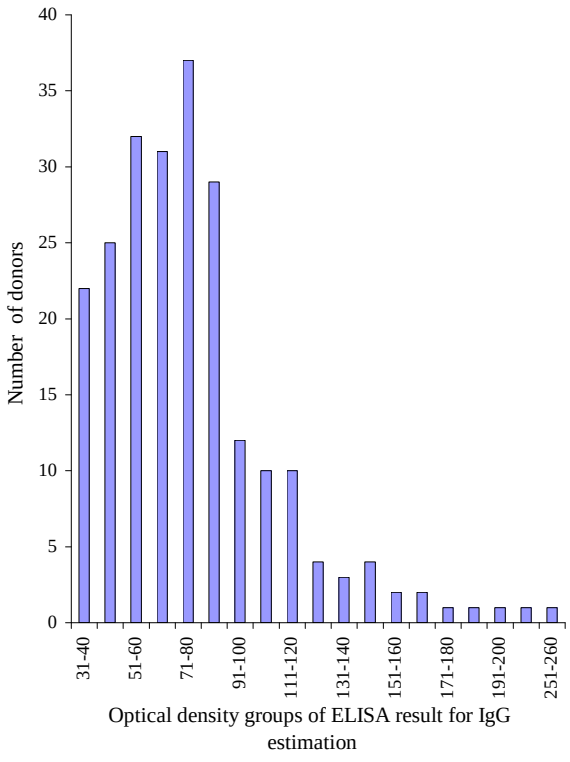

Figure 5.IIc: anti-027 SLPs IgG % in standard serum

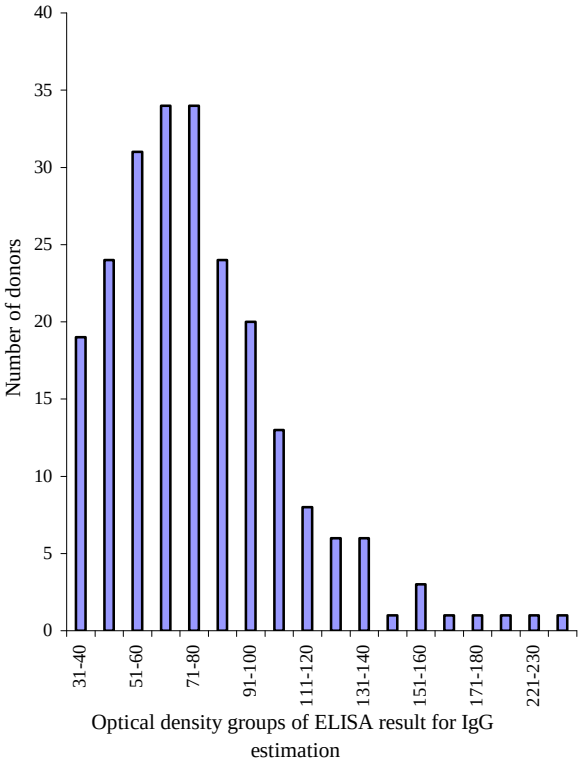

Figure 5.IId: anti-078 SLPs IgG % in standard serum

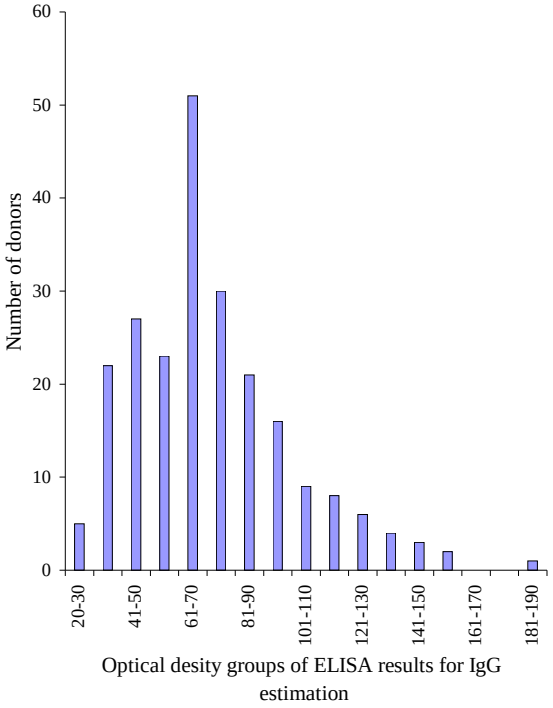

Figure 5.IIe: anti-106 SLPs IgG % in standard serum

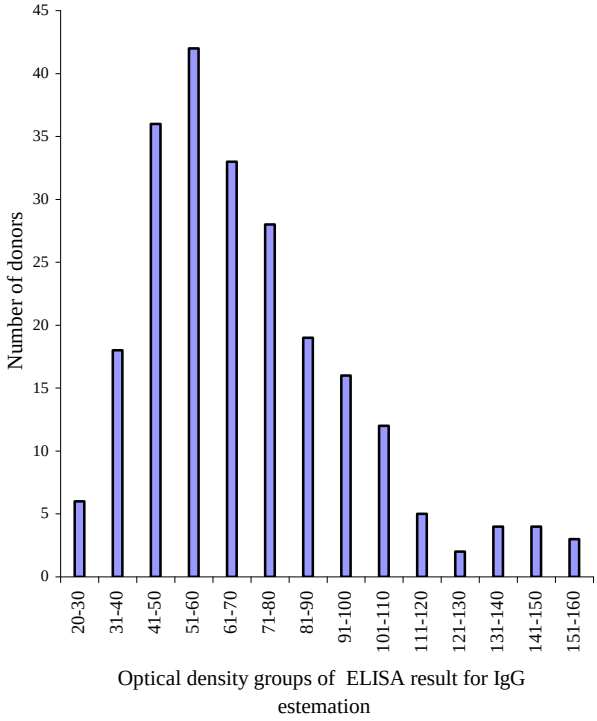

Figure 5.IIIa: anti-001 SLPs IgG % in standard serum

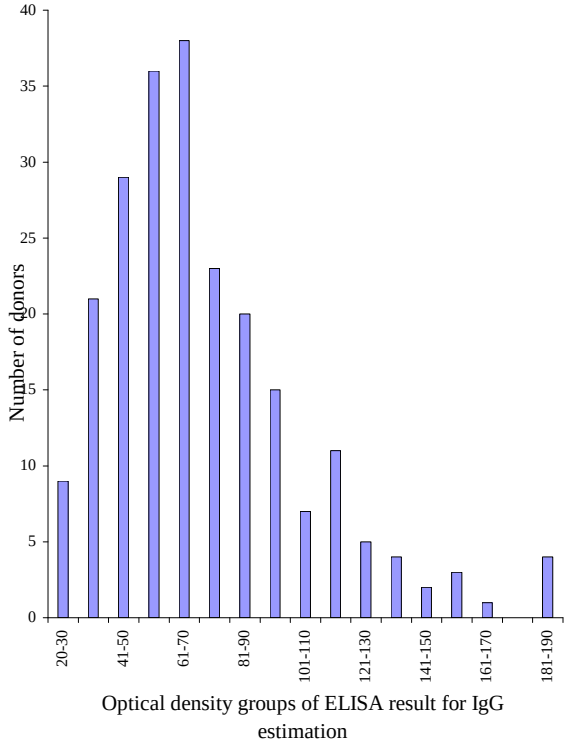

Figure 5.IIIb: anti-002 SLPs IgG % in standard serum

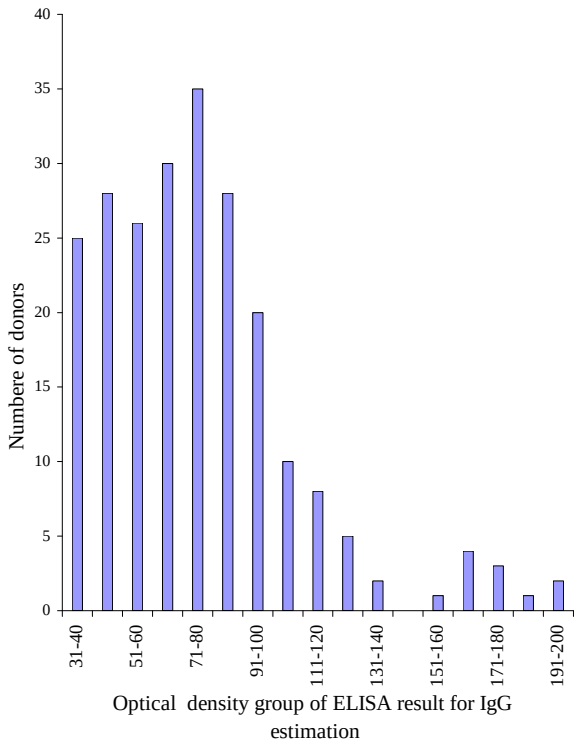

Figure 5.IIIc: anti-027 SLPs IgG % in standard serum

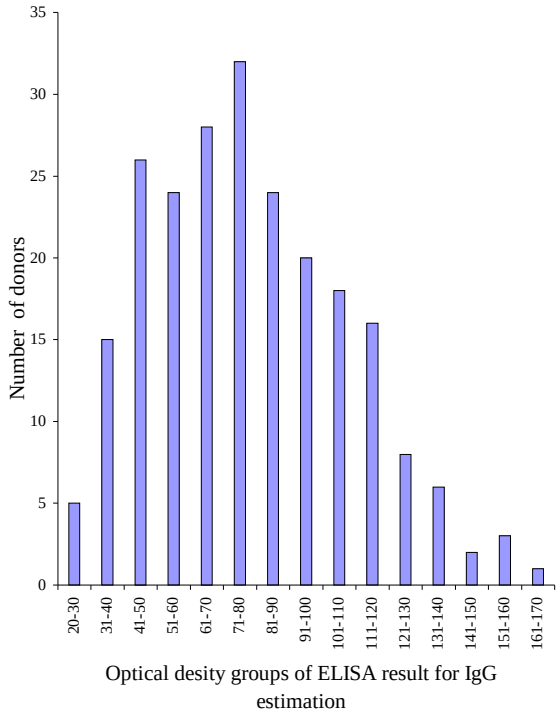

Figure 5.IIId: anti-078 SLPs IgG % in standard serum

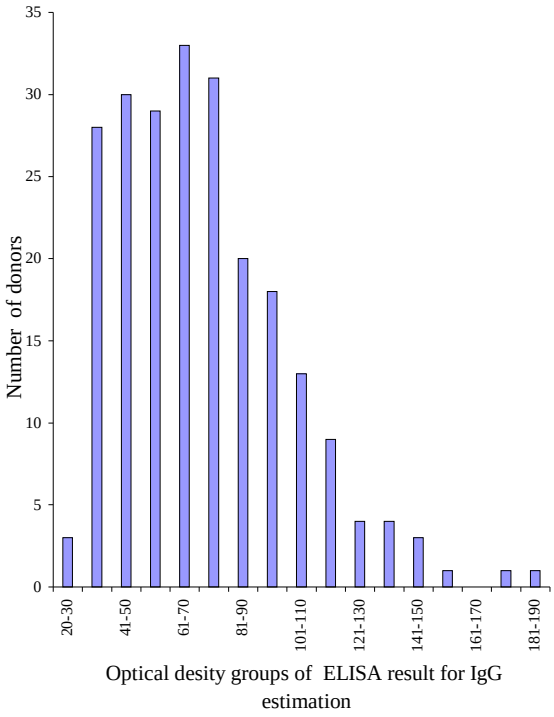

Figure 5.IIIe: anti-106 SLPs IgG % in standard serum

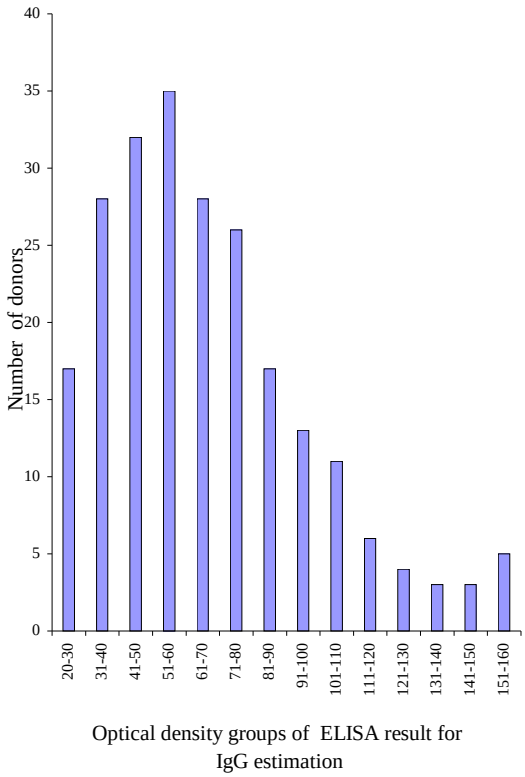

Figure 5.IVa: anti-001 SLPs IgG % in standard serum

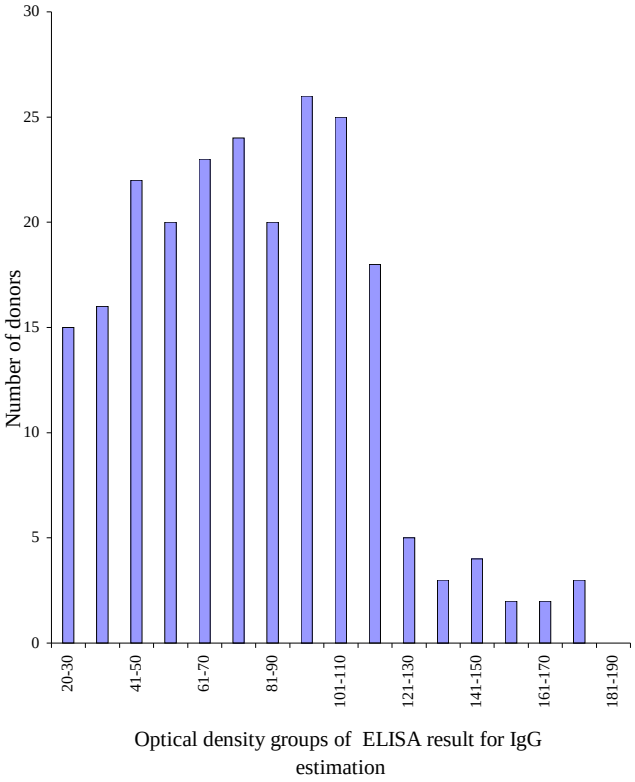

Figure 5.IVb: anti-002 SLPs IgG % in standard serum

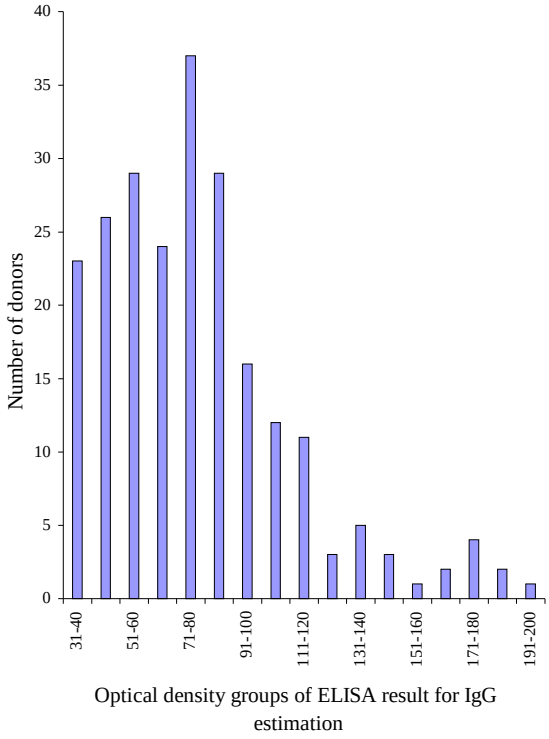

Figure 5.IVc: anti-027 SLPs IgG % in standard serum

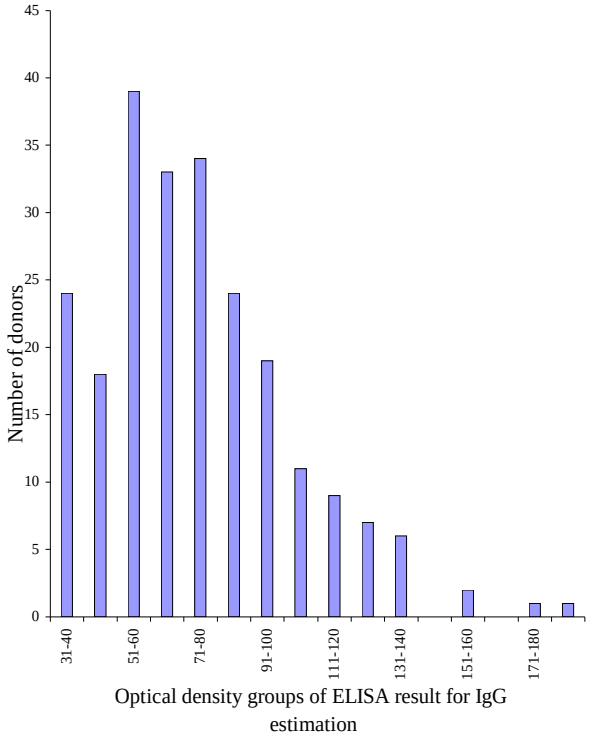

Figure 5.IVd: anti-078 SLPs IgG % in standard serum

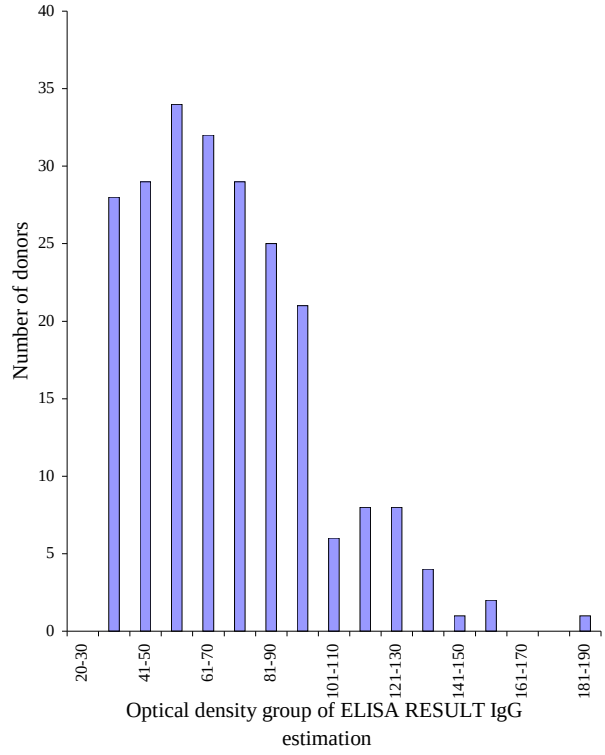

Figure 5.IVe: anti-106 SLPs IgG % in standard serum

These figures indicate that anti-106 SLPs and anti-001 SLPs accounted for the highest percentages. Table 5.1 offered statistical values as standard error of the mean (SEM) which was about 3.4 for anti-106 SLPs and 4.5 for anti-001 SLPs. The confidence interval at 95% was more than 6.0 for anti-027 SLPs and 9.7 for anti-001SLPs, whilst the range of prevalence in all anti-SLPs ranged from 33 to 51. The coefficient of variation with the exception of the 002 ribotype was 0.8.

Statistical parameters for SLPs	Ribotype				
	001	002	027	078	106
Range	47	33	36	33	51
Variance	283.4505	168.9231	164.7778	154	194.882
Standard deviation	16.836	12.997	12.8366	12.4097	13.96
Coefficient of variation	1.0338	0.8123	1.0697	0.9797	1.0409
Standard error of the mean	4.4996	3.4736	2.9449	2.925	3.3858
95% confidence interval	9.7208	7.5043	6.187	6.1712	7.1776
Upper 95% confidence limit	26.00653	23.50427	18.18703	18.83785	20.5893
Lower 95% confidence limit	6.564903	8.495732	5.812967	6.495481	6.23418

Table 5.1: Statistical values of distribution of anti-SLPs IgG in healthy donor serum

The 95% confidence interval ranged from 5.7 to 7.8 in anti-flagellar IgG and from 4.98 to 7 in anti-LC IgG. However, the coefficient variations were mainly around one, with the exception of anti-078 flagellar proteins (about 0.7) as shown in Table 5.2.

Statistic parameters for flagellar	Ribotype				
	001	002	027	078	106
Range	40	38	35	31	33
Variance	185.4505	152.5074	160.0074	108.1714	159.007
Standard deviation	13.618	12.3494	12.6494	10.4005	12.6098
Coefficient of variation	0.8362	0.9208	0.9432	0.6842	0.9402
Standard error of the mean	3.6396	2.9952	3.0679	2.6854	3.0583
95% confidence interval	7.8628	6.3495	6.5037	5.7596	6.4834
Upper 95% confidence limit	24.14853	19.76123	19.91549	20.95963	19.8951
Lower 95% confidence limit	8.422902	7.062296	6.908043	9.440368	6.92839

Table 5.2: Statistical values of distribution anti-flagellar IgG in healthy donor serum

For anti-LC IgG distribution, the coefficient of variation was between 0.7 and 0.99 for the 001 and 106 ribotypes respectively. The most normal distribution was found in anti-027LC IgG.

Statistical parameters for LC	Ribotype				
	001	002	027	078	106
Range	32	26	36	39	34
Variance	134.0659	94.0074	147.7574	176.4667	177.5074
Standard deviation	11.5787	9.6957	12.1555	13.2841	13.3232
Coefficient of variation	0.711	0.7229	0.9063	0.9322	0.9934
Standard error of the mean	3.0945	2.3516	2.9482	3.321	3.2313
95% confidence interval	6.6853	4.9851	6.2498	7.0786	6.8502
Upper 95% confidence limit	22.97105	18.39685	19.66157	21.32858	20.26192
Lower 95% confidence limit	9.600381	8.426676	7.161959	7.171416	6.561613

Table 5.3: Statistical values of anti-LC IgG distribution in healthy donor serum

5.3.3 Contrast between IgG level averages against SLPs, LC and flagellar in the community samples (healthy donors):

Immune response results in donor's serum showed that the SLPs had the highest averages in all ribotypes, with the exception of IgG anti-flagellar, which exceeded the IgG anti-SLPs in the 027 ribotype (Figure 5.V). However, IgG anti-LC was the lowest for all five ribotypes (figure 5.V). On the other hand, the wider range of IgG concentration related to anti-LC was about 0.19. IgG anti-SLPs had the least variation (0.084) between the five ribotypes in an almost similar reaction (Table 5.4). Also there was a significant variation in immune response between different LC from different ribotypes. Donor serum had the best immune reaction to SLPs and flagellar proteins.

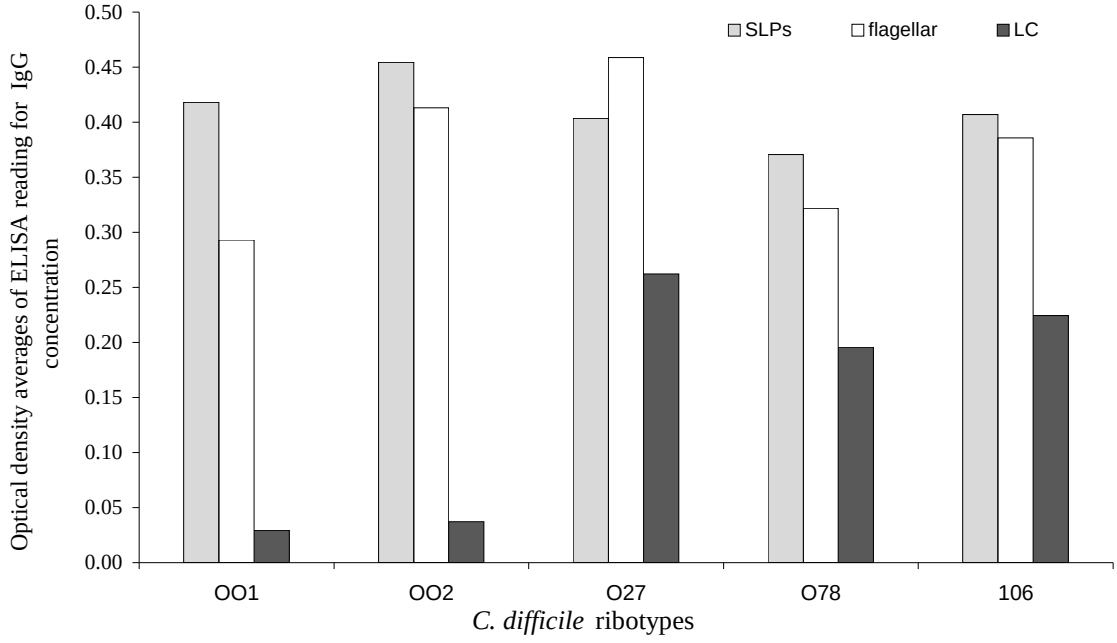

Figure 5.V: Histogram showing the contrast between averages of anti-SLPs, anti-flagellar and anti-LC IgG from five ribotypes in donors samples.

Statistical parameters	SLPs average	Flagellar average	LC average
Arithmetic mean	0.41068	0.374478	0.14975
Geometric mean	0.4098	0.369562	0.104751
Median	0.4069	0.385763	0.195575
Range	0.0837	0.1656	0.2328
Variance	0.000908	0.004525	0.0119
Standard deviation	0.0301	0.0673	0.1089
Coefficient of variation	0.0135	0.1796	0.7271
Standard error of the mean	0.0374	0.0301	0.0487
95% confidence interval	0.448085	0.0835	0.1352
Upper 95% confidence limit	0.373275	0.458001	0.284955
Lower 95% confidence limit	0.373275	0.290955	0.290955
P value at DF = 5	0.0001	0.0001	0.0101

Table 5.4: Statistical values of IgG levels in healthy donors serum.

5.3.4 Spread of IgG in *C. difficile* patterns:

The spread of IgG levels for each pattern was displayed in a trend chart. The R-squared (R^2) value was estimated to indicate the coefficient and effectiveness. The spread of IgG against the three antigens for different ribotypes is presented in figures 5.VIa, 5.VIb. 5. VIc, 5.VId, 5.VIe, 5.VIIa, 5.VIIb, 5.VIIc, 5.VIId, 5.VIIe, 5.VIIIa, 5, VIIIb, 5.V.IIIc, 5.VIIId and 5.VIIIe. All values were represented in relation to all antigens in the study.

Coefficient of R^2 was determined in a statistical analysis that shows the effectiveness of one changeable at predicting another variable. The R^2 value ranged from 1 to 0 and the higher the R^2 value highlights the closer the values are to each other.

The results showed that R^2 ranged from 0.044 – 0.515, these results demonstrate that the most influential results were found with the anti-LC IgG 106 ribotype from all responses to the antigens.

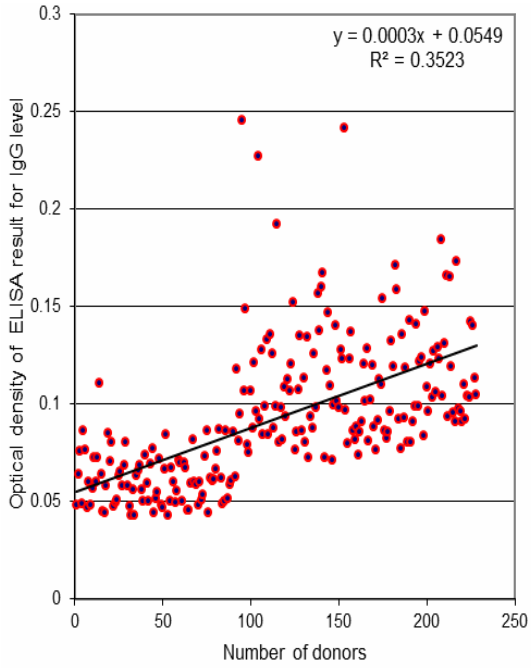

Figure 5.VIa: Anti- 001SLPs IgG distribution

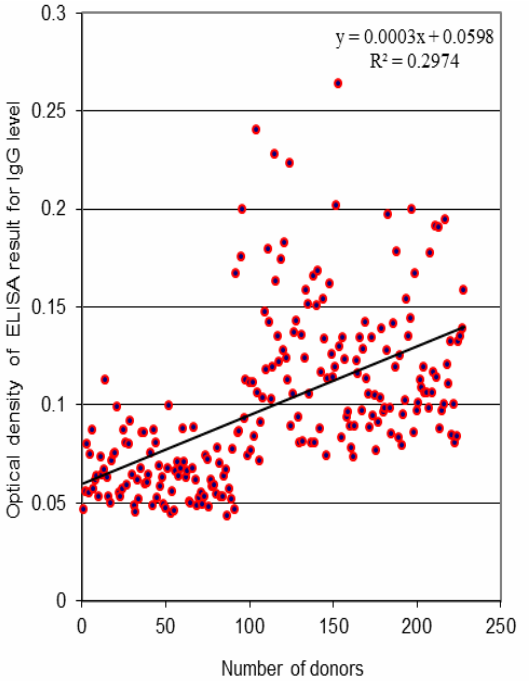

Figure 5.VIb:Anti- 002 SLPsIgG distribution

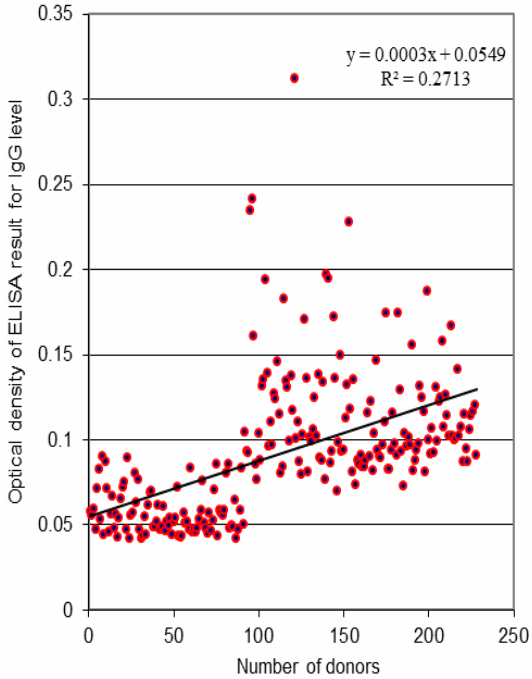

Figure5.VIc:Anti-027 ribotype SLPs IgG distribution

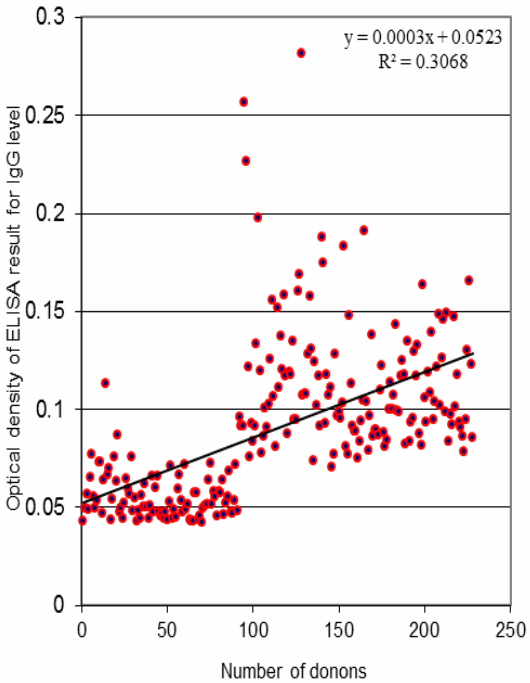

Figure 5.VId: Anti-078 SLPs IgG distribution

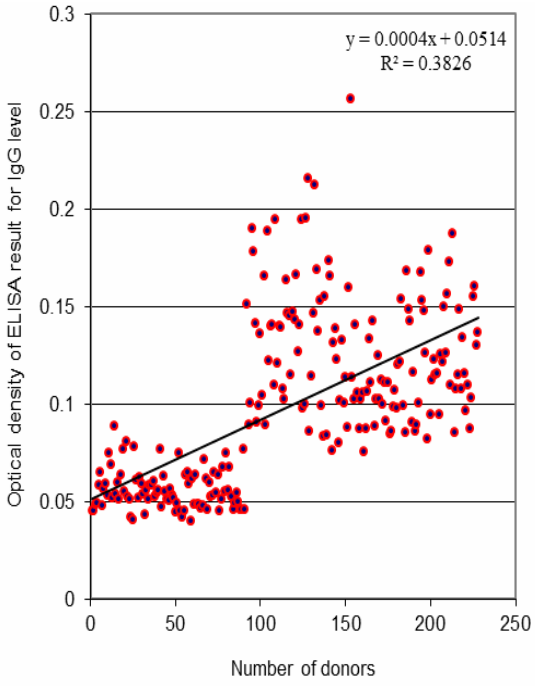

Figure 5.VIe: Anti-106 SLPs IgG distribution

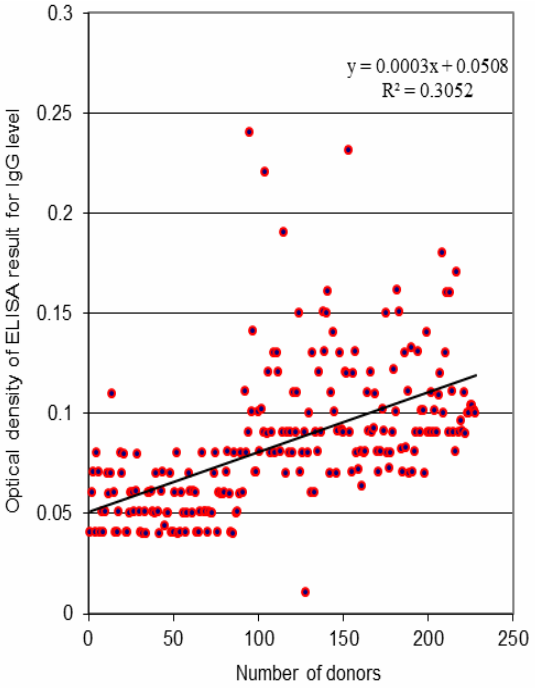

Figure 5.VII:Anti-flagellar 001 IgG distrbution

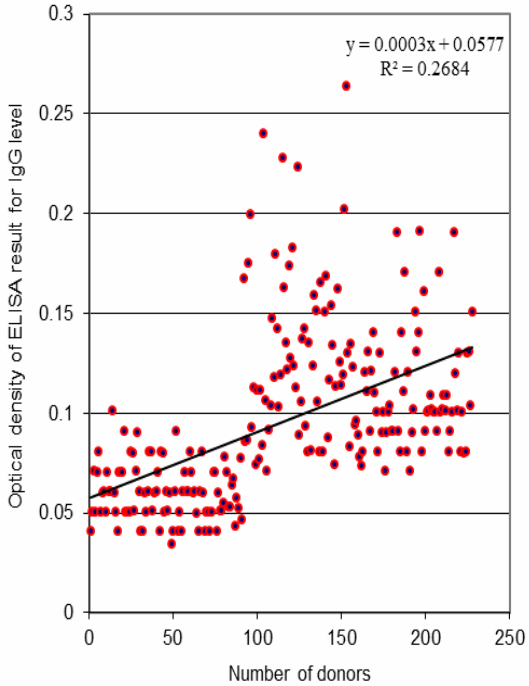

Figure 5.VIIb:Anti flagellar 002IgG distrbution

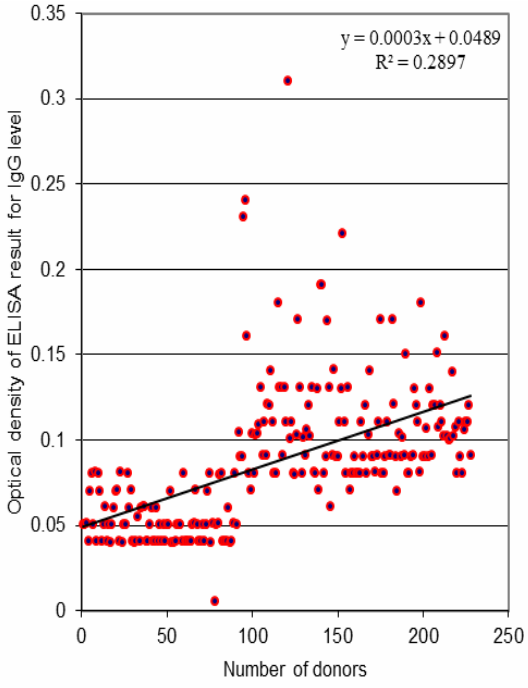

Figure 5.VIIIc:Anti- 027 flagellar IgG distrbution

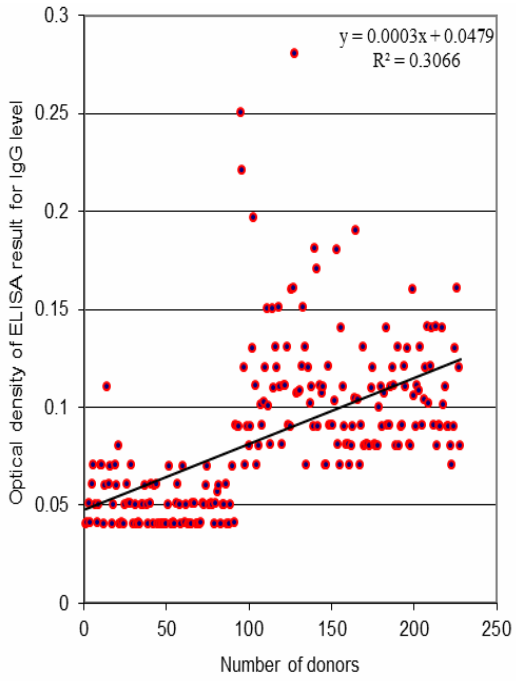

Figure 5.VIId: Anti- 078 flagellar IgG distrbution

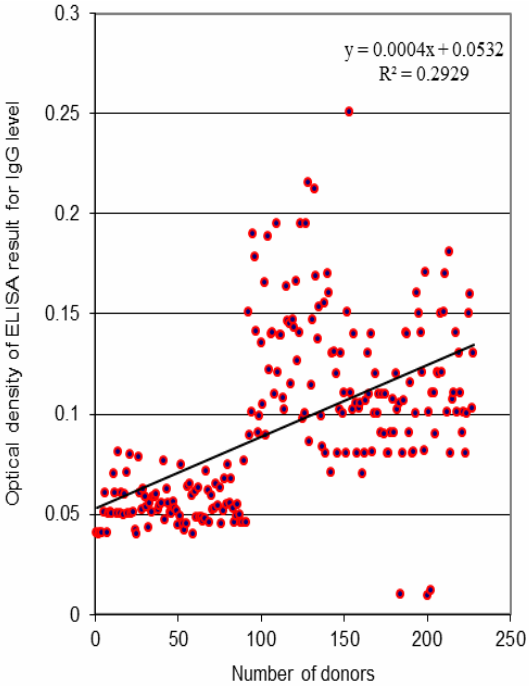

Figure 5.VIIe: Anti-106 flagellar IgG

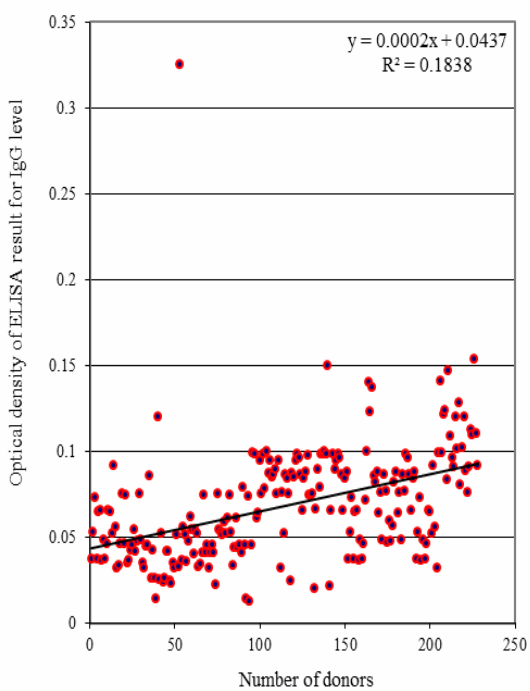

Figure 5.VIIIa: Anti-001LC IgG distribution

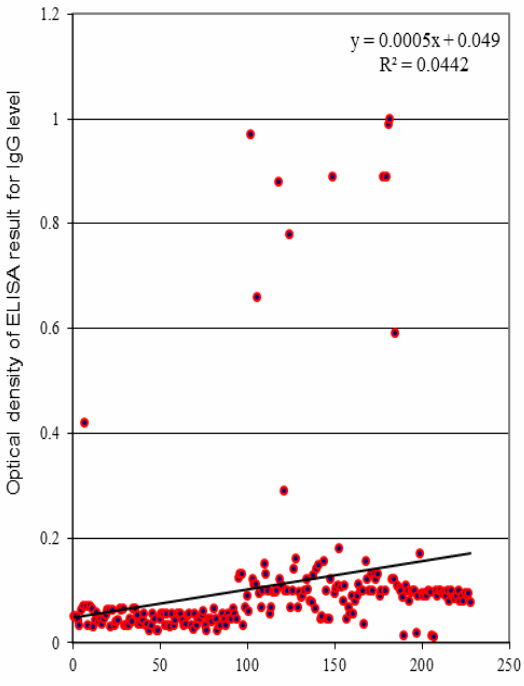

Figure 5.VIIIb: Anti-002 LC IgG distribution

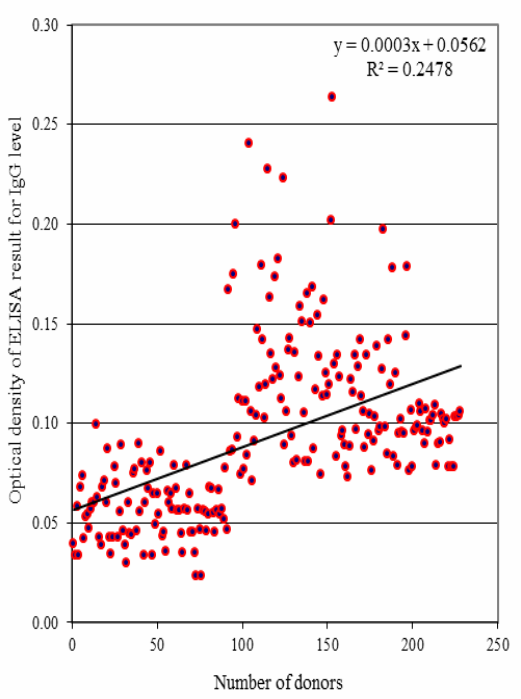

Figure 5.VIIIc: Anti-027 LC IgG distribution

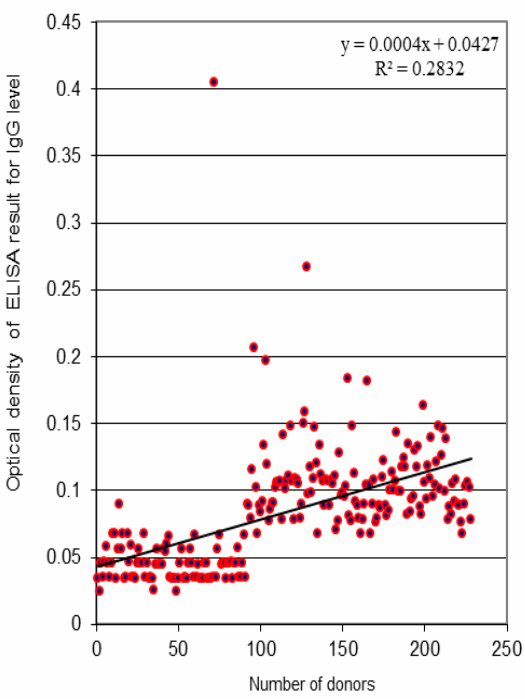

Figure 5.VIIId: Anti-078 LC IgG distribution

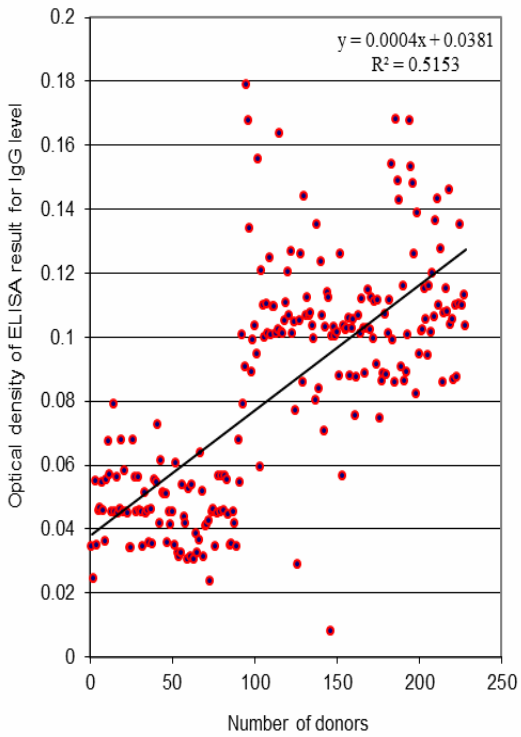

Figure 5.VIIIe: Anti-106 LC IgG distribution

5.3.4.1 Anti-SLP IgG level distribution in donors:

Figures 5.VIa-5.Vi show that the anti-SLPs for ribotype 106 values had the highest R^2 value (0.38), whilst the lowest R^2 assessed was for anti-SLPs 027 ribotype with 0.27.

5.3.4.2 Anti-flagellar IgG level distribution in donors:

Data in figures 5.VIIa-5.VIIe characterise that the anti-flagellar IgG level for ribotype 078 has the highest R^2 value (0.31), whilst ribotype 002 had the smallest (0.27).

5.3.4.3 Anti-LC IgG level distribution:

The statistics in Figures 5.VIIIa-5.VIIIe show that there was a high degree of variation in R^2 values, whereas R^2 for IgG against LC from ribotype 106 distribution reached a peak of 0.52. Conversely, for anti-002 LC IgG levels R^2 fell to 0.04.

5.3.6 Comparison of immune response of patients, carriers, donors and controls to SLPs, LC and flagellar proteins from five dominant ribotypes:

The serum samples used in this study were divided into four groups: symptomatic patients, carriers, controls and community donors, as described in Section 5.2.5.

The averages of anti-IgG were compared, as shown in Figures 5.IX, 5.X and 5.XI. These graphs data show that donor samples had the lowest degree of variation (0.76-0.98) for the three antigens of five ribotypes, followed by the serum samples from the control group. For the symptomatic patients, the corresponding figures were between 0.1 - 0.219, more than five times the range observed in the donor samples.

The highest average value occurred in symptomatic samples due to the three types of antigens: SLPs, LC and flagellar proteins. Statistical analysis of the differences in the means in symptomatic cases and donors indicated there was a significant difference between them (P=0.0345; degree of freedom D.F. = 5) as well with controls. On the other hand, the differences between symptomatic patients and carriers were not considered to be statistically significant (P= 0.73). The error bars correspond to the standard error of the mean (SEM).

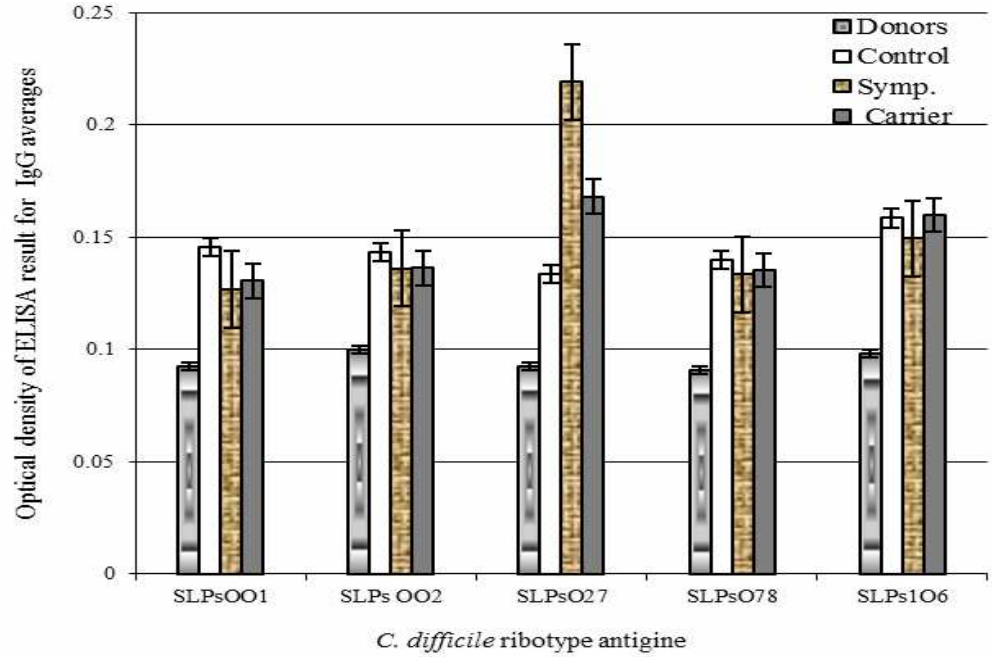

Figure 5.IX : Anti-SLPs IgG from different ribotypes in serum

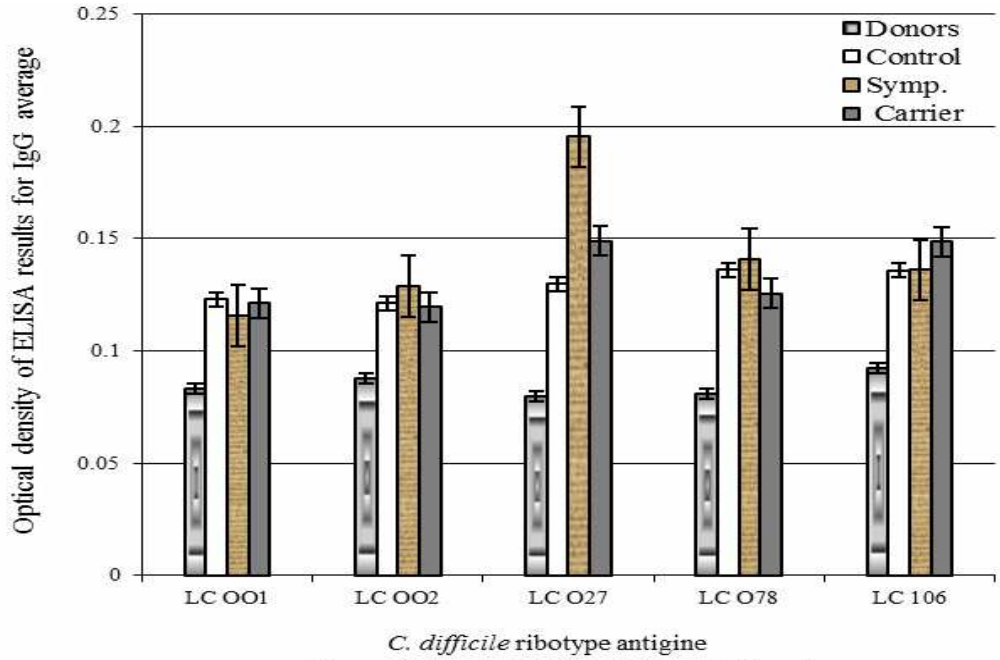

Figure 5.X : Anti-LC IgG from different ribotypes in serum

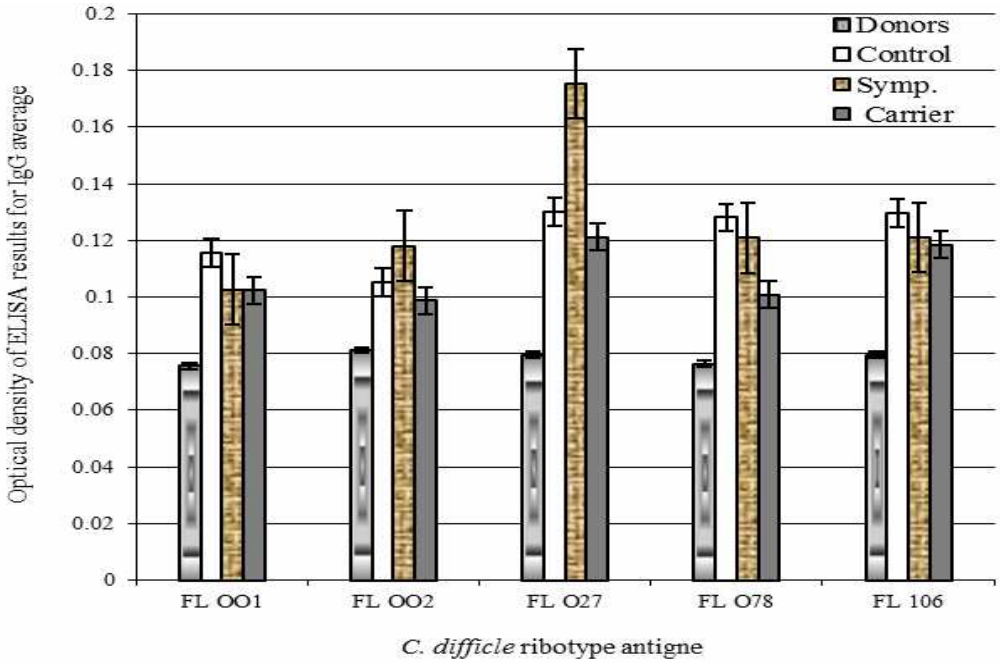

Figure 5.XI : Anti-flagellar proteins IgG from different ribotypes in serum

5.4 Discussion:

This study aimed to extract three antigens from the cell wall and flagellar proteins of the four predominant *C. difficile* ribotypes in South Eastern Scotland along with 027. The objective was to determine the suggestion that the *C. difficile* cell wall molecules were involve in systemic immune responses or not, by estimating the IgG level against these antigens in the people in the community in Edinburgh and in hospitalised individuals. It also aimed to determine the immunogenic components from these three antigens and the relation between cell wall antigens and virulence and to begin to examine the hypothesis that recurrent infection occurs due to a lack of antibody responses .

The three antigens, SLPs, LC and flagellar proteins were found to exhibit immunogenic activities at different levels dependent on the type of antigen and the source.

O'Brien *et al*., (2005) claimed that anti-SLP serum was implicated in the protection of hamsters during lethal exposure to *C. difficile* infection. Kyne *et al*., (2001) recorded that the reduction in recurrent CDI in humans was associated with IgM anti-SLPs. SLPs are probably involved in the *C. difficile* colonisation mechanism in the host bowel and are the main immunogenic component of the *C. difficile* surface cell wall, which can be used in vaccination (Spigaglia *et al*., 2011). Calabi *et al*., (2002) suggested that SLPs have a role in microbial adhesion to epithelial cells and may lead to epithelial inflammation. Wright *et al*., (2007) argued that SLPs functioned as

immunodominant antigens when serum from six patients infected by *C. difficile* 078 ribotypes against SLPs was analysed. Sanchez-Hurtado *et al*., (2008) claimed that the presence of IgG antibodies against SLPs was high in CDI cases compared to asymptomatic and control patients in their study.

The results of this study were corroborated with previous results for SLPs, as immune reactions for all ribotypes-hypervirulent and non-hypervirulent – were found, but at different levels. The immune response of anti-SLP IgG level for 027 were considerably higher in symptomatic serum compared to carrier, control and donor samples with a coefficient of variation (CV) of 0.0135. This outcome did not support Kyne *et al*. (2001) hypothesis, which states that patients colonised by *C. difficile* without associated diarrhoea developed a higher level of antibodies than did symptomatic patients.

One of applications of the immune response studies against SLPs was preventing the host from developing CDI or at least decreasing colonisation by vaccination using SLPs (Kelly and Kyne, 2011; Sandolo *et al*., 2011; Péchiné *et al*., 2011).

Research on the host immune response to flagellar antigens does not yet provide a full understanding of colonisation, pathogenicity and CDI prevention. Tasteyre *et al*., (2001) claim that the flagellated *C. difficile* isolates have the ability to adhere to the bowel mucus tissue of germ-free mice ten times more than non-flagellated strains, which is attributed to the flagella involved in bacterial adhesion to the mucus layer in vivo.

Péchiné *et al*., (2005) also examined *C. difficile* flagella proteins antibodies during the infection, the antibodies were at detectable levels 14 days after diagnosis of CDI. Furthermore, immunising with flagellar proteins in the mouse model, in combination with other surface antigens, was effective when inoculated by the rectal route and then exposing to a hypervirulent *C. difficile* strain. Colonisation was considerably lower in immunised mice compared to controls which received a PBS injection.

Conversely, Dingle *et al*., (2011) rejected the hypotheses concerning the role of flagellar proteins in adherence and virulence when exposed to Caco-2 and epithelial cells *in vitro*. The wild-type strain *C. difficile* 630 was found to have lower adherence compared to mutant strains. As the mutant strains were more virulent in the hamster module, it was suggested that the flagellar proteins did not affect a strain's adherence and virulence. Dingle *et a*l., (2011) propose that suppression of motility is a form of pathogenic strategy for CDI.

The anti-flagellar IgG levels for the five most frequent ribotypes in Scotland were detected by ELISA after exposing to serum from symptomatic, asymptomatic carriers, controls and donors to *C. difficile* flagella proteins. The results indicate that the anti-flagellar IgG distribution in healthy donors was in an almost normal mode and was at a lower level compared to averages of SLPs for four ribotypes, with the exception of 027 ribotypes, which had the highest figures. It was also significantly high for 027 and 002 ribotypes in symptomatic patients compared to carriers,

controls and healthy donors with a coefficient of variation (CV) of 0.179. This may reflect the immunogenicity of flagellar proteins from 027 ribotypes.

Poxton and Cartmill (1981) noted that the lipocarbohydrate polymers present in the *C. difficile* cell wall expressed immunogenic activity. Sanchez-Hurtado and Poxton (2008) noted that the combination of EDTA extract, crude LC with a suboptimal level of toxin had a considerably improved cytotoxic activity on Caco-2 cells and on cells from the Vero cell line *in vitro*. Sanchez-Hurtado *et al*., (2008) investigated anti-LC IgG levels in the serum of symptomatic patients, carriers and control individuals using LC from endemic 001 ribotype and toxinotype 0. Results showed anti-LC IgG level in the controls was higher than in symptomatic samples and non-symptomatic carriers.

Lipocarbohydrate LC antigens from the five endemic *C. difficile* ribotypes in Scotland were challenged to serum samples from Edinburgh residents and hospitalised patients as well as controls to assess the anti-LC IgG levels and compare these to anti-SLPs IgG and anti-flagellar IgG. The amount of anti-LC IgG in donor serum was lower than IgG for SLPs and flagellar proteins from all five ribotypes, although LC from ribotypes 027, 078 and 106 was about twice as high as anti-LC of 001 and 002 ribotypes. For anti-LC IgG from 001 ribotype, similar results to Sanchez-Hurtado *et al*., (2008) were obtained. For LC ribotype 027, the symptomatic serum samples were the highest, with a significant variation in anti-LC IgG levels compared to serum from other groups of individuals. The effect of LC varied

between ribotypes. The symptomatic serum response led to suggest that the LC may have a role in aspects of virulence of the strain and its pathogenicity.

Recognition of the relationship between the lack of production of specific antibodies after initial infection and recurrences was suggested by Drudy *et al*., (2004). The lower level of IgG in community individuals, controls and carriers compared to symptomatic patients does not indicate that production of antibodies has been disabled in these cases, but rather that the pre-existing level of antibodies in normal healthy donors and carriers is normally sufficient to allow the host to protect itself until it can resume the production of specific IgG antibodies after exposure to aetiologic agents.

The weakness in the immune response of elderly people and immunocompromised patients was reflected in their being the most common hosts of CDI, mainly because their immune systems were worn out as a result of ageing and severe or chronic illness.

The paucity of information available about the bowel microbiota in the cases used in the study, as well as patients' history, namely whether or not they had experienced recurrent cases of CDI or had undergone other treatments or operations that may have disrupted the immune system, should also be borne in mind and could clarify the incompatibility with some studies. On the other hand, the sample size may have affected the statistical analysis of results.

To conclude, the relationship between host immune system and the appearance of illness requires further investigation. Immune responses to *C. difficile* cell wall components in normal healthy individuals and patients in this study indicate the immunogenicity of SLPs, LC and flagellar proteins. SLPs from the hypervirulent ribotype 027 had had the highest immune response, as did the LCs and flagellar proteins, but with lower levels, indicating the relationship with virulence. The hypothesis of recurrent infection due a shortage of antibodies was not confirmed in this study as immune responses to non-hypervirulent strains varied between symptomatic cases and carriers. However, this hypothesis does not account for the 027 ribotype which is very uncommon in SE Scotland.

CHAPTER VI INFLUENCE OF *C. difficile* ANTIGENS ON LEUKOCYTES

6.1 Introduction:

As stated in chapter I, the disease symptoms caused by *C. difficile* are extremely wide-ranging. These signs range from mild symptoms to severe illness with difficult-to-treat complications, and may be life threatening in compromised people.

One of the primary barriers in the bowel is the innate immune system. The innate immune cells require immunogenic molecules for recognition and activation. The detection of foreign molecules by the presence of the mucosal layer in the bowel comprises three steps: the creation of an antimicrobial response, the stimulation of the epithelial cells and the activation of immune cells.

This chapter is a comparative investigation of six cytokine levels which are enhanced by three antigens from five dominant *C. difficile* ribotypes in Scotland. The cell line was for in-vitro experiments and the cell wall antigen activities were compared to LPS for the production of cytokines.

The aim of this study was to clarify the immunogenicity of the three antigens (SLPs, LC and flagella) from the dominant *C. difficile* strains, as well as assessing their effectiveness in the innate immunity by stimulating the THP-1 cell line to produce cytokines, and comparing their results to LPS as a control. The production levels of six cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12) were estimated by ELISA, and verified which antigen was hyper immunogenic by stimulating cytokine

secretion. In addition, an attempt was made to clarify the role of these antigens in innate human immunity.

6.1.1 Microbiota and the role of cytokines in controlling innate immunity:

The human bowel is able to be colonised by an enormous number of microorganisms. The normal relationship between the host and the microbes is either symbiotic or commensal on the area of contact, but it may convert to pathogenicity.

Caio *et al*. (2012) suggested that the symbiotic microorganisms have a role in the balance of the microbiota and in supporting the host via many functions. The symbiotic microbes were able to change the innate immune system from an inactive anti-inflammation level condition to an active position by continuous signalling from microbiota inhabitants. This causes the innate immune cells to move and respond rapidly to invasive aetiologic or damaged tissue indicators.

The quick-recruitment innate immune lines are essential for an effective human response to gastrointestinal pathogens. The damaged epithelial tissues include antigenic molecules of the pathogen. After detecting the immunogenic molecules through cellular immunity in the bowel, pro-inflammatory mediators like cytokines are rapidly discharged, which is the primary response to infection. These cytokines play a role in inducing antimicrobial factors and activating immune cells. The pro-inflammatory and regulatory mediators include TNF-α, IL-1-β, IL-6, IL-8, IL-10 and IL-12, which are secreted from leukocytes (Kinnebrew and Pamer, 2012; Lin and Karin, 2007).

6.1.2 Interleukin -β (IL-1-β):

Cytokine protein (IL-1-β) is known as catabolin. The macrophage is the producer of IL-1β preprotein, and is then activated as active cytokine. IL-1β is involved in many cellular activities, such as cell proliferation, differentiation, apoptosis and inflammatory pain hypersensitivity (Pesce *et al*., 2011).

6.1.3 Interleukin 6 (IL-6):

IL-6 has multiple effects, as it is responsible for stimulating acute phase protein synthesis, and stimulates the production of neutrophils in the bone marrow. It also supports B cell growth and is antagonistic to regulatory T cells. IL-6 is relevant to many chronic diseases, including diabetes, and is suggested to have a critical involvement in the development of colon cancer and the pathogenesis of Kaposi sarcoma (Lin and Michael, 2007; Kristiansen and Mandrup-Poulsen, 2005).

6.1.4 Interleukin 8 (IL-8):

IL-8 is a chemokine produced by macrophages and other cell types, such as epithelial and endothelial cells. The endothelial cells store IL-8 in their storage vesicles. The essential role of IL-8 is the induction of chemotaxis such as neutrophils and granulocytes in its target cells, as well as attracting neutrophils to the site of inflammation (Wolff *et al*., 1998).

6.1.5 Interleukin 10 (IL-10):

The effect of IL-10 is immunosuppressive and anti-inflammatory activity, in significant contrast to IL-6. IL-10 inhibits NF-kB activation through ill-defined mechanisms, and as a result inhibits the production of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-12. Consequently, the other role of IL-10 is the inhibition of tumour development and progression, as suggested by Lin and Karin (2007).

6.1.6 Interleukin 12 (IL-12):

IL-12 is a pro-inflammatory heterodimeric cytokine. The dendritic cells (DC), macrophages and human B-lymphoblastoid cells were the creator of IL-12. IL-12 plays an essential role in the activities of natural killer (NK) cells and T lymphocytes, as well as mediating the enhancement of cytotoxic activity. Its other function is the stimulation of the production of interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) from T and natural killer (NK) cells (Hamza *et al*., 2010).

6.1.7 Tumour necrosis factor-alpha (TNF-α):

TNF-α is a cytokine produced by activated macrophages, inflammatory cells and tumour cells. It has a role in systemic inflammation and in stimulating acute phase reactions, and its essential role is the organisation of immune cells. The ability to induce fever and sepsis has been suggested as a TNF function, especially in chronic cases such as inflammatory bowel disease (IBD) (Brynskov *et al*., 2002; Lin and Karin, 2007).

6.1.8 The core of *E. coli* LPS:

Many immunological studies have used *E. coli* LPS as a control antigen because it is readily obtainable and due to its immunogenicity. LPS contains three parts: Lipid A (the endotoxic part), a core oligosaccharide and O-polysaccharide, which is the important antigenic component and is extremely variable. In contrast the Lipid A component is the major biologically active part and is limited in structure and conserved. However, the antibody response acts against oligosaccharide and O-polysaccharide together (Gibbs *et al*., 2004), while the inflammatory response is due to Lipid A.

6.2 Materials and Methods:

6.2.1 The sandwich enzyme-linked immunosorbent assay (ELISA):

The ELISA procedure, following the technique outlined in Section 2.4.1, is summarised in Table 6.1.

Well contents	Antigen	Antigen diluent	Serum	Serum diluent	Conjugate	Substrate
Blank (B)	-	+	-	+	+	+
Antigen control (Ag)	+	-	-	+	+	+
Serum control (S)	-	+	+	-	+	+
Test (T)	+	-	+	-	+	+

Table 6.1: Summary of the ELISA procedure

6.2.2 THP-1 cell line:

The THP-1 cell line was used, as described in Section 2.5, to estimate the activity of *C. difficile* on this part of the immune system. PMA (50ng/ml) was added as a supplement to induce differentiation.

6.2.3 Exposure of THP-1 to *C. difficile* antigens:

The challenge procedure to cell wall antigens which is described in Section 2.6.3 was followed to enhance the THP-1 cell line in order to produce cytokines after induction with *C. difficile* antigens.

6.2.4 Assessment and evaluation of cytokines:

Primary and secondary antibodies dilutions, as well as standard cytokine preparations, were followed as in Section 2.6.4. The steps in the Materials and Methods (Chapter II) were used to detect and evaluate the cytokine concentrations.

6.3 Results:

The in-vitro exposure of *C. difficile* antigens to leukocytes induced cytokine production. The cytokines were captured by specific anti-cytokine antibodies via the ELISA technique. The cytokine concentration (ng/ml) was evaluated by converting the optical density reading of ELISA results to ng/ml using the standard curve given for all standard cytokines used in this study, and the calculation based on a line between the points.

Three *C. difficile* antigens (SLPs, LC and flagella) from six dominant ribotypes (001, 002, 012 027, 078, 106) were used in this study, as well as *E. coli* LPS as a control. The human leukocytes cell line was challenged with these antigens.

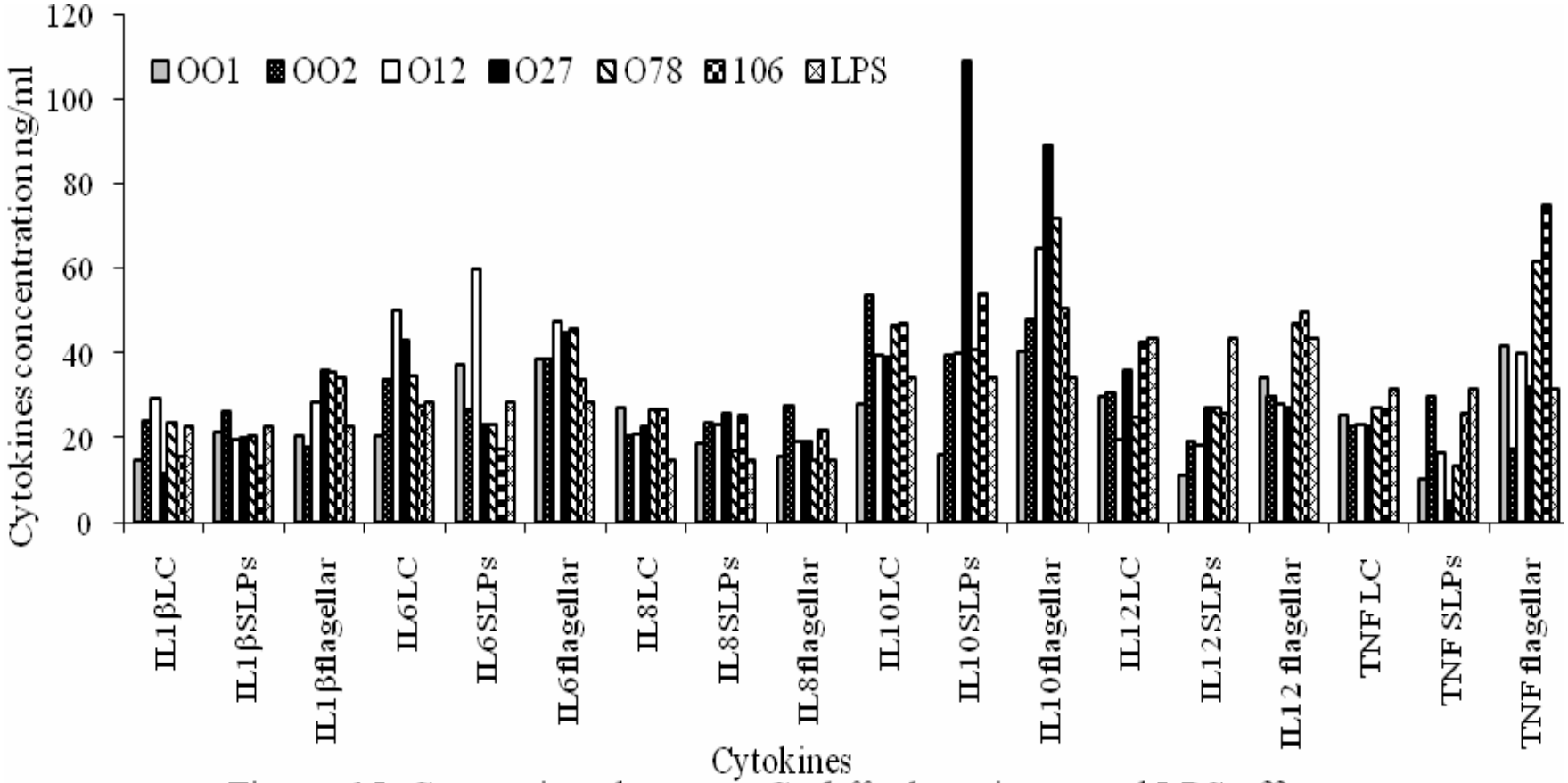

Figure 6.I: Comparison between *C. difficile* antigens and LPS effects on cytokines production

6.3.1 Innate immune response:

Figure 6.1 presents the leukocyte response when exposed to *C. difficile* antigens from six dominant ribotypes and LPS as a control by estimating cytokine production. The highest concentration of antigens was 50µg/ml, and PMA was used at 50ng/ml, which gave more detectable levels than when 10ng/ml of PMA was used.

There was a large variation between the effects of the different antigens. The cytokines induced in the leukocytes cell line ranged between 4.7 ng/ml for TNF-α from LC of 027 ribotype and 109.1 ng/ml for IL-10 after being treated by SLPs from 027 ribotype. The lowest effect of LPS was 14.5ng/ml for IL-8 secretion, and the maximum value was 43.3 ng/ml for IL-12. Generally IL-8 has the lowest level of induction. Nevertheless, TNF-α showed the smallest amount of secretion from the cell line when exposed to 027 ribotype flagellar proteins.

6.3.2 Interleukin 1-β:

The highest level of IL-1β was produced by treating with flagellar antigens of ribotypes 027, 078 and 106; generally IL-1β levels ranged from 11.14ng/ml to 35.5ng/ml, and LC and flagellar from 012 ribotype had a similar effect on IL-1β production. SLPs gave around 20ng/ml from ribotypes 001, 012 and 078, while a level of 27ng/ml was reached with 027 ribotype, dropping to 13ng/ml with 106 ribotype (see figure 6.2).

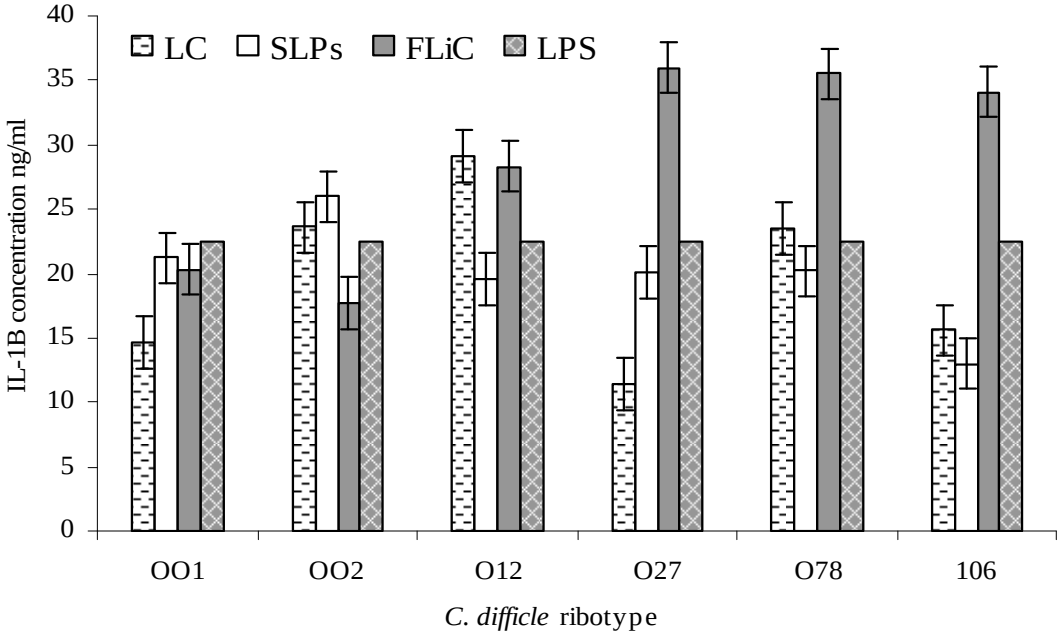

Figure 6.2: Comparison between antigens of six ribotypes for stimuating IL-β production and LPS

Figures 6.2a, 6.2b, and 6.2c present the relationship between IL-1β production and increasing LC, SLPs and flagellar concentrations of the six ribotypes. The enhancement of 5µg concentration of 027-LC on IL-β was not detectable.

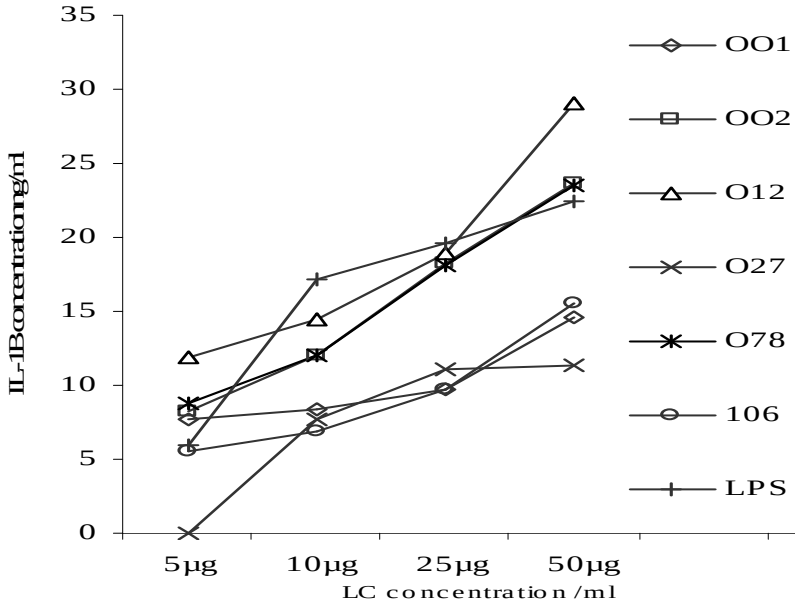

Figure 6.2a: IL-β production by LC from six ribotypes and LPS

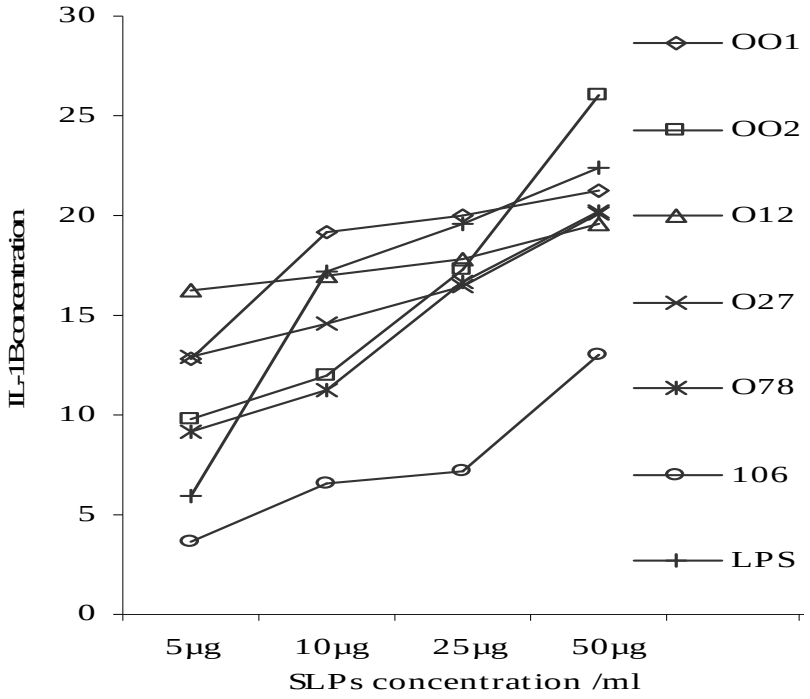

Figure 6.2b: IL-β production by SLPs from six ribotypes and LPS

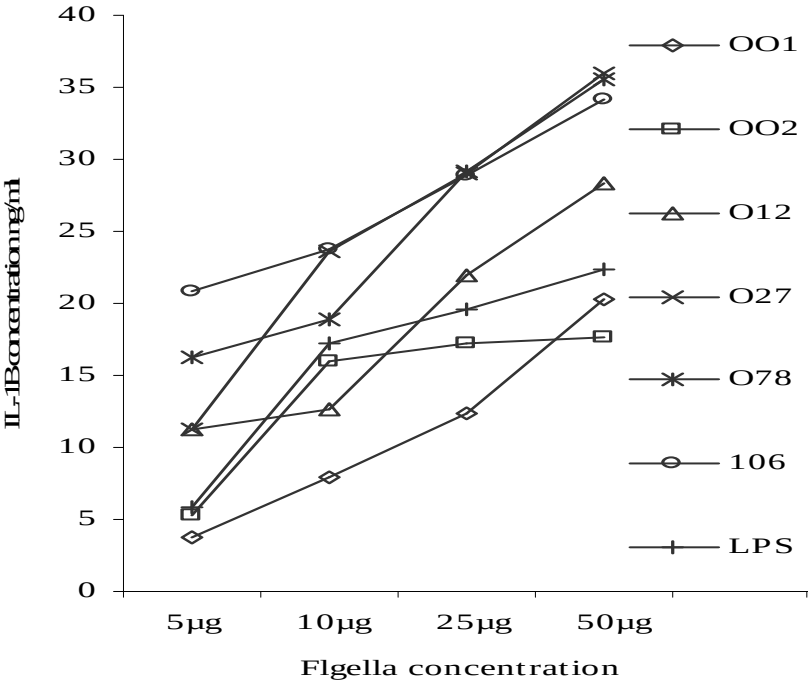

Figure 6.2c:IL-β production by flagella from six ribotype and LPS

LPS 50µg enhancement of induction of IL-1β exceeded that by SLPs from 001, 012, 027, 078 and 106 ribotypes, as well LC from 001, 027 and 106 ribotypes. Also flagellar proteins from 001 and 002 ribotypes were lower than LPS activity.

6.3.3 Interleukin 6:

Generally, IL-6 has a higher result for SLPs than IL-1β and IL-8, but there was considerable variation between the effects on the antigens from different ribotypes; 012 ribotype antigens (SLPs, LC and flagellar) consequently gave higher concentrations than the other ribotypes. On the other hand, IL-6 levels due to flagellar proteins from four ribotypes were superior to SLPs except for ribotype 012, and the IL-6 level ranged from 30ng/ml to 45ng/ml. IL-6 from LC ranged from 20ng/ml to 42ng/ml. The control LPS antigen exhibited a higher value than SLPs from 002, 027, 078 and 106 ribotypes (see figure 6.3).

Figures 6.3a, 6.3b and 6.3c illustrate the relationship between IL-6 production development and increasing LC, SLPs and flagellar concentrations of the six ribotypes. The highest amount was 50µg of antigen used to represent the highest effect on leukocytes to produce IL-6. The LC and SLPs from six ribotypes and LPS at 5µg stimulate IL-6 in the range 10-20ng/ml, but flagellar proteins from 002, 027, 078 and 106 ribotypes effect exceed 25ng/ml of IL-6. 012 antigens have the greatest effects at the concentration 50µg.

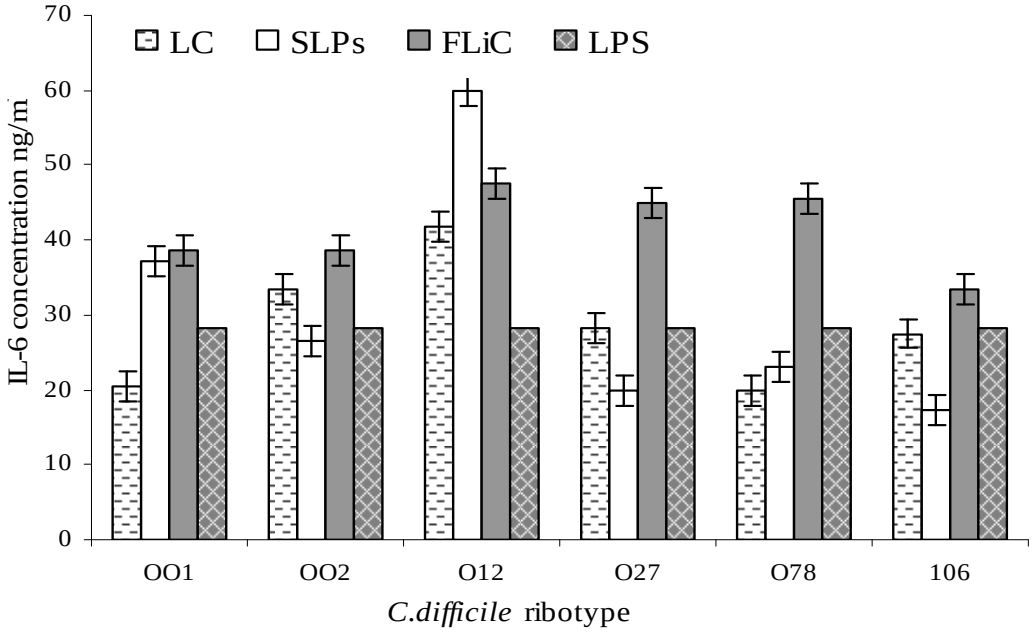

Figure 6.3: Comparison between antigens of six *C. difficile* ribotypes stimulating IL-6 production and LPS

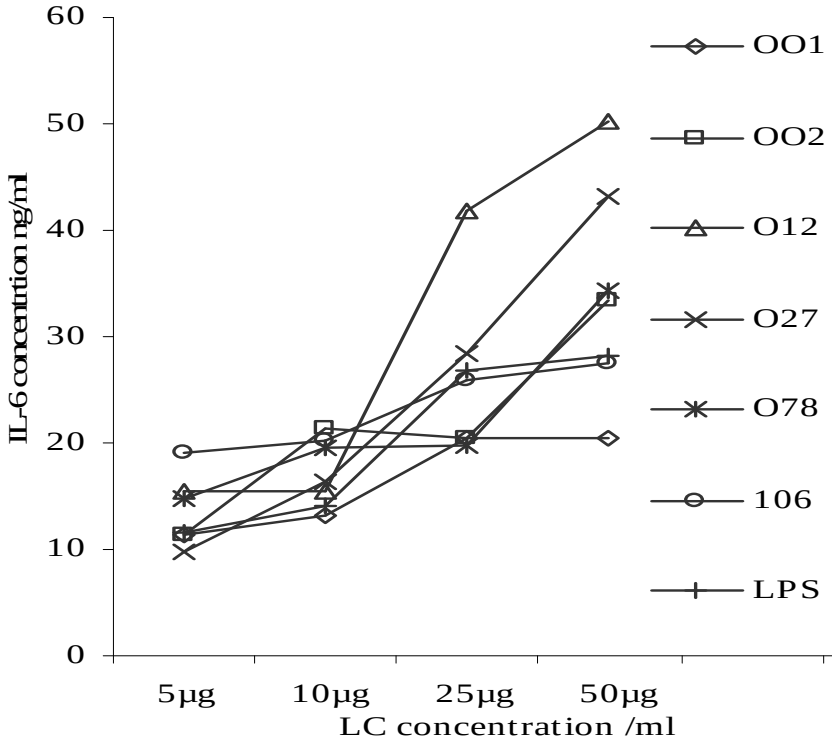

Figure 6.3a: IL-6 production by LCfrom six ribotypes and LPS

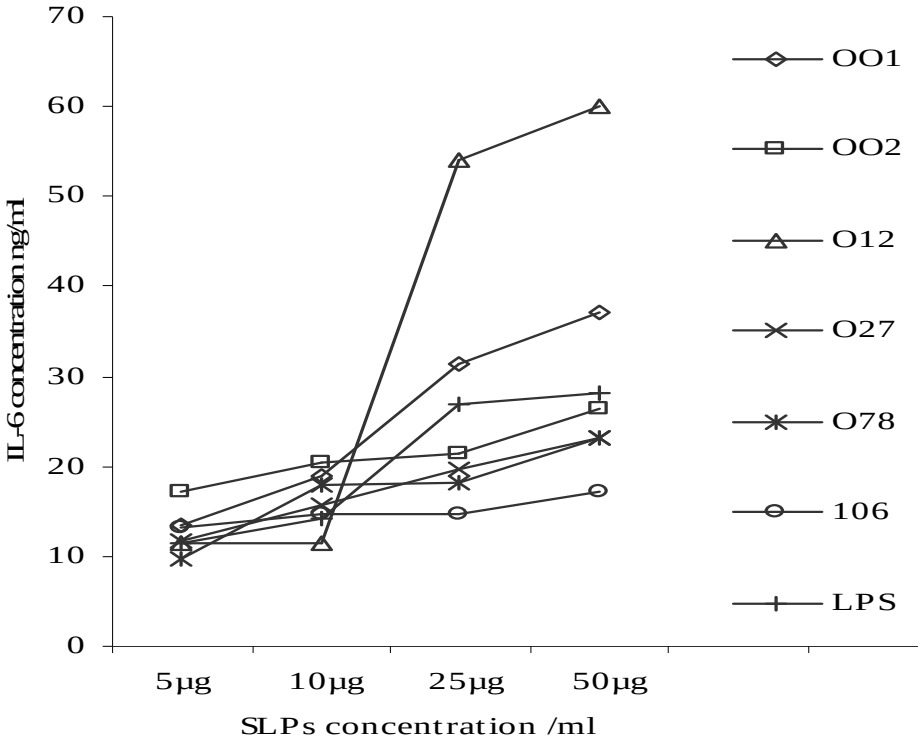

Figure 6.3b: Il-6 production by SLPs from six ribotypes and LPS

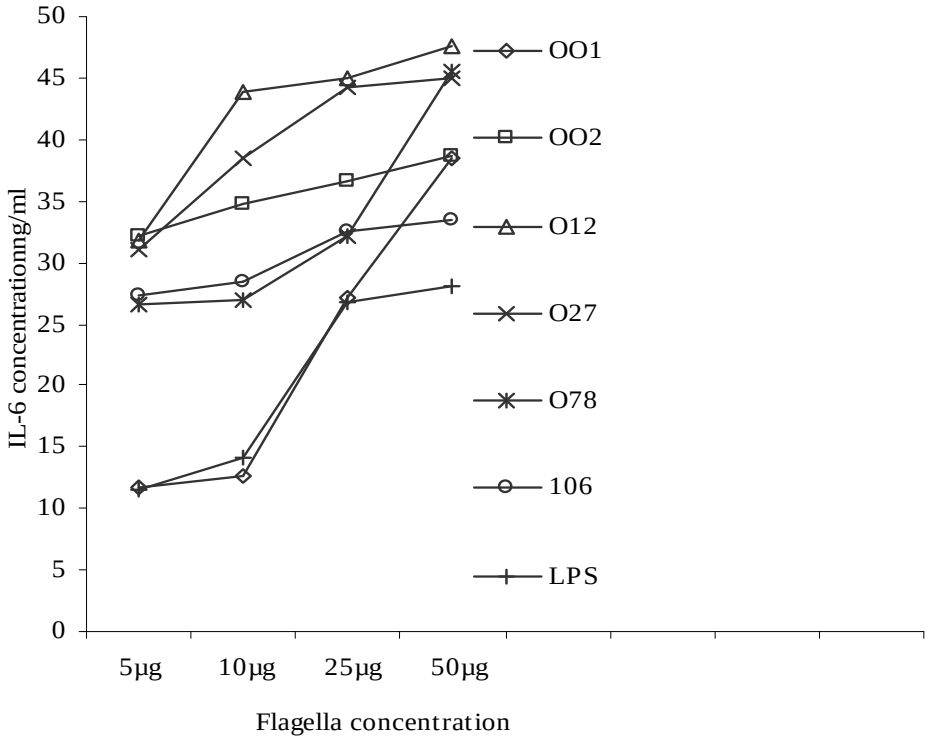

Figure 6.3c: Il6 production by flagella from six riboype and LPS

6.3.4 Interleukin 8:

The production of IL-8 was generally weak for all *C. difficile* antigens in the study as well as for the control. The largest amount of IL-8 was related to flagellar protein from 002 and 001 ribotypes by about 27ng. LPS showed the lowest influence; less than 15ng.

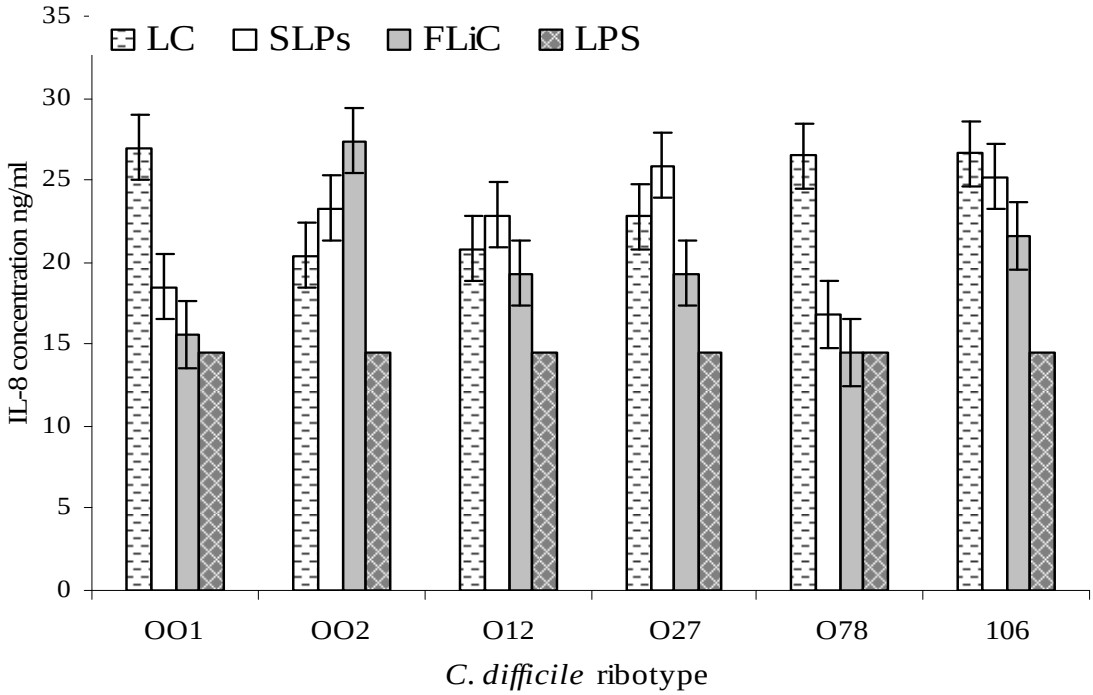

Figure 6.4: Comparison between antigens of six *C. difficile* ribotypes for stimulating IL-8 production and LPS

The relationship between concentration of antigens and cytokines production are summarised in figures 6.4a, 6.4b and 6.4c. The lower concentrations of *C. difficile* antigens effect on stimulating IL-8 remain less than 20 ng/ml and did not go beyond 30ng/ml with 50µg of antigen.

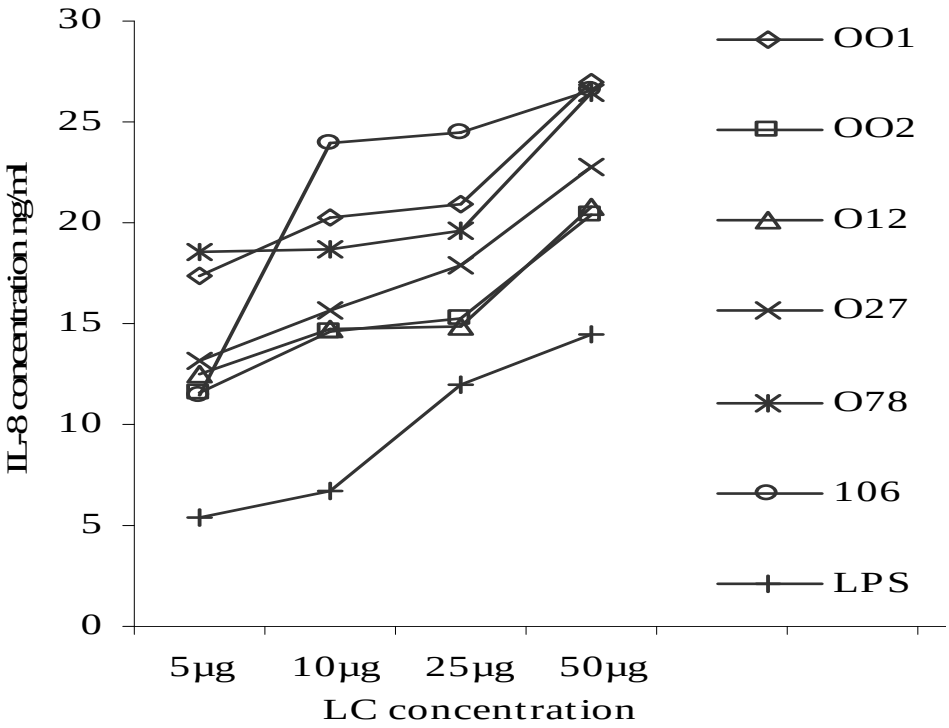

Figure6.4a: IL-8 prodction by LC from six ribotypes

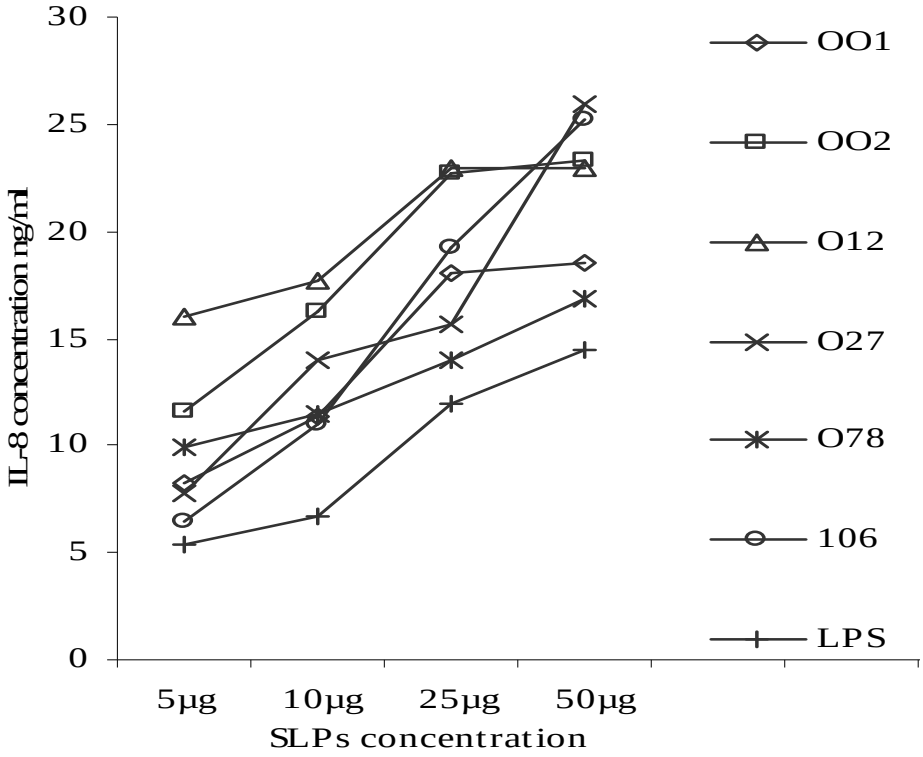

Figure6.4b: IL18 production by SLPs from six ribotypes

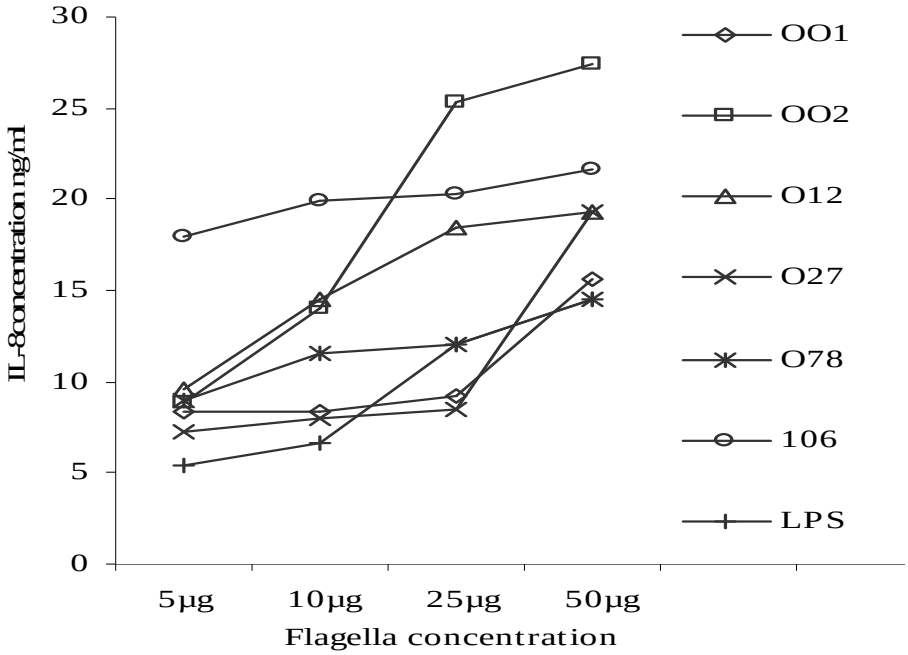

Figure 6.4c: IL-8 production by flagella from six ribotypes

6.3.5 Interleukin 10:

Interleukin-10 has the highest production level in general, and in particular was extremely sensitive to SLPs from 027 ribotype (109.1 ng/ml). However, the flagellar proteins from 027, 078 and 012 ribotypes were at the lower level for stimulation of leukocytes to induce IL-10. Although LPS enhanced leukocytes to produce about 34ng, this was still lower than all the *C. difficile* antigens except for LC and SLPs from 001 ribotype (see Figure 6.5).

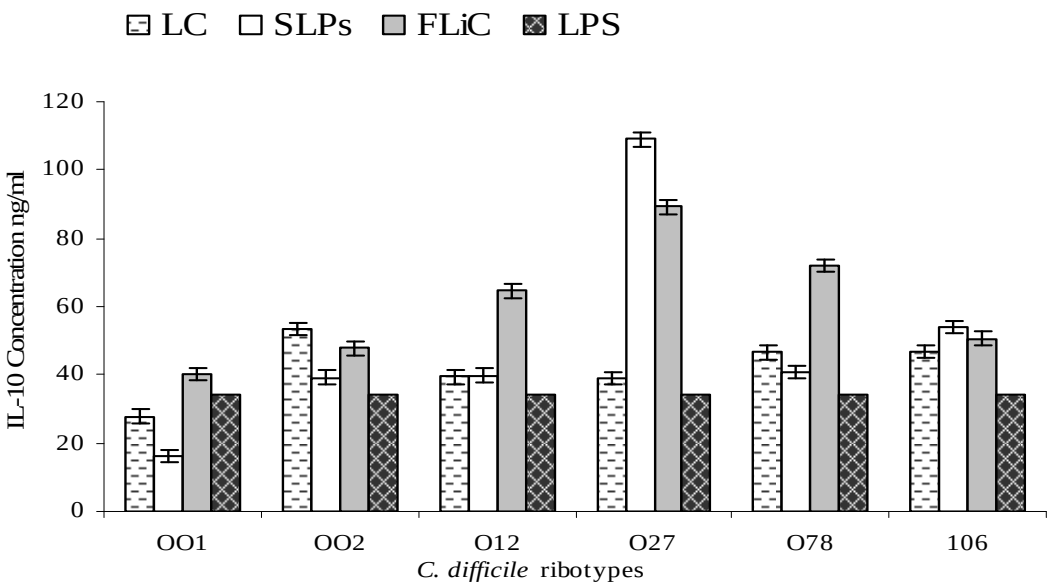

Figure 6.5: Comparison between antigens of six *C. difficile* ribotypes stimulating IL-10 production and LPS

The study were compared the effect of the concentration of *C. difficile* antigens on production IL-10. The result were shown in figures 6.5a, 6.5b and 6.5c. These figures were illustrate that the 50µg of LC from 002 ribotype stimulate IL-10 production by more than 55ng/ml, although IL-10 was not detectable when was used 5µg of LC from 002 and 001 ribotypes.

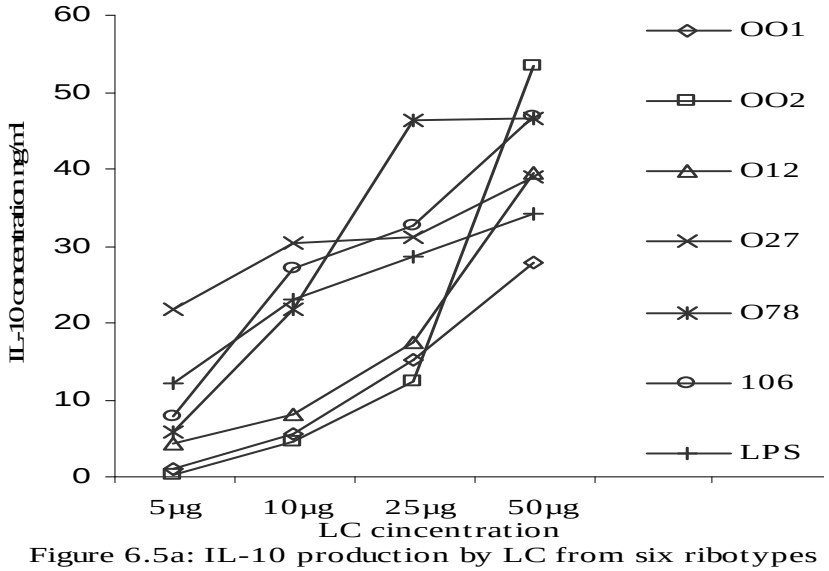

Figure 6.5a: IL-10 production by LC from six ribotypes

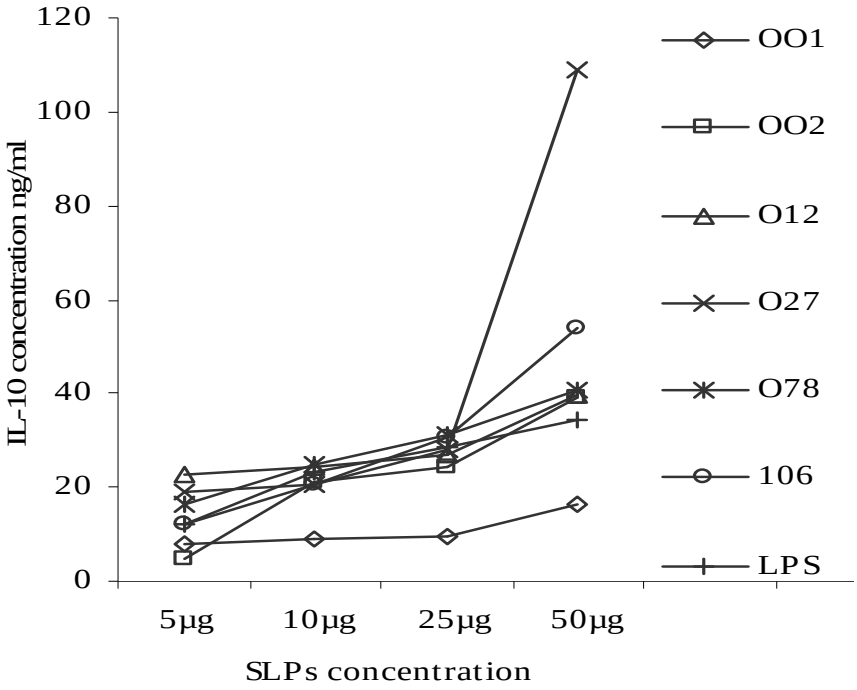

Figure 6.5b: IL-10 production by SLPs from six ribotypes

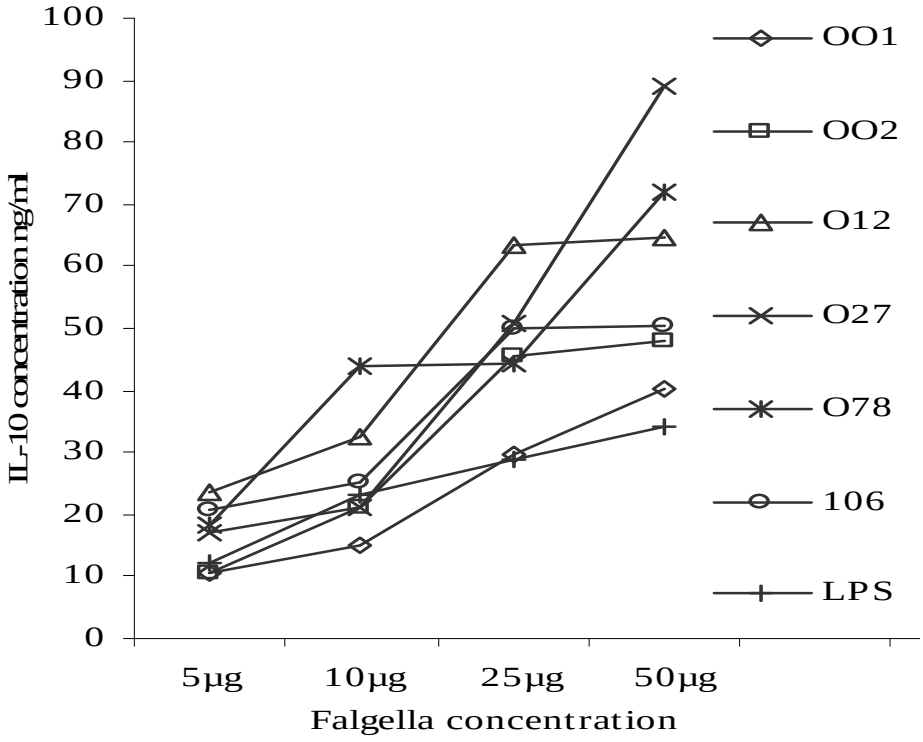

Figure 6.5c: IL10 production by flagella from six ribotypes

6.3.6 Interleukin 12:

Figure 6.6 shows that the flagellar protein antigen from ribotype 106 clearly appeared to be the best at enhancing IL-12 production, followed by ribotype 078. However, leukocytes were not sensitive to SLPs for producing IL-12. On the other hand, the highest influence of LPS was on IL-12 secretion, at 43.3 ng (Figure 6.6).

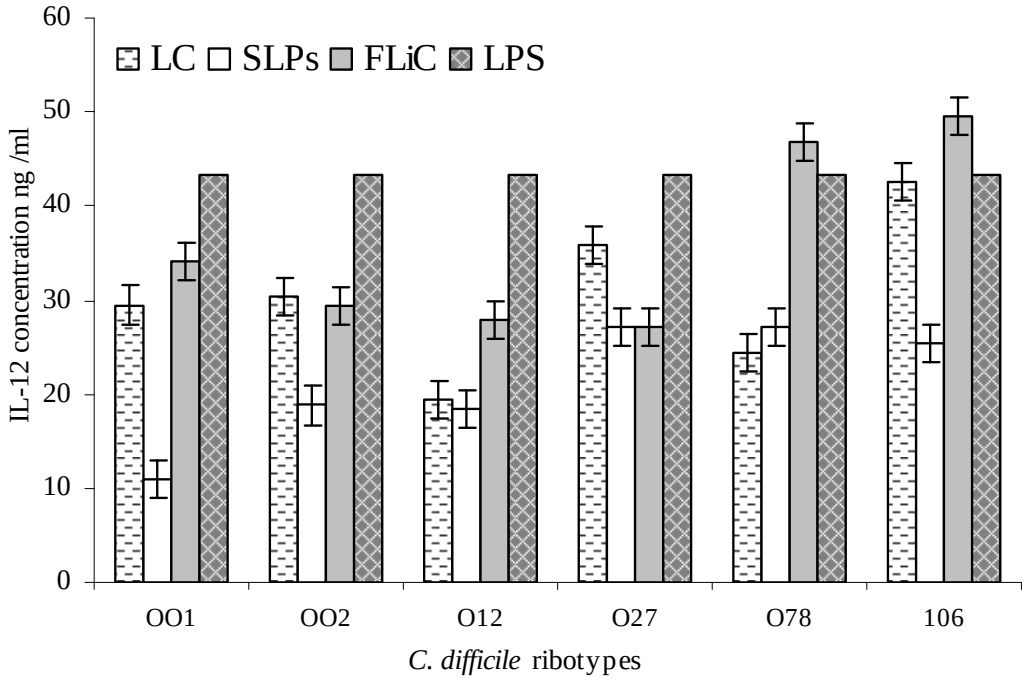

Figure 6.6: Comparison between six *C. difficile* ribotypes antigens stimulating IL-12 production and LPS

As for other interleukins the greatest stimulation was due to exposure to 50µg of antigens. However, the 5µg of SLPs from 001, 002, 012 and 078 ribotypes and also LPS were unable to enhance leukocytes to produce detectable amount of IL-12. As well flagellar proteins from 027 ribotype (see figures 6.6a, 6.6b and 6.6c).

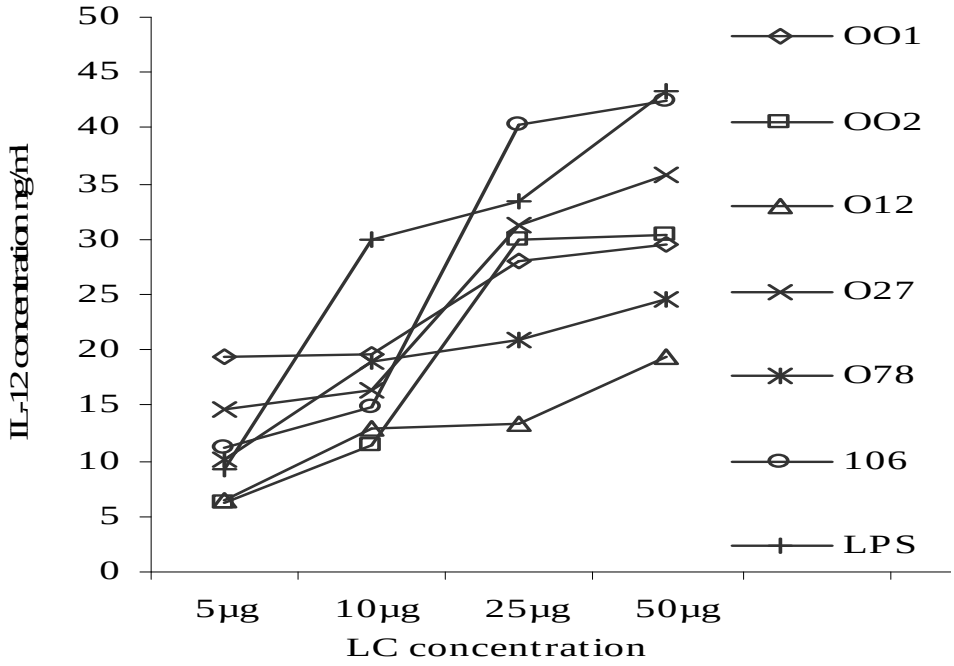

Figure 6.6a: IL-12 production by LC from six ribotypes

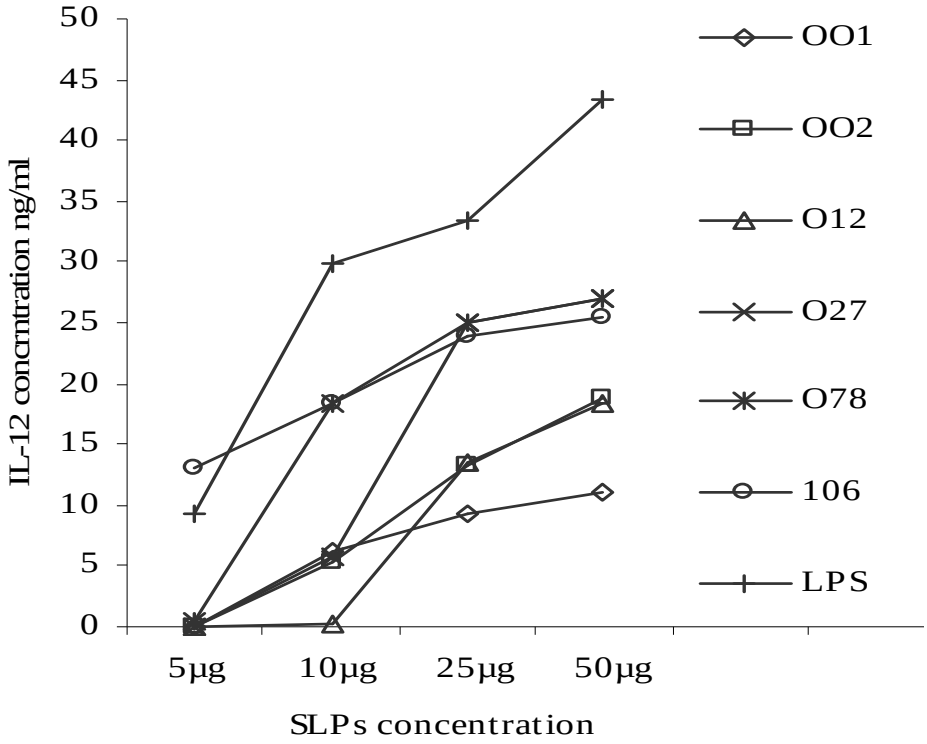

Figure 6.6.b: IL-12 production by SLPs from six ribotypes

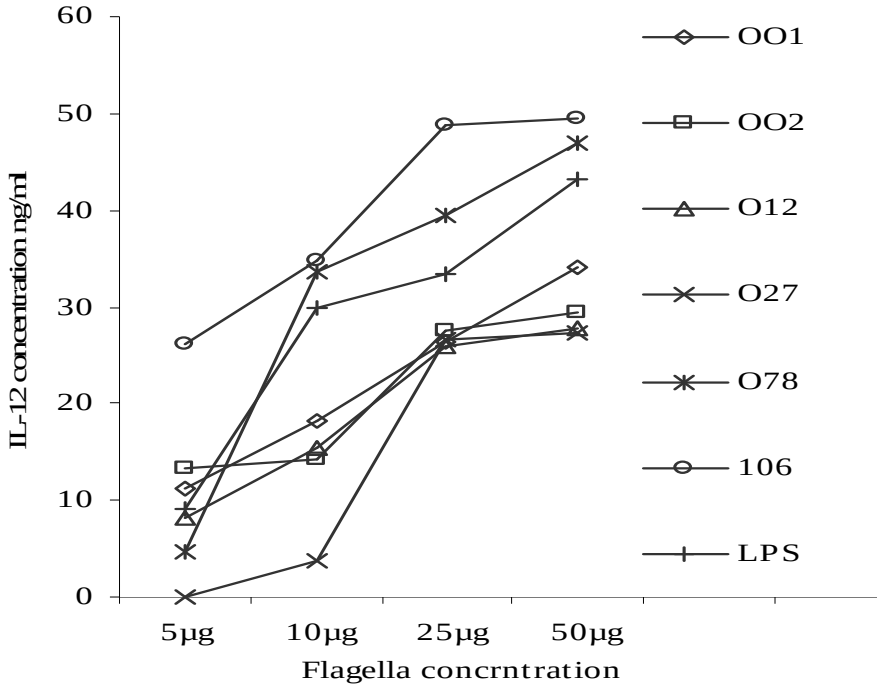

Figure 6.6c: IL-12 production by flagella from six ribotypes

6.3.7 TNF-α:

Figure 6.7 illustrates the elevated effect of flagellar protein antigens from 106, 078, 001, 012 and 027 ribotypes on leukocytes to generate TNF- α, but the LC remains at a steady level for virtually all ribotypes, and SLPs shows the lowest value, while LPS exceeds LC and SLPs at stimulating leukocytes to produce TNF-α.

TNF response to different concentrations of antigens have been similar to other interleukins, which gave the highest concentration when exposed to 50µg of antigen (see figures 6.7a, 6.7b and 6.7c).

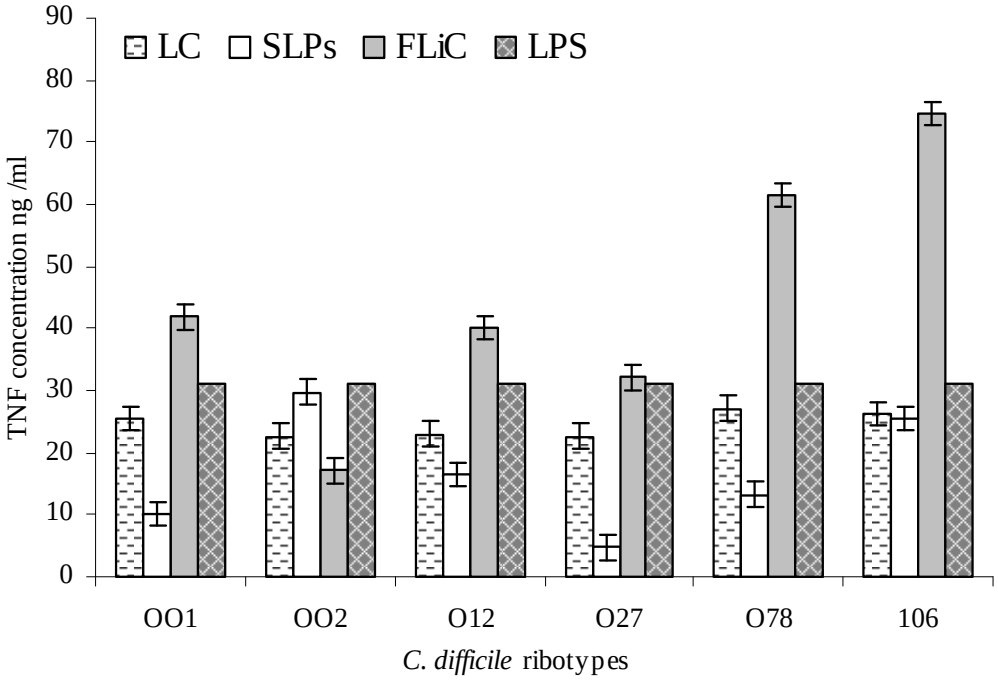

Figure 6.7: Comparison between antigens of *C. difficile* ribotypes stimulating TNF production and LPS

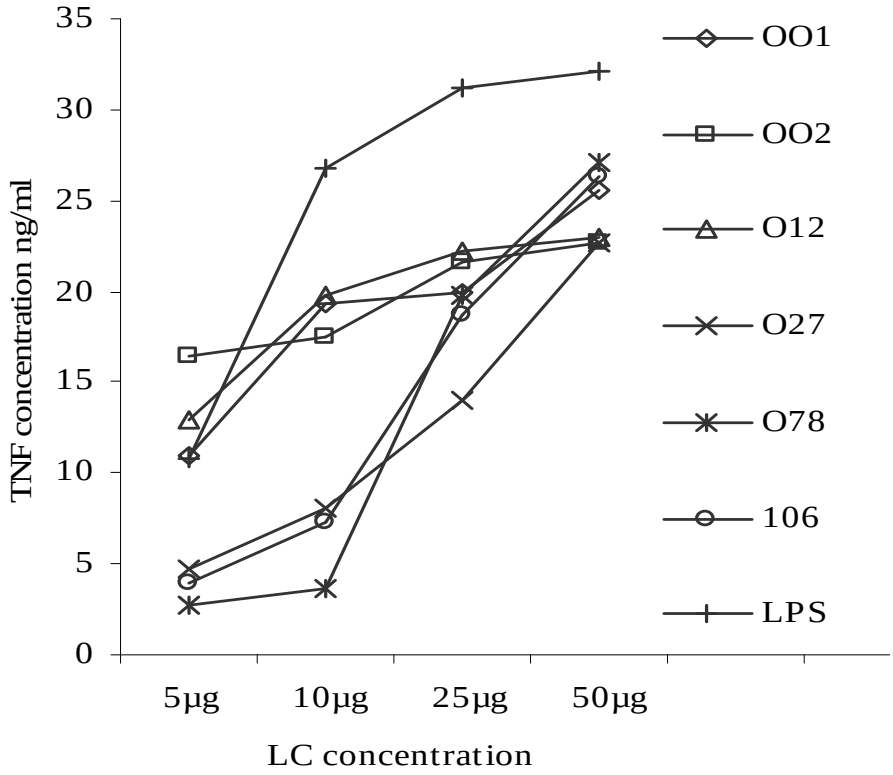

Figure 6.7a: TNF production by LC from six ribotypes

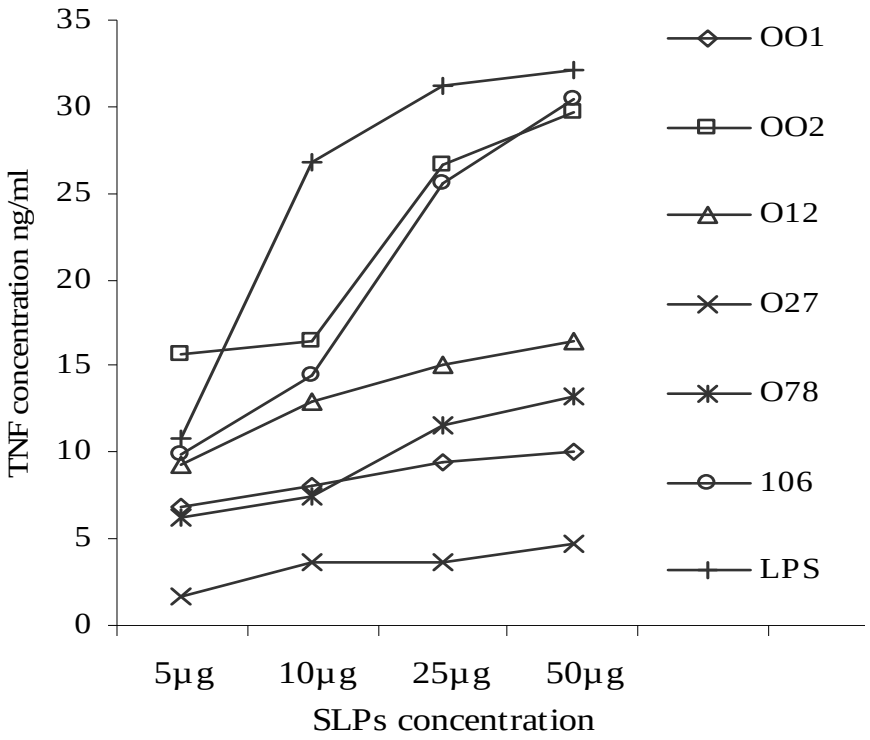

Figure 6.7b: TNF production by SLPs from six ribotypes

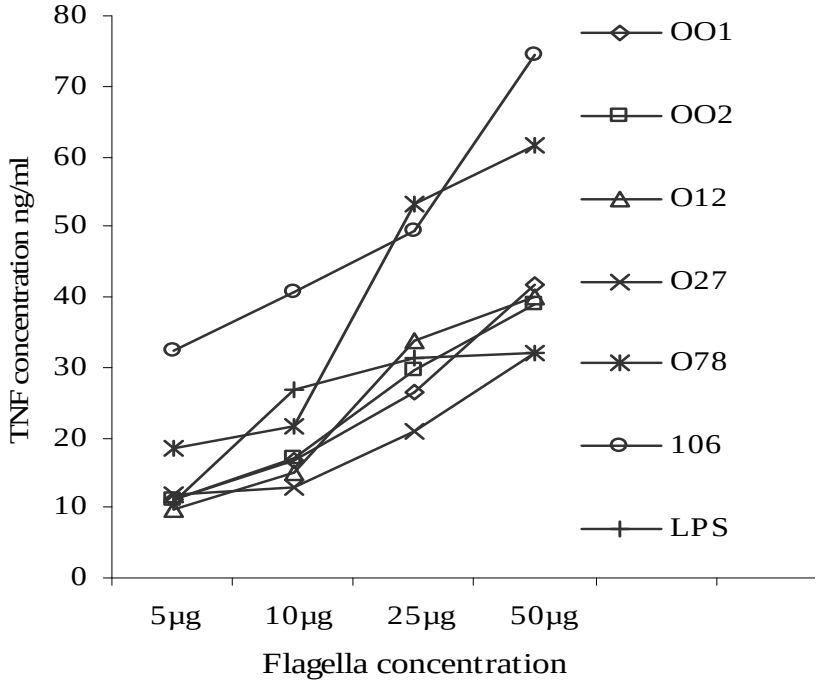

Figure6.7c: TNF production by flagella from six ribotypes

To sum up, ribotype 027 and SLPs influence on IL-10 production reached a peak of 109 ng/ml. Ribotypes 027, 078 and 012 and flagellar proteins induced over 60 ng/ml in IL-10, and TNF-α production by ribotype 106 and flagellar proteins in excess of 60 ng/ml. However, SLPs from 012 and 106 ribotypes only moderately induced cytokine production. The LPS control antigen doesn't go beyond *C. difficile* antigens in terms of enhancing leukocytes except with SLPs and LC in IL-12 secretion. The maximum cytokine secretion occurred with IL-10 with SLPs and crude flagellar from 027 ribotype. The optimum concentration of PMA used was 50 ng, and the highest cytokine levels induced by using 25 µg and 50 µg of antigens.

6.4 Discussion:

This study was designed to investigate the immune activity of three kinds of cell wall molecules from six endemic *C. difficile* ribotypes. The immune activities were assessed by in-vitro stimulation of human leukocytes to produce cytokines after being challenged with immunogenic molecules. An attempt was also made to clarify the role of these antigens in innate human immunity, and to determine the hyper immunogenic fragment from the three molecules used in the study.

O'Brien *et al*. (2005) accepted that the SLPs from different ribotypes were heterogeneous. Sánchez-Hurtado and Poxton (2008) and Wright *et al*. (2008) suggested the role of SLPs in CDI. Ausiello *et al*. (2006) suspected that *C. difficile* infections interrupt the inflammatory and regulatory cytokine stability, as it enhances the production of high volumes of IL-1β and IL-6 as well as IL-10 combined with

IL-12p70, and proposed that there is an opportunity for multi-component vaccines, including SLPs, for protection against CDI. Spigaglia *et al*. (2011) suggested that the SLPs from *C. difficile* 027 and 001 ribotypes give these ribotypes advantages in the pathogenesis of CDI. Noticeably, this study showed that the SLPs from 027 ribotype produced a much more elevated level of IL-10 than with other ribotypes.

However, Bianco *et al*.'s (2011) study used SLPs from *C. difficile* with 027, 001 and 012 ribotypes and evaluated the immunogenicity patterns by exposing SLPs to monocytes in order to produce IL-1β, IL-6 and IL-10. They supposed that the SLPs would stimulate the monocytes to discharge high quantities of IL-10, which indicates the role of SLPs in host immunity against *C. difficile*. But they recommended that the SLPs were not implicated in the rising severity of infection by hypervirulent endemic strains, because they discovered that the immunogenic activity of SLPs extracted from the hypervirulent epidemic *C. difficile* strain has a similar action to non-hypervirulent strains.

The common points were the contribution of SLPs to the human innate immune response and that SLPs stimulate immune cells to produce IL-10 in abundance; these results indicated the effectiveness of SLPs on the pro-inflammatory and regulatory immune response. However, the hypotheses that the type of SLPs affects the aggressiveness of hypervirulent ribotypes requires further investigation. Many factors may have caused this variation in the results of the study; they could be due to the variation in the concentration of the SLPs and PMA used in the study, the

purity of the SLPs and the time of exposure to the antigen, the leukocyte type or anything else that had not been noticed.

Ramos *et al*. (2004) indicated that flagellin plays a role in microbial motility, adherence and pathogenicity. Tasteyre *et al*. (2001) studied the sticking activities of crude flagellin from pathogenic *C. difficile* on sterilised faecal tissue of mice, as well as the activity of flagellated strains. They recorded that adhesion was elevated ten times higher in flagellated isolates than strains without flagella. *C. difficile* flagellar proteins in this study showed high stimulation with IL-10, TNF-α and IL-6. The flagellar proteins from 027 ribotype gave the greatest performance.

In 1981, Poxton and Cartmill purified two types of cell wall carbohydrates from *C. difficile*; they suggested immunogenic activity for both molecules and noted a symmetry of wall and membrane teichoic acid. Sánchez-Hurtado and Poxton (2008) studied the effect of lipocarbohydrate on enhancing the cytotoxicity of suboptimal levels of toxin A. Their results showed notable improvement in the activity on Caco2 and Vero cells, which suggested that LC has a enhancing effect on toxin A activity. Claes *et al*. (2011) pointed out that the lipoteichoic acid stimulatory activity in the bowel immune response was not elevated. In this research, lipocarbohydrate does not show a high effect on immune enhancement in terms of interleukin production except on IL-10 and IL-6, with moderate achievement.

Interleukins 10 production was highly sensitive to the three antigens, and was stimulated more than the other interleukins in this study. Lin and Karin, (2007)

claimed that the IL-10 was recognised as being a regulatory interleukin, which has considerably divergent action compared to pro-inflammatory interleukins such as IL-6 and TNF-α. The decrease in the pro-inflammatory cytokine level and the lower level of IL-1β for all three antigens may indicate the microbial infection strategy illustrated by symptoms, as IL-1β was being responsible for inflammation, pain and hypersensitivity (Pesce *et al.*, 2011). In general, the elevated level of IL-10 by flagellar proteins and SLPs of 027 ribotype may support the hypothesis regarding aggressiveness, as 027 ribotype has a link to hyper-virulence.

To recap, the SLPs and flagellar proteins from the *C. difficile* cell wall were immunogenically active molecules, which subvert the regulatory and pro-inflammatory cytokines, and both were involved in the host innate immune response. The contribution of these in *C. difficile* infection is suspected, thus the involvement of these proteins in vaccines is acceptable. Also their coded genes may have a role in the probiotic effect of non-pathogenic bacteria such as *Lactobacillus* spp Although 027 ribotype, SLPs and flagellar antigens were shown to elevate IL-10 production by human leukocytes, the hypothesis concerning the hypervirulent ribotype characters being responsible alone for the virulence require more investigation before it can be determined conclusively.

CHAPTER VII CONCLUSIONS

This chapter summarises the conclusions that can be drawn from the studies conducted for this thesis, and proposes points that could usefully be expanded through further study on these subjects.

As mentioned at the end of Chapter I, four main aims were being investigated:

1. To determine the distribution of *C. difficile* ribotypes by capillary gel electrophoresis and distinguish the predominant ribotypes.
2. To estimate the antibiotic susceptibility in the strains isolated above to six antibiotics (ampicillin, erythromycin, tetracycline, metronidazole, moxifloxacin and vancomycin) and detect the MICs for these six antibiotics against the *C. difficile* isolates.
3. To determine the hypothesis of the immunogenicity of non-toxin antigens of *C. difficile* cell protein: SLPs, lipocarbohydrate (LC) and flagellar proteins.
4. To verify the hypothesis that the SLPs, LC and flagellar proteins from *C. difficile* are involved in the host innate immune response and have a role in the virulence of the hypervirulent ribotype during the infection.

In the results chapter these were explained extensively and the answers achieved were explained.

Prevalence susceptibility of *C. difficile* ribotypes:

Determining the prevalence susceptibility of *C. difficile* was complicated due to many factors being involved; some were related to the host and others to the aetiologic agents, and in addition the healthcare provider carried a percentage of the responsibility.

The healthcare establishment is responsible for the condition of the community's health, and its strategies offer protection for mankind. It is predominantly the elderly and compromised individuals who are the victims of CDI, either in the wider community or hospitalised individuals. Consequently, infection control and preventive medicine must play a significant role in protecting people. The continuous investigation of aetiologic agents such as *C. difficile* is required to predict outbreaks, along with developing the methodology to obtain truthful results. Thus, novel methods of ribotyping, such as Capillary Gel Electrophoresis Sequencing PCR, have been developed; this is one of the recommended techniques to determine the epidemic strains as it is of a high quality and easy to work with, requires only a short time for investigation and standard data can be contributed from an easily obtainable website (Indra *et al*., 2011).

Elderly people and compromised individuals were the most frequent group of people to be infected by *C. difficile*; this group is the most likely to be hospitalised and consequently to face nosocomial infection. Prevention begins by hand washing and isolation in hospital, and as the *C. difficile* ecology is wide and the spores survive

many harsh conditions, an effective disinfectant is recommended to clean surfaces and linens in hospitals, along with other infection control precautions which it is essential to pursue.

Efficiency of symptomatic CDI patients in producing specific antibodies to immunogenic molecules of *C. difficile*:

There has previously been a debate on whether or not cases of CDI have had the ability to create specific antibodies to *C. difficile* antigens. The ELISA results in this study indicated there were a variety of antibody quantities in the investigated serum samples without a minimum stage.

There was no significant variation in antibody levels for LC and flagellar proteins between symptomatic and non-symptomatic cases. The control SLPs antigens from 027 ribotype had a slightly higher response in symptomatic cases, but the donor-sampled antibody levels were much lower compared to other groups in the study, which was unexpected.

The response which was not clarified was the carrier response, which had almost the same symptomatic level without developing symptoms. It was suggested that the carrier response was faster than the symptoms developed, or that they had recurrent infections, meaning their immune memory of the same antigens was still at a high level; alternatively there may have been some external factors not mentioned.

However, it should be recognised that the study estimated systemic antibodies, while the mucosal antibody levels were more pertinent to developing symptoms.

Influence of *C. difficile* cell wall molecules in immune response:

C. difficile toxins were the most effective factors in creating symptoms, but the other cell wall molecules supported the cytotoxicity affect. These cell wall and flagellar components could have disrupted the immune system while the invasion was underway, as well as being involved in the aetiologic adherence to epithelial cells. Therefore these may have a role in the immune response.

The study outcome indicated the elevated response level to SLPs antigens of hypervirulent ribotype 027. On the other hand, the influence of SLPs from 027 ribotype on leukocytes in enhancing cytokine production shows an increased IL-10 level, which has an anti-inflammation influence as it suppresses pro-inflammatory cytokines and delays symptoms. This may explain the virulence of the ribotypes, as well as the effectiveness of possible vaccines to prevent infection. The other point is the role of this antigen in the probiotic method of CDI prevention. The lipocarbohydrate proteins and flagellar proteins illustrate similar effects on IL-10 production.

To recap, elderly and compromised people are now challenged by multi-resistant aetiologic agents which causes CDI and these spread rapidly worldwide; it also has a complicated set of multiple effects. This organism may have increased virulence

when faced with a challenging ecology, and employs a complicated strategy to invade the host cells. The study aim was to gain an overview of the prevalence of *C. difficile* ribotypes in surgical patients and environments, and to determine the changing nature of antibiotic susceptibility. In addition it was hoped to detect the systemic immune responses to cell wall molecules in patients and donors, and to determine the influence of the immunogenic molecules on human innate immunity.

CHAPTER VIII REFERENCES

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