MOLECULAR IDENTIFICATION AND TYPING OF *CAMPYLOBACTER CONCISUS*

Maitshwarelo Ignatius Matsheka

Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

In the Department of Medical Microbiology

Faculty of Medicine

UNIVERSITY OF CAPE TOWN

September 2000

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

BRIEF CONTENTS

Acknowledgements i
Abbreviations ii
Figures iv
Tables v
Abstract vii
Contents viii

CHAPTER 1: Introduction 1

CHAPTER 2: Isolation, characterisation of a DNA sequence from *C. concisus* and its use as an identification probe 29

CHAPTER 3: Identification of *Campylobacter concisus* using polymerase chain reaction 57

CHAPTER 4: Genotypic typing of *C. concisus* using pulsed field gel electrophoresis and restriction fragment end labelling 68

CHAPTER 5: Random amplified polymorphic DNA analysis of *C. concisus* using $(GTG)_5$ as primer 86

CHAPTER 6: Summary and general conclusions 96

APPENDIX A: Bacterial strains, plasmids and isolation of *Campylobacter* 99

APPENDIX B: Media, buffers and solutions 100

APPENDIX C: Recombinant plasmids 102

LITERATURE CITED 103

ABBREVIATIONS

bp	Base pair
°C	Degrees Centigrade
Ci	Curie
cm	Centimetre
DNA	Deoxyribonucleic acid
DATP	Deoxyadenosine triphosphate
DCTP	Deoxycytidine triphosphate
DNTP	Deoxynucleotide triphosphate
DTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
FIGE	Field inversion gel elctrophoresis
g	Grams
H_2	Hydrogen
H_2S	Hydrogen sulfide
kb	Kilobase
L	Litre
M	molar
Mb	Megabase
mg	Milligram
ml	Millilitre
µg	Microgram
µl	Microlitre
ng	Nanogram
NaCl	Sodium Chloride
nm	Nanometre
nt	Nucleotide
ORF	Open reading frame
OD	optical density
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
RFEL	Restriction fragment end labelling

RFLP	Restriction fragment length polymorphism
RDNA	ribosomal DNA
secs	Seconds
subsp	Subspecies
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
W	Watts
U	Units
Xgal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YT	Yeast tryptone
V	Volts

FIGURES

Figure 1.1	Phylogenetic tree of rRNA clusters within Superfamily VI	6
Figure 2.1	Partial digestion of *C. concisus* genomic DNA	39
Figure 2.2	Genomic DNA from *Campylobacter* species hybridised to DNA fragments from *C. concisus*	40
Figure 2.3	Restriction map of insert in pB3C	41
Figure 2.4	Genomic DNA *Campylobacter* type species hybridised with the 1.6kb fragment from pNeo	43
Figure 2.5	Autoradiographs of *C. concisus* DNA digested with *Hin*dIII following hybridisation with 1.6kb fragment	45
Figure 2.6	Genomic DNA from genetically diverse *C. concisus* strains hybridised to the 1.6kbfragment from pNeo	46
Figure 2.7	The nucleotide sequence of the 1.595kb insert cloned into pUC19 (pNeo)	50
Figure 2.8	Autoradiographs of *Hin*dIII digested genomic DNA from *Campylobacter* species shown in Fig2.4A hybridised to fragments internal to ORF1,ORF2 and ORF3	51
Figure 2.9	Genomic DNA from *C. concisus* probed with portion of ORF3	53
Figure 3.1	DNA from *Campylobacter* species and other bacteria amplified with 16S universal primers	62
Figure 3.2	Amplicons obtained using pcisus1 and pcisus6	63
Figure 3.3	PCR amplication with C. *concisus* specific primers pcisus1 and pcisus6	64
Figure 3.4	Hybridisation with 1.6kb *C. concisus* species specific probe	64
Figure 3.5	Amplified ribosomal DNA restriction analyses with *Dde*I	66

Figure 4.1	PFGE of *Not*I restricted *C. concisus* DNA	75
Figure 4.2	PFGE of *Not*I restricted DNA from grouped *C. concisus* strains	77
Figure 4.3A	Restriction fragment end labelling of *Bgl*II digested DNA with duplicate samples labelled in separate assays	79
Figure 4.3B	Dendrogram of duplicate samples shown in Fig. 4.3A	80
Figure 4.4	Dendrogram of relatedness for RFEL repeated samples run on different gels	81
Figure 4.5	Dendrogram of relatedness for *C. concisus* isolated using RFEL	83
Figure 5.1	RAPD typing using (GTG)5 on isolates found to be genetically diverse by PFGE	89
Figure 5.2	RAPD analysis using (GTG)5 primer	90
Figure 5.3	Numerical analysis of RFEL patterns from *C. concisus* strains	92
Figure 5.4	Field inversion gel electrophoresis of *C. concisus* DNA restricted with *Not*I	93

TABLES

Table 1.1	*Campylobacter* hosts and associated diseases	7
Table 1.2	Growth and biochemical tests for the identification of *Campylobacter*	13
Table 1.3	Flagellin gene typing methods for *Campylobacter*	24
Table 1.4	Distribution of *Campylobacter* and related species isolated from 20123 diarrhetic stools of pediatric patients at the Red Cross Children's Memorial Hospital, Cape Town, South Africa, from October 1 1990 to May 31, 2000	27
Table 2.1	Bacterial reference strains used in study	31
Table 2.2	Bacterial clinical strains used in DNA: DNA hybridisation studies	32
Table 2.3	Summary of RFLP groups identified by probing with 1.6kb fragment	44
Table 3.1	Bacterial clinical strains used in *C. concisus* PCR identification assay	59
Table 4.1	*C. concisus* strains used for PFGE	70
Table 4.2	Bacterial strains used for restriction fragment end labelling	71
Table 4.2	Calculation of *C. concisus* genome size	74

ABSTRACT

A 1.6kb DNA fragment isolated from a *C. concisus* genomic library was found to give *C. concisus* specific restriction fragment length patterns when used as probe in Southern hybridisation studies. In all of the strains tested the probe hybridised to a 500bp *Hin*dIII fragment. Signals were obtained from *C. curvus* and *C. sputorum* subsp *faecalis* but could not be confused with the patterns obtained for *C. concisus*. DNA sequencing of this fragment revealed three ORFs. The first open reading frame encodes the 3' end of the gyrase B subunit. The translation products of the two other ORFs showed similarity to hypothetical proteins, previously identified in *C. jejuni*. DNA: DNA hybridisation studies using a fragment internal to ORF3 demonstrated that this sequence was responsible for the signals obtained with the 500bp fragment. Significantly, no hybridisation signals were obtained with DNA from all the all other *Campylobacter* species tested, suggesting that this sequence was unique to *C. concisus* and could be used as a species specific marker. A rapid PCR assay based on the 1.6kb DNA sequence was developed. Using primers designed to anneal to the extremities of this sequence, an amplicon of the correct size was obtained for *C. concisus* reference strains, local clinical isolates, and clinical isolates from Denmark. PCR products of the correct size were not obtained from other *Campylobacter* species.

In the second part of the study, genetic variability within *C. concisus* isolates from children with diarrhoea was demonstrated using the established genotyping methods PFGE and RFEL. In addition RAPD analysis of *C. concisus* isolates was developed using a simple $(GTG)_5$ oligonucleotide repeat as a primer. Although the study was small, there was good agreement between the fingerprints generated by RAPD, PFGE and RFEL. The technique was found to be rapid and ideal for the primary characterisation of isolates, especially in the absence of an established typing technique for *C. concisus*.

CONTENTS

Chapter 1

Literature review

1.1 Introduction 1

1.1.2 Morphology and isolation 1

1.1.2 Taxonomy 2

1.1.2.1 Campylobacter taxonomic history 2

1.2.2.1 Current taxonomy 4

1.1.3 Ecology and Habitats 6

1.1.4 Campylobacter as Pathogens 8

1.1.4.1 Pathogenic mechanisms 10

1.2 Identification of Campylobacter 12

1.2.1 Biochemical testing 12

1.2.2 Serological tests 14

1.2.3 Whole cell protein analysis 15

1.2.4 Cellular fatty acid analysis 15

1.3 The typing of Campylobacter 16

1.3.1 Serotyping 16

1.3.2.Molecular typing (Genotyping) 17

1.3.2.1 Plasmid typing 17

1.3.2.2. Ribotyping 19

1.3.2.3. Ribosomal DNA analysis 20

1.3.2 4 Chromosomal restriction fragment length polymorphism analysis 20

1.3.2.5 Pulsed field gel electrophoresis 21

1.3.2.6 Random Amplified Polymorphic DNA analysis 22

1.3.2.7 Flagellin typing 23

1.3.2.8 Amplified fragment length polymorphism analysis 25

1.4 Aim of study 26

Chapter 2

Isolation, characterisation of a DNA sequence from *C. concisus* and its use as an identification probe

2.1 Introduction 29

2.2 Experimental procedures 31

2.2.1 Bacterial strains and plasmid vectors 31

2.2.2 Solutions and reactions 33

2.2.3 Extraction of DNA 33

2.2.3.1 Isolation of plasmid DNA 33

2.2.3.2 Genomic DNA extraction 33

2.2.4.Ligations 34

2,2,5 Transformations 34

2.2.6 Analysis of DNA 34

2.2.6.1 Restriction endonuclease digestion 34

2.2.6.2 Agarose gel electrophoresis 34

2.2.6.3 Determination of DNA concentration 35

2.2.7 PCR Amplification 35

2.2.8. DNA:DNA hybridisation 36

2.2.8.1 Preparation of probes 36

2.2.8.2. Hybridisation 36

2.2.9. DNA sequencing 36

2.2.10 Sequence analysis 37

2.2.11 Construction of *C. concisus* genomic library 37

2.3 Results 38

2.3.1 Construction of *C. concisus* genomic library 38

2.3.2 Screening of recombinant clones 38

2.3.3 Specificity of the 1.6kb insert 42

2.3.4 Hybridisation profiles among *C. concisus* clinical strains 43

2.3.5 Sequence analysis of 1.6kb fragment from *C. concisus* 47

2.3.6 Hybridisation studies using DNA sequences internal to ORF 1, 2 and 3 as probes 50

2.4. Discussion 54

Chapter 3

Identification of *Campylobacter concisus* using polymerase chain reaction

3.1 Introduction 57

3.2 Experimental procedures 59

3.21 Bacterial strains 59

3.2.1 PCR assay for the identification of *C. concisus* using pcisus1 and pcisus6 60

3.2.2 Identification of *Campylobacter* using 16S-rDNA restriction fragment length polymorphism 60

3.3 Results and discussion 61

3.3.1 Specificity of PCR for the identification of *C. concisus* 61

3.3.2 Evaluation of PCR for the identification of clinical isolates of *C. concisus* 63

Chapter 4

Genotypic typing of *C. concisus* using pulsed field gel electrophoresis and restriction fragment end labelling

4.1.Introduction 68

4.2 Experimental procedures 69

4.2.1.Bacterial strains 69

4.2.2 Preparation of DNA 71

4.2.3 Restriction endonuclease digestion 72

4.2.4 Separation and detection of restriction of fragments 72

4.2.5 Calculation of genome size 72

4.2.6 Restriction fragment end labelling 73

4.2.7 Numerical analysis of restriction fragment end labelling data 73

4.3 Results 74
4.3.1 PFGE typing of *C. concisus* 74
4.3.2 Restriction fragment end labelling analysis 77
4.3.3 Reproducibility of restriction fragment end labelling patterns 78
4.3.3 Numerical analysis of RFEL profiles for strains grouped by PFGE 82
4.4 Discussion 84

Chapter 5

Random amplified polymorphic DNA analysis of *C. concisus* using $(GTG)_5$ as primer

5.1 Introduction 86
5.2 Methods 87
5.2.1 Bacterial strains 87
5.2.2 RAPD analysis 87
5.2.3 Filed inversion gel electrophoresis 87
5.3 Results 88
5.3.1 RAPD analysis of clinical isolates 88
5.3.2 Restriction fragment end labelling of identical RAPD isolates 91
5.3.3 FIGE analysis of RAPD profile I and II 93
5.4 Discussion 94

Chapter 6: Summary and general conclusions 96

Appendix A: Bacterial strains, plasmids and isolation of *Campylobacter* 99

Appendix B: Media, buffers and solutions 100

Appendix C: Recombinant plasmids 102

Literature cited 103

CHAPTER 1
LITERATURE REVIEW

1.1 INTRODUCTION

Traditionally the genus *Campylobacter* has been recognised in veterinary medicine as a cause of sporadic abortion in cattle and sheep reviewed by Skirrow (1984). More recently some species of this genus have been shown to be responsible for human gastrointestinal disease. This has resulted in renewed interest in the group, with most research concentrating on the species *Campylobacter jejuni*, a bacterium now recognised as one of the prime causes of acute and infectious enteritis in humans (Healing *et al.*, 1992; Skirrow and Blaser, 1992).

1.1.2 Morphology and isolation

Campylobacter are gram-negative, non-spore-forming rods with a length that varies from 0.5 µm to 6.0 µm and a width of 0.2 µm to 0.5 µm. The cells may occur in short or long chains and appear coccoid on exposure to oxygen or in old cultures. The latter form, which is difficult to subculture, is also found in bodies of water, and is thought to represent the dormant stage in the life cycle of the bacteria (Rollins and Colwell, 1986). *Campylobacter* have an optimum growth temperature range of between 30°C and 42°C and are motile through the use of a single polar or dipolar flagella. Members of this genera are defined as non-saccharolytic, micro-aerophilic vibrios with a low G + C DNA base composition ranging from 30 to 46%. Inability to break down complex sugars, coupled with a non-fermentative type metabolism, indicates the dependence of the bacteria on amino acids and intermediates of the citric acid cycle as an energy source.

The isolation of *Campylobacter* is cumbersome as the bacteria have fastidious growth requirements, are inhibited at normal atmospheric oxygen tension and are slow growing, taking up to three days to form colonies on agar plates. Selective media have been used to enhance the growth of *C. jejuni* and *C. coli*, at the expense of other campylobacters (Corry *et al.*, 1995). Due to the fastidious nature of *Campylobacter*, it remains a distinct possibility that the current culture media may not support the growth of other potentially pathogenic species. The recent

This era was also characterised by the isolation and description of a number of microaerophilic vibros living as saprophytes or commensals in a wide variety of domesticated animals and man. The present day *C. jejuni* was isolated from faeces of cattle by Smith and Orcutt (1927). Jones and co-workers (1931) named the bacteria *Vibrio jejuni*, after a causal relationship between these bacteria and bovine dysentery was established. The closely related species *Vibrio coli,* subsequently named *Campylobacter coli,* was initially isolated from pigs with diarrhoea by Doyle (1944). Due to a limited set of differentiating phenotypes this period was also characterised by the description of novel species based on different biotypes of a pre-existing taxon. *Vibrio bubulus*, an isolate from bull semen (Florent, 1953), *Vibrio sputorum* a bacteria named by Prevot (1940) and initially isolated from the sputum of a patient suffering from bronchitis by Tunicliff, in 1914, and *Vibrio fecalis*, a catalase positive isolate, from ovine faeces (Firehammer, 1965), were thought to be closely related. Nevertheless, the bacteria were thought to constitute separate species.

In 1963 Sebald and Véron transferred *V. fetus* and *V. bubulus* into a new genus *Campylobacter*. Ten years later, Véron and Chatelain published a more comprehensive study on the taxonomy of the microaeriphilic Vibrio-like organisms. They considered the genus *Campylobacter* to have four distinct species: *Campylobacter fetus* (the type species), *Campylobacter coli, Campylobacter jejuni* and *Campylobacter sputorum* (comprised of two subspecies, *C sputorum* subsp. *sputorum* and *C. sputorum* subsp. *bubulus*).

On the realisation that catalase activity was not taxonomically significant and with the subsequent aid of DNA: DNA hybridisation studies (Roop *et al.*, 1985), the then known species *C. sputorum* and *C. fecalis* were found to be the same species. They considered all three species as biovars of *Campylobacter sputorum*. The species designations were then changed to reflect these findings, and were consequently known as *C. sputorum* biovar *sputorum, C. sputorum* biovar *bubulus* and *C. sputorum* biovar *fecalis* respectively. *C. sputorum* has recently undergone a taxonomic revision and based on catalase and urease activity, the biovars have currently been amended to *C. sputorum* biovar *paraureolyticus*, for a urease producing variants, *C. sputorum* biovar *sputorum* for catalase negative strains *and C. sputorum* biovar *fecalis* for catalase positive strains (On *et al.*, 1988).

The early years of the newly created genus *Campylobacter* were plagued by confusion with regards to the nomenclature. A situation perpetuated by the loss of the of the original viable cell cultures of *Vibrio fetus* described by Smith and Taylor (1919). In the eighth edition of Bergey's manual of determinative Bacteriology, Smibert (1974) erroneously published the strains causing infertility as *C. fetus* subsp. *fetus* and those causing sporadic abortions as *C. fetus* subsp *intestinalis*. More confusion was sown by referring to the two species of *C. jejuni* and *C. coli* as one species, C. *fetus* subsp *jejuni.* Currently the accepted nomenclature is as outlined by Véron and Chatelain (1973) with modern amendments as outlined by Vandamme and De Ley (1991).

Most of the taxa that make up the genus *Campylobacter* were assigned fairly recently. These consist of *C. concisus, C. rectus* (Tanner *et al.*, 1981) and *C. curvus;* bacteria isolated from the human oral cavity that thrive in a H_2 rich atmosphere during culture. *Campylobacter* strains associated with adenomatosis in pigs, *C. mucosalis* were isolated in 1981 (Lawson *et al.*, 1981) and *C. hyointestinalis* in 1983 (Gebhart *et al.*, 1985). A recent addition, *C upsaliensis,* was first isolated from dogs (Sandstedt *et al* 1983) and is now a recognised emerging pathogen associated with human disease (Goosens *et al*, 1990a, Goosens *et al.*, 1990b; Lastovica *et al.*, 1989). Due to the limited set of differential phenotypic characteristics, most species could not be identified fully, so some taxa were conveniently referred to by vernacular names. In most cases these names pertained to aberrant phenotypes displayed by these species in question (Vandamme *et al.*, 1996). The members of present day genus *Helicobacter*, a genus closely related to that of *Campylobacter* were loosely referred to as gastric *Campylobacter*-like organisms (GCLO) (Warren and Mashall, 1983). Atypical variants of *Campylobacter lari* were referred to as urease positive thermophilic Campylobacter (UPTC group) and nalidixic acid-sensitive Campylobacters (NASC-group)

1.1.2.2 Current taxonomy

The advent of nucleic acid techniques such as 16S rRNA sequencing and their use in classification, revealed the genus *Campylobacter* to be genotypically diverse and helped to draw major border lines across the taxonomically hierarchy of this genus and related genera. New genotypic approaches, such as DNA base ratios helped describe taxa from species to

generic level while DNA: DNA hybridisation studies became benchmarks for describing species.

The genera *Campylobacter* and *Arcobacter* together make up the family *Campylobacteraceae* (Vandamme and De Ley, 1991). The two genera share similar structural, morphological and genotypic characteristics, but differ in physiological properties, in that members of the genus *Arcobacter* are psychrophilic and more aerotolerant. The genus *Arcobacter* presently comprises of 4 species. *A. nitrofigilis* (the type strain), which colonises the roots of the salt marsh plant *Spartina alterniflora* (McClung *et al.*, 1983), *A. cryaerophilus*, *A. butzleri* and *A. skirrowii*. While the presence of a flagella sheath was used to separate *Helicobacter* from *Campylobacter* and *Arcobacter*, more recent data has demonstrated several non-sheathed *Helicobacter* species. Through 16S sequencing the genus *Helicobacter* has been found to be different from true *Campylobacter*, with a closer relationship to *Wolinella succinogenes* than campylobacters (Paster and Dewhirst, 1988).

Modern *Campylobacter* taxonomy was resolved after extensive analysis of partial 16S rRNA homology studies subdivided the pre-scribed genus into three main homology groups corresponding to the present day genera *Helicobacter*, *Arcobacter* and *Campylobacter* (Paster *et al.*, 1988; Romaniuk *et al.*, 1987; Thompson *et al.*, 1988). The general taxonomic structure based on partial 16S rRNA has been found to be in overall agreement with partial sequences of 23S RNA (Van Camp *et al.*, 1993) and also with DNA-rRNA hybridisation analysis studies (Vandamme *et al.*, 1995). In 1987 Romaniuk and associates (1987), found *Campylobacter* to belong to a distinct phylogenic group among the gram-negative group of the eubacteria. It was within this group, now known was as RNA superfamily six, which three rRNA homology groups comprising of the genus *Campylobacter, Arcobacter* and *Helicobacter* were described (Thompson *et al.,* 1988).

The three related genera *Campylobacter*, *Arcobacter* and *Helicobacter* presently comprise 14, 4 and 18 validly described species respectively. Even within these three genera, there is great sequence variability in the 16S rRNA data of each genus, *Campylobacter* has a 5.7% difference, *Helicobacter* 8.6% and *Arcobacter* 5.7% (Vandamme *et al.*, 1996). To date the genus *Campylobacter* comprises 14 species and 6 subspecies (Table 1.1). *C. lanienae*, an

isolate from human faeces has recently been described (Logan *et al.*, 2000). This review will concentrate only on the species of the genus *Campylobacter* while the term *Campylobacteria* will refer to all the genera in rRNA superfamily six. A phylogenic tree, extracted from Vandamme *et al* (1995), indicating relationships among members of the *Campylobacter* is shown in Fig 1.1.

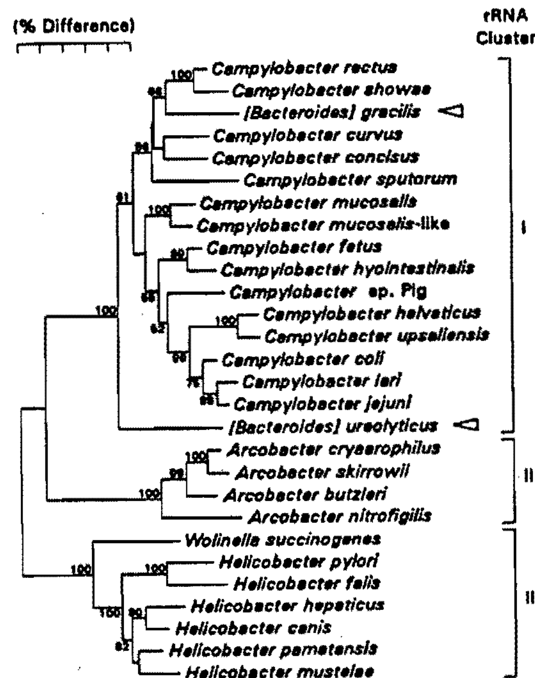


Fig. 1.1. Phylogenetic tree of rRNA clusters within Superfamily VI (taken from Vandamme *et al.*,1995). Scale bar = 5% difference in nucleotide sequence, determined by measuring the lengths of the horizontal lines connecting two species. The values to the left of the nodes are the percentage of times that the strains to the right of the nodes occur together by bootstrapping. Bootstrapping values less than 50% are not shown.

1.1.3 Ecology and habitats

Campylobacters are ubiquitous in nature, colonising a diverse range of host species (table 1.1). They also thrive in a host of aquatic environments and may be isolated from rivers, ponds (Healing *et al.,* 1992) and seawater (Hernandez *et al.,* 1995a; Hernandez *et al.*, 1995b). A free-

Table 1.1 *Campylobacter* Hosts and Associated Diseases (taken from On, 1996)

Species	Subspecies	biovar	Main hosts	Disease in humans	Disease in animals
C. jejuni	*jejuni*		Birds, mammals	Gastroenteritis, septicemia, Gullian Barre syndrome	Gastroenteritis, Avian hepatitis
C. jejuni	*doylei*			Gastroenteritis, septicemia, gastritis	
C. fetus	*fetus*		Sheep, cattle	Gastroenteritis, septicemia	Spontaneous abortion in ovine + bovine
C. fetus	*venerealis*		Cattle	Septicemia	Bovine infectious infertility
C. coli			Pig, birds, bulls, sheep	Gastroenteritis, septicemia	Gastroenteritis
C. lari			Birds, dogs, cats, monkeys, horses	Gastroenteritis, septicemia	Avian gastroenteritis
C. hyointestinalis	*hyointestinalis*		Pig, cattle, hamster	Gastroenteritis	Porcine and bovine enteritis
C. hyointestinalis	*lawsonii*		Pigs		
C. concisus			Humans	Periodontal disease, gastroenteritis	
C. mucosalis			Pigs		Porcine enteritis
C.curvus			Humans	Periodontal disease, gastroenteritis	
C. rectus			Humans	Periodontal disease	
C. upsaliensis			Dogs, cats	Gastroenteritis, septicemia, abscesses	Canine and feline gastroenteritis
C. helveticus[a]			Dogs, cats		Canine and feline gastroenteritis
C. showae[a]			Humans	Periodontal disease	
C. sputorum		*sputorum*	Humans, cattle, pigs	Abscesses, gastroenteritis	
C. sputorum		*paraureolyticus*	Cattle		
C. sputorum		*fecalis*	Cattle, sheep		
C. gracilis			Humans	Periodontal disease, Deep tissue infection	

misleading incidence rate. While reporting of *Campylobacter* infection is compulsory in some states in the United States and in the United Kingdom, inappropriate culture techniques will not detect fastidious *Campylobacter* species such as *C. concisus.*

Campylobacter enteritis is characterised by severe stomach cramps, the disease is usually self-limiting lasting approximately 5 days. In developed countries the pathology of infection manifests itself as inflammatory diarrhoea and affects all age groups, with the majority of infections occurring in young children, with little asymptotic carriage (Skirrow and Blaser, 1992). Complications rarely occur, although infections are sometimes followed by an onset of bacteremia, or very uncommonly a serious condition of paralysis known as Gullian-Barré syndrome (GBS) (Kuroki *et al.,* 1991). This condition is thought to be brought about by cellular or humoral immunopathogenic response to outer membrane lipopolysacharides of *C. jejuni*, which mimic certain human gangliosides (Allos, 1997). The most frequently associated antibody to GBS is anti-GM1, an antibody which has been linked to *C. jejuni* infection in several studies (Kuroki *et al.*, 1991; Rees *et al.*, 1995; Yuki *et al.*, 1990). *Campylobacter* strains with O:19 and O:41 serotypes have been associated with GBS (Fujimoto *et al.*, 1992; Kuroki *et al.*, 1993) The O:19 serotype has been found to be genetically homogenous group (Nishimura *et al.*, 1997), but no differences have been identified between isolates from GBS symptomatic patients and from isolates from patients without GBS. This has led to the assumption that GBS is primarily host dependent rather than strain specific (Wassenaar and Newell, 2000)

In developing countries diarrhoea is usually inflammatory and predominates in the infant population (Skirrow and Blaser, 1992). There is also a noticeably higher incidence rate, but a lower number of symptomatic infections. This is probably due to congenital protection, where protection is conferred by immune mothers to their young through breast-feeding. This line of reasoning is yet to be proven. The observed difference in age distribution and susceptibility to disease between children in developing countries and developed is thought to be accounted for by the early acquisition of immunity by children in developing counties living in hyper-endemic areas of infection (Taylor, 1992).

Apart from the well documented roles of *C. jejuni*, and *C. coli* as causes of gastroenteritis *C. lari*, and very recently *C. upsaliensis*, are also known to be gastrointestinal pathogens (Patton *et al* 1989; Tauxe *et al* 1985) albeit contributing a small percentage of total cases of *Campylobacter* induced enteritis. Other campylobacters are also known to be opportunistic pathogens exerting their effects on immuno-compromised patients to cause bacteremia, peritonitis, meningitis and septic arthritis and abortions (Mishu *et al.,* 1992). *C. fetus* subsp. *fetus* is well known as a cause of septic abortions in domestic animals and as a frequent cause of bacteremia in elderly man. *C. sputorum* and its biovars have been predominately isolated from abscesses. The pathogenic role of *C. concisus* has yet to be defined although it has been implicated in periodontal infection and associated with diarrhoea.

1.1.4.1 Pathogenic mechanisms

Two types of diarrhoea are observed with *C. jejuni* infection. Inflammatory diarrhoea is characterised by slimy stools with pus from disrupted leukocytes. This condition is usually accompanied by fever and the stools have traces of blood. Then there is non-inflammatory diarrhoea, with watery stools, with no pus or blood. Although there have been several reviews on the pathogenesis of *Campylobacter* (Ketley, 1997; Wassenaar, 1997; Wooldridge and Ketley, 1997), the exact pathogenic mode of infection in these bacteria still remains unclear.

The flagella seem to be an important virulence determinant, as it is needed for motility, a crucial activity required for the colonisation of the mucous lining of the gut and intestine. Flagella are also thought to play a direct role in the active invasion of the epithelial cells. Non-motile mutants have been shown to have a lowered invasive capacity (Grant *et al.*, 1993; Wassenaar *et al.*, 1991). However it is doubtful that the disruptive effect caused by invasion of the epithelial cells induces diarrhoea as low levels of bacteria are known to invade a mono-layer of in-vitro cells and the efficient intracellular killing capacity of epithelial cell is well documented (Wassenaar *et al.*, 1991). It therefore seems reasonable to assume that the production of toxins seems to be the most virulent mode of activity.

Members of the pathogenic enterobacteria can draw one of the causes of diarrhoea characterised by watery stools, from enterotoxin production. *Vibrio cholerae* toxin (CT) and *Escherechia coli* heat-labile toxin (LT), deregulates the andenylate cyclase regulatory system of host cells, causing an excess secretion of fluid. Although several studies have reported on enterotoxin production in *Campylobacter* (Nachamkin *et al.*, 1992), enterotoxin production as a virulence factor has been shrouded in controversy. Most research groups have been unable to show enterotoxin activity in *Campylobacter* strains and the homologue enterotoxin gene to those of *Vibrio cholerae* and *E. coli* have yet to be shown to be present in *Campylobacter* (Wassenaar, 1997).

Cytotoxins produced by *Campylobacter* have been described in the literature and are best covered in depth by Wassenaar (1997). Cytotoxins have specific target cells and can either act by disrupting the cell membrane or by intracellular inactivation. In the case of the latter cytotoxins have to be processed by the host cell before eliciting their cytotoxic effects in the cytoplasm. Wassenaar (1997) reports that cytotoxins produced by *Campylobacter* are not well classified, with little comparative studies against the cytotoxic effects of well-characterised cytotoxins. A 70-kd cytotoxin, which is toxic to Chinese hamster ovary cells (CHO), human tumour epithelial cells (HeLa), Hep-2 cells and INT407 cells but not African green monkey kidney cells (Vero) has been described (Wong et al., 1983; Mahajan and Rodgers, 1990). Cytolethal distending toxin (CDT) in Campylobacter was first described by Johnson and Lior (1988). The toxin acts by arresting the phase prior to cell division, causing extensive cell elongation that culminates in cellular disintegration. Sensitive cells include CHO, VERO, HeLa, and Hepp-2 cell lines. Recently human colon carcinoma cell lines (Caco-2 cells) have also been shown to be sensitive to *C. jejuni* CDT (Whitehouse *et al.*, 1998). Hemolytic toxins and shiga-like toxin are also described for *C. jejuni* and *C. coli* strains (Wassenaar, 1997). The combination of flagella assisted invasion of epithelial cells and the production of cytotoxins are the most likely pathogenic determinants in the onset of diarrhoea. Direct linkage of the cytotoxic effect to inflammatory diarrhoea has yet to be established.

Table 1.2 Growth and biochemical tests for the identification of *Campylobacter* (Adapted from Allos *et al.*, 1995)

Campylobacter	Growth tests				H^2S production			Inhibition		Biochemical tests					
Species/subsp	H^2	25°C	37°C	42°C	Rapid[m]	Lead ace[n]	TSI[o]	Nal[k]	Ceph[l]	Cat[e]	Nit red[f]	Ind ace[g]	Pyr[i]	Aryl[h]	Hipp[j]
C. jejuni jejuni 1	-	-	+	+	-	+ +	-	S	R	+	+	+	+	-	+
C. jejuni jejuni 2	-	-	+	+	+	+ +	-	S	R	+	+	+	+	+	+
C. jejuni doylei	d	-	+	(+)	-	-	-	S	(S)	(+)	-	+	+	-	(+)
C. fetus fetus	-	- (+)	+	+ (-)	-	+	-	R	S	+	+	-	-	-	-
C. fetus venerealis	-	+	+	-	-	+	-	R	S	+	+	-	-	-	-
C. coli	-	-	+	+	-	+ +	-	S	R	+	+	+	+	-	-
C. lari	-	-	+	+	+	+	-	R	R	+	+	-	+	-	-
C. hyointestinalis hyointestinalis	d	(+)	+	+	-	5+	3+	R	S	+	+	-	-	-	-
C. hyoitestinalis lawsonii	d	(+)	+	+	-	5+	3+	R	S	+	+	-	-	-	-
C. concisus	+	-	+	(-)	-	3+	(+)	(R)	S	-	+	-	+	+	-
C. mucosalis	+	(-)	+	(-)	-	5+	+	R	S	-	+	-	-	-	-
C.curvus	+	-	+	+	-	5+	+	R	S	-	+	+	+	+	-
C.rectus	+	-	+	+	-	3+	+	S	R	-	+	+	+	+	-
C. upsaliensis	d	-	+	(+)	-	(+)	-	S	S	(+)	+	+	+	-	-
C. helviticus	+	-	+		-	2+	-			-	+	+	+	-	-
C. sputorum (bv) sputorum	-	-	+	+	+	5+	3+	R	S	-	+	-	+	+	-
C. sputorum (bv) bubulus	-	-	+	(+)	+	5+	3+	R	S	-	+	-	-	+	-
C. sputorum (bv) fecalis	-	-	+	+	+	5+	3+	R	S	+	+	-	+	+	-

+, Positive; (+), most strains positive;-, negative;(-), most strains negative; R, Resistant;(R), most strains resistant; S, susceptible;(S), most strains susceptible; [d] some isolates grow much better in H_2- enhanced growth conditions; [e] catalase ; [f] nitrate; [k] nalidixic acid resistance; [l] cephalothin resistance; [m] Rapid H_2-S method of Skirrow and Benjamin. Journal of Clinical Pathology (1980) 33:1122; [n] lead acetate; [o] triple sugar iron .NB *C. jejuni* subsp *jejuni* biotypes 1and 2 refer to Skirrow's biotyping scheme. Susceptibilities are based on 30µg disks.

the atypical members of the thermophilic *Campylobacter*. However, biochemical analysis, is still reliable in the identification of species to the subspecies level a fact given credence by On and Holmes (1995) when they examined 67 phenotypic characteristics in 47 different species encompassing the genera *Campylobacter,* closely superfamily members and the genus *Anaeriospirillum*. By applying numerical analysis to the data, significant correlation between identification based on phylogenetic rDNA data and biochemical analysis data was achieved.

Apart from the problem of atypical strains the main problem with the effectiveness of biochemical testing in the identification of *Campylobacter* is in the lack of standardisation of the tests used. Different schemes and tables in biochemical identification have been published comprising different procedures unique to each laboratory. Seemingly insignificant differences in inoculum size and quantities in basal medium have been shown to have a bearing on results, especially growth inhibition tests (On and Holmes, 1991a; On and Holmes, 1991b). Despite some of the problems discussed above, biotyping still serves as an invaluable tool in the identification of *Campylobacters*. It has a low operational cost and its relative ease of use compared to more sophisticated tests like DNA:DNA hybridisation and 16S-rDNA analysis makes this method indispensable and essential for routine diagnostic work.

1.2.2 Serological tests

Through the years commercial identification systems based on antibody mediated agglutination to *Campylobacter* antigens have been developed. The methodologies are extensively covered in the review by On (1996). These systems which consist of latex particles coated with immunoglubins are principally designed for the presumptive identification of *Campylobacter*. Although a number of false positive results and failure to detect some strains of the intended target species have been reported (Hodinka and Gilligan, 1988; Nachamkin and Barbagallo, 1990), the commercial kits are useful in preliminary diagnosis of *Campylobacter* infection. Effectiveness of these systems in direct detection of *Campylobacter* in faecal and food samples also suggested a lack of sensitivity (Griffiths *et al*., 1989; Hazeleger *et al*., 1992,). Apart from the lack of sensitivity care should be taken when evaluating results in clinical material when using agglutination assays, as positive latex tests have been recorded with nonviable cells (Griffiths *et al*., 1989).

The use of enzyme-linked immunosorbent assays (ELISAs) has also been described in the identification of *Campylobacter* species. Griffiths *et al* (1992) have reported tests capable of differentiating between *C. jejuni, C. coli, C. lari* and C. *upsaliensis.* While antibodies specific to outer membrane proteins (OMPs) have been used by Taylor and Chang (1987) to detect *C. coli, C. jejuni* subsp. *jejuni* and *C. lari*. Complex immunodiffusion assays with taxonomic value have been developed around a method originally described by Falsen (1983), but this method has had little success in the definitive identification of *Campylobacter.*

1.2.3 Whole cell protein analysis

Sodium deodecyl sulphate–polyacrylamide gel electrophoresis of whole cell proteins, depends on the differential electrophoretic mobility of cellular proteins to discriminate between isolates. This method has been tried and tested for the differentiation of the so called asaccharolytic nonpigmented gram negative members of the human oral cavity (Tanner, 1986) and specifically for the identification of the species *C. concisus*. The similar morphology and biochemical characteristics amongst the members of the EF 22 rRNA homology group some times results in the misidentification of *C. concisus* as *C. mucosalis* (Figura *et al.*, 1993; Lastovica *et al.*, 1993). By comparing electrophoregrams of whole cell protein extracts, improved differentiation between these two species has been achieved (Vandamme *et al.*, 1989; On, 1994). This method has also been used in confirming the reclassification of *C. sputorum* sub

sp. *mucosalis* as a separate species, *C. mucosalis* (Costas *et al.*, 1987).

1.2.4 Cellular fatty acid analysis

Cellular fatty acid profiles of bacterial cells have proved to have limited use in the identification and classification of *Campylobacter*. Whole cell extracts of fatty acid methyl esters are used to generate species specific profiles by separation using gas chromatography. With the aid of computers or by visual assessment, *C. jejuni*, *C. fetus*, *C. sputorum*, *C. mucosalis* and *C. coli* can be differentiated (Blaser *et al.*, 1980; Leaper and Owen, 1981; Moss *et al.*, 1984). However, at times more than one species can occur within a defined fatty acid

group and conversely, strains from a single species may have fatty acid profiles designating them to different fatty acid groups.

1.3 THE TYPING OF CAMPYLOBACTER

The main challenges in the epidemiological investigation of *Campylobacter* related infections are due to a number of factors. The bacteria are widely distributed making the routes of transmission complex, involving a wide range of zoonotic hosts and environmental reservoirs. It is widely believed that the major source of infection is food borne, with contamination coming by the way of ingestion of undercooked poultry and unpasteurised milk (Healing *et al*, 1992). Most cases of human infection are sporadic, making the accurate identification of the source of infection difficult. As previously described, the phenotypic methods such as biochemical testing and serotyping are useful for epidemiological monitoring but usually lack the adequate discriminatory power. To provide detailed epidemiological information, high-resolution methods of identification are needed. DNA based molecular typing methods are thought to be the answer, especially as they have the prospect of universal availability and standardisation of protocols.

1.3.1 Serotyping

Serotyping is one of the most commonly used phenotypic typing schemes for *C. jejuni*. Based on immunological properties, this method exploits the variation in bacterial antigenic determinants to discriminate between strains. The serotyping scheme developed by Lior (Lior *et al.*, 1982) detects heat labile (HL) surface based determinants by a passive slide agglutination technique. At present, 108 HL serotypes based on this technique are used to type *C. jejuni* and *C. coli*. An alternative serotyping scheme described by Penner and Henessey (1980) identifies 47 heat stable serotypes of *C. jejuni*, and 19 serotypes of *C. coli* and is commonly referred to as heat stable (HS) or O serotyping scheme. Although both methods have been used with success in the tying of *C. jejuni* and *C. coli*, the latter technique has enjoyed extensive usage with HS1, HS4 and HS2 being the most common serotypes isolated from man (Gibson *et al.*, 1995; Owen *et al.*, 1995). However, in certain cases serotyping is rendered redundant, as some strains cannot be serotyped (Asrat *et al.*, 1997; Jacobs-Reitsma *et*

al., 1995). At times cross reactivity between serotypes, as is the case with the HS4 complex of *C. jejuni*, causes serotyping to be ineffective. Only a few reference laboratories are capable of serotyping *Campylobacter* at the present time.

The Laboratory of Enteric Pathogens (LEP), Public Health Laboratory Service in the United Kingdom has developed an improved serotyping scheme based on the method by Penner. This scheme identifies 48 HS serotypes in *C. jejuni* and 17 in *C. coli*. The method is more reproducible in that direct agglutination to the bacterial sample is carried out, and nonspecificity is reduced by taking readings immediately after the agglutination procedure. By using absorbed antisera instead of unabsorbed antisera, problems with cross-reactivity of isolates have been eliminated, although problems with non-typeability of some isolates still persists (Newell *et al.*, 2000). However, some Campylobacter strains autoagglutinate, and may have to be subcultured up to ten times before the LEP serotyping system can be applied.

1.3.2 Molecular typing (genotyping)

Unlike phenotypic properties which are generally influenced by environmental factors, DNA based techniques are relatively stable from external forces. In molecular typing bacterial isolates are assigned to clonally related lineages by the determination of the underlying DNA sequence polymorphism. The determination of nucleotide sequence variation between isolates can be tackled through various approaches. The most direct method involves sequencing of the target gene, this is a long, laborious and expensive method. What most typing techniques seek to establish is an estimation of genetic polymorphism in a rapid and cost effective way.

Some genotypic typing techniques sample part of the genome to get a representation of the overall genomic variation. Specific gene loci are targeted and either sequenced or analysed by restriction fragment length polymorphism (RFLP) to determine genetic polymorphism. Critical to the success of the method is the appropriate selection of the genetic marker, to attain this a few guidelines are followed. Ideal genes are the ones least affected by recombination and void of mutational hot spots, as these tend to undergo rapid base substitution, resulting in homoplasy (Aquodro and Greenberg, 1983). Again genes that are too conserved like 16S RNA suffer from lack of polymorphisms resulting in low discriminatory power and are therefore

appropriate for speciation. Genes involved in housekeeping activities such a DNA replication, transcription and translation, preferably occurring in single copies have been found to be useful. For typing purposes genes with an ideal mutation rate depends on whether the study is a long-term surveillance study or a short-term outbreak case.

In general an ideal typing method should have the following properties. A high discrimination potential, in that the target gene should be highly polymorphic. However the gene should also display stability of the genotype over time. Apart from the method having high reproducibility, it should also have the ability to assign an ambiguous genotype to each isolate, or what is known as the typeability factor. For a method to be routinely applicable in clinical or reference laboratory it should also be time and cost effective.

1.3.2.1 Plasmid typing

In general plasmid typing has not been used to differentiate members of the genus *Campylobacter*, primarily because plasmids have been found to occur in low frequency, in members of the well recognised thermophillic species (Bradbury *et al.*, 1984; Mayer, 1988). However, many of the catalase negative or weakly positive *C. upsaliensis* strains do carry plasmids (da Silva Tatley *et al.*, 1992; Owen and Hernandes 1990). For this reason most plasmid profiling studies of campylobacters are related to this species.

By comparing plasmid profile types of *C. upsaliensis* isolates from patients in distinct geographical regions, an association between specific plasmid profiles and defined geographical proximity was drawn (Owen and Hernandes 1990). Using plasmid profiling in combination with 16S RNA typing, distinct genotypes of *C. upsaliensis* were also found to be prevalent in canine and humans, suggesting the existence of host-specific strains (Stanley *et al.*, 1994). In a study in which isolates from human faeces were analysed no correlation was found between plasmids containing strains of *C. upsaliensis* and antibiotic resistance (Goosens *et al.*, 1990b). Although plasmids often encode virulence or antibiotic resistance determinants and would ideally be suited for epidemiological typing purposes, their use as genetic markers is not recommended due to their susceptibility to horizontal transfer.

1.3.2.2 Ribotyping

The ribosomal ribonucleic acid (rRNA) genetic locus displays a high degree of conservation across both prokaryotes and eukaryotic organisms and therefore has significant implications on the organisational and evolutionary relationship of living organisms (Cedergren *et al.*, 1988). While sequence analysis of 16S rRNA has helped redefine phylogenetic relationships at species level, rRNA cistron gene restriction fragment analysis, commonly referred to as ribotyping, relies on the different genetic location of the multiple copies of rRNA genes, as well as the surrounding restriction sites in differentiating between genotypes. In *Campylobacter* species there are about 2-4 copies of the rRNA genes in the chromosome (Romaniuk and Trust, 1987). Although some studies have successfully used ribotyping to differentiate between *C. jejuni* in an outbreak situation (Tee *et al.*, 1992), generally ribotyping has limited discriminatory power and is best suited for speciation (Kielbauch *et al.*, 1991). By analysis with a combination of 16S plus 23S RNA gene cistrons, subspecies differentiation of *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* has been achieved (Hernandez *et al.*, 1991). Limited subtyping has also been achieved with *C. jejuni* and *C. coli* isolates (Fayos *et al.*, 1992). In both cases the differentiation of *C. coli* from *C. jejuni* was not attained and was only possible through the utilisation of two restriction enzyme in genomic digests and a 16S RNA gene specific probe (Fitzgerald *et al.*, 1996). Stanley et al (1995) also carried out 16S rRNA gene specific ribotyping of *C. coli* with a clear distinction of *C. jejuni* isolates. With respect to the subtyping of *Campylobacter Pst*I and *Hae*III seem to be the enzymes of choice.

Ribotyping compared to other molecular typing techniques is relatively laborious and time consuming, taking up to a week to attain results. The method is also technically demanding and expensive and cannot be carried out in a routine clinical laboratory. The use of biotinelated probes has meant the use of radioactive labelled probes can be avoided making the method safer. A number of rapid PCR based methods based on rRNA restriction polymophisms have been derived. Amplified ribosomal DNA restriction analysis looks at restriction length polymorphisms, intraspecific to the 16S rRNA gene.

1.3.2.3 Ribosomal DNA analysis

PCR mediated analysis of ribosomal genes, has also been used for epidemiological and identification purposes. In analogy to ribotyping, the different length or sequence polymorphism between 16S and 23S spacer regions of rRNA operons are detected by amplification with primers located in conserved regions of the 16S and 23S genes. There is enough sequence length variation in this region to generate profiles useful for epidemiological analysis (Kostman *et al.,* 1992) and for taxonomic purposes (Jensen *et al.*, 1993).

Primers designed to amplify 16S or 23S rDNA, or both these regions have largely been used for rapid speciation. PCR products are digested with an array of restriction endonucleases and to generate restriction fragment length polymorphisms of rRNA genes in a method called amplified rDNA restriction analysis (ARDRA). Due to the conserved nature of the rRNA genes species specific DNA profiles have been generated for a number of bacteria (Gurtler *et al.*, 1991; Laguerre *et al.*, 1994; Marshall *et al.*, 1999; Vaneechoutte *et al.*, 1992).

1.3.2.4 Chromosomal restriction fragment length polymorphism analysis

Genomic restriction fragment length polymorphism analysis allows for the determination of genetic variation by the mapping of restriction endonuclease sites thoughout the genome. Using 4-6 base pair recognition restriction endonucleases, chromosomal changes brought about by DNA recombination, loss or gain of a restriction site by a mutational event is detected by resolving the restriction fragment patterns using gel electrophoresis. This is one of the early methods applied to various members of the genus *Campylobacter* (Bradbury *et al.*, 1984; Collin and Ross, 1994; Kakoyiannis, 1988; Owen *et al.*, 1985). Enzymes found to yield stable restriction profiles are *Hae*III, *Hin*dIII and *Xho*I (Owen *et al.*, 1989). The only setback of this technique is that it results in the production of numerous bands, which are not distinct and are difficult to resolve by conventional gel electrophoresis and the use of ethidium bromide stained agarose gels. The recent development in numerical methods of analysis has enabled limited determination of relationships between *Campylobacter*.

1.3.2.5 Pulsed field gel electrophoresis

A method, which samples the whole genome for restriction endonuclease sites, is pulsed field gel electrophoresis (PFGE). The method first described by Schwartz and Cantor, (1984) and later developed by Smith *et al.,* 1987 to map the *E. coli* genome, uses rare base cutters to produce genotypic restriction profiles also called macrorestriction profiles (MRP) (Owen *et al.*, 1995; Stanley *et al.*, 1995). Unlike conventional agarose gel electrophoresis which cannot separate fragments larger than 20 kilobase pairs (kb) (Fangman, 1978), PFGE uses a variation in the direction of the electric current across multiple electrodes to separate large DNA fragments of between 20kb and 1000kb (Smith *et al.*, 1987). The technique produces fewer fragments than conventional chromosomal RFLP making analysis easier and as a result has been used in numerous epidemiological studies of Campylobacter (On and Vandamme, 1997; Stanley *et al.*, 1995; Yan *et al.*, 1991). However, due to the fewer number of fragments generated, the method carries a notable risk of homoplasy, in that DNA bands that co-migrate at the same molecular weight are not necessarily the same DNA molecule. To avoid this error, restriction with an additional restriction endonuclease is recommended (Gibson *et al.,* 1997).

PFGE in typing exercises has been found to be highly discriminatory in the analysis of *Campylobacter* isolates (Owen *et al*, 1995; Yan *et al.*, 1991). Compared with other typing techniques, the method has been found to have more discriminatory power than phagetyping, ribotyping (Gibson *et al.,* 1995) serotyping and fatty acid profiling (Steele *et al.,* 1998). A direct comparison between PFGE and Penner heat stable serotypes has indicated some degree of correlation between some MRP types with HS9 and HS38 serotypes (Lorenz *et al.,* 1998). However, isolates with some serotypes in general do not necessarily reflect the same genetic similarity and conversely different serotypes have been found to belong to the same genetic lineage (Gibson *et al*, 1997). *C. jejuni*, which was serologically nontypeable, could also be assigned to known serotypes using this technique (Gibson *et al.*, 1997). Suggesting that non-expression of genetically encoded antigenic properties rendered serotyping redundant.

1.3.2.6 Random amplified polymorphic DNA analysis.

The polymerase chain reaction (PCR) methodology has had a significant impact on the identification and typing of microorganisms. One method which has gained appeal as a DNA fingerprinting tool due to its simplicity, rapid turn over of results and prospect of universal applicability is random amplified polymorphic DNA (RAPD) analysis or arbitrarily primed PCR (AP-PCR). Both methods, which were simultaneously described by Williams *et al.* (1990) and Welsh and McClelland (1990) respectively, generate strain-specific DNA fingerprints by selective amplification of genomic DNA, using a single primer of arbitrary choice. PCR amplification is carried out at low primer annealing temperature. While the method described by Welsh and McClelland (1990) used primers of 20 bp, that of Williams *et al.* (1990) uses primers of 10 to 12 bp. A variation of the PCR method called DNA amplified fingerprinting (DAF), described a year later by Caetano-Anolles *et al.* (1991), used a short primer of 5bp.

The main attraction with the methods described above is that no prior DNA sequence of the microorganism to be typed is required and thus the selection or synthesis of specific primers is avoided. This is attributed to the low stringency primer annealing temperature conditions during PCR amplification which do not require perfect primer matches with the complementary target sequence, for synthesis of product. However, slight variation of the methodology whereby limited knowledge of the genetic characteristics of the test microorganism is required has been described. Collectively, these techniques design primers to consensus motifs of sequences occurring in multiple copies in genomes and exploit these as priming sites. Complementary sequences to conserved motifs in class I repetitive extragenic palindromic elements (REPs) (Stern *et al.*, 1984) and class II enterobacterial repetitive intergenic consensus sequences (ERIC) (Sharples and Lloyd, 1990) have been used to detect DNA sequences diversity among bacterial isolates (de Bruijn, 1992; Endtz *et al.*, 1993; Giesendorf *et al.*, 1993; Versalovic *et al.*, 1991). Targets have also included conserved areas of tRNA fragments (McClelland *et al.*, 1992) which have been used to differentiate at the intraspecies level (Welsh and McClelland, 1992).

One of the greatest challenges of RAPD analysis is its universal application as a typing technique. The generation of reproducible DNA fingerprints remains a problem, which is attributable to many factors, such as the amount of DNA template, the molar quantity of primers and the particular brand of *Taq* polymerase used in the reaction. Although, reproducible profiles are attainable when the method is carried out according to carefully optimised conditions, standardisation of the technique for inter-laboratory comparisons is elusive. A problem linked to the sensitive nature of the PCR assay itself, where, running identical protocols in different thermocyclers results in aberrant results. (MacPherson *et al.*, 1993; Meunier and Grimont, 1993).

1.3.2.7 Flagellin gene typing

One of the most extensively used typing technique for *C. jejuni* and *coli* is flagellin gene typing. Flagellin is the immunodominant antigen and essential structural component of the flagella filament (Cover and Blaser, 1989; Guerry *et al.*, 1991). Flagellin is transcribed from two genes designated *fla*A and *fla*B. These genes are approximately 1730 base pairs long and arranged in tandem with a 170 nucleotide spacer region. The conserved nature of the flagellin gene (Nuijten *et al.*, 1990) and the role of flagella motility as an important pathogenicity factor (Black *et al.*, 1988; Cover *et al.*, 1989; Morooka *et al.*, 1985) makes flagellin an ideal molecular typing marker.

Most of flagellin gene typing has been carried out on *C. jejuni* using two primers designed to the conserved flanking regions of *fla*A (Alm *et al.*, 1993a; Nachamkin *et al.*, 1993; Nishimura *et al.*, 1996). *fla* A typing has also been reported for other species like *C. lari*, *C. coli* and *C. helveticus* (Owen *et al.*, 1993). Most flagellin typing methods amplify an approximate 1.5kb fragment of *fla*A which is digested with an array of enzymes with *Dde*I, *Hae*III, *Hinf*I and a combination of *Ecor*I and *Pst*I being the most popular (Alm *et al.*, 1993a; Burnens *et al.*, 1995; Nachamkin *et al.*, 1993; Owen *et al.*, 1993). Most of the described flagellin typing methods have slight variations in DNA preparations, areas to which primers are designed and the restriction enzymes used, making consolidation of data between different laboratories difficult (Table 1.3). Flagellin gene restriction fragment typing carried out by Ayling *et al.* 1996 is distinct in that both *fla*A and *fla*B gene products are amplified for analysis. This is achieved by

the use of three primers, with one primer designed to the identical conserved 5'end of *fla*A and *fla*B and the other two designed to specific 3' ends of both genes (Table 1.3).

The immunogenic property of the flagellin and its heat labile nature has led to the speculation that it might contribute to the number of antigens detected in the Lior typing scheme (Wenman *et al.*, 1985). However, the contribution of flagellin genes to the Lior agglutination reactions remains controversial as limited correlation between flagellin typing and Lior serotyping has been shown (Alm *et al.*, 1991). In a study by Nachamkin *et al.* (1993), flagellin gene typing could be used to differentiate within a particular serotype and correlation between flagellin gene types and some HL and O serotypes in investigating *C. jejuni* outbreak strains was shown. However, little correlation between flagellin RFLP and Lior serogroups has been reported (Burnens *et al.*, 1995).

Table 1.3 Flagellin Gene Typing Methods for *Campylobacter*.

Authors	Primers		PCR product and size	Restriction endonucleases	Species typed
	Forward, 5'-3' end.	Reverse 5'-3' end.			
Alm *et al.*, (1993a)	ATGGGATTTCGTATTAAC AAGGATTTAAAATGGGTTTTAGAATAAACACC	GCACC[CT]TTAAG[AT]GT[AG]GTTACACCTGC	fla A and fla B 1448bp	*Pst*I, *Eco*RI	*C. jejuni* *C. coli*
Nishimuru *et al.*, (1996)	TACTACAGGAGTTGAGCTT	GTTGATGTAACTTGATTTTG	*fla*A 702bp	*Hae*III, *Afa*I, *Mbo*I	*C. jejuni*
Nachamkin *et al.*, (1993)	GGATTTCGTATTAACACAAATGGTGC	CTGTAGTAATCTTAAAACATTTTG	*fla*A 1728bp	*Dde*I	*C.jejuni*
Ayling *et al.*, (1996)	AAAGGATCCGCGTATTAACACACAAATGTTGCAGC AAAGGATCCGAGGATAAACACCAACATCGGT	GATTTGTTATAGCAGTTTCTGCTATATCC	*fla*A and *fla*B 1490bp	*Dde*I, *Alu*I, *Hinf*I	*C. jejuni*
Burnens *et al.*,(1995)	ccggatcccATGGCATTTCGTATT	ttcgaattCTATTGTAATAATCTTAAAACAT	*fla*A 1731bp	*Alu*I, *Dde*I, *Hae*III, *Hinf*I	*C. jejuni* *C. coli*
King and Clayton (1991)	ATGGGATTTCGTATTAACAC	CTATTGTAATAATCTTAAAA	*fla*A and *fla*B 1723bp	*Hae*III, *Bgl*II	*C. jejuni*

Regions of DNA homology are indicated in red for forward primers and blue for reverse primers. Lower case nucleotide bases indicate sequences used for cloning purposes.

1.3.2.8 Amplified fragment length polymorphism analysis

Amplified fragment length polymorphism (AFLP) analysis is a patented method originally developed for the fingerprinting of plants (Vos *et al.*, 1995), and is currently showing great promise as a universal method for bacterial typing and evolutionary studies (Keim *et al.*, 1997; Kokotovic and On, 1999; Janssen *et al.*, 1996; Valsangiacomo *et al.*, 1995). This method is essentially similar to restriction fragment length polymorphism in that it detects variation in restriction fragments caused by DNA recombination events or point mutations in restriction sites. Slight variations to the technique have been described in different studies but in general the methodology is essentially the same.

Genomic DNA of the test organism is digested with a restriction endonuclease of choice. Usually the choice of enzyme is dependent on the G +C content and the size of the genomic DNA. This information is used to select an average cutting enzyme paired with a frequent cutter or at times only one restriction endonuclease is used (Gibson *et al.*, 1998; Valsangiacomo *et al.*, 1995). The sticky ends of the restriction fragments are ligated to adapters synthesised from double stranded oligonucleotides, designed with compatible ends of the restriction endonuclease used. Selective amplification of restriction fragments is achieved by stringent PCR conditions in combination with the use of primers that extend beyond the adapter and the restriction site sequence. Only a perfect match at the 3' end of the primer and the sequence flanking the restriction site results in a product. The PCR products are either resolved in agarose by staining with ethidium bromide (Gibson *et al.*, 1998; Valsangiacomo *et al.*, 1995) or in polyacrylamide gels after labelling one of the primers with a radioactive nucleotide (Vos *et al.*, 1995: Huys *et al.*, 1996) or fluorescent dyes (Kokotovic and On, 1999). The results allow for computer assisted numerical analysis using software such as GelCompar (Applied Maths, Kortrijk, Belgium). This enables the method to be used in species identification or strain typing.

AFLP has been used with success in determining genetic diversity and evolutionary implications in the spread of *Bacillus anthrax* (Keim *et al.*, 1997), the differentiation between isolates of *H. pylori* and *Campylobacter* (Gibson *et al.*, 1998; Kokotovic and On, 1999). The method has high discriminatory power (Huys *et al.*, 1996; Savelkoul *et al.*, 1999) and has been

found to correspond well with classification results obtained by ribotyping and DNA-DNA hybridisation studies (Clerc *et al.*, 1998). Although the method is reproducible, partially digested genomic DNA may complicate results. Overall the method fulfils all the requirements of an ideal typing technique, and has the additional advantage of generating fingerprints without the prior knowledge of sequence using minimal amounts of DNA. The technique also has the potential for universal standardisation.

1.4 AIM OF STUDY

Traditionally *Campylobacter concisus* has exclusively been recovered from, and considered as part of the bacterial flora, of the human oral cavity (Tanner *et al.*,1979; Tanner *et al.*, 1981; Tanner *et al.*, 1987). *C. concisus* was predominantly isolated from gingival crevices, associated with the onset of periodontal disease (Moore *et al.*, 1985; Tanner *et al.*, 1981; Tanner *et al.*, 1987). However, the pathogenic role of *C. concisus* with respect to a causative role in the onset of periodontal disease is still unknown (Johnson *et al.*, 1985). Subsequently strains from the extra-oral cavity have been isolated; however these were initially misidentified or not classified. The strains include: Nine strains from faeces; two strains from an antrum biopsy: one strain from a blood sample from an adult male, and one strain from the oesophagus (Vandamme *et al.*, 1989).

Recent studies have increasingly reported on the isolation of *C. concisus* from the stools of patients with diarrhoea (Engberg *et al.*, 2000; Lauwers *et al.*, 1991; Van Etterijck *et al.*, 1996). This has largely been attributed to an improvement in culture techniques, where the adoption of the membrane filter technique has improved isolation rates (Lauwers *et al.*, 1991). At the Red Cross Children's War Memorial Hospital in Cape Town, South Africa, since the inception of 'The cape Town Protocol' (Le Roux and Lastovica, 1998), which utilises the membrane filter technique in combination with a H_2 enriched microaerobic environment, the prevalence of *C. concisus* has increased to 23% of the total *Campylobacter* isolates. This is only second to that of *C. jejuni* subsp *jejuni* (Table.1.4). Similar results were obtained by Engberg *et al.* 2000 in a reevaluation of conventional culturing methods, whereby using the filtration technique, yielded a significant increase in *C. concisus* isolates. This indicates that currently the *Campylobacter*

isolation protocols used in most laboratories are inefficient, and in fact, the prevalence of *C. concisus* amongst children is higher than anticipated.

Table 1.4 Distribution of *Campylobacter* and related species isolated from 20 123 diarrhetic stools of paediatric patients at the Red Cross Children's Memorial Hospital, Cape Town, South Africa, from October 1 1990 to May 31, 2000.

Species/subspecies	Number	Percentage (%)
C. jejuni subsp. jejuni biotype 1	1179	28.06
C. concisus	993	23.64
C. upsaliensis	978	23.28
C. jejuni subsp. doylei	383	9.11
H. fennelliae	265	6.30
C. coli	119	2.84
C. jejuni subsp jejuni biotype 2	119	2.84
C. hyointestinalis	53	1.26
H. cinaedi	42	1.00
†CLO/HLO	35	0.83
Arcobacter butzleri	16	0.38
C. fetus subsp.*fetus*	7	1.16
"H. rappini"	4	0.10
C. lari	2	0.05
C. curvus	2	0.05
C. rectus	2	0.05
C. sputorum biovar sputorum	2	0.05
TOTAL	4 201	100

Data from Lastovica and Le Roux (2000)
* Biotype of Skirrow and Benjamin (1980)
† CLO/ HLO *Campylobacter* 'like' organisms or *Helicobacter* 'like' organisms

Whether *C. concisus* is associated with diarrheal disease is still a matter for conjecture. In a study carried out by Van Etterijck (1996), the differences in isolation rates of *C. concisus* from Belgian children with and without diarrhoea, was found to be statistically insignificant, suggesting that *C. concisus* played no pathogenic role and was most probably not a primary pathogen. This was also demonstrated in the study by Engberg *et al.*, (2000), where carriage rates from healthy children were on par with diarrhoeic patients. The results are similar to the enteric *Campylobacter* isolation rates observed in developing countries, including South Africa

where although there is a significantly higher prevalence rate, there is no difference in carriage rate among healthy children and children with diarrhoea above 8 months (Asrat *et al.*, 1997; Bokkenheuser *et al.*, 1979; Taylor, *et al.*, 1988). This finding, together with the apparent lack of epidemics, combined with the milder forms of diarrhoea as witnessed in developing countries, has led to the speculation that *Campylobacter* species as a whole may not be pathogens in developing countries (Taylor, 1992). However, the lack of epidemics may also be attributed to lack of poor surveillance, a problem prevalent in most of the developing world.

In Cape Town, *C. concisus* displays clinical and seasonal characteristics similar to that of *C. jejuni*, an established gastrointestinal pathogen (Lastovica and Engel, 2000). Monthly isolation frequencies showed that both *C. concisus* and *C. jejuni* were notably higher in the three-month period towards the end of summer (February-April). Clinical symptoms of diarrhoea, such as severity and stool consistency were similar to that of *C. jejuni*. However, *C. concisus* was predominantly found in children 1 year or older, while *C. jejuni* was mostly found in children under 12 months. *C. concisus* was also more likely to be associated with children of mixed descent, than indigenous black children. The latter could not be explained by age, gender differences, genetic susceptibility, eating habits or geographical location (Lastovica and Engel, 2000). *C. jejuni* is recognised as one of the main leading causes of gastro-enteritis in the world (Healing *et al.*, 1992; Skirrow and Blaser, 1992), the role of *C. concisus* and its association with diarrhoea has yet to be clearly defined

The study by Van Etterijck (1996) is the only epidemiological surveillance study published to date in which genotyping of *C. concisus* was carried out. As *C. jejuni* and *C. coli* are important human pathogens, most epidemiological typing studies have concentrated on these species. At present, there is no established typing procedure available for *C. concisus*. As a result, little is known about *C. concisus* with regards to reservoirs, mode of transmission and its potential as a pathogen. Like other campylobacters, *C. concisus* is also fastidious and biochemically inactive, making identification by the few prescribed phenotypic tests difficult. The aim of the study was therefore two fold, one was to devise a rapid and specific identification method for *C. concisus*. The second objective was to evaluate potential genotypic typing techniques for prospective epidemiological studies and to gain insight into the genetic characteristics of *C. concisus* isolates in paediatric patients.

CHAPTER 2

ISOLATION, CHARACTERISATION OF A DNA SEQUENCE FROM *C. CONCISUS* AND ITS USE AS AN IDENTIFICATION PROBE

2.1 INTRODUCTION

Like other members of the genus, *C. concisus* is fastidious and is characterised by a relatively low biochemical activity, properties that make the identification of *C. concisus* problematic using conventional phenotypic techniques. Within the genus *Campylobacter, C. concisus* is phenotypically most similar to the species *C. mucosalis.* Both species require H_2 and are reductase, nitrate and oxidase positive. They both produce H_2S, tolerate 1% glycine and are sensitive to 1.5% NaCl. Using the prescribed phenotypic tests sometimes results in the misidentification of these species (Figura *et al.*, 1993). Although susceptibility to cephalothin and differences in growth, temperature and colour can be used to distinguish *C. concisus*, the problem associated with strain variability makes these tests unreliable (Lastovica *et al.*, 1993). Numerical analysis of sixty four phenotypic properties, was used to unequivocally differentiate between *C concisus* and *C. mucosalis* (On, 1994). However, no phenotypic test on its own was sufficient to differentiate between the two species and instead a few tests based on the differential growth ability of strains on a number of inhibitory media were recommended.

Apart from extensive phenotypic testing described above, the most common methods used to identify *C. concisus* have been DNA-DNA hybridisation assays, immunotyping and whole cell protein electrophoresis (Lastovica *et al.,* 1994; Tanner, 1986, Vandamme *et al.*, 1989). Identification using methods such as DNA-DNA hybridisation and whole cell protein electrophoresis requires considerable technical expertise. In addition, the techniques are time consuming and labour intensive.

To date, only one method for the rapid identification of *C. concisus* has been published (Bastyns *et al.,* 1995). This method depends on the PCR amplification of 23S rDNA, and is different from the one described by Marshall *et al.* (1999), in which differentiation of

2.2 EXPERIMENTAL PROCEDURES

2.2.1 Bacterial strains and plasmid vectors

Campylobacter reference strains used in the study are given in Table 2.1. *Campylobacter* clinical isolates (Table 2.2) from the Children's Red Cross Memorial Hospital in Cape Town were isolated using cultural conditions delineated in Appendix A, and identified by E. Le Roux. using the biotyping scheme of Skirrow and Benjamin (1980a). Also included in Table 2.2 are two Danish clinical isolates of *C. concisus* from children, and two *C. concisus* reference strains provided by Dr S. L.W. On from the Veterinary research laboratory in Denmark.

Table 2.1. Bacterial reference strains used in study

Bacterial strains	Other strain designations	Sources
C concisus NCTC 11485	ATCC 33237	Human gingival sulcus
C concisus NCTC 11684		
C concisus CCUG 13144	LMG 7788, FDC 484	Gingival sulcus
C concisus CCUG 19995	LMG 7966	Human faeces
C curvus NCTC 11649		
C rectus NCTC 11489	CCUG 20446, FDC 371	Human periodontitis
C sputorum bv *fecalis* NCTC 11415	CCUG 17761, LMG8531	Ovine faeces
C coli CCUG 11283	LMG 6440, CIP 7080	Porcine faeces
C lari NCTC 11352	LMG 8846, CCUG 23947	Cloacal swab of a herring gull
C upsaliensis NCTC 12183		Human blood
C helvitivus NCTC 12470		Feline faeces
Bacteroides ureolyticus NCTC 10941	CCUG 7319, LMG 6451	Amniotic fluid
C jejuni subsp *doylei* NCTC 11847	CCUG 18265, LMG7790	Human gastric biopsy
*C.jejuni*subsp *jejuni* NCTC 11168	CCUG 6824, LMG 8553	Human faeces
C mucosalis NCTC 11000	CCUG 6822, LMG 6448	Porcine small intestine

[a]FDC- Forsyth Dental Center, Boston, massachusetts; LMG-Culture Collection of the Laboratorium voor Microbiology, University of Ghent, Ghent Belgium; CCUG- Culture collection of the University of Gotenborg, Department of Clinical Bacteriology. University of Gotenborg, Gotenburg, Sweden; NCTC National collection of Type Cultures, Central public Health Laboratory, London, U.K; CIP- Collection de l Institute Pasteur, Paris, Paris, France; ATCC-American Type Culture Collection, Rockville, Md.

Table 2.2 Bacterial clinical strains used in DNA: DNA hybridisation studies

No	*C. concisus* strain	Sex of patient	Age (months)	Clinical condition
1	204-92	N/A	N/A	N/A
2	291-92	M	18	Diarrhoea
3	20-93	M	6	Bilary atresia
4	58-93	N/A	18	N/A
5	271-93	F	14	Twin A watery stool
6	282-93	F	14	Twin A watery stool
7	285-93	F	14	Twin B
8	286-93	F	14	Twin B watery stool
9	361-93	F	66	Chronic colitis
10	25-94	F	24	Microcytic anaemia
11	339-94	M	12	Loose anaemia
12	68-95	F	12	Loose dysentery
13	157-95	F	48	Dysentery
14	133-95	M	25	Diarrhoea +convulsions
15	238-95	M	48	Typhoid
16	1-96	M	60	Bloody diarrhoea
17	207-96	M	60	Bloody diarrhoea
18	211-96	F	38	Diarrhoea
19	214-96	N/A	7	Diarrhoea
20	215-96	M	57	Loose stool
21	218-96	M	37	Dysentery
22	275-96	F	37	Diarrhoea
23	297-96	F	84	Chronic dysentery
24	314-96	F	12	Dysentery
25	317-96	F	24	Dysentery
26	330-96	N/A	N/A	N/A
27	334-96	M	21	Dysentery
28	337-96	F	56	N/A
29	341-96	F	18	Loose stools
30	343-96	N/A	N/A	N/A
31	361-96	N/A	12	Watery stool
32	370-96	F	132	Chronic diarrhoea
33	377-96	F	5	Chronic diarrhoea
34	392-96	M	12	well child
35	393-96	F	5	Loose stools
36	394-96	M	132	Dysentery
37	395-96	F	13	Pneumonia gastroenteritis
38	396-96	F	6	Diarrhoea + vomiting
39	389-96	F	20	Chronic diarrhoea
40	402-96	M	19	Bloody stools
41	404-96	M	33	Hirshsprung disease
42	406-96	F	3	Loose stools
43	415-96	F	1	Bloody stools
44	426-96	M	11	Diarrhoea
45	173-97	M	49	Loose stool + Blood
46	182-97	M	13	Chronic diarrhoea
47	199-97	N/A	N/A	N/A
48	308-98	F	9	Dysentery
49	311-98	F	14	gastroenteritis
50	316-98	M	4	Diarrhoea
51	146-99	N/A	N/A	N/A
52	199-99	F	26	Persistent Diarrhoea

N/A Clinical records not available

E. coli strains used in transformation studies are also given in Appendix A

Plasmid vectors used in the study were pUC19 (Messing 1983; Yanisch-Perron *et al.*, 1985) and the suicide vector pEcoR251 (M. Zabeau, Plant Genetics systems N.V., Gent. Belgium) (Appendix A). A schematic representation of the recombinant plasmids constructed are presented in Appendix C.

2.2.2 Solutions and reagents

Solutions and reagents are presented in Appendix B. All of the reagents were of analytical grade or higher.

2.2.3 Extraction of DNA

2.2.3.1 Isolation of plasmid DNA

Bacterial cells were harvested from overnight cultures grown in 5 ml 2 x yeast-tryptone (2xYT) broth (Appendix B) containing ampicillin (100 µl/ml) and plasmid DNA was isolated using the alkaline lysis method of Ish-Horowicz and Burke (1981).

Large-scale plasmid preparations were carried out using 100 ml overnight cultures containing the appropriate antibiotic. Plasmid DNA was isolated using the Nucleobond AX 100 PC Kit (Macherey-Nagel, Germany), according to the manufacturer's instructions.

2.2.3.2 Genomic DNA extraction

Bacterial genomic DNA was extracted from *Campylobacter* using the scaled up guanidium thiocyanate method as described by Pitcher *et al.* (1989). A pellet of approximatly100 µg of cells was re-suspended in 100 µl of Tris-EDTA (TE) buffer (Appendix B) and lysed with 500 µl of guanidium thiocyanate. The rest of the protocol was carried out as described; using proportionally scaled up solutions. The DNA pellet was washed four times to remove traces of guanidium thiocyanate and the pellet air dried, before re-suspending in 50 µl of TE buffer.

2.2.4 Ligations

Ligations were essentially carried out by the method specified in Sambrook *et al.* (1989), in 20 μl reactions, containing 1x T4 DNA ligase buffer and 10 units of T4 DNA ligase (Boehringer and Mannheim). Typically, an approximate ratio of 3 to 1 molar concentration of insert and vector were used in ligations, and the reactions incubated at 16°C for 4 to 16 hrs.

2.2.5 Transformation

Competent LKIII (Zabeau and Stanley, 1982) and DH5α (Hanahan, 1983) *E. coli* cells were prepared using the method of Dagert and Ehrlich (1979). Ligation mix (20 μl) was added to 100 μl of competent cells and kept on ice for 20 to 30 min, before heat shocking at 42°C for 2 min. A volume (900 μl) of 2xYT broth was added and cells incubated at 37°C for 45 min to allow for gene expression. The cells were plated on 2xYT agar plates containing the appropriate antibiotic for selection of transformants.

2.2.6 Analysis of DNA

2.2.6.1 Restriction endonuclease digestion

Restriction endonuclease digestion of plasmid DNA was typically carried out in 20 to 50 μl reactions, in buffer conditions specified by the manufacturers'. Genomic DNA was restricted in 50 μl reactions using 40 units of restriction endonuclease, at 37°C for 24 hrs.

2.2.6.2 Agarose gel electrophoresis

DNA fragments were separated on a horizontal flat bed gel electrophoresis system in 0.8- 1.2% agarose gels immersed in 0.4 M Tris-acetate and 0.01 M EDTA buffer. Gels were stained with ethidium bromide (Sigma chemicals) and DNA visualised at 302 nm. Photographs were taken using the Mamiya RB67 pro-S camera or captured on the Kodak DC 120 zoom digital camera.

2.2.8 DNA:DNA hybridisation

2.2.8.1 Preparation of probes

DNA of interest was eluted from agarose gels and purified using a phenol extraction procedure described by Seth (1984). DNA was labelled with horseradish peroxidase as described in the ECL direct nucleic acid labelling and detection system kit (Amersham Life Science).

A probe for 16S rDNA was amplified using universal primers (Edwards *et al.*, 1989) and provided by E. Nelson, Department of Medical Microbiology, University of Cape Town.

2.2.8.2 Hybridisation

Probes were hybridised to genomic DNA restricted with *Hin*dIII endonuclease and transferred by capillary action to Hybond-N^+ membranes (Amersham International, UK) using the method of Southern (1975). Hybridisation was carried out as described in the ECL direct nucleic acid labelling and detection system kit manual (Amersham Life Science). Pre-hybidisation was carried out at 42°C for an hour in a rotisserie oven (Techne hybridiser HB-1D). Hybridisation was carried out at 42°C for 14-16 hrs, followed by either low or high stringency washes as delineated in the protocol, without the use of urea. The signal generated was detected by autoradiography, adhering to the instructions delineated in the protocol booklet.

2.2.9 DNA sequencing

Automated sequencing (Phamacia Biotech AB S-751 82 Uppsala Sweden) was done at the Department of Molecular Biology, University of Cape Town. Sequencing of clones in M13 based vectors was carried out using Thermo Sequenaseflourescent labelled primer cycle sequencing kit with deaza-dGTP (Amersham Phamacia Biotech). For internal primers, the Cy™ Thermo Sequenase Dye terminator kit (Amersham Pharmacia Biotech). All reactions were performed to the Manufacturer's instructions using the Chain termination technique of Sanger *et al.*(1977). Reactions were cycle sequenced on a GeneAmp PCR System 9700, perkin Elmer, Applied Biosystems.

2.2.10 Sequence analysis

The DNA sequence of the 1595 bp insert of pNeo was analysed by DNAMAN version 4.0 (Lynnon BioSoft) computer analyses software for nucleic acid and amino acid sequences.

Protein alignments were carried out by the fast alignment method of Wibur and Lipman (1983) using the Blosum matrix with a k-tuble value of 2 and gap penalty of 4.

Sequence similarity searches for nucleic acids and deduced amino acid sequence, was carried out against existing nucleotide and protein databases using Blast (Altschul *et al.*, 1990); Basic Local Alignment Tool at the National Centre for Biotechnology Information (NCBI), National Institute of Health.

2.2.11 Construction of *C. concisus* genomic library

The genomic library was prepared using the method described in Ausebel *et al* (1987), with modifications. To determine the ideal enzyme concentration for partial digestion, serial dilutions at one half the concentration of the *Sau*3AI (5 U/μl) restriction endonuclease (Boeringer Mainnheim, Germany) were set up, ranging from 0.11 to 3.95 x 10^{-4}U/μl. Having determined the ideal concentration for partial digestion, genomic DNA digests (200 μl) with 50 mg of DNA and 3 units of *Sau*3AI were prepared in pairs and digestion was carried out at 37°C for 30 min. The digests were pooled together and extracted once with an equal volume of phenol/chloroform. The DNA was precipitated with isopropanol and washed with 70% ethanol. The DNA pellet was dried and re-suspended in 500μl of TE Buffer overnight.

Sucrose solutions 10% and 40% were prepared in separate cylinders linked by a clamped capillary tube. With continuous stirring in the 40% sucrose cylinder the clamp in the capillary tube was released and a sucrose gradient gently layered into an open top polyallomer tube (Beckman), from a tap leading out of the 40% sucrose solution cylinder.

The partially digested DNA sample was heated at 65°C for 10 min and kept at 20°C before loading on the sucrose gradient. The sucrose gradient was spun at 22000 revolutions per minute (rpm) for 25 hrs 30 min in an ultracentrifuge (Beckman L-70 ultracentrifuge). The bottom of the polyallomer tube was punctured and approximately 1 ml aliquots eluted into 36 eppendorf tubes. An aliquot (20 µl) of each fraction was electrophoresed in a 0.6% agarose gel and fractions containing the same size fragments, pooled together. The DNA was precipitated by adding an equal volume of isopropanol, washed twice with 70% ethanol and each DNA fraction sample re-suspended in 1000 µl TE Buffer.

The 4 kb-5 kb *Sau*3AI fragments were cloned into the *Bgl*II restriction endonuclease site of pEcoR251 suicide vector. The recombinant plasmids were transformed into competent *E. coli* LKIII cells. Isolation of recombinants indicated inactivation of the *Eco*RI endonuclease gene following insertion of DNA at the *Bgl*II site contained within this gene.

2.3 RESULTS

2.3.1 Construction of *C. concisus* genomic library

To determine the concentration of *Sau*3AI suitable for partial restriction of *C. concisus* genomic DNA, serial dilutions ranging from 0.1 to 3.95 $\times 10^{-4}$ U/µg of DNA were set up. Digestion was carried out at 37°C for 30 min. As can be seen Fig. 2.1A (lane 5) partial digestion was achieved using 6.25×10^{-3} U/µg of DNA.

Partially digested DNA was prepared and added to a sucrose gradient. Thirty-six aliquots were collected, and the fractions collected from eppendorfs 9-16 are shown in Fig. 2.1B (lanes 2-9). Fragments presumed to be of the same or similar size were pooled together. Fractions containing fragments equal to or greater than 14 kb (Fig. 2.1B lanes 3 to 5) were pooled and similarly, fractions containing DNA fragments of 8 to 5 kb (Fig. 2.1B, lanes 6-9) were also pooled. The fragments contained in the 8 to 5 kb fractions were cloned into pEcoR251 suicide vector, generating the plasmid library.

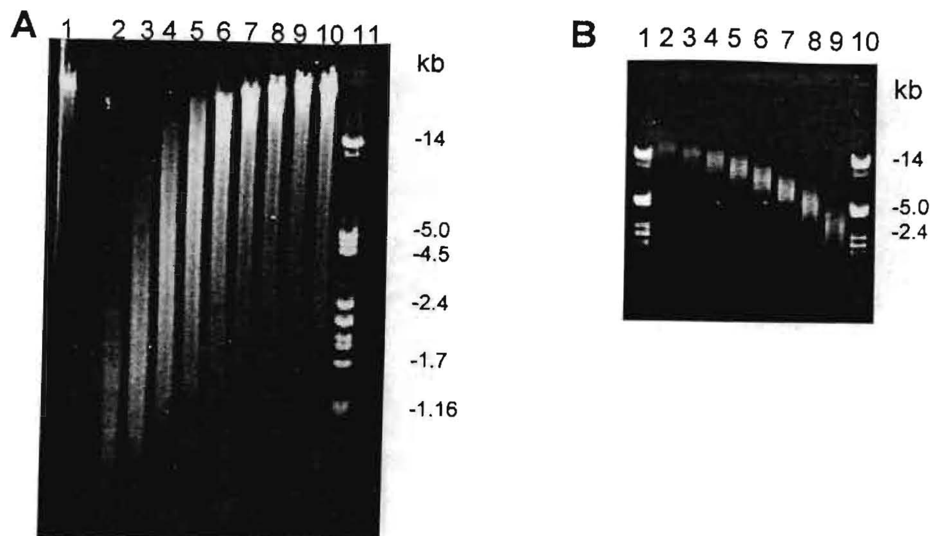


Fig 2.1 Partial digestion of *C. concisus* genomic DNA.
A. DNA partial digests, with serial dilutions of *Sau*3A1. Lane 1, undigested DNA; lane 2,DNA digested with 0.1 U/μg; lane 3, 0.05U /μg; lane 4, 0.025U/μg; lane 5, 0.0125 U/μg; lane 6, 6.25 10^{-3} U/μg; lane 7, 3.125 10^{-3} U/μg; lane 8, 1.562 10^{-3} U/μg; lane 9, 7.813 10^{-4} U/μg; lane 10, 3.95 x 10^{-4} U/μg; lane 11 lambda DNA digested with *Pst*I.
B. Sucrose gradient DNA fractions collected in tubes. Lane 1, lambda DNA digested with *Pst*I; lane 2, DNA fraction in tube 9: lane 3, tube 10; lane 4, tube 11; lane 5, 12; lane 6, tube 13; lane 7, tube 14; lane 8, tube 15, lane 9, tube 16; lane 10, lambda DNA digested with *Pst*I.

2.3.2 Screening of recombinant clones

The *C concisus* DNA library was screened for a DNA sequence that would be useful for the identification of this species. Inserts from a random selection of clones were excised by digestion with *Bgl*II, gel-purified, labelled and hybridised to *Hin*dIII restricted genomic DNA from a panel of *Campylobacter* species, in separate experiments. The species comprised *C. jejuni*, *C. mucosalis*, *C. concisus* type strains and two pairs of clinical *C. concisus* strains isolated from Cape Town and Denmark.

After screening approximately 200 clones, one of the inserts hybridised strongly to three bands of 2.7, 2.6 and 2.2 kb in *C. mucosalis* (Fig 2.2.A, lane 2). An additional signal was obtained with a 8 kb band in this strain; however, this is assumed to be undigested DNA. A weak signal was obtained with a 4.1 kb fragment in *C. jejuni*. Strong hybridisation signals were obtained with four bands (3.0, 1.16, 0.5 and 0.3 kb) with *C. concisus* NCTC 11485. Weak signals obtained with two additional bands (1.5 and 1.8 kb) in this strain probably represent

incompletely digested DNA. With respect to the 4 clinical isolates of *C. concisus*, signals were obtained with bands of 1.6 kb and 0.5 kb only. The presence of common bands in the *C. concisus* isolates, combined with the fact that these bands were not present in *C. jejuni* and *C. mucosalis* suggested that that they could be used as identification markers.

The recombinant plasmid containing the *Bgl*II insert responsible for the observed hybridisation patterns was designated pB3C. To refine the probe further, a limited restriction map of pB3C was determined (Fig. 2.3).

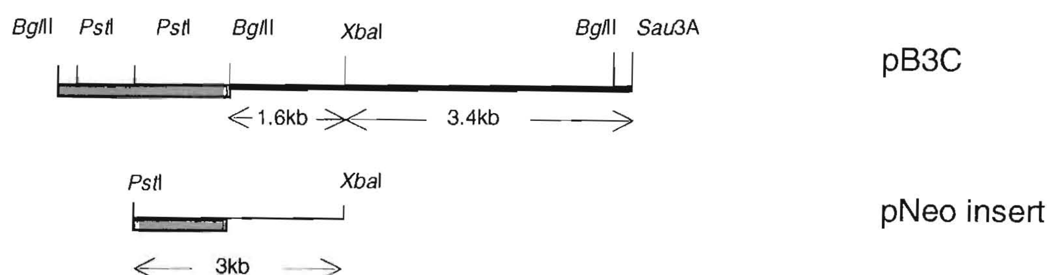


Fig 2.3. Restriction map of insert in pB3C. Red indicates pEcoR251 sequence; 3.0 kb fragment (red/black) was cloned into pUC19, generating pNeo.

Plasmid pB3C contains an insert of 5kb. Limited restriction mapping showed that the insert has an internal *Bgl*II site 400bp from one of the *Sau3*AI sites (Fig 2.3). Restriction of the 5 kb *Sau*3AI fragment with *Bgl*II and *Xba*I dissected the insert into two fragments of 1.6 kb and 3.0 kb. The fragments were separated on a 0.8% agarose, gel purified, labelled and used to probe the same blot shown in Fig 2.2A, in separate assays. The 3.0 kb fragment gave a strong hybridisation signal with a 3.0 kb band, and weak hybridisation signals with 0.5 kb and 0.3 kb bands in *C. concisus* NCTC11485. Hybridisation signals, albeit weak, were also obtained with 1.16 kb bands in two of the Danish clinical isolates (Fig. 2.2B lanes 4 and 5).

When the 1.6 kb *XbaI*/*Bgl*II fragment was used as a probe, strong signals were obtained with bands of 1.16 kb and 0.5 kb, and a weak signal was obtained with a 0.3 kb band in *C. concisus* NCTC 11485. A hybridisation signal was obtained with a 1.6 kb band in all the clinical *C.*

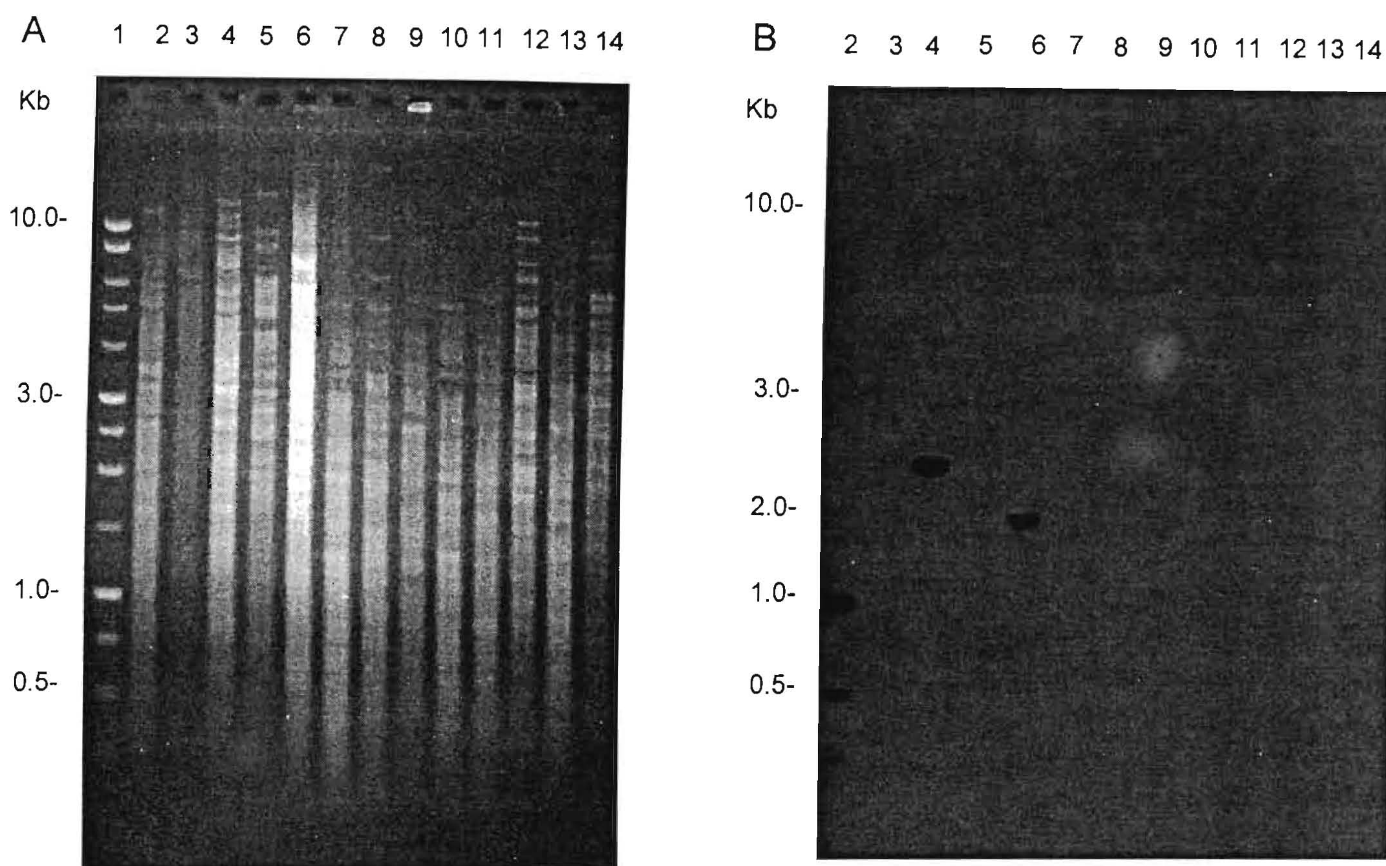


Fig.2.4 Genomic DNA from *Campylobacter* type species hybridised with the 1.6 kb fragment from pNeo.
A. DNA from *Campylobacter* species digested with *Hin*dIII; lane 1, 1 kb marker, lane 2, *C. concisus* NCTC11485; lane 3, *C. mucosalis* NCTC11000; lane 4, *C. curvus* NCTC11649; lane 5, *C. rectus* NCTC11489; lane 6, *C. sputorum* subsp *fecalis* NCTC11415; lane 7, *C. coli* CCUG11283; lane 8, *C. jejuni* NCTC11168; lane 9, *C. lari* NCTC11352; lane 10, *C. upsaliensis* NCTC12183; lane 11, *C. helveticus* NCTC12470; lane 12, *Bacteroides uroelyticus* NCTC10941; lane 13, *C. jejuni* subsp *doylei* NCTC10847; lane 14, *C. fetus* subsp *fetus* NCTC10842.
B Autoradiograph of DNA shown in (A) probed with the 1.6 kb *Bgl*II-*Xba*I fragment.

2.3.4 Hybridisation profiles among *C. concisus* clinical strains

To evaluate further the 1.6 kb fragment as an identification tool for *C. concisus*, DNA from 52 clinical isolates of *C. concisus* from the Children's Red Cross Memorial Hospital was hybridised to the 1.6 kb probe. Analysis of all the hybridisation profiles showed a limited number of restriction fragment length polymorphisms among the isolates. It is important to

note that the profiles described below were reproducible. Four profiles could be identified amongst the 52 isolates (Table 2.2).

Table 2.2 Summary of RFLP groups identified by probing with 1.6kb fragment

Profile group	I	II	III	IV
Fragments to which probe hybridised	1.16kb 0.5kb 0.3kb	1.6kb 0.5kb 0.3kb	0.8kb 0.5kb	1.16kb 0.5kb
Isolates	314-96 392-96 393-96 389-96 182-97	1-96 215-96	199-97 25-94 271-93 286-93 311-98 199-99	157-95, 238-95 218-96, 207-96 275-96, 297-96 330-96, 334-96 337-96, 341-96 343-96, 361-96 316-98, 133-95 291-92, 58-93 204-92, 285-93 282-93, 361-93 339-94, 68-95 211-96, 377-96 394-96, 395-96 396-96, 402-96 404-96, 406-96 415-96, 20-93 426-96, 173-97 308-98, 146-99

The first group, designated profile I comprised 5 isolates, which had a profile identical to NCTC 11485. The probe hybridised to bands of 1.16 kb, 0.5 kb and 0.3 kb in strains 314-96, (Fig. 2.5C, lane 8), 392-96, 393-96, 389-96, 182-97 (Fig. 2.5B, lane 3, 4, 8 and 15).

Profile II, was present in only two isolates. In these isolates the probe hybridised to fragments of 1.6 kb, 0.5 kb and 0.3 kb in strains 1-96 and 215-96 (Fig. 2.5B, lanes 1 and 2 and repeated in panel 2.5D, lanes 6 and 7).

Profile III was observed in six strains. This profile was characterised by fragments of 0.8 kb and 0.5 kb that hybridised to the probe. The isolates with this profile are 199-97, 25-94, (Fig. 2.5B, lanes 5 and 7), 271-93, 286-93, 311-98 and 199-99 (Fig. 2.5D, lanes 3, 4, 13 and 15). The largest group (36 strains) comprised profile IV. All of these strains contained bands of 1.16 and 0.5 kb, which hybridised to the probe.

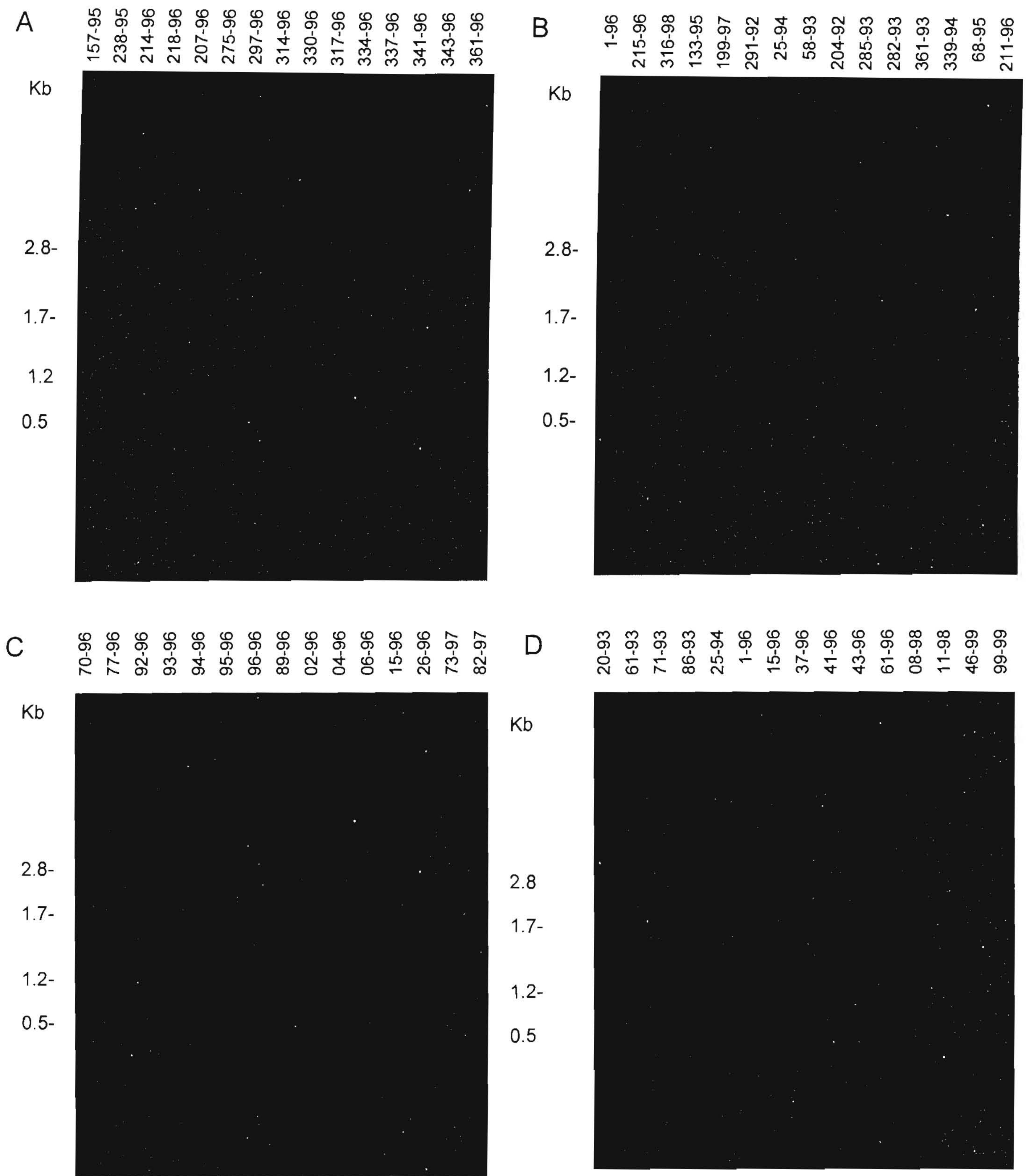


Fig. 2.5. Autoradiograph of *C. concisus* DNA digested with *Hin*dIII following hybridisation with 1.6 kb fragment.

It is not possible to assign a profile to strain 214-96, (Fig 2.5A lane 3) because the DNA from this strain was not digested to completion. Interestingly no hybridisation signals were obtained for strains 317-96 (Fig. 2.5A lane 10) and 370-96 (Fig. 2.5C lane1), presumably because they are not *C. concisus*. These strains are addressed in Chapter 3.

Similar hybridisation studies were carried out on an additional 15 clinical *C. concisus* strains taken to represent genetically diverse groups by pulsed field gel electrophoresis (PFGE) typing (Chapter 4). Strains 205-94, 47-99, CCUG 13144, CCUG 19995, 38-99, 64-99 and 207-94 (Fig. 2.6 lane 1, 2, 4, 6, 11, 12 and 13) have profile type I. One isolate 24-99 (Fig. 2.6 lane 14) had a unique profile: the 1.6 kb probe hybridised to fragments of 1.6 kb, 1.16 kb and 0.5 kb. Seven strains demonstrated profile type II.

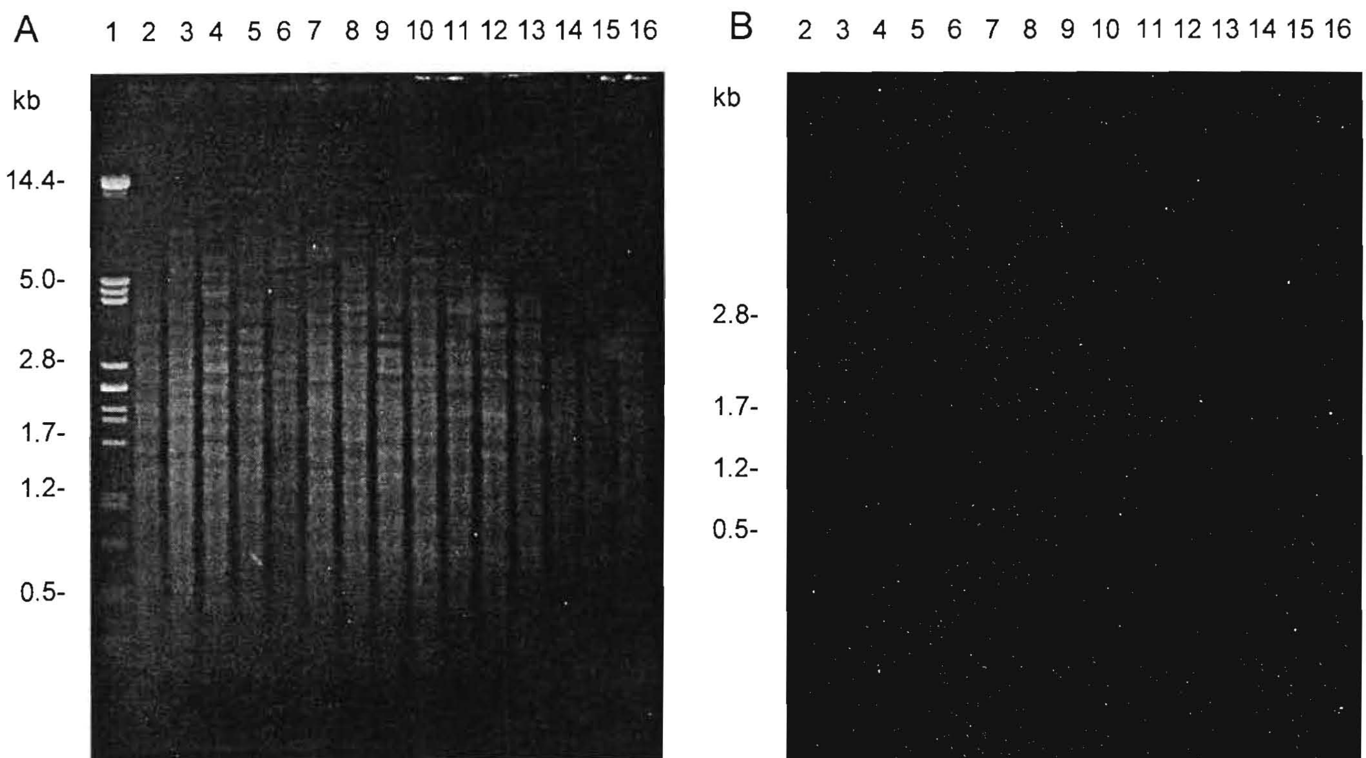


Fig. 2.6. Genomic DNA from genetically diverse *C. concisus* strains hybridised to the 1.6 kb fragment from pNeo.
A. DNA from *C. concisus* isolates digested with *Hin*dIII; lane 1, PstI digest of lambda,; lane 2, 205-94; lane 3, 47-99; lane 4, 204-94; lane 5, CCUG13144; lane 6, 51-99; lane 7, CCUG19995; lane 8, 52-99; lane 9, 61-99; lane 10, 45-99; lane 11, D9; lane 12, 38-99; lane 13, 64-99; lane 14, 207-94; lane 15, 24-99; lane 15, 204-93.
B. Autoradiograph of DNA shown in (A) probed with the 1.6 kb fragment.

Significantly, all of the strains examined contained a 0.5 kb *Hin*dIII fragment which hybridised to the 1.6 kb probe, suggesting a genetic marker for the identification of *C. concisus*. As a first step to gaining further insight into the DNA sequence of the 0.5 kb fragment, the DNA sequence of the 1.6 kb fragment was determined. To facilitate sequencing, a 3.0 kb *Pst*I-*Xba*I fragment (Fig. 2.3), which included a portion of pEcoR251 (1.375kb *Pst*I-*Bgl*II fragment) and the 1.6 kb *Bgl*II-*Xba*I fragment from *C. concisus* was subcloned into the *Pst*I–*Xba*I site of pUC19 and introduced into *E. coli* LKIII. The recombinant plasmid was designated pNeo.

2.3.5 Sequence analysis of 1.6 kb fragment from *C. concisus*

The DNA sequence of the 1.6 kb fragment from *C. concisus* was determined on both strands (Fig. 2.7). Three major open reading frames (ORF) were located on the sense strand, while no significant coding region was apparent on the negative strand. The first ORF (ORF1), extends from the start of the *C. concisus* sequence at nucleotide 2 and ends with a stop codon (TAA) at nucleotide 370. The second open reading frame ORF2, immediately downstream of ORF1, is located at nucleotides 424 to 858 and the third, ORF3 starts with ATG (nt. 1064) and terminates in TAG (nt. 1537). The deduced amino acid sequence for each ORF is given below the nucleotide sequence.

Analysis of the DNA sequence upstream of ORF1 did not identify any bacterial transcription signals; this combined with the size of the ORF (nt. 2-370) suggested that the ORF extends upstream and beyond the start site of the cloned fragment. A comparison of the deduced amino acid sequence of this ORF with the existing sequence databases (Swissprot release) using Blastx (Altschul *et al.*, 1990) showed that the gene encoded the 3' end of the GyrB subunit. An alignment of the amino acid sequences with corresponding regions of GyrB proteins of *C. jejuni* and *Helicobacter pylori* showed 76% and 58% similarity to the proteins of *C. jejuni* and *H. pylori*, respectively.

The second and third ORFs (ORF2 and ORF3) are preceded by ribosomal binding sites (RBS) upstream (6 bp) of the ATG transcription initiation sites. The sequences are identical to the *C. jejuni* consensus sequence AAGGA described by Wosten *et al.*, 1998, which is shorter than that (AGGAGG) proposed by Kim *et al.*, (1995) for *C.jejuni*.

The sequences immediately upstream of the start codons of ORF2 and ORF3 are AT rich. Putative promoter sites resembling the –35 (TTTAAGTNTT) and –10 (TATAAATT) consensus sequences described for *C. jejuni* (Wosten *et al.*, 1998) were identified for each ORF. The putative promoter for ORF2, consists of a -35 sequence (TTTAAAAGG), separated by 20 bp from a –10 (TAGTAA) (Fig. 2.7). ORF3 is preceded by the –35 TTTAAACAAA, and -10 TGATAA promoter sequence. The -16 (TTTTTTTG)N consensus promoter sequence for *C. jejuni* as proposed by Wosten *et al.* 1998, was found to be lacking for both ORFs.

The translation product of ORF2 was found to share significant similarity to a conserved hypothetical protein of 127 amino acids in *C. jejuni* (CDS Cj 1724c). Alignment of the hypothetical protein with deduced amino acid sequence of ORF2 showed 70% sequence identity. The product of ORF3 was found to share 70% similarity with the first 142 amino acids at the amino terminal of a 408 amino acid conserved hypothetical protein of *C. jejuni* (CDS Cj 0015c).

From the DNA sequence it is clear that there is an internal *Hin*dIII-*Hin*dIII fragment that corresponds to the 300bp hybridisation signal. Since the fragment to the left of this (towards the *Xba*I) is too long to contain the *Hin*dIII 500bp fragment, this must be the sequence hybridising to the 1.16 *Hin*dIII-*Hin*dIII fragment. The other end hydridises to the 500bp *Hin*d III-*Hin*dIII fragement. This was confirmed by PCR derived probes [Chapter 2.3.6].

```
      Pcisus1 ------------------>
1     TCTAGAAGAGCTTGTGGTAAATGAAAATTTATTCACAAATCCACTTTATGAAGAGGCACT
        L  E  E  L  V  V  N  E  N  L  F  T  N  P  L  Y  E  E  A  L
      XbaI
61    TTATATCAGCCAAAAGATAAAAGAGCGCGGCCTAGACTTGCATAGTGACGTTATAGACGT
        Y  I  S  Q  K  I  K  E  R  G  L  G  L  H  S  D  V  I  D  V

121   GCTTGATGAAGTAGAGAAAAATGCGAAAAAAGGTGCATATATCCAGCGTTATAAAGGTCT
        L  D  E  V  E  K  N  A  K  K  G  A  Y  I  Q  R  Y  K  G  L

181   TGGTGAGATGAACCCTGAGCAGCTTTGGGAGACTACGATGAACCCTGAGAACAGAAGACT
        G  E  M  N  P  E  Q  L  W  E  T  T  M  N  P  E  N  R  R  L

241   TTTAAAGATCGATATAAACGACGCTATAAGCGCCTCTGATACGTTTAATCTCTTTATGGG
        L  K  I  D  I  N  D  A  I  S  A  S  D  T  F  N  L  F  M  G
                                 <------------------ Pcisus2
301   CGATGAGGTCGAGCCAAGAAGAAACTATATCCAAGACCACGCAAAAGACGTTAAACACTT
        D  E  V  E  P  R  R  N  Y  I  Q  D  H  A  K  D  V  K  H  L

361   AGATATTTAAAAGGTGATCAAATTTAAATTTGACCTAGTTAGTAAAAGTATAAAGGATAA
        D  I  *-35                        -10                RBS
                                                   Pcisus3 ------
421   AAAATGAGCGAAGAGCTAGATATGAAATATGGCGAGAAAATTTTGAAAGAATTTGACGTA
          M  S  E  E  L  D  M  K  Y  G  E  K  I  L  K  E  F  D  V
      ----------->
481   GAGAGTGACCTTGAGGTCTGGGAAAATAAGCAAACAAGAGACTATGTCATAAAGATTACT
       E  S  D  L  E  V  W  E  N  K  Q  T  R  D  Y  V  I  K  I  T

541   CTGCCTGAGTTTTGCTGCCTTTGCCCTCGCTCTGGTTATCCTGACTTTGCGACGATCTAT
       L  P  E  F  C  C  L  C  P  R  S  G  Y  P  D  F  A  T  I  Y

601   CTTGAGTATATCCCAAATAAGTTAGTTGTTGAGCTAAAAGCGATAAAGCTTTATATAAAT
       L  E  Y  I  P  N  K  L  V  V  E  L  K  A  I  K  L  Y  I  N
                                                  HindIII
661   AGTTTTATGAACCGCAACATCAGCCATGAAGATAGTATAAATGGAATTTACTCTGTTTTA
       S  F  M  N  R  N  I  S  H  E  D  S  I  N  G  I  Y  S  V  L

721   GAGAAAAAGCTAGAGCCAAAATTTATGAAGATAGTTGGCGACTTTAACCCACGTGGAAAT
       E  K  K  L  E  P  K  F  M  K  I  V  G  D  F  N  P  R  G  N
                <------------------- Pcisus4
781   GTCCATACAGTTATCGAGATCAGCTCTGATCTAGTGGTGAAAAGCCAGTTGAGGAGAAGA
       V  H  T  V  I  E  I  S  S  D  L  V  V  K  S  Q  L  R  R  I

841   ATTTACTCCAAGAACTAGAGAGAGAAGTAGCTTTGGTGATAAGCCACGTGAGAGATCGTA
       Y  S  K  N  *

901   GCACGAGCTATCGTGGCAGTAGTAGAGCTGGTGGCAGCAGAGGTGGCAGAGATGATAAAT


961   TTCAAAAAGATGACAAACCAAGAAGAAGTTTAAACAAAGAGGGCTTTAGAAAAATAAGCT
                                    -35

1021  ATGCCGATGATAAGAAGCCAAAAGTAGTCAAAAAGGATAAATTATGATAAGTGCTAAGCT
               -10                     RBS            M  I  S  A  K  L
            Pcisus5 ----------------->                            HindIII
1081  TATAGAACATATCTTTAAAGCAGCATCTATATCACGTTGGAACGACTATCCAAAGATGGC
        I  E  H  I  F  K  A  A  S  I  S  R  W  N  D  Y  P  K  M  A

1141  AAATTTAGTCGAGCTTGATAAACAGGCTCATAAATTTATCATCGCTTATTTCATAGCAAA
        N  L  V  E  L  D  K  Q  A  H  K  F  I  I  A  Y  F  I  A  K

1201  ACAAGGGCAAAATGCCGACATAAACTATATCATAGAGGCTGGAATTTTTGAGTTTTTAAG
```

```
        Q  G  Q  N  A  D  I  N  Y  I  I  E  A  G  I  F  E  F  L  S
1261    CAGGGTCGTAGTCACAGACATACGACCAGATGTCTTTCATCACATCCAAAAGACAAAAAA
        R  V  V  V  T  D  I  R  P  D  V  F  H  H  I  Q  K  T  K  K
1321    AGGGCAGATAAATAGCTGGGTCTTAAGCAACCTAGAGGAGCTGATCTCGGAAATAGAAGA
        G  Q  I  N  S  W  V  L  S  N  L  E  E  L  I  S  E  I  E  D
1381    TGGCAAATTTTTAGAGAGATTTAAAAATCACTTTAAAAACGACAAAACCCACGAAAAAGA
        G  K  F  L  E  R  F  K  N  H  F  K  N  D  K  T  H  E  K  E
                                                           <--------
1441    GCGTCTCATCCTAAAAGCAGCGAGCTATCTTGCCACGGTGGGAATTTTCTATCGCCTAT
        R  L  I  L  K  A  A  S  Y  L  A  T  G  G  N  F  L  S  P  I
  ---------Pcisus6
1501    CAAACGGGCCAATTTTAAGCGATATCGACGAGCTTAGGCGAAGGTTGAAGAGGAGATGGA
        K  R  A  N  F  K  R  Y  R  R  A  *
1561    GGATTATTATGAACTAATTGGCGTTAGAAAGATCT
                                    BglII
```

Fig.2.7 The nucleotide sequence of the 1.595 kb insert cloned into pUC19 (pNeo). The deduced amino acid sequence of ORF1 (3' end of gyrase B subunit), ORF2 and ORF3 are indicated below the nucleotide sequence. Potential ribosomal binding sites (RBS) are underlined. The stop codons are indicated by an asterix. The *Xba*I and *Bgl*II restriction sites indicating the extent of the 1.595 kb insert are indicated in bold type. The location of the internal *Hin*dIII restriction sites is also shown in bold text.

2.3.6 Hybridisation studies using DNA sequences internal to ORF, 1, 2 and 3 as probes.

To identify the DNA sequence responsible for the hybridisation signal observed with 500 bp band in all isolates of *C. concisus* studied, primers were designed to amplify portions of each ORF shown in Fig.2.7. This approach was necessary because of the lack of appropriately located restriction sites in the 1.6 kb sequence. The PCR products obtained were separated on a 0.8% agarose, gel purified, labelled and hybridised to the membrane containing DNA from the panel of *Campylobacter* species used previously for determining the specificity of the 1.6 kb insert in Fig. 2.4.

When the fragment internal to ORF1 (*gyr*B) was used to probe the blot, a strong signal was obtained with a 1.16 kb band in *C. concisus* (Fig.2.8B, lane 1). In addition a hybridisation signal was obtained with the 2.4 kb band in *C. curvus* (Fig. 2.8B, lane 3), a 2.1 kb band in *C. sputorum* bv. *fecalis* (Fig.8B, lane 5) and a 0.5 kb band in *C. helveticus*. Weak hybridisation

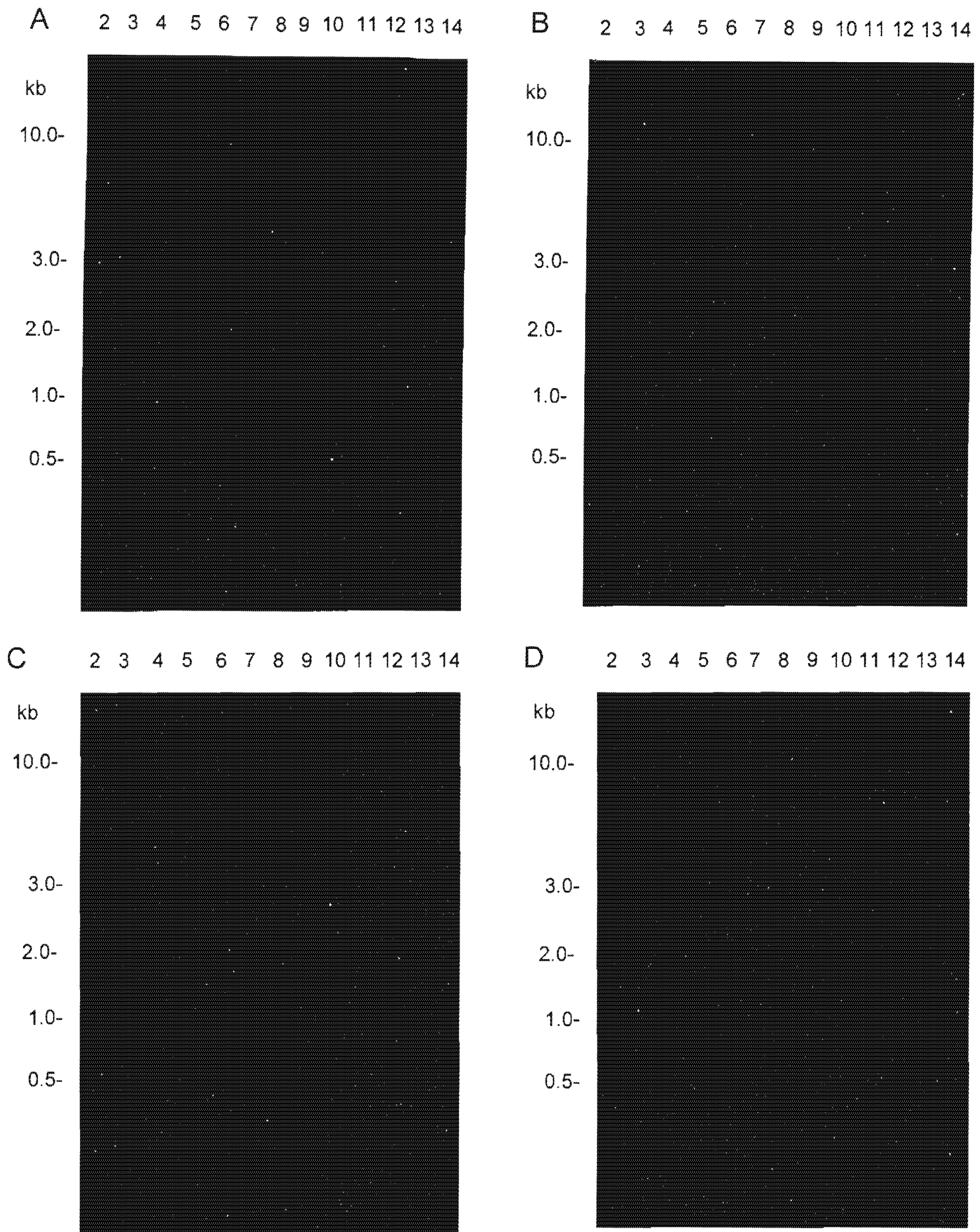


Fig. 2.8 Autoradiographs of *Hin*dIII digested genomic DNA from *Campylobacter* species shown in Fig.2.4A hybridised to fragments internal to ORF1, ORF2 and ORF3.
A. Autoradiograph of DNA hybridised with 1.6kb fragment from pNeo.
B. Autoradiograph of DNA hybridised with 0.335 kb fragment internal to ORF1
C. Autoradiograph of DNA hybridised with 0.338 kb fragment internal to ORF2
D. Autoradiograph of DNA hybridised with 0.414 kb fragment internal to ORF3

signals were obtained with 1.5 kb and 1.8 kb fragments in *C. concisus* and with fragments larger than 1.2 kb in *C. mucosalis, C. cuvus, C. rectus, C. sputorum* bv. *fecalis, C. coli, C. upsaliensis, C. helveticus, Bacteroides ureolyticus*, and *C. jejuni doylei*. No hybridisation signals were obtained for *C. jejuni, C. lari* and *C. fetus* subsp *fetus*.

Similar studies using the probe for ORF2 resulted in strong signals with bands of 1.16 kb and 0.3 kb in *C. concisus* (Fig. 2.8C, lane 1), indicating that the amplified sequence contains a *Hin*dIII site. Analysis of the DNA sequence showed this to be the case: a *Hin*dIII site is present at nucleotides 641-646 in ORF2. Weak signals were observed with bands (0.5 kb, 1.5 kb and 1.8 kb) in this strain. A strong hybridisation signal was also obtained with a 2.4 kb band in *C. curvus*. Weak signals were obtained with DNA from *C. rectus, C. sputorum* bv. *fecalis* and *Bacteroides ureolyticus*.

As the membrane shown in Fig 2.4 had been used in a number of experiments, a fresh blot was prepared. Conditions with respect to the amount of DNA and hybridisation were the same as those used in the preparation of the blot shown in Fig. 2.4. Probing with the PCR product of ORF3 resulted in a strong signal with the 500 bp fragment in *C. concisus* (Fig 2.8D). Weak signals were obtained with bands of 0.7 kb and 5 kb in this strain (probably undigested DNA). Significantly, no signals were observed with DNA from *C. curvus* and *C. sputorum* bv. *fecalis,* or indeed with DNA from any of the other *Campylobacter* species.

In another hybridisation experiment, the internal fragment from ORF3 was used to probe membranes containing DNA from isolates representing three of the profile groups (Fig 2.5D) described in subsection 2.3.4. A strong hybridisation signal was obtained with a 500 bp band in all of the *C concisus* strains (Fig. 2.9), indicating that ORF3 was responsible for the signal with the 0.5 kb fragment observed in earlier experiments (2.3.4). As the 0.5 kb hybridisation band was also present in the fourth profile group represented by the NCTC11485 type strain (Fig. 2.8D lane 1), it is conceivable that ORF3 hybridises to the 0.5 kb DNA fragment in all hybridisation profiles observed for *C. concisus*. The presence of the 0.5 kb fragment in all C. concisus investigated, and its absence in the other campylobacters, suggests that it is unique to *C. concisus* and could be used as an identification marker.

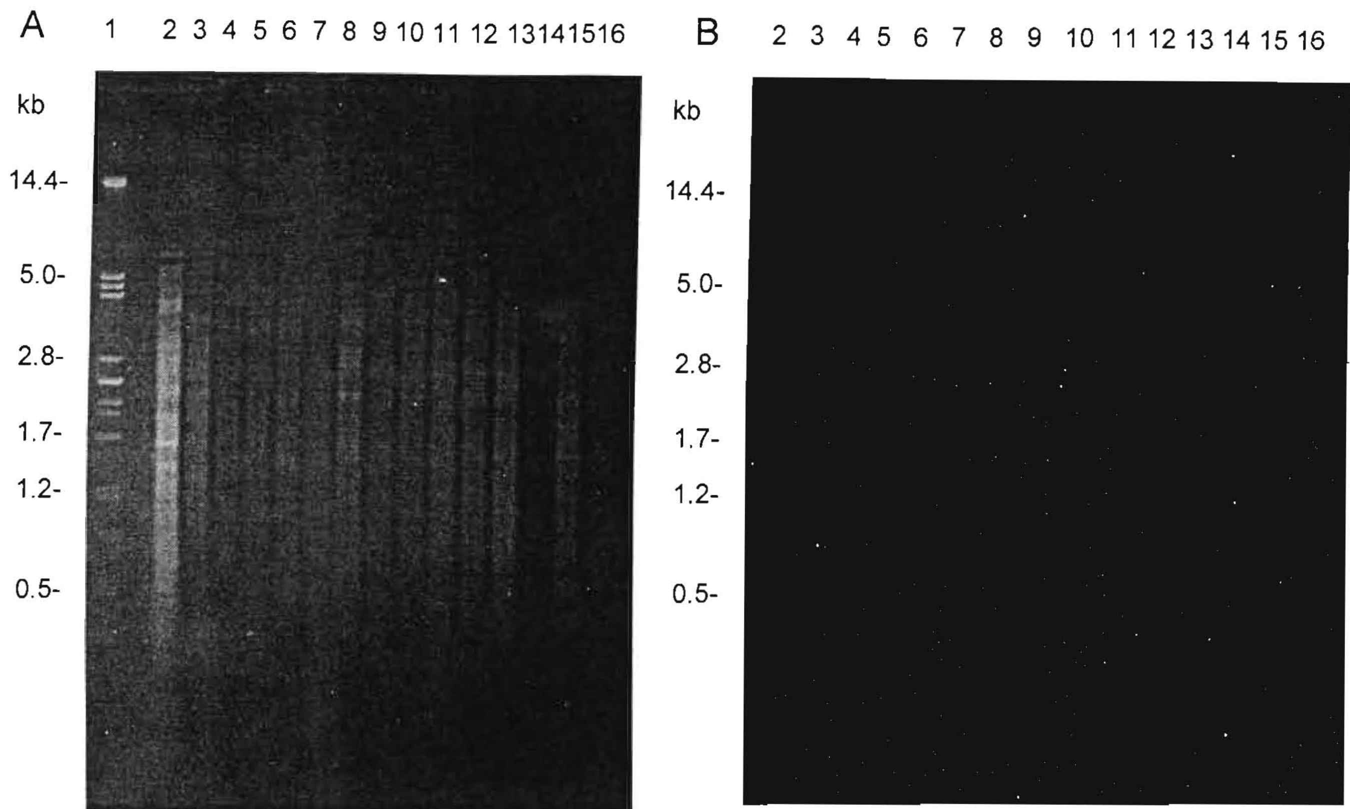


Fig. 2.9. Genomic DNA from *C. concisus* probed with portion of ORF3
A. Genomic DNA from *C. concisus* isolates digested with *Hin*dIII; lane 1, *Pst*I digest of lambda; lane 2, 20-83; lane 3, 361-93; lane 4, 271-93; lane 5, 286-93; lane 6, 25-94; lane 7, 1-96; lane 8, 215-96; lane 9, 337-96; lane 10, 341-96; lane 11, 343-96; lane 12, 361-96; lane 13, 308-98; lane 14, 311-98; lane 15, 146-99; lane 16, 199-99
B Autoradiogram of DNA shown in (A) hybridised to 0.414 kb fragment internal to ORF3.

2.4 DISCUSSION

The fastidious nature and relatively inert biochemical activity of *Campylobacteria* makes them difficult to identify using phenotypic and biochemical tests. The increase in incidence of *C. concisus* in faecal samples of children with diarrhoea has highlighted the necessity for a rapid and reliable identification technique. In the absence of such a technique, the epidemiological and clinical significance of *C. concisus* cannot be investigated. Molecular techniques offer an alternative approach to identification.

A 1.6 kb fragment, containing a portion of *gyr*B (ORF1) and two other ORFs, isolated from a *C. concisus* genomic library, generated hybridisation patterns unique to *C. concisus* strains. This pattern was observed in 63 clinical isolates examined as well as in three reference strains which included *C. concisus* NCTC 11485 type strain. When the same DNA sequence was used to probe DNA from other *Campylobacter* species, signals were obtained with only two species: *C. sputorum* bv. *fecalis* and *C. curvus*. The hybridisation profiles observed in these two strains could not be confused with the profiles identified in *C. concisus* (Fig 2.5). However, that a signal was generated with the DNA from *C. sputorum* bv. *fecalis* and *C. curvus* suggests that these species may be more closely related to *C. concisus* than are the other species. This is supported by phylogenetic rRNA studies (Vandamme *et al.*, 1995) which demonstrated an 86% similarity level between the sequences of the two species. Significantly, no signal was detected for *C. mucosalis* a bacteria phenotypically similar and sometimes misidentified as *C concisus* (Figura *et al.*, 1993).

A total of five RFLP profile patterns were observed in the *C. concisus* strains probed. All of the strains contained a 0.5 kb band that hybridised to the probe. The profiles obtained were consistent for both local and Danish clinical isolates. In addition, a profile indicative of *C. concisus* was observed in two reference strains CCUG 13144 and CCUG 19995, identified by Vandamme *et al.* (1989) as falling into two DNA: DNA hybridisation homology groups. This is an indication that this hybridisation pattern is probably universal among *C. concisus* and not confined to a specific geographic region.

DNA sequence analysis of the 1.6 kb fragment revealed a total of three open reading frames. ORF1 comprised the 3' end of the *gyr*B gene, which included the conserved domain of the carboxyl–terminal, belonging to the protein family of prokaryotic DNA topoisomerase II (Pfam:PF00986). This region of the carboxyl terminus is thought to support complex formation with the DNA gyrase A protein and assists in ATP-independent relaxation of supercoiled DNA (Roca, 1995). In this respect, that signals were obtained with DNA from the majority of *Campylobacter* species, following hybridisation with a portion of ORF1, was not surprising. Strong signals were obtained with DNA from *C. curvus*, *C. sputorum* and *C. helveticus* (Fig 2.8B), suggesting that the *gyr*B genes of these species are closely related to their counterpart in *C. concisus*.

The fragment internal to ORF2 hybridised to DNA from *C. concisus* and *C. curvus*. As ORF2 contains a *Hin*dIII site, two bands (1.16 kb and 0.3 kb) in *C. concisus* hybridised to the probe. On the other hand, only one band (2.4 kb) in the DNA from *C. curvus* hybridised to the probe. Since ORF 1 also hybridised to a fragment of 2.4kb in this strain it is likely that as in *C. concisus*, ORF1 and ORF2 are contiguous in *C. curvus*. Weak hybridisation signals were observed with DNA restriction fragments from *C. rectus*, *C. sputorum* bv. *fecalis* and *Bacteroides ureolyticus*, but no hybridisation signals were detected in other *Campylobacter* species.

Hybridisation studies using a fragment internal to ORF3, showed that this sequence was responsible for the signal obtained with the 0.5 kb band in *C. concisus*. That all of the *C. concisus* strains investigated contained this 0.5 kb fragment suggests that the *Hin*dIII sites at the 5' and 3' sites of ORF 3 are conserved. Signals were not obtained with the DNA from any of the other *Campylobacter* species.

Other workers have developed species specific probes from randomly cloned genomic fragments (Gebhart *et al.*, 1989; Stanley *et al.*, 1992; Bustamante *et al.*, 1995). In these studies probes were used in colony blot hybridisation assays, with the aim of making them appropriate for routine diagnostic purposes. The assay described in this work does not depend on the specific sequence homology for identification. Rather, the gene arrangement and loci of the three open reading frames identified seem to be unique to *C. concisus*. With species-specific

probes designed as described above, chances of cross-hybridisations in closely related species is always a possibility. In the study by Bustamante *et al.* 1995, Cj01 a species specific probe for *C. jejuni* was found to hybridise to some clinical isolates of *C. coli*, while in another study, two of the three probes developed for the specific identification of *C. helveticus*, were found to cross-hybridisation with *C. upsaliensis* DNA (Stanley *et al.*, 1992).

Campylobacter have been described as having fairly fluid genomes susceptible to recombination with extragenic DNA (Wang and Taylor, 1990). This is thought to be responsible for the observed high level of genetic heterogeneity among most members of this genus. This raises the question of the stability of the locus of the 1.6 kb fragment and in turn, the stability and reliability of the hybridisation patterns identified. The complete genome map of *C. jejuni* suggests that the counterparts of *gyr*B, ORF2 and ORF3 identified in *C. concisus* are located close to the origin of replication, a conserved region of the bacterial chromosome (Ogasawara and Yoshikawa, 1992). Thus it could be that the patterns observed in *C. concisus* may be stable, although this would have to be monitored over time.

CHAPTER 3

IDENTIFICATION OF *CAMPYLOBACTER CONCISUS* USING POLYMERASE CHAIN REACTION

3.1 INTRODUCTION

The polymerase chain reaction (PCR), due to its relative ease and speed, has become an attractive option for diagnostic purposes as a rapid identification tool for microorganisms (Thiele *et al.*, 1990). PCR has also been exploited in numerous studies for the identification of *Campylobacter* (Bastyns *et al.*, 1995; Cardarelli-Leite *et al.*, 1996; Eyes *et al.*, 1993; Hurtardo and Owen, 1997; Linton *et al.*, 1997; Marshall *et al.*, 1999). Essential to the development of most species-specific PCR identification methods, is prior knowledge of the target DNA sequence. Consequently, most of the PCR identification methods are based on rRNA gene sequences, because these sequences are readily available due to their taxonomic value as phylogenetically conserved genetic loci.

PCR methods targeting rRNA sequences rely on primers designed to anneal to specific sequences of conserved nucleotides within rDNA, and exploit the variable regions within this locus to generate species specific amplicons. Methods targeting 23S rRNA genes have been described for the identification of thermophillic campylobacters (Eyes *et al.*, 1993) and for *C. concisus* (Bastyns *et al.*, 1995). However, for some PCR methods targeting 23S rRNA (Fermer and Engvall, 1999; Hurtardo and Owen, 1997) and 16S rRNA genes (Cardarelli-Leiti *et al.*, 1996; Marshall *et al.*, 1999), species differentiation requires an additional step of restriction fragment length analysis of the amplicons.

Apart from ribosomal genes some of the genes which have proved useful for PCR identification purposes, and in most cases for the differentiation of the phenotypically closely related species, *C. jejuni* and *C. coli*, are hippuricase (Linton *et al.*, 1997), flagellin (Oyofo *et al.*, 1992), GTP binding protein (Van-Doorn *et al.*, 1997) membrane protein (MapA) (Stucki *et al.*, 1995) and an iron transport protein (CeuE) (Gonzalez *et al.*, 1997).

3.2.2 PCR assay for the identification of *C. concisus*, using pcisus1 and pcisus6

Oligonucleotide primer pcisus1 (5'-GAGCTTGTGGTAAATGA-3') which anneals to nucleotides 5-24 of the *gyr*B gene in ORF1 and pcisus6 (5'-CCCGTTTGATAGGCGATAG-3'), complementary to nucleotides 1508-1489 in ORF3 (Chapter 2 Fig 2.7) were used in amplification assays.

PCR mix was made up to 50 μl and consisted of 50 ng of genomic DNA, 2.5 U of Thermus aquaticus (Taq) polymerase (TaKaRa, biochemicals), 50 pM of primer, 2.5 nM of each dNTP in TaKaRa buffer (100 mM Tris-HCL (pH8.3), 500 mM KCL, 15 mM) (TaKaRa, biomedicals). The PCR was carried out in a Perkin Elmer (Gene Amp 2400) thermocycler, using the following parameters, an initial denaturation step at 94°C for 2 min, followed by 30 consecutive cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min. A final extension step at 72°C was carried out for 10 min.

3.2.3.Identification of Campylobacter using 16S-rDNA restriction fragment length polymorphism

Identification of *Campylobacter* species, was carried out using the amplified ribosomal DNA restriction analysis (ARDRA) method as described by Marshall *et al.* (1999).

Oligonucleotide primers CAH16S 1A (5'-AATACATGCAAGTCGAACGA-3') and CAH16S 1B (5'-TTAACCCAACATCTCACGAC-3') were synthesized by GIBCO-BRL (Life Technologies). Amplification reactions (50 μl) consisted of 50 ng of DNA, primers at 50 pM, 2.5 nM of each dNTP in TaKaRa buffer (TaKaRa biomedicals) and 1.25 units of Taq polymerase (TaKaRa biomedicals). The amplification cycle consisted of initial denaturation at 95°C for 2 min, followed by 30 cycles at 94°C for 1 min, 52°C for 45 secs and 72°C for 2 min. The PCR was completed by a final extension at 72°C for 10 min.

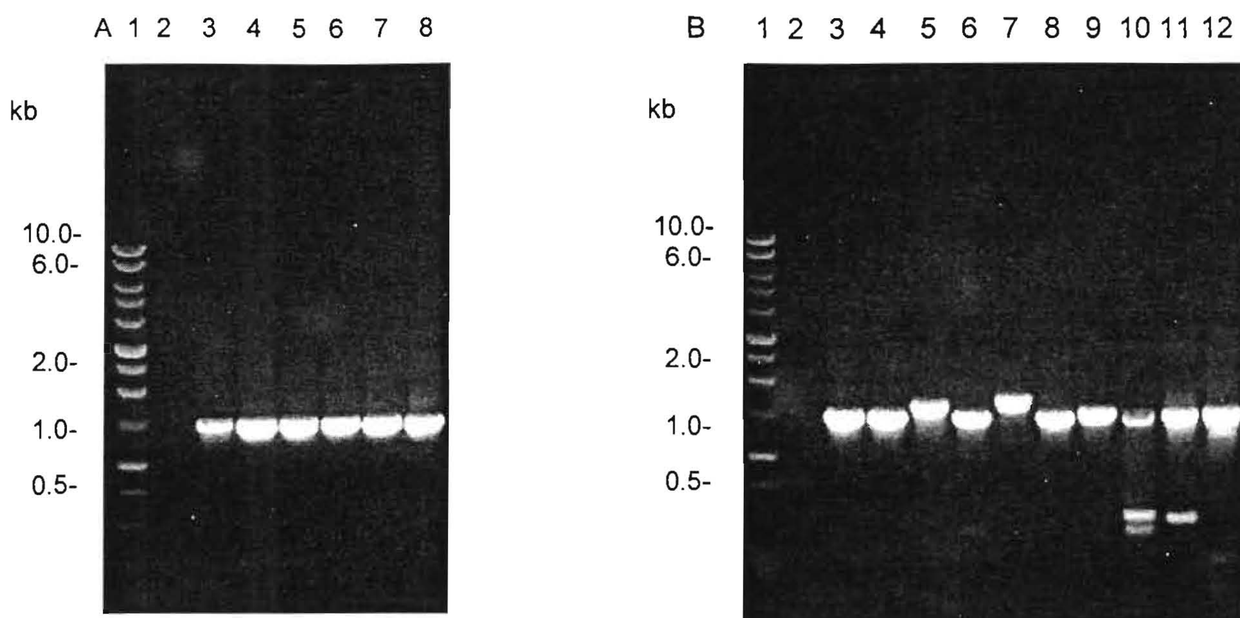


Fig 3.1 DNA from *Campylobacter* species and other bacteria amplified with 16S universal primers
A. lane 1, 1 kb DNA ladder (Promega); lane 2, No DNA template; lane 3, *C. concisus* NCTC11485; lane 4, *C. jejuni* NCTC11168; lane 5, *C. lari* NCTC11352; lane 6, *C. upsaliensis* NCTC12183; lane 7, *C. helveticus*12470; lane 8, *Bacteroides ureolyticus* NCTC 10941.
B. lanes 1, 1kb DNA Ladder (Promega); lane 2, No DNA template; lane 3, *C. concisus* NCTC11485; lane 4, C. *sputorum fecalis* NCTC11415; lane 5, *C curvus* NCTC116490; lane 6, *C. mucosalis* NCTC11000; lane 7, *C. rectus* NCTC11489; lane 8, *Helicobacter pylori*; lane 9, *Acinetobacter*; lane 10, *Mycobacterium BCG*; lane 11, *Esherichia coli*; lane 12, *Salmonella typhi.*

Using the primers directed against sequences of *gyr*B (pcisus1) and ORF3 (pcisus6) of the 1.6 kb *Xba*I-*Bgl*II fragment, originating in *C. concisus* [Chapter 2], a PCR product of the expected size (1.5 kb) was obtained for *C. concisus* type strain NCTC11485 (Fig 3.1A lane 3). No PCR product was obtained from *C. jejuni* NCTC11168, *C. lari* NCTC11352, *C. upsaliensis* NCTC12183, *C. helveticus* NCTC 12470, and *Bacteroides ureolyticus* NCTC 10941 (Fig. 3.2B lanes 4-8). Similarly in additional assays no PCR product were obtained for *C. sputorum fecalis* NCTC11415, *C curvus* NCTC11649, *C. mucosalis* NCTC11000, *C. rectus* NCTC11489 (Fig. 3.2B lanes 4-7) and from super-family member, *Helicobacter pylori,* (Fig 3.2B lane 8) or from the distantly related *Acinetobacter, Mycobacterium BCG, Esherichia coli,* and *Salmonella typhi.* (Fig 3.2B from lanes 9-12). It must be noted that PCR products were not obtained with *C. curvus and C. sputorum* subsp. *fecalis,* even though a hybridisation signal was obtained

when these strains were probed with the 1.6 kb fragment [Chapter 2 Fig 2.4]. This was an expected result as no hybridization signal was obtained with the probe for ORF3 and the DNA from these organisms [Chapter 2 Fig. 2.8D]. Thus, a primer set that includes a primer targeting ORF3, makes the assay specific for *C. concisus*.

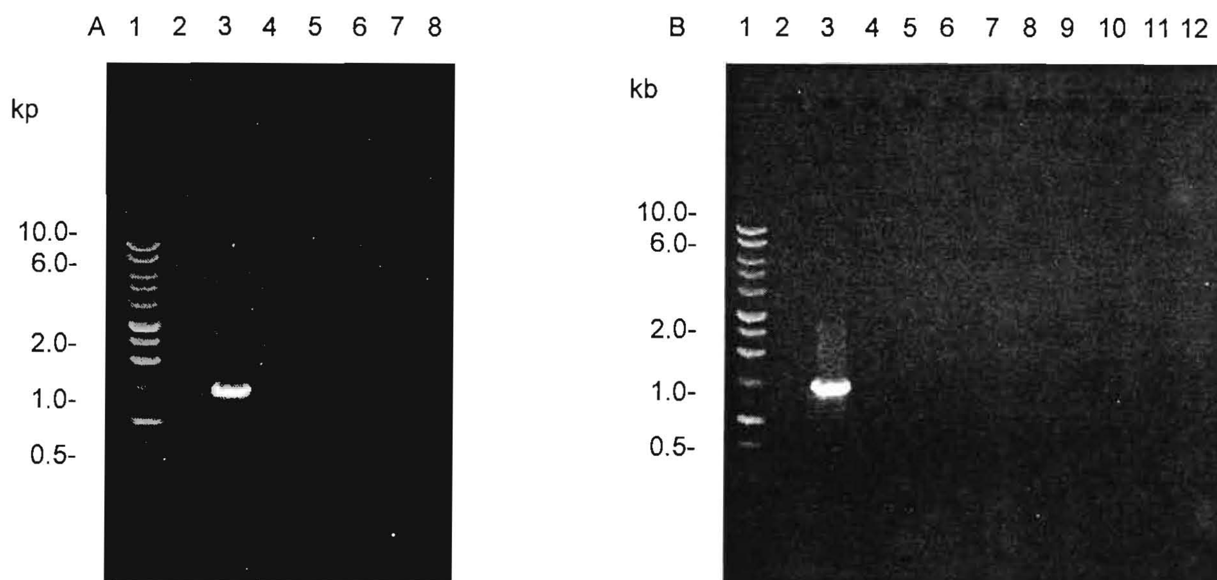


Fig.3.2. Amplicons using primers pcicus1 and pcisus6
A. lane 1, 1 kb DNA ladder (Promega); lane 2, No DNA template; lane 3, *C. concisus* NCTC11485; lane 4, *C. jejuni* NCTC11168; lane 5, *C. lari* NCTC11352; lane 6, *C. upsaliensis* NCTC12183; lane 7, *C. helveticus*12470; lane 8, *Bacteroides ureolyticus* NCTC 10941.
B. lanes 1, 1kb DNA Ladder (Promega); lane 2, No DNA template; lane 3, *C. concisus* NCTC11485; lane 4, C. *sputorum fecalis* NCTC11415; lane 5, *C curvus* NCTC116490; lane 6, *C. mucosalis* NCTC11000; lane 7, *C. rectus* NCTC11489; lane 8, *Helicobacter pylori*; lane 9, *Acinetobacter*; lane 10, *Mycobacterium BCG*; lane 11, *Esherichia coli*; lane 12 *Salmonella typhi*.

3.3.2 Evaluation of PCR for the identification of clinical isolates of *C. concisus*

Using pcisus1 and pcisus6, PCR assays were carried out on 92 clinical isolates, including the 52 clinical isolates previously used in hybridisation studies. With respect to the latter group, a single PCR product of the expected size (1.5 kb) was obtained from each of the 49 isolates which demonstrated a *C. concisus* specific hybridisation profile, showing good agreement between the hybridisation studies and the PCR assay. Although isolate 297-96, demonstrated a

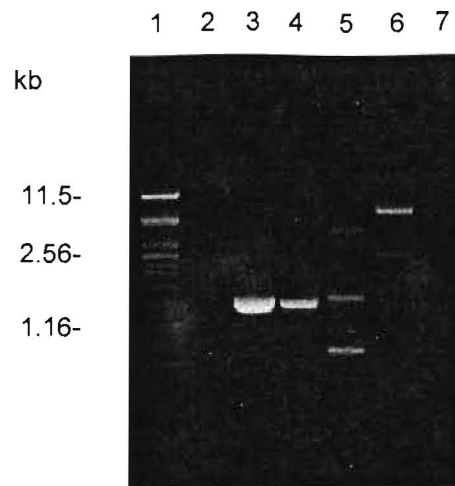


Fig. 3.3. PCR amplification with *C. concisus* specific primers pcisus1 and pcisus6.
Lane, 1 Lambda digested with *Pst*I; No DNA template; lane 3, *C. concisus* NCTC11485; lane 4,- 296-96; lane 5, 297-96; lane 6, 317-96; lane 7, 370-96,

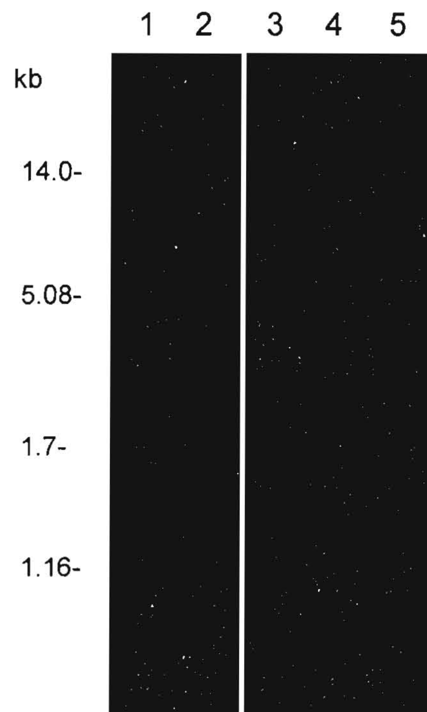


Fig. 3.4. Hybridisation with 1.6 kb *C concisus* species specific probe.
Lane 1-*C concisus* NCTC11485, 2- *C. curvus* NCTC 11649; lane 3, 297-96; lane 4, 317-96; lane 5, 370-96,

C. concisus specific hybridisation profile, 3 PCR products, of which one was the correct size, were obtained from this strain (Fig 3.3 lane 5). This strain is assumed to be *C. concisus*. Due to incomplete digestion of DNA, it was impossible to determine the hybridisation profile of 214-96, (Chapter 2 Fig.2.5A lane 2) however, an amplicon was obtained from this isolate (Results not shown) and it too is assumed to be *C. concisus*. In the preceding chapter no hybridization signals were obtained with DNA from 317-96 and 370-96 when these strains were probed with the 1.6 kb fragment [Fig.2.5A lane 10 and Fig.2.5C lane 1, respectively].On repeating the experiment, signals were obtained (Fig 3.4 lane 4 and 5), but not with fragments associated with *C. concisus*. The results of the PCR assay were consistent with this finding as amplicons were either not obtained or were not of the correct size (Fig. 3.3 lanes 6 and 7), suggesting that these strains are not *C. concisus* or the locus to which *gyr*B or ORF1 and ORF3 belong is not conserved in these strains. Additional studies using ARDRA (Marshall *et al.*, 1999) were performed on these two strains. Following amplification of rDNA and digestion with *Dde*I, the restriction profile of 317-96 (Fig 3.5 lane 7) was consistent with that for *C. upsaliensis* as indicated by Marshal *et al,* (1999). The restriction profile of 370-97 (Fig 3.5 lane 8) did not facilitate its identification to species level. These data suggests that both these strains were originally misidentified as *C. concisus* and highlights the shortcomings of phenotypic testing.

With respect to the additional 42 clinical isolates (Table 3.1), a product of the correct size was obtained from all but 2 isolates: 36-93 and 318-96. These strains were subjected to the hybridization assay described in Chapter 2. The hybridization profiles obtained were not consistent with those for *C. concisus* isolates (Results not shown). To confirm these findings the 16S rDNA-based method (ARDRA) of Marshall *et al.* 1999 was performed. The resulting *Dde*I restriction profiles of the amplicons were different to all the *Campylobacter* profiles specified by the authors (Fig. 3.5, lane 3 and 4). Thus, these strains are not *C. concisus*. For some samples the PCR assay had to be repeated, for isolate 296-96 an amplicon of the correct size was obtained on repeating the assay (Fig 3.3 lane 4). The identity of this isolate was confirmed by restriction fragment polymorphism analysis method of Marshall *et al.* 1999 (Fig 3.5 lane 5).

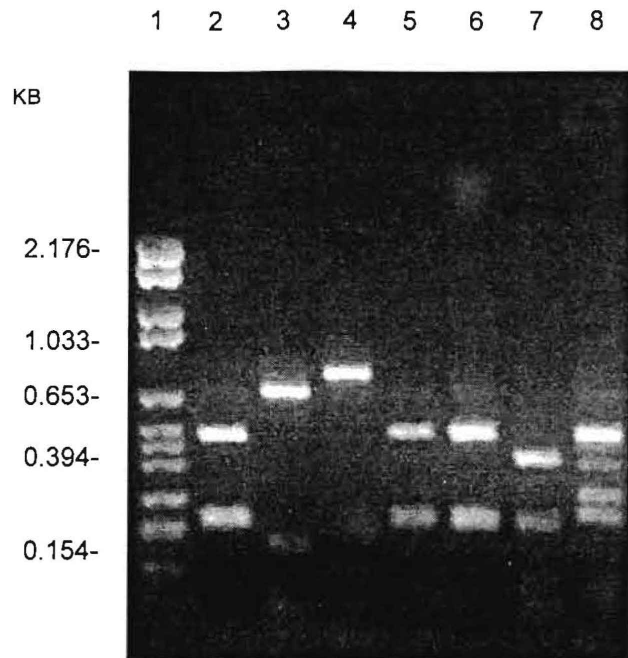


Fig. 3.5. Amplified ribosomal DNA restriction analyses with *Dde*I. Lane 1, DNA marker VI (Boehringer Mannheim): lane 2, *C concisus* NCTC11485; lane 3, Isolate 36-93; lane 4, Isolate 318-96; lane 5, Isolate 296-96; lane 6, Isolate 297-96; lane 7, Isolate 317-96; lane 8, Isolate 370-96.

A rapid PCR assay for the identification of *C. concisus* was developed and evaluated using primers that annealed to the 3'end of *gyr*B and ORF3, respectively [Chapter 2]. The assay was specific for *C. concisus* suggesting that it could be used to identify this organism. Unlike the method of Marshall *et al,* (1999), amplicons did not require an additional restriction endonuclease analysis step, making the method rapid. Although most of the strains tested were isolated in Cape Town, amplicons were obtained from two Danish clinical isolates and importantly, from two reference strains shown to be genetically diverse (Vandamme *et al.*, 1989).

Sequences from randomly selected DNA fragments have also been used to develop PCR based identification schemes for *C. jejuni* and *C. coli* (Bustamente *et al.*, 1995), and for *Helicobacter* (Valentine *et al.*, 1991). In the study by Bustamente *et al.* 1995 one of the DNA probes for *C. jejuni* cross hybridised with five clinical strains of *C. coli*. However, using primers based on the DNA sequence of the probe in a PCR assay, a false positive was obtained from only one of

the *C. coli* isolates. This indicates genetic conservation within the region to which the primers were designed for the two species. Genomic stability and specificity of the target sequence is therefore important in PCR assays used to identify organisms.

The PCR assay described in this study offers the possibility of investigating the epidemiology and clinical significance of *C. concisus*. It has the advantage of requiring minute quantities of DNA and assays can be performed using stored, frozen bacterial pellets. It was this property that enabled the processing of the additional 42 clinical isolates. Although the assay described here was not used on clinical material, similar PCR assays have been used to detect *Campylobacter* species directly from stool samples (Oyofo *et al*., 1992). Direct amplification from boiled pure bacterial colonies, offers a prospect of speeding up the identification assay, although this remains an option yet to be investigated

Overall the PCR method was found to be fast, cheap and simple when compared to Southern blot technique described in Chapter 2 and is more suitable for routine application. However the results obtained do not differentiate between the genotypes identified in Chapter 2. In order to identify potential pathogenic strains of *C. concisus*, subtyping within the species is desirable. Since the polymorphism of the locus identified in Chapter 2 is not sufficient for typing purposes with 74% of the 49 strains investigated falling into one genotype (profile IV). Alternative genotyping methods with higher discrimination were investigated.

CHAPTER 4

GENOTYPIC TYPING OF *C. CONCISUS* USING PULSED FIELD GEL ELCTROPHORESIS AND RESTRICTION FRAGMENT END-LABELLING

4.1 INTRODUCTION

Conventional typing methods such as biotyping and serotyping have been used with success in the epidemiology of *Campylobacter* (Owen *et al.*, 1994: Owen *et al.*, 1995; Patton *et al.*, 1991). Although these methods are cost effective and play a useful role in the primary characterisation of isolates, they have a major weakness in that they lack sufficient discriminatory power. Furthermore, phenotypic properties are prone to environmental pressure and therefore may be unstable. This has called for effective strain typing methods, with high discriminatory power because many *Campylobacter* infections are sporadic and need robust typing techniques to trace the source of infections. As a result many molecular typing methods have been utilised for the study of *Campylobacter* isolates: a few examples being, flagellin gene typing (Ayling *et al.*, 1996; Nachamkin *et al.*, 1993), PFGE (Gibson *et al.*, 1997) and amplified fragment length polymorphism analysis (Duim *et al.*, 1999; Kokotovic and On, 1999). The methods vary in their ability to differentiate between isolates and the ease with which the results can be generated and interpreted.

Pulsed field gel electrophoresis, because of its ability to detect restriction fragment length polymorphism across the entire genome, is documented as being a highly sensitive technique for bacterial strain differentiation (Tenover *et al.*, 1995). The technique also fulfils most of the criteria, required from an ideal typing system, in being able to type a wide variety of bacterial species and generating highly reproducible results which are unambiguous and easy to interpret. As a result this technique is now regarded as the 'gold standard' in the typing of microbial pathogens (Maslow and Muligan, 1996; Tenover *et al.*, 1997). The method has been used to determine the genome sizes of *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. fetus*, (Bourke *et al.*, 1996; Chang and Taylor, 1990; Stanley *et al.*, 1995). It has also been used in

numerous epidemiological investigations of *C. jejuni* and *C. coli* (Owen *et al.*, 1997: Stephens *et al.*, 1998; Yan and Taylor, 1991), *C. fetus* (Rennie *et al.*, 1994) and in the mapping of *C. jejuni* strain UA580 (Chang and Taylor, 1990). However, this method has never been applied to *C. concisus.*

The aim of the study was to investigate the genotypic variation of *C. concisus* isolates in a population of paediatric patients with diarrhoea, using pulsed field gel elctrophoreis and a relatively new technique, restriction fragment end labelling. The latter technique has previously been used in the typing of *Actinobacillus actinomycetemcomitans* (Van Steenbergen *et al.*, 1995), and essentially analyses restriction fragment length polymorphisms in the lower molecular weight level (100-500 bp) by electrophoreses on a polyacrylamide sequencing gel. The authors claim the method compares favourably with other restriction fragment length polymorphism DNA analysis techniques, but has since been used to a limited degree, with the only other known study being that of the typing of *Helicobacter pylori* (Van Doorn *et al.*, 1998). This method as a result has not been fully evaluated as a typing technique by other workers and one of the intentions of the study was to assess its potential as typing technique for *C. concisus*.

The preparation of DNA for PFGE and the PFGE assay was carried out by myself with the assistance of P. Jordan at the Danish Veterinary Laboratory, Copenhagen, Denmark, under the supervision of Dr S.L.W. On.

4.2 Experimental procedures

4.2.1 Bacterial strains

Bacterial strains used in this study are listed in Table 4.1 and Table 4.2. From the 46 *C. concisus* strains used for PFGE analysis (Table 4.1), 41 were isolated from children with diarrhoea admitted to the Red Cross Children's Memorial Hospital in Cape Town. One of the isolates was from an adult patient who also had diarrhoea. Two strains from adults with dental problems and two *C. concisus* reference strains were also included in the study.

Table 4.1 *C. concisus* strains used for PFGE

No	*C. concisus* strains	Sex of patient	Age (Months)	Clinical symptoms
1	20-93	M	6	Bilary atresia
2	104-93	F	33	Loose stools
3	204-93	M	9	Diarrhoea
4	25-94	F	24	Microcytic anaemia
5	204-94	NA	12	Diarrhoea
6	205-94	F	19	Diarrhoea with blood
7	207-94	M	3	Chronic gastroenteritis
8	219-96	F	11	Prolonged diarrhoea
9	220-96	M	9	Dysentery
10	377-96	F	5	Dysentery
11	389-96	F	20	Chronic diarrhoea
12	393-96	F	5	Loose stools
13	396-96	F	6	Diarrhoea and vomiting
14	305-98	F	17	Dysentery
15	306-98	M	19	Hirsprung's disease
16	311-98	F	14	Gatroenteritis
17	312-98	M	6	Diarrhoea 2 weeks
18	313-98	M	9	Abdominal pain, loose stool
19	318-98	M	21	Hiv+ and Diarrhoea
20	328-98	M	24	Dysentery, co-isolate *C. upsaliensis*
21	331-98	F	18	Diarrhoea with blood
22	8-99	M	adult	Diarrhoea
23	15-99	M	7	Hiv+ and Diarrhoea
24	17-99	M	36	Acute gatroenteritis
25	24-99	F	40	Fever ventilated
26	28-99	F	14	Dysentery
27	35-99	F	12	Diarrhoea, co-isolate Crptosporidium
28	38-99	M	13	Protein losing enteropathy,
29	45-99	M	12	Persistent loose diarrhoea
30	47-99	M	15	Gastroenteritis
31	51-99	M	7	Post liver transplant
32	52-99	M	18	Diarrhoea
33	59-99	F	9	Chronic diarrhoea, single granulomar
34	61-99	M	12	Dysentery
35	62-99	F	13	Chronic diarrhoea
36	64-99	F	27	Dysentery
37	113-99	F	15	Prolonged diarrhoea
38	115-99	M	11	Aplastic anaemia
39	126-99	M	10	Chronic diarrhoea with blood
40	131-99	F	25	Dysentery
41	135-99	F	6	Mal-absorption
42	140-99	F	17	Dysentery
43	26A#		Adult	Dental isolate
44	D9#		Adult	Dental isolate
45	CCUG 19995*	NA	Adult	Faeces of patient with fever
46	CCUG 13144*	NA	NA	

C. concisus dental isolates from adults.* *C. concisus* reference strains. N/A Information not available.

Table 4.2 Bacterial strains used for restriction fragment end labelling

No	*C. concisus* strain	Sex of patient	Age (months)	Clinical condition
1	222-94	M	15	Chronic diarrhoea
2	62-95	F	12	Ongoing diarrhoea
3	79-95	M	9	Chronic diarrhoea
4	86-95	M	7	kwashiorkor
5	89-95	N/A	N/A	N/A
6	207-95	M	60	Bloody diarrhoea
7	211-96	F	38	Diarrhoea
8	214-96	N/A	7	Diarrhoea
9	215-96	M	57	Loose stool
10	219-96	N/A	11	Prolonged diarrhoea
11	222-96	M	26	Dysentery
12	247-96	M	23	Diarrhoea + vomiting
13	250-96	F	22	Watery stool
14	259-96	M	35	Diarrhoea +vomiting
15	260-96	M	26	Diarrhoea
16	265-96	F	3	Diarrhoea
17	315-96	M	16	Chronic diarrhoea

N/A Clinical information not available

4.2.2 Preparation of DNA

Chromosomal DNA embedded in agarose blocks was prepared as described by Gibson and fellow workers (Gibson *et al.*, 1994) with modifications. A loopful of cells grown for 48-72 hrs on BHI medium was harvested and resuspended in 2 ml of Pett IV buffer. Formaldehyde (300 µl) was added and the cell suspension was left for 90 min at room temperature. Absorbance values at 450 nm (MR 7000 Dynatech laboratories, Denkendorf, Germany) for 150 µl cell suspension aliquots in microtitre plates (Nunc, A/S Rosklide, Denmark) were determined. Volumes were adjusted to give an absorbance value of 1.2. The cells were washed three times with 1 ml of Pett IV buffer and finally resuspended in 600 µl of the same buffer. Cell suspension aliquots (300 µl) were mixed with 700µl of molten 1% (w/v) chromosomal grade agarose (Bio-rad #162-0135) and cooled to 65°C. The mixture was dispensed into plastic moulds (Phamacia) using a pipette. For each sample 5 DNA blocks were prepared and allowed to set at 4°C for 15 min.

To lyse the cells, the blocks were incubated in 3 ml of 0.5M EDTA, 1% Sarkosyl 5 mg/ml Proteinase K (ESP) buffer for 48 hrs at 56°C. Six consecutive washes with 2 ml of TE pre-

warmed to 56°C were carried out at 30 min intervals. Each set of blocks was stored at 4°C in vials containing TE.

4.2.3 Restriction endonuclease digestion

Agarose slices (approximately 1.0-1.5 mm) were cut from DNA-containing blocks and pre-incubated for 1 hr in a 100 μl of the appropriate restriction buffer, with BSA and Triton X (Amersham life sciences). Digestion was carried out at 37°C for 5 hrs in a 50 μl buffer mix with 20 U of *Not*I, *Sal*I, *Kpn*I and *Sma*I (Amersham life sciences).

4.2.4 Separation and detection of restriction of fragments

The digested agarose blocks were loaded on a 1.0% agarose gel (Bio-Rad #162-0137 made in 0.5 x TBE) and the wells sealed with molten 1.0% (w/v) agarose (Bio-Rad # 162-0165 made up in H_2O). DNA Restriction fragments were separated in 0.5 X TBE by gel electrophoresis using a contour–clamped homogenous electric field apparatus (Bio-Rad model DR-III, Copenhagen, Denmark). For the first phase, pulse times were ramped from 3-10 sec for 4 hrs and the second phase from 10-15 sec for 18 hrs.

DNA was visualised by staining the gel in ethidium bromide for 10-15 min and de-staining in water. Photographs were taken under Ultraviolet light (302 nm) using a Polaroid camera.

4.2.5 Calculation of genome size

Photographic images were captured using Kodak DC120 digital camera (Kodak digital science). The detection of restriction fragments bands for each lane was carried out by computer analysis using the 1D image analysis software, version 3.0 (Kodak digital science). Using the lambda ladder PFGE marker (New England BioLabs) loaded with the samples, and following instructions delineated in the software booklet, molecular weights were assigned to each restriction fragment. The molecular weights of the fragments in each lane were added up, to obtain the total size of each DNA sample. The mean of the total sizes calculated for each DNA sample was taken as the genome size.

4.2.6 Restriction fragment end Labelling

Restriction fragment end-labelling (RFEL) was carried out as described by Van Steenbergen *et al.* 1995, with few modifications. Genomic DNA (2 μg) from *C. concisus* isolates was restricted to completion overnight at 37°C in a 30 μl volume, with 10 units of *Bgl*II, (Boeringer Mannheim). The enzyme was inactivated by phenol/chloroform extraction and the DNA precipitated with ethanol. The 3' recessed termini of the restricted DNA were filled in and labelled with [α-^{32}P]dATP (2.5mCi per reaction; 1μCi = 37kBq), using 1 unit of Klenow fragment of DNA polymerase (Boehringer Mannheim) in a 20μl reaction containing1mM of dCTP, dTTP. The reaction was incubated at 37°C for 30 min, unincorporated dNTPs were removed by washing in 70% ethanol. The DNA was precipitated and re-dissolved in 6 μl of TE buffer (pH 7.6). Efficiency of the labelling was determined by taking readings of 1 μl sample, using a scintillation counter. Equal quantities of labelled samples, were mixed with an equal volume of stop solution (Phamacia Biotech) and denatured by heating at 90°C for 3 min. Samples were loaded on a 6% polyacrylamide sequencing gel and restriction fragments separated by electrophoresis for approximately 3 hrs at 1600V with the dye front acting as guide for the length of the run. The polyacrylamide gel was transferred onto Whatman 3M paper and vacuum dried. The restriction fragment (RFEL) profiles generated detected by autoradiography.

4.2.7 Numerical analysis of restriction fragment end-labelling data

Resulting autoradiograms were captured using a Kodak DC120 digital camera (Kodak Digital Science) and images stored as TIFF files. Images were processed and analysed using GelCompar 3.1 software (Applied Maths, Kortrijk, Belgium). Lanes were assigned to each profile according to the instructions in the supplier's manual (GelCompar). DNA restriction profile patterns were normalised by alignment using a labelled PB322 *Hinf*I digest or ϕ 174 *Hin*fI (Promega) included at regular intervals as a reference standard. Using the curve-fitting algorithm, the background was subtracted from all lanes and selection of bands achieved by selecting a minimal profiling value of 4%. For band comparison, band position tolerance

value of 1.0% was arrived at, to compensate for aberrant gels after running duplicate samples on the same gel.

4.3 RESULTS

4.3.1 PFGE typing of *C. concisus*

For the generation of restriction profiles suitable for pulsed field gel electrophoresis analysis, several enzymes were evaluated. The enzymes *Sal*I and *Sma*I were found to cut *C. concisus* DNA inefficiently (data not shown), while digestion with *Kpn*I generated too many bands and separation was poor, with fragments clustering at low molecular weight levels (data not shown). On the other hand, the restriction fragments generated by *Not*I (GC/GGCCGC) and separated by PFGE were well dispersed, with most DNA samples having 5-12 bands ranging from 45 kb to 530 kb (Fig.4.1A and 4.1B).

Based on a selection of *Not*I DNA digests that generated DNA fragments from approximately 10 kb to 600 kb the genome size of *C. concisus* was calculated (See Table 4.3).

Table 4.3 calculation of *C. concisus* genome size

	C. concisus strain in Fig 4.1.A				*C. concisus* strains in Fig 4.1B					
Lanes	**5**	**8**	**9**	**13**	**1**	**2**	**3**	**10**	**11**	**12**
Strains	**17-99**	**377-96**	**24-99**	**59-99**	**131-99**	**35-99**	**140-99**	**318-99**	**305-98**	**396-96**
Molecular weight of fragments	360.6	430.1	625.0	561.8	590.3	600.9	568.6	484.4	600.9	570.6
	307.6	259.2	422.7	424.2	446.3	394.7	333.9	365.6	485.2	456.8
	245.6	216.2	192.0	262.4	209.8	345.3	216.2	255.2	254.7	341.7
	236.3	197.1	177.5	230.2	205.3	214.3	163.2	180.0	176.5	188.2
	228.2	174.1	174.4	157.9	92.7	103.6	100.7	110.4	166.4	150.9
	106.5	159.6	95.0	66.1	78.0	75.2	83.5	86.1	154.4	128.3
	94.3	112.7	71.6	49.4	73.1	52.7	75.5	65.1	117.5	108.1
	37.0	50.3	66.2		67.0	46.6	68.8	57.7	76.1	101.3
	22.4	37.2	59.2		38.3	26.7	63.1	9.9	56.3	76.9
	7.0				28.0	11.6	59.6		45.4	63.1
					11.6		32.1		37.9	
									17.2	
Total (kb)	1645.5	1636.5	1883.6	1752.0	1840.4	1871.6	1766.0	1614.4	2188.5	2185.9
Mean	1.84Mb									

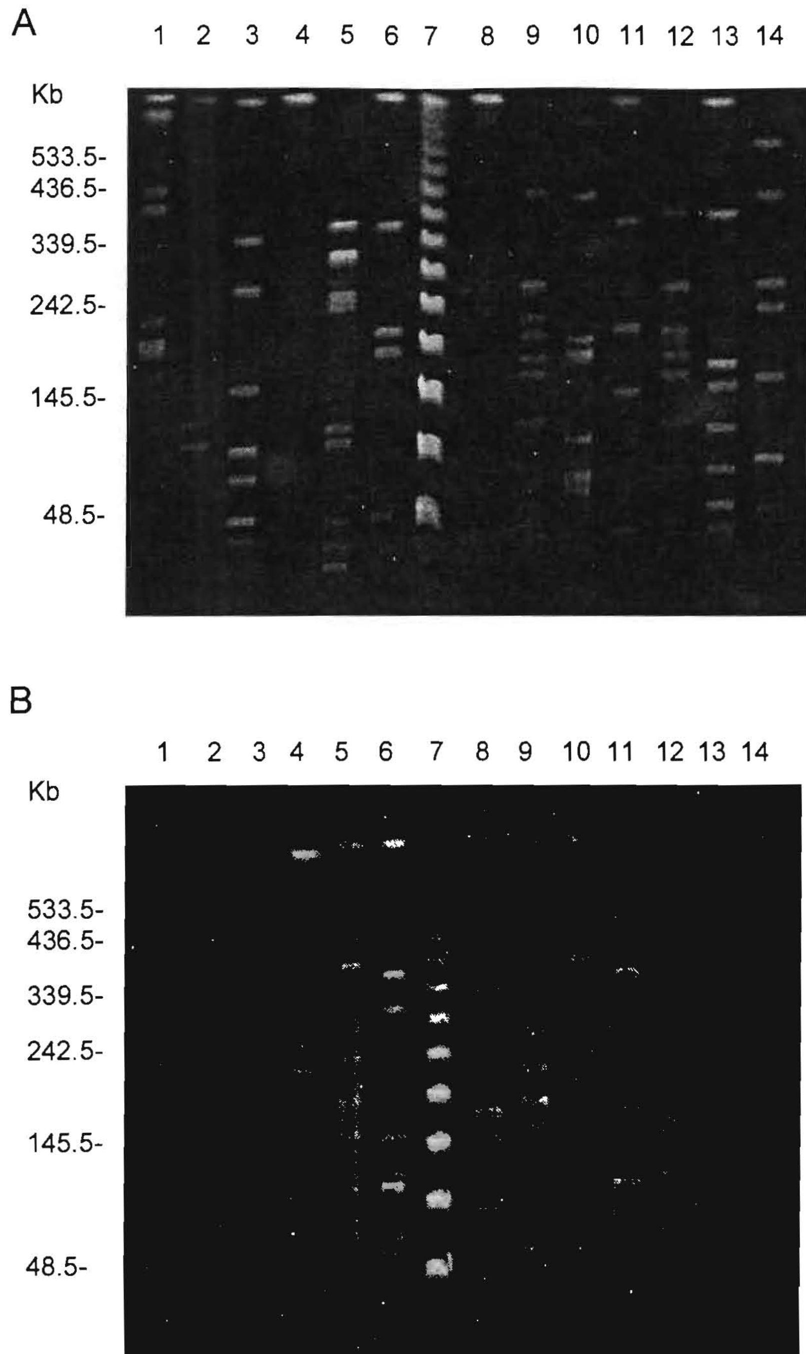


Fig 4.1. PFGE of *Not*I restricted *C. concisus* DNA
A. Lane 1, 389-96; lane 2, 51-99; lane 3, 28-99; lane 4, 45-99; lane 5, 17-99; lane 6, 64-99,; lane 7, lambda Ladder PFGE standard (New England BioLabs); lane 8, 15-99; lane 9, 377-96; lane 10, 24-99; lane 11, 61-99; lane 12, 328-98; lane 13, 8-99, and lane14, 59-99.
B. Lane 1, 131-99; lane 2, 35-99; lane 3, 140-99; lane 4, 113-99; lane 5, 135-99; lane 6, 207-94;lane 7 lambda ladder PFGE standard (New England BioLabs); lane 8, 306-98; lane 9, 331-98; lane 10, 393-96; lane 11, 318-98; lane 12, 305-98; lane 13, 396-96, and lane 14, 312-98.

The mean genome size *of C. concisus* calculated from 10 isolates is 1.84 Mb with a range in sizes between 1.64 Mb and 2.19 Mb. Using *Not*I, restriction patterns were obtained for 42 of the 44 clinical isolates, including the two dental isolates. The DNA from the remaining two isolates (312-98, 45-99) was refractory to digestion with *Not*I (Fig. 4.1B lane 14; Fig. 4.2 lane 3; Fig. 4.2 lane 2) and profiles were not obtained for these two strains.

Macro-restriction profiles generated by *Not*I restriction showed considerable heterogeneity of the 42 isolates. Variability was observed with respect to both the number of restriction fragments (Fig. 4.2) and the distribution of restriction fragments. Only isolates 26A and 104-93 (Fig 4.2 lane 17 and 20) had identical fingerprints, the profiles of the remaining 42 isolates were unique.

Despite the heterogeneity, based on the number and distribution of *Not*I fragments, it was possible to identify three distinct groups. Fig. 4.2 lanes 1-5 represents the first group characterised by relatively few restriction fragments. Lanes 6-14 depict a group of strains, which can be described in terms of even band distribution. All members of this group have well separated fragments ranging from 45 kb to 530 kb. The last group is shown in lanes 16-20: The common denominator of this group being that most of the restriction fragments cluster below 300kb. Representatives from each of the three groups were selected for subsequent comparisons with other genotypic typing techniques. From the first group, three strains were selected 51-99, 45-99, 38-99 (Fig 2.4 lanes 1, 2 and 5), six strains CCUG 13144, 205-94, 64-99, D9, 61-99, 207-94 (Fig 4.2 lanes 6, 7, 8, 10 and 11; Fig 4.1B lane 6) were selected from the second group and six representative strains from the last group 204-93, 52-99, 204-94, 47-99, CCUG 19995 and 24-99 (Fig 4.2 16, 18, and 21-24) were chosen.

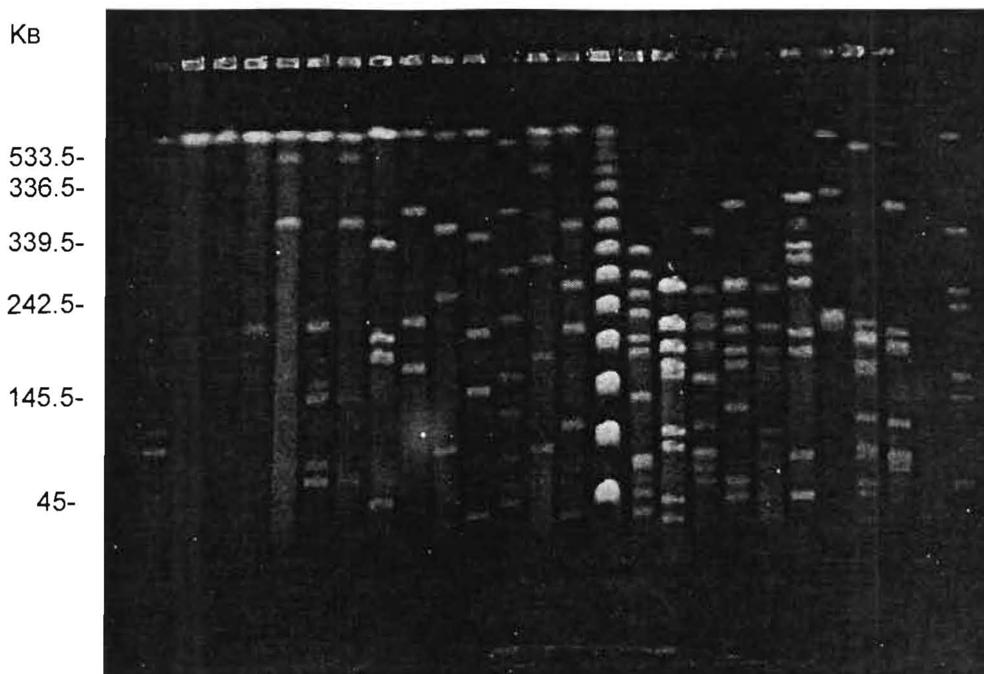


Fig 4 2. PFGE of *Not*I restricted DNA from grouped *C. concisus* strains.
Lane 1, 51-99; lane 2, 45-99; lane 3, 312-98; lane 4, 15-99; lane 5, 38-99; lane 6, CCUG 13144; lane 7, 205-94; lane 8, 64-99; lane 9, 220-96; lane 10, D9; lane 11, 61-99; lane 12, 25-94; lane 13, 115-99; lane 14, 219-96; lane 15, lambda Ladder PFGE standard (New England BioLabs); lane 16, 204, 93; lane 17, 26A; lane 18, 52.99; lane 19, 377-96; lane 20, 104-93; lane 21, 204-94; lane 22, 47-99; lane 23, CCUG 19995; lane 24, 24-99; lane 25, 402-96; lane 26, 20-83.

Given the genetic variability observed, the identical profiles observed in the unrelated strains 26A and 104-93, were unexpected. The strains were isolated approximately four months apart; strain 104-93 was isolated from a patient with gastro-enteritis, while 26A was a dental isolate.

4.3.2 Restriction fragment end labelling analysis

Several restriction endonucleases that result in 5' overhangs were tested for their ability to generate RFEL profile patterns. The enzymes *Eco*RI and *Bam*HI were found to restrict the *C. concisus* DNA inefficiently while, *Hin*dIII resulted in numerous bands which were too complex for analysis. Following restriction with *Bgl*II the patterns consisting of 30 fragments

between 75 bp and 421 bp could be analysed. An intermittent problem with the analysis of restriction fragment end labelling patterns was the high background noise or feint profiles in some samples. For some samples, high background noise was attributed to degradation during DNA extraction, but for some other samples, inefficient labelling and high background was not consistent between consecutive experiments and could not be attributed to any reason. However, this did not hinder data interpretation for most samples as the autoradiograms of the restriction profiles generated were characterised by distinct band signals. A typical gel indicating restriction profiles obtained is shown in Fig 4.3A.

4.3.3 Reproducibility of restriction fragment end labelling patterns

To determine if the restriction fragment end labelling profiles generated were reproducible, DNA samples from 5 strains of *C. concisus,* labelled in separate assays were run in duplicate on the same gel (Fig 4.3A). PBR322 digested with *Hin*fI was loaded on the gel to act as a molecular weight standard. The resulting autoradiogram was analysed using Gelcompar numerical analysis program. Although weak bands were obtained for duplicate samples 222-94a and 222-94b (Fig. 4.3A lane 2 and 7), manual editing within the band search window of Gelcompar, enabled analysis. All duplicate samples clustered together at a correlation level 90% or above, with the exception of samples 222-94a and 222-94b which clustered above the 87% similarity level. (Fig. 4.2.B). The lower similarity level value observed for 222-94 is probably due to inefficient labelling attained for the samples and this result was disregarded in arriving at a intragel correlation value 90% or greater for identical or clonal isolates.

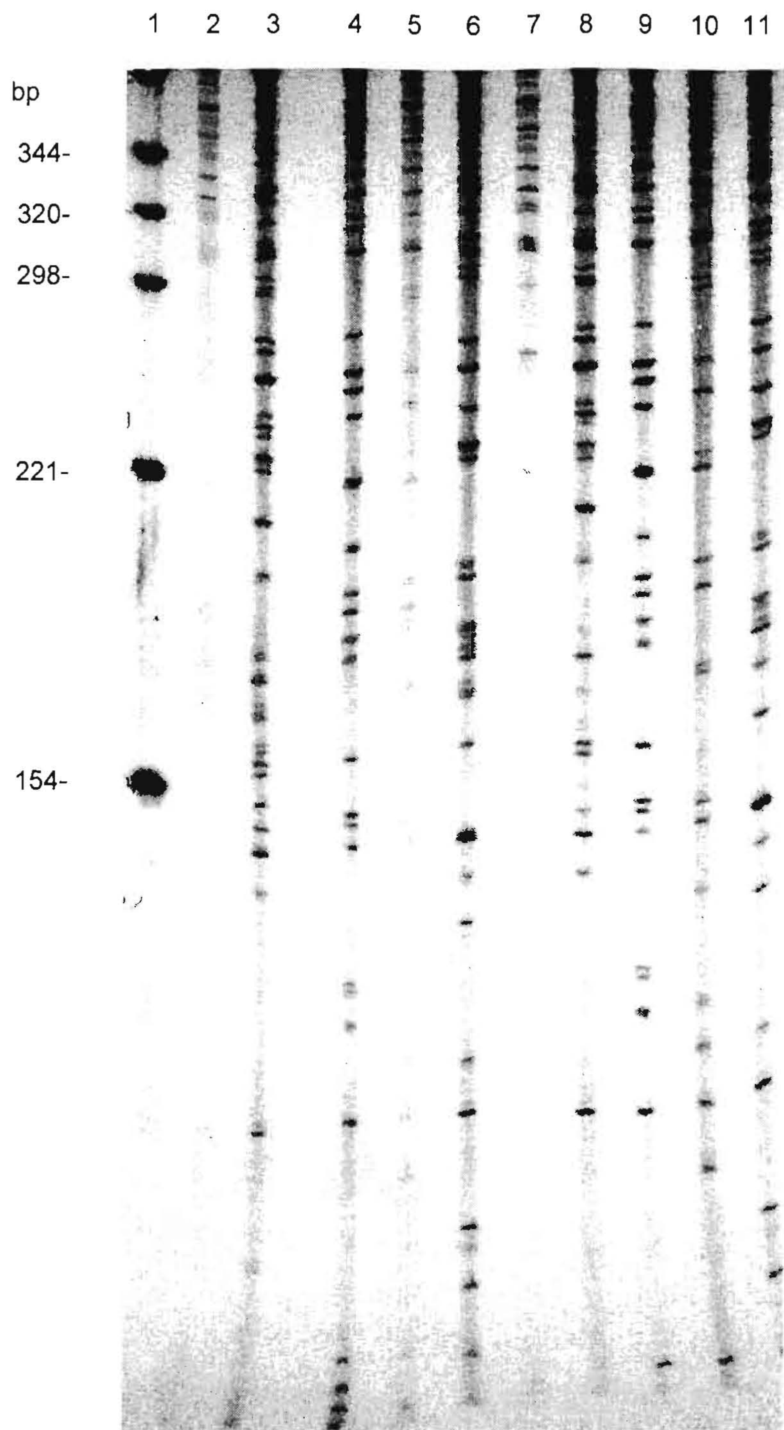


Fig 4.3A Restriction fragment end labelling of *Bgl*II digested DNA with duplicate samples labelled in separate assays.
Lane 1, pBR322 digested with *Hin*fI; lane 2, 222-94; lane 3, 260-96; lane 4, 219-96; lane 5, 86-95; lane 6, 247-96; lane 7, 222-94; lane 8, 260-96; lane 9, 219-96; lane 10, 86-95; lane 11, 247-96.

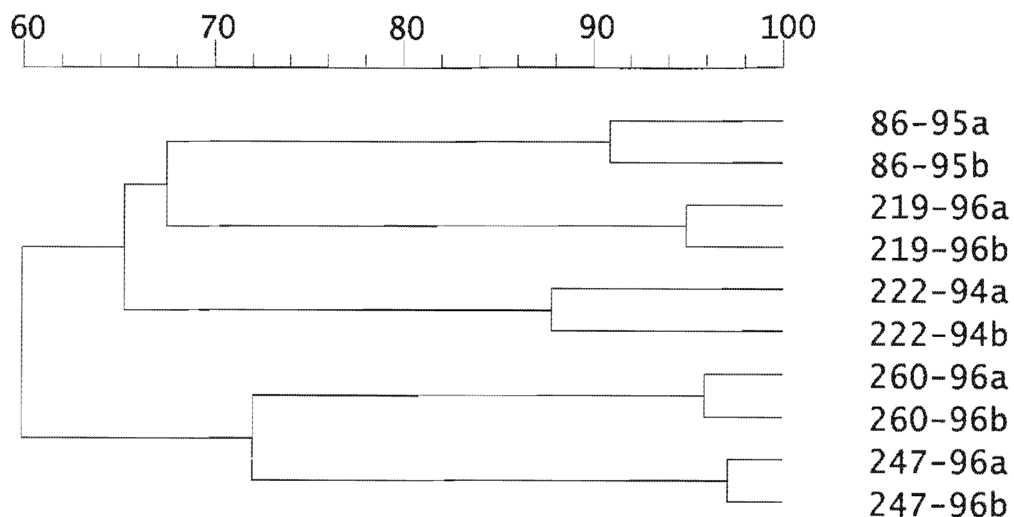


Fig 4.3B. Dendrogram of duplicate samples shown in Fig.4.3A.Correlation is by the Dice coefficient using UPGMA, with a tolerance of 1.0%. and optimisation of 0.50%.

To evaluate the inter-gel reproducibility, or whether RFEL data from separate experimental assays could be correlated, DNA samples from the isolates given above were again subjected to RFEL along with another selection of isolates. The results from the two gels were combined and analysed. Clustering between individual isolates run on different gels was observed only between two (219-96 and 260-96) of the five replicated samples (Fig. 4.4). Even then different labelled reactions of 219-96a and 219-96b run on the same gel clustered at a correlation level of about 95%. This was higher than the 73% linkage level to sample 219-96, which was run on a different gel. Following analysis of the profile of 260-96c, this strain was linked at the 86% similarity to corresponding duplicate samples (260-96a and 260-96b) loaded on the same gel. Correlation of samples on different gels was consistently lower than when samples were loaded on the same gel. This was not due to generation of inconsistent profiles, but rather to minor shift in length of the run between the two gels. This could not be compensated by the normalisation function, in the GelCompar program or by the position tolerance value of 1.0%, set to compensate for aberrant running gels. A case in point being sample 247-96c, which has an identical restriction profile to other replicate samples run on the same gel (247-96a and 247-96b), but due to a shift in the running of the gel, appears unrelated to the other isolates in the

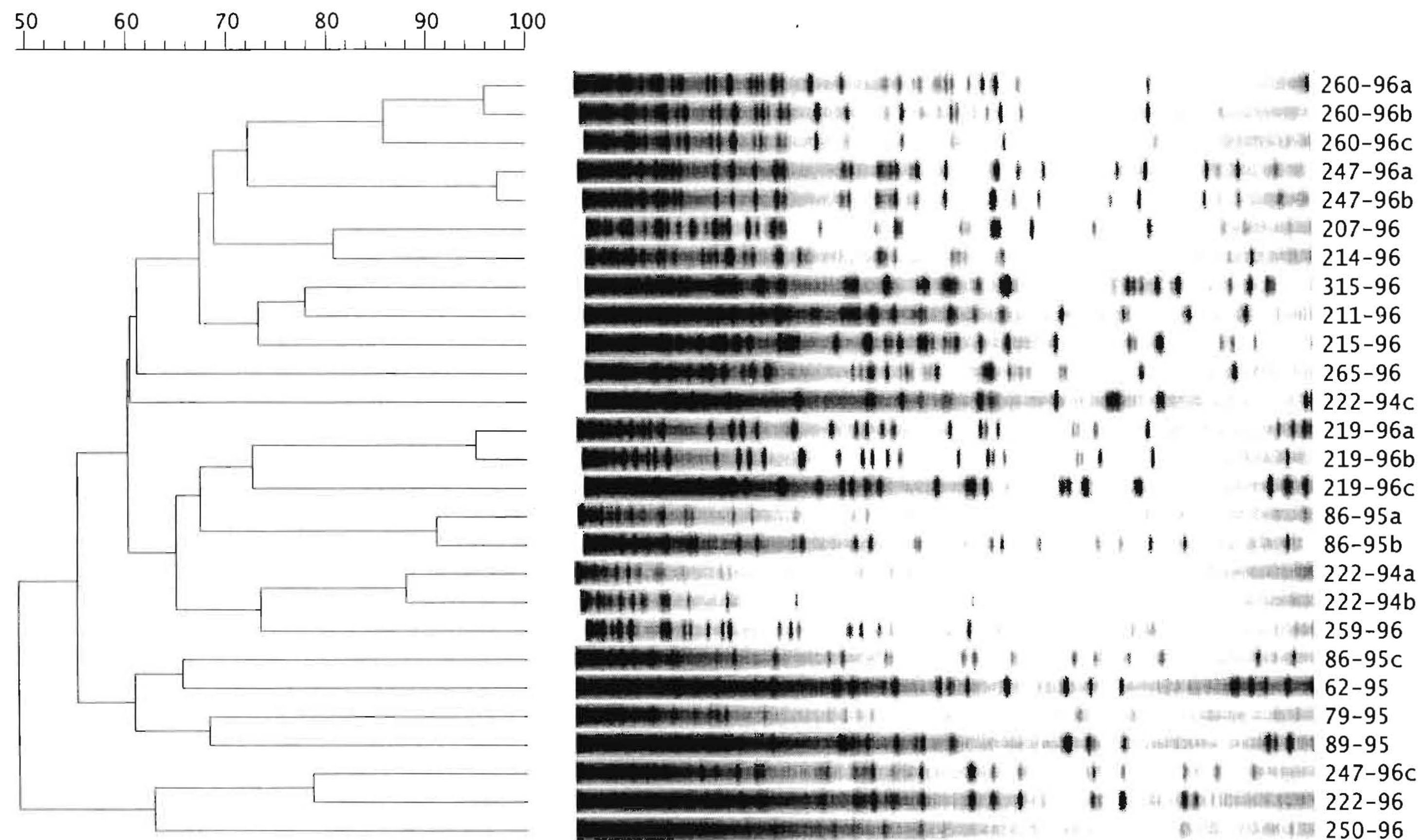


Fig. 4.4 Dendrogram of relatedness for RFEL repeated samples run on different gels . Isolates labelled a and b are samples used in intra-gel analysis shown in Fig 4.3A. Isolates labelled c represent repeat samples loaded on a different gel. Similarity between samples was calculated using the Dice coefficent and the dengrogram drawn by UPGMA with a tolerance value of 1.0% and optimisation of 0.50%.

dendrogram generated. Inter-gel comparison of data generated using FREL under the conditions specified, was therefore misleading and not practised for subsequent data analyses.

4.3.4 Numerical analyses of RFEL profiles for strains grouped by PFGE

Representatives of the three subgroups (51-99, 45-99, CCUG 13144, 205-94, 64-99, D9, 61-99, 207-94, 204-93, 204-94, 47-99 and 24-99) identified following PFGE [section 4.3.1] were subjected to RFEL. As was observed with PFGE, each of the strains had unique profile with isolates with clustering starting below a similarity level of 78% (Fig 4.5). The grouping observed within the PFGE patterns was not apparent in the clustering of RFEL profiles of the 12 selected strains. *C. concisus* CCUG 13144 and 204-93 were least related to other isolates.

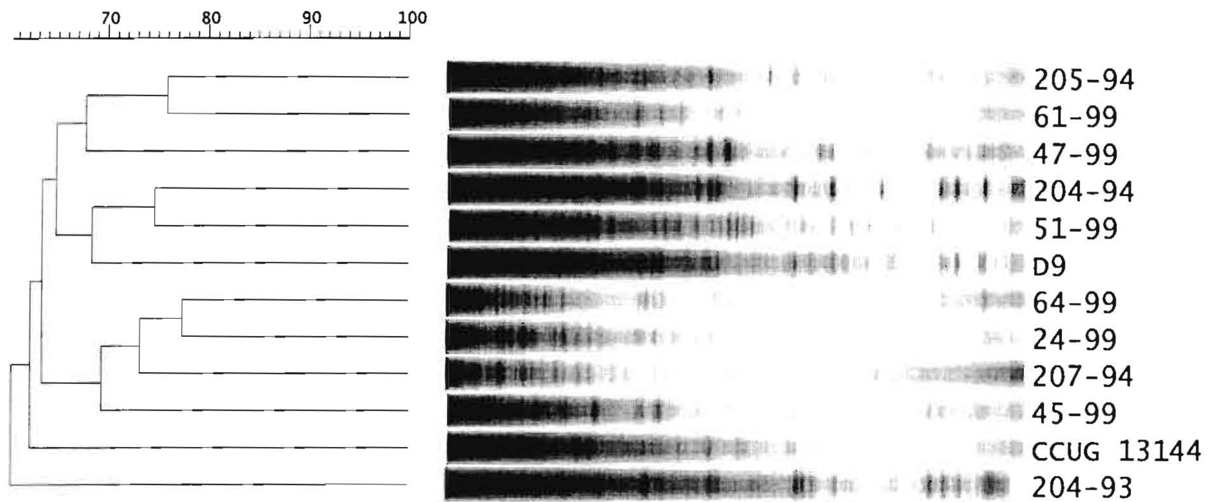


Fig. 4.5 Dedrogram of relatedness for *C. concisus* isolates using RFEL carried out on isolates found to represent genetically diverse strains using PFGE. Strain relatedness was calculated using the Dice coefficient and the dendrogram constructed using UPGMA with a tolerance value of 1.0% and optimisation of 0.50%.

4.4 DISCUSSION

To study the genomic diversity of *C. concisus*, DNA from 46 isolates was digested with *Not*I and the fragments separated by PFGE. It is noteworthy that for the preparation of DNA blocks, inactivation of exonucleases with formaldehyde was necessary, a procedure described for Dnase positive strains of *C. jejuni* (Gibson *et al.*, 1994). It was not possible to obtain suitable restriction patterns following digestion with *Kpn*I, *Sal*I and *Sma*I; however, fingerprints of 42 of the 44 clinical isolates were obtained following digestion with *Not*I. Except for two isolates all the strains had unique *Not*I profiles, an indication of marked genetic variability within the species. An observation, which was reinforced by the fact with the exception of CCUG 13144 and CCUG 19995 reference strains, all the isolates studied were obtained from the same geographic locale. It is possible that the high genomic variability observed could have been amplified by the long time frame (1993-1999) during which the samples were taken. However, marked variability was still evident within the 21 strains collected in 1999. This genotypic heterogeneity is greater than that observed for *C. jejuni, C. coli* (Chang and Taylor, 1990, Yan *et al.*, 1991), and of *H. mustalae* (Taylor *et al.*, 1994). Similar genotypic diversity has been observed *in H. pylori* (Taylor *et al.*, 1992) and *C. upsaliensis* (Bourke *et al.*, 1996).

The genome of *C. concisus* was found to vary between 1.64 to 2.1 Mb, which is not surprising considering the amount of genotypic heterogeneity evident in the species. The mean genome size of *C. concisus* was approximated to be 1.84 MB which is very close to 1.7 Mb as estimated for *C jejuni* and *C coli* (Chang and Taylor, 1990).

Restriction fragment end labelling was evaluated as a typing technique for *C. concisus*. Using the restriction nuclease *Bgl*II, 20-30 restriction fragments ranging from 75 bp to 421 bp were generated. This number was large enough to give sufficient discrimination without being too cumbersome. This allowed for implementation of numerical analysis and the subsequent plotting of a similarity dendrogram for related strains. The restriction fragment end-labelling profiles obtained were distinct and reproducible although the presence of doublets arising from complementary DNA fragments at times could confuse data interpretation. An intra-gel comparison of duplicate samples was linked at a correlation value of greater than 90%, while gels from separate experiments could not be compared due to aberrant migration achieved by

vertical flat back sequencing electrophoresis system used. Results could probably be improved by using a more efficient cooling electrophoresis system.

By subjecting 12 of the 15 strains suggested by PFGE to represent three subgroups of genetic diversity, a comparison between PFGE and RFEL was carried out. Although both techniques look for restriction fragment length polymorphism, they contrast in that PFGE looks for macro restriction patterns in the whole genome, while RFEL samples the genome for RFLP in small fragments below 500 bp. The 12 strains were found to be genetically diverse by using RFEL with all strains having a similarity of less than 78%. Based on these results, it can be assumed that RFEL has a discriminatory power on par with that of PFGE. However, no correlation was found between the subgroups identified by PFGE and clustering achieved by RFEL. The only major drawback of the RFEL was the intermittent, occasional high background noises and feint profiles obtained due to inefficient labelling. This warranted repetition of samples and was time consuming. The method also has the additional hazard of working with radioactivity. Overall RFEL was reliable and is suitable as a secondary technique where rigorous discrimination is required.

The depth in *C. concisus* variability has been alluded to by Vandamme *et al* (1989), when DNA: DNA hydridisation values were compared among *C. concisus* isolates collected from diverse sources. This study is the first report demonstrating strain variability within *C. concisus* isolates. A number of theories have been advanced to account for this genomic variabilty. *C. coli* and *C. jejuni* have been shown to be naturally competent and readily take up naked DNA by natural transformation (Wang and Taylor, 1990). Genomic variability could also be achieved by spontaneous genomic rearrangements, a phenomenon that has been described in *C. coli* (On, 1998). Following successive passages, the restriction profile of *C. coli* was different from that of the parent strain, suggesting intracellular molecular events (On, 1998). These events may play a role in the observed genetic variation prevalent in *C. concisus*.

CHAPTER 5

RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS OF *C CONCISUS* USING $(GTG)_5$ AS PRIMER

5.1 INTRODUCTION

DNA fingerprinting techniques such as PFGE and RFEL as described in the previous Chapter, although reliable and highly discriminatory, are time consuming and laborious. A number of techniques have been used to type *Campylobacter* species (Wassenaar and Newell, 2000), including *fla*A typing, a rapid technique which has been used extensively in the typing of *C. jejuni* (Alm *et al.*, 1993; Nachamkin *et al.*, 1993; Nishimura *et al.*, 1996) and to a limited degree in typing *C. coli, C. lari*, and *C. helveticus* (Owen *et al.*, 1993). During the course of these studies, the latter technique was used in a small study to type *C. concisus*. Using primers described by Owen *et al.* 1993 no amplicons were obtained. Heterogeneity of the target sequences (Harrington *et al*, 1997; Wassenaar *et al.*, 1995) could explain this result and the technique was not evaluated further.

Random amplified polymorphic DNA (RAPD) analysis (Welsh and McClelland, 1990) which is essentially the same as arbitrarily primed PCR (AP-PCR) described by Williams *et al.*, 1990, is a rapid technique that has been used to type many microbial pathogens, including *Campylobacter* (Hernandez *et al.*, 1995b; Iriarte and Owen, 1996; Madden *et al.*, 1998). The method uses arbitrary primers to amplify DNA products under low stringency PCR conditions to generate strain-specific patterns.

A poly-GTG oligonucleotide [$(GTG)_5$] has previously been used for the identification of *Mycobacterium tuberculosis* when used as a radiolabelled probe in genomic Southern hybridisation analysis of *Hin*fI restricted DNA (Wiid *et al.*, 1994). The oligonucleotide has also been used as a probe in the fingerprinting of nontuberculosis mycobacteria (Cilliers *et al.*, 1997) as well as in *Salmonella* and *Shigella* isolates (Doll *et al.*, 1993). According to our knowledge, it has not yet been used before as a RAPD primer. Following modification of the amplification protocol for AP-PCR analysis as described by Williams *et al.* 1990, the $(GTG)_5$ oligonucleotide was used as a primer in PCR assays for the typing of *C. concisus*.

5.2 METHODS

5.2.1 Bacterial strains

A total of 92 *C. concisus* strains (listed in Table 2.2 and Table 3.1) collected from the Red Cross Children's Memorial Hospital bacterial culture collection were typed using RAPD technique. This collection comprised both frozen bacterial samples and fresh samples. Before typing, the isolates were identified by biochemical testing and by the rapid PCR identification protocol based on the 1.6 kb fragment described in Chapter 3.

5.2.2 RAPD analysis

The PCR reactions were carried out in 25 µl reactions containing approximately 500 ng of DNA template and 25 pmole of $(GTG)_5$ primer. To improve reproducibility The Ready To Go RAPD analysis beads (Amersham Pharmacia biotech) were used to provide a consistent and uniform source of *Taq* polymerase, dNTPs and buffer conditions. Amplification was performed starting with 4 cycles of low specificity at 95°C for 2 min, 36°C for 2 min and 72°C for 2 min were followed by 30 consecutive cycles of high specificity at 95°C for 1 min, 50°C for 1 min and 72°C for 2 min. A final extension step at 72°C for 5min was carried out. PCR products were separated by electrophoresis in 1.4% agarose (Techcomp Ltd). The gels were run at 80 V for 2 hrs in 1X TAE, stained with ethidium bromide (Sigma chemicals) and visualised using an ultraviolet transilluminator (302 nm).

5.2.3 Field inversion gel electrophoresis

Preparation of genomic DNA agarose blocks and restriction with *Not*I endonuclease was essentially as described previously [Chapter 4], with minor modifications in preparations of agarose blocks. After washing with formaldehyde, *C. concisus* cells were re-suspended in 1ml PettV buffer and mixed with 1ml of 2% (w/v) low melting point agarose (FMC seaplaque) before pouring into moulds.

Restriction endonuclease digestion of agarose embedded DNA with *Not*I was as described previously [Chapter 4]. The digested agarose blocks were loaded on a 1.0% (w/v) agarose gel (Agarose MP. Boehringer Mannheim) made in 0.5 X TBE and the wells sealed with 2.0% (w/v) low melting point agarose. Separation of DNA restriction fragments was carried out in 0.5 X TBE by field inversion gel electrophoresis (FIGE) using a horizontal flatbed submarine gel electrophoresis unit (Hoeffer Scientific Instruments) and a PC 750 pulse controller (Hoeffer Scientific Instruments). Conditions for FIGE included a voltage gradient of 5 V/cm with an initial forward pulse time of 2.4 sec and a reverse pulse time of 0.8 sec. A forward to reverse 3:1 pulse ratio was maintained for a total run time of 40 hrs with a ramp factor of 1.0. Gels were stained with ethidium bromide (Sigma chemicals) for 10 min and de-stained in water. The DNA was visualised under ultraviolet light (302 nm) and photographs taken using a Kodak DC 120 zoom digital camera (Kodak digital science).

5.3 RESULTS

5.3.1 RAPD analysis of clinical isolates

A total of 98 *C.concisus* isolates from children with diarrhoea and two dental isolates from adult patients were subjected to RAPD analysis. Isolates were collected over an 8 year period (1992-1999), with 44% of the isolates collected in the year 1996. Using the $(GTG)_5$ primer, PCR reaction conditions were optimised by varying the amount of primer and DNA template. Varying the DNA template between 10 ng and 100 ng did not significantly affect the profile patterns obtained (results not shown), while using a primer concentration of 50 pm per reaction, resulted in brighter and discrete bands than using primer concentrations of 25 and 100 pm. To obtain reproducible and informative patterns high stringency cycles had to be preceded by an initial 4 cycles at low stringency, with a primer annealing temperature of 36°C. The resultant DNA fingerprints were characterised by a low intensity smeary background and 2 to 7 discrete bands ranging between 0.15 kb and 1.2 kb. A typical example of the profiles obtained is shown in Fig 5.1.

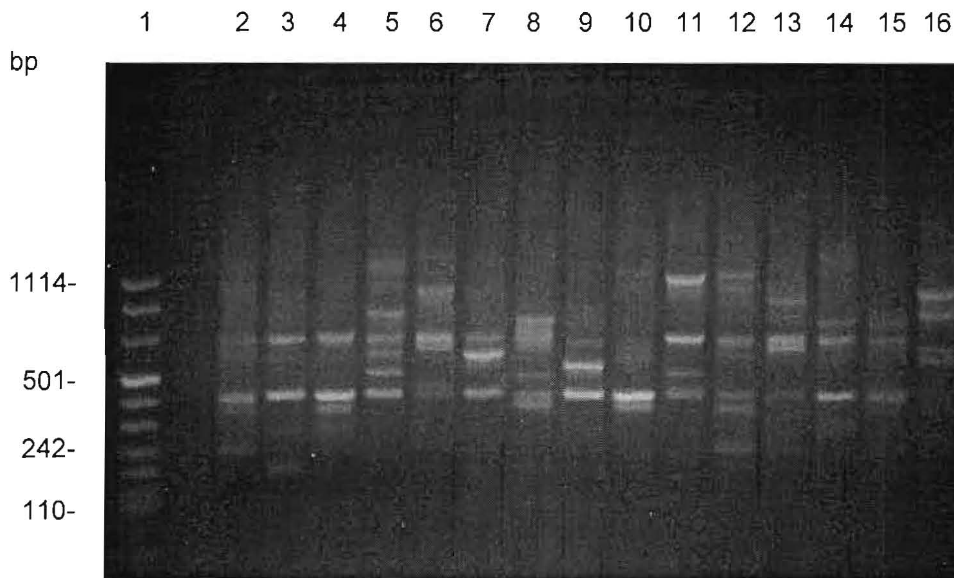
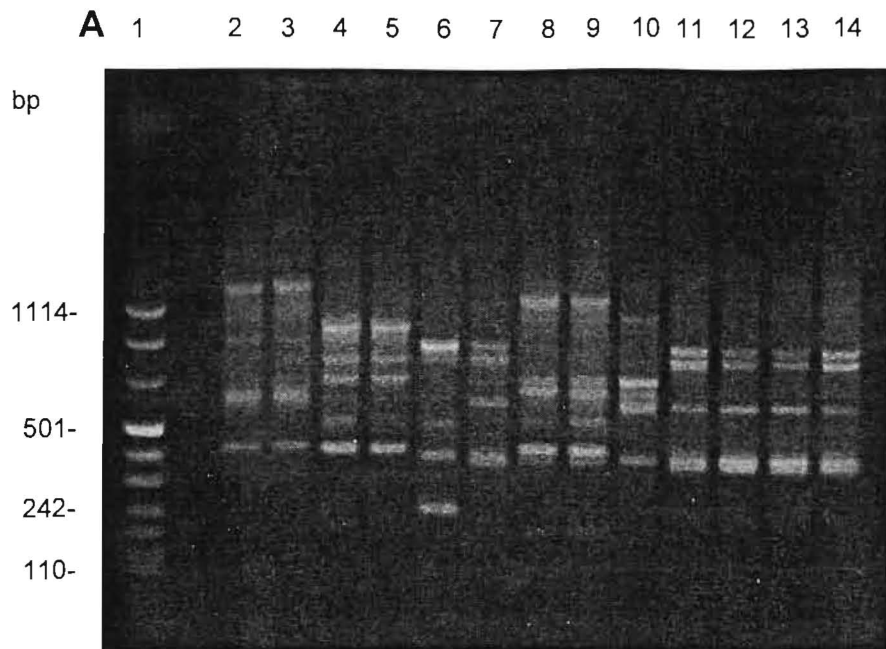


Fig 5.1. RAPD typing using $(GTG)_5$ on isolates found to be genetically diverse by PFGE Lane 1, DNA marker VIII (Boehringer Mannheim); lane 2 , 205-94; lane 3, 47-99; lane 4, 204-94; lane 5, CCUG 13144; lane 6, 51-99; lane 7, CCUG 19995; lane 8, 52-99lane 9, 61-99; lane10, 45-99; lane 11, D9; lane 12, 38-99; lane 13, 64-99; lane 14, 207-94; lane 15, 24-99; lane 16, 204-93.

The RAPD fingerprints obtained were scored by visual analysis. There was a high degree of genotypic diversity evident among *C. concisus* strains, 86 of the 100 isolates had unique RAPD profile types, including in the 15 strains identified by PFGE [Chapter 4] as representing heterogeneous lineages. The profiles of the strains are shown in Fig 5.1. Isolates 207-94 and 24-99 (Fig. 5.1 lanes 14 and 15) appear to have similar profile types, but close inspection suggests that 24-99 contains a doublet of 350 bp, whereas 207-94 contains a single band of that size.

Five profile groups could be identified in 14 of the remaining isolates. Isolate 29-94 and 417-93 (Fig 5.2A lanes 4 and 5) obtained from the same paediatric patient admitted at different times, had the same profile (RAPDI). Strain 271-93 (Fig 5.2A lane 8), isolated from a girl and strain 286-93 (Fig 5.2A lane 9), isolated from her twin sister had identical profiles designated



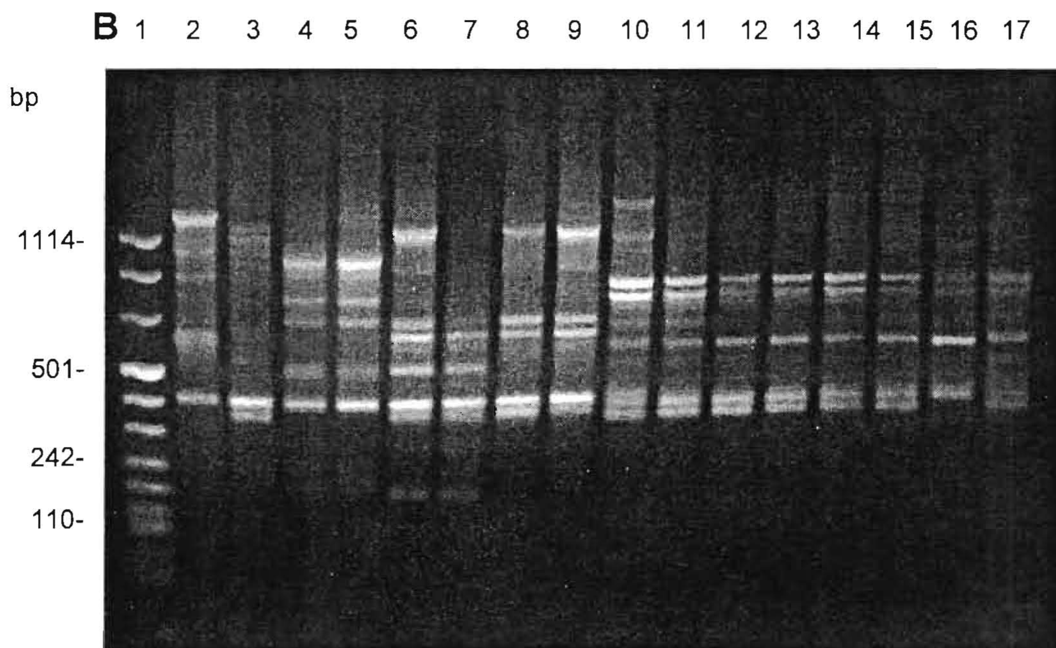


Fig 5.2. RAPD analysis using (GTG)5 primer.
A. Lane 1, Marker VIII; lane 2, 20-83; lane 3, 20-93;lane 4 29-94;lane 5, 417-93; lane 6, 36-93; lane 7, 157-94;lane 8, 271-93; lane 9, 286-93; lane 10, 263-94; lane 11, 223-96; lane 12, 62-93; lane 13, Q-26; lane 14, 26A.
B. Repeat of RAPD analysis. Lane 1, Marker VIII (Boehringer Mannheim); lane 2, 20-93; lane 3, 87-94; lane 4, 29-94; lane 5, 417-93; lane 6, 271-93; lane 7, 286-93; lane 8, 331-96; lane 9, 311-96; lane 10, 157-94; lane 11, 223-96; lane 12, 62-95; lane 13, Q26; lane 14, 26A; lane 15, 337-96; lane 16, 84-95; lane 17, 104-93.

RAPD II. Interestingly, *C. concisus* strains with unique profiles were isolated from each of the twins (data not shown). Identical RAPD profile patterns (RAPDIII) were also obtained from an additional pair of isolates 331-96 and 311-96 (Fig 5.2B lanes 8 and 9). The fourth and largest group of identical isolates contains RAPDIV. Strains, 157-94, 223-96, 62-95, Q-26, 26A, 337-96 and 104-93 (Fig 5.2B lanes10-15 and 17) have the same profile. The profile of 84-94 was originally thought to be identical to RAPDIV, but close inspection suggested that it does not contain a doublet (404 bp), implying it has a unique profile (RAPDV). For each of these isolates, RAPD typing was repeated at least twice. In most cases the profiles were reproducible, but in some cases different amplifications resulted in the disappearance of bands greater than 900 bp. For example, the 1114 bp band is present in 286-93 (Fig 5.2A lane 9), but it is absent in this strain (Fig 5.2B lane 7), following a repeat PCR. Thus, based on the profile observed in Fig 5.2B, strains 286-93 and 271-93 could be construed as different however; Fig 5.2A, suggests that they are clonal. Similarly, the profile of 157-94 shown in Fig 5.2A (lane 7) looks differently to that shown in Fig 5.2B (lane 10).

5.3.2 Restriction fragment end labelling of identical RAPD isolates

Representatives of the RAPD profiles identified above were subjected to RFEL as described in Chapter 4. Included in the analysis were a selection of random isolates, 395-96, 87-94, 307-96 and 20-93 which had unique RAPD profiles. For restriction fragment end labelling, isolates with a linkage level greater than 80% were regarded as being related or similar and therefore belonging to the same RFEL type. For isolates 271-93 and 286-93 (RAPDII) the linkage value was 82%. Thus for these isolates there was good agreement between RAPD and RFEL analysis. In contrast, strains 29-94 and 417-93 (RAPDI) clustered at a low similarity value (73%), suggesting that the two strains are not related. However, this data may be misleading because the RFEL obtained for 29-94 was feint and therefore not ideal for analysis. Four of the seven strains suggested to be clonal by RAPD (RAPDIV) were analysed using RFEL. Because of the similarities between the profiles of strains in RAPDIV and that of 84-95 [section 5.3.1], the latter strain was also included in this study. All of the isolates, including 84-95, were found to be similar, clustering above the 80% correlation value.

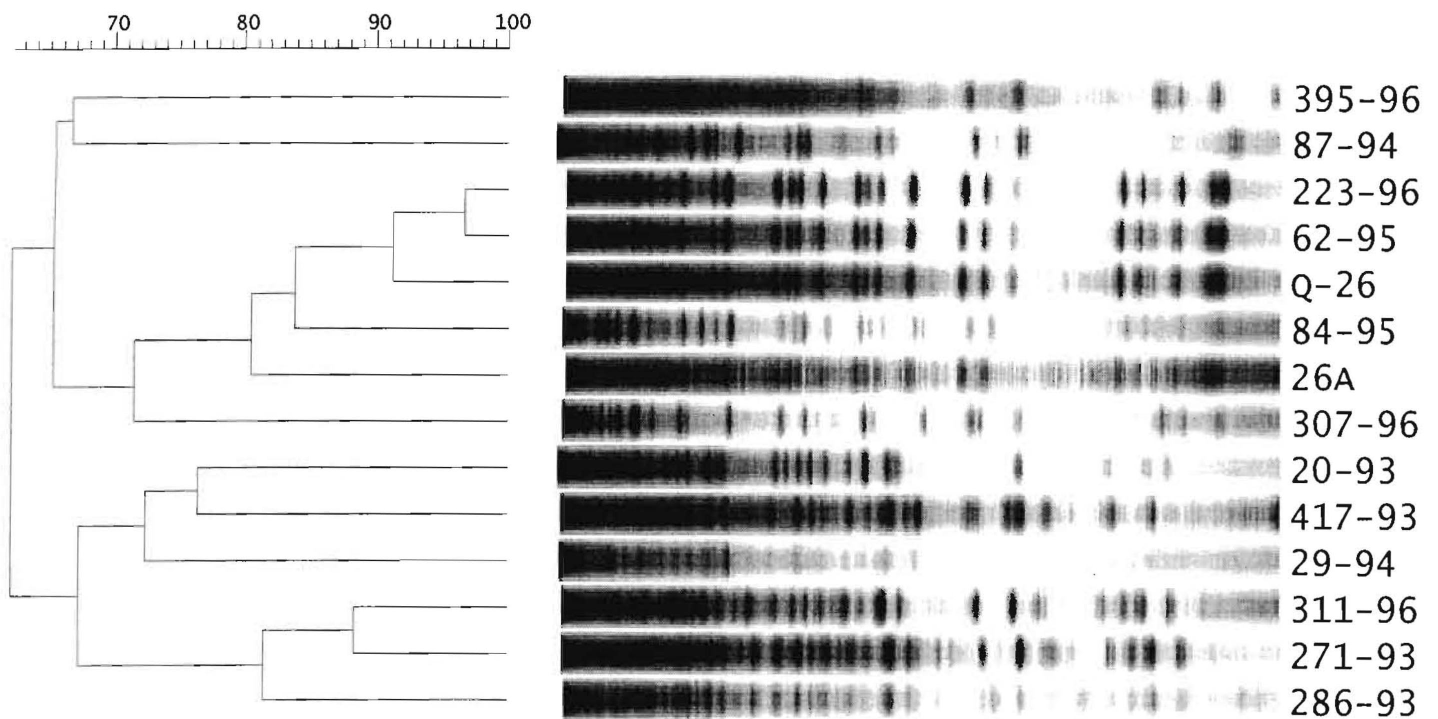


Fig. 5.3 Numerical analysis of RFEL patterns from *C. concisus* strains . Clustering of the *C. concisus* isolates is based on the Dice coefficient and the dendrogram constructed using UPGMA with a tolerance of value of 1.0% and optimiation of 0.50%.

5.3.3 FIGE analysis of strains with RAPD profiles I and II.

Strains 417-93, 29-94 (RAPDI) and strains 271-93, 286-93 (RAPDII) were analysed using FIGE. Four isolates with unique RADP profiles were also included in the study. The FIGE profiles of the isolates belonging to RAPDI (Fig 5.4 lanes 4 and 5) and RADP II (Fig 5.4 lane 2 and 3) were identical. Thus, there was perfect agreement between RAPD, RFEL and FIGE analysis on these strains. All of the strains with unique RAPD profiles also had unique FIGE profiles.

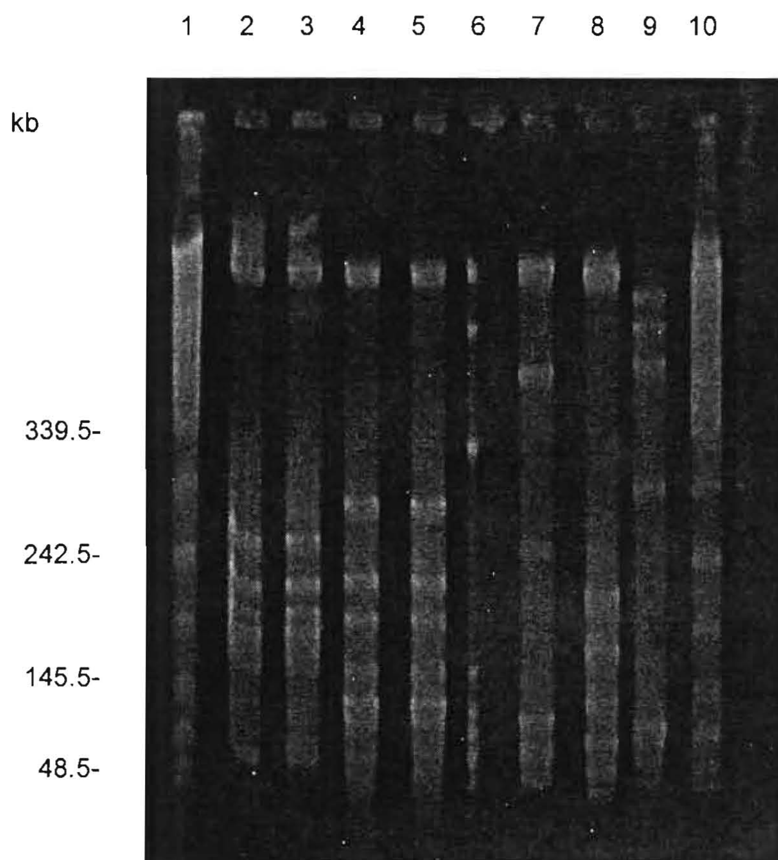


Fig 5.4 Field inversion gel electrophoresis of *C. concisus* DNA restricted with *NotI*. Lane 1 lambda ladder PFGE marker (New England BioLabs); Lane 2, 271-93; lane 3, 286-93; lane 4, 417-93; lane 5, 29-94; lane 6, 361-93; lane 7, 285-93; lane 8, 58-93; lane 9, 282-93; lane 10, lambda ladder PFGE marker.

DISCUSSION

As PFGE and RFEL are not suitable techniques for large epidemiological investigations , an alternative rapid technique was evaluated. The oligonucleotide [$(GTG)_5$] has been used as a probe to type a number of different bacteria (Doll *et al.*, 1993; Cilliers *et al.*, 1997), but has not been used as a primer in a RAPD assay. The data presented in this Chapter suggests that RAPD using this primer is a useful typing tool.

RAPD typing using $(GTG)_5$ olgonucleotide as primer yielded a simple banding patterns which were nevertheless very discriminatory, with 86% of all the strains typed yielding unique profiles. To test the level of typing ability and discrimination the technique was used to type a selection of isolates found to be diverse by PFGE [Chapter 4]. All the PFGE types gave unique profile patterns when typed with RAPD. In addition, isolate 51-99, which was non-typable by restricting with *Not*I using PFGE, was typed using this technique.

RAPD analysis identified related strains. Repetition in some samples resulted in variation in DNA fingerprints obtained, although the assay was carried out in carefully controlled conditions. This lack of reproducibility and the subjectivity in the interpretation of the results has been sighted as being the major achilles heel of RAPD typing (Olive and Bean, 1999;Wassenaar and Newell, 2000). The lack of reproducibility has been attributed to the low annealing temperature required in the assay and numerous other factors relating to the purity of DNA, inconsistency in reagents and thermocyclers (MacPherson *et al.*, 1993; Meunier and Grimont, 1993; Micheli *et al.*, 1994; Penner *et al.*, 1993). To minimise the problem some workers have used primers for enterobacterial repetitive intergenic consensus (ERIC) sequence in combination with a randomly chosen primer for typing campylobacters at higher annealing temperature (Giesendorf *et al.*, 1994). However, this did not eliminate the problem, as reproducibility of bands less than 300 bp has been reported in subsequent studies using the same technique (Iriarte and Owen, 1996). In our study steps taken to improve reproducibility concerned the preparation of DNA limiting the amount of contaminants. Genomic DNA was wound on a glass rod, instead of precipitating the DNA by centrifugation thereby eliminating the contamination with precipitates (Micheli *et al.*, 1994).

In our study in which the collection of isolates spanned a period of eight years, identical isolates were identified using RAPD typing. Although the number investigated was small there was good correlation between RADP, RFEL and FIGE, indicating the credibility of the results obtained using $(GTG)_5$ for RAPD typing.

Restriction fragment end labelling was used to great effect as a secondary typing technique. Isolates identified as identical by RAPD where confirmed using this method. Furthermore, there was good correlation between the RADP profile groups and the clustering of isolates based on dendograms from RFEL results. In some instances RAPD appeared more discriminatory than RFEL. For example, isolate 311-96, 271-93 and 286-93 were all grouped at a correlation of over 82% using RFEL. Although the latter two strains had identical RAPD profiles (RADPII), the profile of 311-96 was found to belong to RAPDIII. No attempt was made to determine the similarity of isolates based on the RAPD banding profile due to the small number of bands generated per isolate (less than 10). This was to avoid an error introduced by homoplasy .

Consolidation of all the data from the two supporting techniques corroborated results obtained using $(GTG)_5$ RADP typing. So our results, suggest that using $(GTG)_5$ as primer RAPD typing, could be used to type *C. concisus*. The method was rapid and easy to perform and the results obtained correlated well with those generated by RFEL data and PFGE analyses. The advantages of the method, far outweigh the drawbacks of the technique, highlighted earlier. This typing technique offers the possibility of carrying out regional surveillance of *C. concisus* isolates and can be used in prospective epidemiological studies to determine the pathogenic potential of *C. concisus*, routes of transmission and the identification of potential reservoirs.

CHAPTER SIX

SUMMARY AND GENERAL CONCLUSIONS

Campylobacter jejuni has recently been recognised world wide as one of the leading causes of gastroenteritis (Healing *et al.*, 1992; Skirrow and Blaser, 1992), especially in the developed countries where *C. jejuni* is considered a primary pathogen. However, the true incidence of human *Campylobacter* infection may be underestimated due to the fastidious nature of the bacteria and the limitations in routine culture methods currently in use. Most cultivation methods are tailor made for isolation of *C. jejuni*, and as a result may inhibit the growth of other potentially pathogenic *Campylobacter* species (Corry *et al.*, 1995). An improvement in culture techniques has seen an emerging class of *Campylobacter* associated gastrointestinal pathogens, the latest addition being *C. upsaliensis* (Goosens *et al.*, 1990).

The inception of the membrane filter technique in conjunction with increased hydrogen tension in culture conditions, has resulted in a higher yield in the number of *C. concisus* isolates in children with diarrhoea in Cape a Town. Unlike *C. jejuni* where most infections are associated with children under eight months (Bokkenheuser *et al.*, 1979), *C. concisus* is prevalent in children over a year old (Lastovica and Engel, 2000). This scenario does not seem to conform to the early acquisition of immunity thought to occur in children living in hyperendemic areas of infection, a theory postulated for *C. jejuni* infections in developing countries. Limited work on *C. concisus* has been carried out, and the association of this bacterium with diarrhoea is still unclear. Epidemiological work is hampered by the lack of adequate identification methods and established typing techniques. The purpose of this study was to address these shortcomings by developing an identification technique for *C. concisus* and evaluating potential molecular typing techniques for future epidemiological work on this bacterium.

Like other members of the genus *Campylobacter C. concisus* is relatively biochemically inactive, making identification difficult, using phenotypic identification schemes. A *C. concisus* genomic library was screened for a DNA sequence that could be used to identify this species. Following Southern hybridisation studies a 1.6 kb DNA from this library generated *C. concisus* specific *Hin*dIII fragment length patterns. Significantly all of the strains contained a 500 bp fragment, which hybridised to the 1.6 kb probe. From the panel of *Campylobacter* type species probed, hybridisation signals were only obtained with two

species *C. sputorum faecalis* and *C. curves.* However, the hybridisation patterns for these two species could not be confused with the profiles obtained for *C. concisus*. No signals were obtained with DNA from *C. mucosalis, C. rectus, C. coli, C. jejuni, C. lari, C. upsaliensis, C. helveticus, Bacteroides ureolyticus, C. jejuni doylei* and *C. fetus* subsp *fetus.*

An analysis of the sequencing data of the 1.6 kb fragment identified 3ORFs. The first ORF encodes the 3' end of the gyrase B subunit. The translational products of ORF two and three showed similarity to hypothetically proteins identified in *C. jejuni*, although the product of ORF3 was smaller than its counterpart in *C. jejuni*. Both these ORF were preceded by ribosomal binding sites previously described for *C. jejuni.* Putative –35 and –10 promoter sites resembling the consensus sequences described for *C. jejuni* (Wosten *et al.*, 1998) were also identified. Unlike the promoters of *C. jejuni*, the regulatory sequences described here lacked the –15 consensus promoter sites. Apart from published sequencing data on rRNA genes, to the best of our knowledge this is the first characterisation of a DNA sequence from *C. concisus.*

Probing with a fragments internal to each of the ORFs, showed that the ORF3 sequence was responsible for the signal obtained with the 500 bp DNA fragment. Significantly, no hybridisation signals were obtained with all the other *Campylobacter* species tested: *C. curvus* and *C. sputorum* subsp. *fecalis C. mucosalis, C. rectus, C. coli, C. jejuni, C. lari, C. upsaliensis, C. helveticus, C. jejuni* subsp *doylei* and *C. fetus* subsp *fetus.* This suggested that the fragment internal to ORF3 was unique to *C. concisus* and could be used as a species specific marker.

As Southern hybridisation studies are laborious, time consuming and not ideal for large scale studies that demand a large number of isolates to be processed, a rapid PCR identification assay based on the 1.6 kb DNA fragment was developed. Using primers that annealed to the extremities of this fragment a 1.5 kb product was obtained from local and Danish clinical isolates, as well as from reference strains. No PCR products were not obtained from *C. curvus* and *C. sputorum* subsp. *fecalis C. mucosalis, C. rectus, C. coli, C. jejuni, C. lari, C. upsaliensis, C. helveticus, Bacteroides ureolyticus, C. jejuni doylei* and *C. fetus* subsp *fetus*, making the assay specific to *C. concisus.* Clearly, molecular identification of *C. concisus* is dependent on the genomic stability of the 1.6 kb fragment within the genome. As *gyr*B in *C. jejuni* lies adjacent to the origin of replication (Parkhill *et al.*, 2000),

a region known to be highly conserved in bacteria (Ogasawara and Yoshikawa, 1992), it is possible to assume that the 1.6 kb fragment in *C. concisus* is stabley located.

Using macro-restriction fragment patterns obtained following PFGE the genome size of *C. concisus* was approximated to be 1.84 MB, which is close to the genome sizes estimated for *C jejuni* and *C coli* (Chang and Taylor, 1990). Using the same technique variability within *C. concisus* was demonstrated. This variability was observed in *C. concisus* isolates from children with diarrhoea collected over a period of eight years, and strains isolated within the same year. Similar results were obtained following a small study using RFEL, a relatively new technique, which has yet to evaluation extensively. Although there was good agreement between the results obtained with PFGE and RFEL, the latter technique was not 'user friendly'. The requirement for ^{32}P radionucleotide, intermittent cases of inefficient labelling, necessitating the repetition of experiments, made the technique arduous. Limitations in the gel electrophoresis apparatus used did not allow for the analysis of samples run in different gels, although excellent intragel comparison of samples was obtained. This limited the number of samples that could be analysed, as only samples loaded on the same gel could be compared. Nevertheless, RFEL was found to be reliable and informative as a typing technique and is recommended for small samples where rigorous discrimination is required.

With a view to recommending a rapid typing method, a simple $(GTG)_5$ oligonucleotide repeat was used as a primer in RAPD assays. Profiles consisting of two to seven bands were obtained for 92 isolates. The technique was highly discriminatory, 87 of the 92 strains had unique profiles. The method was rapid and easy to perform, and except in isolated cases, the fingerprints were reproducible. The clonality of isolates suggested to be identical using RAPD was confirmed with RFEL and PFGE. When tested, there was perfect agreement between RAPD, RFEL and PFGE. Armed with the ability to unequivocally identify and rapidly type *C. concisus*, it is hoped that our knowledge of this organism with regards to its potential as a pathogen, its reservoirs and mode of transmission will advance.

APPENDIX A

BACTERIAL STRAINS, PLASMIDS AND ISOLATION OF *CAMPYLOBACTER*

A.1 Bacterial strains

Table A 1. *E coli* strains used for DNA manipulations

E coli strain	Genotype	Reference
LKIII	*recA, lacI⁻, lacZ, lacy⁺*	Zabeau and Stanley, 1982
DH5α	*recA⁻*	Hanahan, 1983

A.2 Plasmids

Table A.2. Plasmids used in this study

Plasmids	Relevant characteristics	Reference
pEcoR251	Amp^R, *Eco*RI	Provided by M. Zabeau
pUC19	Amp^R, *lacZ⁺*	Messing, 1983; Yaniisch-Perron *et al.*, 1985
pB3C	Amp^R	This study
pNeo	Amp^R	This study

A.3 Isolation of *Campylobacter*

Stool samples were diluted 1:5 with sterile saline. The emulsion was filtered through a 0.6 mm pore size membrane filter (ME 26, Schleider &Schuell, Dassel Germany) onto Tryptose blood agar plates (Oxoid CM 233, Bassingstoke, UK) without any antibiotic. Plates were incubated at 37°C in an enhanced H_2 microaerophillic atmosphere. The atmosphere was generated by use of an Oxoid BR 38 or BBL 70304 (Cockeysville , Maryland) gaspak with out any catalyst.

APPENDIX B

MEDIA, BUFFERS AND SOLUTIONS

B.1 Media

2 X yeast-Tryptone (YT) broth

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g
Distilled water	1000 ml
Autoclave	

2 X YT agar

2 X YT	100 ml
Agarose	1.5 g
Autoclave	

TBA agar

Tryptose blood agar (Oxoid CM233, Bassingstoke, UK)

Fresh horse blood	10%

B.2 Buffers

Tris-EDTA (TE) buffer

Tris-Cl (1M, pH7.6)	1 ml
EDTA (0.5M pH7.6)	0.2 ml
Distilled water	98.8 ml
Autoclave	

ESP buffer

EDTA (0.5M pH)	18.61 g
NaOH	2.0 g
Sarkosyl	1 g
Distilled water	to 100 ml
Autoclave	
Add	
Proteinase K	2.5 g

Pett IV buffer

NaCl	5.84 g
Tris-Cl (1M, pH8)	1 ml
EDTA (0.5M pH8)	2 ml
Autoclave	

0.5 X TBE buffer

	Tris	8.1 g
	Boric acid	1.38 g
	EDTA	0.47 g
	Distilled water	to 100 ml
	Autoclave	

50 X TAE buffer

	Tris	242 g
	Glacial acetic acid	57.1 ml
	EDTA (0.5M, pH8.0)	100 ml
	Distilled water	to 1000 ml
	Autoclave	

10 X NNB buffer

	Tris	162 g
	Boric acid	27.5 g
	EDTA	9.3 g
	Distilled water	to 1000 ml
	Autoclave	

B.3 Solutions

6% polyacrylamide gel mix

	Acrylamide 50% (w/v)	12 ml
	Bis-acrylamide 2.5% (w/v)	1 ml
	10 X NBB	10 ml
	Urea	48 g
	Distilled water	to 100 ml

Guanidinium Thiocyanate solution

	Guanidium thiocynate	60 g
	EDTA (0.5M, ph8.0)	20 ml
	Distilled water	20 ml
	N-Lauryl-sarcosine (w/v)	5 ml
	Filter sterilise	

Ampicillin

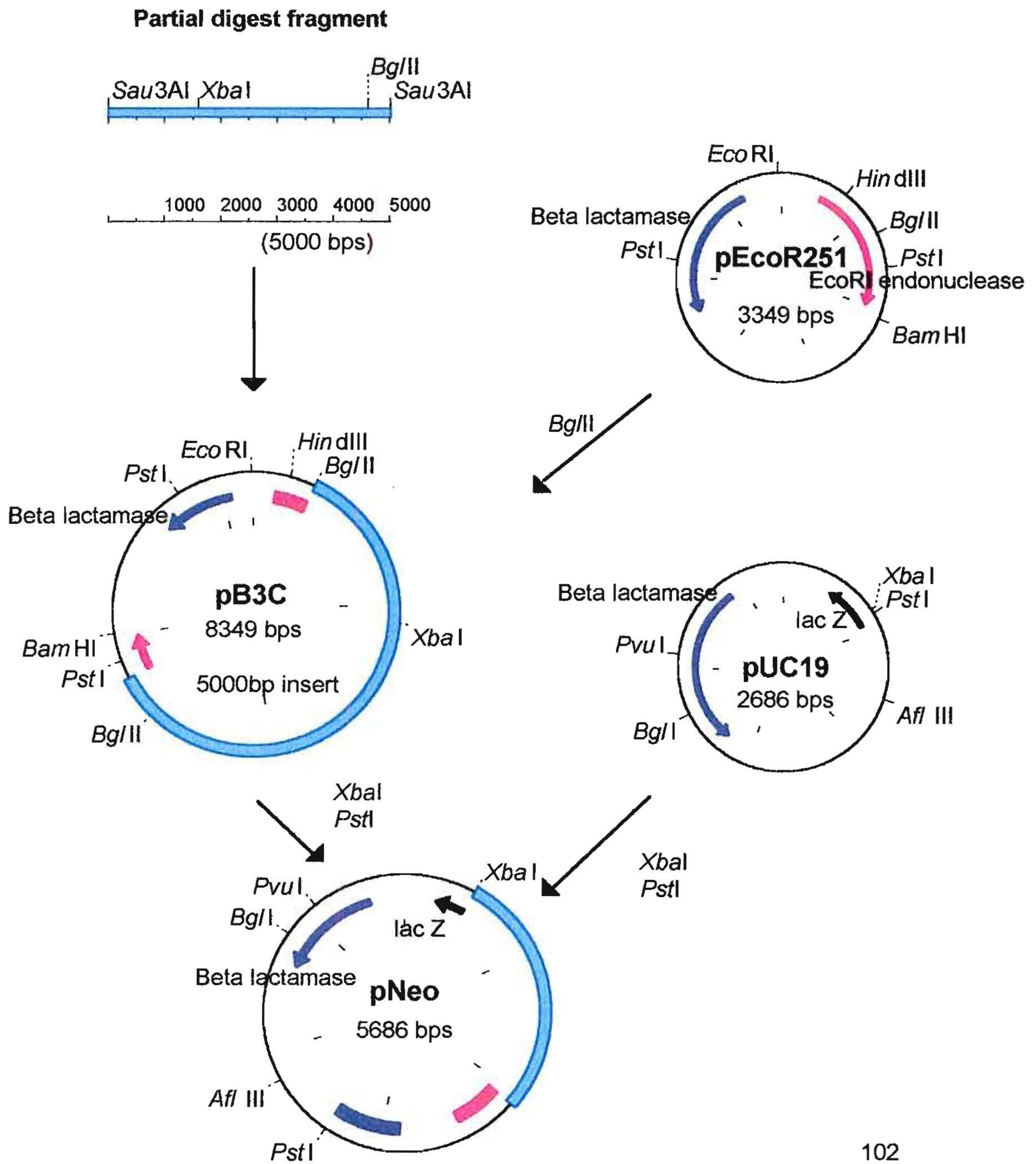
	Ampicillin (100mg/ml)	2 g
	Distilled water	20 ml
	Filter sterilise	

Gel tracking Dye

	Bromophenol blue	0.25 g
	Sucrose	40.0 g
	EDTA (0.5M, pH8.0)	4 ml
	Distilled water	to 100 ml

APPENDIX C

RECOMBINANT PLASMIDS



LITERATURE CITED

Allos, B.M. (1997) Association between *Campylobacter* infection and Guillain-Barre syndrome. *Journal of infectious Diseases.* **176**:S125-S128.

Allos, B.M., A.J. Lastovica, and M.J. Blaser. (1995) Atypical campylobacters and related microorganisms, pp. 849-865. In : *Infections of the gastrointestinal tract.* M. J. Blaser, P.D. Smith, J. I. Ravdin, H. B. Greenberg and R. L Guerrant (eds), Raven Press Ltd., New York.

Alm, R.A., P. Guerry, M.E. Power, H. Lior, and T.J, Trust. (1991) Analysis of the role of flagella in the heat-labile Lior serotyping scheme of thermophilic campylobacters by mutant allele exchange. *Journal of Clinical Microbiology.* **29**:2438-2445.

Alm, R.A., P. Guerry, and T.J. Trust. (1993) Distribution and polymorphism of the flagellin genes from isolates of *Campylobacter coli* and *Campylobacter jejuni. Journal of Bacteriology.* **175**:3051-3057.

Aquadro, C.F. and B.D. Greenberg. (1983) Human mitochondrial DNA variation and evolution: analysis of nucleotide sequence from seven individuals. *Genetics.* **103**:287-312.

Asrat, D.A., A. Hathaway, E. Sjogren, E. Ekwall, and B. Kaijser (1997) The serotype distribution of *Campylobacter jejuni* and *C. coli* isolated from patients with diarrhoea and controls at Tikur Anbassa Hospital, Addis Ababa, Ethiopia. *Epidemiology and Infection.* **118**:91-95.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and J. Lipman (1990) Basic local alignment search tool. *Journal of Molecular Biology.* **215**:403-410

Altwegg, M., A. Burnens, T Zollinger-Iten, and J.L. Penner. (1987) Problems in identification of *Campylobacter jejuni* associated with acquisition of resistance to nalidixic acid. *Journal of Clinical Microbiology.* **25**:1807-1808.

Ausebel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds) (1987) Current Protocols in Molecular Biology Vol 1.

Ayling, R.D., M.J. Woodward, S. Evans, and D.G. Newell. (1996) Restriction fragment length polymorphism of polymerase chain reaction products applied to the differentiation of poultry campylobacters for epidemiological investigations. *Research in Veterinary Science.* **60**:168-172.

Bastyns, K., S. Chapelle, P. Vandamme, H. Goossens, and R De Wachter. (1995) Specific detection of *Campylobacter concisus* by PCR amplification of 23S rDNA areas. *Molecular and Cellular Probes.* **9**:247-250.

Black, R.E., M.M. Levine, M.L. Clements, T.P. Hughes, and M.J. Blaser. (1988) Experimental *Campylobacter jejuni* infection in humans. *Journal of Infectious Diseases.* **157**:472-479.

Blaser, M.J. , C.W. Moss and R.E. Weaver. (1980) Cellular fatty acid composition of *Campylobacter fetus. Journal of Clinical Microbiology.* **11**:448-451.

Bokkenheuser, V.D., N.J. Richardson, J.H. Bryner, D.J Roux, A.B. Schutte, H. J. Koornhof, I. Freiman and E. Hartman. (1979) Detection of enteric Campylobacteriosis in children . *Journal of Clinical Microbiology. 9:227-232.*

Bourke B, Sterman PM, Woodward D, Lior H and Chan VL (1996). Pulsed field gel elctrophoresis indicates genotypic heterogeneity among *Campylobacter upsaliensis* strains. *FEMS Microbiology Letters* **143**:57-61.

Bradbury, W.C., A.D. Pearson , M.A. Marko, R.V. Congi, and J.L. Penner . (1984) Investigation of a *Campylobacter jejuni* outbreak by serotyping and chromosomal restriction endonuclease analysis. *Journal of Clinical Microbiology.* **19**:342-346.

Burnens, A.P., J. Wagner, H. Lior, J. Nicolet, and J Frey. (1995) Restriction fragment length polymorphisms among the flagellar genes of the Lior heat-labile serogroup reference strains and field strains of *Campylobacter jejuni* and *C. coli*. *Epidemiology and Infection.* **114**:423-431.

Bustamante, V.H., J.L. Puente, F. Sanchez-Lopez, M. Bobadilla, and E. Calva. (1995) Identification of *Campylobacter jejuni* and *C. coli* using the rpoB gene and a cryptic DNA fragment from *C. jejuni*. *Gene.* **165**:1-8.

Caetano- Anolles, G., B. Bassam and P. M. Grresshoff. (1991). DNA amplification using very short arbitrary oligonucleotide primers. *Bio/Technology.* **9**:553-557.

Cardarelli-Leite, P., K. Blom, C.M. Patton, M.A. Nicholson, A.G. Steigerwalt, S.B. Hunter, D.J. Brenner, T.J. Barrett, and B. Swaminathan. (1996) Rapid identification of *Campylobacter* species by restriction fragment length polymorphism analysis of a PCR-amplified fragment of the gene coding for 16SrRNA. *Journal of Clinical Microbiology.* **34**:62-67.

Cedergren, R., M.W. Gray, Y. Abel, D. Sankoff. (1988) The evolutionary relationships among known life forms. *Journal of Molecular Evolution.* **28**:98-112.

Chang, N., and D.E. Taylor. (1990) Use of pulsed-field agarose gel electrophoresis to size genomes of *Campylobacter* species and to construct a *Sal*I map of *Campylobacter jejuni* UA580. *Journal of Bacteriology.* **172**:5211-5217.

Cilliers, F.J., R.M. Warren, J.H. Hauman, I.J. Wiid, and P.D. van Helden. (1997) Oligonucleotide (GTG)5 as an epidemiological tool in the study of nontuberculous *mycobacteria*. *Journal of Clinical Microbiology.* **35**:1545-1549.

Clerc., A., C. Manceau, and X. Nesme. (1998) Comparison of randomly amplified polymorphic DNA with amplified fragment length polymorphism to assess genetic diversity and genetic relatedness within genospecies III of *Pseudomonas syringae*. *Applied and Environmantal Microbiology.* **64**:1180-1187.

Collin, D.M., and D.E. Ross. (1984) Restriction endonuclease analysis of *Campylobacter* strains with particular reference to *Campylobacter fetus* ss. *fetus*. *Journal of Medical Microbiology.* **18**:117-124.

Corry, J.E., D.E. Post, P. Colin, and M.J. Laisney. (1995) Culture media for the isolation of campylobacters. *International Journal of Food Microbiology.* **26**:43-76.

Costas., M., R.J. Owen , and P.J.H. Jackman. (1987) Classification of *Campylobacter sputorum* and allied campylobacters based on numerical analyses of electrophoretic protein patterns. *Systematic and Applied Micriobiology.* **9**:125-131.

Cover, T.L., and M.J. Blaser. (1989) The pathobiology of *Campylobacter* infections in humans. *Annual Review of Medicine.***40**:269-285.

Da Silva Tatley, F. M., A.J. Lastovica and L. M. Steyn (1992) Plasmid profiles of "*Campylobacter upsaliensis*" isolated from blood cultures and stools of pediatric patients. *Journal of Medical Microbiology.* **37**: 8-14.

Dagert, M., and S,D, Ehrlich. (1979) Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* **6**:23-28.

de Bruijn, F.J. (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Applied and Environmental Microbiology.* **58**:2180-2187.

Doll, L., S. Moshitch, and G. Frankel. (1993) Poly(GTG)5-associated profiles of *Salmonella* and *Shigella* genomic DNA. *Research in Microbiology.* **144**:17-24.

Doyle, L.P. (1944). A *Vibrio* associated with swine dysentery. *American Journal of Veterinary Research.* **5**:3-5

Doyle, M.P., and J.L. Schoeni. (1986) Isolation of *Campylobacter jejuni* from retail mushrooms. *Applied and Environmental Microbiology.* **51**:449-450.

Duim, B., T.M. Wassenaar, A. Rigter, and J Wagenaar. (1999) High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting. *Applied and Environmental Microbiology.* **65**:2369-2375.

Edwards, U., T. Rogall, H. Blocker, M. Emde and E. Bottger (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of gene coding for 16S ribosomal RNA. *Nucleic Acids Research* **17**: 7843-7853

Endtz, H.P., B.A. Giesendorf, A. van Belkum, S.J. Lauwers, W.H. Jansen, and W.G. Quint. (1993) PCR-mediated DNA typing of *Campylobacter jejuni* isolated from patients with recurrent infections. *Research in Microbiology.* **144**:703-708.

Engberg, J., S.L. On, C.S. Harrington, and P. Gerner-Smidt. (2000) Prevalence of *Campylobacter, Arcobacter, Helicobacter*, and *Sutterella* spp. in human fecal samples as estimated by a reevaluation of isolation methods for Campylobacters. *Journal of Clinical Microbiology.* **38**:286-291.

Eyers, M., S. Chapelle, G. Van Camp, H. Goossens, and R. De Wachter. (1993) Discrimination among thermophilic *Campylobacter* species by polymerase chain reaction amplification of 23S rRNA gene fragments. *Journal Clinical Microbiology.* **31**:3340-3343.

Falsen, E. (1983) Immunodiffusion as an aid in routine identification of uncommon aerobic gram negative bacteria p.477-483. In *H. lederc (ed), Gram negative bacteria of medical and public health importance : Taxonomy , identification , applicationres edition del. Institut National de la Sante et de la Recherche medicale.* Paris. France.

Fangman WL (1978) Separation of very large DNA molecules by gel electrophoresis. *Nucleic Acids Research* 5:653-665.

Fayos, A., R.J. Owen, and J. Hernandez. (1992) Ribosomal RNA gene restriction fragment diversity amongst Lior biotypes and Penner serotypes of *Campylobacter jejuni* and *Campylobacter coli. FEMS Microbiology Letters.* **95**: 87-94.

Fermer, C., and E.V. Engvall (1999) Specific PCR identification and differentiation of the thermophilic campylobacters, *Campylobacter jejuni, C. coli, C. lari,* and *C. upsaliensis. Journal of Clinical Microbiology.* **37**: 3370-33373.

Figura, N., P. Guglielmetti, A. Zanchi, N. Partini, D. Armellini, P.F. Bayeli, M. Bugnoli, and S. Verdiani. (1993) Two cases of *Campylobacter mucosalis* enteritis in children. *Journal of Clinical Microbiology.* **31**:727-728.

Firehammer, B.D. (1965) The isolation of vibrios from ovine feces. *Cornell Vet.* **55**:482-494.

Fitzgerald, C., R.J. Owen, and J. Stanley. (1996) Comprehensive ribotyping scheme for heat-stable serotypes of *Campylobacter jejuni. Journal of Clinical Microbiology.* **34**:265-269.

Florent, A. (1953) Isolement d'un vibrion saprophyte du sperme du taureau et du vagin de la vache (*vibrio bubulus*). *C.R. Soc Biol.* **147**:2066-2069.

Florent, A. (1959) Les deux vibrioses genitales: la vibriose due a *Vibrio fetus venerealis* et la vibriose d' origine intestinale due a *V. fetus intestinalis. Meded. Veeartsenijsch. Rijks-univ. Gent.* **3**:1-60.

Fricker, C.R. and R.W.A. Park (1989) A two year study of the distribution of 'Thermophilic' campylobacters in human environmental and food samples from the Reading area with particular reference to toxin production and heat stable serotype. *Journal of Applied Bacteriology.* **66**:477-490.

Fujimoto, S., N. Yuki, T. Itoh, and K. Amako (1992) Specific serotypes of *Campylobacter jejuni* associated with Gullian--Barré syndrome. *Journal of Infectious Diseases.* **165**: 613-618.

Gebhart, C.J., P. Edmonds, G.E. Ward, H. J. Kurtz, and D.J. Brenner. (1985) *'Campylobacter hyointestinalis'* sp nov.: a new species of *Campylobacter* found in the intestines of pigs and other animals. *Journal of Clinical Microbiology.* **21**:715-720.

Gebhart, C.J., M.P. Murtaugh, G.F. Lin, and G.E. Ward. (1990) Species-specific DNA probes for *Campylobacter* species isolated from pigs with proliferative enteritis. *Veterinary Microbiology.* **24**:367-379.

Gebhart, C.J., G.E. Ward, and M.P. Murtaugh. (1989) Species-specific cloned DNA probes for the identification of *Campylobacter hyointestinalis. Journal of Clinical Microbiology.* **27**:2717-2723.

Gibson, J.R., C. Fitzerald and R.J. Owen. (1995) Comparison of PFGE, ribotyping and phage-typing in the epidemiological analysis of *Campylobacter jejuni* serotype HS2 infections. *Epidemiology and Infection.* **115**:215-225.

Gibson, J., E. Lorenz, and R.J Owen. (1997) Lineages within *Campylobacter jejuni* defined by numerical analysis of pulsed-field gel electrophoretic DNA profiles. *Journal of Medical Microbiology.* **46**:157-163.

Gibson, J.R., E. Slater, J. Xerry, D.S. Tompkins, and R.J. Owen. (1998) Use of an amplified-fragment length polymorphism technique to fingerprint and differentiate isolates of *Helicobacter pylori. Journal of Clinical Microbiology.* **36**:2580-2585.

Gibson, J.R., K. Sutherland, and R.J. Owen (1994) Inhibition of DNAse activity in PFGE analysis of DNA from *Campylobacter jejuni. Letters in Applied Microbiology.* **19**:357-358.

Giesendorf, B. A., H. Goossens, P. Vandamme , L. Vlaes, C. Van den Borre, A. Koeken, H.P. Endtz, H. Stegeman, and W.G.V. Quint (1994) Polymerase chain reaction–mediated DNA fingerprinting for epidemiological studies on *Campylobacter* species. *Journal of Medical Microbiology.* **40**: 141-147

Giesendorf, B.A., and W. G. Quint (1995) Detection and identification of Campylobacter species using the polymerase chain reaction. *Cell Molecular Biology.* **41**: 625-638.

Gonzalez, I., K.A. Grant, P.T. Richardson, S.F. Park, and M.D. Collins.(1997) Specific identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* by using a PCR test based on the *ceuE* gene encoding a putative virulence determinant. *Journal of Clinical Microbiology.***35**:759-763.

Goossens H, Giesendorf BA, Vandamme P, Vlaes L, Van den Borre C, Koeken A, Goossens, H., L. Vlaes, M. De Boeck, B Pot, K. Kersters, J Levy, P De Mol, J.P Butzler, and P Vandamme. (1990a) Is "*Campylobacter upsaliensis*" an unrecognised cause of human diarrhoea?. *Lancet.* **335**:584-586.

Goosens, H., B. Pot, L. Vlaes, C. Van den Barre, R. Van den Abbele, C. Van Naelten, J. Lery, H. Cogniau, P. Marbehant, J. Verhoef, K. Kersters, J.-P. Butzler, and P. Zvandamme. (1990b) Characterisation and description of *Campylobacter upsaliensis* isolated from human feces. *Journal of Clinical Microbiology.* **28**:1039-1046

Grant, C.C., M.E. Konkel, W. Cieplak and L.S. Tompkins. (1993) Role of flagella in adherence , internalization and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures. *Infection and Immunity* **61**:1765-1771.

Griffiths, P.L., I Hooper, C.R. Fricker and R.W.A. Park. (1989) Rapid serological detection of campylobacters in stools and stool cultures using a latex agglutination tests p125-126 In *G.M. Ruiz-Palacios., L. Calva and B.R. Ruiz-Palacios (ed) Campylobacter V proceedings of the Vth international workshop on Campylobacter infections.* National Institute of Nutrition. Mexico city.

Griffiths, P.L., G.S. Moreno and W.W.A. Park. (1992) Differentiation between thermophillic *Campylobacter* species by species-specific antibodies. *Journal of Applied Bacteriology.*

Guerry, P., R.A. Alm, M.E. Power, S.M. Logan, and T.J. Trust. (1991) Role of two flagellin genes in *Campylobacter* motility. *Journal of Bacteriology.* **173**:4757-4764.

Gurtler, V., V.A. Wilson, and B.C. Mayall. (1991) Classification of medically important *Clostridia* using restriction endonuclease site difference of PCR-amplified 16S rDNA. *Journal of General Microbiology.* **137**: 2673-2679.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**:557-5580.

Hani, E.K. and V.L. Chan (1995) Expression and characterisation *of Campylobacter jejuni* Benzoylglycine Amiclohydrolase (Hippuricase) gene in *E. coli. Journal of Bacteriology* **177**:2396-2402.

Harrington, C.S., F.M. Thompson-Carter and P.E.Carter. (1997) Evidence for recombination in the flagellin locus of *Campylobacter jejuni*: implications for the flagellin gene typing scheme . *Journal of Clinical Microbiology.* **35**: 2386-2392.

Harvey, S.M. (1980) Hippurate hydrolysis by *Campylobacter fetus. Journal of Clinical Microbiology.* **11**:435-437.

Hazeleger, W.C. R.R. Beumer and F. M. Rombouts (sp). (1992) The use of latex agglutination tests for determining *Campylobacter* species. *Letters in Applied Microbiology.* **14**:181-184

Healing T.D., M.H. Greenwood and A.D. Pearson (1992) *Campylobacter* and enteritis. *Reviews in Medical Microbiology.***3**:150-167.

Hebert, G.A., P. Edmonds, and D.J. Brenner. (1984) DNA relatedness among strains of *Campylobacter jejuni* and *Campylobacter coli* with divergent serogroup and hippurate reactions. *Journal of Clinical Microbiology.* **20**:138-140.

Hernandez, J. (1993) Incidence and control of *Campylobacter* in foods. *Microbiologia* (SEM). **9**: 57-65

Hernandez, J., J.L. Alonso, A. Fayos, I. Amoros, and R.J. Owen. (1995a) Development of a PCR assay combined with a short enrichment culture for detection of *Campylobacter jejuni* in estuarine surface waters. *FEMS Microbiology Letters.* **127**:201-206.

Hernandez, J., A. Fayos, M.A. Ferrus, and R.J. Owen. (1995b) Random amplified polymorphic DNA fingerprinting of *Campylobacter jejuni* and *C. coli* isolated from human feces, seawater and poultry products. *Research in Microbiology.* **146**:685-696.

Hernandez, J., R.J. Owen, M. Costas, and A. Lastovica. (1991) DNA-DNA hybridization and analysis of restriction endonuclease and rRNA gene patterns of atypical (catalase-weak/negative) *Campylobacter jejuni* from paediatric blood and faecal cultures. *Journal of Applied Bacteriology.* **70**:71-80.

Hodinka, R.L. and P.H. Gilligan. (1988) Evaluation of the campyslide agglutination test for confirmatory identification of selected *Campylobacter* species. *Journal of Clinical Microbiology.* **26**:47-59

Hurtado, A., and R.J. Owen. (1997) A molecular scheme based on 23S rRNA gene polymorphisms for rapid identification of *Campylobacter* and *Arcobacter* species. *Journal of Clinical Microbiology.* **35**:2401-2404.

Huys, G., R. Coopman, P Janssen, and K. Kersters. (1996) High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. *International Journal of Systematic Bacteriology.* **46**:572-580.

Hwang , M.N. and G.M. Ederer. (1975) Rapid hippurate hydrolysis method for presumptive identification of group B *Streptococci. Journal of Clinical Microbiology* **1**:114-115.

Iriarte, M.P., and R.J. Owen. (1996) Repetitive and arbitrary primer DNA sequences in PCR-mediated fingerprinting of outbreak and sporadic isolates of *Campylobacter jejuni. FEMS Immunology and Medical Microbiology.* **15**:17-22.

Ish-Horowicz, D., and J.F. Burke (1981) Rapid and efficient cosmid cloning. *Nucleic Acids Research* 9:2989-2998.

Jacobs-Reitsma, W.F., H.M.E. Maas and W.H. Jansen. (1995) Penner serotyping of *Campylobacter* isolates from poultry with absorbed pooled antisera. *Journal of Applied Bacteriology* **79**: 286-291.

Janssen, P., R. Coopman, G. Huys, J. Swings, M. Bleeker, P. Vos, M. Zabeau, and K. Kersters. (1996) Evaluation of the DNA fingerprinting method AFLP as an new tool in bacterial taxonomy. *Microbiology.* **142**:1881-1893.

Jensen, M.A., J.A. Webster, N. Straus. (1993) Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Applied and Environmental Microbiology.* **59**:945-952.

Johnson , W.M., and H. Lior.(1988) A new heat-labile cytolethal distending toxin (CDTL) produced by *Campylobacter* spp. *Microbial Pathology.* **4**: 115-126.

Johnson, B.W., Y. Lino and R.M. Roop. (1985) Bone resorption in priodontal diseases: role of bacterial factors. *International Endod Journal,.* **18**: 152-157.

Jones, F.S., M. Orcutt and R.B. Little. (1931) Vibrios (*V. jejuni* n. sp.) associated with intestinal disorders in cows and calves. *Journal of Experimental Medicine.* **53**:853-863.

Kakoyiannis, C.K., P.J. Winter, and R.B. Marshall. (1988) The relationship between intestinal *Campylobacter* species isolated from animals and humans as determined by BRENDA. *Epidemiology and Infection.* **100**: 379-387.

Keim, P., A. Kalif, J. Schupp, K. Hill, S.E. Travis, K. Richmond, D.M. Adair, M. Hugh-Jones, C.R. Kuske, and P. Jackson. (1997) Molecular evolution and diversity *in Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *Journal of Bacteriology.***179**:818-824.

Ketley, J.M. (1997) Pathogenesis of enteric infection by *Campylobacter. Microbiology.* **143**:5-21.

Kiehlbauch, J.A., D. J. Brenner, M.A. Nicholson, C.N. Baker, C.M. Patton, A.G. Steigerwalt, and I.K. Wachsmuth. (1991) *Campylobacter butzleri* sp. Nov. isolated from humans and animals with diarrhoeal illness. *Journal of Clinical Microbiology.* **29**: 376-385.

Kim, N.W., R.R. Gutell and V.L. Chan (1995) Complete sequences and organization of the rrnA operon from *Campylobacter jejuni* TGH9011 (ATCC43431). *Gene.* **164**: 101-106.

King, V., and C.L. Clayton. (1991) Genomic investigation of phenotypic variation in *Campylobacter jejuni* flagellin. *FEMS Microbiology Letters.* **68**:107-111.

Kokotovic, B,, and S.L. On. (1999) High-resolution genomic fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* by analysis of amplified fragment length polymorphisms. *FEMS Microbiology Letters.* **173**:77-84.

Korolik, V., P.J. Coloe, and V.A. Krishnapillai. (1988) A specific DNA probe for the identification of *Campylobacter jejuni. Journal of General Microbiology.* **134**:521-529.

Kostman, J.R., T.D. Edlind, J.J. LiPuma, and T.L. Stull. (1992) Molecular epidemiology of *Pseudomonas cepacia* determined by polymerase chain reaction ribotyping. *Journal of Clinical Microbiology.* **30**:2084-2087.

Kuroki S., T. Haruta, M. Yushioka, Y. Kohayashi, M. Nukima and H. Nakamishi. (1991) Guillian barré syndrome associated with *Campylobacter* infection. *Pedriatic Infectious Diseases.* **10**:149-151

Kuroki, S., T. Saida, M. Nukina, T. Haruta, M. Yoshioka, Y. Kaboyashi and H. Nakanishi. (1993) *Campylobacter jeuni* strains from patients with Guillain Barre syndrome belong mostly to penner serogroup 19 and contain β-*N*-acetylglucosamine residues. *Annales of Neurology* **33**:243-247.

Laanbroek, H,J., W. Kingma, and H. Veldkamp. (1997) Isolation of an aspartate-fermenting , free-living *Campylobacter* species. *FEMS Microbiology Letters.* **1**:99-102.

Laguerre, G., M. Allard, F. Revoy and N. Amarger. (1994) Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes . *Applied and Environmental Microbiology.* 60:56-63.

Lastovica, A.J. and M.E. Engel (2000) *Campylobacter concisus*:Pathogen or commensal? Results of a five year Study of pediatric patients in South Africa [D-250]. In *Abstract book and programe, American society for Microbiology 100th general meeting.* Los Angeles, California. American society for Microbiology, Washington, DC.

Lastovica, A.J., and E. Le. Roux. (2000) Efficient isolation of campylobacteria from stools. *Journal of Clinical Microbiology.* **38**: 2798-2799.

Lastovica, A.J., E. Le Roux, and J.L Penner . (1989) "*Campylobacter upsaliensis*" isolated from blood cultures of pediatric patients. *Journal of Clinical Microbiology.* **27**:657-659.

Lastovica, A., E. Le Roux, R. Warren, and H. Klump. (1993) Clinical isolates of *Campylobacter mucosalis. Journal of Clinical Microbiology.* **31**:2835-2836.

Lastovica, A.J., E. Le Roux, R. Warren, and H. Klump. (1994) Additional data on clinical isolates of *Campylobacter mucosalis. Journal of Clinical Microbiology.* **32**:2338-2339.

Lauwers S, Devreker T, Van Etterijck R et al (1991) Isolation of *Campylobacter concisus* from human faeces. *Microbial Ecology in Health and Disease* **4** (suppl.), S91.

Lawson, G.H.K., J.L. Leaver, G.W. Pettigrew and A.C. Rowland (1981). Some features of *Campylobacter sputorum* subsp *mucosalis* subsp nov., nom. rev. and their taxonomic significance. *International Journal of Systematic Bacteriology.* **31**:385-391.

Leaper, S. and R.J. Owen. (1981) Identification of catalase producing *Campylobacter* species on biochemical characteristics and on cellular fatty acid composition. *Current Microbiology.* **6**:31-35.

Le Roux, E., and A.J. Lastovica. (1998) The Cape Town protocol: How to isolate the most campylobacters for your dollar, pound, franc, yen, etc. In *The proceedings of the 9th International Workshop on Campylobacter, Helicobacter, and related organisms* (Cape Town) Institute of Child Health, Red Cross Children's Hospital.

Linton, D., A.J. Lawson, R.J. Owen, and J Stanley. (1997) PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. *Journal of Clinical Microbiology.* **35**:2568-2572.

Lior, H., D.L. Woodward, J.A. Edgar, L.J. Laroche, and P. Gill. (1982) Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. *Journal of Clinical Microbiology.* **15**:761-768.

Logan, J.M., A. Burnens, D. Linton, A.J. Lawson, and J. Stanley. (2000) *Campylobacter lanienae* sp. nov., a new species isolated from workers in an abattoir. *International Journal of Systematic and Evolutionary Microbiology.* **50**:865-872.

Lorenz, E., A. Lastovica, and R.J. Owen . (1998) Subtyping of *Campylobacter jejuni* Penner serotypes 9, 38 and 63 from human infections, animals and water by pulsed field gel electrophoresis and flagellin gene analysis. *Letters in Applied Microbiology.* **26**

MacPherson, J.M., P.E. Eckstein, G.J. Scoles, and A.A. Gajadhar. (1993) Variability of the random amplified polymorphic DNA assay among thermal cyclers, and effects of primer and DNA concentration. *Molecular and Cellular Probes.* 7:293-299.

Madden, R.H., L. Moran, and P. Scates (1998) Frequency of occurrence of *Campylobacter* spp. redmeats and poultry in Northern Ireland and their subsequent subtyping using polymerase chain reaction –restriction fragment length polymorphism and random amplified polymorphic DNA method. *Journal of Applied Microbiology.* **84**:703-708.

Mahajan , S., and F. G. Rodgers. (1990) Isolation, characterisation and host-cell-binding properties of a cytotoxin from *Campylobacter jejuni. Journal of Clinical Microbiology.* **28**:1314-1320.

Maniatis, T.,E.F. Fritsch and J. Sambrook. (1989) Molecular Cloning-A Laboratory Manual, 2nd Ed. N. Ford, C. Nolan,and M. Ferguson *(eds)*, Cold Spring Harbour Laboratory Press, USA.

Marshall, S.M., P.L. Melito, D.L. Woodward, W.M. Johnson, F.G. Rodgers, and M.R. Mulvey. (1999) Rapid identification of *Campylobacter, Arcobacter*, and *Helicobacter* isolates by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene. *Journal of Clinical Microbiology.* **37**:4158-4160.

Maslow, J., and M.E. Mulligan. (1996) Epidemiologic typing systems. *Infection Control and Hospital Epidemioliogy.* **17**:595-604.

Mayer L.W. (1988) Use of plasmid profiles in epidemiologic surveillance of disease outbreaks and in tracing the transmission of antibiotic resistance. *Clinical Microbiology Reviews.* **1**: 228-243.

McClelland, M., C. Petersen, and J. Welsh. (1992) Length polymorphisms in tRNA intergenic spacers detected by using the polymerase chain reaction can distinguish streptococcal strains and species. *Journal of Clinical Microbiology.* **30**:1499-1504.

McClung, C.R., D.G. Patriquin and R.E.Davis. (1983) *Campylobacter nitrofigilis* sp. nov., a nitrogen–fixing bacterium associated with roots of *Spartina alterniflora . International Journal of systematic Bacteriology.* **33**:605-612.

McFadyean, J. and S. Stockman (1913) Report of the Departmental Committee appointed by the Board of Agriculture and Fisheries to inquire into epizootic Abortions Part III. Abortion in sheep H.M.S.O. London.

Messing, J. (1983) New M13 vectors for cloning . *Methods in Enzymology.* **101**:20-78

Meunier, J.R., and P.A. Grimont. (1993) Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. *Research in Microbiology.* **144**:373-379.

Micheli, M.R., R. Bova, E. Pascale, and E. D. Ambrosio (1994) Reproducible DNA fingerprints with the random amplified polymorphic DNA (RAPD) method. *Nucleic Acids Research.* **22**: 1921-1922.

Mishu, B., C.M. Patton and R.V. Tauxe (1992) Clinical and Epidemiological features of *non-jejuni. Non-coli Campylobacter* species. In: *Campylobacter jejuni Current Status and Future Trends* ASM Press Washington DC.

Moore, W.E., L.V. Holdeman, E.P. Cato, R.M. Smibert, J.A. Burmeister, K.G. Palcanis, and R.R. Ranney RR. (1985) Comparative bacteriology of juvenile periodontitis *.Infection and Immunity.* **48**:507-519.

Morooka, T., A. Umeda, and K. Amako. (1985) Motility as an intestinal colonization factor for *Campylobacter jejuni. Journal of General Microbiology.* **131**:1973-1980.
Moss, C.W., A. Kai, M.A. Lambert and C. Patton. (1984) Isoprenoid quinine content and cellular fatty acid composition of *Campylobacter* species. *Journal of Clinical Microbiology.* **19**:772-776.

Nachamkin, I., and S. Barbagallo. (1990) Culture confirmation of Campylobacter spp. by latex agglutination. *Journal of Clinical Microbiology.* **28**:817-818.

Nachamkin, I., M.J. Blaser, and L.S. Tompkins eds. (1992). *Campylobacter jejuni*: Current Status and Future Trends, ASM Press. Washington DC.

Nachamkin, I., K. Bohachick, and C.M. Patton. (1993) Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. *Journal of Clinical Microbiology.* **31**:1531-1536.

Nishimura, M., M. Nukina, S. Kuroki, H. Obayashi, M. Ohta, J.J. Ma, T. Saida and T Uchiyama . (1997) Characterization of *Campylobacter jejuni* isolates from patients with Guillain-Barre syndrome. *Journal of Neurological Science.* **153**:91-99.

Nishimura, M., M. Nukina, J.M. Yuan, B. Q. Shen, J.J. Ma, M. Ohta, T. Saida and T. Uchiyama. (1996) PCR-based restriction fragment length polymorphism (RFLP) analysis and serotyping of *Campylobacter jejuni* isolates from diarrheic patients in China and Japan. *FEMS Microbiology Letters.* **142**:133-138.

Newell, D.G. J.A. Frost, B. Duim, J.A. Wagenaar, R.H. Madden, J. van der Plas and S.L.W On (2000) New developments in the subtyping of *Campylobacter* species. In: *Campylobacter.* Nachamkin, I., M.J. Blaser eds 2nd Edition. Washington D.C. ASM Press.

Nuijten, P.J., F.J. van Asten, W. Gaastra, and B.A. van der Zeijst. (1990) Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. *Journal of Biological Chemistry.* **265**:17798-17804.

Ogasawara, N., and H. Yoshikawa. (1992) Genes and their organization in the replication origin region of the bacterial chromosome. *Molecular Microbiology.* **6**:629-634.

Olive, D.M., and P. Bean (1999) Principles and applications of methods for DNA-based typing of Microbial organisms (Minireview). *Journal of Clinical Microbiology.* **37**: 1661-1669.

On SLW (1994) Confirmation of human *Campylobacter concisus* isolates misidentified as *Campylobacter mucosalis* and suggestions for improved differentiation between the species. *Journal of Clinical Microbiology* **32**:2305-2306.

On, S.L.W. (1996) Identification methods for *Campylobacter, Helicobacters,* and related organisms. *Clinical Microbiology Reviews.* **9**:405-422.

On, S.L.W. (1998) Invitro genotypic variation of *Campylobacter coli* documented by pulsed field gel electrophoretic DNA profiling: implications for epidemiological studies. *FEMS Microbiolgy letters* **165**:341-346.

On, S.L., H.I. Atabay, J.E. Corry, C.S. Harrington, and P. Vandamme. (1998) Emended description of *Campylobacter sputorum* and revision of its infra-subspecific (biovar) divisions, including *C. sputorum* biovar *paraureolyticus*, a urease-producing variant from cattle and humans. *International Journal of Systematic Bacterioliogy.***48**:195-206.

On, S.L.W., and B. Holmes. (1991a). Effect of inoculum size on the phenotypic characterization of Campylobacter species. *Journal of Clinical Microbiology.* **29**:923-926.

On, S.L.W., and B. Holmes. (1991b) Reproducibility of tolerance tests that are useful in the identification campylobacteria. *Journal of Clinical Microbiology.* **29**: 1785-1788.

On, S.L.W., and B. Holmes. (1995) Classification and identification of *Campylobacters* , *Helicobacters* and allied taxa by numerical analyses of phenotypic characters. *Systematic and Applied Microbiology*. **18**:374-390.

On, S.L.W., and P. Vandamme. (1997) Identification and epidemiological typing of *Campylobacter hyointestinalis* subspecies by phenotypic and genotypic methods and description of novel subgroups. *Systematic and Applied Microbiology* **20**:238-247.

Owen, R.J., A. Beck, and P Borman. (1985) Restriction endonuclease digest patterns of chromosomal DNA from nitrate-negative *Campylobacter jejuni*-like organisms. *European Journal of Epidemiology*. **1**:281-287.

Owen, R.J., M. Costas and C. Dawson. (1989) Application of different chromosomal DNA restriction digests fingerprints to specific and subspecific identification of *Campylobacter* isolates. *Journal of Clinical Microbiology*. **27**:2338-2619.

Owen, R.J., A. Fayos, J. Hernandez, and A. Lastovica. (1993) PCR-based restriction fragment length polymorphism analysis of DNA sequence diversity of flagellin genes of *Campylobacter jejuni* and allied species. *Molecular and Cellular Probes*. 7:471-480.

Owen, R.J., C. Fitzgerald, K Sutherland, and P. Borman. (1994) Flagellin gene polymorphism analysis of *Campylobacter jejuni* infecting man and other hosts and comparison with biotyping and somatic antigen serotyping. *Epidemiology and Infection*. **113**:221-234.

Owen, R.J., and J. Hernandez. (1990) Occurrence of plasmids in "*Campylobacter upsaliensis*" (catalase negative group or weak group) from geographically diverse patients with gastroenteritis or bacteremia. European *Journal of Epidemiology*. 6:111-117.

Owen, R.J., K. Sutherland, C. Fitzgerald, J. Gibson, P. Borman, and J. Stanley. (1995) Molecular subtyping scheme for serotypes HS1 and HS4 of *Campylobacter jejuni*. *Journal of Clinical Microbiology*. **33**:872-877.

Oyofo, B.A., S.A. Thornton, D.H. Burr, T.J. Trust, O.R. Pavlovskis, and P. Guerry. (1992) Specific detection of *Campylobacter jejuni* and *Campylobacter coli* using polymerase chain reaction . *Journal Clinical Microbiology*. **30**:2613-2619.

Park, R.W.A., P.L. Griffiths and G.S. Moreno. (1991) Sources and survival of campylobacters: relevance to enteritis and the food industry. *Journal of Applied Bacteriology* symposium supplement.**20**:975-1065.

Paster, B.J. and F.E. Dewhirst (1988) Phylogeny of campylobacters , wolinellas, *Bacteroides gracilis* and *Bacteroides ureolyticus* by 16S ribosomal ribonucleic acid sequencing. *International Journal of Systematic Bacteriology*. **38**:56-62.

Patton , C.M., N. Shaffer, P. Edmonds, T.J. Barret, M.A. Lambert, C. Baker, D.M. Derlman and P.J. Bremner (1989) Human disease associated with *Campylobacter upsaliensis* (catalase- negative or weakly positive *Campylobacter* species) in the United States. *Journal of clinical Microbiology* 27:66-73

Patton, C.M., I.K. Wachsmuth, G.M. Evins, J.A. Kiehlbauch, B.D. Plikaytis, N. Troup, L. Tompkins, and H. Lior. (1991) Evaluation of 10 methods to distinguish epidemic-associated *Campylobacter* strains. *Journal of Clinical Microbiology*. **29**:680-688.

Penner, G.A., A. Bush, R. Wise, W. Kim, L. Domier, K. Kasha, A. Laroche, G. Scoles, S. J. Molnar, G. Fedak (1993) Reproducible of random amplified DNA (RAPD) analysis among laboratories. In: methods and Applications. Cold spring Harbor Laboratory Press.

Penner, J.L., and J.N. Hennessy. (1980) Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp.*jejuni* on the basis of soluble heat-stable antigens. *Journal of Clinical Microbiology*. **12**:732-737.

Picken, R.N., Z. Wang, and H.L. Yang. (1987) Molecular cloning of a species-specific DNA probe for *Campylobacter jejuni*. *Molecular and Cellular Probes*. **1**:245-259.

Pitcher, P.G.N., N.A. Saunders and R.J. Owen. (1989) Rapid extraction of bacterial genomic DNA with Guanidium thiocynate. *Letters in Applied Microbiology* **8**: 151-156.
Prevot, A.R. (1940) Etudes de systematique bacterienne V.Essai de classificatio des vibrions anaerobies. *Annals institut Pasteur* **64**:117-125.

Rees, J.H., N.A. Gregson, and R.A Hughes. (1995) Anti-ganglioside GM1 antibodies in Guillain-Barré syndrome and their relationship to *Campylobacter jejuni* infection. *Annales of Neurology*. 38:809-816.

Rennie, R.P., D. Strong, D.E. Taylor, S.M. Salama, C. Davidson, and H. Tabor (1994) *Campylobacter fetus* diarrhea in a hutterite colony: epidemiological observations and typing of the causative organism. *Journal of Clinical Microbiology*. **32**: 721-724

Roca, J. (1995) The mechanisms of DNA topoisomerases. *Trends in Biochemical Science.* **20**: 156-160.

Rollins, D.M., and R.R. Colwell. (1986) Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Applied and Environmental Microbiology*. **52**:531-538.

Romaniuk, P.J. and T.J. Trust (1987) Identification of *Campylobacter* species by southern hybridization of genomic DNA using an oligonucleotide probe for 16S rRNA genes. *FEMS Microbiology Letters* **43**: 331-335.

Romaniuk, P.J., B. Zoltowska, T.J. Trust, D.J. Lane, G.J. Olsen, N.R. pace, and D.A. Stahl. (1987) *Campylobacter pylori*, the spiral bacterium associated with human gastritis , is not a true *Campylobacter* sp. *Journal of Bacteriology*. **169**:2137-2141.

Roop II, R.M., R.M. Smibert, J.L. Johnson and N.R. Krieg. (1985) *Campylobacter mucosalis* (Lawson, Leaver, Pettigrew, and Rowland 1981) comb. nov.: emended description . *International Journal of Systematic Bacteriology*. **35**:189-192.

Sambrook, J., E.F. Fritsch and T. Maniatis (1989) Molecular cloning : a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sandstedt, K., J. Ursing and M. Walder. (1983) Thermotolerant *Campylobacter* with no or weak catalase activity isolated from dogs. *Current Microbiology*. **8**:209-213.

Sanger, F., S. Nicklen and A.R. Coulson (1977) DNA sequencing with chain –termination inhibitors. *Proceedings of the National Academy of Science*.USA 74:5463-5467.

Savelkoul, P.H., H.J. Aarts, J. de Haas, L. Dijkshoorn, B. Duim, M. Otsen, J.L. Rademaker, L. Schouls, and J.A. Lenstra. (1999) Amplified-fragment length polymorphism analysis: the state of an art. *Journal of Clinical Microbiology*. 37:3083-3091.

Schwartz D.C., and C.R. Cantor.(1984) Separation of yeast chromosome–sized DNAs by pulsed-field electrophoresis. *Cell* **37**: 67-75.

Sebald, M. and M.C. Véron (1963) Teneur en bases de I.A.D.N et classification des vibrios. *Annals Institut Pasteur* **105**:897-910.

Seth, A. (1984) A new method for linker ligation. *Gene Analytical Techniques* **1**:99-103.

Sharples, G.T. , and R.G. Lloyd. (1990). A novel repeated DNA sequence located in the intergenic regions of bacterial chromosomes. *Nucleic Acids Research*. **18**:6503-6508.

Skirrow, M.B.(1984)Diseases due to *Campylobacter, Helicobacter* and related bacteria. *Journal of Comparitive Pathology* **111**:113-149.

Skirrow, M.B., and J. Benjamin. (1980a) Differentiation of enteropathogenic *Campylobacter*. *Journal of Clinical Pathology*. **33**:1122.

Skirrow, M.B., and J. Benjamin (1980b) "1001" *Campylobacter* cultured characteristics of intestinal Campylobacter from man and animals *Journal of Hygiene* **85**:427-442.

Skirrow, M.B., and M.J. Blaser. 1992. Clinical and epidemiological considerations p3-8 in Nachamkin I, Blaser MJ and Tomkins L S (ed*). Campylobacter jejuni Current Status and future trends*. ASM Press washington DC.

Slater, E., and R.J Owen. (1998) Subtyping of *Campylobacter jejuni* Penner heat-stable (HS) serotype 11 isolates from human infections. *Journal of Medical Microbiology*. **47**:353-357.

Smibert, R.M. (1974) Genus II. *Campylobacter* Sebald and Veron 1963, 907, pp.207-212. *In Bergey's manual of determinative bacteriology, 8th Ed., R.E. Buchanan and N.E. Gibbons* (eds), The Williams and wilkins Co., Baltimore.

Smith, T. (1918) *Spirilla* associated with disease of the fetal membrane in Cattle (infectious abortion). *Journal of Experimental Medicine*. **28**:701-721.

Smith CL, Econome JG, Schatt A, Klco S, Cantour CR (1987) A physical map of the *Escherichia coli* K12 genome. *Science* **236**:1448-1453

Smith, T., and M.L. Orcutt. (1927) *Vibrios* from calves and their serological relation to *Vibrio fetus*. *Journal of Experimental Medicine* **45**: 391-397.

Smith, T. and M.S. Taylor. (1919) Some morphological and biochemical characteristics of the *spirilla* (*Vibrio-fetus* n. sp.) associated with disease of the fetal membrane in cattle . *Journal of Experimental Medicine*. 30:299-312.

Southern , E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**: 503-517

Stanley, J., A.P. Burnens, D. Linton, S.L. On, M. Costas, and R.J. Owen . (1992) *Campylobacter helveticus* sp. nov., a new thermophilic species from domestic animals: characterization, and cloning of a species-specific DNA probe. *Journal of General Microbiology*. **138**:2293-2303.

Stanley, J., C. Jones, A. Burnens, and R.J. Owen . (1994) Distinct genotypes of human and canine isolates of *Campylobacter upsaliensis* determined by 16S rRNA gene typing and plasmid profiling. *Journal of Clinical Microbiology*. **32**:1788-1794.

Stanley J, D. Linton, K. Sutherland, C. Jones and R.J. Owen. (1995) High-resolution genotyping of *Campylobacter coli* identifies clones of epidemiological and evolutionary significance. *Journal of Infectious Diseases* **172**:1130-1134.

Steele, M., B. McNab, L. Fruhner, S. DeGrandis, D. Woodward and J.A. Odumeru. (1998) Epidemiological typing of Campylonacter isolated from meat processing plants by pulsed–field gel electrophoresis, fatty acid profile typing, serotyping and biotyping. *Applied and Environmental Microbiology*. **64**:2346-2349.

Stephens, C.P., S.L.W. On, and J.A. Gibson (1998) An outbreak of infectious hepatitis in commercially reared ostriches associated with *Campylobacter coli* and *Campylobacter jejuni*. *Veterinary Microbiology*. **61**:183-190.

Stern, M.J., G.F. Ames, N.H. Smith, E.C Robinson, and C.F. Higgins. (1984) Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell*. **37**:1015-1026.

Stucki, U., J. Frey, J. Nicolet, and A.P. Burnens. (1995) Identification of *Campylobacter jejuni* on the basis of a species-specific gene that encodes a membrane protein. *Journal of clinical Microbiology*. **33**:855-859.

Tanner, A.C. 1986 Characterization of *Wolinella* spp., *Campylobacter concisus, Bacteroides gracilis*, and *Eikenella corrodens* by polyacrylamide gel electrophoresis. *Journal of Clinical Microbiology*. **24**:562-565.

Tanner AC.R, Badger S, Lai CH, Listgarten M A, Visconti RA and Socransky SS (1981) *Wolinella* ge nov., *Wollinella succinogenes* (*Vibrio succinogens* Wolin *et al*) comb.nv.and description of *Bacteriodes graciles* sp nov., *Wolinella recta* sp nov., *Campylobacter concisus* sp nov., and *Eikenella corrodens* from humans with periodontal disease. *International Journal of Systematic Bacteriology* **31**:432-445.

Tanner, A.C., J.L. Dzink, J.L. Ebersole, and S.S. Socransky. (1987) *Wolinella recta, campylobacter concisus, bacteroides gracilis*, and Eikenellacorrodens from periodontal lesions. *Journal of Periodontal Research*. **22**:327-330.

Tanner, A.C.R, Haffer C, Bratthall GT, Visconti RA, Socransky SS (1979) A study of the bacteria associated with advancing periodontitis in man. *Journal of Clinical Periodontology* **6**: 278-307

Tauxe, R.V. (1992) Epidemiology of *Campylobacter jejuni* infection in the United states and other industrialised nations. In: *Campylobacter jejuni current status and future Trends*. Niachamkin, I., M.J. Blaser, and L.S. Tompkins eds. (1992). ASM Press. Washington DC.

Tauxe, R.V., C.M. Patton, P. P. Edmonds, T.J. Barret, D.J. Brenner and P.A. Blake (1985) Illness associated with *Campylobacter laridis*, a newly recognized *Campylobacter* species. *Journal of clinical Microbiology* **21**:222-225.

Taylor D.N. (1992) *Campylobacter* infection in Developing countries :In *Campylobacter jejuni current status and future trends*. Niachamkin, I., M.J. Blaser, and L.S. Tompkins eds. (1992). ASM Press. Washington DC.

Taylor, D.E. and N. Chang (1987) Immunoblot and enzyme–linked immunosorbent assays of *Campylobacter* major outer-membrane protein and application to the differentiation of *Campylobacter* species. *Molecular and cellular probes*.**1**:261-274.

Taylor, D.E., N. Chang, N.S. Taylor, and J.G. Fox. (1994) Genome conservation in *Helicobacter mustelae* as determined by pulsed-field gel electrophoresis. *FEMS Microbiology Letters*. **118**:31-36.

Taylor DE, M. Eaton, M. Chang, and S.M. Salama (1992) Construction of a *Helicobacter pylori* genomic map and demonstration of diversity at the genomic level. *Journal of Bacteriology* **174**:6800-6806

Taylor, D.E., P. Echeverria, C. Pitarangsi, J. Seriwatana, L. Bodhidatta and M.J. Blaser. (1988) Influence of strain characteristics and immunity on the epidemiology of *Campylobacter* infections in Thailand. *Journal of Clinical Microbiology*. **26**:863-868.

Taylor, D.E., and K. Hiratsuka. (1990) Use of non-radioactive DNA probes for detection of *Campylobacter jejuni* and *Campylobacter coli* in stool specimens. *Molecular and Cellular Probes*. **4**:261-271.

Tee, W., B. Mayall, C.R. Lucas, R. Rayner, S. Pearson and B. Dwyer. (1992) Use of ribosomal RNA gene restriction patterns to investigate two outbreaks of *Campylobacter* enteritis in Melbourne, Australia. *Journal of Medical Microbiology*. **37**:385-388.

Tenover, F.C. R.D. Arbeit, and R.V. Goering.(1997) How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. *Infection Control and Hospital Epidemiology* **18**: 426-439.

Tenover., F.C., R.D. Arbeit, R.V. Goering, P.A. Mickelsen, B.A. Murray, D.H. Persing and B. Swaminathan. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. *Journal of Clinical Microbiology*. **33**:2233-2239.

Thiele, D. (1990) The technique of polymerase chain reaction-a new diagnostic tool in microbiology and other scientific fields (review). *Zentralbl Bakteriol.* **273**: 431-454.

Thompson III, L.M., R.M. Smibert, J.L. Johnson and N.R. Krieg: (1988) Phylogenetic study of the genus *Campylobacter. International Journal of Systematic Bacteriology.* **38**:190-200.

Totten, P.A., C.M. Patton, F.C. Tenover, T.J. Barrett, W.E. Stamm, A.G. Steigerwalt, J.Y. Lin, K.K. Holmes, and D.J Brenner. (1987) Prevalence and characterization of hippurate-*negative Campylobacter jejuni* in King County, Washington. *Journal of Clinical Microbiology.* **25**:1747-1752.

Tunicliff, R. (1914). An anaerobic *Vibrio* isolated from a case of acute bronchitis. *Journal of Infectious Diseases.* **15**:350-351.

Valentine, J.L., R.R. Arthur, H.L.T. Mobley, and J.D. Dick (1991) Detection of *Helicobacter pylori* by polymerase chain reaction. *Journal of Clinical Microbiology.* **29**:689-695.

Valsangiacomo, C., F. Baggi, V. Gaia, T. Balmelli, R. Peduzzi, J.C. Piffaretti . (1995) Use of amplified fragment length polymorphism in molecular typing of *Legionella pneumophila* and application to epidemiological studies. *Journal of Clinical Microbiology.* **33**:1716-1719.

Van Camp, G., Y.V. De Peer, S. Nicoli, J.-M. Neefs, P. Vandamme, and R. De Wachter. (1993) Structure of 16S and 23S ribosomal RNA genes in *Campylobacter* species: phylogenetic analysis of the genus *Campylobacter* and presence of internal transcribed spacers. *Systematic and Applied Microbiology.* **16**:361-386.

Vandamme, P., M.I. Daneshvar, F.E. Dewhirst, B.J Paster, K. Kersters, H. Goossens, and C.W. Moss. (1995) Chemotaxonomic analyses of *Bacteroides gracilis* and *Bacteroides ureolyticus* and reclassification of *B. gracilis* as *Campylobacter gracilis* comb. nov. *International Journal of Systematic Bacteriology.* **5**:145-152.

Vandamme, P., H. Goossens. (1992) Taxonomy of *Campylobacter, Arcobacter,* and *Helicobacter*: a review. *Zentralbl Bakteriol.* **276**:447-472.

Vandamme, P., E. Falsen, B. Pot, B. Hoste, K. kesters , and J de Ley. (1989) Identification of EF group 22 campylobacters from gastroenteritis cases as *Campylobacter concisus. Journal of Clinical Microbiology.* **27**:1775-1781.

Vandamme, P and J. De Ley J (1991) Proposal for a new family ; *Campylobacteraceae. International Journal of Systematic Bacteriology* **41**:451-455.

Vandamme, P., B. Pot, M. Gillis, P. de Vos, K. Kersters, and J. Swings. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiology Reviews.* **60**:407-438.

van Doorn, L.J., B.A. Giesendorf, R. Bax, B.A. van der Zeijst, P. Vandamme, and W.G. Quint. (1997) Molecular discrimination between *Campylobacter jejuni, Campylobacter coli, Campylobacter lari* and *Campylobacter upsaliensis* by polymerase chain reaction based on a novel putative GTPase gene. *Molecular and Cellular Probes.* **11**:177-185.

van Doorn, N.E., F. Namavar, J.G. Kusters, E.P. van Rees, E.J. Kuipers, and J. de Graaff. (1998) Genomic DNA fingerprinting of clinical isolates of *Helicobacter pylori* by REP-PCR and restriction fragment end-labelling. *FEMS Microbiology Letters.* **160**:145-150.

Vaneechoutte, M., R. Rossau, P. De Vos, M. Gillis, D. Janssens, N. Paepe, A. De Rouck, T. Fiers, G. Claeys, and K. Kersters K. (1992) Rapid identification of bacteria of the *Comamonadaceae* with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS Microbiology Letters.* **72**:227-233.

Van Etterijck, R., J. Breynaert, H. Revets, T. Devreker , Y. Vandenplas, P. Vandamme, and S. Lauwers. (1996) Isolation of *Campylobacter concisus* from feces of children with and with out diarrhea. *Journal of Clinical Microbiology.* **34**:2304-236.

van Steenbergen, T.J., S.D. Colloms, P.W. Hermans, J. de Graaff, and R.H. Plasterk. (1995) Genomic DNA fingerprinting by restriction fragment end labeling. *Proceedings National Academy of Science U S A.* **92**:5572-5576.

Véron, M., and R. Chatelain. (1973) Taxonomic study of the genus *Campylobacter* Sebald and Veron and designation of the neotype strain for the type species *Campylobacter fetus* (Smith and Taylor) Sebald and Véron. *International Journal of Systematic Bacteriology.* **23**:122-134.

Versalovic, J., T. Koeuth, and J.R. Lupski. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Research. **19**:6823-6831.

Von Graevenitz, A. (1990) A revised nomenclature of *Campylobacter laridis, Enterobacter intermedium* and *Flavobacterium branchiophila. International journal of Systematic Bacteriology* **40**:211.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van der Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research.* **23**:4407-4414.

Warren , J.R. and B. Marshall (1983) Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* I 1273-1275.

Wang, Y. and D.E. Taylor. (1990) Natural transformation in *Campylobacter* species. *Journal of Bacteriology.* **172**:949-955.

Wassenaar, T.M.. (1997) Toxin production by *Campylobacter* spp. *Clinical Microbiology Reviews.* **10**:466-476.

Wassenaar, T.M., N.M. Bleumink-Pluym, and B.A. van der Zeijst. (1991) Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. *EMBO Journal.* **10**:2055-2061.

Wassenaar, T.M., B.N. Fry, and B.A. van der Zeijst. (1995) Variation of the flagellin gene locus of *Campylobacter jejuni* by recombination and horizontal gene transfer. *Microbiology.* **141**:95-101.

Wassenaar, T.M., and D.G. Newell. (2000) Genotyping of *Campylobacter* spp. *Applied and Environmental Microbiology.* **66**:1-9.

Welsh, J., and M. McClelland. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research.* **18**:7213-7218.

Welsh, J., and M. McClelland. (1992) PCR-amplified length polymorphisms in tRNA intergenic spacers for categorizing staphylococci. *Molecular Microbiology.* **6**:1673-1680.

Wenman, W.M., J. Chai, T.J. Louie, C. Goudreau, H. Lior, D.G. Newell, A.D. Pearson, and D.E. Taylor. (1985) Antigenic analysis of *Campylobacter* flagellar protein and other proteins. *Journal of Clinical Microbiology.* **21**:108-112.

Whitehouse, C.A., P.B. Balbo, E.C. Pesci, D.L. Cottle, P.M. Mirabito, and C.L. Pickett. (1998) *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block. *Infection and Immunity.* **66**:1934-1940.

Wibur, W.J., and D.J. Lipman. (1983) Rapid similarity searches of nucleic acid and protein data banks. *Proceedings of the National Academy of Science.* USA **80**:726-730.

Wiid, I.J., C. Werely, N. Beyers, P. Donald, and P.D. van Helden. (1994) Oligonucleotide (GTG)5 as a marker for Mycobacterium tuberculosis strain identification. *Journal of Clinical Microbiology.* **32**:1318-1321.

Williams, J.G., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research.***18**:6531-6535.

Wong, P. Y., S. D. Puthucheary and T. Pang. (1983) Demonstration of a cytotoxin from *Campylobacter jejuni. Journal of Clinical Pathology.* **36**:1237-1240.

Wooldridge, K.G., and J.M. Ketley. (1997) *Campylobacter*-host cell interactions. *Trends in Microbiology.* 5:96-102.

Wosten, M.M.S.M., M. Boeve, M.G.A. Koot, A.D.C. Van der Nuenen and B. A. M Zeijst (1989) Identification of *Campylobacter jejuni* promoter sequences. *Journal of Bacteriology.* **180**: 594-599.

Yan W, Chang N and Taylor DE (1991) Pulsed-field gel electrophoresis of *Campylobacter jejuni* and *Campylobacter coli, Genomic DNA and its Epidemiological Applications. Journal of Infectious Diseases.*163: 1068-1072.

Yanisch-Perron, C., C. Vieira and J. Messing (1985) Improved M13 phage cloning vectors and host strains : nucleotide sequences of the M13 mp18 anf pUC19 vectors. *Gene* **33**: 103-119.

Yuki, N., H. Yoshino, S. Sato, and T. Miyatake. (1990) Acute axonal polyneuropathy associated with anti-GM1 antibodies following *Campylobacter* enteritis. *Neurology.***40**:1900-1902.

Zabeau, M., and K.K. Stanley (1982) Enhanced expression of Cro-β-galactosidase fusion proteins under the control of the Pr promoter of bacteriophage lambda. *EMBO Journal.* **1**: 1217-1224