The prevalence of *Campylobacter* species in human gastroenteritis: a molecular approach

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The prevalence of *Campylobacter* species in human gastroenteritis: A molecular approach

Andrew Jeffrey Lawson, BSc (Hons), MSc

A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

The Open University

Central Public Health Laboratory

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DATE OF SUBMISSION: 24 DECEMBER 2000

DATE OF AWARD: 18 JUNE 2001
I dedicate this thesis to my parents
Abstract

A rapid extraction protocol to facilitate the recovery of bacterial DNA from faecal material for use as a template for PCR was developed. This consisted of a basic guanidine-silica extraction technique with an additional PVP/TE wash step to remove PCR inhibitors. The efficacy of the new extraction protocol in combination with Campylobacter species-specific PCR assays was demonstrated using seeded faecal material and compared favourably with detection by existing culture-based methodologies. A series of pilot studies using clinical samples were then performed; firstly a study of 25 samples of known Campylobacter status; and then a comparison of PCR assay with selective culture in a blind study of 200 samples. These experiments confirmed the effectiveness of PCR assays for the detection of Campylobacter species and consequently a major, multi-centre, investigation was undertaken. Over the course of two years, a total of 3,738 faecal samples from seven contributing centres were examined. This is the largest molecular-based survey of Campylobacter species yet undertaken. The detection rates for PCR and culture were similar and in accord in ~ 78% of C. jejuni and C. coli infections. However, a significant proportion were PCR-positive only (12%) or culture-positive only (10%).

Detection rates of non-jejuni/non-coli Campylobacter species were significantly higher using PCR assay and C. lari (1), C. upsaliensis (9) and C. hyointestinalis (4) were found in culture-negative faeces. The detection of C. hyointestinalis by PCR assay at an incidence of 0.13% is the highest yet recorded from human gastroenteritis.

In a study of faeces from healthy humans, 16S rDNA amplicons originating from a previously undescribed and initially uncultivable Campylobacter species, termed 'Candidatus C. hominis', were discovered. Phylogenetic analysis of 16S rDNA suggested a relationship between the novel species and existing anaerobic Campylobacter species. A membrane filtration technique modified for the recovery of anaerobic bacteria with a novel selective dilution and immunomagnetic separation procedure were developed and pure cultures of the bacteria from which the 'Candidatus C. hominis' 16S rDNA sequences originated were obtained. These strains were characterised and the new species described as C. hominis sp. nov.
Acknowledgements

I am indebted to my principal supervisor, Dr John Stanley for giving me the opportunity to undertake this thesis and for his supervision, encouragement and direction during the course of this work.

I would like to thank the staff of CPHL in general, but especially my colleagues Dr Meeta Desai, Dr Dennis Linton, Dr Julie Logan and Ms Gael O’Neill. Thanks also to Dr Robert Owen (with whom I began this work), Dr Philip Mortimer (director of the laboratory where the bulk of this work was done), Dr Jon Clewley (my second supervisor), Dr Tyrone Pitt and Dr Henrick Chart (Open University representatives at CPHL), Dr Stephen On (for the phenotypic analysis) and Dr John Threlfall (who kindly proof read this document).

I also thank the staff of the PHLS laboratories that contributed clinical samples and culture data for this study – Tooting (former) PHL, Preston PHL, Central Middlesex PHL, Exeter PHL Chelmsford PHL, Ashford PHL, Dorchester PHL and Bangor PHL.

This work was supported by grants from the Department of Health, London, United Kingdom.

Lastly and most importantly, a special thank you to Heather and Rhiannon for their support and tolerance over the past four and a half years.
Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of any other university or institute of learning. The work of this thesis was carried out by myself (unless otherwise stated in the text), under the supervision of Dr. John Stanley of the Central Public Health Laboratory.

Andrew J. Lawson

December 2000
Publications arising from this thesis


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


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<td>AFLP</td>
<td>Amplified Fragment Length Polymorphism</td>
</tr>
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<td>BB</td>
<td>Brucella broth</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-Bromo-4-chloro-3-indolylphosphate p-Toluidine</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CAT</td>
<td>Cefoperazone Amphotericin Teicoplanin agar</td>
</tr>
<tr>
<td>CBA</td>
<td>Columbia Blood Agar</td>
</tr>
<tr>
<td>CCDA</td>
<td>Modified Charcoal Cefoperazone Desoxycholate Agar</td>
</tr>
<tr>
<td>CDCP</td>
<td>Center for Disease Control and Prevention</td>
</tr>
<tr>
<td>CDSC</td>
<td>Communicable Disease Surveillance Centre</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming unit</td>
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<tr>
<td>CNW</td>
<td>Catalase Negative or Weak</td>
</tr>
<tr>
<td>CPHL</td>
<td>Central Public Health Laboratory</td>
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<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
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<td>dATP</td>
<td>2'-deoxyadenosine 5'-triphosphate</td>
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<td>DHB</td>
<td>Digoxigenin Hybridization Buffer</td>
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<td>dCTP</td>
<td>2'-deoxycytidine 5'-triphosphate</td>
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<td>2'-deoxyguanosine 5'-triphosphate</td>
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<td>Digoxigenin</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
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<td>DS</td>
<td>Diatom Suspension</td>
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<td>dTTP</td>
<td>2'-'deoxythymidine 5'-triphosphate</td>
</tr>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid disodium salt</td>
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<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<td>EMBL</td>
<td>European Molecular Biology Laboratory</td>
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<td>FAA</td>
<td>Fastidious Anaerobe Agar</td>
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<td>FITC-HRP</td>
<td>Fluoroisothiocyanate-Horse Radish Peroxidase</td>
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<td>GES</td>
<td>Guanidine EDTA Sarkosyl reagent</td>
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<tr>
<td>G + C</td>
<td>Guanine plus Cytosine</td>
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<td>Guanidine thiocyanate</td>
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<td>HAZ-CHEM</td>
<td>Hazardous Chemical</td>
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<td>HB</td>
<td>Hybridization Buffer</td>
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<td>Healthy Subject</td>
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<td>Kilobase pair</td>
</tr>
<tr>
<td>LB</td>
<td>Lysis Buffer</td>
</tr>
<tr>
<td>MF</td>
<td>Membrane Filter</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue Tetrazolium chloride</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Collection of Type Cultures</td>
</tr>
<tr>
<td>NPI</td>
<td>No Pathogens Isolated</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline Tween</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PHL</td>
<td>Public Health Laboratory</td>
</tr>
<tr>
<td>PHLS</td>
<td>Public Health Laboratory Service</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinyl Pyrrolidone</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal Deoxyribonucleic Acid (i.e. the gene encoding RNA)</td>
</tr>
<tr>
<td>RDP</td>
<td>Ribosome Database Project</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal Ribonucleic acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SDW</td>
<td>Sterile Distilled Water</td>
</tr>
<tr>
<td>SLS</td>
<td>Sodium Lauryl Sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline Sodium Citrate</td>
</tr>
<tr>
<td>Ta</td>
<td>Annealing temperature</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em> DNA polymerase</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate EDTA buffer</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethyl Benzedine</td>
</tr>
<tr>
<td>TSI</td>
<td>Triple Sugar Iron agar</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Unweighted Pair Group Mathematical Average</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>WB</td>
<td>Wash Buffer</td>
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Chapter 1

Introduction
Chapter 1. Introduction

1.1 Early history, spirilla and anaerobic vibrios

Since the nineteenth century organisms resembling *Campylobacter* have been reported from the gastrointestinal tract of man and animals. Because of their relatively fastidious growth requirements, they were at that time uncultivable. They were generally described as 'spirilla' or 'vibrio' on the basis of morphology. *Campylobacter* cells were probably first observed as early as 1886 by Theodore Escherich. He reported non-cultivable spiral bacteria in diarrhoeic stool specimens from kittens and human infants. These organisms were found mainly in the colon or associated with mucus in diarrhoeal stool specimens. The morphology, association with enteritis in infants and young animals, and failure to grow on existing culture media, suggest retrospectively that these microorganisms were *Campylobacter*, no other bacteria with comparable morphology have been associated with human enteric infections. Prior to the 1900's a number of German language publications described the occurrence of 'spirilla' in cases of 'cholera-like' and 'dysenteric disease'. These articles escaped the attention of a wider audience for many years, until they were recently reviewed (Kist, 1986).

In 1913, McFadyean and Stockman were the first to isolate 'vibrio-like bacteria' from the uterine mucus of a ewe and the stomach contents of her foetus (McFadyean & Stockman, 1913). It was found that material from aborted lambs also contained similar bacteria. The organism was capable of transmission and causing subsequent abortion. A similar organism was later isolated from foetuses of aborting cows (Smith, 1918) and named 'Vibrio fetus' (Smith & Taylor, 1919); the disease came to be known as 'vibrionic abortion'. In the 1950's Florent showed that one form of infectious bovine infertility, was caused by
a variety of 'V. fetus', which he termed 'Vibrio foetus var venerealis' (Florent, 1959). In the course of his investigations he had previously isolated a saprophytic 'microaerobic vibrio' which he termed 'Vibrio bubulus' (Florent, 1953) which was later found to be closely related to 'V. sputorum' (Prevot, 1940; Loesche et al., 1965).

In 1914 Tunicliff isolated an 'anaerobic vibrio' from the sputum of a patient with acute bronchitis (Tunicliff, 1914). Eventually this organism was named 'Vibrio sputorum' (Prevot, 1940). In 1927 Smith and Orcutt isolated a 'vibrio' implicated in epidemic 'winter dysentery' in cattle and enteritis in calves, by a process of washing and diluting scrapings of intestinal mucosa (Smith & Orcutt, 1927). Although similar to 'V. fetus' it was antigenically distinct. As it was associated with jejunitis it was named 'Vibrio jejuni' (Jones et al., 1931). Doyle isolated similar 'vibrios' from pigs suffering from swine dysentery (Doyle, 1944); he later named it 'Vibrio coli' (Doyle, 1948). Ironically, neither 'V. jejuni' nor 'V. coli' proved to be the causative agents of the pathologies that they were originally associated with. The 'winter dysentery' of cattle is now thought to have a viral aetiology, and swine dysentery is now known to be caused by the Treponema-like bacterium, Sepulina hyodysenteriae (Skirrow, 1994). It is now apparent that 'V. jejuni' and 'V. coli' (later Campylobacter jejuni and Campylobacter coli) constitute part of the normal flora of the gastrointestinal tract of cattle, pigs and many other animals. Their significance in human illness was not fully appreciated for many years. Sadly, due to the difficulties in preserving these relatively fastidious bacteria, no examples of the original Campylobacter strains isolated by Jones, Doyle and the other early workers have been preserved.
1.2 The genus *Campylobacter*

The genus *Campylobacter* was first proposed and validated in 1963 by Sebald and Véron, to accommodate the 'microaerobic vibrios' *V. fetus* and *V. bubulus* (Sebald & Véron, 1963). These were transferred from the genus *Vibrio* primarily due to their low DNA base composition (mol% G + C 30 to 34), microaerobic growth requirements, and non-fermentative metabolism. It was a further ten years before a comprehensive and systematic study of this new genus was published (Véron & Chatelain, 1973). That study proposed the names *Campylobacter fetus*, *Campylobacter coli*, *Campylobacter jejuni* and *Campylobacter sputorum* (with biotypes *sputorum* and *bubulus*). *C. fetus* was proposed as the type species, a nomenclature finally accepted and included in the 'Approved Lists of Bacterial Names' in 1980 (Skerman *et al.*, 1980).

1.3 *Campylobacter* species as agents of human disease

*Campylobacter* species were first implicated as possible agents of human enteritis in 1938 during an outbreak of apparently milk-borne diarrhoea in two adjacent U.S. prisons (Levy, 1946). At this point campylobacters could not be grown from human faeces. However, several of the victims developed bacteraemia, which led to the culture of *'V. jejuni'*'. Since the blood culture isolates could not be grown on solid media, a full description of the bacteria was not possible.

King carried out the first systematic study of these few 'microaerobic vibrios' isolated from cases of bacteraemia in humans (King, 1957; King, 1962). Crucially, she recognised the similarity between recent isolates of *'V. jejuni'* from poultry and human isolates, which she termed 'related vibrios'. She also observed that human isolates from blood culture were all from patients with acute diarrhoea. Isolation techniques borrowed from veterinary
microbiology, employing filtration (Smibert, 1965), provided an effective means of isolating campylobacters from human faecal samples (Cooper & Slee 1971; Dekeyser et al., 1972). Using this approach Butzler demonstrated that campylobacters were frequently present in the faeces of children with diarrhoea (Butzler et al., 1973).

The major breakthrough in the study of Campylobacter in human disease came in 1977 when Skirrow developed an antibiotic-containing agar medium selective for C. jejuni and C. coli (Skirrow, 1977). As far as public health microbiology was concerned this paper was a watershed: it described the clinical entity, suggested appropriate antibiotic therapy and reported an easy culture method which resulted in a high isolation rate from sporadic community-based diarrhoeal cases. It further demonstrated that there was a serological response to the infection, and confirmed the previously suspected link between disease in humans and the presence of Campylobacter in poultry.

1.4 The Campylobacter taxonomic explosion, 1970 – 1987

Throughout the 1970's and 1980's interest in campylobacters grew, and it became apparent that C. jejuni was a major cause of bacterial enteritis. Many new species of Campylobacter and campylobacter-like bacteria were isolated and described. Campylobacter concisus was isolated from the human oral cavity (Tanner et al., 1981); Campylobacter mucosalis (Lawson et al., 1981) and Campylobacter hyointestinalis (Gebhart et al., 1985) from the intestines of pigs; 'Campylobacter cryaerophila' from aborted bovine foetuses (Ellis et al., 1977; Neill et al., 1985) and 'Campylobacter nitrofigilis' from the roots of a salt-marsh plant, Spartina alterniflora (McClung et al., 1983). 'Campylobacter butzleri' was isolated from humans and nonhuman primates with diarrhoea (Kiehlbauch et al., 1991). Campylobacter lari was isolated from the intestine.
and faeces of a variety of animals, notably seagulls (Skirrow & Benjamin, 1980a). *Campylobacter jejuni* subspecies *doylei* was the designation given to nitrate-negative strains resembling *C. jejuni* (subspecies *jejuni*), isolated from antral gastric biopsies from adults and cases of gastroenteritis in children (Steele & Owen, 1988). *Campylobacter upsaliensis* was found in dogs (Sandstedt et al., 1983); 'Campylobacter pylori' (Marshall et al., 1984; Marshall & Goodwin, 1987) and 'Campylobacter mustelae' (Fox et al., 1988) in the gastric mucosa of humans and ferrets respectively, and 'Campylobacter cinaedi' and 'Campylobacter fennelliae' from a study of enteritis in homosexual males (Fennell et al., 1984; Totten et al., 1985). At this point the taxonomy of the *Campylobacter* genus had become unwieldy, and the relationship between the genus and other apparently similar, genera was unclear. The situation was exacerbated by the fact that *Campylobacter* express relatively few physical or biochemical characteristics suitable for differentiation or classification.

### 1.5 A DNA-based phylogenetic paradigm

*Campylobacter* taxonomy was revolutionised in 1987 when Romaniuk examined partial 16S ribosomal RNA (rRNA) sequences from *Campylobacter* species and found that they constituted a distinct and previously undescribed eubacterial group, only distantly related to other Gram-negative bacteria (Romaniuk et al., 1987). This observation was confirmed by Lau (Lau et al., 1987) who demonstrated that *Campylobacter* species formed a distinct cluster within the phylum of the purple photosynthetic bacteria or *Proteobacteria* (Stacklebrandt et al., 1988). The *Proteobacteria* contain the majority of Gram-negative bacteria and were subdivided by 16S rRNA sequencing into alpha, beta, gamma and delta subclasses (Woese, 1987). On the basis of rRNA-DNA hybridisation, they were also divided into rRNA superfamilies I, II, III, IV and V (De Ley, 1978). The campylobacters
Introduction
together with *Wolinella succinogenes*, an organism originally isolated from the bovine rumen (Wolin *et al.*, 1961; Tanner *et al.*, 1981), were shown to constitute a new subdivision, epsilon, (Lane *et al.*, 1992; Rainey *et al.*, 1993) broadly synonymous with the new rRNA superfamily VI (Vandamme *et al.*, 1991).

Further examination of this growing database of 16S rRNA sequences revealed three major rRNA homology groups within rRNA superfamily VI (Paster & Dewhirst, 1988; Thompson *et al.*, 1988), which were considered substantial enough to constitute separate genera (Thompson *et al.*, 1988). In 1991 Vandamme therefore proposed a revision of taxonomy within rRNA superfamily VI (Vandamme *et al.*, 1991). The newly defined genus *Campylobacter* was restricted to the *Campylobacter* species belonging to the rRNA homology group containing the type species of the *Campylobacter* species: *C. fetus*, *C. hyointestinalis*, *C. sputorum*, *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. concisus*, *C. mucosalis*, together with the generically misnamed *'Wollinella curva'* (Tanner *et al.*, 1984) and *'Wollinella recta'* (Tanner *et al.*, 1981) now reassigned as *Campylobacter curvus* and *Campylobacter rectus*. The name *Arcobacter* was proposed for the second rRNA homology group, which contained *'C. nitrofigilis'*, *'C. cryaerophila'* and *'C. butzleri'*, now termed *Arcobacter nitrofigilis* (the type species), *Arcobacter cryaerophilus* and *Arcobacter butzleri*. There remain many genotypic and phenotypic similarities between *Campylobacter* and *Arcobacter*, and this was reflected in their inclusion in a new eubacterial family, the *Campylobacteraceae* (Vandamme & de Ley, 1991). A third genus, *Helicobacter*, had previously been proposed for *'C. pylori'* and *'C. mustelae'* (Goodwin *et al.*, 1989). Along with *'C. cinaedi'* and *'C. fennelliae'* these formed the third rRNA homology group, containing, initially, *Helicobacter pylori* (the type species), *Helicobacter mustelae*, *Helicobacter cinaedi* and *Helicobacter fennelliae*. *Wolinella succinogenes*
appeared to be related to this third rRNA homology group but was sufficiently distinct genetically and phenotypically to merit genus status of its own.

1.6 Recent additions to the genus Campylobacter

The generically misnamed 'Bacteroides gracilis' and 'Bacteroides ureolyticus' were found to fall within the rRNA homology group of Campylobacter. 'B. gracilis' was renamed Campylobacter gracilis, but 'B. ureolyticus' has a proteolytic metabolism and fatty acid cell wall components not known elsewhere in the genus Campylobacter and is still considered a species incertae sedis pending the isolation and characterization of similar bacteria (Vandamme et al., 1995). Campylobacter helveticus was isolated in Switzerland from faeces of domestic cats and dogs by filtration and on isolation media containing the antibiotic cefoperazone. This species, first characterised at the Central Public Health Laboratory (CPHL), is a catalase-negative, thermophilic campylobacter, most closely related phylogenetically to C. upsaliensis (Stanley et al., 1992). Campylobacter showae was the name given to nine campylobacter-like strains isolated from human gingival crevices by a group working at Showa University Japan. These strains were similar to, but distinct from C. curvus and C. rectus. (Etoh et al., 1993). Campylobacter lanienae was isolated from the faeces of three healthy individuals during a hygiene survey of abattoir workers. These isolates were fully characterised at CPHL, the nearest phylogenetic neighbours were C. hyointestinalis, C. fetus and C. mucosalis (Logan et al., 2000). To illustrate the relationships between the members of the genus, a phylogenetic tree derived from an alignment of published Campylobacter 16S rDNA sequence data is shown in Figure 1.1.
Figure 1.1 Phylogenetic tree derived from an alignment of published Campylobacter 16S rDNA sequence data.
1.7 Description of the genus *Campylobacter*

The name *Campylobacter* comes from the Greek 'campyo' meaning curved or crooked and 'bacter' meaning staff or rod. Generally they are small Gram-negative bacilli (typically 0.5 to 5.0 μm long and 0.2 to 0.5 μm wide), with a spiral curve and tapering ends. Cells usually possess a polar flagellum at one or both ends and this, in combination with the spiral morphology, imparts a high degree of motility to the cell. Most species are microaerobic, requiring an O$_2$ concentration of between 3 to 15 % and CO$_2$ concentration of 3 to 5 %. Members of the genus will grow over a range of temperatures, but certain species, such as *C. jejuni* and *C. coli*, are often termed 'thermophilic', because their optimum growth temperature is 42°C. This probably reflects an adaptation to the temperatures found in their normal habitat, the intestine of warm-blooded animals and birds (Skirrow, 1994; Ketley, 1997).

*Campylobacter* species have a comparatively small AT-rich genome sized between 1600 and 1700 kb (Ketley, 1997). Across the genus the mol% G + C ratio ranges from 29% to 46%. In comparison, the *Escherichia coli* genome is approximately 4600 kb with a mol% G + C ratio of 50%. The small size of the *Campylobacter* genome may be reflected in their requirements for complex media for growth and their inability to ferment carbohydrates or degrade complex substrates. In 2000 the genus *Campylobacter* comprises 16 species, whose physical and biochemical characteristics are summarised in Table 1.1.

In October 1999, sequencing of the entire genome of a *C. jejuni* strain (NCTC 11168) was completed at the Sanger Centre, Cambridge, UK. The circular chromosome was 1640 kb long with a mol% G + C ratio of 30.6%. The genome had the capacity to encode 1,654 proteins and 54 RNA species and was unusual in that it had few insertion sequences,
Table 1.1 Phenotypic characteristics of *Campylobacter* species

<table>
<thead>
<tr>
<th>Species</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Urease</th>
<th>Nitrate reduction</th>
<th>Nitrile reduction</th>
<th>Hippurate hydrolysis</th>
<th>Indoxyl acetate hydrolysis</th>
<th>H₂S production (TSI)*</th>
<th>Growth at 25°C</th>
<th>Growth at 42°C</th>
<th>Growth requires H₂</th>
<th>Growth in 1% glycine</th>
<th>Sensitivity to nalidixic acid</th>
<th>Sensitivity to cephalothin</th>
<th>Sensitivity to metronidazole</th>
<th>Presence of Flagella</th>
<th>% G + C content</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. sputorum</em></td>
<td>+</td>
<td>v</td>
<td>v</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>v</td>
<td>R</td>
<td>v</td>
<td>S</td>
<td>v</td>
<td>29-32</td>
</tr>
<tr>
<td><em>C. gracilis</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>v</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>v</td>
<td>R</td>
<td>v</td>
<td>S</td>
<td>44-46</td>
</tr>
<tr>
<td><em>C. showae</em></td>
<td>v</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>v</td>
<td>R</td>
<td>v</td>
<td>S</td>
<td>44-46</td>
</tr>
<tr>
<td><em>C. curvus</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>-</td>
<td>+</td>
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<td>+</td>
<td>S</td>
<td>v</td>
<td>v</td>
<td>45-46</td>
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<td><em>C. rectus</em></td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>w</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>S</td>
<td>v</td>
<td>v</td>
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<td>-</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>v</td>
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<td>-</td>
<td>-</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>v</td>
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<td>-</td>
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<td>-</td>
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<td>S</td>
<td>v</td>
<td>33-36</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<td>+</td>
<td>R</td>
<td>S</td>
<td>v</td>
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<td>+</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>S</td>
<td>v</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
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<td>v</td>
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<td>S</td>
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<td>R</td>
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<td>+</td>
<td>+</td>
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<td>S</td>
<td>R</td>
<td>v</td>
<td>30-33</td>
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<tr>
<td><em>[B.] ureolyticus</em></td>
<td>+</td>
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<td>R</td>
<td>v</td>
<td>S</td>
<td>28-30</td>
</tr>
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*+, positive reaction; -, negative reaction; v, variable reaction; w, weak reaction, R, resistant; S, sensitive

*, TSI = Triple Sugar Iron medium
phage-associated sequences and repeat sequences. It was estimated that 94.3% of the genome codes for proteins, making it the densest bacterial genome yet sequenced. Another distinct feature (shared with the *H. pylori* genome) was the presence of hypervariable sequences found in certain genes encoding elements of surface structure synthesis, presumably representing a means of immunological variation (Parkhill *et al.*, 2000). However, despite the close relationship suggested by 16S rRNA phylogeny and shared biological properties, only 55% of the sequenced *C. jejuni* genes showed closest similarity with *H. pylori*. The complete genomic sequence of *C. jejuni* is a valuable tool for further analysis of an important human pathogen, but also demonstrates the unique nature of the *Campylobacter* genus.

### 1.8 Campylobacter enteritis in humans

*Campylobacter* enteritis is a major public health problem in the United Kingdom and other industrialised countries. Since 1981 in England and Wales the number of laboratory reports of intestinal infection due to *Campylobacter* species have exceeded those of any other enteric pathogen including salmonellae (Figure 1.2). This increased incidence probably reflects both changes in modern food consumption patterns and a continuing refinement of laboratory isolation techniques. Within the timescale of this thesis the relative proportion of cases of acute gastroenteritis attributed to *Campylobacter* in England and Wales has risen from ~39% in 1997 (Figure 1.3) to ~50% in 1999 (Figure 1.4). This is due to a combination of the continued rise of *Campylobacter* cases and a corresponding fall in *Salmonella* reports (PHLS, 2000).

Within the context of human enteritis, the term 'Campylobacter' is often used loosely and is generally assumed to indicate *C. jejuni* enteritis (specifically that caused by *C. jejuni*
Figure 1.2 *Campylobacter* and *Salmonella* isolations in England and Wales 1977 to 1999

- **Campylobacter**
- **Salmonella**

(Source: PHLS, 2000)
Introduction

subspecies *jejuni*). While *C. jejuni* is undoubtedly the single most important bacterial cause of human gastroenteritis worldwide (Skirrow, 1994; Tauxe, 1992), it should be remembered that other *Campylobacter* species are capable of causing gastrointestinal infection. Unfortunately, the majority of diagnostic microbiology laboratories do not routinely identify *Campylobacter* isolates to species level. During the period 1989 to 1990, 17% of campylobacters reported to the Public Health Laboratory Service Communicable Disease Surveillance Centre (PHLS – CDSC) by laboratories in England and Wales were reported by species, while for the remainder the genus name only was given. Where the species was stated, 89.5% were *C. jejuni*, 10.3% *C. coli* and 0.2% were other named species (Anonymous, 1993).

1.9 Symptoms of *Campylobacter* enteritis in humans

In cases of human illness, the first indication of *Campylobacter* infection is a prodrome characterised by acute abdominal pain often with fever and general malaise, which then progresses to profuse diarrhoea. These prodromal symptoms are present in about half of infected patients. The incubation period prior to the first appearance of symptoms varies from one to seven days, so that the source of the infection and, therefore the exact timing, are often difficult to establish. The diarrhoeic stools often contain fresh blood, mucus and an inflammatory exudate containing leucocytes. Rapidly motile campylobacters may be seen in fresh faecal samples. In the gut mucosa changes occur, ranging from oedema and hyperaemia with petechial haemorrhage to friability. Inflammation of some areas of the ileum and jejunum with mesenteric adenitis usually occurs. Bacteraemia is rarely reported, but a large proportion of cases may be missed, especially in the earlier stages of infection, due to infrequent sampling or inappropriate culture conditions by frontline diagnostic microbiology laboratories. The acute diarrhoea commonly lasts for two to three
Figure 1.3 Campylobacter in human gastroenteritis: England and Wales 1996

Introduction

(Source: PHLS, 2000)
Figure 1.4 Campylobacter in human gastroenteritis: England and Wales 1999

(Source: PHLS, 2000)
days with the patient becoming dehydrated and exhausted. Abdominal pain and discomfort persist during, and sometimes after the diarrhoea has stopped. Relapses may occur but are generally less severe than the first attack. Patients may remain culture-positive for several weeks after clinical symptoms have subsided, but long-term carriage is unknown in people who have a normal immune system (Skirrow, 1994). Although Campylobacter infection can result in a serious illness that may last for a week or more, it is usually self-limiting. Complications are uncommon and the mortality rate is low. The most notable sequela is Guillain-Barré Syndrome (GBS), an acute inflammatory polyneuropathy that has a complex aetiology. Up to two thirds of cases of GBS develop one to three weeks after a gastrointestinal or upper respiratory tract infection. Although many infectious agents have been implicated, 14% of cases have recent evidence of Campylobacter infection (Winer & Hughes, 1988). Infection by some strains of C. jejuni (for example Penner serotype 19) is more likely to precede GBS than others (Fujimoto et al., 1992). In one model, antigenic cross-reactivity between C. jejuni and human peripheral myelin protein is postulated to be the cause of this condition (Fujimoto & Amako, 1990).

Each year, approximately 30 cases of Campylobacter bacteraemia are reported in England and Wales (as received by PHLS – CDSC). By comparison, about 300 bacteraemia cases result from infection with non-typhoidal Salmonella species in the same period. Invasive Campylobacter infection is most common in the elderly (older than 65 years) and least common in children (aged one to 14 years). Only 30% of these cases have underlying conditions that might have predisposed them to infection. Most serotypes are equally common among blood and faecal isolates, but strains of Penner serotype 4 were most frequently reported, possibly indicating a greater propensity for invasive
disease (Skirrow et al., 1993). Only one or two deaths attributed to *Campylobacter* infection are reported each year to CDSC, compared with an annual mortality of between 30 and 70 for non-typhoidal salmonellae. Other complications occur in about 1% of cases, including urticaria (auto-immune skin condition), reactive arthritis and Reiter’s syndrome (Healing et al., 1992).

1.10 Incidence of *Campylobacter* in human disease

*Campylobacter* enteritis is the most frequent cause of acute bacterial diarrhoea worldwide. Since the routine introduction of selective isolation media, the number of isolates in England and Wales has risen steadily to over 50,000 cases each year (see Figures 1.2, 1.3 and 1.4). Since infected people often do not seek medical attention and since case reporting is voluntary, the true incidence is probably significantly higher. Detailed small-scale studies suggest an incidence in the order of ~ 500,000 cases per year in the United Kingdom (Kendall & Tanner, 1982). The vast majority of *Campylobacter* infections appear to be sporadic; less than 2% are associated with outbreaks. Infection occurs in all age groups, but is most common in young adults (Healing, et al., 1992). Another key feature of *Campylobacter* enteritis is that marked seasonal peaks of infection occur in late spring and autumn.

In developing countries the disease manifests itself in a different manner to that in industrialised nations (Taylor, 1992). It is usually restricted to children, causes little apparent effect in adults, and exhibits no seasonal pattern. There is a higher incidence of infection, a much higher rate of asymptomatic carriage and a larger proportion of infections resulting in milder forms of illness with watery, non-inflammatory diarrhoea. These differences of epidemiology and clinical patterns of disease are thought to be due
to much higher exposure and infection rates early in life, resulting in a different pattern of immunity. Although strain differences have been correlated with clinical symptoms in some studies (Taylor, 1992) there is no clear evidence that strains in developing countries differ from those found in the industrialised nations. C. jejuni is an important cause of traveller’s diarrhoea, and it is notable that the spectrum of disease observed in these cases is similar to that described domestically (Taylor, 1992). Approximately 10% of Campylobacter isolates reported in England and Wales are thought to be acquired abroad (Pearson & Healing, 1992).

Campylobacter infections are usually self-limiting. Antibiotics have little effect on the duration of illness or on the severity of symptoms, although they may prevent relapse. Erythromycin is the antibiotic of choice for serious infection, and ciprofloxacin is a suitable alternative (Healing, et al., 1992). However antibiotic-resistant strains are found, including some resistant to ciprofloxacin (Threlfall et al., 1999; Thwaites & Frost, 1999).

1.11 Other Campylobacter species causing human enteritis

There is growing awareness that besides C. jejuni subspecies jejuni a number of other Campylobacter species cause human enteritis. The importance of these other species is probably underestimated because isolation methods have naturally focused primarily on C. jejuni subspecies jejuni (and C. coli), and the selective antibiotics used in isolation media are often inhibitory to the other members of the genera described below (Anonymous, 1993).
1.11.1 *C. jejuni* subspecies *doylei*

*C. jejuni* subspecies *doylei* was isolated from the gastric antrum of adult patients with upper gastrointestinal symptoms in the UK (Kasper & Dickgiesser, 1984), and from the diarrhoeic faeces of children in Australia (Steele *et al.*, 1985). Strains grow slowly at 37°C and not at all at 42°C, and are sensitive to many of the antibiotics used in selective media (Mishu *et al.*, 1992). Besides its relatively poor growth, subspecies *doylei* is distinguished from subspecies *jejuni* by the former's inability to reduce nitrate to nitrite (Steele and Owen, 1988). Studies using the non-inhibitory membrane filter technique frequently isolate *C. jejuni* subspecies *doylei* at low levels (between 0.1% and 2% of total *Campylobacter* isolates) (Goossens *et al.*, 1990a; Musmanno *et al.*, 1998), but despite this its role in human disease remains unknown.

1.11.2 *C. coli*

*C. coli* is probably the third most common single bacterial species causing human gastroenteritis worldwide, after *C. jejuni* and *Salmonella enterica*. Despite this, *C. coli* remains overshadowed by *C. jejuni*, and the two are often considered as a single entity or complex. *C. coli* was first isolated from piglets with swine dysentery (Doyle, 1944), and pigs are still thought to be the principal host although it is also found in avian and other animal species. Strains of *C. coli* are distinguished from the phenotypically similar *C. jejuni* primarily by the former's inability to hydrolyse hippurate (Véron and Chatelain, 1973). However, hippurate-negative strains of *C. jejuni* have been reported (Nicholson & Patton, 1993). The pathogenicity of *C. coli* is likewise similar to *C. jejuni*, although anecdotal evidence suggests *C. coli* enteritis is less severe (Popovic-Uroic *et al.*, 1988). Erythromycin resistance is more prevalent in *C. coli* than *C. jejuni*, possibly reflecting the use of macrolide antibiotics as growth promoters on pig farms (Threlfall, *et al.*, 1999).
1.11.3 *C. lari*

*C. lari* was first isolated by Skirrow and Benjamin from a child with mild diarrhoea, and from the cloacal contents of seagulls (genus *Larus*) (Skirrow and Benjamin, 1980a). Between 1982 and 1983 the Campylobacter Reference Laboratory at the Centres for Disease Control and Prevention (CDSCP), Atlanta, USA, reported six isolates, five from faeces and one from a fatal bacteraemia in an immunocompromised patient (Tauxe, 1992). Symptoms included diarrhoea, cramping abdominal pain and occasionally fever. A large outbreak of waterborne enteritis in Canada was attributed to *C. lari* (Borczyk et al., 1987): contaminated drinking water was implicated as the cause of 162 cases of non-bloody diarrhoea and abdominal pain. Vomiting and nausea were reported in 50% of cases and fever in 20%. *C. lari* was isolated from the faeces of seven patients. *C. lari* grows readily on most *Campylobacter* isolation media, but although regularly isolated from birds and other animals, it constitutes less than 0.1% of human *Campylobacter* isolates (Anonymous, 1993). It is biochemically and morphologically similar to *C. jejuni* and *C. coli*. It is possible that some *C. lari* isolates may have been incorrectly identified, or not identified at all, and its incidence thereby underestimated.

1.11.4 *C. upsaliensis*

*C. upsaliensis* was initially described as 'CNW' (catalase-negative or weakly reacting) *Campylobacter* and was first isolated from the faeces of dogs with and without diarrhoea (Sandstedt, et al., 1983; Sandstedt & Ursing, 1991). Subsequently a number of workers isolated *C. upsaliensis* from cases of human gastroenteritis, by employing a non-inhibitory membrane-filter technique (Albert et al., 1992; Goossens, et al., 1990a; Megraud & Bonnet, 1986) or by modifying existing *C. jejuni* isolation media (Aspinall et al., 1993; Endtz et al., 1991). In the largest study, constituting more than 15000 samples,
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(Goossens, et al., 1990a) *C. upsaliensis* was isolated from 99 patients, 73 of whom were children. Other studies also claimed an association with illness in children (Albert, et al., 1992; Megraud and Bonnet, 1986), especially those from socially disadvantaged groups. It has been suggested that there may be a link between carriage of this organism by dogs and disease in humans (Goossens et al., 1990b).

1.11.5 *C. fetus*

First isolated from an aborted sheep foetus (McFadyean and Stockman, 1913), *C. fetus* is divided into two subspecies; *C. fetus* subspecies *fetus* - a cause of septic abortion in cattle and sheep, and *C. fetus* subspecies *venerealis* - associated with infectious infertility and genital tract infections in cattle. Only *C. fetus* subspecies *fetus* is known to infect humans, where it can cause septicaemic illness in cases of immunosuppression or underlying illness and shows, a marked preference for males (Guerrant et al., 1978; Rettig, 1979). In up to half of these septicaemic patients, diarrhoea is also reported (Franicoli et al., 1985). Despite this, *C. fetus* subspecies *fetus* is only rarely isolated from human faeces; perhaps because its growth is inhibited at the 42°C incubation temperature often used in selective isolation regimes for *Campylobacter*. However, even in studies using membrane-filtration incubated at 37°C (Bolton et al., 1988; Goossens, et al., 1990a) its incidence does not increase. Some rare manifestations of *C. fetus* subspecies *fetus* infection include meningitis, pericarditis, peritonitis, salpingitis, septic arthritis, septic abortion and abscesses (Brown & Sautter, 1977; Lee et al., 1985).

1.11.6 *C. hyointestinalis*

*C. hyointestinalis* was first isolated from the intestines of pigs with proliferative enteritis (Gebhart, et al., 1985). However, while *Campylobacter* species are frequently isolated in
association with this disease, the causative agent is now known to be *Lawsonia intracellularis* (McOrist *et al.*, 1995). In humans, *C. hyointestinalis* has been cited rarely as a cause of gastroenteritis (Fennell *et al.*, 1986; Edmonds *et al.*, 1987; Minet *et al.*, 1988). This species causes infrequent non-bloody diarrhoea often with vomiting, abdominal pain and fever. While the strains in these reports were isolated using conventional techniques, and were evidently resistant to the antibiotics used in selective media, they may not be representative of the species as a whole (Mishu, *et al.*, 1992). Isolates of *C. hyointestinalis* that survive on selective media may not be routinely distinguished from more commonplace species, thus the incidence of this species in human gastroenteritis remains unknown at the outset of this study.

1.1.7 Other *Campylobacter* species

*C. mucosalis* is associated with, but not the causative agent of, porcine proliferative enteropathy (Lawson, *et al.*, 1981). *C. helveticus* was originally isolated from the faeces of domestic cats and dogs (Stanley, *et al.*, 1992), and *C. lanienae* from the faeces of healthy abattoir workers (Logan, *et al.*, 2000). None of these 'enteric' species has been reported in association with human gastrointestinal illness.

*C. concisus* is the most commonly reported *Campylobacter* species from the human gingival crevice, and it is probably a major component of the healthy gingival flora in man (Tanner, *et al.*, 1981; Macuch & Tanner, 2000). A number of studies have reported the isolation of *C. concisus* from diarrhoeic human faeces, and on this basis alone have suggested that it is enteropathogenic in man (Lindblom *et al.*, 1995; Engberg *et al.*, 2000). However, when faeces from both diarrhoeic and healthy cases are examined, *C. concisus* can be recovered from both groups (Van Etterijck *et al.*, 1996). Significantly, none of
these studies investigated the distribution of *C. consisus* in the gingival flora of these cases.

Certain strains of *C. sputorum* are also part of the normal gingival flora of man, while other strains are components of the genital and intestinal flora of cattle (Skirrow, 1994). In one report *C. sputorum* was isolated from the faeces of Swedish children with diarrhoea (Lindblom, et al., 1995). However, the presence or absence of *C. sputorum* in the gingival crevice of these cases was not investigated.

*C. rectus*, *C. curvus*, *C. showae* and *C. gracilis* are also components of the gingival flora of humans. Of these, only *C. rectus* is implicated as a potential human pathogen on the basis that it is detected in higher levels from diseased rather than healthy periodontal and gingival samples (Ashimoto *et al.*, 1996; Macuch and Tanner, 2000). However, the aetiology of periodontal infection is complex and is probably the result of a combination of factors and microorganisms.

The generically misnamed *B. ureolyticus* is currently considered to be a *Campylobacter* on the basis of 16S rDNA analysis. Strains of this bacterium have been recovered, often as part of a mixed bacterial population, from patients with superficial ulcers, soft tissue infections and from cases of non-gonococcal urethritis. Its role as a human pathogen remains unclear (Vandamme, *et al.*, 1995).

### 1.12 Sources of *Campylobacter* infection

The most commonly described sources of *Campylobacter* infection are poultry, red meats, milk, untreated surface water and domestic pets (Tauxe, 1992). The consumption of
infected poultry is thought to account for a large proportion of infections, both sporadic and outbreak-associated. The connection between human illness due to *Campylobacter* and poultry was made as early as 1962 (King, 1962), and in 1977 Skirrow's paper describing the role of *Campylobacter* in human gastroenteritis, also noted that in 10% of campylobacter enteritis cases, contaminated poultry was the suspected vehicle of infection (Skirrow, 1977).

1.12.1 Poultry and red meats

Campylobacters have been reported to be commonplace and present in high numbers in both live chickens (Humphrey *et al.*, 1993) and their carcasses (Park *et al.*, 1981). Studies have demonstrated that *Campylobacter* serotypes most commonly found in chickens are also the most frequently occurring strains in isolates from human gastroenteritis (Munroe *et al.*, 1983; Patton *et al.*, 1985; Rogol & Sechter, 1987; Humphrey, *et al.*, 1993). Case-control studies also strongly associate consumption of poultry with *Campylobacter* infection (Harris *et al.*, 1986; Deming *et al.*, 1987). Cross-contamination from raw chicken to cutting boards, other utensils and food handlers during food preparation constitutes a serious risk of *Campylobacter* infection (Cogan *et al.*, 1999).

Other meat products have also been implicated as potential sources of *Campylobacter* infection. In one study, campylobacters were isolated from 20% of chicken, 5% of pork chops, 4% of pork sausage, 4% of ground beef, 5% of beef flank and 8% of lamb stew (Stern *et al.*, 1985). In another study, samples of animal offal collected from abattoirs and retail butchers were positive for *C. jejuni* or *C. coli* in 31% of sheep, 11% of cattle and 6% of pig offal samples. The isolates were characterised by biotype (Skirrow and Benjamin, 1980b) and serotype (Penner & Hennessey, 1980), see section 1.6.3. The majority of
offal isolates (89.5%) were C. jejuni biotype 1 and serotypes 1 and 2, phenotypes commonly isolated from human beings with Campylobacter enteritis (Bolton et al., 1985). In a study of the prevalence and distribution of Campylobacter serotypes among food animals, 96% of the chicken isolates and 67% of the cattle isolates belonged to the 11 C. jejuni serotypes most frequently implicated in human gastroenteritis (serotypes 1, 2, 3, 4, 5, 13/16, 18, 21, 23, 31, and 36). Serotype 8, a relatively common human isolate, was not recovered. However, isolates from pigs belonged to serotypes uncommon among human isolates (Munroe, et al., 1983).

1.12.2 Milk

Unpasteurised milk is the most commonly reported food vehicle associated with Campylobacter outbreaks in England and Wales. It has been implicated in several major outbreaks, sometimes involving hundreds of people (Hutchinson et al., 1985b; Humphrey & Beckett, 1987; Humphrey & Hart, 1988). In Scotland the sale of unpasteurised milk has been banned since 1983, resulting in a reduction of milk-associated Campylobacter outbreaks (Pearson and Healing, 1992). Campylobacter is introduced into milk either through contamination during milking with bovine faecal material (Humphrey and Hart, 1988) or from the milk of cows with Campylobacter mastitis (Hutchinson et al., 1985a). Other outbreaks have occurred due to failure of the pasteurisation process or due to post-pasteurisation contamination of milk. Bottled pasteurised milk may also be contaminated with campylobacters by wild birds, particularly jackdaws (Corvus monedula) and magpies (Pica pica) piercing foil-capped bottles on household doorsteps (Hudson et al., 1991). In one report an outbreak of C. jejuni affected 11 children in a day nursery. Milk consumed by the children was known to have been pecked/drunk by magpies on occasions. Illness was significantly associated with consumption of milk on a single morning. Examination of
milk from a bottle pecked after the outbreak yielded campylobacters. The level of contamination was approximately six cells of *C. jejuni* per 500 ml of milk (Riordan *et al.*, 1993).

1.12.3 Water

Isolates of *C. jejuni* and *C. coli* have been cultured from rivers, lakes and the sea at many sites in England and Wales (Pearson and Healing, 1992). There is a marked seasonal variation in the levels of campylobacters in natural waters, perhaps reflecting contamination with sewage. In a twelve-month study of one river system, campylobacters were found in 43% of samples. Campylobacters were least likely to be detected in samples collected from rural sites and fast-flowing stretches of river, while the highest counts were obtained from samples taken downstream of sewage works. A seasonal trend was apparent, with peak isolation rates in late autumn and winter, and the lowest in spring and summer. The most prevalent *Campylobacter* species was *C. jejuni* belonging to serotypes commonly isolated from human enteritis. *C. coli* and *C. lari* were also isolated (Bolton *et al.*, 1987). Outbreaks have been associated with drinking untreated water, or may result from contamination of potable water by birds, sewage or untreated water (Palmer *et al.*, 1983).

1.12.4 Animals

In nature *Campylobacter* species require a living avian or mammalian host if they are to multiply, unlike salmonellae, which are able to multiply on any suitable nutrient source at ambient temperatures. Many strains from animal sources are isolated from wild birds, but these are often serotypes not prevalent in human enteritis. Domestic pets, particularly cats and dogs are commonly colonised by campylobacters (Nair *et al.*, 1985) and may act
as reservoirs for human infection. In a case-control study of a *C. jejuni* outbreak on a University campus, infection in 30% of the students was attributed to contact with domestic cats (Deming, et al., 1987). Another case-control study demonstrated a significant association between cases of *Campylobacter* enteritis in infants and the presence of dog puppies in their homes (Salfield & Pugh, 1987).

### 1.12.5 Other sources

Besides its link to poultry and other meats, *Campylobacter* enteritis has been associated with other food vehicles including mushrooms (Harris, et al., 1986) and raw or poorly cooked fish and shellfish (Fricker & Park, 1989).

*Campylobacter* enteritis has been associated with faecal contamination of fresh watercourses. However, in a study of sand from UK seaside bathing beaches, campylobacters were isolated from 50% of samples from non-EEC standard and from 40% of EEC standard beaches. *C. jejuni* and *C. coli* of serotypes associated with human infections were more prevalent from non-EEC standard beaches, while *C. lari* (often associated with seagulls) was more prevalent in sand from EEC standard beaches (Bolton *et al.*, 1999).

Perhaps surprisingly considering its ubiquity, transmission of *Campylobacter* enteritis by person-to-person is uncommon, but has been described in young children (Skirrow, 1994).
1.13 *Campylobacter* infections of animals

*Campylobacter* enteritis is a zoonosis. The principal reservoir for human infection with *C. jejuni* and *C. coli* is poultry where carriage is as asymptomatic (Skirrow, 1994). Here the caecum is heavily colonised with *Campylobacter* and during the mass production process extensive cross contamination of the carcass occurs. Control of *Campylobacter* colonisation in poultry would be a long-term means of reducing infections in humans. *C. jejuni* and *C. coli* are thought to cause mild enteritis in cattle, sheep and pigs, usually affecting young animals. Colonisation in adult animals is usually asymptomatic but provides a significant reservoir for human infection (Skirrow, 1994). *Campylobacter* species have also been associated with bovine mastitis (Hutchinson, et al., 1985a).

*Campylobacter* enteritis has also been reported in domestic animals such as cats and dogs (Nair, et al., 1985; Deming, et al., 1987; Salfield & Pugh, 1987). However, other studies have reported no significant difference between the prevalence in normal animals and those suffering from diarrhoea. It seems that while most animals are unaffected by infection with *Campylobacter*, a few suffer an illness like that seen in man. A small proportion of human infections are acquired from cats and dogs and the victims are often small children (Skirrow, 1981).

*Campylobacter* species are an economically important cause of abortion in sheep and cattle. There are also isolated reports of abortion in other animals such as goats, horses and dogs. *Campylobacter* species are the third most common cause of ovine abortion (after *Chlamydia psittaci* and *Toxoplasma gondii*) accounting for 4% to 13% of cases in England and Wales. Elsewhere in the world, infection rates as high as 65% have been reported in sheep-rearing areas. *C. fetus* accounts for 40% of the outbreaks investigated,
the remainder being attributed to *C. jejuni* and *C. coli* (Skirrow, 1994). Bovine infectious infertility caused by *C. fetus* subspecies *venerealis* remains an economically significant infection in animal husbandry, especially in developing countries with limited access to artificial insemination technology (Skirrow, 1994).

1.14 Pathogenesis of *Campylobacter*

The pathogenesis of *Campylobacter* infection is still poorly understood. This is partly due to the lack of a suitable animal model. Although intestinal colonisation occurs in many animal species such as mice, rabbits and chickens, none of these reproduce the clinical symptoms of acute gastroenteritis seen in humans (Ketley, 1997).

It is unclear how campylobacters disrupt intestinal epithelial cell function to produce diarrhoeal symptoms. *Campylobacter* multiply rapidly after colonisation and patients typically excrete $10^8$ to $10^9$ organisms per gram of faeces (Skirrow, 1994). The watery diarrhoea often associated with *Campylobacter* infection suggests the involvement of an enterotoxin. However, the existence and pathogenic role of a putative *Campylobacter* toxin remains controversial (Ketley, 1997). Significantly, the fully sequenced genome of *C. jejuni* (NCTC 11168) does not contain a cholera-like toxin gene, although other pathogenicity-associated genes encoding a cytolethal-distending toxin (*cdtA-C*), a phospholipase (*pldA*) and haemolysins (Cj0588 and Cj0183) are present (Parkhill, et al., 2000).

In *C. jejuni* lipopolysaccharide (LPS) is thought to be involved in the pathogenesis of both uncomplicated infection and more serious sequelae. Studies suggest that all strains produce lipoooligosaccharide (LOS), with about one-third of strains also producing high-
molecular-weight LPS (Karlyshev et al., 2000). The LPS is biochemically and genetically unrelated to LOS and is similar to group II and group III capsular polysaccharides and correlates with serotype-specific antigens used for serotyping of C. jejuni (Chart et al., 1996; Karlyshev, et al., 2000). The exact role in pathogenesis and distribution of the Campylobacter LPS capsule remains to be determined.

Campylobacter motility is imparted by one or more terminal flagella. Non-flagellated mutants are unable to colonise animals (Caldwell et al., 1985) or humans (Black et al., 1988) and there is evidence that flagella contain adhesins that mediate adherence to human epithelial cells (Newell et al., 1985). There is experimental evidence that flagella are necessary for cell invasion, enabling C. jejuni to cross between epithelial cells grown in monolayers in vitro (Grant et al., 1993). The inflammation and bacteraemia associated with Campylobacter enteritis suggest that invasion is an important pathogenic mechanism. However, while Campylobacter species are able to invade cells in tissue culture, there is as yet little direct evidence of how or if this occurs in vivo (Ketley, 1997).

To successfully colonise the intestine Campylobacter must be able to acquire iron from the host or competing bacterial cells. Iron-deficiency has been shown to increase oxidative stress in Campylobacter (Ketley, 1997). While there is no evidence of siderophore production by Campylobacter, they do possess an iron transport system encoded by the ceu operon that facilitates the utilisation of exogenous siderophores (Richardson & Park, 1995).
1.15 Isolation of *Campylobacter* species

1.15.1 Atmosphere and temperature

All *Campylobacter* species are microaerobic – they grow only at lower oxygen tensions than that in air. Tolerance to oxygen varies greatly, depending on the species and the strain. For *C. jejuni* an atmosphere of 5% O₂, 10% CO₂ and 85% N₂ is optimum (Bolton & Coates, 1983b). Hydrogen concentrations of 5 to 10% have been shown to be a growth requirement for certain species and a growth enhancer for others (Skirrow, 1991). *Campylobacter* species differ in their critical and optimum temperature requirements. *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* grow well at 42°C to 43°C and are often referred to as 'thermophilic' campylobacters – although 'thermotolerant' would be a more accurate term. They will grow at temperatures up to 48°C, but not below 30°C. *C. jejuni* subspecies *doylei* will not grow at 42°C. *C. fetus* grows at both 25°C and 37°C. *C. concisus* and *C. rectus* grow only poorly at 42°C, but well at 37°C.

1.15.2 Culture using filtration

Filtration through a syringe was first used to isolate 'Vibrio fetus' (i.e. *C. fetus* subsp *venerealis*) from samples of bull's semen (Plummer *et al.*, 1962). Similar techniques were used to isolate campylobacters from animal faeces (Smibert, 1965) and the first reported *Campylobacter* isolate direct from human faeces was obtained in this manner (Dekeyser, *et al.*, 1972). However, with the development of selective media this somewhat cumbersome approach was set aside.

A simplified filtration-based methodology was described by Steele and McDermott whereby a faecal suspension was applied to 0.45 μm pore size membrane filters, placed
on an agar base medium. The filter was discarded after 30 minutes and the plate incubated (Steele & McDermott, 1984). This technique has been used to assess the prevalence of Campylobacter species thought to be sensitive to the antibiotics contained in selective agar (Bolton, et al., 1988; Goossens, et al., 1990a). Unfortunately, the sensitivity of detection of filtration methods is limited and bacterial concentrations lower than $10^5 \text{ cfu g}^{-1}$ of faeces cannot be detected (Goossens & Butzler, 1992).

1.15.3 Culture using selective media

The first widely used selective medium for the isolation of campylobacters was that described by Skirrow. It consisted of: a nutrient base, 5% lysed horse blood which had been shown to promote the growth of campylobacters, the antibiotic vancomycin to inhibit the growth of Gram-positive bacteria, plus trimethoprim and polymyxin B which effectively inhibit Gram-negative bacteria (Skirrow, 1977). This medium was designed specifically for the isolation of \textit{C. jejuni} and \textit{C. coli}. The recommended incubation temperature was 42°C, which in itself was inhibitory to many bacteria. Skirrow's medium has proved extremely successful. However, it is prone to overgrowth with certain competing organisms notably swarming \textit{Proteus} species and oxidase-positive \textit{Pseudomonas aeruginosa}. Variations on this medium include Blaser-Wang medium that includes amphotericin B to inhibit \textit{Candida albicans}, and cephalothin to reduce Gram-negative contamination (Blaser et al., 1980). Several workers explored other antibiotic combinations that might inhibit contaminating faecal flora. Butzler's medium contained bacitracin, cycloheximide, colistin, novobiocin and cephalothin in a \textit{Brucella} agar base with 5% horse blood and was designed for incubation at 37°C to facilitate the isolation of \textit{C. fetus} (Lauwers et al., 1978). The Preston \textit{Campylobacter}-selective medium uses polymyxin B, rifampicin, trimethoprim and cycloheximide with lysed blood in either agar or broth, incubated at 42°C. It was
specifically formulated for the isolation of campylobacters from human, animal and environmental samples and for the recovery of low numbers from heavily contaminated samples (Bolton & Robertson, 1982).

Later formulations have used charcoal as a replacement for blood in *Campylobacter* culture media. Both blood and charcoal promote the growth of *Campylobacter* by neutralising toxic oxygen species (Bolton & Coates, 1983a). This was combined with cefoperazone (a broad-spectrum cephalosporin with activity against *Pseudomonas* species) in Charcoal Cefoperazone Desoxycholate Agar (CCDA), which also contains the bile salt desoxycholate and the antifungal amphotericin B (Bolton et al., 1984b). This medium may be incubated between 35°C and 42°C. Studies suggest that optimum isolation is achieved with incubation at 37°C (Bolton et al., 1988). This medium is probably the most widely used in England and Wales (Anonymous, 1993). A modification of this medium, Cefoperazone Amptotericin Teicoplainin (CAT) agar contains a reduced cefoperazone concentration (8 mg l⁻¹ rather than 32 mg l⁻¹) and the antibiotic teicoplanin to reduce overgrowth with *Enterococcus* species (Aspinall, et al., 1993). CAT was developed to enhance the isolation of *C. upsaliensis*, which like many non-jejuni/non-coli campylobacters is sensitive to the antibiotics used in campylobacter-selective isolation media.

1.16 Identification and subtyping of *Campylobacter* species

1.16.1 Phenotypic identification

The Identification of *Campylobacter* can be difficult since strains have relatively fastidious growth requirements and are asaccharolytic. Useful discriminating characteristics are
listed in Table 1.1 and are described in more detail in section 2.6. Speciation relies on relatively few phenotypic tests. For example, *C. jejuni* and *C. coli* are distinguished only by hippurate hydrolysis, while *C. coli* and *C. upsaliensis* are distinguished by the weak catalase activity and sensitivity to cephalothin of the latter. In many clinical laboratories identification to genus level by a combination of growth on selective media, positive oxidase reaction and Gram-stain, is the only identification attempted. In 1989 to 1990 only 17% of campylobacters reported to CDSC by laboratories in England and Wales were identified to species level (Anonymous, 1993). Taxonomic classification of *Campylobacter* species based on a combination of biochemical characteristics and tolerance to antimicrobial substances has been developed (On & Holmes, 1995). However, many of these tests are susceptible to inoculum size and medium composition (On & Holmes, 1991a; On and Holmes, 1991b) and thus remain the province of specialist laboratories.

1.16.2 Subtyping

Serotyping is the principal means of subtyping *Campylobacter*. Two distinct systems predominate: The Penner system is based on passive haemagglutination and identifies 42 *C. jejuni* and 18 *C. coli* serotypes (Penner and Hennessey, 1980). At CPHL an adaptation of this scheme employing direct bacterial agglutination is used, which identifies 47 *C. jejuni* and 15 *C. coli* serotypes, approximately 19% of strains are not typeable (Frost et al., 1998). The second major serotyping system is the Lior scheme (Lior et al., 1982). This system is also based on passive haemagglutination and identifies 108 serotypes including eight among *C. lari* strains, again a significant proportion of strains (17%) are not typable.
A number of biotyping schemes based on variable phenotypic features have been proposed (Skirrow & Benjamin, 1980b; Bolton et al., 1984a). These techniques have proved useful in distinguishing epidemiologically related strains. The advantage of the biotyping approach is that all isolates are typable.

Macro-restriction techniques such as ribotyping and Pulsed-Field Gel Electrophoresis (PFGE) have been employed to subtype Campylobacter strains in some laboratories (Wassenaar & Newell, 2000). Restriction Fragment Length Polymorphism (RFLP) analysis of PCR products of the flagellin genes has been more widely used although its resolution is relatively limited (Alm et al., 1993; Stanley et al., 1995; Waegel & Nachamkin, 1996). Another PCR-based technique, Amplified Fragment Length Polymorphism (AFLP), has proved useful both in epidemiological and taxonomic investigations among Campylobacter species (On & Harrington, 2000; Wassenaar & Newell, 2000). The resolution of all of these techniques is dependent on the choice of restriction enzyme. Potentially they could be applied to any Campylobacter isolate, although certain strains have been shown to produce potent nucleases that may render them untypable by ribotyping and PFGE (Wassenaar & Newell, 2000).

1.16.3 Identification and detection using molecular methods

Molecular techniques such as DNA-DNA hybridisation have been used as a successful reference method for identification and in taxonomic studies (Vandamme & Goossens, 1992). However, perhaps the most widely applicable identification methodologies involve the Polymerase Chain Reaction (PCR). Target genes employed for PCR identification of Campylobacter species from cultured isolates have included 16S rRNA (Giesendorf & Quint, 1995; Linton et al., 1996), 23S rRNA (Eyers et al., 1993), flaA (flagellin) (Waegel &
Nachamkin, 1996), GTP-binding protein (van Doorn et al., 1997), ceuE (iron transport protein) (Gonzalez et al., 1997) and hip (hippuricase) (Linton et al., 1997).

There are few examples of PCR applied to the detection of Campylobacter, without a requirement for culture, directly from substrates such as faeces or foodstuffs. This is because many potential substrates contain substances that inhibit or reduce the sensitivity of PCR assays. For example, faecal material invariably contains numerous nucleases that readily degrade target DNA, together with significant quantities of bile salts, bilirubin, urobilinogens and polysaccharides, all of which can be inhibitory to PCR (Widjojoatmodjo et al., 1992). The small sample volume of the PCR (typically 2.5 to 5.0 μl) is also problematical and can lead to sampling errors (Giesendorf and Quint, 1995). Notwithstanding these difficulties, Flagellin gene and 16S rDNA PCR assays have been applied to foodstuffs (Giesendorf and Quint, 1995; Wegmuller et al., 1993), and a flaA PCR has been applied directly to small numbers of faecal samples without culture of an isolate (Oyofo et al., 1992). The largest PCR-based study (outside of the work described in this thesis) of Campylobacter in clinical faecal samples was of 493 samples examined using selective-enrichment culture and subsequent 16S rDNA-based PCR detection of C. jejuni, C. coli and C. lari (Vanniasinkam et al., 1999).

1.17 The Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR), the process of in vitro synthesis of an essentially unlimited number of copies of a DNA sequence using DNA polymerase, was discovered by Mullis in 1983 (Mullis & Faloona, 1987). Since its introduction, the PCR has revolutionised biomedical research and diagnosis. Its usefulness was first demonstrated
when it was applied to diagnosis of sickle-cell anaemia by amplification of β-globin genomic sequences (Saiki et al., 1985).

PCR uses repeated cycles of oligonucleotide-directed DNA synthesis to replicate a target nucleic acid sequence delimited by the specific hybridisation of the oligonucleotides. This results in an exponential increase in the number of copies of the target DNA. Typically a PCR consists of three stages. The first is denaturation, in which the double-stranded target DNA is melted at 94°C to obtain single strands of DNA. The second is annealing where the oligonucleotide primers bind to their complementary target sequences, this is achieved by lowering the reaction temperature to a point where double stranded DNA is reformed, typically between 40°C and 65°C; The third stage is extension of the oligonucleotide primers to form a complementary DNA strand, synthesized by DNA polymerase (the optimum temperature for Thermus aquaticus DNA polymerase is 72°C). These three steps are thus repeated in a thermal cycler (a typical PCR protocol consisting of between 25 and 35 thermal cycles). The thermal cycler is essentially a programmable heating block, capable of conducting successive heating and cooling cycles. Each cycle results in the duplication of the number of target molecules and thus over the course of 25 or more cycles there is an exponential increase in the copies of synthesized target DNA (see Figure 1.5).

Mullis originally used the Klenow fragment of E. coli DNA polymerase I in his first experiments (Mullis & Faloona, 1987). However, this enzyme was inactivated during the denaturation stage and, fresh DNA polymerase had to be added after each round of amplification. This cumbersome process was alleviated by the discovery and purification of a DNA polymerase from Thermus aquaticus, an extremely thermophilic bacterium.
Figure 1.5 Diagram showing how the repetition of the process of denaturation, primer annealing and extension (PCR) results in the exponential amplification of the target sequence DNA flanked by primers. The amplification of only one of the products of cycle 2 is shown in cycles 3 to 5.

- Forward primer;  
- Reverse primer;  
- Template DNA;  
- Newly synthesised DNA.
growing in hot springs with temperatures in excess of 70°C (Saiki et al., 1988). Taq (T. aquaticus) DNA polymerase is thus resistant to prolonged exposure to high temperatures (it has a half-life of 40 minutes at 95°C). With the discovery of Taq DNA polymerase, PCR could be automated, using a programmable thermal cycler capable of cycling temperatures automatically.

1.18 Ribosomal DNA

Ribosomes are vital components of all living cells and genetic analysis of the genes encoding their production has proved an invaluable tool in the understanding of evolutionary taxonomy at all levels. Ribosomal sequence data has been used extensively to elucidate the interrelationships of the genus Campylobacter and its relatives and consequently provides an ideal target for PCR-based detection and identification schemes.

1.18.1 The ribosome – structure and function

The bacterial (70S) ribosome is made up of ribosomal proteins and ribosomal RNA (rRNA), assembled in two main elements, the 50S and 30S subunits. There are three species of rRNA, designated according to their sedimentation coefficients (S) as 23S, 16S and 5S. In E. coli these are 2904, 1542 and 120 nucleotides long respectively. The 50S subunit is composed of 23S and 5S rRNAs together with 34 intimately associated ribosomal proteins (Guttell et al., 1994). The 30S subunit is composed of 16S rRNA and 21 proteins (Figure 1.6). All three rRNAs are single-stranded molecules with specific and extensive secondary structure, as illustrated in Figure 1.7.
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Figure 1.6 A schematic representation of the structure of the bacterial ribosome.

- 21 ribosomal proteins and 16S rRNA
- 30S subunit
- 34 ribosomal proteins, 23S and 5S rRNA
- 50S subunit

70S ribosome

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Figure 1.7. Secondary structure of the small subunit (16S) rRNA of Bacteria.
(adapted from Neefs et al. 1990)

The 5'-terminus is marked by a filled circle and the 3'-terminus by an arrowhead. Helices bearing a single number are common to both Bacteria and Eukarya. Numbers preceded by P are Bacterial-specific helices. Conserved areas are drawn in bold lines, areas of variable sequence or length in thin lines. Variable regions numbered V1 to V9 are distinguished (region V4 is absent in prokaryotes). Helices drawn in broken lines are present only in a small number of known structures. Archaeal sequences form similar structures to those of Bacteria except for helix 35, which is unbranched as in the Eukarya.
The ribosomal RNA species collectively are encoded by *rrn* genes. The 23S, 16S and 5S rRNAs are encoded by the *rrl*, *rrs*, and *rrf* genes respectively and these are typically arranged in a single operon with the order 16S-23S-5S (Figure 1.8). The number of rRNA operons varies between bacterial species, *Campylobacter* species contain three operon copies, whereas *E. coli* has seven copies (Kim *et al.*, 1993). Transcription of *rrn* operons proceeds away from the origin of replication and is tightly regulated in response to the growth rate of the cell. The *rrn* transcript is processed by RNAases to yield both functional rRNAs and transfer RNAs (tRNAs). The region cleaved between the *rrs* and *rrl* genes is called the internally transcribed spacer. This and the region distal to the *rrf* gene contain genes encoding tRNAs. The structural rRNA genes may be interrupted by intervening sequences that are later excised, which have been observed in the *rrl* gene of *Campylobacter* (Hurtado & Owen, 1997), and the *rrs* genes of *C. sputorum* and *C. helveticus* (Linton *et al.*, 1994).

The full-length 16S and 23S sequences of *E. coli* were first determined by Brosius (Brosius *et al.*, 1978; Brosius *et al.*, 1980). In subsequent years, with the increased availability of DNA sequencers and PCR-based sequencing protocols a huge number of representative sequences have been published and made available for comparative analysis. The 16S sequence in particular has proved extremely valuable for determining the phylogenetic relationships between bacterial species. In both 16S and 23S rRNA 60% of the nucleotides are involved in secondary structure base pairings and about 40% are unpaired (Guttell *et al.*, 1994). 16S rRNA consists of around 48 hairpin-loops and helical regions with interconnecting single strand regions (Figure 1.7). Thus in ‘double stranded’ areas changes in one part of the molecule may require matching substitutions in an
Figure 1.8 Typical arrangement of bacterial rRNA operon.
analogous region so as to maintain base-pair complementarity. This is termed positional covariance (Neefs et al., 1990).

The functional constraints on the ribosome mean that certain regions of the 16S rRNA sequence are highly conserved, being almost invariable for all bacteria. In other regions some variation is possible, allowing differences to evolve between the sequences found in different bacterial genera and species. In a few hypervariable regions, variations occur even within sequences of a single species. In 16S rRNA, eight variable regions have been identified – they are termed V1 to V3 and V5 to V9 (region V4 occurs only in eucaryotic ribosomes). The distribution of these variable regions is illustrated in Figures 1.7 and 1.9 (Neefs et al., 1990).

1.18.2 Ribosomal RNA and bacterial phylogeny

The major phylogenetic discovery derived from rRNA gene sequence comparisons is that living organisms cannot be divided simply according to the eukaryote and prokaryote cell types, as had previously been supposed, but rather into three domains Bacteria, Archaea and Eukarya (Woese, 1987). Analysis of 16S rRNA sequences further divides the Bacteria into a number of divisions termed Phyla (Woese, 1987). Morphological features that had formed the basis of previous classifications were now found to be dispersed among several phyla. The majority of Gram-negative taxa are included in the Proteobacteria (Stackebrandt, et al., 1988), which may be further subdivided into 'rRNA superfamilies'. Campylobacter along with Arcobacter, Wolinella and Helicobacter belong to the epsilon subdivision (rRNA superfamily VI) of the Proteobacteria (Vandamme, et al., 1991; Lane, et al., 1992; Rainey, et al., 1993). The epsilon subdivision (rRNA superfamily VI) can be further subdivided into the family Campylobacteraceae (containing
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Campylobacter species.

Figure 1.9 Arrangement of variable (V) regions within the 16S rRNA (rrs) gene of Campylobacter species.
Campylobacter and Arcobacter), the genera Sulfurospirillum, Helicobacter, Wolinella and Thiovulum, and the generically misnamed 'B. ureolyticus' (Vandamme and de Ley, 1991; Lane, et al., 1992; Vandamme & Goossens, 1992; Vandamme, et al., 1995). Regardless of how informative these 16S rDNA sequence derived relationships and phylogenetic trees appear it is important to bear in mind that these inferences are drawn from the evolution of a single molecule. Strictly speaking, they should be thought of as gene trees rather than organism trees. Recent evidence that horizontal gene transfer between bacterial species is more commonplace than previously appreciated has cast doubts on the validity of gene-based phylogeny. This model presents the bacterial genome as a mosaic of endogenous and 'acquired' genes each with their own evolutionary history, rather than as a discrete, gradually evolving entity (Pennisi, 1999). However, it seems likely that rDNA would, in most instances, evolve in parallel with the host organism being as it is integral to the life of the cell. Evidence of this is provided by the genus Buchnera. These bacteria are obligate endosymbionts found in the gut of aphids and white fly (superfamily Aphidoidea). Here the 16S rDNA-derived phylogenetic tree of Buchnera species from different Aphidoidea genera exactly matches the inferred relationships between their hosts based on the observed physical taxonomy of the insects from both living and ancient (preserved in amber) specimens (Munson, et al., 1991; Baumann, et al., 1995).

1.18.3 Non-cultivable bacteria

Our current knowledge of the presence and diversity of microorganisms associated with the 'human ecosystem' in health and disease is almost entirely derived from cultivation-based methodologies. The gastrointestinal tract is estimated to contain \(~10^{14}\) bacteria belonging to many species and it is thought that only \(~60\%\) of the bacterial forms
observed during microscopic examination of human faecal samples have been cultured (Berg, 1996).

Uncultured microorganisms associated with certain pathological processes have previously been characterised by analysis of 16S rDNA sequences. For example in Whipple's disease monomorphic bacilli were observed in pathological tissues, but all attempts to culture them failed, until their recent propagation in cell culture (Schoedon et al., 1997). Nonetheless, amplification of prokaryotic 16S rDNA from infected eukaryotic host tissue, and subsequent sequence analysis of the amplicon, had allowed the taxonomic position of the bacilli, now named *Tropheryma whippelii*, to be deduced (Reiman et al., 1992). In a similar manner, *'Gastrospirillum hominis'*, a culture-resistant bacterium observed in histological samples from the human gastric mucosa, was identified as a *Helicobacter* and provisionally named *'H. heilmannii'* (Solnick et al., 1993). Likewise, in the analysis of complex bacterial communities such as dental plaque, molecular identification of the uncultivable bacterial components has been achieved by representative cloning of prokaryotic 16S rDNA amplicons, followed by sequence analysis (Wilson et al., 1997).

The examples above illustrate the power of 16S rDNA-based analysis to extend our perception of microbial diversity in pathological and complex ecologies. In a recent study of infectious intestinal disease in England, microbial aetiologies could only be established in ~45% of cases of apparent gastrointestinal infection (Wheeler et al., 1999). Given the relatively fastidious growth requirements of the genus, perhaps other, as yet undescribed, *Campylobacter* species await discovery.
1.19 Aim and objectives

The aim of this study is to investigate the prevalence of *Campylobacter* species in human gastroenteritis using the techniques of molecular microbiology – principally PCR. Within this aim are several objectives:

- To develop a rapid extraction protocol to recover bacterial DNA from faeces – a prerequisite for the pursuit of the further goals.

- To develop and evaluate PCR assays for the detection of *Campylobacter* species recognised as human enteropathogens, namely: *C. jejuni*, *C. coli* and *C. lari*.

- To develop and evaluate PCR assays for the detection of those *Campylobacter* species, namely: *C. fetus*, *C. hyointestinalis*, *C. upsaliensis* and *C. helveticus*, whose role in the aetiology of human gastroenteritis remains uncertain.

- To undertake the development and application of various PCR formats to facilitate the application of the above PCR-assays to epidemiological surveys.

- To conduct a large-scale epidemiological survey of the prevalence of all potentially enteropathogenic *Campylobacter* species in human gastroenteritis.

- To investigate the possible existence of, as yet undiscovered *Campylobacter* species.
Chapter 2

Materials and Methods
Chapter 2. Materials and Methods

Reagents were prepared with and reactions performed in sterile distilled water (SDW) unless otherwise stated. Latex gloves were worn for all procedures. Manufacturers and suppliers are indicated in squared parenthesis [ ]. Reference strains of bacteria are listed in Appendix A.

2.1 Culture and maintenance of bacterial isolates

2.1.1 Sources of bacterial isolates

Reference strains used in this study were obtained from The National Collection of Type Cultures (NCTC) as freeze-dried ampoules. Strains and isolates are listed in the corresponding Chapters.

2.1.2 Culture and maintenance conditions

All strains were cultured on Columbia Blood Agar (CBA) - Columbia agar base with 5% v/v defibrinated horse blood [Oxoid]. Microaerobic bacteria (Campylobacter, Arcobacter and Helicobacter species) were incubated in a Variable Atmosphere Incubator [Don Whitley Scientific] which maintained an atmosphere of 5% O₂, 5% CO₂, 2% H₂ and 88% N₂ (by volume) at 37°C, with the exception of Arcobacter nitrofigilis, which was grown microaerobically in a gas jar at 25°C. Anaerobic bacteria (C. curvus, C. gracillis, C. rectus, C. showae and B. ureolyticus) were incubated in an anaerobic cabinet [Don Whitley Scientific] which maintained an atmosphere of 5% CO₂, 5% H₂ and
90% N₂ (by volume). *E. coli* control strains were cultured aerobically at 37°C. Primary stock cultures were maintained using the 'Protect ' bacterial bead system [Technical Service Consultants Ltd.], at -80°C.

### 2.1.3 Other non-selective growth media

Besides CBA, two other non-selective growth media were used in the course of this study:

Fastidious Anaerobe Agar (FAA) [Lab M] was used as an alternative to CBA in order to enhance the growth of anaerobic *Campylobacter* species. This is a rich growth medium containing per litre volume:

- Peptone 23.0 g
- Sodium chloride 5.0 g
- Soluble starch 1.0 g
- Agar no.2 12.0 g
- Sodium bicarbonate 0.4 g
- Glucose 1.0 g
- Sodium pyruvate 1.0 g
- Cysteine hydrochloride monohydrate 0.5 g
- Haemin 0.01 g
- Vitamin K 0.001 g
- L-arginine 1.0 g
- Sodium pyrophosphate 0.25 g
- Sodium succinate 0.5 g
- Sterile distilled water (SDW) 1000 ml
- To this base 5% v/v defibrinated horse blood was added.
Brucella broth (BB) [Difco] was used to dilute faecal material and *Campylobacter* cultures in a variety of procedures. It gave improved recovery of *Campylobacter* cells compared to saline or peptone recovery diluents. BB was also used to culture *Campylobacter* isolates in a liquid medium to encourage motility (see section 2.6.2). BB consists of per litre:

- Tryptone 10 g
- Peptamin 10 g
- Dextrose 1.0 g
- Yeast extract 2.0 g
- Sodium chloride 5.0 g
- Sodium bisulphite 0.1 g
- SDW 1000 ml

### 2.2 Selective isolation of *Campylobacter* species

#### 2.2.1 Antibiotic containing selective media

Selective media containing antibiotics were used to isolate *Campylobacter* species from faecal material. Two formulations were used, both based on campylobacter blood-free selective agar base [Oxoid], which consists of:

- Nutrient broth (no.2) 25.0 g
- Bacteriological charcoal 4.0 g
- Casein hydrolysate 3.0 g
- Sodium desoxycholate 1.0 g
- Ferrous sulphate 0.25 g
- Sodium pyruvate 0.25 g
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- Agar 12.0 g
- SDW 1000 ml

Modified Charcoal Cefoperazone Desoxycholate Agar (CCDA) was made by the addition of 32 mg l⁻¹ of cefoperazone (Bolton et al., 1984b).

Cefoperazone Amphotericin Teicoplanin agar (CAT) was made by the addition of 8 mg l⁻¹ cefoperazone; 10 mg l⁻¹ amphotericin; and 4 mg l⁻¹ teicoplanin (Aspinall et al., 1993).

Both agars were inoculated with either a 10 µl plastic disposable loop of faecal material or with 100 µl of diluted faecal suspension. Plates were incubated in a microaerobic atmosphere at 37°C (section 2.1.2) for 24 to 48 hours (or longer in some instances). Campylobacter colonies were silver/grey. Identity was confirmed by the oxidase test and Gram stain (see section 2.6). Strains C. jejuni NCTC 11351 and C. helveticus NCTC 12470 were used as positive controls for CCDA and CAT respectively. E. coli NCTC 9001 was used as a negative control for both media.

2.2.2 Isolation by selective filtration

Filtration techniques were among the first selective isolation methods to be applied to Campylobacter species. These methods rely on the fact that campylobacters are relatively small compared to obligate aerobes (such as Pseudomonas species) and facultative anaerobes (such as E. coli) that are often found in faecal material, and would otherwise outgrow any campylobacters present. This simplified membrane filtration method is widely
used in the study of non-*jejuni/non-coli* campylobacters (Steele & McDermott, 1984).

Required:

- BB
- CBA
- 0.45 or 0.65 μm cellulose acetate filter [Sartorius]

Protocol:

1. A 42 mm diameter cellulose acetate filter (either 0.45 or 0.65 μm) was placed on the surface of a pre-dried CBA plate.
2. Approximately 100 mg of faecal sample was diluted 1:10 (w/v) in 1 ml BB and homogenised by vortex mixing to facilitate filtration.
3. 500 μl of the faecal suspension was carefully placed on the surface of the filter.
4. The suspension was allowed to pass through the filter at room temperature for 30 to 40 minutes (or until the filter was dry).
5. The filter was then discarded. Care was taken to avoid contaminating the CBA plate with unfiltered faecal matter.
6. The inoculated CBA plate was incubated under micro-aerobic conditions (see section 2.1.2) for 48 hours.

Controls:

Positive: *C. jejuni* NCTC 11351; Negative: *E. coli* NCTC 9001.
2.2.3 Immunomagnetic Separation (IMS)

IMS employs antibodies attached to magnetic beads to selectively bind target cells, which can then be removed from a complex matrix using a magnet. In this study the magnetic beads were coated with protein G, which binds the Fc portion of the antibody molecule. These were used in conjunction with two commercially prepared antibodies specific for the *Campylobacter* genus.

Required:

- Anti-campylobacter antibodies, either:
  - Anti-flagella mouse monoclonal antibody [Chemicon International Inc.]
  - Anti-cell wall goat polyclonal antibody [Kirkegaard and Perry Labs Inc.]
- Protein G-coated, magnetic beads [PerSeptive Diagnostics Inc.]
- BB
- CBA or FAA
- Magnetic particle concentrator

Protocol:

1. A bacterial suspension was made in 50 μl BB and mixed with 100 μl of an antibody preparation pre-diluted to a working concentration of 20 mg ml\(^{-1}\). This was incubated at 40°C with occasional mixing for 30 minutes.
2. Cells were pelleted by centrifugation at 13000 g for one minute, and the supernatant was discarded.
3. The pellet was washed three times with 1 ml BB.
4. The washed pellet was resuspended in 900 μl BB to which 100 μl of a 5 mg ml\(^{-1}\) suspension of protein G-coated magnetic beads was added.
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This suspension was incubated at room temperature for 15 minutes with gentle mixing.

5. Beads were recovered with a magnetic particle concentrator, and washed three times in BB.

6. The bound bacterial cells were then plated out and incubated as described above (see section 2.1.2).

Controls: Positive: *C. jejuni* NCTC 11351; Negative: *E. coli* NCTC 9001.

2.3 Collection and processing of clinical samples

Faecal samples were collected from both healthy volunteers (free from gastroenteritis) and from clinical cases of acute diarrhoea. For certain cases saliva samples were collected as described by Mortimer & Parry (Mortimer & Parry, 1994) and examined for the presence of *Campylobacter* species.

2.3.1 Faecal Samples

Required:

- Faecal sample containers [Sterilin]
- BB, 9 ml aliquots in sterile universals
- 10 μl disposable loops [Technical Services Consultants Ltd.]
- Vortex mixer

Protocol:

1. Healthy volunteer samples were collected and processed immediately. Clinical faecal samples from cases of acute gastroenteritis were submitted to regional Public Health Laboratories (PHL) and passed on...
for the investigations undertaken herein, not later than one week after initial receipt.

2. A 1 g aliquot of each faecal sample was homogenised in 9 ml of BB by vortex mixing.

3. This Homogenate was then immediately used for either culture (section 2.2) or DNA extraction (section 2.7).

2.3.2 Saliva samples

Required:

- Sponge swab and collection tube [Sterilin]
- BB
- Vortex mixer

Protocol:

1. The sponge swab was rubbed firmly along the gums at the base of the subject's teeth for about a minute.

2. The swab was then vortexed in 0.5 ml of diluent in the close fitting tube provided.

3. The swab was then inverted and the tube centrifuged at 5000 g for five minutes, to collect the supernatant.

4. The supernatant was stored at 4°C. DNA was extracted within 48 hours (see section 2.7).
2.4 Seeding of faecal samples

In order to create positive control faecal samples containing known numbers of bacterial cells, culture-negative stools from healthy (gastroenteritis-free) individuals, were seeded with *Campylobacter*. Such seeded samples were used to determine the relative sensitivities of the *Campylobacter* detection methodologies employed.

Required:

- BB
- CBA

Protocol:

1. Log phase cultures (24 to 48 hour growth) of *Campylobacter* isolates were suspended in BB to produce turbidity equal to that of a 0.5 McFarland standard – equivalent to approximately $1.5 \times 10^8$ colony forming units ml$^{-1}$ (cfu ml$^{-1}$).  
2. Suspensions were serially diluted to give a range of cell concentrations from approximately $10^8$ to $10^2$ cfu ml$^{-1}$.  
3. Estimated cell concentrations were confirmed by viable count on CBA plates incubated at 37°C in a microaerobic atmosphere for 48 hours.  
4. Nine ml of each dilution (for each isolate) was used to homogenise 1 g of faecal material (from a healthy, campylobacter culture-negative individual).  
5. The seeded sample was then used for selective culture (section 2.2) or DNA extraction (section 2.7).
2.5 Minimum Inhibitory Concentration (MIC) determination

The MICs of certain *Campylobacter* strains to the antibiotic cefoperazone were determined as follows:

Required:

- Campylobacter blood-free selective agar base [Oxoid]
- Cefoperazone [Sigma]
- BB

Protocol:

1. Doubling dilutions of cefoperazone from 64 mg l\(^{-1}\) to 2 mg l\(^{-1}\) were prepared in campylobacter blood-free selective agar base.

2. Twenty-four hour cultures of the twelve strains were suspended in BB to produce turbidity equivalent to a 0.5 McFarland standard (1.5x10\(^8\) cfu ml\(^{-1}\)). This suspension was diluted 1:1000 and 10 µl inoculated onto the antibiotic-containing medium and a comparable antibiotic-free medium. The initial concentration of each suspension was confirmed by a viable count.

3. The cultures were incubated at 37°C in a microaerobic atmosphere and growth was recorded after 48 and 96 hours.

4. The MIC was defined as the lowest concentration of antibiotic at which growth was completely inhibited.

2.6 Phenotypic identification

The phenotypic identification of *Campylobacter* species can be difficult since strains have relatively fastidious growth requirements and are asaccharolytic, and since only a limited number of phenotypic characteristics give any
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discrimination. Those characteristics most useful in distinguishing *Campylobacter* from other bacterial genera and *Campylobacter* species from each other are presented below (see Table 1.1)

2.6.1 Gram stain

Due to differences in cell wall structure, most bacteria can be assigned to one of two classes by their Gram reaction. Gram-positive bacteria retain the dark purple stain crystal violet and are not decolourised with acetone, whilst Gram-negative bacteria are decolourised and appear red when counter-stained with carbol fuchsin. The campylobacter Gram stain is characteristic; typically they appear as small, delicate, spiral curved, Gram-negative bacteria (often likened to gull wings or S-shapes).

Required:

- 10 μl disposable loops
- Crystal violet [Severn Biotechnologies]
- Lugol's iodine [Severn Biotechnologies]
- Acetone [BDH]
- Carbol fuchsin [Severn Biotechnologies]
- Microscope slides
- Microscope with 40 x and 100 x oil immersion objective lens

Protocol:

1. A suspension of bacteria was prepared in a drop of SDW, smeared onto a microscope slide and allowed to dry.
2. The slide was flooded with crystal violet for 30 to 60 seconds, and then washed with tap water.

3. The slide was flooded with Lugol's iodine for 30 to 60 seconds, and then washed with tap water.

4. The smear was then decolourised with acetone (a few seconds), which was washed off immediately with tap water.

5. The slide was flooded with carbol fuchsin for one minute, then washed with tap water and allowed to dry.

6. The smear was examined under a microscope under the 100 x oil-immersion objective lens.

2.6.2 Motility

Campylobacters typically exhibit a characteristic darting motility. However, because of their sensitivity to oxygen, they require careful handling.

Required:

- CBA or FAA
- Sterile disposable scalpel [Sterilin]
- BB, 9 ml aliquots in universals
- 10 μl disposable loops
- Microscope slides and cover-slips
- Plasticine
- Microscope with 40 x objective lens
Protocol:

1. A 1 cm by 2 cm chip of agar (either CBA or FAA) was aseptically cut out and placed in sterile universal containing 9 ml of BB.
2. The agar chip was inoculated with the test organism and incubated in appropriate conditions (see section 2.1.2) for 24 to 72 hours.
3. A drop of the liquid culture media was placed on a plastic cover-slip.
4. The cover-slip was then carefully inverted (and the hanging drop suspended) over a microscope slide with plasticine.
5. The edge of the drop was then examined under the 40 x objective lens.

Controls:

Positive: C. jejuni NCTC 11351; Negative: C. gracillis NCTC 12738.

2.6.3 Oxidase

Test strips are impregnated with tetramethyl p-phenylenediamine, which is oxidized to iodophenol blue in the presence of bacterial cytochrome oxidase. A positive oxidase reaction together with the characteristic Gram stain is usually considered sufficient for provisional identification as 'Campylobacter species'.

Required:

- Oxidase test strips [Technical Service Consultants Ltd.]
- 10 μl disposable loops

Protocol:

1. A 24 to 48 hour culture of the test bacteria, grown on either non-selective or selective solid culture medium, was spotted onto the test strip with a 10 μl plastic loop.
2. The strip was observed for a colour change around the inoculated region occurring within one minute.

Results:
Positive: Development of a purple colour.
Negative: The inoculated region remains colourless.

Controls:
Positive: *C. jejuni* NCTC 11351; Negative: *E. coli* NCTC 9001.

2.6.4 Catalase

Two molecules of H$_2$O$_2$ are converted to two molecules of H$_2$O and one molecule of O$_2$ in the presence of the bacterial enzymes catalase or superoxide dismutase. This test is useful for differentiation of certain *Campylobacter* species.

Required:
- 3% (v/v) hydrogen peroxide (H$_2$O$_2$) solution [BDH], stored at 4°C
- Plastic Petri dish [Sterilin]
- 10 μl disposable loops

Protocol:

1. A 24 to 48 hour culture of the test bacteria, grown on either non-selective or selective solid culture medium, was spotted onto the base of an empty Petri dish with a 10 μl plastic loop.
2. A drop of 3% H$_2$O$_2$ was placed over the bacteria and the Petri dish lid was replaced immediately to avoid aerosols.

Results:
Positive: Immediate vigorous production of gas bubbles.
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Weak positive: A few gas bubbles produced (allow 5 minutes before recording a negative result).

Negative: No bubbles formed.

Controls:
Positive: *C. jejuni* NCTC 11351; Weak positive: *C. upsaliensis* NCTC 11541; Negative: *C. concisus* NCTC 11485.

2.6.5 Hippurate hydrolysis

A positive result with this test is characteristic of *C. jejuni*.

Required:

- 1% (w/v) Sodium hippurate solution.
  - Sodium hippurate [BDH] 100 mg
  - SDW. 100 ml
  The solution was filter sterilized and stored at 4°C.

- 3.5% (w/v) Ninhydrin solution.
  - Ninhydrin [BDH] 3.5 g
  - Acetone 50 ml
  - Butanol [BDH] 50 ml
  The solution was filter sterilized and stored at 4°C.

- 10 μl disposable loops

Protocol:

1. 0.4 ml of 1% sodium hippurate was dispensed into a sterile plugged glass tube.

2. A milky suspension of a 24 to 48 hour culture was prepared in 1.0 ml SDW.
3. The 0.4 ml 1% sodium hippurate was mixed with 0.8 ml bacterial suspension and incubated at 37°C for four hours, with occasional shaking.

4. 0.2 ml ninhydrin solution was added, slowly down the side of the tube. (Avoiding mixing or shaking as this may lead to false positives).

Results:
Positive: Development of a deep purple colour within 10 minutes.
Negative: A pale purple or colourless tube.

Controls:
Positive: C. jejuni NCTC 11351; Negative: C. coli NCTC 11366.

2.6.6 Indoxyl acetate hydrolysis

This test is useful for differentiation of certain Campylobacter species.

Required:
- Paper discs impregnated with 10% (w/v) indoxyl acetate.
  - 100 mg of indoxyl acetate [BDH] were dissolved in 1 ml acetone. 50 μl of this solution was applied to a 6 mm diameter 3MM filter paper disc [Whatman] and allowed to air dry at room temperature prior to use. The discs were stored at 4°C in a foil wrapped universal containing a desiccant, prior to use.
- 10 μl disposable loops

Protocol:
1. An indoxyl acetate impregnated disc was placed in the base of an empty Petri dish.
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2. A 24 to 48 hour culture of the test bacteria was spotted onto the disc with a 10 \( \mu l \) plastic loop.

3. The disc was then moistened with a drop of SDW and incubated at room temperature for ten minutes.

Results:

Positive: Appearance of a blue/grey colour within 10 minutes.

Negative: No colour.

Controls:

Positive: C. jejuni NCTC 11351; Negative: C. lari NCTC 11352.

2.6.7 Hydrogen sulphide production

This test is useful for differentiation of certain Campylobacter species, notably C. hyointestinalis from C. fetus.

Required:

- Triple Sugar Iron (TSI) agar slope [Oxoid]
- 1 \( \mu l \) disposable loops strips [Technical Service Consultants Ltd.]

Protocol:

1. A 24 to 48 hour culture of the test bacteria was used to inoculate a TSI agar slope by stabbing the butt and streaking the slope with a 1 \( \mu l \) disposable loop.

2. The inoculated slope was incubated microaerobically and observed daily for up to seven days.

Results:

Positive: Blackening of the butt or the area around the stab.
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Negative: The slope remains unaltered or blackening of the slope surface alone occurs.

Controls:
Positive: *C. hyointestinalis* NCTC 11608; Negative: *C. fetus* subsp *fetus* NCTC 10842

2.6.8 Growth at 25°C and 42°C

Cultures were inoculated onto CBA plates, which were incubated in a microaerobic atmosphere (see section 2.1.2) generated in a gas jar and gas pack [Oxoid]. Jars containing plates were placed in incubators [LEEC] maintained at either 25°C or 42°C for 48 hours, when they were examined for bacterial growth.

Controls:
25°C: Positive: *C. fetus* subsp *fetus* NCTC 10842; Negative: *C. jejuni* NCTC 11351.
42°C: Positive: *C. jejuni* NCTC 11351; Negative: *C. fetus* subsp *fetus* NCTC 10842.

2.7 Extraction of bacterial nucleic acid

The preparation of pure, high molecular mass DNA is essential for analysis of bacteria by molecular techniques. DNA was extracted from pure bacterial cultures by harvesting a rice grain sized clump of cells from a plate culture and resuspending it in 100 µl TE buffer. For DNA extraction directly from faeces, 1 g of faecal sample was homogenised in 9 ml of BB and a 100 µl aliquot of this suspension used.
2.7.1 Phenol-chloroform method

This phenol-chloroform DNA extraction methodology was devised specifically for Gram-negative bacteria (Wilson, 1987). It features the addition of cetyltrimethylammonium bromide (CTAB), a cationic detergent, which precipitates polysaccharides and residual proteins.

Required:

- Tris-EDTA (TE) buffer: pH 8.0; stored at 4°C.
  - Tris HCl 0.1 M pH 8.0 [Severn Biotechnologies] 100 ml
  - EDTA 0.2 M pH 8.0 [Life Technologies] 5.0 ml
  - Make up to 1000 ml in SDW.
- 10% (aqueous) Sodium Dodecyl Sulphate (SDS) [BDH], stored at 37°C.
- Proteinase K, 20 mg/ml [Sigma], stored in 500 µl aliquots at -20°C.
- NaCl 5.0 M, stored at room temperature.
  - NaCl, Analar grade [BDH] 29.22 g
  - Make up to 100 ml in SDW
- CTAB/NaCl (10% w/v CTAB in 0.7 M NaCl) solution, stored at room temperature.
  - NaCl 4.1 g
  - CTAB [BDH] 10 g
  - The NaCl was dissolved in 80 ml SDW heated to 65°C. The CTAB was added gradually whilst stirring continuously. The final volume was adjusted to 100 ml.
- Chloroform:iso-amyl alcohol (24:1) [Fluka], stored at 4°C.
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- Phenol:chloroform:iso-amyl alcohol (25:24:1) [Fluka], stored at 4°C.
- Iso-propanol, Analar grade [BDH], stored at 4°C.
- 70% ethanol, Analar grade [BDH], stored at 4°C.

Protocol:

1. To each 100 μl sample (see section 2.3), 30 μl of 10% SDS and 3 μl proteinase K was added (to give a final concentration of 100 μg/ml proteinase K in 0.5% SDS). The sample was vortex mixed thoroughly, and incubated at 37°C for one hour.

2. 100 μl of 5 M NaCl was added and mixed thoroughly.

3. To this 80 μl of CTAB/NaCl solution was added and the tubes vortexed thoroughly. The sample was incubated at 65°C for 1 hour. At NaCl concentrations greater than 0.5M CTAB complexes with cell wall debris, denatured protein and polysaccharides while the nucleic acids are retained in solution.

4. An approximately equal volume of chloroform reagent was added and the suspension mixed thoroughly and centrifuged at 13000 rpm for five minutes. (The CTAB-protein-polysaccharide complexes are removed by emulsification and extraction with chloroform reagent. A white interface should be visible after centrifugation).

5. The aqueous, viscous supernatant was removed to a clean Eppendorf tube, leaving the interface behind.

6. An equal volume of phenol-chloroform reagent was added to the supernatant, mixed well and centrifuged at 13,000 rpm for 5 minutes. (Any remaining CTAB precipitate is removed).
7. The supernatant was again removed to a clean Eppendorf tube, to which iso-propanol equivalent to 0.6 volumes of supernatant was added. The tube was shaken gently until a stringy white DNA precipitate was visible.

8. The tube was centrifuged at 5000 g for two minutes and the iso-propanol removed.

9. The DNA was washed with 70% ethanol to remove residual CTAB and centrifuged once more at 5000 g for two minutes.

10. The pellet was briefly dried in a lyophilizer.

11. The pellet was redissolved in 100 µl TE buffer and stored at -20°C for later use.

2.7.2 Guanidine-chloroform nucleic acid extraction

This rapid, phenol-free method employs the chaotropic agent Guanidine thiocyanate (GuSCN) to disrupt proteins and inactive nucleases (Pitcher et al., 1989).

Required:

- TE buffer

- Guanidine/EDTA/Sarkosyl (GES) reagent
  - GuSCN [Fluka] 60 g
  - EDTA 0.5M pH 8.0 [Life Technologies] 20 ml
  - 5% (v/v) sarkosyl [BDH] 5 ml
  - SDW 100 ml

The GuSCN and EDTA were mixed well and dissolved in 20 ml SDW, heated to 65°C in an extraction hood (in certain conditions
GuSCN may liberate cyanide). The mixture was allowed to cool. The sarkosyl was added and the total volume made up to 100 ml with SDW. The reagent was filter sterilized and stored at room temperature.

- Ammonium acetate 7.5 M, stored at room temperature.
  - Ammonium acetate, Analar grade [BDH] 77 g
    Made up to 100 ml in SDW.
- Chloroform:iso-amyl alcohol (24:1)
- Iso-propanol
- 70% ethanol

Protocol:

1. 500 µl of GES was added to each 100 µl sample, which was then vortexed briefly. Check for lysis, which should occur in 5 to 10 minutes.

2. The lysed sample was cooled on ice. To this 250 µl ice cold ammonium acetate was added and mixed gently for 10 minutes.

3. 500µl chloroform was added and the phases mixed thoroughly by inversion. The emulsion was then centrifuged at 12000 g for 10 minutes.

4. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube using a wide bore pipette (to avoid shearing the DNA).

5. To this 0.6 volumes of ice cold iso-propanol was added and mixed by inversion.

6. The DNA precipitate was pelleted by centrifugation at 6500 g for 20 seconds. (GES contains GuSCN, which liberates cyanide in acid
conditions. Discarded GES solution was collected in a HAZ-CHEM bottle containing 10 M NaOH).

7. The pellets of DNA were washed five times in 70% ethanol.

8. The pellet was briefly dried in a lyophilizer

9. The DNA pellet was redissolved in 100 μl TE buffer and stored at -20°C for later use.

2.7.3 Guanidine-silica

This rapid method also relies on the chaotropic properties of GuSCN, together with the nucleic acid-binding properties of silica, in the form of celite (the fossilized cell walls of diatoms). Although this method may give rise to significant shearing of purified DNA, it still provides a suitable substrate for PCR analysis (Boom et al., 1990).

Required:

- TE buffer
- Diatom suspension (DS)
  - Celite (diatom powder, analytical grade filter agent) [Sigma] 10 g
  - HCl conc (32% w/v) [BDH] 500 μl
  - SDW 50 ml
  - Mix well and divide into 1 ml aliquots and autoclave (121°C for 20 minutes). Store at room temperature.
- Lysis buffer (LB)
  - GuSCN 120 g
  - Tris HCl 0.1 M, pH 6.4 [Severn Biotechnologies] 100 ml
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- EDTA 0.2 M, pH 8.0 22 ml
- Triton X-100 [BDH] 2.6 g
  - Mix well and dissolve by warming in an extraction hood. Store at room temperature.

• Wash buffer (WB)
  - GuSCN 120 g
  - Tris HCl 0.1 M, pH 6.4 100 ml
  - Mix well and dissolve by warming in an extraction hood. Store at room temperature.

• 70% Ethanol

• Acetone

Protocol:

1. The DS suspension was vortexed until all the particles were completely resuspended. 40 μl of DS and 900 μl of LB were added to a 1.5 ml Eppendorf tube, and vortexed briefly.

2. The 100 μl sample was added to the tube, vortexed for five seconds, incubated at room temperature for 10 minutes, then vortexed once more and centrifuged at 11600 g for 15 seconds.

3. The supernatant was carefully removed and the pellet washed five times as follows:
   - Washes 1 and 2: WB
   - Washes 3 and 4: 70% ethanol
   - Wash 5: acetone
(One ml of each wash was added, vortexed for 5 seconds and then centrifuged at 11600g for 15 seconds. The supernatant was removed and the next wash added. Wash steps 2 and 4 may be omitted depending on the substrate. LB and WB contain GuSCN, which liberates cyanide in acid conditions (see section 2.7.2)

4. After the last wash the pellet was dried at 60°C for 10 minutes.

5. 100 µl TE buffer was added to resuspend the DNA, mixed by vortexing and incubated at 60°C for 10 minutes.

6. The pellet was vortexed until completely resuspended then centrifuged at 11600 g for two minutes.

7. The resuspended nucleic acid was stored at -20°C.

2.8 Removal of inhibitory substances

Faecal samples contain numerous substances inhibitory to PCR, some of these are co-purified with the DNA in the extraction procedures described in section 2.7 (Widjojoatmodjo et al., 1992). The following techniques were employed to reduce this inhibition.

2.8.1 Dilution

The inhibitory effect may be diminished by dilution. However, available target DNA is also reduced.

Required:

- SDW or TE buffer
- Eppendorf tubes
Materials and Methods

Protocol:

1. Nucleic acid extracts were decimally diluted in either SDW or TE buffer to create dilutions of $10^{-1}$, $10^{-2}$ and $10^{-3}$.

2.8.2 Polyvinyl Pyrrolidone (PVP) treatment

PVP incorporated in agarose gels has been used to electrophoretically purify DNA extracted from soil samples (Young et al., 1993). It is thought that PVP 'removes phenolic components' often co-purified with DNA. During the course of this study, it was found that a simple wash step with a buffer containing PVP was effective in removing some inhibitory components present in DNA extracted from faeces (Lawson et al., 1997).

Required:

- PVP/TE solution (10% w/v)
  - PVP [BDH] 10 g
  - TE buffer 100 ml
    Mix well, store at room temperature.
- Ammonium Acetate 7.5 M
- Iso-propanol
- TE buffer

Protocol:

1. A 50 μl aliquot of DNA extracted from a faecal sample was added to 150 μl PVP/TE in a 1.5 ml Eppendorf tube.

2. This was vortexed for five seconds, incubated at room temperature for 10 minutes, and vortexed once more for five seconds.
3. 100 µl of ammonium acetate was added and mixed for five seconds.
4. 600 µl of ISO-propanol was added and mixed for five seconds.
5. The preparation was stored at -20°C for at least 20 minutes.
6. Centrifuge for 10 minutes at 11600 g, discard the supernatant and vacuum dry.
7. The DNA was then reconstituted in 50 µl TE buffer.

2.9 Polymerase chain reaction (PCR)
PCR is a simple, fast and sensitive method of amplifying a segment of target DNA lying between two regions of known sequence, using specific oligonucleotide primers and a thermostable DNA polymerase (see section 1.17). Dedicated equipment and clean preparation areas were used to avoid the risk of contamination.

Required:

- Primers [Cruachem], (see section 2.10) diluted to 100 µM stock solution and 20 µM working concentration.
- Deoxyribonucleotide triphosphates (dNTP) 10 mM [Life Technologies]
  Bases supplied as separate 100 mM solutions:
  - 2'-deoxyadenosine 5'-triphosphate (dATP): 10 µl
  - 2'-deoxycytidine 5'-triphosphate (dCTP): 10 µl
  - 2'-deoxyguanosine 5'-triphosphate (dGTP): 10 µl
  - 2'-deoxythymidine 5'-triphosphate (dTTP): 10 µl
  - SDW 60 µl
- *Thermus aquaticus* (Taq) DNA polymerase [Life Technologies]
• 10x *Taq* polymerase buffer [Life Technologies]
• MgCl₂ 50 mM [Life Technologies]
• Nuclease-free SDW [Promega]
• Robocycler Gradient 96 Hot Top Thermal Cycler [Stratagene]
• PCR reaction tubes, either:
  o 200 μl Thermo-Fast PCR tubes [ABgene]
  o 96 well Thermo-Fast microtitre plate [ABgene]

Protocol:

1. All reactions were prepared in a 'clean room' using dedicated micropipettes and reagents to avoid contamination with DNA.

2. A 'master mix' was made composed of the following multiplied by the number of samples and controls to be examined:

  o 10x *Taq* polymerase buffer 2.5 μl
  o MgCl₂ 50 mM 1.25 μl
  o dNTPs 10 mM 0.5 μl
  o Primers 20 μM 0.5 μl of each
  o *Taq* polymerase 0.125 units
  o DNA sample 2.5 μl
  o Nuclease free SDW to a final volume of 25.0 μl

DNA samples were either faecal extracts or extracts from control strains, in which case the total amount of DNA examined was diluted to a final total of 50 to 100 ng. At least one appropriate positive and negative (SDW) control were included in each run.
3. The samples were amplified using the Robocycler Gradient 96 Hot Top Thermal Cycler (thus a mineral oil overlay was not necessary). Optimum amplification conditions were determined empirically using the 12°C thermal gradient on the Robocycler. Unless otherwise stated, the following set of standard PCR parameters were used:

- Denaturation temperature 94°C for 1 minute
- Variable annealing temperature (see section 2.10) for 1 minute
- Extension temperature 72°C for 1 minute
- For a total of 30 cycles.
- Samples were held at 4°C.

4. PCR reactions were stored at -20°C and examined using agarose gel electrophoresis (see section 2.11).

### 2.10 PCR assays

The following PCR assays were used in the course of this study. The reaction conditions listed in section 2.9 were used together with the specific annealing temperatures (T_A) and primer combinations given in Tables 2.1 to 2.8 below. Primer names ending F and R are forward and reverse primers respectively.

#### 2.10.1 Assays using 16S rDNA-based PCR

16S rDNA is an ideal target for PCR-based identification assays: it is present in all living bacterial cells, sequence variation forms the basis of phylogenetic classification, and it is often a multi-copy gene (three copies in *Campylobacter*) sensitivity will be increased (see section 1.18).
2.10.1.1 Genus-specific PCR

Two *Campylobacter* genus-specific PCR assays were used cgen (Linton et al., 1996) and cgena (Lawson et al., 1998a). The latter was designed during the course of this study.

| Table 2.1 *Campylobacter* genus-specific 16S rDNA-based PCR primers |
|-------------------------|----------------------|-----------------|-----------------|
| Name | Sequence (5' → 3') | T<sub>A</sub> | Product size |
| cgen | CGF | GGA TGA CAC TTT TCG GAG C | 55°C | 816 bp |
| | CGR | CAT TGT AGC ACG TGT GTC | | |
| cgena | CGAF | TTG ATC CTG GCT CAG AGT | 60°C | 1495 bp |
| | CGAR | TTC ACC CCA GTC GCT GAT | | |

2.10.1.2 Group-specific PCR

Primers specific for a group of *Campylobacter* species were developed during the course of this study (Lawson et al., 1999). These primers co-detected, but did not distinguish, *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. helveticus*.

| Table 2.2 *Campylobacter* group-specific 16S rDNA-based PCR primers |
|-------------------------|----------------------|-----------------|-----------------|
| Name | Sequence (5' → 3') | T<sub>A</sub> | Product size |
| cpg | PGF | ACA TGC AAG TCG AAC GAT GAA GC | 68°C | 1195 bp |
| | PGR | TAT AGA TTT GCT CCA CCT CGG | | |
2.10.1.3 Species-specific PCR

These primers were capable of distinguishing *Campylobacter* 16S rDNA sequence variation at the species level. The *cjcc* PCR assay co-detected but did not differentiate *C. jejuni* and *C. coli* (Linton et al., 1997). The *C. lari* (*cla*),

| **Table 2.3 Campylobacter species-specific 16S rDNA-based PCR primers** |
|---|---|---|---|
| **Target species** | **Sequence (5' → 3')** | **T<sub>A</sub>** | **Product size** |
| *C. jejuni/C. coli* (*cjcc*) | CCCJF: AAT CTA ATG GCT TAA CCA TTA  
CCCJR: GTA ACT AGT TTA GTA TTC CGG | 58°C | 854 bp |
| *C. lari* (*cla*) | CLF: CAA GTC TCT TGT GAA ATC CAA C  
CLR: ATT TAG AGT GCT CAC CCG AAG | 64°C | 561 bp |
| *C. upsaliensis* & *C. helveticus* (*chcu*) | CHCUF: GGG ACA ACA CTT AGA AAT GAG  
CUR: CAC TTC CGT ATC TCT ACA GA  
CHR: CCG TGA CAT GGC TGA TTC AC | 60°C or 65°C | 878 bp or 997 bp or 1225 bp |
| *C. fetus* & *C. hyointestinalis* (*chycf*) | CFCHF: GCA AGT CGA ACG GAG TAT TA  
CFR: GCA GCA CCT GTC TCA ACT  
CHR: GCG ATT CCG GCT TCA TGC TC | 65°C or 60°C | 997 bp or 598 bp |
| *C. rectus* (*rec*) | CRF: TTT CGG AGC GTA AAC TCC TTT TC  
CRR: TTT CTG CAA GCA GAC ACT CTT | 60°C | 598 bp |
| *C. hominis* (*hs*) | HSF: GCT AAT CTG CCT CTT AGT AGA  
HSR: CTG TGG AGG GTA GCA AAT TTT | 60°C | 1356 bp |
C. upsaliensis and C. helveticus (chcu), and C. fetus and C. hyointestinalis (chycf) PCR's were designed for the rapid identification of Campylobacter species enteropathogenic for man and animals (Linton, et al., 1996). The chcu and the chycf PCR's consisted of a common forward primer and two species-specific reverse primers, yielding products of different sizes. The C. rectus - specific (rec) PCR was designed to investigate the causes of gingivitis (Ashimoto et al., 1996), while the C. hominis-specific (hs) PCR was designed during the course of this study (Lawson, et al., 1998a).

2.10.2 Campylobacter species-specific 23S rDNA-based PCR

The following species-specific PCR assays were designed from 23S rDNA sequence data to identify C. concisus (con) (Bastyns et al., 1995) and C. sputorum (spt) (Bastyns et al., 1994). The C. concisus-specific PCR used a single forward primer and two reverse primers to take account of sequence variation in this species.

**Table 2.4 Campylobacter species-specific 23S rDNA-based PCR primers**

<table>
<thead>
<tr>
<th>Target species</th>
<th>Sequence (5’ → 3’)</th>
<th>T_A</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. concisus (con)</td>
<td>MUC1 ATG AGT AGC GAT AAT TGG G CAG TAT CGG CAA TTC GCT GAC AGT ATC AAG GAT TTA CG</td>
<td>60°C</td>
<td>306 bp</td>
</tr>
<tr>
<td>C. sputorum (spt)</td>
<td>SPUT1 ATA AGT ACC GAA GTC GTA GG TCT AGG GCT TTA ACA CCC</td>
<td>58°C</td>
<td>588 bp</td>
</tr>
</tbody>
</table>
2.10.3 PCR based on the flaA gene

The PG50 and RAA19 primers were originally developed to sequence C. jejuni flagellin genes (Alm et al., 1993). Sequence variation (polymorphism) between flagellin genes of different strains of C. jejuni and C. coli provide the basis of Campylobacter subtyping schemes (see sections 1.6.3 and 2.14). During the course of this study a modified primer PG50 (PG50A) was developed to optimise the primer pair for use in PCR applied to DNA extracted directly from clinical faecal samples (Linton, et al., 1997).

Table 2.5 Campylobacter flaA gene-base PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
<th>$T_A$</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG50 or PG50A</td>
<td>ATG GGA TTT CGT ATT AAC</td>
<td>60°C</td>
<td>1500 bp</td>
</tr>
<tr>
<td>RAA19</td>
<td>ATG GGA TTT CGT ATT AAC AC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCA CCY TTA AGW GTR GTT ACA CCT GC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Where Y = C or T, W = A or T, and R = A or G

2.10.4 PCR based on the hip gene

The ability to hydrolyse hippurate is characteristic of C. jejuni (see section 2.6.5). The gene encoding the enzyme responsible for this (benzoylglycine amidohydrolase) has been sequenced (Hani & Chan, 1995) and a C. jejuni-specific PCR assay (hip) designed from the data (Linton, et al., 1997).
Table 2.6 *C. jejuni*-specific *hip* gene-based PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
<th>$T_A$</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIPF</td>
<td>GAA GAG GGT TTG GGT GGT G</td>
<td>66°C</td>
<td>735 bp</td>
</tr>
<tr>
<td>HIPR</td>
<td>AGC TAG CTT CGC ATA ATA ACT TG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.10.5 PCR based on the *aspK* gene

This PCR assay (asp) was based on a cloned genomic DNA sequence specific for *C. coli* and putatively identified as an aspartokinase (*aspK*) gene (Linton, et al., 1997).

Table 2.7 *C. coli*-specific *aspK* gene-based PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
<th>$T_A$</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCF</td>
<td>GGT ATG ATT TCT ACA AAG CGA G</td>
<td>60°C</td>
<td>500 bp</td>
</tr>
<tr>
<td>CCR</td>
<td>ATA AAA GAC TAT CGT CGC GTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.10.6 PCR based on the *ceuE* gene

These primers were developed from sequence data of a gene encoding a protein involved in siderophore transport (*ceuE*) in *Campylobacter*. The nucleotide sequence divergence of approximately 13% in the *ceuE* genes of *C. jejuni* and *C. coli* facilitated the design of two species-specific PCR assays (Gonzalez et al., 1997).
Table 2.8 C. jejuni and C. coli-specific ceuE gene-based PCR primers

<table>
<thead>
<tr>
<th>Target species</th>
<th>Sequence (5' → 3')</th>
<th>T_A</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni</td>
<td>JEJ1 CCT GCT ACG GTG AAA GTT TTG C</td>
<td>57°C</td>
<td>793 bp</td>
</tr>
<tr>
<td></td>
<td>JEJ2 GAT CTT TTT GTT TTG TGC TGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. coli</td>
<td>COL1 ATG AAA AAA TAT TTA GTT TTT GCA</td>
<td>57°C</td>
<td>894 bp</td>
</tr>
<tr>
<td></td>
<td>COL2 ATT TTA TTA TTT GTA GCA GCG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.11 Agarose gel electrophoresis

DNA amplicons and fragments were separated and visualised by electrophoresis through agarose gels. At neutral pH the DNA molecule has a net negative charge. When placed in an electrical field DNA will migrate towards the anode (+). The rate at which this migration occurs is dependent on the applied voltage, the salt concentration of the buffer, the pore size of the agarose gel and the size and conformation of the DNA molecule. Smaller DNA molecules will migrate more quickly than larger ones. Agarose gel electrophoresis may be used to examine DNA molecules of between 100 and 25000 bp. The gel is stained with an intercalating dye to allow the DNA molecules to be visualised with an UV light source.

Required:

- Electrophoresis equipment [Life Technologies]
- Camera and 667 or 55 instant sheet film [Polaroid]
- UV Transilluminator (254nm) [Upland]
- 0.5 x TBE buffer
Materials and Methods

- **10 x TBE buffer (1.0 M Tris, 0.9 M Boric acid, 0.01 M EDTA)**
  - [Life Technologies] 50 ml
  - made up to 1000 ml with SDW

- **Loading buffer**
  - 1 % (w/v) bromophenol blue [BDH] 2.5 ml
  - Ficoll [Sigma] 2.5 g
  - 0.5 M EDTA (pH 8.0) 10 ml
  - Made up to 10 ml with SDW

- **Agarose [Life Technologies]**

- **Molecular markers:**
  - Marker λ. Bst E1 digest [Life Technologies]
  - BioMarker Ext 50 to 2000 bp DNA molecular weight marker [Cambio]

- **DNA stains:**
  - Ethidium bromide solution [Life Technologies]
  - SYBR Green I DNA gel stain [Flowgen]

**Protocol:**

1. A 1.5 % (w/v) agarose gel in 0.5 x TBE buffer was prepared by heating in a microwave oven until melted. This was cooled to 55°C in a water bath and poured.

2. The gel was transferred to the electrophoresis tank, the well-forming comb removed and 0.5x TBE buffer added until the gel was covered to a depth of 1 mm.

3. The DNA sample was mixed 1:10 with loading buffer and carefully pipetted into the wells and a voltage applied.
4. Following electrophoresis the gel was stained in either ethidium bromide (1 μl ml⁻¹) for 30 minutes or SYBR Green I (10 μl ml⁻¹) for 15 minutes.

5. The gel was viewed under UV light on a transilluminator and photographed.

2.12 Estimation of DNA concentration

The DNA concentration was measured by taking absorbance readings on a spectrophotometer. The peak absorption of pure DNA occurs with light of a wavelength of 260 nm, such that an optical density (OD) of one is equivalent to 50 μg ml⁻¹ of double stranded DNA (Sambrook et al., 1989). Co-purified contaminants such as proteins and low molecular weight components (RNA's and carbohydrate) have a peak absorbance at 280 nm and 230 nm respectively. The relative purity of a DNA sample can therefore, be determined by the ratio of the absorbance at 260 nm to the absorbances at 280 nm and 230 nm

Required:

- SP1800 UV spectrophotometer [Unicam]
- Disposable cuvettes [Sigma]
- TE Buffer or SDW

Protocol:

1. Switch on spectrophotometer and allow to warm up

2. Take a blank reading with a cuvette containing 500 μl SDW
3. Add 10 μl of DNA sample to 490 μl SDW in a cuvette and read absorbance at 260 nm. DNA estimation is most accurate in the range 0.5 to 1.0 OD, thus the dilution of each sample was adjusted to fall within this range.

4. Absorbances were also measured at 230 nm and 280 nm. The ratio A260/A280 should fall between 1.8 and 1.9, ratios below 1.7 indicate significant protein contamination. The ratio A260/A230 should be ≥ 2.0, lower ratios indicate contamination by polysaccharide.

5. DNA concentration was determined as follows:

An OD of 1 at 260 nm is equivalent to 50 μg ml⁻¹

Thus: A260 x dilution factor x 50/1000 = DNA μg ml⁻¹

### 2.13 Purification of PCR-generated amplicons

In certain experiments PCR amplicons were used as probes in hybridisation experiments (section 2.17) or were sequenced (section 2.19). In these cases it was necessary to remove unincorporated primers and nucleotides from the amplicons. This was achieved using the commercially available 'GeneClean' kit, which consists of a silica matrix that binds DNA fragments larger than 500 bp in size.

**Required:**

- GeneClean II kit [Bio 101 Inc.] consisting of:
  
  - 'TBE modifier'
  
  - Nal 6.0 M
  
  - 'Glass milk': Silica matrix in water.
Materials and Methods

1. 'New wash': A solution of NaCl, Tris and EDTA in water and ethanol

Protocol:

1. The PCR amplicon was electrophoresed on an agarose gel stained and viewed on a UV transilluminator (as described in section 2.11). The required DNA band was excised and placed in a 1.5 ml Eppendorf tube.

2. The volume of the agarose slice was approximated by weight (1 g approximates to 1 ml).

3. 0.5 volume of 'TBE modifier' and 4.5 volumes of NaI were added to the agarose slice, and the tube incubated at 55°C until the agarose was completely dissolved.

4. The 'Glass milk' was vortexed and 10 µl added to each tube. The tubes were vortexed and kept on ice for 10 minutes with occasional mixing.

5. The tubes were centrifuged for five seconds and the supernatant removed and kept to one side in case the DNA was not bound to the silica matrix.

6. 700 µl of ‘New wash’ solution was added to the pellet. The tubes were vortexed, and centrifuged for five seconds, and the supernatant discarded. This step was repeated twice.

7. The pellet was resuspended in 50 µl SDW and incubated at 55°C for three minutes. The tubes were centrifuged for 30 seconds, and the DNA containing supernatant transferred to a clean Eppendorf tube.
6. A 5 μl aliquot of the DNA sample was electrophoresed (see section 2.11) to check the purity.

2.14 Phylogenetic analysis and primer design

16S rDNA sequence data was obtained either from the European Molecular Biology Laboratory (EMBL) sequence database (Baker et al., 2000) or by sequencing 16S rDNA PCR amplicons (see section 2.19).

For phylogenetic analysis, 16S rDNA sequences of E. coli, H. pylori, A. skirrowii, B. ureolyticus, and the remaining 15 Campylobacter species were aligned by the clustal W method (Thompson et al., 1994) using Megalign (part of the Lasergene suite of programs [DNAstar]). The 16S rDNA reference sequences used, and the resulting alignments are listed in appendices A and B. Newly sequenced 16S rDNA data was added to the reference alignment for comparison. Regions of uncertain sequence designation were removed from the alignment and the edited data used as input for phylogenetic analysis, using the neighbour-joining method (Saitou & Nei, 1987) as implemented by the Treecon package (van de Peer & de Wachter, 1993). Data were corrected for multiple base changes using the method of Jukes & Cantor (Jukes & Cantor, 1969), and bootstrap analysis was performed with 1000 re-sampled data sets. The E.coli sequence was used to root the resulting phylogenetic trees.
Materials and Methods

Novel primers were created using the alignments generated with the Megalign software together with the Primer 3 internet design tool (Rozen & Skaletsky, 1997).

2.15 PCR-RFLP flagellin gene subtyping

The flagellin gene (flaA) of C. jejuni and C. coli was amplified using the PCR primers described in section 2.10.3. The amplified product, of approximately 1500 bp was double-digested with the enzymes Pst I and Eco RI (Alm, et al., 1993). Sequence differences (polymorphisms) resulting in altered restriction sites lead to different restriction patterns. This is referred to as Restriction Fragment Length Polymorphism (RFLP). These patterns provide a basis for 'finger printing' strains within a species.

Required:

- Restriction endonuclease Pst I [Life Technologies]
- Restriction endonuclease Eco RI [Life Technologies]
- Reaction buffer (React 2) [Life Technologies]
- Spermidine [Sigma]
- 0.5x TBE
- 3% NuSieve agarose gel [FMC Bioproducts]
- Loading buffer (see section 2.11)
- Molecular marker (see section 2.11)
- 37°C water bath [Grant]
- Electrophoresis equipment
- Polaroid camera and film
Materials and Methods

Protocol:

1. The following reagents were added to a sterile 500 μl Eppendorf tube:
   - flaA PCR amplicon: 13 μl
   - 10 x restriction buffer (React 2): 2 μl
   - Spermidine: 1 μl
   - Pst I: 2 μl
   - Eco RI: 2 μl

2. The tubes were incubated in a 37°C water bath overnight. The reaction was stopped by the addition of 4 μl of loading buffer.

3. A 3% gel prepared using Nusieve agarose was poured and allowed to set.

4. 15 μl of the digested amplicon and 5 μl of the molecular marker were loaded into the well and electrophoresed at 100 v for 3 hours

5. The gel was stained in ethidium bromide solution and viewed under UV light (section 2.11).

2.16 DNA Blotting

For hybridisation-based experiments DNA was fixed to nylon membranes by the following methodologies:

2.16.1 Vacuum Southern blotting

DNA fragments were depurinated, denatured and transferred from agarose gels to nylon membranes by a modification of the method of Southern
Materials and Methods

(Southern, 1975). The single-stranded DNA was fixed to the membrane by UV cross-linking.

Required

- 1 x Saline Sodium Citrate (SSC)
  - 20x SSC (3.0 M NaCl, 0.3 M Sodium citrate) [Life Technologies]
    - 50 ml
  - Distilled water 950 ml

- Loading buffer (see section 2.11)

- Depurinating solution (0.25 M HCl)
  - HCl (concentrated) 21.5 ml
  - Make up to 1000 ml with distilled water

- Denaturing solution (0.5 M NaOH, 1.5 M NaCl)
  - NaOH [BDH] 20 g
  - NaCl 87.66 g
  - Make up to 1000 ml with distilled water

- Neutralising solution (1.5 M NaCl, 0.5 M Tris, 10 mM EDTA)
  - NaCl 87.66 g
  - Tris [BDH] 60.84 g
  - EDTA [BDH] 0.37 g
  - Make up to 1000 ml with distilled water

- Vacu-Gene blotting apparatus [Pharmacia]

- Hybond-N, nylon blotting membrane, 0.45 µm [Amersham]

- UV Stratalinker 1800 [Stratagene]
Protocol:

1. A portion of nylon membrane slightly larger than the gel was cut out, pre-wet in 1 x SSC and placed on the porous bed of the blotting apparatus.

2. A polythene gasket was placed around the membrane to restrict the area of suction to that of the filter. The gel was placed on top of the filter and 10 μl of loading buffer added to each well of the gel.

3. The gel was covered with depurinating solution and a vacuum of 20 to 35 KPa was applied.

4. After 30 minutes excess solution was gently removed with absorbent paper and replaced with denaturing solution for a further 30 minutes.

5. The denaturing solution was replaced with neutralising solution and left for one hour.

6. The vacuum was turned off and the remaining neutralising solution removed. The gel was re-stained with ethidium bromide to confirm the transfer of the DNA to the membrane.

7. DNA was cross-linked to the membrane in a UV Stratalinker 1800.

2.16.2 DNA Slot-blotting

Single stranded DNA can be directly bound to nylon membrane without prior electrophoresis using slot-blotting apparatus.

Required:

- 20 x SSC
- 10 x SSC
- Hybond-N, nylon blotting membrane, 0.45 μm
Materials and Methods

- Sonicator [Heat Systems]
- Slot-blot vacuum-blotting apparatus [Bethesda Research Laboratories]
- UV Stratalinker 1800

Protocol:

1. Genomic DNA samples were prepared from log phase cultures.
2. These were diluted in water to a concentration of 0.2 µg µl⁻¹ and an equal volume of cold 20 x SSC added.
3. The diluted samples were sonicated for three minutes to give an average fragment length of 1 kb.
4. Samples were denatured, by boiling in a water bath for 10 minutes, and then snap cooled on ice.
5. A piece of Hybond-N membrane was cut to size, pre-wet in 10 x SSC and placed in the slot-blot apparatus.
6. A vacuum of between 20 to 30 KPa was applied and the slots washed through twice with 200 µl of 10 x SSC.
7. 40 µl (4 µg) of sonicated single stranded DNA was applied and washed through twice with 200 µl of 10x SSC.
8. DNA was cross-linked to the membrane in a UV Stratalinker 1800.

2.17 Labelling, hybridisation and detection of DNA probes

2.17.1 Digoxigenin (DIG) labelling of DNA probes

DNA probes were generated using random sequence hexamers to prime DNA synthesis from template DNA by the Klenow fragment of DNA
polymerase I which retains 5'→ 3' polymerase activity, but lacks 5'→ 3' exonuclease activity. This procedure is called random-primed labelling.

Required:

- DIG-High Prime [Roche] consisting of:
  - Random hexamers, 1 mM
  - dATP 1 mM
  - dCTP 1 mM
  - dGTP 1 mM
  - dTTP 0.65 mM
  - Alkali-labile-digoxigenin-11-dUTP 0.35 mM
  - Klenow polymerase 1Uµl⁻¹
  - 5 x reaction buffer

- EDTA 200 mM (pH 8.0)

- SDW

Protocol:

1. An aliquot of between 10 ng and 3 µg DNA was prepared in SDW to a total volume of 16 µl.
2. This was boiled in a water bath for 10 minutes and then snap cooled on ice to produce single-stranded template.
3. A total of 4 µl DIG-High Prime was added to a 0.5 ml Eppendorf tube, mixed and centrifuged briefly.
4. The tube was incubated at 37°C for between one and 20 hours.
5. 2 µl EDTA was added to terminate the reaction.
6. The labelled probe was stored at -20°C.
2.17.2 Hybridisation of DIG-labelled probes

Filter hybridisation involves the immobilisation of single-stranded nucleic acid onto a solid support (nitrocellulose or nylon) membrane and hybridisation of digoxigenin labelled probe DNA.

Required:

- **2 x SSC**
  
  20 x SSC (NaCl 3 M. sodium citrate 0.3 M):
  
  - NaCl 175.3 g
  - Sodium citrate 88.2 g
  - Dissolve in 800 ml SDW, adjust pH to 7.0 and make up to 1000 ml. This may be diluted to give other concentrations as required

- **DIG hybridisation buffer (DHB)**
  
  - 20x SSC 125 ml
  - 10% (w/v) Sodium Lauryl Sarcosine (SLS) [BDH] 5 ml
  - 20% (w/v) Sodium Dodecyl Sulphate (SDS) 0.5 ml
  - Blocking reagent [Roche] 5 g
  - Make up to 500 ml with SDW, heat while stirring at 60°C until dissolved. Store at -20°C in 100 ml aliquots.

- **Wash buffer 1 (2 x SSC, 0.1% SDS)**
  
  - 20 x SSC 50 ml
  - 20% SDS 2.5 ml

- **Wash buffer 2 (0.5 x SSC, 0.1% SDS)**
  
  - 20 x SSC 12.5 ml
  - 20% SDS 2.5 ml
Materials and Methods

- Hybridisation oven [Techne]
- Hybridisation tubes [Techne]

Protocol:

1. Membranes were pre-wetted with 2 x SSC

2. The membranes were placed in hybridisation tubes and 5 to 10 ml DHB added. The tubes were placed in the hybridisation oven and pre-hybridised at 65°C for two hours

3. The pre-hybridisation DHB was poured away

4. 5 to 10 ml of fresh DHB containing 250 ng of digoxigenin-labelled probe per 100 cm² of membrane was added and the membrane hybridised for 18 hours at 65°C.

5. Following hybridisation, membranes were washed twice in wash 1 for 5 min at room temperature

6. The membranes were then washed twice more in wash 2 for 15 min at 68°C. Oligonucleotide DNA probes are sensitive to temperature and salt concentration. These final washes define the stringency of hybridisation so that low salt or high temperature increases the stringency of the wash and thus the specificity of the probes. The above standard wash conditions were used for most procedures. However, for DNA-DNA hybridisation, more stringent conditions were required (Owen & Pitcher, 1985), and were calculated with the following formula:

\[
T_m = 81.5 + 16.6 (\log_{10} [Na^+]) + 0.41 (\text{mol}\% \text{ G + C}) - 600/n
\]

where \(Na^+\) = salt concentration and \(n\) = probe length
2.17.3 Detection of hybridised DIG-labelled probes

Once bound to target DNA, hybridised DIG-labelled probes were visualised using the DIG nucleic acid detection kit [Roche].

Required:

- **Buffer 1** (maleic acid 100 mM, NaCl 150 mM, pH 7.5)
  - Maleic acid [BDH] 11.61 g
  - NaCl 8.77 g
  - Dissolve in 800 ml SDW, and adjust the pH to 7.5. Make up to 1000 ml, filter and store at room temperature.

- **Buffer 2** (1% (w/v) blocking agent in buffer 1)

- **Buffer 3** (Tris 100 mM, NaCl 100 mM, MgCl₂ 50 mM, pH 9.5)
  - Tris 12.12 g
  - NaCl 5.84 g
  - MgCl₂ [BDH] 10.16 g
  - Dissolve in 800 ml SDW, and adjust the pH to 9.5. Make up to 1000 ml, filter and store at 4°C.

- **Colour solution**
  - Nitroblue tetrazolium chloride (NBT) [Roche] 45 µl
  - 5-Bromo-4-chloro-3-indolylphosphate (BCIP) [Roche] 35 µl
  - Buffer 3 10 ml

- **Anti-digoxigenin-alkaline phosphatase conjugate** [Roche]

Protocol:

1. Membranes were washed briefly in buffer 1
2. The buffer was poured away, 100 ml buffer 2 added and the membranes incubated at room temperature for 30 minutes on a shaker.

3. Membranes were washed briefly in buffer 1

4. 4 μl of conjugate was diluted in 20 ml buffer 1 to a final concentration of 50 mU ml⁻¹.

5. Membranes were incubated in 20 ml conjugate solution at room temperature for 30 minutes on a shaker.

6. The membranes were washed twice for 15 minutes in 100 ml buffer 1.

7. Membranes were equilibrated in buffer 3 for two minutes.

8. The membranes were then placed in a clean box, 10 ml of colour solution was added and allowed to develop in the dark for 30 minutes to two hours.

9. Development was stopped by washing for five minutes in SDW. Membranes were dried and stored in the dark.

2.18 PCR-Enzyme Linked Immunosorbent Assay (PCR-ELISA)

The method described identifies *Campylobacter* species by capture hybridisation of single stranded 16S rRNA gene amplicons with species-specific probes in a microtitre plate format (Metherell et al., 1999).

2.18.1 Asymmetric PCR

Asymmetric PCR, where one strand of DNA is amplified preferentially to the other, was used to generate fluorescein labelled single stranded DNA complementary to the capture probes.
Materials and Methods

Required:

- *Campylobacter* genus-specific 16S rRNA PCR amplicon (see 2.10.1.1)
- 5' end fluorescein labelled primer (AGA TAC CCT GGT AGT CCA CG)
- PCR reagents and thermal cycler as described in section 2.9

Protocol:

1. A first round product of the *Campylobacter* genus-specific 16S rRNA PCR (cgen) was generated as described in sections 2.9 and 2.10.1.1.
2. 5 µl of the first round product was used as template for a second round PCR reaction, performed in a 25 µl total volume as described in section 2.9, but with the addition of the single fluorescein labelled asymmetric PCR primer.
3. Asymmetric amplification took place using standard conditions (see section 2.9) with an annealing temperature of 60°C

2.18.2 Immobilisation of capture probes

Required:

- 5' biotinylated capture probes [Cruachem] see Table 2.9
- Breakable streptavidin-coated microtitre plate strips [Labsystems]
- PBST
  - PBS [Oxoid] 500 ml
  - Tween 20 [BDH] 0.5 ml
- Microtitre plate washer [Denley]
Protocol:

1. For each well of the microtitre plate 100 μl of a 1 μg ml⁻¹ suspension of each of the eight 5' biotinylated capture probes was prepared in PBST.
2. The probe suspensions were added to the wells of the streptavidin coated microtitre plate and incubated at 37°C for 30 minutes.
3. The wells were then washed three times with PBST.
4. Plates were then held on ice for use the same day.

Table 2.9 Probes used with PCR-ELISA

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence*</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>JEJ/COL 1</td>
<td><strong>TTT GCG GTA CAC TTA ATG CGT T</strong></td>
<td>C. jejuni</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. coli</td>
</tr>
<tr>
<td>JEJ/COL 2</td>
<td><strong>TAA GCT CGG CCG AAC CGT TA</strong></td>
<td>C. jejuni</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. coli</td>
</tr>
<tr>
<td>COL</td>
<td><strong>AAA CCC TGA CTA GCA GAG CAA</strong></td>
<td>C. coli</td>
</tr>
<tr>
<td>LAR</td>
<td><strong>TAA GCT CAC CCG AAG TGT TAG</strong></td>
<td>C. lari</td>
</tr>
<tr>
<td>UPS</td>
<td><strong>AAA CTA CAG AAT TTG TTG GAT ATC</strong></td>
<td>C. upsaliensis</td>
</tr>
<tr>
<td>UPS/HEL</td>
<td><strong>TAA GCT CGA CCG AAT CGT TAG</strong></td>
<td>C. upsaliensis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. helveticus</td>
</tr>
<tr>
<td>HYO</td>
<td><strong>ATA CTC TAA GAT GTT ATT AGG ATA T</strong></td>
<td>C. hyointestinalis</td>
</tr>
<tr>
<td>FET</td>
<td><strong>AAA CTA AGA GAT TAG TTG GAT ATC</strong></td>
<td>C. fetus</td>
</tr>
</tbody>
</table>

Bold type bases represent non-hybridising spacer regions.

2.18.3 Hybridisation and detection

Required:

- Fluorescein-labelled PCR product (see section 2.18.1)
- Breakable microtitre plate strips with immobilised probes (see 2.18.2)
Materials and Methods

- Hybridisation buffer (HB)
  - 20 x SSC 25 ml
  - Marvel milk powder 1 g
  - 10% sarkosyl 10 ml
  - SDW 65 ml
- PBST
- Anti-fluoroisothiocyanate-horse radish peroxidase (FITC-HRP) conjugate [Amersham]
- Tetramethyl benzedine (TMB) solution [Europa Research Products Ltd]
- 2M Sulphuric acid [BDH]
- ELX800 microtitre plate reader [Biotek Instruments Inc]

Protocol:

1. Fluorescein-labelled PCR product (see 2.18.1) was heated to 94°C for 10 minutes, and then snap cooled on ice.
2. 50 µl of PCR product was added to 800 µl HB, well mixed, and 100 µl added to wells containing each of the probes.
3. This procedure was repeated for each sample to be tested.
4. The microtitre plate was incubated at 55°C for 30 minutes.
5. The wells were emptied and then washed three times with PBST.
6. Anti-FITC-HRP conjugate was diluted 1:1000 in HB, and 100 µl added to each well.
7. The microtitre plate was incubated at 37°C for 30 minutes.
8. The wells were emptied and then washed three times with PBST.
9. 100 μl of TMB solution was added to each well and colour allowed to develop for between 40 and 120 minutes, until no further darkening of the solution was apparent.

10. The reaction was stopped by the addition of 50 μl sulphuric acid.

11. The OD of the resulting yellow colour was measured at dual wavelength 450/620 nm on the plate reader and results compared with positive control strains.

2.19 Sequencing *Campylobacter* 16S rDNA

A commercially available dye terminator cycle sequencing kit was used. This employs AmpliTaq DNA polymerase, FS, a form of Taq DNA polymerase lacking 5' → 3' nuclease activity, together with dye-labelled terminator nucleotide base analogues.

Required:

- Target 16S rDNA amplicon (see section 2.10.1)
- Dye Terminator Cycle Sequencing kit [Perkin Elmer Biosystems]
- Sequencing primers see (Table 2.10) diluted to 100 μM stock and 1 μM working concentration.
- Electrophoresis equipment (section 2.11)
- GeneClean II kit (section 2.13)
- PCR grade mineral oil [Sigma]
- Perkin Elmer 9600 Thermal Cycler [PE Biosystems]
- Sodium acetate, 3 M, pH 4.6 [BDH].
- 95% ethanol
Materials and Methods

- 70% ethanol
- Lasergene analysis software

### Table 2.10 Sequencing primers for *Campylobacter* 16S rDNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>£ (F)*</td>
<td>AAG AGT TTG ATC CTG GCT CAG</td>
</tr>
<tr>
<td>339F</td>
<td>TGG AAC TGA GAC ACG GTC CA</td>
</tr>
<tr>
<td>550F</td>
<td>TTA CTC GGA ATC ACT GGG CGT</td>
</tr>
<tr>
<td>887F</td>
<td>GGA GTA CGG TCG CAA GAT TA</td>
</tr>
<tr>
<td>1195F</td>
<td>CGA CGT CAA GTC ATC ATG GC</td>
</tr>
<tr>
<td>1510R*</td>
<td>GGT TAC CTT GTT ACG ACT T</td>
</tr>
<tr>
<td>1044R</td>
<td>CGA CAC GAG CTG ACG ACA GC</td>
</tr>
<tr>
<td>771R</td>
<td>GCG TGG ACT ACC AGG GTA TC</td>
</tr>
<tr>
<td>479R</td>
<td>GGC ACG GAG TTA GCC GGT GC</td>
</tr>
<tr>
<td>320R</td>
<td>TGG ACC GTG TCT CAG TTC CA</td>
</tr>
</tbody>
</table>

* £ and 1510R are universal 16S rDNA primers (Brosius *et al.*, 1978), the remainder were designed from *Campylobacter* species sequence data (Linton, 1997).

**Protocol:**

1. The DNA to be sequenced was amplified using appropriate 16S rDNA primers (section 2.10.1) and diluted to a final concentration of between 10 and 30 ng ml⁻¹.
2. The PCR product was electrophoresed on an agarose gel and the band produced and excised and purified using the GeneClean II kit (see section 2.13).

3. Purified PCR products were sequenced using a dye terminator cycle sequencing kit and the 16S rDNA sequencing primers listed in Table 2.10. For each cycle sequencing reaction the following reagents were mixed in a 0.5 ml Eppendorf tube:
   - Terminator Ready Reaction Mix: 8.0 μl
   - Template (16S rDNA PCR product): 6.0 μl
   - 1 μM primer: 3.2 μl
   - SDW: 2.8 μl
   - Final reaction volume: 20.0 μl. The tubes were overlaid with 50 μl of mineral oil.

4. PCR reactions were performed in a PE 9600 Thermal Cycler as follows: Denaturation 96°C for 10 seconds, annealing 50°C for five seconds, extension 60°C for four minutes for 25 cycles. The reactions were then held at 4°C. This was repeated with each of the sequencing primers.

5. 50 μl PCR reaction was mixed with 2.0 μl sodium acetate and 20 μl of 95% ethanol in a 1.5 ml Eppendorf tube by vortexing, and placed on ice for 10 minutes. This was to allow the removal of excess dye terminators by selective ethanol precipitation of extension products.

6. The tube was centrifuged at 13000g for 30 minutes and the ethanol carefully removed with a pipette as completely as possible.
Materials and Methods

7. The DNA pellet was washed with 150 μl of 70% ethanol. The ethanol was again carefully removed with a pipette.

8. The pellet was dried at room temperature in the dark and stored at 4°C.

9. Sequencing amplification products were separated on a 6% denaturing (sequencing) polyacrylamide gel on an ABI Prism 373A DNA automated sequencer [Cruachem].

10. Sequence data was analysed and aligned using Lasergene software as described in section 2.15.

2.20 Mol% G + C determination

The DNA base composition (mol% G + C content) was estimated from the thermal denaturation temperature (Tm) (Owen & Pitcher, 1985).

Required:

- 0.33 x SSC

- SP1800 UV spectrophotometer with heating block and SP 876 Series 2 temperature programme controller [Unicam]

- Thermistor probe with squirrel data logger [Grant]

- Quartz cuvettes 1 ml capacity [Sigma]

Protocol:

1. Genomic DNA (see section 2.7) was resuspended in 0.33 x SSC.

2. Samples were carefully pipetted (to avoid shearing) into 1 ml quartz cuvettes with a thermistor probe suspended in the solution.
3. A stable baseline absorbance was obtained and the temperature increased at the rate of 1°C per minute.
4. The temperature and the corresponding increase in absorbance of the DNA sample was graphically recorded at regular intervals.
5. Once the DNA was completely denatured (i.e. the absorbance ceased to increase), the cuvette was removed and the apparatus allowed to cool.
6. From the plot of absorbance and temperature the Tm of the DNA was determined and the mol% G + C calculated.
7. Control strains with known mol% G + C values (E. coli NCTC 1009 and C. jejuni NCTC 11351) were used for comparison.
8. All samples (novel strains and controls) were measured three times and an average taken.

2.21 Electron microscopy

Required:
- 1% (v/v) Formalin solution [BDH]
- 2% (w/v) Ammonium molybdate solution [Sigma]
- SDW
- 'Formvar' coated grids [Sigma]
- Filter paper [Whatman]
- EM420 electron microscope [Phillips]
Protocol:

1. Cells were taken from a 48 hour culture on CBA or in BB and resuspended in a 1% formalin solution.

2. A 'Formvar'-coated grid was placed on a drop of the bacteria-formalin suspension for two minutes.

3. The grid was dabbed dry and transferred to a drop of 2% (w/v) ammonium molybdate solution for a further two minutes.

4. The grid was again dabbed dry and transferred to a drop SDW solution for two minutes, dabbed dry and stored prior to examination.

5. Grids were examined in an EM420 electron microscope at 80 kv.
Chapters 3 to 8

Results and Discussion
Chapter 3: Development of a rapid protocol to recover bacterial DNA from human faecal material: Comparison of direct PCR detection with culture techniques for \textit{C. upsaliensis} and \textit{C. helveticus}

3.1 Background

A prerequisite for a PCR-based investigation of the prevalence of \textit{Campylobacter} in human gastroenteritis was the evolution of a suitable bacterial DNA extraction technique. Such methodology must be rapid (to facilitate the processing of a large number of samples in a timely manner) and able to produce nucleic acid sufficiently free of inhibitory substances so as not to adversely affect the sensitivity of the PCR assay.

This chapter presents the development of a rapid PCR protocol, based on 16S rRNA gene primers and a DNA extraction methodology able to recover relatively pure target DNA from faecal material. The aim was to produce a PCR assay capable of detecting and differentiating the phenotypically similar species \textit{C. upsaliensis} and \textit{C. helveticus} directly from seeded human faeces without the need for selective or enrichment culture. The sensitivity of the PCR assay was assessed by comparison with two established culture-based techniques commonly used for the isolation of non-\textit{jejuni}/non-\textit{coli} \textit{Campylobacter} species, namely a selective isolation medium containing cefoperazone (Cefoperazone Amphotericin Teicoplanin agar – CAT) and the 'non-selective' membrane filter technique (MF).
3.2 Results

Bacterial strains and seeding
Twenty-four hour cultures of six strains each of *C. upsaliensis* and of *C. helveticus* were used to 'seed' fresh, campylobacter-free faeces as described in section 2.4. The strains of *C. upsaliensis* and *C. helveticus* used in the seeding experiments are listed in Table 3.1. The MIC of the antibiotic cefoperazone was determined for each of these strains as described in section 2.5. These results are recorded in Table 3.1.

PCR amplification
PCR amplification was performed as described in section 2.9, using the *chcu* PCR assay (Linton et al., 1996) for the detection and differentiation of *C. helveticus* and *C. upsaliensis* (see section 2.10.1.3). This was a duplex assay, consisting of a single forward primer (CHCUF) common to both *C. helveticus* and *C. upsaliensis* and two species-specific reverse primers – CUR, producing a 878 bp *C. upsaliensis*-specific amplicon, and CHR which produced a 1225 bp *C. helveticus*-specific amplicon. In certain isolates the *C. helveticus* amplicon produced was 1375 bp, due to the presence of the atypical intervening sequence (IVS) of 150 bp within this gene (Linton, et al., 1994). The *chcu* PCR assay was applied to DNA extracted from seeded faecal samples (see below). PCR amplicons were electrophoresed and visualized using a UV transilluminator after ethidium bromide staining (see section 2.11).
Table 3.1: Bacterial strains.

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Reference* number</th>
<th>Origin</th>
<th>Cefoperazone MIC (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. helveticus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>C121¹</td>
<td>Feline</td>
<td>8</td>
</tr>
<tr>
<td>H2</td>
<td>C130¹</td>
<td>Canine</td>
<td>16</td>
</tr>
<tr>
<td>H3</td>
<td>C132¹</td>
<td>Canine</td>
<td>16</td>
</tr>
<tr>
<td>H4</td>
<td>C133¹</td>
<td>Canine</td>
<td>32</td>
</tr>
<tr>
<td>H5⁺</td>
<td>C144¹</td>
<td>Feline</td>
<td>32</td>
</tr>
<tr>
<td>H6</td>
<td>NCTC12470T²</td>
<td>Feline</td>
<td>16</td>
</tr>
<tr>
<td><strong>C. upsaliensis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U1</td>
<td>C123¹</td>
<td>Canine</td>
<td>4</td>
</tr>
<tr>
<td>U2</td>
<td>C126¹</td>
<td>Canine</td>
<td>32</td>
</tr>
<tr>
<td>U3</td>
<td>C128¹</td>
<td>Feline</td>
<td>8</td>
</tr>
<tr>
<td>U4</td>
<td>C131¹</td>
<td>Canine</td>
<td>16</td>
</tr>
<tr>
<td>U5</td>
<td>C1231³</td>
<td>Human</td>
<td>16</td>
</tr>
<tr>
<td>U6</td>
<td>NCTC11541T⁴</td>
<td>Canine</td>
<td>≤2</td>
</tr>
</tbody>
</table>

*Strain numbers from the corresponding publications: 1, (Linton et al., 1994); 2, (Stanley, et al., 1992); 3 a laboratory isolate; 4, (Sandstedt, et al., 1983).

⁺ This strain contained a 150-bp IVS within its 16S rRNA genes.

¹ Type strain of the species.
Extraction of bacterial DNA from seeded faeces for PCR

Three principal DNA extraction procedures were investigated to assess the quality of the final (DNA) product, its suitability as a substrate for PCR, its easy of use and speed with which extractions could be performed. These extraction methods were phenol-chloroform (Wilson, 1987), guanidine-chloroform (Pitcher et al., 1989) and guanidine-silica (Boom et al., 1990), and are described in section 2.7. DNA was extracted from freshly seeded faecal samples as described above. These results are summarised in Table 3.2.

Removal of inhibitory substances

To reduce the presence of inhibitory substances co-extracted with DNA during extraction, two additional treatments were used. These were decimal dilution of the DNA extract in TE or SDW (see section 2.8.1) or a brief wash in a TE/PVP buffer (see section 2.8.2). A detailed comparison of these additional treatments was performed with DNA extracted using the guanidine-silica protocol. Results are shown in Table 3.3.

Detection by culture

Seeded faeces of various concentrations were applied to the CAT selective media and the membrane filtration technique as described in sections 2.2.1 and 2.2.2 respectively. The inoculated plates were incubated at 37°C in a microaerobic atmosphere and growth was recorded after 48, 72 and 96 hours. All of these experiments were performed in triplicate and a mean sensitivity calculated. The results are recorded in Table 3.4.
Table 3.2: Comparison of extraction methods

<table>
<thead>
<tr>
<th>Extraction Method</th>
<th>Ease of use</th>
<th>Speed of use</th>
<th>Hazards</th>
<th>Sensitivity (cfu g⁻¹ faeces)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol-chloroform</td>
<td>+</td>
<td>+</td>
<td>Phenol</td>
<td>~10⁶</td>
</tr>
<tr>
<td>GuSCN-chloroform</td>
<td>++</td>
<td>++</td>
<td>GuSCN</td>
<td>~10⁶</td>
</tr>
<tr>
<td>GuSCN-Silica</td>
<td>+++</td>
<td>+++</td>
<td>GuSCN</td>
<td>10⁷ - 10⁸</td>
</tr>
</tbody>
</table>

1. Ease of use: ++++, easiest → +, least easy.
2. Speed of use: ++++, quickest → +, most time consuming.
3. Hazardous substances used.
4. Relative sensitivity of method (cfu g⁻¹ faeces)
Table 3.3: Comparison of dilution and PVP treatment of DNA extracted by the GuSCN-silica extracted method (mean* (S.D.) cfu g⁻¹)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dilution</th>
<th>PVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. helveticus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>5.07 (6.25) x 10⁶</td>
<td>8.97 (6.14) x 10⁴</td>
</tr>
<tr>
<td>H2</td>
<td>2.41 (1.21) x 10⁶</td>
<td>5.04 (4.17) x 10⁵</td>
</tr>
<tr>
<td>H3</td>
<td>2.20 (0.81) x 10⁶</td>
<td>2.20 (0.44) x 10⁵</td>
</tr>
<tr>
<td>H4</td>
<td>2.30 (4.17) x 10⁶</td>
<td>2.30 (2.34) x 10⁵</td>
</tr>
<tr>
<td>H5</td>
<td>2.83 (1.17) x 10⁶</td>
<td>2.80 (2.06) x 10⁵</td>
</tr>
<tr>
<td>H6</td>
<td>1.47 (3.76) x 10⁶</td>
<td>6.67 (4.25) x 10⁴</td>
</tr>
<tr>
<td>C. upsaliensis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U1</td>
<td>1.63 (0.79) x 10⁶</td>
<td>1.03 (0.72) x 10⁵</td>
</tr>
<tr>
<td>U2</td>
<td>1.77 (0.65) x 10⁶</td>
<td>1.77 (0.35) x 10⁵</td>
</tr>
<tr>
<td>U3</td>
<td>1.83 (1.23) x 10⁶</td>
<td>1.38 (1.09) x 10⁵</td>
</tr>
<tr>
<td>U4</td>
<td>1.93 (3.29) x 10⁶</td>
<td>1.89 (0.79) x 10⁵</td>
</tr>
<tr>
<td>U5</td>
<td>1.71 (4.09) x 10⁶</td>
<td>1.17 (1.19) x 10⁵</td>
</tr>
<tr>
<td>U6</td>
<td>3.90 (1.14) x 10⁶</td>
<td>1.86 (1.21) x 10⁵</td>
</tr>
</tbody>
</table>

* Mean of three samples.
Table 3.4: Sensitivities of different methods for detection of *C. helveticus* and *C. upsaliensis* in seeded faeces (mean* (S.D.) cfu g⁻¹)

<table>
<thead>
<tr>
<th>Strain</th>
<th>CAT</th>
<th>MF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. helveticus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>1.47 (0.38) x 10⁵</td>
<td>1.08 (0.89) x 10⁶</td>
</tr>
<tr>
<td>H2</td>
<td>5.71 (4.70) x 10³</td>
<td>3.34 (5.24) x 10⁵</td>
</tr>
<tr>
<td>H3</td>
<td>7.30 (8.40) x 10³</td>
<td>1.19 (1.10) x 10⁵</td>
</tr>
<tr>
<td>H4</td>
<td>8.00 (3.00) x 10³</td>
<td>4.73 (3.51) x 10⁵</td>
</tr>
<tr>
<td>H5</td>
<td>7.30 (6.93) x 10³</td>
<td>5.68 (8.08) x 10⁵</td>
</tr>
<tr>
<td>H6</td>
<td>6.67 (6.01) x 10³</td>
<td>3.04 (3.23) x 10⁶</td>
</tr>
<tr>
<td><strong>C. upsaliensis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U1</td>
<td>1.18 (0.94) x 10⁵</td>
<td>5.83 (7.08) x 10⁴</td>
</tr>
<tr>
<td>U2</td>
<td>1.35 (1.06) x 10³</td>
<td>5.97 (6.96) x 10⁴</td>
</tr>
<tr>
<td>U3</td>
<td>4.79 (6.32) x 10⁵</td>
<td>1.37 (1.10) x 10⁵</td>
</tr>
<tr>
<td>U4</td>
<td>1.20 (1.10) x 10⁴</td>
<td>4.30 (4.83) x 10⁴</td>
</tr>
<tr>
<td>U5</td>
<td>1.71 (0.83) x 10⁴</td>
<td>1.71 (0.83) x 10⁵</td>
</tr>
<tr>
<td>U6</td>
<td>&lt; 10⁶</td>
<td>2.83 (2.70) x 10⁵</td>
</tr>
</tbody>
</table>

* Mean of three samples.
Results and Discussion

Sensitivity of detection

The sensitivity of each extraction technique was defined as the lowest number of viable bacteria that could be detected from the seeded samples and was expressed as cfu g⁻¹ of faeces. Three replicates of each detection technique were performed for each strain tested and the mean sensitivity calculated. The degree of reproducibility obtained with each method was expressed by the standard deviation. A comparison of the relative sensitivity of each method for the detection of *C. upsaliensis* and *C. helveticus* was made using the Wilcoxon rank sum test.

3.3 Discussion

Of the three DNA extraction methodologies investigated, the GuSCN-silica protocol proved to have considerable advantages over the others in terms of ease and speed of use – DNA could be extracted from 12 samples in two hours. The sensitivity of detection of both *C. upsaliensis* and *C. helveticus* by PCR from DNA extracted from seeded faecal samples by each protocol was 100 to 1000 times less sensitive than culture using either CAT or MF. PCR using template DNA extracted using the GuSCN-silica protocol had marginally greater sensitivity than was the case for DNA extracted by the other methods.

The relative lack of sensitivity of the PCR assays was largely due to the presence of inhibitory substances co-extracted with the DNA. This inhibitory effect could be reduced to some extent by either diluting the DNA extracts or by brief treatment with a PVP-containing buffer. Both of these additional
Results and Discussion

treatments improved the sensitivity of the PCR assay when it was applied to DNA from each of the three extraction methods. However, PCR performed on GuSCN-silica extracted DNA after PVP/TE treatment was the most sensitive combination, producing results that were comparable with the culture-based methods using CAT or MF for detection of *C. helveticus* and *C. upsaliensis* (see below). The mechanism by which PVP removes PCR inhibitors from DNA is not precisely known, but it has been suggested by studies of DNA extracted from soils with high organic contents, that PVP absorbs phenol compounds covalently bound to DNA (Young, et al., 1993).

PCR assays performed on GuSCN-silica extracted DNA following PVP treatment, detected all twelve strains seeded in faecal samples with a mean sensitivity of 2.4 (2.04 S.D.) x 10⁶ cfu g⁻¹ after dilution and 1.9 (1.17 S.D.) x 10⁵ cfu g⁻¹. For the seeded faecal samples examined, the sensitivity ranged from 6.7 x 10⁴ to 5.0 x 10⁵ cfu g⁻¹, equivalent to between 34 and 250 cfu per PCR reaction. For comparison PCR was performed on strains suspended in SDW and was able to detect the equivalent of between 5 and 50 cfu per reaction, indicating that some inhibition remained. By PCR *C. helveticus* was detected with a mean sensitivity of 2.3 (1.57 S.D.) x 10⁵ cfu g⁻¹ and *C. upsaliensis* of 1.52 (0.37 S.D.) x 10⁵ cfu g⁻¹. There was no significant difference between the levels of detection for the two species (P > 0.05). PCR consistently produced a single amplicon, appropriate for the species identity of the seeded strain. An example of the PCR assay result is shown in Figure 3.1.
Figure 3.1: Examples of PCR assay products from 'seeded' faecal samples. M, molecular size marker λ BstE.

Lanes 1-4, *C. upsaliensis* (U6) ‘seeded’ at $10^7$, $10^6$, $10^5$ and $10^4$ cfu g$^{-1}$ faeces.
Lanes 5-8, *C. helveticus* (H6) ‘seeded’ at $10^7$, $10^6$, $10^5$ and $10^4$ cfu g$^{-1}$ faeces.
Lanes 9-12, *C. helveticus* (H5) ‘seeded’ at $10^7$, $10^6$, $10^5$ and $10^4$ cfu g$^{-1}$ faeces.
Lane 13, ‘unseeded’ negative control.
Of the culture-based methodologies included for comparison, CAT supported the growth of eleven of the twelve strains of *Campylobacter* examined. The sensitivity of CAT was dependent on the MIC of individual strains to cefoperazone. Strain U6 had an MIC to cefoperazone of 2 mg l⁻¹ and was unable to grow on CAT. Strains H1, U1 and U3 had MICs of 4 to 8 mg l⁻¹ and were detected over a range of 1.5 x 10⁴ to 1.2 x 10⁶ cfu g⁻¹ with a mean of 2.5 (2.48 S.D.) x 10⁵ cfu g⁻¹. The remaining strains had MICs of 16 to 32 mg l⁻¹ and were detected from faeces seeded with between 1.4 x 10² to 2.5 x 10⁴ cfu g⁻¹ with a mean of 8.2 (4.68 S.D.) x 10³ cfu g⁻¹. In most cases colonies were visible on the agar after 48 hours incubation. However, seeded samples yielding scanty growth, and colonies from strains with low MICs to cefoperazone, required incubation for 72 or 96 hours before detection was possible. Those strains of *C. upsaliensis* and *C. helveticus* that grew on SA were detected with a mean sensitivity of 1.3 (0.92 S.D.) x 10⁵ cfu g⁻¹ and 7.7 (11.26 S.D.) x 10⁵ cfu g⁻¹ respectively. The difference between the species was not significant (P > 0.05).

The MF method was the first successful technique for isolating *C. upsaliensis* from clinical samples (Steele & McDermott, 1984; Megraud & Bonnet, 1986). In the present study it detected all the strains of *C. helveticus* and *C. upsaliensis* tested. In most cases colonies were visible on the agar after 48 hours incubation. However, seeded samples yielding scanty growth, particularly of *C. helveticus* required incubation for 72 or 96 hours before all colonies were detectable. Sensitivities ranged from 6.5 x 10⁶ to 1.1 x 10⁴ cfu g⁻¹ with a mean of 4.5 (8.35 S.D.) x 10⁵ cfu g⁻¹ for all strains. Strains of *C.
*upsaliensis* with a mean sensitivity of 1.3 (0.92 S.D.) x 10^5 cfu g\(^{-1}\) were more readily detected than the strains of *C. helveticus*, mean 7.7 (11.26 S.D.) x 10^5 cfu g\(^{-1}\), although the difference between the species was not significant (P > 0.05). These results match previously reported sensitivities (Goossens, et al., 1990a), and were similar to the level of sensitivity achieved with the PCR assay. Although MF is often regarded as a relatively insensitive means of isolation (Goossens & Butzler, 1992), it does have the advantage of facilitating isolation of organisms susceptible to antibiotics commonly used in *Campylobacter*-selective media (Bolton, et al., 1988; Endtz, et al., 1991). For example, in a comparative study of *Campylobacter* isolation techniques MF detected only 60% of the total *Campylobacter* isolates compared with 89% for CCDA (the most successful selective medium), nevertheless, MF was the only method that isolated catalase-negative *Campylobacter* species such as *C. upsaliensis* (Bolton, et al. 1988).

In this study, the selective agar CAT proved to be the most sensitive detection technique for both species, detecting as few as 10^3 cfu g\(^{-1}\) faeces. However, CAT was less sensitive than PCR in detecting strains of both species that had MICs to cefoperazone of less than or equal to 8 mg l\(^{-1}\), notably strain U6, which did not grow on that medium. It is thought that cefoperazone may be inhibitory to certain strains of both species. For example, Aspinall, et al.(1993) were unable to detect 18% of *C. upsaliensis* strains seeded at a level of 10^5 to 10^6 cfu g\(^{-1}\) faeces with an agar containing 8 mg l\(^{-1}\) of cefoperazone. Similarly, Goossens et al. (1990a) reported minimum bactericidal concentrations to
cefoperazone of 6.25 mg l\(^{-1}\) in 48 of 91 (53\%) strains of \textit{C. upsaliensis} tested in Mueller-Hinton broth.

The PCR assay was rapid (small batches of samples could be processed in a single working day) and able to detect and correctly identify all of the strains of \textit{C. helveticus} and \textit{C. upsaliensis} tested. Phenotypically these two species are very similar and are distinguished only by tolerance to selenite and growth on potato starch agar (Stanley et al., 1992). It should be noted that in this study, faeces were seeded with fresh log phase cultures to facilitate accurate determination of cell concentrations and sensitivities. The seeded organisms were probably more readily cultivable than the bacteria in clinical faecal material. It seems reasonable to expect that when the PCR assay is applied to clinical samples, its sensitivity would increase relative to culture-based techniques, due to the presence of additional target DNA from dead, damaged or viable but non-cultivable campylobacters.

In conclusion, these results demonstrate that a PCR assay targeted at conserved 16S rRNA genes in combination with an effective DNA extraction protocol is an effective means of detecting \textit{Campylobacter} in faeces.
Chapter 4: PCR detection, speciation and fingerprinting of C. \textit{jejuni} and C. \textit{coli} direct from diarrhoeic samples

4.1 Background

In the previous chapter it was established that a PCR assay performed on DNA appropriately extracted from faeces could correctly detect and identify strains of phenotypically similar \textit{Campylobacter} species from seeded faecal samples. The next step was to apply this technique to actual clinical samples.

\textit{Campylobacter} enteritis is currently diagnosed by isolation of the organism, which requires inoculation of faecal samples onto selective medium, followed by micro-aerobic incubation at 37°C or 42°C for 48 hours. A further 24 to 48 hours is required for full phenotypic identification (Goossens & Butzler, 1992). Although 16 species of \textit{Campylobacter} are known, \textit{C. jejuni} and \textit{C. coli} account for the vast majority of human infections, with \textit{C. jejuni} isolated in greater than 90% of cases (Anonymous, 1993; Skirrow, 1994; Linton, 1996). In current laboratory practice, speciation of campylobacters relies on relatively few phenotypic tests. For example, \textit{C. jejuni} and \textit{C. coli} are distinguished only by hippurate hydrolysis, while \textit{C. coli} and \textit{C. upsaliensis} are distinguished by the weak catalase activity and sensitivity to cephalothin of the latter. Due to these limitations and the constraints of time and expense, clinical laboratories often report these enteropathogens simply as ‘\textit{Campylobacter} species’ (Anonymous, 1993; Skirrow, 1994). Even when a rapid hippurate hydrolysis phenotypic test is performed to identify \textit{C. jejuni} isolates, there remains
significant difficulty in identification of hippurate-negative isolates, which could belong to other *Campylobacter* species or indeed be hippurate-negative strains of *C. jejuni* (Nicholson & Patton, 1993).

In this chapter PCR assays specific for *C. jejuni* and *C. coli* used in combination with the DNA extraction protocol were applied to human faecal specimens from a clinical laboratory. They were compared with isolation using selective medium and subsequent speciation and typing using established methods.

### 4.2 Results

**Faecal samples**

Faecal samples were provided by Tooting PHL, from 25 cases of acute gastroenteritis, no later than seven days after initial receipt (stools were stored at 4°C in the interim). ‘*Campylobacter species*’ had been isolated from 20 of these, by culture on modified Charcoal Cefoperazone Desoxycholate Agar (CCDA, 32 mg l⁻¹ cefoperazone), incubated microaerobically at 37°C for 48 hours. The remaining five were reported as ‘No Pathogens Isolated’ (NPI) and were *Salmonella, Shigella* and *Campylobacter* free by standard culture techniques at Tooting PHL. A further 20 faecal samples were also provided by healthy volunteers at CPHL.
Results and Discussion

Culture and biochemical identification

All 45 stool samples were re-cultured at CPHL as described in section 2.2.1, on CCDA. After 48 hours incubation at 37°C, isolates were identified phenotypically to the species level using the following procedures: Gram stain; oxidase and catalase activity; hippurate hydrolysis; indoxyl acetate hydrolysis; hydrogen sulphide production from triple sugar iron agar; and susceptibility to nalidixic acid and cephalothin (see section 2.6). 'Campylobacter-like' colonies were grown from all 20 of the previously 'Campylobacter species' positive clinical samples. Eighteen were identified as \textit{C. jejuni} and two as \textit{C. coli}. 'Campylobacter-like' colonies were not detected from the 'NPI' clinical samples or from healthy volunteer stool samples. These results are summarised in Table 4.1.

DNA extraction and PCR assay

Nucleic acids were extracted from faecal material using GuSCN-silica protocol followed by treatment with PVP/TE buffer as described in Chapter 3. In addition, DNA was extracted from cultured campylobacter isolates using the GuSCN-silica protocol alone. The following species-specific PCR assays were used: 16S rDNA-based PCR primers (cjcc) that co-detected, but did not differentiate \textit{C. jejuni} and \textit{C. coli} (see section 2.10.1.3); \textit{C. jejuni}-specific PCR primers (hip) based on the hippuricase gene (see section 2.10.4); \textit{C. coli}-specific PCR primers (asp) based on the aspartokinase gene (see section 2.10.5); and 16S rDNA-based PCR primer sets for other potentially enteropathogenic campylobacters, namely: \textit{C. helveticus} and \textit{C. upsaliensis}.
Table 4.1: Detection and identification of *Campylobacter* species by culture and PCR assay

<table>
<thead>
<tr>
<th>Faecal samples</th>
<th>Culture on CCDA</th>
<th>Phenotypic identification</th>
<th>16S rDNA-based PCR detection/identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C. jejuni</td>
<td>C. coli</td>
</tr>
<tr>
<td>Clinical Samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 'Campylobacter species' positive</td>
<td>20</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>5 'No pathogens isolated'</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CPHL Samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 'Healthy volunteers'</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*One sample positive for C. jejuni (by culture and PCR) produced a 1287 bp amplicon with the chycf primers indicating the presence of C. hyointestinalis*


Results and Discussion

(chcu), C. hyointestinalis and C. fetus (chycf) and C. lari (cla), see section 2.10.1.3.

The 20 samples from healthy volunteers and the five 'NPI' clinical samples were all negative for the above PCR assays. The 20 'Campylobacter species' culture-positive clinical samples, all produced 854 bp amplicons with the cjcc primers, indicating the presence of C. jejuni and/or C. coli. Eighteen of these samples also produced 735 bp amplicons with the hip primers, indicating the presence of C. jejuni, while the remaining two samples produced 500 bp amplicons with the asp primers indicating the presence of C. coli. These identifications matched those made by biochemical phenotyping of the Campylobacter isolates from the clinical samples. The C. jejuni and C. coli-specific PCR assays were also applied to Campylobacter isolates, and gave identical results to direct examination of the faecal samples. The chcu, chycf and cla primers produced no amplicons in all but one case – in a sample culture and PCR positive for C. jejuni a 1287 bp amplicon was produced with the chycf primers indicating the presence of C. hyointestinalis. These results are presented in Figures 4.1a and 4.1b, and summarised in Table 4.1.

Isolation of C. hyointestinalis

The faecal sample found to be PCR-positive for both C. jejuni and C. hyointestinalis was re-examined. This sample had previously been cultured at Tooting PHL and CPHL on both occasions using CCDA incubated at 37°C and only colonies of C. jejuni were apparent. The faecal sample (by now 14 days old) was re-cultured using CCDA, CAT and MF (see section 2.2).
Figure 4.1a: PCR identification to species level in 25 acute diarrhoeic stools

M, molecular size marker λ BstE III, lanes 1-20, ‘Campylobacter species’ culture-positive samples, lanes 21-25, ‘No pathogens isolated’ samples.

A. *cjcc* 16S rDNA PCR co-detecting *C. jejuni* and *C. coli* in the first 20 samples.

B. *hip* gene PCR identifying 18 of the above samples as *C. jejuni* – the exceptions being samples 9 and 14.
Results and Discussion

Figure 4.1b: PCR identification to species level in 25 acute diarrhoeic stools

M, molecular size marker λ BstE III, lanes 1-20, ‘Campylobacter species’ culture-positive samples, lanes 21-25, ‘No pathogens isolated’ samples.

C. *asp* gene PCR identifying the samples in lanes 9 and 14 as *C. coli*.

D. 16S rDNA PCR identifying sample 6 as positive for *C. hyointestinalis*. 
In addition a colony sweep from the primary CCDA culture was subcultured on CCDA, CAT and CBA. Plates were incubated at both 37°C and 25°C for 48 to 96 hours (a minority of \textit{C. hyointestinalis} strains are reported to grow at 25°C, whereas \textit{C. jejuni} will not). The re-cultured faeces produced a scanty growth of colonies identified as \textit{C. jejuni} on those plates incubated at 37°C, there was no growth on the faecal cultures incubated at 25°C. The re-cultured primary CCDA culture produced an apparently pure growth of colonies of \textit{C. jejuni} when incubated at 37°C (30 individual colonies biochemically identified). However the plates incubated at 25°C produced a good, pure growth of campylobacter-like colonies, which were confirmed by phenotypic tests and PCR assay as \textit{C. hyointestinalis}.

\textbf{Flagellin gene fingerprinting by PCR}

The 20 \textit{C. jejuni} and \textit{C. coli} isolates were genotyped by PCR-RFLP of the \textit{flaA} gene using the method of Alm et al. (1993) as described in section 2.15. The \textit{flaA} gene fragments were as shown in Figure 4.2A (lane order as in Figure 4.1). The same experiment was performed with faecal DNA as the substrate. Initially no \textit{flaA} amplicons were produced, but following the redesign of one of the primers (see section 2.10.3) identical \textit{flaA} fingerprints were obtained for matching faecal samples (Figure 4.2B) and isolates (Figure 4.2A). The \textit{flaA} fingerprints of the two \textit{C. coli} isolates (Fig. 4.2A, lanes 9 and 14) matched those of a previously reported \textit{C. coli} \textit{flaA} fingerprint, designated fVI (Stanley \textit{et al.}, 1995). Specific profile designations for \textit{C. jejuni} restriction patterns were not available. The \textit{flaA} primers produce amplicons from the \textit{flaA} genes of \textit{C. jejuni}, \textit{C. coli}, \textit{C. lari}, \textit{C. helveticus} and \textit{C. upsaliensis}.
Figure 4.2: PCR-RFLP of the *Campylobacter flaA* genes from stool samples and the corresponding *Campylobacter* isolate.

M - molecular size marker (123 bp ladder), lanes 1-20 correspond to the twenty *Campylobacter*-positive samples.

A. PCR-RFLP from DNA extracted from the *Campylobacter* isolates.

B. PCR-RFLP from DNA extracted directly from faecal material.
DNA extracted from the *C. hyointestinalis* culture did not produce an amplicon with these primers.

**4.3 Discussion**

The PCR assays described in this Chapter were able to rapidly detect and definitively identify the major human enteropathogens *C. jejuni* and *C. coli*, directly from diarrhoeic stools. By current isolation and culture methods these bacteria are estimated to cause approximately 99% of *Campylobacter* infections in England and Wales or the USA (Tauxe, 1992; Anonymous, 1993). Clearer understanding of the relative disease significance of members of the genus *Campylobacter* requires reliable identification to the species level. The application of PCR-RFLP to obtain *fla* gene fingerprints of isolates and of *Campylobacter* cells in faecal samples demonstrated that in each case both were the same genotype (shared the same fingerprint). Accurate speciation and genetic subtyping are important tools in epidemiological studies of *Campylobacter* infection of man.

Negative PCR results were obtained from twenty-five control stool samples (20 from healthy individuals and five from diarrhoeic patients), which were negative, by culture for *Campylobacter* species. These samples would have contained an extensive range of bacterial species native to the human gastrointestinal tract, as undefined faecal flora. This demonstrates the species-specificity of the PCR assays employed. Cultivable bacteria represent only a proportion of the total flora native to the gastrointestinal tract (Berg,
1996), and *Campylobacter* culture-negative faeces thus represent a comprehensive specificity control for these PCR assays.

The relative frequency of *C. jejuni* (18 per 20 clinical samples) and *C. coli* (2 per 20 faecal samples) in this study is in agreement with previous prevalence data (Tauxe, 1992; Anonymous, 1993). It is worthy of note that laboratory identification of *C. coli* is problematical since the species is generally distinguished from *C. jejuni* by negative hippurate hydrolysis and there is no single positive, phenotypic test specific for *C. coli*. Both the *C. coli*-specific PCR assay and *flaA* gene fingerprinting were able to substantiate the phenotypic identification of the *C. coli* isolates.

Surprisingly, in this somewhat limited sample an 'uncommon' *Campylobacter* species — *C. hyointestinalis* — was identified by PCR, and a corresponding isolate was retrospectively cultured. *C. hyointestinalis* is considered to be only a rare cause of human enteritis (Fennell et al., 1986; Edmonds et al., 1987; Minet et al., 1988). However, in this small study PCR assay from faecal DNA detected *C. hyointestinalis* although this organism went unrecognised by standard culture protocols — masked as it was by a heavier growth of *C. jejuni*. The clinical significance of this isolate of *C. hyointestinalis*, particularly in mixed culture is difficult to assess. However, this finding suggests that PCR-based analysis of DNA extracted directly from faecal material could, if applied to large numbers of samples, quantify the proportion of human diarrhoeic stools containing non-*jejuni*/non-*coli* *Campylobacter* species and contribute to our knowledge of the role of these campylobacters in human enteric disease.
Chapter 5: Detection of *Campylobacter* in gastroenteritis – a pilot study:
Comparison of direct PCR assay of faecal samples with selective culture.

5.1 Background

The preceding Chapters have demonstrated that PCR assays could correctly detect and identify *Campylobacter* species from DNA extracted directly from seeded faecal samples and from (known positive) clinical faecal samples. The next challenge was to apply the methodology to clinical samples in a prospective study. The first aim was to assess the practicality of the PCR assay on a significant number of samples (>100). The efficacy of the PCR assay was assessed by comparison with standard isolation procedures i.e. selective isolation on CCDA (Anonymous, 1993).

A true comparison of the PCR assay with culture is problematical since they have each been developed to achieve different goals. Selective culture in the diagnostic laboratory has evolved to isolate the principal human enteropathogen, *C. jejuni*; the detection of other ‘potentially pathogenic’ species and their identification beyond genus level is not a priority. By contrast the PCR assays were designed to simultaneously detect and identify all ‘potentially pathogenic’ *Campylobacter* species with equal sensitivity. Precise identification to the species level is a prerequisite to accurately define the disease spectrum and microbial ecology of campylobacters – a major aim of this thesis.
5.2 Results

Clinical samples
Faecal samples were provided by Central Middlesex PHL, from 200 cases of acute gastroenteritis and were tested no later than seven days after initial receipt (stools were stored at 4°C in the interim). Samples were examined for the presence of *Campylobacter*, *Salmonella* and *Shigella* species by standard laboratory culture techniques at Central Middlesex PHL. Detection of *Campylobacter* species was made by culture on modified CCDA for 48 hours at 37°C under microaerobic conditions. The identity of colonies with characteristic *Campylobacter* morphology was confirmed by Gram stain and positive cytochrome oxidase test.

DNA extraction and PCR assay
The DNA extraction procedure and PCR assays employed were as described in Chapter 4. The following species-specific PCR assays were used: 16S rDNA-based PCR primers (*cjcc*) that co-detected, but did not differentiate *C. jejuni* and *C. coli* (see section 2.10.1.3); *C. jejuni*-specific PCR primers (*hip*) based on the hippuricase gene (see section 2.10.4); *C. coli*-specific PCR primers (*asp*) based on the aspartokinase gene (see section 2.10.5); and 16S rDNA-based PCR primer sets (see section 2.10.1.3) for other potentially enteropathogenic campylobacters – *C. helveticus* and *C. upsaliensis* (*chcu*), *C. hyointestinalis* and *C. fetus* (*chycf*) and *C. lari* (*cla*).
Southern blotting and hybridisation with DIG-labelled probes

Southern blots were made of PCR products run out on agarose gels as described in section 2.16.1, DNA-DNA hybridisation was performed as described in section 2.17. For the 16S rDNA-based PCR assays, a single probe was produced from \textit{C. jejuni} NCTC 11351 using the \textit{Campylobacter} genus-specific PCR assay \textit{cgen} described in section 2.10.1.1. These primers produced a 780 bp amplicon from a region of 16S rDNA conserved among all seven \textit{Campylobacter} species investigated, and which overlapped the area amplified by the species-specific primers. Probes for the \textit{hip} and \textit{asp} PCR amplicons were prepared by using the original assay primers to amplify probe DNA from \textit{C. jejuni} NCTC 11351 and \textit{C. coli} NCTC 11366 respectively. All PCR-generated probes were purified before use as described in section 2.13.

PCR assays and culture applied to clinical samples

From 18 of 200 faecal DNA extracts, PCR amplicons were produced with the \textit{cjcc} primers. Of these PCR-positive samples, 16 also gave an amplicon with \textit{C. jejuni}-specific \textit{hip} primers, while two gave an amplicon with \textit{asp} primers specific for \textit{C. coli}. One of the \textit{C. jejuni} and one of the \textit{C. coli} PCR-positives were detected only after hybridisation. One of the 200 samples yielded a 1287 bp amplicon with the \textit{chycf} primers indicating the presence of \textit{C. hyointestinalis}. PCR assays for \textit{C. lari}, \textit{C. upsaliensis}, \textit{C. helveticus} and \textit{C. fetus} were negative. Culture on CCDA yielded colonies identified as '\textit{Campylobacter} species' from 16 of the 200 samples. Three samples which were PCR-positive for \textit{C. jejuni} and one sample PCR-positive for \textit{C. hyointestinalis} were culture-negative for '\textit{Campylobacter} species'. One
sample culture-positive for 'Campylobacter species' was negative by PCR. These results are summarised in Table 5.1 and an example of the PCR assay is shown in Figure 5.1.

The difference in the rates of detection between culture and PCR evaluated by McNemar's test, (P value 0.18) was not statistically significant.

**Sensitivity of the PCR assays**

The relative sensitivity of culture on CCDA versus PCR assay for each potentially enteropathogenic *Campylobacter* species, was determined in seeding experiments for the following type strains: *C. jejuni* NCTC 11351, *C. coli* NCTC 11366, *C. lari* NCTC 11352, *C. upsaliensis* NCTC 11541, *C. helveticus* NCTC 12470, *C. hyointestinalis* NCTC 11608 and *C. fetus* subspecies *fetus* NCTC 10842. All were cultured at 37°C on CBA plates under microaerobic conditions. As previously described (in Chapter 3), 24 hour cultures of each strain were used to 'seed' fresh, *Campylobacter*-free faeces to produce a suspension with a cell concentration range equivalent to $10^2 - 10^7$ cfu g$^{-1}$ of faeces. 100μl aliquots of the freshly prepared seeded samples and an unseeded control were used in the DNA extraction procedure described above and inoculated on CCDA plates. Three replicates of each seeded sample were tested. Each of the species-specific PCR assays detected the appropriate target *Campylobacter* species in artificially seeded faeces with a sensitivity of $\sim 10^5$ cfu g$^{-1}$ of faeces.
Table 5.1: *Campylobacter* - positives among 200 acute diarrhoeal stool samples: Identification and speciation by PCR and culture.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Culture (CCDA)</th>
<th>PCR assays</th>
<th>PCR speciation</th>
</tr>
</thead>
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<td></td>
<td></td>
<td>cjcc</td>
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<tr>
<td>6</td>
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</tr>
<tr>
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<td>41</td>
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<td>+</td>
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<table>
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<th>2</th>
<th>0</th>
<th>0</th>
<th>1</th>
<th>19</th>
</tr>
</thead>
</table>

/200

* Positive only after Southern blotting and hybridisation (cf. Figure 5.1).
Figure 5.1: Examples of results obtained by PCR assay.

Data obtained from clinical faecal samples (numbered as in Table 5.1) with C. coli / C. jejuni-specific PCR assay cjcc. M, molecular size marker λ BsfE III; lanes 30-45, faecal samples tested; -, negative control (extracted from control faeces); + positive control (genomic DNA of NCTC 11168).

A, Amplicons visualised on agarose gel by ethidium bromide staining. Sample 32 and positive control show specific amplicons.

B, Same gel transferred by Southern blotting and hybridised with 16S rDNA-specific probe. The assay is positive for samples 32 and 41 as well as the positive control.
Given the initial 1:10 dilution of faecal material, this was equivalent to approximately 25 cfu present in each 25 μl PCR volume. Following Southern blotting and hybridisation with DIG-labelled DNA probes, the threshold of detection was lowered to between $10^4$ and $10^3$ cfu g$^{-1}$ of faeces (between 2.5 and 0.25 cfu per 25 μl PCR volume). The sensitivity of detection of different *Campylobacter* species type strains on CCDA fell between 10 and 100 cfu g$^{-1}$ of faeces (type strains of *C. jejuni*, *C. coli*, *C. lari* and *C. hyointestinalis*). It was less sensitive for *C. helveticus* and *C. fetus* – these type strains were detectable at $10^3$ cfu g$^{-1}$ of faeces. The type strain of *C. upsaliensis* was not detectable on CCDA (>10$^8$ cfu g$^{-1}$ of faeces).

5.3 Discussion

In this study, 16 samples (8%) of 200 unselected faecal samples sent for testing at a clinical laboratory proved to be culture-positive for *Campylobacter* species while 19 samples (9.5%) were found positive by PCR. In addition, PCR assay simultaneously provided identification to species level lacking from culture data. It showed that 16 samples contained *C. jejuni*, two contained *C. coli* and one contained *C. hyointestinalis*. Two of the PCR-positive samples were positive only after Southern blotting and hybridisation, included to maximise the sensitivity of the PCR. This additional hybridisation step did improve the detection rate of the PCR assays (although not significantly), but was time consuming and labour intensive.
In seeding experiments employing the type strains of *C. jejuni* and *C. coli*, for which CCDA was designed (Bolton et al., 1984b), the sensitivity of selective culture was superior to that of PCR. This may in part be due to the difference in sample size examined - while plates were inoculated with 100μl of diluted faecal material, the sample volume for PCR was forty-fold less. It is thought that certain *Campylobacter* species may be susceptible to the antibiotics incorporated in selective media (Goossens & Butzler, 1992; Anonymous, 1993), or there may be variable susceptibility to a selective antibiotic across a species. While the *C. lari*, and *C. hyointestinalis* type strains were detected by culture on CCDA with the same sensitivity as *C. jejuni*, those of *C. fetus* and *C. helveticus* grew less well and that of *C. upsaliensis* did not grow at all. The *C. upsaliensis* type strain had been isolated using the 'non-selective' membrane filtration technique (Sandstedt et al., 1983). In practice, the sensitivity of PCR assay for actual clinical samples is probably greater than that achieved in seeding experiments with log phase cultures of type strains. *Campylobacter* cells in faecal material will exist in a variety of metabolic states, some of which may not be amenable to culture – direct PCR will detect both culturable and non-culturable cells.

Three samples PCR-positive for *C. jejuni* were negative by culture. Since PCR detection and identification was achieved by sequential assays targeted at two different genes (the 16S rRNA gene of *C. jejuni*/*C. coli*, followed by the hippuricase gene) there is little likelihood of false PCR-positivity due to contamination. These PCR-positives may represent strains that were non-viable at the time of culture, or strains susceptible to components of the
selective isolation medium. It is also possible that these \textit{C. jejuni} amplicons might have been produced from strains of \textit{C. jejuni} subspecies \textit{doylei}, since our PCR assays detect both subspecies of \textit{C. jejuni}. This nitrate-negative subspecies grows more slowly at 37°C than \textit{C. jejuni} subspecies \textit{jejuni}. While \textit{C. jejuni} subspecies \textit{doylei} has been isolated from cases of gastroenteritis in children and from gastric biopsies in adults, its exact role as an agent of human disease is not yet understood (Steele & Owen, 1988).

\textit{C. hyointestinalis} was detected by PCR in one sample, but was not found by culture. This species is associated with proliferative enteritis in pigs and has only rarely been cited as a cause of gastroenteritis in humans (Fennell et al., 1986; Edmonds et al., 1987; Minet et al., 1988). In those cases \textit{C. hyointestinalis} was isolated on conventional selective agar, as could be done for the type strain in our seeding experiments. While some such strains are evidently resistant to the antibiotics used in selective media, they may not be representative of the species as a whole (Mishu \textit{et al}., 1992). This may have been the case with clinical sample # 121, which failed to yield colonies on CCDA, but gave a strong positive signal by species-specific PCR assay for \textit{C. hyointestinalis}.

In one case, \textit{Campylobacter} was detected by culture but not by PCR. This isolate was unfortunately not recovered later from storage. Among the possible explanations for the negative PCR result are that \textit{Campylobacter} cells may have been present at less than the detection threshold of the PCR assay. Again, the cells may have lysed \textit{in situ} in the interval between culture
Results and Discussion

and nucleic acid extraction, leaving genomic DNA susceptible to degradation by the diverse nucleases present in faecal material. There may, again, have been sufficient sequence diversity in the 16S rDNA of this particular isolate to have introduced primer mismatching.

There was no evidence of *Campylobacter* infection by either culture or PCR for 180 of the 200 faecal samples. Individual PCR assays for *C. lari*, *C. fetus*, *C. upsaliensis* and *C. helveticus* were all negative.

Detection of *Campylobacter* species by culture requires 48 hours to isolate the organism and assign a putative identification to genus level. Identification to the species level, which requires further investigation, is not normally undertaken as it is considered inconvenient or non cost-effective by diagnostic laboratories (Goossens & Butzler, 1992). While PCR is relatively labour-intensive, and costly compared to culture, direct PCR assay of faecal DNA simultaneously detects and identifies *Campylobacter* species, and a typical batch of 48 samples can be processed within eight hours (or longer if blotting and hybridisation are employed). The ‘non-selective’ detection and accurate identification provided by the PCR assay makes it a useful tool for epidemiological investigation of non-jejuni/non-coli *Campylobacter* species, as evidenced by the detection of the ‘uncommon’ species *C. hyointestinalis* in this relatively small survey (and in the even smaller study in Chapter 3). However, meaningful investigation of the aetiology of non-jejuni/non-coli *Campylobacter* species would require the application of the PCR assays to a much larger sample set.
Chapter 6: A large-scale survey of Campylobacter species in human gastroenteritis by PCR and PCR-ELISA

6.1 Background

In previous Chapters, it was shown that the non-selective detection and accurate identification offered by PCR-based analysis provides a valuable tool for epidemiological investigation of Campylobacter species. This is especially true of non-C. jejuni/non-C. coli Campylobacter species, the aetiology of which in human gastrointestinal illness has been poorly understood (Anonymous, 1993; Linton, 1996).

In the present Chapter the molecular-based approach evolved in Chapters 3, 4 and 5, was applied in a 'large-scale' study (> 3,000 samples) of the role of Campylobacter species in human gastroenteritis. Such a large undertaking was necessary to ensure adequate representation of 'uncommon' Campylobacter species and involved the analysis of clinical samples over a two-year period. To reduce the number of PCR's necessary for a comprehensive analysis of each sample a system of 'screening' PCR's (Lawson, et al., 1999) and species identification by PCR-ELISA (Metherell, et al., 1999) were employed. While in an effort to improve the sensitivity of the PCR assays the nucleic acid dye SYBR Green I was used instead of ethidium bromide (Karlsen, et al., 1995).

This study would not have been possible without the cooperation and interest of the staff of Ashford, Bangor, Central Middlesex, Chelmsford, Dorchester,
Exeter and Preston PHL's. At CPHL, Dr Meeta Desai, Dr Julie Logan and Ms Gael O'Neill also contributed to the logistical and analytical aspects of this study.

6.2 Results

Study design

Faecal samples were collected over a two-year period by seven PHLs in England and Wales - Preston PHL, Central Middlesex PHL, Exeter PHL, Chelmsford PHL, Ashford PHL, Dorchester PHL and Bangor PHL, (termed in this study A, B, C, D, E, F and G respectively). The samples were from cases of acute gastroenteritis submitted from general practice and outpatient departments or collected by environmental health officers. Repeat and follow-up samples, and samples from in-patients were not examined.

DNA was extracted from aliquots of faecal samples sent to the CPHL, not later than 10 days after initial receipt and culture at the collaborating laboratory. Samples were cultured at the source laboratory on selective media and no attempt was made at quantifying Campylobacter cells present. The lag between specimen culture and DNA extraction precluded direct quantitative comparison. Culture data collected by the collaborating laboratories were withheld until completion of 'blind' PCR assays on DNA extracted from corresponding faecal samples at CPHL. PCR data were then compared with the results of culture performed by the contributing laboratories.
Bacteriological investigation of clinical samples

The following investigations were performed at the contributing PHLS laboratories: Faecal samples were examined for *Campylobacter* species, *Clostridium difficile*, *E. coli* O:157, *Salmonella* species, *Shigella* species and ova, cysts and parasites by standard methods. With the exceptions of laboratories A and F, campylobacters were cultured on Campylobacter Blood-free Selective Agar Base (Oxoid CM739) with Charcoal Cefoperazone Desoxycholate Agar supplement (Oxoid SR155). Laboratory A used CAT supplement (Oxoid SR174) while laboratory F used a cefoperazone and amphotericin supplement (Prolab, Neston, UK). Plates were incubated for 48 hours at 37°C (labs A and B) or 42°C (labs C, D, E, F and G) under microaerobic conditions. Isolates were identified to the genus level by morphology and Gram stain. Laboratory A further identified isolates by hippurate, indoxyl acetate hydrolysis and urease production.

Extraction of nucleic acid from faeces

Upon receipt at CPHL, nucleic acid was extracted as previously described. The nucleic acid extracts were stored at -20°C prior to PCR screening. The screening PCR and subsequent identification steps are shown in the algorithm of Figure 6.1.

Screening primers

The first stage of the PCR algorithm was the application of screening primers which could co-detect (but not differentiate) multiple *Campylobacter* species, in this case *C. jejuni*, *C. coli*, *C. upsaliensis*, *C. lari* and *C. helveticus*. These
Figure 6.1. Algorithm for *Campylobacter* detection and speciation by PCR and PCR-ELISA

- **PCR screening**
  - cpg PCR
  - chycf PCR
  - Negative
  - Positive

- **PCR-ELISA**
  - Negative
  - C. jejuni/coli positive
  - non-C. jejuni/coli positive
  - Speciation
    - C. upsaliensis
    - C. hyointestinalis
    - C. lari
    - C. fetus
    - C. helveticus

- **Supplementary PCR tests**
  - hip PCR
  - asp PCR
  - hip or asp PCR positive
  - hip or asp PCR negative
  - hip and asp PCR positive
  - hip and asp PCR negative
  - Speciation
    - C. jejuni
    - C. coli
  - Confirm ELISA
    - cJcc PCR
  - Mixed infection?
    - ceuE PCRs
were termed the 'pathogenic group' or *cpg* primers (see section 10.1.2) and were designed in the course of this study as described in section 2.14. These yielded an amplicon of 1195 bp from DNA prepared from reference strains of the five species above, but not from the remaining species type strains of *Campylobacter* and of other enteropathogenic species referred to Appendix A. *C. hyointestinalis* and *C. fetus* were detected using the previously described *chycf* primers, employed at the screening stage. Attempts to design a single screening PCR for all potentially enteropathogenic *Campylobacter* species were not successful.

The nucleic acid extracts examined in the survey were screened in batches of 96 samples (including positive and negative controls) with both the *cpg* and *chycf* PCR assays. PCR was performed in a 96-well format microtitre plate as described in section 2.9. Products were analysed by 96-well format electrophoresis and gels were stained with SYBR green I and visualised under UV light as described in section 2.11.

At the screening stage, an inclusive approach was adopted and any amplicon of approximately appropriate size was investigated. On further investigation many of these proved to be false or spurious products. In the survey of 3738 samples, the *cpg* PCR was positive for 720 samples, while the *chycf* PCR was positive for 29 samples.
Results and Discussion

Species identification

Samples positive in the screening PCR assays were identified by PCR-ELISA employing capture probes specific for C. jejuni/C. coli, C. upsaliensis, C. hyointestinalis, C. lari, C. fetus and C. helveticus as described in section 2.18. The PCR-ELISA co-identified but did not distinguish samples containing C. jejuni and C. coli. These samples were further examined by the C. jejuni-specific hip PCR (section 2.10.4), the C. coli-specific asp PCR (section 2.10.5) and by way of confirmation the C. jejuni and C. coli-specific cjcc PCR (section 2.10.1.3). In cases where a mixed infection was suspected, two sets of ceuE gene primers (section 2.10.6) capable of distinguishing C. jejuni from C. coli were also used. The steps involved in species identification are summarised in the algorithm (Figure 6.1).

Of the 749 samples positive by the screening PCR assays (cpg and chycf), 492 samples were confirmed as Campylobacter-positive by PCR-ELISA as follows: C. jejuni/C. coli (477); C. jejuni/C. coli and C. upsaliensis (one); C. upsaliensis (10); C. hyointestinalis (three); and C. lari (one). The remaining 257 samples positive by screening PCR were PCR-ELISA negative. C. fetus and C. helveticus were not detected. The 478 samples positive for C. jejuni/C. coli by PCR-ELISA were investigated further as described above. Of these 408 were positive by the C. jejuni-specific hip primers and 16 were positive by the C. coli-specific asp primers. A further 19 samples were positive for both hip and asp PCRs, indicative of a mixed infection. These 19 samples were therefore investigated with the ceuE primers, one specific for the C. jejuni ceuE gene and one specific for the corresponding C. coli ceuE gene. All 19
were positive by both, confirming mixed infections. From the remaining 35 samples positive by PCR-ELISA for *C. jejuni/C. coli*, no *hip* or *asp* amplicon was obtained. These were also negative with *ceuE* primers. Although it was not possible to distinguish the two species in these 35 samples, the PCR-ELISA result was confirmed by a positive *cjcc* PCR.

**Comparison of PCR-based and culture-based detection**

The combination of results from PCR screening, PCR-ELISA and supplementary PCR assays allowed each sample to be assigned an overall molecular identification. This was then compared with culture data: ‘*Campylobacter* species’ were cultured by the seven contributing laboratories from 464 of the 3738 samples. Of these, 413 (410 *C. jejuni/C. coli*, two *C. upsaliensis* and one *C. hyointestinalis*) had been identified by PCR-ELISA from the corresponding faecal sample. The remaining 51 were culture positive but PCR-ELISA-negative.

A total of seventy-nine culture-negative samples were positive by screening PCR and by PCR-ELISA. They comprised 67 *C. jejuni/C. coli*, one mixture of *C. jejuni/C. coli* and *C. upsaliensis*, eight *C. upsaliensis*, two *C. hyointestinalis* and one *C. lari* – cf. Table 6.1). Thus the combination of ‘screening PCR’ and PCR-ELISA detected *Campylobacter* species in 543 of the 3738 samples (528 *C. jejuni/C. coli*, one mixture of *C. jejuni/C. coli* and *C. upsaliensis*, and 14 other non-*C. jejuni/C. coli*).
### Table 6.1. Detection of Campylobacter species

<table>
<thead>
<tr>
<th>Source</th>
<th>Samples examined</th>
<th>C. jejuni/C. coli&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C. upsaliensis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C. hyointestinalis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C. lari&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCR-ELISA culture</td>
<td>PCR-ELISA culture</td>
<td>PCR-ELISA culture</td>
<td>PCR-ELISA culture</td>
<td>PCR-ELISA culture</td>
</tr>
<tr>
<td>Lab A</td>
<td>1178</td>
<td>186/203</td>
<td>189/203</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Lab B</td>
<td>1107</td>
<td>106/121</td>
<td>108/121</td>
<td>3&lt;sup&gt;b&lt;/sup&gt;/3</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;/3</td>
</tr>
<tr>
<td>Lab C</td>
<td>725</td>
<td>63/73</td>
<td>65/73</td>
<td>1/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Lab D</td>
<td>300</td>
<td>55/57</td>
<td>35/57</td>
<td>1/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Lab E</td>
<td>166</td>
<td>20/21</td>
<td>12/21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lab F</td>
<td>162</td>
<td>31/36</td>
<td>35/36</td>
<td>1/1</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;/1</td>
</tr>
<tr>
<td>Lab G</td>
<td>100</td>
<td>17/18</td>
<td>17/18</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>3738</strong></td>
<td><strong>478/529</strong></td>
<td><strong>461/529</strong></td>
<td><strong>11/11</strong></td>
<td><strong>2/11</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup> Number found by specific technique/total found by both techniques. For example in the case of C. jejuni/C. coli, a total of 461/3738 were isolated by culture while 478/529 were identified by the PCR algorithm of Figure 1 (PCR-ELISA' above). A total of 529 were found by a combination of methods. i.e. 68 were not detected by culture and 51 were not identified by PCR-ELISA.

<sup>b</sup> Includes one mixed infection of C. upsaliensis and C. jejuni detected by PCR-ELISA.

<sup>c</sup> In these cases the culture isolated was identified only as ‘Campylobacter species’ - Subsequent species identifications are from PCR-ELISA.
Figure 6.2. Detection of *Campylobacter* species by PCR and PCR-ELISA and comparison with culture: A study of 3137 faecal samples.

Results and Discussion

**CO**

**O**

**CD**

**One C.** *Campylobacter jejuni/coli* positive 529

**C. upsaliensis**

PCR-ELISA & Culture 2

PCR-ELISA only 2

**C. hyointestinalis**

PCR-ELISA & Culture 1

PCR-ELISA only 1

**C. lari**

PCR-ELISA only 1

PCR-ELISA & Culture 9*

Campylobacter species negative 3194

**PCR-ELISA only** was found in a sample also positive for *C. jejuni* (PCR-ELISA & Culture).

* One *C. upsaliensis* (PCR-ELISA only) was found in a sample also positive for *C. jejuni* (PCR-ELISA & Culture).
The detection of Campylobacter species in this study by PCR-based analysis versus selective culture, is compared in Figure 6.2. A breakdown, by sending laboratory, of data for C. jejuni/C. coli and non-C. jejuni/non-C. coli Campylobacter species is provided in Table 6.1.

Statistical analysis

The results of Campylobacter detection by PCR screening and PCR-ELISA were compared with those obtained by culture on selective agar at contributing laboratories using McNemar's test. There was no statistical difference between the number of C. jejuni/C. coli positive samples detected by PCR-ELISA or by culture in the study as a whole (0.5 > P > 0.1). In terms of individual sending laboratories, there was no statistical difference between the culture and PCR-ELISA for laboratories A, B, C, (P > 0.5), F (0.5 > P > 0.1) and G (P = 0.5). However, the detection rate was significantly higher by PCR-ELISA than culture for laboratories D (0.001 > P) and E (0.02 > P > 0.01).

6.3 Discussion

A key development in this study was the design of PCR assays specific for groups of enteropathogenic species, rather than employing series of individual species-specific PCRs as previously described. This was intended to reduce the number of PCR assays performed in the course of a large-scale survey. The basis for these screening PCRs (Figure 6.1) was the phylogenetic tree drawn from the alignment of Campylobacter 16S rDNA sequences (Figure 6.3). This tree shows three distinct clades (species groups). The first contains
Results and Discussion

Figure 6.3. 16S rDNA phylogenetic tree of the genus *Campylobacter* showing three major clades.
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C. gracilis, C. sputorum, C. curvus, C. concisus, C. rectus and C. showae, organisms principally associated with niches in the periodontal cavity of man and animals, and which have as yet no association with human gastroenteritis. The second clade consists of C. fetus, C. hyointestinalis, C. lanienae and C. mucosalis, which are associated with disease and colonisation of farm animals (the first two have also been occasionally implicated in human disease). The third clade consists of C. jejuni, C. coli, C. lari, C. upsaliensis and C. helveticus, species (other than C. helveticus) known to cause gastroenteritis in man. In accordance with the aims of this investigation, primers were developed for the third clade, and an existing set of primers was used for C. fetus and C. hyointestinalis, the only other putative causative agents of human gastroenteritis.

The use of PCR-ELISA for primary species identification was largely successful, C. jejuni/C. coli and five other Campylobacter species could be differentiated following a single PCR reaction. However, the PCR-ELISA was unable to differentiate C. jejuni and C. coli, which in the vast majority of cases have remarkably similar 16S rDNA sequences. The notable exception is the C. coli type strain (NCTC 11366) the 16S rDNA sequence of which is clearly divergent from that of C. jejuni (see Figure 6.3). A C. coli type strain-specific probe (COL) was included in the PCR-ELISA (see section 2.17), but was negative with all other C. coli tested. Clearly NCTC 11366 is not representative of the majority of current C. coli strains (Metherell, et al., 1999). Differentiation of C. jejuni and C. coli was possible using either the hip, asp or ceuE PCR assays. However, these assays based on single copy genes were
not as sensitive as the 16S rDNA-based (three gene copies per genome) cjcc PCR, consequently 35 samples *C. jejuni/C. coli*-positive by PCR-ELISA remained undifferentiated.

SYBR Green I did improve sensitivity the sensitivity of the PCR assays slightly. Amplicons produced from seeded faecal samples stained with SYBR Green I were detected between $10^4$ and $10^5 \text{ cfu g}^{-1}$ as compared with ~$10^5 \text{ cfu g}^{-1}$ when stained with ethidium bromide. However, SYBR Green I was considerably more expensive, had a much shorter half-life and was more prone to deteriorate due to light and temperature than ethidium bromide.

In the course of the study several important practical issues were identified, which would be of importance to any large-scale PCR-based survey. They included the necessity of a dedicated PCR suite to reduce the risk of contamination, robust thermocycling apparatus and reagents, and regular monitoring of the performance and detection threshold for PCR primers. It was found that the titre and quality of these primers could deteriorate over time, and that variation between batches and between manufacturers was commonplace.

*C. jejuni* and *C. coli*, as expected, contributed the largest proportion of PCR-positive samples. There was congruence between PCR and culture for 77.5% of the 529 positives. 12.9% were found only by PCR and 9.6% only by culture. In any comparison of two detection methods on a sizeable sample number, one would not expect complete correlation. Nonetheless, there are several
Results and Discussion

noteworthy factors to consider. 'Culture-positive only' samples may have been PCR-negative due to degradation of *Campylobacter* cells and DNA in the period (up to ten days) between culture and receipt of the faecal sample for DNA extraction. In some cases inhibitory substances present in faeces may have reduced the sensitivity of the PCR assays. There is also the possibility that certain wild type *C. jejuni* or *C. coli* strains might have 16S rDNA sequences that are sufficiently divergent not to be detected by the PCR-ELISA. A proportion of isolates (supplied by laboratory C) from culture-positive/PCR-negative samples, were all successfully identified as *C. jejuni* by the PCR algorithm upon retesting. This suggests that culture-positive PCR-negative samples sometimes could occur due to sampling factors, rather than variation in target DNA sequences.

In previous chapters selective culture was found to be more sensitive than PCR in seeding experiments with log phase cultures of laboratory strains of *C. jejuni*. This observation may be due to the amount of faecal material that is sampled in inoculating a selective agar plate, as opposed to the smaller volume of diluted material (2.5 µl) sampled by PCR. Nonetheless more positive samples were detected by PCR-ELISA than by culture alone. This probably reflects the detection of *Campylobacter* cells in metabolic states that are less amenable to culture on selective media (sub-lethally damaged, viable but non-culturable or even 'dead' cells) and the detection and identification of non-jejuni/non-coli campylobacters. A key feature of the PCR algorithm was that it provided both detection and identification.
The eleven *C. upsaliensis* detected by PCR-ELISA represent an incidence of 0.29%. Only two of these samples had been positive by culture, and in each case, the isolates were reported as 'Campylobacter species' and were not saved for further analysis. The sex/age distribution from the *C. upsaliensis* positives (m/2, m/5, m/10, f/23, m/25, f/27, f/33, m/78, m/u, m/u, u/u, where m = male, f = female, u = unspecified) showed no evidence of association with paediatric gastroenteritis, as has been previously reported (Megraud & Bonnet, 1986; Goossens et al., 1990a).

The three cases of *C. hyointestinalis* represent an incidence of 0.08%. Only one of these samples was reported as 'Campylobacter species' positive by culture (the isolate was not available for further investigation). The sex/age distributions were m/39, f/66, f/u. This represents the highest incidence of *C. hyointestinalis* yet reported from human gastroenteritis. The only other incidence figure available, based on culture, was 0.01% - two out of 15,185 cases (Goossens et al., 1990a). In chapters 4 and 5, *C. hyointestinalis* was detected in one out of 25 and one out of 200 gastroenteritis cases, respectively (Linton et al., 1997; Lawson et al., 1998b). Altogether, this would give an incidence of 0.13%, representing five out of the 3,963 cases of human gastroenteritis examined by PCR-based analysis in the course of this thesis.

Although *C. hyointestinalis* is associated with proliferative enteritis in pigs (Gebhart et al., 1985), it has previously been considered to be only a very rare cause of human gastroenteritis (Fennell et al., 1986; Edmonds et al., 1987; Minet et al., 1988). These results suggest that the association of *C. hyointestinalis* with human gastroenteritis is stronger than previously thought.
C. lari (by PCR-ELISA alone) was detected in only one sample (an incidence of 0.03%) although this species is often cited (Linton, 1996) as the third most commonly isolated enteropathogenic campylobacter in man. The low incidence in this survey suggests that C. lari is less important than C. upsaliensis and C. hyointestinalis in human gastroenteritis.

In 19 samples there was evidence from supplementary PCR assays of a mixed infection of C. jejuni and C. coli not apparent from culture. Seven of these occurred in a laboratory (A) which had the capacity to identify some isolates to species level, and reported five as C. jejuni and two as C. coli. It is likely therefore that only the predominant colony type had been selected for identification. It is interesting that there were slightly more mixed C. jejuni and C. coli infections than C. coli infections alone. Mixtures of Campylobacter species (and serotypes) in human infection may be more common than previously assumed (Ruberg et al., 1998). Another mixed Campylobacter infection noted in this survey was of C. upsaliensis and C. jejuni co-detected by PCR-ELISA, here, only the C. jejuni component was found by culture.

There were also instances of co-infections of C. jejuni/C. coli with other enteropathogens detected by culture and/or microscopy at the contributing laboratories. There were mixtures of C. jejuni with both Sh. sonnei and Blastocystis hominis cysts (one), with Salmonella species (two), and with Cryptosporidium parvum oocysts (three). The Cryptosporidium mixtures are of interest since the presence of this coccidian parasite, which is most frequently associated with water-borne infection (Tzipori & Griffiths, 1998), suggests that the route of transmission of C. jejuni may have been water in these cases.
PCR is more expensive and labour-intensive than selective culture, but does offer a non-selective means of monitoring the incidence of enteropathogenic bacteria. This facet is particularly valuable for epidemiological investigations. Ultimately PCR-based analysis will become amenable to automation and this will reduce the labour component of the final cost. The use of broad-specificity ‘screening’ PCRs greatly reduces the number of assays required for a comprehensive survey.

In summary, the PCR algorithm presented here offers a different perspective on Campylobacter gastroenteritis than that provided by culture, giving information on the identity and occurrence of species that are not detected by standard methodologies employing selective culture. At the time of writing, this study remains the largest molecular survey of Campylobacter gastroenteritis yet undertaken and represents the first application of a PCR-based protocol to investigate the incidence of enteropathogenic Campylobacter species in an epidemiologically valid context.
Chapter 7: *Campylobacter* and the gastrointestinal tract of healthy humans: Discovery of 16S rRNA gene sequences of a novel species – 'Candidatus Campylobacter hominis'.

7.1 Background

Members of the genus *Campylobacter* occupy differing niches in a variety of hosts. *C. jejuni* and *C. coli* colonise the intestinal tract of poultry, pigs and cattle asymptotically, but cause gastroenteritis in humans, while *C. concisus* and *C. sputorum* are isolated from the human gingival crevice, where they are sometimes associated with periodontal disease (Skirrow, 1994). Campylobacters have relatively fastidious growth requirements and require a microaerobic atmosphere, and certain members of the genus are unable to grow on selective media (Goossens & Butzler, 1992). It has been estimated that up to 40% of bacterial forms observed in microscopic examination of human faeces remain uncultured in the laboratory (Berg, 1996). It is therefore possible that some of these may represent undetected *Campylobacter* species in the human gastrointestinal tract.

PCR amplification of the 16S rRNA gene, and subsequent phylogenetic analysis of the sequence data, has facilitated a new approach to the phylogenetic identification of bacteria directly from their natural habitat or area of pathology. Strategies based upon this approach have been used to identify uncultivable bacteria, such as the agents of human ehrlichiosis (Anderson et al., 1991) and Whipple's disease (Relman et al., 1992). Another application
has been in the analysis of complex bacterial communities such as dental plaque (Wilson et al., 1997).

The aim of the work described in this chapter was to assess the incidence of Campylobacter species in the gastrointestinal tract of healthy humans using a 'non-selective' 16S rDNA PCR-based molecular approach. I thank Dr Dennis Linton for his help with various aspects of the sequence analysis portion of this chapter.

7.2 Results

Sample collection from healthy human subjects

Faecal and saliva samples for investigation were provided by 20 healthy human subjects (12 male, 8 female; age range 6 months to 45 years) without current or recent gastrointestinal symptoms. Additionally, five faecal samples from cases of acute gastroenteritis from which no pathogens were isolated ('NPI' samples of Chapter 4) were also examined. All samples were collected and processed as described in section 2.4. Nucleic acid was extracted from a 100 μl aliquot of homogenised faeces or saliva using the GuSCN-silica method (section 2.7.3) followed by treatment with PVP (section 2.8.2). Extracted nucleic acid was stored at -20°C.

PCR analysis

Nucleic acid samples were examined using previously described PCR primers specific either for the genus Campylobacter or for individual species therein.
The following PCR's were employed: 16S rDNA-based PCR primers \((cgen)\) designed to detect all members of the genus *Campylobacter* (see section 2.10.1.1); 16S rDNA-based PCR primers \((cjjc)\) that co-detected *C. jejuni* and *C. coli* (see section 2.10.1.3), 16S rDNA-based primers for other *Campylobacter* species, namely: *C. helveticus* and *C. upsaliensis* \((chcu)\), *C. hyointestinalis* and *C. fetus* \((chycf)\), *C. lari* \((cla)\) and *C. rectus* \((rec)\) - section 2.10.1.3; or primers based on the 23S rRNA genes of *C. sputorum* \((spu)\) and *C. concisus* \((con)\) - see section 2.10.2. PCR was carried out as described in section 2.9.

From the 'healthy subjects' (termed 'HS'), 18 of 20 faecal samples yielded an amplicon with the \(cgen\) genus-specific primers. Four of these were also positive with the species-specific PCR assays: three with \(con\) (*C. concisus*) and one with \(rec\) (*C. rectus*). Nineteen of the 20 saliva samples produced amplicons with \(cgen\) genus-specific primers. These same 19 samples also yielded amplicons with \(con\) (*C. concisus*-specific) primers. One individual \((#14)\) was also positive for *C. rectus* (positive in saliva and faecal samples).

All of the faecal and saliva samples were PCR-negative for *C. upsaliensis*, *C. helveticus*, *C. lari*, *C. fetus*, *C. hyointestinalis*, *C. jejuni*, *C. coli* and *C. sputorum*. These results are summarised in Table 7.1.

From the diarrhoeic 'NPI' faecal samples, two of the five were positive with the \(cgen\) genus-specific primers, but all five were negative with the species-specific primers (see Table 7.1).
Table 7.1 Comparative PCR assay of faecal and saliva samples.

<table>
<thead>
<tr>
<th>No.</th>
<th>PCR from faeces</th>
<th>PCR from saliva</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>cgen</td>
<td>con</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
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</tr>
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</table>

Total: 18/20 3/20 10/20 1/20 19/20 19/20 0/20 1/20
%
90 15 50 5 95 95 0 5

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<tr>
<th>No.</th>
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<th>Not examined</th>
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<td>-</td>
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</table>

Total: 2/5 0/5 0/5 0/5
%
40 0 0 0

cgen - Campylobacter genus-specific PCR assay
con - C. concisus-specific PCR assay
hs - PCR assay specific for the 'HS' 16S rDNA sequence
other - PCR assays specific for C. jejuni, C. coli, C. lari, C. upsaliensis,
       C. helveticus, C. hyointestinalis, C. fetus, C. rectus and C. sputorum
* - PCR-positive for the C. rectus-specific assay
† - Faecal sample culture-positive for C. concisus
Culture of *Campylobacter* species

All faecal samples were examined for the presence of *Campylobacter* species by culture on CCDA (section 2.2.1) and by the membrane filter method (section 2.2.2). Inoculated plates were incubated for up to seven days at 37°C under microaerobic conditions. Only one of the faecal samples (#7) from a healthy subject was culture-positive. Scanty colonies of *C. concisus* were detected using the membrane filter method. This sample was also positive for *C. concisus* by PCR assay. No other *Campylobacter* species were isolated by membrane filter or CCDA culture. Saliva samples were not examined by culture.

Sequence analysis

A new set of *Campylobacter* genus-specific primers (*cgena*) (see section 2.10.1) were designed (as described in section 2.14) to generate a 1450 bp amplicon suitable for sequence analysis. Eighteen 'HS' and two 'NPI' faecal samples previously positive with the *cgen* primers were reanalysed and all yielded 1495 bp amplicons with the new *cgena* primers. The six strongest *cgena* amplicons were produced from four 'HS' samples (HS-A, B, C and D) and two 'NPI' samples (NPI-A and B). These samples were all negative by species-specific PCR. The amplicons were sequenced (section 2.19) and compared with a database of known 16S rRNA sequences as described in section 2.19.

All six sequences readily aligned within the genus *Campylobacter* and a neighbour-joining tree was derived from phylogenetic analysis (Figure 7.1).
Figure 7.1 Campylobacter 16S rDNA phylogenetic tree showing positions of sequences from 'HS' and 'NPI' faecal samples.
The sequences of HS-D, NPI-A and B clustered together with the *C. concisus* rDNA sequence and were more than 95% similar to each other. The HS-A, B and C sequences were more than 99% similar to each other and clustered together within *Campylobacter*, but were distinct from any previously described species. A sequence dissimilarity matrix was constructed from the phylogenetic data (Table 7.2). The percentage dissimilarity between the HS sequences was 0.2% (between HS-A and HS-B) to 0.8% (between HS-A and HS-C). Dissimilarities between HS-A and the most closely related known species were much greater: *C. sputorum* 6.2%, *C. gracilis* 6.6%, *C. rectus* 7.2%, *C. concisus* 7.3%, *C. curvus* 7.5% and *C. showae* 7.7%. The percentage dissimilarity between HS-A and *C. jejuni* was 10.1% and the intergeneric value between HS-A and *H. pylori* was 17.6%.

The HS-A, B and C sequences were analysed with the program ‘Check_Chimera’ available from the Ribosomal Database Project website (Maidak *et al.*, 1997), and this experiment excluded a chimaeric origin for the sequences. A secondary structure model of the rRNA sequence of the PCR generated amplicon of HS-A was prepared, based on a model of the 16S rRNA of *C. sputorum* subsp. *sputorum*. This model (Figure 7.2) confirmed the integrity of the HS-A sequence rRNA secondary structure. Additionally, the secondary structure of the V2 region of the HS-A 16S rRNA model was compared with known conformations of this region (Figure 7.3). This (cf. boxed area of Figure 7.2) was characteristic of the *Campylobacter* subgroup within the epsilon subdivision of the *Proteobacteria*, and was distinct from that of the *Helicobacter* subgroup (Lane *et al.*, 1992).
Table 7.2 Dissimilarity matrix of *Campylobacter* 16S rDNA sequences

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Figure 7.2 Proposed 16S rRNA secondary structure of sequence HS-A (‘Candidatus C. hominis’), based on a model retrieved from the Ribosomal Database Project (Maidak et al. 1997). The characteristic V2 region is boxed (cf Figure 7.3).
Figure 7.3 Secondary structures of the V2 region of 16S rRNA for the five subdivisions of the Proteobacteria, adapted from Lane et al. (1992). Differences formed in the structures of *Campylobacter* and *Helicobacter* are indicated. The 'HS' sequences exhibit the structure typical of *Campylobacter*. 
These data all suggest that the origin of the HS-A, B and C sequences was a novel, previously undescribed, *Campylobacter* species.

**Further investigation of the novel *Campylobacter* species**

A set of PCR primers (*hs* – see section 2.10.1) specific for the HS-A, B and C sequences were designed (as described in section 2.14.1). These *hs* primers produced a predicted 1356 bp amplicon from ten of the 20 'HS' faecal samples previously examined, including the three that yielded the HS-A, B and C sequences (see Table 7.1). The ‘NPI’ samples were all negative with the *hs* primers as were the reference strains of *Campylobacter*, *Helicobacter* and *Arcobacter*. The *hs* PCR assay did not produce an amplicon when it was applied to DNA extracted from 'healthy volunteer' saliva (see Table 7.1).

Follow-up faecal samples were obtained from individuals PCR-positive for the HS sequence. Further attempts to isolate *Campylobacter* species by the membrane filter method were made. Filtrates were cultured on CBA and FAA incubated at 25°C, 37°C and 42°C, in both microaerobic and anaerobic atmospheres (section 2.1). No *Campylobacter* colonies were seen after seven days incubation under microaerobic conditions. Under anaerobic conditions, a mixed growth of bacteria was obtained, but neither individual colonies nor sweeps of the plate(s) yielded PCR-positives (*Campylobacter* genus-specific or ‘HS’ sequence-specific assays).

Both the *hs* and *con* PCR assays were applied to DNA extracted from 200 cases of acute gastroenteritis (the samples collected and examined in chapter
4). Of these 50/200 samples were positive with the con primers and 22/200 were positive with the hs primers. A summary of the distribution of C. concisus and the 'HS' sequence Campylobacter in faeces and saliva as determined by PCR is shown in Table 7.3.

7.3 Discussion

Uncultivable microorganisms associated with certain pathological processes have previously been characterised by analysis of 16S rRNA gene sequences. For example Tropheryma whippelii the agent of Whipple's disease (Relman, et al., 1992) and 'Gastrospirillum hominis', (Solnick et al., 1993). In these pathological conditions a microbial aetiology based on microscopic observation of histological tissue sections had already been established. PCR amplification and sequence analysis of prokaryotic 16S rDNA from infected eukaryotic tissue, which was relatively free of other bacteria then established the species identity of the infective agent. By contrast, in the analysis of complex bacterial communities such as dental plaque (Wilson, et al., 1997), molecular identification of the uncultivable bacterial components has been made possible by representative cloning of prokaryotic 16S rRNA amplicons, followed by sequence analysis. It has been recommended that novel taxa identified in this culture-free manner be accorded provisional status (Candidatus) as incompletely described procaryotes (Murray & Schleifer, 1994; Murray & Stacklebrandt, 1995). The experiments described in this thesis represent a distinct approach to the
Table 7.3 Relative distribution of the ‘HS’ and *C. concisus* sequences in healthy faeces, saliva and diarrhoeic faeces as determined by PCR assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PCR assays</th>
<th><em>C. concisus</em></th>
<th>'HS' sequence</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td><em>Campylobacter</em></td>
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<tr>
<td>'Healthy Subject'</td>
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<td>95%</td>
<td>0%</td>
</tr>
<tr>
<td>Saliva</td>
<td></td>
<td>(19/20)</td>
<td>(0/20)</td>
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<tr>
<td>'Healthy Subject'</td>
<td></td>
<td>15%</td>
<td>50%</td>
</tr>
<tr>
<td>Faeces</td>
<td></td>
<td>(3/20)</td>
<td>(10/20)</td>
</tr>
<tr>
<td>Diarrhoeic</td>
<td></td>
<td>25%</td>
<td>11%</td>
</tr>
<tr>
<td>Faeces</td>
<td></td>
<td>(52/205)</td>
<td>(22/205)</td>
</tr>
</tbody>
</table>
Results and Discussion

molecular ecology of complex communities. In the present study the specificity of PCR primer design at the level of a bacterial genus was employed to selectively amplify target genes from a microbial ecosystem, in this case *Campylobacter* genes from faecal material from healthy humans. The same approach might have diverse applications in the investigation of the molecular ecology of enteric disease. This is perhaps especially relevant to the genus *Campylobacter* which has relatively fastidious growth requirements and is thought to have viable but non-culturable states (Jones et al., 1991).

The human gastrointestinal tract has been estimated to contain ~$10^{14}$ bacteria belonging to many species. These occupy a progressively changing microbial habitat from the oral cavity through the stomach and small bowel to the large bowel (Berg, 1996). Several *Campylobacter* species are associated with the periodontal niche within the oral cavity, notably *C. concisus*, *C. gracilis*, *C. rectus* (Tanner et al., 1981) and *C. showae* (Etoh et al., 1993). However, little is known of the role of *Campylobacter* species as components of the microflora of the lower gastrointestinal tract of healthy humans. In this Chapter 16S rRNA sequences originating from a previously undescribed and at the time non-cultivable *Campylobacter* species were to be found in faecal material from half of the healthy humans studied, irrespective of their age or sex. The provisional new species was therefore termed ‘*Candidatus* Campylobacter hominis’ (L. gen. n. *hominis* of man, host where first isolated).

A total of 25 faecal samples from healthy humans and ‘NPI’ gastroenteritis were initially examined. Of these 20 samples yielded *Campylobacter* genus-
specific amplicons of which only four were positive with published species-specific PCR assays (for 10 of the 15 species) – three C. concisus and one C. rectus. Sequencing of six of the non-attributed genus amplicons revealed that while three probably originated from C. concisus, the origin of the remainder was a novel species, 'Candidatus C. hominis'. Specific primers designed from the 'Candidatus C. hominis' sequences showed that this novel species was present in ten of the 25 faecal samples initially examined. In 16 of 20 genus-positive faecal samples it was possible to account for the genus signal – nine contained 'Candidatus C. hominis', one contained both 'Candidatus C. hominis' and C. concisus, five C. concisus alone (including three identified by sequencing) and one C. rectus (Table 7.1). In four samples the genus amplicon could not be accounted for. This might reflect a difference in sensitivity between the genus and species-specific PCR's, as with the cgena and con primers in HS-D, NPI-A and B. Alternatively, these genus amplicons might have originated from Campylobacter species for which PCR assays are not presently available, such as C. showae or C. gracilis.

'Candidatus C. hominis' was found in 50% of healthy human faeces, 11% of diarrhoeic faeces and was absent from human saliva. From this it would seem that 'Candidatus C. hominis' is an inhabitant of the gut rather than the oral cavity, that it is normally present in the healthy gut and that its numbers drop below the detection threshold when the gut flora is disturbed by diarrhoea.

By contrast, C. concisus was detected in 15% of healthy human faeces, 25% of diarrhoeic faeces and 95% of saliva samples. From this it would appear
that *C. concisus* is an oral cavity microbe which is present in the lower gastrointestinal tract less frequently and probably transiently. In cases of diarrhoea, the PCR detection rate of *C. concisus* increase may be due to either a quicker transit time through the gut (with less opportunity for degradation of *C. concisus* DNA) or to a reduction in competitive DNA sequences/inhibitors of PCR. It is possible than in certain cases *C. concisus* is able to opportunistically colonise the recovering gut.

The normal habitat of *C. concisus* and *C. rectus* is the oral cavity. *C. rectus* has been implicated as a periodontal pathogen because it is isolated in higher levels from diseased rather than healthy periodontal sites (Ashimoto et al., 1996; Macuch & Tanner, 2000). *C. concisus* is commonly isolated from relatively shallow and healthy periodontal sites (Macuch and Tanner, 2000). *C. concisus* is occasionally isolated from faeces (when non-selective methods are employed) and some workers have suggested that *C. concisus* might be enteropathogenic (Lindblom et al., 1995; Van Etterijck et al., 1996; Engberg et al., 2000). However, in these studies it appears that *C. concisus* is isolated equally readily from both diarrhoeal cases and from the faeces of healthy people.

The presence of apparently non-pathogenic *Campylobacter* species in a significant proportion of human faeces might have certain consequences for the development of molecular-based detection strategies. *Campylobacter* genus-based approaches must now be treated with some caution. For
Figure 7.4 Schematic representation of the *Campylobacter* 16S rRNA gene showing the position of the *cgena* genus-specific PCR amplicon and other species-specific PCR amplicons.

- **cgena** - *Campylobacter* genus-specific PCR amplicon
- **cjcc** - *C. jejuni* or *C. coli* amplicon
- **cla** - *C. lari* amplicon
- **chcu** - *C. upsaliensis* amplicon
- **chycf** - *C. hyointestinalis* amplicon
- **C. fetus** amplicon
example, it was hoped that the large amplicon produced by the *Campylobacter* genus-specific primers *cgena* could be used as a target for the enteropathogenic species-specific primers (see Figure 7.4.), effectively creating a nested PCR, which offered both a commensurate increase in sensitivity and a means of reducing the number of necessary PCR's by employing the first round genus PCR as a screen. However, from the data gathered in this Chapter, at least 36% of diarrhoeic faeces contain non-enteropathogenic members of the genus *Campylobacter*. 
Chapter 8: Isolation and characterisation of a new species, Campylobacter hominis sp. nov., from the human gastrointestinal tract

8.1 Background

In Chapter 7, 16S rDNA sequences corresponding to a previously undescribed species of Campylobacter were amplified by genus-specific PCR from human faecal material. The analysis of these amplicons characterised their source as a new taxon, 'Candidatus Campylobacter hominis'. Initial attempts to isolate this organism were unsuccessful.

In the present Chapter further efforts to cultivate and characterise 'Candidatus Campylobacter hominis' are described, culminating in its isolation and full description as Campylobacter hominis sp. nov.

8.2 Results

PCR analysis of faecal samples

Fresh faecal samples were collected from 18 human subjects with no current or recent gastrointestinal symptoms. All samples were collected and processed as described in section 2.4. Nucleic acid extraction was performed by the GuSCN-silica method (section 2.7.3) followed by treatment with PVP (section 2.8.2).
Results and Discussion

The nucleic acid extracts were examined using a 16S rDNA PCR assay specific for ‘Candidatus C. hominis’ (hs) and with a Campylobacter genus-specific assay (cgena) see section 2.10. Amplicons specific for ‘Candidatus C. hominis’ were found in five out of 18 healthy faecal samples examined (26.8%). Fresh faecal samples were obtained from these five individuals for culture.

Isolation procedures

The faecal samples were examined for Campylobacter species by culture on CCDA and CAT (section 2.2.1) and by the membrane filter (MF) method followed by culture on both CBA and FAA (section 2.2.2). All samples were cultured and incubated at 37°C for up to 21 days under either microaerobic or anaerobic conditions, and were examined at regular intervals. Mixtures of bacterial growth or Campylobacter-like colonies were screened by PCR assays for the genus (cgena) and the Candidatus taxon (hs).

After 21 days, all plates (CCDA, CAT and MF) incubated microaerobically showed little or no growth, and any growth found was negative by Campylobacter genus-specific and by ‘Candidatus C. hominis’-specific PCR assays. CCDA and CAT plates incubated anaerobically were also negative. When incubated anaerobically plates inoculated using the MF method produced confluent mixed bacterial growth. Four out of five samples from anaerobic FAA colony sweeps were positive by both Campylobacter genus-specific PCR and ‘Candidatus C. hominis’-specific PCR. These plates were
further examined by the dilution and IMS methods described below. All anaerobic BA plates were PCR-negative by both assays.

**Isolation by dilution and immunomagnetic separation**

Pure cultures of *Campylobacter*-like colonies were obtained by either dilution or immunomagnetic separation (IMS). In the former case colony sweeps were simply resuspended and diluted $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ in brucella broth. The diluted suspensions were inoculated on FAA and incubated anaerobically for 10 to 20 days. This simple approach was successful in one of the five samples. In that case, three distinct colony types were detected from a single faecal sample, all '*Candidatus C. hominis'*-specific PCR-positive. These were termed CH001A (1.0 mm diameter, grey, flat, spreading colony), CH001B (0.5 mm diameter, grey, convex, entire colony) and CH001C (1.0 mm diameter, grey, convex, entire colony).

'*Candidatus C. hominis'*-specific PCR-positive mixed colony sweeps were also investigated using the IMS technique (section 2.2.3). A suspension of the PCR-positive mixed bacterial growth was mixed with either a *Campylobacter* flagella-specific antibody or a *Campylobacter* cell wall-specific antibody. The antibody-bound *Campylobacter* cells were then removed using protein G-coated magnetic beads. Using this approach, the cell wall-specific IMS yielded '*Candidatus C. hominis' PCR-positive colonies, CH002 and CH003, each from further individual faecal samples. Both isolates were similar in appearance to colony type CH001A. IMS with flagellin-specific antibody was unsuccessful.
Either dilution or cell wall-specific IMS strategies thus yielded ‘Candidatus C. hominis’ PCR-positive colonies from three of the five individuals whose faecal samples were positive in the same PCR assay performed directly on DNA extracted from those faecal samples. These strains initially grew very slowly (ten days minimum) and only on FAA. After a few subcultures on FAA they could be successfully grown on CBA; anaerobic conditions were essential. Either very feeble growth or no growth of the isolate at all was observed under microaerobic conditions.

**Sequencing of 16S rDNA and phylogenetic analysis.**

DNA was extracted from one of the putative C. hominis isolates (strain CH001A) using the guanidium-chloroform method (section 2.7.2). From this 16S rDNA was amplified using the genus-specific PCR cgena. The resulting 1414-nucleotide amplicon was sequenced and analysed as described in sections 2.14 and 2.19.

Phylogenetic analysis showed that the 16S rDNA sequence of CH001A clustered among the sequences of ‘Candidatus C. hominis’ previously found only from faecal material (Figure 8.1). It was 0.7% divergent from HS-A, 0.1% from HS-B and HS-C. Dissimilarities between CH001A and the most closely related *Campylobacter* species were as follows: C. sputorum 6.2%; C. gracilis 6.7%; C. rectus 7.0%; C. concisus 7.4%; C. showae 7.4%; and C. curvus 8.3%. Other dissimilarities included: C. jejuni 10.5%; A. skirrowii 15.7%; *H. pylori* 18.1%; and *E. coli* 28.7%.
Figure 8.1. *Campylobacter* 16S rDNA phylogenetic tree showing the positions of uncultured 'Candidatus C. hominis' sequences and the sequence of isolate CH001A.
Results and Discussion

Mol% G + C determination
Genomic DNA samples from isolates CH001A, CH002 and CH003 were prepared using the guanidine-chloroform method (section 2.7.2). DNA base composition (mol% G + C content) was estimated using the thermal denaturation temperature method (Owen & Pitcher, 1985) section 2.20. The DNA base composition of these genomic DNA extracts were determined as 32 to 33 mol% G + C.

DNA-DNA homology
DNA-DNA slot-blot hybridisation was performed on genomic DNA as described in section 2.17, using DNA of CH001A as the probe. Hybridisation was performed under optimal renaturation conditions; 2 x SSC (0.3 M NaCl, 0.3 M sodium citrate) at 62°C. Density analysis was used to determine homology values relative to self-hybridisation of the probe with target DNA of CH001A (Figure 8.2). The relative homology values obtained by DNA-DNA hybridisation in this manner were as follows: CH001A; 100%; CH002; 91.4% and CH003; 98.7%. All other Campylobacter type species, H. pylori, A. skirrowii and E. coli, showed no detectable homology.

Electron microscopy
Electron micrographs of cells from colonies of C. hominis sp. nov. were prepared as described in section 2.21. Preparations of closely related species were also made. Electron micrographs of C. gracilis and C. sputorum cells are shown in Figures 8.3 and 8.4 respectively, while cells of C. hominis sp. nov. are shown in Figures 8.5 and 8.6 (Photography by Dr Henrik Chart).
## Results and Discussion

<table>
<thead>
<tr>
<th>Species</th>
<th>DNA-DNA Homology</th>
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<tr>
<td>CH001A</td>
<td>100%</td>
</tr>
<tr>
<td>CH002</td>
<td>91.4%</td>
</tr>
<tr>
<td>CH003</td>
<td>98.7%</td>
</tr>
<tr>
<td>C. gracilis</td>
<td></td>
</tr>
<tr>
<td>C. sputorum bv. sputorum</td>
<td></td>
</tr>
<tr>
<td>C. sputorum bv. faecalis</td>
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</tr>
<tr>
<td>C. sputorum bv. bubulus</td>
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</tr>
<tr>
<td>C. curvus</td>
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</tr>
<tr>
<td>C. concisus</td>
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</tr>
<tr>
<td>C. showae</td>
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<td>[Bacteroides] ureolyticus</td>
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<tr>
<td>C. mucosalis</td>
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<tr>
<td>C. lanienae</td>
<td></td>
</tr>
<tr>
<td>C. fetus ssp. fetus</td>
<td></td>
</tr>
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<td>C. hyointestinalis</td>
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</tr>
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<td>C. lari</td>
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</tr>
<tr>
<td>C. coli</td>
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</tr>
<tr>
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</tr>
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<td>Arcobacter skirrowii</td>
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<td>Helicobacter pylori</td>
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<td>Esherichia coli</td>
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</table>

**Figure 8.2.** Relative DNA-DNA homology using CH001A genomic DNA as a probe. Homology values were: CH001A, 100%; CH002, 91.4% and CH003, 98.7%. All other *Campylobacter* type species, *A. skirrowii*, *H. pylori* and *E. coli*, showed no detectable homology.
Figure 8.3. Electron micrograph of *C. gracilis* (NCTC 12738<sup>T</sup>).
Bar equals 0.1 μm.
Figure 8.4. Electron micrograph of C. sputorum bv. sputorum (NCTC 11528T). Bar equals 0.1 μm.
Figure 8.5. Electron micrograph of *C. hominis* sp. nov. (CH001 colony type A - NCTC 13146\(^T\)). Bar equals 0.1 \(\mu\)m.
**Figure 8.6.** Electron micrograph of *C. hominis* sp. nov. (CH001 colony type A - NCTC 13146<sup>T</sup>) showing fimbriae-like structures.

Bar equals 0.1 μm.
Results and Discussion

Cells of C. hominis sp. nov. were typically blunt ended bacilli or coccobacilli, 0.25 to 0.5 μm wide and 0.5 to 1.8 μm long. There was no evidence of spiral morphology or flagella. Cells of colony types that exhibited a spreading morphology (CH001A, CH002 and CH003) were found to possess numerous irregular fimbriae-like structures 4 to 8 nm wide and > 1.0 μm long (Figure 8.6). Fimbriae-like structures were not found in the non-spreading colony types (CH001B and CH001C) of the first strain.

Prevalence of C. hominis sp. nov. in diarrhoeic faeces.

Nucleic acid samples extracted from diarrhoeic faecal samples in Chapter 6 were re-examined using the PCR assay for C. hominis. A subset was selected: this consisted of 114 samples previously positive for C. jejuni both by culture and C. jejuni-specific PCRs, and 95 containing no enteropathogenic Campylobacter species either by culture or PCR. C. hominis-specific PCR was positive in 10 of 114 (8.8%) diarrhoeic faecal samples positive for C. jejuni. It was also positive in eight of 95 (8.4%) diarrhoeic faecal samples that contained no enteropathogenic Campylobacter species. There was thus no statistical difference in the detection rate for C. hominis sp. nov. in the two groups (McNemars test, P > 0.5).

8.3 Discussion

Chapter 7 described the presence in human faeces of novel 16S rDNA sequences of Campylobacter origin. These were non-chimaeric, folded to a rRNA secondary structure typical of the genus, and apparently originated from
a novel, as yet uncultured taxon of *Campylobacter*. In accordance with recommendations for the description of incompletely described prokaryotes (Murray & Schleifer, 1994; Murray & Stackebrandt, 1995), the provisional taxon was named ‘*Candidatus* Campylobacter hominis’ (Lawson et al., 1998a). At that point it was not possible to isolate *C. hominis* sp. nov. by conventional *Campylobacter* isolation methods; this was most likely related to the fastidious growth requirements of the organism.

In the present Chapter, culture from PCR-positive faecal samples was achieved using a membrane filtration technique cultured on FAA (a rich medium which contains vitamin K, haemin, cysteine hydrochloride and L-arginine) and incubated under anaerobic conditions, in combination with methodologies of dilution or IMS to isolate the bacteria from mixed culture. It is noteworthy that 16S rDNA sequence analysis of *C. hominis* identified its closest phylogenetic relatives as *C. gracilis*, *C. rectus*, *C. curvus*, *C. showae*, *C. concisus* and *C. sputorum*. These species form a distinctive clade, which includes *C. hominis*, within the genus *Campylobacter* (see Figures 6.3 and 8.1). They all show a requirement for growth in an anaerobic environment, and/or require H₂ for optimum growth (Tanner et al., 1981; Vandamme & Goossens, 1992; Etoh et al., 1993). Furthermore, most of these species are associated with the periodontal crevice of humans and are rarely associated with gastroenteritis. Several share similar cell morphologies: for example, both *C. hominis* sp. nov. and *C. gracilis* are aflagellate straight rods, whilst *C. showae* and *C. concisus* are flagellate straight rods (Tanner, et al., 1981; Etoh, et al., 1993). Thus the overall phylogenetic, physiological, morphological
and ecological properties of these species suggest that they may share a
common lineage within the genus *Campylobacter*. The relatively deep
branching of *C. hominis* sp. nov. and *C. gracilis* observed in the phylogenetic
tree (Figure 8.1), as well as their unusual aflagellate rod-like cell structure,
may indicate these two species represent a distinct group within
*Campylobacter*. Notwithstanding the superficial resemblance of *C. hominis* sp.
and to *C. gracilis*, the mol% G + C value and 16S rDNA sequence of the
former are closer to that of *C. sputorum*. However, despite these apparent
similarities, the bootstrap value for the branching order of *C. hominis* sp. nov
to its two nearest neighbours (*C. gracilis* and *C. sputorum*) by 16S rDNA
analysis is low (478 and 376 respectively, see Figure 8.1) and DNA-DNA
hybridisation (see Figure 8.2) showed no relative homology with other
*Campylobacter* species. Thus the relative importance of any inferred
phylogenetic relationships must remain speculative. The numerous irregular
fimbriae-like structures produced by certain colony types of *C. hominis* sp.
nov. are also distinct from the few examples of fimbriae reported in *C. jejuni*
(Dolg *et al.*, 1996).

Previously, in Chapter 7, *'Candidatus* C. hominis' 16S rDNA sequences were
found to be present in 50% of faecal samples, but absent in saliva samples of
healthy individuals (Lawson *et al.*, 1998a). In the present chapter, *C. hominis-
specific PCR was positive in 27.8% of healthy human faecal samples but only
in 8.6% of diarrhoeic faeces. These findings support the supposition (made in
Chapter 7) that *C. hominis* sp. nov. is a commensal of the human
gastrointestinal tract, which may be displaced or drop below the threshold of
detection during episodes of acute gastroenteritis. A possible consequence of carriage of a commensal *Campylobacter* species in the lower gastrointestinal tract might be conferred protective immunity or resistance to colonisation by pathogenic campylobacters such as *C. jejuni* and *C. coli*. However, the finding of no significant difference in detection rates of *C. hominis* sp. nov does not support this in *Campylobacter* enteritis or non-*Campylobacter* enteritis.

The results described above provide evidence that the strains isolated and examined in this chapter (CH001A, CH001B, CH001C, CH002 and CH003) represent a distinct species within the genus *Campylobacter*, for which the proposed name *C. hominis* sp. nov. has now been accepted (Lawson et al., 2001). The type strain is CH001A<sup>T</sup> and has been deposited in the National Collection of Type Cultures and designated NCTC 13146<sup>T</sup>.

### 8.4 A phenotypic footnote

To complete the description of *C. hominis* sp. nov., thorough phenotypic characterisation and analysis were necessary. This was undertaken by Dr S.L.W. On at the Danish Veterinary Laboratory, Copenhagen. A brief description of this work is included here for completeness.

**Phenotypic characterisation and numerical analysis**

Analysis of the phenotypic characteristics of the *C. hominis* sp. nov isolates and control strains was undertaken using 47 phenotypic tests with media and methodologies as previously described (On & Holmes, 1991a; On & Holmes,
Numerical analysis of the phenotypic data was performed as previously described (On & Holmes, 1995) and a dendrogram indicating levels of similarity was constructed. Strains were clustered by the unweighted pair group mathematical average (UPGMA) method.

The isolates of *C. hominis* were phenotypically homogeneous, with infraspecific variance detected in only 4/47 test results. These were nitrate reduction (CH002 and CH003 positive), tolerance to 1.0% and 2.0% bile (CH002 positive), and resistance to nalidixic acid (CH002 sensitive). Biochemically, *C. hominis* was most similar to the principally anaerobic hydrogen-requiring species *C. concisus*, *C. curvus*, *C. gracilis*, *C. rectus*, *C. showae* and *C. sputorum*. The dendrogram derived from a numerical analysis of 47 phenotypic characteristics shows that the *C. hominis* sp. nov. strains form a distinct cluster at the 92.0% similarity level and are readily distinguished from all other *Campylobacter* species (Figure 8.7).

The phenotypic characteristics of *C. hominis* sp. nov. are listed in the formal description below. The most useful characteristics differentiating *C. hominis* sp. nov. from other *Campylobacter* species are summarised in Table 8.1.

It is interesting to note that some isolates of *C. hominis* sp. nov. were unable to tolerate 1.0% bile in laboratory conditions. In contrast other enteric *Campylobacter* species, such as *C. jejuni*, tolerate bile concentrations of between 1.5% and 2.0% (On et al., 1996). In humans the initial site of
Figure 8.7. Dendrogram of cluster analysis based on 47 phenotypic characteristics of Campylobacter strains (Created by Dr Stephen On). The numbers on the horizontal axis indicate the percentage similarities as determined by simple matching coefficient and UPGMA linkage clustering.

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Table 8.1 Characteristics differentiating *C. hominis* sp. nov. from other *Campylobacter* species

<table>
<thead>
<tr>
<th></th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Urease</th>
<th>Nitrate reduction</th>
<th>Nitrite reduction</th>
<th>Hippurate hydrolysis</th>
<th>Indoxyl acetate hydrolysis</th>
<th>H₂S production (TSA)</th>
<th>Alkaline phosphatase</th>
<th>Growth at 25°C</th>
<th>Growth at 42°C</th>
<th>Growth requires H₂</th>
<th>Sensitivity to nalidixic acid</th>
<th>Sensitivity to cephalothin</th>
<th>Sensitivity to metronidazole</th>
<th>Presence of flagella</th>
<th>mol% G + C content</th>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>S</td>
<td>-</td>
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<td>v</td>
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<td>-</td>
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<td>v, S</td>
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<td>v</td>
<td>v</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>v, R</td>
<td>v</td>
<td>S</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>v, R</td>
<td>S</td>
<td>R</td>
<td>44-46</td>
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<td>R, v</td>
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<td>R, v</td>
<td>+</td>
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<td>+</td>
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<td>R, R</td>
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<td>v</td>
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<td>R</td>
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+, positive reaction; -, negative reaction; v, variable reaction; w, weak reaction, R, resistant; S, sensitive

*TSI = Triple Sugar Iron medium*
infection of *C. jejuni* is the jejunum and upper ileum (Skirrow, 1994), where bile salt concentrations are high. Bile salts are largely absorbed in the terminal ileum (Percy-Robb & Collee, 1972), suggesting that the primary niche of *C. hominis* sp. nov. is the large intestine.

8.5 Formal description of *C. hominis* sp. nov.

*C. hominis* sp. nov. (*hominis*, Latin genitive noun, of man; after host where first detected). Gram-negative, non-spore-forming rods 0.25 to 0.5 µm wide and 0.5 to 1.8 µm long after for 10 days incubation (first subculture following initial isolation) on FAA in an anaerobic atmosphere. Colonies pinpoint to 1.0 mm in diameter, grey, some convex and entire, others flat and spreading. Cells were straight, blunt ended bacilli or coccobacilli. Non-motile: flagella were not evident but certain isolates produce numerous irregular fimbriae-like structures (4 to 8 nm wide and > 1.0 µm long). Optimal growth achieved on FAA under anaerobic conditions at 37°C, but strains will grow on standard 5% blood agar media after adaptation to laboratory conditions. Neither green nor blue-green pigmented growth is observed on blood agar. Pitting of the agar growth medium is absent. Non-haemolytic. No, or extremely poor growth, is attained on either FAA or BA when incubated microaerobically at 37°C. No growth under aerobic conditions at either 25°C or 37°C. Oxidase positive. Catalase, hippuricase, urease and alkaline phosphatase are not produced. Hydrogen sulphide not produced in TSI medium. Indoxyl acetate is not hydrolysed. Neither triphenyl-tetrazolium chloride nor selenite is reduced. All strains grow under anaerobic conditions on media containing 1.0% glycine, 5-fluorouracil (100 U l⁻¹), 0.1% sodium fluoride and 0.1% trimethylamine M-
Results and Discussion

oxide. No growth observed under anaerobic conditions at room temperature (18 – 22°C), 25°C or 42°C, or on media containing 4.0% NaCl, 0.04% TTC, 0.1% potassium permanganate, 0.001% sodium arsenite, 32 mg l⁻¹ cephalothin, 4 mg l⁻¹ metronidazole, 32 mg l⁻¹ carbenicillin (nutrient and blood agar bases), 0.005% basic fuchsin, 0.0005% crystal violet, 0.1% janus green, 0.1% sodium deoxycholate, or 0.02% pyronin. No growth similarly observed on a minimal medium, or MacConkey, casein or tyrosine media. Strains may differ in their ability to reduce nitrate, and grow on media containing 1.0% and 2.0% bile, and nalidixic acid (32 mg l⁻¹).

Formal description of the type strain

NCTC 13146ᵀ is the type strain of C. hominis sp. nov. This strain conforms to the species description given above. The mol% G + C content of NCTC 13146ᵀ DNA was 32.5%. The strain was isolated from the faeces of a healthy adult human male (London, UK) in 1998.
Chapter 9

General Conclusions
Chapter 9: General conclusions

Members of the genus *Campylobacter* occupy numerous ecological niches in the gastrointestinal and genital-urinary tracts of man and animals. *C. jejuni* a major human pathogen and the principal cause of bacterial gastroenteritis worldwide, dominates our perception of the genus as a whole. In contrast certain other *Campylobacter* species present in the human body may be commensal flora or, possibly opportunistic pathogens (Skirrow, 1994). The role in human gastroenteritis of certain other *Campylobacter* species, such as *C. upsaliensis*, is unclear and remains so due to the justifiable public health preoccupation with *C. jejuni*. Further elucidation of the role of *Campylobacter* species in the aetiology of human gastroenteritis has been handicapped by their relatively fastidious nature, the inability of some species and strains to grow on existing selective isolation media, and the paucity of distinctive phenotypic characteristics exhibited by the genus (Linton, 1996).

While cultivation remains the principal means of diagnosis for pathogenic bacteria, the advent of molecular techniques such as PCR and DNA-DNA hybridisation offer the possibility of a different perspective on the microbial world. This new molecular-based perspective need not be hampered by a prerequisite for cell growth on artificial media, or survival in the presence of selective antibiotics. Nevertheless, a significant hurdle to the practical application of molecular techniques to microbial detection exists in the form of the complex matrices that microorganisms naturally inhabit. Substances such as blood, urine and various foodstuffs all contain components that interfere with optimal conditions for molecular techniques. No
sample type is more refractory to the processes of molecular detection than faecal material. For example numerous substances inhibitory to PCR, such as bile salts, bilirubin, urobilinogens and polysaccharides are present in faeces (Widjojoatmodjo et al., 1992), and this is exacerbated by variability of consistency and constituents due to diet.

In this thesis the first goal was the development of a nucleic acid extraction protocol to facilitate rapid recovery of bacterial DNA from faecal material for use as a template for PCR. The optimum protocol was determined to be a rapid GuSCN-silica extraction methodology modified by an additional PVP wash step to remove substances inhibitory to PCR. The new extraction protocol was used in combination with Campylobacter species-specific PCR and initially tested with seeded faecal material where it achieved a level of detection comparable with that of the culture-based methodologies. However, selective culture remained superior for the detection of C. jejuni and C. coli. It was expected that the detection rate of the PCR assays would be greater when applied to actual clinical samples, where Campylobacter cells would be in a range of metabolic states, some less amenable to culture than others.

*Campylobacter* species-specific PCR assays were subsequently applied to bacterial DNA prepared using the new extraction protocol in a series of studies of clinical faecal samples, culminating in a two-year, large-scale, multi-centre investigation. In this major study, a total of 3,738 faecal samples from seven contributing centres, were examined for the presence of enteropathogenic *Campylobacter* species by PCR assay. This is the largest molecular-based survey of *Campylobacter* species yet undertaken (at the time of writing). In the course of the studies conducted in this
thesis a total of 3,963 diarrhoeic faecal samples were examined for *Campylobacter* species by PCR assay. Overall the detection rates of *C. jejuni* and *C. coli* were comparable between PCR assay and culture. However, while PCR and selective culture were in agreement for the detection of ~ 78% of *C. jejuni* and *C. coli* infections, a significant proportion were either culture-positive only (10%) or PCR-positive only (12%). The samples that were culture-positive/PCR-negative may represent relatively low level *Campylobacter* infections, detected due to the greater potential sensitivity of selective culture for *C. jejuni* and *C. coli*. While the cases that were PCR-positive/culture-negative may reflect the ability of the PCR assays to detect *Campylobacter* cells irrespective of metabolic state, including 'dead' or 'non-culturable' cells. Either methodology; PCR or selective culture, used alone would have missed a significant number of *C. jejuni* or *C. coli* infections.

Overall the detection of non-jejuni/non-coli *Campylobacter* species by PCR assay was significantly better than selective culture. Altogether a total of 17 non-jejuni/non-coli *Campylobacter* species were detected by PCR assay, comprising 11 *C. upsaliensis*, five *C. hyointestinalis* and one *C. lari*, only three of these samples were culture-positive (two *C. upsaliensis* and one *C. hyointestinalis*), and the isolates were not identified to species level. The incidence of *C. upsaliensis* in this study was 0.28%, which is higher than previously reported in culture-based studies of the general population (Bolton, et al., 1988; Aspinall, et al., 1993), but less than in studies which included children from socially disadvantaged groups (Goossens, et al., 1990a; Albert, et al., 1992). The detection of *C. hyointestinalis* at an incidence of 0.13% was the highest prevalence yet reported from any group, and indicates that the role of this bacterium in human disease deserves further investigation. Despite
the increased detection of non-jejuni/non-coli Campylobacter species by PCR assay, the incidence of these species as a whole is not great and they must constitute only a very small proportion of the estimated 45% of apparent gastroenteritis cases that remain undiagnosed by microbiology laboratories (Wheeler, et al., 1999). While these studies demonstrate the value of PCR-based methodologies as an epidemiological tool for the investigation of the aetiology of Campylobacter species, this approach remains uneconomical as standard diagnostic technique.

Another interesting feature of the PCR-based approach was the detection of mixed infection in 4% (21 out of 517) of clinical samples Campylobacter-positive by PCR in this study (19 C. jejuni/C. coli, one C. jejuni/C. upsaliensis and one C. jejuni/C. hyointestinalis). This would be difficult to observe using selective culture. There is a growing body of evidence that mixed infections, either of species or serotype, are a significant feature of Campylobacter infection (Ruberg, et al., 1998). In future this phenomenon might be investigated using modifications of the methodologies reported in this thesis.

Campylobacter genus and species-specific PCR assays were also used to investigate the prevalence of Campylobacter species in the gastrointestinal tract of healthy humans. Using this approach, it was determined that C. concisus is widely distributed in the upper gastrointestinal tract whereas 16S rDNA amplicons originating from a previously undescribed Campylobacter species were obtained from faecal material. These amplicons were sequenced and the originating, initially uncultivable bacterium was termed 'Candidatus C. hominis', a provisional taxonomic status. Phylogenetic analysis of 'Candidatus C. hominis' indicated that it was most
closely related to a group of principally anaerobic hydrogen-requiring *Campylobacter* species. Based on this evidence, further isolation experiments were undertaken using the 'non-selective' membrane filtration technique modified for the recovery of anaerobic bacteria, and augmented by a novel selective dilution and immunomagnetic separation procedure to isolate *Campylobacter* cells from a mixture of bacteria. In this manner, pure cultures of the bacteria from which the 'Candidatus C. hominis' 16S rDNA sequences originated were obtained and made amenable to conventional characterisation. This new *Campylobacter* species, now termed *C. hominis* sp. nov., appears to be a component of the normal flora of the human gastrointestinal tract. The significance of this species in human health and disease has yet to be determined.

Besides from the investigations described above, the rapid GuSCN-silica plus PVP wash nucleic acid extraction protocol, developed in the course of this thesis has found wider application. This technique has been successfully applied in a recent study of *Cryptosporidium* species in human and animal faeces using both direct PCR detection and PCR-RFLP (McLauchlin, et al., 2000).
Appendices
## Appendix A: Type and reference strains of bacteria used in this study and accession numbers for their 16S rDNA sequences

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Aeromonas hydrophila

E.coli 0:157

Salmonella enteritidis

Salmonella typhimurium

Salmonella virchow

Shigella flexneri

Shigella sonnei

Vibrio cholerae

Vibrio parahaemolyticus

Yersinia enterocolitica

* 16S rRNA sequences for these strains were retrieved from the EMBL database.

† Strains identified from 16S rDNA sequence data only (Lawson et al., 1998a).

NCTC, National Collection of Type Cultures.

<sup>T</sup> Denotes type strain.
Appendix B: 16S rDNA sequence alignment of Campylobacter species and other bacteria used in this study.
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Appendices
Appendices
Bibliography
Chapter 10: Bibliography


• Skirrow, M. B. (1994). Diseases due to *Campylobacter, Helicobacter* and related bacteria. *Journal of Comparative Pathology* 111, 113-149.


