

**PHENOTYPIC AND MOLECULAR ANALYSIS OF
HELICOBACTER SPP. AND RELATED MICRO-
ORGANISMS IDENTIFIED IN CLINICAL &
ENVIRONMENTAL SPECIMENS**

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**Thesis presented for the Degree of
Master of Science
In the Department of Clinical Laboratory Sciences
Division of Medical Microbiology
Institute of Infectious Diseases and Molecular Medicine
Faculty of Health Sciences
University of Cape Town
August 2006**

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ACKNOWLEDGEMENTS

I wish to thank my supervisor, Prof. A.J. Lastovica, and my co-supervisor, Dr. H. Smuts, for all the support, encouragement, guidance and advice on writing my thesis.

A special thanks to E. le Roux for her technical assistance and teaching me all that I know on culturing *Helicobacter* and related micro-organisms. I will be forever grateful. To my colleagues in the laboratory as well as all staff and the Head of the Medical Microbiology Department, Prof. L. Steyn, thank you for your friendship, advice and making my stay a very enjoyable one.

To my loving parents, siblings, niece and other family, thank you for your understanding and support through the years. It is greatly appreciated!

In addition, I would like to extend my sincere gratitude to my financial assistants: University of Cape Town; Prof. A.J. Lastovica and the Department of Science and Technology (South Africa) as a partner in the European Commission's Fifth Framework Programme "Quality of Life and Management of Living Resources" CAMPYCHECK Project (QLK1 CT 2002 02201); Medical Research Council (MRC) of South Africa; Duncan Baxter and Marion Beatrice Waddell. I would also like to thank Oxoid for supplying certain laboratory chemicals and the Oxoid Biochemical Identification System (O.B.I.S.) kits for the study.

Thank you all!

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ABBREVIATIONS

2xYT	2xYeast Tryptone
AFLP	Amplified fragment length polymorphisms
AMP	Ampicillin
ATP	Adenosine triphosphate
β -galactosidase	Beta-galactosidase
β -globin	Beta-globin
BLAST	Basic Local Alignment Search Tool
$^{\circ}$ C	Degrees Celsius
CaCl ₂	Calcium chloride
Cag A	Cytotoxin associated gene A
cfu	colony forming units
CLO	<i>Campylobacter</i> -like organism
CO ₂	Carbon dioxide
CTAB	Hexadecyltrimethyl ammonium bromide
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dNTP	Deoxynucleotide triphosphate
ddNTP	Dideoxynucleoside triphosphate
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra acetic acid (disodium salt)
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
GHLOs	Gastric <i>Helicobacter</i> -like organisms
H ₂	Hydrogen
H ₂ S	Hydrogen sulphide

HLOs	<i>Helicobacter</i> -like organisms
IBD	Inflammatory bowel disease
IF Primer	Inner forward primer
IgG	Immunoglobulin G
IPTG	Isopropyl- β -D-thiogalactopyranoside
IR Primer	Inner reverse primer
KOH	Potassium hydroxide
KNO ₃	Potassium nitrate
lac Z	lactose Z
L-ALA	L-alanine aminopeptidase
LANA	L-alanine-4-nitroanilide
LB	Luria broth
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MgCl ₂	Magnesium chloride
M.W.	Molecular weight
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
O ₂	Oxygen
O.B.I.S.	Oxoid Biochemical Identification System
OD	Optical density
OF.Primer	Outer forward primer
OR Primer	Outer reverse primer
PbAc	Lead acetate
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCR-RFLPs	Polymerase chain reaction-restriction fragment length polymorphisms
PIPES	Piperazine-1, 4-Bis (2-Ethanesulphonic acid)
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism

rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulphate
sp.	Species
TAE	Tris-acetate
TBA	Tryptose blood agar
TE	Tris-EDTA
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris (hydroxymethyl)-methylammonium chloride
TSI	Triple Sugar Iron
ureC	urease C
UV	Ultraviolet
Vac A	Vacuolating Cytotoxin A
VBNC	Viable but non-culturable
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
λ -DNA	Lambda DNA

UNITS

bp	base pairs
g	gram
hr	hours
kb	kilobase
M	Molar
mM	millimolar
mg	milligrams
ml	millilitre
mm	millimeters
μ l	microlitre
μ m	micrometer
μ g	micrograms
min	minutes
ng	nanograms
nm	nanometers
rpm	revs per minute
sec	seconds
U	Units
V	Volts
vols	volumes
wt / vol	weight per volume

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ABSTRACT

Interest in the *Helicobacter* genus has intensified since the discovery of *Helicobacter pylori* and its association with human disease in 1982, but knowledge of the disease potential of many non-*pylori Helicobacter* spp. and species of the related genera *Campylobacter* and *Arcobacter* is lacking. Due to the frequent isolation of *H. fennelliae* and *H. cinaedi* from paediatric patients suffering from diarrhoea and septicaemia in Cape Town, an attempt was made to determine the environmental reservoir of these pathogens. Species of *Helicobacter*, of which *H. pylori* is the most commonly isolated, have been detected in various human tissues. In the first part of the project, the 16S rRNA gene of *Helicobacter* was amplified from various cadaver tissues to determine etiological niches other than the recognized sites. Sequence and phylogenetic analysis of the amplified fragments indicated the presence of *H. pylori* in all *Helicobacter*-positive tissues, most of which was supported by the literature while others appeared to be a result of contamination.

The second part of the project included the amplification of the 16S rRNA gene from clinical and environmental isolates belonging to the genera *Helicobacter*, *Arcobacter* and *Campylobacter* to confirm the species preliminarily identified by biochemical and phenotypic testing. Although, *Helicobacter* was not isolated from the environment, a related micro-organism, *Arcobacter butzleri*, was isolated from raw and treated sewage / sludge samples. All clinical and environmental isolates were used to validate the L-ALA kit (O.B.I.S. Ltd., U.K.) and confirmed by the Fluka Aminopeptidase Test (Code 75554) (Fluka, Germany). The results were as expected; however, those of the 42 strains of 10 species of *Helicobacter* illustrated the absence of L-alanine aminopeptidase (pepN) in this genus. Analysis of the available full genomic sequences on the NCBI database confirmed these results. The same clinical and environmental isolates were used to validate the CampyCheck *Campylobacter* latex agglutination kit (CampyCheck, Microgen Bioproducts, U.K.). This prototype kit was specifically designed to react with *Campylobacter* species, however, the results illustrated that isolates of *Helicobacter* and other non-related bacteria could also react with these reagents, suggesting the possibility of a common surface antigen amongst these micro-organisms. The 16S rRNA gene

sequences of the clinical and environmental isolates and various reference sequences were used to generate phylogenetic trees. The *Helicobacter*-based phylogenetic tree illustrated the presence of several putative genomo-species; while the *Arcobacter*-based phylogenetic tree illustrated that the 4 sewage / sludge isolates clustered with *A. butzleri* suggesting that they are isolates of this micro-organism.

CHAPTER 1:

LITERATURE REVIEW

1.1. Introduction

Interest in the *Helicobacter* genus has intensified since the discovery of *Helicobacter pylori* (*H. pylori*), the first *Helicobacter* known to successfully colonize the gastric environment (Marshall & Warren, 1984). Most of the research relating to *Helicobacter* is based on *H. pylori*. A search on the Pubmed database revealed that more than 22 000 *Helicobacter* publications are focused on the gastric bacterium, *H. pylori*. In comparison, fewer publications are focused on the enteric *Helicobacter* spp. with approximately 33 on *H. fennelliae* and 55 on *H. cinaedi* illustrating that the disease potential of these micro-organisms are just beginning to be understood. Other species of *Helicobacter* have been identified in human and animal digestive tracts and are thought to be associated with human and animal diseases of gastric, enteric or systemic origin (Totten *et al.*, 1985; Fox *et al.*, 1989; Paster *et al.*, 1991; Fox *et al.*, 1998; De Groote *et al.*, 1999; Simmons *et al.*, 2000; Tee¹ *et al.*, 2001; Tee² *et al.*, 2001; and Gueneau & Loiseaux-De Goër, 2002). *H. pylori* is now known to be an etiological agent of gastritis, gastric and duodenal ulcers and is also associated with the development of gastric cancers and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Jukes & Cantor, 1969; Parsonnet *et al.*, 1991; Lee *et al.*, 1993; Parsonnet *et al.*, 1994; Goteri *et al.*, 1997; Fox *et al.*, 1998; McGee & Mobley, 1999; and Gueneau & Loiseaux-De Goër, 2002; Perez-Perez & Blaser, 2004). *Campylobacter jejuni* is globally recognized as one of the major causes of gastro-enteritis and diarrhea in humans and animals (Lambert *et al.*, 1987; Tee *et al.*, 1987; Tee *et al.*, 1988; Griffiths & Park, 1990; Kiehlbauch *et al.*, 1991; Lior, 1996; Le Roux & Lastovica, 1998; Allos, 2001). *Campylobacter jejuni* has also been associated with septicaemia, meningitis, the Guillain-Barré syndrome and reactive arthritis (Lastovica, 2006). *Arcobacter* spp. have also been associated with human and animal disease (Vandamme *et al.*, 1992¹; Vandamme *et al.*, 1992²; Lastovica & Skirrow, 2000).

1.2. Morphology

Helicobacter and *Campylobacter* are microaerophilic micro-organisms, i.e. they require O₂ levels below the range of 2 to 10 % for growth as their cells are damaged by the normal atmospheric level of O₂ (20 %) (Prescott *et al.*, 1996). Their colonies are generally domed, 0.5 – 1.0 mm in diameter and buff, dirty yellow or cream in colour (Le

Roux & Lastovica, 1998). *Arcobacter* are aerotolerant micro-organisms and their colonies tend to be whiter than those of *Campylobacter* (Le Roux & Lastovica, 1998). Some species of *Helicobacter* such as *H. fennelliae* and *H. cinaedi* may exhibit a thin flat film-like growth, while majority display colonial growth (personal communication: E. le Roux). A Gram stain would confirm any *Campylobacter*-like micro-organism as Gram-negative (Le Roux & Lastovica, 1998; Dewhirst *et al.*, 2000; Fox *et al.*, 2002). *Campylobacter* cells can be polymorphic, and can either be comma, gull-winged shaped (most *Campylobacter* spp.); short and stubby (*C. mucosalis*); or tiny and hardly curved (*C. concisus*, *C. curvus*), while *C. hyointestinalis* exhibit a slightly curved morphology similar to *Arcobacter* spp. (personal communication: E. le Roux). These variations in morphology between species may be a result of Gram-staining an older culture (Le Roux & Lastovica, 1998). *Helicobacter* are generally helical (fig. 1.1), curved or fusiform rods with a width of 0.3 - 0.6 μm and length of 1.0 - 5.0 μm (Dewhirst *et al.*, 2000). However, as the culture becomes older the cells may transform into a spherical or coccoid shape (fig. 1.2) as demonstrated by *H. pylori* (Bode *et al.*, 1993; Nilius *et al.*, 1993; Benaïssa *et al.*, 1996; and Costa *et al.*, 1999) and *H. cholecystus* (Franklin *et al.*, 1996). *Helicobacter* is motile by means of bipolar, single or multiple flagellae, which in most species is sheathed (Prescott *et al.*, 1996; Dewhirst *et al.*, 2000).



Figure 1.1: Transmission electron micrograph of *H. salomonis*. Note the multiple sheathed flagella and spiral morphology. Bar = 1 μm (Jalava *et al.*, 1997)



Figure 1.2: A negatively stained preparation of the coccoid (c) and short curved rod (r) forms of *H. cholecystus*. Bar = 1 μm (Franklin *et al.*, 1996)

1.3. Brief taxonomy of *Helicobacter*

H. pylori, the first type species of the genus *Helicobacter*, was initially described as a species of the genus *Campylobacter* (Marshall & Warren, 1984; Marshall *et al.*, 1984). However, due to marked differences between the 16S ribosomal RNA (rRNA) sequences of *Campylobacter pylori* (*C. pylori*) and various other *Campylobacter* species it was placed within the genus *Helicobacter* (Romaniuk *et al.*, 1987; Paster & Dewhirst, 1988; Goodwin *et al.*, 1989; Dewhirst *et al.*, 2000). The genus *Helicobacter* along with the related bacterial genera *Campylobacter*, *Arcobacter* and *Wolinella* are placed within a distinct group known as the rRNA superfamily VI, more commonly known as the epsilon division of the class *Proteobacteria* (On, 2001) (fig. 1.3). The taxonomic diversity of the group is matched by the broad spectrum of disease it is associated with, as well as the diverse ecological niches in which the organisms survive (On, 2001).

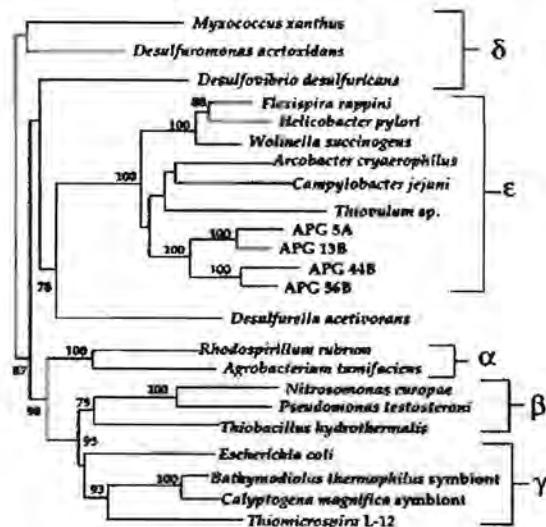


Figure 1.3: Phylogenetic tree illustrating the various sub-groups of *Proteobacteria*. The *Helicobacter* genus belongs to the epsilon sub-group (Haddad *et al.*, 1995)

The association of *H. pylori* with peptic ulcer, gastritis and gastric neoplasia (Parsonnet *et al.*, 1991) in humans has prompted detailed investigations into the clinical significance and incidence of gastric bacteria in humans and various animals (Lee, 1989; Blaser, 1993; Fox & Lee, 1993; Lee *et al.*, 1993). These findings have led to research in determining whether domestic pets, specifically dogs and cats (Eaton *et al.*, 1996), could

act as reservoirs of *Helicobacter*. As a result, the taxonomy of *Helicobacter* has swiftly expanded, in the past decade, with an average of two or three new species added to the genus per annum (Melito *et al.*, 2001). The exact number of species within the *Helicobacter* genus is contradictory. According to On *et al.*, (2002), the growing *Helicobacter* genus consists of 31 species of which 22 are primarily associated with extragastric niches whereas Whary & Fox (2004) approximates that 26 species have been fully named and states that various additional novel species are pending characterization. This deviation is indicative that different techniques are used by various research groups to identify a new *Helicobacter* species, of which the most common technique is the amplification of the 16S rRNA gene by the Polymerase Chain Reaction (PCR). PCR is a sensitive molecular technique that can be used to detect small quantities of DNA from a micro-organism such as *Helicobacter* by using genus-or-species-specific primers. The amplified fragments may then be compared to known sequences in the National Centre for Biotechnology Information (NCBI) database to help determine the species of bacteria present. However, Fox *et al.*, (1992) noted that the sequencing data of the 16S rRNA gene may not be sufficient to guarantee the identity of a species. Thus a new species cannot be proposed on the basis of a single technique such as the amplification of the 16S rRNA gene (Dewhirst *et al.*, 2000). A range of different techniques including biochemical and phenotypic tests as well as other molecular techniques such as Southern Hybridization, Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs), amplified fragment length polymorphisms (AFLP)-based fingerprints, whole-cell protein profiles, etc. are essential to alleviate any discrepancies and unquestionably determine whether the specific *Helicobacter* species is novel (Vandamme *et al.*, 1996; Vandamme *et al.*, 2000; Dewhirst *et al.*, 2000; On, 2001). This was illustrated by Goodwin *et al.*, (1989) who proposed the novel *Helicobacter* genus on the basis of 5 major taxonomic features, namely, ultrastructure and morphology; growth characteristics; enzyme capabilities; cellular fatty acid profiles; and menaquinones. A new species cannot be assigned on the basis of a single isolate. At least 5 strains, preferably more, must be critically examined. There remains an ecological division within the *Helicobacter* genus and taxa are generally, either, gastric or lower enteric in

origin (On, 2001) (table 1.1 & table 1.2). However a few enteric species which are often associated with hepatic disease have also been noted to colonize the stomach (On, 2001).

Table 1.1: Gastric-and-enteric grouped species of *Helicobacter* isolated from animals

Taxon	Source	Isolation niche	Isolated / detected / described by
<i>H. nemestrinae</i>	Monkey	Gastric	Brondson <i>et al.</i> , 1991
<i>H. muridarum</i>	Mouse	Gastric	Lee <i>et al.</i> , 1992
<i>H. acinonyx</i>	Cheetah	Gastric	Eaton ¹ <i>et al.</i> , 1993
<i>H. bizzozeronii</i>	Dog	Gastric	Hänninen <i>et al.</i> , 1996
<i>H. salomonis</i>	Dog	Gastric	Jalava <i>et al.</i> , 1997
<i>H. felis</i>	Cats & Dogs	Gastric	Paster <i>et al.</i> , 1991
<i>H. marmotae</i>	Woodchucks	Gastric	Fox <i>et al.</i> , 2002
<i>H. canis</i>	Dogs	Enteric	Stanley ¹ <i>et al.</i> , 1994
<i>H. hepaticus</i>	Mice	Enteric	Fox <i>et al.</i> , 1994
<i>H. bilis</i>	Mice	Enteric	Fox <i>et al.</i> , 1997
<i>H. trogontum</i>	Mice	Enteric	Mendes <i>et al.</i> , 1996
<i>H. cholecystus</i>	Mice	Enteric	Franklin <i>et al.</i> , 1997
<i>H. rodentium</i>	Rat	Enteric	Shen <i>et al.</i> , 1997
<i>H. pametensis</i>	Birds	Enteric	Dewhirst <i>et al.</i> , 1994
<i>H. pullorum</i>	Chickens	Enteric	Stanley ² <i>et al.</i> , 1995
<i>H. mesocricetorum</i>	Syrian hamsters	Enteric	Simmons <i>et al.</i> , 2000
<i>H. aurati</i>	Syrian hamsters	Enteric	Patterson <i>et al.</i> , 2000
<i>H. marmotae</i>	Cats & woodchucks	Enteric	Fox <i>et al.</i> , 2002

In addition, two uncultured strains have provisionally been proposed as *candidatus* spp, namely, *candidatus H. bovis* (cattle) and *candidatus H. suis* (pigs) (as listed by Dewhirst *et al.*, 2000; On, 2001).

Table 1.2: *Helicobacter* species isolated from humans

Taxon	Symptoms / illness of the patient	Source	Isolated / detected / described by
<i>Helicobacter</i> sp. with 99 % homology to <i>H. pylori</i>	Cancer	Liver biopsy sample	Fan <i>et al.</i> , 2002
<ul style="list-style-type: none"> • <i>H. cinaedi</i> • <i>H. heilmannii</i> • <i>H. pullorum</i> 	<ul style="list-style-type: none"> • Gastritis • Gastro-enteritis 	Stomach	<ul style="list-style-type: none"> • Peña <i>et al.</i>, 2002 • Dieterich <i>et al.</i>, 1998 • Stanley² <i>et al.</i>, 1994
<ul style="list-style-type: none"> • <i>H. winghamensis</i> • <i>H. canadensis</i> • <i>H. fennelliae</i> 	<ul style="list-style-type: none"> • Gastro-enteritis • Diarrhoea • Septic shock 	<ul style="list-style-type: none"> • Stool • rectal swab 	<ul style="list-style-type: none"> • Hsueh <i>et al.</i>, 1999 • Melito <i>et al.</i>, 2001 • Fox <i>et al.</i>, 2000
<ul style="list-style-type: none"> • <i>H. westmeadii</i> • <i>H. rappini</i> • <i>H. pullorum</i>-like • <i>Helicobacter</i> sp. Flexispira • <i>H. cinaedi</i> • <i>H. fennelliae</i> • <i>Helicobacter</i> sp. 	<ul style="list-style-type: none"> • AIDS • HIV positive • Bacteremia • Immuno-compromized • Septic Shock 	<ul style="list-style-type: none"> • Blood 	<ul style="list-style-type: none"> • Trivett-Moore <i>et al.</i>, 1997 • Iten <i>et al.</i>, 2001 • Tee¹ <i>et al.</i>, 2001 • Tee² <i>et al.</i>, 2001 • Murakami <i>et al.</i>, 2003 • Hsueh <i>et al.</i>, 1999 • Weir <i>et al.</i>, 1999
<i>Helicobacter</i> sp. strain Mainz	AIDS	Joint effusions	Husmann <i>et al.</i> , 1994
<i>Helicobacter</i> sp.	X-Linked hypogammaglobulinemia	Abdominal abscess	Han <i>et al.</i> , 2000
<ul style="list-style-type: none"> • <i>Helicobacter</i> spp. • <i>H. hepaticus</i> • <i>H. pylori</i> 	<ul style="list-style-type: none"> • Chronic cholecystitis • Extrahepatic biliary disease • Gallbladder neoplasm 	<ul style="list-style-type: none"> • Bile • Gallbladder • Gallstones 	<ul style="list-style-type: none"> • Fox <i>et al.</i>, 1998 • Kobayashi <i>et al.</i>, 2005 • Pradhan & Dali, 2004 • Apostolov <i>et al.</i>, 2005 • Abayli <i>et al.</i>, 2005
<i>H. pylori</i>	Tonsillitis	Tonsils	Cirak <i>et al.</i> , 2003

1.4. Host range and pathogenicity of *Helicobacter*

The presence of gastric spiral shaped micro-organisms has been noted in animals and humans for more than a century (Fox *et al.*, 2002). The Italian anatomist, Giulio Bizzozero, was one of the first observers of gastric spiral shaped bacteria in animals (Bizzozero, 1893; Marshall, 2002), while Freedberg & Barron (1940) and Steer & Colin-Jones (1975) were among the first to report its presence in the human stomach. These spiral micro-organisms were generally ignored by the scientific community until the isolation of *H. pylori* from the human gastric mucosa of patients suffering from chronic gastritis and peptic ulcers (Marshall & Warren, 1984). Since then, *H. pylori* have also been associated with gastric and duodenal ulcers and the development of gastric cancers (Jukes & Cantor, 1969; Parsonnet *et al.*, 1991; Lee *et al.*, 1993; Parsonnet *et al.*, 1994; Fox *et al.*, 1998; McGee & Mobley, 1999; and Gueneau & Loiseaux-De Goër, 2002). For many years, *Helicobacter* was thought to only colonize the stomach of animals and humans. *Helicobacter* can also colonize sites other than the stomach in man and a variety of animals.

1.4.1. Animals as a host

Various species of *Helicobacter* are present in specific animal hosts including: woodchucks, cats, cheetahs, lions, dogs, cattle, mice, Syrian hamsters, ferrets, rhesus monkeys and baboons, to name a few (table 1.1). *Helicobacter* is also known to infect a variety of aquatic species such as seals, dolphins and whales.

Helicobacter species which are often found in the stomachs of carnivorous pets have traditionally been referred to as “gastric *helicobacter*-like organisms” (GHLOs). These *Helicobacter* species display characteristic morphology and in addition also encompass a high level of urease activity which allows the bacteria to survive within an acidic environment (Lecoindre *et al.*, 2000).

1.4.1.1. The presence of *Helicobacter* within cats

Lecoindre *et al.*, (2000) have observed a difference in the prevalence rate of GHLO in cats, ranging between 45 and 100 %. Differences may be attributed to varying detection techniques, selection of animal populations and geographical variation (Lecoindre *et al.*, 2000). There are two GHLOs which are most

frequently found in cats, *Helicobacter felis*, which was initially cultured from the gastric tissues of cats (Lee *et al.*, 1988; Paster *et al.*, 1991), and an unculturable species related to both *H. heilmannii* and *H. bizzozeronii* (Lecoindre *et al.*, 2000). Handt *et al.*, (1994) have also detected *H. pylori* in cats, while Dieterich *et al.*, (1998) and Norris *et al.*, (1999) have detected, by PCR, an unculturable species of *Helicobacter* which is closely related to *H. heilmannii*. Dieterich *et al.*, (1998) have also provided molecular evidence, using PCR, that cats can concurrently be infected with more than one strain of “*H. heilmannii*”. In addition, Fox *et al.*, (2002) have isolated four bacterial strains, from cat faeces, which were characterized by phenotypic, biochemical as well as 16S rRNA analysis and determined to be a novel *Helicobacter* species, *Helicobacter marmotae*, which was first isolated from the livers of woodchucks.

1.4.1.2. The presence of *Helicobacter* within cheetahs and lions

Globally, most cheetahs in captivity suffer from a progressive gastritis resulting in vomiting, weight loss and inability to thrive and are associated with *Helicobacter* infection (Eaton² *et al.*, 1993; Munson, 1993; Munson *et al.*, 1999; Terio *et al.*, 2005). Many captive cheetahs also develop systemic amyloidosis (type AA) resulting in renal failure and ultimately death (Papendick *et al.*, 1997). In addition, it was noted that moderate to severe gastritis was present in more than 70 % of cheetahs that had died since 1995 within the North American captive population (Terio *et al.*, 2005). Gastritis was also noted to be the leading cause of death or the reason for euthanasia in 69 % of South African cheetahs in captivity (Munson *et al.*, 1999). In 1991, Eaton *et al.*, identified a spiral bacterium, morphologically similar to “*H. heilmannii*”, using electron microscopy on gastric biopsy samples from captive cheetahs. In addition, Eaton¹ *et al.*, (1993) also isolated four strains of a novel *Helicobacter* species, *Helicobacter acinonyx*, from the stomachs of cheetahs suffering from gastritis. Since the first isolation of *H. acinonyx* by Eaton¹ *et al.*, in 1993, many culture attempts from cheetahs have been unsuccessful despite the presence of spiral bacteria demonstrated histologically (Terio *et al.*, 2005). This may suggest the absence of *H. acinonyx* from the biopsy samples of cheetahs or inefficient culturing techniques. Furthermore, it may suggest that *H.*

acinonyx is not the most common *Helicobacter* species infecting captive cheetahs and that other non-culturable species of *Helicobacter* could play a role in the development of gastritis (Terio *et al.*, 2005). In 2005, Terio *et al.*, identified multiple types of *Helicobacter* (most homologous to *H. pylori*, *H. felis* and *H. heilmannii*) in captive cheetahs suffering from gastritis. Although Terio *et al.*, (2005) also detected *Helicobacter* in wild cheetahs (most homologous to *H. pylori* and *H. heilmannii*); *H. acinonyx* was not detected in any of the cheetahs. However, the samples from wild cheetahs were all obtained from one geographic region in Namibia and thus may have resulted in a biased identification of *Helicobacter* species (Terio *et al.*, 2005). The presence of similar *Helicobacter* species in captive (with gastritis) and wild (without gastritis) cheetahs suggests that various host factors play a role in *Helicobacter* infection in cheetahs and in addition it provides a natural disease model to study the effect various host factors have on the development of gastritis (Terio *et al.*, 2005). Kinsel *et al.*, (1998) have observed *Helicobacter*-like organisms (HLOs) in the stomachs of African lions from Namibia.

1.4.1.3. The presence of *Helicobacter* within dogs

Infection associated with HLOs was observed in 61 – 80 % of dogs suffering from vomiting (Geyer *et al.*, 1993; Hermanns *et al.*, 1995; Yamasaki *et al.*, 1998); 67 – 86 % of clinically healthy pet dogs (Eaton *et al.*, 1996; Yamasaki *et al.*, 1998) and nearly 100 % of shelter dogs and laboratory Beagles (Henry *et al.*, 1987; Eaton *et al.*, 1992; Eaton *et al.*, 1996). Various gastric bacteria with differing ultrastructural morphologies have been isolated or identified from domestic carnivores, however, their prevalence in different animal populations and clinical significance is unknown (Eaton *et al.*, 1996). Gastric bacteria are common in various mammals, including symptomatic and asymptomatic dogs (Weber *et al.*, 1958; Henry *et al.*, 1987; Geyer *et al.*, 1993; Lee *et al.*, 1993; Otto *et al.*, 1994; Hermanns *et al.*, 1995; Eaton *et al.*, 1996). For example, Hänninen *et al.*, (1996) recently cultured a novel canine *Helicobacter* sp., *Helicobacter bizzozeronii*, from the stomachs of dogs. Although Simpson *et al.*, (1999) were experimentally successful in infecting dogs with *Helicobacter felis*; their findings suggested that

H. felis might not be a gastric pathogen in this host. This is in agreement with the Eaton *et al.*, (1996) study where another gastric *Helicobacter*, *H. pylori*, could not be isolated from domestic dogs. However, the latter scenario may have been due to inefficient culturing conditions and the fact that *H. pylori* can transform from a spiral to coccoid form which is believed to be viable but non-culturable (Bode *et al.*, 1993; Cellini *et al.*, 1994; Dunn *et al.*, 1997; Mizoguchi *et al.*, 1999; Ren *et al.*, 1999). Buczolitis *et al.*, (2003) have provided PCR-based genetic evidence that *H. pylori* occurs in the gastric mucosa of dogs. This is indicative that the stomachs of dogs may provide an unfavourable environment for *H. pylori* thus resulting in the transformation from spiral to coccoid form and coincidentally also switching to the viable but non-culturable state (Buczolitis *et al.*, 2003). Radin *et al.*, (1990) have observed that *H. pylori* persistently colonized the gastric mucosa of gnotobiotic dogs for a period of at least one month with the resultant disease syndrome resembling that of humans. In addition, Jalava *et al.*, (1997) have successfully isolated a novel *Helicobacter* sp., *Helicobacter salamonis*, from biopsy samples obtained from healthy pet and adult experimental Beagle dogs.

1.4.1.4. The presence of *Helicobacter* within cattle

Two independent research groups (Günther & Schulze, 1992; Haringsma & Mouwen, 1992) reported their visual observations of helically shaped bacteria in the abomasums of calves and adult cattle using histological data and considered the bacteria to be of *Helicobacter* origin. De Groote *et al.*, (1999) validated these observations by amplifying the bacterial 16S rDNA from biopsy samples of cattle and using comparative 16S rDNA sequence analysis was able to determine that the specific spiral bacteria in the abomasums of adult cattle was a novel *Helicobacter* sp. and consequently proposed the name “*candidatus Helicobacter bovis*”. Unfortunately, *in vitro* isolation of these bacteria has, as yet, been unsuccessful (Jelinski *et al.*, 1995).

1.4.1.5. The presence of *Helicobacter* within mice and Syrian hamsters

To date, a few *Helicobacter* species as well as the genetically related bacterium “*Flexispira rappini*” have been isolated from rodents (Phillips *et al.*, 1983; Lee *et*

al., 1992; Schauer *et al.*, 1993; Fox *et al.*, 1994; Fox *et al.*, 1995). Lee *et al.*, (1992) isolated a novel *Helicobacter* sp., *Helicobacter muridarum*, from the intestinal mucosa of rodents, while Fox *et al.*, (1994) isolated a novel *Helicobacter* sp., *Helicobacter hepaticus*, from the livers and intestinal mucosal scrapings of mice. Various novel *Helicobacter* species have also been isolated from Syrian hamsters. These include, among others, *Helicobacter cholecystus* (Franklin *et al.*, 1996) from the gallbladder; *Helicobacter mesocricetorum* (Simmons *et al.*, 2000) from faeces of asymptomatic Syrian hamsters; and *Helicobacter aurati* (Patterson *et al.*, 2000) from the gastro-intestinal tissues.

1.4.1.6. The presence of *Helicobacter* within rhesus monkeys and baboons

Fernandez *et al.*, (2002) recently stumbled upon an unexpected finding that asymptomatic captive rhesus monkeys are commonly infected with *Helicobacter cinaedi*. In another unrelated study, Mackie & O' Rourke (2003) observed numerous spirally-shaped bacteria which were morphologically similar to *H. pylori*, in antral glands; and *H. heilmanni*, in the fundic glands of baboons. This initial observation was further confirmed by the amplification of both the urease and 16S rRNA genes and was consequently the first report of HLOs in symptomatic baboons (Mackie & O' Rourke, 2003).

1.4.1.7. The presence of *Helicobacter* within sea lions and seals

Recently, Harper¹ *et al.*, (2003) were successful in culturing 2 novel *Helicobacter* spp. from 1 of 4 symptomatic harp seals studied, while the bacterium was only detected in 2 of 4 harp seals using a PCR technique. One of the 2 bacterial isolates, also classified by 16S rRNA analysis was urease-negative and clustered with *H. canis* while the second, urease-positive, isolate clustered with isolates from a sea lion and sea otters. Similarly, Oxley & McKay (2004) detected, by PCR, *Helicobacter* species in the faeces of several captive Australian sea lions and Australian fur seals. From all the animals investigated in their study, only 1 Australian sea lion was previously diagnosed with a suspected *Helicobacter* related infection.

1.4.1.8. The presence of *Helicobacter* within dolphins and whales

Over the decades, there have been numerous reports of gastric ulcerations observed in captive and wild dolphins (Geraci & Gerstmann, 1966; Ridgway, 1968; Ridgway, 1972; Sweeney, 1978; Leatherwood & Reeves, 1990; Gaskin, 1992; Abollo, 1998; Harper *et al.*, 2000) some of which were associated with parasitic infections and the consumption of foreign objects (Sweeney & Ridgway, 1975; Coffey, 1977; Gallo Reynoso, 1991; Herreras *et al.*, 1997), while others were associated with non-parasitic infections (Geraci & Gerstmann, 1966; Sweeney & Ridgway, 1975; Sweeney, 1978; Harper *et al.*, 2000). Consequently, there exists some speculation as to whether dolphins suffer from gastric and peptic ulcer diseases associated with the *Helicobacter* bacterium (Harper *et al.*, 2000). In 2000, Harper *et al.*, managed to successfully culture a novel *Helicobacter* species, most closely related to *H. pylori*, from the gastric mucosa of 2 of 8 dolphins. The presence of *Helicobacter* was confirmed, by PCR, in 7 of the 8 symptomatic dolphins. Their findings suggested that the novel *Helicobacter* sp. played a causative role in the pathogenesis of gastritis in these dolphins and may ultimately play a role in gastric ulcer development (Harper *et al.*, 2000). *Helicobacter* is not only confined to the stomachs of dolphins as observed by Goldman *et al.*, (2002). They have provided evidence of a *Helicobacter* sp. which has been detected, by PCR, in the dental plaque of 2 captive dolphins which may be a direct result of their diet. In addition, these results may also suggest that their oral cavities act as a reservoir of *Helicobacter* (Goldman *et al.*, 2002). Recently, a novel *Helicobacter* sp., *Helicobacter cetorum* (*H. cetorum*), was successfully cultured from the main stomach of two stranded wild Atlantic white-sided symptomatic dolphins (Harper¹ *et al.*, 2002; Harper² *et al.*, 2003) and from the faeces of three symptomatic captive cetaceans, namely, a Pacific white-sided dolphin; Atlantic bottlenose dolphin and Beluga whale (Harper¹⁺² *et al.*, 2002; Harper² *et al.*, 2003). Although the prevalence of *H. cetorum* infection in wild populations is unknown, results within this study suggest a 50 % prevalence of *H. cetorum* infection in the dolphin population (Harper² *et al.*, 2003). However, given the fact that *H. cetorum* is a gastric *Helicobacter* and the fact that faecal and sera

testing have limitations, the true *H. cetorum* prevalence in this dolphin population may even be higher than observed (Harper² *et al.*, 2003).

1.4.2. Humans as a host

Since the discovery of *H. pylori* in the gastric mucosa of patients with chronic gastritis by Warren & Marshall (1983), *H. pylori* has been associated with various gastro-duodenal diseases such as peptic ulcer; gastritis; and gastric neoplasia (Goodwin *et al.*, 1986; Parsonnet *et al.*, 1991; Wee *et al.*, 1992; Boyanova *et al.*, 2003). The eradication of *H. pylori* has been associated with a histological improvement of gastritis; lower re-lapse rate and a lower risk of bleeding from duodenal ulcer (Pakodi *et al.*, 2000). Generally, *H. pylori* infections are prevalent in 50 – 90 % of the population in developing countries compared to 5 – 20 % in developed countries (Marshall, 2002). This marked difference is attributed to a low socio-economic status during childhood, overcrowded dwellings and unsanitary conditions (Marshall, 2002). The survival of *H. pylori* in the human host is ensured by the production of a copious amount of urease enzyme and several cytotoxins such as the vacuolating cytotoxin A (Vac A) and cytotoxin associated gene A (Cag A) proteins. The urease enzyme is essentially used to lower the intracellular pH of the stomach to below 5.0 (Melchers *et al.*, 1998) by breaking down urea in the gastric juice and extracellular fluid. Thus *H. pylori* can survive for a sufficient amount of time to ensure the colonization of the gastric mucosa (Marshall *et al.*, 1990). Variants of the Vac A cytotoxin are activated in low pH (De Bernard *et al.*, 1995) of which the most aggressive forms are more likely to be associated with peptic ulcer whereas the more benign forms may be associated with gastritis in asymptomatic people (Atherton *et al.*, 1995; Marshall, 2002). The Vac A gene encodes the vacuolating cytotoxin A protein which induces the formation of intracellular vacuoles in epithelial cells, while the Cag A protein plays a role in preventing the maintenance for the normal cytoskeletal structure of the epithelium and thought to enhance the attachment and survival of nearby *H. pylori* (Segal *et al.*, 1999; Marshall, 2002). *H. pylori* colonizes the gastric mucus-secreting cells, located beneath the gastric mucous layers, by adhering to the surface pili of gastric cells (Prescott *et al.*, 1996). *H. pylori* binds

to the Lewis antigens and monosaccharide sialic acid of glycoproteins located on the surface of gastric epithelial cells (Prescott *et al.*, 1996). *H. winghamensis* (Melito *et al.*, 2001), *H. pullorum* (Stanley² *et al.*, 1994) and *H. cinaedi* (Quinn *et al.*, 1984; Peña *et al.*, 2002) have also been isolated from patients with gastroenteritis suggesting that entero-hepatic *Helicobacter* may also be etiologic agents of human gastro-duodenal diseases (Peña *et al.*, 2002). In addition, Fox *et al.*, (2000) have successfully isolated a novel *Helicobacter* species, *H. canadensis*, from humans suffering from diarrhoea.

However, *H. pylori* and other *Helicobacter* species are not only confined to the stomach. In recent years, various novel and diverse *Helicobacter* species have been associated with the pathogenesis of human entero-hepatic diseases (Nilsson *et al.*, 1999; Nilsson *et al.*, 2000; Queiroz & Santos, 2001; Solnick & Schauer, 2001; Rocha *et al.*, 2005). Although a small proportion of the global population is infected with the hepatitis C virus (HCV), chronic hepatitis is known to occur in up to 75 % of all cases; 20 % will develop cirrhosis; and chronic liver disease will evolve into hepato-cellular carcinoma in 3 – 5 % of patients each year (Seeff, 2002). Fan *et al.*, (2002) detected *Helicobacter* species with a 99 % homology to the 16S rRNA gene of *H. pylori* in liver tissues of HCV positive Chinese patients with primary hepatocellular carcinoma. In addition, Nilsson *et al.*, (2000) detected *H. pylori* and other *Helicobacter* species in the liver tissues of patients suffering from liver diseases. Recently, the results of Rocha *et al.*, (2005) suggest an association of *H. pylori*-and-*H. pullorum*-like micro-organisms with hepatitis C cirrhosis in patients with or without hepato-cellular carcinoma. It is thought that *Helicobacter* might cause various structural changes within the liver ultimately resulting in cirrhosis (Villeneuve *et al.*, 1996; Hano *et al.*, 2003; Rocha *et al.*, 2005). Alternatively, *Helicobacter* might only cause these structural changes to ensure its survival but due to the counter-action executed by the host immune response, these changes ultimately result in liver-related diseases. This is supported by the fact that *Helicobacter* species may also be detected in the liver of hepatitis C cirrhosis negative patients (Avenaude *et al.*, 2000; Rocha *et al.*, 2005). Thus the presence of *Helicobacter* can be seen as a possible co-risk factor

in chronic liver diseases. However, this speculation requires further exploration, given that *Helicobacter* has the ability to produce toxins, such as the Cag A and Vac A proteins, which may interfere with the normal functioning of hepatic cells (Rocha *et al.*, 2005). Apostolov *et al.*, (2005) have detected *H. pylori* and other *Helicobacter* species in the liver and gallbladder of patients with chronic cholecystitis using immunological and molecular techniques. Data by Abayli *et al.*, (2005) supported the possibility of *H. pylori* playing an imperative role in the formation of cholesterol gallstones. Using specific staining techniques Pradhan & Dali (2004) observed that the presence of *H. hepaticus* was linked to gallbladder neoplasm. They also state that further studies are required to conclusively illustrate that *H. hepaticus* is a risk factor for the pathogenesis of gallbladder carcinoma. Data from Fox *et al.*, (1998) supported an association of various bile-resistant *Helicobacter* species with gallbladder disease, while data by Kobayashi *et al.*, (2005) indicated that *Helicobacter* may be associated with extrahepatic biliary diseases.

Helicobacter have also been detected in other organs of the human body. The evidence implicating *Helicobacter* to heart diseases is controversial. Mendall *et al.*, (1994) found serologic evidence directly linking *H. pylori* seropositivity to coronary heart disease; while the results by Patel *et al.*, (1994) indicated that *H. pylori* infection was associated with increased fibrinogen levels, considered to be a vital risk factor for coronary heart disease. However, a study (Danesh & Peto, 1998) concluded that coronary heart disease was not confined to an independent effect by *H. pylori* but more likely due to a result of a combination of risk factors. This suggestion was supported by a large, independent study by Wald *et al.*, (1997). Using immuno-histochemical staining, Koullias *et al.*, (2004) found no evidence of *H. pylori* in specimens of aortic aneurysms and penetrating aortic ulcers. However, different strains of *H. pylori* are known to exist of which the more virulent Cag A strain has been associated with ischemic heart disease (Pasceri *et al.*, 1998). Kaplan *et al.*, (2006) have detected *H. pylori* in the atherosclerotic plaques of the carotid artery using PCR, while using an enzyme immunoassay Masoud *et al.*, (2005) provided evidence for the association of *H.*

transmission. Fan *et al.*, (1998) illustrated that *H. pylori* could survive up to 10 days in milk stored at 4⁰C but only 4 days in tap water. However using electron microscopy, the coccoid form could still be detected up to 7 days in tap water, stored at 4⁰C. In addition, Poms & Tatini (2001) successfully isolated *H. pylori* from spiked pasteurized skim milk and tofu samples up to 5 days; and from spiked raw chicken and lettuce leaves up to 2 days of refrigeration at 4⁰C. However, *H. pylori* could not be isolated from the spiked yoghurt samples suggesting that the *H. pylori* strain (NCTC 11637), and perhaps other strains of *Helicobacter*, are unable to survive in an organic acid environment provided by the lactic acid bacteria present in yoghurt fermentations (Midolo *et al.*, 1995). Although, heat treatment (pasteurization of milk, preparation of tofu, etc.) would kill *H. pylori*, it is possible that secondary contamination could occur when handled in an unsanitized manner (Poms & Tatini, 2001). This transmission route could also be achieved by the inadvertent ingestion of food contaminated with excreta from insects living in unsanitary environments (Imamura *et al.*, 2003; Allen *et al.*, 2004). In addition, the consumption of raw vegetables and fruit fertilized with organic manures (including raw sewage / sludge and human / animal faeces infected with *H. pylori*) has been found to be a risk factor for *H. pylori* infection (Hopkins *et al.*, 1993; Herrera, 2004; Nicholson *et al.*, 2005).

1.6.2.7. Environment-to-human route

Animals and humans shed *H. pylori* in their faeces and the mechanisms for entry into various water sources, survival, ingestion and infection are consequently dependent upon a range of environmental factors (Bellack *et al.*, 2006). As wild birds are recognized as probable zoonotic agents of *H. pullorum* it is possible that the faecal contamination of pond water and parks could expose people to infection (Waldenström *et al.*, 2003). *H. pylori* have been detected, by PCR, in various Japanese water sources including: river; pond; and well water (Sasaki *et al.*, 1999; Fujimura *et al.*, 2004) suggesting that drinking water may be a vehicle of *Helicobacter* infection. However, *H. pylori* contaminated water is not only confined to drinking water in Japan.

Using PCR, Park *et al.*, (2001) and Watson *et al.*, (2004) also detected *H. pylori* in water and water bio-films in the U.K. *H. pylori* transmission via water sources was given further support by a molecular study based on PCR of Mexican water sources, including raw municipal wastewater from an open wastewater canal (Mazari-Hiriart *et al.*, 2001; Lu *et al.*, 2002); and a German epidemiological study (Krumbiegel *et al.*, 2004). Using an enzyme-linked immunosorbent assay (ELISA) to detect *H. pylori* antibodies in various water sources in Kazakhstan, Nurgalieva *et al.*, (2002) found that poor sanitary practices relating to waste disposal and household hygiene may serve as contributing factors for the transmission of *H. pylori*. However, *H. pylori* have rarely been isolated from contaminated water sources. Recent data (Adams *et al.*, 2003) suggest that environmental exposure could induce *H. pylori* to enter a viable but non-culturable (VBNC) state thus preventing the isolation of this micro-organism through culture and allowing it to persist until a suitable host is acquired. The detection of *H. pylori* genes in sea water along the Italian coast (Cellini *et al.*, 2004; Carbone *et al.*, 2005) suggests that the marine environment could act as a vector for infection, especially since the ocean is frequented for swimming and fishing purposes. Parsonnet *et al.*, (1999) provided intriguing evidence suggesting that the aerosol droplets around a vomiting patient, infected with *H. pylori*, could potentially act as a vehicle of transmission to uninfected humans. Although Snaidr *et al.*, (1997) had not detected *Helicobacter* in their activated sludge samples a related micro-organism, *Arcobacter*, was isolated.

The exact mode for *Helicobacter* transmission cannot be conclusively demonstrated and currently remains an open topic. The available evidence seems to be in agreement with Goodman *et al.*, (1996) suggesting a multiple-pathway phenomenon for *H. pylori* infection, in both human-to-human transmission and via external sources. The dominant mode for *Helicobacter* transmission may be variable upon comparison with different countries or populations (Stone, 1999). Nonetheless, it is evident that the true prevalence of *Helicobacter* in human disease; and in animal, environmental and other sources can only be determined by using appropriate detection techniques. It is also important to note

that the association of *Helicobacter* with human disease is dependent on several factors such as: strain variability, the amount of *Helicobacter* present and host immune response.

1.7. Identification of *Helicobacter*

The isolation of *Helicobacter* is imperative (especially if a novel species is suspected) to ensure the correct characterization of an isolate by using biochemical, phenotypic, serologic and molecular tests.

1.7.1. Primary isolation of *Helicobacter*

The successful isolation of *Helicobacter* from various sources including: clinical and environmental, through culture, is demanding and generally incorporates the use of specific media and culturing conditions. Although strains of several *Helicobacter* species are capable of growing on simple nutritional agar media, the majority require media supplemented with serum or blood for successful culture (Dewhirst *et al.*, 2000). *H. pylori* may be isolated from gastric tissue as well as from biopsies of duodenal or oesophageal tissue, containing gastric metaplasia, using non-selective media such as chocolate agar or antibiotic-containing selective media such as those of Goodwin or Skirrow (Perez-Perez & Blaser, 2004). However, many *Helicobacter* species are unable to grow on blood-free culture media and are sensitive to the cefoperazone antibiotic supplement used in certain media (On *et al.*, 1996). In addition, the presence of hydrogen in the atmospheric gas mixture enhances the successful culturing of all known *Helicobacter*, *Arcobacter* and *Campylobacter* species (Lastovica & Skirrow, 2000; Lastovica, 2006; Appendix D).

The isolation of *Helicobacter* from the environment remains problematic using traditional media for culture. To date, several media have been assessed for the isolation of *H. pylori* from different environments including: infected individuals (Hachem *et al.*, 1995; Piccolomini *et al.*, 1997); ground beef (Stevenson *et al.*, 2000); rumen and abomasum samples of cattle (Stevenson *et al.*, 2000); and non-sterile well water containing native flora and further contaminated with a few common waterborne micro-organisms (Degnan *et al.*, 2003). However, the first three matrices are nutrient rich and in order for *H. pylori* to survive in different

water sources they must be able to physiologically adapt to a depleted-nutrient environment (Azevedo *et al.*, 2004). Thus traditional isolation media may be too rich in nutrients resulting in a condition known as nutrient shock and ultimately preventing the optimum culturability of *Helicobacter* (Reasoner & Geldreich, 1985; Azevedo *et al.*, 2004). In addition, the primary growths of certain *Helicobacter* species such as *H. pylori* is generally slow and usually require about 4 days to develop discernible colonies (Degnan *et al.*, 2003). Although the results of Van der Hulst *et al.*, (1996) illustrated that an incubation of up to 10 days is required to detect all *H. pylori* infections with the use of non-selective media as opposed to 7 days using selective media; Boyanova *et al.*, (2003) illustrated that an incubation period of 11 days is vital to detect the growth of all *H. pylori* isolates. Due to the slow growth of *H. pylori*, they may be out-competed by various faster growing micro-organisms, particularly fungi, which could ultimately conceal the presence of this slow-growing pathogen on various media. Thus Degnan *et al.*, (2003) developed an *H. pylori* selective medium containing a novel mixture of growth supplements; amphotericin B and polymixin B antibiotics; as well as a phenol red indicator to detect the production of urease. Using aliquots of non-sterile well water containing various native flora and further inoculating it with eight common waterborne micro-organisms, their data illustrated that the colour indicator system could presumptively identify *H. pylori* colonies up to 12 - 20 hr sooner than other conventional media. However, further studies are required to validate the success of this medium using various water sources.

Special conditions are required to successfully culture *Helicobacter* from various sources. All known *Helicobacter* species are unable to survive an O₂ tension of 20 % at normal atmospheric pressure and are, therefore, known to be micro-aerophilic as they require special conditions during incubation to ensure their optimal growth. These include: a temperature of 37⁰C and atmospheric conditions of 5 % O₂; 10 % CO₂; and 85 % N (Lecoindre *et al.*, 2000) during incubation.

1.7.2. Tests to identify and confirm the presence of *Helicobacter*

In addition to the Gram-stain, other techniques have also been used to determine the Gram-status of a micro-organism, especially in cases where Gram variability have been recorded; these include the use of the KOH (Carlone *et al.*, 1983); L-alanine-4-nitroanilide (Carlone *et al.*, 1983); vancomycin and colimycin susceptibility testing (Von Graevenitz & Bucher, 1983); and the Gram-Sure test (Remel, U.S.A.). The data by Gregersen (1978) suggested that the dissolution of the cell wall and cytoplasmic membrane, by 3 % KOH, should be a reliable marker for the detection of Gram-negative micro-organisms (Carlone *et al.*, 1983). However, Blachman *et al.*, (1980) and Halebian *et al.*, (1981) have both reported that the KOH test and Gram stain do not always correlate. The L-alanine aminopeptidase has been suggested for differentiating between Gram-positive and Gram-negative micro-organisms, with the enzyme being more prevalent in the latter group (Cerny, 1976, 1978). This is in agreement with data by Carlone *et al.*, (1983) suggesting that the KOH and L-alanine-4-nitroanilide (LANA) tests could be useful in characterizing clinical isolates. They found that both the KOH and LANA tests correlated with the Gram staining technique when non-fermentative bacilli and *Bacillus* species were used but did not correlate with non-sporulating anaerobic micro-organisms. However, upon testing various *Campylobacter* species their data illustrated that although the micro-organisms were Gram-negative and LANA negative the KOH test indicated a positive result. In addition, Carlone *et al.*, (1983) illustrated that vancomycin and colimycin susceptibility could also be used to determine the Gram-status of anaerobic micro-organisms, although the reaction is not as quick as the KOH and LANA tests. However, Geraci & Hermans (1983) and Catchpole *et al.*, (1997) illustrated that these susceptibility tests are not definitive as some Gram-positive cultures are vancomycin resistant while others are susceptible to vancomycin. By using the Gram-Sure test (Remel, U.S.A.): a commercially available disk test to detect the presence of L-alanine aminopeptidase, generally present in the cell walls of Gram-negative micro-organisms, Fenollar & Raoult (2000) illustrated that it could be useful in identifying the Gram-status of unusual micro-organisms. Despite the

availability of various diagnostic tests, the Gram stain remains the technique of choice, worldwide; to determine the Gram-status and to observe the basic morphology of a micro-organism. Once the identification of *Helicobacter* and related micro-organisms have been confirmed as Gram-negative various well-described standardized tests (Appendix D) are used to characterize it to the species level (Mégraud *et al.*, 1985; Popovic-Uroic *et al.*, 1990; Kiehlbauch *et al.*, 1991; Barrow & Feltham, 1993; On & Holmes, 1995; On *et al.*, 1996; Atabay *et al.*, 1998; Le Roux & Lastovica, 1998; Dewhirst *et al.*, 2000; On *et al.*, 2000; Lastovica, 2006).

Due to difficulties in isolating *Helicobacter* (including *H. pylori*) from various environmental sources, only expensive and sophisticated techniques including: immuno-separation; PCR and Southern Hybridization with specific probes have been successful in detecting *H. pylori* from samples other than clinical origin (Velazquez & Feirtag, 1999). Despite the fact that *H. pylori* is difficult to isolate from the environment, a PCR technique was shown to be sensitive enough to detect a concentration of 0.1pg (Goosen *et al.*, 2002) of *Helicobacter* DNA in various environmental samples including drinking water in Peru (Hulten *et al.*, 1996), Japan (Sasaki *et al.*, 1999) and England (Park *et al.*, 2001; Watson *et al.*, 2004); freshwater environments in Mexico City (Mazari-Hiriart *et al.*, 2001); sea water along the Italian coast (Cellini *et al.*, 2004; Carbone *et al.*, 2005) and in insects (Grübel *et al.*, 1998; Imamura *et al.*, 2003). Other current molecular techniques include the use of ELISA, immuno-staining, autoradiography and ATP bioluminescence.

1.7.3. Amplification of the 16S rRNA gene

The amplification of the desired fragment is obtained through PCR, a sensitive molecular technique to detect low quantities of bacteria, especially non-viable bacteria, in various clinical and environmental sources (Persing *et al.*, 1993; Vaneechoutte *et al.*, 1997). The successful amplification of a desired PCR product is determined by various critical factors, including: choice of primers; annealing time; annealing temperature; number of cycles within a reaction; and various inhibitors within a sample.

In recent years, the majority of publications have relied on the amplification of the 16S rRNA gene and subsequent sequencing thereof to identify the species of *Helicobacter*, *Arcobacter* and *Campylobacter*; with few studies reporting on the usefulness of amplifying and sequencing the 23S rRNA gene (Eyers *et al.*, 1993; Bastyns *et al.*, 1994; Kiehlbauch *et al.*, 1995; Harmon & Wesley, 1996; Germani *et al.*, 1997; Hurtado & Owen, 1997; Fermer & Engvall, 1999; Vandamme & On, 2001; Kuijper *et al.*, 2003). Due to the high sequence similarity of the 16S rRNA gene between *H. felis*, *H. bizzozeronii* and *H. heilmanni* Jalava *et al.*, (1997) stated that this gene might not be suitable for species-specific PCR in *Helicobacter*. However, the 16S rRNA gene may still be suitable for genus-specific PCR in *Helicobacter* as illustrated by Shen *et al.*, (2001) and Garcia *et al.*, (2002). *Helicobacter* has been identified to the species level, in a cat suffering from a mixed bacterial infection, using the C97 / C05 *Helicobacter* genus-specific primer set generating a 1 200 bp fragment followed by restriction fragment length polymorphism (RFLP) analysis with the *Hha* I or *Alu* I restriction enzymes (Shen *et al.*, 2000, 2001). Using the C97 / C98 *Helicobacter* genus-specific primer set, a 400 bp fragment was successfully amplified from DNA extracted from the faeces of ferrets (Garcia *et al.*, 2002).

Several other genes have also been used to detect the presence of *Helicobacter* (specifically *H. pylori*) in various samples. In addition to using the 16S rRNA primer set, Cirak *et al.*, (2003) also used primers to detect the presence of the *cag* A gene of *H. pylori* in tonsil and adenoid tissues. Similarly, several independent environmental studies have also based the detection of *H. pylori* on the amplification of the: 16S rRNA; *cag* A; urease A; urease C and *vac* A genes (Mazari-Hiriart *et al.*, 2001; Lu *et al.*, 2002; Cellini *et al.*, 2004; Carbone *et al.*, 2005).

Nevertheless, a positive PCR result should be confirmed by other results such as DNA sequence analysis, as false positive results could occur (Buczolits *et al.*, 2003) through non-specific amplification of the desired product DNA.

1.7.4. Sequencing of the 16S rRNA gene

During the 1980's-and-90's various molecular techniques were developed in genetics. As a result the ribosomal RNA molecules were recognized as suitable indicators in determining the phylogenetic relationships between micro-organisms (Woese, 1987). The 5S; 23S; and the most frequently used 16S rRNA gene sequences were proposed to illustrate the best phylogenetic relationships among bacteria due to their presence in all organisms; high degree of stability; slow evolutionary change rate; and functional constancy (Woese, 1987; Jalava, 1999). The 16S rRNA gene is \pm 1500 bp in length and is generally present as several copies in a bacterial cell (Woese, 1987; Jalava, 1999). A comparison of nearly the entire sequence of the 16S rRNA gene is undoubtedly one of the most powerful techniques to determine the phylogenetic relationship of an unknown organism (Fox *et al.*, 1998; Vandamme *et al.*, 2000) largely due to the conserved and variable regions within the gene (De Rijk *et al.*, 1992; Gurtler & Stanisich, 1996). The similarities and differences within the 16S rRNA gene sets the foundation for most phylogenetic dendograms to determine a bacterial species (Marshall, 2002) and in addition could possibly indicate a common origin among various prokaryotic organisms (Melito *et al.*, 2001). Although various studies have illustrated that this technique, on its own, is not sensitive enough to correctly identify strains to the species level (Stackebrandt & Goebel, 1994; Stanley² *et al.*, 1994; Fox *et al.*, 2000; Shen *et al.*, 2001; Fox *et al.*, 2002) it can unequivocally differentiate the *Helicobacter* genus from all other genera (Dewhirst *et al.*, 2000). It has previously been reported that strains belonging to different species could have identical 16S rRNA gene sequences; and strains of one species could have 16S rRNA gene sequences that differ by 3 % (Stackebrandt & Goebel, 1994) or even 4 % (Harrington & On, 1999) of the total 16S rRNA gene sequence (Vandamme *et al.*, 2000). Thus the 16S rRNA gene sequences can occasionally be very misleading; therefore, conclusions should not be made in the absence of appropriate complementary data (Vandamme *et al.*, 2000).

1.8. Taxonomy

Taxonomy is a science which attempts to arrange the constant expanding information pertaining to the natural diversity of organisms into a more defined and meaningful hierarchical system. This science includes three major areas: classification of organisms into groups based on similarity; nomenclature of various groups according to international rules; as well as the identification of unknown organisms each of which is linked to the other (Krieg & Holt, 1984; Vandamme *et al.*, 1996; On, 2001). Micro-organisms are identified by comparing data of an unknown strain with those of known taxa (On, 2001). Strains are therefore identified when the two data sets match at an acceptable level (On, 2001). The criteria used for matching may include biochemical test results, electrophoretic patterns, whole-cell pyrolysis mass spectra or comparison of sequences of the 16S rRNA gene (On, 2001). In 2002, Gueneau & Loiseaux-De Goër used sequences of the 16S rRNA gene to infer the phylogeny of the *Helicobacter* genus and illustrated that the gastric species are divided in at least two phylogenetic groups; one homogenous and clearly separated from enteric species while the other formed a tight cluster within the enteric species group. As *Wolinella succinogenes* is the closest neighbour of the *Helicobacter* genus, it was used as a root / outgroup of the phylogenetic tree (Gueneau & Loiseaux-De Goër, 2002).

1.9. Aim and objectives of the project

Aim:

- ❖ To attempt to isolate *Helicobacter* spp. and related genera from various environmental and clinical sources to gain insight into one or more of the following: prevalence, persistence, disease potential, transmission routes or reservoir of *Helicobacter* and related genera.

Objectives:

- ❖ To culture, isolate, revitalize and identify *Helicobacter* and related micro-organisms from clinical and environmental specimens using phenotypic, biochemical and serological tests.

- ❖ To validate the Oxoid Biochemical Identification System (O.B.I.S. Ltd., U.K.) for the differentiation of *Campylobacter* and *Arcobacter* from other Gram-negative micro-organisms, including *Helicobacter* and confirming the results with the Fluka Aminopeptidase Test (Code 75554) (Fluka, Germany).
- ❖ To validate the CampyCheck *Campylobacter* latex agglutination kit (Microgen Bioproducts, U.K.) using pure cultures of *Campylobacter*, *Helicobacter*, *Arcobacter* and other non-related micro-organisms.
- ❖ To detect, by PCR, the presence of *Helicobacter* and related micro-organisms in various clinical and environmental specimens as well as in DNA extracted from various tissues of two cadavers.
- ❖ To generate phylogenetic trees, inferred by the 16S rRNA gene sequences, to confirm the species of *Helicobacter* and related micro-organisms within the clinical and environmental specimens upon comparison with reference strains.

CHAPTER 2:

EXPERIMENTAL PROTOCOLS

2.1. Background to Experimental Procedures

2.1.1. Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is an *in vitro* technique, developed by Kary Mullis and Fred Faloona between 1983 and 1985 (Mullis & Faloona, 1987), which is used to amplify large quantities of a specific nucleotide sequence from small quantities of DNA without cloning it into a vector (Prescott *et al.*, 2002). For a PCR to work, it is vital that the ends of the target sequence are known in sufficient detail in order to synthesize oligonucleotide primers that will hybridize to the target sequence to initiate the PCR (Mullis & Faloona, 1987). In addition, the amplification process also requires deoxynucleoside triphosphates (dNTPs), namely deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP) and deoxycytidine triphosphate (dCTP); 1x PCR buffer (without MgCl₂) (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, stabilisers and 50 % glycerol) (Southern Cross Biotechnology); MgCl₂; two oligonucleotide primers, complimentary to a specific sequence within the target gene, and a thermostable DNA polymerase. The PCR consists of three major steps, namely, denaturation; annealing and extension. During denaturation, the complementary strands of the double-stranded DNA (dsDNA) are separated at a high temperature. The second step entails the lowering of the temperature to allow the primers to anneal to the complementary target sequence on both single strands of the separated dsDNA (Mullis & Faloona, 1987; Prescott *et al.*, 2002). However, too low an annealing temperature would result in non-specific amplification while too high an annealing temperature would inhibit the primers from annealing, by means of hydrogen bonds, to the target sequence. Thus a temperature gradient PCR should be carried out to determine the optimal annealing temperature for the amplification of the desired product. Finally, once annealed the primers are extended by the thermostable DNA polymerase using the available dNTPs. These three steps are repeated in cycles resulting in the exponential synthesis of the target DNA (Prescott *et al.*, 1996) followed by a 4°C step, for an indefinite time, to prevent the degradation of the amplified products whilst in the thermocycler. A schematic diagram of the Polymerase Chain Reaction is illustrated in fig. 2.1.

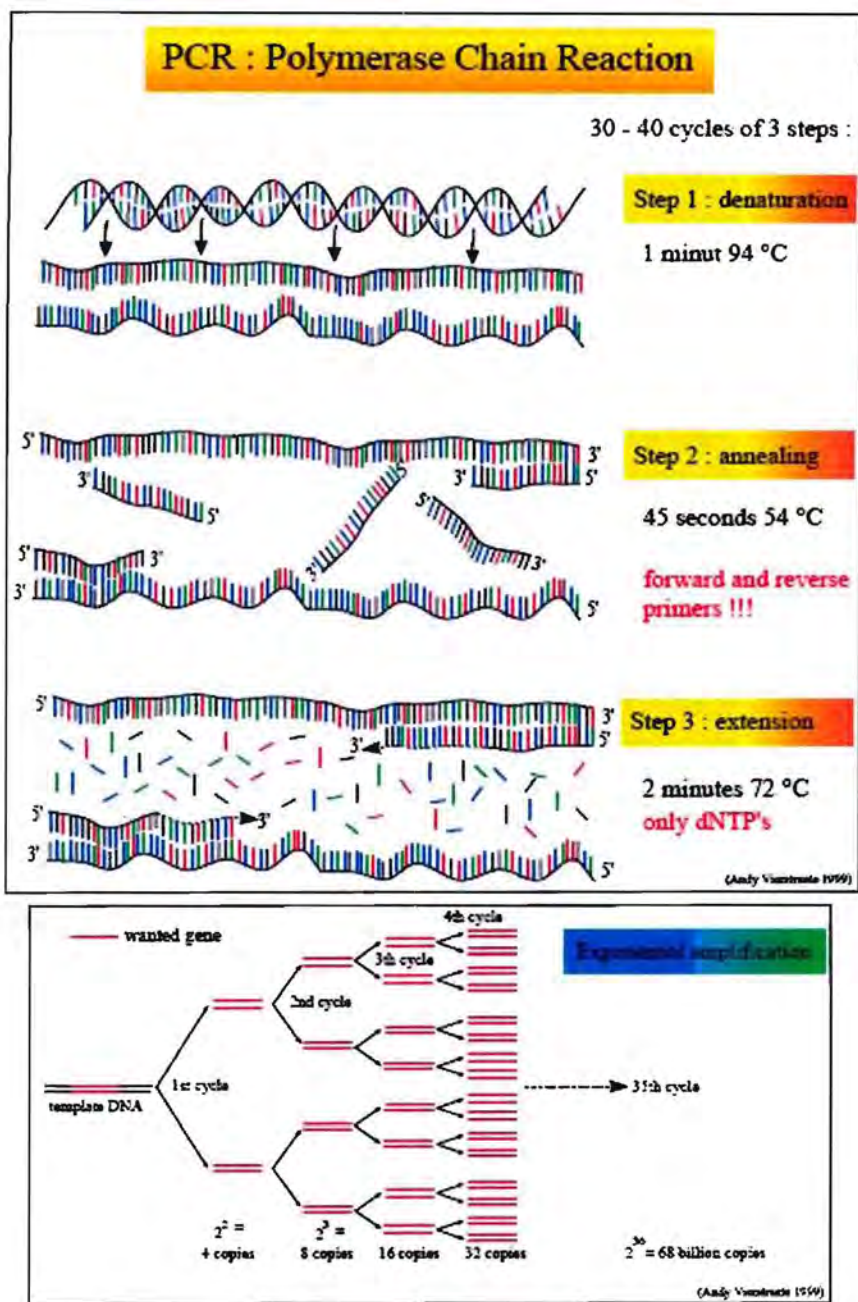


Figure 2.1: A schematic representation of the Polymerase Chain Reaction (PCR) (<http://users.ugent.be/~avierstr/pdf/PCR.pdf>).

Another form of PCR, namely nested PCR, was carried out in the first part of this study using cadaver DNA as a template.

2.1.1.1. Nested PCR

Nested PCR involves the use of two rounds of PCR, an outer and an inner PCR. Nested PCR is required for the preparation of sufficient quantities of desired fragments such as the small (± 350 bp) and large (± 1161 bp) fragments of the 16S rRNA gene of cadaver DNA to ensure efficient cloning and optimal direct sequencing reactions. During the first round of PCR the outer forward (OF) and outer reverse (OR) primers, designed to anneal to the outer complementary target sequence, are used. The PCR products are loaded into the wells of an agarose gel and electrophoresed alongside a molecular weight marker (M.W.) to determine whether the desired outer fragment can be seen on an agarose gel. If the outer PCR products cannot be seen on an agarose gel, an inner PCR is carried out using the inner forward (IF) and inner reverse (IR) primers and the outer PCR products as a template, i.e. the inner primers are designed to anneal within the outer complementary target sequence (figure 2.2). All inner PCR products as well as the outer PCR positive control are loaded into the wells of an agarose gel and electrophoresed alongside a M.W. to determine whether the correct sized fragment was amplified during the inner PCR, using the outer positive control as a guide.

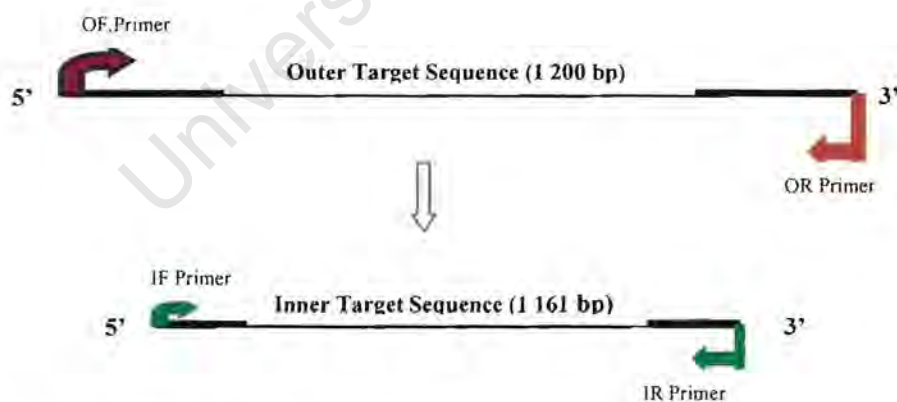


Figure 2.2: A schematic representation of a nested PCR.

2.1.2. Cloning

Molecular cloning is a technique that employs plasmids as vehicles to multiply the desired fragment by introducing foreign DNA into bacterial cells (Schlegel, 1990). The

DNA of interest is inserted into a vector by cutting both the DNA of interest and the vector with the same restriction enzyme. This causes the vector to linearize and also creates “sticky ends” on both pieces of DNA. Due to the palindromic nature of the restriction enzyme recognition sites, the sticky ends will be complimentary to each other. The linearized vector and DNA of interest will interact with each other and join by means of the hydrogen bonds, forming a recombinant DNA molecule. However, the hydrogen bonds are not strong enough and can only hold the recombinant molecule together for a few milliseconds. Thus the phosphodiester backbones of the DNA molecules are permanently joined together with the aid of DNA ligase. However, if DNA ligase is present in a mixture of two different DNA fragments, any of the two DNA fragments could transiently join with the linearized vector and form a recombinant DNA molecule (<http://www.emunix.emich.edu/~rwinning/genetics/tech.htm>). A schematic diagram of this process is outlined in fig. 2.3. In many cases, a commercial expression vector, for example, the pGEM-T Easy vector (Promega, U.S.A) is used to detect whether an insert is present. Here, the DNA of interest is targeted to the multiple cloning site within the lactose Z (*lac Z*) gene. The successful cloning of an insert into the pGEM-T Easy vector interrupts the coding sequence of the beta-galactosidase (β -galactosidase) gene (Schlegel, 1990; Prescott *et al.*, 1996). By plating transformants onto an agar plate containing ampicillin; a histochemical stain: 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) and an inducer: isopropyl- β -D-thiogalactopyranoside (IPTG) it is possible to identify the clones containing the insert, i.e. the recombinants (www.promega.com). Recombinants appear white in colour due to the presence of the insert resulting in an interruption in the β -galactosidase gene (Koenen *et al.*, 1986; Prescott *et al.*, 1996). Non-recombinants, however, appear blue in colour due to the expression of the β -galactosidase gene thus illustrating that the insert is not present (Schlegel, 1990).

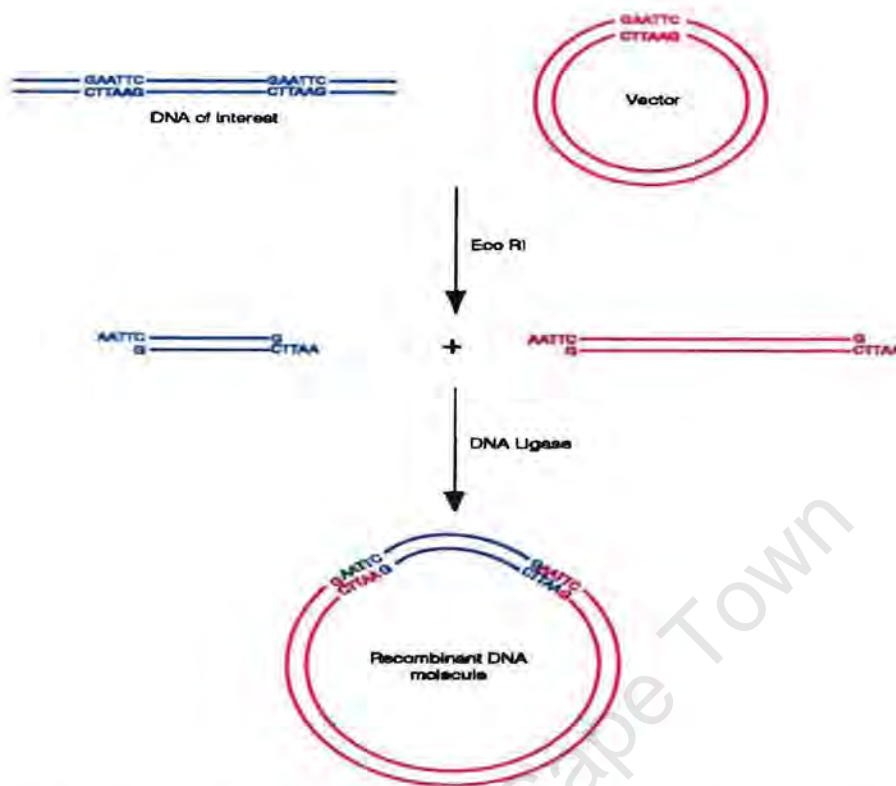


Figure 2.3: A diagrammatic representation illustrating the construction of a recombinant plasmid. (<http://www.emunix.emich.edu/~rwinning/genetics/tech.htm>).

2.1.3. DNA Sequencing

DNA sequence analysis is a rapid and accurate technique to enrich knowledge about a particular gene structure. In addition, sequencing can also be used to determine whether the amplified DNA fragment is the desired fragment (Davis, 1986) by comparing it to known sequences within the database. Two DNA sequencing methods were developed. The first method, developed by Maxam and Gilbert in 1977 (Davis, 1986) entails the chemical modification and cleavage of specific nucleotides followed by electrophoresis on high-resolution denaturing acrylamide gels. The second method, developed by Sanger *et al.*, in 1977, is based on the generation of DNA fragments through the controlled interruption of enzymatic replication (Davis, 1986). The cycle sequencing method was used in this study.

2.1.3.1. Cycle Sequencing

The Cycle Sequencing method was derived from the Sanger method (PE Applied Biosystems, 1998). This is a simple technique in which successive rounds of denaturing, annealing and extension, in a thermocycler, result in the linear amplification of extension products. During the denaturing step, the complementary strands of the dsDNA are separated at a high temperature. This is followed by an annealing step where the temperature is lowered to allow the primer to anneal to the complementary target sequence (Mullis & Faloona, 1987; Prescott *et al.*, 2002). Thereafter, the annealed primer is extended by the thermostable DNA polymerase using the available dNTP's and terminated by the incorporation of a dideoxynucleoside triphosphate (ddNTP). There are two different cycle sequencing strategies: the difference is in the cycle sequencing mixture. The cycle sequencing mixture could either contain dye-labelled primers (fig. 2.4 A) or dye-labelled ddNTP's (fig. 2.4 B) (<http://www.ibt.lt/sc/files/QIAGEN%20Guide%20to%20Template%20Purification%20and%20DNA%20Sequencing.pdf>). After the cycle sequencing reaction has been completed, the products are loaded into sequencing reaction sample tubes and placed in an autosampler tray capable of holding 48-96 samples and electrophoresed on a urea-acrylamide gel. Once the DNA fragments reach a detector window in the capillary, a laser excites the fluorescent dye labels. The emitted fluorescence is collected once per second by a cooled, charge-coupled device camera at specific wavelength bands and stored as digital signals on a Power Macintosh computer for processing. Thereafter, sequence analysis software interprets the results by detecting the bases from the fluorescence intensity at each data point. For consistency, the software always displays the analysed data in the following way: dATP as green, dCTP as blue, dGTP as black or yellow and dTTP as red in the electropherogram view (fig. 2.4 C) (<http://www.public.iastate.edu/~jsteime1/ITS%20Sequence%20Demo.html>; PE Applied Biosystems, 1998).

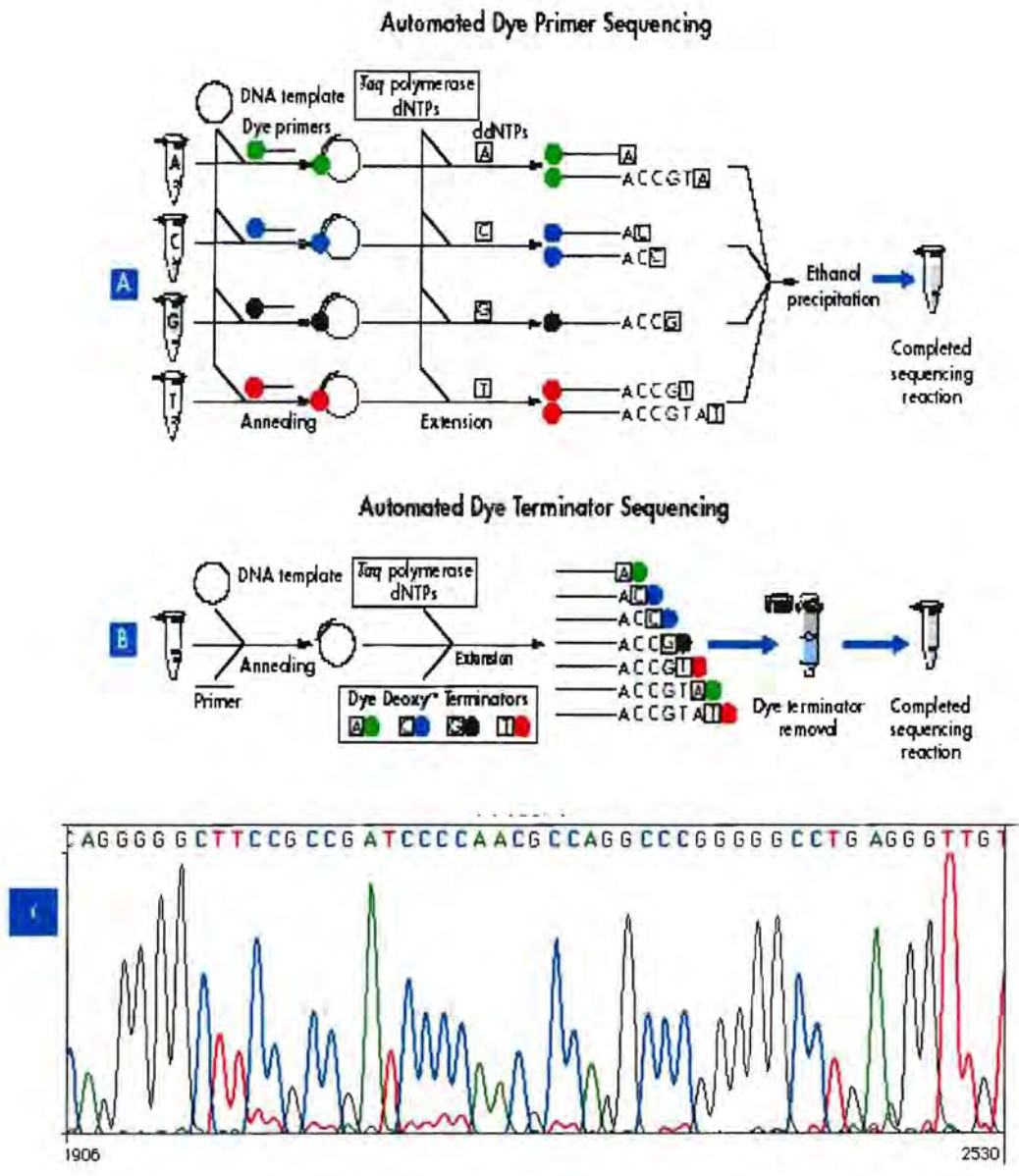


Figure 2.4: A schematic representation of the cycle sequencing reaction using dye-labelled primers (A) or dye-labelled terminators (ddNTP's) (B) and a representative electropherogram (C).

2.2. Experimental Procedures

2.2.1. Revitalisation of *Helicobacter* isolates stored at -70°C

A 100 µl aliquot of sterile 2xYT broth (NHLS media lab, Cape Town) was added to 2 ml sterile Eppendorf tubes (Eppendorf AG, Germany). Thereafter, five to seven PL160M Microbank Beads (Microbank™ Pro-Lab Diagnostics, Canada), of a specific isolate were added to the allocated Eppendorf tubes using a sterile loop. The Eppendorf tubes were incubated at 37°C for 10 min at 700 rpm in an Eppendorf Thermomixer compact (Eppendorf AG 22331, Germany) to wash the bacteria from the beads into the 2xYT broth. The entire volume of broth was then dispensed; drop wise, onto an allocated tryptose blood agar (TBA) (NHLS media lab, Cape Town) plate using a sterile Pasteur pipette (LP Italiana Spa, Italy). The TBA plates remained at room temperature (RT) for approximately 30 min to allow the drops to dry. Thereafter, the TBA plates were inverted and incubated for two days at 37°C in a H₂-enriched microaerophilic atmosphere generated by an Oxoid BR 38 gas generating kit (Oxoid Ltd., U.K.).

2.2.2. Isolation of *Helicobacter* and related micro-organisms from various environmental sources using various culturing techniques.

By consulting the literature it was noted that *Helicobacter* and related genera have been detected in some extraordinary environmental niches such as sea water, rivers, etc. Thus it was decided to sample some extraordinary sites in the environment. A once-off 50 ml sample of each site was obtained in a sterile manner to screen for the presence of *Helicobacter* and related genera and to attempt to culture these isolates, if present. If the screening process was successful another two samples of the same site would have been obtained and processed in the same manner to confirm its presence.

All experiments were done in triplicate. All samples were collected over the weekend and stored at 4°C for a maximum of 2 days before the initial experiment was carried out. All samples were collected and handled in a sterile manner.

2.2.2.1. Sea water samples using TBA plates

a. Direct Filtration Samples

The experimental sample consisted of 400 µl of sea water only. The positive control consisted of 400 µl of sea water spiked with two 5 µl loopfuls of 2-3 day old *H. pylori* culture. As a negative control, the 0.6 µm and 1.2 µm ME 26 Mixed cellulose ester membrane filters (Schleicher and Schuell MicroScience, Germany) were placed on allocated TBA plates without having anything filtered through it to determine whether the filters could be a source of contamination.

b. Enrichment Technique Samples

The experimental sample consisted of 200 µl of sterile 2xYT broth, 100 µl of sea water and 5 M Equine hemin (Sigma, Germany). The positive control consisted of 200 µl of sterile 2xYT broth, 100 µl of sea water and 5 M Equine hemin spiked with two 5 µl loopfuls of 2-3 day old *H. pylori* culture. The negative controls consisted of sterile 2xYT broth only and sterile 2xYT broth and 5 M Equine hemin, respectively, to determine whether the 2xYT broth or hemin were a source of contamination. The samples and controls were incubated at 37⁰C at 350 rpm on a Hagär shaker (Pinelands, Cape Town) for 48hr.

2.2.2.2. Dry and wet sea sand samples using TBA plates

Dry sea sand refers to the area of sand that was not exposed to sea water whereas the wet sea sand refers to the area of sand that was in contact with sea water and was still moist.

a. Direct Streaking Technique

A sterile inoculating loop was placed and swirled around in the dry sea sand sample, which was stored at 4⁰C, and streaked onto a TBA plate. Similarly, the same procedure was used for the wet sea sand sample.

b. Enrichment Technique Samples

A 10 g sample of both dry and wet sea sand was weighed and transferred into allocated sterile 100 ml conical flasks. To each of the samples, 50 ml of sterile 2xYT broth and 5 M equine hemin was added. The positive controls consisted of

10 g of wet or dry sea sand, 50 ml of sterile 2xYT broth and 5 M Equine hemin spiked with two 5 µl loopfuls of 2-3 day old *H. pylori* culture. The negative controls consisted of 50 ml of sterile 2xYT broth only and 50 ml of sterile 2xYT broth and 5 M Equine hemin to determine whether the 2xYT broth or hemin were a source of contamination. All samples and controls were incubated at 37°C at 350 rpm on a Hagär shaker for 48hr.

2.2.2.3. Sea water samples using *H. pylori* selective media (Oxoid Columbia Agar Base (CM 0331) and Oxoid *Helicobacter pylori* Selective Supplement (Dent) SR0147E) (Oxoid Ltd., U.K.)

a. Direct dispensing of the sea water sample onto *H. pylori* selective media

The experimental sample consisted of an aliquot of 200 µl of sea water which was directly dispensed onto an *H. pylori* selective plate (Appendix B). As a positive control, a 200 µl aliquot of sea water was seeded with two 5 µl loopfuls of 2-3 day old *H. cinaedi* and *H. fennelliae* cultures to determine if the sea water would inhibit their growth. Similarly, a 200 µl aliquot of sea water was seeded with two 5 µl loopfuls of 2-3 day old *H. pylori* culture.

b. Direct Filtration Technique Samples

The experimental sample consisted of 200 µl of sea water only (see 2.2.2.5.). As a negative control, a 1.2 µm membrane filter was placed onto an *H. pylori* selective plate without having anything filtered through it to determine if the filter was a source of contamination. The positive controls consisted of a 200 µl aliquot of sea water spiked with two 5 µl loopfuls of 2-3 day old cultures of *H. cinaedi*, *H. fennelliae* or *H. pylori* to determine if the 1.2 µm filter would prevent filtration of any of the three species of *Helicobacter*.

2.2.2.4. Duck Pond Water Experiment using *H. pylori* selective media

a. Direct dispensing of the Duck Pond Water sample onto *H. pylori* selective media

An aliquot of 200 µl of duck pond water was directly dispensed onto a *H. pylori* selective plate. The positive controls consisted of 200 µl aliquots of duck pond water spiked, either, with two 5 µl loopfuls of 2-3 day old *H. cinaedi* and *H.*

fennelliae cultures or an *H. pylori* culture only to determine if the duck pond water would hinder their growth and survival.

b. Direct Filtration Technique Samples

The experimental sample consisted of 200 µl of duck pond water only, while the positive control samples consisted of 200 µl of duck pond water spiked with two 5 µl loopfuls of 2-3 day old *H. cinaedi* and *H. fennelliae* cultures or *H. pylori* culture only (see 2.2.2.5.). As a negative control, a 1.2 µm membrane filter was placed onto a *H. pylori* selective plate without having anything filtered through it.

2.2.2.5. Filtration Technique and Incubation

a. Filtration Technique

Each of the direct filtration technique and enrichment technique samples and controls were filtered through 0.6 µm and 1.2 µm ME 26 membrane filters onto allocated TBA or *H. pylori* selective plates at RT. Thereafter, filters were removed within 20 min to ensure optimal filtration. All plates were allowed to dry at RT for 30-40 min.

b. Incubation

All plates were incubated at 37°C in a H₂-enriched microaerophilic atmosphere generated by an Oxoid BR 38 generating kit.

2.2.2.6. Dry sea sand, wet sea sand and duck pond sand experiments using *H. pylori* selective media and the Pulsification Technique

During pulsification the Pulsifier (Filtaflex Ltd, Canada) very rapidly beats the sample in the sterile pulsifying bag (Microgen Bioproducts Ltd., U.K.) using an oscillating metal ring. During this process intense turbulence is generated within the sample resulting in the creation of shear forces across the surfaces of various objects or particles within the sample which drive the bacteria off various sample matrices and into the broth or sterile phosphate buffer saline (PBS) (pH 7.2) in which they are suspended. The pulsification process results in a minimal breakdown of the sample matrix and thus the sample is essentially intact. These pulsified samples are therefore generally easier to pipette and filter, allowing

more of the sample to be concentrated (http://ift.confex.com/ift/2004/techprogram/paper_26721.htm).

a. Pulsification Technique using dry sea sand, wet sea sand and duck pond sand samples

The experimental samples consisted of 25 g of dry sea sand, wet sea sand or duck pond sand dispensed into an allocated pulsifier bag. Thereafter, 30 ml of sterile PBS was added. The positive controls consisted of 25 g of dry sea sand, wet sea sand or duck pond sand; 30 ml of sterile PBS and spiked with two 5 µl loopfuls of 2-3 day old *H. femelliae*, *H. cinaedi* and *H. pylori* cultures. The negative control consisted of 30 ml of PBS only to check for any contamination. Samples and controls were pulsified for 30 sec using a Pulsifier. Each sample and control was filtered through an allocated piece of single-layered cheesecloth and into a CellStar sterile non pyrogenic 50 ml PP-tube (DNase and RNase free) (Greiner Bio-One GmbH, Germany). Filtrates were centrifuged at 1 000 rpm using a GH-3.7 Beckman rotor in a Beckman GS-6 centrifuge (Beckman, U.S.A.). The supernatants were poured into 50 ml Oakridge centrifuge tubes (Nalgene, U.S.A) while the pellets were discarded. Thereafter, supernatants were centrifuged at 14 000 rpm for 10 min using a JA-20 rotor in a Beckman J2-21 centrifuge (Beckman, U.S.A.). Supernatants were discarded and each pellet re-suspended in 500 µl of sterile PBS and filtered through a 1.2 µm membrane filter onto an allocated *H. pylori* selective plate. Thereafter, the filtration technique as described in 2.2.2.5.a. was followed. As an additional negative control, a 1.2 µm membrane filter was placed onto a *H. pylori* selective plate to determine if the filter was a source of contamination.

2.2.2.7. Melkbos 4X4 water Experiment

a. Direct Streaking Technique

A sterile inoculating loop was placed in the Melkbos 4X4 water sample, which was stored at 4⁰C for 2 days, and streaked onto a *H. pylori* selective plate followed by incubation as in 2.2.2.5.b.

b. Direct Filtration Technique

The experimental sample consisted of 300 µl of Melkbos 4X4 water sample only. The two positive controls consisted of 300 µl of Melkbos 4X4 water sample spiked with two 5 µl loopfuls of 2-3 day old *H. cinaedi* and *H. fennelliae* cultures; and *H. pylori* culture only, respectively. As negative controls, 0.6 µm and 1.2 µm membrane filters were placed onto *H. pylori* selective plates without having anything filtered through it to check for any filter contamination. Thereafter, the procedures in 2.2.2.5.a. and 2.2.2.5.b. were followed.

2.2.2.8. Melkbos 4X4 gravel and muddy sand experiment

The experimental samples consisted of 25 g of gravel or muddy sand dispensed into allocated pulsifier bags. Thereafter, 30 ml of sterile PBS was added. The positive controls consisted of 25 g of gravel or muddy sand, 30 ml of sterile PBS and spiked with two 5 µl loopfuls of 2-3 day old cultures of *H. cinaedi*, *H. fennelliae* and *H. pylori*. The negative control consisted of 30 ml of PBS only. Samples and controls were pulsified for 30 sec in a Pulsifier. An inoculum of each sample and control was streaked onto an allocated *H. pylori* selective plate to determine whether the bacteria of interest were blocked by the cotton gauze, used in the following step. Samples and controls were filtered through individual Smith and Nephew Natuuril (100 mm X 75 mm X 75 mm X 8 ply) cotton gauze, placed over the mouth of a sterile CellStar 50 ml PP-tube (DNase and RNase free). The filtrate was centrifuged at 1 000 rpm for 10 min using a GH-3.7 Beckman rotor in a Beckman GS-6 centrifuge. Supernatants were poured into 50 ml Oakridge centrifuge tubes, while the pellets were discarded. Thereafter, supernatants were centrifuged at 14 000 rpm for 10 min using a JA-20 rotor in a Beckman J2-21 centrifuge. Supernatants were discarded and pellets re-suspended in 500 µl of

sterile PBS. An inoculum of each sample and control was streaked onto an allocated *H. pylori* selective plate to determine if any bacteria were blocked by the 0.6 µm ME 26 membrane filter, used in the following step. The remaining volume of each re-suspended pellet, consisting of concentrated bacterial cells, was dispensed onto an allocated 0.6 µm membrane filter placed on a *H. pylori* selective plate. Thereafter, filtration and incubation, as described in 2.2.2.5.a. and 2.2.2.5.b., respectively were followed.

2.2.2.9. Compost and mountain soil experiment

The mountain soil was obtained from a hiking trail at Lions Head. A 25 g sample of mountain soil and compost was placed into allocated pulsifying bags to which 30 ml of sterile PBS was added. The positive controls consisted of 25 g of compost and mountain soil, respectively; 30 ml of sterile PBS and spiked with two 5 µl loopfuls of 2-3 day old cultures of *H. fennelliae*, *H. cinaedi* and *H. pylori*. The negative control consisted of 30 ml of PBS only. Samples and controls were pulsified for 30 sec with a Pulsifier. Each sample and control was streaked onto an allocated *H. pylori* selective plate to determine if the cotton gauze, in the next step, would retain any bacteria of interest. Samples were filtered through a Smith and Nephew Natuuril (100 mm X 75 mm X 75 mm X 8 ply) pure cotton gauze, placed over the mouth of a sterile CellStar 50 ml PP-tube (DNase and RNase free). The filtrates were centrifuged at 1 000 rpm for 10 min using a GH-3.7 Beckman rotor in a Beckman GS-6 centrifuge. Supernatants were transferred to 50 ml Oakridge centrifuge tubes and pellets were discarded. Supernatants were centrifuged at 14 000 rpm for 10 min using a JA-20 rotor in a Beckman J2-21 centrifuge. Supernatants were discarded and pellets re-suspended in 500 µl of sterile PBS and filtered through a 0.6 µm membrane filter on a *H. pylori* selective plate followed by procedures described in 2.2.2.5.

2.2.2.10. Mountain water and laboratory tap water experiment

The mountain water sample was obtained from a hiking trail at Lions Head. The experimental samples consisted of 1 ml of laboratory tap water and mountain water, respectively, filtered through a 0.6 µm membrane filter. Similarly, a 1 ml

aliquot of each sample was filtered through a 1.2 μm membrane filter. This was done to determine if the 0.6 μm filter would prevent the bacteria of interest from filtering through. The positive controls consisted of 1 ml of laboratory and mountain water, respectively, spiked with two 5 μl loopfuls of 2-3 day old cultures of *H. fennelliae*, *H. cinaedi* and *H. pylori* each of which were filtered through a 0.6 μm and 1.2 μm filter. As negative controls, 0.6 μm and 1.2 μm membrane filters were placed onto *H. pylori* selective plates to check for contamination followed by filtration and incubation in 2.2.2.5.a. and 2.2.2.5.b., respectively.

2.2.2.11. Treated and untreated sewage / sludge experiment

a. Direct Streaking and Filtration Techniques

Inoculums of the unprocessed (initial) untreated and treated sewage / sludge samples were streaked onto allocated TBA and *H. pylori* selective plates to compare bacterial growth between the two plates. Volumes of 40 ml of untreated and treated sewage / sludge samples were centrifuged at 14 000 rpm at RT for 7 mins in a JA-20 rotor in a Beckman J2-21 centrifuge. Supernatants were discarded and pellets retained and re-suspended in 500 μl of sterile PBS. Inoculums of re-suspended (processed) treated and untreated sewage / sludge samples were streaked onto allocated TBA and *H. pylori* selective plates to determine if any bacteria of interest were lost after centrifugation. Volumes of 200 μl of re-suspended treated and untreated sewage / sludge samples were filtered through 0.6 μm membrane filters on TBA and *H. pylori* selective plates. As a negative control, a 0.6 μm membrane filter was placed onto a *H. pylori* selective plate to detect any filter contamination. The two positive controls consisted of 100 μl of re-suspended sample spiked with two 5 μl loopfuls of 2-3 day old cultures of *H. fennelliae* and *H. cinaedi*; and *H. pylori*, respectively. The positive controls were filtered through a 0.6 μm membrane filter onto a *H. pylori* selective plate. In addition, inoculums of each of the positive controls were streaked onto a *H. pylori* selective plate to determine if the filter was preventing filtration of any bacteria of interest. Thereafter, the filtration and incubation procedures, as in 2.2.2.5.a. and 2.2.2.5.b., respectively were followed.

b. Enrichment Technique

A 20 ml volume of treated and untreated sewage / sludge was dispensed into a sterile 50 ml conical flask and incubated at 37°C in a H₂-enriched microaerophilic atmosphere, generated by an Oxoid BR 38 generating kit, for 2 days. After 2 days, 200 µl aliquots of treated and untreated sewage / sludge samples were filtered through 0.6 µm membrane filters onto TBA and *H. pylori* selective plates. The remainder of the samples were centrifuged at 14 000 rpm at RT for 10 mins in a JA-20 rotor in a Beckman J2-21 centrifuge. Supernatants were discarded and pellets re-suspended in 500 µl of PBS. A 200 µl aliquot of each re-suspended sample was filtered through 0.6 µm membrane filters onto TBA and *H. pylori* selective plates. As positive controls, a 100 µl aliquot of treated and untreated sewage / sludge sample was spiked with two 5 µl loopfuls of 2-3 day old cultures of *H. fennelliae*, *H. cinaedi* and *H. pylori*. In addition, each enriched positive control was streaked onto TBA and *H. pylori* selective plates to determine if the 0.6 µm filter blocked filtration of *H. fennelliae*, *H. cinaedi* or *H. pylori*. As a negative control, a filter was placed directly onto a *H. pylori* selective plate without having anything filtered through it to determine whether the filter was a source of contamination. Filters were removed within 20 min and the filtrates spread evenly on the surfaces of TBA and *H. pylori* selective plates using a sterile hockey-stick spreader. Plates were allowed to dry at RT and then inverted and incubated at 37°C in a H₂-enriched microaerophilic atmosphere generated by an Oxoid BR 38 generating kit.

2.2.3. Culture Maintenance

Samples and cultures were re-incubated every second day at 37°C in a H₂-enriched microaerophilic atmosphere generated by using an Oxoid BR 38 gas generating kit in a clean gas jar.

Cultures intended for short-term storage (less than 5 days) were stored at 37°C under appropriate atmospheric conditions.

Pure cultures intended for long-term storage involved the sweeping up of 2-3 old healthy-growing cultures, using a sterile loop, off TBA plates. The mass of bacteria was sterilely

deposited into a Microbank tube containing broth and beads. The Microbank tube was shaken sufficiently to ensure that the bacterial mass was broken down and evenly displaced. It was also done to allow the bacteria to enter the hole in the centre of the bead and adhere to the rest of the bead. Thereafter, the broth was swiftly removed using a sterile Pasteur pipette after flaming the mouth of the Microbank tube. These tubes were immediately snap-frozen and stored at -70°C .

2.2.4. Biochemical and phenotypic characterization of *Helicobacter* and related micro-organisms

Originally, the “Cape Town protocol” was developed to isolate and identify to species level a variety of *Campylobacter*, *Helicobacter* and *Arcobacter* from diarrhoeic stool but it can be adapted to work for other samples as well. The protocol consists of membrane filtration followed by incubation in a H_2 -enriched microaerophilic atmosphere. Various biochemical and phenotypic tests, based on and outlined in the “Cape Town protocol” (Appendix D), were carried out to identify the bacteria of interest, namely *Helicobacter*; *Campylobacter* and *Arcobacter* species, from a diverse range of bacteria within a specific sample. In addition, all clinical; environmental; and revitalized isolates of *Helicobacter* and related genera were also characterized using biochemical and phenotypic tests to identify the species present. All the isolates were later used to validate kits and to generate phylogenetic trees based on the amplification of the 16S rRNA gene.

2.2.4.1. Phenotypic characterisation

The phenotypic characterization of a bacterium includes the observation of its colony morphology and growth characteristics.

a. Preliminary identification procedure based on colony morphology

For the purpose of this study, only colonies resembling the recognized morphology of *Helicobacter*, *Campylobacter* and *Arcobacter* were picked and streaked on fresh TBA plates for single colonies. Generally, *Campylobacter* colonies appear dirty yellow or cream in colour. *Helicobacter* colonies are generally light yellow to off-white in colour and grow as tiny, isolated, round, translucent colonies, a common trait of gastric *Helicobacter* e.g. *H. pylori*, or as a thin flat film as *H. cinaedi* and *H. fennelliae*. In addition, *H. fennelliae* possesses a

distinct odour resembling that of hypochlorite. *Arcobacter* appear to be whiter than those of *Campylobacter* and grow and survive under aerobic conditions (Le Roux and Lastovica, 1998). Once the colonies of interest were selected, bacterial smears were prepared for a Gram stain.

b. Preparing a good bacterial smear for a Gram stain

A drop of water was placed onto a microscope slide using a sterile Pasteur pipette (LP Italiana Spa, Italy). A sterile inoculating loop was used to pick up a colony of interest from a TBA plate and mixed with the drop of water to create a milky suspension. The smear was allowed to air-dry. Thereafter, the smear was heat-fixed by swiftly passing the slide, 3-5 times, over the flame of the Bunsen burner. The slide was allowed to cool before a Gram stain was prepared.

c. The Gram stain

A Gram stain (developed in 1884 by Christian Gram, a Danish physician) is used to determine the Gram-status of a young cultured micro-organism; a purple-blue colour indicates the presence of a Gram-positive micro-organism, while Gram-negative micro-organisms such as *Helicobacter*, *Campylobacter*, *Arcobacter*, etc are stained pink or red (Prescott *et al.*, 1996).

The Gram-staining technique was used to confirm that cultures were pure and for preliminary identification of the bacteria of interest. Using the Gram stain, *Campylobacter*-like organisms appeared short, stubby or comma-shaped; *Helicobacter*-like organisms appeared to thin, long and spiral in shape; while *Arcobacter*-like organisms were bigger than *Campylobacter*, and slightly curved (Le Roux and Lastovica, 1998). The Gram-staining technique indicated that all of the above micro-organisms were Gram-negative.

Procedure

The microscope slide was flooded with crystal violet stain for 1 min. Crystal violet stain was gently washed off with water followed by flooding with Gram's iodine solution for 1 min. The slide was gently rinsed with water and blot dry with Kimwipe to remove excess water. The slide was flooded with acetone for 15-20 sec to wash off the excess crystal violet dye from the cells and immediately rinsed

with water to seize the decolourization process. Thereafter, the slide was flooded with dilute carbol fuchsin for 30 sec, immediately rinsed with water and allowed to air-dry (<http://web.indstate.edu/thcme/micro/basic.html>). The results of the Gram stain was observed with an Olympus microscope, model: CH20BIMF200 (Olympus Optical Co, Ltd, Japan) using the 10 X objective lens to focus in on the sample. Thereafter, higher resolution was used to gain a more detailed view of bacteria morphology and to preliminary identify these bacteria to the genus level, e.g. *Helicobacter*, *Campylobacter* and *Arcobacter*.

d. Growth Conditions

The type of growth conditions required for the optimal growth and survival of specific cultures helps to determine the bacteria to the genus level, e.g. *Arcobacter* cultures grow under aerobic conditions. *Helicobacter* and *Campylobacter* require microaerophilic or hydrogen-enhanced microaerophilic conditions for growth.

Procedure

Each culture was streaked, in triplicate, onto TBA plates and incubated in H₂-enriched microaerophilic, CO₂-enriched and normal aerobic environments to determine their growth and survival patterns under these conditions.

2.2.4.2. Biochemical characterization

Various biochemical tests, indicated below, and in Appendix D, were used to identify bacteria to the species level. Each biochemical test was performed in duplicate. Positive and negative controls were included in each test.

a. The Pyrazinamide Test

A sterile 5 µl inoculating loop was used to generously sweep up bacterial cells, grown in H₂, and emulsified in the top third of the pyrazinamide media (NHLS media lab, Greenpoint) creating a heavy inoculum. One tube containing semi-solid pyrazinamide media was used per sample. The inoculated pyrazinamide tubes were incubated in a H₂-enriched microaerophilic atmosphere generated by an Oxoid BR 38 generating kit for 2 days (indoxyl acetate positive cultures) or 4 days (indoxyl acetate negative cultures). After incubation, 1 ml of freshly

prepared 10 % ferrous sulphate (FeSO_4) was added along the wall of the tube and allowed to settle at RT for a few minutes before results were recorded. The formation of a brown ring was indicative of a positive result, while a colourless reaction symbolized a negative result.

b. The Urease Test

A sterile 5 μl inoculating loop was used to transfer bacterial cells, grown in appropriate atmospheric conditions, to a small allocated area on a urea agar base plate (NHLS media lab, Greenpoint). The inoculums were heavily spread onto the surface and stabbed into the media and left at RT before the results were recorded. Appearance of a bright pink colour was indicative of a positive result e.g. *H. pylori*, while a negative result was illustrated by a colourless reaction e.g. *H. cinaedi*.

c. The Rapid H_2S Test

A sterile 5 μl inoculating loop was used to generously sweep up 2-3 day old bacterial cells, grown in H_2 , and deposited into the semi-solid iron broth (NHLS media lab, Cape Town) as an intact “blob”. One tube was used per sample. The inoculated tubes were incubated overnight in a H_2 -enriched microaerophilic environment. A positive result was indicated by the blackening of the area around “blob” e.g. *C. jejuni* subsp. *jejuni*, while a negative result was indicated by an unchanged colour around the “blob” e.g. *C. upsaliensis*.

d. The H_2S production on Triple Sugar Iron (TSI) Test

A sterile 5 μl inoculating loop was used to sweep up a generous amount of 2-3 day old bacterial cells, grown in appropriate atmospheric conditions, spread onto the slanted TSI agar (NHLS media lab, Cape Town) followed by stab-inoculation of the concave end of the agar. A lead acetate (PbAc) strip was suspended over the slant. The tubes were incubated for 48 hr in appropriate atmospheric conditions before the results were recorded. The blackening of the PbAc strip was indicative of a positive result e.g. *A. butzleri*, while a colourless reaction indicated a negative result.

e. The Oxidase Test

A sterile 5 µl inoculating loop was used to sweep up a generous amount of 2-3 day old bacterial cells, grown in a favourable environment, and spread in an allocated area on an oxidase strip (<http://medic.med.uth.tmc.edu/path/oxidase.htm>). Bacteria possessing the cytochrome oxidase enzyme oxidized the strip resulting in a dark purple colour, indicative of a positive result e.g. *H. pullorum*, while a colourless reaction indicated a negative result.

f. The Antibigram Test

Inoculums of 2-3 day old bacterial cultures, grown in appropriate conditions, were streaked in duplicate on TBA plates. One antibiotic disc was used per plate. After streaking, the nalidixic acid and cephalothin antibiotic discs were placed on allocated TBA plates in an area where heavy growth was expected. Cultures were incubated in required environments for 48 hr. Thereafter, culture growth was analyzed to determine whether inhibition zones were present.

2.2.5. Validating kits using pure cultures of *Helicobacter* and other micro-organisms

Only pure cultures were used to validate kits, and each sample was tested in triplicate to determine reproducibility.

2.2.5.1. Validating the Oxoid Biochemical Identification System (O.B.I.S.

Ltd., U.K.) for the differentiation of *Campylobacter* and *Arcobacter* from other Gram-negative organisms

The O.B.I.S. kit was specifically designed to rapidly differentiate *Campylobacter* and *Arcobacter* from other Gram-negative micro-organisms. Prior to the use of the O.B.I.S. kit, a (0.5 M) NaOH or KOH test was required to determine the Gram-status of the tested bacteria.

Table 2.1: Expected reactions for the O.B.I.S. test kit

Micro-organism	KOH Test	L-ALA Test
<i>Campylobacter</i> species	+	-
<i>Arcobacter</i> species	+	-
Gram- positive species	-	-
Other Gram-negative species	+	+

a. The (0.5M) NaOH or KOH Test Procedure

One to two loops of (0.5 M) NaOH or KOH solution was placed onto a clean glass microscope slide. Using a sterile 5 µl loop, a few colonies of a pure 2-3 day old bacterial culture were mixed into the (0.5 M) NaOH or KOH solution. The loop was carefully raised, at intervals, from the mixture to check for the formation of a DNA “string” between the loop and the mixture. A negative result was characterized by the formation of a suspension of cells without the presence of a DNA “string”, indicative of a Gram-positive bacterium. A positive result was characterized by a high viscosity in the mixture in addition to the formation of a DNA “string”, indicative of a Gram-negative bacterium.

b. O.B.I.S. Test Procedure

Using a sterile 5 µl loop, a few colonies of a pure 2-3 day old bacterial culture were spread across the reaction zone, inside a circle, of a test card. One drop of O.B.I.S. buffer solution was dispensed onto each of the inoculated reaction zones. After 30 sec, one drop of O.B.I.S. developing solution was dispensed onto each of the reaction zones. The appearance of a purple colour, within 20 seconds, indicated a positive L-ALA reaction and the presence of L-ALA, as illustrated for *E. coli*. A colourless reaction, after 20 sec, indicated a negative reaction and that the bacterium was a presumptive *Campylobacter* or *Arcobacter* species (O.B.I.S. Instruction Leaflet).



Figure 2.5: The O.B.I.S. test card illustrating the presence or absence of L-ALA.

2.2.5.2. The L-ALA Confirmatory Test using the Fluka Aminopeptidase Test (Code 75554) (Fluka, Germany)

A sterile 5 µl loop was used to transfer a few colonies of pure culture onto a test strip and placed in a sterile plastic culture tube, with cap, (12 X 75 mm) (Elkay Laboratory Products Ltd., U.K.) containing 0.2 ml of distilled water. The tubes were incubated at 37°C for 10-30 min. The appearance of a yellow suspension illustrated that L-ALA was present, indicative that the micro-organism was Gram-negative as illustrated by *E. coli* (left tube, fig. 2.6). (Exceptions include *Bacteroides vulgatus*, *Bacteroides fragilis*, *Veillonella parvula*, *Campylobacter* spp., *Arcobacter* spp.). A sample without yellow colouration illustrated that L-ALA was absent, indicative of a Gram-positive micro-organism, for example, *Staphylococcus* sp. (right tube, fig. 2.6). This test does not recommended the picking of any pigmented colonies (Fluka Aminopeptidase Test Instruction Leaflet). In addition, the growth medium on which colonies are grown should not contain any dyes or indicators as it may result in a “false” positive reaction.



Figure 2.6: The Fluka Aminopeptidase Test indicating the presence or absence of L-ALA.

2.2.5.3. Validating the CampyCheck *Campylobacter* latex agglutination kit (Microgen Bioproducts, U.K.) using pure cultures of *Campylobacter*, *Helicobacter*, *Arcobacter* and other non-related micro-organisms.

This prototype CampyCheck *Campylobacter* latex agglutination kit is a rapid latex agglutination test intended for the presumptive identification of *Campylobacter* colonies isolated on selective or non-selective solid media from clinical, food or environmental samples (Campycheck *Campylobacter* Leaflet, Microgen, U.K.). In this study, colonies of *Campylobacter*; *Helicobacter*, *Arcobacter* and various Gram-negative and Gram-positive bacteria were tested to validate the specificity of the kit.

Principle of the CampyCheck *Campylobacter* latex agglutination test

An agglutination reaction occurs when an immune complex is formed by cross-linking cells or particles with specific antibodies resulting in the formation of visible clumps (aggregates) that can be seen with the naked eye (Prescott *et al.*, 1996). Recently agglutination techniques that employ microscopic synthetic latex spheres, coated with antigens, have been developed. The latex particles of this prototype kit were coated with rabbit immunoglobins which were raised against antigen preparations from selected *Campylobacter* spp. When the sensitized latex particles are mixed with a solution containing specific *Campylobacter* antigens, an immunochemical reaction occurs causing the finely dispersed latex particles to

agglutinate into aggregates which are clearly visible to the naked eye (*CampyCheck Campylobacter* Leaflet, Microgen, U.K.).

a. Quality Control

The following quality control procedure was carried out using the positive control, provided in the kit, to confirm that the reagents were functioning optimally.

Procedure

An aliquot of 50 µl of positive control was dispensed onto each of the three adjacent areas on the test slide and tested with both of the test and control latex reagents as described in the “Test Procedure” below. Deterioration of a reagent was indicated when:

- a. There was no reaction between the test latex reagents and the positive control or when a reaction illustrated a significant loss of strength over time.
- b. The control latex reagent reacted with the positive control.
- c. A latex reagent became discoloured or formed lumps which did not disappear upon shaking.

b. Test Procedure

Prior to the testing of a range of samples, the *Campycheck Campylobacter* latex agglutination reagents were taken out of the refrigerator and allowed to reach RT. A 50 µl aliquot of isotonic saline was dispensed onto each of the three ovals of the agglutination slide. Using an inoculating loop, several colonies with *Campylobacter*-like morphology were mixed into each of the three ovals containing isotonic saline to form even suspensions on the slide. A 50 µl aliquot of control latex reagent was dispensed onto one of the bacterial suspensions on the slide. Similarly, 50 µl aliquots of each of the two test latex reagents were dispensed onto the other two bacterial suspensions. The bacterial suspensions were mixed with the latex reagents using mixing sticks, starting with the control latex reagent, and spread to the peripheries of the oval test areas. The slide was rocked gently at 150 rpm on an Innova 2 000 Platform shaker (New Brunswick Scientific Co., U.S.A.) for 2 min to keep the fluid suspensions in constant

movement. Thereafter, results were recorded. The test procedure was also carried out on colonies unrelated to *Campylobacter* to confirm the specificity of the kit.

- **Interpretation**

An agglutination reaction is illustrated by the visible aggregation of latex particles. The strength of the reaction may vary and it may be assessed according to the following guidelines.

- + reaction : Fine but readily discernible granularity against a milky background.
- ++ reaction : Coarse granularity against a milky background.
- +++ reaction : Heavy clumping of particles around the periphery of the test oval, against a clear background.

Table 2.2: An interpretation of the CampyCheck *Campylobacter* latex agglutination results

Thermophilic Latex	Species Latex	Control Latex	Interpretation
+	-	-	Thermophilic <i>Campylobacter</i>
+	+	-	Thermophilic <i>Campylobacter</i>
-	+	-	Non-Thermophilic <i>Campylobacter</i>
+ or --	+ or -	+	Auto-agglutination*

*An isolate that causes the Control Latex reagent to agglutinate cannot be tested by CampyCheck *Campylobacter* latex agglutination kit (CampyCheck *Campylobacter* Instruction Leaflet, Microgen, U.K.).

2.2.6. Preparation of Genomic DNA for amplification

The DNA obtained from pure cultures of *Helicobacter* and related genera were extracted using a modification of the hexadecyltrimethyl ammonium bromide (CTAB) method (Current Protocols in Molecular Biology, volume 1, Unit 2.4) while the DNA obtained from human samples were extracted by Dr. Smuts using

the Qiagen tissue kit (Southern Cross Biotechnology, South Africa). Due to the presence of *Helicobacter* DNA in the oesophageal tissue samples of cadavers 5 and 6, it was decided to extract DNA from various tissues of the above mentioned cadavers to determine whether *Helicobacter* DNA was also present within them.

2.2.6.1. DNA extracted using the CTAB method

The original CTAB method was modified. Various samples were prepared differently. The first preparation entailed the use of fresh cultures where 1-2 loopfuls of 2-3 day old pure bacterial cultures were re-suspended in 567 μ l of Tris-EDTA (TE) buffer (Appendix A). The second preparation entailed the use of environmental samples where 5 g of each of the wet and dry sea sand; pond sand; "Melkbos 4 X 4" gravel and muddy sand; compost; and mountain soil samples were dispensed into allocated pulsifying bags and pulsified in 7 ml of PBS (pH 7.2) for 30 sec using a Pulsifier. In addition, the positive controls of the latter samples (spiked with 2-3 day old cultures of *H. fennelliae*, *H. cinaedi* and *H. pylori*), were prepared for the CTAB DNA extraction in the same manner. Thereafter, the pulsified solutions were centrifuged at 14 000 rpm in an Eppendorf centrifuge 5417C (Gerätebau Eppendorf GmbH, Germany) at RT and the pellets re-suspended in 567 μ l of TE buffer. The third preparation entailed the use of treated and untreated sewage / sludge samples, where 12 ml of each sample was centrifuged at 14 000 rpm in an Eppendorf centrifuge 5417C at RT and the pellets re-suspended in 567 μ l of TE buffer. An additional 6 ml sample of each sewage sample was spiked with 2-3 day old cultures of *H. fennelliae*, *H. cinaedi* and *H. pylori* and prepared in the same manner as above. Thereafter, 30 μ l of 10 % SDS (Appendix B) and 3 μ l of 20 mg/ml of proteinase K (Appendix B) were added to the re-suspended pellets to give a final concentration of 100 μ g/ml of proteinase K in 0.5 % SDS. Samples were incubated and mixed at 37⁰C for 1 hr at 700 rpm in an Eppendorf Thermomixer compact to lyse the bacterial cell walls. A volume of 100 μ l of 5 M NaCl (Appendix B) was added to each sample and mixed thoroughly to prevent a CTAB-nucleic acid precipitate from forming. Thereafter, 80 μ l of CTAB / NaCl (Appendix B) solution was

added, mixed thoroughly and incubated for 10 min at 65°C. An equal volume of 24:1 chloroform / isoamyl alcohol (Appendix B) was added, mixed thoroughly and centrifuged at RT for 7 min at 14 000 rpm in an Eppendorf centrifuge 5417C to remove CTAB-protein / polysaccharide complexes. Each supernatant was transferred to a sterile 2 ml Eppendorf tube. An equal volume of 25:24:1 phenol / chloroform / isoamyl alcohol (Appendix B) was added and centrifuged at RT for 7 min at 14 000 rpm in an Eppendorf centrifuge 5417C to remove the remaining CTAB precipitate. Each supernatant was transferred to a sterile 2 ml Eppendorf tube, a 0.6 volume of isopropanol was added to precipitate the DNA and centrifuged at RT for 7 min at 14 000 rpm in an Eppendorf centrifuge 5417C. The DNA was washed with 200 µl of 70 % ethanol to remove residual CTAB solution followed by centrifugation at RT for 10 min at 14 000 rpm in an Eppendorf centrifuge 5417C. The pellets were re-suspended in 100–500 µl of TE buffer, depending on the size of the pellet, for 2 days at 4°C. After 2 days, the pellets were checked, in a well lit area, to determine if the pellets were totally re-suspended. In rare cases where the pellets were not totally re-suspended after the 2 day incubation period, 100 µl of TE buffer was added and incubated at 37°C for 30 min to allow the compact mass of DNA to loosen and re-suspend in TE buffer. Tubes were gently shaken to increase the rate of re-suspension and if necessary returned to 37°C for another 30 min incubation period. For short-term storage (e.g. when the DNA was frequently used), the DNA was stored at 4°C to prevent shearing or degradation of DNA due to constant freeze-thaw processes. For long-term storage (e.g. when DNA was only required in a few weeks or months), the DNA was stored at -20°C.

2.2.7. Determining the concentration of DNA samples

The DNA concentrations were determined by a 1 % agarose gel (Appendix A) electrophoresis. To visualize the DNA, 6.25 µl of ethidium bromide (EtBr) (100 µg/ml) was added to 50 ml of a 1 % agarose solution, mixed and poured into a desired gel tray containing a comb of the desired number of wells. Aliquots of 1 µl of each DNA sample was mixed with 1 µl of 6X loading buffer (Appendix A) and loaded into the wells of a 1

% agarose gel alongside titrated concentrations (5 ng/μl, 10 ng/μl, 20 ng/μl and 100 ng/μl) of the lambda DNA (λ-DNA) marker (Roche, South Africa). A 15 μl aliquot of EtBr (100 μg/ml) was mixed in the 1X Tris-acetate (TAE) buffer (Appendix A) at the bottom end of the submerged gel to ensure upward transfer and thus efficient intercalation of EtBr within DNA. Thereafter, the 1 % agarose gel was electrophoresed at 100 V for 10 min. The DNA concentration of each sample was estimated by comparison with known concentrations of the λ-DNA marker. The DNA was visualized under an ultra violet light source (302 nm) and photographed using a Kodak Digital DC 120 camera (Kodak, U.S.A.).

2.2.8. Primers used to amplify the 16S rRNA and the *L-ALA* (pep N) genes

The Department of Molecular and Cell Biology, Sequencing Unit at the University of Cape Town synthesized the primers in a Beckman Oligo 1000M DNA Synthesizer (Beckman, U.S.A.).

2.2.8.1. *Helicobacter* genus-specific primers used to amplify the 16S rRNA gene

The primers that were used to amplify the short (±350 bp and 400 bp) and large (1 161 bp or 1 200 bp) fragments of the 16S rRNA gene are illustrated in table 2.2. In cases where the outer PCR product could not be seen on an agarose gel, an inner PCR was done using the outer PCR products as a DNA template. The C97 / C98 (Garcia *et al.*, 2002) primer set was used to amplify the short outer PCR product (400 bp), as observed for the cadaver 6 tissue samples. As the short outer PCR products of cadaver 5 could not be seen on the agarose gel the S1 / AS1 (Designed by Dr. Smuts) primer set was used to amplify the short inner PCR product (±350 bp). Furthermore, the C97 / C05 (Shen *et al.*, 2001) primer set was used to amplify the outer (1.2 kb) fragment as observed for DNA samples of cadaver 5 tissues and pure *Helicobacter* cultures. Due to unsuccessful detection of the large outer PCR product in cadaver 6 tissue samples the S1 / AS2 (Designed by Dr. Smuts) primer set was used to amplify the large inner PCR product (1 161 bp).

2.2.8.2. The use of the CAH 16S 1a and CAH 16S 1b general primer set to amplify the 16S rRNA gene of *Campylobacter*, *Arcobacter* and *Helicobacter*.

The CAH 16S 1a and CAH 16S 1b primer set (Marshall *et al.*, 1999), illustrated in table 2.2, was used to amplify a 1004 bp fragment of the 16S rRNA gene in *Campylobacter*, *Arcobacter* and *Helicobacter* species. Using the CAH 16S 1a / CAH 16S 1b primer set, together with C97 / C05 *Helicobacter* genus-specific primer set it was possible to confirm the presence of *Campylobacter*, *Helicobacter* or *Arcobacter* in a DNA sample.

Table 2.3: Primers used to amplify the short and large fragments of the 16S rRNA gene

Target Fragment (bp)	Primer Name	Primer Sequence (5' – 3')	Direction	Annealing Temperature (°C)	Reference
400	C97	GCT ATG ACG GGT ATC C	Forward	50	Garcia <i>et al.</i> , (2002)
400	C98	GAT TTT ACC CCT ACA CCA	Reverse	52	Garcia <i>et al.</i> , (2002)
±350	S1	GAG AGG GTG AAC GGA CAC ACT	Forward	66	Designed by Dr. Smuts
±350	AS1	CCC ACA CTC TAG GAT AGC AG	Reverse	62	Designed by Dr. Smuts
1 200	C97	GCT ATG ACG GGT ATC C	Forward	50	Garcia <i>et al.</i> , (2002)
1 200	C05	ACT TCA CCC CAG TCG CTG	Reverse	58	Shen <i>et al.</i> , (2001)
± 1 161	S1	GAG AGG GTG AAC GGA CAC ACT	Forward	66	Designed by Dr. Smuts
± 1 161	AS2	GCC GTG GGC (AG)GT AGC	Reverse	56	Designed by Dr. Smuts
1 004	CAH 16S 1a	AAT ACA TGC AAG TCG AAC GA	Forward	52	Marshall <i>et al.</i> , (1999)
1 004	CAH 16S 1b	TTA ACC CAA CAT CTC ACG AC	Reverse	52	Marshall <i>et al.</i> , (1999)

Key:

- The C97 / C98 primer set was used to amplify the short (400 bp) fragment of the 16S rRNA gene of cadaver 6 DNA.
- The S1 / AS1 primer set was used to amplify the short (±350 bp) fragment of the 16S rRNA gene of cadaver 5 DNA.
- The C97 / C05 primer set was used to amplify the large (1 200 bp) fragment of the 16S rRNA gene from cadaver 5 DNA and pure *Helicobacter* cultures.

- The S1 / AS2 primer set was used to amplify the large (1 161 bp) fragment of the 16S rRNA gene of cadaver 6 DNA.
- The CAH 16S rRNA 1a / CAH 16S rRNA 1b primer set was used to amplify the 1 004 bp fragment of the 16S rRNA gene from *Campylobacter*, *Arcobacter* and *Helicobacter*.

2.2.8.3. Designing primers to amplify the *L-ALA* gene

Upon use of the O.B.I.S. kit, it was observed that *L-ALA* was absent in various *Helicobacter* species and it was decided to design *L-ALA* primers, illustrated in table 2.3, to confirm the observations of the O.B.I.S. kit. The *L-ALA* sequences of various bacteria (from GenBank, NCBI database) were aligned and compared to identify suitable conserved regions where the *L-ALA* primers could be designed. The primers were also designed to flank regions of diversity within the *L-ALA* gene among the different bacteria tested.

Primer design

The *L-ALA* primers were designed using the Primer Designer for Windows programme, Version 2.0 (Scientific and Educational Software, 1994).

Table 2.4: Primers used to amplify the *L-ALA* gene of various micro-organisms

Target Fragment (bp)	Primer	Primer Sequence (5' – 3')	Direction	Annealing Temperature (°C)	Reference
Variable due to insertions and deletions	L-ALA 1	ACA AAC GAA ATC ACC G	Forward	41	Designed for the study
Variable due to insertions and deletions	L-ALA 2 ¹	G(AG)T CAC G(CG)(CT) (AG)T(CA) (AG)AA (AG)(TA)T G	Reverse	56	Designed for the study
Variable due to insertions and deletions	L-ALA 1*	T(GA)T TTG C(TG)T T(GA)G TGG C	Forward	55	Designed for the study
Variable due to insertions and deletions	L-ALA 2 ²	A(AG)(CA) CC(CT) (CT)(TG)(AG) TT(CT) TCC AT(CT) AGC AC	Reverse	69	Designed for the study

Key:

L-ALA 1: First forward primer designed to amplify the aminopeptidase N gene, however there was no significant match to the gene of interest.

L-ALA 1*: This forward primer was designed to specifically recognize and bind to the aminopeptidase N gene. Upon comparison to known sequences in the NCBI database it was determined that this sequence should bind the aminopeptidase N gene.

L-ALA 2¹ & L-ALA 2²: These reverse primers were designed to bind to the aminopeptidase N gene.

2.2.9. Amplification of *Helicobacter* and related micro-organisms

Two primer sets could detect *Helicobacter* in samples. The first two primer sets, C97 / C98 and C97 / C05, are genus-specific and will only amplify the 16S rRNA gene of *Helicobacter*. However, the CAH 16S 1a / CAH 16S 1b primer set will amplify the 16 S rRNA genes of *Helicobacter*, *Campylobacter* and *Arcobacter*. Furthermore, the L-ALA primer set was designed to confirm the presence or absence of the *L-ALA* gene.

2.2.9.1. Amplification of the 16S rRNA and *L-ALA* genes

a. Amplification of 16S rRNA gene of *Helicobacter* and related micro-organisms.

The polymerase chain reaction (PCR) was used to amplify DNA sequences of interest in an Applied Biosystems 2 720 Thermal Cycler (Applied Biosystems, Singapore). Prior to amplification of the short (± 350 bp or 400 bp) fragment of the 16S rRNA gene, all DNA samples obtained from various tissues of the two cadavers were amplified using the β -globin forward and reverse primers (gH20 / PC04) (Bell *et al.*, 1993) to determine whether any PCR inhibitors were present in any of the DNA samples, thus assessing the quality of each DNA sample. Good quality DNA was indicated by a bright 250 bp fragment due to the amplification of the β -globin gene. Only good quality DNA was used as a template to screen for the presence of *Helicobacter*, through amplification of a short (± 350 bp or 400 bp) fragment in the various tissue samples. Positive reactions were confirmed by the successful amplification of the large (1 161 or 1 200 bp) fragment in each of the samples. However, upon using DNA from pure cultures of *Helicobacter* and related micro-organisms, the 1.2 kb fragment was of interest. The amplification of the 1.2 kb fragment of *Helicobacter* and 1 004 bp fragment of related micro-organisms were also attempted using DNA extracted from various environmental sources. Each reaction mix consisted of 0.8 mM dNTPs (Bioline, U.S.A); 1 X PCR buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, stabilisers and 50 % glycerol) (Southern Cross Biotechnology, South Africa); 1.5 mM MgCl₂ (Southern Cross Biotechnology, South Africa); 50 pmol/ μ l of, both, the forward and reverse primers; and 1.75 U of Super-Therm

Taq polymerase (Southern Cross Biotechnology, South Africa) made up to a volume of 50 μ l with sterile PCR water in an autoclaved 0.2 ml thin wall PCR tube which was RNase and DNase free (Quality Scientific Plastics, U.S.A). The average human DNA concentration used to amplify the short and large fragments of the 16S rRNA gene ranged between 200-300 ng/ μ l. However, the average bacterial DNA concentration used to successfully amplify the large fragment of the 16S rRNA gene ranged between 100-500 ng/ μ l. The same concentration of bacterial DNA was used to amplify the aminopeptidase N (*L-ALA*) gene. A negative control consisting of PCR mix only was included in each PCR run to detect contamination in PCR reagents and PCR water. Similarly, a positive control consisting of PCR mix and 80 ng/ μ l of plasmid 1738 E6 (known to contain the full length C97 / C05 target sequence of *Helicobacter*) was also included to illustrate if the primers worked efficiently.

b. Amplification of the *L-ALA* gene from various micro-organisms.

The PCR reaction mixes were prepared as in 2.2.9.a. In addition varying MgCl₂ titrations were also included.

2.2.9.2. PCR Conditions

a. PCR conditions for the amplification of 250 bp fragment of the β -globin gene

The initial denaturation was carried out at 95^oC for 2 min, followed by 40 amplification cycles, each consisting of 95^oC for 20 sec, 50^oC for 30 sec and 72^oC for 1 min. A final primer extension at 72^oC for 7 min was included followed by storage at 4^oC for an indefinite period to prevent degradation of PCR products.

b. PCR conditions for the amplification of the short outer (400 bp) fragment of the 16S rRNA gene

The PCR conditions for the cadaver 6 tissue samples were similar as in 2.2.9.b.i. except for the annealing temperature of 52^oC for 30 sec. However, due to the unsuccessful detection of cadaver 5 outer PCR products (400 bp fragment), on an agarose gel, an inner PCR was required to amplify the \pm 350 bp fragment of the

16S rRNA gene using the outer PCR products as a template and the conditions described in 2.2.9.b.iii.

c. PCR conditions for the amplification of the short inner ±350 bp fragment of the 16S rRNA gene

The PCR conditions were similar as in 2.2.9.b.i. except for the annealing temperature of 60⁰C for 30 sec.

d. PCR conditions for the amplification of the large outer (1 200 bp) fragment of the 16S rRNA gene

The PCR conditions were similar as in 2.2.9.b.i. except for the annealing temperature of 58⁰C for 30 sec. However, due to the unsuccessful detection of cadaver 6 outer PCR products (1 200 bp fragment), on an agarose gel, an inner PCR was required to amplify the ±1 161 bp fragment of the 16S rRNA gene using the outer PCR products as a template and the conditions described in 2.2.9.b.v. However, upon using the DNA obtained from cadaver 5 tissues and from pure bacterial cultures the outer PCR was successful and thus an inner PCR was not necessary.

e. PCR conditions for the amplification of the 1 161 bp (S1 / AS2 primer set) fragment of the 16S rRNA gene

The PCR conditions were similar as in 2.2.9.b.i. except for the annealing temperature of 61⁰C for 30 sec.

f. PCR conditions for the amplification of the 1 004 bp (CAH 16S rRNA 1a / CAH 16S rRNA 1b primer set) fragment of the 16S rRNA gene

The PCR conditions were as follows: an initial denaturation carried out at 95⁰C for 2 min, followed by 30 amplification cycles, each consisting of 94⁰C for 30 sec, 52⁰C for 30 sec and 72⁰C for 90 sec. A final primer extension at 72⁰C for 10 min was included followed by storage at 4⁰C for an indefinite period to prevent degradation of PCR products.

g. PCR conditions for the amplification of the *L-ALA* gene using the L-ALA

1 / L-ALA 2 primer set

The PCR conditions were as follows: an initial denaturation carried out at 95°C for 2 min; followed by 30 amplification cycles, each consisting of 94°C for 30 sec; a range of annealing temperatures to optimize the PCR; and 72°C for 1 min. A final primer extension at 72°C for 7 min was included followed by storage at 4°C for an indefinite period to prevent degradation of PCR products.

2.2.10. Agarose gel electrophoresis of PCR products

Gel electrophoresis was carried out using 2 % agarose gels (Appendix A) dissolved in 1X TAE buffer. To visualize the DNA, 25 µl of ethidium bromide (EtBr) (100 µg/ml) was added to 200 ml of 2 % agarose solution, mixed and poured into the desired gel tray containing the comb with the desired number of wells. A volume of 10 µl of each PCR sample was mixed with 2 µl of 6X loading buffer (Appendix A) and loaded into the wells. A 20 µl aliquot of EtBr (100 µg/ml) was mixed in the 1X TAE buffer at the bottom end of the submerged gel to ensure upward transfer and thus efficient intercalation of EtBr within DNA. Gels were electrophoresed at 120 V for 2 hr alongside a molecular weight marker. DNA was visualized under an ultra violet light source (302 nm) and photographed using a Kodak Digital DC 120 camera (Kodak, U.S.A.).

2.2.11. Cloning of the amplified 16S rRNA products from cadaver DNA

2.2.11.1. The preparation of competent cells

Competent cells are bacterial cells that are capable of taking up and incorporating DNA fragments into its genome during transformation (Prescott *et al.*, 1996). The calcium chloride method (Current Protocols in Molecular Biology, volume 1, Unit 1.8) was used to prepare competent cells. A single colony of *E. coli* JM109 was inoculated into 50 ml of Luria broth (LB) (NHLS media lab, Cape Town), in a sterile 100 ml conical flask, and incubated overnight at 37°C at 250 rpm. A 1:100 dilution of overnight culture was prepared in 400 ml of LB in a sterile 2 L flask and incubated at 37°C @ 250 rpm until the culture reached an optical density (OD) reading of 0.375 at the 590 nm wavelength. Thereafter, the culture was dispensed into eight sterile, pre-chilled 50 ml Oakridge centrifuge tubes, incubated on ice for 10 min and centrifuged for 7 min @ 3 000 rpm @

4°C. Supernatants were discarded and pellets gently re-suspended in 10 ml of sterile ice-cold CaCl₂ solution (Appendix B) followed by centrifugation @ 2 500 rpm @ 4°C for 5 min. Supernatants were discarded and pellets gently re-suspended in 10 ml of sterile ice-cold CaCl₂ solution and incubated on ice for 30 min. Re-suspended pellets were centrifuged at 2 500 rpm @ 4°C for 5 min. Supernatants were discarded and pellets gently re-suspended in 2 ml of sterile ice-cold CaCl₂ solution and incubated on ice for 6 hr to increase the competency of cells. The competent cells were stored at -70°C in 200 µl aliquots mixed with 20 µl of 80% glycerol in pre-chilled, sterile 1.5 ml Eppendorf tubes. Glycerol was added to the competent cells before storage to prevent the competent cells from shearing during the freeze-thaw process.

2.2.11.2. Transformation efficiency of competent cells

The transformation efficiency was determined during each cloning process to determine the competency of the cells at that specific time. To determine the transformation efficiency of the competent cells a 100 µl aliquot of competent cells mixed with 10 ng of uncut pcDNA 3.1 plasmid was transferred to a sterile, pre-chilled 1.5 ml Eppendorf tube and stored on ice for 10 min. Cells were heat-shocked @ 42°C in a water bath (Mettler, Germany) for 2 min. Thereafter, a volume of 0.9 ml of LB was added, mixed and incubated @ 37°C for 1 hr. A 100 µl aliquot of the transformation culture was spread on the surface of a 2xYT agar plate containing ampicillin (AMP) (100 mg/ml) (Appendix B), diluted to a final concentration of 100 µg/ml. The plate was allowed to dry for 30 min, inverted and incubated for 12-16 hr @ 37°C. The competency of cells is determined by counting the number of colonies and expressing it as $n \times 10^8$ colony forming units (cfu)/µg DNA where n represents an unknown number, for example: $200 \text{ cfu} \div 0.001 \text{ ng} = 2 \times 10^5 \text{ cfu/ng} = 2 \times 10^8 \text{ cfu/}\mu\text{g DNA}$. The ideal competency of $\pm 10^8 - 10^9$ is required to ensure efficient transformation.

2.2.11.3. Ligation reactions using the pGEM-T Easy vector (Promega, U.S.A.)

The ligation reagents were briefly thawed. The pGEM-T Easy vector and control insert DNA tubes were briefly centrifuged to collect the contents at the bottom of the tubes, while the 2X Rapid Ligation buffer was thoroughly mixed before use.

a. Ligation Controls

i. Positive Control

This control was used to determine whether the ligation proceeded efficiently. The Promega ligation reaction mix consisted of 5 µl of 2X Rapid Ligation buffer, T4 DNA ligase; 50 ng/µl of pGEM-T Easy vector; 8 ng/µl of control insert DNA; and 3U of T4 DNA ligase made up to a final volume of 10 µl with sterile water (www.promega.com).

ii. Background Control

This control was used to determine the amount of background blue colonies as a result of the ligation of non-T-tailed or digested pGEMT-Easy vector without the insert. This reaction mix consisted of 5 µl of 2X Rapid Ligation buffer, T4 DNA ligase; 50 ng/µl of pGEMT-Easy vector; and 3 U of T4 DNA ligase made up to a final volume of 10 µl with sterile water (www.promega.com).

b. Experimental Ligation

i. Standard ligation reaction with amplified short (± 350 bp or 400 bp) and large (1 161 bp) fragments of the 16S rRNA gene from various human tissue samples extracted from cadavers.

This reaction was used to insert the amplified product of interest into the pGEM-T Easy vector. The reaction consisted of 5 µl of Rapid Ligation buffer, T4 DNA ligase; 50 ng/µl of pGEM-T Easy vector; 20 ng (± 350 bp or 400 bp) – 60 ng (1 161 bp) of PCR product; and 3 U of T4 DNA ligase made up to a final volume of 10 µl with sterile water (www.promega.com).

Thereafter, all the ligation reactions, described above, were mixed and incubated overnight @ 4°C.

2.2.11.4. Transformations using the pGEMT-Easy vector (Promega) and amplified products of the 16S rRNA gene from cadaver DNA.

A sufficient quantity of 2xYT / AMP / IPTG / X-Gal plates (Appendix B) were prepared and equilibrated to RT prior to plating. In addition, a 2xYT plate without AMP and one

with AMP only was also prepared. The tubes containing the ligation reactions were centrifuged, after overnight incubation @ 4⁰C, to collect the contents at the bottom of the tube. An aliquot of 2 µl of each ligation control and 7 µl of each experimental ligation were dispensed into sterile, pre-chilled 1.5 ml Eppendorf tubes and kept on ice. Similarly, 10 ng of circular pcDNA3.1 and linear PUC 19 plasmids were dispensed into allocated sterile, pre-chilled Eppendorf tubes to determine the transformation efficiency and confirm that linear plasmids were not transformed, respectively. Thereafter, 100 µl of recently thawed competent cells was added; mixed and kept on ice for 30 min. In addition, 100 µl of competent cells was dispensed into a sterile, pre-chilled Eppendorf tube without DNA. The competent cells were heat-shocked @ 42⁰C for 2 min in a water bath (Mettmert, Germany). A volume of 0.9 ml of 2xYT broth (NHLS Media Lab, Green point) was added and incubated for 1 hr @ 37⁰C to allow for the transformation of DNA into the bacterial genome before plating 100 µl of each transformation culture onto duplicate 2xYT / AMP / IPTG / X-Gal plates. A 100 µl aliquot of competent cells, without DNA, was spread onto a 2xYT / AMP and 2xYT only plate to detect contamination and the presence of an AMP resistance gene within the genome of competent cells. Plates were allowed to dry for 30 min, followed by incubation @ 37⁰C for 12-16 hr.

2.2.12. Direct screening of clones

The PCR mixes prepared for the screening of clones containing the short (±350 bp or 400 bp) and large (1 161 bp) amplified fragments of the 16S rRNA gene were prepared as described in 2.2.9.a except the mixes were made up to a final volume of 25 µl instead of 50 µl. After streaking each clone onto a 2xYT plate to prevent loss of a clone of interest, the remainder of each clone was swirled into the allocated PCR mix. The PCR conditions for the amplification of the short 16S rRNA fragments were as follows: 2.2.9.b.i and 2.2.9.b.ii for the short 400 bp fragment and 2.2.9.bi and 2.2.9.biii for the ±350 bp fragment, respectively. Furthermore, the PCR conditions for the amplification of the large 16S rRNA fragments were as follows: 2.2.9.b.i and 2.2.9.b.iv for the 1 200 bp fragment and 2.2.9.b.i and 2.2.9.b.v for the 1 161 bp fragment, respectively. Mini scale plasmid preparations were carried out for each positive clone prior to sequencing.

2.2.13. Mini scale plasmid preparations of desired clones to be sequenced

Mini scale preparations were carried out using the Plasmix plasmid DNA purification system (Talent, Italy). Each clone was mixed in 10 ml of 2xYT broth containing 10 µl of AMP (100 mg/ml) (Appendix B) and incubated overnight at 37⁰C. A volume of 2 ml of bacterial culture was dispensed into a sterile 2 ml Eppendorf tube and centrifuged at 14 000 rpm for 5 min at RT in an Eppendorf centrifuge 5417C. Supernatants were discarded, while the pellets were re-suspended in 200 µl of re-suspension solution (Talent, Italy). A volume of 200 µl of lyzing solution (Talent, Italy) was added and mixed by inverting the tube until the suspension cleared. Thereafter, 200 µl of neutralizing solution (Talent, Italy) was added and mixed, as above, until a white precipitate formed followed by centrifugation at 14 000 rpm for 5 min at RT. Supernatants were decanted into sterile 2 ml Eppendorf tubes followed by the addition and mixing of 1 ml of Plasmix-high capacity purification resin (Talent, Italy). One Plasmix filter was used per sample, placed into an allocated sterile 2 ml Eppendorf tube. A 750 µl aliquot of each sample was transferred into an allocated filter and centrifuged at 14 000 rpm for 1 min at RT. The filters were washed with 750 µl of washing solution (Talent, Italy), containing 1 volume of EtOH prior to use, and centrifuged at 14 000 rpm for 1 min at RT. Solutions were decanted from the 2 ml collection tubes, followed by a repeat of the above step. Thereafter, filters were washed with 80 % EtOH and dried by centrifugation at 14 000 rpm for 1 min at RT. The filters were transferred to sterile 1.5 ml Eppendorf tubes. Plasmids were eluted by adding 30 µl of TE buffer (Appendix A), pre-heated at 65-70⁰C. DNA was allowed to re-suspend for 15 min followed by centrifugation at 14 000 rpm for 30 sec at RT.

2.2.14. Determining the DNA concentration of clones for sequencing

A UV spectrophotometer was used to determine the DNA concentrations of pGEM-T Easy plasmids containing the insert, i.e. clones. A 1:20 dilution of each sample was prepared and the DNA concentration was determined by measuring the absorbance at 260 nm in a Beckman DU-40 Spectrophotometer (Beckman, U.S.A.). Once the DNA concentrations were calculated, 20 µl of each sample was sent for sequencing to the DNA Sequencing Unit, Molecular and Cell Biology, U.C.T.

2.2.15. Direct sequencing of PCR products

Due to difficulties experienced in cloning the large (1 161bp) fragments of 16S rRNA gene of cadaver 6, it was decided to perform direct sequencing on every PCR product thereafter. The PCR products were prepared for direct sequencing in the following way. Gel electrophoresis was performed on the PCR products using a 2 % agarose gel as described in 2.2.10., except the total PCR reaction volume was loaded for each sample and thereafter excised under a low UV light source using a sterile surgical blade for each sample. Once excised the Qiagen QIAquick Gel Extraction kit (Southern Cross Biotechnology, South Africa) was used to extract and purify the amplified DNA from the 2 % agarose gel in TAE buffer. Gel slices were weighed and transferred to allocated sterile 2 ml Eppendorf tubes followed by the addition of 3 volumes of buffer QG (Southern Cross Biotechnology, South Africa) to each sample and incubation at 50⁰C for 10 min in an Eppendorf Thermomixer compact. After the gel slices were completely dissolved the colour of the mixture was checked to determine whether the pH was optimal for binding DNA. One volume of isopropanol was added to each sample and mixed thoroughly. One QIAquick spin column, per sample, was placed in a 2 ml collection tube. The DNA sample was added to the QIAquick column and centrifuged at 14 000 rpm for 1 min at RT in an Eppendorf centrifuge 5417C. The flow-through was discarded followed by the addition of 0.5 ml of buffer QG to remove all traces of agarose and centrifugation at 14 000 rpm for 1 min at RT. A volume of 0.75 ml of buffer PE (Southern Cross Biotechnology, South Africa) was added. The column was allowed to stand for 5 min followed by centrifugation at 14 000 rpm for 1 min at RT. The flow-through was discarded and the column centrifuged for an additional 3 min to remove residual ethanol. The QIAquick columns were transferred to allocated sterile 1.5 ml Eppendorf tubes. DNA was eluted by adding 30 µl of buffer EB (10 mM Tris-Cl, pH 8.5) to the centre of the QIAquick membrane and incubated at RT for 15 min to increase DNA concentration. Thereafter, the columns were centrifuged at 14 000 rpm for 1 min at RT. DNA concentration was determined, as described in 2.2.7., and 20 µl of each sample was sent for sequencing to the DNA Sequencing Unit, Molecular and Cell Biology, U.C.T.

2.2.16. Sequence analysis and the generation of phylogenetic trees

2.2.16.1. Sequence analysis

The DNAMAN (Version 4.0, Lynnon BioSoft, 1994-1997) and Chromas (Version 1.43, Conor McCarthy, School of Biomolecular and Biomedical Science, Faculty of Science, Griffith University, Australia) programmes were used to analyze and manually correct DNA sequences. Thereafter, the programme ClustalG (Version 1.4) (obtained via Dr. Smuts) was used to carry out complete multiple alignments on the corrected sequences. All sequences were cut to ensure an optimal alignment.

2.2.16.2. Generation of phylogenetic trees

The phylogenetic trees were drawn using the Treecon for Windows (Treeconw) software programme (http://bioinformatics.psb.ugent.be/psb/Userman/treecon_intro.html). These phylogenetic trees are based on the evolutionary distances computed from the amino and nucleic acid sequences. The evolutionary distance is computed by taking all the pairs of sequences into account while the phylogenetic tree is “inferred” by taking the relationship between these distance values into account, i.e. the more closely related sequences would be grouped together as a cluster (http://bioinformatics.psb.ugent.be/psb/Userman/treecon_intro.html). The Treeconw software programme also has an option to place a root on the tree by using the Root Option (Van de Peer Y & De Wachter R, 1993). For the purpose of this study, *Wolinella succinogenes* was used as a root for each phylogenetic tree.

CHAPTER 3:

RESULTS

University of Cape Town

3. Experimental Results

3.1. Isolation of *Helicobacter* and related micro-organisms from various environmental sources

Although *Helicobacter* was not successfully isolated from the environmental samples that were tested, a related micro-organism, *Arcobacter* was isolated. Detailed results are depicted in table 3.1 below.

Table 3.1: Isolation results from various environmental sources

Environmental Source	Isolation Method	Isolation of <i>Helicobacter</i> or related micro-organisms	Number of colonies isolated
Sea Water	Direct Filtration on TBA media	None	0
Sea Water	Enrichment technique with hemin & 2xYT broth plated on TBA media	None	0
Dry & Wet Sea Sand	Direct Streaking on TBA media	None	0
Dry & Wet Sea Sand	Enrichment Technique with hemin & 2xYT broth and plated on TBA media	None	0
Sea Water	Direct Dispensing on <i>H. pylori</i> selective media	None	0
Sea Water	Direct Filtration on <i>H. pylori</i> selective media	None	0
Duck Pond Water	Direct Dispensing on <i>H. pylori</i> selective media	None	0
Duck Pond Water	Direct Filtration on <i>H. pylori</i> selective media	None	0
Dry & Wet Sea Sand	Pulsification and Filtration on <i>H. pylori</i> selective media	None	0
Duck Pond Sand	Pulsification and Filtration on <i>H. pylori</i> selective media	None	0
Melkbos 4x4 Water	Direct Streaking on <i>H. pylori</i> selective media	None	0

Melkbos 4x4 Water	Direct Filtration on <i>H. pylori</i> selective media	None	0
Melkbos 4x4 Gravel and Muddy Sand	Pulsification & Filtration on <i>H. pylori</i> selective media	None	0
Compost	Pulsification & Filtration on <i>H. pylori</i> selective media	None	0
Mountain Soil	Pulsification & Filtration on <i>H. pylori</i> selective media	None	0
Mountain Water & Laboratory Tap Water	Direct Filtration on <i>H. pylori</i> selective media	None	0
Treated Sewage/Sludge	Direct Streaking on <i>H. pylori</i> selective media	Overgrowth of contaminants	0
Untreated Sewage/Sludge	Direct Streaking on <i>H. pylori</i> selective media	Overgrowth of contaminants	0
Treated Sewage/Sludge	Filtration on <i>H. pylori</i> selective media	Overgrowth of contaminants	0
Treated Sewage/Sludge	Filtration on TBA media	<i>Arcobacter</i>	2
Untreated Sewage/Sludge	Filtration on <i>H. pylori</i> selective media	Overgrowth of contaminants	0
Untreated Sewage/Sludge	Filtration on TBA media	<i>Arcobacter</i>	2
Treated Sewage/Sludge	Enrichment & Filtration on <i>H. pylori</i> selective media	Overgrowth of contaminants	0
Treated Sewage/Sludge	Enrichment & Filtration on TBA media	Overgrowth of contaminants	0
Untreated Sewage/Sludge	Enrichment & Filtration on <i>H. pylori</i> selective media	Overgrowth of contaminants	0
Untreated Sewage/Sludge	Enrichment & Filtration on TBA media	Overgrowth of contaminants	0

- All positive and negative controls gave the expected result in the above experiments.

3.2. Biochemical and phenotypic characterization of *Helicobacter* and related micro-organisms

Biochemical and phenotypic characterization was carried out on a range of samples to identify *Helicobacter* and related micro-organisms to the species level.

3.2.1. Phenotypic characterization

All bacteria of interest, namely, *Helicobacter*, *Campylobacter* and *Arcobacter* were preliminary identified by their Gram-negative status (data not shown); motility capability (data not shown); conditions for optimal growth; colony and bacterial morphology; as shown in table 3.2 below.

Table 3.2: Phenotypic characterization of *Helicobacter* and related bacteria

Micro-organism	Growth	Colony morphology	Bacterial morphology
12 <i>H. cinaedi</i>	H ₂ only	Thin, off-white spreading film	Short, spiral-shaped
12 <i>H. fennelliae</i>	H ₂ only	Thin, off-white spreading film with a hypochlorite resembling odour	Short, spiral-shaped
11 <i>H. pylori</i>	H ₂ and slow growth in CO ₂	Small, isolated, round, translucent colonies	Polymorphic shape
1 <i>H. acinonyx</i>	H ₂ and slow growth in CO ₂	Small, isolated, round, off-white colonies	Short, spiral-shaped
3 <i>H. pullorum</i>	H ₂ only	Small, isolated, round, off-white colonies	Short, spiral-shaped
2 <i>H. canadensis</i>	H ₂ and slow growth in CO ₂	Small, isolated, round, off-white colonies	Short, spiral-shaped
2 <i>H. cholecystus</i>	H ₂ only	Small, isolated, round, off-white colonies	Short, spiral-shaped
1 <i>H. winghamensis</i>	H ₂ only	Small, isolated, round, off-white colonies	Short, spiral-shaped
1 <i>H. pametensis</i>	H ₂ and slow growth in CO ₂	Small, isolated, round, off-white colonies	Short, spiral-shaped
1 <i>H. hepaticus</i>	H ₂ only	Small, isolated, round, off-white colonies	Short, spiral-shaped
1 <i>C. upsaliensis</i>	H ₂ & CO ₂	Small, isolated, round, buff coloured colonies	Short, stubby and u-shaped
1 <i>C. jejuni jejuni</i>	H ₂ & CO ₂	Small, isolated, round, buff coloured colonies	Short, stubby and u-shaped
1 <i>C. jejuni</i>	H ₂ & CO ₂	Small, isolated, round, buff coloured colonies	Short, stubby and u-shaped
1 <i>C. concisus</i>	H ₂ only	Small, isolated, round, buff coloured colonies	Short, stubby and u-shaped
5 <i>Arcobacter butzleri</i>	H ₂ , CO ₂ and aerobic conditions	Small, isolated, round, white colonies spreading film	Small, cigar-shaped, slightly curved

3.2.2. Biochemical characterization

Biochemical characterization was carried out on all cultures to identify *Helicobacter* and related micro-organisms to the species level. The results are shown in table 3.3.

Table 3.3: Biochemical test results identifying *Helicobacter* and related bacteria to the species level

Micro-organism	Ind. Ac	N.R	Cat	Pyraz	Aryl Sylf.	Urease	Oxid	Hip	Rapid H ₂ S	Mac	Antibio-gram
12 <i>H. cinaedi</i>	-	+	+	-	-	-	+	0	0	0	0
12 <i>H. fennelliae</i>	+	-	+	-	+	-	0	0	0	0	0
11 <i>H. pylori</i>	0	0	0	0	0	+++	0	0	0	0	0
1 <i>H. acinonyx</i>	-	-	+	-	-	+	+	-	0	0	Ceph ^S , Nal ^R
3 <i>H. pullorum</i>	-	+	+	-	-	-	+	0	0	0	Ceph ^R , Nal ^S
2 <i>H. canadensis</i>	+	-	+	-	-	-	+	0	0	0	Ceph ^R , Nal ^R
2 <i>H. cholecystus</i>	-	+	+	-	-	-	+	0	0	0	Ceph ^R , Nal ^S
1 <i>H. winghamensis</i>	+	-	+	-	-	-	+	0	0	0	Ceph ^S , Nal ^S
1 <i>H. pametensis</i>	-	+	+	-	-	-	+	0	0	0	Ceph ^R , Nal ^S
1 <i>H. hepaticus</i>	-	+	+	+	+	+	+	0	0	0	Ceph ^R , Nal ^R
1 <i>C. upsaliensis</i>	+	+	-	0	-	-	0	-	-	-	Ceph ^S , Nal ^S
1 <i>C. jejuni jejuni</i>	+	+	+	0	+	-	0	+	+	+	Ceph ^R , Nal ^S
1 <i>C. jejuni</i>	+	+	+	0	-	-	0	+	-	+	Ceph ^R , Nal ^S
1 <i>C. concisus</i>	-	+	-	0	+	-	0	-	-	-	Ceph ^R , Nal ^R
5 <i>Arcobacter butzleri</i>	+	+	+	-	-	-	+	-	- But + for H ₂ TSI	+	Ceph ^R , Nal ^S

Key:

Ind. Ac	: Indoxyl Acetate
N.R.	: Nitrate Reductase
Cat.	: Catalase
Pyraz.	: Pyrazinamide
Aryl. Sulf.	: Aryl Sulfatase
Oxid	: Oxidase
Hip	: Hippurate
Mac	: MacConkey
0	: Not Tested
+++	: Very Strong positive reaction within seconds
Ceph ^R	: Cephalothin Resistant
Ceph ^S	: Cephalothin Sensitive
Nal ^R	: Nalidixic Acid Resistant
Nal ^S	: Nalidixic Acid Sensitive

3.3. Validating kits using pure cultures of *Helicobacter* and other micro-organisms

3.3.1. Validating the Oxoid Biochemical Identification System (O.B.I.S.) for the differentiation of *Campylobacter* and *Arcobacter* from other Gram-negative organisms

Tests were done in triplicate.

O.B.I.S. = Oxoid Biochemical Identification System: (0.5 M) NaOH or KOH Test and the L-alanine aminopeptidase Test.

Prior to testing for the presence of L-ALA, the Gram-status of all isolates was determined by Gram-staining. A total of 157 fresh and revitalized strains of *Campylobacter* and 12 isolates of *Arcobacter* were tested, all of which were positive for the NaOH and negative for the L-ALA (O.B.I.S. and Fluka) tests (table 3.4). The positive NaOH results indicated that *Campylobacter* and *Arcobacter* were Gram-negative micro-organisms, while the negative L-ALA results indicated the absence of L-ALA in these micro-organisms.

Table 3.4: The NaOH and L-ALA (O.B.I.S. and Fluka) test results for *Campylobacter* and *Arcobacter*

Organism	# Strains	Gram-status by Gram-staining	0.5 M NaOH	O.B.I.S. L-ALA	Fluka L-ALA
<i>C. jejuni</i> subsp. <i>jejuni</i>	32	Gram-negative	+	-	-
<i>C. jejuni</i> subsp. <i>doylei</i>	12	Gram-negative	+	-	-
<i>C. coli</i>	10	Gram-negative	+	-	-
<i>C. concisus</i>	31	Gram-negative	+	-	-
<i>C. upsaliensis</i>	24	Gram-negative	+	-	-
<i>C. hyointestinalis</i>	1	Gram-negative	+	-	-
<i>C. lari</i>	2	Gram-negative	+	-	-
<i>C. sputorum</i>	3	Gram-negative	+	-	-
<i>C. fetus</i> subsp. <i>fetus</i>	7	Gram-negative	+	-	-
<i>C. fetus</i> subsp. <i>venerealis</i>	3	Gram-negative	+	-	-
<i>C. helveticus</i>	1	Gram-negative	+	-	-
<i>C. lanenae</i>	1	Gram-negative	+	-	-
<i>C. mucosalis</i>	2	Gram-negative	+	-	-
<i>C. gracilis</i>	2	Gram-negative	+	-	-
<i>C. showae</i>	1	Gram-negative	+	-	-
<i>C. curvus</i>	3	Gram-negative	+	-	-
<i>C. insulaenigrae</i>	5	Gram-negative	+	-	-
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>	4	Gram-negative	+	-	-
<i>C. hyointestinalis</i> subsp. <i>Lawson</i>	2	Gram-negative	+	-	-
<i>A. butzleri</i>	7	Gram-negative	+	-	-
<i>A. butzleri</i> -like	4*	Gram-negative	+	-	-

Key:

- 4*: Environmental isolates # 7 & # 8; # 10 & # 12 from untreated and treated sewage/sludge samples, respectively.

Note: Both the O.B.I.S. and Fluka L-ALA test results were in perfect agreement, indicating the absence of the L-ALA enzyme in *Campylobacter* and *Arcobacter* (O.B.I.S. manual, 2005).

A total of 42 fresh and revitalized strains of 10 species of *Helicobacter* were tested. As expected, the NaOH test results were positive (table 3.5) indicating that these micro-organisms are Gram-negative. However, all *Helicobacter* strains displayed negative results for the L-ALA (O.B.I.S. and Fluka) tests (table 3.5) suggesting the absence of L-ALA in these micro-organisms.

Table 3.5: The NaOH and L-ALA (O.B.I.S. and Fluka) test results for *Helicobacter*

Organism	# Strains	Gram-status by Gram-staining	0.5 M NaOH	O.B.I.S. L-ALA	Fluka L-ALA
<i>H. pylori</i>	10	Gram-negative	+	-	-
<i>H. jennelliae</i>	12	Gram-negative	+	-	-
<i>H. cinaedi</i>	11	Gram-negative	+	-	-
<i>H. acinonyx</i>	1	Gram-negative	+	-	-
<i>H. winghamensis</i>	1	Gram-negative	+	-	-
<i>H. hepaticus</i>	1	Gram-negative	+	-	-
<i>H. pullorum</i>	2	Gram-negative	+	-	-
<i>H. canadensis</i>	2	Gram-negative	+	-	-
<i>H. mesocricetorum</i>	1	Gram-negative	+	-	-
<i>H. pamatensis</i>	1	Gram-negative	+	-	-

The O.B.I.S. test was further validated against Gram-positive and other Gram-negative micro-organisms and confirmed with the Fluka Aminopeptidase test. As expected, the NaOH and L-ALA (O.B.I.S. and Fluka) test results of the 96 Gram-negative clinical isolates were positive (table 3.6) indicating that the micro-organisms were Gram-negative and that L-ALA was absent. However, the results of the 49 Gram-positive clinical isolates were unexpected. All of the test results of the NaOH and L-ALA (O.B.I.S. and Fluka) were positive (table 3.6), suggesting the presence of L-ALA in these micro-organisms.

Table 3.6: The NaOH and L-ALA (O.B.I.S. and Fluka) test results for the Gram-negative and Gram-positive clinical isolates

Organism	# Strains	Gram- status by Gram-staining	(0.5 M) NaOH / KOH	O.B.I.S. L-ALA	Fluka L-ALA
<i>Acinetobacter</i> spp.	8	Gram-negative	+	+	+
<i>Salmonella</i> spp.	11	Gram-negative	+	+	+
<i>E. coli</i>	25	Gram-negative	+	+	+
<i>Shigella</i> spp.	11	Gram-negative	+	+	+
<i>Klebsiella</i> spp.	9	Gram-negative	+	+	+
<i>Haemophilus</i> spp.	13	Gram-negative	+	+	+
<i>Streptococcus</i> spp.	19	Gram-positive	+	+	+
<i>E. faecalis</i>	11	Gram-positive	+	+	+
<i>Staphylococcus</i> spp.	19	Gram-positive	+	+	+
<i>Serratia marcescens</i>	5	Gram-negative	+	+	+
<i>Burkholderia</i>	1	Gram-negative	+	+	+
<i>Pseudomonas</i> spp.	3	Gram-negative	+	+	+
<i>Proteus</i> spp.	3	Gram-negative	+	+	+
<i>Stenotrophonus maltophilia</i>	1	Gram-negative	+	+	+
<i>Enterobacter cloacae</i>	3	Gram-negative	+	+	+
<i>Citrobacter</i>	2	Gram-negative	+	+	+
<i>Morganella</i>	1	Gram-negative	+	+	+

3.3.2. Validating the CampyCheck *Campylobacter* latex agglutination kit (Microgen Bioproducts, U.K.) using pure bacterial cultures

Tests were done in triplicate. This prototype kit was validated against pure cultures of *Campylobacter* (positive control), *Arcobacter*, *Helicobacter* and other non-related micro-organisms. Although the results of *Campylobacter* were as expected, the results of other micro-organisms were variable (table 3.7).

Table 3.7: The CampyCheck *Campylobacter* Latex Agglutination kit results

Micro-organism	Total # of Strains Tested	Gram-status by Gram-staining	Thermophilic <i>Campylobacter</i>	Non-Thermophilic <i>Campylobacter</i>	Auto-agglutination
<i>C. jejuni</i> subsp. <i>jejuni</i>	2	Gram-negative	1	0	0
<i>C. upsaliensis</i>	1	Gram-negative	1	0	0
<i>C. concisus</i>	1	Gram-negative	0	1	0
<i>H. cinaedi</i>	11	Gram-negative	0	11	0
<i>H. fennelliae</i>	12	Gram-negative	0	12	0
<i>H. pylori</i>	10	Gram-negative	0	10	0
<i>H. acinonyx</i>	1	Gram-negative	0	1	0
<i>Arcobacter butzleri</i>	5	Gram-negative	1	0	4
<i>E. coli</i>	13	Gram-negative	0	0	13
<i>Enterococcus faecalis</i>	11	Gram-positive	0	0	11
<i>Shigella flexneri</i>	11	Gram-negative	0	0	11
<i>Haemophilus influenzae</i>	8	Gram-negative	5	1	2
<i>Staphylococcus</i> spp.	18	Gram-positive	0	0	18
<i>Streptococcus</i> spp.	9	Gram-positive	0	0	9
<i>Salmonella</i> spp.	10	Gram-negative	0	0	10
<i>Acinetobacter</i>	4	Gram-negative	0	0	4
<i>Serratia marcescens</i>	4	Gram-negative	0	0	4
<i>Stenotrophomonas maltophilia</i>	1	Gram-negative	0	0	1
<i>Burkholderia</i>	1	Gram-negative	0	0	1
<i>Pseudomonas</i>	3	Gram-negative	0	0	3
<i>Proteus mirabilis</i>	3	Gram-negative	0	0	3
<i>Enterobacter</i> spp.	3	Gram-negative	0	0	3
<i>Citrobacter</i>	2	Gram-negative	0	0	2
<i>Morganella</i> spp.	1	Gram-negative	1	0	0

3.4. Amplification of *Helicobacter* and related micro-organisms

DNA from all tissues from cadaver 5 and cadaver 6 illustrated the amplification of the β -globin gene. A representative gel is shown in figure 3.1.

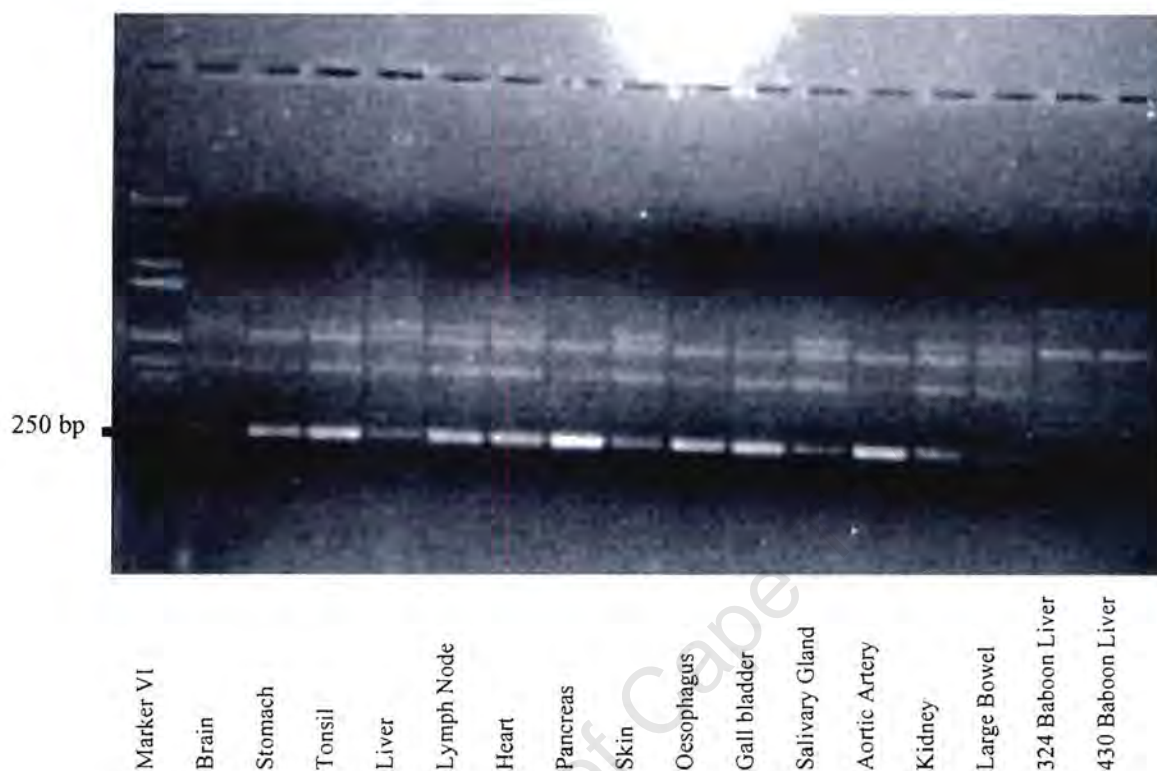


Figure 3.1: A representative gel depicting the amplification of the 250 bp fragment of the β -globin gene.

3.4.1. Amplification of the 16S rRNA gene using cadaver DNA as a template

3.4.1.1. Amplification of the short (\pm 350 or 400 bp) fragment

The 400 bp fragment of the 16S rRNA gene could be amplified from 14 of 22 tissue samples of cadaver 6 (table 3.8). Those tissues which showed no amplification were also negative when the nested PCR was used. The amplification of the 16S rRNA gene from cadaver 5 tissues was only successful after nested PCR, yielding a \pm 350 bp fragment which could be amplified from 7 of 25 tissue samples (table 3.8). Figure 3.2 is a representative gel illustrating the amplification of the 400 bp fragment of cadaver 6. The 400 bp fragment from lanes spleen to bone illustrated a positive reaction and confirmed by sequencing. Samples of the liver and small bowel appeared to be positive but found to be non-specific by sequencing. Samples from the lanes heart to skeletal muscle were negative by PCR.

Although, bands in the heart and skeletal muscle lanes were present they could not be seen in figure 3.2, below.

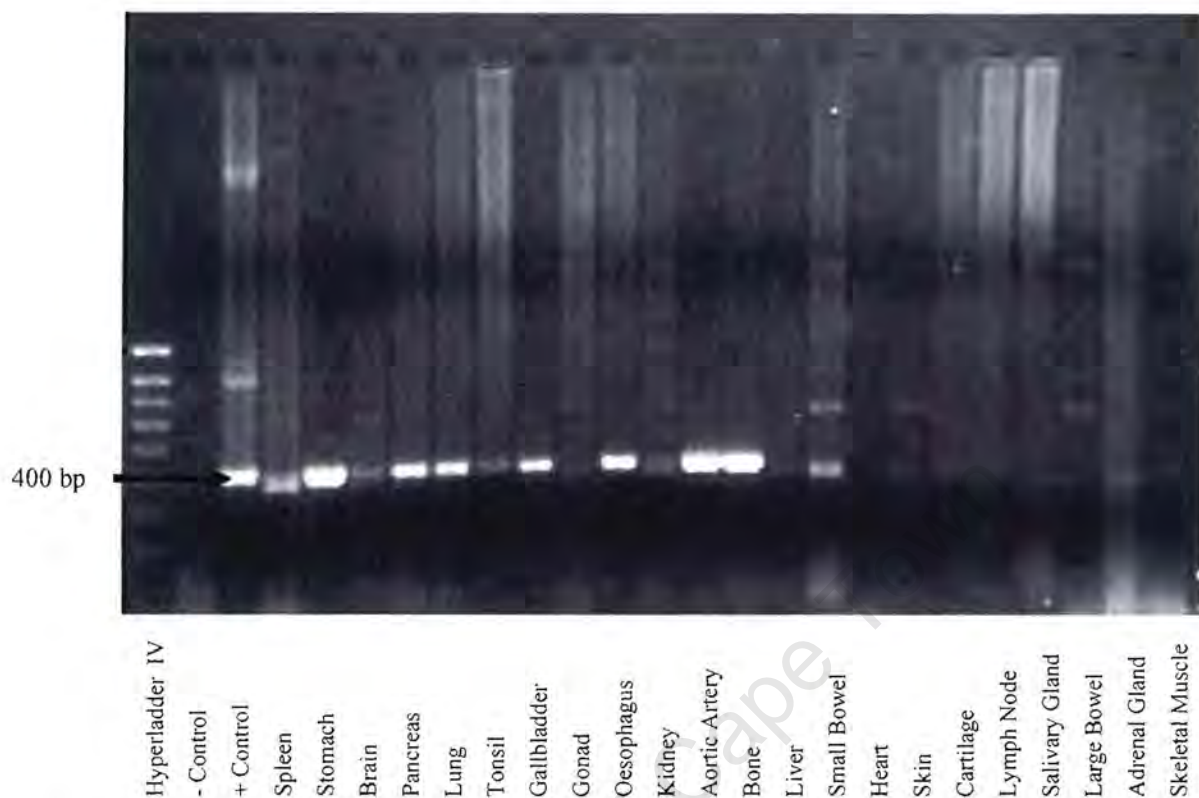


Figure 3.2: A representative gel depicting the amplification of the 400 bp fragment of the 16S rRNA gene of cadaver 6. – Control: no DNA template; + Control: Plasmid E6 1738 containing the full C97 / CO5 sequence.

3.4.1.2. Amplification of the large (± 1 161 or 1 200 bp) fragments

The 1 200 bp fragment could be amplified from 2 of 25 tissue samples of cadaver 5 (table 3.8). The 1 200 bp fragment of tissues other than the stomach and oesophagus, of cadaver 5, were found to be non-specific by PCR and sequencing (results not shown). The amplification of the large fragment of the 16S rRNA gene of cadaver 6 was only successful after nested PCR, yielding a ± 1 161 bp fragment which could be amplified from 8 of 22 tissue samples (table 3.8). A representative gel illustrating the ± 1 161 bp fragment of the 16S rRNA gene of cadaver 6 tissues can be seen in figure 3.3. The band above the 1 161 bp fragment in the 4th lane, containing the amplified sample of stomach, depicts the outer 1 200 bp fragment. The 1 161 bp fragment from the lanes labelled stomach to the aortic artery illustrated a positive reaction (fig. 3.3) and were confirmed by sequencing. The pancreas and bone appeared to be

positive (fig. 3.3) but found to be non-specific when sequenced; while the lanes from the spleen to the small bowel indicated a negative result by PCR (fig. 3.3).



Figure 3.3: A representative gel depicting the amplification of the 1 161 bp fragment of the 16S rRNA gene of cadaver 6. – Control: no DNA template; + Control: Plasmid E6 1738 containing the full C97 / CO5 sequence.

Table 3.8, below, is a summary of the amplification results for the short and large 16S rRNA fragments of all tissues of cadavers 5 and 6.

Table 3.8: Amplification results of *Helicobacter* using the C97/C98; S1/AS1; C97/C05 and S1/AS2 primer sets and confirmation with sequencing

Tissue Samples	Short outer (400 bp) fragment of cadaver 6 (C97/C98)	Short inner (\pm 350 bp) fragment of cadaver 5 (S1/AS1)	Large outer (1 200 bp) fragment of cadaver 5 (C97/C05)	Large inner (\pm 1161 bp) fragment of cadaver 6 (S1/AS2)
Heart	-	-	-	-
Liver	**	-	-	-
Skin	-	-	-	-
Cartilage	-	-	-	-
Spleen	+	+	-	-
Stomach	+	+	+	+
Brain	+	-	-	+
Pancreas	+	+	-	**
Lung	+	-	-	+
Tonsil	+	-	-	-
Gallbladder	+	-	-	+
Lymph Node	-	-	-	-
Salivary Gland	-	-	-	-
Gonad	+	-	-	-
Oesophagus	+	+	+	+
Kidney	+	-	-	-
Large Bowel	-	+	-	-
Aortic Artery	+	+	-	+
Adrenal Gland	-	-	-	-
Bone	+	-	-	**
Small Bowel	**	-	-	-
Skeletal Muscle	-	+	-	-
Ovary / Testis	0	-	-	0
Thyroid	0	-	-	0
Bladder	0	-	-	0

Key:

- + : Detection of *Helicobacter* sp. by PCR and sequencing.
- : No detection of *Helicobacter* sp. by PCR thus no need for sequencing.
- ** : Detection of *Helicobacter* sp. by PCR but not by sequencing.
- 0 : No DNA extracted from the tissue.

3.4.2. Amplification of the 16S rRNA gene using DNA isolated from revitalised *Helicobacter* species as a template

The large (1 200 bp) fragment of the 16S rRNA gene from various *Helicobacter* species was successfully amplified using the genus-specific primers of Shen *et al.*, 2001 and Garcia *et al.*, 2002. A representative gel (fig. 3.4) shows that the *Helicobacter* genus-specific primers did not amplify species of *Campylobacter* and *Arcobacter*.

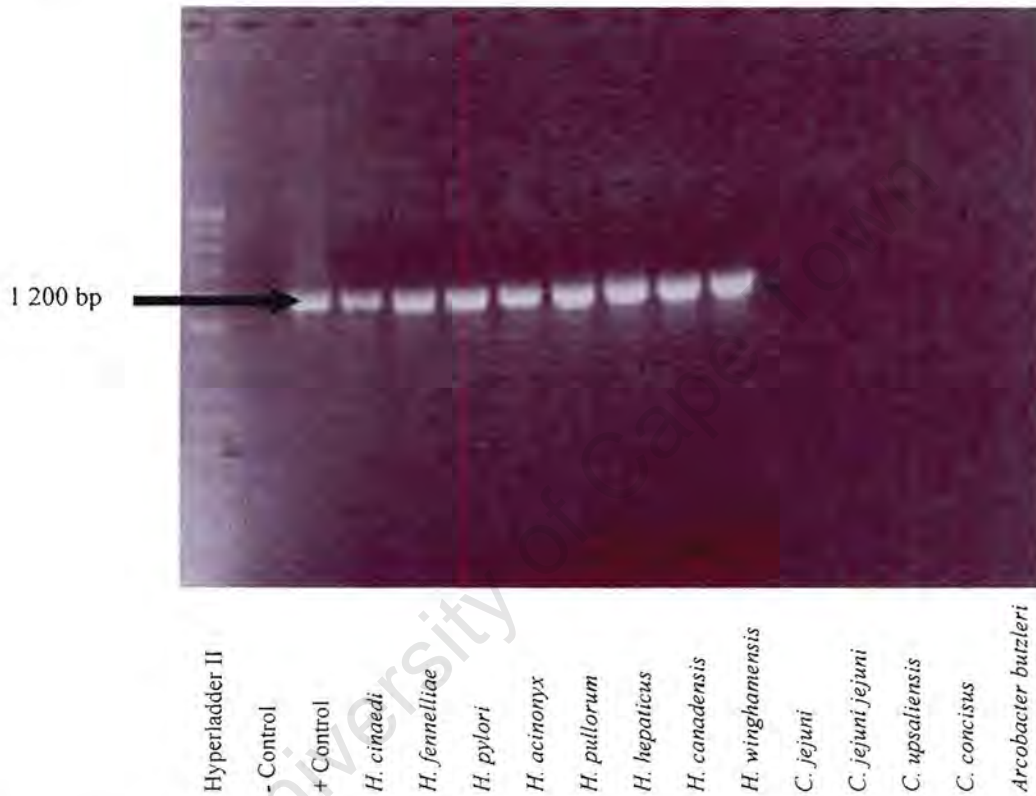


Figure 3.4: A representative gel depicting the amplification of the 1 200 bp fragment of the 16S rRNA gene of pure *Helicobacter*, *Campylobacter* and *Arcobacter* cultures. – Control: no DNA template; + Control: Plasmid E6 1738 containing the full C97/CO5 sequence.

3.4.3. Amplification of the 16S rRNA gene using DNA extracted from sewage / sludge isolates (7, 8, 10 and 12) as a template

Following the adapted procedure of the “Cape Town Protocol” (Appendix D), 4 colonies (isolates 7 & 8, 10 & 12) were successfully isolated from the sewage / sludge samples, 2 from the treated (isolates 7 & 8) and 2 from the untreated (isolates 10 & 12) samples. These isolates were preliminarily identified by phenotypic and

biochemical tests and confirmed by sequence analysis as *Arcobacter butzleri*. The 16S rRNA gene, of all 4 isolates, was successfully amplified using Marshall *et al.*, 1999 *Campylobacter*, *Arcobacter* and *Helicobacter* group-specific primers and yielded a 1 004 bp fragment (fig. 3.5).

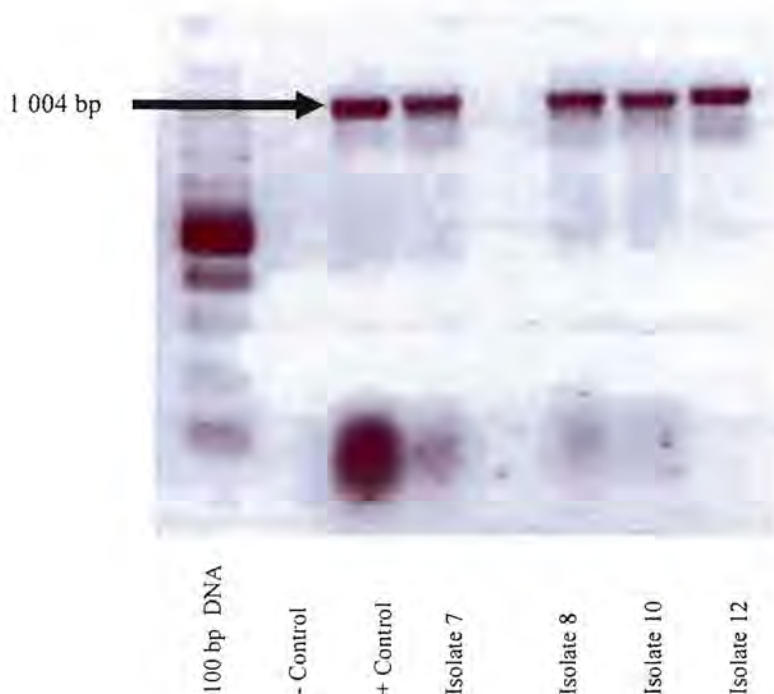


Figure 3.5: A representative gel depicting the amplification of the 1 004 bp fragment of the 16S rRNA gene from isolates of *Arcobacter*. – Control: no DNA template; + Control: DNA from a pure culture of *Arcobacter butzleri*.

3.4.4. Amplification of the aminopeptidase (L-ALA) gene from various micro-organisms

Using the L-ALA primers designed in this study, the *L-ALA* gene could not be amplified from a variety of Gram-positive and Gram-negative micro-organisms. A representative gel (fig. 3.6) illustrates the results of the L-ALA PCR. The expected L-ALA amplification product was \pm 380 bp as the gene is known to have several insertions and deletions. PCR products within the expected size range were observed in the lanes labelled *C. jejuni*, *Klebsiella* and *Enterobacter* but were non-specific when sequenced.



Figure 3.6: A representative gel depicting the amplification results of the L-ALA PCR. – Control: no DNA template; + Control: was not available.

3.5. Direct Screening of clones

3.5.1. Direct screening of the short (± 350 bp or 400 bp) fragment of the 16S rRNA gene

The S1/AS1 primer set (designed by Dr. Smuts) was used to screen for positive clones containing the ± 350 bp fragment (short inner nested PCR) of cadaver 5, while the C97/C98 (Garcia *et al.*, 2002) *Helicobacter* genus-specific primer set was used to screen for positive clones containing the 400 bp fragment of cadaver 6. A representative gel depicting the amplification of the ± 350 bp fragment of the 16S rRNA gene of cadaver 5 is shown in figure 3.7. The clones: St (Stomach) 1, St 3, St 4, St 5, St 6, St 7, St 8, St 10 and H (Heart) 1 appeared to be positive for the ± 350 bp fragment (fig. 3.7). Thus these bands were excised from the gel and cleaned up for sequencing. The band above the ± 350 bp fragment (in the + control lane) corresponds to the amplified 400 bp fragment, a product of the outer PCR reaction.

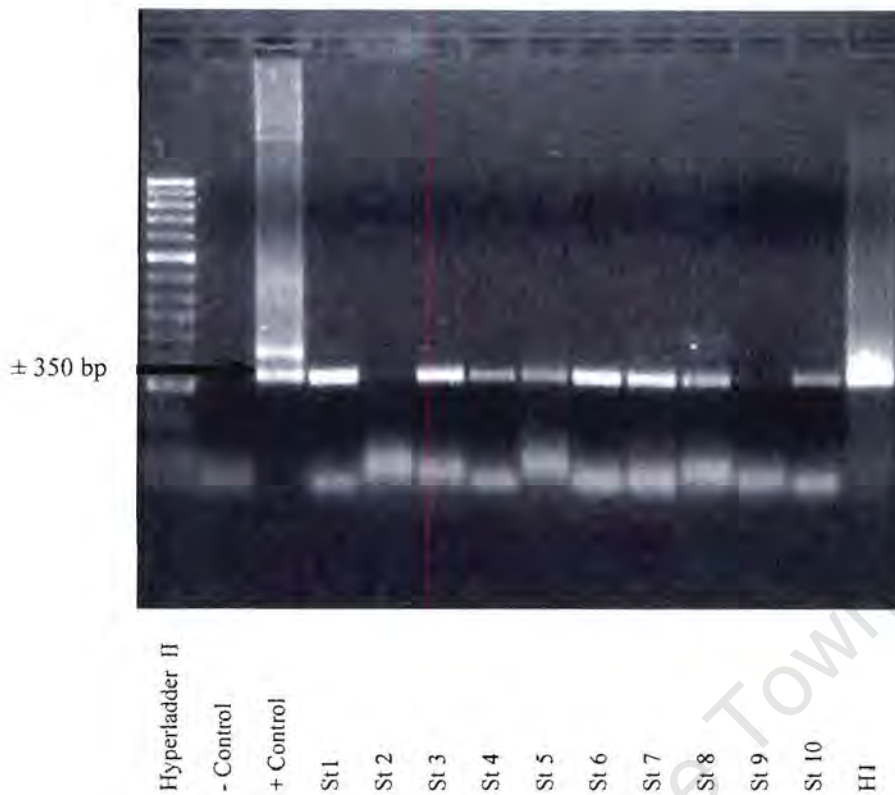


Figure 3.7: A representative gel depicting the amplification of the ± 350 bp fragment of the 16S rRNA gene of cadaver 5 clones. – Control: no DNA template; + Control: Plasmid E6 1738 containing the full C97 / CO5 sequence.

3.5.2. Direct screening of the large (1 200 bp) fragment of the 16S rRNA gene

The C97 / C05 primer set was used to screen for positive clones containing the 1 200 bp fragment of cadaver 5 DNA (fig. 3.8), while the S1 / AS2 primer set was used to screen for positive clones containing the ± 161 bp fragment of cadaver 6 DNA (gel not shown). In figure 3.8, clone 5L.Stomach was the only clone that showed a specific band for the 1 200 bp fragment and was excised from the gel and cleaned up for sequencing. The only other clone, containing the large fragment of the 16S rRNA gene, of cadaver 5 that was successfully sequenced was 5L.Oesophagus (gel not shown). The others were all found to be non-specific PCR products.

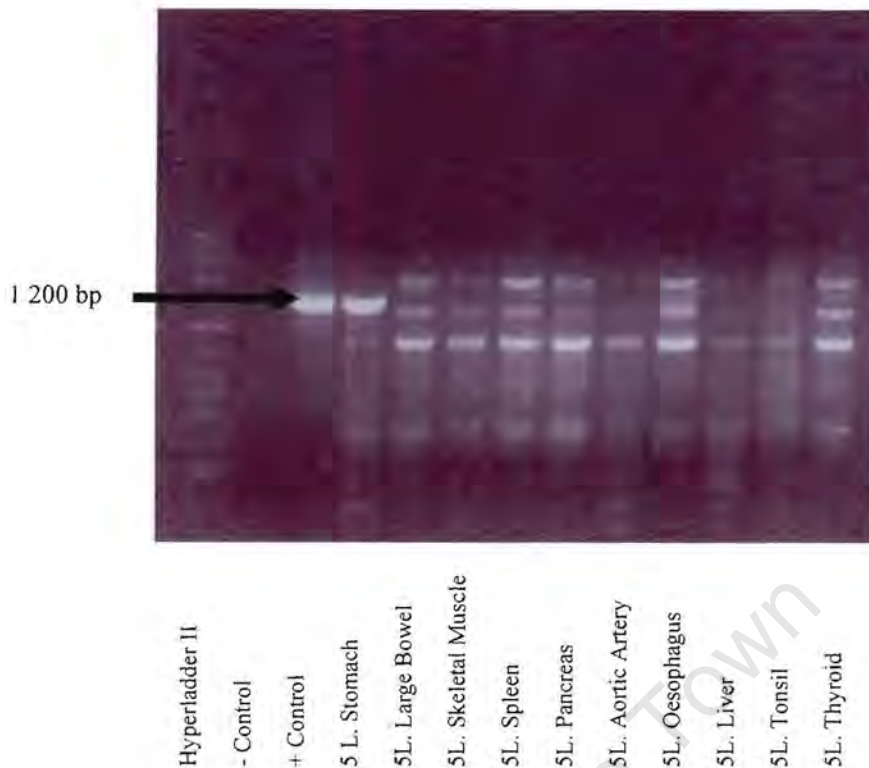


Figure 3.8: A representative gel depicting the amplification of the 1200 bp fragment of the 16S rRNA gene of cadaver 5 clones. – Control: no DNA template; + Control: Plasmid E6 1738 containing the full C97 / CO5 sequence.

3.6. Phylogenetic trees

3.6.1. Phylogenetic tree of the corrected short (± 350 bp) sequences of the 16S rRNA gene of cadaver 5 tissue samples

A phylogenetic tree was constructed using the corrected short sequences of various tissue samples of cadaver 5 to determine the species of *Helicobacter* present in each of the tissues. All tissue samples of cadaver 5 (marked with an asterisk): stomach, aortic artery, pancreas, spleen, large bowel, skeletal muscle and oesophagus including two South African strains, SA-4 and SA-3, cluster with various isolates of *H. pylori* in the gastric group I (sub-group IA) (fig. 3.9) suggesting that each of the tissue samples of cadaver 5 were infected with *H. pylori*. Bootstrap values above 75 % are significant and thus only these percentage values were included in the tree (fig. 3.9). A related micro-organism of *Helicobacter*, *Wolinella succinogenes* was used to root the tree (fig. 3.9).

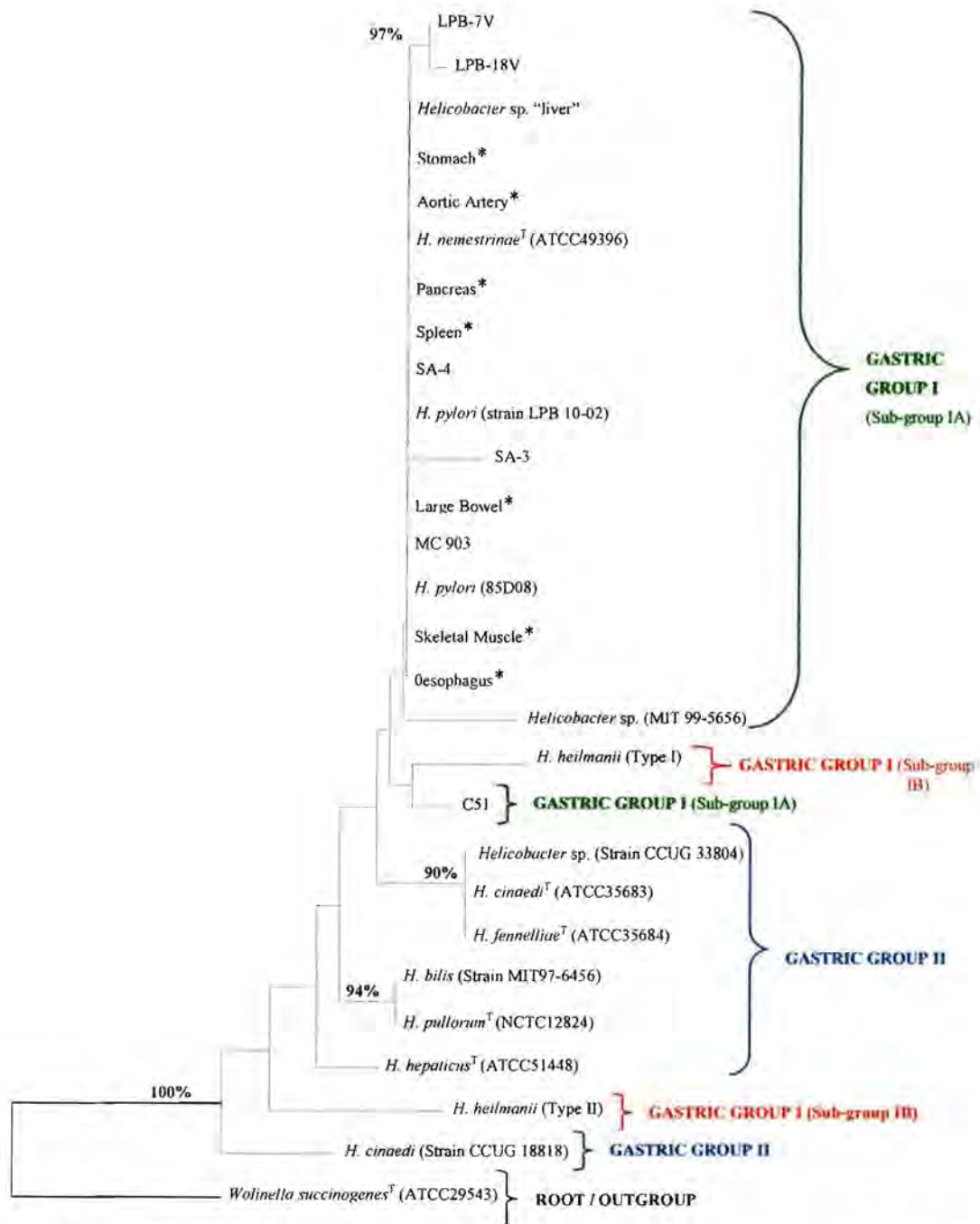


Figure 3.9: Phylogenetic tree illustrating the clustering of the corrected short (\pm 350 bp) sequences of various tissues of cadavers 5 (*) in relation to known *Helicobacter* sequences in the NCBI database.

3.6.2. Phylogenetic tree of the corrected short (400 bp) sequences of the 16S rRNA gene of cadaver 6 tissue samples

The corrected short sequences of various tissue samples of cadaver 6 (marked with an asterisk): kidney, oesophagus, lung, stomach, pancreas, brain, bone, spleen, tonsil, aortic artery, gonad, and gallbladder clustered with various isolates of *H. pylori* in the

gastric group I (sub-group IA) (fig. 3.10) suggesting that each of the tissue samples of cadaver 6 were infected with *H. pylori*. Bootstrap values above 75 % are significant and thus only these percentage values were included in the tree (fig. 3.10). *Wolinella succinogenes* was used to root the tree.

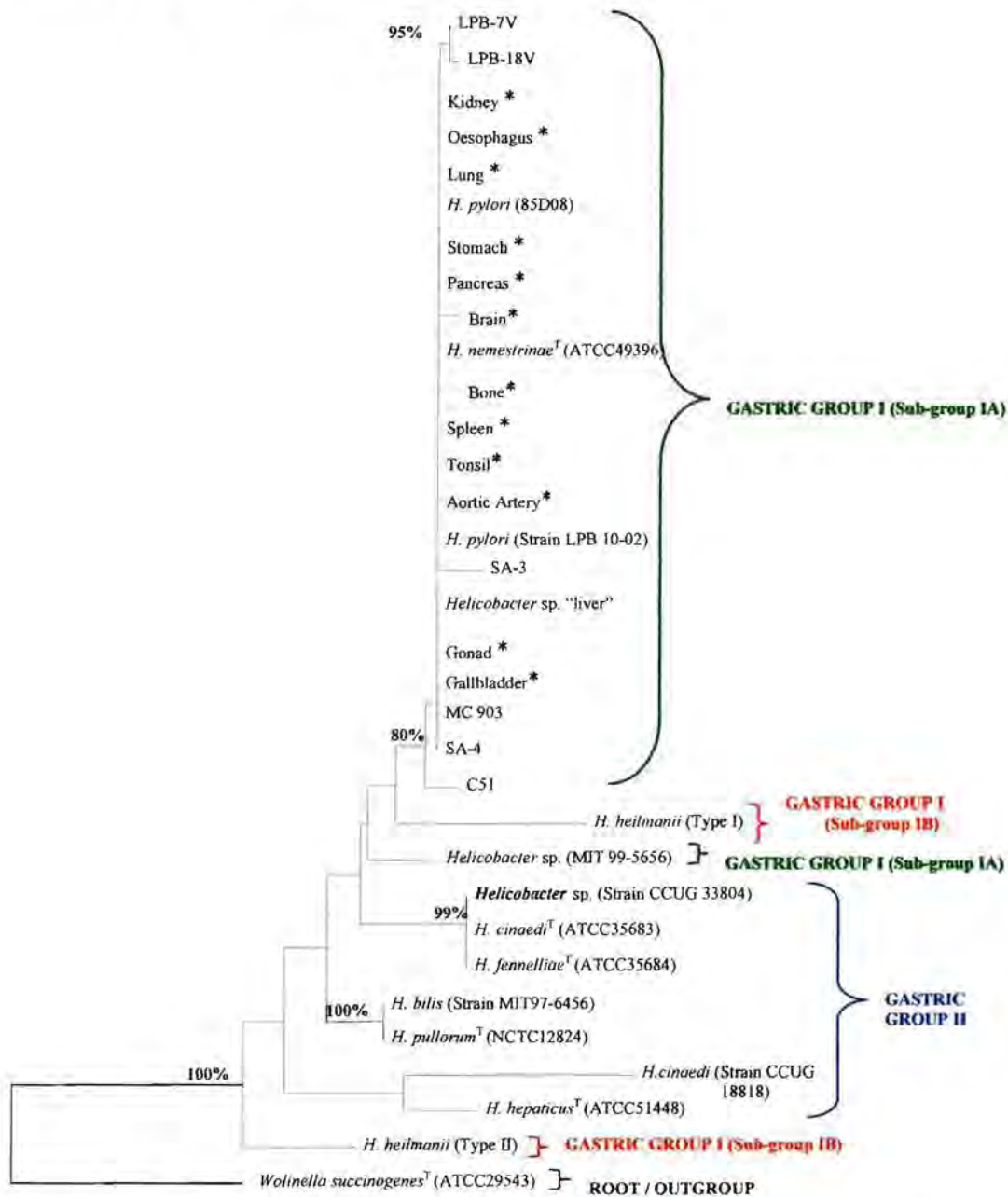


Figure 3.10: Phylogenetic tree illustrating the clustering of the corrected short (400 bp) sequences of various tissues of cadavers 6 (*) in relation to known *Helicobacter* sequences in the NCBI database.

3.6.3. Phylogenetic tree of the corrected large sequences ($\pm 1\ 161$ bp) of the 16S rRNA gene of various tissue samples of cadavers 5 and 6

The cadaver 5 sequences; stomach and oesophagus cluster together, while those of cadaver 6 form several groupings and all appear to be closely related to C51 (*H. pylori* isolate) within gastric group I (sub-group IA), supported by high bootstrap values (fig. 3.11). C51 was isolated from a patient suffering from liver cancer.

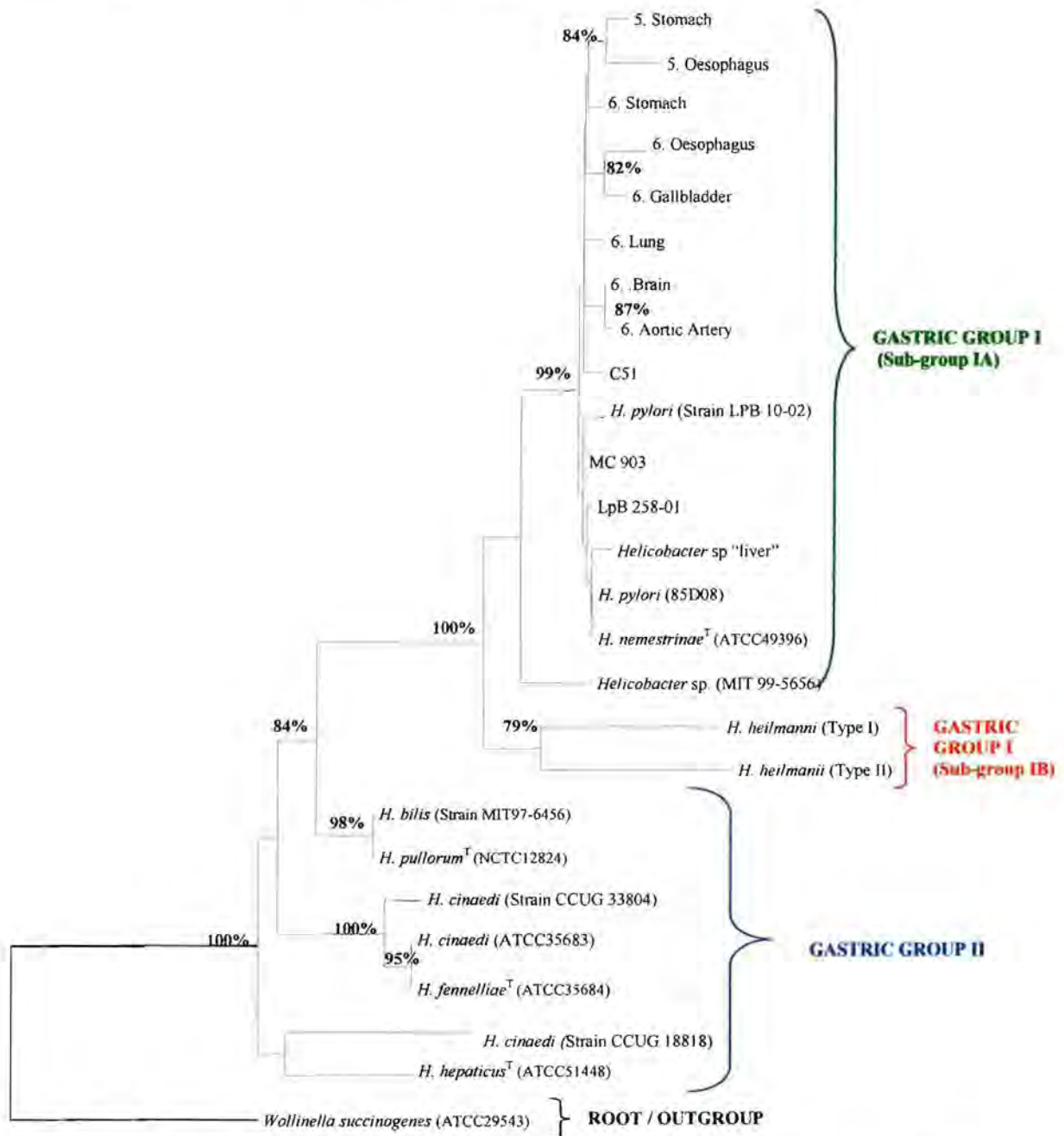


Figure 3.11: Phylogenetic tree illustrating the clustering of the corrected large ($\pm 1\ 161$ bp) sequences of various tissues of cadavers 5 and 6 in relation to known *Helicobacter* sequences in the NCBI database.

3.6.4. Phylogenetic tree of the corrected large sequences (1 200 bp) of the 16S rRNA gene of various pure *Helicobacter* isolates

The phylogenetic tree illustrates the presence of 3 putative genomo-species within *H. pylori* (fig. 3.12). The *H. pylori* isolates: Z28, WT9 and Y28 branches off from the other strains suggesting the presence of a putative genomo-species, supported by a bootstrap value of 82 % (fig. 3.12). In addition, isolates Y27; Y37; Y31; Y30; Y38; and Y29 cluster together and appear to be closely related to the reference strain of *H. pylori* (ATCC43504) forming the second putative genomo-species, while isolate U24 branches off separately to all other *H. pylori* strains suggesting the presence of a third putative genomo-species, supported by a bootstrap value of 100 % (fig. 3.12). All the strains from Denmark group correctly with the reference strains of: *H. hepaticus* (ATCC35684), *H. cholecystus* (Hkb-1T), *H. pametensis* (ATCC51478), *H. winthamensis* (NFLP 97-1090), *H. canadensis* (ATCC700968) and *H. pullorum* (NCTC12824) confirming that they are isolates of these species (bootstrap values of 100 %) (fig.3.12). As expected, *H. acinonyx*, an isolate of this study, groups correctly with the *H. acinonyx* (ATCC43504) reference strain (fig. 3.12). The tree suggests the presence of 4 putative genomo-species within *H. cinaedi*. The *H. cinaedi* isolates: W34, 34.94, C48, 60.05, U25 and Y15 form a separate cluster to isolates 142, X11 and R50, while isolates P5 and 130.94 branch off separately, supported by high bootstrap values. Surprisingly, reference strains of *H. cinaedi* (ATCC35683) and *H. fennelliae* (ATCC35684) group together (fig. 3.12). The *H. fennelliae* group is divided into two main sub-groups: isolates that were identified as *H. fennelliae* by phenotypic and biochemical tests and sequence analysis (58.05; L38; 384.96; Li 78.94; and 225.04) and those isolates identified as *H. fennelliae* (Q22; 166.02; 145.02; P27; 57.02; 194.02; and 55.02) by phenotypic and biochemical tests, but using Basic Local Alignment Search Tool (BLAST) analysis were indicated to be more closely aligned with *Helicobacter* sp. Wee Tee. Isolates previously characterized as *H. pylori* (Y36) and *H. cinaedi* (S2) cluster with the *H. fennelliae* grouping, however BLAST analysis indicated that they were also more closely aligned with *Helicobacter* sp. Wee Tee. In addition, isolates S2 and 225.04 appear to form a distinct group from the other strains, supported by a high bootstrap value of 100 %.

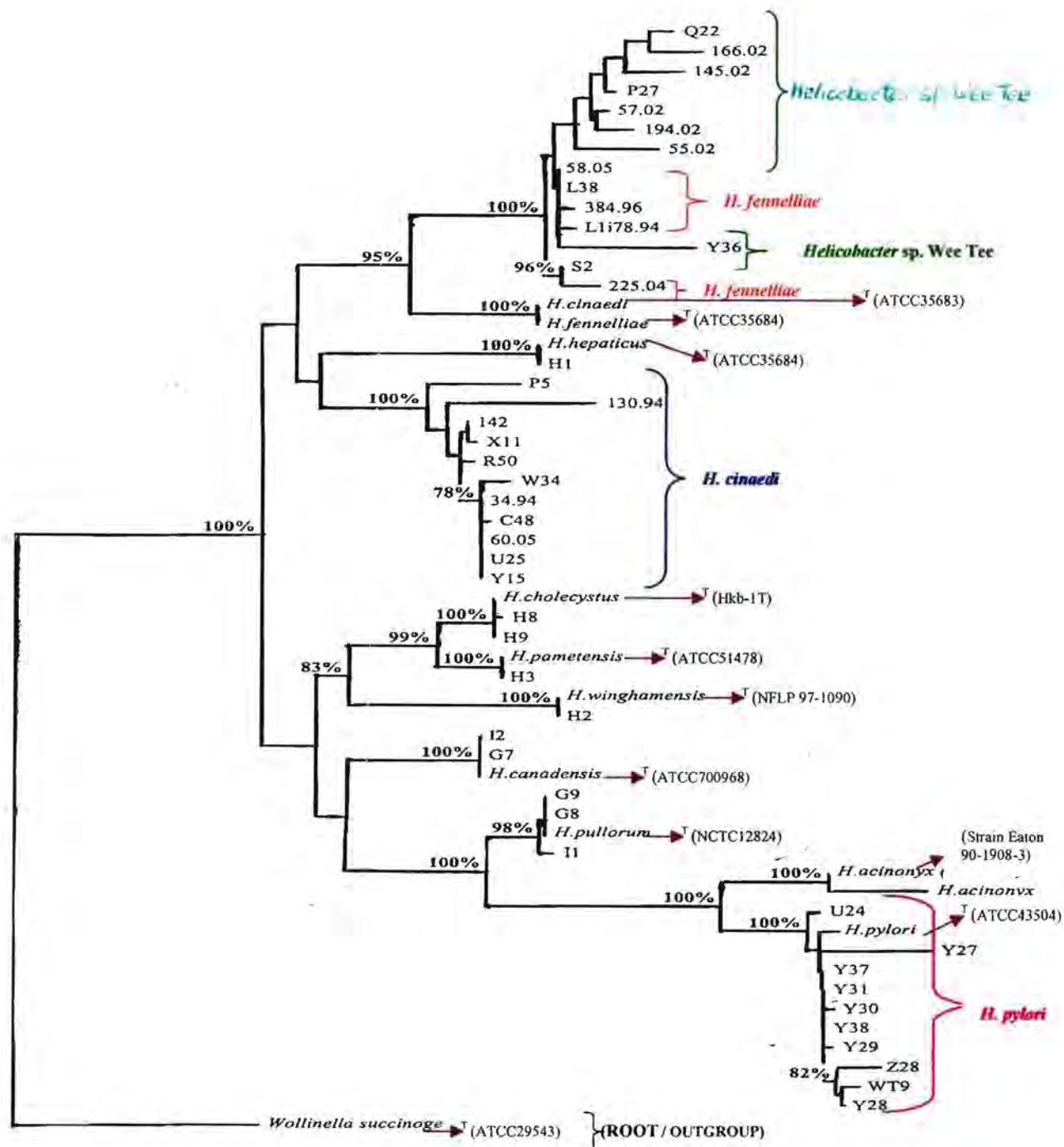


Figure 3.12: Phylogenetic tree inferred by the 1 200 bp fragment of the 16S rRNA gene of *Helicobacter*. Denmark strains are labelled as: H1, H8, H9, H3, H2, I2, G7, G9, G8, and I1 while all the other isolates are South African strains, excluding *H. acinonyx*. Bootstrap values above 75 % are regarded to be significant. All type strains are indicated by a plum arrow, followed by a superscripted T and the type strain number.

3.6.5. Phylogenetic Tree of the corrected large sequences ($\pm 1\ 042$ bp) of the 16S rRNA gene of *Arcobacter* isolates isolated from the sewage / sludge environmental source

All 4 isolates cluster with *Arcobacter butzleri* (*A. butzleri*); however differences among the isolates are noted (fig.3.13).

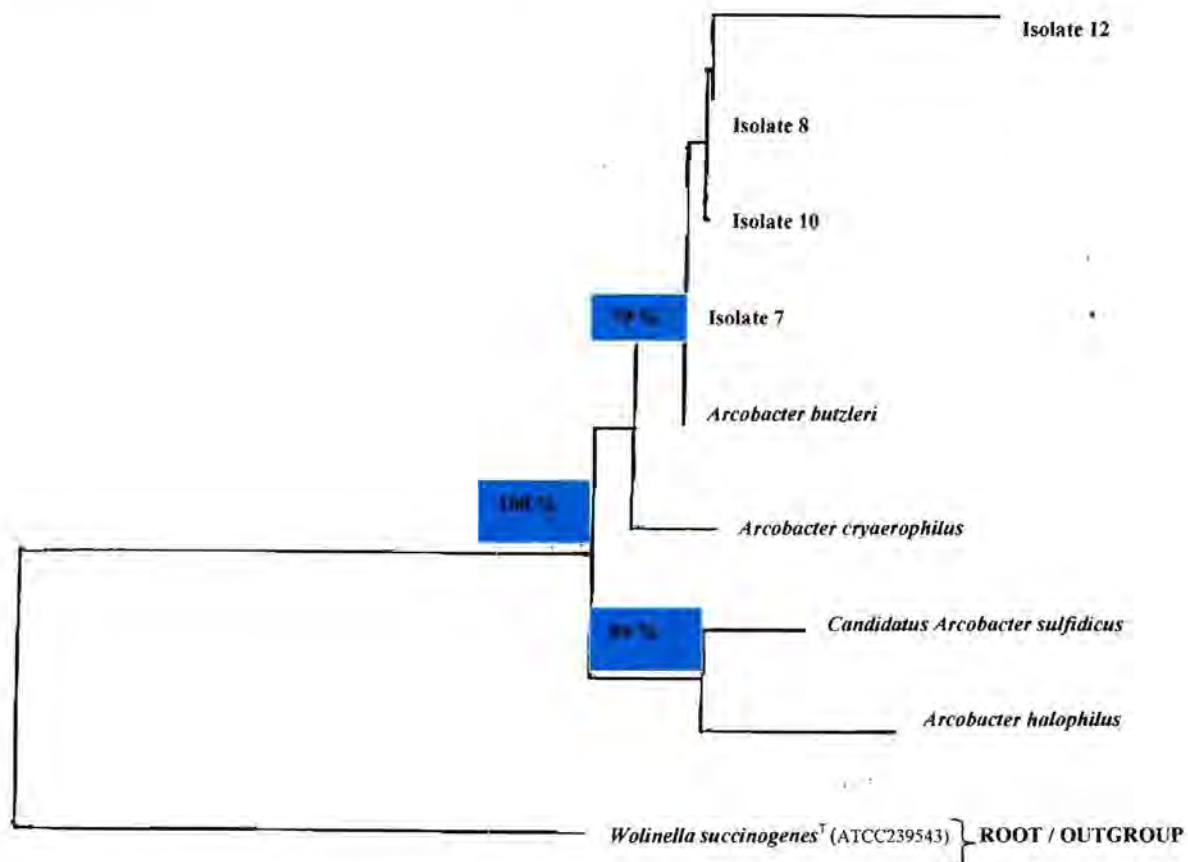


Figure 3.13: Phylogenetic tree inferred by the 1 004 bp fragment of the 16S rRNA gene of *Arcobacter*. The accession numbers for the reference strains of *Arcobacter* are as follows: *A. butzleri* (U34388); *A. cryaerophilus* (U34387); *Candidatus Arcobacter sulfidicus* (AY035822); and *Arcobacter halophilus* (*Arcobacter* sp. LAB31B) (AF513455).

CHAPTER 4:

DISCUSSION

University of Cape Town

4.1. Isolation of *Helicobacter* and related micro-organisms from various environmental sources

Campylobacter has been isolated from river, lake, untreated well and surface waters in Greece, Ireland, Israel and the U.K. (Ghinsberg *et al.*, 1994; Arvanitidou *et al.*, 1995; Bolton *et al.*, 1999; Moore *et al.*, 2001). Free-living and plankton-associated *Helicobacter pylori* were detected by PCR in sea water along the Italian coast of the Adriatic Sea and Straits of Messina (Cellini *et al.*, 2004; Carbone *et al.*, 2005).

Based on these studies it was decided to obtain a sample of sea water along the west coast of Cape Town, between Strandfontein and Sunrise Beach (a famous fishing site) as this stretch of beach occasionally washes out sea waste resulting in brown coloured waves, presumably due to sewage effluent from nearby sewage outflow pipes. However, at the time of sampling no “brown waves” were present. Nevertheless, it was decided to take a sample of the sea water, as well as wet beach sand (area of beach sand which was still moist due to recent contact with sea water) and dry beach sand (the sea shore). These samples were vertically in line with one another and collected in the early evening just before sunset; however, re-sampling could not be carried out at the same location as a permanent marker was not made. In addition, samples of other recreational sites were also obtained: a sample of duck pond water; duck pond sand; muddy water, gravel and muddy sand from the Melkbos 4x4 off-road challenge track; and mountain soil and water obtained from Lion’s Head hiking trail. All of these samples were collected in the latter part of the morning, around lunch time.

All samples were collected over the weekend, during the autumn and winter seasons, but were only processed the next working day (1 – 2 days later). These samples were stored at 4 °C after a period of 3 – 5 hr as these samples were collected on an outing. These conditions could have been contributing factors for the unsuccessful isolation of *Helicobacter*. All samples were processed in duplicate, and all positive and negative controls gave the expected results.

When an aliquot of sea and duck pond water was directly dispensed onto *H. pylori* selective media, *Helicobacter* isolation remained unsuccessful. Similarly, *Helicobacter* isolation remained unsuccessful for the wet beach sand, dry beach sand and Melkbos 4x4 off-road challenge track water samples which were directly streaked onto TBA plates. Despite direct streaking of the gravel and muddy sand from the Melkbos 4x4 off-road challenge track and mountain soil samples (prior to all filtration stages in the pulsification technique) *Helicobacter* could not be successfully isolated. However, this could not be confirmed for the mountain water and duck pond sand samples as neither the direct dispensing nor direct streaking techniques were carried out. Despite this, samples remained stored at 4 °C for later processing, using PCR, to detect whether *Helicobacter* DNA was present in the samples.

In addition, a sample of fresh compost (obtained immediately after opening of the bag) and laboratory tap water was analyzed, as indicated in table 3.1, but proved to be unsuccessful in isolating *Helicobacter* despite using the direct streaking technique.

Although Diergaardt *et al* (2003) were unsuccessful in isolating *Campylobacter* from various water sources (drinking, surface, ground and sewage sources) in South Africa, they were, however, successful in obtaining 4 *Arcobacter butzleri* isolates in total, 3 from surface water and 1 from raw sewage.

With these results in mind it was decided to obtain a sample of raw and treated sewage / sludge. The isolation of *Helicobacter* and related micro-organisms was carried out as indicated in table 3.1. The direct streaking and filtration methods on *H. pylori* selective media were both unsuccessful due to an overgrowth of contaminants despite the presence of various antibiotics in the media. This illustrates that the dosage of antibiotics in the media could have been insufficient or the incorrect antibiotics were used. Similarly, the enrichment technique (as described in 2.2.2.11.b.) followed by filtration on *H. pylori* selective and TBA media was unsuccessful for the isolation of *Helicobacter* and related micro-organisms due to the overgrowth of contaminants. *Helicobacter* was not isolated upon direct filtration on TBA media. This was anticipated as the raw and treated sewage / sludge samples were collected in July (winter in South Africa and the period when Cape Town

generally receives the most rain). During the winter months, more surface run-off occurs as a result of a heavy rainfall. It is at this time that the surface run-off from adjacent farmlands (Bolton *et al.*, 1987), abattoir and animal processing plants (Jones¹ *et al.*, 1990) reach various water sources but may dilute the amount of bacteria in the process. However, due to minimal and delayed winter rainfall during July 2005 minimal surface run-off occurred. Temperatures are also known to drop drastically during the winter months and thus only bacteria that are cold tolerant, such as species of *Arcobacter*, can survive.

Despite using various isolation techniques on the samples listed above (as indicated in table 3.1) the isolation of *Helicobacter* and related micro-organisms was unsuccessful. If *Helicobacter* cells were present, they may not have been isolated due to: a prolonged delay period before plating; storage and processing of samples under unfavourable conditions resulting in the shearing / lysing of bacterial cells; nutrient depletion or release of inhibitory substances resulting in the overgrowth of competing micro-organisms; exposure to O₂ and other physiologically stressing agents; minimal quantity of *Helicobacter* within the sample (900 – 1000 bacteria are required before the bacteria can pass through the filter and onto the agar plate) (personal communication with Prof. Lastovica); or an inefficient filtration technique where the filter could have retained *Helicobacter* due to the auto-agglutination of cells.

Despite the unsuccessful isolation of *Helicobacter*, a total of four isolates displaying typical *Arcobacter*-like morphology were obtained. Two were isolated from the treated and 2 from the untreated sewage / sludge samples. In 1991, Lior and Woodward identified sewage as one of the most frequent non-human sources of *Arcobacter butzleri*. *Arcobacter* species have the ability to grow aerobically at lower temperatures (15 °C – 30 °C) (Vandamme *et al.*, 1991; Ursing *et al.*, 1994) unlike other related micro-organisms such as *Helicobacter* and *Campylobacter*.

Future work relating to the isolation of *Helicobacter* from environmental samples should include the spiking of samples with various strains of *Helicobacter* at the sampling site to determine if the bacterium could survive in that milieu during storage, transportation and processing (discussion with Prof. Lastovica).

4.2. Biochemical and phenotypic characterization of *Helicobacter* and related micro-organisms

Biochemical and phenotypic characterization was carried out on a range of isolates obtained from revitalized, clinical and environmental samples to identify *Helicobacter* and related micro-organisms to the species level.

4.2.1. Phenotypic characterization

The “Cape Town Protocol” (Appendix D) and Gram-staining protocol gave the expected phenotypic reactions for all bacterial strains tested.

4.2.2. Biochemical characterization

Biochemical characterization, based on the tests outlined in Appendix D and in chapter 2, was carried out on all cultures to identify potential *Helicobacter*, *Campylobacter* and *Arcobacter* isolates to the species level.

The results, in table 3.3, for *H. cinaedi*, *H. fennelliae*, *H. pylori*, *H. acinonyx*, *H. pullorum*, *H. cholecystus*, *H. pametensis*, *H. hepaticus* and *H. canadensis* were in agreement with published results (Le Roux & Lastovica, 1998; Jie-Song *et al.*, 1999; Melito *et al.*, 2001 and Waldenström *et al.*, 2003). The *H. winghamensis* strain of this study gave the expected results except for positive catalase activity (Melito *et al.*, 2001). The 7 strains of *H. winghamensis* tested by Melito *et al.*, (2001) were all catalase negative. This discrepancy could be due to strain variability for catalase production.

The *Campylobacter* results were in agreement with the “Cape Town Protocol” and Steinbrueckner *et al.*, 1999. Although the results were generally in agreement with Burnens² *et al.*, 1993, there were some deviations among the results of *C. concisus* and *C. jejuni* subspecies *jejuni*. Burnens² *et al.*, (1993) indicated that H₂S production (TSI) for *C. concisus* was positive, contrary to the result of this study. This deviation may, however, be due to the use of different media to the Burnens² *et al.*, (1993) study or strain variation. In this study semi-solid iron broth (prepared by the NHLS media lab, Greenpoint, Cape Town) was used for the rapid H₂S production test. It has been shown that different types of media used for the H₂S production test may give

different results for the sub-species (Appendix D), as illustrated for *A. butzleri* in table 3.3. *C. jejuni* subsp. *jejuni* also illustrates deviations in results upon comparison to Burnens² *et al.*, 1993 where the micro-organism does not display arylsulphatase activity. However, according to the “Cape Town Protocol”, *C. jejuni* subspecies *jejuni* biotype 1 does not display arylsulphatase activity but *C. jejuni* subspecies *jejuni* biotype 2 does. Similarly, *C. jejuni* subspecies *jejuni* biotype 1 does not produce H₂S but *C. jejuni* subspecies *jejuni* biotype 2 does upon use of rapid H₂S media (Le Roux & Lastovica, 1998).

The results for the 4 strains of *Arcobacter butzleri* were in perfect agreement with the “Cape Town Protocol” and Burnens² *et al.*, 1993.

4.3. Validating kits using pure cultures of *Helicobacter* and other micro-organisms

4.3.1. Validating the Oxoid Biochemical Identification System (O.B.I.S.) for the differentiation of *Campylobacter* and *Arcobacter* from other Gram-negative organisms and confirmation with the Fluka Code no. 75554 Aminopeptidase Test (Fluka, Germany)

The L-alanine aminopeptidase has been suggested for differentiating between Gram-negative and Gram-positive bacteria, being more prevalent in the former group (Cerny, 1976, 1978; Manafi *et al.*, 1991). *Campylobacter* and *Arcobacter* are Gram-negative bacteria and thus do not possess the L-ALA enzyme (Carlone *et al.*, 1983; O.B.I.S. Instructions Leaflet, 2005).

The test results for the strains of *Campylobacter* and *Arcobacter* were as expected (table 3.5). However, the results obtained from all 42 strains of the 10 *Helicobacter* spp. tested were a surprise, as these Gram-negative species were also negative using the L-ALA test. It was suspected that the O.B.I.S. chromogen may be unable to enter the *Helicobacter* cell wall and thus be inaccessible to the L-ALA enzyme of *Helicobacter*. Thus a different, commercially available Aminopeptidase Test System (Fluka Code no: 75554) was used, in duplicate, along with the O.B.I.S. kit. Both of these

different L-ALA systems confirmed the lack of L-alanine aminopeptidase in *Helicobacter* for every strain tested (table 3.6).

As these results have not previously been reported, this is a novel finding which has important taxonomic and diagnostic implications. BLAST analysis of the available complete genomic sequences from *Campylobacter* and *Helicobacter* species, on the NCBI database, confirmed that the L-alanine aminopeptidase enzyme is lacking in the *Helicobacter* genus, as has been found for the *Campylobacter* and *Arcobacter* genera. This could be expected as *Campylobacter*, *Arcobacter* and *Helicobacter* all belong to the epsilon division of the class *Proteobacteria*.

A total of 96 fresh and revitalized Gram-negative and 49 Gram-positive clinical isolates were tested to validate the O.B.I.S. kit. As expected, all Gram-negative micro-organisms tested were positive for the KOH and L-ALA (O.B.I.S. & Fluka) tests, as illustrated in table 3.4. However, the results for the Gram-positive micro-organisms were different to the expected results of the O.B.I.S. The KOH test was done in triplicate and the results for all 49 Gram-positive micro-organisms were positive. These results were not in agreement with the data of Gregersen (1978) suggesting that the dissolution of the cell wall and cytoplasmic membrane, by 3 % KOH, should be a reliable marker for the detection of Gram-negative micro-organisms (Carlone *et al.*, 1983). However, Blachman *et al.*, (1980) and Halebian *et al.*, (1981) have both reported that the KOH test and Gram reaction do not always correlate (Carlone *et al.*, 1983). In addition, the L-ALA (O.B.I.S. & Fluka) test results were positive for each of the 49 Gram-positive micro-organisms. These results may however, be indicative that the L-ALA enzyme is present in some Gram-positive bacteria. This is in agreement with Cerny, (1976, 1978) who stated that the L-alanine aminopeptidase enzyme could be present in both Gram-positive and Gram-negative micro-organisms but is more prevalent in the latter group. Perhaps, the presence of the L-ALA enzyme is predetermined by the type of

environment in which the bacterium dwells. It may be that the presence of L-ALA in a specific bacterium may assist in obtaining vital nutrients from the surrounding environment. The L-ALA enzyme may be involved in the activation mechanism or transport of antibiotics into the cell (Braun *et al.*, 1983); degradation of various toxic peptides; or inactivation of physiologically vital proteins or peptides (Lazdunski, 1989 & Miller, 1975; Gonzales & Robert-Baudouy, 1996).

4.3.2. Validating the CampyCheck *Campylobacter* latex agglutination kit

RO3613 (Microgen Bioproducts Ltd, Camberley, U.K.)

The CampyCheck *Campylobacter* latex agglutination kit is designed to differentiate *Campylobacter* spp. from other bacteria, as well as differentiate thermophilic and non-thermophilic *Campylobacter* species. This is useful as *Campylobacter*, *Helicobacter* and other bacteria similar in cellular and colonial morphology can be isolated together from the environment, food and clinical specimens. As this latex agglutination test is designed to react with the surface antigens of *Campylobacter* species it is useful as a pre-screening test

The *Campylobacter* results, in table 3.7, are in agreement with previous work with *C. jejuni* subsp. *jejuni* and *C. upsaliensis* reacting with the thermophilic latex reagent (Campycheck, 2004) but not with the species latex reagent suggesting that they are thermophilic *Campylobacter*.

The results of various *Helicobacter* species (table 3.7) were not as expected. A total of 34 strains of *Helicobacter* spp. were tested: 11 *H. cinaedi*; 12 *H. fennelliae*; 10 *H. pylori* and 1 *H. acinonyx*. All *Helicobacter* species and 1 strain of *Haemophilus influenza* reacted with the species latex reagent and none of the *Helicobacter* species reacted with the thermophilic latex reagent.

Five strains of *Haemophilus* and a strain each of *Arcobacter butzleri*; and *Morganella* were tested and each reacted with the thermophilic latex reagent

(table 3.7). This was not in agreement with the CampyCheck *Campylobacter* kit (Campycheck, 2004).

In addition, 4 *A. butzleri* and 2 *Haemophilus* strains; as well as strains of *E. coli*; *Enterococcus faecalis*; *Shigella flexneri*; *Staphylococcus* spp; *Streptococcus* spp; *Salmonella* spp; *Acinetobacter*; *Serratia marscescens*; *Stenotrophanus maltophilia*; *Burkholderia*; *Pseudomonas*; *Proteus mirabilis*; *Enterobacter* spp; and *Citrobacter* (table 3.7) all reacted with the control latex reagent indicating that non-specific reactions had occurred and are in agreement with Campycheck, 2004.

As all of these tests were carried out in triplicate and the same results were obtained each time, experimental error does not seem to be a contributing factor for the results described above. The CampyCheck *Campylobacter* latex agglutination kit results indicate that there are several problem areas with this system. Firstly, upon validation it was observed that the reagents in this kit also react with bacteria other than *Campylobacter*. Secondly, the species latex reagent of the kit reacts with various *Helicobacter* species. Currently, the test can only confirm the identification of *Campylobacter* once biochemical and phenotypic characterization have been carried out on pure cultures of the isolates.

Theoretically, if the antigenic sequences are highly specific for *Campylobacter*, the kit should be an ideal test for the identification of *Campylobacter*. However, this is not always the case as observed with the CampyCheck *Campylobacter* kit. Latex agglutination tests are based on the membrane components of bacteria. Lipopolysaccharides (LPSs) are one of the main antigens in the outer membrane of *Campylobacter* and although they are similar, they are not identical in all *Campylobacter* species. LPSs are present in all Gram-negative bacteria and are composed of: lipid A, core polysaccharide and the O side chain. Lipid A binds the LPS in the outer membrane and is the endotoxic portion of the complex; the core polysaccharide is joined to lipid A; and the O side chain is a short

polysaccharide chain extended outward from the core and appears on the cell surface, serving as an antigenic determinant (O antigen) (<http://www.cehs.siu.edu/fix/medmicro/genmicr.htm>; Prescott *et al.*, 1996). Gram-negative bacteria have the ability to rapidly change the nature of their O side chains to avoid detection by host defence mechanisms through the molecular mimicry of host structures (Moran, 1996; Prescott *et al.*, 1996; Vandenbrouke-Grauls & Appelmelk, 1998; Moran & Prendergast, 2001). Antibodies have a specific affinity to a particular site on an antigen, namely the antigenic determinant (epitope) (Stryer, 1997) and as *Helicobacter* species and other bacteria were found to react with the latex agglutination reagents in the CampyCheck *Campylobacter* latex agglutination kit, it suggests that these bacteria may possess similar epitopes to *Campylobacter*.

4.4. Amplification of *Helicobacter* and related micro-organisms

4.4.1. Amplification of 16S rRNA gene using cadaver DNA / *Helicobacter* DNA as a template

The access to a range of tissue samples, collected from post mortems, provided an opportunity to identify other ecological niches of *H. pylori* in the human body. Upon screening of the oesophageal tissue samples from over 30 cadavers, Dr. Smuts identified cadavers 5 and 6 positive for *Helicobacter* DNA using the S1 / AS1 or C97 / C98 primer sets (listed in table 2.3).

The screening of various tissue samples, listed in table 3.8, for *Helicobacter* DNA confirmed the presence in sites which have previously been noted to be colonized with *Helicobacter*. These include the: stomach (Marshall & Warren, 1984; Blaser, 1990; Forman *et al.*, 1991; Parsonnet *et al.*, 1994; Dunn *et al.*, 1997; Goteri *et al.*, 1997; Pakodi *et al.*, 2000; Ierardi *et al.*, 2001; Perez-Perez & Blaser, 2004), gallbladder (Fox *et al.*, 1998; Pradhan & Dali, 2004; Abayli *et al.*, 2005; Apostolov *et al.*, 2005) and pancreas (Pakodi *et al.*, 2000; Nilsson *et al.*, 2006).

A number of surprising sites were also identified. These include: the brain, aortic artery, tonsils, lung, gonad, bone and skeletal muscle. The presence of *H. pylori* DNA in these

samples could have been a result of contamination through the accidental inclusion of fragments from surrounding and broken blood vessels, blood or tissue containing *Helicobacter*. As described below, there is evidence that support some of these unusual findings.

The stomach is considered to be the primary niche for *Helicobacter* colonization and the detection in both cadavers supports this. *H. pylori* is known to establish a chronic infection which is associated with an inflammatory response of the gastric epithelium (Costa *et al.*, 1999) and recognized as a risk factor for gastric adenocarcinoma (Forman *et al.*, 1991; Parsonnet *et al.*, 1994) and peptic ulcer disease (Marshall & Warren, 1984; Blaser, 1990; and Dunn *et al.*, 1997). Histology studies have provided evidence for the association of *H. pylori* with chronic active gastritis with successful isolation in 70 % of gastric and greater than 90 % of duodenal ulcer patients (Pakodi *et al.*, 2000).

H. pylori was successfully amplified in the large bowel of cadaver 5 of this study. For years, the colon and small bowel were known to be populated with a complex microbial system comprising mainly of anaerobic bacteria which thrive in the environment of the bowel (Solnick & Schauer, 2001). The anaerobic bacteria of the bowel include members of the genus *Helicobacter* which are ubiquitous colonizers of the enteric mucosal surface (Solnick & Schauer, 2001). Previously, the pathogenesis of many *Helicobacter* species isolated from the intestinal contents of humans was doubted as many of them were isolated in the absence of disease (Melito *et al.*, 2001). As a consequence, little had been done to attempt to elucidate the disease mechanisms of intestinal *Helicobacter* species; until recently when *H. cinaedi*, *H. fennelliae*, *H. pullorum*, *H. canadensis* and *H. winghamensis* were among those isolated from humans and associated with gastroenteritis and enteric diseases (Totten *et al.*, 1985; Archer *et al.*, 1988; Burnens *et al.*, 1994; Stanley *et al.*, 1994; Steinbrueckner *et al.*, 1997; Fox *et al.*, 2000; Melito *et al.*, 2001; Lastovica, 2006).

Although *H. pylori* was consistently amplified from the oesophagus in both cadavers, the evidence in the literature is controversial. Some evidence suggests that the presence of *H. pylori* in this tissue is a result of contamination. As everybody occasionally experience gastro-oesophageal reflux, it is not surprising that *H. pylori* and more commonly its DNA

have been detected in the oesophageal area (Mapstone *et al.*, 1993; Li *et al.*, 1996). In South Africa, *H. pylori* and *Wolinella* have also been detected in oesophageal tissue samples of patients with oesophageal carcinoma (Bohr *et al.*, 2003).

Although the detection of *Helicobacter* in the tonsils is relatively uncommon, *H. pylori* was amplified from tonsils DNA of cadaver 6. Recently, Cirak *et al.*, (2003) detected a virulent strain of *H. pylori* possessing the *Cag A* gene in tonsil and adenoid tissue using PCR; while Unver *et al.*, (2001) detected these micro-organisms using the chromogenic *Campylobacter*-like organism (CLO) test. However, Yilmaz *et al.*, (2004) illustrated that the CLO test was unsuccessful in detecting *H. pylori* in tonsil and adenoid tissue despite confirming the presence of *H. pylori* in stool samples from the same children. This may indicate that *H. pylori* does not colonize tonsil and adenoid tissues and that these tissues are not reservoirs for *H. pylori* in children.

As the pancreas is a vital organ, located near the stomach, which supplies digestive fluid and insulin to control the sugar levels in the body, it may be that *H. pylori* dissemination from the stomach could result in an infection in the pancreas. Previous literature has suggested an association of *H. pylori* with various cancers in humans, including pancreatic adenocarcinoma (Pakodi *et al.*, 2000). In addition, Kountouras *et al.*, (2005) proposed the possibility that *H. pylori* might trigger autoimmune pancreatitis via induction of autoimmunity and apoptosis. A more recent study by Nilsson *et al.*, (2006) showed the presence of *Helicobacter* DNA in pancreatic cancer and surrounding normal tissue. The finding of *Helicobacter* DNA in the pancreatic tissue of both apparently healthy cadavers of this study contradicts Nilsson's work where *Helicobacter* DNA was detected in pancreatic cancer patients but not in the control patients.

The same could be true for the presence of *H. pylori* in the spleen, of both cadavers, and kidney of cadaver 6. The spleen is an organ located between the stomach and the diaphragm and produces cells involved in the immune response such as lymphocytes. The kidneys filter waste from blood and excrete these waste products and water in urine. Thus the presence of *Helicobacter* in the blood, stomach or any other organ in the vicinity may result in a disseminated infection. A mouse study to determine tissue tropism indicated a transient colonization of *H. pylori* in multiple sites including the

spleen and kidney (McCathey *et al.*, 1999). However, *H. pylori* was only isolated from the stomach 30 days after inoculation suggesting a preferential site for colonization. Thus the amplification of *H. pylori* DNA from tissues of the spleen and kidney in this study may be indicative of a transient infection or blood contamination.

The presence of *H. pylori* in the gallbladder of apparently healthy cadaver 6 is in support of the study by Chen *et al.*, (2003) where *Helicobacter* DNA was detected in 40.5 % of the gallbladder tissue samples obtained from their control patients. This suggests that *Helicobacter* may be present at this site in patients without gallbladder disease.

H. pylori DNA was consistently amplified in the aortic artery of both cadavers. Controversial evidence exist implicating *H. pylori* in coronary and ischemic heart diseases (Mendall *et al.*, 1994; Patel *et al.*, 1994; Wald *et al.*, 1997; Danesh & Peto, 1998; Pasceri *et al.*, 1998; Koullias *et al.*, 2004). The presence of *H. pylori* DNA in the atherosclerotic plaques of the carotid artery as detected by PCR and absence thereof in the control samples suggested that *H. pylori* could play a role in the pathogenesis of atherogenesis (Kaplan *et al.*, 2006). Although there was no evidence of atherosclerotic plaques in the two cadavers of the study, infection of *H. pylori* at this site cannot be ruled out. Alternatively, the presence of *H. pylori* DNA at this site may be a result of blood contamination.

Interestingly, *H. pylori* was also detected in lung tissue of cadaver 6. *H. pylori* infection is a common disease worldwide with *H. pylori* prevalence ranging from 25 % in developed countries to greater than 80 % in developing countries (Pounder, 1995; Ece *et al.*, 2005) and recently it has been associated with the development of many respiratory illnesses (Rehfeld *et al.*, 1989; Sellers *et al.*, 1990; Weinberg *et al.*, 1991; Moss, 1999) Gocyk *et al.*, 2000; Roussos *et al.*, 2003; Philippou *et al.*, 2004; Ece *et al.*, 2005). Evidence from a mouse study (McCathey *et al.*, 1999) suggests that the presence of *H. pylori* DNA at this site may be a result of a transient infection.

Although both cadavers were healthy, i.e. their tissues showed no signs of disease; it is possible that the presence of *H. pylori* in their tissues, as described above, could have led to the progression of various diseases (Marshall & Warren, 1984; Parsonnet *et al.*, 1994;

Fox *et al.*, 1998; Pakodi *et al.*, 2000; Bohr *et al.*, 2003; Perez-Perez & Blaser, 2004; Abayli *et al.*, 2005; Nilsson *et al.*, 2006).

In the present study, *H. pylori* was amplified in tissues of the brain, gonad and bone of cadaver 6 and in skeletal muscle tissue of cadaver 5. The evidence for the association of *Helicobacter* with cerebro-vascular disease; development of reproductive disorders and skin / bone infections is controversial and not based on PCR but on a rapid enzyme linked immunosorbent assay (ELISA) (Hugh & Mike, 1998; Cuccherini *et al.*, 2000; Heuschmann *et al.*, 2001; Figura *et al.*, 2002; Masoud *et al.*, 2005). Due to a lack of evidence for the presence of *Helicobacter* in the tissues described above, it suggests that the amplification is a result of contamination.

4.4.2. Amplification of the 16S rRNA gene using DNA from various environmental sources as a template

As culturing could prove to be tedious, many labs resort to the use of quicker molecular techniques, of which PCR is the most common, to detect the presence of *Helicobacter* or other bacteria of interest.

The *Helicobacter* genus-specific primer set (C97 / C05) was used to amplify the 16S rRNA gene in sea water; wet beach sand and dry beach sand samples. However, non-specific amplification was obtained (data not shown) and subsequent sequencing of these non-specific bands confirmed that *Helicobacter* was not present in the sea water, wet beach sand and dry beach sand samples. The lack of amplification could have been influenced by various factors such as time of collection, depth of sample (some bacteria have a better survival rate further below the surface), unfavourable environmental conditions, low number or absence of *Helicobacter* in the sample, etc.

All samples were collected below the surface layer of the sampling sites during the early to late afternoon. Even though the samples were collected early in March, the beginning of autumn, the climate was still relatively warm and the hours of daylight were still relatively long, accompanied by high incandescent light (Diergaardt *et al.*, 2003). Experiments focusing on the effects of light intensity on *Campylobacter* illustrated that *Campylobacter* could not survive at high temperatures in water over prolonged periods

and is easily damaged by exposure to UVB rays (Korhonen and Martikainen, 1991; Obiri-Danso *et al.*, 2001; and Diergaardt *et al.*, 2003) and as a result they may be eliminated from sea water within 30 min of intense light exposure but can survive 24 hr in darkness (Jones² *et al.*, 1990). This may prove to be the same for *Helicobacter*. However, this contradicts the findings of Cellini *et al.*, (2004) where free *H. pylori* were detected during the summer season while plankton-associated *H. pylori* were detected during November, December and March (Cellini *et al.*, 2004). This contradiction may, however, be a direct result of geographical change as the summer climate or U.V. exposure levels in Italy may not be as intense as in South Africa.

The pond water and pond sand samples analyzed by PCR was also unsuccessful (data not shown). Lack of amplification could have been due to the presence of inhibiting elements, the absence or low numbers of *Helicobacter* in the sample.

The detection of *Helicobacter* in mountain soil and water samples was also unsuccessful (data not shown). This may have been contributed by a heavier rainfall which could have diluted out *Helicobacter* species and other related bacteria at the time of sampling (Bolton *et al.*, 1987) or other factors.

4.4.3. Amplification of the 16S rRNA gene using pure DNA from sewage / sludge isolates (7, 8, 10 and 12) as a template

Sequence analysis of the 16S rRNA gene confirmed that all 4 sewage / sludge isolates were homologous to *A. butzleri*. This is in agreement with a study (Lior & Woodward, 1991) where sewage was identified as one of the most frequent non-human sources of *Arcobacter butzleri*.

4.4.4. Amplification of the aminopeptidase (*L-ALA*) gene from various micro-organisms

During amplification of the *L-ALA* gene, non-specific products were obtained (fig. 3.6) and sequence analysis of these products indicated that the amplification was unsuccessful. Lack of amplification could have been due to the level of degeneracy within the primers. Although, the L-ALA1* (forward) and L-ALA2² (reverse) primers have 3'-ends with GGC and GCAC, respectively, to increase binding of the primers to

the DNA template (Roche Molecular Biochemicals PCR applications manual, 1999) and do not have runs of 3 or more G or C bases to prevent possible stabilization of non-specific annealing of the primers (Roche Molecular Biochemicals PCR applications manual, 1999) non-specific amplification still occurred in some samples.

A comparison of the L-ALA1* primer sequence to known sequences within the NCBI database revealed that although the primer would bind to the *L-ALA* gene of some bacteria, it was not *L-ALA* specific. Thus the non-specificity of the primer sequences in combination with the level of degeneracy in both *L-ALA* primers resulted in the unsuccessful amplification of the *L-ALA* gene.

4.5. Phylogenetic trees

Phylogenetic trees provide an insight into the relatedness of organisms and can also be used to confirm the species of an isolate.

4.5.1. Phylogenetic analysis of the 16S rRNA gene from tissue samples of cadavers 5 and 6

The phylogenetic trees inferred by the short 16S rRNA sequences of cadavers 5 and 6 showed similar clustering within the *H. pylori* group. The scattering effect of gastric sub-groups IA and IB of gastric group I are indicative that the sequences of the 16S rRNA gene may be too short thus resulting in the improper grouping of gastric groups within phylogenetic trees. This probability is supported by Gueneau & Loiseaux-De Goër (2002) who used aligned sequences consisting of ± 960 nucleotides to generate a phylogenetic tree.

The phylogenetic tree inferred by the large 16S rRNA sequences of cadavers 5 and 6 of this study favourably compared to Gueneau & Loiseaux-De Goër (2002). The gastric groupings are in agreement with Gueneau & Loiseaux-De Goër (2002). As the *H. pylori* sequences of the South African strains (SA-4 and SA-3) were too short these could not be included in the phylogenetic tree inferred by the larger sequences of the 16S rRNA gene. All cadaver sequences grouped separately to *H. nemestrinae* and all *H. pylori*, except that of strain C51. C51 is a *H. pylori* strain amplified from

the liver of a French patient with hepatitis C virus associated cirrhosis (Rocha *et al.*, 2005).

Although the longer sequences of cadaver 5 are not significantly different to those of cadaver 6 they branch separately illustrating that the *H. pylori* sequences of cadaver 5 are more similar to each other. This may be indicative that different *H. pylori* strains are present in cadavers 5 and 6. *H. pylori* in the stomach and oesophagus of cadaver 5 differed from each other suggesting that the cadaver may have been infected with two different strains of *H. pylori*. The same is suggested for cadaver 6 tissues of the oesophagus and gallbladder as well as the brain and the aortic artery which are also significantly different to each other. The grouping of the brain and aortic artery may suggest a common source of contamination, e.g. the same blood vessel. The stomach and lung sequences are seen as isolated branches and if the presence of *H. pylori*, in the lung, is a source of contamination it may suggest that the lungs were contaminated by a source other than the stomach.

4.5.2. Phylogenetic analysis of the 16S rRNA gene of various pure *Helicobacter* isolates

As all of the *H. pylori* strains (Appendix E) were isolated from similar sites and sources, i.e. from patients with gastric carcinoma, the reason for the presence of 3 putative genomo-species remains unclear. Perhaps, it is a result of varying host immune responses.

The *H. acinonyx* (NCTC12686) (Appendix E) isolate of this study was obtained from Eaton *et al.*, (1993), while the 16S rRNA gene sequence of strain Eaton 90-1908-3 was used as a reference. Eaton *et al.*, (1993) initially isolated 4 strains from cheetahs with gastritis but the complete 16S rRNA gene sequences were only determined for two of the isolates. The differences in the two *H. acinonyx* 16S rRNA gene sequences, shown in the tree, confirm that the strains are different.

Most of the *Helicobacter* strains from Denmark: G9, G7, H9, H3, H2, H1, I2 and I1 (Appendix E) grouped with the reference strains confirming the species of the strain. This is contrary to G8 and H8 which were labeled as *H. mesocricetorum* and

Wolinella succinogenes, respectively and biochemical and phenotypic testing indicated that these strains were *H. pullorum* and *H. parmatensis*, respectively. Sequence analysis confirmed this. These discrepancies may have been a result of incorrect labeling or false identification. However, other molecular techniques should be included to elucidate this as sequence analysis has its limitations (Vandamme *et al.*, 2000).

The phylogenetic tree further illustrated the possibility of 4 putative genom-species within *H. cinaedi*. The clinical source of these *H. cinaedi* strains varied from blood and stools. Isolate P5 displayed a reddish colony colour, an uncommon trait of *Helicobacter*. (by personal communication with Prof. Lastovica). Despite this, the isolate was identified as *H. cinaedi* using various biochemical and phenotypic tests. P5 was isolated from human stool of a patient experiencing abdominal pain, while the other strains of *H. cinaedi* were isolated from patients exhibiting varying symptoms (Appendix E). It has recently been noted that the reference strain of *H. cinaedi* (ATCC35683), used in this study was misidentified and now known to be *H. fennelliae* (Kuhnert & Burnens, 2001). As this is the same type strain used by Gueneau, P. & S. Loiseaux-De Goër, (2002) it is possible that they were not aware of this finding.

BLAST analysis revealed that several strains within the *H. fennelliae* grouping and a strain each of *H. pylori* (Y36) and *H. cinaedi* (S2) aligned more closely to the *Helicobacter* sp. Wee Tee 16S rRNA sequence. However, Vandamme *et al.*, (2000) has stressed the importance of using other molecular techniques in conjunction with the 16S rRNA sequence analysis to avoid misidentification of an isolate.

4.5.3. Phylogenetic analysis of the 16S rRNA gene of *Arcobacter* isolates isolated from the sewage / sludge environmental source

The data is in agreement with Lior and Woodward (1991) who identified sewage as one of the most frequent non-human sources of *Arcobacter butzleri*.

4.6. Conclusions

All of the objectives of this project have been met. Using PCR and confirming the results with sequence and phylogenetic analysis, *H. pylori* DNA was detected in all *Helicobacter*-positive tissues of cadavers 5 and 6. The presence of *H. pylori* DNA in tissues of the stomach, gallbladder, pancreas, tonsils and oesophagus have been supported by the literature, while the amplification of *H. pylori* in tissues of the brain, aortic artery, lung, bone, skeletal muscle, kidney and spleen may have been a result of blood contamination. However, it is possible that a transient infection of *H. pylori* could have occurred in the latter tissues. Although both cadavers were apparently healthy, it is possible that the presence of *H. pylori* in their tissues could have led to the progression of various diseases later in life.

This project has illustrated the difficulty in isolating *Helicobacter* from the environment. Although *Helicobacter* was not isolated from any of the environmental samples a related micro-organism, *Arcobacter butzleri*, was isolated. All clinical and environmental isolates of *Helicobacter*, *Campylobacter* and *Arcobacter* were successfully characterized using biochemical and phenotypic tests. The identification of all clinical and environmental isolates of *Helicobacter* and *Arcobacter* were further confirmed with sequence and phylogenetic analysis of the 16S rRNA gene.

Using the O.B.I.S. and Fluka L-alanine aminopeptidase systems it was discovered that *Helicobacter*, as with *Campylobacter* and *Arcobacter*, do not possess the L-alanine aminopeptidase (pepN) enzyme. These results were confirmed by comparing the full genomic sequences of bacteria known to contain the enzyme with those of *Campylobacter* and *Helicobacter*.

Data from the Campycheck *Campylobacter* latex agglutination kit suggested the presence of a common surface antigen among *Campylobacter*, *Helicobacter*, *Arcobacter* and non-related micro-organisms.

Appendix A

Gels & Buffers

1. 1 % Agarose Gel

- Weigh out 2 g agarose and make up to a final volume of 200 ml with 1X TAE buffer (recipe to follow).
- Heat and boil in a microwave until the agarose has dissolved.
- Once dissolved, add 25 μ l of EtBr (100 μ g/ml) to the agarose solution.
- Cool to 55 – 60⁰C before pouring.
- Pour the agarose solution into the required gel tray containing the desired comb size and allow setting for 30 min.

2. 2 % Agarose Gel

- Weigh out 4 g agarose and make up to a final volume of 200 ml with 1X TAE buffer.
- Heat and boil in a microwave until the agarose has dissolved.
- Once dissolved, add 25 μ l of EtBr (100 μ g/ml) to the agarose solution.
- Cool to 55 – 60⁰C before pouring.
- Pour the agarose solution into the required gel tray containing the desired comb size and allow setting for 30 min.

3. 50X Tris-Acetate EDTA (TAE) Buffer

- Weigh out 242 g Tris, add 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA.
- Make up to a final volume of 1000 ml with Millique H₂O.
- Mix thoroughly.
- Autoclave at 121⁰C for 20 min at 15 pounds of pressure.

4. 1X TAE Buffer

- Measure out 200 ml 50X TAE.
- Make up to a final volume of 10 L with Millique H₂O.
- Mix thoroughly

5. 6X Loading Buffer (Gel Tracking Dye)

- Weigh out 25 mg bromophenol blue, 4.0 g sucrose and measure 0.4 ml EDTA (0.5 M, pH 8.0).
- Make up to a volume of 10 ml with Millique H₂O.
- Mix thoroughly.

6. Tris-EDTA (TE) Buffer

- Measure out 1 ml (0.5M) EDTA and 5 ml (1M) Tris-Cl (pH7.6) and make up 500 ml with Millique H₂O.
- Mix thoroughly.
- Autoclave at 121⁰C for 20 min at 15 pounds of pressure.

Appendix B

Solutions, Media And Media Additives

1. 1 M Tris-Cl

- Dissolve 121 g Tris base in 800 ml Millique H₂O.
- Adjust to pH 7.6 with HCl pellets.
- Mix thoroughly.
- Once the desired pH is obtained, make up to 1L with Millique H₂O.
- Autoclave at 121⁰C for 20 min at 15 pounds of pressure.

2. 10 mM Tris-Cl

- Aliquot 5 ml 1 M Tris-Cl (pH 7.6) and make up to 500 ml with Millique H₂O.
- Mix thoroughly.
- Autoclave at 121⁰C for 20 min at 15 pounds of pressure.

3. 0.5 M EDTA

- Dissolve 93.05 g EDTA in 400 ml Millique H₂O by stirring vigorously while adding NaOH pellets (EDTA will not dissolve until the pH is raised to 8.0).
- Make up to a final volume of 500 ml with Millique H₂O.
- Autoclave at 121⁰C for 20 min at 15 pounds of pressure.

4. 10 % Sodium dodecyl sulphate (SDS) (w/v)

- Weigh out 40 g SDS (wearing a mask and gloves) and dissolve thoroughly in 400 ml Millique H₂O.
- Heat to ± 80⁰C to dissolve.

5. 20 mg/ml Proteinase K

- Weigh out 20 mg proteinase K and dissolve thoroughly in 1 ml Millique H₂O.
- Filter sterilize.
- Store in 20 µl aliquots at -20⁰C.

6. 5 M Sodium Chloride (NaCl)

- Weigh out 14.61 g NaCl and dissolve thoroughly in 50 ml Millique H₂O.
- Autoclave at 121⁰C for 20 min at 15 pounds of pressure.

7. 2 M Sodium Hydroxide (NaOH)

- Weigh out 8 g NaOH and dissolve thoroughly in 100 ml Millique H₂O (To be used to make up phenol, below).

8. Hexadecyltrimethyl ammonium bromide (CTAB) / NaCl

- Dissolve 4.1 g NaCl in 80 ml Millique H₂O.
- Slowly add 10 g CTAB while heating and stirring.
- If necessary, heat to 65⁰C to dissolve.
- Adjust to a final volume of 100 ml with Millique H₂O.

9. 24:1 Chloroform / isoamyl alcohol

- Measure out 4 ml isoamyl alcohol and add 96 ml chloroform to make a final volume of 100 ml.

10. Phenol

- Weigh out 500 g commercial crystallized phenol.
- Add 0.6 g 8-hydroxyquinoline, 7.5 ml (2M) NaOH, 130 ml Millique H₂O and 6 ml Tris-Cl (1 M, pH 7.6). Leave overnight to liquefy.
- Mix thoroughly.
- Dispense in 50 ml aliquots and store at -20⁰C. (The result is a phenol solution in 10 mM Tris, pH 7.6).

11. 25:24:1 Phenol / chloroform / isoamyl alcohol

- Measure out 50 ml phenol (as prepared above), 48 ml chloroform and 2 ml isoamyl alcohol.
- Mix thoroughly.
- Cover the container in foil as it is light sensitive.

12. CaCl₂ (for the preparation of competent cells)

- Weigh out 0.8821 g CaCl₂.
- Measure out 18.75 ml 80 % glycerol (80 ml glycerol mixed with 20 ml Millique H₂O), creating a 15 % glycerol solution.
- Weigh out 30.23 g PIPES and mix in 100 ml Millique H₂O, creating a 1 M Piperazine-1, 4-Bis (2-Ethanesulphonic acid) (PIPES) solution.
- Mix: weighed CaCl₂ (above), 15 % glycerol (prepared above) and 1 ml of 1 M PIPES solution.
- Make up to 100 ml with Millique H₂O.
- Mix thoroughly and autoclave at 121⁰C for 20 min at 15 pounds of pressure.

13. 100 mg/ml Ampicillin (AMP)

- Dissolve 2 g AMP in 20 ml Millique H₂O.
- Filter sterilize.
- Store in 200 µl aliquots at -20⁰C.
- **N.B.** Dilute 1/1000 into media (1µl/ml) for a final concentration of 100 µg/ml.

14. 200 mg/ml Isopropyl-β-D-thio-galactopyranoside (IPTG)

- Mix 2 g IPTG in 8 ml Millique H₂O.
- Make up to a final volume of 10 ml.
- Store in 2 ml aliquots at -20⁰C.

15. 20 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal)

- Make a 40 mg/ml solution in dimethyl sulfoxide (DMSO).
- After the X-gal has completely dissolved, add 1 volume of water.
- Store in 1 ml aliquots at -20°C and wrap tubes in foil as it is light sensitive.

Media

1. 2xYT /AMP / IPTG / X-gal

- 200 ml 2xYT agar (NHLS media lab, Cape Town) is heated until completely dissolved.
- Cool to 50°C (AMP is heat inactivated).
- Thereafter 200 μl AMP, 100 μl IPTG and 1 ml X-gal is added and mixed thoroughly.
- Approximately, 20 ml media is poured, in a laminar flow hood wiped down with 70 % EtOH, in each Petri dish and allowed to solidify with the lid slightly ajar.
- Once solidified, the media may be used.

2. *Helicobacter pylori* (*H. pylori*) selective media

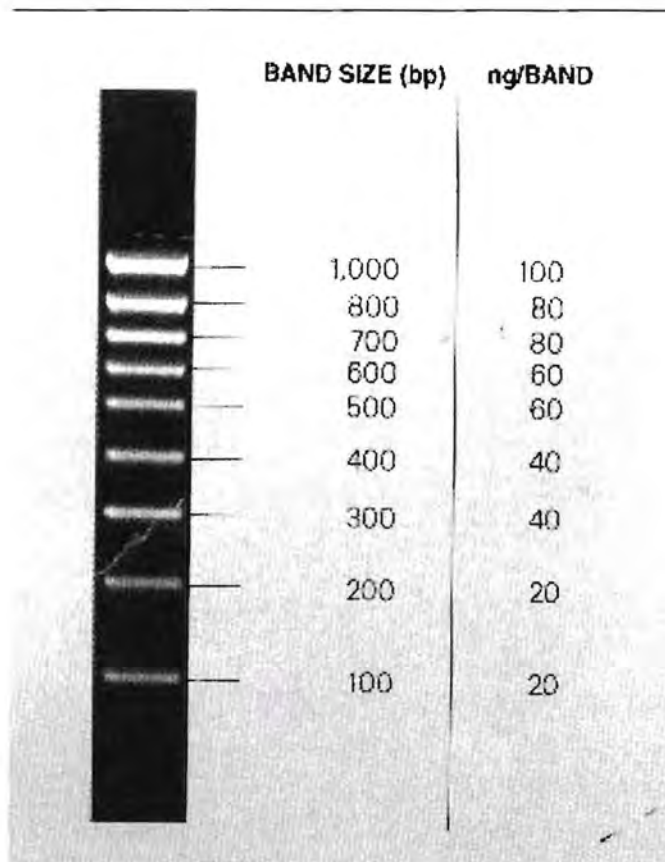
- Weigh out 39 g Columbia Agar Base and dissolve in 1 L Millique H_2O .
- Autoclave at 121°C for 15 min at 15 pounds of pressure.
- Cool to 50°C .
- Add 70 ml Laked Horse Blood (Oxoid Ltd., U.K.) to the autoclaved Columbia Agar Base solution and mix thoroughly.
- Add 4 ml *H. pylori* Selective Supplement (Dent) SR0147E.
- Mix thoroughly before pouring 20 ml *H. pylori* selective media into sterile Petri dishes.
- Allow to dry for 30 min before use.

Appendix C
Molecular Weight Markers
and
Cloning Vector

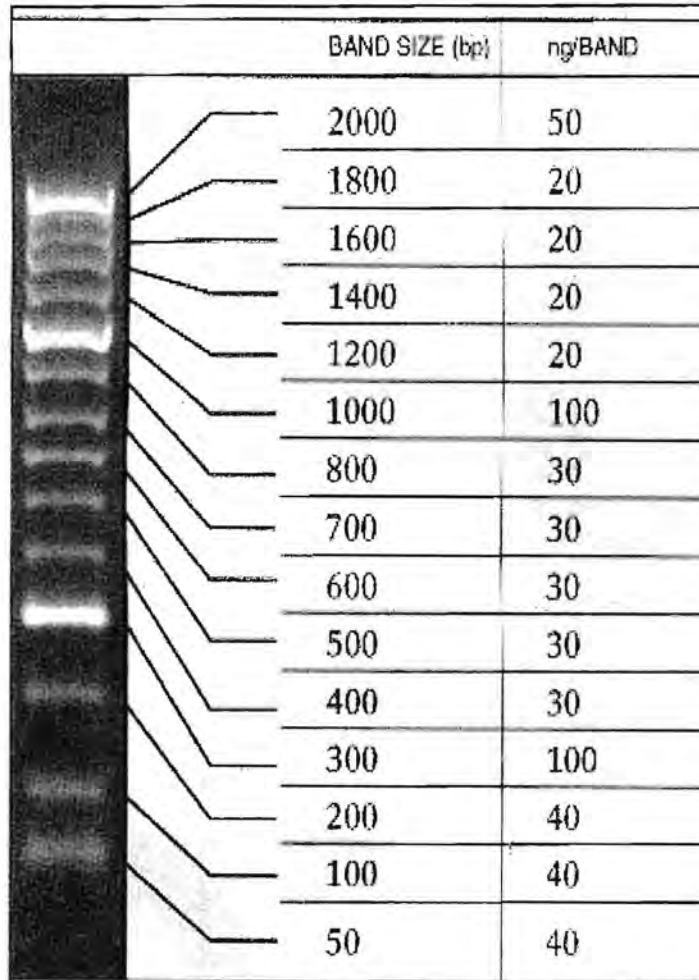
Marker VI (Roche, South Africa)

- 2176 (55ng) 22%
- 1766 (45ng) 18%
- 1230 (31ng) 12.5%
- 1033 (26ng) 10.5%
- 653 (16ng) 6.5%
- 517 (13ng) 5%
- 453 (11ng) 4.5%
- 394 (10ng) 4%
- 298/298 (15ng) 6%
- 234/234/220 (17ng) 7%
- 154/154 (8ng) 3%

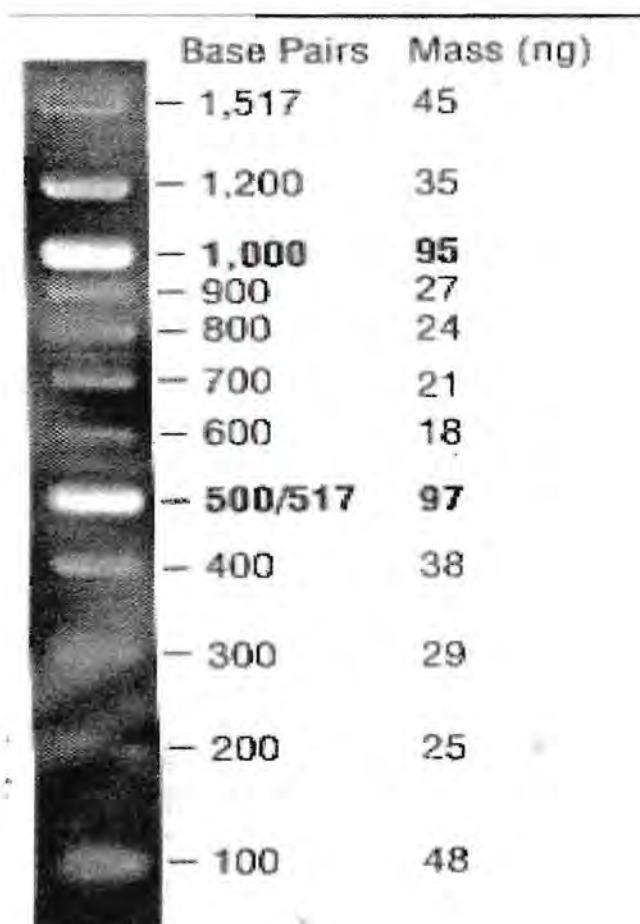
Hyperladder IV (Bioline, U.S.A.)



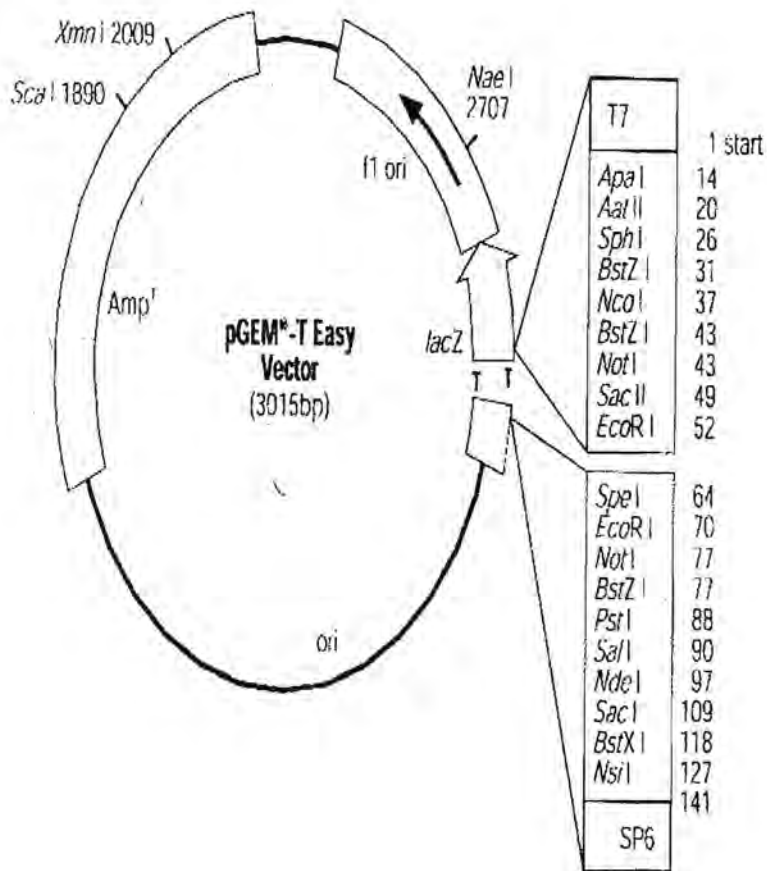
Hyperladder II (Bioline, U.S.A.)



100 bp DNA marker (New England Biolabs, U.K.)



pGEM-T Easy Cloning Vector



Appendix D

The Cape Town Protocol for the isolation and identification of *Campylobacter*, *Arcobacter* and *Helicobacter* (short version)

PRIMARY ISOLATION PROCEDURE

By using the **membrane filter technique** for processing stool samples, blood agar plates (no antibiotics added) and incubating in an **increased H₂-microaerophilic atmosphere**, virtually all known species of *Campylobacter*, *Helicobacter* and *Arcobacter*.

Required materials:

Tryptose Blood agar: Oxoid CM233

Membrane filter: 47 mm diameter, pore size 0.6 μ m, Schleicher & Schuell ME26

Hydrogen enhanced atmosphere: Anaerobic sachets **NO CATALYST** (Oxoid BR38 or BBL 70304). One Sachet per small jar (~12 plates) or 4 sachets per large jar (~36 plates) or Evacuate jar to 560 mm Hg and replace with 15% CO₂ and 85% H₂ gas mixture.

1. Specimen preparation

a) Stools: Prepare a watery emulsion of stools in sterile saline. Mucoid samples should be vortexed.

b) Intestinal scrapings (PM specimens) and sheath washings (bulls): shake up in saline.

c) Gastric Biopsy Material: Roll gently over the surface of 2 or 3 TBA plates using a swab dipped in Tryptic Soy Broth.

d) Blood cultures: Squirt ~0.2 ml of the mixture taken from the blood culture bottle over the surface of a TBA plate. **DO NOT USE THE FILTER METHOD.**

2. Place a 0.6 micron pore-size membrane filter (Schleicher & Schuell ME26) directly on to a TBA plate using sterile forceps.

3. Flood the central area of the filter with the emulsion using a transfer pipette. **Do not splash or spill beyond the filter margin.** Re-flood 2 or 3 times.

4. Remove and discard the filter **within 15 minutes**. Incubate the plate as soon as possible in a CO₂ incubator for the time being, before incubating in the H₂ enriched atmosphere generated by the BR 38 gaspak. Sterilize the forceps between specimens (heat- then cool in 70 % alcohol).

5. Incubate the plate in H₂ for 6 days, examining **every two days**. Do not discard or ignore the primary plate once growth has been obtained, as several *Campylobacter* species may be present with different growth rates. The H₂ atmosphere is obtained by the use of an Oxoid BR38 gaspak **without catalyst** or a 15 %CO₂ and an 85 % H₂ gas mixture.

6. Morphologically different colonies (shape, size, time needed for growth to appear, etc.) could indicate a mixed infection of two or more *Campylobacter* species.

IDENTIFICATION PROCEDURE

Campylobacter colonies are mostly buff coloured or dirty yellow. Exceptions are *C. helveticus*, *H. fennelliae*, *H. cinaedi*, and *H. rappini*; they have a thin flat film-like growth, which initially can even look like a swarming *Proteus* and can take up to six days to become visible to the naked eye on initial isolation, especially in a mixed culture. The gastric helicobacters form tiny, translucent colonies. Colonies of *Arcobacter* tend to be whiter than *Campylobacter*.

H. fennelliae is the only species that has an odour - hypochlorite without the "sting".

A gram stain will confirm any *Campylobacter*-like organism as either comma, or gull-winged shaped, or very thin, long spirals (*H. cinaedi*, *H. fennelliae*), short and stubby (*C. mucosalis*) big and slightly curved (*C. hyointestinalis* and *Arcobacter* spp.) tiny, hardly curved (*C. concisus*, *C. curvus*, *C. rectus*, *C. ureolyticus*) or gigantic (*Anaerobiospirillum*) gram negative bacilli. *Campylobacter* are easily "wiped off the plate" - do not plate out "heavy handedly". Two loops used alternatively are vital for ensuring complete cooling after flaming.

Subculturing onto two TBA plates should yield enough culture material to perform all the tests necessary for identification. The antibiogram can be done at the same time by putting one antibiotic disc on each isolation plate (on the streak lines). Incubate plates in H₂ for 48 hours. Prepare slides at the same time for motility and a Gram stain.

Do not leave the culture plates on the bench for a prolonged period of time, place in CO₂ until the H₂ jar is put up. Time is of the essence, so leave the staining of the slides until later.

This whole procedure might have to be repeated, if at a later stage, a mixed growth becomes evident on either the primary or subsequent plates.

ONCE PURE GROWTH HAS BEEN OBTAINED, PROCEED AS FOLLOWS:

Step	Procedure	Use growth from	Incubate in	Comments
1	Prepare 2 subculture plates		CO ₂ and H ₂	Keep CO ₂ plate up to 4 days before scoring "no growth". Examine H ₂ plate after 48 hr.
2	Indoxyl acetate		aerobic	10 minutes
3	Antibiogram ¹	CO ₂	H ₂	48 hours
4	Catalase		aerobic	immediate
5	Nitrate reductase	CO ₂	anaerobically ²	overnight
6	Aryl sulphatase	only from H₂	H ₂	48 hr for In Ac +ve strains 96 hr for In Ac -ve strains
7	Rapid H ₂ S	only from H₂	H ₂	overnight
8	Hippurate	CO ₂	CO ₂	overnight
9	Aerobic 1/4 plate	CO ₂	aerobic	48 hours

1. Antibiogram: Nalidixic Acid, Cephalothin, Ciprofloxacin. Never use more than 2 or 3 antibiotic discs per plate, preferably 1 per plate. It is more practical to incubate sensitivity plates in H₂ unless dealing with a known CO₂ organism. Larger inhibition zones are observed on CO₂.

2. Anaerobically or in a H₂ enriched atmosphere *Incubate in H₂ ONLY

NOTE: *C. hyointestinalis* catalase +ve *C. concisus* catalase -ve.

Media and reagents required

ITEM
Acetone
Butanol
Hydrogen peroxide (20 vol)
Potassium nitrate
Indoxyl acetate Sigma I 3500
Sodium hippurate Sigma H9380
Ninhydrin Merck 6762/BDH 10132 4E
McConkey Oxoid CM7b
Brucella base Oxoid CM 169
Technical Agar #3 Oxoid LI 3
Phenolphthalein disulphate Sigma P0251
Na ₂ CO ₃ 15 % solution
Nutrient Broth # 2 Oxoid CM67
TSI Oxoid CM 277
Ferrous Sulphate
Sodium metabisulphite
Sodium pyruvate

The Cape Town Protocol (short version) TESTS and REAGENTS

INDOXYL ACETATE HYDROLYSIS

Preparation:

A 10 % (w/vol) solution of Indoxyl acetate (Sigma 13500) in acetone. Saturate filter paper strips with the above mixture and air dry. Store in amber bottle in the fridge.

The test:

Rub a loopful of bacteria onto a small area of the strip. Wet strip thoroughly (keep moist for up to 10 minutes) with distilled water. A number of tests can be done on the same strip, store partly used strips in a petri dish in the refrigerator.

Dark blue colour e.g. *C. jejuni* = positive. No colour change e.g. *C. fetus* = negative.

NOTE: Some campylobacteria produce a bacterial esterase, which, in the presence of oxygen, will hydrolyse indoxyl (a breakdown product of tryptophan) to indigo and indigo white.

NITRATE REDUCTION

Preparation:

0.1 % (wt/vol) Potassium Nitrate in Tryptose Blood agar plates

0.5 g KNO₃ dissolved in 3 ml distilled water

Filter aseptically into 500 ml prepared TBA media

Seal plates into plastic bags, plates will keep for weeks at 4°C

The test:

Stab and spread a large loopful of bacteria over a small area (do no more than 4 tests per plate). Incubate overnight, preferably under anaerobic conditions (essential for some species) or under H₂ enhanced conditions for 48 hours. Aerate (30 minutes) on bench before scoring.

Dark green brown zone (e.g. *C. jejuni jejuni*) = positive. No colour change (e.g. *C. jejuni doylei*) = negative.

Note: The formation of a green-brown zone indicates that the KNO₃ has been reduced to KNO₂, resulting in the oxidation of haemoglobin to methaemoglobin. Inoculate a known positive control onto any nitrate plate containing less than 4 tests, particularly when nitrate negative *C. jejuni doylei* or *Helicobacter fennelliae* is suspected. It is essential to incubate anaerobically for 24 hours or in H₂ for 48 hours without delay. *C. mucosalis* and *C. concisus* give very weak reactions.

CATALASE

The test:

A loopful of bacteria (taken carefully from a TBA plate - do not stab to cool) is picked up with a capillary tube containing Hydrogen peroxide (20 vols). Oxygen bubbles are trapped in the tube.

C. jejuni jejuni will give a positive result. *C. upsaliensis* will give a negative / weakly positive result.

Notes:

Catalase breaks down hydrogen peroxide into oxygen and water. A positive reaction can be so strong that large bubbles are formed - this can block the capillary tube and prevent

the ascent of any bubbles. On close scrutiny the trapped bubbles can be seen stuck to the surface of the bacteria or forced out at the bottom.

ARYLSULPHATASE ACTIVITY

Preparation:

Brucell broth (Difco 0495-17-3)	14.00 g
Bacto Agar or Technical No 3	1.75 g
Phenolphthalein Disulphate (Sigma P 0251)	0.325 g
Dist H ₂ O	500 ml

Steam to dissolve, tube in 4 ml amounts. Autoclave 15 ib/15 min. Prepare small batches to prevent false positives

Sodium Carbonate solution

Na ₂ CO ₃ anhydrous	15 g
Distilled H ₂ O	100 ml

Store on the bench, do not refrigerate, as it will solidify.

The test:

USE ONLY ON A CULTURE GROWN IN H₂

Emulsify in the top 1/3 of the substrate using a very heavy inoculum (turbid).

Incubate in H₂ 48 hours for Indoxyl acetate POSITIVE cultures

96 hours for Indoxyl acetate NEGATIVE cultures

Add about 1 ml of 15 % sodium carbonate (Na₂CO₃) solution - can give the tube a shake.

The breakdown product of phenolphthalein forms a pink colour in the presence of Na₂CO₃.

A positive result produces a bright pink colour, e.g. *C. jejuni jejuni* 2. A negative result produces no colour change, e.g. *C. jejuni jejuni* 1.

HIPPURATE HYDROLYSIS

Preparation:

Stock = 5 % Hippuric acid (Sodium salt Sigma H 9380, or Merck 820648) in dist H₂O.

Store frozen

Hippurate broth

Hippuric acid stock solution 25 ml

Dist H₂O 100ml

Filter ~1 ml amounts into **glass tubes. Do not autoclave.**

Ninhydrin (Merck 6762, BDH 10132 4E) 3.5 g

50:50 Butanol:Acetone 100ml

Store Ninhydrin solution in freezer

The test:

Only *C. jejuni jejuni* and *C. jejuni doylei* are positive. Culture: from CO₂ plate ONLY. Incubate test in CO₂ ONLY. Thaw hippurate broth and inoculate heavily. Incubate overnight (more practical than a 2 hour test). Gently add ~0.5 ml ninhydrin solution. Do not shake (aerate) the tube. Read within 10 minutes (the 2 hour test needs 30 minutes incubation).

C. jejuni jejuni and *C. jejuni doylei* will give a positive result which is a strong purple colour. Other *Campylobacter* species will give a negative result, either colourless or very light purple.

Notes:

Hippuricase hydrolyses hippurate to benzoic acid and glycine. Glycine is deaminated by the oxidising agent, ninhydrin. Ninhydrin becomes reduced in the process and a purple coloured dye is formed. Sometimes H₂ cultures may give false positive results - Essential to use a CO₂ grown culture and to incubate the test in a CO₂ environment.

AEROBIC GROWTH AT ROOM TEMPERATURE

Squiggle-inoculate a positive control and three tests onto a TBA plate divided in 4. Leave on the laboratory bench for 48 hours. Only *Arcobacter* species will grow.

GROWTH ON MACCONKEY

Preparation:

MacConkey without NaCl or Crystal Violet (**ONLY Oxoid CM 7b** gives consistent results)

The test:

Inoculate lightly and streak out for single colonies on 1/2 plates. Incubate in H₂ for 48 hours.

A positive result is the growth of visible **single** colonies. If unsure, and for slow growing organisms, incubate for a further 48 hours.

SEMI-SOLID IRON BROTH FOR RAPID H₂S PRODUCTION

Preparation:

Bacto or Technical Agar	1.75 g
Nutrient Broth No. 2 (Oxoid CM67)	12.50 g
Dist H ₂ O	400 ml

Steam above to dissolve

Sodium metabisulphite Na ₂ S ₂ O ₅	0.5 g
Sodium pyruvate CH ₃ COCOONa	0.5 g
Ferrous sulphate FeSO ₄ .7H ₂ O	0.5 g
Dist H ₂ O	50 ml

Dissolve the above

Combine the 2 solutions and bring the volume up to 500 ml with dist H₂O. Autoclave 15 lb/15 min in 100 ml amounts. Store this stock in the dark at room temperature. Dispense 2 ml amounts in sterile small screw capped plastic tubes. Store in the dark and at room temperature.

GROWTH ON MACCONKEY

Preparation:

MacConkey without NaCl or Crystal Violet (**ONLY Oxoid CM 7b** gives consistent results)

The test:

Inoculate lightly and streak out for single colonies on 1/2 plates. Incubate in H₂ for 48 hours.

A positive result is the growth of visible single colonies. If unsure, and for slow growing organisms, incubate for a further 48 hours.

TROUBLE SHOOTING AND USEFUL HINTS

Incubate for 48 hour periods

Some of the more fastidious species may be lost if the jar is opened after only 24 hours of incubation, especially during initial isolation. All the enzymes are at their peak and best results are obtained from 48 hr cultures. Both arylsulphatase and indoxyl acetate tend to give false negative results with old cultures – even a 3 day old culture. Rapid H₂S can also give problems, especially for *C. lari*. Hippurate is an exception to the rule, and older cultures can be used for these tests.

All the enzymes are better developed in H₂ than in CO₂

Sometimes too well - e.g. excess H₂S production of CO₂ species on PbAc and TSI, resulting in false positive reactions if performed from an H₂ culture. Occasionally, otherwise obvious *C. coli* or *C. upsaliensis* cultures seem to be hippurate positive. Repeat from a culture grown in CO₂.

False positive hippurate reactions can also occur if: plastic tubes are used, the medium is defrosted a third time (i.e. re-freeze unused hippurate solutions only once), the incubated tube is aerated either by shaking or if left standing for too long after the ninhydrin has been added.

Rapid H₂S and arylsulphatase tests should always only be done from cultures grown in H₂-very poor or inconsistent results are obtained from CO₂ plates. It is more practical to use H₂ grown cultures to do all the tests (except for TSI and PbAc) while establishing if the isolate is capable of growth in CO₂.

Mixed infections

Very little variation will occur in results for the tests chosen for this identification scheme. Usually discrepancies in the results are indicative of a mixed infection.

Fresh TBA plates

The isolation rate of *Campylobacteraceae* strains is very poor on TBA plates that are older than 10 days. *Helicobacter* species are even more fastidious. Fresh plates, poured

twice weekly, and using older plates for subculture only, is recommended. Even on 3 week-old plates established cultures are often lost.

Alternate jars

With 4 sachets in a 36 plate anaerobic jar, more H₂ is available for the organisms. (Two sachets in a small [3 liter] jar produce too much pressure for efficient growth of the bacteria). It is preferable to use the large jars, otherwise alternate the culture plates - 2 days in a small jar, 2 days in a large jar.

New specimens

Culture plates must be incubated in H₂ as quickly as possible, e.g. the morning specimens by midday and the afternoon specimens by the end of the day. H₂-dependent organisms do not last too well in a CO₂ atmosphere over a prolonged period of time.

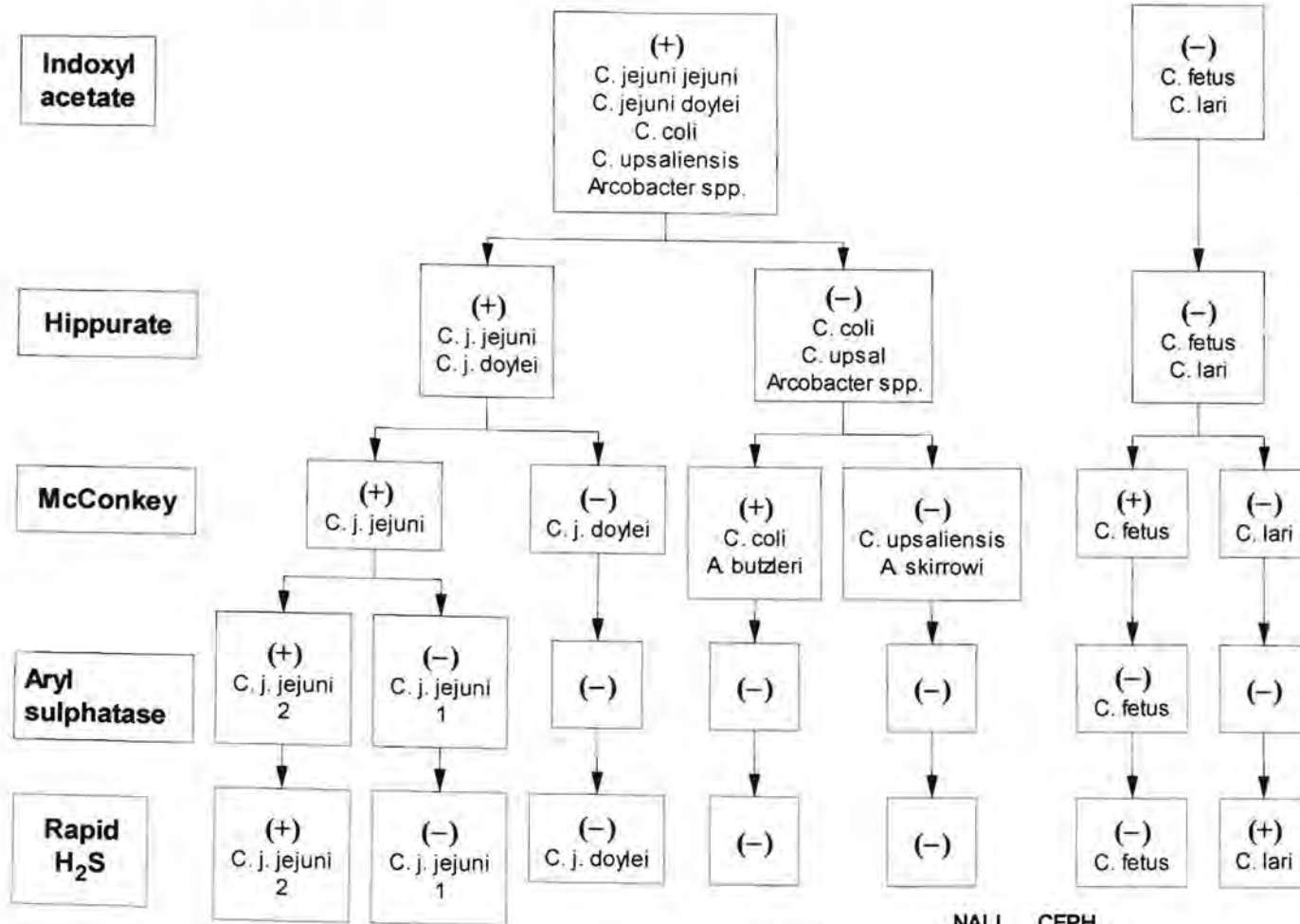
Distribution of *Campylobacter* and related species isolated from diarrhoeic stools at the Red Cross Children's Hospital, Cape Town, South Africa Oct. 1, 1990-Sept 30, 2005

Species	Number	%
<i>C. jejuni</i> subsp. <i>jejuni</i>	1794	32,37
<i>C. concisus</i>	1364	24.61
<i>C. upsaliensis</i>	1304	23.53
<i>C. jejuni</i> subsp. <i>doylei</i>	418	7.54
<i>H. fennelliae</i>	319	5.75
<i>C. coli</i>	170	3.06
<i>C. hyointestinalis</i>	53	0.96
<i>H. cinaedi</i>	48	0.87
CLO / HLO*	28	0.51
<i>Arcobacter butzleri</i>	20	0.37
<i>C. fetus</i> subsp. <i>fetus</i>	9	0.16
<i>H. rappini</i> / <i>C. rectus</i> / <i>C. curvus</i>	8	0.14
<i>C. lari</i> / <i>C. sputorum</i> bv <i>sputorum</i>	7	0.13
Total	5542	100.00

*CLO / HLO: *Campylobacter* or *Helicobacter* organisms that could not be fully characterized.

Cape Town Protocol for the Isolation of *Campylobacter* spp.

Microaerophilic Atmosphere

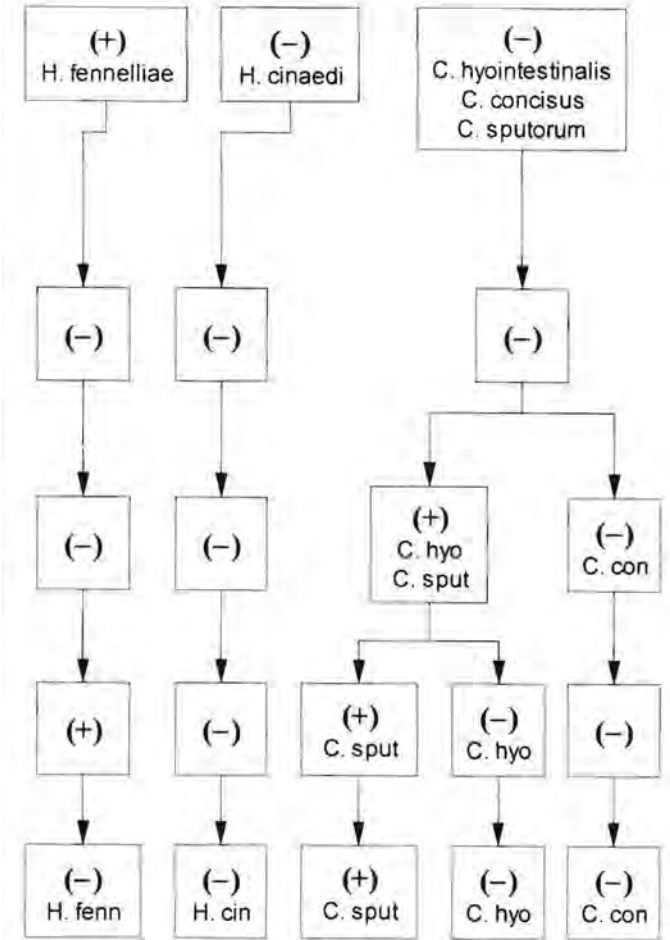


- *C. doylei* is Nitr Red -ve; *C. upsaliensis* is Nitr Red +ve
- *C. coli* & *C. lari* produce +++ H₂S (lead acetate strip)
- *Arcobacter* spp. are aerotolerant & grow at room temperature

	NALI	CEPH
<i>C. j. jejuni</i>	S/R	R
<i>C. j. doylei</i>	S	S
<i>C. upsaliensis</i>	S	S
<i>C. coli</i>	S	R
<i>C. fetus</i>	R	S
<i>C. lari</i>	R	R
<i>Arcobacter</i> spp.	S/R	S/R

S = Sensitive
 R = Resistant

H₂-enriched Microaerophilic Atmosphere



- Only *H. fennelliae* (& *H. rappini*) Nitr Red -ve
- *H. cin*, *H. fenn* (& *H. rapp*) produce very little, or no, H₂S on lead acetate strip

Appendix E

Microbank No.	Strain No.	Species (initial identification)	Source / clinical (if known)
South African Strains			
Q22	71.95	<i>H. fennelliae</i>	Hs: diarrhoea
	166.02	<i>H. fennelliae</i>	Hs: gastroenteritis
	145.02	<i>H. fennelliae</i>	Hs: chronic gastritis
P27	381.94	<i>H. fennelliae</i>	Hs: dysentery
	57.02	<i>H. fennelliae</i>	Hs: gastroenteritis
	194.02	<i>H. fennelliae</i>	Hs: chronic diarrhoea
	55.02	<i>H. fennelliae</i>	Hs: gastroenteritis
	58.05	<i>H. fennelliae</i>	
L38	95.94	<i>H. fennelliae</i>	Hs: acute diarrhoea
	384.96	<i>H. fennelliae</i>	Hs: diarrhoea
	Lli78.94	<i>H. fennelliae</i>	Hs: loose stool
	225.04	<i>H. fennelliae</i>	Hs: dysentery
S2	242.95	<i>H. cinaedi</i>	Hs: FTT diarrhoea
P5	348.94	<i>Helicobacter</i> sp.	Hs: abdominal pain
	130.94	<i>H. cinaedi</i>	
	142	<i>H. cinaedi</i>	
X11	99.97	<i>H. cinaedi</i>	Hs: dysentery, anaemia
R50	247.95	<i>H. cinaedi</i>	Hs: loose stools
W34	40.97	<i>H. cinaedi</i>	Bc
	34.94	<i>H. cinaedi</i>	Hs: diarrhoea
C48	28.93	<i>H. cinaedi</i>	
	60.05	<i>H. cinaedi</i>	BC
U25	326.96	<i>H. cinaedi</i>	Hs: diarrhoea
Y15	297.97	<i>H. cinaedi</i>	Hs: Kwashiorkor sepsis
Y36	Hp Ca-13	<i>H. pylori</i>	Normal
U24	Hp 483	<i>H. pylori</i>	
Y27	Hp Ca-1	<i>H. pylori</i>	Gastric carcinoma
Y37	Hp Ca-14	<i>H. pylori</i>	Gastric carcinoma
Y31	Hp Ca-8	<i>H. pylori</i>	Gastric carcinoma
Y30	Hp Ca-7	<i>H. pylori</i>	Gastric carcinoma
Y38	Hp Ca-15	<i>H. pylori</i>	Gastric carcinoma
Y29	Hp Ca-6	<i>H. pylori</i>	Gastric carcinoma
Z28	Hp Ca-17	<i>H. pylori</i>	Gastric carcinoma
WT9	BT 84	<i>H. pylori</i>	
Y28	Hp Ca-5	<i>H. pylori</i>	Gastric carcinoma
Denmark Strains			
H1	CCUG 33637	<i>H. hepaticus</i>	
H8	CCUG 13145	<i>Wolinella succinogenes</i>	
H9	H166-2	<i>H. cholecystus</i>	
H3	CCUG 29255	<i>H. pametensis</i>	
H2	ATCC BAA-430	<i>H. winghamensis</i>	
I2	15L 787/92	<i>H. canadensis</i>	
G7	CCUG 47163	<i>H. canadensis</i>	
G9	CCUG 22837	<i>H. pullorum</i>	
I1	C12	<i>H. pullorum</i>	
G8	ATCC 700932	<i>H. mesocricetorum</i>	
U.S.A. Strains			
M27	NCTC 12686	<i>H. acinonyx</i>	Cheetah: gastritis

Hs: human stool; Bc: Blood culture

ABSTRACT

&

POSTER PRESENTATIONS

L-ALA poster presentations

Evaluation of the Oxoid Biochemical Identification System (O.B.I.S.) for the differentiation of *Campylobacter* and *Arcobacter* from other Gram-negative organisms

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The poster was presented at two international scientific meetings:

CHRO 2005

13th International Workshop on
Campylobacter, *Helicobacter* and Related Organisms
Gold Coast, Queensland, Australia
September 4 - 8, 2005

Emerging *Campylobacter* spp. in the food chain: CAMPYCHECK

Feb 8th 2006
Croke Park Conference Centre
Dublin, Ireland

CHAPTER 5:

LITERATURE CITED

University of Cape Town

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A list of corrections done for the Masters thesis of Nisreen Hoosain

From Prof. E. Wasserman:

- An overall aim for the project was included and the bulleted “aims” at the end of the first chapter have now been formulated as different objectives (pp. 32-33).
- The reason why specific environmental sites were sampled; collected and how the number of samples to be processed were determined are indicated in chapter 2 (pg. 41).
- Clarity is given on which samples the biochemical and phenotypic characterization were performed, where they were obtained and why they were included in the study in chapter 2 (pg. 50).
- The reason why cadaver DNA was included in this study is indicated in chapter 2 (pg. 60).
- Tables 3.2 and 3.3 were left in the results section as they constitute original observations of this study.
- A reason for the brown colour of the waves is further stressed in chapter 4 (pg. 99).
- A brief referral to the two sets of primers which were used by Dr. Smuts to detect the presence of *Helicobacter* DNA in oesophageal tissue samples are included in chapter 4 (pg. 107).
- References are included to strengthen the possibility that the presence of *H. pylori* in the different tissues of cadavers could have led to the progression of “various” diseases if they were to live longer in chapter 4 (pp. 110-111).

From Prof. P. Gouws:

- Done all typographical corrections in the contents pages and throughout the thesis.