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The detection and characterisation of Helicobacter species in Australian marsupials

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The Detection and Characterisation of *Helicobacter* Species in Australian Marsupials

Thosaporn Coldham

A thesis submitted for the degree of Doctor of Philosophy

School of Biotechnology and Biomolecular Sciences
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Sydney, Australia

July, 2004

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I dedicate my work to the memory of my Father. If, as a child I had not received the support from him "*to go out study*" I would never have been in the position to attempt these studies. Thanks Dad.

Certificate of Originality

I hereby declare that this submission is my own work and to the best of my knowledge it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

Thosaporn Coldham

July, 2004

Abstract

This thesis examined the hypotheses that the mucus lining of the gastrointestinal tract (GIT) of Australian marsupials is colonised with large populations of spiral and fusiform shaped bacteria, many of which belong to the genus *Helicobacter* and that these *Helicobacter* species are likely be unique.

The presence of spiral and fusiform shaped bacteria in the GIT of 8 Australian marsupial species (32 animals in total) was examined using microscopy, culture and *Helicobacter* genus specific PCR. The marsupials studied included the brushtail possum, ringtail possum, koala, wombat, Eastern grey kangaroo, Tasmanian devil, Eastern quoll and long nosed bandicoot. The spiral and fusiform shaped isolates were characterised and identified using morphological appearance, *Helicobacter* genus specific PCR and 16S rRNA gene sequence comparisons. The spatial distribution of *Helicobacter* species in the GIT sections was examined microscopically in silver stained sections of the GIT and using Fluorescent *in situ* hybridisation (FISH) with a *Helicobacter* genus specific probe.

Spiral and/or fusiform shaped bacteria were detected and/or isolated from all marsupials studied. The prevalence and bacterial load of these organisms was found to differ in each marsupial species. These bacteria were found to belong to 3 different genera (*Helicobacter, Campylobacter* and *Desulfovibrio*). Each marsupial species appeared to be colonised with one or more unique *Helicobacter* species. Comparison of the detection of *Helicobacter* species in different groups of marsupials (herbivores, omnivores and carnivores) suggests that diet as well as the function and structure of the GIT may have a significant impact on their colonisation.

Phylogenetic analysis of the new possum *Helicobacters* showed that they shared a common ancestor. Comparison of *Helicobacter* species isolated from different species of marsupial and placental mammals, as well as birds, showed

that differences in environmental location i.e. gastric vs lower bowel had a major impact on the position of the *Helicobacters* on the phylogenetic tree.

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Chapter 1

Literature Review

1.1 The mucus-associated microbiota

A complex population of microorganisms has been found to inhabit and multiply at the surface of the gastrointestinal mucosa of many mammals and birds. At the time of birth, a mammalian foetus is free of cultivable microorganisms. Within 24 hours after birth, several bacterial species become established throughout the gastrointestinal tract (GIT) [1]. According to a defined time sequence, different species of microbiota colonise different areas of the GIT [2-4]. The surface of the gastrointestinal mucosa and its associated microorganisms are important components of the gastrointestinal ecosystem. Bacteria can be found attaching to the epithelial surface, in the adjacent mucus layers, or within intestinal crypts. The composition of this microbiota is profoundly influenced by that of the mother at the time of birth [5]. The development of this mucosa-associated microbiota in the GIT is very important for survival as it provides a functional barrier to colonisation by pathogens, plays an important role in normal nutrition and metabolism, and helps to shape the development of the intestine's mucosal immune system [6, 7].

The structure of the GIT dictates the localisation of the microbiota as well as the composition of the microbiota [8]. All vertebrates have a digestive tract and accessory glands (pancreas). The digestive tract can be separated into four major regions: headgut (oral and throat), foregut (esophagus and stomach), midgut (small intestine), and hindgut (large intestine). Major structural differences between the GIT of mammals are observed in the stomach and hindgut [9]. The stomach of some mammals is further compartmentalised into sub-areas depending upon the animal species. In ruminants the stomach is enlarged and divided into compartments which essentially act as a fermentation vat. Microbes within the vat have the first opportunity to utilise the animal's food. Indeed in ruminants, the host is largely

dependent for its nutrition upon these microbes and their metabolic end products [8]. Most mammals have a distinct hindgut which consists of the colon, rectum and often a caecum. The hindgut serves as the final site for storage of digesta and retrieval of dietary or endogenous electrolytes and water. It is also a principal site of microbial fermentation in herbivores [9]. Some herbivorous mammals have an extremely large caecum while others have an extremely large proximal colon which has developed as a microbial fermentation site. Indeed in some mammals, microbial activity in the caecum may provide as much as 25-35% of the animal's nutrition [8]. Most omnivorous and carnivorous animals have a simple stomach and a short intestinal tract. They either have no caecum or one diminished in size, as in these animals extensive microbial degradation is not required as most of their dietary intake is easily digestible [8].

1.1.1 The gastrointestinal ecosystem

At any point in time the GIT is populated by both autochthonous (indigenous) microbiota and a variable set of allochthonous (non-indigenous) microbiota. Autochthonous species have a symbiotic relationship and have evolved with their host over a long period, and generally persist throughout the life span of the animal [10]. The autochthonous microbiota of animals differs from species to species and in addition, within a species, varies from individual to individual [11]. In contrast, the allochthonous microbiota is derived from food, water, soil, air, or alternatively may be derived from another site within the GIT [8]. In a perturbed gastrointestinal ecosystem, the allochthonous microbiota are transient and are found temporarily colonising habitats vacated by their autochthonous inhabitants [2, 3]. In studies of the gastrointestinal ecosystem an important distinction between the autochthonous and allochthonous is that an autochthonous microbe colonises the habitat natively whereas an allochthonous microbe cannot colonise (i.e. multiply in it) except under abnormal situations. Criteria for determining whether an organism is autochthonous or not are not completely defined. However, in general, most of the autochthonous microorganisms of the GIT that can grow anaerobically, are always found in normal adults, colonise particular areas of the gastrointestinal tract, colonise their habitats during succession in infant animals, maintain stable population

levels in climax (balanced) communities in normal adults and may be associated with the mucosal epithelium [8].

In a well functioning gastrointestinal ecosystem, all available habitats and niches are occupied by autochthonous microbiota, thus prohibiting the establishment and multiplication of allochthonous organisms which are passing through the GIT. As the autochthonous microbiota act as a defence mechanism, most pathogens or their toxic products must be able to get very close to the gut surface in order to invade and cause tissue damage in the GIT. This phenomenon has been called ‘bacterial antagonism’ [12], ‘bacterial interference’ [13] or ‘colonisation resistance’ [14]. A number of autochthonous species however appear to posses both commensal and/or opportunistic traits. “Opportunistic infections” are caused, not by extraneous pathogens, but by bacteria that are autochthonous in adjacent tissues or in neighbouring organs [15]. In some circumstances, the autochthonous microbiota can translocate across the intestinal epithelial barrier to cause infection in extra-intestinal sites. The autochthonous microbiota is believed to be continuously translocating in low numbers from the GIT to extra-intestinal sites even in healthy immunocompetent hosts. However they are usually killed *en route* or *in situ* in the lymphoid organs by the host‘s reticuloendothelial system and mesenteric-lymph node (MLN) complex [16]. Studies using mono-associated ex-germ free mice would suggest that *Pseudomonas aeruginosa* and the Gram-negative facultative anaerobic *Enterobacteriaceae* have the greatest efficiency for translocating from the GIT to the MLNs. Gram-positive oxygen tolerant bacteria, such as *Staphylococcus epidermidis* and *Lactobacillus brevis* translocate at an intermediate level. The least effective in translocation to the MLNs are the obligate anaerobic bacteria such as *Bacteroides fragilis* and *Fusobacterium russii* which colonise the GIT at very high levels [16]. A number of mechanisms have been observed that appear to promote bacterial translocation from the GIT in animal models. The first mechanism is when intestinal overgrowth in the GIT occurs following disruption of the gastrointestinal (GI) ecology by oral antibiotic treatment, protein malnutrition, shock or other conditions. The second mechanism occurs when there is physical damage to the mucosal barrier as a result of ischemia/reperfusion

injury during endotoxic or hemorrhagic shock. The third mechanism occurs when there is a decrease in the immune defences resulting from immunosuppressive drugs or disease syndromes such as cancer and AIDS [16, 17].

The relationship between a mammalian host and its intestinal microbiota is generally symbiotic. From birth, a process develops that usually benefits both the microbes involved and the colonised host, each provides something essential to the other and receives something essential in return [6]. The mammalian gut has adapted to its microbiota so that it can cope with it, or benefit from it [18]. All gastrointestinal bacteria are present due to their ability to utilise one or a small number of substrates better than other organisms. These bacteria carry out a range of biochemical functions [7]. For example *E. coli, B. cereus, S. faecalis, Bacteroides spp., Eubacterium spp.* and *Clostridium spp*. are involved in the deconjugation and dehydroxylation of bile acids [19]. *Bacteroides, Eubacterium, Propionibacterium, Fusobacterium, Bifidobacterium, Lactobacillus, Clostridium, Enterobacterium, Veillonella, Enterococcus, Enterobacteria, and Streptococcus* all are involved in the production of menaquinones such as vitamin K [20]. One or more types of volatile and non-volatile acids can be found in any region of the tract colonised by microorganisms. These substances produced by gut anaerobes are the major source of energy for the colonic mucosa [8]. The fusiform anaerobes are responsible for the presence of volatile fatty acids, especially butyric acid, which exerts an inhibitory effect on coliform bacteria [21]. Indeed in rats, long-chain fatty acids present in the intestine have been shown to be a factor in controlling the localisation and population levels of some strictly anaerobic indigenous bacteria [22]. The composition of the microbiota in climax communities is regulated by multifactorial processes exerted by the animal host. These include diet and environmental factors such as hydrogen ion (pH) concentration, presence of bile acids, mucin, antibodies, phagocytes, peristalsis, as well as body temperature. The microbial population is also affected by forces resulting from the activities of microbes themselves, including nutrition competition, the production of toxic metabolic end products, such as volatile fatty acids and

hydrogen sulphide (H_2S), and the maintenance of a low oxidation-reduction potential.

To date the influence of any particular factor on the composition of the microbiota in any particular habitat is unclear. A simplified model of host-microbial interactions in the mammalian intestine was introduced by Bry *et al.* (1996) [23]. In this study the interaction that allows microbes to modify cellular differentiation programs and create a favourable niche was investigated. It was found by a comparison of conventionally housed and germ-free mice (NMRI strain) that production of fucosylated glycoconjugate and an alpha 1, 2-fucosyltransferase mRNA in the small-intestinal epithelium required normal microflora. A component of the microflora was identified by inoculation of germ-free mice with *Bacteroides thetaiotaomicron,* a selected, single genetically manipulatable resident bacterial species, which is also a prominent component of the normal mouse and human microbiota. It was revealed that *B. thetaiotaomicron* has a capacity of utilising L-fucose and induces fucosylated glycoconjugate production in the small intestine of its host. Colonisation of germ-free mice with *B. thetaiotaomicron* restored the fucosylation program, whereas an isogenic *B. thetaiotaomicron* strain which carried a transposon insertion that disrupts its ability to use L-fucose as a carbon source, did not. It was suggested by Bry *et al.* that this simplified model should aid the study of open microbial ecosystems.

In a subsequent study, Hooper *et al.* (1999) showed that *B. thetaiotaomicron* regulated production of ileal epithelial fucosylated glycans for its own nutritional benefit by using a repressor, FucR, as a molecular sensor of L-fucose availability [24]. A subsequent study examined the essential nature of the interactions between resident microorganisms and their hosts by examining global intestinal transcriptional responses using DNA micro arrays in germfree mice colonised with *B. thetaiotaomicron* [25]. This study revealed that *B. thetaiotaomicron* modulated the expression of genes involved in several important intestinal functions including nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and postnatal intestinal maturation while *Bifidobacterium infantis* (a prominent component of the

preweaning human and mouse ileal flora, and a commonly used probiotic) and *Escherichia coli* K12 (a normal component human intestinal flora) did not. It was suggested by Hooper *et al*. that the species selectivity of some of the colonisation-associated changes in gene expression, emphasises how the physiology can be affected by changes in the composition of the indigenous microbiota.

Few studies have examined the number and type of bacteria from human intestinal samples. In 1983, Croucher *et al.* reported a detailed study of the microorganisms associated with the colon surface obtained from four sudden-death subjects [26]. In this study the anaerobic and facultative organisms associated with the human colonic mucosa were enumerated and characterised. In three of the four subjects studied, the colony counts for total anaerobes, lactobacilli, total aerobes, faecal streptococci and enterobacteria were found to be similar along the length of the colon. The predominant anaerobes isolated in this study were *Bacteroides* spp. and *Fusobacterium* spp. These composed over 50% of the microbiota, as determined by morphology and glucose fermentation products. This study also showed that the microorganisms associated with the colonic mucosa of each individual were distinct and complex. In addition, scanning electron microscopy showed that the majority of microorganisms were located in the mucus layer of the colon, with spiral-shaped organisms located deep within the mucin layer and above the surface of the epithelium in two of the four subjects. These spiral-shaped organisms were not however isolated during the bacteriological investigations [26]. Due to the need for invasive procedures to obtain suitable materials from the gastrointestinal tract, this study is one of very few studies in which direct culture of human intestinal flora has been attempted.

Given this, in many studies faeces have been used to examine the composition of the microbiota of the human GIT. For example, Moore and Holdeman (1974) examined the faecal flora of 20 Japanese-Hawaiians using anaerobic tube culture techniques [27]. In this study 113 distinct types of organisms from a total of 1,147 isolates were observed and were estimated to account for 94% of the viable cells in the faeces. Based on statistical calculations, these authors

estimated that the total number of different types of bacteria present in the intestinal tract, at any time, would be likely to exceed 400 to 500 species, the predominant genera being *Bacteroides, Fusobacterium, Eubacterium, Bifidobacterium, Clostridium, Ruminococcus, Lactobacillus, Peptococcus,* and *Peptostreptococcus*.

More recently, culture-independent techniques based on the sequence variability of 16S rRNA genes have been used to examine the faecal flora of humans. These studies showed that the majority of bacteria in human faeces have yet to be obtained in culture. In addition when specific bacterial groups have been successfully cultured the numbers present do not appear to reflect the situation in the GIT. For example in a study by Langendijk *et al.* (1995), the enumeration of *Bifidobacterium* species in human faeces was investigated using fluorescent *in situ* hybridisation (FISH) with three genus-specific probes targeting regions V2, V4 and V8 of the 16S rRNA gene [28]. To determine quantification of bifidobacteria in the faeces, the results of 16S rRNA hybridisation were compared with that of culture on *Bifidobacterium*-selective agar. The comparison showed that there was no significant difference between the numbers determined and implied that all bifidobacteria in faeces were culturable. This study showed that the contribution of bifidobacteria in the total culturable intestinal microflora was almost 10-fold overestimated, when cultural methods were used as the sole method for enumeration.

In another study by Wang *et al.* (1996), twelve predominant anaerobic bacterial types in the faeces of both humans and animals (rat, mouse, cat, dog, monkey, and rabbit) were quantitated using PCR (polymerase chain reaction) [29]. The results of this study showed that *Fusobacterium prausnitzii, Peptostreptococcus productus,* and *Clostridium clostridiiforme* could be detected in high numbers (dilutions for positive PCR results ranging from 10^{-3} to 10^{-8} cells) in all of the human and animal faecal samples tested. *Bacteroides thetaiotaomicron, Bacteroides vulgatus,* and *Eubacterium limosum* also could be detected in relatively high numbers (10^{-2} to 10^{-6}) in adult human faeces. In comparison *Escherichia coli, Bifidobacterium adolescentis, Bifidobacterium longum,*

Lactobacillus acidophilus, Eubacterium biforme, and *Bacteroides distasonis* were detected at low levels (less than 10^{-2}) or were not detected in any of the faecal samples studied.

Variations in bacterial populations present in human faeces have also been examined by Franks *et al.* [30]. In their study, the faecal flora of nine volunteers was followed over a period of 8 months using six group-specific 16S rRNA-targeted oligonucleotide probes. Using a combination of probes, these authors were able to detect, using FISH, at least two-thirds of the bacterial population of the faeces. The bacteria detected included the genera *Streptococcus* and *Lactococcus,* the *Bacteroides fragilis* group, the *Clostridium lituseburense* group and *Clostridium coccoides–Eubacterium rectale* groups, and the species *Bacteroides distasonis* and *Clostridium histolyticum*. The *Bacteroides* and *Clostridium coccoides–Eubacterium rectale* groups constituted half of the bacterial composition of the faeces. In addition, to obtain a better coverage of the bacterial composition, two more specific probes were used to detect *Bifidobacteria* as well as an unknown group of gram-positive bacteria (low G+C #2) which were related to members of the *Clostridium leptum* group. This latter group were difficult to culture but represented a significant proportion of the 16S rRNA extracted from faecal samples. In individual volunteers the bacterial composition of the faeces was found to fluctuate over time. Of all the bacterial groups examined the *Bifidobacteria* showed the greatest variation over time.

The diversity of the predominant bacteria present in human faeces has also been analysed by Zoetendal *et al.* using temperature gradient gel electrophoresis (TGGE) of PCR amplicons of the V6 to V8 regions of the 16S rRNA gene [31]. While TGGE analysis of faecal 16S rDNA amplicons from 16 individuals showed that each subject had a different profile, some bands were shown to be common in all subjects. This study underlined and further supported the fact that each individual has a unique microbial community and that over time the dominant bacteria are stable.

Although analysis of faecal samples provides an indirect picture of the intestinal microbiota, information on the total number and types of microbes in faeces is

not necessarily indicative of the composition of communities within a particular site in the gastrointestinal tract. For example, in a study by Marteau *et al.* the composition of the caecal and faecal microbiota of 8 healthy volunteers were examined using culture and dot blot hybridisation [32]. Using both viable counts and dot blot hybridisation, the strictly anaerobic, *Bifidobacteria, Bacteroides*, and members of the *Clostridium coccoides* group and *Clostridium leptum* subgroup were found to be consistently lower in caecal samples as compared with faecal samples. In contrast facultative anaerobes represented by the *Lactobacillus-Enterococcus* group and *E. coli* showed much higher rRNA proportions in caecal contents. This study showed that using culture-dependent (total viable count) and culture-independent (dot blot hybridisation) methods, the caecal microbiota differed greatly from the faecal microbiota.

In a recent study, Zoetendal *et al.* (2002) compared bacterial communities present in faeces and biopsy samples obtained from the evacuated ascending, transverse, and descending colons of 10 individuals using denaturing gradient gel electrophoresis (DGGE) [33]. DGGE analysis of 16S rDNA amplicons was used to determine, compare, and visualise the composition of the predominant bacteria in these sites. Interestingly, the profiles which represented the predominant bacterial communities obtained from the biopsy specimens of different individuals varied significantly from those of the faecal samples. Since the biopsy samples were taken after evacuation of the colon it is possible that the bacteria detected are mucosa-associated and in close contact with the host. These results underline the fact that the mucosa-associated bacteria in the colon differ from those within the faeces. Moreover the predominant community in biopsy samples from all locations in the colon gave very similar profiles in each individual, distributed equally along the complete colon and appeared to be unique or host specific. Zoetendal *et al.* concluded that the observed host specific DGGE profiles of the mucosa-associated bacterial community in the colon supported the hypothesis that host-related factors are involved in the determination of the GIT microbial community.

1.1.2 Spiral and fusiform shaped mucosa-associated microorganisms

A vast array of microorganisms has been observed in close association with the epithelial surfaces of all parts of the GIT. These microorganisms may colonise and multiply on the surface of gastrointestinal mucosa, or alternatively, multiply in the lumen of the tract and simply adhere to some structure on an epithelial surface without multiplying there. To be able to colonise the mucosal surface microorganisms must be able to thrive in the environment and utilise nutritional conditions that exist there [34]. In addition, to remain associated with the mucus, microorganisms need to be able to withstand the flow of intestinal chyme. Three mechanisms of association with the intestinal mucosa have been described [35]. The first is adhesion, in which bacteria attach to the epithelium by means of specific adhesins, or by the development of specialised insertion structures. The second is surface mucus colonisation, in which certain organisms have the ability to survive in and presumably multiply in the outer areas of the mucus layer covering the gut surface (mucus blanket) [36]. The third is deep mucus and crypt association, in which the mucus-filled crypts of Lieberkuhn and crypts of the large bowel are colonised by dense aggregates of bacteria [18, 37].

Mucosa-associated microorganisms are defined as, any organism that is seen in significant numbers in specimens of gastrointestinal tissue following vigorous washing [38]. They are generally considered to be members of the autochthonous flora. Direct electron microscopic examination of well-washed GIT tissues has shown the presence of attached bacteria of different morphological types on the surface of different regions of the intestinal tract of a variety of birds, mammals and insects species. The major morphological types of highly adapted bacteria found within the mucus layers and the intestinal crypts of rats, dogs and other animals have a spiral or fusiform morphology [3, 11, 39-41]. Fusiform bacteria having an ecological preference for the mucus blanket [42].

During the first week of life spiral-shaped anaerobes have been detected colonising the mucus layers of the epithelium of the large bowel of baby mice. In the second week of life fusiform-shaped anaerobes have been found to co-habit the mucus layer with these spiral-shaped organisms, the fusiform-shaped organisms reaching climax levels by the end of the second week. These spiral and fusiform-shaped organisms remained associated in the mucus of the caecal and colonic mucosal epithelium in adult mice [3, 42, 43]. The location of mucosa-associated microorganisms in the intestinal tract of rats as well as the descriptive names given to the different morphological types of bacteria observed by microscopic examination have been reviewed and summarised by Phillips *et al.* and shown in Figure 1.1 [37.] This review showed a wide range of spiral-shaped bacteria to be present in rats. For example, short, fat spiral bacteria (St) [44] and crescent-shaped bacteria (Cr) [45] have been visualised throughout the ileal crypts, while filamentous organisms (Fil) have been observed attached to the villous surface of the small intestine and thin rigid spiral bacteria (Rs) [39] in the caecal mucosa. Flexible spiral organisms (B) [40] have also been found in the caecal mucosa, while fusiform shaped organisms (Fu) [3] have been observed lying in a parallel sheet in the colon, but never in the crypts. Small *Borrelia*–like organisms with long tapered ends (Bi) have also been visualised in the colonic region [37] and small S-shaped bacteria (Ss) have been found inhabiting the caecal mucosa and colon [39]. Bacteria colonising the mucus layer share two common properties, a spiral morphology and enhanced motility in viscous environments that allows the organisms to move in the mucus [46, 47]. The ability to swim towards the intestinal cell surface enables these organisms to avoid washout due to the peristaltic movement of the intestinal contents.

Descriptions of mucosa-associated bacteria are not restricted to the intestinal tract. Spiral shaped bacteria have also been shown to colonise the gastric mucosa. These bacteria were first observed in the stomach of dogs and were originally described by Rappin who initially referred to these as spirochaetes [48]. Rappin’s discovery was confirmed by Bizzozero who reported these spirochaetes inhabit the mucus layer covering the mucosa and penetrate into the lumen of the pyloric and fundical glands [49]. In 1896 Salomon reported

spiral shaped organisms to be present in the stomachs of dogs, cats, and a wide variety of animals. Salomon was the first to propagate these spiral organisms by feeding gastric mucosa, obtained from cats and dogs, to uninfected mice [50].

With the advent of the electron microscope, morphological descriptions of gastric bacteria became more precise [51, 52]. For example, in a study of the external form and structure of spiral shaped organisms present in the gastric mucosa of dogs and cats Weber *et al.* showed that at least two morphologically distinct types of spiral shaped bacteria were present, one of which was thick and had 4 to 10 coils and the other was a thinner form, with fewer than 10 coils, [51]. The thicker form was found almost exclusively in dogs, while in cats although both morphological types were found, approximately 85 to 95% of the spiral shaped organisms were of the thinner type. Study of the fine structure of these gastric organisms by Lockard and Boler, using thin sections of mucosa, showed that in dogs three morphological forms of spiral shaped bacteria were present. At this time Lockard and Boler suggested that these three morphological forms (Figure 1.2) were the same organism and simply represented different stages in the mechanism of movement [52]. The first type was described as a straight cylinder, with periplasmic fibrils tightly coiled around the entire surface of the organism. The second type was described as a loose spiral bacterium, with periplasmic fibrils surrounding the cell. The third type was a tight spiral bacterium without external fibrils. Subsequently, these three morphological types were shown to be three different organisms [53-55].

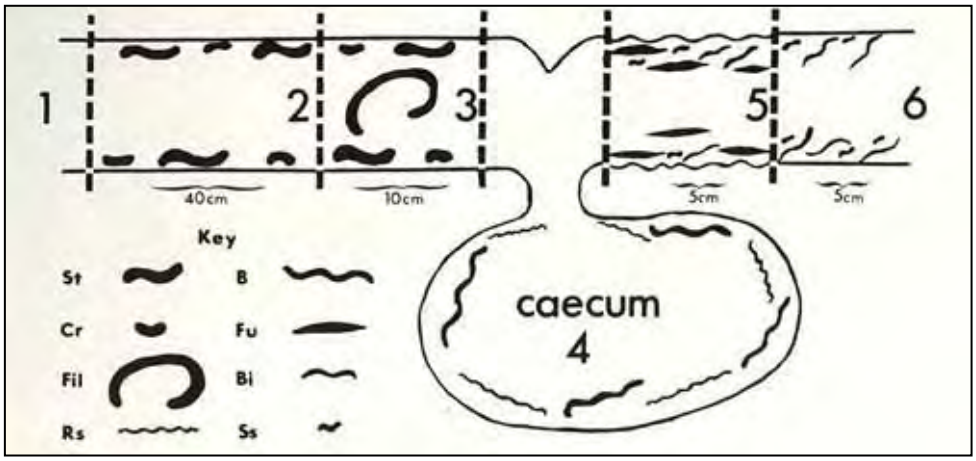


Figure 1.1 The location of mucosa-associated microorganisms in the intestinal tract of rats and the descriptive names given to the different morphological types of bacteria observed by microscopic examination [37].
(St) = Short, fat spiral, (Cr) = Crescent-shaped, (Fil) = Filamentous organism, (Rs) = Thin rigid spiral bacteria, (B) = Flexible spiralled organism, (Fu) = Fusiform, (Bi) = Small *Borrelia* –like organism with long tapered ends, and (Ss) = Small S-shaped bacteria

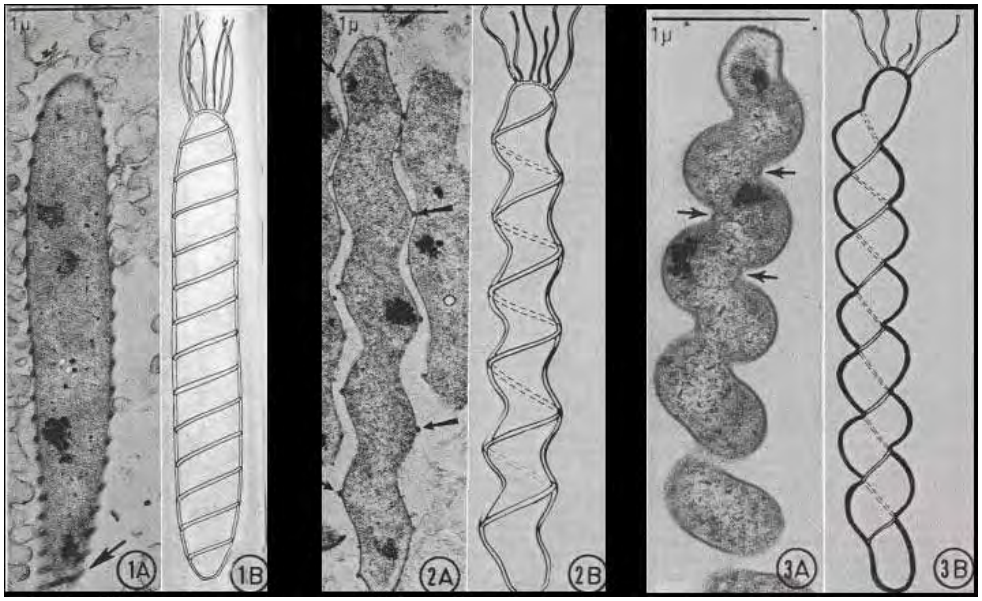


Figure 1.2 Lockard's three morphological forms [52]

A = A micrograph of a thin section, B = A drawing as it would appear in its whole, un-sectioned form

1. The organism characterised as a straight cylinder with periplasmic fibrils tightly coiled around the entire surface of the organism.

2. The organism characterised as a loose spiral bacterium with periplasmic fibrils surrounding the cell.

3. The organism characterised as a tight spiral bacterium without external fibrils.

In humans, spiral bacteria were first observed in 1906 in patients with gastric carcinoma [56]. In a later extensive study conducted by Doenges, spiral shaped bacteria were observed in 43% of 242 human stomachs examined at autopsy as well as in 19 of 19 Macacus rhesus monkeys examined. In this study, Doenges was however unable to relate the presence of these organisms to any particular type of stomach disease [57]. In contrast, in 1954 following an examination of more than 1,000 human gastric biopsies, Palmer reported no spirochetes to be present in the human stomach [58]. Following this report very little attention was given to the study of the stomach as a niche for autochthonous microbiota. However in 1967, some of the first detailed anatomical descriptions of the gastric mucosa, as viewed by the electron microscope, were published by Ito [59]. As part of this study, Ito published photographs and drawings of the structure of the parietal cell and secreting glands of the gastric corpus. Interestingly these photographs showed a spiral shaped bacterium with several sheathed flagella to be present within a parietal cell.

In 1975, in a study of inflammatory cell migration through the gastric epithelium and its relationship to bacteria, Steer demonstrated that, in a number of patients, migration of polymorphonuclear leukocytes could be partially attributed to bacteria observed on the mucosal surface [60]. In the same year, in an examination of the relationship between the activity of gastritis, bacteria, and gastric ulceration Steer and Colin-Jones reported 81% of patients with gastric ulcers to have spiral shaped bacteria present on their gastric mucosa [61]. On culture, a *Pseudomonas* species was grown and these workers suggested that this organism was a possible aetiological factor in gastric ulceration. These findings aroused little interest in the scientific community until 1982 when Marshall successfully cultivated a spiral organism, which had been observed by his colleague Warren, on the gastric epithelium of patients suffering from active chronic gastritis from gastric biopsy specimens [62]. Speculation of the role that such spiral bacteria might play in gastrointestinal disease followed.

1.2 The genus *Helicobacter*

Cultivation of this spiral or curved bacterium from the gastric mucosa of humans presenting with dyspepsia was initially reported by Marshall *et al.* in 1983 [62]. This bacterium was first named *Campylobacter pyloridis*, as it resembled *Campylobacter* spp. in morphology, growth requirements, G+C content (34 %) and sensitivity to metronidazole. In 1987 however, the name *C. pyloridis* was revised to *C. pylori* to correspond with the correct Latin genitive of the noun pylorus [63]. In 1989, *C. pylori* was subsequently transferred to a new genus, *Helicobacter*, and renamed *Helicobacter pylori* due to differences in important features such as flagella, fatty acid content and 16S rRNA sequence. *Helicobacter pylori* was the first member and is the type species of the genus *Helicobacter* [64].

Subsequent studies went on to show that *H. pylori* played a critical role in peptic ulcer disease, gastric cancer and B-cell mucosa associated lymphoid tissue (MALT) lymphoma [62, 65-68]. The recognition of the role of this bacterium in gastric pathophysiology led not only to a fundamental change in our understanding of gastroduodenal disease but also of gastrointestinal microbial ecology.

As a result of the discovery of *H. pylori,* interest in bacteria associated with the gastrointestinal tract of humans and other animals was renewed. In 1987 a bacterium with a morphology similar to that of Lockard' s type 1 was isolated from aborted ovine foetuses and classified as "*Flexispira rappini*" [69]. "*Flexispira rappini*" was the provisional name given to Gram-negative, microaerophilic, motile, spindle-shaped organisms with spiral periplasmic fibres and bipolar tufts of sheathed flagella. The phylogenetic position of "*Flexispira rappini*" falls within the genus *Helicobacter,* according to 16S rRNA sequence analysis. Recent evidence suggests that this group of organisms contains at least 10 *Helicobacter* taxa, including two named species, *H. trogontum* (flexispira taxon 6) and *H. bilis* (flexispira taxon 9) [70]. Recently, two more *Helicobacter* species with a similar morphology to flexispira*,* have been isolated from hamsters and cotton-top tamarins. One was named *Helicobacter aurati*

[71] and the other is referred to as *Helicobacter* sp. cotton-top tamarin [72]. As bacteria with this characteristic morphology do not belong to a single well defined species, in this thesis they will be referred to as "flexispira" or flexispira-like organisms.

In 1988 Lee *et al.*, cultured a bacterium with a morphology very similar to that of Lockard's type 2-organism from the stomach of cats [54]. This organism later named *Helicobacter felis,* was a long spiral-shaped organism which had periplasmic fibrils that usually occurred in pairs [73].

Attempts to culture gastric spiral organisms with Lockard type 3-morphology from dogs, cats, pigs and humans has had limited success. Organisms with this morphology observed in the stomach of humans were originally referred to as "*Gastrospirillum hominis"* [55]. Subsequently, Solnick *et al.* cloned and sequenced the 16S rRNA gene from 2 human isolates and showed that they belonged to the *Helicobacter* genus, and were closely related to *H. felis.* These isolates were given the provisional name of *"Helicobacter heilmannii*" [74]. A bacterium with a similar morphology to *"H. heilmannii*" has recently been cultured from dogs. The results of electron microscopy, biochemical characteristics, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles clearly showed that these organisms differed from *H. felis*, "*Flexispira rappini"* and other previously cultured gastric *Helicobacter* spp. resulting in their classification as *Helicobacter bizzozeronii* [75].

Subsequently, many studies have shown that *Helicobacter* species are not restricted to the gastric mucosa, indeed many strains have been isolated from the intestinal tract of humans and animals. Early observations of spiral-shaped organisms in the GIT of several mammals including humans were originally reported in 1923 by Parr [76]. In 1983, a group of *Campylobacter*-like organisms (CLOs) were isolated from rectal swabs taken from 26 of 158 homosexual men with proctitis, proctocolitis and enteritis and from 6 of 75 asymptomatic homosexual men [77]. These CLOs were further characterised by Fennell and Totten [78] and were phenotypically separated into 3 groups, CLO-1 (25 isolates), CLO-2 (5 isolates) and CLO-3 (1 isolate) [79]. Subsequently the name

Campylobacter cinaedi was given to the CLO-1 strains and *Campylobacter fennelliae* to the CLO-2 strains [79]. *Campylobacter cinaedi* and *C. fennelliae* were subsequently transferred to the genus *Helicobacter* and named *Helicobacter cinaedi* and *Helicobacter fennelliae* respectively [80]. At present the CLO-3 strain is referred to as "*Helicobacter* sp. strain CLO-3".

Fusiform and spiral-shaped organisms from the intestinal tract of rodents have been cultured and described by several investigators, however many of these investigators did not classify these organisms into known species [5, 11, 41, 81, 82]. For example, in 1980 Phillips *et al.* isolated a spiral-shaped bacterium with distinctive morphology from the intestinal crypts of rats [41]. This spiral shaped organism was one of the first *Helicobacter*-like species to be cultivated in artificial media. It was not until 1992 however that further investigation of this spiral shaped organism showed that it belonged to the genus *Helicobacter.* This bacterium was subsequently named *Helicobacter muridarum* [83].

1.2.1 Current knowledge of the *Helicobacte*r genus

The *Helicobacter* genus currently comprises 23 bacterial species (Last full up date: March 18, 2004) according to the list of bacterial names with standing in the nomenclature (http:// www.bacterio.cict.fr/h/helicobacter.html). The cellular morphology of members of this genus is varied and includes curved rods, spiral rods or fusiform-shaped rods ranging in size from 0.2 to 1.2 μm wide and from 1.5 to 10 μm long. Examples of the morphology of *Helicobacter* species isolated to date are shown in Figure 1.3.

They are non-spore forming and Gram-negative. Most are motile by means of a single polar flagella or bipolar tufts of up to 20 flagella. *Helicobacter mustelae* also has lateral flagella. In most species the flagella are sheathed, however several species that have non-sheathed flagella have been described including *Helicobacter pullorum, Helicobacter rodentium, Helicobacter ganmani* and *Helicobacter mesocricetorum*. *Helicobacter* species have an optimum temperature for growth of 37^0C and are usually microaerophilic with a

respiratory type of metabolism. They produce oxidase and most strains produce catalase. The G+C content of the DNA is 24-48% [80, 84].

The majority of members of the genus *Helicobacter* are found naturally colonising the mucus layer covering the epithelial surface of the gastrointestinal tract. *Helicobacter* species can be separated into two main groups, gastric and lower bowel *Helicobacters*. A few *Helicobacter* species such *as Helicobacter bilis* [85, 86], *Helicobacter aurati* [71]*, Helicobacter muridarum* [87] can however colonise both the stomach and lower bowel. There are currently 7 validated *Helicobacter* species that have been isolated from gastric tissue and 16 validated lower bowel species. Two as yet uncultured putative new species, for which 16S rRNA data are available, have been proposed as *Candidatus* spp. (the official naming of incompletely described prokaryotes): "*Candidatus* Helicobacter bovis" (from cattle), and "*Candidatus* Helicobacter suis" (from pigs). The validly named *Helicobacter* species, the putative novel species and *Candidatus* spp. with their known host(s) are shown in the Tables 1.1, 1.2 and 1.3 respectively.

Table 1.1 Gastric *Helicobacter* species with their known host(s) and the site/source from which they were isolated.

Name	Site/source	Host(s)	Reference(s)
Validated taxa			
H. acinonychis	Stomach	Cheetah	[88]
H. bizzozeronii	Stomach	Dog	[75]
H. felis	Stomach	Dog, cat	[73]
H. mustelae	Stomach	Ferret	[64, 89]
H. nemestrinae	Stomach	Pigtail macaque	[90]
H. pylori	Stomach	Human	[64]
H. salomonis	Stomach	Dog	[91]
"*Candidatus*" sp. and unvalidated taxa			
"H. cetorum"	Stomach, faeces	Dolphin	[92]
"H. suncus"	Stomach	Musk shrew	[93]
"Candidatus Helicobacter bovis"	Abdomasal stomach	Cattle	[94]
"H. heilmanii" type 1	Stomach	Human	[74]
"H. heilmanii" type 2	Stomach	Human	[74]
"Candidatus Helicobacter suis"	Stomach	Pig	[95]

Table 1.2 Validated taxa of lower bowel *Helicobacter* species with their known host(s) and the site/source from which they were isolated.

Name	Site/source	Host(s)	Reference(s)
H. aurati	Stomach and caeca	Syrian hamster	[71]
H. bilis	Colon, caeca, liver & bile	Mice	[96]
H. canadensis	Faeces	Human	[97]
H. canis	Faeces, liver (1 puppy)	Dog, puppy & Human	[98, 99]
H. cholecystus	Gallbladder, liver	Hamster	[100]
H. cinaedi	Intestine, Rectal swab (human)	Hamster, human	[78, 79]
H. fennelliae	Rectal swab	Human	[78, 79]
H. ganmani	Small & large intestine, caeca & liver	Mice	[101]
H. hepaticus	Colon, caeca & liver	Mice	[102]
H. mesocricetorum	Faeces	Hamster	[103]
H. muridarum	Intestine, stomach	Mice, rat	[41, 83]
H. pametensis	Faeces	Bird (Tern, gull), pig	[104, 105]
H. pullorum	Liver, duodenum & caeca (chicken), Faeces (human)	Chickens, human	[106]
H. rodentium	Faeces, colon & caeca	Mice	[107]
H. trogontum	Colonic mucosa	rat	[108]
H. typhlonius	Intestine	Mice	[109]

Table 1.3 Unvalidated taxa of lower bowel *Helicobacter* species with their known host(s) and the site/source from which, they were isolated.

Name	Site/source	Host(s)	References
Helicobacter sp. Bird B	Faeces	Bird (Tern)	[105]
Helicobacter sp. Bird C	Faeces	Bird (house sparrow)	[105]
"H. colifelis"	Faeces	Kitten	[110]
Helicobacter sp. cotton-top tamarin	Faeces	Cotton-top tamarins	[72]
Helicobacter sp. strain CLO3	Rectal swab	Human	[78, 79]
"H. marmotae"	Liver (woodchuck), Faeces (cat)	Woodchuck, cat	[111]
"*H. muricola"*	Caeca and faeces	Korean wild mouse	[112]
"Flexispira rappini"	Aborted sheep foetus, Stomach (pig, dog), Faeces (human, dog), Blood (human)	Aborted sheep foetus, sheep, pig, dog, human,	[53, 70]
Helicobacter sp. rhesus Type 1&2	Anterior, transverse & descending portion of large intestine	Rhesus monkey	[113]
"H. winghamensis"	Faeces	Human	[114]

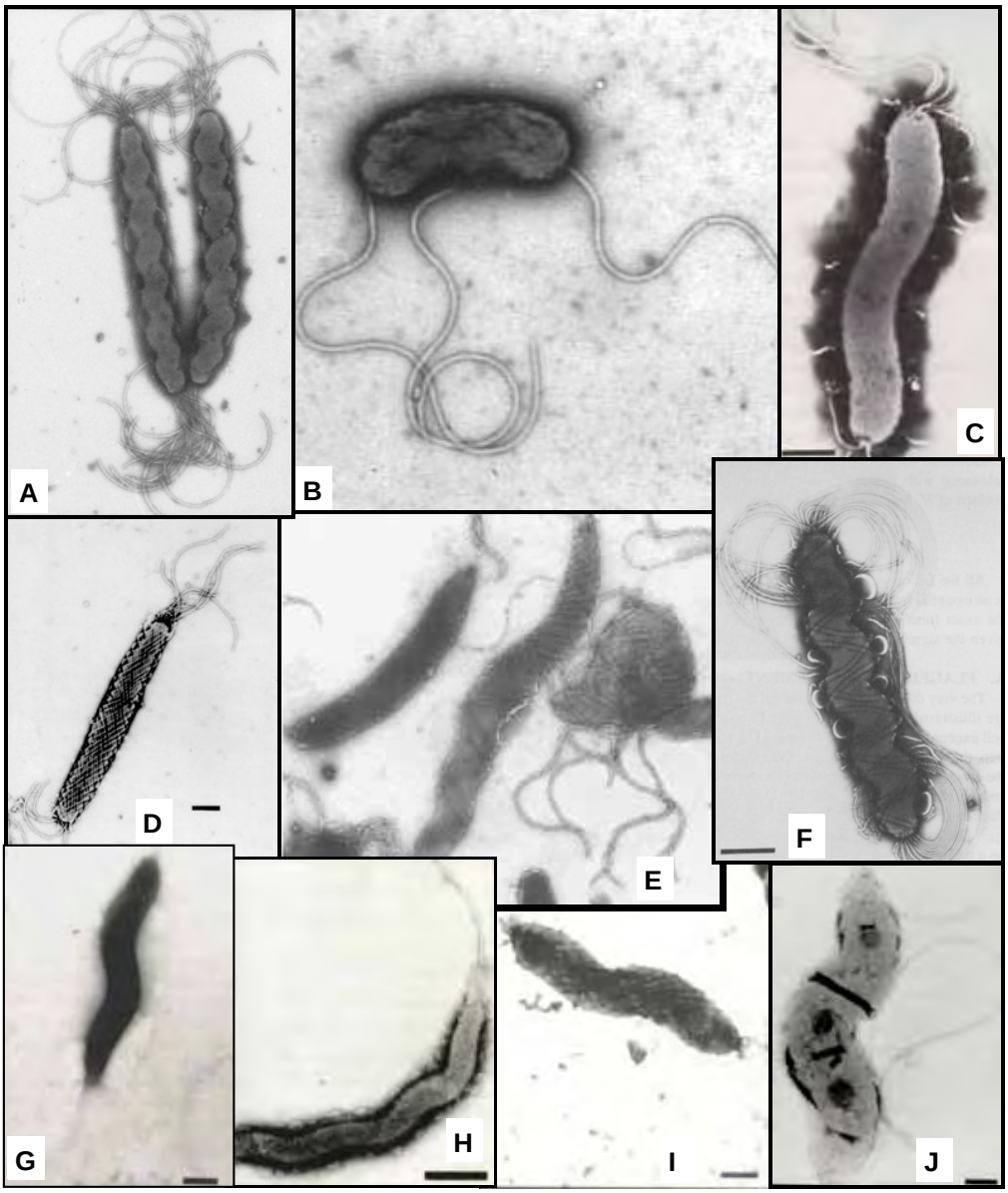


Figure 1.3 Examples of the morphology of *Helicobacter* species

A) *Helicobacter bizzozeronii*, magnification 20 000. (Micrograph courtesy of K. Jalava, University of Helsinki, Finland)

B) *Helicobacter mustelae,* magnification 30 000. (Micrograph courtesy J. O'Rourke)

C) *Helicobacter pylori,* Bar = 0.5 μm. (Micrograph reproduced from reference [115])

D) *Helicobacter aurati,* Bar = 0.5 μm. (Micrograph reproduced from reference [71])

E) *Helicobacter bilis,* Bar = 0.5 μm. (Micrograph reproduced from reference [96])

F) *Helicobacter felis,* Bar = 0.5 μm. (Micrograph reproduced from reference [73])

G) *Helicobacter ganmani,* Bar = 0.5 μm. (Micrograph reproduced from reference [101])

H) *Helicobacter hepaticus,* Bar = 0.5 μm. Micrograph reproduced from reference [102])

I) *Helicobacter muridarum,* Bar = 0.4 μm. (Micrograph courtesy J. O'Rourke)

J) *Helicobacter rodentium,* Bar = 0.2 μm. (Micrograph reproduced from reference [107])

1.2.2 *Helicobacter* Taxonomy

Bacterial taxonomy is comprised of three principle areas: classification, nomenclature and identification, each linked to each other [116]. Classification is the orderly arrangement of organisms into taxonomic groups (taxa) on the basis of similarities or relationships. Nomenclature is the assignment of names to the taxonomic groups according to international rules. Identification is the process of determining if a new isolate belongs to one of the established named taxa. The basic taxonomic group in bacterial systematics is the species, which is defined as a group of strains that share many features in common. One strain of a species is designated as the type strain. It serves as the reference strain for a name and is the permanent example of the species. Strains are identified when data from an unknown isolate matches with a known strain to an acceptable level [117]. An identification scheme for a group of organisms can be devised only after that group has first been classified. If the organism is new and cannot be identified as belonging to existing taxa, they are named according to the rules of nomenclature and placed in an appropriate position in an existing classification [116].

The use of the 16S rRNA gene for determining phylogenetic relationships among all living organisms has played a major role in the rearrangement of bacterial taxonomy. Because of its conserved nature and universal distribution, phylogenetic comparison of small-subunit rRNA (SSU rRNA) sequences has become a powerful method for the systematic classification of microbial organisms at the family, genus, species, and subspecies levels. Presently, the most useful and extensively investigated phylogenetic marker molecules are 16S rRNAs and to a lesser extent, 23S rRNAs [118, 119].

The genus *Helicobacter* was assigned to the family *Campylobacteriaceae,* class *Proteobacteria* within the division *Epsilonproteobacteria* (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/). The closest taxonomic relatives of the genus *Helicobacter* are *Campylobacter, Wolinella, Arcobacter, Thiovulum* and *Sulfurospirillum* [120]. The identification of *Helicobacter* species is extremely difficult due to the biochemical inertness and variation in phenotypic results within the species. The primary identification and classification of new

Helicobacter species, and even the genus itself, has relied heavily upon molecular techniques, in particular sequencing of the 16S rRNA gene. However there are a number of limitations regarding the identification of new *Helicobacter* species using 16S rRNA gene sequencing data. Although 16S rRNA sequences can be used routinely for the identification of new isolates, the effective identity of 16S rRNA sequences is not necessarily a sufficient criterion to guarantee species identity [121]. Strains belonging to different species may have almost identical 16S rRNA gene sequence. For example *H. felis, H. bizzozeronii* and *H. salomonis* have virtually identical 16S rRNA gene sequence [122]. In contrast strains of one species may have a 16S rRNA gene that differs by up to 3% and even greater than 4% of the total gene sequence [123].

Extensive sequence diversity in the 16S rRNA gene of *Helicobacter cinaedi* has been documented in a polyphasic taxonomic study of members of this species [123]. Polyphasic taxonomy is the integration of different types of data and information on microorganisms and essentially indicates a consensus type of taxonomy [124]. As a result of these studies, strains described previously as "*Helicobacter* sp. strain Mainz" and "*Helicobacter westmeadii*" have now been shown to represent additional strains of *H. cinaedi* [123, 125, 126]. Another example is the gastric *Helicobacter* species isolated from a pigtailed macaque, *H. nemestrinae,* which was named based on only a single isolate, the only available strain of this taxon being the type strain [90]. Subsequently seven housekeeping genes, *atpA, efp, mutY, ppa, trpC, ureI* and y*phC*, and two flagellin genes *flaA* and *flaB* of the *H. nemestrinae* type strain were sequenced and were shown to cluster together with sequences obtained from 20 or more *H. pylori* isolates [127]. Furthermore the 16S rDNA sequence of *H. nemestrinae* was found to be similar to that of *H. pylori* strain 85D08 and differed by less than 1% from the 16S rDNA sequences of numerous other *H. pylori* strains. These data indicate that *H. nemestrinae* is a strain of *H. pylori* and as a result the *H. nemestrinae* name is now referred to as a junior heterotypic synonym of *H. pylori*.

Thus 16S rDNA sequence analysis cannot be regarded as the gold standard for species-level identification of helicobacters and other epsilon *Proteobacteria*

species. Problems encountered with the taxonomy of this genus, such as the misidentifying of species and species based on only a single isolate has resulted in the recommendation (by the International Committee of Systematic Bacteriology on the taxonomy of *Campylobacter* and related species) of minimal standards for describing new species in the genus *Helicobacter* [128]. These standards have been applied to avoid the confusion that sometimes accompanies premature formal naming of a species. In order to provide a measure of phenotypic and genotypic variation, the examination should be based on five or more strains from different sources. Where too few strains are available for formal naming, a description can be usefully published. Further descriptions of putative new species should be made according to the inter-strain relationships described by means of a polyphasic taxonomic analysis. The minimal standards recommended by the International Committee include:
Cell and colony morphology, motility and Gram reaction

- Growth conditions and characteristics
- Biochemical properties
- Resistance to antimicrobial agents
- Molecular data such as 16S rRNA data (at least 1450 bp of a minimum of three stains), DNA-DNA hybridisation, G + C content, whole cell protein profiles
- Ecology

Putative new species of uncultured organisms for which 16S rRNA data are available may be assigned to "*Candidatus"* status. Standards for new "*Candidatus*" species were also described and include:
Molecular data e.g. 16S rRNA gene sequence

- Probe for *in situ* identification
- Morphology, Gram reaction and preliminary metabolic data

1.2.3 Coevolution between *Helicobacter* species and their hosts

The gastrointestinal tract of an animal is home for a diverse community of microorganisms, as discussed in section 1.1. The complex relationship between these bacteria and their host is of considerable importance. Little is known

about how members of the indigenous microbiota interact with their vertebrate hosts or their coevolution. Vertebrate evolution has been derived from fossil records. Indeed fossils are the primary basis for the definition of the geological time scale, which divides the earth's history into a series of large time slices: eons, eras, periods and epochs. The geological time scale of the Phanerozoic eon to the present time, 570 million years (myr), including the names of eras, subera, epochs and the length of time of each is shown in Figure 1.4. The Phanerozoic eon is subdivided into three eras; the Phalaeozoic era or era of 'Ancient life', the Mesozoic era or 'Age of reptiles' and the Cainozoic era or 'Age of mammals'. The first appearance of vertebrates on earth, based on fossil records, was as early as 550 myr ago in the Cambrian period [129]. The divergence of birds and mammals was approximately 310 myr ago. The earliest ancestors of mammals (synapsids) and birds (diapsids) are lizard-like and first appeared in the Carboniferous period (354 myr ago). The estimated time for the marsupial-placental separation is estimated to be 135-173 Myr ago [130, 131]. Thus the diverse vertebrate species have evolved over a long period of time.

By comparison very little is known about the evolution of microorganisms. The earliest bacterial fossils were found in 3.5 billion years old stromatolites at North Pole in Western Australia. These fossil bacteria resemble *Cyanobacteria* [132, 133].

Currently *Helicobacter* species have been detected colonising the digestive system of many mammals and birds. In addition there is evidence that *Helicobacter* species may be present in reptiles. For example gastric spiral bacteria (possibly more than one *Helicobacter* species) have been observed microscopically in reptiles [122]. *Helicobacter* DNA has also been detected in the faeces of zoo animals, including reptiles (Nile crocodile and Taiwan beauty snake), using polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) [134]. *Helicobacters* are believed to be indigenous microorganisms with many *Helicobacter* species being shown to be host specific. This indicates that helicobacters and their hosts may have naturally coadapted to each other or coevolved over a long period of time. However some *Helicobacter* species are known to be able to colonise a number

of different host species. Thus there is always a possibility that helicobacters may have passed from one host species to another via the food chain or other mechanisms.

Animals have adapted to environmental change as well as to food sources. They can be simply divided into carnivores, omnivores and herbivores based on the general type of diet. The diet and foraging strategies used by animals also affects the morphology and function of the animal's digestive system. Gastrointestinal microbial communities are believed to have adapted together with changes to the host.

In an attempt to understand how *Helicobacter* species may have adapted to the digestive systems of a variety of animal hosts, the currently named *Helicobacter* species were grouped by the author according to the classification of their host animals, as shown in Table 1.4 and Figure 1.5.

Gastric *Helicobacters* have been found to colonise the stomach of ruminants such as sheep and cattle in the order Artiodactyla, as well as the stomach of animals such as whales and dolphins in the order Cetacea. These animals have a stomach that is enlarged and sacculated or compartmentalised. Gastric *Helicobacters* have also been found colonising the stomach of humans, a member of the order Primate, as well as animals such as dogs, cats, and ferrets, members of the order Carnivora. The common feature of the members of the order Artiodactyla, Primate and Carnivora is they mostly digest food in their stomach. In contrast lower bowel *Helicobacters* are more likely to colonise animals such as mice, rats, and hamsters in the order Rodentia. These animals have in general a very simple stomach but an enlarged caecum or proximal colon. The orders Carnivora, Primate, Artiodactyla, Cetacea, Insectivora and Rodentia all belong to the subclass eutherian (or placental); class Mammalia [135]. At the commencement of the current study no information concerning *Helicobacters* in one of the major class of Mammalia, the Marsupialia, was known.

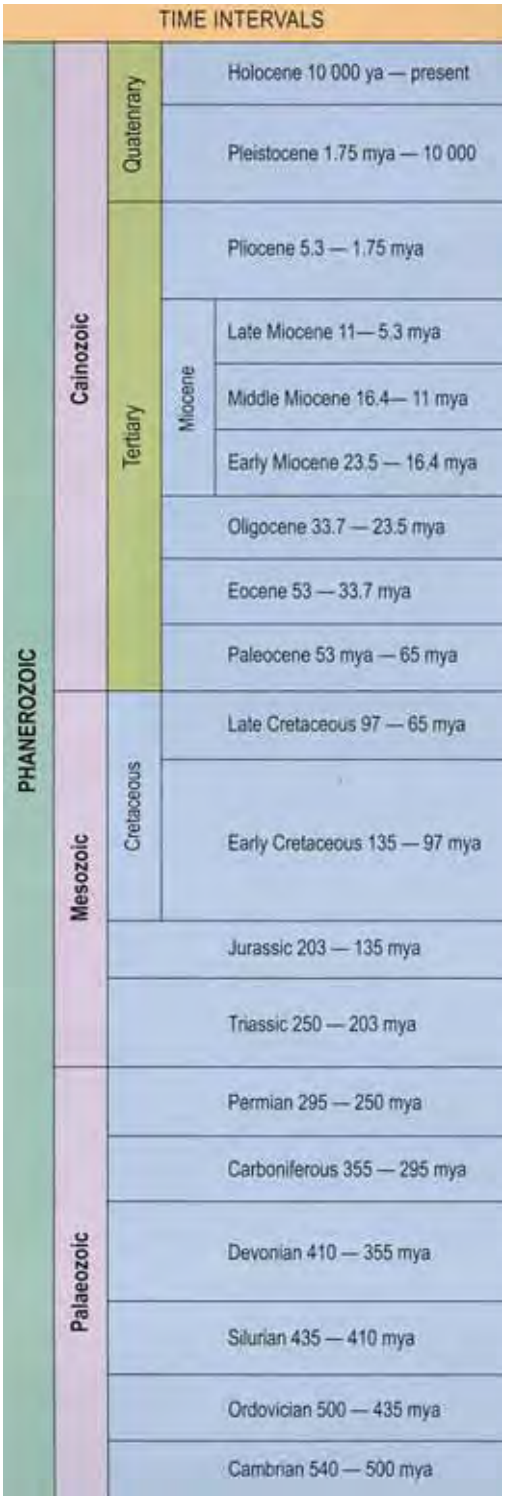


Figure 1.4 Geological time scale of the Phanerozoic eon

Host Classification	Animal hosts	*Helicobacter* species
Class Aves	Bird (tern, house sparrow), chicken	**Lower bowel *Helicobacter*** *Helicobacter* sp. Bird B & Bird C, *H. pametensis, H. pullorum*
Class Mammalia Order Primate	Human, pigtailed macaque, rhesus macaque, cotton-top tamarin	**Gastric *Helicobacter*** *H. pylori, "H. heilmannii"* , *H. nemestrinae* **Lower bowel *Helicobacter*** *H. canadensis, H. fennelliae, "H. winghamensis", H. cinaedi, Helicobacter* sp. cotton-top tamarin
Class Mammalia Order Carnivora	Dog, cat, ferret, cheetah	**Gastric *Helicobacter*** *H. bizzozeronii, H. mustelae, H. felis,* *H. salomonis, H. acinonychis* **Lower bowel *Helicobacter*** *H. canis, " H. colifelis", "Flexispira rappini", Helicobacter sp. rhesus*
Class Mammalia Order Rodentia	Mouse, rat, & hamster	**Lower bowel *Helicobacter*** ***H. aurati, H. bilis, H. cholescystus, H. cinaedi, H. ganmanii, H. hepaticus,* *H. mesocricetorum, H. muridarum,* *H. rodentium, H. trogontum," H. typhlonius",* *"Flexispira rappini"*
Class Mammalia Order Artiodactyla	Cattle, pig, & sheep	**Gastric *Helicobacter*** "*Candidatus* Helicobacter bovis", "*Candidatus* Helicobacter suis" **Lower bowel *Helicobacter*** *"Flexispira rappini"*
Class Mammalia Order Insectivora	House musk shrew	**Gastric *Helicobacter*** *"H. suncus"*
Class Mammalia Order Cetacea	Dolphin,& whale	**Gastric *Helicobacter*** ***"H. cetorum"*

Table 1.4 The animal hosts, host classification of currently known *Helicobacter* species

** *H. aurati* and ***"H. cetorum"* found colonised both stomach and lower bowel,

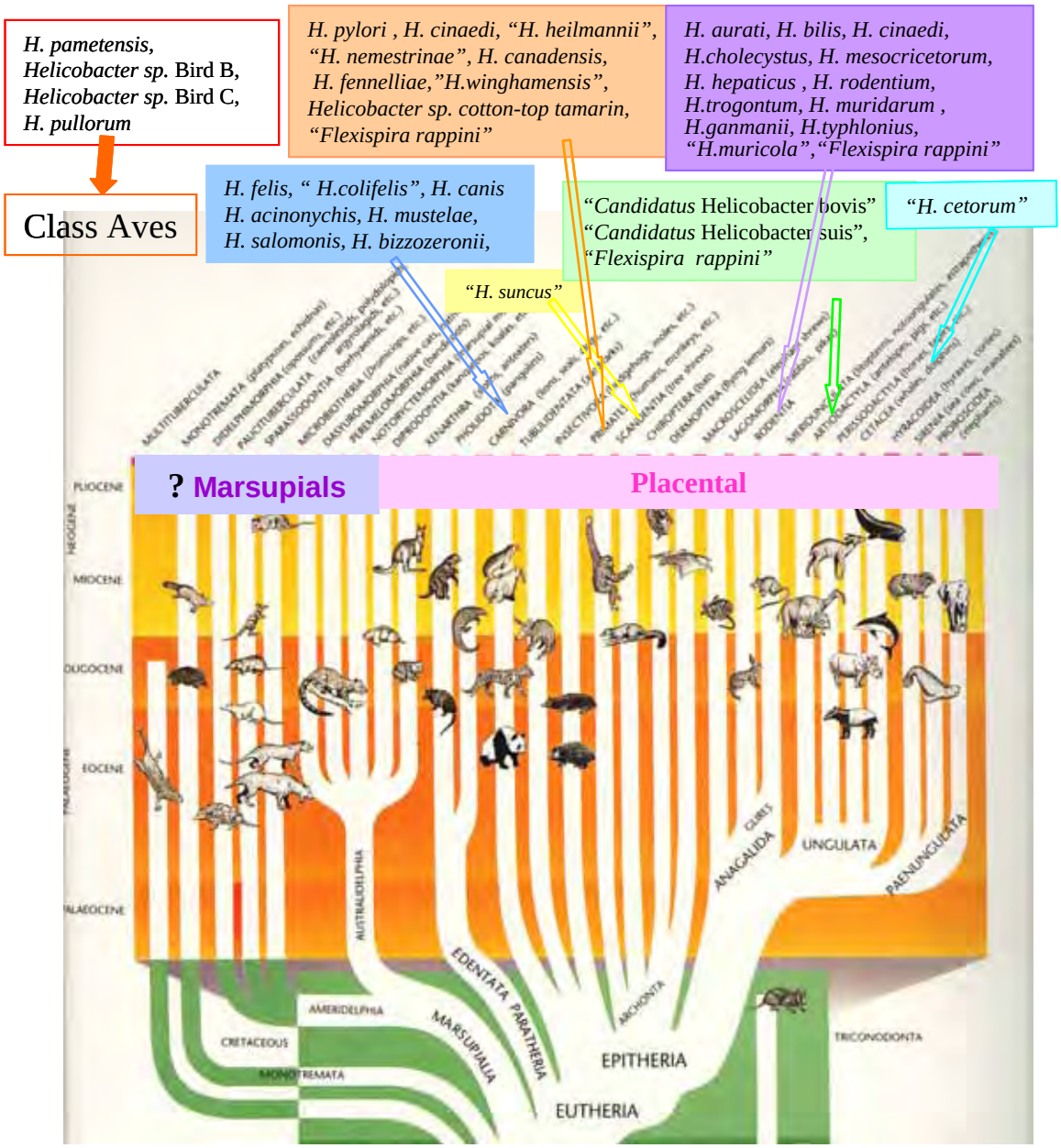


Figure 1.5 The grouping of *Helicobacter* species in relation to the classification of host animals. The diagram of the mammal classification was taken from "Hypothesis of the interrelationships of living and fossil mammals" by Vickers-Rich [129].

1.3 Marsupials

The most striking feature of marsupials is that they are born in an embryonic condition: tiny, naked and with hind limbs and tail still undeveloped. Monotremes (subclass Prototheria) and marsupials (subclass Metatheria) are the oldest group of native animals living on the Australian continent. No fossil monotremes have been found outside Australasia. An opalised-jaw fragment of *Steropodon galmani,* a 110-million year old monotreme, was discovered in Lightning Ridge, NSW [133]. This is the first and so far the only known Mesozoic mammal found in Australia.

In contrast to the monotremes, dental fossils of the oldest marsupials in Australia, Tingamara Fauna, were found in southern Queensland and are thought to be at least 54 million years old (myr), from the early Eocene epoch [129]. Marsupial fossils approximately 100 myr old however have also been found in North America [136]. In addition, extremely primitive marsupials from the Cretaceous period have been found in both North America and South America, thus making these a more likely point of origin than Australia [137]. According to fossil records, marsupials seem to have moved from North America into Europe at the end of the Cretaceous period, then to South America across Antarctica and finally to Australia. Dispersal to Australia was complete soon after the sinking of the South Tasman Rise which resulted in the final separation of Australia from Antarctica about 50 myr ago (see Figure 1.6). Subsequently, whilst marsupials became extinct in Europe and North America, they survived in South America and in Australia [130]. While there are several species still present in South America with a few species re-invading North America, over 60% of all living marsupials are found in Australia where they represent the most important group of terrestrial mammals of this continent. The movement of the Australian continent as well as the changes in global climate have affected the unique nature of Australian vertebrates and marsupials. Australia has been isolated from the rest of the world for more than 50 myr, which has led to the evolution of its biota quite independent [129]. Currently

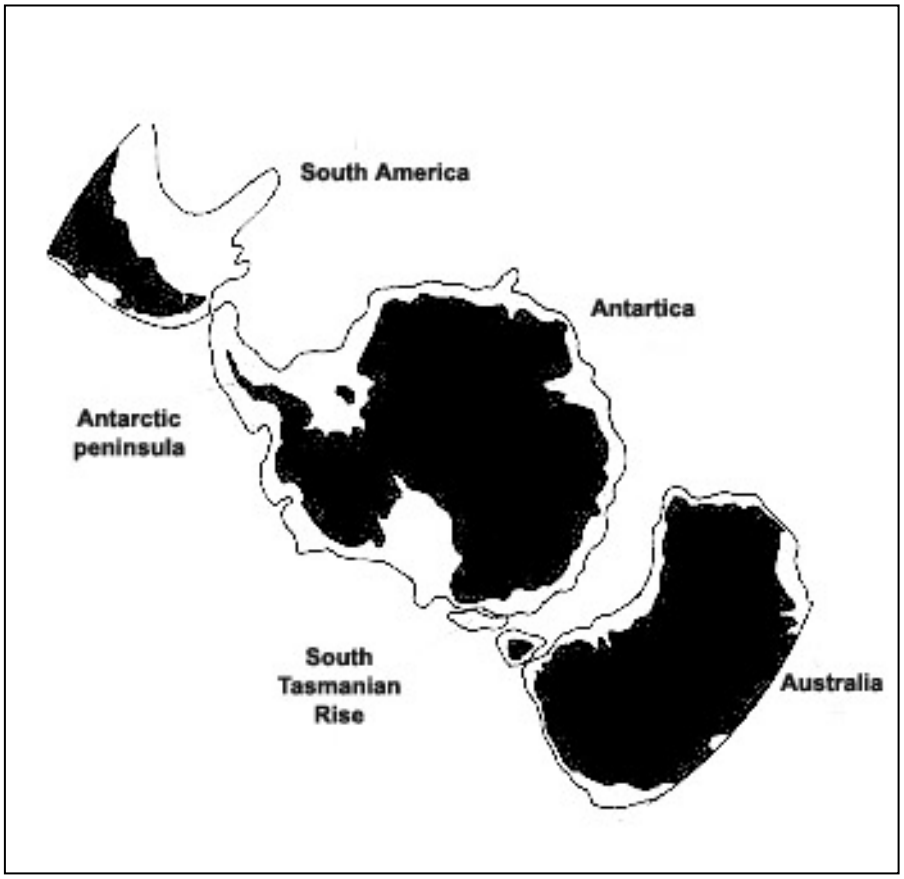


Figure 1.6 The connection between South America, Antarctica and Australia 50 million years ago [138].

there are approximately 180 species of living marsupials in Australia and New Guinea, 78 in South America and 1 in North America [136].

1.3.1 The classification of Marsupials

As discussed previously, classification is the grouping of species into a hierarchy of categories. It may reflect the common usage or the evolutionary relationship of a species and is subject to revision when new information is obtained [136]. A number of criteria have been used to classify marsupials. These include anatomical information such as dentition, geography and diet. Marsupials are separated by dentition into 2 main groups, polyprotodonts (having many teeth and always three pairs of incisors on the lower jaw) and diprotodonts (having fewer teeth and only a single pair of long, strong, pointed incisors in the lower jaw). Geographically, they are separated into two cohorts: the Ameridelphia, restricted to the Americas, and the Australidephia, comprising all species found in Australia, New Guinea and nearby islands. Marsupials can be grouped into three types based on their dietary categories and gastrointestinal tract specialisation, carnivores/ insectivores, omnivores and herbivores. In general all carnivores are polyprotodonts. Omnivores are also characterised by polyprotodont dentition and all herbivorous marsupials are diprotodonts.

Comparative anatomical studies of marsupials have been going on since the mid 1800s, however at that time these studies were carried out to understand the structure and function of these animals rather than marsupial phylogeny. Since the 1960s our understanding of marsupial phylogeny has increased due to the improved knowledge of fossil records, new studies of little-examined tissue systems such as the brain structure, tarsal bone morphology and sperm ultrastructure, and advances in molecular systematics [139]. Comprehensive examination of the relationships between Australian marsupials began with the serological studies of Kirsch in 1968 and 1977 [140, 141], followed by micro-complement fixation by Maxson [142], and Baverstock *et al.* (1990) [reviewed by Kirsch *et al.* [143]. However the most critical information has been based on molecular studies of amino acid sequences by Air *et al.* in 1971 [reviewed by

Kirsch *et al.* [143]. In 1997 Kirsch *et al.* examined past DNA-hybridisation studies of marsupials and presented a reanalysis of the data [143]. The 102-taxon tree presented in Kirsch's study is the most species-rich phylogeny of marsupials ever constructed on the basis of a single data type. From this data Kirsch suggested an approximate time-scale for the radiation of marsupials, and offered some recommendations for the classification of Marsupialia based on both molecular and anatomical features. The 102- taxon tree (101 marsupial taxa and an out group placental mammal) as shown in Figure 1.7, indicates that marsupials can be separated into 13 presumptive monophyletic groups of taxa. Groups 1-8 and 10-11 refer to Australian marsupials, groups 9 and 12-13 refer to American marsupials, with group 14 the out-group. A simplified tree illustrated by pictures is shown in Figure 1.8. This tree suggests that no existing marsupial lineage originated before the late Cretaceous period and that all existing marsupials, together with most South American and all Australian fossils, should be recognised as a monophyletic group. This detailed study indicated that the misleading 'Australia' v 'American' distinction should be abandoned, even as a geographic convenience.

The marsupial classification used in this thesis (shown in Table 1.5) is based on the latest information obtained from anatomical studies, geographic distribution and molecular analysis. This classification was simplified from the classification of Hume [130].

Figure 1.7 DNA-hybridisation relationships of extant marsupial families [143]

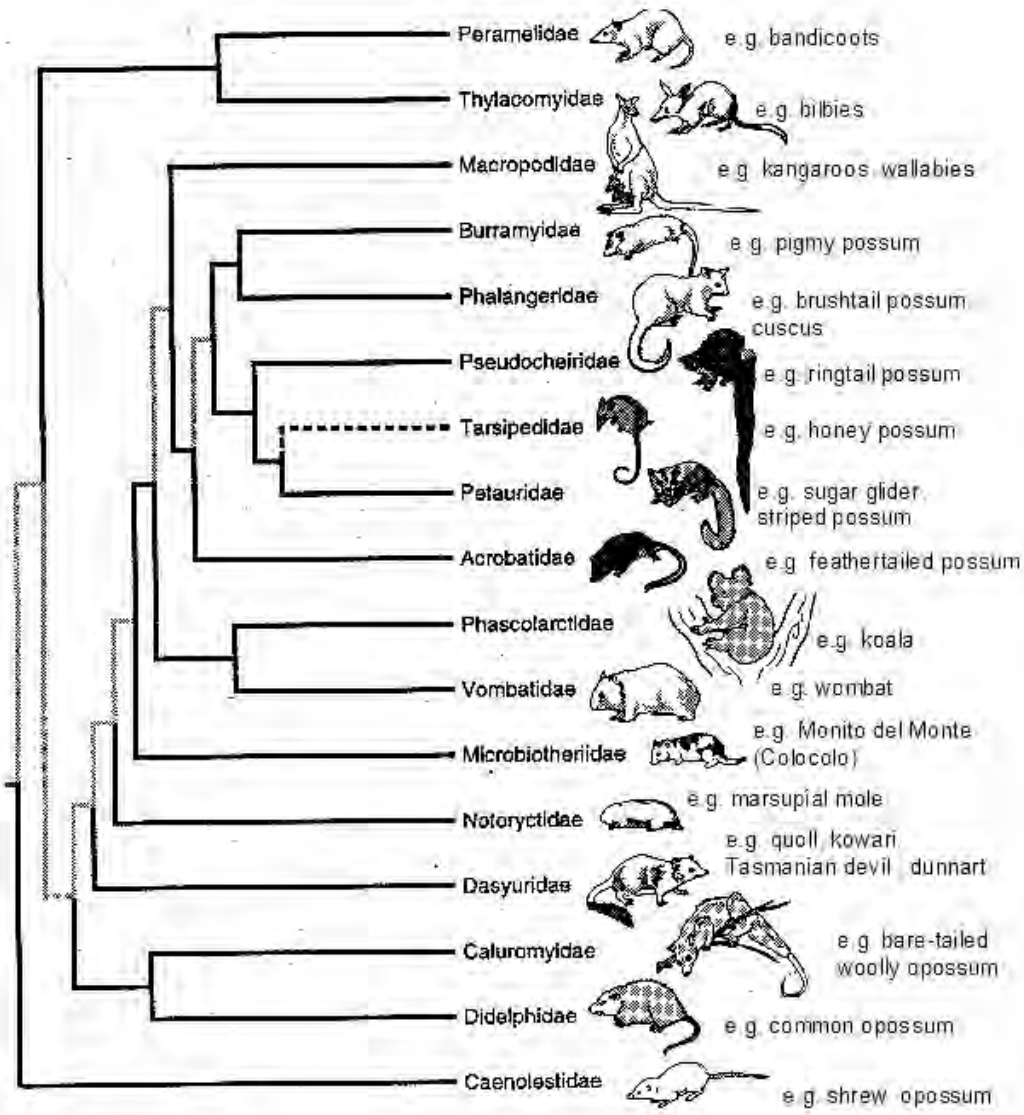


Figure 1.8 A simplified tree of the DNA–hybridisation relationships of representatives of the extant marsupial families abstracted from Figure 1.7. The common names of animal species were taken from the classification of Strahan [136].

Class	**Mammalia**		
Subclass	Metatheria (Marsupialia)		
Cohort	**Australidelphia**	**Cohort**	**Ameridelphia**
Order	Microbiotheria	Order	Didelphimorphia
Family	Microbiotheriidae -Monito del Monte	Family	Didelphidae -bare-tailed woolly opossum -Common opossum
Order	Dasyurida	Order	Paucituberculata
Family	Dasyuridae -kultarr, dunnart, antechinus, **Eastern quoll,** kowari, **Tasmanian devil**	Family	Caenolestidae -rat opossum -shrew opossum
Family	Myrmecobiidae -numbat		
Family	Thylacinidae -Tasmanian tiger		
Order	Notoryctemorphia		
Family	Notoryctidae -marsupial mole		
Order	Peramelina		
Family	Peramelidae -**bandicoot**, bilby		
Family	Peroryctidae -echymipera, rufous spiny bandicoot		
Order	Diprotodontia		
Family	Phascolarctidae -**koala**		
Family	Vombatidae **-wombat**		
Family	Burramyidae -pygmy possum		
Family	Petauridae -striped possum, sugar glider		
Family	Pseudocheiridae -**ringtail possum**, greater glider		
Family	Tarsipedidae -honey possum		
Family	Acrobatidae -feather tail possum, feather tail glider		
Family	Phalangeridae -**brushtail possum**, cuscus		
Family	Potoroidae -rat kangaroo, bettong, potoroo		
Family	Macropodidae -tree kangaroo, dorcopsis, wallaby, hare-wallaby wallaroo, **Eastern grey kangaroo**, euro, quokka, pademelon		

Table 1.5 Classification of Mammals based on the classification of Hume [130]. The animals available for study in this thesis are shown in bold.

1.3.2 The digestive system of Australian marsupials

The classification of marsupials used in this thesis is also based on their dietary requirements and the structure of the gastrointestinal tract. This is because the natural niche of *Helicobacter* spp. is the gastrointestinal tract and the structure of the gastrointestinal tract dictates the location of the microbiota and the composition of the microbiota [8]. As mentioned previously, Marsupials can be simply divided into three feeding types, carnivores, omnivores and herbivores [9]. The characteristics of gastrointestinal tract structure, function and forage strategies of each type of marsupials are outlined below:

1.3.2.1 Carnivorous marsupials

Marsupial carnivores comprise members the orders Microbiotheriidae, Dasyurida, Notoryctemorphia, Paucituberculata and some members of the order Didelphimorphia [9, 144]. Carnivorous marsupials mainly eat animal material; their diet is characterised by a high content of protein, water, vitamins and minerals, a variable amount of fat and a low level of carbohydrate. Carnivores are distinguished from nearly all omnivores and herbivores not only by their dentition, but also by the morphology of their gastrointestinal tracts. The digestive system of a carnivorous marsupial is very simple. The stomach is basic and in those species that consume large prey it may be quite voluminous. The small intestine is short, as is the large intestine. Although the small intestine is short, it dominates the digestive tract, constituting approximately 87% of the total GIT length. The colon is very short, constituting approximately 7% of the GIT length. No Australian marsupial carnivore has a caecum [144]. Examples of the gastrointestinal tract of carnivorous marsupials are shown in Figure 1.9 A.

1.3.2.2 Omnivorous marsupials

Omnivorous marsupials comprise members of the order Peramelina and some members of the orders Diprotodontia and Didelphimorphia. Omnivorous marsupials ingest plants and/ or fungal materials as well as animal materials. A feature of many omnivorous marsupials is their ability to switch between different animal and plant food resources as the availability of these foods change between seasons. The digestive system of omnivorous marsupials is more complex than that of carnivores. As omnivores consume more indigestible

material than carnivores, they require more lubrication to protect the gut lining from physical trauma during the passage of plant residues. Plant residues provide an additional substrate for bacteria and other microorganisms resident in the gut. It is assumed that in a number of species these microorganisms degrade the structural polysaccharides of gums and chitin of insect exoskeletons. In comparison to carnivores the digestive tract of omnivores has a caecum and a longer small intestinal and a longer and larger colon. The small intestine makes up 63% of the total length of the GIT and the colon 26% of the total tract length. The caecum is moderate in size being 7% of the total length [145]. The gastrointestinal tract of the long nosed bandicoot is shown in Figure 1.9 B.

1.3.2.3 Herbivorous marsupials

Herbivorous marsupials are plant eaters and comprise most members of the order Diprotodontia. The digestive system of herbivorous marsupials is the most complex. This can conveniently be divided into two groups, the foregut fermenters and the hindgut fermenters. The term ‘foregut’ refers to oesophagus and stomach. The term ‘hindgut’ refers to the entire large intestine including the caecum, colon and rectum.

Foregut fermenters comprise members of the family Potoroidae and Macropodidae. In foregut fermenters, food is retained and subjected to microbial attack in the forestomach, an expanded area of the stomach proximal to the site of hydrochloric acid secretion. Plants toxins such as alkaloids, but not phenolics or terpenes, are degraded by forestomach bacteria. Only hindgut fermenters are able to utilise *Eucalyptus* leaves which contain both phenolics and terpenes. Potoroid and Macropodid marsupials are two of several groups of mammalian foregut fermenters which include ruminants, camelids, hippos, sloths and colobine monkeys. The forestomach can be divided into an enlarged forestomach and hindstomach. The enlarged forestomach can be divided into sacciform and tubiform regions where the microbial fermentation of ingested food occurs. The hindstomach is the site where hydrochloric acid and pepsinogen are secreted. The gastrointestinal tract of an Eastern grey kangaroo, an example of a forestomach fermenter, is shown in Figure 1.9 C.

In hindgut fermenters, the principal site of microbial fermentation is either the caecum or the proximal colon. The hindgut serves as the final site for storage of digesta, retrieval of dietary and endogenous electrolytes and water [9]. Hindgut fermenters have a very simple and small stomach. The arboreal folivores (animals that live in trees and eat tree leaves) with the exception of the tree kangaroo, are caecum fermenters. These include the families Phalangeridae (brushtail possum), Pseudocheiridae (ringtail possum) and Phascolarctidae (koala). While there can be some microbial fermentation in the proximal colon, in the majority of caecum fermenters microbial fermentation is almost always confined to the caecum. The koala's small intestine is relatively short, being only 29% of the total intestine. It is the main site of energy absorption in this animal. The caecum is enormous in size being approximately 1.3 m, 23% of the total intestinal length. The ringtail possum has a short small intestine but a large, strong haustrated (a non-permanent sacculation) caecum. The tissue mass of the caecum of a brushtail possum is about half the size of the caecum of a ringtail possum. It has been suggested that there is more emphasis on the enzymatic digestion of cell contents in the small intestine and less in the caecum of the brushtail possum [146]. The gastrointestinal tract of a koala, ringtail possum and brushtail possum are shown in Figures 1.9 D, E and F respectively.

The only marsupial colon fermenter, the wombat, is a member of the family Vombatidae. Other mammalian colon fermenters include the equids (horse, donkey, zebra), tapirs, rhinos, elephants and sirenians (dugong and manatees). The wombat has a very simple and small stomach. The small intestine is 36% of the total GIT length and the caecum is extremely small [147]. The haustrated colon is about 60% of total tract length. The gastrointestinal tract of a wombat is shown in Figure 1.9 G.

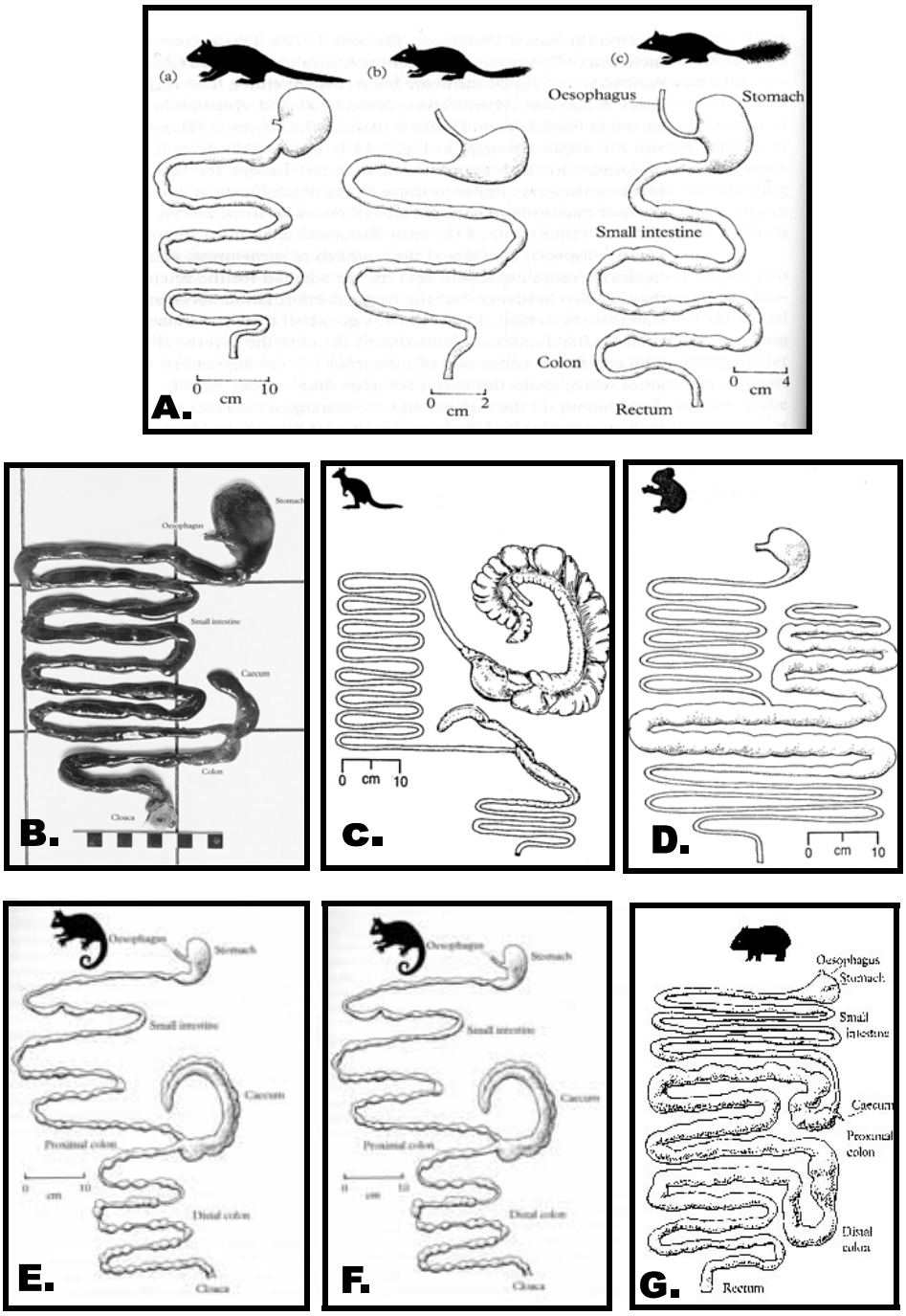


Figure 1.9 The gastrointestinal tract (GIT) of carnivorous, omnivorous, and herbivorous marsupials [9, 144, 145, 146]

(A) = the GIT of a number of carnivorous marsupials, a =spotted-tail quoll, b =kowari, c = brush-tail phascogale, (B) = the GIT of an omnivorous marsupial, the long nosed bandicoot, (C) = the GIT of a foregut fermenter herbivorous marsupial, the Eastern grey kangaroo, (D) = the GIT of a colon fermenter herbivorous marsupial, the koala, (E) = the GIT of a caecum fermenter herbivorous marsupial, the brushtail possum, (F) = the GIT of a caecum fermenter herbivorous marsupial, the ringtail possum, (G) = the GIT of a caecum fermenter herbivorous marsupial, the wombat.

1.3.3 Current knowledge of the gastrointestinal microbial community in marsupials

To date very few studies have examined the number and types of microorganisms in the gastrointestinal tract of Australian marsupials. The microorganisms belonging to the family *Enterobacteriaceae* were investigated by Gordon *et al.* In this study faeces of 642 mammalian hosts, representing 16 families and 79 species were collected in Australia [148, 149]. The animals studied included Eutherian mammals (bats, and rodents), Monotremes (platypus and echidna), as well as a diverse array of marsupial species. Carnivorous marsupials studied included planigales, dunnarts, antechinus, quolls, and the Tasmanian devil. The only omnivorous marsupials studied were bandicoots. The herbivorous marsupials included koalas, wombats, ringtail possums, gliders, brushtail possums, pygmy possums, bettong, potoroos, rat–kangaroos, wallabies and kangaroos. In this study the enteric bacterial community of bats (insectivorous animal) was found to be very different from that in other families. The enteric bacterial community of carnivorous marsupials were found to be similar to that of rodents but different from herbivorous marsupials, all of which were found to be quite similar. The enteric microbiota of carnivores and insectivores were found to be the most diverse. By contrast the enteric microbiota of herbivorous marsupials, which are exclusively vegetarian, exhibited a low level of diversity. The composition of enteric bacteria was found to be determined by both the taxonomic family to which the host belonged, and the geographical area from which the host was collected. The authors suggested that these differences in enteric communities and the distribution of a particular bacterial species, may be a result of many factors including, differences in the normal core temperature (30°C for monotremes, 35°C for marsupials and 38°C for eutherian mammals), differences in gut morphology and diet amongst the host species.

Knowledge regarding the mucosa-associated bacterial communities in different parts of the GIT of marsupials is very limited. This knowledge has been derived indirectly from studies of the digestion and metabolism of marsupials' digesta.

For example, in a study of the morphology and physiology of koalas in 1978, McKenzie examined the caecum epithelium using light, scanning and transmission electron microscopy [150]. McKenzie observed that the lumen of the koala was lined by a pseudostratified columnar epithelium. Various kinds of bacteria could be seen arranged perpendicular to and adhering to the luminal borders of the epithelial cells. Both Gram-positive and Gram-negative bacteria were seen in palisade arrays in the mucus layer. Scanning EM revealed that the luminal surface of the caecum was completely covered by a mat of bacteria, predominately bacilli but with some cocci. No spirochaetes or fusiform bacilli were seen in the koala studied. It was suggested that the close association of bacteria with caecal epithelial absorptive cells could aid absorption of the products of microbial fermentation of the eucalypt leaf material. Further investigations of enteric bacteria from the koala have been carried out by Osawa. The koala is a folivore, feeding almost exclusively on the *Eucalyptus* leaves, which are known to have a high concentration of tannins. Tannins are a diverse group of soluble phenolic compounds that form a chemical complex with proteins called tannin-protein complex (T-PC). They are resistant to degradation in the gut of mammals. Osawa first described microbial degradation of T-PC in 1990, for a strain of S*treptococcus bovis* biotype I isolated from the faeces of koala [151]. In later studies, tannin-protein complex-degrading enterobacteria (T-PCDE) were isolated from the faeces and from a layer of bacteria attached to the caecal epithelial of a koala. Osawa suggested that these bacteria played a more important role than *S. bovis* in the koala's ability to obtain dietary protein from tannin-rich eucalypt leaves, the food source of koalas [152, 153].

Microscopic investigations of the digesta derived from eucalypt leaves obtained from the caecum of ringtail possums have also been described. O'Brien *et al.* (1986) observed a large population of bacteria that were associated with the eucalypt leaf fragments and appeared to attack selected cell-walls and protoplasts [154]. A year later, Foley *et al.* observed extensive numbers of bacteria attaching to lignified tissues of plant fragments in the caecum and faeces of brushtail possums [155]. The attachment of bacteria to the forestomach epithelial surface of quokkas and kangaroos were also observed by Hume *et al.* (1999) [146].

Compared to the knowledge obtained from human and placental mammals, very little is known regarding the mucosa-associated bacterial communities in different parts of the GIT of marsupials. As mentioned above, there have been very few studies investigating the bacterial composition of the GIT in marsupials. The bacterial composition of the mucosa-associated microorganisms in the GIT has mainly been examined microscopically. Cultivation studies have focused on selective studies such as those of tannin-protein complex-degrading bacteria in koalas. Mucosa-associated microorganisms are regarded as constituting part of the indigenous microbiota of an animal species and thus important to the well being of the host as discussed previously. To date there has been no comprehensive investigation of the mucosa-associated microorganisms of Australian marsupials. Thus it seemed timely to conduct a systematic investigation of the mucosa-associated microorganisms in Australian marsupials utilising recent advances in culture methodologies and molecular detection techniques. These studies have the potential to provide important information relating to the bacteriology, ecology and bacterial-host relationship of Australian marsupials. Furthermore the investigation of the presence of *Helicobacter* species in Marsupials has the potential to add interesting insights into the bacterial evolution as well as the co-evolution of *Helicobacter* species and their host.

Hypothesis to be tested

The mucus lining of the gastrointestinal tract of Australian marsupial species are colonised with large populations of spiral/helical and fusiform-shaped bacteria, many of which belong to the genus *Helicobacter.* Furthermore these bacteria are likely to constitute unique *Helicobacter* species.

Specific aims:

- To culture spiral/helical and fusiform-shaped bacteria from specific regions of the gastrointestinal tract of different Australian marsupials.

- To identify and characterise the spiral/helical and fusiform isolates, in particular *Helicobacter* species, both phenotypically and genetically.

- To conduct a phylogenetic analysis of the marsupial *Helicobacter* isolates in relation to other *Helicobacter* species.

- To undertake a systematic study of the location of *Helicobacter* species within the gastrointestinal tract of marsupials.

Chapter 2

Materials and Methods

2.1 Bacterial culture Media

2.1.1 Brain heart Infusion broth (BHI)

Brain heart infusion (BHI) powder (Oxoid, Basingstoke, UK)	3.7 g
Distilled water	100 ml

The BHI powder was added to the water and then sterilised in an autoclave at 121°C for 15 minutes.

2.1.2 Brain heart Infusion–Glycerol medium (BHIG)

Brain heart infusion broth	100 ml
Glycerol	31 g

Brain heart infusion broth and glycerol were autoclaved separately at 121°C for 15 minutes. Once cooled the BHI broth was aseptically added to the glycerol and mixed and stored at 4°C.

2.1.3 Horse blood agar (HBA)

Blood Agar Base No. 2 (Oxoid)	18 g
Sterile defibrinated horse blood (Oxoid)	25 mL
Amphotericin (Fungizone, E. R. Squibb & Sons, Princeton, NJ)	2.5 μg/mL
Distilled water	500 mL

2.1.4 Campylobacter selective agar (CSA)

HBA	~525 mL
Skirrow's selective supplement	
Polymyxin B (Sigma, St. Lois, Mo)	2.5 μg/mL
Vancomycin (Eli Lilly &Co, Australia)	10 μg/mL
Trimethoprim (Sigma)	5 μg/mL

HBA and CSA were prepared by suspending Blood Agar Base No. 2 in distilled water and sterilising by autoclaving at 121°C for 15 minutes. The agar was then allowed to cool to 47°C after which sterile defibrinated horse blood, Fungizone

and, in the case of CSA, Skirrow's selective supplement was added. The media was then mixed and approximately 25 mL poured into sterile petri dishes. The plates were then allowed to set for approximately 2 hours after which they were wrapped in polyethylene food wrap to prevent moisture loss. Plates were stored upright at 4°C for up to 2 weeks.

2.2 The collection of specimens

Gastrointestinal tract (GIT) samples were collected from thirty-two marsupials. These animals were deceased animals from Taronga Zoo and wild animals delivered to the Zoo, which had had to be put down for compassionate reasons (see Table 4.2, 5.2 and 6.2). These animals consisted of:

- Eleven brush tail possums (*Trichosurus vulpecula*),
- Ten ringtail possums (*Pseudocheirus peregrinus*),
- Three koalas *(Phascolarctos cinereus cinereus*),
- Two wombats (*Vombatus ursinus*),
- One Eastern grey kangaroo (*Macropus giganteus*),
- One eastern quoll (*Dasyurus viverrinus*),
- One Tasmanian devil (*Sarcophilus harrisii*),
- Three long nosed bandicoots (*Perameles nasuta).*

The animals used in this study, classified by their dietary preference, are shown in Figure 2.1. For each animal, three samples of tissue from each of the following sites were collected: the liver, stomach, mid ileum, ileum at 3 cm above the caecum (3-ileum), caecum, colon and rectum. The first sample from a particular location was frozen at -70°C for DNA extraction. The second sample was frozen in 1 mL of BHIG and kept at -70°C until cultured. The third sample was fixed in formalin for histology. All specimens, except those from the kangaroo were collected by Dr Karrie Rose, Pathologist, Veterinary & Quarantine Centre, Taronga Zoo, Sydney. The specimen processing included in this study is shown in Figure 2.2.

2.3 Isolation of *Helicobacter* and other spiral bacteria

2.3.1 Direct culture

Liver samples were aseptically removed and homogenised in BHI broth. A sample of the homogenate was then inoculated onto moist CSA plates and streaked out for single colonies. Gastrointestinal samples were washed by shaking vigorously in physiological saline several times. The mucosa was then scraped off using a no. 11 surgical scalpel blade. These scrapings were then inoculated onto moist CSA plates as described for the liver samples. Plates were incubated, lids uppermost, in an anaerobic jar (HP 11, Oxoid) containing a microaerobic gas generating kit (BR 56, Oxoid) or with an anaerobic gas generating kit (BR 38, Oxoid) at 37°C. The plates were checked for growth every 3 to 4 days after inoculation and re-incubated for up to 10 days.

2.3.2 Culture using selective filtration

Nitrocellulose membrane filters with a pore size of 0.65 μm (Millipore, Bedford, MA) were placed on the surface of moist HBA plates. The gastrointestinal mucus scrapings or homogenised livers were inoculated onto the centre of membranes and the plates were then placed in a CO_2 incubator set at 37°C, 10% CO_2 and 95% humidity for 2 hours. After this time the membranes were removed and the plates were incubated in anaerobic jars as described in section 2.3.1.

For both the direct and filter culture techniques, the suspected *Helicobacter spp* were subcultured onto HBA or CSA to obtain pure isolates. Bacterial selection for subculture was based on colony morphology and microscopic appearance. The selected colonies appeared as a thin watery film or translucent colonies, 0.5-2 mm in diameter. *Helicobacter* species are generally spiral/helical to curved shape or tapered rods when viewed by phase contrast microscopy. The isolation protocol is illustrated in Figure 2.3. Pure isolates of the different bacteria were stored in BHIG medium in liquid nitrogen for further identification.

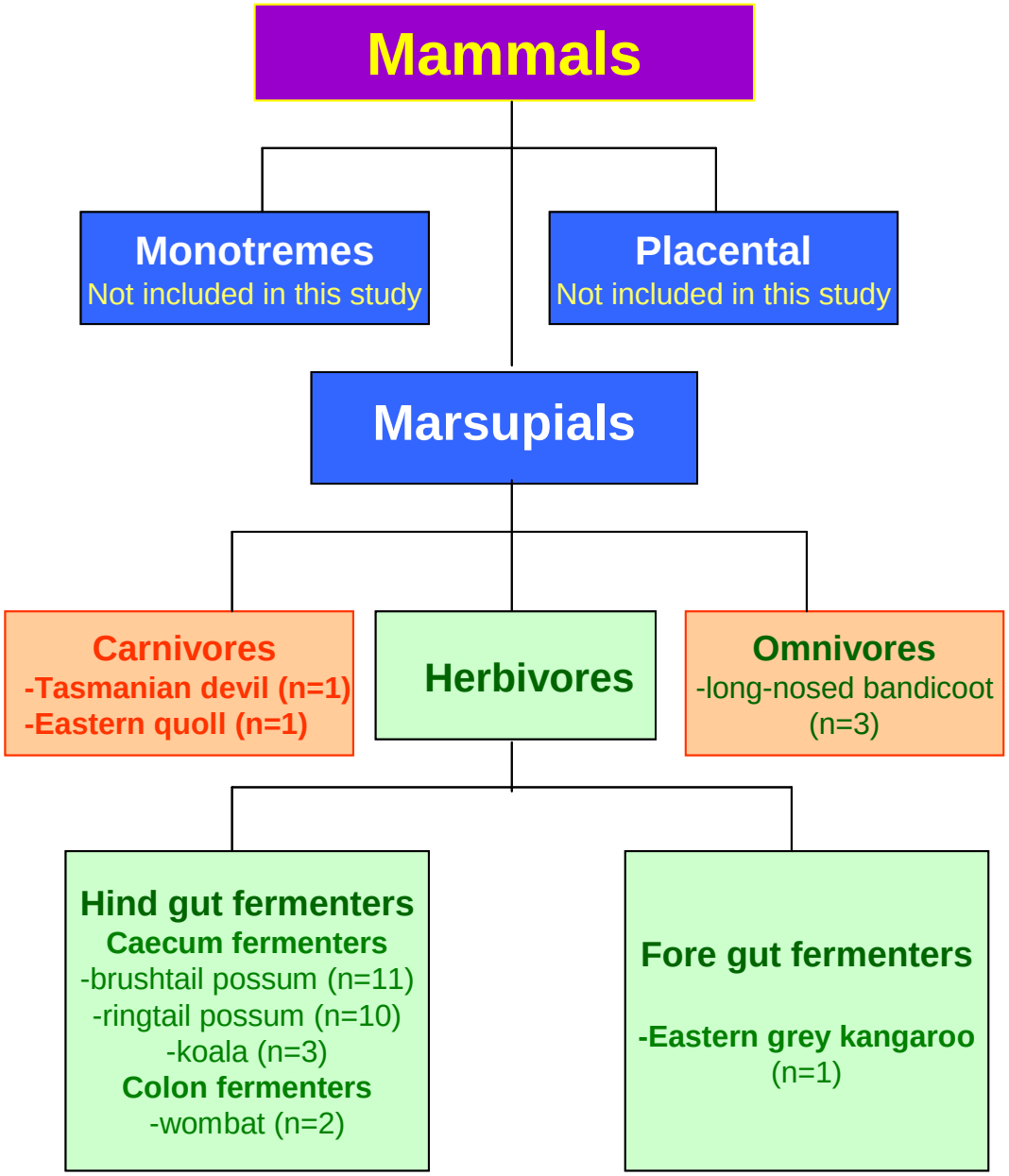


Figure 2.1 Animal samples used in this study

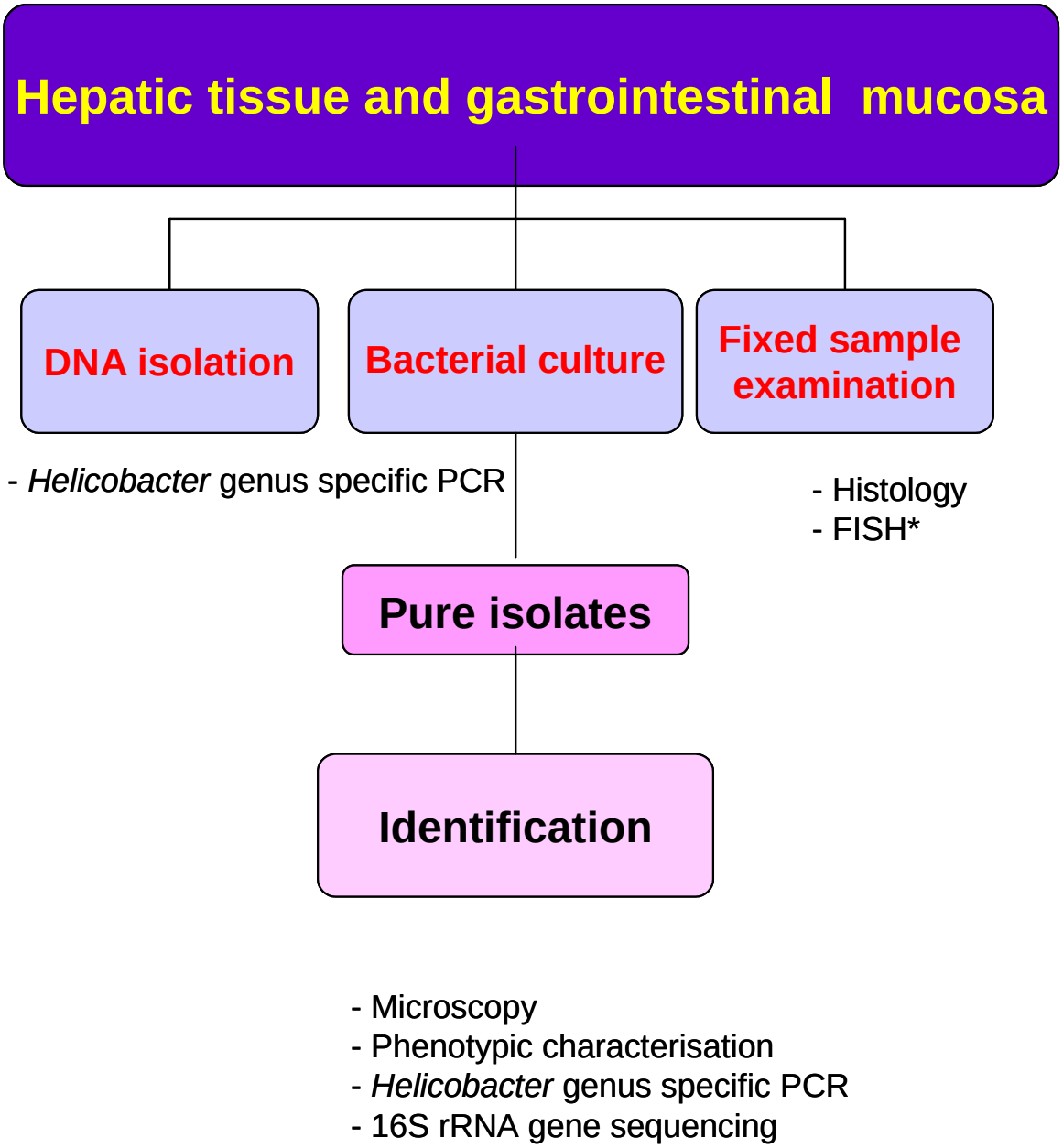


Figure 2.2 Specimen processing

**FISH = Fluorescent *in situ* hybridisation (Chapter 3)

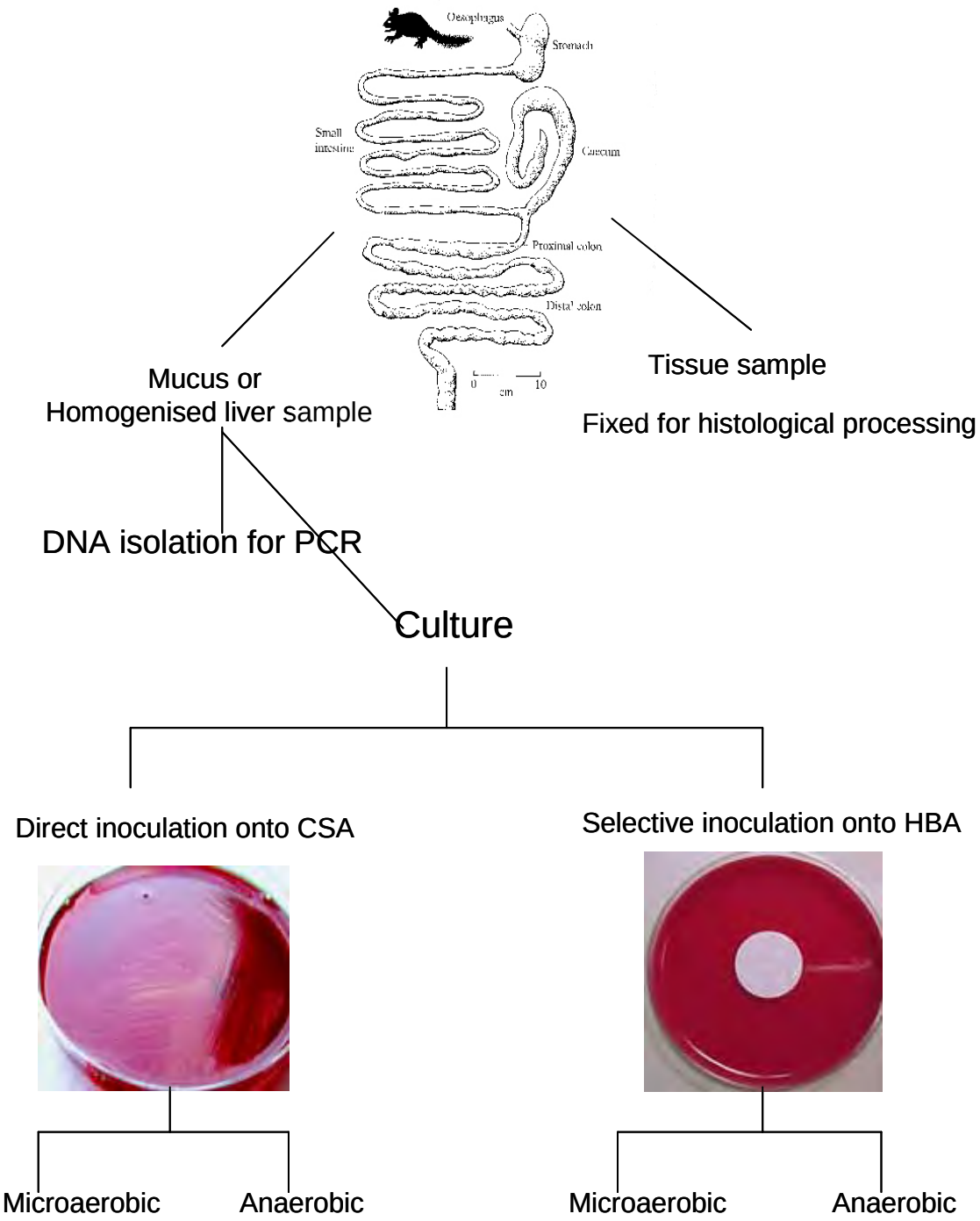


Figure 2.3 The isolation protocol for spiral bacteria

2.4 Electron microscopy

Freshly grown bacteria from HBA plates were mixed with a drop of sterile water on a clean microscope slide to obtain a slightly turbid suspension. A drop of 2% uranyl acetate stain was then added to the bacterial suspension and mixed. A collodion/carbon coated grid (483 or 400 mesh) was then touched onto the surface of the bacteria/stain suspension with the filmed side (dull side) of the grid face down and left for approximately 30 seconds. Excess stain suspension was absorbed with a filter paper wedge and the grid left to dry. The stained grid was then viewed by transmission electron microscopy (H7000-Hitachi, Tokyo, Japan).

2.5 Biochemical testing of bacterial isolates

2.5.1 Rapid urease

Two to three drops of urease reagent was placed into a well of a microtitre tray. A loop full of bacteria from a culture plate was inoculated into the well. The presence of the enzyme urease was indicated by a colour change in the medium from yellowish orange to dark red due to the hydrolysis of urea and the liberation of ammonia.

2.5.1.1 Urease reagent

Urea	2 g
Phenol red (0.5% w/v)	10 mL
$Na_2HPO_4.12\ H_2O$	0.157 g
$Na_2HPO_4.2\ H_2O$	0.08 g
NaN_3 (0.02 % w/v)	0.02 g
Distilled water to	100 mL
Adjust to pH 6.3-6.5	

2.5.2 Catalase

Bacteria were suspended in a drop of 10% (v/v) H_2O_2 on a glass slide. Rapid formation of bubbles indicated liberation of O_2 and the presence of the catalase enzyme.

2.5.3 Oxidase

A filter paper strip, moistened with a drop of Oxidase Reagent (1% (w/v) tetra-methyl-p-phenylene-diamine di-hydrochloride) (DIFCO, Becton Dickinson and Company) was inoculated with a loop full of bacteria. The indophenol oxidase enzyme oxidises the phenylene diamine to form indophenol. Bacteria were designated as positive if a dark purple colour appeared due to the oxidation of phenylene diamine by indophenol oxidase.

2.5.4 API-Campy Identification System

Biochemical tests for nitrate reduction and gamma-glutamyl transferase were conducted using a commercial kit, API Campy identification system for *Campylobacter* species (bioMurieux, Marcy-l' Etoile, France) according to the manufacturer's protocol. These tests were conducted to determine the presence of a range of enzymes, not for identification purposes. Briefly, the bacterial culture was suspended in 0.85 % NaCl to a turbidity equivalent of McFarland 6. The suspension was then distributed into the wells on the test strip and incubated for 24 hours at 35-37°C in aerobic conditions. The metabolic end products produced during the incubation period were revealed through colour changes, which were either spontaneous or apparent after the addition of reagents. The reactions were read visually and interpreted according to the reading table provided.

2.5.5 Indoxyl acetate hydrolysis

The indoxyl acetate hydrolysis test was performed using the disk method of Mills and Gherna with slight modifications [156]. Indoxyl acetate differential disks were prepared by placing sterile disks into a dark coloured bottle containing 0.25 g of indoxyl acetate (Sigma) dissolved in 2.5 ml of acetone. Saturated disks were placed in a glass petri dish and allowed to air dry away from direct light. A dried disk was then placed on a glass microscope slide and moistened with 1 to 2 drops of sterile distilled water. A heavy inoculum of the test culture was then smeared onto the disk and this was observed for up to 20 min at room temperature for the development of a blue colour. The test was considered negative if no colour change occurred within 20 minutes.

2.5.6 Alkaline phosphatase

The alkaline phosphatase test was performed using Rosco Diagnostic tablets (#55921, DUTEC Diagnostics A/S ROSCO-2630TAASTRUP, Denmark). This tablet contains the chromogenic substrate: 4-nitrophenyl phosphatebis (2-amino-2-ethyl-1, 3-propandiol) salt that, in the presence of alkaline phosphatase, releases free 4-nitrophenol (yellow colour). A dense bacterial suspension of the strain to be tested was prepared to a turbidity equivalent of McFarland 4 with 0.25 mL saline in a tube. One Alkaline phosphatase diagnostic tablet was added to the tube and incubated at 35-37°C for 4 hours. The test was considered positive if a strong yellow colour developed. A negative result appeared as slight yellow to colourless.

2.5.7 Hippurate hydrolysis

The hippurate hydrolysis test was performed using Rosco Diagnostic tablets (#56721, DUTEC Diagnostics). This tablet contains sodium hippurate, which is split into benzoic acid and glycine by the action of the hippurate hydrolase. The glycine product was detected using ninhydrin reagent which deaminates glycine and released ammonia to react with residual ninhydrin to form a purple colour.

A dense bacterial suspension of the strain to be tested was prepared to a turbidity equivalent of McFarland 4 in 0.25 mL saline. One diagnostic tablet was added to the tube and incubated at 35-37°C for 4 hours. After incubation 5 drops of 3.5% Ninhydrin solution was added after which the tube was closed and re-incubated for 10 minutes at 35-37°C. The result was then read within 5 minutes. The test was considered positive if a deep purple-blue colour developed. A negative result appeared light yellow to colourless or if there was only a faint tinge of purple.

2.6 Susceptibility to antimicrobial agents

Susceptibility to antimicrobial agents was determined by disk diffusion. The *Helicobacter* species obtained in this study were fastidious and only grew well on HBA plates by streaking the entire surface of the plate directly. Attempts to

standardise the inoculum by inoculating the plate with a known concentration of bacterial cells in liquid broth were not successful. To overcome this, the surface of a HBA plate was streaked with an inoculum of bacteria approximately equivalent to a standard inoculum (10^8-10^9 cfu/mL) to ensure even bacterial growth. Individual susceptibility disks containing nalidixic acid (30 μg, NA 30, Oxoid), cephalothin (30 μg, KF 30, Oxoid) and metronidazole (5 μg, MTZ 5, Oxoid) were placed onto the agar surfaces. The zone of inhibition was measured for each strain. Sensitivity or resistance to above antimicrobial agents was determined. Strains were determined as sensitive if there was a zone of inhibition of 2 cm or more and resistant if there was no zone or a zone less than 2 cm.

2.7 Histology

Samples were fixed in 10% buffered formalin and then dehydrated and embedded in paraffin wax using standard techniques. Four-micron sections were cut and stained using a modified Steiner silver stain and a hematoxylin & eosin (H & E) stain. Paraffin embedding and staining was carried out by the School of Pathology, UNSW.

The silver stained sections were examined for the presences of spiral/helical shaped bacteria by the author. The H&E stained sections were examined for histopathological changes by Dr. Karrie Rose, Pathologist, Veterinary & Quarantine Centre, Taronga Zoo.

2.7.1 10% Buffered formalin

40% Formaldehyde	100 ml
$NaH_2PO_4.2H_2O$	4.52g
Na_2HPO_4	6.5g
Distilled water	900 ml

2.8 Preparation of PCR template

DNA from the GIT mucus or liver was extracted using the Phenol chloroform method. DNA from pure bacterial cultures was extracted using either Xanthogenate (XS) buffer [157] or the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN).

2.8.1 Phenol chloroform method

Each homogenised liver or mucus sample was suspended in a 2 mL centrifuge tube containing 900 μL of STE buffer (150 mM NaCl, 10 mM tris pH 8, 1 mM EDTA), 20 μL proteinase K (5 mg/mL), 50 μL/ml 10%SDS and then incubated overnight at 55°C. An equal volume (1 mL) of saturated phenol (Sigma) was then added to the cell lysate. The mixture was mixed and centrifuged at 12,000 rpm for 10 minutes, the top layer of the solution was then transferred into a new tube. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) (Sigma) was then added to the solution in the new tube, mixed and centrifuged at 12,000 rpm for 10 minutes. The top layer was then transferred to a fresh tube and an equal volume of chloroform (Sigma) added. The mixture was then mixed and centrifuged at 12,000 rpm for 10 minutes. The extraction step with chloroform was the repeated one more time. The solution in the top layer was then transferred to a final tube. The DNA in this solution was then precipitated for 10 minutes at room temperature with one-tenth volume of 3 M sodium acetate, pH 5.2 and 1 volume of 100% ethanol after which it was centrifuged at 12,000 rpm for 15 minutes at 4°C. The DNA pellet was washed with approximately 400 μL of 70% ethanol and then dried under vacuum using a DNA Speed Vac Vacuum centrifuge (Savant Instruments Inc., Farmingdale, NY). The dried DNA pellet was dissolved in 100μL TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA) and stored at 4°C.

2.8.2 Cell lysis using Xanthogenate (XS) [157]

Bacterial cultures were harvested and suspended in a minimal volume of TE buffer in a 2 mL microcentrifuge tube. One mL of XS buffer was added to the

tube and this was incubated at 70°C for 30 minutes. The mixture was then incubated on ice for 30 minutes, after which it was centrifuged at 12,000 rpm for 10 minutes. The supernatant was then carefully removed into a new tube. The genomic DNA in the supernatant was precipitated by the addition of 1 mL of isopropanol then incubated at room temperature for 10 minutes. The DNA was pelleted by centrifugation at 12,000 rpm for 20 minutes and the supernatant was discarded. The pellet was washed with 70% ethanol and then dried under vacuum. The dried DNA pellet was dissolved in 200μl TE buffer, pH 8.0, and stored at 4°C.

2.8.2.1 XS buffer

Potassium ethyl xanthogenate (Fluka Chemika, Buchs, Switzerland)	0.5g
4 M Ammonium acetate	10 mL
1 M Tris-HCL pH 8.0	5 mL
0.5 M EDTA pH 8.0	2 mL
20 % (w/v) SDS	2.5 mL
Distilled water to	50 mL

2.8.3 The Puregene DNA isolation (Gentra Systems)

The DNA extraction method was carried out according to the manufacturer's instructions for Gram-negative bacteria. Briefly, bacterial cells were harvested from agar plates and placed in a tube containing 600 μL of cell lysis solution and then incubated at 80°C for 5 minutes. After incubation, the cell lysate was precipitated with protein precipitation solution. The DNA in the supernatant was then precipitated with isopropanol and centrifuged. The DNA pellet was washed with 70% ethanol and dried under vacuum for 10 minutes, and then re-suspended in 100 μL of DNA Hydration solution.

2.9 DNA amplification by Polymerase Chain Reaction (PCR)

DNA from either tissue or a pure bacterial culture was used as template for the *Helicobacter* genus specific or nested PCR. All PCRs were performed in a

reaction volume of 20 μL. Thermal cycling was carried out in a PCR Sprint Temperature Cycling System (Hybaid, Middlesex, UK.) or GeneAmp PCR System 2400 (Perkin Elmer, Emeryville, CA) in 0.2 mL microcentrifuge tubes.

The PCR amplification mixture contained reaction buffer (67 mm Tris, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.45% Triton X-100 and 0.2% gelatin), 2 or 3 mM $MgCL_2$, 1 unit Taq polymerase (Fisher, Perth, Australia), 100μM deoxynucleotide triphosphate (Boehringer), 10 pmol of each oligonucleotide primer and 1 μL of diluted DNA (usually a 1:10 dilution of original sample containing approximately 20-100 ng/μL). The final volume was made up to 20 μL with sterile distilled water. All PCR studies included a “no DNA” negative control reaction to test for the presence of contaminating DNA.

2.9.1 *Helicobacter* genus specific PCR

The PCR cycling conditions for the *Helicobacter* genus specific PCR were initially performed as described by Riley *et al.* using primers H276f and H676r (see Table 2.1) [158]. However, attempts to amplify DNA from the GIT of the wombat from which *Helicobacter* species had been successfully isolated by culture were unsuccessful. Thus this PCR was optimised for amplifying helicobacter DNA from the GIT of the wombat as well as all other marsupials (described in chapter 3). The successful amplification of helicobacter DNA was achieved when the annealing temperature of the reaction was raised from 53°C to 57°C and the $MgCl_2$ concentration increased to 3 mM. The PCR reactions underwent an initial denaturation period at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 5 seconds, annealing at 57°C for 5 seconds and extension at 72°C for 30 seconds followed by a final extension at 72°C for 2 minutes.

2.9.2 Nested PCR

Nested PCR was conducted by amplifying the prokaryote 16S RNA gene using the universal bacterial primers, F27 and R1494 [159, 160]. Reactions in the first round of PCR underwent an initial denaturation period at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 10 seconds, annealing at 50°C for 15 seconds, extension at 72°C for 2 minutes and a final extension at 72°C

for 7 minutes. The PCR product (1:10 dilution) of this first round PCR was then used as a template to amplify *Helicobacter* genus specific DNA in the second round PCR using primers H276f and H676r as above.

2.9.3 Agarose gel electrophoresis and DNA visualisation

Four microlitres of PCR product was added to approximately 1 μl of 10x loading dye (25% glycerol, 0.4% bromphenol blue, 0.4% xylene cyanol) prior to electrophoresis in a 1.5 % agarose gel immersed in TAE buffer (40 mM Tris acetate, 1 mM EDTA). The electrophoresis was run at 75 V for 30 to 45 minutes. Agarose gels were then stained with ethidium bromide and the DNA visualised by UV transillumination. Each gel was photographed using the Gel Doc ™ (Bio-Rad) 2000 Gel Documentation System.

2.10 DNA sequencing

Sequencing of the 16S rRNA gene from pure bacterial cultures isolated from all animals was conducted as outlined below.

2.10.1 DNA Preparation for 16S rRNA sequencing

DNA from pure cultures of *Helicobacter* species cultivated in this study was amplified using the following protocol. Initially the DNA was subjected to a *Helicobacter* genus specific PCR using primers H267f and H676r, as described above. The PCR product from this reaction was then sequenced with primer H267f. A second PCR reaction was conducted if the sequencing data obtained from the *Helicobacter* genus specific PCR product was shown to belong to the *Helicobacter* genus or closely related genera when compared with known sequences from GenBank databases using BLASTN at the National Centre for Biotechnology Information (NCBI) site (hptt://www.ncbi.nlm.nih.gov/BLAST/).

The second PCR reaction utilised the universal bacterial primers F27 and R1494, as described above in Section 2.9.2. The PCR product from this reaction was sequenced using six sequencing primers (F27, R341, F530, F1115, R1220 and R1494) [159] (see Table 2.1 and Figure 2.4).

PCR products were purified from contaminants, including primer-dimers and amplification primers by ethanol precipitation prior to the DNA sequencing application. Briefly, PCR products from several identical reactions (approximately 3 to 5 x 50μl reactions) were combined in a clean 1.5 ml microcentrifuge tube containing two volumes of 80% ethanol and one-tenth volume of 3 M sodium acetate. The solution was mixed, centrifuged at 14000 rpm for 15 min at room temperature and then the pellet dried under vacuum for 15 min. The dried DNA was re-suspended in 12 μl of sterile milli Q water. To determine the amount of DNA to be added for sequencing reaction a 2 μl sample was run on a 1.5% agarose gel.

2.10.2 DNA Sequencing

Purified DNA was directly sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. Briefly, 4 μl of BigDye terminator (BD, PE Applied Biosystems), 1μl of sequencing primer and 1 to 5 μl of purified PCR product were added to a 0.2 ml microcentrifuge tube. Reactions were subjected to thermocycling using the GeneAmp PCR System 2400 (Perkin Elmer) and the following conditions; an initial denaturation period at 96°C for 1 minute followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds and extension at 60°C for 4 minutes.

Completed sequencing reactions were purified by ethanol precipitation to remove unincorporated dye-labelled terminators that can obscure data at the beginning of the sequence. Briefly, the reaction was pipetted into a 1.5 mL microcentrifuge tube containing 8 μl of sterile distilled water, 32 μl of 95% ethanol and 5 μl of 3 M sodium acetate. The solution was then vortexed briefly and left to stand at room temperature for 15 minutes after which it was centrifuged at 14,000 rpm for 20 minutes. The supernatant containing the unincorporated dye terminator was removed completely. Two hundred and fifty microlitres of 70% ethanol was then added to the tubes, vortexed briefly and then centrifuged for 10 minutes at 14,000 rpm. The supernatant was aspirated carefully and then dried in a vacuum centrifuge for 15 minutes.

Sequencing products were separated on an ABIPRISM model 377 DNA sequencer machine and analysed using the Perkin Elmer ABI Prism Sequencing Analysis Software Version 3.3 (PE Biosystems, Foster City, California USA). Sequencing samples were run on 4.25% polyacrylamide gels at 51°C with 1 X TBE Buffer pH 8.3. Blue Dextran/ Formamide (1:5) dye (4 μL) added to the samples, vortexed, spun briefly, then heated for 3 minutes at 95°C, placed on ice and then loaded on the gel. Samples were run for 9 hours at 1,200 volts. Sequencing was conducted by the Automated DNA Analysis Facility, UNSW.

Primer	Sequence (5' to 3')	Tm	References
27F	AGAGTTGATCCTGGCTCAG	60	[159, 160]
1494R	TACGGCTACCTTGTTACGAC	56	[159, 160]
H276f	CTATGACGGGTATCCGGC	58	[158]
H676r	ATTCCACCTACCTCTCCCA	58	[158]
341R	CTGCTGCCTCCCGTAGG	58	[159]
530F	GTGCCAGCAGCCGCGG	58	[159]
1115F	CAACGAGCGCAACCCT	54	[159]
1220R	ATTGTAG(T/C)ACGTGTGTAGC	58-60	[159]

Tm: Theoretical temperature for primer/template disassociation.

Table 2.1 Oligonucleotide primers used for PCR amplification and sequencing reactions

The first PCR product

H276f _____H676r ~374bp

H267f ----------→

-Sequence with primer H276f

The second PCR product

F27_____R1494 ~1.4Kb

F27 ----------→

←----------R341

F530----------→

←----------R1220

F1115----------→

←----------R1494

-Sequencing primers: F27, R341, F530, F1115, R1220, and R1494

Figure 2.4 16S rRNA gene PCR/Sequencing Strategies

2.10.3 Phylogenetic analysis

16S rDNA sequences were assembled using the ABI Prism ™ Sequencing 2.1.1 INHERIT ™f auto-assembler program and the consensus compared with the sequences of other bacteria in the GenBank databases using BLASTN (hptt://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned using the GCG program Pile up, version 8 (Genetics Computer Group, Program manual for the Wisconsin Package, 1994) and the multiple sequences alignment and profile alignment tools in the ClustalX package [161, 162]. Aligned sequences were checked manually and nucleotide positions that contained ambiguities were removed from further analysis. The phylogenetic tree was constructed by the neighbour-joining method of Saitou & Nei [163] and the tree plotted using NJ plot in the ClustalX package. Bootstrap values were obtained from analysis of one thousand re-samplings of the corrected alignment which was created using the program SEQBOOT [164] and analysed using parsimony (DNAPARS) and maximum likelihood (DNAML) algorithm and CONSENSE, all from the PHYLIP package. The level of similarity of sequences was determined using a multiple sequence alignment as input in the HOMOLOGIES program (GCG). All programs in the PHYLIP package used in the sequence manipulation and phylogenetic analyses were accessed via the ECGC extensions to the Wisconsin Package, version 8.1.0, 1996 via the Australian National Genomic Information Service (ANGIS, www.angis.org.au/WebANGIS).

Chapter 3

Development and optimisation of experimental methods for the detection and isolation of spiral and fusiform shaped organisms, in particular *Helicobacter* species, from the gastrointestinal tract of Australian marsupials

3.1 General Introduction

As discussed in chapter 1, spiral and fusiform shaped microorganisms have been observed by microscopy colonising the mucus layer overlying the surface of the digestive tract of many animals. Australian marsupials are a unique group of mammals which have evolved separately to other mammals. The overall goal of this thesis is to show that a large population of spiral and fusiform shaped microorganisms, many of which may belong to the genus *Helicobacter*, can also be found within a similar niche of Australian marsupials. In order to investigate the presence of these microorganisms the first steps of this thesis were to develop suitable methods for the detection and/or isolation of spiral and fusiform shaped bacteria, in particular *Helicobacter* species, from the GIT of Australian marsupials.

To date a range of methods have been used to investigate whether such organisms are present in the GIT of both humans and animals. The most common methods used have been microscopic examination, bacterial cultivation and molecular techniques such as polymerase chain reaction (PCR) and fluorescent *in situ* hybridisation (FISH).

In early studies of the gastrointestinal microbiota of humans and animals, microscopic examination of fresh or fixed gastrointestinal specimens was the principal method used to detect the presence of microorganisms including spiral and fusiform shaped bacteria. Although this approach allows for the observation of these bacteria the identity of such organisms cannot be determined using microscopy alone. Furthermore these morphologically based studies cannot distinguish between the various bacteria constituting the *Helicobacter* genus as

these exhibit a wide range of morphologies which cannot readily be distinguished from members of a number of other genera including *Campylobacter, Spirillum, Spirochaeta* and *Desulfovibrio*. Thus given this paucity of morphologic distinction among bacteria, such simple microscopic methods are useful only for the presumptive detection of organisms.

In microbiology, bacterial culture is considered to be the fundamental method for the identification of microorganisms. Once an organism is isolated in pure culture, it can be phenotypically and genotypically characterised. However in the case of microorganisms colonising the GIT, in particular *Helicobacter* species, obtaining a pure culture of a specific organism is often difficult due to the presence of large numbers of other organisms at this site. This difficulty arises from the fact that *Helicobacter* species are extremely fastidious, requiring a period of three to ten days for bacterial growth to occur. Thus given that the vast majority of normal GIT microbiota multiplies more rapidly than *Helicobacter* species, visualisation and subsequent isolation of these spiral and fusiform organisms is often unsuccessful. Indeed even within the *Helicobacter* genus, a number of species have been characterised that as yet have not been able to be cultured on artificial media, for example "*Helicobacter heilmannii*", "*Candidatus* H. bovis" and "*Candidatus* H. suis".

The advent of molecular biology has revolutionised all fields of microbiology. In particular polymerase chain reaction (PCR) has allowed the detection of microorganisms in mixed populations without the requirement for bacterial culture. Currently a number of PCR methods have been developed for the detection of *H. pylori* in clinical samples. Primers used in such studies have targeted a number of sites including the 16S rRNA gene [165, 166], a 26-kDa species specific antigen (SSA) [167], the urease A subunit (*ureA)* gene [168] and the gene encoding the phosphoglucosamine mutase gene *(glmM)* [169]. To date, the only PCR that has been shown to be capable of detecting all possible *Helicobacter* spp. present in clinical samples are those that target the 16S rRNA gene, due to its universal and conserved nature in all bacteria species. Thus this thesis will rely on a genus specific PCR, that targets a specific region of the

16S rRNA gene of members of the *Helicobacter* genus, to detect these organisms.

While culture and PCR allow the detection of helicobacter species, neither of these methods can predict the location of these organisms within the host. Fluorescent *in situ* hybridisation (FISH), which utilises a combination of microscopic and molecular based methodologies, is a technique which can provide information on the presence, number, morphology and spatial distribution of microorganisms within their natural microhabitat [170]. This technique provides the precision of molecular genetics as well as the visual information that can be derived from microscopy. Microscopic identification of single microbial cells using such techniques was first introduced into bacteriology by Giovannoni *et al.* in 1988, who used radioactively labelled rRNA-directed oligonucleotide probes in *in situ* hybridisation (ISH) [171]. More recently radioactively labelled probes have been supplanted by fluorescently labelled probes, non-isotopic dyes, which are safer and provide better resolution [172]. The use of fluorescently labelled probes was extended by Amann *et al.* (1990) who has demonstrated that most cells in complex bacterial communities, such as the ruminal environment, are permeable to short-oligonucleotide probes [173]. Furthermore Amann *et al.* developed the EUB338 probe, the universal probe most commonly used to detect all eubacterial cells. This probe has been widely used as a control probe for the hybridisation reaction in the FISH technique.

FISH has the ability to detect specific nucleic acid sequences by the use of a fluorescently labelled probe that hybridises specifically to a complementary target sequence within intact cells. The availability of this technique has made whole cell-hybridisation with rRNA-targeted probes a useful and suitable tool for determinative, phylogenetic, and environmental studies in microbiology. To date, FISH has been used successfully to rapidly identify many pathogenic bacteria including streptococci, enterococci, staphylococci and Gram-negative rods in clinical samples such as blood [174, 175], sputum and throat swabs [176] and faeces [28, 30]. FISH has also been applied to paraffin sections of fixed tissue samples. For example *Salmonellae* species have been detected in

paraffin sections of liver, colon and lung tissue obtained from experimentally infected mice, as well as from animals with a history of Salmonellosis [177].

While microscopy, cultivation, PCR and FISH all provide important information regarding specific organisms within an ecological niche, no one particular method alone is sufficient for the detection, isolation and localisation of *Helicobacter* species in the GIT. Thus in the current study all the above methods were used.

Thus the aims of the initial part of this thesis were (1) to optimise cultivation strategies for the isolation of spiral and fusiform shaped organisms, in particular *Helicobacter* species, from the GIT of marsupials (2) to optimise a genus specific PCR for the rapid detection of *Helicobacter* species in the GIT of marsupials as well as (3) to develop a FISH technique that would allow the detection and determination of the localisation of *Helicobacter* species within the marsupial GIT.

3.2 Bacterial Cultivation

3.2.1 Introduction

Helicobacter species, including the 23 validated and several unvalidated species, have been successfully isolated from the stomach, lower bowel and faeces of humans and animals (described in Chapter 1). Isolation of these organisms was achieved through the selection of optimal growth conditions, culture media and isolation strategies. The majority of *Helicobacter* species grow at 37^0C, under microaerobic and/or anaerobic conditions. Primary isolation of *Helicobacter* spp. is generally performed using moist solid media supplemented with 5-10% sheep or horse blood, or bovine serum. Base media such as Blood agar base, Columbia agar base, Brucella medium base, Brain heart infusion, Mueller-Hinton and trypticase soy agar have been commonly used [87, 178]. Studies have shown that the yield from culture may be improved if both selective and non-selective agar is used [179]. To suppress the growth of luminal organisms antibiotic supplements have been commonly used. These supplements include:

-Skirrow's (Vancomycin 10 mg/L, Trimethoprim 5 mg/L, and Polymyxin B 2,500IU/L),

-Dent's (Vancomycin 10 mg/L, Trimethoprim 5 mg/L, Cefsulodin 5 mg/L and Amphotericin B 2 mg/L),

-Blaser-Wang's (Vancomycin 10 mg/L, Trimethoprim 5 mg/L, Polymyxin B 2,500IU/L, Cephalothin 15 mg/L, and Amphotericin B 2 mg/L).

Many years of experience in our laboratory of cultivating *Helicobacter* species has shown that while the majority of *Helicobacter* species grow well in the presence of Skirrow's supplement, some *Helicobacter* species, such as *H. pullorum,* are sensitive to Polymyxin B and cannot grow in the presence of Skirrow's supplement [180]. For this reason it was considered essential to always include non-selective agar in isolation attempts. The major problem however in using non-selective media is overgrowth by other microorganisms which may mask the *Helicobacter* species. This can be overcome to some degree by using a filtration method which can eliminate the growth of non-motile organisms. The filtration method was initially developed for the cultivation of

Campylobacter species from faeces [181]. In this technique a membrane filter with a pore size of 0.45 to 0.65 μm is placed onto the surface of an agar plate. Liver and GIT samples are then placed on top of the filter and any motile bacteria can pass through the filter. The filter containing most of the luminal bacteria is then removed and the motile bacteria, including *Helicobacter* species, are then able to grow in the absence of other non-motile luminal bacteria. Optimisation of the cultivation method for *Helicobacter* organisms is described below.

3.2.2 Bacterial Cultivation methods and Microscopic examination

Cultivation strategies for the isolation of spiral and fusiform shaped organisms, in particular *Helicobacter* species, from marsupials were initially applied to the liver and GIT samples obtained from the first 2 brushtail possums (BTP1 and 2) and a wombat (wombat 1). Prior to examination and cultivation of these samples, luminal organisms were reduced by washing the collected tissues in physiological saline. Scrapings of gastrointestinal mucus, which contain the mucus-associated organisms, were then examined microscopically and inoculated onto culture media.

3.2.2.1 Phase contrast microscopy

Homogenised livers and mucus scrapings from the GIT of the BTP1, BTP2, and wombat 1 were emulsified in physiological saline and observed under phase contrast microscopy for the detection of spiral and fusiform-shaped organisms.

3.2.2.2 Bacterial cultivation

The combination of direct inoculation onto CSA plates (HBA with Skirrow's supplement) and the filtration method using HBA plates (as described in chapter 2) were used to culture spiral organisms. In all 3 animals examined in this chapter liver and GIT samples including stomach, mid ileum, ileum at 3 cm above the caecum, caecum, colon and rectum were examined.

Direct inoculation

The homogenised liver and scrapings of gastrointestinal mucus were inoculated onto moist CSA plates and streaked out for single colonies. Plates were incubated, lids uppermost, in an anaerobic jar (HP 11, Oxoid) with a microaerobic gas generating kit (BR 56, Oxoid) or anaerobic gas generating kit (BR 38, Oxoid) at 37°C. The plates were checked for growth every 3 to 4 days after inoculation and re-incubated for up to 10 days.

Selective filtration culture

Nitrocellulose membrane filters with a pore size of 0.65 μm (Millipore, Bedford, MA) were placed on the surface of moist HBA plates. The gastrointestinal mucus scrapings or homogenised livers were inoculated onto the centre of membranes and the plates were then placed in a CO_2 incubator set at 37°C, 10% CO_2 and 95% humidity for 2 hours. After this time the membranes were removed and the plates were incubated in anaerobic jars as described above.

3.2.3 Results

3.2.3.1 Phase contrast microscopy

Microscopic examination of mucus scrapings obtained from the 2 brushtail possums and the wombat showed that many spiral or fusiform shaped organisms were present in the ileum, caecum, colon and rectum of the animals.

3.2.3.2 Bacterial cultivation

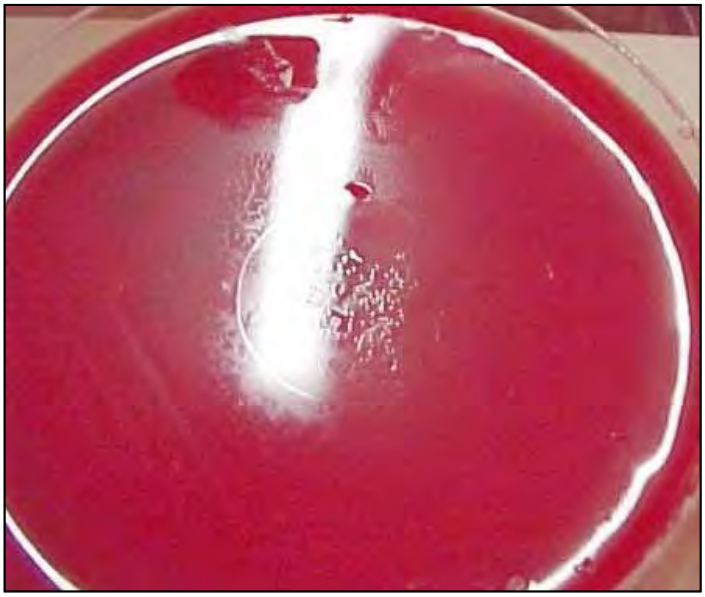
Following incubation, any colonies that appeared as a thin water-like film (see Figure 3.1 A) or as tiny pinpoint colonies on the HBA or CSA plates (see Figure 3.1 B) were examined using phase contrast microscopy. The majority of organisms from these colony types appeared to possess a spiral or fusiform morphology.

By using a combination of culturing methods (direct inoculation and the membrane filtration method) *Helicobacter* species were isolated from both brushtail possums and the wombat. *Helicobacter* species were isolated from the rectum of BTP1 and from the caecum of BTP2. No *Helicobacter* species were

isolated from the other sites of these brushtail possums. From the wombat *Helicobacter* species were isolated from the mid ileum, ileum at 3 cm above the caecum, caecum, colon and rectum. Furthermore *Campylobacter* species were isolated from the colon of wombat. The cultivation results correlated with the observation of spiral and fusiform shape organisms using phase contrast microscopy.

The results of phase contrast microscopy and cultivation from the liver and GIT from the two brushtail possums and the wombat are summarised in Table 3.1.

A.



B.

Figure 3.1 The appearance of a thin water-like film (A) and tiny pinpoint colonies on the HBA or CSA plates (B).

Specimen	BTP 1		BTP 2		Wombat 1	
	Microscopy	Culture	Microscopy	Culture	Microscopy	Culture
Liver	NS	-	NS	-	*	-
Stomach	NS	-	NS	-	NS	-
Mid ileum	NS	-	CR	-	F	F
3-ileum	Rod	-	Rod	-	F	F
Caecum	Rod, CR	-	Sp, Rod & CR	Sp	F & Sp	F
Colon	Rod, CR	-	Rod & CR	-	F, Sp & Rod	F & Sp
Rectum	Sp, Rod & CR	Sp	Rod, CR & F	-	Rod & CR	F

Table 3.1 The summary of the results obtained from the phase contrast microscopy and the cultivation of the liver and GIT samples of the two brushtail possums and the wombat.

3-ileum = Ileum at 3 cm above the caecum, - = no isolate obtained, Rod = rod shaped but not curved,* = 1-2 bacteria found, CR = curved rod, Sp = spiral, F = fusiform, NS = Rod, CR, Sp, & F not detected.

3.3 *Helicobacter* genus specific PCR

3.3.1 Introduction

While a number of primers have been described as being specific for the *Helicobacter* genus, the primers chosen for this study, H276f and H676r, are those originally designed by Riley *et al.* for the detection and identification of murine *Helicobacter* species [158]. These primers target a region of the 16S rRNA gene specific for the genus *Helicobacter* with a predicted product size of 374 bp. In his original study Riley *et al.* tested the sensitivity of this assay using 10 fold serial dilutions of *H. hepaticus* and *H. muridarum* DNA, ranging from 100 ng to 100 fg as template in the *Helicobacter* PCR assay. To simulate diagnostic conditions, this assay was performed in the presence of 1.25 μg of DNA extracted from the caecum of a mouse which was negative for *Helicobacters.* An amplification product of the expected size was detected when as little as 5 pg of DNA from either *H. hepaticus* or *H. muridarum* was used as template. Since its publication this sensitive and specific PCR has been extensively used for the screening and preliminary identification of *Helicobacter* DNA in the *Helicobacter* research laboratory at the University of NSW and thus was chosen to screen the marsupial samples.

Important parameters that influence the specificity and efficiency of a PCR reaction include the annealing temperature and the magnesium ion concentration. In theory, the concentration of $MgCl_2$ should be in the range of 1 to 6 mM $MgCl_2$. The annealing temperature, which is dependent on the size and nucleotide composition of the oligonucleotide used, can vary between 55°C and 65°C [182]. As a rough guide, this temperature should be ~5°C below the melting temperature (Tm) of the primer. The Tm for both H2767f and H676r is 58°C, thus the annealing temperature used in the published reaction, 53°C, was the ideal temperature. Indeed differences in the annealing temperature of as little as 1°C can affect the specificity of a reaction [182]. However, the clinical performance of the assay also depends on other factors, including the DNA extraction method used and the presence of inhibitors in the sample. Thus prior to the use of a PCR with a new sample type, the annealing temperature for the

combination of each individual primer pair and template should be tested and optimised.

3.3.2 Preliminary study

Prior to the commencement of the marsupial study, this PCR protocol was tested with *Campylobacter jejuni* and a number of *Helicobacter* reference strains including *H. pylori, H. hepaticus, H. bilis, H. trogontum, H. felis, H. muridarum,* and *H. mustelae.* In addition, DNA extracted from the liver and mucus scrapings of GIT samples including stomach, mid ileum, ileum at 3 cm above the caecum, caecum, colon and rectum obtained from a mouse, 2 brushtail possums (BTP1 and BTP2) and a wombat (wombat 1) was also tested.

3.3.2.1 Method

The PCR protocol used in this preliminary study was the protocol previously used in the *Helicobacter* research laboratory, BABS, UNSW which was slightly modified from that of Riley *et al*. The conditions of the PCR were as follows: the initial denaturation period was 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 5 seconds, annealing at 53°C for 5 seconds and extension at 72°C for 30 seconds followed a final extension at 72°C for 2 minutes.

3.3.2.2 Results

Using the *Helicobacter* genus specific PCR, as described above, it was possible to amplify a 374 bp PCR product from all *Helicobacter* reference strains tested (Figure 3.2 A) as well as from DNA extracted from the mouse, BTP1 and BTP2. A PCR product was amplified from the colon and rectum of BTP1 and from the caecum, colon and rectum of BTP2. However the amplification of *Helicobacter* DNA from wombat 1 samples, from which *Helicobacter* spp were isolated by cultivation, was unsuccessful (Figure 3.3 A).

Due to the above discrepancies it was considered essential to optimise the amplification reaction of the *Helicobacter* genus specific PCR to allow for the maximum detection of *Helicobacter* species in the marsupials.

3.3.3 Optimisation of PCR condition

3.3.3.1 Method

Optimisation of the *Helicobacter* genus specific PCR was carried out by varying the annealing temperature and the $MgCl_2$ concentration. The annealing temperatures tested were 53^oC, 55^oC, 57^oC and 61^oC and the $MgCl_2$ concentrations tested were 1.5, 2.0, 3.0 and 4 mM. DNA extracted from GIT samples from BTP1, BTP2 and wombat 1, were used as template for the optimisation reactions, *H. pylori* DNA was used as positive control and water (no DNA) was used as negative control in all reactions.

3.3.3.2 Results

A PCR product of the expected size was obtained with the positive control DNA at all $MgCl_2$ concentrations used (1.5, 2.0, 3.0 and 4 mM) and at annealing temperatures of 53^oC, 55^oC, 57^oC and 61^oC.

The amplification of *Helicobacter* DNA from the GIT of the brushtail possums appeared to be improved when the $MgCl_2$ concentration was increased to 3 mM as the product was easier to detect in a gel. There was no obvious difference in the PCR product obtained when the $MgCl_2$ concentration was increased from 3 mM to 4 mM at an annealing temperature of 53^oC or when annealing temperatures of between 53^oC and 57^oC were used.

Helicobacter DNA was successfully amplified from the wombat GIT using a $MgCl_2$ concentration of 3 mM at 55^oC and 57^oC (see Figure 3.3 B) but not at 53^oC. Thus the annealing temperature had a major impact on the amplification reactions. Given these results an annealing temperature of 57^oC was chosen for use in this study.

The thermocycling profile for the *Helicobacter* specific PCR used in the optimised reaction was: an initial denaturation at 94^oC for 5 minutes followed by 35 cycles of denaturation at 94^oC for 5 seconds, annealing at 57^oC for 5 seconds and extension at 72^oC for 30 seconds, followed by a final extension at

72°C for 2 minutes. The $MgCl_2$ concentration used was 3.0 mM, as described in Chapter 2.

As the annealing temperature for this PCR was changed from 53°C to 57°C and the $MgCl_2$ concentration used was 3.0 mM, the specificity of the amplification reaction was retested with the *Helicobacter* reference strains using the optimised protocol. The results showed that there was no difference in the amplification of *Helicobacter* reference strains as shown in Figure 3.2 B.

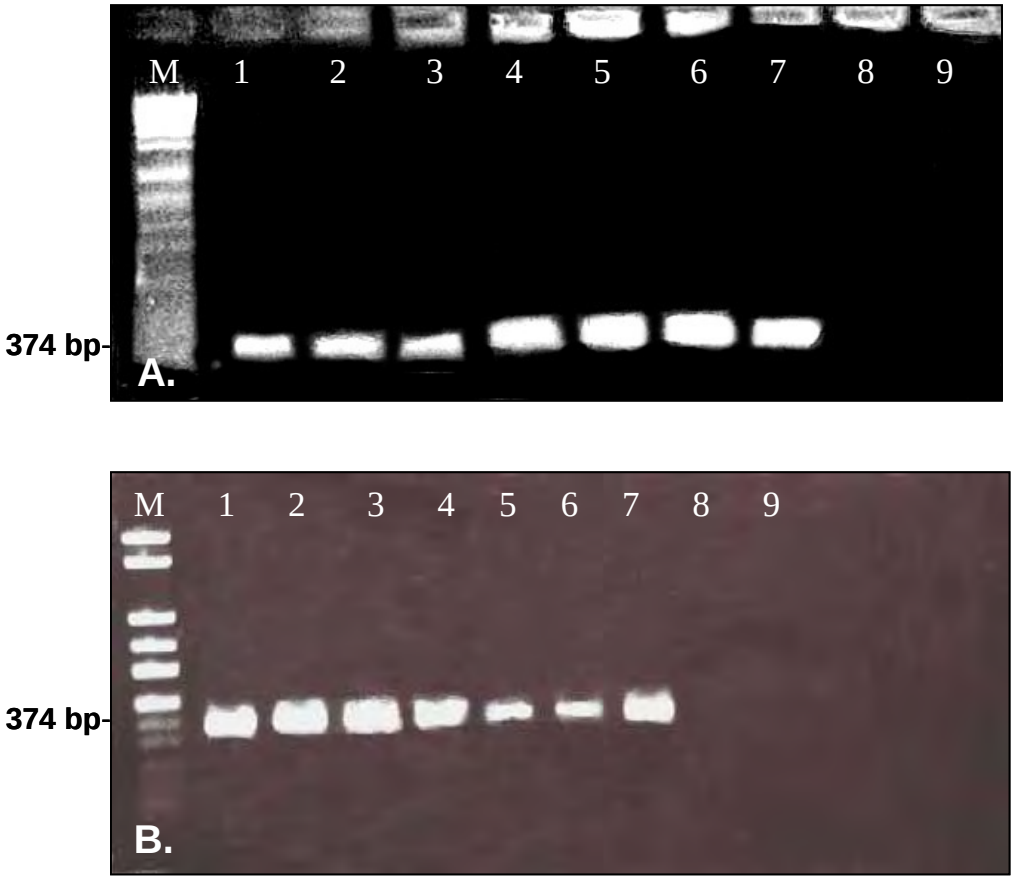


Figure 3.2 *Helicobacter* genus specific PCR with *Helicobacter r*eference strains using primers H276f and H676r,

A) Amplification using the PCR protocol used in *Helicobacter* laboratory, BABS, UNSW (annealing temperature = 53°C, $MgCl_2$= 2 mM)

B) Amplification using the optimised PCR protocol (annealing temperature = 57°C, $MgCl_2$= 3 mM)

For both A and B

M = SPP1/*Eco* R1 marker (A), FN1 (B)

Lane 1 = *H. pylori,*

Lane 2 = *H. hepaticus,*

Lane 3 = *H. bilis,*

Lane 4= *H. trogontum,*

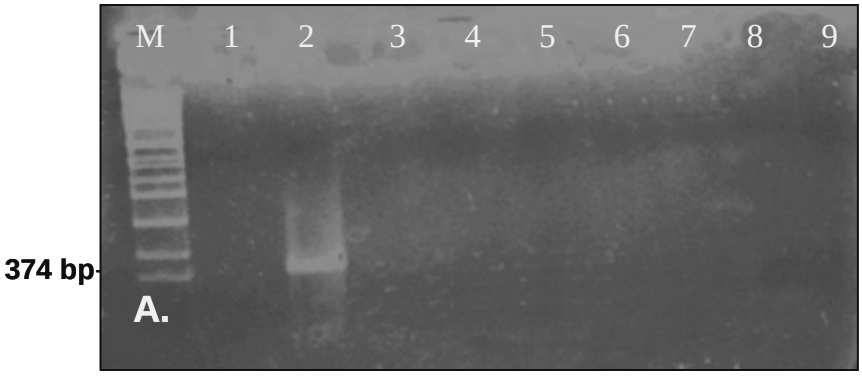
Lane 5 = *H. felis,*

Lane 6= *H. muridarum,*

Lane 7 = *H. mustelae*

Lane 8 = *C. jejuni*

Lane 9 = no DNA



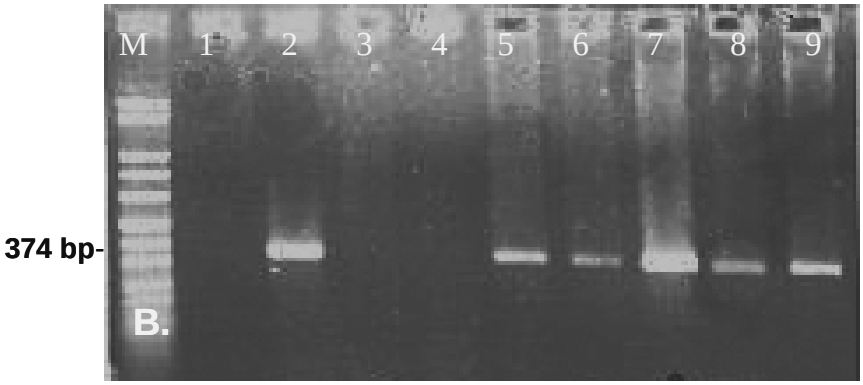


Figure 3.3 Optimisation of *Helicobacter* genus specific PCR using primers H276f and H676r. DNA extracted from the GIT of wombat 1 was used as the template.

A) Amplification using the PCR protocol used in the *Helicobacter* laboratory, BABS, UNSW (annealing temperature = 53°C, $MgCl_2$= 2 mM)

B) Amplification using the optimised PCR protocol (annealing temperature = 57°C, $MgCl_2$ = 3 mM)

For both A and B

M = SPP1/*Eco* R1 marker (A), FN1 (B)

Lane 1 = no DNA

Lane 2 = *H. pylori*, positive control

Lane 3 = DNA extracted from the liver,

Lane 4 = DNA extracted from the stomach

Lane 5 = DNA extracted from the mid ileum

Lane 6 = DNA extracted from the ileum at 3 cm above the caecum

Lane 7 = DNA extracted from the caecum

Lane 8 = DNA extracted from the colon

Lane 9 = DNA extracted from the rectum

3.3.4 Limit detection of *Helicobacter* genus specific PCR

The limit of detection of *Helicobacter* species using the optimised PCR was also determined. DNA extracted from the GIT of mice known to be negative for *Helicobacters* but which had been spiked with *H. hepaticus*, was used as a simulated diagnostic sample. It was not possible to use spiked marsupial DNA to simulate the diagnostic sample as all the marsupials obtained for study at this stage were shown to be colonised by *Helicobacter* species. Serial 10-fold dilutions of *H. hepaticus* ranging from 10^2 to 10^9 cell/ mL of *H. hepaticus* were used to determine the limit of detection. *H. hepaticus* was selected to determine the limit of detection as it is a recognised intestinal *Helicobacter* and is able to form discrete colonies after incubation of 3 to 5 days on HBA with 2.0% agar.

3.3.4.1 Method

3.3.4.1.1 *H. hepaticus* suspension preparation

H. hepaticus was cultured on HBA plates incubated microaerobically at 37°C for 3 days. A stock solution was prepared by harvesting the cells into sterile tubes containing 5 mL BHI broth and adjusting the cell concentration to a turbidity equivalent of McFarland 3 (bacterial concentration of approximately 9.0×10^8 cells/ mL). Ten fold serial dilutions of the stock suspension were then prepared to obtain dilutions down to approximately 10^2 cell/ mL. To determine the number of colonies forming units (CFU), 100μL of each dilution was spread on an HBA plate containing 2% agar and incubated in microaerobic conditions at 37°C for 4 days. From the CFU count, the *H. hepaticus* cell concentration in the stock suspension was calculated and adjusted to approximately 10^9 cells/ mL.

3.3.4.1.2 The preparation of *H. hepaticus* DNA from pure culture

The *H. hepaticus* stock suspension was 10 fold serially diluted from 10^9 to 10^2 cells/ mL. One milliliter from each of the bacterial dilutions was centrifuged at 14,000 rpm for 10 minutes and the BHI broth removed. The DNA from the centrifuged cells was extracted using XS buffer as described in section 2.8.2. The dried DNA products were dissolved in 200 μL TE buffer. At this stage it was assumed that the number of *H. hepaticus* cells in 200 μL DNA was equal to the number of *H. hepaticus* cells in 1 mL of the BHI broth at a particular dilution. For example in a *H. hepaticus* suspension containing 10^9 cells/ mL, the number

of *H. hepaticus* cells in 2 μL of the DNA suspension was equal to 10^7 cells/ mL. Two microlitres of DNA was used for the amplification in all *Helicobacter* genus specific PCRs.

3.3.4.1.3 The preparation of DNA from a *H. hepaticus* spiked mucus sample

Mucus scrapings from the GIT of mice known to be free of helicobacters was divided into ten 50 mg portions and placed into separate sterile 2 mL tubes. Apart from the control sample, these samples were then spiked with 1 mL each of serial 10-fold dilutions of *H. hepaticus* ranging from 10^2 to 10^9 cell/ mL. Each spiked sample was centrifuged at 14,000 rpm for 10 minutes to remove all BHI broth and the DNA was extracted using the phenol-chloroform method as described in section 2.8.1. The dried DNA products were then dissolved in 200 μL TE buffer. Two microlitres of DNA was used in the *Helicobacter* genus specific PCRs.

3.3.4.2 Results

The limit of detection of the optimised *Helicobacter* genus specific PCR tested with a pure culture of *H. hepaticus* was 10-100 CFU as shown in Figure 3.4.

The limit of detection of the optimised *Helicobacter* genus specific PCR tested using a GIT mucus sample spiked with *H. hepaticus* was 100-1000 CFU as shown in Figure 3.5.

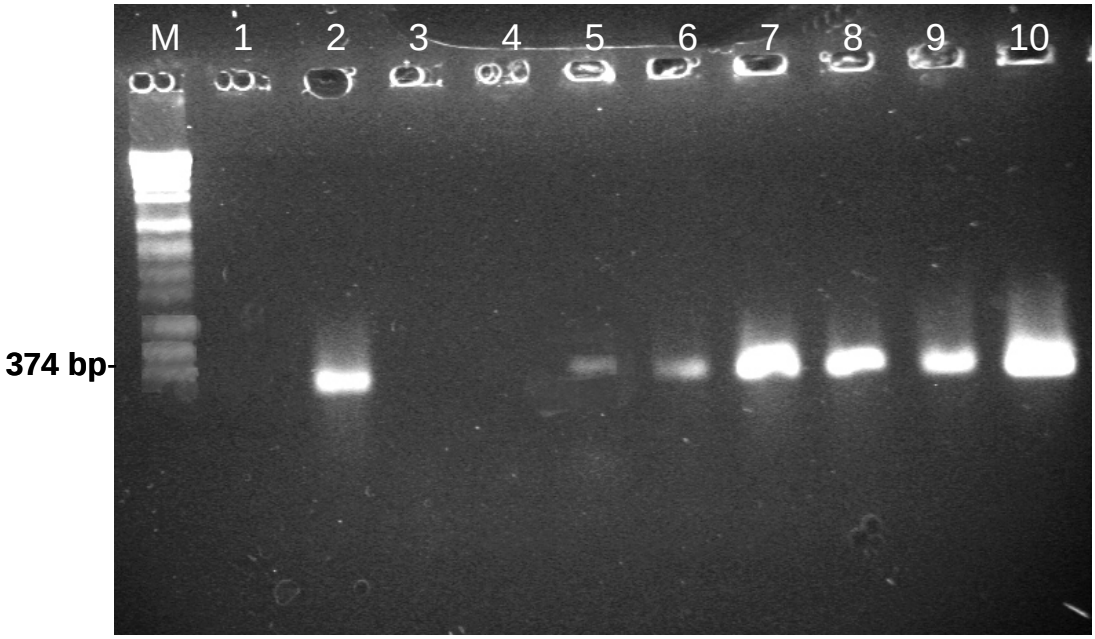


Figure 3.4 *Helicobacter* genus specific PCR using primers H276f and H676r amplifying 10-fold dilutions of *H. hepaticus* pure culture DNA.

M = SPP1/*Eco* R1 marker

Lane 1 = No DNA

Lane 2 = *H. pylori* control

Lane 3 = 0-1 cell of *H. hepaticus*

Lane 4 = 1-10 cells of *H. hepaticus*

Lane 5 = 10 - 10^2 cells of *H. hepaticus*

Lane 6 = 10^2 –10^3 cells of *H. hepaticus*

Lane 7 = 10^3 – 10^4 cells of *H. hepaticus*

Lane 8 = 10^4 – 10^5 cells of *H. hepaticus*

Lane 9 = 10^5 – 10^6cells of *H hepaticus*

Lane 10 =10^6 – 10^7 cells of *H. hepaticus*

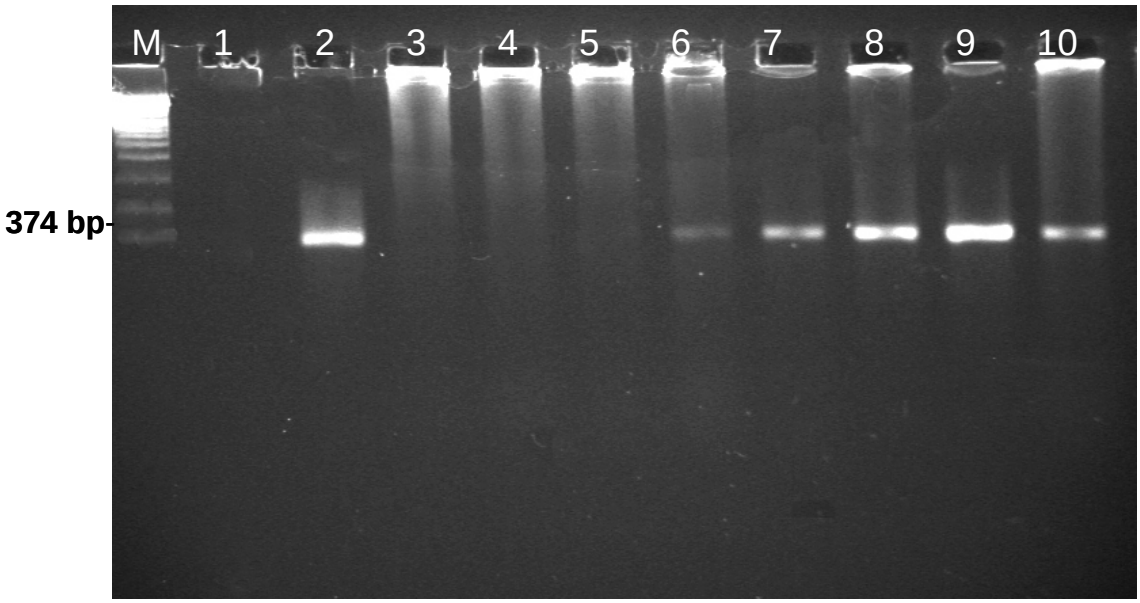


Figure 3.5 *Helicobacter* genus specific PCR using primers H276f and H676r amplifying DNA from mice GIT mucus spiked with 10-fold dilutions of *H. hepaticus* DNA.

M = SPP1/*Eco* R1 marker

Lane 1 = No DNA

Lane 2 = *H. pylori* control

Lane 3 = 0-1 cell of *H. hepaticus*

Lane 4 =1-10 cells of *H. hepaticus*

Lane 5 =10 – 10^2 cells of *H. hepaticus*

Lane 6 =10^2 –10^3 cells of *H. hepaticus*

Lane 7 =10^3 – 10^4 cells of *H. hepaticus*

Lane 8 =10^4 – 10^5 cells of *H. hepaticus*

Lane 9 =10^5 – 10^6 cells of *H. hepaticus*

Lane 10 = 10^6 - 10^7 cells of *H. hepaticus*

3.4 Fluorescent *in situ* hybridisation (FISH)

3.4.1 Background

The first description of the visualisation of *H. pylori* in paraffin embedded sections of human gastric biopsies using *in situ* hybridisation (ISH) was reported by Van den Berg *et al.* in 1989 [183]. In this study whole genomic DNA of *H. pylori* was labelled with biotin and used as a non-radioactive labelled specific probe. Hybridisation was detected using anti-biotin and horseradish peroxidase (HRP) conjugated anti-Ig. It was concluded by Van den Berg *et al.* that this method was sensitive and the bacteria which do hybridise were unequivocally identified as *H. pylori.*

A few years later, Bashir *et al.* applied an ISH method to detect *H. pylori* in paraffin embedded gastric biopsy specimens using a PCR-generated biotinylated probe [184]. A biotinylated 109 bp PCR product of a selected region of the 16S rRNA gene of *H. pylori* was generated by PCR using a mixture of dTTP and biotin-11-dUTP in a ratio of 3 to 1, and primers HP1 (5' CTGGAGAGACTAAGCCCTCC 3') and HP2 (5' ATTACTGACGCTGATTGTGC) in the PCR reaction mix. The biotin labelled hybridisation product was detected by the use of a biotin-strepavidin-alkaline phosphatase sandwich technique. Using this probe the hybridisation reaction was sensitive and specific for *H. pylori*. However some problems occurred because the access of this probe to the bacterial nucleic acids was limited by the bacterial cell wall and there was nonspecific binding of biotin with the bacterial cell walls.

In 1996 Karttunen *et al.* developed a non-radioactive ISH method for detection of *H. pylori* in paraffin embedded gastric biopsy specimens using a digoxigenin labelled oligonucleotide probe [185]. This method allowed specific hybridisation with target RNA and thus reduced/eliminated binding to other bacterial structures. In this study a different primer pair described as HP1 (5'-TGGCAATCAGCGTCAGGTAATG-3') and HP2 (5'-GCTAAGAGA TCAGCCTA TGTCC-3') were used to amplify a segment between nucleotide 219 and 740 of the 16S rRNA gene from *H. pylori* using reverse transcription PCR (RT-PCR). The PCR product (520 bp) obtained was labelled with digoxigenin and used as

a specific probe. Hybridisation was detected using anti-digoxigenin antibodies conjugated to alkaline phosphatase. By comparison to culture and histology it was claimed by Karttunen *et al.* that this ISH provided a sensitive and specific method for the detection and confirmation of *H. pylori* infection.

As outlined above the detection of hybridisation using non-radioactive labeling methods based on reporter molecules (such as biotin and digoxigenin) are involved in many hybridisation detection processes however the use of fluorescent dye-labelled probes in FISH is a straightforward method and the hybridised cells can be visualised directly by fluorescent microscopy. Recently Trebesius *et al.* successfully used FISH to detect *H. pylori* within gastric tissue and simultaneously identified clarithromycin resistance genotypes by using a set of fluorescently labelled short-oligonucleotide probes which bound to either the *H. pylori* 16S rRNA gene or regions of the 23 rRNA gene containing specific point mutations responsible for clarithromycin resistance [186]. This method was subsequently successfully applied to paraffin-embedded and shock-frozen gastric biopsy specimens that had been prepared for pathological examination [187]. The validly of this FISH based method in detecting clarithromycin resistance in 109 *H. pylori* cultures was compared with E-test and disk diffusion [188]. In this study it was shown that there were no discrepancies between the three methods, with FISH shown to be the most rapid (approximately 3 hours) and accurate method. In addition this same group used the FISH technique to detect "*H. heilmannii*"-like organisms (HHLO) in the human gastric biopsies [189]. This latter study highlights an advantage of FISH as not all subtypes of HHLO's can be cultivated *in vitro*.

To date, the use of FISH in to examine *Helicobacters* has been limited to investigations of gastric organisms, primarily *H. pylori*. In the current study FISH, using a *Helicobacter* genus specific probe, was optimised to allow for the detection and determination of the spatial localisation of *Helicobacter* species in representative paraffin sections of the marsupial gastrointestinal tract.

3.4.2 Experimental method

The FISH technique used in this thesis was modified from that described by Amann *et al.* [173, 190]. The procedures for the FISH technique are outlined below and in Figure 3.6.

The specificity of the *Helicobacter* specific probe was tested using pure cultures of *Psychrobacter* sp. strain SW5 [191], *C. jejuni* and *H. pylori* strain SS1. Prior to the commencement of studies in marsupials the FISH technique was trialed on paraffin embedded, formalin fixed gastrointestinal tissue samples from a *H. muridarum* infected mouse and tissue sections of the rectum of a brushtail possum (BTP10). Hybridisation buffers containing either 30% or 40% formamide were used in these preliminary studies.

3.4.2.1 Sample preparation

3.4.2.1.1 Fixation of cell controls

Bacterial cells in the exponential growth phase were harvested in 1 mL of BHI. The cell suspension was then centrifuged at 14,000 rpm for 3 to 4 minutes and 750 μl of the supernatant removed. To each of the control cells, 750 μl of fixative (33 mL milli Q water at 60°C, 1 drop 10M NaOH, 2 g paraformaldehyde and 16.5 mL 3 x PBS) was added and this was vortexed for 1 minute. The cell suspension was then incubated at 4°C for up to 24 hours. After incubation the cells were centrifuged at 6,000 rpm for 5 minutes and the supernatant removed. Nine hundred microlitres of 1x PBS buffer and 100 μl of 0.1% non-ionic detergent (Igepal CA-630; Sigma chemical Co., ST Louis, MO.) were added into the cells and this was then centrifuged at 6,000 rpm for 5 minutes. Five hundred microlitres of 0.1% non-ionic detergent was then added to the pellet. The cell suspension was again centrifuged at 6,000 rpm for 5 minutes. The pellet was then re-suspended in 200 μl of storage buffer (40 mm Tris buffer pH 7.2 and 0.2 % Igepal CA-630) and 200 μl of 96% ethanol. The fixed cells were stored at - 20°C.

3.4.2.1.2 Pure culture

One to three microlitres of a suspension of the fixed cells (*H. pylori* strain SS1, *Psychrobacter* sp. strain SW5 and *C. jejuni*) were applied to each well on a

Poly-L-lysin coated slide, and left to air dry. The cells were then dehydrated in 50, 80, and 96 % ethanol for 3 min each and left to air dry. Fixed *H. pylori* strain SS1 (positive control) and *Psychrobacter* spp strain SW5 (negative control) were used as bacterial cell controls in every test.

3.4.2.1.3 Fixed tissue

Briefly, 2 x 5μm serial sections were cut from paraffin embedded tissue (PET). One section was immobilised on an Aminopropyltriethoxysilane (TES)-coated slide (routinely used in the Histopathology Laboratory, UNSW) for FISH and the other was stained with a modified Steiner silver stain. The section prepared for FISH was de-paraffinised by placing it in xylene for 2 x 10 minutes and submerging it in 100% ethanol for 3 x 10 dips, followed by air drying. Examination of the silver stained slide allowed for the identification of a suitable area of tissue for further study. Once this was determined a hole was cut in the middle of a pressure seal and this was pressed onto the slide to surround the area of interest (see Figure 3.6).

3.4.2.2 Oligonucleotide probes

The Eubacterial 16S rRNA probe, EUB338 [192], labelled with fluorescein-isothiocyanate (FITC), referred to as EUB338-FITC, and a *Helicobacter* genus specific probe labelled with tetramethyl-rhodamine-isothiocyanate (TRITC) (GENSET pacific Pty. Ltd. Lismore, Aust.), referred to as HRh, were used in this study (see Tables 3.2 and 3.3). The HRh probe was designed by Dalton and Neilan (School of BABS, UNSW) based on the oligonucleotide specific for the *Helicobacter* genus published by Fox *et al.* [193]. The specificity of *Helicobacter* specific probe was assessed by Dalton and Neilan (School of BABS, UNSW) by comparing the probe's sequence with entries in the GenBank database for prokaryotes and eukaryotes.

3.4.2.3 Fluorescent *in situ* hybridisation method

3.4.2.3.1 Hybridisation of control cells

Nine microlitres of hybridisation solution (the same stringency as used in the tissue section) and 1 μl of the appropriate probe, EuB338-FITC (0.64 μg/μL) or HRh (0.8 μg/μL), were added to wells containing the control cells and mixed

gently. The slides were then placed into a dark moist chamber and incubated overnight at 37^0C.

3.4.2.3.2 Hybridisation of section

Eighteen microlitres of the optimised hybridisation solution (washing solution I, as shown in Table 3.4) and 2 μl of each probe, EuB338-FITC and HRh, were added to the sample well and mixed. The slides were then incubated in a dark sealed moist chamber overnight at 37^oC (hybridisation temperature).

3.4.2.3.3 Washing

After hybridisation the slides were rinsed with 50-100 μL of pre-warmed milli Q water, followed by pre-warmed hybridising solution (40% formamide) and then incubated for 20 minutes at 37^oC. Pre-warmed washing solution II (10 ml 1 M Tris pH 7.2, 18 ml 5M NaCl and 72 ml milli Q water) was then added and the slides were incubated for a further 15 minutes at 37^oC. Finally the slides were rinsed in milli Q water and air-dried in the dark.

3.4.2.3.4 Photomicroscopy

Once the slides were dry and ready to be visualised, a drop of an anti-fade agent, Citiflour (Citiflour UKC, Canterbury, UK) was added. Epi-fluorescent microscopic examination of the slides was conducted using a Zeiss Axioskop microscope fitted with an HBO 50-W mercury lamp and equipped with filter sets 10 and 15 (Carl Zeiss, Oberkochen, Germany) for detecting the green FITC signal (excitation of 490 nm and emission of 520 nm) and the red TRITC signal (excitation of 541 nm and emission of 572 nm), respectively. Photographs were taken using a 400 ASA colour slide film.

In addition to epi-fluorescent microscopy slides were also visualised using scanning confocal laser microscopy (SCLM). Use of the confocal microscope allowed for better resolution of hybridised bacteria as this technique allows for visualisation of three dimensional images and the sub-cellular location of labeling [194]. Furthermore digital image collection is approximately 1000 times more sensitive than the film used in epi-fluorescent microscopy. In this thesis SCLM images were obtained using an Olympus GB200 microscope (Olympus

Optical Company Ltd., Tokyo, Japan) fitted with a piezo-electric z stage. The microscope was fitted with a 60 X, 1.4-numerical oil immersion lens. An argon laser was used as the excitation source for the fluorochromes used. The images obtained were analyzed using Photoshop 6.0 (Adobe Systems Inc., Mountain Vies, CA).

Probe	Target	Location	Sequence	Reference
Eu338-FITC	Eukaryote	338-355	5’ gCTgCCTCCCgTAggAgT 3’	[192]
HRh	*Helicobacter* sp.	274-300	5’ CTCAggCCggATACCCgTCAT AgCCT 3 ’	Dalton & Neilan*

Table 3.2 DNA Probes used for FISH

* = designed by Dalton and Neilan (School of BABS, UNSW) based on the oligonucleotide specific for the *Helicobacter* genus published by Fox *et al.* [193].

	Wave length		
Fluorochromes	**Excitation (nm)**	**Emission (nm)**	**Colour**
Fluorescence-isothiocyanate (FITC)	490	520	Green
Tetramethyl-rhodamine-isothiocyanate (TRITC)	541	572	Red

Table 3.3 Fluorochromes used in this study

% Formamide	30%	40%
5M NaCl	18	18
1M Tris pH 7.2	10	10
Formamide	30	40
10% SDS	1	1
Milli Q water	41	31

Adjust pH to 7.2 using HCl

Table 3.4 Hybridisation and Washing solution I (in mL)

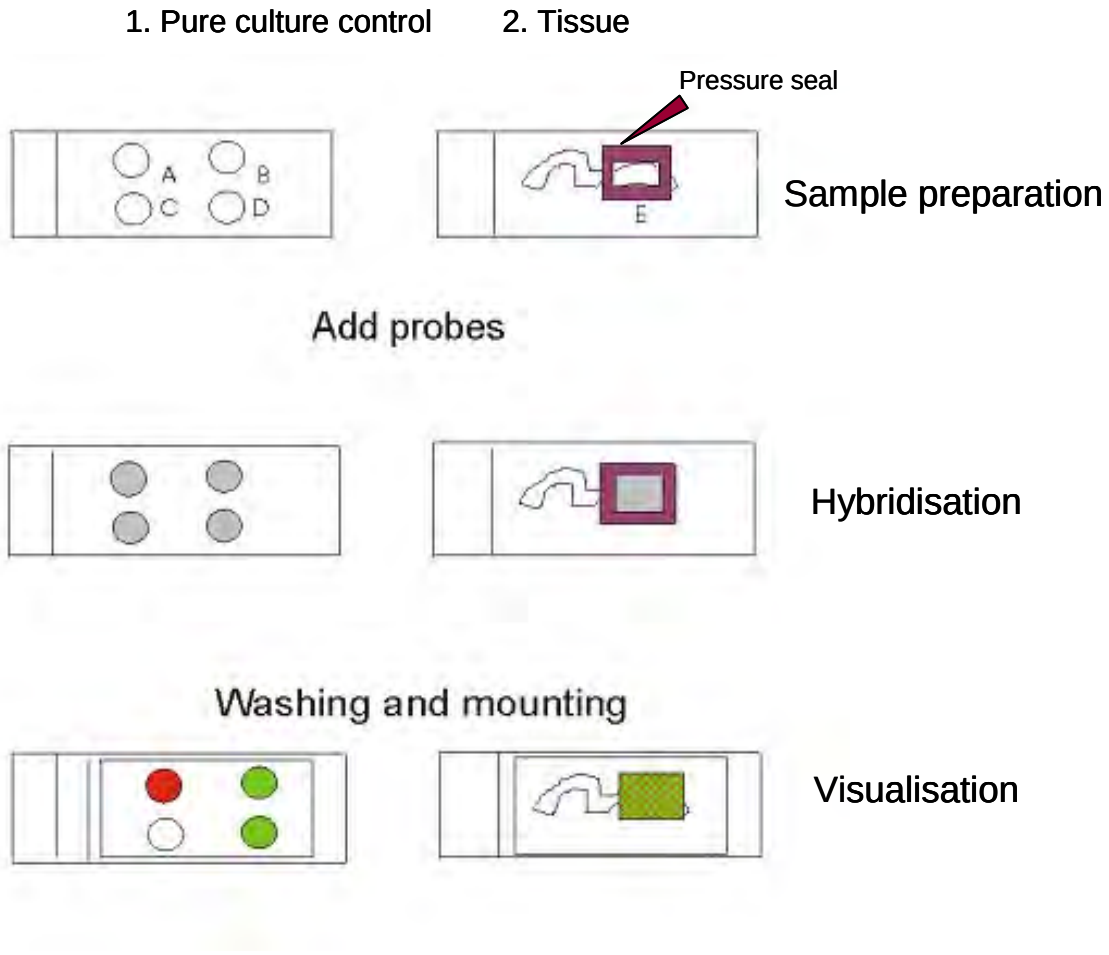


Figure 3.6 Flow chart of the FISH procedure

1) Hybridisation of control cells,

A= *H. pylori* cell hybridised with HRh probe,

B= *H. pylori* cell hybridised with Eu338-FITC probe,

C= SW5 cell hybridised with HRh probe,

D= SW5 cell hybridised with Eu338-FITC probe,

2) Hybridisation of paraffin embedded tissue (E)

See section 3.4.2.3 for detail

3.4.3 Results

The hybridisation of control cells using stringency condition at 30% formamide solution showed that all control bacterial cells (*H. pylori* strain SS1, *Psychrobacter* sp. strain SW5 and *C. jejuni*) are able to hybridise with the Eubacterial probe (EUB338-FITC) resulting in the emission of a green signal. *H. pylori* cells were also able to hybridise with the *Helicobacter* genus specific probe (HRh) resulting in the emission of a red signal (Figure 3.7 A). *Psychrobacter* sp. strain SW5 and *C. jejuni* cells did not hybridised with the HRh probe, neither of these cells emitting a red signal. When both EUB338-FITC and HRh probes were applied to a mixture of *H. pylori* and *Psychrobacter* sp. strain SW5, *H. pylori* cells hybridised with both the EUB338-FITC and the HRh probes. The dual signal of *H. pylori* cells hybridised with both probes results in the simultaneous emission of an orange signal (a mixture of red and green) (Figure 3.7 B) while the *Psychrobacter* sp. strain SW5 hybridised only with the EUB338-FITC and emitted a green signal.

Given that the pure control cells were hybridised well with EUB338-FITC and/or HRh probes using 30% formamide solution, this condition was applied in the preliminary studies conducted with GIT sections from a *H. muridarum*-infected mouse. As non-helicobacters and other interference was more likely to be present in the fixed tissue as compared to the pure culture cells, hybridisation conditions using a 40% formamide solution was also examined. This study showed that using both 30% and 40% formamide the spiral shaped bacterium (*H. muridarum*) produced a green fluorescent signal when the EUB338-FITC probe was applied and a red signal when the HRh probe was applied to the slide, while the other bacteria on the same slide bound only to the EUB338-FITC but not the HRh probe. Similar results were obtained when these probes were applied to a section of rectum from the brushtail possum (BTP6 & 10). Samples from both the mouse (not shown) and brushtail possum (Figures 3.8 and 3.9) showed a large number of bacteria to be present with *Helicobacter* species being predominant in the mucus layer overlying the intestinal epithelium. No auto-fluorescent was observed from other substances in the tissue sections.

Given that the hybridisation reaction was successful using 30% and 40% formamide with fixed tissue sections, the hybridisation reaction was conducted using 40% formamide solution (a higher stringency) with the sections of fixed specimens obtained from the marsupials in this thesis. The specimens studied were the rectum of 4 brushtail possums and 1 ringtail possum, the stomach of a kangaroo and a Tasmanian devil and the colon of a long nosed bandicoot. The results obtained from these studies will be discussed in detail in Chapters 4, 5 and 7.

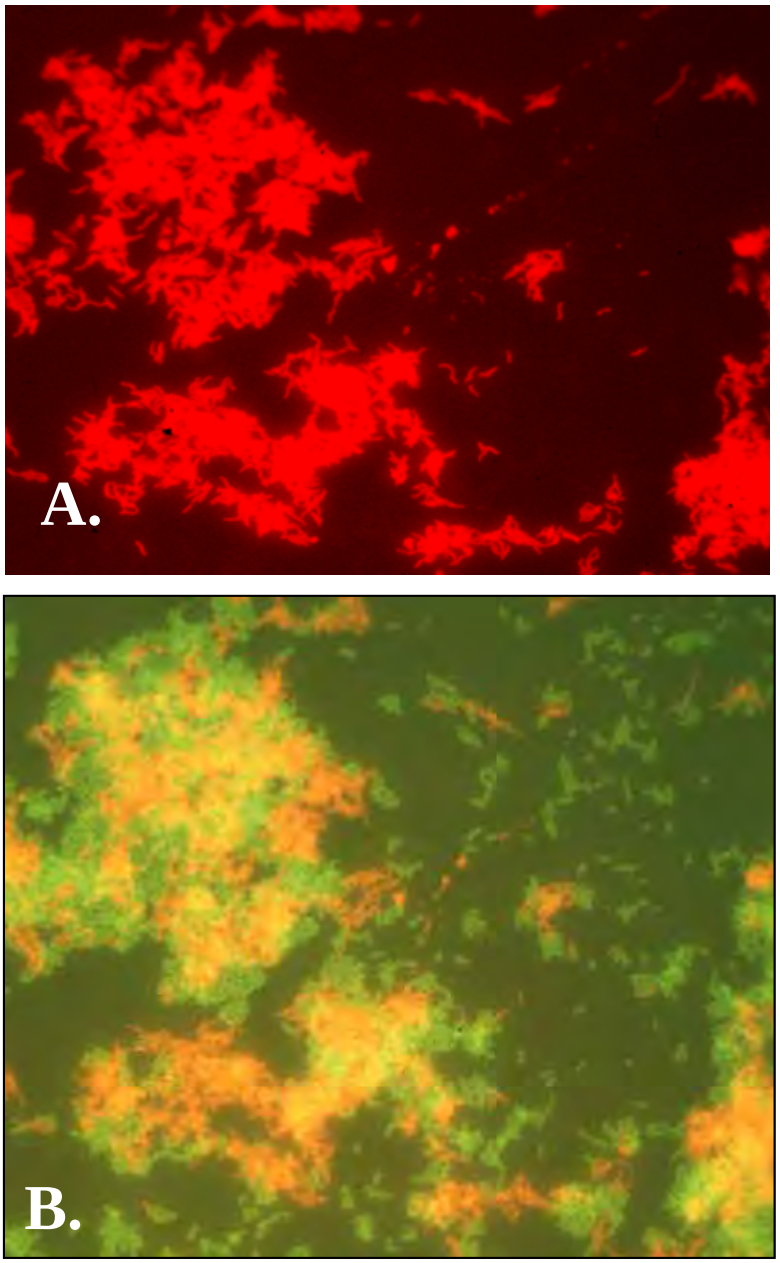


Figure 3.7 Epi-fluorescent photomicrographs of a mixture of *H. pylori* strain SS1 and the *Psychrobacter* sp. strain SW5.

A) *H. pylori* strain SS1 hybridised with the HRh probe (*Helicobacter* genus specific probe labelled with tetramethyl-rhodamine-isothiocyanate) showing a red signal.

B) *H. pylori* strain SS1 hybridised with both the HRh and the EUB338-FITC probes (Eubacterial probe labelled with fluorescein-isothiocyanate) showing an orange colour (mixture of red and green colours) and *Psychrobacter* sp. strain SW5 hybridised with EUB338-FITC probe showing a green signal.

(Magnification X 630)

Photographs taken by Helen Dalton (School of BABS, UNSW).

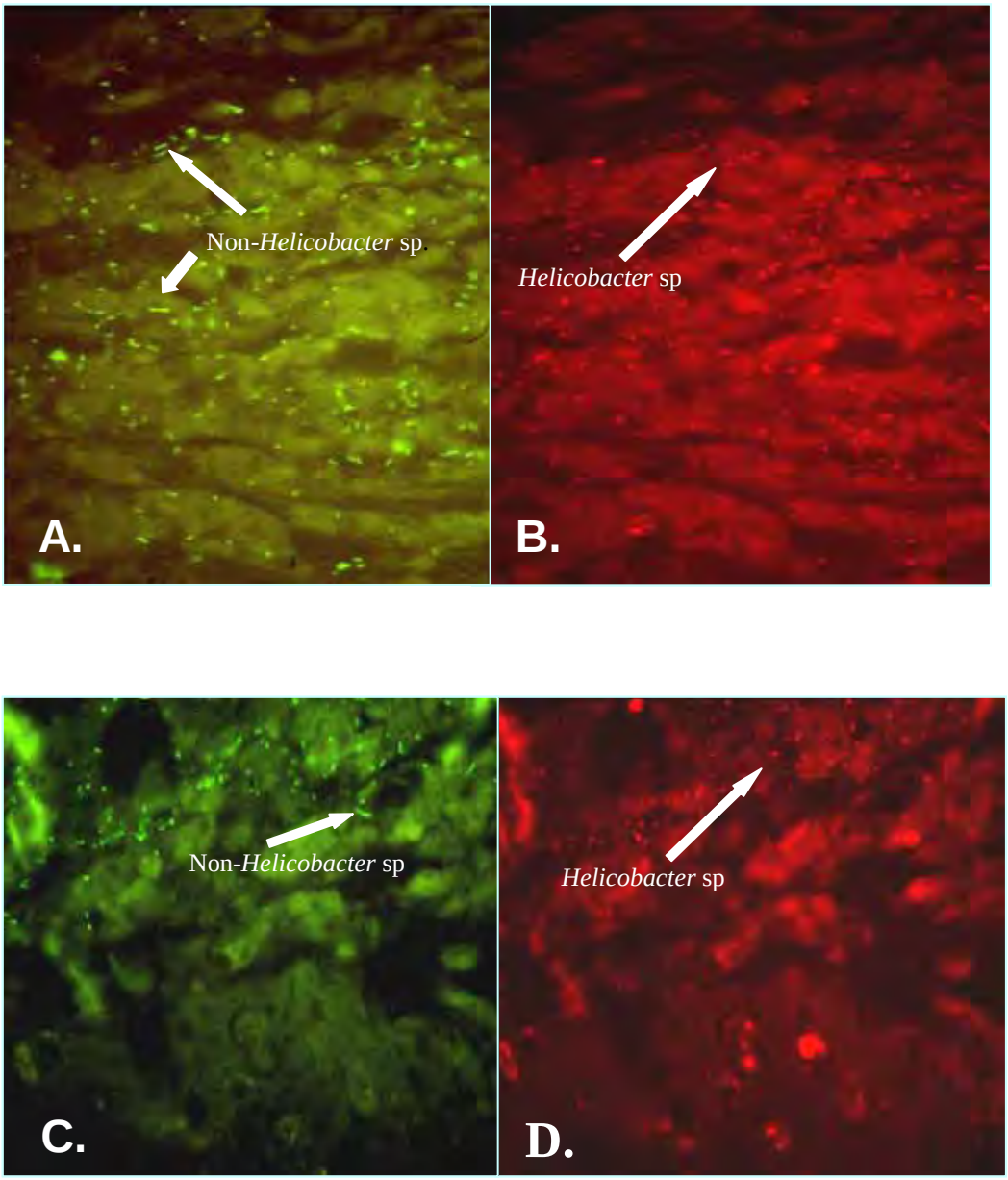


Figure 3.8 Epi-fluorescent photomicrographs of a section taken from the rectum of brushtail possums (A & B from BTP6), (C & D from BTP 10).

A) All bacteria hybridised with the EUB338-FITC probe showing a green signal.

B) Bacteria belonging to the *Helicobacter* genus hybridised with the HRh probe showing a red signal.

C) All bacteria hybridised with the EUB338-FITC probe showing a green signal.

D) Bacteria belonging to the *Helicobacter* genus hybridised with HRh probe showing a red signal.

Photographs taken by the author (Magnification x 630).

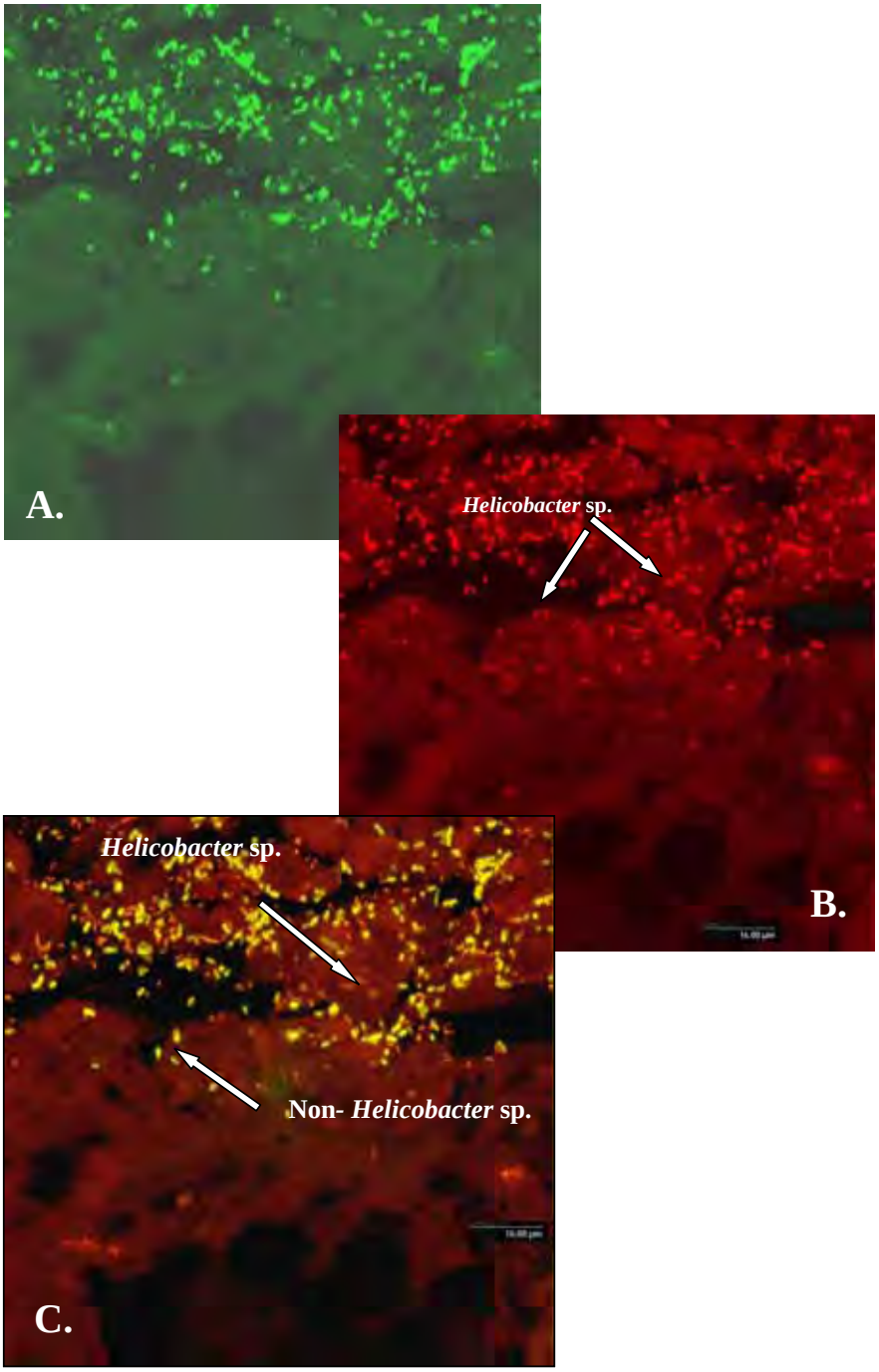


Figure 3.9 Confocal-fluorescent photomicrographs of a section taken from the rectum of a brushtail possum (BTP10).

A) All bacteria hybridised with the EUB338-FITC probe (showing a green signal.

B) Bacteria belonging to the *Helicobacter* genus hybridised with the HRh probe showing a red signal.

C) Bacteria belonging to the *Helicobacter* genus hybridised with both the HRh and the EUB338-FITC probes showing an orange colour (mixture of red and green colours). Photograph taken by Helen Dalton (School of BABS, UNSW). Bar = 16.00μm

3.5 Discussion and summary

In this study, bacterial cultivation, *Helicobacter* genus specific PCR and FISH were optimised to allow for the detection and/or isolation of spiral and fusiform shaped bacteria, in particular *Helicobacter* species, from Australian marsupials.

Bacterial cultivation

Initial examination by phase contrast microscopy was included in this study as it allowed for the preliminary detection of spiral and fusiform shaped organisms in intestinal mucus samples.

In previous studies successful culture of many of the currently known *Helicobacter* species and isolates has been due to either the use of a non-selective agar coupled with a membrane filter to reduce contamination or a selective agar, or a combination of both. Campylobacter selective agar containing Skirrow's supplement (Vancomycin, Trimethoprim and Polymyxin B) has generally been the selective agar of choice as this medium is suitable for cultivation of most *Helicobacter* and *Campylobacter* species including *H. felis* [54], *H. muridarum* [83], *H. hepaticus* [102], *H. bilis* [96] and *H. trogontum* [108]. The filtration method has been used successfully to isolate other *Helicobacter* species including *H. rodentium* [107] and *H. mesocricetorum* [103]. Recently the combination of direct inoculation and filtration method was successfully used to isolate newer *Helicobacter* species such as *H. ganmani* [101], "*H. marmotae"* [111] and *H. cetorum* [92]. Furthermore, in a study by Engberg *et al.* the prevalence of *Campylobacter, Arcobacter, Helicobacter,* and *Sutterella* species in human faecal samples was estimated and the efficacies of conventional selective methods for the isolation of *Campylobacter* were reevaluated [195]. While two charcoal-based selective agars, modified charcoal cefoperazone deoxychocolate agar (mCCDA) and cefoperozone-amphotericin-teicoplanin (CAT) agar, recovered significantly more thermophilic *Campylobacter* species than Skirrow's medium the most common *Helicobacter* species found in human faeces, *H. cinaedi*, was only successfully detected using Skirrow's medium.

In this preliminary study, *Helicobacter* species were successfully isolated from both BTPs examined and a wombat. Positive culture in these animals correlated both with the presence of spiral and fusiform organism observed by microscopy and the results of *Helicobacter* genus specific PCR. As will be discussed in the following chapters a large number of isolates from marsupials were cultivated using these two culture methods which correlates with previous experience in our laboratory (J. O'Rourke, B. Robertson, A. Lee, personal communications) and with published studies described above. This success in the isolation of spiral and/or fusiform shaped bacteria, in particular *Helicobacters,* by cultivation has also allowed for the further characterisation of these microorganisms, some of which constitute new species, as will be described in the subsequent chapters.

***Helicobacter* genus specific PCR**

The *Helicobacter* genus specific PCR which utilised primers H276f and H676r is well accepted as a specific PCR for the detection of members of the genus *Helicobacter* [158]. As mentioned above the high degree of concordance between the detection of helicobacters by PCR and the success in their cultivation indicates that, by using the modifications described here in the methodology, this PCR could reliably detect helicobacters in the GIT of marsupials. However, recently, in a study by Buczolits *et al.* it was found that this primer pair can also amplify a member of the genus *Brevudimonus (*strain H2/98-FUBDUS), which was isolated from the stomach of a dog [196]. *Brevudimonus* strains have often been isolated from freshwater, including tap water. It is possible that the *Brevudimonus* strain was transferred to the stomach of the dog from drinking water. This is the first example of a problem with the specificity of the *Helicobacter* genus specific primer pair used in this study (H276f and H676r) and it shows that a positive signal after application of the *Helicobacter* genus specific PCR may not necessarily indicate the presence of helicobacters in the specimen. The discrepancies in the specificity of PCR using *Helicobacter* genus specific primer pair, H276f and H676r, also showed that no particular method alone is sufficient for the detection of *Helicobacter* spp.

Currently there is no standard protocol for the reporting of the sensitivity of a PCR assay. In many reports, the sensitivity is stated in picogram of DNA or the number of cells per milliliter (CFU/mL). In this study the sensitivity assay (limit detection of PCR) was described as CFU/mL. The limit of detection of the *Helicobacter* genus specific PCR achieved in the present study was 10-100 cells of a *H. hepaticus* pure culture and 100-1000 cells from the spiked GIT samples from the mice. The limit of detection determined by Riley *et al.* for the *Helicobacter* genus specific PCR was 5 pg of DNA [158]. In comparison to the limit detection determined by Riley *et al.*, a more biologically meaningful data point involves the conversion of a weight value to a cell number. If it assumed that most bacteria have a genome roughly the size of that of *E. coli*, then 5 fg is equivalent to about one genome. Therefore, 1 pg is equivalent to 200 cells [197]. However the genomic size of *H. pylori* (1.66 Mb) is 2.8 times smaller than *E. coli* (4.64 Mb). The limit detection determined by Riley *et al.* would be 1000-2800 cells. Though this conversion is not very accurate the determination of the limit of detection in our study compared favorably to that of Riley *et al.* The limit of detection in the intestinal samples was 10 fold less sensitive than that in the pure bacterial culture. This reduction in the sensitivity of the detection of *Helicobacter* spp. in clinical specimens agrees with the report of the detection of *H. pylori* using PCR by Bamford *et al.* [198]. In this study the limit of detection was 10 CFU when using pure culture suspension of *H. pylori*. However the sensitivity of the detection was reduced when a PCR was used to detect *H. pylori* in faces and dental plaque. The limit of detection in these latter samples ranged from 10^2 to 10^4 depending upon the actual specimen. Bamford *et al.* suggested that a variety of substances present in faeces, dental plaque as well as foods can inhibit polymerase enzymes.

Fluorescent *in situ* hybridisation

Both Eubacterial (EUB338-FITC) and *Helicobacter* genus specific (HRh) probes were used to evaluate the application of FISH for the determination of the spatial distribution and localisation of *Helicobacter* species in formalin fixed tissue sections. FISH was applied to a section of rectal tissue obtained from a brushtail possum known to be *Helicobacter* positive by cultivation and PCR. Large numbers of bacteria, many of which were identified as *Helicobacter*

species, were found in the mucus layer overlying the epithelium of the GIT of the marsupials examined. Under optimised conditions, the specificity of FISH is dependant on the specificity of the probe used. The specificity of the hybridisation in a FISH reaction in this study is roughly equivalent to that of a *Helicobacter* genus specific PCR. The *Helicobacter* genus specific probe used in FISH was based on the region of 16S rRNA gene specific for *Helicobacter* species at the same region as the complementary sequence of primer H267f used in *Helicobacter* genus specific PCR as demonstrated below:

Sequence of probe used in FISH - 5' TCTCAGGCCGGATACCCGTCATAGCCT 3'

Complementary sequence of H267f - 5' GCCGGATACCCGTCATAG 3'

Sequence of H267f - 5' CTATGACGGGTATCCGGC 3'

As the specificity of the probe used in the FISH is expected to be similar to the probe used in PCR, the specificity of this probe was tested only with pure cultures of *H. pylori* strain SS1 as a positive control and *Psychrobacter* sp. strain SW5 and *C. jejuni* as negative controls. In addition the aims of using FISH in this study were to determine the locations of *Helicobacters* in the *Helicobacter* species positive sections of marsupials GIT.

It is generally considered that the detection of *Helicobacter* spp. is conclusive if all detection methods used provide the same result. In cases where cultivation is not successful it is still very important to know if there is any helicobacters present. This can be assessed by the use of microscopy and/or molecular methods such as PCR and FISH. Though the success of PCR and FISH are heavily dependant on the specificity of the oligonucleotide primers or probe used, in general the detection of *Helicobacter* by PCR is very sensitive. FISH may not be as sensitive as PCR due to the limitations in the detection of *Helicobacter* in their natural microhabitat. The visualisation of *Helicobacters* is dependent on the detection of the organisms in a 5 μm section of paraffin embedded tissue. The value of FISH over PCR is that FISH provides visual information about the presence, number, morphology and spatial distribution of microorganisms, within their natural microhabitat.

If no spiral and fusiform shaped bacteria are detected by phase contrast microscopy or by *Helicobacter* genus specific PCR or cultivation it is unlikely that there are any *Helicobacter* species present in the particular sample. In the current study however helicobacters could be readily identified in samples obtained from the lower bowel of most of the animals examined. In general, helicobacters were not seen in the small intestine, stomach and liver, though there were exceptions. *Helicobacters* were detected in the stomachs of a kangaroo and a Tasmanian devil.

If spiral and fusiform shaped bacteria were detected by phase contrast microscopy, but no helicobacters were successfully cultivated or detected by PCR then it is possible that the organisms seen by microscopy do not belong to the genus *Helicobacter.* Previous studies in our laboratory have shown that spiral-shaped bacteria isolated from the GIT of mammals can also belong to *Campylobacter* genus or as yet unclassified genera.

Overall while no single method is perfect for the detection and isolation of *Helicobacter* species from a mixed microbial community, each method is able to provide useful information. Thus use of the combination of several methods to provide the best picture of the colonisation of Australian marsupials by *Helicobacter* spp. has been adopted for further in-depth studies of these animals, as will be described in the following Chapters.

Chapter 4

The detection and isolation of spiral and fusiform organisms, in particular *Helicobacter* species, from the gastrointestinal tract of the brushtail possum

4.1 Introduction

Australian possums are a diverse group ranging from tiny gliding pygmy-possums to large agile climbing brushtail possums and cuscuses [136]. They are all diprotodonts feeding mainly on leaves, shoots, flowers and fruit. As a group, they have successfully adapted to the changes brought about by human impact on their natural environment and share many similar characteristics. The common brushtail possum (*Trichosurus vulpecula*) is the largest arboreal (tree-dwelling) marsupial herbivore. It is equivalent to a domestic cat in size (Figure 4.1). Common brushtail possums (BTPs) are frequent in urban areas and are distributed throughout the Australian (including Tasmanian) woodland. They take advantage of the shelters provided for them in household roofs and sheds. They feed on a variety of leaves, particularly eucalypts. Although to some extent the livers of the BTPs are able to detoxify the poisons in eucalypt leaves, they cannot cope with an exclusive diet of this abundant food and must supplement their diet with fruit, buds, barks, and occasionally clover and other pasture plants. They also eat a variety of foods provided unintentionally by humans in their gardens and rubbish bins, and occasionally they may eat meat. Physiologically the BTP is a ‘caecum fermenter’ [146]. Their digestive tract consists of a simple stomach and a well developed caecum and proximal colon (Figure 4.2).

Many studies have examined the immunobiology, anatomy, physiology, nutrition, digestion, morphology and function of the GIT of BTP’s and compared this with that found in other related marsupials [146, 155, 199-201]. By comparison, only a few studies have investigated pathogenic organisms and their association with brushtail possum pathology. A wide range of helminths have been found, mostly associated with the small intestine of the BTP [146]. However no details are available on the pathology associated with these

helminths. In New Zealand, the brushtail possum has been implicated as the principal wildlife reservoir for *Mycobacterium bovis,* the causative agent of bovine tuberculosis (TB) [202, 203]. It has been suggested that *Mycobacterium bovis* was first introduced into possums from infected livestock and that they now constantly reinfect pastures causing problems for the livestock industry. This organism has been most commonly found in the lungs and lymph nodes and occasionally within generalised lesions in the liver, spleen and kidney of possums [204]. The immunobiology of mycobacterial infections in the brushtail possum and other marsupials has been reviewed by Buddle *et al.* (2000) [199]. They suggested that the susceptibility to mycobacterial infections in possums and other marsupials may be linked to deficiencies in their cellular immunity.

In a study carried out by Canfield *et al.* (1991), Tyzzer's disease (*Bacillus piliformis* infection) was diagnosed in nine marsupials (two brushtail possums, three ringtail possums, an unspecified possum species, a koala, a wombat and a dasyurid) [205]. In these infected animals it was apparent that the liver and heart were the common sites for both gross and microscopic lesions associated with this disease. No macroscopic changes were present in the gastrointestinal tract of these marsupials. Since *B. piliformis* could not be grown on conventional bacteriological media, the diagnosis of Tyzzer's disease in this study was based on a combination of clinical history, appearance of lesions and the presence of typical organisms, assessed using a silver stain, in the affected sites.

Prior to the current study, very little was known about the normal gastrointestinal microbiota of the brushtail possum. The aim of this study was to investigate the gastrointestinal microbiota of the BTP for the presence of spiral and fusiform shaped bacteria, and in particular *Helicobacter* spp., colonising gastrointestinal mucus in different regions of the GIT. This study has the potential to provide important information relating to the specific natural niche of these bacteria and the co-relationship between mucus-associated microorganisms and their brushtail possum hosts and substantially increase our understanding of the ecology of *Helicobacter* species.

Figure 4.1 The brushtail possum (*Trichosurus vulpecula*) photographed by 'The Parks and Wildlife Service', Tasmania.

4.2 Materials and experimental methods

4.2.1 Animal history and collection of specimens

The animals examined in this study were obtained from the Veterinary & Quarantine centre, Taronga Zoo, Sydney, Australia. All BTPs were wild animals living freely in the Taronga Zoo area (non caged), in surrounding National Parks (NP) such as Ku-ring-gai Chase National Park, Lane Cove National Park or in suburban areas of Sydney, New South Wales (e.g. Mosman and North Sydney). All BTPs examined had been injured or were in ill health and had been taken to the Veterinary & Quarantine centre for care and had subsequently died or had been euthanased for compassionate reasons. The animal history of the 11 BTPs is summarised in Table 4.1.

Specimens from the 11 BTPs were collected as described in section 2.2. For each animal, three samples of tissue from each of the following sites were collected: the liver, stomach, mid ileum, ileum at 3 centimetres (cm) above caecum, caecum, colon and rectum (Figure 4.2). The first sample from a particular location was frozen at -70°C for DNA extraction. The second sample was frozen in one mL of BHIG and kept at -70°C until cultured and the third sample was fixed in formalin for histology and FISH. The frozen samples were placed on dry ice during delivery to the laboratory.

4.2.2 Bacterial cultivation

Prior to culture the presence of spiral and/or fusiform shaped bacteria was determined in homogenised livers and mucus scrapings obtained from the different regions of the GIT. To aid in visualisation, the samples were mixed with normal saline and examined by phase contrast microscopy at 1000x magnification.

Following microscopic examination, samples were cultured on CSA and HBA using both the direct inoculation method and the selective filtration method (see Figure 2.3). Following incubation at 37°C, clear colourless colonies with a diameter less than 0.5 -1.0 mm or any thin water-like films on the CSA and HBA

plates were examined by phase contrast microscopy. Bacteria with a spiral to curved morphology and those with a straight fusiform morphology were subcultured onto fresh CSA and HBA plates to obtain pure cultures. Following isolation these pure cultures were then screened for *Helicobacter* species by the use of a *Helicobacter* genus specific PCR. Isolates positive in the *Helicobacter* genus specific PCR, as well as some of the *Helicobacter* genus specific PCR negative colonies that had a spiral morphology, were further analysed by sequencing of their 16S rRNA gene. The morphology of representative organisms was also examined using transmission electron microscopy.

4.2.3 Detection of *Helicobacter* species from mucus scrapings of the GIT by *Helicobacter* genus specific PCR

DNA from the liver and GIT mucus scrapings of all 11 BTPs was extracted using the phenol chloroform method (Section 2.8.1). The DNA was then used in a direct and a nested *Helicobacter* genus specific PCR as described in Chapter 2. The universal bacterial primers, F27 and R1494, were used in the first round of the nested PCR, *Helicobacter* genus specific primers, H276f and H676r, were used in the second round of the nested PCR and in the direct genus specific PCR (Table 2.1). Both the direct and nested *Helicobacter* specific PCR's were performed in parallel to the bacterial culture. Preparation of the PCR template and the PCR protocol, including all the primers used in the PCR reactions, are described in Sections 2.8, 2.9 and Table 2.1, respectively.

4.2.4 Histopathology

Formalin fixed samples were embedded in paraffin and 5-μm sections were cut and stained with hematoxylin and eosin (H&E) for histopathological analysis and the modified Steiner silver stain for bacterial observation. The histopathology of the liver and gastrointestinal tract was analysed by Dr. Karrie Rose, Pathologist, Veterinary & Quarantine Centre, Taronga Zoo.

4.2.5 The spatial distribution of bacteria in fixed sections of the liver and different regions of the GIT

The morphology and distribution of the bacteria in the liver, stomach and different regions of the GIT was observed in the silver stained sections using light microscopy.

The spatial distribution of bacteria belonging to the genus *Helicobacter* was further observed using fluorescent *in situ* hybridisation (FISH) with a *Helicobacter* genus specific probe (Section 3.4) in formalin-fixed rectal sections of 4 brushtail possums in which *Helicobacter* species had been isolated and detected by PCR. Prior to performing the FISH technique, two sequential sections of each of the four formalin-fixed rectal samples were cut. One was silver stained and the other de-waxed for FISH. Areas observed by light microscopy of silver stained rectal sections to have a large number of bacteria present were marked on the slides. FISH was then performed on the de-waxed sequential sections on this marked location.

In order to observe the distribution of *Helicobacter* species in relation to the other indigenous microbiota, two probes, a *Helicobacter* genus specific probe, labelled with tetramethyl-rhodamine-isothiocyanate (HRh), and a eubacterial probe, labelled with fluorescein-isothiocyanate (Eu338-FITC), were both applied to the same de-waxed slide. The eubacterial probe hybridises with all bacteria resulting in a green colour which is observed using an appropriate filter set. Bacteria that hybridise with the *Helicobacter* genus specific probe show a red colour which is observed using an appropriate filter set. As *Helicobacter* species are also eubacteria, these bacteria hybridise with both the HRh and Eu338-FITC probes and both red and green signals are detected at the same time. This results in *Helicobacter* species giving a yellow to orange fluorescent signal (a combination of the red and green signals). The sequences of both the eubacterial probe and the *Helicobacter* genus specific probes used in the FISH reaction are shown in Table 3.1.

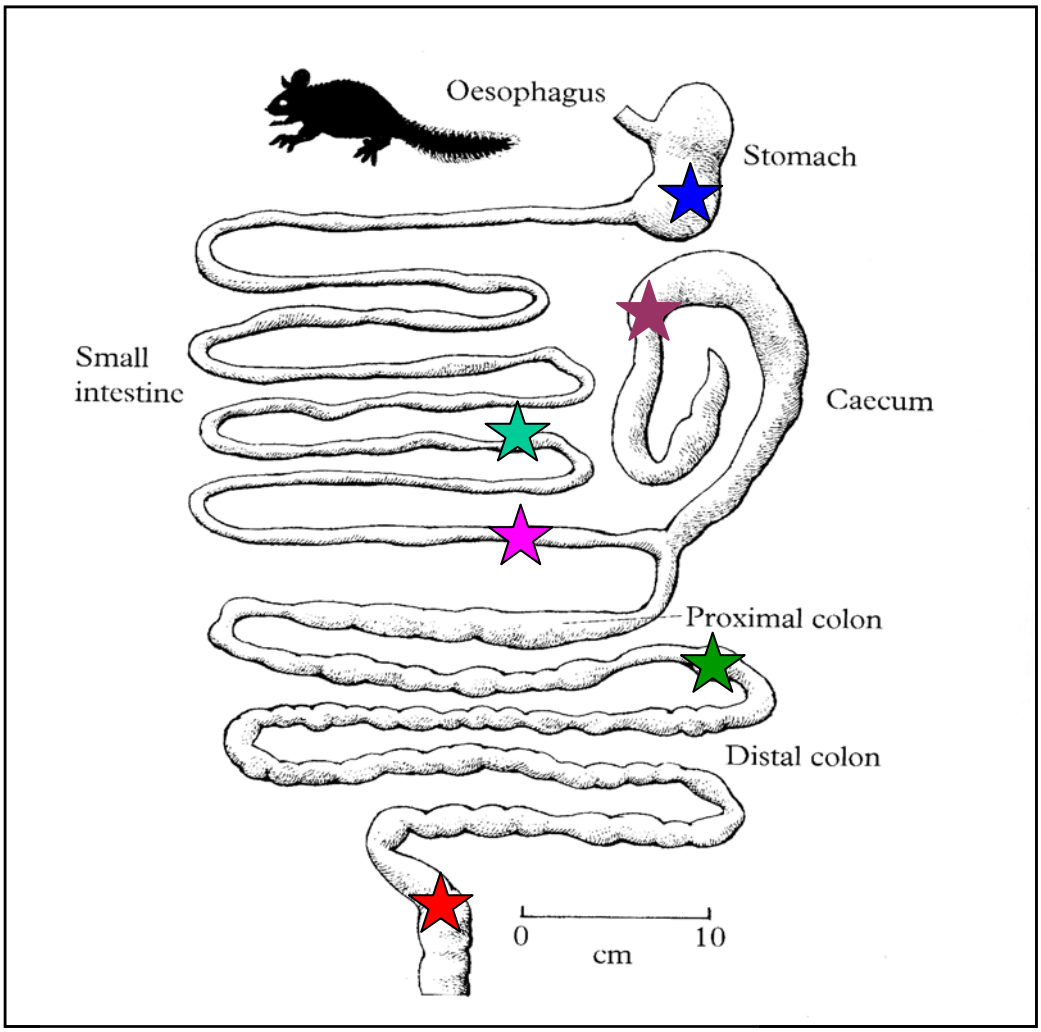


Figure 4.2 Regions of the gastrointestinal tract of the brushtail possum from which specimens were collected.

★ = Stomach

★ = Mid ileum

★ = Ileum at 3 cm above the caecum

★ = Caecum

★ = Colon

★ = Rectum

Animal	Habitat	Sex	Age	Cause of dead	Period of trauma (days)	Gross pathology
BTP1	Mosman	M	A	Accident/ Euthanased	4	Subacute fracture, right radius & tibia
BTP2	Taronga Zoo	M	A	Diseased/ Euthanased	4	Focal spinal abscess-haemolytic *Streptococcus* gr. C found in liver, spleen, shoulder and spine
BTP3	Taronga Zoo	F	A	Accident/ Euthanased	10	Sub acute ulcer-anterior left carpus (caught in a fox jaw trap)
BTP4	Mosman	M	A	Poisoned	1	Marked extensive subcutaneous haemorrhage, most consistent with the ingestion of rat bait poison
BTP5	Taronga Zoo	-	A	Attacked/ Euthanased	<1	Amputated right hind foot (predation by carnivore)
BTP6	Ku-ring-gai Chase NP	-	A	Diseased/ Euthanased	2	Large, chronic ulceration at left and right tail base with patchy alopecia at ears and thorax
BTP7	North Sydney	-	A	Diseased/ Euthanased	3	Extensive multifocal ulceration and dermatitis of face, rump and scrotum
BTP8	Lane Cove	M	A	Attacked/ Euthanased	<1	Missing left eye and other injuries
BTP9	Mosman	F	A	Diseased/ Euthanased	-	Large mammary mass
BTP10	Taronga Zoo	F	A	Found dead	-	Fractured spine and pucture wounds
BTP11	Mosman	F	A	Found dead	-	Acute traumatic injuries

Table 4.1 The habitat, sex, age, cause of death, period in care and gross pathology of the brushtail possums examined in this study.

F = female, M = male, A = adult, - = no details available, NP = National Park

4.3 Results

4.3.1 Bacterial cultivation

Preliminary phase microscopic examination showed motile, curved to spiral rods to be present in the majority of the mucus scrapings obtained from the GIT of the possums. In all 11 BTPs high numbers of spiral shaped bacteria were observed in mucus scrapings obtained from the caecum, colon and rectum. In addition to the spiral shaped bacteria, fusiform shaped bacteria were observed in the rectal and colonic areas of the GIT of 3 of the 11 BTPs.

Bacterial culture of mucus scrapings obtained from the lower bowel of all 11 BTPs showed either clear colourless, small to pinpoint colonies or thin water-like films on both the CSA and HBA plates after 5 to 7 days incubation, under either microaerobic or anaerobic conditions. Microscopic examination of these colonies showed two morphological types to be present. Those with a spiral to curved morphology were designated ‘Sp’ and those with a straight fusiform rod-like morphology were designated ‘F‘. Only bacteria which had a morphological appearance of ‘Sp’ and ‘F’ were subcultured onto fresh CSA and HBA plates to obtain pure cultures. Of these colonies, 48 separate cultures of ‘Sp’ and ‘F’ shaped bacteria were obtained. From the caecum of one brushtail possum an isolate which was a tightly coiled rod was also cultivated. This was referred to as ‘Tcr’.

4.3.1.1 Screening of pure isolates for the presence of *Helicobacter* species using the *Helicobacter* genus specific PCR

DNA extracted from the above 48 isolates was amplified using a *Helicobacter* genus specific PCR (Section 2.9.1). Of these 48 isolates, a total of 28 were identified as *Helicobacter* species. These isolates were cultivated from the ileum at 3 cm above the caecum (n=2), caecum (n=7), colon (n=8) and rectum (n=11). No *Helicobacter* species were isolated from the liver, stomach or mid ileal regions. In some animals only one morphological type was detected, whereas in others both morphological types could be seen (Sp and F). The regions in each of the brushtail possums from which each of the isolates was cultivated and their respective morphologies are shown in Table 4.2. Examples of the

PCR reactions from the *Helicobacter* genus specific reaction amplifying DNA from examples of isolates with different morphology types is shown in Figures 4.4, 4.6 and 4.8.

Sequencing of the near complete 16S rRNA gene of 18 of the 28 *Helicobacter* isolates was undertaken. The rationale for selecting the 18 isolates to be further characterised by sequencing was as follows. In animals in which more than one morphological type was cultured at least 2 isolates with different morphologies were selected from each animal (BTP3, BTP5, and BTP8). However from animals (BTP4, BTP6, BTP9 and BTP10) in which only one morphological type was isolated, 1 to 3 isolates of the same morphological type were selected from different locations. As only one isolate was cultured from each of the remaining animals (BTP1, BTP2, BTP7 and BTP11) all these isolates were sequenced.

The ‘Tcr’ isolate cultured from the caecum of one brushtail possum did not belong to the genus *Helicobacter* as no PCR product was obtained after amplification with the *Helicobacter* genus specific primers. Sequencing of the near complete 16S rRNA gene of the ‘Tcr’ isolate was also undertaken.

Comparison of the 16S rRNA gene sequences obtained in this study with other sequences from GenBank confirmed that the 18 isolates were potentially novel *Helicobacter* species. The ‘Tcr’ isolate was shown to be a *Campylobacter* species. Phylogenetic analysis of all 16S rRNA gene sequences will be discussed in greater detail in Chapter 6.

4.3.1.2 Morphological analysis of the pure isolates using TEM

Fifteen of the 18 potentially novel *Helicobacter* species described above and the ‘Tcr’ isolate were successfully recultivated for further detailed morphological analysis by TEM. The analysis showed that these 15 negatively stained isolates fell into three morphological types:

1. ‘Comma’ shape; 0.3-0.5 x 1.6-2.2 μm in size; no apparent flagella (Figure 4.3).

2. ‘Fusiform’ shape; 0.5-0.8 x 2.6-3.4 μm in size; 6-12 bipolar sheathed flagella; entwined with periplasmic fibres which appeared to cover the whole cell (Figure 4.5).
3. ‘S’ shape; 0.3-0.5 x 2-3 μm in size; bipolar sheathed flagella; no periplasmic fibrils (Figure 4.7).

Thus using TEM, the spiral shaped bacteria which had been observed by light microscopy could now be separated into two types, ‘comma’ and ‘S’ shaped bacteria. Morphologically the ‘Tcr’ isolate was shown to be a tightly coiled rod with no periplasmic fibrils, 0.5 x 3-4 μm in size and bipolar flagella (Figure 4.9). The 18 isolates and the regions of the GIT from which the *Helicobacter* species were isolated and their bacterial morphology are shown in Table 4.3.

Animals	3-ileum	Caecum	Colon	Rectum
BTP1	ND	ND	ND	**Sp**
BTP2	ND	**Sp**	ND	ND
BTP3	**Sp**	**Sp**	**Sp**	**Sp, F**
BTP4	**Sp**	**Sp**	**Sp**	**Sp**
BTP5	ND	ND	**Sp, F**	**Sp, F**
BTP6	ND	**F**	**F**	ND
BTP7	ND	ND	ND	**F**
BTP8	ND	**Sp**	**Sp**	**F**
BTP9	ND	**Sp**	**Sp**	**Sp**
BTP10	ND	**Sp**	**Sp**	**Sp**
BTP11	ND	**Tcr**	ND	**Sp**

Table 4.2 The regions of the brushtail possum GIT from which *Helicobacter* species were isolated and their bacterial morphology based on light microscopy. (3-ileum = ileum at 3 cm above the caecum, Sp = Spiral shape, F = fusiform shape, Tcr = tightly coiled rod, ND = not detected)

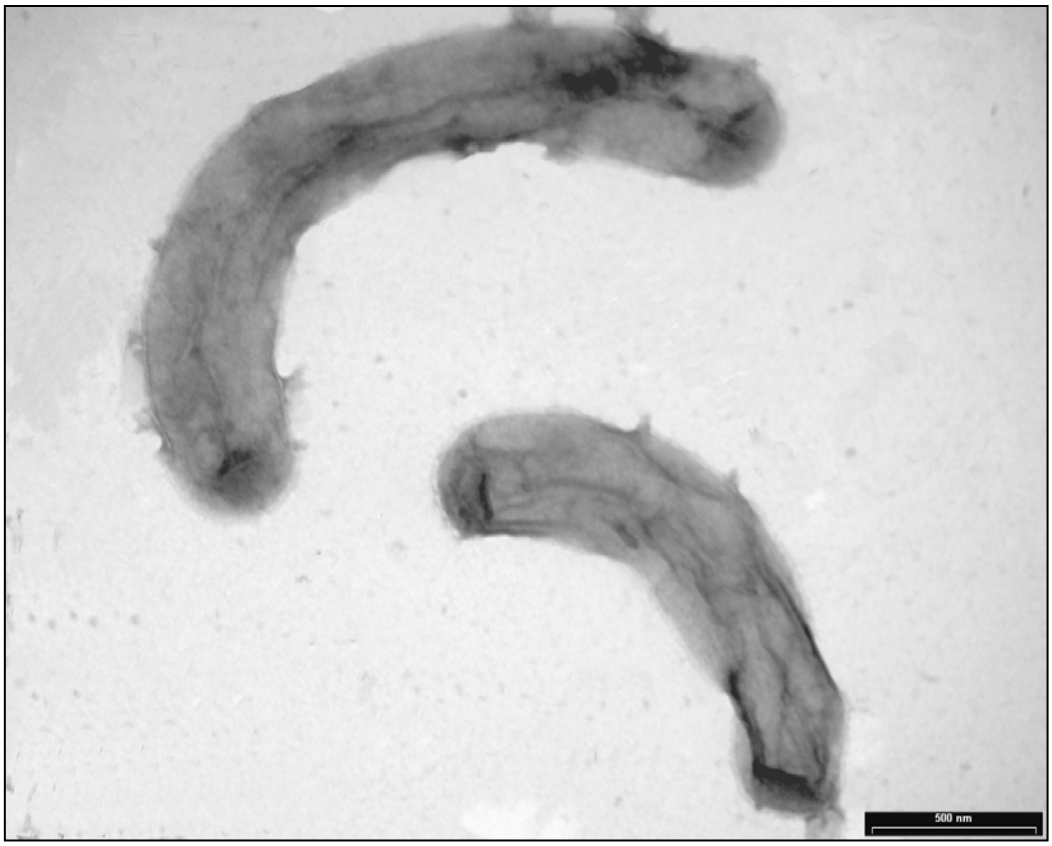


Figure 4.3 A transmission electron micrograph of a 'comma' shaped *Helicobacter* isolate, 'BTP5C'. This bacterium measured 0.3 by 1.5-2.2 μm. No flagella were apparent. (Bar = 500 nm)

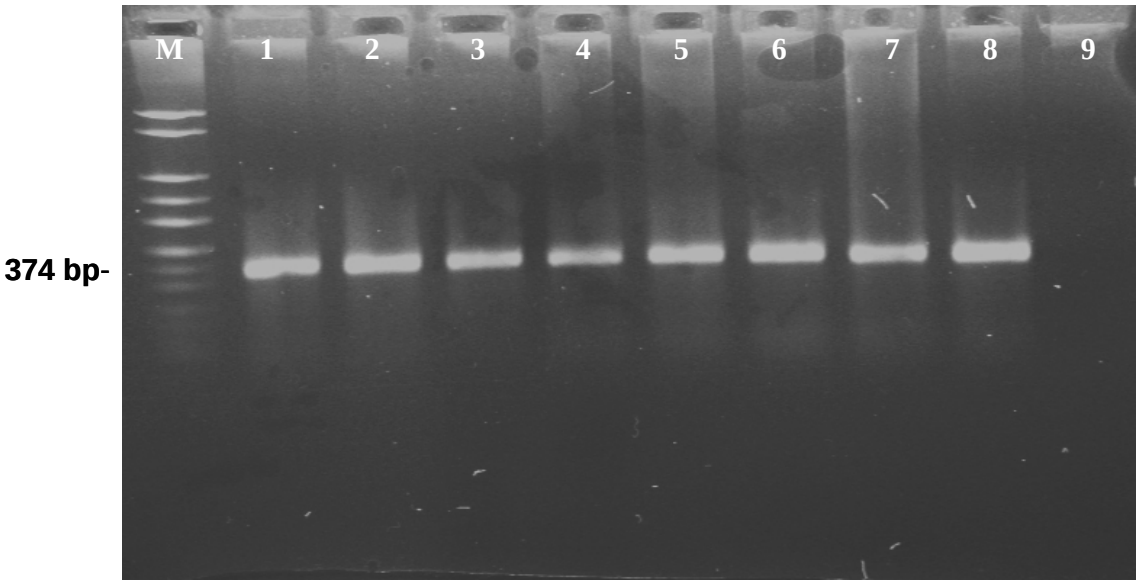


Figure 4.4 The PCR products obtained from the 'comma' shaped bacteria using the *Helicobacter* genus specific PCR, separated on a 1.5 % agarose /TAE gel. (BTP = Brushtail possum)

M = Molecular weigh marker (FN1)
Lane 1 = BTP1C isolate
Lane 2 = BTP2C isolate
Lane 3 = BTP3C isolate
Lane 4 = BTP4C isolate
Lane 5 = BTP6C isolate
Lane 6 = BTP8C isolate
Lane 7 = BTP9C isolate
Lane 8 = *Helicobacter pylori* positive control
Lane 9 = 'No DNA' negative control

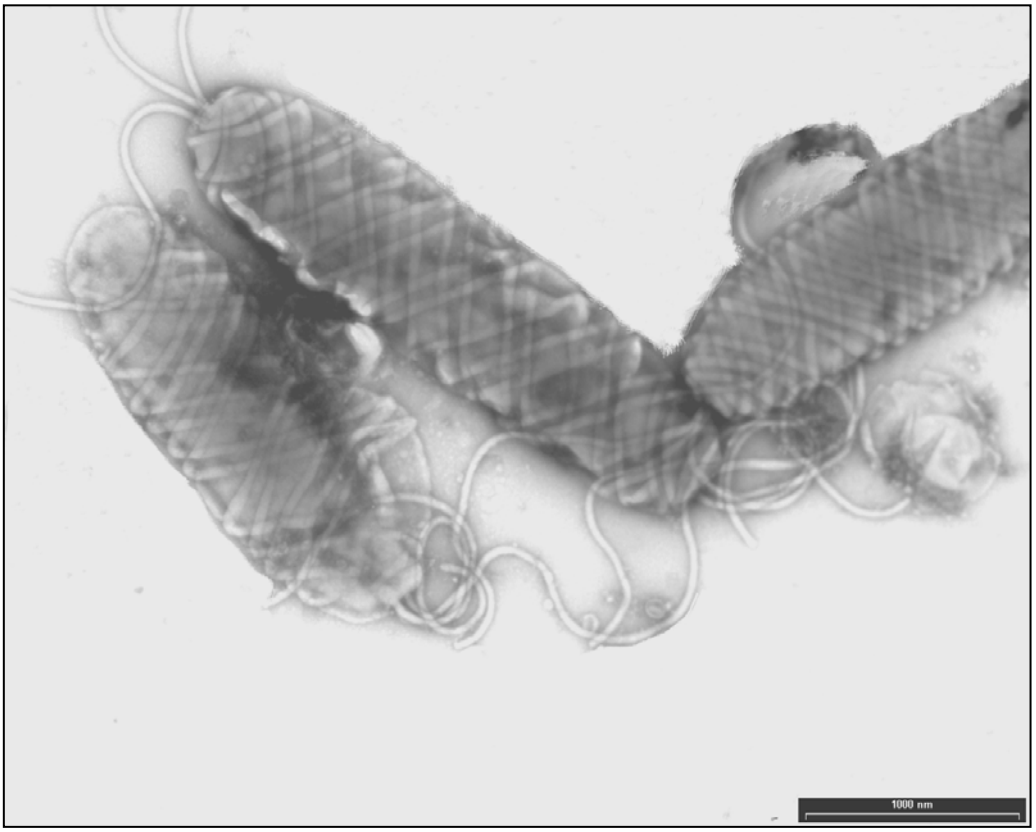


Figure 4.5 A transmission electron micrograph of a 'fusiform' shaped *Helicobacter* isolate, 'BTP4F'. The bacterium measured 0.8 by 2.3-3.0 μm and was entwined with periplasmic fibres which appeared to cover the whole cell. It had multiple (6-12) bipolar flagella. (Bar = 1000 nm)

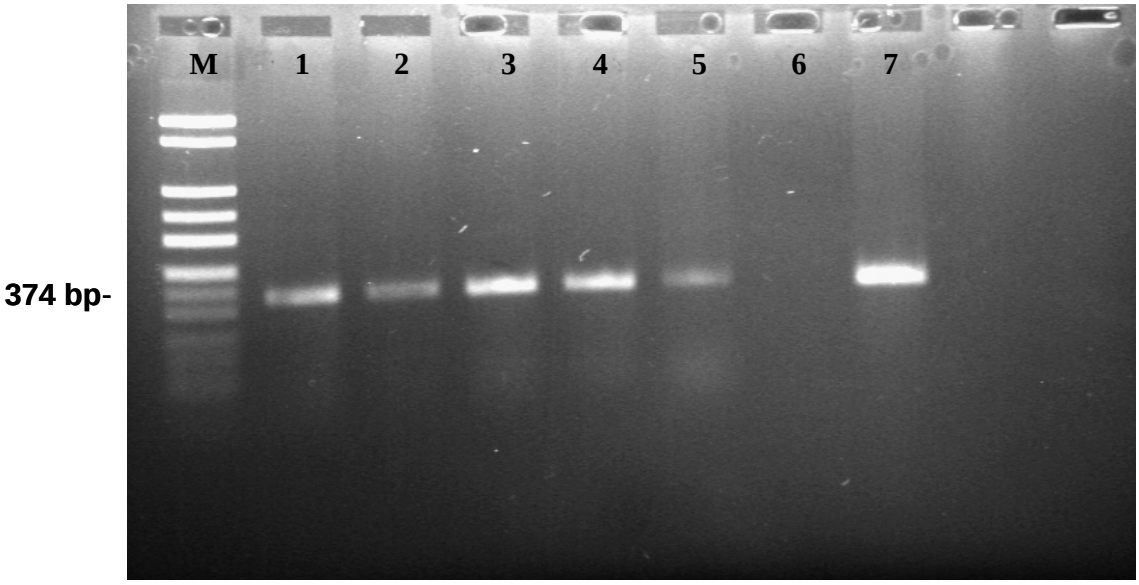


Figure 4.6 The PCR products obtained from the 'fusiform' shaped bacteria using the *Helicobacter* genus specific PCR, separated on a 1.5 % agarose /TAE gel. (BTP=Brushtail possum)

M	=	Molecular weigh marker (FN1)
Lane 1	=	BTP1F isolate
Lane 2	=	BTP2F isolate
Lane 3	=	BTP3F isolate
Lane 4	=	BTP5F isolate
Lane 5	=	BTP6F isolate
Lane 6	=	'No DNA' negative control
Lane 7		*Helicobacter pylori* positive control

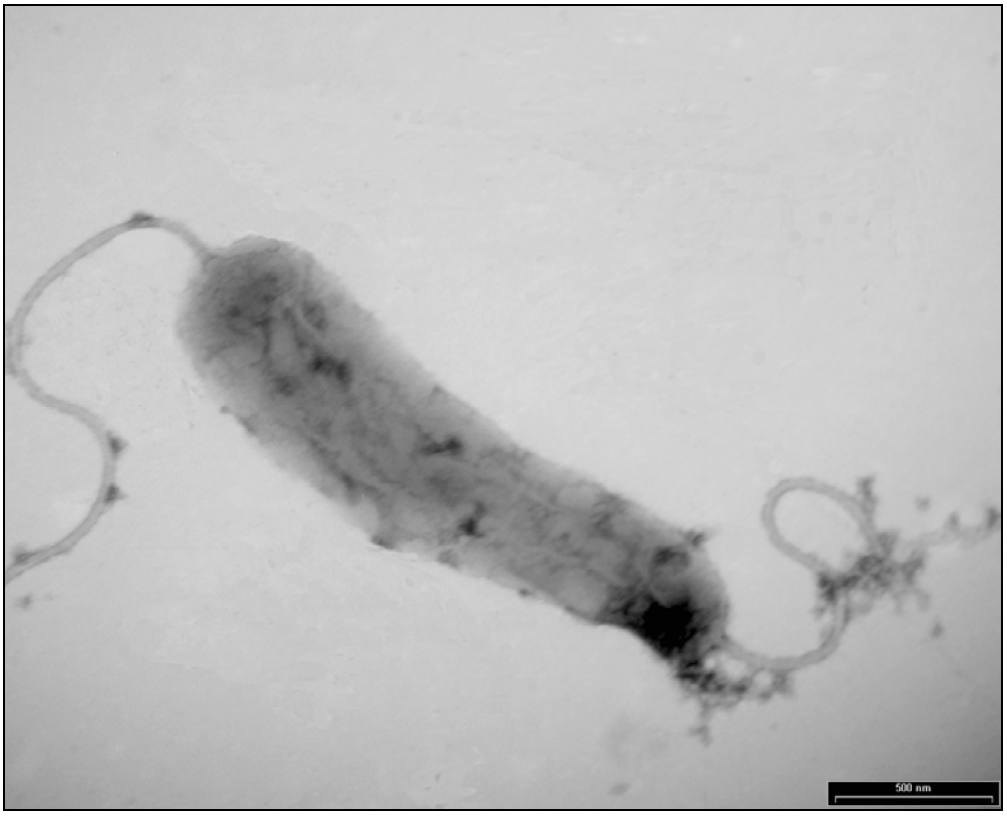


Figure 4.7 A transmission electron micrograph of an 'S' shaped *Helicobacter* isolate, 'BTP1S'. The bacterium measured 0.3 by 2.3 μm and had bipolar, sheathed flagella. (Bar = 500 nm)

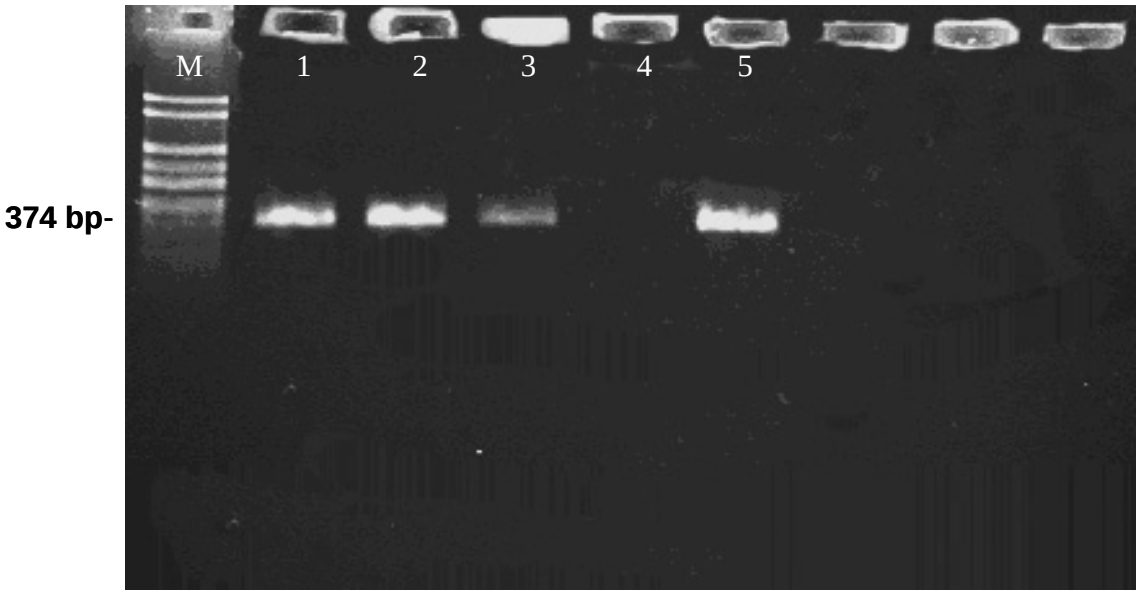


Figure 4.8 The PCR products obtained from the 'S' shaped bacteria using the *Helicobacter* genus specific PCR, separated on a 1.5 % agarose /TAE gel. (BTP = Brushtail possum)

M	=	Molecular weigh marker (FN1)
Lane 1	=	BTP1S isolate
Lane 2	=	BTP2S isolate
Lane 3	=	BTP3S isolate
Lane 4	=	'No DNA' negative control
Lane 5	=	*Helicobacter pylori* positive control

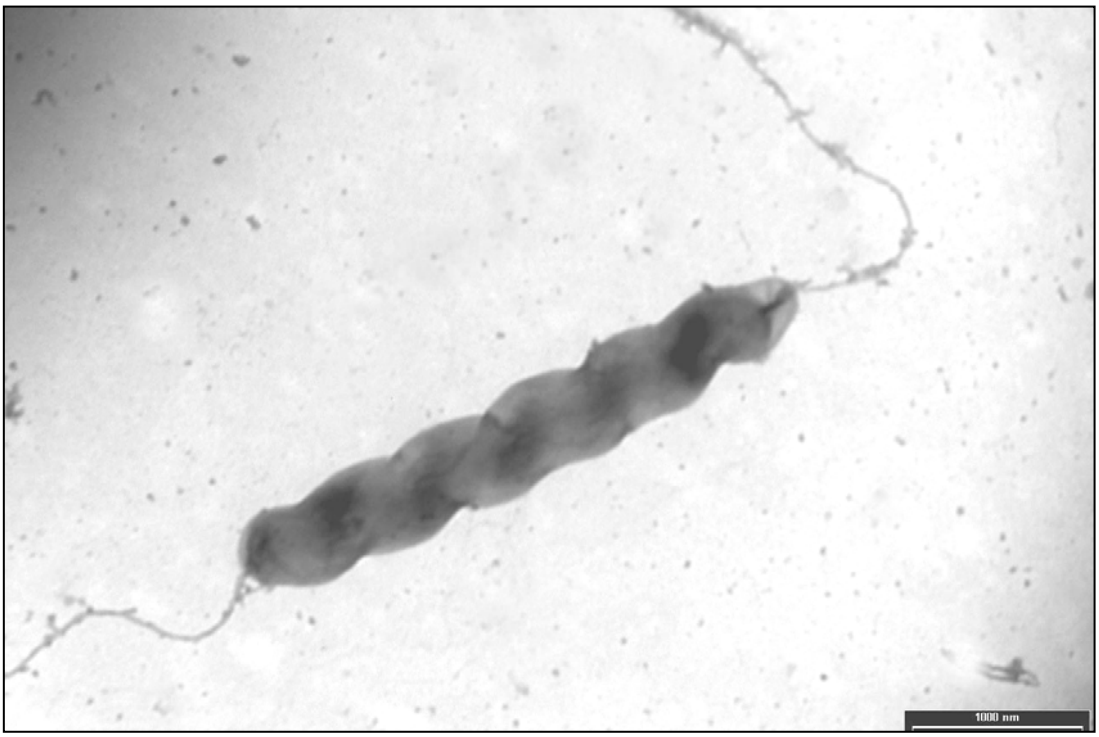


Figure 4.9 A transmission electron micrograph of the tightly coiled rod *Campylobacter* sp. 'Tcr'. The bacterium measured 0.5 by 4 μm and had bipolar flagella. (Bar = 1000 nm)

Name	Origin	Location	Light microscopy	TEM
BTP1C	BTP1	Rectum	Spiral	Comma
BTP2C	BTP3	Rectum	Spiral	Comma
BTP3C	BTP5	Colon	Spiral	Comma
BTP4C	BTP8	Colon	Spiral	Comma
BTP5C	BTP9	Colon	Spiral	Comma
BTP6C	BTP9	Rectum	Spiral	Comma
BTP7C	BTP10	Colon	Spiral	Comma
BTP8C	BTP10	Colon	Spiral	Comma
BTP9C	BTP11	Rectum	Spiral	Comma
BTP1F	BTP3	Rectum	Fusiform	Fusiform
BTP2F	BTP5	Rectum	Fusiform	Fusiform
BTP3F	BTP6	Caecum	Fusiform	ND
BTP4F	BTP6	Colon	Fusiform	Fusiform
BTP5F	BTP7	Rectum	Fusiform	ND
BTP6F	BTP8	Rectum	Fusiform	Fusiform
BTP1S	BTP2	Caecum	Spiral	S
BTP2S	BTP4	Caecum	Spiral	ND
BTP3S	BTP9	Caecum	Spiral	S

Table 4.3 The name, origin, location, and morphology (light microscopy and TEM) of the 18 *Helicobacter* species isolated from the 11 BTPs.

4.3.2 Detection of *Helicobacter* species in the liver fragments and in mucus scrapings of the GIT using direct and nested *Helicobacter* genus specific PCR

4.3.2.1 Detection using direct *Helicobacter* genus specific PCR

Amplification of DNA extracted from the homogenised livers and mucus scrapings of the GIT from all 11 BTPs (Table 4.4) showed a PCR product of the expected size (374 bp) to be present in the following samples:

- Ileum at 3 cm above the caecum (n=2),
- Caecum (n=5),
- Colon (n= 10)
- Rectum (n= 11)

None of the liver, stomach or mid ileum samples were positive in the direct genus specific PCR.

4.3.2.2 Detection using nested *Helicobacter* specific PCR

Amplification of DNA extracted from the homogenised livers and mucus scrapings of the GIT from all 11 BTPs (Table 4.4) showed a PCR product of expected size (374 bp), in the following samples:

- Liver (n= 6),
- Stomach (n=4),
- Mid ileum (n= 5),
- Ileum at 3 cm above the caecum (n= 7),
- Caecum (n= 9),
- Colon (n=10),
- Rectum (n= 11)

Helicobacter DNA was detected by nested PCR in the liver (BTP1, 2, 3, 8, 9 and 11) and all regions of the GIT in four of the brushtail possums studied (BTP1, 2, 3 and 11).

Regions / Animals	Liver	Stomach	Mid ileum	3-ileum	Caecum	Colon	Rectum
BTP1	*	*	*	*	+ *	+ *	~+*
BTP2	*	*	*	+*	~+ *	+ *	+ *
BTP3	*	*	*	~+ *	~+ *	~+ *	$\sim_2$+ *
BTP4	–	–	–	~ *	~+ *	~+ *	~+ *
BTP5	–	–	–	–	–	$\sim_2$+ *	$\sim_2$+ *
BTP6	–	–	–	–	~*	~+*	+ *
BTP7	–	–	–	–	–	–	~+ *
BTP8	*	–	–	*	~*	~+ *	~+ *
BTP9	*	–	–	–	~*	~+ *	~+ *
BTP10	–	–	*	*	~*	~+ *	~+ *
BTP11	*	*	*	*	+ *	+ *	~+ *

Table 4.4 The results of *Helicobacter* culture, direct and nested PCR obtained from the 11 BTP's.

3-ileum = ileum at 3 cm above the caecum,

~ = *Helicobacter* culture positive,

$\sim_2$ = two isolates with different morphologies cultured,

+ = direct PCR positive, * = nested PCR positive,

- = *Helicobacter* culture, direct and nested PCR, all negative.

4.3.2.3 Comparison of the detection of *Helicobacter* species by bacterial culture and PCR

A comparison of the number and percentage detection of *Helicobacter* species in different regions of the gastrointestinal tract of the 11 brushtail possums by direct and nested *Helicobacter* genus specific PCR and culture is shown in Table 4.5.

The isolation of *Helicobacters* by the culture method was shown to be compatible with the detection of *Helicobacter* DNA using the direct PCR method. No helicobacters were isolated or detected using these 2 methods from the liver, stomach and mid ileum of all 11 BTP's (Table 4.5). The same isolation and PCR detection results were obtained from the ileum at 3 cm above the caecum, that is, helicobacters were isolated and detected from 2 of the 11 BTPs (18.2%). With respect to the caecal samples, culture seemed to be more sensitive than direct PCR as helicobacters were isolated from 7 of 11 BTPs (63.6%), while they were detected in only 5 of 11 BTPs (45.5%) using direct PCR. However from the colonic and rectal samples, direct PCR was more sensitive in the detection of helicobacters, 10 of the 11 BTPs (90.9%) and 11 of 11 the BTPs (100%) respectively, than the culture method, 7 of 11 BTPs (63.6%) and 9 of 11 BTPs (81.8%) respectively.

Nested PCR was shown to be a sensitive method for the detection of *Helicobacter* DNA, with *Helicobacter* DNA detected from the caecal, colonic and rectal samples from which helicobacters were isolated by culture, or detected by direct PCR. From the ileum at 3 cm above the caecum samples *Helicobacter* DNA was detected in 7 of the 11 BTPs using nested PCR. Of these 7 BTPs, 4 had no evidence of *Helicobacter* species by culture and/or direct PCR (see Table 4.4). Furthermore, while helicobacters were neither isolated by culture nor detected by direct PCR, *Helicobacter* DNA was detected in the liver (6 of the 11 BTPs, 54.5%), stomach (4 of the 11 BTPs, 36.4%) and mid ileum (5 of the 11 BTPs, 45.5%) of some of the possums (Table 4.5).

Region	Cultivation	Direct PCR	Nested PCR
Liver	0/11 (0%)	0/11 (0%)	6/11 (54.5%)
Stomach	0/11 (0%)	0/11 (0%)	4/11 (36.4%)
Mid ileum	0/11 (0%)	0/11 (0%)	5/11 (45.5%)
Ileum at 3 cm above the caecum	2/11 (18.2%)	2/11 (18.2%)	7/11 (63.6%)
Caecum	7/11 (63.6%)	5/11 (45.5%)	9/11 (81.8%)
Colon	7/11 (63.6%)	10/11 (90.9%)	10/11 (90.9%)
Rectum	9/11 81.8%)	11/11 (100%)	11/11 (100%)

Table 4.5 A comparison of the number and percentage of *Helicobacter* species detected in different regions of the gastrointestinal tract of the 11 brushtail possums using culture and direct and nested *Helicobacter* genus specific PCR.

4.3.3 The spatial distribution of mucus-associated bacteria in fixed sections of the liver and different regions of the GIT of BTPs

From the silver stained liver sections only a few curved to spiral shaped bacteria were observed in 2 of the 11 BTPs (BTP 1 and BTP 6) (see Figure 4.10). A very low number of bacteria were observed in the stomach, mid ileum and ileum at 3 cm above the caecum sections of most of the BTP's studied. Large numbers of curved to spiral shaped bacteria were observed in the mucus layer overlying the epithelium and in the crypts of the rectal, colonic and caecal samples (see Table 4.6). Examples of the distribution of mucus-associated bacteria in the rectal regions of BTPs 6, 7, 8, 9, 10 and 11 in silver stained sections are shown in Figures 4.11 A, B, C, D, E, and F, respectively.

The spatial distribution of bacteria belonging to the genus *Helicobacter* was further observed using fluorescent *in situ* hybridisation (FISH) with a *Helicobacter* genus specific probe (Section 3.4) from the formalin-fixed rectal sections of 4 brushtail possums (BTP 6, 7, 8 and 10) in which *Helicobacter* species had been isolated and detected by PCR. In these 4 rectal sections *Helicobacter* species were shown to localise predominantly in the mucus layer overlying the surface epithelium and within the crypts of the rectum. Images of the hybridised bacteria taken using epi-fluorescent microscopy and confocal scanning microscopy are shown in Figure 3.8 and Figures 3.9 and 4.12, respectively. Most of the bacteria present in the mucus lining the epithelium and in the rectal crypts were shown to belong to the genus *Helicobacter* (red colour in Figure 3.8 B & D, Figure 3.9 B and Figure 4.12 B and orange colour in Figure 3.10 C and Figure 4.12 C).

4.3.4 Histopathology

Histopathological examination of liver sections from all eleven brushtail possums showed that all were within the normal range with only a mild multifocal portal infiltration of small mononuclear cells being evident. No significant lesions were observed in the stomachs of 9 of the 11 BTPs. In 2 of the 11 BTPs studied (BTP5 and BTP9), scattered lymphocytes, plasma cells

and eosinophils were observed in the lamina propria of the stomach. The lower bowel of all BTPs was normal with no infiltration being observed in the intestinal mucosa. Full details of the histopathology observed in the hematoxylin and eosin stained sections of the liver and all specimens collected from the GIT of all animals studied is shown in Appendix 1.

Regions / Animals	Liver	Stomach	Mid ileum	3-ileum	Caecum	Colon	Rectum
BTP1	+	+	+	+	++++	++++	++++
BTP2	-	+	+	+	+++	++++	++++
BTP3	-	+	+	+	++++	++++	++++
BTP4	-	-	-	+	+++	++++	++++
BTP5	-	-	-	+	+++	+++	+++
BTP6	+	+	+	+	++	+++	++++
BTP7	-	-	-	+	++	++	+++
BTP8	-	-	-	+	+++	++++	++++
BTP9	-	-	-	++	+++	++++	++++
BTP10	-	-	+	++	++++	++++	++++
BTP11	-	-	+	++	++++	++++	++++

Table 4.6 The grading of curved to spiral shaped microorganisms detected by histology in the liver, epithelial mucus layer and crypts of the gastrointestinal tissue of the 11 BTPs.

- = no bacteria found

+ = very few of curved to spiral shaped microorganisms present

++ = a few curved to spiral shaped microorganisms present

+++ = a moderate number of curved to spiral shaped microorganisms present

++++ = numerous curved to spiral shaped microorganisms present

(3-ileum = ileum at 3 cm above the caecum)

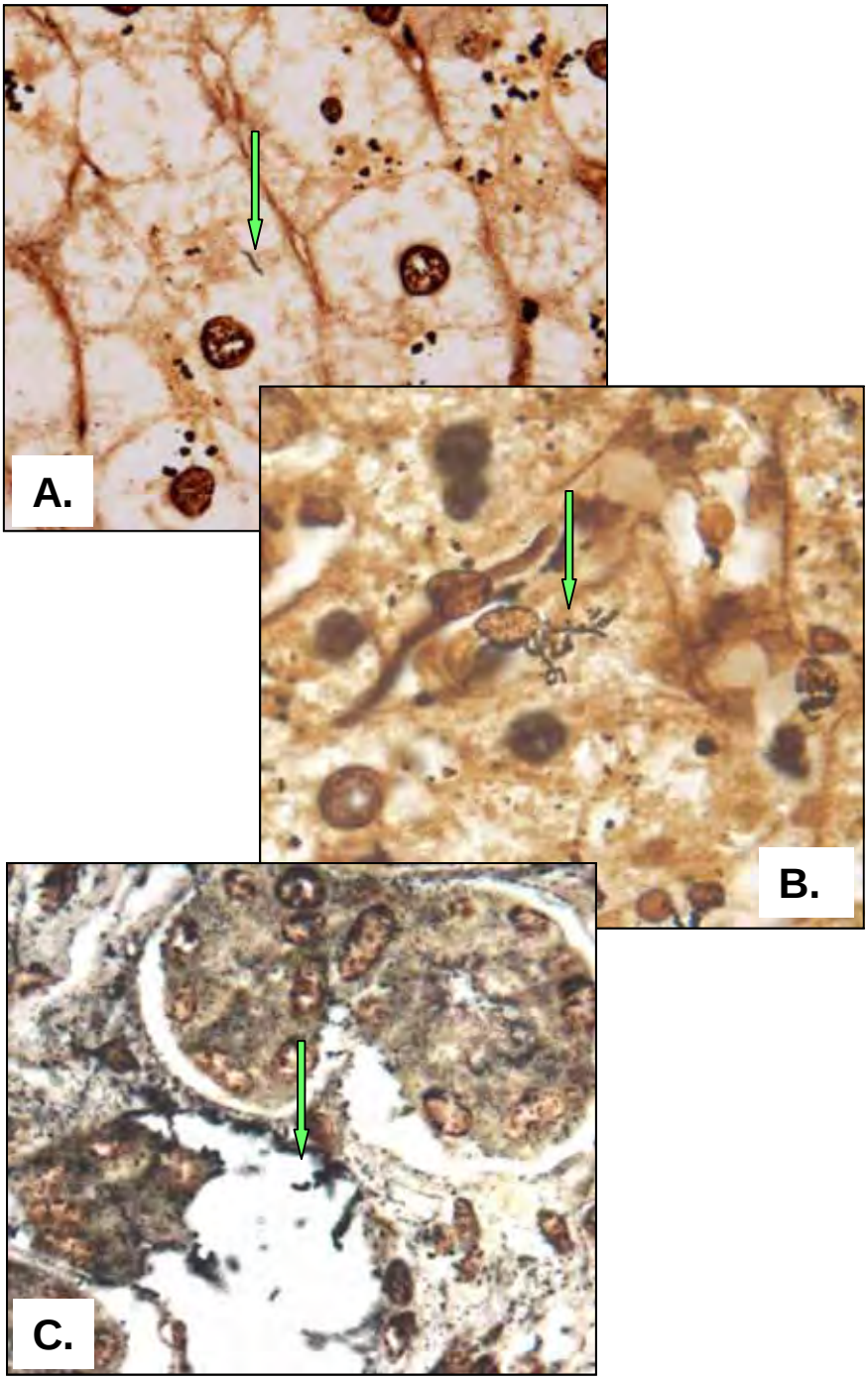


Figure 4.10 Photomicrographs of silver stained liver and stomach samples showing very few numbers of curved to spiral shaped organisms.

A = the liver of BTP 1, B = the liver of BTP 6, C = the stomach of BTP 3

Magnification = 1000x

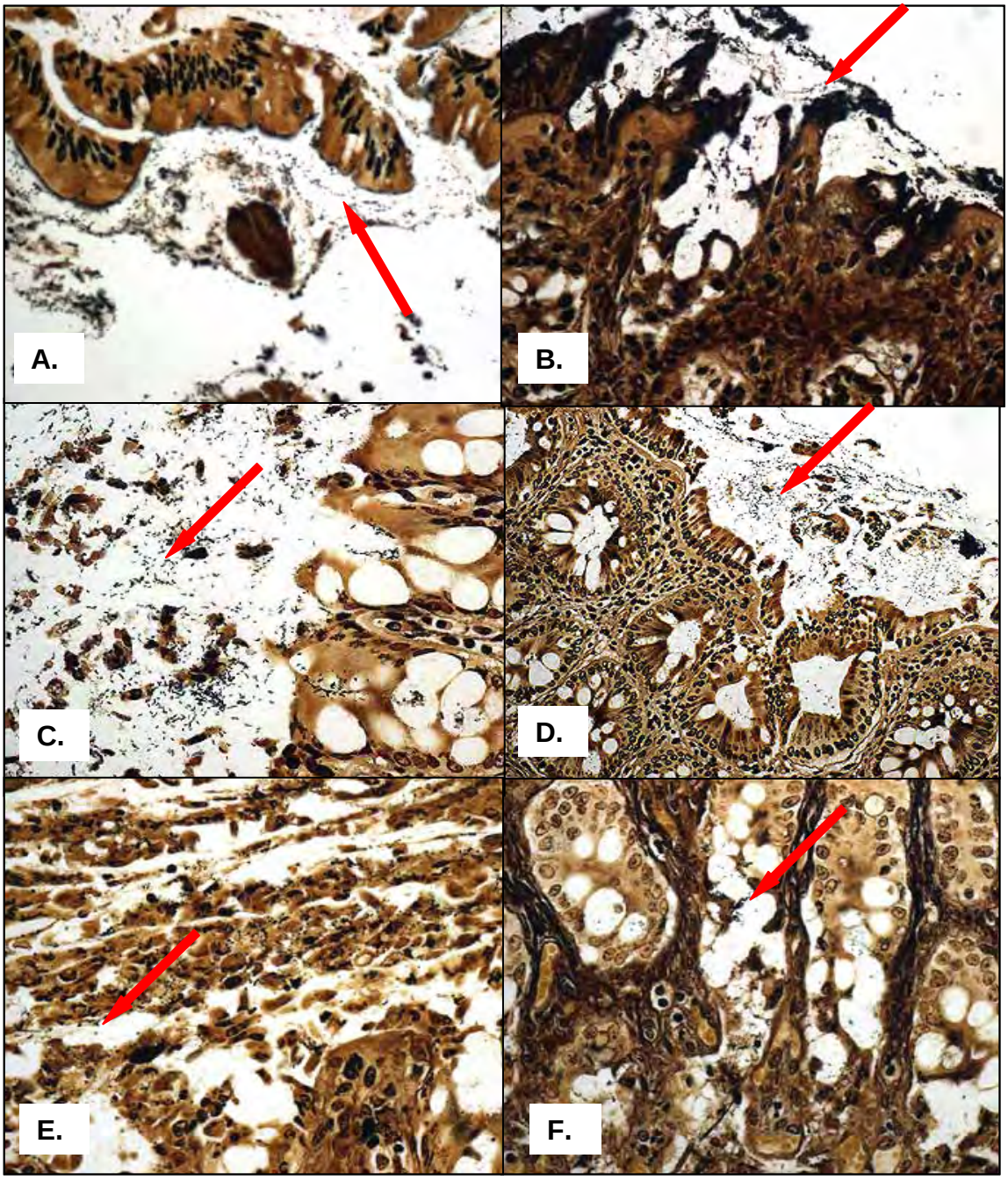


Figure 4.11 Photomicrographs of silver stained rectal samples showing high numbers of curved to spiral shaped organisms in the mucus layer lining the rectum of the brushtail possums (A = BTP 6; B = BTP 7; C = BTP 8; D = BTP 9; E= BTP10 and F= BTP11). Magnification = 400x

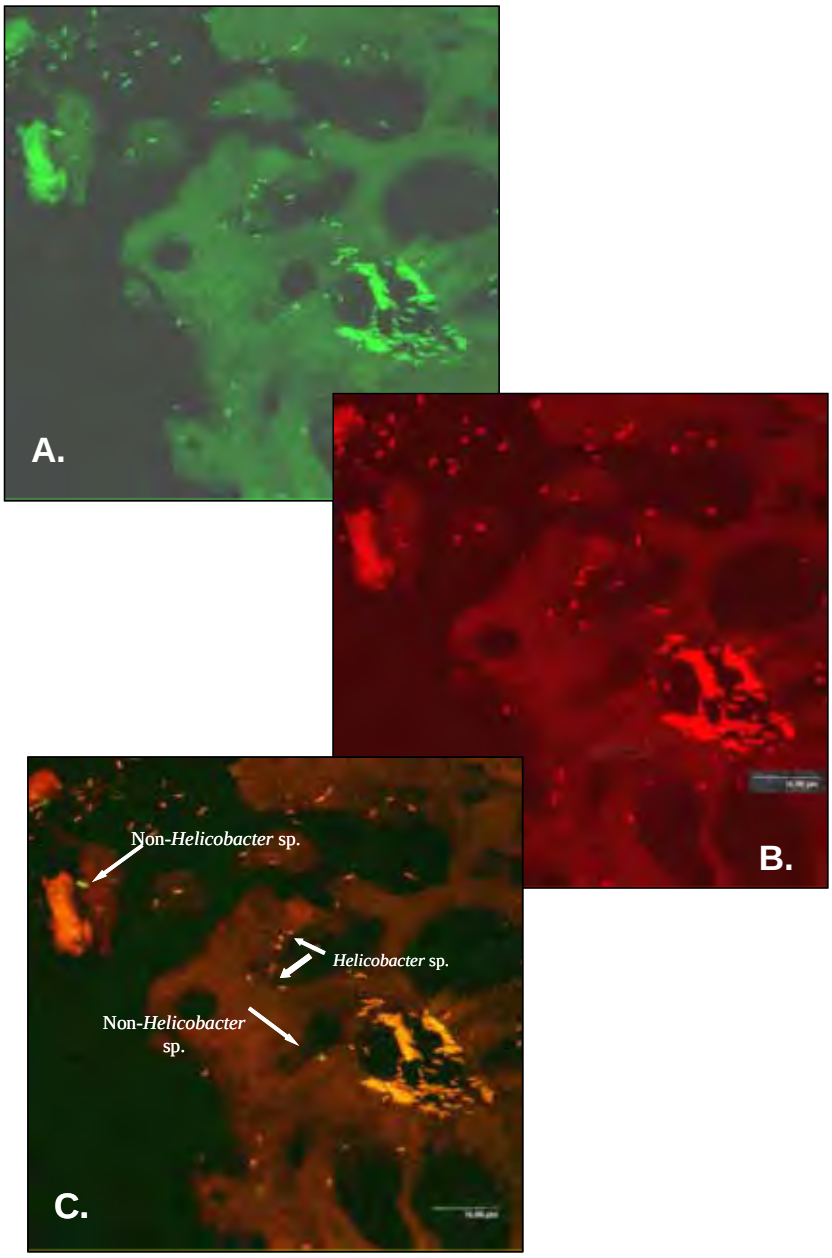


Figure 4.12 Confocal-fluorescent photomicrographs of a rectal sample taken from BTP8 showing that the majority of curved to spiral bacteria colonising the mucus layer belong to the *Helicobacter* genus.

(A) Bacteria hybridised with the Eu338-FITC probe (Eubacterial probe labelled with fluorescein-isothiocyanate) showing a green colour.

(B) Bacteria hybridised with the HRh probe (*Helicobacter* genus specific probe labelled with tetramethyl-rhodamine-isothiocyanate) showing a red colour.

(C) Bacteria hybridised with both Eu338-FITC and HRh probe showing an orange colour (mixture of red and green colours).

Photomicrograph taken by Helen Dalton (School of BABS, UNSW). (Bar = 16.00 μm)

4.4 Summary and Discussion

The brushtail possum is one of the most common Australian marsupials and one of the four marsupial species (the greater glider, common ringtail possum, common brushtail possum and the koala) that feed on eucalypt leaves [206]. Prior to the study described in this thesis, the mucus-associated microorganisms of BTPs had never been investigated. Indeed there have only been a few studies examining the microbiota of the GIT of these animals. In a study which investigated the digestion and metabolism of high-tannin *Eucalyptus* foliage by brushtail possums, Foley *et al.* found no microorganisms attaching to fragments of partly digested eucalypt leaves in the stomach [155]. In contrast, large numbers of bacteria were observed colonising the cell walls of fragments of eucalypt leaves in the caecum and proximal colon. One possible conclusion from these results is that many microorganisms are able to colonise the lower bowel of BTPs, and that these are involved in the fermentation of foliage. In another study, Gordon *et al.* investigated the effect of the host and geographical location on the distribution of enteric bacteria in the faeces of Australian mammals. This study showed that the composition of the community of enteric bacteria was significantly different among host families and host localities [149].

There are numerous reports describing the isolation of *Helicobacter* species from the stomach, lower bowel and faeces of humans and animals [84, 207]. However few studies have systematically examined the presence of *Helicobacter* species along different regions of the GIT. To address this issue, in the present study, samples were taken from different locations within the gastrointestinal tract of the BTP's, including the stomach and 5 regions of the lower bowel. Given that a number of studies have described the isolation of *Helicobacter* species from non-gastrointestinal sites such as the liver and gall bladder, the former of these sites was also examined in the present study [100, 102]

The current study, which directly investigated the presence of mucus-associated bacteria in the GIT, has shown for the first time that *Helicobacter*

species are present in the GIT of Australian marsupials. Of the 11 wild BTP's examined, *Helicobacter* species were isolated and detected from the lower bowel of all animals by culture and PCR amplification. Of these isolates, 18/28 were shown to belong to the *Helicobacter* genus by *Helicobacter* genus specific PCR and the 16S rRNA gene sequences analysis. The *Helicobacters* isolated from the BTP's differed in morphology and could be separated into three morphological types by TEM: comma, fusiform and S-shaped. The level of similarity of the 16S rRNA gene sequences of each morphological type was >99% (Chapter 6) with each morphological type representing a possible novel *Helicobacter* species. The fusiform shaped organisms possessed periplasmic fibres that entwined the bacterial cell in a similar fashion to those described for the so called "*Flexispira rappini*" or flexispira–like organisms [70, 84]. Further comparison of the BTP fusiform shaped isolates to other flexispira–like organisms will be discussed in Chapter 6.

Despite the fact that the cultivation of helicobacters is acknowledged to be complicated, difficult, laborious and time-consuming, in the present study a high number of *Helicobacter* species with different morphologies were isolated from all BTPs by culture. While bacterial culture detects only viable organisms, detection of *Helicobacter* DNA can represent viable and/or non-viable cells. A high level of concordance was found between the *Helicobacter* cultivation results and the direct PCR results as shown in Table 4.5. *Helicobacter* species were cultured from the rectum of 9 BTPs and detected by both direct and nested PCR from all 11 BTPs. From the colon, *Helicobacter* species were cultured from 7 BTPs and detected by both direct and nested PCR in 10 BTPs. From the caecum, *Helicobacter* species were cultured from 7 BTPs and detected by both direct and nested PCR in 5 and 9 BTPs respectively. From the ileum at 3 cm above the caecum, *Helicobacter* species were cultured and detected by direct PCR from 2 of 11 BTPs and by nested PCR from 7 of 11 BTPs. This result suggests that in most BTPs the number of helicobacters present in the caecal region, and the regions above this, is very low and below the limit of detection of direct PCR. The decreased detection of *Helicobacter* species in the caecal samples using direct PCR as compared with culture might reflect a variation in numbers of *Helicobacter* spp. present in different areas of

the caecum. This suggestion is supported by the fact that the numbers of curved to spiral shaped organisms in the silver stained sections of the caecum ranged from few to numerous. Given that three separate sections of the caecum were used for DNA extraction, culture and histology, it may be that the number of spiral shaped organisms in the tissue collected for DNA extraction was low. However the low sensitivity of PCR *Helicobacter* DNA detection in the caecal samples may be more likely to be due to PCR inhibitors in the caecal samples rather than small number of organisms, given that the sensitivity of culture in general is equal or lower than that of PCR methods.

Nested PCR is considered to be more sensitive than direct PCR. This view is supported by the current study in which *Helicobacter* species were detected using nested PCR in samples which were negative by direct PCR (the liver, stomach, mid ileum and ileum at 3 cm above the caecum) (Table 4.4). Although the liver and GIT samples used for DNA extraction, culture and histology were from the same region, they were not the same piece of tissue. The results of the nested PCR were in some instances supported by the findings of histology. Indeed, curved to spiral shaped bacteria were observed in silver stained sections of almost all of these nested PCR positive samples, except for the liver (Table 4.6). There are two possible explanations for the observed discrepancies between the detection of *Helicobacters* in the liver by nested PCR (6/11 BTPs) and histology (1/11 BTPs). The first is that in the liver, the bacteria are randomly distributed (usually in small numbers) and are thus difficult to detect when they are present. This contrasts with the detection of bacteria in the GIT where bacteria are primarily located in the mucus layer lining the epithelium, as can be seen in Figure 4.10. The second reason is that nested PCR only detects *Helicobacter* DNA, which may in these cases have translocated from the GIT.

While the reliability of nested PCR can be questioned, a negative and positive control was included in all reactions to exclude the possibility of contamination in the PCR reaction. The fact that only low numbers of spiral organisms were observed by histology and that *Helicobacter* species were only detected by nested PCR in the liver, stomach and mid ileum could suggest that following the

animal's death these organisms spread to these sites from the lower bowel. In some cases, collection of samples was not always possible directly following the death of the animal, especially in cases where the animals were found already dead (BTP10 and BTP11). A number of mechanisms have been described which indicate that bacterial translocation from the GIT to extra-intestinal sites may be promoted in specific situations. These include disruption of the gastrointestinal (GI) ecology when intestinal overgrowth in the GIT occurs, physical damage to the mucosal barrier as well as a decrease in the immune defenses [16]. Given that a high number of helicobacters were found colonising the lower bowel of the BTP's, a number of these mechanisms might have occurred, especially as the animals were all under some form of stress prior to their death. For example, BTP1, BTP2 and BTP3 were suffering and under medical care for between 4 and 10 days before death (see Table 4.1).

The situation in BTP6 was however different. Low numbers of curved to spiral shaped bacteria were observed in the silver stain sections of the liver (see Figure 4.10), stomach, mid ileum and ileum at 3 cm above the caecum of BTP6, however no *Helicobacters* were detected either by culture or direct and nested PCR. While it may be argued that the numbers of *Helicobacter* species may be too low to be detected by culture or direct PCR, they are sufficiently high to be detected microscopically in silver stained sections. These results would suggest that the observed organisms in BTP6 may not belong to the *Helicobacter* genus.

The detection of *Helicobacter* species in the liver of these animals is not without precedent. For example, several *Helicobacter* species have been isolated or detected in extra gastric or intestinal sites including the liver, bile, gall bladder and blood of animals and humans [96, 100, 102, 126]. The mechanisms whereby *Helicobacters*, whose normal ecological niche is the lower bowel, are able to colonise the liver and gall bladder is unknown. It has been suggested that the bacteria may access the liver by initial M-cell uptake, with spread to the liver via the portal circulation and finally discharge of the bacteria into the biliary tract. Another possibility is the direct translocation through enterocytes or migration of helicobacters from the lumen of the gut into the bile duct [16, 208].

Studies have shown that a number of different rodent species (rat, mice and Syrian hamsters) from different animal facilities can be colonised by a range of different *Helicobacter* species including *H. hepaticus, H. cinaedi, H. trogontum, H. bilis, H. muridarum, H. ganmani, H. rodentium*, *H. typhlonius, H. aurati, H. cholecystus, H. mesocricetorum* and *"Flexispira rappini'* (Table 1.2 and 1.3). However, few studies to date have described a systematic investigation of the entire GIT of any animal. In the current study more than one morphological type of *Helicobacter* species was isolated from a single specimen in some of the brushtail possums. This would suggest that in a single animal, more than one species of *Helicobacter* can naturally colonise the same location of the gastrointestinal tract. For example, both 'comma' and 'fusiform' shaped helicobacters were found co-inhabiting the colonic and rectal areas of a number of the BTP's. In contrast 'S' shaped *Helicobacter* isolates mainly colonised the caecum. The presence of more than one morphological type of spiral shaped bacterium in a single location of some of these brushtail possums is consistent with previous findings in rodents [37, 71, 179, 209]. For example, more than 25 years ago, Phillips *et al.* provided one of the first extensive descriptions of the distribution of spiral shaped bacteria in the GIT [37]. These studies, which were based on bacterial morphology, showed that different types of spiral and fusiform bacteria colonised different niches within the GIT. However, at that time these different bacterial types were not fully characterised.

Subsequent studies have shown that some of the bacterial types described by Phillips *et al*. correlate with *Helicobacter* species [83]. For example, in one of the most comprehensive studies conducted to date, Robertson *et al*. (1998) investigated the mucus-associated bacteria present in 23 Quackenbush/Swiss (Q/S) mice originating from 4 different animal facilities in Sydney (PhD thesis, UNSW, Sydney) [179]. This study examined 4 different regions of the GIT (stomach, small bowel, caecum and large bowel) and the liver of these mice using culture and characterisation methodologies similar to those described in the present study. Of the 110 isolates cultivated, 68 (62%) were identified as *Helicobacter* species. The species cultivated included *H. bilis, H. muridarum, H. ganmani* and a number of other uncharacterised species. Of the remaining

isolates, 30 constituted a new genus, (proposed name *Mucispirillum*), and the remaining 12 belonged to the genus *Desulfovibrio*. Mice from 2 of the 4 animal houses were shown to be colonised with multiple (7-8) species, while animals from the other 2 facilities were colonised with only 1 or 2 species. Furthermore, mice were often found to be colonised in a specific region of their GIT by more than one type of organism.

In a similar study, Patterson *et al.* (2000) described the successful culture of three morphological types of *Helicobacters* from a single tissue specimen obtained from a Syrian hamster [71]. These were fusiform shaped rods, long slender curved bacteria and short curved bacteria. The fusiform shaped organism was the most common phenotype in these animals and was subsequently named *H. aurati*. Thus it would appear that co-colonisation by a number of *Helicobacter* species is likely to be more common than reported.

Approximately 42% (20/48) of the isolates cultivated from the possums in this study were shown not to be *Helicobacter* species. The majority of these 20 isolates were curved rods. Given that the aim of this study was to characterise spiral/helical and fusiform isolates identified as *Helicobacter* species both phenotypically and genetically, these non *Helicobacters* were not further identified. One exception was the 'Tcr' isolate (tightly coiled rod), which was further identified and characterised due to its unique morphology. This isolate was subsequently identified as a *Campylobacter* species. Thus *Helicobacter* spp. and *Campylobacter* spp. were isolated from the rectum and caecum of a brushtail possum, respectively.

Campylobacter species are commonly isolated from faecal samples of both humans and animals [195, 210]. The natural habitat of most *Campylobacter* spp., including *Campylobacter jejuni*, is the intestinal tract of warm-blooded animals. Co-infection with enteric *Helicobacter* spp. and *Campylobacter* spp. in cats has been reported by Shen *et al.* (2001) [209]. In their study 64 campylobacter-like organisms (CLOs) were isolated from the faeces of 64 of 227 cats studied. Fifty one of the 64 cultures were further examined using a *Campylobacter* genus specific PCR (primers C98 and C99) and a *Helicobacter*

genus specific PCR (primers C97 and C05). Seventeen of the 51 cultures were shown to be positive for both *Campylobacter* spp. and *Helicobacter* spp., 30 of the 51 cultures being positive only for *Campylobacter* spp. and 4 of the 51 cultures positive only for *Helicobacter* spp. The detection of both *Campylobacter* spp. and *Helicobacter* spp. in 17 of the 51 cultures showed that these cultures contained a mixture of both genera. Shen *et al.* suggested that *Helicobacter* spp. may be present in smaller numbers and grow at a slower rate than *Campylobacter* spp. and thus they may be missed in primary isolation. The study of Shen *et al.* demonstrates a high prevalence of mixed infections of *Campylobacter* and *Helicobacter* species, which is probably more common than reported in the literature.

The spatial distribution of mucus-associated bacteria observed from the silver stained sections from different regions of the GIT using light microscopy showed that a high number of curved to spiral shaped rods were present at the mucosal surface as well as in the caecal, colonic and rectal crypts. These curved to spiral shaped microorganisms could possibly be Helicobacters, Campylobacters or belong to one or more unknown genera. To investigate whether any of the spiral shaped bacteria belonged to the genus *Helicobacter* a FISH technique, using a *Helicobacter* genus specific probe, was applied to rectal sections from 4 brushtail possums in which *Helicobacter* spp. had been detected and isolated by PCR and culture respectively. The results of FISH showed that the majority of these mucus-associated bacteria belonged to the genus *Helicobacter*. Given that histopathological examination of sections obtained from the liver and gastrointestinal tract of the brushtail possums showed all animals to be in general, healthy, these *Helicobacter* species are likely to represent indigenous microorganisms which colonise the lower bowel of wild, normal, healthy brushtail possums.

Brushtail possums are caecum fermenters with microbial fermentation taking place in the caecum/proximal colon site. As yet, the bacteria inhabiting the caecum of brushtail possums responsible for microbial fermentation have not been investigated. From this study, the ecological niche of *Helicobacter* species was shown to be the lower bowel covering the areas between the caecum and

rectum and in some animals expanding to the ileum at 3 cm above the caecum. Though the colonisation of *Helicobacter* species is not confined to the caecal-proximal colonic area, an important role of *Helicobacte*r species in the fermentation of the food contents cannot be excluded. It is also possible that *Helicobacters* gain an optimal nutritional benefit from this area, as this region is the preferred ecological niche for *Helicobacter* species colonisation in most mammals. Currently there is no evidence indicating whether the host gains any reciprocal benefit from these bacteria.

The discovery of *Helicobacter* spp. inhabiting the lower bowel of the brushtail possum adds to the growing list of animals in which *Helicobacters* have been found in the intestine of the animals. This finding is likely to contribute important information relating to the evolutionary aspect of these bacteria with their host.

Chapter 5

The detection and isolation of spiral and fusiform shaped organisms, in particular *Helicobacter* species, from the gastrointestinal tract of the ringtail possum

5.1 Introduction

The common ringtail possum (*Pseudocheirus peregrinus)* is one of Australia's most familiar marsupials and the smallest arboreal folivore (leaf-eater) (Figure 5.1). It can be found along the Eastern coast of Australia, from Cape York extending through Victoria, South-Eastern South Australia and Tasmania [206]. It is distinguished from the brushtail possum by the light covering of fur on its tail, its white tail tip, and shorter, less pointed ears.

The ringtail possum (RTP) is a caecum fermenter and well adapted to a diet of eucalypt leaves. Although eucalypt leaves are a major food source for RTPs they also feed on flowers, particularly those of eucalypts and fruits [146]. Its dietary preferences vary geographically. As distinct from the BTP, the RTP is strictly herbivorous and never eats insects. The RTP also eats its own soft faeces (caecotrophs), a behavior called coprophagy [206]. Soft faeces are excreted during daylight and the RTP eats these immediately. As these soft faeces pass through the gut for the second time, additional nutrients are absorbed.

Given its diet it is apparent that the RTP is capable of detoxifying the tannins and phenols derived from ingestion of eucalypt leaves. Although a number of studies have investigated the detoxifying role of microorganisms in the caecum on the tannins and phenols present in eucalypt leaves, little is known about the overall ecology of the GIT. The main focus of this current research is the study of mucus-associated bacteria, an area that has not been directly investigated in the RTP. Thus the aim of this study was to determine if any spiral and fusiform shaped microorganisms, in particular *Helicobacter* spp., are present in the different regions of the GIT of RTP and if so, to characterise them. As the strategies and methods used for the detection and isolation of *Helicobacter* spp.

were successful for the study of BTPs (Chapter 4) these methods were also applied to the study of RTPs. A second aim is to compare the detection and isolation rates of these bacteria in the RTP with that in the BTP and evaluate the impact of differences in diet, feeding strategies and the digestive system between these two animals on the colonisation of the GIT by spiral and fusiform shaped microorganisms, in particular *Helicobacter* spp.

Figure 5.1 The common ringtail possum (*Pseudocheirus peregrinus)*
Photograph by 'The Parks and Wildlife' Service division, Department of Environment and Management, Tasmania.

5.2 Materials and experimental methods

5.2.1 Animal history and collection of specimens

All ringtail possums included in this study were obtained from the Veterinary & Quarantine centre, Taronga Zoo, Sydney, Australia. All animals were living freely in the Taronga Zoo area (non-caged) and in suburban areas of Sydney, New South Wales (e.g. Mosman, Beecroft, St. Leonards, Cammeray and Middle Cove). All RTPs examined had been injured or were in ill health. They had been taken to the Veterinary & Quarantine centre for care and had subsequently died or had been euthanased for compassionate reasons. The history of the 10 RTPs examined is summarised in Table 5.1.

Specimens from the 10 RTPs were collected as described in section 2.2. For each animal, three samples of tissue from each of the following sites were collected: the liver, stomach, mid ileum, ileum at 3 centimeters (cm) above caecum, caecum, colon and rectum (Figure 4.2). The first sample from a particular location was frozen at -70°C for DNA extraction. The second sample was frozen in one mL of BHIG and kept at -70°C until cultured and the third sample was fixed in formalin for histology and FISH. The frozen samples were placed on dry ice during delivery to the laboratory.

All methods used including bacterial cultivation, *Helicobacter* genus specific PCR, histopathology and FISH, are as outlined in Chapters 2 and 4. The FISH technique was performed on a formalin-fixed colonic section of one RTP (RTP 9) in which *Helicobacter* species were isolated and detected by PCR.

Animal	Habitat	Sex	Age	Cause of dead	Period in care (days)	Gross pathology
RTP1	Mosman	F	A	Found dead	<1	Marked haemorrhagic duodenitis
RTP2	Mosman	-	J	Attacked/ Euthanased	<1	Cranial puncture wounds (predation by dog)
RTP3	Beecroft	F	A	Attacked/ Euthanased	6	Necrotic right paw and puncture below left eye (predation by cat)
RTP4	Taronga Zoo	F	A	Diseased/ Euthanased	1	Dental attrition and swollen paws
RTP5	Mosman	M	A	Diseased/ Euthanased	1	Neurological signs (possibly hit by car)
RTP6	St Leonards	F	A	Accident/ Euthanased	1	Spinal trauma
RTP7	Cammeray	-	-	Diseased/ died	3	Cranial contusion and oral haemorrhage
RTP8	Middle Cove	M	A	Attacked/ Euthanased	<1	Multiple puncture wounds, (predation by dog)
RTP9	Mosman	F	A	Diseased/ Euthanased	-	Neurological signs
RTP10	Mosman	F	A	Diseased/ died	-	Neurological signs

Table 5.1 The habitat, sex, age, cause of death, period in care and gross pathology of the ringtail possums examined in this study.

F = female, M = male, A = adult, J = juvenile, - = no details available, Neurological signs = fits/ seizures, head tilt, tremors or opisthotonos

5.3 Results

5.3.1 Bacterial cultivation

Preliminary phase microscopic examination showed no microorganism to be present in the homogenised liver samples of any of the RTPs. A mixture of microorganisms of various morphologies i.e. curved rods, short rods, cocci and diplococci, were observed in the mucus scrapings of GIT samples from all regions. In addition a number of long curved to spiral shaped organisms were seen in the mucus scrapings obtained from the colon of RTP1 and RTP9. Fusiform shaped organisms were observed in mucus scrapings obtained from the colon and rectum of RTP4, the ileum at 3 cm above the caecum, caecum, colon and rectum of RTP5 and the rectum of RTP7.

Bacterial culture of the liver and the GIT mucus scrapings of the 10 RTPs revealed either very low numbers of bacterial colonies or no growth on both HBA and CSA plates, incubated under both microaerobic and anaerobic conditions after 3 to 7 days. Attempts to subculture from the clear, colourless, small to pinpoint colonies obtained from the 10 RTPs, resulted in the growth of 13 separate cultures of spiral to curved shaped bacteria. No fusiform shaped organisms were isolated from any of the RTP’s.

5.3.1.1 Screening of pure isolates for the presence of *Helicobacter* species using the *Helicobacter* genus specific PCR

DNA extracted from the above 13 isolates was amplified using a *Helicobacter* genus specific PCR. Of these 13 isolates, 5 were identified as *Helicobacter* species (Figure 5.2). These isolates were cultured from the ileum at 3 cm above the caecum (n=1), colon (n=3) and rectum (n=1) of 3 RTPs. Of these 5 isolates only a spiral morphological type was detected. No *Helicobacter* species were isolated from the liver, stomach, mid ileum and caecum regions of any RTP studied.

The five isolates, which were confirmed as *Helicobacter* species using the *Helicobacter* genus specific PCR, were designated as strains RTP1S to RTP5S (see Table 5.2 and 5.3). These isolates originated from 3 different animals.

- In the first RTP (RTP1), *Helicobacter* species were isolated from the ileum at 3 cm above the caecum (RTP1S) and the colon (RTP2S).
- In the second RTP (RTP5), a *Helicobacter* species was isolated from the colon (RTP3S).
- In the third RTP (RTP9), *Helicobacter* species were isolated from the colon (RTP4S) and the rectum (RTP5S).

The PCR products from the *Helicobacter* genus specific reaction amplifying DNA from these 5 isolates is shown in Figure 5.2.

Sequencing of the near complete 16S rRNA gene (~1500 bp) of these 5 isolates was also undertaken. These were then compared to other sequences from the GenBank database and all were confirmed to be *Helicobacters*. The similarity among the 16S rRNA gene sequences of the five isolates was 99.6 to 99.9%, indicating the five isolates potentially belong to a novel *Helicobacter* species. A more detailed phylogenetic analysis of these isolates will be described in Chapter 6.

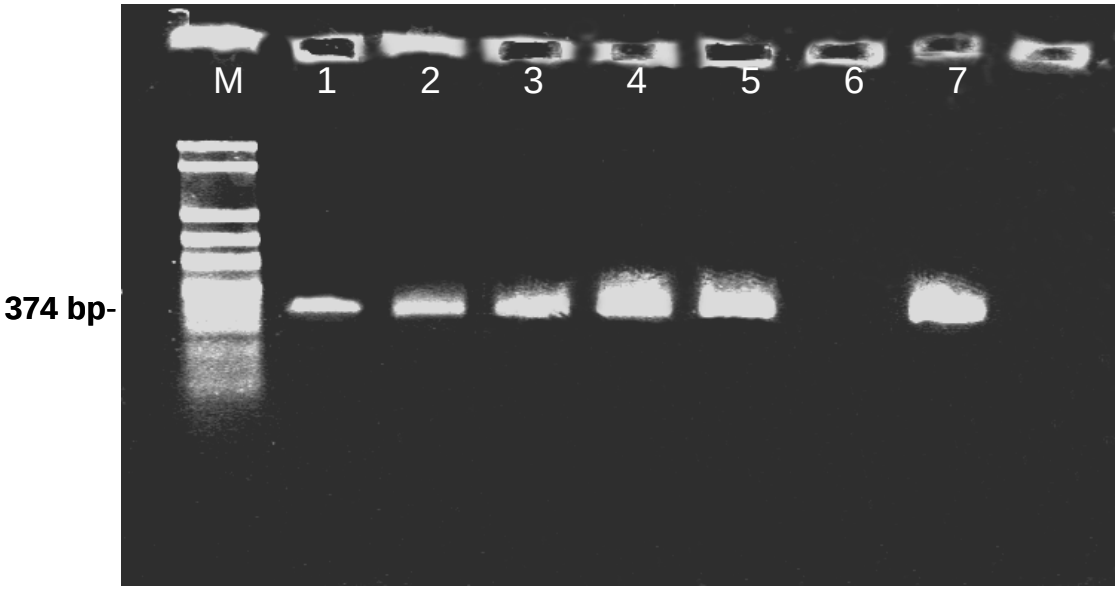


Figure 5.2 The PCR products obtained from the 'S' shaped bacteria using the *Helicobacter* genus specific PCR, separated on a 1.5 % agarose /TAE gel. (RTP=Ringtail possum)

M	=	Molecular weigh marker (FN1)
Lane 1	=	RTP1S isolate
Lane 2	=	RTP2S isolate
Lane 3	=	RTP3S isolate
Lane 4	=	RTP4S isolate
Lane 5	=	RTP5S isolate
Lane 6	=	'No DNA' Negative control
Lane 7	=	*'H. pylori'* Positive control

5.3.1.2 Morphological analysis of the pure isolates using TEM

The 5 isolates were further examined using transmission electron microscopy (TEM). Negative stains showed that these five isolates were all ‘S’ shaped; measuring 0.3 x 2.5 μm in size; with bipolar flagella and no periplasmic fibrils (Figure 5.3).

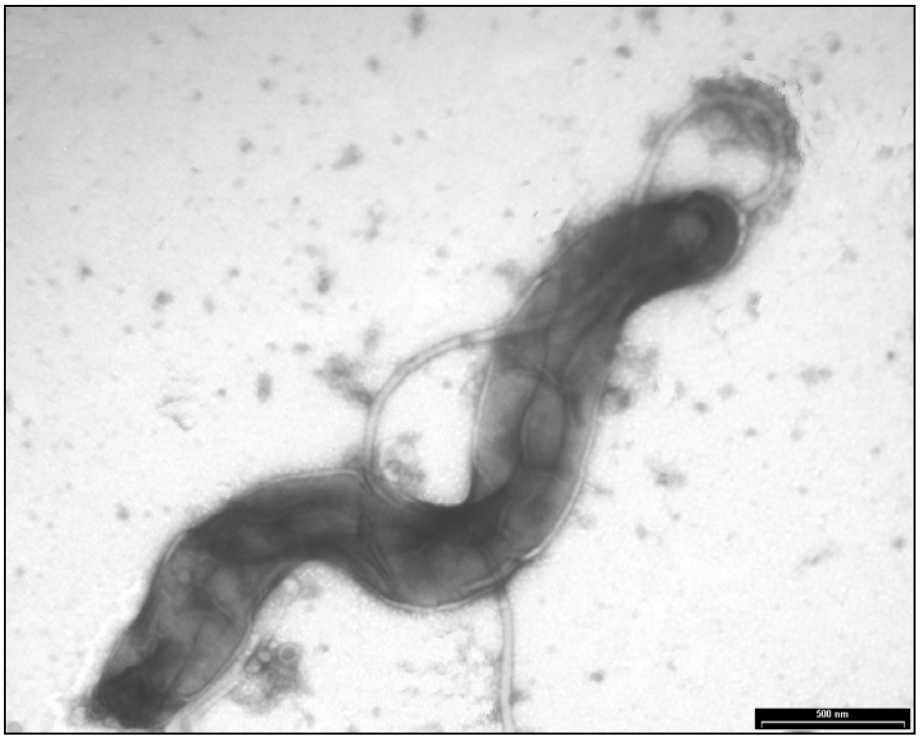


Figure 5.3 A transmission electron micrograph of an 'S' shaped *Helicobacter* isolate, 'RTP5S'. The bacterium measured 0.3 x 2.5 μm and had bipolar sheathed flagella. (Bar=500 nm)

Name	Origin	Location	Light microscopy	EM
RTP1S	RTP1	3-ileum	Spiral	S
RTP2S	RTP1	Colon	Spiral	S
RTP3S	RTP5	Colon	Spiral	S
RTP4S	RTP9	Colon	Spiral	S
RTP5S	RTP9	Rectum	Spiral	S

Table 5.2 The name, origin, location, and morphology (light and electron microscopy) of the 5 *Helicobacter* species isolated from 3 ringtail possums.
3-ileum = ileum at 3 cm above the caecum.

5.3.2 Detection of *Helicobacter* species in the liver and mucus scrapings of the GIT using direct and nested *Helicobacter* genus specific PCR

5.3.2.1 Detection using direct *Helicobacter* genus specific PCR

Amplification of DNA extracted from the homogenised livers and mucus scrapings of the GIT from all 10 RTPs (Table 5.3) showed a PCR product of the expected size (374 bp) to be present in the following samples:

- Colon (n= 3)
- Rectum (n= 7)

None of the liver, stomach, mid ileum, ileum at 3 cm above the caecum or caecum samples were positive in the direct genus specific PCR.

5.3.2.2 Detection using nested *Helicobacter* specific PCR

Amplification of DNA extracted from the homogenised livers and mucus scrapings of the GIT from all 10 RTPs (Table 5.3) showed a PCR product of expected size (374 bp) in the following samples:

- Stomach (n=1),
- Mid ileum (n= 1),
- Ileum at 3 cm above the caecum (n= 2),
- Caecum (n= 3),
- Colon (n=8)
- Rectum (n= 10)

No *Helicobacter* DNA was detected using nested genus specific PCR from any liver samples.

Regions / Animals	Liver	Stomach	Mid ileum	3-ileum	Caecum	Colon	Rectum
RTP1	–	*	*	~*	*	~+ *	+*
RTP 2	–	–	–	–	–	–	*
RTP 3	–	–	–	–	–	*	*
RTP 4	–	–	–	–	–	+ *	+ *
RTP 5	–	–	–	*	*	~ *	+ *
RTP 6	–	–	–	–	–	*	*
RTP 7	–	–	–	–	–	*	+ *
RTP 8	–	–	–	–	–	*	+ *
RTP 9	–	–	–	–	*	~+ *	~+ *
RTP 10	–	–	–	–	–	–	+ *

Table 5.3 The results of *Helicobacter* culture, direct and nested PCR obtained from the 10 RTP's.

3-ileum = ileum at 3 cm above the caecum,

~ = *Helicobacter* culture positive,

+ = direct PCR positive, * = nested PCR positive,

- = *Helicobacter* culture, direct and nested PCR, all negative.

5.3.2.3 Comparison of the detection of *Helicobacter* species by bacterial culture and PCR

Helicobacter species were isolated from 3 of the 10 ringtail possums using culture while *Helicobacter* DNA was detected from 7 of the 10 RTPs using direct PCR and from 10 of the 10 RTPs using nested PCR (Table 5.3).

From the 5 *Helicobacter* isolates, RTP2S, RTP4S and RTP5S were cultured from the colon of RTP1, and the colon and the rectum of RTP9 respectively, where *Helicobacter* DNA was detected using both direct and nested PCR. Of the 2 remaining isolates, RTP1S was isolated from the ileum at 3 cm above the caecum of RTP1 and RTP3S was isolated from the colon of RTP5 where *Helicobacter* DNA was detected using nested PCR but not direct PCR (Table 5.3). The percentage of animals in which *Helicobacter* species were isolated using culture and *Helicobacter* DNA was detected using direct and nested PCR is shown in Table 5.4.

Region	Cultivation	Direct PCR	Nested PCR
Liver	0/10 (0%)	0/10 (0%)	0/10 (0%)
Stomach	0/10 (0%)	0/10 (0%)	1/10 (10%)
Mid ileum	0/10 (0%)	0/10 (0%)	1/10 (10%)
3-ileum	1/10 (10%)	0/10 (0%)	2/10 (20%)
Caecum	0/10 (0%)	0/10 (0%)	3/10 (30%)
Colon	3/10 (30%)	3/10 (30%)	8/10 (80%)
Rectum	1/10 (10%)	7/10 (70%)	10/10 (100%)

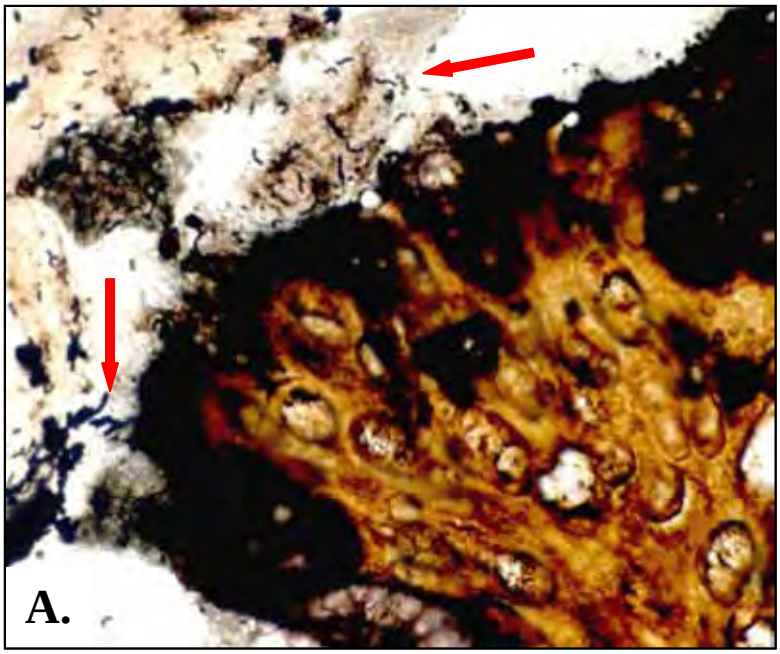
Table 5.4 Number and percent detection of *Helicobacter* species in different regions of the gastrointestinal tract of the 10 ringtail possums using culture, direct and nested *Helicobacter* genus specific PCR.

(3-ileum = ileum at 3 cm above the caecum)

5.3.3 The spatial distribution of microorganisms in fixed sections of the liver and different regions of the GIT of RTPs

No microorganisms were observed in any silver stained sections of the liver using light microscopy. Examination for the presence of mucus-associated microorganisms from the silver stained sections of the different regions of the ringtail possum GIT was problematic. Large numbers of clumps of bacteria-like particles in the luminal contents of the RTPs obscured the presence of mucus-associated bacteria. These clumps are likely to be the bacteria present in the soft faeces (caecotrophs) ingested by the ringtail possum, fibres or other food particles. Apart from these clumps, very low numbers of a mixture of rods and cocci were observed in the stomach, mid ileum and ileum at 3 cm above the caecum of most of the RTP studied, except for RTP1 and RTP5. In these latter animals, higher numbers of long curved rods were observed in mid ileum and ileum at 3 cm above the caecum. Small numbers of long, curved to spiral shaped organisms were observed in the mucus layer overlying the epithelium and in the crypts of the caecum, colon and rectum of RTP1, RTP5, and RTP 9. Scattered short curved and long curved to spiral shaped organisms were observed mixed with other microorganisms such as cocci, short rods and fat rods, in the mucus layer overlying the epithelium of the colon and rectum of RTP4, RTP7, RTP8 and RTP10. No curved to spiral shaped organisms were observed in the caecal, colonic and rectal samples of RTP2, RTP3 and RTP6. A mixture of cocci, short rods and fat rods were seen in these latter animals. Examples of the distribution of mucus-associated bacteria in the colonic region of RTP9 are shown in Figure 5.4.

The FISH technique was performed on a formalin-fixed colonic section of RTP 9 in which *Helicobacter* species had been isolated and detected by PCR. Bacteria belonging to the *Helicobacter* genus were observed predominantly colonising the mucus layer lining the epithelium of the colon (Figure 5.5) and in the crypt of the colon (Figure 5.6).



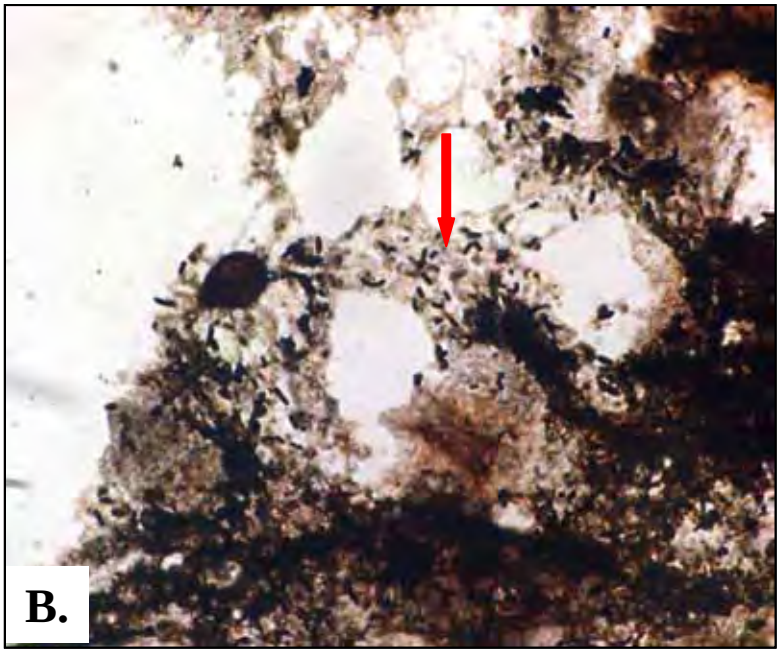


Figure 5.4 Photomicrographs of a silver stained colonic sample obtained from RTP 9 showing a small number of long curved to spiral shaped organisms (arrows) in the mucus layer lining the epithelium of colon. (Magnification = 1000 for A and B)

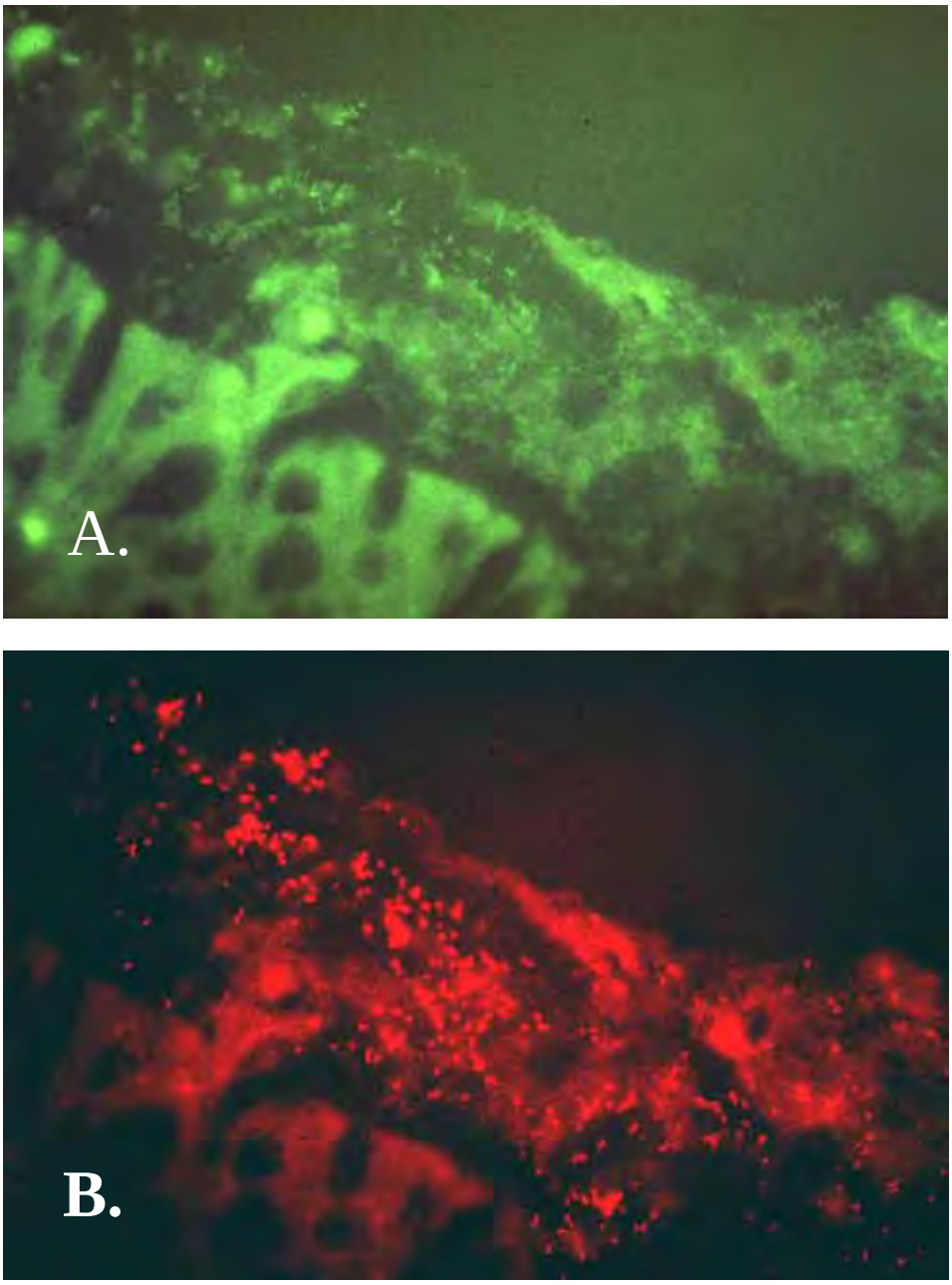


Figure 5.5 Epi-fluorescent photomicrographs of a colonic section taken from RTP 9 showing that the bacteria colonising the mucus layer overlying the epithelium of colon belong to the *Helicobacter* genus.

A - Bacteria hybridised with Eu338-FITC probe (Eubacterial probe labelled with fluorescein-isothiocyanate) showing a green signal.

B - Bacteria hybridised with HRh probe (*Helicobacter* genus specific probe labelled with tetramethyl-rhodamine-isothiocyanate) showing a red signal.

Photographs taken by the author (Magnification = 630).

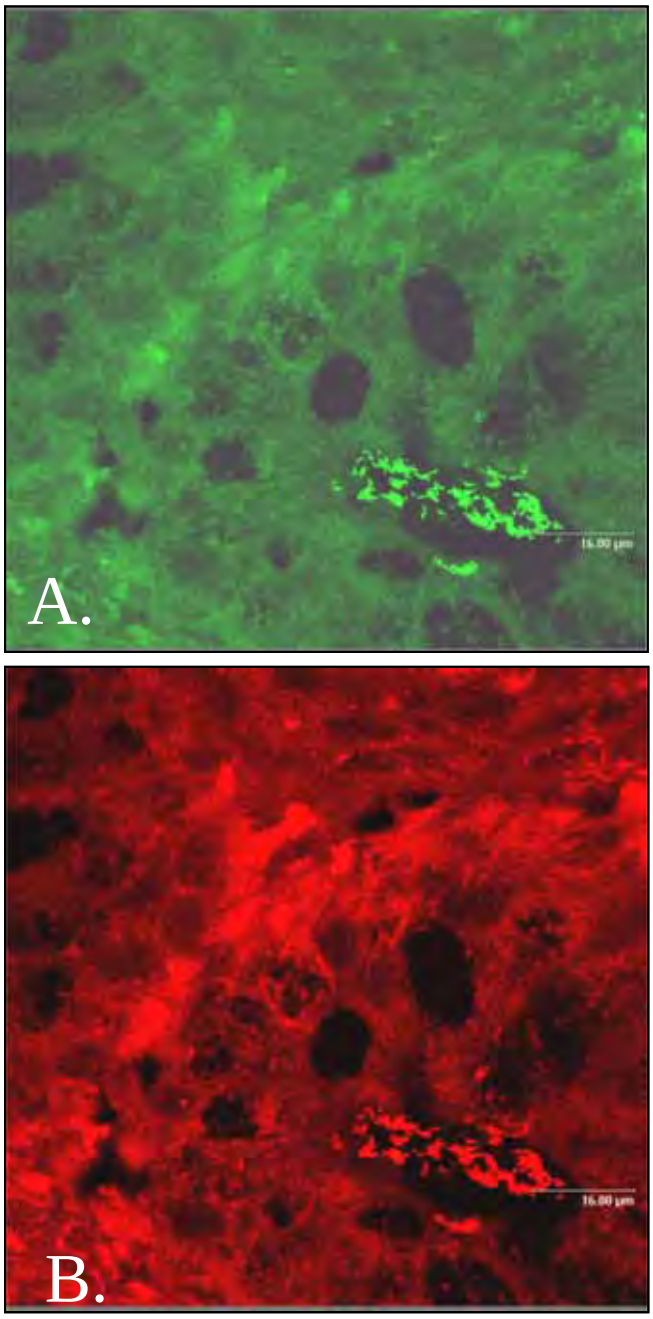


Figure 5.6 Confocal-fluorescent photomicrographs of a colonic sample taken from RTP9 showing that the bacteria colonising the crypt belong to the *Helicobacter* genus.

A) Bacteria hybridised with the Eu338-FITC probe showing a green colour.

B) Bacteria hybridised with the HRh probe showing a red colour.

Photographs taken by Helen Dalton (School of BABS, UNSW) (Bar=16.00 μm).

5.3.4 Histopathology

Histopathological examination of liver sections obtained from the ringtail possums showed these to be normal with no significant lesions except for the RTP 2, which had a mild focal portal mononuclear aggregation and multifocal neutrophilic aggregations.

No significant lesions were observed in the stomachs of 9 of the 10 RTPs. In 1 of the 10 RTPs (RTP1) there was a mild focal aggregation of mononuclear cells and neutrophils in the lamina propria.

The lower bowel of all RTPs was normal with few infiltrations being observed in the intestinal mucosa.

The histopathology observed in the hematoxylin and eosin stained sections of the liver and all specimens collected from the GIT of all RTPs studied is shown in Appendix 1.

5.4 Summary and Discussion

A potentially new *Helicobacter* species was isolated from the lower bowel of the ringtail possum. A combination of microscopic examination, culture and PCR showed that there were *Helicobacter* species colonising the lower bowel of all the ringtail possums studied. *Helicobacter* DNA was detected from 7 of the 10 RTPs (70%) by direct PCR and 10 of the 10 RTPs (100%) by nested PCR. Five isolates of *Helicobacter* spp. were cultured from 3 of the 10 RTPs. All isolates had a similar 'S' shaped morphology. 16S rRNA gene sequence analysis showed that the similarity of the 5 isolates ranged from 99.6 to 99.9% (Chapter 6). A comparison of the 16S rRNA gene sequences of the isolates with other *Helicobacters* in GenBank showed that the 5 isolates were most similar to *H. canis* and *H. hepaticus* (94%). Thus, based on the similarity between the 5 RTP isolates and the fact that they were only 94% related to other *Helicobacter* species, it would appear that the RTP isolates probably constitute a new species.

Microscopic examination of the silver stained colonic sections of the RTP's showed curved rods and long curved to spiral shaped bacteria to be present in the mucus layer and in the crypts of the colon. By use of the FISH technique, the majority of the spiral shaped bacteria colonising the mucus layer overlying the epithelium, as well as in the crypts of the colon, were shown to belong to the *Helicobacter* genus.

Helicobacter species were found colonising the distal end (colon and rectum) of the GIT of all RTP's except RTP1 where one *Helicobacter* spp. was isolated from the ileum at 3 cm above the caecum. In this animal *Helicobacter* DNA was detected in all regions of the GIT except for the liver. According to this animals' history, it was found dead and thus it is possible that, as discussed in Chapter 4, bacteria from the distal end of the GIT had translocated to other sites (the stomach and small intestine) following death and prior to sample collection.

In the RTP, the cultivation rate, as compared with detection using PCR, was low. In the rectal region of the RTP, only one *Helicobacter* isolate was cultured

(RTP9) while *Helicobacter* DNA was detected in the rectal region of 7 RTPs using direct PCR and in 10 using nested PCR. This low cultivation rate in the RTP's might relate to the fact that these *Helicobacters* have specific nutritional requirements. For example, they may require specific plant secondary metabolites found in the GIT for growth, and hence have failed to grow on artificial media. Another factor that may have contributed to the low cultivation rate of *Helicobacter* species in these animals could be that some *Helicobacter* species in the RTP may be sensitive to the aerobic conditions created during sample collection.

Interestingly, the cultivation rate of *Helicobacter* spp. in the RTPs (3 of 10 animals) was considerably lower than that in the BTP's (11 of 11 animals). Furthermore only 5 cultures of *Helicobacter* spp. were successfully isolated from 3 RTPs as compared with 28 isolates from the 11 BTPs. In contrast the rate of detection using direct PCR was only slightly lower than that of the BTP's (7 of 10 RTPs as compared with 11 of 11 BTPs). While the failure to isolate *Helicobacters* may be due to the requirement for specific nutrients as discussed above it is also possible that in the RTP, the number of *Helicobacter* spp. present in the lower bowel is very low thus making it only detectable by PCR.

Despite this, the lower bowel of both the RTP and the BTP were found to be colonised with phylogenetically very closely related *Helicobacter* species. All of the *Helicobacter* species isolated from the RTPs and BTPs formed a monophyletic cluster in the phylogenetic tree reconstructed from their 16S rRNA gene sequences (as will be described in chapter 6). Given that the RTP and BTP are very closely related species, believed to have diverged approximately 55 million years ago [211], the lower cultivation and detection rates of *Helicobacter* spp. in the RTP's as compared with the BTP's is somewhat surprising. The difference in the *Helicobacter* species detection rate in the GIT of the RTP and BTP may result from either differences in the ability of *Helicobacter* species in these two marsupials to utilise the available nutrients and to tolerate the toxins produced within the GIT of the host animals, or differences in nutrients available to the *Helicobacters* in each individual host. For example the differing foraging and digestive strategies of the two possum

species may have an impact on the level of nutrients and/or toxins present in the GIT.

The development of three basic strategies by possums for obtaining sufficient nutrition and energy from food sources has recently been described by Kerle [206]. **The first strategy** relates to food preferences and behavioural mechanisms such as choosing very specific foods and reducing their energy needs. **The second strategy** is the physical adaptation of the teeth and gut to effectively break down plant fibres. **The third strategy** is the development of metabolic processes for energy conservation and detoxification of food. Thus, it is possible that a comparison of the developed strategies for obtaining sufficient nutrition and energy from food between the RTPs and BTPs may provide an insight into the impact of these factors on the colonisation of *Helicobacter* species in the GIT of these animals. These comparisons are described below:

Food preferences.

The RTP and BTP are two of only four mammalian species that utilise eucalypt leaves as their primary food source. The RTP is a strict herbivore, its diet consists almost entirely of eucalypt leaves, shoots or flowers, often derived from only one or two plant species. The BTP is more flexible in its food choices with a diet consisting not only of eucalypt leaves but fruits, grasses, herbs, insects and meat [206]. Eucalypt leaves have a low concentration of nutrients, are very fibrous and contain anti-nutrients or plant secondary metabolites (allelochemicals) [146, 206]. The plant secondary metabolites found in eucalypt leaves are of two types;

The first type interferes with the digestive process of the consumer. Examples of this type include lignin and tannins. These compounds are considered to be resistant to degradation within the gut of animals and interfere with digestion and the utilization of dietary protein**.** Lignin interferes with the microbial digestion of plant cell-wall polysaccharides, and condensed tannins form complexes with proteins making them less available to the animal.

The second type is toxic to animal tissues and includes hydrolysable tannins, low molecular weight phenolic compounds and essential oils

(terpenoids) [146]. Several hydrolysable tannins, such as tannic acid have been shown to have an antibacterial action [212]. One of the distinctive essential oils found in eucalypt leaves are the terpenes. The terpenes are aromatic compounds (which account for the eucalypt smell) and thus may be toxic to the gut microbes that are important for the digestion of plant cell walls [206].

The feeding behaviour of possums indicates that they can be very specific in their selection of foods so as to ensure that they obtain sufficient nutrition by avoiding poorer quality plant foods [146, 206]. The presence of secondary metabolites in individual plant/eucalypt species has been shown to have an effect on the eucalypt leaves chosen to be eaten. The role of plant secondary metabolites (PSMs) in the food choice of marsupials feeding on eucalypts was investigated by Lawler *et al.* (1998) [213]. These investigators found that two groups of PSMs, terpenes and DFPs (diformylphlorglucinols, a phenolic compound) may have a major effect on the feeding choice of the ringtail possum. In this study Lawler observed that ringtail possums, when feeding on eucalypt leaves, appear to smell the leaves (*Eucalyptus* terpenes) carefully before ingestion. The authors hypothesised that the terpenes present in the eucalypt leaves may act as a cue in foliage choice. Hume however suggested that in general, foliage acceptability is likely to be determined by the relative proportions of nutrients and plant secondary metabolites, not just the concentrations of one or the other [146].

Higher colonisation rates of *Helicobacter* spp. were detected in the BTP in comparison with the RTP. Thus it is possible that the higher intake of eucalypt leaves and the higher level of anti-nutrients in the RTP limits the ability of *Helicobacter* species to colonise their GIT.

Physical Adaptations.

RTP's and BTP's, both being herbivorous, have similar basic dentition (diprotodonts) and digestive tracts (caecum fermenters), however there are a number of differences which relate to their respective food preferences. Comparison of the molars in RTPs and BTPs has shown that the former possess crescent shaped molars which reflect their ability to grind leaves into

very fine particles in preparation for bacterial fermentation of fibre in the gut ,while the BTP's have four pointed cusp shaped molars that are less efficient at this process [206]. Furthermore, the ability to grind leaves into very fine particles may result in the release of high amounts of anti-nutrients or plant secondary metabolites present in the eucalypt leaves, which may be toxic to some bacteria including *Helicobacter* species.

The structure of the RTP and BTP digestive tracts also differs. The RTP possesses a specific mechanism for controlling the flow of different sized particles between the caecum and the colon. Separation of different sized food particles occurs in the proximal colon, with small particles being pushed back into the caecum, whilst large particles continue their passage through the colon [146]. The selective retention of solutes and fine particles (including plant secondary metabolites) in the caecum, maximises the opportunity for their microbial degradation. The low level of *Helicobacter* detection in the caecum of the RTP suggests that *Helicobacter* species may not have the ability to utilise the solutes and fine particles, or to tolerate the plant secondary metabolites implying that they may not be involved with fermentation. Furthermore RTPs have a mechanism by which they can gain additional nitrogen content and nutritional needs by ingesting material derived from their caecum, which is high in nitrogen, directly from the cloaca (caecotrophy) [146, 206].

In contrast, the BTP has a relatively simple structural and functional caecum and proximal colon that work together as a single fermentation chamber. There is no selective retention of digesta in the GIT of the BTP, thus different sized particles do not move through the gut at different rates. In addition there is no evidence that BTP's are caecotrophic. It was suggested by Hume that the BTP depends more on the enzymatic digestion of food contents in the small intestine than microbial digestion in the caecum [146].

The ability to grind leaves into fine particles and the selective retention of digesta in the GIT may have an impact on the colonisation of *Helicobacter* spp. The RTP has the ability to grind leaves into fine particles and the selective retention of digesta, including toxins, in the caecum. Thus the environment in

the caecum may not be suitable for helicobacters, a concept reflected in the above results that showed *Helicobacter* spp. were restricted to the distal end (the colon and rectum) of the GIT in this animal. In comparison, the BTP eats less eucalypt leaves and has less ability to grind leaves into fine particles. Furthermore, it has no selective retention of the digesta, so there are less toxins in any specific areas in the GIT, thus this allows *Helicobacter* species to colonise regions between the caecum to rectum of the BTP and in some BTPs, to expand to the ileum at 3 cm above the caecum.

Digestive Mechanisms.

The RTP and BTP have developed different mechanisms for obtaining sufficient nutrition and energy from their plant foods and for overcoming the toxic effects of plant secondary metabolites. How these mechanisms operate is still unclear. Investigation of the effects of tannin on the digestion of high-tannin *Eucalyptus melliodora* (yellow box) leaves in the BTP was investigated by Foley *et al.* (1987) [155]. In this study the intake and digestibility of tannin-rich eucalypt leaves was examined and compared with the results of a diet in which tannin had been inactivated with polyethylene glycol. It was found that within the BTP's digestive tract, tannin significantly reduced fibre digestibility and tended to lower nitrogen digestibility. The authors suggested that the inability of the BTP to intake only eucalypt leaves is due to the lack of a mechanism to selectively retain fluid and fine particles, including bacteria, in the caecum and thus microbial matter is lost in the faeces. Thus to be able to obtain sufficient nutrition and energy the BTP has to overcome these problems by using a behavioural solution rather than a physiological one. This results in the BTP spending more feeding time on the ground eating other plants, even although this increases the risk of predation [155].

In a similar study to that described in the BTPs, the effects of tannins on the digestion of high-tannin *Eucalyptus ovata* (swamp gum) leaves in the RTP were investigated by McArthur *et al.* (1991) [214]. This study found that there was no reduction in digestibility of fibre and nitrogen in the RTP. McArthur *et al.* suggested that RTPs have some mechanisms to counteract the digestion-inhibiting effect of eucalypt tannin. The specialised caecum of the RTP, which is

the site of fermentation, is the most likely site for the dissociation of tannin-protein complexes. This suggestion was also supported by a microscopic investigation of the digesta derived from *Eucalyptus ovata* leaves in the GIT of the RTP by O'Brien *et al.* (1986) [154]. These authors observed high numbers of bacteria attaching to plant fragments in the RTP's caecum. Many of these bacteria are ingested due to the caecotrophic behaviour of the RTP. It was suggested that the RTP feeding coprophagically is living off two sorts of protein: leaf protein and microbial protein. The source for the nitrogen content of the microbial protein is almost certainly leaf nitrogen, which is converted into microbial nitrogen by detanning of leaf protein in the caecum and lower colon by microbial action [154].

Two additional studies of marsupial herbivores by McArthur *et al.* have investigated the nutritional effects of quebracho (*Schinopsis* spp.), a condensed-tannin, in two foregut fermenters and two hindgut fermenters. The two foregut fermenters studied were a grazing parma wallaby, *Macropus parma,* (which mainly eats grasses) and a browsing red-bellied pademelon, *Thylogale billardierii,* (which mainly eats trees and shrubs) [215]. The two hindgut fermenters were brushtail possums and ringtail possums [200]. The quebracho used in these studies was composed of a low molecular weight fraction (non-tannin) and a higher molecular weight protein-binding fraction which was known to reduce protein digestibility in a range of placental mammals. This study showed that quebracho had the greatest effect on the digestibility of protein by the parma wallaby with only a minor effect being observed in the red-bellied pademelon. As tannins are widespread in dicotyledenous forbs, trees and shrubs (the normal diet of browsers) but absent in grasses (the normal diet of grazers), it is believed that browsers have evolved mechanisms to reduce the effect of tannin on the digestibility of protein or fibre. In contrast, quebracho did not reduce the digestibility of protein or fibre in either of the hindgut fermenters (BTPs and RTPs).

The results of the above studies by McArthur *et al.* showed that in the RTP neither tannin nor quebracho had an effect in reducing the digestibility of protein whereas in the BTP, quebracho also had no effect but tannin was found to

reduce digestibility of protein. McArthur *et al.* suggested that the impact of tannin can vary in consumers depending on their diet and their gut structure and function. Furthermore this response is not due to the function of hindgut vs. foregut fermenters as other hindgut fermenters such as rats and prairie voles are severely affected by quebracho, both showing reduced protein digestibility and toxicity levels high enough to cause death [216, 217] (reviewed by McArthur *et al.* [200]. It was also suggested by McArthur *et al.* that the reactivity of the tannin to protein may be minimised by certain gut conditions such as detergency in the caecum, and its degradation to smaller phenolic subunits by caecal microbes [200]. These small phenolic compounds may be retained in the caecum and proximal colon for extended periods which would maximise the opportunity for their microbial degradation [146].

The actual mechanisms of digesting food to obtain sufficient nutrition and energy, as well the ability to tolerate the toxins produced within the GIT of possums is complex and unclear. Comparison of the three basic strategies used by RTPs and BTPs for obtaining sufficient nutrition and energy from food suggests that diet and the mechanisms involved in the digestion of food may have a significant impact on the colonisation by *Helicobacter* species. Based on the findings of the current study, one possible hypothesis could be that the higher the intake of eucalypt leaves by the host, the lower colonisation of *Helicobacter* species in their GIT.

The presence of *Helicobacter* spp. in both the BTPs and RTPs raises questions as to their impact on the digestive system. To be able to thrive in the GIT of animals feeding on plant foods high in antinutrients (such as eucalypt leaves), possum *Helicobacters* must possess a specific ability or have evolved mechanisms that allow them to tolerate the toxins of plant secondary metabolites, as well as utilise the nutrients available in this region. Investigation of the levels and effects of plant secondary metabolites on *Helicobacters* in different locations along the GIT of the RTP and the BTP, as well as how these organisms respond to specific compounds present in eucalypt leaves, may provide a greater understanding of how these digestive mechanisms impact on the colonisation by microbes. In particular investigation of the ability of

Helicobacter species to utilise tannin-treated nutrient media or their tolerance of the toxic effects of tannins would provide an important insight into the role of *Helicobacter* spp. in both the BTP and RTP gastrointestinal tracts.

Chapter 6

Characterisation and identification of new *Helicobacter* species isolated from the GIT of Possum

6.1 Introduction

Since the initial discovery that in humans *H. pylori* plays a critical role in peptic ulcer disease, gastric cancer and MALT lymphoma the number of species in the genus *Helicobacter* has expanded rapidly [62, 65-68]. Currently there are 23 formally named *Helicobacter* species (Last full update: March 18, 2004) as well as many more putative species awaiting formal classification (http:// www.bacterio.cict.fr/h/helicobacter.html). However, problems have been encountered in the formal classification of several species within the *Helicobacter* genus. In these cases, classification was based either solely on 16S rRNA gene sequence data, or on a single isolate. For example, "*Helicobacter* sp. strain Mainz" [125] and "*H. westmeadii*" [126] have now been shown to represent additional strains of *H. cinaedi* [123] while *H. nemestrinae,* whose formal classification was based on only a single isolate, has subsequently been described as a junior heterotypic synonym of *H. pylori* [127].

The taxonomy of the morphologically similar group of organisms described as *'Flexispira rappini'* is based on 16S rRNA gene sequence data. This complicated group of organisms is now known to contain at least 10 possible taxa. Two of these flexispira taxa were previously named, *Helicobacter bilis* (flexispira taxon 9) and *Helicobacter trogontum* (flexispira taxon 6). Recently, using polyphasic characterisation, including DNA-DNA hybridisation together with the recommended criteria for the description of novel *Helicobacter* species, flexispira taxa 1, 4 and 5 and the Finnish porcine *Helicobacter i*solates have been proven to be members of the species *Helicobacter trogontum* (flexispira taxon 6) [218].

To avoid the confusion resulting from the premature formal naming of a species, the International Committee of Systematic Bacteriology on the taxonomy of *Campylobacter* and related species has agreed, in principle, on minimum

requirements for the description of new species in the genus *Helicobacter* [128]. These requirements are consistent with a polyphasic approach to bacterial systematics [124]. It is recommended that the description of a putative new species or subspecies is based on the examination of as many strains as possible, preferably not fewer than 5 isolates from different sources, in order to provide a measure of phenotypic and genotypic variation. Where too few strains are available for formal naming, a description can be usefully published. The requirements include:

- Cell and colony morphology, motility and Gram reaction
- Growth conditions and characteristics
- Susceptibility to antimicrobial agents
- Biochemical properties including oxidase, catalase, urease, nitrate reduction, indoxyl acetate hydrolysis, alkaline phosphatase activity and gamma glutamyl transferase
- Molecular data such as 16S rRNA data (at least 1450 bp of a minimum of three stains), DNA-DNA hybridisation, G + C content, whole cell protein profiles
- Ecology

As described in chapters 4 and 5, 18 new BTP *Helicobacter* isolates and 5 new RTP *Helicobacter* isolates were isolated from the lower bowel of 11 brushtail possums and 3 ringtail possums, respectively. Cell and colony morphology, motility, growth conditions, 16S rRNA gene sequence and the ecology of these 23 isolates has been described in chapters 4 and 5. According to the recommended minimal requirements for describing new species in the genus *Helicobacter,* formal naming for the comma and fusiform shaped helicobacters from the BTP and the S-shaped helicobacters from the RTP can be undertaken. There are however too few strains for formal classification of the S-shaped helicobacters isolated from BTP can also to be described.

For the formal naming of the possum isolates, additional information such as biochemical properties, susceptibility to antimicrobial agents, phylogenetic analyses and other molecular data such as DNA-DNA hybridisation is required.

Thus the aim of this study is to obtain the information required for the formal naming of the three potentially new *Helicobacter* species isolated from possums.

In addition, as the fusiform shaped organisms isolated from the brushtail possums were shown to be flexispira-like organisms (fusiform shaped with periplasmic fibres around the cells and bipolar tufts of sheathed flagella), these isolates will be compared phenotypically and genotypically with the other 10 flexispira taxa and *Helicobacter aurati* (flexispira taxon 11).

6.2 Materials and methods

6.2.1 New isolates obtained from possums

Twenty three *Helicobacter* isolates cultured from the BTPs and RTPs were analysed phylogenetically. These isolates were as follows:

- ‘Comma’ shaped isolates cultured from the BTP: ‘BTP1C’ to ‘BTP9C’, (n=9),
- ‘S’ shaped isolates cultured from the BTP: ‘BTP1S’ to ‘BTP3S’, (n=3),
- ‘Fusiform’ shaped isolates cultured from the BTP: ‘BTP1F’ to ‘BTP6F’, (n=6),
- ‘S’ shaped isolates cultured from the RTP: ‘RTP1S’ to ‘RTP5S’, (n=5).

Due to the laborious nature of cultivation of these new isolates and the limited success in re-growing them from frozen cultures, the phenotypic tests including biochemical tests, growth on other media and resistance to antimicrobial agents was performed on only 7 of the 9 comma-shaped isolates (one isolate per BTP), 5 of the 6 fusiform-shaped isolates (one isolate per BTP) and 5 of the ‘S’ shaped isolates cultured from the 3 RTPs.

6.2.2 Phylogenotypic analysis

6.2.2.1 rRNA sequence homology and phylogenetic tree reconstruction

The inference of 16S rRNA gene sequence homology (common ancestry) was made based on the level of sequence similarity. 16S rRNA gene sequences of

the possum isolates (Table 6.1) and the sequences from the *Helicobacter* reference strains and closely related bacteria representing species of the genera *Campylobacter, Arcobacter* and *Wolinella* (Table 6.2) were aligned using the GCG program, Pile up. The multiple aligned sequences (GCG/MSF) were used as an input in the HOMOLOGIES program (GCG) and in the phylogenetic tree reconstruction. All the methods used in the phylogenetic analysis are described in section 2.10.4, Chapter 2.

Isolate	Animal	Morphology	Region of isolation	Accession No.
BTP1C	BTP1	Comma	Rectum	AY554115
BTP2C	BTP3	Comma	Rectum	AY554116
BTP3C	BTP5	Comma	Colon	AY554117
BTP4C	BTP8	Comma	Colon	AY554118
BTP5C	BTP9	Comma	Colon	AY554119
BTP6C	BTP9	Comma	Rectum	AY554120
BTP7C	BTP10	Comma	Colon	AY554121
BTP8C	BTP10	Comma	Rectum	AY554122
BTP9C	BTP11	Comma	Rectum	AY554123
BTP1F	BTP3	Fusiform	Rectum	AY554124
BTP2F	BTP5	Fusiform	Rectum	AY554125
BTP3F	BTP6	Fusiform	Caecum	AY554126
BTP4F	BTP6	Fusiform	Colon	AY554127
BTP5F	BTP7	Fusiform	Rectum	AY554128
BTP6F	BTP8	Fusiform	Rectum	AY554129
BTP1S	BTP2	S	Caecum	AY554135
BTP2S	BTP3	S	Caecum	AY554136
BTP3S	BTP9	S	Caecum	AY554137
RTP1S	RTP1	S	Ileum	AY554130
RTP2S	RTP1	S	Colon	AY554131
RTP3S	RTP5	S	Colon	AY554132
RTP4S	RTP9	S	Colon	AY554133
RTP5S	RTP9	S	Rectum	AY554134

Table 6.1 Origin of the *Helicobacter s*pecies obtained from the possums and the accession numbers of their 16S rRNA sequence data.

Taxon	Accession No.
Helicobacter pylori	M88157
Helicobacter acinonychis	M88148
Helicobacter felis	M57389
Helicobacter bizzozeronii	Y09404
Helicobacter mustelae	M35048
Helicobacter pametensis	M88147
Helicobacter aurati	AF297868
Helicobacter fennelliae	M88154
Helicobacter trogontum	U65103
Helicobacter canis	L13464
Helicobacter bilis	U18766
Helicobacter cinaedi	M88150
Helicobacter muridarum	M80205
Helicobacter hepaticus	L39122
Helicobacter canadensis	AF26037
Helicobacter pullorum	L36141
Helicobacter rodentium	U96296
Helicobacter sp. rhesus type 2	AF333340
Arcobacter butzleri	L14626
Wolinella succinogenes	M88159
Campylobacter jejuni	L04315

Table 6.2 Accession numbers of the reference strains used for determination of 16S rRNA position

6.2.3 Phenotypic analysis

Phenotypic tests used for the typing of the putative new *Helicobacter* spp. included Gram stain, activities of urease, catalase, oxidase, gamma-glutamyl transferase and alkaline phosphatase, and hydrolysis of indoxyl acetate and hippurate. These were conducted as described in Section 2.5, Chapter 2. Tolerance to 1.0% (w/v) glycine and 1.5% (w/v) NaCl was determined by culturing the bacteria on HBA supplemented with each of these compounds. Growth at 25°C and 42°C was tested by culturing the isolates on HBA at these incubation temperatures. Susceptibility to nalidixic acid, cephalothin and metronidazole was determined as described in Section 2.6, Chapter 2.

6.3 Results

6.3.1 Phylogenotypic analysis

The near complete 16S rRNA gene sequences (~1450 bp) obtained from 18 BTP isolates and 5 RTP isolates were compared to those sequences in GenBank. Their closet homologies to valid *Helicobacter* spp. are described below. Sequences from the comma shaped BTP isolates were 94 to 95% similar to *H. trogontum* and *H. muridarum.* Sequences from the 'S' shaped BTP isolates were 95% similar to *H. canadensis* and *H. canis.* Sequences from the fusiform shaped BTP isolates were 95% similar to *H. canadensis* and *H. muridarum.* Sequences from the S-shaped RTP isolates were 94% similar to *H. canis* and *H. hepaticus.* The names of the isolates and the accession numbers are shown in Table 6.1. A multiple sequence alignment of the determined 16S rRNA gene sequences of these isolates is presented in Appendix 2.

6.3.1.1 rRNA sequence homology

The sequences from each group of the possum isolates showed a high level of homology within each group. The percentage similarity of each of the isolates, 'comma' shaped BTP isolates, 'S' shaped BTP isolates, 'fusiform' shaped BTP isolates and 'S' shaped RTP isolates, was 98.8%-99.6 % within each group of isolates. These results would suggest that members of each of these 4 groups belong to the same species. The percentage similarity of each of the groups ('comma' shaped BTP, 'S' shaped BTP, 'fusiform' shaped BTP and 'S' shaped

RTP) was <95.3% among the groups. The level of sequence similarity of the 16S rRNA sequences is presented in similarity matrices (Appendix 3).

The low percentage of similarity between each of the groups (<95.3%) indicated that these 4 groups probably constitute 4 different species. In addition, with the exception of the 'comma' shaped isolates none of these 4 groups showed levels of similarity of >97% to any other validly named *Helicobacter* in GenBank. Indeed the only one stain in the GenBank database, shown to be 97.1 to 97.9 % closely related to the ‘comma’ shaped isolates from BTP was *Helicobacter* sp. rhesus type 2 (accession number AF333340), spiral shaped organisms with bipolar sheathed flagella, which were isolated from colonic biopsy of rhesus monkey with diarrhoea.

6.3.1.2 Phylogenetic tree reconstruction

Phylogenetic trees reconstructed from the 16S RNA gene sequence data of the *Helicobacter* species obtained from the BTPs and the RTPs and related reference strains, using the distance, parsimony and maximum likelihood methods, were all found to be similar. A phylogenetic tree reconstructed using the distance method is shown in Figure 6.1. All possum *Helicobacter* sequences formed a monophyletic cluster and within the possum *Helicobacter* cluster the sequences obtained from ‘BTP1C to BTP9C’, ‘BTP1F to BTP6F’, ‘BTP 1S to BTP3S’ and ‘RTP1S to RTP5S’ isolates clustered into 4 groups in the phylogenetic tree. The bootstrapping values among the groups of ‘comma’ shaped BTP, ‘S’ shaped BTP, ‘fusiform’ shaped BTP and ‘S’ shaped RTP, were equal to 1000. These results further support the view that these 4 groups constitute 4 different species.

Thus, the results of the phylogenetic studies further confirm that the 3 groups of isolates from the possums could constitute 3 novel *Helicobacter* species. To further investigate this possibility, additional characteristics of each group were examined. These characteristics included the criteria required for the naming of new species as previously described [128]. In addition PCR primers specific for each of the proposed new possum *Helicobacter* species were designed and

validated. What follows is a description of the putative new species which have been named as:

- *“Helicobacter vulpecula”*; isolates BTP1C- BTP9C, from the brushtail possum (named after the brushtail possum, *Trichosurus vulpecula*).
- *“Helicobacter peregrinus”;* isolates RTP1S-RTP5S from the ringtail possum (named after the ringtail possum, *Pseucheirus peregrinus*).
- “*Helicobacter kirkbridei*”, isolates BTP1F -BTP6F from the brushtail possum (named after Kirkbride who isolated the first “flexispira” strain from an aborted lamb foetus) [53].

Figure 6.1 The phylogenetic tree constructed from *Helicobacter* spp. obtained from the brushtail and ringtail possums and the references strains of *Helicobacter* and related genera using the distance method [162].

6.3.2 Phenotypic analysis of "*Helicobacter vulpecula*" sp. nov.

6.3.2.1 Growth and morphological characteristics

All 7 'comma' shaped isolates from the brushtail possums (BTP1C-BTP4C, BTP6C, BTP8C and BTP9C) grew on HBA and CSA at 37°C under microaerobic and anaerobic conditions, but not at 25°C or 42°C. The growth appeared as pinpoint colonies after 4-5 days of incubation.

Phase contrast microscopic examination showed that "*Helicobacter vulpecula"* sp. nov were motile curved to spiral rods (0.3-0.5 x 1.6-2.2μm) however no flagella were apparent by transmission electron microscopy. A transmission electron micrograph of "*Helicobacter vulpecula"* sp. nov is shown in Figure 6.2.

6.3.2.2 Biochemical and physiological characteristics

All 7 isolates examined were Gram negative, oxidase and catalase positive and urease negative. They grew in the presence of 1% glycine and 1.5% NaCl. They were resistant to nalidixic acid (30 μg disc) (inhibition zone = 0 mm) and cephalothin (30 μg disc) (inhibition zone = 0 mm) but sensitive to metronidazole (5 μg disc) (inhibition zone >50 mm). They reduced nitrate to nitrite. They did not hydrolyse hippurate. Alkaline phosphatase and gamma-glutamyl transferase activities were negative. Indoxyl acetate hydrolysis was variable (positive in 4/7 strains). A comparison of a number of these characteristics with other *Helicobacter* species is shown in Table 6.3.

6.3.2.3 Identification of strains by PCR with specific primers

PCR primers BC1405f and BC1914r were designed for rapid identification of "*Helicobacter vulpecula"* sp. nov. The multiple sequence alignment created for the phylogenetic analysis was used to locate these oligonucleotide primers. The sequence of the primers was checked for probe match against small subunit rRNAs of the prokaryotes using the Check Probe version 2.1r3, http://rdp.cme.msu.edu/cgis/probe_match.cgi. The thermocycling profile used is as follows: an initial denaturation at 94°C for 5 minutes followed by 35 cycles of

denaturation at 94°C for 5 seconds, annealing at 57°C for 5 seconds and extension at 72^{0}C for 30 seconds, followed by a final extension at 72°C for 2 minutes. The $MgCl_2$ concentration used was 2 mM. The expected size of the PCR product is 510 bp. The sequence of the BC1405f and BC1914r primer pair is shown below:

BC1405f	5' CGCTAATGTGCGAAAGCAT 3'	forward (Tm = 63.8^{o}C)
BC1914r	5' TCTTACGATATTGCTTCTCGT 3'	reverse (Tm = 58.0^{o}C)

The specificity of the amplification using this primer pair and the thermocycling profile was tested against the 5 strains of "*Helicobacter peregrinus"*", the 5 strains of "*Helicobacter kirkbridei"*" and *Helicobacter* sp. 'BTP1S'–'BTP3S' (Figures 6.3) as well as *H. pylori, H. bilis, H. mustelae, H. trogontum, H. felis, H. hepaticus, H. rodentium* (Figures 6.8 B). No products were obtained from any of these isolates or species. In contrast, amplification of "*Helicobacter vulpecula"* was achieved from DNA extracted from the 7 strains of "*Helicobacter vulpecula"*. Amplification of "*Helicobacter vulpecula"* was also achieved from DNA extracted from the original GIT samples from the BTPs from which "*Helicobacter vulpecula*" had been isolated.

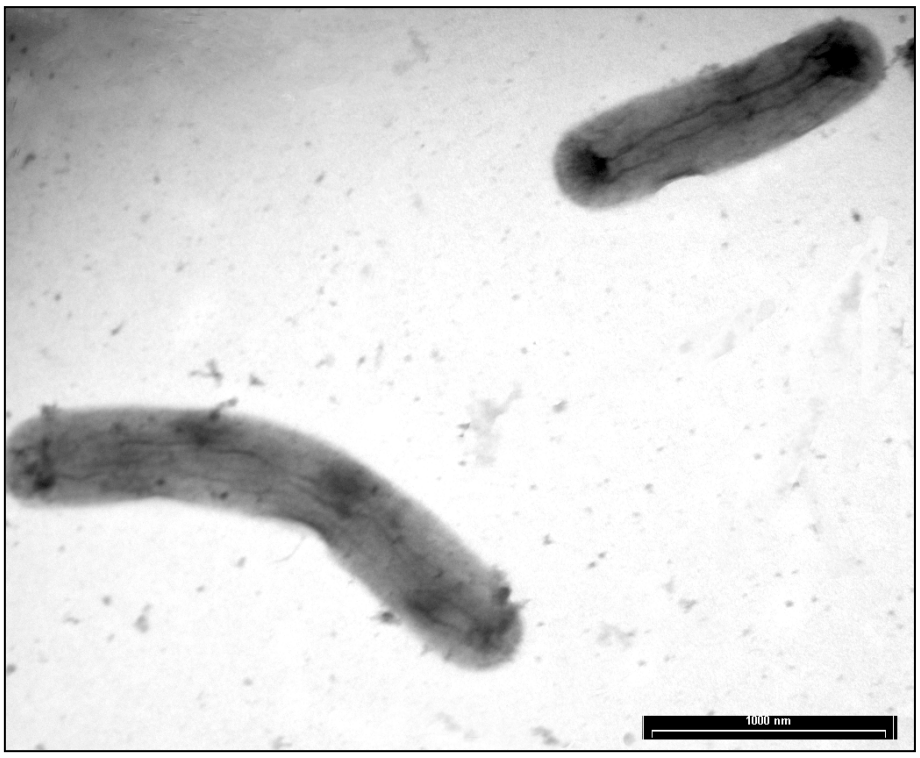


Figure 6.2 A transmission electron micrograph of "*Helicobacter vulpecula*", type strain 'BTP2C". These bacteria measured 0.3 by 1.5-2.2 μm. No flagella were apparent. (Bar =1000 nm)

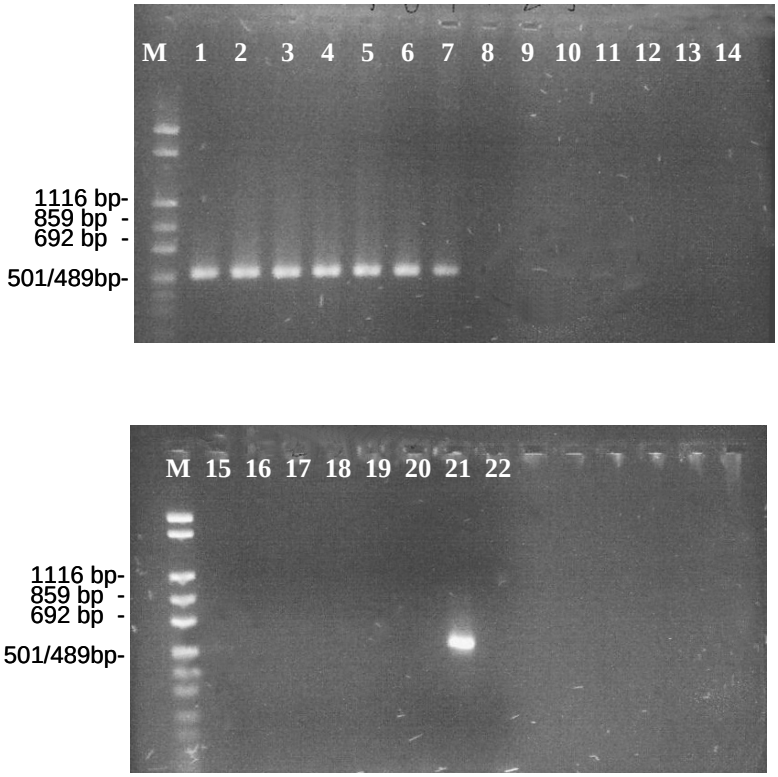


Figure 6.3 The results of a *Helicobacter vulpecula"* species specific PCR showing a 510 bp product where DNA from the RTP and other BTP *Helicobacter* isolates was amplified using the primers BC1405f and BC1914r, and then separated on a 1.5 % agarose /TAE gel.

Lane M = FN1	Lane 8 = BTP1F	Lane 16 = RTP1S
Lane 1 = BTP1C	Lane 9 = BTP2F	Lane 17 = RTP2S
Lane 2 = BTP2C	Lane 10 = BTP4F	Lane 18 = RTP3S
Lane 3 = BTP3C	Lane 11 = BTP5F	Lane 19 = RTP4S
Lane 4 = BTP4C	Lane 12 = BTP6F	Lane 20 = RTP5S
Lane 5 = BTP6C	Lane 13 =BTP1S	Lane 21 = BTP1C
Lane 6 = BTP8C	Lane 14 = BTP2S	Lane 22 = 'No DNA' control
Lane 7 = BTP9C	Lane 15 =BTP3S	

6.3.3 Phenotypic analysis of "*Helicobacter peregrinus*" sp. nov.

6.3.3.1 Growth and morphological characteristics

All 5 'S' shaped isolates from the ringtail possums (RTP1S-RTP5S) grew on HBA and CSA at 37°C under microaerobic and anaerobic conditions, but not at 25°C or 42°C. The growth appeared as pinpoint colonies.

Phase contrast microscopic examination showed that "*Helicobacter peregrinus*" sp. nov were motile spiral rods (0.3 x 2.5 μm). A transmission electron micrograph of "*Helicobacter peregrinus*" sp. nov is shown in Figure 6.4.

6.3.3.2 Biochemical and physiological Characteristics

The 5 isolates examined were Gram negative, oxidase and catalase positive and urease negative. They grew in the presence of 1% glycine (w/v) and 1.5% NaCl (w/v). They were resistant to nalidixic acid (30 μg) (inhibition zone = 0 mm) but sensitive to metronidazole (5 μg) (inhibition zone >50 mm) and cephalothin (30 μg) (inhibition zone >25 mm). Some strains (3/5) reduced nitrate to nitrite. All isolates were negative for alkaline phosphatase and gamma-glutamyl transferase activities, and hippurate and indoxyl acetate hydrolyses. A comparison of some of these characteristics with other *Helicobacter* species is shown in Table 6.3.

6.3.3.3 Identification of strains by PCR with specific primers

PCR primers RS1469f and RS2120r were designed for rapid identification of the "*Helicobacter peregrinus"* as described in section 6.3.2.3. The expected size of the PCR product is 650 bp. The thermocycling profile was the same as described in section 6.3.2.3, except that the annealing temperature was 55°C. The sequences of the RS1469f and RS2120r primers are shown below;

RS1469f	5' CTATGGATGCTAGTTGTTGCT 3'	forward (Tm = 58.5^{o}C)
RS2120r	5' CCGTAGACAGTAGTTATTTTAA 3'	reverse (Tm = 52.3^{o}C)

The specificity of the amplification using this primer pair and the thermocycling profile outlined above were tested against the 7 strains of "*Helicobacter vulpecula*"", the 5 strains of "*Helicobacter kirkbridei*", and *Helicobacter* sp. 'BTP1S'–'BTP3S' (Figures 6.5) and *Helicobacter* reference species as listed in section 6.3.2.3 (Figures 6.8 A). No products were obtained from any of these isolates or species. In contrast, amplification of "*Helicobacter peregrinus"* was achieved from DNA extracted from the 5 strains of "*Helicobacter peregrinus"* and also from DNA extracted from the original GIT samples from the RTPs from which "*Helicobacter peregrinus*" had been isolated.

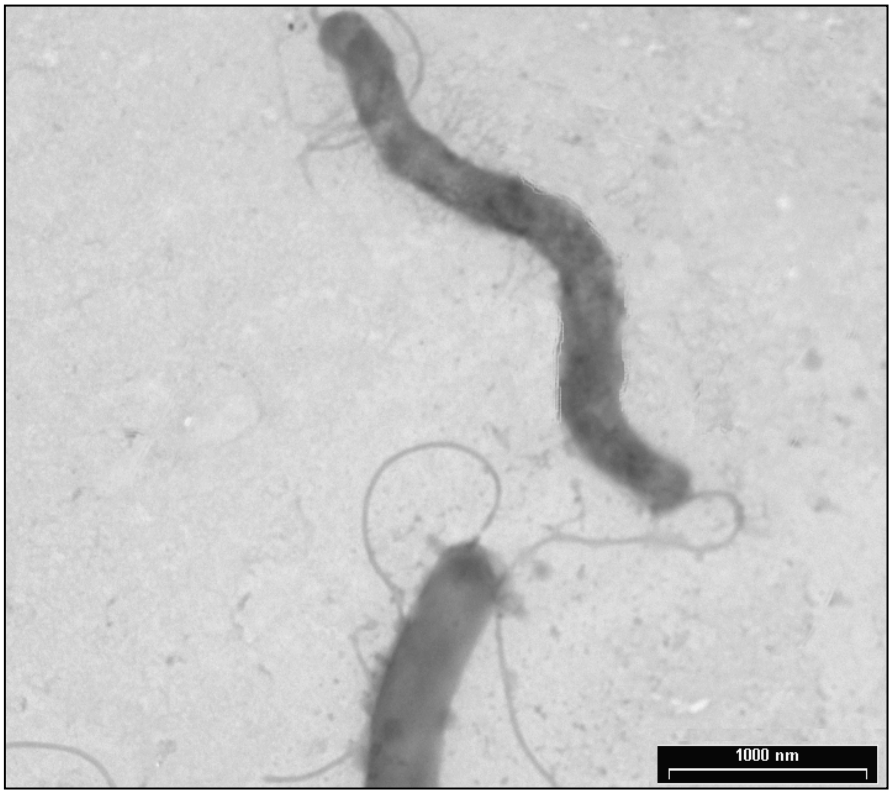


Figure 6.4 A transmission electron micrograph of "*Helicobacter peregrinus*", type strain (RTP4S). This bacterium measured 0.3 by 2.5 μm and had bipolar flagella. (Bar =1000 nm)

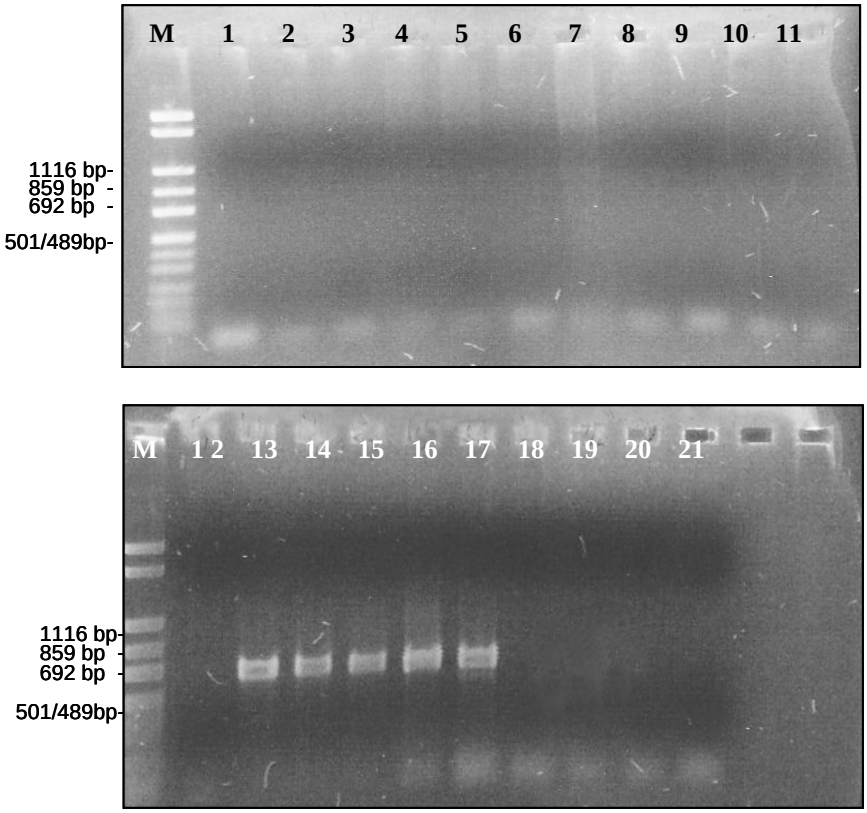


Figure 6.5 The results of a "*Helicobacter peregrinus"* species specific PCR showing a 650 bp product where DNA from the BTP and RTP *Helicobacter* isolates was amplified using the primers RS1469f and RS2120r, and then separated on a 1.5 % agarose /TAE gel.

Lane M = FN1	Lane 8 = BTP1F	Lane 16 = RTP4S
Lane 1 = BTP1C	Lane 9 = BTP2F	Lane 17 = RTP5S
Lane 2 = BTP2C	Lane 10 = BTP4F	Lane 18 = BTP1S
Lane 3 = BTP3C	Lane 11 = BTP5F	Lane 19 = BTP2S
Lane 4 = BTP4C	Lane 12 = BTP6F	Lane 20 = BTP3S
Lane 5 = BTP6C	Lane 13 = RTP1S	Lane 21 = 'No DNA' control
Lane 6 = BTP8C	Lane 14 = RTP2S	
Lane 7 = BTP9C	Lane 15 = RTP3S	

6.3.4 Phenotypic analysis of "*Helicobacter kirkbridei*" sp. nov.

6.3.4.1 Growth and morphological characteristics

All 5 'fusiform' shaped isolates grew on HBA and CSA at 37°C under microaerobic and anaerobic conditions, but not at 25°C or 42°C. The growth appeared as thin water-like films after 4-5 days of incubation.

Phase contrast microscopic examination showed that "*Helicobacter kirkbridei*" sp. nov were motile fusiform shaped rods (0.5-0.8 x 2.6-3.4 μm). A transmission electron micrograph of a "*Helicobacter kirkbridei*" isolate is shown in Figure 6.6.

6.3.4.2 Biochemical and physiological Characteristics

All 5 isolates were Gram negative, oxidase, catalase and urease positive. They grew in the presence of 1% glycine (w/v) and 1.5% NaCl (w/v). They were resistant to nalidixic acid (30μg) (inhibition zone = 0 mm) and cephalothin (30μg) (inhibition zone = 0 mm) but sensitive to metronidazole (5μg) (inhibition zone >30 mm). They did not reduce nitrate. Alkaline phosphatase activity was negative but gamma-glutamyl transferase activity was positive. All isolates hydrolysed hippurate but not indoxyl acetate. A comparison of some of these characteristics with other *Helicobacter* species is given in Table 6.3.

6.3.4.3. Identification of strains by PCR with specific primers

PCR primers BF1295f and BF1905r were designed for rapid identification of *Helicobacter kirkbridei*" sp. nov as described in section 6.3.2.3. The expected size of the PCR product is 710 bp. The thermocycling profile was the same as described in section 6.3.2.3. The sequences of primers BF1295f and BF1905r are shown below:

BF1295f	5' TGCATTTGAAACTATTAACCTA 3'	forward (Tm = 55.7^{o}C)
BF1905r	5' TCACAGTATTGCATCTCCTT 3'	reverse (Tm = 57.2^{o}C)

The specificity of the amplification, using this primer pair and the thermocycling profile, was tested against the 7 strains of "*Helicobacter vulpecula*"", the 5 strains of "*Helicobacter peregrinus*" and *Helicobacter* sp. 'BTP1S'–'BTP3S'

(Figures 6.7) and *Helicobacter* reference species as listed in section 6.3.2.3 (Figures 6.8 C). No products were obtained from any of these isolates or species. The amplification of "*Helicobacter kirkbridei*"" was achieved from DNA extracted from the 5 strains of "*Helicobacter kirkbridei*" and also achieved from DNA extracted from the GIT sample of the BTPs from which "*Helicobacter kirkbridei*"" had been isolated.

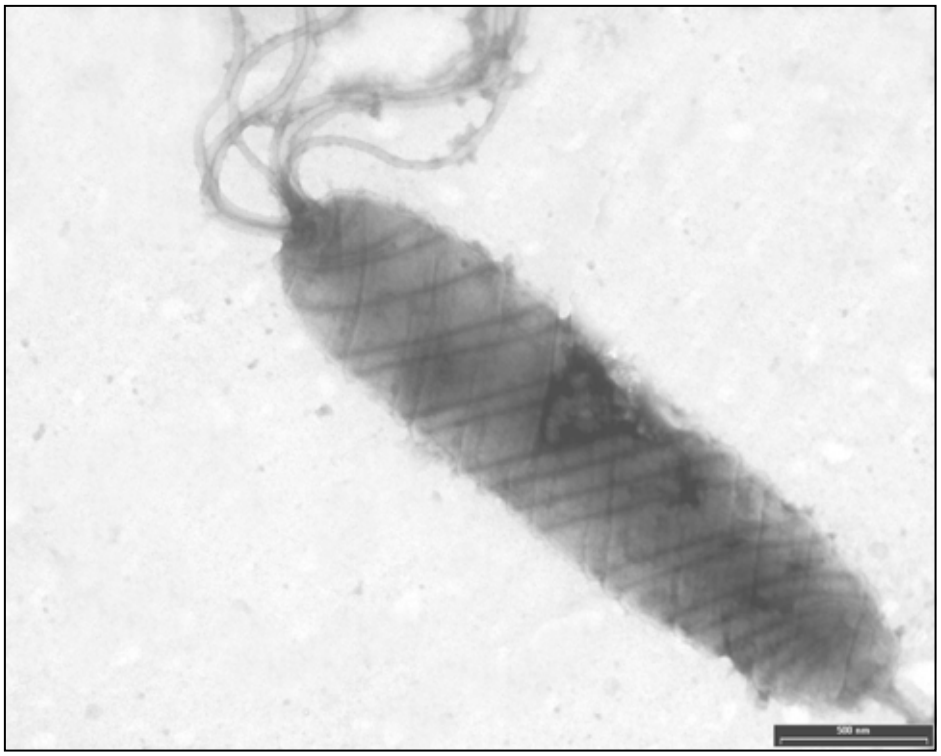


Figure 6.6 A transmission electron micrograph of "*Helicobacter kirkbridei*", type strain (BTP1F). The bacterium measured 0.6 by 2.5 μm and was entwined with periplasmic fibres, which appear to cover the whole cell. It had multiple bipolar sheathed flagella. (Bar = 500 nm)

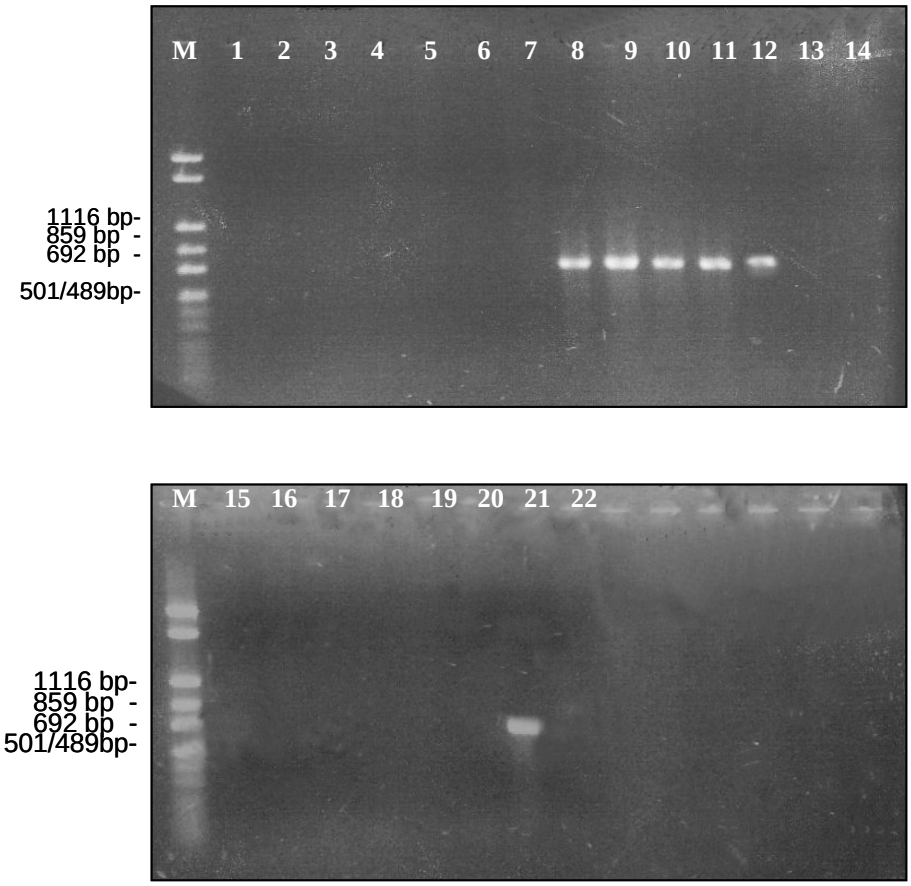


Figure 6.7 The results of a "*Helicobacter kirkbridei*" species specific PCR showing a 710 bp product where DNA from the RTP and other BTP *Helicobacter* isolates was amplified using the primers BF1295f and BF1905r, and then separated on a 1.5 % agarose /TAE gel.

Lane M = FN1	Lane 8 = BTP1F	Lane 16 = RTP1S
Lane 1 = BTP1C	Lane 9 = BTP2F	Lane 17 = RTP2S
Lane 2 = BTP2C	Lane 10 = BTP4F	Lane 18 = RTP3S
Lane 3 = BTP3C	Lane 11 = BTP5F	Lane 19 = RTP4S
Lane 4 = BTP4C	Lane 12 = BTP6F	Lane 20 = RTP5S
Lane 5 = BTP6C	Lane 13 =BTP1S	Lane 21 = BTP1F
Lane 6 = BTP8C	Lane 14 = BTP2S	Lane 22 = 'No DNA' control
Lane 7 = BTP9C	Lane 15 =BTP3S	

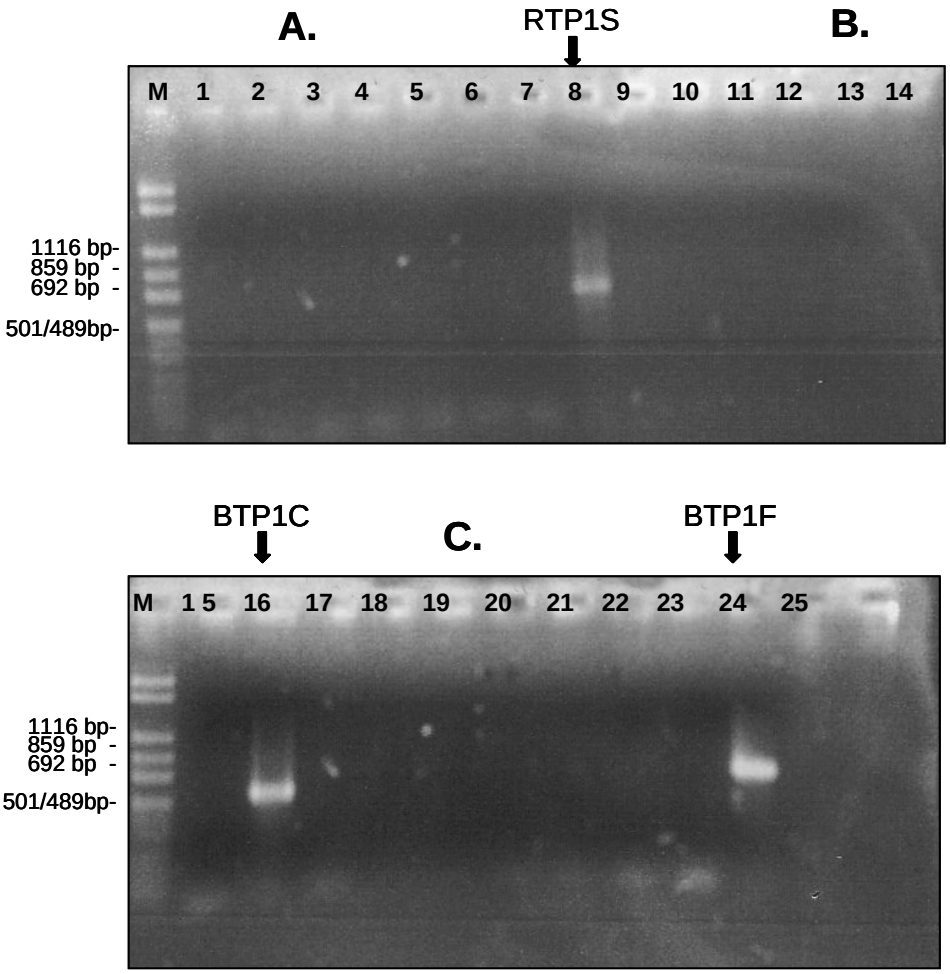


Figure 6.8 The results of *Helicobacter* species specific PCR amplifying DNA from the *Helicobacter* reference strains using the primers A) RS1469f & RS2120r, (expect size = 650 bp), B) BC1405F & BC1914R, (expect size = 510 bp), C) BF1295f & BF1905r, (expect size = 710 bp), separated on a 1.5 % agarose /TAE gel.

A)	B)	C)
Lane M = FN1		Lane 17 = *H. pylori*
Lane 1 = *H. pylori*	Lane 9 = *H. pylori*	Lane 18 = *H. bilis*
Lane 2 = *H. bilis*	Lane 10 = *H. bilis*	Lane 19 = *H. mustelae*
Lane 3 = *H. mustelae*	Lane 11 = *H. mustelae*	Lane 20 = *H. trogontum*
Lane 4 = *H. trogontum*	Lane 12 = *H. trogontum*	Lane 21 = *H. felis*
Lane 5 = *H. felis*	Lane 13 = *H. felis*	Lane 22 = *H. hepaticus*
Lane 6 = *H. hepaticus*	Lane 14 = *H. hepaticus*	Lane 23 = *H. rodentium*
Lane 7 = *H. rodentium*	Lane 15 = *H. rodentium*	Lane 24 = BTP1F (control)
Lane 8 = RTP1S (control)	Lane 16 =BTP1C (control)	Lane 25 = “No DNA” control

		Flagella				
Characteristics	**Size(μm)**	**No.of flagella/cell**	**Distribution**	**Periplasmic fibers**	**Growth at 42°C**	**Growth on 1% glycine**
"H. vulpecula"	**0.3-0.5 x 1.6-2.2**	**NA**	**NA**	**NA**	**-(0/7)**	**+(7/7)**
"H. kirkbridei"	**0.5-0.8 x 2.6-3.4**	**6-12**	**Bp**	**+(5/5)**	**-(0/5)**	**+(5/5)**
"*H. peregrinus*"	**0.3 X 2.5**	**2**	**Bp**	**-(0/5)**	**-(0/5)**	**+(5/5)**
H. acinonychis	0.3 x 1.5-2	2-5	Bp	-	(-)	-
H. aurati	0.6 x 4-8	7-10	Bp	+	+	-
H. bilis	0.5 x 4-5	3-14	Bp	+	+	+
H. bizzozeronii	0.3 x 5-10	10-20	Bp	-	V	(-)
H. canadensis	0.3 x 1.5-4	1-2	Bp	-	+	ND
H. canis	0.25 x 4	2	Bp	-	+	-
H. cinaedi	0.3-0.5 x 1.5-5	1-2	Bp	-	V	-
H. felis	0.4 x 5-7.5	14-20	Bp	+	V	-
H. fennelliae	0.3-0.5 x 1.5-5	2	Bp	-	(-)	-
H.ganmani	0.3 x 2.5	2	Bp	-	-	-
H. hepaticus	0.2-0.3 x 1.5-5	2	Bp	-	-	+
H. rodentium	0.3 x 1.5-5	2	Bp	-	+	+
H. cholecystus	0.5-0.6 x 3-5	1	Po	-	+	+
H. trogontum	0.6-0.7 x 4-6	5-7	Bp	+	+	ND
H. pullorum	0.5 x3-4	1	Mp	-	+	-
H. mesocricetorum	0.5 x 2.5	2	Bp	-	+	-
H. muridarum	0.5-0.6 x 3.5-5	10-14	Bp	+	-	-
H. mustelae	0.5-1 x 2-5	4-8	Pt	-	V	-
H. nemestrinae	0.2-0.3 x 2-5	4-8	Bp	-	+	-
H. pametensis	0.4 x 1.5	2	Bp	-	+	V
H. pylori	0.5 x 2.5-5	4-8	Bp	-	(-)	-
H. salomonis	0.8-1.2 x 5-7	10-23	Bp	-	-	-

Table 6.3 **Characteristics that differentiate comma shaped BTP isolate, fusiform shaped BTP isolate and S-shaped RTP isolates from other *Helicobacter.* Data was obtained from references and this study. +, positive reaction; -, negative reaction; S, susceptible; R, resistant; ND, not determined; NA, not applicable; Bp, bipolar; Po, polar; Mp, monopolar; Pt, peritrichous. The numbers in parenthesis are numbers of strains positive/number of strains tested.**

Characteristics	Catalase production	Nitrate production	Urease activity	Hippurate hydrolysis	Alkaline phosphatase activity	Glutamyl transferase activity	Indoxyl-acetate hydrolysis	Susceptibility to	
								cephalothin	Nalidixic acid
"H. vulpecula"	**+(7/7)**	**+(7/7)**	**-(0/7)**	**-(0/7)**	**-(0/7)**	**-(0/7)**	**V (4/7)**	**R(7/7)**	**R(7/7)**
"H. kirkbridei"	**+(5/5)**	**-(0/5)**	**+(5/5)**	**+(5/5)**	**-(0/5)**	**+(5/5)**	**-(0/5)**	**R(5/5)**	**R(5/5)**
"H. peregrinus"	**+(5/5)**	**V(3/5)**	**-(0/5)**	**-(0/5)**	**-(0/5)**	**-(5/5)**	**-(0/5)**	**S(5/5)**	**R(5/5)**
H. acinonychis	+	-	+	-	+	+	(-)	S	R
H. aurati	+	-	+	ND	-	+	+	R	S
H. bilis	+	+	+	-	-	+	-	R	R
H. bizzozeronii	+	+	(+)	-	V	+	+	S	R
H. canadensis	+	V	-	ND	-	-	+	R	R
H. canis	-	-	-	-	+	+	+	I	S
H. cinaedi	(+)	+	-	-	(-)	-	(-)	I	S
H. felis	+	+	(+)	-	V	+	(-)	S	R
H. fennelliae	(+)	-	-	-	(-)	-	+	S	S
H.ganmani	(-)	-	-	-	-	-	-	R	S
H. hepaticus	+	+	+	-	-	-	+	R	R
H. rodentium	+	+	-	-	-	-	-	R	R
H. cholecystus	+	+	-	-	+	-	-	R	I
H. trogontum	+	+	+	-	V	+	-	R	R
H. pullorum	+	+	-	-	-	ND	-	S	R
H. mesocricetorum	+	+	-	-	+	-	ND	R	S
H. muridarum	+	-	+	-	+	+	-	R	R
H. mustelae	+	+	-	-	+	+	+	R	S
H. nemestrinae	+	-	+	-	+	ND	-	S	R
H. pametensis	(+)	+	-	-	(+)	-	-	S	S
H. pylori	+	-	+*	-	+	+	(-)	S	R
H. salomonis	+	+	+	-	+	+	+	S	R

Table 6.3 Continued

6.3.5 Comparison of "*Helicobacter kirkbridei*" to the existing flexispira-like organisms.

"Helicobacter kirkbridei" was compared phylogenetically and phenotypically to the reference strains of the 10 flexispira taxa described by Dewhirst *et al*.[70] and *H. aurati* [71]. In this thesis, fusiform shaped *Helicobacters* were isolated from the lower bowel of a wombat (WB1F & WB2F) and 2 long nosed bandicoots (LNB1F & LNB2F) (these will be described in Chapter 7). These isolates were also included in this phylogenetic comparison.

6.3.5.1 Materials and methods

6.3.5.1.1 Phylogenetic comparison

The near complete 16S rRNA gene sequences of *"Helicobacter kirkbridei"* strains BTP1F to BTP6F, *Helicobacter* sp. strains WB1F, WB2F, LNB1F and LNB2F, flexispira taxa 1 to 10, *H. aurati* and reference strains of *Helicobacter* as well as the closely related bacteria representing *Campylobacter, Arcobacter* and *Wolinella* genera (Tables 6.1, 7.3, 6.4 & 6.2 respectively), were used in the sequence similarity determination (Homologies program) and phylogenetic tree reconstruction (distance method). All methods used in the phylogenetic analysis are described in Section 2.10.4, Chapter 2.

6.3.5.2.2 Phenotypic comparison

Phenotypic characteristics of flexispira taxa 1 to 10 and *Helicobacter aurati* (flexispira taxa 11) as described in the original publications, were compared with those of the 5 flexispira-like *Helicobacters* (BTP1F, BTP2F and BTP4F-BTP6F) [70, 71, 96, 108].

6.3.5.2 Results

6.3.5.2.1 Phylogenetic comparison

Percentage sequence similarities for the species and isolates described above are shown in Appendix 4 and summarise below.

- The 16S rRNA sequences of "*Helicobacter kirkbridei"* strains BTP1F-BTP6F were <95.8 % similar to all flexispira taxa compared.

- The 16S rRNA sequences of LNB1F was most similar (99.2%) to flexispira taxa 9 (*H. bilis*) and >97% similar to flexispira taxa 2 (97.5%), taxa 4 (97.4%), taxa 7 (97.2%) and taxa 8 (98.9%) and <97% similar to the remaining flexispira taxa that were compared.
- The 16S rRNA sequences of LNB2F was 97.3 % and 97.0% similar to flexispira taxa 6 and 7 respectively, and <97% similar to the remaining flexispira taxa.
- The 16S rRNA sequences of WB1F and WB2F was <97% similar to all flexispira compared (93.1% - 96.7%).

The phylogenetic tree constructed from the 16S rRNA gene sequence data of all isolates included in this comparison, is shown in Figure 6.8.

6.3.5.2.2 Phenotypic comparison

A comparison of some of the phenotypic characteristics of flexispira taxa 1 to 10 and *H. aurati* with those of "*Helicobacter kirkbridei"* (BTP1F, BTP2F & BTP4F-BTP6F) are presented in Table 6.5. This comparison showed that all flexispira-like *Helicobacters* (except for flexispira taxon 10 and *H. aurati*), were urease and gamma-glutamyl tranferase activity positive, alkaline phosphatase activity and indoxyl acetate hydrolysis negative and resistant to nalidixic acid and Cephalothin. Flexispira taxon 10 was urease and gamma-glutamyl tranferase negative. *H. aurati* hydrolysed indoxyl acetate and sensitive to nalidixic acid. Furthermore, incubation at 42°C did not support the growth of "*Helicobacter kirkbridei*".

Flexispira taxa	Sequences accession no.	Sources	Isolated by (References)
Flexispira taxon 1	U96300	Pig	C.E. Gates [70]
Flexispira taxon 2	AF225546	Sheep	C.E. Gates [70]
Flexispira taxon 3	AF22547	Pig stomach: aborted foetus	C.E. Gates [70]
Flexispira taxon 4	AF22548	Sheep	C.E. Gates [70]
Flexispira taxon 5	M88137	Aborted sheep foetus	C.A Kirkbride [53, 70]
Flexispira taxon 6 (*H. trogontum*)	U65103	Rat intestine	E.N. Mendes [108]
Flexispira taxon 7	U51874	Dog stomach	K.A. Eaton [85]
Flexispira taxon 8	M88138	Human stool	J.R. Archer [220]
Flexispira taxon 9 (*H. bilis*)	U18766	Bile, liver & intestine of aged mice	J.G. Fox [96]
Flexispira taxon 10	AF107494	Cotton-top tamarin faeces	J.G. Fox [72]
Helicobacter aurati	AF297868	Stomach and caeca of Syrian hamster	M.M. Patterson [71]

Table 6.4 Accession numbers and source of isolates of flexispira taxa 1 to 10 and *Helicobacter aurati*

Figure 6.9 Phylogenetic tree constructed from "*Helicobacter kirkbridei*" (strains BTP1F-BTP6F), Flexispira taxa 1-10, *H. aurati* and reference strains of *Helicobacter* and related genera using the distance method [162].

Characteristics	*"H. kirkbridei"*	Flexispira taxon										*H. aurati*
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	
Nitrate reduction	- (0/5)	-	+	+	+	+	+	-	-	+	-	-
Alkaline phosphatase activity	- (0/5)	-	-	-	-	-	-	-	-	-	-	-
Urease activity	+ (5/5)	+	+	+	+	+	+	+	+	+	-	+
Indoxyl acetate hydrolysis	- (0/5)	-	-	-	-	-	-	-	-	-	-	+
Glutamyl transferase activity	+ (5/5)	+	+	+	+	+	+	+	+	+	-	+
Growth at 42 ^{0}C	- (0/5)	+	+	+	+	+	+	+/-	+	+	+	+
Growth with 1% glycine	+ (5/5)	-	-	-	-	w	-	-	-	+	+	-
<u>Susceptibility to</u>												
Nalidixic acid (30 ug)	R (5/5)	R	R	R	R	R	R	R	R	R	R	S
Cephalothin (30 ug)	R (5/5)	R	R	R	R	R	R	R	R	R	R	R
Periplasmic fibres	+ (5/5)	+	+	+	+	+	+	+	+	+	+	+
Distribution of flagella	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Bp

Table 6.5 **Comparison of physiological and biochemical characteristics of the "*Helicobacter kirkbridei*" determined during this study with *Helicobacter* spp., flexispira taxa 1 to 10, and *H. aurati*.**

+ = 80-100% strains positive, - = 0-17% strains negative, S = susceptible, R = resistant, W = weak, Bp = Bipolar.

6.4 Summary and Discussion

Twenty three *Helicobacter* isolates were isolated and characterised from the lower bowel of 11 brushtail possums and five isolates from the lower bowel of 3 ringtail possums. On the basis of phylogenetic and phenotypic characteristics these isolates could be separated into four groups. Adequate numbers of isolates and detailed information were available from 3 of these 4 groups to allow for the proposal that these three groups constitute putative novel *Helicobacter* species. These were given the names "*Helicobacter vulpecula*" and "*Helicobacter kirkbridei"* from the brushtail possum and "*Helicobacter peregrinus*" from the ringtail possum. These possum isolates are the first group of *Helicobacter* species isolated from Australian marsupials and this is the first time that more than one potentially new *Helicobacter* species has been isolated in a single study.

The morphologic appearance of the possum *Helicobacter* species was observed to be similar to that of other *Helicobacter* spp. i.e. curved, spiral and fusiform shaped rods with or without periplasmic fibres. Morphologically "*Helicobacter vulpecula*" is a curved rod. Using TEM, flagella were not observed in "*Helicobacter vulpecula*", although they appeared to be motile using phase contrast microscopy. Thus there is the possibility that the flagella of these organisms may have been lost during preparation of the cells for negative staining. However in the current study the presence of flagella in this species was not further investigated. The morphology of "*Helicobacter peregrinus"* is common among *Helicobacter* species, i.e. 'S' shaped without periplasmic fibres and with single bipolar flagella. *"Helicobacter kirkbridei"* isolates have a fusiform shape with periplasmic fibres and multiple tufts of sheathed flagella, similar to the Flexispira.

Prior to the isolation of *Helicobacter* species from the possums, phylogenetic analysis of sequences from all *Helicobacters* (mainly from placental mammals) had shown that they could be separated into two main groups, the lower bowel and the gastric *Helicobacters*. In accordance with the previous findings phylogenetic analysis of the new possum *Helicobacter* isolates revealed that

they grouped with the lower bowel *Helicobacters.* In the phylogenetic tree (Figure 6.1), all of the possum isolates including the fusiform shaped "*Helicobacter kirkbridei"* were found to be closely related and grouped into one cluster.

Interestingly *Helicobacter* sp. rhesus type 2 (3 strains with identical 16S rRNA gene sequences) also clustered with "*Helicobacter vulpecula*" and shared 97.1 to 97.9% similarity. In general, the level of 16S rRNA gene sequence similarity between the species is found to be <97%, however strains belonging to different species may have almost identical 16S rRNA gene sequences and strains of one species may differ by up to 4.5% [123, 218]. Thus similarity comparisons of 16S rRNA gene sequence do not provide conclusive evidence for species delineation. In this situation, further comparison of "*Helicobacter vulpecula*" with *Helicobacter* sp. rhesus type 2 showed that they were obviously different. *Helicobacter* sp. rhesus type 2 is a spiral shaped bacteria with bipolar sheathed flagella while "*Helicobacter vulpecula"* is comma shaped bacterium with no apparent flagella. In addition *Helicobacter* sp. rhesus type 2 is sensitive to cephalothin while "*Helicobacter vulpecula*" is resistant. Based on phenotypic characteristic, *Helicobacter* sp. rhesus type 2 is unlikely to constitute the same species with "*Helicobacter vulpecula"*.

In contrast to "*Helicobacter vulpecula*", phylogenetic and phenotypic characteristics of *"Helicobacter kirkbridei"* when compared to those of flexispira taxa 1 to 10 and *H. aurati* (taxon 11) showed that these strains were phenotypically similar but phylogenetically different (<95.8% similar to flexispira taxa 1-11). In addition, *"Helicobacter kirkbridei"* had a unique position in the phylogenetic tree and clustered with flexispira taxa 2 and 3. However it is apparent that additional investigations are required to support tentative identification results obtained by comparison of complete 16S rRNA genes. This was evident in the misidentification of *Helicobacter* sp. strain Mainz [125] and *"Helicobacter westmeadii"* [126], which have now been shown to be additional strains of *H. cinaedi* [123]. These additional investigations included DNA-DNA hybridisation, numerical analysis of whole-cell protein or fatty acid profile, or

high-resolution amplified fragment length polymorphism (AFLP)-based fingerprints [128]. By using these methodologies, flexispira taxa 1, 4 and 5 and the Finnish porcine *Helicobacter* isolates were identified as members of *H. trogontum* species [218].

The phylogenetic differences among the possum isolates and other *Helicobacter* species allowed the design of species specific primers. In this study, primers specific for each of the three possum *Helicobacter* species were designed and validated. The development of these primers is particularly important as they will allow the screening of larger numbers of BTPs and RTPs for the presence of *Helicobacter* species and thus the prevalence of the new *Helicobacter* species can be determined in future studies.

As yet, DNA-DNA hybridisation, G + C content or whole cell protein profiles have not been examined and thus each of the novel isolates has not conclusively been showed to belong to a particular *Helicobacter* species. To date the following information has been determined for each of the putative species: cell and colony morphology, motility, Gram reaction, growth conditions and characteristics, resistance to antimicrobial agents, biochemical properties including oxidase, catalase, urease, nitrate reduction, indoxyl acetate hydrolysis, alkaline phosphatase activity and gamma-glutamyl transferase, ecology and 16S rRNA data (at least 1450 bp of a minimum of three stains). This information indicates that each of the putative species described has the potential to be formally proposed as a new *Helicobacter* species.

Description of "*Helicobacter vulpecula*" sp. nov.

"*Helicobacter vulpecula*" [named after the brushtail possum, *Trichosurus vulpecula*]. Cells are curved to spiral rods (0.3-0.5 x 1.6-2.2 μm). Cells appear motile by phase contrast microscopy but no flagella are apparent by transmission electron microscopy. After 4 to 5 days, small, pinpoint, colourless, translucent colonies are observed on 5% horse blood agar (HBA) or *Campylobacter* selective agar (CSA). Growth occurs at 37°C but not at 25 or 42°C under anaerobic and microaerobic, but not aerobic conditions. All isolates

grow on media containing 1.0% glycine (w/v) and 1.5% NaCl (w/v). All isolates are positive for catalase and oxidase but negative for urease activity. Tests for gamma-glutamyl transferase and alkaline phosphatase activity and hippurate hydrolysis are negative. Indoxyl acetate hydrolysis is variable (positive in 4/7 strains). Nitrate is reduced to nitrite. All isolates are sensitive to metronidazole (5 μg) but resistant to cephalothin (30 μg) and nalidixic acid (30 μg). "*Helicobacter vulpecula"* was isolated from the caecum, colon and rectum of brushtail possums.

Description of "*Helicobacter peregrinus*" sp. nov.

"*Helicobacter peregrinus*" [named after the ringtail possum, *Pseudocheirus peregrinus*]. Cells are spiral rods (0.3 x 2.5 μm) with bipolar flagella and are motile under phase contrast microscopy. After 4 to 5 days, small, pinpoint, colourless, translucent colonies are observed on 5% horse blood agar (HBA) or Campylobacter selective agar (CSA). Growth occurs at 37°C but not at 25 or 42°C under anaerobic and microaerobic conditions, but not under aerobic condition. These isolates can grow weakly on media containing 1.0% glycine (w/v) and 1.5% NaCl (w/v). All isolates are positive for catalase and oxidase but are negative for urease and gamma-glutamyl transferase activities. Tests for nitrate reduction are variable (positive in 3/5 strains). Indoxyl acetate hydrolysis, hippurate hydrolysis and alkaline phosphatase activity are negative. All isolates are sensitive to metronidazole (5 μg) and cephalothin (30 μg) but resistant to nalidixic acid (30 μg). "*Helicobacter peregrinus*" was isolated from the ileum, colon and rectum of ringtail possums.

Description of "*Helicobacter kirkbridei*" sp. nov.

"*Helicobacter kirkbridei*" [named after C. A. Kirkbride who isolated the first "flexispira" strain from an aborted lamb foetus [53]. Cells are fusiform shaped rods with periplasmic fibrils (0.5-0.8 x 2.6-3.4 μm) and bipolar multiple flagella (6-12). They are motile under phase contrast microscopy. After 4 to 5 days, a thin water-like film is observed on 5% horse blood agar (HBA) or Campylobacter selective agar (CSA). Growth occurs at 37°C but not at 25 or

42°C under anaerobic and microaerobic, but not under aerobic conditions. All isolates grow on media containing 1.0% glycine (w/v) and 1.5% NaCl (w/v). All isolates are positive for urease, catalase, and oxidase activities. Tests for hippurate hydrolysis and gamma-glutamyl transferase activities are positive. Indoxyl acetate hydrolysis, reduction of nitrates and alkaline phosphatase activity are all negative. The isolates are all sensitive to metronidazole (5 μg) but resistant to cephalothin (30 μg) and nalidixic acid (30 μg). "*Helicobacter kirkbridei*" was isolated from the caecum, colon and rectum of brushtail possums.

Chapter 7

The detection and isolation of spiral and fusiform shaped organisms, in particular *Helicobacter* species, from the gastrointestinal tract of other marsupials

7.1 Introduction

Given the high detection rate of spiral and fusiform shaped bacteria from the brushtail and ringtail possums, this study was broadened to cover additional Australian marsupial species. The marsupials available during the examination period of this study included the koala, wombat and Eastern grey kangaroo (herbivores), the Eastern quoll and Tasmanian devil (carnivores) and the long nosed bandicoot (omnivores). Each of these marsupials is shown in Figure 7.1.

Koala (*Phascolarctos cinereus*)

Koalas are tree-dwelling marsupials that feed solely on the foliage of a few species of eucalypt trees [146]. They eat approximately one kilogram of eucalypt leaves per day. Although eucalypt leaves are poisonous to most mammals, the liver of the koala is capable of detoxifying the oils and phenols present in these leaves. Koalas are 'caecum fermenters', having an enlarged caecum, which contains cellulose-digesting microorganisms. The caecal epithelium of the koala has been shown to be covered with Gram positive and Gram negative bacilli, cocci and actinomycete-like organisms which appear to be firmly attached. To date no spirochaetes or fusiform bacilli have, however, been observed [150]. The bacteria present in the lumen of the caecum and proximal colon of koalas has been characterised by London in his M.Sc. Thesis [221], and this work was later reviewed by Hume [146]. London presumptively grouped these bacteria into the genera *Bacteroides, Eubacterium, Peptococcus, Peptostreptococcus, Propionibacterium* and an unidentified group of Gram-positive rods [146]. Koalas are caecophagic and young koalas feed on their mother's faeces. This faecal material contains the microorganisms that will colonise their own caecum and aid in the digestion of eucalypt leaves.

Wombat (*Vombatus Ursinus*)

Wombats are the largest burrowing marsupial [136, 147]. They are heavily built animals with a flattened head and rump, and stumpy powerful limbs. They have one pair of large upper and one pair of large lower incisors. Unlike most other mammals their teeth grow continuously, with those that are worn down by gnawing on roots that obstruct their burrows being replaced. Wombats feed on grasses and herbs as well as the roots of shrubs and trees. They are 'colon fermenters' having a very long and highly developed colon. In these animals, markedly higher numbers of bacteria have been detected in the digesta obtained from the colon, as compared with that from the stomach and small intestine [147]. This finding would suggest that microbial fermentation of plant cell walls in the wombat is confined to the colon. To date, no reports on the composition of the colonic bacterial population of wombats are available, however, anophocephalid cestodes and strongyloid nematodes have been reported to be common in the small intestine and colon respectively**.**

There are two types of wombats, the common wombat and the hairy-nosed wombat. In the current study, the common wombat, which is a forest dweller and frequently found in burrows on the slopes above creeks and gullies in the rocky and mountainous country in the Southeast part of mainland Australia and Tasmania, was examined.

The Eastern grey kangaroo (*Macropus giganteus*)

The kangaroos and wallabies (family Macropodidae), and the rat–kangaroo, bettongs and potoroos (family Potoroidae), are two herbivorous marsupial families in which the primary site of microbial fermentation is the forestomach (foregut fermenters) [222]. "Macropod" means long foot and all members of this family have disproportionately large hindlimbs and reduced forelimbs. Eastern grey kangaroos are large grazing macropodid marsupials whose specific food preference is grass [136]. Eastern grey kangaroos inhabit most areas of the Eastern states of Australia and Tasmania. They have an enlarged forestomach,

where microbial fermentation of ingested food occurs, and a hind stomach that secretes hydrochloric acid and pepsinogen. The forestomach is separated into a sacciform and tubiform stomach [222].

The microbiology of the oesophageal region of the Eastern grey kangaroo has been examined by Obendorf [223]. In this study, which used light microscopy, a dense layer of mixed bacteria were found covering the epithelial cells lining the oesophagus. In Gram stained sections and smears, bacteria of various types were observed, including Gram-negative rods and fusiform shaped organisms arranged in short chains and compact palisades, Gram-positive cocci, coccobacilli and occasional rods arranged in clusters and chains. In a study by Dellow *et al.* the microbial activity in the forestomach of free-living macropodid marsupials, including the Eastern grey kangaroo, was examined [224]. This investigation showed that ciliate protozoa were present in the forestomach, with the greatest number of these being found in the sacciform stomach of all species. The bacterial flora in the forestomach (both sacciform and tubiform) digesta of the Eastern grey kangaroo was found to be complex, with the total bacterial count being reported to range from 1.8 to 4.3 x 10^{11} per gram. Where the pH of the tubiform forestomach digesta was found to lie between 5.7- 6.7, no detail on the specific bacterial groups in the forestomach of the Eastern grey kangaroo was reported.

The Tasmanian devil and Eastern quoll

The Tasmanian devil and Eastern quoll are large dasyurids (family Dasyuridae), and are known to be carnivorous marsupials. Carnivores are believed to be the earliest marsupials that developed at the beginning of the group's history [144]. All animals in this group have five digits on their front feet and never less than four on their hind feet. Carnivores are distinguished from nearly all omnivores and herbivores by their dentition and gastrointestinal tract morphology. Unlike other marsupials the pouches of most carnivores are not pocket-like but shallow. Many species however do not have any pouch at all. They are polyprotodonts and have many sharp teeth that are useful for eating other animals. They eat mainly, although not solely, animal materials consisting of

either vertebrate or invertebrate prey, or both, such as small mammals, birds, lizards and insects. As carnivores, the GITs of Tasmanian devils and Eastern quolls are simple and dominated by the small intestine. No Australian carnivorous marsupials have a caecum. To date, no investigation of the bacterial flora of this group of animals has been undertaken. However, it has been reported that dasyurids harbour the widest range of helminth families of any group of marsupials [144].

The Tasmanian devil (*Sarcophilus harrisii*)

The Tasmanian devil is the largest of the surviving Australian carnivores. It eats any material of animal origin, ranging from corbie grubs to mammals larger than itself. Although the Tasmanian devil has a fierce appearance and awesome name, it is primarily a carrion-eater and is a poor hunter [136]. With its powerful jaws and sharp sectorial teeth, it can consume every part of a dead kangaroo or sheep, including the skull.

The Eastern quoll (*Dasyurus vierrinus*)

The Eastern quoll is a medium sized carnivore. It superficially resembles the European polecat. Eastern quolls have white-spotted black or olive-brown fur that is soft, thick and short [136]. While the Eastern quoll once ranged over most of south-eastern Australia, they are now confined to Tasmania. They are opportunistic carnivores and feed on a wide range of prey, including ground-nesting birds and small mammals such as bandicoots, rabbits, lizards and rats. They also scavenge the carcasses of large animals, such as wallabies, possums and sheep when available. Grasses are eaten regularly and fruit, such as the blackberry, is seasonally popular.

Long nosed bandicoot (*Perameles nasuta*)

Bandicoots are omnivorous marsupials which represent the evolutionary link between the preceding carnivorous group of marsupials and the herbivorous or plant-eating marsupials [145]. Bandicoots possess the polyprotodont teeth arrangement of carnivores, but have a similar foot structure to kangaroos and other diprotodonts. Long nosed bandicoots are slender, medium sized

marsupials and are common in the coastal areas of Eastern Australia [136]. They are solitary ground dwelling animals which feed in all seasons, mainly on ants, insect larvae, cockroaches and plant material. The caecum of long nosed bandicoots is moderate in size. The colon is short relative to the small intestine. Although the bacteriology of the long nosed bandicoot has not been investigated, a diverse array of helminthic parasites, and in particular trichostrongyloid nematodes, have been observed in the intestinal tract of these animals [145].

Thus to date, it is clear that for the majority of marsupials, little is known of their bacterial microbiota, and in particular those bacteria present within the gastrointestinal tract**.**

The aims of the current study were therefore to attempt to detect spiral and fusiform shaped bacteria, in particular *Helicobacter* spp., in different regions of the GIT of the above marsupials and to culture these organisms, if present. Given the success of the methods used for the detection and isolation of *Helicobacter* spp. in both the brushtail and ringtail possum (Chapter 4 and Chapter 5, respectively) identical methods were applied to the study of these animals.

A second aim of this study was to compare the detection and isolation rate of spiral and fusiform shaped bacteria, in particular *Helicobacter* spp. in a variety of marsupials and to determine the association, if any, between their presence and the diet, feeding strategies and digestive system of these marsupials.

Figure 7.1 Animal species from which samples were obtained for the study A) Koala, B) Wombat, C) Eastern grey kangaroo, D) Eastern quoll, E) Tasmanian devil, F) Long nosed bandicoot

7.2 Materials and methods

7.2.1 Animal histories and collection of specimens

All animals included in this study (except for an Eastern grey kangaroo) were obtained from the Veterinary & Quarantine centre, Taronga Zoo, Sydney, Australia. The koalas (n=3), Eastern quoll (n=1) and Tasmanian devil (n=1) were zoo animals living in Taronga Zoo, Sydney (one koala lived in Western Plains Zoo, Dubbo, NSW). The wombats (n=2) were wild animals, living freely in country areas of New South Wales (Kulnura and Cobbity). All long nosed bandicoots (n=3) were wild animals living freely in the North Head National Park, Sydney. The Eastern grey kangaroo (n=1) was an animal living in a semi natural enclosure, Cowan Research Station, which is located in the outskirts of Sydney and had been euthanased for a study of the salivary gland by Associate Professor Mike Beal (School of BEES, UNSW). All animals, except for the Eastern grey kangaroo, had been injured or were in ill health and had been taken to the Veterinary & Quarantine centre for care. Subsequently these animals had died or were euthanased for compassionate reasons. The history of each of the 11 animals is summarised in Table 7.1.

Specimens from these 11 marsupials were collected as described in section 2.2. For each animal, three samples of tissue from each of the following sites were collected: the liver, stomach, mid ileum, ileum at 3 cm above the caecum (3 cm ileum), caecum, colon and rectum. The first sample from a particular location was frozen at -70°C for DNA extraction. The second sample was frozen in one mL of BHIG and kept at -70°C until cultured and the third sample was fixed in formalin for histology and FISH. In all cases except for the kangaroo, frozen samples were placed on dry ice during delivery to the laboratory.

All specimens were examined using phase microscopy, bacterial cultivation, *Helicobacter* genus specific PCR, FISH and histopathology, as outlined in Chapters 2 and 4. Cultivation from the Eastern grey kangaroo was conducted using fresh specimens. At the time of specimen collection, additional specimens from the Eastern grey kangaroo were collected, frozen and stored in liquid nitrogen for later use.

FISH was performed on formalin-fixed stomach sections from the kangaroo and Tasmanian devil, as well as colonic sections from one of the long nosed bandicoots. In all these animals, *Helicobacter* species had been isolated and also detected by PCR in the regions examined.

Animal	Habitat	Sex	Age	Cause of dead	Period in care (days)	Gross pathology
Koala 1	Taronga Zoo	M	J	Diseased/ Euthanased	29	Bacterial pneumonia (*Klebsiella pneumoniae*)
Koala 2	Taronga Zoo	F	A	Diseased/ Euthanased	0	Multi-organ failure, marked cervical and meningeal haemorrhages. No infectious disease noted.
Koala 3	Western Plains Zoo	M	A	Diseased/ Euthanased	-	Bilateral cataracts and retinal atrophy
Wombat 1	Kulnura	M	A	Diseased/ Euthanased	1	Severe sarcopic mange
Wombat 2	Cobbity	M	A	Accident/ Euthanased	1	Marked pulmonary oedema and congestion. Cervical & humeral fractures (probably struck by car)
Eastern grey kangaroo 1	Cowan	F	2	Euthanased	0	Euthanased for salivary gland study
Long nosed bandicoot 1	North Head NP	M	A	Predation/ Euthanased	0	Multiple puncture wounds (probably predated by fox)
Long nosed bandicoot 2	North Head NP	F	A	Predation/ Euthanased	0	Multiple puncture wounds (probably predated by dog). Three to four cestodes found in small intestine.
Long nosed bandicoot 3	North Head NP	M	A	Predation/ Euthanased	0	Multiple puncture wounds (probably predated by fox). Intestinal cestoidiasis and gastric nematoidiasis.
Eastern quoll 1	Taronga Zoo	M	A	Found dead	0	Marked pulmonary congestion. Focal nasal abrasion.
Tasmanian devil 1	Taronga Zoo	F	A	Diseased/ Euthanased	10	Metastatic neoplasia

Table 7.1 The habitat, sex, age, cause of death, period in care and gross pathology present in the koalas, wombats, Eastern grey kangaroo, long nosed bandicoots, Eastern quoll and Tasmanian devil examined in this study.
F = female, M = male, A = adult, J = juvenile, - = no details available,
NP = National Park

7.3 Results

7.3.1 Bacterial cultivation

7.3.1.1 Bacterial cultivation of the herbivorous marsupials (the koala, wombat and Eastern grey kangaroo)

Preliminary phase contrast microscopy of the homogenised livers and GIT mucus scrapings of the 3 **koalas** showed cocci, diplococci, thick curved rods and fat spirals to be present in all regions of the GIT, with large numbers of these bacteria being present in the caecum and colon of all 3 koalas. No bacteria were observed in the liver samples. In **wombat** 1, long thin rods or fusiform shaped rods as well as spiral shaped organisms were present in the GIT mucus scrapings from the mid ileum, ileum, caecum, colon and rectum. In wombat 2, mainly curved rods were observed in the GIT mucus scrapings. In the **Eastern grey kangaroo**, spiral shaped organisms with three to five irregular turns were observed in the mucus scrapings of the stomach, predominantly in the tubiform stomach. A high number of motile curved rods were also observed in the caecum, colon and rectum.

Bacterial culture of the liver and the GIT mucus scrapings of all 3 **koalas** showed a few colourless, small colonies on CSA plates, however, no growth was present on the majority of HBA plates after 3 to 7 days incubation, under either microaerobic or anaerobic conditions. Microscopic examination of the colonies on CSA plates showed cocci and curved rods to be present. From these colonies, 6 separate isolates of curved rod shaped organisms were obtained.

Cultures from the 2 **wombats** showed clear, colourless, small colonies and thin water-like films to be present on both CSA and HBA plates after 3 to 7 days incubation, under either microaerobic or anaerobic conditions. Microscopic examination of these colonies showed them to be of two morphological types, curved to spiral (Sp) and fusiform (F). Of these colonies, 18 separate cultures of Sp and F shaped bacteria were obtained.

Cultures from the **Eastern grey kangaroo** showed clear, colourless, small colonies and thin water-like films on both the CSA and HBA plates after 3 to 7 days incubation, under either microaerobic or anaerobic conditions. Microscopic examination of these colonies showed these to be of the Sp (curved to spiral) morphological type. A total of 9 separate isolates were obtained. A spiral shaped organisms with irregular turns was grown from the tubiform stomach. Unfortunately, this died following attempts to subculture them onto fresh plates to obtain a pure isolate. As a result these bacteria could not be examined in detail.

7.3.1.1.1 Screening of pure isolates for the presence of *Helicobacter* species using the *Helicobacter* genus specific PCR

DNA extracted from the 6 koala isolates, 18 wombat isolates and 8 Eastern grey kangaroo isolates, was amplified using a *Helicobacter* genus specific PCR (Section 2.9.1). None of the koala isolates or the Eastern grey kangaroo isolates, were identified as *Helicobacter* species. Of the 18 wombat isolates, 5 isolates were identified as *Helicobacter* species. These 5 cultures were fusiform shaped and had been isolated from the mid ileum, ileum at 3 cm above the caecum, caecum, colon and rectum of wombat 1, respectively. No helicobacters were isolated from wombat 2.

Of the 5 *Helicobacter* spp. isolates identified using the *Helicobacter* genus specific PCR, the near complete 16S rRNA gene of 2 isolates was sequenced. These isolates were named WB1F and WB2F and had been isolated from the caecum and colon of wombat 1 respectively. In addition, the near complete 16S rRNA gene of a spiral shaped isolate grown from the colon of wombat 1 (named WB1), which was negative by *Helicobacter* genus specific PCR, was also sequenced. The PCR product obtained from WB1F is shown in Figure 7.5

The 16S rRNA gene sequence data obtained from WB1F (1474 bp) and WB2F (1484 bp) was 99.7% similar, suggesting these may belong to a single species. The sequences of both isolates were compared to others in the GenBank database and were confirmed to be *Helicobacter* species. These isolates were

most closely related to *H. canadensis* (96%). The isolate WB1 (1463 bp) was most closely related to *Campylobacter hyointestinalis* (94%) and thus was possibly a new *Campylobacter* species.

7.3.1.2 Bacterial cultivation of the carnivorous marsupials (the Tasmanian devil and Eastern quoll)

Preliminary phase microscopic examination of the homogenised liver and the GIT mucus of the **Tasmanian devil** showed spiral shaped bacteria to be present in the colon and rectum. In the **Eastern quoll**, a large number of bacillus-like organisms were observed in the mucus scrapings of the mid ileum and ileum at 3 cm above the colon. In addition, a mixture of thin and short curved rods and cocci were present in the stomach, colon and rectum.

Bacterial culture of the liver and the GIT mucus scrapings of the **Tasmanian devil** showed clear, colourless small colonies and thin water-like films on both CSA and HBA plates after 3 to 7 days incubation under either microaerobic or anaerobic conditions. Microscopic examination of these colonies showed them to be of the Sp (curved to spiral) morphological type. Of these colonies, 5 separate cultures were obtained. Culture of samples from the **Eastern quoll** showed a few clear, colourless small colonies on both CSA and HBA plates after 3 to 7 days incubation, under either microaerobic or anaerobic conditions. Again microscopic examination of these colonies showed them to be of the Sp (spiral) morphological type. Of these colonies, 10 separate cultures were obtained.

7.3.1.2.1 Screening of pure isolates for the presence of *Helicobacter* species using the *Helicobacter* genus specific PCR

DNA extracted from the 5 Tasmanian devil isolates and 10 Eastern quoll isolates, was amplified using a *Helicobacter* genus specific PCR (Section 2.9.1).

Of the 5 Tasmanian devil isolates, 2 were identified as *Helicobacter* species. These isolates were cultured from the stomach and rectum and were named TD1S and TD2S, respectively. The PCR product obtained from the TD1S

isolate using the *Helicobacter* genus specific PCR is shown in Figure 7.5. Sequencing of the 16S rRNA gene of both TD1S and TD2S was undertaken. The sequence data obtained from TD1S (1466 bp) and TD2S (305 bp) was compared to other sequences in the GenBank database and both were confirmed to be *Helicobacter* species. TD1S was found to be most closely related to *Helicobacter* sp. 'MIT99-5660-6' (98%) and *H. pylori* isolate MC238 (97%). Only part of the 16S rRNA gene (305 bp) from the TD2S isolate was successfully sequenced. The sequence of TD2S was shown to be closely related to *Helicobacter* sp. strain B9A Seymour (97%). The morphology of the TD1S isolate was examined using TEM and is shown in Figure 7.2.

Of the 10 Eastern quoll isolates, none were identified as *Helicobacter* species. A spiral shaped isolate obtained from the colon, termed as 'EQ1', which was negative for *Helicobacter* genus specific PCR, was further examined by 16S rRNA gene sequencing. The sequence data obtained from EQ1 (1387 bp) was compared to other sequences in the GenBank database and shown to be most closely related to *Campylobacter jejuni* NCTC11168 (98%). The morphology of the EQ1 isolate was examined using TEM and is shown in Figure 7.3.

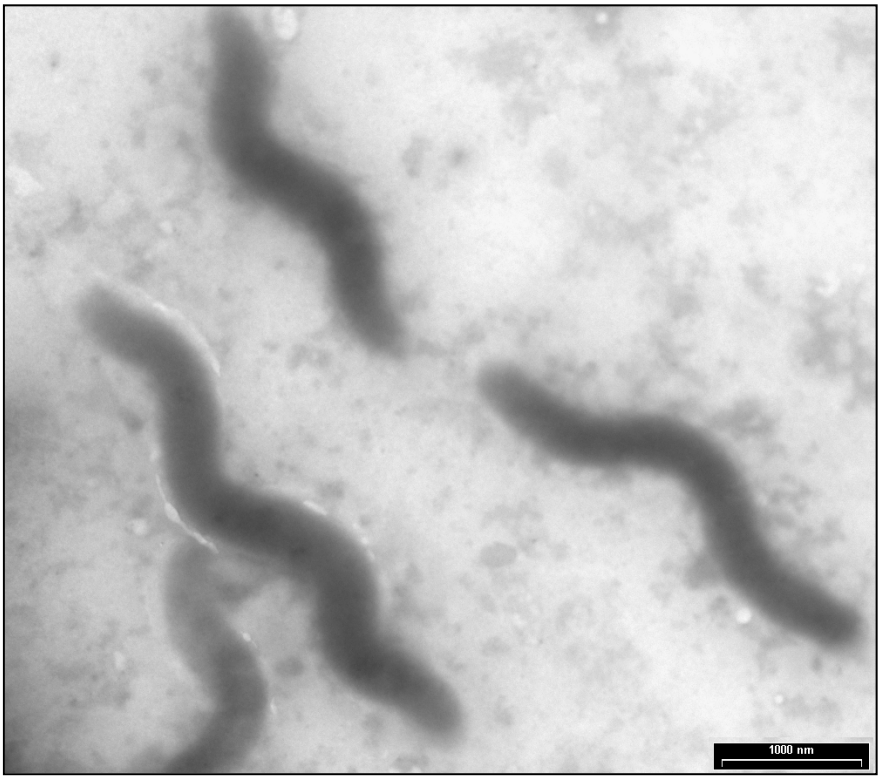


Figure 7.2 A transmission electron micrograph of the spiral shaped *Helicobacter* isolate (TD1S) cultured from the stomach of the Tasmanian devil. The bacterium measured 0.5 – 0.6 by 4-5 μm. (Bar = 1000 nm)

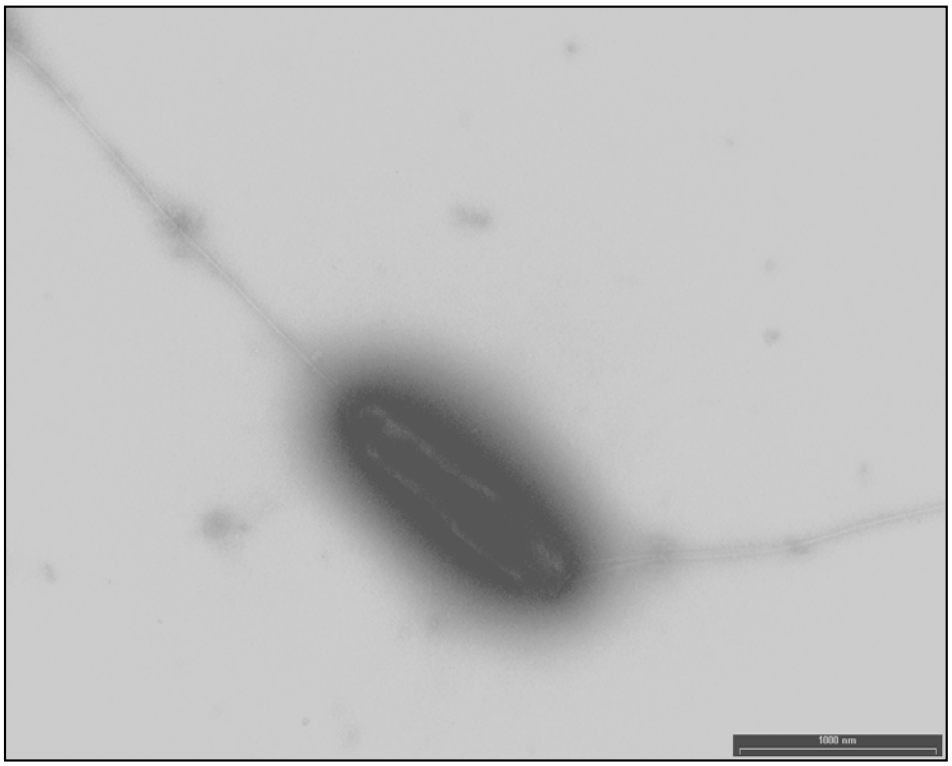


Figure 7.3 A transmission electron micrograph of the curved rod shaped *Campylobacter* isolate (EQ1) cultured from the colon of the Eastern quoll. The bacterium measured 0.23 by 1.3 μm and had bipolar flagella. (Bar = 1000 nm)

7.3.1.3 Bacterial cultivation of the omnivorous marsupial (the long nosed bandicoot)

Preliminary phase microscopic examination of the homogenised liver and GIT mucus scrapings from all 3 **long nosed bandicoots**, showed a high number of actively motile thick and thin spiral-shaped shaped bacteria to be present in the mucus scrapings from the caecum, colon and rectum.

Bacterial culture of the mucus scrapings of the 3 long nosed bandicoots showed heavy growth spreading all over both the CSA and HBA plates from all animals, after 3 to 7 days incubation under either microaerobic or anaerobic conditions. Microscopic examination of the growth on these plates showed a mixture of highly active motile spiral shaped bacteria of various forms, including long, thin, thick, short and fat spiral shaped organisms. Initial attempts to isolate each of these organisms in pure culture proved to be extremely difficult. A further attempt was therefore made to isolate these highly motile organisms. In this case, a bacterial suspension obtained from a CSA plate which was first diluted in BHI broth after which it was streaked onto fresh CSA and HBA plates. This approach allowed 18 separate cultures to be obtained from two of the long nosed bandicoots (1 and 2).

7.3.1.3.1 Screening of pure isolates for the presence of *Helicobacter* species using the *Helicobacter* genus specific PCR

DNA extracted from the above 18 isolates was amplified using a *Helicobacter* genus specific PCR (Section 2.9.1). Of these 18 isolates, 4 were identified as *Helicobacter* species. These isolates were cultured from the caecum and colon of long nosed bandicoot 1 and the colon and rectum of long nosed bandicoot 2. These were named LNB1S and LNB1F, and LNB2F and LNB3F, respectively. PCR products obtained from the LNB1F and LNB2F isolates using the *Helicobacter* genus specific PCR, are shown in Figure 7.4.

Attempts were made to sequence the 16S rRNA gene of the 4 *Helicobacter* isolates. Near complete 16S rRNA gene sequences were obtained from only

LNB1F and LNB2F. Only partial sequence was obtained from the other 2 isolates with the sequence data indicating that these latter 2 strains were not pure cultures. No further identification of these isolates was undertaken. In addition, the near complete 16S rRNA gene of 2 spiral shaped non-helicobacter isolates was also undertaken. These 2 non-helicobacter isolates had been isolated from the caecum and rectum of long nosed bandicoot 2 and were named LNB1D and LNB2D respectively.

The sequences obtained from the 2 helicobacters and the 2 non-helicobacters were compared to other sequences in the GenBank database. The results of these comparisons are as follows:

-LNB1F (1571 bp) was found to be closely related to Helicobacter sp. 'Flexispira taxon 9' (AF047851) (99%) which was isolated from dog faecal samples and LNB2F (1452 bp) was found to be closely related to *H. pametensis* (AF302105) (96%).

-Both of the non-helicobacter isolates were confirmed to be *Desulfovibrio* spp. LNB1D (1439 bp) was found to be (although not closely) related to *Desulfovibrio* sp. D4 (AF192155) (99%) which was isolated from the blood of patient with a history of gastric ulcer. LNB2D (1441 bp) was found to be closely related to *Desulfovibrio* sp. UNSW3caefatS (AF056091) (97%), a spiral organism isolated from mouse caecum.

The morphology of *Helicobacter* sp. ‘LNB1F’ and *Helicobacter* sp. ‘LNB2F’ were examined using TEM, and are shown in Figure 7.5 A and B.

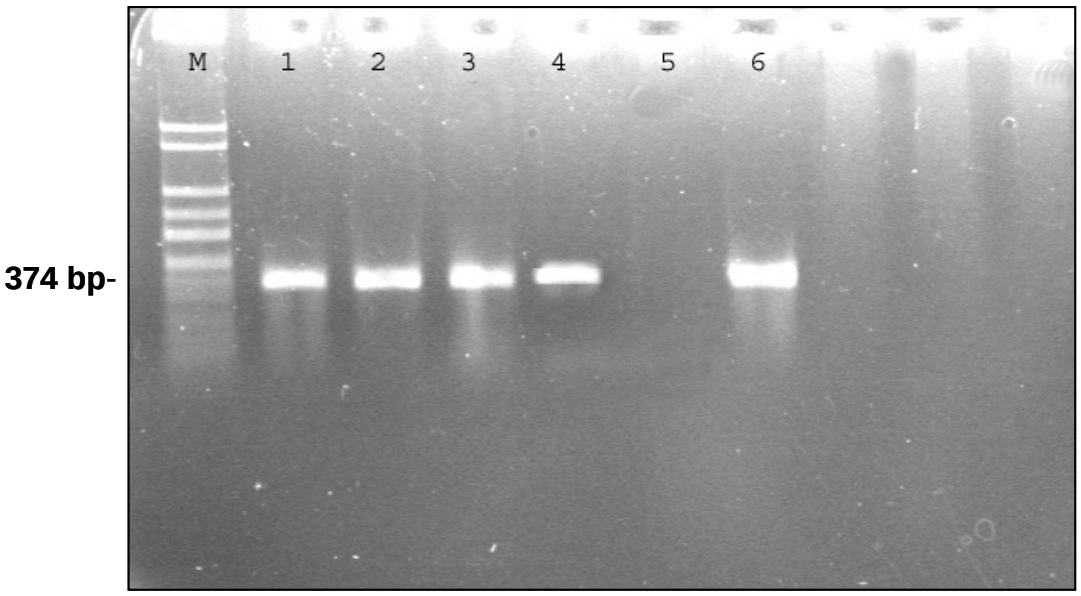


Figure 7.4 The PCR products obtained from the WB1F, TD1S, LNB1F and LNB2F isolates using the *Helicobacter* genus specific PCR, separated on a 1.5 % agarose /TAE gel.

Lane M = Molecular weight marker (FN1)

Lane 1 = WB1F isolate

Lane 2 = TD1S isolate

Lane 3 = LNB1F isolate

Lane 4 = LNB2F isolate

Lane 5 = 'No DNA' Negative control

Lane 6 = '*H. pylori'* Positive control

(TD = Tasmanian devil, LNB = Long nosed bandicoot)

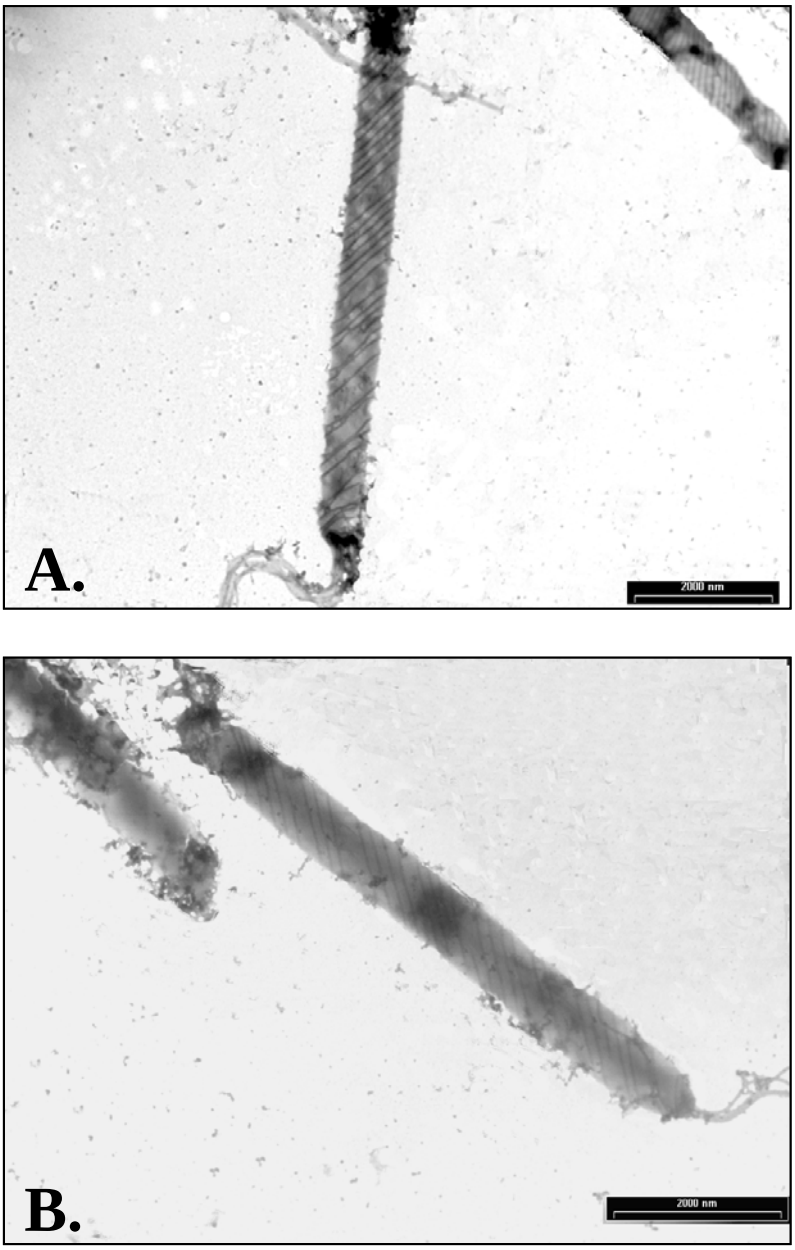


Figure 7.5 A transmission electron micrograph of the fusiform shaped *Helicobacter* isolates, LNB1F (A) and LNB2F (B). Each bacterium measured 0.8-1.3 by 4.5-5.3 μm and was entwined with periplasmic fibres, which appeared to cover the whole cell and had multiple bipolar flagella. (Bar=2000 nm)

7.3.2 Detection of *Helicobacter* species in the liver and mucus scrapings of the GIT using direct and nested *Helicobacter* genus specific PCR

7.3.2.1 Herbivorous marsupials (the koala, wombat and Eastern grey kangaroo)

7.3. 2.1.1 Detection using direct *Helicobacter* genus specific PCR

Amplification of DNA extracted from the homogenised livers and mucus scrapings of the GIT of all 3 koalas, 2 wombats and the Eastern grey kangaroo showed none of the samples from the above marsupials (with the exception of wombat 1) to be positive in the direct PCR. However, a PCR product of the expected size (374 bp) was obtained from the mid ileum, ileum at 3 cm above the caecum, caecum, colon and rectum of wombat 1 (Table 7.2).

7.3.2.1.2 Detection using nested *Helicobacter* specific PCR

Amplification of DNA extracted from the homogenised livers and mucus scrapings of the GIT from all 3 koalas, 2 wombats and the Eastern grey kangaroo (Table 7.2) showed a PCR product of the expected size (374 bp) to be present in the following samples:

- The 3 koalas: liver (n=3), stomach (n=2), mid ileum (n=1) and colon (n=1). None of the caecal or rectal samples were positive.
- The 2 wombats: liver (n=1), stomach (n=1), mid ileum (n=1), ileum at 3 cm above the caecum (n=2), caecum (n=2), colon (n=2), and rectum (n=2).
- Eastern grey kangaroo: stomach (n=1), colon (n=1), and rectum (n=1). None of the liver, ileum or caecal samples were positive.

The PCR products obtained from the stomach of the Eastern grey kangaroo were sequenced and compared to other sequences from GenBank. Partial 16S rRNA gene sequence (613 bp) was obtained and was shown to be most closely related to *Helicobacter pylori* strain SS1 (98%).

7.3.2.2 Carnivorous marsupials (the Tasmanian devil and Eastern quoll)

7.3. 2.2.1 Detection using direct *Helicobacter* genus specific PCR

Amplification of DNA extracted from the homogenised livers and mucus scrapings of the GIT of the Tasmanian devil showed a PCR product of the expected size (374 bp) in samples from the stomach, colon and rectum. No PCR product was obtained from the liver, mid ileum and ileum at 3 cm above the colon (no caecum in this marsupial).

PCR products of the expected size (374 bp) were obtained from the stomach, ileum at 3 cm above the colon (no caecum), colon and rectum of the Eastern quoll. No PCR product was obtained from either the liver or mid ileum (Table 7.2).

7.3.2.2.2 Detection using nested *Helicobacter* specific PCR

Amplification of DNA extracted from the homogenised livers and mucus scrapings of the GIT of the Tasmanian devil showed a PCR product of the expected size (374 bp) to be present from the stomach, mid ileum, ileum at 3 cm above the colon, colon and rectum. No PCR product was obtained from the liver.

A PCR product of the expected size (374 bp) was obtained from the liver, stomach, mid ileum, ileum at 3 cm above the colon, colon and rectum of the Eastern quoll (see Table 7.2).

7.3.2.3 Omnivorous marsupials (the long nosed bandicoot)

7.3.2.3.1 Detection using direct *Helicobacter* genus specific PCR

Amplification of DNA extracted from the homogenised livers and mucus scrapings of the GIT of all 3 long nosed bandicoots showed a PCR product of the expected size (374 bp) in samples from the caecum (n=1), colon (n=1), and rectum (n=3) (Table 7.2). No PCR product was obtained from the liver, stomach, mid ileum and ileum at 3 cm above the caecum.

7.3.2.3.2 Detection using nested *Helicobacter* specific PCR

Amplification of DNA extracted from the homogenised livers and mucus scrapings of the GIT of all 3 long nosed bandicoots showed a PCR product of the expected size (374 bp) to be present from the ileum at 3 cm above the caecum (n=2), caecum (n=3), colon (n=3), and rectum (n=3). None of the liver, stomach and mid ileum samples from the long nosed bandicoots were positive in the nested PCR (Table 7.2).

The cultivation and PCR results obtained from all animals studied in this chapter are summarised in Table 7.2.

Regions / Animals	Liver	Stomach	Mid ileum	3-ileum	Caecum	Colon	Rectum
Koala 1	*	*	*	–	–	*	–
Koala 2	*	–	–	–	–	–	–
Koala 3	*	*	–	–	–	–	–
Wombat 1	–	–	~+ *	~+ *	~+ *	~+ *	~+ *
Wombat 2	*	*	–	*	*	*	*
Kangaroo	–	~1 *	–	No sample	–	*	*
T. devil	–	~+ *	*	*	No sample	+*	~+ *
E. quoll	*	+ *	*	+ *	No sample	+ *	+ *
LNB 1	–	–	–	*	~*	~*	+ *
LNB 2	–	–	–	–	+*	~+ *	~+ *
LNB 3	–	–	–	*	*	*	+ *

Table 7.2 The results of *Helicobacters* culture, direct and nested PCR obtained from the 11 marsupials studied.

T. devil = Tasmanian devil, E. quoll = Eastern quoll, LNB = long nosed bandicoot,

~ = *Helicobacters* culture positive, ~1 = *Helicobacters* culture positive but died during subculture,

+ = direct PCR positive**, *** = nested PCR positive,

- = *Helicobacters* culture, direct PCR and nested PCR, all negative

7.3.3 The spatial distribution of the bacteria in fixed sections of the liver and different regions of the GIT

7.3.3.1 Herbivorous marsupials (the koala, wombat and Eastern grey kangaroo)

Microscopic examination of silver stained sections obtained from the 3 **koalas** showed 2 predominant morphological types of microorganisms, cocci and filamentous bacillary forms. Cocci were found mainly in the mucus and the surface epithelial layer of the stomach, mid ileum, ileum at 3 cm above the caecum, colon and rectum. Filamentous bacilli were present in large numbers covering the epithelial layer of the caecum of koala 1 and 3 and covering the epithelial layer of the ileum at 3 cm above the caecum in koala 2. Given that isolation and detection of *Helicobacters* using culture and direct PCR was unsuccessful, the spatial distribution of bacteria belonging to the genus *Helicobacter,* using FISH, was not performed in these animals.

In **wombat 1**, a small number of long curved to spiral shaped bacteria were observed in the mucus layer overlying the epithelial cells of the caecum, colon and rectum. In wombat 2, a large number of long thick curved to spiral shaped organisms were observed in the mucus layer of the mid ileum, ileum at 3 cm above the caecum, colon and rectum. Cocci, short curved rods, long thin spiral and small spiral shaped organisms were also observed in the mucus layer of the caecum, colon and rectum of both wombats. Although *Helicobacters* were isolated from the lower bowel of wombat 1, there were very low numbers of mucus-associated bacteria observed in the silver stained sections of the lower bowel. Furthermore, the isolation and detection of *Helicobacters* using culture and direct PCR was unsuccessful in wombat 2. Given this, the spatial distribution of bacteria belonging to the genus *Helicobacter* was not examined in the wombats. Examples of photomicrographs of silver stained sections from the caecum and colon of wombat 1 are shown in Figure 7.6.

In the **Eastern grey kangaroo**, a large number of long spiral-shaped bacteria with irregular turns were observed in the mucus layer overlying the epithelium, as well as in the crypts of the stomach. A large number of long curved to spiral shaped organisms, different to those spiral shaped organisms observed in the stomach, were observed in the mucus layer overlying the epithelium and in the crypts of the caecum. A mixture of curved rods and cocci were present in small numbers in the mucus layer overlying the epithelium of other regions of the GIT. Silver stained sections from the tubiform forestomach of the Eastern grey kangaroo are shown in Figure 7.7.

The spatial distribution of bacteria belonging to the genus *Helicobacter* was examined using fluorescent *in situ* hybridisation (FISH) with a *Helicobacter* genus specific probe, in a section of tubiform forestomach obtained from the Eastern grey kangaroo (Section 3.4, Chapter 3). The majority of spiral shaped organisms present in the mucus layer overlying the epithelium and within the crypts of the stomach were shown to belong to the genus *Helicobacter*. Images of the hybridised bacteria are shown in Figure 7.8.

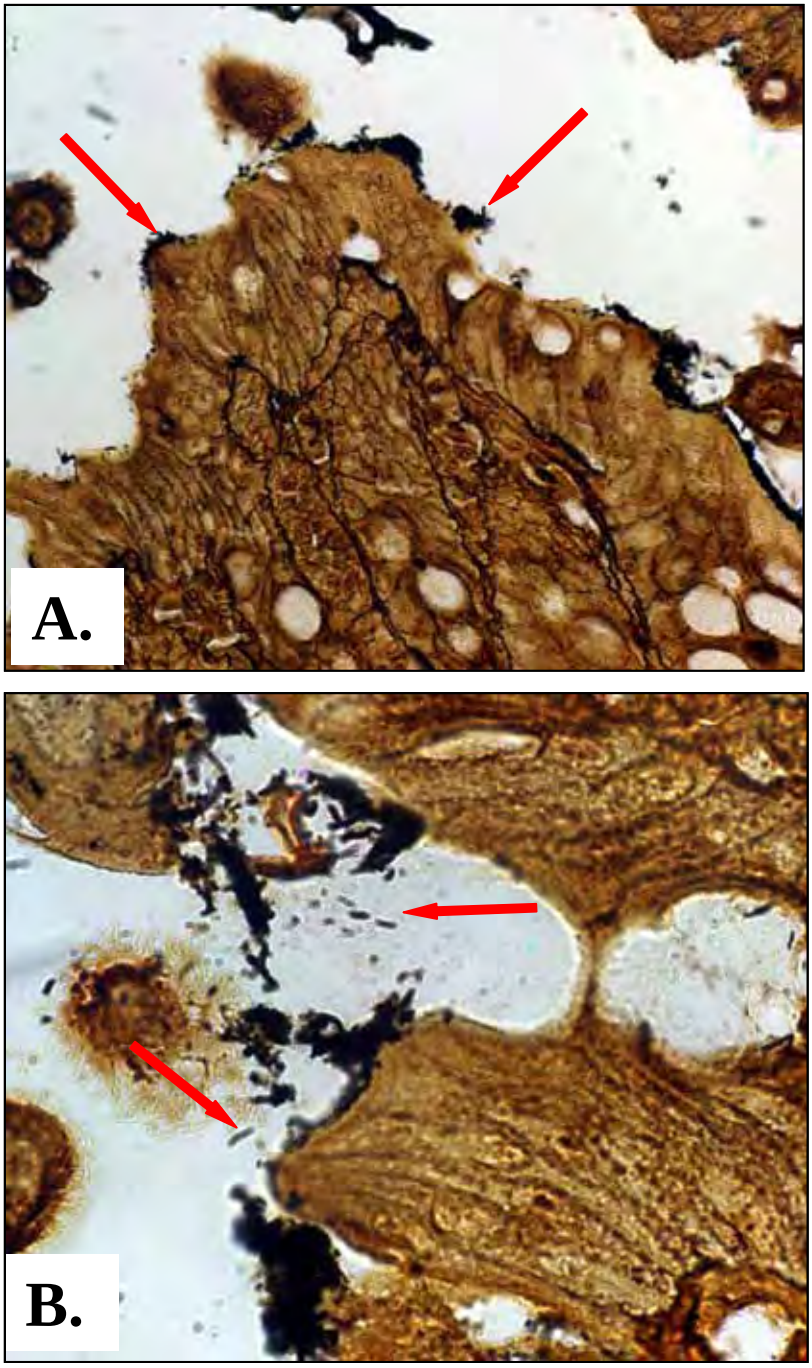


Figure 7.6 Photomicrographs of silver stained sections from different regions of the caecum of wombat 1 (A & B), showing a small number of spiral and fusiform shaped organisms located in the mucus layer overlying the epithelium. Magnification = 400 (A), = 1000 (B).

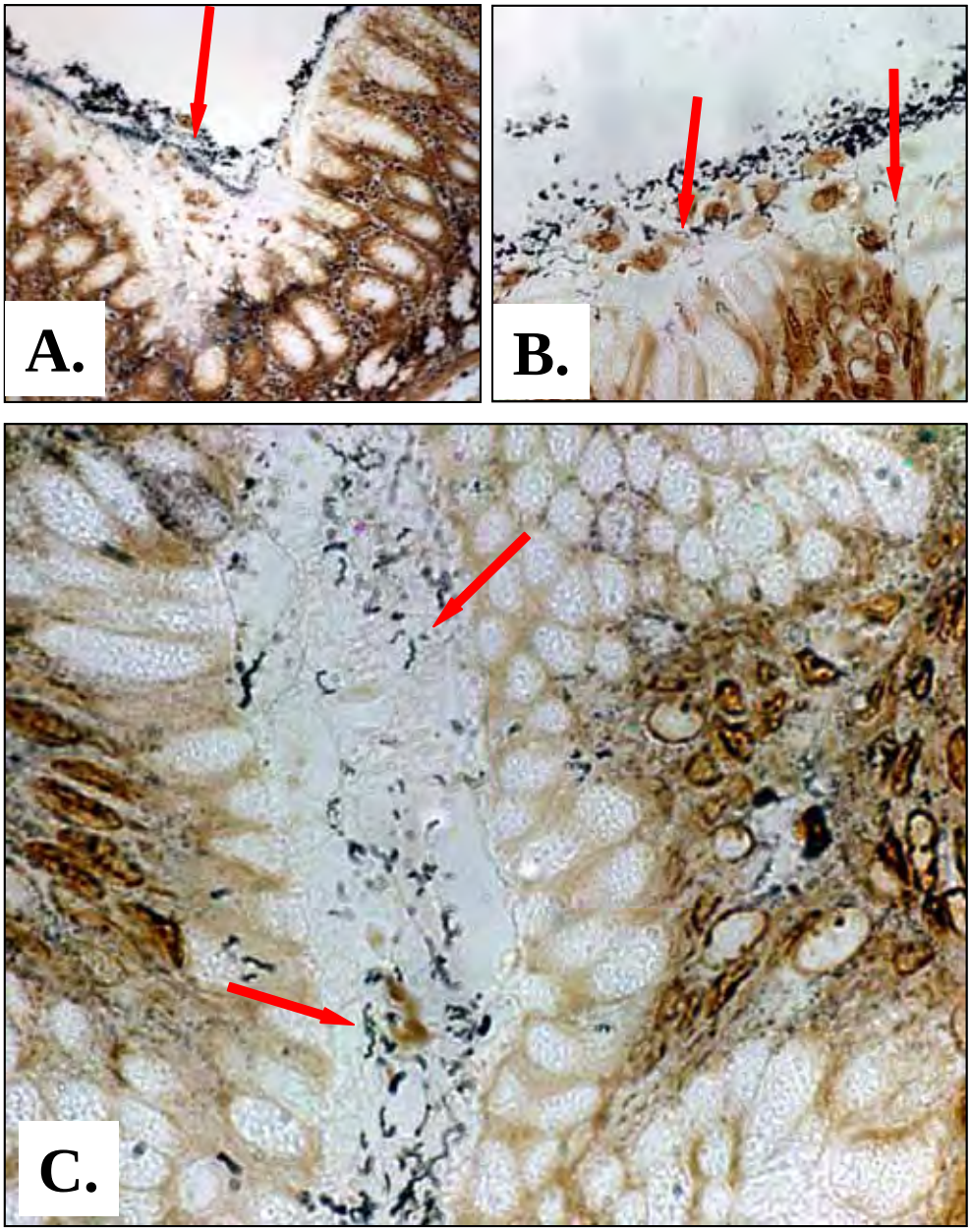


Figure 7.7 Photomicrographs of silver stained sections of the stomach of the Eastern grey kangaroo showing high numbers of long spiral shaped organisms (arrows) located in the mucus layer overlying the epithelial cells and within the crypts at different regions of the stomach.

Magnification = 200 (A), = 1000 (B, C).

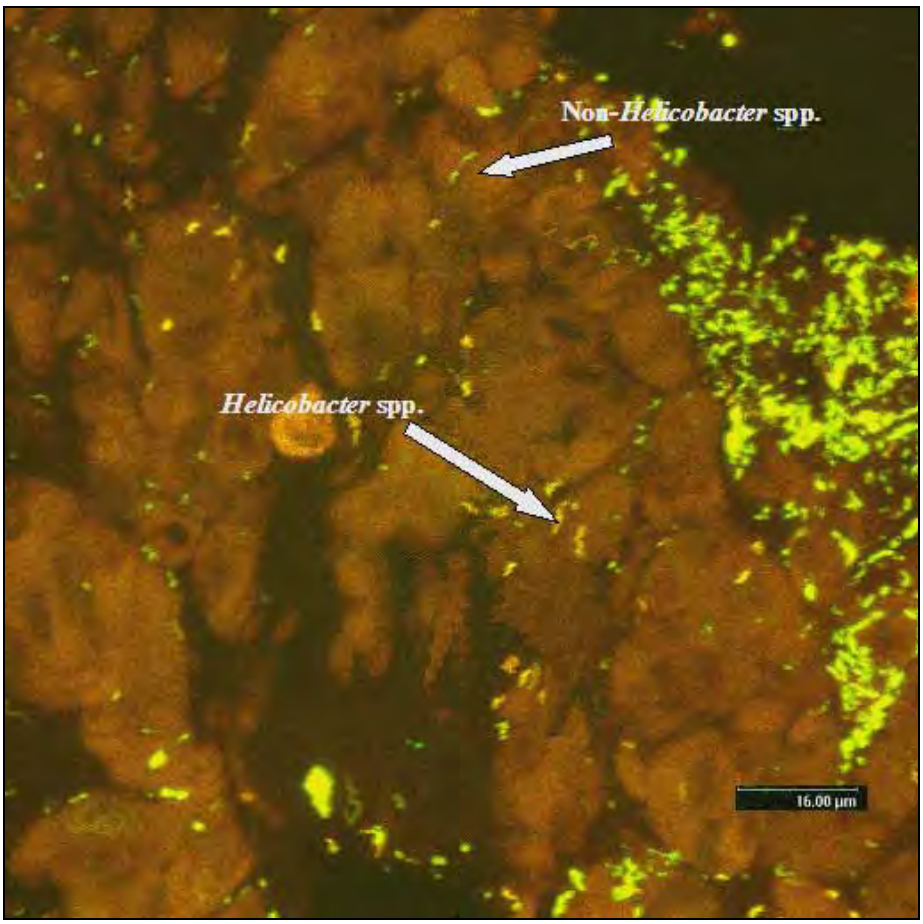


Figure 7.8 Confocal-fluorescent photomicrographs of fixed stomach samples taken from the Eastern grey kangaroo showing that a large number of the spiral shaped bacteria colonising the mucus layer of the stomach belonged to the *Helicobacter* genus.

-Bacteria hybridised with both Eu338-FITC (Eubacterial probe labelled with fluorescein-isothiocyanate) and HRh probes (*Helicobacter* genus specific probe labelled with tetramethyl-rhodamine-isothiocyanate) showing a yellow-orange colour (mixture of red and green colours).

-Non-helicobacter bacteria hybridised with the Eu338-FITC probe only, showing a green colour.

Photomicrographs taken by Helen Dalton (School of BABS, UNSW). (Bar=16.00 µm)

7.3.3.2 Carnivorous marsupials (the Tasmanian devil and Eastern quoll)

Microscopic examination of silver stained sections from the **Tasmanian devil** showed a high number of long spiral shaped rods, long tightly coiled rods and short curved rods to be present in the crypts of the stomach (Figure 7.9). A small number of short curved rods were observed in the mid ileum and ileum at 3 cm above the colon. A large number of long thick curved to spiral organisms were observed in the mucus layer overlying the epithelial cells of the colon and rectum. Using FISH, with a *Helicobacter* genus specific probe (Section 3.4, Chapter 3), the spatial distribution of bacteria belonging to the genus *Helicobacter* was observed in a section of stomach from the Tasmanian devil from which TD1S had been isolated. The two types of spiral shaped organisms present within the crypts of the GIT were shown to belong to the genus *Helicobacter*. The small spiral shaped bacteria are likely to be TD1S. Images of the hybridised bacteria are shown in Figure 7.10.

In silver stained sections of the **Eastern quoll**, large numbers of bacillus-like organisms were observed in the mucus layer overlying the epithelium and in the crypts of the stomach, mid ileum and ileum at 3 cm above the colon. A high number of mixed curved to spiral shaped bacteria and cocci were present in the mucus layer overlying the epithelium and in the crypts of the colon and rectum. Given that the isolation of *Helicobacter* species using culture was unsuccessful in this animal, the spatial distribution of bacteria belonging to the genus *Helicobacter* using FISH was not examined.

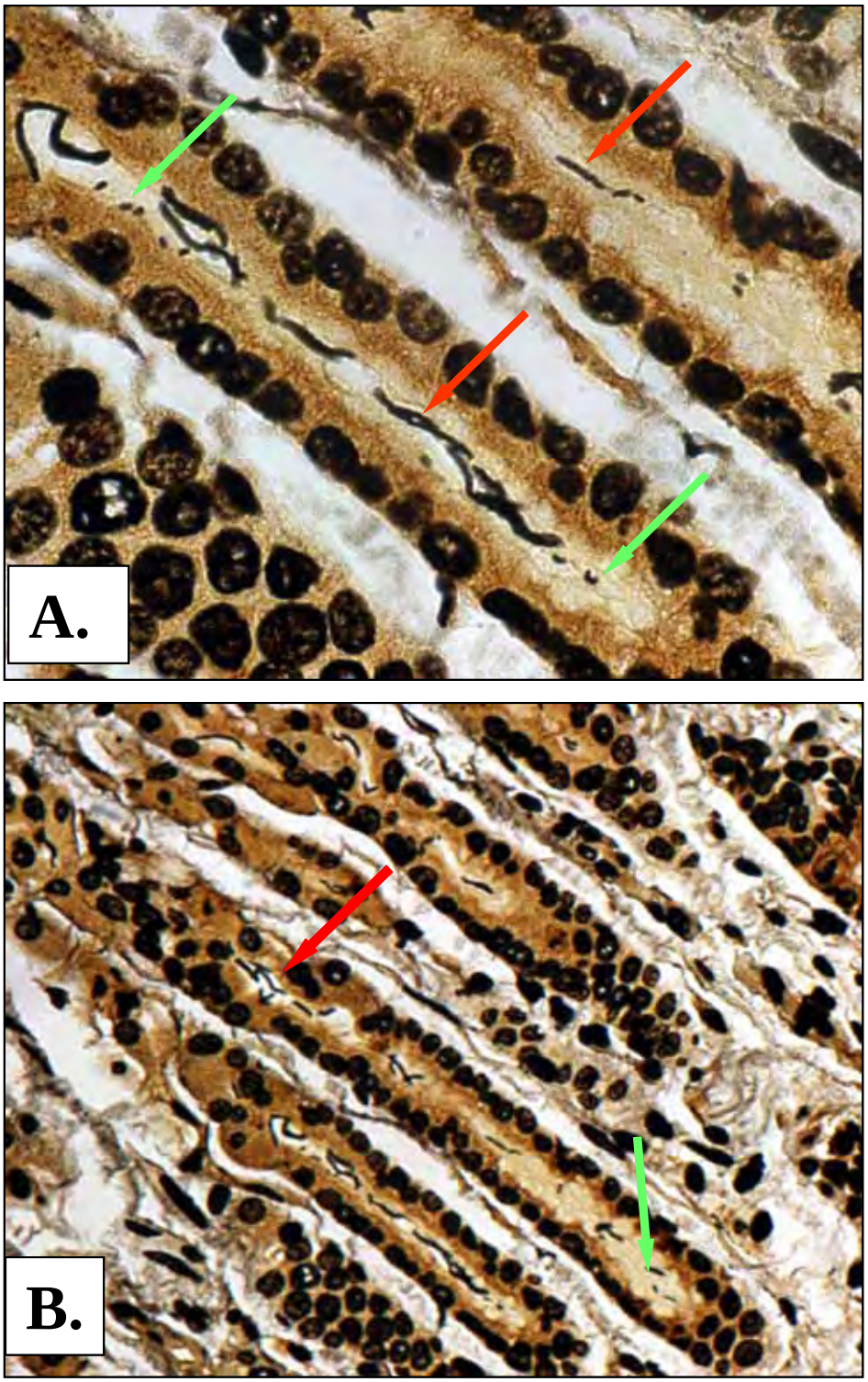


Figure 7.9 Photomicrographs of a silver stained section of the stomach from the Tasmanian devil showing two morphological types of organisms, short curved rods (green arrow) and long tightly coiled rods (red arrow), located within the crypts of the stomach. Magnification =1000(A), =400 (B).

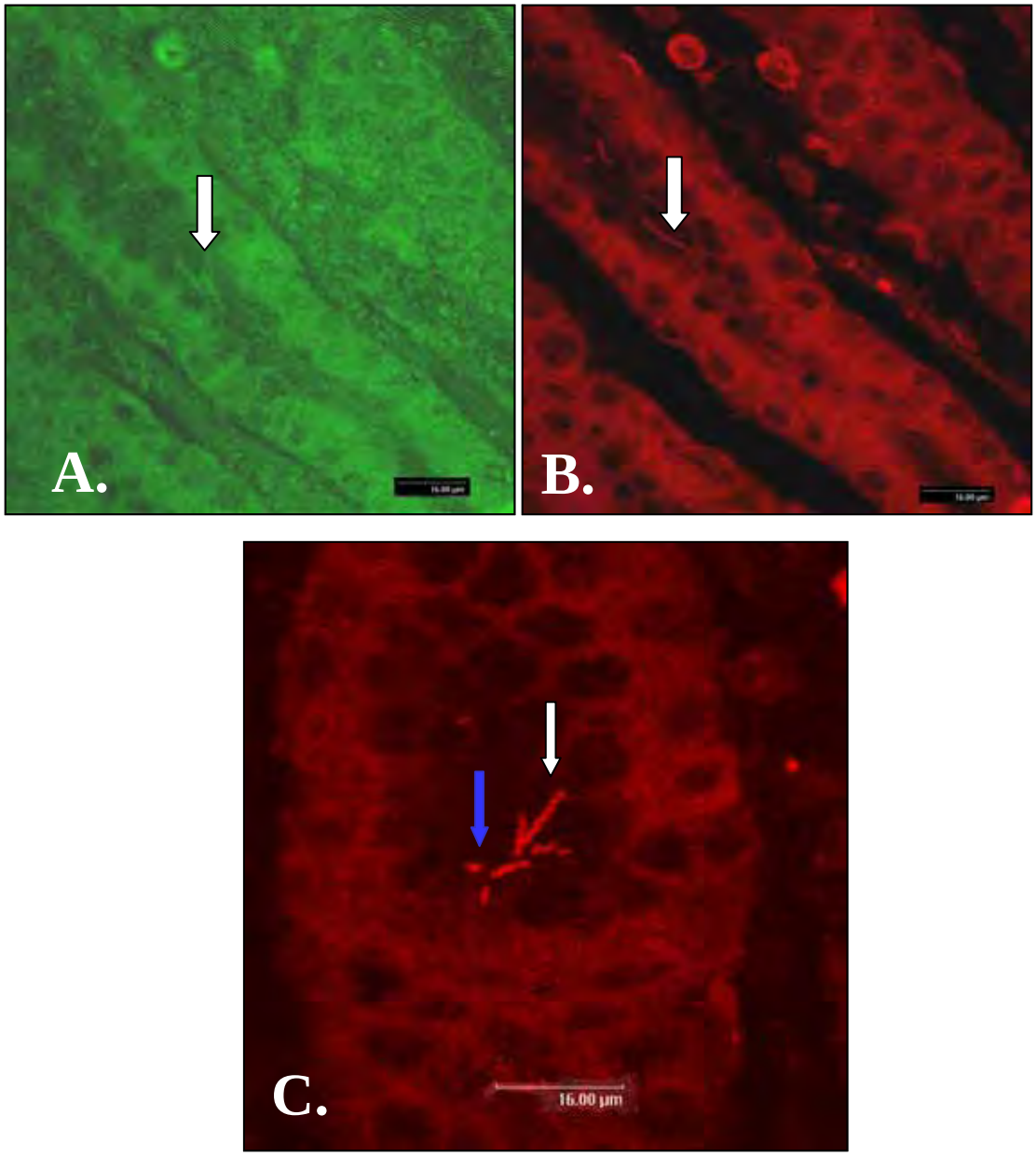


Figure 7.10 Confocal-fluorescent photomicrographs of a stomach section taken from the Tasmanian devil showing that the majority of spirals colonising the crypts of the stomach, belonged to the *Helicobacter* genus.

(A) Long tightly coiled rods hybridised with the Eu338-FITC probe showing a green colour.

(B) Long tightly coiled rods hybridised with the HRh probe showing a red colour.

(C) A mixture of long tightly coiled (white arrows) and short curved rods (blue arrows) hybridised with the HRh probes showing a red colour.

7.3.3.3 Omnivorous (the long nosed bandicoot)

Microscopic examination of silver stained sections from the 3 long nosed bandicoots showed a large number of short curved rods and long curved to spiral shaped bacteria to be present in the mucus layer overlying the epithelium, and in the crypts of the caecum, colon and rectum. Small numbers of these bacteria were also observed in the ileum. A very small number of short curved to spiral rods and cocci were also seen in the stomach and mid ileum. No bacteria were observed in the liver of any of the long nosed bandicoots. Photomicrographs of silver stained sections obtained from the colon of long nosed bandicoot 1 are shown in Figure 7.11.

The spatial distribution of bacteria belonging to the genus *Helicobacter* in a section of colon taken from long nosed bandicoot 1 was observed using FISH with the *Helicobacter* genus specific probe (Section 3.4). A small number of spiral shaped organisms present in the mucus layer and in the mucosal crypts of the colon were shown to belong to the genus *Helicobacter*. Images of the hybridised bacteria are shown in Figure 7.12.

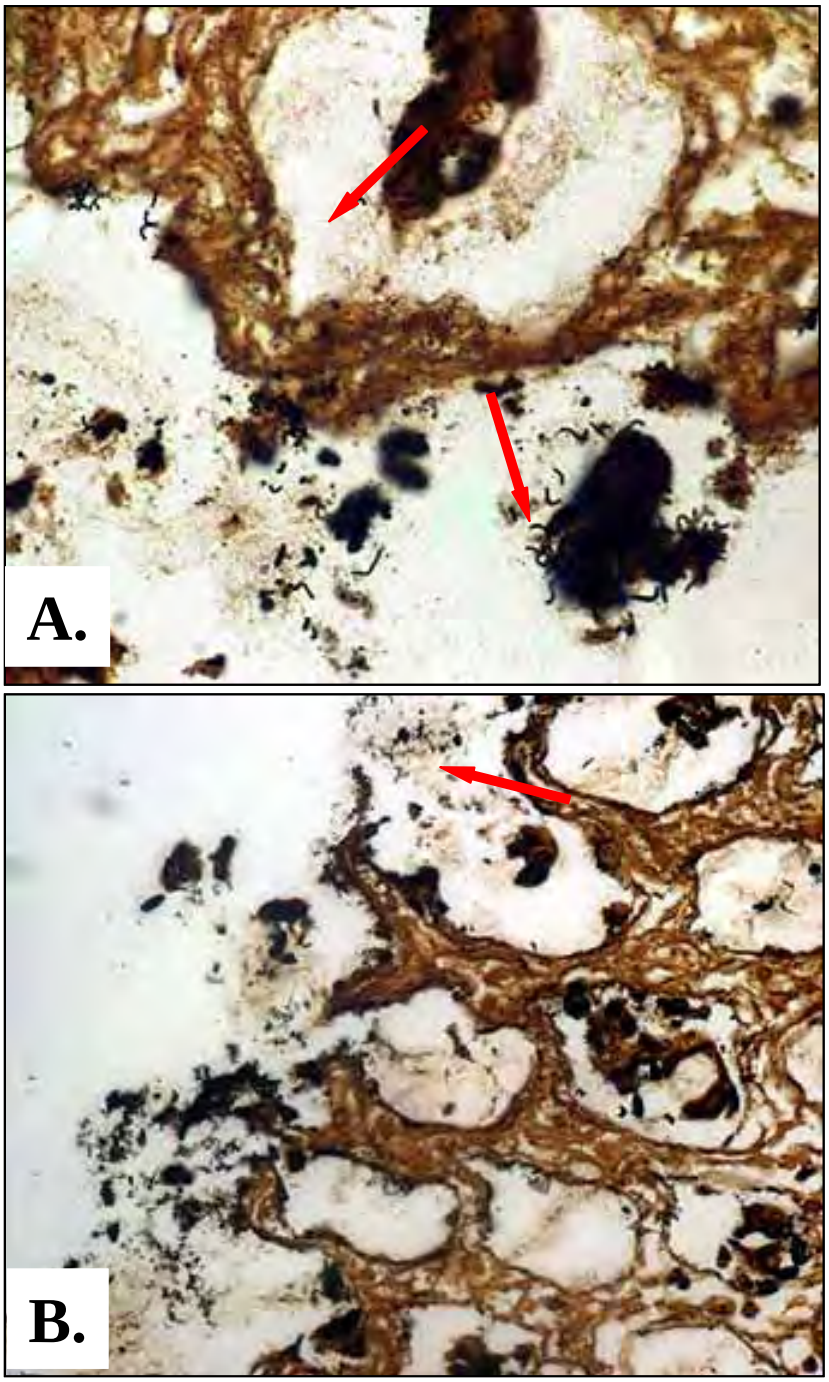


Figure 7.11 Photomicrographs of silver stained sections obtained from the colon of the long nosed bandicoot showing a high number of long spiral shaped organisms present in the mucus layer lining the epithelium at different regions of the colon. Magnification =1000(A), =400 (B).

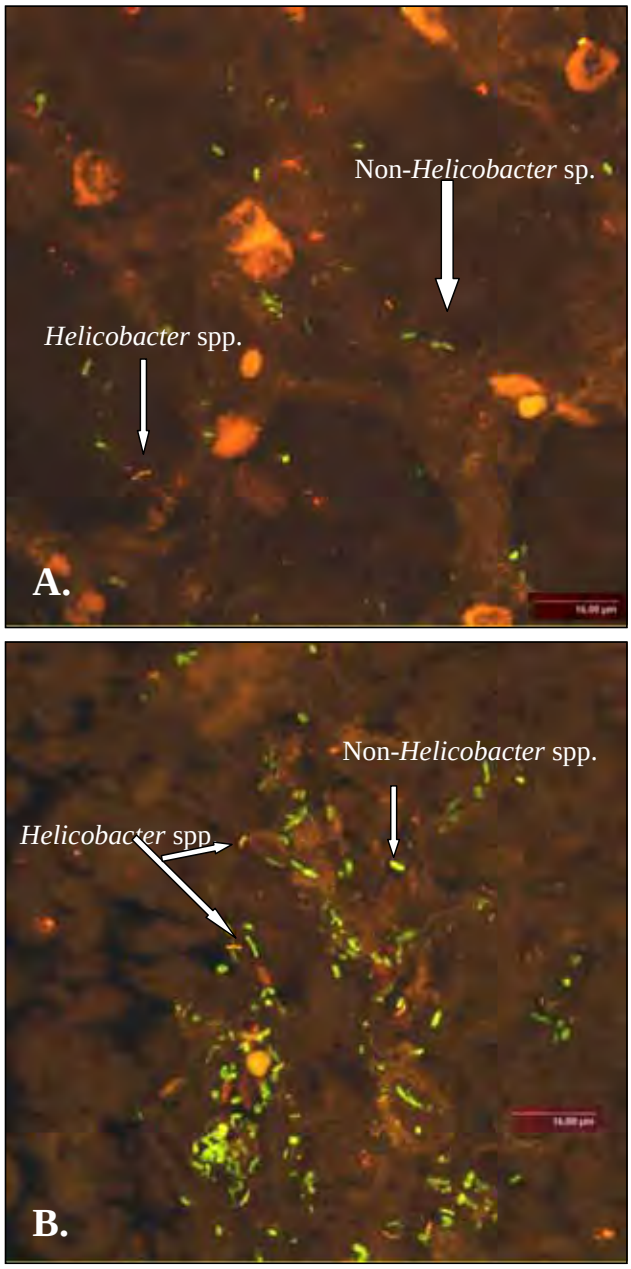


Figure 7.12 Confocal-fluorescent photomicrographs at different regions of the colon of long nosed bandicoot 1 showing a mixture of spiral shaped organisms colonising the mucus layer overlying the epithelium. A number of these organisms were shown to belong to the *Helicobacter* genus (A & B).

-Bacteria hybridised with both Eu338-FITC and HRh probes showing a yellow-orange colour (mixture of red and green colours).

-Non-helicobacter bacteria hybridised with the Eu338-FITC probe only, showing a green colour.

Photomicrograph taken by Helen Dalton (School of BABS, UNSW). (Bar=16.00 μm)

7.4 Phylogenetic analysis of 16S rRNA gene

In this chapter a total of 9 possible new *Helicobacter, Campylobacter* and *Desulfovibrio* isolates were obtained as a result of the isolation of spiral and fusiform shaped organisms. One *Helicobacter* sp. 'TD1S' was isolated from a Tasmanian devil, two *Helicobacters* (WB1F, WB2F) and 1 *Campylobacter* (WB1) were isolated from a wombat, two *Helicobacters* (LNB1F and LNB2F) and 2 *Desulfovibrio* (LNB1D and LNB2D) were isolated from the long nosed bandicoots and one *Campylobacter* (EQ1) was isolated from an Eastern quoll.

The phylogenetic relationship of these isolates was determined from analysis of their near complete 16S rRNA gene sequences using the distance method and this was compared to a number of *Helicobacter* reference strains and closely related genera (species and accession numbers are listed in Table 7.3 and Table 7.4). The distance between all pairs of sequences was calculated and a phylogenetic tree plotted using Bootstrap NJ TREE (1,000 bootstrap replicates). All programs were from the ClustalX package [162]. In this phylogenetic analysis, the representative strains of *"Helicobacter vulpercula*" (BTP2C), *"Helicobacter peregrinus"* (RTP4S), *"Helicobacter kirkbridei"* (BTP1F) and 'S' shaped BTP *Helicobacter* (BTP1S) as well as *Campylobacter* sp. 'BTP1', were included. All methods used for the phylogenetic analysis are described in Section 2.10.

Isolate	Animal	Morphology	Region of isolation	16S rRNA gene Accession No.
WB1F	Wombat 1	Fusiform	Caecum	AY554140
WB2F	Wombat 1	Fusiform	Colon	AY554141
LNB1F	Long nosed bandicoot 1	Fusiform	Colon	AY554138
LNB2F	Long nosed bandicoot 2	Fusiform	Colon	AY554139
TD1S	Tasmanian devil	S-shaped	Stomach	AF506794
Campylobacter sp. 'BTP1'	Brushtail possum	Tightly coiled rod	Caecum	AY554142
Campylobacter sp. 'EQ1'	Eastern quoll	S-shaped	Colon	AY554143
Campylobacter sp. 'WB1'	Wombat 1	S-shaped	Colon	AY554144
Desulfovibrio sp. 'LNB1'	Long nosed bandicoot 1	Fat-S	Colon	AY554145
Desulfovibrio sp. 'LNB2'	Long nosed bandicoot 2	Fat-S	Colon	AY554146

Table 7.3 Origin, morphology and accession numbers of the near complete 16S rRNA gene of the new *Helicobacter s*pp., *Campylobacter* spp. and *Desulfovibrio* spp. obtained in this chapter.

Taxon	Accession No.	Taxon	Accession No.
Helicobacter felis	M57389	*Desulfovibrio dsulfuricans*	M34113
Helicobacter pylori	M88157	*Desulfovibrio fairfieldensis*	U42221
Helicobacter mustelae	M35048	*Desulfovibrio halophilus*	X99237
Helicobacter pametensis	M88147	*Desulfovibrio intestinalis*	Y12254
Helicobacter aurati	AF297868	*Desulfovibrio termitidis*	X87409
Helicobacter trogontum	U65103	Desulfovibrio sp. ABHU1SB	AF056088
Helicobacter hepaticus	L39122	Desulfovibrio sp. UNSW3caefatS	AF056091
Helicobacter muridarum	M80205	*Campylobacter helveticus*	CHU03022
Helicobacter canis	L13464	*Campylobacter fetus*	CAJRRDAC
Helicobacter cinaedi	M88150	*Campylobacter hyointestinalis*	AF219235
Helicobacter bilis	U18766	*Campylobacter rectus*	CAJRRDAF
Helicobacter pullorum	L36141	*Campylobacter curvus*	CAJRRDAB
Helicobacter canadensis	AF26037	*Campylobacter coli*	CAJRRDAA
Helicobacter rodentium	U96296	*Campylobacter lari*	CAJRRDAE
Arcobacter butzleri	L14626	*Campylobacter jejuni*	L04315

Table 7.4 Accession numbers of the 16S rRNA gene sequence of reference strains of *Helicobacter*, *Desulfovibrio* and *Campylobacter* species used in the phylogenetic analysis.

7.4.1 Results

The phylogenetic position of all the new *Helicobacter* isolates derived from the study of the phylogenetic tree as shown in Figure 7.13 confirmed the BLAST results which showed that TD1S, WB1F, WB2F, LNB1F and LNB2F, all belong to the *Helicobacter* genus. From the phylogenetic tree, *Helicobacter* sp. 'TD1S' isolated from the stomach of a Tasmanian devil clustered with all other gastric *Helicobacter* species previously isolated from placental animals. All *Helicobacter* spp. isolated from the lower bowel of Australian marsupials clustered with lower bowel *Helicobacters* previously isolated from placental animals. BTP1, WB1 and EQ1 isolates, and LNB1D and LNB2D isolates clustered with other *Campylobacter* species and *Desulfovibrio* species, respectively.

The percent similarity among the marsupial *Helicobacter* species isolated in this study is summarised in Table 7.5 (the percent similarity of these marsupial isolates to other closely related bacteria is shown in Appendix 5). From these results it can be seen that the level of sequence similarity among each of the marsupial *Helicobacters* is less than 97% (except for the WB1F, WB2F, and LNB2F isolates). This indicates that each of the new isolates obtained from individual animal species potentially belong to a different or new species. The sequences from WB1F and WB2F isolates were shown to be 99.7% similar. This level of similarity indicates that both isolates constitute the same species. The sequences from LNB1F and LNB2F isolates obtained from 2 different long nosed bandicoots were shown to be 95.5% similar. Both isolates were which may explain their low level of similarity. Interestingly one of the fusiform isolates from the long nosed bandicoot (LNB2F) was ~97% similar to the fusiform isolates obtained from the wombats WB1F and WB2F. Further analysis using a polyphasic approach, as described by Vandamme, is required to confirm if there is any significant relationship between the wombat and the long nosed bandicoot fusiform isolates [124]. By comparison the other long nosed bandicoot fusiform isolate (LNB1F) showed a lower level of similarity (95.7%) to either of the both wombat isolates, WB1F & WB2F.

In summary the results of the phylogenetic analysis of the 16S rRNA gene sequence of all the marsupial *Helicobacter* species suggest that each of the marsupial species, examined in this study, may be colonised with a unique *Helicobacter* species. In addition, there is evidence that the animals are colonised by more than one *Helicobacter* species.

	TD1S	WB1F	WB2F	LNB1F	LNB2F	*"H. vulpecula"*	*"H. kirkbridei"*	*"H. peregrinus"*	BTP1S
TD1S	100								
WB1F	92.5	100							
WB2F	92.5	**99.7**	100						
LNB1F	93	95.7	95.7	100					
LNB2F	93.2	**97.1**	**97**	95.5	100				
"H. vulpecula"	91.7	93.5	93.5	93.2	94.4	100			
"H. kirkbridei"	92.5	94.6	94.6	94.5	95.4	94.9	100		
"H. peregrinus"	91.8	93.7	93.7	93.9	94	94.9	95.4	100	
BTP1S	92.7	94.6	94.6	94.8	95.1	95.3	94.9	96.1	100

Table 7.5 The percent similarity among the marsupial *Helicobacter* species isolated in this study

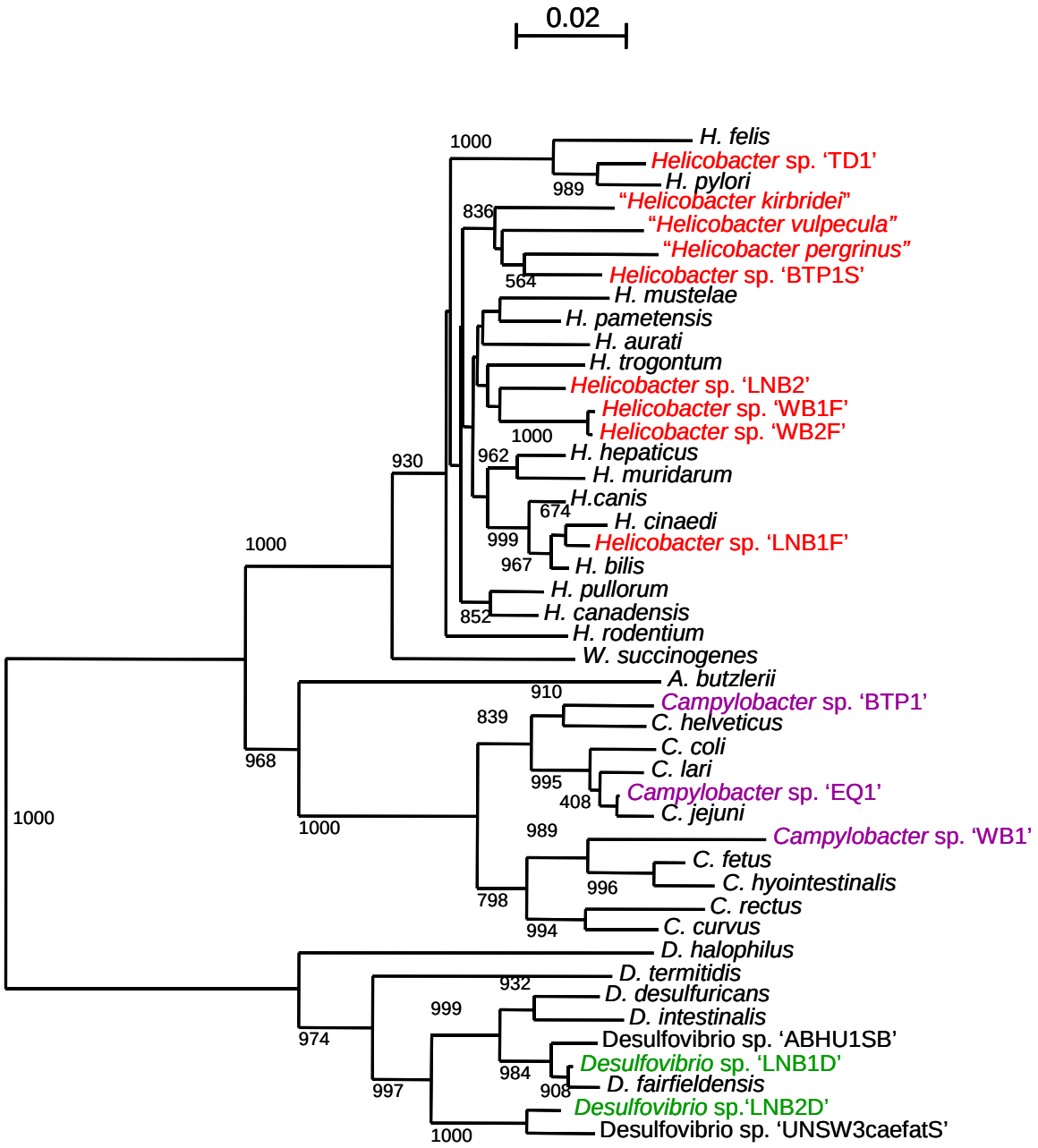


Figure 7.13 The phylogenetic tree derived from the near complete 16S rRNA gene of *Helicobacter* spp., *Campylobacter* spp. and *Desulfovibrio* spp. obtained from all marsupials studied, together with the related reference strains. The phylogenetic tree was reconstructed using the distance method [162].

7.5 Summary and Discussion

Using a combination of culture, PCR, microscopic examination and FISH, *Helicobacter* species were detected in all the marsupials studied, which included three koalas, two wombats, one Eastern grey kangaroo, one Tasmanian devil, one Eastern quoll and three long nosed bandicoots. One *Helicobacter* isolate was cultured from the stomach of the Tasmanian devil (carnivores) and one from the Eastern grey kangaroo (foregut fermenter herbivore). Two *Helicobacter* isolates were cultured from the lower bowel of one wombat, and one isolate from each of the two long nosed bandicoots. The morphology of these *Helicobacter* isolates fell into two types, spiral and fusiform shaped. In addition, a *Campylobacter* isolate was cultured from the lower bowel of the same wombat as referred to above, and two *Desulfovibrio* isolates were cultured from one of the long nosed bandicoots from which a *Helicobacter* isolate had been cultured. Further analysis of the 16S rRNA and partial sequence of the urease gene of the organism isolated from the stomach of the Tasmanian devil (TD1S) was undertaken by Jani O'Rourke (PhD thesis) [225]. In this analysis comparison of TD1S with a large number of known gastric *Helicobacters* including *H. pylori*, *H. bizzozeronii*, *H. felis* and *H. salomonis,* and other uncultured gastrospirilla, showed that this isolate clustered with *H. pylori* on the basis of 16S rRNA and urease gene sequences and morphology.

In general the isolation of *Helicobacters* by culture correlated with the detection of *Helicobacter* DNA using direct PCR. Interestingly in the Eastern quoll, we were unable to culture helicobacters from any sample while a *Campylobacter* species was isolated by culture from the colon and *Helicobacter* DNA was detected in the stomach and all regions of the lower bowel. The inability to cultivate a *Helicobacter* species in the Eastern quoll may have been due to the overgrowth of other species such as *Campylobacters,* which obscured the presence of the *Helicobacters* as has been discussed previously in Chapter 4 [209]. In the Eastern quoll only one morphological type, a spiral shaped bacterium, was initially observed by microscopy. This organism was successfully cultured and subsequently identified as a *Campylobacter* species.

In comparison to the Eastern quoll, initial observations by phase microscopy of the mucus-associated bacteria in the long nosed bandicoot revealed the presence of a variety of morphological types including fat-S, short spirals and long thin spirals. As a result of this observation additional steps were taken to increase the chance of culturing each of these morphological types. In this case, colonies growing on the initial culture medium were diluted with BHI broth in an attempt to isolate each of the morphological types in pure culture. Although laborious and time consuming, this approach resulted in a number of pure cultures from these mixed cultures. Subsequently, two pure isolates of fusiform shaped bacteria from the colon of the two bandicoots, and two pure isolates of fat-S shaped bacteria from the caecum and rectum of one of the bandicoots were identified and shown to belong to the *Helicobacter* and *Desulfovibrio* genera, respectively.

Helicobacter species were detected in differing locations within the GIT of the marsupial species studied (Table 7.2). It is possible that colonisation of the GIT of marsupials by *Helicobacter* species may be impacted upon by a number factors including diet, structure and function of the GIT as well as the mechanisms the host animals use for the digestion and extraction of nutrition from food sources. Based on their diet and GIT structure, the marsupials examined in this study could be separated into three groups, herbivores, omnivores and carnivores. The gastrointestinal tracts of these different groups of marsupials are shown diagrammatically in Figure 1.9, Chapter 1. As can be seen in this figure, the GIT of these groups of marsupials differs substantially.

The **Tasmanian devil and Eastern quoll** are carnivores eating mainly other animals. Their diet contains a high level of protein and a low level of carbohydrates. Given this, they are not dependent on microbial fermentation and thus have a simple digestive system with a short GIT (contains no caecum) through which the rate of passage of food is rapid [144]. In the Tasmanian devil a *Helicobacter* species was isolated from the stomach, in addition, *Helicobacter* DNA was detected in the lower bowel. In the Eastern quoll *Helicobacter* DNA was detected in the lower bowel. The detection of *Helicobacter* spp. in the lower

bowel of carnivores is not without precedent. For example, enteric *Helicobacter* species (*Helicobacter* sp. rhesus type 2) have been isolated from the colon of rhesus monkeys with chronic idiopathic colitis [226]. The presence of either viable or non-viable *Helicobacter* species in the stomach and at the distal end of the GIT indicates that the ecological niche of *Helicobacter* species in these animals may be both the stomach and the lower bowel. From these results we are unable to speculate on the role of *Helicobacter* spp. in the GIT of marsupial carnivores.

The **long nosed bandicoot** is an omnivore and has a flexible dietary strategy. It eats a variety of invertebrates, plant materials and fungi. Its digestive system is more complex than that of a carnivore. The most obvious feature of the omnivore's hindgut is a moderate sized caecum combined with a short colon, relative to the small intestine. Being an omnivore the rate of passage of digesta through the bandicoot gut is a little slower than in the carnivore, thus enabling them to maximise the use of plant material by selectively retaining solutes and small particles in both the caecum and proximal colon, the major site for microbial fermentation. However in mealworm-fed bandicoots, the major sites of digesta retention are the distal colon and rectum [145]. Hume has suggested that the flexibility of the bandicoot digestive tract is probably an important factor for these small marsupials allowing them to switch between different animal and food plant resources according to changes between seasons. The location of the *Helicobacters* detected by culture or PCR in these animals was mainly in the lower bowel, the major sites of digesta retention and microbial fermentation. This location appears to be the natural ecological niche of the *Helicobacters* in the long nosed bandicoots.

The most complex digestive systems are found in herbivores, where microbial fermentation of food intake is very important. Herbivores can be classified into two main groups, foregut fermenters and hindgut fermenters. the latter can be separated into caecum fermenters. (e.g. koalas, ringtail and brushtail possums) and colon fermenters (e.g. wombats), based on microbial fermentation sites.

The **Eastern grey kangaroo** is a foregut fermenter. The advantage of having microbial fermentation in the foregut is that microbial cells, high in quality protein and vitamin B, are synthesised in the foregut and can be digested in the small intestine, whereas microbes synthesised in hindgut are lost with the faeces unless the faeces are ingested (coprophagy) [222]. *Helicobacter* species were detected by culture and FISH from the stomach and *Helicobacter* DNA was detected by nested PCR in the stomach and lower bowel of the Eastern grey kangaroo. Thus in the kangaroo, the natural ecological niche of the *Helicobacters* appears to be the stomach and the lower bowel.

The **wombat** is a colon fermenter. Its natural diet is perennial native grasses. The most striking feature of the wombat is the development of a very large colon which is its main microbial fermentation site [147]. *Helicobacter* species were mainly detected by culture and/or PCR in the lower bowel of the two wombats. This location appears to be the natural ecological niche of the *Helicobacters* in these animals.

The **koala** is a unique herbivore with a complex digestive system containing an enormous sized hindgut. This enables it to cope with its unique diet which consists virtually of only eucalypt leaves. The koala's daily energy requirements are met by digestion in the small intestine of the cell content of eucalypt leaves. Furthermore a colonic separation mechanism in the koala's GIT results in the selective retention of solutes and fine particles in the caecum and the proximal colon which is important for microbial fermentation in these locations [146]. No *Helicobacter* species were isolated from the koalas with only *Helicobacter* DNA being detected, in most cases in the liver and/or stomach samples of the three koalas, using nested PCR. The significance of these finding requires further investigation.

Among the marsupials studied the koala is the only animal feeding solely on eucalypt leaves. These are known to be low in protein content and high in antinutrients as well as toxic to animal tissues and microbes. It is believed that gut microbes in the koala play an important role in the extraction of sufficient

nutrition from plant foods and overcoming the toxic effects resulting from plant secondary metabolites. In koalas, there have been extensive studies examining microbial degradation of the tannin-protein complex (T-PC). In 1990, *Streptococcus bovis* biotype 1 was isolated from the faeces of koalas and it was suggested by Osawa that these organism have a role in the microbial degradation of T-PC [151]. These bacteria have the unique characteristic of degrading the tannin-protein complex, as shown by the formation of a distinct clear zone on tannin-treated brain heart infusion agar. As a result of these studies Osawa suggested that the koala has a microbiological mechanism for deriving dietary proteins from the T-PC. More recently, facultative anaerobic T-PC-degrading enterobacteria (T-PCDE) have been isolated by Osawa from the faeces and from a layer of bacteria attached to the caecal wall of koalas [152]. In most of the koalas studied, the number of T-PCDE was found to be higher than the number of T-PC-degrading *S. bovis.* This led Osawa to suggest that T-PCDE played a more active role in digesting T-PC in the alimentary tracts of koalas. The low incidence of detection of *Helicobacter* species in the koalas suggests that a high intake of eucalypt leaves may limit the ability of *Helicobacter* to colonise the GIT of these marsupials. This suggestion is supported by the fact that the detection rate of *Helicobacter* species in the ringtail possum which have a diet containing significant amounts of eucalypt leaves was lower than that in the brushtail possum.

The phylogenetic study of the 16S rRNA gene sequence of all potentially new marsupial *Helicobacters* supported the finding that differences in environmental location i.e. gastric vs lower bowel has a major impact on the position of *Helicobacter* species on the phylogenetic tree. As can be seen in Figure 6.1, the lower bowel marsupial *Helicobacters* fell within the existing group of lower bowel *Helicobacters* while the *Helicobacter* isolate from the stomach of the Tasmanian devil fell within the existing group of gastric *Helicobacters*. These findings are consistent with the hypothesis proposed by Cohan that sequence similarity clusters in the bacterial world should correspond to an ecologically distinct population [124, 227, 228].

To date, there are 23 validly named *Helicobacte*r species, 2 '*Candidatus'* Helicobacter sp. (List of bacterial names with standing in nomenclature, last full update: March 18, 2004) and many more putative *Helicobacter* species in the genus *Helicobacter*. Fifteen of the 23 validly named *Helicobacters* are lower bowel *Helicobacters* (Table 1.2, Chapter 1) with majority isolated from rodents, which are hindgut fermenting herbivores. This is similar to the findings in marsupials, as most *Helicobacters* were isolated from the lower bowel of hindgut fermenters. This would suggest that the number of *Helicobacter* species in the hindgut fermenters of both placental mammals and marsupials is very high.

In the current study only two marsupial carnivores (the Tasmanian devil and Eastern quoll) were examined. Interestingly *Helicobacters* were isolated or detected by PCR in the stomach of both of these marsupials. This finding is consistent with isolation of *Helicobacters* from the stomach of primate and carnivores as reviewed in Chapter 1 (Table 1.4). For examples *H. pylori* was isolated from the stomach of humans. *H. bizzozeronii, H. salomonis* and *H. felis* were isolated from the stomach of dogs and cats. The common feature of these animals is their high protein diet. In addition *Helicobacters* were also detected and isolated from the stomach of the kangaroo which is herbivore. This finding is also consistent with the detection of *Helicobacters* from the stomach of cattle ("*Candidatus* Helicobacter bovis"). The common feature of kangaroos and cattle is that they are all herbivores which have a compartmentalised stomach.

The success in the detection and/or isolation of many new *Helicobacter* species from Australian marsupials including the wombat, the Eastern grey kangaroo, the Tasmanian devil, the Eastern quoll and the long nosed bandicoot, brushtail possum and ringtail possum indicates that *Helicobacter* species are common in Australian marsupials. The diversity of *Helicobacter* species within the marsupial group, is similar to that has been reported in placental mammals.

Chapter 8

Summary, General Discussion and Future Directions

8.1 Summary of major findings

The aim of this study was to investigate whether or not spiral and/or fusiform shaped bacteria, and in particular *Helicobacter* species, were present in the mucus layer overlying the epithelium of the gastrointestinal tract of Australian marsupials. If *Helicobacter* species were present, they were to be identified and characterised phylogenetically and phenotypically. The natural ecological niche of these organisms was investigated in different regions of the GIT using the combination of microscopy, culture and PCR amplification. A range of representative marsupial species was investigated and the bacteria from these marsupials were compared with *Helicobacter* isolates from animals at different positions on the evolutionary tree. The findings are described below.

1. Examination of a variety of Australian marsupial species has revealed that spiral and fusiform shaped bacteria colonise the mucus layer overlying the epithelium of the gastrointestinal tract of all marsupials. However the prevalence and bacterial load are different in each of the marsupial species examined. A number of bacteria with spiral shaped morphology proved not to belong to the genus *Helicobacter* but rather belonged to the genera *Campylobacter* and *Desulfovibrio*. However all fusiform shaped bacteria isolated in this study proved to be members of the genus *Helicobacter.*

2. Each marsupial species was found to be colonised by one or more unique *Helicobacter* species. The natural niche of *Helicobacter* species in Australian marsupials appeared to be impacted upon by the host's diet and the structure and function of the gastrointestinal tract. The detection rate of *Helicobacters* was higher in the brushtail possum than that in the ringtail possum but was very low in the koala. This suggested that a high

intake of eucalypt leaves by the marsupial host possibly limits the colonisation ability of *Helicobacters* in its gastrointestinal tract.

3. Phylogenetic analysis of all the new *Helicobacter* isolates showed that differences in environmental location i.e. gastric vs lower bowel appeared to have a major impact on the position of these *Helicobacters* on the phylogenetic tree. The *Helicobacters* isolated from the lower bowel of marsupials fell within the existing group of lower bowel *Helicobacters*. A *Helicobacter* strain isolated from the stomach of a Tasmanian devil also fell within the existing group of gastric *Helicobacters*. Host phylogeny also had some impact on the position of the *Helicobacters* on the phylogenetic tree. For example, the possum novel *Helicobacter* isolates were found to be closely related, and formed a monophyletic cluster separate from other lower bowel *Helicobacters*.

8.2. General discussion

Recent advances in Palaeontology and molecular science have substantially increased our understanding of the evolution of mammals. While primitive mammals are believed to have appeared sometime in the Triassic period (205-248 million years ago, mya) these species are now all extinct. Subsequent to this, during the Jurassic and Cretaceous periods (67–205 mya), the more modern mammals evolved.

Of the three groups of mammals in existence today, the monotremes (Prototherians) were believed to be the first to diverge, followed by the divergence of the placental mammals (Eutherians) and marsupials (Metatherians). The estimated time of the marsupial-placental split is controversial. Based on phylogeny and timescales for mammalian evolution developed by McKenna and Bell (1977) the divergence of the marsupials and placental mammals is reported to be 125 mya [238]. However, more recent molecular dating analyses estimate that the split between the marsupials and placental mammals occurred earlier [232, 239]. For example, recently Woodburne *et al.* evaluated previous molecular data using a relaxed molecular clock approach, multiple constraints from fossil records and two different amino acid data sets (IGF2 receptor and BRCA1 protein). As a result of these studies these authors suggested that the marsupial and placental mammal split occurred during the Jurassic period (182-190 mya) [235, 232, 239].

In addition, the origin of the different mammals also is controversial. In a recent study Luo *et al.* analysed tribosphenic molar fossils to determine the origins of monotremes, marsupials and placental mammals [234]. This study suggests that marsupials and placental mammals shared a common ancestor upon the Laurasian continents (Northern continents), while living monotremes originated in the Gondwana landmasses (Southern continents). In agreement with this is the recent fossil evidence from two early mammals, *Eomaia* and *Sinodelphys*, which exhibit classical features of the eutherian and metatherian mammals

respectively. They were discovered in China in the same Early Cretaceous (125 mya) geological layer suggesting a Eurasian origin (part of Laurasian supercontinent) for these mammals [236, 237].

The oldest fossil records of monotremes, *Steropodon galmani*, obtained from Flat Rocks, Victoria and Lightning Ridge, New South Wales, suggest that monotremes have inhabited Australia for 110-120 mya [133, 233]. The oldest marsupial fossils (isolated teeth) in Australia, which were obtained from the Tingamarra deposit in Murgon, south-eastern Queensland, are from the late Palaeocene epoch (dated at ~55 mya)[129, 233]. Fossil evidence of Australia's earliest known bat, also discovered in deposits in Queensland, also comes from this same time period. In contrast, fossil records from rodents (rats and mice) indicate that rodents did not invade Australia from Asia until the early Pliocene epoch (4-5 mya) [233]. Current evidence would also suggest that the invasion of Australia by humans from South-East Asia occurred approximately 50,000-68,000 years ago, although it is possible that this may have occurred much earlier [233].

While previous studies have shown that *Helicobacter* species are able to colonise a wide range of placental mammals, it was unknown whether these bacteria could colonise marsupials. The current study has shown that marsupials are colonised by unique *Helicobacter* species. Indeed *Helicobacter* species were found colonising the GIT of a range of Australian marsupials including the Eastern quoll, Tasmanian devil, long nosed bandicoot, wombat, possum and kangaroo. This finding suggests that as has been found in placental mammals, the diversity of *Helicobacters* within Australian marsupials is very high (Table 8.1 and Figure 8.1). The ability of *Helicobacter* species to colonise a wide variety of placental mammals, marsupials and birds, suggests that *Helicobacter* species may be of ancient origin and that they may have coevolved with their animal hosts over a long period of time.

Evidence supporting the concept that *Helicobacter* species may have coevolved with their hosts is the fact that *Helicobacters* have a very small genome (<2 Mb)

[84], a characteristic common to many symbiotic and pathogenic bacteria. Indeed Ochman *et al*. have suggested that small genome size is a neutral or deleterious consequence of long-term evolution under conditions imposed by the symbiotic and pathogenic life-styles [231]. In addition, these authors suggest that once genome reduction has occurred, bacteria are unable to re-acquire the multitude of eliminated genes, and as a consequence of this are unable to revert to a life-style independent from their hosts [231]. The fact that *Helicobacter* species have been shown to be incapable of long term survival in the natural environment outside their hosts would further support the view that *Helicobacters* have evolved in response to direct adaptations required for their survival in the gastrointestinal tract of specific hosts.

Many factors may have impacted upon the evolution of *Helicobacter* species and influenced their co-relationship with their hosts. The term “evolution” has been described by Hanson *et al.* as “the mechanism producing the diversity of life” [229]. Thus it is possible that the ancestors of the *Helicobacter*s may have evolved to be compatible with the evolutionary changes occurring in the GIT of their animal hosts. This reciprocal change, as an outcome of the co-relationship between *Helicobacter* species and their hosts, has resulted in the subdivision of these bacteria over evolutionary time into numerous species. Support for the concept of a ‘primordial helicobacter’ is evidenced by the fact that to date all the *Helicobacters* isolated from placental and marsupial mammals and birds, group together in a phylogenetic tree, thus making it likely that they share the same ancestor. This is particularly significant if we consider the fact that the marsupial *Helicobacters,* described in this study, have almost certainly evolved with their hosts in isolation, as a consequence of the separation of the Australian continent from other land masses approximately 50 million years ago. If marsupials had acquired their *Helicobacter* species more recently, we would not expect to see such a high level of diversity within the marsupial *Helicobacters,* or for these species to show such a high degree of specificity for their individual host species.

This reciprocal change in interacting species has been termed coevolution. In a recent study, Thompson reviewed and summarised the concepts of coevolution based on a variety of mechanisms and outcomes of reciprocal evolutionary change [230]. In this study, Thompson discussed 5 modes of coevolution, Gene-for gene coevolution, Specific coevolution, Guild coevolution, Diversifying coevolution and Escape and radiation coevolution. Three of these modes of coevolution do not involve speciation. Of the remaining 2 modes, “the diversification coevolution” mode best explains the mechanisms and outcomes of reciprocal evolutionary change between *Helicobacter* species and their hosts. Diversification coevolution is defined as “reciprocal evolution between species in which the interaction causes at least one of the species to become subdivided into two or more reproductively isolated populations” [230], a situation we observe in the genus *Helicobacter*.

While the discovery that *Helicobacter* species can colonise marsupials has significantly expanded our existing knowledge of the geographic distribution and diversity of *Helicobacter* species, additional studies in other animal species has the potential to provide further insights into the evolution of these bacteria. Two groups of animals, for which little is known with respect to their *Helicobacter* status, are the reptiles and the monotremes. To date two studies have reported the presence of *Helicobacters* in reptiles. One study was based on the detection of *Helicobacter* DNA in faecal samples of the Nile crocodile and Taiwan beauty snake, using PCR-DGGE technique [134]. The other study (unpublished) was based on microscopic evidence of gastric spiral bacteria in reptiles [122]. The significance of these findings is questionable and thus further investigations to detect the presence of *Helicobacter* species in reptiles are required.

Of more interest however is the question “Do *Helicobacter* species colonise monotremes?”, a group believed to have diverged prior to the divergence of placental mammals and marsupials. Prior to the commencement of the current study in marsupials, specimens from two short-beaked echidnas (animals resident in Taronga Zoo) were collected and examined in our preliminary investigations. In this early study, *Helicobacter* DNA was detected using nested

PCR only in the rectum of one echidna. This preliminary finding suggests that monotremes may also be colonised by *Helicobacter* species. While we planned to further examine this issue, unfortunately additional suitable specimens from either the echidna or platypus, the only two members of the monotremes, were unavailable during the course of this thesis. Given our initial findings in the echidna, further studies in monotremes would certainly be warranted.

As discussed in Chapter 1, investigation of the microbiota of the gastrointestinal tract of mammals has been undertaken since the early days of bacteriology. These bacteria are essential for the health of humans and animals. In the current study characteristics of marsupials such as their diet and the structure and function of their gastrointestinal tracts, have been shown to impact upon their colonisation by *Helicobacters.* This finding is an example of the knowledge that we can obtain from the study of the co-relationship between microbes and their animal host. Currently, there is an abundance of phenotypic and genotypic information available on *Helicobacter* species as well as on their animal hosts. Examination of the phenotypic and genotypic characteristics of these *Helicobacter* species could provide an insight into what factors have influenced the coevolution of *Helicobacter* species and their hosts. In many mammals *Helicobacter* species are more likely to be symbiotic rather than pathogenic bacteria. A question that remains to be answered, however, is “What is the benefit that *Helicobacters* gain from their hosts and what is the reciprocal benefit that the hosts gain from the *Helicobacters?*”

Class **Mammalia** Subclass **Marsupialia**	**Animal hosts**	***Helicobacter* species**
Order **Dasyurida**	Tasmanian devil (carnivore)	**Gastric *Helicobacter*** *Helicobacter* sp. 'TD1'
Order **Peramelina**	Long nosed bandicoot (omnivore)	**Lower bowel *Helicobacter*** *Helicobacter* sp. 'LNB1F', *Helicobacter* sp. 'LNB2F'
Order **Diprotodontia**	Wombat (colon fermenter herbivore)	**Lower bowel *Helicobacter*** *Helicobacter* sp. 'WB1F', *Helicobacter* sp. 'WB2F'
	Ringtail possum (caecum fermenter herbivore)	**Lower bowel *Helicobacter*** *"Helicobacter peregrinus"*
	Brushtail possum (caecum fermenter herbivore)	**Lower bowel *Helicobacter*** *"Helicobacter vulpecula"* *"Helicobacter kirkbridei"* *Helicobacter* sp. 'BTP1S'-'BTP5S'

Table 8.1 The grouping of marsupial *Helicobacter* species in relation to the classification of marsupial hosts.

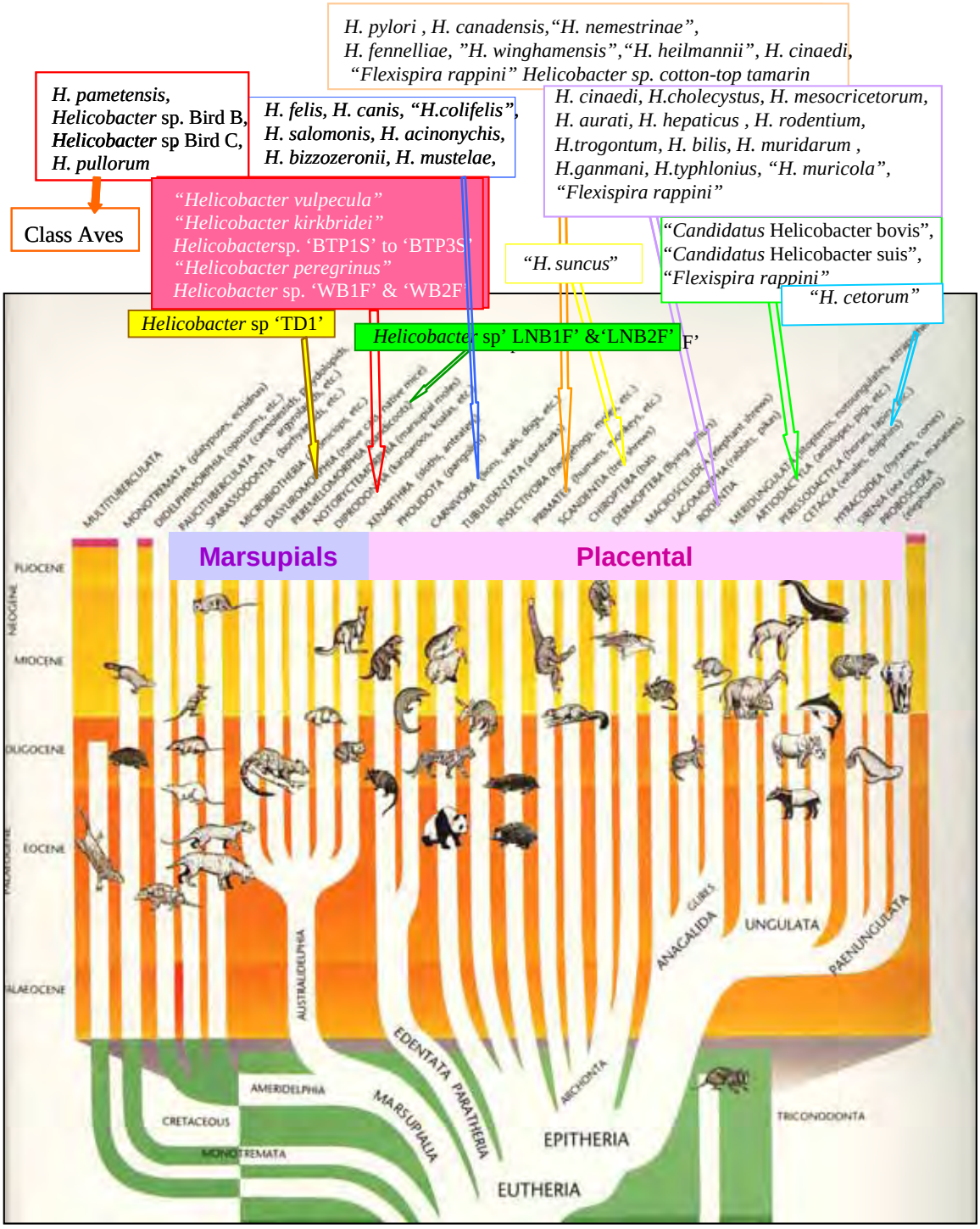


Figure 8.1 The grouping of *Helicobacter* species in relation to the classification of marsupial and placental hosts. The diagram of the mammal classification was taken from "hypothesis of the interrelationships of living and fossil mammals by Vickers-Rich [129].

8.3 Future Directions

This thesis has provided important preliminary data on the diversity and localisation of *Helicobacter* species in a limited number of marsupial species. As such it provides a small but significant insight into microbial diversity, microbial evolution and the co-relationship between these bacteria and their hosts.

Further investigation into the prevalence and diversity of *Helicobacter* species in a larger number of brushtail and ringtail possums, as well as a more comprehensive range of marsupial species, would clearly provide a greater insight into the role of *Helicobacters* in the GIT. Investigation of the specific characteristics of marsupial *Helicobacter* species to different components of the hosts' diets (such as tannin and other plant secondary metabolites from eucalypt leaves) may provide important information on the impact of diet on the colonisation of the marsupial GIT by *Helicobacters.* In addition, such studies would provide additional material for the elucidation of the evolution of the *Helicobacter* species.

The availability of primers, designed in this study, specific for the BTP and RTP *Helicobacter* species, will facilitate a more efficient screening of a large number of BTP's and RTP's for the presence of these *Helicobacter* species. Use of molecular techniques such as PCR-DGGE and in *situ* hybridisation using genus and species specific probes, also have the potential to facilitate the detection of *Helicobacter* species in a larger number of samples and a wide range of sample types such as biopsy specimens, faeces, etc. or from archival specimens such as paraffin embedded tissues.

Understanding the significance of the detection of spiral and fusiform bacteria other than *Helicobacter* species i.e. *Campylobacter* spp. and *Desulfovibrio* spp., commonly found co-colonising with *Helicobacter* species could also increase our knowledge of the inter-relationship between bacteria of similar morphology in the same GIT environment.

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Appendix 1 The histopathology observed in the hematoxylin and eosin stained sections of the liver and all specimens collected from the gastrointestinal tract of all marsupials studied.

Animals	Regions	Histopathology
BTP 1	liver	well preserved, multifocal, mild portal small mononuclear cells
	stomach	well preserved, no significant lesions
	mid-ileum	well preserved ,very mild scattered small mononuclear cells throughout lamina propria
	3cm-ileum	well preserved, rare small mononuclear cells in lamina propria
	caecum	well preserved, very mild eosinophils &small mononuclear cells in lamina propria
	colon	well preserved, rare eosinophils & small mononuclear cells in lamina propria
	rectum	well preserved, very mild small mononuclear cells scattered in lamina propria
BTP 2	liver	well preserved, mild multifocal portal small mononuclear cells, mild neutrophil scattered throughout sinusoids
	stomach	well preserved, no significant lesions
	mid-ileum	autolytic vilar tips & bodies, mild difuse small mononuclear cells throughout lamina propria focally extensive lymph aggregate in deep lamina propria sub mucosa & focally distending the body of avillus
	3cm-ileum	well preserved, very mild small mononuclear cells & eosinophils scattered throughout lamina propria
	caecum	well preserved, mild diffuse small mononuclear cells, neutrophil & plasma cell throughout lamina propria, small number eosinophils throughout lamina propria
	colon	well preserved, mild diffuse lamina propria, small number neutrophil throughout lamina propria
	rectum	well preserved, mild small mononuclear cells throughout lamina propria & focal aggregates small mononuclear cells lamina propria
BTP 3	liver	well preserved, rare lymphocyte & plasma cells in portal tracts, lymphocytes with intracellular inclusion bodies (either viral or host protein)
	stomach	well preserved, no significant lesions
	mid-ileum	well preserved,nmild diffuse lymphocyte & plasma cells neutrophil throughout lamina propria ,high number of coccidial, gametocytes & oocysts with intracytoplasmic vacuoles in enterocytes at villi or tips.
	3cm-ileum	well preserved, mild small mononuclear cells, neutrophil & eosinophils throughout lamina propria &mucosa
	caecum	well preserved, mild lymphocyte, plasma cells, neutrophil & eosinophils throughout lamina propria
	colon	well preserved, mild lymphocyte, plasma cells & eosinophils
	rectum	well preserved, mild lymphocyte, plasma cells & eosinophils throughout lamina propria
BTP 4	liver	well preserved, diffuse periacinar hepatocell necrosis, small number neutrophil & small mononuclear cells in necrotic area.
	stomach	mild autolytic, no significant lesions
	mid-ileum	mild autolytic, mild lymphocyte & plasma cells neutrophil eosinophils in lamina propria, multifocal clusters eosinophils in internal lumen between villi
	3cm-ileum	well preserved, mild small number eosinophils & neutrophil in deep lamina propria
	caecum	mild-moderate autolytic, mild lymphocyte, plasma cells & eosinophils in lamina propria
	colon	moderate autolytic, mild lymphocyte & plasma cells small mononuclear cells
	rectum	well preserved, no significant lesions

Animals	Regions	Histopathology
BTP 5	liver	well preserved , no significant lesions
	stomach	mild autolytic , duodenum mild-moderate lamina propria mononuclear cells & eosinophils
	mid-ileum	well preserved,mild lamina propria monocytes,eosinophils &neutrophil
	3cm-ileum	mild autolytic,mild lamina propria monocytes, eosinophils & neutrophil, focal lamina propria monocytes aggregate.
	caecum	mild autolytic, mild lamina propria monocytes & moderate lamina propria eosinophils, mild eosinophils in sub mucosa.
	colon	well preserved, mild eosinophils & mononuclear cells in lamina propria.
	rectum	well preserved, scattered lamina propria mononuclear cells in lamina propria.possible increase lamina propria fibrosis.
BTP 6	liver	well preserved, mild diffuse portal lymphocyte & plasma cells , multifocal hepatocellular intracellular inclusion bodies (either viral or host protein)
	stomach	mild-moderate autolytic, no significant lesions
	mid-ileum	moderate autolytic,small mononuclear cells scattered throughout lamina propria
	3cm-ileum	moderate autolytic, scattered lymphocyte, plasma cells & eosinophils in lamina propria
	caecum	moderate autolytic,mild lymphocyte & plasma cells eosinophils throughout deep lamina propria
	colon	moderate autolytic, much of mucosa missing, scattered lymphocyte & plasma cells throughout lamina propria
	rectum	moderate autolytic, lymphocyte & plasma cells scattered throughout lamina propria
BTP 7	liver	well preserved, mild multifocal portal lymphocyte & plasma cells & smallnumber eosinophils, many binucleate hepatocytes
	stomach	mild autolytic, no significant lesions
	mid-ileum	mild autolytic, scattered lymphocyte & plasma cells in lamina propria, scattered coccidial, gametocytes & oocysts in cytoplasmic vacuoles of enterocytes near the tips of villi.
	3cm-ileum	well preserved, as per mid-ileum
	caecum	mild-moderate autolytic,increas number coccidial oocysts in lumen, scattered lymphocyte & plasma cells & eosinophils in lamina propria
	colon	moderate autolytic, scattered eosinophils lymphocyte & plasma cells in lamina propria
	rectum	moderate autolytic, no significant lesions
BTP 8	liver	well preserved, mild multifocal portal mononuclear cells (lymphocyte & plasma cells), multifocal hepatocellular intracellular inclusion bodies (either viral or host protein)
	stomach	well preserved, no significant lesions
	mid-ileum	well preserved, scattered small mononuclear cells in deep lamina propria
	3cm-ileum	well preserved, as mid-ileum
	caecum	mild autolytic,mild lymphocyte, plasma cells & eosinophils throughout lamina propria
	colon	well preserved, mild mononuclear cells throughout lamina propria with multifocal larger lamina propria mononuclear cell aggregates.
	rectum	well preserved, scattered lymphocyte & plasma cells throughout lamina propria

Animals	Regions	Histopathology
BTP 9	liver	well preserved, small foci haematopoiesis scattered throughout parenchyma, mild multifocal mononuclear cells aggregrates in portal tracts.
	stomach	mild autolytic, multifocal small clusters lymphocyte, plasma cells 7 eosinophils in lamina propria
	mid-ileum	moderate autolytic, mild lymphocyte, plasma cells & small number of eosinophils in deep lamina propria
	3cm-ileum	mild autolytic, mild lymphocyte, plasma cells & eosinophils in deep lamina propria
	caecum	mild autolytic, scattered lymphocyte & plasma cells in lamina propria
	colon	mild autolytic, scattered lymphocyte & plasma cells in lamina propria, multifocal small aggregates mononuclear cell in lamina propria, focal sub mucosa aggregate mononuclear cells & small number eosinophils
	rectum	mild autolytic, as colon, focal sub mucosa lymphoid aggregates.
BTP 10	liver	well preserved, mild multifocal lymphocyte, plasma cells & small number of eosinophils scattered haematopoietic cells throughout sinusoids, multifocal hepatocellular intracellular inclusion bodies (either viral or host protein), multifocal periacinar hepatocellular degeneration.
	stomach	mild autolytic, no significant lesions
	mid-ileum	well preserved, scattered lymphocyte & plasma cells in deep lamina propria
	3cm-ileum	well preserved, scattered small mononuclear cells in deep lamina propria
	caecum	well preserved, mild lymphocyte, plasma cells & eosinophils throughout lamina propria, eosinophils scattered throughout mucosal epithelial.
	colon	mild autolytic, mild lymphocyte,plasma cells & eosinophils in deep lamina propria
	rectum	mild-moderate autolytic, mild lymphocyte,plasma cells & eosinophils primarily in deep lamina propria
BTP 11	liver	well preserved, mild multifocal portal aggregates small mononuclear cells, high number of binucleate hepatocytes.
	stomach	mild autolytic, no significant lesions
	mid-ileum	mild autolytic, segmental deep lamina propria mononuclear cell infiltrate, remaining sections No significant lesions
	3cm-ileum	mild autolytic, focal lymphoid aggregates in deep lamina propria. There are centrally depleted of lymphocytes& contain tingible body macrophages, remaining sections No significant lesions
	caecum	mild autolytic,scattered lymphocyte & plasma cells eosinophils in lamina propria
	colon	well preserved, very small number eosinophils in lamina propria
	rectum	mild-moderate autolytic, scattered lymphocyte & plasma cells eosinophils in lamina propria

Animals	Regions	Histopathology
RTP 1	liver	mild autolytic, no significant lesions
	stomach	mild autolytic, mild focal mononuclear cells & neutrophils in lamina propria
	mid-ileum	mild autolytic, mild small mononuclear cells in deep lamina propria
	3cm-ileum	mild autolytic, mild small mononuclear cells throughout lamina propria
	caecum	moderate-marked autolytic, mucosa missing some areas, mild mononuclear cell & eosinophils in intact lamina propria
	colon	well preserved, scattered small mononuclear cells & eosinophils in lamina propria
	rectum	well preserved, scattered mononuclear cell in lamina propria
RTP 2	liver	well preserved, hepatocytes are moderately microresiculated & vacuolated. Mild focal portal mononuclear cells aggregate, multifocal neutrophil aggregate.
	stomach	well preserved, no significant lesions
	mid-ileum	well preserved, multifocal large lamina propria mononuclear cell nodules, mild mononuclear cells scattered throughout the lamina propria.
	3cm-ileum	well preserved, multifocal extensive well developed lymphoid follicles. Mild small mononuclear cells throughout the lamina propria.
	caecum	well preserved, no significant lesions.
	colon	well preserved, moderate rugae formation of the mucosa, mild lamina propria oedema with mild neutrophil in oedematous areas.
	rectum	well preserved, mild lamina propria oedema.
RTP 3	liver	segmental autolytic, no significant lesions
	stomach	mild-moderate autolytic, no significant lesions
	mid-ileum	mild autolytic, short villi in 2 sections, scattered lymphocyte, plasma cells 7 eosinophils in lamina propria, focal lymphoid aggregate in lamina propria
	3cm-ileum	moderate-marked autolytic, large section of mucosa missing, multifocal lamina propria lymphoid aggregates.
	caecum	moderate-marked autolytic, segments of mucosa missing, remaining mucosa is very flat.
	colon	mild autolytic, scattered lymphocyte, plasma cells & eosinophils in lamina propria
	rectum	mild autolytic, focal very small mononuclear cell aggregates in lamina propria
RTP 4	liver	well preserved, no significant lesions
	stomach	well preserved,segmental increase lamina propria connective tissue
	mid-ileum	well preserved, enterocytes at the tips of villi contain intracellular inclusion bodies (either viral or host protein), mild small mononuclear cells throughout deep lamina propria
	3cm-ileum	well preserved, as per mid ileum
	caecum	well preserved, flat epithelium, intracellular inclusion bodies (either viral or host protein) in enterocytes
	colon	well preserved, scattered lymphocyte & plasma cells in lamina propria, possible mild lamina propria oedema
	rectum	well preserved, as per colon
RTP 5	liver	central autolytic, no significant lesions
	stomach	mild autolytic, no significant lesions
	mid-ileum	moderate autolytic, no significant lesions
	3cm-ileum	mild autolytic, mild lamina propria mononuclear cell infiltrates
	caecum	well preserved, flat mucosa, small numberscat small mononuclear cells in lamina propria
	colon	well preserved, multifocal large lamina propria mononuclear cellaggreates & lymphoid follicles, many follicles centrally depleted of lymphocytes
	rectum	well preserved, scattered lamina propria small mononuclear cells, possible mild lamina propria oedema.

Animals	Regions	Histopathology
RTP 6	liver	well preserved, no significant lesions
	stomach	mild autolytic, no significant lesions
	mid-ileum	moderate-marked autolytic, no significant lesions
	3cm-ileum	mild-moderate, no significant lesions
	caecum	mild autolytic, small number enterocytes with intracellular inclusion bodies (either viral or host protein), small number small mononuclear cells scattered in lamina propria
	colon	moderate-marked autolytic, no significant lesions
	rectum	moderate autolytic, no significant lesions
RTP 7	liver	well preserved, no significant lesions
	stomach	mild-moderate autolytic, no significant lesions
	mid-ileum	moderate autolytic, scattered lamina propria small mononuclear cells
	3cm-ileum	moderate autolytic, scattered small mononuclear cells in lamina propria
	caecum	mild autolytic, flat epithelial, scattered mononuclear cells & eosinophils in lamina propria
	colon	mild-moderate autolytic, possible mild lamina propria oedema
	rectum	mild autolytic as per colon
RTP 8	liver	well preserved, no significant lesions
	stomach	mild autolytic, no significant lesions
	mid-ileum	mild autolytic, segmental well developed lymphoid follicles in lamina propria, follicles centrally depleted of lymphocytes & contain karyotic, pykotic cell debries & tingible body macrophages(sign of acute lymphoid necrosis), scattered small mononuclear cells in deep of remaining intestine in enterocyte at tips of some villi
	3cm-ileum	well preserved, very small number small mononuclear cells & eosinophils scattered in lamina propria
	caecum	mild autolytic, flat epithelial multifocal intracellular inclusion bodies (either viral or host protein) in enterocytes
	colon	mild autolytic, no significant lesions
	rectum	mild autolytic , very smallnumber scattered small mononuclear cells & eosinophils in lamina propria
RTP 9	liver	well preserved, no significant lesions
	stomach	mild autolytic, no significant lesions
	mid-ileum	mild autolytic, scattered small mononuclear cells & rare eosinophils in lamina propria ,intracellular inclusion bodies (either viral or host protein) in many enterocytes at villar tips
	3cm-ileum	well preserved, no significant lesions
	caecum	well preserved, flat mucosa, scattered lamina propria small mononuclear cells, intracellular inclusion bodies (either viral or host protein) in scattered enterocytes
	colon	well preserved, no significant lesions
	rectum	well preserved, no significant lesions
RTP 10	liver	well preserved, no significant lesions
	stomach	well preserved, no significant lesions
	mid-ileum	mild -marked autolytic, small mononuclear cells eosinophils scattered in deep lamina propria, much of mucosa missing.
	3cm-ileum	well preserved,very small number small mononuclear cells & eosinophils scattered in deep lamina propria, increase number intracellular inclusion bodies (either viral or host protein) in villar enterocytes.
	caecum	well preserved, primarily flat mucosa, increase intracellular inclusion bodies (either viral or host protein) in enterocytes
	colon	well preserved, very small number eosinophils & small mononuclear cells in lamina propria, increase number intracellular inclusion bodies (either viral or host protein) in enterocytes
	rectum	well preserved, small number of mononulear cells scattered throughout lamina propria, possible mild lamina propria oedema

Animals	Regions	Histopathology
Wombat 1	liver	well preserved, mild biliary hyperplasia, neutrophil are scattered throughout the sinusoids, multifocal hepatocytes have large nuclei with peripheralised chromatin & eosinophils intracellular inclusion bodies (either viral or host protein) (papova virus?).
	stomach	well preserved, no significant lesions
	mid-ileum	well preserved, mild mononuclear cells throughout lamina propria, mild ciliate parasite along mucosal surface.
	3cm-ileum	well preserved, mild mononuclear cells throughout lamina propria, mild ciliates along mucosa.
	caecum	well preserved, moderate irregular mucosal depth.moderate small mononuclear cells & mild eosinophils throughout lamina propria, marked cilate parasites along mucosal surface & in multifocal crypts. Probable *Balantidium* sp.
	colon	well preserved, mild mononuclear cells throughout the lamina propria, mild cilate parasites along the surface of the mucosa.
	rectum	well preserved, focal lamina propria lymphoid nodules.
Wombat 2	liver	well preserved, no significant lesions
	stomach	mild autolytic, no significant lesions.
	mid-ileum	moderate autolytic, mild lamina propria mononuclear cell.
	3cm-ileum	moderate autolytic, cross sections of a nematode larvae in the lumen, scattered mild mononuclear cells throughout lamina propria.
	caecum	moderate autolytic, no significant lesions.
	colon	moderate autolytic, no significant lesions.
	rectum	mild-moderate autolytic, mononuclear cells scattered throughout lamina propria.

Animals	Regions	Histopathology
Koala 1	liver	well preserved, kupffer cells contain small quantities of brown cytoplasmic pigment.
	stomach	a) well preserved, no significant lesions. b) mild autolytic, no significant lesions
	mid-ileum	well preserved, no significant lesions.
	3cm-ileum	mild autolytic, moderate rugae formation throughout the mucosa.
	caecum	mild autolytic, no significant lesions.
	colon	well preserved, the lamina propria is mildly oedematosis, small numbers of neutrophil are multifocal evident in the superficial lamina propria.mild multifocal necrosis & exfoliation of superficial epithelial
	rectum	as tissue above.
Koala 2	liver	well preserved,hepatocytes contain moderate gum titics of brown cytoplasmic pigment, moderate binucleate hepatocytes, scattered hepatocytes with very large nuclei & eosinophils intracellular inclusion bodies (either viral or host protein). Hepatocytes are mildly to moderately vacuolated.
	stomach	well preserved, no significant lesions
	mid-ileum	well preserved, mild rugae formation, entercytes at the tips of villi contain eosinophils intracellular inclusion bodies (either viral or host protein).
	3cm-ileum	well preserved, no significant lesions,intracellular inclusion bodies (either viral or host protein) in enterocytes at the tips of villi, scattered eosinophils in mucosa.
	caecum	well preserved, no significant lesions.
	colon	well preserved, the lamina propria is moderately oedematous, the pattern of mucosal crypts is irregular
	rectum	well preserved, as colon.
Koala 3	liver	well preserved, multifocal marked vacuolated hepatocytes
	stomach	well preserved, no significant lesions
	mid-ileum	mild-moderate autolytic, scattered small mononuclear cells in lamina propria
	3cm-ileum	mild autolytic, segmental sub mucosa chain of discrete lymphoid follicles & lymphoid aggregates, follicles are mild centrally depleted of lymphocytes& contain small number of macrophages with cytoplasmic brown pigment, mild small mononuclear cells in deep lamina propria, many villar enterocytes with large intracellular inclusion bodies (either viral or host protein)
	caecum	well preserved, no significant lesions
	colon	well preserved, scattered small mononuclear cells in lamina propria ,mild lamina propria oedema
	rectum	well preserved, many vacuolated enterocyte in crypts

Animals	Regions	Histopathology
Kangaroo 1	liver	well preserved, mild portal mononuclear cells , mild neutrophil & smaller number of eosinophils in sinusoids
	stomach (blind sac)	well preserved, squamous stomach-1st section-No significant lesions , 2nd section-moderate eosinophils & small mononuclear cells in lamina propria & mucosa Glandular stomach-1) focal submucosal necrosis infiltrated with eosinophils multifocal submucosal lymphoid follicles with diffuse mild eosinophils & small mononuclear cells through out remaining submucosa especially perivascularly. 2)Marked lymphocytes, plasma cell&eosinophils in lamina propria near lumen all areas of stomavh & diffusely in lamina propria overlying submucosal lymph follicles.3)multifocal cross sections of small nematodes in mucosa. several gastric pits filled with multifocal. 4) multifocal coat mucosal surface focally.
	tubiform stomach	well preserved, multifocal marked lamina propria sub mucosa lymphocyte & plasma cells eosinophils in areas where mucosal glands contain cross sections of nematode parasites or nematode ova, small number cryts contain clusters of eosinophils , other areas mild lymphocyte & plasma cells eosinophils throughout lamina propria
	hind stomach	well preserved, glandular mucosa, 1st section mild lymphocyte & plasma cells eosinophils throughout deep lamina propria across section of a nematode is present in the lumen adjacent to this section. 2nd sections, no significant lesions.
	ileum	mild-moderate autolytic, moderate diffuse lymphocyte & plasma cells eosinophils throughout lamina propria & mild in sub mucosa, section of nematode in the mucosa
	caecum	well preserved, segmental moderate lamina propria lymphocyte & plasma cells eosinophils, focal lymphoid aggregate beneath this segment, diffuse moderate lymphocyte & plasma cells eosinophils in superficial sub mucosa .
	colon	well preserved, mild-moderate eosinophils throughout lamina propria , mild-moderate eosinophils throughout superficial sub mucosa with scattered lymphocyte & plasma cells , multifocal sub mucosa lymphotic vessel filled with lymphocytes, small number of eos scat over mucosal surface.
	rectum	well preserved, mild lymphocyte & plasma cells eosinophils throughout lamina propria & superficial sub mucosa.
Eastern quoll 1	liver	well preserved, mild biliary hyperplasia, small basophilic round structures line the biliary mucosa- the structures look like old nuclei & may indicate increase cell turnover.
	stomach	mild autolytic, no significant lesions.
	mid-ileum	moderate-marked, no significant lesions.
	3cm-ileum	moderate-marked, no significant lesions.
	colon	mild autolytic, no significant lesions.
	rectum	mild autolytic, no significant lesions.
Tasmanian devil 1	liver	well preserved, mild portal mononuclear cell aggregates.
	stomach	well preserved, no significant lesions.
	mid-ileum	well preserved, no significant lesions.
	3cm-ileum	well preserved, scattered mononuclear cells throughout the lamina propria.
	colon	mild autolytic, segmentally extensive submucosal lymphoid follicles. Scattered mononuclear cells in lamina propria.
	rectum	mild autolytic, scattered mononuclear cells in lamina propria.Thick colonies of bacilli are present in multifocal crypts.

Animals	Regions	Histopathology
Long nosed bandicoot 1	liver	well preserved, moderate-marked multifocal portal small mononuclear cells & eosinophils aggregates, increase portal & periportal eosinophilic amorphous material-probable amyloid, multifocally extensive periacinar and random eosinophils infiltrates.
	stomach	mild autolytic, mild infiltration eosinophils smaller number small mononuclear cells,multifocal lamina propria deposits eosinophilic amorphous material-probable amyloid (along basement membranes of gastric crypts).
	mid-ileum	moderate autolytic, mild diffuse lamina propria eosinophils & small mononuclear cells, multifocal lymphoid aggregates in deep lamina propria
	3cm-ileum	moderate-marked autolytic, nematode ova in intestinal lumen, mild eosinophils & small mononuclear cells throughout lamina propria & sub mucosa .
	caecum	moderate autolytic, nematod eova in lumen, mild-moderate eosinophils & small mononuclear cells throughout lamina propria & sub mucosa
	colon	moderate autolytic, mild eosinophils & small mononuclear cells in lamina propria & sub mucosa.
	rectum	moderate autolytic, mild eosinophils & small mononuclear cells in lamina propria
Long nosed bandicoot 2	liver	well preserved, diffuse mild periacinar eosinophils infiltrate
	stomach	well preserved, diffuse mild eosinophils throughout lamina propria
	mid-ileum	moderate-marked autolytic,mild diffuse eosinophils in lamina propria, scattered eosinophils in sub mucosa & serosa
	3cm-ileum	mild autolytic, as per midileum
	caecum	well preserved, no significant lesions
	colon	mild autolytic, mild diffuse eosinophils in lamina propria, cross sections of nematode in mucosa.
	rectum	mild autolytic, as per colon & eosinophils scattered in serosa
Long nosed bandicoot 3	liver	well preserved, moderate-periacinar eosinophils infiltrates, multifocal moderate portal eosinophils & small mononuclear cells infiltrates
	stomach	mild autolytic, mild eosinophils throughout lamina propria & sub mucosa
	mid-ileum	marked autolytic, most of mucosa is missing
	3cm-ileum	moderate autolytic, segmental sub mucosa long chain of discret lymphoid fillicles. Distending sub mucosa, diffuse mild eosinophils infiltrates lamina propria, multiple sections of nematode parasites in deep mucosa.
	caecum	mild-moderate autolytic, mild eosinophils throughout lamina propria
	colon	mild autolytic, mild eosinophils throughout lamina propria ,multifocal nematode ova & nematode in mucosa
	rectum	mild autolytic, mild eosinophils throughout lamina propria, multifocal nematodes in mucosa

Appendix 2 Multiple sequence alignment of 16S rRNA gene sequences of all *Helicobacter* species isolated from brushtail possums and ringtail possums

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                         *        20         *        40         *
H. vulpecula
BTP1C        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP2C        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP3C        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACg
BTP4C        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP5C        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP6C        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP7C        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP8C        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP9C        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
H. kirkbridei
BTP1F        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP2F        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP3F        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP4F        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP5F        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP6F        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
Helicobacter sp.
BTP1S        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP2S        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
BTP3S        : CTGGCTCAGAGTGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACG
H. peregrinus
RTP1S        : CTGGCTCAGAGTGAACGCTGGCGGTGTGCCTAaTACATGCAAGTCGAACG
RTP2S        : CTGGCTCAGAGTGAACGCTGGCGGTGTGCCTAATACATGCAAGTCGAACG
RTP3S        : CTGGCTCAGAGTGAACGCTGGCGGTGTGCCTAATACATGCAAGTCGAACG
RTP4S        : CTGGCTCAGAGTGAACGCTGGCGGTGTGCCTAATACATGCAAGTCGAACG
RTP5S        : CTGGCTCAGAGTGAACGCTGGCGGTGTGCCTAATACATGCAaGTCGAACG


                         60         *        80         *       100
H. vulpecula
BTP1C        : ATGAAGATTCTAGCTTGCTAGAGTTGGATTAGTGGCGCACGGGTGAGTAA
BTP2C        : ATGAAGATTCTAGCTTGCTAGAGTTGGATTAGTGGCGCACGGGTGAGTAA
BTP3C        : ATGAAGaTTCTAGCTTGCTAGAGTtGGATTAGTGGCGCACGGGTGAGTAA
BTP4C        : ATGAAGATTCTAGCTTGCTAGAGTTGGATTAGTGGCGCACGGGTGAGTAA
BTP5C        : ATGAAGATTCTAGCTTGCTAGAGTTGGATTAGTGGCGCACGGGTGAGTAA
BTP6C        : ATGAAGATTCTAGCTTGCTAGAGTTGGATTAGTGGCGCACGGGTGAGTAA
BTP7C        : ATGAAGATTCTAGCTTGCTAGAGTTGGATTAGTGGCGCACGGGTGAGTAA
BTP8C        : ATGAAGATTCTAGCTTGCTAGAGTTGGATTAGTGGCGCACGGGTGAGTAA
BTP9C        : ATGAAGATTCTAGCTTGCTAGAGTTGGATTAGTGGCGCACGGGTGAGTAA
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H. kirkbridei

BTP1F : ATGAAACTTCTAGCTTGCTAGAAGTGGATTAGTGGCGCACGGGTGAGTAA
BTP2F : ATGAAGCTTCTAGCTTGCTAGAAGTGGATTAGTGGCGCACGGGTGAGTAA
BTP3F : ATGAAACTTCTAGCTTGCTAGAAGTGGATTAGTGGCGCACGGGTGAGTAA
BTP4F : ATGAAACTTCTAGCTTGCTAGAAGTGGATTAGTGGCGCACGGGTGAGTAA
BTP5F : ATGAAGCTTCTAGCTTGCTAGAAGTGGATTAGTGGCGCACGGGTGAGTAA
BTP6F : ATGAAGCTTCTAGCTTGCTAGAAGTGGATTAGTGGCGCACGGGTGAGTAA

***Helicobacter* sp.**

BTP1S : ATGAAGATTCTAGCTTGCTAGAGTTGGATTAGTGGCGCACGGGTGAGTAA
BTP2S : ATGAAGATTCTAGCTTGCTAGAATTGGATTAGTGGCGCACGGGTGAGTAA
BTP3S : ATGAAGATTCTAGCTTGCTAGAGTTGGATTAGTGGCGCACGGGTGAGTAA

H. peregrinus

RTP1S : ATGAAGCTTCTAGCTTGCTAGAAGTGGATTAGTGGCGCACGGGTGAGTAA
RTP2S : ATGAAGCTTCTAGCTTGCTAGAAGTGGATTAgTGGCGCACGGGTGAGTAA
RTP3S : ATGAAGCTTCTAGCTTGCTAGAAGTGGATTAGTGGCGCACGGGTGAGTAA
RTP4S : ATGAAGCTTCTAGCTTGCTAGAAGTGGATTAGTGGCGCACGGGTGAGTAA
RTP5S : ATGAAGCTTCTAGCTTGCTAGAAGTGGATTAGTGGCGCACGGGTGAGTAA

* 120 * 140 *

H. vulpecula

BTP1C : TACATAGGTAACATGCCCTTTAGTCTGGAATAGCCACTGGAAACGGTGAT
BTP2C : TACATAGGTAACATGCCCTTTAGTCTGGAATAGCCACTGGAAACGGTGAT
BTP3C : TACATAGGTAACATGCCCTTTAGTCTgGaATAGCCACTGGAAACGGTGAT
BTP4C : TACATAGGTAACATGCCCTTTAGTCTGGAATAGCCACTGGAAACGGTGAT
BTP5C : TACATAGGTAACATGCCCTTTAGTCTGGAATAGCCACTGGAAACGGTGAT
BTP6C : TACATAGGTAACATGCCCTTTAGTCTGGAATAGCCACTGGAAACGGTGAT
BTP7C : TACATAGGTAACATGCCCTTTAGTCTGGAATAGCCACTGGAAACGGTGAT
BTP8C : TACATAGGTAACATGCCCTTTAGTCTGGAATAGCCACTGGAAACGGTGAT
BTP9C : TACATAGGTAACATGCCCTTTAGTCTGGAATAGCCACTGGAAACGGTGAT

H. kirkbridei

BTP1F : TACATAGGTAACATGCCCTTTAGTCTAGGATAGCCACTGGAAACGGTGAT
BTP2F : TACATAGGTAACATGCCCTTTAGTCTAGGATAGCCACTGGAAACGGTGAT
BTP3F : TrCATAGGTAACATGCCCTTTAGTCTAGGATAGCCACTGGAAACGGTGAT
BTP4F : TACATAGGTAACATGCCCTTTAGTCTAGGATAGCCACTGGAAACGGTGAT
BTP5F : TACATAGGTAACATGCCCTTTAGTCTAGGATAGCCACTGGAAACGGTGAT
BTP6F : TACATAGGTAACATGCCCTTTAGTCTAGGATAGCCACTGGAAACGGTGAT

***Helicobacter* sp.**

BTP1S : TGCATAGGTTATGTGCCCTTTAGTCTAGGATAGCCACTGGAAACGGTGAT
BTP2S : CGCaTAGGTTATGTGCCCCATAGTCTAGGATAGCCACTGGAAACGGTGAT
BTP3S : TGCATAGGTTATGTGCCCTTTAGTCTAGGATAGCCACTGGAAACGGTGAT

H. peregrinus

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RTP1S        : TGCATAGGTAACATGCCCTTTAGTCTGGGATAGCCACTGGAAACGGTGAT
RTP2S        : TGCATAGGTAACATGCCCTTTAGTCTGGGATAGCCACTGGAAACGGTGAT
RTP3S        : TGCATAGGTAACATGCCCTTTAGTCTGGGATAGCCACTGGAAACGGTGAT
RTP4S        : TGCATAGGTAACATGCCCTTTAGTCTGGGATAGCCACTGGAAACGGTGAT
RTP5S        : TGCATAGGTAACATGCCCTTTAGTCTGGGATAGCCACTGGAAACGGTGAT
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                      160         *        180         *        200
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H. vulpecula

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BTP1C        : TAATACCGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGTC
BTP2C        : TAATACCGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGTC
BTP3C        : TAATACcGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGTc
BTP4C        : TAATACCGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGCC
BTP5C        : TAATACCGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGCC
BTP6C        : TAATACCGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGCC
BTP7C        : TAATACCGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGTC
BTP8C        : TAATACCGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGTC
BTP9C        : TAATACCGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGCC
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H. kirkbridei

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BTP1F        : TAATACTGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGCC
BTP2F        : TAATACTGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGCC
BTP3F        : TAATACTGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGCC
BTP4F        : TAATACTGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGCC
BTP5F        : TAATACTGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGCC
BTP6F        : TAATACTGGATACTCCCTACGGGGGAAAGAATTTCGCTAAAGGATTGGCC
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Helicobacter sp.

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BTP1S        : TAATACTGGATATTCCCTACGGGGGAAAGAATTTCGCTAAAGGATCAGCC
BTP2S        : TAATACTGGATACTCCCTACGGGGGAAAGAATTTCGCTATGGGATCAGCC
BTP3S        : TAATACTGGATATTCCCTACGGGGGAAAGAATTTCGCTAAAGGATCAGCC
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H. peregrinus

```
RTP1S        : TAATACTAGATACTCCCTACGGGGGAAAGAATTTTGCTAAAGGATTGGCC
RTP2S        : TAATACTAGATACTCCCTACGGGGGAAAGAATTTTGCTAAAGGATTGGCC
RTP3S        : TAATACTAGATACTCCCTACGGGGGAAAGAATTTTGCTAAAGGATTGGCC
RTP4S        : TAATACTAGATACTCCCTACGGGGGAAAGAATTTTGCTAAAGGATTGGCC
RTP5S        : TAATACTAGATACTCCCTACGGGGGAAAGAATTTTGCTAAAGGATTGGCC
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                         *        220         *        240         *
H. vulpecula
BTP1C        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP2C        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP3C        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP4C        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP5C        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP6C        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP7C        : TATGTCCTATCAGCTTGTTGGTGAGGtAAtGGCTTACCAAGGCTATGACG
BTP8C        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP9C        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
H. kirkbridei
BTP1F        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP2F        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP3F        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP4F        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP5F        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP6F        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
Helicobacter sp.
BTP1S        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP2S        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATGACG
BTP3S        : TATGTCCTATCAGCTTGTTGGTGAGGTAATGGCTTACCAAGGCTATgACG
H. peregrinus
RTP1S        : TATGTCCTATCAGTTTGTTGGTGAGGTAATGGCTTACCAAGaCTATGACG
RTP2S        : TATGTCCTATCAGTTTGTTGGTGAGGTAATGGCTTACCAAGACTATGACG
RTP3S        : TATGTCCtATCAGTTTGTTGGTGAGGTAATGGCTTACCAAGACTATgACG
RTP4S        : TATGTCCTATCAGTTTGTTGGTGAGGTAATGGCTTACCAAGACTATGACG
RTP5S        : TATGTCCTATCAGTTTGTTGGTGAGGTAATGGCTTACCAAGACTATGACG


                        260         *        280         *        300
H. vulpecula
BTP1C        : GGTATCCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAGATACGGTC
BTP2C        : GGTATCCGGCCTgAGAGGGTGAACGGACACACTGGAACTGAGATACGGTC
BTP3C        : GGTATCCGGCCTGAGAGGGTGAaCGGACACACTGGAACTGAGAtACGGTC
BTP4C        : GGTATCCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAGATACGGTC
BTP5C        : GGTATCCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAGATACGGTC
BTP6C        : GGTATCCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAGATACGGTC
BTP7C        : GGTATCCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAGATACGGTC
BTP8C        : GGTATCCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAGATaCGGTC
BTP9C        : GGTATCCGGCCTGAGAGGGTGAACGGACACACtGGAACTGAGATACGGTC
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H. kirkbridei

BTP1F : GGTATCCGGCCTGAGAGGGTGATCGGACACACTGGAACTGAGACACGGTC
BTP2F : GGTATCCGGCCTGAGAGGGTGACCGGACACACTGGAACTGAGACACGGTC
BTP3F : GGTATCCGGCCTGAGAGGGTGATCGGACACACTGGAACTGAGACACGGTC
BTP4F : GGTATCCGGCCTGAGAGGGTGATCGGACACACTGGAACTGAGACACGGTC
BTP5F : GGTATCCGGCCTGAGAGGGTGACCGGACACACTGGAACTGAGACACGGTC
BTP6F : GGTATCCGGCCTGAGAGGGTGACCGGACACACTGGAACTGAGACACGGTC

Helicobacter sp.

BTP1S : GGTATCCGGCCTGAGAGGGTGATCGGACACACTGGAACTGAGACACGGTC
BTP2S : GGTATCCGGCCTGAGAGGGTGATCGGACACACTGGAACTGAGACACGGTC
BTP3S : GGTATCCGGCCTGAGAGGGTGATCGGACACACTGGAACTGAGACACGGTC

H. peregrinus

RTP1S : GGTATCCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAGACACGGTC
RTP2S : GGTATCCGGCCTgAGAGGGTGAACGGACACACTGGAACTGAGACACGGTC
RTP3S : GGtATCCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAGACACGGTC
RTP4S : GGTATCCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAgACACGGTC
RTP5S : GGTATCCGGCCTGAGAGGGTGAACGGACACACTGGAACTGAgACACGGTC

* 320 * 340 *

H. vulpecula

BTP1C : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP2C : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP3C : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP4C : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP5C : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP6C : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP7C : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP8C : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP9C : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG

H. kirkbridei

BTP1F : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP2F : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP3F : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP4F : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP5F : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG
BTP6F : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGCGAAAG

Helicobacter sp.

BTP1S : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGGGAAAC
BTP2S : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGGGAAAC
BTP3S : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGGGAAAC

H. peregrinus

```
RTP1S        : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGGGAAAC
RTP2S        : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGGGAAAC
RTP3S        : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGGGAAAC
RTP4S        : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGGGAAAC
RTP5S        : CAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCTCAATGGGGGAAAC
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                       360         *        380         *        400
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H. vulpecula

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BTP1C        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP2C        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP3C        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP4C        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP5C        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP6C        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP7C        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP8C        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP9C        : CCTGAAGCAGCAACGCCGcGTGGAGGATGAAGGTTTTAGGATTGTAAACT
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H. kirkbridei

```
BTP1F        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP2F        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP3F        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP4F        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP5F        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP6F        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
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Helicobacter sp.

```
BTP1S        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP2S        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
BTP3S        : CCTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
```

H. peregrinus

```
RTP1S        : CCTGAAGCAGCAACACCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
RTP2S        : CCTGAAGCAGCAACACCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
RTP3S        : CCTGAAGCAGCAACACCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
RTP4S        : CCTGAAGCAGCAACACCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
RTP5S        : CCTGAAGCAGCAACACCGCGTGGAGGATGAAGGTTTTAGGATTGTAAACT
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                        *        420         *        440         *
H. vulpecula
BTP1C        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
BTP2C        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
BTP3C        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
BTP4C        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
BTP5C        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
BTP6C        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
BTP7C        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
BTP8C        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTa
BTP9C        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
H. kirkbridei
BTP1F        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
BTP2F        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
BTP3F        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
BTP4F        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
BTP5F        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
BTP6F        : CCTTTTGTTAGAGAAGATTATGACGGTATCTAACGAATAAGCACCGGCTA
Helicobacter sp.
BTP1S        : CCTTTTATAAGAGAAGATTATGACGGTATCTTATGAATAAGCACCGGCTA
BTP2S        : CCTTTTATAAGAGAAGATTATGACGGTaTcTTATGAATAAGCaCCGGCTA
BTP3S        : CCTTTTATAAGAGAAGATTATGACGGTATCTTATGAATAAGCACCGGCTA
H. peregrinus
RTP1S        : CCTTTTATAAGAGAAGATTATGACGGTATCTTATGAATAAGCACCGGCTA
RTP2S        : CCTTTTATAAGAGAAGATTATGACGGTATCTTATGAATAAGCACCGGCTA
RTP3S        : CCTTTTATAAGAGAAGATTATGACGGTATCTTATGAATAAGCACCGGCTA
RTP4S        : CCTTTTATAAGAGAAGATTATGACGGTATCTTATGAATAAGCACCGGCTA
RTP5S        : CCTTTTATAAGAGAAGATTATGACGGTATCTTATGAATAAGCACCGGCTA


                      460         *        480         *        500
H. vulpecula
BTP1C        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
BTP2C        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
BTP3C        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
BTP4C        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
BTP5C        : ACTCCGTGCcAGCAG-CCGCGGTAATACGgAGGGTGCAAGCGTTACTCGG
BTP6C        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
BTP7C        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
BTP8C        : ACTCCGTGcCAGCAg-CCGCGGTAATACGgAGGGTGCAAGCGTTACTCGG
BTP9C        : ACTCCGTGCCAGCAGcCCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
```

H. kirkbridei

```
BTP1F        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
BTP2F        : ACTCCGTGCCAGCAGcCCGCGGTAATACGGAGGGtGCAAGCGTTACTCGG
BTP3F        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
BTP4F        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
BTP5F        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
BTP6F        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
```

Helicobacter sp.

```
BTP1S        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
BTP2S        : ACTCcGTGCCAGCAG-CCGCGGTAATACGgAGGGTGCAAGCGTTACTCGG
BTP3S        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
```

H. peregrinus

```
RTP1S        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
RTP2S        : ACTCCGTGCCAGCAG-CCgCGGTAATACGGAGGGTGCAAGCGTTACTCGG
RTP3S        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
RTP4S        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
RTP5S        : ACTCCGTGCCAGCAG-CCGCGGTAATACGGAGGGTGCAAGCGTTACTCGG
```

```
                        *        520         *        540         *
```

H. vulpecula

```
BTP1C        : AATCACTGGGCGTAAAGAGCACGTAGGCGGGATAATAAGTCAGATGTGAA
BTP2C        : AATCACTGGGCGTAAAGAGCACGTAGGCGGGATAATAAGTCAGATGTGAA
BTP3C        : AATCACTGGGCGTAAAGAGCaCGTAGgCGGGATAATAAGTCAGATGTGAA
BTP4C        : AATCACTGGGCGTAAAGAGCACGTAGGCGGGATAATAAGTCAGATGTGAA
BTP5C        : AATCACTGGGCGTAAAGAGCACGTAGGCGGGATAATAAGTCAGATGTGAA
BTP6C        : AATCACTGGGCGTAAAGAGCACGTAGGCGGGATAATAAGTCAGATGTGAA
BTP7C        : AATCACTGGGCGTAAAGAGCACGTAGGCGGGATAATAAGTCAGATGTGAA
BTP8C        : AATCACTGGGCGTAAAGAGCACGTAGGCGGGATAATAAGTCAGATGTGAA
BTP9C        : AATCACTGGGCGTAAAGAGCACGTAGGCGGGATAATAAGTCAGATGTGAA
```

H. kirkbridei

```
BTP1F        : AATCACTGGGCGTAAAGAGTGCGTAGGCGGGTTAATAAGTCAGATGTGAA
BTP2F        : AATCaCTGGGCGTAAAGAGTGCGTAGGCGGGtTAATAAGTCAGATGTgAA
BTP3F        : AATCACTGGGCGTAAAGAGTGCGTAGGCGGGTTAATAAGTCAGATGTGAA
BTP4F        : AATCACTGGGCGTAAAGAGTGCGTAgGCGGGTTAATAAGTCAGATGTGAA
BTP5F        : AATCaCTGGGCGTAAAGAGTGCGTAGGCGGGTTAATAAGTCAGATGTGAA
BTP6F        : AATCACTGGGCGTAAAGAGTGCGTAGGCGGGTTAATAAGTCAGATGTGAA
```

Helicobacter sp.

```
BTP1S        : AATCACTGGGCGTAAaGAGCGCGTAGGCGGAATAGTAAGTCAGATGTGAA
BTP2S        : AATCACTGGGCGTAAAGAGCGCGTAGGCGGAATAGTAAGTCAGATGTGAA
BTP3S        : AATCaCTGGGCGTAAAGAGCGCGTAGGCGGAATAGTAAGTCAGATGTGAA
```

H. peregrinus

```
RTP1S        : AATCACTGGGCGTAAAGAGCGCGTAGGCGGAATAATAAGTCAGATGTGAA
RTP2S        : AATCACTGGGCGTAAAGAGCGCGTAGGCGGAATAATAAGTCAGATGTGAA
RTP3S        : AATCACTGGGCGTAAAGAGCGCGTAGGCGGAATAATAAGTCAGATGTGAA
RTP4S        : AATCACTGGGCGTAAAGAGCGCGTAGGCGGAATAATAAGTCAGATGTGAA
RTP5S        : AATCACTGGGCGTAAAGAGCGCGTaGGCGGAATAATAAGTCAGATGTGAA
```

```
                       560         *        580         *        600
```

H. vulpecula

```
BTP1C        : ATCCTATGGCTTAACCATAGAACTGCATTTGAAACTATTATTCTGGAGTA
BTP2C        : ATCCTATGGCTTAACCATAGAACTGCATTTGAAACTATTATTCTAGAGTA
BTP3C        : ATcCTATGGCTTAACCATAGAACTGCATTTGAAACTATTATTCTAGAGTA
BTP4C        : ATCCTATGGCTTAaCCATAGAACTGCATTTGAAACTATTATTCTAGAGTA
BTP5C        : ATCCTATGGCTTAACCATAGAACTGCATTTGAAACTATTATTCTAGAGTA
BTP6C        : ATCCTATGGCTTAACCATAGAACTGCATTTGAAACTATTATTCTAGAGTA
BTP7C        : ATCCTATGGCTTAACCATAGAACTGCATTTGAAACTATTATTcTGgAGTA
BTP8C        : ATCCTATGGCTTAACCATAGAACTGCATTTGAAACTATTATTCTGGAGTA
BTP9C        : ATCCTATGGCTTAACCATAGAACTGCATTTGAAACTATTATTCTAGAGTA
```

H. kirkbridei

```
BTP1F        : ATACTATGGCTTAACTATAGAACTGCATTTGAAACTATTAACCTAGAGTA
BTP2F        : ATACTATGGCTTAACTATAGaACTGCATTTGAAACTATTAACCTAgAgTA
BTP3F        : ATACTATGGCTTAACTATAGAACTGCATTTGaAACTATTAAcCTAgAGTa
BTP4F        : ATACTATGGCTTAaCTATAGaACTGCATTTGAAaCTATTaACCTAGAGTA
BTP5F        : ATACTATGGCTTAACTATAGaACTGCATTTGAAACTATTAaCCTAGAGTA
BTP6F        : ATACTATGGCTTAACTATAGAACTGCATTTGAAACTATTAACCTAGAGTA
```

Helicobacter sp.

```
BTP1S        : ATCCTATAGCTTAACTATAGAATTGCATTTGAAACTATTATTCTAGAGTG
BTP2S        : ATCCTATAGCTTAACTATAGAATTGCATTTGAAACTATTATTCTAGAGTG
BTP3S        : ATCCTATAGCTTAACTATAGAATTGCATTTGAAACTATTATTCTAGAGTG
```

H. peregrinus

```
RTP1S        : ATACTATGGCTTAACCATAGAACTGCATTTGAAACTGTTATTCTAGAGTG
RTP2S        : ATACTATGGCTTAACCATAGAACTGCATTTGAAACTGTTATTCTAGAGTG
RTP3S        : ATACTATGGCTTAaCCATAGAaCTGCATTTGAAaCTGTTATTCTAGAGTG
RTP4S        : ATACTATGGCTTAACCAtAgAaCTGCATTTGAAACTGTTATTCTAGAgTG
RTP5S        : ATACTATgGCTTAACCATAGAACTGCATTTGAAACTGTTATTCTAGAGTG
```

```
                         *        620         *        640         *
H. vulpecula
BTP1C        : TGGGAGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP2C        : TGGGAGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP3C        : TgGGAGAGGTAgGTGGAATtCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP4C        : TGGgAGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP5C        : TGGGAGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP6C        : TGGgAgAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP7C        : TGGgAgAGGTAGGTGgAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP8C        : TGGGAGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP9C        : TGGGAGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
H. kirkbridei
BTP1F        : TGGGAGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP2F        : TGGGaGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP3F        : TGGGAGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP4F        : TgGGAgAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP5F        : TGGGAGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP6F        : TGGGAGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
Helicobacter sp.
BTP1S        : TGGGAGAGGTAAGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
BTP2S        : TGGGAGAGGTAAGTGGAATTCTTGGTGTAGGGGTAAAATCCgTAGAGATC
BTP3S        : TGGGAGAGGTAAGTGGAATTCTTGGTGTAgGGGTAAAATcCGTAGAGATC
H. peregrinus
RTP1S        : TGGGAGAGGTAAGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
RTP2S        : TGGGAGAGGTAAGTGGAATTCTTGGTGTAgGGGTAAAATCCGTAgAGATC
RTP3S        : TGGGAGAGGTAAGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
RTP4S        : TGGgAgAgGTAAgTgGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC
RTP5S        : TGGGAGAGGTAAGTGGAATTCTTGGTGTAGGGGTAAAATCCGTAGAGATC


                        660         *        680         *        700
H. vulpecula
BTP1C        : AAGAGGAATACTCATTGCGAAGGCGACTTACTGGAACATTACTGACGCTA
BTP2C        : AAGAGGAATACTCATTGCGAAGGCGACTTACTGGAACATTACTGACGCTA
BTP3C        : AAGAGGAaTACTCATTGCGAAGGCGACtTACTGGAACATTACTGACGCTa
BTP4C        : AAGAGGAAtACTCATTGCGAAGGCGACTTACTGGAACATTACTGACGCTA
BTP5C        : AAGAGGAAtACTCATTGCGAAGGCGACTTACTGGAACATTACTGACGCTA
BTP6C        : AAGAGGAATACTCATTGCGAAGGCGACTTACTGGAACATTACTGaCGCTA
BTP7C        : AAGAGgAATACTCATTGCGAAGGCGACTTACTGGAACATTACTGACGCTA
BTP8C        : AAGAGGAATACTCATTGCGAAGGCGACTTACTGGAACATTACTGACGCTA
BTP9C        : AAGAGGAATACTCATTGCGAAGGCGACTTACTGGAACATTACTGACGCTA
```

H. kirkbridei

BTP1F : AAGAGGAATACTCATTGCGAAAGCGACCTACTGGAACATTACTGACGCTC
BTP2F : AAGAGGAATACTCATTGCGAAAGCGACCTACTGGAACATTACTGACGCTC
BTP3F : AAGAGGAATACTCATTGCGAAAGCGACCTACTGGAACATTACTGACGCTC
BTP4F : AAGAGGAATACTCATTGCGAAAGCGACCTACTGGAACATTACTGACGCTC
BTP5F : AAGAGGAATACTCATTGCGAAAGCGACCTACTGGAACATTACTGACGCTC
BTP6F : AAGAGGAATACTCATTGCGAAAGCGACCTACTGGAACATTACTGACGCTC

Helicobacter sp.

BTP1S : AAGAGGAATACTCATTGCGAAGGCGACTTACTGGAACATTACTGACGCTG
BTP2S : AAGAGGAATACTCATTGCGAAgGCGACTTACTGGAACATTACTGaCgCTG
BTP3S : AAGAGGAATACTCATTGCGAAGGCGACTTACTGGAACATTACTGACGCTG

H. peregrinus

RTP1S : AAGAGGAATACTCATTGCGAAGGCGACTTACTGgAACATTACTGACGCTg
RTP2S : AAGAGGAATACTCATTGCGAAgGCGACTTACTGGAACATTAcTGACGCTG
RTP3S : AAGAGGAATACTCATTGCGAAGGCGACTTACTGGAACATTACTGACGCTG
RTP4S : AAGAGGAATACTCATTGCGAAGGCGACTTACTGGAACATTACTGACGCTG
RTP5S : AAGAGGAATACTCATTGCGAAGGCGACTTACTGGAACATTACTGACGCTG

* 720 * 740 *

H. vulpecula

BTP1C : ATGTGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAT
BTP2C : ATGTGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAT
BTP3C : ATGtGCGAAAGCATGGgGAGCAAACAGGATTAGATACCCTGGTAGTCCAT
BTP4C : ATGTGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAT
BTP5C : ATGTGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAT
BTP6C : ATGTGCGAAAGCATGGGGAGCAAACAGGATTAGATaCCcTGGTAGTCCAT
BTP7C : ATGTGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAT
BTP8C : ATGTGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAT
BTP9C : ATGTGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAT

H. kirkbridei

BTP1F : ATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
BTP2F : ATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
BTP3F : ATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
BTP4F : ATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
BTP5F : ATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
BTP6F : ATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC

Helicobacter sp.

BTP1S : ATGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
BTP2S : ATGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
BTP3S : ATGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGtCCAC

H. peregrinus

```
RTP1S        : ATGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
RTP2S        : ATGTGCGAAAGCGTGGGgAGCAAACAGgATTAGATACCcTGGTAGTCCAC
RTP3S        : ATGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
RTP4S        : ATGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
RTP5S        : ATGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
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                       760         *        780         *        800
```

H. vulpecula

```
BTP1C        : GCCCTAAACGATGAATGCTAGTTGTTGCCCTGCTTGTCAGGGCAGTAATG
BTP2C        : GCCCTAAACGATGAATGCTAGTTGTTGCCCTGCTTGTCAGGGCAGTAATG
BTP3C        : GCCCTAAACGATGAATGCTAGTTGTTGCCCTGCTTGTCagGGCAGTAATG
BTP4C        : GCCCTAAACGATGAATGCTAGTTGTTGCCCTGCTTGTCAGGgCAGTAATG
BTP5C        : GCCCTAAACGATGAATGCTAGTTGTTGCCCTGCTTGTCAGGGCAGTAATG
BTP6C        : GCCcTAAaCGATgAATGCTAGTTGTTGCCcTGCTTGTCAgGGCAGTAATG
BTP7C        : GCCCTAAACGATGAaTGCTAGTtGTtGCCCTGCTtGTCAGGgCAGTAATg
BTP8C        : GCCcTAAaCGATGAATGCTAGTTGTTGCCCTGCTTGTCAGGgCAGTAATG
BTP9C        : GcCCTAAACGATGAATGCTAGtTGtTGCCCTGCtTGTCAgGGCAGTAaTG
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H. kirkbridei

```
BTP1F        : GCCCTAAACGATGAATGCTAGTTGTTGTGAGGCTTGTCCTTGCAGTAATG
BTP2F        : GCCCTAAACGATGAATGCTAGTTGTTGTGAGGCTTGTCCTTGCAGTAATG
BTP3F        : GCCCTAAACGATGAATGCTAGTTGTTGtgAGGCTTGTCCTTGCAGTAATG
BTP4F        : GCCCTAAACGATGAATGCTAGTTGTTGTGAGGCTTGTCCTTGCAGTAATG
BTP5F        : GCCCTAAACGATGAATGCTAGTTGTTGTGAGGCTTGTCCTTGCAGTAATG
BTP6F        : GCCCTAAACGATGAATGCTAGTTGTTGTGAGGCTTGTcCTTGCAGTAATG
```

Helicobacter sp.

```
BTP1S        : GCCCTAAACGATGAATGCTAGTTGTTGCTCTGCTTGACAGAGCAGTAATG
BTP2S        : GCCCTAAACGATGAATGCTAGTTGTTGCTCTGCTTGACAGAGCAGTAATG
BTP3S        : GCCCTAAaCGATGAATGCTAGTTGTTGCTCTGCTTGACAGAGCAGTAATG
```

H. peregrinus

```
RTP1S        : GCCCTAAACTATGGATGCTAGTTGTTGCTCTGCTTGTCAGGGCAGTAATG
RTP2S        : GCCcTAAACTATGgATGCTAGTTGTTGCTCTGCTTGTCAGGGCAGTAATG
RTP3S        : GCCCTAAaCTATGGATGCTAGTTGTTGCTCTGCTTGTCAgGGCAGTAATG
RTP4S        : GCCCTAAACTATGGATGCTAGTTGTTGCTCTGCTTGTCAGGGCAGTAATG
RTP5S        : GCCCTAAACTATGGATGCTAGTTGTTGCTCTGCTTGTCAGGGCAGTAATG
```

```
                    *       820         *       840         *
H. vulpecula
BTP1C        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAA
BTP2C        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAA
BTP3C        : CAGCTAACGCATTAAGCATTCCGCcTGGGgAGtACGGTCGCAAGATTAAA
BTP4C        : CAGCTaACGCATTAAGCATTCCGCcTGGGGAGtaCGGTCGCAAGATTAAA
BTP5C        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAA
BTP6C        : CAGCTAaCGCATTAAGCATTCCGCcTGGGgAGtACGGTCGCAAGATTAAA
BTP7C        : CAGCTAACGCATTAaGCATtCCGCcTGGGgAGTACGGTCGCAAGATTAAA
BTP8C        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAA
BTP9C        : CAGCTaACGCATTAAGCATTCCGCCTgGGGAGTACGGTCGCAAGATTaAA
H. kirkbridei
BTP1F        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTcGCAAGATTAAA
BTP2F        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAA
BTP3F        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGtACGGTCGCAAGATTAAA
BTP4F        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAA
BTP5F        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAA
BTP6F        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAA
Helicobacter sp.
BTP1S        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAA
BTP2S        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAA
BTP3S        : CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAA
H. peregrinus
RTP1S        : TAGCTAACGCATTAAGCATCCCGCCTGGGGAGTACGGTCGCAAGATTAAA
RTP2S        : TAGCTAACGCATTAAGCATCCCGCCTGGGGAGTACGGtCGCAAGATTAAA
RTP3S        : TAGCTAACGCATTAAGCATCCCGCcTGGGGAGTACGGTCGCAAGATTAAA
RTP4S        : TAGCTAACGCATTAAGCATCCCGCCTGGGGAGTACGGTCGCAAGATTAAA
RTP5S        : TAGCTAACGCATTAAGCATCCCGCCTGGGGAGTACGGTCGCAAGATTAAA


                    860         *       880         *       900
H. vulpecula
BTP1C        : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
BTP2C        : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
BTP3C        : aCTCAAAGgAATAGaCGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
BTP4C        : ACTCAAAGgAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
BTP5C        : ACTCAAAGGAATAGACGGGGACCCGCACAAgCGGTGGAGCATGTGGTTTA
BTP6C        : ACTCAAAGGAAtAGACgGGGACCCGCACAAgCGGTGGAGCATGTGGTTTA
BTP7C        : aCTCAAAGgAATAGaCGGGGaCCCGCACAAGCGGTGGAGCATGTGGTTTA
BTP8C        : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
BTP9C        : ACTCAAAgGAATAgACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
```

H. kirkbridei

BTP1F : ACTCAAAGGAATAgACGGGGACCCGCaCaAgCGGTGGAGCATGTGGTTTA
BTP2F : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
BTP3F : ACTCaAAGGAATAgACGGGGaCCCGCaCAAgCGGTGGAGCATGTGGTTTA
BTP4F : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTgGAGCATGTGGTTTA
BTP5F : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
BTP6F : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA

Helicobacter sp.

BTP1S : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
BTP2S : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
BTP3S : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA

H. peregrinus

RTP1S : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
RTP2S : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
RTP3S : ACTCAAAGgAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
RTP4S : ACTCAAAGGAATAGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTA
RTP5S : ACTCAAAGGAATAGAcGGGGACCCGCaCAAGCGGTGgAGCATGTGGTTTA

* 920 * 940 *

H. vulpecula

BTP1C : A-TTCGAAGATACGCGAAGAACCTTACCTAGGCTTGACATTGATAGAATC
BTP2C : A-TTCGAGGATACGCGAAGAACCTTACCTAGGCTTGACATTGATAGAATC
BTP3C : A-TTCGAAGATACGCGAAGAaCCTTACCTAGGCTTGACATTGATAGAATC
BTP4C : A-TTCGAAGATACGCGAAGAACCTTACCTAGGCtTGACATTGATAGAATC
BTP5C : A-TTCGAAGATACGCGAAGAACCTTACCTAGGCTtGACATTGATAGAATC
BTP6C : A-TTCGAAGATACGCGAAGAACCTTACCTAGGCTTGaCATTGATAGAATC
BTP7C : A-TTCGAAGATACGCGAAGAACCTTACCTAGGCTTGACATTGATAGAATC
BTP8C : A-TTCGAAGATACGCGAAGAACCTTACCTAGGCTTGACATTGATAGAATC
BTP9C : A-TTCGAAGATACGCGAAGAACCTTACCTAGGCTTGACATTGATAGAATC

H. kirkbridei

BTP1F : A-TTcGAAgATACGCGAAGAACCTTACCTAGGCTTGACATTGATAGAATc
BTP2F : A-TTCGAAGATACGCGAAGAaCCTtACCTAGGCTTGACATTGATAGAATC
BTP3F : A-TTCGAAGAtACGCGAAGAACCTTACCTAGGCTtGACATTGATAGAATC
BTP4F : A-TTCGAAGATACGCGAAGAACCTTACCTAGGCTTGACATTGATAGAATC
BTP5F : A-TTCGAAGATACGCGAAGAaCCTTACCTAGGCTTGACATTGATAGAATC
BTP6F : A-TTCGAAGATACGCGAAGAACCTTACCTAGGCTTGACATTGATAGAATC

Helicobacter sp.

BTP1S : A-TTCGAAGATACGCGAAGAACCTTACCTAGTCTTGACATTGATAGAATC
BTP2S : A-TTCGAAGATACGCGAAGAACCTTACCTAGTCTTGACATTGATAGAATC
BTP3S : A-TTCGAAGATACGCGAAGAACCTTACCTAGTCTTGACATTGATAGAATC

H. peregrinus

```
RTP1S        : ATTtCGAAGATACGCGAAGAaCCTTACCTAGGCTTGACATTGATAGAATT
RTP2S        : A-TTCGAAGATACGCGAAGAACCTTACCTAGGCTTGACATTGATAGAATT
RTP3S        : A-TTCGAAGATACGCGAAGAaCCTTACCTAGGCTTGACATTGATAGAATT
RTP4S        : A-TTCGAAGATACGCGAAGAACCTTACCTAGGCTTGACATTGATAGAATT
RTP5S        : A-TTCGAAGATACGCGAAGAACCTTACCTAGGCTTGACATTGATAGAATT
```

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                       960          *        980          *       1000
```

H. vulpecula

```
BTP1C        : TGCTAGAGATAGC-AGAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
BTP2C        : TGCTAGAGATAGC-AGAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
BTP3C        : TGCTAGAGATAGC-AGAGTGCCACGtAAGTGGAGCTTGAAAACAGGTGCT
BTP4C        : TGCTAGAGATAGC-AgAGCGCCACGCAAGTGGAGCTTGAAAACaGGTGCT
BTP5C        : TGCTAGAGATAGC-AGAGCGCCACGCAAGTGGAGCTTGAAAACaGGTGCT
BTP6C        : TGCTAGAgATAGC-AGAGCGCCACGCAAGTGGAGCTTGAAAACAGGTgCT
BTP7C        : TGCTAGAGATAGC-AGAGTGCCACGCAAGTGGAGCTTGAAAACaGGTGCT
BTP8C        : TGCTAGAGATAGC-AGAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
BTP9C        : TGCTAGAGATAGC-AGAGCGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
```

H. kirkbridei

```
BTP1F        : TGCTAgAGATAgC-GGAGTGCCACGCAAGTGGAGCTTgAAAACAGGTGCT
BTP2F        : TGCTAGAGATAGC-GGAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
BTP3F        : TGCTAgAGATAgC-GGAGtGCCACGCAAGTGGAGCTTgAAAACAGGTGCT
BTP4F        : TGCTAGAGATAGC-GGAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
BTP5F        : TGCTAGAGATAGC-GGAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
BTP6F        : TGCTAGAGATAGC-GGAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
```

Helicobacter sp.

```
BTP1S        : TGCTAGAGATAGC-GGAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
BTP2S        : TGCTAGAGATAGC-GGAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
BTP3S        : TGCTAGAGATAGC-GGAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
```

H. peregrinus

```
RTP1S        : TGTTAGAGATAGCAAAaGTGCCACGCAAGTGGAGCTTGAAAaCAGGTgCT
RTP2S        : TGTTAGAGATAGC-AAAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
RTP3S        : TGTTAGAGATAGC-AAAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
RTP4S        : TGTTAGAGATAGC-AAAGTGCCACGCAAGTGGAGCTTGAAAACAGGTGCT
RTP5S        : TGTtAGAGATAGC-AAAGTGCCACgCAaGTGGAGCTTGAAAACAGGTGCT
```

```
                        *      1020         *      1040         *
H. vulpecula
BTP1C        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
BTP2C        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
BTP3C        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
BTP4C        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
BTP5C        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTcCCGCA
BTP6C        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
BTP7C        : GCaC-GGCTGTCGtCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
BTP8C        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
BTP9C        : GCAC-GGCTGTCGtCAGCTCGTGTCGTGAGATGTTGGGTTAAGTcCCGCA
H. kirkbridei
BTP1F        : GCaC-GGCTGTCGTCAGCTCGTGTCGTgAGATGTTGGGTTAAGTCCCGCA
BTP2F        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
BTP3F        : GCAC-GGCTGTCGTCAGCtCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
BTP4F        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
BTP5F        : GCAC-GGCTGTCGTCAGCTCGTGTCGTgAgATGTTgGGTTAAGTCCCGCA
BTP6F        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
Helicobacter sp.
BTP1S        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
BTP2S        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
BTP3S        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTcCCGCA
H. peregrinus
RTP1S        : GCAC-GGcTGTCGTCAGCTCGTGTCGTgAGATGTTGGGTTAAGTCCCGCA
RTP2S        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
RTP3S        : GCAC-GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
RTP4S        : GCaC-GgcTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
RTP5S        : GCACGGgCTGTCGTCAGCTCGTGTCGTGAGATGTtGggTtAaGtCCCGCA


                      1060         *      1080         *      1100
H. vulpecula
BTP1C        : ACGAGCGCAACCCTTGTCCTTAGTTGCTAACAGTTAG--CTGAGCACTCT
BTP2C        : ACGAGCGCAACCCTTGTCCTTAGTTGCTAACAGTTAG--CTGAGCACTCT
BTP3C        : ACGAGCGCAACCCTTGTCCTTAGtTGCTaACAGTTAG--CTGAGCACTCT
BTP4C        : ACGAGCGCAACCCTTGTcCTTAGTTGCTAACAGTTAG--CTGaGcACTCT
BTP5C        : ACGAGCGCAACCCTTGTCCTTAGTTGCTAACAGTTAG--CTGAGCACTCT
BTP6C        : aCGAGCGCAACCCTTGTCCTTAGTTGCTAACAGTTAG--CTGAGCaCTCT
BTP7C        : ACGAGCGCAACCCTTGTCCTTAGtTGCTAACAGtTAG--CTGAGCACTCT
BTP8C        : ACGAGCGCAACCCTTGTCCTTAGTTGCTAACAGTTAG--CTGAGCACTCT
BTP9C        : ACGAGCGCAACCCTTGTCCTTAGTTGCTaACAGTTAG--CTGAGCACTCT
```

H. kirkbridei

```
BTP1F        : ACGAGCGCAACCCTCGTCCTTAGTTGCTAACAGTTCGGCTTGAGCACTCT
BTP2F        : ACGAGCGCAACCCTCGTCCTTAGTTGCTaACAGTTCGGCTTGAGCACTCT
BTP3F        : ACGAGCGCAACCCTCGtCCTTAGTTGCTAACAGtTCGGCTTGAGCACTCT
BTP4F        : ACGAGCGCAACCCTcGTCCTTAGTTGCTaACAGTTCGGCTTGAGCACTCT
BTP5F        : ACGAGCGCAACCCTcGTCCTTAGtTGCTaACAGTTCGGCTTGAGCACTCT
BTP6F        : aCGAGCGCAACCCTCGTCCTTAGTTGCTAACAGTTCGGCTTGAGCACTCT
```

Helicobacter sp.

```
BTP1S        : ACGAGCGCAACCCTCGTCATTAGTTGCTAGCAGTTCGGC-TGAGCACTCT
BTP2S        : ACGAGCGCAACCCTCGTCATTAGTTGCTAGCAGTTCGGC-TGAGCACTCT
BTP3S        : ACGAGCGCAACCCTcGTCATTAGtTGCTAGCAGTTCGGC-TGAGCACTcT
```

H. peregrinus

```
RTP1S        : ACGAGCGCAACCCTCGTCCTTAGTTgcTAGCAGTTCGGC-TGAGCACTCT
RTP2S        : ACGAGCGCAACCCTCGTCCTTAGTTGCTAGCAGTTCGGC-TGAGCaCTCT
RTP3S        : ACGAGCGCAACCCTCGTCCTTAGtTGCTAGCAGTTCGGC-TGAGCACTCT
RTP4S        : ACGAGCGCAACCCTCGTCCTTAGTTGCTAGCAGTTCGGC-TGAGCACTCT
RTP5S        : ACGAGCGCAACCCTCGTCCTTAGTTGCTAGCAGTTCGGC-TGAGCACTCT
```

```
                        *      1120         *      1140         *
```

H. vulpecula

```
BTP1C        : AAGGAGACTGTCTTCGTAAGAAGAAGGAAGGTGAGGA-CGACGTCAAGTC
BTP2C        : AAGGAGACTGTCTTCGTAAGAAGAAGGAAGGTGAGGA-CGACGTCAAGTC
BTP3C        : AAGGAGACTGTCTTCGTAAGAAGAAGGAAGGTGAGGA-CGACGTCAAGTC
BTP4C        : AAGGAGACTGTCTTCGTAAGAAGAAGGAAGGTGAGGA-CGACGTcAAGTC
BTP5C        : AAGGAGACTGTCTTCGTAAGAAGAAGGAAGGTGAGGA-CGACGTCAAGTC
BTP6C        : AAGGAGACTGTCTTCgTAAGAAGAAgGAAgGTGAGGA-CGACgTCAAGTC
BTP7C        : AAGGAGACtGTCTTCGTAAGAAGAAGGAAGGTGAGGA-CGACGTCAAGTC
BTP8C        : AAGGAGACTGTCTTCgTAAGAAGAAGGAAgGTGAGGA-CgACGTCAAGTC
BTP9C        : AAGGAGACTGTCTTCGTAAGAAGAAGGAAGGTGAGGA-CGACGTCAAGTc
```

H. kirkbridei

```
BTP1F        : AAGGAGACTGCTTCTGTAAGGAAGAGGAAGGTGAGGA-CGACGTCAAGTC
BTP2F        : AAGGAGACTGCTTCTGTAAGGAAGAGGAAGGTGAGGA-CGACGTCAAGTC
BTP3F        : AAGGAGACTGCTTCTGTAAGGAAGAGGAAGGTGAGGA-CgACgTCAAGTC
BTP4F        : AAGGAGACTGCTTCTGTAAGGAAGAGGAAGGTGAGGA-CGACGTCAAGTC
BTP5F        : AAGGAGACTGCTTCTGTAAGGAAGAGGAAGGTGAGGAcCgACGTCAAGTC
BTP6F        : AAGGAGACTGCTTCTGTAAGGAAGAGGAAGGTGAGGA-CGACGTCAAGTC
```

Helicobacter sp.

```
BTP1S        : AATGAGACTGCCTTCGTAAGGAGGAGGAAGGTGAGGA-CGACGTCAAGTC
BTP2S        : AATGAGACTGCCTTCGTAAGGAGGAGGAAGGTGAGGA-CGACGTCAAGTC
BTP3S        : AATGAGACTGCCTTCgTAaGGAGGAGGAAGGTGAGGA-CgACgTCAAGTC
```

H. peregrinus

```
RTP1S        : AAGGAGACTGCTTCTGTAAGGAAGAGgAAGGTGAGga-CGACGTCAAGTC
RTP2S        : AAGGAGACTGCTTCTGTAAGGAAGAGGAAGGTGAGGA-CGACGTCAAGTC
RTP3S        : AAGGAGACTGCTTCTGTAAGGAAGAGGAAGGTGAGGA-CGACGTCAAGTC
RTP4S        : AAGGAGACTGCTTCTGTAAGGAAGAGGAAGGTGAGGA-CGACGTCAAGTC
RTP5S        : AAGGAGACTGCTTCTGTAAGGAAGAGGAAGGTGAGGA-CGACGTCAAGTC
```

```
                     1160         *      1180         *      1200
```

H. vulpecula

```
BTP1C        : ATCATGGCCCTTACGCCTAgGGCTACACACGTGCTACAATGGGGTGTACA
BTP2C        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGGTGTACA
BTP3C        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGGTGtACA
BTP4C        : ATCATGGcCCTTACGCCTAGGGCTACACACGTGCTACAATGGGGTGTACA
BTP5C        : ATcaTGGcCCTTACGCCTAGGGCTAcACACGTGCTACAATGGGGTGTACA
BTP6C        : ATCATGGcCCTTACGCCTAGGGCTACACACGTGCTACAATGGGGTGTACA
BTP7C        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGGTGTACA
BTP8C        : ATCATGGcCCTTACgcCTAGGGCTACACACGTGCTACAATGGGGTGTACA
BTP9C        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGGTGTACA
```

H. kirkbridei

```
BTP1F        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGATACACA
BTP2F        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGATACACA
BTP3F        : ATcATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGATaCACA
BTP4F        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGATACACA
BTP5F        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGATACACA
BTP6F        : ATCATGGCCCTTACGCCTAGGGCTaCACACGTGCTACAATGGGATACACA
```

Helicobacter sp.

```
BTP1S        : ATCATGGCCCTTACGACTAGGGCTACACACGTGCTACAATGGGGTGCACA
BTP2S        : ATCATGGCCCTTACGACTAGGGCTACACACGTGCTACAATGGGATGCACA
BTP3S        : ATcATGGcCCTTACGACTAGGGCTACACACGTGCTACAATGGGGTGCACA
```

H. peregrinus

```
RTP1S        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGATATACA
RTP2S        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGATATACA
RTP3S        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGATATACA
RTP4S        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGATATACA
RTP5S        : ATCATGGCCCTTACGCCTAGGGCTACACACGTGCTACAATGGGATATACA
```

```
                        *      1220         *      1240         *
H. vulpecula
BTP1C        : ACGAGAAGCAATATCGTAAGATGGAGCAAATCTCCAAAACATCTCTCAGT
BTP2C        : ACGAGAAGCAATATCGTGAGATGGAGCAAATCTCCAAAACATCTCTCAGT
BTP3C        : AcGAGAAGCAATATcGTAAGATGGAGCaAATCTCCAAAACATCTCTCAGT
BTP4C        : ACGAGAAGCAATATCGTAAGATGGAGCAAATCTCCAAAACAtCtCTCAGT
BTP5C        : ACGAGAAGCAATATCGTAAGATGGAGCAAATCTCCAAAACATCTCTCAGT
BTP6C        : ACGAGAAGCAATATCGTAAGATGGAGCAAATCTCCAAAACATCTCTCAGT
BTP7C        : ACGAGAAGCAATATCGTAAGATGGAGCAAATCTCCAAAACATCTCTCAGT
BTP8C        : ACGAGAAGCAATATCGTAAGATGGAGCAAATCTCCAAAACATCTCTCAGT
BTP9C        : ACGAGAAGCAATATCGTAAGATGGAGCAAATCTCCAAAACATCTCTCAGT
H. kirkbridei
BTP1F        : AGGAGATGCAATACTGTGAGGTGGAGCTAATCTTAAAAATATCTCTCAGT
BTP2F        : AGGAGATGCAATACTGTGAGGTGGAGCTAATCTTAAAAATATCTCTCAGT
BTP3F        : AGGaGATGCAATACTGTGAGGtGGAGCTAATcTTAAAAATATCTcTCAGT
BTP4F        : AGGAGATGCAATACTGTGAGGTGGAGCTAATCTTAAAAATATCTCTCAGT
BTP5F        : AGGAGATGCAATACTGTGAGGTGGAGCTAATCTTAAAAATATCTCTCAGT
BTP6F        : AGGAGATGCAATACTGTGAGGTGGAGCTAATCTTAAAAATATCTCTCAGT
Helicobacter sp.
BTP1S        : AAGAGACGCAATACTGCGAAGTGGAGCAAATCTCTAAAACATCTCTCAGT
BTP2S        : AAGAGATGCAATACTGCGAGGTGGAGCAAATCTCTAAAACATCTCTCAGT
BTP3S        : AAGAGATGCAATACTGCGAAGTGGAGCAAATCTCTAAAACATCTCTCAGT
H. peregrinus
RTP1S        : AAGAGATGCAATACTGCGAAGTGGAGCTAATCTCAAAAATATCTCTCAGT
RTP2S        : AAGAGATGCAATACTGCGAAGTGGAGCTAATCTCAAAAATATCTCTCAGT
RTP3S        : AAGAGATGCAATACTGCGAAGTGGAGCTAATCTCAAAAATATCTCTCAGT
RTP4S        : AAGAGATGCAATACTGCGAAGTGGAGCTAATCTCAAAAATATCTCTCAGT
RTP5S        : AAGAGATGCAATACTGCGAAGTGGAGCTAATCTCAAAAATATCTCTCAGT


                      1260         *      1280         *      1300
H. vulpecula
BTP1C        : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP2C        : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP3C        : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP4C        : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP5C        : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP6C        : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP7C        : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP8C        : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP9C        : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
```

H. kirkbridei

BTP1F : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP2F : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP3F : TCGGATTGTAGTCTGCAACTCGACTaCATGAAGCTGGAATCGcTAGTAAT
BTP4F : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP5F : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP6F : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT

Helicobacter sp.

BTP1S : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP2S : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
BTP3S : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT

H. peregrinus

RTP1S : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
RTP2S : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
RTP3S : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
RTP4S : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT
RTP5S : TCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAAT

* 1320 * 1340 *

H. vulpecula

BTP1C : CGCAAATCAGCCATGTTGCGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP2C : CGCAAATCAGCCATGTTGCGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP3C : CGCaAATCAGCCATGTtGCGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP4C : CGCAAATCAGCCATGTTGCGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP5C : CGCAAATCAGCCATGTTGCGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP6C : CGCAAATCAGCCATGTTGCGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP7C : CGCAAATCAGCCATGTTGCGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP8C : CGCAAATCAGCCATGTTGCGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP9C : CGCAAATCAGCCATGTTGCGGTGAATACGTTCCCGGGTCTTGTACTCACC

H. kirkbridei

BTP1F : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP2F : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP3F : CGTGAATCAGCCATGTCACGGTGAATaCGTTCCCGGGTcTTGTACTCACC
BTP4F : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP5F : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP6F : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC

Helicobacter sp.

BTP1S : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP2S : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC
BTP3S : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC

H. peregrinus

```
RTP1S        : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC
RTP2S        : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC
RTP3S        : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC
RTP4S        : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC
RTP5S        : CGTGAATCAGCCATGTCACGGTGAATACGTTCCCGGGTCTTGTACTCACC
```

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                     1360         *      1380         *      1400
```

H. vulpecula

```
BTP1C        : GCCCGTCAAACCATGGGAGTTGTATTCGCCTTAAGCCGGGATGCTAAATT
BTP2C        : GCCCGTCAAACCATGGGAGTTGTATTCGCCTTAAGCCGGGATGCTAAATT
BTP3C        : GCCCGTCAaACCATGGGAGTTGtATTCGCCTTAAGCCGGGATGCTAAATT
BTP4C        : GCCCGTCAAACCATGGGAGTTGTATTCGCCTTAAGCCGGGATGCTAAATT
BTP5C        : GCCCGTCAAACCATGGGAGTTGTATTCGCCTTAAGCCGGGATGCTAAATT
BTP6C        : GCCCGTCAAACCATGGGAGTTGTATTCGCCTTAAGCCGGGATGCTAAATT
BTP7C        : GCCCGTCAAACCATGGGAGTTGTATTCGCCTTAAGCCGGGATGCTAAATT
BTP8C        : GCCCGTCAAACCATGGGAGTTGTATTCGCCTTAAGCCGGGATGCTAAATT
BTP9C        : GCCCGTCAAACCATGGGAGTTGTATTCGCCTTAAGCCGGGATGCTAAATT
```

H. kirkbridei

```
BTP1F        : GCCCGTCACACCATGGGAGTTGTATTCGCCTTAAGTCGGGATACTAAATT
BTP2F        : GCCCGTCACACCATGGGAGTTGTATTCGCCTTAAGTCGGGATACTAAATT
BTP3F        : GCCCGTcACACCATGGGaGTtGTaTTCGCCTTAAGTCGGGATACTAAATT
BTP4F        : GCCCGTCACACCATGGGAGTTGTATTCGCCTTAAGTCGGGATACTAAATT
BTP5F        : GCCCGTCACACCATGGGAGTTGTATTCGCCTTAAGTCGGGATACTAAATT
BTP6F        : GCCCGTCACACCATGGGAGTTGTATTCGCCTTAAGTCGGGATACTAAATT
```

Helicobacter sp.

```
BTP1S        : GCCCGTCACACCATGGGAGTTGTATTTGCCTTAAGTCGGCATGCTAAAAT
BTP2S        : GCCCGTCACACCATGGGAGTTGTATTTGCCTTAAGCCGGGATGCTAAAAT
BTP3S        : GCCCGTCACACCATGGGAGTTGTATTTGCCTTAAGTCGGCATGCTAAAAT
```

H. peregrinus

```
RTP1S        : GCCCGTCACACCATGGGAGTTGTATTCGCCTTAAGCCGGGATGTTAAAAT
RTP2S        : GCCCGTCACACCATGGgAGTTGTATTCGCCTTAAGCCGGGATGTTAAAAT
RTP3S        : GCCCGTCACACCATGGGAGTTGTATTCGCCTTAAGCCGGGATGTTAAAAT
RTP4S        : GCCCGTCACACCATGGGAGTTGTATTCGCCTTAAGCCGGGATGTTAAAAT
RTP5S        : GCCCGTCACACCATGGGAGTTGTATTCGCCTTAAGCCGGGATGTTAAAAT
```

```
                        *      1420         *      1440         *
H. vulpecula
BTP1C        : GGCTACCGTCTACGGTGGGTGCAGCGACTGGGGTTAAGTCGTAACAAGGT
BTP2C        : GGCTACCGTCTACGGTGGGTGCAGCGACtGGGGTTAAGTCGTAACAAGGT
BTP3C        : GGCTACCGTCTACGGTGGGTGCAGCGACTGGGGTTAAGTCGTAACAAGGT
BTP4C        : GGCTACCGTCTACGGTGGGTGCAGCGACTGGGGTTAAGTCGTAA------
BTP5C        : GGCTACCGTCTACGGTGGGTGCAGCGACTGGGGTTAAGTCGTAACAAGGT
BTP6C        : GGCTACCGTCTACGGTGGGTGCAGCGACTGGGGTTAAGTCGTAACAAGGT
BTP7C        : GGCTACCGTCTACGGTGGGTGCAGCGACTGGGGTTAAGTCGTAACAAGG-
BTP8C        : GGCTACCGTCTACGGTGGGTGCAGCGACTGGGGTTAAGTCGTAACAAGGT
BTP9C        : GGCTACCGTCTACGGTGGGTGCAGCGACTGGGGTTAAGTCGTAACAAGGA
H. kirkbridei
BTP1F        : GGTTACTGCCCACGGCGGATGCAGCGACTGGGGTGAAGTCGTAACAAGGT
BTP2F        : GGTTACTGCCCACGGCGGATGCAGCGACTGGGGTGAAGTC----------
BTP3F        : GGTTACTGCCCACGGCGGATGCAGCGACTGGGGTGAAGTCGTA-------
BTP4F        : GGTTACTGCCCACGGCGGATGCAGCGACTGGGGTGAAGTCGTAACAAGGT
BTP5F        : GGTTACTGCCCACGGCGGATGCAGCGACTGGGGTGA--------------
BTP6F        : GGTTACTGCCCACGGCGGATGCAGCGACTGGGGTGAAGTCGTAACAAGGT
Helicobacter sp.
BTP1S        : AGCTACCGCCTACGGCAGATGCAGCGACTGGGGTGAAGTCGTAACAAGGT
BTP2S        : AGCTACCGTCTACGGCGGATGCAGCGACTGGGGTGAAGTCGTAACAAGG-
BTP3S        : AGCTACCGCCTACGGCAGATGCAGCGACTGGGGTGAAGTCGTAAC-----
H. peregrinus
RTP1S        : AACTACTGTCTACGGCGGATACAGCGACTGGGGTGAAGTCGTAACAAGGA
RTP2S        : AACTACTGTCTACGGCGGATACAGCGACTGGGGTGAAGTCGTAACAAGGT
RTP3S        : AACTACTGTCTACGGCGGATACAGCGACTGGGGTGAAGTCGAAGA-----
RTP4S        : AACTACTGTCTACGGCGGATACAGCGACTGGGGTGAAGTCGTAACAAGG-
RTP5S        : AACTACTGTCTACGGCGGATACAGCGACTGGGGTGAAGTCGTAAC-----
```

Appendix 3 Similarity matrices showing percentage sequence similarities for *Helicobacter* species obtained from the brushtail and ringtail possums, and the references strains of *Helicobacter* and related genera used in the phylogenetic reconstruction of the 16S ribosomal RNA gene.

Similarity Matrix Part: 1

	1	2	3	4	5	6	7	8	9	10	11	12
1 *H. acinonychis*	100.0%	96.1%	94.2%	94.4%	91.9%	91.7%	92.6%	92.7%	89.3%	91.9%	90.6%	91.1%
2 *H. pylori*		100.0%	93.8%	94.6%	93.8%	93.4%	94.1%	94.0%	90.6%	93.2%	91.5%	92.3%
3 *H. felis*			100.0%	96.8%	90.2%	90.3%	92.2%	92.1%	89.5%	91.1%	90.1%	90.9%
4 *H. bizzozeronii*				100.0%	91.9%	92.4%	93.7%	93.0%	90.4%	92.6%	91.4%	91.6%
5 *H. mesocricetorum*					100.0%	97.1%	96.0%	95.8%	92.8%	94.8%	94.4%	93.6%
6 *H. rodentium*						100.0%	95.9%	95.3%	92.4%	95.3%	94.5%	93.3%
7 *H. pullorum*							100.0%	96.6%	93.3%	96.2%	94.7%	93.6%
8 *H. pametensis*								100.0%	93.0%	95.7%	94.4%	95.7%
9 *H. fennelliae*									100.0%	92.9%	93.1%	91.4%
10 *H. canis*										100.0%	96.8%	94.7%
11 *H. cinaedi*											100.0%	93.6%
12 *H. mustelae*												100.0%
13 *H. hepaticus*												
14 *H. trogontum*												
15 *H. aurati*												
16 *H. canadensis*												
17 *H. muridarum*												
18 BTP4C												
19 BTP5C												
20 BTP6C												
21 BTP7C												
22 BTP1C												
23 BTP8C												
24 BTP2C												
25 BTP3C												
26 BTP9C												
27 *H.sp rhesus type2*												
28 BTP1F												
29 BTP4F												
30 BTP6F												
31 BTP3F												
32 BTP2F												
33 BTP5F												
34 BTP2S												
35 BTP4S												
36 BTP3S												
37 BTP5S												
38 BTP1S												
39 BTP1S												
40 BTP3S												
41 BTP2S												
42 *W. succinogenesis*												
43 *H. bilis*												
44 *C. jejuni*												
45 *A. butzleri*												

Similarity Matrix Part: 2

	13	14	15	16	17	18	19	20	21	22	23	24
1 *H. acinonychis*	91.9%	91.1%	92.1%	92.3%	91.6%	90.0%	90.0%	90.0%	90.0%	89.7%	90.0%	89.7%
2 *H. pylori*	93.0%	92.3%	93.2%	93.6%	92.4%	91.9%	91.9%	91.9%	91.7%	91.4%	91.9%	91.5%
3 *H. felis*	91.3%	90.8%	90.7%	91.5%	90.9%	89.7%	89.7%	89.8%	89.9%	89.6%	89.9%	89.6%
4 *H. bizzozeronii*	92.8%	92.4%	92.3%	92.9%	91.5%	91.3%	91.3%	91.3%	91.4%	91.4%	91.4%	91.4%
5 *H. mesocricetorum*	94.6%	95.6%	96.3%	95.9%	93.5%	93.6%	93.6%	93.6%	93.5%	93.5%	93.5%	93.6%
6 *H. rodentium*	95.2%	95.3%	95.5%	96.5%	93.9%	93.2%	93.2%	93.2%	93.1%	93.1%	93.1%	93.2%
7 *H. pullorum*	95.8%	96.0%	95.3%	97.9%	94.4%	94.3%	94.3%	94.3%	94.2%	94.2%	94.2%	94.2%
8 *H. pamatensis*	95.8%	95.6%	96.4%	96.5%	95.3%	93.8%	93.8%	93.9%	93.7%	93.4%	93.7%	93.5%
9 *H. fennelliae*	93.2%	93.6%	93.5%	92.6%	92.4%	92.3%	92.3%	92.4%	92.3%	92.0%	92.4%	92.1%
10 *H. ozanis*	97.1%	96.0%	95.6%	96.3%	95.5%	94.1%	94.1%	94.1%	93.9%	93.9%	93.9%	94.0%
11 *H. cinaedi*	96.0%	95.4%	94.4%	94.6%	94.4%	92.5%	92.5%	92.5%	92.3%	92.1%	92.4%	92.3%
12 *H. mustelae*	94.7%	94.9%	94.2%	94.2%	94.0%	92.0%	91.9%	92.0%	91.8%	91.5%	91.9%	91.6%
13 *H. hepaticus*	100.0%	97.0%	96.8%	97.4%	97.0%	94.3%	94.2%	94.3%	94.3%	94.4%	94.3%	94.3%
14 *H. trogontum*		100.0%	96.1%	95.8%	95.6%	94.9%	94.9%	94.9%	94.8%	94.8%	94.8%	94.9%
15 *H. aurati*			100.0%	96.6%	95.5%	93.7%	93.7%	93.8%	93.7%	93.7%	93.7%	93.7%
16 *H. canadensis*				100.0%	95.6%	94.1%	94.0%	94.1%	93.9%	93.3%	93.9%	93.5%
17 *H. muridarum*					100.0%	93.8%	93.7%	93.8%	93.6%	93.5%	93.7%	93.6%
18 BIP4C						100.0%	100.0%	100.0%	99.8%	99.8%	99.8%	99.7%
19 BIP5C							100.0%	100.0%	99.8%	99.8%	99.8%	99.5%
20 BIP6C								100.0%	99.8%	99.8%	99.8%	99.7%
21 BIP7C									100.0%	100.0%	100.0%	99.8%
22 BIP1C										100.0%	100.0%	99.8%
23 BIP8C											100.0%	99.8%
24 BIP2C												100.0%
25 BIP3C												
26 BIP9C												
27 H.sp rhesus type2												
28 BIP1F												
29 BIP4F												
30 BIP6F												
31 BIP3F												
32 BIP2F												
33 BIP5F												
34 BIP2S												
35 BIP4S												
36 BIP3S												
37 BIP5S												
38 BIP1S												
39 BIP1S												
40 BIP3S												
41 BIP2S												
42 *W. succinogenesis*												
43 *H. bilis*												
44 *C. jejuni*												
45 *A. butzleri*												

Similarity Matrix Part: 3

	25	26	27	28	29	30	31	32	33	34	35	36
1 *H. aoinonyobis*	89.9%	89.9%	90.5%	90.1%	90.4%	90.5%	90.1%	90.5%	90.5%	89.8%	90.1%	90.1%
2 *H. pylori*	91.8%	91.8%	92.3%	91.5%	91.8%	91.9%	91.5%	91.9%	91.9%	91.0%	91.3%	91.3%
3 *H. felis*	89.8%	89.7%	90.4%	90.1%	90.5%	90.6%	90.2%	90.5%	90.5%	89.2%	89.6%	89.5%
4 *H. bizzozeronii*	91.4%	91.3%	91.7%	92.2%	92.2%	92.2%	92.2%	92.2%	92.2%	91.2%	91.2%	91.2%
5 *H. mesocricetorum*	93.6%	93.6%	94.5%	93.8%	93.8%	93.9%	93.7%	93.9%	93.9%	92.7%	92.7%	92.7%
6 *H. rodentium*	93.2%	93.2%	94.6%	94.2%	94.2%	94.3%	94.2%	94.3%	94.3%	93.4%	93.4%	93.4%
7 *H. pullorum*	94.2%	94.2%	95.6%	95.2%	95.2%	95.2%	95.1%	95.2%	95.2%	94.0%	93.9%	93.9%
8 *H. pametensis*	93.8%	93.7%	94.3%	94.2%	94.5%	94.6%	94.2%	94.5%	94.5%	93.3%	93.6%	93.5%
9 *H. fennelliae*	92.4%	92.3%	93.4%	91.1%	91.5%	91.6%	91.2%	91.5%	91.5%	90.8%	91.2%	91.1%
10 *H. canis*	94.0%	94.0%	95.8%	94.5%	94.5%	94.5%	94.4%	94.5%	94.5%	94.2%	94.1%	94.1%
11 *H. cinaedi*	92.4%	92.4%	94.2%	92.8%	93.1%	93.1%	92.8%	93.1%	93.1%	91.9%	92.2%	92.1%
12 *H. muridarum*	92.0%	91.9%	93.0%	92.3%	92.6%	92.6%	92.3%	92.7%	92.7%	92.0%	92.4%	92.4%
13 *H. hepaticus*	94.2%	94.2%	95.8%	95.4%	95.3%	95.3%	95.2%	95.2%	95.2%	94.5%	94.5%	94.2%
14 *H. trogontum*	94.9%	94.9%	95.9%	94.4%	94.4%	94.4%	94.4%	94.4%	94.4%	94.0%	94.0%	94.0%
15 *H. aurati*	93.6%	93.7%	95.1%	94.7%	94.6%	94.7%	94.5%	94.7%	94.7%	93.6%	93.6%	93.3%
16 *H. canadensis*	94.0%	94.0%	95.7%	95.3%	95.6%	95.6%	95.7%	95.7%	95.6%	93.9%	94.5%	94.3%
17 *H. muridarum*	93.7%	93.7%	95.0%	94.2%	94.4%	94.5%	94.1%	94.5%	94.5%	92.8%	92.9%	92.9%
18 BTP4C	99.8%	100.0%	97.8%	95.2%	95.2%	95.3%	95.1%	95.3%	95.2%	94.8%	94.8%	94.6%
19 BTP5C	99.7%	99.9%	97.7%	95.1%	95.2%	95.3%	94.9%	95.3%	95.2%	94.7%	94.8%	94.5%
20 BTP6C	99.7%	99.9%	97.9%	95.2%	95.2%	95.3%	95.1%	95.3%	95.2%	94.8%	94.8%	94.5%
21 BTP7C	99.8%	99.7%	97.8%	95.2%	95.2%	95.3%	94.8%	95.2%	95.2%	94.8%	94.7%	94.5%
22 BTP1C	99.7%	99.7%	97.1%	94.9%	95.1%	95.2%	94.8%	95.2%	95.2%	94.8%	94.7%	94.5%
23 BTP8C	99.8%	99.7%	97.8%	95.2%	95.2%	95.3%	94.9%	95.2%	95.2%	94.8%	94.7%	94.5%
24 BTP2C	99.7%	99.6%	97.3%	95.0%	95.2%	95.3%	94.8%	95.3%	95.2%	94.9%	94.8%	94.5%
25 BTP3C	100.0%	99.7%	97.8%	95.0%	95.1%	95.2%	95.0%	95.2%	95.2%	94.6%	94.7%	94.4%
26 BTP9C		100.0%	97.7%	95.1%	95.1%	95.2%	94.8%	95.3%	95.2%	94.7%	94.8%	94.5%
27 H.sp rhesus type2			100.0%	94.8%	95.0%	95.1%	95.0%	95.1%	95.1%	94.6%	95.1%	94.8%
28 BTP1F				100.0%	99.9%	99.8%	99.6%	99.9%	99.9%	95.5%	95.8%	95.5%
29 BTP4F					100.0%	99.9%	99.9%	99.9%	99.9%	95.7%	95.8%	95.5%
30 BTP6F						100.0%	99.6%	100.0%	100.0%	95.8%	95.9%	95.6%
31 BTP8F							100.0%	99.8%	99.8%	95.4%	95.8%	95.6%
32 BTP2F								100.0%	100.0%	95.8%	95.8%	95.8%
33 BTP5F									100.0%	95.8%	95.8%	95.8%
34 RTP2S										100.0%	100.0%	99.7%
35 RTP4S											100.0%	99.7%
36 RTP3S												100.0%
37 RTP5S												
38 RTP1S												
39 BTP1S												
40 BTP3S												
41 BTP2S												
42 *W. succinogenesis*												
43 *H. bilis*												
44 *C. jejuni*												
45 *A. butzleri*												

Similarity Matrix Part: 4

	37	38	39	40	41	42	43	44	45
1 *H. acinonychis*	89.8%	90.1%	90.5%	90.5%	90.3%	89.0%	90.3%	82.5%	83.1%
2 *H. pylori*	91.0%	91.2%	91.6%	91.6%	91.5%	90.2%	91.9%	83.6%	84.3%
3 *H. felis*	89.2%	89.5%	89.2%	89.3%	89.0%	88.3%	89.7%	81.6%	81.8%
4 *H. bizzozeronii*	91.2%	91.2%	91.0%	91.1%	91.0%	88.9%	91.2%	83.1%	82.5%
5 *H. mesocricetorum*	92.7%	92.6%	93.6%	93.7%	93.9%	91.3%	94.4%	85.1%	85.3%
6 *H. rodentium*	93.4%	93.3%	94.3%	94.4%	94.1%	92.6%	94.9%	85.9%	85.6%
7 *H. pullorum*	93.6%	93.9%	94.3%	94.2%	94.2%	91.9%	95.1%	85.4%	85.6%
8 *H. pametensis*	93.2%	93.5%	93.8%	93.7%	94.5%	93.0%	95.3%	85.6%	85.2%
9 *H. fennelliae*	90.9%	91.1%	91.2%	91.2%	91.1%	89.7%	92.9%	83.7%	83.3%
10 *H. canis*	93.8%	94.1%	95.2%	95.2%	95.0%	92.2%	97.6%	85.4%	84.7%
11 *H. cinaedi*	91.8%	92.1%	92.7%	92.7%	92.7%	91.7%	97.1%	84.6%	84.0%
12 *H. mustelae*	92.1%	92.3%	93.1%	93.0%	93.8%	91.9%	94.1%	84.2%	84.7%
13 *H. hepaticus*	94.1%	94.3%	95.5%	95.2%	95.2%	92.2%	96.4%	85.0%	84.8%
14 *H. trogontum*	94.0%	93.9%	94.6%	94.6%	94.9%	92.7%	95.3%	84.4%	85.3%
15 *H. aurati*	93.2%	93.4%	94.8%	94.6%	95.0%	92.5%	95.0%	85.3%	84.6%
16 *H. canadensis*	94.5%	94.3%	95.3%	95.7%	95.3%	92.5%	95.4%	85.5%	85.1%
17 *H. muridarum*	92.6%	92.8%	94.2%	94.0%	94.0%	92.0%	94.6%	84.0%	84.5%
18 BTP4C	94.7%	94.8%	95.1%	95.1%	95.1%	91.7%	93.6%	84.1%	84.4%
19 BTP5C	94.5%	94.7%	95.0%	94.9%	95.1%	91.7%	93.6%	84.2%	84.4%
20 BTP6C	94.5%	94.8%	95.2%	95.1%	95.1%	91.8%	93.6%	84.2%	84.4%
21 BTP7C	94.4%	94.8%	95.1%	94.8%	95.0%	91.6%	93.5%	84.2%	84.3%
22 BTP1C	94.4%	94.6%	95.2%	94.8%	95.1%	91.4%	93.5%	83.9%	84.2%
23 BTP8C	94.4%	94.6%	95.1%	94.9%	95.0%	91.7%	93.5%	84.2%	84.4%
24 BTP2C	94.5%	94.7%	95.3%	94.8%	95.0%	91.5%	93.6%	84.0%	84.4%
25 BTP3C	94.7%	94.5%	95.0%	95.0%	94.9%	91.7%	93.6%	84.2%	84.4%
26 BTP9C	94.4%	94.8%	95.1%	94.8%	95.0%	91.6%	93.5%	84.1%	84.3%
27 *H.sp rhesus type2*	94.8%	95.0%	94.9%	95.1%	95.3%	92.5%	95.1%	84.9%	84.8%
28 BTP1F	95.4%	95.7%	95.0%	95.0%	95.3%	90.7%	94.6%	84.6%	85.0%
29 BTP4F	95.5%	95.7%	95.2%	95.3%	95.2%	91.0%	94.6%	84.8%	85.1%
30 BTP6F	95.5%	95.7%	95.2%	95.1%	95.3%	90.9%	94.6%	84.7%	85.2%
31 BTP3F	95.5%	95.5%	94.9%	95.3%	94.9%	90.7%	94.6%	84.5%	84.9%
32 BTP2F	95.8%	95.8%	95.2%	95.3%	95.2%	90.8%	94.6%	84.6%	85.1%
33 BTP5F	95.8%	95.8%	95.2%	95.2%	95.2%	90.8%	94.6%	84.6%	85.1%
34 RTP2S	99.7%	99.9%	96.1%	95.8%	96.3%	90.5%	93.9%	84.0%	85.1%
35 RTP4S	99.8%	100.0%	96.1%	96.1%	96.2%	90.8%	93.9%	84.2%	85.2%
36 RTP3S	99.6%	99.7%	95.8%	95.8%	95.9%	90.7%	93.9%	84.2%	85.2%
37 RTP5S	100.0%	99.7%	95.7%	95.9%	95.9%	90.4%	93.6%	83.9%	84.9%
38 RTP1S		100.0%	96.0%	95.9%	96.2%	90.7%	93.9%	84.2%	85.3%
39 BTP1S			100.0%	99.6%	99.0%	92.0%	95.1%	84.6%	85.1%
40 BTP3S				100.0%	98.8%	91.9%	95.1%	84.4%	84.9%
41 BTP2S					100.0%	92.4%	95.1%	84.9%	85.1%
42 *W. succinogenesis*						100.0%	92.5%	84.9%	85.4%
43 *H. bilis*							100.0%	85.1%	84.5%
44 *C. jejuni*								100.0%	86.1%
45 *A. butzleri*									100.0%

Appendix 4 Similarity matrices showing percentage sequence similarities for "*Helicobacter kirkbridei*", flexispira taxa 1-10 and the related species used in the phylogenetic reconstruction of the 16S ribosomal RNA gene.

Similarity Matrix Part: 1

	1	2	3	4	5	6	7	8	9	10	11	12
1 LNB1F	100.0%	99.2%	97.5%	96.3%	90.3%	91.7%	89.6%	91.4%	94.6%	94.6%	94.6%	94.6%
2 Flexispira taxon 9		100.0%	96.9%	95.9%	90.7%	92.4%	90.1%	91.7%	94.7%	94.7%	94.7%	94.6%
3 Flexispira taxon 2			100.0%	98.1%	90.8%	92.3%	90.7%	92.1%	95.8%	95.8%	95.7%	95.6%
4 Flexispira taxon 3				100.0%	90.8%	92.3%	90.7%	92.2%	94.9%	94.9%	94.8%	94.9%
5 H.acinonychis					100.0%	96.1%	94.2%	94.4%	90.4%	90.7%	90.8%	90.4%
6 H.pylori						100.0%	93.8%	94.6%	91.8%	92.1%	92.2%	91.8%
7 H.felis							100.0%	96.8%	90.4%	90.8%	90.9%	90.5%
8 H.bizzozeronii								100.0%	92.4%	92.4%	92.5%	92.4%
9 BTP1F									100.0%	99.9%	99.8%	99.6%
10 BTP4F										100.0%	99.9%	99.9%
11 BTP6F											100.0%	99.6%
12 BTP3F												100.0%
13 BTP2F												
14 BTP5F												
15 H.mesocricetorum												
16 H.rodentium												
17 H.pullorum												
18 H.pametensis												
19 H.canadensis												
20 Flexispira taxon 1												
21 Flexispira taxon 5												
22 Flexispira taxon 6												
23 Flexispira taxon 4												
24 H.hepaticus												
25 H.aurati												
26 WB1F												
27 WB2F												
28 LNB2F												
29 Flexispira taxon 8												
30 H.cinaedi												
31 H.canis												
32 Flexispira taxon 7												
33 H.fennelliae												
34 H.muridarum												
35 H.mustelae												
36 W.succinogenes												
37 Flexispira taxon10												
38 C.jejuni												
39 A.butzleri												

Similarity Matrix Part: 2

	13	14	15	16	17	18	19	20	21	22	23	24
1 LN21F	94.6%	94.6%	94.6%	95.1%	95.1%	95.4%	95.9%	95.3%	95.7%	95.6%	97.4%	96.3%
2 Flexispira taxon 9	94.7%	94.7%	94.8%	95.3%	95.5%	95.8%	95.8%	95.3%	96.3%	95.8%	97.4%	96.9%
3 Flexispira taxon 2	95.7%	95.7%	94.4%	95.1%	96.5%	95.3%	96.6%	95.4%	96.4%	95.3%	95.8%	96.1%
4 Flexispira taxon 3	94.8%	94.8%	94.6%	94.7%	95.8%	94.2%	96.6%	95.8%	96.2%	96.0%	95.7%	95.6%
5 H.acinonychis	90.7%	90.8%	91.8%	91.5%	92.5%	92.6%	92.2%	91.5%	90.6%	90.9%	91.0%	91.8%
6 H.pylori	92.2%	92.2%	93.7%	93.4%	94.1%	94.0%	93.5%	92.9%	91.9%	92.3%	92.2%	93.0%
7 H.felis	90.8%	90.8%	90.1%	90.6%	92.1%	91.9%	91.3%	91.4%	90.7%	90.7%	90.3%	91.2%
8 H.bizzozeronii	92.5%	92.5%	91.9%	92.3%	93.5%	92.9%	92.8%	93.4%	92.2%	92.2%	91.8%	92.6%
9 BT91E	99.9%	99.9%	94.2%	94.6%	95.7%	94.6%	95.4%	94.9%	94.3%	94.5%	95.1%	94.6%
10 BT94E	99.9%	99.9%	94.2%	94.6%	95.6%	94.9%	95.8%	94.9%	94.9%	94.5%	95.1%	94.7%
11 BT96E	100.0%	100.0%	94.3%	94.7%	95.7%	95.0%	95.8%	94.9%	94.9%	94.5%	95.1%	94.6%
12 BT93E	99.8%	99.8%	94.2%	94.6%	95.5%	94.6%	95.8%	94.9%	95.3%	94.5%	95.0%	94.9%
13 BT92E	100.0%	100.0%	94.3%	94.7%	95.6%	95.0%	95.8%	94.9%	95.5%	94.5%	95.1%	95.2%
14 BT95E		100.0%	94.3%	94.7%	95.6%	95.0%	95.8%	94.9%	95.7%	94.5%	95.1%	95.4%
15 H.mesocricetorum			100.0%	97.1%	96.0%	95.6%	95.5%	96.1%	95.6%	95.6%	95.2%	94.6%
16 H.rodentium				100.0%	95.9%	95.3%	96.4%	95.9%	95.6%	95.3%	96.0%	95.2%
17 H.pullorum					100.0%	96.6%	97.8%	96.9%	96.3%	96.0%	95.7%	95.8%
18 H.pametensis						100.0%	96.4%	95.8%	96.2%	95.6%	95.7%	95.8%
19 H.canadensis							100.0%	96.3%	96.0%	95.6%	97.2%	96.5%
20 Flexispira taxon 1								100.0%	95.7%	95.8%	95.9%	95.4%
21 Flexispira taxon 5									100.0%	97.4%	98.0%	96.5%
22 Flexispira taxon 8										100.0%	96.8%	97.0%
23 Flexispira taxon 4											100.0%	97.4%
24 H.hepaticus												100.0%
25 H.aurati												
26 WB1F												
27 WB2F												
28 LN52F												
29 Flexispira taxon 8												
30 H.cinaedi												
31 H.canis												
32 Flexispira taxon 7												
33 H.fennelliae												
34 H.muridarum												
35 H.mustelae												
36 W.succinogenes												
37 Flexispira taxon10												
38 C.jejuni												
39 A.butzleri												

Similarity Matrix Part: 2

	25	26	27	28	29	30	31	32	33	34	35	36
1 LNB1F	94.7%	95.5%	95.5%	95.3%	98.9%	97.8%	97.7%	97.2%	92.5%	95.4%	94.0%	92.2%
2 Flexispira taxon 9	95.5%	96.2%	96.2%	95.9%	98.1%	97.1%	98.1%	97.3%	93.0%	95.6%	94.2%	92.5%
3 Flexispira taxon 2	94.6%	95.6%	95.6%	96.3%	97.1%	95.7%	96.4%	96.6%	92.5%	94.6%	94.0%	91.6%
4 Flexispira taxon 3	94.4%	95.8%	96.1%	96.3%	95.8%	95.2%	95.3%	95.4%	92.3%	94.3%	93.3%	91.5%
5 H.acinonychis	91.9%	91.0%	90.6%	91.1%	91.3%	90.8%	91.7%	91.4%	88.9%	91.3%	91.1%	89.2%
6 H.pylori	93.2%	92.5%	92.2%	92.9%	92.7%	91.8%	93.2%	93.1%	90.4%	92.1%	92.4%	90.5%
7 H.felis	90.6%	90.3%	89.9%	90.6%	90.7%	90.3%	90.9%	91.3%	89.2%	90.5%	90.9%	88.5%
8 H.bizzozeronii	92.1%	91.9%	91.9%	92.4%	92.0%	91.4%	92.5%	93.1%	90.1%	91.2%	91.5%	89.2%
9 BTP1F	94.1%	93.5%	93.6%	94.6%	94.9%	93.6%	94.9%	95.4%	91.3%	94.3%	92.7%	90.4%
10 BTP4F	94.1%	94.0%	93.9%	94.6%	95.2%	93.9%	94.9%	95.4%	91.6%	94.5%	93.0%	90.7%
11 BTP6F	94.1%	94.0%	93.9%	94.6%	95.2%	93.9%	94.9%	95.4%	91.7%	94.6%	93.1%	90.6%
12 BTP3F	94.2%	94.2%	94.0%	94.6%	94.9%	93.6%	94.8%	95.4%	91.3%	94.2%	92.7%	90.4%
13 BTP2F	94.7%	94.5%	94.5%	95.1%	95.2%	93.9%	94.8%	95.4%	91.7%	94.5%	93.1%	90.5%
14 BTP5F	94.9%	94.7%	94.7%	95.3%	95.2%	93.9%	94.8%	95.4%	91.7%	94.5%	93.1%	90.5%
15 H.mesocricetorum	96.3%	95.6%	95.6%	95.7%	94.9%	94.5%	94.8%	94.6%	92.5%	93.4%	93.8%	91.7%
16 H.rodentium	95.5%	95.5%	95.5%	95.4%	95.5%	94.9%	95.3%	95.1%	92.2%	93.6%	93.6%	92.9%
17 H.pullorum	95.8%	95.5%	95.8%	96.5%	95.9%	95.0%	96.2%	96.2%	93.0%	94.1%	93.9%	92.2%
18 H.pametensis	96.4%	96.0%	95.6%	96.7%	96.1%	94.6%	95.7%	95.7%	92.7%	95.1%	95.9%	93.4%
19 H.canadensis	95.8%	95.1%	95.4%	96.2%	96.8%	95.0%	96.3%	96.4%	92.3%	95.2%	94.4%	92.6%
20 Flexispira taxon 1	95.4%	95.0%	95.0%	96.4%	95.8%	95.0%	95.3%	96.0%	93.0%	93.9%	94.0%	92.3%
21 Flexispira taxon 5	95.6%	96.7%	96.7%	96.8%	96.4%	95.9%	96.1%	96.0%	92.1%	94.7%	94.5%	92.7%
22 Flexispira taxon 6	96.1%	95.9%	95.9%	97.3%	95.7%	95.7%	96.0%	96.6%	93.3%	95.1%	95.1%	93.0%
23 Flexispira taxon 4	95.9%	96.3%	96.4%	95.7%	97.6%	96.9%	97.5%	96.6%	92.7%	95.6%	94.4%	93.2%
24 H.hepaticus	96.8%	96.0%	95.9%	96.5%	97.5%	96.4%	97.1%	97.9%	92.9%	96.5%	94.9%	92.5%
25 H.aurati	100.0%	95.9%	95.8%	96.5%	95.8%	94.6%	95.6%	95.9%	93.2%	95.1%	94.4%	92.8%
26 WB1F		100.0%	99.7%	97.1%	96.6%	95.7%	96.4%	95.7%	91.7%	94.0%	94.0%	92.9%
27 WB2F			100.0%	97.0%	96.3%	95.4%	96.4%	95.7%	91.3%	93.7%	93.7%	92.6%
28 LNB2F				100.0%	96.2%	94.8%	96.3%	97.0%	92.7%	94.1%	94.7%	92.6%
29 Flexispira taxon 8					100.0%	98.1%	97.9%	97.9%	92.7%	95.3%	94.6%	92.7%
30 H.cinaedi						100.0%	97.1%	96.6%	92.9%	94.6%	93.5%	92.2%
31 H.canis							100.0%	97.4%	92.6%	95.3%	94.5%	92.6%
32 Flexispira taxon 7								100.0%	93.7%	95.6%	94.5%	92.2%
33 H.fennelliae									100.0%	92.2%	91.2%	90.1%
34 H.muridarum										100.0%	93.9%	92.4%
35 H.mustelae											100.0%	92.3%
36 W.succinogenes												100.0%
37 Flexispira taxon10												
38 C.jejuni												
39 A.butzleri												

Similarity Matrix Part: 4

	37	38	39
1 LNB1F	88.6%	84.8%	84.2%
2 Flexispira taxon 9	89.1%	85.0%	84.6%
3 Flexispira taxon 2	88.3%	85.2%	85.4%
4 Flexispira taxon 3	88.2%	84.1%	84.5%
5 H.acinonychis	91.3%	82.6%	83.6%
6 H.pylori	92.8%	83.8%	85.0%
7 H.felis	91.3%	81.6%	82.4%
8 H.bizzozeronii	93.1%	83.2%	83.1%
9 BTP1F	94.3%	84.7%	85.0%
10 BTP4F	94.6%	85.0%	85.1%
11 BTP6F	94.6%	84.9%	85.2%
12 BTP3F	94.6%	84.7%	84.9%
13 BTP2F	94.7%	84.7%	85.1%
14 BTP5F	94.7%	84.8%	85.1%
15 H.mesocricetorum	94.1%	85.2%	85.7%
16 H.rodentium	94.0%	85.9%	86.0%
17 H.pullorum	95.1%	85.5%	86.0%
18 H.pametensis	94.1%	85.7%	85.7%
19 H.canadensis	94.5%	85.7%	85.4%
20 Flexispira taxon 1	94.7%	85.6%	85.2%
21 Flexispira taxon 5	93.5%	85.3%	85.7%
22 Flexispira taxon 6	95.3%	84.6%	85.6%
23 Flexispira taxon 4	95.0%	85.3%	85.0%
24 H.hepaticus	94.5%	85.2%	85.0%
25 H.aurati	93.8%	85.5%	84.9%
26 WB1F	93.1%	85.3%	85.2%
27 WB2F	93.2%	85.1%	85.0%
28 LNB2F	94.3%	85.2%	85.0%
29 Flexispira taxon 8	95.1%	85.7%	85.0%
30 H.cinaedi	94.9%	84.7%	84.2%
31 H.canis	96.0%	85.4%	85.2%
32 Flexispira taxon 7	96.4%	84.9%	84.7%
33 H.fennelliae	93.7%	84.1%	83.8%
34 H.muridarum	94.7%	84.5%	85.1%
35 H.mustelae	93.5%	84.3%	85.3%
36 W.succinogenes	90.6%	85.1%	85.4%
37 Flexispira taxon10	100.0%	84.6%	83.9%
38 C.jejuni		100.0%	86.1%
39 A.butzlerii			100.0%

Appendix 5 Similarity matrices showing percentage sequence similarities for all *Helicobacter, Campylobacter* and *Desulfovibrio* isolated obtained from marsupials studied, and the references strains used in the phylogenetic reconstruction of the 16S ribosomal RNA gene.

Similarity Matrix Part: 1

	1	2	3	4	5	6	7	8	9	10	11	12
1 UNSW3caefat3	100.0%	97.7%	92.9%	92.7%	93.2%	91.2%	92.3%	88.8%	85.0%	76.9%	76.4%	78.3%
2 LNB2		100.0%	93.2%	93.3%	93.0%	92.2%	92.4%	89.0%	85.9%	78.2%	77.5%	79.0%
3 ASHU18B			100.0%	98.0%	98.2%	94.4%	95.9%	89.5%	86.0%	77.2%	76.8%	78.4%
4 D.fairfieldensis				100.0%	99.2%	95.3%	97.0%	90.3%	86.4%	77.4%	77.1%	78.2%
5 LNB1					100.0%	95.1%	96.4%	90.0%	86.8%	78.3%	77.7%	79.1%
6 D.intestinalis						100.0%	96.6%	89.5%	87.4%	76.5%	76.3%	78.1%
7 D.desulfuricans							100.0%	90.4%	87.0%	77.6%	77.1%	78.6%
8 D.termitidis								100.0%	86.4%	76.7%	76.4%	77.7%
9 D.halophilus									100.0%	76.4%	76.1%	77.5%
10 H.bilis										100.0%	99.2%	92.8%
11 LNB3F											100.0%	92.2%
12 H.pylori												100.0%
13 TD18												
14 H.felis												
15 BTP4F												
16 H.muridarum												
17 BTP4S												
18 BTP2S												
19 BTP1C												
20 WB2F												
21 WB1F												
22 H.hepaticus												
23 H.trogontum												
24 H.aurati												
25 LNB2F												
26 H.canadensis												
27 H.pametensis												
28 H.pullorum												
29 H.canis												
30 H.cinaedi												
31 H.mustelae												
32 H.rodentium												
33 W.succinogenes												
34 C.curvus												
35 C.rectus												
36 EQ1												
37 C.jejuni												
38 C.lari												
39 C.coli												
40 C.helveticus												
41 BTP1												
42 C.hyointestinalis												
43 C.fetus												
44 WB1												
45 A.butzleri												

Similarity Matrix Part: 2

	13	14	15	16	17	18	19	20	21	22	23	24
1 UNSW3caefar3	78.1%	77.0%	77.5%	77.8%	77.9%	77.7%	78.0%	78.3%	78.6%	78.5%	78.6%	78.6%
2 LNB2	79.6%	77.9%	78.3%	78.8%	78.5%	78.6%	78.4%	79.4%	79.5%	79.6%	79.4%	79.5%
3 ASHU13B	78.4%	77.2%	77.2%	77.5%	78.0%	77.4%	78.2%	78.2%	78.3%	78.6%	79.1%	78.4%
4 D.fairfieldensis	78.5%	77.2%	76.2%	77.5%	77.0%	76.3%	77.1%	77.2%	77.3%	77.9%	79.2%	77.8%
5 LNB1	79.1%	78.1%	78.2%	78.5%	78.8%	78.2%	78.7%	79.1%	79.2%	79.5%	79.7%	79.1%
6 D.intestinalis	78.6%	76.9%	76.1%	76.8%	77.0%	76.1%	76.7%	76.5%	76.6%	77.3%	78.6%	77.4%
7 D.desulfuricans	79.0%	77.6%	76.2%	77.8%	77.3%	76.3%	77.1%	77.2%	77.5%	78.2%	79.3%	78.0%
8 D.termitidis	77.4%	76.1%	75.4%	76.6%	76.0%	74.9%	75.9%	76.3%	76.4%	77.0%	78.1%	76.1%
9 D.halophilus	77.6%	75.8%	75.4%	76.3%	76.3%	75.3%	75.4%	76.2%	76.3%	77.1%	77.8%	77.0%
10 H.bilis	93.6%	90.6%	95.2%	95.5%	95.8%	94.6%	94.3%	96.3%	96.3%	97.1%	96.0%	95.6%
11 LNB1F	93.0%	90.1%	94.5%	95.3%	94.8%	93.9%	93.2%	95.7%	95.7%	96.5%	95.7%	94.8%
12 H.pylori	97.2%	93.8%	92.0%	92.6%	92.3%	91.6%	92.0%	92.3%	92.7%	93.0%	92.4%	93.3%
13 TD1S	100.0%	93.6%	92.5%	92.6%	92.7%	91.8%	91.7%	92.5%	92.5%	92.7%	92.9%	93.3%
14 H.felis		100.0%	90.6%	91.0%	89.9%	89.7%	90.2%	90.1%	90.4%	91.3%	90.8%	90.7%
15 BIP1F			100.0%	94.7%	94.9%	95.4%	94.9%	94.6%	94.6%	95.7%	94.7%	95.0%
16 H.muridarum				100.0%	94.7%	93.2%	94.0%	94.0%	94.3%	96.9%	95.3%	95.3%
17 BIP1S					100.0%	96.1%	95.3%	94.6%	94.6%	95.8%	95.0%	95.3%
18 RIP2S						100.0%	94.9%	93.7%	93.7%	94.7%	94.2%	93.9%
19 BIP1C							100.0%	93.5%	93.5%	94.8%	93.2%	94.2%
20 WB2F								100.0%	99.7%	95.9%	95.9%	95.3%
21 WB4F									100.0%	96.0%	95.9%	95.9%
22 H.hepaticus										100.0%	97.0%	96.3%
23 H.trogontum											100.0%	98.1%
24 H.aurati												100.0%
25 LNB2F												
26 H.canadensis												
27 H.pametensis												
28 H.pullorum												
29 H.canis												
30 H.cinaedi												
31 H.mustelae												
32 H.rodentium												
33 W.succinogenes												
34 C.curvus												
35 C.rectus												
36 ZQ1												
37 C.jejuni												
38 C.lari												
39 C.coli												
40 C.helveticus												
41 BIP1												
42 C.hyointestinalis												
43 C.fetus												
44 WB1												
45 A.butzleri												

Similarity Matrix Part: 8

	25	26	27	28	29	30	31	32	33	34	35	36
1 UNSW3caefat3	78.9%	78.6%	78.3%	78.0%	78.5%	77.2%	77.8%	78.1%	78.8%	75.4%	75.4%	78.5%
2 LNB2	79.9%	79.5%	79.3%	79.2%	79.3%	78.3%	78.5%	79.3%	79.5%	76.8%	76.9%	78.3%
3 ASHU18B	79.1%	78.4%	78.3%	78.0%	78.4%	77.4%	78.2%	78.5%	79.3%	75.7%	75.6%	77.8%
4 D.fairfieldensis	78.7%	77.4%	78.4%	78.0%	78.5%	77.5%	78.2%	78.3%	79.0%	75.4%	75.1%	77.9%
5 LNB1	80.0%	79.3%	79.1%	79.3%	79.0%	78.5%	78.8%	78.8%	79.9%	76.5%	76.4%	78.0%
6 D.intestinalis	78.1%	76.9%	77.6%	77.3%	78.0%	76.7%	77.2%	77.3%	78.5%	75.0%	74.8%	77.9%
7 D.desulfuricans	78.7%	77.6%	78.5%	78.4%	78.8%	77.6%	77.9%	78.7%	79.2%	75.3%	75.0%	77.6%
8 D.termitidis	77.5%	76.2%	77.3%	77.1%	77.9%	76.7%	77.3%	77.2%	77.5%	74.0%	73.7%	76.5%
9 D.halophilus	77.4%	76.2%	77.0%	77.3%	77.9%	76.3%	76.4%	76.9%	77.0%	74.5%	74.2%	77.5%
10 H.bilis	96.0%	96.2%	95.9%	95.8%	98.2%	97.1%	94.2%	95.6%	92.5%	84.7%	83.8%	86.1%
11 LNB1F	95.5%	95.7%	95.6%	95.4%	97.9%	97.7%	94.1%	95.4%	92.2%	84.5%	83.6%	86.1%
12 H.pylori	93.0%	93.7%	94.0%	94.2%	93.3%	91.6%	92.3%	93.5%	90.5%	84.0%	83.0%	85.7%
13 TD13	93.2%	94.0%	94.6%	94.7%	94.1%	91.6%	93.1%	94.4%	90.2%	83.7%	83.0%	86.4%
14 H.felis	90.8%	91.5%	92.1%	92.2%	91.1%	90.1%	90.9%	90.8%	88.5%	82.1%	81.3%	83.6%
15 BTP1F	95.4%	95.5%	94.7%	95.8%	95.0%	93.3%	92.5%	94.7%	90.6%	83.6%	83.7%	86.4%
16 H.muridarum	94.4%	95.6%	95.3%	94.5%	95.6%	94.4%	93.9%	93.9%	92.6%	84.4%	83.9%	85.8%
17 BTP1S	95.1%	95.6%	94.5%	95.0%	95.8%	93.3%	93.4%	94.8%	91.9%	84.1%	83.6%	86.7%
18 BTP2S	94.0%	94.1%	93.8%	94.5%	94.6%	92.4%	92.3%	93.7%	90.4%	83.3%	82.9%	86.5%
19 BTP1C	94.4%	93.6%	94.1%	94.9%	94.6%	92.7%	91.9%	93.7%	91.4%	83.5%	83.5%	85.9%
20 WB2F	97.0%	96.2%	95.6%	95.8%	96.4%	95.1%	93.5%	95.5%	92.6%	84.5%	84.1%	87.2%
21 WB1F	97.1%	95.9%	96.0%	95.8%	96.4%	95.5%	93.8%	95.5%	92.8%	84.8%	84.5%	87.2%
22 H.hepaticus	96.5%	97.4%	95.8%	95.8%	97.1%	96.0%	94.7%	95.2%	92.4%	84.6%	84.5%	86.7%
23 H.trogontum	97.3%	95.6%	95.6%	96.0%	96.0%	95.4%	94.9%	95.3%	93.0%	84.7%	84.1%	86.6%
24 H.aurati	96.5%	96.6%	96.4%	95.8%	95.6%	94.4%	94.2%	95.5%	92.7%	85.2%	85.0%	87.2%
25 LNB2F	100.0%	96.9%	96.7%	96.5%	96.3%	94.5%	94.5%	95.4%	92.5%	85.0%	84.4%	86.9%
26 H.canadensis		100.0%	96.5%	97.9%	96.3%	94.6%	94.1%	96.5%	92.7%	84.9%	84.6%	87.5%
27 H.pametensis			100.0%	96.6%	95.7%	94.4%	95.7%	95.3%	93.3%	85.7%	85.0%	87.1%
28 H.pullorum				100.0%	96.2%	94.7%	93.6%	95.9%	92.2%	85.2%	84.7%	87.2%
29 H.canis					100.0%	96.8%	94.7%	95.3%	92.5%	85.0%	84.8%	86.9%
30 H.cinaedi						100.0%	93.6%	94.5%	91.9%	84.6%	84.6%	86.3%
31 H.mustelae							100.0%	93.3%	92.2%	84.4%	83.4%	86.0%
32 H.rodentium								100.0%	92.8%	85.7%	85.3%	87.6%
33 W.succinogenes									100.0%	85.1%	84.8%	85.7%
34 C.curvus										100.0%	94.8%	90.7%
35 C.rectus											100.0%	91.0%
36 EQ1												100.0%
37 C.jejuni												
38 C.lari												
39 C.coli												
40 C.helveticus												
41 BTP1												
42 C.hyointestinalis												
43 C.fetus												
44 WB1												
45 A.butzleri												

Similarity Matrix Part: 4

	37	38	39	40	41	42	43	44	45
1 UNSW3caefat3	78.9%	77.9%	76.2%	77.6%	77.2%	75.5%	75.8%	75.4%	77.0%
2 LNB2	77.5%	78.6%	76.7%	78.3%	77.5%	76.5%	77.2%	75.5%	77.5%
3 ABHU18E	76.4%	77.1%	75.7%	76.8%	76.7%	74.9%	75.2%	74.9%	76.3%
4 D.fairfieldensis	76.2%	77.3%	75.4%	76.0%	76.3%	75.6%	75.1%	75.3%	76.0%
5 LNB1	77.3%	78.3%	76.5%	77.5%	76.9%	76.0%	76.0%	75.9%	77.1%
6 D.intestinalis	75.9%	76.8%	75.4%	75.9%	76.3%	75.9%	75.6%	74.7%	75.8%
7 D.desulfuricans	76.1%	77.3%	75.5%	75.5%	76.1%	75.7%	75.2%	75.1%	76.3%
8 D.termitidis	74.8%	75.7%	74.0%	74.2%	75.1%	74.1%	73.9%	73.7%	74.7%
9 D.halophilus	75.6%	76.5%	75.1%	75.7%	75.5%	75.3%	74.8%	74.3%	75.2%
10 H.bilis	85.1%	85.7%	85.3%	85.8%	85.4%	84.7%	84.6%	83.2%	84.7%
11 LNB1F	84.9%	85.5%	85.2%	85.4%	85.4%	84.7%	84.4%	83.5%	84.3%
12 H.pylori	84.2%	84.5%	83.9%	84.7%	84.9%	83.4%	83.8%	82.4%	85.2%
13 TD18	84.9%	85.2%	84.6%	85.4%	86.1%	84.1%	84.0%	83.8%	85.5%
14 H.felis	82.2%	82.9%	81.3%	82.8%	82.9%	81.7%	82.7%	81.1%	83.0%
15 BTP1F	85.0%	85.6%	83.0%	85.7%	86.0%	83.6%	84.2%	82.8%	85.4%
16 H.muridarum	85.0%	85.4%	84.8%	85.7%	85.1%	84.1%	84.9%	82.9%	85.4%
17 BTP1S	85.1%	85.5%	85.4%	85.5%	85.5%	83.8%	84.6%	82.7%	85.7%
18 BTP2S	84.8%	85.2%	85.2%	85.2%	85.5%	83.2%	83.9%	82.6%	85.7%
19 BTP1C	84.5%	85.0%	84.8%	84.7%	85.5%	83.1%	84.0%	82.9%	84.9%
20 WB2F	85.7%	86.2%	85.6%	85.9%	85.7%	84.7%	85.3%	84.3%	85.6%
21 WB1F	85.9%	86.5%	85.8%	85.9%	85.7%	84.5%	85.5%	84.2%	85.7%
22 H.hepaticus	85.7%	86.2%	85.8%	86.0%	86.0%	84.9%	85.3%	83.5%	85.5%
23 H.trogontum	85.2%	85.8%	85.4%	86.2%	86.0%	85.2%	85.0%	84.7%	86.1%
24 H.aurati	86.1%	86.6%	86.3%	86.3%	86.4%	85.0%	85.5%	83.8%	85.5%
25 LNB2F	85.7%	86.6%	85.5%	86.6%	86.1%	85.1%	85.4%	84.3%	85.6%
26 H.canadensis	86.3%	86.9%	86.1%	86.8%	86.6%	84.1%	85.1%	84.0%	85.8%
27 H.pametensis	86.3%	86.7%	86.0%	86.6%	86.3%	85.1%	85.6%	84.6%	86.2%
28 H.pullorum	86.1%	86.6%	85.9%	86.7%	86.2%	85.0%	84.9%	84.4%	86.5%
29 H.canis	86.9%	86.3%	86.1%	86.8%	86.2%	85.3%	85.2%	83.9%	85.6%
30 H.cinaedi	85.4%	85.9%	85.7%	86.0%	85.2%	85.2%	85.3%	84.0%	84.3%
31 H.mustelae	84.9%	85.6%	84.6%	85.2%	85.0%	83.9%	84.3%	83.1%	85.6%
32 H.rodentium	86.3%	87.0%	86.2%	86.9%	86.4%	85.7%	85.7%	85.0%	86.4%
33 W.succinogenes	85.2%	85.8%	85.0%	85.1%	84.4%	84.1%	84.6%	84.2%	85.6%
34 C.curvus	90.5%	90.3%	91.3%	89.9%	88.4%	91.5%	92.8%	91.8%	85.3%
35 C.rectus	91.0%	90.9%	91.8%	90.1%	88.5%	91.7%	93.0%	90.9%	85.3%
36 EQ1	98.3%	97.8%	97.0%	97.0%	95.2%	93.9%	92.9%	92.8%	86.4%
37 C.jejuni	100.0%	98.0%	98.3%	96.0%	94.2%	93.5%	92.8%	91.7%	85.9%
38 C.lari		100.0%	97.1%	95.5%	93.8%	93.7%	93.0%	91.9%	85.7%
39 C.coli			100.0%	95.0%	93.5%	94.8%	93.9%	92.8%	85.4%
40 C.helveticus				100.0%	96.4%	92.2%	91.7%	91.2%	85.9%
41 ECP1					100.0%	91.0%	90.0%	90.5%	86.6%
42 C.hyointestinalis						100.0%	97.3%	94.6%	85.4%
43 C.fetus							100.0%	93.2%	85.3%
44 WB1								100.0%	84.7%
45 A.butzleri									100.0%