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MORPHOLOGY, HAEMOLYSIS AND ENVIRONMENTAL SOURCES OF CATALASE-POSITIVE CAMPYLOBACTERS

By

SAMUEL MUTWIRI ARIMI BVM., M.Sc.

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*HUNTER et al. (1983); and HUTCHINSON & BOLTON (1983).

ABSTRACT

Literature relating to the isolation, identification and importance of catalase-positive campylobacters is reviewed and the need for further work emphasized. Campylobacters have been isolated directly, or after enrichment, from faeces of diarrhoeic patients, from piglets and from sewage and the efficiency of various procedures compared. Attempts to devise a selective medium using dyes were unsuccessful. Campylobacters occur commonly in Reading sewage, the sewage treatment works removes 99.9%, but about 10^{10} campylobacters/day are released into the nearby river from the works. Biotyping and serotyping showed that the types in sewage were similar to those isolated from human faeces and in beef, lamb and chicken meats on sale in Reading.

Differentiation betwen campylobacters was studied in two regards. After developing a method involving treatment with Mitomycin to facilitate production of long helical organisms, measurements were made of wavelength and amplitude. It was confirmed that, in general, the thermophilic campylobacters have smaller wavelength and amplitude than the C.fetus subspecies but there was an important exception in that the long organisms of the type strain of C.laridis (NCTC 11352) had wavelength indistinguishable from C.fetus. Separation of the two subspecies of C.fetus could not be achieved using this characteristic. Four strains

representing different campylobacters all showed right-handed helix when examined by scanning electron microscopy. Haemolysis hitherto generally regarded as not a property of catalase-positive campylobacters, has been demonstrated by most C.jejuni and a few C.coli strains when cultured on heart infusion agar (Difco) at 42°C for 4 days. None of the campylobacters gave a positive CAMP reaction with either Staphylococcus aureus or Streptococcus agalactiae.

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CHAPTER 1

GENERAL INTRODUCTION

Pathogenic campylobacters cause a variety of diseases in both animals and human beings. The most notable condition in human beings is the self-limiting, acute diarrhoea often accompanied by blood and mucus in the stool (Skirrow, 1977; Butzler & Skirrow, 1979). The main causes of the disease are Campylobacter jejuni and Campylobacter coli. Over the last ten years, C. jejuni has overtaken Salmonella to become the commonest detected cause of bacterial enteritis in Britain (Skirrow, 1982; Communicable Disease Report (CDR) 86/53). Reports from other countries indicate the extensive distribution of the problem (Steele & McDermott, 1978; Blaser et al., 1980; Richardson et al., 1981; Riley & Finch, 1985).

Although some members of Campylobacter (Sebald & Véron, 1963) previously known as Vibrio fetus (Smith & Taylor, 1919; Véron & Chatelain, 1973) have been known to cause abortion in animals since 1913 (McFadyean & Stockman, 1913), members of the genus were not clearly recognized as serious pathogens of man until recently, largely due to difficulties in culturing and isolating the organisms, especially from faeces. The first isolation of campylobacters from human beings was made in 1947 from the blood of a pregnant woman who subsequently aborted (Vinzent et al., 1947, quoted by Doyle, 1981). Ten years later King (1957) studied human strains in detail and recorded a close association of thermophilic campylobacters, which she referred to as "related vibrios", with human enteritis. This observation was supported by Dekeyser et al.

(1972) in Belgium, who used a membrane filter technique to isolate "related vibrios" from human diarrhoeal stools. Then followed what is regarded as the most important paper in the subject in which Skirrow (1977) described a selective medium for isolating campylobacters directly from faeces and its use to show the importance of campylobacters in human diarrhoea. Thus the gates were opened to many fields of Campylobacter research.

Development of a selective medium was not by itself a solution to campylobacter problems, but a major step forward in the struggle to solve the mysteries surrounding the organism. A lot has been achieved over the last ten years an substantial data accumulated in many areas, for example, isolation media, techniques and methods of isolation, diagnostic methods, characterization and classification, serology pathogenicity and epidemiology.

As we look back at what has been achieved, we nonetheles realise the challenges that campylobacters continue to pose and the problems we have to solve. Continued development of serotyping and biotyping methods is helping us to understand the epidemiology of the disease and particularly the role of animals as sources of infection; but the number of reported cases of campylobacter gastroenteritis continues to rise unabated (CDR 86/53). It can reasonably be argued that this rise in numbers reflects increased efficiency in isolation t

an increasing number of laboratories, but it could also be that there is an increase in occurrence of the disease, which continues to emphasise the widespread occurrence and importance of campylobacter enteritis.

There are still many gaps in our knowledge. Efficient and effective culture media and techniques have been developed, yet problems in developing countries continue to hamper progress in research since most of these media and techniques are unsuitable for a variety of reasons. For example, basic requirements like lysed horse blood which is needed in Skirrow's medium to neutralize trimethoprim antagonists that are present in most media, are not readily available and antibiotics used in the media are prohibitively expensive. Skirrow's medium is one of the most widely used commercially available selective media and although it has been said that blood from other animal species could be used instead of horse blood without compromising the activity of trimethoprim (Bopp et al., 1982,quoted by Goossens et al., 1984), availability of suitable sterile blood is, in general, a problem in many countries. Differentiation between strains and even between species of Campylobacter still poses problems because the organisms are negative in many traditional tests including ability to attack carbohydrates (Smibert, 1984).

Study of topics reported in this thesis were chosen to cover several areas namely: (1) epidemiology; (2) isolation

from human, animal, food and environmental sources; (3) diagnostic and differential tests for catalase-positive campylobacters pathogenic for man and animals. It is hoped that the findings reported here will help to increase our understanding of campylobacters and will thereby contribute to the drive to reduce the suffering caused by these organisms.

CHAPTER 2

LITERATURE REVIEW

Campylobacters are Gram-negative, polarly flagellate, curved and helical microaerophilic bacteria. They are divided into two groups according to whether they are catalase-positive or negative. Only one member of the catalase-negative group is thought to be pathogenic (Butzler & Skirrow, 1979); C. sputorum subsp. mucosalis (C. mucosalis comb.nov.: Roop et al., 1985) is associated with proliferative enteritis of swine (Lawson & Rowland, 1974; 1984). The catalase-positive group contains species pathogenic for man and animals. Many general reviews and monographs on these organisms are now available (Smibert, 1978; Doyle, 1981; Garcia et al., 1983; Butzler, 1984; Lander, 1985; Axon, 1986; Walker et al., 1986). I have therefore reviewed literature on selected topics related mainly to C. fetus, C. jejuni, C. coli, C. laridis and C. pylori, formerly C. pyloridis (Marshall & Goodwin, 1987).

2.1 THE ORGANISM CAMPYLOBACTER

2.1.1 Classification

1. Which Family?

In the eighth edition of Bergey's Manual of Determinative Bacteriology the genus Campylobacter is grouped in the family Spirillaceae which comprises bacteria with helical or vibrioid cells, having from less than one complete helical turn to many turns (Krieg & Smibert, 1974). This

classification is now in question. According to Krieg (1984) in the recently published Bergey's Manual of Systematic Bacteriology, it is difficult to decide which genera of helical organisms should presently be included in the family Spirillaceae because of considerable phylogenetic heterogeneity although some exhibit certain similarities. He proposes a mere loose assemblage of taxa that exhibit some morphological and/or physiological similarities pending future studies of genetic relatedness which should help to provide a more satisfactory and suitable arrangement.

2. Genus

McFadyean & Stockman (1913) described a "vibrio" apparently responsible for abortion in pregnant ewes, and six years later Smith & Taylor (1919) recovered from aborted calves a microaerophilic "spirillum", identical to the vibrio of McFadyean & Stockman, and named it Vibrio fetus. The assigning of this organism to the genus Vibrio was based on the morphological appearance of curved cells predominant over spiral ones, particularly in young cultures.

Although similar in more regards than cell curvature (Park, 1961), vibrios and campylobacters differ greatly in many phenotypic respects and also in guanine + cytosine (G•C) content of the DNA (Sebald & Véron, 1963; Feeley, 1966; Baumann et al., 1984; Smibert, 1984). The property of cell

curvature is considered to be a characteristic of dubious taxonomic value (Davis & Park, 1962) and when one considers the finding of straight rod variants in certain Spirillum species (Krieg, 1984) the importance of helical/vibrioid morphology loses some of its force. Sebald & Véron (1963) placed Vibrio fetus in a new genus, separated from Vibrio partly on the basis of a low G+C content of the DNA, and called it Campylobacter (Greek word meaning a curved rod) with Vibrio fetus (renamed Campylobacter fetus) as the type species. They found G+C content of the DNA of Vibrio cholerae, Vibrio metchnikovii, Vibrio anguillarum, Vibrio costicolus, Vibrio succinogenes and several other Vibrio spp. to be about 47 mol % and that of the so called vibrios related to veterinary pathology to be 32 mol %. Recently quoted figures show G+C content of the DNA of Campylobacter spp. to range from 30 to 38 mol% (Smibert, 1984) but this does not include the G+C content of the DNA of the free-living aspartate-fermenting Campylobacter spp. (41.6 mol %; Laanbroek et al., 1977), which is unusually high for the genus Campylobacter and which in fact overlaps with the G+C content of the DNA of Vibrio spp. which ranges from 38 to 51 mol % (Baumann et al., 1984). Laanbroek et al. (1977) considered the difference in G+C content of the DNA of the 'free-living aspartate-fermenting' Campylobacter spp. and that of the Campylobacter spp. of human and animal origin not to be large enough to exclude it from the genus Campylobacter. C.pylori

has a DNA base composition of G+C 35.8 - 37.1 mol% (Marshall et al.,1984, cited by Goodwin et al., 1985), which is within the Campylobacter range.

3. Species

Before 1980 there were two systems of naming Campylobacter species i.e. the 'French' version of Véron & Chatelain (1973) and the 'American' version of Smibert (1974). These two naming systems created a lot of confusion. This confusion was resolved when French system was officially accepted following the publication of approved lists of bacterial names (Skerman et al., 1980) and will be used throughout this thesis unless otherwise stated.

Names of catalase-positive species of Campylobacter in both French and American systems are shown in Table 2.1 which, unfortunately, is still required for interpretation of literature both before, and in some cases, since 1980. Catalase-positive campylobacters and the diseases they are associated with in man and animals are summarized in Table 2.2. The delineation of species is now supported by information on per cent G+C content of the DNA and DNA hybridization (Harvey & Greenwood, 1983a).

Table 2.1: French and American classification of catalase-positive *Campylobacter* spp.

French version (Véron & Chatelain, 1973)*	American version (Smibert, 1974)
Campylobacter fetus subsp. *fetus*	*Campylobacter fetus* subsp. *intestinalis*
Campylobacter fetus subsp. *venerealis*	*Campylobacter fetus* subsp. *fetus*
Campylobacter jejuni)) *Campylobacter coli*)	*Campylobacter fetus* subsp. *jejuni*

* = Officially recognized nomenclature in the "Approved Lists" of bacterial names (Skerman *et al*. 1980)

Table 2.2: Catalase-positive *Campylobacter* spp. associated with disease conditions in man and/or animals

Campylobacter spp.	Disease in man	Disease in animals					
		Cattle	Sheep	Pigs	Minks	Poultry	Dogs
1. *C.fetus* subsp. *fetus*	Septicaemia and various localised conditions e.g. arthritis, meningitis, endocarditis etc.; enteritis*	Sporadic abortion, diarrhoea in milk-fed and ruminating calves	enzootic abortion				
2. *C.fetus* subsp. *venerealis*		Abortion and infertility					
3. *C.jejuni*	enteritis, occasionally septicaemia and various localised conditions	Mastitis, sporadic abortion			diarrhoea, abortion	vibrionic hepatitis in chickens, blue comb in turkeys	diarrhoea, abortion in the bitch

Table 2.2 Continued.

Campylobacter spp.	Disease in man
4. *C.coli*	enteritis
5. *C.laridis*†	enteritis
6. *C.pylori*	strongly associated with gastritis
7. *C.fecalis*	
8. *C.hyointestinalis*	
9. Aerololerant campylobacters (*C.cryaerophila*)	
10. *C.cinaedi*, *C.fennelliae*	enteritis

Disease in animals					
Cattle	Sheep	Pigs	Minks	Poultry	Dogs
		may be involved in dysentery			
enteric lesions					
		associated with swine proliferative enteritis			
implicated as a cause of mastitis and abortions		isolated from aborted foetus			

Table 2.2 contd.

* Although considered a non-enteric pathogen, isolations associated with diarrhoea have been reported.

† Isolations from a few cases of gastroenteritis have been reported.

This table has been compiled from the following references: Morris & Park (1973); Ellis et al. (1977); Skirrow (1977); Blaser et al. (1978); Butzler (1978); Ellis et al. (1978); Lander & Gill (1980); Devlin & McIntyre (1983); Garcia et al. (1983); Gebhart et al. (1983); Harvey & Greenwood (1983b); Hunter & Prescott (1983); Marshall (1983); Warren (1983); Bulgin et al. (1984); Taylor & Al-Mashat (1984); Tauxe et al. (1985); Totten et al. (1985); Axon (1986); Klein et al. (1986).

2.1.2 General Characteristics

Campylobacters are small, non-sporeforming, Gram-negative bacteria that have a characteristic curved, S-shaped or spiral morphology (Karmali & Skirrow, 1984; Smibert, 1984). Cells are said to vary in size from 0.2 to 0.5 um wide to 6.5 to 8 um long. The cells, particularly the shorter forms, are highly motile with a characteristic rapid corkscrew-like motion by means usually of a single polar flagellum at one or both ends of the cell (but see page 18 for *C. pylori*). Darting and oscillation movements with rotations on axis are also common. Motility diminishes as the cultures get older and cells convert to coccoid bodies which are considered degenerative and non-viable (Karmali *et al.*, 1981; Moran & Upton, 1986), similar to those formed by vibrios (Baker & Park, 1975), because they do not show growth on culture media. There are now reports indicating existence of viable but non-culturable stage of *C. jejuni* (Rollins & Colwell, 1986) which means that methods presently used for detection and enumeration must be re-evaluated, new techniques devised and viability of the coccoid forms investigated further.

Catalase-positive campylobacters are oxidase positive; they reduce nitrate to nitrite but there may exist within the *C.jejuni*/*C.coli* group a subgroup of nitrate-negative strains (Karmali & Skirrow, 1984). Metabolism is entirely respiratory and carbohydrates are neither fermented nor oxidized (Alexander, 1957; Smibert, 1984). A low oxygen tension is

required for growth but there are, however, aerotolerant campylobacters (Neill et al.,1985). Some species can grow anaerobically but only if they are able to respire using substances including fumarate, nitrate, L-aspartate and trimethylamine-n-oxide (Park et al., 1980; Razi et al., 1981; Skirrow et al., 1982).

Considered as a group, campylobacters can grow over a wide range of temperatures, for instance, C. cryaerophila (aerotolerant campylobacters) can grow at as low as 15°C (Neill et al., 1985) and C. laridis can grow at as high as 45.5°C (Skirrow & Benjamin, 1980). C. fetus grows at as low as 25°C and at 37°C but not normally at 42°C although some strains will grow at this temperature (Smibert, 1984). Thermophilic campylobacters (C.jejuni, C.coli and C.laridis) grow at 37°C and 42-43°C but not at 25°C. Growth takes 24h to 48h to show on blood agar and sometimes longer incubation period is required especially for C. fetus subsp. venerealis strains which grow more slowly (Skirrow & Benjamin, 1980). The appearance of colonies varies. In general, C. fetus produces small, round and convex colonies which grow to 1-2 mm on blood agar in 48h at 37°C while the thermophilic group produces typically flat, glossy and effuse colonies with a tendency to spread along the direction of the streak marks of the inoculating wire loop (Butzler & Skirrow, 1979; Skirrow & Benjamin, 1980; Smibert, 1984). Swarming on moist agar to form a thin film is common with thermophilic campylobacters

especially C. jejuni, and young colonies have watery to greyish appearance. As colonies age they develop a metallic surface sheen.

2.1.3 Differentiation between catalase-positive Campylobacter spp.

There is no single test which can be reliably used for differentiation and it is therefore a continuing challenge to find differential phenotypic tests for campylobacters, partly because these organisms metabolize no carbohydrates, and partly because they are all negative in many of the physiological tests of differential value in other groups of bacteria. As with all groups of bacteria, the importance of G+C ratios and nucleic acid hybridization studies have increased to become crucial in the definitive distinction between species.

2.1.4 Differentiation between C. fetus and thermophilic campylobacters

King (1957) differentiated C. fetus strains from C.jejuni/C.coli group using growth temperatures because the former grew at 25°C and not at 42°C and the latter at 42°C and not at 25°C. Skirrow & Benjamin (1980) tested 1220 campylobacter strains from man, animals and the environment for growth at various temperatures and found growth at 25°C to distinguish clearly strains of the C. fetus from those of C.

jejuni, C. coli and C. laridis group. However, maximum growth temperature is unreliable because some strains of C.fetus grow at 42°C (Skirrow & Benjamin, 1980; Smibert, 1984). C.fetus is also different from the thermophilic types in being sensitive to cephalothin (30 ug disc or 64 mg l^{-1}) (some strains of C.coli are, however, said to be sensitive) and growing anaerobically in presence of fumarate (Razi et al.,1981; Skirrow et al.,1982; Karmali & Skirrow, 1984).

2.1.5 Differentiation between C.fetus subsp. fetus and C.fetus subsp. venerealis

The number of tests for differentiating between C. fetus subsp. fetus and C. fetus subsp. venerealis is very limited; in fact the two cannot be easily distinguished with certainty possibly because of the existence of more than two types (Park et al.,1962; Florent, 1963). Colonies of C.fetus subsp. fetus are well developed by 48h while slow growing C. fetus subsp. venerealis takes at least 72h (Karmali & Skirrow, 1984). Growth in presence of 1% glycine and production of hydrogen sulphide in sensitive cystine-containing media detected by absorbent paper strips impregnated with lead acetate (Park et al.,1962; Karmali & Skirrow, 1984), are two biochemical tests commonly used for differentiation. C. fetus subsp. fetus is positive in both tests and C.fetus subsp. venerealis in neither. Some C.fetus subsp. venerealis that

were found to be H_2S positive were classified by Véron & Chatelain (1973) as C.fetus subsp. venerealis biotype intermedius. According to Karmali et al. (1981) C.fetus subsp. fetus and C.fetus subsp. venerealis can clearly be separated using cell morphology.

2.1.6 Differentiation between C.jejuni, C.coli and C.laridis

There are a few tests for differentiating between the thermophilic campylobacters, and whilst these tests have proved useful, some do not always discriminate well between strains. Hydrolysis of sodium hippurate by C.jejuni, first described in campylobacters by Harvey (1980) and extensively applied by Skirrow & Benjamin (1982), distinguishes C.jejuni from C.coli and C. laridis which are negative. However, some strains of C.jejuni are hippurate negative or only weakly positive when tested by the tube method, which detects presence of glycine, one of the products of hippurate hydrolysis, by the purple colour produced in the ninhydrin reaction (Hébert et. al.,1984). Detection of hippurate hydrolysis by two-dimensional thin-layer chromatography is reportedly more specific (Lin et al.,1986).

Growth at 45.5°C and resistance to nalidixic acid (30 ug disc) distinguishes C.laridis from C.coli and C.jejuni which can grow at 43°C but not at 45.5°C and are sensitive to nalidixic acid. C.laridis and C.jejuni biotype II produce H_2S

in a medium supplemented with ferrous sulphate, sodium pyruvate and sodium metabisulphite (FBP). C.coli do not produce H_2S in FBP medium (Skirrow & Benjamin, 1980).

Other tests which have been used for differentiation include DNA hydrolysis, alkaline phosphatase and growth in charcoal-yeast extract agar (Hébert et al.,1982; Roop et al.,1984).

2.1.7 Consideration of C.pylori

Warren (1983) observed numerous small and S-shaped bacilli (C.pylori), which resembled C.jejuni in stomach biopsies of patients with gastritis. Marshall (1983) cultured these organisms using campylobacter techniques and studied them in detail. He observed certain morphological differences with Campylobacter, which included possession of multiple unipolar flagella of the sheathed type, each with a proximal terminal bulb. Campylobacters have predominantly a single polar flagellum, usually of the non-sheathed type, at one or both ends of the cell. In a detailed study, Curry et al.,(1985) compared the ultrastructure of C.pylori with that of other campylobacters and Aquaspirillum serpens (NCTC 10593) and found C.pylori to resemble Spirillum morphologically more than Campylobacter. Based on this evidence, together with other biological attributes, they suggested placing of C.pylori in another genus.

2.2 CAMPYLOBACTER INFECTIONS

2.2.1 Infection of animals

1. Infection with C.fetus subsp. venerealis

C.fetus subsp. venerealis appears to infect only cattle. The organisms specifically occur in the prepuce of the bull and in various reproductive organs of the cows and heifers, causing serious reproductive problems, and huge economic losses to farmers. Although it is said that about 95% of cases of enzootic infertility in cattle are caused by C.fetus subsp. venerealis while C.fetus subsp. fetus is responsible for about 5% (Dekeyser, 1984), the picture is not clear (Park et al.,1962). Infection is venereally transmitted and bulls may acquire infection from infected cows, contaminated bedding or equipment used for collecting semen - one reason why up to 50% or more of the bulls in an artificial insemination centre may be affected (Clark, 1971, quoted by Collins et al.,1985; Dekeyser, 1984; Collins et al.,1985). The mucosa of the prepuce, glans penis and distal urethra is colonized with no local or general symptoms (Clark, 1971). Infected asymptomatic bulls are very difficult to detect and they remain carriers for long periods. Infection does not affect the quality of spermatozoa; treatment of semen with antibiotics to remove the campylobacter results in semen of normal fertility. Susceptible cows are infected by carrier bulls during service or via semen during artificial insemination. C. fetus subsp.

venerealis quickly proliferates in the vagina and the infection ascends into the cervix, uterus and oviducts causing cervico-vaginitis, endometritis and salpingitis (Clark, 1971; Collins et al.,1985). Whereas inflammatory reactions may contribute to the death of the embryo, it is suggested (Ware, 1980) that campylobacters use up the dissolved oxygen and possibly other nutrients which are essential pre-implantation requirements for the blastocyst which subsequently dies before implantation. Heifers are more susceptible to infection than are older cows because they lack immunity. Infected cows may remain for months before showing signs of heat, and when they do, many services are required to get them pregnant and carry foetus to term.

2. Infection with C. fetus subsp. fetus

C.fetus subsp. fetus has been recovered from the faeces of apparently normal cattle and sheep. Although it has been considered as a non-enteric pathogen, it may cause diarrhoea in milk-fed and ruminating calves (Al-Mashat & Taylor, 1983; Taylor & Al-Mashat, 1984). The organisms, however, are responsible for causing non-venereal enzootic abortions in sheep and sporadic abortions in cows (Jensen et al., 1957;Firehammer, 1979). C.fetus subsp. fetus from the intestines enter the blood stream causing bacteraemia and invade an already well developed placenta causing placentitis. Subsequently the foetus dies of anoxia followed by a late

abortion (Dekeyser, 1984). The different modes of infection by the two biotypes was first recognized by Florent (1959).

3. Infection with C.jejuni

C.jejuni is commonly isolated from faeces of apparently normal cattle but the organism may cause diarrhoea in calves and older cattle (Al-Mashat & Taylor, 1980). It occasionally causes sporadic bovine abortion as a result of systemic disease due to bacteraemia apparently following ingestion of the organism in fodder or water (Collins et al., 1985). The ability of C.jejuni to infect the bovine udder and cause clinical mastitis was experimentally demonstrated by Lander & Gill (1980) in an important study with relevance to epidemiology. Since then a few cases of what has been considered natural Campylobacter mastitis have been reported (Hudson et al.,1984; Hutchinson et al.,1985; Morgan et al.,1985). In the Netherlands, Boer et al.(1984) examined 750 mastitic milk samples for C.jejuni and in England Waterman et al.(1984) examined 1214 samples and none of them yielded C.jejuni. Lovett et al. (1983) described a sensitive enrichment method involving bubbling microaerobic gas mixtures through the enrichment for recovering very low numbers of C.jejuni from milk and in light of their work, the results of Boer et al. and Waterman et al. may need reinterpretation (but see page 34 and 36).

In some instances C.jejuni may cause non-venereal systemic abortion in sheep (Berg et al.,1971; Firehammer, 1979). The organisms cause vibrionic hepatitis in chickens and blue-comb in turkeys (Garcia et al.,1983) and although common in the intestine, enteritis is not a feature.

Infection of other animal species has been reported to be associated with many syndromes including diarrhoea in dogs especially puppies (Blaser et al.,1978; Fox et al.,1983), abortion in bitches (Bulgin et al.,1984), diarrhoea and abortion in minks (Hunter & Prescot, 1983; Hunter et al.,1983) and diarrhoea in cats (Fox et al.,1985).

4. Infection with C.coli

C.coli is common in small numbers in the faeces of clinically normal pigs and appears in the diarrhoeic faeces of pigs in much larger numbers (Taylor & Al-Mashat, 1984). Originally it was thought to cause swine dysentery (Doyle, 1944) but its causative role in disease was subsequently discounted when anaerobic spirochaete (Treponema hyodysenteriae) was shown to be the cause of swine dysentery (Taylor & Alexander, 1971). Nonetheless, involvement of campylobacters in the infection in some way has been reported by Morris & Park (1973), Fernie et al. (1975) and Taylor & Olubunmi (1981).

5. Infection with other catalase-positive campylobacters

C.laridis is common in the intestinal tracts of seagulls of the genus Larus (Skirrow & Benjamin, 1980; Fricker et al.,1983) and possibly in other birds (Fricker & Metcalfe, 1984), but its pathogenicity to animals and birds is not known. C.fecalis originally isolated from the faeces of clinically normal sheep (Firehammer, 1965) has been reported to cause enteritis in calves orally inoculated with pure culture of the organisms (Al-Mashat & Taylor, 1981). C.hyointestinalis has been associated with porcine proliferative enteritis (Gebhart et al.,1983); its pathogenic significance is yet to be determined. Aerotolerant campylobacters (C.cryaerophila) have been isolated from, and have been presumed to be the cause of, aborted pig and bovine foetuses (Ellis et al. 1977; Ellis et al.,1978) and they have also been implicated as the cause of bovine mastitis (Logan et al.,1982).

2.2.2 Infection in man

The history of campylobacter enteritis in man (and also in animals) is apparently much older than recent literature has indicated. In a recent review article by Kist (1985), Escherich (1886a; 1886b, quoted by Kist, 1985) described existence of vibrios, measuring 2000-5000 nm in length and consisting of two to six coils, in the intestines and faeces of babies with diarrhoea and also in kittens which had died of

diarrhoeal disease. Sixty years later Levy (1946) described presence of vibrio-like organisms in the stools of patients following an outbreak of gastroenteritis in which milk was epidemiologically implicated as the source of the causative agent. Vinzent *et al.* (1947) was successful in culturing *C.fetus* from the blood of three pregnant women. These studies and more recent work by King (1957), Dekeyser *et al.*(1972), Butzler (1973) and Skirrow (1977) brought campylobacters from obscurity to be recognized as the commonest detected bacterial cause of diarrhoea in human beings. Campylobacteriosis in man is basically of two types: gastrointestinal and extra-gastrointestinal (Butzler & Skirrow, 1979; Mandal *et al.*,1984; Axon, 1986). Gastrointestinal campylobacteriosis includes gastritis, enteritis and enterocolitis. The last two conditions will hereafter be referred to as enteritis.

1. Gastrointestinal campylobacteriosis

a. Infection with *C.jejuni* and *C.coli*

C.jejuni and *C.coli* infect otherwise healthy persons, adults and children, causing acute enteritis characterized by watery and foul smelling self-limiting diarrhoea often accompanied by blood and mucus in the stool (Skirrow, 1977; Butzler & Skirrow, 1979; Doyle, 1981; Mandal *et al.*,1984). Clinical aspects of enteritis are dealt with by Skirrow (1977; 1982), Butzler & Skirrow (1979), Hay & Ganguli (1980) and

Mandal et al. (1984), and are essentially as follows. The incubation period is on the average 3-5 days. Other symptoms include nausea, anorexia, abdominal pain and discomfort. Sometimes patients present with acute abdominal pain and they are admitted to hospital generally misdiagnosed as cases of acute appendicitis, though a few of them do indeed have acute appendicitis. Falsely, intussusception is also sometimes diagnosed and laparatomy performed on babies with campylobacter enteritis. Morbidity is high but mortality is low, death usually occurring as a result of complications in otherwise weakened persons.

C.coli accounts for only 3 to 5% of the campylobacter isolates from cases of human enteritis (Karmali & Skirrow, 1984), but in certain countries percentage is much greater.

b. Infection with C.fetus subsp. fetus

Butzler & Skirrow (1979), in a survey in Belgium of 22,000 stools, found C.fetus subsp. fetus in only 3 of the stools, and there was no association with diarrhoea. From then on, C.fetus was considered unimportant in gastroenteritis and hence few laboratories bother to look for it. Routine isolation procedure often involves incubation at 43°C which would inhibit any C.fetus subsp. fetus. Recently, however, several cases of gastroenteritis associated with C.fetus subsp. fetus have been reported, mostly in homosexuals (Devlin & McIntyre, 1983; Harvey & Greenwood, 1983b; Klein et

al.,1986), making it necessary for the routine isolation strategy to be reconsidered. The strains so far detected are thermotolerant, being capable of growth at 42°C, the temperature used for cultivation of C.jejuni and other thermophilic campylobacters.

c. Infection with C.pylori

C.pylori, originally seen in large numbers in biopsies taken from the stomachs of patients with gastritis (Warren, 1983), is now widely considered to be associated with gastritis, although the role of the organism in the pathogenesis of the disease is not yet established (Axon, 1986; McNulty et al.,1986). Presence of C.pylori is strongly correlated with histologically confirmed gastritis, while its disappearance is strongly correlated with application of treatment which leads to improvement of the gastritis (McNulty et al.,1986). Whilst the role of C.pylori in the pathogenesis of gastritis is yet to be established, production of acute self-limiting dyspeptic syndrome with acute gastritis in a normal volunteer after swallowing the bacteria adds support to the possible aetiologic role of C. pylori (see Axon, 1986).

2. Extra-gastrointestinal campylobacteriosis

Campylobacter infections occurring outside the gastro-intestinal tract are most commonly caused by C.fetus subsp.

fetus, which is an opportunistic pathogen. Usually patients are debilitated persons whose body defences have been weakened by underlying conditions such as alcoholism, cirrhosis, diabetes and leukaemia (Butzler, 1978; Mandal et al.,1984). C.jejuni occasionally causes extra-gastrointestinal campylobacteriosis.

Extra-gastrointestinal campylobacteriosis takes different forms, some of which are regarded as complications of gastro-enteritis. Septicaemia without localization of infection may occur either during or after campylobacter enteritis, or without gastroenteritis. It may persist and the patient may register elevated body temperature. At this febrile stage the organisms are culturable from blood. Localization of infection can result in suppurative arthritis, meningitis, endocarditis, pericarditis, peritonitis, thrombo-phlebitis, cholecystitis, abscesses and abortion (King, 1957; Butzler, 1978; Doyle, 1981; Mandal et al.,1984). Infants and pregnant women are especially susceptible to infection by C.fetus subsp. fetus.

2.3 MEDIA AND ISOLATION TECHNIQUES FOR THE THERMOPHILIC CAMPYLOBACTERS AND C.FETUS

Advances in development of culture media and isolation techniques for campylobacters have largely favoured C.jejuni, and the other thermophilic campylobacters, because of its

important aetiologic role in acute enteritis in human beings. Sadly, little has been done for C.fetus in this area and recent literature on the subject is therefore mainly on thermophilic campylobacters. Because of this, although the purpose of this section was to review the literature on media and isolation techniques for both C.fetus and thermophilic campylobacters, it will be seen that most of the references particularly on selective media and enrichments are on thermophilic campylobacters.

2.3.1 Media and growth requirements

Campylobacters obtain their energy by oxidizing tricarboxylic acid cycle intermediates or amino acids and other compounds which can easily be introduced into this cycle (Alexander, 1957; Smibert, 1963). Their inability to utilize carbohydrates either oxidatively or fermentatively is one of the characteristics which differentiates them from vibrios. Campylobacters grow well on several laboratory media including nutrient agar or blood agar. Difficulties experienced in culturing them in the earlier days was due more to lack of understanding of the gaseous requirements, physico-chemical conditions of the growth medium, and the effect of other competing micro-organisms, than to the inability of the medium to supply nutrients.

Campylobacters generally require oxygen for growth and yet they are poisoned by it at atmospheric partial pressure. Studies have incriminated several oxygen derivatives, including superoxide ion, hydrogen peroxide, hydroxyl radical and singlet oxygen (Hoffman et al., 1979; Bolton et al.,1984; Juven & Rusental, 1985). However, the answer may be simpler in that key enzymes may need to be kept in the reduced state (Lascelles & Calder, 1985). Exposure of media to air and light, especially daylight and fluorescent light, which encourages formation of oxygen derivatives in the medium makes it increasingly difficult for campylobacters to grow. Use of freshly prepared media, and if this is not possible, storage of such media in the dark and/or anaerobically is recommended (Hoffman et al.,1979; Fricker et al., 1985). Addition of ferrous sulphate, sodium metabisulphite and sodium pyruvate (FBP) supplement (George et al.,1978) to the culture medium enhances aerotolerance of campylobacters and improves growth.

2.3.2 Isolation methods

1. Membrane filter technique

Before Skirrow (1977) described a selective medium for direct isolation of campylobacters from faeces, isolations were only possible from 'clean' sites (blood) or by use of membrane filter technique (Morris & Park, 1971; Dekeyser et al.,1972; Butzler et al., 1973). Using a membrane filter of

mean pore size 0.65 um, Dekeyser et al. (1972) were able to isolate thermophilic campylobacters from human diarrhoeal faeces for the first time and to correlate diarrhoea with septicaemia by showing faecal isolates to be identical to blood isolates from the same patients. Because of their small size campylobacters are able to pass through the membrane filter while most other bacteria are retained. Some other micro-organisms that pass through the pores may necessitate the use of a selective medium in conjunction with filtration.

The membrane filter technique has a number of disadvantages. It is cumbersome to handle for routine use and is not very sensitive. It is therefore only suitable for isolations from samples containing large number of campylobacters, as for instance, in acute diarrhoeic stools. Recently, however, a filter technique involving application of a sample directly on to a 0.45 um Gelman cellulose acetate membrane filter placed on blood agar plate instead of using filtration equipment has been reported as quick and sensitive (Steele & McDermott, 1984). Steele & McDermott (1984) examined 1000 human faecal specimens for C.jejuni comparing this technique and selective medium, and out of the 56 positive specimens, 89% were positive using the 0.45 um filter and 80% by selective medium. The 0.45 um filter was so successful with very few contaminants that a 0.65 um pore size was unnecessary. Since pore sizes for most types of filter are 'mean' pore size, this 0.45 um may have some larger ones that the campylobacters get

through but not very large ones to increase the risk of other organisms getting through.

The advantage of the membrane filter is its ability to recover campylobacter strains which are sensitive to one or more of the inhibitory substances used in the selective medium (Steele & McDermott, 1984; Ng, et al.,1985) or to high temperature.

2. Use of selective media

Today selective media are extensively used in isolation of thermophilic campylobacters from faeces, foods, water and other environmental materials. They have proved extremely useful in recovering small numbers of campylobacters from contaminated food samples, faeces and water (Skirrow, 1977; Doyle & Roman, 1982; Lovett et al.,1983; Ribeiro & Price, 1984). Several selective media containing different combinations of inhibitory substances have been developed for isolation of thermophilic campylobacters. The following four examples are most commonly used: Skirrow's medium (Skirrow, 1977); Butzler's medium (Butzler & Skirrow, 1979); Preston medium (Bolton & Robertson, 1982); Campy-BAP (Blaser et al., 1979). These media are supplemented with haemolysed or whole blood for enhancement of campylobacter growth. Recently charcoal-based, blood-free selective media have been described (Bolton & Coates, 1983; Bolton et al., 1984; Karmali et al.,

1986). Preston medium and the charcoal-based, blood-free selective medium described by Bolton et al.(1984) have been reported to give similar rates of isolation of C.jejuni from human faeces (Bolton et al.,1984). In another comparison, Karmali et al.(1986) have reported a higher recovery rate of C.jejuni and C.coli from human faeces with their blood-free, charcoal-based selective medium (CSM) than with Skirrow's medium.

Selective media differ in their performance as far as isolation of campylobacters is concerned. Bolton et al. (1983) compared Skirrow's, Butzler's, Campy-BAP and Preston media for Campylobacter spp. using human, animal and environmental specimens and found Butzler's medium to give the lowest isolation rate and Preston medium, which was most selective, to give the highest isolation rate. These observations were confirmed by Fricker et al. (1983) when comparing the four media for their efficiency in recovering campylobacters from 389 freshly voided seagull faeces. It is reasonable to conclude that Preston medium is the most suitable medium for isolation of thermophilic campylobacters from contaminated specimens.

Selective media have some disadvantages. They are expensive and not always easy to prepare in the laboratory. Subsequently injured C.jejuni and C.coli have been shown to be sensitive to some of the antimicrobial substances used in the selective media (Ray & Johnson, 1984; Humphrey & Cruickshank,

1985) and some strains of apparently healthy C.fetus, C.coli and other campylobacters have also been reported to be sensitive (Steele & McDermott, 1984; Ng et al., 1985). Efficiency of the selective media is reduced by these factors especially when used for cultivating stored or processed specimens. For these reasons pre-enrichement may be necessary.

Selective media owe their inhibitory effects against contaminating organisms partly due to the added antibiotics and partly due to the high incubation temperature. Most C.fetus will not grow at 42°C and some of the antibiotics used in the media, for example cephalothin in Campy-BAP medium (Blaser et al.,1979), are inhibitory to C.fetus (Karmali et al., 1982), which makes some of the selective media unsuitable for isolation of C.fetus.

3. Selective enrichment and culture techniques

Various types of isolation techniques have been described, depending on the source of specimen being examined and the number of expected campylobacters in the specimen. With faeces from acute cases of diarrhoea where campylobacter numbers are usually large, direct plating on selective medium may suffice, but this is not always the case (Fricker & Girdwood, 1984). However, specimens from convalescent patients or from patients who have had antibiotic treatment, or specimens which have not been promptly processed, require

enrichment to increase recovery rate (Hutchinson & Bolton, 1983; Rogol et al.,1985). Enrichment and, if necessary, pre-enrichment of faecal, food and environmental samples before plating is now accepted as an essential requirement (Chan & Mackenzie, 1982; Doyle & Roman, 1982; Fricker et al.,1983; Ribeiro & Price, 1984; Rogol et al.,1985). Enrichment is usually carried out at 42-43°C. At this temperature, thermophilic campylobacters grow best and some of the competing micro-organisms as well as most C.fetus (See page 15) are inhibited. Surprisingly, Rubin & Woodard (1983) reported increased isolation rate of C.jejuni from human faeces enriched in Campy-Thio broth at 4°C. Campylobacters do not grow at 4°C and so it is unclear how this enrichment worked. Fricker (1984) suggested that it worked by selectively killing the competing micro-organisms.

To enrich for very low numbers of C.jejuni in milk, Lovett et al. (1983) described a sensitive method involving bubbling of microaerobic gas mixture (5% O_2 + 10% CO_2 + 85% N_2) through the enrichment broth incubated statically at 42°C for 24h before plating. They reported the method to be very effective in detecting $\leq$1 organism per ml. Sensitivity of this method was confirmed by Hunt et al.(1985). These findings suggest that a reassessment of importance of mastitis caused by C.jejuni may be appropriate (See page 21).

2.4 EPIDEMIOLOGY OF CAMPYLOBACTER ENTERITIS IN HUMAN BEINGS

The various disease conditions caused by campylobacters in both human beings and animals have been examined in the preceding sections under 'campylobacter infections' in man and animals of which enteritis in human beings is undoubtedly the most important and therefore of the greatest concern. I have therefore decided to devote this section mainly to the epidemiology of campylobacter enteritis in human beings.

Considerable advances have been made in recent years in our understanding of campylobacter enteritis. We know that C. jejuni is the main cause of enteritis and that the organism has been found in many countries (Butzler & Skirrow, 1979; Skirrow, 1982). Possibly partly because of better resources and awareness, prevalence in technologically developed countries is better understood than in developing countries. However, the situation in developing countries appear more complex because there is not a simple correlation between presence of campylobacters in human faeces and enteritis.

2.4.1 Sources of infection for human beings

1. Infection by contaminated foods

a. Milk

Many outbreaks of campylobacter enteritis involving large number of persons have been associated with consumption of

unpasteurized cows' milk (Taylor et al., 1979; Jones et al.,1981; Hutchinson et al.,1985); some cases involving consumption of goats' milk have also been reported (Hutchinson et al.,1985). How C.jejuni gains access into the milk is still unclear. The organism has rarely been recovered from the epidemiologically incriminated milk in an outbreak, making it difficult to elucidate clearly the source although insufficiently sensitive culture technique might be involved (See page 34). Failure to recover the organism from milk and its recovery from the faeces of dairy cows supplying the incriminated milk (Robinson et al., 1979; Pearson et al.,1983; Waterman et al.1984) has led to the general conclusion that milk is contaminated by faeces. However, Lander and Gill (1980) doubted whether faecal contamination could be a source of large number of campylobacters without gross appearance of faecal material in milk. They were of the opinion that a purer and more abundant source was necessary and therefore proposed udder infection as a possible source. They showed experimentally that C.jejuni was capable of causing bovine mastitis and that the organisms were excreted in large numbers in milk before there were any obvious signs of disease in the cow. However, only a few of what have been considered natural cases of campylobacter mastitis have so far been reported (Hudson et al.,1984; Morgan, 1984) and therefore there is no clear evidence yet to show that the infected udder is an important source of C.jejuni in milk. Finding of C.jejuni in relatively large numbers in the faeces of normal cows and none in

mastitic milk samples supports the theory of faecal contamination (Boer et al.,1984; Waterman et al.,1984). However, the general conclusion that the udder is not an important source of C.jejuni should be considered in light of the probable insensitive culture techniques for recovering the organisms from the milk and the minimum infective dose to establish an infection.

The mode of infection in human beings is by ingestion of the organisms in contaminated water or foodstuffs, for example milk in this case. Perhaps a large dose is necessary for a sizeable number of organisms to reach the intestines and establish infection when it is considered that a large proportion would be killed in the stomach by the hydrochloric acid. Rapid destruction of C.jejuni by hydrochloric acid pH 2.3 was demonstrated by Blaser et al.(1980) indicating that gastric acid is an effective barrier against infection. The type of food taken with the campylobacters would offer protection and milk which has a very strong buffering capacity serves that purpose. As few as 500 C.jejuni organisms swallowed in 180 ml of milk by a volunteer have been reported to cause clinical symptoms of enteritis (Robinson, 1981). However, it is not known for sure what the minimum infective dose is.

Recently, Klein et al.(1986) reported an outbreak of campylobacter enteritis involving both C.jejuni and C.fetus subsp. fetus in which raw bovine milk was epidemiologically

implicated. This was the first time that enteritis involving C.fetus subsp. fetus had been associated with consumption of raw milk. The strain of C.fetus subsp. fetus involved was thermotolerant being capable of growing at 42°C. Perhaps the importance of C.fetus subsp. fetus as an enteric pathogen is underestimated since isolation methods commonly used do not cater for its isolation.

b. Poultry

Poultry constitutes by far the greatest potential source of campylobacters. Since a large percentage of chickens (Grant et al.,1980) and turkeys (Luechtefeld & Wang, 1981) harbour large numbers of C.jejuni in their intestinal tracts, the chances of contamination of carcasses during evisceration are similarly high. Grant et al. (1980) reported recovery of as many as 4.4×10^6 C.jejuni/g faeces of broiler chicken. The extent of contamination of poultry with C.jejuni was shown by Dawkins et al.(1984) in a study of the spread of C.jejuni through four large kitchens. The organism was isolated from fresh birds, frozen birds, giblets, thawed juices and work surfaces. This study shows the kind of risk that exists in catering establishments handling raw chicken. Improper cooking or cross contamination from raw to cooked foods can be envisaged as occurring with important consequences similar to those that have been demonstrated in some outbreaks of salmonella food poisoning.

Sporadic cases of enteritis in human beings that have been traced to poultry have probably arisen through handling the raw product rather than from eating the cooked flesh (Skirrow, 1977). A small outbreak attributed to eating undercooked chicken at a dinner dance was reported by Skirrow et al.(1981). Brouwer et al. (1979) reported a large outbreak involving army recruits who had themselves to kill, prepare and cook a chicken in the field.

c. Red meats

Contamination with C.jejuni at slaughter may be large but numbers found on carcasses especially in the retail shops are generally very low (Skirrow, 1982; Turnbull & Rose, 1982), an indication of low survival rate. Drying in air during cooling in abattoirs has been reported to reduce substantially the number of campylobacters on the surface of pig carcasses (Oosterom, 1985).

2. Transmission by contaminated water

Unchlorinated water is an important vehicle for transmission of campylobacter infection when one considers the large number of people who can become infected. Outbreaks which have occurred have proved this point. Vogt et al.(1982) reported a large outbreak of acute gastroenteritis in Vermont, U.S.A. involving about 2,000 of the town's 10,000 inhabitants.

The illness was strongly associated with consumption of water. Other outbreaks include one affecting about 2,000 people in central Sweden (Mentzing, 1981) and one affecting 234 pupils and 23 staff at a boarding school in England (Palmer et al., 1983). In this last case, C.jejuni was recovered from the water supply. Faeces from birds roosting on water tanks, or contamination by sewage have been suggested as the most likely sources of contamination of water.

3. Transmission by direct animal contacts

Apparently campylobacter infections occasionally can be acquired through direct contact with carrier or sick animals, mainly household dogs and cats. Campylobacter enteritis has been reported in children and adults who have had close contacts with dogs suffering from diarrhoea (Skirrow, 1977; Blaser et al.,1978). Recently acquired puppies are more commonly involved than fully grown dogs. Cats may also transmit Campylobacter to human beings, but they appear less important in this respect (Blaser et al.,1984).

Infection can be acquired from farm animals. Perhaps the best evidence of transmission of Campylobacter to man is the case reported by Duffel & Skirrow (1978) in which a farmer gave "a kiss of life" to a moribund born lamb and came down with acute enteritis. The organisms from both farmer and lamb

belonged to the C.coli/C.jejuni groups of Véron & Chatelain (1973). Serotypes and biotypes were apparently not determined.

4. Person to person transmission

Cases of person to person transmission of Campylobacter infection indicate transmission from adults to infants and vice versa. In utero transmission from mother to the baby (CDR 79/17, quoted by Blaser et al, 1984) has been indicated by isolation of C.jejuni from the blood of a baby shortly after delivery by caesarean section. The baby developed diarrhoea three days after delivery and C.jejuni was isolated from the faeces of both mother and baby. New-born infants may also acquire C.jejuni infection from their mothers, most likely at the time of birth (Veskari et al., 1981).

Blaser et al. (1981) reported cases of campylobacter enteritis in two extended families. Family members had close contact with each other, particularly babies with diarrhoea. In these cases the method of spread of the disease among family members was apparently by person to person transmission rather than by food or drink. These are rare and on balance one can say person to person contact is of no importance in spread of the disease.

Much epidemiological information has become available which in fact often emphasizes inadequacies of existing cultural or typing techniques. But it is fair to say that we are still a long way from a comprehensive understanding of the spread of enteritis due to C.jejuni and its relatives.

CHAPTER 3

GENERAL METHODS

3.1 MEDIA

3.1.1 Sterilization by autoclaving

Unless otherwise stated, sterilization by autoclaving was at 121°C (15 lb./in ^{2}) for 20 min.

3.1.2 Sterilization by filtration

Items to be sterilized by filtration were usually passed through a 0.2 um pore size membrane filter (Schleicher & Schill, Dassel, W.Germany). Antibiotic preparations which contained methanol were sterilized using a seitz filter (Carlson-Ford Ltd., Ashton).

3.1.3 Pouring and drying of plates

Molten agar, cooled to 50°C in a waterbath after autoclaving, and supplemented with the additives as required, was poured into petri dishes (90 mm diameter; Sterilin Ltd.) in a laminar-flow cabinet, 15 to 20 ml per plate (unless otherwise stated) and allowed to solidify and dry for 45 to 60 min. Plates were used fresh, or stored at 4°C in the dark and used within 2 days.

3.1.4 Preparation of routine media

Blood agar base (BAB; Oxoid No.2), nutrient broth (NB; Oxoid No.2), heart infusion broth (HI; Difco) and brain heart

infusion broth (BHI; Oxoid) were prepared according to the instructions of the manufacturers. Dispensing before autoclaving was into bottles in appropriate volumes, usually 200ml or 400ml for pouring plates or in 5ml volumes in 28g McCartney bottles for other purposes. Heart infusion agar (HIA) was made by solidifying HI with 12 gl^{-1} agar (Oxoid No.3) before autoclaving. Each medium was mixed well after autoclaving to distribute the agar evenly. When a medium was required for counting colonies, agar content was increased to 1.5% (w/v) to restrict swarming of colonies.

1. Plain agar

Agar No.3 (Oxoid), 12g, in demineralized water, 1l, was adjusted to pH 7.0 and distributed into bottles in 50 ml volumes before autoclaving.

2. Peptone water (PW; 0.1%)

Peptone (Oxoid), 1g and NaCl, 5g were mixed in 1 l of demineralized water, adjusted to pH 7.2 and distributed into 15mm x 150mm test-tubes in 9 or 10 ml volumes before autoclaving.

3.1.5 Media containing blood

Defibrinated blood (horse or sheep;Tissue Culture Services, Slough), was added whole, unless specified, to give

a final concentration of 5% (v/v) in various media. These were horse blood agar (HBA; BAB + blood); horse blood heart infusion agar (HBHA; HI + blood); sheep blood agar (SBA; BAB + blood) and sheep blood heart infusion agar (SBHA; HIA + blood).

When washed horse or sheep red blood cells (HRBC or SRBC) were required, they were sedimented by centrifugation, washed three times in sterile saline (0.85% NaCl) and re-suspended to the original volume in the same saline before adding to media in the same concentration as above.

1. Lysis of blood for media

For some purposes, lysed blood was required. Saponin powder (Sigma; 10g) was dissolved in 100 ml of demineralized water in a 250 ml conical flask, filter sterilized and dispensed into bijoux bottles in 5ml volumes for storage at -20^{o}C. After preliminary experiments, the following procedure was used for lysing blood. When required, the saponin solution (1 part) was added to blood (50 parts), mixed and incubated at 37^{o}C in a waterbath for 5 min, usually before addition to media.

2. Media containing lysed blood (5% v/v)

NB was supplemented with saponin-lysed horse blood and ferrous sulphate, sodium metabisulphite and sodium pyruvate (FBP) to make a non-selective enrichment broth (NSEB),

and with antibiotics to make selective Preston broth (PB; Bolton & Robertson, 1982). The broths were aseptically dispensed into bottles in the required amounts.

NA was supplemented with saponin-lysed horse blood, FBP and antibiotics to make Preston agar (PA).

3.1.6 Preparation of FBP supplement (George et al., 1978)

Ferrous sulphate, 4g; sodium metabisulphite, 4g and sodium pyruvate, 4g were dissolved together in 100ml demineralized water, sterilized by filtration and dispensed into bijoux bottles in 5ml volumes, stored frozen at -20°C and thawed when required. For media, the supplement was added to give 0.05% (w/v) final concentration of each ingredient.

3.1.7 Preparation of antibiotics supplement (Bolton & Robertson, 1982)

Stock solution was prepared as follows: rifampicin (Sigma), 100 mg, was first dissolved completely in 50 ml methanol. Then polymyxin B sulphate (Sigma), 50,000 iu; trimethoprim lactate (Sigma), 100 mg and cycloheximide (Sigma), 1000 mg were added and the solution made to 100 ml with demineralized water. The solution was filter sterilized, dispensed into bijoux bottles in 4 ml amounts and frozen at -20°C. It was used in the media to give final concentration of: rifampicin, 10 mg l^{-1}; polymyxin B, 5000 iu l^{-1}; trimethoprim, 10 mg l^{-1} and cycloheximide, 100mg l^{-1}.

3.1.8 Ferrous sulphate, sodium metabisulphite and sodium pyruvate broth (FBP)

NB was supplemented with agar (Oxoid No.3; 1.2 g l^{-1}) and FBP. For testing H_2S production (Skirrow & Benjamin, 1982) the medium was dispensed into bijoux bottles in 4 ml volumes. When required for storage of campylobacters at -70°C or -20°C, the ingredients were added to 85% NB plus 15% glycerol (v/v).

3.1.9 Yeast extract aspartate nutrient agar (YNA) and semisolid YNA (SYNA)

NB was supplemented with (g l^{-1}): agar (Oxoid No.3), 15 (YNA) or 2 (SYNA); yeast extract (Difco), 3; potassium-L-aspartate (Sigma), 2; haematin (Sigma; Razi & Park, 1979), 0.001 (YNA). SYNA was dispensed into 28g McCartney bottles in 15 ml volumes before autoclaving. If not used on the day of preparation, before inoculation it was steamed for 10 min and cooled. Filter sterilized stock solution of haematin was made by dissolving 0.1g in 1 ml 1M NaOH and making up to 100 ml with sterile demineralized water. It was stored at 4°C in 10 ml volumes in 28g McCartney bottles and for routine use 1 ml was added to 100 ml of YNA.

3.1.10 Media for H_2S production and glycine tolerance in brucella broth

Brucella broth (BB; Difco) was made semisolid by adding 3g l^{-1} agar (Oxoid No.3). For various tests ingredients (Park et al., 1962) were added and media dispensed into 28g McCartney bottles in 15 ml volumes before autoclaving. If not used on the day of preparation, before inoculation media were steamed for 10 minutes and cooled.

3.2 GASEOUS CONDITIONS FOR INCUBATION

Inoculated solid or liquid media were incubated under a microaerobic condition created by evacuating an anaerobic jar (without a catalyst) to 500 mm Hg below atmospheric pressure and refilling with 95% H_2/5% CO_2 or alternatively 95% N_2/5% CO_2.

For some purposes media were incubated aerobically with bottle caps screwed tight. Sometimes anaerobic incubation was required. This was achieved by evacuating an anaerobic jar with a catalyst to 650 mm Hg below atmospheric pressure and refilling with 95% H_2/5% CO_2 three times, unless stated otherwise. BA freshly inoculated with Pseudomonas aeruginosa was included each time as a control for anaerobiosis.

3.2.1 Temperature of incubation

Cultures were routinely incubated at 42°C (37°C for C. fetus subspecies).

3.3 BACTERIAL STRAINS

3.3.1 Source of Campylobacter strains

A large collection of strains was available in the department, kindly provided by Dr. Fricker, Mrs. Georges, Dr. Park all of this department, and Mr. Morgan of I.R.A.D. Compton. Some strains were obtained from the National Collection of Type Cultures (NCTC). In addition, a large number of strains was isolated during this study from various sources. Species and subspecies represented were:

1. C.jejuni human isolates and NCTC 11168 and 11322
2. C.coli pig isolates and NCTC 11350 and 11366*
3. C.laridis seagull isolates and NCTC 11352* and 11458
4. C.fetus subsp. fetus bovine and ovine isolates and NCTC 10842* and 10348
5. C.fetus subsp. venerealis bovine genital strains and NCTC 10354 (designated G212).

* indicates type strain.

3.3.2 Receipt of the bacterial cultures

Freeze dried campylobacters were suspended in NB, then streaked on HBA and also inoculated into NSEB in bijoux bottles. The NSEB was incubated with bottle caps screwed tight for 24h at 37°C and then streaked on HBA. Inoculated plates were incubated microaerobically at 37°C for 48h or longer if necessary.

Frozen cultures (from -20°C or -70°C) were thawed in air at room temperature and promptly plated on HBA.

3.3.3 Storage and maintenance of campylobacter cultures

A lawn of a 24h or 48h culture was removed with a wire loop and inoculated as a lump into FBP broth in a 2 ml storage vial. This was then frozen at -70°C and/or -20°C. Cultures were also maintained at 37°C in SYNA with bottle caps screwed tight and used as readily available stock. They were subcultured fortnightly. Bottles opened for use were replaced with freshly inoculated ones.

3.4 DIAGNOSTIC AND DIFFERENTIAL TESTS

3.4.1 Recognition of catalase-positive campylobacters

To be recognized as a catalase-positive Campylobacter a culture had to consist of Gram-negative, curved, S-shaped or

helical organisms, to possess a darting, corkscrew-like motility, to not attack carbohydrates, to show growth only at or near the surface of semisolid medium and to be oxidase positive and catalase positive.

3.4.2 Differential tests

Unless otherwise stated differential tests were as described by Skirrow & Benjamin (1982) and Skirrow *et al.* (1982). Tests were performed on young (usually 24h) cultures.

1. Hippurate hydrolysis

A uniformly inoculated HBA was swabbed and a dense suspension of the test organism made by adding growth to 2.0 ml 0.1M phosphate buffer pH 7.0 + 0.5 ml freshly made 5% (w/v) aqueous sodium hippurate and mixed by rotamixing. After incubation aerobically at 37°C in a waterbath for 2h, 1 ml freshly made ninhydrin solution (3.5g ninhydrin in 100 ml 1:1 mixture of acetone and butanol) was added gently without mixing and bottles left at room temperature for 2h. Development of a deep purple colour was recorded as positive. Positive and negative NCTC strains, usually *C.jejuni* 11168 and *C.coli* 11350 were included as controls in each test.

2. H_2S production in FBP broth culture

Growth from a 24h (or older if growth was not enough at 24h) was inoculated as a lump into FBP broth, incubated at 37°C for 4h and then examined for blackening around the lump of bacteria, an indication of a positive reaction.

I often found it difficult to pick a lump of bacteria with a wire loop from a swarming culture and place it successfully inside the medium, so I overcame this problem by swabbing the growth with a cotton wool swab moistened in sterile saline and breaking it inside the bottle of FBP broth. Blackening occurred around the cotton swab in positive cases.

3. Resistance to nalidixic acid (NX) and cephalothin

A zone of inhibition equal to or greater than 6 mm around a 30 ug disc of NX or cephalothin (Sigma) on a uniformly inoculated HBA plate was considered to indicate sensitivity.

4. H_2S production in cystine-containing medium and glycine tolerance (Park et al., 1962)

The test organism was inoculated into (a) BB alone (control) (b) BB + 0.02% (w/v) L-cystine (Sigma) and (c) BB + 1% (w/v) glycine (Sigma). Lead acetate paper was hung above the medium in (a) and (b), held in place by the bottle

cap screwed down tight. Bottles were incubated aerobically at 37°C and examined for growth and blackening of the paper strips daily, changing the blackened papers, for seven days.

5. Growth at various temperatures

The strains to be tested were inoculated on freshly prepared HBA plates and incubated in a microaerobic environment containing hydrogen in an incubator at 25°C, or in stirred water-baths set at 37°C (control), 42°C and 45°C using mercury thermometers of certified accuracy. Temperatures were checked at least once daily; plates were incubated for up to 5 days.

3.4.3 Serotyping of C.jejuni by passive haemagglutination (PHA)

The procedure was as described by Penner & Hennessy (1980) with some modifications recommended by Fricker et al. (1987).

1. Antisera

Titrated Penner antisera raised in rabbits against Penner strains of C.jejuni were kindly provided by Dr. Fricker,

Department of Microbiology, University of Reading, who helped by giving advice and by typing some of the strains.

2. Preparation of soluble heat-stable antigens

Bacterial growth from a swabbed HBA plate incubated for 24h at 42°C was harvested into 2 ml saline (0.85% NaCl) and heated for 1h at 100°C. Cells were sedimented by centrifugation and the supernatant fluid used in the PHA.

3. Sensitization of sheep red blood cells (SRBC)

To I ml 1% SRBC, washed three times in PBS, was added 0.25 ml of the antigen preparation. After rotamixing and incubating at 37°C for 1h, the sensitized SRBC were washed three times in PBS and made up to the original volume.

4. Testing procedure

Antiserum, 25 ul, was mixed with an equal volume of sensitized SRBC in a round bottomed well of a microplate with 96 wells (Flow Laboratories). Plates were then shaken, incubated at 37°C for 1h and left at 4°C overnight before examining for haemagglutination.

The campylobacter strains were tested against 25 Penner antisera which were available in our laboratory at the time and which included serotypes most common in both man and

animals. Each strain was first tested against pools of antisera, five in a pool, constituted as follows:- pool one: 1, 2, 3, 4 and 5; pool two: 6, 7, 8, 9 and 10; pool three: 11, 15, 16, 18 and 19; pool four: 20, 23, 24, 27 and 30 and pool five: 31, 35, 37, 44 and 55. A strain which reacted with a pool of antisera was then tested against all the individual antisera in the pool to identify the serotype.

3.5 COUNTING METHODS

3.5.1 Colony count by using spread plate

Decimal dilutions of the sample were made in 0.1% PW unless stated otherwise and 0.1 ml spread on the agar surface using a sterile curved glass rod and incubated. Plates with countable colonies i.e. between 30 and 300 per plate were chosen for counting.

3.5.2 Colony count by using a spiral plater

Spiral plater model D (Don Whitley Scientific Ltd.) was used. About 2-3 ml of the liquid culture or bacterial suspension were placed in a spiral-plater cup and plated as described by the manufacturer. If dilution of the culture was not already done and was deemed necessary before spiral

plating, one drop (0.02 ml) from a 50-dropper pasteur pipette was mixed with 2 ml diluent in a spiral plater cup to make 100 fold dilutions. After incubation of plates colonies were counted according to the instructions of the manufacturer.

3.6 ATMOSPHERE FOR INCUBATION OF THE ENRICHMENTS FOR THERMOPHILIC CAMPYLOBACTERS

Enrichment of samples prior to plating has been shown to increase recovery rates of thermophilic campylobacters from a number of sources (Fricker et al., 1983; Hutchinson & Bolton, 1983; Rosef et al., 1983; Wesley et al., 1983) and this is epidemiologically very useful when the number of organisms in the sample is small. Incubation is either in air with bottle caps screwed tight or in a microaerobic condition created by replacing part of the air with a mixture of hydrogen and carbon dioxide, or nitrogen and carbon dioxide. These gaseous conditions were investigated, using campylobacter positive human faecal samples, to see which one would be most suitable for enrichment of samples by increasing recovery rates.

Each sample was diluted tenfold up to 10^{-9} in PB in triplicate and one set each of bottles incubated in (a) air alone (b) 1/3 air + 2/3 95% H_2/5% CO_2 and (c) 1/3 air + 2/3 95% N_2/5% CO_2 at 42°C. The dilutions 10^{-1} to 10^{-9} were also plated directly on PA, incubated at 42°C and the highest

positive dilutions after 24h used to choose the enriched dilutions (24h) for colony counting on PA using a spiral plater. Plates were incubated for 48h in a microaerobic condition containing hydrogen or nitrogen, or duplicate plates were incubated each in one of the two conditions.

The suitability of the enrichment method was judged by the number of colony forming units (cfu) of campylobacters after 24h enrichment of the sample. Differences in cfu under hydrogen and nitrogen enrichment conditions were small although enrichment under hydrogen appeared slightly better. The two methods were considered to be similar to one another in performance and better than aerobic enrichment (Table 3.1). The effect of enriching broths in one gaseous condition and incubating plates inoculated with the broths in a different gas mixture was tested (Table 3.2). The number of cfu of the two samples used showed no marked differences. It was concluded that there is no advantage of using two different gaseous conditions, one for enrichment of broths and another for incubation of plates. Hereafter in this thesis, either hydrogen and carbon dioxide or nitrogen and carbon dioxide gas mixtures were used in microaerobic conditions.

Table 3.1 Suitability of three gaseous conditions for enrichment of samples for thermophilic campylobacters as compared using human faeces

Plates incubation gas mixtures: 1/3 air+2/3 of →	Enriched dilutions which were counted		cfu ml^{-1} after 24h enrichment in air (1/3) plus 2/3 of:-			
			N_2/CO_2 #	H_2/CO_2 *	air	
	N_2/CO_2 #	H_2/CO_2 *	N_2/CO_2 #	H_2/CO_2 *	N_2/CO_2 #	H_2/CO_2 *
Sample						
BH 25	10^{-3}	10^{-3}	$4.1x10^6$	$1.1x10^7$	$3.2x10^6$	$2.5x10^4$
BH 26	10^{-2}	10^{-3}	$4.1x10^6$	$1.8x10^8$	$9.5x10^7$	$1.1x10^7$
BH 27	10^{-2}	10^{-3}	$1.3x10^7$	$1.1x10^7$	$3.4x10^7$	$5.5x10^4$
BH 28	10^{-3}	10^{-3}	$1.6x10^6$	$1.5x10^6$	$8.2x10^5$	$1.8x10^3$
BH 29	10^{-5}	10^{-5}	$9.3x10^6$	$4.4x10^6$	$2.7x10^4$	$3.5x10^6$
BH 30	10^{-3}	10^{-3}	$1.5x10^5$	$5.5x10^7$	$1.3x10^5$	$4.4x10^7$
BH 31	10^{-3}	10^{-4}	$1.5x10^7$	$7.6x10^7$	$1.5x10^7$	$5.6x10^4$
BH 32	10^{-2}	10^{-3}	$1.8x10^8$	$3.3x10^8$	$5.4x10^7$	$4.0x10^5$
BH 34	10^{-6}	10^{-5}	$1.1x10^7$	$3.4x10^7$	$5.3x10^4$	$4.0x10^3$
BH 39	10^{-4}	10^{-4}	$8.4x10^7$	$1.5x10^8$	$1.4x10^4$	$1.0x10^7$
BH 40	10^{-4}	10^{-4}	$3.4x10^8$	$8.0x10^8$	$9.3x10^7$	$1.3x10^5$
BH 41	10^{-3}	10^{-3}	$4.3x10^7$	$4.9x10^7$	$9.4x10^4$	$1.5x10^4$
BH 47	10^0	10^{-2}	$1.4x10^6$	$1.4x10^6$	$1.3x10^5$	$1.2x10^5$
BH 68	10^{-2}	10^{-2}	$8.6x10^4$	$1.5x10^3$	N/G	N/G
BH 74	10^{-4}	10^{-5}	$2.0x10^7$	$3.5x10^7$	$1.4x10^4$	$1.3x10^4$

cfu = Colony forming units; # = 95% N_2/5% CO_2;

* = 95% H_2/5% CO_2; N/G = No growth.

Plates incubation gas mixtures 1/3 air + 2/3 of:- →	Enrichment dilu--tions which were counted		cfu ml^{-1} after 24h enrichment in air (1/3) + 2/3 of:-					
			N_2/CO_2 #		H_2/CO_2 *		air	
	N_2/CO_2 #	H_2/CO_2 *	N_2/CO_2 #	H_2/CO_2 *	N_2/CO_2 #	H_2/CO_2 *	N_2/CO_2 #	H_2/CO_2 *
Sample								
BH 233	10^{-4}	10^{-4}	$1.5x10^8$	$1.3x10^8$	$2.7x10^8$	$1.8x10^8$	$1.1x10^8$	- †
BH 236	10^{-4}	10^{-4}	$7.6x10^7$	$3.1x10^8$	$4.6x10^8$	$3.9x10^8$	$1.8x10^7$	$2.0x10^7$

cfu = Colony forming units

= 95% N_2/5% CO_2

* = 95% H_2/5% CO_2

† = Spoilt plates

CHAPTER 4

ISOLATION OF CAMPYLOBACTERS

SECTION 1

4.1 OCCURRENCE OF CAMPYLOBACTERS IN MAN, ANIMALS, MEAT AND OFFAL, AND SEWAGE

4.1.1 INTRODUCTION

Campylobacter enteritis in man has emerged in recent years as the commonest detected bacterial enteritis. C.jejuni and C.coli, the commonest causative agents, have been isolated from a wide range of animal species (Luechtefeld et al., 1981; Rosef et al., 1983), their products, and water (Knill et al., 1982; Turnbull & Rose, 1982; Bolton et al., 1985). C.jejuni biotype I is reportedly more common in both human beings and animals than biotype II, and serotypes 1, 2 and 4 are more prevalent in both situations (Jones et al., 1984; Lauwers & Penner, 1984; Bänffer, 1985; Rosef et al., 1985; Lastovica et al., 1986). Bolton et al. (1985) reported that C.jejuni strains isolated from river water downstream from sewage effluent discharge sites were frequently of the same serotypes as those isolated from human faeces. Despite some well documented epidemiological studies incriminating milk (Jones et al., 1981; Hutchinson et al., 1985) or water (Mentzing, 1981; Palmer et al., 1983) as the vehicle of spread, the vast majority of cases of campylobacter enteritis in man has no proven source; the complex cycle of campylobacter infections is not yet well understood.

It was decided to look at the occurrence of thermophilic campylobacters in various situations, partly to assess how often they occur, and partly to obtain strains for detailed

biotyping and serotyping studies. In this way I hoped to obtain a reasonable idea of the distribution and the types of the organism in some important situations in the cycle of campylobacter infections. The samples chosen for this study were from several sources. (1) Human diarrhoeal faeces, readily obtainable from the Royal Berkshire Hospital, Reading. (2) Guts from piglets; nutritional experiments at Food Research Institute (F.R.I.), Reading, involving feeding very young piglets with yoghurt offered me the opportunity to examine their guts. (3) Packaged beef, pork, lamb and chicken meat and offal from food stores. (4) Sewage.

4.1.2 MATERIALS AND METHODS

1. Samples

a. Human faeces

Faecal samples from known cases of gastroenteritis positive for campylobacters were obtained from the Department of Pathology, Royal Berkshire Hospital, Reading on a weekly basis over a period of eighteen months. Samples had been suspended in 3 ml of 0.1% PW and stored at $4^{o}C$ for up to one week. In addition, campylobacter negative samples were collected.

b. Pig guts

Pieces of ileum and colon (ca 15cm to 20cm long) complete with gut contents, from each of the 12 piglets used in yoghurt feeding experiments at F.R.I., Reading, were kindly provided by Dr. R. Fuller. The piglets were weaned two days after birth onto yoghurt (6) and base milk (6; controls) and killed after 14 days. The guts were received in our laboratory within 3 hours of killing of the piglets.

c. Meat and offal

Frozen or refrigerated beef, lamb, pork and chicken were purchased from Reading food stores.

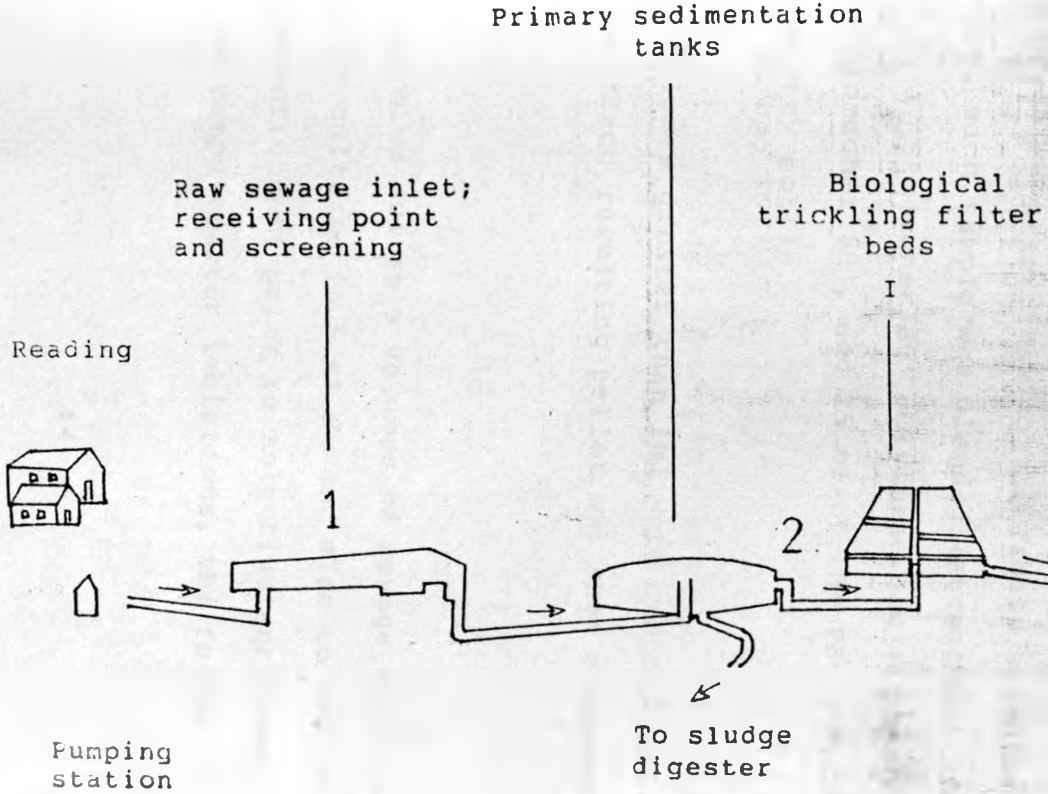
d. Sewage

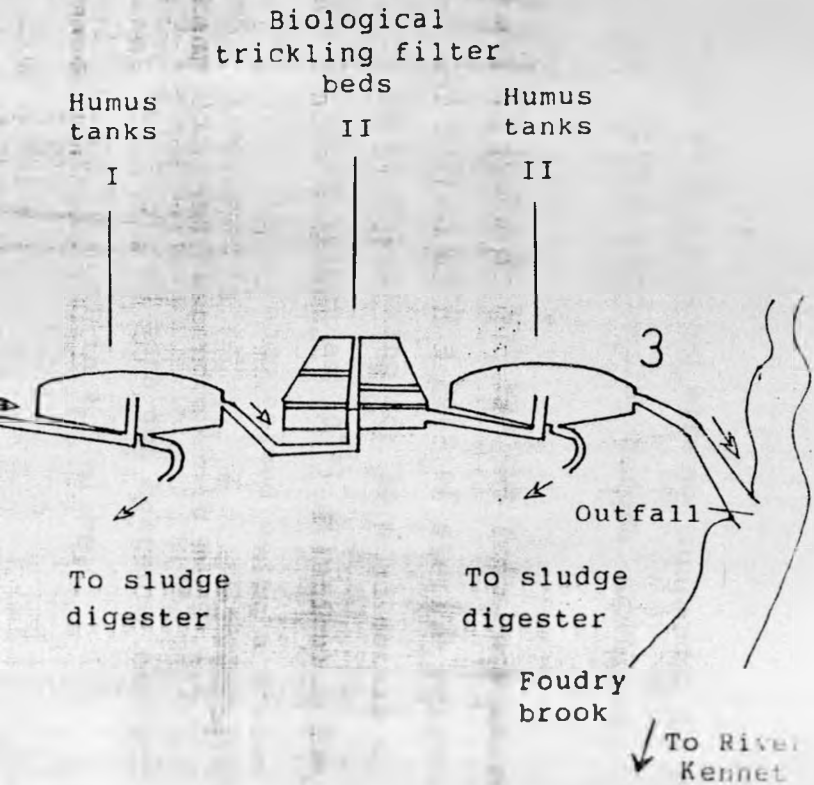
Samples (500ml) of incoming sewage, primary sedimentation effluent and final effluent were collected at the points of turbulence in sterile bottles from Reading trickling filter sewage treatment works as shown in Fig. 4.1.

2. Processing of samples

All the samples were processed and cultured within 2h of arrival in this laboratory.

Fig. 4.1 : A plan of Manor farm, Reading trickling filter sewage treatment works showing sample collection points for examination for thermophilic campylobacters. Sampling points are indicated as 1, 2 and 3. Raw sewage is pumped to the receiving point from which it flows through the system by gravity with a mean residence of 7h.


Primary sedimentation tanks
Raw sewage inlet; receiving point and screening
Biological trickling filter beds
I
Reading
1
2
Pumping station
To sludge digester


Biological
trickling filter
beds
II
Humus
tanks
I
Humus
tanks
II
3
Outfall
To sludge
digester
To sludge
digester
Foudry
brook
To Rive
Kennet

Human faeces. These were received already suspended in PW and did not require further processing before culturing.

Pig guts. A 3cm long piece of gut was cut from each ileum and colon sample, split open with a pair of sterile scissors and washed in 10 ml PB by rotamixing for 30 s to obtain a suspension of gut contents (taken as 10^{-1}) dilution). The gut wall was washed two more times, homogenized using a tissue grinder (Gallenkamp Co.) and suspended in 10 ml PB (10^{-1} dilution). Decimal dilutions of both gut contents and gut wall were then made in PB (0.5 ml in 4.5 ml PB).

Meat and Offal. After thawing the frozen samples at room temperature, each sample was blended (stomached) for 30 s in 50 ml PB in a 'Stomacher 80' polythene bag (10cm x 15cm) using Colworth 'Stomacher 80', and 25 ml of the PB transferred into a 28g McCartney bottle.

Sewage. Volumes greater than 1ml were centrifuged at 20,000g for 15 min. Each resulting pellet was then suspended in 5 ml NSEB.

To determine suitable volumes of sewage which could efectively be cultured in 5 ml PB and also to determine whether pre-enrichment prior to selective enrichment increases frequency of campylobacter isolations, the following sets of

broths (NSEB) were prepared for each sample: (1) four sets of four broths each containing the bacteria in 10 ml, 1.0 ml, 0.1 ml and 0.01 ml of incoming sewage (2) four sets of three broths each containing the bacteria in 10 ml, 1.0 ml and 0.1 ml of primary sedimentation efluent and (3) four sets of three broths each containing the bacteria in 100 ml, 10 ml and 1.0 ml of final effluent. A total of 25, 22 and 25 samples of incoming sewage, primary effluent and final effluent respectively were examined.

Hourly samples taken from 0600 to 2000 daily for 5 consecutive days were processed within one hour after collection for enumeration of campylobacters by the most probable number (MPN) method (Bolton et al., 1982) to assess reductions in numbers by the sewage treatment processes at different stages. Each sample was inoculated into 10x5ml NSEB. On the basis of experience from preliminary experiments volumes used per bottle were: incoming sewage, 5ul; primary sedimentation effluent, 50 ul and final effluent, 10 ml (centrifuged).

c. Culture of samples

Human faecal samples known to be positive for campylobacters were streaked directly on PA. Samples received as negative were each enriched in PB (0.5 ml in 4.5 ml PB) under each of the three gaseous conditions (see Chapter 3.6)

to compare further the ability of the three enrichment method to recover campylobacters. The number of campylobacters in the pig gut contents and gut wall was estimated by enriching the dilutions in PB, plating on PA and noting the highest positive dilution from which campylobacters were isolated. Colony counts were also made by spreading 0.1ml on PA.

To determine suitable inoculum sizes for and the effect of pre-enrichment on the recovery of thermophilic campylobacters from incoming sewage, primary sedimentation effluent and final effluent, processed samples were treated a follows. To one set of each dilution, antibiotics were added at time zero (0h) and all four sets of dilutions incubated at 42°C. Of the remaining three sets of broths, one was removed from the incubator after each of 1h, 2h and 4h, antibiotics added and then the broths re-incubated. Enrichments were plated on PA after 24h and 48h.

For enumeration of campylobacters by the MPN method, inoculated NSEB were pre-enriched for 2h, antibiotics added, and the broths re-incubated and plated on PA after 48h. The 2h pre-enrichment was used because preliminary tests showed i to be slightly better than direct enrichment in recovering th campylobacters from sewage.

Unless stated otherwise, incubation of broths and plate in the above situations was done microaerobically in gas

mixtures containing hydrogen at 42°C for 24h (broths) or 48h (plates).

4.1.3 RESULTS

1. Campylobacters in human faeces

Of the 105 samples received as negative for campylobacters from the hospital, which were examined by the enrichment method, five were detected as positive for C.jejuni (referred to as 'late positive'). The methods by which recoveries were made are shown in Table 4.1. All these five 'late positive' samples were positive by hydrogen enrichment after 24h which showed a slightly better recovery than nitrogen or air enrichment, although, because of the small number of positives, it is not possible to make a good comparison. Clearly it is useful to enrich when attempting to recover campylobacters from human faeces. It is possible that the isolation procedure used at the hospital was not able to detect campylobacters in the five samples. I do not know if any other cause of the diarrhoea was found by the hospital for these five cases.

Biotyping of 107 isolates from 102 campylobacter positive and 5 campylobacter 'negative' (late positive) diarrhoeal faeces was done and 106 (99.1%) were hippurate positive indicating that they were C.jejuni. Of these, 75 (70.1%) were

biotype I (Table 4.2). C.coli was not common; in fact only one was isolated.

Of the 73 C.jejuni strains which were serotyped with the 25 Penner antisera, 67.1% were typable. Strains which reacted with antisera to Penner 4 almost invariably reacted with antisera to Penner 16 and vice versa. Thus these strains were grouped together and were the most frequent (20.5%). The next most frequent was serotype 2 (12.3%), followed by serotypes 6 and 19 (6.8% each). Distribution of the serotypes, together with those of isolates from sewage, is shown in Table 4.3.

2. Campylobacters in pigs

Campylobacters were commonly recovered from eight of the 12 piglets (Table 4.4) either from the ileum or colon or both and often in relatively low numbers (range 20 to 10^6) per 3cm long gut (Table 4.5). The colon appeared to be more heavily colonized and although feeding yoghurt to the piglets was reported to suppress Escherichia coli count in the stomach and duodenum (Fuller, 1986), the campylobacters in the ileum and colon were apprently unaffected by the yoghurt diet. However, in the cases of the animals we examined, there was some uncertainty about some prior treatment of the piglets. It may be that some had received prior antibiotic treatment which

might well remove campylobcters (Fernie et al., 1975) thus making our negative results unreliable.

The 16 isolates were biotyped and all were C.coli (hippurate -; H_2S -).

3. Campylobacters in meat and offal

C.jejuni and C.coli were recovered from a relatively large proportion of samples (Table 4.6). Chicken had the greatest proportion of positive samples, 29/33 (88%) and pork the smallest, 5/20 (25%). C.jejuni biotype I was the commonest isolate from chicken, lamb and beef. Pork yielded only C.coli. Although some of the samples were received in frozen condition and others in chilled condition, no attempt was made to categorize them as either frozen or chilled because it was not known whether the chilled samples had been frozen previously, a situation which is apparently common with this type of material.

4. Campylobacters in sewage

(i) Effect of inoculum size, direct enrichment and pre-enrichment on recovery rates.

The effects of inoculum size, direct enrichment and pre-enrichment on the recovery of campylobcters from sewage are

shown in Table 4.7. In general, the results show that:-

1. More positive results occur earlier (24h) than later (48h) with large inocula possibly because campylobacters are already present in large numbers.

2. Samples rich in contaminating bacteria tend to become positive earlier and negative later.

3. Early positive samples tend to become negative at 48h presumably because contaminating microorganisms outgrow them producing an inhibitory effect.

4. More positive results occur late (48h) with small inocula; the number of campylobacters is presumably small and so a long enrichment period is required for multiplication to detectable level.

5. Both direct enrichment and the 2h pre-enrichment methods appear suitable for recovering campylobacters from sewage. However, 2h pre-enrichnment seems more suitable for small inocula containing few contaminants, and direct enrichment more suitable for large inocula containing large number of contaminants.

The results (Table 4.7) suggest that to achieve good recovery of campylobacters from sewage (using 5ml of PB) the

following combinations of parameters would be suitable prior to plating.

a. Crude sewage: 24h direct enrichment of 1.0ml

b. Primary sedimentation effluent: 2h pre-enrichment of 0.1ml for a total of 48h incubation time.

c. Final effluent: 24h direct enrichment of precipitate from 100ml.

These suggestions, which reflect the situation of the sewage during a dry week in September 1985 when sampling was done, may be subject to variations in concentrations of campylobacters and contaminating microorganisms.

ii Number of campylobacters in the sewage and their removal by treatment processes.

The mean number (MPN) and ranges of campylobacters in sewage at the three stages of treatment (plus the incoming sewage flow rates) are shown in Table 4.8 and their hourly fluctuations illustrated in Fig. 4.2. Campylobacter numbers reached their peaks at 1200, 1400 and 1900h in incoming sewage (2h after peak influent flow), primary sedimentation effluent

and final effluent respectively. The peak hours suggested the following mean sewage retention times: (i) from crude influent to primary sedimentation effluent 2h, (ii) from crude influent to final effluent 7h. These times correspond with the mean flow times of sewage through the stage of the works as provided by the sewage works Manager. The sequential reductions in numbers are shown in Table 4.9 using, as an example, 5 day means of campylobacter numbers at the peak hours. The organisms were detected in large numbers in the influent sewage and were reduced in numbers by the treatment processes by 99.9%.

Campylobacters were found in all but six samples studied. One isolate was taken from each positive sample in the survey, and from some isolates in the preliminary work and biotyped (Table 4.10). Of the 232 isolates tested, 226 (97%) were recognized as C.jejuni (hippurate +) of which 189 (81.5%) were biotype I. Serotyping of 95 of the C.jejuni strains was attempted with 25 Penner antisera as described in page 53. Over 68% were typable. It was found that those serotypes common in man were frequently common in sewage (Table 4.3). This is consistent with humans being the source of the isolates from sewage, but does not prove that they were.

Table 4.1 : Recovery of C.jejuni by enrichment methods from of the 105 human faecal samples received as negative.

	Enrichment					
	24h			48h		
Sample	N_2/CO_2 #	H_2/CO_2 *	air †	N_2/CO_2 #	H_2/CO_2 *	air †
1	+	+	+	+	+	+
2	+	+	-	-	+	-
3	+	+	+	+	+	+
4	-	+	+	-	-	-
5	+	+	+	+	+	+

= Incubation in microaerobic condition containing 1/3 air + 2/3 95% N_2/5% CO_2

* = Incubation in microaerobic condition containing 1/3 air + 2/3 95% H_2/5% CO_2

† = Incubation aerobically with bottle caps screwed tight

+ = positive

- = negative

Table 4.2 : Types of thermophilic campylobacters isolated from human faeces.

Number of strains	Hippurate hydrolysis	H_2S	Designation
75 (70.1%)	+	-	C.jejuni biotype I
31 (29%)	+	+	C.jejuni biotype II
1 (0.9%)	-	-	C.coli

Total = 107

+ = positive

- = negative

Table 4.3 : Serotypes of C.jejuni isolated from human faeces (73) and sewage (95) typed by PHA using 25 Penner antisera.

Serotype	Human isolates		Sewage isolates	
	No.	%	No.	%
1	2	2.7	8	8.4
2	9	12.3	1	1.1
3	0	0	1	1.1
4, 16	15	20.5	37	38.9
6	5	6.8	4	4.2
8	3	4.1	2	2.2
9	1	1.4	0	0
11	1	1.4	0	0
15	1	1.4	0	0
19	5	6.8	3	3.2
23	0	0	1	1.1
24	0	0	1	1.1
27	0	0	1	1.1
31	4	5.5	3	3.2
37	0	0	1	1.1
44	2	2.7	1	1.1
55	1	1.4	1	1.1
Untypable	24	32.9	30	31.6
Total	73	100	95	100

Table 4.4 : Recovery† of campylobacters from the guts of piglets.

	Fed base milk#			Fed yoghurt##		
	Piglet identity	ileum	colon	Piglet identity	ileum	colon
batch 1*	1A/AR292	+	+	6A/AR292	+	+
	1B/AR292	+	-	6A/AR292	+	+
batch 2**	1A/306	-	-	2A/306	-	-
	1B/306	-	-	2B/306	-	-
batch3**	1C/306	+	+	2C/306	+	+
	1D/306	+	+	2D/306	+	+

† = from gut contents or gut wall or both

\# = skimmed milk (liquid) + skimmed milk powder to give 16% total solids.

= base milk fermented with Lactobacillus sp. and Streptococcus sp.

\+ = positive for campylobacters

\- = negative

* = examined by both direct plating and enrichment

** = examined by enrichment alone.

Table 4.5 : Number of campylobacters in 3cm of gut of the piglets estimated by dilution and enrichment (plate count) of the samples.

fed base milk*				fed yoghurt			
Piglet identity	section of the gut	gut contents	gut wall	Piglet identity	section of the gut	gut contents	gut wal
1A/AR292	ile	0	$2x10^2$	6A/AR292	ile	0	2x10
		(0)	(0)			$(8.5x10^2)$	(3x10
	col	$2x10^4$	$2x10^2$		col	$2x10^3$	2x10
		$(8x10^3)$	$(3x10^2)$			$(2.5x10^2)$	(2x10
1B/AR292	ile	0	20	6B/AR292	ile	0	2x10
		$(1x10^2)$	(0)			(0)	(0
	col	0	0		col	0	2x10
		(0)	(0)			$(2x10^2)$	(0
1C/306	ile	$2x10^2$	$2x10^2$	2C/306	ile	$2x10^5$	2x10
	col	$2x10^6$	$2x10^5$		col	$2x10^6$	2x10
1D/306	ile	$2x10^4$	$2x10^4$	2D/306	ile	$2x10^3$	2x10
	col	$2x10^6$	$2x10^6$		col	$2x10^6$	2x10

ile = ileum

col = colon

* = controls

Table 4.6 : Detection and typing of campylobacters from meat and offal

Samples			Isolates		
Type	Number examined	Number positive (%)	*C.jejuni* biotype I (%)	*C.jejuni* biotype II (%)	*C.coli* (%)
Chicken	33	29 (88)	21 (72)	6 (21)	2 (7)
Pork	20	5 (25)			5 (100)
Beef	15	8 (53)	7 (87.5)		1 (12.5)
Lamb	8	3 (38)	3 (100)		
Total	76	45 (59)	31 (69)	6 (13)	8 (18)

Table 4.7 : The effect of different inoculum sizes, direct enrichment and pre-enrichment on the recovery of campylobacters from sewage.

	Percentage recovery (i.e. percentage of those examined that were positive by the method stated							
Enrichment ⟶	24h				48h			
Pre-enrichment ⟶	0h*	1h	2h	4h	0h*	1h	2h	4h
Sewage inoculum (ml/5ml broth)								
Incoming sewage								
10.0	52	16	24	24	8	0	0	4
1.0	80	67	80	50	33	40	47	8
0.1	73	60	73	25	80	27	53	33
0.01	50	38	38	0	75	50	50	40
Primary effluent								
10.0	77	36	64	28	30	22	17	0
1.0	69	69	54	33	71	64	79	22
0.1	46	62	62	0	64	64	86	40
Final effluent								
100.0	57	0	43	0	43	0	57	0
10.0	40	10	28	24	20	0	12	0
1.0	40	0	10	10	0	0	0	0

* = Antibiotics (for Preston medium) added before inoculation i.e. direct enrichment

Table 4.8 : Number of campylobacters (MPN) in incoming crude sewage, primary sedimentation effluent and final effluent (minimum, maximum and mean of 5 days) at Reading sewage treatment works, sampled from 16th to 20th September, 1985.

Sampling time	Incoming sewage flow rate (l/sec)			Campylobacters/l in								
				Incoming sewage			Primary sedimentation effluent			Final effluent		
	min	max	mean	min	max	mean	min	max	mean	min	max	mean
				$x10^4$	$x10^4$	$x10^4$	$x10^4$	$x10^4$	$x10^4$			
0600	22.2	372.2	160	1.0	4.4	2.1	0.4	2.4	1.3	22	92	41
0700	66.7	388.9	225.6	<1.0	2.0	1.8*	<0.2	3.2	1.7*	10	69	41
0800	127.8	527.8	357.8	2.0	5.1	2.7	0.2	2.4	1.0	10	69	35
0900	422.2	911.1	624.4	1.0	10.0	4.0	0.4	1.0	0.8	22	69	43
1000	572.2	900.0	728.9	1.0	13.8	7.7	0.7	2.4	1.4	10	92	46
1100	483.3	855.6	668.9	2.0	7.2	4.9	0.4	4.6	2.2	22	36	
1200	616.7	800	684.3	5.4	46.0	18.0	0.7	4.6	1.8	51	96	

Table 4.8 continued.

Sampling time	Incoming sewage flow rate (l/sec)			Campylobacters/l in								
				Incoming sewage			Primary sedimentation effluent			Final effluent		
	min	max	mean	min	max	mean	min	max	mean	min	max	mean
				$x10^4$	$x10^4$	$x10^4$	$x10^4$	$x10^4$	$x10^4$			
1300	494.4	588.9	545.6	2.0	24.0	11.0	2.4	4.6	3.3	22	230	10[illegible]
1400	511.1	783.3	628.3	<1.0	2.2	1.8*	2.4	4.6	3.9	10	>230	96*
1500	433.3	738.9	493.3	2	10.0	4.2	1.8	>4.6	3.2*	36	230	109
1600	350.0	650.0	526.7	<1.0	2.2	1.8*	1.4	>4.6	1.9*	36	230	109
1700	355.6	666.7	473.3	2.0	7.2	5.3	1.8	2.4	2.3	22	>230	130*
1800	333.3	750.0	554.4	1.0	4.4	2.3	1.4	3.2	2.0	10	230	150
1900	311.1	488.9	363.3	<1.0	2.0	1.7#	0.7	3.2	1.9	51	230	167
2000	283.3	338.9	312.0	<1.0	2.2	2.1*	0.2	2.4	1.3	22	160	76

MPN = Most probable number

min = minimum

max = maximum

* = based on 4 counts

\# = based on 3 counts

Fig. 4.2 Hourly fluctuations of campylobacters (means of 5 days) in (a) incoming crude sewage (-X-), (b) primary sedimentation effluent (-0-) and (c) final effluent (-Δ-). Samples were taken from 16th to 20th of September 1985.

Maximum number of campylobacters detected:

X_1 $10^{5.66}/l$

X_2 $10^{4.66}/l$

X_3 $10^{2.36}/l$

Minimum number of campylobacters detected:

Y_1 $10^{4}/l$

Y_2 $10^{3.3}/l$

Y_3 $10^{1}/l$

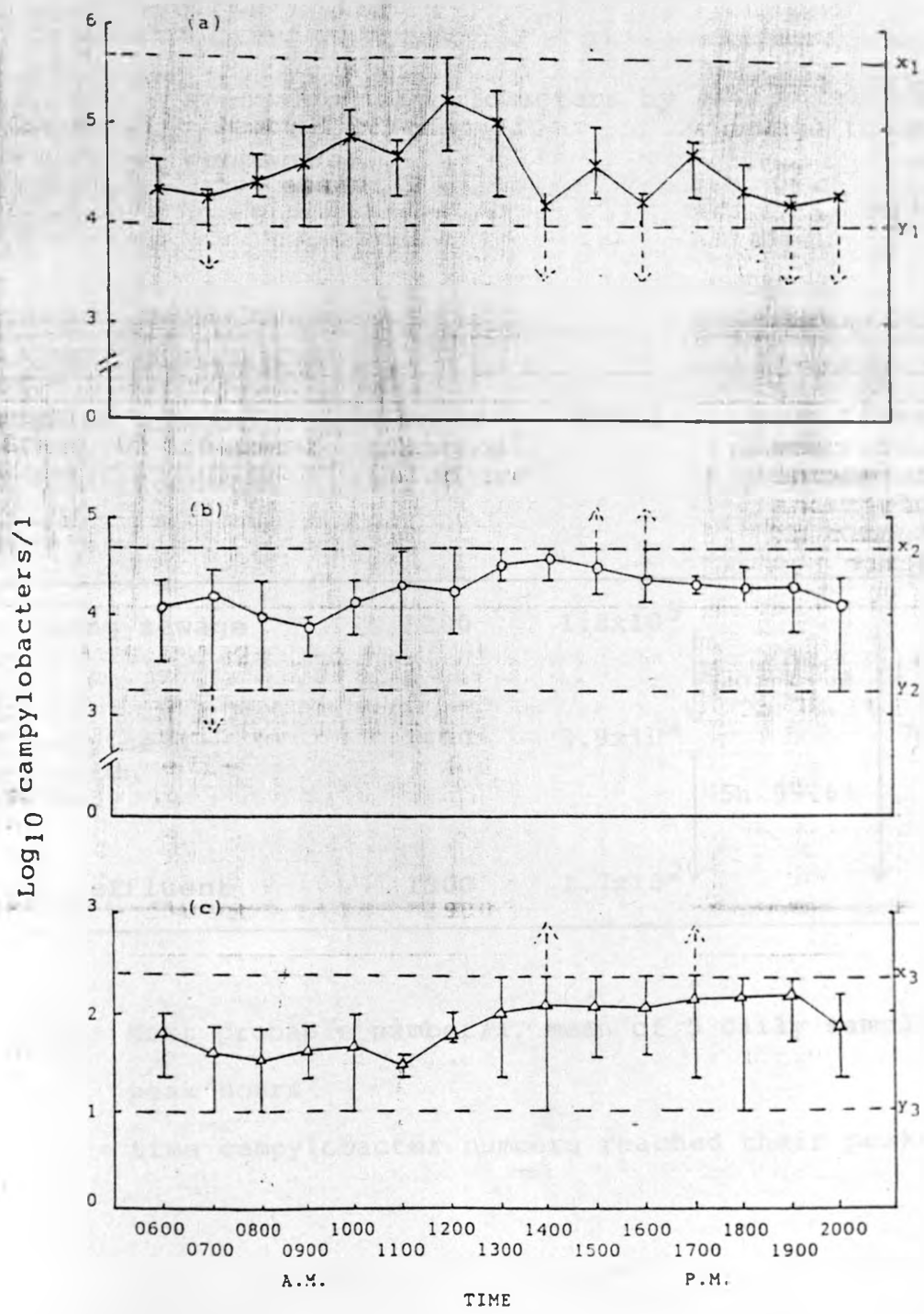


Table 4.9 : Removal of campylobacters by sewage treatment processes.

Stage of treatment	Time of sampling*	MPN/l	mean time from intake to works and reductions in campylobacter numbers
Incoming sewage	1200	1.8×10^5	
			2h 78.3%
Primary sedimentation effluent	1400	3.9×10^4	7h 99.9%
			5h 99.6%
Final effluent	1900	1.7×10^2	

MPN/l = Most probable number/l, mean of 5 daily samples at peak hours

* = time campylobacter numbers reached their peaks.

Table 4.10 : Types of thermophilic campylobacters received by Reading sewage treatment works from 16th to 20th September 1985.

Number of strains	Hippurate hydrolysis	H_2S	Designation
189 (81.5%)	+	-	C.jejuni biotype I
37 (15.9%)	+	+	C.jejuni biotype II
6 (2.6%)	2 ±	-	C.laridis or C.coli
	4 -	-	

+ = positive

± = weak positive

- = negative

4.1.4 DISCUSSION

A large percentage of the campylobacter isolates from human faeces (99.1%), meat and offal (chicken, beef, lamb and pork; 82%) and sewage (97.4%) were C.jejuni, biotype I being most predominant (Table 4.2, 4.6 & 4.10). C.coli and C.laridis constituted the remaining small proportion. The serotyping scheme of Penner and Hennessy (1980) has been extended to include 60 typing antisera (Penner et al., 1983). Of the isolates examined by serotyping, 67.1% of human strains and 68.4% of sewage strains were typable using 25 Penner antisera. The most common serotypes (in descending order) were: 4/16, 2, 1, 6, 19, 31 and 44 in both human faeces and sewage in Reading. These results show that C.jejuni types common in faeces of patients with diarrhoea in Reading area are also common in sewage and animal meat products (except pork). The faeces examined at the Royal Berkshire hospital laboratory are from inpatients and outpatients of the hospital, from patients visiting other hospitals and from patients visiting general practitioners served by the laboratory. Whereas the data represent the situation in Reading area, there is a possibility that campylobacter infection in some people could have been acquired from other parts of Britain or abroad.

Among foods of animal origin, poultry is considered to be the main source of campylobacter infections in human beings

(Skirrow, 1982; Oosterom et al., 1985). C.jejuni is found in large numbers in the intestinal tract of a high percentage of chickens (Ribeiro, 1978), and usually the dressed poultry and poultry products are highly contaminated with the organism (Simmons & Gibbs, 1979; Hartog & Boer, 1982). Of the four types of meat products which I examined, chicken is the commonest source of C.jejuni. Pork (Table 4.6) seemed an insignificant source of campylobacters in Reading as found by other workers in most other places studied. For example Bänffer (1985) reported that in the region of Rotterdam in the Netherlands chickens contributed more to campylobacter infections in humans than did pigs.

Campylobacters contaminate poultry meat and other meats presumably through contact with intestinal contents during slaughtering and evisceration. Because of the poor survival rate of campylobacters on some meats, numbers may be small by the time the meat reaches the retail shops (Turnbull & Rose, 1982; Oosterom et al., 1983). However, campylobacters survive better in foods at refrigeration temperatures than at ambient temperature and in an atmosphere of CO_2 than in air (Svedhem et al.,1981; Blankenship & Craven, 1982). Depending possibly on the strain of C.jejuni, the initial number of cells and the environmental conditions of storage, particularly temperature, campylobacters may survive in raw foods for long periods. Individuals may thus become infected via (a) foods of animal origin that have not been heated sufficiently to inactivate Campylobacter, or (b) sufficiently

heated foods which have been contaminated post heating via raw foods containing campylobacters. The high incidence of campylobacters in the meat and offal samples examined in this study, particularly in chicken (88%) underlies the potential risk of infection for human beings.

C.coli commonly occurs in the intestines of pigs as commensals, usually in small numbers and in close association with the gut mucosa (Kang, 1981; Morris & Park, 1971). In this study C.coli was the only type found in the guts of the piglets. The regular presence of the organisms incriminates the pig as a potentially common source of infection for human beings, but isolations from pork from retail shops showed pork not to be a numerically important source. One reason may be the rapid death of the organisms on the carcasses (Hudson & Roberts, 1982). However, this study shows that pigs acquire campylobacters at a very early age and tolerate them as commensals without showing clinical symptoms of disease. C.coli was thought to be the cause of swine dysentery (Doyle, 1944). However, the role of C.coli in the disease was discounted when the organisms were found in healthy pigs as well as diseased ones, and when attempts to reproduce the disease by feeding the organisms alone to susceptible pigs failed (Deas, 1960; Morris & Park, 1971). Later Taylor & Alexander (1971) incriminated T.hyodysenteriae as the causative agent of swine dysentery. However, the situation is not yet clear. C.coli is still considered to play a role in

causing diarrhoea in pigs in certain circumstances and the two organisms, C.coli and T.hyodysenteriae, could be involved (Morris and Park, 1973; Fernie et al., 1975; Taylor & Olubunmi, 1981).

A lot of knowledge has been gained about the occurrence of campylobacters in human beings, animals and animal products through biotyping and serotyping. We also know something about campylobacters in water, mostly through epidemiological studies. However, little, if anything, is known about campylobacters in sewage. Sewage contains human, animal and industrial wastes and many microorganisms pathogenic for man and animals (Jones & Watkins, 1985). It seems likely that many of the human pathogens are contributed by human faeces and urine, but this is by no means certain. This study, in showing that those biotypes and serotypes of campylobacters common in human faeces are also common in sewage, provides evidence consistent with human faeces being a major source. The campylobacter strains could also have originated from other sources, for example a poultry farm or slaughter house discharging into the sewage system.

Sewage is potentially an important source of campylobacter infection. Those who work routinely with sewage and sewerage systems will be at greatest risk. A case of campylobacter enteritis attributed to falling in sewage has been reported (Sumathipala & Morrison, 1983). The indirect potential risks to human beings and animals are through :

(a) sewage sludge disposed on arable land and pastures (Carrington, 1981; Jones & Watkins, 1985); (b) discharge of treated sewage effluent into rivers.

The fate of the large percentage of campylobacters (99.9%) removed from the fluid component by sewage treatment was not studied and information from elsewhere, is scanty or non-existent. The organisms may be killed, or injured so as to be sensitive to isolation techniques used, during the treatment process; or concentrated and removed in the sludge. Bearing in mind the disposal practices for sewage sludge on arable land and pastures, it is important that further studies should be made. For example more information is needed on the survival time of campylobacters in sludge applied to land and the hazards that survivors might pose to both man and animals. Jones & Watkins (1985) stated that campylobacters present in sludge spread on land die more rapidly than salmonellas but they did not present data. The infective dose of C.jejuni in milk is reported to be as low as 500 organisms (Robinson, 1981). Bearing this in mind, it is possible that human beings can acquire Campylobacter infection from crops which are eaten raw and which are obtained from agricultural land treated with sewage sludge.

Some campylobacters are present in the final effluent which is discharged into the river. Although the percentage is small, the numbers are substantial. I estimate (Fig. 4.3) that approximately 10^{10} campylobacters are released from

Reading sewage works into nearby river daily. In a survey of campylobacters in a river system subject to sewage effluent discharge, Bolton et al. (1985) concluded that sewage is an important source of C.jejuni in river water and that biotypes and serotypes common in humans were also common in river water, especially at sampling points downstream of sewage effluent discharge sites. The findings of the present survey of campylobacters in sewage in Reading strongly supports these observations.

Biotyping and serotyping are not measures of pathogenicity of Campylobacter types. However, they do help in epidemiological studies in indicating the types which are common to different situations. Fig.4.4 gives a proposed pattern of occurrence of campylobacters in man, animals and the environment. It seems reasonable to assume that the contributors of the greatest number of campylobacters in sewage are individuals with acute gastro-enteritis. Since human excreta is an important component of sewage, it is interesting to consider how many persons in Reading may be suffering with campylobacter enteritis at any one time, assuming, of course, that all the thermophilic campylobacters in sewage are contributed by such persons. The calculation in Fig. 4.5 based on numbers found in influent sewage and assumptions of several parameters, show that 6 persons with campylobacter enteritis in Reading could account for the numbers found. However, this assumes no death prior to receipt of sewage at the works. The fact that 15 serotypes

(Table 4.3) were isolated indicates that this is a gross under-estimate and that probably considerable death of organisms occurs during transport to the works.

4.1.5 CONCLUSION

The campylobacter biotypes and serotypes that are commonly reported as associated with enteritis in man, are also present in human faeces examined at the Royal Berkshire Hospital, Reading, in foods and in sewage. Sewage treatment does not remove all the campylobacters and so could be partly responsible for maintaining the organisms in the cycle of campylobacter infection. Campylobacters are regularly present in healthy piglets, are well established in the gut from a very early age and are not affected by a diet of yoghurt, but these are not the type commonly associated with human disease in the U.K.

Figure 4.3 : Estimated number of campylobacters discharged from Reading sewage treatment works into nearby river daily.

Mean number of campylobacters in sewage effluent approximately 85/l

Mean daily sewage effluent flow from Reading sewage works (supplied by the management) ..... $6x10^7$l

. . Total discharge of campylobacters is approximately ..... $5.1x10^9$

Fig. 4.4 Proposed pattern of occurrence of campylobacters in man, animals and the environment.

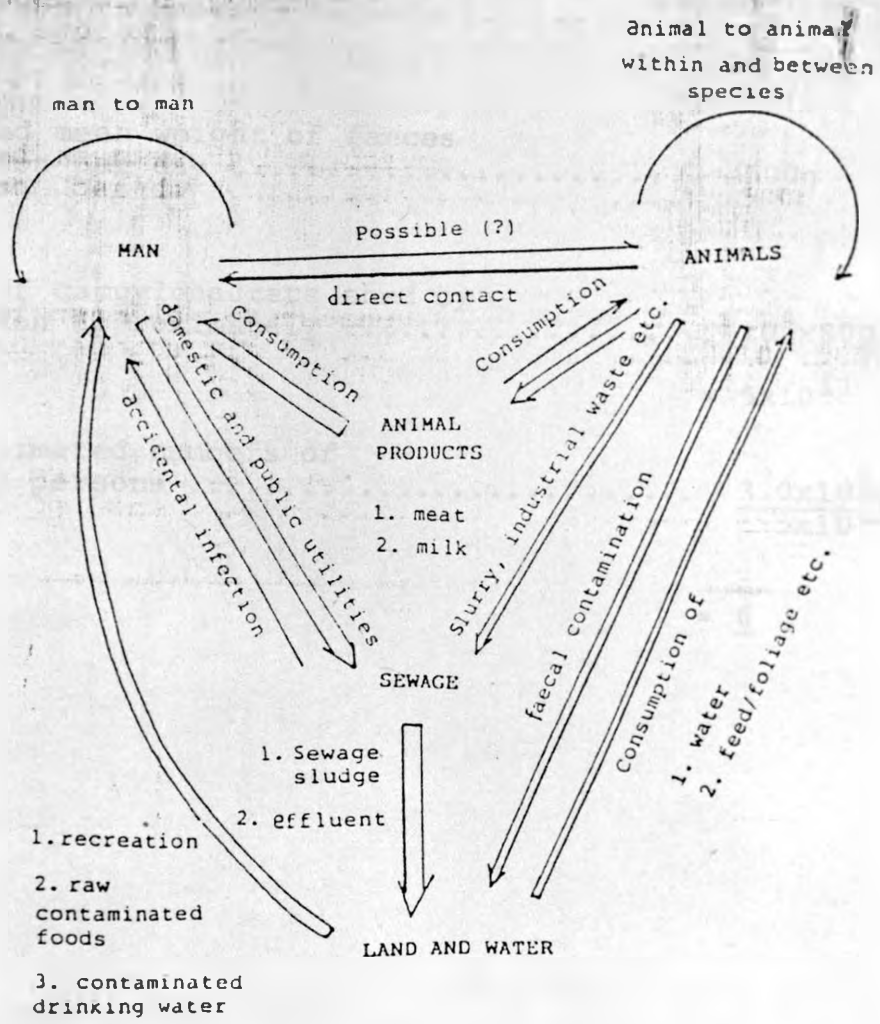


Figure 4.5 : Estimated number of diseased persons shedding campylobacters into Reading sewage, using numbers found in sewage.

Estimated daily sewage flow into the works (data given by the Manager) ..... 6×10^7 l

Mean number of campylobacters in the influent approximately ..... $5 \times 10^4 \times 6 \times 10^7$

$= 3.0 \times 10^{12}$

Estimated mean weight of faeces per person per day 500g

Number of campylobacters shed by one person approximately ..... $10^9 \times 500$

$= 5 \times 10^{11}$

. . Estimated numbers of diseased persons ..... $\frac{3.0 \times 10^{12}}{5 \times 10^{11}}$

$= \underline{6}$

SECTION 2

4.2 AN ATTEMPT TO DEVELOP A SELECTIVE MEDIUM USING DYES AND THALLOUS ACETATE

4.2.1 INTRODUCTION

Selective media for campylobcters contain expensive antibiotics, which are not readily available in developing countries, and rely on incubation at 42°C - 43°C, which inhibits C.fetus. For these reasons it was decided to see if certain dyes and/or other substances could be used in place of antibiotics so as to produce a cheaper and more useful selective medium. It was hoped that addition of an inhibitor to existing selective media might facilitate recovery of C.fetus through incubation at 37°C, which otherwise allows growth of many contaminants. There have been a few reports of occurrence of C.fetus subsp. fetus in human faeces and in association with enteritis (Devlin & McIntyre, 1983; Klein et al.. 1986), but study is impeded by lack of a suitable isolation method for these organisms. C.fetus subspecies and the thermophilic campylobacters grow at 37°C and a few strains of C.fetus subsp. fetus grown at 42°C (Skirrow & Benjamin, 1980). The two types can occur together and recoveries of some C.fetus subsp. fetus have been made using media and techniques intended for thermophilic campylobacters (Klein et al. 1986). It was hoped also that if the thermophilic campylobacters were sensitive to the inhibitors to be tested, a medium enriching specifically for C.fetus subspecies could be developed.

An almost infinite number of inhibitors is available for study. However, malachite green, brilliant green and thallous

acetate were chosen, for the following reasons. Brilliant green and malachite green are both triphenylmethane dyes which also include crystal violet and fuchsin. At very low concentrations these dyes are bacteriostatic for many Gram-positive bacteria, though relatively inactive against a range of Gram-negative species and acid-fast bacteria (Singleton & Sainsbury, 1980). There is some indication in the literature that attempts have been made by some workers to use brilliant green for isolation and differentiation of campylobacters. Florent (1959) used brilliant green selective medium to isolate C.coli from the intestines of pigs. However, he noticed from cultural point of view that C.coli was strongly sensitive to brilliant green. Soderlind (1965) used solid agar medium containing 1:60,000 brilliant green, to suppress growth of contaminants, for isolation of C.coli from pig intestines. Ability of C.fetus to grow on brilliant green agar containing 1/33,000 brilliant green was tested by Véron & Chatelain (1973). They reported tolerance, indicated by good growth of the organism. However, Skirrow & Benjamin (1980) did not find tolerance to brilliant green helpful in distinguishing C.jejuni and C.coli strains. Razi (1982) tested sensitivity of campylobacters to a number of dyes which included brilliant green, crystal violet and fuchsin, but malachite green was not examined. Malachite green has been used in selective media for isolation of salmonellas (Rappaport et al. 1956), but the dye does not seem to have been studied for campylobacters.

Thallous acetate was included in the study because it inhibits many Gram-negative bacteria. It has long been used in selective media for isolation of streptococci in the dairy industry (Mckenzie, 1941). Since Gram-negative bacteria are major contaminants in faeces, it was considered worthy examining the effect of thallous acetate.

4.2.2 EXPERIMENTS

Resistance of campylobacters to malachite green, brilliant green and thallous acetate.

C.jejuni NCTC 11322, C.coli NCTC 11350, C.laridis NCTC 11458 and C.fetus subsp. fetus NCTC 10348 (see Chapter 3 page49) were used in the study.

a. Resistance of campylobacters to various concentrations of the three substances impregnated in paper discs.

Decimal dilutions of filter sterilized aqueous malachite green (BDH), brilliant green (BDH) and thallous acetate (BDH) were prepared and stored at 4°C in the dark for up to one month. Sterile 6mm Whatman AA discs were impregnated with different concentrations of the three substances and dried at 42°C overnight in glass petri dishes which allow even drying of the discs. The impregnated discs were applied on the

surface of HBA inoculated uniformly by swabbing with the test organisms and the plates incubated microaerobically (1/3 + 2/3 95% N_2/5% CO_2) at 37°C for 48h. Zones of inhibition around the discs equal to or more than 3mm were easy to read and were therefore considered to indicate sensitivity; zones less than 3mm were considered to indicte slight sensitivity and no zone to indicate resistance.

Malachite green was more inhibitory to C.jejuni, C.coli and C.laridis than to C.fetus subsp. fetus (Table 4.11). Ignoring diffusion effect differences and using zone size as an indicator it seemed that brilliant green was less inhibitory to C.fetus than either malachite green or thallous acetate. All four species showed surprisingly uniform resistance to thallous acetate; they were sensitive only at the highest concentration (1.0% w/v).

b. Resistance of campylobacters to the three substances incorporated in blood agar.

HBA containing various concentrations of the three substances were streaked to obtain isolated colonies, incubated at 37°C for 48h and examined for growth in the streaked areas. Good growth i.e. colonies 1-2 mm in the streaked areas was considered a good indication of tolerance. Reduced size of colonies was considered poor growth and absence of visible colonies as complete inhibition.

Table 4.11 : Resistance of campylobacters to various concentrations of malachite green, brilliant green and thallous acetate impregnated in absorbent paper discs and tested by placing them on horse blood agar inoculated uniformly with campylobacter test strains.

Discs impregnated with solutions of the test compounds of the following strengths (% w/v)	Campylobacter strains			
	C.jejuni NCTC 11322	C.coli NCTC 11350	C.laridis NCTC 11458	C.fetus subsp. fetus NCTC 10348
Malachite green				
1.0	S	S	S	S
0.1	S	S	S	I
0.01	S	S	S	R
0.001	R	R	R	R
0.0001	R	R	R	R
Brilliant green				
1.0	S	S	S	I
0.1	I	I	I	R
0.01	I	R	R	R
0.001	R	R	R	R
0.0001	R	R	R	R

Table 4.11 continued.

Discs impregnated with solutions of the test compounds of the following strengths (% w/v)	Campylobacter strains			
	C.jejuni NCTC 11322	C.coli NCTC 11350	C.laridis NCTC 11458	C.fetus subsp. fetus NCTC 10348
Thallous acetate				
1.0	S	S	S	S
0.1	R	R	R	R
0.01	R	R	R	R
0.001	R	R	R	R
0.0001	R	R	R	R

S = sensitive (zones of inhibition around the disc more than 3mm).

I = slight sensitivity (zones of inhibition around the disc less than 3mm).

R = Resistant (no zone of inhibition).

Table 4.12 : Effect of malachite green, brilliant green or thallous acetate incorporated in horse blood agar on the growth of campylobacters.

Concentration in the medium of test compounds (% w/v)	Growth*			
	C.jejuni NCTC 11322	C.coli NCTC 11350	C.laridis NCTC 11458	C.fetus subsp. fetus NCTC 10348
Malachite green				
0.01	-	-	-	-
0.001	-	-	-	±
0.0001	+	+	+	+
Brilliant green				
0.01	-	-	-	-
0.001	±	+	+	+
0.0001	+	+	+	+
Thallous acetate				
0.01	-	-	-	-
0.001	-	±	-	-
0.0001	+	+	+	+

+ = good growth; ± = poor growth;

- = no growth; * = repeat experiment showed the same results.

Results (Table 4.12) showed brilliant green to be least inhibitory to all the four species of Campylobacter and indicated that it could possibly be suitable for isolation provided contaminants in the samples were sufficiently suppressed by it. Malachite green was less inhibitory to C.fetus than to C.jejuni, C.coli and C.laridis and could therefore possibly be useful in selecting for C.fetus. Thallous acetate was inhibitory to all the species and therefore unsuitable. However, it was interesting to see if the three substances had any synergistic or antagonistic effect when used together in a medium, either for suppressing growth of contaminants or for allowing growth of campylobacters.

c. Combined effect of malachite green, brilliant green and thallous acetate on the growth of contaminants and campylobacters.

Two sets of HBA each containing 0.001% (w/v) and 0.0001% (w/v) of each of the three substances were prepared and inoculated with three human faecal samples, two known to be positive for C.jejuni and one negative, and with the four strains of campylobacters. In addition Preston agar plates were similarly inoculated. Plates were examined for growth of campylobacters and contaminants after incubation for 48h at 37°C.

Table 4.13 Combined effect of malachite green, brilliant green and thallous acetate in horse blood agar (HBA) on the growth of faecal flora and campylobacters.

	HBA plus malachite green, brilliant green and thallous acetate (% w/v)		Preston agar
	0.001*	0.0001*	
Campylobacter strains			
C.jejuni NCTC 11322	-	+	+
C.coli NCTC 11350	-	+	+
C.laridis NCTC 11458	-	+	+
C.fetus subsp. fetus NCTC 10348	-	+	+
Faecal samples			
No. 14001†	-#	-#	(+)**
No. 12849†	-#	-#	-**
No. 14146§	-#	-#	-##

HBA = horse blood agar

* = concentration of each substance in HBA.

\+ = growth of campylobacters

(+) = few colonies of campylobacters

\- = no growth of campylobacters

† = known campylobacter positive faecal samples.

§ = " " negative " samples.

\# = heavy growth of contaminating organisms.

= few contaminating organisms.

** = no contaminants.

Concentrations of the three compounds inhibiting growth of campylobacters allowed heavy growth of contaminants from the faeces (Table 4.13). C.jejuni was recovered from the two positive samples on PA but not on HBA containing malachite green, brilliant green and thallous acetate.

4.2.3 CONCLUSION

The three compounds studied cannot be recommended for use for isolating campylobacters from faeces at 37°C. It was decided not to proceed further with this line of work.

CHAPTER 5

STUDY OF THE CELL MORPHOLOGY AS AN AID IN DIFFERENTIATION AND CHARACTERIZATION OF CATALASE-POSITIVE CAMPYLOBACTERS

5.1 INTRODUCTION

Campylobacter fetus (Vibrio fetus; Smith & Taylor, 1919) was originally classified in the genus Vibrio simply because the curved nature of the organism resembled that of vibrios (Park, 1961). Although morphological appearance is no longer an inviolate criterion for placing an organism in a certain genus, and it is now known that campylobacters are different from vibrios in many ways, it is nevertheless a characteristic of some differential and taxonomic value.

Campylobacters appear as small, curved or S-shaped or spiral rods (Butzler & Skirrow, 1979; Smibert, 1984), a characteristic which is generally very helpful in recognition of the members of the genus during isolation. However, certain differences exist between groups of campylobacters; for example helical forms of both subspecies of C.fetus have larger wavelengths and amplitudes than do the C.jejuni/C.coli/C.laridis organisms (Skirrow & Benjamin, 1980; Karmali et al., 1981; Skirrow et al., 1983). Karmali et al. (1981) reported an interesting study of the wavelengths and amplitudes of helical forms of C.jejuni, C.fetus subsp. fetus and C.fetus subsp. venerealis and the measurements obtained indicated clear differences among the three groups of organisms. The work thus showed the potential usefulness of wavelength and amplitude in differentiation of species and subspecies.

However, helical forms of some types of campylobacters are not readily produced (Butzler & Skirrow, 1979; Skirrow et al., 1983) and so measurements of wavelength and amplitude are difficult. Inspired by the findings of Karmali et al. (1981) I decided to develop a technique for producing long helical forms of campylobacters with the aims of (a) facilitating measurements of campylobacter helices which are otherwise sometimes difficult to study because helical forms are rare (b) measuring cell wavelength and amplitude of some of the campylobacter strains we have in our stock and extending the study to other species not examined by Karmali et al. (1981) (c) studying helix handedness of the organisms i.e., the direction of rotation of the spirals, clockwise or anticlockwise. The type of handedness may be an important means of classification especially if the helix handedness is a stable genetic characteristic and different handedness occurs within the genus. Konishi & Yoshii (1986) found Aquaspirillum itersonii, Aquaspirillum peregrinum and Aquaspirillum metamorphum to maintain their right-handed helix after antibiotic treatment with cephalexin.

Long forms of Gram-negative bacteria can be produced by a number of agents especially antibiotics at low concentrations (Lastovica, 1982; Park, 1982; Konishi & Yoshii, 1986). For the purpose of this study, the methods described by Park (1982) using penicillin and Mitomycin-C to induce long forms

in Escherichia coli were adopted and modified for campylobacters.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains

1. Source

The campylobacter strains used in this study were chosen to include five species and subspecies as follows: (i) C. jejuni, National Collection of Type Cultures (NCTC) 11168, NCTC 11322 and seven strains designated 'SH' isolated by Mrs. S. Georges from faeces of human beings suffering from diarrhoea; (ii) C.coli NCTC 11350, NCTC 11366 and 10 strains designated 'P' isolated from pigs; (iii) C.laridis NCTC 11458, NCTC 11352 and seven strains designated 'C' isolated from seagulls by Dr. C. Fricker (iv) C.fetus subsp. fetus, NCTC 10348, NCTC 10842 and nine ovine and bovine isolates designated 'D' provided by Mr. J. Morgan, I.R.A.D., Compton; (v) C. fetus subsp. venerealis, two bovine genital strains designated 'G', including NCTC 10354, provided by Mr.J. Morgan, I.R.A.D. Compton. For more information see section 3.3 (general methods).

2. Confirmation of identity of strains

Identity of the strains was determined as described in chapter three. When individual strains gave soma measurements that were at odds with the general pattern or disagreed with those of Karmali et al. (1981), they were checked for identity using key physiological tests which comprised growth temperatures, resistance to nalidixic acid, hippurate hydrolysis, glycine tolerance, and H_2S production in sensitive medium containing cystine and in FBP broth.

5.2.3 Antibiotics

Sodium benzyl penicillin (500,000 iu; Sigma) and crystalline Mitomycin-C (2mg; Kyowa Hakko Kagyo Co. Ltd) were each dissolved in small amounts of demineralized water, filter sterilized, diluted, and added to cooled molten media to give the final concentrations indicated.

5.2.4 Methods for production of helical long forms

In order to establish a suitable low antibiotic concentration for growth and production of long forms by the campylobacters on solid media, two culture methods were investigated.

1. Gradient plates

The method of Spooner & Sykes (1972) was used with modification. YNA (10ml) was poured into a 90 mm diameter petri dish placed in a slanting position. When the agar had set the plate was placed horizontally and 10 ml of the same medium containing penicillin or Mitomycin was added. Thus each plate had deep antibiotic-containing agar on one side and deep antibiotic-free agar on the other side with varying amounts across the plate. Plates containing different amounts of penicillin (62.5 iu to 312.5 iu per plate) or Mitomycin (2.5 ug to 12.5 ug per plate) were made and equilibrated for 18h at 4°C before inoculation.

2. Trough plate

A trough approximately 15mm wide was cut through the middle of a plate containing approximately 20ml YNA and filled with molten agar (plain) containing penicillin or Mitomycin. Molten agar was cooled to 47°C in a waterbath before adding the antibiotics. Several plates containing different concentrations of each antibiotic were prepared and tested for production of long helical forms by campylobacters. After preliminary tests, trough plates and Mitomycin were chosen for the main study. The standard method was then to add 0.2ml of

0.1mg ml^{-1} solution of Mitomycin to 10ml of molten plain agar to give a final concentration of 2.0ug ml^{-1} and to use this for filling the troughs as described above.

3. Inoculation and incubation of plates

Plates were streaked along the antibiotic gradient (gradient plates) or across the trough (trough plates) with heavy inocula of 24h BA cultures, incubated at 37°C in microaerobic condition containing nitrogen and examined for growth and production of helical forms after 24h.

5.2.5 Examination using phase contrast microscopy

1. Preparation of bacteria for microscopic examination

Growth was removed with a wire loop from the side near the trough (Fig. 5.1) and suspended in a small volume (0.2ml) of sterile saline (0.85% NaCl) by rotamixing to give light turbidity in order to obtain separated organisms. Following the method of Cure & Keddie (1973) a loopful of suspension was then transferred to a thin layer of plain agar prepared and dried on a glass slide, spread out with sterile curved glass rod to retain a smooth surface and the smear allowed to stand for 5 to 10 minutes for moisture to be absorbed into the agar

and immobilise the organisms. A 1cm square agar containing the bacterial smear was then cut, transferred to a clean glass slide and a coverslip applied. Edges between the coverslip and slide were sealed with molten Vaseline to prevent evaporation and drying of the smear. Control smears were prepared from growth taken from areas not exposed to antibiotics (Fig. 5.1).

2. Photography

Examination and photography were undertaken using an M20 microscope (Wild) with x100 phase contrast oil immersion objective. Fields containing helical organisms were focused and photographed at random using the microphotoautomat (Wild) with Panatomic x 135 mm film (Kodak). A slide micrometer scale was photographed under the same magnification for obtaining measurements of organisms.

3. Measurements of wavelength and amplitude

After development the negatives were projected using an enlarger on to plain paper and focused to give the same final magnification of 7700. The helical shape of each organism was recorded by drawing of a line along the middle of the image of the organism on the paper (Fig.5.2 and 5.3). Analysis of the images consisted of measurement of wavelength, i.e. the full

horizontal distance between adjacent crests and amplitude, i.e. the full vertical distance between the two adjacent crests and the trough (Fig. 5.2 and 5.3). Because of the technique used, only part of the organism was measured for amplitude. At least ten helical organisms per strain representing a large number of wavelengths and amplitudes were measured. Images of lines of the slide micrometer were drawn on paper under the same conditions as the organisms and used to determine the magnification of the organisms. Extended multiple range test (Kramer, 1956) or t-test was used for separation of means.

5.2.6 Examination using scanning electron microscopy (SEM)

1. Preparation of bacteria for examination

Growth was taken from the trough agar plate as described previously (see section 5.2.5 and Fig. 5.1) and organisms suspended in 2ml of 0.1M sodium phosphate buffer pH 7.2, rotamixed to wash and centrifuged (5000g for 10 min) to sediment the organisms. The supernatant fluid was removed, organisms resuspended in 4ml of the phosphate buffer and washed two more times. Washed organisms were fixed in 1ml glutaraldehyde fixative (5ml of 25% glutaraldehyde stock; BDH, in 20ml 0.1M sodium phosphate buffer pH 7.2) overnight. The

fixed organisms were washed three times in sterile demineralized water and resuspended in demineralized water to give a slight turbidity. The bacterial suspension was then transferred to a piece of glass coverslip, mounted on a grid, using a thin tipped pasteur pipette, and allowed five minutes for the bacteria to settle. The excess fluid was then removed using a thin strip of blotting paper and the smear dried overnight in a desiccator under partial vacuum.

2. Determination of helix handedness

The fixed bacterial smears were coated with gold and examined using JEOL scanning electron microscope (JSM-T20). The microscope produced real images of the helical organisms. It was therefore possible to determine helix handedness directly on the screen of the TV monitor as well as on printed pictures as described by Charleton et al. (1979), and Konishi & Yoshii (1986) i.e. by noting the rotation of the helix (clockwise or anticlockwise) while moving along the spiral away from the observer. The method used is illustrated diagramatically in Fig. 5.4.

Fig. 5.1 Trough agar plate showing growth of campylobacters (a & b) on YNA and the areas from which bacteria were taken for examination of long helical forms. Plates were incubated for 24h at 37°C microaerobically (1/3 air + 2/3 95% N_2/5% CO_2).

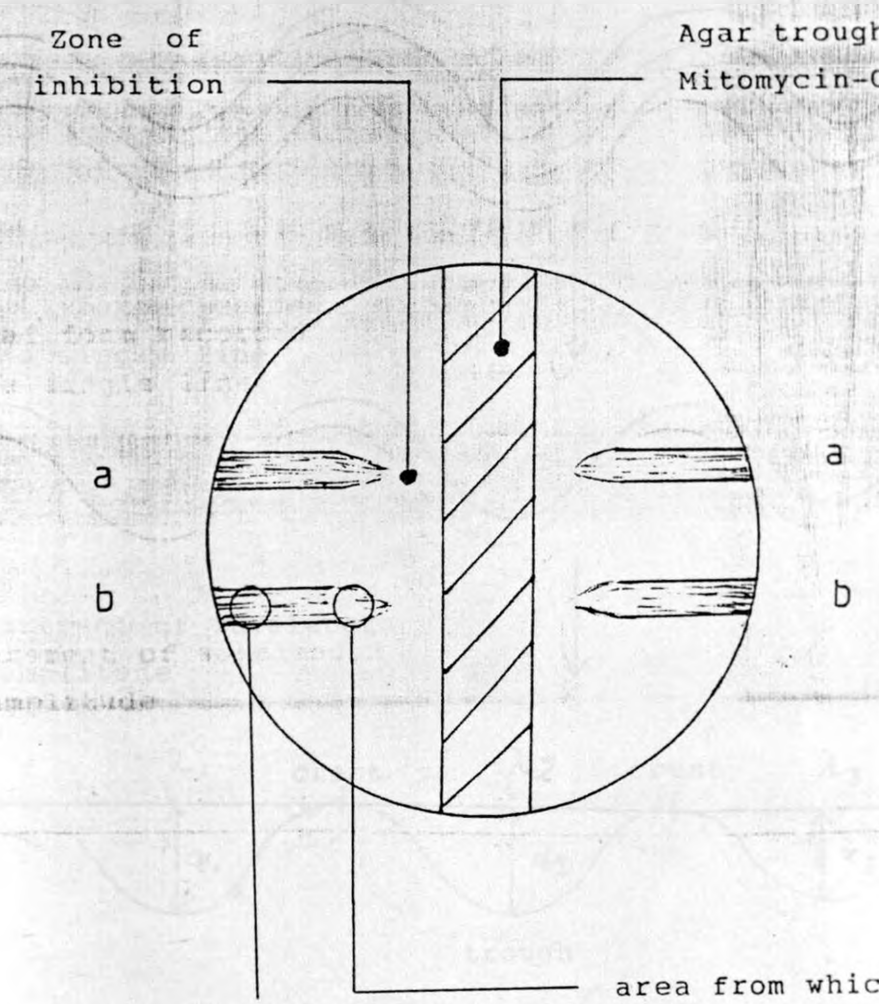
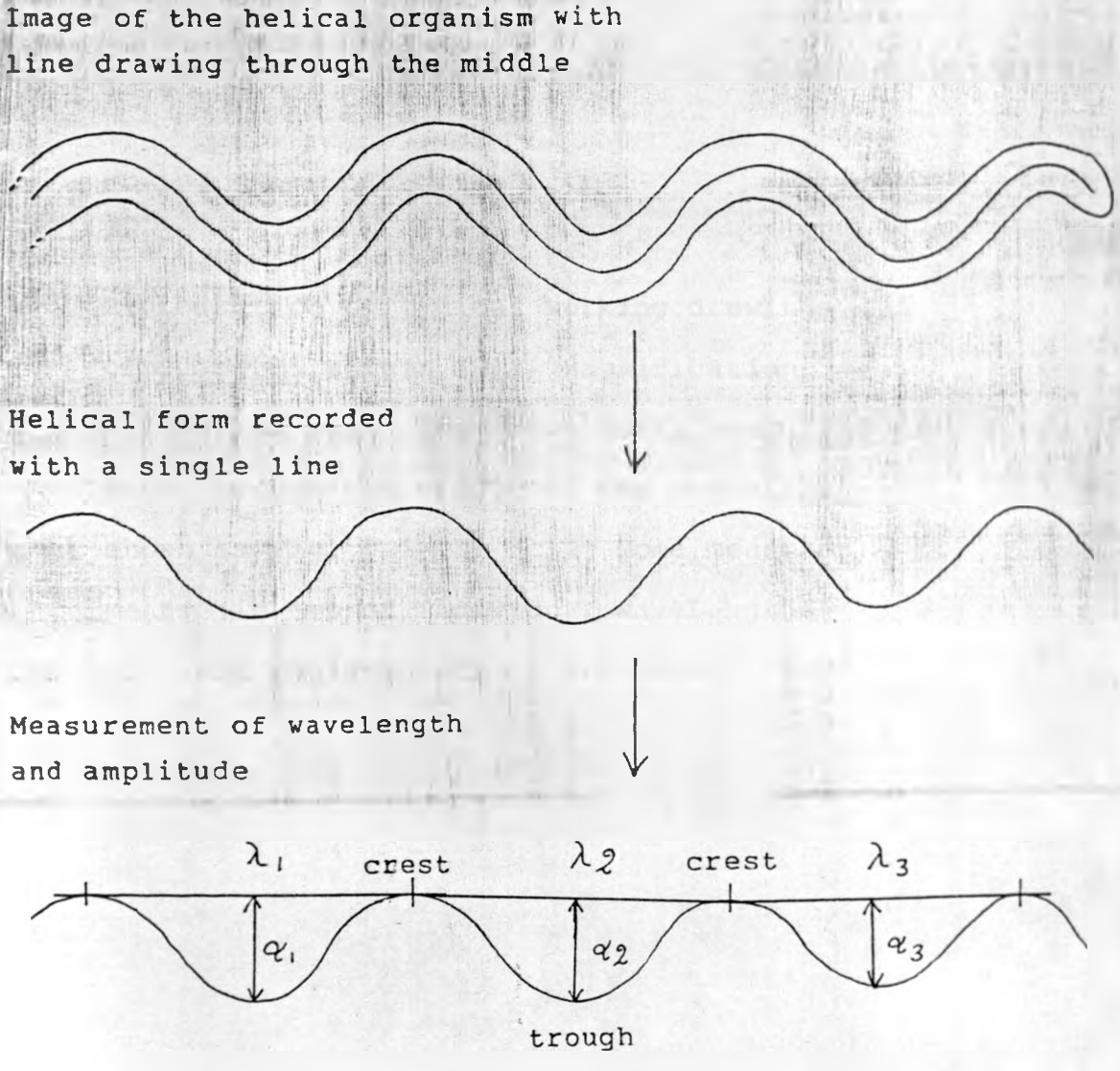


Fig. 5.2 Schematic presentation of the recording of the helical form of the campylobacter by a single line through the middle of the organism and measurement of the wavelength and amplitude.



λ = wavelength

α = amplitude

Fig. 5.3 Reproduction of typical working drawings of C.jejuni strain SH11 (Magnification x 7700) showing line drawings (1 and 2) made through the middle of the organisms shown in Fig. 5.1 (i) (c). Some measurements (in mm) of individual wavelengths (λ) and amplitudes ($\propto$) are shown.

1

λ 9, 9, 8, 8, 8, 7·5,

α 2·5, 2·5, 2·5, 2, 2·5, 3,

2

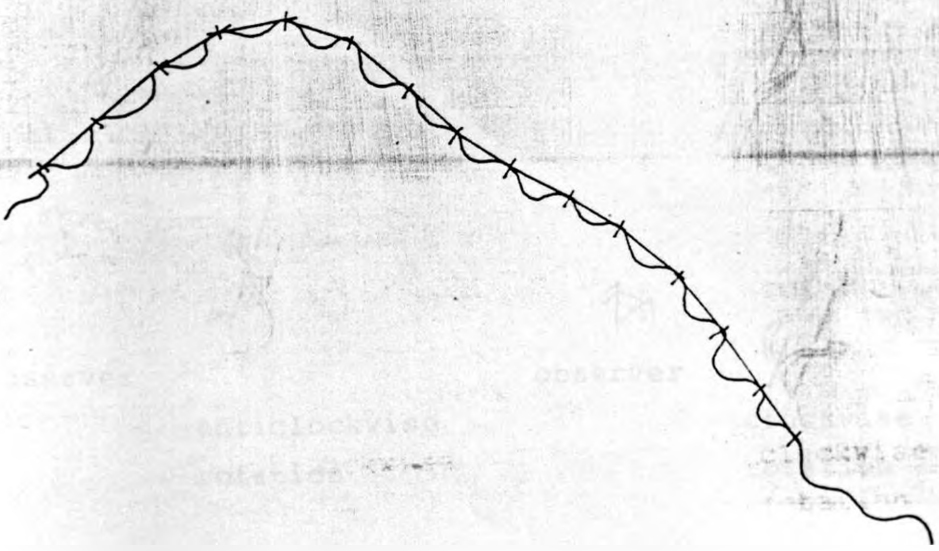
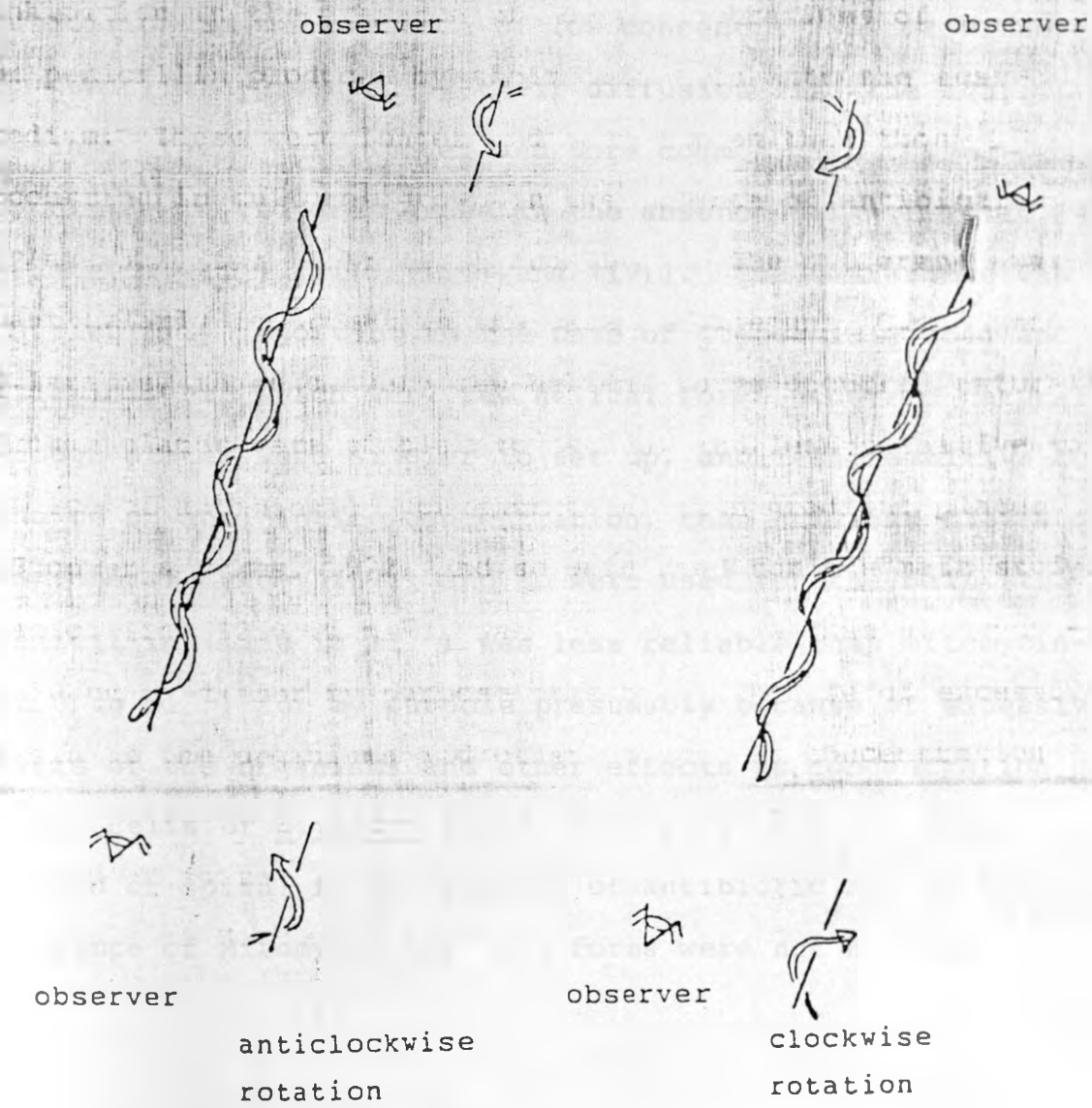


Fig. 5.4 Diagram to show how right or left handedness (clockwise or anticlockwise rotation) of campylobacters was determined.



a) left handed helix

b) right handed helix

5.3 RESULTS

5.3.1 Induction of long forms

Long forms, usually helical, were produced by campylobacters in the region of growth next to the zone of inhibition in the presence of low concentrations of Mitomycin or penicillin produced by their diffusion into the agar medium. These were longer and more common than those occurring in cultures grown in the absence of antibiotic [Plate 5.1 (i); (ii); (iii) and (iv)]. The difference was particularly important in the case of C.jejuni, C.coli and C.laridis, in which very few helical forms occurred naturally. Trough plates were simpler to set up, and less sensitive to choice of antibiotic concentration, than gradient plates (Spooner & Sykes, 1972) and so were used for the main study. Penicillin (1250 iu ml^{-1}) was less reliable than mitomycin-C (2.0 ug ml^{-1}) for my purpose presumably because of excessive lysis of the organisms and other effects as concentration rose. Cells of C.jejuni strain NCTC 11322 did not appear curved or spiral in the absence of antibiotic and in the presence of Mitomycin the long forms were not helical.

5.3.2 Effect of Mitomycin on soma wavelength and amplitude

Nineteen strains representing all the five species and subspecies studied were grown in the presence and absence of Mitomycin and measurements made. No difference or only small differences in soma wavelength and/or amplitude were detected. The data (Table 5.1) indicated that the use of Mitomycin-induced helical long forms for measurement of wavelength and amplitude was justified.

5.3.3 Soma measurements of various species and subspecies

Mean wavelengths and amplitudes of 42 Mitomycin treated-strains including those used above are given in Table 5.2. Strain NCTC 11322 was ignored as it showed no curvature. Two groups emerged: *C.jejuni*, *C.coli* and *C.laridis* group with exception of *C.laridis* NCTC 11352, the type strain, which will be considered later; and *C.fetus* subspecies. Soma measurements of strains of *C.jejuni*, *C.coli* and *C.laridis* are smaller than those of *C.fetus* subspecies. The ranges of wavelength and amplitude means of strains in the former group overlap extensively showing it is not possible to differentiate between the species using these features.

Scatter plots, on the same scale, of wavelengths and amplitudes of the strains for the three species, are compared in Fig. 5.5a (a set of plots on transparent paper is in the pocket at the rear of the thesis). The presentation emphasises further the overlap.

C.laridis NCTC 11352 showed wavelength and amplitude (2.25 um and 0.5 um respectively), which were much larger than any other member of the species. The measurements closely resembled those of C.fetus. When this discrepancy was detected, detailed checking of identity of the strain was undertaken. This confirmed its identity as C.laridis.

Only two C.fetus subsp. venerealis strains were available for study. Soma measurements of these two strains were found to be different (Table 5.2; Fig. 5.5b). The wavelength of strain NCTC 10354 (2.63um) was close to that obtained by Karmali et al. (1981) (2.56um) with the same strain, and the spiral pattern of the organism [Plate 5.1 (iv), (l) and (m)] fitted the description of C.fetus subsp. venerealis (Karmali et al., 1981). Wavelengths and amplitudes of the other strain (G214) were smaller, and similar to those of C.fetus subsp. fetus strains. This overlap meant that unlike Karmali et al. (1981) I could not differentiate between the two subspecies on the basis of soma wavelength.

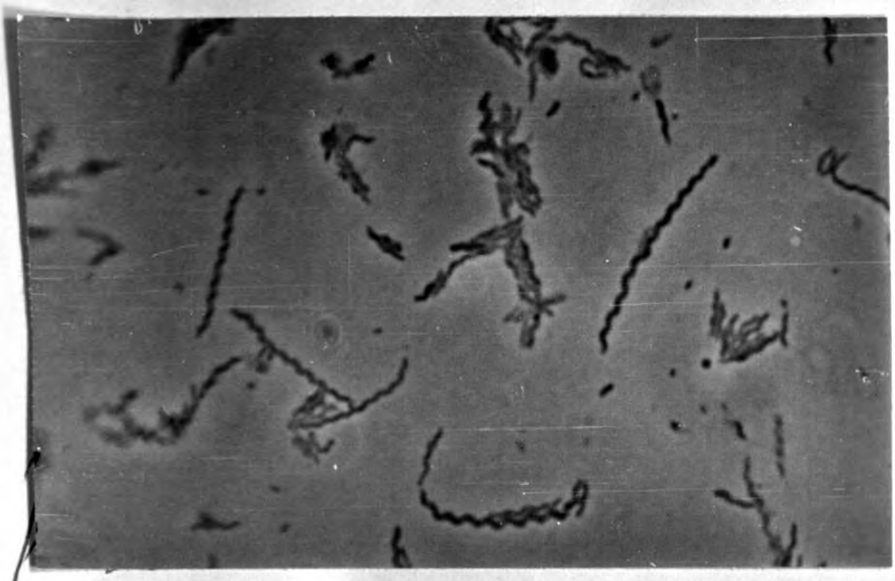
5.3.4 Helix handedness

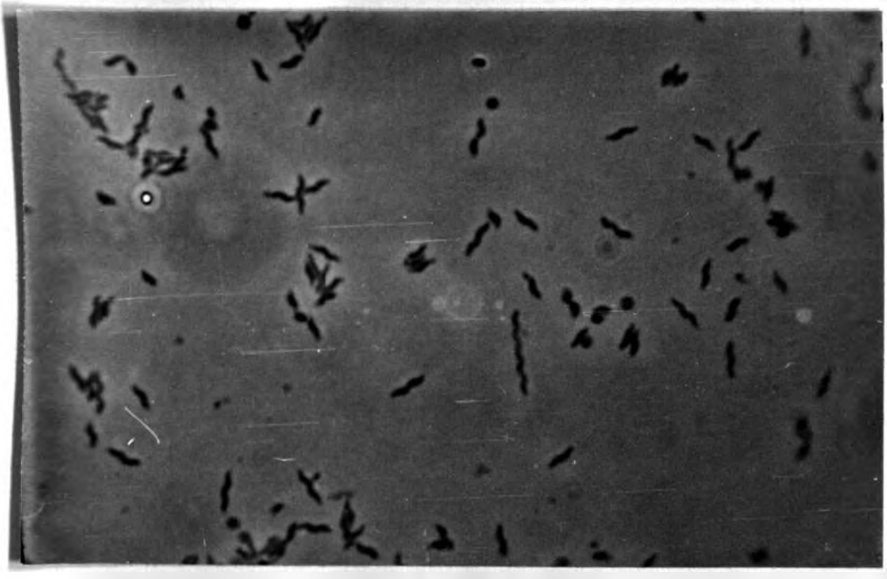
Four campylobacter strains, C.jejuni NCTC 11168, C.laridis NCTC 11352, C.fetus subsp. fetus NCTC 10842 and C.fetus subsp. venerealis NCTC 10354 (G212) were treated with Mitomycin to induce helical long forms and examined for helix handedness. All four had a right handed helix (clockwise rotation) as shown in Plate 5.2 a, b, c and d. The organisms showed good helical morphology which allowed determination of the direction of rotation of helices. Thus the method used to prepare bacteria for examination was adequate for the purpose, although some of the organisms appeared collapsed because of the drying in air under partial vacuum. Absence of differences between the strains studied suggested that further work was not justifiable.

Plate 5.1 Phase contrast photomicrographs of campylobacters showing many long helical forms in Mitomycin-treated cultures compared with the untreated (controls); Magnification x2300. The organisms were exposed to Mitomycin for 24h at $37^{o}C$ under microaerobic incubation (1/3 air + 2/3 95% N_2/5% CO_2).

Plate 5.1 (i)

C.jejuni: (a) NCTC 11168 treated with Mitomycin and (b) untreated (control); (c) SH11 strain treated with Mitomycin as used for drawing shown as Fig.5.3.





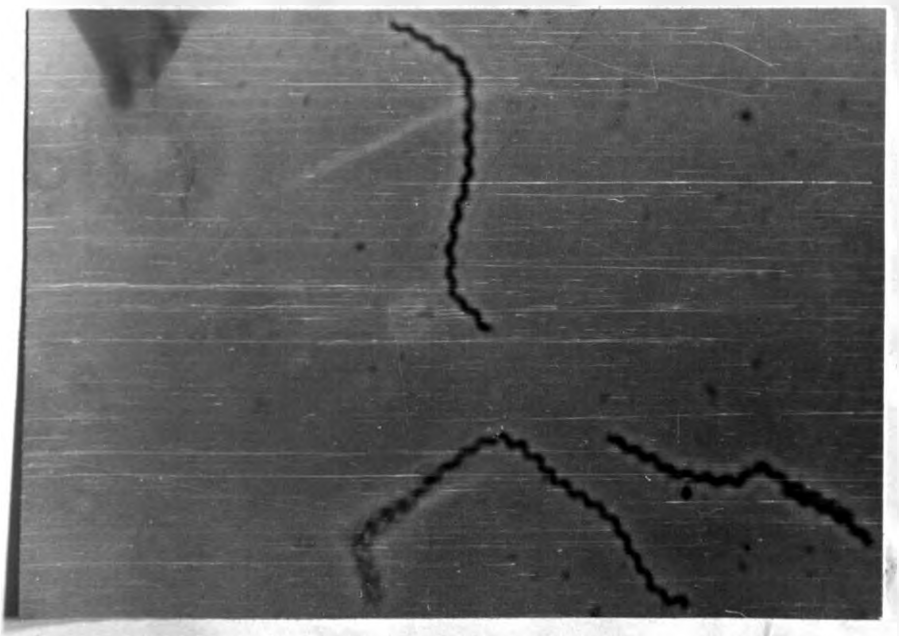
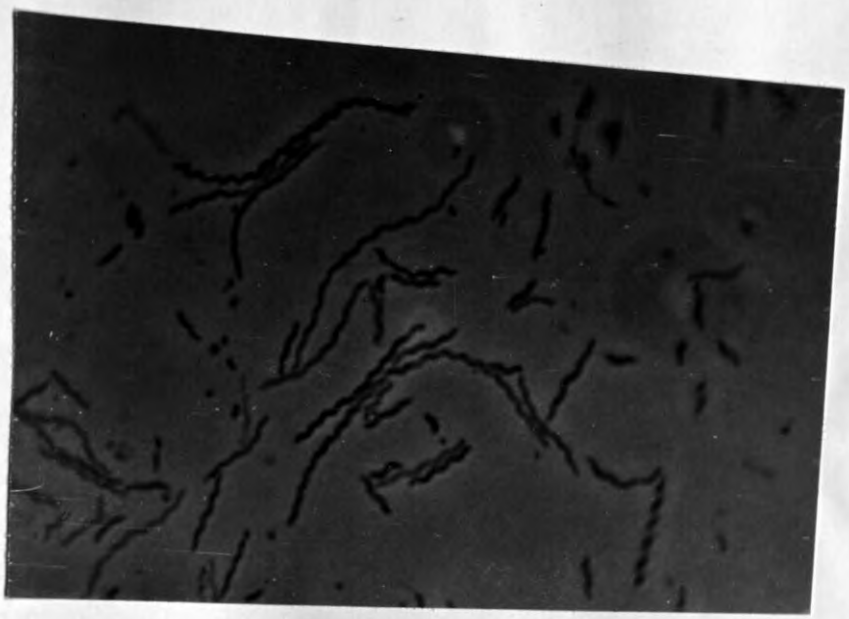


Plate 5.1 (ii)

C.coli: NCTC 11350 (d) treated with Mitomycin and (e) untreated (control).



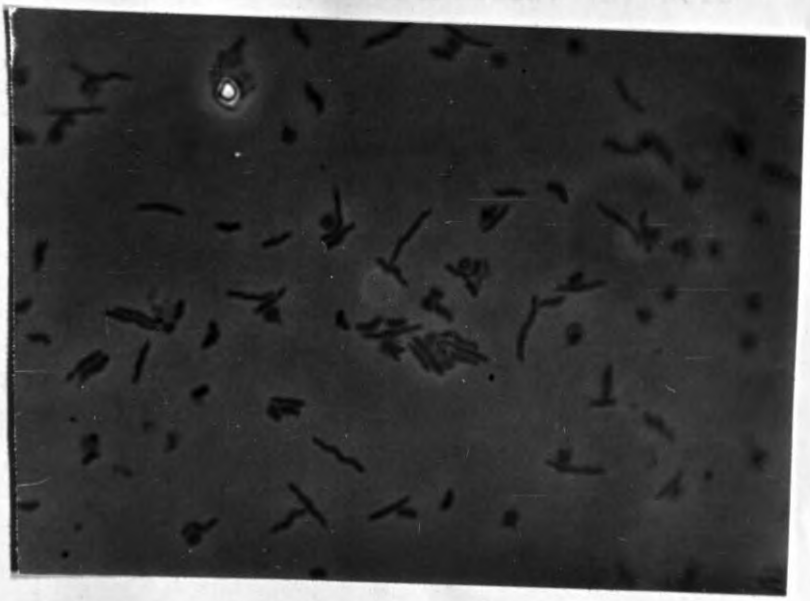
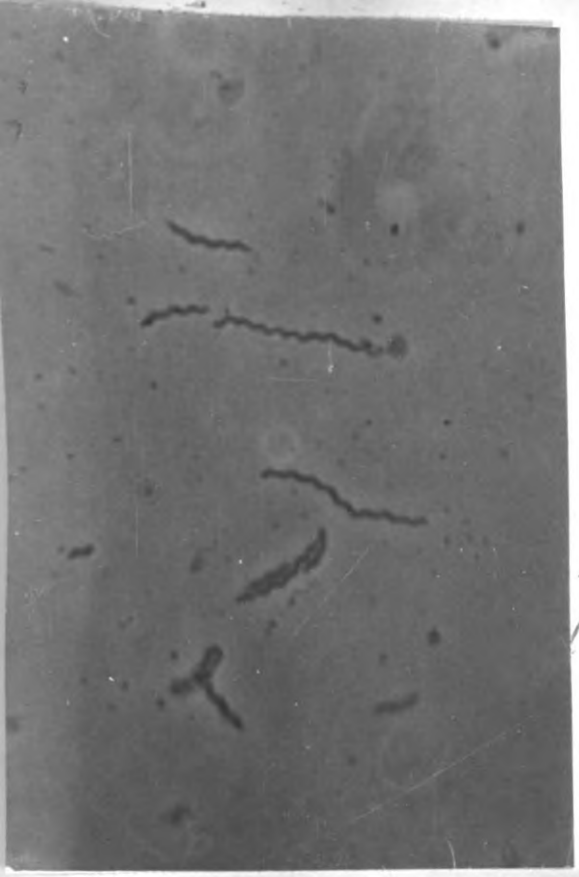
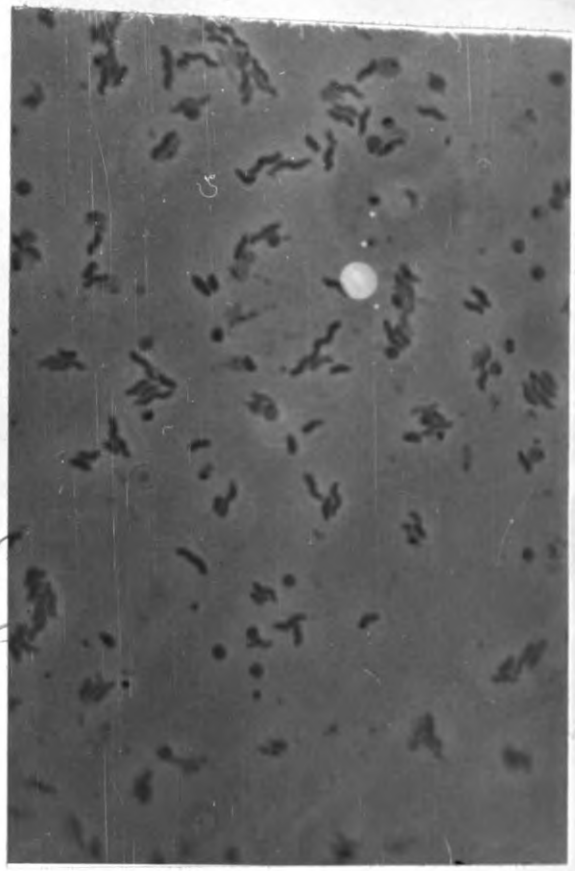
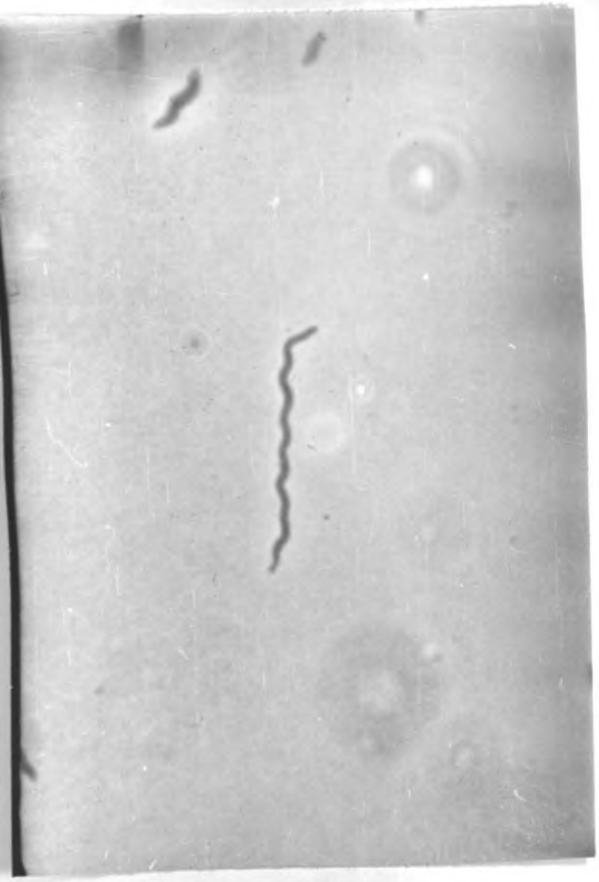


Plate 5.1 (iii)

C.laridis: (f) strain C40 treated with Mitomycin and (g) untreated; (h) NCTC 11352 the type strain treated with Mitomycin and (i) untreated. Note the markedly greater wavelength of NCTC 11352 (h) compared with strain C40.







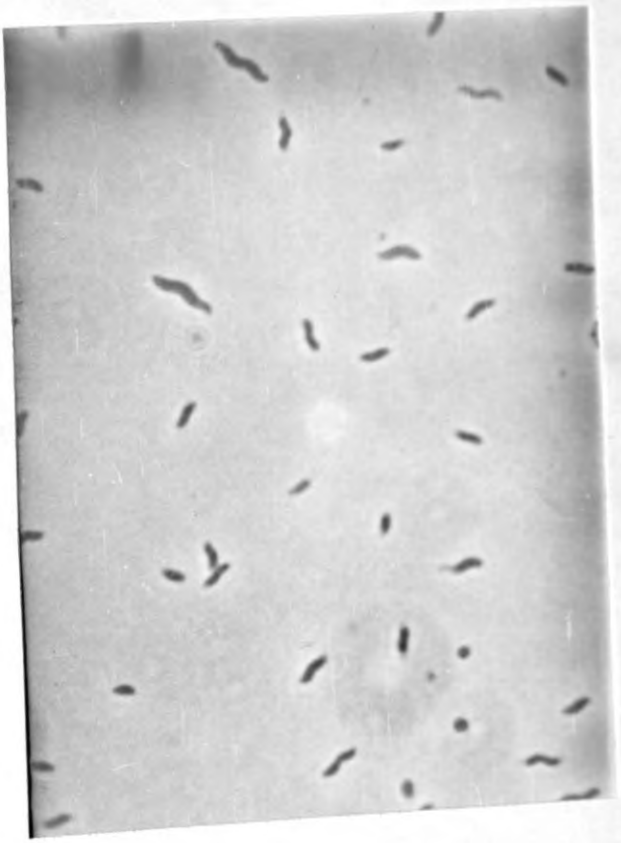
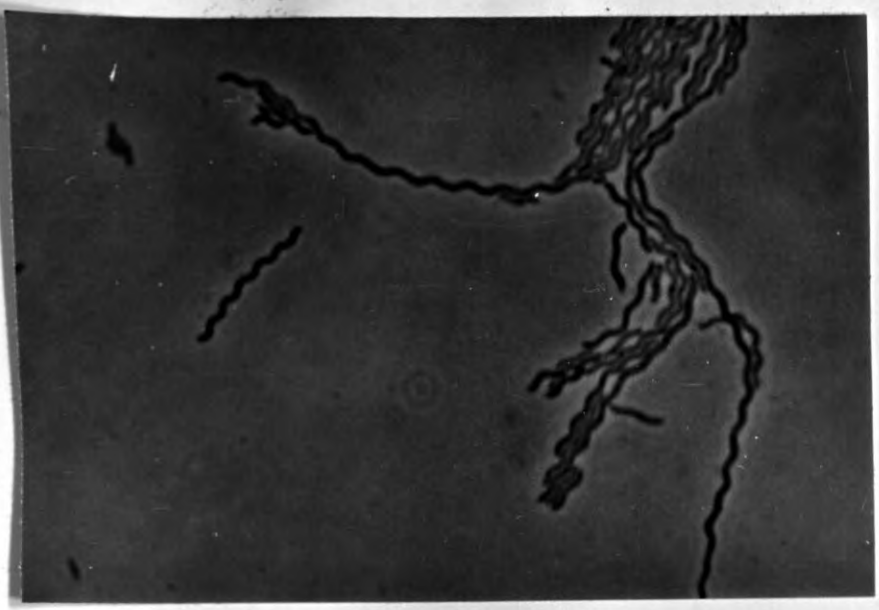


Plate 5.1 (iv)

C.fetus subsp.*fetus*: (j) NCTC 10842 treated with Mitomycin and (k) untreated.



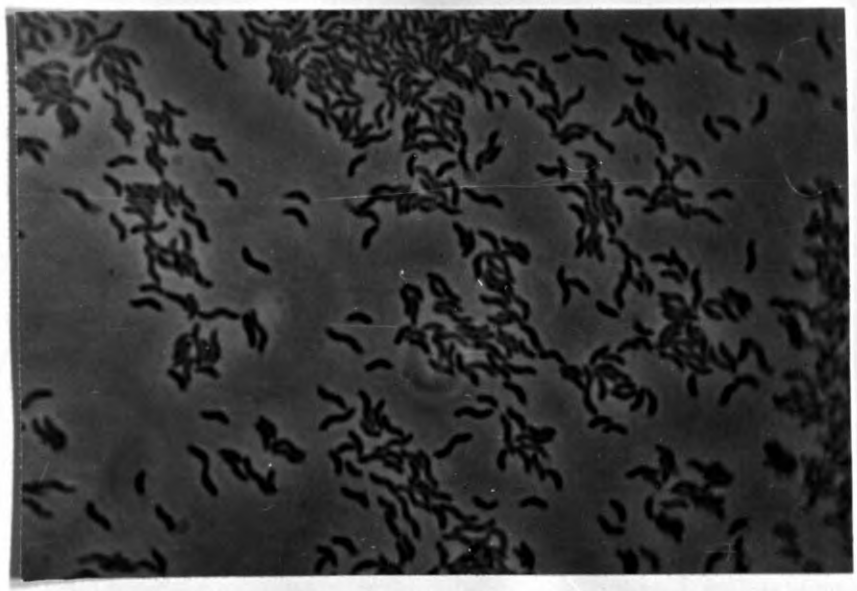
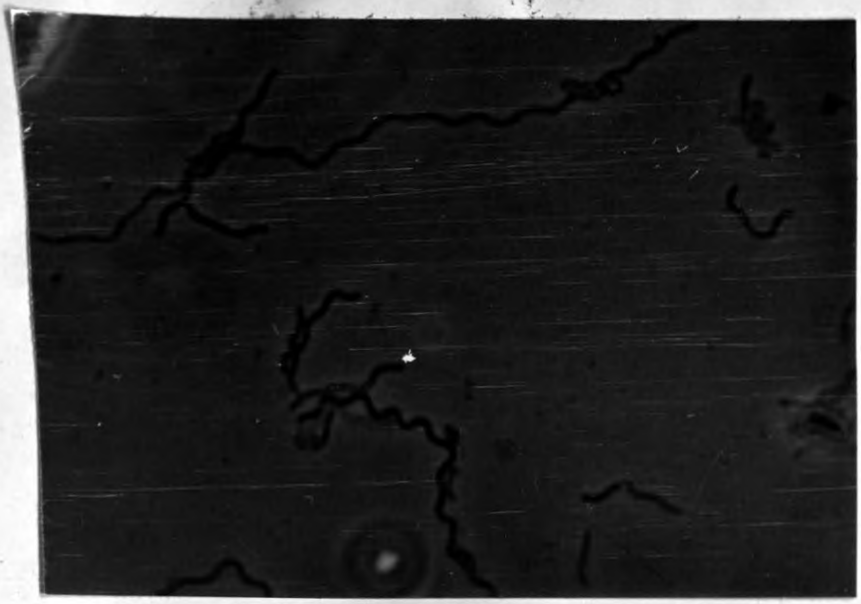


Plate 5.1 (iv) contd.

(l) C.fetus subsp. venerealis NCTC 10354 (G212) treated with Mitomycin and (m) untreated.



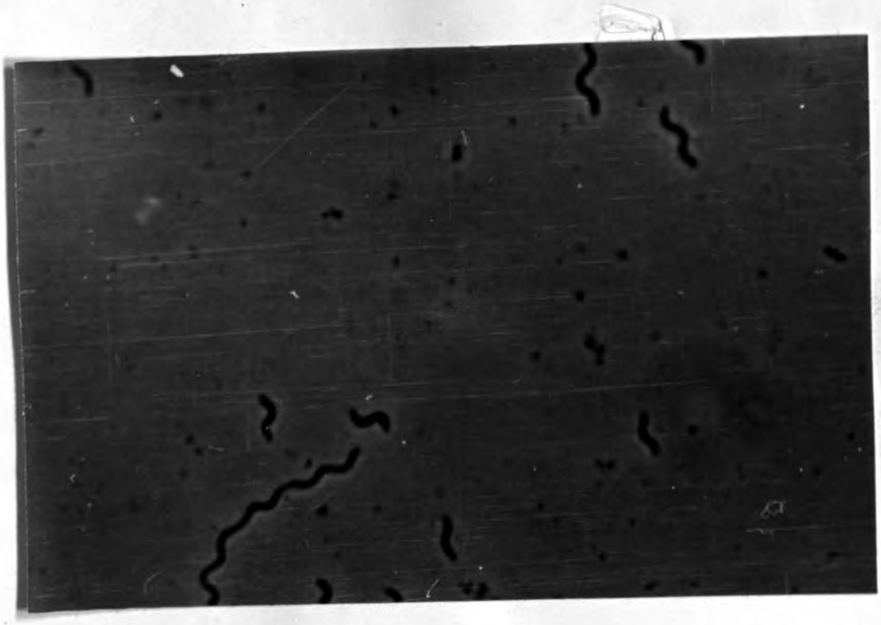


Table 5.1 Wavelength and amplitude of campylobacters treated with Mitocycin and and untreated

Strain	treatment with Mitomycin			untreated		
	n	$\bar{X}$	SD	n	$\bar{X}$	SD
C.jejuni			λ (um)			
SH 9	98	1.17	0.13	17	1.19	0.19
SH 11	136	1.19	0.15	23	1.06	0.11
SH 38	107	1.05	0.12	45	1.08	0.12
NCTC 11168	114	1.19	0.14	20	1.11	0.10
			α (um)			
SH 9	98	0.34	0.07	17	0.30	0.06
SH 11	136	0.38	0.08	23	0.41	0.07
SH 38	107	0.25	0.04	45	0.25	0.04
NCTC 11168	114	0.31	0.07	20	0.28	0.07

Table 5.1 contd.

Strain	Treatment with Mitomycin			Untreated		
	n	$\bar{X}$	SD	n	$\bar{X}$	SD
C.coli						
			λ (um)			
P1	102	1.18	0.13	28	1.20	0.11
P3	46	1.22	0.11	23	1.27	0.11
P23	47	1.22	0.13	21	1.21	0.11
NCTC 11350	80	1.33	0.19	32	1.25	0.20
			α (um)			
P1	102	0.32	0.07	28	0.32	0.05
P3	46	0.33	0.07	23	0.33	0.07
P23	47	0.27	0.06	21	0.31	0.06
NCTC 11350	80	0.39	0.09	32	0.28	0.06
C.laridis						
			λ (um)			
C27	49	1.23	0.15	7	1.27	0.13
C31	48	1.14	0.12	60	1.16	0.14
C40	77	1.19	0.15	41	1.25	0.16
NCTC 11458	49	1.38	0.16	21	1.36	0.16

Table 5.1 contd.

Strain	Treatment with Mitomycin			Untreated		
	n	$\bar{X}$	SD	n	$\bar{X}$	SD
			α (um)			
C27	49	0.42	0.10	7	0.42	0.14
C31	48	0.28	0.05	60	0.28	0.05
C40	77	0.31	0.07	41	0.34	0.08
NCTC 11458	49	0.37	0.10	21	0.34	0.10
C.fetus subsp. fetus						
			λ (um)			
D15	69	2.23	0.25	49	2.02	0.28
D23	98	1.98	0.31	61	1.81	0.22
D217	69	2.20	0.23	31	2.23	0.26
NCTC 10348	46	2.06	0.27	26	2.15	0.24
NCTC 10842	63	1.87	0.22	18	1.88	0.18
			α (um)			
D15	69	0.53	0.12	49	0.47	0.11
D23	98	0.46	0.11	61	0.45	0.10
D217	69	0.40	0.08	31	0.46	0.10
NCTC 10348	46	0.51	0.15	26	0.58	0.16
NCTC 10842	63	0.37	0.08	18	0.39	0.11

Table 5.1 contd.

Strain	Treatment with Mitomycin			Untreated		
	n	$\bar{X}$	SD	n	$\bar{X}$	SD
C.fetus subsp. *venerealis*						
			λ (um)			
NCTC 10354 (G212)	65	2.63	0.37	53	2.62	0.37
G214	52	1.93	0.22	38	2.01	0.26
			α (um)			
NCTC 10354 (G212)	65	0.59	0.15	53	0.63	0.16
G214	52	0.36	0.09	38	0.47	0.08

λ = wavelength

α = amplitude

$\bar{X}$ = mean of λ or α

SD = standard deviation

n = number of measurements of λ and α

Table 5.2 : Wavelength and amplitude of 8 strains of C.jejuni, 12 strains of C.coli, 9 strains of C.laridis, 11 strains of C.fetus subsp.fetus a strains of C.fetus subsp. venerealis treated w Mitomycin, including those used in Table 5.1.

Bacterial Strains	n	λ (um)		α (um)	
		$\bar{X}$	SD	$\bar{X}$	SD
C.jejuni					
SH 11	136	1.19	0.15	0.38	0.08
NCTC 11168	114	1.19	0.14	0.31	0.07
SH 9	98	1.17	0.13	0.34	0.07
SH 32	105	1.17	0.15	0.29	0.07
SH 3	87	1.08	0.11	0.31	0.05
SH 38	107	1.05	0.12	0.25	0.04
SH 8	40	1.03	0.16	0.22	0.04
SH 14	117	0.98	0.10	0.32	0.06
Maximum		1.19		0.38	
Minimum		0.98		0.22	
Mean		1.12		0.31	
SD		0.15		0.08	

Table 5.2 contd.

Bacterial Strains	n	λ (um) $\bar{X}$	λ (um) SD	∝ (um) $\bar{X}$	∝ (um) SD
C.coli					
NCTC 11350	80	1.33	0.19	0.39	0.09
P8	110	1.27	0.12	0.38	0.07
P43	66	1.25	0.15	0.36	0.07
P6	79	1.24	0.11	0.35	0.06
P3	46	1.22	0.11	0.33	0.07
P23	47	1.22	0.13	0.27	0.06
P49	69	1.22	0.14	0.35	0.09
P1	102	1.18	0.13	0.32	0.07
P2	51	1.18	0.13	0.35	0.06
P55	45	1.15	0.13	0.27	0.06
P48	78	1.11	0.17	0.30	0.07
NCTC 11366	52	1.11	0.12	0.32	0.07
Maximum		1.33		0.39	
Minimum		1.11		0.27	
Mean		1.22		0.34	
SD		0.15		0.08	

Table 5.2 contd.

Bacterial Strains	n	λ (um) $\bar{X}$	λ (um) SD	α (um) $\bar{X}$	α (um) SD
C.laridis					
NCTC 11352	47	2.25#	0.30	0.50#	0.15
C44	69	1.38	0.18	0.29	0.06
NCTC 11458	49	1.38	0.16	0.37	0.08
C81	85	1.36	0.18	0.33	0.08
C11	59	1.30	0.16	0.43	0.14
C27	49	1.23	0.15	0.42	0.14
C72	68	1.23	0.24	0.41	0.11
C40	77	1.19	0.15	0.31	0.07
C31	48	1.14	0.12	0.28	0.05
Maximum		2.25		0.50	
Minimum		1.14		0.28	
Mean		1.28*		0.36*	
SD		0.19		0.12	
C.fetus subsp. *fetus*					
D43	84	2.31	0.29	0.51	0.12
D15	69	2.23	0.25	0.53	0.11

Table 5.2 contd.

Bacterial Strains	n	λ (um)		∝ (um)	
		$\bar{X}$	SD	$\bar{X}$	SD
D19	27	2.22	0.24	0.51	0.12
D217	69	2.21	0.23	0.40	0.08
55/30D	74	2.17	0.23	0.37	0.08
V28D	78	2.13	0.25	0.41	0.12
D215	92	2.11	0.21	0.41	0.11
50/1D	92	2.07	0.25	0.33	0.08
NCTC 10348	46	2.06	0.27	0.51	0.15
D23	98	1.98	0.31	0.47	0.11
NCTC 10842	63	1.87	0.22	0.37	0.08
Maximum		2.22		0.51	
Minimum		1.87		0.33	
Mean		2.12		0.43	
SD		0.28		0.12	

Table 5.2 contd.

Bacterial Strains	n	λ (um) $\bar{X}$	λ (um) SD	∝ (um) $\bar{X}$	∝ (um) SD
C.fetus subsp. *venerealis*					
NCTC 10354 (G212)	65	2.63	0.37	0.59	0.15
G214	52	1.93	0.22	0.36	0.09

λ = wavelength

∝ = amplitude

n = number of wavelength and amplitude measurements

$\bar{X}$ = mean

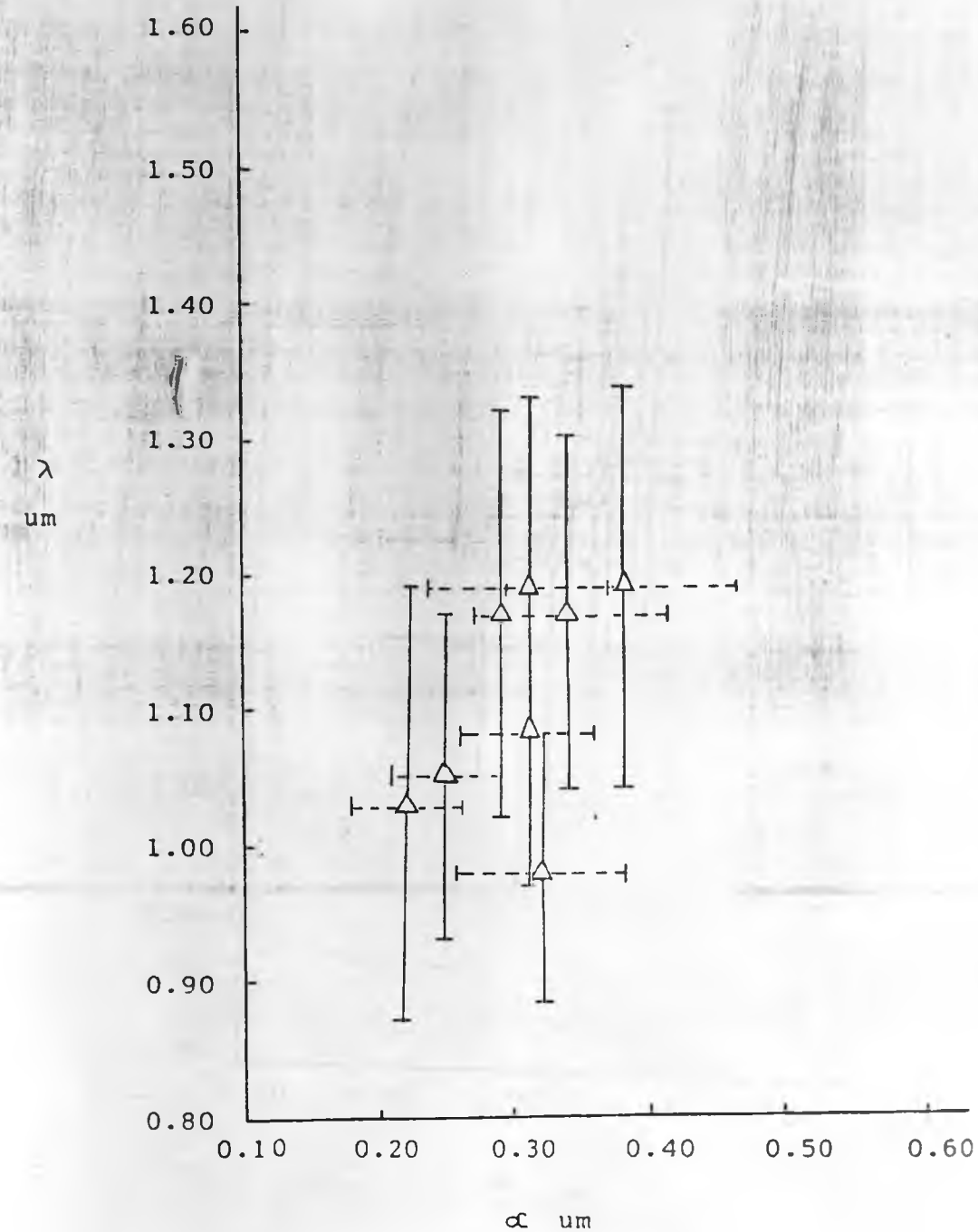
SD = standard deviation

= soma measurements larger than any other member of *C.laridis*

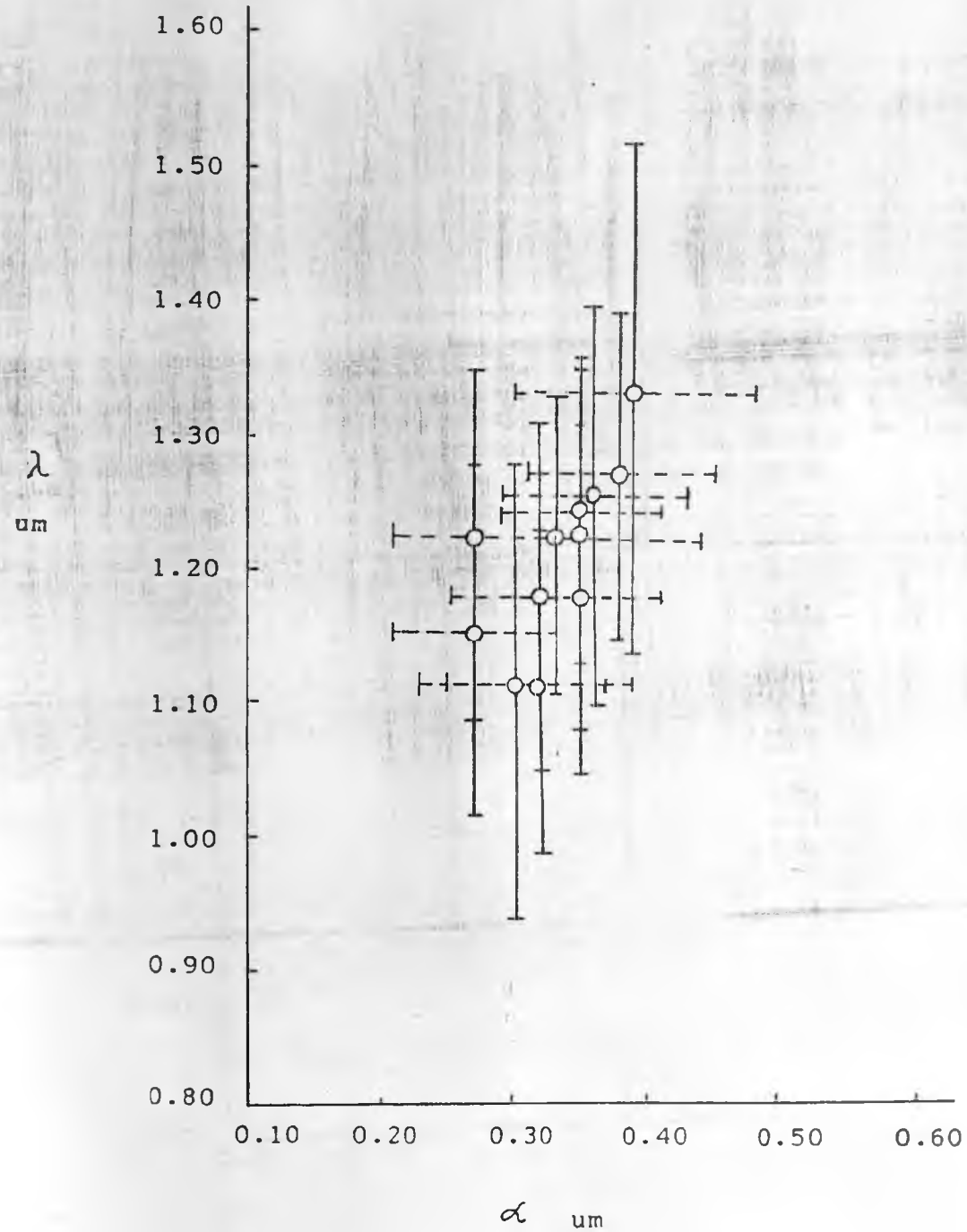
* = means excluding NCTC 11352 values.

Fig. 5.5 a

Scatter plots of the means of wavelengths and amplitudes of the strains of C.jejuni, C.coli and C.laridis group to give a pictorial comparison of the three species (NCTC 11352, the type strain of C.laridis whose wavelength and amplitude are exceptionally large, is not included). Since the scale is the same for the three species, comparisons can be done by placing the graphs on top of each other to see spread of the measurements. For this purpose a set of plots on transparent sheets is placed in the pocket at the rear of the thesis. It can be seen that the scatter points of wavelengths and amplitudes for the three species overlap extensively.


1.60
1.50
1.40
1.30
λ
um
1.20
1.10
1.00
0.90
0.80
0.10
0.20
0.30
0.40
0.50
0.60
α um

C. coli


1.60
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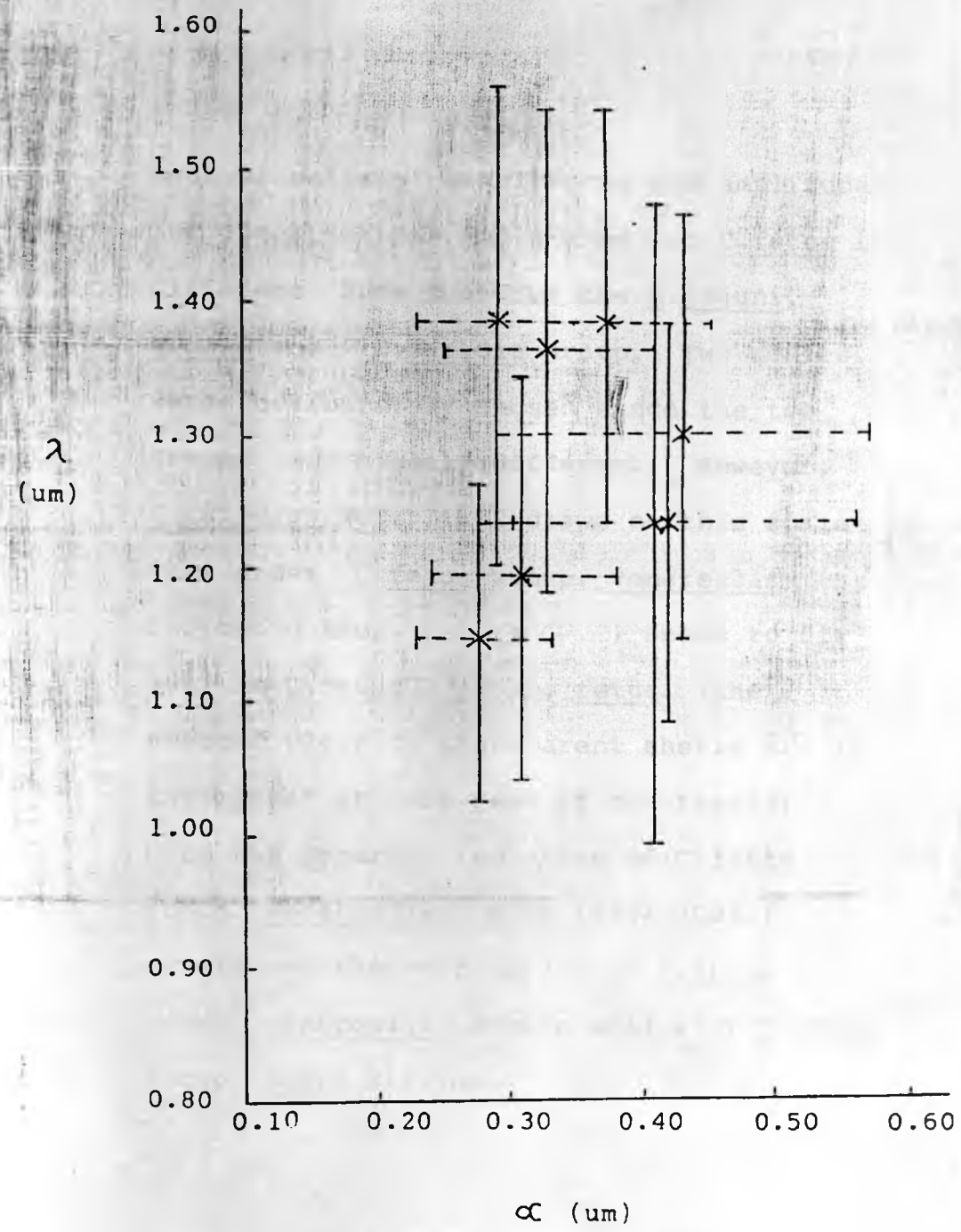
α um


1.60
1.50
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1.10
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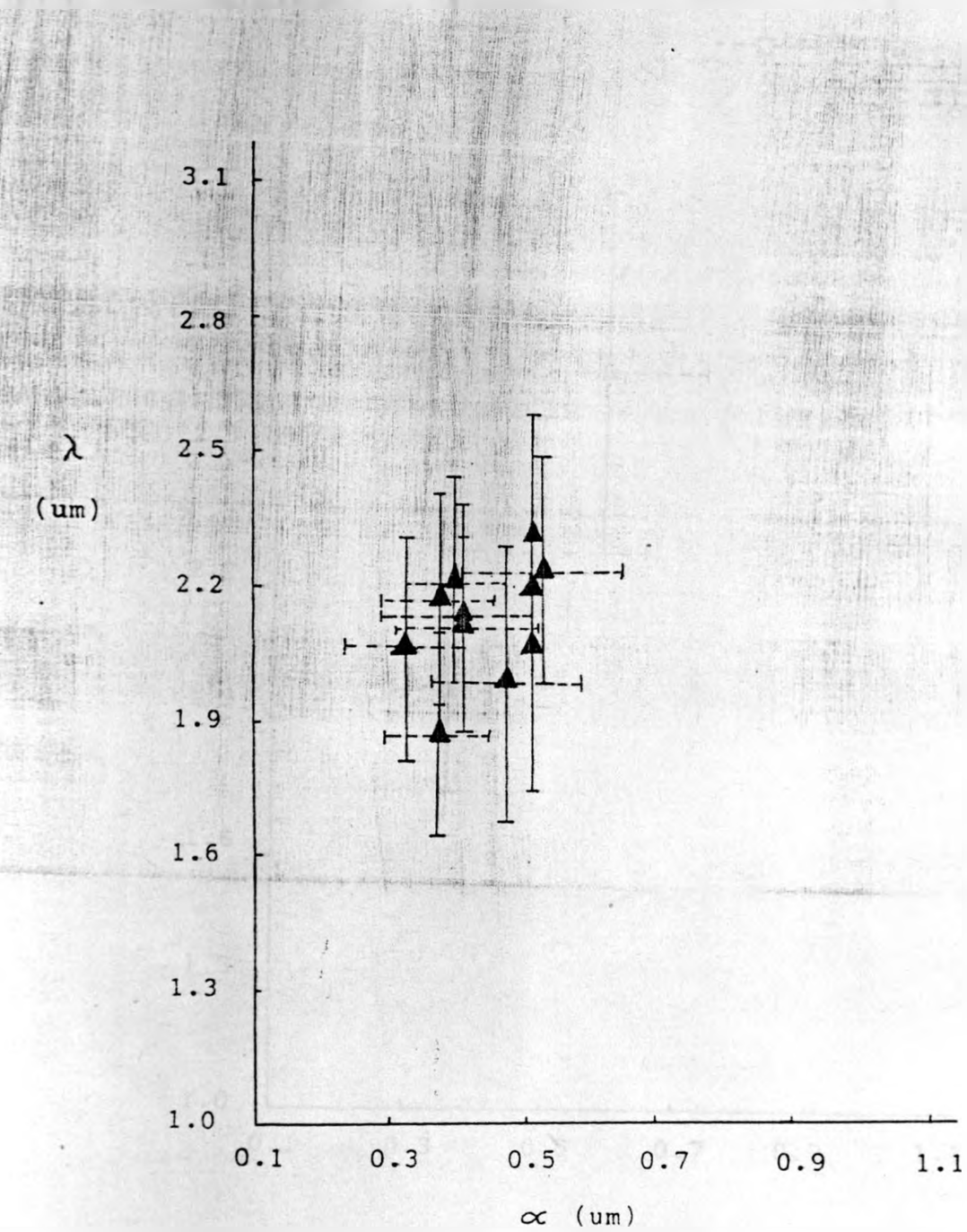
0.90
0.80
λ
(um)
0.10
0.20
0.30
0.40
0.50
0.60
α (um)

Fig. 5.5 b.

Scatter plots of wavelengths and amplitudes for C.fetus. The scale used for C.fetus is different from that for the C.jejuni, C.coli and C.laridis group. Two scales were deliberately chosen since the two groups are clearly different. However, C.laridis NCTC 11352 drawn on this scale (see under C.fetus subsp. venerealis indicated thus ) seems to fit well in C.fetus subsp. fetus. (the scatter plots on transparent sheets are in the pocket at the rear of the thesis). Note the apparent isolation of C.fetus subsp. venerealis NCTC 10354 (G212) strain and the overlapping of C.fetus subsp. venerealis strain G214 with C.fetus subsp. fetus strains.

C.fetus subsp. fetus



C.fetus subsp. venerealis

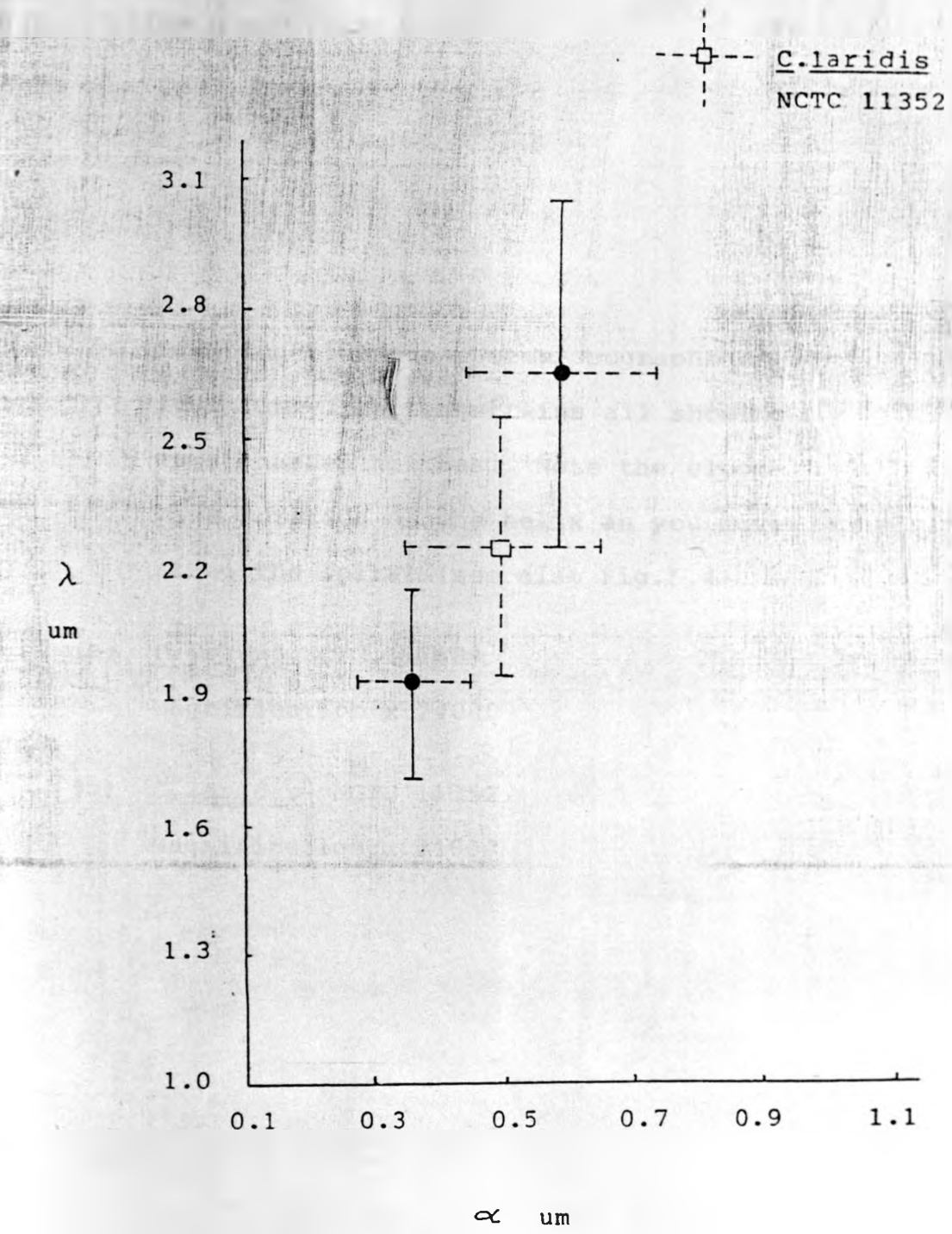


Plate 5.2 Scanning electron photomicrographs of four campylobacter strains all showing right-handed helices. Note the clock-wise rotation of the helix as you move along the spiral (see also Fig.5.4).

(a) C.jejuni NCTC 11168
Magnification x 24000

(b) C.laridis NCTC 11352
Magnification x 24000

Plate 5.2

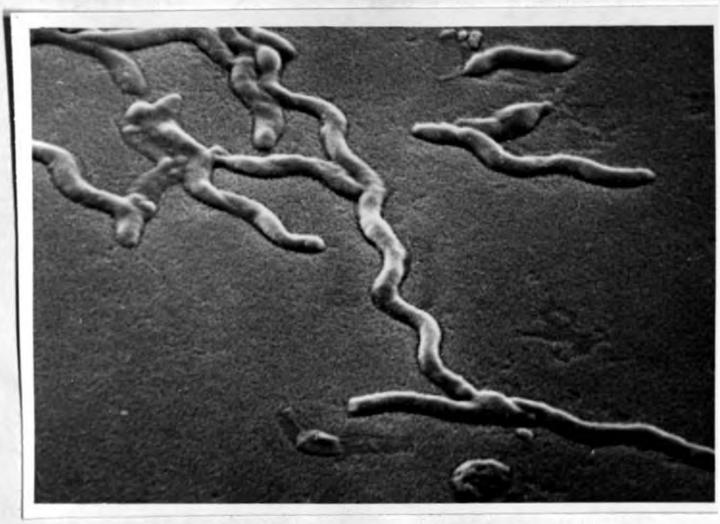
(c) C.fetus subsp. fetus NCTC 10842

Magnification x 8400

(d) C.fetus subsp. venerealis

NCTC 10354 (G212)

Magnification x 12000



5.4 DISCUSSION

Treatment of campylobacters with penicillin or Mitom caused formation of helical long forms so facilitating measurement of soma wavelength and amplitude. This was specially useful for C.jejuni, C.coli and C.laridis strai which do not readily produce helical forms without antibiotics. Long forms of bacteria have been produced w number of antibacterial agents at low concentrations for different purposes. For example Park (1982) used penicil a β - lactam antibiotic, which blocks incorporation of peptidoglycan units into murein of the bacterial cell wal thereby interfering with its synthesis, and Mitomycin-C, binds DNA preventing its replication yet allowing elongat to produce long forms of E.coli for teaching of bacterial physiology. Konishi & Yoshii (1986) have used cephalex β -lactam antibiotic, to produce long helical forms of Aquaspirillum spp. whose short cells do not normally form complete spirals, to facilitate determination of helix handedness. Nalidixic acid which binds DNA preventing replication, yet allowing elongation of the cells, has be used in the study of viable but non-culturable stage of C.jejuni (Rollins & Colwell, 1986) and in direct microsco count of viable bacterial cells (Kogure et al., 1979).

Both penicillin and Mitomycin were used in this stud and found to be suitable in inducing formation of helical

forms, but Mitomycin produced less changes in the culture and was therefore used for the main study. The trough agar plate was easy to prepare and convenient for culture of campylobacters and production of long forms. Since a heavy inoculum was used, there was ample growth and a good crop of helical forms for both C.fetus subspecies and the thermophilic campylobacters after incubation for 24h at 37°C. All the strains were incubated for 24h at 37°C in order to equalize test conditions. Treatment produced plenty of helical forms as shown in Plate 5.1 (i) to (iv).

The C.jejuni, C.coli and C.laridis group has been described as having helical cells with tight spirals (short wavelength) and C.fetus as having open undulations (long wavelength), which enables easy differentiation between the two groups (Skirrow & Benjamin, 1980). Karmali et al. (1981) went a stage further to show a clear distinction in wavelength and amplitude of C.jejuni, C.fetus subsp. fetus and C.fetus subsp. venerealis by measuring the organisms. In the study presented here, a clear distinction was found between C.jejuni, C.coli and C.laridis group on the one hand, and C.fetus subspecies on the other, except for C.laridis NCTC 11352, the type strain, which is discussed later. These findings agree with and expand those of Skirrow & Benjamin (1980), Skirrow et al. (1983) and Karmali et al. (1981). The wavelength and amplitude data for C.jejuni, C.coli and C.laridis (Table 5.2) do not help in differentiating between

species. There is a wide variability of wavelength and amplitude between strains within a species as indicated by the range of means (Table 5.2; Fig. 5.5 a & b). The overlap of means of strains of different species emphasises the difficulty of distinguishing between closely related groups.

Karmali et al. (1981) reported a clear distinction between C.fetus subsp. fetus (12 strains) and C.fetus subsp. venerealis (4 strains) using wavelength and amplitude. I was only able to study two strains of C.fetus subsp. venerealis, one of which was NCTC 10354 (G212) which Karmali et al. (1981) had also examined. This number of C.fetus subsp. venerealis was too small to make a good comparison with C.fetus subsp. fetus (11 strains), but important observations were made. Although the technique I used for obtaining cell measurements was different from the one used by Karmali et al. (1981), the soma wavelength (2.63um) of C.fetus subsp. venerealis NCTC 10354 (G212) was found to be close to that obtained by Karmali et al.(1981) i.e. 2.56um. However, soma measurements of the other strain (G214) were smaller; wavelength and amplitude means readily overlapped with those of C.fetus subsp. fetus (Table 5.2; Fig. 5.5b) making it impossible to differentiate between the two subspecies. The findings of Karmali et al. (1981) encouraged the continued use of wavelength and amplitude as differential characteristics for C.fetus subsp. fetus and C.fetus subsp. venerealis. However, my work shows

that undue reliance cannot be placed on this feature for differentiating between the two subspecies.

Quite unexpectedly, C.laridis NCTC 11352, the type strain, was found to have wavelength and amplitude (Table 5.2) completely different from those of other strains of the species and closely similar to those of C.fetus subspecies. This organism has never been measured before. Attempts by Skirrow (Skirrow, 1987, personal communication) to measure the cell wavelength failed because the organisms were too short and Skirrow could only describe them as 'oval and curved bacilli'. I was able to measure the cell wavelength and amplitude because treatment with Mitomycin induced formation of helical forms. However, compared with other strains, long helical forms were not as plentiful. A check of identity by physiological tests confirmed that the strain used was C.laridis.

As this is the first time the strain has been measured for wavelength and amplitude, a question one asks is whether other batches of NCTC 11352 are the same or different. There are two possibilities: either (1) C.laridis NCTC 11352 has a large wavelength and amplitude markedly different from other members of the species or (2) the large measurements observed are not those of NCTC 11352. Observations in this study supports the former possibility, since the culture behaved physiologically as C.laridis, and thus emphasises the fact

that within species, there is wide diversity in wavelength and amplitude between strains.

Another morphological characteristic of campylobacters examined during this study was helix handedness (clockwise or anticlockwise rotation) of the spirals. The main aim was to find whether the characteristic could be useful in differentiating between groups and in classification. However, the results obtained in this study do not suggest that the characteristic is of any differential value since all the four strains studied representing C.jejuni, C.laridis, C.fetus subsp.fetus and C.fetus subsp. venerealis showed a right-handed helix (clockwise rotation; Plate 5.2 a, b, c and d). The right handed helix is also possessed by other spiral bacteria, for example Aquaspirillum metamorphum (Konishi & Yoshii, 1986).

Only Mitomycin-treated cells were examined for helix handedness. Since untreated cells were not examined, it is not known whether antibiotic treatment had any effect on the helix handedness. However, in a study similar to this one, Konishi & Yoshii (1986) reported no effect of antibiotic treatment on the left handed helix of A.itersonii and A.peregrinum, both spiral bacteria, using a β-lactam antibiotic cephalexin to induce long helical forms to facilitate determination of helix handedness.

5.5 CONCLUSIONS

1. Use of Mitomycin greatly facilitated the determination of wavelength and amplitude of the soma of campylobacters.

2. In general, the thermophilic campylobacters have smaller wavelength and amplitude than C.fetus.

3. Exceptions mean that soma measurements cannot be relied upon for grouping campylobacters or for distinguishing between the subspecies of C.fetus.

4. There is no evidence to indicate that helical handedness is a useful differential feature within the genus.

CHAPTER 6

HAEMOLYTIC ACTIVITY OF CAMPYLOBACTERS

6.1 INTRODUCTION

Bacteria produce many types of toxins among which are haemolysins which damage red blood cell membrane causing lysis (Bernheimer, 1976). Bacterial haemolysins are produced mainly by the Gram-positive bacteria. Some Gram-negative bacteria have also been shown to produce haemolysins, for example some strains of Escherichia coli (Short & Kurtz, 1971; Rennie et al., 1974); Vibrio parahaemolyticus (Miyamoto et al., 1969; Sakurai et al., 1971); Vibrio cholerae biotype eltor (Sakazaki et al., 1971); and Aeromonas hydrophila (Bernheimer & Avigad, 1974). The ability to cause haemolysis is an important differential characteristic of the organism, and for some, for example V. parahaemolyticus (Miyamoto et al., 1969), production of haemolysin has been claimed to be associated with human pathogenicity. Similarly, haemolysin production by E.coli has been associated with strains that produce enteric disease in some species of animals e.g. oedema in the intestines of pigs and also extra-intestinal diseases in man (Short & Kurtz, 1971).

We know that catalase-positive campylobacters are important enteric and extra-intestinal pathogens of both man and animals. They are generally reported as not haemolytic but little work has been reported concerning this characteristic. We had often observed, in this laboratory,

some haemolytic changes on horse blood agar inoculated with campylobacters. A study by Fricker *et al.* (1985) using a tube test and horse erythrocytes showed hot-cold type haemolysis by *C.jejuni* strains. This interesting finding prompted us to investigate further the haemolytic activity of several catalase-positive *Campylobacter* species on blood agar plates.

A number of factors which might influence *in vitro* expression of haemolysis, including synergistic effect with the organisms responsible for the CAMP reaction (Christie *et al.*, 1944) were examined. The CAMP reaction, named after the initials of the authors (Christie, Atkins & Munch - Petersen) who first described it, is shown by using a *Staphylococcus aureus* strain that produces only the β-lysin. This is a hot-cold lysin i.e. it does not lyse erythrocytes until they have been chilled. When *Streptococcus agalactiae*, normally non-haemolytic, is growing near such a *Staph.aureus* on a blood agar plate it causes lysis of the erythrocytes from some animal species e.g. sheep, goat and cattle if they have been sensitized by the β-lysin without the need for chilling.

It was hoped that a study of these phenomena would yield interesting results of differential or diagnostic value, and in addition would increase our understanding of the interaction of campylobacters with mammalian cells.

6.2 MATERIALS AND METHODS

6.2.1 BACTERIAL STRAINS

1. Campylobacter strains

The 152 strains used in this study representing four species, were from a number of different sources and mostly isolated in this department. These were:

1. C.jejuni. Eighty three human strains, isolated in this department from faeces of human beings suffering from gastroenteritis; 19 environmental strains isolated from sewage; plus NCTC 11168 and NCTC 11322.
2. C.coli. Four human strains; 17 pig strains; and NCTC 11350 and NCTC 11366.
3. C.laridis. Eighteen seagull strains plus NCTC 11458 and NCTC 11352.
4. C.fetus subsp. fetus. Three bovine and ovine strains plus NCTC 10842 and NCTC 10348.

C.fetus subsp. venerealis was not tested for haemolysis because strains received as C.fetus subsp. venerealis, and which had been chosen for testing for haemolysis, turned out to be C.fetus subsp. fetus when checked for identity by physiological tests.

2. Other bacterial strains

1. Staphylococcus aureus producing only β -lysin, National Collection of Dairy Organisms (NCDO) 1245.
2. Non-haemolytic CAMP-positive Streptococcus agalactiae NCDO 1348.
3. Pseudomonas aeruginosa IA from our departmental stock.

6.2.2 MEDIA FOR TESTING HAEMOLYSIS

Sakazaki et al. (1971) found peptone and extracts from animal tissue to stimulate haemolytic activity of eltor strains of V.cholerae in culture media. They found that suitable conditions were provided by heart infusion broth consisting of those two ingredients, by commercially available dehydrated heart infusion broth and by commercially available dehydrated brain heart infusion broth.

Although Vibrio spp. are different from campylobacters, it was decided to experiment with HI (Difco) and BHI (Oxoid), which each contains a high concentration of animal tissue extracts (Table 6.1) to see whether they were suitable for studying haemolytic activity of campylobacters and to compare them with the commonly used blood agar base (BAB) No.2 (Oxoid). Oxoid agar No.1 was used to solidify HI broth to make

HIA because of its high gel strength at low concentration, purity and clarity. RBC of two blood types, sheep and horse, which are commonly used for bacterial cultivation were chosen for the study. To facilitate diffusion out in the hope of achieving a better expression of haemolysis by producing wider zones, less agar than usual, ca 12 ml of HBHA, HBA, SBHA or SBA containing 5% (v/v) washed or unwashed RBC, prepared as described in Chapter 3, was used. Preliminary experiments were undertaken to see the effect of using lower erythrocyte concentrations than 5%. It was hoped that lower concentrations would increase sensitivity of the test, but in fact, when the concentration of washed RBC was less than 5%, haemolysis was less easy to detect because the RBC did not darken the plates sufficiently.

6.2.3 TEST PROCEDURES

1. Cultivation of bacteria for testing

Usually bacteria were prepared as follows. Test organisms were inoculated on YNA, incubated microaerobically for 24h and cultures used for inoculating blood agar plates directly for haemolysis testing, or broths. Inoculated HI or BHI broths (5ml) in 28g McCartney bottles were incubated

Table 6.1 : Composition of heart infusion (Difco), brain heart infusion (Oxoid) and blood agar base No.2 (Oxoid) when prepared for use.

Ingredients	Media		
	Difco HI*	Oxoid BHI	Oxoid BAB No.2
	% w/v	% w/v	% w/v
Beef heart infusion	1.0	0.5	
Bacto-tryptose	1.0		
Calf brain infusion		1.25	
Proteose peptone		1.0	1.5
Dextrose		0.2	
Sodium chloride	0.5	0.5	0.5
Liver digest			0.25
Yeast extract			0.5
sodium phosphate		0.25	
Agar			1.2

* = Solidified with 1.2% (w/v) agar No.1 (Oxoid) to make heart infusion agar

HI = Heart infusion

BHI = Brain heart infusion

BAB = Blood agar base

microaerobically for 48h with bottle screw-caps loose. For some purposes, these HI or BHI broth cultures were used to inoculate blood agar plates and to test for haemolysis in a tube.

2. Plate haemolysis test

a. Inoculation and Incubation of Plates

A well dried blood agar plate was inoculated, in a line covering a small area, with a loopful of broth culture or with a heavy inoculum from a 24h YNA culture in a similar manner. Four to six strains were tested on one plate. After preliminary tests, one strain of C.jejuni SH5, showing distinct haemolysis and scored +, was chosen as a positive control and was always inoculated on plates together with the test organisms. Frankly, results were difficult to categorize. It was decided to use the following categorization. Organisms showing a transparent non-red zone around and beneath the growth after incubation for 4 days were considered positive. Strains showing an area slightly less opaque than uninoculated medium, but still somewhat red beneath and close to the growth were considered weak positive. Strains were regarded as negative if they caused only greening or little or no change of the medium.

Except where indicated, incubation of plates was under microaerobic environment containing hydrogen at 42°C for 4 days.

b. Tests under different conditions

The appropriate incubation time was determined by incubating inoculated sheep blood heart infusion agar (SBHA) plates at 42°C and examining them for haemolysis after 48h, 72h and 96h. C.fetus subsp. fetus strains were always incubated at 37°C, the thermophilic campylobacters were tested for haemolysis at 37°C and 42°C. Washed and unwashed sheep and horse RBC, HIA and BAB No.2 (Oxoid) and the incubating gas mixtures containing nitrogen or hydrogen were compared for their ability to affect expression of haemolysis.

3. Synergistic haemolysis

a. Christie, Atkins & Munch-Petersen (CAMP) phenomenon

Christie et al. (1944) observed the ability of β -lysin producing Staph.aureus and Strep. agalactiae to cause synergistic haemolysis of sheep and bovine erythrocytes. The phenomenon became a very useful test for detecting Strep. agalactiae quickly and accurately on blood agar plates and for differentiating it from other streptococci (Munch-

Petersen & Christie, 1947). Similar synergistic reactions have been reported between other types of bacteria, for example Clostridium perfringens and Strep. agalactiae (Hansen & Elliot, 1980); Staph. aureus and Mobiluncus mulieris, and M.curtisii (Spiegel & Roberts, 1984); Staph. aureus and some Aeromonas spp. (Figura & Guglielmetti, 1987). The phenomenon has been found useful in differentiating between A.hydrophila (positive aerobically or anaerobically), A. sobria (positive aerobically) and A. caviae (negative) in only 18 to 24h whereas it may take up to 5 days using biochemical tests (Figura & Guglielmetti, 1987).

Campylobacters may be producing haemolysins (Fricker et al., 1985), but might not be able to lyse the RBC without a secondary factor. It was therefore decided to test the ability of campylobacters to perform synergistic haemolysis with either β-lysin producing Staph. aureus or Strep. agalactiae.

b. CAMP test demonstration using Staph. aureus and Strep. agalactiae

Preliminary tests showed it was necessary to wash RBC used to detect the CAMP reaction so as to remove inhibitory substances in the blood, for example antibodies. SBHA and SBA plates containing washed SRBC were streaked across the centre

with Staph. aureus and then with Strep. agalactiae at right angles, close to but not touching the Staph. aureus streak. After aerobic incubation at 37°C for 24h, a typical CAMP reaction-arrow-head haemolysis (Plate 6.1) within the zone of erythrocytes darkened by Staph. aureus β-lysin was produced. Similar haemolysis was produced whether incubated aerobically, microaerobically or anaerobically and whether at 37°C or 42°C. Campylobacter strains were similarly tested against both the Staph.aureus and Strep.agalactiae.

The CAMP reaction is reportedly enhanced by anaerobic incubation (Christie et al., 1944). It was therefore decided to attempt the test anaerobically by using pre-grown cultures of campylobacters.

c. Preparation of crude staphylococcal β-lysin (Davis et al., 1984)

Twelve NA plates were streaked across the surface, each plate 6 to 8 times to give parallel lines, with Staph. aureus and then incubated aerobically at 37°C for 48h. The 48h cultures were frozen overnight at -20°C and the agar thawed at room temperature. The resultant released fluid, containing β-lysin, was collected, clarified by centrifugation and then sterilized by filtration through 0.2 um pore size filter. Sterility was tested by inoculating 0.1ml into 10ml NB and incubating at 37°C for 48h. The sterile haemolysin was

dispensed into bijoux bottles in 5ml amounts and if not used immediately, was stored at 4°C. Activity remained for at least 3 months.

d. Efficiency of crude β-lysin, and the test for synergistic haemolysis with campylobacters under anaerobic conditions

SBHA plates were swabbed through the middle with a cotton wool swab soaked in β-lysin to sensitize the erythrocytes and the surface allowed to dry. Strep. agalactiae was streaked at right angles to the hopefully sensitized zone and partly within it. Plates were incubated at 37°C and 42°C. Good CAMP reactions were obtained aerobically, microaerobically and anaerobically at both temperatures. BHI culture broths (48h) of the campylobacter test organisms were similarly streaked and plates incubated anaerobically for four days with Strep.agalactiae included as a positive control for haemolysis and Pseudomonas aeruginosa as a control for anaerobiosis.

4. Tube haemolysis test

Campylobacters were checked for haemolysis by using a tube test. For each test organism, 2ml of 48h BHI broth culture was pipetted into a centrifuge tube followed by 0.1ml of 10% washed SRBC. A control tube in which haemolysis would not occur and against which haemolysis was visually judged was

prepared in the same way but using sterile broth. Prepared tubes were shaken to mix the contents, incubated in a waterbath at 37°C for 2h and then kept at 4°C overnight before reading. The tubes were first examined visually without disturbing the contents (to check for haemagglutination) then centrifuged and the clear supernatant fluids visually compared with the control. Haemolysis was scored positive, weak positive or negative depending on the amount of haemoglobin detected in the supernatant fluid and hence released from the cells.

5. Attempt to use plate haemolysis to distinguish C.jejuni from other thermophilic campylobacters

The experiment was designed to see whether haemolysis could be used to distinguish C.jejuni from C.coli and C.laridis. Forty strains representing the three species (C.jejuni, 14; C.coli, 13; and C.laridis, 13) were grown on YNA for 24h and plates, together with strain details, were given to Mr. S. Illingworth, a colleague in the department. He then coded the cultures so that I would not know their identity. He returned the newly labelled plates to me to test and identify the strains by haemolysis. Results were given back to him to mark the correctly identified strains. Each strain was then examined for purity and tested for hippurate hydrolysis, resistance to nalidixic acid and cephalothin as described in Chapter 3 to confirm identity by generally accepted criteria.

6.3 RESULTS

6.3.1 PLATE HAEMOLYSIS

1. Incubation period

Initial tests with 20 strains of C.jejuni including NCTC 11168 and NCTC 11322 at 42°C indicated haemolytic activity on SBHA. There was little or no haemolysis after incubation for two days, but clearing on plates was obvious around some strains after three days. It was easiest to detect haemolysis after incubation for four days. This incubation period was subsequently used for all the tests. A period of refrigeration to detect hot-cold effects reported by Fricker et al. (1985) in tube tests was not undertaken. It was thought that this would be cumbersome for routine use and might confuse the clear positive results that emerged.

2. Haemolytic activity of C.jejuni, C.coli, C.laridis and C.fetus subsp. fetus on sheep blood heart infusion agar (SBHA)

All the C.jejuni strains (hippurate +), from both human beings and sewage, showed some haemolysis on SBHA at 42°C. Of the 104 strains examined, 96 (92%) were positive and 8 (8%) weak positive (Table 6.2). The agent was diffusible and caused clearing of the medium, which was easily detected, starting at the middle of the bacterial growth i.e. the area

of ageing cells and often extending beyond the margins of growth. The types of haemolysis expressed by C.jejuni (scored +) at 42°C, compared with C.coli and C.laridis are shown in Plate 6.2 a, b, c and d. Behaviour of C.jejuni, C.coli, C.laridis and C.fetus subsp. fetus at 37°C is shown in Plate 6.3. When swarming occurred with C.jejuni strains (see Plate 6.2 a, strain SH6 and SH28) the whole area covered by the growth was haemolysed.

A number of C.coli strains were haemolytic , some giving only a weak reaction, often with partial clearing at the poles of the streak while the centre was apparently unchanged. C.laridis strains did not generally cause clearing. However, zones of medium around the growth were commonly green. Such an effect is reminiscent of α-haemolysis by streptococci but was considered as negative haemolysis in this study.

3. Haemolysis under different conditions

Haemolysis on BA prepared using BAB No.2 (Oxoid) was poor and unsatisfactory even after incubation for four days. But haemolysis of sheep blood in agar consisting of HIA (50% v/v), BAB No.2 (50% v/v) and 5% (v/v) washed RBC, was better than in sheep BA, but not so good as in SBHA. This indicated a possible lack of essential ingredients or presence of inhibiting substance(s) in BAB No.2.

HI and BHI broth cultures and YNA cultures were equally suitable for the cultivation of campylobacters for haemolysis testing. It is therefore equally satisfactory to use inocula from any of these sources. It was thought that pre-incubation using ingredients in test media might have been important but this was not the case.

Haemolysis occurred with both sheep and horse blood. To exclude the possibility of inhibitory substances in the blood (antihaemolysin antibodies, for example) which might antagonise haemolysis, washed erythrocytes were tested but there was no difference from whole blood. Plates prepared with horse erythrocytes were much lighter in colour than those prepared using sheep erythrocytes. This may present difficulties in reading haemolysis; sheep erythrocytes are therefore preferable.

Haemolysis at 37°C (Plate 6.3) was detected with only one strain of C.jejuni, which was only weakly positive at this temperature but strongly positive at 42°C. C.fetus subsp. fetus, which grows at 37°C and not at 42°C, C.coli and C.laridis were not haemolytic.

Haemolysis was detected equally well under incubation gas mixture containing nitrogen as under a mixture containing hydrogen. However, plates usually darkened under nitrogen which is disadvantageous with regard to reading them.

4. Synergistic haemolysis

No synergistic haemolysis was observed with either β-lysin producing Staph. aureus or Strep. agalactiae, microaerobically or anaerobically.

6.3.2 TUBE TEST

1. Haemolysis

Haemolysis in the tube was tested using BHI broth cultures to check for hot-cold haemolysis and to see whether there was correlation with haemolysis in the plate. Some C.jejuni strains (17% +; 35% weak +) and a large proportion of C.coli (67% +; 14% weak +) showed hot-cold haemolysis while all C.laridis and C.fetus subsp. fetus were negative (Table 6.2).

Table 6.2 : Haemolytic behaviour of 104 strains of *C.jejuni*, 23 strains of *C.coli*, 20 strains of *C.laridis* and 5 strains of *C.fetus* subsp. *fetus* on sheep blood heart infusion agar (SBHA) or in tubes containing washed sheep erythrocytes.

Species	Number of strains examined	Numbers giving stated reaction on SBHA after 4 days incubation								Numbers giving stated reaction in tubes				Numbers showing haemagglutination in tubes			
		42°C				37°C											
		+	±	-	ND	+	±	-	ND	+	±	-	ND	+	±	-	ND
C.jejuni	104	96	8	0	0	0	1	49	54	17	35	48	4	25	0	75	4
C.coli	23	5	6	12	0	0	0	20	3	14	3	4	2	0	0	23	0
C.laridis	20	0	0	20	0	0	0	18	2	0	0	18	2	0	0	18	2
C.fetus subsp. *fetus*	5				5	0	0	5	0	0	0	5	0	0	0	5	0
Total	152																

SBHA = Sheep blood heart infusion agar containing washed erythrocytes

\+ = Haemolysis, shown on plates as distinct clearing, and in tube as red colour in supernatant fluid.

± = Weak haemolysis; - = Little or no haemolysis; ND = Not done

2. Haemagglutination

One interesting observation during the tube haemolysis test was the haemagglutination of RBC by some C.jejuni strains. After overnight at 4°C, the contents of centrifuge tubes were examined for haemagglutination before centrifugation and checking the supernatant fluids for haemolysis. Haemagglutination was revealed by clusters of erythrocytes settling over a large area at the bottom of the centrifuge tube. In a negative case erythrocytes settle as a small button.

6.3.3 ATTEMPT TO USE PLATE HAEMOLYSIS TO DISTINGUISH C.JEJUNI FROM C.COLI AND C.LARIDIS

Fifteen of the 40 strains of C.jejuni, C.coli and C.laridis strains, coded to hide their identity and tested at 42°C, were haemolysis positive. The positives included all the 14 C.jejuni strains (correctly identified) and one C.coli strain. Thus I was able to correctly identify all of the C.jejuni strains using the haemolysin assay, but discrimination between C.jejuni and C.coli was not possible.

Plate 6.1 Christie, Atkins & Munch-Petersen (CAMP) reaction on sheep blood agar containing 5% (v/v) washed RBC after aerobic incubation for 24h at 37°C: A = Staph.aureus NCDO 1245 producing only β-lysin; B = non-haemolytic CAMP-positive Strep.agalactiae NCDO 1348. Note the zone of changed RBC and the arrow-head haemolysis within the zone.

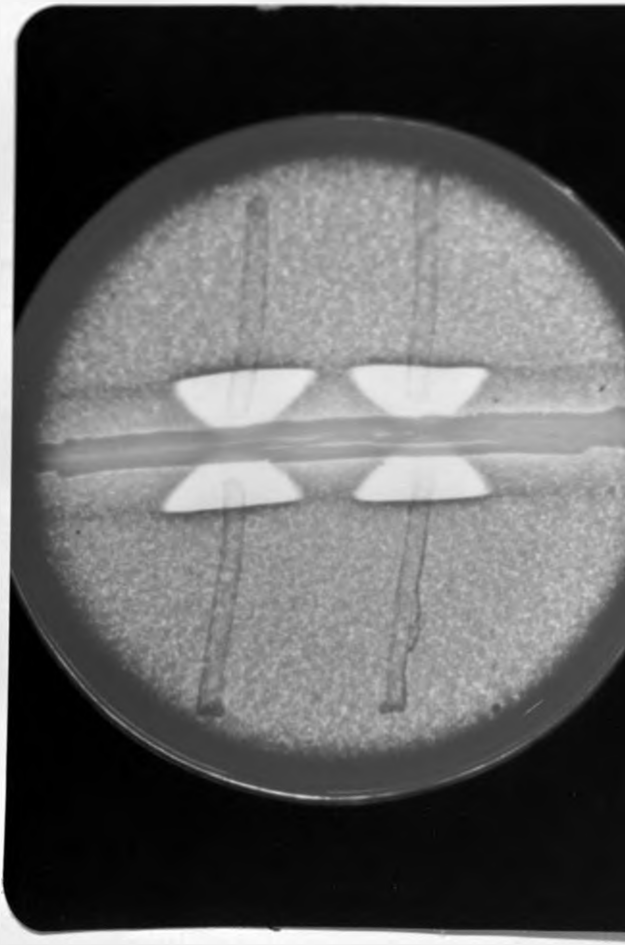


Plate 6.2 Haemolytic activity of C.jejuni, C.coli and C.laridis on SBHA containing 5% (v/v) washed sheep erythrocytes after incubation for 4 days at 42°C in microaerobic condition containing hydrogen.

a. C.jejuni strains SH5, SH6, SH11 and SH28 (all human) showing strong haemolysis. SH5 was used as a positive control throughout the study. The apparently extended haemolytic zones of SH6 and SH28 are due to swarming of the organisms and haemolysis of RBC in the areas covered.

b. C.coli strains P2, P6 and NCTC 11350 compared with C.jejuni SH5.

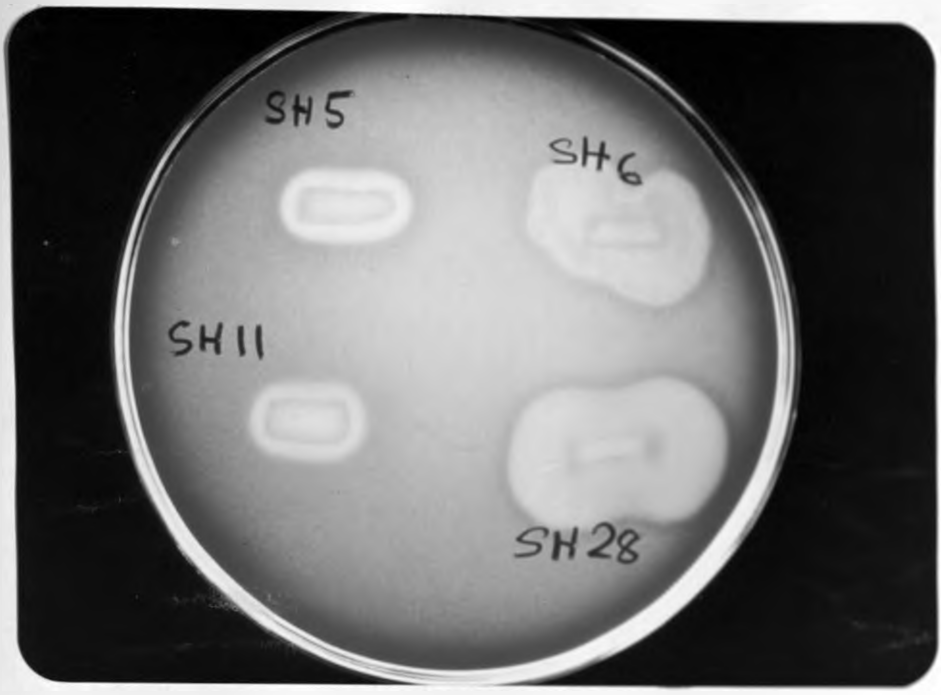


Plate 6.2 continued.

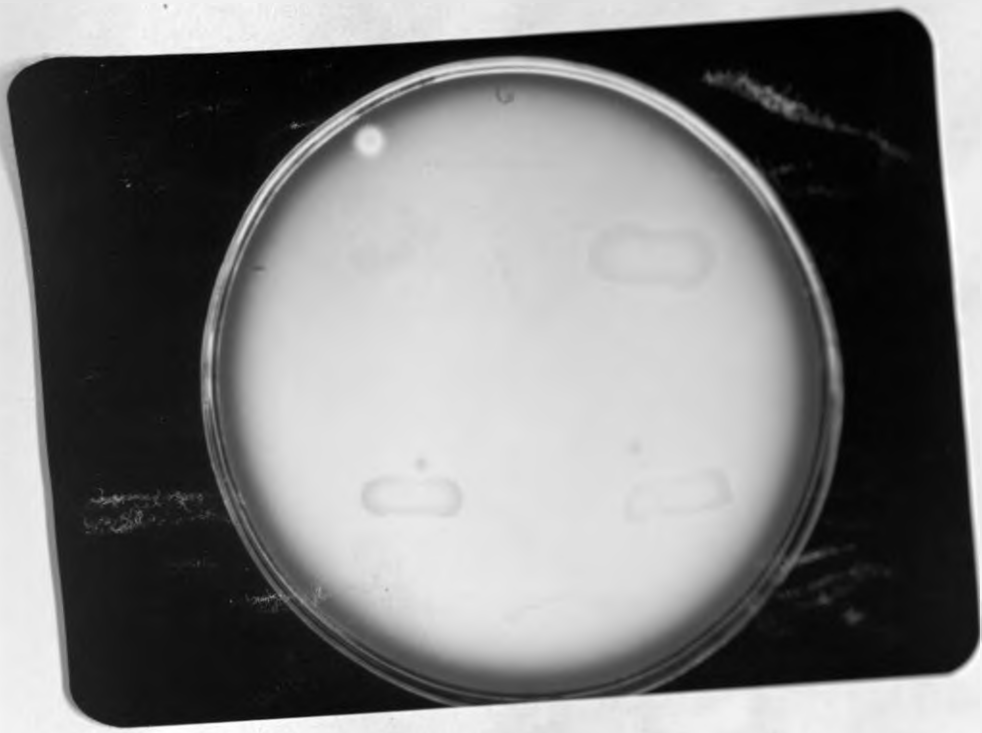
c. C.laridis strains CO2, CO4 and NCTC 11458 compared with C.jejuni.

d. Comparison of C.jejuni (SH5, human isolate and 4P10, sewage isolate), C.coli (P2 and NCTC 11350) and C.laridis (CO2 and NCTC 11458) on one SBHA plate.

SH5
CO2
11458
CO4

SH5
CO2
P2
11458
11350
4P10

Plate. 6.3 SBHA plate showing absence of haemolysis at 37°C after incubation for 4 days in a microaerobic condition containing hydrogen. SH5 = C.jejuni (control); P1 = C.coli; CO2 = C.laridis; and NCTC 10842 = C.fetus subsp. fetus. (ignore contaminant).



6.4 DISCUSSION

Catalase-positive campylobacters are usually reported as not haemolytic. In this study, it has been shown that C.jejuni and some C.coli strains are haemolytic when tested at 42°C. There is apparently no previous study of haemolysis by campylobacters on blood agar plates from which lessons can be learnt. However, it seems, from this study, that growth media and incubation conditions are of key importance. In a similar study of haemolysis by V.cholerae (Sakazaki et al., 1971), it was found that basal media containing a high content of animal tissue extract stimulated haemolysis in culture. This appeared to be the case with campylobacters; C.jejuni showed stronger haemolysis on HIA, which contains a high content of animal tissue extracts (Table 6.1) than on blood agar (BAB No.2), which contains a relatively low content of animal tissue extract. When haemolysis was tested on a medium consisting of HIA 50 % (v/v) plus BAB No.2 50% (v/v), haemolysis was greatly improved compared with BAB. This suggested either that ingredients in HIA are essential for expression of strong haemolysis or that inhibitors may be present in BAB, but can be diluted out. Evidence from work with V.cholerae (Sakazaki et al., 1971) and E.coli (Short & Kurtz, 1971), reporting the importance of animal tissue extract for haemolysis, favour the former explanation. This aspect was not investigated further.

Incubation for three to four days was required before haemolysis on the blood agar plates developed clearly. The start of the haemolysis was at the middle of the streak of growth, the area of ageing bacterial cells. These two observations suggested that the haemolysin is probably an intracelluar substance which is released as the organisms age, possibly involving conversion to coccoid forms, death and lysis. Failure to detect haemolysis on blood agar plates by C.jejuni and the other Campylobacter species at 37°C might mean either that the temperature is not suitable for production of haemolysin or that any haemolysin produced is unable to act on and lyse the RBC or that because growth is quicker at 42°C haemolysin is released sooner. These possibilities were not investigated further. The cytolytic bacterial toxins with known enzymatic modes of action are the two phospholipases: the β -lysin of Staph.aureus (sphingomyelinase C) and the α -toxin of Cl.perfringens type A (Phospholipase C; Freer & Arbuthnott, 1976). These two toxins bring about lysis of treated cells by hydrolysis of membrane phospholipids. The full effect of phospholipid degradation (particularly in the case of β -lysin) on membrane stability is evident only after β -lysin treated cells have been chilled below 10°C, usually 4°C or when the β -lysin treated cells are acted upon by the CAMP factor of Strep.agalactiae (Christie et al., 1944; Munch-Petersen & Christie, 1947). The hot-cold haemolysis observed with C.jejuni and C.coli in the tube test indicated a haemolysin

similar to staphylococcal β-lysin, but synergistic haemolysis with Strep.agalactiae was absent on blood agar. Although it is thermostable and extractable by boiling in saline, the haemolysin is not a lipopolysaccharide (Fricker et al., 1985).

The results of the tube test were in some ways similar to those reported by Fricker et al. (1985). However, in the present case, assessments were made by eye not by spectrophotometer, and so fewer positives were detected. The phenomenon detected in the tube was hot-cold lysis and was shown by most C.coli and some C.jejuni (Table 6.2). Other reasons for discrepancies are that in this study tube haemolysis was tested using whole broth culture not cell suspension in phosphate buffer saline, and cultures were grown in BHI broth not blood agar plates. Incubation of plates for 96h (48h for BHI broth cultures used for the tube test) may have caused so many C.jejuni strains to show plate haemolysis and not tube haemolysis, but the cause of the observed discrepancies, for example why a substantial number of C.coli strains showed tube haemolysis but not plate haemolysis is not clear. However, it must be borne in mind that the tube test relies on detection of coloured released haemoglobin from RBC, whereas the plate test relies on decolouration of released haemoglobin. Thus a variety of factors may be involved in producing these discrepancies.

The clear haemolysis shown on SBHA by C.jejuni strains, by a few C.coli strains but by no others (Table 6.2; Plate 6.2 a,b, c and d), raises the question as to whether this characteristic is of any differential or diagnostic value within genus. An experiment done to see if it was easy to distinguish C.jejuni strains by using haemolysis showed that reliance on clear expression of haemolysis was more likely to indentify a C.coli strain as C.jejuni than to miss a C.jejuni strain. However, 8% of C.jejuni strains were only weakly haemolytic and so could be misidentified. The feature does not correlate 100% with other features, but clearly plate haemolysis is a useful differential characteristic for C.jejuni. Whereas C.jejuni showed clear plate haemolysis, most C.coli and all C.laridis showed little or no activity.

C.jejuni is the main cause of campylobacter diarrhoea in human beings. About 95% of the isolates from cases of campylobacter enteritis are C.jejuni, most of the remainder being C.coli (Karmali & Skirrow, 1984), and one wonders whether production of haemolysin(s) by C.jejuni is correlated with its pathogenicity for human beings and animals, as has been claimed for various other bacteria (see page149). For example, in a study of food poisoning by V.parahaemolyticus, Miyamoto et al. (1969) reported production of thermostable haemolysin by the organism to be strongly correlated with

human pathogenicity. My work has shown that there is a definite interaction between C.jejuni and mammalian cells (RBC).

During this study, it was observed that several C.jejuni strains (Table 6.2) caused haemagglutination. Using a more elaborate method, Morris (1973) tested catalase-positive campylobacters (species not specified) from (a) prepuce of a bull; (b) fOetus of sheep; (c) faeces and duodenum of pigs, and found all of them able to haemagglutinate RBC of guinea pig, man and sheep regardless of their source. Going by the sources of the strains he tested, they most likely included C.fetus subsp. venerealis, C.fetus subsp. fetus and C.coli. In this study haemagglutination was obtained only with a few C.jejuni strains and not with other species possibly because the test was unsuitable for haemagglutination. It should be noted that haemagglutination was observed during checking of haemolysis by tube test and was not specifically studied. However, the observation further emphasises aspects of the interaction between C.jejuni and mammalian cells.

6.5 CONCLUSION

C.jejuni strains and a few C.coli strains haemolyse sheep and horse erythrocytes on suitable blood agar plates at 42°C.

Haemolysis is not shown at 37°C. C.fetus which cannot be tested at 42°C, shows no haemolysis at 37°C. Microaerobic gas mixture containing hydrogen is preferable for incubation of plates. Synergistic haemolysis with Staph.aureus or Strep.agalactiae is absent. The plate haemolysis test as described shows promise as a potential differential characteristic within the thermophilic campylobacters.

CHAPTER 7

GENERAL DISCUSSION

The once obscure campylobacters, associated with diseases only in animals, have now been recognized as important pathogens of both man and animals. There have been tremendous achievements in many areas of campylobacter research since 1977, which have shown C.jejuni and to a lesser extent C.coli, as major bacterial causes of enteritis in human beings in many parts of the world. The widespread occurrence of the organisms in a wide range of animal species, which includes pets, and meat and milk producing animals, most of which carry them as commensals in their intestines, shows the diversity of ready sources of infection for human beings. Recently reported cases of human enteritis involving strains of C.laridis (Simor & Wilcox, 1987) and C.fetus subsp.fetus (Edmonds et al., 1985; Klein et al., 1986), bring to light the role of other Campylobacter spp. in enteric disease in man.

Differentiation between campylobacter strains has been difficult because of the limited number of tests in which any strains gave positive results, but over the last ten years, divisions have become clearer with new physiological tests, for example hippurate hydrolysis which distinguishes C.jejuni from C.coli (Skirrow & Benjamin, 1982). News of new tests or improvement of the existing ones to help in differentiation is therefore welcome. Serotyping is one area which is finding increasing usefulness in epidemiological studies. As part of my work, C.jejuni strains isolated from various situations were serotyped using heat-stable antigens. Serotypes common

in human faeces were shown to be also common in sewage, thereby indicating that sewage is an important potential reservoir of campylobacters that can infect man. Improvements in serotyping techniques to make them simpler and easier to perform, for example slide coagglutination (Fricker et al., 1986) means that many unsophisticated laboratories can now perform serotyping with ease and so can look forward to much new information on epidemiology of campylobacter infections.

Morphology is a characteristic that has been important in recognition of campylobacters ever since their first detection and various workers have noted subtle differences between types. Helices are often seen in many cultures and these have been characterized. However, some campylobacters do not readily produce helical long forms which can be measured for wavelength and amplitude. A convenient, easy to perform technique for producing helical long forms, by treating cultures with antibiotics, is reported here and facilitates measurement of wavelength and amplitude. Using the technique it was possible to confirm the generally clear distinction between the thermophilic campylobacters and C.fetus. However, differentiation between the thermophilic campylobacters or between the subspecies of C.fetus was found not to be possible. The large soma helix measurements of C.laridis strain NCTC 11352, which has not been measured before, emphasises the diversity of soma measurements of strains within a species and cautions that absolute reliance must not be placed on these features.

The helix handedness (right handed) which can easily be determined directly on the TV monitor using SEM, which produces real images, does not appear useful for differentiation between species.

Catalase-positive campylobacters are generally reported as not haemolytic, but this is wrong. Using suitable media and incubating conditions, it was shown during these studies that C.jejuni strains and a few C.coli strains are distinctively haemolytic on appropriate blood agar plates. C.laridis strains show greening or little haemolysis. This characteristic holds promise as a useful differential test for C.jejuni but it may not hold any relevance in pathogenicity.

It is largely the improvements in isolation methods i.e. media and techniques starting with Skirrow's (1977) report, which have enabled us to understand so much about epidemiology of the organisms in the genus Campylobacter. However, the selective media which were developed, were meant for isolation of mainly thermophilic campylobacters and generally require incubation at high temperatures ($42^{o}C$-$43^{o}C$). Some campylobacters are sensitive to some of the antibiotics used in the selective media, and others will not grow at $42^{o}C$. The antibiotics used in the media are expensive and blood, until recently generally considered an essential requirement in the media, is expensive and difficult to obtain in some countries. Efforts are now being made to improve the methods in order to

increase isolation efficiency, to reduce costs, and to make media and techniques readily available to many countries. Notable advances in this area include development of blood-free media and attempts to improve efficiency at 37°C, a temperature which allows growth of a wider range of campylobacters. For workers who cannot afford gas mixtures in cylinders for microaerobic incubation and have to use candle jar method, burning methylated spirit might be a useful alternative (Ribeiro et al., 1985; Skirrow et al., 1987). It was found during the present work that microaerobic enrichment of faecal samples in an environment containing hydrogen or nitrogen, was superior to aerobic incubation (with bottle caps screwed tight), indicating that microaerobic conditions are an advantage for enrichment as well as for plating. The limited trial to look for selective agents as alternatives or additions to antibiotics, using brilliant green, malachite green and thallous acetate, gave disappointing results. It seems that for selection we still have to rely on expensive antibiotics or expensive membrane filtration.

C.jejuni was found to be the commonest campylobacter type in human faeces, sewage and animal food products in Reading. Infection is far more likely from consuming raw or contaminated chicken than pork. Although during these studies it was found that piglets acquire C.coli infections from a very early age, and that a variation in diet has no effect on the colonization of the gut, pork is not a major source of campylobacters in Reading. However, the situation is by no

means similar in all other countries. Here pork may be a more important source of campylobacter infections in humans (usually due to C.coli) than in U.K.

Treatment of sewage removes substantial numbers of campylobacters but many remain in the effluent, which is discharged into the river. This potentially endangers people who engage in recreational activities in the rivers or who drink unchlorinated water contaminated with sewage. The incrimination of sewage or faeces as sources of contamination of water involved in outbreaks of campylobacter enteritis (Mentzing, 1981; Vogt et al., 1982), is further strengthened by these findings. The large number of campylobacters removed in the sludge, which is spread on land and which we do not know much about, points to another potential source of infections for human beings and animals.

Integration of available epidemiological data shows a complex pattern of occurrence of campylobacters in human beings, domestic and wild animals, food, sewage and water. Bearing this in mind, production of campylobacter-free flocks of, say, chickens by instituting control measures is a noble idea, but a difficult one to implement because it requires complete understanding of the epidemiology of the disease and possibly prohibitively expensive rearing conditions. Although the organisms occur in so many different situations, their survival in the environment is generally poor. However, the theory of viable but non-culturable bacteria being potentially

an important source of infection (Rollins & Colwell, 1986) raises questions of a fundamental nature as to how long and in what form campylobacters may survive in the environment and how able they are to give rise to infection in animals and human beings. Could it be that in those situations where food-borne or water-borne outbreaks of enteritis have been reported and organisms have not been recovered from the implicated food item, the campylobacters were viable but non-culturable and after ingestion, they converted to a form which could multiply in the gastrointestinal tract and cause diarrhoea? It may be so. What is certain is that bacteria in the Campylobacter genus become more intriguing the more we study them. I hope that my contribution helps and encourages others to look closely at these fascinating bacteria and the problems they cause.

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