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Isolation of Thermotolerant Campylobacter – Review & Methods for New Zealand Laboratories

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1 Introduction

Campylobacter is a leading cause of enteric illness in many developed countries, with particularly high rates occurring in New Zealand. Thermotolerant campylobacters, those species that grow at 42°C, include the two species implicated in most cases of human disease, C. jejuni subsp. jejuni (referred to as C. jejuni in this report) and C. coli. Some strains of C. jejuni are highly infectious, with the infective dose reported to be as low as 800 cells (Black et al., 1988). Other Campylobacter species associated with human disease include C. lari and C. upsaliensis (Lastovica & Skirrow, 2000).

The family Campylobacteriaceae contains 18 species and subspecies of Campylobacter (Nachamkin et al., 2000). The majority of species of Campylobacter are motile, curved S- or spiral shaped Gram negative rods, 0.2 to 0.8 µm wide and 0.5 to 5 µm long. In some old cultures cells become coccoid in shape, but as only a limited number of isolates adopt this morphology, it is apparently not an essential feature of a “viable but non culturable state” for Campylobacter (Park, 2002).

Campylobacter do not ferment carbohydrates and usually obtain energy from amino acids or tricarboxylic acid cycle intermediates. Typical biochemical reactions include the reduction of fumerate to succinate and negative methyl red, acetoin and indole production reactions. Most species reduce nitrate and are oxidase positive but only C. jejuni is hippurate positive (Vandamme, 2000). Campylobacters are resistant to a wide range of antibiotics, a feature that is exploited in the design of isolation media. Thermophilic campylobacters do not grow at temperatures of less than 30°C and their optimal growth temperature is 42°C (Park, 2002). Campylobacters do not grow in air but require microaerophilic (5% O₂ is optimal) and capnophilic (3 to 5% CO₂) conditions for growth (Doyle & Jones, 1992).

Unlike many enteric pathogens there is limited spread of Campylobacter within families and the main reservoir of infection is animals. There are well-documented cases of common-source outbreaks associated with foods, unpasteurised milk and water. Although C. jejuni does not survive particularly well on foods due to sensitivity to drying, temperature, ambient oxygen concentration and heat, refrigeration conditions promote survival and long-term survival is probable on frozen food. In many countries routine surveillance of microorganisms in food and water increasingly includes thermotolerant campylobacters (FDA BAM, 1998).

Although there is no generally accepted ‘standard’ method of isolating campylobacters from food, faeces or environmental samples, protocols have been published by recognized authorities including the International Standards Organisation (ISO), the UK Public Health Laboratory Services (PHLS) and the US Food and Drug Administration
The UK Microbiological Safety of Food Funders Group (MSFFG) has commissioned publicly-funded research on Campylobacter and has recommended methods both for highly contaminated samples and for food surveillance. The MSFFG endorsed a WHO recommendation that there is a need for collaborative international research on techniques for the isolation of campylobacters from food and environmental samples (MSFFG, 2001).

A review of methods was published by Corry et al. (1995) and these workers concluded that although there is no generally accepted “standard” method but there are some generally agreed procedures. These include the use of a preliminary (resuscitation) period of incubation at reduced temperature (37°C) for about 4 h prior to increasing the temperature to 42°C for the remainder of the 48 h of incubation time. The inclusion of oxygen quenching supplements in pre-enrichment media (Bolton et al., 1984) seems to be a widely adopted practice, probably because it allows broth cultures to be incubated in air. For analysis of faecal samples from infected patients, direct plating onto either blood agar or a Campylobacter selective agar is often preferred to pre-enrichment. It appears from the scientific literature that a consensus is developing on a number of issues related to the isolation of Campylobacter. The ISO Standard method (ISO, 1995) is currently under revision (Jacobs-Reitsma & de Boer, 2001) and proposed changes include an extension of incubation time from 18 h to 24 - 48 h and emphasising the importance of microscopic examination in identifying campylobacters.

Reports in the literature suggest that a number of laboratories are using molecular methods to either directly identify Campylobacter species in samples or to confirm the species identity of isolates (Eyers et al., 1993; Ayling et al., 1996; Gonzales et al., 1997; Wallace, 1997; Fermer & Engvall, 1999; Savill et al., 2001). Recently Sails et al. (2001) described a combined PCR-ELISA method that they found to be highly sensitive and specific for identification of C. jejuni and C. coli. Molecular methods can include a conventional enrichment step to increase the recovery of injured cells and to allow isolates to be obtained in pure culture for subsequent typing. At present there are only a limited number of New Zealand laboratories that are routinely using PCR-based methods for determination of Campylobacter in samples (e.g. ESR). Therefore, it is likely that conventional cultural methods will continue to be needed to ensure that campylobacters are isolated from all regions of the country.

The New Zealand Food Safety Authority, Ministry of Health and Environmental Science and Research Institute are currently implementing a National Microbial Sub-typing Database to facilitate epidemiological understanding of zoonotic disease in New Zealand. An ancillary part of this project, and indeed for all Campylobacter research in New Zealand, is the standardisation, where possible, of methods for detection, isolation
and identification of thermotolerant Campylobacter from a range of sample types including food, water and clinical specimens. The purpose of this Report is to review current isolation methods for Campylobacter under New Zealand conditions and, after widespread stakeholder consultation, to adopt a National Reference Method for research projects. Recognising that modified and new methodologies may at times be required, alternative methods will be assessed against this National Reference Method.

2 Review of Isolation Methods

The development of Skirrow medium was the key to successful study of thermotolerant campylobacters. This medium enabled successful recovery of thermotolerant Campylobacter and therefore provided evidence linking disease to the contamination of food, particularly chicken (Skirrow, 1977). Skirrow agar contains peptones as a nutrient source, lysed horse blood and antibiotics to prevent growth of other microorganisms. These ingredients form the basis of most Campylobacter media in common use (Post, 1995). Plates are incubated in the microaerophilic atmosphere (5-6% oxygen, 10% carbon dioxide and 84-85% nitrogen) required by Campylobacter and at the elevated temperature of 42-43°C to select for thermotolerant species.

2.1 Media components

All Campylobacter media contain peptones and antibiotics, most contain blood, and as well, many include oxygen quenching agents to overcome the adverse effects of toxic oxygen species (e.g. hydrogen peroxide and superoxide).

Nutrient Sources

As Campylobacter do not ferment carbohydrates, peptones are included in media as a nutrient source. Preston broth (Bolton & Robertson, 1982) and Exeter broth (Martin et al., 1996) contain meat extract (or Lab Lemco, Oxoid) at 10 g/l and peptone at 10 g/l. Bolton’s broth and Campylobacter enrichment broth have a nutrient formulation that consists of peptones, yeast extract and a tri-carboxylic cycle intermediate, alpha-ketoglutaric acid.

Blood

Many Campylobacter media contain blood (at 5-7% (v/v)) to quench toxic oxygen compounds (e.g. hydrogen peroxide, Bolton et al., 1984) that can form when media are exposed to light. In addition, blood neutralizes trimethoprim antagonists (Corry et al., 1995). Saponin-lysed horse blood has generally been found to be the most suitable.
Tran (1995) described a blood-free medium for isolation of campylobacters under normal aerobic incubation conditions that included an oxygen quenching compound, Oxyrase® (Tran, 1995). Subsequently Tran (1998) evaluated the efficiency of this medium without the inclusion of Oxyrase®. Although comparable results to those obtained using Bolton broth (FDA BAM, 1998) were reported, it was noted that efficiency varied for different foods. Another blood-free enrichment broth, modified charcoal cefoperazone deoxycholate broth (mCCDB), has been reported to be less selective than Preston broth (Jacobs-Reitsma & de Boer, 2001). Blood free media would be very convenient but it appears that further refinements together with inter-laboratory studies are needed before laboratories could be confident in using these media for isolation of Campylobacter.

**Antibiotics**

The inclusion of antibiotics in isolation media is crucial to the recovery of Campylobacter. Campylobacter are resistant to several antibiotics including: vancomycin (inhibits Gram-positive cocci); polymyxin B (inhibits Enterobacteriaceae and Pseudomonas spp.); Trimethoprim (inhibits Proteus spp. and Gram positive cocci) and cephalosporins (inhibit Enterobacter spp., Serratia spp., Pseudomonas aeruginosa, some Proteus spp., Yersinia enterocolitica). Rifampicin was substituted for vancomycin in Preston media (Bolton & Robertson, 1982) but later studies (Humphrey & Cruikshank, 1985; Humphrey, 1990) found rifampicin to be inhibitory to stressed cells of *C. jejuni*.

Antibiotics which inhibit yeasts and moulds are usually included in Campylobacter media. Until recently cycloheximide was the most widely used antifungal antibiotic but it is now considered too toxic for inclusion in microbiological media. Substitutes include amphotericin B or natamycin. Amphotericin B has been demonstrated to be a satisfactory substitute for cycloheximide (Martin et al., 2002).

A number of antibiotics that are commonly used in Campylobacter media can adversely affect recovery of some species or strains. For example, Nachamkin et al. (2000) reported that cephalothin, colistin and polymyxin B may inhibit some strains of *C. jejuni* and *C. coli* and also *C. fetus* subsp *fetus*, *C. jejuni* subspp. *doylei* and *C. upsaliensis*.

Delayed addition of antibiotics may enhance recovery of thermotolerant Campylobacters. For example, in some protocols inoculated broths are incubated for 4 h at 37°C followed by addition of antibiotics and then transfer to a microaerophilic atmosphere at 42°C for 44 h (Wallace, 1997; Jones et al., 1999).

It is likely that future developments in Campylobacter media will be based on changes to optimize recovery of species other than *C. jejuni* (Post, 1995) e.g. supplement (CAT) designed for selective isolation of *C. upsaliensis* (Oxoid Manual, 1998).
2.2 Methods that include pre-enrichment

Food and environmental samples are usually contaminated with relatively low numbers of Campylobacter. The incorporation of pre-enrichment procedures into laboratory protocols has been found to increase recovery of Campylobacter from most sample types (Bolton et al., 1984) and is generally recommended for analysis of food, water and other environmental samples.

Pre-enrichment usually starts with a resuscitation procedure that is included to overcome damage to cells caused by drying, heating, starvation, freezing and/or oxygen radicals. Probably the most widely used resuscitation procedure consists of 4 h incubation at 37°C (Humphrey, 1989; Bolton, 2000) after which the pre-enrichment broths are transferred to 42°C. It is recommended that resuscitation be limited to 4 h to prevent overgrowth by contaminants (Goosens & Bultzer, 1992). However Humphrey and Muscat (1989) reported optimal recovery of C. jejuni from river water by selective isolation in broth for 48 h at 37°C. Following pre-enrichment, an aliquot of growth from enrichment tubes is sub-cultured to a chosen selective agar that is usually incubated for 48 h at 42°C to confirm the presence of thermotolerant Campylobacter and to yield single colonies for species identification and for sub-species typing.

There are many pre-enrichment broths described in the literature but five in particular seem to be in common use: Preston broth, Exeter broth, Bolton broth, CEB broth (Campylobacter enrichment broth) and Park & Sanders broth.

**Preston broth**

Bolton & Robertson (1982) found Skirrow agar insufficiently selective for recovering campylobacters from animal specimens and environmental samples and described Preston medium as an effective alternative. Preston medium can be used as either a broth for pre-enrichment or as an agar for selective isolation of single colonies. Preston medium is based on a nutrient broth that does not include yeast extract, a known trimethoprim antagonist and includes 5% (v/v) lysed horse blood. The antibiotics are: polymyxin B (5 IU/ml), rifampicin 10 µg/ml), trimethoprim 10 (µg/ml) and cycloheximide (100 µg/ml). Rifampicin was found to be very effective in suppressing Gram-positive bacteria. Incubation is in a microaerophilic atmosphere at 42°C (Post, 1995). This formulation of Preston is specified in an International Standards Organisation Method (1995).

A modification to the original Preston formulation was the inclusion of sodium pyruvate, sodium metabisulphite and ferrous sulphate (FBP) to improve the quenching of toxic oxygen derivatives (Bolton et al., 1984). The inclusion of FBP allowed aerobic incubation and also storage of broth for up to 7 days at 4°C. For successful aerobic
incubation containers must have tightly fitting lids and a head space of less than 1 cm. Nowadays, the formulation of Preston broth plus FBP supplements appears to be the mostly widely (Donnison, 1998; Baylis et al., 2000).

The components of Preston broth are available commercially and comprise: dehydrated Preston broth, FBP supplement and antibiotic supplement (in which cycloheximide has been replaced by amphotericin B (10 mg/l) and 5% (v/v) lyed horse blood.

The protocol for pre-enrichment in Preston broth is aerobic incubation (in containers with a very small head space) at 37°C for 4 h (to allow resuscitation) followed by incubation at 42°C for 48 h (Baylis et al., 2000).

Exeter broth

The original formulation of Exeter medium was based on a nutrient broth and included 5% (v/v) lyed horse blood. Later this formula was amended (Humphrey, 1986) to include oxygen quenching agents (the FBP mixture of Bolton et al., 1984), allowing Exeter broth to be incubated aerobically. A further amendment was to change the base to a nutrient broth that does not contain yeast extract, a known trimethoprim antagonist (Mason et al., 1996).

Humphrey and Cruikshank (1985) tested the susceptibility of both injured and non-injured C. jejuni to a number of antibiotics and found that stressed cells of C. jejuni are damaged by rifampicin and that cefoperazone optimized recovery of C. coli (Humphrey & Cruikshank, 1985). These observations have directed the antibiotics for incorporation in Exeter medium: cefoperazone (15 µg/ml), Polymixin B (5 IU/ml), trimethoprim (10µg/ml), rifamapicin (10 µg/ml) and amphotericin (2 µg/ml) (Humphrey et al., 1995; Martin et al., 2002).

As currently formulated, Exeter broth is essentially Preston broth including the growth and selective supplements (Oxoid Manual, 1998) plus cefoperazone (added as a filter sterilized solution to a final concentration of 15 µg/ml).

Exeter can be prepared from commercially available Preston ingredients, FBP supplement, antibiotic supplement and 5% (v/v) lyed horse blood and a filter sterilised solution of cefoperazone (added to a final concentration of 15 µg/ml). Exeter broth can be solidified by the addition of 15 g/l of agar the basal medium prior to autoclaving. The complete medium contains FBP supplement, blood and the antibiotics.

The protocol for pre-enrichment in Exeter broth is aerobic incubation (in containers fitted with a screw cap with a head space < 1 cm) for 4 h at 37°C (to allow resuscitation of injured cells (Humphrey, 1986) followed by 24 - 48 h at 42°C (Martin et al., 2002).
Exeter broth is specified in two Standard Operating Procedures for Campylobacter (F 21 and W 8) published by the UK Health Protection Agency formerly the Public Health Laboratory Service (http://www.phls.org.uk/dir/hq/sops).

**Bolton broth**

Bolton Broth (Campylobacter enrichment broth - Bolton formula) is recommended in protocols produced by the U.S. Food and Drug Administration (FDA BAM, 1998) for recovery of campylobacters from wide range of sample types. The base medium and supplements are available commercially.

Dehydrated Bolton broth contains peptone and yeast extract, alpha-ketoglutaric acid, sodium pyruvate, sodium metabisulphite and haemin. Haemin is included to overcome trimethoprim antagonism as a result of inclusion of yeast extract. Sodium pyruvate and sodium metabisulphite are included to allow aerobic incubation, sodium carbonate is included to provide carbon dioxide during growth (Post, 1995). The complete medium also includes 5% (v/v) lysed horse blood and the antibiotics: cefoperazone (20 µg/ml), vancomycin (10 µg/ml), trimethoprim (10 µg/ml) and cycloheximide (50 µg/ml).

In the U.S. FDA protocol (FDA BAM, 1998) a microaerophilic atmosphere is specified for incubation, achieved using either using commercial Campylobacter gas paks, or an incubator gassed with a flowing mixture of 5% oxygen, 10% carbon dioxide and 85% nitrogen. For most types of sample, the resuscitation period consists of incubation for 4 h at 37°C. Resuscitation is modified for analysis of shellfish to 3 h at 30°C followed by 2 h at 37°C. Following resuscitation, enrichment broths are transferred to 42°C. FDA BAM (1998) includes protocols for incubation without shaking. Incubation at 42°C is for 28 - 29 h for most sample types but is extended to 44 h for dairy products and 48 h for shellfish.

**Campylobacter enrichment broth (CEB)**

A commercially available enrichment broth called Campylobacter Enrichment Broth (CEB, e.g. LAB M, 135) has the same base formulation as Bolton broth and the complete medium varies only in the substitution of natamycin for cycloheximide in the antibiotic supplement (e.g. LAB M X132).

Pre-enrichment broths that contain FBP supplements can be incubated aerobically (in containers with a very small head space, <1 cm) for 4 h at 37°C to allow resuscitation, followed by incubation for a further 20 to 44 h at 42°C (Baylis *et al.*, 2000).
Park and Sanders broth

The base formula of Park and Sanders broth is Brucella broth (Difco) containing peptones, glucose, yeast extract, sodium pyruvate and sodium metabisulphite. Prior to inoculation with sample, 5% (v/v) lysed horse blood and two antibiotics, vancomycin and trimethoprim (both added at a concentration of 10 mg/l). The initial period of incubation is 4 h at 32°C after which two further antibiotics, cefoperazone (32 mg/l) and cycloheximide 100 mg/l) are added and the enrichment broths transferred to 37°C for 4 hours followed by transfer to 42°C for 40 - 42 h. All three incubation steps are carried out in a microaerophilic atmosphere (ISO, 1995). The use of Park and Sanders is recommended (ISO, 1995) for samples that have received treatment expected to cause stress, for example freezing.

In an inter-laboratory study (Scotter et al., 1993) the protocol using Park and Sanders broth was modified so that the first incubation, 32°C for 4 h, was aerobic. After 4 h cefoperazone and cycloheximide were added, a microaerophilic atmosphere established and the enrichment incubated for 2 h at 37°C. The enrichments were then transferred to 42°C for up to 48. These variations from the ISO procedure were presumably to reduce the cost of establishing a microaerophilic atmosphere twice and to allow the two resuscitation steps to be completed in a normal working day.

2.3 Selective agars

Following pre-enrichment the isolation procedure continues with sub-culture to Campylobacter selective agar plates. In most protocols, a loopful of growth from a pre-enrichment culture is streaked to a plate of Campylobacter selective agar in such as way as to obtain single colonies. There are a variety of selective agars and some protocols specify more than one to be used for selective enrichment. Combinations of pre-enrichment broth and one or more selective agars are described in different protocols.

The growth of campylobacters is reduced when plates are stored in the presence of air and also light (Bolton et al., 1984). Therefore, it is preferable to use agar plates immediately after preparation. In cases where storage cannot be avoided, the maximum period should not exceed 5 days. This applies to both anaerobic storage at room temperature and aerobic storage in a refrigerator (Corry et al., 1995).

Immediately before use selective agar plates should be dried to remove any surface water. Plates can be dried at 42°C for the minimum time needed to remove surface water or overnight at room temperature but should not be dried in a laminar flow cabinet because even brief drying in flowing air with the lids off inhibits recovery of Campylobacter (FDA BAM, 1998).
Campylobacter selective agars may include blood but blood-free formulations are also available. Although numerous agars have been reported in the literature the following is a selection of those that have been reported recently and/or frequently. Agars that include blood (usually as 5-7% (v/v) lysed horse blood) include: Skirrow, Campy-Cefex, Butzler (or modified Butzler), Preston and Exeter. Blood-free selective agars can include charcoal as an oxygen quencher, for example Karmali or mCCDA (modified Charcoal Cefoperazone, Deoxycholate agar). Selective agars that contain neither charcoal nor blood include semisolid blood-free medium (SSM, Goosens et al., 1989) and Abeyta-Hunt-Bark Agar (A-H-B agar, FDA BAM, 1998).

2.4 Sub-culture from pre-enrichment broths

Specific selective agars may be specified in isolation protocols. For example, in the ISO (1995) protocol subculture from Preston or Park and Sanders broth to Karmali agar is specified together with subculture to any other agar chosen from the following list: Butzler, Skirrow, CCDA or Preston agar. A proposed amendment to ISO 1995 is plating to only one agar, either Karmali or mCCDA (Jacobs-Reitsma & de Boer, 2001). Plating to mCCDA is specified in the Methods Manual prepared for the New Zealand Freshwater Microbiological Study (Donnison, 1998), the UK PHLS and the Australian AIFST Group protocol (Wallace, 1997) subculture is to both Preston and Skirrow agars. A choice of two isolation agars to be used following pre-enrichment in Bolton broth is given in the FDA protocol (FDA BAM, 1998), one of which is mCCDA and the other Abeyta-Hunt-Bark agar.

Sub-culture to a non-selective agar after pre-enrichment in Park and Sanders broth is described by Scotter et al. (1993). The non-selective agar, nutritive blood agar (NBG), was reported to consist of Nutrient agar No 2 (Oxoid CM689), FBP supplement (Oxoid SR84) and 5% (v/v) lysed horse blood. Ten or twelve drops (Corry et al., 1995) of pre-enrichment culture are pipetted to the centre of a 0.65 µm membrane filter placed on the surface of an NBG plate. The filter is left in place for 30 minutes at room temperature and then plates are closed and incubated in a microaerophilic atmosphere at 42°C for 24 - 48 h. Plates are only scored negative after 5 days incubation. The protocol combining step-wise increase in incubation temperature with step-wise addition of antibiotics to Park and Sanders broth together with filtration to remove competing microorganisms, followed by incubation on non-selective blood agar, was found to be very sensitive and capable of recovering 2-10 cells per 10 g sample. However the method is cumbersome and not well suited to the routines of a busy laboratory.
2.5 Direct plating of samples onto selective agars

Direct plating onto Campylobacter selective agar is widely used by clinical laboratories examining human faeces. Wells et al. (1982) compared the effectiveness of four agar media: Skirrow; Campy-BAP; Butzler and BU40 (modified Butzler), for direct plating of faecal samples by streaking faecal swabs to agar plates and incubating at 42°C for 24, 48 & 72 h. Campy-BAP and Butzler (both versions) gave similar results with respect to recovery and ease of recognition of Campylobacter and suppression of contaminants, but Skirrow was the most sensitive for human specimens and BU40 for dog and cat specimens. For best results these workers recommended streaking onto more than one medium and daily examination of plates although overall, 48 h was the best single incubation time.

A relatively simple, inexpensive, blood-free semi-solid medium, containing Mueller Hinton broth and 0.4% agar, was developed by Goossens et al. (1989) to exploit the ability of Campylobacter to swarm. In this medium growth occurs below the surface so that there is no requirement for blood, charcoal or FBP agents. The selective agents are cefoperazone (30 mg/l) and trimethoprim (50 mg/l). For use, undiluted faecal samples are inoculated to the edge of Petri plates that are incubated microaerophically at 42°C for 42 h. The authors reported that the medium was both sensitive and selective.

Examples of plating media that include charcoal instead of blood include modified CCDA (mCCDA) and Karmali agars (Corry et al., 1995). It has been reported that these two media are essentially similar in sensitivity and productivity (Jacobs-Reitsma & de Boer, 2001). The selective agents in mCCDA are cefoperazone (32 mg/l) and amphotericin B (10 mg/l). Karmali agar includes haematin instead of ferrous sulphate as one of the oxygen quenching (FBP) agents and the antibiotics vancomycin (20 mg/l) and cycloheximide (100 mg/l). A modification of the antibiotics in mCCDA that consists of cefoperazone (8 mg/l), teicoplanin (4 mg/l) and amphotericin B 10 mg/l has been reported to improve the recovery of C. upsaliensis from faeces while at the same time isolating equivalent numbers of other Campylobacter spp. to mCCDA (Corry et al., 1995).

In a comparative study of direct plating of faeces onto Campylobacter selective agar Gun-Munro et al. (1987) found that more strains of both C. jejuni and C. coli were isolated more frequently on charcoal based media than on Skirrow agar. They also reported better suppression of normal faecal microbiota on charcoal based media.

Fricker (1987) reported that storage of solid Campylobacter media in air at room temperature results in a marked drop in sensitivity and advised that agars be freshly prepared. Where this is not practical Campylobacter selective agar can be stored satisfactorily in an anaerobic atmosphere at 4°C.
Direct plating to selective agar is a protocol included in ISO (1995). It is recommended for use in testing samples in which a high level of contamination by thermotolerant Campylobacter is suspected. In the ISO protocol direct plating onto two different agars is specified, one of which should be Karmali. Lastovica & le Roux (2000) compared direct plating onto a selective agar to filtration onto non-selective agar for analysis of human stool samples and found a substantially higher rate of isolation of Campylobacter from diarrhoeic children and also recovery of a wider range of species. Engberg et al. (2000) observed that it is well known that no single method will successfully recover all campylobacters from clinical samples.

Although direct plating is mostly used for faecal samples (human and chicken), Moore (2001) found that disruption of liver tissue by homogenizing or stomaching can release inhibitory compounds and recommended swabbing of freshly eviscerated liver and inoculation onto Preston agar within 3 h. Other workers, Line et al. (2001) have also reported satisfactory recovery of Campylobacter from a specific sample type by direct plating. In this case the samples were carcass rinses obtained from freshly processed broiler chickens plated directly onto Campy-cefex agar (Stern et al., 1992).

2.6 Comparisons of method performance

A number of workers have compared the performance of different media and protocols for isolating thermotolerant Campylobacter. Recent studies include those of Baylis et al. (2001) and Line et al. (2001). The results of an extensive inter-laboratory study, in which 14 laboratories participated, are reported by Scotter et al. (1993). In this study, the ability to detect Campylobacter at two different, low levels of contamination was compared and the findings are described in detail later in this Section.

Hudson (1997) compared recovery by two different methods that included pre-enrichment. These were Preston broth → mCCDA agar and Exeter broth → Exeter agar. Comparisons were done using naturally contaminated samples, including offal and chicken. Hudson reported a higher rate of recovery using the Exeter protocol and recommended the adoption of this method by New Zealand Public Health Laboratories.

Baylis et al. (2000) compared the performance of pre-enrichment media for recovery of campylobacters from food using both artificially and naturally contaminated samples. All pre-enrichments included an initial period of resuscitation, 4 h at 37°C, after pre-enrichment all were subcultured to mCCDA agar. The pre-enrichment broths were: Bolton broth, Campylobacter enrichment broth (CEB) and Preston broth. A variety of poultry, offal and meat samples were artificially contaminated with reference cultures (including typical competing microflora) to determine quantitative recovery of Campylobacter. The mean population (expressed as logs) recovered was: Preston broth
7.11, Bolton broth 7.09 and CEB 6.57. Preston broth supported growth of the greatest number of Campylobacter strains but it failed to inhibit some competitor organisms. In contrast CEB inhibited all competitors but failed to support all of the Campylobacter strains.

Several enrichment media were tested for their ability to recover Campylobacter from fresh poultry meat at the Institute for Animal Science and Health, ID-Lelystad, The Netherlands. The media were Bolton broth, CEB broth and two formulations of Preston broth, one as described in ISO 10272 (ISO, 1995) and the other without the inclusion of horse blood. Samples were added to complete broths, rinsed and then incubated for 24 to 48 h at 41.5 °C, following which broths were plated on mCCDA. The findings were that Preston broth minus horse blood (but with the inclusion FBP supplement and antibiotics) and incubation for 24 h at 41.5°C gave the best results. (W. Jacobs-Reitsma pers. comm.).

When recovery from 100 naturally contaminated samples was compared the number of samples confirmed positive was 66 for Bolton broth, 53 for CEB broth and 53 for Preston broth. The difference between recoveries on Bolton and CEB broths was unexpected as both have an identical media composition. The authors speculated that the difference might be due to differences in the peptones used. Baylis et al. (2000) concluded that Bolton broth represents the best overall compromise between growth and inhibition for the analysis of food samples.

Line et al. (2001) compared recovery and enumeration of Campylobacter in carcass rinse samples obtained from freshly processed broiler chickens in a study done in parallel in two different laboratories. Two MPN methods, Hunt selective enrichment broth followed by plating onto mCCDA and Rosef enrichment broth followed by plating onto Mueller-Hinton blood agar with antibiotics (MHBA) were compared to direct plating onto Campy-Cefex agar. The Rosef-MHBA method compared poorly to the other two but Hunt-mCCDA MPN and direct plating on Campy-Cefex were not significantly different suggesting that direct plating is a satisfactory, rapid alternative for this type of sample. As there are no reports of the effectiveness of applying direct plating using Campy-Cefex to other types of sample, further investigation is needed before it could be considered a “general” method.

An Inter-laboratory study

A reasonably large-scale inter-laboratory study involving 14 laboratories was undertaken to compare the recovery of Campylobacter from naturally contaminated samples (chicken skin) at low rates of contamination. The two chosen levels of contamination were approximately 2 cells per g and approximately 10 cells per g. Isolation rates were
compared by three different protocols (Scotter et al., 1993) that were: primary enrichment in Park and Sanders broth followed by secondary enrichment on a non-selective agar; or one of two formulations/protocols based on Preston broth with secondary enrichment on Preston agar or Skirrow agar.

One formulation of Preston broth, PB mod, included FBP ingredients (oxygen quenchers) and the other was Preston broth, PB, (without FBP ingredients). Both Preston broth formulations included the antibiotic mixture (Polymixin B 5000 IU/l, rifampicin 10 mg/l, trimethoprim 10 mg/l, cycloheximide 100 mg/l). PB mod was incubated aerobically, presumably in tubes with a minimal headspace, but PB was incubated in a microaerophilic atmosphere. The enrichment incubation protocol for PB mod included a pre-enrichment step of 4 h incubation at 37°C followed by transfer to 42°C for a further 44 h. For PB incubation was at 42°C for 48 h. Primary enrichment in PB mod was followed by selective enrichment on Skirrow agar and Preston agar. Primary enrichment in unmodified Preston broth (PB) was followed by selective enrichment on Skirrow agar plus any other agar of choice. All agars were incubated at 42°C for 24 - 48 h, with incubation extended to up to 5 days.

Primary enrichment in Park and Sanders broth was in a microaerophilic atmosphere for a total of 48 h and involved a stepwise increase in temperature and of addition of antibiotics. The first antibiotic mixture (vancomycin and trimethoprim, both at 10 mg/l), was added prior to addition of the test sample and the mixture subjected to pre-enrichment at 32°C for 2 h. This was followed by addition of a second antibiotic mixture (cefoperazone 32 mg/l and cycloheximide 100 mg/l) and a second pre-enrichment step, incubation at 37°C for 2 h. After the second period of incubation, broths were transferred to a 42°C incubator for further 40 – 42 h. Following enrichment, a few drops of enrichment culture were applied (with a Pasteur pipette) to a membrane filter (0.65 μm) placed on a plate of non-selective nutritive blood agar (NBG – nutrient agar + FBP + 5% (v/v) lysed horse blood). The membrane was left on the plate for about 30 minutes, removed, and the plate incubated microaerophilically at 42°C for up to 5 days.

Twelve laboratories returned valid results from blind analyses of 3 sets of five samples in duplicate, 15 in total. The results demonstrated that the Park and Sanders protocol was the most effective for recovery of Campylobacter at both levels of contamination tested. Confirmed thermotolerant Campylobacter were detected in 82% of all samples tested by the Park and Sanders protocol compared to 54% for PB and 48% for PB mod. At the higher level of contamination, approximately 10 cells per g, rates of recovery for Park and Sanders and PB were similar at 77% and 75% respectively but rates were lower at 63% for PB mod. At the lower level of contamination, approximately 2 cells per g, recovery for Park and Sanders was 86% compared to 33% for both PB and PB mod.
The authors concluded that the primary enrichment step was the critical one for recovery of Campylobacter as the results demonstrated that none of the selective agars used influenced the actual recovery of Campylobacter. However, those laboratories that used CCDA agar reported there were fewer contaminants that with Skirrow agar. As a result of the study, Scotter et al. (1993) recommended the Park and Sanders protocol for testing food with low level of natural contamination. The necessity to establish a microaerophilic atmosphere 3 times during the protocol and the labour-intensive nature of the method were noted.

2.7 Standard methods for food and water

Recognised authorities that publish standard microbiological methods include the International Standards Organisation (ISO), the US Food and Drug Association (FDA) and the UK Health Protection Agency (PHLS). Both authorities have produced standard methods for the isolation of thermotolerant Campylobacter in 1995 and 1998 respectively. The ISO standard is currently under review (Jacobs-Reitsma & de Boer, 2001: D. Wareing, Preston, UK, pers. comm.).

ISO (1995) methods

The International Standards Organisation method (ISO, 1995) is a horizontal method for the detection and enumeration of campylobacters in food and animal feedstuffs. Three methods are included. Two methods that include a pre-enrichment procedure are intended for general use while a third, direct plating, is designated only for products in which a large quantity of Campylobacter are suspected.

The pre-enrichment protocols are based on either Preston broth or Park and Sanders broth. The Preston broth protocol is very straightforward – the formulation does not include FBP nor a resuscitation step. Samples are added to x 9 (weight or volume/volume) Preston broths and incubated at 42°C in a microaerophilic atmosphere for 48 h. The Park and Sanders protocol has as the first step, addition of only a portion of the antibiotics included in the complete formula and incubation at 32°C for 2h. The subsequent step consists of addition of the remaining antibiotics, reestablishment of a microaerophilic atmosphere and transfer to 37°C for 2 h. Step 3 consists of transfer to 42°C (maintaining a microaerophilic atmosphere) for 40 – 42 h.

After pre-enrichment by either protocol, selective enrichment is done by plating to two agars in parallel, one of which must be Karmali. Recipes for a number of other agars are given including: modified Butzler, Skirrow, CCDA and Preston. The protocol for incubation of all types of agar plates involves establishing a microaerophilic atmosphere and incubation at 42°C for 24 – 72 h.
The direct plating protocol involves spread-plating of an aliquot of sample to two agars in parallel, one of which must be Karmali. Agar plates are incubated in a microaerophilic atmosphere at 42°C for 24 – 72 h.

For all three protocols, there is a requirement for confirmation of thermotolerant Campylobacter. Five characteristic colonies from each selective agar plate are examined and subjected to a series of biochemical tests.

Note: the ISO protocol recommends lysis of horse blood by freezing and thawing before use.

FDA (1998) methods

The Bacteriological Analytical Manual (BAM) produced by the US Food and Drug Authority includes protocols for the isolation of Campylobacter from food and water (FDA BAM, 1998). There is a general method for most types of sample and in addition, specific methods for water, shellfish, milk and cheese products. The methods include both sample preparation and isolation protocols.

All incubations are done in a microaerophilic atmosphere that can be established in different ways for enrichment broths. One way involves using a flowing gas stream (bubbler system) in conjunction with incubation in flasks placed in a shaking waterbath. The other method applies to both enrichment broths and agar plates, and involves static incubation in an anaerobic jar. The microaerophilic atmosphere is established either by evacuation followed by gassing with the appropriate mixture of gases, or by use of a suitably sized commercial “gas pak”.

The specified enrichment broth for all sample types is Bolton broth and all protocols include a pre-enrichment procedure to increase recovery of stressed cells.

One of two specified agars can be chosen for the selective isolation step that follows enrichment. The agars are either Abeyta-Hunt-Bark or modified CCDA. The specified procedure is to inoculate the chosen agar after 24 h enrichment and again after 48 h enrichment in broth. Both an undiluted and a diluted (1:100 in 0.1% peptone) aliquot of enrichment broth are streaked to selective agar. Agars are incubated (preferably in anaerobic jars that are half full) in a microaerophilic atmosphere at 42°C for 24 h to 48 h. It is advisable to check after 24 h.
UK Methods

The UK Microbiological Safety of Food Funders Group (MSFFG, 2001) reported a collaborative trial comparing the Park and Sanders and Preston isolation protocols given in ISO (1995) with the Exeter protocol (Martin et al., 1996). As a result, MAFF recommended that the Park and Sanders protocol (ISO, 1995) continue to be used for food surveillance monitoring.

An investigation of methods for isolation of thermotolerant Campylobacter from surfaces contaminated by raw chicken, determined that the best recovery was obtained by placing swabs directly into Exeter broth and that this medium was better for these samples than either Preston broth or Park and Sanders broth (MSFFG, 2001). Exeter broth is specified as the enrichment medium in 2002 protocols produced by the UK Health Protection Agency for analysis of food and water samples. These protocols recommend 24 h incubation at 37 °C followed by a further 24 h at 41.4 °C. Enrichment is followed by plating to mCCA agar and incubation of plates in a microaerophilic atmosphere for up to 48 h at 41.5 °C.

Australian methods

An Australian standard method is described by Wallace (1997). This method is based on Preston media and includes an enrichment broth containing FBP. An aerobic pre-enrichment step is performed for 2 h at 37°C. After pre-enrichment, the Preston antibiotic mixture (Polymixin B 5000 IU/l, rifampicin 10 mg/l, trimethoprim 10 mg/l and cycloheximide 100 mg/l) is added, a microaerophilic atmosphere established, and incubation continued at 42°C for 46 h. Selective enrichment is by streaking to both Preston and Skirrow agars and incubating in a microaerophilic atmosphere at 37°C for 48h. A direct plating procedure, presumably included for samples suspected to contain large numbers of Campylobacter, uses Preston agar and incubation in a microaerophilic atmosphere at 42°C for 48h. After selective enrichment, typical Campylobacter colonies (grey to light tan, flat to slightly raised, with a diameter of 1 to 2 mm) are streaked to blood agar plates (non-selective agar) and incubated in a microaerophilic atmosphere at 42°C for 48 h. The species identity is determined by carrying out antibiotic susceptibility tests (cephalothin and nalidixic acid) and hippurate hydrolysis.

New Zealand method

Hudson (1997) recommended that all sample types be analysed using Exeter media. Incubation included a resuscitation step for 4 h at 37°C prior to transfer to 42°C for 44 h. Selective plating was by subculture to Exeter agar (Exeter broth solidified by addition of 15 g/l agar) with incubation in a microaerophilic atmosphere at 42°C for 24 – 48 h.
3 Sample Preparation

A variety of protocols are used for sample preparation and although protocols are given for a range of food and water samples by FDA (BAM, 1998) there appears to be considerable variability in methods used in different laboratories. Aeration may cause Campylobacter to enter a non-culturable state therefore, it would seem advisable to use sample preparation techniques that minimise aeration (Gauthier, 2000). The effects of aeration may be reduced by homogenising samples in enrichment broth that contains oxygen quenching agents (e.g. the FDA general method, BAM, 1998).

3.1 Swab samples

A procedure for swab samples is given by the FDA (BAM, 1998). Swabs are returned to the laboratory and placed into sterile flasks containing 10 ml of enrichment broth. Flasks are then incubated in a microaerophilic atmosphere suitable for Campylobacter. In a variation that allows for aerobic incubation, swabs are placed in small sterile vials, sticks broken off below the top of the vial, and enrichment broth added to just below the surface. Vials are capped and incubated in a normal air incubator.

Some workers prefer to add swabs to a transport medium for return to the laboratory. For example, Moore (2001) reported optimal recovery of thermotolerant Campylobacter from naturally contaminated pig liver by swabbing (not homogenising) freshly eviscerated liver. Swabs were pre-moistened in Cary-Blair transport medium (this medium is available commercially) and an aliquot of this medium inoculated to selective agar (Oxoid Manual, 1998).

3.2 Faecal Samples

The presence of Campylobacter in faecal samples is usually determined by direct plating onto a suitable agar. To quantify campylobacter, a measured aliquot of an emulsion of faeces (usually 1 g faeces mixed with 9 ml buffer) can be spread onto surface-dried plates of campylobacter agar. If the concentration is required for samples requiring enrichment (e.g. animal faeces) serial dilutions of faeces are inoculated to tubes of a campylobacter enrichment broth set out in a most probable number format.

Some laboratories favour the use of rapid methods for analysis of faecal samples. Although rapid methods such as enzyme immunoassay methods (e.g. ngaio Diagnostics, Nelson, New Zealand) can be a useful addition to the suite of methods available to laboratories it needs to be noted that a conventional culture technique is also required for “positive” samples in order to obtain isolates for subsequent speciation and sub-species typing.
3.3 Food samples

A method suitable for most samples, including food, is described in the FDA BAM (1998). In this method 25 g sample (50 g for vegetables) is added to 100 ml Bolton broth plus antibiotics in a net lined bag and gently shaken for 5 min. The bag is removed and the mixture subjected to microaerophilic enrichment with resuscitation (4 h at 37 °C followed by 20 - 44 h at 42°C. Hudson (1997) also reported direct homogenisation of 10 g food sample in 90 ml of enrichment medium, in this case, Exeter broth. Sample-enrichment broth homogenates can be diluted in maximum recovery diluent (Martin et al., 1991), as for example, when enumeration by the most-probable-number technique is required.

Methods are also given for sample types that require special treatment including milk products and shellfish. Milk product samples are centrifuged (12,000 x g for 40 minutes) the supernatant discarded and the pellet re-suspended in 100 ml enrichment broth. Shellfish samples are analysed as both 1:10 (w/v) and 1:100 (w/v) mixtures of homogenate and enrichment broth (FDA BAM, 1998).

Park and Sanders (1991) described a method for vegetables in which rinsing was done in buffer rather than enrichment broth. For fresh vegetables, a 50 g aliquot was used for each sample and each aliquot cut into pieces (2 cm²) prior to addition to 250 ml of sterile 0.1% peptone. For tuberous vegetables the sample size was 200 g and these vegetables were not cut prior to rinsing. Vegetable/peptone water mixtures were shaken for 5 min., strained through cheese cloth, centrifuged at 16 000 x g at 4°C for 10 min., the supernatant discarded and the “sediment” re-suspended in 100 ml of enrichment broth. A feature of the method given by these workers was the stepwise increase in temperature and addition of antibiotics. This enrichment format is included in the 1995 ISO Standard (ISO, 1995).

3.4 Water samples

For detection of low numbers of Campylobacter in water samples some form of concentration technique, usually filtration, is required. Mathewson et al. (1983) investigated three types of 0.45µm filters for their ability to recover C. jejuni from water samples, Millipore HA, Gelman GN6 and positively charged Zetapor (Cuno, Meriden). Recovery was substantially better using Zetapor filters and could be increased further by using Zeta Plus 50S filters. When filters of different pore size, 0.65µm, 0.45µm and 0.22µm were compared it was found that 0.45 µm gave satisfactory results.

Blaser and Cody (1986) tested whether pre-filtration of samples through larger pore size filters prior to filtration through 0.45µm filters would result in a satisfactory protocol. However, these workers reported the best recovery of Campylobacter was achieved by
filtration through a 0.45μm filter (without pre-filtration) and incubation of filters face down on selective agar. They noted that this method did not allow a count to be obtained and that an MPN (most-probable-number) protocol would be needed to achieve this. Dousse et al. (1993) filtered wastewater samples through 0.45 μm Millipore HA filters and compared recovery on filters placed face up, face down or pre-enriched in Preston broth for 6 h at 42°C. The best recovery was found to occur after pre-enrichment, followed by placement of filters face down on Preston agar.

Most New Zealand laboratories filter water samples through 0.45 μm membrane filters, including for analysis of Campylobacter. Although some cells in natural samples will be attached to particles that will be retained on filters this will not necessarily always be the case. To evaluate the ability of filters to retain Campylobacter, aliquots of a suspension of C. jejuni were filtered through both a 0.45 μm filter and a 0.2 μm filter and the filters examined under a scanning electron microscope. As shown in Plate 1 and Plate 2 most of the cells passed through the pores of the 0.45 μm filter but many were retained on the 0.22 μm filter.

Plate 1. Scanning electron micrograph of a 0.45 μm membrane filter used to filter a suspension of Campylobacter jejuni. Note only one cell has been retained on the filter and others that are in the process of passing through the pores.
Plate 2. Scanning electron micrograph of a 0.2 µm membrane filter used to filter the same suspension of *Campylobacter jejuni* as used for the filter shown in Plate 1. Note the large number of cells retained on the surface of this filter.

The procedure described in the FDA BAM (1998) is to filter 2 – 4 litres (if possible) through 0.45 µm (Zetapor) filters. These filters have a positive charge which is presumably why Campylobacter cells are retained on the filter. In this method, filters (fragmented if necessary) are placed in 100 ml of Bolton enrichment broth, taking care to immerse the filter completely in the broth. For this method, the pre-enrichments are incubated in a microaerophilic atmosphere over 3 stepwise increases in temperature. The first step is at 30°C for 2 h after which enrichments are transferred to 37°C for a further 2 h and finally as a third step to 42°C for 20 – 44 h and finally selective plating. A recommendation that large volumes of water sample (at least 1 – 2 litres) be filtered for analysis of Campylobacter was also made by Hanninen et al. (2003).

C. Ross compared recovery of Campylobacter from turbid water samples by an MPN technique in which specified volumes of water samples were filtered sequentially through glass fibre filters (Whatman GFC) either with or without filter aid (Kieselguhr, Rhone Polenc). This step removed large particles and made the next filtration step, which involved filtration through a 0.2 µm membrane filter (Sartorius CN), much faster. Without the use of pre-filters a large number of membrane filters was required to filter the required volume of sample for each MPN tube. Both the pre-filter and the filter were incubated in enrichment broth as Campylobacter were found to have been trapped on both the pre- and membrane filters. The main advantage of the procedure was a greatly decreased sample processing time.
Concentration by centrifugation rather than by filtration was used by Fricker and Park (1989). These workers concentrated samples (e.g. 100 ml) by centrifugation at 20 000 g for 10 minutes. The pellet was re-suspended in Preston broth and cultured as for food samples (ISO, 1995). Raw sewage was analysed by addition of 1 ml aliquots to 50 ml Preston broth, subjected to the same protocol.

Overall, it appears that most useful concentration technique for water samples is membrane filtration through cellulose ester filters of 0.2 μm pore size or 0.45 μm Zetapor filters. Filters should be completely immersed in enrichment broth. The use of pre-filters may be useful when water samples contain particles, in which case both the pre-filter and membrane filter are incubated in the enrichment broth.

3.5 Soil samples

There are few reports of isolation of Campylobacter from soil. Bolton et al. (1999) added 25 g aliquots of beach sand directly to 225 ml aliquots of Exeter broth (Martin et al., 1996) followed by subculture to CCDA agar.

Ross (manuscript in preparation) used an MPN format to enumerate thermotolerant Campylobacter in soils irrigated with farm dairy effluent. Exeter broth (Martin et al., 1996; Martin et al., 2002) was the primary enrichment medium and mCCDA (Oxoid) was the secondary enrichment medium. Weighed aliquots of soil (usually 10 g, 1 g and 0.1 g) were inoculated directly into Exeter broth.

To determine whether Exeter broth gave satisfactory recovery of thermophilic Campylobacter from soil, five-tube series MPN counts from Exeter broth → mCCDA were compared to those from parallel series using CEB broth (Lab M, 135) → modified CCDA improved (Lab M, 112). No significant difference was found between the two methods (Ross, unpublished data).

4 Methods for Species Identification

The Standard Methods for Campylobacter (ISO. 1995; FDA, 1998) include protocols for confirming isolation of thermotolerant campylobacter and, after isolates have been obtained in pure culture, a set of tests to allow species identification.

ISO (1995)

The ISO protocol requires 5 typical colonies from each selective enrichment plate to be examined to confirm isolation of thermotolerant Campylobacter. These colonies are tested by: Gram stain and microscopic examination of a wet mount to examine motility, for production of oxidase and for their ability to grow at 25°C. The last test is done in
non-selective medium (Brucella broth) that is incubated microaerophilically for 2 – 5 days. Biochemical tests proceed for all isolates that are oxidase positive, curved Gram-negative rods with spiralling "corkscrew" motility. If growth is recorded at 25°C and the other tests suggest the presence of Campylobacter, further plating (e.g. on blood agar) is probably required to obtain a pure culture.

The biochemical tests are: $\text{H}_2\text{S}$ production (in triple sugar iron agar) resistance to nalidixic acid, resistance to cephalothin, hydrolysis of hippurate and catalase. Typical reactions for 4 commonly isolated species of thermotolerant Campylobacter are given in Table 1.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>C. jejuni</th>
<th>C. coli</th>
<th>C. lari</th>
<th>C. upsaliensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at 25°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\text{H}_2\text{S}$</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>- or slight</td>
</tr>
<tr>
<td>Hippurate hydrolysis</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

(+) means slightly positive

Table 1. Typical biochemical reactions of commonly isolated species of thermotolerant Campylobacter

FDA (1998)
For confirmation of thermotolerant Campylobacter a series of procedures are given. Firstly a wet mount from at least one colony on each agar plate is examined microscopically for typical morphology and motility. If organisms appear typical, then two colonies from each plate are streaked onto a plate of Abeyta-Hunt-Bark agar prepared without antibiotics. Streaking is continued until a pure culture is obtained. Pure cultures are subjected to a series of biochemical tests carried out in parallel with suitable control cultures (e.g. C. jejuni and C. lari).

The FDA specifies 16 biochemical tests. This is a more extensive range than those given in the ISO (1995) and is designed to identify a wider range of species.

Bolton et al. (1992) developed a suite of 19 tests designed to differentiate 18 species (or sub-species) of Campylobacter.
4.1 New Zealand protocols for confirmation and speciation

A selection of the preliminary identification protocols used in New Zealand laboratories demonstrates that most follow the ISO (1995) protocol reasonably closely. Two laboratories, Otago University and ESR routinely use a PCR based system to identify C. jejuni and C. coli.

**Otago University** growth from colonies with typical Campylobacter morphology is tested for oxidase and catalase activity and Gram stained. At Otago the counterstain used in the Gram stain test is carbol fuchsin not saffranin as the latter does not stain these bacteria well. A wet mount is not routinely done. C. jejuni is initially distinguished from C. coli by the hippurate test and all isolates are routinely tested using a multiplex PCR system that includes fla A. C. jejuni isolates are typed by pulse-field gel electrophoresis. Sample types include human and animal faecal samples and food samples.

**LabNet (Invermay)** applies the following tests to all Campylobacter isolates: catalase, growth in 1% glycine, hippurate, H₂S, nalidixic acid, cephalothin and growth at both 25°C and 42°C. This laboratory specialises in veterinary specimens but also analyses food and water.

**Massey University** the following tests are applied: microscopic examination is followed by a Gram stain and oxidase testing. The followed tests are then performed: hippurate, H₂S, catalase, nitrate (37°C for 72 h), nalidixic acid, cephalothin and growth at both 25°C and 42°C. For biochemical tests requiring incubation, either blood agar or tryptic soy agar is used and unless otherwise specified plates are incubated at 37°C for 48 h. C. jejuni isolates are typed by pulse-field gel electrophoresis. This laboratory analyses veterinary specimens and also food, water and wastewater samples.

**AgResearch** all isolates are examined using a wet mount and phase contrast microscopy. Isolates identified as Campylobacter are subjected to the following tests: Gram stain, oxidase, hippurate, nalidixic acid and cephalothin. C. jejuni isolates are typed by pulse-field gel electrophoresis. This laboratory analyses animal faecal samples and water and wastewater samples.

**ESR (KSC) National Campylobacter Reference Laboratory** isolates that are received for typing are identified by an extensive range of biochemical tests that include: growth in oxygen, microaerophilic growth at 25°C, 37°C and 42°C, hippurate, nitrate, urea, indoxyl acetate, catalase, H₂S production, growth in 1% glycine, growth in 3.5% NaCl, growth on McConkey agar, and sensitivities to nalidixic acid and cephalothin. Isolates are presumed to have been subjected to microscopic examination and oxidase testing by the isolation laboratory but these tests may be checked as well. Sometimes a flagella stain is done. ESR laboratories analyse a very wide range of sample types.
C. jejuni isolates are typed by both Penner serotyping and pulse-field gel electrophoresis.

**Diagnostic Medlab (Auckland)** reported procedures carried out both in their own laboratory and in five similar hospital and community laboratories. Isolates that have appropriate morphology on campylobacter specific agar are confirmed by microscopic examination and biochemical tests. The tests that are routinely done are a Gram stain and/or a wet mount to confirm characteristic Campylobacter cell morphology and an oxidase test to confirm a positive reaction. If required, speciation is done by testing for hippurate hydrolysis and cephalothin susceptibility.

**Valley Diagnostic Laboratory (Lower Hutt)** the Charge Microbiologist summarized information gathered from ten New Zealand laboratories that analyse clinical specimens. The majority of these laboratories apply a routine confirmation procedure that consists of an oxidase test and a Gram stain and some also do a motility test. However, only a few of the laboratories that were questioned perform a hippurate test to identify C. jejuni.

### 5 Sample Holding and Transport

Studies have shown Campylobacter retain culturability for a considerable period when held at 4°C (Gauthier, 2000) but most investigators prefer to analyse them as soon as possible after collection (Goosens & Butzler, 1992). Same day analysis should be a priority for samples that are likely to contain stressed organisms, including those that could dry out, contain >2% (w/v) salt, have a pH < 5 (e.g. yoghurt) and those that have been aerated.

Water samples are either immersed in crushed ice (R. Eyles, Wellington School of Medicine, pers. comm.) or placed on ice packs in an insulated container (e.g. chilly-bin) for transport to an analytical laboratory. Usually analysis is commenced within 24 h of collection but should probably not be delayed for more than 48 h.

Faecal samples are brought to 4°C as quickly as possible and held at 4°C; usually analysis is commenced within 24 h of collection. However some laboratories have found that if faecal swabs are transported in Cary-Blair medium at 4°C analysis can commence 3 - 11 days after collection.

Food samples suspected as having only small numbers of campylobacters are held at 4°C before being analysed as soon as possible. For samples that require longer than 48 h transport, or are likely to contain highly stressed Campylobacter, a transport medium should be used.
When a transport medium is required, the commercially available Cary-Blair medium is frequently chosen. An alternative medium, Lander transport and storage medium (Lander, 1982) has been reported to maintain the viability of campylobacters for up to 7 days prior to incubation.

6 Storage and Transport of Isolates

It is very important that isolates of thermotolerant Campylobacter are carefully stored and transported to ensure that viable isolates are received by the specialist laboratory undertaking the typing. Only isolates that have been established as pure cultures should be stored and transported. In New Zealand isolates are sent to the national Campylobacter spp. Reference Laboratory, ESR Porirua for typing. Campylobacter jejuni isolates received by ESR Porirua are typed by Penner serotyping and pulse-field gel electrophoresis to allow source tracing in outbreaks and to identify patterns of strain occurrence in New Zealand.

Laboratories may also wish to store isolates that have been subjected to preliminary tests (e.g. microscopic examination) to enable biochemical testing to be done on batches of isolates. As a general rule, isolates should be obtained in pure culture prior to storage.

Storage at -70°C: The most generally successful method for storing isolates is to remove a very generous amount of pure culture from an agar plate using a sterile swab or plastic loop, to a cryovial containing about 1 ml of sterile nutrient broth containing 15 - 20 % (v/v) glycerol. Cryovials can be stored indefinitely in an ultra-freezer at -70 to -80°C. The nutrient broth used can be any non-selective formulation including: commercial preparations of nutrient broth, tryptic soy broth, brain heart infusion broth or Brucella broth.

Isolates can also be stored successfully at -70°C when a generous inoculum from an agar plate is recovered on an Amies charcoal swab.

Storage at -20°C: Many laboratories will not have ready access to an ultra-freezer so alternative storage methods are required.

Otago University successfully stores clinical isolates for 3 – 6 months using the same procedure as for -70°C storage. The nutrient medium is tryptic soy broth with 20% (v/v) glycerol, in 3 ml aliquots in bijoux bottles. Inoculated Bijoux are stored in a freezer at -20°C for up to 6 months.

Some laboratories have reported problems when Campylobacter is stored in a nutrient broth at -20°C and Otago University has another method that may be more generally useful. For this storage method, a generous amount of growth from an agar plate is re-
suspended in 3 ml of sterile skim medium (available commercially) with 20% (v/v) glycerol in bijoux and stored at -20°C. Maximum storage time for this protocol is being investigated (C. Pope, pers. comm.).

Note: At Otago University storage media are prepared within a few days of use and stored (with loose lids on bijoux) in a microaerophilic atmosphere until required (C. Pope, pers. comm.). The use of this protocol is highly recommended.

AgResearch has tested storage of three C.jejuni isolates, NZRM 1958, ATCC 33291 and a wild strain in skim milk medium plus glycerol in a household freezer (-20°C). All three strains grew satisfactorily on non-selective blood agar plates that were incubated in a microaerophilic atmosphere for 24h – 48h at 37°C (C. Ross unpublished data).

Transport

Overall the most satisfactory method for transport of isolates is the use of Amies charcoal swabs. These swabs should be inoculated with a generous amount of culture from a plate and placed in a properly labelled approved Biohazard container. Approved procedures must be followed for the transport of any infectious material. Containers should be sent to their destination by an approved same-day or overnight courier, with appropriate documentation (IATA regulations). It is advisable to give the receiving laboratory prior notice of their arrival.

7 Isolation of Campylobacter in New Zealand

7.1 University and Crown Research Institute Laboratories

Researchers in Universities and Crown Research Institutes isolate Campylobacter from a wide range of sample types. Although some researchers use PCR methods to detect specific species of campylobacter they also use laboratory culture to obtain isolates for sub-species typing.

Massey University

At Massey University different methods are used for different sample types. As well many samples are analysed in parallel for both thermotolerant Campylobacter and non-thermotolerant species of veterinary importance.

Faecal samples

For isolation of thermotolerant Campylobacter, faecal samples are analysed within 90 min of collection wherever possible, using an enrichment broth culture and selective plating protocol. The general method for animal faeces is to add a 1 g sample (wet
weight) to 9 ml of enrichment broth. For chicken faeces 1 g faeces is added to 9 ml Bolton enrichment broth and incubated in a microaerophilic atmosphere for 48 h at 42°C. Selective enrichment is by subculture to mCCDA plates, incubated for 48 – 72 h at 42°C. For cattle faeces, 1 g faeces is added to 9 ml Preston enrichment broth and incubated in a microaerophilic atmosphere for 48 h at 42°C. Selective enrichment is by subculture to mCCDA plates, incubated for 48 – 72 h at 42°C.

**Clinical Samples**

A range of Campylobacter is sought in clinical samples and the choice of methods reflects this. Samples include human faeces, intestinal swabs, blood, heart contents and tissues and the standard protocol is to inoculate 3 media in parallel: mCCDA agar and non-selective blood agar plates (included for recovery of *C. fetus*) and additionally, enrichments are set up in Bolton broth. All incubations are for 48 h at 37°C in a microaerophilic atmosphere. After 48 h the agar plates used in the two direct plating procedures are examined for the presence of Campylobacter. The Bolton enrichment broth is subcultured to both mCCDA and blood agar and incubated for 48 h at 37°C. Because all incubations are done at 37°C, the ability to grow at 42°C is a routine test for all isolates.

**Media preparation and storage**

Lysed horse blood is purchased from a commercial supplier. Broths (Preston and Bolton) are prepared with all ingredients added, aliquotted into tubes and if not used immediately, stored at –20°C until required. The agars used are mCCDA and blood agar (Columbia agar base). Blood agar plates are prepared as a Columbia agar (minus blood) base with an overlay of Columbia agar + blood added when plates are required.

**ESR**

ESR (Christchurch Science Centre) has a standard enrichment → selective isolation protocol that is applied to all samples analysed. These include faeces, water, and offal and chicken carcasses. The protocol consists of pre-enrichment in Exeter broth with resuscitation for 4 h at 37°C followed by incubation for 44 h at 42°C. Selective plating is on Exeter agar, incubated microaerophilically at 42°C for 24 - 48 h. Exeter agar is Exeter broth with the addition of 15 g/l of agar (Humphrey, 1995). This procedure is used for obtaining isolates for typing.

For detection and enumeration (in an MPN format) a PCR-based method is routinely used to identify the presence of specific species of thermotolerant Campylobacter, principally *C. jejuni* and *C. coli* in samples. This method is 30% more sensitive that direct isolation (M. Savill, pers. comm.) and reduces laboratory analytical time and
materials because plating and isolation in pure culture is only performed for samples in which Campylobacter has been positively identified. Once in pure culture, isolates are typed by Penner serotyping and pulse-field gel electrophoresis by the national Campylobacter Reference Laboratory at ESR (Kenepuru Science Centre).

**Otago University**

Otago University protocols are generally based on ISO methods (1995), updated to include recent information from the scientific literature. Faecal samples are analysed by direct plating to Skirrow agar (ISO, 1995); a Campylobacter count can be obtained after 4 h using this method (C. Pope, pers. comm.).

For poultry samples two isolation methods are used. One method includes pre-enrichment of an aliquot of sample added to Preston enrichment broth followed by selective plating on mCCDA agar. A second method is direct plating to a non-selective blood agar. The formulation of Preston broth used included the FBP growth supplement; lysed horse blood and antibiotic supplement (Polymixin B 5000 IU/l, rifampicin 10 mg/l, trimethoprim 10 mg/l and amphotericin B 10 mg/l, Oxoid SR204E). Broths are incubated at 42°C for 24 h. After incubation, 20 µl of enrichment broth is streaked onto mCCDA agar (Oxoid CM739) with the antibiotic supplement that contains cefoperazone (32 mg/l) and amphotericin B (10 mg/l) (Oxoid SR155E). Plates are incubated microaerophilically at 37°C for 48 h. The other isolation procedure is by direct plating method and involves washing a chicken meat sample in 50 ml 0.1% (w/v) peptone, centrifuging the mixture and re-suspending the pellet in 1 ml 0.1% (w/v) peptone. Analysis is by spreading 100 µl aliquots of the re-suspended mixture onto a blood agar plate.

All plates with growth are stored at 4°C for examination and biochemical testing, isolates do not store well at room temperature.

**AgResearch**

AgResearch uses Exeter broth for all sample types (Martin et al., 1996). For examination of environmental samples (animal faeces, soil, water and sediments) Nutrient broth No 2 (Oxoid CM67), with the addition of FBP supplement (Oxoid SR 84), 5% (v/v) lysed horse blood and an antibiotic supplement that contains amphotericin B instead of cycloheximide (Oxoid SR 204E) is used (Martin et al., 2002). In the protocol for examination of water and wastewater samples (MIMM, 2000, Chapter 13, Section 1) this medium is called “modified Preston broth” because it differs from Preston broth only in the addition of 15 µg/ml cefoperazone (Humphrey, 1995). Exeter broth enrichments are incubated aerobically in containers with a head space of less than 1 cm. The first
stage is a resuscitation procedure that consists of incubation at 37°C for about 16 - 20 h before transfer to 42°C for 32 - 28 h (48 h total). Selective plating is onto mCCDA (Oxoid CM739) with antibiotic supplement (Oxoid SR155) and incubation of plates in a microaerophilic atmosphere at 42°C for 24 h followed by a further 24 h if necessary.

For examination of meat and meat products large samples (0.5 – 1.0 kg) are tested. These samples are either rinsed or homogenised in 100 ml of maximum recovery diluent (0.1% peptone and 0.85% NaCl). A 10 ml aliquot is inoculated into a flask containing 90 ml Exeter broth that has been prepared according to the ESR formulation. The enrichment broth is incubated microaerophilically at 37°C for 5 h and then transferred to 42°C for a further 44 h. Selective isolation is on Exeter agar plates incubated at 42 °C for 48 h (MIMM, 2000, Chapter 7, Section 3).

LabNet (Invermay)

LabNet analyses a variety of sample types, particularly veterinary samples. When Campylobacter of animal clinical significance e.g. C. fetus, are sought, samples are received in transport medium and analysed by direct plating to Skirrow agar with incubation for 48 h at 37°C. Different procedures and media are used for the food and water samples that are analysed for thermotolerant Campylobacter. For these samples an enrichment procedure is carried out using Bolton broth followed by plating on both Skirrow and mCCDA agars. Bolton broth is incubated at 42°C for 48 hours and the selective agars at 42°C for 48 hours.

7.2 Clinical Laboratories

Two clinical laboratories reported the procedures carried out in their own laboratories that they compared to those of other clinical laboratories that analyse human faecal samples for campylobacters.

Diagnostic Medlab (Auckland)

The Diagnostic Medlab (Auckland) reported that clinical laboratories in the Auckland region test for Campylobacter in clinical samples (i.e. human faeces) collected from symptomatic patients. Samples are processed on the day that they are collected and analysis is by direct plating onto mCCDA plates. Plates are incubated in a microaerophilic atmosphere at 42°C for 24 – 48 h. Only one sample per patient is plated as experience has shown that there is no advantage in using more that one agar plate or in using different types of agar.
Valley Diagnostic Laboratory (Lower Hutt)

Valley Diagnostic Laboratory surveyed ten New Zealand clinical laboratories. These laboratories all reported that they analyse human faecal samples by direct plating. Most use mCCDA but a few use non-selective blood agar. Plates are incubated in a microaerophilic atmosphere, generally at 42°C (sometimes 35°C); for at least 48 h (larger laboratories use a dedicated microaerophilic incubator).

One laboratory reported that it uses a rapid enzyme immunoassay method (EIA) (ngaio Diagnostics, Nelson, NZ) that gives results in 2 hours.

8 Recommendations for New Zealand Laboratories

Laboratories need robust, sensitive methods for isolation of thermotolerant campylobacters. The methods discussed in this Report are based on traditional laboratory culture and are suitable for identifying or enumerating thermotolerant Campylobacter in samples of various types.

At the request of the New Zealand Enteric Diseases Research in New Zealand Steering Committee, information was sought to allow selection of an analytical method that would be reliable and cost-effective for New Zealand laboratories. The Committee expressed a preference for one recommended medium for enrichment and one recommended medium for selective plating. Exeter broth is the preferred enrichment medium recommended, after discussion and consultation, by the Methodology sub-group of the N.Z. Enteric Zoonotic Disease Research Group and mCCDA agar is the preferred selective plating medium.

In certain cases a particular protocol may be required for a specific purpose. For example, the US FDA procedure that specifies Bolton/CEB broth as an enrichment medium may be required for export food. In this case the customer’s requirement is paramount. A further consideration is the bias that Exeter broth and mCCDA give to recovery of *C. jejuni* (Silley, 2003). Where a wider range of thermotolerant campylobacters is sought the use additional media is recommended.

For analysis of food, water, sediments, soil and animal faeces the recommendation in this Report is that an enrichment procedure, that includes pre-enrichment, be carried out. The exception is for human faecal specimens from symptomatic patients that are a special case in that enrichment is not required.
8.1 Selection of enrichment broth

The first step in the isolation of thermotolerant campylobacters from most samples is an enrichment procedure. Internationally the most widely-used enrichment media seem to be Preston, Exeter and Bolton/CEB broths. The Preston broth formulation containing FBP (e.g. Wallace, 1997) differs only from Exeter broth in that the latter broth includes an additional antibiotic, cefoperazone (Martin et al., 1996; Martin et al., 2002). Theoretically, Bolton broth has some advantages as it contains defined growth substrates specifically designed to limit trimethoprim antagonism and also haem in as well as FBP and blood to increase oxygen quenching. Bolton broth also contains vancomycin to suppress Gram positive cocci as it is considered less inhibitory to campylobacters than the rifampicin included in Preston and Exeter broths.

All these broths are included in various standard methods published by recognised authorities. Bolton/CEB is the enrichment medium in protocols published by US FDA (BAM, 1998); Preston broth is included in the ISO Standard method (ISO, 1995) and Exeter broth is the enrichment medium in Standard Operating Procedures published by the UK Health Protection Agency (PHLS, 2002). In a New Zealand study, Hudson (1997) examined comparative performance of these media for isolating thermotolerant Campylobacter from a variety of food samples and reported best overall performance from Exeter broth.

8.2 Recommendation for routine use

Exeter broth is recommended for routine use in New Zealand laboratories. This medium is already widely used for food and water samples, and was the primary enrichment medium used for samples analysed in the Freshwater Microbiological Study (McBride et al., 2002).

Non-routine recovery of highly stressed campylobacters

Particular care is required to assure recovery of highly stressed Campylobacter expected to be present in processed foods and treated drinking water. For these samples the recommended procedure is enrichment in Park and Sanders broth according to the protocol given in the ISO Standard (1995). As this is a time consuming and expensive procedure it may not be realistic for routine use in small New Zealand laboratories. However, it is recommended in outbreak situations in which foods that have been dried, salted or frozen are implicated.
8.3 Protocol for enrichment

The initial enrichment procedure consists of inoculation of samples/diluted samples in a liquid (broth) medium. Incubation of inoculated broths can either be in a microaerophilic atmosphere suitable for campylobacters or, if screw-capped containers with a minimal head-space are used, in an air incubator. The broth should be at room temperature prior to inoculation of samples.

Exeter broth can be stored in a refrigerator at 4°C for up to 7 days before use. However, for incubation in air it is recommended that the broth be freshly prepared, preferably on the day of use.

Pre-enrichment

Inclusion of pre-enrichment step is highly recommended. Some researchers favour a period of 4 h incubation at 35 – 37°C prior to transfer to 41.5 - 42°C for the remaining 44 h of incubation. However, PHLS Standard Operating Procedures (2002) specify pre-enrichment for 22 h at 37 °C followed by transfer to 41.5°C for a further 22 h.

The recommendation in this Report is that 4 h at 35 – 37°C is the minimum pre-enrichment procedure for samples. For samples in which there are likely to be low numbers of stressed campylobacter and relatively low numbers of contaminating microorganisms (e.g. many water samples and processed foods) a longer, up to 24 h, period of pre-enrichment is preferable.

8.4 Selective plating medium

The mCCDA medium in widespread use in New Zealand laboratories and is included in international standard protocols (PHLS, 2002; FDA BAM, 1998) gives satisfactory results and is recommended in this Report for selective plating.

If laboratories wish to choose alternative plating media from the range of well documented types available they should demonstrate that this does not compromise performance. Scotter et al. (1993) reported that primary enrichment not secondary is the most important stage in the isolation of campylobacters from samples.

The ISO (1995) protocol specifies that selective plating be done on two media in parallel but FDA (BAM, 1998) requires only one agar to be used. The use of only one selective agar (probably blood free) is under discussion for the proposed revision of the 1995 ISO standard (Jacobs-Reitsma & de Boer, 2001).
Protocol for selective plating

Agar plates need to be brought to room temperature prior to inoculation.

Plates are incubated in a microaerophilic atmosphere suitable for campylobacters, either in gas-tight containers, or in an incubator with the required atmosphere. Incubation is at 41.5 - 42°C for 18 - 48 h. Plates can be stored for up to 2 weeks in sealed bags at 4°C, if possible in an anaerobic atmosphere, but preparation on the day of use is preferable.

8.5 Direct plating

There is widespread consensus that direct plating of faeces from infected patients is satisfactory for recovery of thermotolerant Campylobacters. Aliquots of faeces/diluted faeces are spread directly to mCCDA plates at room temperature. Plates are incubated for up to 48 h at 41.5 - 42°C.

Some laboratories prefer to incubate mCCDA at 37°C. This procedure does not guarantee recovery of thermotolerant species and if results are to be reported as thermotolerant campylobacters a confirmation procedure is required. This involves subculture of isolates to a second plate of campylobacter agar that is incubated at 41.5 - 42°C for up to 48 h.

Although direct plating is the method of choice for specimens from patients with acute Campylobacter enteritis, a liquid enrichment step is recommended for specimens from patients with Guillain-Barré syndrome (Kist & Bereswill, 2001).

8.6 Enumeration protocols

Enumeration protocols can be applied to both enrichment and direct plating methodologies.

Most-probable-number technique

When an estimation of the number of campylobacters in a sample is required, the enrichment stage can be arranged in a most-probable-number (MPN) format. Currently most NZ laboratories use a 3-tube per row, three consecutive dilution MPN method which means that 9 enrichment tubes are required per sample. Although this format is economical terms of time and materials, poor precision limits its usefulness. When a higher level of precision is required, a 5-tube per row MPN method (15 tubes per sample) is preferable as it provides better quality data. For treated drinking water a 10-tube single dilution format may be suitable.
Incubation of enrichment broths is always preceded by pre-enrichment. Following enrichment, each tube is sub-cultured to a parallel plate of mCCDA agar that is labelled with the tube number. The number of positive plates in each row is counted and the MPN score obtained from the results for 3 consecutive rows. The MPN score is referred to MPN Tables to obtain the concentration of campylobacters in the sample. Results should be expressed per volume of water, per g wet weight of food and per g dry weight of soil or sediments.

**Plating methods**

Clinical specimens from symptomatic patients usually contain high numbers of Campylobacter and can be enumerated by direct plating. A measured volume of diluted faeces (e.g. 1:10 w/v) is spread to the surface of an air dried plate of mCCDA agar and the colonies counted after incubation. Drying of plates is required to limit the spreading of colonies. As over-drying inhibits colony growth the use of a laminar flow cabinet is not recommended.

Some authorities have protocols for direct enumeration of thermotolerant campylobacters by membrane filtration, such as Standard Operating Procedure W 8 (PHLS, 2002). However, in general MPN protocols are both more convenient and more accurate due to the spreading nature of campylobacter colonies and the necessity to filter large volumes of water that may often be turbid.

**8.7 Gaseous Atmosphere**

Enrichment broths can either be incubated in air or in a microaerophilic atmosphere suitable for campylobacters. A suitable microaerophilic atmosphere contains 5% oxygen, 10% carbon dioxide and 85% nitrogen. If incubation is done in air then the use of screw-capped containers with a very small head space after addition of sample is mandatory. If available, incubation in a microaerophilic atmosphere is preferable.

Plates need to be incubated either in air-tight containers (e.g. anaerobic gas jars) in a microaerophilic atmosphere or in loose lidded containers in an incubator with a microaerophilic atmosphere. The microaerophilic atmosphere in gas jars is usually produced by using commercial gas-generating kits. It is most important that a kit appropriate for the volume of the gas jar is chosen.
8.8 Rapid Methods

Laboratories are increasingly seeking rapid methods for routine screening of samples. Methods that may be useful for human faecal specimens include enzyme immuno-assay (e.g. Campylobacter EIA - Ngaio Diagnostics, NZ) a multi-well system (e.g. Campylobacter Simplate, IDEXX, US) or in-house or proprietary PCR methods. Clinical laboratories will generally find routine plating of faecal specimens onto selective media the most appropriate method for their requirements. For sample types other than human faeces PCR methods are probably the most useful as they can be used in association with enrichment, concentration and enumeration protocols. The use of PCR is limited because it could detect non-viable cells and lack of a bacterial isolate will hamper further public health investigations. When introducing a rapid method it is advisable to carry out parallel testing with a conventional method.

Most rapid methods only detect the presence of thermotolerant campylobacters, although with appropriate choice of primers PCR methods can detect different Campylobacter species. If a rapid method is used, positive samples need to be subjected to a conventional culture procedure to obtain isolates for sub-species typing. A national database of Campylobacter spp. is being set up in New Zealand to improve the epidemiology of Campylobacter infection. This is an integral component of the national strategy for reducing campylobacteriosis in New Zealand.

8.9 Analysis of specific types of sample

Human Faecal Specimens

Human faecal specimens collected from symptomatic patients can be analysed by direct plating without enrichment. For recovery of *C. jejuni* and probably most strains of *C. coli*, plating to mCCDA medium is satisfactory. However, as mCCDA contains selective agents that bias against species such as *C. upsaliensis*, parallel inoculation onto additional media (e.g. CAT and/or blood agar) is required when a wider range of species is targeted.

Veterinary Specimens

In contrast to faeces from symptomatic humans, thermotolerant campylobacters may not be present in large numbers in animal faeces, therefore, inclusion of a pre-enrichment step is recommended.

For veterinary laboratories it may be a priority to test animal clinical specimens (including faeces, intestinal swabs, blood, and stomach contents) for *C. fetus* subspecies *fetus* or *venerealis*. Protocols for thermotolerant species are not useful for
C. fetus which is usually sought by direct plating of faecal samples onto a non-selective blood agar with incubation for 48 h at 35 – 37°C in a microaerophilic atmosphere. If thermotolerant Campylobacter are sought then a parallel enrichment and isolation procedure is required.

**Food and other “solid” samples**

Analysis of food and other solid samples is preceded by homogenisation, usually in enrichment broth. A 1:10 (w/v) mixture of sample and broth is homogenised as gently as possible, either in a stomacher (mesh-lined bags are convenient) or in a Waring blender operated on low speed to avoid excess aeration. Volumes of ≥10 ml are incubated directly but smaller volumes, and aliquots of dilutions, are usually inoculated into x10 (v/v) of enrichment broth.

**Milk samples**

Milk samples (usually 50 ml aliquots) are concentrated by centrifugation in a refrigerated centrifuge at 12 000 g for 40 min. The pellet is re-suspended in 10 ml of enrichment broth (FDA BAM, 1998).

**Water samples**

Water samples, including drinking water, surface water, ground water and treated wastewaters require concentration, usually by filtration. Cellulose nitrate filters, 0.45 µm, can allow campylobacters to pass through and should not be used. Two types of membrane filter that retain campylobacters are recommended, 0.2 µm pore size cellulose ester membrane filters of (PHLS, 2002) or positively charged 0.45 µm Zetapor filters (US FDA (BAM, 1998; Meriden).

The volume depends on the water type but for all but highly contaminated samples (e.g. wastewater) a total volume at least 1 litre should be processed. Filtration of larger volumes may be advantageous for treated drinking water. To assist filtration, turbid samples can be pre-filtered through a coarse filter (e.g. Whatman GFC), using Filter-aid if necessary. The GFC filtrate is then passed through a membrane filter. Both pre-filters (plus Filter-aid if used) and membrane filters are added to sufficient enrichment broth to immerse all filters used completely.
9 Acknowledgements

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10 References


Humphrey, T.J. & Muscat, I. 1989. Incubation temperature and the isolation of *Campylobacter jejuni* from food, milk or water. Letters in Applied Microbiology, 9, 137-139.


Park, P. 2002. The physiology of Campylobacter species and its relevance to their role as foodborne pathogens. International Journal of Food Microbiology, 74, 177-188.


Appendix I: Isolation of Thermotolerant Campylobacter by a Pre-enrichment Method

1.1 Background and Rationale

The genus *Campylobacter* is recognised as an increasing cause of human foodborne enteritis worldwide. Two species of this genus, *C. jejuni* and *C. coli*, are isolated from almost all clinical cases of Campylobacter infection, with the proportion of each species differing from country to country.

This method described consists of initial selective enrichment in Exeter broth followed by secondary selective enrichment on mCCDA plates.

In some circumstances alternative methods may be required such as those recommended in FDA protocols (BAM, 1998). In this protocol the primary enrichment medium is Bolton broth and selective plating agar can be either mCCDA or Abeyta-Hunt-Bark agar.

Samples for Campylobacter analysis should be collected using aseptic techniques, placed on ice for transport, and analysed as soon as possible after collection.

1.2 Procedure

Use a reference strain of *C. jejuni* (e.g. NZRM 1958) for quality control. If a negative quality control strain is required a strain of *E. coli* (e.g. NZRM 916) is satisfactory.

**Sample preparation**

*Most sample types*

For most solid samples 25 g (wet weight) should be tested but the amount should be increased to 50 g for vegetables. Rinse the sample in 225 g of Exeter broth either by gentle shaking (25 rpm) on a bench top shaker or by homogenising in a stomacher for 15 – 30 seconds. If the sample is placed in a net lined bag, the solid residue can be separated from the liquid portion. The entire liquid portion, with or without solids, is incubated.

*Swab samples for meat and meat products*

Use a sterile swab and swab a measured area of test sample. Swab a large area for test samples not expected to have high numbers of Campylobacter (e.g. at least 100 cm$^2$). Prior to swabbing, moisten swabs with either Lander transport medium or Cary-Blair transport medium. For transport, swabs should be placed into sterile containers,
on ice, in an insulated box. On receipt by the analytical laboratory transfer the swab (including any unabsorbed transport medium) to a tube of Exeter broth (9 or 10 ml tubes) and incubate as soon as possible.

**Shellfish**

Shuck sufficient shellfish to yield at least 100 g composite of liquor and meat. Homogenise in either a stomacher or a Waring blender (operated at low speed) for 60 seconds. For analysis remove a 25 ml aliquot of homogenate and add to 225 ml Exeter broth. As well, dilute an aliquot of the homogenate 1:10 in MRD and add 25 ml of diluted homogenate to 225 ml Exeter broth.

**Milk**

Milk samples are centrifuged before inoculation into enrichment medium. Aliquots of 50 – 100 ml are centrifuged in a refrigerated centrifuged (12 000 x f for 40 minutes). The supernatant is discarded and the pellet resuspended in 90 – 100 ml Exeter broth.

**Faeces**

The two-stage process involving enrichment procedure is recommended for animal faeces but human faeces are usually analysed by direct plating (see Appendix III).

*Enrichment:* Aliquots of faeces are added directly to Exeter enrichment broth. The normal test portion is 1 g (wet weight) but if numbers are expected to be low 10 g aliquots should be used. Add the weighed aliquot of faeces to x9 or x 10 (w/v) Exeter broth. After incubation, sub-culture growth to a mCCDA plate.

**Primary Selective Enrichment**

Primary enrichment in Exeter broth is done in two stages. Stage one is a pre-enrichment period that is designed to assist recovery of stressed and injured Campylobacter cells. Stage two is selective enrichment at 41.5 - 42°C. Primary enrichment cultures in Exeter broth can be incubated aerobically in screw-capped containers provided containers are long and narrow in shape and there is only a very small head space (< 1 cm) after addition of sample. Alternatively broth enrichments can be incubated in containers with loose caps that are placed in a microaerophilic atmosphere. Incubate without shaking.

**Microaerophilic incubation atmosphere:** Either use an anaerobic jar and commercial kits that will generate a microaerophilic atmosphere consisting of 5-6% oxygen, 10% carbon dioxide and 84-85% nitrogen or use an incubator with a flowing stream of the same gases in the same proportions. If enrichments are incubated in a flowing gas stream the microaerophilic atmosphere must be maintained during all stages of incubation.
Pre-enrichment

- Incubate Exeter pre-enrichment broth at 37°C for at least 4 hours. If both campylobacter and competing microorganisms are expected to be present in low numbers (e.g. processed foods) then extend pre-enrichment incubation for up to 24 h.

Enrichment

- Transfer the pre-enrichments to an incubator at 41.5 - 42°C and incubate for a further 44 hours (24 h following extended pre-enrichment incubation). If loose capped containers are used, maintain the microaerophilic atmosphere.

Secondary Selective Enrichment

Selective plating

- Sub-culture an aliquot of growth, either a loopful or pipette 0.1 µl, from the enrichment broth onto a mCCDA plate*. Spread to obtain single colonies. Incubate at 41.5 - 42°C for 48 hours under a microaerophilic atmosphere.

- After incubation examine plates for typical Campylobacter colonies to confirm isolation.

* prior to plating, agar plates should be dried in air to remove surface moisture, do not over dry and do not dry in a laminar flow cabinet.

1.3 Confirmation of thermotolerant Campylobacter

Typically thermotolerant Campylobacter colonies appear as flat, moist, grey-white colonies with irregular spreading margins on mCCDA.

To confirm the presence of Campylobacter, select one or more typical colonies from each mCCDA plate for further examination. The (minimum) required tests are:

- microscopic examination that consists of examination of a wet mount
- traditional Gram stain some workers prefer to counterstain with saffranin rather than rather than carbol fuschin.
- oxidase test.
**Oxidase test procedure**

- Place 2 or 3 drops of reagent to the centre of a filter paper (e.g. Whatman No 1) and allow a few seconds for absorption.
- Using a platinum or plastic loop smear a loopful of test colony onto the filter paper over a line 3 – 6 mm long. Examine the smear immediately.
- A positive test is the production of a dark purple colour within 5 – 10 seconds.

Include the reference culture (*C. jejuni* NZRM 1958) as a positive control when carrying out the oxidase test.

**Score the sample positive or negative according to the results of the reactions.**

**Positive Thermotolerant Campylobacter reactions**

- **Gram stain**
  - Gram-negative, small "gull" shaped rods
- **Motility (wet mount)**
  - + (typically “corkscrew”)
- **Oxidase test**
  - +

1.4 **Reporting results**

Express the result as presence or absence of thermotolerant Campylobacter in the portion of sample that was tested (i.e. per test unit, unit weight, unit area or unit volume).

1.5 **Media**

**Primary selective enrichment medium**

**Exeter broth base**

**Basal medium**

<table>
<thead>
<tr>
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<th>g/950 ml</th>
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<tr>
<td>Meat extract</td>
<td>10.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0</td>
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<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>950 ml</td>
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</table>

pH 7.5 ± 0.2

Dissolve the ingredients in the distilled water. Sterilise at 121°C for 15 minutes.

Cool to 50°C before addition of blood and supplements.

* Dehydrated broth base is available commercially.
Additives

i. Lysed horse blood (5% v/v)

**Horse blood (sufficient for 1000 ml)**

- Lysed (defibrinated/laked) horse blood 50 ml

**ii. Growth Supplement**

**Campylobacter Growth Supplement** (available commercially)

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<thead>
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<td>Sodium pyruvate</td>
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</tr>
<tr>
<td>Sodium metabisulphite</td>
<td>0.125 g</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
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</table>

To reconstitute, add 2 ml of sterile distilled water and mix to dissolve. Use immediately.

**Note:** 2 vials are required for 1 litre of medium

**iii. Antibiotics**

(a) **Campylobacter Selective Supplement** (available commercially)

<table>
<thead>
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</thead>
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<tr>
<td>Rifampicin</td>
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<tr>
<td>Trimethoprim</td>
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<tr>
<td>Amphotericin B</td>
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To reconstitute, add 2 ml sterile distilled water and mix to dissolve. Use immediately.

**Note:** 2 vials are required for 1 litre of medium

(b) **Cefoperazone stock solution**

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</tr>
</tbody>
</table>

Stir to dissolve (if desired, larger amounts can be prepared, dispensed as 2 ml aliquots and stored frozen at -20°C).
### Complete Exeter Broth

**Ingredients**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g or ml/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal medium (sterile)</td>
<td>950 ml</td>
</tr>
<tr>
<td>lysed horse blood</td>
<td>50 ml</td>
</tr>
<tr>
<td>Campylobacter Growth Supplement</td>
<td>2 vials</td>
</tr>
<tr>
<td>Cefoperazone stock solution</td>
<td>2 ml</td>
</tr>
<tr>
<td>Campylobacter Selective Supplement</td>
<td>2 vials</td>
</tr>
</tbody>
</table>

- Cool the sterile basal medium to 50°C.
- Add 50 ml of lysed horse blood, 2 vials of Campylobacter growth supplement, 2 ml of stock solution of cefoperazone (7.5 mg/ml) and two vials of *Campylobacter* Selective Supplement.
- Mix well and dispense as required.

Store at 4°C for up to 2 weeks, but preferably use on the day of preparation if incubating in air.

### Secondary Selective Enrichment Medium

**Campylobacter mCCDA**

**Basal medium** (available commercially)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat extract</td>
<td>10.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Bacteriological charcoal</td>
<td>4.0</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>3.0</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>1.0</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.25</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>0.25</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH 7.4 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Suspend 22.75 g of dehydrated mCCDA agar base in 500 ml of distilled water, mix well and boil to dissolve the agar. Sterilise by autoclaving at 121°C for 15 min.
mCCDA Selective Supplement (available commercially)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Vial contents (sufficient for 500 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefoperazone</td>
<td>16 mg</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>5 mg</td>
</tr>
</tbody>
</table>

To reconstitute, add 2 ml sterile distilled water and mix to dissolve.

Complete mCCDA medium

Cool autoclaved basal medium to about 50°C. Aseptically add 2 vials of mCCDA Selective Supplement per litre and pour to Petri plates.

Plates can be stored for up to 2 weeks in sealed containers at 4°C (if possible in an anaerobic atmosphere).

Prior to use, air dry plates (do not use a laminar flow cabinet), either by leaving unopened on the bench overnight or, when plates are used on the day of preparation, in an incubator at 42°C.

Maximum Recovery Diluent (MRD)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>8.5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH 7.0 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Dissolve ingredients in distilled water. Dispense as required at autoclave at 121°C for 15 minutes (volumes up to 500 ml) and for 30 min (volumes > 500mL)

If prepared in screw capped containers MRD can be stored in a dark cupboard for up to 12 weeks.

Lander Transport and Storage Medium

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto Veal infusion broth</td>
<td>25</td>
</tr>
<tr>
<td>Lysed horse blood</td>
<td>7% (v/v)</td>
</tr>
<tr>
<td>Bacteriological charcoal</td>
<td>0.5% (w/v)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.04</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>0.01</td>
</tr>
<tr>
<td>Polymixin B</td>
<td>50 000 IU</td>
</tr>
<tr>
<td>Actidione</td>
<td>0.1</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>0.1</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH 7.4 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>
Prepare veal infusion + charcoal bases and autoclave at 121°C for 15 min. Cool and add the remaining ingredients as filter-sterilized solutions. Dispense into screw-capped containers, for example: 5 ml in bijoux or 10 ml in McCartney bottles. Store tubes at room temperature in the dark for up to 90 days.

**Cary-Blair Transport Medium** (formulation for Campylobacter)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissodium hydrogen phosphate</td>
<td>1.1</td>
</tr>
<tr>
<td>Sodium thioglycollate</td>
<td>1.5</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.09</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>10.0</td>
</tr>
<tr>
<td>Agar</td>
<td>1.6</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

pH 8.4 ± 0.2

Suspend dry ingredients in the distilled water and bring gently to the boil to dissolve the agar. Distribute in small screw-capped containers and sterilise by immersing in flowing steam for 15 minutes. Cool and tighten screw caps. Store the medium in the dark in either a refrigerator or at room temperature for up to 19 months.

**Oxidase Reagent**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Para-aminodimethylaniline oxalate</td>
<td>1.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Dissolve the para-aminodimethylaniline oxalate in the distilled water by gently heating. The reagent can be stored in the refrigerator, or dispensed as small aliquots and frozen at -20°C. Commercial preparations are available, usually as test strips or paper discs. These are both stable and convenient.
**Isolation of Thermotolerant Campylobacter**

**by an Enrichment Method**

1. **25g portion**
   - Prepare sample by gentle shaking in 225ml Exeter broth

2. **50g portion**
   - Transfer to a 250ml screwtop bottle ensuring <1cm headspace
   - Incubate aerobically for 4h at 37°C
   - Transfer to 42°C for a further 44h

3. **Inoculate from the enrichment broth to an mCCDA plate**

4. **Incubate for 48h at 42°C in a microaerophilic atmosphere**

**Confirm as thermotolerant Campylobacter**

- Gram stain
- Microscopic examination of wet mount
- Oxidase
Appendix II: Enumeration of Thermotolerant Campylobacter in Water by Most-Probable-Number (MPN)

2.1 Background and Rationale

The genus *Campylobacter* has been established as a common, if not the major cause, of human diarrhoeal disease. Most of the *Campylobacter* spp. associated with human disease are thermotolerant with the principal species involved being *C. jejuni* and *C. coli*. Campylobacters are widespread in the environment, usually as a result of relatively recent contamination with animal or avian faeces, agricultural run-off or sewage effluent.

This method described consists of primary selective enrichment in Exeter broth followed by secondary selective enrichment on a blood-free agar, mCCDA.

Bolton broth may be requested as an alternative primary enrichment medium in some circumstances. An alternative blood-free secondary enrichment medium is commercially available Karmali agar.

Samples for Campylobacter analysis should be collected using aseptic techniques, placed on ice for transport, and analysed as soon as possible after collection.

2.2 MPN Procedure for the enumeration of thermotolerant Campylobacter

**Quality Control**

Quality control of media is essential and a reference strain of *Campylobacter jejuni* is required (e.g. NZRM 1958). If desired *Escherichia coli* (e.g. NZRM 916) can be used as a negative control.

**MPN Format for water or effluent**

Choose an appropriate MPN format according to the water or effluent to be analysed, for example:

*For most natural waters and well-treated effluents:*

3 x 100 ml, 3 x 10 ml, 3 x 1 ml or 1 x 500 ml, 5 x 100 ml, 5 x 10 ml.

*For effluents and highly polluted waters:*

3 x 10 ml, 3 x 1 ml, 3 x 0.1 ml or 1 x 50 ml, 5 x 10 ml, 5 x 1 ml

Very polluted samples may require a tenfold smaller series that is prepared by diluted samples in ten-fold dilution series in a suitable buffer such as sterile 0.1% peptone (pH 6.8 ± 0.2).
Note 1  An MPN Table gives the concentration of bacteria per 100 ml, based on aliquots of 10 ml, 1 ml and 0.1 ml in each row for the 3-tube MPN format or of 50 ml, 10 ml and 1 ml for the 5-tube format. When ten-fold larger aliquots are inoculated, the concentration read from the MPN Table will give the number of Campylobacter per litre.

Note 2  Aliquots of greater than 1 ml are usually concentrated by filtration through one or more 0.45 µm membrane filters. For very turbid samples the use of a pre-filter (e.g. Whatman GFC) followed by a membrane filter may be more satisfactory. For either pre-filters or membrane filters, the use of filter aid (e.g. Kieselgurh) can be helpful. Following filtration, pre-filters (plus filter aid if used) and all the membrane filters used for that sample aliquot are added to a single tube of medium. The medium must completely cover all filters.

MPN format for Solid or Semi-solid Samples

The same MPN format can be used for solid or semi-solid samples such as faeces, soil and sediment as is used for water and effluent. The chosen format and the MPN series will depend on the expected level of contamination in the sample. Usually the maximum sized aliquot of sample tested is 50 g. 50 g aliquots should be weighed and inoculated directly into at least 200 ml Exeter broth. 10 g aliquots should be treated in the same way and are inoculated into volumes of 50 – 100 ml of Exeter broth. Aliquots of 1 g are weighed and inoculated into 10 ml of Exeter broth. For amounts less than 1 g ten-fold dilution series can be prepared in sterile diluent (e.g. 0.1% peptone buffer at pH 6.8 ± 0.2 or sterile distilled water) and 1 ml aliquots inoculated into 10 ml Exeter broth. For aerobic incubation, ensure that only a minimal headspace will remain after addition of sample and close tubes with a screw cap.

When Campylobacter are enumerated in solid or semi-solid solids samples, results can be expressed either on a wet or dry weight basis. If the concentration is to be reported on the basis of dry weight, then it will be necessary to determine the water content of the sample of soil or sediment. Procedures are available in a number of texts (e.g. Standard Methods for the Examination of Water & Wastewater, published by the American Public Health Association).

Procedure

Primary enrichment in Exeter broth is done in two stages. Stage one is a pre-enrichment period that is designed to assist recovery of stressed and injured Campylobacter cells. Stage two is selective enrichment at 42°C. Primary enrichment cultures in Exeter broth can be incubated aerobically in screw-capped containers. These containers should be long and narrow in shape and there should only be a very small head space (< 1cm) after addition of sample. Alternatively broth enrichments can be incubated in containers with loose caps that are placed in a microaerophilic
environment, either in an anaerobic gas jar with activated Campylobacter gas generation pack or in an incubator with a flowing microaerophilic gas atmosphere. Incubate without shaking.

**Primary Selective Enrichment**

- Add aliquots of sample or diluted sample, or filters, to Exeter broth ensuring there is a minimal headspace in tubes that are to be incubated aerobically.
- As a general rule, samples are added to x9 or x10 (v/v) of enrichment broth.
- To add filters to broth, roll each filter carefully with sterile forceps, taking care not to lose any suspended material retained on the filter surface. Filters must be completely immersed in the broth.

**Note:** All tubes must be carefully labelled to ensure their identity in the MPN series.

**Pre-enrichment**

An initial pre-enrichment step is always included. The minimum pre-enrichment period is incubation of tubes at 37°C for 4 h.

**Enrichment**

After the pre-enrichment step, transfer tubes to 42°C and incubate for a further 44 h.

**Note:** For samples in which campylobacters are likely to be stressed and the background contamination low, for example treated drinking water or high quality natural waters, the initial pre-enrichment step can be extended to 24 h at 37°C, or if preferred, the entire enrichment step can be carried out at 37°C (i.e.48 h) (Humphrey & Muscat, 1989). If the pre-enrichment period is extended the subsequent enrichment period can be reduced so that the total period will be 48 h.

**Secondary Selective Enrichment**

**Selective plating**

- Subculture from each primary selective enrichment tube by streaking a loopful of growth, or a 0.1 µl aliquot, from an Exeter broth tube to a plate of mCCDA* that is labelled to maintain its identify in the MPN series.
- Invert plates and place in a microaerophilic atmosphere.
- Incubate plates at 42°C for 48 h.
- Prior to plating, agar plates should be air dried to remove surface moisture but do not over dry and do not dry in a laminar flow cabinet.
Microaerophilic atmosphere: Either use an anaerobic jar and commercial kits that will generate a microaerophilic atmosphere consisting of 5-6% oxygen, 10% carbon dioxide and 84-85% nitrogen or use an incubator with a flowing stream of the same gases in the same proportions.

Confirmation of thermotolerant Campylobacter

Typically thermotolerant Campylobacter colonies appear as flat, moist, grey-white colonies with irregular spreading margins on mCCDA.

To confirm the presence of Campylobacter, select one or more typical colonies from each mCCDA plate for further examination. The (minimum) required tests are:

- microscopic examination that consists of examination of a wet mount
- traditional Gram stain some workers prefer to counterstain with safranin rather than rather than carbol fuschin.
- oxidase test.

Oxidase test procedure

- Place 2 or 3 drops of reagent to the centre of a filter paper (e.g. Whatman No 1) and allow a few seconds for absorption.
- Using a platinum or plastic loop smear a loopful of test colony onto the filter paper over a line 3 – 6 mm long. Examine the smear immediately.
- A positive test is the production of a dark purple colour within 5 – 10 seconds.

Include the reference culture (C. jejuni NZRM 1958) as a positive control when carrying out the oxidase test.

Score the sample positive or negative according to the results of the reactions.

<table>
<thead>
<tr>
<th>Positive Thermotolerant Campylobacter reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
</tr>
<tr>
<td>Motility (wet mount)</td>
</tr>
<tr>
<td>Oxidase test</td>
</tr>
</tbody>
</table>

2.3 Reporting results

Count the positive plates in each MPN row and add them up for each row. Select the combination of scores that is neither all positive nor all negative for the three rows and refer the resulting MPN score to an appropriate MPN Table to obtain the concentration of thermotolerant Campylobacter. Express the concentration as either the number of Campylobacter per 100 ml or per litre as required.
2.4 Media

Primary selective enrichment medium

Exeter broth

**Exeter broth base**

**Basal medium**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat extract</td>
<td>10.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>950 ml</td>
</tr>
<tr>
<td>pH 7.5 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Dissolve the ingredients in the distilled water. Sterilise at 121°C for 15 minutes.

Cool to 50°C before addition of blood and supplements.

* Dehydrated broth base is available commercially.

**Additives**

*i. Growth supplement (available commercially)*

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Vial contents (sufficient for 500 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium pyruvate</td>
<td>0.125 g</td>
</tr>
<tr>
<td>Sodium metabisulphite</td>
<td>0.125 g</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.125 g</td>
</tr>
</tbody>
</table>

To reconstitute, add 2 ml of sterile distilled water and mix gently to dissolve. Use immediately.

*ii. Lysed/defibrinated horse blood (5% v/v)*

**Lysed/defibrinated horse blood (sufficient for 500 ml)**

Lysed/defibrinated (laked) horse blood 25 ml

Lysed horse blood should be used within the date specified by the supplier.

*iii. Antibiotics*

Two antibiotic solutions are added to each inoculated MPN tube after 4-6 h incubation at 37°C. These are Campylobacter Selective Supplement (Preston) and Cefoperazone solution.

(a) Campylobacter Selective Supplement (available commercially)
### Ingredients

<table>
<thead>
<tr>
<th>Vial contents (sufficient for 500 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymyxin B</td>
</tr>
<tr>
<td>Rifampicin</td>
</tr>
<tr>
<td>Trimethoprim</td>
</tr>
<tr>
<td>Amphotericin B</td>
</tr>
</tbody>
</table>

To reconstitute, add 2 ml of sterile distilled water to each vial of Selective Supplement (as supplied by the manufacturer) and mix gently to dissolve: Use immediately.

(b) Cefoperazone solution

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>mg/2ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefoperazone</td>
<td>15 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

Stir to dissolve (If desired larger amounts can be prepared and 2 ml aliquots stored in the freezer until required). Add to 1 litre of Exeter medium.

**Complete Exeter broth**

Dissolve the basal ingredients in 950 ml of water and sterilise by autoclaving at 121°C for 15 min. Cool to 50°C or below and add 2 vials of growth supplement and 50 ml of lysed horse blood. Add 2 vials of antibiotic supplement and 2 ml of cefoperazone solution. Aseptically dispense the required aliquots to containers of a size to ensure a small head space after addition of sample. Medium can be stored for up to 2 weeks at 4°C. However, for optimum results, particularly when incubating tubes in air, use on the day of preparation.

**Secondary Enrichment Medium**

**Campylobacter mCCDA**

**Basal medium** (available commercially)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat extract</td>
<td>10.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Bacteriological charcoal</td>
<td>4.0</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>3.0</td>
</tr>
<tr>
<td>Sodium desoxycholate</td>
<td>1.0</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.25</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>0.25</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

pH 7.4 ± 0.2

Suspend 22.75 g of dehydrated mCCDA agar base in 500 ml of distilled water, mix well and boil to dissolve the agar. Sterilise by autoclaving at 121°C for 15 min.
**mCCDA Selective Supplement** (available commercially)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Vial contents (sufficient for 500 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefoperazone</td>
<td>16 mg</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>5 mg</td>
</tr>
</tbody>
</table>

To reconstitute, add 2 mL of sterile distilled water and mix to dissolve.

**Complete mCCDA medium**

Cool autoclaved basal mCCDA medium to about 50°C. Add 2 vials of antibiotic supplement to a litre of mCCDA. Pour to Petri plates. Plates can be stored for 2 weeks in sealed containers at 4°C (in an anaerobic atmosphere if available).

**Oxidase Reagent**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Para-aminodimethyanaline oxalate</td>
<td>1.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Dissolve the para-aminodimethyanaline oxalate in the distilled water by gently heating. The reagent can be stored in the refrigerator, or dispensed as small aliquots and frozen at -20°C.

Commercial preparations are available, usually as test strips or paper discs. These are both stable and convenient.
**Most Probable Number Tables**

The multi-tube Most Probable Number (MPN) method gives a statistical number of bacteria in a sample. MPN tests can be carried out in a number of different formats including multiple tubes in a single dilution (e.g. 10 tubes inoculated with 1 ml of undiluted sample) or a series of tubes in each of three sequential dilutions (e.g. 5 tubes in three rows, each row inoculated with 1 ml of a sequential 10-fold dilution series). An MPN index is obtained from the number of tubes scored positive for the target bacteria according to the chosen format and the index referred to appropriate MPN tables. MPN tables are published by a number of authorities including WHO (1984) – see Tables 2 & 3. Recently MPN tables that increase the accuracy of estimation, i.e. Xact MPN tables were published by McBride (2003) and the use of these is **highly recommended**. McBride (2003) also includes MPN scores for formats that test an entire litre of water, allowing a negative result to be reported as zero Campylobacter per litre.

**Table 2.** MPN and 95% confidence limits for various combinations of positive results when three 10 ml portion, three 1 ml portions and three 0.1 ml portions are used

<table>
<thead>
<tr>
<th>Number of tubes giving a positive reaction</th>
<th>MPN per 100 ml</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 of 10 ml each</td>
<td>3 of 1 ml each</td>
<td>3 of 0.1 ml each</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
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<td>1</td>
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<td>1</td>
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<td>3</td>
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<tr>
<td>3</td>
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<tr>
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<tr>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 3. MPN and 95% confidence limits for various combinations of positive and negative results when one 50 ml portion, five 10 ml portions and five 1 ml portion is used

<table>
<thead>
<tr>
<th>Number of tubes giving a positive reaction out of</th>
<th>MPN per 100 ml</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 of 50 ml</td>
<td>5 of 10 ml</td>
<td>5 of 1 ml</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
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<td>0</td>
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<td>5</td>
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</tbody>
</table>
References
Humphrey, T.J. & Muscat, I. 1989. Incubation temperature and the isolation of
Campylobacter jejuni from food, milk or water. Letters in Applied Microbiology, 9,
137-139.
McBride, G. 2003. Preparing exact most probable number (MPN) tables using
occupancy theory, and accompanying measures of uncertainty. NIWA Technical
Report 121, pp 62.
PHLS 2002. Detection of Campylobacter species. PHLS Standard Operating
**Enumeration of Thermotolerant Campylobacter in Water by MPN**

1. Add filter(s) or 1 ml aliquots of sample to Exeter broth. Incubate for 4h at 37°C then transfer to 42°C for a further 44h ensuring a <1cm headspace.

2. Plate a loopful of growth from each tube to a parallel mCCDA plate.

3. Incubate for 48h at 42°C in a microaerophilic atmosphere.

4. Confirm thermotolerant Campylobacter on each plate.

   - Gram stain
   - Microscopic examination of wet mount
   - Oxidase

5. Calculate the MPN score and refer to MPN tables to obtain the number of *Campylobacter* spp. per litre of water.
Appendix III: Isolation of Thermotolerant Campylobacter in Clinical Samples by Direct Plating

1.1 Background and Rationale

The genus *Campylobacter* is recognised as an increasing cause of human foodborne enteritis worldwide. Two species of this genus, *C. jejuni* and *C. coli*, are isolated from most cases of Campylobacter infection, with the proportion of each species differing from country to country. Faecal samples collected from symptomatic patients contain large numbers of campylobacters that can be readily recovered by a single stage isolation technique.

This method described consists of direct plating of sample (or diluted sample) onto the Campylobacter selective enrichment medium mCCDA. Alternative secondary enrichment media include commercially available Karmali agar, also a blood-free medium.

Faecal samples for Campylobacter analysis should be and analysed as soon as possible after collection, preferably on the same day.

1.2 Procedure

Use a reference strain of *C. jejuni* (e.g. NZRM 1958) for quality control. If a negative quality control strain is required a strain of *E. coli* (e.g. NZRM 916) is satisfactory.

**Sample preparation**

An aliquot of faeces can be spread directly to an agar plate but it is usually emulsified in a buffer solution (e.g. maximum recovery diluent or 0.1% peptone water). For presence/absence testing it is not necessary to weigh faeces or accurately measure volumes but faeces is generally added to approximately x10 (w/v) of buffer solution. If single colonies, or a count of campylobacter, are required the mixture is further diluted as a ten-fold dilutions series and a range of dilutions plated out.

If the concentration is required then an aliquot of faeces should be weighed on a balance and added to a measured volume of buffer. Serial dilutions should be accurately done using a pipette. Aliquots to be plated are also pipetted (usually 0.1 ml is added to an agar plate).
Plating to detect Campylobacter

Choice of laboratory protocol

For most laboratories direct plating is done by streaking a test sample to mCCDA with incubation in a microaerophilic atmosphere at 41.5 – 42°C for up to 48 h.

However, some laboratories may wish to isolate a wide range of campylobacters and achieve this by either incubating mCCDA agar plates at the lower temperature of 37°C or by the use of a non-selective agar such as blood agar with incubation at either 37°C or 42°C. In either of these cases a further plating step is mandatory if results are to be reported as thermotolerant Campylobacter. This additional step consists of sub-culture of isolates obtained following incubation at 37°C to a second agar plate and incubating this at 41.5 – 42°C for 24 to 48 h.

Presence/Absence plating

To detect the presence of Campylobacter in the test sample either spread an aliquot of faeces/diluted faeces over the surface of an air dried mCCDA plate or streak a loopful so as to obtain single colonies. Inoculated plates are then incubated without delay in a microaerophilic atmosphere at 41.5-46°C for up to 48 h.

Enumeration by direct plating

To obtain the concentration of Campylobacter in the test sample mCCDA plates must be dried to facilitate the growth of single colonies. Although it is necessary to remove surface moisture it is important not to reduce productivity by over-drying. Do not dry plates in a laminar flow cabinet, either leave plates (lids on) overnight at room temperature for dry for a few hours at 42°C.

- Pipette 0.1 µl of diluted faecal suspension over the surface of an air dried mCCDA plate. Leave upright until the inoculum has been absorbed
- Repeat for at least 2 serial dilutions
- Incubate plates at 41.5 - 42°C for 48 hours in a microaerophilic atmosphere
- After incubation examine plates for typical Campylobacter colonies.

Microaerophilic incubation atmosphere: Either use an anaerobic jar and commercial kits that will generate a microaerophilic atmosphere consisting of 5-6% oxygen, 10% carbon dioxide and 84-85% nitrogen or use an incubator with a flowing stream of the same gases in the same proportions. If enrichments are incubated in a flowing gas stream the microaerophilic atmosphere must be maintained during all stages of incubation.
1.3 Confirmation of thermotolerant Campylobacter

Typically thermotolerant Campylobacter colonies appear as flat, moist, grey-white colonies with irregular spreading margins on mCCDA.

To confirm the presence of Campylobacter, select one or more typical colonies from each mCCDA plate for further examination. The (minimum) required tests are:

- **microscopic examination** that consists of examination of a wet mount
- **traditional Gram stain**, some workers prefer to counterstain with saffranin rather than carbol fuschin.
- **oxidase test**.

**Oxidase test procedure**

- Place 2 or 3 drops of reagent to the centre of a filter paper (e.g., Whatman No 1) and allow a few seconds for absorption.
- Using a platinum or plastic loop smear a loopful of test colony onto the filter paper over a line 3 – 6 mm long. Examine the smear immediately.
- A positive test is the production of a dark purple colour within 5 – 10 seconds.

Include the reference culture (*C. jejuni* NZRM 1958) as a positive control when carrying out the oxidase test.

**Score the sample positive or negative according to the results of the reactions.**

**Positive Thermotolerant Campylobacter reactions**

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td>Gram-negative, small &quot;gull&quot; shaped rods</td>
</tr>
<tr>
<td>Motility (wet mount)</td>
<td>+ (typically “corkscrew”)</td>
</tr>
<tr>
<td>Oxidase test</td>
<td>+</td>
</tr>
</tbody>
</table>

1.4 Reporting results

Express the result as presence or absence of thermotolerant Campylobacter in the portion of sample that was tested (i.e. per test unit, unit weight, unit area or unit volume).
1.5 Media

Selective Enrichment Medium

Campylobacter mCCDA

*Basal medium* (available commercially)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab-Lemco powder</td>
<td>10.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Bacteriological charcoal</td>
<td>4.0</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>3.0</td>
</tr>
<tr>
<td>Sodium desoxycholate</td>
<td>1.0</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.25</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>0.25</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

pH 7.4 ± 0.2

Suspend 22.75 g of dehydrated mCCDA agar base in 500 ml of distilled water, mix well and boil to dissolve the agar. Sterilise by autoclaving at 121°C for 15 min.

*mCCDA Selective Supplement* (available commercially)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Vial contents (sufficient for 500 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefoperazone</td>
<td>16 mg</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>5 mg</td>
</tr>
</tbody>
</table>

To reconstitute, add 2 ml sterile distilled water and mix to dissolve.

*Complete mCCDA medium*

Cool autoclaved basal medium to about 50°C. Aseptically add 2 vials of mCCDA Selective Supplement per litre and pour to Petri plates.

Plates can be stored for up to 2 weeks in sealed containers at 4°C (if possible in an anaerobic atmosphere).

Prior to use, air dry plates (do not use a laminar flow cabinet), either by leaving unopened on the bench overnight or, when plates are used on the day of preparation, in an incubator at 42°C.
Maximum Recovery Diluent (MRD)

**Ingredients** | **g/l**
---|---
Peptone | 1
Sodium chloride (NaCl) | 8.5
Distilled water | 1000 ml

pH 7.0 ± 0.2

Dissolve ingredients in distilled water. Dispense as required at autoclave at 121°C for 15 minutes (volumes up to 500 ml) and for 30 min (volumes > 500mL)

If prepared in screw capped containers MRD can be stored in a dark cupboard for up to 12 weeks.

Oxidase Reagent

**Ingredients** | **g/100 ml**
---|---
Para-aminodimethyanaline oxalate | 1.0
Distilled water | 100 ml

Dissolve the para-aminodimethyanaline oxalate in the distilled water by gently heating. The reagent can be stored in the refrigerator, or dispensed as small aliquots and frozen at -20°C.

Commercial preparations are available, usually as test strips or paper discs. These are both stable and convenient.
**Isolation of Thermotolerant Campylobacter**

1. **Faecal sample from symptomatic patient** mixed 1:10 (w/v) in buffer

2. **Spread to MCCDA**

3. **Spread to MCCDA or blood agar**

4. **Incubate for 48h at 42°C in a microaerophilic atmosphere**

5. **Incubate for 48h at 37°C in a microaerophilic atmosphere**

6. **Subculture to MCCDA or blood agar**

7. **Incubate for 24h at 42°C in a microaerophilic atmosphere**

8. **Microscopic examination**

9. **Oxidase**

10. **Confirm as thermotolerant Campylobacter**
Appendix IV: Species Identification of Thermotolerant Campylobacter Isolates

4.1 Introduction

It is desirable that thermotolerant Campylobacter isolated from samples be obtained in pure culture and identified to species level as a routine step in surveillance monitoring. Once obtained in pure culture, isolates should be stored in a way that will ensure that they are available for typing. Although there is no standardised set of tests for identifying Campylobacter, methods for species identification have been published by a number of workers and authorities e.g. the International Standards Organization (ISO, 1995). A simplified set of tests is given in this Appendix based on publications by Bolton et al. (1992), On & Holmes (1992), MiMM (2001) and Post (1995).

*Campylobacter jejuni* NZRM 1958 and *C. coli* NZRM 2607 are suitable strains for quality control in identification tests. Additional species, such as *C. lari* NZRM 2622, may be used if required.

When carrying out differential tests the inclusion of a positive control culture is advisable and that of a negative control culture desirable.

**Note:** If cultures have been stored prior to species identification the basic tests should be done to confirm their identity as Campylobacter and to check culture purity: The basic tests are a Gram stain and wet mount to check morphology and motility and an oxidase test (see Appendix I, Section 1.3).

**Positive Campylobacter reactions**

<table>
<thead>
<tr>
<th>Test</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td>Gram-negative, small “gull” shaped rods</td>
</tr>
<tr>
<td>Motility (wet mount)</td>
<td>+ (typically “corkscrew”)</td>
</tr>
<tr>
<td>Oxidase test</td>
<td>+</td>
</tr>
</tbody>
</table>

4.2 Differential tests

The tests in this section include temperature tolerance, hippurate hydrolysis, indoxyl acetate hydrolysis and antibiotic sensitivity (nalidixic acid and cephalothin).

To prepare an isolate for species identification, inoculate an agar plate containing a suitable medium for Campylobacter, preferably a non-selective blood agar. Incubate
plates at 37°C for 18-24 hours in a microaerophilic atmosphere. If necessary, continue re-streaking and incubating until an isolate has been obtained in pure culture.

**Temperature Tolerance**

Inoculate a loopful of growth to each of three blood agar plates and incubate one plate at each of the following temperatures: 25°C, 37°C and 42 - 43°C for up to 3 days in a microaerophilic atmosphere. Examine plates for typical Campylobacter colonies.

**Growth in air**

Inoculate a loopful of standard suspension onto a blood agar plate and incubate in air at 37°C for 48 h. Examine plates the presence of typical Campylobacter colonies.

*Note*: Some strains of *C. jejuni* show slight growth in air.

**Hippurate Hydrolysis Test**

- Suspend sufficient bacterial cells in 0.8 ml sterile distilled water in a small tube to form a milky suspension. Add 0.2 ml of a 5% (5 g/100 ml) sodium hippurate aqueous solution and incubate at 37°C for 4 h.
- Add 0.4 ml of ninhydrin solution (3.5 g in 100 ml of a 1:1 (v:v) acetone and butanol mixture). DO NOT SHAKE TUBES AFTER NINHYDRIN ADDITION. Incubate for up to 20 min. at room temperature.
- Hydrolysis of hippurate is indicated by a colour change to dark purple due to the release of glycine. A purple colour is a positive result. No colour or a faint trace of purple is a negative result.

*Note*: Hippurate Test Kits are available commercially.

**Indoxyl Acetate Test**

- Saturate discs of Whatman AA filter paper with a 10% solution of indoxyl acetate in acetone and allow to air dry.
- Apply a loopful of growth (from a 24 h culture on blood agar) to the disc.
- Add sterile distilled water – just sufficient to moisten the disc.
- Hydrolysis of indoxyl acetate is indicated by the development of a blue-grey colour within 10 minutes at room temperature.

**Nitrate Reduction Test**

- Place a 6 x 1 cm filter-paper strip soaked in 40% (w/v) potassium nitrate solution down the centre of a blood agar plate (standard size Petri dish).
• Inoculate a portion of growth from a 24 h culture over a 1 cm diameter, 2 cm from the paper strip.

• Incubate the plate in a microaerophilic atmosphere at 37°C and inspect plates daily for up to 5 days.

• A greening of the medium surrounding the inoculum indicates a positive result.

*Note*: no more than 4 strains (including control strains) can be inoculated to one plate.

**Antibiotic Susceptibility Tests (Nalidixic acid and Cephalothin)**

• Make a slightly turbid suspension by emulsifying colonial growth in 5 ml 0.1% (w/v) sterile peptone water.

• Charge a swab with the peptone water suspension and disperse over the surface of a blood agar plate (to produce a lawn of confluent bacteria on the surface of the agar after incubation).

• Using sterile tweezers, aseptically place one nalidixic acid (30 µg) disc and one cephalothin (30 µg) disc on the surface, one on each side of the agar plate. Ensure that the discs are widely spaced. Tweezers should be re-flamed after each disc.

• Incubate plates in a microaerophilic atmosphere at 37°C for 24 - 48 h and up to 3 days if necessary.

• Examine for zones of inhibited bacterial growth around the antibiotic discs. A zone of inhibition indicates the sensitivity (S) of the isolate to the antibiotic whereas growth right up to the disc indicates resistance (R).

*Note 1*: Two isolates can be tested on one plate of blood agar to reduce costs. Divide a blood agar plate in half by removing a strip of agar across the plate with a sterile scalpel (or use divided Petri dishes) and inoculate each isolate to the surface of a “half” plate.

*Note 2*: An alternative to using swabs is to prepare a slightly turbid suspension in 1-2 ml of sterile peptone water. Pipette 100 µl onto one side of the plate and disperse over the entire surface with a sterile spreading rod. This method can be used for divided plates if the inoculum size is decreased to 50 µl.

**H₂S in Triple sugar iron (TSI) agar**

• Inoculate slants of TSI from the bottom to the top in a zig-zag pattern then stab the butt.

• Cap the slants loosely and incubate at 37°C in a microaerophilic atmosphere for up to 5 days.
• A positive result for H₂S is indicated by blackening of the medium around the inoculum.

• A positive result for glucose fermentation is a yellow colour in the butt with or without bubbles or cracks in the agar.

• A positive result for lactose and/or sucrose is a yellow colour on the slope surface.

**Catalase Test**

• Place a drop of 3% (v/v) hydrogen peroxide on a glass slide

• Using a platinum or plastic loop add a loopful of growth from the TSI slant

• A positive test is the formation of bubbles within 30 seconds.

**Species Identification reactions of some Thermotolerant Campylobacter**

<table>
<thead>
<tr>
<th></th>
<th><em>C. jejuni</em></th>
<th><em>C. coli</em></th>
<th><em>C. lari</em></th>
<th><em>C. upsaliensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippurate hydrolysis</td>
<td>+/v</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
| Antibiotic sensitivity
  Nalidixic acid (30 µg) | S/v        | S         | R         | S                |
  Cephalothin (30µg)     | R          | R         | R         | S                |
| Catalase              | +          | +         | +         | - (or weak)      |
| Nitrate reduction     | +          | +         | +         | +                |
| Indoxyl acetate hydrolysis | +       | +         | -         | +                |
| Growth in air at 37°C | -/sl       | -         | -         | -                |
| 25°C - microaerophilic| -          | -         | -         | -                |
| 37°C – microaerophilic| +          | +         | +         | +                |
| 42 - 43°C - microaerophilic | +    | +         | -         | +/-              |

* C. *jejuni* subsp *jejuni*

v = variable  sl = slight

**Note:** Some strains of Campylobacter are aerotolerant and may exhibit slight growth under aerobic conditions.

**Blood Agar Medium** (non-selective)

**Basal medium**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat extract</td>
<td>10.0</td>
</tr>
<tr>
<td>Peptones</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Agar 15.0
Distilled water 1000 ml
pH 7.3 ± 0.2

Suspend dehydrated ingredients in the distilled water and heat to boiling to completely dissolve agar. Dispense as required and sterilize by autoclaving at 121°C for 15 minutes. Cool to about 47°C and aseptically add 5% (v/v) lysed horse blood, mix by swirling and pour to Petri plates.

Note: dehydrated medium is commercially available.

Additive

**Horse blood (5% v/v)**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>ml/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysed horse blood</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

*Note: some workers prefer to use whole blood.*

**Complete Blood Agar**

Cool sterile basal agar to about 47°C. To 950 ml of basal agar add 50 ml of blood and dispense to Petri dishes. Plates can be stored for up to 7 days at 4°C although use on the day of preparation is recommended.

Immediately before use dry plates to remove surface moisture. Do not over dry and do not use a laminar flow cabinet.

**Triple Sugar Iron Agar**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Peptones</td>
<td>20.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Lactose</td>
<td>10.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>0.3</td>
</tr>
<tr>
<td>Sodium thiosulphate</td>
<td>0.3</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.024</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH 7.4 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Add the ingredients to the distilled water, boil to dissolve the agar, mix thoroughly and dispense in 10 ml portions into capped test-tubes. Sterilise by autoclaving at 121°C for
15 minutes. Allow the medium to cool and set in sloped form with a butt of about 2.5 cm. This medium should be used on the day of preparation.
References


Appendix V: Storage of Thermotolerant Campylobacter Isolates

Prior to storage isolates should be obtained in pure culture. All isolates prepared for storage should be sourced from freshly grown cultures (e.g. on blood agar).

Methods are given for short term storage (3 months or less) and long term storage at -70°C to –80°C.

5.1 Short term storage

Cultures can be stored at room temperature for 1-2 months in semi-solid medium or for up to 3 months at -20°C in skim milk medium.

I. Semi-solid medium

**Basal medium**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat extract</td>
<td>10.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Agar</td>
<td>1.8</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

pH 7.5 ± 0.2

Dissolve the ingredients in the distilled water and dispense 10 ml to 16 x125 mm screw-capped tubes. Sterilize at 121°C for 15 minutes.

* Dehydrated broth is available commercially.

**Additives**

**Campylobacter Growth Supplement** (available commercially)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Vial contents (sufficient for 500 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium pyruvate</td>
<td>0.125 g</td>
</tr>
<tr>
<td>Sodium metabisulphite</td>
<td>0.125 g</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.125 g</td>
</tr>
</tbody>
</table>

To reconstitute, add 2 ml of sterile distilled water and mix to dissolve. Use immediately

**Note:** 2 vials are required for 1 litre of medium
Complete Semi-solid medium

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g or ml/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal medium (sterile)</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Campylobacter Growth Supplement</td>
<td>2 vials</td>
</tr>
</tbody>
</table>

- Cool the sterile basal medium to 50°C.
- Add 2 vials of Campylobacter growth supplement.
- Mix well and dispense as required.

*Note:* Preferably use on the day of preparation

**Procedure for storage in semi-solid medium at room temperature**

(i). Inoculate semi-solid medium at the surface with a loopful of growth. Ensure that caps are loose and incubate tubes in a microaerophilic atmosphere at 37°C for 24-48 h.

(ii). Immediately after incubation tighten caps and store cultures in the dark at room temperature.

*Note 1:* Cultures will remain viable for one to two months.

*Note 2:* Only sub-culture once from each tube.

II. Skim milk medium with glycerol

**Basal medium**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk powder</td>
<td>100</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH 6.8 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Dissolve skim milk in water and strain through muslin if necessary. Dispense 2 - 3 ml aliquots to capped tubes (e.g. bijoux) and sterilise by autoclaving at 121°C for 5 minutes. **The medium should not be subjected to extended heating.**

Cool to room temperature and aseptically add 20% sterile glycerol (v/v). Either use immediately or loosen the caps and place tubes in an anaerobic atmosphere for use within 1 week.
Procedure for storage in skim milk medium at -20°C
Add a generous loopful of growth to a tube of skim milk medium with 20% glycerol. Cap tube and place in a freezer at -20°C for up to 3 months.

5.2 Long term storage of thermotolerant Campylobacter

Campylobacter can be stored for extended periods in an ultrafreezer at -70°C to -80°C. Storage can either be on Amies charcoal sticks or in non-selective broth supplemented with glycerol.

Amies charcoal sticks: inoculate sticks with a generous amount of growth and place in an ultrafreezer.

These sticks are recommended for transporting isolates to another laboratory. Fresh sticks (not frozen sticks) should be used for transport.

Glycerol broth: inoculate small tubes of any peptone based non-selective broth medium with a generous amount of fresh growth.

Suitable broths include: brain heart infusion broth, tryptic soy broth, Mueller-Hinton broth or Brucella broth. Broths are prepared according to the manufacturer’s specifications, tubed and autoclaved. After cooling, 15%-20% sterile glycerol is added and mixed thoroughly but without excessive aeration. Either use immediately or loosen the caps on the tubes and place in an anaerobic atmosphere for use within 1 week.

For laboratories that have the facilities, freeze drying and/or storage in liquid nitrogen are satisfactory techniques for long term storage.

Note: when transporting isolates of Campylobacter approved Biohazard containers and approved transport companies must be used and IATA Dangerous Goods Regulations followed.

5.3 Reference