

**ZOONOTIC MICRO-ORGANISMS:  
RESERVOIRS AND POTENTIAL  
TRANSMISSION ROUTES**

**Objectives 2 & 3**

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contract for scientific services

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## **OBJECTIVE 2**

ASSESSMENT OF THE VIABILITY ASSAY (INCLUDING  
ASSESSMENT OF ITS APPLICABILITY IN THE NATURAL  
ENVIRONMENT)

## **OBJECTIVE 3**

INVESTIGATION OF THE POTENTIAL FOR SEDIMENTS TO  
ACT AS RESERVOIRS FOR *CAMPYLOBACTER*

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## SUMMARY

### Objective 2

Current methods for the detection of *C. jejuni* are of long duration (four days to get a result), labour intensive, expensive and do not detect viable but non-culturable cells. The objective of this research was to investigate a viability marker for *Campylobacter jejuni* that would enable the rapid detection of viable cells only.

Conventional PCR detection targets DNA, and the initial intention of this research was to target mRNA which is a less stable molecule than DNA. However our earlier work showed that mRNA can persist in cells for extended periods after cell death. As a consequence we have developed an assay based on the *induction* of mRNA, with the logic being that only viable cells will be able to synthesise new mRNA.

We report here further refinements of the methodology to make it more robust and reproducible. We have also evaluated the use of centrifugation to concentrate cells prior to viability assay. Key findings of the study are:

- Using a laboratory strain of *C. jejuni*, we are able to detect as few as 1.5 viable cells per RT-PCR or 15 cells in the extraction volume.
- Spiked river water samples were processed using the viability assay, and detection and enumeration of viable *C. jejuni* achieved. In spiked river water samples 3.5 viable *C. jejuni* per RT-PCR could be detected.

### Objective 3

Methodology developed at ESR for detection of *Campylobacter* was applied to the enumeration of *C. jejuni* and *C. coli* from sediment samples from the Ashburton River while conventional methods were used to allow isolations to be made. Findings are:

- *C. jejuni* was isolated from four of six sediment samples tested, while *C. coli* was isolated from one sample.
- In general *C. jejuni* isolates in sediment and the overlying water were not of the same type.

This is consistent with a hypothesised system where sediment absorbs *Campylobacter* cells and therefore provides a cumulative record of types deposited from the water above.

## RECOMMENDATIONS

### Objective 2

The developed assay appears technically feasible, and we make the following recommendations to enable its routine use in the enumeration of viable *C. jejuni*:

- Establish meanings of cell death, using means recognised for eukaryotes and transfer to prokaryotes where appropriate.
- Evaluate the assay's effectiveness with cells killed by U.V., chlorine, heat and starvation.
- Evaluate the assay with a wider range of environmental *Campylobacter* isolates and other organisms.
- Investigate membrane filtration and elution for the concentration of cells from large volumes of water.
- Evaluate the assay comparatively with double enrichment MPN-PCR in a range of environmental samples (alongside planned 2002-2003 research).

### Objective 3

In order to understand the relationship between *Campylobacter* strains from sediment and water it will be necessary to do more extensive sampling of sediment, interstitial water and the water column for a range of weather conditions, flow conditions and farming practices. Particular recommendations are:

- Investigate the relationship between *Campylobacter* from sediment and water samples at a number of sites in a river to include various river flows, rain events and farming practices.
- Establish the number of *Campylobacter* cells in sediment, interstitial water and the water column at a range of sites and over time to allow evaluation of the relative loading of these environmental sample types.
- Determine the relationship between *Campylobacter* cells associated with suspended sediment particles, sediment and the water column during high rainfall events.

## **OBJECTIVE 2: DEVELOPMENT OF A VIABILITY ASSAY FOR *CAMPYLOBACTER JEJUNI***

### **1.1 Introduction**

Traditional microbiological isolation and identification methods for *Campylobacter* can often take several days to complete. For example the current gold standard used in this transmission routes study (CTR) takes four days to obtain a result. As described in Appendix 1, a filtered sample is enriched for 48 hours, a portion transferred to new broth and enriched for a further 24 hours. The broth is then centrifuged, washed and polymerase chain reaction (PCR) performed. This assay detects only living, culturable *Campylobacter* cells, and has a detection limit as low as one cell/100mL. When performed in an MPN format (not used in the CTR study) it is enumerative. The MPN format is however expensive, requiring the analysis of ideally three different volumes for each sample, preferably each in triplicate.

There is therefore a need for a more rapid specific method for detecting viable enteropathogenic campylobacters in environmental samples to further understanding of the epidemiology of infection, and more simply for the identification of living cells. In addition, Jones *et al.* (1991) demonstrated that environmentally stressed campylobacters might persist in the environment in a 'viable but non-culturable' (VBNC or VNC) form that can be recovered *in vivo*. These VNC cells would not be detected using the current double enrichment PCR assay.

The inherent specificity, high sensitivity, and rapidity of PCR for *in vitro* enzymatic DNA amplification has been applied previously as an alternative to conventional microbiological culture methods to detect specific types of micro-organisms in environmental and clinical samples. There have been several reports of using PCR methods for the detection of *C. jejuni* in water (Bej *et al.*, 1990; Hernandez *et al.*, 1995), chicken (Giesendorf *et al.*, 1992; Itoh *et al.*, 1995; Winters and Slavik, 1995; Winters *et al.*, 1997; O'Sullivan *et al.*, 2000) and dairy or other food products (Wegmuller *et al.*, 1993; Allmann *et al.*, 1995; Jackson *et al.*, 1996; Winters *et al.*, 1998; Thunberg *et al.*, 2000). However, these

conventional PCR methods detect chromosomal gene sequences that can be present in non-viable cells and therefore cannot determine viability (Josephson *et al.*, 1993).

Messenger RNA (mRNA) is a more labile molecule than DNA or rRNA. In living cells it is rapidly degraded with a typical half-life of two to three minutes, therefore dead cells should contain no mRNA. Detection of viable cells using mRNA as the target for reverse transcriptase PCR (RT-PCR) has been demonstrated in *Legionella pneumophila* (Bej *et al.*, 1991), *Mycobacterium leprae* (Patel *et al.*, 1993), *Vibrio cholerae* (Bej *et al.*, 1996), *Cryptosporidium parvum* (Stinear *et al.*, 1996; Kaucner and Stinear, 1998; Widmer *et al.*, 1999; Jenkins *et al.*, 2000; Baeumner *et al.*, 2001), *Listeria monocytogenes* (Herman, 1997; Klein and Juneja, 1997), *Mycobacterium tuberculosis* (Jou *et al.*, 1997; Hellyer *et al.*, 1999); (Pai *et al.*, 2000), thermophilic *Campylobacter* spp. (Sails *et al.*, 1998), *Giardia intestinalis* (Kaucner and Stinear, 1998), *Escherichia coli* (Sheridan *et al.*, 1998; Sheridan *et al.*, 1999; Birch *et al.*, 2001), *Salmonella enteritidis* (Szabo and Mackey, 1999), *Salmonella enterica* (Simpkins *et al.*, 2000), *Enterococcus faecalis* (del Mar Lleo *et al.*, 2000), and *Pneumocystis carinii* (Maher *et al.*, 2001). These RT-PCR assays were however not quantitative as they were traditional gel based presence/absence RT-PCRs. Most reports were preliminary and do not seem to take account of the decay of mRNA except in cells killed by extreme measures. None of these studies appear to have been followed up by publications demonstrating use in environmental studies. More specifically, the *Campylobacter* study only reported results using  $10^8$  cells, was not quantitative, and required a restriction digest for identification of *C. jejuni*.

The aim of this project was to develop a TaqMan based quantitative RT-PCR assay for the detection of mRNA and apply it to discriminate between viable and dead cells of *C. jejuni*. The method needs to be reliable, robust, and suitable for routine testing a range of environmental water types for viable *C. jejuni*.

Previous research by ESR on a viability assay for *Campylobacter*

ESR's June 2001 report to the Ministry of Health (FW0149) described:

- The establishment of RT-PCR methods for the detection of the heatshock mRNA *groEL* and *dnaJ*.
- The development, testing and sensitivity determination of a heatshock induction assay based on *groEL*.

The key conclusion was that, mRNA could persist in dead cells for one month or more after cell death. Assays based on the direct detection of mRNA therefore have the potential to be affected by high levels of false positives from mRNA in dead cells.

Applying the logic that only viable cells are able to synthesise new mRNA, and dead cells are unable to, we developed a viability assay for *C. jejuni* by measuring the induction by heatshock of *groEL* mRNA.

#### 1.1.1 2001-2002 Research

The aim for the for 2001-2002 research was to refine further the viability assay and advance understanding of its potential applicability in the natural environment.

The following milestones were completed:

- Design and optimisation of a minor groove binding (MGB) probe for the *groEL* TaqMan system.
- Determination of the specificity of *groEL* TaqMan primers and probe.
- Determination of the sensitivity of the developed viability assay using serial dilutions of cells.
- Investigation of the use of centrifugation to concentrate environmental samples.
- Evaluation of the effectiveness of the method in spiked river samples.

## 1.2 Methods and Results

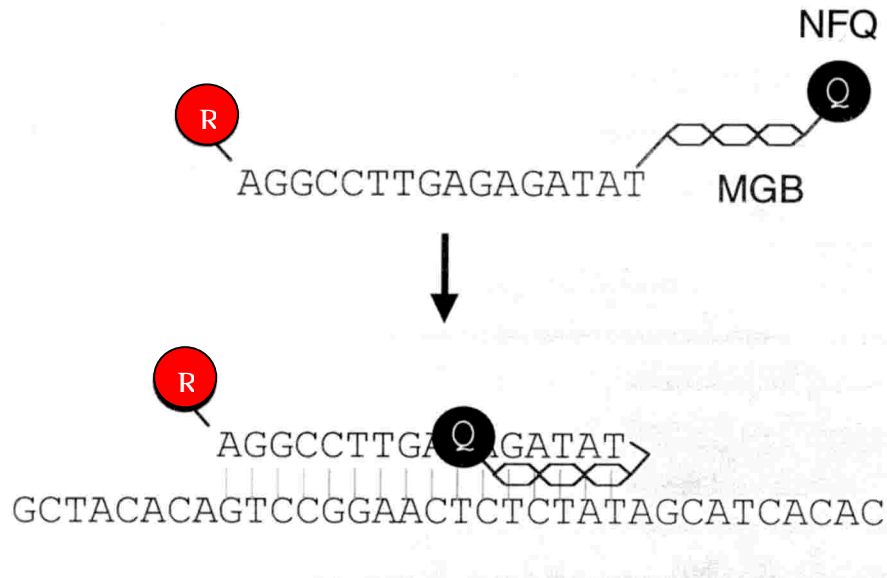
### 1.2.1 Design and optimisation of an MGB probe and new primers for *groEL*

Applied BioSystems released the third generation of TaqMan probes in 2001. This new chemistry, termed MGB probes, uses a non-fluorescent quencher (NFQ) to replace the previous TAMRA quencher dye, and incorporates MGB probe chemistry to increase dramatically the stability of probe hybridisation. Figure 1 illustrates the chemistry of the probe, and the hybridisation of a probe to target DNA. The effects of these enhancements are:

- Enhanced fluorescent performance of the probe
- Ability to use shorter probes increasing hybridisation efficiency, and allowing greater flexibility in design of amplicons.

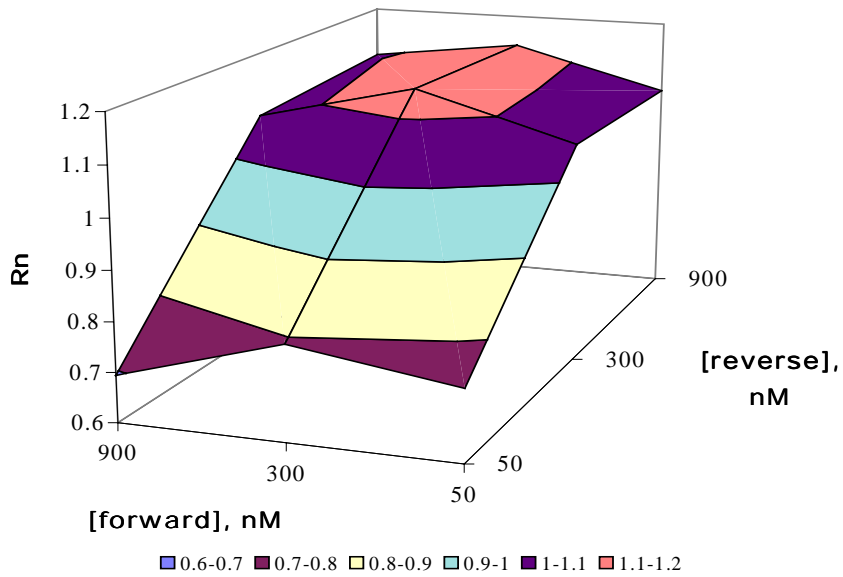
This offers the opportunity to design and optimise an MGB probe and new primers for *C. jejuni groEL*.

**Figure 1 TaqMan MGB Probe.** A specific oligonucleotide with fluorescent reporter (R, usually FAM), non-fluorescent quencher (NFQ), and minor groove binder (MGB), hybridises to a complementary region of target nucleotides.

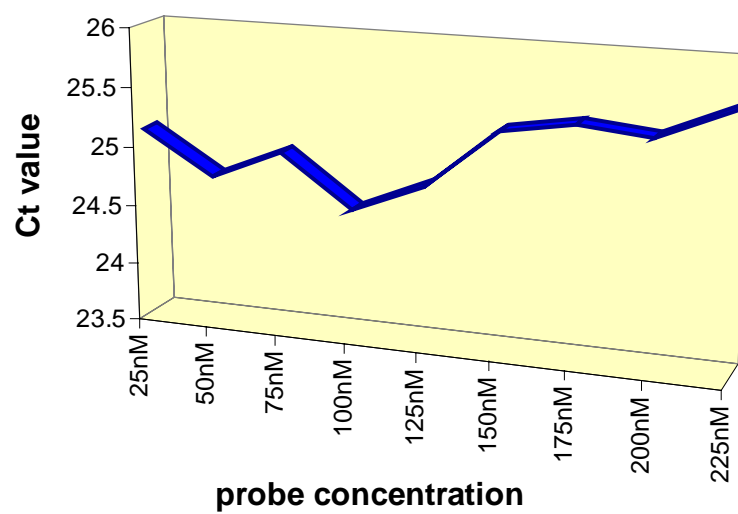


To improve further the *groEL* TaqMan PCR system for the detection of *C. jejuni* new primers and probe were designed for amplifying the *C. jejuni groEL* mRNA. The optimum primer concentrations were determined as 300 nM of each of primers (Figure 2) and the optimum probe concentration was determined to be 100 nM (Figure 3). The narrow range in variation of Ct (the PCR cycle at which amplification exceeds a given threshold) with changing concentration of probe emphasises the robustness of the MGB probe.

**Figure 2 *groEL* Primer Optimisation.** The highest Rn value, and therefore the best amplification, was observed with 300 nM of each primer.



**Figure 3 *groEL* Probe Optimisation.** The optimal level of probe is that which gives the lowest Ct value – in this case it was 100 nM.



### 1.2.2 Specificity of *groEL* TaqMan system

The aim was to determine the specificity of *groEL* TaqMan system. The *groEL* TaqMan primers and probe were tested in a PCR with DNA extracted from a range of organisms. As illustrated in Table 1, of the organisms tested, the *groEL* primers and probe amplified with highest efficiency from the *C. jejuni* templates. Amplification was also observed from several other organisms, but with lower efficiency.

**Table 1 Specificity of *groEL* TaqMan Primers and Probe**

Organism	Lab stock Number	TaqMan result	Ct
<i>Campylobacter jejuni</i> F38011	SS0139	+	23
<i>Campylobacter jejuni</i> Type strain	SS0214	+	17
<i>Campylobacter coli</i>	SS0215	-	60
<i>Campylobacter fetus</i>	SS0120	+	38
<i>Campylobacter hyoilei</i>	SS0119	-	60
<i>Campylobacter lari</i>	SS0141	+	38
<i>Campylobacter upsaliensis</i>	SS0136	+	34
<i>Arcobacter butzleri</i>	SS0121	-	60
<i>Arcobacter cryaerophilus</i>	SS0122	+	43
<i>Bacillus cereus</i>	SS0152	-	60
<i>Bacillus subtilis</i>	SS0108	-	60
<i>Enterobacter aerogenes</i>	SS0112	+	41
<i>Enterobacter faecalis</i>	SS0158	-	60
<i>Escherichia coli</i>	SS0205	-	60
<i>Helicobacter pylori</i>	SS0118	-	60
<i>Klebsiella pneumoniae</i>	SS0161	-	60
<i>Listeria innocua</i>	SS0115	+	35
<i>Listeria ivanovii</i>	SS0149	-	60
<i>Listeria monocytogenes</i>	SS0203	-	60
<i>Morganella morganii</i>	SS0111	-	60
<i>Proteus vulgaris</i>	SS0103	-	60
<i>Pseudomonas aeruginosa</i>	SS0153	-	60
<i>Saccharomyces cerevisiae</i>	SS0106	+	36
<i>Salmonella menston</i>	SS0105	+	41
<i>Shigella flexneri</i>	SS0114	+	33
<i>Staphylococcus epidermidis</i>	SS0186	-	60
<i>Staphylococcus aureus</i>	SS0151	-	60
<i>Streptococcus bovis</i>	SS0193	-	60

This specificity assay should be extended to a wider range of organisms, and a variety of *C. jejuni* isolates, including newly isolated environmental isolates from the Ashburton River. The amplification from the non-*C. jejuni* isolates is a concern. It may either indicate low levels of contamination of the bacterial stocks with *C. jejuni* DNA, or low level non-

specificity of the primers and probe. Further work is in progress to clarify this lack of specificity.

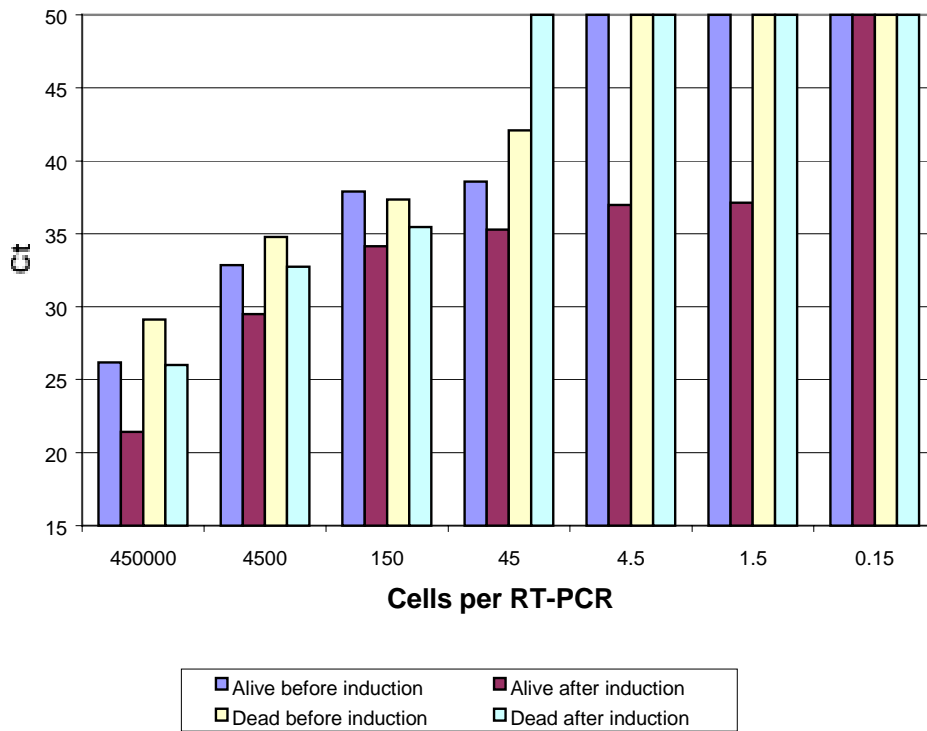
### 1.2.3 Sensitivity of the viability assay

The aim was to determine the sensitivity of the developed viability assay using serial dilutions of cells and the new MGB probe.

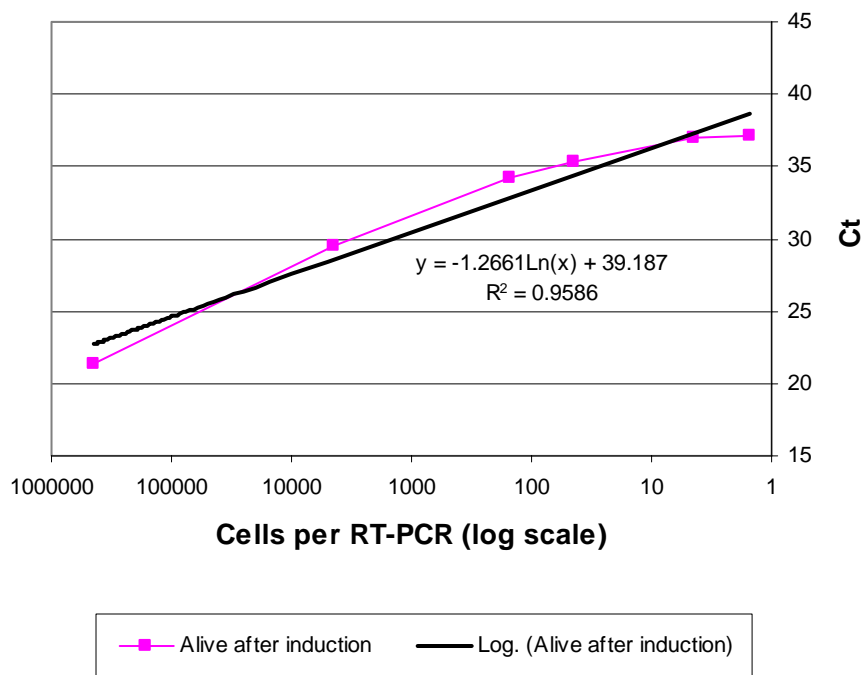
*C. jejuni* F38011 cells were grown overnight at 37°C in a CO<sub>2</sub> incubator on blood agar. The cells were harvested in Exeter broth, serially diluted, and the cells in each dilution enumerated by plate count on blood agar. Aliquots (200 µL) with between 4.5 x 10<sup>6</sup> and 1.5 colony forming units (cfu) were then processed in duplicate. Half were killed by chlorine (incubation with chlorine at final concentration of available chlorine 580µg/mL for 45 min, followed by addition of sodium thiosulphate to 10mM) to give “dead cells”, or else left at room temperature to give “living cells”. Both dead and living cells were incubated at 37°C for 2 hours then heat-shocked at 48°C for 30 min. Total RNA was extracted with TRIzol LS Reagent and DNA was digested with DNase. One-step TaqMan RT-PCR was performed using optimised *groEL* primers and probe concentrations. In all experiments, each and every extracted sample was tested by TaqMan PCR without the addition of reverse transcriptase. No amplification from this control was taken as evidence that all DNA had been removed from the extract, and that amplification from the RT-PCR was due to amplification of RNA.

At each cell dilution the maximum signal was detected from induced living cells (Red bars, Figure 4). Figure 4 is a composite figure derived from two independent experiments. The high degree of linearity from independent experiments (Figure 5) indicates the quantitative nature of this assay. Amplification could be observed from as few as 1.5 cells per RT-PCR or 15 cells per extraction. At the lowest dilution amplification was observed from only one of the duplicates (at a Ct of 41), but as our assay requires amplification from duplicates for confirmation, this is reported as no amplification. The sensitivity of the assay with serial dilutions of cells appears to be very good and is 1.5 cells per RT-PCR or 15 cells per extraction.

**Figure 4 Amplification of *groEL* mRNA from Living and Dead *Campylobacter jejuni*, Before and After Heatshock Induction.** The lower the Ct (threshold cycle) the more mRNA detected. Value of 50 indicates no detectable amplification.



**Figure 5 Linearity of mRNA Amplification of *groEL*.**



#### 1.2.4 Concentration of samples by centrifugation

The aim of this experiment was to investigate the potential of centrifugation to concentrate water samples for use in the viability assay.

To concentrate *C. jejuni* in water samples by centrifugation, a range of G-forces and times were tested. Briefly,  $10^4$  *C. jejuni* F38011 cells were inoculated into 50 mL of either sterile distilled water, buffered peptone or river water. Cells were then recovered from the liquid medium by centrifugation at 1900 – 4300 × G for between 5 and 45 min. Cell pellets were resuspended in 0.5 mL of Exeter broth. Cell recoveries were estimated by counting culturable colonies on Columbia blood agar (CBA) or Exeter agar plates, both before and after centrifugation.

The highest recoveries of cells were observed using 4,300 × G for 10 min. Centrifugation times between 7 and 25 min were not found to affect recoveries. Therefore a centrifugation time of 10 min was used in subsequent experiments. Table 2, gives the recoveries observed from each liquid type. The best liquid medium was buffered peptone water, which gave approximately 50% recovery. A problem with this type of experiment is that spiking into water samples results in a seemingly instant loss of culturability of many of the cells. This was most dramatically seen with sterile water where 90% loss of cells was observed. Exeter agar is more selective than CBA, which is able to revive 50% more of these damaged cells.

**Table 2 Effect of Centrifugation Conditions on Recovery**

Water type	Cell death	Recovery	
		CBA	Exeter
Buffered peptone	0%	50%	-
River water	50%	-*	10%
Sterile ddH <sub>2</sub> O	90%	45%	20%

\* Recovery could not be estimated on CBA plates with spiked river water due to the low selectivity of the media, and overgrowth with other organisms present in the water.

In summary, centrifugation was able to recover culturable cells but with low efficiencies.

It is noted that higher recoveries of cells were observed when, after centrifugation, the cells were incubated for 2 hours at 37°C before plating. This increased recovery is most likely due to recovery or resuscitation of cells damaged in either the centrifugation process or by osmotic shock when inoculated into the sample. This provides support for the value of the 2 hours incubation in this viability protocol, but this time period needs to be investigated further. Viable cells in river samples should not have the osmotic shock problems experienced with these laboratory strains.

### 1.2.5 Evaluation with environmental samples

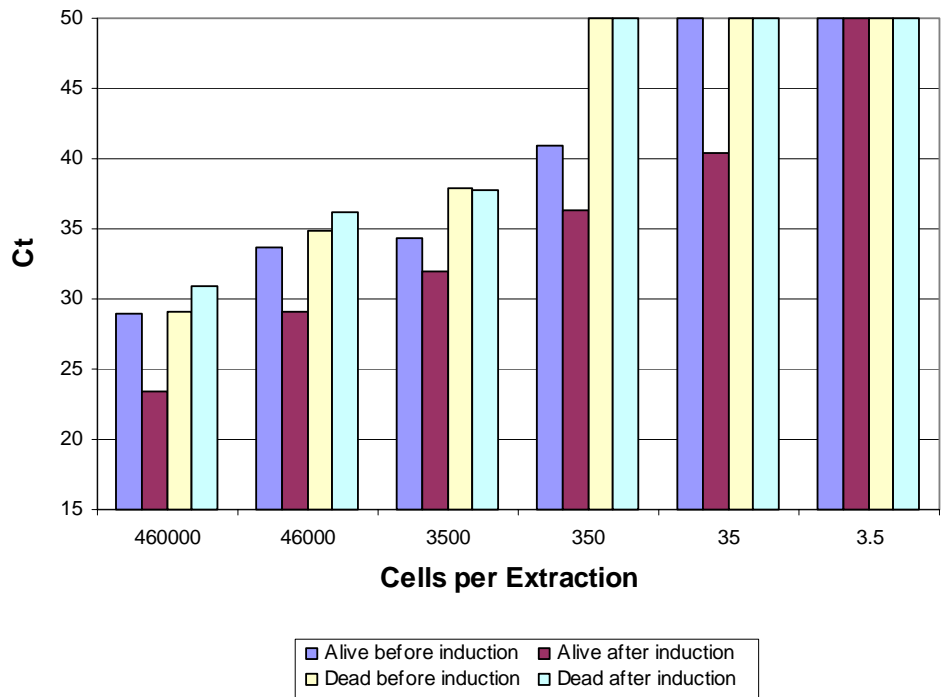
The viability method was evaluated in spiked river water and municipal effluent.

#### 1.2.5.1 *Spiked river water*

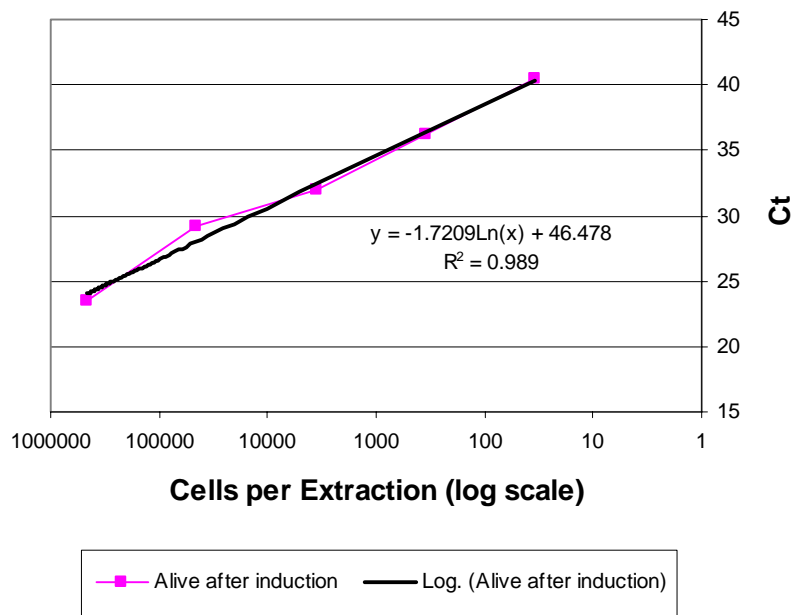
To investigate the potential of the viability method by spiking river water samples, river water was collected from a stream adjacent to ESR, Christchurch. Serial dilutions of *C. jejuni* F38011 were inoculated into 50 mL of river water and centrifuged at  $4,300 \times G$  for 10 min. The supernatant was discarded, and pellets were resuspended in 0.5 mL Exeter broth (without antibiotics), and the viability assay performed as described (section 1.2.3).

At each cell dilution the maximum signal was detected from induced living cells (Red bars, Figure 6). Figure 6 is a composite figure derived from two independent experiments, one at the upper end of the scale, and the other covering the lower four dilutions. The high degree of linearity from independent experiments (Figure 7) confirms the quantitative nature of this assay. Amplification could reliably be observed from as few as 3.5 cells per RT-PCR or 35 cells per extraction.

**Figure 6 Sensitivity of the Viability Assay in Spiked River Water**



**Figure 7 Linearity of mRNA Amplification of *groEL*.**



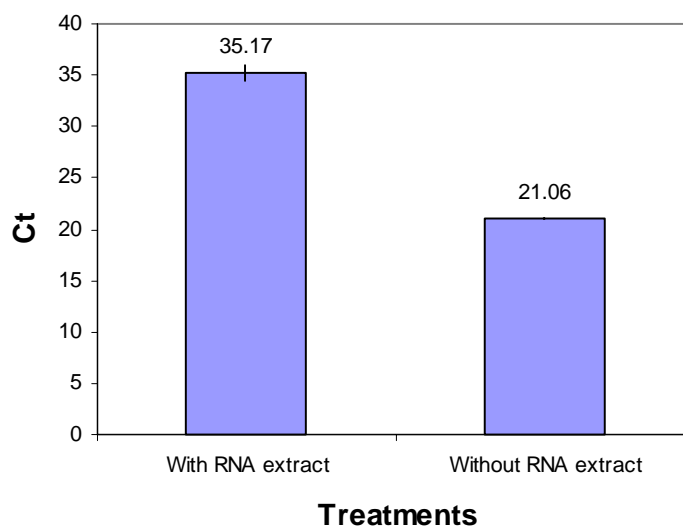
### 1.2.5.2 Unspiked Municipal effluent

To evaluate the effectiveness of the viability method with raw effluent, municipal effluent from primary treatment tanks was collected from the Christchurch City Wastewater treatment plant (CWTP) at Bromley. Samples were centrifuged at  $4,300 \times G$  for 10 min. Samples of 50 mL were concentrated to 0.5 mL, and 200  $\mu$ L aliquots use for the viability assay (section 1.2.3).

No TaqMan amplification was detected in any of the samples. Analysis by double enrichment culturing identified that *C. jejuni* was present in the samples, although the number of *C. jejuni* was not determined (at least one *C. jejuni* cell per 100 mL must have been present). In a subsequent experiment *C. jejuni* F38011 DNA was amplified with and without the addition of the municipal effluent RNA extract. Significant inhibition of approximately 14 cycles was observed (Figure 8.). Each 3 cycle reduction in CT is equivalent to approximately a log reduction in DNA detected. Therefore a reduction in CT of 14 cycles indicates that the inhibitors present would reduce sensitivity by at least 10,000-fold.

The viability method was not as sensitive as the double enrichment PCR method at detecting *C. jejuni* in municipal effluent. It would appear that materials, which significantly inhibit the PCR, are present in the extract.

**Figure 8 Effect of Municipal Effluent RNA Extract on Ct**



#### 1.2.5.3 Spiked municipal effluent

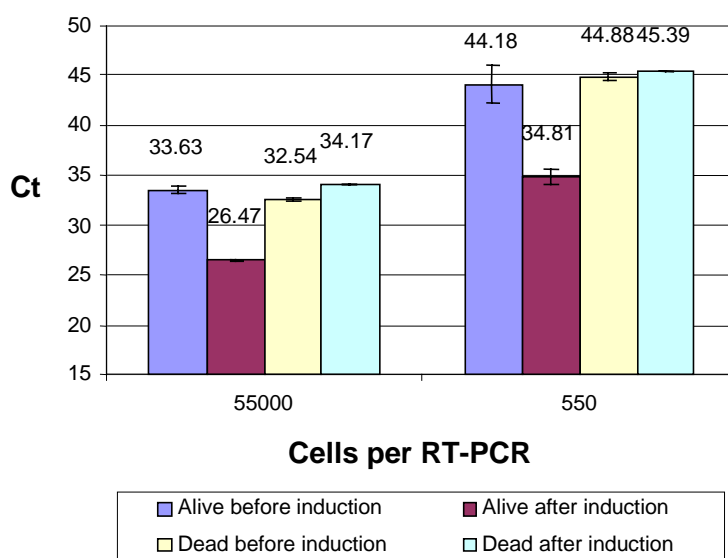
To further investigate the degree of inhibition, effluent was spiked with *C. jejuni*.

Municipal effluent described above was autoclaved for 20 min. The sample was then centrifuged in 50 mL tubes, supernatants discarded and the pellets resuspended in 0.25 mL Exeter broth (without antibiotics). Pre-diluted *C. jejuni* F38011 cells (at two dilution levels) were added into pellet suspensions at a ratio of 1:1 (v/v), aliquoted, and the viability assay performed as described in section 1.2.3.

Significant induction of *groEL* mRNA was observed in the living cells, while dead cells maintained nearly the same level. This indicates that the large amount of suspended solids had little effect on *groEL* mRNA induction and total RNA extraction. But the sensitivity of TaqMan RT-PCR on mRNA extracted from these environmental samples seems to be decreased compared with that on mRNA extracted from pure culture (Figure 4) by a factor of approximately 10 (Figure 9).

In summary, autoclaving appears to have reduced much of the inhibitory effect present in the effluent. The nature of the inhibitors, and how to remove them without destructive autoclaving, needs to be investigated further.

**Figure 9 Spiked Municipal Effluent (autoclaved)**



### 1.3 Assessment of Applicability in the Natural Environment

#### 1.3.1 *Campylobacter* levels in environmental water samples

There is little available information on the levels of *Campylobacter* in water samples. A previous pilot study for the MoH (E80) using MPN-PCR, identified low levels of *Campylobacter* in the water samples analysed (Table 3).

**Table 3 *Campylobacter jejuni* in Selected Water Types**

Water type	MPN/100 mL
Surface water	<0.12 - >11
Shallow groundwater	<0.06 - 0.72
Roof water	<0.06 - 0.56
Drinking water	<0.06 - 0.3

*C. jejuni* have previously been identified in raw sewage from the Christchurch Wastewater Treatment Plant (CWTP) at concentrations between 2,400 and >11,000 *C. jejuni* per 100 mL (Horswell *et al.*, 2001). Overseas studies have measured between 50 and 500,000 *Campylobacter* per 100 mL of raw sewage (Koenraad *et al.* 1997). The maximum level detected in the oxidation ponds at the end of the treatment process at CWTP was 9 *C. jejuni* per 100 mL. At a different wastewater treatment plant without oxidation ponds,

approximately 900 *C. jejuni* per 100 mL were identified in the wastewater discharge (Personal communication).

So while raw sewage may contain up to  $5 \times 10^5$  *Campylobacter* per 100 mL, levels much lower than this are likely to be encountered in environmental, drinking, or treated waste water. With currently available knowledge, *C. jejuni* is likely to be present in water used for drinking at less than one *C. jejuni*/100mL, while in river waters less than 500 *C. jejuni*/100 mL is likely.

### 1.3.2 Samples with low concentrations of *C. jejuni*

When the total number of living and/or dead *C. jejuni* in the extracted sample is less than approximately 100 (based on observations from experiments described earlier) then a signal will only be detected from induced, living *C. jejuni*. The assay could therefore be performed by concentrating a sample, splitting it in half, and assaying before and after induction. Alternatively duplicate samples could be concentrated and assayed one before, and one after induction. The amplification level of the induced sample could therefore be compared with standard curve, and number of cells derived.

Our current detection limit is close to 1 cell per RT-PCR reaction in pure culture. The key to assaying samples with low levels of *C. jejuni* will be to concentrate larger volumes, and then to extract and RT-PCR amplify the complete sample. Figure 10 illustrates two potential workflows for analysing a 1-litre sample. Provided inhibition was not a factor this assay could be used to detect 0.1 *C. jejuni*/100mL.

### 1.3.3 Samples with higher concentrations of *C. jejuni*

The same strategy as outlined above can be used for samples with 100 *C. jejuni* or more per 100 mL. Amplification in this case will occur from the preinduced cells, but provided an induction is seen the number of viable cells can be determined. If no induction is observed it would indicate that non-viable cells predominate.

When living *C. jejuni* outnumber the dead *C. jejuni* the assay will always function well. When however dead cells outnumber the living by perhaps 10-fold or more, then masking of living cells may occur. This theoretical ratio needs to be investigated experimentally. This would be an unusual situation (such as in wastewater treatment plant after U.V. treatment) which could usually be predicted.

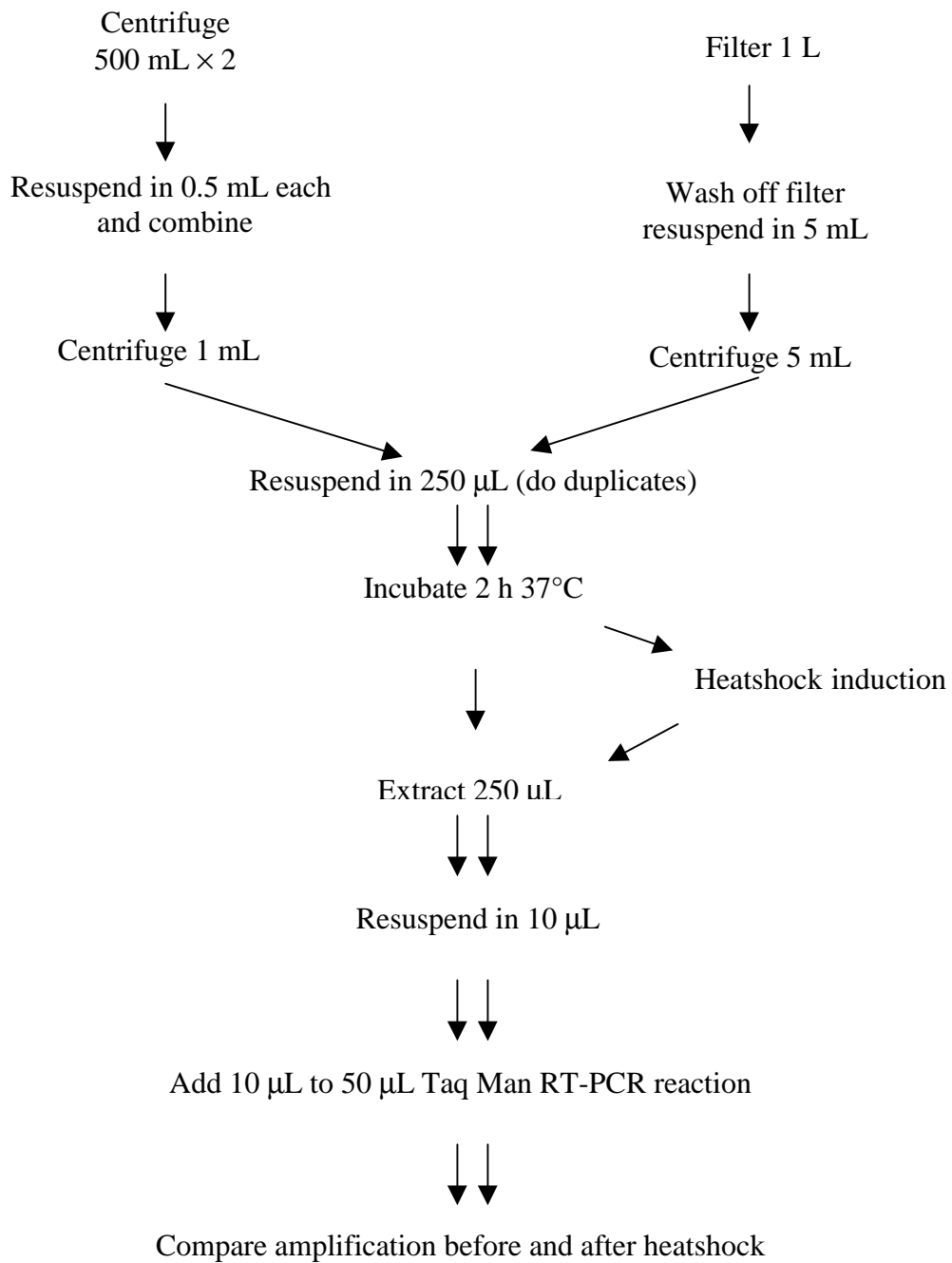
#### 1.4 Conclusions

We have further refined the viability assay and made it more reproducible. We have also evaluated the use of centrifugation to concentrate cells prior to viability assay. Using a laboratory strain of *C. jejuni*, we are able to detect as few as 1.5 viable cells per RT-PCR or 15 cells in the extraction volume. Spiked river water samples were processed using the viability assay, and detection and enumeration of viable *C. jejuni* achieved. In spiked river water samples 3.5 viable *C. jejuni* per RT-PCR could be detected.

Clear progress has therefore been made, and we make the following recommendations to enable its routine use in the enumeration of viable *C. jejuni*.

- Establish meanings of cell death, using means recognised for eukaryotes and transfer to prokaryotes where appropriate.
- Evaluate the assay's effectiveness with cells killed by U.V., chlorine, heat and starvation.
- Evaluate the assay with a wider range of environmental *Campylobacter* isolates and other organisms.
- Investigate membrane filtration and elution for the concentration of cells from large volumes of water.
- Evaluate the assay comparatively with double enrichment MPN-PCR in a range of environmental samples (alongside planned 2002-2003 research).

**Figure 10 Potential Flow Chart for Analysis of a 1-Litre Sample**



### **OBJECTIVE 3: INVESTIGATION OF THE POTENTIAL FOR SEDIMENTS TO ACT AS RESERVOIRS FOR *CAMPYLOBACTER***

#### **1.5 Introduction**

The presence of faecal bacteria in surface waterways is likely to be from one of three sources: groundwater seepage, overland flow washing contaminants off the land or resuspension of sediment. As higher concentrations of faecal indicator bacteria are recorded during and after periods of rainfall when compared to base flow conditions, the contributions from overland flow and resuspended sediment are generally likely to be more important than contaminated groundwater seepage. Grazing cattle and sheep are potential sources of contamination of waterways indirectly from overland flow which washes faecal material into the waterway during storm events (wash in) or directly from defecation in the waterway creating “hot spots”.

In order to differentiate between the effect of wash in and resuspension of sediment Nagels *et al.* (in press) created three artificial floods and compared the concentrations of *E. coli*, flow and turbidity (a measure of the finer sediment fraction) with a natural flood event recorded within the same catchment. Results showed increases in concentrations of *E. coli* of 2 orders of magnitude during the artificial floods. These increases in *E. coli* were similar in magnitude to those measured during the natural flood event. A correlation number between turbidity and *E. coli* was established ( $r=0.93$ ).

From analysis of stream substrate and the loadings calculated for faecal indicators, it was proposed that mobilisation of sediment containing high concentrations of faecal bacteria (hot spots) during the peak flow of a flood event could be as important as a source of faecal indicators, or more important, than wash in from overland flow (Davies-Colley *et al.* 2001). If most of the faecal contamination is in the sediment then routine water quality sampling, which is usually undertaken on a random basis with few flood events, may significantly underestimate the bacterial loading (Nagels *et al.* in press).

Similarly, *Campylobacter* in the water column may be removed through adsorption to sediment, or released to the water column from the sediment, either during storm events when sediment is mobilised or by stock disturbing the sediment. Removal of bacteria to the sediment and resuspension during high rain events has been proposed as a method of concentrating *E. coli* (Nagels *et al.* in press). As adsorption to the sediment may occur over a period of time, it is likely that the *Campylobacter* strains in the sediment are not necessarily the same as those currently in the water.

Determining the presence and survival of *Campylobacter* in sediment at three sampling sites in the Ashburton River is Objective 3 of this part of the *Campylobacter* Transmission Routes Study (CTR), this is a preliminary study only a limited number of samples were tested.

#### 1.5.1 Aims

The aims of this objective were to establish whether the predominant strains of *Campylobacter* in sediment and associated overlying water samples are the same, and to determine the concentrations of *Campylobacter* in sediment and associated water phase.

### 1.6 Methods

Rainfall data for the period June to August 2001 at Winchmore, the closest weather station, was obtained from the National Institute of Water and Atmospheric Research. Daily mean river flow data for the South Branch of the Ashburton River at Mt Somers were provided by Environment Canterbury for the year 2001, with monthly averages for the years 1982-2001 (South Branch).

#### 1.6.1 Enrichment/PCR

Duplicate samples of the top sediment were collected in 50 mL sterile containers by scooping the container into the top fraction of the sediment (upper 5-10 mm) from the following sites:

- Ashburton Inlet (after the confluence of the North and South Branch)
- Ashburton Forks - Taylors Stream (South Branch)
- Ashburton Forks - Valetta Bridge (South Branch).

Samples were transported to the laboratory and allowed to settle at room temperature for at least an hour and the water pipetted to another sterile container. The resultant water and sediment portions were tested separately as described in Appendix 1.

### 1.6.2 Serotype comparison

Penner serotyping and pulse field gel electrophoresis (PFGE) were carried out on one *Campylobacter jejuni* isolate per positive water and sediment sample. PFGE was also carried out on one *C. coli* per positive sample. Penner serotyping was performed by the passive haemagglutination technique described by Penner and Hennessy (1980) to determine the heat stable serotypes of *C. jejuni* isolates. Antisera were produced at the Enteric Reference Laboratory (ESR-KSC) by the methods described by Penner and Hennessy (1980) using their reference strains for antisera production. Penner serotyping was not performed on *C. coli* isolates as the requisite antisera were not available.

### 1.6.3 Quantitative comparison

One composited Ashburton Inlet sediment sample was separated into sediment and water portions and the number of *Campylobacter* was estimated in both, using the most probable number (MPN) method. Briefly one 50 mL, three 10 mL, three 1 mL, three 0.1 mL and three 0.01 mL volumes of the water portion and three 10 g, three 1 g, three 0.1 g and three 0.01 g wet weights of the sediment portion were tested for *Campylobacter* using the method described in Appendix 1. No strain typing was carried out. The sediment fraction was analysed for total solids.

## 1.7 Results

### 1.7.1 Serotype comparison

Samples for serotyping were collected by Ashburton District Council in sterile control tubes on 7/8/01. The flow at for the South Branch was 5601 l/s, which was similar to the previous day (5637 l/s), but less than the mean flow for the month (6508 l/s). The most recent rain (1.6mm) was 5 days prior to sampling on 2/8/01.

Of the 50 mL sample, the volume of water associated with each sample was around 15-30 mL with 35-20 mL of sediment. Table 4 gives the species identified in the water and sediment samples. Table 5 summarises the Penner and PFGE typing results for the *C. jejuni* isolates.

**Table 4 Presence of *Campylobacter* in Sediment and Water Phases**

Site	Positive samples			
	Water		Sediment	
	<i>C. jejuni</i>	<i>C. coli</i>	<i>C. jejuni</i>	<i>C. coli</i>
Ashburton Inlet	2/2	0/2	2/2	1/2
Ashburton Forks – Taylors Stream	½	0/2	0/2	0/2
Ashburton Forks – Valetta Bridge	2/2	0/2	2/2	0/2

**Table 5 Penner Serotype and PFGE Pulsetype Results for *Campylobacter jejuni* Isolated from Sediment and Water Samples**

Site	Phase	Penner serotype	PFGE pulsetype
Ashburton Inlet a	Water	Untypable	201
Ashburton Inlet a	Sediment	45	25
Ashburton Inlet b	Water	Untypable	237
Ashburton Inlet b	Sediment	Untypable	237
Ashburton Forks – Taylors Stream	Water	8, 17	239
Ashburton Forks – Valetta Bridge a	Water	57	60b
Ashburton Forks – Valetta Bridge a	Sediment	25	Not cutting
Ashburton Forks – Valetta Bridge b	Water	Untypable	25d
Ashburton Forks – Valetta Bridge b	Sediment	45	25

### 1.7.2 Quantitative comparison

Samples for quantitative comparison were collected on 25/6/01. There was no rain on the day of sampling, but 3.4 mm of rain was recorded on the previous day. Within the previous week the only other rainfall event was 0.2 mm on 19/6/01. The river flow at Mt Somers on the day of sampling was 6155 l/s which was lower than the previous day 6795 l/s. Higher flow the previous day is consistent with rainfall on that day. The average flow for June was 4959 l/s.

The sediment at the sampling site was described as sandy with variable amounts of organic material present as vegetative matter. Total moisture in the sediment fraction was 25.1% (w/w).

The water phase contained 150 *C. jejuni* per 100 mL (by MPN) and the sediment contained 75 *C. jejuni* per 100 g (by MPN).

## 1.8 Discussion

Ready access to the Ashburton River by farmed animals is typical of farmed river catchments in New Zealand. The Ashburton Intake site is reported as being a strategic cattle crossing for the district (Durie, pers. com.). The sediment is therefore potentially contaminated by wash in from overland flow and from direct defecation in the stream bed. Table 4 shows that there is *Campylobacter* present in the sediment and the water with *C. jejuni* being more prevalent than *C. coli*. This is consistent with the prevalence observed in other environmental matrices the CTR study. *C. coli* was only detected in sediment at the Ashburton Intake, and this could be owe to input from the North Branch of the Ashburton River, or from the intake being a cattle crossing point. All water samples contained *C. jejuni*, but this species was not present in all sediment samples (e.g. absent from Taylors Stream). This suggests that the diversity of *Campylobacter* in the water and sediment may be different, possibly owing to differences in survival characteristics in the two phases or because the *Campylobacter* in the sediment has accumulated over time.

Different serotypes in sediment and water were generally observed (Table 5). Except for Ashburton Inlet b where the PFGE pulsetype was the same, different types were generally

found in water and sediment. Two sediment isolates were typable by both systems. However a third sediment isolate was of a different serotype.

As there had been recent rain the concentration of *Campylobacter* in the water is likely to be elevated owing to processes such as wash-off of contaminants from land or increased concentrations of suspended sediment with absorbed contaminants in the water column. However, within the limits of the MPN test, the concentrations of *C. jejuni* were similar in the sediment and water.

Testing more samples of sediment, sediment water and the water column would be needed to confirm the relative concentrations of *C. jejuni* in water and sediment. Concentrations may vary dependent on changes to river flow (e.g. high flows caused by high rainfall, or snow melt, or low flow) or farming practices (e.g. before and after stock movements across the river). Analysis of suspended sediment concentrations in the water would be useful in elucidating any relationship between suspended sediment and *Campylobacter* concentrations in the water column.

## **1.9 Conclusions**

In general *C. jejuni* isolated from sediment were of distinguishable serotypes. This is consistent with a hypothesised system where sediment absorbs *Campylobacter* and therefore provides a cumulative record of types that have been in the water above.

The concentrations of *C. jejuni* in the sediment and water were similar on this occasion. More sampling is required to provide sufficient data to determine whether the sediment is a sink for *Campylobacter*.

In order to understand the relationship between *Campylobacter* strains from sediment and water it will be necessary to do more extensive sampling of sediment, interstitial water and the water column for a range of weather conditions, flow conditions and farming practices.

## 1.10 Recommendations

- Investigate the relationship between *Campylobacter* from sediment and water samples at a number of sites in a river to include various river flows, rain events and farming practices.
- Establish the number of *Campylobacter* cells in sediment, interstitial water and the water column at a range of sites and over time to allow evaluation of the relative loading of these environmental sample types.
- Determine the relationship between *Campylobacter* cells associated with suspended sediment particles, sediment and the water column during high rainfall events.

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## APPENDIX 1

### LABORATORY PROTOCOLS FOR DETECTION OF *CAMPYLOBACTER* FROM ENVIRONMENTAL MATRICES

#### SECTION A: LABORATORY PROTOCOLS FOR ENRICHMENT OF *CAMPYLOBACTER* FROM ENVIRONMENTAL MATRICES

##### A.1 Procedure For *Campylobacter* Isolation From Samples Of Sediment and Water

*Lab coats, gloves and eye protection must be worn at all times when carrying out this procedure. Wherever it is practical sample manipulations involving open tubes must be carried out in an approved biohazard cabinet.*

##### A.2 Procedure for Sediment Samples (Figure 12)

###### A2.1 Materials and Equipment

35 ml Universal bottles (# LBS 3722W)

Whirl-Pak Bags (# BO1020 WA, Nasco, Life Technologies)

Gas jar (Oxoid, Basingstoke, Hampshire, England)

CampyGen™ system (Oxoid, Basingstoke, Hampshire, England) or 10% CO<sub>2</sub> incubator

Incubators: 42 ± 1°C, 37 ± 0.5°C

“Exeter” agar plates (Section F)

“Exeter” enrichment broth

Columbia Blood Agar plates with 5% defibrinated sheep blood

**Please do not dispose of any of the original samples until two weeks after initial arrival. Samples should be stored at 4°C.**

A2.2.a **Sediments:** Using sterile utensils, prepare a homogenate by weighing 10 g of diced meat (e.g. offal) into a sterile “Whirl Pak” bag and adding 90ml of “Exeter”

enrichment broth (Section F). Stomach for 20 sec to mix in a Colworth Stomacher 400.

**A2.2.b Primary Enrichment Broth:** Incubate enrichment broth at  $37 \pm 0.5^{\circ}\text{C}$  for a minimum of 4 hours in a microaerophilic atmosphere. Transfer the jar containing enrichments to an incubator operating at  $42 \pm 0.5^{\circ}\text{C}$  as soon as possible after the 4 hours and continue incubation up to a total of 48 h.

**A2.2.c Secondary Enrichment Broth:** Using a sterile pipette, transfer 0.1 ml of the enrichment broth into a 10 ml “Exeter” enrichment broth and incubate in a microaerophilic atmosphere at  $42 \pm 0.5^{\circ}\text{C}$  for 24 h.

**A2.2.d Performance of controls is checked before any results of samples are recorded (refer Section B).**

### **A.3 Procedure for Water Analysis**

#### **A3.1 Materials and Equipment**

Vacuum/pressure pump

Filter apparatus, sterile

Membrane filters,  $0.45\mu\text{m}$ , sterile

Forceps: smooth-tipped

100  $\mu\text{L}$  pipettes

Incubators:  $42 \pm 1^{\circ}\text{C}$ ,  $37 \pm 0.5^{\circ}\text{C}$

Anaerobic jar

Gas jar (Oxoid, Basingstoke, Hampshire, England)

CampyGen™ system (Oxoid, Basingstoke, Hampshire, England) or 10%  $\text{CO}_2$  incubator

Incubators:  $42 \pm 1^{\circ}\text{C}$ ,  $37 \pm 0.5^{\circ}\text{C}$

#### **A3.2 Procedure for water analysis (Figure 12)**

**Please do not dispose of any of the original samples until two weeks after initial arrival. Samples should be stored at 4°C.**

**A sterile funnel must be used for each sample. Alternatively, funnels can be disinfected between samples by immersing in boiling distilled water for 5 minutes and allowing to cool or by immersing in alcohol and flaming.**

A3.2.a Using sterile forceps, place a sterile membrane filter on a filter-support base and attach the funnel.

A3.2.b Shake the sample bottle at least 25 times.

A3.2.c Filter 10ml or less of water. Apply vacuum to draw sample through. Rinse the sides of the funnel twice with 20-30 ml of sterile rinsing buffer (BPW) and turn the vacuum off once the rinsing buffer has passed through the filter. Due to the large volume of water use as many filters as necessary if the first filter becomes clogged with debris. Maintain aseptic techniques during the changing of any filters.

A3.2.d Carefully remove the filter with sterile forceps and place into a 100 ml “Exeter” enrichment broth in a “Whirl-Pak” Bag.

A3.2.e **Primary Enrichment Broth:** Incubate enrichment broth at  $37 \pm 0.5^{\circ}\text{C}$  for a minimum of 4 hours in a microaerophilic atmosphere. Transfer the jar containing enrichment broths to an incubator operating at  $42 \pm 0.5^{\circ}\text{C}$  as soon as possible after the four hours and continue incubation up to a total of 48 h in a microaerophilic atmosphere.

A3.2.f **Secondary Enrichment Broth:** After the 48 hour incubation, mix each of the enrichment broths by inversion or gentle swirling then. Using a sterile pipette, transfer 0.1 ml of the enrichment broth into a 10 ml “Exeter” enrichment broth and incubate in a microaerophilic atmosphere at  $42 \pm 0.5^{\circ}\text{C}$  for 24 h.

A3.2.g Set up controls for all enrichment batches as outlined in Section B.

## SECTION B: CONTROLS

- B.1 All media is to be validated as required for the MfE project.
- B.2 Test each batch of Exeter broth (Section F) against our *Campylobacter* multiplex primer set as for the Mfe project:
- B.3 Set up controls for enrichment batches (Figure 11)  
For each batch of samples processed, positive, negative and sterility controls are to be included at all stages in the procedure as listed below:

For primary enrichment:

### Positive controls

- A) “Exeter” enrichment broth spiked with 100 µL aliquot *Campylobacter jejuni* (NCTC 11351) grown 48 hours in “Exeter” broth
- B) 100 µL aliquot of *Campylobacter coli* (NCTC 11366) grown 48 hours in “Exeter” broth.

**Negative control** “Exeter” enrichment broth spiked with *Escherichia coli* (ATCC 25922) grown 24 hours in Nutrient Broth.

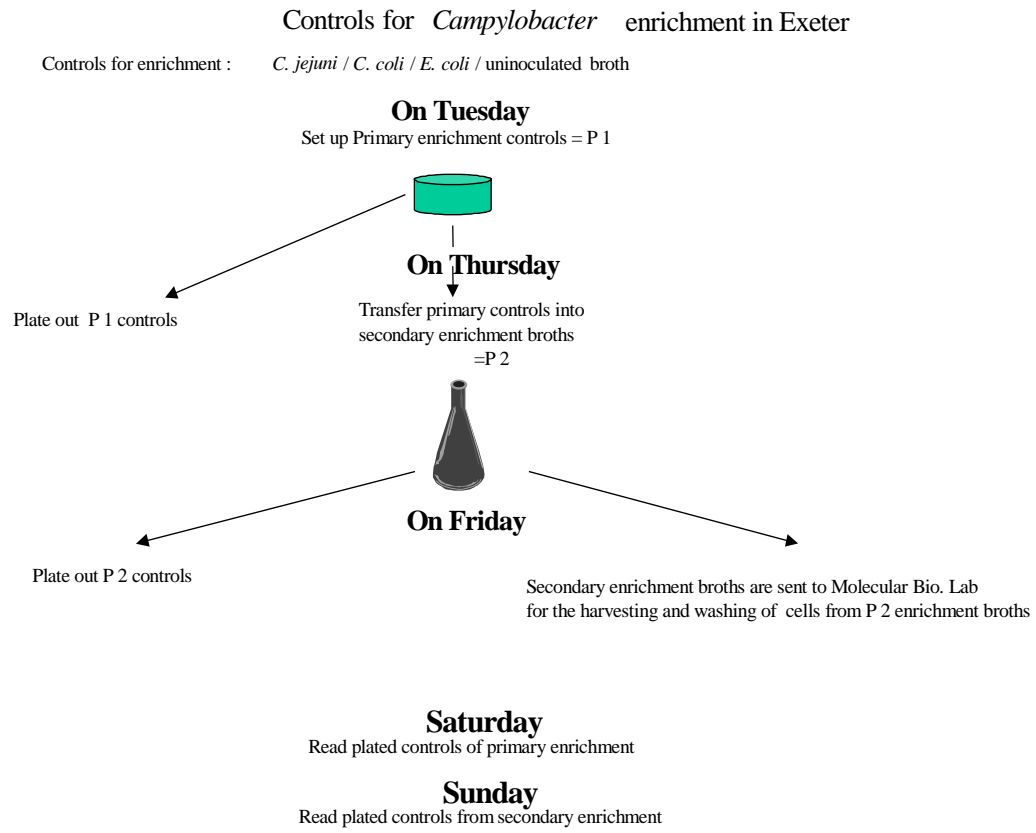
**Sterility control** Uninoculated “Exeter” enrichment broth

**Refrigerated** Uninoculated “Exeter” enrichment broth stored at 4°C

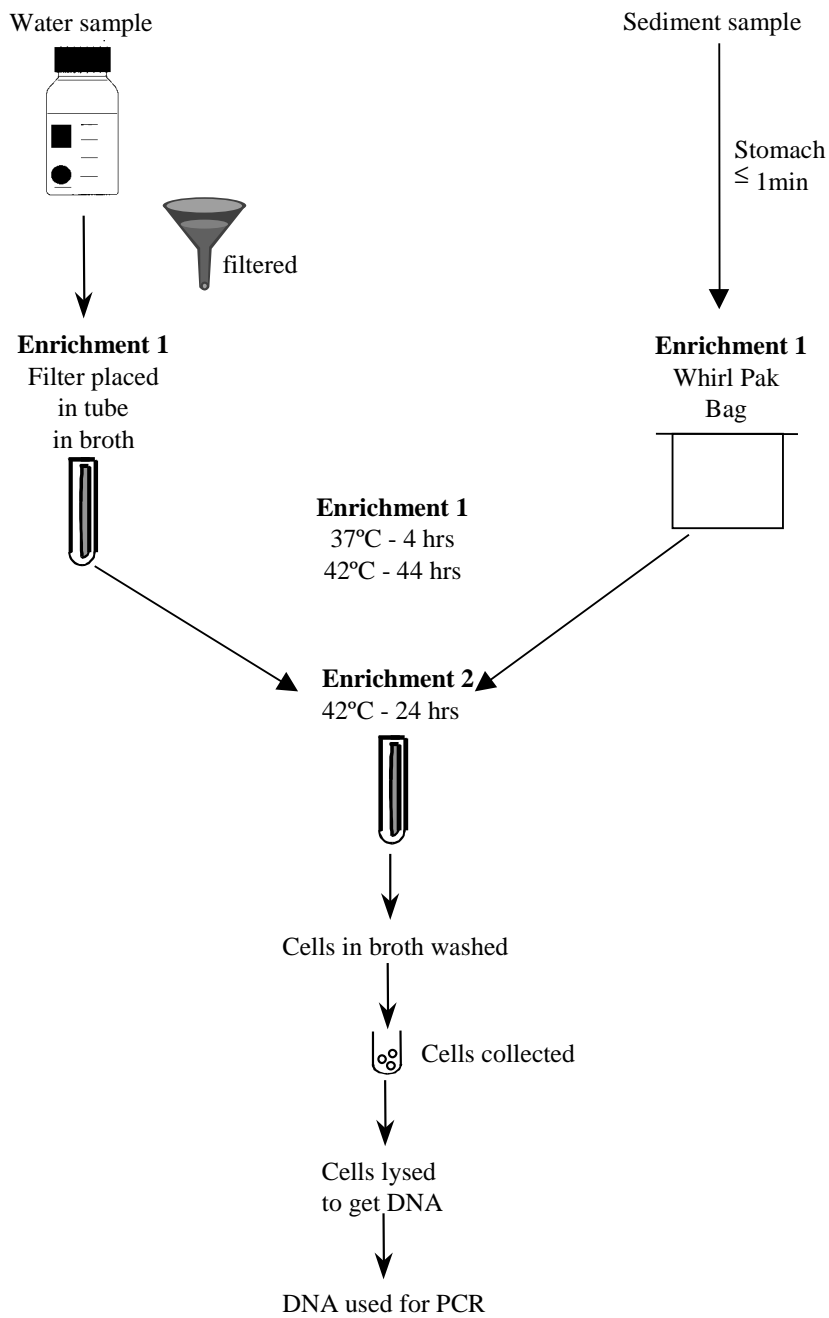
Please plate out all controls to check their culturability on Exeter plates.

**Performance of controls is checked before the results of any samples are recorded.**

**Figure 11**      **Controls for *Campylobacter* Enrichment Process**



**Figure 12** Procedure for Enrichment of *Campylobacter* cells



## **SECTION C: PREPARATION OF ENRICHMENT BROTH CELLS FOR TESTING BY PCR**

Secondary “Exeter” enrichment broths are received from the Public Health Lab (PHL) on the Friday morning and the cells present in the enrichment broths are washed as outlined below.

### **C.1 Cell Harvest and Washing (for further details refer to Figure 13).**

*Lab coats, gloves and eye protection must be worn at all times when carrying out this procedure. Wherever it is practical sample manipulations involving open tubes must be carried out in an approved biohazard cabinet.*

C1.1 Label 1.5ml microcentrifuge tubes (2 for each sample).

C1.2 Add 1ml of the secondary “Exeter” enrichment broth sample to each of the two tubes and repeat for all samples. Note: The two sample sets are processed separately from here on.

C1.3 Centrifuge one of the 1 ml sample sets at 7000 rpm for 20 minutes at 4°C.

C1.4 Remove supernatant from each tube and add 1ml of Phosphate Buffered Saline (PBS), vortex to resuspend cells. Centrifuge 7000 rpm for 10 minutes.

C1.5 Repeat 1.4.

C1.6 Remove supernatant and add 400µl of PBS. Resuspend cells in the PBS and store at -20°C until required.

### **C.2 Long Term Sample Storage**

C2.1 Prior to commencing cell washing, six glass balls are added to sufficient microcentrifuge tubes for the number of samples to be processed. The tubes are

then autoclaved, and dried before adding 500µl BHI broth containing 20% glycerol to each tube.

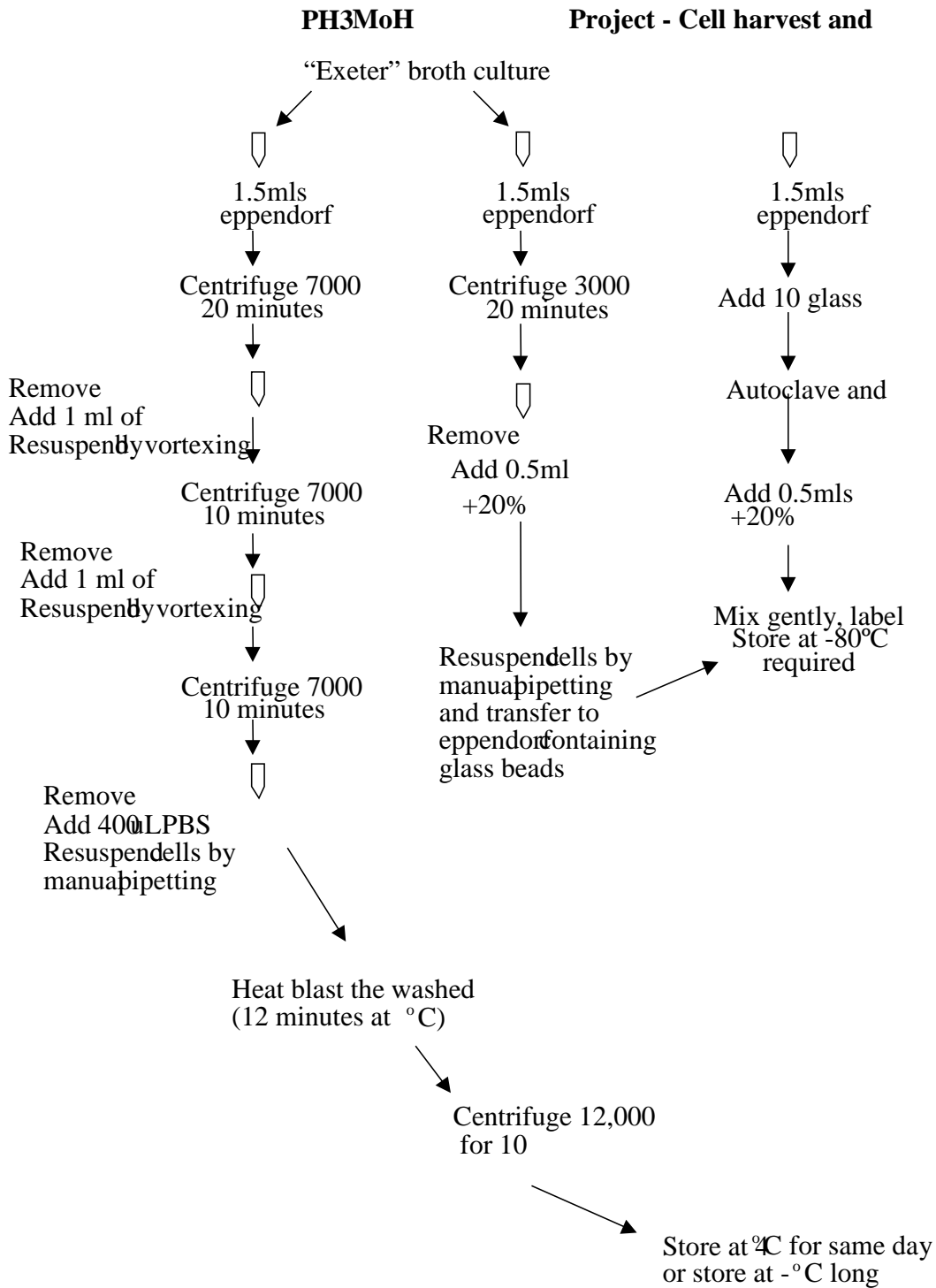
C2.2 Centrifuge the second set of samples at 3000 rpm for 20 minutes.

C2.3 Remove supernatant from each tube and add 500µl Brain Heart Infusion (BHI) broth to which 20% glycerol has been added. Resuspend cells.

C2.4 Transfer the resuspended cells to the microcentrifuge tube containing the glass balls and broth.

C2.5 Label with sample number, gently mix and store at -80°C until required.

**Figure 13 Bacterial Cell Harvest and Washing**



### C.3 PCR Detection of Viable *Campylobacter* Cells

The secondary enrichment step was introduced to ensure that only viable cells were detected. For example, in a water sample, the number of *Campylobacter* cells required to be present in the original sample to give a positive result has been calculated as follows:

Volume of water sample	Number of <i>Campylobacter</i> cells/100ml
10 ml	$2.9 \times 10^6$
100 ml	$2.9 \times 10^5$
500 ml	$5.8 \times 10^4$

1.8 It was calculated from these figures that at least  $5.8 \times 10^4$  non-viable cells would have to be present in the original sample to produce a false positive result, MOH report FW9948, 1999.

**SECTION D: STANDARD PROTOCOL FOR THE DETECTION OF  
*CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER  
COLI* BY THE POLYMERASE CHAIN REACTION**

**D.1 Sample Preparation**

This step is to be performed immediately before PCR. Heat-treated samples are not very stable and must be amplified as soon as possible or stored at  $-20^{\circ}\text{C}$ .

Wear safety glasses during this procedure as heated tubes can explode spilling their contents.

D1.1 Turn on 0.5 ml tube heating block and set at  $100^{\circ}\text{C}$ .

D1.2 Defrost samples to be tested.

D1.3 Heat-treat samples at  $100^{\circ}\text{C}$  for 12 minutes (check temperature on thermometer).

D1.4 Centrifuge samples at 12,000 rpm for 10 minutes at  $4^{\circ}\text{C}$  while preparing the premixes.

**CONTROLS for the PCR reaction**

D1.5 Preparation of a positive PCR control. Measure 80  $\mu\text{l}$  of ddH<sub>2</sub>O into a 0.5 ml tube. Add 10  $\mu\text{l}$  of each working solution (100  $\mu\text{g}/\text{ml}$ ) of DNA from *C. jejuni* and *C. coli* to give a final concentration of 10  $\mu\text{g}/\text{ml}$  DNA per bacterial species. Mix and aliquot 33  $\mu\text{l}$  of PCR positive control into 2 further tubes. Store in  $-20^{\circ}\text{C}$  freezer. Add 10  $\mu\text{l}$  of this solution to the premix for positive control.

Negative PCR control. Add 10  $\mu\text{l}$  of autoclaved deionised water to PCR negative control premix.

## D.2 Preparation of Premix

Prepare sufficient premix for all samples, and positive and negative PCR controls plus a spare tube (Table 6).

### D2.1 Primer preparation

All stock solutions of primers prepared (in manufacturer's tubes) are at 100 nmoles/ml (100 picomoles/ $\mu$ l). To prepare a working solution for PCR, dilute all stock primers 1:10 (10  $\mu$ l primer + 90  $\mu$ l ddH<sub>2</sub>O), to produce a working concentration of 10 pmoles/ $\mu$ l.

### D2.2 Nucleotides (dNTPs)

These are purchased individually from Life Technologies as dATP, dCTP, dGTP and dTTP each at a concentration of 100mM. To prepare a 25 mM solution of dNTP's add equal volumes of each (eg 25  $\mu$ l) to a 0.5 ml tube.

### D2.3 PCR Buffer (supplied by PE Biosystems with the Taq Polymerase enzyme)

10 X Buffer containing 50 mM KCl, 10 mM Tris, pH 8.4, with no MgCl<sub>2</sub> is used.

### D2.4 Polymerase Enzyme

Taq Polymerase enzyme was purchased from PE Biosystems: Amplitaq, 5.0 units/ $\mu$ l.

### D2.5 Magnesium Chloride

MgCl<sub>2</sub> is purchased from PE Biosystems as a stock concentration of 25 mM. It is added to the premix to give a final concentration of 4.0 mM. BSA is also added to help prevent any inhibition, (refer Section F for preparation).

### D2.6 Distilled Water

Dnase / Rnase- Free Water purchased from Life Technologies, Gibco #10977-015.

**Table 6 Template of the Premix for *C. jejuni* and *C. coli* specific PCR**

Reagents	Concentration per reaction tube (µl)	Volume per reaction tube (µl)
dd H <sub>2</sub> O to make final volume of 50µl		e.g. 17.25
25 mM MgCl <sub>2</sub>	4 mM	8
BSA 2 mg/ml	0.2 mg/ml	5
10 x PCR buffer	1 x	5
DNTPs (25 mM each)	250 µM	0.5
Taq Polymerase Amplitaq (5 Units/ µl)	1.25 Units	0.25
<b>Primer stock solutions 10 picomoles/µl</b>		
CeuE forward <i>C. coli</i>	10 picomoles	1.0
CeuE reverse <i>C. coli</i>	10 picomoles	1.0
LpxA forward <i>C. jejuni</i>	5 picomoles	0.5
LpxA reverse <i>C. jejuni</i>	5 picomoles	0.5
Therm 1M Forward Thermophilic <i>Campylobacter</i>	5 picomoles	0.5
Therm 2M Reverse Thermophilic <i>Campylobacter</i>	5 picomoles	0.5
<b>Volume of master mix Per reaction</b>		40 µl
Amount of DNA template		e.g. 10 µl (for heat blasted cells)
Total volume		50 µl

### D.3 Amplification of DNA

Forty µl of premix was aliquoted into each 0.2ml PCR tube without oil. The Perkin Elmer 9700 thermal cycler has a hot top, which negates the need for an oil overlay. Ten µl of sample or control DNA was added to the premix and tubes gently mixed. Tubes were briefly centrifuged to ensure the entire sample was in the bottom of the tube and run on a Perkin Elmer 9700 thermal cycler under the following conditions:

94°C for 3 minute      cycle 1                      (denaturing)

94°C for 1 minute }                              (denaturing)

60°C for 1 minute }      cycles 2-41              (annealing)

74°C for 1 minute }                              (extension)

74°C for 8 minutes      cycle 42                      (extension)

### D.4 Detection of PCR Product

#### Agarose gel:

#### D4.1 Gel Casting

D4.1.1 Prepare individual sterile 100ml Schott bottles each containing 1 gram of agarose.

When ready to pour gel add 50 ml of 1 x Tris Borate EDTA (TBE) running buffer (Section F). Loosen cap and heat in microwave on high power for 1 minute, swirl gently and repeat heating for another 20 seconds or until the gel is homogeneously melted. Wear gloves and avoid contact with steam as the agarose mixture contains ethidium bromide. Allow the gel to cool for 15 - 20 minutes.

D4.1.2 While gel is cooling set up the gel casting tray, ensuring it is level and using the 22 lane comb for the Midicell system.

D4.1.3 Pour the gel into the casting chamber and allow to set - approximately 15 minutes.

D4.1.4 Prepare running buffer (1 X TBE) by diluting 200 ml of 10X TBE with 1800 ml of deionised water. Add 100 µl of ethidium bromide and mix thoroughly. Add enough of the running buffer to the gel chamber to just cover the gel.

#### D4.2 Gel Loading

D4.2.1 Dot 3µl of loading buffer onto a piece of parafilm according to the number of samples to be run.

D4.2.2 Add 10 µl of the PCR product to the dot of loading buffer.

D4.2.3 Mix each dot with pipette tip and load carefully into well, minimising DNA spillage. Load 10 µl of the stock solution of the '1kb plus' DNA ladder (Section F) at the ends of the gel, either side of the samples.

D4.2.4 Once all samples are loaded, close the cover of the tank, plug in the electrodes, set the voltage at 100 Volts and run the gel for one hour and forty minutes or until the blue dye is at the front edge of the gel.

*NOTE:* The steps in B4.2 should be performed without delay and interruption.

D4.2.5 When the time is up, unplug gel tank, and with gloves remove the gel from the tank.

D4.2.6 Transfer to the UV transilluminator and examine gel for DNA bands. Wear UV protective visor for eye and skin protection.

D4.2.7 Take a polaroid photo. Setting red filter, exposure f 5.6 for ½ sec. using black and white film. Double expose the film.

D4.2.8 Remove photograph from the film cassette by pulling the white tab and then the black tab in one sweeping movement.

D4.2.9 Leave the photo on the bench to develop for 45 seconds. Peel away the backing to view the photograph.

D4.2.10 Examine the bands and identify the *Campylobacter* species present.

**Positive Controls:** The following bands must be present in the positive control.

LpxA = 99 bp *C. jejuni*

Therm = 246 bp Thermophilic *Campylobacter*

Ceu = 695 bp *C. coli*

**NB** To confirm the presence of *C. jejuni* in a sample, 2 bands must be visualised on the agarose gel: LpxA band at 99 bp and Therm band at 246 bp.

To confirm the presence of *C. coli* in a sample 2 bands must be visualised on the agarose gel: Ceu band at 695 bp and Therm band at 246 bp.

**Negative Controls:** No bands should be present in the negative controls except for the primer –dimer band. This band confirms that the PCR reaction was not inhibited.

**SECTION E:           PROCEDURE FOR ISOLATION AND RESUSCITATION OF  
*C. JEJUNI* AND/OR *C. COLI***

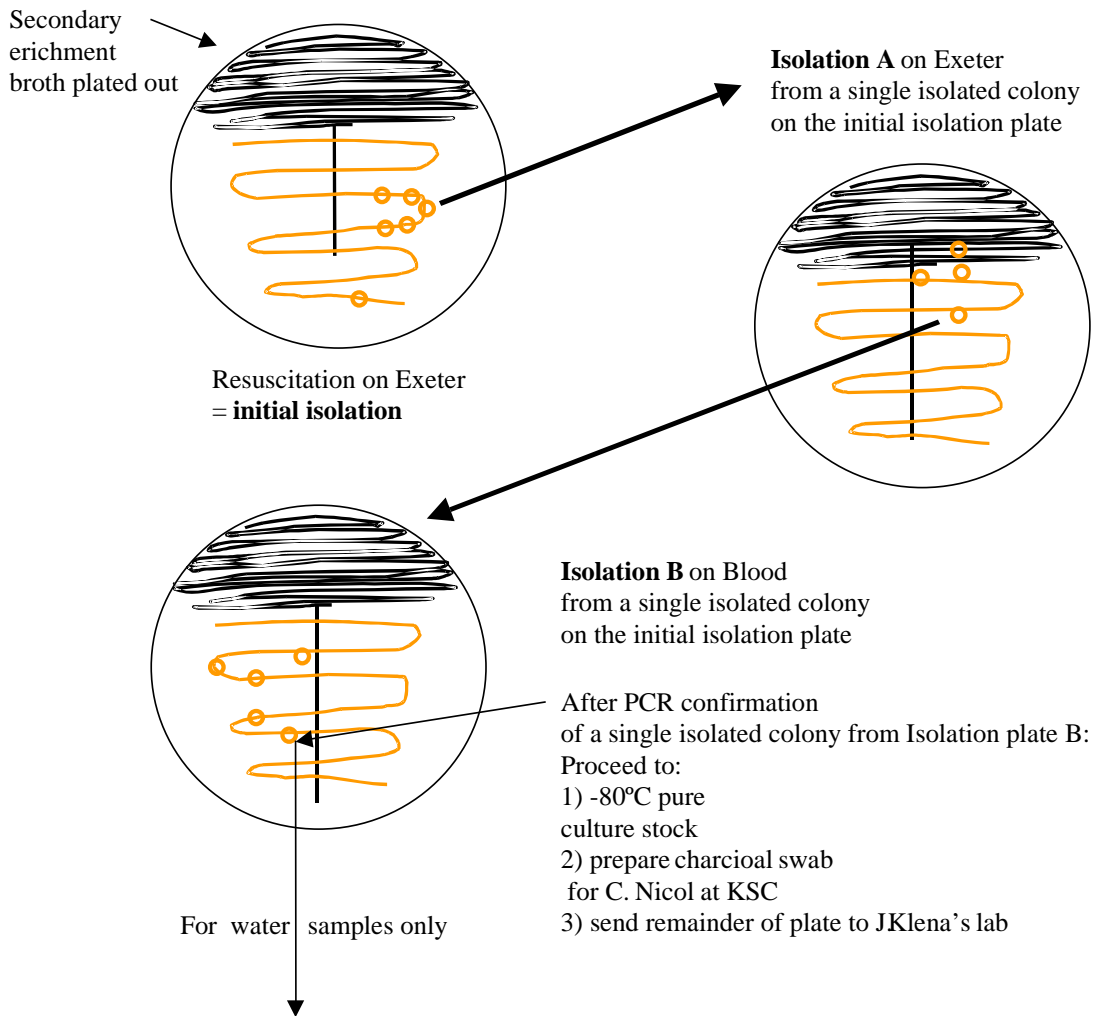
**E.1    Procedure for Isolation and Resuscitation of *C.jejuni* and/or *C. coli***

- E1.1   Secondary Exeter broths that have tested positive for *C. jejuni* and/or *C. coli* by the *Campylobacter* multiplex PCR method, are plated onto Exeter agar and incubated microaerophilically for 48 hours at 42°C.
- E1.2   Isolated colonies from these initial Exeter plates are streak isolated onto Columbia Blood Agar (CBA) for 2 consecutive times, for 48 hours each, to ensure the purity of the isolate
- E1.3   The identity of the selected purified isolate is confirmed by the *Campylobacter* Multiplex PCR of a single isolated colony. The methodology for the PCR reaction is the same as presented in Table 6, with the exception that the distilled water increases to 27.25 µl. This is because whole cells are being added as the DNA template instead of a suspension of washed cells.
- E1.4   The double distilled water (DDW) is added to the PCR tube and a small portion of a single isolated colony is added to the distilled water.
- E1.5   The bacterial cells are lysed by heating in a thermal cycler (Perkin Elmer 9700) at 94°C for 3 minutes and held at 4°C until the PCR premix is added. Thereafter the PCR cycle conditions and the running of the gel and visualisation of the DNA are the same as the methods outlined in Section D.

## E.2 Mixed *C.jejuni* and *C. coli* Samples

- E2.1 A few samples contain both *C. jejuni* and *C. coli* bacteria. These samples are initially isolated onto Exeter agar from the secondary Exeter broth or from the secondary Exeter broth stored in the -80°C freezer. The plates are incubated microaerophilically for 48 hours at 42°C. Faecal samples can also be used to isolate *C. jejuni* and *C. coli* from mixed samples by direct plating of faeces onto Exeter agar. Recovery of *Campylobacter* is enhanced by suspending approximately one gram of faeces in 10 ml of Exeter broth prior to plating out loopfuls onto Exeter agar and incubating under microaerophilic conditions at 42° C for 48 hours.
- E2.2 The *Campylobacter* Multiplex PCR then putatively identifies 8 isolated colonies as. At the same time as a portion of the isolated colony is being added to the DDW for the PCR reaction another portion of the same colony is plated out onto a CBA plate. The plates are incubated microaerophilically for 48 hours at 42°C. One of each *C. jejuni* and *C. coli* identified by PCR, is further purified by streak isolating two consecutive times onto CBA plates. At this stage the *Campylobacter* Multiplex PCR confirms the identification of the bacteria.
- E.3 When the isolates have been identified as either *C. jejuni* or *C. coli* they are prepared for transportation to Kenepuru Science Centre (KSC) for serotyping and PFGE analysis and/or to PaMS Microbiology Laboratory at the University of Canterbury for PFGE analysis. *Campylobacter* strains sent to KSC are transported as Charcoal swabs, whereas, the PaMS Microbiology lab receives *Campylobacter* strains on CBA plates.

Figure 14 *Campylobacter* Isolation and Resuscitation



**Water samples require a further isolation from a single colony**

**Isolation C :**

isolation streak of part of the same colony identified by PCR from isolation plate C.

After PCR confirmation proceed with steps 1-3 as outlined above for isolation B plate

## SECTION F: MEDIA AND REAGENTS

dd H<sub>2</sub>O = double distilled water

### 2% Agarose gel

1 g	Agarose	2%
5 mls	10 x TBE	1 x TBE
45 mls	dd H <sub>2</sub> O	
2.5 µl	Ethidium Bromide	0.5 µg/ml (10 mg/ml stock)

Heat in microwave until all agarose has dissolved. (for details, refer to section on gel casting).

### Brain Heart Infusion (BHI) Broth containing 20% Glycerol

Brain Heart Infusion Broth (Merck 1.10493)	3.7 g
Glycerol (BDH # 10118 4K)	20 ml
Deionised water	80 ml

Weigh the required amount of broth into a Schott bottle and add the water. Mix thoroughly to dissolve broth, microwaving if necessary. Autoclave at 121°C for 15 minutes. Allow to cool and check the pH is  $7.4 \pm 0.2$ . Store at 2-8°C for up to 3 months.

### Bovine serum albumin (BSA) preparation (2 mg/ml)

Weigh out 100 mg BSA (Albumin, Sigma A-4503 from Global Science) into a Falcon tube

Add 50 ml sterile dd H<sub>2</sub>O (2 mg/ml)

Dissolve by shaking. Filter sterilise through a 0.2 µm filter

Dispense aseptically in 1ml aliquots into sterile 1.5 ml tubes

Store in freezer at -20°C

### Buffered Peptone Water 1% (BPW)

Peptone Water (Merck # 1.07228)	25.5 gram
Distilled Water	1 litre
pH	7.2
Autoclave at 121°C for 21 minutes	

### DNA Ladder (1kb plus supplied by Life Technologies)

Make stock solution:

1kb plus DNA (1.0 µg/µl)	30 µl
(Gibco # 10787-018, Life Technologies)	
1 x TBE Buffer	150 µl
Loading Dye	40 µl

Aliquot into Eppendorf tubes and store at -20°C

### Ethidium bromide (10 mg/ml)

Weigh 10 mg Ethidium bromide (Sigma E-8751) into an Eppendorf tube. Add 1 ml of dd H<sub>2</sub>O. Store at 2-8°C.

### “Exeter” medium

Nutrient broth No. 2 (Oxoid) made according to instructions per one litre volumes. After autoclaving, add the following per litre:

- 50 ml lysed horse blood,
- 5 ml filter-sterilised solution containing 4% sodium metabisulphite, 4% sodium pyruvate and 10% iron sulphate solution, (aseptically dispense solution in 5 ml amounts and store in the freezer) 15 mg cefaperazone, (add 2ml/litre of filter sterilised stock solution, 7.5 mg/ml) 2 vials Oxoid supplement SR117E.

Each vial of supplements supplies 2500 i.u. polymixin B, 5 mg rifampicin, 5 mg trimethoprim and 50mg actidione. These components vary from the “Exeter” formulation by the inclusion of actidione, but it provides the convenience of the commercial availability of the antibiotic supplement.

#### “Exeter” agar

Add 15 g of agar to a litre of nutrient broth No. 2 and boil to dissolve before autoclaving. Proceed as above for “Exeter” medium.

#### Gel-loading buffer –for agarose gels

0.25% bromophenol blue (Sigma #B0128)

30% glycerol (BDH #10118 4K) in water

Store at 4°C

#### Phosphate buffered saline (PBS)

Dissolve one PBS tablet (Oxoid) in 100ml of dd H<sub>2</sub>O. Autoclave and store at 4°C.

#### 10 x TBE Buffer

108 g	Trizma Base (Sigma T-8524)	0.9M
55 g	Boric acid (Sigma B-6768)	0.9M
40 mls	0.5 M EDTA pH 8.0	0.02M

Dissolve the above in 900 ml dd H<sub>2</sub>O and make up to 1L;

or purchase 10 x TBE powder from USB # 70454 which comes makes 200ml aliquots when reconstituted.

1 x TBE (working TBE)

200 ml	10 x TBE	0.09M
1800 ml	dd H <sub>2</sub> O	
100 µl	Ethidium Bromide	0.5 µg/ml
	(10 mg/ml stock)	

or dilute 10 x TBE stock solution 1:10 in distilled water