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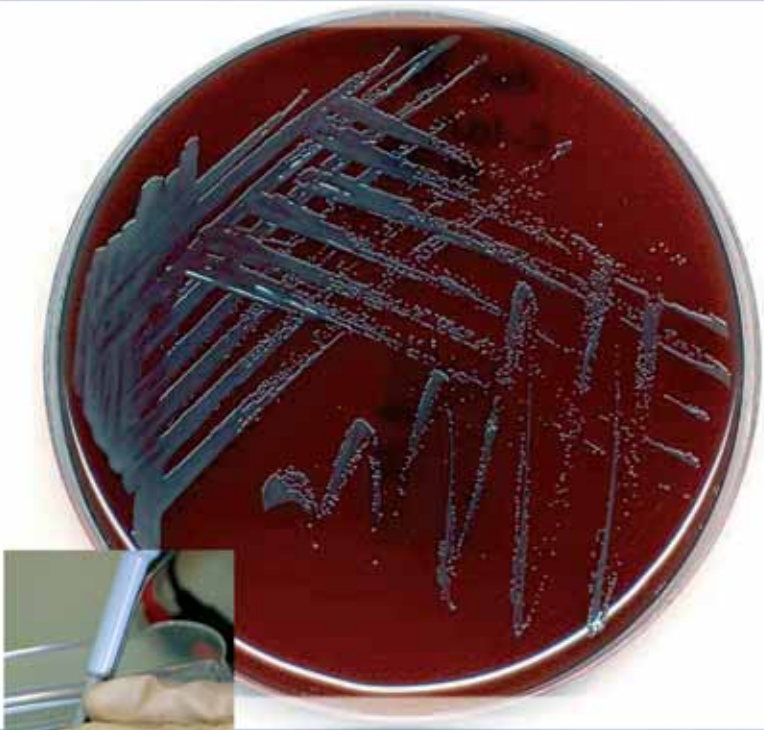
Recovery and identification of emerging *Campylobacteraceae* from food



Ashtown Food Research Centre

RESEARCH & TRAINING FOR THE FOOD INDUSTRY

RESEARCH REPORT NO 85

RECOVERY AND IDENTIFICATION OF EMERGING *CAMPYLOBACTERACEAE* FROM FOOD

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SUMMARY

The family *Campylobacteraceae* includes 23 different species of *Campylobacter* and *Arcobacter*. To date, clinical and epidemiological interest has focused almost exclusively on just two of these species, *C. jejuni* and *C. coli*. Current routine examination methods for both clinical and food samples look exclusively for these two species. Recent clinical research indicates that some of the other, previously ignored *Campylobacter* species may be linked to human infection. The focus of this research was to develop a routine procedure which would allow recovery of all 23 species of *Campylobacteraceae* from food samples.

The method developed involved an initial enrichment of test sample (25 g) in *Campylobacter* Enrichment Broth (225 ml) with 5% laked horse blood. The sample was pulsified for 15 sec and incubated for 24 h at 37°C under modified atmosphere conditions (2.5 % O_2, 7 % H_2, 10 % CO_2 and 80.5 % N_2). After incubation, 10 ml of broth was centrifuged at 2500 rpm for 10 sec. A mixed ester membrane was placed on the surface of an Anaerobe Basal Agar (ABA) plate with 5 % laked horse blood. An aliquot of supernatant (200 µl) was placed on the membrane surface for 15 min during which time motile *Campylobacteraceae* passed through to the agar surface. Plates were incubated for up to 6 days in the modified atmosphere described above. Suspect colonies were confirmed by a number of biochemical tests including two novel procedures developed within this EU-funded project. The first is a kit which examines bacteria for the presence of L-alanaine aminopeptidase (OBIS L-ALA kit, Oxoid), and the second is a Latex agglutination test against all *Campylobacteraceae* (Microgen Bioproducts, UK). The presence of bacteria belonging to the family *Campylobacteraceae* was confirmed by examining colonies for a unique gene (16S- rRNA) using a Polymerase Chain Reaction (PCR). Individual species of *Camplyobacter* and *Arcobacter* were identified using a series of further single and multiplex PCR reactions, each of which targeted a gene unique to the particular species.

The protocol developed has been shown to successfully recover all 23 species of *Campylobacteraceae* at a detection level of 10 cfu g^{-1} from both fresh meat (beef, pork and poultry) and bovine faeces. The method has shown the presence of a

range of naturally-occurring *Campylobacteraceae* (*C. jejuni, C. coli, C. lari, C. upsaliensis, C. mucosalis, C. sputorum*) in chicken, pork, beef and bovine faeces.

INTRODUCTION

Campylobacter spp. are recognised as a leading cause of foodborne illness and are the commonest cause of bacterial gastro-enteritis in Ireland. In 2005, there were 1803 human cases (Crude Incidence Rate, 46/100,000) of Campylobacteriosis in the Republic of Ireland with the highest burden of infection in children under 5 years of age (HPSC, 2005). While these figures are high, it should be noted that the isolation and identification methods used to examine clinical and food samples for *Campylobacter* are designed primarily to detect only two species, *C. jejuni* and *C. coli*, which up to now have been assumed to be the key clinically relevant *Campylobacteraceae*. The current routine procedure for *Campylobacter* involves enrichment under microaerophilic conditions at 42°C, recovery on culture media containing blood, detoxifying charcoal and selective antibiotics, and confirmation by motility, hippurate hydrolysis, catalase and nitrogen production. It is now recognised that these procedures inhibit the recovery of many of the 23 species of *Campylobacteraceae.* However, isolation using a gas atmosphere containing hydrogen with recovery by filtration onto blood agar at a lower temperature of 37°C (to facilitate recovery of thermo-sensitive strains) reportedly detects a much wider range of *Campylobacteraceae* spp. (known and emergent) from clinical specimens (Lastovica and Le Roux, 2000). Using this modified methodology, epidemiological evidence is now accumulating from studies in South Africa and Europe that emergent *Campylobacter* species including *C. concisus* and *C. upsaliensis* are being isolated at high rates from human stool samples and may be as clinically significant as *C. jejuni* and *C. coli*. (Engberg *et al.*, 2000; Aabenhus *et al.*, 2002; Maher *et al.*, 2003). Information on the reservoirs of emerging *Campylobacteraceae* in the agri-food chain is very sparse and generally related to sources of infection in outbreaks. It is, therefore, essential to modify current detection methods for the isolation of *Campylobacteraceae* in food and environmental samples. Successful modification will also allow effective monitoring and surveillance together with

a better understanding of the epidemiology and transmission routes of these pathogens and an assessment of the public health risk that they may pose.
The focus of the research described in this report was to improve the isolation, detection and identification of emerging *Campylobacteraceae* from fresh meat and animal faeces.

CAMPYLOBACTERACEAE

Campylobacteraceae species (n = 23) of human, animal and clinical origin used in this study were obtained from the Danish Food and Veterinary Research Institute in Copenhagen and maintained at –80ºC (Table 1)

Table 1: *Campylobacteraceae* spp. used in the study

C. jejuni subsp. *jejuni*	*C. hyointestinalis*
C. jejuni subsp. *doylei*	*C. mucosalis*
C. coli	*C. concisus*
C. upsaliensis	*C. sputorum* biovar. *sputorum*
C. fetus subsp. *fetus*	*C. ureolyticus*
C. fetus subsp. *venerealis*	*C. gracilis*
C. lari	*C. rectus*
C. hyoilei	*C. helveticus*
C. showae	*C. hominis*
C. lanienae	*C. curvus*
Arcobacter butzleri	
Arcobacter skirrowi	
Arcobacter cryaerophila	

OPTIMISATION OF ENRICHMENT PROCEDURE

Growth of the *Campylobacteraceae* spp. (Table 1) was examined in a number of different media {Hunts broth (Ransom and Rose, 1998); Bolton broth (Oxoid, U.K.); *Campylobacter* Enrichment broth (CEB)(LAB M, U.K.)} incubated at

37ºC in an atmospheric cabinet (Don Whitley) containing 2.5 % O_2, 7 % H_2, 10 % CO_2 and 80.5 % N_2 (Keevil *et al.*, 2006). During the enrichment period (72 h), *Campylobacter* were plated out at various intervals onto different agar plates (Tryptose blood agar with 5 % lysed horse blood or Anaerobe Basal Agar (ABA) (Oxoid) with 5 % lysed horse blood) and enumerated. Motility was also determined at these intervals using NanoOrange staining (Molecular Probes, The Netherlands) according to the method of Alonso *et al.* (2002). The results indicated that enrichment in CEB broth and plating onto ABA with 5% lysed horse blood yielded good growth of all species examined with motility maintained; this latter factor is believed to be critical for the next phase of extraction.

OPTIMISATION OF ISOLATION PROCEDURE

Meat pieces or bovine faeces (25 g) were inoculated with selected *Campylobacter* species (Table 1) to a level of 10 or 100 cfu g^{-1}, placed in a filter stomacher bag with 225 ml of CEB and 5 % lysed horse blood, and incubated for 24 h or 48 h at 37 ºC in the gas atmosphere described above. Options were investigated for recovery of *Campylobacter* from the enrichment broth, alone and in combination, including (i) stomaching versus pulsification (Microgen Bioproducts, U.K.); (ii) centrifugation of enrichment broth to remove food debris (different times and speeds); (iii) membrane filtration (different membrane materials and pore sizes: cellulose nitrate 0.45 µm; cellulose acetate 0.45 µm; cellulose nitrate 0.60 µm; mixed ester 0.60 µm; polycarbonate 0.60 µm) and (iv) the volumes of enrichment broth to be placed on the membrane surface (0.2, 0.3, 0.4 ml). The optimised method is shown in Figure 1.

IDENTIFICATION OF *CAMPYLOBACTERACEAE*

Biochemical tests

Colonies considered to be presumptive *Campylobacteraceae* were confirmed by a number of initial biochemical tests including the Gram reaction, hippurate test, catalase and oxidase tests in addition to two novel biochemical tests designed within the EU project consortia. These were (i) kits which examine

bacteria for the presence of L-alanine aminopeptidase (OBIS L-ALA kit, Oxoid, U.K.) and (ii) a Latex agglutination test against all *Campylobacteraceae* as described by Clark *et al.* (2005) (Microgen Bioproducts, U.K.).

Polymerase Chain Reaction (PCR)

Initially, bacteria were confirmed as members of the family *Campylobacteraceae* using a Polymerase Chain Reaction via the presence of the 16S- rRNA gene which is unique to the family *Campylobacteraceae* (Linton *et al.*, 1996). The amplicon product size was 816-bp. All bacteria positive for this gene were then identified to species level.

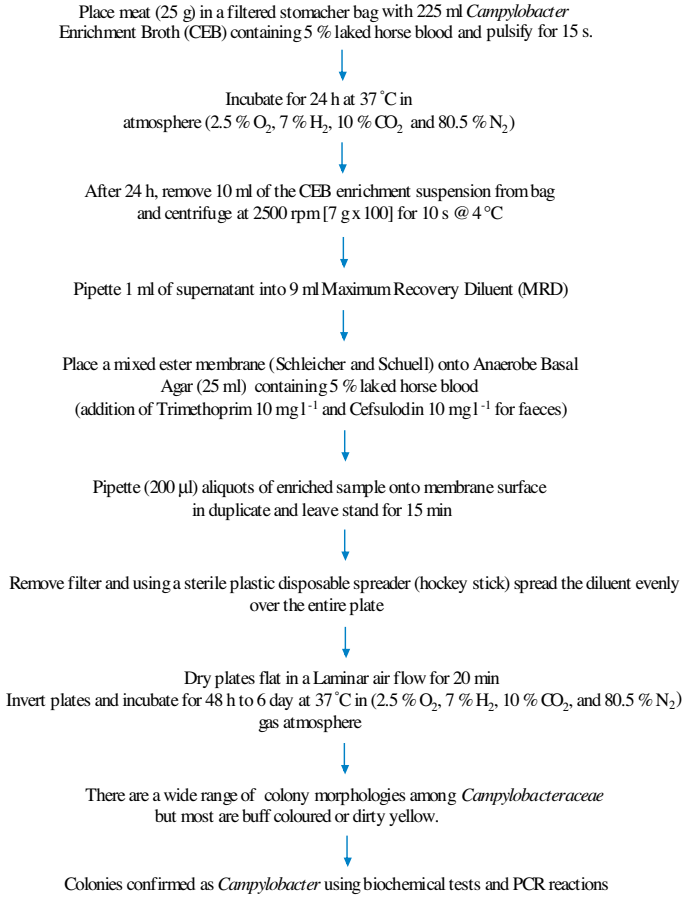
Campylobacter and *Arcobacter* species-specific genes and primers were selected and applied in a number of multiplex or singleplex PCR reactions. These included a multiplex PCR for *C. jejuni* subsp *jejuni, C. coli, C. lari*, and *C. upsaliensis* (Klena *et al.*, 2004). This PCR targets differences in the nucleotide sequence of the *IpxA* gene to distinguish between these 4 species. The *IpxA* encodes for the enzyme LpxA which catalyses the first step in lipid A biosynthesis and subsequently lipopolysaccharide biosynthesis. A further multiplex PCR, developed by the EU Project consortia (USDA, Personal communication) and due for publication in 2007, identified five species, *C. helveticus, C. fetus* subsp. *fetus, C. curvus, C. lanienae* and *C. hyointestinalis*. Single PCR reactions were conducted to identify *C. concisus* (Bastyns *et al.*, 1995) and *Arcobacter* spp. (Brightwell *et al.*, 2006). As yet, there is no routine PCR test for the remaining 6 species (*C. gracilis, C. hyoilei, C. showae, C. lanienae, C. rectus, C. hominis*). These can be identified by performing a full biochemical matrix analysis (Lastovica, 2006), by ribotyping (DeCesare *et al.*, 2006) or by gene sequencing technologies. Routine PCR methods are under development for these species.

VALIDATION OF THE DEVELOPED METHOD

Beef, pork and chicken (25 g) were inoculated individually with each of the above 23 *Campylobacteraceae* spp. to a level of (10 cfu g^{-1} or 100 cfu g^{-1}) and their recovery examined using the protocol illustrated in Figure 1. This experiment was conducted in triplicate; results showed that the method

facilitated recovery of all species of *Campylobacteraceae* from each of the three fresh meat types at both inoculum levels. Bovine faecal samples (25 g) were each inoculated individually with the 23 *Campylobacteraceae* spp. at 10 cfu g^{-1} or 100 cfu g^{-1} and recovery tested using the protocol described in Figure 1 but with the addition of Trimethoprim (10 mg l^{-1}) and Cefsulodin (10 mg l^{-1}) into the ABA plates to control growth of the background micro-flora. This experiment was also conducted in triplicate. Results showed that the method facilitated recovery of all species of *Campylobacteraceae* from faecal material at both inocula (Duffy *et al.*, 2006).

Fig 1. Flow diagram of method for isolation and detection of *Campylobacteraceae* from fresh meat



APPLICATION OF METHOD TO FOOD CHAIN SAMPLES IN IRELAND

The protocol developed was applied in a snapshot study to examine fresh meats (beef, pork and chicken) and bovine faeces in Ireland for the presence of *Campylobacteraceae*. In 32 samples positive for *Campylobacter* (chicken, pork, beef), the following *Campylobacteraceae* were found: *C. jejuni* (n = 13), *C. coli* (n = 12), *C. lari* (n = 7), *C. upsaliensis* (n = 2), *C. mucosalis* (n = 4) and *C. sputorum* (n = 2). Overall, 40 isolates were recovered from the 32 samples with a number of samples containing more than one *Campylobacter* species.

CONCLUSION

A method was developed for the isolation, detection and identification of emerging *Campylobacter* in meat (beef, pork and chicken). This included a modified atmosphere enrichment system, extraction technique, plating agar and molecular identification tools. The method has been successfully transferred to both national and international laboratories working in this area and biochemical and antibody-based tests developed by the EU project consortia will be launched commercially in 2007. The outcome of this project is the availability of tools with which the public health risk posed by emergent *Campylobacteraceae* may be assessed. Ongoing research (below) will establish the risk posed by these emergent *Camplyobacter* species in Irish food.

FUTURE RESEARCH

Follow-on research is being undertaken at Ashtown Food Research Centre (Teagasc) and the Public Health Laboratory (Health Service Executive, Dublin Mid Leinster, Cherry Orchard Hospital, Dublin) and is funded under the Food Institutional Research Measure. This is initially aimed at establishing the frequency with which these species are present in food and human diarrhoeal samples in Ireland. Studies will then be conducted to establish if these emergent species are as virulent as the well-recognised *C. coli* and *C.*

jejuni species. Genomic technologies will be used to compare isolates from food and clinical sources and will establish if they contain known or novel genes linked to virulence. Additionally, the fitness of selected isolates to typical foodborne stresses will be established to determine whether additional controls are needed to address these new species. Stakeholders will be kept fully informed of developments and on the risks posed by these newly emergent *Campylobacter* species.

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