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of LIVERPOOL

The Molecular Epidemiology of *Campylobacter jejuni* in Ruminants.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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Abstract.

The primary objective of this study was to describe the temporal and spatial aspects of the epidemiology of ruminant *Campylobacter jejuni*. The work presented in this thesis is nested within a larger multidisciplinary study investigating the epidemiology of human campylobacteriosis in Lancashire and will be used to quantify and describe the contribution of ruminant derived campylobacters to the human disease burden.
Fifteen dairy and four sheep farms in three geographically distinct areas of Lancashire namely the southern Fylde, the Pennines east of Lancaster and the southern Pennines around Clitheroe were sampled at eight week intervals over a two year period. Twenty random freshly voided faecal samples were collected from lactating cows or adult sheep at each sampling event. Isolation of up to four putative campylobacters per faecal sample was performed using standard microbiological techniques. Speciation was performed using a number of PCR assays.
We investigated the test performance of the hippurate hydrolysis test, aerobic non-growth and a number of PCR assays in order to optimise our laboratory protocol. This was carried out at the start of the study during February – March 2006 when all cattle were in winter housing. The high prevalence of *Arcobacter* spp found in bovine faecal pats was responsible for the poor specificity observed with the16S rRNA PCR assay for identification of the genus *Campylobacter* (Linton *et al.* 1996) such that its use was subsequently abandoned. The hippurate hydrolysis test was relatively sensitive and specific for identification of *C. jejuni* with both false positives and false negatives occurring, but perhaps, with the advent of PCR assays, it should no longer be considered to be a "gold standard" for identification of *C. jejuni*. The ability of *Arcobacter* spp to grow in air coupled with the inability of almost all campylobacters to grow in air provided a highly sensitive and specific means of differentiating campylobacters from arcobacters and was subsequently included in our routine speciation protocol.
Campylobacter jejuni was isolated from every farm although not on every sampling occasion. There was considerable variation in the faecal pat prevalence of *C. jejuni* both between farms and temporally. Peak prevalence in both cattle and sheep was observed during the summer but in dairy cattle this peak was associated with grazing at pasture rather than season *per se* although in sheep it was truly seasonal. We hypothesise that in dairy cattle this may be related to the very different diets fed to housed cattle compared to grazed grass. This finding deserves further investigation since it might offer the prospect of dietary interventions to control this zoonotic pathogen. Both increase herd size and milk yield were associated with increased prevalence whilst in sheep both increased group size and pasture quality were associated with increased prevalence.
Multi-locus sequence typing (MLST) was performed on 1000 of the 2,300 *Campylobacter jejuni* isolates collected. Full allelic profiles were obtained for 94% of the isolates sequenced. 86 previously unrecorded sequence types were identified. Eighty seven percent of isolates were assigned to the following clonal complexes (C-C) using BURST at http://pubmlst.org/ : C-C 21, C-C 42, C-C 45, C-C 48, C-C 52, C-C 61, C-C 206, C-C 257 and C-C 403, in broad agreement with previous ruminant studies. All of these clonal complexes have been associated with human disease. There was considerable farm level variation at clonal complex level both in the occurrence and prevalence of clonal complexes. In cattle C-C 61, C-C 21, C-C 403 and C-C 45 were the most prevalent clonal complexes isolated whilst in sheep C-C 42, C-C 21, C-C 48 and C-C 52 were the most prevalent. Multivariate analysis

suggested a seasonal trend in the case of isolates belonging to C-C 45 with peak prevalence observed during the summer.
We employed measures of gene flow, linkage disequilibrium, and overall diversity to describe the genetic diversity present in ruminant *C. jejuni* isolates. Gene flow analysis demonstrated a high level of genetic homogeneity between populations at all levels of analysis suggesting that much of the considerable diversity observed was generated prior to establishment of the farms sampled. Farm livestock purchase policy did not appear to influence farm level diversity with the greatest diversity observed on farms that did not purchase stock, implying that between farm transmission is relatively unimportant in generating diversity. The presence of linkage disequilibrium was demonstrated, with the standardised indices of association (I_A^S) observed being consistent with a weakly clonal population structure as has been described previously for *C. jejuni.* Diversity, as demonstrated by the number of different sequence types present, appeared to be greater amongst cattle isolates compared to sheep isolates. Similarly there appeared to be greater diversity amongst isolates collected from farms in the Pennines compared to those from the Southern Fylde. At clonal complex level, diversity was greater amongst isolates belonging to C-C 21, C-C 45 and C-C 206. These findings suggest that acquisition of *Campylobacters* from wildlife may play a role in generating diversity amongst ruminant *C. jejuni* populations.

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Chapter One.

Introduction and Literature review.

1. 1 Introduction

Infection with *Campylobacter* spp is recognised as a major cause of infectious gastroenteritis worldwide. It is estimated to cause between 5 – 14% of cases of diarrhoea globally (WHO 2005). In the UK, *Campylobacter* spp are the most frequently diagnosed cause of infectious diarrhoea. Peak incidence of confirmed cases was in 1998 when 58,059 cases were diagnosed microbiologically (Frost *et al.* 2002), although the incidence of confirmed cases appears to be falling currently (Fig 1.1) with 44,343 cases being reported in 2005 (DEFRA 2005) in England and Wales. This equates to an incidence of ~ 80 cases/100,000 people. There is considerable variation in reported incidence rates world-wide with the reported incidence rate varying from 21.9 cases/100,000 (Samuel *et al.* 2004) in the USA to 400/100,000 people in New Zealand (Baker *et al.* 2006) . However since the majority of human cases are not reported, these figures are likely to be a gross under-estimate of the true incidence rates (Tauxe 1992). *Campylobacter* spp are hyper-endemic in developing countries with community based case-control studies providing incidence rate estimates of 40,000 – 60,000 / 100,000 for children under 5 years of age (Oberhelman & Taylor 2000, Rao *et al.* 2001).

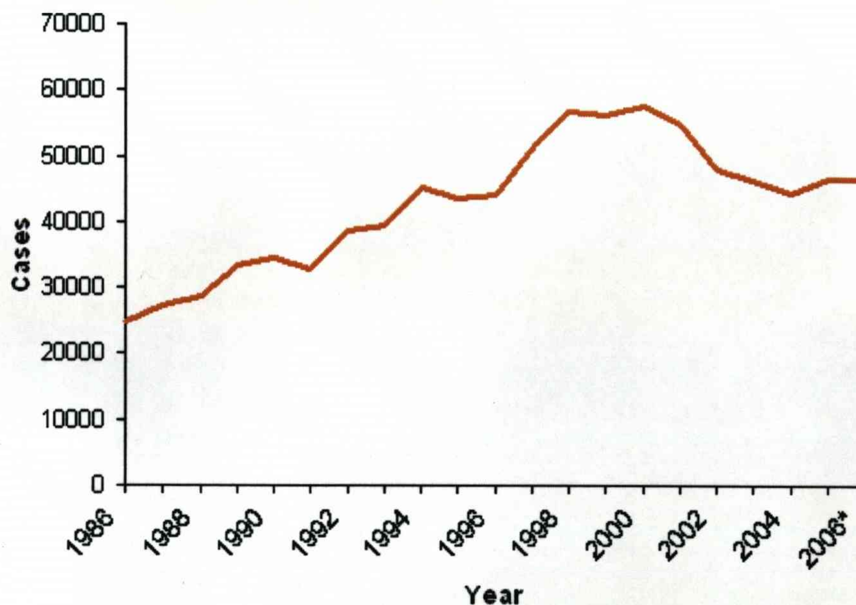


Figure 1.1. Number of laboratory reports of confirmed isolation of *Campylobacter* spp from human faecal samples from England and Wales between 1986 – 2006. Source: Health Protection Agency.

Most cases of human campylobacteriosis are self limiting with fever, abdominal pain, diarrhoea and nausea with vomiting being the chief clinical signs observed. The incubation period is between 1 – 10 days (median 2 days) with illness usually lasting for about 7 days (Chin 2000). Treatment consists of fluid therapy although antibiotics are used in some cases.

Guillian Barre syndrome (GBS) and Reiters syndrome (reactive arthritis) have been reported as auto-immune related sequelae to *Campylobacter* spp infection (Altekruse *et al.* 1999). One case of GBS is estimated to occur per 1000 cases of campylobacteriosis with 40% of cases of GBS showing evidence of recent campylobacter infection (Allos 1997) whilst Reiter's syndrome is believed to occur in up to 1% of campylobacteriosis cases (Peterson 1994).

1.2 Microbiology

The genus *Campylobacter* was first proposed in 1963 by Sebald and Veron and originally contained two species, namely *C fetus* and *C bubulus* (now known as *C sputorum* var *sputorum*). Currently there are 16 species and six subspecies within the genus. *Campylobacter* spp are slender Gram negative curved rods (Campynet 2005). Most species are motile by virtue of an unsheathed flagellum at one or both ends of the cell. This is thought to allow the organism to colonise mucus within the intestinal and caecal crypts (Lee et al 1986) and is considered to be a major virulence factor. Campylobacters are micro-aerophillic, growing best in an atmosphere containing 5 – 10% oxygen. Campylobacters are acid sensitive and will not grow below pH 4.9 with optimal growth of *C. jejuni* occurring in the pH range 6.5 – 7.5. Most campylobacters of medical importance are referred to as thermophillic reflecting not only their ability to grow at high temperatures but also their inability to grow at temperatures below 30^0C (Stanley & Jones 2003). The optimal temperature for growth is 42^0C reflecting the adaption to temperatures within the avian gut.

This combination of micro-aerophillic and thermophillic growth requirements means that their chief reservoir are the intestines of warm blooded animals and birds and they do not multiply in the environment or in foodstuffs or water although biochemical investigations (Kelly et al 2001) suggest that *C. jejuni* can in fact exploit more diverse environments than previously thought.

It would appear that the main survival strategy for campylobacters is production of vast numbers such that enough will survive in the environment to infect new hosts (Jones 2001).

In light of their thermophillic nature and inability to amplify outside their host species, it is suggested that they are considered as "food-borne" rather than "food-poisoning" pathogens.

1.3 Isolation and speciation.

Campylobacter spp were first isolated from human blood cultures of diarrhoeic patients in 1938. Isolation from faeces was first achieved in 1972 using the technique of membrane filtration. This was followed by the development by Skirrow (1977) of a selective medium utilising a cocktail of antibiotics designed to isolate *C. jejuni* and *C. coli*. However this did not allow isolation of other less common campylobacters due to their sensitivity to the antibiotics used. Since then a large number of isolation and enrichment media for *Campylobacter* spp have been developed. These are extensively reviewed by Corry *et al.* (1995). Being micro-aerophillic, *Campylobacter* spp are best grown in an atmosphere with increased CO_2 and decreased O_2 tension, typically 6% O_2, 10% CO_2 and 84% N supplied by a commercially available generator envelope. Bolton *et al.* (1997) investigated the efficacy of three commercial gas generating systems and a novel evacuation-replacement technique for isolation of campylobacters and found no significant differences in performance. Whilst most campylobacters grow at 37^0C, *C jejuni* is best grown at 42^0C (Quinn *et al.* 2002), corresponding to avian body temperature.

Until the advent of DNA based technologies, species identification of campylobacters utilised biochemical testing (Penner 1988). However identification at the species level alone is inadequate for detailed epidemiological investigations and considerable effort has gone into the development of tools for sub-typing *Campylobacter* spp. One of the most widely used typing method is serotyping utilising both heat labile and heat stable antigens (Penner & Hennessey 1980, Lior *et al.* 1982). The Penner scheme is a modified direct method of typing heat stable antigens routinely used in the UK for surveillance purposes (Frost *et al.* 1998). Another typing method is Preston biotyping (Bolton *et al.* 1992) which uses the H_2S production test and the deoxyribonuclease test together with tests for susceptibility to a variety of agents incorporated in the

culture medium. This allows isolates to be typed using a four digit biotype code. Phage typing (Salama 1990) may also be used for sub typing purposes.

Wareing *et al.* (2002) used serotyping, biotyping and phage typing in combination to investigate phenotypic diversity in isolates from human cases. Using all three methods together, only 2.5% of isolates were untypable whereas the use of only one method alone would result in approximately 15% of isolates being untypable. This combination of methods now forms the basis of the Health Protection Agency (HPA) national reference typing scheme for the UK. However phenotypic typing methods suffer from a number of disadvantages, including the high number of untypable strains, cost of reagents and the technically demanding requirements of these techniques.

Genotyping techniques are increasingly used and offer considerable advantages including potential universal availability and increased discriminatory power. The chief techniques currently in use are:

- **Identification via polymerase chain reaction (PCR) amplification** of specific DNA sequences unique to a species. PCR methodologies allow rapid screening and identification of large numbers of isolates. Numerous PCR assays have been developed and include:
    - Pan-Campylobacter 16S rRNA PCR for identification of the genus *Campylobacter* (Linton *et al.* 1996).
    - Colony multiplex PCR (Wang *et al.* 2002) for identification of 23S rRNA from *Campylobacter* spp, *hipO* gene (hippuricase) from *C. jejuni* subsp *jejuni*, and *GlyA* gene (serine hydroxymethyltransferase) from *C coli* and *C lari*.
    - 16S rRNA duplex PCR for identification of *C. hyointestinalis* and *C. fetus* (Linton *et al.* 1996).
    - *ceuE* gene PCR (Gonzalez *et al.* 1997) for identification of *C. jejuni*.
    - *ceuE* gene PCR (Gonzalez *et al.* 1997) for identification of *C. coli*.

On and Jordan (2003) investigated the test performance of 11 PCR assays for the identification of *C. jejuni* and *C. coli* and found no one test to be 100% sensitive or specific. They suggest that a polyphasic strategy involving series testing using more than one PCR should be adopted for maximum accuracy in assignment to species.

- **Flagellin typing** (*fla* typing). The flagellin gene locus of *C. jejuni* contains 2 flagellin genes (*flaA* and *flaB*) arranged in tandem with both highly conserved and variable regions present. Restriction Fragment Length Polymorphism (RFLP) analysis based on the flagellin gene *flaA* (Nachamkin *et al.* 1993) is widely used. Essentially this involves amplification of the *flaA* gene by PCR followed by use of a restriction enzyme to generate PCR product fragments which are then separated by electrophoresis. At least seven *fla* typing procedures utilising both different primers and restriction enzymes have been developed (Wassenaar & Newell 2000) and there is a need for standardisation of techniques including primers and restriction enzymes in order to compare results from different laboratories. Harrington *et al.* (2003), in an attempt to address this problem, used the CAMPYNET (http://campynet.vetinst.dk/) sample set of 100 isolates of *C. jejuni* and *C. coli* to compare three fla typing methods. They suggested using the full *flaA* gene followed by restriction digestion with the enzyme *Dde*1 for optimal discrimination and inter-laboratory reproducibility. Attempts have been made to correlate flagellin genotypes with Lior serotype in light of the putative role of flagellin as the dominant heat labile antigen in the Lior serotyping scheme; results suggest only weak correlations between serotype and *fla* type (Nachamkin *et al.* 1996).
- **Amplified Fragment Length Polymorphism** (AFLP) fingerprinting is a technique for sub-typing bacterial species. It is based on the selective amplification of restriction fragments of bacterial chromosomal DNA following complete digestion of the DNA with two restriction enzymes, one with a 4 base-pair (bp) recognition site and one with a 6 bp recognition site. It is designed so that only fragments flanked by both recognition sites are amplified. These fragments are labelled with a fluorescent or radio-active marker and separated on polyacrylamide gels (Wassenaar & Newell 2000). The technique was developed for *Campylobacter* spp by Duim *et al.* (1999). It has the advantage that it can be easily automated allowing standardisation and high throughputs of strains making it suitable for epidemiological investigations.

- **Pulsed-Field Gel Electrophoresis** (PFGE). In this technique the bacterial DNA is cleaved using by restriction enzymes that cut infrequently. The large DNA fragments are separated by electrophoresis under conditions in which the orientation of the electric field is changed in a pulsed manner (Wassenaar & Newell 2000). In order to protect the bacterial DNA from shearing, it is immobilised in agarose blocks prior to cell lysis. All further steps are carried out by means of enzymatic diffusion into the blocks. The blocks are directly loaded onto agarose gels for electrophoresis. A number of different protocols have been developed by different laboratories making inter-laboratory comparison hard. In an attempt to address this problem, standardised protocols have been developed under the auspices of PulseNet (www.cdc.gov/pulsenet). Ribot *et al.* (2001) describe such a protocol using the restriction enzyme *Sma*1.
- **Multi-Locus Sequence Typing** (MLST). The afore-mentioned genotyping methods all suffer from a lack of standardisation making comparisons between different laboratories or countries challenging. MLST was developed in part to address this problem of standardisation. A major advantage of MLST is that sequence data obtained from different laboratories can be readily compared. The data lends itself to electronic storage and distribution thus allowing international comparison. An internet site has been set up as a repository and database for *Campylobacter* spp MLST data (http://campylobacter.mlst.net). . Since its inception in 2001, MLST is increasingly recognised as the "gold standard" for molecular typing of *C. jejuni* and *C. coli*. MLST is well suited for long term and global epidemiology since it identifies variation which is accumulating slowly in the population. Essentially the technique involves the amplification and sequence typing of seven housekeeping genes present in the *Campylobacter* chromosome (Dingle *et al.* 2001).

  The following seven loci were chosen for the *Campylobacter jejuni* MLST scheme (protein products are shown in parantheses).

  - *aspA* (aspartase A)
  - *glnA* (glutamine synthetase)
  - *gltA* (citrate synthase)

- *glyA* (serine hydroxymethyl transferase)
- *pgm* (phosphoglucomutase)
- *tkt* (transketolase)
- *uncA* (ATP synthase α subunit)

The loci are all sufficiently separated on the bacterial chromosome, with the minimum distance between loci being 70 kb (Fig 1.2), such that coinheritance of two or more loci in a recombination event is considered unlikely.

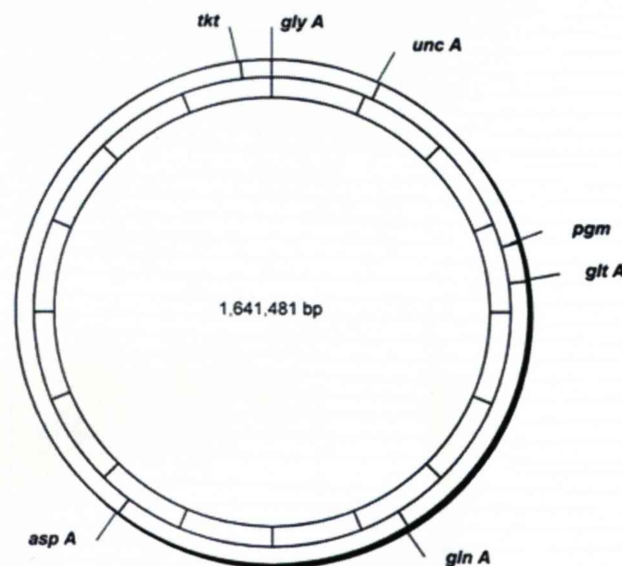


Figure 1.2. Chromosomal locations of MLST loci. The positions of the seven loci are shown on a map of the *C. jejuni* chromosome derived from the genome sequence of isolate NCTC 11168 (http:sanger-.ac.uk/Projects/C_jejuni). The 1,641,481 –bp genome is divided into 10 segments (indicated on the inner circle) with each segment representing 164,148 bp (from Dingle *et al.* 2001).

Such genes evolve slowly since they are essential for metabolic functions. The technique was developed for *C. jejuni* by Dingle *et al.* (2001) based on data from 194 isolates from a number of sources. A total of 155 sequence types (STs) were identified which were placed in 62 clonal lineages or complexes. The members of a clonal complex were defined, using the computer program BURST (http://pubmlst.org) as two or more independent isolates with an ST that shared identical alleles at four or more loci (of the seven loci sequenced). The sequence data for the seven housekeeping genes suggested that horizontal gene exchange,

including import of alleles from other *Campylobacter* spp including *C. coli* has a major influence on the structure and evolution of *Campylobacter jejuni* populations. MLST data suggests that *C. jejuni* whilst being genetically diverse has a weakly clonal population structure with a number of clonal complexes (C-C) corresponding to "lineages" i.e. they are derived from a common ancestor (Dingle *et al.* 2002). A number of studies (Dingle *et al.* 2002, Colles *et al.* 2003, Manning *et al.* 2003) have found that certain clonal complexes are associated with given hosts suggesting that isolates within clonal complexes may have undergone niche adaption. Other mechanisms postulated to influence this population structure include clonal expansion, geographical or ecological isolation, host immune response or barriers to genetic exchange (Manning *et al.* 2003). The scheme has been extended to *C. coli* (Dingle *et al.* 2005) allowing comparison between *C. jejuni* and *C.coli*. The two species showed an 86.5% homology at the nucleotide level within the MLST loci, with evidence of genetic exchange of the housekeeping genes albeit at a very low rate. Studies suggest that there is considerable overlap between strains isolated from humans and animals (Dingle *et al.* 2002) with 6 clonal complexes namely C-C 21, C-C 45, C-C 206, C-C 61, C-C 48 and C-C 257 accounting for 60% of human disease isolates. A 2 year study in Northwest England (Sopwith *et al.* 2006) demonstrated the value of MLST in elucidating the epidemiology of human disease due to *C. jejuni*. The authors found the majority of cases to be associated with clonal complexes C-C 21 and C-C 45, with the latter clonal complex having a significantly higher case incidence rate in rural areas compared to urban areas raising the possibility that infection with C-C 45 may be associated with an environmental exposure route in rural areas.

An MLST scheme for *Campylobacter fetus* has been set up utilising the same loci with a database hosted at http://pubmlst.org/cfetus (van Bergen *et al.* 2005). The MLST scheme has been further extended to encompass *C.lari, C. upsaliensis* and *C. helveticus* by using 2 additional loci, namely *adk* and *pgi* (Miller *et al.* 2005) together with degenerate primers for the other 7 alleles. MLST has also been used in conjunction with both *flaA* RFLP (Djordjevic *et al.* 2007) and *flaA* short variable region (SVR) sequencing (Clark *et al.* 2005) who used both MLST and *flaA*-SVR sequencing in a waterborne outbreak of *C. jejuni*. The latter authors suggested that whilst MLST is more suitable for determining population changes

on a large scale, *flaA* SVR sequence typing may produce information that correlates more closely with epidemiological findings during outbreaks of human disease. Similarly Sails *et al.* (2003) found that use of MLST in conjunction with *flaA* SVR sequencing provided a level of discrimination equivalent to that achieved with PFGE in outbreak investigations with the advantage that sequence based methods allow global comparison via the central database.

With the recognition that clonal complexes represent important epidemiological groupings, efforts are being made to develop rapid clonal complex identification methods based on identification of single-nucleotide polymorphisms (SNPs) (Best *et al.* 2005). Such techniques would be well suited for outbreak investigation.

1.4 Epidemiology of human campylobacteriosis.

Campylobacter spp were first recognised as human enteric pathogens in the 1970s. They cause disease in persons of all ages but peak incidence is seen in the young, especially male teenagers (Skirrow 1977). The reported incidence of human cases in England and Wales has increased year on year (Fig 1.1.) with the peak incidence of confirmed cases in 1998 when 56,852 cases were confirmed microbiologically. The true incidence has been estimated to be considerably higher and in the region of 300,000 cases annually in England and Wales (Adak *et al.* 2005) with an annual cost of approximately £70M in England (Roberts *et al.* 2003). The incidence has fallen dramatically since then with 42,146 cases confirmed in 2004 (HPA 2005) and 44,343 cases confirmed in 2005 (DEFRA 2005). The reasons for this decline are not known or understood.

Human campylobacteriosis shows strong seasonality, in common with other food-borne infections with a peak in the spring and/or summer months, although there is wide variation between countries (Kovats *et al.* 2005). In the UK, Skirrow (1991) reported a marked peak in May with a smaller secondary increase in the autumn, whilst a more recent study in the NW of England (Sopwith *et al.* 2003), found 47% of cases occurring between May and August with a minor peak of cases in March although this peak has not been observed elsewhere. Similar trends have been reported from other temperate countries (Nyelen *et al.* 2002). It has been suggested that this seasonal trend may be driven primarily by environmental temperature (Louis *et al.* 2005) or by the ecology of animal reservoirs of *Campylobacter* (Stanley & Jones 2003).

The majority of human cases of campylobacteriosis (93%) are attributed to infection with *C. jejuni* with 7% being due to *C. coli* (Gillespie et al 2002). Minor causes of human campylobacteriosis include *C. lari*, *C fetus*, *C. upsaliensis* and *C. hyointestinalis* all of which have been isolated from domestic animals (Stanley & Jones 2003).

Most cases of human infection are considered to be single sporadic cases or small family clusters (Skirrow 1991) with outbreaks being relatively uncommon and often associated with food-handling faults in commercial catering establishments (Frost *et al.* 2002). Contaminated water sources, both municipal and private (Duke *et al.* 1996, Said *et al.* 2003) have also been implicated in outbreaks. A large outbreak affecting 3000 people in Vermont USA was attributed to contamination of the town's water supply (Vogt *et al.* 1982) most likely from agricultural run-off, whilst sewage contamination of a municipal water supply led to a prolonged outbreak of campylobacteriosis in Denmark (Engberg *et al.* 1998).

Campylobacteriosis is generally considered a food borne disease and numerous risk factors for acquisition of infection have been suggested. A case control study (Rodrigues *et al.* 2001) of cases presenting with *C. jejuni* isolated from stool samples identified overseas travel and consumption of chicken in a restaurant as risk factors for infection. Most cases were unexplained although the authors suggest that, in light of the low infective dose required, cross-contamination resulting from kitchen hygiene practices usually regarded as acceptable may be a route of infection in some sporadic cases. An association with eating chicken, either in the home or in a catering establishment, has been reported in many other studies (e.g. Friedman *et al.* 2004, Kappersud *et al.* 2003, Neimann *et al.* 2003).

Other suggested food sources are raw milk (Gillespie *et al.* 2003, Peterson 2003, Neimann *et al.* 2003), pork meat, attending barbecues (Kappersud *et al.* 2003, Neimann *et al.* 2003), eating raw clams (Griffin *et al.* 1983) and salad vegetables (Evans *et al.* 2003). Unchlorinated water has also been implicated (Kappersud *et al.* 2003, Kussi *et al.* 2004) as has bottled water (Evans *et al.* 2003) as a source of infection.

As is apparent from numerous case control studies, some of which are cited above, exposure to poultry products represent an important risk factor for infection suggesting poultry are a significant reservoir for *Campylobacter* spp pathogenic to humans. This was well illustrated in a "natural experiment" in Belgium in 1999 when

it was found that livestock feedstuffs had become contaminated with dioxins. Contamination started in January 1999 but the authorities were not informed until May. On June 2nd the authorities ordered the immediate withdrawal from sale of all Belgian eggs and poultry products, with other European countries and Russia rapidly following suit. For the period that contaminated products were off the market (4 weeks), the incidence of reported human campylobacteriosis fell by 40% compared to the expected number as predicted by a model using historical data from the Belgian surveillance system. The decline in cases lasted as long as the period that poultry products were withdrawn (Vellinga & Van Loock 2002).

Broiler chickens are frequently infected with *Campylobacter* spp (Corry & Abatay 2001) with infected birds excreting large numbers of campylobacters in their faeces such that 100% infection rates are rapidly achieved in infected sheds. There is considerable research being carried out investigating the acquisition and spread of campylobacters in broiler flocks and methods to reduce this. Vertical transmission from parent flocks, carryover from previous *Campylobacter* positive batches, airborne transmission from neighbouring broiler houses, and horizontal transmission from water, environmental sources, domestic and wild animals have all been implicated (Bull *et al.* 2006). This high rate of infection of birds is compounded by poor hygiene and cross contamination at slaughter such that up to 80% - 90% of carcasses sold are contaminated (Bolton *et al.* 1999).

Freezing of poultry carcasses significantly reduces *Campylobacter* spp contamination of poultry products. This was well illustrated in Iceland where prior to 1996, only frozen chicken could be sold. Following a change in regulations allowing sale of fresh chicken, sales increased as did incidence of human campylobacteriosis from an incidence rate of 14.6 cases/100,000 in 1995 to 33.1, 34.7, 80.4, and 157 cases/100,000 people in 1996, 1997, 1998 and 1999 respectively. In 2000, following interventions including the freezing of all chicken from *Campylobacter* positive flocks, the incidence fell to 75.4 cases/100,000 people in 2001 (Reiersen *et al.* 2002). Further insight into the potential importance of chicken as a source of human infection comes from genotyping studies. MLST data suggests that C-C 45 is the most commonly isolated *C. jejuni* clonal complex from poultry (Colles *et al.* 2003, Manning *et al.* 2003) and is also frequently isolated from human cases (Dingle *et al.* 2002) providing strong evidence of an association between human disease and poultry.

Conversely, the isolation of other MLST types from human cases but not from poultry suggests that other reservoirs of *C. jejuni* infection exist.

There is an increasing body of evidence suggesting that environmental exposure and/or contact with animals is an important route of infection. In their retrospective UK study, Louis *et al.* (2005) found significant correlations at district level between campylobacteriosis incidence rates and agricultural data for the district namely total agricultural area, agricultural labour force and number of cattle, sheep, pigs or poultry. Campylobacteriosis incidence rates were also positively correlated with percentage of rural wards in a district. The authors conclude there is a linkage between human campylobacteriosis cases and environmental factors rather than just food sources *per se*. Similar conclusions have been drawn in recent studies from Denmark (Ethelberg *et al.* 2005) and Quebec (Michaud *et al.* 2004).

1.5 Epidemiology of *Campylobacter* spp in ruminants

Campylobacter spp are well recognised as both commensals and pathogens of livestock. *C. fetus* is recognised as a cause of reproductive failure in both cattle and sheep whilst *C. jejuni*, *C. coli* and *C. hyointestinalis* can be isolated from the intestinal tract of healthy cattle sheep and pigs (Radostits *et al.* 2000). *C. jejuni* has been implicated as an infrequent cause of bovine mastitis with excretion of the organism in milk (Orr *et al.* 1995) with the potential to cause human disease if drank. Although considered as a commensal in ruminants, *C. jejuni* has been associated with outbreaks of diarrhoea in lambs and abortion in beef cattle, whilst *C. hyointestinalis* has been associated with outbreaks of diarrhoea in young calves (Radostits *et al.* 2000).

The public health significance of cattle and sheep associated *Campylobacter* spp relates not only to carcass or milk contamination but also to environmental and water contamination via animal slurries and abbatoir effluents (Stanley *et al.* 1998b). It has been estimated that on average a dairy cow will produce upwards of 57 litres of faeces per day thus an average 100 cow dairy herd will produce in the region of 40,000 litres of faeces weekly (MAFF 1991) affording considerable potential for environmental contamination.

Information on the survival of campylobacters in faeces is scant but one experimental study suggested that 90% inactivation of *C. jejuni* occurred within 6 days of deposition in the case of bovine faeces on pasture (Sinton *et* al 2007).

A number of studies investigating the prevalence of thermophillic campylobacters have been carried out at various locations world-wide and are summarised in Table 1. An important point to bear in mind when interpreting such studies is the exact methodology used, in particular the techniques used for isolation. Issues to consider include the use of enrichment broth versus direct inoculation, the use of filtration methods and the exact composition of media used (Atabay and Corry 1998). Whilst the majority of studies involve culture of the organism, a number of studies in Canada (Inglis *et al.* 2003, 2004a, 2004b) have utilised faecal DNA extraction followed by standard PCR or Real-Time Quantatative PCR. These studies suggest that the most prevalent species are *C. lanienae* and *C. jejuni* in Canadian beef cattle with 49% and 38% of beef cattle shedding, respectively. *C. lanienae* was first isolated from 2 abattoir workers in the UK during a routine hygiene screen (Logan *et al.* 2000) and has also been isolated from pig faeces in Japan (Sasaki *et al.* 2003).

Few studies have been carried out in the UK investigating the prevalence and diversity of *Campylobacter* spp. in cattle. In a two year study of dairy cattle (on-farm) and beef cattle (at slaughter) in Lancashire thermophillic campylobacters were isolated from 89.4% of beef cattle (Stanley *et al.* 1998b). A study based on sampling of freshly voided cattle faeces in the Wirral, Merseyside reported a *C. jejuni* prevalence of 32.4% (Robinson *et al.* 2005) which is in agreement with a previous study involving intensive environmental sampling of a 100 square Km area of Cheshire which reported a *C. jejuni* bovine faecal pat prevalence of 36% (Brown *et al.* 2004).

Faecal shedding of *Campylobacter* spp is intermittent with young animals having a higher prevalence than older animals (Stanley *et al.* 1998b, Nielsen 2002) with up to 80% of calves shedding at age 91 -120 days. Not only is the prevalence higher in young animals but the numbers of *Campylobacters* excreted per gram of faeces (faecal concentration) are considerably higher (Nielsen 2002), such that the *Campylobacter* numbers in faeces of calves aged between 30 – 60 days are of a similar magnitude to that seen in broiler chickens before slaughter at 40 days (Stanley & Jones 2003). There is no vertical transmission of *Campylobacter* spp but calves and lambs become colonised rapidly within a few days of birth via acquisition from the contaminated environment and horizontal spread (Stanley & Jones 2003).

Management practices have a significant effect on the magnitude of *Campylobacter* spp excretion in two ways. Firstly poor hygiene practices will allow re-infection of

animals from bedding or water troughs thus increasing the *Campylobacter* load. Secondly, the diet fed can affect magnitude of excretion with feedlot cattle on high grain diets having a higher prevalence than grass fed animals (Garcia *et al.* 1985). An experimental study (Berry *et al.* 2006) investigating dietary and genetic influences on carriage of *E. coli* and *Campylobacter* spp. found diet to be only weakly associated with campylobacter excretion. Robinson *et al* (2005) found the presence of whole grain in the faeces of young cattle to be associated with an increased risk of isolating *Campylobacter* from faeces. Presence of whole grain in faeces is considered to be indicative of sub-acute ruminal acidosis, a common nutritional disorder in dairy cattle of all ages (Grove-White 2004).

Seasonality in shedding has been observed in a two year longitudinal study of dairy cattle in Lancashire with peak shedding in spring and autumn (Stanley & Jones 1998b). Although no significant effect of any environmental parameters on timing of the peaks could be detected, there did appear to be a spatial component in that peaks coincided in herds on close neighbouring farms. The peaks on northern farms preceded those on farms that were 20 miles south by two months in spring and one month in autumn. These 2 peaks roughly coincide with the transition from housing to grazing in spring and *vice versa* in autumn. These two periods are well recognised as periods of dietary change in which nutritional disorders are common, due in part to disturbances in gut flora. It has been hypothesised that these peaks could be associated with calving and associated hormonal changes or stress, but this is unlikely since dairy farmers tend to calve their cows all the year round. No seasonality was observed in beef cattle at slaughter which would support the hypothesis that seaonality in excretion is associated with nutritional changes since it is unlikely that beef cattle would be subject to nutritional changes prior to slaughter (personal observation).

It has been suggested that the seasonality of common source campylobacteriosis outbreaks in humans associated with raw milk or contaminated water may be a reflection of the ecology of the bovine *Campylobacter* reservoir (Tauxe 1992).

The role of sheep in the epidemiology of *Campylobacter* has not been investigated to the same degree as for cattle. A study of lambs at slaughter using enrichment media showed up to 91% of lambs' intestines to be colonised (Stanley *et al.* 1998c). The numbers of campylobacters in lamb intestines is considerably higher than in cattle intestines (Stanley *et al.* 1998b) but lower than reported for broiler chickens (Wallace

et al. 1997). There was a significant seasonal peak in the spring but this could not be correlated with environmental conditions. The faecal carriage rate of campylobacters was significantly lower than the intestinal carriage rate with shedding being intermittent, as in cattle, with rates varying from zero to almost 100%. Highest rates were seen coinciding with lambing, weaning and nutritional changes such as moving to new pastures. As with calves, baby lambs were rapidly colonised soon after birth (Jones *et al.* 1999). The authors suggest that shedding of campylobacters by sheep has considerable potential for contamination of pasture and surface water and that the role of sheep in the epidemiology of campylobacteriosis has been underestimated in the past.

Recent studies using MLST have provided further evidence on the role of ruminants in the epidemiology of human campylobacteriosis. Both Colles *et al.* (2003) and Manning *et al.* (2003) found C-C 61 and C-C 42 to be widespread in ruminant isolates whilst C-C 45 appears to be the predominant clonal complex isolated from poultry sources. C-C 61 was also found in all groups of sheep whilst C-C 48 was found in adults but not in lambs. Both groups of workers found C-C 21 to be the most widespread clonal complex. Colles *et al.* (2003) isolated it from slurry and starlings amongst other sources and suggested that members of the C-C 21 may be particularly well suited for long term survival since slurry is likely to have a large number of different genotyped added to it on a regular basis, yet members of C-C 21 were the most commonly isolated from it.

French *et al.* (2005) carried out intensive structured sampling of an area of farmland in Cheshire involving livestock and wild animal faeces, soil and environmental water. The most common isolates from bovine faeces were members of C-C 21 (39%), C-C 61 (22%) and C-C 45 (11%). Statistical analysis showed C-C 61 to be over-represented in bovine faeces whilst the prevalence of C-C 21 was consistent with the null hypothesis of it being no more likely to appear in cattle faeces than other hosts. Studies suggest that there is considerable overlap between strains isolated from humans and animals (Dingle *et al.* 2002) with 6 clonal complexes (C-C 21, C-C 45, C-C 206, C-C 61, C-C 48 and C-C 257) accounting for 60% of human disease isolates.

1.6 The role of wildlife and pets in the epidemiology of campylobacteriosis.

Whilst there is considerable evidence accumulating on the role of poultry and ruminants, little is known on the role of wildlife. *Campylobacter* spp have been isolated from wild birds such as pigeons, ducks, crows, geese and starlings (Stanley & Jones 1998). French *et al.* (2005) isolated members of C-C 21, C-C 45 and a number of previously unidentified STs from wild birds, other wildlife and water. Wild birds have been implicated in the dissemination of *Campylobacter* spp over large areas, which can introduce new strains into herds or flocks. They have also been implicated in the contamination of coastal bathing water, shellfish beds and human water supplies (Jones 2001) as well as contaminating bottled milk by pecking at the bottle tops (Hudson *et al.* 1990).

Contact with pet dogs and cats is recognised as a potential risk factor for human campylobacteriosis (Deming *et al.* 1987, Adak *et al.* 1995). *C. jejuni*, *C. coli* and *C. upsaliensis* can frequently be isolated from both cats and dogs (Baker *et al.* 1999). Hald & Masden (1997) in a cross sectional study of puppies and kittens reported 29% of puppies to be infected with the chief species isolated being *C. jejuni*. Only 2 (5%) kittens were infected; both with *C. upsaliensis*. *Campylobacter* spp are a recognised cause of diarrhoea in dogs with diarrhoeic animals in the household likely to represent a greater risk than apparently healthy animals as demonstrated by Nair *et al.* (1985) who isolated *C. jejuni* from 21.7% of diarrhoeic dogs but only from 3.1% of apparently healthy dogs. Interestingly they isolated *C. jejuni* from 6.7% of "non diarrhoeic but unhealthy" dogs suggesting that any "sick dog" represents a significantly greater risk than "healthy dogs" irrespective of diarrhoea.

1.7 *Campylobacter* spp in water and the environment.

The natural reservoir of *Campylobacter* spp is the intestinal tract of birds and mammals, being unable to replicate outside the intestinal tract due to their thermophillic and micro-aerophillic requirements. The environmental load is therefore dependant on firstly shedding by hosts and secondly the ability of the micro-organism to survive in the environment. The presence of *Campylobacter* spp in environmental samples is indicative of recent contamination since they are unable to survive for long periods; their survival time being shorter than that of the usual indicators of faecal contamination namely faecal coliforms and streptococci.

The presence of *Campylobacter* spp in rivers, streams and lakes is dependant on the surrounding environment and the degree of contamination by birds or animals. It has been suggested that survival of *Campylobacter* spp in water can be influenced by the presence of waterborne protozoa. An in-vitro study demonstrated that *C. jejuni* remained viable for up to 36 hours longer after internalisation by *Tetrahymena pyriformis* or *Acanthamoeba castellanii* than when they were in a free state in water; they also showed increased resistance to disinfection when internalised (Snelling *et al.* 2005). Conversely, grazing by the freshwater planktonic crustacean *Daphnia carinata* has been shown to reduce the survival of *C. jejuni* (Schallenberg *et al.* 2005).

In New Zealand, Savill *et al.* (2001) using PCR and Most Probable Number (MPN) techniques found significant numbers of campylobacter in 60% of river samples and 75% of shallow ground water samples. Very low numbers were detected in 29% of drinking water samples despite these samples complying with New Zealand's water hygiene directives regarding detection of *E. coli*. Similar results have been obtained for UK (Bolton *et al.* 1987) and German (Stelzer & Jacob 1991) river water.

A study of the river Conder in Lancashire showed that whilst the upper reaches were free of *Campylobacter* spp it became progressively more contaminated as it flowed through grazed farmland. A two year study of the river Lune in Lancashire showed *Campylobacter* spp to be present all year but with lower numbers isolated in the summer. It is suggested that this is due to increased die off resultant on higher environmental temperatures and increased ultra-violet light. Sources of contamination for the Lune included sewage works, sheep and cattle grazing, agricultural run-off and indigenous wild waterfowl (Jones 2001). In a study in Cheshire involving intensive environmental sampling of a 100 square kilometre area, *Campylobacter* spp were isolated from 15% of water samples but only 0.9% of soil samples (Brown *et al.* 2004).

Groundwater is normally considered to be microbiologically clean although it has been implicated as a source of *Campylobacter* for poultry flocks (Pearson *et al.* 1993). Stanley *et al.* (1998d) investigated groundwater contamination arising from a nearby dairy farm. They cultured identical *C. jejuni* biotypes from the groundwater and the dairy herd supporting the theory that groundwater is a potential route for *Campylobacter* spp transmission.

Epidemiological studies have implicated private water supplies as a source of human infection in some cases (Duke *et al.*, 1993, Said *et al.* 2003) with the source of

infection usually attributed to contamination with animal waste, although large outbreaks have occurred resultant on contamination of municipal water supplies (Vogt *et al.* 1982).

Contamination of ponds, lakes and coastal waters is widespread and associated with wild birds such as ducks, geese, seagulls and oystercatchers. Sewage is not believed to be important in contamination of coastal waters with *Campylobacter* spp (Jones 2001).

Survival time of *Campylobacter* spp, as measured by the time required for a 1-$\log_{10}$ reduction in bacterial numbers (D-value), has been investigated in the case of both farmyard manure and liquid slurry inoculated with *C. jejuni* and then spread on pasture (Hutchinson *et al.* 2005). Results were similar for both materials with D-values between 2 – 3 days, although viable *C. jejuni* could still be isolated from the pasture for up to 63 days. This study suggests that farmyard manure represents no lesser risk than liquid slurry.

Stanley et al (1998a) suggested that there was little die-off of campylobacters in stored dairy slurry although aerobic digestion of slurry reduced campylobacter populations. They found that campylobacters could not be detected 24 hours after spraying aerated slurry on land, whereas they could be detected up to 20 days after spraying of unaerated slurry on the land. This finding suggests that unaerated slurry application to land can result in substantial contamination which can lead to contamination of water sources posing a risk to both animals and man. There was a marked seasonal variation with higher numbers of campylobacters isolated from stored slurry during the winter months compared to the summer. This was attributed to increased survival with lower environmental temperatures.

An experimental study from New Zealand (Ross & Donnison 2006) suggested that *C. jejuni* can survive in soil for up to 25 days after application of farm dairy effluent. The authors comment that this may have implications for pasture management practices since the usual NZ practice of 14 day rotational grazing would allow opportunities for maintaining cycles of re-infection.

Table 1.1. Reported estimates for *Campylobacter* spp prevalence in cattle and sheep: non – UK studies.

Country (region)	Number of Herds sampled	Production group sampled	Total Campylobacter Prevalence (%) (animal level)	*C. jejuni* Prevalence (%) (animal level)	*C. coli* Prevalence (%) (animal level)	Reference
USA	96	Lactating adults	51.2	n/a	n/a	Englen *et al.* 2007
USA (Wisconsin)	60	Lactating adults	26.7 organic 29.1 convent	n/a	n/a	Sato *et al.* 2007
USA	31	Lactating adults	n/a	37.7	n/a	Wesley *et al.* 2000
USA (Washington)	15	Lactating adults Feedlot Calf rearing Cow-calf	46.5 46.9 43.8 49.4	31.2 31.6 23.8 47.1	5.8 13.3 20 0.6	Bae *et al.* 2005
USA	1 feedlot	Feedlot	n/a	44.6	n/a	Besser *et al.* 2005
USA	n/a	Cull dairy cows	n/a	7	n/a	Dodson & LeJeune 2005
Finland	n/a	Slaughter	31.1	19.5	2.2	Hakkinen *et al.* 2007
Norway	333	Slaughter adult calves	29 46	26 (all cattle)	3.0 (all cattle)	Johnsen *et al.* 2006
Denmark	24	Lactating adults Calves< 4 months Calves > 4months	9.2 42.1 20	n/a n/a n/a	n/a n/a n/a	Nielsen 2002
Iran	n/a	Meat samples	10	n/a	n/a	Taremi *et al.* 2006
Thailand	n/a	Lactating adults Milk samples	14 10	8.8 n/a	n/a n/a	Padungtod & Kaneene 2005
New Zealand	1	Lactating	54	54	n/a	Adhikari *et al.* 2004
Switzerland	67	Cow-calf pre-wean weaning	65.3 44.9	35.3 13.3	2.6 1.7	Busato *et al.* 1999
Spain	120 124 82	Sheep Beef Lactating adults	8.8 5.4 66.7	n/a	n/a	Oporto *et al.* 2007

Chapter Two.

General Materials and Methods.

2.1 Recruitment and sampling strategy.

Three farm animal veterinary practices in Lancashire were invited to participate. These were selected based on their geographical position in the county and on their likely willingness to cooperate in recruitment of farms. The three practices selected were in Longbridge, Lancaster and Clitheroe and served the Southern Fylde (Zone 1), N. Lancashire (Zone 2) and S.E. Lancashire (Zone 3) respectively (Figure 2.1.). The veterinary practices were requested to supply client lists detailing the farm enterprises and approximate numbers of animals. . Both dairy and sheep farms were selected from Zones 2 & 3 whilst only dairy farms were selected from the Zone 1 (on account of there being no eligible sheep farms in that area). Eligible farms were defined as being dairy farms with over 100 adult cows with or without a sheep enterprise and sheep farms with over 150 breeding ewes and no other livestock enterprises. Eligible farms were entered on a database and selected randomly, stratifying by veterinary practice and farm type using Survey Toolbox V.1.0 (Cameron 1999). Selected farms were contacted and asked to participate in the study. If a selected farm was unwilling to participate, another farm would be drawn at random and asked to participate. All farms were visited prior to sampling and a questionnaire (Appendix A.) regarding farming practices completed. A total of 18 farms were selected and agreed to participate in the study. The distribution of farms within zones was as follows

- Zone 1
    - 4 dairy only farms
    - 2 dairy farms with sheep enterprises
- Zones 2 & 3
    - 2 dairy only farms
    - 2 dairy with sheep enterprises
    - 2 sheep farms

One farm (No. 8) from Zone 2 ceased trading in December 2006 thus left the study. A neighbouring farm (No 19) was recruited *in lieu* and sampled for the remainder of the study. Another farm (No 11) ceased keeping cattle in June 2007 thus sampling on this farm was incomplete. This farm embarked on a dairy goat enterprise, purchasing does from all over the UK. Twenty faecal samples were collected from the goats in April 2007.

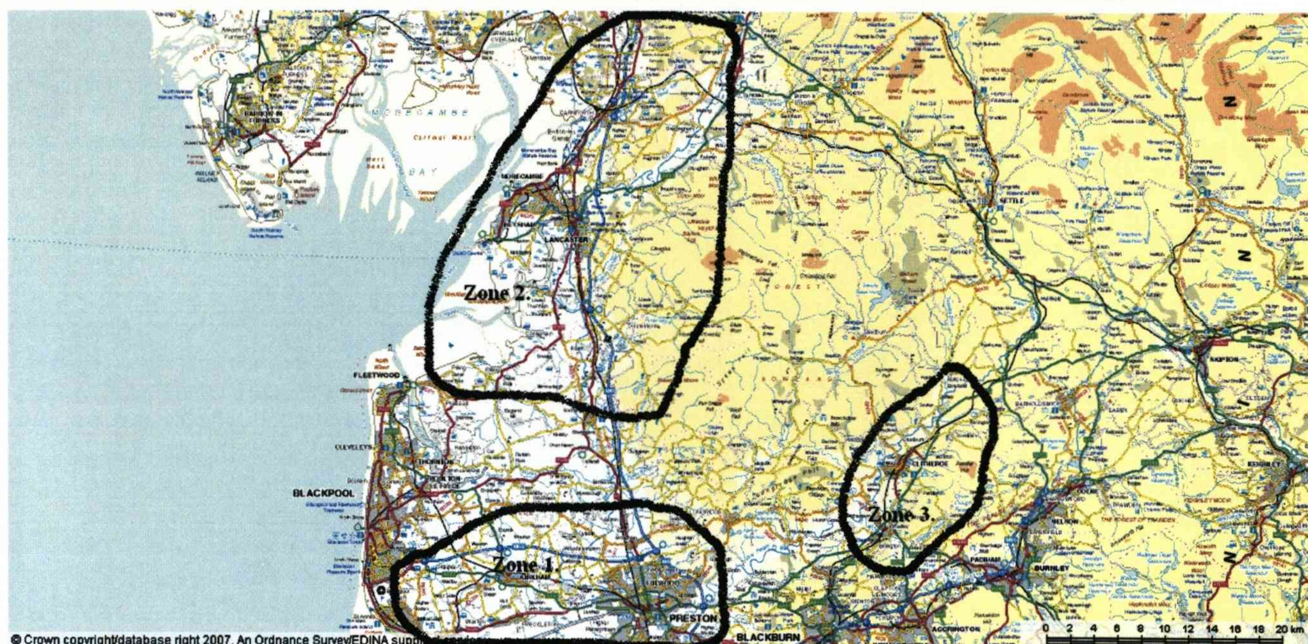


Fig 2.1. Map of Lancashire showing approximate bounds of sampling zones.

The study was a repeated cross sectional study with each farm being sampled at 8 week intervals. Three farms (all from one geographical zone) were sampled each week. Since one of the objectives of this study was to quantify the contribution of farm animals to the environmental campylobacter load, samples were obtained from the numerically predominant production group i.e. lactating dairy cows in the case of dairy farms and adult ewes in the case of sheep farms. At each visit, current management and production details were obtained (Appendix B.) as follows:

Cows.

- Number of cows in lactating group
- Where sampled
    - Housed
    - At pasture
- Average daily milk yield
- Number of cows that calved within the last month
- Date of turn-out to pasture or housing
- Feeding system currently in use
- Length of time cows have been grazing current pasture
- Details regarding any slurry application to the pasture currently grazed
- Details of co-grazing with sheep

Sheep.

- Number of sheep in sampled group
- Where sampled
  - Housed
  - At pasture
- Type of animals in sampled group
  - Ewes & baby lambs
  - Ewes & fattening lambs
  - Ewes only
  - Fattening lambs only
- Length of time in the sampled environment
- Type of pasture
  - Rough pasture
  - Mediocre pasture
  - Lush pasture
- House hygiene (if applicable)
- Is sampling occurring during lambing season
- Details of any medication given within the last two weeks

In the case of cattle, a faecal sample was collected from the ground after a cow had been observed defecating whilst in the case of sheep, samples were collected from the ground providing they were warm to the touch indicating they had been recently voided. To ensure that duplicate sheep samples were not collected, no two samples were collected unless separated by a minimum distance of 15 metres. Each faecal pat was sampled from at least 3 sites within the pat and mixed thoroughly in a sterile sample pot to minimise possible within-pat variation. In the case of sheep faecal pellets, at least 3 were collected. Twenty faecal samples were collected at each visit. Samples were transported to the laboratory on ice.

In the case of dairy cows faecal consistency was scored using a score from 1 – 5 (Hughes 2001) and a larger sample was collected from the same faecal pat in an arm-length plastic glove for further analysis by sieving to assess fibre length and presence of partially digested grains (Grove-White 2004).

2.2 Microbiology.

One gram of faeces was placed in 9 ml of *Campylobacter* enrichment broth (IDG Ltd, Bury, UK) with cefoperazone, vancomycin, trimethoprim and cycloheximide (CVTC supplement: IDG Ltd, Bury, UK) and after homogenising for 30 seconds in a Colworth 80 stomacher (A. J. Seward & Co. Ltd, London, UK) was incubated in a plastic universal bottle for 24 hours at 37^0C in a in a "Variable Atmosphere Incubator" (VAIN, Don Whitley Scientific Ltd, UK) which maintained a microaerobic atmosphere (CO_2 12%, H_2 3%, O_2 11%. N_2 74%). After incubation, 50µl of the enrichment broth was inoculated onto a *Campylobacter* blood-free selective agar (CSA) plate (IDG Ltd, Bury, UK) enriched with cefoperazone and amphotericin (CA supplement: IDG Ltd, Bury, UK). A 30µg nalidixic acid sensitivity disc (Mast Group, Merseyside, UK) was placed on the heaviest part of the inoculum. Similarly a 5µl loopful of enrichment broth was inoculated onto a second CSA plate without a nalidixic acid disc. The CSA plates were incubated at 37^0C in a microaerobic atmosphere for 60-72 hours after which time plates were examined and up to 4 putative campylobacter colonies (per faecal sample) were sub-cultured onto blood agar plates and incubated at 37^0C under microaerobic conditions as described earlier. Putative campylobacters were identified on colony morphology (silvery metallic sheen, "flying saucer" appearance) on CSA plates: Plate2.1). After 72 hours incubation, single colonies were sub-cultured onto two blood agar plates. One plate was incubated for 48 hours under microaerobic conditions as described and the other plate incubated for 48 hours at 30^0C in air.

Putative *Campylobacter* spp on a CSA plate.

A rapid hippurate hydrolysis test (Harvey 1980) was performed in 96-well plates by inoculating 100 µl 1% sodium hippurate solution with a small amount ("leading edge") of the culture, incubating for 2 hours at 37^0C in microaerobic conditions and

then adding 50µl of Ninhydrin solution (3.5% in 1:1 acetone:butanol) and incubating in air for 20 minutes at room temperature at which time plates were read. A positive result was indicated by a purple colour which varied in intensity from faint to deep purple. The intensity of a positive result was recorded on a scale of 1+ to 3+ with 3+ being taken as a positive result indicating hydrolysis had occurred.

2.3 Species assignment protocol.

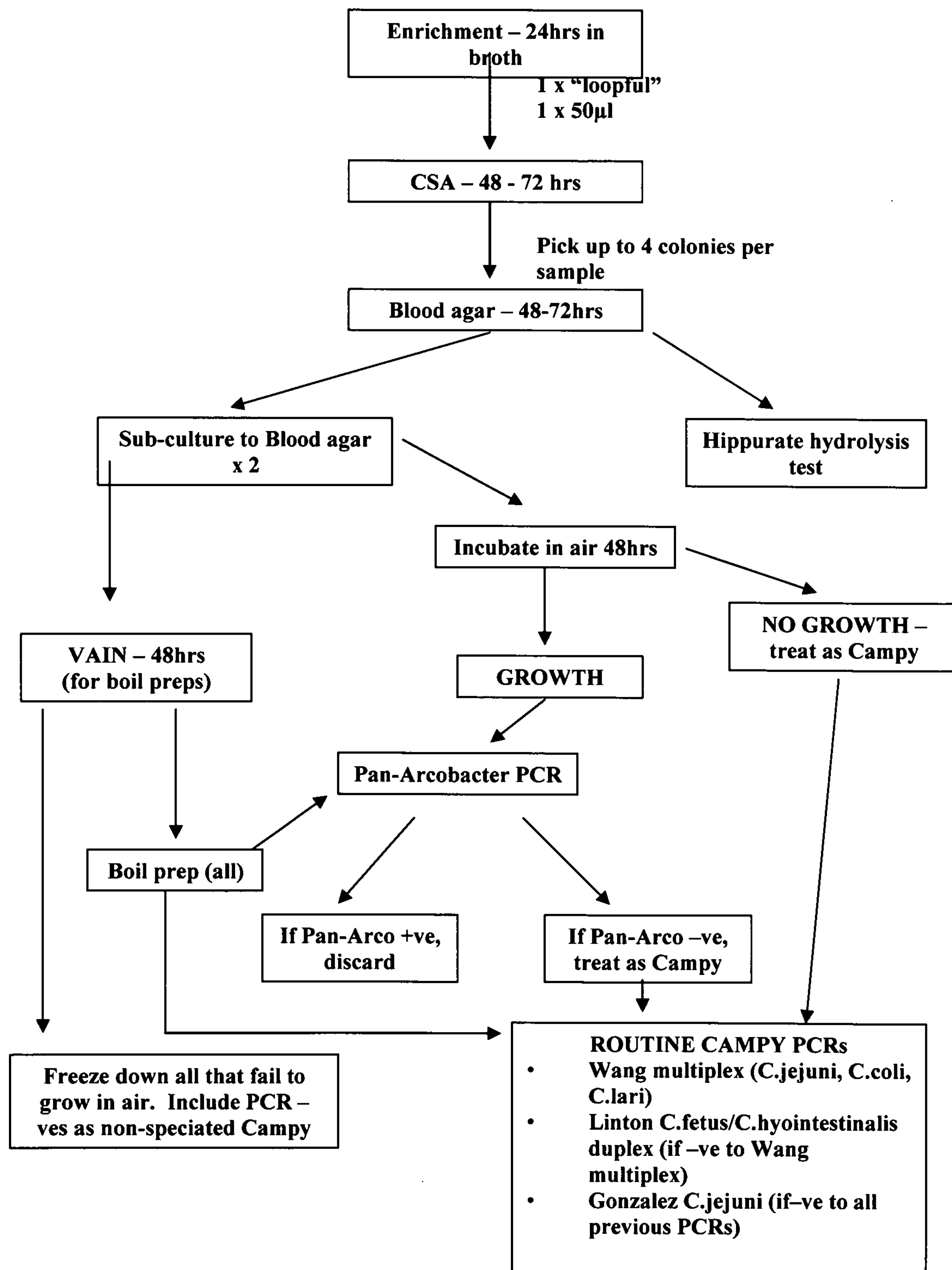
A crude DNA aqueous lysate ("boiled preparation") was prepared by inoculating 200µl of distilled water with a small amount of the culture ("leading edge of a loop"), heating at 100^0 C for 15 minutes followed by centrifugation at 1300 rpm for 10 minutes. All putative campylobacter isolates were frozen in Microbank tubes (Pro-Lab Diagnostics, Neston, United Kingdom) and stored at -80^0C.

Assignment to species was performed by PCR assay in 96-well plates as follows:

- Isolates which grew in air were subjected to the pan-arcobacter 16S rRNA PCR assay for identification of the genus *Arcobacter* (Gonzalez *et al.* 2000). Any isolates which were negative to this PCR were considered to be putative campylobacters and tested further by PCR.
- Isolates which failed to grow in air were considered to be putative campylobacters together with any isolates that grew in air but were negative to the pan-arcobacter 16S rRNA PCR assay. These isolates were then assigned to campylobacter species as follows:
    - All isolates were subjected to a colony multiplex PCR assay (Wang *et al.* 2002) for identification of *Campylobacter jejuni*, *C. coli* and *C. lari*.
    - Isolates negative to the aforementioned PCR were subjected to a duplex PCR assay (Linton *et al.* 1996) for identification of *Campylobacter fetus* and *C. hyointestinalis*.
    - Any remaining unspeciated isolates were subjected to a monoplex PCR assay (Gonzalez *et al.* 1997) for identification of *Campylobacter jejuni*.

This serial protocol was adopted in the light of the results from an initial pilot study in which all putative campylobacter isolates were subjected to all the PCR assays. This study is described in Chapter 3. The protocol is represented as a flow chart in Fig 2.2.

Fig 2.2. Flow chart of *Campylobacter* spp speciation protocol.



2.4 PCR Methodologies.

All PCR assays were performed in 25µl volumes comprising 1µl DNA lysate and 24µl of mastermix. In addition to a negative control of purified water, two positive DNA controls were used for each 96-well plate namely a DNA extract from a type strain (obtained from the HPA) purified using a commercial kit (Nucleospin: Macherey-Nagel GmbH & Co. KG.) and an "extract control" i.e. DNA from the type strain extracted as an aqueous lysate along with the batch of samples under test. The following PCR assays were used for assignment to species during the entire study.

- **16S rRNA PCR** assay for identification of the genus *Arcobacter* (Gonzalez *et al.* 2000). This PCR utilises the primers Arc 1 and Arc 2. These primers specifically amplify a 181-bp DNA fragment of the 16S rRNA gene from *Arcobacter* spp.

ARC1	5'- AGAACGGGTTATAGCTTGCTAT - 3'
ARC2	5'- GATACAATACAGGCTAATCTCT - 3'

Mastermix:

dNTPs (10mM of each)	0.5 µl
10x PCR buffer	2.5µl
$MgCl_2$ 25mM	1.5µl
Primers (100µM) – each	0.25µl
Taq polymerase (5Uµl^{-1})	0.1µl
Molecular grade water	18.9µl

Reaction conditions:

- initial heating for 3 minutes at 94°C followed by:
- 25 amplification cycles consisting of
  - 1 minute at 94°C (denaturation)
  - 1 minute at 59°C (primer annealing)
  - 1 minute at 72°C
- A final elongation step (72°C for 7 minutes) followed the final amplification cycle.

- **Colony multiplex PCR** assay (Wang *et al.* 2002) for identification of *Campylobacter jejuni*, *C. coli* and *C. lari*. The following primers were utilised:
  - 23S rRNA - generating a 650-bp amplicon.

23SF	5'- TATACCGGTAAGGAGTGCTGGAG -3'
23SR	5'- ATCAATTAACCTTCGAGCACCG -3'

  - *C. jejuni hipO* gene –generating a 323-bp amplicon

CJF	5'-ACTTCTTTATTGCTTGCTGC -3'
CJR	5'- GCCACAACAAGTAAAGAAGC -3'

  - *C. coli glyA* gene –generating a 126-bp amplicon

CCF	5'- GTAAAACCAAAGCTTATCGTG -3'
CCR	5'- TCCAGCAATGTGTGCAATG -3'

- *C. lari glyA* gene –generating a 251-bp amplicon

CLF	5'- TAGAGAGATAGCCAAAAGAGA -3'
CLR	5'- TACACATAATAATCCCACCC -3'

Mastermix:

dNTPs (10mM of each)	0.5 μl
10x PCR buffer	2.5μl
$MgCl_2$ 25mM	2.5μl
Primers (100μM) – each	
CJF, CJR, CLF,CLR	0.125μl
CCF, CCR	0.25μl
23SF, 23SR	0.05μl
Taq polymerase ($5U\mu l^{-1}$)	0.25μl
Molecular grade water	15.65μl

Reaction conditions:

- initial heating for 6 minutes at 96°C followed by:
- 30 amplification cycles consisting of
  - 0.5 minute at 95°C (denaturation)
  - 0.5 minute at 59°C (primer annealing)
  - 0.5 minute at 72°C
- A final elongation step (72°C for 7 minutes) followed the final amplification cycle.

- **16S rRNA duplex PCR** assay for identification of *Campylobacter fetus* and *C. hyointestinalis* (Linton *et al.* 1996). This PCR utilises a common forward primer primers ChyoF and 2 reverse primers namely ChyoR and CfetR. These primers generate 1287-bp *C. hyointestinalis* and 997-bp *C. fetus* amplicons.

ChyoF	5'- GCAAGTCGAACGGAGTATTA --3'
ChyoR	5'- GCGATTCCGGCTTCATGCTC --3'
CfetR	5'- GCAGCACCTGTCTCAACT --3'

Mastermix:

dNTPs (10mM of each)	0.5 μl
10x PCR buffer	2.5μl
$MgCl_2$ 25mM	2.5μl
Primers (100μM) – each	0.25μl
Taq polymerase ($5U\mu l^{-1}$)	0.1μl
Molecular grade water	17.9μl

Reaction conditions:

- initial heating for 3 minutes at 94°C followed by:
- 25 amplification cycles consisting of
  - 1 minute at 94°C (denaturation)
  - 1 minute at 65°C (primer annealing)
  - 1 minute at 72°C
- A final elongation step (72°C for 7 minutes) followed the final amplification cycle.

- ***ceuE* gene PCR** assay for identification of *Campylobacter jejuni* (Gonzalez *et al.* 1997). This PCR utilises the primers JEJ1 and JEJ2. These primers specifically amplify a 793-bp DNA fragment of the *ceuE* gene from *C. jejuni.*

JEJ1	5'- CCTGCTACGGTGAAAGTTTTGC -3'
JEJ2	5'-GATCTTTTTGTTTTGTGCTGC -3'

Mastermix:

dNTPs (10mM of each)	0.5 μl
10x PCR buffer	2.5μl
$MgCl_2$ 25mM	3.5μl
Primers (100μM) – each	0.25μl
Taq polymerase ($5U\mu l^{-1}$)	0.1μl
Molecular grade water	16.9μl

Reaction conditions:

- initial heating for 3 minutes at 94^{0}C followed by:
- 30 amplification cycles consisting of
  - 0.5 minute at 94^{0}C (denaturation)
  - 0.5 minute at 57^{0}C (primer annealing)
  - 1 minute at 72^{0}C
- A final elongation step (72^{0}C for 5 minutes) followed the final amplification cycle.

During the initial pilot study described in Chapter 3, two additional PCR assays were used, namely:

- **16S rRNA PCR** assay for identification of the genus *Campylobacter* (Linton *et al.* 1996). This PCR utilises the primers CampyF and CampyR. These primers specifically amplify a 816-bp DNA fragment of the 16S rRNA gene.

  CampyF 5'- GGATGACACTTTTCGGAGC -3'

  CampyR 5'- CATTGTAGCACGTGTGTC -3'

  Reagent concentrations and reaction conditions are as described earlier for the 16s rRNA *C. fetus* and *C. hyointestinalis* PCR, but substituting the above set of primers.

- ***ceuE* gene PCR** assay for identification of *Campylobacter coli* (Gonzalez *et al.* 1997). This PCR utilises the primers COL1 and COL2. These primers specifically amplify a 894-bp DNA fragment of the *ceuE* gene from *C. jejuni.*

  COL1 5'- ATGAAAAAATATTTAGTTTTTGCA -3'

  COL2 5'- ATTTTATTATTTGTAGCAGCG -3'

  Reagent concentrations and reaction conditions are as described earlier for the *ceuE* gene PCR for *C. jejuni*, but substituting the above set of primers.

The following *arcobacter* and *campylobacter* isolates, obtained from the HPA, were used as positive controls: *C. jejuni* NCTC 11168, *C. coli* NCTC 11366, *C. hyointestinalis* NCTC 11608, *C. fetus fetus* NCTC 10842, *C. fetus venearalis* NCTC 10354, *C. lanienae* NCTC 13004, *Arcobacter butzleri* NCTC 12481. MolTaq (Molzym GmbH & Co.KG, Bremen, Germany) taq polymerase was used in all PCR assays. dNTPs (deoxyribonucleotide triphosphates) were supplied as individual reagents Rovaleb: Teltow, Germany) and made up to a solution containing 10mM of each dNTP. Magnesium Chloride solution and X10 PCR buffer were supplied by Molzym GmbH. Composition of the X10 buffer was 600mM Tris-sulphate (pH 9.1) and 180mM Ammonium sulphate. All PCRs were performed in a programmable thermal cycler (ABI 20720: Applied Biosystems). PCR products (20μl) were analysed by electrophoresis on 1.5% or 2% (in the case of the colony multiplex PCR)

agarose gel stained with ethidium bromide. Gels were run for 75 minutes at 120V DC current after which they were read and photographed using a GelDoc 2000 (Biorad)

2.5 Multilocus Sequence Typing.

One thousand *C. jejuni* isolates were subject to MLST. Isolates were randomly selected using Survey Toolbox (Cameron 1999) stratifying by Zone and Sampling Round (defined as the 8 week period during which each farm was sampled once). Isolates were processed in batches of 250. In the case of the first 250 isolates, the original DNA aqueous lysate was used, whilst for the second batch, the isolate was grown up on Blood Agar and an aqueous lysate prepared as described earlier. In the case of the last 500 isolates, these were grown up on Blood Agar and DNA extracts prepared using a commercial kit (Prepman: Applied Biosystems). These changes in extraction methodology were introduced in an attempt to maximise the initial PCR yield.

Essentially sequencing for MLST involves 3 steps, namely:

- PCR amplification of the gene product.
- Dideoxy-termination sequencing reaction performed on each DNA strand (forward and reverse) using internal nested primers and Big Dye Ready Reaction Mix (Applied Biosystems).
- Removal of unincorporated dye terminators by precipitation with 95% ethanol-3M sodium acetate followed by one wash with ice-cold 70% ethanol.
- DNA sequencing.

The first 500 isolates were processed using primers (Dingle *et al* 2001) and reaction conditions developed by Dr MCJ Maiden's group at the Wellcome Trust Centre for the Epidemiology of Infectious Disease, Department of Zoology, University of Oxford (personal communication: Dr Howard Leatherbarrow), henceforth referred to as "The Oxford Protocol", whilst the remaining isolates were processed using primers and reaction conditions ("The Miller Protocol") as described by Miller *et al.* (2005). This change in protocol was adopted in an attempt to optimise the entire process. In the event of failure to obtain sequence data for a given allele, the entire process would be repeated for up to a maximum of 6 times.

2.5.1 Oxford Protocol.

The following primers were used for amplification:

Table 2.1. Primers used for amplification in the Oxford MLST protocol.

Locus	Primer	Sequence (5′ - 3′)	Product Size (bp)
aspA	aspA9 (forward)	5′- AGT ACT AAT GAT GCT TAT CC -3′	941
	aspA10 (reverse)	5′- ATT TCA TCA ATT TGT TCT TTG C -3′	
glnA	glnA1	5′- TAG GAA CTT GGC ATC ATA TTA CC -3′	1305
	glnA2	5′- TTG GAC GAG CTT CTA CTG GC -3′	
gltA	gltA1	5′- GGG CTT GAC TTC TAC AGC TAC TTG -3′	1112
	gltA2	5′- CCA AAT AAA GTT GTC TTG GAC GG -3′	
glyA	glyA1	5′- GAG TTA GAG CGT CAA TGT GAA GG -3′	1052
	glyA2	5′- AAA CCT CTG GCA GTA AGG GC -3′	
pgm	pgmS5	5′- GGT TTT AGA TGT GGC TCA TG -3′	680
	pgmS2	5′- TCC AGA ATA GCG AAA TAA GG -3′	
tkt	tktS5	5′- GCT TAG CAG ATA TTT TAA GTG -3′	692
	tktS4	5′- ACT TCT TCA CCC AAA GGT GCG -3′	
uncA	uncAS5	5′- TGT TGC AAT TGG TCA AAA GC -3′	631
	uncAS4	5′- TGC CTC ATC TAA ATC ACT AGC -3′	

Amplification reactions were performed in 50µl volumes comprising 2µl of DNA lysate and 48µl of mastermix, using a programmable thermal cycler (ABI 20720: Applied Biosystems) as follows:

Mastermix:

dNTPs (10mM of each)	1.0 µl
10x PCR buffer	5.0µl
$MgCl_2$ 25mM	3.0µl
Primers (10µM) – each	1.0µl
Taq polymerase ($5U\mu l^{-1}$)	0.25µl

Reactions Conditions:

- initial heating for 3 minutes at 95°C followed by:
- 35 amplification cycles consisting of
  - 20 seconds at 94°C (denaturation)
  - 20 minute at 50°C (primer annealing)
  - 1 minute at 72°C
- A final elongation step (72°C for 5 minutes) following the final amplification cycle.

- Amplification was performed in 45 sample batches together with one negative and two positive controls as follows: DNA from *C. jejuni* strain NCTC 11168 (HPA) extracted using a commercial kit (Nucleospin: Macherey-Nagel GmbH & Co. KG.)), DNA from *C. jejuni* strain NCTC 11168 extracted in parallel with the DNA from the isolates under investigation ("extract control"). Thus 2 alleles from 45 isolates were amplified on one 96-well plate at a time.

- Gel electrophoresis to confirm that the reaction had worked and assess intensity of PCR products was performed on 5μl of product from each of the first 12 wells of the 96-well plate i.e. on the DNA controls and 4 isolates for each allele. A 2% agarose gel was run for 20 minutes at 120V DC.
- Following confirmation of successful PCR, products were purified by precipitation using 20% polyethylene glycol- 2.5M NaCl followed by 2 washes using ice-cold 70% ethanol.
- Products were then re-suspended in up to 50μl molecular grade water. Re-suspension volume was dependant on intensity of PCR product as visualised following gel electrophoresis with barely visible products being re-suspended in 10μl whilst more intense products were re-suspended in volumes up to 50μl. Following re-suspension products were stored at -20^{0}C until required.
- Sequencing reactions were performed in a programmable thermal cycler (ABI 20720: Applied Biosystems) using the following primers:

Table 2.2. Primers used for sequencing in the Oxford MLST protocol.

Locus	Primer	Sequence (5′ - 3′)
aspA	aspS3 (forward)	5′- CCA ACT GCA AGA TGC TGT ACC AGC -3′
	aspS6 (reverse)	5′- TTC ATT TGC GGT AAT ACC ATC -3′
glnA	glnS1	5′- GCT CAA TTC ATG CAT GGC -3′
	glnS6	5′- TTC CAT AAG CTC ATA TGA AC -3′
gltA	gltS1	5′- GTG GCT ATC CTA TAG AGT GGC -3′
	gltS6	5′- CCA AAG CGC ACC AAT ACC TG -3′
glyA	glyS3	5′- AGC TAA TCA AGG TGT TTA TGC GG -3′
	glyS4	5′- AGG TGA TTA TCC GTT CCA TCG C -3′
pgm	pgmS5	5′- GGT TTT AGA TGT GGC TCA TG -3′
	pgmS2	5′- TCC AGA ATA GCG AAA TAA GG -3′
tkt	tktS5	5′- GCT TAG CAG ATA TTT TAA GTG -3′
	tktS4	5′- ACT TCT TCA CCC AAA GGT GCG -3′
uncA	uncAS5	5′- TGT TGC AAT TGG TCA AAA GC -3′
	uncAS4	5′- TGC CTC ATC TAA ATC ACT AGC -3′

Sequencing reactions were performed in 10μl volumes in a programmable thermal cycler (ABI 20720: Applied Biosystems) as described below:

Reaction mix.	
Amplified DNA	1.50μl
5x Buffer	1.87μl
Primer F or R (0.67μM)	4.0μl
Big Dye	0.25μl
Molecular grade water	2.38μl

Reaction conditions:

- initial heating for 3 minutes at 94^{0}C followed by:
- 25 amplification cycles consisting of
  - 1 minute at 94^{0}C (denaturation)
  - 1 minute at 65^{0}C (primer annealing)
  - 1 minute at 72^{0}C
- A final elongation step (72^{0}C for 7 minutes) followed the final amplification cycle.

2.5.2 Miller *et al.* (2005) Protocol.

In contrast to the Dingle protocol, the same primers are used for both amplification and sequencing.

Table 2.3. Primers used for amplification and sequencing in the Miller MLST protocol.

Locus	Primer	Sequence (5′ - 3′)
aspA	aspAF1 (forward)	5′- GAGAGAAAAGCWGAAGAATTTAAAGAT -3′
	aspAR1 (reverse)	5′- TTTTTTCATTWGCRSTAATACCATC -3′
glnA	glnAF	5′- TGATAGGMACTTGGCAYCATATYAC -3′
	glnAR	5′- ARRCTCATATGMACATGCATACCA -3′
gltA	gltAF	5′- GARTGGCTTGCKGAAAAYAARCTTT -3′
	gltAR	5′- TATAAACCCTATGYCCAAAGCCCAT -3′
glyA	glyAF	5′- ATTCAGGTTCTCAAGCTAATCAAGG -3′
	glyAR	5′- GCTAAATCYGCATCTTTKCCRCTAAA -3′
pgm	pgmF1	5′- CATTGCGTGTDGTTTTAGATGTVGC -3′
	pgmR1	5′- AATTTTCHGTBCCAGAATAGCGAAA -3′
tkt	tktF1	5′- GCAAAYTCAGGMCAYCCAGGTGC -3′
	tktR	5′- TTTTAATHAVHTCTTCRCCCAAAGGT -3′
uncA (atpA)	atpAF	5′- GWCAAGGDGTTATYTGTATWTATGTTGC -3′
	atpAR	5′- TTTAADAVYTCAACCATTCTTTGTCC -3′

The above primers are described as "degenerate primers" i.e. a mixture of primers differing at specific bases. Coding is as follows

R=A+G K=G+T H=A+T=C N=A+C+T+G Y=C+T

S=G+C B=G+T+C M=A+C W=A+T V=G+A+C D=G+A+T

Amplification reactions were performed in 50µl volumes comprising 2µl of DNA lysate and 48µl of mastermix, using a programmable thermal cycler (ABI 20720: Applied Biosystems) as follows:

Mastermix:

dNTPs (10mM of each)	1.0 µl
10x PCR buffer	5.0µl
$MgCl_2$ 25mM	4.5µl
Primers (10µM) – each	5.0µl
Taq polymerase (5Uµl^{-1})	0.25µl

Reactions Conditions:

- initial heating for 3 minutes at 95^0C followed by:
- 35 amplification cycles consisting of
  - 20 seconds at 94^0C (denaturation)
  - 20 minute at 50^0C (primer annealing)
  - 1 minute at 72^0C
- A final elongation step (72^0C for 5 minutes) following the final amplification cycle.

Gel electrophoresis, precipitation, re-suspension and sequencing reaction conditions were identical to those used in the Oxford protocol (apart from choice of sequencing primers).

Following the sequence reactions, irrespective of protocol used up to this point, removal of unincorporated dye terminators was achieved by precipitation with 95% ethanol-3M sodium acetate followed by one wash with ice-cold 70% ethanol.

Plates were prepared for loading onto an ABI 3130xl Genetic Analyser by the addition of 10μl of Hi-Di Formamide (Applied Biosystems) to each well and heating at 94^0C for 2 minutes. Samples were run on the ABI 3130xl using a 50cm array and POP-7 polymer (Applied Biosystems).

Complete laboratory protocols including precipitation and clean-up methodologies are in Appendix 3.

Sequence data assembly, alignment, and interrogation of the *Campylobacter jejuni* Multi Locus Sequence Typing website (http://pubmlst.org/ campylobacter/) for assignment of sequence type was performed using the STARS computer program (Chan M.S. & Ventress N. 2001) running under Biolinux (http://nebc.nox.ac.uk/biolinux.html)

Chapter Three.

Identification of ruminant campylobacters: Test characteristics of some phenotypic and genotypic methods.

3.1 Introduction

Infection with *Campylobacter spp* is recognised as a major cause of infectious gastroenteritis worldwide. It is estimated to cause between 5 – 14% of cases of diarrhoeal disease globally (WHO 2005). In the UK, campylobacteriosis is the most frequently diagnosed cause of infectious diarrhoea. The peak incidence of confirmed cases was in 1998 when 58,059 cases were diagnosed microbiologically (Frost et al 2002) whilst 45,290 cases were diagnosed in 2006 (HPA 2007). However the true incidence has been estimated to be considerably higher and in the region of 300,000 cases annually in England and Wales (Adak et al 2005) with an annual cost of approximately £70M in England alone (Roberts et al 2003). Whilst poultry and poultry products are believed to be the main source of human infection with up to 80% - 90% of carcasses sold being contaminated with *Campylobacter spp*, chiefly *C. jejuni* and *C. coli* (Bolton *et al.* 1999), there is increasing evidence suggesting that environmental exposure and/or contact with animals is an important route of infection (Louis *et al.* 2005, Ethelberg *et al* 2005, Michaud *et al* 2004).

Determination of the extent and importance of potential environmental reservoirs such as ruminant meat products or faeces necessitates the screening of large numbers of samples for the presence of campylobacters and their accurate identification at a species level. Identification of campylobacters using phenotypic methods is problematic because of their fastidious growth requirements and biochemical inertness (On 1996).

The hippurate hydrolysis test (Harvey 1980) is considered the "gold standard" for identification of *C. jejuni* although both false positives and false negatives have been reported (Morris *et al* 1985, Totten *et al* 1987, Denis *et al* 1999). Consequently there has been considerable interest in the development of molecular identification techniques based on the polymerase chain reaction (PCR).

PCR techniques offer advantages in terms of low cost, high sensitivity and specificity and the ability to rapidly screen large numbers of samples. A number of PCR assay have been developed for identification of both the genus *Campylobacter* and of species within the genus (e.g. Linton *et al.* 1996, Gonzalez *et al* 1997, Wang *et al.* 2002). On and Jordan (2003) investigated the test performance of 11 PCR assays for identification of *C. jejuni* and *C. coli* and found no one test to be 100% sensitive or specific. Furthermore they found that the use of aqueous lysates could result in sub-

optimal performance – an important point in studies involving large numbers of isolates where DNA purification is not carried out. They suggest that a polyphasic strategy involving series testing using more than one PCR assay should be adopted for maximum accuracy in identification of *C. jejuni.*

Arcobacter spp are increasingly recognised as being widely distributed in both poultry and cattle (Snelling *et al* 2006, Wesley *et al* 2000) and PCR assays have been developed for their identification and assignment to species ((Gonzalez *et al.* 2000, Harmon & Wesley 1997).

The purpose of this study was to investigate the test performance of "failure to grow in air", the hippurate hydrolysis test and a number of PCR assays on putative *Campylobacter* isolates from ruminant faeces utilising aqueous lysates rather than purified DNA extracts.

3.2 Materials and Methods.

In an on-going longitudinal study of 14 dairy and four sheep farms, 280 freshly voided faecal samples were collected from dairy cows and 80 similar samples collected from adult sheep during February and March 2006. Between 3 - 10 grams of faeces were collected from at least 3 sites within the faecal pat and mixed thoroughly in a sterile sample pot. In the case of sheep faecal pellets, at least 3 were collected. Samples were transported to the laboratory on ice. One gram of faeces was placed in 9 ml of *Campylobacter* enrichment broth (IDG Ltd, Bury, UK) with cefoperazone, vancomycin, trimethoprim and cycloheximide (CVTC supplement: IDG Ltd, Bury, UK) and after homogenising for 30 seconds in a Colworth 80 stomacher (A. J. Seward & Co. Ltd, London, UK) was incubated in a plastic universal bottle for 24 hours at 37^0C in a microaerobic atmosphere (CO_2 12%, H_2 3%, O_2 11%. N_2 74%). After incubation, 50µl of the enrichment broth was inoculated onto a *Campylobacter* blood-free selective agar (CSA) plate (IDG Ltd, Bury, UK) enriched with cefoperazone and amphotericin (CA supplement: IDG Ltd, Bury, UK). A 30µg nalidixic acid sensitivity disc (Mast Group, Merseyside, UK) was placed on the heaviest part of the inoculum. Similarly a 5µl loopful of enrichment broth was inoculated onto a second CSA plate without a nalidixic acid disc. The CSA plates were incubated at 37^0C in a microaerobic atmosphere for 72 hours after which time plates were examined and up to 4 putative campylobacter colonies were sub-cultured

onto blood agar plates and incubated at 37^{0}C under microaerobic conditions as described earlier. Putative campylobacters were identified on colony morphology (silvery metallic sheen, "flying saucer" appearance) on CSA plates. After 60 -72 hours incubation, single colonies were sub-cultured onto two blood agar plates. One plate was incubated for 48 hours under microaerobic conditions as described and the other plate incubated for 48 hours at 37^{0}C in air.

A crude DNA aqueous lysate ("boiled preparation") was prepared by inoculating 200µl of distilled water with a small amount of the culture ("leading edge of a loop"), heating at 100^0 C for 15 minutes followed by centrifugation at 1300 rpm for 10 minutes. All presumptive campylobacter isolates were frozen in Microbank tubes (Pro-Lab Diagnostics, Neston, United Kingdom) and stored at -80^{0}C.

A rapid hippurate hydrolysis test (Harvey 1980) was performed in 96-well plates by inoculating 100 µl 1% sodium hippurate solution with a small amount ("leading edge") of the culture, incubating for 2 hours at 37^{0}C in microaerobic conditions and then adding 50µl of Ninhydrin solution (3.5% in 1:1 acetone:butanol) and incubating in air for 20 minutes at room temperature at which time plates were read. A positive result was indicated by a purple colour which varied in intensity from deep to faint purple. The intensity of a positive result was recorded on a scale of 1+ to 3+ with 3+ being taken as a positive result indicating hydrolysis had occurred. In the case of 436 isolates, the hippurate hydrolysis test was carried out after growing up the isolates from frozen beads (Microbank tubes) rather than on the original cultures.

All isolates were subjected to the following PCR assays for speciation purposes: pan-campylobacter 16S rRNA PCR assay for identification of the genus *Campylobacter* (Linton *et al* 1996): pan-arcobacter 16S rRNA PCR assay for identification of the genus *Arcobacter* (Gonzalez *et al.* 2000): colony multiplex PCR assay (Wang *et al* 2002) for identification of 23S rRNA from *Campylobacter* spp, *hipO* gene (hippuricase) from *C. jejuni* subsp *jejuni*, and *glyA* gene (serine hydroxymethyltransferase) from *C. coli* and *C. lari*: 16S rRNA duplex PCR assay for identification of *C. hyointestinalis* and *C. fetus* (Linton *et al* 1996): *ceuE* gene PCR assay (Gonzalez *et al* 1997) for identification of *C. jejuni*: *ceuE* gene PCR assay (Gonzalez *et al* 1997) for identification of *C. coli*.

In all cases of isolates cross-reacting with more than one assay, the PCR assays were repeated on a fresh culture grown up on blood agar from a frozen isolate (via a further

sub-culture step from a single colony) in order to rule out the possibility of mixed cultures being tested.

3.3 Results.

Two hundred and fifty six (71%) of the 360 faecal samples collected yielded 995 putative campylobacters based on morphological appearance on CSA plates. These nine hundred and ninety five isolates were sub-cultured onto blood agar. In the case of 25 isolates, fungal or bacterial overgrowth occurred such that DNA extracts could not be prepared. A total of 970 isolates were subjected to incubation in air and to PCR assays.

3.3.1 Air growth.

Fungal or bacterial overgrowth occurred on 83 (8.5%) of the isolates during air growth such that the plates could not be read. Of the remaining 887 isolates, 471 (53.1%) did not grow in air suggesting they were putative *Campylobacter* spp. Subsequently 397/471 (84.3%) of these isolates were identified by PCR assay as being a speciated campylobacter (*C. jejuni, C. coli, C. fetus* or *C. hyointestinalis*). Of the 74 isolates that failed to grow in air in air but were not identified as *Campylobacters,* 25 were identified as *Arcobacter* spp with no identity being assigned to the remaining 49 isolates. In addition, 4 isolates which grew in air were identified by PCR assay as *Campylobacters* with 2 isolates identified as *C. coli*, one as *C. hyointestinalis* whilst one isolate was positive for both the *C. jejuni* hipO gene and the pan-arcobacter PCR assay. Thus "failure to grow in air" has a test sensitivity for detection of the afore-mentioned *Campylobacter* spp of 99% (95% CI 97.5 – 99.7) and a specificity of 84.8% (95% CI 81.3 – 87.8).

3.3.2 Hippurate Hydrolysis.

The hippurate hydrolysis test was carried out on a total of 830 isolates with 562 (67.7%) of isolates demonstrating no detectable hippurate hydrolysis (Table 1). Of the 268 isolates showing a colour change, 211 (78.7%) were classified as 3+ i.e. a strong positive reaction indicating hydrolysis. Taking PCR identification of *C. jejuni* as the "gold standard" and a strong colour change (3+) as a positive result, the test sensitivity of the hippurate hydrolysis test for detection of *C. jejuni* is 94.1% (95% CI 89.4 – 97.1) and the specificity is 92.2% (95%CI 89.9 – 94.2). Ten *C. jejuni* isolates

failed to hydrolyse hippurate whilst one *C. coli,* four *C. fetus* and fourteen *Arcobacter* spp isolates, together with 32 unidentified isolates, hydrolysed hippurate.

Table 3.1. Distribution of isolates showing hippurate hydrolysis.

Number of isolates (n = 830)	**Result**	**Description**
562 (67.6%)	0	No colour change
211 (25.3%)	+++	Deep purple colour
39 (4.7%)	++	Faint purple colour
18 (2.2%)	+	Very Faint purple colour

A total of 451 isolates were identified as campylobacters using the PCR assays for assignment to species namely *C jejuni*, *C coli*, *C. hyointestinalis* and *C. fetus*. The individual PCR test results are summarised in Table 3.2.

3.3.3. Performance of the *Campylobacter* 16S rRNA PCR assay (Linton *et al* 1996).

Seven hundred and sixteen isolates (73.8%) were positive for this PCR assay suggesting they were *Campylobacter* spp. Four hundred and twenty nine of the 451 speciated campylobacter isolates, identified using specific speciation PCR assays, were positive to the *Campylobacter* 16S rRNA PCR assay equating to a test sensitivity of 95.1% (95% CI 92.7 – 96.9) for detection of *Campylobacter* spp. Two hundred and eighty seven isolates were positive to the *Campylobacter* 16S rRNA PCR assay but negative to all the speciation PCR assays performed. Calculation of the true specificity is problematic but if it is assumed that none of these 287 "false positives" were unspeciated *Campylobacters* (that had not been tested for e.g. *C. lanienae*) this equates to a specificity of 44.7% (95% CI 40.4% - 49.1%) In fact, two hundred and twenty four (78%) of the 287 "false positive" isolates were positive by the *Arcobacter* spp 16S rRNA PCR assay suggesting considerable cross-reaction between *Arcobacter* spp and the *Campylobacter* 16S rRNA PCR assay. Alternatively, if it is assumed that the remainder of the 287 "false positives" (n = 63) were unspeciated *Campylobacters* this would equate to a specificity of 50.9% (95% CI 46.2

– 55.6). It is likely that the true specificity lies between these two values since neither assumption is likely to be absolutely correct.

3.3.4 Performance of the *Campylobacter* 23S rRNA PCR assay (Wang *et al* 2002).

Eight hundred and eleven isolates (83.6%) were positive by this PCR assay suggesting they were either *Campylobacter* spp or *Arcobacter* spp. Three hundred and sixty eight of the 451 speciated *Campylobacter* isolates, identified using specific speciation PCR assays, were positive to the *Campylobacter* 23S rRNA PCR assay whilst 364 of the 400 *Arcobacter spp* isolates were positive equating to a test sensitivity of 85.9% (95% CI 83.4 – 88.2) for detection of *Campylobacter* spp. The specificity was 32.3% (95% CI 24.1 – 41.2).

3.3.5 Performance of the *Arcobacter* 16S rRNA PCR assay (Gonzalez *et al.* 2000).

Four hundred isolates (41.2%) were positive by this PCR assay suggesting they were *Arcobacter* spp. Of these 400 isolates, only five were identified as *Campylobacter* spp namely one *C. jejuni*, one *C. coli*, two *C. hyointestinalis* with one isolate being positive to both the *C. hyointestinalis* and the *C. jejuni ceuE* gene PCR assays , suggesting that the *Arcobacter* 16S rRNA PCR assay is highly specific for *Arcobacter* spp. No estimation can be made of sensitivity of the PCR assay since there is no absolute "gold standard" for identification of *Arcobacter* spp in this study.

3.3.6 Performance of *C. jejuni* speciation PCR assays.

One hundred and seventy five (18%) isolates were identified as *C. jejuni* by the *C. jejuni hipO* gene PCR assay (Wang *et al* 2002) whilst 179 (18.4%) were identified as *C. jejuni* by the *C. jejuni ceuE* gene PCR assay (Gonzalez *et al* 1997). If it is assumed a positive reaction to either PCR assay ("gold standard") is diagnostic for presence of *C. jejuni* then the total number of *C. jejuni* isolates in this study is 196 representing 20.2% of the 970 isolates. One hundred and fifty eight *C. jejuni* isolates were positive by both PCR assays whilst 17 were positive only by the *hipO* gene PCR assay with 21 being positive only by the *ceuE* gene PCR assay. For the *ceuE* gene PCR assay, this equates to a test sensitivity of 91.3% (95% CI 86.5% - 94.9%) whilst test sensitivity of the *hipO* gene PCR assay is 89.3% (95% CI 84.1% - 93.2%). There were 3 cases of cross reactions with other PCR assays; namely one isolate was positive to both *C. jejuni* PCR assays and to the 16S rRNA *C fetus* PCR assay, one isolate was positive to

both *C. jejuni* PCR assays and to the 16S rRNA *C. hyointestinalis* PCR assay whilst one isolate was positive to the *hipO* gene *C. jejuni* PCR assay and to the 16S rRNA *C hyointestinalis* PCR assay.

3.3.7 Performance of *C. coli* speciation PCR assays.

Ninety nine (10.2%) isolates were identified as *C. coli* by the *C. coli glyA* gene PCR assay (Wang *et al* 2002) whilst 107 (11%) were identified as *C. coli* by the *C. coli ceuE* gene PCR assay (Gonzalez *et al* 1997). If it is assumed a positive reaction to either PCR assay ("gold standard") is diagnostic for presence of *C. coli* then the total number of *C. coli* isolates in this study is 110 representing 11.3% of the 970 isolates. Ninety seven *C. coli* isolates were positive by both PCR assays whilst 2 were positive only with the *glyA* gene PCR assay with 10 being positive only by the *ceuE* gene PCR assay. This equates to test sensitivities of 97.3% (95% CI 92.2% - 99.4%) for the *ceuE* gene PCR assay and 90% (95% CI 82.8% - 94.9%) for the *glyA* gene PCR assay. There was only one cross reaction with one isolate being positive by both the *C. coli ceuE* gene PCR assay and the 16S rRNA *C. hyointestinalis* PCR assay.

3.3.8 Performance of *C. hyointestinalis* and *C. fetus* 16S rRNA duplex PCR assay (Linton *et al* 1996).

No estimate of the test sensitivities of this PCR assay may be made due to the absence of any "gold standards". There was a total of six isolates, namely two *C. jejuni*, one *C. coli* and three *Arcobacter spp* which were also positive by any one of the primers in this PCR assay suggesting the specificity is high.

Table 3.2. Results of speciation PCR assays.

PCR	n	Positive
Campylobacter 16S rRNA (Linton *et al* 1996)	970	716 (73.8%)
Arcobacter 16S rRNA (Gonzalez *et al.* 2000)	970	400 (41.2%)
Campylobacter 23S rRNA (Wang *et al* 2002)	970	811 (83.6%)
C. jejuni HipO gene (Wang *et al* 2002)	970	175 (18.0%)
C. coli GlyA gene (Wang *et al* 2002)	970	99 (10.2%)
C. lari GlyA gene (Wang *et al* 2002)	970	0
16s rRNA *C. hyointestinalis* (Linton *et al* 1996)	970	48 (4.9%)
16s rRNA *C. fetus* (Linton *et al* 1996)	970	102 (10.5%)
C. jejuni ceuE gene (Gonzalez *et al* 1997)	970	179 (18.4%)
C. coli ceuE gene (Gonzalez *et al* 1997)	970	107 (11%)
Total *C. jejuni* (positive to either speciation PCR)	970	196 (20.3%)
Total *C. coli* (positive to either speciation PCR)	970	109 (11.2%)

3.4 Discussion.

Elucidating the potential role of ruminants in the epidemiology of human campylobacteriosis necessitates the screening of large numbers of faecal samples for the presence of *Campylobacter* spp, for example Brown *et al* (2004) examined 1,014 faecal samples whilst Wesley *et al* (2000) examined 2,085. A genus-specific PCR assay would be of considerable value in such studies enabling both better estimation of overall campylobacter prevalence and mitigating the high workload involved in such studies. Brown *et al* (2004) used the *Campylobacter* 16S rRNA PCR assay (Linton *et al* 1996) to estimate the prevalence of *Campylobacter* spp other than *C. jejuni*, *C. coli*, C. *lari* and *C. hyointestinalis* and found 41% of isolates tested positive for one of the *Campylobacter* species whilst 15% were classified as "unspeciated" *Campylobacters*. Extrapolating the findings of the present study it is highly possible that a large proportion of these were *Arcobacter* spp.

Two such PCR assays have been investigated in the present study, namely the *Campylobacter* 16S rRNA PCR assay (Linton *et al* 1996) and the *Campylobacter* 23S rRNA PCR assay (Wang *et al* 2002). Neither PCR assay would appear to perform adequately on ruminant derived samples. Whilst the sensitivity of the *Campylobacter* 16S rRNA PCR assay is reasonable for detection of *C. jejuni*, *C. coli*, C. *fetus* and *C. hyointestinalis* (95.1%), the specificity is poor with estimates of between 44.7% and

50.9%, severely limiting its use as a screening test. The poor specificity is due in large part to the considerable cross-reaction seen between it and *Arcobacter* spp. In addition to failing to detect almost 5% of campylobacters, its use would result in grossly over-inflated estimates of *Campylobacter spp* prevalence. The *Campylobacter* 23S rRNA PCR assay is designed to detect *Campylobacter, Arcobacter and Helicobacter* spp. Thus it is not suitable for screening of samples for *Campylobacter spp* alone. For this test the sensitivity was fair (85.9%) but the specificity was poor (32.3%). Both these PCR assays have been validated using moderately large numbers of isolates from a variety of sources both clinical and environmental and included well-established laboratory isolates (Linton *et al* 1996, Wang *et al* 2002) so it is perhaps surprising that they performed so poorly on ruminant derived isolates. It is possible that the poor test performances demonstrated in this study compared to the validation studies is a reflection of the genetic diversity present in *Campylobacter* spp from different animal species since it is likely that the majority of isolates used in the validation studies were derived from human and poultry sources rather than ruminants.

None of the *C. jejuni* or *C. coli* PCR assays in this study were100% sensitive thus reinforcing the conclusions drawn by On and Jordan (2003) who advocate serial testing of isolates for maximum sensitivity.

Two phenotypic tests were investigated in this study namely "ability to grow in air at 37^0C" and the hippurate hydrolysis test. Air growth showed 99% sensitivity for detection of *Campylobacter spp* with only 4 isolates identified as *Campylobacter* spp growing in air. Specificity was 84.8% with 25 *Arcobacter* spp and 49 unidentified isolates apparently failing to grow in air. However it must be borne in mind that a limited number of *Campylobacter* species specific PCR assays were used thus it is possible that a proportion of these 49 unspeciated "false positives" were in fact *Campylobacters*. If this were the case then the estimate for specificity would be higher. In light of the apparent high prevalence of *Arcobacter* spp in ruminant faeces, the inability to grow in air offers a simple screening test for identification of putative *Campylobacter* spp.

The ability to hydrolyse hippurate is considered as a "gold standard" test for identification of *C. jejuni*, although both false negatives i.e. hippurate negative *C. jejuni* (Totten *et al* 1987) and false positives (Denis *et al* 1999) have been reported. The present study found both false positives (n = 51) and false negatives (n = 10). In

the present study, 94% of *C. jejuni* isolates were identified correctly by their ability to hydrolyse hippurate but there were a number of false positives such that the specificity was 92.2%. These results call into question the wisdom of using the hippurate hydrolysis test alone as a gold standard for identification of *C. jejuni* in ruminant studies.

All the cattle sampled in this study were housed, suggesting a relatively high faecal prevalence of *Arcobacter* spp in housed cattle.

This study has demonstrated the poor test performance of the 16S rRNA *Campylobacter* genus PCR assay in studies involving ruminant samples. In large part this is consequent on the cross-reaction between the 16s rRNA *Campylobacter* PCR assay and *Arcobacter* spp.

Chapter Four.

Descriptive results.

Summary statistics for study population.

Fifteen dairy farms and four sheep farms participated in the study. One farm (No. 8) from Zone 2 ceased trading in December 2006 and left the study. A neighbouring farm (No 19) was recruited *in lieu* and sampled for the remainder of the study. Another farm (No 11) ceased keeping cattle in June 2007 thus sampling on this farm was incomplete. Farm details were collected with a questionnaire (Appendix A.) prior to starting sampling and relevant details are summarised below together with additional information acquired during sampling visits.

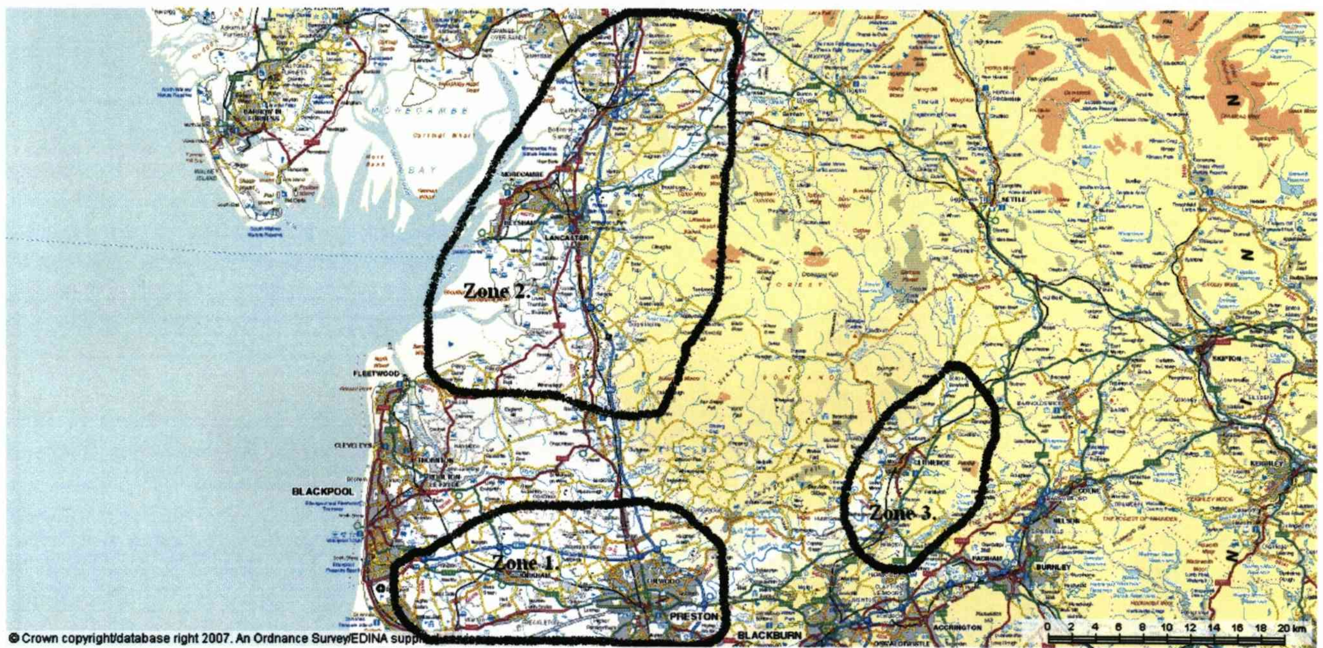


Figure 4. 1. Map showing approximate bounds of sampling areas.

4.1 Dairy herds.

Fourteen of the herds were of the Holstein Friesian breed whilst one was Ayrshire and Ayrshire X Friesian.

4.1.1 Replacement policy. Thirteen farms bred their own replacements whilst two were "flying herds" i.e. all lactating cows were purchased either from herd dispersal sales or from livestock markets. Six farms could be considered "closed" in that they did not buy-in stock at all whilst 6 reported occasional cattle purchases with 3 farms reporting frequent purchases.

4.1.2 Herd Size. This was defined as the total number of adult cows in the herd, both lactating and dry. The median herd size was 145 (inter-quartile range 104 – 200), ranging from 71 – 280 cows.

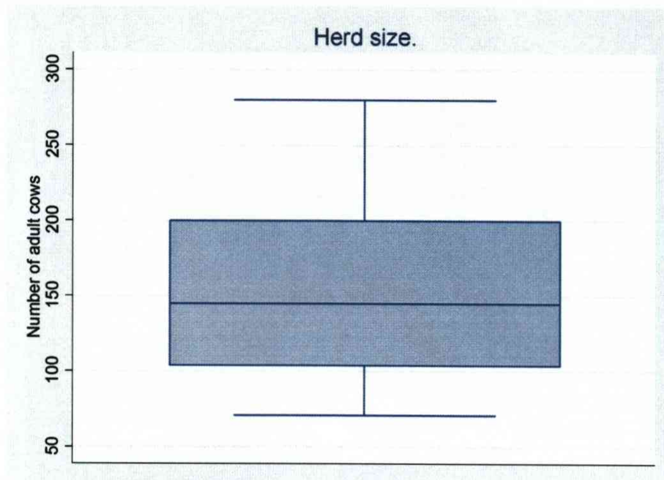


Figure 4. 2. Box plot of number of adult cows in participating herds (n = 15).

4.1.3 Annual milk yield. Estimates were obtained for 365 day milk yield. Median annual milk yield was 8,000 (inter-quartile range 7200 – 9000) litres, ranging from 6000 to 9600 litres per annum.

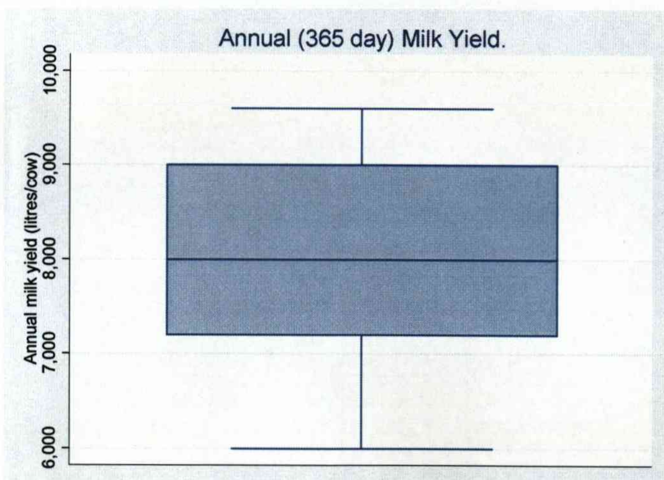


Figure 4. 3. Box plot of annual milk yield in participating herds.

4.1.4 Grazing practices. Whilst 6 of the dairy farms had concurrent sheep enterprises varying in size from 60 – 700 breeding ewes, 7/9 of the remainder over-wintered sheep. Thus 13/15 dairy farms also grazed sheep for all or part of the year. None of the dairy farms practiced co-grazing of adult cattle and sheep although on the 6 farms which had a sheep enterprise, the sheep would on occasions graze pastures previously grazed by the adult dairy cows, whilst co-grazing of sheep and young stock was frequently practiced, although not consistently.

All the dairy farms practiced set-stocking of pastures, i.e. the cows would graze an entire field for a variable period of time depending on the size of field and stock numbers. The time cows were grazing a field would vary from 3 – 20 days depending on number of cows, grass growth etc. On occasions, some farmers would graze two fields concurrently with cattle grazing one pasture at night and one in the day.

During the winter season when cows were housed slurry was spread on fields irrespective of their future use (grazing or silage) by all farmers. During the summer grazing period, slurry was spread on grazing pastures by 12/15 farmers and manure spread by six. The criteria by which it was decided to allow cows to graze a pasture previously spread with slurry or manure varied between farmers with 7 stating that they waited a fixed time of at least 6 weeks before allowing cows to graze whilst 4 allowed cows to graze when the pasture "looked clean" whilst one allowed cows to graze only after a period of rain. All farmers spread slurry and manure on fields destined for ensiling or arable use.

Three farmers reported having spread domestic sewage on grazing pastures although it was not a consistent management practice.

In five herds, lactating cows were housed all year, although other classes of stock grazed outside in summer. On all farms that grazed lactating cows during the summer, "spring turn-out" was gradual in that initially cows would be turned out during the day and housed at night for a period of up to 1 – 3 weeks. A similar procedure was practised in the autumn at housing. During the summer of 2007, some farmers would bring cows in during periods of inclement weather for varying periods, either completely or at night only.

In summary, there was considerable variation in grazing practices both between farms and within farms depending on local weather conditions, especially during the wet summer of 2007.

All farms had water troughs in some or all of their grazing fields with 2 farmers using streams or rivers in some fields as the main water source. On six farms the water was supplied from a borehole whilst on the remainder it was supplied via the mains supply. Eight herds had regular access to ponds in grazing fields whilst nine had access to streams or ditches.

4.1.5 Housing practices. Fourteen of the dairy herds were housed in cubicles whilst one herd was loose housed on straw bedding. There was considerable variation in overall standard of housing and cubicle design. There were sufficient numbers of cubicles for all cows on all farms. All herds fed housed cows at a feed barrier with food being put out once daily.

Water was supplied via troughs in all houses and was sourced from the mains supply on 9 farms and from farm boreholes on 6 six farms. Water troughs were cleaned routinely at varying intervals on 14 farms but not on the remainder.

4.1.6 Feeding practices.

Ten farms made clamp grass silage only whilst 5 made both big-bale and clamp silage. Eleven farms stored purchased foodstuffs in rodent-proof bins whilst 5 stored foodstuffs loose on the floor.

- **Winter feeding.** One farm fed all constituents as a total mixed ration (TMR) at the feed barrier whilst 12 fed a TMR and additional concentrate in the milking parlour ("hybrid TMR"). Two farms fed grass silage at a feed barrier and concentrate in the milking parlour, although on occasions additional "straights" such as brewers grains would also be fed at the barrier. All farmers fed grass silage with ten also feeding maize silage and four feeding whole-crop silage. Straw was included in the diet on eight farms whilst haylage was included on four farms. Pea silage was fed on one farm. All farmers purchased parlour concentrate, if fed, whilst 12 purchased a proprietary "protein blend" for inclusion in the TMR. Only 2 farmers fed home-mixed protein blend. Other feeds utilised included sugar beet pulp, brewer's grains and potatoes.
- **Summer Feeding.** In the case of the five herds that were housed throughout the year, the summer diet was the same as was fed in winter. Of the 10 herds

that grazed, all bar one fed a buffer feed, generally consisting of one or more forages with or without a protein blend. There was considerable variation both between and within farms with regards to buffer feeding. In all cases the buffer feed was supplied to the cows twice daily after milking in the farm buildings, although the time cows were allowed to eat it varied considerably. All farmers who grazed cattle fed proprietary concentrates in the milking parlour with the amount being fed varying according to grass quality and milk yield.

4.1.7 Calf rearing and young stock management. This was broadly similar, albeit to varying standards, on all farms. On seven farms, cows calved in group housing whilst eight farms used individual calving pens. The calf remained with its dam for a period varying from 1 -4 days before being removed to calf housing which consisted of individual pens on 12 of the farms with group pens (up to 10 calves) on 5 farms. Milk replacer was fed on 7 farms whilst all farms fed waste milk either as a sole feed or in conjunction with milk replacer. Milk or milk replacer was fed twice daily via a bucket on 14 farms whilst one farm used an automatic group feeder with teats. Solid food (concentrate and fodder) was introduced to the calves in the first week of life on 12 farms whilst on the remaining three it was introduced in the second week. Calves were weaned at between 6 – 10 weeks of age then group housed and fed on a variety of forages, chiefly grass silage and concentrates.

Hygiene protocols and standards varied considerably between farms with 7 farmers stating they cleaned feeding utensils at least once daily whilst the others stated they cleaned utensils weekly.

In summer young stock over 4 months of age were grazed outside. No young stock co-grazed with adult cattle although on 5 farms young stock co-grazed with sheep. Two farms did not breed dairy replacements at all and all calves were sold either at a few days of age or after weaning.

4.1.8 Wildlife and other animals. All farmers reported seeing wild birds on pastures whilst 12 reported their presence in cattle housing with 5 commenting that large numbers would visit on occasions. The predominant species visiting buildings were sparrows, pigeons and pheasants. Five farmers reported that deer would visit their fields on occasions. All farmers reported seeing rodents in their farm buildings with 6

reporting large numbers being present on occasions. Fourteen farmers routinely carried out rodent control. Free range poultry were kept on 6 farms whilst 14 kept at least one dog on the farm with cats being present on 10 farms.

4.2 Sheep farms. Two of the sheep farms were lowland with one farm grazing on the saltmarshes of the River Lune estuary whilst 2 were upland with summer grazing on moorland. On both upland farms, the chief breeds of sheep kept were Swaledale and North Country Mules whilst on the lowland farms the predominant breed was North Country Mule. The 2 upland farms kept 1000 and 700 ewes respectively whilst one lowland farm had 700 ewes whilst the other kept 150 ewes. Lambing took place solely outdoors on the smaller lowland farm and solely indoors on the other. On both upland farms twin-bearing ewes were lambed indoors whilst singleton bearing ewes lambed outdoors. All farms lambed their ewes between February and April and fat lambs were sold from June onwards.

4.3 Summary of baseline microbiological data.

- 19 farms sampled
    - 15 dairy farms
    - 4 sheep farms

All farms were sampled 12 times at 8 weekly intervals with the following exceptions

- Farm 8 – sampled 6 times during the first year then ceased trading.
- Farm 19 – recruited in the second year as a replacement for Farm 8 and sampled 6 times in the second year.
- Farm 11 – ceased keeping cattle in June 2007 – sampled 9 times up till then.

Twenty faecal samples were collected at each visit, yielding a total of 4260 samples. Four potential isolates were taken from each sample yielding 17040 potential bacterial isolates. 9499 putative *Campylobacter* spp isolates were grown and identified as follows (Table 4.1.):

Table 4.1. Distribution of *Campylobacter* and *Arcobacter* isolates by species sampled.

	Cattle	Sheep
Number of faecal pats collected	3300	960
Number of potential isolates (4 per pat)	13200	3840
Number of isolates grown (% of potential isolates)	7779 (58.9%)	1720 (44.8%)
Arcobacter spp (% of actual isolates)	4299 (55.3%)	236 (13.7%)
Campylobacter jejuni (% of actual isolates)	1857 (23.9%)	450 (26%)
Campylobacter coli (% of actual isolates)	346 (4.4%)	815 (47.4%)
Campylobacter fetus (% of actual isolates)	871 (6.8%)	211 (12.3%)
Campylobacter hyointestinalis (% of actual isolates)	380 (4.9%)	0
Campylobacter lari (% of actual isolates)	26 (0.33%)	8 (0.05%)

Table 4.2. Distribution of *Campylobacter* and *Arcobacter* isolates by species sampled - faecal pat level.

	Cattle (%)	Sheep (%)
Number of faecal pats collected	3300	960
Number of pats with putative campylobacter	2230 (67.6%)	494 (51.4%)
Nos. pats with *Arcobacter spp* only	1138 (51%)	66 (13.4)
Nos. pats with *Campylobacter jejuni* only	453 (20.3%)	118 (23.9)
Nos. pats with *Campylobacter coli* only	67 (3%)	200 (40.5)
Nos. pats with *Campylobacter fetus* only	191 (8.6%)	51 (10.3%)
Nos. pats with *Campylobacter hyoint.* only	90 (4%)	0
Nos. pats with *Campylobacter lari* only	2 (0.09%)	2 (0.4%)
Nos. pats with *Arcobacter* & *C. jejuni*	86 (3.9)	6 (1.2%)
Nos. pats with *Arcobacter* & *C. coli*	20 (0.9%)	3 (0.6)
Nos. pats with *Arcobacter* & *C. fetus*	57 (2.6%)	0
Nos. pats with *Arcobacter* & *C. hyointestinalis*	19 (0.8%)	0
Nos. pats with *C. jejuni* & *C. coli*	19 (0.8%)	23 (4.7%)
Nos. pats with *C. jejuni* & *C. fetus*	49 (2.2)	15 (3.0%)
Nos. pats with *C. jejuni* & *C. hyoint.*	17 (0.8%)	0
Nos. pats with *C. coli* & *C. fetus*	8 (0.4%)	10 (2.0%)
Nos. pats with *C. coli* & *C. hyoint.*	3 (0.1%)	0
Nos. pats with *C. fetus* & *C. hyoint.*	5 (0.3)	0
Nos pats with *C. jejuni*, *C. coli* & *arcobacter*	3	0
Nos pats with *C. jejuni*, *C. fetus* & *arcobacter*	1	0
Nos pats with *C. jejuni*, *C. fetus* & *C. hyoint.*	1	0

Table 4.3. Overall faecal pat prevalence, over the sampling period of *Campylobacter* spp and *Arcobacter* spp by species sampled.

	Cattle (n = 3300)	Sheep (n = 960)	P value chi^2
Arcobacter spp	40.1% (95% CI: 38.5 – 41.8)	8.0% (95% CI:6.3 – 9.7)	0.0001
C. jejuni	19.1% (95% CI: 17.7 – 20.4)	17.0% (95% CI:14.6 – 19.4)	0.145
C. coli	3.7% (95% CI: 3.0 – 4.3)	24.6% (95% CI: 21.8 – 27.3)	0.0001
C. fetus	9.5% (95% CI: 8.5 – 10.5)	7.9% (95% CI: 6.2 – 9.6)	0.138
C. hyointestinalis	4.1% (95% CI: 3.4 – 4.8)	0	
C. lari	0.06% (95% CI: -0.02% – 0.14)	0.21% (95% CI:-0.008 – 0.04)	0.188

At a pat level (Tables 4.2. & 4.3.), 67.6% of cow pats and 51.4% of sheep faecal samples yielded at least one putative campylobacter isolate. 288 (12.9%) of cow pats yielded more than one species of *Campylobacter*. Two hundred and eighty three of these pats yielded 2 different species of *Campylobacter* whilst five pats yielded three species. Similarly 57 (5.9%) sheep faecal samples yielded two *Campylobacter* species whilst 437 yielded one species only.

Simple univariate analysis (chi^2 test) was performed to investigate any species differences in faecal pat prevalences of the micro-organisms (Table 4.3). There was no apparent difference in *C. jejuni*, *C. fetus* or *C. lari* prevalence between cattle and sheep faeces whilst *arcobacter* spp prevalence was significantly higher (P = 0.0001) in cattle faeces compared to sheep faeces. Conversely *C. coli* prevalence was significantly higher in sheep faeces compared to cattle faeces (P = 0.0001). *C. hyointestinalis* was only isolated from cattle faeces, albeit at a low level.

Chapter Five.

Temporal and spatial variation in the faecal pat prevalence of *Campylobacter jejuni* in ruminants in Lancashire.

5.1 Introduction.

Human infection with *Campylobacter jejuni* is a major public health problem world-wide (WHO 2005) and is estimated to cause between 5 – 14% of cases of diarrhoea globally. In the United Kingdom, campylobacteriosis is the most common cause of infectious diarrhoea with a reported incidence rate of approximately 80 cases/100,000 population (DEFRA 2005). However the actual incidence is likely to be much higher due to considerable under-reporting (Tauxe 1992) and some estimates place the true UK incidence in the region of 300,000 cases per annum (Adak *et al.* 2005).with approximately 80 deaths per annum. Campylobacteriosis is considered to be a food-borne disease with the majority of cases resulting from ingestion of contaminated foodstuffs. Most cases are sporadic single cases or family clusters (Skirrow 1991) although outbreaks occur associated with food handling faults in commercial catering establishments (Frost *et al.* 2002). Exposure to poultry products has been identified as the major infection route for human cases in numerous case control studies e.g. Rodrigues *et al.* 2001, Kappersud *et al.* 2003, Neimann *et al.* 2003, Friedman *et al.* 2004.

It is well established that poultry are often heavily infected with *Campylobacter jejuni* such that 100% infection rates have been reported in broiler chicken populations (Corry & Abatay 2001), with up to 90% of poultry carcasses at the point of sale being infected (Bolton *et al.* 1999). However, poultry products whilst being the major source do not account for all cases. There are well documented cases associated both with other foodstuffs such as raw milk (Gillespie *et al.* 2003), raw clams (Griffin *et al.* 1983), salad vegetables (Evans *et al.* 2003) and contamination of water sources, both private and municipal (Duke *et al.* 1996, Said *et al.* 2003).

Molecular biological techniques, in particular multi-locus sequencing (MLST) have provided valuable insight into the sources of human infection (Dingle *et al.* 2002, Colles *et al.* 2003, Manning *et al.* 2003), for example Sequence Type 45 (ST-45) is frequently isolated from both human cases and poultry products suggesting that poultry is the most probable source of infection with ST-45, whilst ST-61 is common in ruminants but rare in poultry thus suggesting a ruminant source for human cases with ST-61.

A number of studies focusing on infection in people living in rural areas have suggested a role for environmental factors or infection from sources other than

poultry. In their retrospective UK study, Louis *et al.* (2005) found significant correlations at district level between campylobacter rates and agricultural data for the district namely total agricultural area, agricultural labour force, number of cattle, sheep, pigs or poultry. Campylobacter rates were also positively correlated with percentage of rural wards in a district. The authors concluded that there is a linkage between human campylobacter cases and environmental factors rather than just food sources *per se*. A study in a defined rural area of New Zealand (Garrett *et al.* 2007) utilising both serotyping and *Sma*I macro-restriction profiling via pulse-field gel electrophoresis found that human isolates tended to be more similar to those from ruminant faeces and offal than from chicken carcasses, pork offal or duck faeces suggesting that acquisition of infection via environmental exposures or contact with animals is important in rural settings.

Seasonal trends in human cases are well recognised with peak cases occurring in the spring and or summer months (Sopwith *et al.* 2003, Kovats *et al.* 2005). The exact reasons for this seasonality are unknown although environmental temperature (Louis *et al.* 2005) has been suggested as a driver as has the ecology of animal reservoirs of *Campylobacter* spp (Stanley & Jones 2003).

Campylobacter jejuni is a well recognised commensal of the gastro-intestinal tract of ruminants and numerous studies world-wide,(e.g. Wesley *et al.* 2000, Bae *et al.* 2005, Hakkinen *et al.* 2007, Adhikari *et al.* 2004), have produced prevalence estimates with the majority based on faecal sampling and subsequent culture with final speciation performed either by classical phenotypic methods or, more recently, molecular techniques utilising PCR. Care is required in comparing results from different studies since differing culture techniques (Corry *et al.* 1995) are likely to produce different estimates.

Three studies investigating the prevalence in cattle have been conducted within the last ten years in the U.K. In a two year study of beef cattle at slaughter in Lancashire thermophillic campylobacters were isolated from 89.4% of beef cattle (Stanley *et al.* 1998a). The same authors obtained representative pooled faecal samples from entire dairy herds by collecting faeces from the floor of the collecting yard after morning milking. They found evidence of seasonal periodicity in the number of thermophillic campylobacters being excreted with peaks in the spring and autumn. They also suggested that there was a spatial component with the peaks being observed one to two months earlier on northerly farms compared to farms approximately 25 miles

south. Species identification was not performed in this study thus estimates of *C. jejuni* prevalence cannot be inferred.

A study based on sampling of freshly voided cattle faeces in the Wirral, Merseyside reported a *C. jejuni* prevalence of 32.4% (Robinson *et al.* 2005), which is in agreement with a previous study involving intensive environmental sampling of a 100 square Km area of Cheshire which reported a bovine *C. jejuni* prevalence of 36% (Brown *et al.* 2004).

A number of studies (Stanley *et al.* 1998a, Nielsen 2002) have shown not only that the faecal prevalence of *Campylobacter* spp is higher in young animals, but that larger numbers of campylobacters are excreted per gram of faeces by young animals compared to adults.

There is little information regarding *Campylobacter* spp and sheep. One study (Stanley *et al.* 1998b) investigated thermophillic campylobacters in sheep in Lancashire U.K. and suggested a faecal pat prevalence of 30%. However no speciation of campylobacters was performed. No seasonal or grazing associated variation in prevalence was observed although a significant seasonal trend was seen in numbers of campylobacters isolated from the intestine of lambs at slaughter with peak numbers observed in the spring.

The present study investigated the faecal pat prevalence of dairy cattle and sheep in Lancashire in order to gain further information regarding the potential contribution of ruminants to the environmental load of *Campylobacter jejuni.*

5.2 Materials and methods.

Full details of all methods are given in Chapter 2. The study design was a repeated cross-sectional study over a 2 year period starting in January 2006. Fourteen dairy and four sheep farms were recruited via three veterinary practices in Lancashire. The three practices selected were in Longbridge, Lancaster and Clitheroe and served the Southern Fylde (Zone 1), N. Lancashire (Zone 2) and S.E. Lancashire (Zone 3) respectively. Six dairy farms were recruited in Zone 1 whilst four dairy and two sheep farms were recruited in each of the other zones. One farm in Zone 2 ceased trading in December 2006 and was replaced with a neighbouring farm for the rest of the study. Another farm in Zone 2 ceased keeping cattle in June 2007 thus sampling on this farm was incomplete.

Eligibility criteria for entry were:

- Dairy farms with over 100 adult cows with or without a sheep enterprise
- Sheep farms with over 150 breeding ewes and no other livestock enterprises.

Farms were visited at 8 week intervals when 20 freshly voided faecal samples would be collected from the lactating cows on dairy farms or adult sheep on sheep farms. Samples were only collected from animals observed to defecate by the author. Three farms from one zone would be sampled at each occasion. Each faecal pat was sampled from at least 3 sites within the pat and mixed thoroughly in a sterile sample pot to minimise possible within-pat variation. In the case of sheep faecal pellets, at least 3 were collected. Twenty faecal samples were collected at each visit. Samples were transported to the laboratory on ice.

In the case of dairy cows faecal consistency was scored using a score from 1 – 5 (Hughes 2001) and a larger sample was collected from the same faecal pat in an arm-length plastic glove for further analysis by sieving to assess fibre length and presence of partially digested grains (Grove-White 2004).

At each visit, current management details were obtained via a short questionnaire (Appendix B).

In the laboratory, one gram of faeces was placed in 9 ml of *Campylobacter* enrichment broth (IDG Ltd, Bury, UK) with cefoperazone, vancomycin, trimethoprim and cycloheximide (CVTC supplement: IDG Ltd, Bury, UK) and after homogenising for 30 seconds in a Colworth 80 stomacher (A. J. Seward & Co. Ltd, London, UK) was incubated in a plastic universal bottle for 24 hours at 37^0C in a in a "Variable Atmosphere Incubator (VAIN, Don Whitley Scientific Ltd, UK) maintaining a microaerobic atmosphere (CO_2 12%, H_2 3%, O_2 11%. N_2 74%). After incubation, 50µl of the enrichment broth was inoculated onto a *Campylobacter* blood-free selective agar (CSA) plate (IDG Ltd, Bury, UK) enriched with cefoperazone and amphotericin (CA supplement: IDG Ltd, Bury, UK). A 30µg nalidixic acid sensitivity disc (Mast Group, Merseyside, UK) was placed on the heaviest part of the inoculum. Similarly a 5µl loopful of enrichment broth was inoculated onto a second CSA plate without a nalidixic acid disc. The CSA plates were incubated at 37^0C in a microaerobic atmosphere for 60-72 hours after which time plates were examined and up to 4 putative campylobacter colonies (per faecal sample) were sub-cultured onto blood

agar plates and incubated at 37^{0}C under microaerobic conditions as described earlier. Putative campylobacters were identified on colony morphology. After 72 hours incubation, single colonies were sub-cultured onto two blood agar plates. One plate was incubated for 48 hours under microaerobic conditions as described and the other plate incubated for 48 hours at 30^{0}C in air.

A crude DNA aqueous lysate ("boiled preparation") was prepared by inoculating 200µl of distilled water with a small amount of the culture ("leading edge of a loop"), heating at 100^0 C for 15 minutes followed by centrifugation at 1300 rpm for 10 minutes. All putative campylobacter isolates were frozen in Microbank tubes (Pro-Lab Diagnostics, Neston, United Kingdom) and stored at -80^{0}C.

Assignment to species of putative campylobacters was by the following PCR assays in 96-well plates:

- 16S rRNA PCR assay for identification of the genus *Arcobacter* (Gonzalez *et al.* 2000). Any isolates which were negative to this PCR were considered to be putative campylobacters and tested further by PCR.
- colony multiplex PCR assay (Wang *et al.* 2002) for identification of *C. jejuni*, *C. coli* and *C. lari.*
- duplex PCR assay (Linton *et al.* 1996) for identification of *C. fetus* and *C. hyointestinalis*.
- monoplex PCR assay (Gonzalez *et al.* 1997) for identification of any remaining *C. jejuni* that failed to be identified by the colony multiplex PCR assay.

All covariate data was entered in an Access 2003 database (Microsoft) whilst laboratory data was entered into an Excel2003 (Microsoft) spreadsheet. Data analysis was performed using Stata v10 (StataCorp: Texas) and MLwiNv2.02 (Centre for Multilevel Modelling: Bristol)

The following covariates (Tables 5.1 & 5.2) were recorded at each sampling visit and were considered for inclusion in later statistical analyses:

Table 5.1. Description of variables collected at cattle sampling visits for possible inclusion in statistical analyses.

Variables (cattle)	Type	Description and coding of variable
Farm identity	categorical	
Husbandry_type	categorical	0 = cows housed all year with no access to pasture
		1 = cows graze outside in summer, housed in winter
Purchase policy	categorical	0 = no purchased stock (closed herd)
		1 = occasional purchase of cows
		2 = frequent purchase
Group size	continuous	Number of animals in sampled group
Where sampled	categorical	Inside = 0 or Outside = 1
Zone	categorical	1 = Longbridge
		2 = Lancaster
		3 = Clitheroe
Date of sampling	dd/mm/yy	
Av_daily_milk	continuous	Average daily milk yield on sampling day
Feeding_type	categorical	Feeding system used as follows
		1 = TMR – total mixed ration
		2 = Hybrid_TMR – TMR & parlour feed
		3 = grazing & buffer feed & parlour feed
		4 = grazing & parlour feed
		5 = silage & parlour feed
Turn_out	dd/mm/yy	Date of turn out from housing to pasture
House_in	dd/mm/yy	Date of housing
Number_fresh_cows	continuous	Number of cows calved within last month
Slurry_spread	categorical	Was any slurry spread on pasture currently grazed within the last month 1 = Yes 2 = No
		No farmers answered Yes to this question
Faecal_score	categorical	Score of 1 – 5 depending on consistency with score 1 being very firm & score 5 being liquid (Hughes 2001)
Sieve_score	categorical	Score of 1 – 3 being a composite score for presence of grains & long fibre (>1") with 1= no grains or long fibre, 3 = large amounts of grains and presence of many long fibres (Grove-White 2004)

Table 5.2. Description of variables collected at sheep sampling visits for possible inclusion in statistical analyses.

Variables (sheep)	Type	Description and coding of variable
Farm identity	categorical	
Group size	continuous	Number of animals in sampled group
Type_sheep sampled	categorical	Type of sheep sampled 1 = Ewes only 2 = Ewes & baby lambs 3 = Ewes & fat lambs
Where_sampled	binary	Inside = 0 or Outside = 1
House_hygiene	categorical	Scored 1 – 5 with 1 = excellent to 5 = filthy
Zone	categorical	1 = Longbridge 2 = Lancaster 3 = Clitheroe
Date of sampling	dd/mm/yy	
How_long_in_field	continuous	Length of time sheep have been grazing pasture
Stocking_density	continuous	Number of sheep per hectare
Pasture type	categorical	Quality of pasture scored 1 - 3 1 = poor 2 = mediocre 3 = lush
Lambing _season	categorical	Were the flock lambing at the time of sampling
Concentrates_fed	categorical	Were concentrates fed at the time of sampling
Slurry_spread	categorical	Details of any slurry spread on pasture currently grazed within the last month No farmers answered Yes to this question

Univariate analysis. Simple univariate analysis (chi^2 test) was performed to investigate both any associations between *C. jejuni* faecal pat prevalence and any of the covariates measured and also between covariates to identify potential confounders. A new variable "Proportion of fresh calved cows" was calculated by dividing "the number of cows that calved in last four weeks" by the total size of the lactating group. The continuous variables "Lactating cow group size", "Daily average milk yield", "Proportion of fresh calved cows" and "Sheep stocking density" were transformed into quintiles for future analysis. The sampling period was split into "Summer" and "Winter" with the winter period defined as being from 1st October – 30th April. The term "Sampling event" is defined as "a visit to a farm to collect samples." Box plots were drawn showing median values and quartiles.

Multivariate analysis. Choice of covariates to include in regression models was made on both biological and statistical grounds. A conceptual hierarchical framework (Figure 5.1) was developed utilising the concept of proximate and distal determinants (Victora *et al* 1997). Covariates for which P was less than 0.2 in univariate analysis were considered suitable for initial inclusion in a multivariate regression model. Collinearity between covariates was investigated using Cramer's phi statistic with significant collinearity suggested by a phi statistic greater than 0.6. If significant collinearity was demonstrated, one of the covariates was discarded with the exact decision being made on grounds of biological plausibility. A backward and forward stepwise method was used to assess which covariates to retain in a model with a likelihood ratio test being performed to compare models at each stage. A likelihood ratio P value less than 0.05 was taken to indicate that addition or exclusion of a covariate had a significant effect on the model.

Time was included in the model as a composite of four sine and cosine functions (Stolwijk *et al.* 1999). Four time covariates (x_1 x_2 x_3 x_4) were generated as follows:

$x_1 = \cos(2\pi t/52)$

$x_2 = \text{sine}(2\pi t/52)$

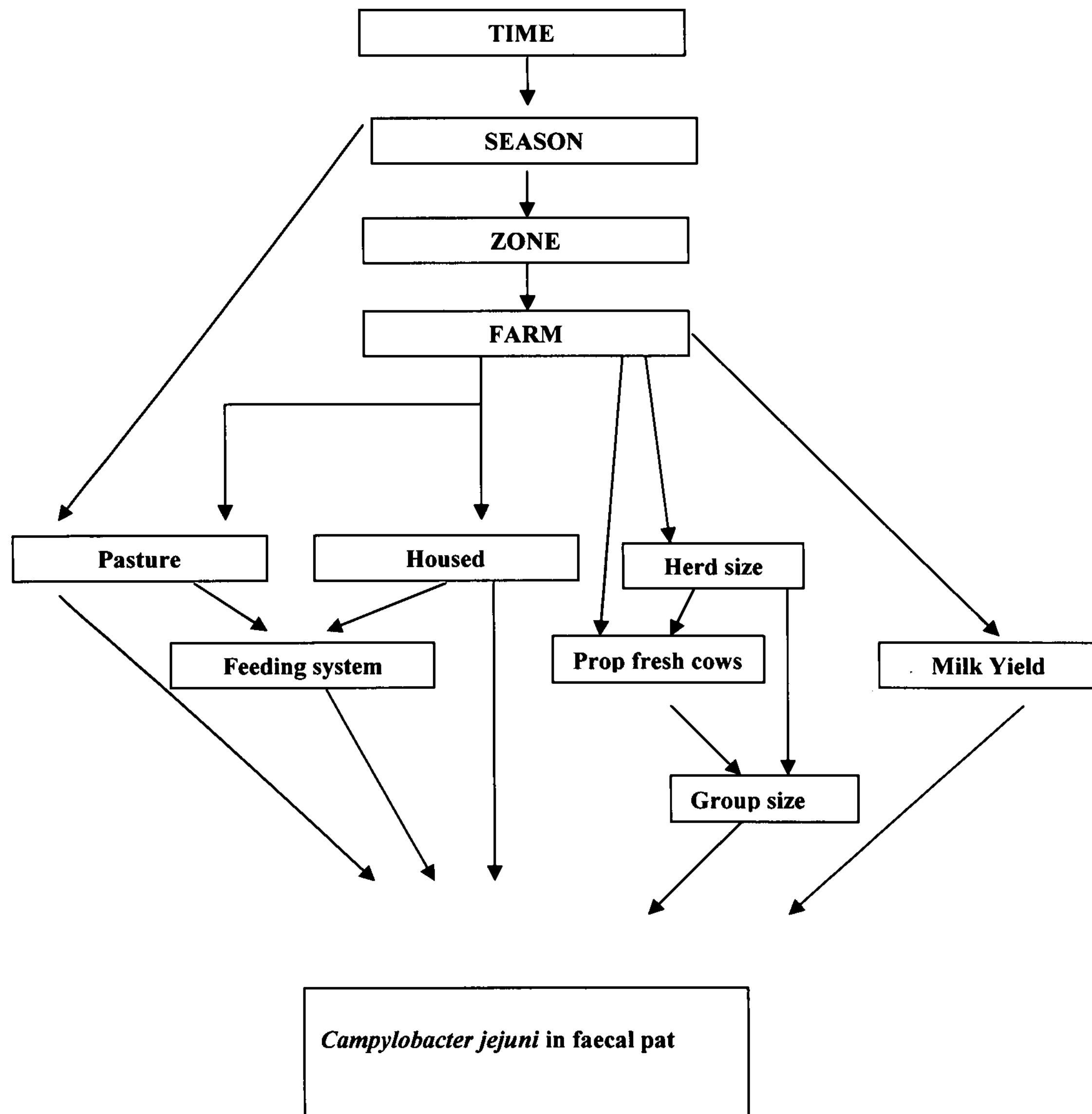
$x_3 = \cos(4\pi t/52)$

$x_4 = \text{sine}(4\pi t/52)$

where t = week number with week 1 being the first week in January 2006 when sampling commenced (on the 9th January)

A logistic regression model was chosen with the outcome being *C. jejuni* present / absent. Thus the actual predicted outcome variable is expressed as the log of the odds $\pi/(1-\pi)$. Interactions between variables were considered for inclusion if they were biologically plausible and retained if they improved the fit of the model. The data has a hierarchical structure in that each faecal pat is nested within a farm, with each farm being nested within a zone. Since there were only 19 farms, these were initially fitted as fixed effects in the models investigating the influence of covariates on the pat prevalence of *Campylobacter jejuni*.

Figure 5.1. A conceptual hierarchical framework of risk factors for *Campylobacter jejuni* in dairy cows in Lancashire.



5.3 Results.

The median herd size, defined as total number of lactating and dry cows, was 145 (inter-quartile range 104 – 200) ranging from 71 – 280 cows. Fourteen of the herds were Holstein Friesians whilst one herd was Ayrshire and Ayrshire X Friesian. Median annual milk yield was 8,000 (inter-quartile range 7200 – 9000) litres, ranging from 6000 to 9600 litres per annum.

Two of the sheep farms were lowland with one farm grazing on the saltmarshes of the River Lune estuary whilst 2 were upland with one utilising summer grazing on moorland. The chief breeds of sheep kept were Swaledale and North Country Mules. The 2 upland farms kept 1000 and 700 ewes respectively whilst one lowland farm had 700 ewes and the other kept 150 ewes.

Twenty faecal samples were collected at each sampling visit, yielding a total of 4260 samples. Four potential isolates were taken from each sample yielding 17040 potential bacterial isolates. 9499 putative *Campylobacter spp* isolates were grown and 2307 (24.3%) were identified as *C. jejuni*. (Table 5.3).

Table 5.3. Distribution of *Campylobacter spp* and *Arcobacter spp* isolates by host species.

	Cattle	**Sheep**
Number of faecal pat samples	3300	960
Number of potential isolates (4 per pat)	13200	3840
Number of isolates grown (% of potential isolates)	7779 (58.9%)	1720 (44.8%)
Arcobacter spp (% of actual isolates)	4299 (55.3%)	236 (13.7%)
Campylobacter jejuni (% of actual isolates)	1857 (23.9%)	450 (26%)
Campylobacter coli (% of actual isolates)	346 (4.4%)	815 (47.4%)
Campylobacter fetus (% of actual isolates)	871 (6.8%)	211 (12.3%)
Campylobacter hyointestinalis (% of actual isolates)	380 (4.9%)	0
Campylobacter lari (% of actual isolates)	26 (0.33%)	8 (0.05%)

5.3.1 Univariate analysis

At a pat level, this equated to a *C. jejuni* faecal pat prevalence of 19.1% (95% CI: 17.7 – 20.4) and 17.0% (95% CI: 14.6 – 19.4) for cattle and sheep respectively. There was

no species difference in pat prevalence (P = 0.145). Results of simple univariate analysis for covariates recorded are presented below:

- **Geographical location.**

Cattle faecal pat prevalence of *C. jejuni* was higher (P = 0.0001) in samples collected from Zone 2 compared to samples from the other zones. Similarly sheep faecal pat prevalence was higher (P = 0.0001) in Zone 2 compared to Zone 3 (Table 5.4, Figure 5.2).

Table 5.4. Faecal pat prevalence of *C. jejuni* by sampling zone.

	Cattle. Prevalence % (95% CI)	Sheep Prevalence % (95% CI)
Zone 1	16.7 (14.8 – 18.7)	n/a
Zone 2	24.2 (21.4 – 27)	21.5 (17.8 – 25.1)
Zone 3	17.7 (15.3 – 20.1)	12.5 (9.5 – 15.5)

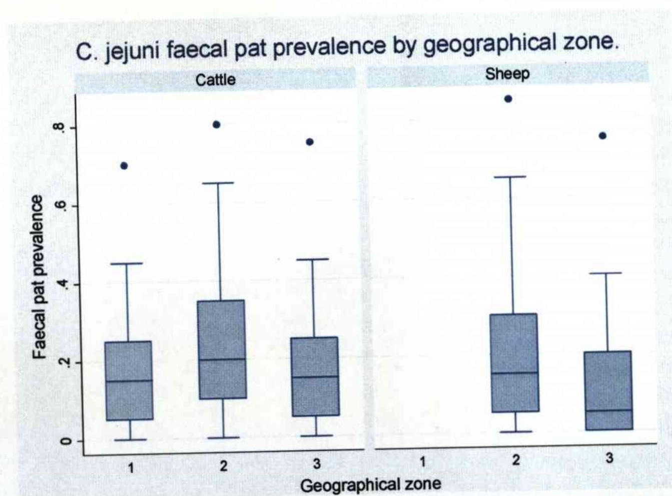


Figure 5.2. Box plot of *C. jejuni* faecal pat prevalence by sampling zone.

- **Animal environment at time of sampling.**

The prevalence of *C. jejuni* was higher in faecal samples collected from cattle at pasture from both cattle (P = 0.0001) and sheep (P = 0.001) compared to housed animals (Table 5. 5, Figure 5. 3).

Table 5.5. Faecal pat prevalence of *C. jejuni* by sampling environment.

	Cattle. Prevalence % (95% CI)	Sheep Prevalence % (95% CI)
Housed	16.6 (15.1 – 18.2)	6.7 (2.1 – 11.2)
Pasture	23.4 (21.0 – 25.8)	18.4 (15.8 – 21.1)

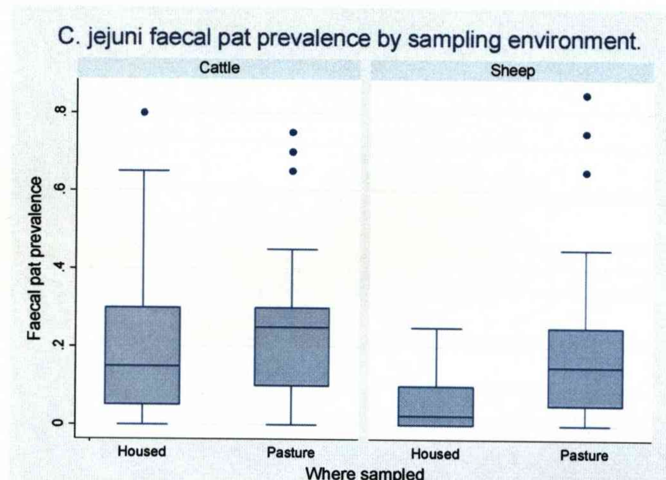


Figure 5.3. Box plot of *C. jejuni* faecal pat prevalence by sampling environment.

- **Season.**

Faecal pat prevalence was higher in both cattle and sheep (P = 0.0001) during summer compared to winter (Table 5.6, Figure 5.4).

Table 5.6. Faecal pat prevalence of *C. jejuni* by season.

	Cattle. Prevalence % (95% CI)	Sheep Prevalence % (95% CI)
Winter	16.4 (14.7 – 18.1)	9.2 (8.8 – 9.3)
Summer	22.3 (20.2 – 24.4)	26.1 (22.0 – 30.3)

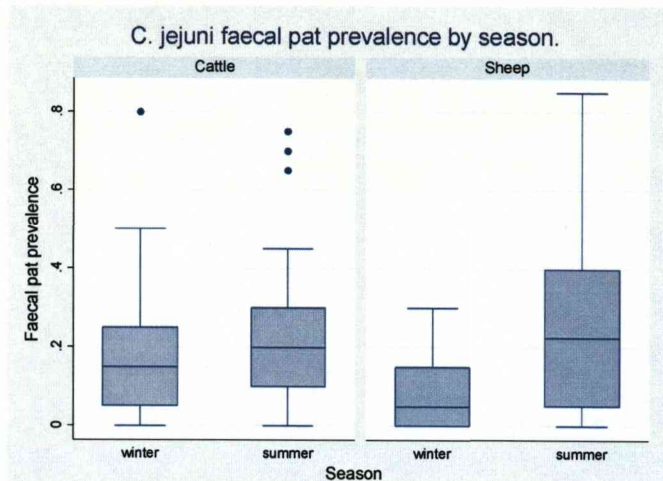


Figure 5.4. Box plot of *C. jejuni* faecal pat prevalence by season.

- **Type of feeding system (cattle only).**

There were differences (P = 0.0001) in *C. jejuni* faecal pat prevalence associated with the feeding system used for cattle (Table 5.7). However it must be borne in mind that the feeding system used depends on the season and the sampling environment.

Table 5.7. Faecal pat prevalence of *C. jejuni* by feeding system.

Feeding system	Number of sampling events.	Mean prevalence (%)	95% C I
TMR	7	17.5	15.3 – 19.8
Hybrid TMR	95	17.3	15.6 – 19.0
Grazing & buffer & parlour	47	22.9	20.2 – 25.6
Grazing & parlour	10	23.5	17.6 – 29.5
Silage & parlour	6	7.5	2.7 – 12.3

- **Lactating animal group size at time of sampling.**

Group size at time of sampling varied from 31 – 273 animals (median 103 inter-quartile range 84 – 140). There was a non significant (P= 0.07) trend score for increasing *C. jejuni* prevalence with increasing group size (Fig 5.5.).

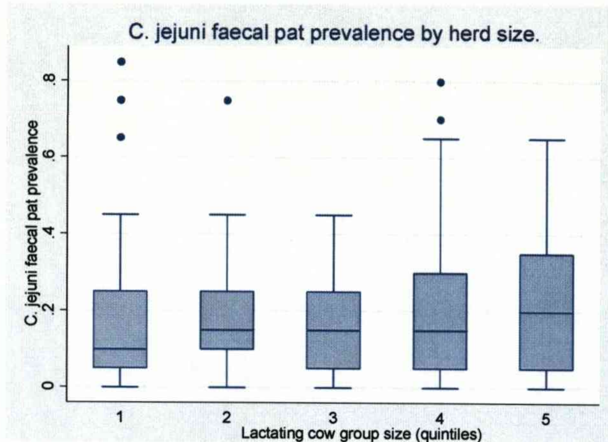


Figure 5.5. Box plot of *C. jejuni* faecal pat prevalence by group size.

- **Proportion of freshly calved cows in sampled lactating group.**

. The proportion of freshly calved cows in sampled groups varied from 0 – 0.42 (median 0.086 inter quartile range 0.05 – 0.125). Univariate analysis suggested a (P = 0.006) trend towards increasing pat prevalence with increasing proportion of fresh cows in the group (Figure 5.6.).

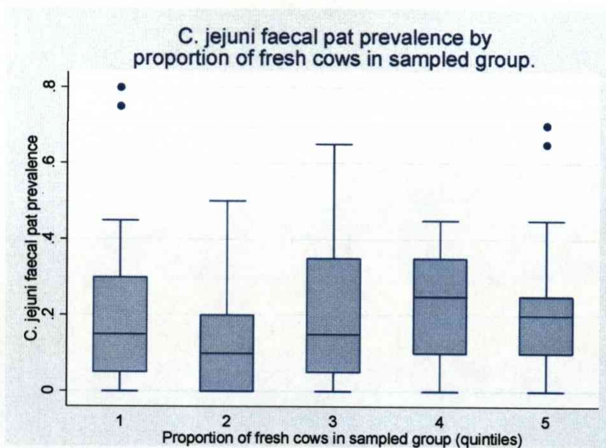


Figure 5.6. Box plot of *C. jejuni* faecal pat prevalence by the proportion of fresh cows in sampled lactating group.

- **Average daily milk yield of sampled group.**

This ranged from 11 – 35 litres/cow/day (median 25 inter-quartile range 21 – 28). Univariate analysis suggested a (P = 0.006) trend towards increasing pat prevalence with increasing milk yield (Figure 5.7.).

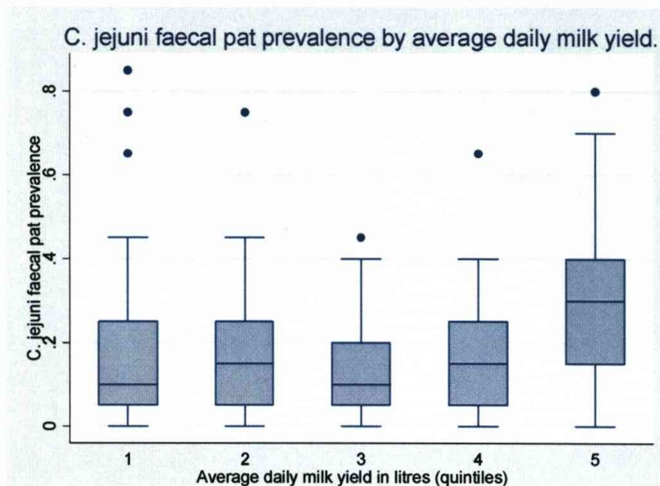


Figure 5.7. Box plot of *C. jejuni* faecal pat prevalence by the average daily milk yield of the sampled group.

- **Cattle faecal score and faecal sieve score.**

There was no significant association between either faecal score (P = 0.689) or faecal sieve score (P = 0.979) and *C. jejuni* pat prevalence.

- **Cattle purchase policy.**

Six farmers reported that they bought no stock in (i.e. were closed herds) whilst 6 reported occasional purchases with 3 farms reporting frequent stock purchases. Two of the latter group could be considered to be "flying herds" in that all lactating animals were purchased with no replacements being bred on the farm. There was no significant association between purchase policy (P = 0.875) and *C. jejuni* pat prevalence.

- **Sheep stocking density.**

There was a wide variation in stocking density both between and within farms which was consistent with the seasonal nature of sheep husbandry. There was an

association (P = 0.001) between increasing stocking density and *C. jejuni* faecal pat prevalence (Figure 5.8).

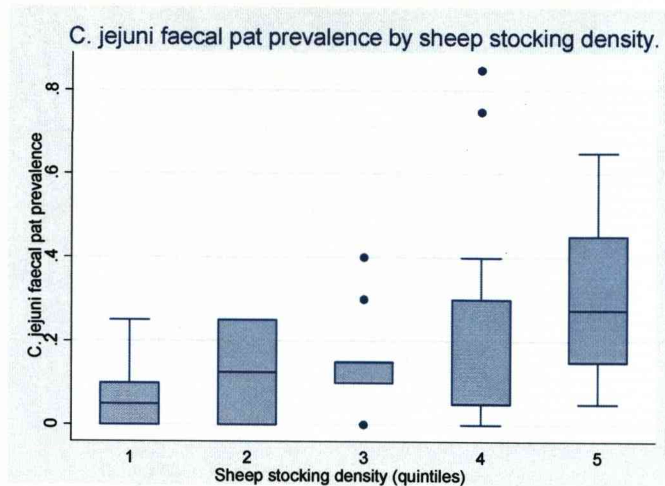


Figure 5.8. Box plot of *C. jejuni* faecal pat prevalence by sheep stocking density (sheep/ha).

- **Lambing season.**

The lambing season occupies a relatively short time period on most farms of between 4 – 6 weeks (personal observation) thus bi-monthly sampling as in this study will not capture the event in all cases. In this study, it was captured in both years for 2 of the 4 farms sampled occurring between mid-January and mid-April. Both flocks were housed. Overall there was a weak trend for the *C. jejuni* faecal pat prevalence to be lower during the lambing season (P = 0.055)

- **Pasture Type (sheep).**

There was a significant (P = 0.0001) positive association between *C. jejuni* faecal pat prevalence and pasture type with the highest prevalences being observed in the case of sheep grazing lush pastures (Figure 5.9.).

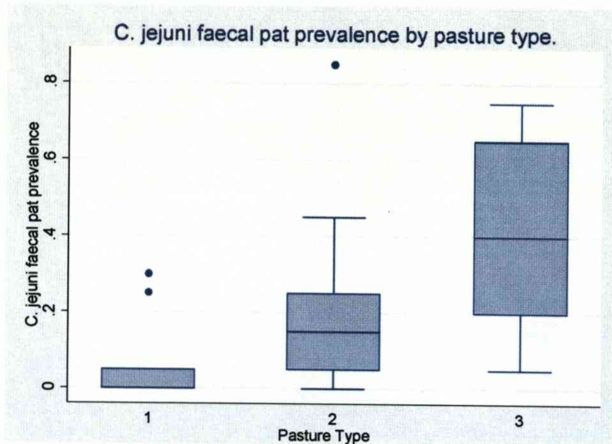
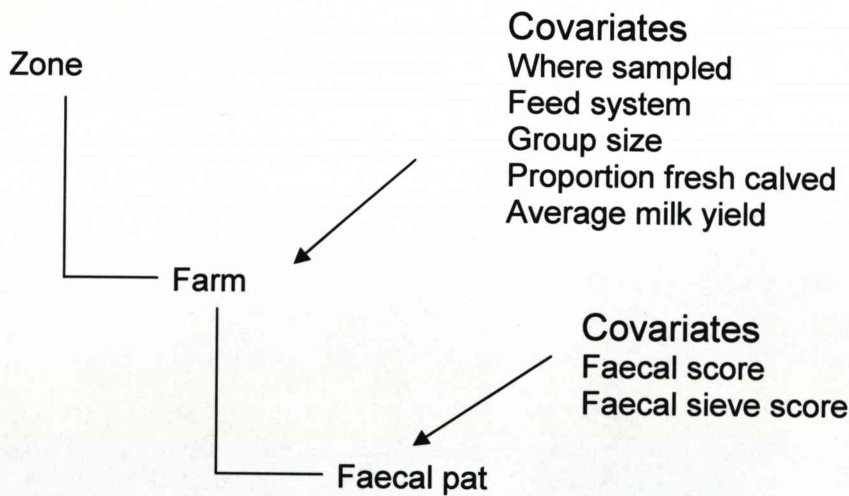


Figure 5.9. Box plot of *C. jejuni* faecal pat prevalence by pasture type.

5.3.2 Multivariate analysis.

The data is hierarchical in structure as shown in Figure 5.10.

Figure 5.10. Hierarchical structure of dataset for cattle farms.



Model 1. Dairy cattle. (Table 5.8)

A logistic regression model was fitted with the underlying *a priori* hypothesis being that time or season is a primary determinant of the probability of a bovine faecal pat being colonised by *Campylobacter jejuni*, with other more proximal covariates also having an effect. It attempts to estimate the significance and magnitude of these potential confounders.

The covariate Farm was treated as a fixed effect. The Stata command for generating the model was:

xi:logit jej_present i.farm in_out group_size milk_yield tcos2 tsin2 tcos4 tsin4

where xi: and i. are terms used to represent fixed effects.

Deviance = 3050 df = 3278

Hosmer-Lemeshow chi^2 statistic = 7.36 P = 0.6 for 11 groups (df = 9)

- **Farm.** This was considered as a fixed effect with Farm 1 taken as baseline. There was considerable variation in the effect of farm with odds ratios ranging from 0.4 (95% CI 0.20 – 0.0.81) [Farm 14] to 2.24 (95% CI 1.39 – 3.61) [Farm 18], suggesting that even having adjusted for the recorded management associated covariates in the model there remains unexplained variation due to farm.
- **Where Sampled.** This was coded as a binary variable (in_out) with 0 coding for "housed" and 1 coding for "at pasture". The odds ratio was 2.14 (95% CI 1.57 - 2.92) (Wald Test P = 0.0001) suggesting that dairy cows kept outside have almost double the odds of excreting *C. jejuni* in their faeces after adjusting for other covariates including time of year with which it is strongly associated since dairy cows are not kept outside during the winter months.
- **Average daily milk yield.** There was a significant (Wald Test P = 0.033) although small (O.R. 1.05 (95% CI 1.02 – 1.09) effect of increasing milk yield on the odds of a cow excreting *C. jejuni*. This describes the marginal effect of increasing yield by one unit i.e. one litre whilst the actual range of yields was11 – 35 litres/cow/day (median 25 inter-quartile range 21 – 28).
- **Group Size.** As with milk yield there was a significant (Wald Test P = 0.001) although small (O.R. 1.008 (95% CI 1.004 – 1.01)) effect. Again this was a measure of the marginal effect of increasing group size by one cow

only. Group size at sampling is primarily a function of overall herd size although the calving pattern will also impact on this measure.

Model 2. Dairy cattle. (Table 5.9.)

This model was fitted with farm as a random effect in light of the hierarchical nature of the data. The spatial term "Zone" was included initially as a fixed effect but did not improve the model fit (Likelihood Ratio test $chi^2 = 0.48$ df = 2 P= 0.78) thus was excluded from the model. It may be concluded that in this study geographical location of the farms within Lancashire had no association with *C. jejuni* faecal pat prevalence. The possible effect of variation in the effect of in_out (i.e. where cattle were sampled) on different farms was investigated by fitting it as a random slope to the afore-mentioned variable in a mixed effects logistic model. However it had no effect on model fit, thus was removed from the model, suggesting that the effect of sampling environment on the faecal pat prevalence of *C. jejuni* is fairly constant (allowing for random error) irrespective of which farm was sampled.

The odds ratios for the covariates "in_out" (2.11 95% CI 1.57 – 2.84), milk yield (1.05 95% CI 1.02 – 1.08) and group size (1.005 95% CI 1.00 – 1.01) were all of similar magnitude to that in the previous model with Wald test P values < 0.05. The Stata command for generating the model was:

xtmelogit jej_present in_out group_size milk_yield tcos2 tsin2 tcos4 tsin4 ||farm: , or

The command xtmelogit refers to a mixed effects logit model with farm as a panel variable. Model fit was checked by visual examination of a plot of the residuals versus time (Figure 5.12).

The seasonal component of the model, after adjusting for the other covariates in the model, was investigated by plotting logit predictions using time covariates only (Figure 5.11.). The magnitude of these seasonal trends is slight with the log odds fluctuating around 0 equating to an odds ratio of 1, i.e. suggesting that season has little effect on *C. jejuni* faecal pat prevalence after adjusting for other covariates. Removal of the time covariates from the model had no effect on model fit (Likelihood ratio test chi = 4.57 with 4df P=0.3343) confirming there is no association between bovine *C. jejuni* faecal pat prevalence and seasonality *per se*.

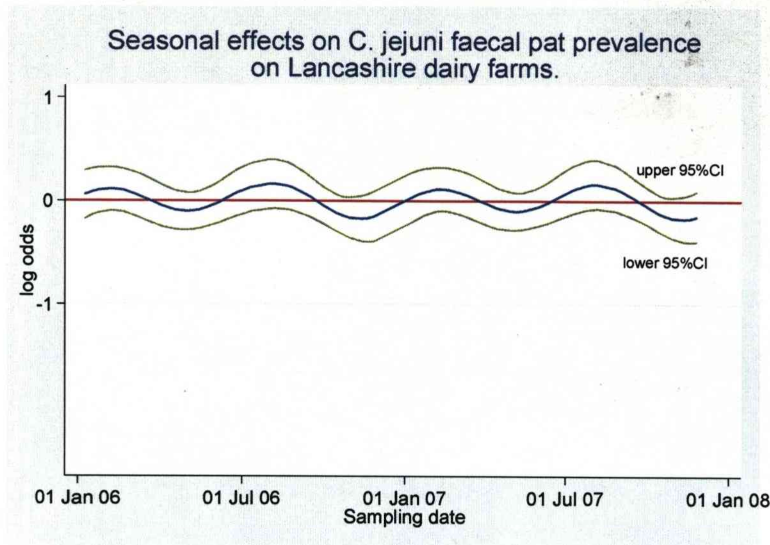


Figure 5.11. The seasonal component to variation in *C. jejuni* faecal pat prevalence on Lancashire dairy farms (n = 15).

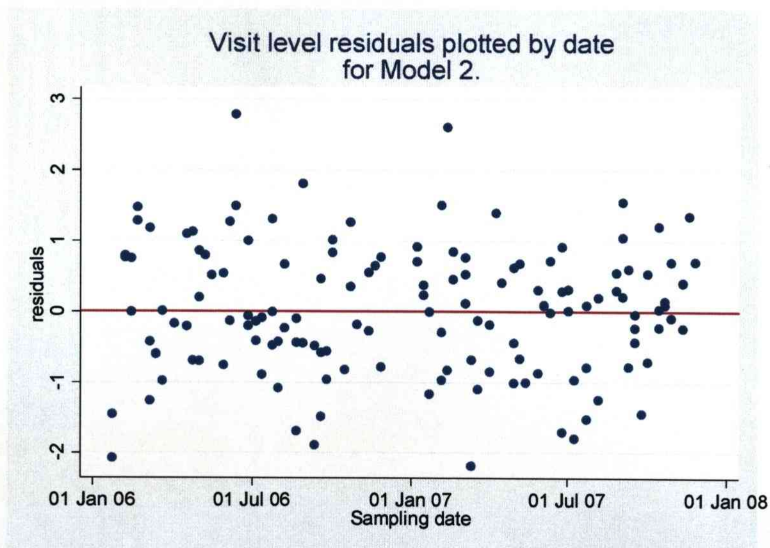


Figure 5.12. Visit level residuals (observed log odds minus predicted log odds) for Model 2. (Random effects model for *C. jejuni* faecal pat prevalence on Lancashire dairy farms).

Model 3. Sheep. (Table 5.10.)

This may be considered to be the ovine equivalent of Model 1. It was built with the underlying *a priori* hypothesis being that time or season is a primary determinant of the probability of a sheep faecal pat being colonised by *Campylobacter jejuni*, with other more proximal covariates also having an effect. It attempts to estimate the significance and magnitude of these potential confounders. The covariate Farm was considered as a fixed effect. The Stata command for generating the model was:

xi: logit jej_present i.farm pasture_type quin_density lambing_season tcos2 tsin2 tcos4 tsin4, or

where xi: and i. are terms used to represent fixed effects.

Deviance = 681 df = 809

Hosmer-Lemeshow chi^2 statistic = 7.01 P = 0.5361 for 10 groups (df = 8)

- **Farm.** This was considered as a fixed effect with Farm 9 taken as baseline. There was considerable variation in the effect of farm with odds ratios ranging from 0.45 (95% CI 0.26 – 0.81) [Farm 15] to 1.05 (95% CI 0.63 – 1.76) [Farm 12], suggesting that even having adjusted for the recorded management associated covariates in the model there remains a considerable amount of unexplained variation due to farm. It is of note that Farms 15 and 16 are in Zone 3 whilst Farms 9 and 12 are in Zone 2 suggesting that sheep farms in Zone 2 (Lancaster) have approximately double the odds of an ovine faecal pat containing *C. jejuni* compared to sheep farms in the Clitheroe area. However the reliability of this finding must be questioned in light of the small number of farms sampled.
- **Pasture type.** There was a strong association (Wald Test P = 0.001) between pasture type and faecal pat prevalence (O.R. 2.16 95%CI 1.39 – 3.35) suggesting that increasing lushness of pasture is associated with increased odds of a sheep excreting *C. jejuni* in its faeces.
- **Stocking density.** Increased stocking density was strongly associated (Wald Test P = 0.004) with increased odds of a sheep excreting *C. jejuni* in its faeces (O.R. 1.29 95%CI 1.07 – 1.55). It is likely that stocking density (a management decision made by the farmer) will be a reflection of time of year,

amount of grass growth (i.e. pasture type) and production targets being aimed for.

- **Lambing season.** Sampling during the lambing season was associated (Wald Test P = 0.02) with increased odds of a sheep excreting *C. jejuni* in its faeces (O.R. 4.68 95%CI 1.28 – 17.17). The relative paucity of samples taken during this relatively short time period is reflected in the wide confidence intervals.

The seasonal component of the model, after adjusting for the other covariates in the model, was investigated by plotting logit predictions using time covariates (Figure 5.13.). This suggests that there are seasonal trends in the probability of a sheep faecal pat being colonised by *C. jejuni*, with the log odds varying from approximately -1 to +1 with peaks during the summer months. This was confirmed by examining model fit with and without the time covariates using a likelihood ratio test. Inclusion of the time covariates significantly improved model fit (Likelihood ratio test chi = 34.07 with 4 df P = 0.00001)

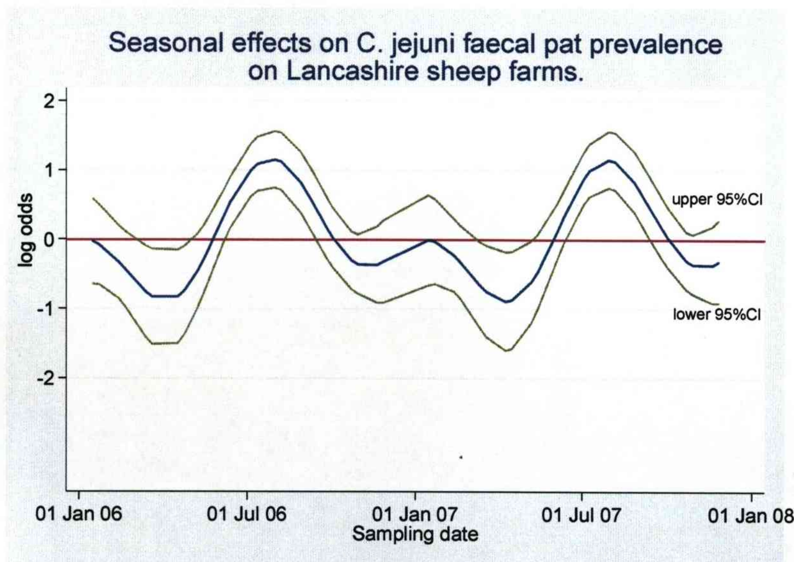


Figure 5.13. The seasonal component to variation in *C. jejuni* faecal pat prevalence on Lancashire sheep farms (n = 4).

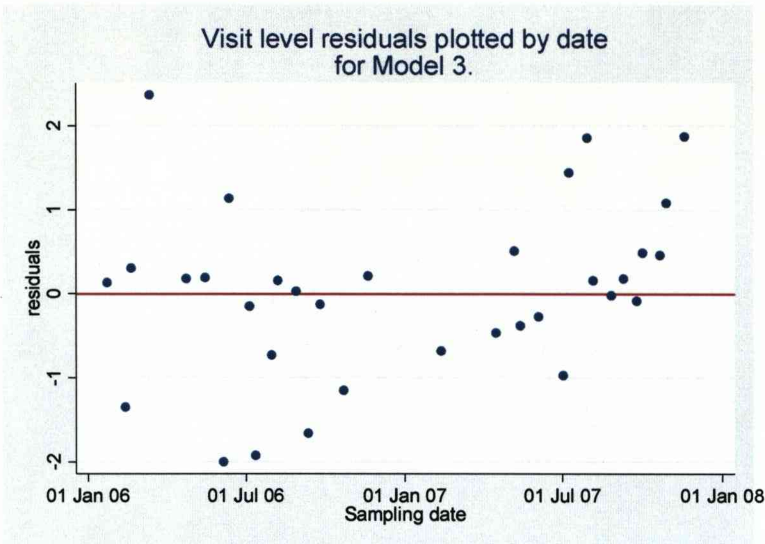


Figure 5.14. Visit level residuals (observed log odds minus predicted log odds) for Model 3. (Fixed effects model for *C. jejuni* faecal pat prevalence on Lancashire sheep farms).

Table 5.8. Fixed effects multivariate logistic regression model including covariates associated with the probability of isolating *Campylobacter jejuni* from cattle faeces on Lancashire dairy farms. Farm is considered as a fixed effect (n = 15).

Covariate	Estimate β	95% CI	Odds Ratio	95% CI	Wald test P value
Baseline (farm1 in_out=0)	-3.97	-4.98 - -2.95			0.0001
Farm					
Farm 2	0.47	-0.38 - 0.98	1.60	0.96 - 2.67	0.069
Farm 3	-0.62	-1.13 - -0.11	0.54	0.32 - 0.89	0.017
Farm 4	-0.44	-1.00 - 0.11	0.64	0.37 - 1.12	0.117
Farm 5	-0.39	-0.92 - 0.15	0.68	0.40 - 1.16	0.155
Farm 6	0.24	-0.31 - 0.78	1.27	0.74 - 2.18	0.394
Farm 7	-0.51	-1.31 - 0.28	0.60	0.27 - 1.33	0.205
Farm 8	-0.14	-0.74 - 0.46	0.87	0.48 - 1.59	0.649
Farm 10	-0.26	-0.78 - 0.25	0.77	0.46 - 1.28	0.313
Farm 11	0.40	-0.14 - 0.94	1.49	0.86 - 2.57	0.151
Farm 13	-0.73	-1.27 - -0.19	0.48	0.28 - 0.82	0.008
Farm 14	-0.92	-1.63 - -0.21	0.40	0.20 - 0.81	0.011
Farm 17	0.31	-0.17 - 0.77	1.36	0.84 - 2.22	0.210
Farm 18	0.80	0.33 - 1.28	2.24	1.39 - 3.61	0.001
Farm 19	-0.06	-0.75 - 0.64	0.94	0.47 - 1.89	0.873
Other Covariates					
in_out	0.76	0.45 - 1.07	2.14	1.57 - 2.92	0.0001
Group size	0.01	0.003 - 0.013	1.01	1.00 - 1.01	0.001
Milk Yield	0.05	0.02 - 0.09	1.05	1.02 - 1.09	0.003
tcos2	-0.04	-0.23 - 0.15	0.96	0.79 - 1.17	n/a
tsin2	0.02	-0.12 - 0.15	1.02	0.89 - 1.16	n/a
tcos4	0.07	-0.12 - 0.15	1.07	0.94 - 1.22	n/a
tsin4	0.12	-0.01 - 0.25	1.13	0.98 - 1.29	n/a

Table 5.9. Random effects multivariate logistic regression model including covariates associated with the probability of isolating *Campylobacter jejuni* from cattle faeces on Lancashire dairy farms. Farm is considered as a random effect.

Covariate	Estimate β	95% CI	Odds Ratio	95% CI	Wald test P value
Baseline (in_out=0)	-3.72	-4.57 - -2.87			0.0001
In_out	0.75	0.45 - 1.04	2.11	1.57 - 2.84	0.0001
Group size	0.005	0.002 - 0.01	1.01	1.00 - 1.01	0.002
Milk yield	0.052	0.02 - 0.08	1.05	1.02 - 1.08	0.0001
tcos2	-0.16	-0.20 - 0.17	0.98	0.82 - 1.18	n/a
tsin2	0.02	-0.12 - 0.15	1.02	0.89 - 1.16	n/a
tcos4	0.06	-0.07 - 0.19	1.06	0.93 - 1.21	n/a
tsin4	0.12	-0.01 - 0.26	1.13	0.99 - 1.29	n/a

Table 5.10. Multivariate logistic regression model including covariates associated with the probability of isolating *Campylobacter jejuni* from sheep faeces on Lancashire sheep farms. Farm is considered as a fixed effect (n = 4).

Covariate	Estimate β	95% CI	Odds Ratio	95% CI	Wald test P value
Baseline (farm9)	-4.11	-5.43 - -2.79			0.0001
Farm					
Farm 12	0.048	-0.47 - 0.56	1.05	0.63 - 1.76	0.856
Farm 15	-0.79	-1.36 - -0.21	0.45	0.26 - 0.81	0.007
Farm 16	-0.43	-1.12 - 0.26	0.65	0.33 - 1.29	0.221
Other Covariates					
Pasture type	0.77	0.33 - 1.21	2.16	1.39 - 3.35	0.001
Stocking density (quintiles)	0.31	0.10 - 0.52	1.37	1.11 - 1.69	0.004
Lambing season	1.54	0.24 - 2.84	4.68	1.28 - 17.17	0.020
tcos2	-0.51	-0.89 - -0.13	0.60	0.41 - 0.88	n/a
tsin2	-0.39	-0.82 - 0.03	0.67	0.44 - 1.03	n/a
tcos4	0.49	0.09 - 0.89	1.64	1.10 - 2.45	n/a
tsin4	0.26	-0.04 - 0.56	1.30	0.96 - 1.76	n/a

Derivation of point prevalence estimates for *C. jejuni* in bovine and ovine faecal pats is problematic due to the considerable variation observed. "Sampling environment" is the prime determinant in the case of cattle, with time of year or season being the prime determinant in sheep. Using these two covariates and allowing for clustering at farm level may allow the "best overall estimates" to be obtained producing estimates as follows:

Cattle.

- Range 0 – 80%
- Housed 16.6% (95% CI 15.1 – 18.2)
- At pasture 23.4% (95% CI 20.0 – 25.8)

Sheep.

- Range 0 – 85%
- Summer 26.1% (95% CI 22.0 – 30.3)
- Winter 9.2% (95% CI 6.7 – 11.7)

5.4 Discussion.

This study demonstrates that both dairy cattle and sheep act as significant reservoirs of *Campylobacter jejuni* with all herds and flocks in the study showing evidence of being colonised, although not at every sampling event during the two year study. Other studies have suggested much lower between-herd prevalence estimates for example, Oporto *et al.* (2007) in a cross-sectional study in the Basque country of Spain, utilising both pooled and individual faecal samples, found 67.1% of dairy herds and 55% of sheep flocks colonised with thermophillic campylobacters with *C. jejuni* being found in 20% of herds. The disparity between that study and the present findings may well be a reflection of a likely intermittent excretion pattern such that one sampling event at one point in time has a relatively low sensitivity resulting in under-estimation of the true prevalence.

Since the present study was a longitudinal study demonstrating considerable variation in *C. jejuni* faecal pat prevalence estimates, it may be argued that derivation of a single point estimate for within-herd *C. jejuni* prevalence has limited meaning and value. Other studies have produced prevalence estimates ranging from 54% in New Zealand dairy cattle (Adhikari *et al.* 2004) to 5.8% in US dairy cattle (Bae *et al.* 2005) whilst two recent UK studies carried out in the Northwest of England suggest point

prevalences of 32.4% (Robinson *et al.* 2005) and 36% (Brown *et al.* 2004) respectively. It is interesting to note the wide variation between the New Zealand and US estimates in light of the differing husbandry practices in those countries. In New Zealand dairy cattle are kept at pasture all year, whilst in the west coast states of the US, where Bae and colleagues carried out their study, the majority of dairy cattle are housed all year with no access to pasture. The two point estimates for prevalence in dairy cattle (housed and at pasture) derived in this study are both lower than those obtained in previous UK studies but not markedly so. It is worth bearing in mind that both these studies were conducted at the same laboratory as the present study using identical culture and PCR techniques, thus results are likely to be comparable.

Seasonal trends in human cases of campylobacteriosis have been identified with peak numbers of reported case occurring during the summer months (Kovats *et al.* 2005). A study in the Northwest of England encompassing the present study area found 47% of human cases occurring between May and August (Sopwith *et al.* 2003). It has been speculated that this seasonality may in part be a reflection of the ecology of the various animal reservoirs, including ruminants, although human behavioural factors are also likely to play a part e. g. foreign travel, summer barbeques, holidays resulting in exposure to rural environments and watercourses.

The present study identified apparent strong seasonality in *C. jejuni* faecal pat prevalence with highest prevalences in both cattle and sheep recorded during the summer months.

In the case of dairy cattle, this apparent seasonality was a reflection of where the animals were sampled with higher prevalences recorded in cattle at pasture. In fact, in our multivariate model, sampling environment was the recorded covariate with the strongest effect (Odds Ratio 2.11 95%CI 1.57 - 2.84) on *C. jejuni* faecal pat prevalence in dairy cattle. The actual seasonal effect having adjusted for the other covariates was not significant (P = 0.3343).

Two possible explanations may be generated. Firstly that dairy cattle are at a greater risk of exposure to, and thus colonisation by, *C. jejuni* when outside at pasture. This increased exposure could be due to both presence of wildlife and drinking from natural watercourses. These are both well-established exposure routes (French *et al.* 2005). However, the risk of a cow acquiring *C. jejuni* from a herd mate would likely be significantly reduced at pasture since faecal contamination and exposure to faeces is considerably less at pasture compared to when housed. Cattle are known to avoid

grazing grass which has faecal contamination and in the present study no slurry was spread on grazing pastures during the grazing season. The second explanation is that faecal pat prevalence is a reflection of *C. jejuni* excretion rather than colonisation *per se* and this is influenced by other factors acting at an intestinal level in the animal. The diet received by housed cattle is markedly different from that received when grazing at pasture, in particular with regards to carbohydrates with grazing cattle ingesting high levels of soluble sugars but low levels of starches, whilst housed animals on a diet of conserved forages and grain base products ingest high levels of starches but minimal levels of sugars (Chamberlain & Wilkinson 1996). Thus it may be hypothesised that the observed *C. jejuni* faecal pat prevalence could be a reflection of these very different diets which would likely impact on the intestinal ecosystem in different ways.

In the present study we were not able to show any effect of feeding system on faecal pat prevalence, but this may be due to considerable confounding by other covariates and also the relatively small number of herds sampled. Furthermore no attempt was made to record actual feeds utilised due to the dynamic nature of practical cattle nutritional management.

We were not able to demonstrate any significant association between faecal characteristics, namely consistency and sieve score (Grove-White 2004) after adjusting for confounding, and faecal pat prevalence. However these techniques are utilised primarily to assess rumen function (detection of sub-acute ruminal acidosis) rather than intestinal function.

There is little data regarding the influence of diet on excretion of campylobacters in the faeces. A study in feedlot cattle suggested that high levels of grain feeding was associated with increased excretion of campylobacters (Garcia *et al.* 1985), whilst Robinson *et al.* (2005) found the presence of whole grain in the faeces of young cattle to be associated with an increased risk of isolating *Campylobacter* from faeces. The current findings, namely that faecal pat prevalence increases in grass fed animals would appear to contradict these findings. However the findings of Robinson and colleagues refer to young animals in which rumen development is incomplete and the finding of grain in the faeces of these animals suggests a degree of rumen dysfunction. The study by Garcia and colleagues was carried out on feedlot cattle which by definition receive no grass, thus their findings can be interpreted as the effect of

increased starch levels in animals already fed a high starch low sugar diet. Thus neither study is comparable to the present study of adult dairy cattle.

There was a positive association between faecal pat prevalence and increased group size. This may be due to increased exposure of individual animals to *C. jejuni* from their herd-mates. Increased prevalence of infectious agents is commonly associated with increased group size in cattle, for example, Paratuberculosis prevalence is strongly associated with increased herd size (Muskens *et al.* 2003).

There was no association between faecal pat prevalence and the proportion of freshly calved cows in the sampled group, although there was an association with increased milk yield. Increased milk yield in a dairy cow is often interpreted as a proxy for "increased metabolic stress" on the animal due to the increased metabolic demands placed on her. However it is well recognised (Chamberlain & Wilkinson 1996) that this "metabolic stress" is maximal in the freshly calved cow at the start of lactation. Thus there will be considerable confounding between these two variables, which may explain the lack of association between faecal pat prevalence and the proportion of freshly calved cows in the sampled group. Notwithstanding this confounding, it may be inferred that increased stress, as shown by increased milk production, is associated with increased faecal pat prevalence of *C. jejuni*. It has been demonstrated that stress, in its broadest terms, may increase susceptibility to bacterial infections such as *Salmonella* and *Campylobacter* (Humphrey 2006) and furthermore may increase excretion of bacteria such as *Salmonella* spp (Corrier *et al.* 1990).

Few studies have been carried out in sheep, although high intestinal carriage rates of thermophillic campylobacters (91%) have been demonstrated in lambs at slaughter in Lancashire (Stanley *et al* 1998b), with higher counts being recorded than in cattle at slaughter. However the same authors found faecal carriage in grazing sheep to be considerably lower (29.3%) which they attributed to intermittent excretion patterns. They found 87% of the campylobacters isolated from sheep faeces to be *C. jejuni* suggesting a *C. jejuni* faecal pat prevalence of 25%. In their study, samples were collected during late spring and early autumn. Our *C. jejuni* prevalence estimates for sheep in summer (26.1% 95% CI 22.0 – 30.3) are in close agreement with their findings although in the present study, *C. jejuni* represented only 26% of total sheep isolates with *C. coli* accounting for 47% and *C. fetus* accounting for 12% (Table 3.).

In the present study, the prevalence of *C. jejuni* in sheep at grass was similar to that of dairy cattle, although the prevalence of *C. coli* was considerably higher. As with

cattle, the *C. jejuni* faecal pat prevalence during winter months was significantly lower despite not being housed, as is the case with dairy cattle in winter. Multivariate modelling suggested that this is a true seasonal effect in sheep unlike in cattle where the observed seasonal changes are associated primarily with the sampling environment (housed or at pasture).

It could be hypothesised that this apparent species difference is a reflection of the differing physiology of these two species. In the case of Holstein dairy cattle there are no seasonal adaptive physiological features as demonstrated by the complete lack of seasonality in reproductive behaviour and appetite. On the other hand, the sheep is a highly seasonal animal with marked seasonality both in reproduction and in appetite such that marked changes in gut function, as demonstrated by changes in digestibility, are observed during winter (Argo *et al.* 1999).

Increased stocking density was positively associated with increased ovine pat prevalence. It could be argued that this is a reflection of increased exposure risk from other animals, i.e. a similar effect to increasing group size in cattle. There was also a positive association between increased pasture quality and faecal pat prevalence, which may reflect a dietary effect. There was a strong positive effect of lambing with sheep during the lambing season having an increased faecal pat prevalence. However cautious interpretation of these associations is required due to the small number of flocks sampled and the relatively infrequent sampling interval. With regards to lambing, only two flocks were sampled during the lambing season and they were both housed. One of these flocks was housed in poor, dirty conditions, which would likely be conducive to both high transmission rates between animals, and high excretion rates associated with stress as a result of the sub-optimal housing conditions.

Geographical zone within Lancashire had no influence on bovine faecal pat prevalence although it appeared that sheep farms in the Lancaster area had a higher pat prevalence. However this finding was based on only two farms in each zone and may be a reflection of the individual farms rather than true spatial variation. A previous study in Lancashire (Stanley *et al.* 1998a) suggested a spatial component to the timing of peak thermophillic campylobacter excretion with peaks observed 1-2 months earlier on more northerly farms. The present study would be unlikely to detect such trends due to the relatively infrequent sampling interval (8 weeks) adopted.

In conclusion, this study has demonstrated that both cattle and sheep represent a significant reservoir of *C. jejuni* especially during the summer months when

prevalence is highest in grazing cattle and sheep. Whilst the variation observed in cattle faecal pat prevalence is associated with sampling environment rather that season *per se*, in sheep it is truly seasonally driven. The association between cattle faecal pat prevalence and sampling environment deserves further investigation to elucidate the mechanisms involved, with the possibility that it could lead to control strategies based on nutritional interventions.

Chapter Six.

Molecular epidemiology of *Campylobacter jejuni* in ruminants in Lancashire: a two year longitudinal study utilising multi-locus sequence typing.

6.1 Introduction.

Infection with *Campylobacter* spp, chiefly *C. jejuni*, is reported to be the major cause of human bacterial gastro-enteritis world-wide (WHO 2005) accounting for 5% - 14% of cases. In the UK, the estimated disease burden due to infection with *Campylobacter* is estimated to be about 300,000 cases per annum (Adak *et al.* 2005), although the reported incidence rate is considerably less at approximately 80 cases/100,000 population (DEFRA 2005). Exposure to poultry products has been identified as the major infection route for human cases in numerous case control studies (e.g. Rodrigues *et al.* 2001, Kappersud *et al.* 2003, Neimann *et al.* 2003, Friedman *et al.* 2004). It is well established that poultry are often heavily infected with *Campylobacter jejuni* with 100% infection rates reported in broiler chicken populations (Corry & Abatay 2001). Up to 90% of poultry carcasses at the point of sale may be infected (Bolton *et al.* 1999).

However, poultry products whilst being the major source do not account for all cases and there is increasing interest in the role of other exposure routes including ruminants. A recent study in a defined rural area of New Zealand (Garrett *et al* 2007) utilising both serotyping and *Sma* I macro-restriction profiling via pulse-field gel electrophoresis found that human isolates tended to be more similar to those from ruminant faeces and offal than from chicken carcasses, pork offal or duck faeces, suggesting that acquisition of infection via environmental exposures or contact with animals is important in rural settings, whilst other studies have also suggested a role for other non-poultry sources of infection, especially in rural settings (Sopwith *et al.* 2006).

Historically, the elucidation of the sources of human infection with *C. jejuni* and subsequent transmission routes has been hampered by a lack of suitable universally comparable molecular typing methods. The development of multilocus sequence typing (MLST) (Dingle *et al.* 2001) has largely overcome this problem since it allows direct comparison of typing data between laboratories world-wide and storage of data on a public database (http://campylobacter.mlst.net). Essentially MLST involves the sequencing of seven housekeeping genes which are highly conserved and evolve slowly. Isolates are thus defined as sequence types (STs) by the allelic profiles of these seven genes. Sequence types may then be allotted to membership of a clonal

complex defined as two or more independent isolates with an ST that share identical alleles at four or more loci (of the seven loci sequenced).

Dingle *et al.* (2002) found 6 clonal complexes (C-C 21, C-C 45, C-C 206, C-C 61, C-C 48 and C-C 257) accounting for 60% of human disease isolates. A number of studies using MLST data have suggested that sequence types associated with human disease are widespread in the rural environment (French *et al.* 2005) and found in ruminants and wildlife (Dingle *et al.* 2002, Colles *et al.* 2003, Manning *et al.* 2003 Karenlampi *et al.* 2007, Kwan *et al.* 2008a, Kwan *et al.* 2008b). Whilst there is considerable overlap between hosts, these studies and others would suggest that sequence types belonging to the following clonal complexes (C-C) predominate in cattle and sheep: C-C 61, C-C 42, C-C 21, C-C 48, whilst C-C 45 is found predominantly in chickens but also in ruminants.

It has been suggested that these apparent species associations may represent niche adaption by *C. jejuni* to certain hosts, although other mechanisms including clonal expansion, geographical or ecological isolation, host immune response or barriers to genetic exchange may also operate to produce these apparent host preferences (Manning *et al.* 2003).

The present study is nested in the previous study reported in Chapter Five which describes the temporal and spatial variation in overall *C. jejuni* faecal pat prevalence found during the period of this study. The chief findings were that in both species, *C. jejuni* faecal pat prevalence was higher during the summer months, but in dairy cattle this change was driven primarily by turn-out to pasture, whilst in sheep it was truly seasonal and unrelated to sampling environment or management practices.

The objective of the present study is to describe the diversity and prevalence of *C. jejuni,* as defined by membership of a given MLST clonal complex in cattle and sheep in Lancashire and furthermore to investigate temporal and spatial variation in *C. jejuni* prevalence within these populations.

6.2 Materials and Methods.

Full details of all methods are given in Chapter 2. The study design was a repeated cross-sectional study over a 2 year period starting in January 2006. Fourteen dairy and four sheep farms were recruited via three veterinary practices in Lancashire. The three practices selected were in Longbridge, Lancaster and Clitheroe serving the

Fylde (Zone 1), N. Lancashire (Zone 2) and S.E. Lancashire (Zone 3) respectively. Six dairy farms were recruited in Zone 1 whilst four dairy and two sheep farms were recruited in each of the other zones. One farm in Zone 2 ceased trading in December 2006 and was replaced with a neighbouring farm for the rest of the study. Another farm in Zone 2 ceased keeping cattle in June 2007 thus sampling on this farm was incomplete. Eligibility criteria for entry were: Dairy farms with over 100 adult cows with or without a sheep enterprise; Sheep farms with over 150 breeding ewes and no other livestock enterprises.

Farms were visited at 8 week intervals when 20 freshly voided faecal samples would be collected from the lactating cows on dairy farms or adult sheep on sheep farms. Samples were collected from animals observed to defecate by the author. Three farms from one zone would be sampled at each occasion. Each faecal pat was sampled from at least 3 sites within the pat and mixed thoroughly in a sterile sample pot to minimise possible within-pat variation. In the case of sheep faecal pellets, at least 3 were collected. Twenty faecal samples were collected at each visit. Samples were transported to the laboratory on ice.

At each visit, current management details were obtained via a short questionnaire (Appendix B).

In the laboratory, one gram of faeces was placed in 9 ml of Campylobacter enrichment broth (IDG Ltd, Bury, UK) with cefoperazone, vancomycin, trimethoprim and cycloheximide (CVTC supplement: IDG Ltd, Bury, UK) and after homogenising for 30 seconds in a Colworth 80 stomacher (A. J. Seward & Co. Ltd, London, UK) was incubated in a plastic universal bottle for 24 hours at 37^0C in a in a "Variable Atmosphere Incubator (VAIN, Don Whitley Scientific Ltd, UK) maintaining a microaerobic atmosphere (CO_2 12%, H_2 3%, O_2 11%, N_2 74%). After incubation, 50µl of the enrichment broth was inoculated onto a Campylobacter blood-free selective agar (CSA) plate (IDG Ltd, Bury, UK) enriched with cefoperazone and amphotericin (CA supplement: IDG Ltd, Bury, UK). A 30µg nalidixic acid sensitivity disc (Mast Group, Merseyside, UK) was placed on the heaviest part of the inoculum. Similarly a 5µl loopful of enrichment broth was inoculated onto a second CSA plate without a nalidixic acid disc. The CSA plates were incubated at 37^0C in a microaerobic atmosphere for 60-72 hours after which time plates were examined and up to 4 putative campylobacter colonies (per faecal sample) were sub-cultured onto blood agar plates and incubated at 37^0C under microaerobic conditions as described earlier.

Putative campylobacters were identified on colony morphology. After 72 hours incubation, single colonies were sub-cultured onto two blood agar plates. One plate was incubated for 48 hours under microaerobic conditions as described and the other plate incubated for 48 hours at 30^0C in air.

A crude DNA aqueous lysate ("boiled preparation") was prepared by inoculating 200μl of distilled water with a small amount of the culture ("leading edge of a loop"), heating at 100^0 C for 15 minutes followed by centrifugation at 1300 rpm for 10 minutes. All putative campylobacter isolates were frozen in Microbank tubes (Pro-Lab Diagnostics, Neston, United Kingdom) and stored at -80^0C. In order to maximise sensitivity, two PCR assays were performed for identification of *C. jejuni* isolates: a colony multiplex PCR assay (Wang *et al.* 2002) and a monoplex PCR assay (Gonzalez *et al.* 1997).

Of the 2307 *C. jejuni* isolates identified, 1003 isolates were selected for sequencing and subsequent assignment to ST. Isolates were randomly selected using Survey Toolbox (Cameron 1999) stratifying by Zone and Sampling Round (defined as the 8 week period during which each farm was sampled once).

Isolates were processed in batches of 250. In the case of the first 250 isolates, the original DNA aqueous lysate was used, whilst for the second batch, the isolate was grown on Blood Agar and an aqueous lysate prepared as described earlier. In the case of the last 500 isolates, these were grown on Blood Agar and DNA extracts prepared using a commercial kit (Prepman: Applied Biosystems). These changes in extraction methodology were introduced in an attempt to maximise the initial PCR yield.

Essentially sequencing for MLST involves 3 steps, namely:

- PCR amplification of the gene product.
- Dideoxy-termination sequencing reaction performed on each DNA strand (forward and reverse) using internal nested primers and Big Dye Ready Reaction Mix (Applied Biosystems).
- DNA sequencing.

The first 500 isolates were processed using primers and reaction conditions as described by Dingle *et al* (2001) whilst the remaining isolates were processed using primers and reaction conditions as described by Miller *et al.* (2005). This change in protocol was adopted in an attempt to optimise the entire process. In the event of

failure to obtain sequence data for a given allele, the entire process would be repeated for up to a maximum of 6 times.

PCR amplification was performed in 50µl volumes comprising 2µl of DNA lysate and 48µl of mastermix, using a programmable thermal cycler (ABI 20720: Applied Biosystems). Sequencing was performed using an ABI 3130xl automatic sequencer with a 50cm array and POP-7 polymer (Applied Biosystems).

Complete laboratory protocols including precipitation and clean-up methodologies are in Appendix C.

Sequence data assembly, alignment, and interrogation of the *Campylobacter jejuni* Multi Locus Sequence Typing website (http://pubmlst.org/ campylobacter/) for assignment of sequence type was performed using the STARS computer program (Man Suen Chan & Nicki Ventress 2001) running under Biolinux (http://nebc.nox.ac.uk/biolinux.html).

All covariate data was entered in an Access 2003 database (Microsoft) whilst laboratory data was entered into an Excel2003 (Microsoft) spreadsheet. Data analysis was performed using Stata V10 (StataCorp: Texas)

The following covariates (Tables 6.1 & 6.2) were considered for inclusion in later statistical analyses:

Table 6.1. Description of variables collected at cattle sampling visits for possible inclusion in statistical analyses.

Variables (cattle)	Type	Description and coding of variable
Farm identity	categorical	
Purchase policy	categorical	0 = no purchased stock (closed herd)
		1 = occasional purchase of cows
		2 = frequent purchase
Group size	continuous	Number of animals in sampled group
Where sampled	categorical	Inside = 0 or Outside = 1
Zone	categorical	1 = Fylde
		2 = Lancaster
		3 = Clitheroe
Date of sampling	dd/mm/yy	
Av_daily_milk	continuous	Average daily milk yield on sampling day
Number_fresh_cows	continuous	Number of cows calved within last month

Table 6.2. Description of variables collected at sheep sampling visits for possible inclusion in statistical analyses.

Variables (sheep)	Type	Description and coding of variable
Farm identity	categorical	
Group size	continuous	Number of animals in sampled group
Where_sampled	binary	Inside = 0 or Outside = 1
Zone	categorical	1 = Longbridge
		2 = Lancaster
		3 = Clitheroe
Date of sampling	dd/mm/yy	

A new variable "Proportion of fresh calved cows" was calculated by dividing "the number of cows that calved in the previous four weeks" by the total size of the lactating group. The continuous variables "Lactating cow group size", "Daily average milk yield", "Proportion of fresh calved cows" and "Sheep stocking density" were transformed into quintiles. The sampling period was split into Summer and Winter with the winter period defined as being from 1st October – 30th April.

The *C. jejuni* isolates in this study were selected for sequencing by a process of stratified random sampling, stratified by sampling round and zone (giving 36 strata), from the larger population of isolates collected during the study period. In order to obtain true estimates allowing for the sampling scheme, the survey (svy) commands in Stata 10 were employed for all analyses with strata defined and using sampling probability weights defined as the inverse of the probability of a faecal pat being selected for MLST taking ruminant species sampled into account, whilst "farm" was specified as the primary sampling unit (psu).

Since the *C. jejuni* isolates in this study were selected from the larger population collected during the study, the term "faecal prevalence of a given clonal complex" is strictly defined as the proportion of *C. jejuni* positive pats which were colonised by a given clonal complex.

Univariate analysis was performed to investigate any associations between *C. jejuni* clonal complex faecal pat prevalences and any of the covariates measured.

Choice of covariates to include in multivariate regression models was made on both biological and statistical grounds. Covariates for which P was less than 0.2 in univariate analysis were considered suitable for initial inclusion in a multivariate

regression model. Collinearity between covariates was investigated using Cramer's phi statistic with significant collinearity suggested by a phi statistic greater than 0.6. If significant collinearity was demonstrated, one of the covariates was discarded with the exact decision being made on grounds of biological plausibility. Interactions between variables were considered for inclusion if they were biologically plausible and retained if they improved the fit of the model. A backward and forward stepwise method was used to assess which covariates to retain in a model. Model fit at each stage was assessed using the Wald test together with the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) (Long 1996) and the fitstat command in Stata since the likelihood ratio test is invalid for models with robust variance estimates as is the case when using probability weights.

Time was included in models as a composite of four sine and cosine functions (Stolwijk *et al.* 1999). The following four time covariates (x_1 x_2 x_3 x_4) were generated: $x_1 = \cos(2\pi t/52)$, $x_2 = \text{sine}(2\pi t/52)$, $x_3 = \cos(4\pi t/52)$, $x_4 = \text{sine}(4\pi t/52)$ where t = week number with week 1 being the first week in January 2006 when sampling commenced (on the 9th January)

Logistic regression models were fitted with the outcome being probability of the particular clonal complex under investigation being present in a faecal pat. Therefore individual models were fitted for each clonal complex. Multivariate regression was not attempted if the number of faecal pats containing the C-C of interest was less than 20, thus no modelling of sheep data was performed. This decision was made in order to avoid spurious results due to small sample numbers. The data has a hierarchical structure, with each faecal pat nested within a farm and with each farm being nested within a zone.

6.3 Results.

One thousand and three of the 2307 *C. jejuni* isolates identified were sequenced. Full allelic profiles were obtained for 939 isolates whilst partial profiles were obtained for 64 isolates. A total of 154 sequence types were identified with 86 being new sequence types. Of the 939 isolates with full allelic profiles, 856 were assigned to existing clonal complexes (*C. jejuni* MLST database interrogated on the 19th June 2008) (Tables 6.3. & 6.4, Figures 6.1. & 6.2.).

Table 6.3. Distribution by host species of *C. jejuni* isolates selected for multilocus sequencing.

	Number of isolates	Number of isolates with incomplete allelic profiles	Number of isolates with full allelic profiles & ST assigned	Number of isolates assigned to a known clonal complex	Number of isolates with full allelic profiles but not assigned to a clonal complex
Cow	841 (83.8%)	56 (0.07%)	785 (93.3%)	712 (85%)	73 (0.09%)
Sheep	162 (16.2%)	8 (0.05%)	154 (95.1%)	144 (88.9%)	10 (0.06%)
Total	1003	64	939	856	83

Table 6.4. Distribution by host species of assigned clonal complex membership.

Clonal Complex	Cattle isolates (n = 785)	Sheep isolates (n = 154)
21	125 (15.9%)	21 (13.6%)
22	7 (0.9%)	3 (1.9%)
42	61 (7.8%)	27 (17.5%)
45	45 (5.7%)	6 (3.9%)
48	21 (2.7%)	22 (14.3%)
49	5 (0.6%)	0
52	18 (2.3%)	16 (10.4%)
61	210 (26.7%)	20 (13%)
177	1 (0.1%)	0
206	36 (4.6%)	16 (10.4%)
257	30 (3.8%)	0
354	7 (0.9%)	6 (3.9%)
403	137 (17.4%)	4 (2.6%)
443	3 (0.4%)	2 (1.3%)
508	1 (0.1%)	0
658	2 (0.2%)	0
692	3 (0.4%)	1 (0.6%)
Unassigned C.C.	73 (9.3%)	10 (6.5%)

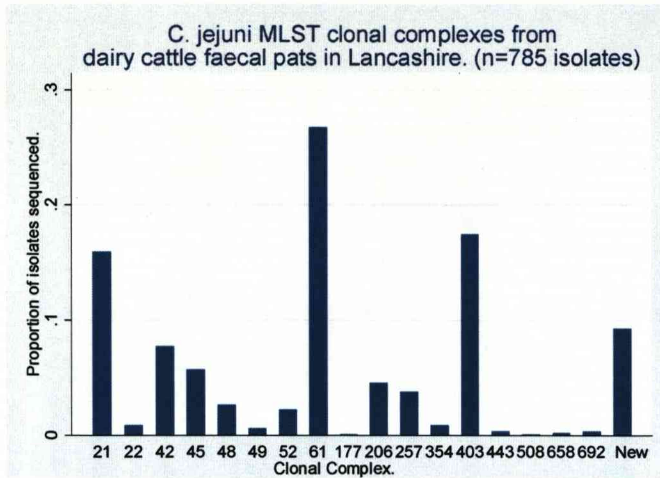


Figure 6.1. Distribution of *C. jejuni* clonal complexes from dairy cattle faecal pats in Lancashire (n=785 isolates).

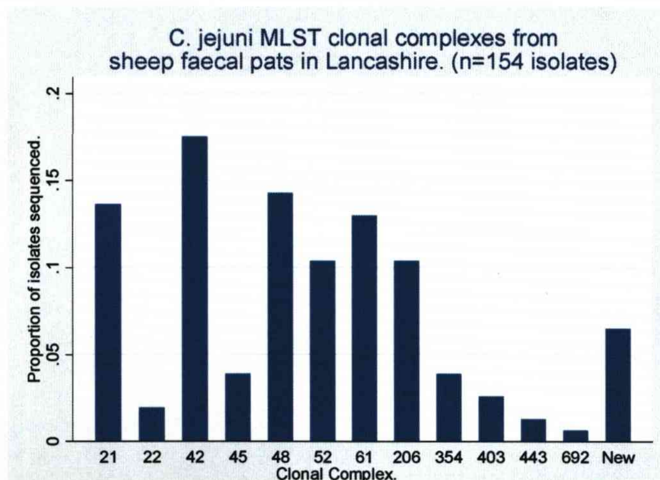


Figure 6.2. Distribution of *C. jejuni* clonal complexes from sheep faecal pats in Lancashire (n=154 isolates).

The 785 bovine isolates with full allelic profiles were obtained from 485 faecal pats whilst the 154 ovine isolates were obtained from106 pats, making an overall total of 591 pats. The majority of faecal pats sampled only contained members of one clonal complex with 30 (6%) bovine pats and 4 (3.8%) ovine pats containing members of more than 1 clonal complex.

The distribution of C. *jejuni* clonal complexes by faecal pat is given in Table 6.5. and represented graphically in Figure 6.3. & 6.4.

Table 6.5. Pat level distribution of *C. jejuni* MLST clonal complexes by species.

Clonal Complex	Cattle (n = 485)	Sheep (n = 106)
21	81 (16.7%)	14 (13.2%)
22	5 (1.0%)	3 (2.8%)
42	37 (7.6%)	15 (14.1%)
45	33 (6.8%)	5 (4.7%)
48	14 (2.9%)	14 (13.2%)
49	5 (1.0%)	0
52	12 (2.5%)	14 (13.2%)
61	123 (25.4%)	15 (14.1%)
177	1 (0.2%)	0
206	17 (3.5%)	10 (9.4%)
257	18 (3.7)	0
354	3 (0.6%)	3 (2.8%)
403	81 (16.7%)	3 (2.8%)
443	2 (0.4%)	2 (1.9%)
508	1 (0.2%)	0
658	2 (0.4%)	0
692	1 (0.2%)	1 (0.94%)
Unassigned clonal complexes	53 (10.9)	7 (6.6)
Incomplete allelic profiles	24 (4.9%)	5 (4.7%)
Number of faecal pats with 1 clonal complexes	431 (88.9%)	97 (91.5%)
Number of faecal pats with 2 clonal complexes	29 (6.0%)	3 (2.8%)
Number of faecal pats with 3 clonal complexes	1 (0.21%)	1 (0.94%)

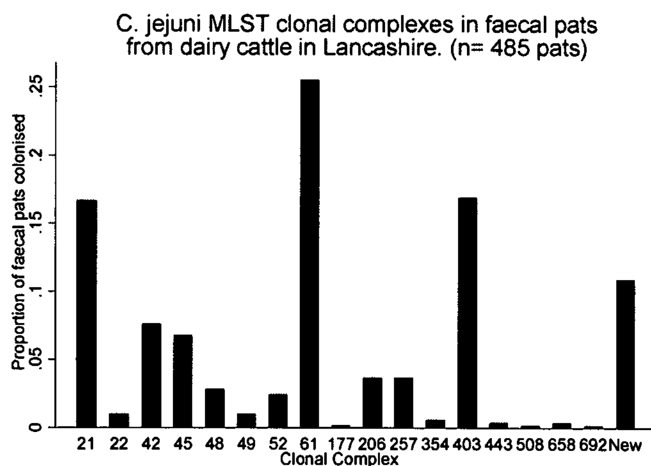


Figure 6.3. Pat level distribution of *C. jejuni* MLST clonal complexes from dairy cattle in Lancashire.

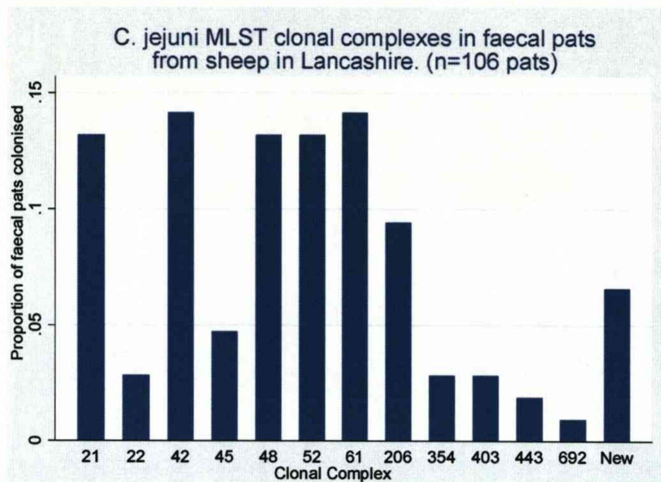


Figure 6.4. Pat level distribution of *C. jejuni* MLST clonal complexes from sheep in Lancashire.

6.3.1 Univariate analysis.

Univariate analysis was performed for all clonal complexes with a prevalence of greater than 3% in either host species, henceforth referred to as the "major clonal complexes" namely C-C 21, C-C 42, C-C 45, C-C 48, C-C 52, C-C 61, C-C 206, C-C 257, C-C 403. These clonal complexes represent 87% of all isolates. Faecal pat prevalences in Tables 6.6 – 6.10 are estimated accounting for the stratified random sampling strategy employed.

In cattle faeces, C-C 61 (25.5%) is the predominant clonal complex, followed by C-C 21 (19.8%) and C-C 403 (15.9%). In sheep faeces, the predominant clonal complexes are C-C 21, C-C 42, C-C 48, C-C 52 which all have faecal pat prevalences of between 14% – 18%. There are apparent host species differences in clonal complex faecal pat prevalences with C-C 403 being higher in cattle faeces (P = 0.008) whilst C-C 48 and C-C 52 are found at higher prevalences in sheep faeces (P = 0.007 & P = 0.001 respectively). C-C 257 was only found in cattle faeces (Table 6.6).

The bovine faecal pat prevalence of C-C 45 was significantly higher (P = 0.007) both in cattle at pasture compared to when housed, and in summer compared to winter (P = 0.0005). No other environmental or seasonal differences in C-C prevalences were observed (Tables 6.7 & 6.8).

In sheep, it was not possible to perform any meaningful analyses investigating associations between season, sampling environment or geographical zone and sheep faecal pat clonal complex prevalences due to the relative paucity of data.

The bovine faecal pat prevalences of C-C 21 and C-C 45 were significantly (P = 0.022 & P = 0.0003)) higher in Zone 1 compared to the other two zones (Table 6.9).

Table 6.6. Estimated faecal pat prevalence of the major *C. jejuni* MLST clonal complexes in cattle and sheep faecal pats from Lancashire.

Clonal Complex	Cow (n = 485)	Sheep (n = 106)	P value
21	19.8% (95%CI 14.4 – 25.2)	14.5% (95%CI 6.5 – 22.5)	0.379
42	6.7% (95%CI 3.9 – 9.5)	17.3% (95%CI -3.9 – 38.4)	0.159
45	8.5% (95%CI 4.6 – 12.3)	1.6% (95%CI 0.4 – 2.8)	0.054
48	2.6% (95%CI 1.0 – 4.2)	14.4% (95%CI -0.5 – 33.8)	0.007
52	2.2%(95%CI 0.6 – 3.7)	14.3% (95%CI 1.1 – 27.5)	0.001
61	25.5% (95%CI 19.0 – 32.1)	9.7% (95%CI -0.1 – 19.4)	0.122
206	3.7% (95%CI 1.4 – 6.0)	7.1% (95%C -0.1 – 14.9)	0.068
257	2.5 (95%CI 0.6 – 4.3)	0	
403	15.9% (95%CI 9.7 – 22.2)	3.7% (95%CI -3.4 – 12.4s)	0.008

Table 6.7. Estimated faecal pat prevalence of the major *C. jejuni* MLST clonal complexes in cattle by season.

Clonal complex	Season.		
	Summer	Winter	P value
21	22.4% (95%CI 14.3 – 30.5)	16.1% (95%CI 9.9 – 22.3)	0.217
42	4.8% (95%CI 1.4 – 8.3)	9.4% (95%CI 4.9 – 14.0)	0.123
45	12.4% (95%CI 6.1 – 18.6)	2.9% (95%CI 0.8 – 5.0)	0.0005
48	1.7% (95%CI -0.01 – 3.6)	3.8% (95%CI 1.01 – 6.6)	0.215
52	2.2% (95%CI -0.01 – 4.4)	2.1% (95%CI -0.01 – 4.2)	0.983
61	22.3% (95%CI 14.9 – 29.7)	30.2% (95%CI 19.1 – 41.2)	0.229
206	2.9% (95%CI -0.01 – 5.8)	4.9% (95%CI 1.1 – 8.8)	0.394
257	3.4% (95%CI 0.5 – 6.3)	1.2% (95%CI -0.2 – 2.7)	0.163
403	15.6% (95%CI 7.0 – 24.1)	16.4% (95%CI 7.5 – 25.3)	0.898

Table 6.8. Estimated faecal pat prevalence of the major *C. jejuni* MLST clonal complexes in cattle by sampling environment.

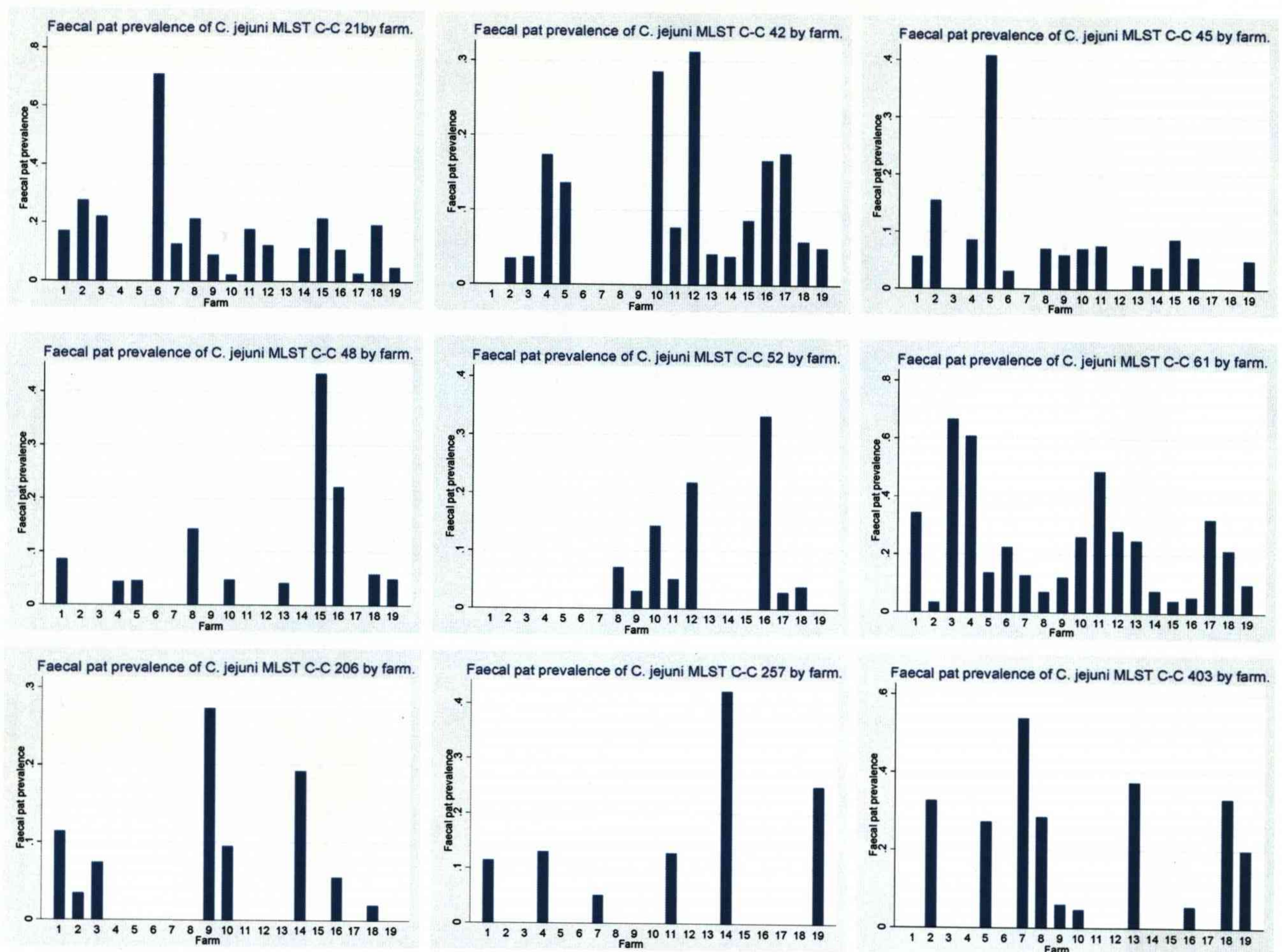
Clonal complex	Sampling Environment.		
	At pasture	Housed	P value
21	23.6% % (95%CI 15.1 – 32.1)	15.5% % (95%CI 9.5 – 21.4)	0.112
42	4.8% % (95%CI 0.7 – 9.0)	8.9% % (95%CI 4.4 – 13.4)	0.249
45	12.4% % (95%CI 5.8 – 19.1)	4.0%% (95%CI 1.2 – 6.7)	0.007
48	2.2% % (95%CI -0.01 – 4.5)	3.0% % (95%CI 0.9 – 5.1)	0.643
52	2.7% % (95%CI -0.2 – 5.1)	1.6% % (95%CI -0.01 – 3.2)	0.441
61	26.4% % (95%CI 17.9 – 34.9)	24.5% % (95%CI 14.5 – 34.5)	0.774
206	3.1% % (95%CI -0.01 – 6.3)	4.5% % (95%CI 1.1 – 7.8)	0.561
257	1.0% % (95%CI -0.01 – 2.6)	4.2% % (95%CI 0.7 – 7.6)	0.087
403	12.8% % (95%CI 5.4 – 20.3)	19.3% % (95%CI 9.4 – 29.3)	0.286

Table 6.9. Estimated faecal pat prevalence of the major *C. jejuni* MLST clonal complexes in cattle by geographical zone.

Clonal complex	Zone			
	Fylde (Zone 1)	Lancaster (Zone 2)	Clitheroe (Zone 3)	P value
21	26.5% (95%CI 17.4 – 35.7)	14.2% (95%CI 7.0 – 21.4)	12.1 % (95%CI 4.3 – 19.9)	0.022
42	3.9% (95%CI 1.0 – 6.8)	10.8% (95%CI 3.9 – 17.7)	7.6% (95%CI 2.4 – 12.8)	0.083
45	13.9% (95%CI 6.8 – 21.1)	4.8% (95%CI 1.0 – 8.7)	1.1% (95%CI -0.4 – 2.6)	0.0003
48	2.3% (95%CI -0.1 – 4.7)	3.1% (95%CI 0.1 – 6.1)	2.7% (95%CI 0.2 – 5.0)	0.88
52	1.5 (95%CI -0.4 – 3.5)	3.2 % (95%CI 0.5 – 5.8)	3.8% (95%CI -2.5 – 10.1)	0.58
61	26.5% (95%CI 17.6 – 35.4)	26.9% (95%CI 12.6 – 41.1)	21.5% (95%CI 10.0 – 32.9)	0.80
206	4.5% (95%CI 0.6 – 8.4)	2.5% (95%CI -0.5 – 5.6)	3.5% (95%CI -0.7 – 7.7)	0.72
257	1.6% (95%CI -0.4 – 3.5)	3.2% (95%CI 0.5 – 5.8)	3.8% (95%CI -2.5 – 10.1)	0.58
403	11.7% (95%CI 2.8 – 20.6)	18.1% (95%CI 6.0 – 20.1)	22.5% (95%CI 10.4 – 34.6)	0.35

There was considerable between-farm variation in both presence and prevalence (defined as the proportion of pats collected over the two year period which were colonised), of specific clonal complexes (Figure 6.5).

Figure 6.5. Faecal pat prevalence of the major clonal complexes by farm.



6.3.2 Multivariate analysis.

In the case of cattle isolates, multivariate logistic regression with farm specified as a fixed effect was performed to investigate any associations between farm identity, purchase policy, geographical zone, time of sampling, sampling environment and presence of a given clonal complex. The term "prevalence" is used in the following section as referring to "the proportion of *C. jejuni*-positive pats collected over the two year period which were colonised by a given clonal complex", rather than in its usual context of point prevalence.

- C-C 21. A total of 81 bovine and 14 ovine faecal pats were colonised. This clonal complex was not isolated from 3 of the dairy farms (4, 5, 13) but was present on all of the sheep farms. There was no association between bovine faecal pat prevalence and either time of sampling, purchase policy, geographical zone, sampling environment. There was a strong association

between farm identity and bovine faecal pat prevalence (Wald chi^2 test 35.77 with 11df P = 0.0002) Mean estimated within farm bovine faecal pat prevalence was 23.1% (95%CI 17.5 – 28.7) on the farms from which it was isolated, with a range from 3.% to 71%.

- C –C 42. A total of 37 bovine and 15 ovine faecal pats were colonised. This clonal complex was not isolated from four of the dairy farms (1, 6, 7, 8), nor from one sheep farm (9). In the case of dairy farms there was no association between either purchase policy, sampling environment, or time of sampling and faecal pat prevalence but there was a strong association between farm identity and faecal pat prevalence (Wald chi^2 test 22.79 with 10df P = 0.0115). Mean estimated within farm bovine faecal pat prevalence on the farms from which it was isolated was 9.2% (95%CI 5.9 – 12.5) with a range from 3% to 28%.
- C –C 45. A total of 33 bovine and 5 ovine faecal pats were colonised. This clonal complex was not isolated from four of the dairy farms (3, 7, 17, 18), nor from one sheep farm (12). In the case of dairy farms there was no association between faecal pat prevalence and purchase policy, geographical zone or sampling environment but faecal pat prevalence was strongly associated with both time of sampling and farm identity (Fig. 6.6) (Wald chi^2 test 40.4 with 5df P = 0.0001). Mean estimated within farm bovine faecal pat prevalence on the farms from which it was isolated was 11.8% (95%CI 6.8 – 16.8) with a range from 4% to 40%.
- C-C 48. Only 14 bovine and 14 ovine pats were colonised thus no multivariate modelling was attempted. This clonal complex was not isolated from seven of the dairy farms (2, 3, 6, 7, 11, 14, 17), nor from two sheep farms (9, 12). Mean within farm bovine faecal pat prevalence on the farms from which it was isolated was 5.6% (95%CI 2.4 – 8.9) with a range from 4% to 9%.
- C-C 52. Only 12 bovine and 14 ovine pats were colonised thus no multivariate modelling was attempted. This clonal complex was not isolated from ten of the dairy farms (1, 2, 3, 4, 5, 6, 7, 13, 14,19), nor from one sheep farms (15). Mean within farm bovine faecal pat prevalence on the farms from which it was isolated was 6.5% (95%CI 1.8 – 11.3) with a range from 3% to 14%.

- C-C 61. A total of 124 bovine and 15 ovine faecal pats were colonised. This clonal complex was present on all farm sampled. There was no association between bovine faecal pat prevalence and either time of sampling, geographical zone, sampling environment or purchase policy, but there was a significant association (Wald chi^2 test 64.38 with 14df $P = 0.0001$) between prevalence and individual farm identity. Mean within farm bovine faecal pat prevalence on the farms from which it was isolated was 25.5% (95%CI 19.0 – 32.1) with a range from 4.8% to 72.1%.
- C-C 206. Only 18 bovine and 10 ovine pats were colonised thus no multivariate modelling was attempted. This clonal complex was not isolated from nine of the dairy farms (4, 5, 6, 7, 8, 11, 13, 14, 18), nor from two sheep farms (12, 15). Mean within farm bovine faecal pat prevalence on the farms from which it was isolated was 5.6% (95%CI 2.4 – 8.9) with a range from 2% to 19%.
- C-C 257. Only 18 bovine pats were colonised with no sheep pats colonised thus no multivariate modelling was attempted. This clonal complex was not isolated from nine of the dairy farms (2, 3, 5, 6, 8, 10, 13, 17, 18), nor from any of the sheep farms. Mean within farm bovine faecal pat prevalence on the farms from which it was isolated was 6.8% (95%CI 1.6 – 12) with a range from 4% to 23%.
- C-C 403. Eighty two bovine and three ovine pats were colonised. However it was not isolated from 6 of the dairy farms (1, 3, 4, 6, 11,17) or from 2 of the sheep farms (12, 15) in the study. There was no association between bovine faecal pat prevalence and time of sampling, purchase policy, geographical zone or sampling environment, but there was a significant association (Wald chi^2 test 19.38 with 7df $P = 0.0071$) between prevalence and individual farm identity. Mean within farm bovine faecal pat prevalence on the farms from which it was isolated was 30.2% (95%CI 21.5 – 39) with a range from 4% to 54%.

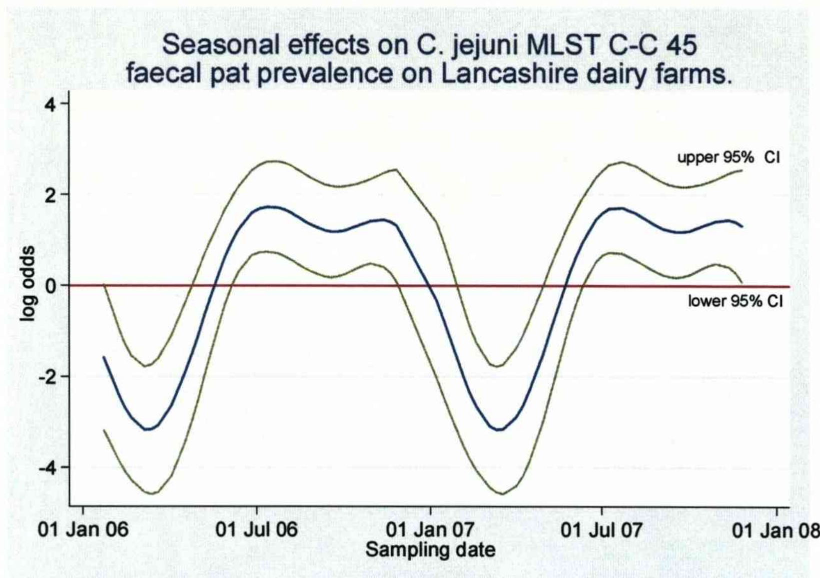


Figure 6.6. The seasonal component to variation in *C. jejuni* MLST C-C 45 faecal pat prevalence on Lancashire dairy farms.

In summary, the chief source of variation in bovine faecal pat prevalence of the major *C. jejuni* MLST clonal complexes would appear to be the farm from which the samples were collected, apart from C-C 45 where a seasonal trend was apparent with highest prevalences observed during the summer months.

6.4 Discussion.

The use of MLST allows a given campylobacter isolate to be described at two levels in terms of the genetic diversity present in the seven housekeeping genes sequenced. Firstly assignment to a specific sequence type (ST) and secondly assignment of the sequence type to a specific clonal complex (C-C), which is defined as containing sequence types that differ by no more than three of the seven alleles sequenced. Clonal complex membership is increasingly being used as a tool to describe the epidemiology of *C. jejuni* in both man and animals.

The present study investigates the distribution of *C. jejuni* on dairy and sheep farms in Lancashire at the clonal complex level, and represents the largest MLST based study of *C. jejuni* in cattle and sheep at the present time, with 939 isolates assigned to sequence type, representing 41% of the total *C. jejuni* isolates collected over a two year period from 15 dairy and four sheep farms. The 939 isolates were assigned to 68 existing and 86 new sequence types. This assignment of 86 new sequence types

suggests that there is considerably more diversity amongst cattle and sheep *C. jejuni* isolates than is currently recorded on the pubmlst database (http://pubmlst.org/campylobacter/).

A number of previous studies (Dingle *et al.* 2002, Colles *et al.* 2003, Manning *et al.* 2003, French *et al.* 2005, Karenlampi *et al.* 2007, Kwan *et al.* 2008a) have suggested that C-C 61, C-C 21, C-C 42, C-C 48, C-C 257, C-C 403 are commonly isolated from ruminants although in one of these studies C-C 403 was found predominantly in pigs (Manning *et al.* 2003).

The present study is in broad agreement with these findings with C-C 21, C-C 42, C-C 45, C-C 48, C-C 52, C-C 61, C-C 206, C-C 257, C-C 403 comprising 83% of the isolates sequenced with the remainder assigned to one of seven other clonal complexes or to none. In contrast to previous studies (Dingle *et al.* 2002, Manning *et al.* 2003, French *et al.* 2005), which have suggested that C-C 45 is associated primarily with poultry, wildlife, water and human cases, the present study found it to be well represented amongst bovine faecal isolates. The afore-mentioned clonal complexes identified in the present study have all been found in association with human disease (Dingle *et al.* 2002, Sopwith *et al.* 2006).

A marked finding in the present study was the considerable between-farm variation in terms of both the presence of a clonal complex and its prevalence, for example, C-C 61 was the only clonal complex to be isolated from all farms, whilst C-C 257 was only isolated from 6 of the 15 dairy farms and none of the sheep farms and C-C 206 was only isolated from 6 dairy and two sheep farms. Similar findings have been observed in a 12 month longitudinal study of 5 dairy farms in the Wirral, Cheshire (Kwan *et al.* 2008a).

Whilst care must be taken in the interpretation of any species differences in clonal complex prevalence, due to the relatively small number of isolates per complex, there appeared to be genuine species differences in prevalence, with C-C 45, C-C 257 and C-C 403 being more frequent amongst cattle isolates, whilst C-C 48 and C-C 52 were more widespread amongst the sheep isolates. However, there was no difference in the frequency of isolation of C-C 21, C-C 42, C-C 61 or C-C 206 between cattle and sheep.

C-C 61 was the most frequently isolated complex overall, confirming it's pre-eminence in ruminants and adding weight to the hypothesis that it is a ruminant adapted strain.

The findings regarding C-C 21 support the observations of French *et al.* (2005) who found it to have no apparent host preference, being isolated at similar frequencies from cattle and wildlife samples in a cross sectional study of a 100 km^2 area of farmland in Cheshire. A similar conclusion was drawn by Colles and colleagues (2005) who speculated that members of C-C 21 may be well adapted for long term survival given their apparently ubiquitous distribution.

Multivariate analysis was carried out to investigate any temporal or spatial associations influencing the prevalence of clonal complexes C-C 21, C-C 42, C-C 45 and C-C 61 on dairy farms. It was not carried out for any other clonal complexes or for sheep farms due to the small numbers of isolates belonging to any given clonal complex. In the case of C-C 21, C-C 42 and C-C 61, it suggested that the variation in prevalences observed was associated primarily with farm identity alone i.e. localised spatial variation. In the case of C-C 45, in addition to localised spatial variation, there was also a significant temporal association with a higher prevalence observed during the summer months.

Whilst univariate analysis suggested that both C-C 21 and C-C 45 were both more prevalent in the Fylde area, this was not the case in the multivariate analyses suggesting, in this study at least, that there are no large scale spatial effects on *C. jejuni* MLST clonal complex prevalence.

The study reported in Chapter 5. suggested that whilst *C. jejuni* faecal pat prevalence in dairy cattle was higher in the summer months, this was a reflection primarily of the sampling environment rather than time *per se*. i.e. faecal pat prevalence was higher during the summer months since cattle were outside grazing. At first sight, the results presented here do not appear to support that hypothesis since "sampling environment" had no significant effect in any of the multivariate models. Furthermore there were no apparent seasonal effects observed with the predominant cattle associated clonal complexes, namely C-C 61 and C-C 403, whilst the seasonal change in C-C 45 prevalence was associated with time *per se* and not sampling environment. However, it should be borne in mind that in the previous study the denominator used in analyses was the total number of faecal pats collected, with the modelling outcome being "the log odds of a faecal pat being colonised by *C. jejuni*", whilst in the present study the denominator is the number of faecal pats from which *C. jejuni* was isolated, with the outcome being "log odds of a faecal pat being colonised by a particular C-C given that it is colonised by *C. jejuni*". Thus the analysis in this study may be considered as

being conceptually similar to so-called case-case studies (Gillespie *et al.* 2002), with "cases" being specific clonal complexes and "controls" being the remainder of the population of *C. jejuni* from which the controls originate, and so should be interpreted in the light of the previous study describing the overall epidemiology of *C. jejuni.*

A study of 493 human *C. jejuni* isolates collected during 2003-2004 from Lancashire (Sopwith *et al.* 2006) found C-C 21 followed by C-C 45 and C-C 257 to be the chief MLST clonal complexes causing notified cases of human disease. The incidence of reported disease appeared to be higher in rural compared to urban areas, although this is likely to be confounded by other factors such as possible differences in health seeking behaviour. This apparent difference was not observed in the 0 -14 year age group. Whilst illness associated with C-C 21 appeared to be spatially uniformly distributed between both urban and rural areas, cases associated with C-C 45 were most often reported from rural areas during the summer months in particular during June and July. This time period corresponds to the period of increased prevalence of C-C 45 observed in cattle in the present study.

It may be speculated that the apparent increase in human C-C 45 cases in rural Lancashire is a reflection of the seasonal increase in bovine faecal pat prevalence observed in the present study. Further evidence for this hypothesis is supplied by the observation that C-C 45 has been shown to be the predominant *C. jejuni* clonal complex to be isolated from recreational surface water in the same study area ((Sopwith *et al* 2007), suggesting a means whereby the increased bovine faecal load of C-C 45 seen in summer could be a causal factor in the observed increase in human illness observed. It is worth bearing in mind that 100 cows will produce upwards of 5,000 litres of faeces daily (MAFF 1991) thus offering considerable potential for contamination of surface water sources.

Chapter Seven.

Genetic diversity amongst *Campylobacter jejuni* isolates from cattle and sheep in Lancashire.

7.1 Introduction.

Infection with *Campylobacter* spp, chiefly *C. jejuni*, is the major cause of human bacterial gastro-enteritis world-wide (WHO 2005) accounting for 5% - 14% of cases. In the UK, the estimated disease burden due to infection with *Campylobacter* is estimated to be about 300,000 cases per annum (Adak *et al.* 2005) although the reported incidence rate is considerably less at approximately 80 cases/100,000 population (DEFRA 2005). Whilst poultry and poultry products are well recognised sources of human infection, there is increasing evidence for a role for ruminants in the epidemiology of human campylobacteriosis (Dingle *et al.* 2002, Colles *et al.* 2003, Manning *et al.* 2003, French *et al.* 2005, Karenlampi *et al.* 2007, Kwan *et al.* 2008a). Multilocus sequence typing (Dingle *et al* 2001) is a high resolution bacterial genotyping technique, which as well as providing insight into the epidemiology of human and animal campylobacteriosis, allows investigation of the genetic diversity within, and evolution of, campylobacters.

MLST involves the sequencing of seven housekeeping genes which by definition are highly conserved and evolve slowly. Since there is no evolutionary pressure *per se* on these housekeeping genes, the allelic variation present may be considered to be neutral and as such can provide valuable information on the processes operating to produce genetic diversity amongst campylobacters. Isolates are defined as sequence types (ST) by the allelic profiles of these seven genes. Sequence types may then be allotted to membership of a clonal complex, defined as two or more independent isolates with an ST that share identical alleles at four or more loci (of the seven loci sequenced), using the software program eBURST (Feil *et al.* 2004) . The following seven loci were chosen (Dingle *et al.* 2001) for the *Campylobacter jejuni* MLST scheme.

- *asp*A (aspartase A)
- *gln*A (glutamine synthetase)
- *glt*A (citrate synthase)
- *gly*A (serine hydroxymethyl transferase)
- *pgm* (phosphoglucomutase)
- *tkt* (transketolase)
- *unc*A (ATP synthase α subunit)

The loci are all sufficiently separated on the bacterial chromosome, with the minimum distance between loci being 70 kb (Figure17.1), such that coinheritance of two or more loci in a recombination event is considered unlikely.

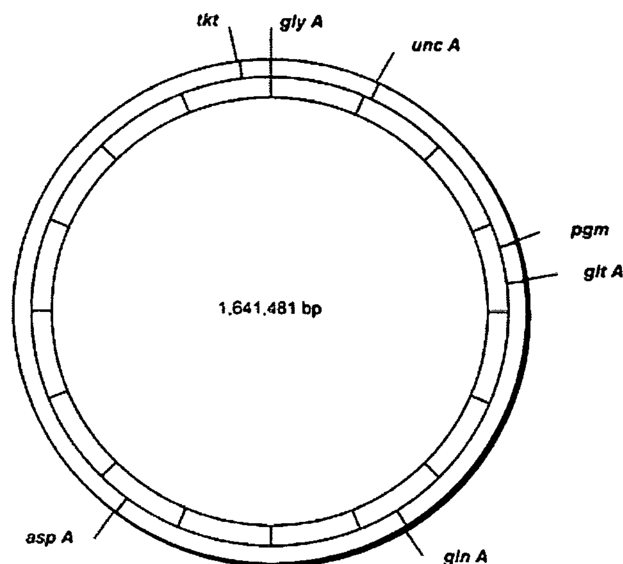


Figure 7.1. Chromosomal locations of MLST loci. The positions of the seven loci are shown on a map of the *C. jejuni* chromosome derived from the genome sequence of isolate NCTC 11168 (http//:sanger-.ac.uk/Projects/C_jejuni). The 1,641,481 –bp genome is divided into 10 segments (indicated on the inner circle) with each segment representing 164,148 bp (from Dingle *et al.* 2001).

In the simplest model of bacterial population structure, haploid bacteria reproduce asexually by binary fission with each cell producing two identical daughter cells. In such a situation, chromosomal variation occurs by *de novo* mutations which thus are only spread to direct descendants of the mother cell, i. e. vertical transmission of genetic information. In such a population, the distribution of chromosomal polymorphisms will be non-random and will lead to the development of distinct clones, each with their own "set" of polymorphisms. Such a situation is described as being purely clonal with the polymorphisms being in linkage disequilibrium. This is in marked contrast to a population of sexual organisms where mutations are continually reassorted resulting in linkage equilibrium, with mutations at different sites occurring in random combinations (Spratt & Maiden 1999). It is now appreciated that whilst bacteria are largely asexual, there also operate a number of processes for horizontal exchange of genetic material (recombination) such as

conjugation, transformation, transduction and these have been referred to as "localised sex" (Maynard Smith *et al.* 1991). Such localised sex will result in transfer of fragments of DNA between bacteria of the same species and between different species. The frequency of localised sex can vary from very low (or zero) to very high and will depend both on the species involved and the frequency at which they meet. Such localised sex will disrupt the clonal population structure resulting from purely asexual reproduction.

It is now recognised that the two extremes of strictly clonal or non-clonal bacterial population structures rarely exist with most bacterial populations occupying a middle ground. *Campylobacter jejuni* is recognised as being a weakly clonal organism (Dingle *et al.* 2002) in which recombination is an important mechanism in producing genetic diversity.

Departure from linkage disequilibrium suggests that a bacterial population is not wholly clonal in structure. However linkage disequilibrium may appear to be present in a weakly clonal population if sampling is biased in favour of certain clones e.g. if only isolates from cases of disease are sampled whereas in reality only a few disease causing clones are present within the wider population.

On the other hand, linkage equilibrium is less likely to result from sampling artefacts and thus in a sufficiently large population, the lack of association between alleles at different loci can be taken to imply that recombination is present and that apparently distinct lineages share a common gene pool (Spratt & Maiden 1999).

The standardised (for number of alleles) Index of Association (I_A^S) is a function of the rate of recombination and equal to zero for linkage equilibrium (Maynard Smith *et al* 1993). In this procedure, I_A is defined as V_O/V_E-1 where V_O is the sum of the observed variance of K which is the "distance between two individuals" (the number of loci at which two alleles differ) and V_E is the sum of the expected variance if the alleles present at different loci in an individual are independent i.e. if there is no linkage disequilibrium. To determine the presence of linkage disequilibrium, the observed variance is compared to the expected variance. A significant difference indicates the presence of linkage disequilibrium. It may be calculated using MLST data in the software package LIAN (Haubold & Hudson 2000), thus affording a means of demonstrating the presence of linkage disequilibrium.

Recombination within DNA sequences results in different parts of a gene having different evolutionary histories whereby mosaic genes are present in which one

portion of the gene may be identical between isolates whereas other parts differ. This non-random distribution of polymorphic sites may be detected by a number of procedures (Maynard Smith 1992, Sawyer 1999) thus allowing the recognition and quantification of recombination in bacterial populations

Estimates of gene flow such as Wright's F_{ST} statistic (Slakin & Barton 1989) provide a measure of gene flow or transfer between geographically or otherwise distinct populations where a value of 1 implies two populations are completely distinct with no flow of genes between them whilst a value of 0 implies the two populations are homogenous with similar allelic frequencies. This value may be calculated with the DNAsp software program (Rozas *et al.* 2003) using concentated sequence data for the 7 alleles sequenced.

Analysis of **Mo**lecular **V**ariance (AMOVA: Excoffier *et al.* 1992) is a least-squares methodology akin to ANOVA for partitioning the variance in molecular diversity within and between populations. It uses information on the allelic content of haplotypes i.e. sequence types, and the frequency of occurrence of haplotypes within a population. It has been used extensively to investigate diversity between populations e.g. Walton (1997) investigated the population structure of Harbour Porpoises from British waters using mitochondrial DNA data and demonstrated significant differences between porpoises from the northern and southern North Sea and between the Northern North Sea and the Celtic/Irish Sea. It may be performed using the Arlequin Version 3.1 software program (Schneider *et al.* 2000).

Estimation of the diversity or "taxonomic richness" present amongst populations from different environments and sources, taking differing sample sizes into account, may be performed by using analytical rarefaction to produce rarefaction curves (Hughes *et al.* 2001). Rarefaction not only takes the sample size into account but by means of extrapolation of the rarefaction curve indicates whether further sampling would be likely to increase the number of taxa (or sequence types in the case of MLST data) observed. Examination of the 95% confidence intervals allows inference regarding statistical significance when comparing curves. Analytical rarefaction may be performed in the PAST software package (Hammer *et al.* 2008). Analytical rarefaction has been used previously to investigate the epidemiology of human cryptosporidiosis using multilocus genotyping data (Grinberg *et al* 2008) and to compare the sequence type diversity of *C. jejuni* in wildlife compared to cattle (French 2007).

Classically, genotypic data are typically displayed visually by means of dendrograms e.g. the unweighted pair-group method with arithmetic averages (UPGMA), on the basis of a matrix of pairwise differences in the allelic profiles of the isolates. Whilst this is highly effective in establishing that isolates are identical or closely related, dendrograms provide no information on the patterns of evolutionary descent of isolates within a clonal complex and furthermore, since the tree produced by such methods is rooted, they assume a bifurcating tree-like phylogeny with a common founder strain. This tree like structure is incorrect for weakly clonal or non clonal organisms where diversity is produced by recombination in addition to mutation. Alternative approaches include split decomposition, using Splitrees (Huson & Bryant 2006) which use the concept of phylogenetic networks rather than phylogenetic tree, and eBURST.

The software program eBURST (Feil *et al.* 2004) places isolates in clonal complexes having first identified the likely founder genotype of the clonal complex on the basis of parsimony as the sequence type that has the most single locus variants (SLV) in the group or clonal complex. Similarly subgroups and subgroup founders are identified. Statistical confidence in founding genotypes is provided by means of bootstrapping. eBURST diagrams can be produced which display the pattern of descent of all sequence types within a clonal complex from the founder sequence type via SLVs i.e. 2 STs will be linked if they differ by only one SLV but not if they differ by more e.g. if one is a DLV (double locus variant) of the other, they will not be linked.

MLST data, both at ST and C-C level, provide a powerful tool for increasing our knowledge of the transmission routes in human campylobacteriosis (Sopwith *et al* 2006) and source attribution models have been constructed using a number of approaches (French 2007). Previous studies (Dingle *et al.* 2002, Colles *et al.* 2003, Manning *et al.* 2003 Karenlampi *et al.* 2007) have found sequence types belonging to the following clonal complexes (C-C) to predominate in cattle and sheep: C-C 61, C-C 42, C-C 21, C-C 48, C-C 45 and furthermore it has been suggested that the widespread occurrence in ruminants of sequence types belonging to C-C 61 may represent adaption to the host species (Manning *et al.* 2003). There are few data on the diversity of sequence types within localised populations although three recent studies have investigated sequence type diversity in cattle at farm level (Kwan *et al.* 2008a) and in cattle, wildlife and the environment in a farmland area (French *et al.* 2005, Kwan *et al.* 2008b).

MLST data has provided valuable insight into the population structure and evolution of *C. jejuni*. For example, Dingle *et al.* (2002) and French *et al.* (2005) suggest that the *uncA*17 allele found in the founder strain of C-C 61 differs markedly from the majority of other *uncA* alleles and suggested that it originated in *Campylobacter coli.* Further, it has recently been suggested on the basis of MLST data that *C. jejuni* and *C. coli* are converging to form one single species (Sheppard *et al.* 2008) and that this may be a consequence of ecological factors namely the development of intensive agriculture allowing the two bacterial species to come into close contact. Additional evidence of recombination comes from McCarthy *et al.* (2007) who demonstrated substantial import of genetic material into isolates belonging to the ST-21 clonal complex from other *Campylobacters* in the same host species.

The present study is nested within, and builds upon the studies reported in Chapters 5 and 6 which investigate aspects of the epidemiology of *C. jejuni* in cattle and sheep in Lancashire at both microbial species level and MLST clonal complex level.

7.2 Materials and Methods.

Full details of all methods are given in Chapter 2. The study design was a repeated cross-sectional study over a 2 year period starting in January 2006. Fourteen dairy and four sheep farms were recruited via three veterinary practices in Lancashire. The three practices selected were in Longbridge, Lancaster and Clitheroe and served the Fylde (Zone 1), N. Lancashire (Zone 2) and S.E. Lancashire (Zone 3) respectively. Six dairy farms were recruited in Zone 1 whilst four dairy and two sheep farms were recruited in each of the other zones. One farm in Zone 2 ceased trading in December 2006 and was replaced with a neighbouring farm for the rest of the study. Another farm in Zone 2 ceased keeping cattle in June 2007 thus sampling on this farm was incomplete. Eligibility criteria for entry were: Dairy farms with over 100 adult cows with or without a sheep enterprise; Sheep farms with over 150 breeding ewes and no other livestock enterprises.

Farms were visited at 8 week intervals when 20 random freshly voided faecal samples would be collected from the lactating cows on dairy farms or adult sheep on sheep farms. Samples were collected from animals observed to defecate by the author. Three farms from one zone would be sampled at each occasion. Each faecal pat was sampled from at least 3 sites within the pat and mixed thoroughly in a sterile sample

pot to minimise possible within-pat variation. In the case of sheep faecal pellets, at least 3 were collected. Twenty faecal samples were collected at each visit. Samples were transported to the laboratory on ice.

At each visit, current management details were obtained via a short questionnaire (Appendix B).

In the laboratory, one gram of faeces was placed in 9 ml of *Campylobacter* enrichment broth (IDG Ltd, Bury, UK) with cefoperazone, vancomycin, trimethoprim and cycloheximide (CVTC supplement: IDG Ltd, Bury, UK) and after homogenising for 30 seconds in a Colworth 80 stomacher (A. J. Seward & Co. Ltd, London, UK) was incubated in a plastic universal bottle for 24 hours at 37^0C in a in a "Variable Atmosphere Incubator" (VAIN, Don Whitley Scientific Ltd, UK) maintaining a microaerobic atmosphere (CO_2 12%, H_2 3%, O_2 11%, N_2 74%). After incubation, 50µl of the enrichment broth was inoculated onto a *Campylobacter* blood-free selective agar (CSA) plate (IDG Ltd, Bury, UK) enriched with cefoperazone and amphotericin (CA supplement: IDG Ltd, Bury, UK). A 30µg nalidixic acid sensitivity disc (Mast Group, Merseyside, UK) was placed on the heaviest part of the inoculum. Similarly a 5µl loopful of enrichment broth was inoculated onto a second CSA plate without a nalidixic acid disc. The CSA plates were incubated at 37^0C in a microaerobic atmosphere for 60-72 hours after which time plates were examined and up to 4 putative campylobacter colonies (per faecal sample) were sub-cultured onto blood agar plates and incubated at 37^0C under microaerobic conditions as described earlier. Putative campylobacters were identified on colony morphology. After 72 hours incubation, single colonies were sub-cultured onto two blood agar plates. One plate was incubated for 48 hours under microaerobic conditions as described and the other plate incubated for 48 hours at 30^0C in air for detection of *Arcobacter* spp.

A crude DNA aqueous lysate ("boiled preparation") was prepared by inoculating 200µl of distilled water with a small amount of the culture ("leading edge of a loop"), heating at 100^0 C for 15 minutes followed by centrifugation at 1300 rpm for 10 minutes. All putative isolates were frozen in Microbank tubes (Pro-Lab Diagnostics, Neston, United Kingdom) and stored at -80^0C. In order to maximise sensitivity, two PCR assays were performed for identification of *C. jejuni* isolates: a colony multiplex PCR assay (Wang *et al.* 2002) and a monoplex PCR assay (Gonzalez *et al.* 1997).

Of the 2307 *C. jejuni* isolates identified, 1003 isolates were selected for sequencing and subsequent assignment to ST. Isolates were randomly selected using Survey

Toolbox (Cameron 1999) stratifying by Zone and Sampling Round (defined as the 8 week period during which each farm was sampled once).

Isolates were processed in batches of 250. In the case of the first 250 isolates, the original DNA aqueous lysate was used, whilst for the second batch, the isolate was grown on blood agar and an aqueous lysate prepared as described earlier. In the case of the last 500 isolates, these were grown on blood agar and DNA extracts prepared using a commercial kit (Prepman: Applied Biosystems). These changes in extraction methodology were introduced in an attempt to maximise the initial PCR yield.

Essentially sequencing for MLST involves 3 steps, namely:

- PCR amplification of the gene product.
- Dideoxy-termination sequencing reaction performed on each DNA strand (forward and reverse) using internal nested primers and Big Dye Ready Reaction Mix (Applied Biosystems).
- DNA sequencing.

The first 500 isolates were processed using primers and reaction conditions as described by Dingle *et al* (2001) whilst the remaining isolates were processed using primers and reaction conditions as described by Miller *et al.* (2005). This change in protocol was adopted in an attempt to optimise the entire process. In the event of failure to obtain sequence data for a given allele, the entire process was repeated for up to a maximum of 6 times.

PCR was performed in 50µl volumes comprising 2µl of DNA lysate and 48µl of mastermix, using a programmable thermal cycler (ABI 20720: Applied Biosystems). Sequencing was performed using an ABI 3130xl automatic sequencer with a 50cm array and POP-7 polymer (Applied Biosystems).

Complete laboratory protocols including precipitation and clean-up methodologies are in Appendix C.

Sequence data assembly, alignment, and interrogation of the *Campylobacter jejuni* MLST database (http://pubmlst.org/ campylobacter/) for assignment of sequence type was performed using the STARS computer program (Man Suen Chan & Nicki Ventress 2001) running under Biolinux (http://nebc.nox.ac.uk/biolinux.html).

All covariate data was entered in an Access 2003 database (Microsoft) whilst laboratory data was entered into an Excel2003 (Microsoft) spreadsheet. Data analysis was performed using Stata V10 (StataCorp: Texas).

Gene flow was assessed by calculating Wright's F_{ST} using the DNAsp software package (Rozas *et al.* 2003) and performing Analysis of Molecular Variance with the Arlequin V3.0 (Schneider *et al.* 2000) software package.
Linkage disequilibrium was investigated by calculating the standardised Index of Association (I_A^S) (Maynard Smith *et al* 1993) in the software package LIAN (Haubold & Hudson 2000).
Genotypic diversity, at sequence type level, was estimated by performing rarefaction analysis with the production of rarefaction curves using the PAST (Hammer *et al.* 2008) software package.

7.3 Results.

The 1003 isolates sequenced were obtained from 591 faecal pats (from which 4 putative *campylobacter* isolates were cultured per pat). Nine hundred and thirty nine (93.6%) full allelic profiles were obtained from the 1003 isolates sequenced. The 785 bovine isolates were obtained from 485 faecal pats whilst the154 ovine isolates were from 106 faecal pats. A total of 154 sequence types were identified with 86 being new sequence types. Of the 939 isolates with full allelic profiles, 856 were assigned to existing clonal complexes (*Campylobacter jejuni* MLST database at http://pubmlst.org/ interrogated on the 19th June 2008).
Only one bacterial species was isolated from 457 (77.3%) of the 591 pats with two species isolated from 131 (22.2%) of the pats and three species isolated from three pats. Three hundred and six pats (52%) yielded one isolate of *C. jejuni* with 181 yielding 2 isolates, 81 yielding 3 isolates whilst 23 pats yielded 4 isolates. 489 pats contained one sequence type only whilst 65 contained 2 STs, 6 pats contained 3 STs and 2 pats contained 4 STs i.e. 73 pats contained multiple sequence types (Table 7.1)

Table 7.1. Pat level distribution of sequence types.

Number of pats	Nos. *C. jejuni* isolates obtained per pat	Number of different sequence types found in each pat				
		0 (incomplete)	1	2	3	4
306	1	20	286			
181	2	5	135	41		
81	3	2	55	19	5	
23	4	2	13	5	1	2
Total						
591 pats		**29**	**489**	**65**	**6**	**2**

Of the 73 pats containing more than one sequence type, 37 (51%) contained sequence types belonging to the same clonal complex with 32 pats (44%) containing sequence types belonging to different clonal complexes whilst 4 pats contained sequence types unassigned to clonal complexes.

The number of isolates obtained from each farm is represented graphically in Figure 7.2.

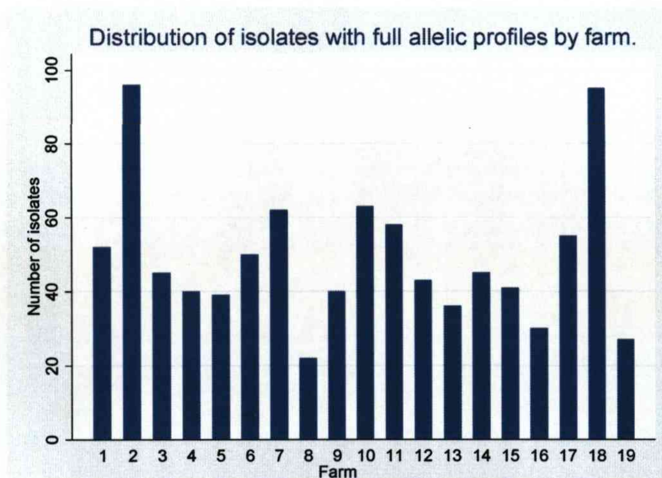


Figure 7.2. Number of isolates with full allelic profiles by farm.

Substantially more isolates were obtained from Farms 2 and18 with substantially fewer from Farms 8 and19 which were only in the study for 12 months. The median number of different sequence types isolated from each farm was 14 (inter-quartile range 10 – 20) (Figure 7.3).

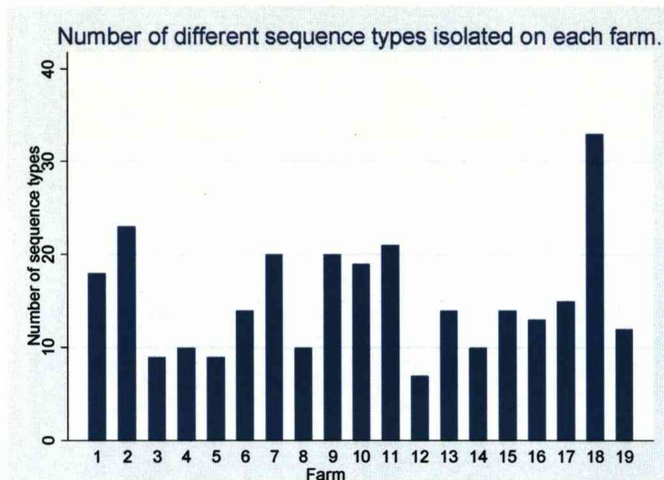
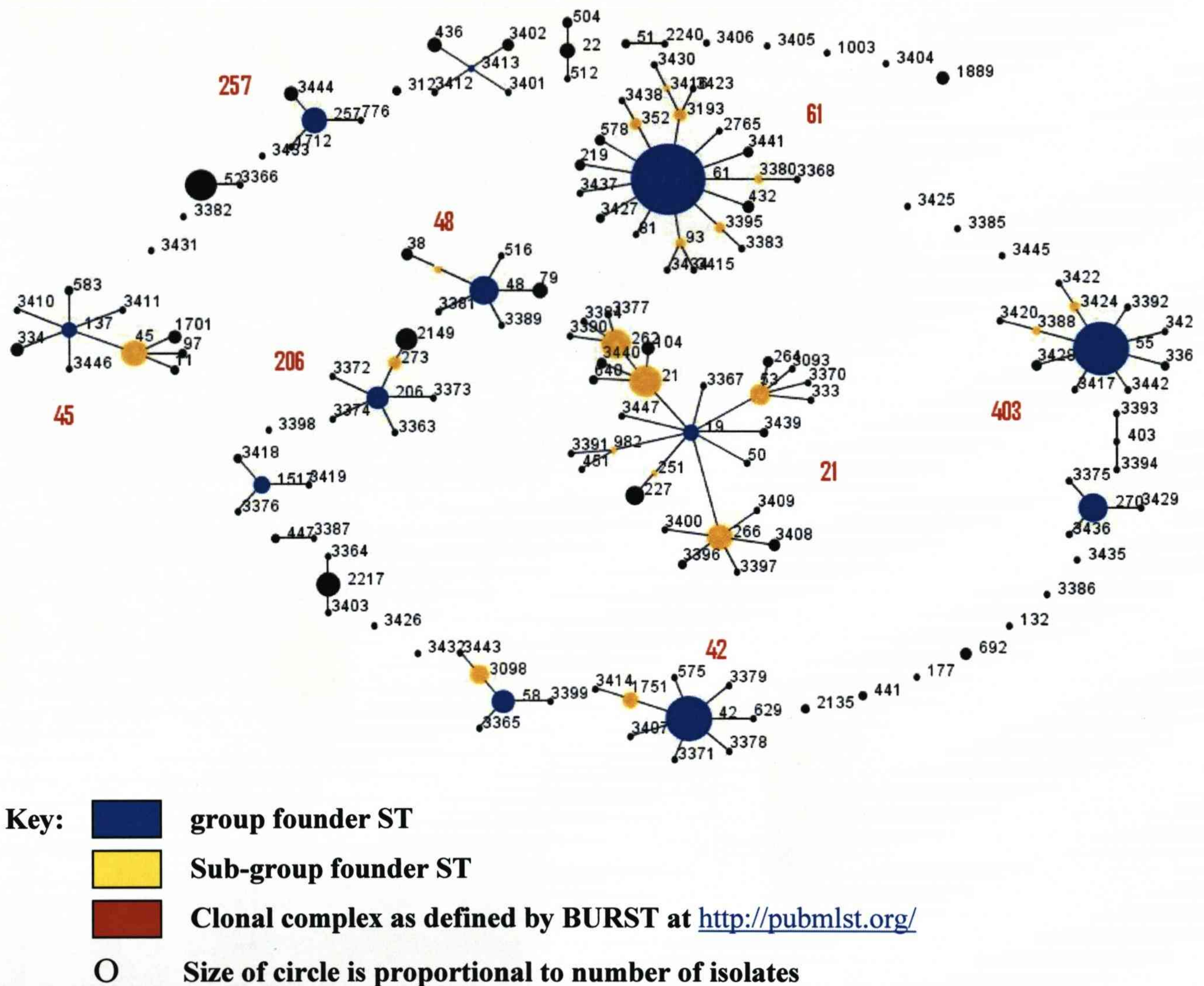


Figure 7.3. Number of different sequence types isolated from each farm.

Fifty nine sequence types were isolated in Zone 1 (Fylde) with 72 isolated in Zone 2 (Lancaster) whilst 69 STs were isolated from Zone 3 (Clitheroe). One hundred and thirty six STs were isolated from cattle faeces with 41 STs isolated from sheep faeces. Eighty three percent of isolates were assigned to the following clonal complexes using BURST at http://pubmlst.org/ : C-C 21, C-C 42, C-C 45, C-C 48, C-C 61, C-C 206, C-C 257& C-C 403, hereafter referred to as "major clonal complexes" (Figure 7.4). Analysis of the present dataset suggests the presence of groups not recognised as clonal complexes by BURST analysis at http://pubmlst.org/ e.g. a group with ST 55 as its founder (Fig 7.4). The STs comprising this group are in fact members of C-C 403 as ascertained by interrogation of the *C. jejuni* database at http://pubmlst.org/ but fail to group with ST 403 in Figure 7.4 due to the absence in the present dataset of an SLV linking any members of the group to ST 403.

Figure 7.4. Population snapshot of *C. jejuni* isolates from cattle and sheep in Lancashire.



Key:

[blue]	**group founder ST**
[yellow]	**Sub-group founder ST**
[red]	**Clonal complex as defined by BURST at http://pubmlst.org/**
O	**Size of circle is proportional to number of isolates**

7.3.1 Gene Flow.

This was assessed by means of Wrights F_{ST} statistic at four levels, namely between farms, between geographical zones, between cattle and sheep and between animals grazing outside and animals kept inside:

- **Farm.** Pairwise F_{ST} values were computed for gene flow between farms. The F_{ST} values computed were small ranging from 0.001 to 0.13 suggesting that the population of *C. jejuni* is homogenous such that individual farm populations are genetically indistinguishable
- **Animal species.** The pairwise F_{ST} statistic was 0.0246 for gene flow between the 2 species. Using permutation testing with 1000 replicates, this was not

significantly different from 0, suggesting that there is no difference between the 2 populations and they are homogenous and genetically indistinguishable

- **Geographical zone**. Pairwise F_{ST} values were 0.0039 (Fylde V Lancaster), -0.0051 (Fylde V Clitheroe) and 0.0166 (Lancaster V Clitheroe) for gene flow between geographical zones. Permutation testing with 1000 replicates failed to demonstrate any statistically significant differences suggesting that the cattle and sheep *C. jejuni* population is homogenous in these 3 areas of Lancashire.
- **Environment.** The pairwise F_{ST} statistic was 0.0007 for gene flow between the two sampling environments with no statistical significance using permutation testing. This suggests that there is no discernible genetic difference between *C. jejuni* populations obtained from animals at pasture and housed animals.

In summary, gene flow analysis demonstrates that all the *C. jejuni* isolates in this study belonged to the same homogenous population.

AMOVA was performed to investigate partitioning of variance at four levels namely: between zone, between farm, between animal species, within farm. In all analyses performed almost all variation (>98%) was at the within farm level, providing further evidence that there were no host species or spatial genetic differences in the *C. jejuni* populations studied.

7.3.2 Linkage Disequilibrium.

The presence or absence of linkage disequilibrium was investigated by calculating the standardised Index of Association (I_A^S) at four levels, namely between farms, between geographical zones, between cattle and sheep and between cattle grazing outside and those kept inside (Table 7.2).

The standardised Index of Association (I_A^S) was significantly greater than zero in all analyses performed demonstrating that linkage disequilibrium was present.

Table 7.2. Standardised Indices of Association for *C. jejuni* isolates from cattle and sheep in Lancashire.

	V_O	V_E	I_A^S	P value
All isolates	4.055	1.016	0.498	0.0001
Zone				
Zone 1 (Fylde)	5.681	1.192	0.628	0.0001
Zone 2 (Lancaster	4.061	1.028	0.492	0.0001
Zone 3(Clitheroe)	3.377	0.921	0.444	0.0001
Species				
Cow	4.451	1.034	0.554	0.0001
Sheep	3.857	1.063	0.438	0.0001
Environment (cattle only)				
At pasture	4.029	1.033	0.483	0.0001
Housed	4.365	1.035	0.537	0.0001
Farm Identity				
Farm 1	5.862	1.337	0.564	0.0001
Farm 2	6.57	1.144	0.791	0.0001
Farm 3	10.428	1.731	0.837	0.0001
Farm 4	8.918	1.731	0.692	0.0001
Farm 5	6.138	1.255	0.648	0.0001
Farm 6	7.461	1.509	0.657	0.0001
Farm 7	8.804	1.545	0.705	0.0001
Farm 8	4.10	1.046	0.487	0.0001
Farm 9	4.623	1.236	0.457	0.0001
Farm 10	5.502	1.305	0.536	0.0001
Farm 11	7.869	1.474	0.723	0.0001
Farm 12	7.364	1.440	0.685	0.0001
Farm 13	6.118	1.216	0.672	0.0001
Farm 14	5.561	1.048	0.717	0.0001
Farm 15	5.980	1.439	0.526	0.0001
Farm 16	4.853	1.061	0.595	0.0001
Farm 17	6.548	1.270	0.693	0.0001
Farm 18	5.161	1.195	0.553	0.0001
Farm 19	4.593	0.828	0.758	0.0001

7.3.4 Population diversity.

This was investigated by performing rarefaction analysis to produce rarefaction curves (Figures 7.5 – 7.10). It was carried out on the entire dataset and at five levels, namely between farms, between cattle and sheep, between geographical zones using cattle isolates only since no sheep were sampled in Zone 1, between isolates from cattle grazing outside and cattle kept inside, and between cattle farms classified according to their purchase policy:

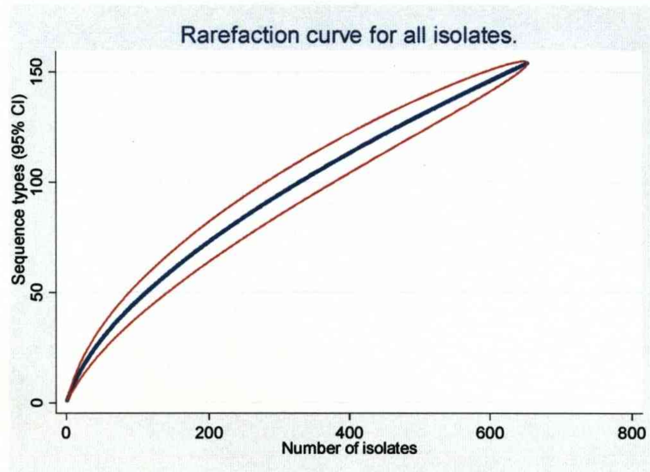


Figure 7.5. Rarefaction curve for all isolates.

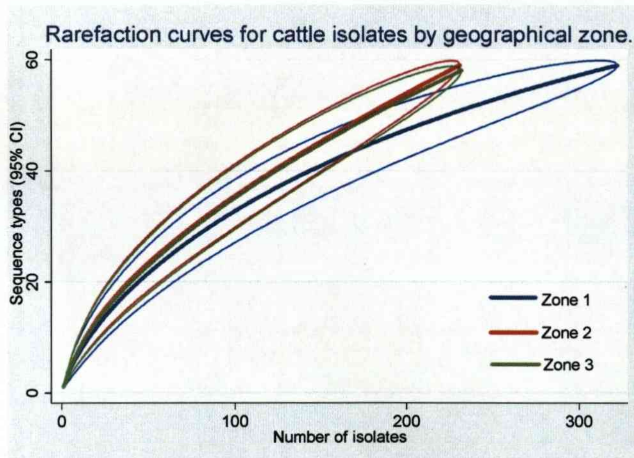


Figure 7.6. Rarefaction curves for cattle isolates by geographical zone.

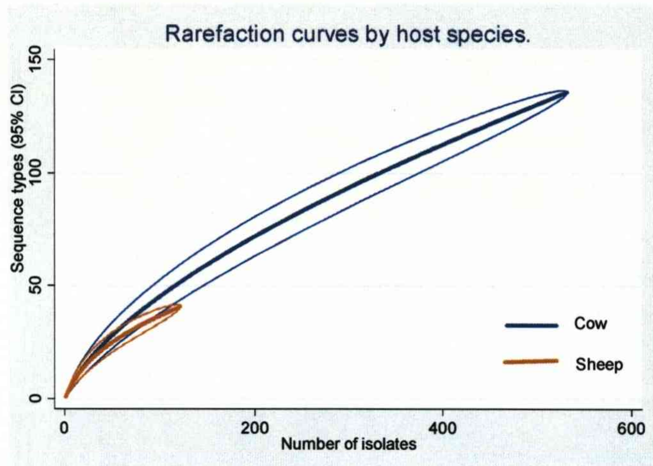


Figure 7.7. Rarefaction curves for all isolates by host species.

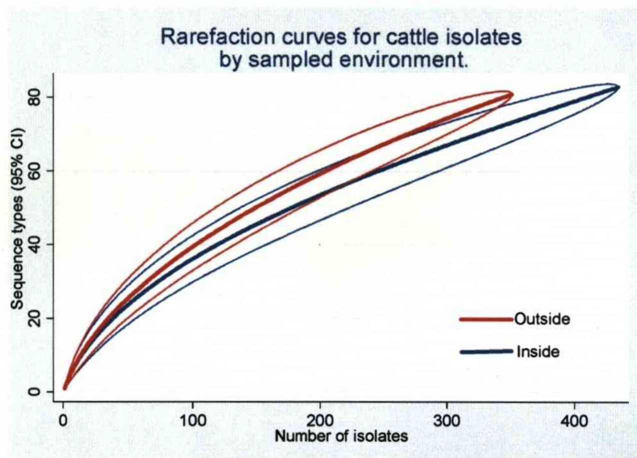


Figure 7.8. Rarefaction curves for cattle isolates by sampled environment.

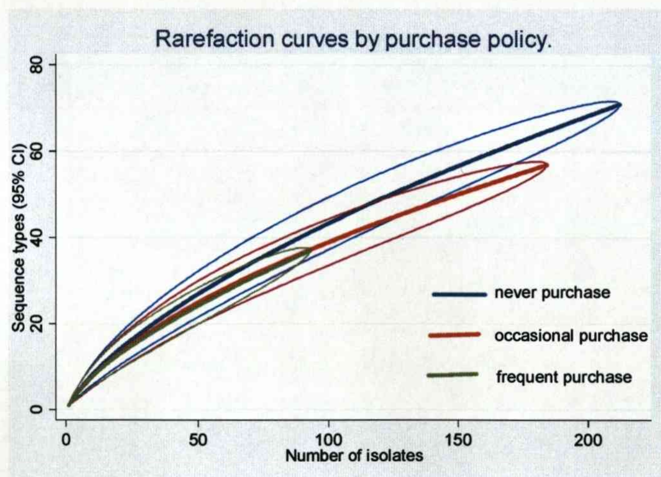
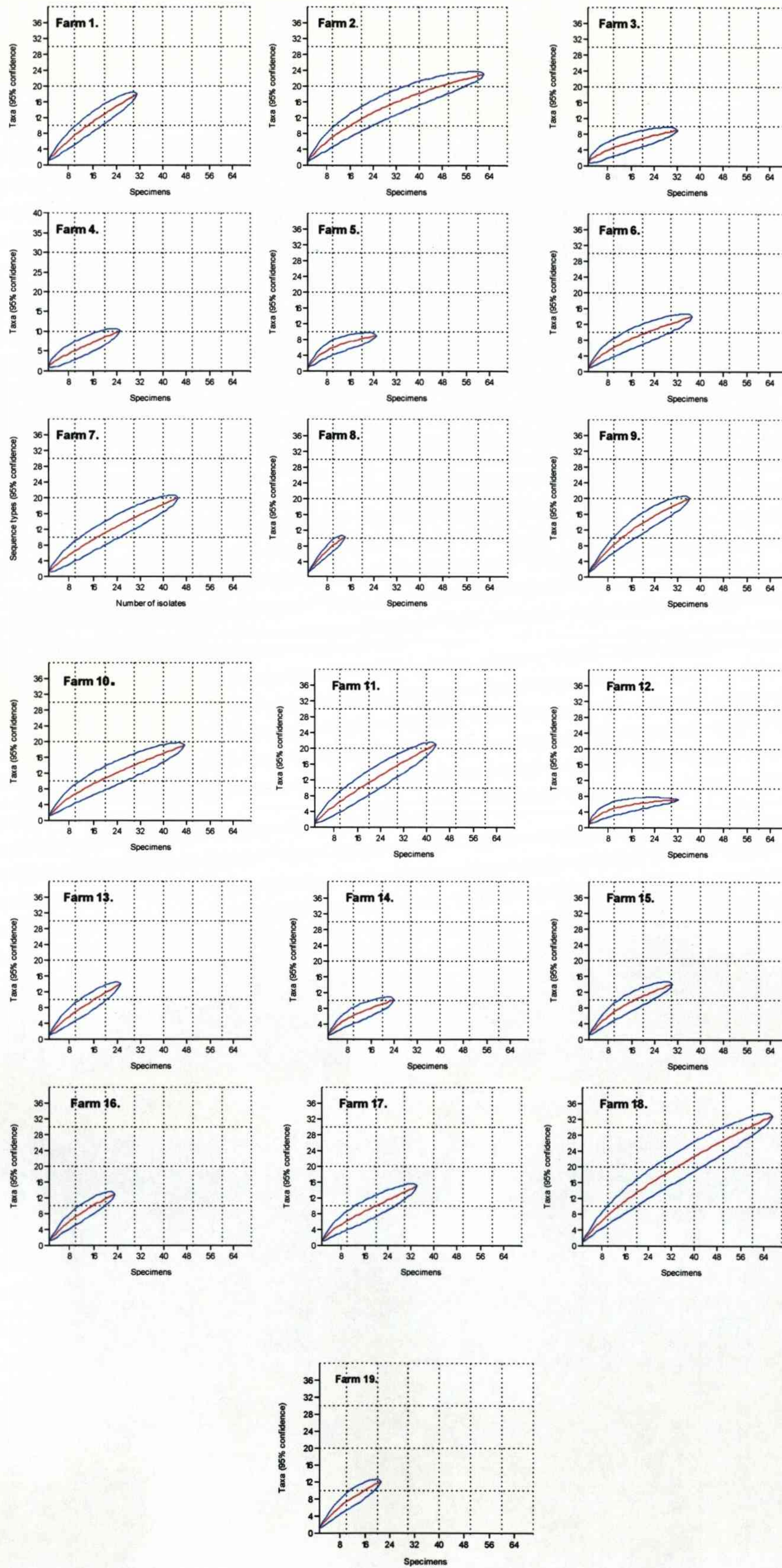


Figure 7.9. Rarefaction curves for cattle isolates by purchase policy.

Figure 7.10. Individual rarefaction curves for all farms.



Examination of the rarefaction curves (Figures 7.5 – 7.10 suggest there is considerable variation in the amount of sequence type diversity present at the different analytical levels investigated, although the sample sizes are small such that statistical significance at the 95% level cannot be demonstrated.

- **Host species.** Examination of the rarefaction curves show they diverge and extrapolation of the "sheep curve" (equivalent to taking more samples) would demonstrate statistical significance by examination of the confidence limits. The sequence type diversity appears to be greater amongst cattle isolates compared to sheep isolates although this is statistically non-significant.
- **Geographical zone.** Examination of the rarefaction curves suggest that the curves diverge and the level of diversity amongst cattle isolates in Zones 2 and 3 are similar but are greater than in Zone 1.
- **Sampling environment.** The sequence type diversity appears to be greater amongst isolates obtained from grazing cattle compared to housed cattle, although this is statistically non-significant.
- **Farm.** There is a considerable variation in the amount of diversity present on farms.
- **Purchase policy.** It would appear that farm purchase policy had an influence on sequence type diversity with greater diversity present on farms that never purchased animals compared to those that did.

7.3.5 Sequence type diversity amongst members of the major clonal complexes.
This is summarised in Figure 7.4 and Table 7.3. C-C 21 and C-C 61 are the most numerous clonal complexes with the largest number of sequence types in them. A total of 86 previously unreported STs were identified of which 14 were in C-C 21 and 15 were in C-C 61. Individual eBURST diagrams for all clonal complexes are in Appendix D.
Rarefaction analysis was performed to investigate sequence type diversity amongst members of the major clonal complexes (Figs 7.11).
Standardised indices of association were calculated for sequence types belonging to the major clonal complexes (Table 7.4) to ascertain whether linkage disequilibrium was present or absent at clonal complex level.

Table 7.3. Distribution of major clonal complex membership amongst cattle and sheep *C. jejuni* isolates from Lancashire dairy and sheep farms.

Clonal complex	Number of isolates	Number of faecal pats	Number of farms	Number of sequence types
C-C 21	146	125	16	29
C-C 42	88	83	14	10
C-C 45	51	46	14	13
C-C 48	43	41	10	7
C-C 61	230	208	19	26
C-C 206	52	48	8	8
C-C 257	30	24	6	4
C-C 403	141	130	10	11

Table 7.4. Standardised Indices of Association for *C. jejuni* isolates from cattle and sheep in Lancashire belonging to the major clonal complexes.

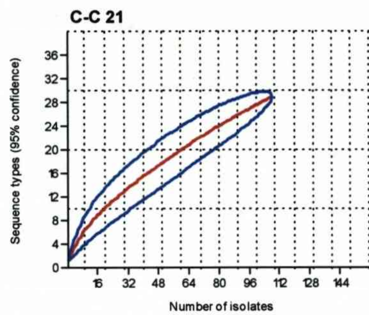
Clonal Complex	Number of isolates	V_O	V_E	I_A^S	P value
C-C 21	146	1.208	1.00	0.035	0.005
C-C 42	88	0.752	0.489	0.089	0.004
C-C 45	51	1.155	1.021	0.022	0.144
C-C 48	43	0.841	0.709	0.031	0.151
C-C 61	230	0.718	0.529	0.059	0.002
C-C 206	52	0.968	0.912	0.010	0.210
C-C 257	30	0.362	0.430	-0.026	1.00
C-C 403	141	1.178	0.701	0.113	0.0001

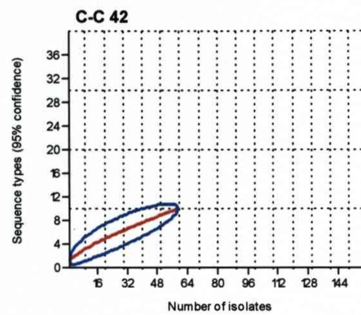
Linkage disequilibrium was demonstrated for C-C 21, C-C 42, C-C 61 & C-C 403 but not for C-C 45, C-C 48, C-C 206 or C-C 257. However the failure to demonstrate linkage disequilibrium in the case of C-C 45, C-C 48, C-C 206 or C-C 257 may be a reflection of the relatively small number of isolates belonging to these clonal complexes. In all case, the clonal complex level I_A^S values (-0.03 – 0.11) are considerably less than the I_A^S values calculated earlier (0.43 – 0.83) for the entire dataset (Table 7.2).

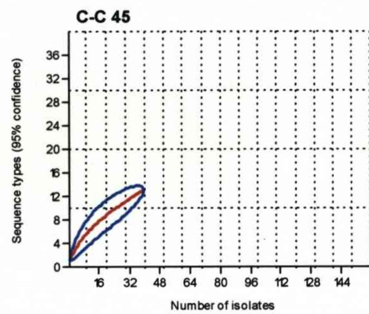
Examination of rarefaction curves suggest that there is greater diversity amongst isolates belonging to C-C 21 and C-C 45 than amongst isolates belonging to the other

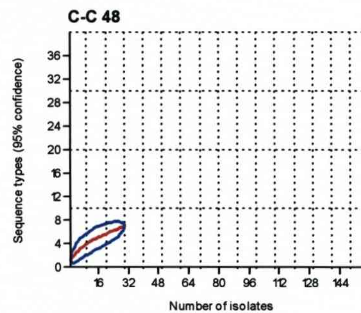
clonal complexes investigated. However sample sizes are small such that statistical significance at the 95% level cannot be demonstrated

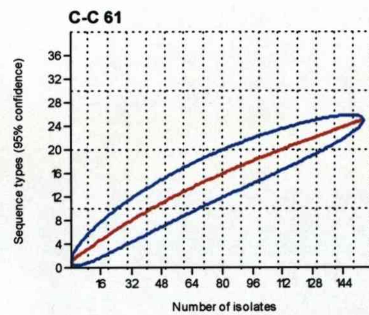
Figures 7.11. Rarefaction curves for isolates belonging to the major clonal complexes.

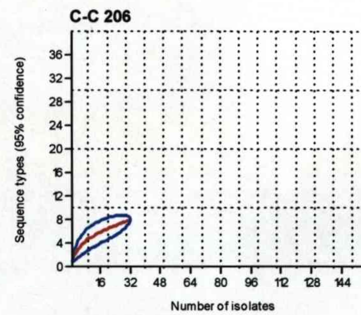


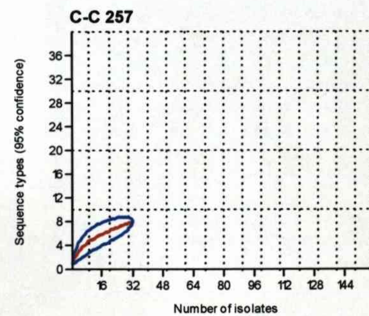


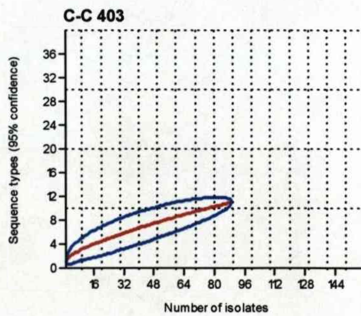












7.4 Discussion.

This study investigates the genetic diversity amongst *C. jejuni* isolates from cattle and sheep faeces on Lancashire dairy and sheep farms and builds on the results reported in previous chapters in this thesis.

Microbial diversity may be considered to occur at two levels namely within-animal and between-animal. Sampling strategy is crucial in recognition and quantification of diversity at both levels. For demonstration and quantification of within-animal diversity this implies taking multiple isolates from the animal or faecal pat.

In the present study four putative campylobacter colonies were selected from each faecal pat. This decision was made primarily on logistical grounds bearing in mind that the primary objective of the study was to describe between animal diversity and to sample the maximum number of animals within time and labour constraints.

Co-colonisation of pats both at species level and at *C. jejuni* sequence type level was observed with more than one bacterial species isolated from 23% of the 591 faecal pats and co-colonisation by more than one *C. jejuni* sequence type occurring in 73 (12%) of the sampled pats. Approximately half of these 73 pats were colonised by sequence types belonging to the same clonal complex with the remainder being colonised by sequence types belonging to different clonal complexes, or in the case of four pats colonised with sequence types unassigned to clonal complex. This would suggest that there is both substantial sequence type and clonal complex diversity present within animals as well as between animals.

Thus it is apparent that ruminants can be colonised by more than one species of *Campylobacter* and by more than one *C. jejuni* sequence type simultaneously. This multiple colonisation is a pre-requisite for recombination events since *Campylobacter* spp do not multiply outside their host and furthermore, the frequency of recombination will depend, in part, on the frequency of co-colonisation both at species level and sequence type level

Further studies are required to quantify this within-animal diversity. To the best of the author's knowledge no such studies have been undertaken, although the issue of optimal microbial sampling strategy has been addressed previously with regards to *E. coli* in poultry (Singer *et al.* 2000) whilst Dopfer *et al* (2008) employed Bayesian techniques to address the issue using a number of micro-organisms as examples and

produced a generic program in WinBUGS (http://www.mrc-bsu.cam.ac.uk/bugs) to perform the necessary sample size calculations.

The animal level (or faecal pat level) sampling strategy employed is crucial in the recognition and quantification of genetic diversity within a bacterial population – biased sampling, e.g. from diseased individuals only might imply a high degree of clonality when in fact wider sampling might reveal the existence of multiple clones not found in diseased individuals, i.e. a weaker clonal population structure would become apparent. The present study employed repeated random sampling of farms, thus minimising potential sampling biases, allowing us to draw valid conclusions about the population structure of *C. jejuni* in dairy cattle and sheep.

We have employed measures of gene flow, linkage disequilibrium, and overall diversity to describe the genetic diversity present in ruminant *C. jejuni* isolates. *Campylobacter jejuni* is considered to be weakly clonal (Dingle *et al.* 2002) with both mutation and recombination generating considerable diversity as demonstrated by the fact that at the time of writing there are 3654 sequence types placed in 43 clonal complexes recorded on the campylobacter MLST database (http://pubmlst.org). Analysis of MLST data from a cross sectional study of an area of Cheshire farmland (Fernhead *et al.* 2005) found substantial evidence for recombination with short tract lengths (225 – 750 base pairs) and estimated the rate of recombination to be of a similar magnitude to the rate of mutation.

In the present study, we calculated the standardised indices of association (I_A^S) both for the entire *C. jejuni* population and at four levels, namely between farms, between geographical zones, between cattle and sheep and between cattle grazing outside and those kept inside. In all cases the index of association was significantly different from zero demonstrating the presence of linkage disequilibrium. The values of I_A^S observed (0.44 – 0.84) were consistent with a limited amount of recombination that does not completely destroy the linkage between alleles i.e. a weakly clonal population structure. Whilst the I_A^S is an indirect measure of the recombination rate, it is of value primarily in qualitatively identifying the presence or absence of linkage disequilibrium rather than quantifying it: however it could be argued that the wide range of values observed may be a reflection of the likely variation present in recombination rates between different sub-populations e.g. between farms.

Gene flow comparison, as measured by Wrights F_{ST} statistic, suggest that at all levels of analysis, the populations may be considered genetically homogenous with similar

allelic frequencies. However this does not necessarily imply that levels of diversity are similar within sub-populations. Furthermore AMOVA demonstrated that almost all (> 98%) of the variance present resided at the within-farm level.

Rarefaction analysis was performed for different levels. In using this technique, no assumptions are made regarding the nature of the diversity present i.e. in the present study sequence types are considered to be "equally different" to each other whereas in reality the difference between any two sequence types is likely to vary depending on the number of base pairs at which they differ (genetic distance).

Estimation of the diversity in any population will depend both on the number of samples taken as well as the underlying true diversity. Rarefaction analysis takes the sample size into account and extrapolation of the rarefaction curves allows the investigator to judge firstly whether further sampling would be likely to result in more sequence types being observed, and secondly whether the underlying true diversity has been fully described. When this has occurred, then by definition, the rarefaction curve will be flat since further sampling would not reveal the presence of any more sequence types.

The rarefaction curves generated in this study suggest that in almost all cases further sampling would demonstrate increased diversity, implying that the actual amount of sequence type diversity present is greater than that demonstrated. Inspection of individual rarefaction curves show there is considerable variation in the diversity present at almost all levels, particularly at farm level.

Of particular interest is the rarefaction curve for Farm 12 (Figure 7.10), a sheep farm, which appears almost horizontal suggesting that further sampling would not demonstrate any greater diversity to be present. Comparison of this curve with those of the other sheep farms in the study namely Farms 9, 15 &16 suggest that there is significantly less diversity present on Farm 12. This farm is a large hill farm where the stock are kept on the open hill at very low stocking densities for much of the year whilst the other sheep farms in the study are at lower altitude with no open hill grazing and so will have higher stocking densities. Between animal transmission of *C. jejuni* is via the oro-faecal route and it is a reasonable assumption that this will be via ingestion of contaminated pasture. It follows that the rate of transmission will be proportional in part to stocking density which may explain the reduced diversity observed on this hill farm compared to the other lowland farms.

Sequence type diversity appears to be greater amongst cattle isolates compared to sheep isolates. This would suggest there may a higher frequency of recombination events amongst cattle isolates compared to sheep isolates. Dairy cattle are generally kept at higher stocking densities than sheep thus increasing the likelihood of acquisition of campylobacters from herdmates. This would likely afford greater opportunities for recombination and thus increased diversity.

There appears to be greater diversity amongst cattle isolates collected at pasture compared to those from housed cattle. In Chapter 5, we reported that the faecal pat prevalence of *C. jejuni* was significantly higher at pasture. However the observation that grazing at pasture increase both prevalence and diversity of *C. jejuni* offers no insight into the direction of any possible causal pathways: does increased prevalence produce increased diversity or *vice versa*?

Amongst the cattle isolates, inspection of the rarefaction curves suggested that there was greater sequence type diversity observed in zones 2 & 3 compared to zone 1. Zone 1 was the southern Fylde, a coastal plain bordered by the River Ribble estuary to the south and the Irish Sea to the west whilst zones 2 and 3 were both in the western Pennines. Biodiversity is likely to be greater in the Pennines which have a greater range of wildlife habitats and are less intensively farmed than the Fylde. It may be hypothesised that the greater ST diversity observed in cattle isolates from the Pennine areas are due to increased transmission from wildlife occurring in these more bio-diverse environments compared to the Fylde.

Introduction of new animals to the herd or flock did not appear to increase the amount of sequence type diversity present: in fact there was greater diversity amongst herds reported as being "closed" compared to herds reporting occasional or frequent purchase of animals. However it is well recognised that self reporting of purchase policy by farmers is often inaccurate with farmers forgetting or ignoring purchases made in the past, thus the finding of greater diversity in closed herds should be treated with scepticism, although it does suggest that frequent purchase of animals does not lead to increased sequence type diversity.

As reported in Chapter 6, the diversity observed at clonal complex level is in agreement with a number of other studies of *C. jejuni* in farm animals ((Dingle *et al.* 2002, Colles *et al.* 2003, Manning *et al.* 2003, French *et al.* 2005, Karenlampi *et al.* 2007, Kwan *et al.* 2008a) confirming the preponderance of isolates belonging to C-C 21, C-C 42, C-C 45, C-C 48, C-C 61, C-C 206, C-C 257 & C-C 403 amongst cattle

and sheep isolates, all of which have been reported as causing human disease in the Northwest of England (Sopwith *et al.* 2006). We investigated the diversity observed amongst members of these clonal complexes.

Inspection of the individual rarefaction curves suggests that there is greater diversity amongst members of C-C 21 and C-C 45 compared to the other major clonal complexes.

Previous studies (French *et al.* 2005, Kwan *et al.* 2008b) have suggested that both C-C 21 and C-C 45 are widespread and found in a wide range of host species and environmental samples. Is the increased diversity observed amongst these clonal complexes in the present study a reflection of cross species transmission between wildlife and farm animals? An alternate hypothesis could be that these clonal complexes represent "founder strains" and have been present for a longer period of time allowing them to acquire more mutations. This is supported by a recent study of Finnish isolates from humans, cattle and poultry (Karenlampi *et al.* 2007) which found greater diversity of sequence types amongst C-C 21 and C-C 45 compared to other clonal complexes. Similarly, both these clonal complexes have the greatest number of sequence types currently on the campylobacter MLST database (http://pubmlst.org), with 370 sequence types belonging to C-C 21 and 204 belonging to C-C 45.

Measurement of gene flow by Wrights F_{ST} statistic suggests that the *C. jejuni* population in this study is genotypically homogenous. This suggests either that much of the diversity observed has been generated prior to the establishment of these farms or alternatively there is a high rate of transmission between farms. The finding that present purchase policy had no influence on farm level diversity would suggest that between farm transmission is not implicated in generation of diversity thus favouring the hypothesis that much of the genetic diversity observed was generated well before the establishment of these individual farms. Sheppard *et al.* (2008) suggested that high rates of recombination between *C. jejuni* and *C. coli* are leading to a merging of the two species and this is a consequence of intensification of agriculture over the last century. Similarly we may hypothesise that much of the diversity observed amongst *C. jejuni* isolates in this study arose during a similar time frame resultant on intensification allowing increased transmission between animals and subsequent increased rates of recombination.

Chapter Eight.

Concluding Discussion.

Infectious diarrhoea is a major cause of morbidity and mortality worldwide with a devastating impact in developing countries. It is estimated that global mortality due to diarrhoea in young children is in the region of 2.5 million deaths per annum (Kosek *et al.* 2003) with almost all these deaths occurring in the developing world. *Campylobacter* spp are the major infectious cause of diarrhoea worldwide and thus a likely major contributor to this global mortality toll.

In the developed world, improvements in housing, sanitation and general health have reduced the toll of diarrhoea due to all causes but infection with *Campylobacter* spp remains an important public health issue with over 40,000 cases diagnosed in the UK in 2005. However the number of reported cases is a gross under-estimate of the actual number and it has been estimated that the actual toll is in the region of 300,000 cases annually in England and Wales (Adak *et al.* 2005) with an annual cost of approximately £70M in England (Roberts *et al.* 2003).

It is acknowledged that human campylobacteriosis is zoonotic and the major sources of human infection are domestic animals and wildlife although there is scant data on asymptomatic human carrier rates. A study of kitchen workers in Brazil (Tosin & Machado 1995) suggested a carrier rate in the region of 6% with highest rates observed in young adult males aged 20 – 35. However there are no peer reviewed accounts of carrier humans acting as primary sources of infection in outbreaks, although localised spread occurs during outbreaks e.g. between family members.

The role of poultry and poultry products in the epidemiology of human campylobacteriosis is well established but that of other sources of infection is less well defined. There is increased recognition that ruminants may play a role both via contaminated meat or dairy products and environmental contamination.

The design of effective science-based interventions to control human campylobacteriosis requires knowledge of the likely sources, the epidemiology of the agent within and between host species and the likely human exposure routes.

The advent of molecular typing methods has increased our knowledge and understanding of the epidemiology of *C. jejuni* considerably. The development of MLST and the Oxford database has been a major step forward since it allows both unequivocal identification of isolates and comparison of isolates worldwide unlike PFGE, for example, which is only applicable on a local scale. The use of MLST has proved a valuable tool for the development of source attribution models (French 2007) which are essential for effective targeting of interventions. A study

investigating source attribution methodology (McCarthy *et al* 2007) found the use of full allelic profiles to be superior to the use of sequence type alone; work is also being carried out (personal communication: Howard Leatherbarrow) investigating the potential of sequencing of the SVR of the *flaA* gene as an adjunct to MLST to further improve source attribution.

The work presented in this thesis is part of a larger collaborative study of *Campylobacter* in Lancashire with the objectives of better understanding the epidemiology of human disease. The project has 2 major components, firstly the MLST typing of 2500 human isolates collected between 2000 – 2005; secondly, a two year prospective study involving collection and MLST typing of all isolates from human cases of disease together with 1000 isolates from retail poultry and 1000 ruminant isolates. Thus the results reported here will be utilised in the development of real time source attribution models encompassing MLST sequence type data together with temporal and spatial data.

Whilst it is increasingly recognised that ruminants are a likely important source of human campylobacter infections, the transmission routes remain unclear. A number of routes are possible including ingestion of contaminated foodstuffs (as is the case with poultry), direct or indirect contact with infected animals or their faeces either through a persons work or leisure activities (farm workers, petting farms, hiking etc). Another poorly defined route of transmission is via environmental exposure although it includes contamination of water sources which is a well recognised route of infection.

One of the objectives of the present study was to investigate the role of ruminants as potential drivers of environmental exposure. For this reason, the study was designed to investigate the contribution of adult dairy cattle to environmental exposure routes since they represent the largest production group numerically in Lancashire compared to e.g. beef cattle. Adults are the largest producers of potentially contaminated faeces compared to other production groups present on a farm such as growing calves, producing upwards of 50 litres of faeces per cow daily. Sheep farms were included for two main reasons, firstly there is a paucity of data regarding sheep and campylobacters and secondly there is a relatively large sheep population in the Pennines, a popular area for leisure pursuits.

In designing the study, we wanted to describe the epidemiology of ruminant campylobacteriosis both spatially and temporally. We considered that temporal

aspects of the epidemiology were of more importance than spatial aspects since people travel widely thus infection could be acquired at some distance from where someone lived and subsequently became ill. The study design reflects this emphasis on temporal events by using repeated cross-sectional sampling of fifteen dairy farms and four sheep farms from three distinct localities. Selection of farms was via the attending veterinary practices and whilst efforts were made to avoid any biases in selection of farm e.g. by type, this study cannot be considered to be "randomised". However the farms selected were considered to be representative of dairy and sheep farms in Lancashire both in terms of stock numbers and husbandry practices. With increased lactational yield, there is a trend in UK agriculture towards all-year housing of dairy cattle in order to better meet their nutritional requirements and three of the recruited farms managed their cattle in this way; the remainder were traditionally managed with stock grazed outside during the summer and housed in winter. This was fortuitous because it meant that a recent UK management trend was represented in the study and it allowed us to quantify the effect of sampling environment on prevalence of *C. jejuni* and adjust for temporal confounding.

Previous epidemiological studies in the northwest of England of *Campylobacter* in cattle and sheep based on faecal sampling have demonstrated seasonal variation in both prevalence and bacterial counts with both being maximal during the summer months (Stanley & Jones 2003, Robinson *et al.* 2005, Kwan *et al.* 2008a).

Our study is in broad agreement but suggests that in cattle the increased prevalence observed during summer is associated with being out at pasture rather than season *per se*. However a summer increase in prevalence is also observed in sheep which are not normally housed apart from during the lambing season.

We hypothesise that in dairy cattle the increased prevalence is associated with grazing pasture rather than the consumption of ensiled forages and grain based products that occurs with housed animals. These two diets are fundamentally different in terms of carbohydrate content with high soluble sugar concentrations but relatively low starch concentrations present in grazed grass whilst ensiled forages and grains provide low levels of sugars but high starch concentrations. It is reasonable to assume that these very different diet types will impact differently on the intestinal environment and ecology.

Other studies (e.g. Garcia *et al.* 1985) have suggested higher prevalences of campylobacters in animals fed high levels of grains whilst a recent study (Krueger *et*

al. 2008) found higher campylobacter counts in grain fed compared to grass fed steers. The latter study used colony counting techniques to enumerate campylobacter concentrations. In Chapter 3 we reported a high prevalence of *Arcobacter* spp in housed dairy cattle fed ensiled forages and high levels of grain based products. The use of classical microbiological techniques for colony counting would likely be confounded by the presence of campylobacter-like organisms such as *Arcobacter* spp giving misleading results as to campylobacter numbers. Furthermore, in our study, we described associations between sampling environment and *C. jejuni* prevalence rather than prevalence of thermophillic campylobacters thus results are not strictly comparable in any case.

In her study (Robinson *et al.* 2005) the author found the presence of whole grains in faeces of young cattle to be associated with the presence of *C. jejuni*. In adult dairy cattle, the presence of whole grains in faeces is indicative of either improperly prepared grain silages in which the grains have been inadequately processed or of sub-acute ruminal acidosis (SARA), a widespread nutritional problem in many dairy herds (Grove-White 2004). In the present study all bovine faecal samples were scored both for liquid consistency and for the presence of whole grains or long fibre in faeces after sieving in cold water, a technique used by veterinarians in herd investigations of nutritional problems. No association could be demonstrated between either of these faecal measures and *C. jejuni* prevalence suggesting that in adult ruminants, at least, there is no association between the faecal pat prevalence of *C. jejuni* and presence of grains in faeces, and by implication SARA.

This possible association between *C. jejuni* prevalence and diet deserves further investigation by means of well controlled feeding trials since it might offer prospects for future interventions to reduce *C. jejuni* faecal pat prevalence and environmental load.

MLST was carried out using both the Oxford protocol and the Miller protocol. There was a marked difference in performance as indicated by the number of "repeat" PCRs required. The Miller protocol performed significantly better such that it has now been adopted as routine in our laboratory.

One thousand and three of the 2,307 *C. jejuni* isolates were selected for sequencing. Since four isolates were grown from each faecal sample and selection of isolates for sequencing was performed at isolate level rather than faecal pat level, this meant that multiple isolates from the same animal were likely to be selected on occasions.

Whilst true random selection, where each isolate has an equal probability of being selected, is usually considered the optimal strategy for epidemiological investigations, we adopted a sampling strategy involving stratification both by geographical zone and "sampling round" a proxy for time. This decision was made since firstly we performed sequencing concurrently with on-farm sampling making true random sampling impossible, and secondly we wished to describe the sequence type diversity present and felt that stratification would be beneficial in this respect.

Although selection at isolate level rather than at faecal pat level reduced the power of the study in describing the epidemiology at clonal complex level, since relatively fewer animals were included in the analyses, it offered insight into the within-animal diversity present with the observation of both multiple species and multiple sequence types in the same pat. This is an area deserving of further investigation both to better inform future study design and increase our understanding of campylobacter diversity at the within animal level.

As has been demonstrated in previous studies (Dingle *et al.* 2002, Colles *et al.* 2003, Kwan *et al.* 2008a), isolates belonging to C-C 21, C-C 61, C-C 42, C-C 45, C-C 48, C-C 206, C-C 257 & C-C 403 predominated although C-C 257 was absent from sheep. All these clonal complexes have been isolated from cases of human disease. The observation that the faecal pat prevalence of isolates belonging to C-C 45 was higher during the summer months was of particular interest since in a study of human cases in Lancashire (Sopwith *et al.* 2006), cases associated with C-C 45 were most often reported from rural areas during the summer months in particular during June and July whilst C-C 45 has also been shown to be the predominant *C. jejuni* clonal complex to be isolated from recreational surface water in the same study area (Sopwith *et al.* 2007). Work in New Zealand has indicated that the predominant genotypes isolated from surface water in dairy and sheep catchments are derived from wildlife rather than ruminants (Personal communication: Nigel French) so it may be unwise to presume a causal relationship regarding ruminant faeces and surface water contamination

MLST provides information on the processes operating at a molecular level such as recombination and mutation which together with selection determine the population structure and evolution of the organism in question. In the case of *Campylobacter jejuni* the use of MLST has demonstrated the weakly clonal population structure of the organism and provided estimates of recombination and mutation rates (Fernhead

et al. 2005) whilst a recent study using MLST demonstrated genetic exchange between *C. jejuni* and *C. coli* such that the authors predict that the two species will converge to become one species at a later date (Sheppard *et al.* 2008).

In the present study we investigated genetic diversity using three methods.

Firstly, estimates of gene flow at various levels of analysis namely species, zone, sampling environment and farm, suggested that there was no underlying heterogeneity in that populations were genetically indistinguishable. We hypothesise that much of the genetic diversity observed was generated well before the establishment of these farms as a consequence of the intensification of agriculture during the last 100 years. Recombination amongst *C. jejuni* in farm animals will be favoured by increased population sizes favouring between animal transmission and co-colonisation by multiple strains. Both herd and flock size have increased dramatically during this period particularly since 1945 when UK agriculture was encouraged to expand and intensify. This trend towards increased dairy herd size accelerated considerably in the 1980s and continues today.

Secondly, linkage analysis was performed to indirectly demonstrate the presence of linkage disequilibrium (or absence of linkage equilibrium). At all levels of analysis, the presence of linkage disequilibrium was demonstrated although there was considerable variation in the estimates of indices of association. The estimates obtained were however consistent with a weakly clonal population structure in which both mutation and recombination act to generate genetic diversity. Recombination is increasingly recognised as an important mechanism in the evolution of many pathogens allowing acquisition of potential virulence factors such as antibiotic resistance. Understanding the factors that may drive high rates of recombination could be of value in controlling future development and spread of such virulence factors – allowing us to "stay ahead of the curve."

Finally, the actual diversity present was quantified at sequence type level. Rarefaction analysis was performed to demonstrate the diversity present taking sample size into account. This suggested that there was more diversity within cattle compared to sheep although more samples would be required to show statistical significance. We suggest this may be a consequence of the greater stocking density that cattle are kept at thus increasing the likelihood of acquisition of *Campylobacters* from herdmates offering greater opportunities for recombination and thus increased diversity.

Both prevalence and diversity appeared to be greater in magnitude in cattle at pasture compared to indoors. Two hypotheses may be generated to explain this apparent association: Firstly, the increased diversity observed is resultant on the increased prevalence observed at pasture with increased recombination occurring due to there being increased numbers of campylobacters. An alternative hypothesis is that there is increased transmission from wildlife and birds to cattle when they are outside compared to when housed. The observation of increased diversity amongst isolates belonging to C-C 21 and C-C 45 compared to other clonal complexes is of particular interest in this respect since both clonal complexes are widespread amongst wildlife and birds.

There was a suggestion of apparently greater sequence type diversity amongst cattle isolates present within zones 2 & 3 compared to Zone 1. Is this a reflection of likely greater biodiversity and increased wildlife in the foothills and valleys of the Pennines compared to the southern Fylde, a low-lying intensively farmed flat grassland and vegetable growing area with relatively little woodland? It is well recognised that intensive farming and monoculture are associated with reduced biodiversity (Krebs *et al.* 1999) and latterly EU and UK government policy in areas such as the Pennines has actively supported farming practices to encourage biodiversity via various financial grants. Increased biodiversity would likely result in higher rates of acquisition of campylobacters from wildlife by cattle and sheep.

Farm purchase policy had no influence on the level of diversity suggesting that herd level diversity is generated at that level rather than being imported. This may suggest that the principles of biosecurity at farm level are irrelevant with regards to bovine colonisation by *C. jejuni* with most diversity being generated at farm level rather than by import of novel sequence types in purchased animals.

At all levels of analysis in this study, namely species level, clonal complex level and sequence type level it is apparent that after adjusting for confounding, much of the variation present resided at farm level. It could be argued that further understanding of this farm level variation is crucial for the development of strategies for reduction of ruminant derived human cases of campylobacteriosis. However whether epidemiological investigations alone have the power to tease out the nature of these "farm effects" remains open to question.

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Appendix.

Appendix A.

Lancashire longitudinal farm study

2006 - 2008

Confidential questionnaire

VTRI Fellowship in Veterinary Epidemiology

University of Liverpool.

THE UNIVERSITY
of LIVERPOOL

Farm Code ______

Farm details.

Name ______________________

Address ______________________

______

____

____

Tel. ____________

Mobile ____________

Herd details

1. Which of the following enterprises do you have. (Tick all that apply)

- Dairy ☐
- Suckler beef ☐
- Beef fattening unit ☐
- Breeding & fattening sheep ☐
- Sheep fattening only ☐

2. How many of the following animals are there on the farm?

- Dairy calves (0-6 months) ______
- Dairy heifers (6months-first calving) ______
- Dry cows ______
- Lactating cows ______
- Adult beef animals ______
- Growing beef animals ______
- Adult breeding sheep ______
- Growing sheep ______

3. Which of the following animals are also kept on the farm?

Geese ☐
Ducks ☐
Chickens ☐
Turkeys ☐
Pigs ☐
Horses ☐
Deer ☐
Dogs ☐
Cats ☐

4. Do you overwinter sheep for another farmer or farmers? Yes☐ No☐

Other Farm Enterprises

5. Do you grow any arable crops for sale Yes☐ No☐

6. What crops do you grow (give details) ____________________

7. Do you grow any vegetables Yes☐ No☐

8. What vegetables do you grow (give details) ____________________

9.Do you sell vegetables to supermarket or wholesaler Yes☐ No☐

Locally eg farmers market Yes☐ No☐

Dairy Herd

10. What is the annual milk yield per cow ______ Litres

11. What is the main breed of dairy cattle ____________

12. Do you calve all year round? Yes☐ No☐

13. If no to above

What is the main calving period? (range of months) ____________

14. Do you buy cattle in Yes☐ No☐

15. If Yes to above

How often do you buy in Frequently (> 2 times annually) ☐
Occasionally ☐

Do you buy Young stock only (< 12 months old ☐
Older stock ☐

Housing

Calving cows.

16. Where do cows calve down Individual calving pens Yes☐ No☐
Dry cow housing Yes☐ No☐

17. After calving – how long is the cow kept separate from main group

0 -12 hours ☐
13 – 24 hours ☐
25 – 48 hours ☐
2 – 4 days ☐
More than 4 days ☐

Lactating cows

18. Are your milking cows housed in

Cubicles only ☐
Cubicles & straw yards ☐
Straw yards ☐

19. If you have straw yards How often do you add more straw bedding _____________

How often do you clean yards out _____________

20. If you have cubicles How many cubicles do you have for the milking cows ________

Dry cows

21. Are your dry cows housed in

Cubicles only ☐
Cubicles & straw yards ☐
Straw yards ☐

22. If you have straw yards   How often do you add more straw bedding ______

______

How often do you clean yards out ______

______

23. If you have cubicles How many cubicles do you have for the milking cows ___

______

Feeding

Dry cows

24. How long is the average dry period in your herd ________________

25. Do you feed a "transition diet" to your close up cows   Yes☐ No☐

26. If you feed a transition diet – how long do you feed it for __________

27. If you feed a transition diet – do you use "leftovers" from the lactating group
    Yes☐ No☐

28. If you feed a transition diet – is it " custom-made" (mixed separately) for the dry cows Yes☐ No☐

29. If you feed a transition diet – do you use dry cow rolls or cobs
    Yes☐ No☐

30. Do dry cows go out to graze in the summer   Yes☐ No☐

Lactating cows

Winter Feeding

31. Which of the following systems do you use:

TMR only   Yes☐ No☐

TMR + parlour cake   Yes☐ No☐

Silage(s) at barrier + parlour cake   Yes☐ No☐

Self feed silage at face + parlour cake   Yes☐ No☐

32. Which of the following ingredients do you feed:

Grass Silage	Yes☐	No☐
Maize silage	Yes☐	No☐
Wholecrop silage	Yes☐	No☐
Alkalage	Yes☐	No☐
Hay / haylage	Yes☐	No☐
Straw	Yes☐	No☐
Other forages	Yes☐	No☐
Home mix "blend"	Yes☐	No☐
Purchased "blend"	Yes☐	No☐

33. If you feed other forages – which forages ____________________ ______________

34. If you feed other straights – which straights eg brewers grains _____ ______________

____ ______

35. Are the milking cows split into groups according to yield or stage of lactation
Yes☐ No☐

If you answered Yes – How many groups ______________

36. What water sources do cows drink from inside

Mains water	Yes☐	No☐
Borehole water	Yes☐	No☐

Summer Feeding

37. Do you turn cows out in summer Yes☐ No☐

38. When do you turn cows out ___________

39. When are cows brought back inside ___________

40. Is turn out abrupt (out in day only at first) or gradual Yes☐ No☐

41. In the summer – do you keep the cows out for 24 hours a day

High yielders	Yes☐	No☐
Low yielders	Yes☐	No☐

If you do not keep cows out all the time – how many hours daily are they out for _ _______

42. Do you buffer feed in summer Yes☐ No☐

43. If you buffer feed – do you feed

In or near the farm buildings Yes☐ No☐

From troughs in field Yes☐ No☐

From round or square feeders in field Yes☐ No☐

Grazing management

44. Which of the following do you use in summer grazing

Set Stocking Yes☐ No☐

Strip grazing Yes☐ No☐

Paddock grazing Yes☐ No☐

45. What water sources do cows drink from outside

Mains water Yes☐ No☐

Borehole water Yes☐ No☐

River or streams Yes☐ No☐

46. Do adult cows ever share grazing with younger stock (excluding heavy pregnant heifers) Yes☐ No☐

47. Do adult stock follow-on after young stock Yes☐ No☐

48. Do young stock follow-on after adult stock Yes☐ No☐

If you keep sheep or over-winter sheep:

49. Do adult cattle ever co-graze with sheep Yes☐ No☐

50. Do young stock ever co-graze with sheep Yes☐ No☐

51. Do you graze adult cattle on pasture after sheep Yes☐ No☐

52. Do you graze sheep on pasture after adult cattle Yes☐ No☐

53. Do you graze young stock on pasture after sheep Yes☐ No☐

54. Do you graze sheep on pasture after young stock Yes☐ No☐

55. Do grazing cattle (any age) have access to; (Tick all that apply)

Ponds ☐

Streams or rivers ☐

Drainage ditches ☐

None of the above ☐

Calves and Young Stock management

Baby calves

56. How long does the newborn calf stay with its dam

Snatch calving	Yes☐	No☐
Less than 24 hours	Yes☐	No☐
24 – 48 hours	Yes☐	No☐
2 – 4 days	Yes☐	No☐
More than 4 days	Yes☐	No☐

57. Are baby calves (pre-weaned) kept in

Individual pens	Yes☐	No☐
Group pens	Yes☐	No☐
Hutches	Yes☐	No☐

58. Which of the following are fed to baby calves

Milk replacer	Yes☐	No☐
Waste milk	Yes☐	No☐
Pooled colostrum	Yes☐	No☐

59. Do you feed acidified milk replacer Yes☐ No☐

60. Are calves fed ad lib Yes☐ No☐

If not fed ad-lib – how many times daily are they fed ___________

61. Are calves fed from a

Nipple ☐
Bucket ☐
Other (please specify) ____________

62. How often are buckets or nipple feeders washed

Between feeds ☐
Daily ☐
Weekly ☐
Other-please specify _______

63. How often are buckets or nipple feeders sterilised

Between feeds ☐
Daily ☐
Weekly ☐
Other-please specify _______

64. Do calves have an individual bucket/nipple that is only used for them

Yes☐ No☐

65. When is concentrate first offered to calves?

0 – 7 days ☐
8 – 14 days ☐
After 2 weeks ☐

66. When is forage first offered to calves?

0 – 7 days ☐
8 – 14 days ☐
After 2 weeks ☐

67. What type of forage is offered to calves before weaning?

Hay ☐
Silage ☐
Straw ☐
Not offered but kept on straw bedding ☐

68. What criteria are used to decide when to wean calves? (Tick all that apply)

Age ☐
Concentrate intake ☐
Body Size ☐
Convenience e.g. to make room in housing ☐
Forage intake ☐

69. What is average age of weaning ____________

70. How are calves weaned? (Tick all that apply)

Abruptly ☐
Gradual reduction in amount ☐
Gradual dilution of milk ☐
Gradual increase in feeding interval ☐

71. Are calves grouped:

Before weaning ☐
After weaning ☐
On the same day as weaning ☐

72. What type of calf concentrate is fed after weaning

Coarse mix ☐
Pellet ☐
Both ☐

73. Is left over TMR fed to unweaned calves?

Often ☐
Occasionally ☐
Never ☐

74. Are calves fed milk or replacer at floor level? All ☐ Some_% None☐

75. Are unweaned calves fed concentrate at floor level? All ☐ Some_% None☐

76. Are unweaned calves fed forage at floor level? All ☐ Some_% None☐

Post-Weaning up to 6 months old

77. What is the average group size when housed inside __________

78. What type of forage is fed to calves after weaning

Hay ☐
Silage ☐
Straw ☐
Not offered but on straw bedding ☐

79. What type of concentrate is fed?

Coarse mix ☐
Pellet ☐
Both ☐

80. Is left over TMR fed to weaned calves

Often ☐
Occasionally ☐
Never ☐

81. Are weaned calves fed concentrate at floor level? All ☐ Some_% None☐

82. Are weaned calves fed forage at floor level? All ☐ Some_% None☐

Feeding of heifers over 6 months

83. What is the average group size when housed inside __________

84. Which of the following types of forage are fed? (Tick all that apply)

Grass silage ☐
Maize silage ☐
Whole crop silage ☐

85. Is concentrate fed Yes☐ No☐

86. If concentrate is fed is it pelleted ☐ coarse mix ☐

87. In summer, are growing heifers outside grazing Yes☐ No☐

88. Do they graze pastures previously (within last month) grazed by adult cows Yes☐ No☐

89. Do they ever co-graze with sheep Yes☐ No☐ Occasionally ☐

90. Do they graze pastures previously (within last month) grazed by sheep Yes☐ No☐ Occasionally ☐

Silage making

91. Do you make

Big bale ☐
Silo ☐
Both ☐

92. Which fields are used to make grass silage? (Tick all that apply)

Dedicated leys never grazed by cattle ☐
Dedicated leys occasionally grazed by cattle ☐
Mixed use pasture ☐

Food storage

93. Where do you store "blends" or "straights"

Storage bins Yes☐ No☐
Loose on floor Yes☐ No☐
Sacks Yes☐ No☐
Other (please state) ____________

Water

94. What is the farm's water supply?

Mains ☐
Spring ☐
Borehole ☐

95. How is domestic sewage dealt with?

Mains sewage ☐
Septic tank ☐
Cesspit ☐

96. Are water troughs in housing routinely cleaned? Yes☐ No☐

Pasture management

97. Do grazing cattle have access to; (Tick all that apply)

Ponds? ☐
Streams? ☐
Drainage ditches? ☐
None of the above ☐

98. Are wild birds often seen on pasture? Yes☐ No☐

99. Are any of the following seen in large numbers on your farm

Geese Yes☐ No☐
Ducks Yes☐ No☐
Seagulls Yes☐ No☐
Rooks / crows Yes☐ No☐
Starlings Yes☐ No☐
Pigeons Yes☐ No☐

Others ____________________

Slurry Management

Slurry

100. For how long can you store slurry in winter before tank / pit is full ________
______

101. Do you aerate slurry Yes☐ No☐
102. Do you use any "slurry treatment" Yes☐ No☐

If Yes: please give details ________

103. Is slurry spread on land used for grazing? Yes☐
No☐

104. Is slurry spread on land used for silage making? Yes☐
No☐

105. Is slurry spread on land used for arable crops fed to cattle e.g. maize etc
Yes☐ No☐

106. At what point after application of slurry are cattle grazed
- Once it appeared clear of manure/slurry ☐
- After a period of rain ☐
- After a set interval ☐ how long ________
- Don't know ☐

107. At what point after application of slurry is silage taken ?
- Once it appeared clear of manure/slurry ☐
- After a period of rain ☐
- After a set interval ☐ how long ________
- Don't know ☐

108. How long is slurry stored before spreading (shortest period) ____________
______

109. Is slurry agitated before spreading?
- Always ☐
- Sometimes ☐
- Never ☐

110. How is slurry spread? (Tick all that apply)
- Tanker ☐

Irrigator ☐
Umbilical cord ☐
Injected ☐
Spreader ☐

Manure

111. Is manure spread on land used for grazing? Yes☐ No☐

112. Is manure spread on land used for silage making? Yes☐ No☐

113. Is manure spread on land used for arable crops fed to cattle Yes☐ No☐

114. At what point after application of manure are cattle grazed?
Once it appeared clear of manure/slurry ☐
After a period of rain ☐
After a set interval ☐ how long ________
Don't know ☐

115. At what point after application of manure was silage taken?
Once it appeared clear of manure/slurry ☐
After a period of rain ☐
After a set interval ☐ how long ________
Don't know ☐

116. How long is manure stored before spreading (shortest period) ______
____

Sewage

117. Is sewage spread on the farm? Yes☐ No☐

118. How often is it spread on land used for grazing? ______
____

119. How often is it spread on land used for silage making? ____________
______

120. How often is it spread on land used for arable crops fed to cattle? ______
____

121. At what point after application of sewage are cattle grazed?

Once it appeared clear sewage ☐
After a period of rain ☐
After a set interval ☐ how long ________
Don't know ☐

122. At what point after application of sewage is silage taken?

Once it appeared clear of manure/slurry ☐
After a period of rain ☐
After a set interval ☐
Don't know ☐

Other waste

123. Is any other type of waste spread on the farm? Yes☐ No☐

What type ______________________

124. How often is it spread on land used for grazing? ____________________

125. How often is it spread on land used for silage making? ____________________

126. How often is it spread on land used for arable crops fed to cattle? ____________________

127. At what point after application of the waste are cattle grazed?

Once it appeared clear of waste ☐
After a period of rain ☐
After a set interval ☐ how long ________
Don't know ☐

128. At what point after application of the waste was silage taken?

Once it appeared clear of waste ☐
After a period of rain ☐
After a set interval ☐ how long ________
Don't know ☐

Pest Control

129. Do you notice rodents around the farm?

Often ☐
Rarely ☐
Never ☐

130. Do you notice wild birds in the buildings?

Often ☐
Rarely ☐
Never ☐

131. What types of birds ____

132. Is active rodent control carried out on the farm? Yes☐ No☐

Sheep management (only to be answered if you have a sheep enterprise)

133.Number of breeding ewes ________________

134. Main breed of ewe ___________

135. How many lambs do you sell each year (approximately) _______________

136. Which period do you sell the majority of lambs: From___________ to ___ _____

137. Do you ever buy in store lambs for fattening Yes☐ No☐

138. When is your lambing season: From_______ to _________

139. Do you lamb indoors Yes☐ No☐

140. If you lamb indoors – how many days after the lamb is borne do you put the ewe & lamb outside _____

141. When do you wean the lambs (month) ___________

142. Do sheep graze over the same pastures as cattle Yes☐ No☐

143. Do any of your sheep graze away from your farm for any period Yes☐ No☐

If Yes: Please give details ________________________________

Do you have any other comments on your farming practices ____________

____

____

____

____

____

____

____

Appendix B.

(Farm visit details form)

Farm ID __________ Visit Code ________

Zone _______ Date _______

Species sampled __________

Cows sampled

Management group sampled ____________ Where sampled ___________

Who is co-grazing in field ______________

Date of turn-out __________ OR Date of housing ______

Number of pats (cows) sampled _________ Number of animals in group _____

Length of time in the sampled environment ____________

Number of cows calved in last 4 weeks __________

Average daily milk yield (total daily production/ number lactating cows) _______

Nutrition

Type of feeding system _________________________________

Feeding changes in last 3 weeks Y N ___________________________

Slurry (only applicable if animals outside)

Has slurry been put on this pasture in last 2 months Y N

If Yes : Date of slurry application ______

How was it applied _____________

Is slurry treated in any way ____

Sheep sampled

Types of animals in sampled group: Ewes & lambs (baby)

Ewes & lambs (fattening)

Ewes only

Fattening lambs only

Type of samples taken ________________

Where sampled ________________

Length of time in the sampled environment ____________

Number of pats sampled _________

If outside Type of pasture: Rough____ mediocre _____ lush ______

Number of sheep (total) ____

Area (ha) __________ Approx stocking density ____

House hygiene (circle) Excellent Good Mediocre Poor Filthy

Is concentrate fed Y N

Any medication (Type?) given in last 2 weeks (type) Y N ___________

Appendix C.

(MLST Protocol)

Defra/VTRI Sequencing Unit

Campylobacter MLST Protocol (manual method)

Version 2

May 2005

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Extract Preparation

Reagents/Equipment required -

sterile phosphate buffered saline (PBS)
Sterile 1.5ml microcentrifuge tubes
Sterile plastic loops
Dry block heater (Grant UBD4)
Microcentrifuge
Storage racks
Freshly grown *Campylobacter* (or beads)

1. Switch block heater on and set temperature to 100°C.
2. Dispense 150µl sterile PBS into sufficient 1.5ml tubes; label tubes with unique identifier (usually culture collection number).
3. Add *either* a light inoculum of bacteria *or* 1 or 2 beads to each labelled tube (SINGLE BEAD IS THE PREFERRED OPTION).
4. Mix by vortexing.
5. Incubate in block heater at 100°C for 15 minutes.
6. Centrifuge at 13000rpm for 10 minutes.
7. Freeze at -20°C until required. Store at 4°C when thawed.

DO NOT VORTEX BEFORE USE. IF PELLET IS DISTURBED, RE-CENTRIFUGE BEFORE USE.

PCR - reactions

Reagents/Equipment required -

PCR reagents (dNTPs [10mM], 10x buffer, $MgCl_2$ [25mM], *Taq* DNA polymerase [$5U\mu l^{-1}$], primers [10µM], molecular grade H_2O)
96-well non-skirted microtitre plates (AbGene)
Adhesive PCR film (AbGene)
DNA extracts
Thermal cycler
Centrifuge with microtitre plate rotor
Plate vortexer

1. In Excel create a spreadsheet to indicate which DNA isolate will be in each well of the half-microtitre plate. Use the blank form "Blank batch form.xls", and fill in the blank fields as appropriate. This sheet will act as a sample tracking sheet throughout the MLST process. Remember to include a negative control. Assign unique identifier to the plate in the format : MLST PCR XXX *A & B*, where *XXX* is the unique PCR batch number, A is the locus on the left hand side of the plate, and B is the locus on the right hand side of the. Record in the comments box if primers used differed from the standard set (Appendix I). Save a copy as the batch name i.e. MLST PCR XXX.

2. Mix PCR reagents together (Master mix) in the following quantities;

	1x Master Mix	52x Master Mix
Sigma molecular grade H_2O	36.75µl	1911µl
10x buffer	5.0µl	260µl
$MgCl_2$ (25mM)	3.0µl	156µl
dNTPs (10mM stock)	1.0µl	52µl
Forward primer (10µM stock)	1.0µl	52µl
Reverse primer (10µM stock)	1.0µl	52µl
Taq polymerase (5 units/µl)	0.25µl	13µl

3. Aliquot 48 µl master mix per microtitre well and tap plate gently to ensure liquid is in the bottom of the well. Pipette 2 µl DNA (boiled lysate) onto the side of each well as per plate layout created in Excel.

4. Gently tap plate to move DNA to well bottom and carefully seal the plate with adhesive film, paying particular attention at the edges. Vortex and spin plate briefly at 500 rpm.

5. Place plate in thermal cycler and load program with the following conditions;

C. jejuni isolates / primers;

95 °C for 3 min
94 °C for 20 sec
50 °C for 20 sec } X 25
72 °C for 1 min
72 °C for 5 min
4 °C forever.

C. coli isolates / primers;

95 °C for 3 min
94 °C for 30 sec
50 °C for 30 sec } X 25
72 °C for 1 min
72 °C for 5 min
4 °C forever.

6. Mix 5 µl of each sample with 1 µl 6x loading buffer and load into wells of a 2% agarose gel containing ethidium bromide 0.5 µg / ml. Electrophorese at about 120 V for 20 min and visualise DNA on a U.V. transilluminator.

The method can be halted here indefinitely, with products being stored at 4°C for up to 2 weeks, or at -20°C for indefinite storage.

See APPENDIX I – Miller Primers for alternative reactions for both *C.jejuni* and *C.coli*.

PCR product clean-up (PEG precipitation)

1. Aliquot 60 µl 20% (w/v) PEG_{8000}, 2.5M NaCl per well, using a multichannel pipette, seal wells with adhesive film, vortex and briefly spin the plate at 500 rcf to ensure mix is at the bottom of the wells. Incubate the plates for either 15 min at 37 °C, 30 min at 20 °C or overnight at 4 °C. (Longer incubations do not have a detrimental effect on the clean up procedure).

2. Spin at 2750 rcf at 4 °C for 60 min.

3. To remove PEG, place folded blue tissue into the bottom of the centrifuge plate holders and gently invert the plate onto blue tissue. Spin at 500 rpm for 60 sec.

4. Wash pellet twice with 150 µl 70% ice-cold ethanol. i.e. add 150 µl per well and spin at 2750 rcf for 10 min. Remove ethanol by inversion of plate onto blue tissues, and then spin inverted plate on folded clean blue at 500 rpm for 60 sec. Repeat.

5. Air dry plate on bench for 10 min.

6. Re-suspend pellet in STERILE milliQ water. Re-suspension volume is dependant on intensity of PCR product observed following PCR e.g. Barely visible products are re-suspended in 5 µl with more intense products re-suspended in volumes up to 50 µl. Volumes for each locus batch are determined with reference to intensity of product band on gel image. Seal lid carefully, vortex and spin briefly.

7. Resuspended products can be stored long-term at -20°C, or short-term at 4°C.

Sequencing reactions

1. Create a speadsheet in Excel to indicate which isolate/primers will be in which wells, such that the PCR product from well A1 will be in A1 and A2, the forward primer will be A1 and the reverse in A2. PCR product from A2 in A3 and A4 etc, according to the sequence plate pipetting guide sheet in Appendix VII. The name of the isolate is unimportant but the extension must be in the format indicated. (*pgm* and *tkt* are expressed as eg. pgm_1 and tkt_1). Use template "Sequencing plate template (aspA).xls". Assign a unique identifier in the format : VTRI_XXX*loc*_dd.mm.yy, where XXX is the unique batch number, *loc* is the locus to be sequenced, and *dd.mm.yy* is the date on which the sequencing reaction was run. Save a copy as the run name. Remember to fill in all the appropriate fields. For subsequent loci, use the "replace" function in Excel to change the locus name, and save a copy under the name of that locus.

	1	2	3	4	5	6	7	8	9	10	11	12
A	806.aspA1	806.aspA2	869.aspA1	869.aspA2	1030.aspA1	1030.aspA2	1200.aspA1	1200.aspA2	1267.aspA1	1267.aspA2	1431.aspA1	143
B	808.aspA1	808.aspA2	875.aspA1	875.aspA2	1062.aspA1	1062.aspA2	1202.aspA1	1202.aspA2	1280.aspA1	1280.aspA2	1434.aspA1	143
C	809.aspA1	809.aspA2	882.aspA1	882.aspA2	1075.aspA1	1075.aspA2	1209.aspA1	1209.aspA2	1291.aspA1	1291.aspA2	1491.aspA1	149
D	815.aspA1	815.aspA2	892.aspA1	892.aspA2	1079.aspA1	1079.aspA2	1210.aspA1	1210.aspA2	1293.aspA1	1293.aspA2	1495.aspA1	149
E	818.aspA1	818.aspA2	912.aspA1	912.aspA2	1094.aspA1	1094.aspA2	1212.aspA1	1212.aspA2	1310.aspA1	1310.aspA2	1506.aspA1	150
F	825.aspA1	825.aspA2	920.aspA1	920.aspA2	1190.aspA1	1190.aspA2	1218.aspA1	1218.aspA2	1417.aspA1	1417.aspA2	1540.aspA1	154
G	834.aspA1	834.aspA2	923.aspA1	923.aspA2	1192.aspA1	1192.aspA2	1219.aspA1	1219.aspA2	1418.aspA1	1418.aspA2	1558.aspA1	155
H	850.aspA1	850.aspA2	935.aspA1	935.aspA2	1196.aspA1	1196.aspA2	1221.aspA1	1221.aspA2	1423.aspA1	1423.aspA1	blank	blan

2. Open the file **plate3700.xls** and enable macros. Copy and paste the data from the above spreadsheet (excluding cell letter/number) into it and select the Save cell. Save as a .txt file. This is required as a list file for STARS to rename the data prior to analysis. Do NOT save changes to plate3700.xls.

3. Make up master mix in required volume. Make two batches of 50 aliquots per sequencing plate :

	1x Master Mix	50x Master Mix
Molecular grade H_2O	2.38 µl	119 µl
5x buffer	1.87 µl	93.5 µl
Big Dye	0.25 µl	12.5 µl
Primer (forward OR reverse) [0.67µM]	4 µl	200 µl

N.B. Sequencing primers are at 0.67µM i.e. 1:15 dilution of PCR primer concentration (see Appendix I). Sequencing primers are not necessarily the same as the PCR primers.

4. Add 8.5µl of master mix containing forward primer to wells of columns 1,3,5,7,9 and 11; 8.5µl of master mix containing reverse primer to wells of columns 2,4,6,8,10 and 12

5. Pipette 1.5 µl of the first PCR product onto the side of wells A1 and A2. Repeat for remainder of wells as per plate layout. Spin briefly to move DNA template to bottom of wells.

6. Place plate in thermal cycler and load program with the following conditions;

   96 °C for 10 sec }
   50 °C for 5 sec } X 30
   60 °C for 2 min }
   4 °C forever.

7. Do not stop at this point. Proceed immediately to precipitation unless sequencing reaction runs overnight.

Sequencing reaction clean-up (ethanol precipitation)

a. Per plate mix 7000 μl 100% ethanol and 280 μl 3M sodium acetate and aliquot 52 μl per well.

b. Replace adhesive film, vortex and briefly spin (500 rpm). Incubate at room temp for 45 min and spin at 2750 rcf (4 °C) for 1 h.

c. Remove adhesive film and gently invert plate onto absorbent tissue. Spin inverted plate on fresh tissue (500 rpm) for < 1min.

d. Wash pellet once by addition of 150 μl ice-cold 70% ethanol per well, cover plate with film and spin at 2750 rcf for 10 min.

e. Remove adhesive film, invert plate onto absorbent tissue and give a final short inverted spin at 500 rpm.

f. Air dry at room temp for 10 minutes. Recover plate with adhesive film and store at –20 °C prior to sequencing (MAXIMUM 72 hours).

g. Aliquot 10μl HiDi (formamide) per well, vortex and briefly spin.

h. Heat denature 2mins at 94°C. Allow to cool, remove film, and load plate onto sequencer.

Reactions in tubes (repeats)

PCR

1. To carry out MLST PCR in tubes use a 0.2 ml thin-walled tube and use the same reaction mixture, quantity and thermal cycler conditions as for a 96-well plate.

2. Run 5 μl out on a gel.

3. To PEG precipitate; add 50 μl water to each tube and transfer total volume to a 1.5 ml tube. Then add 60 μl PEG / NaCl, vortex, incubate as for plates and spin at 13000g for 30 min.

4. Pipette off PEG and wash once with 500 μl 70% ethanol (13000g 10 min). Air dry and resuspend as per usual.

SEQUENCING

1. Use 0.2 ml tubes and set up sequencing reactions as per 96 well plate.

2. Add 10 μl water per tube and transfer reactions to1.5 ml tubes. Add 52 μl ethanol/Na acetate, incubate as per plates and spin 13000g for 30 min.

3. Wash once with 70% ethanol as above.

APPENDIX I – Routine primer sets

aspA	(aspatase)
glnA	(glutamine synthetase)
gltA	(citrate synthase)
glyA	(serine hydroxy methyl transferase)
pgm	(phospho glucomutase)
tkt	(transketolase)
uncA	(ATP synthase alpha subunit)

All primers must be made up in hood.

PCR primers : resuspend as per data sheet (100µM), then dilute 1:10 to provide MLST PCR working stock (10µM).

Sequencing primers : resuspend as per PCR primers (to 10µM), the dilute a further 1:15 to give working sequencing primer stock (0.67µM)

PCR (*C.jejuni*)

***C. jejuni* PCR Primers;**

Locus	Primer	Sequence (5' - 3')	Product Size (bp)
aspA	aspA9 (forward)	5'- AGT ACT AAT GAT GCT TAT CC -3'	941
	aspA10 (reverse)	5'- ATT TCA TCA ATT TGT TCT TTG C -3'	
glnA	glnA1	5'- TAG GAA CTT GGC ATC ATA TTA CC -3'	1305
	glnA2	5'- TTG GAC GAG CTT CTA CTG GC -3'	
gltA	gltA1	5'- GGG CTT GAC TTC TAC AGC TAC TTG -3'	1112
	gltA2	5'- CCA AAT AAA GTT GTC TTG GAC GG -3'	
glyA	glyA1	5'- GAG TTA GAG CGT CAA TGT GAA GG -3'	1052
	glyA2	5'- AAA CCT CTG GCA GTA AGG GC -3'	
pgm	pgmS5	5'- GGT TTT AGA TGT GGC TCA TG -3'	680
	pgmS2	5'- TCC AGA ATA GCG AAA TAA GG -3'	
tkt	tktS5	5'- GCT TAG CAG ATA TTT TAA GTG -3'	692
	tktS4	5'- ACT TCT TCA CCC AAA GGT GCG -3'	
uncA	uncAS5	5'- TGT TGC AAT TGG TCA AAA GC -3'	631
	uncAS4	5'- TGC CTC ATC TAA ATC ACT AGC -3'	

Sequencing (*C.jejuni*)

***C. jejuni* Sequencing Primers;**

Locus	Primer	Sequence (5' - 3')
aspA	aspS3 (forward)	5'- CCA ACT GCA AGA TGC TGT ACC AGC -3'
	aspS6 (reverse)	5'- TTC ATT TGC GGT AAT ACC ATC -3'
glnA	glnS1	5'- GCT CAA TTC ATG CAT GGC -3'
	glnS6	5'- TTC CAT AAG CTC ATA TGA AC -3'
gltA	gltS1	5'- GTG GCT ATC CTA TAG AGT GGC -3'
	gltS6	5'- CCA AAG CGC ACC AAT ACC TG -3'
glyA	glyS3	5'- AGC TAA TCA AGG TGT TTA TGC GG -3'
	glyS4	5'- AGG TGA TTA TCC GTT CCA TCG C -3'

pgm	pgmS5	5'- GGT TTT AGA TGT GGC TCA TG -3'
	pgmS2	5'- TCC AGA ATA GCG AAA TAA GG -3'
tkt	tktS5	5'- GCT TAG CAG ATA TTT TAA GTG -3'
	tktS4	5'- ACT TCT TCA CCC AAA GGT GCG -3'
uncA	uncAS5	5'- TGT TGC AAT TGG TCA AAA GC -3'
	uncAS4	5'- TGC CTC ATC TAA ATC ACT AGC -3'

PCR & Sequencing (*C.coli*)

***C. coli* PCR and Sequencing Primers;**

Locus	Primer	Sequence (5' - 3')
aspA	aspcoli S1 (forward)	5'- CAA CTT CAA GAT GCA GTA CC -3'
	aspcoli S2 (reverse)	5'- ATC TGC TAA AGT ATG CAT TGC -3'
glnA	glncoli S1	5'- TTC ATG GAT GGC AAC CTA TTG -3'
	glncoli S2	5'- GCT TTG GCA TAA AAG TTG CAG -3'
gltA	gltcoli S1	5'- GAT GTA GTG CAC TTT TAC TC -3'
	gltcoli S2	5'- AAG CGC TCC AAT ACC TGC TG -3'
glyA	glycoli S1	5'- TCA AGG CGT TTA TGC TGC AC -3'
	glycoli S2	5'- CCA TCA CTT ACA AGC TTA TAC-3'
pgm	pgmcoli S1	5'- TTA TAA GGT AGC TCC GAC TG -3'
	pgmcoli S2	5'- GTT CCG AAT AGC GAA ATA ACA C -3'
tkt	tktcoli S1	5'- AGG CTT GTG TTT TCA GGC GG -3'
	tktcoli S2	5'- TGA CTT CCT TCA AGC TCT CC -3'
uncA	unccoli S1	5'- AAG CAC AGT GGC TCA AGT TG -3'
	unccoli S2	5'- CTA CTT GCC TCA TCC AAT CAC -3'

flaA locus

***flaA* locus primers;**
Same primer sets used for both *C. jejuni* and *C. coli* isolates.

Locus	Primer	Sequence (5' - 3')
flaA 321bp	fla4F (forward)	5'- GGA TTT CGT ATT AAC ACA AAT GGT GC -3'
	fla625R (reverse)	5'- CAA G(AT)C CTG TTC C(AT)A CTG AAG -3'
	fla242FU	5'- CTA TGG ATG AGC AAT T(AT)A AAA T-3'

Stock primers are kept at 100 μM (100 pmol/μl) and diluted 1:10 for use in PCR and further diluted 1:15 for use in sequencing reactions (0.67 μM).

Miller primers (*C.jejuni* & *C.coli*)

Locus	Primer	Sequence (5' - 3')
aspA	aspAF1 (forward)	5'- GAGAGAAAAGCWGAAGAATTTAAAGAT -3'
	aspAR1 (reverse)	5'- TTTTTTCATTWGCRSTAATACCATC -3'
glnA	glnAF	5'- TGATAGGMACTTGGCAYCATATYAC -3'
	glnAR	5'- ARRCTCATATGMACATGCATACCA -3'
gltA	gltAF	5'- GARTGGCTTGCKGAAAAYAARCTTT -3'
	gltAR	5'- TATAAACCCTATGYCCAAAGCCCAT -3'
glyA	glyAF	5'- ATTCAGGTTCTCAAGCTAATCAAGG -3'
	glyAR	5'- GCTAAATCYGCATCTTTKCCRCTAAA -3'
pgm	pgmF1	5'- CATTGCGTGTDGTTTTAGATGTVGC -3'
	pgmR1	5'- AATTTTCHGTBCCAGAATAGCGAAA -3'
tkt	tktF1	5'- GCAAAYTCAGGMCAYCCAGGTGC -3'
	tktR	5'- TTTTAATHAVHTCTTCRCCCAAAGGT -3'
uncA (atpA)	atpAF	5'- GWCAAGGDGTTATYTGTATWTATGTTGC -3'
	atpAR	5'- TTTAADAVYTCAACCATTCTTTGTCC -3'

The above primers are described as "degenerate primers" i.e a mixture of primers differing at specific bases. Coding is as follows

R=A+G K=G+T H=A+T=C N=A+C+T+G Y=C+T

S=G+C B=G+T+C M=A+C W=A+T V=G+A+C D=G+A+T

Amplification reactions are performed in 50µl volumes comprising 2µl of DNA lysate and 48µl of mastermix, using a programmable thermal cycler (ABI 20720: Applied Biosystems) as follows:

Mastermix:

dNTPs (10mM of each)	1.0 µl
10x PCR buffer	5.0µl
$MgCl_2$ 25mM	4.5µl
Primers (10µM) – each	5.0µl
Taq polymerase ($5U\mu l^{-1}$)	0.25µl

Reactions Conditions:

- initial heating for 3 minutes at 95^{0}C followed by:
- 35 amplification cycles consisting of
  - 20 seconds at 94^{0}C (denaturation)
  - 20 minute at 50^{0}C (primer annealing)
  - 1 minute at 72^{0}C
- A final elongation step (72^{0}C for 5 minutes) following the final amplification cycle.

Gel electrophoresis, precipitation, re-suspension and sequencing reaction conditions were identical to those used in the Oxford protocol (apart from choice of sequencing primers).

APPENDIX II – Alternate primer sets

If no alternates are listed, then the sequencing primers can be used for PCR.

PCR (*C.jejuni*)

Locus	Primer (Forward)		Primer (Reverse)	
aspA	A1	AAAGCTGCAGCTATGGC	A2	AAGCGCAATATCAGCCACTC
	A3	ATGAGGTTTATTATGGAGTGC	A4	CCTCTTTGGCTATAGAAGCTG
tkt	A1	TTTAAGTGCTGATATGGTGC	A4	CATAGCCTGTTCTCTGATACC
	A3	GCAAACTCAGGACACCCAGG	A6	AAAGCATTGTTAATGGCTGC
pgm	A1	TTGGAACTGATGGAGTTCG	A2	AAGAGCTTAATATCTCTGGCTTCTAG
	A3	TCAGGGCTTACTTCTATAGG	A4	AGCTTAATATCTCTGGCTTC
	A7	TACTAATAATATCTTAGTAGG	A8	CACAACATTTTTCATTTCTTTTTC
uncA	A3	AAAGCTGATGAGATCACTTC	A2	GCTAAGCGGAGAATAAGGTGG
	A9	ATGGACTTAAGAATATTATGGC	A4	ATTCTTTGTCCACGTTCAAG
			A8	ATAAATTCCATCTTCAAATTCC

Sequencing (*C.jejuni*)

Locus	Primer (Forward)		Primer (Reverse)	
glnA	S3	CATGCAATCAATGAAGAAAC	S4	GCATACCATTGCCATTATCTCCG
gltA	S3	CTTATATTGATGGAGAAAATGG	S8	TGCTATACAGGCATAAGGATG
tkt	S1	TGCACCTTTGGGCTTAGC	S6	AAGCCTGCTTGTTCTTTGGC
pgm	S3	GCTTATAAGGTAGCACCTACTG		
uncA	S3	AAAGTACAGTGGCACAAGTGG		

APPENDIX III - routine reagent recipes

20% PEG_{8000}/2.5M $NaCl_2$ - 200g PEG_{8000}
146.1g $NaCl_2$

Make up to 1l in Duran bottle with distilled H_2O.

Put in magnetic stirrer and leave to stir overnight.

3M sodium acetate - 24.6g sodium acetate in 100ml of distilled H_2O.

APPENDIX IV - allele start/stop sequences

Locus	Allele length	5' (START)	3' (STOP)
aspA	477bp	ATGATAGGT	
	CAGTTGTA		
glnA	477bp	GATCCTTTT	TGACAATGTT
gltA	402bp	GAGCTTAAAAA GAAATGAAAAA	AACAGTTCGT
glyA	507bp	GGTGGACATT	
	GAACCAAT		
pgm	498bp	GGGCTTAT GGACTTAAT	TTTTAATCAAT
	TTTTAACCAAT		
		GGTTTAAATAT GGACTTAATAT	TTTTGCATAAT
tkt	459bp	TTACATTTAAG TTGCATTTAAG	CAAGCTAAA
uncA	489bp	GCCGGTGC GCTGGTGC	CCTTGCTCAA GACCTTGCTCA
flaA	321bp	AAAGCAACT	
	TTTTCAGTTC		
		AAGGCAACT	
	TTTTAAATTT		
			TTTCAATTT TTTAAATTC TTTTCAGTTT

Appendix D.

(eBURST diagrams for clonal complexes.)

The key to the following eBURST diagrams is given below:

Key:

(blue box)	**group founder ST**
(yellow box)	**Sub-group founder ST**
O	**Previously unreported ST**
O	**Size of circle is proportional to number of isolates**

Appendix D Figure 1. eBurst diagram of C-C 21 isolates from cattle and sheep in Lancashire.

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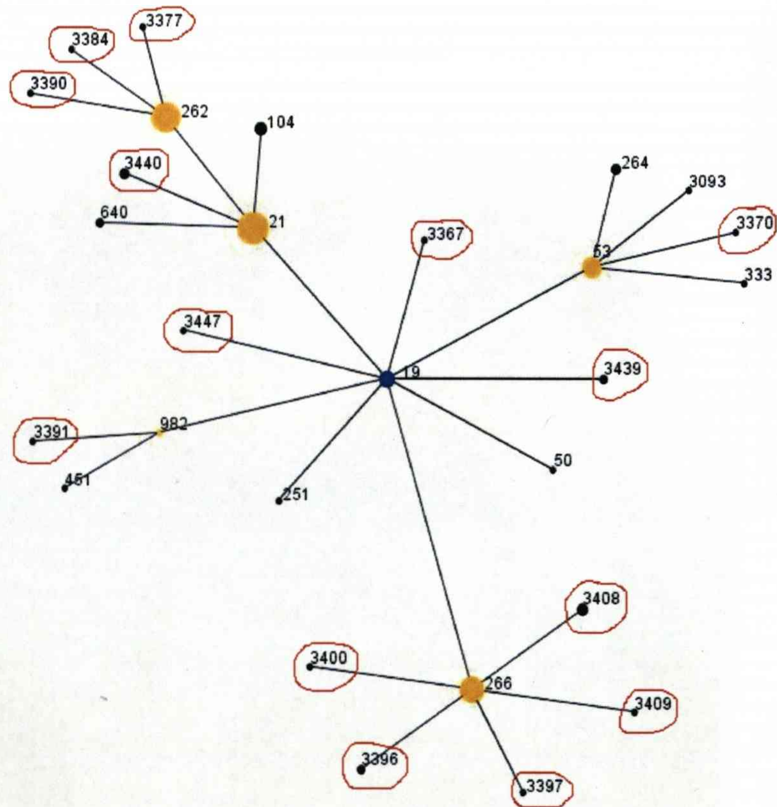
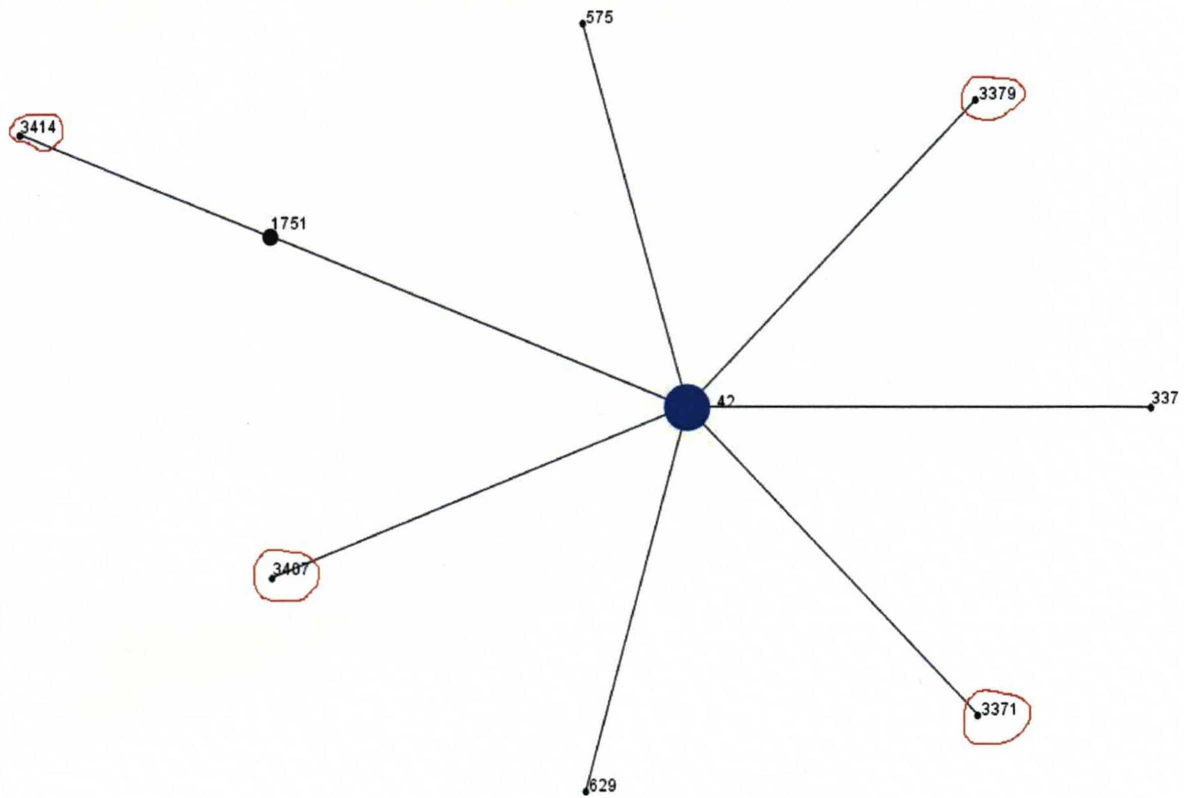
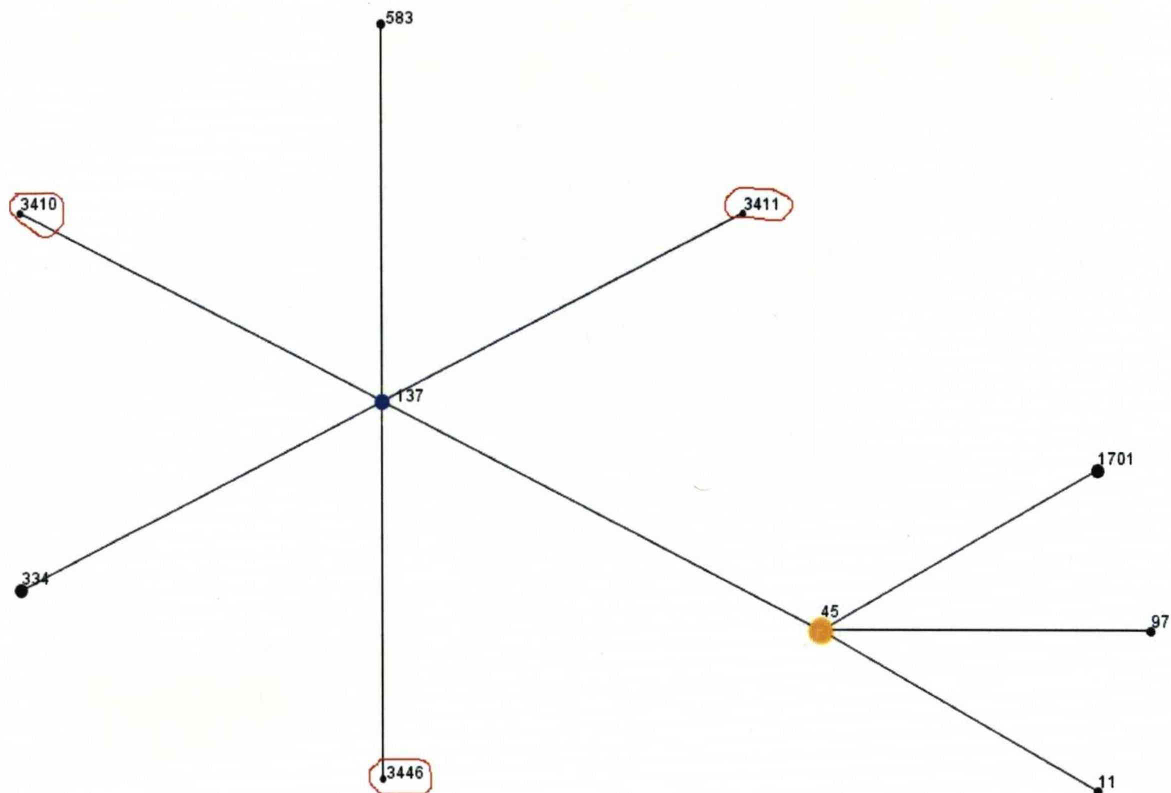


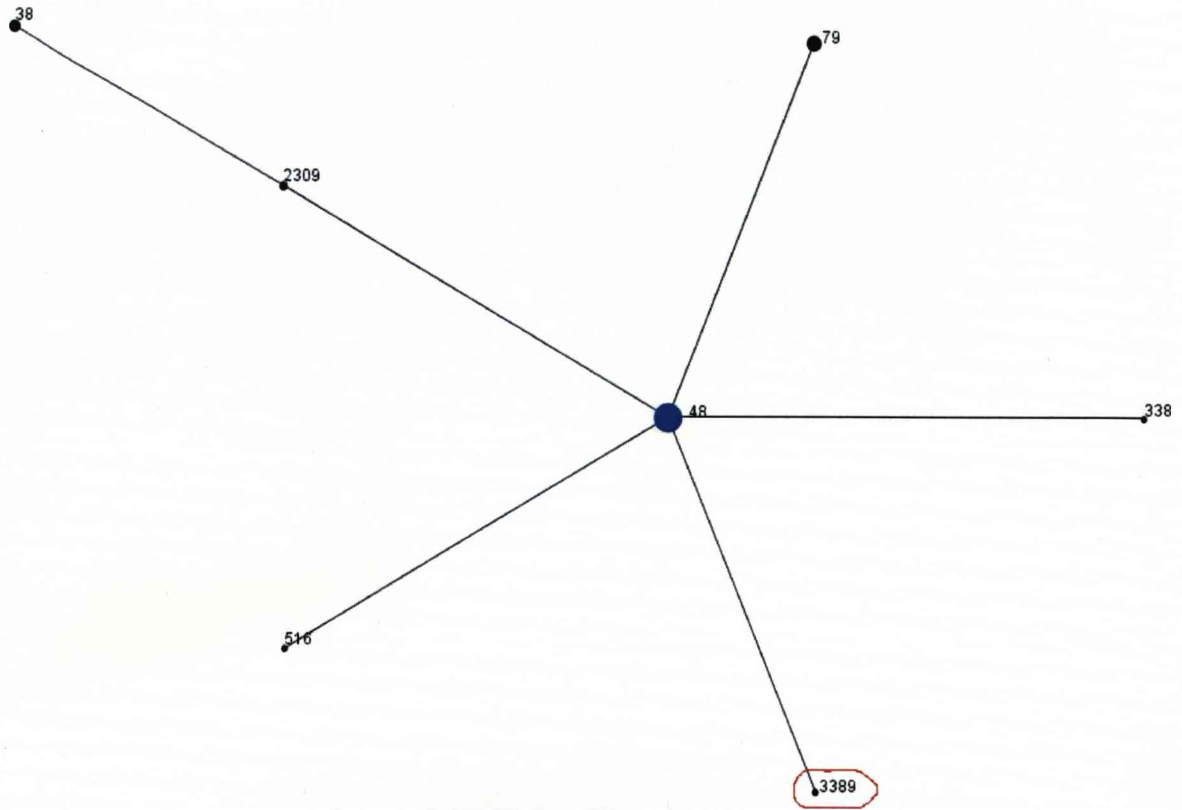
Appendix D Figure 2. eBurst diagram of C-C 42 isolates from cattle and sheep in Lancashire.



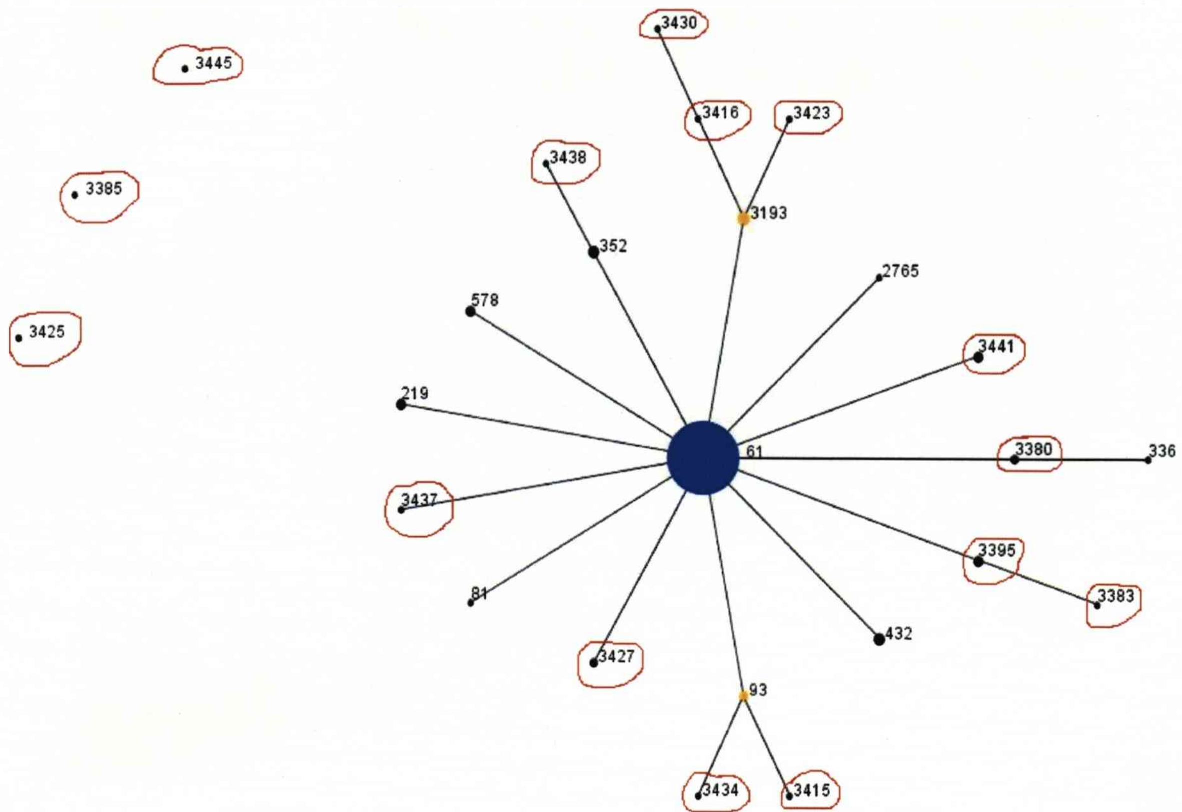
Appendix D Figure 3. eBurst diagram of C-C 45 isolates from cattle and sheep in Lancashire.



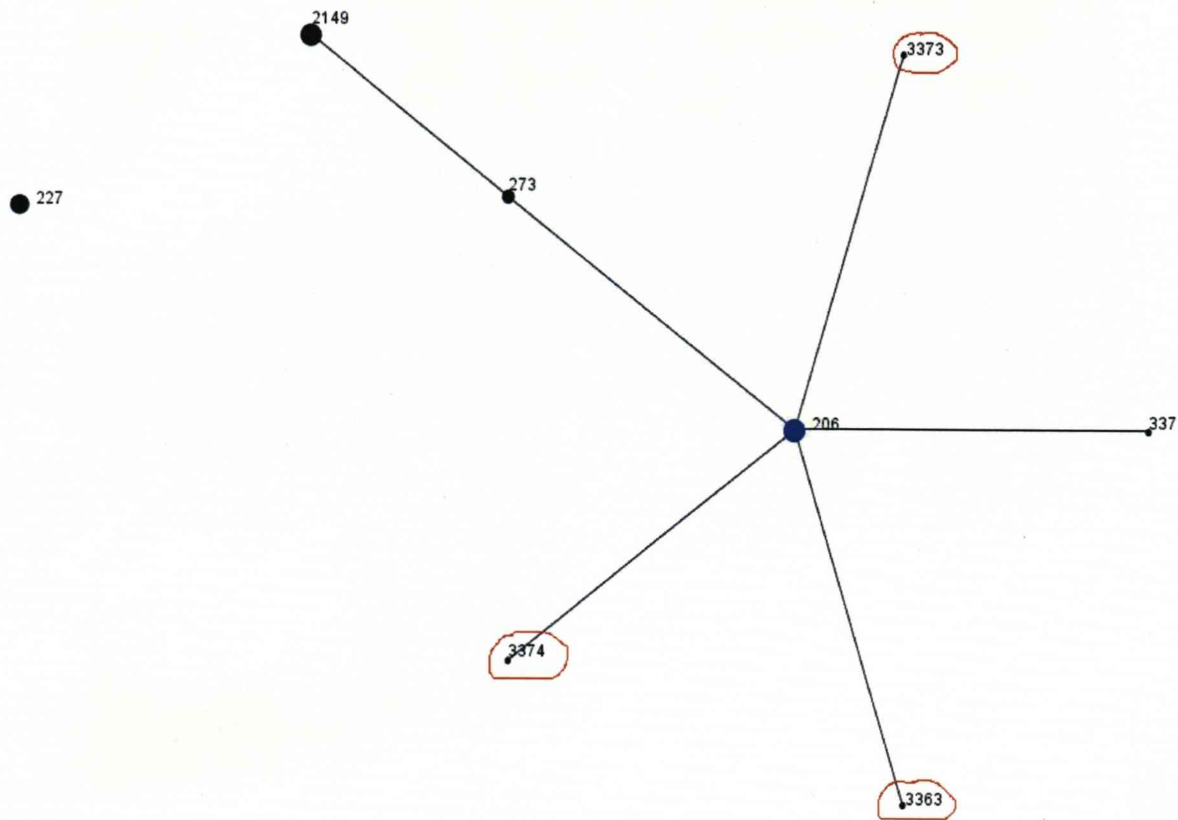
Appendix D Figure 4. eBurst diagram of C-C 48 isolates from cattle and sheep in Lancashire.



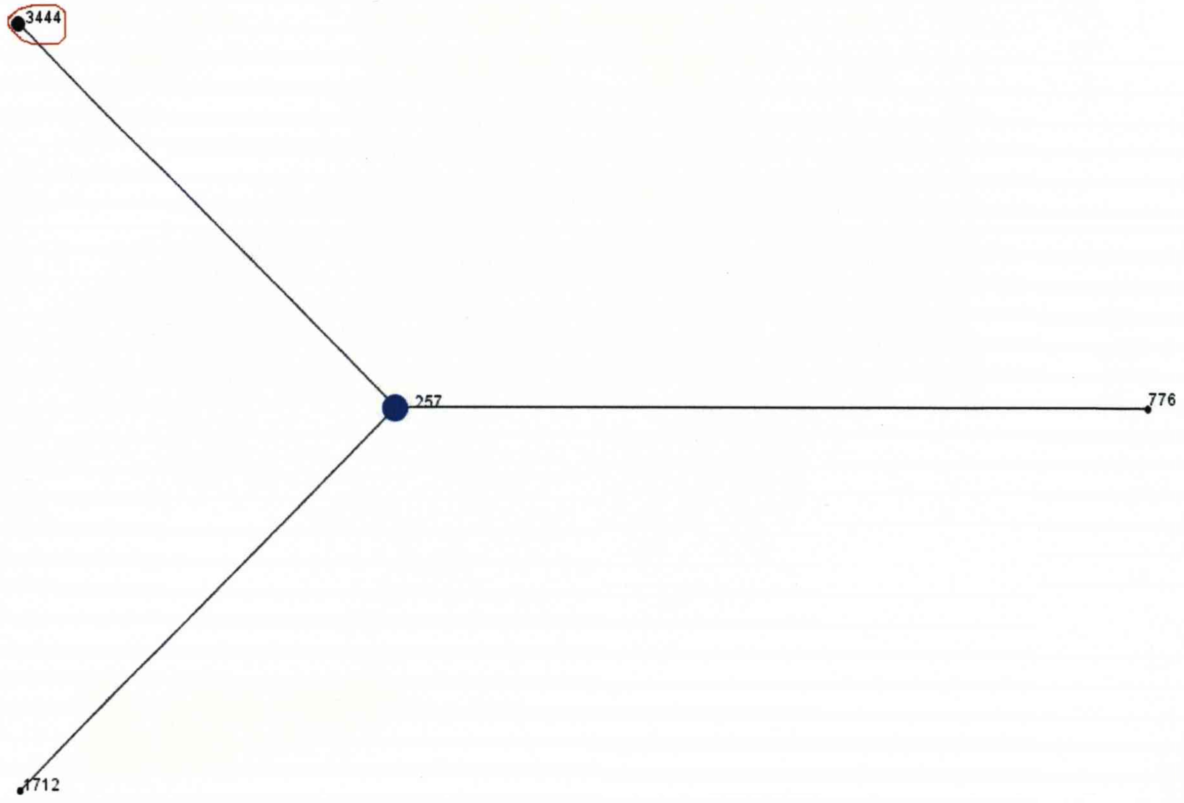
Appendix D Figure 5. eBurst diagram of C-C 61 isolates from cattle and sheep in Lancashire.



Appendix D Figure 6. eBurst diagram of C-C 206 isolates from cattle and sheep in Lancashire.



Appendix D Figure 7. eBurst diagram of C-C 257 isolates from cattle and sheep in Lancashire.



Appendix D Figure 8. eBurst diagram of C-C 403 isolates from cattle and sheep in Lancashire.