The Epidemiology of Campylobacter Infection in Dogs in the Context of the Risk of Infection to Humans

By

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Memorandum

Apart from the help and advice acknowledged, this thesis represents the unaided work of the author.

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Contents

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Abstract

Campylobacter spp. are the most common causes of bacterial gastroenteritis in humans worldwide, and although poultry and cattle are considered major sources of *Campylobacter* spp., infection has also been associated with dogs. In order to investigate the potential zoonotic risk to humans, dog faeces were examined for the presence of *Campylobacter* spp. from several different dog populations including; vet-visiting, boarding, rescue and hunt dogs. The *Campylobacter* spp. prevalence, and species distribution was determined for all studies, and some studies were analysed for possible risk factors for *Campylobacter* spp. carriage in dogs. Longitudinal studies were carried out on kennelled dogs to investigate shedding patterns, and possible transmission. All *C. jejuni,* and 41 *C. upsaliensis* isolates from these studies underwent multilocus sequence typing (MLST), along with nine *C. upsaliensis* isolates originating from human clinical cases, in order to identify possible sources of infection, and assess the potential zoonotic risk to humans. Additionally a pilot study was performed to annotate a plasmid as part of a *C. upsaliensis* genome project.

The findings of this thesis found that the overall prevalence of *Campylobacter* spp. ranged from 0-73%, although the majority of studies had a prevalence greater than 30%. The prevalence and species distribution differed depending upon the dog population. Kennelled dogs generally demonstrated the highest overall *Campylobacter* spp. prevalence, whilst the greatest species diversity was found in hunt dogs. *C. upsaliensis* dominated in most of the populations sampled, except for two hunt kennels where *C. lari* and *C. jejuni* dominated. The prevalence of *C. jejuni* was relatively high in some of the rescue and hunt kennels, reaching 20% and 26% respectively, whereas in vet-visiting and boarding dogs it was relatively low, 1.2-9%.

Longitudinal studies indicated that the majority of dogs entered the kennels already carrying *Campylobacter* spp. but when possible transmission events occurred they often involved *C. jejuni*. Rescue dogs appeared to be exposed to sources of *C. jejuni* before and after entry to the kennel, but boarding dogs were only exposed after entry. The shedding of *C. jejuni* in dogs appeared to be over short durations, whereas dogs that carried *C. upsaliensis* shed the bacterium in nearly every sample. Data suggested that dogs carried the same *C. upsaliensis* strain throughout the study, providing further evidence that the species may act as a commensal in dogs. Further to this no associations could be made between *Campylobacter* spp. carriage, specifically *C. upsaliensis*, and disease in dogs in any of the studies. Younger dogs were significantly more likely to carry *C. upsaliensis* than older dogs in the vet-visiting study (OR for every additional month 0.99) and living with another dog carrying *Campylobacter* spp., was significantly associated with *Campylobacter* spp. carriage in dogs.

A considerable amount of genetic diversity was observed within the *C. jejuni* and *C. upsaliensis* isolates originating from dogs, and MLST results suggested that strains of both species were the same, or highly similar to strains found in humans. This suggests that there may be common sources of infection for both humans and dogs and that dogs remain a potential zoonotic risk to humans. Although only a small number of household dogs carry *C. jejuni*, infected dogs should still be considered a potential zoonotic risk to humans, particularly if the dogs originate from kennelled or hunt kennel populations where the prevalence may be higher. Dogs remain a significant reservoir of *C. upsaliensis*, but the relationship between the presence of *C. upsaliensis* and gastroenteritis in both dogs and humans is still unclear.

1. Chapter one.

Introduction

1.1 *Campylobacter*

Campylobacter spp. are amongst the most commonly reported bacterial cause of human gastroenteritis in the UK and worldwide (Adak *et al*., 2002; CDC., 2008c; DEFRA, 2007; Humphrey *et al*., 2007; Westrell *et al*., 2009). *Campylobacter* spp. are zoonotic bacteria that are often found in the intestine of many animal species (Brown *et al*., 2004; DEFRA, 2007; Wilson *et al*., 2008; Workman *et al*., 2005). In some hosts, these bacteria can cause symptoms such as diarrhoea, but in others it can remain asymptomatic (Acke *et al*., 2009; Feodoroff *et al*., 2009; Guest *et al*., 2007; Jenkin and Tee, 1998; Leblanc Maridor *et al*., 2008; Rossi *et al*., 2008; Smith *et al*., 2008). *Campylobacter* is a spiral, microaerobic, gram negative bacteria that belongs to the 16S rRNA superfamily VI (Vandamme *et al*., 1991).

1.2 Taxonomy

Before 1963, spiral shaped bacteria were recognised as the genus *Vibrio*. These bacteria, particularly *Vibrio fetus*, were sometimes isolated from cattle experiencing abortion and diarrhoea, and were occasionally reported in humans (King, 1962; Moynihan and Stovell, 1955; Smith and Orcutt, 1927). In 1963, Sebald and Veron (1963) reclassified *V. fetus* and *Vibrio bubulus,* and transferred them into the genus *Campylobacter* (Sebald and Veron, 1963). There are currently 18 species, and six subspecies of *Campylobacter* (On, 2001). *Campylobacter fetus,* previously *V. fetus*, remains an important pathogen, especially in sheep and cattle (Campero *et al*., 2005; Fenwick *et al*., 2000). *Campylobacter jejuni* is probably the most ubiquitous of all the *Campylobacter* spp., and is considered to be closely related to *Campylobacter coli* (Dingle *et al*., 2005). *C.*

jejuni comprises of two subspecies, *jejuni* and *doylei*, although the latter is less common and *C. jejuni* subsp. *jejuni* is usually referred to simply as *C. jejuni* (On, 2005). *C. jejuni* and *C. coli* are both thermotolerant, (On, 2005) and have been known to participate in the exchange of genes, to the extent that they have a combined multilocus sequence typing (MLST) scheme (Dingle *et al*., 2005; Jolley and Chan, 2004). It has even been suggested that the two species could be converging (Sheppard *et al*., 2008). *Campylobacter lari*, *Campylobacter upsaliensis* and *Campylobacter helveticus* are also considered to be thermotolerant and closely related to *C. jejuni* and *C. coli* based upon 16S rRNA gene sequence comparisons (On, 2005; Thompson *et al*., 1988). *C. upsaliensis* and *C. helveticus* are closely related, sharing more than 97% homology which may have previously lead to confusion in differentiating between these two species (On, 2005). Linton *et al*, (1996) proposed a PCR assay based on the 16S rRNA gene in order to differentiate between *C. upsaliensis* and *C. helveticus*. This assay was also modified to detect other *Campylobacter* spp. such as *C. lari*, *Campylobacter hyointestinalis*, and *C. fetus* (Linton *et al*., 1996). This assay and other molecular techniques have improved current knowledge about the phylogeny, identity, and epidemiology of various *Campylobacter* spp.

1.2.1 Typing Campylobacter spp.

There are several typing methods currently available for *Campylobacter* spp., some of which will be discussed here. When examining methods, it is worth mentioning that any *Campylobacter* spp. typing technique is likely to encounter limitations regarding time, cost, and the instability of the *Campylobacter* genome, which is prone to interspecies and intraspecies recombination (Sheppard *et al*., 2008; Suerbaum *et al*., 2001). Each method has its advantages and disadvantages, so methods should be selected on the

basis that they are the most appropriate method for the situation, which may entail using two methods in conjunction with each other.

1.2.1.1 PCR Based Methods

There are many different *Campylobacter* spp. genes targeted by different PCR assays. Assays based upon the 16S rRNA gene, *hipO* (hippuricase) encoding gene, 23S rRNA gene, *glyA* (serine hydroxymethyltransferase) gene, and *sapB2* (surface layer protein) gene have been used with success in various studies (Linton *et al*., 1996; Wang *et al*., 2002). Variation in the gene that codes for the filament of the flagellum (*flaA*) has been investigated using restriction fragment length polymorphism analysis (RFLP-*flaA*) (Linton *et al*., 1997; Uzunovic-Kamberovic *et al*., 2007), and short variable region (SVR) sequence typing of the *flaA* gene (Colles *et al*., 2008a; Meinersmann *et al*., 1997; Price *et al*., 2006). RFLP has also been used in conjunction with the partial *groEL* (60-kDa chaperonin heat shock protein) gene to successfully identify species belonging to the genus *Campylobacter* (Karenlampi *et al*., 2004). RFLP involves digesting bacterial chromosomal DNA using a restriction endonuclease which can then be separated by electrophoresis and the gel examined under UV light. The many fragments produced can be made more manageable by selecting a rare cutting enzyme, i.e. a six base cutter, and specialised electrophoresis which is able to separate the larger fragments (as described under Pulsed field gel electrophoresis). Alternatively the fragments can be transferred to membranes and hybridised with a labelled probe, that is specific to certain repetitive DNA fragments, with the number, and size of these fragments in relation to the probe, used to compare strains (Foley *et al*., 2009; Moore *et al*., 2002). Probe targets can include ribosomal RNA genes, also known as ribotyping (Moore *et al*., 2002). This technique can be adapted so that certain genes are targeted by a PCR assay before restriction enzymes are used to digest the PCR products and produce a banding pattern

(Nayak *et al*., 2006). This allows the fragments to be visualised using standard electrophoresis techniques. The RFLP method is occasionally unable to type all isolates (Uzunovic-Kamberovic *et al*., 2007) and some isolates may remain unassigned using the *flaA* SVR method (Colles *et al*., 2008a). However, it has been suggested that both these techniques are useful for investigating short term outbreaks (Clark *et al*., 2005). The largest disadvantage when targeting a single gene is that it may not be truly representive of the whole genome, and if recombination occurs at that particular loci, which has been demonstrated (Harrington *et al*., 1997), it will have a much more significant effect on the data produced than it would if the genome was analysed as a whole.

1.2.1.2 Macro-restriction Analysis of Genomic DNA and Pulsed-field Gel Electrophoresis

Macro-restriction analysis of DNA using pulsed-field gel electrophoresis (PFGE) uses an enzyme, such as *Sma*I or *Kpn*I, to digest genomic DNA which can then be visualised as banding patterns, or fingerprints, on a gel under UV light. This technique is able to separate large molecules of DNA by periodically switching the voltage in the gel tank in three directions, at angles of 120°. Each direction has equal pulse times and the net result is the forward migration of DNA. Computer software such as Bionumerics V. 4.01 software (Applied Maths, Krtrijk, Belgium) is able to analyse this data and interpret phylogenetic relationships. The electrophoresis stage of PFGE can take considerably longer to run than standard electrophoresis, taking over 16 hours, and despite the use of packages such as Bionumerics, it is difficult to make comparisons between gels and between different studies and/or laboratories. There is also the potential for operator error in assigning bands which will also vary between different operators. Some studies also report that not all isolates are digestible by the same enzyme when using PFGE (Broman *et al*., 2002; Damborg *et al*., 2004; Devane *et al*., 2005; Ragimbeau *et al*.,

2008), and although it has been used extensively on *C. jejuni* isolates, modifications may be needed before this technique could be applied to other *Campylobacter* spp. The use of two enzymes, targeting different restriction sites will aid discrimination which is why some studies use two enzymes to obtain one gel and/or dendrogram (Broman *et al*., 2004; Chang and Taylor, 1990; McTavish *et al*., 2007). Damborg, *et al* (2004) reported that isolates with identical or closely related *Sma*I profiles showed more distinct *Kpn*I profiles, and other studies have also found *Kpn*I to be more discriminatory than *Sma*I (Karenlampi *et al*., 2003). In contrast, McTavish *et al*, (2007) initially used *Sma*I and subsequently found isolates could not be distinguished further by *Kpn*I.

PFGE may also be affected by the presence or absence of plasmids, which can be lost or gained, and recombination, which as previously mentioned is not unusual in *Campylobacter* spp. (Barrett *et al*., 2006; Sheppard *et al*., 2008; Suerbaum *et al*., 2001); all of this may result in low reproducibility. The use of more than one enzyme may help to dilute this effect, and most typing techniques will encounter recombination problems when analysing *Campylobacter* spp.

Isolates are considered indistinguishable when there is no difference in the number of fragments observed on a PFGE gel (Tenover *et al*., 1995). When an isolate has two to three different fragments compared to the outbreak strain, this probably resulted from just one genetic difference, caused by a point mutation, insertion or deletion. If a point mutation occurs at a restriction site, then the enzyme will not digest at that position, resulting in the two original fragments appearing as one larger fragment which will appear as a new fragment, hence three fragment changes (Barrett *et al*., 2006). This isolate would therefore be considered as 'probably part of the outbreak', whereas isolates with six different fragments are possibly related to the outbreak strain because

they could have arisen from just two genetic differences. Seven or more differences would be considered different from the outbreak strain (Tenover *et al*., 1995). However Barrett *et al* (2006) suggest that due to the rareness of the restriction sites, several genetic changes would probably have to occur for changes to be visualised on a PFGE gel. Furthermore, it was suggested that when isolates from the same population/outbreak are taken over a period of time, fragments may no longer be indistinguishable from the original outbreak strain because mutations are more likely to occur over longer periods of time, and that this should be taken into consideration (Barrett *et al*., 2006). Overall, PFGE is useful for detecting outbreaks and tracing the source of infection (Fitzgerald *et al*., 2001), and the data obtained are based on genomic DNA so they are likely to be more representive of the isolate as a whole, compared to techniques based on only small sections of the genome, such as PCR assays that target only one gene.

1.2.1.3 Amplified Fragment Length polymorphism

Amplified fragment length polymorphism (AFLP) produces fingerprints similar to those of PFGE and is also based upon genomic DNA. Two restriction enzymes, able to cut at different restriction sites, such as *Bgl*II, *Csp*6I, *Hind*III, and *Hha*I are used to digest the genomic DNA and produce fragments (Duim *et al*., 2003; Hanninen *et al*., 2001; Waldenstrom *et al*., 2007). AFLP adaptors are ligated to the ends of these fragments, and certain selected ligated fragments can be amplified by PCR (Mueller and Wolfenbarger, 1999; Partis *et al*., 2007). These banding patterns can then be observed via gel electrophoresis. AFLP is able to target several restriction sites throughout the genome producing a good representation of the strain, which allows isolates to be compared (Partis *et al*., 2007). AFLP demonstrates high sensitivity and repeatability, is relatively quick and easy to perform, and apart from an automated gene sequencer, costs are relatively similar to other techniques (Mueller and Wolfenbarger, 1999). It is more

robust when recombination occurs compared to other techniques because like PFGE, it analyses genomic DNA. AFLP is usually able to digest all isolates and it has been adapted for species such as *C. upsaliensis* and *C. helveticus*, successfully differentiating between these two closely related species (Damborg *et al*., 2008; Wieland *et al*., 2005). The main draw backs when using AFLP are that; like PFGE, it is difficult to compare gels between different studies, and a large number of bands are produced. This results in AFLP being heavily dependent on software (such as ABI Genescan, PE Applied Biosystems) to assign bands, which may not always be accurate, particularly when there are bands of different intensities.

1.2.1.4 Random Amplified Polymorphic DNA

Random amplified polymorphic DNA (RAPD) analysis uses short primers to amplify random DNA segments which are separated by gel electrophoresis and analysed as banding patterns. Unfortunately this results in low selectivity, primer mismatches which can cause artificial variation in banding patterns, and a low rate of reproducibility (Foley *et al*., 2009; Meunier and Grimont, 1993; Mueller and Wolfenbarger, 1999). Problems with reproducibility can be caused by minor changes to reagents, amplification conditions such as brand of thermocycler, and/or the use of a centrifuge during ethanol precipitation, which is why caution must be taken when comparing results from different laboratories (Meunier and Grimont, 1993; Micheli *et al*., 1994). However this technique is relatively cheaper than other methods, and has been utilised with success in some studies, despite the fact that not all isolates produced a PCR product (Hernandez *et al*., 1995; Workman *et al*., 2005). If reagents and cycling parameters are standardised, this technique can be advantageous because it only requires a small amount of bacterial DNA, it is less labour intensive than other methods, and it does not depend on culturing or the use of selective primers (Franklin *et al*., 1999).

1.2.1.5 Multilocus Sequence Typing

Multilocus sequence typing (MLST) is essentially a set of PCR assays that usually target seven or more housekeeping genes (and in some cases antigenic genes, such as *flaA*, Dingle *et al*., 2008). Variations at any one base or more produce an allele number, and the combination of these allele numbers produce a sequence type (ST). Sequences types are grouped depending upon their phylogeny, essentially if isolates share four or more loci, then the sequence types are grouped into clonal complexes (Dingle *et al*., 2001). MLST is highly reproducible, easy to interpret, and most importantly it provides international nomenclature (Maiden *et al*., 1998). MLST has been reported to be highly discriminatory and produces a result for every isolate, even though no clonal complex may initially be assigned to some isolates (Djordjevic *et al*., 2007; Ragimbeau *et al*., 2008). Examples of *C. jejuni* STs are discussed later in this chapter. Currently there are only a handful of *Campylobacter* spp. which have an established MLST scheme and database, for example *C. jejuni* and *C. coli*, whereas for others such as *C. upsaliensis*, the database has relatively few STs and clonal complexes (Jolley and Chan, 2004). In most situations the advantages outweigh potential problems such as expense, which may include the need for culturing, and the time and expense of sequencing. As discussed in Humphrey *et al*, (2007) MLST is prone to less variation than phenotypic methods, but as a consequence, certain sequence types may need further testing in order to distinguish between them, hence the antigenic genes previously mentioned. Pure colonies and thus culture are required for MLST, as mixed infections (encountered during direct PCRs, where bacterial DNA is extracted directly from faecal samples for example) would have a huge impact on the allele numbers, and sequence types assigned. However, many molecular techniques require the use of culture. Overall, the advantages of MLST suggest it is likely to become the gold-standard for typing *Campylobacter* spp.

1.2.2 Comparison Between Campylobacter Typing Techniques

Several studies have suggested that MLST is more discriminatory for *Campylobacter* spp. than, either RFLP-*flaA* or SVR-*fla* (Djordjevic *et al*., 2007). Duim *et al*, (2003) identified that the *flaA* SVR technique showed the lowest amount of correlation to MLST data, compared with PFGE, AFLP and ribotyping, because variations in the *flaA* SVR amino acid sequence occurred in all major MLST complexes suggesting that horizontal gene transfer for flagella antigens occurs commonly. Several studies have found typing techniques such as PFGE, AFLP and RFLP-*flaA* to be highly correlated with the clonal complexes produced by MLST (Djordjevic *et al*., 2007; Duim *et al*., 2003; Islam *et al*., 2009; Ragimbeau *et al*., 2008). The other advantage MLST has over other techniques is that there is currently a large *C. jejuni* and *C. coli* MLST database available which allows associations to be made between possible sources, hosts and STs/clonal complexes (Jolley and Chan, 2004).

Ragimbeau *et al* (2008) compared MLST to PFGE and *fla* typing, to analyse the same *C. jejuni* strains from humans, poultry, and cattle (Ragimbeau *et al*., 2008). The results indicated that some of the MLST clonal complexes such as clonal complex ST-61, were strongly associated with certain PFGE clusters and there were some instances of this occurring between some MLST clonal complexes and *fla* SVR sequence types. The PFGE method, using *Sma*I digestion, was unable to digest five isolates in that study, whereas sequencing the *fla* SVR was successfully performed for 207 of the 208 isolates. In that study MLST was able to type all isolates, with all except 13 assigned to known clonal complexes (Ragimbeau *et al*., 2008).

There is some evidence to suggest that RFLP-*flaA* provides greater discrimination between *C. jejuni* and *C. coli* isolates than RAPD (Ertas *et al*., 2004), but PFGE has

been shown to have a higher discriminatory power than either RFLP-*flaA* or other techniques such as SVR-*fla* sequencing, in a study investigating a *C. jejuni* outbreak (Fitzgerald *et al*., 2001). In some studies PFGE and AFLP have been shown to produce similar results, demonstrating high levels of discriminatory power, although occasionally AFLP has been able to distinguish further patterns compared to PFGE (Duim *et al*., 2003; Hanninen *et al*., 2001; Islam *et al*., 2009).

PFGE and/or AFLP are particularly useful when investigating isolates from within a population, such as an outbreak, but MLST is probably the most useful technique for comparing isolates from different outbreaks or studies. Since no one technique is likely to be truly accurate in its interpretation, a combination of techniques should be used in order to obtain reliable results, and MLST should be one of these methods. In future studies, genome sequencing may be the most accurate typing technique (discussed in Chapters 7&8), particularly if species such as *C. jejuni* and *C. coli* are converging (Sheppard *et al*., 2008).

1.3 Clinical Importance of *Campylobacter* spp. in Humans

As mentioned above, *Campylobacter* spp. are amongst the most commonly reported bacterial cause of human gastroenteritis world wide (Adak *et al*., 2002; CDC., 2008c; DEFRA, 2005, 2007). In 2007, 57,590 cases were reported in the United Kingdom, which was an increase of 10% compared to the previous year (DEFRA, 2007), and it is probable that many cases are unreported due to the self-limiting nature of the disease in most people (Wheeler *et al*., 1999). Clinical signs of *Campylobacter* spp. in humans include self-limiting diarrhoea, abdominal pains, vomiting, and in rare cases spontaneous abortion, haemolytic-uremic syndrome and Guillain-Barre syndrome (Denneberg *et al*., 1982; Eberhart-Phillips *et al*., 1997; Feodoroff *et al*., 2009; Gillespie

et al., 2006; Ho *et al*., 1995; Jimenez *et al*., 1999; Steinkraus and Wright, 1994). The incubation period can range from one to 10 days, although this is often dose dependent, and symptoms can last from three hours to three days (Blaser *et al*., 1987).

1.4 *Campylobacter* spp. Pathogenesis

Certain species of *Campylobacter*, in particular *C. jejuni*, can cause acute inflammation of the colon, often characterised by infiltration of the mucosa with neutrophils and lymphocytes (Russell *et al*., 1989). Cell cycle arrest caused by some *Campylobacter* spp. has also been observed (Mooney *et al*., 2001). The invasive ability of a *Campylobacter* is strongly affected by its motility, provided by the flagellum. This has been demonstrated by studies that inactivated the *flaA* gene (encodes for the filament of the flagella) or generated mutant bacteria, and found that this affected mobility and thus invasiveness (Konkel *et al*., 2004; Wassenaar *et al*., 1991). Adhesive properties of lipopolysaccharides (LPS) and flagella have been reported in other studies, and are thought to aid the invasion of *Campylobacter* spp. into the mucosa and epithelial cells causing cellular damage and subsequent diarrhoea (McSweegan and Walker, 1986; Wassenaar *et al*., 1991; Wassenaar and Blaser, 1999).

Flagella and LPS are not the only virulence mechanisms utilised by this bacteria, *Campylobacter* spp. also secrete toxins that stimulate an inflammatory response in the host. Cytokines such as interleukin-8 (IL-8) are secreted by host cells in response to bacterial invasion, acting as early warning signs to the host immune system (Jung *et al*., 1995), and *Campylobacter* spp. flagellum and cytolethal distending toxin (CDT) are both thought to stimulate the secretion of IL-8 from host cells (Zheng *et al*., 2008). CDT originating from certain *Campylobacter* spp. has also been shown to induce a DNA

repair response in host cells, suggesting that it causes DNA damage to the host (Hassane *et al*., 2003).

1.5 Sources of *Campylobacter* spp. Infection

Campylobacteriosis cases in humans are usually sporadic, often the result of consuming contaminated food. *Campylobacter* has also been associated with a seasonal peak in reporting rates during the summer months (Frost, 2001; Sopwith *et al*., 2006). In contrast, outbreaks of campylobacteriosis tend to be the result of exposure to contaminated water supplies or dairy products such as unpasteurised, or bird-pecked milk (Blaser *et al*., 1987; CDC., 2002; Frost, 2001; Jakopanec *et al*., 2008; Levesque *et al*., 2008; Riordan *et al*., 1993; Smith *et al*., 2006). The majority of *Campylobacter* spp. infections in humans are caused by *C. jejuni* and to a lesser extent *C. coli*, but in a small proportion of cases *C. upsaliensis* and *C. lari* have been isolated (DEFRA, 2005, 2007, (Goossens *et al*., 1990a; Labarca *et al*., 2002; Lastovica and Le Roux, 2003; Prasad *et al*., 2001).

1.5.1 Poultry and Raw Meat

The primary route of *Campylobacter* spp. infection is via the faecal oral route and poultry meat is considered the most significant source of *Campylobacter* infection in humans (Humphrey *et al*., 2007; Hussain *et al*., 2007; Sheppard *et al*., 2009; Wilson *et al*., 2008). A recent report by the Food Standards Agency found that there was a *Campylobacter* spp. prevalence of 65.2% in retail chicken in the UK (FSA, 2009) and other studies have also found *C. jejuni* in chickens and/or raw poultry (Hussain *et al*., 2007; Little *et al*., 2008; Stoyanchev *et al*., 2007). Additionally epidemiological evidence has found significant associations between campylobacteriosis in humans and eating raw poultry, and this is supported by molecular studies which have identified

similar *C. jejuni* strains in chickens and humans (Eberhart-Phillips *et al*., 1997; Kapperud *et al*., 1992; Ragimbeau *et al*., 2008; Wilson *et al*., 2008; Workman *et al*., 2005). For example one study found that 46% of human and chicken *C. jejuni* strains had overlapping sero- and genotype combinations (Karenlampi *et al*., 2003), and another based in the UK found that chicken was estimated as the source of *C. jejuni* infection in the majority (56.6%) of human cases examined (Wilson *et al*., 2008).

Raw meat such as beef, lamb, rabbit and to a lesser extent pork have also had *Campylobacter* spp. isolated from them, and many studies have found significant associations between *C. jejuni* molecular profiles found in humans and cattle (Brown *et al*., 2004; French *et al*., 2005; Little *et al*., 2008; Wilson *et al*., 2008). *C. jejuni* clonal complex ST-48 was associated with consuming raw minced meat in a study carried out in Finland (Karenlampi *et al*., 2007). Consuming sausages cooked on a barbeque, and eating chicken in a restaurant have also been associated with campylobacteriosis (Eberhart-Phillips *et al*., 1997; Kapperud *et al*., 1992). Undercooking and cross-contamination from raw meat most likely plays an important role in the transmission of *C. jejuni* from meat, particularly poultry, to humans (DEFRA, 2007; Frost, 2001). Further more, poultry meat should be regarded as different to other meat because *Campylobacter* spp. are not restricted to the intestine of chickens, but can be found throughout the meat (Berndtson *et al*., 1992; Katzav *et al*., 2008; Luber and Bartelt, 2007; Stoyanchev *et al*., 2007). *Campylobacter* spp. have also been isolated from fruit salad, sandwiches, cheese, and mayonnaise, the latter being significantly associated with *Campylobacter* infection in some studies (Hussain *et al*., 2007; Tenkate and Stafford, 2001).

1.5.2 Environmental Sources of Campylobacter spp.

Despite the strong associations between consumption of contaminated food or drink, and campylobacteriosis, *Campylobacter* spp. have also been isolated from various other sources and environmental samples such as water (environmental), soil and sand (Brown *et al*., 2004; Dingle *et al*., 2001; French *et al*., 2005; Kemp *et al*., 2005; Wilson *et al*., 2008). One study observed that some strains of *C. jejuni* appeared to move from one area of the country to another, indicating the possibility of environmental factors (Karenlampi *et al*., 2003). *C. jejuni* has even been isolated from flies (Adhikari *et al*., 2004), and there are sequence types found in humans that cannot be associated with cattle or poultry suggesting other possible sources (Ragimbeau *et al*., 2008). This could potentially mean that poultry, raw meat and unpasteurised milk may not be the only significant sources of *Campylobacter* spp. infection for humans.

Other risk factors for human campylobacteriosis include, recent overseas travel, rainwater as a source of water for the home, ingesting untreated water from lakes, rivers and streams, and contact with puppies and cattle (Adak *et al*., 1995; Eberhart-Phillips *et al*., 1997; Kapperud *et al*., 1992; Tenkate and Stafford, 2001). Karenlampi *et al*, (2007) identified that certain MLST clonal complexes and/or sequence types were significantly associated with some of these sources, for example clonal complex ST-677 was associated with drinking non-chlorinated water, ST-45 was associated with pet contact and several were associated with cattle, discussed later in this chapter.

1.5.3 Immunity to Campylobacter spp.

campylobacteriosis is usually self limiting, and there is evidence based on increasing immunoglobulin titers in *Macaca nemestrina* monkeys, to suggest that after an initial experimental *Campylobacter* spp. infection, the host retains some immunity if exposed

to the bacteria again (Russell *et al*., 1989). Humans have also demonstrated immunity to *Campylobacter* spp. in studies investigating outbreaks, particularly those that have had regular contact with farm animals (Blaser *et al*., 1987; Forbes *et al*., 2009; Russell *et al*., 1989). Symptoms may persist for longer in some immuno-compromised patients. Patients found to be infected with both human immunodeficiency virus (HIV) and *C. upsaliensis*, which is normally considered less virulent than *C. jejuni*, suffered from diarrhoea for up to 60 days in one study (Jenkin and Tee, 1998; Jimenez *et al*., 1999).

Interestingly, recent consumption of roast or baked chicken, handling raw chicken with giblets, and occupational contact with livestock and/or their faeces, were found to be protective against *Campylobacter* infection in some studies (Adak *et al*., 1995; Eberhart-Phillips *et al*., 1997). As previously discussed, chicken is a major source of *Campylobacter* spp., as are cattle, so presumably contact with these sources increases the likelihood of exposure to *Campylobacter* spp., facilitating immunity to the bacterium.

1.5.4. Preventing Campylobacteriosis

Acidic conditions, such as those experienced in the stomach are more than unfavourable to some *Campylobacter* spp., and although they are thermotolerant, they are considered susceptible to freeze thawing, oxidative stress, and u.v radiation (Blaser *et al*., 1980; Garenaux *et al*., 2008; Garenaux *et al*., 2009; Obiri-Danso *et al*., 2001). The most effective measures to take against *Campylobacter* infection are personal and kitchen hygiene, thorough cooking of raw meat, particularly poultry, and correct storage. More *Campylobacter* spp. have been isolated from chilled products compared to frozen products, and interestingly heat-treated products (ready-to-eat) showed no evidence of *Campylobacter* spp. in one study (Stoyanchev *et al*., 2007). Since *Campylobacter* spp.

can also be found in muscular tissue and subcutaneous layers (Berndtson *et al*., 1992; Katzav *et al*., 2008; Luber and Bartelt, 2007; Stoyanchev *et al*., 2007), hygiene procedures in abattoirs designed to prevent contamination of intestinal content with meat/muscle, may not be sufficient to remove all risk of infection. Therefore it should be assumed that any poultry brought into the kitchen may contain *Campylobacter* spp.

Studies have demonstrated the importance of hygiene in the kitchen area. *Campylobacter* spp. were still found on plates after washing, although they were very sensitive to air drying or drying with a cloth, and were no longer isolated from the plates after drying (Mattick *et al*., 2003). *Campylobacter* spp. have been isolated from chopping boards, hands, cloths and taps during preparation of raw poultry, although rinsing was found to be a crucial stage of washing up/cleaning surfaces in order to eliminate most of the *Campylobacter* spp. (Cogan *et al*., 2002). Therefore in order to reduce the chances of campylobacteriosis, care should be taken in preparation of poultry, i.e. no cross-contamination, surfaces should be washed and rinsed, plates should be dried, raw meat should be frozen rather than refrigerated if possible, and meat should be thoroughly cooked.

1.6 *Campylobacter* spp. in Other Animals

Campylobacter spp. are found in a wide range of animal hosts and particular species of *Campylobacter* are found more commonly in certain animal hosts than others. *C. jejuni* is probably the most commonly isolated *Campylobacter* spp. overall and it has been isolated from various animals, including cattle, sheep, chickens, wild birds, pigs, dogs and cats (Brown *et al*., 2004; Wilson *et al*., 2008; Workman *et al*., 2005). *C. jejuni* and to some extent *C. coli* are commonly isolated from chickens and *C. coli* is also strongly associated with pigs (Lyngstad *et al*., 2008; Petersen *et al*., 2007; Stoyanchev *et al*.,

2007; Workman *et al*., 2005). *C. upsaliensis* is commonly isolated from dogs, and *C. helveticus* is isolated most frequently from cats, although *C. upsaliensis* can also be found in cats (Engvall *et al*., 2003; Koene *et al*., 2008; Rossi *et al*., 2008; Sandberg *et al*., 2002; Wieland *et al*., 2005; Workman *et al*., 2005). Additionally *C. lari* is commonly isolated from wild birds (Waldenstrom *et al*., 2002; Waldenstrom *et al*., 2007).

Multi-locus sequence typing, reveals that certain *C. jejuni* sequence types are more common than others in humans and certain animal hosts (Table 1.1). In Humans, ST-21 and ST-45 tend to predominate (Table 1.1). They are both found in poultry and cattle, although ST-45 is often associated with the environment (French *et al*., 2005; Sopwith *et al*., 2008). ST-45 is found commonly in water, wildlife and chickens, whilst ST-61, ST-53, ST-58, and ST-883, have been statistically associated with cattle (Colles *et al*., 2003; French *et al*., 2005; Karenlampi *et al*., 2007; Sopwith *et al*., 2008) (Table 1.1). Some of these sequence types are found readily in human cases of campylobacteriosis (Dingle *et al*., 2001; Dingle *et al*., 2002), suggesting either common sources of infection, or possible transmission between animals and humans. Some sequence types appear to be more unique to certain animal hosts, for example Colles *et al*, (2008a) identified sequence types that were highly associated with geese (e.g. ST-702), and found that none of these sequence types were found in starlings or chickens.

Table 1.1. Sources of *C. jejuni* MLST clonal complexes.

Clonal Complex	Sources of Isolation	References
ST-21	Human, Poultry, Cattle, Wild Birds, Rabbits, Sheep, Water	(Colles *et al*., 2003; Dingle *et al*., 2002; French *et al*., 2005; Ragimbeau *et al*., 2008; Sopwith *et al*., 2008)
ST-45	Human, Poultry, Water, Cattle, Wild Birds, Badger, Rabbit, Sheep, Sand, Soil	(Colles *et al*., 2003; Dingle *et al*., 2001; French *et al*., 2005; Sopwith *et al*., 2008)
ST-48	Human, Cattle, Poultry, Sheep, Sand, Water	(Colles *et al*., 2003; Dingle *et al*., 2002; Ragimbeau *et al*., 2008; Sopwith *et al*., 2006, 2008)
ST-257	Human, Cattle, Poultry, Wild Birds, Water	(Colles *et al*., 2003; French *et al*., 2005; Ragimbeau *et al*., 2008; Sopwith *et al*., 2008)
ST-353	Human, Poultry, Cattle	(Colles *et al*., 2003; Duim *et al*., 2003; Karenlampi *et al*., 2007; Kwan *et al*., 2008b; Ragimbeau *et al*., 2008)
ST-206	Human, Poultry, Cattle, Sheep	(Colles *et al*., 2003; Dingle *et al*., 2002; Kwan *et al*., 2008b; Ragimbeau *et al*., 2008)
ST-354	Human, Poultry, Cattle	(Colles *et al*., 2003; Dingle *et al*., 2002; Djordjevic *et al*., 2007; Kwan *et al*., 2008b; Ragimbeau *et al*., 2008)
ST-443	Human, Poultry	(Colles *et al*., 2003; Ragimbeau *et al*., 2008; Sopwith *et al*., 2006)
ST-22	Human, Poultry, Sheep	(Colles *et al*., 2003; Dingle *et al*., 2001; Ragimbeau *et al*., 2008)
ST-61	Human, Cattle, Sheep, Water	(Colles *et al*., 2003; Dingle *et al*., 2002; French *et al*., 2005; Karenlampi *et al*., 2007; Kwan *et al*., 2008b; Ragimbeau *et al*., 2008; Sopwith *et al*., 2008)
ST-52	Human, Poultry, Sheep	(Colles *et al*., 2003; Dingle *et al*., 2001)

ST-42	Human, Cattle, Poultry, Wild Bird	(Dingle *et al*., 2002; Djordjevic *et al*., 2007; Jolley and Chan, 2004; Kwan *et al*., 2008b; Ragimbeau *et al*., 2008)
ST-607	Human, Poultry	(Djordjevic *et al*., 2007; Ragimbeau *et al*., 2008)
ST-403	Human, Porcine	(Dingle *et al*., 2002; Ragimbeau *et al*., 2008; Wilson *et al*., 2008)
ST-658	Human, Water, Cattle	(Djordjevic *et al*., 2007; Karenlampi *et al*., 2007; Kwan *et al*., 2008b; Sopwith *et al*., 2008)
ST-508	Human, Wild Birds, Water, Cattle	(Duim *et al*., 2003; French *et al*., 2005; Kwan *et al*., 2008b; Sopwith *et al*., 2008; Wilson *et al*., 2008)

Complexes displayed according to the frequency of isolation from humans in various studies (Colles *et al*., 2003; Dingle *et al*., 2001; Dingle *et al*., 2002; Djordjevic *et al*., 2007; Duim *et al*., 2003; Karenlampi *et al*., 2007; Ragimbeau *et al*., 2008; Sheppard *et al*., 2009; Sopwith *et al*., 2006), i.e. on average clonal complex ST-21 appears to be the most commonly isolated complex from humans overall.

The majority of studies have found no association between *Campylobacter* spp. and diarrhoea in their preferred animal host, suggesting that this bacteria is a commensal in some animal species (Leblanc Maridor *et al*., 2008; Rossi *et al*., 2008; Smith *et al*., 2008), although in some studies *C. jejuni* and/or *C. coli* have been associated with diarrhoea in cats and dogs (Acke *et al*., 2009; Guest *et al*., 2007). Further studies are needed to investigate this relationship. Additionally, *C. fetus* has been associated with abortion in sheep, cattle, and even humans (Campero *et al*., 2005; Fenwick *et al*., 2000; Steinkraus and Wright, 1994). There is also evidence to suggest that *C. jejuni* may cause abortions in sheep and cattle, and that this consequence is not unique to *C. fetus* infection (Campero *et al*., 2005; Sahin *et al*., 2008).

1.7 *Campylobacter* spp. in Dogs

C. upsaliensis is the most commonly reported *Campylobacter* spp. in the majority of dog populations sampled, particularly in the UK and Europe (Acke *et al*., 2009; Engvall *et al*., 2003; Hald *et al*., 2004; Koene *et al*., 2004; Rossi *et al*., 2008; Sandberg *et al*., 2002; Wieland *et al*., 2005). However some studies have found *C. jejuni* to be the most commonly isolated species in dogs, particularly outside of Europe (Hald and Madsen, 1997; Lopez *et al*., 2002; Tsai *et al*., 2007; Workman *et al*., 2005). This may relate to the difference in temperature observed between Northern Europe and countries such as Barbados, since *Campylobacter* spp. infection is often associated with a summer peak (Frost, 2001; Sopwith *et al*., 2006). Differences in the culture and hygiene practices of these countries may also explain the high *C. jejuni* prevalence found in these dogs compared to European studies. Cultivation techniques also contribute significantly to the isolation of certain *Campylobacter* spp., as discussed later in this chapter. Other species of *Campylobacter* such as *C. coli* and *C. lari* have also been isolated from dogs on occasion, but these species are usually of very low prevalence (Engvall *et al*., 2003; Hald *et al*., 2004; Koene *et al*., 2008; Rossi *et al*., 2008; Tsai *et al*., 2007).

1.7.1 Detection Methods

It is difficult to directly compare the prevalence of *Campylobacter* spp. found in the different studies due to the variety of methods used to isolate the bacteria. It is particularly difficult to compare studies carried out decades apart because of the increased number of methods currently used, and because methods have only recently been optimised for the detection of *C. upsaliensis*, potentially the most prevalent *Campylobacter* spp. found in dogs.

There is currently no gold standard for the isolation of *Campylobacter* spp. so a combination of different methodologies have been used in many studies. In the majority of studies methods have been adapted for *C. jejuni*, the most common *Campylobacter* spp. found in humans, and have only recently been optimised for *C. upsaliensis* (Fernandez and Martin, 1991; Fleming, 1983; Fox *et al*., 1983; Malik and Love, 1989; Nair *et al*., 1985). In other species such as dogs, other methods have been developed to try and optimise isolation rates for samples that may contain various strains of *Campylobacter* spp., such as *C. upsaliensis*.

1.7.1.1 Media

Examples of different culture methods include, *Campylobacter* selective agars such as modified charcoal cefoperazone deoxycholate agar (mCCDA), which in some studies detected more *Campylobacter* spp., and showed higher selectivity than other methods (Engberg *et al*., 2000; Korhonen and Martikainen, 1990), or *Campylobacter* selective blood based agars which are also successful and commonly used (Acke *et al*., 2009; Burnens and Nicolet, 1992; Engvall *et al*., 2003; Fernandez and Martin, 1991; Hald *et al*., 2004; Koene *et al*., 2004; Lopez *et al*., 2002).

Antibiotics such as cefoperazone, amphotericin, and teicoplanin (i.e. CAT) are also used in different combinations (Acke *et al*., 2009; Hald *et al*., 2004; Koene *et al*., 2004; Rossi *et al*., 2008; Steinhauserova *et al*., 2000). The use of CAT supplement, and longer incubation times are optimised for the detection of *C. upsaliensis* but they have not always been included in some studies including those mentioned in this chapter (Hald and Madsen, 1997; Lopez *et al*., 2002), therefore the prevalence of *C. upsaliensis* may have been underestimated. The addition of CAT supplement to *Campylobacter* selective agar produced high isolation rates for detecting *C. upsaliensis,* attributed to a

lower concentration of cefoperazone in CAT media (8µm/ml) than in mCCDA (32µm/ml), and in some studies was superior to other methods (Aspinall *et al*., 1993, 1996; Burnens *et al*., 1992; Burnens and Nicolet, 1992; Byrne *et al*., 2001; Corry and Atabay, 1997). Despite *C. upsaliensis* demonstrating susceptibility to concentrations of 8µm/ml of cefoperazone, (the concentration often present in CAT media), it has been suggested that the deoxycholate in the media may play a role in reducing the effects of this antibiotic (Corry and Atabay, 1997).

1.7.1.2 Filtration

Acke *et al*, (2009) noted that direct plating onto mCCDA and CAT, and filtration onto blood media yielded the highest isolation rates of *Campylobacter* spp. In agreement with Acke *et al*, (2009), several studies have also found that the use of a filter improved the isolation of *Campylobacter* spp. (Modolo and Giuffrida, 2004; Moreno *et al*., 1993), particularly *C. upsaliensis* (Aspinall *et al*., 1996), and reduced contaminants (Korhonen and Martikainen, 1990). However there are limitations associated with filtration. Previous studies have found filtration could not detect co-infection of *Campylobacter* spp. (Koene *et al*., 2004) and Goossens *et al*, (1990b) demonstrated that colonies below 10^5 cfu per g of faeces could not be detected by filtration.

1.7.1.3 Enrichment

Enrichment broths are commonly used to isolate *Campylobacter* spp. (Acke *et al*., 2009; Baker *et al*., 1999; Maher *et al*., 2003; Manfreda *et al*., 2003). Enrichment detected more *C. jejuni* than direct plating in a study examining vet visiting dogs (Fleming, 1983), and improved detection of *Campylobacter* spp. by 30% compared to CCDA alone in another study (Maher *et al*., 2003). However, including an enrichment stage also has its disadvantages as it is known to result in a higher bacterial load of

contaminating flora (Abulreesh *et al*., 2005) which can increase with longer incubation periods (Korhonen and Martikainen, 1990).

1.7.1.4 Incubation Time

Incubation time is important because *C. jejuni* can be cultured in 48hours, however *C. upsaliensis* usually takes 96 hours to grow (Byrne *et al*., 2001; Labarca *et al*., 2002; Moreno *et al*., 1993). An example of this effect can be observed in two studies by Hald *et al* (1997 and 2004). The study published in 1997 found higher isolation rates of *C. jejuni* compared to *C. upsaliensis*, but the reverse was true in the 2004 study, where samples were incubated for 96 hours, rather than the 48 hours used in the first study. However, differences in the sample population may have played a role in these findings.

1.7.1.5 Temperature

In addition to the role of incubation time, temperature may also play a role in detecting different *Campylobacter* spp. The temperature for incubation also tends to vary between studies, usually either 42°C or 37°C to imitate the body temperature of chickens, and humans, respectively (Burnens and Nicolet, 1992; Engvall *et al*., 2003; Hald and Madsen, 1997; Koene *et al*., 2004; Malik and Love, 1989; Modolo and Giuffrida, 2004; Rossi *et al*., 2008; Workman *et al*., 2005). Thirty seven degrees celsius tends to be used most commonly and there does not appear to be any substantial difference between these two temperatures in terms of the frequency of *Campylobacter* spp. isolation (Engvall *et al*., 2003; Hald *et al*., 2004; Rossi *et al*., 2008).

1.7.1.6 Extraction of DNA from Faecal Samples With Subsequent PCR 'Direct PCR'

More recently a number of studies have used direct PCR to detect *Campylobacter* spp. from faecal samples of various species including dogs and humans (Lawson *et al*., 1999;

Linton *et al*., 1997; Maher *et al*., 2003; Persson and Olsen, 2005; Westgarth *et al*., 2009). There is evidence to suggest that direct PCR may be more sensitive than culture, particularly when samples are 'aged', have a low yield of DNA, or are in a viable but non-culturable form (Lawson *et al*., 1999; Maher *et al*., 2003). In a study examining clinical specimens, some of which were 'aged', direct PCR detected *Campylobacter* spp. in 38% of culture negative samples (Maher *et al*., 2003). Direct PCR may also help to identify mixed infections of different *Campylobacter* spp. (Lawson *et al*., 1999). However, some studies suggest that culture may be more effective at isolating *Campylobacter* spp., especially when samples are fresh (Persson and Olsen, 2005), possibly because of degradation of bacterial DNA and inhibitory substances present in faeces which may reduce the sensitivity of direct PCR (Lawson *et al*., 1999). However other reports have found little difference between direct PCR and culture for detecting *Campylobacter* spp. (Linton *et al*., 1997; Westgarth *et al*., 2009), so the use of both methods has been encouraged for maximum recovery (Persson and Olsen, 2005; Westgarth *et al*., 2009). Differences between studies in target genes for PCR may also affect the outcome, although assays based on 16SrRNA have been used in the majority of studies (Lawson *et al*., 1999; Maher *et al*., 2003; Persson and Olsen, 2005; Westgarth *et al*., 2009).

1.7.2 Prevalence of Campylobacter spp. *in Different Dog Populations*

The prevalence of *Campylobacter* spp. in dogs varies considerably, depending upon the population sampled and the detection methods used. The vet-visiting population of dogs in several European countries appear to have a *Campylobacter* spp. prevalence of approximately between 24%-41%, with the majority of these dogs carrying *C. upsaliensis* (Acke *et al*., 2009; Rossi *et al*., 2008; Sandberg *et al*., 2002; Wieland *et al*., 2005). *C. jejuni* was the second most commonly isolated *Campylobacter* spp. in these

studies with a prevalence of between 3% and 10% (Acke *et al*., 2009; Rossi *et al*., 2008; Sandberg *et al*., 2002; Wieland *et al*., 2005).

The pattern observed in household dogs is similar to that of vet-visiting dogs, whereby *C. upsaliensis* is usually isolated more than *C. jejuni* (Engvall *et al*., 2003; Hald *et al*., 2004; Koene *et al*., 2004; Westgarth *et al*., 2009), although there are exceptions where *C. jejuni* dominates (Hald and Madsen, 1997; Lopez *et al*., 2002). The prevalence of *Campylobacter* spp. appears to vary between 17% and 77% within this population of dogs, although isolation methods and geographical locations of the various studies most likely play an important role in the differences observed (Engvall *et al*., 2003; Fernandez and Martin, 1991; Hald *et al*., 2004; Koene *et al*., 2004; Lopez *et al*., 2002). The prevalence of *C. upsaliensis* in some of these studies (excluding Fernandez and Martin, 1991 where methods were optimised for *C. jejuni*) ranges from 3%-59%, whereas the prevalence of *C. jejuni* ranges from 11% to 40% (Engvall *et al*., 2003; Fernandez and Martin, 1991; Hald *et al*., 2004; Koene *et al*., 2004; Lopez *et al*., 2002).

Studies that have sampled both household/vet-visiting and stray/kennelled dogs, consistently identify higher *Campylobacter* spp. prevalences in the stray/kennelled dogs than in the household dogs (Baker *et al*., 1999; Fernandez and Martin, 1991; Malik and Love, 1989; Tsai *et al*., 2007; Workman *et al*., 2005). In Ireland, reports as high as 87% for the prevalence of *Campylobacter* spp. have been found in sheltered dogs (Acke *et al*., 2006), although the species distribution for this study is unknown. In Chile, Fernandez and Martin, (1991) found a *Campylobacter* spp. prevalence of 51% in stray dogs with a *C. jejuni* prevalence of 36%. *C. jejuni* has been found to dominate in some studies that sampled stray dogs (Tsai *et al*., 2007; Workman *et al*., 2005), and even when it does not dominate, the prevalence is high compared to other studies such as vet-

visiting and household studies mentioned above (Fernandez and Martin, 1991). Reasons for this are unclear but may be due to increased environmental, or other animal exposure (as discussed in Chapters 4&8).

1.7.3 Risk Factors for Campylobacter spp. Carriage

1.7.3.1 Age

The majority of studies have identified that younger rather than older dogs are more likely to carry *C. upsaliensis* and *C. jejuni* (Acke *et al*., 2009; Acke *et al*., 2006; Engvall *et al*., 2003; Guest *et al*., 2007; Sandberg *et al*., 2002; Wieland *et al*., 2005; Workman *et al*., 2005). In particular most studies have found that dogs younger than 15 months old are more likely to carry *Campylobacter* spp. than older dogs (Acke *et al*., 2009; Engvall *et al*., 2003; Lopez *et al*., 2002; Nair *et al*., 1985; Sandberg *et al*., 2002; Wieland *et al*., 2005), and in one study increased *C. upsaliensis/C. helveticus* carriage in younger dogs has been observed with dogs ranging from 13-36 months old (Wieland *et al*., 2005). However some studies, including this latter study, did not find significant associations between age and *C. jejuni* carriage (Tsai *et al*., 2007) or *Campylobacter* spp. generally, in dogs (Burnie *et al*., 1983; Tsai *et al*., 2007; Wieland *et al*., 2005). Younger animals may be more susceptible to *Campylobacter* spp. invasion because they are naïve to the bacterium, whereas older animals are more likely to have encountered *Campylobacter* spp. Immunity to *Campylobacter* spp. after previous exposure has been demonstrated in monkeys in another study (Russell *et al*., 1989).

1.7.3.2 Clinical Disease

The majority of studies have found no significant relationship between disease and *Campylobacter* spp. carriage in dogs (Acke *et al*., 2006; Koene *et al*., 2008; Sandberg *et al*., 2002; Workman *et al*., 2005). It has been suggested that *C. upsaliensis* is a

commensal in dogs because it is often the most commonly isolated *Campylobacter* spp. found in dogs, and is not isolated to the same extent from any other animal, except occasionally cats (Acke *et al*., 2006; Engvall *et al*., 2003; Sandberg *et al*., 2002; Workman *et al*., 2005). One study even suggested that diarrhoea in dogs was negatively associated with *Campylobacter* spp. carriage, and that the diarrhoea caused a 'wash-out' effect (Wieland *et al*., 2005). In contrast, other studies have found an association between *Campylobacter* spp. and clinical signs (Guest *et al*., 2007), particularly in younger and kennelled dogs (Burnens *et al*., 1992; Fleming, 1983; Fox *et al*., 1983; Nair *et al*., 1985). When studies have found that *Campylobacter* spp. carriage is associated with diarrhoea in dogs, *C. jejuni* is often the most frequently isolated *Campylobacter* spp. in these studies (Fleming, 1983; Fox *et al*., 1983; Nair *et al*., 1985). A recent study by Acke *et al*, (2009) reported that *C. jejuni* was the most prevalent species in dogs with diarrhoea, that *C. upsaliensis* was significantly more prevalent in the healthy dogs, and dogs with other medical or surgical conditions than in the group of dogs with diarrhoea. However numbers of *C. jejuni* isolates were small in this study.

1.7.3.3 Sources of Campylobacter spp.

Sources of *Campylobacter* spp. for dogs are relatively unknown, but it seems plausible that the sources for dogs would be similar to those previously discussed for humans, at least for *C. jejuni*, i.e. raw meat, raw milk and contact with other animals, particularly their faeces. A study in Switzerland described that contact with poultry or birds was significantly associated with *C. jejuni* carriage in dogs, with an OR of 2.9 (Wieland *et al*., 2005). The same study concluded that feeding chicken or meat to dogs was protective for *C. jejuni* carriage in dogs (Wieland *et al*., 2005). Additionally, open drains, and possibly lakes have been associated with *Campylobacter* spp. carriage in dogs (Baker *et al*., 1999; Wieland *et al*., 2005). Westgarth *et al*, (2008) identified that

the most common sleeping place for household dogs were kitchen areas. This may be significant as raw meat is most likely to be stored, prepared, and disposed of in the kitchen area, providing opportunity for dogs to come into contact with this possible source of *Campylobacter* spp. Sources of *C. upsaliensis* are less clear than those of *C. jejuni*. Transmission between dogs is a possible route of infection/carriage, especially as they partake in coprophagia (Westgarth *et al*., 2008). Interestingly *C. upsaliensis* has been isolated from a poultry abattoir, although at a low prevalence (Stoyanchev, 2004).

1.8 Dogs as a Risk in the Context of Human Infection

Although poultry and cattle are considered the greatest sources of *Campylobacter* spp. infection for humans, there is evidence of an increased risk of *Campylobacter* spp. infection in humans associated with dog or pet ownership (Adak *et al*., 1995; FSA, 2005; Kapperud *et al*., 1992; Salfield and Pugh, 1987; Tenkate and Stafford, 2001). In particular, significant associations have been established between campylobacteriosis in humans, especially young children, and the introduction of a puppy into the household (Blaser *et al*., 1978; Eberhart-Phillips *et al*., 1997; Salfield and Pugh, 1987; Tenkate and Stafford, 2001). This is probably due to contact with canine faeces, and the frequent, and close contacts that occur between dogs and humans (Westgarth *et al*., 2008). The combination of increased *Campylobacter* spp. carriage in younger dogs (Acke *et al*., 2006; Engvall *et al*., 2003; Guest *et al*., 2007; Sandberg *et al*., 2002; Wieland *et al*., 2005), and the increased frequency of contact with puppies (and puppy faeces) found in Westgarth *et al*, (2008) may explain why introducing a new puppy into the house is a risk factor for human campylobacteriosis (Eberhart-Phillips *et al*., 1997; Salfield and Pugh, 1987; Tenkate and Stafford, 2001). Thus, younger dogs are more likely to shed *Campylobacter* spp., and humans are more likely to have increased contact with a young dog.

Several studies have investigated the relationship of *C. jejuni* infection between dogs and humans in order to establish whether or not dogs pose a zoonotic risk of *C. jejuni* infection to humans. Damborg *et al*, (2004) used Pulsed-field gel electrophoresis (PFGE) to examine strains of *C. jejuni* found in both human patients and their dogs, and identified identical *C. jejuni* strains between a two year old girl and her pet dog. Identical *C. jejuni* strains have also been isolated from dogs and humans in other work (Workman *et al*., 2005). Karenlampi *et al*, (2007) found that the ST-45 complex isolated from humans, was significantly associated with contact with pet cats and dogs. Clonal complex ST-45, is considered to be one of the most important sequence types as it is frequently isolated from humans (Colles *et al*., 2003; Dingle *et al*., 2002; Karenlampi *et al*., 2007; Ragimbeau *et al*., 2008; Sopwith *et al*., 2006).

1.9 *Campylobacter upsaliensis*

1.9.1 The Importance of C. upsaliensis Infection in Humans

Campylobacter upsaliensis is predominantly isolated from dogs and to a lesser extent cats (Sandberg *et al*., 2002; Wieland *et al*., 2005; Workman *et al*., 2005). Several studies have isolated *C. upsaliensis* from human stool samples (Byrne *et al*., 2001; Carter and Cimolai, 1996; Goossens *et al*., 1990a; Gurgan and Diker, 1994; Jenkin and Tee, 1998; Jimenez *et al*., 1999; Lawson *et al*., 1999; Patton *et al*., 1989; Prasad *et al*., 2001), with some studies identifying *C. upsaliensis* as the second most common *Campylobacter* spp. isolated from humans after *C.jejuni/coli* (Labarca *et al*., 2002; Lastovica and Le Roux, 2003; Vandenberg *et al*., 2006). *C. upsaliensis* is known to invade human epithelial cells, possibly interacting with cytoskeletal structures, and causes nuclear fragmentation, cell cycle arrest and apoptosis (Mooney *et al*., 2003; Mooney *et al*., 2001).

Although symptoms of *C. upsaliensis* infection in humans may be milder than those caused by *C. jejuni* infection (Goossens *et al*., 1990b; Jimenez *et al*., 1999), it is still associated with the same syndromes that can be brought on by other *Campylobacter* spp. infections as mentioned previously (Carter and Cimolai, 1996; Gurgan and Diker, 1994; Hald and Madsen, 1997; Jimenez *et al*., 1999). In one study, *C. upsaliensis* was the only enteric pathogen isolated from 13 patients with immunodeficiency virus (HIV), causing diarrhoea of a mild to moderate severity, lasting between five weeks and three months (Jenkin and Tee, 1998). With no other significant sources of *C. upsaliensis* presently known, this makes pets, particularly dogs, likely sources of *C. upsaliensis* infection to humans. This is supported by associations found between *C.upsaliensis* infection in humans and dogs living in the same household (Goossens *et al*., 1991; Labarca *et al*., 2002; Lentzsch *et al*., 2004).

The true prevalence of *C.upsaliensis* infection in humans may be underestimated, as most detection methods are optimised for the detection of *C. jejuni* (Byrne *et al*., 2001; Kulkarni *et al*., 2002; Labarca *et al*., 2002; Lastovica and Le Roux, 2003), and if symptoms are milder during *C. upsaliensis* infection, this may result in fewer reported cases.

1.9.2 Transmission of C. upsaliensis Between Dogs and Humans

A recent study by Damborg *et al*, (2008) found no association between the amplified fragment length polymorphism (AFLP) patterns of several *C. upsaliensis* isolates from both humans and dogs, and instead described two distinct groups, one containing only human isolates and one containing mostly canine isolates. However, the origin of the samples used may have played a significant role in these findings. Most of the samples in the ‘dog’ group originated from Denmark and Sweden predominantly between 2000

and 2006, whereas the 'human' group, consisted of isolates from South Africa, Belgium, United Kingdom, Senegal and Denmark, with nearly all samples isolated between 1985 and 1999. Differences in origin can play a significant role, as Lentzsch *et al*, (2004) described distinct genotypic clusters for *C. upsaliensis* samples isolated from different continents, and it could be argued that in the study by Damborg *et al*, (2008), two of the four human UK isolates belonged to the 'dog' group, and that all of the human and dog isolates from Sweden belonged to the same (dog) group. In addition, *C. upsaliensis* isolates originating from a dog and a human have both been shown to invade human epithelial cell lines (Caco-2, T84 and HeLa) in substantial numbers (Mooney *et al*., 2003).

1.10 *Campylobacter* spp. Genomics

Currently there is an MLST scheme and a large MLST database dedicated to *C. jejuni*/*C. coli* (Dingle *et al*., 2001; Jolley and Chan, 2004) and MLST is becoming the gold-standard for typing *Campylobacter* spp. (see section 1.2.1 of this thesis). However, information obtained from isolates based on the full genome, rather than seven loci (MLST) will be considerably more representative for typing and comparative purposes. The genomes of some *C. jejuni* strains, isolated from human clinical cases and also from chicken carcases (NCTC 11168 and RM1221, respectively), have previously been sequenced and are considered complete (Fouts *et al*., 2005; Parkhill *et al*., 2000). The genome of *C. jejuni* RM1221 is 1, 777, 831 bp in length, and putative roles have been assigned to 60% of the open reading frames (ORFS) (Fouts *et al*., 2005). Other *Campylobacter* spp. genomes have been sequenced, but not necessarily completed. These include *C. coli* (RM2228) at 8.5-fold coverage, *C. lari* (RM2100) at 16.5-fold coverage, and *C. upsaliensis* (RM3195) at 9.0-fold coverage (Fouts *et al*., 2005).

Comparison between the genomes of *C. jejuni*, *C. coli* and *C. upsaliensis* has revealed information such as; some differences and similarities in phylogeny, plasmids, metabolism, protein secretion systems, virulence and antibiotic resistance between different *Campylobacter* spp. (Fouts *et al*., 2005). An interesting similarity in this study was that *C. upsaliensis* RM3195 had the third highest amino acid similarity with *C. jejuni* RM1221, after *C. jejuni* NCTC 11168 and *C. coli* RM2100 (Fouts *et al*., 2005). However there were also differences, for example *C. upsaliensis* RM3195 was found to contain two plasmids, unlike either of the two *C. jejuni* strains within this study. Nevertheless, *C. jejuni* plasmids have previously been reported (Bacon *et al*., 2000; Bacon *et al*., 2002; Fouts *et al*., 2005).

Molecular biology may be able to explain differences in virulence, host specificity, and growth rate between *Campylobacter* spp.; for example, and as previously described, *C. jejuni* grows faster than *C. upsaliensis* in culture, has different antibiotic sensitivities, causes symptoms more severe than *C. upsaliensis* in humans, and tends to be found in a greater variety of hosts than *C. upsaliensis*, but the reasons for this are not fully understood (refer to sections 1.6 and 1.7.1 of this thesis). Genome sequencing is particularly useful for *Campylobacter* spp., because this bacteria is prone to intraspecies and interspecies recombination (Sheppard *et al*., 2008; Suerbaum *et al*., 2001), which can make comparisons between isolates difficult.

1.11 *Salmonella*

Salmonella is a Gram negative bacteria with non-sporing rods that belongs to the family Enterobacteriaceae (Hafez and Jodas, 2000). There are two species of *Salmonella*, *S. bongori* and *S. enterica* and the latter species consists of many serovars (Hafez and Jodas, 2000; Leminor and Popoff, 1987; Reeves *et al*., 1989). These serovars have been

divided into three main groups: group one contains highly host-adapted and invasive serovars such as *S.* Pullorum and *S*. Gallinarum found in poultry, and *S*. Typhi found in humans; group two contains non-host adapted, but invasive serovars which possibly infect humans, such as *S*. Typhimurium, *S*. Arizonae and *S*. Enteritidis; group three consists of non-host adapted, non-invasive serovars which represents the majority of *Salmonella* serovars (Hafez and Jodas, 2000).

1.11.1 The Importance of Salmonella in Humans

Salmonella spp. infection in humans is often associated with self-limiting diarrhoea, fever, and abdominal pains (CDC, 2008a; DEFRA, 2007). Although *Salmonella* spp. infection is important, generally the majority of studies have reported that *Campylobacter* spp. are the most commonly reported bacterial cause of gastroenteritis in humans (Adak *et al*., 2002; DEFRA, 2007; Westrell *et al*., 2009). Surveillance data combined from ten states in America in 2008, indicated that *Salmonella* spp. were the most common laboratory-confirmed foodborne pathogen reported in humans, with an incidence of 16.20 per 100, 000 of the population (CDC, 2008c). In the UK, 13, 213 human cases of *Salmonella* infection were reported during 2007 and the most commonly identified serotypes found in humans were *S*. Enteritidis and *S*. Typhimurium (DEFRA, 2007). *Salmonella* serovar Enteritidis infections are usually associated with consumption of raw, or lightly cooked foods containing eggs or chicken (Braden, 2006; Currie *et al*., 2005; DEFRA, 2007).

1.11.2 Animals as a Source of Salmonella

Salmonella enterica has been isolated from many animals such as chickens, cattle, sheep, pigs, horses, dogs, and reptiles (Hidalgo-Vila *et al*., 2008; Oloya *et al*., 2009; Oloya *et al*., 2007; Snow *et al*., 2008). Within most of these animals, serovar

Typhimurium appears to dominate, although in the UK *S.* Dublin is found commonly in cattle, *S*. Enterica Diarizonae has been commonly reported in sheep, and *S.* Enteritidis was reported as the most common serovar found in chickens from Great Britain in 2007 (DEFRA, 2007; Oloya *et al*., 2009; Oloya *et al*., 2007). There have been several cases of human salmonellosis associated with animals, particularly reptiles, including an outbreak amongst children attending a reptile exhibit at a zoo (CDC, 2003; Friedman *et al*., 1998), and between handling pet rodents and salmonellosis in humans (Hargreaves, 2007; Swanson *et al*., 2007).

1.11.3 Salmonella Carriage in Dogs

1.11.3.1 Salmonella Serovars

Salmonella has been isolated from dogs within various populations, although there appears to be no one dominant serovar isolated between studies. *Salmonella* serovars Typhimurium, and Newport were either the most common, or second most common serovars found in dogs from several studies (Bagcigil *et al*., 2007; Fukata *et al*., 2002; Hald *et al*., 2004; Oloya *et al*., 2007; Seepersadsingh *et al*., 2004), although other serovars such as *S*. Javiana, *S*. Arechavaleta, *S*. Montevideo, *S*. Give, *S*. Corvallis, *S*. Enteritidis and *S*. Duesseldorf have dominated more than *S.* Typhimurium and/or *S.* Newport in some instances (Bagcigil *et al*., 2007; Fukata *et al*., 2002; Kocabiyik *et al*., 2006; Schotte *et al*., 2007; Seepersadsingh *et al*., 2004; Tsai *et al*., 2007). Dogs are known to have close contact with humans, and often spend time sleeping and eating in kitchen areas where food is prepared (Westgarth *et al*., 2008), they too could pose a zoonotic risk to humans when they shed *Salmonella* spp.

1.11.3.2 Salmonella Prevalence in Dogs

The prevalence of *Salmonella* spp. isolated from dogs within different dog populations tends to vary. Studies based upon household dogs have found the prevalence can range from 1.1% to 15.4% (Fox *et al*., 1983; Hald *et al*., 2004; Tsai *et al*., 2007), and interestingly this latter study found that the prevalence of *Salmonella* spp. in dogs under 6 months old was 36.9%.

However, higher carriage rates have been reported in other populations of dogs in certain situations. Stray dogs have been consistently identified with higher carriage rates of *Salmonella* spp. compared to other populations (Kocabiyik *et al*., 2006; Seepersadsingh *et al*., 2004; Tsai *et al*., 2007). Although a study comparing 100 vet visiting, and 100 kennel dogs only isolated *Salmonella* spp. once from each group (Bagcigil *et al*., 2007), and in some studies, little or no *Salmonella* spp. have been isolated from diarrhoeic dogs (Fox *et al*., 1983; Hackett and Lappin, 2003). *Salmonella* spp. have been found in 69% of healthy sled dogs (Cantor *et al*., 1997), and during an outbreak in a kennel, the prevalence per sampling day ranged from 5.6 to 77.8%, with two sources of dehydrated dog food suspected as the sources of infection (Schotte *et al*., 2007). *Salmonella* spp. have been isolated from raw dog food and dogs that were fed raw food containing *Salmonella* spp. have been shown to subsequently shed the same *Salmonella* serovar (Finley *et al*., 2008; Finley *et al*., 2007). Dog food/treats have even been implicated in human cases of salmonellosis (Pitout *et al*., 2003).

1.12 Aims of This Thesis

To investigate the epidemiology and risk factors of *Campylobacter* spp. carriage in dogs, in an attempt to assess the potential risk to dogs, and zoonotic risk posed by them to humans. In order to do this, the following were investigated:

- The prevalence and species distribution of *Campylobacter* spp. in different dog populations within the UK; vet-visiting, household dogs boarding at kennels, rescue/stray dogs in kennels, and dogs from hunting kennels.
- Risk factors for *Campylobacter* spp. carriage in dogs.
- Shedding patterns of *Campylobacter* spp. carriage in kennelled dogs observed through longitudinal studies.
- The molecular epidemiology *C. jejuni* and *C. upsaliensis* via the use of MLST (*C. jejuni* and *C. upsaliensis*), PFGE (*C. jejuni*), and a pilot study investigating a *C. upsaliensis* genome, and more specifically a plasmid.
- In addition, all dog populations were screened for the presence of *Salmonella* spp. to investigate whether or not dogs are a significant reservoir of this bacterium for humans.

2. Chapter two

General Materials and Methods

This chapter explains the methods for all laboratory procedures used for the studies described in this thesis. Study designs are included within their relevant chapters, along with details regarding the methods chosen for particular studies.

2.1 *Campylobacter* Culture from Faecal Samples

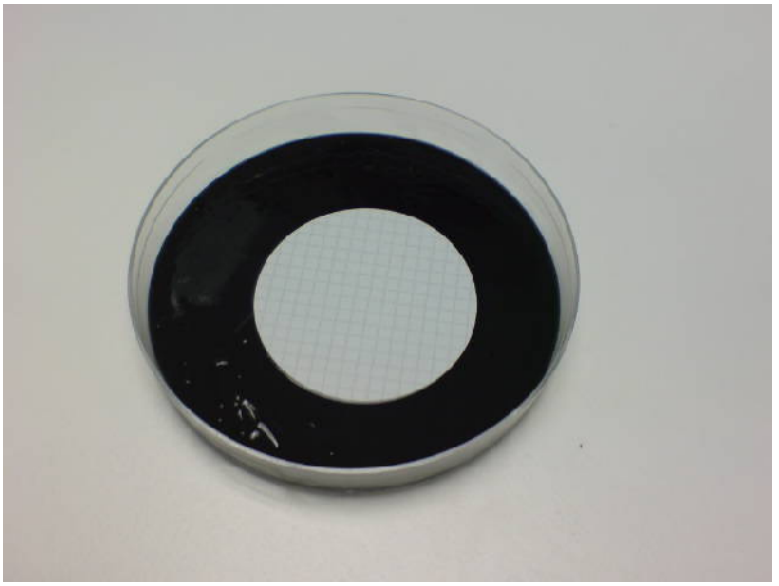
On arrival at the lab faecal samples (including diarrhoeic samples), were diluted 1:10 in 0.85% saline. Each sample was then subject to potentially three methods for *Campylobacter* spp. isolation:

i) Direct plating; using a 5µl loop, onto *Campylobacter* selective agar, modified cefoperazone deoxycholate agar (mCCDA) (Lab M) with the addition of cefoperazone and amphotericin (CA) (Lab M).

ii) Filtration; one to three drops (three drops when samples were diarrhoeic or appeared relatively dilute) of saline–diluted sample through a 0.7 µm nitrocellulose membrane for 10 minutes onto *Campylobacter* selective agar (mCCDA) with the addition of cefoperazone, amphotericin and teicoplanin (CAT) (Oxoid Ltd) supplement, before removal of filter (Plate 2.1).

iii) Enrichment; five drops of the saline–diluted sample were added to 4ml of *Campylobacter* enrichment broth (Lab M) along with 10% lysed horse blood (Southern Group Labs Ltd) and incubating for 24 h prior to inoculation onto *Campylobacter* selective agar as previously in (i).

Plate 2.1. 0.7 µm nitrocellulose membrane on *Campylobacter* selective agar with CAT supplement.



All plates were incubated for 96 h at 37ºC under microaerophilic conditions with an atmosphere of N_2 (74%), O_2 (11%), H_2 (3%) and CO_2 (12%), in a variable atmosphere incubator (Don Whitely Scientific Ltd). Up to four suspect *Campylobacter* spp. colonies (1-3 mm diameter, round, white, cream or silver in colour Plate 2.2) from different locations on each plate were sub–cultured onto Columbia blood agar (CAB) plates containing 5% defibrinated horse blood (Southern Group Labs) and were incubated for a further 48 h. If there were different morphological features observed with the colonies on the plates, such as differences in size, then colonies consisting of the various sizes were chosen where possible. A 'sweep' (attempts were made to include every colony or the majority of colonies on the plate using a 5µl loop) of the whole plate was also taken and sub-cultured onto CAB plates. The sweep was not intended for molecular work but was taken to ensure no other species of *Campylobacter* were overlooked. Suspect *Campylobacter* colonies were collected using a 5µl sterile loop, suspended in 100µl of phosphate buffered saline (PBS) and heated at 100 ºC for 10 minutes to provide cell lysates for use in the subsequent PCR reactions. All isolates that

reached this stage were frozen down in microbank tubes™ (Prolab diagnostics) at -80 ºC.

Plate 2.2. *C. upsaliensis* colonies on *Campylobacter* selective agar with CAT supplement, after prior filtration.

2.1.1 Media

Three different culture methods were chosen for the work within this thesis to maximise recovery, and although blood based agars have been used for initial *Campylobacter* spp. isolation in several studies, particularly with filters (Acke *et al*., 2009; Burnens *et al*., 1992; Koene *et al*., 2004; Modolo and Giuffrida, 2004), they were not used to isolate *Campylobacter* spp. for any study within this thesis, but instead were used to encourage growth after isolation from other non-blood based culture methods. One reason for this selection is because *Campylobacter* spp. colonies tend to swarm blood-based agars more than charcoal-based agars (Karmali *et al*., 1986). Therefore charcoal-based agars were more suitable because pure colonies were desired. As described in chapter one,

charcoal-based agars have demonstrated high degrees of success in the isolation of *Campylobacter* spp. Additionally CAT supplement was chosen specifically to detect *C. upsaliensis*, the most common *Campylobacter* spp. found in dogs (Rossi *et al*., 2008; Sandberg *et al*., 2002).

2.1.2 Filtration

Various success rates have been reported with the use of filters. Since the samples in this study were faecal, the number of contaminants present in the samples was likely to be high, so a filter, which allows passage of small, motile bacteria such as *Campylobacter* spp., was included in an attempt to reduce contamination. Several studies have found that the use of a filter improved the isolation of *Campylobacter* spp. (Modolo and Giuffrida, 2004; Moreno *et al*., 1993), particularly *C. upsaliensis* (Aspinall *et al*., 1996), and reduced contaminants (Korhonen and Martikainen, 1990).

2.1.3 Enrichment

Some studies have found an enrichment stage to be more successful in detecting *Campylobacter* spp. than other isolation methods (Fleming, 1983; Maher *et al*., 2003). For this reason, and despite varying results from different studies, an enrichment stage was initially included for the isolation of *Campylobacter* spp. to ensure maximum recovery of all *Campylobacter* spp.

2.1.4 Incubation Time and Temperature

Despite the fact that *C. jejuni* can be cultured in 48h, *C. upsaliensis* usually takes 96h to grow (Byrne *et al*., 2001; Labarca *et al*., 2002; Moreno *et al*., 1993), so samples described within this thesis were incubated for a minimum of 96h. This incubation period is also suitable for other *Campylobacter* spp. such as *C. coli* and *C. lari* (Hald *et*

al., 2004; Moreno *et al*., 1993). The culture methods described in this thesis were chosen on the basis that they would detect any *Campylobacter* spp., not just *C. jejuni* and *C. upsaliensis*.

Samples were incubated at 37°C for all the studies within this thesis because despite some previous studies using an incubation temperature of 42°C, 37°C tends to be used most commonly (Burnens and Nicolet, 1992; Engvall *et al*., 2003; Hald and Madsen, 1997; Koene *et al*., 2004; Malik and Love, 1989; Modolo and Giuffrida, 2004; Rossi *et al*., 2008; Steinhauserova *et al*., 2000; Workman *et al*., 2005), and there does not appear to be any substantial difference between these two temperatures in terms of the frequency of *Campylobacter* spp. isolation (Engvall *et al*., 2003; Hald *et al*., 2004; Rossi *et al*., 2008). Additionally, an incubation temperature of 42°C would limit the detection to only thermotolerant *Campylobacter* spp., excluding and reducing detection of *C. hominis* and *C. upsaliensis* respectively, whereas the majority of *Campylobacter* spp., such as *C. upsaliensis*, *C. jejuni*, C. *coli, C. fetus, C. lari* and *C. hyointestinalis* will grow at 37°C (Acke *et al*., 2009; Corry *et al*., 1995).

2.2 Extraction of DNA from Faecal Samples with Subsequent PCR 'Direct PCR'

As described in chapter one, direct PCR has been used by other studies to detect *Campylobacter* spp. and has been shown to detect mixed *Campylobacter* spp. carriage (Lawson *et al*., 1999). Persson and Olsen, (2005) recommended that direct PCR was useful for detecting non-culturable bacteria which may have been exposed to unfavourable conditions during transport. Therefore, in an attempt to detect any viable but non-culturable *Campylobacter* spp., direct PCR was performed in addition to culturing in most of the studies within this thesis, with particular importance assigned to

those studies (Chapter 3) which included samples being sent by standard post and therefore such samples may not have been fresh.

2.2.1 DNA Extracted Directly from Faecal Samples

Faecal suspensions were prepared as a 1:10 dilution in viral transport media (VTM) and clarified by centrifugation for 10 minutes at 4000 × *g*. They were stored at -80°C and when required, samples were defrosted and chosen in no particular order. Bacterial DNA was extracted from 140µl of each faecal suspension (previously frozen at -80°C) using a QIAamp® Viral RNA Mini kit (QIAGEN Ltd), according to the manufacturer's instructions and four negative controls of high grade molecular water (Sigma) were included for every 20 samples The purified DNA was eluted in 60µl buffer AVE (RNAase-free water containing 0.04% sodium azide). Samples were stored at -20°C, although PCR was performed on these samples no later than a month after the initial extraction. An RNA kit that extracted both DNA and RNA, was used because the samples were also required for another study on canine corona virus (CCV).

2.3 *Campylobacter* Species Identification for Culture and Direct PCR

A series of PCR assays targeting selected genes were performed to determine the species of *Campylobacter.* A 16S rRNA encoding gene (Linton *et al*., 1996) and *glyA* (Wang *et al*., 2002) gene fragments were utilised for *C. upsaliensis* identification. For the identification of *C. jejuni*, amplification of a *hipO* gene fragment was used (Wang *et al*., 2002). All isolates, whether cultured or extracted directly from faeces, were subjected to these three specific PCR identification assays. Suspect *Campylobacter* spp. colonies which appeared negative in *C. upsaliensis* and *C. jejuni* PCR assays were further analysed by an assay targeting a partial *groEL* gene, optimized to detect the majority of *Campylobacter* spp. (Karenlampi *et al*., 2004).

Table 2.1. Primers used for *Campylobacter* spp. identification. Bases in bold indicate primers used for sequencing.

Species	Locus	Primer	Sequence (5'-3')
C. upsaliensis	16SrRNA	LintonF (forward)	GGGACAACACTTAGAAATGAG
		LintonR (reverse)	CACTTCCGTATCTCTACAGA
C. upsaliensis	*glyA*	WangF (forward)	AATTGAAACTCTTGCTATCC
		WangR (reverse)	TCATACATTTTACCCGAGCT
C. jejuni	*hipO*	HipoF (forward)	ACTTCTTTATTGCTTGCTGC
		HipoR (reverse)	GCCACAACAAGTAAAGAAGC
Campylobacter spp.	*groEL*	M13H60F (forward)	**GAGCGGATAACAATTTCACACA** GGNGAYGGNACNACNACNGCNAC NGT
		T7H60R (reverse)	**TAATACGACTCACTATAGGG**TC NCCRAANCCNGGNGCYTTNACNG

2.3.1 PCR Procedure

The PCR procedures were based on a protocol previously optimised, particularly for the partial *groEL* gene (Karenlampi *et al*., 2004; Westgarth *et al*., 2009), consisting of 41µl Master mix 2.5mM MgCL according to the manufacturers instructions (ABgene™), with the primers as in Table 2.1. Primers were made to a concentration of 15 picomolars per microlitre, and added at a volume of 3µl each for all PCR assays, including *glyA, hipO* and the 16S rRNA encoding gene, and 47µl of master mix with primers was added to each reaction, with 3µl DNA, resulting in a 50 µl reaction. The species specific PCR assays, i.e. *glyA*, 16S rRNA and *hipO* each consisted of 41 µl 1.1x reddyMix™ PCR Master Mix (1.5mM $MgCl_2$) according to the manufacturers instructions (ABgene™), with their specific primers (3µl each) as in Table 2.1. The cycling parameters for all assays in table 2.1 included an initial denaturation at 95°C for 2 minutes, 40 amplification cycles with denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute, extension at 72°C for 3 minutes and a final incubation at 72°C for 5 minutes.

2.3.2 Electrophoresis

Samples were then run on 1% agarose gels in 1×TAE buffer consisting of 40 ml stock buffer (Tris-acetate-EDTA 50× 242g Tris, 57.1mls glacial acetic acid and 100mls 0.5M EDTA to 1 litre of water) and 1960ml double distilled water, with 5µl of Ethidium bromide (500µg/ml Sigma) added for every 100ml of TAE buffer. Marker consisted of 172µl distilled water, 38µl loading buffer and 15µl ØX 174 marker (ABgene™). The loading buffer was added at 1µl per sample for the *groEL* assay before loading the samples onto a gel, and gels were run for 30-46 minutes at 120-150V, depending upon the size of the gel, and visualised under UV light.

2.4 Purification of PCR Products for Sequencing

Initially a QIAquick® PCR gel Extraction Kit (QIAGEN) was used to purify products because of the excess primer in the samples, but a QIAquick® PCR Purification Kit (QIAGEN) was later found to be just as effective and quicker. The protocols for both kits were followed according to the manufacturer's instructions, using a microcentrifuge. Samples were eluted in 30µl elution buffer for both kits and stored at 4°C before usage which was typically less than 24hours later.

2.4.1 Polyethylene Glycol 'PEG' Precipitation

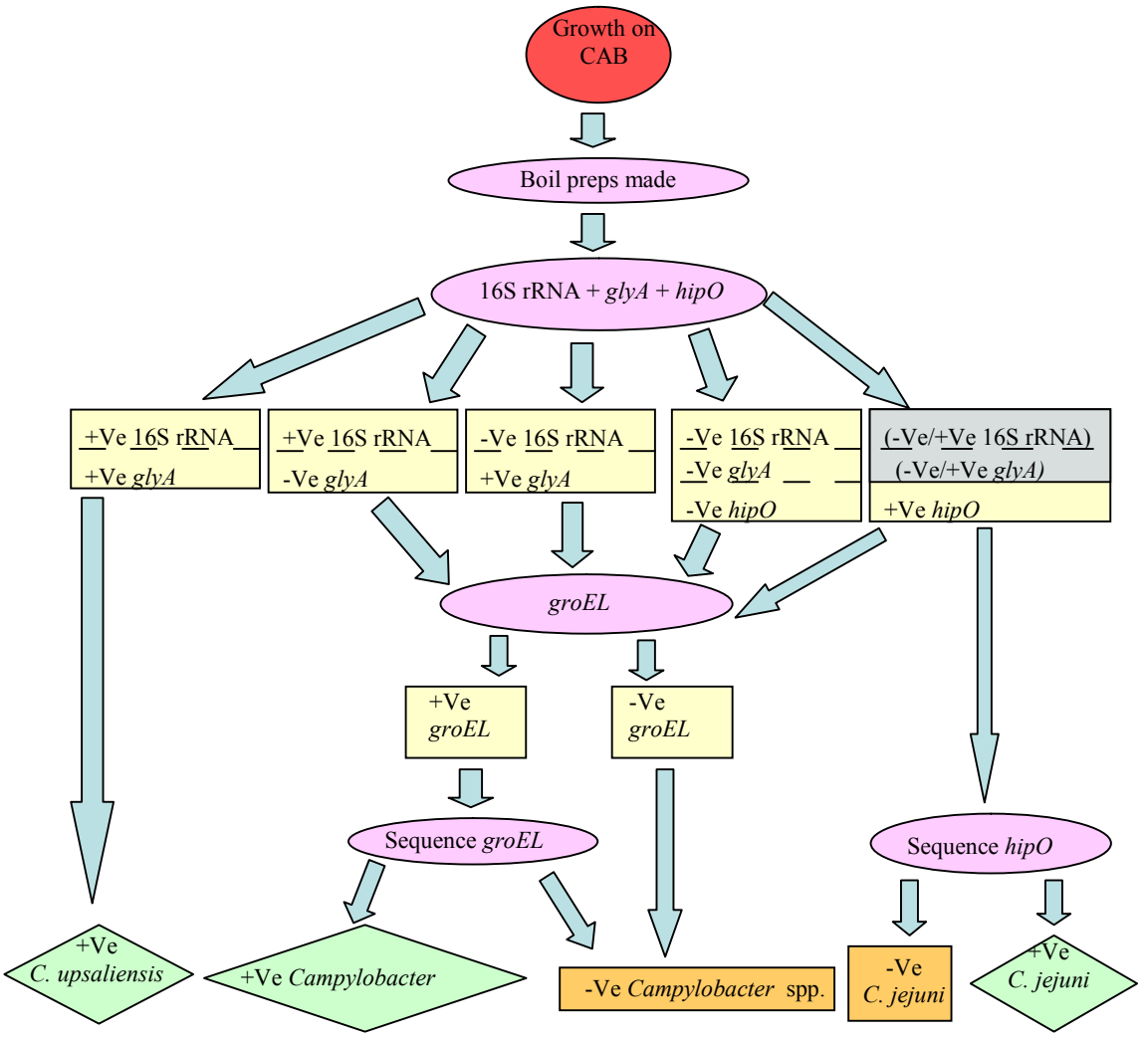
PEG precipitation was used for the purification of PCR products from the majority of studies mentioned in this thesis, with the exception of the vet-visiting cross-sectional (Chapter 3). This method could potentially process four to eight 96 well plates at once if necessary. This method was PEG precipitation which involved adding 60µl of 20% (w/v) PEG_{8000}, 2.5M NaCL to each sample which had previously undergone PCR. Samples (in a 96 well plate) were mixed using a vortex and spun at 500 rpm. The sample mixture was incubated for 15 minutes at 37°C before being spun at 2750 rcf at

4°C for 60 minutes. The PEG was then removed by inverting the samples onto tissue and spinning the inverted plate briefly at 500 rpm for 60 seconds. The pellets were then washed with 150µl 70% ice cold ethanol and spun at 2750 rcf for 10 minutes. The ethanol was removed by another inversion of the samples onto blue tissue and a brief spin. The pellets were then washed with ethanol a second time, and inverted onto tissue a second time as mentioned above. Samples were then left to air dry for 10 minutes. Molecular grade water was then added to the samples before a final vortex and brief spin at 500 rpm for 60 seconds. The amount of molecular grade water added to each sample depended upon the brightness of the DNA on the gel picture produced earlier after the PCR stage. This varied from 10µl for weak bands to 30µl for strong bands, however, the average amount of water added to most of the samples was 25µl as this provided the optimum volume to be sent for sequencing without diluting the DNA below 5 ng/µl, which would have been too low to sequence. A final gel could then be run to ensure samples contained enough DNA for sequencing.

2.5 Sequencing

Culture isolates positive for both the 16S rRNA encoding and *glyA* genes on PCR were considered *C. upsaliensis*, and no further sequencing confirmation was carried out (Fig 2.1.). However, if isolates were negative on one or both of these assays, they were further analysed by PCR amplification of the *groEL* gene (Karenlampi *et al*., 2004), followed by sequencing of the amplicon. *C. jejuni* isolates identified by PCR of the *hipO* gene fragment, were amplified and sequenced using both the *groEL* and *hipO* products (Fig 2.1) to ensure confirmation. Isolates that did not appear to be *C. upsaliensis* or *C. jejuni*, as they were negative on 16S rRNA, *glyA* and *hipO* PCR assays, were also sequenced by targeting the partial *groEL* gene (Fig 2.1).

Figure 2.1. Flow chart demonstrating PCR and sequencing procedure for every suspect *Campylobacter* spp. isolate successfully grown on CAB media.



Key

Procedure: preparing boil prep, PCR assay, or sequencing

Result of a procedure

Confirmation of the presence of *Campylobacter* spp.

The procedure for PCR assays and sequencing was similar for direct PCR, except that sequencing the partial *groEL* gene from direct PCR products was unsuccessful, so the species specific primers that yielded a positive result, i.e. 16S rRNA, *glyA*, and/or *hipO*, were used for sequencing. Sequencing was only performed on amplicons derived from direct PCR samples that did not yield *Campylobacter* spp. on culture.

After purification of PCR products (derived from culture and or direct PCR), purified DNA was sent at a minimum concentration of 5 ng/µl, plus 15pmol primer in a minimum volume of 15 µl and were sent to MWG Bio-tech UK and MACROGEN Korea. Forward and reverse primers were both used for each sample.

2.5.1 In House Sequencing

In house sequencing was also carried out, but results were not as reliable as out sourcing the sequencing. A BigDye® Xterminator™ Purification kit was used in an attempt to reduce unincorporated dye terminators (dye blobs), but this was only occasionally successful and did not increase the length of the product, neither did extending the run time of the sequence reaction. For these reasons the external sequencing labs mentioned above were used for sequencing.

2.5.1.1 Procedure

A master mix containing 2.38µl molecular grade water, 1.87µl 5x buffer, 0.25µl Big Dye and 4µl of forward or reverse primer (0.67µM), per reaction, was added to 1.5µl purified PCR product. Samples/reactions were mixed using a vortex and spun briefly (500rpm) before undergoing the following conditions; 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 2 minutes, which was run for 30 cycles. A mixture of 7000µl 100% ethanol and 280µl 3M sodium acetate was made per 96 well plate, and 52µl was added

to each sample. The samples/reactions then underwent a vortex and a brief spin (500 rpm) before being incubated at room temperature for 45 minutes and then spun at 2750 rcf (4°C) for 1 hour. The samples/reactions were then inverted onto absorbent tissue and spun for 1 minute (500 rpm). The DNA pellet was then washed once by adding 150µl ice-cold ethanol to each sample/reaction, and spinning them at 2750 rcf for 10 minutes. The samples/reactions were again inverted onto absorbent tissue and briefly spun. The samples/reactions were left to air dry for 10 minutes before 10µl HiDi (formamide) was added to each sample/reaction. This was followed by a vortex and a brief spin. Samples/reactions were denatured for 2 minutes at 94°C, after which they were allowed to cool and then loaded onto the sequencer (HITACHI Applied Biosystems 3130×1 and 3100 capillary array) using either a long run or a short run depending upon the size of the expected product, e.g. *groEL* had a longer run time than other PCR products.

2.5.1.2 Xterminator Purification

A BigDye® Xterminator™ Purification kit was used in an attempt to improve the quality of the read for the in house sequencing. It was used in place of the ethanol wash mentioned previously in the protocol for in house sequencing. For a 10µl reaction size, 45µl SAM™ Solution and 10µl BigDye® Xterminator™ were added to the sample and mixed for 30 minutes using a vortex. The samples were then centrifuged at 1000×g for 2 minutes before being analysed by the sequencer.

2.5.2 Sequence Analyses and Phylogenetic Trees

Forward and reverse sequences were checked and complemented using Chromas pro Version 1.34 Copyright © 2003-2006 Technelysium Pty Ltd. A basic alignment search tool (BLAST http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) was used to confirm *Campylobacter* spp. Neighbour-joining trees were produced using the program

Molecular Evolutionary Genetic Analysis, version 3.1 (MEGA) Copyright © 1993-2005 Sudhir Kumar, Koichiro Tamur and Masatoshi Nei. Maximum-likelihood trees were produced using a phylogeny inference package (Phylip) version 3.68 August 2008, Joseph Felsenstein, and all Bootstraps were conducted with 1000 replicates. Trees produced in Phylip were visualised in MEGA, with one exception (Appendix 4; Fig 4.9) where FigTree version 1.2.3, 2006-2009, Andrew Rambaut, Institute of Evolutionary Biology, University of Edinburgh, was used.

2.6 Bacterial Enumeration

Spiral plating was used for quantification of *Campylobacter* spp. One gram of a neat faecal sample was weighed, and a 1:10 dilution was made by adding 9g of buffered peptone water (BPW, Lab046A, LabM, Bury UK) in a stomacher bag. Samples were homogenized in a Cloworth 80 stomacher (A.J. Seward & Co. Ltd., London, UK) for 30 seconds, after which the supernatant was poured into a universal tube, whilst the sediment was left in the bag. The supernatant was plated out onto CAT plates using a Whitley Automatic Spiral plater ('WASP 2'; Don Whitely Scientific Limited, Shipley, UK) set in a logarithmic mode, dispensing 50µl. The dispenser was washed in two separate sterile water pots, and disinfectant between each sample. Fresh disinfectant was made on a regular basis, and new sterile water was supplied every day. Plates were then incubated in a VAIN, as mentioned previously, and left to grow for $\geq$ 96 hours. The colonies were then counted using a colony counter (Stuart Scientific), and a bacterial count was obtained in accordance with the manufacturers instructions (WASP 2'; Don Whitely Scientific Limited, Shipley, UK).

2.6.1 Confirmation of Suspect Campylobacter spp. *Colonies*

Five representational colonies from each plate were transferred to CAB plates and grown in a VAIN for 48 hours. Attempts were made to select colonies on the basis of their morphological features and distribution across the plate, e.g. if the plate consisted of predominantly small colonies with three to four large colonies, then four small colonies from various locations, and one large colony would be selected. The procedure was based on the methods of a previous study which enumerated *Escherichia coli* from cattle faeces (Robinson *et al*., 2004). Apart from different bacteria, the main difference in the methods compared to this present study were that five isolates (if possible) were confirmed by PCR for every plate in the present study, as opposed to 10 colonies from a random selection of plates in Robinson *et al* (2004).

Cell lysates were prepared from these colonies (unless they were overgrown with contaminating bacteria) and five isolates were subject to three PCR assays, i.e. *glyA*, 16S rRNA and *hipO*. The isolates were confirmed as either *Campylobacter* spp. positive or negative. If all five isolates were confirmed as *Campylobacter* spp. then the number of colonies that were originally counted was accepted. However, if only four of the five isolates were confirmed as *Campylobacter* spp. then only 80% of the original count would be accepted, and if three of five were confirmed, then only 60% of the original count would be accepted and so on. When there were too many colonies to count, this was interpreted as greater than 4×10^6 CFU/ml per gram of faeces, since this was the maximum number that could be calculated using the dilution and manufacturers instructions (WASP 2'; Don Whitely Scientific Limited, Shipley, UK), which was entered as 4×10^6 for the purpose of producing graphs. At the dilution used (1:10), and with the dispenser set at 50µl, the method was unable to detect less than 200 CFU/ml of *Campylobacter* spp.

The sequencing procedure for isolates positive for *Campylobacter* spp. via spiral plating was the same as described in figure 2.1 for first and last positive samples in kennel 2, but not for intermediate spiral plating samples in both kennels 1&2, where one positive PCR was accepted and no sequencing was carried out.

2.7 Multilocus Sequence Typing 'MLST'

2.7.1 C. jejuni

Purified DNA was sent to Oxford University, Department of Zoology, along with culture in microbank tubes. MLST PCR assays, and assignment of sequence types was done by Allison Cody. At the University of Liverpool, *C. jejuni* isolates, previously identified by PCR, were re-grown onto CAB plates from beads (in microbank tubes) that were previously frozen at -80°C, and grown in a VAIN for 48hours. Cells were harvested and added to 1ml 1xTE buffer (10mM Tris-HCL, 1mM EDTA, pH 8.0) and genomic DNA was extracted using a Wizard® Genomic DNA Purification kit, according to the manufacturers' instructions. Samples were rehydrated by adding 100µl DNA Rehydration Solution and incubation at 65°C for 60 minutes.

2.7.1.1 MLST PCR Assays and Assignment to Clonal Complexes

Internal fragments of seven housekeeping genes (aspartase A, *aspA*; glutamine synthetase, *glnA*; citrate synthase, *gltA*; serine hydroxymethyl transferase, *glyA*; phosphoglucomutase, *pgm*; transketolase, *tkt* and ATP synthase α subunit, *uncA*) were amplified and sequenced as described by Dingle *et al*, (2001)(Table 2.2), with some minor modifications. When no PCR products could be observed at certain loci on agarose gel electrophoresis, primers were substituted for *C. jejuni* and *C. coli* primers described by Miller *et al*, (2005) in order to amplify these loci. Nucleotide sequencing was carried out at least once on each DNA strand using the same primers as those

employed to obtain the amplicon. Alleles, sequence types and clonal complexes were assigned using the MLST database available at http://pubmlst.org/*Campylobacter*.

Table 2.2. **Primers used for the amplification of *C. jejuni* alleles for MLST, adapted from Dingle *et al*., (2001) (primers used for sequencing not shown).**

Locus	Primer	Sequence (5'-3')	Amplicon size (bp)
asp	aspA9 (forward)	AGTACTAATGATGCTTATCC	899
	aspA10 (reverse)	ATTTCATCAATTTGTTCTTTGC	
gln	glnA1 (forward)	TAGGAACTTGGCATCATATTACC	1,262
	glnA2 (reverse)	TTGGACGAGCTTCTACTGGC	
glt	gltA1 (forward)	GGGCTTGACTTCTACAGCTACTTG	1,012
	gltA2 (reverse)	CCAAATAAAGTTGTCTTGGACGG	
gly	glyA1 (forward)	GAGTTAGAGCGTCAATGTGAAGG	816
	glyA2 (reverse)	AAACCTCTGGCAGTAAGGGC	
pgm	pgmA7 (forward)	TACTAATAATATCTTAGTAGG	1,150
	pgmA8 (reverse)	CACAACATTTTTCATTTCTTTTTC	
tkt	tktA3 (forward)	GCAAACTCAGGACACCCAGG	1,102
	tktA6 (reverse)	AAAGCATTGTTAATGGCTGC	
unc	uncA7 (forward)	ATGGACTTAAGAATATTATGGC	1, 120
	uncA2 (reverse)	GCTAAGCGGAGAATAAGGTGG	

2.7.2 C. upsaliensis

All MLST PCR assays for *C. upsaliensis* were carried out at the University of Liverpool. Isolates were re-grown from frozen (-80°C) onto CAB plates for 48 hours. A Chelex-100 protocol was then used to extract the bacterial DNA, because it prevents degradation of DNA by chelating metal ions, that may otherwise act as catalysts in the

break down of DNA under high temperatures such as boiling (Walsh *et al*., 1991). After initial growth of cells onto CAB plates, cells were harvested and suspended in 300 µl of Chelex solution (20% (w/v) Chelex-100 in 10mM Tris-HCL, 1mM EDTA, pH 8.0). The suspension was incubated at 95°C for 10 minutes, followed by centrifugation at 10K rpm for two minutes. The supernatant was removed and diluted 1:10 for use in further PCR reactions.

2.7.2.1 PCR procedure for C. upsaliensis MLST

The protocol for the MLST method was an adaptation of the methods suggested by Miller *et al*, (2005). Each reaction contained 41µl Master mix 2.5mM MgCL according to the manufacturers instructions (ABgene™), 3µl of forward, and 3µl of reverse primer, each at a concentration of 15 picomolars per microlitre, with 3µl of bacterial DNA added to make a 50µl reaction. Seven sets of primers were used for each isolate (Table 2.3). The cycling parameters were as follows; 30 seconds at 94°C, 30 seconds at 53°C, and 2 minutes at 72°C for 30 cycles. Amplicons were examined via gel electrophoresis and purified using PEG precipitation as described previously. Samples were sequenced using the same primers in table 3 and were sequenced by MACROGEN Korea sequencing lab, and analysed using Chromas pro Version 1.34 Copyright © 2003-2006 Technelysium Pty Ltd. Sequences were then submitted to the *C. upsaliensis* MLST database (http://pubmlst.org/cupsaliensis/) to determine whether or not the allele already had a known allele number and/or sequence type. Sequences representing new alleles and also sequence types were sent to William Miller (United States Department of Agriculture, Agricultural Research Service, Western Regional Research Centre) for submission to the database, where a sequence type and clonal complex was assigned.

Table 2.3. Primers used for the amplification and sequencing of *C. upsaliensis* alleles in MLST, table adapted from Miller *et al*, (2005).

Locus	Primer	Sequence (5'-3')	Amplicon size (bp)
adk	adkF (forward)	TGAAAGAATTRTTTTTAATCATAGG	545-546
	adkR (reverse)	CTTTCATRTCWGCHACGATAGGTTC	
asp	aspAF2 (forward)	GAAGCWAAAGCWAAAGAATAYAAAGAT	690
	aspAR2 (reverse)	GAGTTTTTTGCAWGCTTCWGGATT	
atpA	atpAF (forward)	GWCAAGGDGTTATYTGTATWTATGTTGC	700
	atpAR (reverse)	TTTAADAVYTCAACCATTCTTTGTCC	
glnA	glnAF (forward)	TGATAGGMACTTGGCAYCATATYAC	751
	glnAR (reverse)	ARRCTCATATGMACATGCATACCA	
glyA	glyAF (forward)	ATTCAGGTTCTCAAGCTAATCAAGG	716
	glyAR (reverse)	GCTAAATCYGCATCTTTKCCRCTAAA	
pgi	pgiF2 (forward)	TTTAGTGGGWATGGGTGGKTCAAGT	660
	pgiR3 (reverse)	TCTCTAGCACCAATGAGAGCTATGG	
tkt	tktF1 (forward)	GCAAAYTCAGGMCAYCCAGGTGC	730
	tktR (reverse)	TTTAATHAVHTCTTCRCCCAAAGGT	

2.8 Macro-restriction Pulsed-field Gel Electrophoresis

The macro-restriction pulsed-field gel electrophoresis (PFGE) method was a modified version of the protocol by Ribot *et al*, (2001). Cells of *C. jejuni* were harvested from CAB plates into 2ml sterile Phosphate buffered saline (PBS) in sterile 7ml plastic bijou bottle. Cell density was measured in a 3ml optical cuvette at 610nm, in a spectrophotometer set with a range of '0-2'. A "*Campylobacter* PFGE Absorbance Calculator" Excel spreadsheet was used to calculate the ratio of culture and PBS needed

to result in a bacterial optical density (OD) of OD_{610} 0.4, and a total volume of 400µl. This was then transferred to a fresh Eppendorf tube containing 25µl of a 20mgml^{-1} Proteinase K solution (Sigma) in sterile water (Molecular grade water -Sigma), and was mixed gently. PFGE grade agarose (Bio-Rad) 1% in 1xTE buffer (TE), was added (400µl) to the eppendorf and mixed briefly by pipetting, and transferred to duplicate plug moulds. The plugs were then set at 4°C. Plugs were transferred to sterile 5ml bijoux containing 3ml Cell Lysis Buffer (CLB- 50mM Tris, 50mM EDTA, 1%[w/v] N-lauryl sarcosine, pH 8.0) containing 25µl 20mgml^{-1} Proteinase K and were incubated with shaking at 54°C for 15 minutes. Plugs were washed four times at 54°C for 20 minutes; once with 3ml sterile distilled water, and three times with 3ml TE x1. They were then washed once in 500µl 0.1x TE buffer for 20 minutes at 25°C. Blocks were equilibrated in 200µl 1x Restriction endonuclease & buffer (Sigma) (restriction buffer *Sma*I) for 20 minutes at 25°C. DNA was then digested in 200µl 1x restriction buffer (Violet for *Sma*I) containing 40U *Sma*I for 2 hours at 25°C. A gel was run (150ml 1% PFGE agarose in 0.5x TBE); with an initial switch time of 6.7s, and a final switch time of 38.3s, with a total run time of 16 hours. The gel was stained in ethidium bromide solution, and examined under UV illumination

2.8.1 Analysis

PFGE gels were analysed using BioNumerics V. 4.01 software (Applied Maths, Krtrijk, Belgium) with the Dice similarity coefficient, 0.5% optimisation and 1% tolerance, and dendrograms were done using unweighted-pair group method with average linkages (UPGMA).

2.9 Genome sequencing

Full genome sequencing was carried out on a canine isolate of *C. upsaliensis* (dog 52A). The isolate was inoculated onto a CAB plate and grown in the VAIN for 48 hours. A Wizard® Genomic DNA Purification kit was used to isolate the genomic DNA of the bacteria. The DNA was then sent to the school of Biological sciences, Liverpool University, where Kevin Ashelford and Alistair Darby sequenced the genomic DNA. A Genome Sequencer™ FLX (454 Life Sciences™)(Droege and Hill, 2008) was used to sequence the bacterial DNA.

2.9.1 Sequencing

Genomic DNA Preparation and Sequencing Pyrosequencing was performed by generating a standard fragment and paired-end single-stranded template DNA library using the GS DNA Library Preparation Kits (Roche Applied Sciences) that were then amplified by emPCR and sequenced on a GS-FLX (454 Life Sciences). The 454 reads were assembled with Newbler (v1.1.03.24) using default assembly parameters.

2.9.2 Sequence Analysis, Annotation and Comparative Genomes

Assembly was performed with newbler (Roche, USA) and gap4 (http://staden.sourceforge.net). Protein-coding genes were identified with GLIMMER (Delcher *et al*., 1999) and GENEMARK (Lukashin and Borodovsky, 1998); and tRNA genes by tRNAscan-SE (Lowe and Eddy, 1997). Putative functions were inferred using BLAST against the National Center for Biotechnology Information databases (Altschul *et al*., 1990), InterProScan (Hunter *et al*., 2009). Metabolic pathways were examined by using the SEED (Overbeek *et al*., 2005) and KEGG databases (Kanehisa and Goto, 2000). Pathway figures were constructed using IPATHm (Letunic *et al*., 2008). Artemis v11 was used to organize data and facilitate annotation (Rutherford *et al*., 2000). Repeat

identification was made using MUMmer (Kurtz *et al*., 2004). Orthologs were defined using ORTHOMCL (Li *et al*., 2003).

2.9.2.1 Phylogeny

The phylogeny was reconstructed using orthologous gene sets identified from other bacterial genomes using ORTHOMCL (Li *et al*., 2003), aligned with MUSCLE (Edgar, 2004) and trimmed with GBLOCKS (Castresana, 2000). Gene alignments were then concatenated and maximum likelihood trees calculated by JTT, estimated transition/transversion ratio, fix proportion of invariable sites using PHYML (Felsenstein J. 1993. PHYLIP PHYLogeny Inference Package version 3.6a2, Distributed by the author, Department of Genetics, University of Washington, Seattle, WA.), 1000 boot replicates were performed. The Bayesian MC3 approach was implemented in MrBayes v3.1 (Huelsenbeck and Ronquist, 2001).

2.9.2.2 Primer Design- Closing Gaps

Two possible plasmids were located within *C. upsaliensis* 52A and primers were designed to obtain the unknown sequences within the plasmids (gaps). Primers were initially designed by hand, approximately 20 bases in length, and primer sites were chosen at least 40 bases before or after (depending upon the primer being forward or reverse) the required sequence i.e. gap. Primers were then checked for suitability using a website called Oligo Calc: Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html). A program called Primer 3 was subsequently used to design primers (http://frodo.wi.mit.edu/). DNA had previously been extracted from isolate dog 52A for MLST, so this was used as the template for PCR reactions using the newly designed primers. The same PCR protocol, including reagents and cycling parameters that were used for MLST (on the *C. upsaliensis*

isolates) were used for closing the gaps within the plasmids. Isolates were purified and sequenced as previously described for MLST of *C. upsaliensis* isolates.

2.10 Plasmid Extraction

Culture from dog 52A was grown from microbank beads stored at -80°C on CAB for 72 hours in a VAIN. Extraction was first attempted using a QIAprep® Spin Miniprep Kit (QIAGEN), according to the manufacturers' microcentrifuge instructions. DNA was eluted in 50µl of elution buffer and a 1.5% agarose gel was run for 40 minutes before being examined under UV. No bands could be seen on the gel (including the wells) so a second procedure was implemented.

2.10.1 Modified Kado and Liu Plasmid Isolation Procedure

2.10.1.1 First Procedure

The procedure was adapted from the methods suggested by Kado and Liu, (1981) and Wigley, (1999). Culture grown on CAB plates was added to 1.5ml distilled water and then pelleted at 13, 000×g for 4 minutes in a centrifuge. The supernatant was poured off and the pelleted cells were lysed by agitating the pipette tip into the pellet followed by addition of 150 µl of lysing solution, which was subsequently mixed to form a suspension. Lysing solution consisted of; 10ml distilled water, 0.06g tris (Sigma), 0.3g sodium dodecyl sulphate (SDS, 3g/100ml, BHD Laboratory supplies), and 170 µl 2M sodium hydroxide (NaOH, 0.8g/10ml, BDH Laboratory supplies). Tris was completely dissolved before the addition of SDS and NaOH.

The suspension was heated at 65°C for 90 min before 150 µl of phenol/chloroform, (produced by mixing equilibrated phenol, chloroform and isoamyl alcohol, in a ratio of 25:24:1, Sigma) was added and an emulsion was produced by vigorous shaking of the

sample for 3 minutes. The emulsion was centrifuged at 13, 000×g for 3 min and 75 µl of the upper aqueous layer was removed (pipette tip cut to reduce shearing of large plasmids). Twenty microlitres of the sample was added to 7.5µl of loading buffer prior to immediate loading onto a 1% agarose gel using TBE buffer, with the addition of 2µl ethidium bromide (10x; 109g Tris, 55g Boric acid, 9.3g EDTA supplied by Sigma, and 1 litre of water). The gel was run at 120V for 45 minutes and examined, then a further 45 minutes at 120V, and then additionally was run for 45 minutes at a higher voltage (140V).

2.10.1.2 Second Procedure

This protocol was repeated but with the following modifications; the *C. upsaliensis* isolate was cultured in *Campylobacter* enrichment broth (as previously described) for 24 hours in a VAIN, whilst a marker, *Escherichia coli* R39 was cultured in Luria Bertani (LB, Sigma) broth from beads stored at -80°C, at 37°C, aerobically for 24 hours. The suspensions were vortexted with phenol/chloroform for 10 minutes, before being loaded on a 0.5% agarose gel (50ml), run at 150V, initially for 45 minutes before examination under UV, and then additionally for a further 60 minutes before a second examination.

2.10.1.3 Third Procedure

The suspensions from the second procedure were repeated for the electrophoresis stage, but with the following modifications. A larger gel (100ml) and tank were used, a 0.7% agarose gel with TAE buffer, was run for 45 minutes before examination, and an additional 25 minutes before a second examination.

2.11 *Salmonella* Isolation

For attempted culture of *Salmonella* spp. from faecal samples, samples were initially prepared in saline at a dilution of 1:10 (0.85% NaCl), five drops of the saline solution were placed into 4.5ml of Buffered peptone water (BPW) (Lab046A, LabM), and incubated for 24 hours at 37°C. After incubation, 100µl of the sample was then added to Rappaport-Vassiliadis Medium broth (RVB) (Lab086, LabM) and incubated at 42°C for 24 hours. After which point, 100µl of RVB was then added to a central point on to Rappaport-Vassiliadis semi-solid agar (RVA) (Lab150, LabM) and incubated at 37°C for 24-48h. Plates were then examined for growth to the edges of the petri-dish, indicating swarming and the presence of a motile bacteria. When swarming occurred a loopful of media near the outer edge of the plate was collected using a 5µl loop and was subcultured onto nutrient agar (NA Lab08 LabM) and MacConkey Agar (MAC) (Lab002, LabM) and incubated for 24 hours at 37^{o}C.

Lactose-negative bacteria, such as *Salmonella* spp. do not change colour on MAC agar, so can be distinguished from lactose fermenting bacteria whose colonies appear pink. Any lactose-negative isolates that did not swarm on the NA were then subjected to a slide agglutination test with poly 'O' and poly 'H' antisera (Pro-Lab, Neston UK).

Two drops of sterile saline were placed on a clean glass slide. A drop of poly 'O' antisera (test) was added to one of these saline drops. Growth was taken from the NA using a 5µl and mixed with the 'test' and 'control' (sterile saline alone), before the slide was gently rocked backwards and forwards to observe agglutination; distinct agglutination (granular clumping) within 1 minute. This was then repeated using poly 'H' antisera. Isolates were regarded as *Salmonella* if they agglutinated both the poly 'O' and 'H' tests drops. Isolates were serotyped using the Kauffman-White scheme using

specific somatic and flagella antisera (this serotyping was performed by Dr Nicola Williams). Isolates were also biochemically confirmed as *Salmonella* spp., using an api20E (bioMerieux, France) test strip performed according to the manufacturers instructions and then frozen in Microbank tubes and kept at –80^{o}C.

3. Chapter three

Prevalence of and Risk Factors Associated with the Shedding of *Campylobacter* spp. in a Cross-sectional Study of Dogs Attending Veterinary Practices

3.1 Abstract

campylobacteriosis is a major cause of gastroenteritis in humans. Some studies suggest that ownership of a dog is a risk factor for human infection. This study aimed to determine the prevalence, species distribution, and risk factors for *Campylobacter* spp. in dogs attending veterinary practices.

Faecal samples were collected in a cross-sectional study from 249 asymptomatic and symptomatic dogs attending veterinary practices in the UK, and examined for the presence of *Campylobacter* spp. The *Campylobacter* spp. prevalence was 38% (95 % CI 32, 44), *C. upsaliensis* accounted for 94 (98%) isolates whilst the remainder were *C. jejuni.* Culture detected 61.4% of the 96 *Campylobacter* spp. positive samples, while direct PCR from DNA extract detected 91.6%. Direct PCR positive samples that were negative in culture, were in the post significantly longer than those samples that were positive by culture (P=0.01). Multivariable analysis showed that younger dogs were more likely to carry *C. upsaliensis.*

The high prevalence of *C. upsaliensis* supports the hypothesis that dogs, particularly younger dogs, may be an important source of *C. upsaliensis* infection for humans. However the prevalence of *C. jejuni* in dogs, the most common *Campylobacter* spp. found in humans, in the present study was low (1.2%, 95% CI 0.3, 3).

3.2 Introduction

Campylobacter spp. are the most commonly reported bacterial cause of human gastroenteritis (Adak *et al*., 2002; CDC, 2008c; DEFRA, 2007). The majority of infections are caused by *C. jejuni* and *C. coli*, but in a small proportion of cases *C. upsaliensis* has been isolated (Goossens *et al*., 1990a; Labarca *et al*., 2002; Lastovica and Le Roux, 2003). However the true prevalence of *C.upsaliensis* infection in humans may be underestimated, as most detection methods are optimised for *C. jejuni* detection (Byrne *et al*., 2001; Kulkarni *et al*., 2002; Labarca *et al*., 2002; Lastovica and Le Roux, 2003), and symptoms may be milder in *C. upsaliensis* infections, resulting in fewer reported cases (Goossens *et al*., 1990b; Jimenez *et al*., 1999).

There is some evidence of a risk of *Campylobacter* infection in humans associated with dog or pet ownership (Adak *et al*., 1995; FSA, 2005; Kapperud *et al*., 1992; Tenkate and Stafford, 2001). Some studies have shown an association between *C. jejuni* infection in humans and dogs in the same household (Damborg *et al*., 2004), and similar associations have also been found for *C.upsaliensis* (Goossens *et al*., 1991; Labarca *et al*., 2002; Lentzsch *et al*., 2004).

The reported prevalence of *Campylobacter* spp. carriage found in dogs varies widely, depending on the population sampled and also on the detection methods used (Acke *et al*., 2006; Hald and Madsen, 1997; Hald *et al*., 2004; Rossi *et al*., 2008; Sandberg *et al*., 2002; Wieland *et al*., 2005). Probably the most common species isolated from dogs is *C. upsaliensis* (Hald *et al*., 2004; Koene *et al*., 2004; Rossi *et al*., 2008; Sandberg *et al*., 2002); although in other studies, *C. jejuni* appears to predominate (Hald and Madsen, 1997; Lopez *et al*., 2002; Tsai *et al*., 2007; Workman *et al*., 2005).

Whether or not *Campylobacter* spp. carriage is associated with clinical disease in dogs is not clear. Some studies have found no significant relationship between diarrhoea and *Campylobacter* spp. status (Acke *et al*., 2006; Sandberg *et al*., 2002; Workman *et al*., 2005) suggesting the organism may be a commensal in dogs (Engvall *et al*., 2003). Other studies have found *Campylobacter* spp. associated with clinical signs (Guest *et al*., 2007), particularly in younger dogs (Burnens *et al*., 1992; Fox *et al*., 1983; Nair *et al*., 1985).

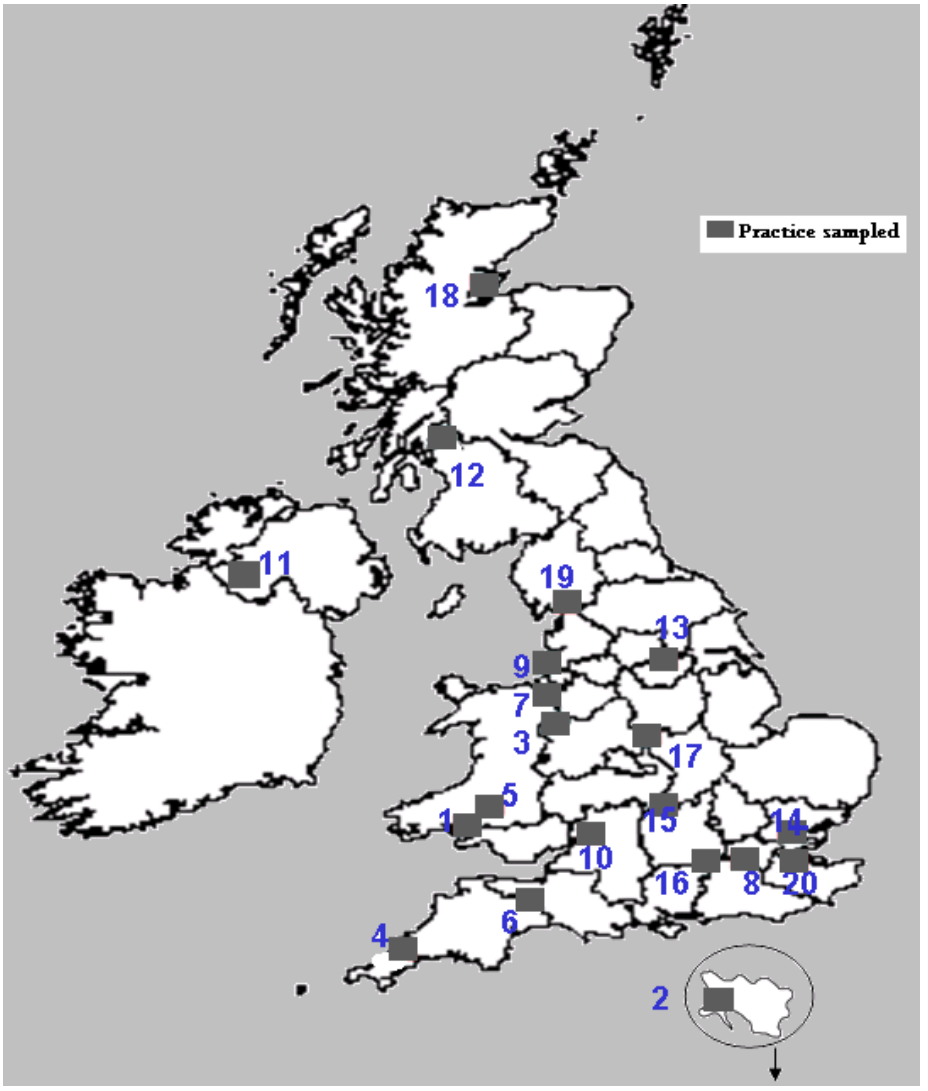
When age has been investigated as a risk factor for *Campylobacter* spp. carriage in dogs, the majority of studies have identified that younger dogs are more likely to carry *C. upsaliensis*, and *C. jejuni,* than older dogs (Acke *et al*., 2006; Engvall *et al*., 2003; Guest *et al*., 2007; Sandberg *et al*., 2002; Wieland *et al*., 2005). However, a small number of studies suggest age is not a risk factor for *C. jejuni* (Tsai *et al*., 2007; Wieland *et al*., 2005). Prevalence of *Campylobacter* spp. carriage also generally appears to be higher in kennelled dogs compared to some other populations (Acke *et al*., 2006; Tsai *et al*., 2007; Workman *et al*., 2005).

The aim of this investigation was to determine the prevalence and species distribution of *Campylobacter* spp. carriage in dogs visiting veterinary practices throughout the UK, and to identify possible risk factors for *C. upsaliensis* carriage.

3.3 Materials and Methods

Dogs visiting veterinary practices were selected as the target population. Veterinary practices were selected using a random number generator from each of the 23 UK regions defined by the Royal College of Veterinary Surgeons. Study design and recruitment of practices were in collaboration with Jenny Stavisky (PhD studies of canine corona virus). We aimed to recruit one practice per region. Of the 23 regions in the UK, three practices were excluded as they had no eligible practices willing to participate. As a result, 20 practices submitted samples to the study (Fig 3.1).

Figure 3.1. Map of the UK displaying the practice locations.



Between August and December 2006 each practice was requested to obtain one faecal sample from each of 25 dogs attending the practice for any reason, including routine checks, neutering or illness; practitioners were requested not to specifically select nor exclude dogs with enteric disease, but to include a number which reflected their representation of dogs attending the practice. Practices were provided with sample pots, questionnaires, tongue depressor, gloves, plastic bag, and reply paid envelopes (Appendix 1, Fig 1.1).

Faecal samples from recruited dogs were collected predominantly by staff at the veterinary practice, but on occasion by the owners. Questionnaires were completed either by the owner, or by the practitioner in the presence of the owner. Neat samples of canine faeces were sent in via standard first class post without transport media, with the number of days in the post recorded. Samples received from dogs living in the same house were sent in separate pots and envelopes with one exception (pots were in the same envelope). For each sampled dog, owner consent was obtained, along with details of signalment and vaccine and health status. Samples were tested for the presence of *Campylobacter* spp. and *Salmonella* spp.

3.3.1 Bacterial Culture

Three culture methods were used to isolate *Campylobacter* spp. The methods are described in chapter two of this thesis but in brief; (i) Direct plating on to *Campylobacter* selective agar (Lab M) with the addition of cefoperazone and amphotericin (CA) (Lab M). (ii) Filtration through a 0.7 µm nitrocellulose membrane onto *Campylobacter* selective agar as in (i) with the addition of cefoperazone, amphotericin and teicoplanin (CAT) (Oxoid Ltd) supplement. (iii) Adding the sample to *Campylobacter* enrichment broth (Lab M) with 10% lysed horse blood (Southern Group

Labs Ltd) incubated for 24 h prior to inoculation onto *Campylobacter* selective agar, as in (i).

3.3.2 Direct Extraction of DNA from Faecal Samples ('Direct PCR')

Bacterial DNA was extracted directly from the faecal samples and direct PCR was performed as described in materials and methods (Chapter 2).

3.3.3 Species Identification

As described in chapter two, a series of PCR assays targeting selected genes were performed to determine the identity of the isolates for *C. upsaliensis* identification, targeting the 16S rRNA encoding gene (Linton *et al*., 1996) and *glyA* gene (Wang *et al*., 2002) whilst for *C. jejuni* identification, amplification of a *hipO* fragment was used (Wang *et al*., 2002). To confirm the identity of selected isolates, both cultured and those extracted directly from faeces, were submitted to the three specific PCR assays. All *C. jejuni hipO* products were confirmed by PCR and sequencing. In some cases the identity of suspect *Campylobacter* spp. was confirmed by amplifying and sequencing the partial *groEL* gene (Karenlampi *et al*., 2004), or the species specific assays for direct PCR products as described in chapter two, 2.3-2.5.

3.3.4 Statistics

Analysis for risk factors was performed for *C. upsaliensis* carriage, where samples were positive by any of the detection methods used. Chi-squared analysis and univariable and multivariable logistic regression analyses were used to investigate the following variables; whether or not the dog lived with another dog or cat, recent (within the past month) antibiotic treatment, diarrhoea or vomiting, vaccine status, sex, neutered status, breed, size, and age of the dog. All variables were tested for correlation using

Spearman's Rank correlation. Age was checked for linearity before it was entered into the final model by use of a generalised additive model (GAM)(Hastie and Tibshirani, 1990). Variables tested during multivariable model building included those with univariable $P<0.3$ and the model was built using backward stepwise elimination. Mann-Whitney test was used to investigate the effect of time in the post on *C. upsaliensis* carriage as these data were not normally distributed. In all the analyses, significant differences were indicated by a $P<0.05$. Statistical analyses were performed with SPSS 15.0, except GAMs, which were performed using S-plus (MathSoft Inc, 2005) and McNemar chi squared tests which were performed using http://www.graphpad.com/quickcalcs/Mc Nemar1.cfm.

Analysis was only performed for *C. upsaliensis* because there were too few *C. jejuni* isolates to perform a valid test with these isolates. These two species were not combined for statistical analysis within this particular study, due to the uncertainty of the niches they occupy. If one (*C. upsaliensis*) is a commensal, and one (*C. jejuni*) is considered pathogenic, then they could have different risk factors, which when combined could mask the true outcome.

3.4 Results

Twenty practices from 20 UK regions (from 36 practices initially approached; 64%) participated and returned samples. In total 249 canine faecal samples were received with the median number of samples returned being 12 per practice. Samples were in the post for an average of 2.5 days (median 2, Standard deviation 1.5), range 1-12 days.

3.4.1 Questionnaire Information

Questionnaires were returned with all the samples, although there was some information missing (Appendix 1, Table 1.1). The date of collection was not recorded on 16 questionnaires, one did not state the age, five did not record the neutered status of the dog, 41 breeds were unknown/not recorded, 13 failed to record the dogs vaccination status, six did not record the antibiotic status of the dogs, ten did not comment on the number of dogs living in the household, 12 did not comment on whether or not any cats lived with the dog, seven failed to comment on the history of diarrhoea and eight did not comment on the history of vomiting for the dog. When a variable for a particular dog was not recorded by the owner/veterinarian this was entered as missing data for statistical analysis. Logistic regression in SPSS automatically excludes any case with missing values in both univariable and multivariable analysis

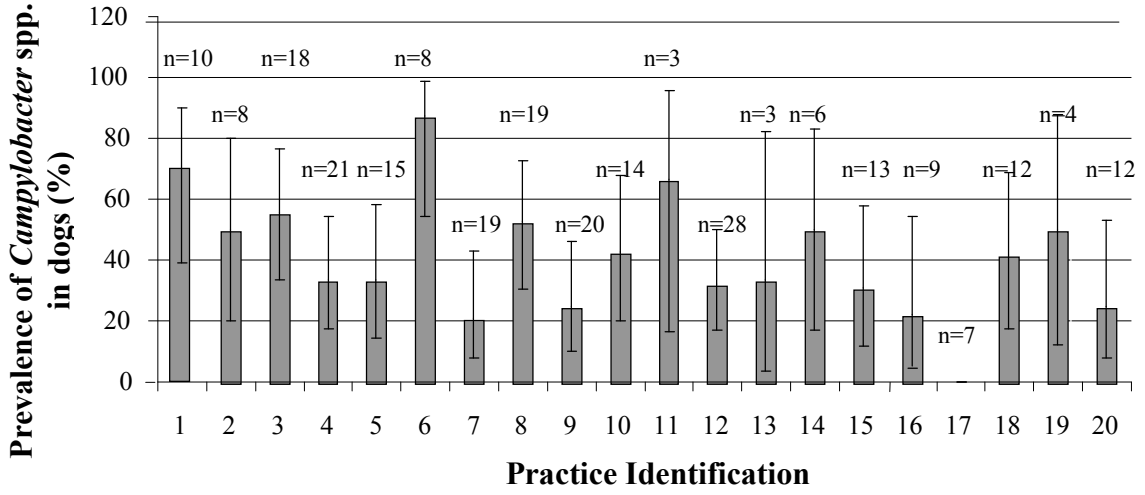
Some forms were filled out incorrectly, for example, next to the question how many dogs are in your household including this one, some questionnaires were filled in as zero, i.e. the owner did not include the dog that the sample came from. These values were assumed to be one dog in the house, but it should be noted that when the answer 'one dog' was given for this question, the owner may have meant that this dog lives with one other dog. It is possible owners incorrectly filled out other areas of the questionnaires as well, i.e. some stated that dogs who are several years old have never received a vaccine, which is unlikely, but not impossible. It is important to bear this in mind as this measurement error may affect results.

3.4.2 Campylobacter spp. Isolation

Campylobacter spp. were detected in 96 samples, giving a prevalence of carriage of 38% (95% CI 32, 44). The prevalence on a practice basis varied from 0-87.5% (Fig 3.2).

C. upsaliensis accounted for 93 (96%), of the *Campylobacter* spp., whilst two samples were identified as carrying *C. jejuni* (2%). One dog (06012) had a mixed infection of *C. upsaliensis* and *C. jejuni.*

Figure 3.2. Prevalence of *Campylobacter* spp. in dogs per practice 95% CI (median number of samples received per practice = 12.5, range 3-28).



n= total number of dogs sampled per practice

3.4.3 Detection Methods

3.4.3.1 Culture

Not all samples positive by one culture method were positive by another, so all three culture methods were needed for maximum recovery (Table 3.1). Comparison between the three different culture methods, showed that direct plating detected significantly more *C. upsaliensis* than prior enrichment $P<0.01$ (Table 3.2). Filtration detected more *C. upsaliensis* than enrichment, although this only approached significance ($P=0.05$). However, of the isolates detected only by enrichment in culture, all were also detected by direct PCR, i.e. if enrichment had not been included, the same number of *Campylobacter* spp. isolates would have been detected, providing culture and direct PCR were both used (Table 3.1).

Table 3.1. **Detection of *Campylobacter* spp. from faecal samples.**

	Culture Methods			Direct PCR of DNA extract straight from faecal sample		
	Campylobacter spp.			*C. upsaliensis*		*C. jejuni*
Dog isolate	Direct	Prior Enrichment	Prior Filtration	*gly A*	16S rRNA	*hipO*
# 01 013	-	-	-	+	+	-
# 01 020	-	-	-	+	+	-
# 01 030	-	-	-	+	+	-
# 02 007	-	-	-	-	+	-
# 02 023	-	-	-	-	+	-
# 02 024	-	-	-	-	+	-
# 03 012	-	-	-	-	+	-
# 03 014	-	-	-	+	+	-
# 03 018	-	-	-	+	+	-
# 03 020	-	-	-	-	+	-
# 03 028	-	-	-	-	+	-
# 04 002	-	-	-	+	+	-
# 04 005	-	-	-	+	+	-
# 04 014	-	-	-	+	+	-
# 04 024	-	-	-	+	+	-
# 05 015	-	-	-	+	+	-
# 07 005	-	-	-	+	+	-
# 07 014	-	-	-	+	+	-
# 08 002	-	-	-	+	+	-
# 08 003	-	-	-	+	+	-
# 08 009	-	-	-	+	+	-
# 09 008	-	-	-	+	+	-
# 09 014	-	-	-	+	+	-
# 09 020	-	-	-	+	+	-
# 10 001	-	-	-	+	+	-
# 10 004	-	-	-	+	+	-
# 10 018	-	-	-	+	+	-
# 12 016	-	-	-	+	+	-
# 12 025	-	-	-	+	+	-
# 12 030	-	-	-	+	+	-
# 12 032	-	-	-	+	+	-
# 13 021	-	-	-	+	+	-
# 14 002	-	-	-	+	+	-
# 15 001	-	-	-	+	+	-
# 18 019	-	-	-	+	+	-
# 19 011	-	-	-	+	+	-
# 20 024	-	-	-	+	+	-
*☼06 012	+	-	-	+	+	-
■ 03 029	-	-	+	-	-	-
■ 05 009	+	-	-	-	-	-
■ 05 027	+	-	-	-	-	-
■ 12 004	+	+	-	-	-	-
*■ 12 028	+	+	-	-	-	-
■ 16 022	+	-	-	-	-	-
■ 18 007	-	-	+	-	-	-
■ 20 012	+	+	+	-	-	-

Culture -Ve Direct PCR +Ve n=37

Direct PCR +Ve *C. upsaliensis* Culture +Ve *C. jejuni* n=1

Culture +Ve Direct PCR -Ve n=8

Continued on next page

*	04 011	+	+	+	-	-	+
	01 031	+	+	+	+	+	-
	01 033	+	-	+	-	+	-
	01 039	+	-	+	-	+	-
	01 041	+	-	-	-	+	-
	02 017	+	-	-	+	+	-
	03 008	+	+	+	+	+	-
	03 009	+	+	+	+	+	-
	03 011	+	-	+	-	+	-
	03 021	+	-	+	+	+	-
	04 003	+	+	+	-	+	-
	04 008	-	-	+	-	+	-
	05 019	-	-	+	+	+	-
	05 025	+	+	-	+	+	-
	06 002	+	-	+	-	+	-
	06 007	+	-	+	+	+	-
	06 008	+	-	+	+	+	-
	06 009	+	+	+	+	+	-
	06 010	+	-	+	+	+	-
	06 018	+	-	+	+	+	-
	07 002	-	+	-	-	+	-
	07 018	+	+	+	+	+	-
	08 001	+	-	+	+	+	-
	08 014	+	+	+	+	+	-
	08 017	+	-	+	+	+	-
	08 018	+	+	-	+	+	-
	08 019	+	-	+	-	+	-
	08 020	+	+	+	+	+	-
	08 024	+	-	+	-	+	-
	09 002	-	+	-	+	+	-
	09 025	+	+	-	+	+	-
	10 006	-	+	+	+	+	-
	10 016	+	+	-	+	+	-
	10 020	+	+	+	+	+	-
	11 002	+	+	+	+	-	-
	11 021	-	+	+	-	+	-
	12 006	+	-	-	+	+	-
	12 012	+	+	+	+	+	-
	12 034	+	+	-	-	+	-
	14 011	-	-	+	-	+	-
	14 017	+	-	+	+	+	-
	15 006	+	+	+	+	+	-
	15 012	+	+	-	+	+	-
	15 027	-	+	+	+	+	-
	16 002	-	-	+	-	+	-
	18 010	-	-	+	-	+	-
	18 016	+	+	+	+	+	-
	18 020	+	+	+	-	+	-
	19 022	+	-	+	+	+	-
	20 021	+	+	-	+	+	-
		47/96=48.9%	30/96=31.2%	41/96=42.7%	65/94=69.1%	86/94=91.4%	1/3=33%

Culture +Ve
Direct PCR +Ve
n=50

Culture combined: 59/96=61.4% Direct PCR combined: 88/96=91.6%

* = Following culture and subsequent sequencing, dog isolate confirmed as *C. jejuni*
# = Dogs positive by direct PCR but negative in culture
■ = Dogs positive by culture but negative by direct PCR
☼ =Dog positive by direct PCR but negative in culture for *C. upsaliensis* only, but positive in culture for *C. jejuni* only

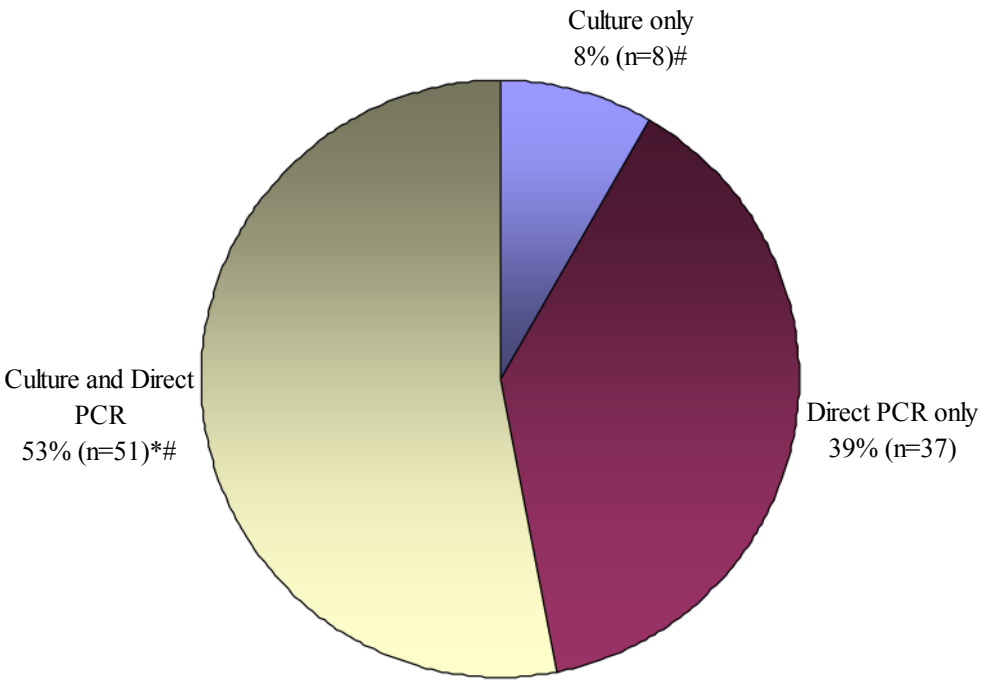
Table 3.2. Comparison of culture detection methods compared for the detection of *C. upsaliensis* (McNemar Chi squared) in vet-visiting dogs.

Variable value		+	-	Coef	OR	95% CI	*P*-
Culture methods	Direct	44	12	0	1		
	Enrich	28	28	8.65	0.23	0.07-0.64	<0.01
	Direct	44	12	0	1		
	Filtration	40	16	0.37	0.71	0.28-1.72	0.54
	Enrich	28	28	0	1		
	Filtration	40	16	3.78	2.20	1-5.20	0.05

3.4.3.2 Comparison of Culture and Direct PCR

Overall, fewer samples were positive by culture compared to direct PCR. Culture detected 61.4% (95% CI 51, 71) of the 96 *Campylobacter* spp. positives, while direct PCR detected 91.6% (95% CI 86, 97) (Table 3.1). Thirty eight additional dogs (15.2%, 95% CI 10, 19) were positive for *C. upsaliensis* by direct PCR, but negative in culture (including dog 06 012) (Fig 3.3). However culture detected seven *C. upsaliensis*, and two *C. jejuni* that direct PCR did not detect. Despite the fact that no mixed infections were found within the culture or direct PCR methods themselves, one dog (06 012) was positive for only *C. jejuni* in culture, and was positive for only *C. upsaliensis* by direct PCR (Table 3.1). In both culture and direct PCR, 16SrRNA encoding gene detected more *C. upsaliensis* isolates than *glyA* (Table 3.1).

Figure 3.3. Comparison of detection methods (%) for *Campylobacter* spp. (*C. upsaliensis* unless otherwise stated) in dogs n=96.



*dog 06012 included once; detected by culture (*C. jejuni*) and direct PCR (*C. upsaliensis*).

one/two dog(s) carried *C. jejuni*.

Culture= detected by any one or more of the three culture methods.

Direct PCR= detected by direct PCR on DNA extracted directly from faeces.

3.4.3.2 Effects of Transportation

There was no association between the number of days that the samples had been in the post and overall *C. upsaliensis* carriage status (*P*=0.5). Culture positive samples were on average in the post for 2.21 days (median 2, range 1-5, IQR 1, 3) and direct PCR 'only' positive samples were in the post on average for 3.2 days (median 2.5, range 1-12, IQR 2, 4). No significant difference was found for days in the post between all negative samples and the culture-positive samples (*P*=0.5) (Tables 3.3 & 3.4). However, the samples positive by direct PCR 'only' had been in the post for longer, than all negative samples (*P*=0.03), and compared to the samples positive by culture (*P*=0.01).

Table 3.3: **Number of days that *C. upsaliensis* positives samples had been in the post: Comparison of culture positive, and direct PCR 'only' positives in vet-visiting dogs.**

Variable	N	Coef	Mean rank	*P*-value
Culture+Ve	53	673	39.7	0.01*
Direct PCR +Ve	36		52.81	
Culture +Ve	53	6.72	104.77	0.03***
Direct PCR +Ve	36		140.00	
Culture and Direct PCR -Ve	142		114.11	

* Mann-Whitney U
*** Kruskal Wallis

Table 3.4: **Post-hoc comparison of culture *C. upsaliensis* positives, and direct PCR 'only' positives, for number of days that samples had been in the post in dogs.**

Variable		Mean difference	S.E.	95.0% C.I. Lower Upper	*P*-value
Culture and Direct PCR –Ve	Culture +Ve	0.24	0.25	-0.35-0.83	0.59
	Direct PCR +Ve	-0.71	0.29	-1.40-0.02	0.03
Culture +Ve	Culture & Direct PCR	-0.24	0.25	-0.83-0.35	0.59
	Direct PCR +Ve	-0.95	0.33	-1.75--0.16	0.01

3.4.4 Unconfirmed Isolates

Eight additional faecal samples had a low yield of DNA and the sequencing was inconclusive or only worked using one primer. These samples were positive on the 16S rRNA PCR, but negative for *glyA* in the direct PCR of DNA extracts, while being negative in culture. For these reasons, these eight dogs were not included in the total number of positive dogs. If these dogs did have *C. upsaliensis*, this would take the overall prevalence from 38.5% (95% CI 32, 44), to 41.7% (104/249, 99% CI 35, 47).

3.4.5 C. jejuni

Two dogs carrying *C. jejuni* were reported as occasionally having slight diarrhoea and soft faeces, although only one of the samples from these two dogs was soft on arrival. The other dog showed no signs of diarrhoea. These three dogs were 12, 110, and 132 months of age, and although numbers were too small to perform statistical analysis on these dogs, analysis was carried out on these variables for *C. upsaliensis* carriage (section 3.4.6.1).

3.4.6 Statistical Analysis of Risk Factors

3.4.6.1 Univariable Analysis

Of the 247 dogs (i.e. excluding those carrying *C. jejuni* only), 179 (72%, 95% CI 66, 77) did not have a history of diarrhoea within the last month prior to the sample being collected, while 62 (25%, 95% CI 19, 30) did (six were unknown). There was no significant association between recent (within the last week/month) diarrhoea or vomiting and the presence or absence of *C. upsaliensis*, (P=0.9 and P=0.8, respectively). There was a trend for dogs that had not received antibiotics in the last month to be more likely to be *C. upsaliensis* positive, but this difference was not significant (P=0.1) (Appendix 1, Table 1.4).

No significant associations were found between the breed (P=0.3) or size (P=0.1) (both based upon kennel club categories), dog gender (P=0.2), neutered status (P=0.5) or vaccination status (P=0.2) of the dogs and their *C. upsaliensis* carriage status.

Living with a positive dog was significantly associated with *C. upsaliensis* carriage (P<0.01; Table 3.2). Some cells had counts less than or equal to five for this variable due to a total of 44 dogs, from 21 different households, of which *C. upsaliensis* status

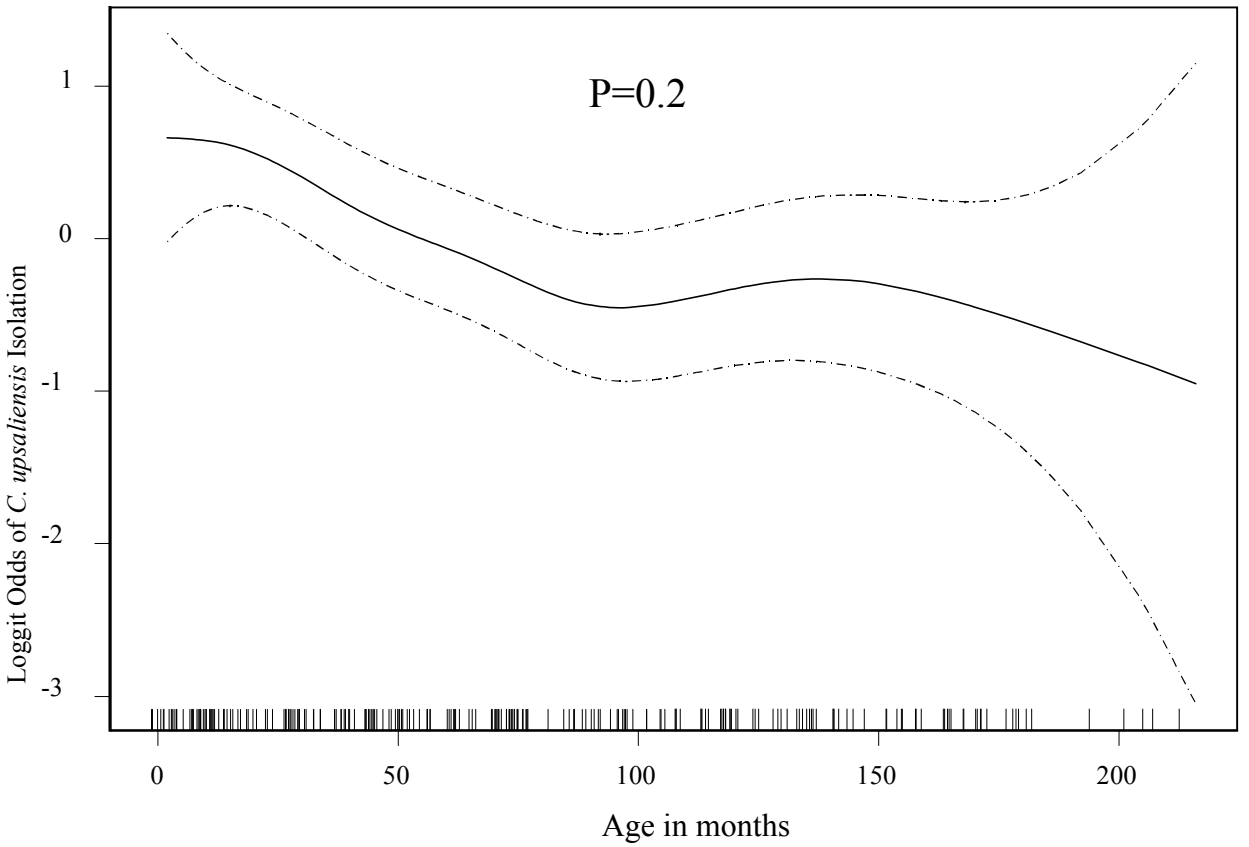
was known. There was no allowance for practice clustering, although dogs originating from the same household seemed fairly well distributed; practices 1, 8 and 9 had 3 households each, practices 5, 6, 10 and 12 each had two, and practices 2, 3, 4 and 20 had one household each. There was no significant association between the *C. upsaliensis* carriage of the dog and whether or not they lived with a cat (*P*=0.4).

Age of the dog was significantly associated with *C.upsaliensis* carriage status and the GAM suggested that this relationship was linear (Fig 3.5) with younger dogs more likely to carry *C. upsaliensis* (Appendix 1, Table 1.1).

Table 3.2. Univariable analysis of dog characteristics/variables and *C. upsaliensis* status, positive or negative by any isolation method (*P*<0.1).

Variable		+	-	Coef	SE	OR	95% CI	*P*-value
							Lower Upper	
Age	Total months	-	-	-0.007	0.003	0.99	0.98-0.99	<0.01
Status of other dogs in the same household	No dog(s)	47	96			1		<0.01
	Positive	15	5	1.81	0.54	6.12	2.10-17.87	<0.01
	Only negative	4	20	-0.89	0.57	0.40	0.13-1.26	0.12
	Unknown	22	29	0.43	0.33	1.55	0.80-2.98	0.19
Antibiotics	Recent	16	40			1		
	None	76	109	0.55	0.331	1.74	0.91-3.33	0.09

Figure 3.5. GAM graph demonstrating linear relationship between age in dogs and *C. upsaliensis* status (data not significantly different from a linear relationship P=0.2).



3.4.6.2 Multivariable Model

The final model generated (Table 3.3) suggested that the risk of being positive for *C. upsaliensis* decreased with increasing age (OR for every additional month 0.99, 95% CI 0.99, 1.00). For example the odds ratio for a ten year old dog compared to a one year old dog would be 0.3. The final model also included the variable; living with another dog or not (*P*=0.06). As this variable approached significance and has been shown in other studies to be associated with *C. upsaliensis* status (Westgarth *et al*., 2009) it was kept in the final model and could not be ruled out as having no effect on *C. upsaliensis* status in dogs sampled. No other risk factors were significant in the multivariable model. The model appeared to fit the data well (Hosmer-Lemeshow *P*=0.7). Two-way interaction terms between biologically plausible variables were tested for in the final model, but were not found to be significant.

Table 3.3: **Multivariable analysis of risk factors for dogs and *C. upsaliensis* infection (n=238, 9 missing values, Hosmer-Lemeshow *P*=0.737).**

Variable		Coef	S.E.	Odds ratio	95.0% C.I. Lower Upper	*P*-value
Age	Age in months	-0.006	0.002	0.99	0.99-1.00	<0.01
Lives with another dog	No			1		
	Yes	0.51	0.27	1.67	0.97-2.89	0.06

Variables tested during model building included: Age of the dog, living with another dog, size, sex of dog, and antibiotic status in the past month.

3.4.7 Salmonella

Two of the dogs sampled were infected with *Salmonella* Newport, giving a prevalence of 0.8%. Both the dogs came from practice four, had received recent antibiotics and were large/giant breeds, and although one had recent diarrhoea, the other had not.

3.5 Discussion

The prevalence of *C. upsaliensis* reported in this study (38%) is in the middle of the range reported for similar dog populations (17%, to 59%) (Rossi *et al*., 2008; Sandberg *et al*., 2002). Different prevalence rates identified in studies may be due to differences in the underlying populations, or in the methods used, which have only recently been optimised for *C. upsaliensis* as well as *C. jejuni* detection (Byrne *et al*., 2001; Guest *et al*., 2007; Kulkarni *et al*., 2002; Labarca *et al*., 2002; Lastovica and Le Roux, 2003). Despite the dog population in this study being a vet-visiting one; the majority of these dogs were healthy (no diarrhoea). These results suggest that dogs may be an important reservoir for *C. upsaliensis*.

Previous studies have reported considerably higher prevalences of *C. jejuni* (3 to 40%) in dogs (Hald and Madsen, 1997; Koene *et al*., 2004; Lopez *et al*., 2002; Tsai *et al*., 2007; Workman *et al*., 2005) than this study (1.2%), this may be due to a number of factors including; the source of the dogs, age and detection methods used. Some studies have found an association with *C. jejuni* and disease in dogs (Burnens *et al*., 1992; Fox *et al*., 1983; Nair *et al*., 1985), which might imply that *C. jejuni* infection in dogs is sporadic, potentially the result of eating contaminated food, as with humans. However other studies have found no association between *C. jejuni* infection and diarrhoea in dogs (Damborg *et al*., 2004; Hald *et al*., 2004; Lopez *et al*., 2002). Most studies have found younger dogs are more likely to carry *Campylobacter* spp. than older dogs, particularly when *C. upsaliensis* is the most common species isolated (Hald *et al*., 2004; Sandberg *et al*., 2002; Wieland *et al*., 2005). This association with younger dogs has not been reported where *C. jejuni* is taken into account, or when the *C. upsaliensis* prevalence is low (Tsai *et al*., 2007; Wieland *et al*., 2005). If indeed *C. jejuni* is sporadic in dogs, then this disassociation with age might be expected. The low prevalence in this present study suggests that this vet visiting population of dogs are unlikely to be an important source of *C. jejuni* infection for humans.

3.5.1 Comparison of Detection Methods

Of the three culture methods, plating after enrichment detected fewer *Campylobacter* spp. than the other two culture methods. This has also been noted by another study (Westgarth *et al*., 2009). One explanation for this might be that the enrichment stage allows for contaminating bacteria to increase and out compete *Campylobacter* spp. (Abulreesh *et al*., 2005; Korhonen and Martikainen, 1990). There appeared to be no significant difference in the numbers of *Campylobacter* spp. detected by direct plating onto mCCDA, and CAT media with prior filtration. Other studies have found similar

findings for the detection of *Campylobacter* spp. (Bourke *et al*., 1998), *C. jejuni* and *C. coli* (Engberg *et al*., 2000), and *C. upsaliensis* (Hald and Madsen, 1997).

3.5.2 Culture Versus Direct PCR

In this study, direct PCR was found to be more sensitive than culture for detecting *C. upsaliensis*. Transportation time appears to have a significant effect on culturable *Campylobacter* spp., and this has been observed in another study (Koene *et al*., 2004). An additional factor may be the existence of viable, but non-culturable forms of *C. upsaliensis* (Murphy *et al*., 2006; Persson and Olsen, 2005), which are thought to occur more frequently when bacteria are exposed to adverse conditions (transportation). Alternatively low level shedding or a past infection may be detectable by direct PCR but not by culture. In the instance of dog 06012, which was cultured as *C. jejuni*, but was found to have *C. upsaliensis* by direct DNA extract, the *C. jejuni* may have out competed *C. upsaliensis* in culture due to its faster growing time (Labarca *et al*., 2002; Lastovica and Le Roux, 2003).

Persson and Olsen, (2005) found that when isolating *C. coli* and *C. jejuni*, direct PCR was inferior compared to culture, particularly with fresh samples. In the current study, the three *C. jejuni* isolates were all detected by culture, whereas only one of these isolates was detected by direct PCR. Samples obtained with minimal transportation time between collection and processing, may yield a different outcome to the one observed in this study. A disadvantage to direct PCR is that specific species primers need to be used instead of degenerate primers, which in this study limited the detection to *C. jejuni* and *C. upsaliensis*. Despite this, it is possible to expand this method with the use of additional primers, multiplex PCR (Grove-White *et al*., 2009) or real-time PCR (Chaban *et al*., 2009).

However, eight faecal samples that yielded *C. upsaliensis* isolates in culture, did not yield an amplification product when tested by direct PCR. This observation has been made previously for *C. jejuni* (Lawson *et al*., 1999) and has been attributed to the degradation of DNA and/or the presence of inhibitory substances present in the faeces that may reduce the sensitivities of the PCR assay. Currently, no 'gold standard' exists for the detection of *Campylobacter* spp., and therefore direct PCR and culture methods should both be used to maximise recovery.

3.5.3 Dog Age, Clinical Signs, and Campylobacter spp. *Status*

Similar to other studies (Acke *et al*., 2006; Engvall *et al*., 2003; Guest *et al*., 2007; Sandberg *et al*., 2002; Wieland *et al*., 2005), younger dogs were found to have a greater risk for *C. upsaliensis* carriage than older dogs. Hald *et al*, (2004) found that the carriage rate of *Campylobacter* spp. in pet dogs in Denmark peaked at 13-15 months of age, especially for *C. upsaliensis*, which is similar to the findings of this present study. Similarly, Guest *et al*, (2007) also found that dogs negative for *Campylobacter* spp. were older (with an average age of 42.5 months) than the positive dogs who had an average age of 13.5 months. The most likely explanation for this effect is that older dogs have probably been exposed to *Campylobacter* spp. previously, and therefore developed a certain level of immunity to the bacterium. Immunity to *Campylobacter* spp. has been observed in *Macaca nemestrina* monkeys based on increasing immunoglobulin titres, which suggested that after an initial *Campylobacter* spp. infection, the host retained some immunity if exposed to the bacteria again (Russell *et al*., 1989).

We did not observe a statistically significant association between *Campylobacter* spp. carriage and clinical presentation/history, as has been reported in other studies (Acke *et al*., 2006; Engvall *et al*., 2003; Sandberg *et al*., 2002; Workman *et al*., 2005). However,

some studies have found *Campylobacter* spp. associated with clinical signs (Acke *et al*., 2009; Guest *et al*., 2007), particularly in younger dogs (Burnens *et al*., 1992; Fox *et al*., 1983; Nair *et al*., 1985), and often for *C. jejuni* (Fox *et al*., 1983; Nair *et al*., 1985). Furthermore the way in which dogs were sampled by the practitioners may have lead to bias, i.e. samples were taken based on the practitioners decision, not by random selection. A case control study would be a more appropriate method to explore this variable.

3.5.4 Dogs Living With Other Dogs and Cats

Dogs that lived with other dogs (not necessarily carrying *Campylobacter* spp.) tended to be more likely to carry *C. upsaliensis* in multivariable analysis, and this has also been found in other work (Westgarth *et al*., 2009). There was a significant association between a dog carrying *C. upsaliensis* and living with another positive dog in univariable analysis, although numbers for this group were small. Acke *et al*, (2006) suggested that dogs who live in groups, such as kennels have a higher prevalence of *Campylobacter* spp. carriage, possibly due to cross-infection, and Damborg *et al*, (2008) found indistinguishable amplified fragment length polymorphism (AFLP) patterns in strains that were isolated from dogs living in the same house or kennel, suggesting transmission. Previous studies have found no association between a dog's *Campylobacter* spp. status, and whether or not they lived with any other animals (Hald *et al*., 2004; Lopez *et al*., 2002); these findings are supported by the current work as we did not find any association between canine *C. upsaliensis* carriage and cohabiting with a cat, possibly because cats predominantly carry *C. helveticus* rather than *C. upsaliensis* (Rossi *et al*., 2008; Wieland *et al*., 2005; Workman *et al*., 2005).

3.5.5 Salmonella

In vet visiting and household dogs, a *Salmonella* spp. prevalence of 1-2% has previously been found (Bagcigil *et al*., 2007; Hald *et al*., 2004; Tsai *et al*., 2007), which is supported by the findings in this study where the prevalence of *Salmonella* spp. in dogs was very low (0.8%, 95% CI -0.3, 1.9). Other studies have found higher prevalences of *Salmonella* spp. in dogs from various populations, ranging from 1-69%, although the majority of studies find a prevalence of less than 10%. (Bagcigil *et al*., 2007; Cantor *et al*., 1997; Hackett and Lappin, 2003; Hald *et al*., 2004; Schotte *et al*., 2007; Tsai *et al*., 2007).

3.5.5.1 Salmonella Serovar Newport

Salmonella Newport was the only serovar found in this study and was found in two dogs. Although there appears to be no one serovar dominant in dogs, Newport has previously been reported as either the most common, or second most common serovar (Hald *et al*., 2004; Oloya *et al*., 2007; Seepersadsingh *et al*., 2004). The most likely source of infection for this serovar is thought to originate from cattle, but it has also been isolated from horses, reptiles, and seafood (CDC, 2008a; Gaertner *et al*., 2008; Karon *et al*., 2007; Khan *et al*., 2009; Oloya *et al*., 2007; Talbot *et al*., 2006). There was evidence of a shared source of infection, or possible transmission between a calf and a dog during an outbreak of *S*. Newport on a farm (Daly and Neiger, 2008). Of the two dogs carrying *S*. Newport in this current study, one experienced diarrhoea in the past week prior to sampling, but the other had no recent history of diarrhoea. Both dogs in this study had received recent antibiotic treatment, which has been associated with increased *Salmonella* spp. isolation (Warnick *et al*., 2003).

3.5.5.2 Salmonella spp. Infections in Humans

The most commonly identified serotypes found in humans appear to be *S.* Enteritidis and *S.* Typhimurium in the UK and some states of America (CDC, 2008c; DEFRA, 2007). In some states of America, *S.* Newport accounted for 10% of salmonellosis cases, which was second only to the servovars previously mentioned (CDC, 2008c; Jones *et al.*, 2008), and in some situations *S.* Newport appears to be the second most commonly isolated serovar after Typhimurium (Oloya *et al.*, 2007; Oloya *et al.*, 2009) (discussed in Chapter 5). Although *S.* Newport is less invasive and results in fewer deaths compared to *S.* Typhimurium, salmonellosis caused by *S.* Newport can still result in hospitalisation (Jones *et al.*, 2008), and outbreaks of this serovar in humans have occurred (CDC, 2008a; Greene *et al.*, 2008; Irvine *et al.*, 2009). The sources of these outbreaks varies, but exposure to cattle, farms, unpasteurised milk, Mexican-style cheese, ham, mung bean sprouts, tomatoes, and lettuce (presumably contaminated with animal faeces whilst growing) have been identified as possible sources of infection (CDC, 2008a; Greene *et al.*, 2008; Irvine *et al.*, 2009; Karon *et al.*, 2007; Lyytikainen *et al.*, 2000; Mohle-Boetani *et al.*, 2009).

3.5.5.3 Salmonella Zoonoses

Associations between reptiles, pet rodents and salmonellosis in humans have been documented, but little is known about the relationship between dogs and humans, regarding *Salmonella* spp. transmission (CDC, 2003; Friedman *et al.*, 1998). Interestingly, dog food/treats have been implicated in human cases of salmonellosis, some of which involved *S.* Newport (CDC, 2008b; Pitout *et al.*, 2003). The results of this study suggest that this population of dogs is not a significant source of *Salmonella* spp. infection for humans. However, caution should still be taken when a dog does shed *Salmonella* spp.

3.6 Conclusions

The high prevalence of *C. upsaliensis* carriage found in dogs in our study and other published work provides some evidence that this species may be a commensal in dogs. Although the relationship between *C. upsaliensis* and gastroenteritis in both dogs and humans is still unclear, given the close contact between them, dogs, and particularly younger dogs may be a potential source of infection for humans (Westgarth *et al*., 2007; Westgarth *et al*., 2008). Dogs that live with other dogs carrying the bacterium, may have an increased likelihood of carrying *C. upsaliensis.* The prevalence of *C. jejuni* in dogs in this study was low, suggesting that this population of dogs is unlikely to be a common source of *C. jejuni* infection for humans.

4. Chapter four

Prevalence of and Risk Factors for *Campylobacter* spp Shedding in Longitudinal Studies of Kennelled Dogs

4.1 Abstract

Campylobacteriosis is a major cause of gastroenteritis in humans. The majority of infections are caused by *C. jejuni* and *C. coli*, although *C. upsaliensis* has also been isolated from stool samples. Some studies suggest that ownership of a dog is a risk factor for human infection, and higher *Campylobacter* spp. isolation rates have been observed in kennelled dogs. This study aimed to determine the prevalence, species distribution, and shedding patterns for *Campylobacter* spp. in kennelled dogs. Faecal samples were collected in longitudinal studies from dogs housed in two kennels, one boarding, and one rescue, and examined for the presence of *Campylobacter* spp.

The *Campylobacter* spp. prevalence in dogs in the boarding kennel ranged from 41% (CI, 95% 22, 61) on entry, to 50% (95% CI 30, 70) overall, and in the rescue kennel ranged from 67% (95%, CI 49, 81) on entry, to 73% (95%, CI 56, 87) overall. In both kennels combined, *C. upsaliensis* was isolated from 32 dogs (62%, 95% CI 48, 73) whilst *C. jejuni* was isolated from 8 (15%, 95% CI 7, 26). Younger dogs were more likely to carry *Campylobacter* spp. than older dogs (P=0.01).

The majority of positive dogs entered the kennels already carrying *Campylobacter* spp. but in some cases shedding appeared to commence after entry into the kennel. The prevalence of *C. upsaliensis* and *C. jejuni* in both kennels was relatively high compared to other dog populations, suggesting kennelled dogs, particularly young dogs, may pose a risk of infection to humans.

4.2 Introduction

The majority of human *Campylobacter* spp. infections are caused by *C. jejuni* and *C. coli*, and to a lesser extent *C. upsaliensis*, which is usually the most commonly isolated *Campylobacter* spp. from dogs (Goossens *et al*., 1990a; Labarca *et al*., 2002; Lastovica and Le Roux, 2003). Although dogs are not considered a major source of *Campylobacter* spp., there is some evidence of an elevated risk of *Campylobacter* spp. infection in humans associated with dog or pet ownership (Adak *et al*., 1995; FSA, 2005; Tenkate and Stafford, 2001).

The prevalence of *Campylobacter* spp. carriage generally appears to be higher in kennelled dogs compared to some other dog populations, such as household dogs/vet-visiting dogs, when studies are conducted from the same country (Acke *et al*., 2006; Acke *et al*., 2009; Tsai *et al*., 2007; Workman *et al*., 2005). In the UK and Ireland, household/vet-visiting dogs appear to have a *Campylobacter* spp. prevalence of between 26.2% and 46.5% (Acke *et al*., 2009; Westgarth *et al*., 2009), whereas the prevalence of *Campylobacter* spp. carriage in kennelled dogs from these two countries ranges from 21% to 87% (Acke *et al*., 2006; Guest *et al*., 2007). A similarly high *Campylobacter* spp. prevalence has also been observed in other countries (Tsai *et al*., 2007; Workman *et al*., 2005) and intensive housing of boarding cats and dogs can increase the carriage rate of *Campylobacter* spp. by 1.98 times (Baker *et al*., 1999; Torre and Tello, 1993). Compared to other dog populations, the prevalence of *C. jejuni* (sometimes combined with *C. coli*) in kennelled/stray dogs, is relatively high ranging from 21% to 44%, and *C. jejuni* is occasionally isolated more often than *C. upsaliensis* in these studies (Fernandez and Martin, 1991; Malik and Love, 1989; Tsai *et al*., 2007; Workman *et al*., 2005).

Although other longitudinal studies have been carried out on kennelled dogs, few have focused on the shedding patterns of *Campylobacter* spp. on a daily basis or explored which *Campylobacter* spp. are carried by dogs on entry compared to several days later. The frequency of *Campylobacter* shedding from dogs has implications for prevalence based studies obtained through cross-sectional studies, and also for the risk of infection to humans.

The aims of this study were to determine the prevalence, species distribution, and shedding pattern for *Campylobacter* carriage in dogs housed in two kennels, one boarding and one rescue. The study also aimed to explore which *Campylobacter* spp. were carried by the dogs on entry to the kennel, and after they had been housed in the kennel for several days. Risk factors of carrying *Campylobacter* spp. were analysed for the dogs, and attempts at quantifying the *Campylobacter* spp. were also made.

4.3 Materials and Methods

Two kennels, one rescue, one boarding were chosen on a convenience basis, and both had approximately 200 dogs. Questionnaires regarding the dogs details (approximate age, neutered status, breed, size, gender, health status, weight, arrival date, departure date and source e.g. dog warden) were filled in by the samplers (Appendix 2; Figure 2.1).

Dogs were recruited on arrival into the kennel and the first faecal sample was collected in the majority of cases within 24 hours, although in some cases dogs did not defecate until several days later, thus it is possible the actual first sample was not collected. If dogs did not produce faeces during collection this was recorded as 'n'. Dogs were sampled daily, and samples were processed on the same day as collection. All samples were tested for the presence of *Campylobacter* spp. and the majority of first and last samples were tested for *Salmonella* spp. (unless the animal was euthanised/collected prematurely) as described in 4.3.3 and chapter 2.

4.3.1 Kennel 1: Rescue Kennel

Kennel 1 consisted of a rescue kennel which held approximately 130 dogs, in the North West of England, with a high intake of dogs. Cats and rodents were also housed within different blocks. The kennel was divided into three main blocks for dogs; holding, re-homing and quarantine, all of which were close together (Appendix 2, Fig 2.2). Both staff and dogs moved within and between the various blocks. Dogs were fed standard commercial dog food and biscuits, apart from those with special dietary requirements. All dogs were vaccinated on arrival against canine distemper, canine adenovirus, canine parainfluenza, leptospira and parvovirus. A cross-sectional pilot study was initially carried out in May 2007 to ensure that *Campylobacter* spp. were present. The

Campylobacter spp. prevalence in the pilot was 56% (95%, CI 25, 83) and no *Salmonella* spp. were isolated from the pilot dog samples (n=9). In the main study, faecal samples were collected from May to June 2007. Once recruited, dogs were sampled daily either until they were re-homed or euthanized or up to a maximum of 15 samples each.

4.3.2 Kennel 2: Boarding Kennel

Kennel 2 consisted of a boarding kennel which held approximately 200 dogs, in the North West. All dogs had up to date vaccination history and owner consent was obtained (Appendix 2, Fig 2.4). Dogs were recruited on the basis of permission obtained by the owners the previous day. Some dogs housed in pairs were excluded due to difficulty in distinguishing between faeces. Once recruited, dogs were sampled daily for a maximum of 15 days. The kennel had 11 main blocks for dogs, relatively close together (Appendix 2, Figure 2.3). Seven of these main blocks housed dogs that were sampled regularly, including blocks A, B, C, D, E, I and J, whilst block G remained predominantly empty, apart from two days where one dog included in the study was moved into this block. The remaining blocks did not house any dogs that were included in the present study. Cats were also kept at the kennel, but in a separate section. Staff moved freely between the blocks. Dogs were fed standard commercial dog food, apart from those who were supplied with their own food from the owners, and tinned sausages were given to most dogs as treats. Sampling took place every day over a period of 15 days, during September and October 2007.

4.3.3 Bacterial Culture

Two culture methods were used to isolate *Campylobacter* spp. The methods are described in chapter two of this thesis but in brief; (i) Direct plating on to

Campylobacter selective agar (Lab M) with the addition of cefoperazone and amphotericin (CA) (Lab M). (ii) Filtration through a 0.7 µm nitrocellulose membrane onto *Campylobacter* selective agar as in (i) with the addition of cefoperazone, amphotericin and teicoplanin (CAT) (Oxoid Ltd) supplement. The prior enrichment method was not included in this study as it had been found previously to have lower sensitivity (Chapter 3). All positive samples were provisionally identified as *Campylobacter* spp. based on morphology (see Chapter 2, 2.1), and all first and last *Campylobacter* spp. positive samples from each dog were confirmed, including species identification. Additionally PCR was also performed on those isolates that did not demonstrate typical morphology.

4.3.4 Direct Extraction of DNA from Faecal Samples ('Direct PCR')

Direct PCR also was performed as previously described in chapter two (and 4.3.5) for detection of *C. upsaliensis* and *C. jejuni* identification. DNA was extracted from the first and last sample of all dogs. In the case of dogs which were negative to culture on their first sample but subsequently produced *Campylobacter* spp. positive samples, DNA was extracted from the first positive and final positive samples; for example, in Table 4.1, dog 8 had direct PCR performed on its first, third and last sample.

4.3.5 Species Identification

As described in chapter two, a series of PCR assays targeting selected genes were performed to determine the identity of the isolates for *C. upsaliensis* identification, targeting the 16S rRNA encoding gene (Linton *et al*., 1996) and *glyA* gene (Wang *et al*., 2002) whilst for *C. jejuni* identification, amplification of a *hipO* fragment was used (Wang *et al*., 2002). To confirm the identity of selected isolates, both cultured and those extracted directly from faeces, were submitted to the three specific PCR assays. All *C.*

jejuni hipO products were confirmed by PCR and sequencing. In some cases the identity of suspect *Campylobacter* spp. were confirmed by amplifying and sequencing the partial *groEL* gene (Karenlampi *et al*., 2004), or the species specific assays for direct PCR products, as described in chapter two, 2.3-2.5.

4.3.6 Phylogeny

Phylogenetic trees were constructed using MEGA version 3.1 and Phylip version 3.68 as described in chapter two, 2.5.2 and were based on the partial *groEL* gene.

4.3.7 MLST

MLST was performed for all *C. jejuni* isolates within this study and on a selection of *C. upsaliensis* isolates chosen using a random number generator (Chapters 2, 2.7, & 6).

4.3.8 Bacterial Enumeration – Spiral Plating

Spiral plating was performed as described in chapter two for a sub-set of dogs from kennel 1 as a pilot study for the technique, and was performed on all dogs in kennel 2. All samples collected during the first five days in kennel 1, were subject to spiral plating. After the five days, the first five dogs which appeared positive in culture, were followed through with spiral plating. Isolates obtained from spiral plating from two dogs in kennel 1 were confirmed as *Campylobacter* spp. by PCR assays (4.3.5 and Chapter 2). In kennel 2, all samples from all dogs underwent spiral plating, with positive samples confirmed by PCR and first and last samples sequenced if necessary. In kennel 2, PCR assays were performed on the intermediate samples enumerated by spiral plating, but these were not confirmed by sequencing (Chapter 2, 2.6).

4.3.9 Salmonella spp.

Every dog's first sample was tested for the presence of *Salmonella* spp. according to the methods described in chapter two. Every dog's last sample was also tested for *Salmonella* spp. where possible, and in the rescue kennel, several dogs were tested for *Salmonella* spp. after they had stayed approximately one week in the kennel.

4.3.10 Statistics

Univariable analysis for risk factors allowing for repeated measures, was performed for *Campylobacter* spp. carriage, where samples were positive by any method. Statistics were not carried out separately for different *Campylobacter* spp. because the majority of the intermediate samples were not identified on the species level, and due to the number of mixed infections observed. Data from both kennels were combined for analysis. Univariable logistic regression analyses with a random effect term to allow for repeated measures within an animal were used to investigate the following variables; kennel, days in kennel, age, breed, size, diarrhoea status, blood in faeces and block type. All variables were tested for correlation using Spearman's Rank correlation. Age was checked for linearity before it was entered into the final model by use of a generalised additive model (GAM)(Hastie and Tibshirani, 1990). Variables tested during multivariable model (with a random effect term) building included kennel, those with univariable $P< 0.3$ and the model was built using backward stepwise elimination. In all the analyses, significant differences were indicated by a $P<0.05$. Statistical analyses were performed with SPSS 16.0 and Egret for windows 0.2, Cytel Corporation 1999, except GAMs which were performed using S-plus (MathSoft Inc 2005) and McNemar tests which were performed using http://www.graphpad.com/quickcalcs/McNemar1.cfm.

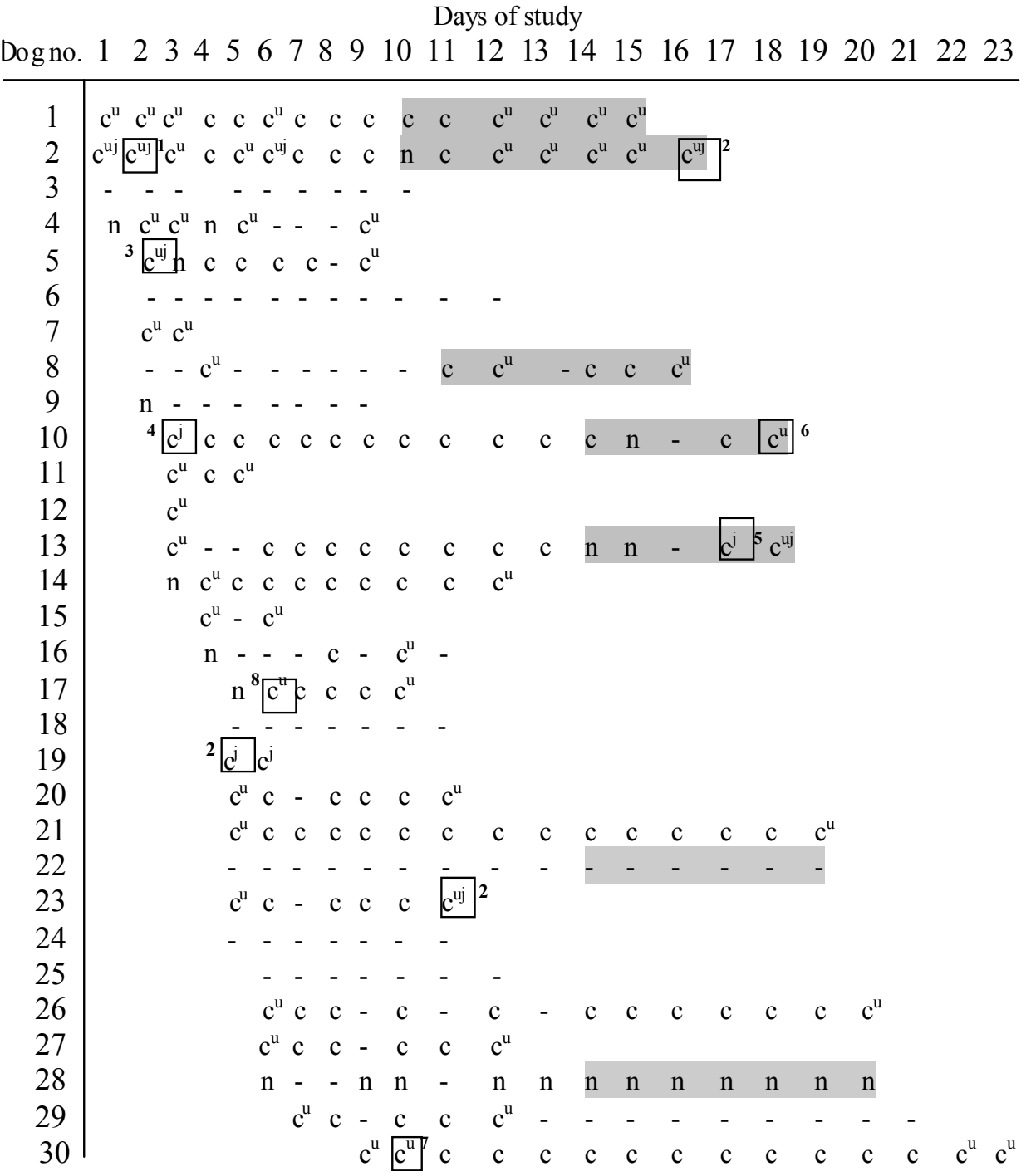
4.4 Results

4.4.1 Kennel 1: Rescue Kennel

Thirty dogs were recruited and followed for a maximum of 15 samples each, range 1-15, mean 8.9. In total, 268 samples were collected (Table 4.1). Nine dogs were put to sleep after seven samples and one dog was followed for 15 days but only three samples were collected. A further six dogs had 1-5 samples taken, while four more reached 8-11 samples, leaving ten dogs with 15 samples each.

The overall sample prevalence for *Campylobacter* spp. was 61% (95%, CI 55, 67) of 268 samples. Of the 62 samples where the species was determined, *C. upsaliensis* accounted for 58, (94%), and *C. jejuni* was detected in 11 (18%). There were seven mixed infections of *C. upsaliensis* and *C. jejuni* (Table 4.1). On entry to the kennel, *Campylobacter* spp. were detected in 20 of the 30 dogs, giving a prevalence of 67% (95%, CI 49, 81) (Table 4.2). *Campylobacter* spp. were detected in 22 of the 30 dogs at some point during their stay, giving an overall dog prevalence of 73% (95%, CI 56, 87). The overall dog prevalence for *C. upsaliensis* carriage was 70% (95% CI 52, 84) and the prevalence for *C. jejuni* carriage in dogs was 20% (95% CI 12, 48). Sixteen of these 22 positive dogs were found to have *C. upsaliensis* only, whilst one was found to have only *C. jejuni*, and five dogs had mixed infections of both these *Campylobacter* spp., although not always within the same sample (Table 4.1). No *Campylobacter* spp. were detected in eight dogs (Table 4.1).

Table 4.1. **Kennel 1 Rescue Kennel: Daily shedding pattern for all C*ampylobacter* spp.**



c =typical *Campylobacter* spp. growth in culture but not confirmed by PCR, - =negative for *Campylobacter* spp. n = not sampled, c^{u} = positive for *C. upsaliensis*, c^{j} = positive for *C. jejuni,* c^{uj} = positive for *C. upsaliensis* and *C. jejuni*, (grey) housed in quarantine.□ = MLST performed, [1]= *C. jejuni* ST-267, [2]= *C.jejuni* ST-45, [3]= *C. jejuni* ST-257, [4]= *C. jejuni* ST-3613, [5]=*C. jejuni* ST-137, [6] =*C. upsaliensis* ST-74, [7]=*C. upsaliensis* ST-83, and [8] = *C. upsaliensis* ST- 93 (Chapter 6).

Table 4.2. Summary of *Campylobacter* spp. shedding patterns in dogs (n=30) from kennel 1.

***Campylobacter* spp. Status**	**Total Dogs**	**Percentage (%)**	**95% CI Lower Upper**
First sample			
-Ve *Campylobacter* spp.	10	33	19-51
+Ve *C. upsaliensis* only	16	53	36-70
+Ve *C. jejuni* only	2	7	1-20
+Ve *C. upsaliensis* + *C. jejuni*	2	7	1-20
Any sample			
-Ve *Campylobacter* spp.	8	27	13-44
+Ve *C. upsaliensis* only	16	53	36-70
+Ve *C. jejuni* only	1	3	0-15
+Ve *C. upsaliensis* + *C. jejuni*	5	17	7-33

4.4.2 Kennel 2: Boarding Kennel

Twenty two dogs were recruited and were followed over a period of 15 days, resulting in 131 samples, range 2-14, mean 5.95. The sample prevalence was 36% (95%, CI 29, 45) for *Campylobacter* spp. Nine dogs had *Campylobacter* spp. detected on entry (41%, CI 95%, 22, 61), all of which were *C. upsaliensis*. Additionally, two of the dogs, 9 and 12, (previously negative on arrival) had mixed infections of *C. upsaliensis* and *C. jejuni* isolated in later samples, on days 11 and 15 respectively (Tables 4.3 and 4.4). This resulted in an overall dog prevalence for *Campylobacter* spp. of 50% (95%, CI 30, 70) and the prevalence for *C. upsaliensis* was the same as *Campylobacter* spp. as it was found in 100% of dogs who had *Campylobacter* spp. detected. The dog prevalence in the kennel for *C. jejuni* was 9% (95%, CI 1.9, 26).

Table 4.3. Kennel 2 Boarding Kennel: Daily shedding pattern for all C*ampylobacter* spp.

	Days of study														
Dog no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1*	-	n	-	-	-	-									
2*	-	-	-	-	-	-									
3#	c^u	c	c	n	c^u	c^u									
4#	c^u	c^u	c	c^u	n	n									
5	-	-	-	-											
6	n	c^u	c	c^u	c	c^u	c^u								
7	n	n	c^u [2]	c^u	c^u	n	-	n	n	n	n	n	n		
8	-	-	-	-	-	-	-	-	-	-	-	-	n	-	-
9	-	-	-	-	-	-	-	-	-	-	c^{uj} [1]	c^{uj}	n	-	
10	c^u	c^u	c^u	c^u	c^u	c	n	c^u							
11		-	n	-	n	-	-	n	-	n					
12		n	n	n	-	-	n	-	-	-	n	-	-	n	c^{uj} [1]
13		n	n	c^u	c^u	n	c^u	c	c	c^u					
14		[3] c^u		c^u	c^u	n	c^u	c	c^u						
15			[4] c^u	c^u	c^u	c^u	c	n	c^u	c^u					
16				-	-	-	-	-	-	-					
17				n	c^u	-	c^u	n	n						
18						n	-	n	-	n	-	-	-	-	-
19						n	n	n	-	-	-	n	n	-	n
20									n	n	-	-	n		
21									n	-	-	-	n	n	-
22									-	n	-	-	-	-	

c=typical *Campylobacter* spp. growth in culture but not confirmed by PCR, - =negative for *Campylobacter* spp., n= not sampled, $c^{u=}$ positive for *C. upsaliensis*, c^{uj} = positive for *C. upsaliensis* and *C. jejuni*. * and # =dogs from the same house. □=MLST performed, [1]=*C. jejuni* ST-508, [2]=*C. upsaliensis* ST-67, [3]=*C. upsaliensis* ST-87, and [4]= *C. upsaliensis* ST-98.

Table 4.4. Summary of *Campylobacter* spp. shedding patterns in dogs (n=22) from kennel 2.

***Campylobacter* spp. Status**	**Total Dogs**	**Percentage (%)**	**95% CI Lower Upper**
First sample			
-Ve *Campylobacter* spp.	13	59	38-77
+Ve *C. upsaliensis* only	9	41	23-62
+Ve *C. jejuni* only	0	0	
+Ve *C. upsaliensis* + *C. jejuni*	0	0	
Any sample			
-Ve *Campylobacter* spp.	11	50	30-70
+Ve *C. upsaliensis* only	9	41	23-62
+Ve *C. jejuni* only	0	0	
+Ve *C. upsaliensis* + *C. jejuni*	2	9	2-26

4.4.3 Kennels 1&2: Rescue and Boarding

4.4.3.1 Shedding Patterns

In both the rescue and the boarding kennels, 27 out of 52 (52%, 95% CI 39, 65) dogs had *C. upsaliensis* isolated from their first sample, and in most cases continued to shed *Campylobacter* spp. in every subsequent sample (Tables 4.1 and 4.3). Phylogenetic analysis based on the partial *groEL* gene suggested that of the dogs examined, individual dogs shed the same *C. upsaliensis* strain throughout their samples in study, indicating that the dogs were not subject to cycles of re-infection with different strains (Fig 4.1). One dog (8) from the rescue kennel had one *C. upsaliensis* positive sample whilst it was in the holding block, but when this dog was moved to the quarantine block, five out of six samples were positive for *Campylobacter* spp. (Table 4.1). Samples taken from this dog in both the holding, and quarantine block could not be distinguished by sequence analysis based on the partial *groEL* gene (Fig 4.1).

Of 52 dogs overall, *C. jejuni* was isolated from the first sample taken after entry from four dogs (8%, 95% CI 3, 17), and all four dogs originated from the rescue kennel. Since only one of these four dogs was found to shed *C. jejuni* in subsequent samples, shedding of *C. jejuni* in dogs from both kennels was over a much shorter duration than *C. upsaliensis* carriage. Of the 52 dogs, seven dogs shed more than one *Campylobacter* spp. and 19 dogs remained negative for *Campylobacter* spp. throughout the study.

Overall 39.9% of the dogs shedding *Campylobacter* spp. also had diarrhoea at some stage of the study (45.4% in kennel 1 and 27.2% in kennel 2). One of the two dogs carrying *C. jejuni* in kennel 2 produced two soft faeces prior to the isolation of *C. jejuni*, but no diarrhoea was recorded for either of these two dogs throughout the study. Four of the six dogs identified as carrying *C. jejuni* in kennel 1 displayed signs of diarrhoea at some point during the study but not necessarily when the sample was positive for *C. jejuni*. Three of the 11 boarding dogs carrying *C. upsaliensis* showed signs of diarrhoea at some point during the study, whilst nine of the 22 dogs carrying *C. upsaliensis* had diarrhoea in the rescue kennel. However 15 of the 30 dogs in the rescue kennel, and six of the 22 boarding dogs showed some degree of diarrhoea at some stage. Diarrhoea was also observed in four dogs from the rescue kennel and three dogs from the boarding kennel, which had no *Campylobacter* spp. isolated throughout the study. Overall there were no significant associations found between the presence of *Campylobacter* spp. and diarrhoea in dogs within this study (see section 4.4.5.2).

4.4.3.2 Possible Transmission Events

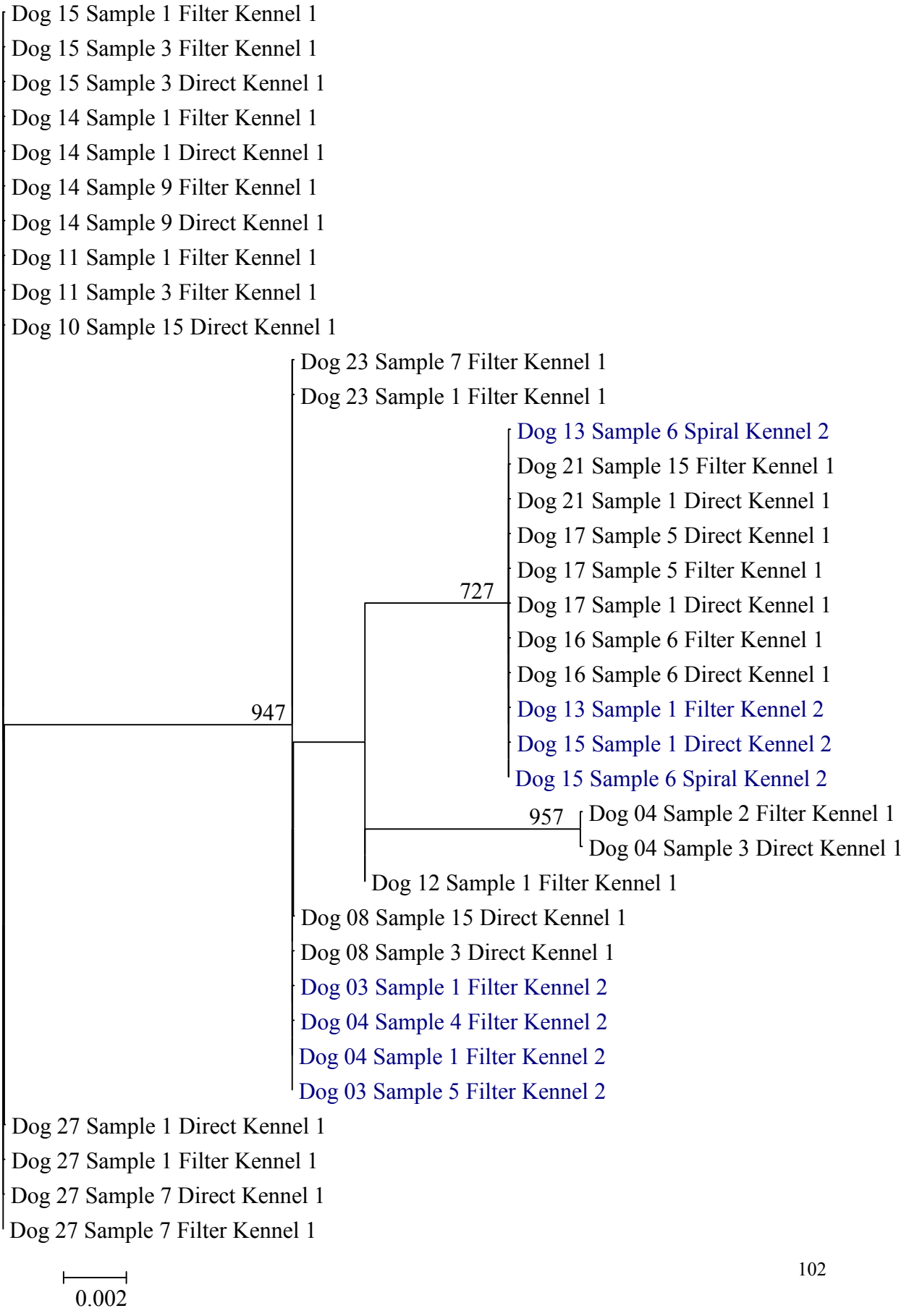
When data from both kennels were combined, 15.6% of dogs who had *C. upsaliensis* detected, only had this species detected after arrival, whereas 62.5% of the dogs that had *C. jejuni* detected, had this species detected only after arrival. Five dogs had *C.*

upsaliensis detected after entry, but not within their first sample (kennel 1 dogs 8, 10 &16 and kennel 2 dogs 9&12) and five dogs (kennel 1 dogs 2, 13& 23 and kennel 2 dogs 9&12) had *C. jejuni*/different strains of *C. jejuni* detected, after entry but not within their first sample, overall in both kennels (Tables 4.1 and 4.3). Thus two of the dogs (dogs 9&12) in the boarding kennel demonstrated mixed infections of *C. upsaliensis* and *C. jejuni* and appeared to possibly begin shedding *Campylobacter* spp. a week after entering the premises (Table 4.3). *C. jejuni* isolates from both these two dogs appeared to be indistinguishable by two molecular typing methods (Chapter 6). In the rescue kennel, dog 2 appeared to shed *C. jejuni* for the duration of the study, however, molecular evidence based on MLST and PFGE suggested that this dog shed different strains of *C. jejuni* in its first and then subsequent samples (Chapter 6, Table 6.1). Both dogs 2 and 13 from the rescue kennel only had *C. jejuni* detected from their samples after being moved to the quarantine block (Table 4.1). However, their MLST sequence types (ST-45 and ST-267) were different (Chapter 6 Table 6.1).

4.4.3.2.1 Dogs from the Same Household

In kennel 2, dogs 3 and 4 originated from the same house, and were housed together in the kennel. Neighbour-joining and maximum likelihood analysis of the partial *groEL* gene phylogenetic trees provided no evidence to suggest that these strains were dissimilar (Fig 4.1, and Appendix 2, Fig 2.5), and although they appeared to have similar strains of *C. upsaliensis*, further analysis would be required to confirm this.

Figure 4.1. Un-rooted bootstrap maximum likelihood tree based upon partial *groEL* gene, from culture isolates in kennels 1 and 2 (kennel 2 in blue) based on 440bp (1000 replicates). Direct= irect plating, filter=filtration, spiral=spiral plating.



4.4.4. Bacterial Enumeration Kennels 1&2

Comparison of bacterial loads between kennels appeared similar overall, although with only two dogs followed successfully from kennel 1, comparisons were difficult. The majority of dogs appeared to shed between 2×10^5 and 4×10^6 CFU/ml faeces, although one dog never shed more than 16×10^4 CFU/ml faeces, and counts as low as 1400 CFU/ml faeces were occasionally observed (Appendix 2, Fig 2.11). The limits of detection ranged from 200 to 4×10^6 CFU/ml so shedding outside these limits may have gone undetected.

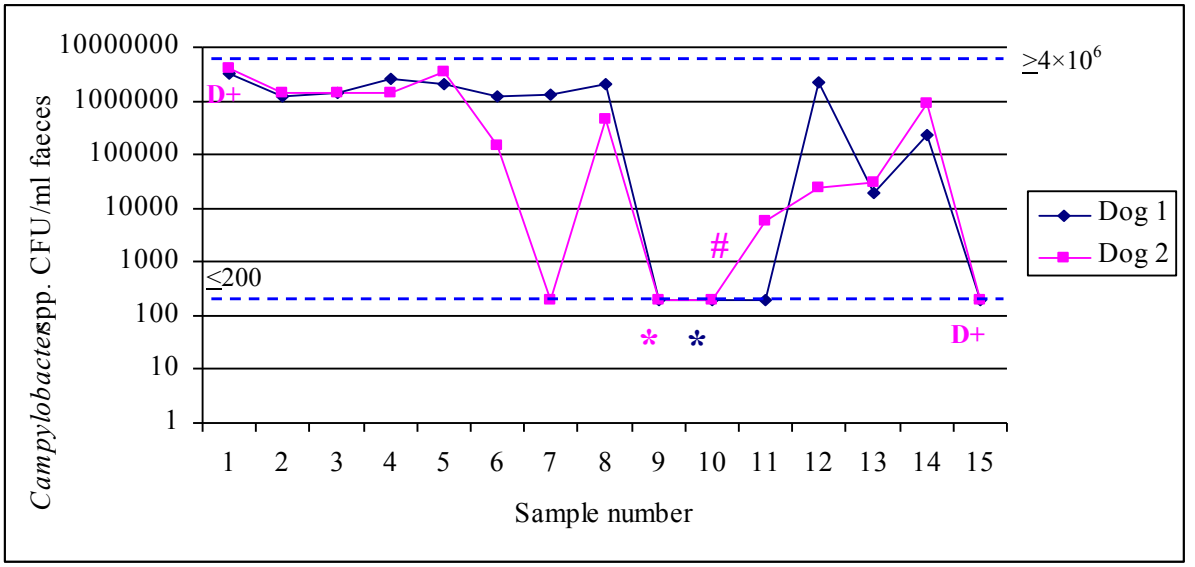
4.4.4.1. Kennel 1: Rescue Kennel

In kennel 1 a pilot study was carried out with samples from five dogs examined using spiral plating. Two dogs were euthanized after their seventh and eleventh sample, whilst three dogs were followed for 15 samples, although one remained predominantly negative (Fig 4.2, and Appendix 2 Fig 2.6). One of these dogs had severe diarrhoea, showing great variation in the number of *Campylobacter* spp. colonies shed between samples, and peaks appeared to correspond with episodes of diarrhoea (Appendix 2, Fig 2.6). However these colony counts were not confirmed by PCR.

Only two dogs were successfully followed for the majority of the study, and were suitable for PCR confirmation, these were dogs 1 and 2, (Fig 4.2). No *Campylobacter* spp. were isolated from either of these two dogs in samples nine and 10 on the spiral plating, despite being positive on other culture plates. At least one dog was treated with antibiotics during this time. The counts that were obtained indicated little variation in shedding for the first few samples, but by the sixth sample, both dogs appeared to be shedding fewer *Campylobacter* spp. than previously, and by dog 1 and dog 2's

thirteenth and eleventh sample respectively the variation appeared considerable (Fig 4.2).

Figure 4.2. Kennel 1: *Campylobacter* spp. colonies (CFU/ml faeces) in dogs 1 and 2 after PCR confirmation (*C. upsaliensis* unless otherwise stated).



* Dog moved to quarantine block with suspected kennel cough.

Dog treated with antibiotics and wormed.

D+ Diarrhoeic sample.

Nb Dog 2 had mixed infections of *C. upsaliensis* and *C. jejuni* in samples 1, 2, 6 and 15

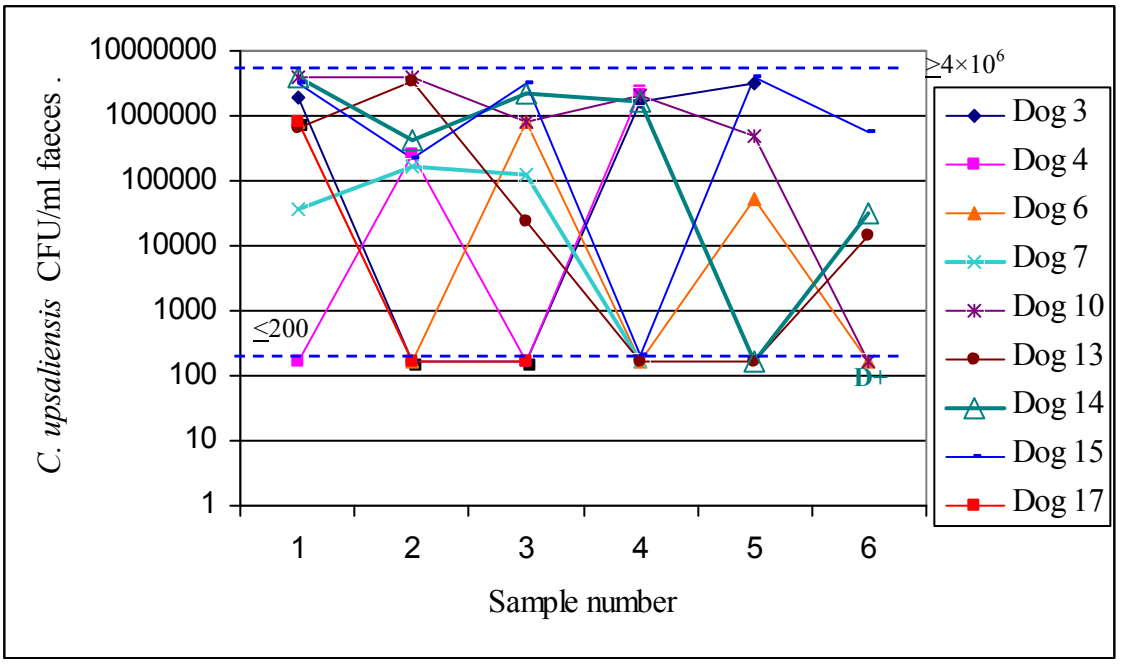
4.4.4.1.1. Limitations

Spiral plating had some limitations. The range that could be detected was between 200 and 4×10^6 CFU/ml faeces, so any counts outside of this range could not be recorded. Not all *Campylobacter* spp. positive samples (via other culture methods) showed growth on the spiral plates, so counts could not be obtained for some positive dog samples. Plates were prone to contamination which made it difficult to count the relatively small *Campylobacter* spp. colonies, and in several cases after the colonies were transferred onto CAB plates, the colonies failed to grow and PCR assays could not be performed. Due to the presence of contaminants, only counts confirmed by PCR could be truly accepted as *Campylobacter* spp.

4.4.4.2 Kennel 2: Boarding Kennel

All dog samples underwent spiral plating throughout the study, regardless of whether or not they were carrying *Campylobacter* spp. on entry, and in general *Campylobacter* spp. positive samples did grow on the spiral plates. The majority of dogs in kennel 2 shed variable amounts of *Campylobacter* spp. throughout the study, with large peaks and troughs (Fig 4.3). Dog 13 appeared to shed greater numbers of *Campylobacter* spp. for samples 1 and 2 compared to subsequent samples, and dog 14 who appeared to shed fewer *Campylobacter* spp. by samples 5 and 6. In general the dogs shed levels between 2×10^5 and the maximum, 4×10^6 CFU/ml faeces. However dogs 6 and 7 shed relatively fewer *Campylobacter* spp. than the other dogs, and *C. upsaliensis* present in dog 7 was only detected by spiral plating (Appendix 2, Table 2.2).

Figure 4.3|. Kennel 2: Counts of *C. upsaliensis* colonies (CFU/ml faeces) in dogs 3, 4, 6, 7, 10, 13, 14, 15, and 17 after PCR confirmation. *



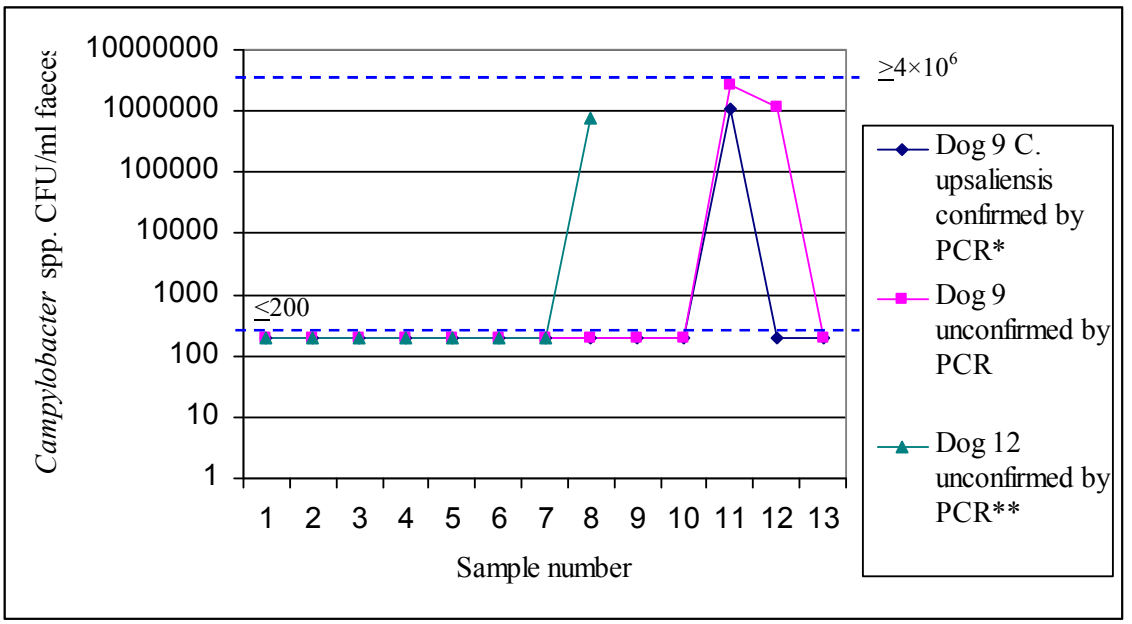
* For clarity, dog data are also presented in appendix 2, figs 2.7-2.10.

D+ Diarrhoeic sample.

4.4.4.2.1 Shedding Patterns in Dogs With Potential Transmission

Results for the two dogs (9&12) only positive for *Campylobacter* spp. after several days in the kennel are presented in Fig.4.4, and are based on both PCR confirmed and PCR unconfirmed results; both dogs were positive for both *C. jejuni* and *C. upsaliensis* at some point during the study (Table 4.2). Dog 9 appeared to have a sudden increase in the number of *Campylobacter* spp. which peaked before very quickly decreasing again (Fig 4.4), whereas the *Campylobacter* spp. shedding in dog 12 may have been increasing more gradually.

Figure 4.4. Kennel 2: *Campylobacter* spp. (CFU/ml faeces) colonies that grew via spiral plating, with and without PCR confirmation in dogs 9 and 12.*



* *C. jejuni* was also detected in the same sample but by direct plating.

***C. jejuni* and *C. upsaliensis* were both detected in the same sample by filtration.

4.4.5 Analysis Kennels 1& 2

Questionnaires were completed for each dog, although some information was missing (Appendix 2, Table 2.4).

The neutered status of many of the females from the rescue kennel was unknown, and the weight of the dogs from the boarding kennel was not recorded by the staff. During the first days of collection, it was unknown whether or not the dogs had previous diarrhoea, bloody faeces or vomiting, so these were often recorded as unknown, however, any of these symptoms observed on the day of collection were recorded.

Despite the advantage of 'known' information provided by the owners of the boarding dogs, if information regarding the dog was not on file at the kennel, it was difficult to record, e.g. neutered status. Although previous vaccination history and actual age were known for the boarding dogs, neither were known for the rescue dogs and the age recorded was based on the kennel staffs evaluation, usually based on dental condition. Only dogs from kennel 1 showed symptoms of kennel cough, and unfortunately the vomiting data from both kennels relied on kennel staff recording this variable or upon the samplers witnessing it during sampling, which provides opportunity for incorrect categorisation.

4.4.5.1 Univariable Analysis at the Dog Level

In a chi squared analysis there was no significant difference between the two kennels and the overall number of dogs positive at any one time (i.e. the dog level prevalence) for *Campylobacter* spp. (P=0.08), *C. upsaliensis* (P=0.1) or *C. jejuni* (P=0.2) (Table 4.5).

Table 4.5. **Chi-squared analysis of the overall number of dogs positive at any one time for *Campylobacter* spp., *C. upsaliensis,* and *C. jejuni* in each kennel.**

Variable	+	-	Coef	OR	95% CI	*P*-value
***Campylobacter* spp.**						
Kennel 1	22	8		1		
Kennel 2	11	11	2.92	0.36	0.09-1.34	0.08
C. upsaliensis						
Kennel 1	21	9		1		
Kennel 2	11	11	2.10	0.42	0.11-1.55	0.14
C. jejuni						
Kennel 1	6	24		1		
Kennel 2	2	20	1.13	0.40	1.13-2.60	0.28

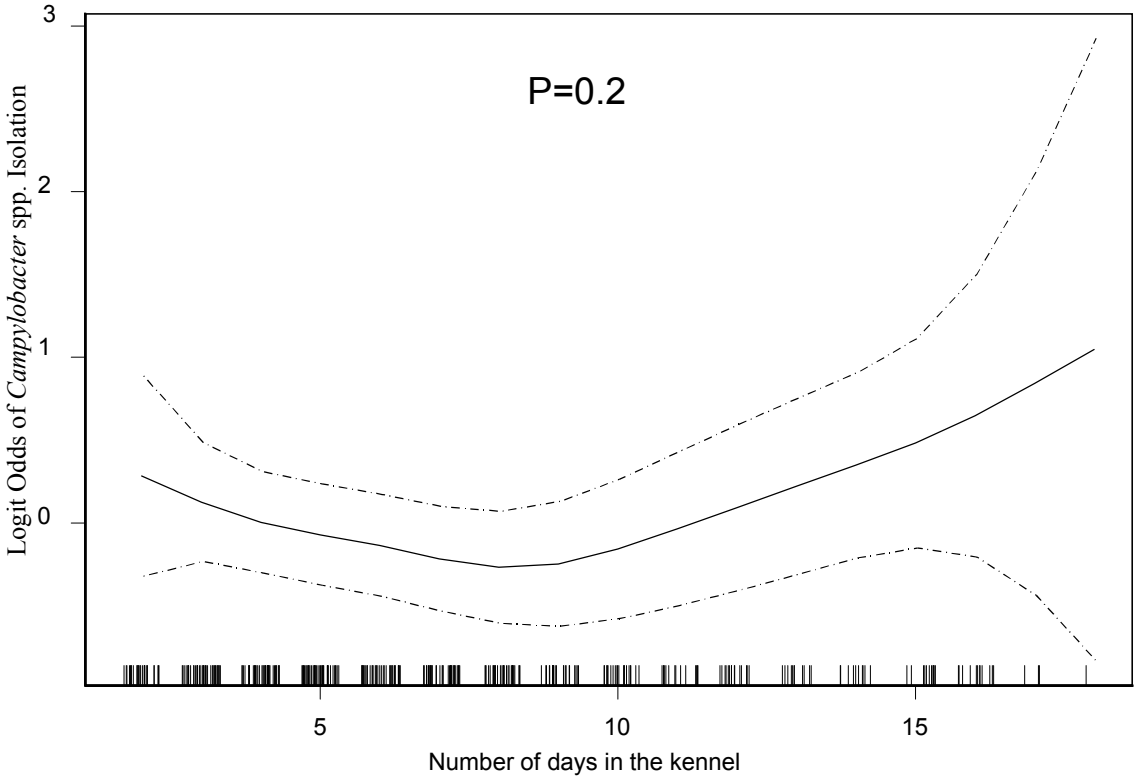
4.4.5.2 Univariable Analysis at the Sample Level

Univariable analysis at the sample level, allowing for clustering within animal, is shown in appendix 1, Table 2.1; there was no significant difference observed for dogs carrying *Campylobacter* spp. between the two kennels (P=0.09), or between different blocks (P=0.1). There were also no significant associations found between the breed, size (based on kennel club categories), or dog gender and their *Campylobacter* spp. carriage status.

Kennel cough was significantly associated with *Campylobacter* spp. carriage (P=0.04), and recent vomiting (within the last month) appeared to be protective against *Campylobacter* spp. carriage (P<0.01). There was no significant association between recent diarrhoea (including soft samples) or blood in the faeces and the presence or absence of *Campylobacter* spp.

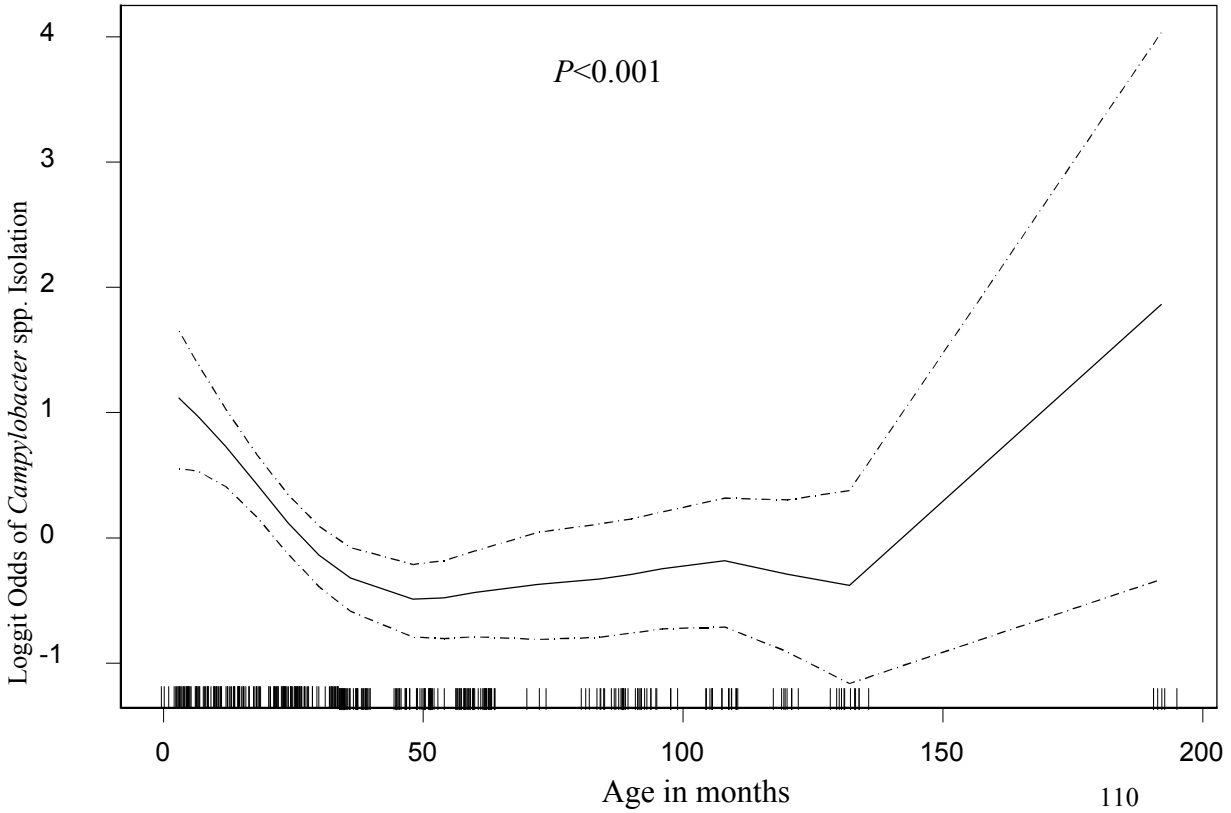
A GAM graph indicated that the relationship between length of stay and *Campylobacter* spp. carriage status was linear (Fig 4.5), however this relationship was not significant in univariable analysis (*P*=0.9). Additionally when the *Campylobacter* spp. status of the first samples taken from all the dogs was compared with the *Campylobacter* spp. status of the samples collected on the remaining days (first sample compared to remainder; i.e. in theory before and after entry) there was no significant difference (*P*=0.1). Whether or not they had been moved in the last 24h (*P*=0.9) or 48h (*P*=0.6) was also not associated with *Campylobacter* spp. carriage, although only eight dogs were moved in kennel 1 and only one dog was moved in kennel 2 (Appendix 2, Table 2.1).

Figure 4.5. GAM graph demonstrating the linear relationship between the number of days dogs were in the kennel and *Campylobacter* spp. status of the sample collected (the graph indicates that the data is not significantly different from that of a linear relationship *P*<0.2).



Sex, weight, breed and size appeared to show no association with *Campylobacter* spp. carriage in dogs (Appendix 2, Table 2.1). Age in months was not significantly associated with *Campylobacter* spp. carriage (allowing for clustering) based on the raw data (Appendix 2, Table 2.1). A major consideration for this variable is that age was estimated in the rescue kennel, so any associations based on this variable may not be truly accurate. However an interesting observation was that a GAM demonstrated that the relationship between age in months and *Campylobacter* spp. status was not linear (Fig 4.6) with the highest risk in the young and old animal. Despite no significant association, when the data was centred and squared to account for increased risk in young and old animals, this relationship was significant (Appendix 2, Table 2.1). Multivariable, multilevel logistic regression resulted in only this variable, as a polynomial squared term, remaining in the model, although for reasons previously mentioned, age cannot be considered a significant finding within this study.

Figure 4.6. GAM graph demonstrating the non-linear relationship between age in dogs and *Campylobacter* spp. status (data is significantly different from that of a linear relationship *P*<0.001).



4.4.6 Detection Methods

The recovery rates for *Campylobacter* spp. for individual culture and direct PCR are shown in Table 4.6. Overall culture detected slightly more *Campylobacter* spp. than direct PCR in both kennels, and detected all strains of *C. jejuni*, whereas direct PCR did not detect any *C. jejuni*. There was no significant difference observed between any of the culture methods for overall detection. However, when the two kennels were combined, *C. upsaliensis* isolates were significantly more likely to be detected by filtration onto CAT, than by direct plating onto mCCDA (Table 4.7).

Table 4.6 Results of all methods used for the recovery of *Campylobacter* spp. from kennels 1&2.

				Culture Methods						Direct PCR		
				Campylobacter spp.			*C. upsaliensis*		*C. jejuni*	*C. upsaliensis*		*C. jejuni*
	Kennel	Dog isolate	Sample	Direct	Filtration	Spiral	gly*A*	16S rRNA	*hipO*	*glyA*	16S rRNA	*hipO*
	1	Dog 1	1	+	+	+	+	+	-	-	+	-
	1	Dog 1	15	+	+	+	+	+	-	+	+	-
■*	1	Dog 2	1	+	+	+	+	+	+	+	+	-
■*	1	Dog 2	15	+	+	-	+	+	+	+	+	-
	1	Dog 4	1	-	+	n/a	-	+	-	-	+	-
#	1	Dog 4	7	-	-	n/a	n/a	n/a	n/a	-	+	-
■*	1	Dog 5	1	+	+	n/a	+	+	+	+	+	-
	1	Dog 5	8	-	+	n/a	+	+	-	+	+	-
■♦	1	Dog 7	1	-	-	+	+	+	-	-	-	-
■♦	1	Dog 7	2	-	-	+	+	+	-	-	-	-
	1	Dog 8	1	-	-	n/a	n/a	n/a	n/a	-	-	-
■	1	Dog 8	3	+	-	n/a	-	+	-	-	-	-
	1	Dog 8	15	+	-	n/a	-	+	-	-	+	-
■*	1	Dog 10	1	+	+	n/a	-	-	+	-	-	-
	1	Dog 10	15	+	-	n/a	**+1**	+	-	-	+	-
	1	Dog 11	1	+	+	n/a	-	+	-	-	+	-
	1	Dog 11	3	+	+	n/a	-	+	-	-	+	-
	1	Dog 12	1	+	+	n/a	-	+	-	-	+	-
	1	Dog 13	1	+	+	n/a	+	+	-	+	+	-
■*	1	Dog 13	15	+	+	n/a	-	-	+	+	+	-
	1	Dog 14	1	+	+	n/a	-	+	-	-	+	-
	1	Dog 14	9	+	+	n/a	**+1**	+	-	-	+	-
	1	Dog 15	1	-	+	n/a	-	+	-	-	+	-
	1	Dog 15	3	+	+	n/a	-	+	-	-	+	-
	1	Dog 16	1	-	-	n/a	n/a	n/a	n/a	-	-	-
■	1	Dog 16	6	+	+	n/a	-	+	-	-	-	-
	1	Dog 16	7	-	-	n/a	n/a	n/a	n/a	-	-	-
	1	Dog 17	1	+	+	n/a	-	+	-	-	+	-
	1	Dog 17	5	+	+	n/a	-	+	-	-	+	-
■*	1	Dog 19	1	+	+	n/a	-	-	+	-	-	-
■*	1	Dog 19	2	+	+	n/a	-	-	+	-	-	-
	1	Dog 20	1	+	-	n/a	+	+	-	+	+	-
	1	Dog 20	7	+	+	n/a	+	+	-	-	+	-
	1	Dog 21	1	+	+	n/a	**+1**	+	-	-	+	-
	1	Dog 21	15	+	+	n/a	**+1**	+	-	-	+	-
	1	Dog 23	1	-	+	n/a	-	+	-	+	+	-
■*	1	Dog 23	7	+	+	n/a	-	+	+	+	+	-
	1	Dog 26	1	+	+	n/a	+	+	-	+	+	-
	1	Dog 26	15	+	+	n/a	+	+	-	+	+	-
	1	Dog 27	1	+	+	n/a	-	+	-	-	+	-
	1	Dog 27	7	+	+	n/a	-	+	-	-	+	-
	1	Dog 29	1	+	-	n/a	+	+	-	+	+	-
	1	Dog 29	15	-	-	n/a	-	-	-	-	-	-
#	1	Dog 30	1	-	-	n/a	n/a	n/a	n/a	+	+	-
	1	Dog 30	2	+	+	n/a	+	+	-	+	+	-
	1	Dog 30	14	+	+	n/a	+	+	-	+	+	-
#	1	Dog 30	15	-	-	n/a	n/a	n/a	n/a	+	+	-
	2	Dog 03	1	-	+	+	-	+	-	-	+	-
	2	Dog 03	5	+	+	+	-	+	-	-	+	-
	2	Dog 04	1	-	+	-	-	+	-	-	+	-
	2	Dog 04	4	+	+	+	-	+	-	-	+	-
	2	Dog 06	1	+	+	+	+	+	-	+	+	-
	2	Dog 06	6	+	+	-	+	+	-	-	+	-

■	2	Dog 07	1	-	-	+	+	+	-	-	-	-
■	2	Dog 07	3	-	-	+	+	+	-	-	-	-
	2	Dog 07	4	-	-	-	n/a	n/a	n/a	-	-	-
	2	Dog 09	1	-	-	-	n/a	n/a	n/a	-	-	-
■*	2	Dog 09	12	+	+	-	+	+	+	+	+	-
	2	Dog 09	13	-	-	-	n/a	n/a	n/a	-	-	-
	2	Dog 10	1	+	+	+	+	+	-	+	+	-
	2	Dog 10	7	+	+	-	+	+	-	+	+	-
	2	Dog 12	1	-	-	-	n/a	n/a	n/a	-	-	-
■*	2	Dog 12	8	-	+	-	-	+	+	-	-	-
	2	Dog 13	1	-	+	+	-	+	-	-	+	-
	2	Dog 13	6	-	-	+	-	+	-	-	+	-
	2	Dog 14	1	-	+	+	+	+	-	+	+	-
	2	Dog 14	6	-	+	+	+	+	-	+	+	-
	2	Dog 15	1	+	-	+	+1	+	-	-	+	-
	2	Dog 15	6	-	-	+	-	+	-	-	+	-
	2	Dog 17	1	-	+	+	+	+	-	+	+	-
#	2	Dog 17	3	-	-	-	n/a	n/a	n/a	+	+	-
				42/57=74%	47/57=82%	19/24=79%	31/57=54%	55/57=96%	10/57=18%	25/53=47%	53/53=100%	0/53=0%

Culture Combined: 57/71=80% Direct PCR combined: 53/71=75%

* *C. jejuni* present in sample
# Direct PCR positive, culture negative for *C. upsaliensis*
+1 Positive for one colony only (out of a maximum of 15)
■ Culture positive, Direct PCR negative for *C. jejuni* and/or *C. upsaliensis*
♦ +Ve only on spiral plating

Table 4.7. Comparison of culture detection methods (McNemar Chi squared) for the detection of *Campylobacter* spp. in dogs from kennels 1&2.

Variable		+	-	Coef	OR	95% CI	*P*-value
Kennels 1&2							
C. jejuni	Direct	10	6	0	1		
	Filtration	5	11	1.77	0.28	0.02-1.50	0.18
C. upsaliensis	Direct	3	13	0	1		
	Filtration	10	6	4.00	8.00	1.07-354.981	0.04

* PCR confirmed isolates from dogs shedding *C. jejuni* at some stage of the study (dogs n=8, samples n=16). Samples included: Kennel 1: dog 2 samples 1&15; dog 5 samples 1&8; dog 10 samples 1&15; dog 13 samples 1, 13&15; dog 19 samples 1&2; and dog 23 samples 1&2. Kennel 2: dog 9 samples 11&12; and dog 12 sample 8).

4.4.7 Salmonella spp.

No *Salmonella* spp. were isolated from any of the samples from any of the dogs within either of the two kennels.

4.5 Discussion

The overall prevalence of *Campylobacter* spp. was relatively high in both kennels (rescue: 73%, 95% CI 56, 87 and boarding 50%, 95% CI 30, 70), with *C. upsaliensis* isolated the most frequently, followed by *C. jejuni*, the only other species isolated within this study. The majority of dogs that gave positive samples entered the kennels already carrying *Campylobacter* spp., with only a small number shedding the bacterium only after arrival and most of these predominantly involved *C. jejuni.* This study found that co-infection with *C. upsaliensis* and *C. jejuni* did occur within the same sample and/or dog (particularly dogs positive for *C. jejuni*), which has been reported in other work (Hald *et al*., 2004; Koene *et al*., 2004) and that detectable levels of *Campylobacter* spp. are not necessarily shed in every faecal sample, also noted by Hald *et al*, (2004) and Newton *et al*, (1988). However in most cases positive dogs shed *Campylobacter* spp. in nearly every sample.

4.5.1 C. upsaliensis

The overall prevalence of *C. upsaliensis* carriage found in dogs during the study in both types of kennel (rescue: 70%, 95% CI 52, 84 and boarding: 50%, 95% CI 30, 69), was higher than in some household/vet visiting (Rossi *et al*., 2008; Sandberg *et al*., 2002), and stray dog populations (Tsai *et al*., 2007; Workman *et al*., 2005). In contrast, the prevalence of *C. upsaliensis* carriage in household dogs in Scandinavia appeared to be similar to the kennel prevalences found in the present study (Engvall *et al*., 2003; Hald *et al*., 2004; Koene *et al*., 2004). However, it is probable that many factors influence the

carriage rate of various species of *Campylobacter* in different dog populations including housing, management, geographical and climatic differences. In addition, sampling strategies and detection methods will play a role. Nevertheless data from this present study suggests that kennels may be an important reservoir of *C. upsaliensis* infection for humans.

The high prevalence of *C. upsaliensis* carried by the dogs in this study and others suggests that this bacterium may be a commensal in dogs (Engvall *et al*., 2003; Hald *et al*., 2004; Koene *et al*., 2004). Further more, the high carriage rate of *C. upsaliensis* in dogs is generally not observed in any other animal, except possibly in some populations of cats (Wieland *et al*., 2005; Workman *et al*., 2005), and the majority of studies have been unable to make significant associations between diarrhoea in dogs and the presence of *Campylobacter* spp., particularly *C. upsaliensis* (Acke *et al*., 2006; Koene *et al*., 2008; Sandberg *et al*., 2002; Stavisky *et al*., 2009; Workman *et al*., 2005).

4.5.2 C. jejuni

The prevalence of *C. jejuni* found in the rescue kennel (20%, 95% CI 12, 48) is similar to the findings of other studies based upon stray dog populations (Fernandez and Martin, 1991; Tsai *et al*., 2007; Workman *et al*., 2005), and the prevalence of *C. jejuni* carriage in the dogs from the boarding kennel (9%, CI 95% 1.9, 26) was in the mid range of other types of dog populations (Burnens *et al*., 1992; Engvall *et al*., 2003; Fox *et al*., 1983; Hald and Madsen, 1997; Lopez *et al*., 2002; Rossi *et al*., 2008; Sandberg *et al*., 2002; Wieland *et al*., 2005).

None of the dogs positive for *C. jejuni* appeared to shed this species over the whole sampling period, except for one dog where two different strains were detected. It has

previously been noted that *C. jejuni* was shed from dogs over a short duration, usually only detected in a single sample from dogs sampled monthly over 12 months (Hald *et al*., 2004). In this present study neither of the two dogs from the boarding kennel who had *C. jejuni* isolated, had the bacterium isolated within the first week, suggesting the possibility that the *C. jejuni* was acquired within the kennel. This hypothesis is supported by the fact that these two *C. jejuni* strains showed identical PFGE fingerprints and both belonged to ST-508, which appears to be relatively uncommon in dogs (Chapter 6). In the one dog (kennel 1) that appeared to be positive for *C. jejuni* in first and last samples, molecular evidence suggested that this dog actually shed two different strains of *C. jejuni*, demonstrated by different PFGE patterns, and different MLST types (complexes ST-283 and ST-45) (Chapter 6). Therefore no single *C. jejuni* strain was shed for the duration of the study for any individual dog in either kennel.

In agreement with the findings of this present study, the majority of other studies have found no association between *Campylobacter* spp. carriage in dogs and diarrhoea (Acke *et al*., 2006; Koene *et al*., 2008; Sandberg *et al*., 2002; Workman *et al*., 2005). However associations have been made when *C. jejuni* was the most commonly isolated *Campylobacter* spp. (Fleming, 1983; Fox *et al*., 1983; Nair *et al*., 1985). *C. jejuni* was significantly associated with diarrhoea in dogs housed in one animal shelter (Sokolow *et al*., 2005), and recently Acke *et al*, (2009) reported that *C. jejuni* was the most prevalent species in dogs with diarrhoea. In a combined population of boarding kennelled, stray and vet-visiting dogs, Baker *et al*, (1999) reported that the presence of *C. jejuni* was 17% in diarrhoeic faeces, whereas the *C. jejuni* prevalence in samples of normal consistency was 5%. Interestingly, all of the *C. jejuni* isolates from this latter group, were isolated from stray dogs, not boarding kennelled, or vet-visiting dogs. Therefore it

is possible that carriage of *C. jejuni* in dogs could be the result of a more transient infection, unlike *C. upsaliensis* which may be considered a commensal in dogs.

Kennelled dogs, both rescue and those housed in boarding kennels, may pose a greater risk of *C. jejuni* infection to humans than other dog populations, such as vet visiting (Chapter 3), and there is some evidence from this present study to suggest that dogs can begin to shed *Campylobacter* spp., particularly *C. jejuni*, and/or different strains of *Campylobacter* spp. after entry into the kennel.

4.5.3 Comparison of Different Dog Populations

Some studies have found stray or shelter dogs tend to shed *Campylobacter* spp. more frequently than household pets (Acke *et al*., 2006; Tsai *et al*., 2007; Workman *et al*., 2005), and intensive housing has been identified as a risk factor for *Campylobacter* spp. carriage in dogs (Baker *et al*., 1999; Torre and Tello, 1993). Reasons for this may include increased exposure to sources of *Campylobacter* spp., both inside and outside the kennel (discussed further in the final discussion chapter eight). Other possibilities include transmission between dogs, mediated by high density housing, and stress experienced by the dogs upon entering the kennelled environment.

There was a higher prevalence of *Campylobacter* spp. carriage in the rescue dogs within this study, although no statistically significant difference could be found between the two kennels for the overall prevalence of *Campylobacter* spp. Interestingly, none of the boarding dogs appeared to shed *C. jejuni* on entry to the kennel, unlike the rescue dogs, where four dogs entered the kennel carrying *C. jejuni*. This suggests that some of the rescue dogs came into contact with sources of *C. jejuni* outside the kennel, whereas the boarding (household) dogs appeared to have less exposure to *C. jejuni* prior to entering

the kennel. This is supported by the findings in a study of household pets in a similar locality, where carriage rates of *C. jejuni* were similarly low (0.5%) compared to the dogs in the present study on entry to the boarding kennel (Westgarth *et al*., 2009).

4.5.4. Possible Transmission Events

There were possible transmission events in both kennels. In both kennels combined, five dogs shed *C. upsaliensis*, and five dogs shed strains of *C. jejuni* that were not detected within the dog's first sample, only subsequent samples. Limitations in the sensitivity of detection methods may partly explain this, but additionally other possibilities, such as increased shedding after stress, are discussed below.

4.5.4.1 Quarantine Block

Of the three dogs in the rescue kennel who appeared to have acquired *C. jejuni* strains, two appeared to have acquired infection during their last six to seven days in the quarantine block. Reasons for this are unclear but may include; transmission between dogs from increased socialising from sharing pens, exposure to a contaminated environment, the effects of stress caused by moving to a different block, or an increase in the burden of other pathogens, presumably transmitted from different dogs with various ailments in the quarantine block. Further to this, many of the dogs housed in the quarantine had kennel cough, and results from this present study indicated that kennel cough may be significantly associated with dogs carrying *Campylobacter* spp., although this may be a confounding factor for other unidentified risk factors. Associations have previously been made between indicators of poor broiler flock health, such as digital dermatitis and increased *Campylobacter* spp. carriage (Bull *et al*., 2008). This has been attributed to poor biosecurity, common environmental effects, increased transmission

(explained further in 4.5.4.2.3), or stress (explained further in 4.5.4.3)(Bull *et al*., 2008), all of which are possible factors in this present study, particularly for the quarantine.

Additionally one dog from the rescue kennel appeared to shed *Campylobacter* spp. (presumed to be *C. upsaliensis*) more frequently once it was moved into the quarantine block. *C. upsaliensis* isolates from this dog, obtained during the dog's stay in both the holding and quarantine block were examined, but phylogenetic analysis based on the partial *groEL* gene sequence could not distinguish between them. This suggests that this dogs did not become re-infected with a different *C. upsaliensis* strain once it entered the quarantine block, but that there were other reasons for the increased shedding frequency, such as stress or exposure to other pathogens.

4.5.4.2 Transmission Within the Kennels

4.5.4.2.1 Food

Food may have contributed to the sources of *Campylobacter* spp. within the kennels. Bacteria such as *Salmonella* spp. have been isolated from dog food and dog treats (CDC, 2008b; Finley *et al*., 2008; Strohmeyer *et al*., 2006; Weese *et al*., 2005). The food in each kennel was not tested, but consisted mostly of standard dried dog food in both kennel, with the boarding kennel also providing the dogs with tinned 'hot dog' sausages (61% mechanically recovered chicken). It seems unlikely that dried dog food or food prepared for human consumption were sources of *Campylobacter* spp. for the dogs in this present study, supported by other work which did not isolate any *Campylobacter* spp. in commercially available raw diets for dogs (Strohmeyer *et al*., 2006; Weese *et al*., 2005). However, risks may have arisen from cross contamination or inadequate storage of the food in the kennels. In this situation diet seems an unlikely source of

Campylobacter spp. but cannot be fully dismissed, particularly for those dogs that started shedding *C. jejuni* after entry.

4.5.4.2.2 Water and Environmental Sources

Other possible sources could include wild birds since all dogs were allocated time in outdoor pens and *Campylobacter* spp. have been isolated from wild birds in several studies (Brown *et al*., 2004; French *et al*., 2005; Waldenstrom *et al*., 2002; Waldenstrom *et al*., 2007; Wilson *et al*., 2008). *Campylobacter* spp. have also been isolated from rodents and even flies, both of which had access to the kennels (Adhikari *et al*., 2004; French *et al*., 2005; Meerburg *et al*., 2006). As mentioned previously *Campylobacter* spp. have also been isolated from water (Brown *et al*., 2004; French *et al*., 2005; Horman *et al*., 2004; Kemp *et al*., 2005), and exposure to open drains have been significantly associated with an increase in *Campylobacter* spp. carriage by up to 2.6 times in intensively housed cats and dogs (Baker *et al*., 1999).

4.5.4.2.3 Transmission Between Dogs

Staff in both kennels had frequent contact between the different dogs of the same kennel, and the dogs in the rescue kennel were all exercised in the same field providing opportunity for transmission between the dogs. Pens were hosed down and disinfected with bleach every day, although the water usually collected into communal drains. Although most of the dogs in this study had individual indoor and outdoor pens for the duration of their stay, occasionally dogs were housed in pairs or rotated every few hours, particularly in the outdoor pens of the quarantine block, and on admission to the rescue kennel. In the boarding kennel, some dogs were housed in pairs, sometimes on a short term basis, but usually on a long term basis if the dogs originated from the same house.

As mentioned in chapter three, Acke *et al*, (2006) suggested that kennels have a higher prevalence of *Campylobacter* spp. carriage, possibly due to cross-infection facilitated by the animals living in groups, and Damborg *et al*, (2008) suggested that transmission of *Campylobacter* spp. between dogs can occur because of indistinguishable AFLP patterns in *C. upsaliensis* strains that were isolated from dogs living in the same house or kennel. Further to this, a study in the UK identified that dogs in a socialising group, had the second highest *Campylobacter* spp. prevalence (after dogs with diarrhoea), compared to kennelled and visiting dogs, suggesting that dog to dog transmission, or effects of socialising may be important (Guest *et al*., 2007). It is unknown what degree of contact, or what time scale would be required for transmission to occur between dogs, but presumably any situation that allows one dog to come into contact with another dog's faeces provides opportunity for *Campylobacter* spp. transmission.

Factors regarding; age, immunity, and the presence of already established *Campylobacter* spp. within a dog (particularly *C. upsaliensis*), will most likely affect a dog's susceptibility to *Campylobacter* spp. invasion. For example, results based on sequence analysis of the partial *groEL* gene suggested that cycles of *C. upsaliensis* re-infection did not occur, and that dogs continued to shed the same strain. In contrast *C. jejuni* was shed over a shorter duration, and different strains were observed within the same dog. A future study may benefit from examining *C. jejuni* and different strains of *C. upsaliensis in vitro* to determine if an established strain can be displaced by another. However, the dogs in this study who did not shed *Campylobacter* spp. for their first two or three samples, may not necessarily have acquired the bacterium from other dogs or sources in the kennel. This could be due to the effects of stress, a failure in the detection methods used, or it could simply be a particular shedding pattern within that dog.

4.5.4.3 Dogs Exposed to Stress

Some studies have found that kennels cause elevated stress levels in dogs, particularly in dogs naïve to kennelled environments (Hiby *et al*., 2006; Rooney *et al*., 2007). Cortisol/creatinine (CC) ratios have been successfully used as a stress indicator in dogs (e.g. under conditions of hypoglycemia)(Beerda *et al*., 1996). Dogs habituated to spatial group living show increased urinary CC ratios when they are exposed to individual, spatially restricted housing, even after five weeks, with the greatest effects observed in bitches (Beerda *et al*., 1999). This latter study also suggested that even bad weather could induce stress in dogs (Beerda *et al*., 1999). When animals experience stress or trauma, neurotransmitters such as noradrenaline (NA) increase (Buhler *et al*., 1978; McCarty *et al*., 1997), and *C. jejuni* cultures exposed to this neurotransmitter in iron-restricted environments show evidence of increased growth rate, motility, and invasion of cultured epithelial cells (Cogan *et al*., 2007; Humphrey, 2006).

Neurotransmitters facilitate this effect because normally animal hosts reduce the amount of iron available in the intestine to levels below the minimum required for bacterial growth (Andrews *et al*., 2003; Bullen *et al*., 1991). Bacteria have therefore evolved strategies to capture iron, such as siderophores which are able to scavenge iron from the environment (Andrews *et al*., 2003; Humphrey, 2006). If high affinity iron-binding proteins such as transferrins or lactoferrins, are present in the intestinal mucosa, the ability of the siderophores to retrieve iron may be reduced (Bullen *et al*., 1991). *C. jejuni* possesses very few or no siderophores (Field *et al*., 1986), and when neurotransmitters are released (e.g. due to a stressful environment), they can mediate the removal of iron from host transferrins (Freestone *et al*., 2002), making iron available for *C. jejuni*.

Thus it is likely that the stress of being in kennels may lead to an increase in the amount of NA levels and *C. jejuni* shedding in the same dog, and that this may have contributed to, or offer an alternative explanation to transmission within the kennel. Although little work has been performed on *C. upsaliensis* and iron acquisition, evidence has been found suggesting that *C. upsaliensis* genes encoding iron uptake regulation such as *fur*, share up to 87% amino acid identity with *C. jejuni* (Bourke *et al*., 1996), which may indicate that similar mechanisms are utilised by these two species for retrieving iron. This may also have been the reason why one dog appeared shed *C. upsaliensis* more frequently once it was moved to the quarantine block. Therefore associations between stress and increased *C. upsaliensis* shedding can not be dismissed.

4.5.5. Risk Factors for Campylobacter spp. *Carriage*

4.5.5.1 Length of Stay

Work by others has suggested that stray, kennelled dogs, shed significantly more *Campylobacter* spp. five to seven days after arrival compared to entry (Burnie *et al*., 1983). However there was no significant association between *Campylobacter* spp. carriage in dogs and length of stay in the kennels, despite the linear relationship shown in the GAM. This result is not surprising since the majority of dogs entered the kennels already shedding *Campylobacter* spp., and furthermore the number of dogs in the present study was relatively low.

Additionally, it has been suggested that stray animals can become infected after arrival, but before sampling (Burnie *et al*., 1983; Gruffydd-Jones *et al*., 1980). The majority of the dogs in this study stayed in the kennel for approximately 24 hours (depending upon the time of day the dog was admitted) before a faecal sample was collected. This was sometimes longer if the dog did not produce a faecal sample at the time of sampling on

the first day, and most of the dogs were housed with other dogs on arrival to the kennel. This time period (24 hours) might be long enough for dogs to acquire and subsequently shed the bacterium, which, coupled with the origin of some of these dogs (i.e. stray), could explain the high prevalence of *Campylobacter* spp. found on entry in this present study. Islam *et al*, (2006) demonstrated that when *Macaca mulatta* (Rhesus macaque) were infected with doses of 10^7 CFU *C. jejuni*, 70% of the monkeys shed the same strain within 24 hours. Although the bacterial enumeration in this study suggested that the majority of dogs shed fewer than 4×10^6 CFU of *Campylobacter* spp., the spiral plating method was unable to differentiate between infections of 4×10^6 CFU and greater, and some counts had to be discarded due to lack of PCR confirmation. Therefore some dogs may have shed bacterial loads of 10^7 CFU or greater. Dogs infected with high doses of *Campylobacter* spp. within the first 24 hours of arrival in the kennel, may have subsequently shed the bacterium in the first sample collected, which may have been mistaken for carriage prior to entering the kennel.

In contrast, the incubation period in humans appears to range from one to ten days, averaging at approximately three to four days, which is also dose dependent (Blaser *et al*., 1987; Wood *et al*., 1992). If this situation is typical for dogs as well as humans, this suggests that the dogs who shed *C. jejuni* within their first sample, probably acquired the bacterium before entering the kennel, but infections acquired in the kennel, prior to sampling, cannot be fully dismissed.

4.5.5.2 Age Associations with Campylobacter spp.

In agreement with a previous study (Chapter 3), younger dogs were found to be significantly more likely to carry *Campylobacter* spp. than older dogs. This has been reported in several studies (Acke *et al*., 2006; Engvall *et al*., 2003; Guest *et al*., 2007;

Sandberg *et al*., 2002; Westgarth *et al*., 2009; Wieland *et al*., 2005), and this may be associated with a developed immunity to the bacterium in older dogs. Evidence has been found in other animals demonstrating that after an initial *Campylobacter* spp. infection, the host retains some immunity if exposed to the bacteria again (Russell *et al*., 1989). Of the six dogs carrying *C. jejuni* in the rescue kennel, all except one (60 months) were 12 months of age or younger.

Although the (age) graph produced did not indicate a linear relationship, results were skewed by a dog of 192 months of age shedding *Campylobacter* spp. in every sample tested, and a dog aged 132 months shedding *C. jejuni* in its last sample (i.e. a late shedder). It is difficult to analyse results based on two dogs but if this trend is representive, and older dogs are at a greater risk of carrying *Campylobacter* spp. than slightly younger adult dogs, this too is probably related to immunity, which can decrease with age (Blount *et al*., 2005; Greeley *et al*., 2001; Greeley *et al*., 1996; Kaszubowska, 2008). There is also evidence to suggest that effects of stress may be more prominent in older adult animals compared to younger adult animals because NA levels in the plasma are higher, spillover into the intestine is greater, and clearance is poorer in older animals at baseline and during stress (McCarty *et al*., 1997). Caution must be taken in interpreting the results of this current study because the majority of the dogs in the rescue kennel had their age estimated, and because *C. jejuni* and *C. upsaliensis*, which may have different roles, were analysed together.

4.5.5.3 Clinical Signs

No associations could be made between diarrhoea and dogs carrying *Campylobacter* spp. in this study, supported by other studies (Acke *et al*., 2006; Koene *et al*., 2008; Sandberg *et al*., 2002; Workman *et al*., 2005), although having kennel cough was

associated with carrying *Campylobacter* spp. and vomiting appeared protective against *Campylobacter* spp. carriage. The data for both vomiting and kennel cough, were based on small data sets, and this coupled with the uncertain accuracy regarding the recording of these variables, makes it difficult to determine the validity of these findings, particularly when there is little or no evidence in other work to support them.

Vomiting may reduce an animal's appetite, and prevent ingestion of *Campylobacter* spp., or clear *Campylobacter* spp. before colonisation in the intestine. Alternatively, the vomiting may be caused by a more established pathogen that prevents *Campylobacter* spp. colonisation, or vomiting may indicate an early acute infection (Chapter 6, section 6.6.2.2.1). Some dogs carrying *Campylobacter* spp., particularly *C. jejuni*, have showed signs of vomiting in another study (Fox *et al*., 1983) which may or may not be related to infection with *Campylobacter* spp. Generally little has been reported concerning vomiting and the presence of *Campylobacter* spp. in dogs, and when it has been reported, no significant associations have been found (Westgarth *et al*., 2009).

Kennel cough was significantly associated with dogs carrying *Campylobacter* spp. A number of pathogens have been implicated in the aetiology of respiratory disease in dogs (kennel cough); canine parainfluenza virus (Erles *et al*., 2004), *Bordetella bronchiseptica* (Chalker *et al*., 2003) and canine coronavirus (Erles and Brownlie, 2005). Although the disease is usually associated with a viral infection, secondary opportunistic bacteria can infect the host, and a multi-factorial pathogenesis has been suggested for this disease (Buonavoglia and Martella, 2007). High density housing of dogs in kennels allows for introduction of different pathogens, including those that are associated with kennel cough and *Campylobacter* spp. which probably explains the apparent association between kennel cough and *Campylobacter* spp. carriage observed within this study.

4.5.6. Bacterial Enumeration

The dogs appeared to shed approximately between 2×10^5 and 4×10^6 CFU/ml or more of *Campylobacter* spp. within their faeces, although was the limits of detection were between 200 and 4×10^6 CFU/ml. Experiments with other animals such as monkeys found that animals had to be infected with doses of $\times10^{11}$of *C. jejuni* in order for 80% of the animals to show signs of mild disease (Islam *et al*., 2006). Conversely, doses as low as 500 bacterial cells (ACMSF, 2005), and 8×10^2 have been demonstrated to infect humans, who subsequently showed signs of disease (Medema *et al*., 1996). A *C. jejuni* outbreak in a nursery, thought to have originated from bird-pecked milk, demonstrated how approximately six *C. jejuni* cells per 500ml could cause disease in children (Riordan *et al*., 1993). However loads of approximately 9×10^4 have been shown to produce disease in more humans than other doses (Medema *et al*., 1996). The enumeration results based on *C. jejuni* were not conclusive within this present study, due to mixed infections and lack of PCR confirmation. Therefore the load required for *C. jejuni* to cause symptoms in dogs, and thus possibly humans via dogs, remains unclear. Levels of approximately 4×10^6 CFU/ml faeces or greater of *C. upsaliensis* in general appear not to induce clinical signs in the dog, as demonstrated by this study. This supports the hypothesis that *C. upsaliensis* may be a commensal in dogs. However there is little information available on the infectious dose required for *C. upsaliensis* infection in humans.

4.5.6.1 Factors Associated with Changes in Shedding

In this study, factors such as diarrhoea, and the presence of *C. jejuni,* appeared to coincide with possible variations in the amount of *Campylobacter* spp. shed within some dog samples, but this effect was not examined statistically as numbers were small. In kennel 1, no *Campylobacter* spp. were isolated from two dogs when they were initially

moved to the quarantine with suspected kennel cough. One dog was treated with antibiotics, and although the second dog did not have antibiotic treatment recorded, it is possible it was also treated with antibiotics. As mentioned previously, the effects of stress can not be dismissed, and stress may be responsible for any instances of dogs shedding higher counts of *Campylobacter* spp. within their first sample(s), compared to later samples, which arguably occurred in six dogs.

Two dogs in kennel 2 appeared to demonstrate an increase in bacterial load when *C. jejuni* and *C. upsaliensis* were detected for the first time. However this increase was only for a short duration, supporting the previous observation that *C. jejuni* was never shed for more than two or three samples consecutively, possibly indicating its role as a transient infection rather than as a commensal. Whether or not infection with *C. jejuni* increased the bacterial load of a pre-existing *C. upsaliensis* colonisation is unclear from this study.

4.5.6.2 Spiral Plating Limitations

Results produced from spiral plating were occasionally difficult to interpret and inconsistent with isolation methods run in parallel. This could be due to variations in the quantity and dilution of the samples applied to them. It was not possible to quantify plates which contained *Campylobacter* counts greater than 4×10^6 CFU/ml faeces because this was this the maximum that could be calculated at the dilution used with the manufacturers instructions (WASP 2'; Don Whitely Scientific Limited, Shipley, UK); in future studies it may be beneficial to use further dilutions. A study based on *E. coli* in cattle determined that counts obtained via spiral plating were accurate between the range of 1×10^3 to 1×10^8 CFU g^{-1} but that the precision of the counts decreased below 1×10^3, indicated by an increase in the coefficient of variation (Robinson *et al*., 2004). Habib *et*

al, (2008) noted that mCCDA often resulted in *Campylobacter* spp. swarming the plate and that the isolation medium chosen was important, especially when trying to generate countable results. The spiral plates were prone to contaminants, which may have been due to lower concentrations of antibiotics compared to direct plating, the lack of a filter, or despite care, insufficient cleaning of the equipment.

An alternative may be the use of real time PCR in order to quantify *Campylobacter* spp. in canine faecal samples. This technique has been successfully used and adapted for several species of *Campylobacter* isolated from canine faecal samples, including some newly identified species in dogs, within the same sample, and can potentially detect up to 10^5 or 10^6 copies of target DNA (Chaban *et al*., 2009). Only *C. upsaliensis* and *C. jejuni* specific primers were used in direct PCR for this present study, but direct PCR can also potentially detect additional species depending on primer design and genes targeted. However, general *Campylobacter* spp. primers based on the partial *groEL* gene were not successful using direct PCR, mainly because they amplified a wide range of other bacterial species. In addition, amplification of the *hipO* gene to detect *C.jejuni* was not successful in the direct PCR assays; possible reasons for this are unclear but may relate to a lower yield of *C. jejuni* DNA in comparison to culture (Chapter 3, 3.5.2). Real time PCR may provide a more accurate quantitative assessment of *Campylobacter* spp. isolated from dogs than spiral plating because it does not depend upon an operator counting colonies, and although background contamination may reduce the sensitivity of the real time assay, it should not affect it to the same extent as it does for spiral plating.

4.6 Conclusion

The prevalence of *C. upsaliensis* and *C. jejuni* was high in both kennels, suggesting that this population of dogs, particularly younger dogs, may be an important source of *C. upsaliensis* and *C. jejuni* infection to dogs and to humans via dogs. Although the rescue kennel had a higher prevalence of both *C. upsaliensis* and *C. jejuni* carriage in dogs, no statistically significant difference was found between the two different kennel types for *Campylobacter* spp. carriage. Neither could this study find any significant differences in the carriage of *Campylobacter* spp. found in dogs on entry compared to the subsequent days of sampling for either of the kennels. Dogs carrying *C. upsaliensis* appeared to shed this species in the majority of samples, whereas the duration of *C. jejuni* shedding appeared to be limited. This suggested a commensal role for *C. upsaliensis*, whereas carriage of *C. jejuni* may have been the result of a transient infection. Some dogs however, had no *Campylobacter* spp. isolated from their faeces at any stage during the study. The majority of positive dogs entered the kennels already carrying *Campylobacter* spp. and although the numbers of dogs who did not shed *Campylobacter* spp. until after arrival were few, when this did occur, it involved *C. jejuni* proportionally more than *C. upsaliensis*.

5. Chapter five

Prevalence of *Campylobacter* spp Shedding in Cross-sectional Studies of Different Kennelled Dog Populations

5.1 Abstract

C. jejuni and *C. coli* cause the majority of *Campylobacter* infections in humans, although *C. upsaliensis* and *C. lari* have also been isolated. Although poultry and cattle are considered the main sources of infection, several studies have indicated that dogs could be a potential source of *Campylobacter* spp. for humans. The prevalence of carriage in dogs varies depending upon the population sampled, with *Campylobacter* spp. isolated more frequently from kennel dogs than household dogs. This study aimed to determine the prevalence and species distribution, for *Campylobacter* spp. from faecal samples of dogs housed in one rescue, one boarding, and four hunt kennels.

The prevalence of *Campylobacter* spp. was high (31%-71%) in four of the kennels, but low in the two other kennels (5% and 0%). *C. upsaliensis* predominated in the boarding and rescue kennels, whilst *C. jejuni* was the most commonly isolated species in two of the four hunt kennels sampled. In one hunt kennel, four different *Campylobacter* spp. were isolated including *C. coli*. Further *C. lari* was isolated more frequently than any other *Campylobacter* spp. within this kennel. The prevalence of *C. jejuni* was relatively high in two hunt kennels (14%, 95% CI 4, 33 and 26%, 95% CI 16, 40). Hunt dogs had the widest *Campylobacter* spp. distribution, possibly as a result of their diet and/or exposure to environmental sources of *Campylobacter* spp. Thus hunt dogs may pose a relatively greater risk of *C. jejuni*, *C. coli* and *C. lari* infection to humans, whereas rescue and boarding dogs remain significant sources of *C. upsaliensis*. However the exposure of humans to these canine populations may vary.

5.2 Introduction

The prevalence of *Campylobacter* spp. carriage in dogs varies depending upon the dog population. In household/vet-visiting dogs the prevalence ranges from 2.7% to 77%, although in most studies the prevalence is between 20-40% for this population (Acke *et al*., 2009; Engvall *et al*., 2003; Fernandez and Martin, 1991; Hald and Madsen, 1997; Koene *et al*., 2004; Lopez *et al*., 2002; Malik and Love, 1989; Rossi *et al*., 2008; Sandberg *et al*., 2002; Tsai *et al*., 2007; Westgarth *et al*., 2009). The prevalence in kennelled dogs ranges from 21% to 87%, and when household and stray/kennelled dogs have been compared, stray dogs consistently carry more *Campylobacter* spp. (Acke *et al*., 2006; Acke *et al*., 2009; Guest *et al*., 2007; Tsai *et al*., 2007; Workman *et al*., 2005).

In most dog populations, *C. upsaliensis* and *C. jejuni* tend to be the most commonly isolated species found in dogs, and occasionally species such as *C. coli*, *C. lari* and *C. helveticus* have been isolated, although in much smaller numbers (Acke *et al*., 2009; Engvall *et al*., 2003; Tsai *et al*., 2007; Wieland *et al*., 2005). Compared to other dog populations, the prevalence of *C. jejuni* (sometimes combined with *C. coli*) in kennelled/stray dogs, is relatively high ranging from 21% to 44% (Fernandez and Martin, 1991; Malik and Love, 1989; Tsai *et al*., 2007; Workman *et al*., 2005). The majority of *Campylobacter* spp. infections in humans are caused by *C. jejuni* and *C. coli*, but in a small proportion of cases *C. upsaliensis* and *C. lari* have been isolated (DEFRA, 2005, 2007; Goossens *et al*., 1990a; Labarca *et al*., 2002; Lastovica and Le Roux, 2003; Prasad *et al*., 2001). Since *C. jejuni* is responsible for the majority of human campylobacteriosis, kennelled dogs may potentially be a greater risk of *Campylobacter* spp. infection to humans than other dog populations.

The aims of this study were to determine the prevalence, and species distribution, of *Campylobacter* spp. in cross-sectional studies of dogs housed in one rescue, one boarding and four different hunt kennels. Little recent work has been done on *Campylobacter* spp. carriage in kennelled dogs within the UK (Burnie *et al*., 1983). Two recent studies in the UK and Ireland indicated a high prevalence of *Campylobacter* spp., but these were not described on a species level (Acke *et al*., 2006; Guest *et al*., 2007). There is a particular lack of information regarding hunt dogs in the UK and other countries. This population of dogs is of interest since these dogs mix in large groups and regularly come into contact with carcasses and other possible sources of *Campylobacter* spp.

5.3 Materials and Methods

Faecal samples were collected from six kennels (A-F). Kennel A was a boarding kennel, kennel B was a rescue kennel, and kennels C-F were hunt kennels. Kennels were chosen on the basis of convenience and the staff's willingness to participate. Questionnaires regarding the dogs' details' (including approximate age, breed sex, health status, and source), were filled in by the samplers for kennel B (Appendix 2, Fig 2.1). For kennel A, the date that the dogs entered the kennel was recorded by the samplers. Other information regarding the dog's sex, breed age etc was not recorded as no owners were present to give permission, however, if faeces appeared loose or bloody, this was recorded. Kennels C, D, E and F had general information recorded about all the dogs regarding breed, age range, diet, antibiotic treatment etc, but no individual information per dog was obtained.

All samples were transported to the laboratory and processed within 24 hours of collection except samples from kennel B which were kept at 4°C over night before processing. The details of the study populations were as follows:

5.3.1 Kennel A: Boarding Kennel

The boarding kennel holding approximately 75 dogs, was located in Birmingham and dogs were recruited and sampled on the same day in October 2007. The kennel was visited and samples were taken from any dog that produced faeces during the visit. The kennel consisted of three main conjoined blocks for dogs, one of which housed a large number of rescue dogs (Appendix 3, Fig 3.1, Block 3). Cats and rodents were also boarded there, but were housed in different areas. Staff moved freely between the different blocks. Dogs were fed standard commercial dog food, apart from those with special dietary requirements, or those who had their own food supplied by the owner.

5.3.2 Kennel B: Rescue Kennel

The rescue kennel holding approximately 170 dogs, was located in Cambridgeshire and dogs were recruited and sampled on the same day in August 2007. The kennel was visited and samples were taken from any dog that produced faeces during the visit. The centre was well funded, spacious and consisted of three main blocks for dogs, admission, re-home, and quarantine (Appendix 3, Figure 3.2). Horses, cats and rodents were housed in separate areas. Staff stayed within their designated block. Dogs were fed standard commercial dog food, apart from those with special dietary requirements.

5.3.3 Kennels C-F: Hunt kennels

In all four hunt kennels the age of the dogs ranged from new born to eight or nine years old. Dogs were housed in open air yards with indoor sleeping pens. During the hunting

season dogs covered ground grazed by livestock twice weekly, apart from kennel C where this occurred daily. Unlike kennels A and B, samples could not be assigned to individual dogs. Faeces were collected from dogs shortly after exercise, and although samples were presumed to be from different dogs, and care was taken to collect distinguishable faeces, some dogs may have been sampled more than once.

5.3.3.1 Kennel C

Kennel C in North Wales was sampled in May 2008. The kennel consisted of 82 hounds (English, American and Welsh foxhound), 28 of which were male and 54 of which were female, and 39 beagles, 18 of which were male and 21 of which were female. Dogs were fed a mixture of raw meat and bone in conjunction with formulated meal ingredients (wheat maize, soya, chicken, turkey, rabbit, green leaf, carrots, peas, oils and fats) which were fed according to appetite or condition depending on the time of year.

5.3.3.2 Kennel D

Kennel D in Cheshire was sampled in July 2008. The kennel consisted of 85 Old English foxhounds, 20 of which were male, and 65 of which were female. The kennel also bred 25-30 puppies per year. Dogs were fed only commercially formulated nutritional product (flake barley, wheat, maize, biscuit- 19% protein, molasses) once daily.

5.3.3.3 Kennel E

Kennel E in the West Midlands was sampled in July 2008. The kennel consisted of 87 dogs, most of which were Old English foxhounds, although a couple were Welsh foxhounds. There were 59 males and 28 females. This kennel had an outbreak of kennel cough (KC) during sampling. The antibiotic administered was; procaine penicillin and

dihydrostreptomycin sulphate BP (penstrep; company unknown). Dogs were fed meat pies and formulated ration (cooked wheat, meat and bone meal, cooked maize and biscuit) once daily according to appetite in close season and according to condition during hunting.

5.3.3.4 Kennel F

Kennel F in Cheshire was sampled in July 2008. The kennel consisted of 70 English foxhounds, 31 of which were male, and 39 were female. There were also an additional 18 puppies. Dogs were fed once a day on a meal based substrate during sampling, but usually dogs were fed on tripe.

5.3.4 Bacterial Culture

The culture methods are described in full in chapter two of this thesis. In brief; faecal homogenates were prepared in saline at a dilution of 1:10 (0.85% NaCl) and *Campylobacter* spp. were detected by one or two of the following culture methods; (i) Direct plating on to *Campylobacter* selective agar (Lab M) with the addition of cefoperazone and amphotericin (CA) (Lab M). (ii) Filtration through a 0.7 μm nitrocellulose membrane onto *Campylobacter* selective agar as in (i) with the addition of cefoperazone, amphotericin and teicoplanin (CAT) (Oxoid Ltd) supplement. Filtration was used to detect *Campylobacter* spp. in all kennels (Table 5.1) because this method was found to be either the most sensitive (Westgarth *et al*., 2009), or amongst the most sensitive detection methods in previous studies (Chapter 3&4). Additionally, direct plating as above was used in kennels A-D.

5.3.5 Direct Extraction of DNA from Faecal Samples ('Direct PCR')

Kennels A and B were also screened for *C. upsaliensis* and *C. jejuni* by using direct PCR from faecal samples as previously described in chapter two (Table 5.1). This procedure was only performed for kennels A and B because the longitudinal kennel studies (Chapter 4) found culture to be the most sensitive detection method when samples were fresh, particularly for species other than *C. upsaliensis*.

5.3.6 Species Identification

As described in chapter two, a series of PCR assays targeting selected genes were performed for species identification. Briefly, 16S rRNA encoding gene (Linton *et al*., 1996) and *glyA* (Wang *et al*., 2002) fragments were targeted for *C. upsaliensis* identification. For *C. jejuni* identification, amplification of a *hipO* fragment was used (Wang *et al*., 2002). All isolates, both cultured and those extracted directly from faeces were submitted to the three specific PCR assays. Suspected *Campylobacter* spp. isolates that were negative on all three assays underwent a partial *groEL* gene PCR assay (Karenlampi *et al*., 2004; Chapter 2), which if positive was sequenced to determine species.

Table 5.1. Detection methods used to detect *Campylobacter* spp. per kennel.

Kennel	Culture: Direct Plating	Culture: Filtration	Direct PCR	Samples Collected	Additional Information
Kennel A (R+B)	YES	YES	YES	per individual dog	
Kennel B (R)	YES	YES	YES	per individual dog	VAIN malfunction
Kennel C (H)	YES	YES	NO	from groups of dogs	
Kennel D (H)	YES	YES	NO	from groups of dogs	
Kennel E (H)	NO	YES	NO	from groups of dogs	KC* outbreak
Kennel F (H)	NO	YES	NO	from groups of dogs	

R=rescue, B=boarding, H=hunt dogs and KC*=Kennel cough treated with penicillin and streptomycin

5.3.7 Salmonella spp. *Isolation*

All samples were tested for the presence of *Salmonella* spp. according to the methods described in chapter two.

5.3.8 Statistics

Univariable logistic regression analysis of *Campylobacter* spp. prevalence was carried out on all six kennels. Analysis for risk factors was performed for *C. upsaliensis* carriage for kennels A and B combined, due to the relatively low sample numbers at each kennel. No risk factors were examined for the hunt kennels (kennels C-F) because individual dog data were not recorded. Samples were classified as positive if

Campylobacter spp. had been detected by any methodology, and for the purpose of the analysis it was assumed that each sample represented one particular dog. Chi-squared analysis and univariable logistic regression analyses were used to investigate the following variables for kennels A and B combined; kennel, diet, length of stay, diarrhoea status, blood in faeces, and vomiting status. Variables that were not recorded for kennel A, such as age, sex etc, could not be used for statistical analysis when both kennels were combined. In all the analyses, significant differences were indicated by a $P<0.05$. Statistical analyses were performed with SPSS 15.0, except GAMs, which were performed using S-plus (MathSoft Inc, 2005).

5.4 Results

Campylobacter spp. were detected in all the kennels sampled except one. The prevalence of carriage ranged from 0%-71%, but was high in the majority of kennels. Overall, *C. upsaliensis* and *C. jejuni* were the most commonly identified species in the kennels. However, the species identified varied depending upon the kennel type, with hunt kennels demonstrating the greatest diversity of *Campylobacter* spp. The prevalence of the different species at each of the different kennels is shown in Table 5.2.

5.4.1 Kennel A: Boarding Kennel

Fifty two samples were collected from the boarding kennel. *Campylobacter* spp. were detected in 37 samples, giving a relatively high prevalence of 71% (95% CI 59, 84) (Table 5.2). *C. upsaliensis* was detected in 36 samples, and *C. jejuni* was detected in one sample. The *C. jejuni* was found in block 3, the block that housed rescue dogs.

5.4.2 Kennel B: Rescue Kennel

Twenty nine samples were collected from the rescue kennel. *Campylobacter* spp. were detected in nine samples, giving a prevalence of 31% (95% CI 14, 48). *C. upsaliensis* accounted for eight of the *Campylobacter* spp., whilst one was identified as *C. jejuni* (Table 5.2).

These results were based on direct PCR alone (except for one *C. jejuni* isolate) due to a VAIN malfunction during the week that the samples were processed, and conditions became more aerobic than the optimum (11%). This resulted in very few plates showing any growth, and most of the ones that did grow, could not be confirmed by PCR or sequencing. The only exception to this was the one *C. jejuni* isolated. The fact that only one culture result could be included allows for the possibility that the actual prevalence could be greater than 31%.

5.4.3 Kennel C: Hunt Kennel

Forty nine samples were collected from a hunt kennel, 15 of which (31%, 95% CI 19, 44) were positive for *Campylobacter* spp. Two samples had *C. upsaliensis* only isolated, nine samples only had *C. jejuni* isolated, three samples had mixed infections of *C. upsaliensis* and *C. jejuni* and one sample had a mixed infection of *C. jejuni* and *C. coli*.

5.4.4 Kennel D: Hunt Kennel

Twenty one samples were collected from a hunt kennel, 10 of which (48%, 95% CI 28, 68) were positive for *Campylobacter* spp. One sample had only *C. upsaliensis* isolated, one sample had only *C. coli* isolated, four samples had only *C. lari* isolated, two samples had mixed infections of *C. upsaliensis* and *C. jejuni* isolated, one sample had a

mixed infection of *C. jejuni* and *C. lari*, and one sample had a mixed infection of *C. coli* and *C. lari*.

5.4.5 Kennel E: Hunt Kennel

Twenty samples were collected from a hunt kennel, one of which (5%, 95% CI 0.5, 21) was positive for *Campylobacter* spp. This sample was positive for *C. jejuni*. No other samples had *Campylobacter* spp. isolated.

5.4.6 Kennel F: Hunt Kennel

Twenty samples were collected from a hunt kennel. No *Campylobacter* spp. were isolated from this kennel.

5.4.7 Univariable Analysis

Univariable analysis using logistic regression based on all six kennels indicated that dogs from kennel A were significantly more likely to be carrying *Campylobacter* spp. than dogs from any other kennel ($P<0.001$), with the exception of kennel D (Appendix 3 Table 3.1).

5.4.7.1 Kennels A&B

Questionnaires were completed for each dog in boarding kennel A and rescue kennel B, although some information was missing, i.e. the neutered status of many females from the rescue kennel was unknown.

Analysis was performed for *C. upsaliensis* only, so two dogs who shed *C. jejuni* were not included in the analysis. Kennel A had a significantly higher number of dogs positive for *C. upsaliensis* than kennel B $P<0.001$ (Table 5.3, Table 5.4 and Appendix 3,

Table 3.1). Dogs who received recent antibiotics were more likely to be negative for *C. upsaliensis* carriage (*P*=0.04), although numbers for this group were small. The number of days that the dogs had been in the kennel for verged on significance when analysed with *C. upsaliensis* carriage status (*P*=0.058), although a GAM graph indicated that this relationship was not linear (Appendix 3, Fig 3.3). When length of stay was divided into two categories, 1-7 days in the kennel, versus ≥8 days in the kennel, the dogs who stayed longer than a week had increased odds (OR 5.2) of carrying *C. upsaliensis* (Table 5.3). No significant association was found between *C. upsaliensis* carriage status and the following variables; diarrhoea/soft faeces (*P*=1.0), vomiting (*P*=0.2), blood in faeces (*P*=0.4), or diet (*P*=0.1) (Appendix 3, Table 3.1). Multivariable analysis was attempted but was inconclusive due to the number of missing values.

5.4.8 Detection Methods Kennels A&B

In kennel A culture detected 92% of the 37 *Campylobacter* spp. positive samples, while direct PCR from DNA extracts detected 89%. Comparison between the two different culture methods, indicate that direct plating was the most sensitive method for detecting *C. upsaliensis* (97%), compared to filtration (70%). Direct plating detected significantly more *C. upsaliensis* than filtration (*P*=0.01). Culture detected four *C. upsaliensis* and one *C. jejuni* that direct PCR did not detect. Three dogs were positive for *C. upsaliensis* by direct PCR of DNA extracts, straight from faecal samples, but negative in culture.

In kennel B, culture only detected 11% of the *Campylobacter* spp. positive samples, whilst direct PCR detected 89% of the positive samples. This was predominantly due to a malfunction with the VAIN.

5.4.9 Salmonella spp.

Three samples from hunt kennel E had *Salmonella* spp. isolated from them, giving kennel E a *Salmonella* spp. prevalence of 15% (95%, CI 4, 35). All three isolates were typed as serovar *S*. Typhimurium, and no other *Salmonella* spp. were isolated from any other kennels.

Table 5.2. Prevalence of *Campylobacter* spp. by kennel.

Kennel	+	-	*Campylobacter* spp. %	CI	*C. upsaliensis* %	CI	*C. jejuni* %	CI	*C. coli* %	CI	*C. lari* %	CI
Kennel A	37	15	**71**	59-84	**69**	56-81	**2**	0.2-9	**0**		**0**	
Kennel B	9	20	**31**	14-48	**28**	14-45	**3**	0.4-15	**0**		**0**	
Kennel C	15	34	**31**	19-44	**10**	4-21	**26**	16-40	**2**	0.2-9	**0**	
Kennel D	10	11	**48**	28-68	**14**	4-33	**14**	4-33	**10**	2-27	**29**	13-50
Kennel E	1	19	**5**	0.5-21	**0**		**5**	0.5-21	**0**		**0**	
Kennel F	0	20	**0**		**0**		**0**		**0**		**0**	

Table 5.3. **Univariable analysis of dog variables and *C. upsaliensis* status from kennels A (boarding) & B (rescue) (*P*<0.1).**

Variable		+	-	Coef	S.E.	OR	95.0% C.I. Lower Upper	*P*-value
Kennel	Boarding	36	15			1		
	Rescue	8	20	-1.79	0.51	0.16	0.06-0.46	<0.001
Length of stay	Days	44	32	0.008	0.004	1.008	1.00-1.01	0.06
	1-7 days	4	11			1		
	≥8 days	40	21	1.65	0.64	5.23	1.48-18.47	0.01
Recent antibiotics	No	43	29			1		
	Yes	1	6	-2.10	1.10	0.11	0.01-0.98	0.04

Recent= Within the past month, S.E = Standard error and OR = Odds ratio. Two dogs which had *C. jejuni* isolated were not included in the analysis.

5.5 Discussion

The prevalence of *Campylobacter* spp. varied between kennels ranging from 0%-71%, but was generally quite high. *C. upsaliensis* and *C. jejuni* were isolated from nearly every kennel, whilst *C. coli* and *C. lari* were only isolated from hunt kennels. Additionally one hunt kennel was dominated by *C. lari*. The prevalence of *C. jejuni* was considerably higher in most of the hunt kennels compared to either the rescue or the boarding kennel, where *C. upsaliensis* dominated.

5.5.1 Rescue and Boarding Kennels

The prevalence of *Campylobacter* spp. found in the rescue kennel (31%) was in the mid range of the kennels within this study and other work (Acke *et al*., 2006; Hald and Madsen, 1997; Rossi *et al*., 2008; Sandberg *et al*., 2002; Tsai *et al*., 2007; Workman *et al*., 2005). The rescue kennel's prevalence may have been lower than the boarding kennel prevalence (71%) because of good hygiene practice/staff restrictions in the rescue kennel, and/or the VAIN malfunction which meant culture results could not be obtained from the rescue kennel. This may have an additional impact because previous studies indicated that culture was the most sensitive detection method when sample were fresh (Chapter 4). Alternatively this may indicate that rescue dogs are of no greater risk to humans in terms of *Campylobacter* spp. infection than boarding dogs. Other factors including the different locations of the kennels might also play a role.

5.5.2 Hunt Kennels

Although no generalisations can be made regarding the different kennel types (i.e. rescue, boarding and hunt) and what *Campylobacter* spp. are isolated, the hunt

dogs appeared to have the widest diversity of *Campylobacter* spp. and *Campylobacter* spp. prevalence. They also had the highest rates of *C. jejuni*, *C. lari* and *C. coli* carriage. The sampling technique could not guarantee that the same dog was not sampled twice. However attempts were made to reduce this possibility by selecting faeces, for example after dogs were walked or that were in different areas in the kennel. Repeat sampling of dogs also appears less likely because molecular based evidence suggested that many of the *C. jejuni* strains were different (Chapter 6), although this would not necessarily take mixed infections into account.

5.5.2.1 Low Campylobacter spp. *Prevalence, Kennels E&F*

There was a low prevalence found in kennels E and F, however sample sizes were small in these kennels and hence confidence intervals were large. It may also be the result of repeat dog sampling, or the methods used to isolate *Campylobacter* spp., because filtration was the only method used in these two kennels. Filtration with CAT supplement is optimised for *C. upsaliensis* detection (Byrne *et al*., 2001; Goossens *et al*., 1990b) so it is possible that it does not select for different *Campylobacter* spp. as efficiently, although evidence from the other kennels and studies in this thesis suggests that it would be unlikely for none of these dogs to carry *C. upsaliensis*. Nevertheless, these two kennels may have been dominated by *Campylobacter* spp. other than *C. upsaliensis*, so consequently filtration onto CAT media may not have been the most appropriate isolation method in this situation and may have missed *Campylobacter* spp. other than *C. upsaliensis*. Filtration also requires bacterial counts greater than 10^5 CFU per g of faeces in order to be detected (Goossens *et al*., 1990b).

Additionally, dogs from kennel E were undergoing treatment for an outbreak of kennel cough. Although the dosage and duration of the antibiotic treatment is unknown, the antibiotics used (e.g. penicillin and dihydrostreptomycin) may have had an effect on any *Campylobacter* spp. present, especially since these antibiotics reduce numbers of *C. fetus* (Shin *et al*., 1988; Sullivan *et al*., 1966). Furthermore, this study found dogs that had recently been treated with antibiotics were less likely to shed *C. upsaliensis*. It is possible that dogs from kennel F had also recently undergone treatment, unknown to the sampler, which may have affected the results. Another explanation might be that this population of dogs has acquired immunity to *Campylobacter* spp., or shed the bacterium in undetectable amounts. As previously mentioned, there was no guarantee that the same dog would not be sampled twice, which may have affected results, particularly if the prevalence was low.

5.5.3 C. upsaliensis

C. upsaliensis was the most commonly isolated *Campylobacter* spp. in kennels A (boarding) and B (rescue), which has also been found in other work based upon household or vet visiting dogs (Engvall *et al*., 2003; Koene *et al*., 2008; Rossi *et al*., 2008; Sandberg *et al*., 2002; Westgarth *et al*., 2009). However in agreement with findings from other kennelled dogs, and some other dog populations (Tsai *et al*., 2007; Workman *et al*., 2005), *C. upsaliensis* was the second most commonly isolated species found in kennels C (hunt) and D (hunt) (jointly with *C. jejuni* in kennel D). *C. upsaliensis* was not isolated from kennels E and F. The majority of kennelled dogs, whether they are rescue, boarding or hunt, appear to be important

reservoirs for *C. upsaliensis* infection for humans, particularly rescue and boarding kennels.

5.5.4 C. jejuni

The prevalence of *C. jejuni* in our study varied between low prevalences of 0%-5%, similar to other work that investigated household/vet visiting dogs (Rossi *et al*., 2008; Sandberg *et al*., 2002), and higher prevalences of 14% and 26% which have also been found in studies based upon various dog populations such as household and stray/sheltered dogs (Hald and Madsen, 1997; Hald *et al*., 2004; Lopez *et al*., 2002; Tsai *et al*., 2007; Workman *et al*., 2005). *C. jejuni* was the most commonly isolated species in kennels C and E, (the only species isolated from kennel E), and it was the second most commonly isolated species (jointly with *C. upsaliensis*) in kennel D. The *C. jejuni* prevalence was considerably higher in the hunt dogs (excluding kennel F) than in the rescue, and boarding dogs, despite a higher overall *Campylobacter* spp. prevalence in the boarding dogs. This suggests hunt dogs may be an important potential source of *C. jejuni* infection for humans, although this will also depend on other factors such as level of contact between the species. However, previous work (Chapter 4), based on longitudinal studies, suggests that certain rescue and boarding kennels could still be a potential risk of *C. jejuni* infection for humans.

5.5.5 C. coli and C. lari

The prevalence of *C. coli* and *C. lari* was also higher in the hunt dogs than in the rescue or boarding dogs, where neither of these two species were isolated. Mixed infections with these species and/or *C. upsaliensis*/*C. jejuni* were observed in

several samples, and this has been reported in other work (Engvall *et al*., 2003; Hald *et al*., 2004; Koene *et al*., 2008). The prevalence of *C. coli* isolated from the dog samples was similar to findings in other studies (Engvall *et al*., 2003; Tsai *et al*., 2007), and like other studies (Koene *et al*., 2008; Rossi *et al*., 2008; Sandberg *et al*., 2002) some kennels had no *C. coli* isolated from the dog samples. *C. lari* was the most frequently isolated species in kennel D, demonstrating a prevalence of 29% and was unique to this kennel. Although this species has been isolated from dogs in other work, the prevalence has been considerably lower, usually between 1-2%, although one study reported a slightly higher prevalence of 10% (Engvall *et al*., 2003; Hald *et al*., 2004; Koene *et al*., 2008; Rossi *et al*., 2008). Very few studies have found *C. lari* to dominate in dog populations, or have a prevalence as high as 29%, suggesting that this kennel may have had particular risk factors for exposure to this organism. Overall these studies on hunt kennels suggest that they may be a possible source of *C. lari* and *C. coli* to dogs and thus possibly humans.

5.5.6. Diet

Diet, particularly the consumptions of raw/undercooked/cross-contaminated poultry plays the most significant role in human campylobacteriosis (Hussain *et al*., 2007; Sheppard *et al*., 2009; Wilson *et al*., 2008). Therefore differences in diet among the kennels may have influenced the *Campylobacter* spp. status of the dogs. For example, kennel E had a very low *Campylobacter* spp. prevalence and these dogs were fed meat pies. Pies will have undergone strict hygiene procedures to make them fit for human consumption, reducing the risk of *Campylobacter* spp. In contrast dogs in kennel C were fed raw meat and bone and dogs from this

kennel had the highest carriage rate of *C. jejuni*; previous studies have isolated *C. jejuni* and *C. coli* from carcasses and meat (De Cesare *et al*., 2008; Workman *et al*., 2005). Raw meat is a source of *Campylobacter* spp. (predominantly *C. jejuni* and *C. coli*) and has been associated with *Campylobacter* spp. infection in humans (Eberhart-Phillips *et al*., 1997; Karenlampi *et al*., 2007; Little *et al*., 2008).

5.5.7 Environmental Sources of Campylobacter spp.

Hunt dogs potentially come into contact with more environmental sources of *Campylobacter* spp. than other dog populations. Several papers have identified environmental sources of *C. jejuni, C. lari* and *C. coli* such as surface water (Horman *et al*., 2004; Kemp *et al*., 2005), cattle faeces, rabbit faeces, badger faeces, and soil (Brown *et al*., 2004; Leatherbarrow *et al*., 2007), and all three of these *Campylobacter* spp. have been isolated from wild birds (Colles *et al*., 2008b; Waldenstrom *et al*., 2002; Waldenstrom *et al*., 2007; Workman *et al*., 2005). In dogs, significant associations have been found between *C. jejuni* isolation and regular contact with birds or poultry (Wieland *et al*., 2005). The majority of the hunt dogs will have potential contact to these sources whilst they are out hunting, being walked, and even in their diets. Additionally this might have implications for household dogs that are exposed to similar possible sources of *Campylobacter* spp.

5.5.8 Salmonella spp.

No *Salmonella* spp. were isolated in any of the kennels investigated apart from hunt kennel E where the prevalence was relatively high (15%) compared to the other kennels in this study and other reports from different countries

(Seepersadsingh *et al*., 2004; Tsai *et al*., 2007). There is currently little UK data available on the prevalence of *Salmonella* spp. in dogs, although a low prevalence (6%) has been observed previously (Burnie *et al*., 1983), and outbreaks have also been reported more recently (Schotte *et al*., 2007). In addition, one study in the UK reported that of 59 dogs carrying *Campylobacter* spp., one also carried *Salmonella* spp. (Fleming, 1983). Seepersadsingh *et al* (2004) examined several different populations of dogs, including vet-visiting, household, pounds/shelters, and hunt dogs, but still found a relatively low, overall *Salmonella* spp. prevalence of 3.6%. Of these dog populations (excluding quarantine dogs), pound dogs rather than hunt dogs, demonstrated the highest prevalence of *Salmonella* spp. carriage (Seepersadsingh *et al*., 2004). However, the prevalence in kennel E (15%) was similar to findings of another study that investigated household dogs (15.4%) (Fox *et al*., 1983).

Salmonella enterica serovar Typhimurium, appears to be one of the most common, or second most common serovars found in dogs from several studies (Bagcigil *et al*., 2007; Fukata *et al*., 2002; Hald *et al*., 2004; Oloya *et al*., 2007; Seepersadsingh *et al*., 2004). *S.* Typhimurium is probably the most ubiquitous *Salmonella enterica* serovar, and tends to dominate in most animal species (Oloya *et al*., 2009; Oloya *et al*., 2007). Sources of *Salmonella* spp. infection for dogs are probably similar to those for humans, such as consumption of raw, or lightly cooked foods containing eggs or chicken (Braden, 2006; Currie *et al*., 2005; DEFRA, 2007).

S. Enteritidis and *S.* Typhimurium are the most commonly identified causes of salmonellosis in humans in the UK (DEFRA, 2007), and so the hunt dogs in this

study cannot be ruled out as a possible zoonotic risk to humans. However, the majority of dogs, from the majority of different dog populations do not appear to be a significant source of *Salmonella* spp. infection to humans.

5.6 Conclusion

Generally in most kennels the prevalence of *Campylobacter* spp. was relatively high. Overall *C. upsaliensis* and *C. jejuni* were isolated the most frequently, although *C. lari* dominated in one hunt kennel. Whether or not *C. upsaliensis* and *C. lari* cause disease in humans is still unclear, although these *Campylobacter* spp. have been isolated from humans in various studies (Goossens *et al*., 1990a; Labarca *et al*., 2002; Lastovica and Le Roux, 2003; Petersen *et al*., 2007; Prasad *et al*., 2001). *C. jejuni* and *C. coli* are much more important causes of campylobacteriosis in humans (DEFRA, 2007; Prasad *et al*., 2001). The prevalence of *C. jejuni* was relatively high in two kennels, and its presence in dogs may be related to diet. Hunt dogs in this study had the greatest species diversity for *Campylobacter* spp., presumably due to their diet and/or increased exposure to environmental sources of *Campylobacter* spp. Compared to other dog populations, hunt dogs may pose the greatest risk of *C. jejuni, C. coli* and *C. lari* infection to humans, whereas rescue and boarding dogs remain significant sources of *C. upsaliensis* infection to humans. The degree to which humans are exposed to these different dog populations may vary however.

6. Chapter six

Typing of *C. jejuni* and *C. upsaliensis* Isolates from Dogs Using Multilocus Sequence Typing and Pulsed Field Gel Electrophoresis

6.1 Acknowledgements

The multilocus sequence typing (MLST) of *C. jejuni* isolates within this study was done in collaboration with The University of Oxford. Allison Cody performed the PCR, sequencing, and assignment of alleles to sequence types, and subsequently clonal complexes. However the collection, species identification and DNA extraction of these isolates were performed at The University of Liverpool. All human *C. upsaliensis* isolates originated from the Health Protection Agency (HPA), with the exception of reference strain RM3195. The PCR and analysis of *C. upsaliensis* alleles were performed at The University of Liverpool, as was the purification of PCR products. Isolates were sent to MACROGEN for sequencing, but in addition, several new alleles with poor traces were sent to Bill Miller (Western Regional Research Centre, USA, curator of *C. upsaliensis* MLST database) for sequencing with subsequent submission to the *C. upsaliensis* MLST database.

6.2 Abstract

Risk of *Campylobacter* infection in humans has been associated with many sources including dogs. This study aimed to investigate whether or not *C. jejuni* and *C. upsaliensis* strains carried by dogs were distinguishable or not from strains carried by humans, and if there were possible common sources of *C. jejuni* infection for both humans and dogs.

MLST together with macro-restriction analysis of genomic DNA using *Sma*I and pulsed-field gel electrophoresis (PFGE), were both used to analyse 33 *C. jejuni* isolates obtained from various dog populations. MLST was also used to type 41 dog, and nine human *C. upsaliensis* isolates from various studies.

MLST data suggested that there was a large amount of genetic diversity amongst canine *C. jejuni* isolates, and that the majority of sequence types found in these dogs were the same as those reported in humans. The main exception was sequence type (ST) 2772, which was isolated from four samples and could not be assigned to a clonal complex. The most commonly identified clonal complex was ST-45 (11 isolates), followed by ST-21 (4 isolates), ST-508 (4 isolates), and ST-403 (3 isolates). The profiles obtained by macro-restriction PFGE were largely in concordance with the MLST results.

All *C. upsaliensis* sequence types were novel apart from the reference strain and only three were found in more than one isolate, ST-72 (2 isolates), ST-98 (2 isolates) and ST-104 (3 isolates). ST-104 was the only sequence type to be isolated from both dogs and humans. The isolates were assigned to 12 clonal complexes,

whilst 15 remained unassigned. Four of these complexes contained isolates originating from both humans and dogs. None of the complexes were exclusive to human isolates and two isolates from dogs within the same kennel belonged to the same complex.

There was considerable genetic diversity amongst both the *C. jejuni* and *C. upsaliensis* isolates from dogs and humans. This suggested that dogs are exposed to various sources of *C. jejuni* infection and the similarity of these sequence types to *C. jejuni* isolated from humans suggests there may be transmission, or common sources of infection for both dogs and humans. Strains of *C. upsaliensis* isolated from humans did not appear to group separately from dog strains, indicating common sources of infection, or possible transmission. Although only a small number of household dogs may carry *C. jejuni*, infected dogs should still be considered a potential zoonotic risk to humans, particularly if the dogs originate from kennelled or hunt dog populations. Dogs also remain a possible zoonotic risk to humans for *C. upsaliensis*, however, further work is needed to investigate the frequency, and severity of *C. upsaliensis* infection in humans.

6.3 Introduction

Campylobacter spp. are amongst the most commonly reported bacterial causes of human gastroenteritis (Adak *et al*., 2002; CDC, 2008c; DEFRA, 2007; Westrell *et al*., 2009). The majority of such infections are caused by *C. jejuni* and *C. coli*, however *C. upsaliensis* is occasionally isolated (Labarca *et al*., 2002; Lastovica and Le Roux, 2003; Vandenberg *et al*., 2006). There is some evidence of an elevated risk of *Campylobacter* spp. infection in humans associated with dog or pet ownership (Adak *et al*., 1995; FSA, 2005; Tenkate and Stafford, 2001). An estimated 6% of *C. jejuni*/*coli* enteritis cases in humans have been attributed to exposure to diarrhoeic animals (Saeed et al., 1993).

Multilocus sequence typing is a tool used to investigate genetic relationships between *Campylobacter* spp., particularly *C. jejuni*. MLST involves the amplification and sequencing of 7 well conserved housekeeping genes. The seven loci are sufficiently separated on the chromosome (70 kb) such that coinheritance of two or more loci in a recombination event is considered unlikely (Dingle *et al*, 2001). Isolates are defined as sequence types based on the allelic profiles of these seven genes. Sequences may the be allotted to membership of a clonal complex defined as two or more independent isolates with a sequence type that share identical alleles at four or more loci using the software program e BURST (Feil *et al* 2004). The technique allows estimation of both mutation and recombination rates. It is useful for investigating possible reservoir hosts, and host associations, and also for studying the epidemiology of the disease (Dingle *et al*., 2005; Ogden *et al*., 2007). *C. jejuni* clonal complexes ST-21 and ST-45, and others such as ST-61, are often isolated from human cases of campylobacteriosis (Dingle *et al*., 2002;

Ragimbeau *et al*., 2008). Complexes ST-21 and ST-45 are commonly found in chickens, cattle, water, and wildlife, and ST-61 has been particularly associated with cattle (Colles *et al*., 2003; French *et al*., 2005; Karenlampi *et al*., 2007; Sopwith *et al*., 2008).

Some other *C. jejuni* sequence types isolated from humans have not yet been associated with cattle and poultry, and the sources of these need further investigation (Ragimbeau *et al*., 2008). In contrast, some sequence types such as ST-702 in geese, are strongly associated with certain animal hosts (Colles *et al*., 2008a), but are not usually isolated from humans (Dingle *et al*., 2001; Dingle *et al*., 2002; Duim *et al*., 2003; Jolley and Chan, 2004; Ragimbeau *et al*., 2008). Compared to *C. jejuni* there is comparatively less genetic information available regarding *C. upsaliensis*, although a *C. upsaliensis* MLST scheme has recently been established (Miller *et al*., 2005), and an MLST database has recently been established (Jolley *et al*., 2004).

Whether or not dogs are a possible source of *C.jejuni* infection for humans is not fully understood. Macro-restriction pulsed-field gel electrophoresis (PFGE) has been used in studies to compare *C. jejuni* isolates found in both dogs and humans (Owen *et al*., 1995) and in some cases dog and owner did share an identical strain (Damborg *et al*., 2004). There are currently few MLST data available for *Campylobacter* spp. isolated from dogs, although *C. jejuni* ST-45 infection in humans has been significantly associated with contact with pet cats and dogs (Karenlampi *et al*., 2007).

The aims of this study were to examine 33 *C. jejuni* isolates obtained from dogs from various populations using MLST in order to (i) determine whether strains of *C. jejuni* carried by dogs could be distinguished from those found in humans and other species; and (ii) to determine possible sources of canine infection. Pulsed-field gel electrophoresis, with *Sma*I digestion was also performed on the isolates in order to investigate the epidemiology of the infection within the different dog populations.

Forty one *C. upsaliensis* isolates obtained from dogs from various populations, and nine human *C. upsaliensis* isolates were analysed using MLST to determine (i) whether strains of *C. upsaliensis* carried by dogs were distinguishable or not from strains found in humans and/or other species; and (ii) to investigate the genetic diversity of *C. upsaliensis* carried by dogs.

6.4 Materials and Methods

6.4.1 Selection of C. jejuni Isolates

Thirty three isolates of *C. jejuni* were isolated in the UK between 2005 and 2008; the origin of the isolates and locations are shown in Table 6.1. In summary, two were obtained from household pets (Westgarth *et al*., 2009; and an additional household dog), three from a cross-sectional study of dogs visiting veterinary practices (Chapter 3), and 11 were from longitudinal and cross-sectional studies of boarding and rescue kennels (Chapters 4&5). A further 17 isolates were obtained from three hunt kennels (Chapter 5). Samples were cultured using several different isolation methods as previously described in chapter two.

6.4.2 Selection of C. upsaliensis Isolates

Forty one isolates of *C. upsaliensis* were isolated from dogs in the UK between 2005 and 2008; the origin of the isolates and locations are shown in Table 6.2. Forty isolates were chosen at random using a random number generator from 227 *C. upsaliensis* isolates, (representing 227 different dogs), grown on culture from previous studies (Westgarth *et al*., 2009, and Chapters 3-5). An additional isolate, chosen for genome analysis (Dog 52 A; Chapter 7) was also included. Nine isolates of *C. upsaliensis*, isolated from human clinical cases were also examined. Eight of these isolates were obtained from the Health Protection Agency (HPA) (Sopwith *et al*., 2006, 2008), and one isolate was the reference strain RM3195 isolated from a human case of Guillain-Barré syndrome. Due to confidentiality, little information was known about the human isolates apart from the region, year of isolation and that all were symptomatic cases.

6.4.3 Bacterial Culture

Cultivation methods are described in chapter two; briefly for culture, *Campylobacter* selective agar (Lab M) with the addition of cefoperazone and amphotericin (CA) (Lab M); and filtration through a 0.7 µm nitrocellulose membrane onto *Campylobacter* selective agar as previously described but with the addition of cefoperazone, amphotericin and teicoplanin (CAT) (Oxoid Ltd) supplement were used to isolate *Campylobacter* spp. in all studies, except hunt kennel 7 where only filtration was used. Additionally *Campylobacter* enrichment broth (Lab M) supplemented with 10% lysed horse blood (Southern Group Labs Ltd) was also used for studies A and B (Table 6.1). All plates were incubated for 96 h at 37ºC under microaerophilic conditions (74 N_2%, 11% O_2, 3% H_2 and 12%

CO_2), in a variable atmosphere incubator (Don Whitely Scientific Ltd). Although up to four colonies and a sweep were taken from each plate (as described in Chapter 2, section 2.1), only one pure colony was chosen per sample for further molecular typing by MLST and PFGE.

6.4.4 Multilocus Sequence Typing

MLST was performed as previously described in chapter two, section 2.7. Briefly, for *C. jejuni,* seven housekeeping genes (*aspA, glnA, gltA, glyA, pgm, tkt* and *uncA*) were sequenced as described by Dingle *et al*, (2001), with some minor modifications. Alleles, sequence types and clonal complexes were assigned using the MLST database available at http://pubmlst.org/*Campylobacter*. MLST on *C. jejuni* isolates was carried out by Dr Allison Cody at Oxford University.

Briefly for *C. upsaliensis*, isolates grown on CAB were extracted using the Chelex protocol (Chapter 2, 2.7.2). Amplified fragments of seven housekeeping genes (*adk, aspA, atpA*, *glnA, glyA, pgi* and *tkt*) (Miller *et al*., 2005) and products were sequenced. Alleles, sequence types and clonal complexes were assigned using the MLST database available at http://pubmlst.org/*Campylobacter*. New alleles were submitted to Bill Miller, the curator of the *C. upsaliensis* MLST database. In some cases where sequence traces could not be submitted (due to double peaks at one base), chelex extracted DNA was sent to Bill Miller for further sequencing. Clonal complexes were assigned based on allelic profiles of the isolates using eburst version 3 (http://eburst.mlst.net/), where isolates within each clonal complex shared at least five alleles, as proposed by Miller *et al* (2005). This analysis was performed for the isolates within this study, and with all currently known *C.*

upsaliensis MLST data. Phylogentic analysis using maximum likelihood and neighbour joining trees, with bootstrap values using MEGA version 3.1 and Phylip version 3.68 (Chapter 2, 2.5.2).

6.4.5 Pulsed-field Gel Electrophoresis

Thirty three *C. jejuni* isolates were examined by macro-restriction PFGE using a modified version of Ribot *et al*., (2001) (Chapter 2, 2.8). Briefly, DNA was digested in 200µl 1x *Sma*I restriction buffer containing 40U *Sma*I for 2 hours at 25°C. A gel was run (1% PFGE agarose in 0.5x TBE); with an initial switch time of 6.7s, and a final switch time of 38.3s, with a total run time of 16 hours. The gel was stained in ethidium bromide, and examined under UV illumination. PFGE gels were analysed with BioNumerics V. 4.01 software (Applied Maths, Kortrijk) using the Dice similarity coefficient with 0.5% optimisation, 1% tolerance, and dendrograms were calculated using unweighted-pair group method with average linkages (UPGMA).

6.5 Results

6.5.1 C. jejuni

6.5.1.1 MLST

Thirty three isolates were assigned to nine different clonal complexes (Table 6.1, Appendix 4, Table 4.1). Overall, ST-45 was the most common clonal complex (11 isolates) identified in the dogs, followed by complexes ST-21 (4 isolates), ST-508 (4 isolates), and ST-403 (3 isolates). ST-2772 was isolated four times, but could not been assigned to a complex.

ST-45, the founder genotype of clonal complex ST-45 (Fig 6.1), was the most common, and was isolated five times. Although numbers were small, ST-45 was isolated from rescue dogs more frequently than other populations of dogs, whilst the three isolates belonging to ST-334 were exclusive to one hunt kennel within this study (Table 6.1 & Appendix 4 Table 4.1). All isolates belonging to clonal complexes ST-21 and ST-22 were obtained from hunt kennel dogs as were the four ST-2772 isolates (Table 6.1 & Appendix 4 Table 4.1).

Figure 6.1. All *C. jejuni* STs isolated from dogs in this current study (eBURST). *Primary founders coloured blue, size of dots is relative to number of isolates.

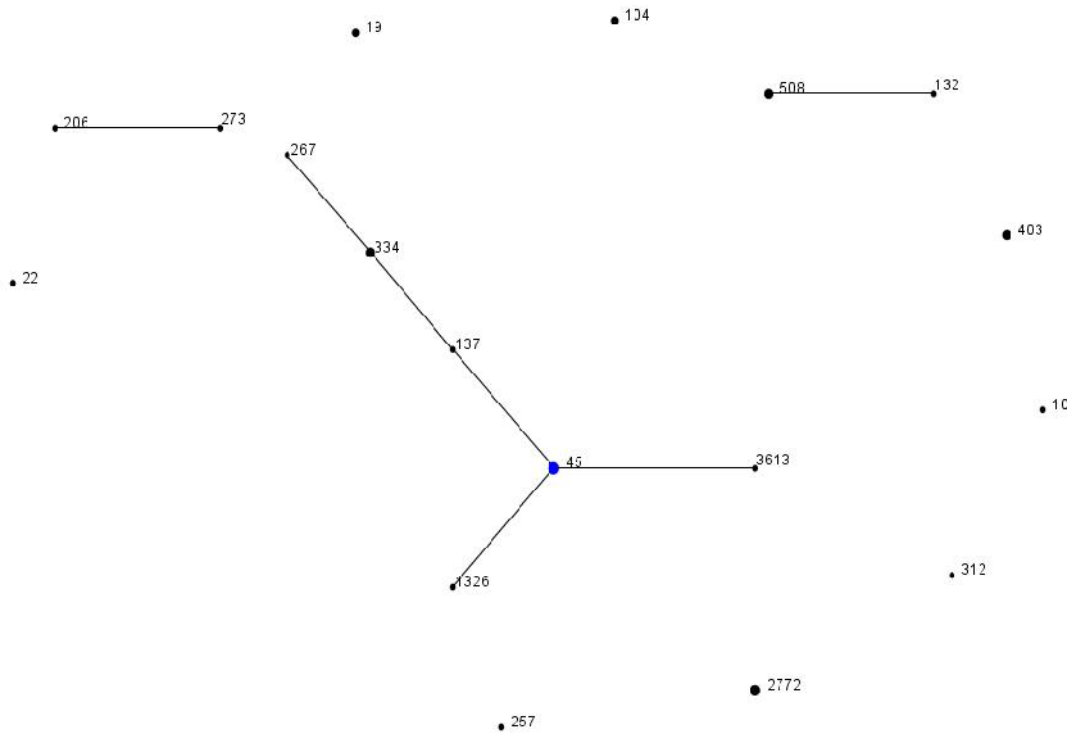


Table 6.1. **Source and Multilocus sequence type of *C. jejuni* isolates from dogs**.

Dog Source	N.o of Dogs	N.o of Isolates	ST	CC	Frequency of Isolation
Household	2	2	403	403	1
			403	45	1
Boarding	2	2	508	508	2
Vet visiting	3	3	273	206	1
			132	508	1
			312	658	1
Rescue	8	9	45	45	4
			137	45	1
			3613	45	1
			257	257	1
			1044	658	1
			267	283	1
Hunt	17*	17	2772	-	4
			104	21	2
			19	21	2
			45	45	1
			334	45	3
			403	403	2
			206	206	1
			508	508	1
			22	22	1

ST= Sequence Type, CC= Clonal Complex, and *= Samples collected from groups of dogs, each sample presumed to originate from a different dog.

6.5.1.2 PFGE

In general the genetic relatedness as assessed by PFGE agreed with the MLST results (Fig 6.2). The dendrogram produced using *Sma*I clustered together all the isolates typed as clonal complexes ST-45, ST-206, ST-508 and ST-403. Unlike MLST, the PFGE method did not differentiate between dog isolates 13 and 16. However, the bottom fragment of isolate 13 did appear a slightly different weight to isolate 16, and the sequence types only varied by one base change (Jolley and Chan, 2004), indicating that these isolates were closely related. PFGE demonstrated distinguishable profiles between the ST-2772 isolates, the ST-403

isolates, the ST-658 isolates, and the ST-21 isolates, whereas the MLST results did not (Fig 6.2).

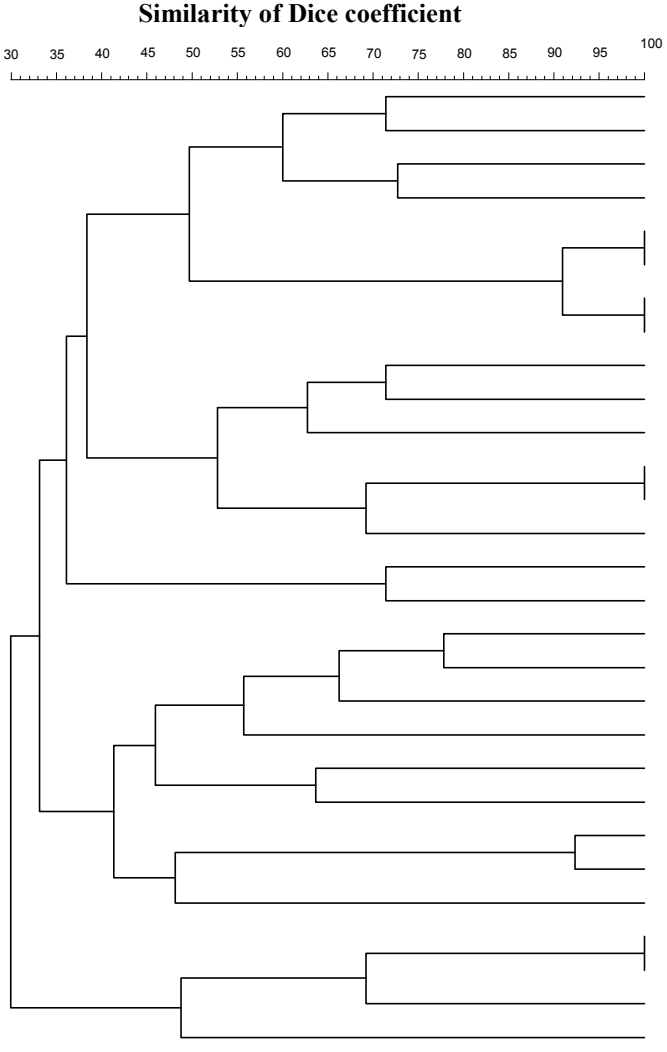
Identical or near-identical PFGE patterns were observed between dogs from within the same population, i.e. isolates 3 and 4 obtained from the same boarding kennel; isolates 25 and 26 from the same hunt kennel, and isolates 11 and 12, and 13 and 16 obtained from a rescue kennel (Fig 6.2). With the exception of isolates 13 and 16 which belonged to ST-3613 (reported for the first time) and ST-45 respectively, all these pairs of isolates were the same sequence type (Fig 6.2). Four isolates (15, 22, 27, and 28) could not be digested with *Sma*I. Three of these isolates (22, 27 and 28), belonged to ST-334, whilst isolate 15 belonged to ST-267.

6.5.1.2 Isolation Methods

There did not appear to be any trends between the isolation methods used and the sequence types observed, although isolates chosen originated from only two isolation techniques, filtration onto CAT media and direct plating onto mCCDA.

Legend for Figure 6.2: ST= Sequence type, CC= Clonal complex and CI= 95% confidence interval. North West= North West England, *same dog (isolate 15= 2nd, isolate 16=15th sample), #= rescue dog visiting a practice, ♦=isolate could not be digested using *Sma*I, Direct=direct plating onto mCCDA and Filtration=filtration onto CAT media. A=national cross sectional study of vet visiting dogs (Chapter 3), B=study of household dogs (Westgarth *et al*, 2009), C=member of staff's dog, D=longitudinal study in a rescue shelter (Chapter 4), E=longitudinal study in a boarding kennel (Chapter 4), F=rescue shelter, G=stray block in a boarding kennel (Chapter 5), H=hunt kennel C (Chapter 5), I=hunt kennel D (Chapter 5), and J=hunt kennel E (Chapter 5).

Figure 6.2. *C. jejuni* dendrogram constructed using Dice (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%] coefficient for PFGE using *Sma*I.



Dog Isolate	ST	Complex	Study
8	45	45	G
2	1326	45	C
10	137	45	D
19	45	45	H
16	45	45	D
13	3613	45	D
12	45	45	D
11	45	45	D
1	403	403	B
20	403	403	H
24	403	403	H
25	2772	-	H
26	2772	-	H
17	2772	-	H
5	273	206	A
29	206	206	H
32	19	21	I
33	19	21	J
14	1044	658	F
21	104	21	H
23	2772	-	H
18	104	21	H
7	312	658	A
9	257	257	D
31	22	22	I
3	508	508	E
4	508	508	E
6	132	508	A#
30	508	508	I

6.5.2 C. upsaliensis

Fifty *C. upsaliensis* isolates were assigned to 46 sequence types, all of which were novel apart from the reference strain RM3195 which was identified as ST-5 (Table 6.2 & Appendix Table 4.2). Sequence types were assigned to clonal complexes by examining their allelic profiles (Table 6.2) and their phylogeny was also examined (Fig 6.3 & Appendix 4, Fig 4.1).

6.5.2.1 Clonal Complexes

The sequence types were assigned to 12 clonal complexes (including ST-106 but excluding ST-72; Appendix 4, Figs 4.2-4.17), although 15 sequence types could not be assigned to any complexes (Table 6.2 & Appendix 4 Table 4.2). Four of these complexes, E, F, H, and J contained isolates originating from both humans and dogs, and this similarity between human and dog derived isolates was supported by high bootstrap values (Fig 6.2). The remainder contained only those isolates that originated from dogs, but none of the clonal complexes were exclusive to human isolates. There did not appear to be any strong commonalities within the clonal complexes, however both the hunt dogs from study I belonged to complex D. All three isolates in complex I were isolated after prior enrichment; both isolates in complex C were isolated by direct plating and both isolates in complex D were isolated via filtration.

Table 6.2. **Source and MLST of *C. upsaliensis* isolates from dogs.**

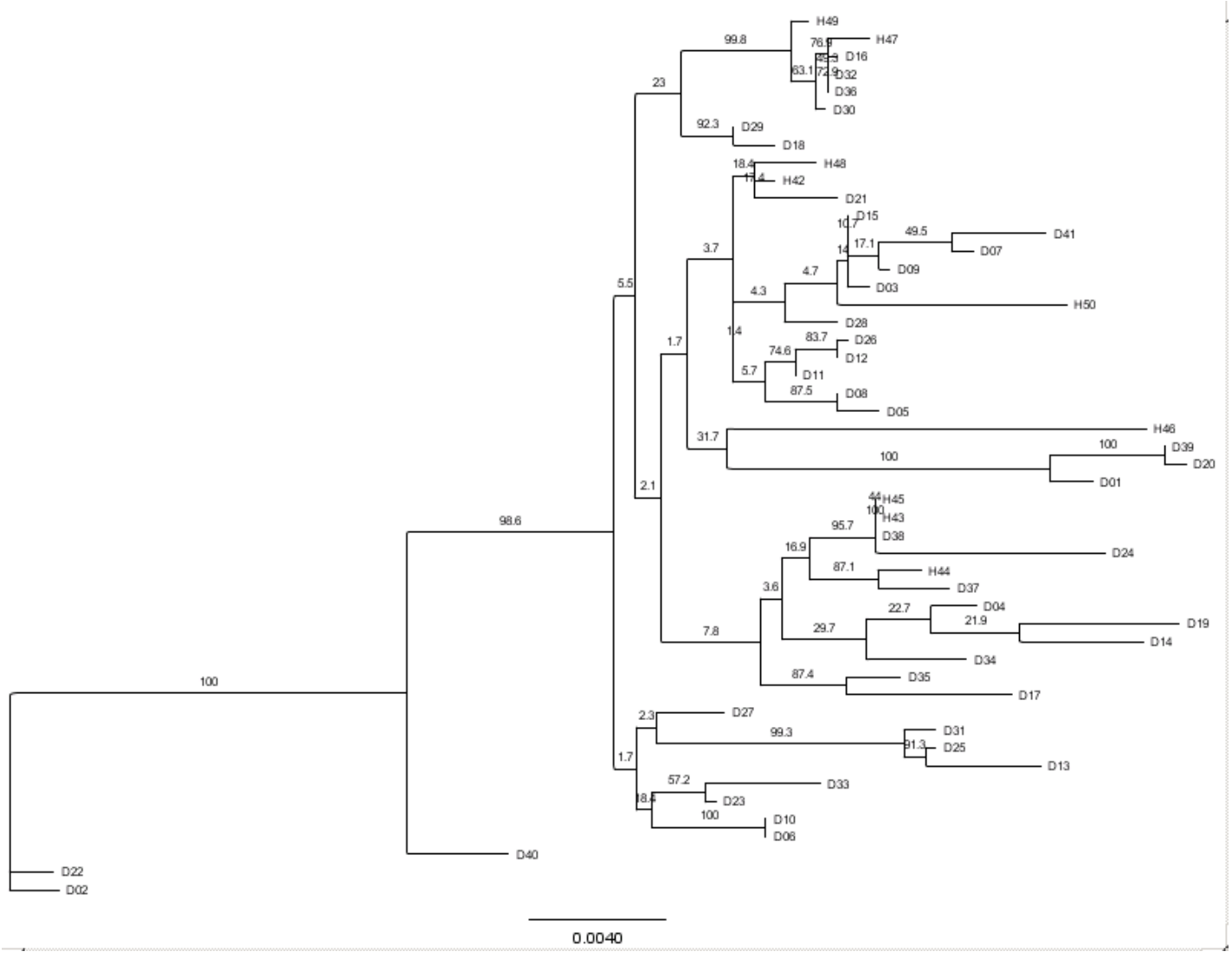
Source	N.o of Isolates	ST	Group/CC	Frequency of Isolation
Household Dogs	9	88	A	1
		79	C	1
		104	E	1
		95	G	1
		69	K	1
		70	Singleton	1
		85	Singleton	1
		100	Singleton	1
		107	Singleton	1
Boarding Dogs	8	97	C	1
		98	F/ST-16	2
		87	H	1
		75	K	1
		99	L	1
		67	Singleton	1
		73	Singleton	1
Vet visiting Dogs	18	68	A	1
		72	B (singletons)	2
		91	C	1
		90	E	1
		96	F/ST-16	1
		82	F/ST-16	1
		84	G	1
		71	H	1
		78	I	1
		77	I	1
		92	I	1
		103	J	1
		76	K	1
		89	L	1
		80	Singleton	1
		101	Singleton	1
		106	Other	1
Rescue Dogs	3	74	H	1
		83	Singleton	1
		93	Singleton	1
Hunt Dogs	3	86	D	1
		105	D	1
		94	K	1
Human	9	104	E	2
		112	F/ST-16	1
		108	H	1
		110	J	1
		5	Singleton	1
		113	Singleton	1
		114	Singleton	1
		115	Singleton	1

Legend for Table 6.2. ST= Sequence Type, CC= Clonal Complex, *= Samples collected from groups of dogs, each sample presumed to originate from a different dog, group B contains only singletons, and Other = belongs to a different complex (Appendix 4, Fig 4.11)

6.5.2.2 Sequence Types

Only three sequence types were found more than once, ST-72 (2 isolates), ST-98 (2 isolates) and ST-104 (3 isolates). ST-104 was the only sequence type to be isolated from both dogs and humans, all located in the North West. Dogs that yielded the same sequence types had no obvious trends, although both dogs with ST-72 were vet-visiting, isolated by direct plating and those belonging to ST-98 were boarding (Table 6.2 & Appendix 4 Table 4.2).

Figure 6.3. Un-rooted bootstrap (%) consensus maximum likelihood tree of *C. upsaliensis* MLST, concatenated sequences based on 3243bp (1000 replicates, D= dog derived isolate, H= human derived isolate).



6.5.2.3 Alleles

For each locus there were up to 17 different alleles although in most cases one or two alleles tended to predominate. For example *pgi* was dominated by allele 12, whereas *adk* was dominated by alleles 1 and 13. Approximately half of the alleles identified for each locus were novel and this effect was slightly greater in *pgi* and *aspA,* but was observed less for *adk*. The locus *glnA,* contained the greatest number of novel alleles in total, but not the greatest variety. The majority of alleles found in the human isolates were also found in the dog isolates, however there were some exceptions, for example human isolate H 50 contained 3 alleles not observed in any dog isolate, and isolate H 46 (RM3195) did not share any alleles with any other isolate.

6.5.2.4 Repeatability

In total 68 reactions were repeated during the study. Several alleles for several isolates were repeated from PCR stage for various reasons; to improve the quality of the sequence trace, to ensure no cross contamination had occurred (at PCR stage or subsequently) when several isolates (belonging to the same batch for sequencing) were assigned to the same allele, and for those alleles which had a large number of base changes (approximately $\geq$20) when compared to their closest allele number. No discrepancies were observed between the 68 repeats and their original results, unless the original results produced a poor unusable sequence trace.

6.6 Discussion

6.6.1 C. jejuni

MLST demonstrated considerable diversity amongst the *C. jejuni* sequence types and clonal complexes isolated from the dogs in this study; the PFGE profiles were largely in concordance with these results, and showed a similar amount of genetic diversity. The majority of clonal complexes found in dogs were the same as those reported in humans, including some of the most frequently isolated complexes in humans i.e. ST-45, ST-21, ST-22, ST-257 and ST-206, (Dingle *et al*., 2002; Duim *et al*., 2003; Jolley and Chan, 2004; Levesque *et al*., 2008; Ragimbeau *et al*., 2008; Sopwith *et al*., 2006, 2008). The only exception was ST-2772 which was isolated from four samples, and could not be assigned to a clonal complex. The most commonly identified clonal complexes in dogs were ST-45, ST-21, ST-508 and ST-403.

6.6.1.1 Sequence Type 45

There are various possible sources from which dogs might acquire clonal complex ST-45. This clonal complex has been isolated from a range of sources such as water, wild birds, cattle, sheep, rabbits, badgers, turkey chicks, broiler chicks, and soil as well as humans (Colles *et al*., 2003; French *et al*., 2005; Ragimbeau *et al*., 2008). Sopwith *et al*, (2008) found that ST-45 was the most commonly isolated sequence type from water, and suggested that it might be better adapted to survive outside a host, and thus might be crucial in the transmission of *C. jejuni* throughout the environment. Interestingly, open drains, and possibly lakes have been associated with *Campylobacter* spp. carriage in dogs (Baker *et al*., 1999; Wieland *et al*., 2005) and the ST-45 complex isolated

from humans has also been significantly associated with contact with pet cats and dogs (Karenlampi *et al*., 2007). This may indicate common sources of infection for humans and dogs, or possibly that dogs may act as conduits of infection from the environment to humans.

6.6.1.2 Clonal Complexes ST-508 and ST-403

Clonal complexes ST-508 and ST-403 were isolated from several dogs within this study. Both complexes have also been isolated from humans, and interestingly both dominated in human *C. jejuni* isolates obtained from a study in Curacao (Dingle *et al*., 2002; Dingle *et al*., 2008; Duim *et al*., 2003). Possible reservoirs of these complexes are unclear, although ST-508 has been isolated from sources such as wild birds and cattle, whilst ST-403 has been found in porcine isolates and also occasionally in cattle (French *et al*., 2005; Jolley and Chan, 2004; Kwan *et al*., 2008b; Wilson *et al*., 2008).

6.6.1.3 Different Dog Populations

The rescue dogs in this study had the lowest diversity of clonal complexes. Clonal complex ST-45 was the most common amongst the rescue dogs, and within this complex the central genotype, ST-45 dominated. In contrast, dogs from hunt kennels had the greatest diversity of clonal complexes, which could be a result of frequent exposure to possible sources of infection since dogs from hunt kennels 1 and 2 were exercised daily through fields grazed by livestock, and dogs in hunt kennel 1 were fed a diet of raw meat and bone. Cattle faeces, carcasses, wildlife, birds, soil and water have all been shown to carry *C. jejuni* (Brown *et al*., 2004; De Cesare *et al*., 2008; Kemp *et al*., 2005; Waldenstrom *et*

al., 2002; Workman *et al*., 2005). Although a variety of sequence types were isolated from hunt kennels, several dogs carried ST-2772, ST-334, or members of the ST-21 complex, none of which were observed in any of the other dog populations within this study. Prior to the present study, the only reported isolation of, ST-2772 has been from cattle (Jolley and Chan, 2004), whereas clonal complex ST-21 has been isolated from poultry, cattle and human disease (Dingle *et al*., 2002; Ragimbeau *et al*., 2008).

6.6.1.4 Zoonotic Risk

The zoonotic risk of *C. jejuni* transmission from dogs to humans must be put into context. The prevalence of *C. jejuni* carriage in vet-visiting, and community dogs in the UK was found to be low in two previous studies (1.2%, 95% CI 0.3, 3 and 0.5%, 95% CI 0.0-3.0) (Chapter 3; Westgarth *et al*., 2009). Therefore the likelihood of a household pet dog carrying *C. jejuni* is low, although when a dog does carry *C. jejuni*, the risk to humans may increase because the data suggests that these strains are similar to those that can infect humans (Dingle *et al*., 2002; Duim *et al*., 2003; Jolley and Chan, 2004; Levesque *et al*., 2008; Ragimbeau *et al*., 2008; Sopwith *et al*., 2006, 2008). Additionally the prevalence of *C. jejuni* in dogs from kennelled and hunt dog populations within this thesis (Chapters 4&5) was higher (9.1%, 95% CI 1.9-26, 10.3%, 95% CI 3-25, 20%, 95% CI 8-36, and 26.5%, 95% CI 16-40) than either the vet-visiting or community based dog populations (Chapter 3; Westgarth *et al*., 2009). Exposure to kennelled and hunt dog populations may pose a greater zoonotic risk to humans than household pets, but providing standard hygiene is practised, i.e. washing hands after contact with

dog and/or dog faeces before mouth-to-hand contact, the risk should be greatly reduced.

6.6.1.4 Molecular Typing Techniques

PFGE was useful in initially examining *C. jejuni* isolates from within a dog population, and in general it agreed with MLST data. This has also been observed in another study where PFGE and MLST complexes were similar (Ragimbeau *et al*., 2008). For example, two dogs (isolates 3 and 4) which had been negative for *Campylobacter* spp. for over a week in a boarding kennel began to shed *C. jejuni* within four days of each other. Results from PFGE indicated that the patterns were indistinguishable, which may suggest possible transmission, or that the two dogs may have shared a common source of infection. This was further supported by MLST data, which also indicated identical sequence types. A similar situation was found for isolates 11 and 12 from a rescue kennel.

DNA from four isolates resisted digestion by *Sma*I. These isolates belonged to ST-334 or ST-267, both of which could not be digested using *Sma*I in another study (Ragimbeau *et al*., 2008). These two sequence types only differ by one base, so despite being undigested, PFGE demonstrated relative clonality for these isolates. These findings support the need for two independent molecular typing techniques, particularly when analysing a bacterium with a potentially unstable genome such as *Campylobacter* spp. (Hanninen *et al*., 1999; Steinbrueckner *et al*., 2001). This is important because a single base change can result in a different

sequence type, or the alteration of a restriction site, which can subsequently lead to a three fragment difference in PFGE (Tenover *et al*., 1995).

6.6.1.5 Conclusion

In conclusion, this study suggests that there is considerable genetic diversity between *C. jejuni* sequence types obtained from dogs from various sources, and on the whole dogs do not have strains of *C. jejuni* particular to them. The majority of sequence types found in dogs within this study have also been isolated from humans. These data may indicate that there are common sources of infection for both humans and dogs, and that dogs remain a possible zoonotic risk of *C. jejuni* infection for humans. However, the exposure risk to dogs, and thus possibly humans, for certain sequence types may differ depending upon the circumstances of the dog (Westgarth *et al*., 2008).

6.6.2 C. upsaliensis

MLST demonstrated considerable diversity amongst the *C. upsaliensis* sequence types and clonal complexes isolated from the dogs and humans in this study. The large number of novel alleles, sequence types, and clonal complexes probably reflects the relatively small *C. upsaliensis* database currently available, in conjunction with the large amount of diversity observed. The diversity of the *C. upsaliensis* isolates appeared to be even greater than the diversity observed within the *C. jejuni* isolates. A similar situation was observed by Miller *et al* (2005) where approximately four times more alleles were recorded at each locus for *C. upsaliensis* compared to *C. coli*. Previous studies utilizing AFLP have also

observed a large amount of genetic diversity in *C. upsaliensis* isolates originating from canine samples (Damborg *et al*., 2008; Koene *et al*., 2009).

6.6.2.1 Possible Transmission Between Dogs

Both isolates from hunt kennel D (study I) belonged to the same clonal complex. This might indicate that the dogs from this kennel maintain a certain strain of *C. upsaliensis* within their population, and that vertical and/or horizontal transmission may occur between the dogs. This has also been suggested in a previous study where AFLP patterns of *C. upsaliensis* strains isolated from dogs living in the same household or kennel were indistinguishable from one another (Damborg *et al*., 2008). Further to this, two dogs originating from the same household both shared the same sequence type (ST-25) in another study (Miller *et al*., 2005).

Koene *et al* (2009) reported that dogs from the same household did not share indistinguishable AFLP patterns, suggesting that transmission may not occur between animals. However, only two dogs originating from the same household both carried *C. upsaliensis* in Koene *et al* (2009), and despite these two *C. upsaliensis* strains only sharing a genetic identity of 80%, these two isolates were more closely related to each other than compared to any other isolate (Koene *et al*., 2009). The two strains may have simply evolved independently over time since other *Campylobacter* spp. such as *C. jejuni* are considered to be a rapidly evolving species (Wilson *et al*., 2009).

In this current study, faecal samples collected from hunt dogs could not be assigned to individual dogs, allowing for the possibility that both isolates originated from the same dog. Despite the work of others suggesting some *Campylobacter* spp. are evolving rapidly, the likelihood of these two samples originating from the one dog seems unlikely because their *atpA* alleles differed by two base changes (positions 150 and 189). There is little work on the rate of mutation in *C. upsaliensis*, but work performed by others suggests *C. jejuni* has a relatively low rate of mutation, approximately 1.9×10^{-6} – 2.77×10^{-6} per kb, compared to other bacteria such as *E. coli* where the rate is approximately 1.9×10^{-3} per kb (Drake, 1991; Wilson *et al*., 2009). Therefore, taking into account the doubling time of *C. jejuni* (112 minutes; Han *et al*., 2009), there does not appear to have been enough time for two point mutations to have occurred at this conserved locus within the present study. However, this effect can not be fully dismissed, and MLST cannot distinguish between effects of recombination or mutation. Further more this could be the result of a mixed infection within one dog (perhaps sampled twice). Mixed infections may also increase the chances of recombination which has been shown to play an important role in *Campylobacter* spp. evolution (Sheppard *et al*., 2008; Wilson *et al*., 2009).

6.6.2.2 Zoonotic Risk

Only three *C. upsaliensis* sequence types were identified more than once, and one of these, ST-104, was found in both human and canine isolates. However there were limited data concerning the isolates originating from humans, including post code information, i.e. it is unclear as to whether or not the two ST-104 isolates originating from humans came from individuals living in the same

household. It was also observed that four of the clonal complexes contained isolates originating from both humans and dogs, whilst none were exclusive to human isolates. In contrast to findings from Miller *et al* (2005), the isolates that grouped into complex F/ST-16 were not unique to dogs/pets, although only two isolates belonged to this complex in Miller *et al*, (2005) and there may be temporal or geographical differences between the two studies. This indicates that humans and dogs may share possible sources of *C. upsaliensis*, or that transmission can occur between the two. Other studies have found associations between *C.upsaliensis* infection in humans and dogs living in the same household (Goossens *et al*., 1991; Labarca *et al*., 2002; Lentzsch *et al*., 2004). A similar situation was also recorded in a case of a human abortion that was associated with a *C. upsaliensis* infection transmitted from a cat (Gurgan and Diker, 1994). In addition, *C. upsaliensis* isolates originating from a dog and a human have both been shown to efficiently invade three different types of human epithelial cells (Caco-2, T84 and HeLa) (Mooney *et al*., 2003), indicating that canine derived *C. upsaliensis* has the same pathogenic potential as strains found in humans.

Conversely, several studies have found no association between *C. upsaliensis* isolated from humans and dogs (Damborg *et al*., 2008; Labarca *et al*., 2002; Stanley *et al*., 1994). A recent study by Damborg *et al*, (2008) found no association between the AFLP patterns of *C. upsaliensis* isolates from both humans and dogs. However, most of the samples in the 'dog' group originated from Denmark and Sweden predominantly between 2000 and 2006, whereas the 'human' group, consisted of isolates from South Africa, Belgium, United

Kingdom, Senegal and Denmark, with nearly all samples isolated between 1985 and 1999. Differences in location can play a significant role, as described by Lentzsch *et al*, (2004) and Miller *et al* (2005). A similar situation was true in the study by Stanley *et al* (1994) where human isolates contained greater numbers of plasmids than the canine strains which did not originate from the same countries as the humans strains. In this present study, human and dog samples that contained ST-104, all originated from the North West and were collected within three years of each other.

It should also be considered that the time lag between the collection of patient and pet dog samples in Labarca *et al* (2002) was three to six months. As previously discussed, PFGE can be affected by the presence or absence of plasmids, and recombination, particularly if a point mutation occurs at a restriction site (Barrett *et al*., 2006; Sheppard *et al*., 2008; Suerbaum *et al*., 2001). This time lag may also explain why Miller *et al* (2005) reported different MLST results for the same *C. upsaliensis* isolates that were used in Labarca *et al* (2002). Other explanations for this might include; that owners acquire immunity to the strain carried by their own pet and are more likely to be infected from an animal they do not have regular contact with, or that co-infection may explain the lack of similarity between human and dog strains originating from the same household.

This possible zoonotic relationship remains unclear due to the low numbers of human *C. upsaliensis* isolates currently available, and the lack of data on human and pet strains of *C. upsaliensis* originating from the same house at the same

time. This effect is only confounded by the large amount of genetic diversity observed between *C. upsaliensis* strains in this study and others (Damborg *et al*., 2008; Koene *et al*., 2009). Since dogs and to a lesser extent cats, are the only significant sources of *C. upsaliensis* currently known, and given the results in this present study, combined with the close contact between humans and pets, dogs remain a potential source of *C. upsaliensis* infection for humans.

6.6.2.2.1 Disease

All isolates originating from humans were recorded as symptomatic, indicating that *C. upsaliensis* may cause disease in humans which has also been found in other work (Byrne *et al*., 2001; Carter and Cimolai, 1996; Goossens *et al*., 1990a; Gurgan and Diker, 1994; Jenkin and Tee, 1998; Jimenez *et al*., 1999; Lawson *et al*., 1999; Patton *et al*., 1989; Prasad *et al*., 2001). However, it is not known whether or not these human derived samples had other additional pathogens isolated from them. Conversely, the majority of canine isolates were asymptomatic, adding further evidence that this species is a commensal in dogs.

6.6.2.3 Isolation Methods

In general human and dog isolates did not group into particular sequence types or complexes depending upon the isolation method used to obtain them. However, there were some exceptions. Complex C contained only isolates (two) that were plated directly onto mCCDA, complex D contained only isolates (two) that had undergone filtration onto CAT media, and complex I contained only isolates (three) that had undergone prior enrichment. Without further isolates it is unknown as to whether or not these associations are genuine, but the latter

association i.e. complex I, may be more reliable since isolates selected by prior enrichment were not generally as common as those selected by either filtration or direct plating. Miller *et al* (2005) described two distinct groups of *C. upsaliensis,* with one group containing strains predominantly isolated with cefoperazone/cephalothin, and with the other group containing those strains isolated without either of these antibiotics, but with filtration instead. Although all of the isolates in this study were exposed to cefoperazone at some stage during their isolation, those isolates cultured with prior enrichment would have been exposed to cefoperazone-free broth for 24 hours, in optimal growing conditions before being exposed to cefoperazone. Other work has observed that when the same *Campylobacter* spp. is isolated from one sample, different sequence types (of the same clonal complex) can be selected depending upon the enrichment method used (personal communication, Williams. L, University of Bristol). Therefore it cannot be ruled out that some MLST results may depend upon the isolation methods used, and standardized methods should ideally be used for comparative purposes to reduce potential bias.

6.6.2.4 Co-infection

Co-infection, particularly of the same *Campylobacter* spp., can have a dramatic effect upon MLST results. A mixed infection may result in the wrong combination of alleles and thus an incorrect/false sequence type. Aggregation of different strains can even occur when single colonies are carefully selected (Miller *et al*., 2000). Regardless of whether or not truly pure colonies are selected, multiple strains of the same *Campylobacter* spp. may be present in the same sample, and it has been suggested that multiple colonies from one sample

should be examined in molecular based studies (Koene *et al*., 2009). This may also explain why owner and pet *C. upsaliensis* isolates are sometimes different. Although care was taken to select pure colonies in the present study, mixed infections may still have occurred, and only one pure colony was selected from each sample. However an additional purification step such as an extra blood agar step may reduce this. Therefore it is possible that some strains (from the same sample) were not examined, but it is difficult to know exactly how many colonies would have to be selected in order to analyze every possible strain.

6.6.2.5 Conclusions

There was considerable genetic diversity between the *C. upsaliensis* sequence types obtained from both dogs and humans. The majority of isolates contained one or more novel alleles, which resulted in a novel sequence type for every isolate (except RM3195). Only three sequence types were isolated more than once, with one of these sequence types being isolated from two humans and one dog. Strains of *C. upsaliensis* isolated from humans did not appear to group separately when compared to strains obtained from dogs, indicating common sources of infection, or possible transmission. There was also some evidence to suggest that transmission can occur between dogs. Dogs remain a possible zoonotic risk to humans, however, further work is needed to investigate the frequency, and severity of *C. upsaliensis* infection in humans.

7. Chapter seven

Pilot study: *Campylobacter upsaliensis* Genome and Plasmid Sequencing

7.1 Acknowledgements

The genome project was done in collaboration with Alistair Darby, Kevin Ashelford and Neil Hall at The University of Liverpool. Genome sequencing and construction of scaffolds was performed by Kevin Ashelford, whilst assembly, comparison to other genomes and initial annotation was done by Alistair Darby.

7.2 Abstract

Genome sequencing has many applications including; identifying virulence genes, investigating evolutionary origins, and providing data on the whole strain. Currently RM3195, isolated from a human, is the only published *C. upsaliensis* genome available. Dogs appear to be significant reservoirs of *C. upsaliensis*, but the likelihood of transmission of this bacterium between dogs and humans is unclear. This pilot study aimed to sequence a common (amongst canine isolates) *C. upsaliensis* strain isolated from a dog in the UK and identify and annotate any large plasmids found. A Genome Sequencer™ FLX (454 Life Sciences™)(Droege and Hill, 2008) was used to sequence the bacterial DNA.

The genome was approximately 1,765,608 bp in length, and at least one large plasmid (126 kb) was identified. The plasmid contained several amino acid sequences coding for Tra proteins indicating the presence of a conjugative type IV secretion system.

7.3 Introduction

Whole genome analysis is a useful molecular tool, providing information based on a whole organism, as opposed to a particular gene or set of alleles which may focus on only an aspect of the organism, which may or may not be representational. Whole genome sequencing has applications for identifying virulence genes in pathogens, which could potentially be targeted for treatment in an infected individual. This technique may also help to explain what adaptations certain bacteria have that enable them to be symptomatic or asymptomatic depending on their host, and why some are host specific. Evolutionary data can also be gathered through the sequencing of several genomes, and may be particularly important for bacteria such as *Campylobacter* spp., since this bacterium has shown evidence of species convergence, which can be problematic for typing techniques (Sheppard *et al*., 2008).

The genomes of some *C. jejuni* isolates, obtained from human clinical cases and also from chicken carcases (e.g. NCTC 11168 and RM1221, respectively), have previously been sequenced and are considered complete (Fouts *et al*., 2005; Parkhill *et al*., 2000). The genomes of up to 18 different strains of *C. jejuni* have been compared in previous work (Pearson *et al*., 2003), but there are relatively few strains of *C. upsaliensis* that have been sequenced to the same extent. The unfinished *C. upsaliensis* genome, RM3195, isolated from a human case of Guillain-Barré syndrome is currently at 9-times coverage (Fouts *et al*., 2005) and provides a good basis of comparison for any future sequencing of *C. upsaliensis* genomes. Currently RM3195 is the only published *C. upsaliensis* genome, and some key features observed within this genome compared to other

Campylobacter spp. were the comparatively fewer virulence genes, the greater numbers of poly G tracts, and the presence of more (two) plasmids (Fouts *et al*., 2005).

Dogs appear to be significant reservoirs of *C. upsaliensis* (Acke *et al*., 2009; Engvall *et al*., 2003; Hald *et al*., 2004; Koene *et al*., 2004; Rossi *et al*., 2008; Sandberg *et al*., 2002; Wieland *et al*., 2005) and although the possible transmission of *C. upsaliensis* from dogs to humans is still unclear (Damborg *et al*., 2008; Stanley *et al*., 1994), there is some evidence to suggest it can occur (Goossens *et al*., 1991; Labarca *et al*., 2002; Lentzsch *et al*., 2004). Differences may or may not exist between *C. upsaliensis* carried by humans and dogs, and these can be investigated by genome sequencing. This genomic information may help to clarify whether or not *C. upsaliensis* transmission can occur between dogs and humans. Further more, the role of *C. upsaliensis* as a commensal in dogs is accepted but not fully understood. Genome sequencing may help to determine what adaptations have evolved that allow *C. upsaliensis* to colonise dogs specifically, and why symptoms in humans appear milder compared to *C. jejuni* infection (Goossens *et al*., 1990b; Jimenez *et al*., 1999). The latter is probably due to the presence of fewer virulence genes in *C. upsaliensis* (RM3195) (Fouts *et al*., 2005), but more *C. upsaliensis* genomes are needed to explore this.

7.3.1 Plasmids

Although distinct from the genomic DNA, plasmids are also identified during genome sequencing. Plasmids can be important with regards to DNA exchange

and have been known to act as vehicles for transmission of virulence and antibiotic resistance genes in some bacteria including *C. jejuni* (Arias *et al*., 2009; Bacon *et al*., 2000). In particular a tetracycline resistance plasmid (*tet*O) has been identified in *C. jejuni* and *C. coli*, and reports suggest that these two species are able to transfer this plasmid between each other (Batchelor *et al*., 2004). Approximately 29%-32% of human *C. jejuni* and *C. coli* isolates harbour plasmids ranging from 2 kb to162 kb (Schmidt-Ott *et al*., 2005; Tenover *et al*., 1985), whereas a larger proportion of *C. upsaliensis* strains isolated from human patients have been shown to contain plasmids (89.9%)(Goossens *et al*., 1990a), ranging in size from 1.5 kb to 110 kb (Fouts *et al*., 2005; Stanley *et al*., 1994). The difference in the carriage of plasmids appears to be one of the major differences between *C. upsaliensis* and *C. jejuni*. This is supported by results from genome sequencing where *C. jejuni* isolates were found to contain no plasmids, as opposed to a *C. upsaliensis* isolate which was found to contain at least two, pCU110 and pCU3 (Fouts *et al*., 2005).

7.3.1.1 Secretion Systems

Large plasmids isolated from different *Campylobacter* spp. such as *C. jejuni, C. coli*, *C. lari*, and *C. upsaliensis* have all provided evidence for the presence of a type IV secretion system (TFSS) (Bacon *et al*., 2000; Fouts *et al*., 2005). Secretion systems in Gram negative bacteria range from relatively simple systems, such as type I (TISS) to more complex systems such as TFSS. A TISS comprises of three main proteins which together usually transport one substrate protein, whilst TFSS are usually more complex, transporting proteins and DNA (Gerlach and Hensel, 2007; Pohlman *et al*., 1994). In particular, TFSS have been

associated with conjugation and transportation of toxins (Sandberg *et al*., 2006; Weiss *et al*., 1993).

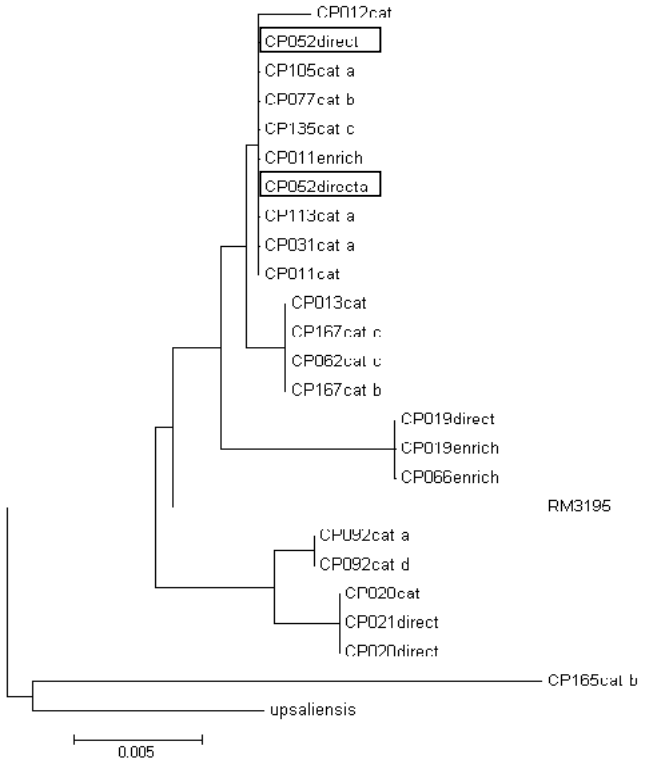
The aims of this pilot study were to sequence a common (amongst canine isolates) *C. upsaliensis* strain isolated from a dog in the UK and identify and annotate any large plasmids found. This study also provides a foundation towards the annotation of the genome itself in future work, enabling future comparisons between this canine isolate and the human *C. upsaliensis* isolate RM3195.

7.4 Materials and Methods

7.4.1 Selection of Isolate

An isolate was selected for full genome sequencing by Dr Alan Radford, Dr Richard Birtles and Dr Carol Porter. The isolate was selected from a cross-sectional study of household dogs where 37 of 183 dogs were positive for carrying *C. upsaliensis* (Westgarth *et al*., 2009). Dog 52A was chosen because its *groEL* sequence was common amongst the household dog population and was the most distinct from RM3195 (Fig 7.1).

Figure 7.1. *groEL* sequences for *C. upsaliensis* from isolates obtained in a study of household dogs. Neighbour joining-distance tree based on 498bp (created by Dr Alan Radford).



Direct= Direct plating onto mCCDA

Cat= Prior filtration onto CAT plates

Enrich= Prior enrichment before direct plating onto mCCDA

RM3195= Reference strain, upsaliensis= strain KO979

7.4.2 Preparation and Sequencing of Dog 52A

The procedure for sequencing the isolate and all other aspects of the methodology for this chapter is described in chapter two of this thesis. In brief, A Wizard® Genomic DNA Purification kit was used to isolate the genomic DNA of the bacteria. The DNA was then sent to the school of Biological sciences, Liverpool University, where Kevin Ashelford and Alistair Darby sequenced the genomic DNA. A Genome Sequencer™ FLX (454 Life Sciences™; Droege and Hill, 2008) was used to sequence the bacterial DNA.

7.4.2.1 Sequence Analysis, Annotation and Comparative Genomes

Assembly was performed with newbler (Roche, USA) and gap4 (http://staden.sourceforge.net). Protein-coding genes were identified with GLIMMER (Delcher *et al*., 1999) and GENEMARK (Lukashin and Borodovsky, 1998); and tRNA genes by tRNAscan-SE (Lowe and Eddy, 1997). Putative functions were inferred using BLAST against the National Center for Biotechnology Information databases (Altschul *et al*., 1990), InterProScan (Hunter *et al*., 2009).

7.4.3 Plasmid

As described in the results section of this chapter, a large plasmid was observed within one of the scaffolds. Attention was focused on producing a full complement of sequences for this large plasmid, and subsequently annotating the plasmid. This work was considered a pilot study, in preparation for future work where the genome its self will be annotated.

7.4.3.1 Primer Design

Primers were designed to close the 12 gaps (including sets designed to join the circular plasmid), using Primer 3 (http://frodo.wi.mit.edu/). Primers were then checked for suitability using Oligo Calc: Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html).

7.4.3.2 Plasmid Isolation

In order to confirm there were no further gaps in the plasmid sequence, the approximate size of the plasmid was visualised on a gel. However before

successful visualisation was achieved, several attempts at isolating the plasmid failed, possibly due to its large size. The various procedures are described in detail in chapter two of this thesis, but are described in brief below:

7.4.3.3 Extraction Using a QIAprep® Spin Miniprep Kit (QIAGEN)

Extraction was first attempted using a QIAprep® Spin Miniprep Kit (QIAGEN), according to the manufacturer's microcentrifuge instructions. No bands could be visualised on the gel (including the wells) so a second procedure was implemented.

7.4.3.4 Modified Kado and Liu Plasmid Isolation Procedure

Three procedures were performed based on phenol/chloroform and adapted from the methods suggested by Kado and Liu, (1981) and Wigley, (1999). The results were inconclusive due to unknown size of the plasmids within the *E. coli* marker i.e. four bands/plasmids should have been identifiable within the *E. coli* strain, but only two were observed (Appendix 5, Figs 5.1-5.3).

7.4.3.6 Visualisation Using PFGE

The isolate was grown on a CAB plate and incubated for 48 hours in a VAIN. The isolate then underwent the methods described for PFGE in chapter two of this thesis, excluding *Sma*I digestion. Two plugs (from the 1 isolate) were inserted into the gel, and this overall method was successful. The plasmid was visualised on the gel, whilst the genomic DNA remained in/near the well.

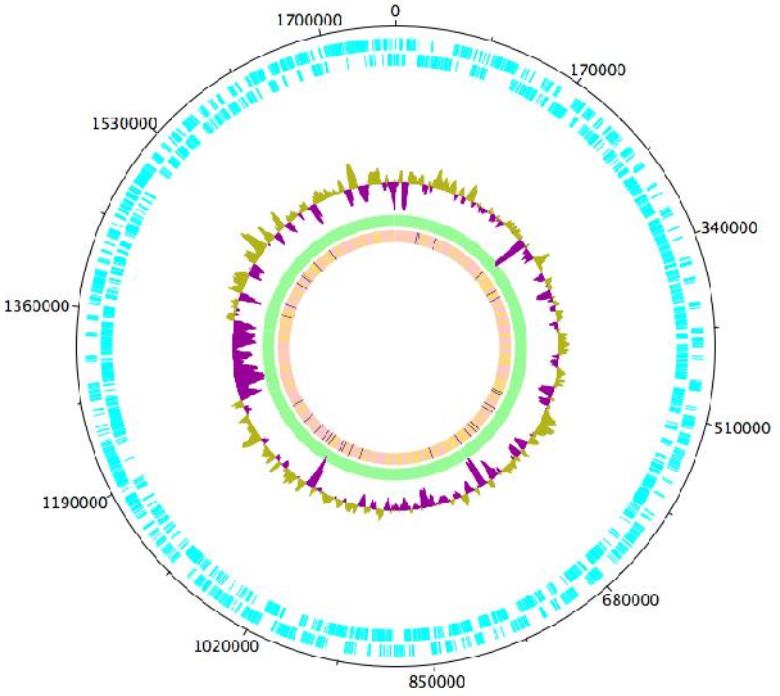
7.5 Results

7.5.1 Genome

The genome sequence obtained for *C. upsaliensis* 52A was 1,765,608 bp in length with an average GC content of 34.7% (Fig 7.2). The genome was divided into 13 scaffolds (scaffold contains contigs of known order) with an average of 135816 bp, the shortest containing 2821 bp and the longest containing 575838 bp. There were 60330 (3.4%) non-ACGT bases. Scaffolds were arranged to make a pseudochromosome based on the genomes of other *Campylobacter* spp.

Of the coding domain sequence (CDS) features without a pseudo qualifier, there were 2311 open reading frames (ORFS) identified containing 1656795 bases, with a density of 1.308 genes per kb (764 bases per gene). The average length was 716 bp, with a coding percentage of 93.7%, and a GC content of 35.03%.

Figure 7.2. Diagram demonstrating ORFs (turquoise), GC% +Ve/-Ve content (olive and purple) and contigs (peach) of the *C. upsaliensis* 52A (pseudo) genome (created by Dr Alistair Darby).



7.5.2 Plasmid pCU120

A large plasmid (>120 kb) was identified within one of the original scaffolds. Initial identification was based on the comparison of scaffolds between *C. upsaliensis* 52A and RM3195, the latter of which contains two plasmids of known sizes (Fig 7.3). The pCU120 plasmid was then visualised on a gel to estimate its size (Fig 7.4). A Lambda Ladder PFG Marker and *E.coli* 39R861 were used as references but the *E. coli* strain did not produce any visible plasmids for comparison.

Figure 7.3. Comparison of scaffolds from RM3195 (X axis) and *C. upsaliensis* 52A (Y axis). Dots indicate similarity between scaffolds, colour indicates orientation of the scaffolds (image produced by Dr Alistair Darby).

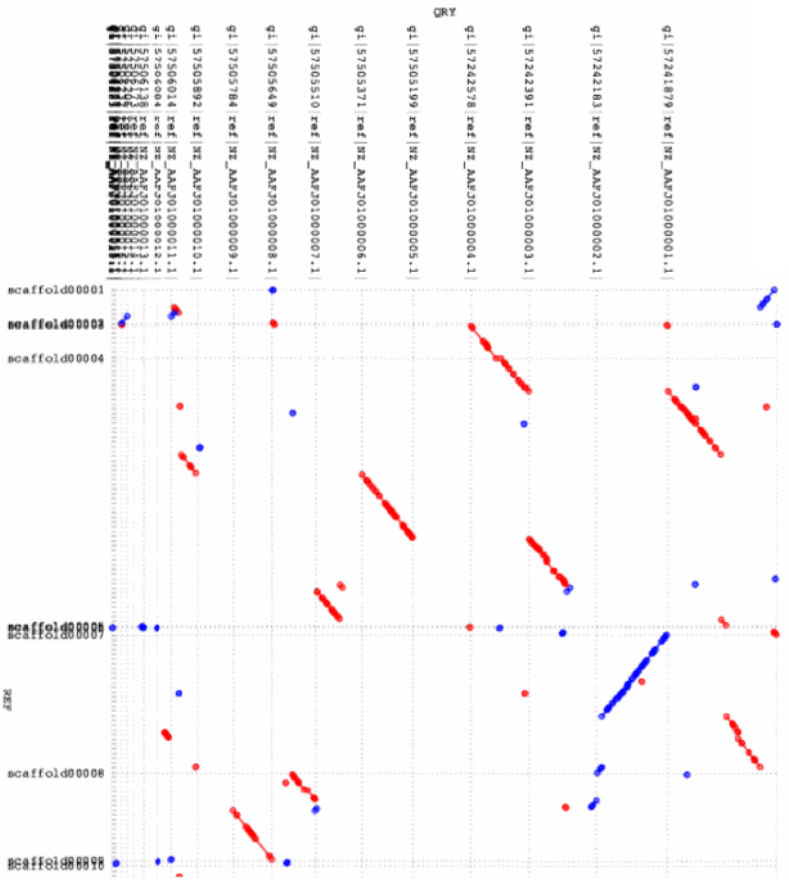
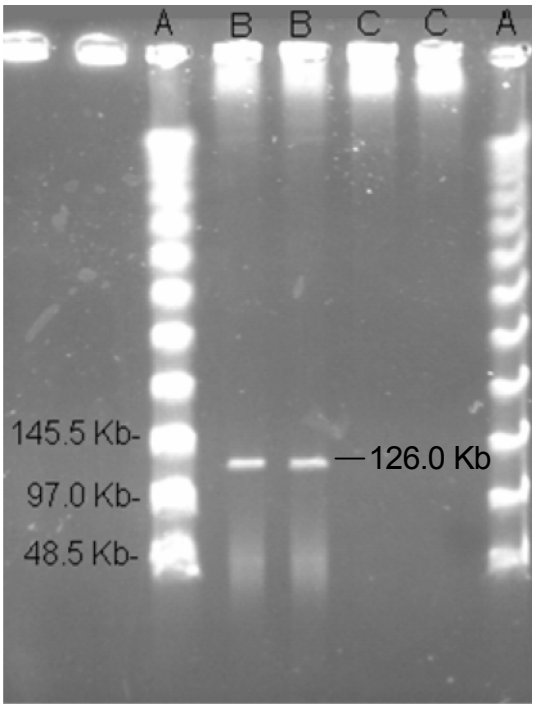


Figure 7.4. PFGE gel indicating the size of plasmid pCU120.



A=Lambda Ladder PFG Marker (Successively larger concatemers of lambda DNA, *cl*857 *ind* 1 *Sam*7, size range 50-1,000 Kb).

B=*C. upsaliensis* isolated from Dog 52A.

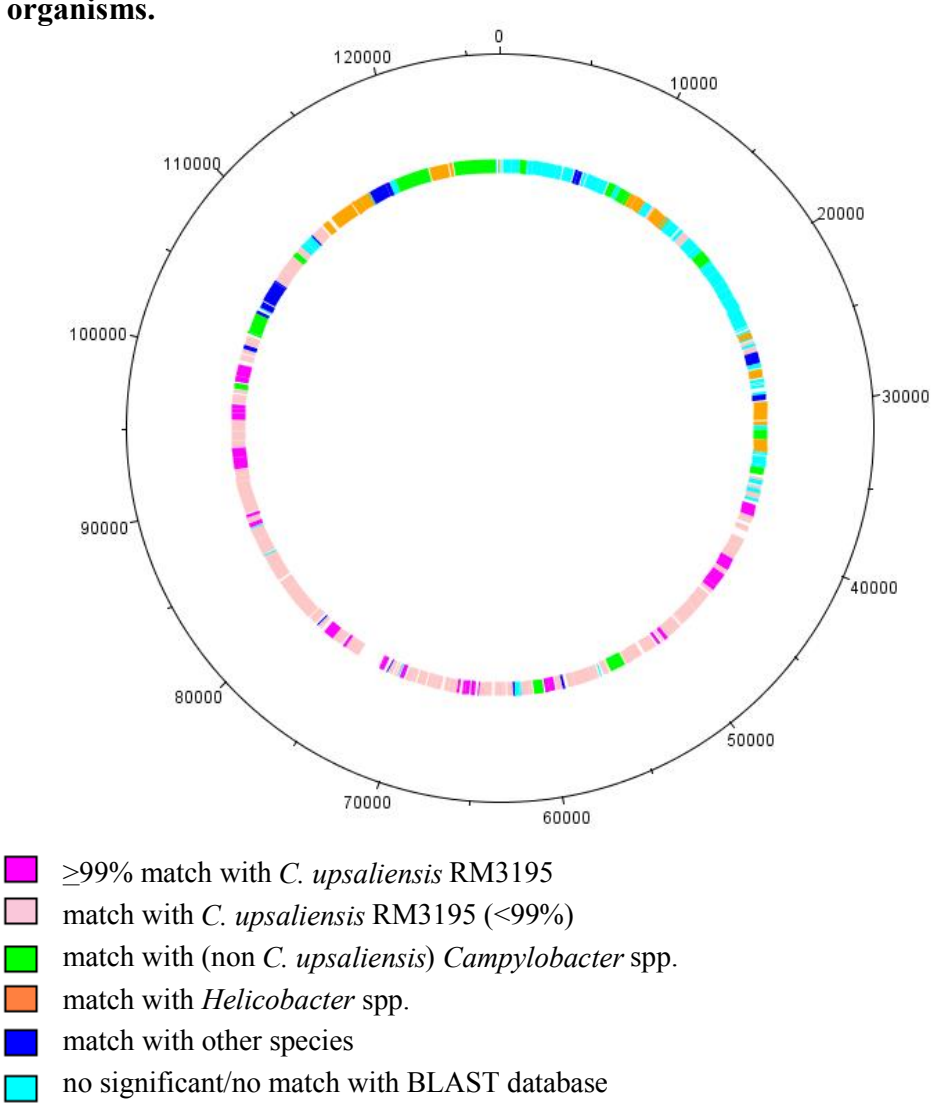
C=*E.coli* 39R861(plasmid sizes: 7.1 kb, 36.8 kb, 65.0 kb and 151.0).

The initial sequence for pCU120 was contained within one scaffold, which consisted of 13 contigs. Primer sets were designed to close the 12 gaps, plus an additional pair designed to join the first contig to the last. In total a combination of 34 PCR assays were used to determine the sequence of these various gaps, the products of which ranged in size from 108-2023bp.

Within pCU120 there were 126, 400 bp. There were 207 ORF (CDS features without a pseudo qualifier) consisting of 116, 199 bases, with an average length of 540.4. The density was 1.632 genes per kb (612 bases per gene), with an average length of 561, and a coding percentage of 91.6%. There appeared to be a slight bias in that the majority of the ORFs were in the reverse orientation.

Approximately half of the ORFs identified in pCU120 were either similar, or highly similar to genes found in RM3195, particularly the plasmid pCU110 (Fig 7.5). These ORFs were located within close proximity to each other in pCU120 (Fig 7.5). The other half of pCU120 contained predominantly non-coding/novel regions, and regions that matched with other species such as *C. jejuni*, *C. coli* and *Helicobacter* spp. Amino acid sequences were matched particularly with the plasmids found in *C. jejuni* and *C. coli*.

Figure 7.5. Diagram indicating the similarity of plasmid pCU120 ORFs to other organisms.



7.5.2.1 Protein Functions

Putative roles were assigned to 46% (95/207) of the ORFs in pCU120 (Appendix Fig 5.4). Twenty five of the 207 ORFs were associated with TFSS, including 20 *Tra*, 2 *Virb*, 2 *Trb* and one *pilT* gene (Fig 7.6). A further 29 ORFs were putatively assigned to roles including; translation, transcription, DNA repair, cell division, plasmid partition, mobilisation or recombination. Nine genes were associated with proteins of phage origin including four *Yop*s (Yersinia outer membrane proteins), one *Bet* (phage recombination protein) coding genes, a site-specific recombinase phage integrase family (XerD and XerC integrases DNA breaking-rejoining enzymes), two phage head morphogenesis proteins SPP1, and bacteriophage L54a single-stranded DNA binding protein. Another six ORFs were possibly periplasmic or membrane proteins.

Twenty seven other ORFs were assigned to various other functions, for example; ATPase, Fic proteins, M protein, flagellar basal-body rod protein, toxin-like proteins, and antitoxin proteins (RelE/StbE family). Several amino acid sequences matched with other hypothetical/conserved hypothetical proteins (47/207, 23%), whilst 31% (64/207) had no significant matches and were assumed to be psedo/non-coding or novel regions.

7.5.2.2 Proteins Associated With Type IV Secretion Systems

Several *Tra* genes were identified within pCU120, including; *TraB, TraC, TraD, TraE, TraF, TraG, TraH, TraK, TraL, TraN, TraU, TraV,* and *TraW.* Additionally *TrbB*, *TrbC, Virb1*, *Virb5* and *pilT* were also identified (Fig 7.6). Functions are indicated in Fig 7.7. There appeared to be a distinctive *Tra* region within pCU120, although some *Tra* and *Virb* genes were found in different locations (Fig 7.6). All these genes, with the exception of *Virb* genes, were located within a region of pCU120 that showed high similarity to RM3195, particularly pCU110 (Figs 7.5& 7.6 and Appendix 5, Fig 5.5). It was noted that the majority of poly G tracts were located within *Tra* ORFs.

Figure 7.6. Location of TFSS coding genes on plasmid pCU120.

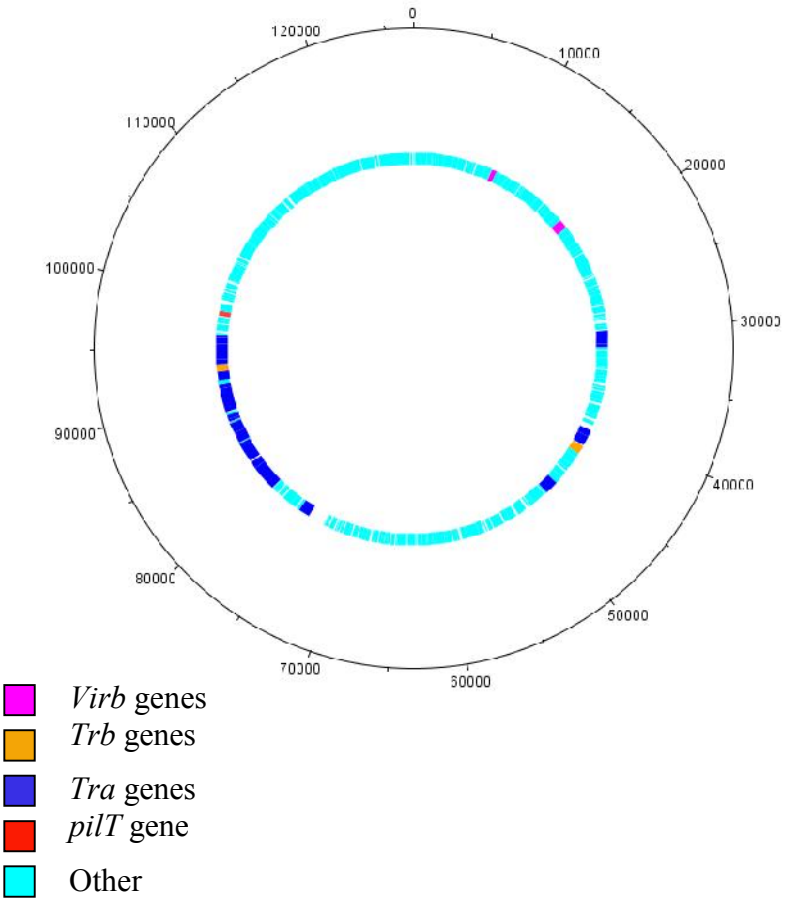
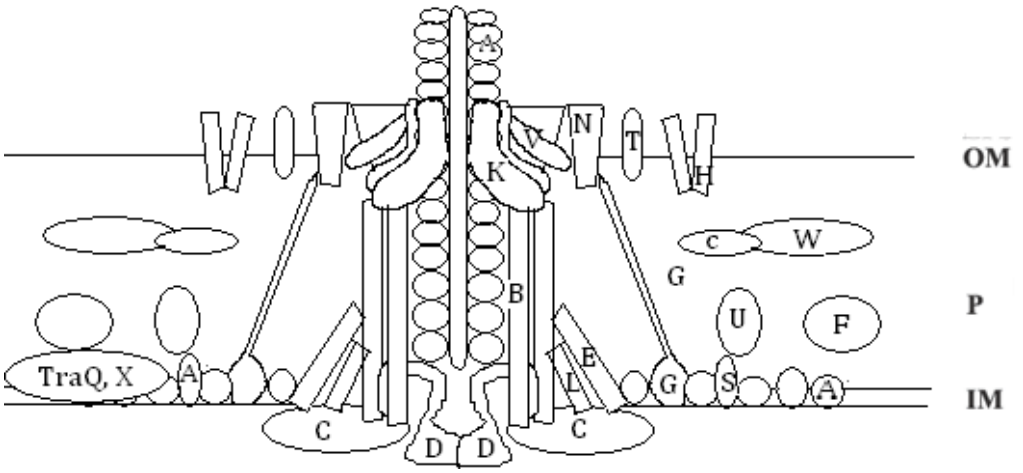


Figure 7.7. Representational diagram of a TFSS adapted from Lawley *et al*., (2003). Upper case letters indicate Tra proteins, lower case indicate Trb proteins (Eisenbrandt *et al*., 1999), OM=outer membrane, P=periplasm, and IM=inner membrane.



7.5.3 Potential Small Plasmid

As well as pCU120, there were also some matches with a second plasmid found in RM3195, suggesting isolate *C. upsaliensis* 52A may contain another, smaller plasmid of approximately 3, 476 bp. This smaller plasmid was identified within one scaffold, containing three contigs, and appeared to have two possible gaps. Primers were designed and despite apparent PCR products, sequencing was not successful and this plasmid was not investigated further. It should be noted that on earlier, 'unsuccessful' gel images (Appendix 5, Figs 5.1-5.3), the DNA band present may have represented this, or other plasmids that may have been present, as opposed to pCU120.

7.6 Discussion

Sequence obtained from *C. upsaliensis* strain 52 A, isolated from a dog, was 1,765,608 bp in size and contained at least one large plasmid (126 kb), and possibly a second, smaller plasmid (3.4 kb). The previously sequenced *C. upsaliensis* genome (RM3195) had a similar sized genome, and a similar number of plasmids of approximately the same sizes i.e. 126 kbp and 3.4 kbp compared to 110 kbp and 3.1 kbp (Fouts *et al*., 2005).

Approximately half of pCU120 was similar or highly similar to amino acid sequences found in RM3195, particularly matching those in pCU110. Interestingly all of these sequences were adjacent to each other which might suggest a common ancestry for these two plasmids, where only certain beneficial genes, such as those coding for TFSS (discussed below, section 7.6.1) have been maintained. Since RM3195 and *C. upsaliensis* 52A were isolated over a decade apart, in South Africa and the UK respectively, the proportion of dissimilarity between the plasmids (which are the most common vehicles of DNA transfer between bacteria, Arias *et al*., 2009) might be expected. Further more, the fact that there were a number of matches with such a high similarity may suggest that these large plasmids code for crucial proteins in *C. upsaliensis* strains. More annotations of plasmids from various *C. upsaliensis* strains would be needed to confirm or dispel this hypothesis. Alternatively it may be due to unsequenced, or unidentified regions within either of the plasmids pCU110 or pCU120 (some pCU110 sequences may be missing or located within different scaffolds).

Other similarities between pCU120 and pCU110 were also observed, for example Fouts *et al*, (2005) observed that single-stranded binding proteins were conserved across all those *Campylobacter* spp. that contained plasmids, suggesting a common ancestry. In agreement with these findings, a putative single-stranded binding protein was also found on plasmid pCU120.

7.6.1 Type IV Secretion System

Also in agreement with findings from Fouts *et al*, (2005), several components of a TFSS were located on plasmid pCU120. The TFSS in pCU120 was located within the region that matched with RM3195, suggesting that this may be an important feature within these two *C. upsaliensis* isolates (and possibly others). TFSSs are utilised in different ways for various bacteria, and consist of approximately 10 proteins or more (Gerlach and Hensel, 2007; Li *et al*., 1998; Winans *et al*., 1996). These proteins are often homologues, providing similar functions across different species of bacteria, but they are not identical on a nucleotide level. In the IncN plasmid pKM101 (isolated from *E.coli*), *Tra* genes are involved in the conjugal transfer system (Pohlman *et al*., 1994), proteins are transferred via the VirB system in *Agrobacterium tumefaciens* (Vergunst *et al*., 2000), and in *Bordetella pertussis,* the Ptl system of proteins is utilised to export the pertussis toxin (Weiss *et al*., 1993).

The plasmid was found to have several *Tra* amino acid sequences, including *TraB*, *TraC*, *TraD*, *TraE*, *TraF*, *TraG*, *TraH*, *TraK*, *TraL*, *TraN*, *TraU*, *TraV* and *TraW*. Additionally *TrbB* and *TrbC* (but not *TrbI*) were also found on the large plasmid. These proteins are associated with F-type TFSS conjugative systems

and with the exception of TraN, TraU (mating pair stabilisation and DNA transfer proteins), TraD and TrbB, are involved in pilus assembly (Anthony *et al*., 1999; Moore *et al*., 1981). However a few *Tra* amino acid sequences were not identified *TraQ* (pilin chaperone), *TraX* (pilin acetylation), and pilin components *TraA* (Minkley *et al*., 1976; Moore *et al*., 1981) and *TraM* (Pohlman *et al*., 1994; Schmidt-Eisenlohr *et al*., 1999). Some of these proteins are essential for F-pilus formation. Previous work demonstrated that mutants lacking one or more of these proteins resulted in an alteration in the number or length of F-pili, and that TraH was the most highly connected node (Harris and Silverman, 2004; Moore *et al*., 1981). The study by Harris *et al*, (2004) also concluded that *TraH*, *TraF*, *TraW*, *TraU* and *TrbB* were all hallmarks for an F-like type IV secretion system (Harris and Silverman, 2004), the genes of which were all found within the plasmid in this current study. The following pilin components, *TraA* (Minkley *et al*., 1976), *TraM* (Pohlman *et al*., 1994) and *Virb2* (Schmidt-Eisenlohr *et al*., 1999), were not located on pCU120. However *TrbC* and *Virb5* were identified on pCU120. *Virb5* may be a minor component of the pilus apparatus (Schmidt-Eisenlohr *et al*., 1999), and there is evidence to suggest that *TrbC* is a pilin subunit its self (Eisenbrandt *et al*., 1999).

Transfer coupling proteins such as TrwB, TraG, TraD, and VirD4 are required for DNA or protein transfer in *Agrobacterium* spp. and conjugation systems in various bacteria (Cabezon *et al*., 1994; Moncalian *et al*., 1999; Vergunst *et al*., 2000). *TraG* and *TraD* were both found in pCU120, and Tra proteins in particular are associated with conjugation in *E. coli* (Pohlman *et al*., 1994), suggesting a conjugative/DNA transfer role in *C. upsaliensis* 52A, as opposed to

toxin secretion. This conjugative role is supported by the findings of Fouts *et al*, (2005), who suggested a similar role for the pCU110 *C. upsaliensis* plasmid. Further work is needed to determine if all TFSS's in *C. upsaliensis* plasmids are utilized in this way.

7.6.1.1 Homopolymeric Repeated Regions

Fouts *et al*, (2005) observed that the genome of *C. upsaliensis* RM3195, contained more homopolymeric regions, particularly G:C tracts, than other *Campylobacter* spp. DNA repeats can result in greater variation and increased recombination (Shak *et al*., 2009), which in turn potentially allow for adaptation to different environments. A large number of poly G tracts might normally be explained by the presence of unique ORFs, but Fouts *et al*, (2005) could not explain why only a minority of these repeats were found in unique ORFs for RM3195. This present study was focused on annotating only one plasmid (as opposed to the genome), but it was noted that several poly G:C tracts were also present in this plasmid, in 'novel', phage, hypothetical and functional proteins. A large number of these G:C tracts were within ORFs that coded for Tra proteins. This is similar to the findings of Fouts *et al*, (2005), but is difficult to explain because *Tra* genes appear to be conserved and are not unique to *C. upsaliensis*. However it should be noted that more than three homopolymeric bases can be misinterpreted by the Genome Sequencer™ FLX (454 Life Sciences™).

7.6.2 Possible Virulence Factors in pCU120

7.6.2.1 Yersinia Outer Proteins (Yop)

Several possible virulence factors were identified within pCU120, including *YopX*. Interestingly, within pCU120 there were four amino acid sequences coding for *YopX*, but all were different. There is currently little information for *Yop*s found in *Campylobacter* spp., although *Yerisinia* spp. invasion proteins were reported in a *C. lari* plasmid (Fouts *et al*., 2005). There is also little information regarding *YopX* specifically in other species, but functions have been assigned to other *Yops* (Andersson *et al*., 1999; Navarro *et al*., 2005). Yops are considered to be virulence factors that evade the host immune system by various methods such as, macrophage apoptosis (Bi *et al*., 2009), inhibition of phagocytosis via disruption of actin cytoskeleton (Adkins *et al*., 2007), interference of calcium signalling in neutrophils (Andersson *et al*., 1999), and inhibition of the inflammatory response by interfering with cytokine production (Navarro *et al*., 2005; Yao *et al*., 1999). *Yop*s, thought to be of phage origin, are usually found on a 70 kb plasmid, and some Yops are thought to be involved in the delivery apparatus, usually transported by a type III secretion system (TTSS), whilst others are effector proteins, secreted into eukaryotic cells in order to disrupt their activity (Andersson *et al*., 1999; Cornelis and Wolf-Watz, 1997). Although no TTSS was found within pCU120, genes coding for a TTSS may have been located on the genome or on another plasmid within *C. upsaliensis* 52A. Further more, a TFSS was identified within pCU120 which might play a role in the transportation of Yops, although as discussed previously, a conjugative role seems the most likely explanation for this apparatus. However due to the phage origin of Yops, it cannot be excluded that these proteins are not

fully utilised by the bacterium. One of the four *YopX* was not observed in pCU110, suggesting it was either lost from this plasmid, or was an insertion to pCU120. The first seems more likely because this amino acid sequence was specifically *YopX*, as opposed to a different *Yop*, although phylogenetic analysis is needed to explore this. This might provide further evidence that *Yop*s are not fully utilised by *C. upsaliensis*, at least in RM3195.

7.6.2.2 M-Protein

A putative M-protein was identified within plasmid pCU120. The amino acid sequence in pCU120 matched 100% with an amino acid sequence of RM3195 that was annotated as M-protein, but no other evidence was found to suggest that this truly was an M-protein, for example the pCU120 'M-protein' sequence did not match M-protein of any other isolate or species on the BLAST database, apart from RM3195, and no conserved domains were observed for this amino acid sequence via BLAST or EMBL-EBI (European Molecular Biology Laboratory European Bioinformatics institute http://www.ebi.ac.uk/). M-protein is a virulence factor released by bacteria such as *Streptococcas* spp. which disrupts the activity of factor H, an inhibitor of the alternative pathway of complement activation (Ashbaugh *et al*., 2000; Horstmann *et al*., 1988). Under activation of factor H can result in auto-immune conditions, such as Guillain-Barré syndrome, which would have been of interest since RM3195 was isolated from a case of Guillain-Barré syndrome. Evidence obtained from the BLAST database suggested that this ORF was probably more similar to 3-deoxy-D-manno-octulosonic-acid transferase.

7.6.2.3 Toxin-Like Proteins

There were several putative toxin associated proteins found within pCU120 including, a toxin ABC transporter, bacteriocin resistance protein, bacteriocin-type signal sequence domain-containing protein, and three amino acid sequences associated with plasmid stabilisation (RelE/StbE), cytotoxic translational repressor of toxin-antitoxin, addiction module antitoxin and a toxin-like protein addiction module toxin.

Plasmid stabilisation proteins are used to ensure that plasmids are maintained in the population and are thought to be affected by stress (Gerdes *et al*., 2005; Pandey and Gerdes, 2005). For this reason, plasmids may contain a toxin and anti-toxin, the latter of which is encoded on the plasmid. Daughter cells may be exposed to the toxin via the parent cell, and will not survive unless they can produce the anti-toxin (Gerdes *et al*., 2005; Pandey and Gerdes, 2005). This is both a virulence and survival feature of bacteria, because if the plasmid also contains virulence genes, such as antibiotic resistance or flagella, then these will be maintained in the population.

Bacteriocins are antagonistic to other bacteria, and have been observed in *Campylobacter* spp. previously (Workman *et al*., 2008). Therefore there was no evidence to suggest that plasmid pCU120 coded for any host associated toxins.

7.6.2.4 Histidine Kinase

One amino acid sequence responsible for the coding of membrane associated signal transduction histidine kinase was located within pCU120. Histidine

kinases are usually part of a two-component regulatory system, which are designed to respond to environmental changes. Two-component regulatory systems are important in *C. jejuni*, and have been shown to be important for temperature-dependent growth and colonisation (Bras *et al*., 1999; Raphael *et al*., 2005).

7.6.2.5 Motility and Adherence

Virulence in *Campylobacter* spp. has been associated with motility and ability to adhere to host cells (Yao *et al*., 1994). However, plasmid pCU120 contained only one gene involved in flagellum synthesis, FlgG, a basal-body, distal rod protein, and no adhesion proteins were observed within pCU120. Other flagellum or adhesion coding genes may be present in the genome or on another plasmid, but pCU120 does not appear to have motility based virulence factors.

7.6.3 Importance of Plasmids in C. upsaliensis Isolated from Humans

Studies by various authors have suggested that *C. upsaliensis* strains may be more likely to contain plasmids than *C. jejuni* strains (Fouts *et al*., 2005; Goossens *et al*., 1990a; Schmidt-Ott *et al*., 2005; Stanley *et al*., 1994; Tenover *et al*., 1985). The majority of *C. upsaliensis* strains isolated from human samples have been shown to contain plasmids, some ranging in size from 1.5 kb to 110 kb (Fouts *et al*., 2005; Goossens *et al*., 1990a; Stanley *et al*., 1994).

One study observed that in general, *C. upsaliensis* strains isolated from humans contained more plasmids than those extracted from dogs, which unlike the findings of this present study, often did not contain any plasmids at all (Stanley

et al., 1994). The conclusions drawn from Stanley *et al*, (1994) were that human and canine *C. upsaliensis* isolates were not related due to differences in plasmid carriage, and because of differing ribotypes. However human and dog isolates in Stanley *et al*, (1994) did not originate from the same countries, and the human strain isolated from the UK contained a ribotype similar to that of a canine ribotype, and like many of the canine isolates, this human strain contained no plasmid. Therefore transmission between dogs and humans, or a common source of *C. upsaliensis* carriage/infection, cannot be ruled out.

Some strains of *C. upsaliensis*, particularly those carrying plasmids, appear to be associated with human disease severe enough to report to a doctor. Up to 89.9% of *C. upsaliensis* isolates from humans have been shown to contain plasmids in one study, and 60% of *C. upsaliensis* strains from patients were found to contain plasmids in another (Goossens *et al*., 1990a; Stanley *et al*., 1994). This may indicate that these plasmids contain certain virulence factors which subsequently lead to symptomatic infection in humans. Since the dog isolate in this study (*C. upsaliensis* 52A) contained at least one large plasmid, this strain, and thus the dog of origin, could be considered a potential risk to humans. However, it should be noted that dogs appear to carry both *C. upsaliensis* strains with and without plasmids (Stanley *et al*., 1994) and no clear virulence factors could be identified within the plasmid of this present study. However, this present study was based on one isolate that did not originate from a human, and only examined one possible plasmid.

In essence, *C. upsaliensis* infection may be more severe (and thus reported) in humans when the strains contain plasmids, but *C. upsaliensis* infection (asymptomatic or symptomatic) may still occur when strains contain no detectable plasmids. Both *C. upsaliensis* strains with and without plasmids are carried by dogs, suggesting either possible transmission between dogs and humans, or a common source of infection.

7.6.4 Conclusions

This presence of a plasmid found within this canine derived isolate may indicate that this strain, and thus the dog of origin, could be a potential zoonotic risk to humans. Only a small number of genes, possibly associated with virulence in *Campylobacter* spp. were found within pCU120. The dominant feature of pCU120 was the presence of a conjugative TFSS. Both of these findings are consistent with information obtained from a previously sequenced *C. upsaliensis* isolate (RM3195), which demonstrated relatively fewer virulence genes than species such as *C. jejuni* (Fouts *et al*., 2005). Further to this, the TFSS was located within a region on pCU120 that was similar, or highly similar to amino acid sequences found in pCU110 (RM3195), supporting the findings of Fouts *et al* (2005) that this system is conserved across many *Campylobacter* spp. and is therefore utilised by this species.

The next stage of the project is to annotate the *C. upsaliensis* 52A genome its self. The *C. upsaliensis* 52A genome will then be examined for similarities and differences between RM3195 and other published *Campylobacter* spp. genomes, with special attention given to investigating possible virulence factors (or lack

of). Comparison of this genome to that of RM3195 may be the beginning of genome sequencing for *C. upsaliensis* isolates. There are still many unanswered questions regarding this species as mentioned previously in section 7.1, which can be explored via genome sequencing. MLST data (Chapter 6) indicated that there was considerable genetic diversity amongst *C. upsaliensis* isolates originating from both humans and dogs. Genome sequencing may improve current understanding of this diversity, and determine whether or not this species is as diverse as current data suggests.

8. Chapter eight

Final Discussion

8.1 Background

Campylobacter spp., are the most common causes of bacterial gastroenteritis in humans worldwide (CDC, 2008c; DEFRA, 2007; Westrell *et al*., 2009). These infections are dominated by *C. jejuni*, and to a lesser extent *C. coli*, however, other species such as *C. upsaliensis* and *C. lari* have also been isolated from human disease (Adak *et al*., 2002; CDC, 2008c; DEFRA, 2007). The true prevalence of *C. upsaliensis* may be underestimated due to the mild nature of the disease in comparison to *C. jejuni* (Goossens *et al*., 1990b; Jimenez *et al*., 1999), and isolation procedures are often optimised for *C. jejuni* as opposed to *C. upsaliensis* (Fernandez and Martin, 1991; Fleming, 1983; Fox *et al*., 1983; Malik and Love, 1989; Nair *et al*., 1985).

Contact with raw poultry, and cattle are considered the main sources of infection for *C. jejuni* in particular (Humphrey *et al*., 2007; Hussain *et al*., 2007; Sheppard *et al*., 2009; Wilson *et al*., 2008), however there are several reports of an increased risk of *Campylobacter* spp. infection for humans associated with dog ownership or contact (Adak *et al*., 1995; FSA, 2005; Kapperud *et al*., 1992; Salfield and Pugh, 1987; Tenkate and Stafford, 2001). Dogs are significant sources of *C. upsaliensis* (Acke *et al*., 2009; Hald *et al*., 2004; Koene *et al*., 2004; Rossi *et al*., 2008; Wieland *et al*., 2005), and in some situations can be considered sources of *C. jejuni* (Hald and Madsen, 1997; Lopez *et al*., 2002; Tsai *et al*., 2007; Workman *et al*., 2005). Kennelled/stray dogs in particular have been associated with a high overall *Campylobacter* spp. prevalence, and demonstrate

some of the highest prevalences of *C. jejuni* (Fernandez and Martin, 1991; Tsai *et al*., 2007; Workman *et al*., 2005).

8.1.1 Aims

The aims of this thesis were to investigate the potential risk to humans that dogs may pose in terms of *Campylobacter* spp. carriage, and to gain further insight into the aetiology of *Campylobacter* spp. within dogs. This was accomplished by determining; the prevalence and species distribution of *Campylobacter* spp. in dogs from different populations including vet-visiting and kennelled dogs, analysing risk factors for canine *Campylobacter* spp. carriage, and recording shedding patterns within dogs. In addition several *Campylobacter* spp. isolates were examined on a molecular level in order to investigate their zoonotic potential, and a pilot study was carried out to annotate a large plasmid found within a *C. upsaliensis* strain isolated from a household dog.

8.2 Findings

The findings of this thesis revealed that a large number of predominantly healthy dogs, potentially between 30%-73%, carry one or more *Campylobacter* spp., and that this prevalence can depend upon the population sampled. The highest overall isolation rates of *Campylobacter* spp. were found in rescue and boarding kennels (Chapter 4&5), whilst the lowest were found in vet-visiting dogs (Chapter 3), some individual hunt kennels (Chapter 5) and one rescue kennel (Chapter 5), although the latter may have been limited by lack of culture results. Additionally the results in the hunt kennels may have been affected by the particular culture methods used. These observations are in agreement with findings from other

studies, and supports the idea that certain dog populations, particularly rescue/kennelled dogs have higher carriage rates of *Campylobacter* spp. than vet-visiting or household dogs (Acke *et al*., 2006; Acke *et al*., 2009; Tsai *et al*., 2007; Westgarth *et al*., 2009; Workman *et al*., 2005).

8.2.1 Campylobacter Species

The species distribution of *Campylobacter* also appeared to be influenced by the origins of the dogs. The studies based upon vet-visiting, boarding and rescue dogs demonstrated that when dogs carried *Campylobacter* spp., the species isolated were only *C. upsaliensis* and *C. jejuni*, whereas other species such as *C. coli* and *C. lari* were only identified when hunt dogs were sampled. The majority of studies have isolated both *C. upsaliensis* and *C. jejuni* from dogs within various populations (Acke *et al*., 2009; Sandberg *et al*., 2002; Westgarth *et al*., 2009; Workman *et al*., 2005), but there is limited literature available on *Campylobacter* spp. in hunt dogs, so it is uncertain as to whether or not these observations are typical for this particular group. Sources of *C. jejuni*, *C. coli* and *C. lari* for hunt dogs probably include their diet which included raw meat in one kennel, and increased environmental exposure to *Campylobacter* spp. No other *Campylobacter* spp. were isolated in any of the studies, despite other reports of *C. helveticus* isolation from dogs, however this species is mainly reported in cats (Engvall *et al*., 2003; Wieland *et al*., 2005).

8.2.1.1 C. upsaliensis

In general *C. upsaliensis* dominated more than any other *Campylobacter* spp. This has been reported in other studies (Acke *et al*., 2009; Engvall *et al*., 2003;

Hald *et al*., 2004; Koene *et al*., 2004; Rossi *et al*., 2008; Sandberg *et al*., 2002; Wieland *et al*., 2005) and confirms that dogs in the UK are significant reservoirs of *C. upsaliensis*. *C. upsaliensis* was isolated from every study, except hunt dogs in kennels E and F where only one *C. jejuni*, and no *Campylobacter* spp. were isolated respectively. This may have been the result of the methods chosen, i.e. CAT with filtration, but this does not fully explain the apparent absence of *C. upsaliensis*. The frequency of *C. upsaliensis* isolation from dogs described in this thesis provides further evidence of a commensal role for this bacterium in dogs.

8.2.1.1.1 Possible Transmission

During this present study, there were several instances where sequential *C. upsaliensis* isolates were characterised from kennelled dogs using the partial *groEL* sequence, and within these, there were no instances of dogs shedding different strains of *C. upsaliensis*. Mixed *C. upsaliensis* infections may have been present but were not detected, therefore longitudinal kennel data indicated that dogs did not encounter cycles of re-infection, but instead continued to shed the same detectable strain of *C. upsaliensis*. Most dogs (carrying *C. upsaliensis*) shed *C. upsaliensis* in every sample, but in cases where shedding appeared to start after entry to the kennels, shedding may have been caused by stress.

MLST data suggested that there was considerable genetic diversity between *C. upsaliensis* isolates originating from both humans and dogs, and this diversity has been reported previously for *C. upsaliensis* (Miller *et al*., 2005), particularly when isolated from canine samples (Damborg *et al*., 2008; Koene *et al*., 2009). These data also suggested that transmission (presumably to a naïve dog), or

common sources of *C. upsaliensis* possibly occur between dogs because despite small numbers, two hunt dogs from the same kennel had strains that belonged to the same complex, whereas no other dogs had *C. upsaliensis* strains that belonged to this complex. Similar situations have previously been noted in dogs originating from the same kennel or household (Damborg *et al*., 2008; Miller *et al*., 2005). This may explain why a dog living with another dog, particularly one carrying *C. upsaliensis*, was a risk factor for vet-visiting dogs, in terms of carrying *C. upsaliensis* (Chapter 3).

8.2.1.1.2 Zoonotic Potential

Although it is unclear as to the frequency and severity of *C. upsaliensis* infection in humans, infection has been reported (Byrne *et al*., 2001; Carter and Cimolai, 1996; Goossens *et al*., 1990a; Gurgan and Diker, 1994; Jenkin and Tee, 1998; Jimenez *et al*., 1999; Lawson *et al*., 1999; Patton *et al*., 1989; Prasad *et al*., 2001). Whether or not any of these cases were the direct result of dog to human transmission is not fully understood. Studies examining isolates on a molecular level have concluded that dog and human *C. upsaliensis* isolates appear distinct, although these studies are often limited by the selection of isolates chosen, due to the relatively few reported cases of *C. upsaliensis* in humans (Damborg *et al*., 2008; Stanley *et al*., 1994). Results from this present study based on MLST data could not rule out the possibility of transmission or a common source of *C. upsaliensis* infection for both dogs and humans, since human and dog isolates did not appear to segregate. Other studies have also suggested possible links between dogs and *C. upsaliensis* infection in humans (Goossens *et al*., 1991; Labarca *et al*., 2002; Lentzsch *et al*., 2004), and since no other significant sources of *C.*

upsaliensis have been currently found (except maybe cats to some extent), this may indicate that dogs are a potential source of *C. upsaliensis* for humans. This possibility is further enhanced by the close contact between dogs and humans (Westgarth *et al*., 2008).

8.2.1.2 C. jejuni

C. jejuni was isolated from every dog population studied (except hunt kennel F where no *Campylobacter* spp. were found), although the prevalence was not as consistently high as *C. upsaliensis*. Despite this, the prevalence of *C. jejuni* was relatively high in some of the rescue and hunt kennels, reaching 20% (95%, CI 8, 36) and 26% (95%, CI 16, 40) respectively. This is consistent with other studies based upon kennelled/stray dogs who found similarly high prevalence's of *C. jejuni* (Malik and Love, 1989; Tsai *et al*., 2007; Workman *et al*., 2005). However this prevalence is considerably higher than other UK based studies, and studies based on household or vet-visiting dogs where the prevalence was low, 1.2% (95%, CI 0.3, 3) (Chapter 3).

8.2.1.2.1 Possible Transmission

When possible transmission events occurred in both the (longitudinal) boarding and rescue kennels, they involved *C. jejuni* proportionately more than *C. upsaliensis* (Chapter 4). Some rescue dogs entered the kennel as carriers of *C. jejuni*, whereas none of the boarding dogs did. Of three dogs in the rescue kennel who appeared to have acquired *C. jejuni* strains within the kennel, two spent the last six to seven days in the quarantine block. It is unclear as to whether or not the quarantine block was associated with these dogs carrying *C. jejuni*, but it

appeared to have a greater potential for disease transmission compared to other blocks for various reasons. Although no direct dog to dog transmission could be confirmed in the quarantine, dogs with potentially increased disease burdens experienced more socialising in the quarantine block, due to the sharing of outdoor pens, than dogs housed in other blocks. This meant that the potential for transmission, or increased stress, due to the presence of other dogs or other pathogens, was present in the quarantine block. Dogs were probably exposed to sources of *C. jejuni* both prior to admission and after entry to the kennel premises, which may or may not have included the quarantine block. However it remains uncertain as to whether or not these sources were other dogs/faeces.

Interestingly two dogs in the boarding kennel that had no *Campylobacter* spp. isolated from them for over a week began to shed *C. upsaliensis*, and *C. jejuni* at a similar point in time. An explanation may be that whatever these dogs were exposed to favoured both *C. jejuni* and *C. upsaliensis*, but effects caused by stress are unlikely for *C. jejuni* because of the molecular evidence suggesting that the two strains were the same in both of these two dogs.

C. jejuni appears to out-compete *C. upsaliensis in vitro*, due to the shorter incubation period required for *C. jejuni* (Byrne *et al*., 2001; Labarca *et al*., 2002; Moreno *et al*., 1993), but little work has been performed *in vivo*. If *C. upsaliensis* is more adapted to survive in a dog than *C. jejuni*, it may be able to out-compete *C. jejuni*, acting as a defence mechanism for the dog. It is unclear as to whether or not all dogs carry *C. upsaliensis*, but shed the bacterium in variable amounts,

some of which may be too low to detect, or if some dogs simply acquire *C. upsaliensis* at some stage of their life, whilst others do not.

8.2.1.2.2 Zoonotic Potential

The strains of *C. jejuni* carried by dogs appeared to show considerable genetic diversity, although they were not as diverse as the strains of *C. upsaliensis* mentioned previously (Chapter 6). With the exception of ST-2772 found in hunt dogs, the majority of sequence types found in the dogs were the same as those reported in humans. Some clonal complexes found in dogs were the same as some of the most common complexes found in humans such as ST-21 and ST-45. These data indicated that there are likely to be common sources of infection, such as poultry, for both humans and dogs, and that dogs remain a possible zoonotic risk of *C. jejuni* infection for humans. However, the human exposure risk may vary depending on the circumstances of the dog. For example young dogs may have more contact with humans (Westgarth *et al*., 2008), and dogs from particular origins may pose a greater risk, such as dogs from rescue or hunt kennels where the prevalence may be higher (Chapters 4&5).

8.2.2 Possible Sources of Campylobacter spp. *for Dogs*

Poultry meat is considered the most significant source of *C. jejuni* for humans (Humphrey *et al*., 2007; Hussain *et al*., 2007; Sheppard *et al*., 2009; Wilson *et al*., 2008) and because dogs and humans live in such close proximity, it is likely that poultry meat contributes to *C. jejuni* carriage in dogs, especially since human titbits are often fed to pet dogs (Westgarth *et al*., 2008). *Campylobacter* spp. have also been isolated from environmental water (Brown *et al*., 2004; French *et*

al., 2005; Horman *et al*., 2004; Kemp *et al*., 2005), presumably contaminated with faecal material. Stray dogs in particular may have more access to surface water such as puddles, and bins containing under cooked or raw poultry compared to household (vet-visiting and boarding) dogs. Despite household dogs receiving titbits, the same survey reported that 83% of household dogs were never fed raw meat (Westgarth *et al*., 2008). This may explain why *C. jejuni* was only found in rescue dogs on entry to the kennel and not household/boarding dogs. However, oral-faecal transmission of *Campylobacter* spp., particularly *C. upsaliensis*, between dogs is likely to play a major role in the epidemiology of canine *Campylobacter* carriage.

8.2.3 Risk Factors for Dogs

Although several risk factors were investigated in the various studies, only; dog age, kennel cough, recent vomiting, living with another dog carrying *Campylobacter* spp., and antibiotic treatment were found to have significant associations for *Campylobacter* spp. carriage in dogs. Length of stay in a kennel verged on significance, as did living with another dog of any *Campylobacter* spp. status. Recent vomiting appeared to be protective, but this category and the kennel cough variable were based on limited data. There was also possible bias in the reporting of certain variables such as kennel cough, age and vomiting as they primarily depended upon the kennel staff recording them.

8.2.3.1 Age

Age was identified as a risk factor for *Campylobacter* spp. carriage in dogs from two studies within this thesis (Chapters 3&4). Vet-visiting dogs and kennelled

dogs indicated a significant trend where younger dogs had increased odds of carrying *Campylobacter* spp. compared to older dogs. Age has been identified as a risk factor for the carriage of *Campylobacter* spp. in several studies, with dogs younger than six months old (Acke *et al*., 2009; Nair *et al*., 1985), 12 months old (Engvall *et al*., 2003; Lopez *et al*., 2002; Sandberg *et al*., 2002; Wieland *et al*., 2005), and even 15 months old (Hald *et al*., 2004) more likely to carry *Campylobacter* spp. than older dogs. In contrast, other studies have not found younger dogs to be significantly more likely to carry *Campylobacter* spp. than older dogs (Burnie *et al*., 1983; Tsai *et al*., 2007; Wieland *et al*., 2005).

8.2.3.2 Disease

Associations have been made between young dogs, clinical signs and the presence of *Campylobacter* spp. in previous studies (Fleming, 1983; Fox *et al*., 1983; Nair *et al*., 1985), however, the studies within this thesis were unable to find any trends or significant relationships regarding diarrhoea. A recent study by Acke *et al*, (2009) noted a trend towards dogs carrying *C. jejuni* and displaying symptoms, but could not perform statistical analysis on such relatively few *C. jejuni* positive samples.

Despite the lack of association between clinical signs and *Campylobacter* spp., there was some evidence to suggest that *C. upsaliensis* acted as a commensal in these dogs. The high prevalence of this species found in nearly every dog population sampled within this study, and the lack of an association with diarrhoea are suggestive of a commensal role. Further to this, it was noted that

when *C. upsaliensis* was shed, the same apparent strain was shed over a long duration.

Conversely, this present study was unable to isolate the same strain of *C. jejuni* for more than a couple of days, indicating short term shedding. Although there was no apparent trend towards diarrhoea in dogs carrying *C. jejuni*, as mentioned previously, trends have been reported in another study (Acke *et al*., 2009). Interestingly, many of the studies which have found younger dogs to be more likely to carry *Campylobacter* spp., reported *C. upsaliensis* as the most common *Campylobacter* spp. in dogs (Acke *et al*., 2009; Engvall *et al*., 2003; Sandberg *et al*., 2002; Wieland *et al*., 2005), whereas other studies have been unable to find significant associations between age and *C. jejuni* carriage (Tsai *et al*., 2007; Wieland *et al*., 2005). This may partly be because *C. jejuni* is not a commensal in dogs, but instead a transient infection, which may or may not be symptomatic. As a result, *C. jejuni* may be able to infect dogs of any age, as opposed to *C. upsaliensis* which colonises young, naïve dogs, who may shed greater numbers of *C. upsaliensis* initially, until they have developed some immunity towards this species.

8.2.4 Isolation Methods

Overall, isolation rates of all *Campylobacter* spp. appeared similar between direct plating onto mCCDA and filtration onto CAT media. Although some studies showed a slight tendency towards one of these methods, i.e. direct plating detected slightly more *Campylobacter* spp. in vet-visiting dogs (Chapter 3), and CAT based methods detected slightly more in kennel 2 (Chapter 4), there were

no significant differences between these two methods and the overall isolation of *Campylobacter* spp. in any of the studies within this thesis. However enrichment appeared to detect significantly less *Campylobacter* spp. then either of these two methods (Chapter 3).

In kennels 1 and 2 (Chapter 4) there was a slight tendency for *C. jejuni* isolates to be detected by direct plating rather than by filtration, whereas the opposite was true for *C. upsaliensis* isolates that originated from these same dogs (i.e. those with overall mixed infections), whereby significantly more *C. upsaliensis* isolates were detected by filtration (Appendix 2, Table 2.4). Since CAT agar is optimised for *C. upsaliensis* detection (Aspinall *et al*., 1993, 1996; Burnens *et al*., 1992; Burnens and Nicolet, 1992; Byrne *et al*., 2001; Corry and Atabay, 1997), it is not surprising that this agar detected the majority of the *C. upsaliensis* isolates from the dogs with mixed infections. The agar used in the direct plating method did not contain either a filter, or the same antibiotics found in CAT agar, which are favourable to *C. upsaliensis*. This, added to the knowledge that *C. jejuni* grows faster in culture, and can therefore outcompete *C. upsaliensis*, explains the differences observed between these two species and detection methods (Byrne *et al*., 2001; Labarca *et al*., 2002).

Culture appeared to detect more *Campylobacter* spp. than direct PCR when samples were fresh, but direct PCR detected more *Campylobacter* spp. when samples had been the post (Chapter 3). Direct PCR was useful for detecting *C. upsaliensis* but did not always identify *C. jejuni* in samples and was not tested on any other species. Koene *et al*, (2004) also noted a similar effect of transportation

time, and another study which extracted *Campylobacter* spp. DNA directly from faeces, found that direct PCR had a higher sensitivity for detecting *Campylobacter* spp. in 'aged' samples, compared to culture with PCR confirmation (63% and 0% respectively for 'aged' samples n=8; Maher *et al*., 2003). Therefore culture would be recommended for detecting a wider range of species and especially when samples are fresh, but direct PCR should be used for samples that have experienced a time delay between collection and processing, and multiplex PCR assays may be successful in targeting several species. Where possible both methods should be used in conjunction with one another in order to maximise recovery.

8.2.5 Salmonella

Overall the prevalence of *Salmonella* spp. was, if detected at all, extremely low (Chapter 3), in every population sampled except for one hunt kennel where the prevalence was high (15%, Chapter 5). The two serovars found in the vet-visiting dogs and hunt dogs respectively were *S.* Newport and *S.* Typhimurium, the latter of which being one of the most commonly identified causes of salmonellosis in humans in the UK (DEFRA, 2007). Therefore the majority of dogs should not be considered a significant source of *Salmonella* spp. for humans, but hunt dogs may pose a slightly greater zoonotic risk.

8.2.6 Conclusions

The findings of this thesis suggest that potentially a large number of dogs in the UK carry *Campylobacter* spp., especially *C. upsaliensis*. This species appears to be a commensal in dogs, and was found more commonly in younger dogs

compared to older dogs. The prevalence of *C. jejuni* was low in vet-visiting dogs, and boarding dogs, particularly on entry to the kennel, but was higher in rescue and hunt dogs. A greater species diversity was observed within the hunt kennels, where *C. coli* and *C. lari* were also observed. No associations could be made between the carriage of *Campylobacter* spp. in dogs, and clinical symptoms.

Longitudinal studies indicated that the majority of dogs carrying *Campylobacter* spp., carried the bacterium before entry to the kennels, and that *C. upsaliensis* was shed over a longer duration than *C. jejuni*. However there were some instances of possible transmission events within both the boarding and rescue kennel, and when they occurred, they often involved *C. jejuni*. Apparent *C. upsaliensis* transmission events were probably caused by fluctuating shedding patterns within that dog exasperated by stress, a failure in detection methods, or a shedding pattern within that dog. There was also some evidence to suggest that dog to dog transmission can occur, especially since a dog living with another dog, particularly one carrying *Campylobacter* spp., was more likely to carry *Campylobacter* spp. itself.

A considerable amount of genetic diversity was observed within the *C. jejuni* and *C. upsaliensis* isolates originating from dogs, and results suggested that strains of both species were the same, or similar to strains found in humans. This suggests that there may be common sources of infection for both humans and dogs and that dogs remain a potential zoonotic risk to humans. Although only a small number of household dogs carry *C. jejuni*, infected dogs should still be considered a potential zoonotic risk to humans, particularly if the dogs originate

from kennelled or hunt kennel populations where the prevalence may be higher. Dogs are a significant reservoir of *C. upsaliensis*, but the relationship between the presence of *C. upsaliensis* and gastroenteritis in both dogs and humans is still unclear.

8.2.7 Future Work

8.2.7.1 C. jejuni and Disease in Dogs

The relatively few examples of *C. jejuni* found in UK dogs, limits the extent to which associations can even be attempted, regarding risk factors and *C. jejuni*. In order to establish the relationship between clinical signs and *C. jejuni* in dogs, a considerable number of samples would be required. Additionally, certain dog populations may need to be targeted in order to increase the likelihood of isolating *C. jejuni*, for example hunt and kennelled dogs appear to have higher carriage rates of *C. jejuni* than vet-visiting dogs. A UK based case control study with presence of diarrhoea as the outcome variable would be the most appropriate way to investigate this. The prevalence of *C. jejuni* also appears to be higher in dogs from other countries, so prevalence based studies on dogs from various populations, such as kennelled, hunt and household could be done outside the UK in order to obtain a greater number of *C. jejuni* isolates for statistical analysis. In this case however the risk factors may not be applicable to UK dog populations.

It may also be of interest to examine the interactions of both *C. jejuni* and *C. upsaliensis in vitro* using canine derived cell culture to determine whether or not the presence of *C. upsaliensis* affects the colonisation potential of *C. jejuni*. This

type of study could also investigate what effects different quantities of both *C. upsaliensis* and *C. jejuni* have on each other, to represent the amount of *C. upsaliensis* typically shed by dogs, and the various infectious dosages of *C. jejuni*.

8.2.7.2 C. upsaliensis Carriage

The evidence from this thesis suggests that *C.upsaliensis* is probably a commensal in dogs, with no associations found between carriage and diarrhoea. However, there are still unanswered questions; at what stage of life do dogs become carriers of *C. upsaliensis*, what are the initial sources of infection, and is this bacteria transmitted horizontally and/or vertically between dogs? Longitudinal studies aimed at examining the presence of *C. upsaliensis* in nursing bitches, their newly born puppies (including swabs/faecal samples, or blood samples to examine antibodies via enzyme linked immunosorbent assay, taken before their first feed, if possible and ethical to do so), and their milk may explain if vertical transmission is a factor in *C. upsaliensis* carriage in dogs. A study examining *Helicobacter* spp. found that puppies may acquire *Helicobacter* spp. during the lactation period, and that puppies are able to infect each other during early life (Hanninen *et al*., 1998). Another study examining *Campylobacter* spp. in a closed breeding colony found that *Campylobacter* spp. were detected in the majority of puppies by eight weeks of age, and that their Ig G titres appeared to correlate with increased *Campylobacter* spp. carriage (Newton *et al*., 1988). This type of study may clarify what potential parameters are involved in dog to dog transmission for *C. upsaliensis*, and if it can occur for

this species, it may be hypothesised that it can also occur for other *Campylobacter* spp. such as *C. jejuni*.

8.2.7.2.1 Investigating Sources of C. upsaliensis

Apart from dogs (and possibly humans), sources of *C. upsaliensis* are relatively unknown. The only other significant sources to date are cats (Sandberg *et al*., 2002; Wieland *et al*., 2005; Workman *et al*., 2005), although one study reported isolating *C. upsaliensis* from a poultry slaughter house (Stoyanchev, 2004). It would be beneficial to know if cats mainly harbour *C. upsaliensis* when they are housed with a dog or not. Cats may shed *C. upsaliensis* for long periods of time, just as the dogs did in the longitudinal studies within this thesis. It is plausible that given the close genetic relationship between *C. upsaliensis* and *C. helveticus*, cats may sometimes carry *C. upsaliensis* as a commensal. Longitudinal studies may help determine if this is the case.

Another interesting aspect may be to examine environmental factors. Wildlife, such as mice, may enter houses, especially kitchens, which may lead to contaminated surfaces and/or food. In addition, mice are often hunted by cats which live in close proximity to humans. Wildlife have been found to carry species such as *C. jejuni* and *C. coli* in some studies, however not all of these studies were optimised for *C. upsaliensis* or *C. helveticus* detection (Brown *et al*., 2004; Kwan *et al*., 2008a; Meerburg *et al*., 2006; Petersen *et al*., 2001).

One study has recently investigated small rodents in farmland and private woodland areas (Williams *et al*., 2009). Bank voles and wood mice were

screened for *Campylobacter* spp., including *C. upsaliensis*, but this species was not identified and MLST data suggested that many *C. jejuni* isolates were unique to voles. This evidence might suggest rodents are an unlikely source of *Campylobacter* spp. for cats and thus humans, particularly wood mice where the *Campylobacter* spp. prevalence was low. However, the small size of rodent faeces may mean that they are more prone to desiccation, and as previously described (Chapter 3) direct PCR (which was not used in Williams *et al*, 2009) may be useful, particularly for detecting *C. upsaliensis* in unfavourable conditions. Additionally other rodent species such as house mice were not tested and further studies, particularly based in household gardens, might be useful to fully explore mice/rodents as a possible source.

Species such as *C. coli* and *C. lari* were isolated from the hunt dogs within this present study, and one possible explanation for this was increased exposure to environmental sources of these *Campylobacter* spp. As mentioned in chapter five, these sources might be birds, cattle faeces, rodents or water. Comparisons of isolates from these various sources, to those found in hunt dogs are needed to determine whether or not transmission is likely to occur. More prevalence studies based on hunt dogs may indicate whether or not hunt dogs regularly carry a greater species distribution of *Campylobacter* spp. than other dog populations.

8.2.7.3 Bacterial Enumeration

To date, no studies have fully explained whether or not all dogs carry *C. upsaliensis*. Dogs may carry variable amounts of this bacterium, some of which may be undetectable. However, some dogs may not carry *C. upsaliensis* at all.

Neither of these situations are fully understood. If some dogs never carry *C. upsaliensis*, then what are the reasons behind this. Is it a lack of exposure, perhaps during a certain age range, to any sources of *C. upsaliensis*, or is it some level of immunity. To answer these questions, firstly it needs to be established if the apparent *C. upsaliensis* negative dogs, are completely clear of the bacterium. Enumeration studies may be the most accurate way of determining this. The spiral plating described within this thesis was unable to detect counts below 200 CFU/ml faeces, and had other limitations, so a more sensitive method, such as real time PCR, may be able to detect smaller quantities of *Campylobacter* spp. Real time PCR has been successfully used in another study and has been optimised to detect several *Campylobacter* spp. (Chaban *et al*., 2009). However the spiral plating method in this present study, and real time PCR in other studies appear to have a similar upper detection limit where real time PCR is able to detect up to 10^5 or 10^6 copies of target DNA (Chaban *et al*., 2009), and the upper limit of spiral plating can be increased via further dilutions.

A culture based enumeration technique may be needed in conjunction with real time PCR, in case viable counts are required, or if PCR inhibitors are present. If spiral plating was repeated, the number of colonies chosen for PCR confirmation could be adjusted to correspond to the total number of colonies on the plate, as five may not be sufficient to give a truly representational selection of the colonies observed. Negative controls could also be dispensed onto the plates, and different types of media should ideally be used.

Currently, real time PCR is probably the most accurate way of determining if all dogs carry *C. upsaliensis* (but in variable amounts which may not always be detected by culture) or not. However it would be difficult to fully establish if a dog was completely free of *C. upsaliensis* because even if the enumeration technique was highly sensitive, *C. upsaliensis* may be present, but not shed in every faecal sample.

8.2.7.4 Effects of Stress and Campylobacter spp. *in Dogs*

Future work regarding *Campylobacter* spp. in kennelled dogs may benefit from measuring stress levels in dogs simultaneously with quantifying *Campylobacter* spp. shedding. Urine samples could be collected with little variation in the study design described in chapter four, as a large number of dogs only urinate once they have been moved to an outside pen. Faecal samples could be collected at the same time, according to the methods in this study. The bacteria could be quantified, whilst the CC ratios could be calculated and compared to the bacterial load. Dogs not exposed to stress, such as household dogs sampled by the owner, or ideally before admission to the kennel, could serve as controls. In theory this would expose any relationship between stress and shedding of *Campylobacter* spp. in dogs.

8.2.7.5 Investigating Zoonotic Relationship Between Dogs and Humans

8.2.7.5.1 Multilocus Sequence Typing

Currently the *C. jejuni*/*C. coli* MLST database has limited isolates originating from dogs, and the *C. upsaliensis* MLST database contains significantly fewer isolates and profiles than the *C. jejuni*/*C. coli* MLST database. Additions to both

these databases, particularly the *C. upsaliensis* database, may suggest possible sources of infection for both dogs and humans, and clarify whether transmission occurs between dogs, i.e. do dogs in similar geographical locations/kennels carry similar strains, as was suggested by two hunt dogs in the present study.

Further work on MLST could also entail investigating whether or not different cultivation methods result in different sequence types from within the same sample, as has been suggested previously (personal communication, Williams. L, Bristol University). Basing results on just one sequence type might be misleading. For this reason it would be beneficial to select more than one colony, from more than one isolation method per dog/sample, since one dog may carry more than one sequence type.

8.2.7.5.2 Genome Sequencing

Sequencing genomes is one of the most accurate typing methods currently available. Further work will be carried out on *C. upsaliensis* 52A, specifically genome annotation, and identification of any further plasmids. Genome sequencing different *C. upsaliensis* strains may be more accurate than MLST for example. However MLST is currently quicker, easier and information obtained through MLST may indicate which strains should be sequenced. Comparisons of human and canine *C. upsaliensis* in particular may help to explain whether or not dogs are the primary source of *C. upsaliensis* infection for humans, whether dogs and humans share similar sources of *C. upsaliensis*, or whether canine and human derived strains are unrelated. This method may also help to determine if

C. upsaliensis strains originating from symptomatic humans are more likely to contain a plasmid than strains that are asymptomatic, or strains found in dogs.

8.2.7.5.3 Sampling Owner and Dog

Ultimately however, further studies are needed where owner and dog from the same household at the same time, are sampled for *Campylobacter* spp., specifically *C. upsaliensis*, to determine if this species can be transmitted from dog to human or vice versa. The main limitation for these studies to date are that they rely on human presentation of disease. As mentioned previously, symptoms may be milder with *C. upsaliensis* infection, and it may be under reported. Sampling owners and pet dogs, regardless of their disease status may indicate whether or not transmission is occurring and simultaneously uncover un-reported *C. upsaliensis* illness in humans. If more cases of *C. upsaliensis* infection in humans are found, the relationship between this organism and disease in humans can also be explored.

8.2.7.6 Improving Techniques

In any future studies, additional cultivation methods could be used to improve the isolation of *Campylobacter* spp. Filtration with a blood based media should be used as an additional method to ensure that no *Campylobacter* spp. are overlooked. Some studies have found filtration onto blood agar, and additionally direct plating onto mCCDA and CAT media to be the most successful method for isolating *Campylobacter* spp. (Acke *et al*., 2006; Acke *et al*., 2009). However this method may result in colonies swarming so should not be used alone, particularly for molecular based work.

Appendix 1.
Figure 1.1. Questionnaire for Vet-visiting cross sectional study, designed by Jenny Stavisky.

DOGS IN THE COMMUNITY SURVEY

The University of Liverpool is conducting a study into the causes of digestive disorders in dogs. In order to do this, we are collecting faecal (poo) samples from healthy and sick dogs throughout the United Kingdom. Your vet has agreed to help us by asking you and your dog to take part.

This involves a member of staff at your veterinary surgery taking a few details from you, and a faecal sample from your dog when he or she toilets. Samples will be returned anonymously to us for analysis, and we will not be able to identify individual participants. We ask for your postcode to enable us to compare results from different regions. Your vet will give you a leaflet explaining more about the study.

Many thanks for taking part.

Name of dog ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Postcode ☐☐☐☐☐☐☐

I consent to a faecal sample being taken from my dog and used for the University of Liverpool's study.

Signed _____ Date ☐☐ / ☐☐ / ☐☐

dd/mm/yy

NOW PLEASE TURN OVER THE PAGE

Surgery no. ☐☐ Dog no. ☐☐☐

For office use

SPONSORED BY SCHERING PLOUGH AND DEFRA

8840

TO BE COMPLETED BY THE DOG'S OWNER, TOGETHER WITH THEIR VETERINARY SURGEON OR VETERINARY NURSE.

How to complete the form:
Please complete the questionnaire using blue or black ink. Indicate your choice or choices for each question by drawing a cross in the box, not a tick: ☒
If you make a mistake or decide to change your answer, please fill in the first box completely and put a clear cross in the new box.

1. Approximately how old is your dog? ☐☐ years and ☐☐ months ☐ Don't know

2. Is your dog a particular breed? ☐ Purebred ☐ Mixed breed ☐ Don't know

3. If known, please state breed. ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

4. What sex is your dog? ☐ Male ☐ Female ☐ Don't know

5. Is your dog neutered? ☐ Yes ☐ No ☐ Don't know

6. Has your dog had a routine vaccination **(not rabies or bordetella)** within the last: ☐ Year ☐ 2 Years ☐ >2 Years ☐ Never/ Don't know

7. What is the reason for today's visit to the vet?

8. Has your dog had any diarrhoea in the last: ☐ Month ☐ Week ☐ No recent diarrhoea

9. Has your dog had any vomiting in the last: ☐ Month ☐ Week ☐ No recent vomiting

10. Has your dog had any antibiotics in the last: ☐ Month ☐ Week ☐ No recent antibiotics

11. If your dog has had antibiotics within the last month, please state which (if known).

12. Any other comments or information:

THANK YOU!

If you have any further queries, comments or problems, please contact Jenny Stavisky at: Dept of Clinical Veterinary Science, Leahurst, Chester High Road, Wirral CH64 7TE. Tel 0151 794 6027; email dogstudy@liverpool.ac.uk

Table 1.1: Univariable analysis of dog characteristics/variables and *C. upsaliensis* status in vet-visiting dogs.

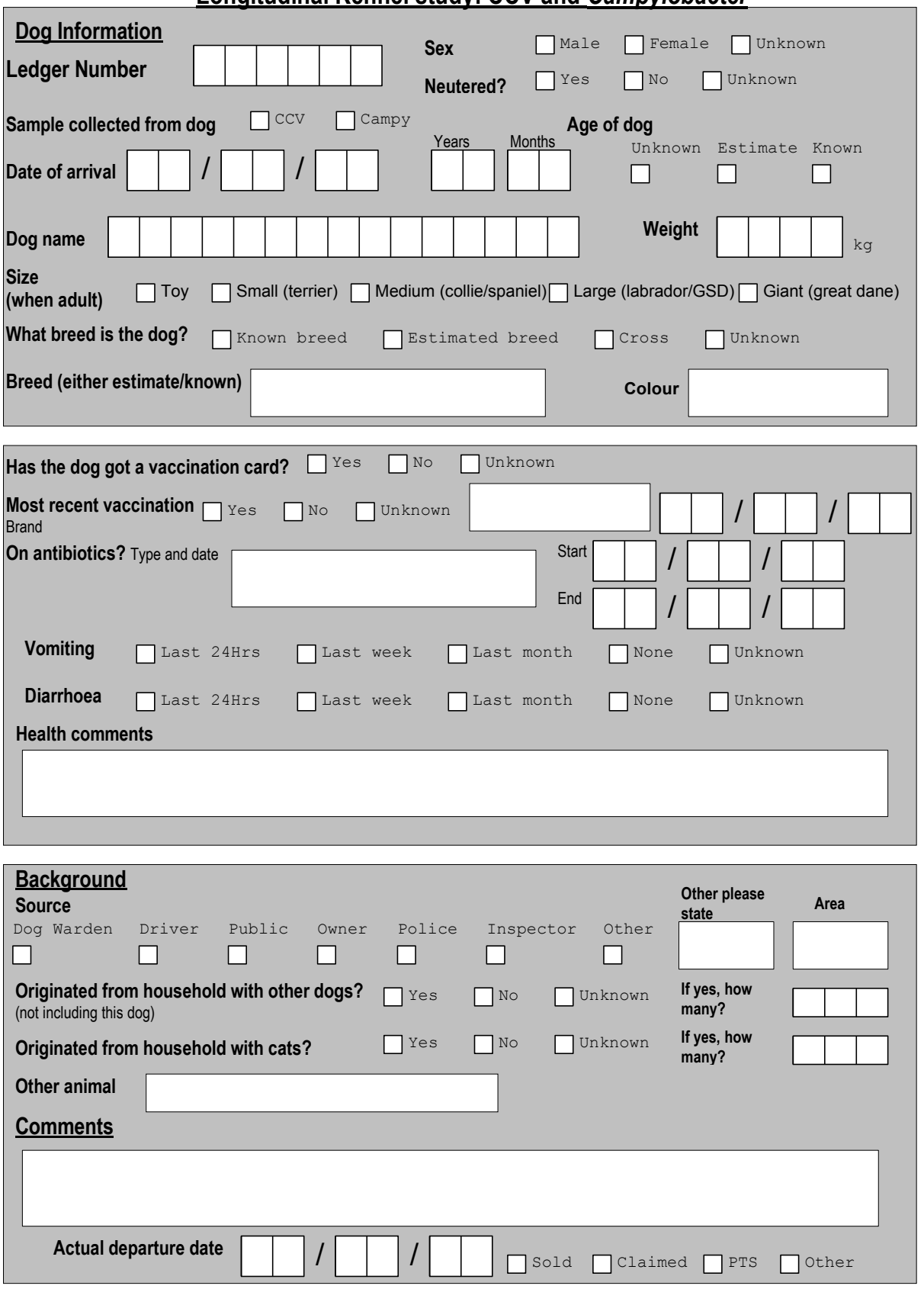
Variable		+	-	Coef	S.E.	Odds ratio	95.0% C.I. Lower	Upper	*P*-value
Month samples were processed	August	13	36			1			0.29
	September	29	43	0.62	0.40	1.868	0.84	4.11	0.12
	October	27	34	0.78	0.41	2.199	0.97	4.94	0.05
	November	13	26	0.32	0.46	1.385	0.55	3.47	0.48
	December	12	14	0.86	0.50	2.374	0.87	6.44	0.09
Days in the post		-	-	0.05	0.08	1.05	0.89	1.25	0.50
Age	≤1 year	16	16			1			
	>1 year	78	136	-0.55	0.38	0.57	0.27	1.21	0.14
	≤ 18 months	25	21			1			
	> 18 months	69	129	-0.800	0.331	0.449	0.235	0.860	0.016
	≤2 years	31	22			1			
	>2 years	63	130	-1.06	0.31	0.34	0.18	0.64	<0.001
	0-24 months	31	22			1			0.01
	25-48 months	16	28	-0.90	0.41	0.40	0.17	0.92	0.03
	49-72 months	17	24	-0.68	0.42	0.50	0.22	1.15	0.10
	73-96 months	5	23	-1.96	0.56	0.15	0.05	0.46	<0.001
	97-120 months	8	18	-1.15	0.50	0.31	0.11	0.85	0.02
	121-144 months	8	14	-0.90	0.52	0.40	0.14	1.13	0.08
	≥ 145 months	9	23	-1.28	0.48	0.27	0.10	0.71	<0.01
	Age in months	-	-	-0.007	0.003	0.99	0.98	0.99	<0.01
Sex	Male	47	64			1			
	Female	47	89	-0.33	0.26	0.71	0.42	1.20	0.21
Neutered	No	31	43			1			
	Yes	63	106	-0.19	0.28	0.82	0.47	1.43	0.49
Breed	Gundog	29	45			1			0.31
	Hound	4	4	0.43	0.74	1.552	0.36	6.69	0.55
	Unrecognised	3	15	-1.17	0.67	0.310	0.08	1.16	0.08
	Terrier	10	7	0.79	0.54	2.217	0.75	6.48	0.14
	Utility	3	9	-0.65	0.70	0.517	0.12	2.07	0.35
	Working	8	12	0.03	0.51	1.034	0.37	2.83	0.94
	Pastoral	8	17	-0.31	0.49	0.730	0.27	1.90	0.52
	Toy	5	5	0.43	0.67	1.552	0.41	5.83	0.51
	Known cross	9	14	-0.002	0.48	0.998	0.38	2.60	0.99
Size	Toy	3	3			1			0.16
	Small	15	28	-0.62	0.87	0.53	0.09	2.98	0.47
	Medium	29	31	0.06	0.85	0.93	0.17	5.01	0.93
	Large	27	62	-0.83	0.84	0.43	0.08	2.29	0.32
	Giant	5	4	0.22	1.05	1.25	0.15	9.91	0.83
	Small	18	31			1			0.14
	Medium	29	31	0.47	0.393	1.61	0.74	3.48	0.22
	Large	32	66	-0.18	0.366	0.83	0.40	1.71	0.62

Appendix

Vaccine Type	No Vaccine	3 11			1			0.74
	Durammune	15 25	0.78	0.729	2.20	0.52	9.17	0.27
	NOBIVAC	26 44	0.77	0.697	2.16	0.55	8.48	0.26
	Other	4 9	0.48	0.886	1.63	0.28	9.25	0.58
	Procyon	5 5	1.29	0.908	3.66	0.61	21.73	0.15
	Vanguard	12 13	1.21	0.765	3.38	0.75	15.14	0.11
	Unknown	24 39	0.81	0.701	2.25	0.57	8.91	0.24
Vaccine	No	3 11			1			
	Yes	86 135	0.84	0.66	2.33	0.63	8.61	0.20
Antibiotic Type	No Antibiotics	76 109			1			0.50
	Amoxicillin	10 22	-0.42	0.41	0.65	0.29	1.45	0.29
	Other	6 16	-0.62	0.50	0.53	0.20	1.43	0.21
	Unknown	0 2	-20.84	28420.7	0	0		0.99
Antibiotics	Recent	16 40			1			
	None	76 109	0.55	0.33	1.74	0.91	3.33	0.09
Diarrhoea	Recent	23 39			1			
	None	68 111	0.03	0.30	1.03	0.57	1.88	0.90
Vomiting	Recent	15 23			1			
	None	76 126	-0.07	0.36	0.92	0.45	1.88	0.82
Number of other dogs in the same house	None	47 96			1			0.38
	One	22 32	0.34	0.32	1.40	0.73	2.67	0.30
	Two	7 5	1.05	0.61	2.86	0.86	9.48	0.08
	Three	7 8	0.58	0.54	1.78	0.61	5.22	0.28
	≥ Four	5 9	0.12	0.58	1.13	0.36	3.57	0.82
	Number of dogs	- -	-0.024	0.038	0.977	0.906	1.053	0.538
Lives with another dog	No	47 96			1			
	Yes	41 54	0.43	0.27	1.55	0.90	2.64	0.10
Status of other dog(s) in the same household	No dog(s)	47 96			1			<0.01
	Positive	15 5	1.81	0.54	6.12	2.10	17.87	<0.01
	Only negative	4 20	-0.89	0.57	0.40	0.13	1.26	0.12
	Unknown	22 29	0.43	0.33	1.55	0.80	2.98	0.19
Lives with Cat (s)	No	60 110			1			
	Yes	27 39	0.23	0.29	1.26	0.70	2.27	0.42

The first category was used as the reference category for each variable

Appendix 2.
Figure 2. 1 Questionnaire used for Kennel studies.



Longitudinal Kennel study: CCV and *Campylobacter*

Dog Information

Ledger Number ☐☐☐☐☐☐

Sex ☐ Male ☐ Female ☐ Unknown

Neutered? ☐ Yes ☐ No ☐ Unknown

Sample collected from dog ☐ CCV ☐ Campy

Date of arrival ☐☐ / ☐☐ / ☐☐

Age of dog Years ☐☐ Months ☐☐ Unknown ☐ Estimate ☐ Known ☐

Dog name ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

Weight ☐☐☐☐ kg

Size (when adult) ☐ Toy ☐ Small (terrier) ☐ Medium (collie/spaniel) ☐ Large (labrador/GSD) ☐ Giant (great dane)

What breed is the dog? ☐ Known breed ☐ Estimated breed ☐ Cross ☐ Unknown

Breed (either estimate/known) _____ **Colour** _____

Has the dog got a vaccination card? ☐ Yes ☐ No ☐ Unknown

Most recent vaccination Brand ☐ Yes ☐ No ☐ Unknown _____ ☐☐ / ☐☐ / ☐☐

On antibiotics? Type and date _____ Start ☐☐ / ☐☐ / ☐☐ End ☐☐ / ☐☐ / ☐☐

Vomiting ☐ Last 24Hrs ☐ Last week ☐ Last month ☐ None ☐ Unknown

Diarrhoea ☐ Last 24Hrs ☐ Last week ☐ Last month ☐ None ☐ Unknown

Health comments

_____

Background

Source

Dog Warden ☐ Driver ☐ Public ☐ Owner ☐ Police ☐ Inspector ☐ Other ☐

Other please state _____ **Area** _____

Originated from household with other dogs? (not including this dog) ☐ Yes ☐ No ☐ Unknown **If yes, how many?** ☐☐☐

Originated from household with cats? ☐ Yes ☐ No ☐ Unknown **If yes, how many?** ☐☐☐

Other animal _____

Comments

_____

Actual departure date ☐☐ / ☐☐ / ☐☐ ☐ Sold ☐ Claimed ☐ PTS ☐ Other

2.1 Specification of Sources

The sources of the dogs from the rescue kennel were also recorded into the following groups; ‘dog warden’ who actively searched for and collected stray dogs; a ‘driver’ who would usually be telephoned about a stray dog by a member of the public and would come and collect it; a dog brought in by the ‘public’ means that a member of the public actually brought the dog down to the kennel themselves or possibly phoned the driver who collected it, and this might be a stray dog on their street, or a deception in that it is their own dog; an ‘owner’ might bring their dog to the kennel because they have decided to give the dog up for some reason, this group is the least likely to consist of stay dogs; an ‘inspector’ is an RSPCA officer who potentially has signed the dog over from the owner or they have found it as a stray; and ‘police’ means that either the police have confiscated the dog, found it as a stray, or perhaps it's been abandoned after the owner has been arrested. The source that was recorded, was to some extent the kennel staffs individual choice, for example, if a member of the public rang the kennel and a driver went out and collected the dog, this could have been recorded as public or driver.

Figure 2.2. Layout of kennel 1 (Illustrated by Jenny Stavisky).

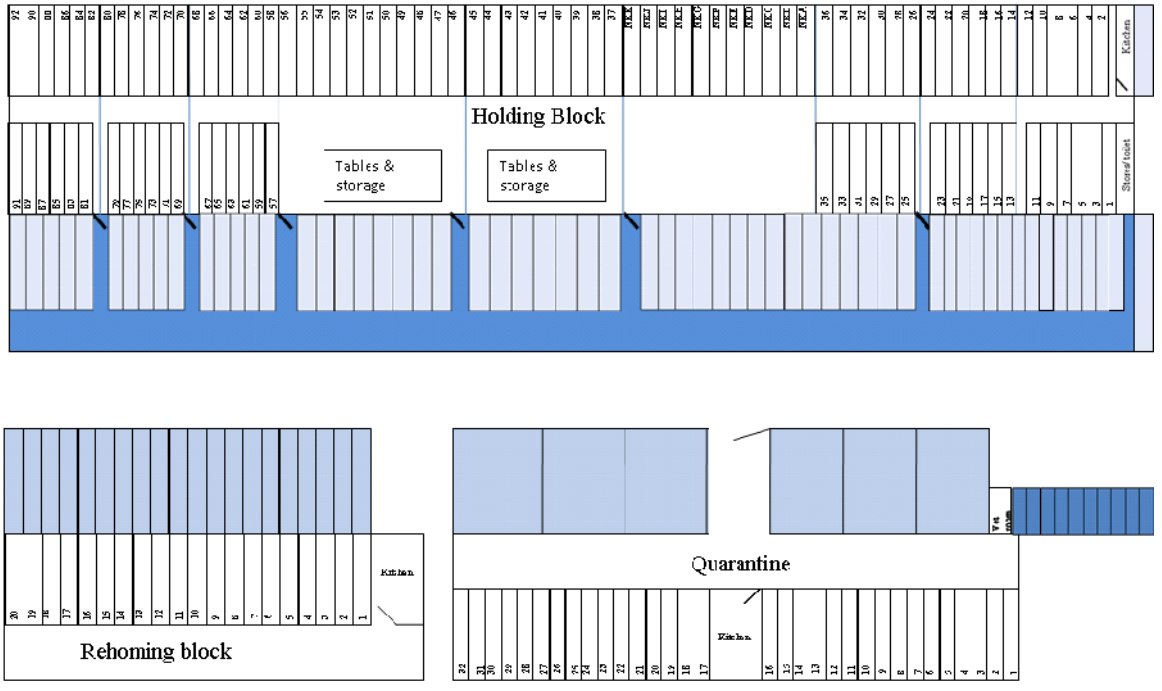


Figure 2.3. Layout of kennel 2 (adapted from an illustration by Jenny Stavisky).

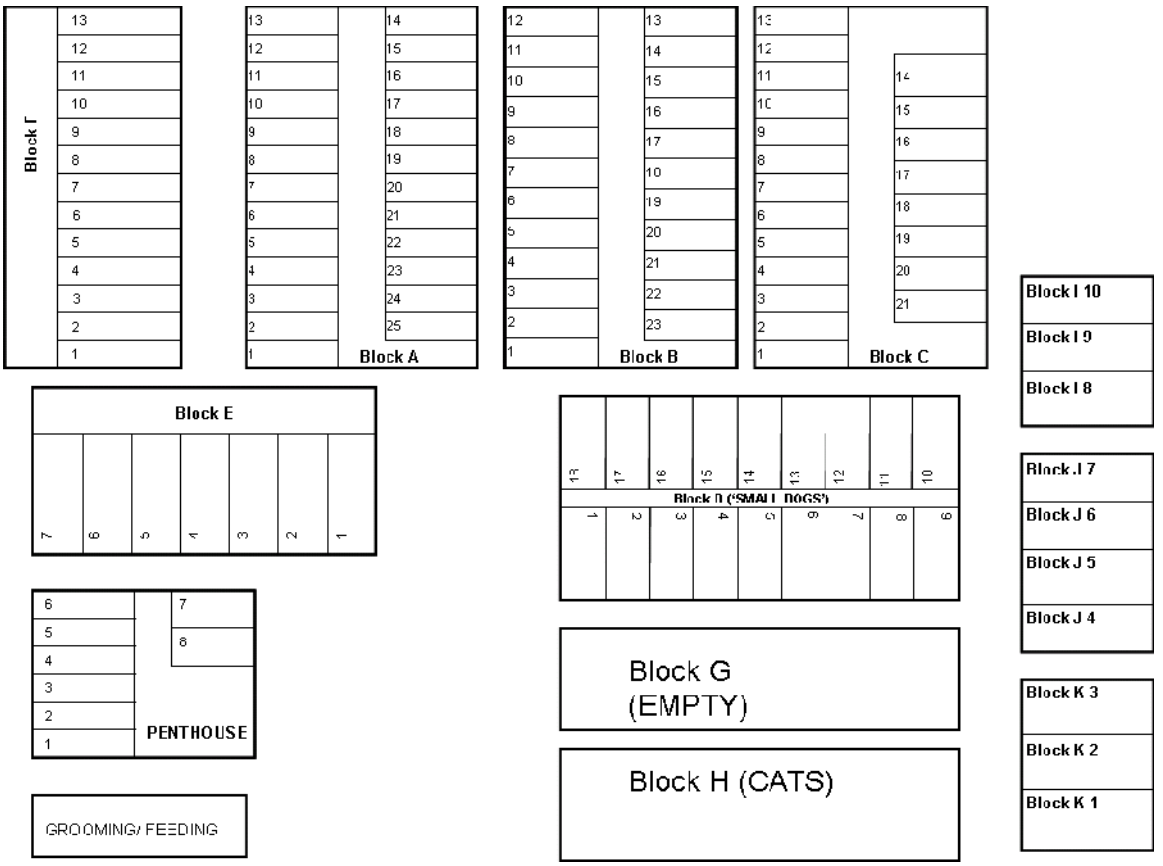


Figure 2.4. Owner consent form for dogs in kennel 2 (boarding).

UNIVERSITY OF LIVERPOOL

Consent form

Dogs in the Community

Leahurst (University of Liverpool) is conducting a study into causes of digestive disorders in dogs, and the kennel has kindly agreed to help us by asking for you and your dog to take part.

The study involves a member of staff from Leahurst collecting a faecal sample left in the kennel from your dog. We would also like to record a few details which will include, the age, sex and breed of your dog, along with the first half of your post code, which allows us to compare results on a regional basis. Samples will be made anonymous before analysis. **Therefore it will not be possible to give results for individual dogs.**

I consent to a faecal sample, and the above details, being taken from my dog and used for the University of Liverpool's study.

Signed Signed

1st half of postal code 1st half of post code

Figure 2.5 Un-rooted neighbour-joining phylogenetic tree with bootstrap values of the partial *groEL* gene for *C. upsaliensis* culture isolates from both kennel 1 and kennel 2, based on 440bp (1000 replicates). Direct= direct plating, filter= filtration, spiral= spiral plating.

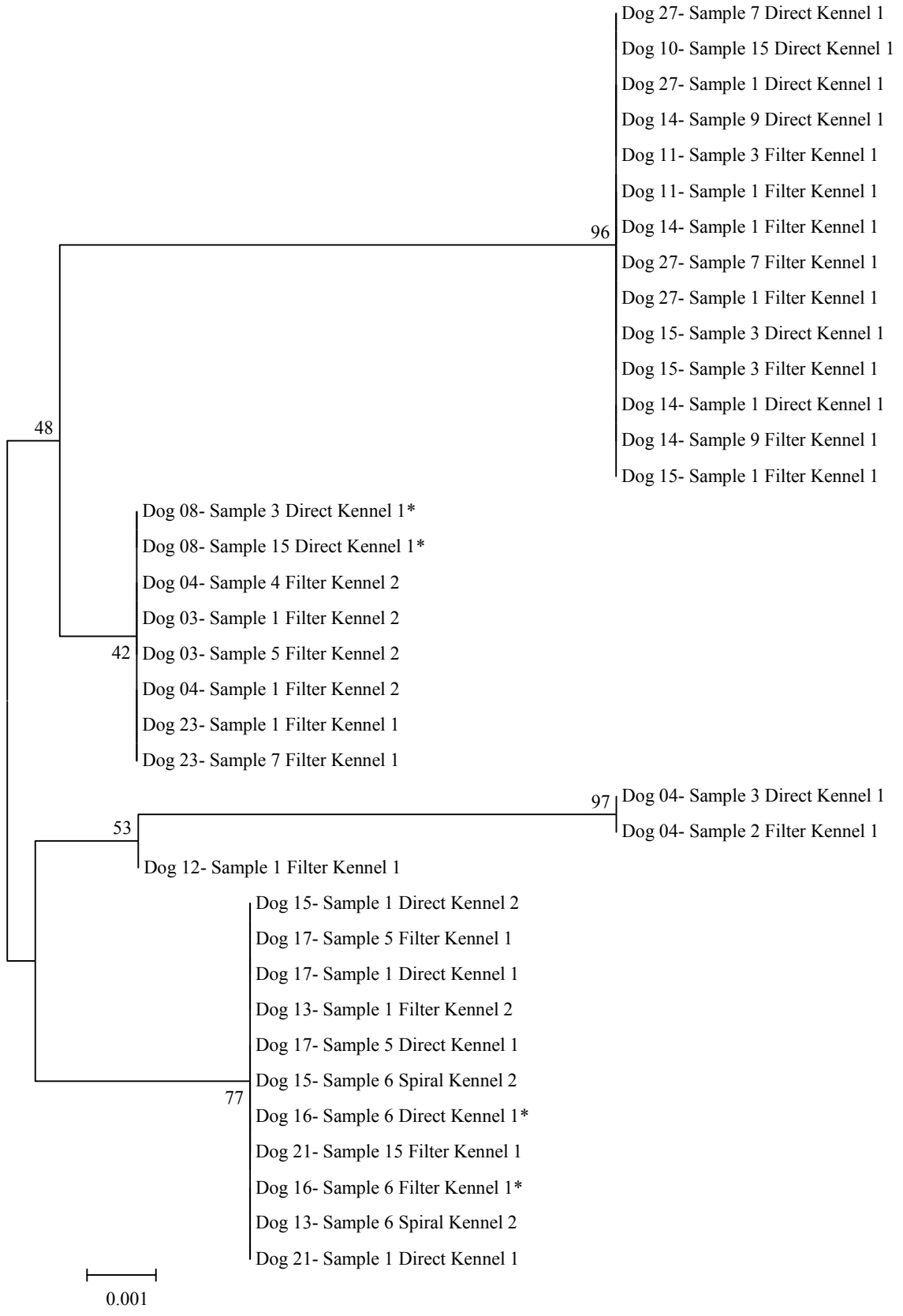
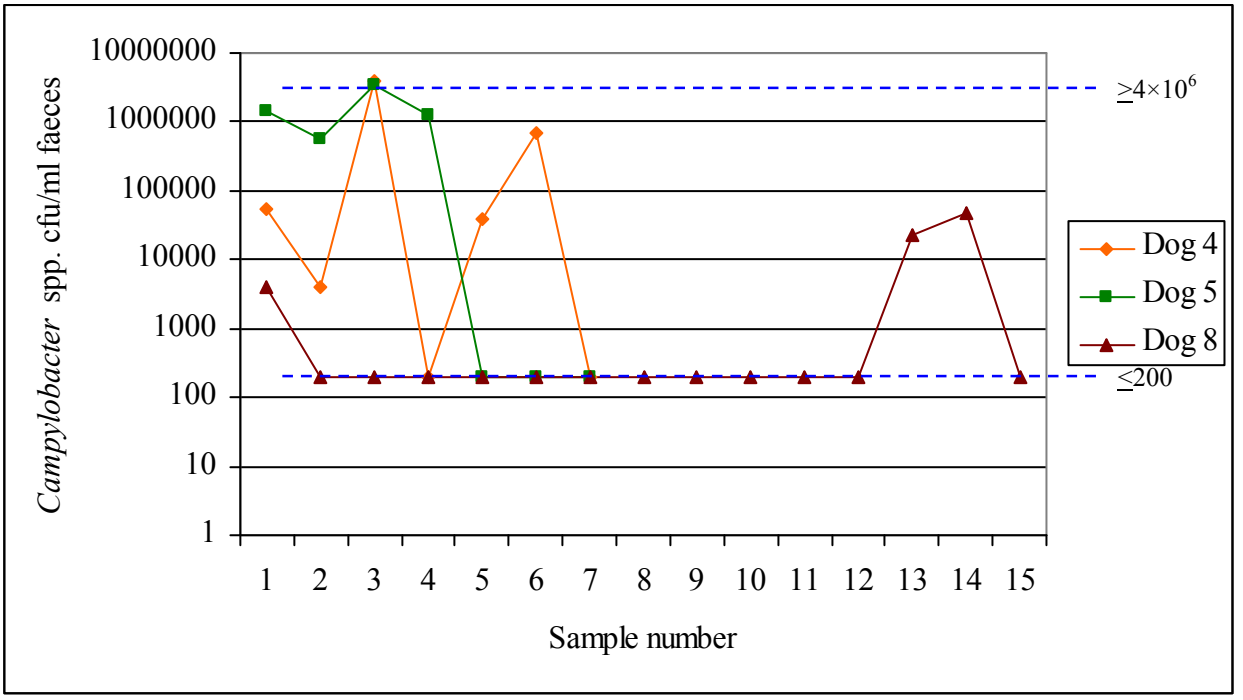


Table 2.1. Univariable analysis allowing for clustering of dog characteristics/variables and *Campylobacter* spp. status (positive or negative by any isolation method) for both kennels 1 (rescue) and 2 (boarding).

Variable	+	-	%SCL Coef	%SCL SE	Coef	SE	OR	95% CI Lower	95% CI Upper	*P*-value
Kennel										
Kennel 1	165	103	3.92	0.50			1			
Kennel 2	48	83			1.36	0.82	3.92	0.78	19.64	0.09
Dog Moved										
No	207	183	4.06	0.51			1			
Yes	6	3			-0.09	1.37	0.90	0.06	13.34	0.94
Dog Moved In last 48h										
No	201	177	4.05	0.51			1			
Yes	12	9			-0.42	0.91	0.65	0.10	3.93	0.64
Sex										
Male	82	99	4.16	0.53			1			
Female	125	81			-1.13	0.77	0.32	0.07	1.46	0.14
Day in Kennel	213	186	4.06	0.51	0.001	0.05	1.00	0.89	1.11	0.97
Remaining	193	176	4.07	0.49						
Second	20	30			1.46	0.92	4.30	0.70	26.4	0.11
Weight	152	67	3.32	0.60	0.009	0.02	1.00	0.95	1.06	0.74
Month Sampled										
May	111	79	3.95	0.50			1			0.14
June	54	24			-0.29	0.54	0.74	0.25	2.17	0.58
September	47	80			1.07	0.90	2.93	0.49	17.37	0.23
October	1	3			3.38	1.62	29.6	1.21	720.29	0.03
Age										
Months	173	160	3.78	0.47	0.01	0.009	1.01	0.99	1.03	0.11
Age centred Squared	173	160	3.64	0.47	0.0006	0.0003	1.00	1.0001	1.0011	0.01
Breed										
Gundog	19	31	4.42	0.58			1			0.06
Hound	17	5			0.34	1.16	1.41	0.14	13.86	0.76
Unrecognised	81	33			1.91	1.04	6.80	0.88	52.29	0.06
Terrier	57	80			1.11	1.02	3.05	0.41	22.75	0.27
Working	4	7			-0.93	1.23	0.39	0.03	4.39	0.44

Pastoral	19	19			1.81	1.26	6.14	0.51-73.83	0.15
Toy	10	11			1.20	2.06	3.35	0.05-190.5	0.55
Kennel Cough									
No	167	172	4.23	0.51			1		
Yes	46	14			1.25	0.61	3.49	1.05- 11.55	0.04
Size									
Small	78	75	3.93	0.45			1		0.07
Medium	105	74			-0.31	0.62	0.73	0.21- 2.50	0.61
Large	30	37			1.37	0.79	3.93	0.82- 18.78	0.08
Blood in Faeces									
No	210	176	4.07	0.51			1		
Yes	3	10			-0.99	1.68	0.36	0.01- 9.94	0.55
Vomiting									
No	206	176	4.27	0.53			1		
Yes	2	10			-2.92	0.98	0.05	0.007- 0.37	0.003
Diarrhoea									
No	173	169	4.06	0.50			1		
Yes	40	17			0.97	0.69	2.65	0.67-10.43	0.16
Block									
Stray	138	73	4.22	0.54			1		0.16
Boarders	48	83			-0.82	0.63	0.43	0.12-1.50	0.18
Quarantine	22	9			1.07	0.84	2.92	0.55-15.28	0.20
Rehome	5	21			0.18	0.74	1.20	0.28-5.13	0.80

Figure 2.6. Counts of *Campylobacter* spp. colonies (CFU/ml faeces) in dogs 4, 5 and 8 from kennel 1, unconfirmed by PCR.*



* Dog 4 had severe diarrhoea in samples 1, 3 and 6.

Dog 5 had diarrhoea for sample 1, and this sample also had *C. jejuni* isolated from it.

Dog 8 was the only dog in this figure not to be euthanized after its seventh sample.

Points on dashed line indicate either a count of 200 CFU/ml (i.e. 1 colony), or no colonies observed i.e. counts below lower detection limit.

Figure 2.7. Kennel 2 *C. upsaliensis* colonies (CFU/ml faeces) in dogs 3 and 4 with PCR confirmation. Samples 3, 4 and 5 for dog 3 were diarrhoeic.

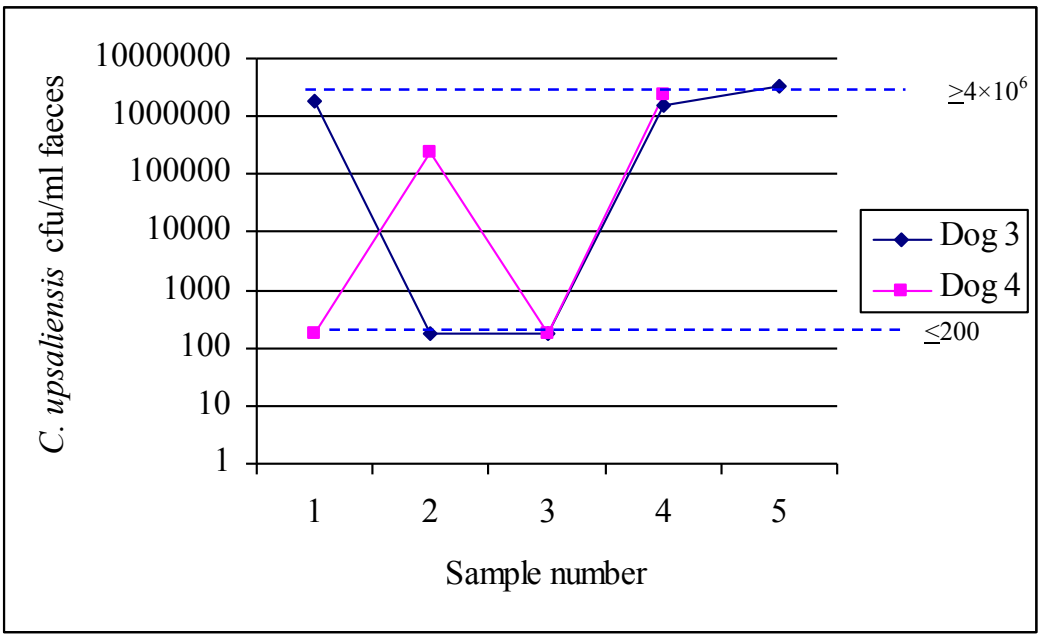


Figure 2.8. Kennel 2. Counts of *C. upsaliensis* colonies (CFU/ml faeces) in dogs 6 and 10 with PCR confirmation. Dog 6 was identified as having mucus in it's 5th sample.

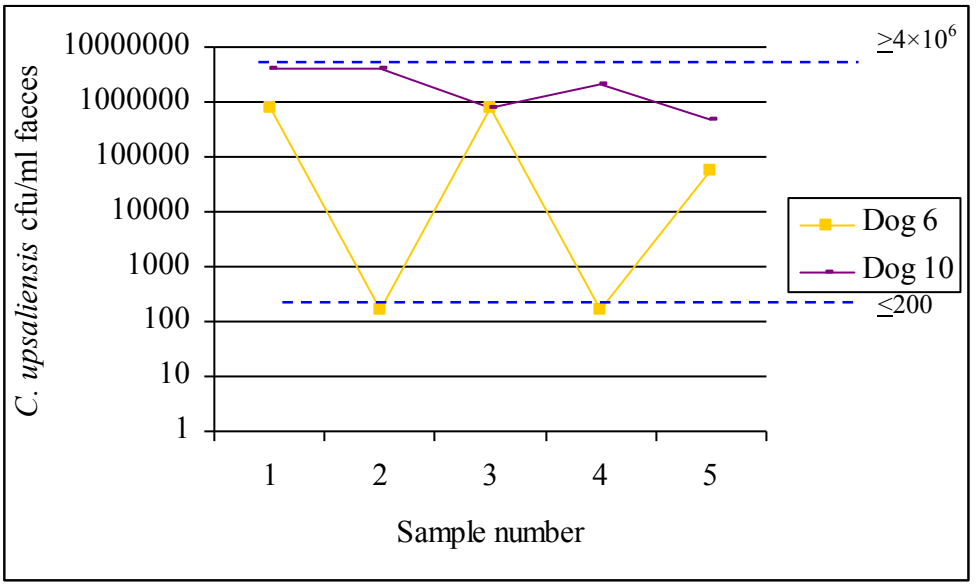


Figure 2.9. Kennel 2. Counts of *C. upsaliensis* colonies (CFU/ml faeces) in dogs 7, 13 and 17 with PCR confirmation.

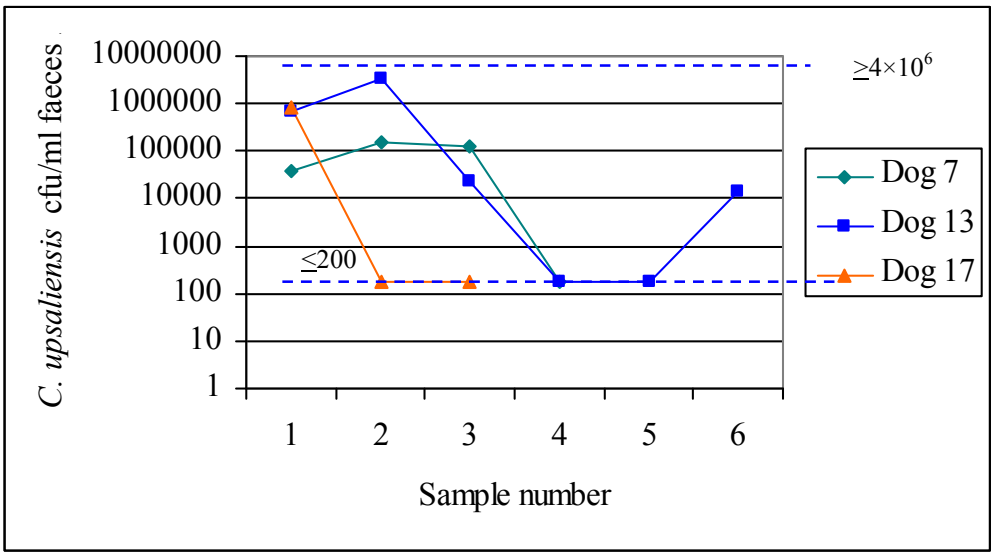


Figure 2.10. Kennel 2. Counts of *C. upsaliensis* colonies (CFU/ml faeces) in dogs 14 and 15, with PCR confirmation. Sample 6 for dog 14 was diarrhoeic.

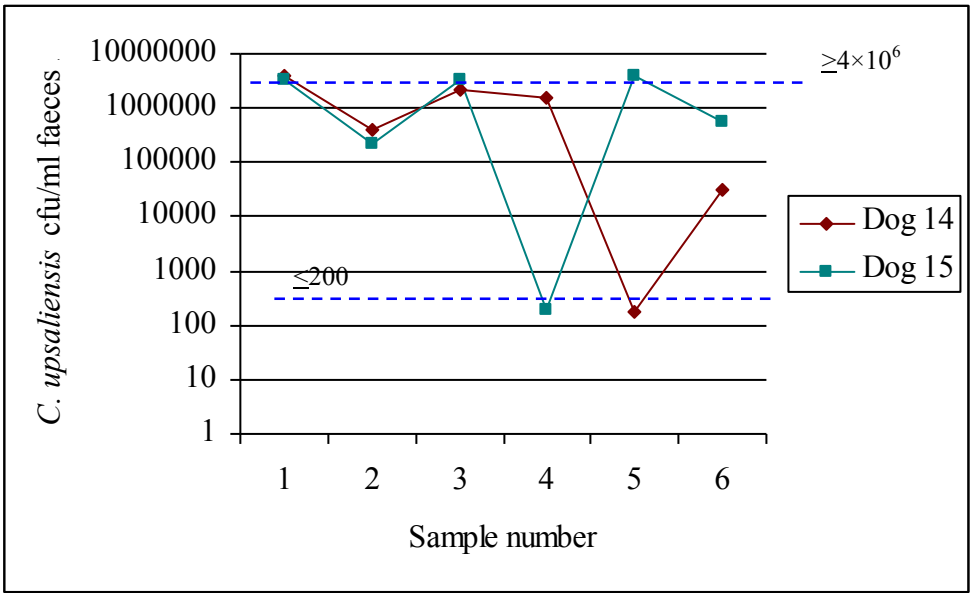
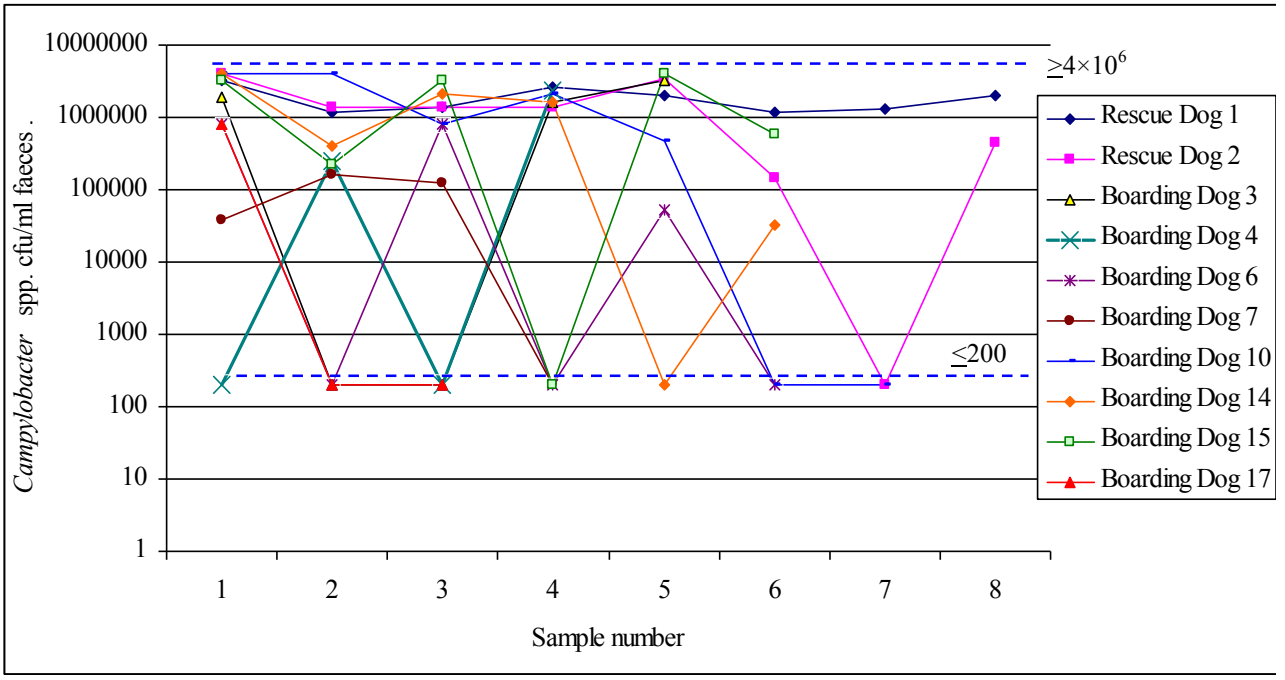


Figure 2.11. Kennels 1&2: *C. upsaliensis* counts (CFU/ml faeces) for dogs from both kennels with PCR confirmation over the first eight samples. Dogs 9 and 12 from kennel 2 not included due to lack of PCR confirmation and shedding after eight days.



Appendix 3.

Table 3.1. Univariable analysis of dog variables and *C. upsaliensis* status from kennels A & B.

Variable		+	-	Coef	S.E.	OR	95.0% C.I. Lower	Upper	*P*-value
Kennel	Boarding	37	15			1			
	Rescue	8	20	-1.81	0.51	0.16	0.05	0.44	<0.001
Length of stay	Days	44	32	0.008	0.004	1.008	1.00	1.01	0.058
	1-7 days	4	11			1			
	≥8 days	40	21	1.65	0.64	5.23	1.48	18.47	0.01
Diarrhoea /Soft stool	No	36	28			1			
	Yes	9	7	0.00	0.56	1.00	0.33	3.01	1.00
Diet	Standard	34	31			1			
	Other	11	4	0.91	0.63	2.50	0.72	8.69	0.14
Blood in faeces	No	43	32			1			
	Yes	2	3	-0.70	0.94	0.49	0.07	3.14	0.45
Recent antibiotics	No	44	29			1			
	Yes	1	6	-2.20	1.10	0.11	0.01	0.96	0.04
Vomiting	No	44	32			1			
	Yes	1	3	-1.41	1.17	0.24	0.02	2.43	0.22

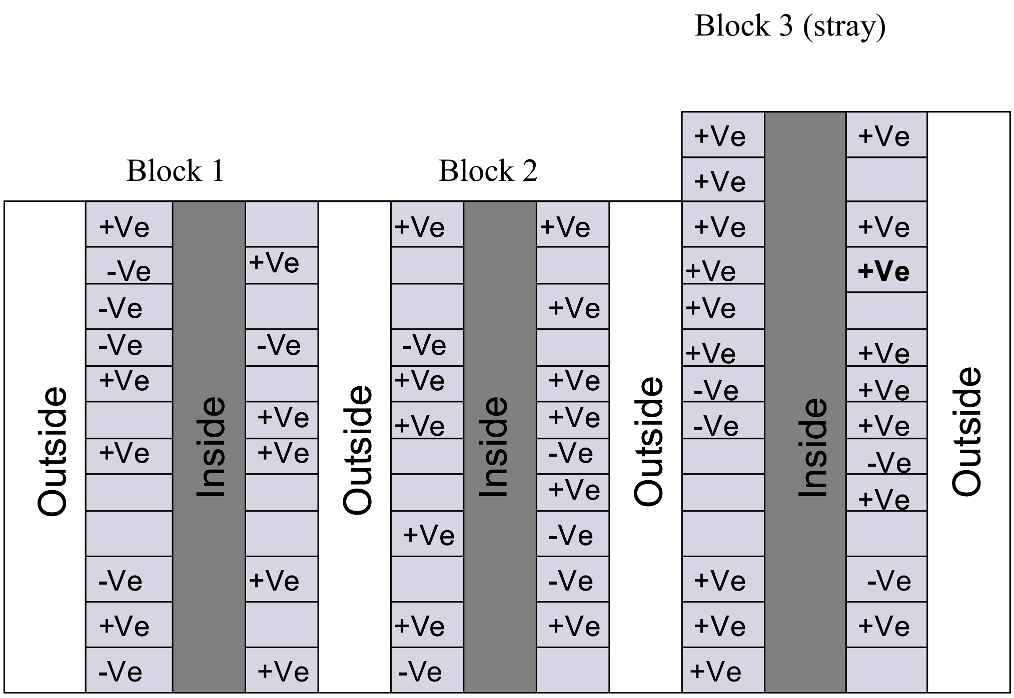
Recent= Within the past month
Standard diet= Fed standard kennel food
Other diet= Fed puppy, special or owners chosen diet
Coef= Coefficient, S.E= Standard error and OR= Odds ratio.

Table 3.2. Univariable analysis of *Campylobacter* spp. status in dogs from all six kennels.

Kennel	+	-	Coef	S.E.	OR	95.0% C.I. Lower Upper	*P*-value
Kennel A	37	15			1		<0.001
Kennel B	9	20	-1.7	0.5	0.18	0.06-0.49	<0.001
Kennel C	15	34	-1.72	0.43	0.17	0.07-0.42	<0.001
Kennel D	10	11	-0.99	0.53	0.36	0.13-1.04	0.06
Kennel E	1	19	-3.84	1.07	0.02	0.003-0.17	<0.001
Kennel F	0	20	-22.1	8987.4	0	0	0.99

Coef= Coefficient, S.E= Standard error and OR= Odds ratio.

Figure 3.1. Layout of boarding kennel A with locations and *Campylobacter* spp. status of dogs shown.



+Ve= *C. upsaliensis,* **+Ve**= *C. jejuni*, and –Ve= negative for *Campylobacter* spp. All kennels had indoor and outdoor compartments.

Figure 3.2. Layout of rescue kennel B (not to scale). Octagonal shaped kennels reduce the number of other dogs viewable by one particular dog, thus reducing noise and stress. All kennels had indoor and outdoor compartments which were all under cover.

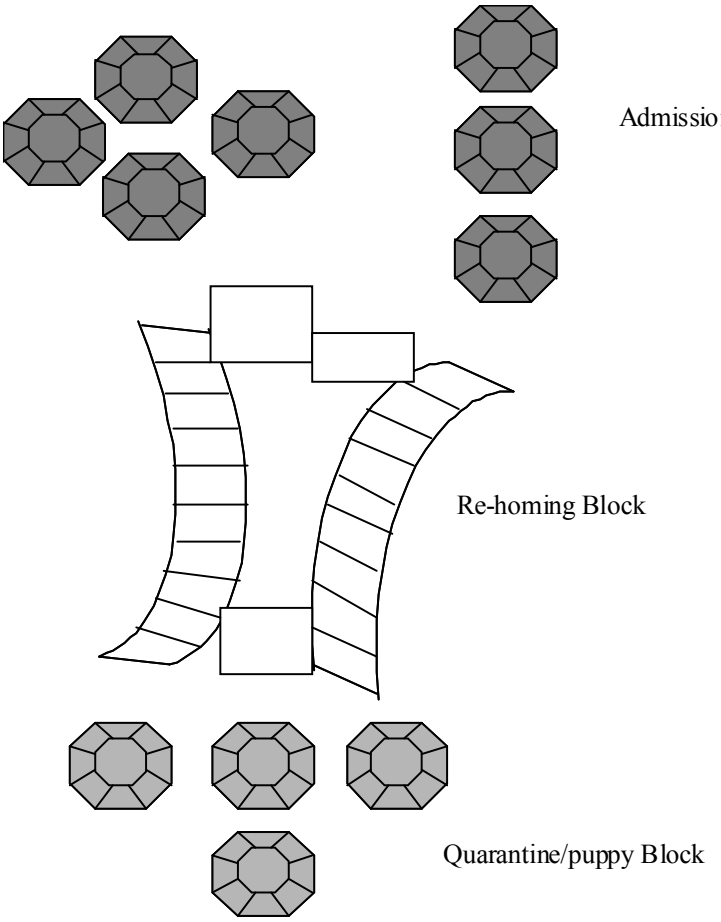
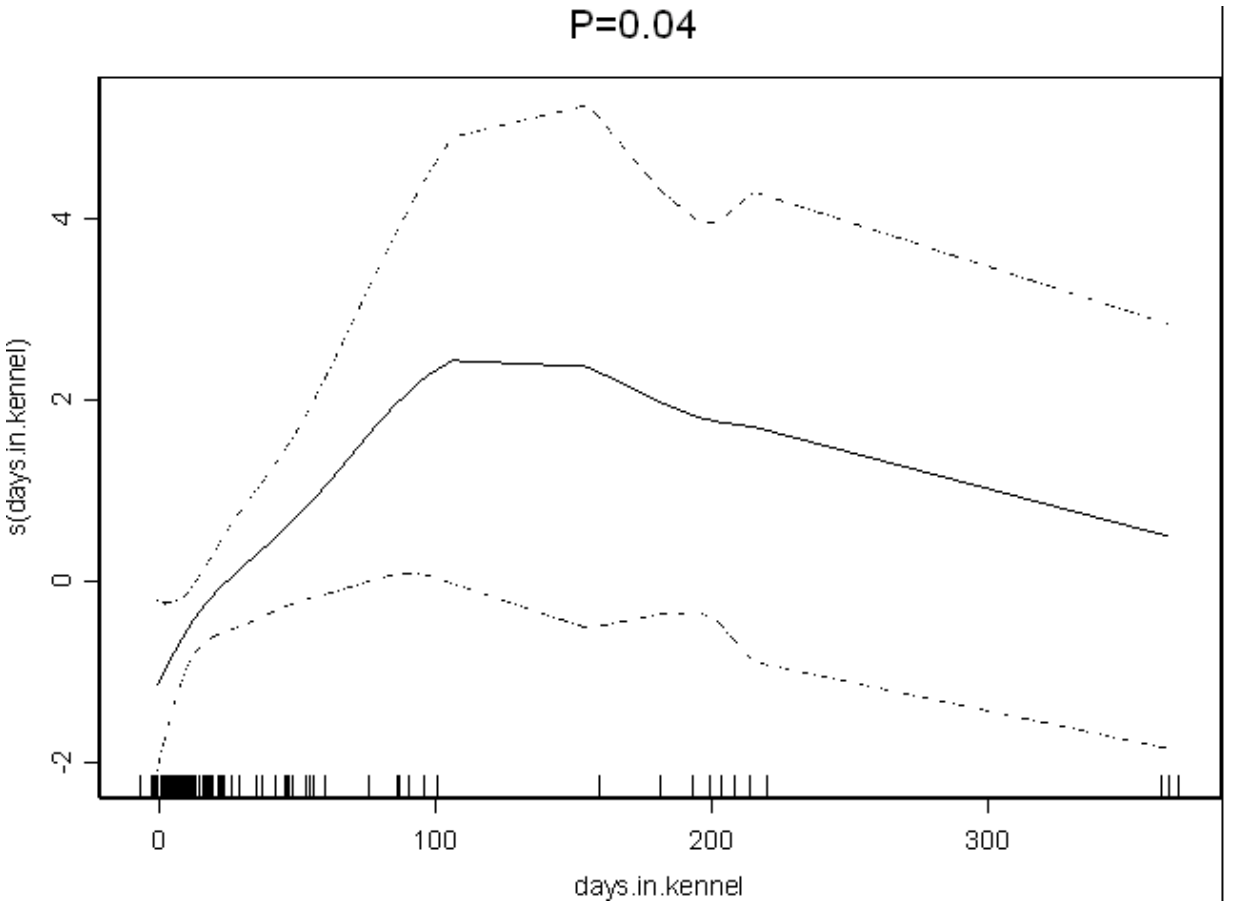


Figure 3.3. GAM graph demonstrating the non-linear relationship between the number of days dogs were in kennels A&B and *C. upsaliensis* status of the sample collected (the graph indicates that the data is significantly different from that of a linear relationship $P<0.04$).



S(days in kennel)= Loggit Odds of *C. upsaliensis* Isolation

Appendix 4.

Table 4.1. Origin and Multilocus sequence type of all *C. jejuni* isolates from dogs in the UK.

Isolate	Origin	Study	Isolation Method	Location	Year	ST	CC	Locus						
								aspA	glnA	gltA	glyA	pgm	tkt	unc
1	Household dog	B	Filtration	North West	2005	403	**403**	10	27	16	19	10	5	7
2	Household dog	C	Direct	North West	2007	1326	**45**	104	7	10	4	1	7	1
3	Boarding dog	E	Direct	North West	2007	508	**508**	1	6	60	24	12	28	1
4	Boarding dog	E	Filtration	North West	2007	508	**508**	1	6	60	24	12	28	1
5	Vet visiting dog	A	Direct	South West	2006	273	**206**	2	21	5	37	60	1	5
6#	Vet visiting dog	A	Filtration	South West	2006	132	**508**	1	6	22	24	12	28	1
7	Vet visiting dog	A	Direct	Glasgow	2006	312	**658**	14	45	2	4	19	3	6
8	Rescue dog	G	Direct	Birmingham	2007	45	**45**	4	7	10	4	1	7	1
9	Rescue dog	D	Direct	North West	2007	257	**257**	9	2	4	62	4	5	6
10	Rescue dog	D	Direct	North West	2007	137	**45**	4	7	10	4	42	7	1
11	Rescue dog	D	Filtration	North West	2007	45	**45**	4	7	10	4	1	7	1
12	Rescue dog	D	Direct	North West	2007	45	**45**	4	7	10	4	1	7	1
13	Rescue dog	D	Direct	North West	2007	3613	**45**	4	296	10	4	1	7	1
14	Rescue dog	F	Direct	Cambridge	2007	1044	**658**	2	10	2	4	19	3	6
15♦	Rescue dog*	D	Direct	North West	2007	267	**283**	4	7	40	4	42	51	1
16	Rescue dog*	D	Direct	North West	2007	45	**45**	4	7	10	4	1	7	1
17	Hunt dog	H	Filtration	North Wales	2008	2772	**-**	10	4	43	19	6	18	7
18	Hunt dog	H	Direct	North Wales	2008	104	**21**	2	1	1	3	7	1	5
19	Hunt dog	H	Direct	North Wales	2008	45	**45**	4	7	10	4	1	7	1
20	Hunt dog	H	Filtration	North Wales	2008	403	**403**	10	27	16	19	10	5	7
21	Hunt dog	H	Filtration	North Wales	2008	104	**21**	2	1	1	3	7	1	5
22♦	Hunt dog	H	Filtration	North Wales	2008	334	**45**	4	7	40	4	42	7	1
23	Hunt dog	H	Filtration	North Wales	2008	2772	**-**	10	4	43	19	6	18	7

24	Hunt dog	H	Direct	North Wales	2008	403	**403**	10	27	16	19	10	5	7
25	Hunt dog	H	Direct	North Wales	2008	2772	**-**	10	4	43	19	6	18	7
26	Hunt dog	H	Filtration	North Wales	2008	2772	**-**	10	4	43	19	6	18	7
27♦	Hunt dog	H	Filtration	North Wales	2008	334	**45**	4	7	40	4	42	7	1
28♦	Hunt dog	H	Direct	North Wales	2008	334	**45**	4	7	40	4	42	7	1
29	Hunt dog	H	Filtration	North Wales	2008	206	**206**	2	21	5	37	2	1	5
30	Hunt dog	I	Filtration	North West	2008	508	**508**	1	6	60	24	12	28	1
31	Hunt dog	I	Filtration	North West	2008	22	**22**	1	3	6	4	3	3	3
32	Hunt dog	I	Filtration	North West	2008	19	**21**	2	1	5	3	2	1	5
33	Hunt dog	J	Filtration	Midlands	2008	19	**21**	2	1	5	3	2	1	5

Legend for Table 4.1 &: ST= Sequence type, CC= Clonal complex and CI= 95% confidence interval. North West= North West England, *same dog (isolate 15= 2nd, isolate 16=15th sample), #= rescue dog visiting a practice, ♦=isolate could not be digested using *Sma*I, Direct=direct plating onto mCCDA and Filtration=filtration onto CAT media. A=national cross sectional study of vet visiting dogs (Chapter 3), B=study of household dogs (Westgarth *et al*, 2009), C=member of staff's dog, D=longitudinal study in a rescue shelter (Chapter 4), E=longitudinal study in a boarding kennel (Chapter 4), F=rescue shelter, G=stray block in a boarding kennel (Chapter 5), H=hunt kennel C (Chapter 5), I=hunt kennel D (Chapter 5), and J=hunt kennel E (Chapter 5).

Table 4.2. Dog and human *C. upsaliensis* isolate information for MLST (Novel ST/alleles indicated in bold).

										Locus					
Group/CC (founder)	**Isolate**	**Origin**	**Study**	**Location**	**Date**	**Isolation**	**Age (m)**	**Disease**	**ST**	**adk**	**aspA**	**atpA**	**glnA**	**glyA**	**pgi**	**tkt**
A (none)	Dog 2	Vet-visiting	A	Wales	2006	Enrich	7	No	**68**	1	**40**	1	13	25	22	26
	Dog 22	Household	B	North west	2005	Filtration	12	No	**88**	1	**40**	**26**	13	1	22	26
B (singletons)	Dog 6	Vet-visiting	A	Jersey	2006	Direct	32	No	**72**	8	**38**	1	11	1	12	22
	Dog 10	Vet-visiting	A	Wales	2006	Direct	138	No	**72**	8	**38**	1	11	1	12	22
C (none)	Dog 13	Household	B	North west	2005	Filtration	96	No	**79**	9	2	9	15	1	14	1
	Dog 25	Vet-visiting	A	London (Bracknel)	2006	Direct	150	No	**91**	9	2	9	15	1	**31**	1
	Dog 31	Boarding	G	Birmingham	2007	Direct	U	No	**97**	9	2	9	3	1	12	1
D (none)	Dog 20	Hunt	I	North west	2008	Filtration	U	U	**86**	**25**	**43**	**24**	27	**33**	**30**	**32**
	Dog 39	Hunt	I	North west	2008	Filtration	U	U	**105**	**25**	**43**	**23**	27	**33**	**30**	**32**
E (none)	H 43	Human	HPA	North west	2002	U	U	Yes	**104**	1	29	1	**31**	**35**	12	26
	H 45	Human	HPA	North west	2002	U	U	Yes	**104**	1	29	1	**31**	**35**	12	26
	Dog 38	Household	B	North west	2005	Direct	168	U	**104**	1	29	1	**31**	**35**	12	26
	Dog 24	Vet-visiting	A	London	2006	Enrich	132	No	**90**	**26**	29	1	**31**	**35**	12	26
F /ST-16 (98)	Dog 30	Vet-visiting	A	South west	2006	Direct	182	No	**96**	17	**45**	1	**33**	9	12	1
	Dog 32	Boarding	E	North west	2007	Filtration	18	No	**98**	7	**45**	1	**33**	9	12	1

	Dog 36	Boarding	G	Birmingham	2007	Direct	U	No	**98**	7	**45**	1	**33**	9	12	1
	Dog 16	Vet-visiting	A	London (Kent)	2006	Filtration	112	No	**82**	7	**45**	1	**33**	9	**29**	1
	H 47	Human	HPA	North west	2007	U	U	Yes	**112**	7	**45**	**23**	**33**	**36**	12	1
G (none)	Dog 18	Vet-visiting	A	South West	2006	Filtration	4	Yes	**84**	10	13	10	**32**	12	12	1
	Dog 29	Household	B	North west	2005	Direct	60	No	**95**	10	13	1	**32**	12	12	1
H (71)	Dog 5	Vet-visiting	A	Scotland	2006	Enrich	16	No	**71**	13	3	9	10	12	12	12
	Dog 8	Rescue	D	North west	2007	Direct	108	No	**74**	13	3	9	1	12	12	12
	Dog 21	Boarding	E	North west	2007	Filtration	U	No	**87**	13	3	**25**	**31**	25	12	**33**
	H 42	Human	HPA	North west	2006	U	U	Yes	**108**	13	3	9	**31**	12	12	**33**
I (none)	Dog 12	Vet-visiting	A	Gloucester	2006	Enrich	48	No	**78**	13	3	1	**28**	20	**28**	9
	Dog 11	Vet-visiting	A	North west	2006	Enrich	204	No	**77**	13	3	1	13	**37**	**28**	9
	Dog 26	Vet-visiting	A	North west	2006	Enrich	120	No	**92**	13	3	1	**28**	20	12	9
J (none)	Dog 37	Vet-visiting	A	N. Ireland	2006	Enrich	72	No	**103**	23	29	18	**31**	25	22	26
	H 44	Human	HPA	North west	2002	U	U	Yes	**110**	1	29	18	**34**	25	22	26
K (76)	Dog 3	Household	B	North west	2005	Filtration	U	No	**69**	1	3	**27**	**30**	20	12	12
	Dog 9	Boarding	G	Birmingham	2007	Direct	U	Yes	**75**	1	**41**	**22**	**30**	20	12	12
	Dog 15	Vet-visiting	A	Gloucester	2006	Enrich	57	No	**76**	1	3	**22**	**30**	20	12	12
	Dog 28	Hunt	H	North Wales	2008	Filtration	U	U	**94**	18	3	**22**	**30**	20	12	26

L (none)	Dog 23	Vet-visiting	A	South Wales	2006	Filtration	76	No	**89**	13	17	1	18	1	1	9
	Dog 33	Boarding	G	Birmingham	2007	Direct	U	No	**99**	13	11	1	18	1	1	**34**
Singletons	Dog 1	Boarding	E	North west	2007	Spiral	U	No	**67**	**24**	**39**	**23**	**29**	1	**27**	**30**
	Dog 4	Household	B	North west	2005	Filtration	24	No	**70**	13	29	1	**31**	**35**	14	4
	Dog 7	Boarding	G	Birmingham	2007	Filtration	U	No	**73**	6	17	**22**	**30**	**34**	12	12
	Dog 14	Vet-visiting	A	South Wales	2006	Direct	5	No	**80**	13	**42**	1	13	1	14	4
	Dog 17	Rescue	D	North west	2007	Direct	60	Yes	**83**	13	29	1	3	**32**	12	**31**
	Dog 19	Vet-visiting	A	Scotland	2006	Enrich	13	No	**85**	1	11	9	3	**38**	14	**33**
	Dog 27	Rescue	D	North west	2007	Direct	U	No	**93**	1	**44**	1	13	1	**32**	4
	Dog 34	Household	B	North west	2005	Filtration	60	No	**100**	**27**	3	1	3	20	**33**	**35**
	Dog 35	Vet-visiting	A	London	2006	Enrich	23	No	**101**	22	29	**22**	3	**32**	12	4
	*Dog 40	Vet-visiting	A	Gloucester	2006	Direct	16	No	**106**	18	**46**	15	13	21	3	23
	Dog 41	Household	B	North west	2005	Direct	60	No	**107**	1	17	**22**	**30**	**34**	**34**	26
	H 46	Human	RM31 95	South Africa	1994	U	U	GB	5	4	4	5	5	4	5	5
	H 48	Human	HPA	North west	2007	U	U	Yes	**113**	10	3	1	**31**	1	12	**33**
	H 49	Human	HPA	North west	2007	U	U	Yes	**114**	**27**	**47**	1	**33**	9	**35**	1
	H 50	Human	HPA	North west	2007	U	U	Yes	**115**	**28**	3	**28**	13	20	**36**	12

GB=Guillain-Barré syndrome

CC= clonal complex

m=months

U=unknown.

Spiral=direct plating onto CAT

Filtration=filtration onto CAT

Enrich=Enrichment+mCCDA

Direct=Direct plating (mCCDA)

A=vet-visiting (Chapter 3)

B=household (Westgarth *et al*, 2009)

C=member of staff's dog

D=longitudinal rescue shelter (Chapter 4)

E=longitudinal boarding kennel (Chapter 4)

F=rescue shelter (Chapter 5)

G=boarding kennel (Chapter 5)

H=hunt kennel C (Chapter 5)

I=hunt kennel D (Chapter 5)

* belongs to other complex (Appendix 4, Fig 4.11)

Figure 4.1. Un-rooted bootstrap (%) consensus neighbour joining tree of concatenated *C. upsaliensis* MLST sequences based on 3243bp (1000 replicates).

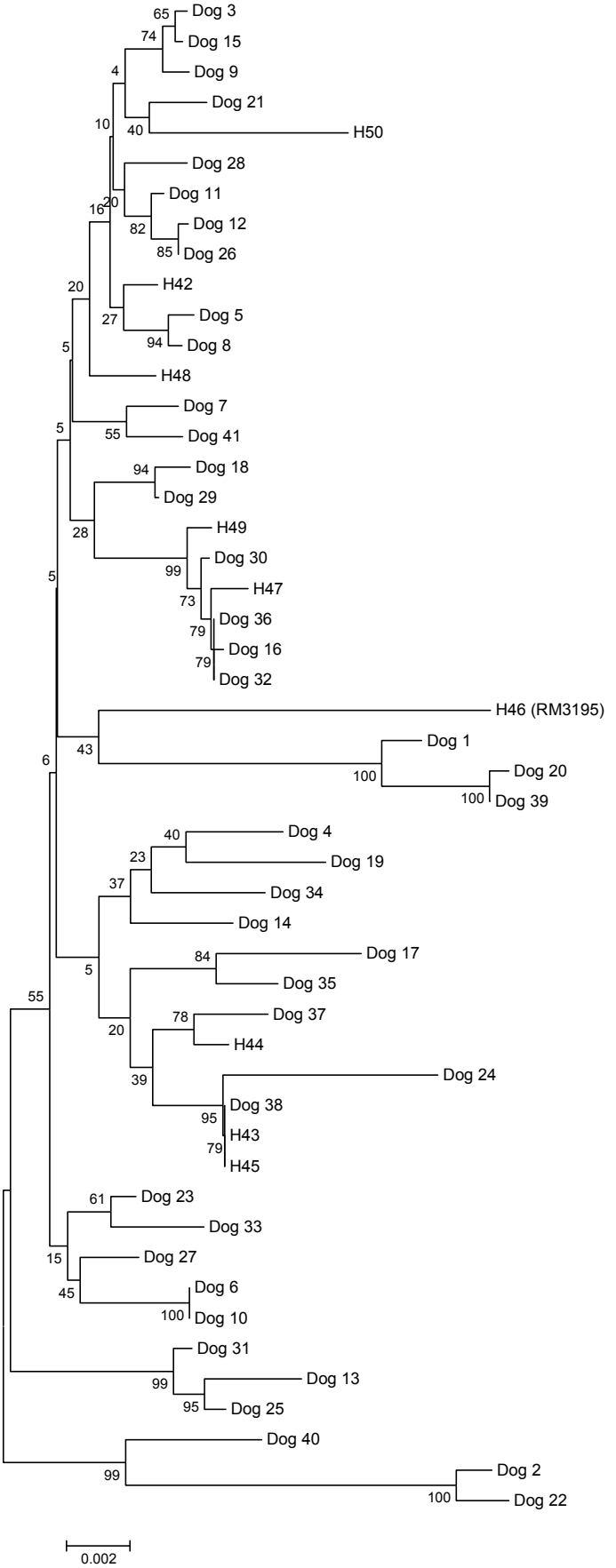


Figure 4.2. Group 1 as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

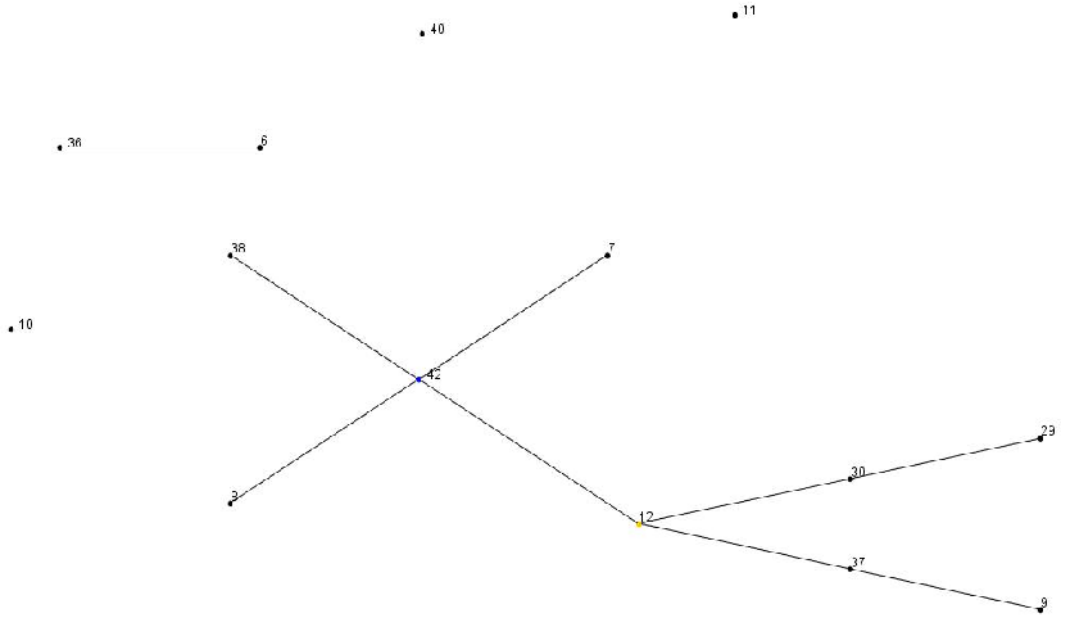


Figure 4.3. Group 2 (complex F/ST-16) as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

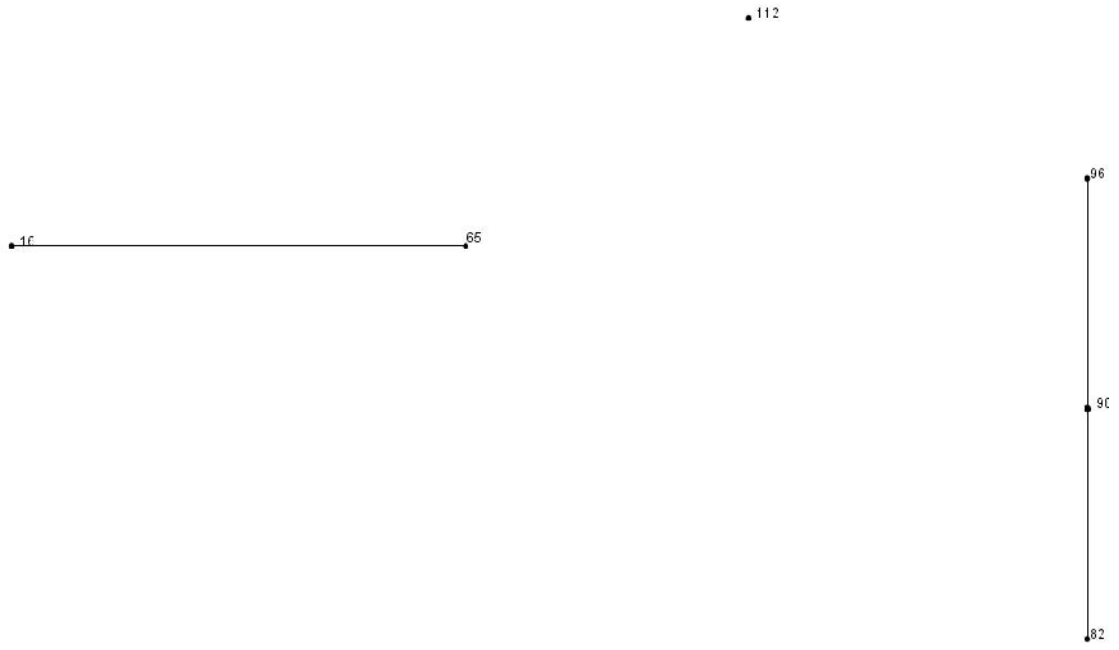


Figure 4.4. Group 3 (complex H) as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

Figure 4.5. Group 4 as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

Figure 4.6. Group 5 (complex K) as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

Figure 4.7. Group 6 as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

Figure 4.8. Group 7 (complex J) as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

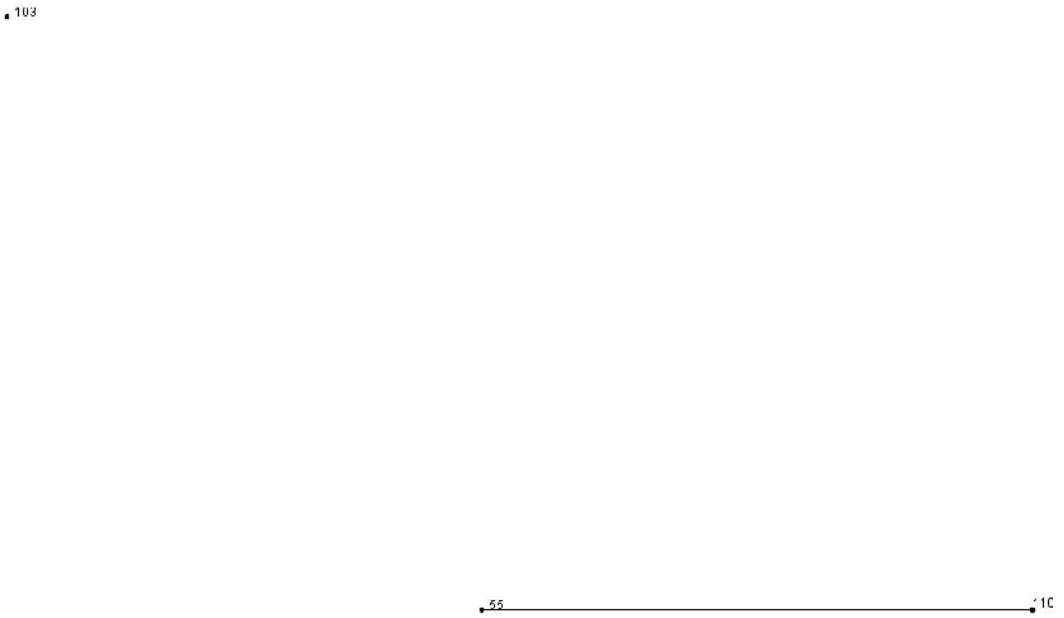


Figure 4.9. Group 8 (complex C) as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

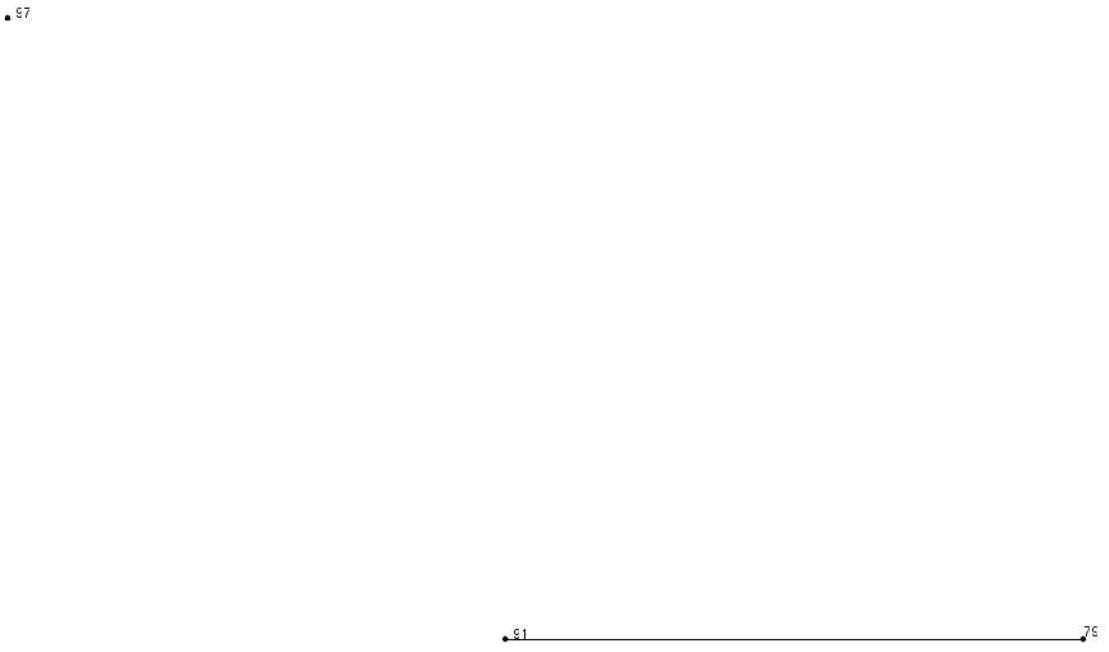


Figure 4.10. Group 9 (complex I) as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

Figure 4.11. Group 10 as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

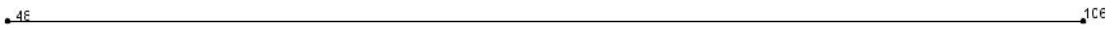


Figure 4.12. Group 11 (complex D) as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

Figure 4.13. Group 12 (complex E) as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

Figure 4.14. Group 13 as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

Figure 4.15. Group 14 (complex L) as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

Figure 4.16. Group 15 (complex G) as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

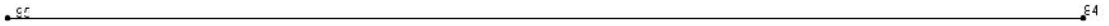
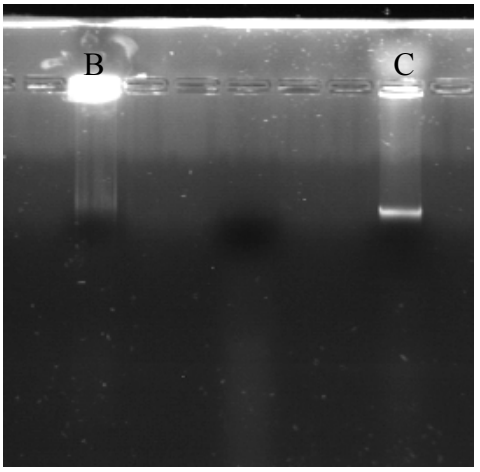


Figure 4.17. Group 16 (complex A) as defined by eBURST, based on all currently known *C. upsaliensis* sequence types. Primary founders coloured blue, and the subgroups founders of these linked clusters coloured yellow.

Appendix 5.

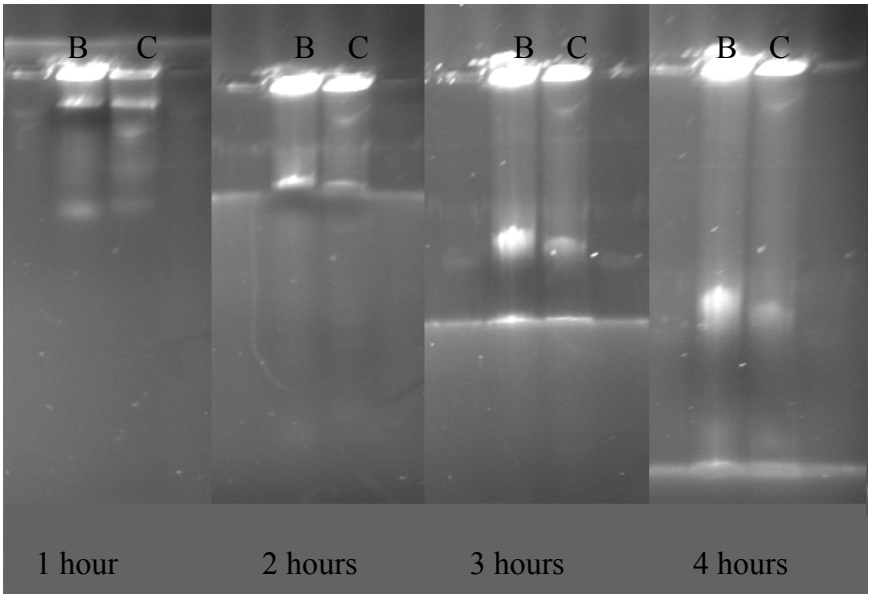
Figure 5.1. Gel image of pCU120 after first modified Kado and Liu plasmid isolation procedure.



B=*C. upsaliensis* isolated from Dog 52A.

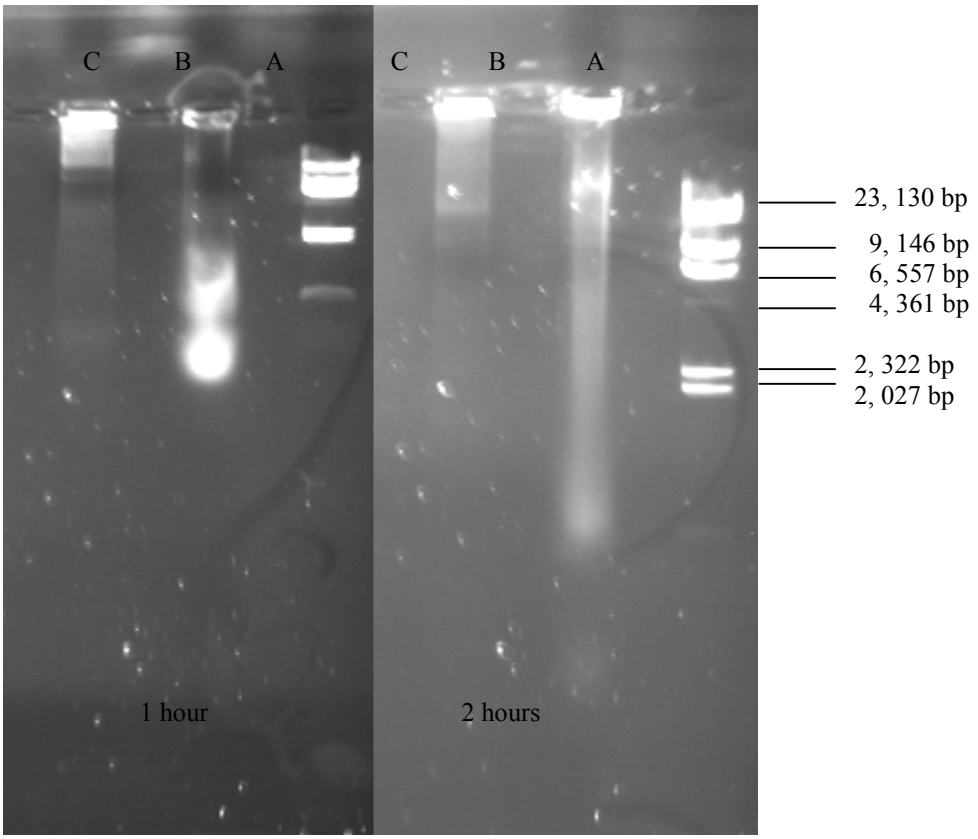
C=*E.coli* 39R861 (plasmid sizes: 7.1 kb, 36.8 kb, 65.0 kb and 151.0)

Figure 5.2. Gel image of pCU120 after second modified Kado and Liu plasmid isolation procedure.



B=*C. upsaliensis* isolated from Dog 52A and C=*E.coli* 39R861 (plasmid sizes: 7.1 kb, 36.8 kb, 65.0 kb and 151.0).

Figure 5.3. Gel image of pCU120 after third modified Kado and Liu plasmid isolation procedure.

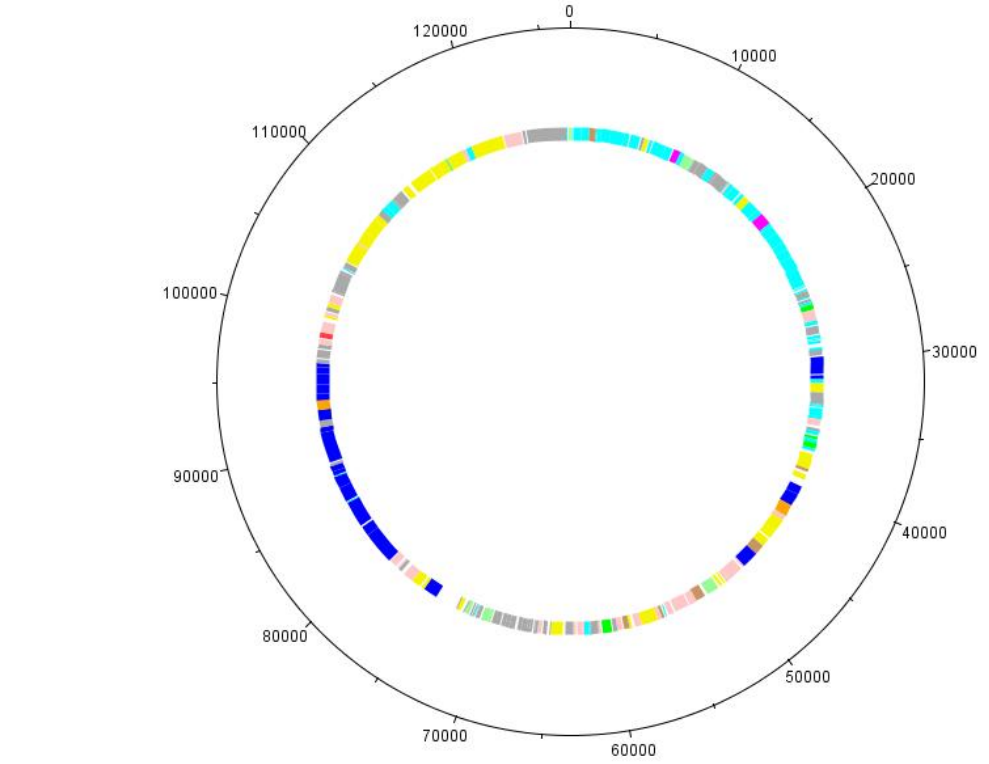


A= Lambda hind III marker (size range 23, 130-125 b)

B=*C. upsaliensis* isolated from Dog 52A

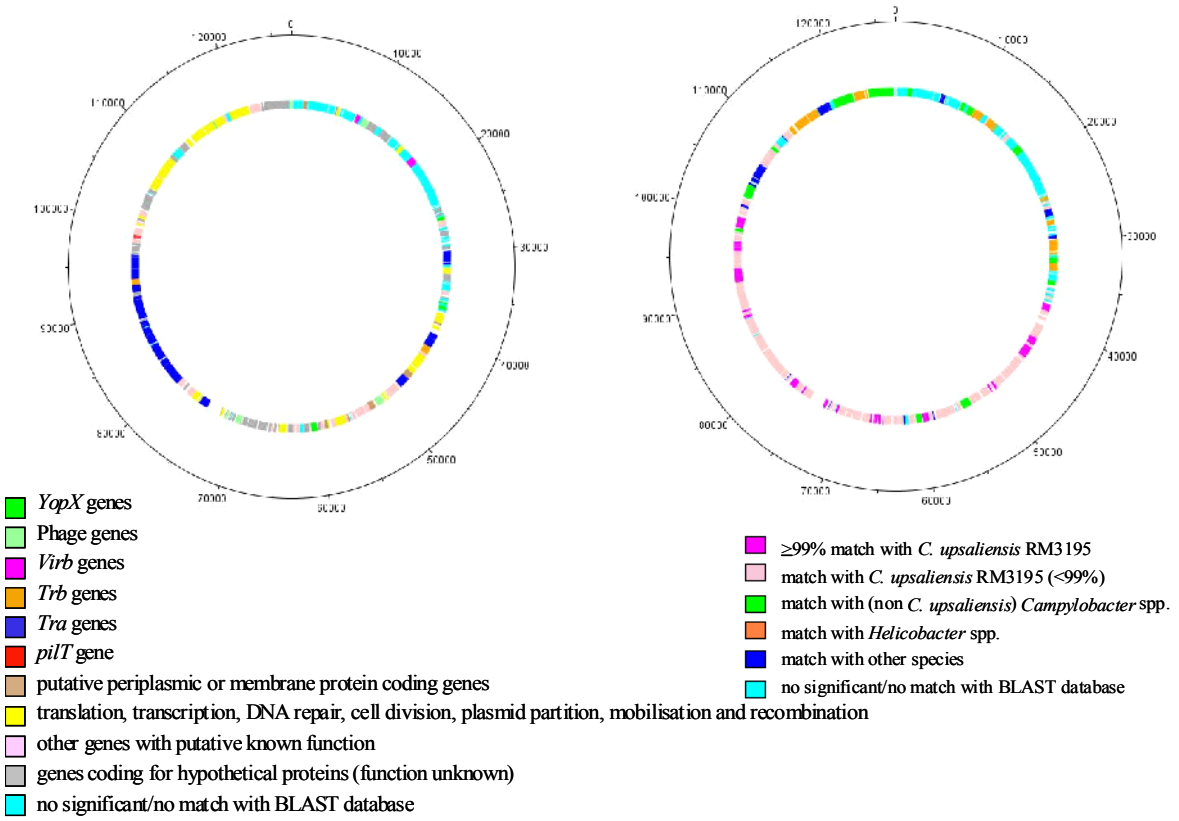
C=*E.coli* 39R861 (plasmid sizes: 7.1 kb, 36.8 kb, 65.0 kb and 151.0).

Figure 5.4. Diagram indicating the putative functions of the amino acids within pCU120.



YopX genes
Phage genes
Virb genes
Trb genes
Tra genes
pilT gene
putative periplasmic or membrane protein coding genes
translation, transcription, DNA repair, cell division, plasmid partition, mobilisation and recombin
other genes with putative known function
genes coding for hypothetical proteins (function unknown)
no significant/no match with BLAST database

Figure 5.5. Comparison of figures 7.5 (Chapter 7) and 5.4 (Appendix 5) demonstrating TFSS coding proteins in a (probable) conserved region.



Appendix 6 Publications

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