The Molecular Epidemiology of Scottish *Campylobacter* Isolates from Human Cases of Infection using Multilocus Sequence Typing (MLST)

CaMPS - *Campylobacter* MLST Project in Scotland

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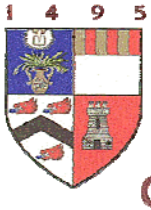

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CONTENTS

FIGURES

TABLES

1 Scientific and lay summaries

1.1 Scientific summary

Clinical *Campylobacter* infections comprise two species, *C. jejuni* and *C. coli*, and contributes to half of all reported cases of infectious intestinal disease in the UK, imposing a continuing healthcare burden. Approximately seven unreported cases occur for each reported case, and the likely total imposes an annual economic burden of £0.5 bn. Potential infection sources occur in animal and bird faeces in the environment and in animal meats in the food chain. Quantitative attribution of clinical infections to infection sources is rudimentary, however, and this makes measures for reducing human infection difficult to implement.

Bacterial strain typing technology has produced multilocus sequence typing (MLST), which categorises each strain as a sequence type (ST). The Food Standards Agency Scotland funded the present project with the main goal of using MLST to provide quantitative attributions of clinical *Campylobacter* infections to infection sources.

The present project comprised (i) contemporaneous collections of *Campylobacter* isolates from clinical infections and potential infection sources in Scotland, (ii) typing isolates by MLST and (iii) analyses of species and strain diversity, spatiotemporal clustering of clinical strains, specificity of non-human strains to source of origin and attribution of clinical strains to potential infection sources.

Campylobacter isolates from clinical infections (N=5672) were obtained from 97.3% of the cases reported from all mainland and island Health Boards to Health Protection Scotland during July 2005 – September 2006. *Campylobacter* isolates were also obtained from host and food sources comprising farm species (N=576), wild birds (N=188), companion animals (N=53) and retail chicken and offal (N=282) during April 2005 – November 2006. Strains from animal and bird hosts were isolated from faeces collected on farms in NE and SW Scotland and in the city centres of Aberdeen Edinburgh and Glasgow and from specimens provided by veterinary laboratories. Strains from food were isolated from chicken and offal portions collected in retail outlets in NE Scotland and from swab specimens provided by public authority laboratories.

Species composition in the clinical isolates averaged 90.4% *C. jejuni* (range across Health Boards 77.8-94.2%), as expected, with *C. coli* accounting for almost all of the remainder.

Species composition in the above four categories of non-human isolates ranged from 78.5% *C. jejuni* in farm species to 96.2% *C. jejuni* in companion animals, with *C. coli* accounting for most of the remaining strains and being especially common in strains from the farm species subcategories of sheep (39.7% *C. coli*) and pigs (91.2% *C. coli*) and the wild bird subcategories of ducks (41.7% *C. coli*) and swans (50.0% *C. coli*).

Strain (ST) diversity was very high in all clinical isolates (mean Hunter-Gaston diversity index (DI) = 0.971, range across Health Boards 0.965-1.000) and in most non-human sources (mean DI=0.975, range across all subcategories except urban pigeons 0.904-0.981) with pigeons having much lower strain diversity than any other source (DI=0.594). Clinical strain diversity was homogeneous across the 12 mainland Health Boards, suggesting that clinical infection is homogeneous across Scotland. Clinical strain diversity was slightly heterogeneous across month, with certain STs (*e.g.*, ST45, ST53, ST2030) having pronounced peaks of occurrence during spring or summer months. Clinical strain diversity was also slightly heterogeneous across patient age class, with older patients having higher proportions of *C. coli* strains. Non-human strain diversity was heterogeneous across source categories, consistent with infection sources having some degree of characteristic strains.

Three different analyses of spatiotemporal clustering, informed by strain ST data, all implied that most clinical cases (93-98%) were sporadic and that only a minority (2-7%) showed significant clustering. The latter group included the STs previously identified as showing pronounced monthly peaks.

Approximately three-quarters of clinical isolates could be attributed to each of six potential infection sources: less than 1% to pigs, 5-6% to wild birds, 12-15% each to cattle, sheep and companion animals, and just over 30% to retail chicken. When companion animals were excluded the attributions were similar: less than 1% to pigs, 7-8% to wild birds, 15-18% each to cattle and sheep, and 35-36% to retail chicken.

The present study clearly identified retail chicken as the single largest source of clinical *Campylobacter* infection in Scotland, consistent with well-known *Campylobacter* prevalence and bacterial loads in broiler chickens and with case-control studies on *Campylobacter* epidemiology. The present study also identified farm ruminants as sources, for which infection routes are uncertain and confirming studies are rare. According to these findings, human *Campylobacter* infection can only be reduced when *Campylobacter* prevalence and concentration in retail poultry are reduced and infection routes from farm ruminants identified.

1.2 Lay summary

Two species of a bacterium called *Campylobacter* (*C. jejuni* and *C. coli*) cause about half of all reported cases of food poisoning in the UK, creating extra work for hospital staff and GPs. Seven further cases of *Campylobacter* food poisoning occur for every reported case, and all these infections cost the UK economy about £0.5 bn each year. We know that infections come from droppings of various species of farm and wild animals and birds, and from contamination of various animal meats in food chains. To reduce *Campylobacter* food poisoning, however, we need to know *how much* infection comes from each source.

Microbiologists have developed a DNA fingerprinting technique for bacteria called "multilocus sequence typing" or MLST, which classes a bacterial strain as a "sequence type" or ST. To the extent that sources of *Campylobacter* infection have characteristic spectra of STs, then strains from each source can (in principle) be matched with cases of *Campylobacter* food poisoning and thereby "judged guilty" of having caused the infections. The Food Standards Agency Scotland funded the present project to evaluate whether this principle is useful in a realistic context for estimating how much infection comes from specific sources.

The present project had three stages (i) acquisition of *Campylobacter* strains from all food poisoning cases reported in Scotland during a 15-month period and from representative sources of infection in the environment and the food chain, (ii) determination of the ST of each strain and (iii) various questions requiring data analysis. The most important questions are: How diverse and distinct are *Campylobacter* strains from different sources? Were all infections sporadic or did some of them occur in clusters? What are the proportions of clinical strains that can be attributed to different sources of infection?

We acquired 5672 strains from almost all the reported cases of *Campylobacter* food poisoning in Scotland during July 2005 – September 2006. We also acquired strains from a range of likely infection sources: 576 from farm species, 188 from wild birds, 53 from cats and dogs, and 282 from chicken meat and offal. Strains were grown from animal and bird droppings collected on farms in NE and SW Scotland and in the city centres of Aberdeen Edinburgh and Glasgow, from meat portions bought in retail outlets, and from swab specimens provided by public authority and veterinary laboratories during April 2005 – November 2006.

Most of the strains from the food poisoning cases were *C. jejuni* (90%) and almost all of the rest were *C. coli*, in line with expectations. Most of the strains from the above four infection

sources were also *C. jejuni*, with *C. coli* accounting for most of the rest, but *C. coli* was more common in strains from ducks, swans and sheep (40-50%) but especially pigs (90%).

The strains from both the food poisoning cases and the infection sources had very diverse ST compositions, the only exception being strains from urban pigeons, most of which were a single, distinct ST. The food poisoning strains had a homogeneous spectrum of STs across the 12 mainland Health Boards, suggesting that infections causing food poisoning originate from the same sources across Scotland. The food poisoning strains had a slightly heterogeneous spectrum of STs across month of the collection period, with certain STs (*e.g.*, ST45, ST53, ST2030) having spikes of occurrence during spring or summer, and across patient age classes, with older patients having more *C. coli* strains.

The strains from infection sources had spectra of STs that were to some extent characteristic of each source. The least overlap was seen in strains from pigs, gulls and urban pigeons, which had almost completely diagnostic STs, and the greatest overlap was seen in strains from cattle and sheep, which shared several STs that were common in both sources. Strains from other wild birds, cats and dogs, and retail chicken were intermediate in terms of ST overlap. It therefore seemed possible to use ST as a means of attributing strains from food poisoning cases to sources to a degree of certainty.

According to three different analyses, only a minority (2-7%) of food poisoning strains were clustered in the same 30-day period while the rest were apparently sporadic. The clustered strains included the STs previously identified as having monthly spikes.

About three-quarters of the food poisoning strains could be attributed to each of six infection sources in the following percentages: less than 1% to pigs, 5-6% to wild birds, 12-15% each to cattle, sheep and companion animals, and just over 30% to retail chicken. Excluding cats and dogs as sources yielded similar attributions: less than 1% to pigs, 7-8% to wild birds, 15-18% each to cattle and sheep, and 35-36% to retail chicken.

Our study clearly identified retail chicken as the single largest source of *Campylobacter* food poisoning in Scotland, consistent with published findings that *Campylobacter* is common and can occur at high levels in broiler chickens and that chicken consumption is a risk factor in food poisoning. We also identified farm ruminants as infection sources. Thus, *Campylobacter* food poisoning can only be reduced when *Campylobacter* in retail poultry is reduced and infection routes from farm ruminants are identified.

1.3 Glossary

Attribution	Inference of the source of human *Campylobacter* infection using **strain type**.
CaMPS	This study. *Campylobacter* MLST Project in Scotland.
cfu	Colony forming units. Typically a measure of the number of live cells in a sample.
Clonal complex (CC)	A group of STs whose members are linked to at least one other member by being identical for six of the seven **MLST** genes.
CPHM	Consultant in public health medicine in Scotland. Similar to (CCDC) Consultant in communicable disease control in England and Wales.
Diversity index (DI)	The Hunter-Gaston diversity index: a measure of the diversity of types in a group that takes account of differences in sample size among groups. DI values range from 0 (all types are the same) to 1 (all types are different).
Host association	The concept of a host species having a characteristic set of *Campylobacter* strains.
IID	Infectious Intestinal Disease
Isolate	A *Campylobacter* culture isolated from a specimen by microbiological methods.
Public Analysts	Local authority food safety surveillance laboratories. In this study, one of the bodies that provided *Campylobacter* isolates from retail foods.
MLST	**M**ulti**L**ocus **S**equence **T**yping.
Orphan ST	An **ST** that (at a specific date of database interrogation) is not part of a **clonal complex**.
Reservoir host	Domesticated and wild animal and bird species, in which *Campylobacter* is maintained as a source of human infection.
SAC	Scottish Agricultural College
Single-locus variant	An **ST** that differs from another ST for one of the seven **MLST** genes while being identical at the other six genes.
Site	Place where a specimen was collected.
Specimen	A swab containing *Campylobacter* growth from clinical, food or veterinary sources, a sample of animal or bird faeces, or a portion of retail food.
ST	**S**equence **T**ype of a *Campylobacter* isolate, defined as its allelic profile for a standard set of seven housekeeping genes. ST is the **strain** used in this project.
Strain	A collection of genotypically indistinguishable isolates. Usually of a common ST
Type / typing	Often genotyping or phenotyping. The process of characterising an **isolate** at the level of its DNA or at the level of its expressed biological characters (biochemical, physiological, antigenic etc)
UoA, UoO	University of Aberdeen, University of Oxford.

2 Introduction

2.1 Background

Campylobacter as a human pathogen

Campylobacter are Gram-negative bacteria that live commensally in the gastrointestinal tracts of a wide range of animals and birds, including farmed species and companion animals. Some *Campylobacter* species are also zoonotic human pathogens. A typical human infection consists of a self-limiting bout of diarrhoea, abdominal cramps and fever lasting about five days. *Campylobacter* infection was implicated in causing human enteritis in the late 1970s (109), and has since become recognised as the commonest known cause of bacterial infectious intestinal disease (IID) worldwide. According to WHO estimates, *Campylobacter*-related illness affects around 1% of populations in developed countries every year.

Campylobacter infection causes almost half of all IID cases in the UK (Figure 1), with *Campylobacter jejuni* causing around 90% of cases and the closely-related *Campylobacter coli* causing almost all the rest. Annual reported incidence of *Campylobacter* in England and Wales increased from approximately 25,000 in 1989 to approximately 50,000 in 2007, reaching a peak of approximately 58,000 in 2001 (Figure 2), and accounting for 32-55% of all reported cases of IID during this period (50). Annual reported incidence of *Campylobacter* in Scotland rose from approximately 3,000 cases in 1989 to approximately 5,200 in 2007, through a peak of approximately 6,500 in 2001 (Figure 2), accounting for 40-54% of all reported IID cases (Figure 3). The annual incidence rates in the 12 mainland Scottish Health Boards during 2005-2006 ranged from 51 to 136 cases/100,000 population (Figure 4) (51). All these reported incidences are likely to be substantial underestimates because of underreporting, which in England is estimated to average 7.6-fold (16, 127).

High rates of *Campylobacter* incidence translate into substantial annual economic costs, estimated at £503M in the UK (all likely cases) (53), EUR9M in the Netherlands (reported cases in 1999) (120), and $4.3bn in the USA (all likely cases) (9). *Campylobacter* infection can also lead to serious longer-term illness. Approximately one case for every 1000 reported cases leads to Guillain-Barré syndrome: a serious condition of reversible or permanent loss

of limb motor function that is the commonest cause of acute flaccid paralysis. *Campylobacter* infection is also associated with the non-paralytic version of GBS, Miller-Fisher syndrome, and with reactive arthritis.

Figure 1. Proportion of reported IIDs during 1996-2005. a: Scotland, b: England and Wales.

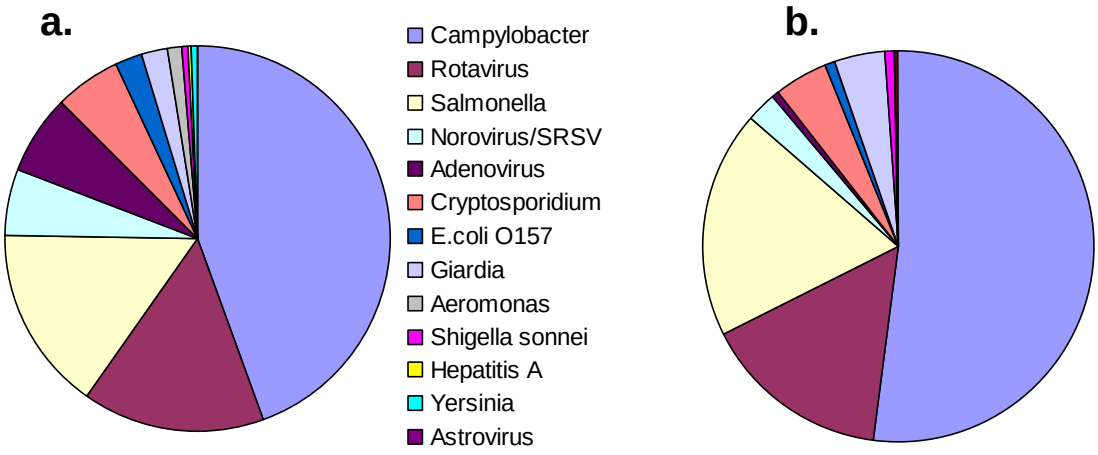


Figure 2. Annual number of *Campylobacter* cases in Scotland and in England and Wales.

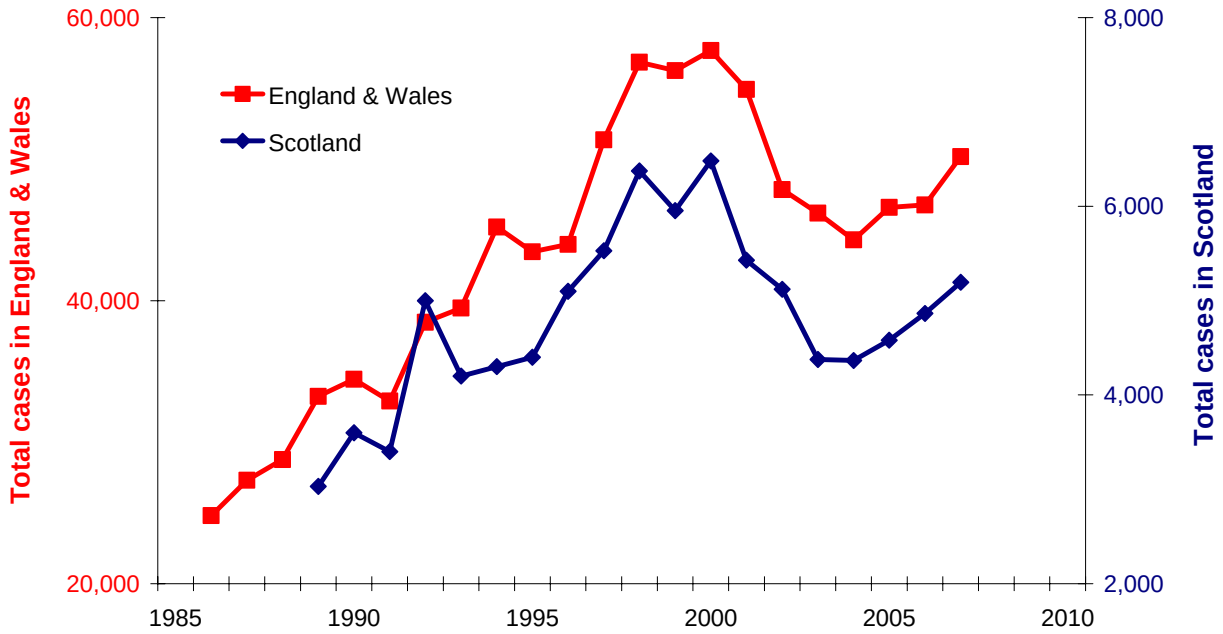


Figure 3. Annual *Campylobacter* incidence in relation to total IID incidence in Scotland

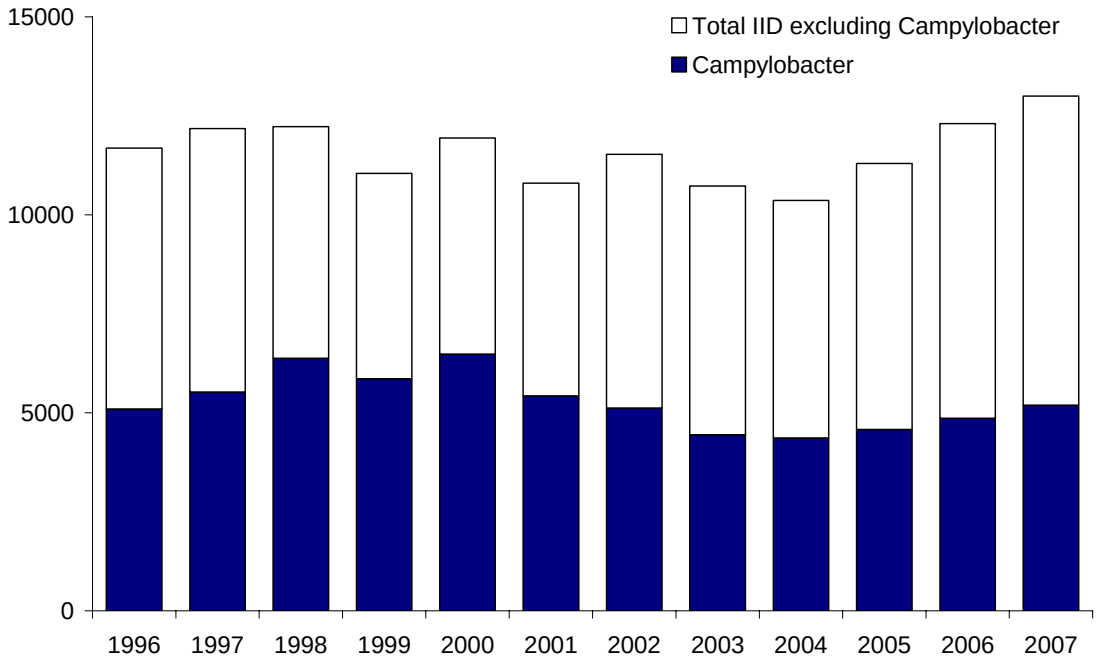
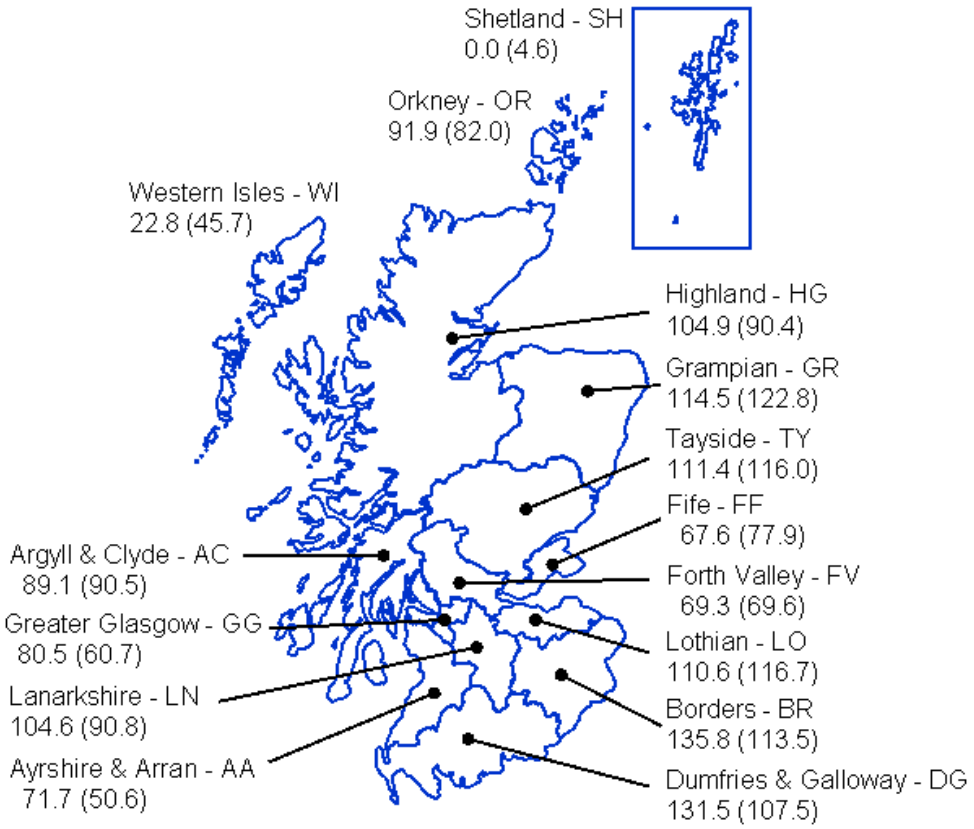


Figure 4. *Campylobacter* incidence across Scottish Health Boards in 2005 and 2006



The incidence values are the annual cases per 100,000 in each Health Board in 2006 (2005) as reported in Health Protection Scotland weekly reports.

Both the UK and Scottish governments have a responsibility to promote health and minimise logistic burden on the health care sector, and therefore want the incidence of human *Campylobacter* infection substantially reduced. Human *Campylobacter* infection is viewed as having a significant food-borne component, and therefore food safety regulation bodies and organisations in the food production sector are best-placed to identify and implement effective interventions.

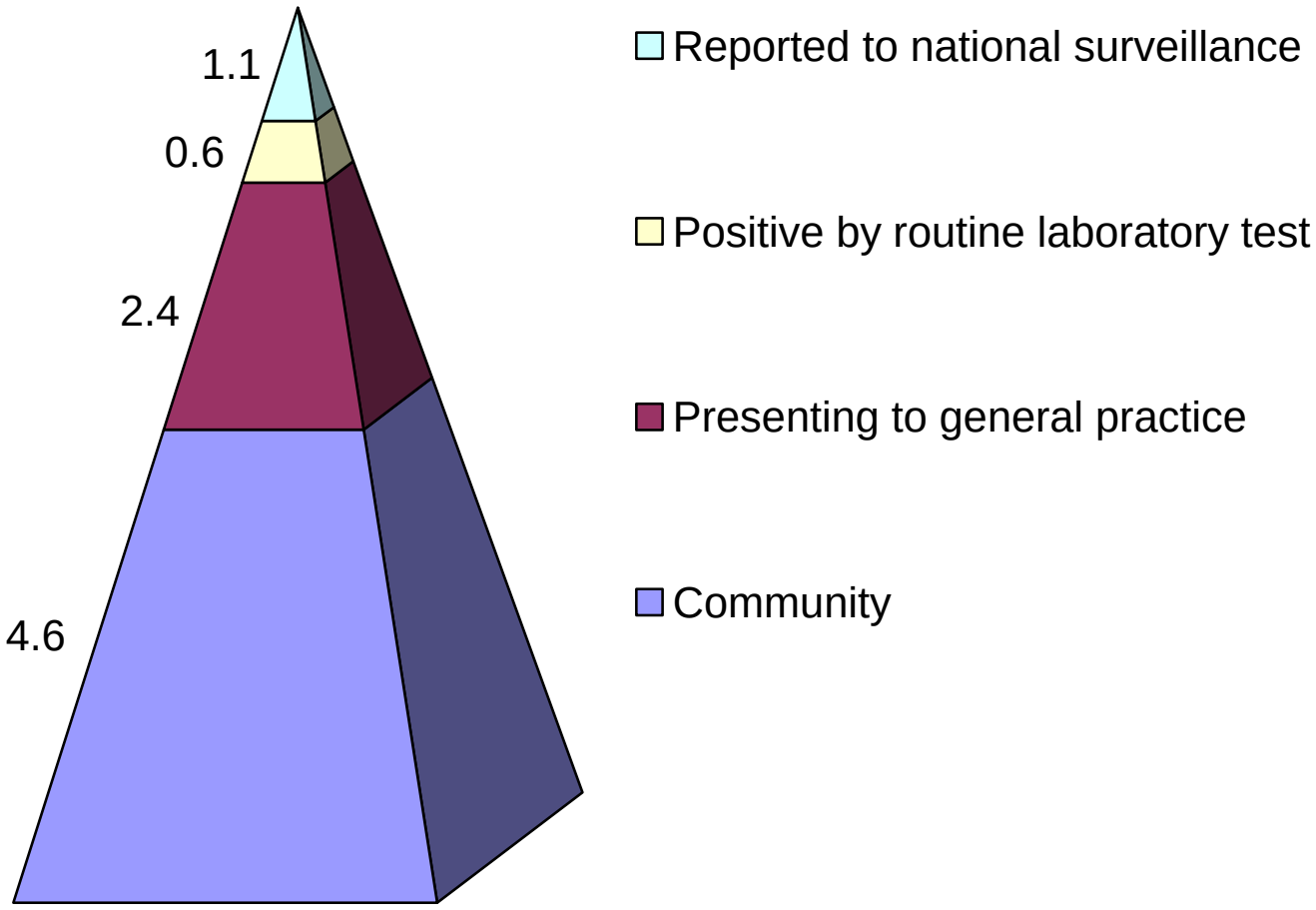
Campylobacter epidemiology – disease incidence

Human *Campylobacter* infection in temperate countries has five characteristic epidemiological features. First, there is a trough during winter followed by a 2- or 3-fold higher peak in spring or early summer. This seasonal pattern occurs consistently in the UK, several other European countries, Canada, Australia and New Zealand. The rise in incidence towards the summer peak tends to show a weak association with ambient temperature (63,73, 118).

Second, incidence shows both fluctuations and consistent trends between years. Annual reported incidence in the UK more than doubled between 1986 and 2000, fell during 2000-2004 then rose during 2005-2007 (Figure 2). The causes of these trends are unclear. The fall during 2000-2004 coincided with the Food Standards Agency Strategic Plan for 2001-2006 and the FSA Foodborne Disease Strategy, which were aimed at reducing food-borne disease by 20%.

Third, reported incidences show regional differences. Annual incidences during 1996-2007 varied more than six-fold among the 12 mainland Scottish Health Boards: from 44/100,000 (Ayrshire & Arran in 2004) to 303/100,000 (Forth Valley in 1998). These differences reflect no obvious general outbreaks, which are rare in *Campylobacter* (see below), and no countrywide N-S or E-W trends: Ayrshire & Arran consistently reports the lowest annual incidences (44-72/100,000) whereas the neighbouring Dumfries & Galloway consistently reports some of the highest (105-160/100,000). These reported rates are likely to be substantial underestimates of total incidence in the general population according to the “reporting pyramid” model, in which reporting rates and levels of disease notification are inversely related (Figure 5).

Figure 5. Schematic representation of differential reporting of *Campylobacter* incidence.



(data are rates per 1000/person years for 70 GP practices in England (128).

Fourth, incidence shows associations with patient age and sex (73). In England and Wales during 1990-1999, incidence was highest for children up to 4 years old (98-226/100,000), lowest for five- to 19-year-olds (34-65/100,000), and intermediate for older age groups and declining for people over 70. The seasonal peak in incidence shows an interaction with age, being most evident in young children aged 1-4 years and least evident in people over 70. Furthermore, incidence was approximately 20% higher in males than in females across all regions and age groups.

Fifth, most human *Campylobacter* infections appear to be sporadic. Only a handful of household or localised outbreaks are identified each year in the UK from spatio-temporal clustering of reported cases (90,103). Cases might nonetheless be linked at household or community levels more so than is presently believed: approximately one in six reported

Campylobacter cases in England and Wales during 2000-2001 occurred at the same time as another case of gastrointestinal disease in the same household (12, 25).

Campylobacter epidemiology – potential causes

The causes of human *Campylobacter* epidemiology are studied using two main approaches, with additional information from reported outbreaks when available. First, case-control studies are used to characterise exposure risk factors for people acquiring infection. Second, microbiological surveys are used to characterise *Campylobacter* prevalence and bacterial load in potential sources of infection.

Case-control studies implicate diverse risk factors, including preparation and consumption of chicken, drinking unpasteurised milk, living in a rural environment, contact with farm and companion animals, travel overseas, drinking bottled water and having an untreated domestic water supply. Consumption of chicken is consistently identified as a risk factor (45, 49, 101), as is consumption of chicken dishes in restaurants (71). Infection sources are most easily identified when infections are clustered in outbreaks, *i.e*., when many cases can be used to implicate the same infection source, and least effective when cases appear sporadically (14, 76).

Microbiological surveys show that *Campylobacter* is widespread in the gastrointestinal flora of farm animals, companion animals and wild birds. In farm animals, intestinal carriage of *Campylobacter* in cattle ranges from 0.8% to 89%, with rates dependent on herd size and type, season, age of animal, sample site, sample frequency, isolation method, geography, diet and husbandry practices, and with higher values resulting from isolation by enrichment growth in addition to direct plating (113). Intestinal carriage of *Campylobacter* in lambs at slaughter can be as high as 91% (113). Prevalence of *Campylobacter* was over 30% in cattle faeces and over 40% in sheep faeces in a farmland area of Cheshire, UK, in 1998-1999, with almost all cattle isolates being *C. jejuni* and sheep having approximately equal proportions of *C. jejuni* and *C. coli* (8).

In companion animals, rates of intestinal carriage of *Campylobacter* in healthy cats and dogs in Norway compared with animals with diarrhoea were similar at 16-18% (cats) and 23-27% (dogs) (105). A longitudinal study in Denmark demonstrated higher carriage rates in healthy pet dogs: from 60% at 3-8 months old to nearly 100% at one year old (40). Approximately

three-quarters of the isolates in both studies were *C. upsaliensis*, which is rare in human infections, with most of the remainder being *C. jejuni*.

In wild birds, *Campylobacter* prevalence is typically lower than in farm and companion animals. Overall prevalence in pooled wild bird faeces collected near four broiler chicken sheds in Georgia, USA in 1997-1999 was 10% (15). Overall prevalence in faeces or cloacal swabs from almost 1800 migratory wild birds representing 26 avian families was 21%: approximately one-quarter (5%) were *C. jejuni*, one-quarter (5%) were *C. lari* and half (11%) were unidentified *Campylobacter* species (124). Overall prevalence in unidentified wild faeces from farmland in Cheshire, UK was approximately one-third, with approximately one-quarter being *C. jejuni* (8).

Microbiological surveys also demonstrate the widespread occurrence of *Campylobacter* in retail meats. A large survey of retail chicken in England carried out during 1998-2000 found that 83% of carcasses were contaminated with *Campylobacter*, with 20% of them having loads greater than 10^5 CFU (59). A second, larger survey that included Wales, Scotland and Northern Ireland carried out during 2001 found that an average of 50% of chicken carcasses were contaminated (27). A recent study in NE Scotland carried out during 2006 made similar findings: 90% of carcasses were contaminated with *Campylobacter*, with 4% of them having loads of 10^5 CFU or greater (35). In comparison, the infectious dose of *Campylobacter* for humans can be as low as 500-800 cells (62). Another study showed that *Campylobacter* occurs in approximately 60% of broiler flocks at slaughter in the UK (5).

Retail red meats typically have lower levels of *Campylobacter* contamination than retail chicken, with meat portions typically having lower levels of contamination than offal. In the UK during 2003-2005, *Campylobacter* occurred in 5% of portions *versus* 12% of offal in retail beef, 7% of portions *versus* 37% of offal in retail lamb and 5% of portions *versus* 18% of offal in retail pork (70). In New Zealand during 2003-2004, *Campylobacter* occurred in 3% of retail beef portions, 7% of lamb and mutton portions, and 9% of pork portions (130).

It is easy to believe that retail chicken is the single biggest source of human *Campylobacter* infection: there is good evidence for high prevalence, high bacterial loads and high consumption (annual production of broilers in the UK is approximately 800 million), and the most obvious routes of *Campylobacter* transfer to humans are cross-contamination from infected meat during food processing or in domestic kitchens, and inadequate cooking of infected meat. This belief is the basis of the current “FSA Strategy for the Control of

Campylobacter in Chickens", whose main goal is a 50% reduction in the prevalence of UK-produced chickens testing positive for *Campylobacter* by 2010.

This strategy seeks to persuade consumers to improve kitchen hygiene and to establish a HACCP framework in abattoirs processing chickens but its main goal is to persuade farmers to practice good biosecurity on broiler farms and in transporting birds to abattoirs. This strategy is based on scientific advice from the Second ACMSF Report on *Campylobacter* that appropriate biosecurity measures can prevent broiler flocks from becoming infected (2).

Two questions arise. First, can strategies aimed at reducing human *Campylobacter* incidence exclude non-foodborne sources from consideration with impunity? If non-foodborne sources do in fact contribute to infection, then stronger evidence for this will be needed. Second, will the FSA Strategy for the Control of *Campylobacter* in Chickens achieve its goal (28)? If retail chicken is indeed the single biggest source of human *Campylobacter* infection in the UK, and the strategy fails, then further action will need to be taken. Options include further improvements in farm biosecurity and flock scheduling in abattoirs, and further research and development of alternative interventions based on elimination. These options are costly, and their implementation will require stronger evidence for the contribution of retail chicken to human *Campylobacter* infection.

Stronger evidence for or against specific sources of human infection could be obtained in two ways. First, evidence for risk factors from case-control studies might be stronger if unrecognised outbreaks could be identified. Second, evidence for transmission routes might be stronger if human *Campylobacter* infections could be attributed to specific sources. Both aims are within the scope of molecular epidemiological analyses based on bacterial strain typing methodologies.

Molecular epidemiology of *Campylobacter*

Molecular epidemiology (as applied to pathogens) involves categorising pathogen isolates as "strains". Strain is a fluid definition that varies according to which assay is used to characterise, type, an isolate. Matching of strains among apparently sporadic cases of human infection is used to infer the occurrence of outbreaks, and matching of strains between infection sources and human cases is used to evaluate the contributions of different sources to human infection.

Campylobacter strain typing

Many different phenotypic and genotypic methods have been used to type *Campylobacter* (28, 126). Two phenotyping methods are referred to in this report. Penner heat-stable (HS) serotyping (94) involves detecting cell-surface antigens using polyclonal antibodies. Phage typing (36) involves detecting different patterns of cell lysis after exposure to different combinations of bacteriophage.

Six genotyping methods are referred to in this report. These in turn fall into two classes according to whether the raw data they yield is inherently continuous or inherently categorical. Pulsed-field gel electrophoresis (PFGE) (110) involves cutting genomic DNA into large fragments using restriction enzymes whose recognition sites are rare in bacterial genomes and separating the fragments by gel electrophoresis using electric fields pulsed in alternating directions. Ribotyping involves digesting bacterial genomic DNA into fragments using restriction enzymes with frequent recognition sites followed by Southern blotting and hybridization with a ribosomal RNA gene (rDNA) probe and visualisation of strain-specific rDNA fragments (117). Detection of *flaA*-RFLP (87) involves PCR amplification of the whole *flaA* gene, cutting the amplicon with restriction enzymes with frequent recognition sites followed by separation of the resulting fragments by gel electrophoresis and fragment visualisation. Detection of amplified fragment length polymorphisms (AFLP) (122)involves cutting genomic DNA into small fragments using a high-frequency cutting restriction enzyme, ligation of PCR primers to each end of the fragments, selective amplification of subsets of the fragments using primers with selective extensions, followed by separation of the resulting fragments by gel electrophoresis and fragment visualisation. *flaA*-SVR sequencing involves PCR amplification and DNA sequencing of the short variable region (SVR) of the *flaA* gene (79). Multi-locus sequence typing (MLST) (74) is similar to *flaA*-SVR sequencing except that short, variable regions of (typically) seven housekeeping genes are analysed. MLST categorises each isolate as a sequence type (ST) according to its allele profile across the set of genes. Isolates matching for the whole set of genes are categorised as being the same ST. Isolates mismatching for one gene of the set are defined as single-locus variants (SLV) and are categorised as being in the same clonal complex (CC). Isolates in the same ST or CC are assumed to have a common ancestor, which is assumed to be more recent for isolates in the same ST than for isolates in the same CC.

PFGE, ribotyping, *flaA*-RFLP and AFLP all yield continuous raw data, *i.e*., a linear series of DNA fragments characterised by their mobility in a gel matrix, although AFLP yields

categorical data when the fragments are sized on a DNA sequencer. In contrast, *flaA*-SVR and MLST both yield categorical raw data, *i.e.*, different DNA sequences. Strain profiles based on continuous data have low transferability, *i.e.*, they are not straightforward to compare between different laboratories or between different periods within the same laboratory. Strain profiles based on categorical data have much higher transferability, and this makes them straightforward to digitise, transfer in electronic form and codify and compare using global databases.

Strain clustering in outbreaks

The use of strain matching for detecting outbreaks of human *Campylobacter* infection has been evaluated by typing isolates from known or suspected local, small-scale outbreaks. In one study, nine isolates from a suspected outbreak in the Scottish Ayrshire and Arran Health Board, in which the patients had all eaten at the same canteen during June 1996, all matched for Penner HS serotype and PFGE, ribotype, and *flaA*-RFLP profiles, but the combined profile also matched with nine other isolates from apparently sporadic cases that occurred in five other Health Boards during 1995 and 1996 (44). In a second study, nine isolates from a suspected outbreak in a school in Kansas, USA in 1998 all matched for Penner HS serotype, PFGE and *flaA*-RFLP profiles and *flaA*-SVR sequence, and mismatched with 13 other isolates from apparently sporadic cases that occurred in the same county during 1998 (26). In a third study, seven isolates from a suspected outbreak in a nursing home in South Wales in 2000 all matched for Penner HS serotype, phage type and PFGE profile and mismatched with one isolate each from two other suspected outbreaks from the same locality during the same period. Nonetheless, the seven outbreak isolates also matched with three other isolates from apparently sporadic cases from the same locality during the same period (11). In a fourth study, 2-5 isolates from each of 12 known outbreaks that occurred in eight US states during 1981-1998 (including the Kansas school outbreak discussed above) either all matched for Penner HS serotype and profiles for PFGE, ST and *flaA*-SVR sequence within each outbreak group or mismatched with one isolate within the group (104). One or two additional isolates associated with seven of the outbreaks, but without epidemiological indication of being part of the outbreak, all mismatched with the isolates from the corresponding outbreak. These evaluation studies all point to the same conclusion: strain typing can be used to identify local outbreaks of human *Campylobacter* infection but outbreak identification based on strain typing data alone will be statistical and quantitative, not qualitative.

Spatio-temporal clusters of matching molecular profiles of *Campylobacter* strains are also evident in apparently sporadic clinical cases in Canada (82) and Denmark (30), potentially indicating outbreaks. Nonetheless, inferring outbreaks according to single-strain matching might fail when the outbreak contains several strains. Chickens (46, 106), pigs (112) and retail meats (64) often harbour multiple *Campylobacter* strains, as do some human patients (34, 98).

Host association of strains

Strain typing data provide evidence for the association of certain *Campylobacter* strains with specific hosts. Four MLST-based studies have evaluated host association in *C. jejuni* strains across farm animals, poultry and wildlife. The first study, involving hosts from NW England, N Wales and SW Scotland, found that strains in CC45 were associated with broiler and turkey chicks, CC42, 48 and 61 strains were associated with cattle and sheep, whereas CC21 strains occurred in all sources analysed (13). The second study of farm animals, mostly from the UK and most of the rest from N Europe, also found that CC45 strains were overrepresented in poultry (along with CC257 and CC283 strains), that CC48 and CC61 strains were overrepresented in cattle, that CC42 strains were overrepresented in sheep, and that CC403 strains were overrepresented in pigs (76). The third study, based in a cattle farming area of Cheshire, UK, found that CC45 strains were overrepresented in wild birds and rabbits and that CC61 strains were overrepresented in cattle (29). The fourth study, based on a global collection of strains, found that approximately 80% of strains could be correctly assigned to chickens or to bovids (cattle and sheep) using the alleles of the MLST profile whereas assignment based on ST or CC was much less accurate (77). One AFLP-based study has evaluated the host association of *C. jejuni* and *C. coli* strains across cattle, pigs, poultry and retail meats, finding evidence for host association for *C. coli* but not for *C. jejuni* (48). One MLST-based study has evaluated the host association of *C. coli* strains across pigs, cattle and poultry from the USA, finding strong evidence for it. One strain (ST1068) accounted for most of the cattle isolates, and approximately three-quarters of common alleles were associated with pigs or poultry, but not both (84).

These studies suggest that the degree of host association varies across *C. jejuni* and *C. coli* strains: some strains show consistent associations whereas others occur in all hosts. They also suggest that the degree of host association detected depends on the level of molecular resolution used in the analysis, being lowest at the level of strains defined as ST or CC using

MLST data and highest at the level of alleles within MLST loci. The MLST studies that discuss the host association of *Campylobacter* strains from specific environmental sampling were all based on collections of a few hundred isolates at most. This sample size limitation meant that host association had to be analysed at the CC, not the ST, level.

Attribution of clinical strains to infection sources

Host associations of *Campylobacter* strains provide the basis for attributing human clinical strains to different infection sources. Several such studies have been carried out, and their main conclusions are summarised in Table 1. One of the first studies, based in the Netherlands and typing strains by AFLP, found that *C. jejuni* and *C. coli* strains from poultry houses overlapped with clinical strains from the same area (21). This finding was confirmed in two studies of strains from retail chicken and human disease, one based in Christchurch, New Zealand and analysing *C. jejuni* and *C. coli* (52) and the other based in Helsinki, Finland and analysing *C. jejuni* (42), with both studies typing strains by PFGE. These studies all suggest that one source of human *C. jejuni* and *C. coli* infection is poultry.

A study based in NW England found that *C. jejuni* and *C. coli* strains, isolated from cattle, sheep and turkeys and typed by PFGE and *flaA*-RFLP, overlapped with strains from human clinical cases occurring in the same area (29). A study based in the UK found that *C. jejuni* strains, isolated from cattle, sheep, pigs, poultry and pets throughout the UK and Europe and typed by MLST, also occurred in human clinical cases (76). Three further studies confirm the idea that most farm species are sources of human infection: a UK-based farm-scale study of *C. jejuni* strains isolated from cattle, sheep, poultry, and human clinical cases and typed by MLST (13); a study based in Japan of *C. jejuni* and *C. coli* strains isolated from cattle, poultry, pigs and human clinical cases and typed by *flaA*-RFLP (56); and a study based in Canterbury, New Zealand of *C. jejuni* and *C. coli* strains isolated from cattle (faeces and offal), sheep (faeces and offal), pigs (offal), chicken carcasses, ducks (faeces), environmental water and human clinical cases, and typed by Penner HS serotyping and PFGE (17). Further analysis of the dataset from the latter study implied that the main sources of human infection were cattle and sheep, with the other sources having only minor contributions (31). This conclusion was partly supported by another New Zealand-based study of *C. jejuni* and *C. coli* strains isolated from retail beef, lamb and mutton, chicken, pork and, unweaned veal, which found that these strains overlapped with human clinical strains in decreasing order of the above list of meat types (130). A counter-example comes from a

comparison of *C. jejuni* and *C. coli* strains from cattle, poultry and human infection from sources in Finland, typed by PFGE, which found that human isolates showed most overlap with poultry isolates and much less overlap with isolates from cattle (60). These studies identify poultry, cattle and sheep as the major overall sources of human *C. jejuni* and *C. coli* infection but they also suggest that the major source can depend on location, such that poultry was the main source in the studies of urban and suburban areas whereas farm ruminants (cattle and sheep) were the main sources in the studies of rural areas.

A study based in Denmark found that *C. coli* strains, isolated from poultry (chicken, ducks, turkey and ostrich), pigs and human clinical cases and typed by Penner HS serotyping and AFLP, showed that the human strains overlapped with the poultry strains but not the pig strains (107). Two other studies strengthen the idea that pigs are not a source of human infection: another study based in Denmark and involving *C. coli* isolates typed by MLST (69) and a study based in Quebec, Canada that found no overlap between *C. coli* strains isolated from pigs and human infections and typed by PFGE (37). According to these studies, pigs are a very minor source of human *C. coli* infection.

Two studies evaluated the contribution of wildlife hosts to *Campylobacter* infection of humans and broiler flocks. The first study, based in Denmark, of *C. jejuni* strains from seven wild animal species, nine wild bird species, broiler chickens and human infections, and typed by Penner HS serotyping, PFGE and *flaA*-RFLP, found little or no overlap between wildlife strains and broiler or human strains (97). The second study, based in Sweden, of *C. jejuni* strains from 20 species of migratory birds and human infections, and typed by PFGE, found little or no overlap between the strains from birds and the strains from human infections (7). According to these studies, wild animals and birds are never more than occasional sources of human *C. jejuni* infection.

Most of these studies provide only a crude, qualitative indication of overlap between clinical and infection source strains of *Campylobacter*. The present study aims to take attribution analysis to the next level by providing quantitative values of attribution of clinical strains to sources.

Limitations of strain typing and potential of MLST

The above studies of *Campylobacter* molecular epidemiology show that strain diversity remains high throughout the infection chain: from sources through infection vehicles to

human clinical cases, consistent with the existence of a very large pool of strains. According to this view, the main features of *Campylobacter* transmission will be evident only at large spatio-temporal scales, *e.g*., at a national scale. Most of the studies conducted to date on *Campylobacter* molecular epidemiology are small-scale because the strain typing methods used are unsuitable for high sample throughput, and this makes it difficult for any one study to yield national-scale conclusions. A larger-scale picture could emerge from combining raw datasets across studies but this is difficult to achieve because most of the strain typing methods used yield non-transferable data. These limitations of strain typing methods explain the dearth of studies conducted at national scales and covering a wide range of reservoir hosts and clinical cases.

MLST methods are well developed for *Campylobacter* (18-20,85). MLST is applicable to all strains of *C. jejuni* and *C. coli*, is suitable for high throughput and yields inherently categorical data that are straightforward to acquire, store, transfer and analyse electronically. Global databases and online analytical programmes are available for all stages of MLST analysis from profiling new isolates to analysis of strain matching. The seven housekeeping genes typed in *Campylobacter* MLST are considered to be largely free of the effects of strong selection (95).

Table 1. Summary of outcomes of studies of overlap between *Campylobacter* strains from clinical infections and from potential infection sources.

					Potential infection source												
Ref	Country	Period of strain isolation	Species	Typing method	Cattle [1]	Beef or veal [2]	Sheep [1]	Lamb or mutton [2]	Pig [1]	Pork [2]	Broiler poultry	Retail chicken	Other poultry [1]	Pets	Wild animals	Wild birds	Water
21	Netherlands	1993	Cc & Cj	AFLP	-	-	-	-	-	-	●	●	-	-	-	-	
52	New Zealand	1996 -97	Cc & Cj	Penner PFGE	●	-	●	-	-	-	-	●	-	-	-	-	●
42	Finland	1996 -98	Cj	PFGE	-	-	-	-	-	-	-	●	-	-	-	-	-
29	UK	1993 -96	Cc & Cj	PFGE flaA-RFLP	●	-	●	-	-	-	-	-	●	-	-	-	-
76	UK, Denmark Czech Republic Netherlands S. Africa France, Sweden	1988 1999 2000	Cj	MLST	●	-	●	-	○	-	●	-	-	●	-	-	-
13	UK	1990s	Cj	MLST	●	-	●	-	-	-	●	-	●	-	-	●	-
56	Japan	2001 -03	Cc & Cj	flaA-RFLP	●	-	-	-	-	-	●	-	-	-	-	-	-
17 31	New Zealand	2001 -02	Cc & Cj	Penner PFGE	●	●	●	●	-	●	-	●	●	-	-	-	●
130	New Zealand	2003 -04	Cc & Cj	Penner PFGE	-	●	-	●	-	●	-	●	-	-	-	-	-
60	Finland	2003	Cc & Cj	PFGE	○	-	-	-	-	-	-	●	-	-	-	-	-
107	Denmark	1999 -2001	Cc	Penner AFLP	●	-	●	-	○	-	●		●	○	-	-	-
69	Denmark	1996 -2002	Cc	MLST	●	-	-	-	○	-	●	●	-	-	-	-	-
37	Canada		Cc & Cj	PFGE	-	-	-	-	○	-	-	-	-	-	-	-	-
97	Denmark	1996 -98	Cj	Penner flaA-RFLP PFGE	-	-	-	-	-	-	●	-	-	-	○	○	-
7	Sweden	2000	Cj	PFGE	-	-	-	-	-	-	-	-	-	-	-	○	-

Notes: Ref, citation reference in Citations section; Cc, *Campylobacter coli*; Cj, *Campylobacter jejuni*; ●, strains from the indicated source overlapped with clinical strains; ○, strains from the indicated source showed little or no overlap with clinical strains; - , source was not sampled; 1: faeces or caeca; 2: retail meat or offal.

2.2 Aims of the study

The 2nd Report on *Campylobacter* to the FSA from the Advisory Committee on the Microbiological Safety of Food published in 2005 (2) discusses using strain typing for improving knowledge of *Campylobacter* epidemiology, and assesses the technical aspects of different *Campylobacter* typing schemes. One of the report's conclusions is that

> *MLST ... offers an opportunity in the short to medium-term to improve our knowledge of what is a very complex epidemiological story*

and recommends that:

> *the FSA needs … to [bring] together laboratories capable of applying MLST so that investigative programmes can be designed to improve our epidemiological understanding …*

The present study implements this recommendation as a national-scale study of *Campylobacter* molecular epidemiology in Scotland. This study was lead by the Department of Medical Microbiology at the University of Aberdeen who have expertise in the epidemiology of human bacterial diseases, in collaboration with the University of Oxford group that developed MLST for *Campylobacter*, the NHS diagnostic laboratories in Scotland and many other providers of specimens and isolates from across Scotland.

Scotland provides an ideal setting for such a study. Human *Campylobacter* infection in Scotland is representative of the rest of the UK. Scotland has a unified public health service that provides routine diagnosis of reported *Campylobacter* infections in hospital bacteriology departments covering the whole country. Scotland also has four public analyst laboratories providing 32 local authorities with analytical services for foodborne contamination. Food sources in Scotland are a representative mix of local, UK-sourced, supermarket, and imported produce. Scotland's population is spread across both urban and rural areas and is thus exposed to sources of infection typical of both environments. Scotland's population (5.5M) is large enough to represent society-level transmission but small enough that total numbers of clinical *Campylobacter* isolates are manageable. The study was designed to include isolates from all reported human *Campylobacter* cases of infection in Scotland during a 15-month period that encompassed a summer infection peak, and isolates from a wide range of infection sources collected during the same period.

Objectives

The project Scope of Work consisted of five objectives subdivided into tasks. These are listed below with summaries of actual activities as modified during the project.

Objective 01. Collect clinical *Campylobacter* isolates and isolate *Campylobacter* from food and environmental samples.

Task 01/01. Collection of clinical isolates.

Task 01/02. Collection of food and environmental isolates.

Approximately 5,500 clinical *Campylobacter* isolates, corresponding to almost all cases reported in Scotland from mid-July 2005 to mid-October 2006, were collected. Approximately 3,000 environmental and food specimens were collected from three urban and two rural settings across Scotland, and approximately 1,100 *Campylobacter* were isolated from these. Basic epidemiological information was collected for the clinical specimens. Prevalence and bacterial load data were collected for the positive food and environmental specimens. The *Campylobacter* isolates from all sources were archived.

Objective 02. Speciate the clinical, food and environmental isolates.

Task 02/01. Speciate clinical isolates and confirm quality of DNA.

Task 02/02. Speciate food and environmental isolates and confirm quality of DNA.

This task became unnecessary in the form originally proposed and was abandoned. The species identifications were actually obtained from typing data (see Objectives 3 and 4).

Objective 03. Undertake multilocus sequence typing (MLST) of *C. jejuni* and *C. coli* clinical isolates.

Task 03/01. MLST of *C. jejuni* clinical isolates.

Task 03/02. MLST of *C. coli* clinical isolates.

Objective 04. Undertake MLST of the *Campylobacter* food and environmental isolates.

Task 04/01. MLST of *C. jejuni* food and environmental isolates.

Task 04/02. MLST of *C. coli* food and environmental isolates.

The MLST profiles of all isolates were obtained using a typing method applicable to strains of both clinical and food and environmental origin, and to both *C. jejuni* and *C. coli* strains. All data were tabulated in a relational database.

Objective 05. Analyse MLST profiles of *Campylobacter* clinical, food and environmental isolates, and other published profiles. Disseminate the results and produce the final report and publications for peer review.

Task 05/01. The molecular epidemiology of *Campylobacter* in human disease in Scotland.

Task 05/02. The food and environmental *Campylobacter* in Scotland.

Task 05/03. The population genetics of *Campylobacter.*

The data analysis focused on five main aspects most relevant to the FSA goal of an improved understanding of *Campylobacter* epidemiology: (i) species and strain diversity of *Campylobacter* isolates from clinical infections, host species and foods, (ii) differences between Health Boards, months of collection and patient age classes in *Campylobacter* species and strain composition, (iii) known outbreaks of human *Campylobacter* infection analysed according to strain clustering and patient co-infection, (iv) unknown outbreaks of infection inferred from incidence peaks and strain clustering in clinical cases, and (v) association of *Campylobacter* species and strains with particular reservoir hosts or foods, and attribution of the likely origins of human *Campylobacter* infection.

3 Methods

3.1 Specimen collection

Clinical specimens

Ethical approval for the collection of the information used in this project was obtained (Appendix A). *Campylobacter* isolates from clinical specimens were obtained from NHS clinical diagnostic laboratories in Scotland during mid-July 2005 to mid-October 2006. All 28 laboratories were contacted before the study, and all agreed to participate. These laboratories use microbiological culture to confirm *Campylobacter* presence in specimens from patients with symptoms typical of *Campylobacter* infection. The University of Aberdeen (UoA) provided the laboratories with transport swabs for returning isolates by post, and paid a small sum of money to cover costs for each isolate. The supplying laboratories staff recorded information about each isolate on the swab label. The information requested was: 1. submitting laboratory; 2. patient date of birth; 3. laboratory specimen number; 4. date that the *Campylobacter*-positive case was reported. On receipt at UoA, all samples were given a unique code number, the swab label information was entered into a Microsoft Access database, and this information was rechecked against the database before disposal of the swab.

Environmental and food specimens

Specimens from potential sources of *Campylobacter* infection were collected from animal and wild bird faeces and retail meats. Faecal specimens of animals and wild birds were collected in rural NE and SW Scotland (the Health Board areas of Grampian, Dumfries & Galloway and Ayrshire & Arran) and in Aberdeen, Edinburgh and Glasgow city centres (Table 2; Figure 6). Glasgow Veterinary School and the SAC regional laboratories also supplied *Campylobacter* isolates from farm and, generally ill, companion animals.

Comprehensive sampling of retail foods was outside the remit of this project. Instead, retail food types that (according to published studies) typically had high *Campylobacter* prevalence (mainly retail chicken but also cattle, sheep and pig offal) were targeted for sampling in NE Scotland. Local authority surveillance programs in Scotland also supplied *Campylobacter*

isolates from retail chicken. Table 3 summarises the sites and the number of specimens collected. The collection schedule spanned the duration of the project with two intensive periods of sampling: one of farms in postcode district AB31, NE Scotland (Figure 7a, green histograms in weeks 25-40 of 2006) and one of bought retail chicken in NE Scotland (Figure 7b, red histograms in weeks 16 and 18 of 2006).

The faecal specimens of animals and wild birds were collected mainly at farms and outdoor, urban areas frequented by people. The farms were chosen as follows. The postcode districts (indicated by the first part of the postcode) in the three rural areas were tabulated, the central town or village was identified, farmers listed in “yell.com” under that location were contacted by phone, and one willing farmer in each postcode district randomly chosen for each collection visit. Intensive, single-week collection trips focused on three or four neighbouring postcode districts, and the trips continued until approximately 500 samples had been collected from 80-100 sites in each Health Board Area (Table 2; Appendix C). SAC staff visited the farms in Ayrshire & Arran and Dumfries & Galloway Health Boards, with nine weeks of sampling effort spread as evenly as possible across both Health Boards. UoA staff visited the farms in Grampian Health Board, with 10 weeks of sampling effort and a final session over a three-week period spread as evenly as possible across the Health Board. All but two farms were visited once, and the exceptions were visited twice. Farms in postcode district AB31 in Grampian Health Board were visited successively in a more intensive schedule to provide temporal coverage in addition to the spatial coverage of the national sampling scheme (Appendix C).

The aim on each farm visit (excluding the visits to farms in AB31) was to collect 13-15 fresh faecal specimens comprising four from cattle, four from sheep and the rest from other available species: mainly poultry, pigs, companion animals (cats, dogs, horses) or wild birds (pigeons, starlings, geese, gulls and corvids). The actual number of specimens collected depended on the species available at each site, and almost all the cattle were beef cattle. All specimens were placed in a cool box containing bubble-wrapped cool packs. SAC staff sent the specimens they collected to UoA by overnight courier. The faecal specimens from the three city centres were collected by UoA staff at public parks and ponds, waterfronts, open-air dining areas and public squares, where wild bird and pet faeces were expected to be common and where people congregate.

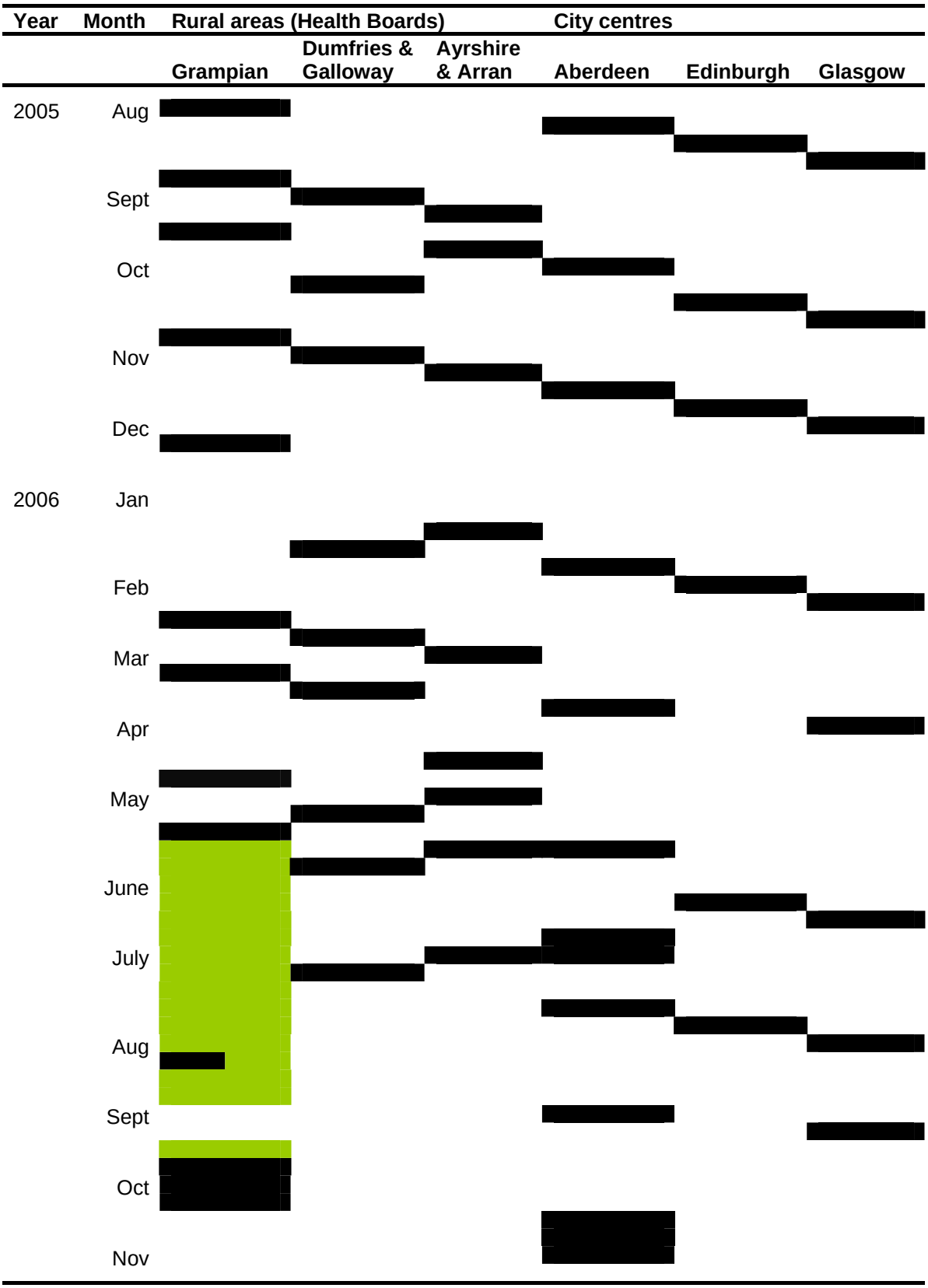
The specimens of large-animal faeces were collected as grab samples of 50-100g. Specimens of smaller, avian, faeces were collected using a sterile swab moistened with

distilled water or saline. Avian faecal specimens were categorised to source species when a flock of a single, visually-identified species was seen leaving the collection site, and as unidentified when no species origin was evident. Gulls were not identified to species but most were probably herring and black-headed gulls in coastal areas, and black-headed or common gulls inland. Faecal specimens were collected fresh whenever possible, and information on the specimen and the site's GPS location was recorded (Appendix C).

Veterinary samples (mainly bacterial cultures) were obtained from diagnostic laboratories at the Glasgow Veterinary School and from SAC regional laboratories in Aberdeen, Edinburgh (including Capital Diagnostics and Penicuik), Perth and St. Boswells.

Retail chicken and raw liver portions were purchased from supermarket outlets and butcher shops in Grampian Health Board during October 2005 to September 2006. Chicken breasts, drumsticks, wings, thighs, legs and whole carcasses were included. Fresh and frozen specimens were collected in an overall 4:1 ratio, as in a previous 2001 FSA study (27). The animal species of the liver specimens was recorded. *Campylobacter* isolates from Public Analyst surveillance programs were obtained from laboratories in Aberdeen, Dundee, Edinburgh and Glasgow. These isolates originated from retail chicken and a few raw liver portions sold at retail food outlets in city centres and towns across Grampian, Tayside, Lothian, Greater Glasgow, Forth Valley and Highland Health Boards.

Table 2. The schedule for collecting environmental specimens.



Collection weeks for the main schedule (black) and the AB31 postcode district (green).

Figure 6. Locations of collection sites of environmental specimens.

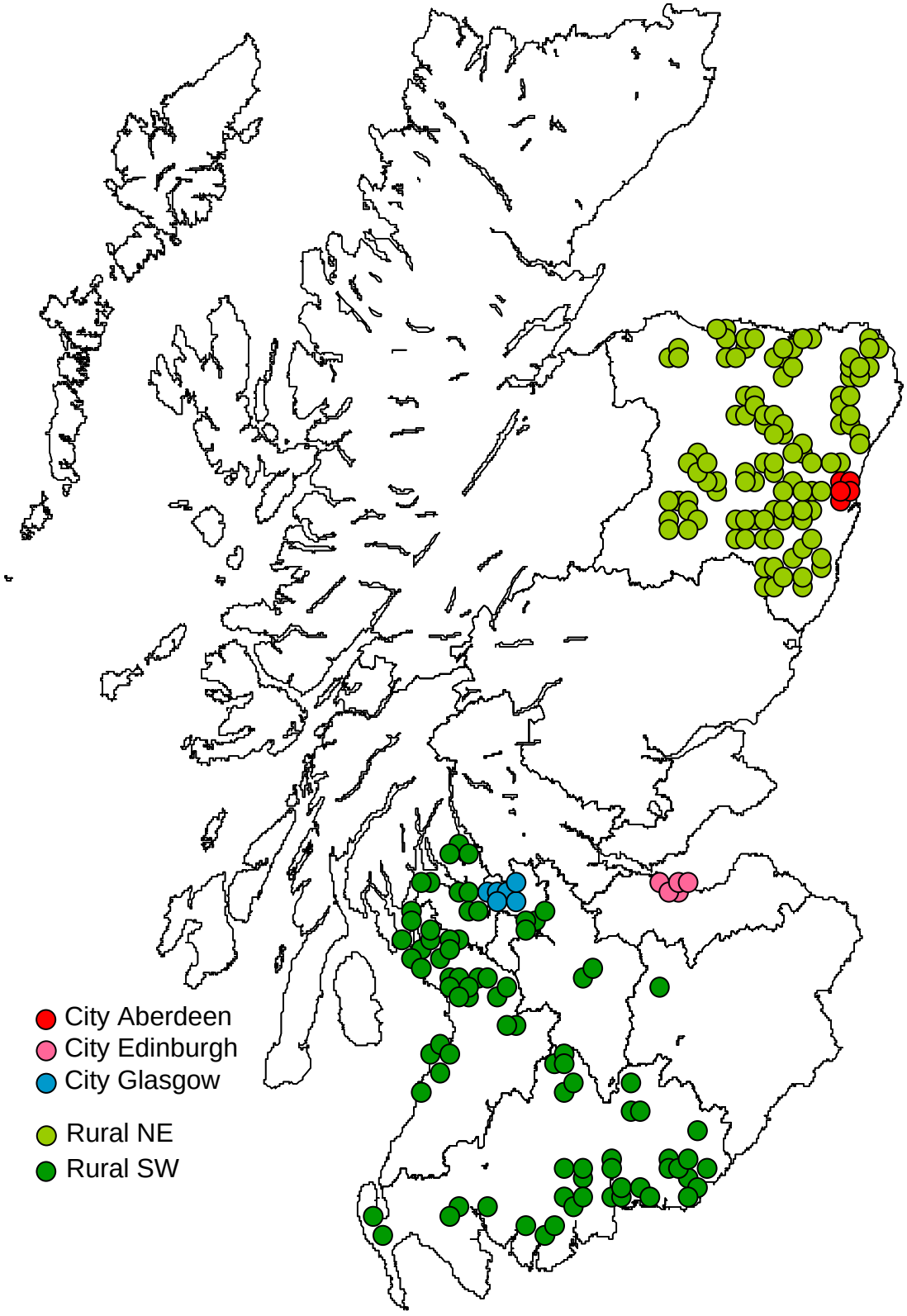
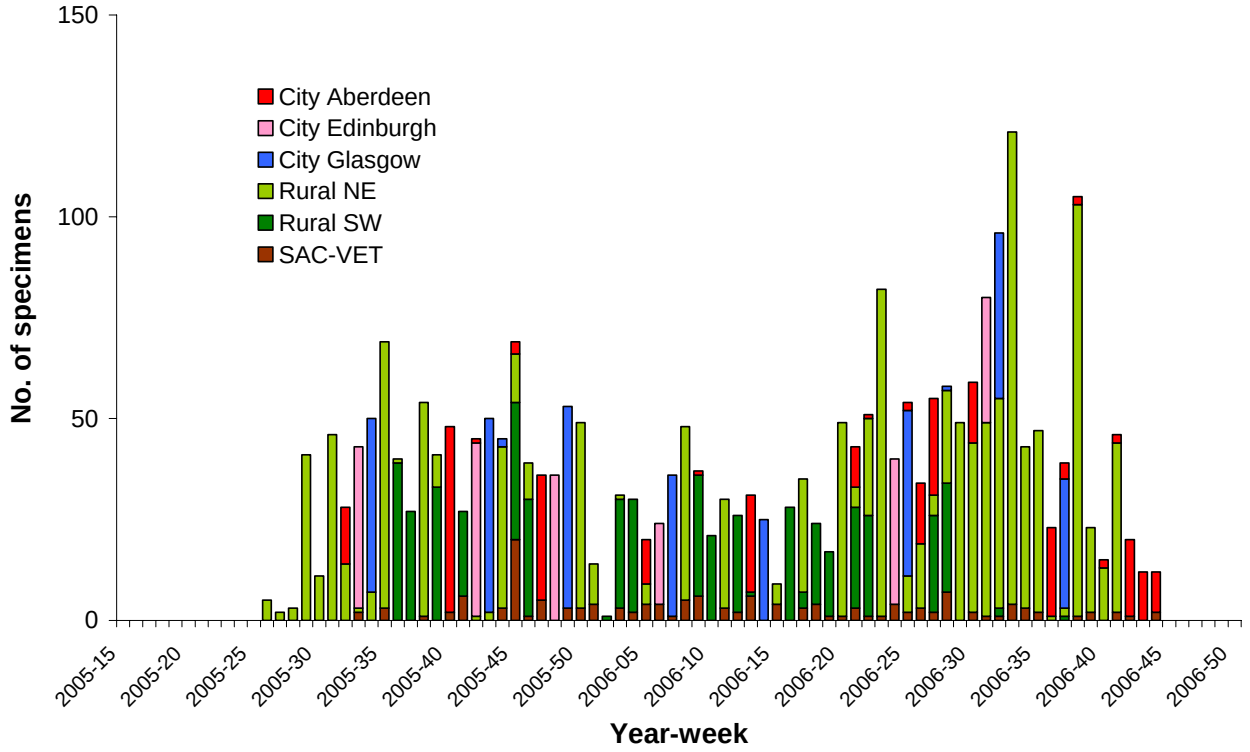


Figure 7. Numbers of environmental and food specimens collected.

(a) Environmental specimens



(b) Food specimens

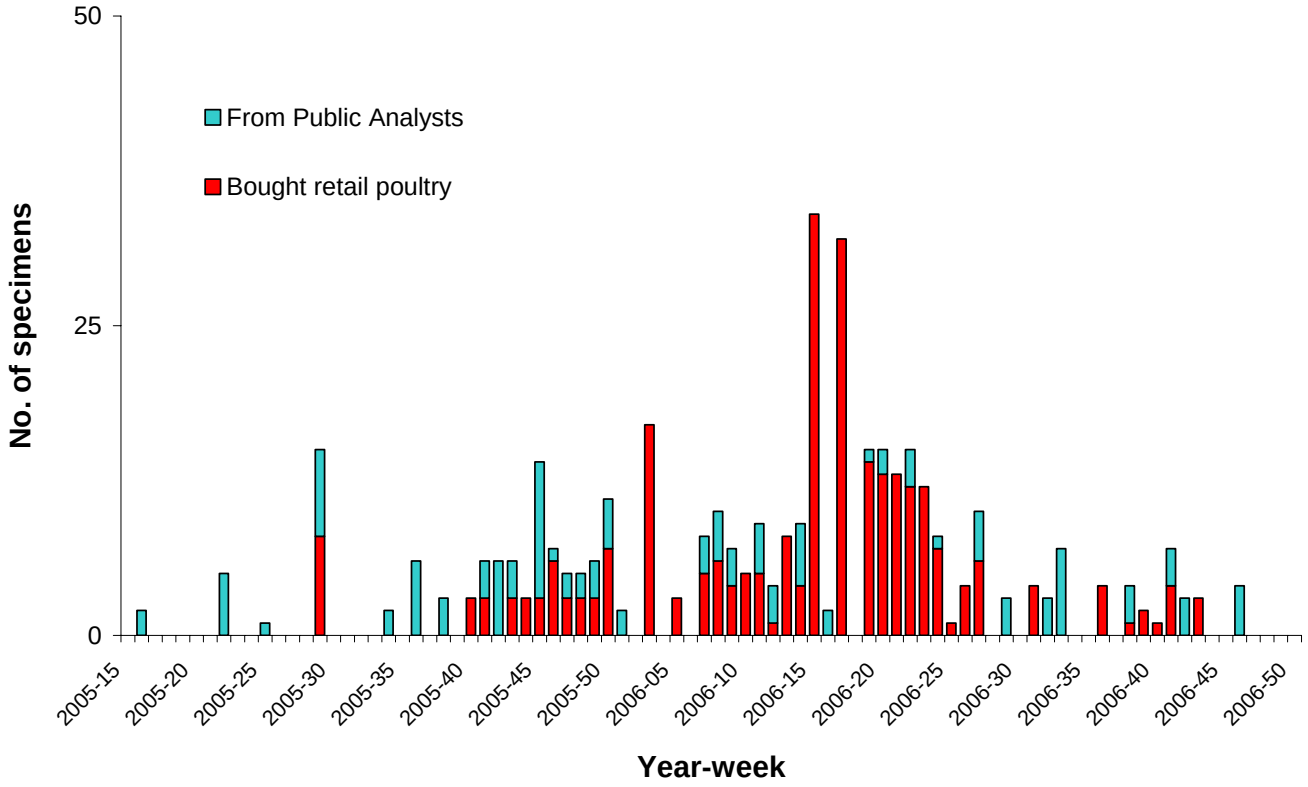


Table 3. Numbers of sites sampled and specimens collected from potential sources of *Campylobacter* infection in Scotland.

	NE Scotland	SW Scotland	Aberdeen	Edinburgh	Glasgow	SAC Vet [1]	Public Analysts [1]	Total
No. of sites	172 [2]	83	27	13	10			305
No. of specimens								
Farm species	1163 [3]	449	14			25		1651
Wild bird	68	19	197	181	282			747
Companion animal	22	7	53	21	31	112		246
Retail chicken and offal	270						124	394
Total specimens	**1523**	**475**	**264**	**202**	**313**	**137**	**124**	**3038**

1: most of the bacterial isolates from SAC and Glasgow Vet School, and all those from Public Analysts, had no supplied information on site of origin; 2: value includes the sites for retail food (N=66); 3: specimens from both the structured and the intensive surveys of farms in NE Scotland.

3.2 Laboratory methods

Microbiology

All the clinical isolates and a minority of the environmental and food isolates were obtained as *Campylobacter* cultures provided by hospital, veterinary or Public Analyst diagnostic laboratories. These laboratories had confirmed the presence of *Campylobacter* in blood, faecal or food-wash specimens by microbiological analysis, followed by observation of Gram-staining morphology typical of *Campylobacter*. The laboratories had prepared the *Campylobacter* isolates as whole-plate sweeps of colony growth harvested onto charcoal transport swabs supplied by UoA, and had posted the isolates as quickly as possible afterwards. Upon receipt at UoA, isolates were subcultured on charcoal cefoperazone deoxycholate agar (CCDA) plates, or on CCDA plates followed by subculture on the more selective modified CCDA (mCCDA) plates in the few cases with high background growth of yeasts. Plates were incubated under microaerobic conditions (2% H_2, 5% O_2, 5% CO_2, balance N_2) at 37°C for 2-4 days. *Campylobacter* colony growth was harvested into storage vials for archiving at –80^{o}C and into buffer for DNA extraction.

All the remaining *Campylobacter* isolates from animal, bird and food sources were obtained at UoA by microbiological culture from faecal or food portion specimens. One 10 g subsample of each large-animal faecal specimen was homogenised in 90 ml of *Campylobacter* enrichment broth (Appendix D), and one 1 g swab or subsample of each small avian faecal specimen was homogenised in 10 ml of broth. Each food specimen was bathed at ambient temperature in enrichment broth for 1 h with occasional agitation; the volumes used were 300 ml for portions and 500 ml for whole carcasses.

Enumeration of *Campylobacter* from faecal and food specimens was by direct plating, and further isolations made after extended growth in enrichment broth. For direct plating, decimal dilutions (10-fold, 100-fold and 1000-fold for both faecal homogenates and food portion washes) were prepared, 0.1 ml of each dilution was plated onto a CCDA plate, and the plates incubated as described. For enrichment growth, the remaining volume of the faecal homogenate or food portion wash was incubated under microaerobic conditions at 37ºC for 2 days. Samples (0.1 ml) were removed, plated onto CCDA plates, and the plates incubated as described. The presence or absence of *Campylobacter* colonies was determined according to a visible agglutination with Microscreen latex (product code M46, Microgen Bioproducts). Colony growth from both isolation procedures was harvested, archived and prepared for DNA extraction as described.

Campylobacter prevalence values were calculated as the percentage of samples (faecal specimens, swabs, carcasses or food portions) that yielded *Campylobacter* presence, irrespective of whether *Campylobacter* was detected using direct plating, enrichment growth or both methods, or of whether a single strain or multiple strains were isolated from a sample.

Campylobacter colonies were counted visually on the plate in the decimal dilution series with a countable number of colonies (1- approx. 200), and were standardised as CFU/g of original specimen for the faecal specimens and as CFU/sample for the food specimens. The range of possible counts for the faecal specimens was 10^2-10^7 CFU/g. The range of possible counts for the food specimens was >$3x10^3$-$3x10^8$ CFU/sample (portions) and >$5x10^3$ -$5x10^8$ CFU/sample (whole carcasses).

DNA extraction

During the first five months of the project, the “Oxford” DNA extraction method (boiling a bacterial suspension in phosphate-buffered saline solution) was used. This method yielded a

high failure rate in MLST so an alternative rapid method involving Chelex resin was tested, found to be effective, and used for all new isolates and all isolates that had previously failed in MLST (Appendix D).

Campylobacter speciation and MLST

The first published schemes for *Campylobacter* MLST featured different PCR primers for *C. jejuni (19)* and *C. coli* (18). These MLST schemes were used initially, and presumptive *Campylobacter* isolates were first speciated by multiplex PCR. Two assays were used: one (125) that was found to yield unreliable results for identifying *C. coli*, and a more recent alternative (61) proving more reliable results (Appendix D). A "second-generation" *Campylobacter* MLST scheme (85) was used subsequently because *C. jejuni* and *C. coli* can be typed with the same "Miller" primers, making speciation unnecessary (Appendix D).

Isolates were typed for two additional genes: the short variable region (SVR) sequence of the flagellin component gene *flaA*, and a variable central region of major outer membrane protein gene *porA*. Virtually all isolates were typed for *flaA* and about 20% were typed for *porA* (primer sequences were unavailable initially). These additional data will be analysed and detailed in a subsequent report.

DNA samples were processed in 96-well plates and a Biomek 2000 liquid-handling robot was used to set up PCR and DNA sequencing reactions. Both measures were highly effective for increasing sample throughput. Sequence electropherograms were assembled to double-strand sequence and assigned to MLST alleles for each batch of DNA samples from one 96-well plate using the semi-automatic processing available in the STARS package (Man-Suen Chan and Nicki Ventress, University of Oxford). A new STARS project template was designed for processing electropherograms generated using the "Miller" primers for the seven standard MLST genes and the primers for *flaA* and *porA* used in this project. Sequence types were assigned from allele profiles at the seven MLST loci using the online facility available at the C. jejuni/C. coli MLST website (http://pubmlst.org/). See Appendix D for the detailed laboratory protocols for MLST and DNA sequencing.

Access database

The information on specimens was tabulated in a Microsoft Access database (Appendix D). Patient dates of birth were obtained by translation of the patient ID code, which in most cases was either a 6-digit code, *i.e*., a date of birth in DDMMYY format, or a 10-digit code, *i.e*., a DDMMYY date of birth with 4 extra terminal digits. Dates of birth and specimen numbers were also provided as independent electronic data for all the isolates from Grampian Health Board. Clinical isolates likely to have originated from the same patient were identified because they could create false clusters: groups of isolates with matching patient ID codes that (i) originated from the same NHS hospital, (ii) were reported during the same 2-week period, and (iii) had the same ST-flaA type were identified. In each group, the isolate with the earliest reporting date was categorised as primary and the others, as duplicates, which were excluded from subsequent analyses. Post codes and Ordnance Survey grid references were obtained from streetmap.co.uk. Molecular data were obtained from the *C. jejuni*/*C. coli* MLST website (pubmlst.org/perl/mlstdbnet/mlstdbnet.pl?file=pub-cj_isolates.xml), the *Campylobacter flaA* variable region website (hercules.medawar.ox.ac.uk/flaA/), and the *Campylobacter porA* website (hercules.medawar.ox.ac.uk/momp/ - ID & password access).

Data analysis

The statistical methods and software packages used are summarized here. Further details are given below in the appropriate results and discussion sections.

The numbers and proportions of *Campylobacter* isolates, species and STs in different groups were calculated and displayed using arithmetic and graphical functions in Microsoft Excel.

Strain diversity was quantified using the Hunter-Gaston diversity index DI (54) calculated using the V-DICE calculator (www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl). DI is a measure of the diversity of types in a group that takes account of differences in sample size among groups. DI values range from 0 (all types are the same) to 1 (all types are different). Strain diversity was further characterized by rarefaction using the RarefactWIN program (www.uga.edu/~strata/software/Software.html).

Evidence for stratification of *Campylobacter* across various categories of grouped isolates was assessed using the software packages ARLEQUIN (24) and GENEPOP 4 (strucFile setting) (102). How much strain diversity was present at different hierarchical levels among categories of grouped isolates was estimated using AMOVA in ARLEQUIN. Monthly variation in the occurrence of strains was quantified as the coefficient of variation of the monthly total of each strain.

Temporal clustering of clinical *Campylobacter* cases within Health Boards was characterised using Fourier time series models (83) and randomisation tests (75).

Spatial and temporal clustering of clinical *Campylobacter* strains with matching ST was characterized using customized randomization procedures and the software package SATSCAN (66).

Attribution of clinical *Campylobacter* isolates to infection sources was done using arithmetic and graphical functions in Microsoft Excel.

4 Results

4.1 Yield of *Campylobacter* isolates, prevalence and bacterial loads

Yield of isolates

Clinical isolates of *Campylobacter* were submitted from 15th July 2005 to 26th September 2006 from all of the hospital diagnostic laboratories in Scotland that deal with cases of clinical infection (Table 4; Appendix E). These isolates represented over 97% of the cases reported to Health Protection Scotland (HPS) during that period (Figure 8; Table 4). Four Health Boards (Borders, Fife, Forth Valley and Greater Glasgow) had lower submissions than the other 11 Health Boards compared to the number of cases recorded by HPS. Discrepancies between the number of isolates collected and the number of cases recorded by HPS were due to a number of different reasons but included the areas covered by some hospitals and HB changing and inconsistent notification of cases.

Environmental and retail food isolates of *Campylobacter* were obtained from every specimen type collected (Table 5a). Approximately half of these isolates were from farm species, just over one-quarter were from retail chicken and offal, one-sixth were from wild birds and the rest from companion animals. The most intensively sampled species within the “farm species” category were cattle and sheep, and within “wild bird”, feral pigeons and gulls (Appendix C).

Campylobacter prevalence

The prevalence of *Campylobacter* in clinical isolates was not determined as all isolates were provided as cultures of *Campylobacter*.

Campylobacter prevalence in environmental sources and retail foods fell into three ranges (excluding isolates from presumably ill animals supplied as veterinary swabs): 0-14% in companion animals, 21-36% in farm species and wild birds and 69-75% in retail chicken and offal (Table 5b). Cattle and sheep faeces were sampled in NE and SW Scotland in a structured survey consisting mostly of a single visit to each farm. Cattle and sheep faeces were also sampled in NE Scotland in postcode district AB31 during the summer months of

2005 and 2006 in a longitudinal survey conducted at six farms. *Campylobacter* prevalence values in the structured survey were similar across the four combinations of host species and area: cattle in NE Scotland (26.4%, N=220), cattle in SW Scotland (24.7%, N=263) sheep in NE Scotland (24.9%, N=217) and sheep in SW Scotland (25.6%, N=184), where N is the number of faecal samples analysed.

The intensive survey in NE Scotland yielded prevalence values for cattle (53.1%, N=516) that were over two times higher, and values for sheep (42.9%, N=70) that were approximately one-and-a-half times higher, than the corresponding values for the structured survey. This might be partly due to higher *Campylobacter* prevalence in cattle and sheep during the summer months, as preliminary results indicate (Figure 9).

The overall prevalence of *Campylobacter* in chicken portions from retail outlets in NE Scotland was 74.2% (N=217), and monthly values were in the range 53-94% for the nine months when at least 10 specimens were collected, with no obvious seasonal variation in prevalence. *Campylobacter* prevalence in liver portions ranged from 50-100% in each of the four source species tested and the overall prevalence was approximately 60% (Table 6).

Campylobacter prevalence in two potential vector groups (flies and slugs) was characterised in a parallel BBSRC Ph.D. project (by E. Sproston). Approximately 10% of the pooled samples for each vector were positive for *Campylobacter* (Table 7). The isolates obtained were typed in the present project. There was no evidence for differentiation in ST frequency between isolates from flies from “Fields with cattle only” and “Fields with sheep only” (P = 0.8, MCMC implementation of Fisher’s exact test).

Campylobacter bacterial counts in potential reservoirs

Among the four most intensively sampled reservoir hosts (cattle, sheep, gull and pigeon), 11-26% of *Campylobacter*-positive specimens yielded bacterial counts in the “high shedder” category of $>10^4$ CFU per gram (Table 8).

There was no evidence that faecal specimens from NE Scotland (excluding the intensive survey) versus SW Scotland had different distributions when compared within cattle and within sheep and categorised according to the 10-fold ranges of bacterial counts (Figure 10; GENEPOP package, Fisher’s exact test implemented using the strucFile setting, $P > 0.05$ in both cases). There was also no evidence that faecal specimens from cattle versus sheep from NE Scotland had different distributions of counts according to the same test ($P > 0.05$).

In contrast, there was evidence that faecal specimens from cattle versus sheep from SW Scotland had different distributions (P = 0.006), with specimens from sheep having higher bacterial counts. There was also evidence that faecal specimens from cattle in NE Scotland from the intensive versus structured surveys had different distributions (P << 0.001), with specimens from the intensive survey having higher bacterial counts.

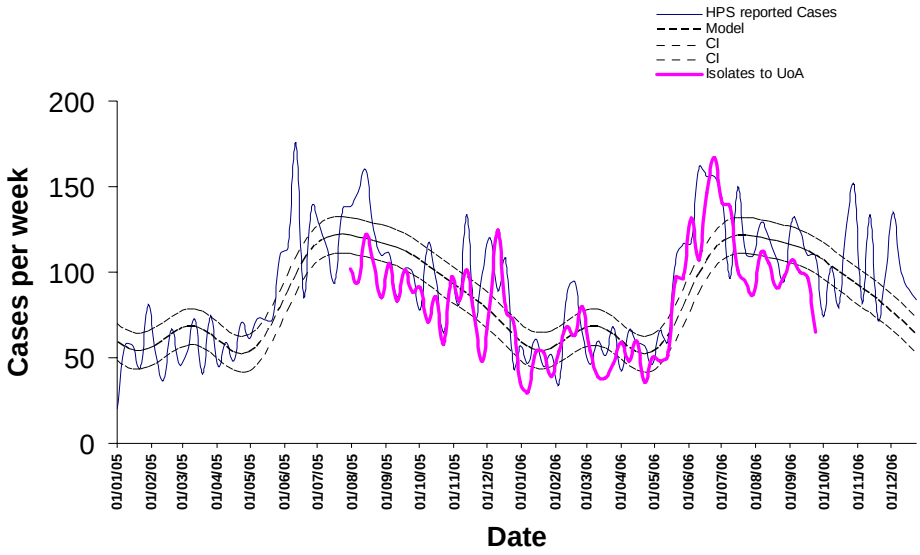
In retail chicken, 25% of specimens had no detectable *Campylobacter* according to either direct plating or enrichment growth, 47% of specimens had *Campylobacter* present according to enrichment but counts were below the detection threshold of 10^3 CFU ($<10^3$ in Figure 11), 14% of specimens yielded bacterial counts of 10^3-10^4 CFU and 14% of specimens yielded bacterial counts of 10^4 CFU and above per carcass or portion (Figure 11). In retail offal, 30 of the 51 specimens tested were positive for *Campylobacter*, of which 27 yielded bacterial counts in the lowest "$<10^3$ CFU/portion" category and the remaining three (lamb and ox liver) had counts in the range 10^4-10^5 CFU/portion.

Table 4. Clinical isolates received from Health Boards.

Health Board	Isolates received	% of *C. jejuni* [1]	Cases reported to HPS	% of HPS cases received	Incidence per 100,000 [2] 2005	2006
Argyll and Clyde [3]	132	92.7	112	>100	90.5	89.1
Ayrshire & Arran	265	93.6	287	92.3	50.6	71.7
Borders	133	92.9	154	86.4	113.5	135.8
Dumfries & Galloway	219	86.4	221	99.1	107.5	131.5
Fife	284	90.6	351	80.9	77.9	67.6
Forth Valley	174	92.7	256	68.0	69.6	69.3
Grampian	820	94.2	771	>100	122.8	114.5
Greater Glasgow	927	89.1	1070	86.6	60.7	80.5
Highland	310	89.6	281	>100	90.4	104.9
Lanarkshire	682	88.7	651	>100	90.8	104.6
Lothian	1150	89.1	1126	>100	116.7	110.6
Orkney	19	77.8	17	>100	82.0	91.9
Shetland	24	86.4	0 [4]	>100	4.6	0 [4]
Tayside	524	90.5	525	99.8	116.0	111.4
Western Isles	9	88.9	9	100	45.7	22.8
Total	5672 [5]	90.4	5831	97.3	90.2	95.3

1: Species identity was inferred only for the 5247 isolates with complete MLST data (Table 10), the rest of such isolates were *C. coli*; 2: data from HPS weekly report (72); 3: the original definition of Argyll and Clyde Health Board was retained for this study despite its split and amalgamation with Greater Glasgow and Highland Health Boards in March 2006; 4: Shetland did not report cases to HPS in 2006; 5: Two of the isolates were split according to colony morphology into two isolates each, yielding a total of 5674 clinical isolates.

Figure 8. Modelled and actual trends in numbers of *Campylobacter* cases in Scotland.



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Modelled trends (details of the modelling method are given in section 6.4.2.1) are based on numbers of cases reported to Health Protection Scotland from Jan. 2003 to July 2005. Actual trends are the numbers of isolates submitted during 15th July 2005 to 26th Sept. 2006 and the corresponding numbers of cases reported to Health Protection-Scotland.

Table 5a. Yield and species composition of *Campylobacter* isolates from composite environmental and retail food sources.

Source type	NE Scotland	SW Scotland	Aberdeen	Edinburgh	Glasgow	SAC Vet [1]	PA [2]	% of *C. jejuni* [4]	Total
Farm species	447 [3]	106	3			20		78.5	576
Wild bird	27	4	42	37	78			93.1	188
Comp-anion animal	1	1	3	0	0	48		96.2	53
Retail chicken & offal	175						107	81.9	282
Total	**650**	**111**	**48**	**37**	**78**	**68**	**107**	**82.7**	**1099**

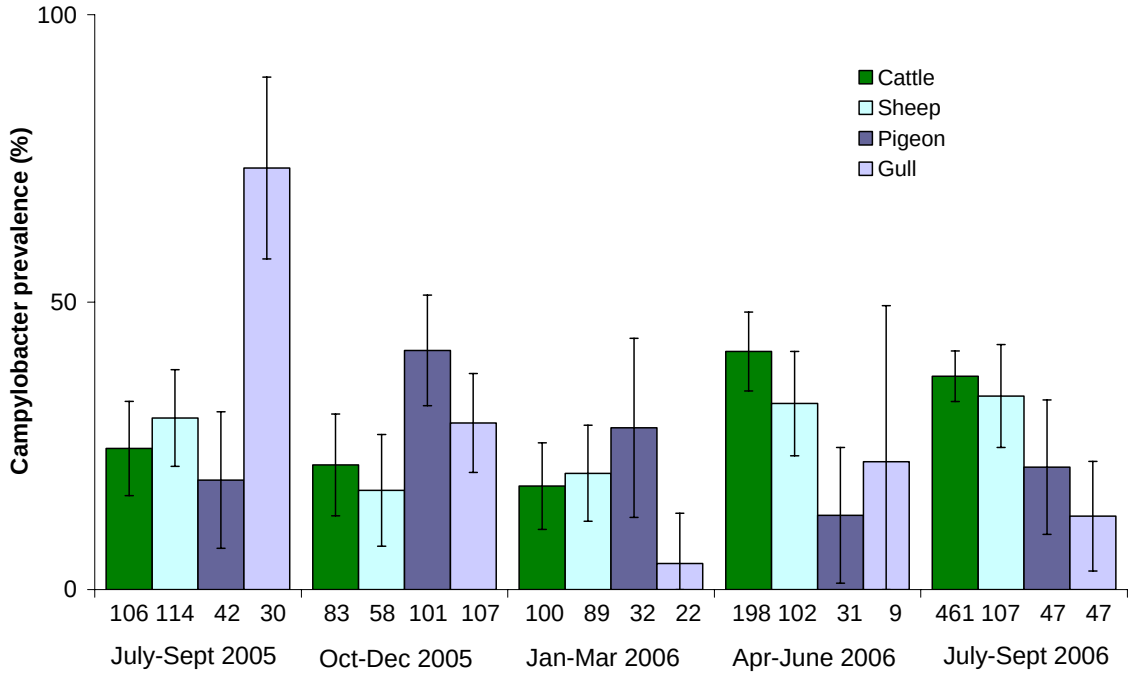
Table entries give the number of *Campylobacter* strains isolated and successfully typed to ST, including multiple strains with different STs from single specimens. 1: isolates supplied by SAC and Glasgow Vet School; 2: isolates supplied by Public Analysts; 3: includes isolates from the structured and intensive surveys of farms in NE Scotland; 4: *C. coli* accounted for almost all of the remaining isolates (in addition, gulls yielded 8 isolates tentatively identified as *C. lari* and dogs yielded 8 isolates tentatively identified as *C. upsaliensis*).

Table 5b. *Campylobacter* prevalence in composite environmental and retail food sources.

Source type	NE Scotland	SW Scotland	Aberdeen	Edinburgh	Glasgow	SAC Vet [1]	PA [2]	Total
Farm species	36.4±1.4 [3]	24.9±2.0	35.7±12.8			80.0±8.0		33.9±1.2
Wild bird	36.8±5.8	21.1±9.4	22.8±3.0	26.0±3.3	30.4±2.7			27.7±1.6
Comp-anion animal	8.3±5.6	12.5±11.7	10.7±4.1	13.6±7.3	0.0	47.3±4.7		25.7±2.7
Retail chicken & offal	69.3±2.8						75.0±3.9	71.1±2.3
Total	**41.8±1.3**	**24.6±2.0**	**21.0±2.5**	**24.6±3.0**	**27.4±2.5**	**53.3±4.3**	**75.0±3.9**	**36.5±0.9**

Table entries give the percentage of specimens positive for *Campylobacter* with standard errors. 1: isolates supplied by SAC and Glasgow Vet School; 2: isolates supplied by Public Analysts; 3: includes isolates from the structured and intensive surveys of farms in NE Scotland.

Figure 9. Quarterly differences in *Campylobacter* prevalence in the four most intensively-sampled reservoir hosts.



Values for farm species include both the structured and intensive surveys in NE Scotland. The values below the X-axis are the specimen sample sizes and the vertical bars depict the 95% confidence intervals.

Table 6. *Campylobacter* prevalence in retail liver portions.

Liver source	Calf	Ox	Lamb	Pig	Chicken	Total
N	4	12	16	10	9	51
Prevalence (%±SE)	100.0	50.0±14.4	62.5±12.1	50.0±15.8	55.6±16.6	58.8±6.9
Counts (CFU/portion)	4 $<10^3$	5 $<10^3$ 1: $9.0x10^3$	8 $<10^3$ 1: $1.5x10^4$ 1: $9.9x10^4$	5 $<10^3$	5 $<10^3$	

Table 7. *Campylobacter* prevalence in groups of pooled flies from one farm in NE Scotland.

	Positive pools	Total pools [1]
Flies		
Fields with cattle only [2]	4	73
Fields with sheep only [3]	5	34
Fields with cattle and sheep	0	48
Slugs [4]	2	15
Total	11	170

1: pools contained approximately 75 flies or 8 slugs; 2: isolates had five known STs; 3: isolates had seven known STs; 4: isolates had one new ST with a closest match to ST954, which was isolated from a wild bird in Cheshire, UK, in 2002.

Table 8. Bacterial counts (CFU/g) in *Campylobacter*–positive environmental specimens.

Source type	$<10^3$	10^3-10^4	10^4-10^5	10^5-10^6	10^6-10^7	10^7-10^8	% high shedders ($>10^4$)	Total
Farm species								
Cattle	223	53	24	8	1	1	11.0	310
Sheep	74	19	19	8	1	4	25.6	125
Pig	15	5	5	4	1		33.3	30
Poultry	11	2	1	1			13.3	15
Wild bird								
Duck	10	3			1		7.1	14
Goose	15	2	1				5.6	18
Gull	41	2	4	6	4		24.6	57
Swan	3	1			1		20.0	5
Corvid	2						0.0	2
Pheasant	4						0.0	4
Pigeon	60	2	4	4	3		15.1	73
Starling	1						0.0	1
Unidentified	18	2	2	3			20.0	25
Companion animal								
Cat [1]	1						0.0	1
Dog [1]	1	2	1		1	1	50.0	6
Horse	1						0.0	1

All specimens yielding counts were positive for *C. jejuni* or *C. coli* according to yield of a complete or partial ST. 1: excludes SAC and Glasgow Vet School isolates because these were supplied as cultures.

Figure 10. Bacterial counts in *Campylobacter*-positive faecal samples from cattle and sheep in NE and SW Scotland.

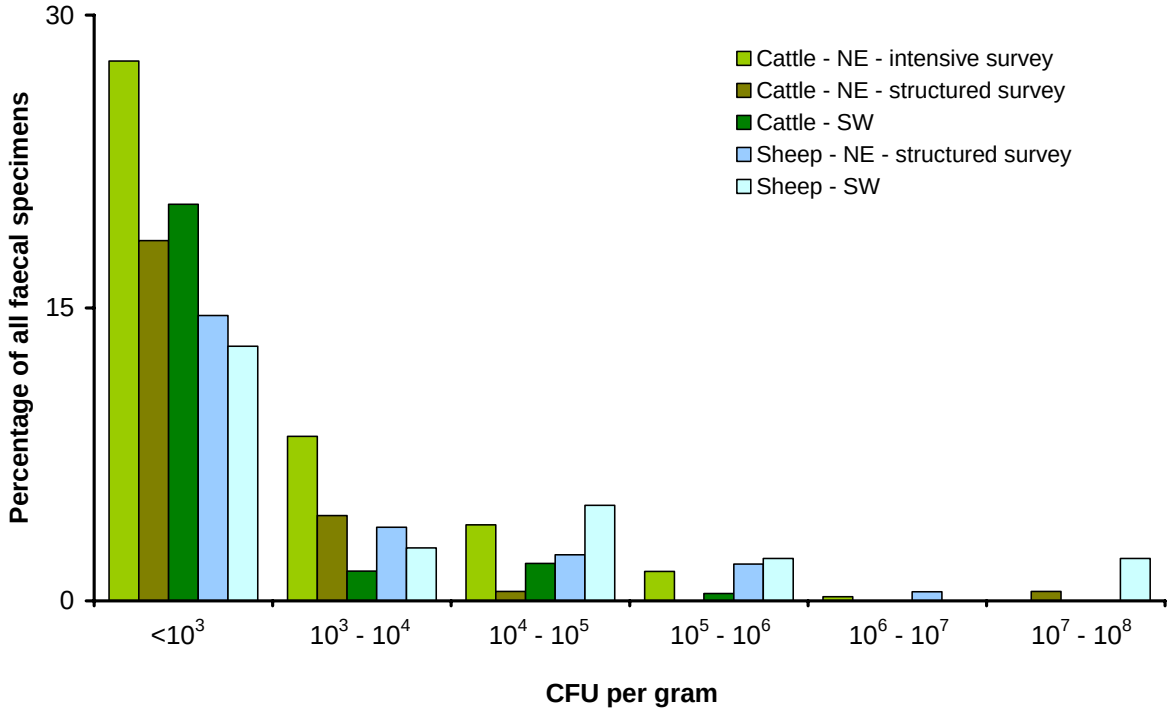
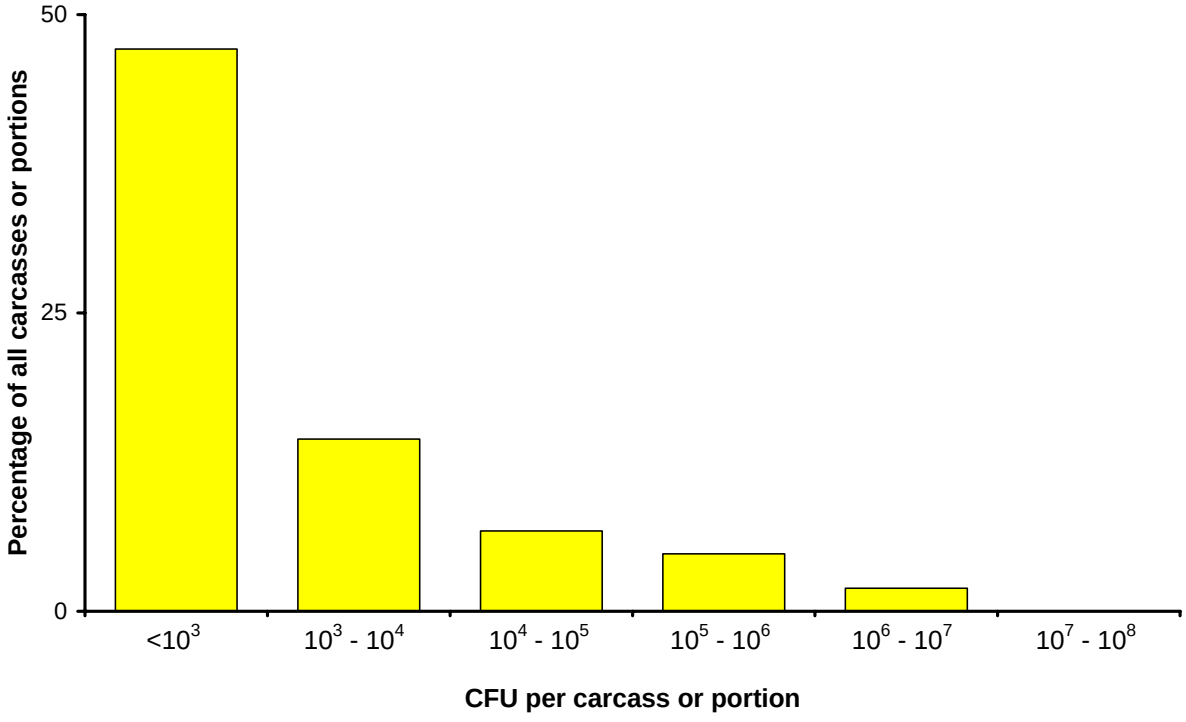


Figure 11. Bacterial counts in *Campylobacter*-positive retail chicken carcasses or portions from NE Scotland.



4.2 Molecular diversity of *Campylobacter* isolates

Most of the clinical (92%) and the environmental and food isolates (93%) yielded a complete ST (Table 9; Full data in Appendices F, G). Incomplete typing accounted for most of the untyped isolates, and the commonest kind of typing failure (all genes missing) probably indicates that an isolate is a bacterial species other than *Campylobacter*. The presence of *Campylobacter* species other than *C. coli* and *C. jejuni* (Table 10), and of mixed strains, accounted for the balance of the untyped isolates. Clinical isolates sharing the same patient identifier and ST, and having reporting dates within the same 2-week period, were inferred to be duplicate specimens from the same patient taken during one clinical episode. These inferred duplicate clinical isolates were excluded from further analysis.

All of the clinical isolates with a complete ST were classified as either *Campylobacter jejuni* or *C. coli* (Table 10) (18). Orkney Health Board had the lowest percentage of *C. jejuni* (77.8%) and Grampian Health Board had the highest (94.2%; Table 4).

Campylobacter isolates from all sources except pigeon contained almost maximum levels of ST diversity (Figures 12-14). Applying the flexible concept of “strain” to this diversity, the number of strains decreased when isolates first categorised as ST were re-categorised as CC or orphan ST. The decrease was approximately 70% in the case of the clinical strains but only approximately 33% in the case of the environmental and food strains. Thus, CC membership characterised the clinical isolates to a greater degree than the environmental and food isolates.

The clinical isolates had almost maximal values of the Hunter-Gaston diversity index (DI). There was almost no evidence that any of the 15 Scottish Health Boards had significantly higher or lower DI values than the others (Figure 14a). The exception was Lothian Health Board, which had a marginally higher DI value with a distinct 95% confidence interval when compared with Lanarkshire and Grampian. Most of the environmental and food isolates also had very high DI values (Figure 14b). Unidentified wild birds, waterfowl (ducks, geese and swans) all had DI values that were as high as the values seen in the clinical isolates. The two major farm species (cattle and sheep), two of the three kinds of identified wild birds (gull and pigeon), cats and dogs combined, and retail foods all had somewhat lower DI values and distinct 95% confidence intervals when compared with clinical isolates. Pigeon isolates had an exceptionally low DI value.

Each of the sources of *Campylobacter* can be viewed as representing a total level of *Campylobacter* strain diversity. This prompts a question: does each sample collection contain all of this diversity, or does more diversity remain unsampled?

The extent to which the isolates from sources represented the maximum hypothetical diversity was characterised using rarefaction. Rarefaction is a data re-sampling technique that indicates whether diversity has reached a plateau or is still rising at the total sample size, *i.e*., at the end of collection. A rarefaction curve that has reached a plateau indicates that all diversity has been sampled whereas an increasing slope indicates that some diversity remains unsampled. This method assumes that the dataset represents a random sample taken from a closed system characterised by a constant, stable spectrum of types.

The rarefaction curves for all clinical, environmental and food sources were still rising, even at the maximum sample sizes (Figure 15a-c). There are two nonexclusive explanations for this result. First, *Campylobacter* strains in Scotland represent a closed system with a constant, stable spectrum of types but none of the specimen collection schemes was sufficiently comprehensive to encompass the total ST diversity present. Second, the concept of a closed system containing a constant, stable spectrum of types is invalid for *Campylobacter* sampled at the spatial and temporal scales used in this project.

The most reasonable interpretation of this tabulation and analysis of strain diversity is that *Campylobacter* present in clinical, environmental and food sources in Scotland represents an extremely large pool of strains that is continually being augmented: internally by mutation and recombination and externally by strain input from human travel and migrating wildlife.

Table 9. Summary of outcomes for isolate typing

ST acquisition	Clinical isolates	%	Environmental & food isolates	%	Total	%
Successful	5247	92.5	1118	93.3	6365	92.6
Unsuccessful [1] or data not used [2]	427	7.5	80	6.7	507	7.4
Total	5674		1198		6872	

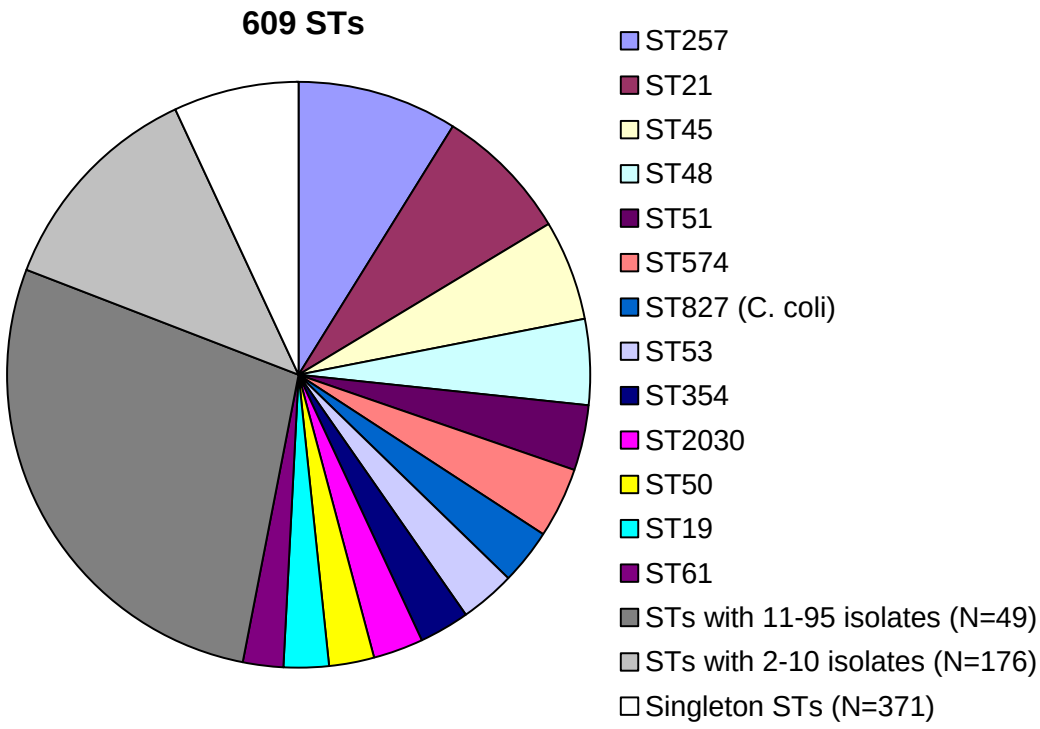
1: typing was unsuccessful when an isolate's ST was partial or missing, the isolate contained mixed strains or it was not *C. coli* or C. *jejuni*. 2: ST data were not used when patient details were wrong or where samples were second or subsequent isolates from a given patient and had the same ST as the patient's first isolate.

Table 10. Species composition of isolates.

Campylobacter species	Clinical isolates	%	Environmental & food isolates	%	Total	%
C. coli [1]	506	9.6	196	17.3	702	11.0
C. jejuni [1]	4741	90.2	922	81.1	5663	88.6
C. fetus [2]			2	0.2	2	0.0
C. lari [2]	6	0.1	8	0.7	14	0.2
C. upsaliensis [2]	5	0.1	8	0.7	13	0.2
Unidentified [3]	416		62		478	
Total	5674		1198		6872	

1: species were identified using known STs according to their species attribution in the PubMLST *Campylobacter jejuni/C. coli* database, and using new STs according to the species attribution of the closest-matching known ST. 2: species were tentatively identified according to clustering of the isolate *glyA* sequences with other *Campylobacter* species in the GenBank database. 3: species were not identified for the following reasons: samples were second or subsequent isolates from a single patient and had the same ST as the first isolate; the patient details were wrong; the ST was partial or missing; the isolate contained more than one strain; the isolate yielded a *glyA* sequence that did not cluster with any *Campylobacter* species in the GenBank database.

Figure 12. Clinical isolates categorised by ST or by CC/orphan ST



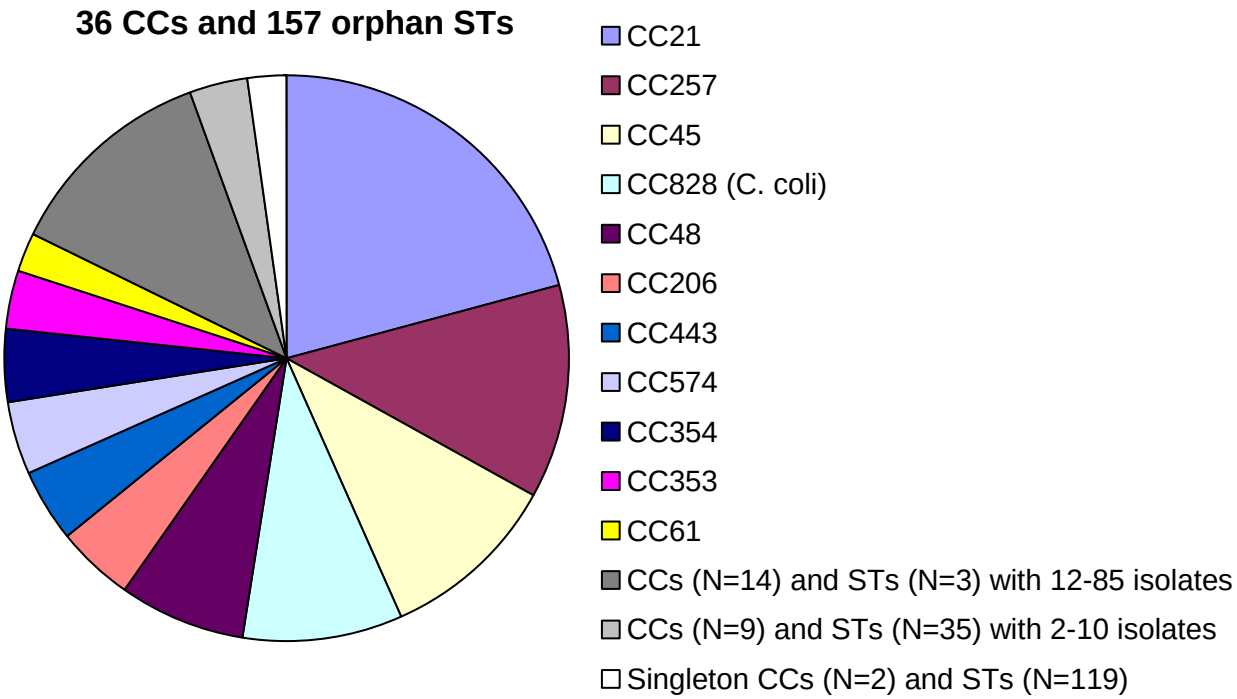


Figure 13. Environmental and food isolates categorised by ST or by CC/orphan ST.

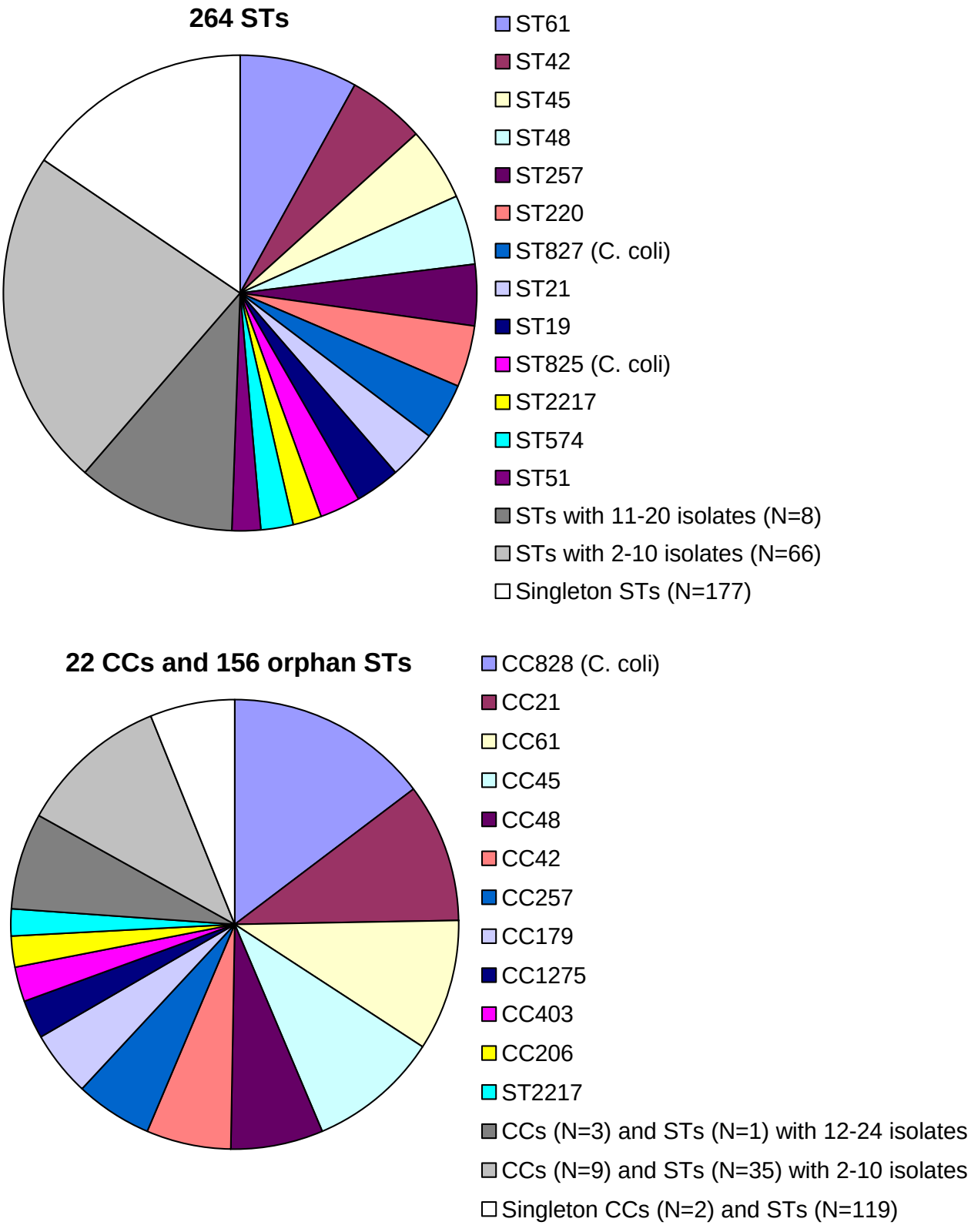
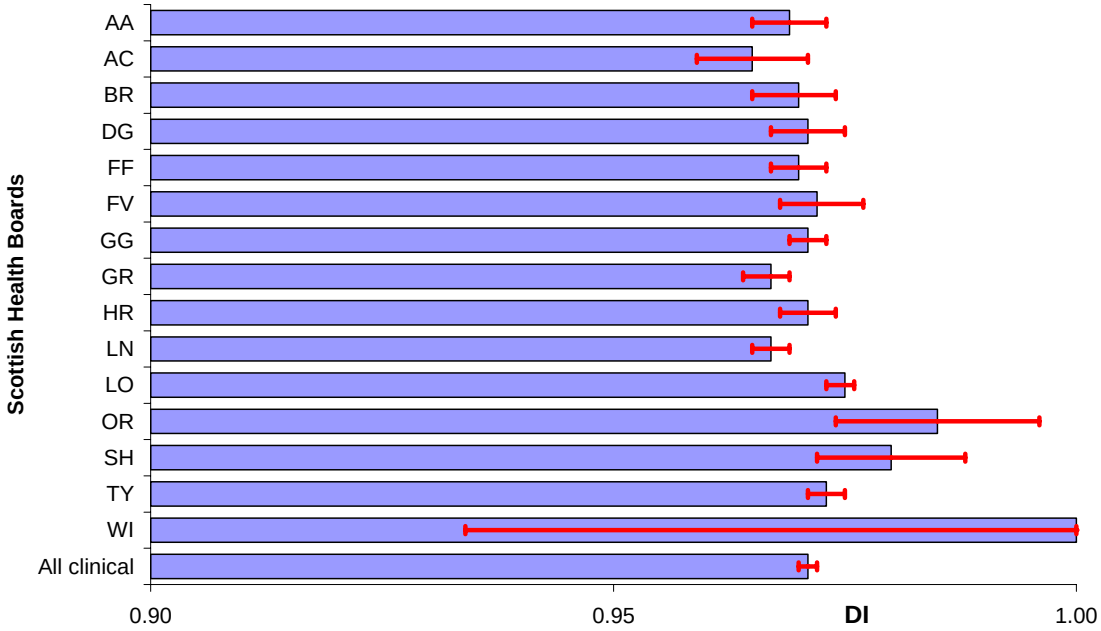
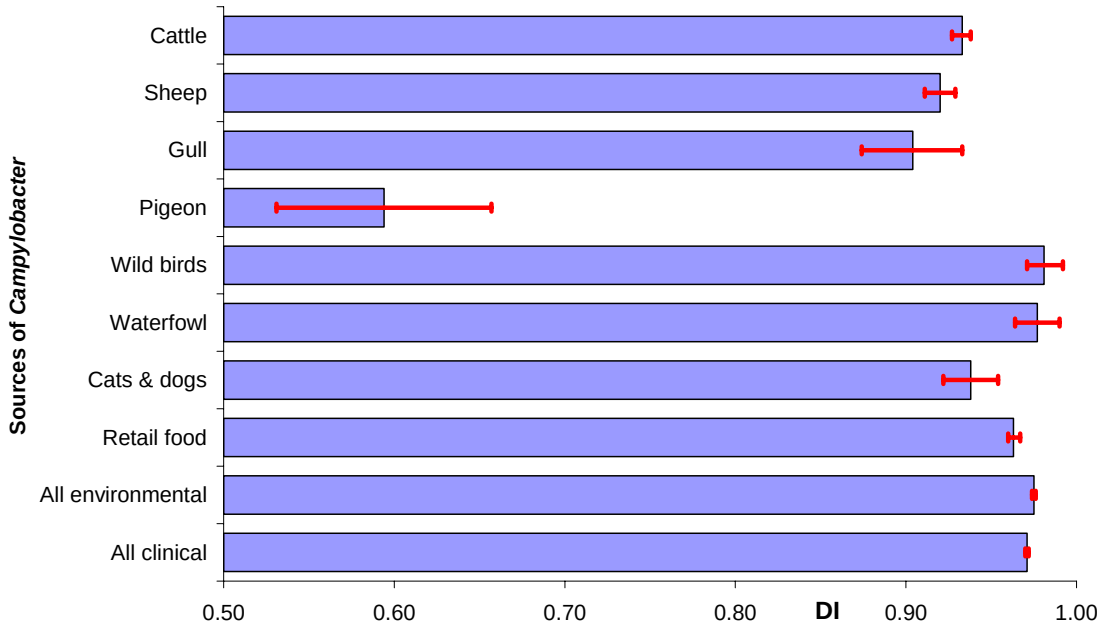


Figure 14. ST diversity in *Campylobacter* isolates from clinical infections, environmental sources and retail foods.

(a) Clinical isolates



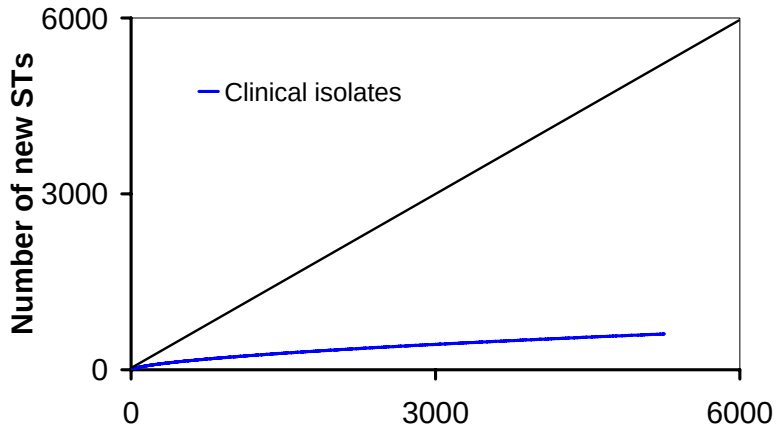
(b) Environmental and food isolates



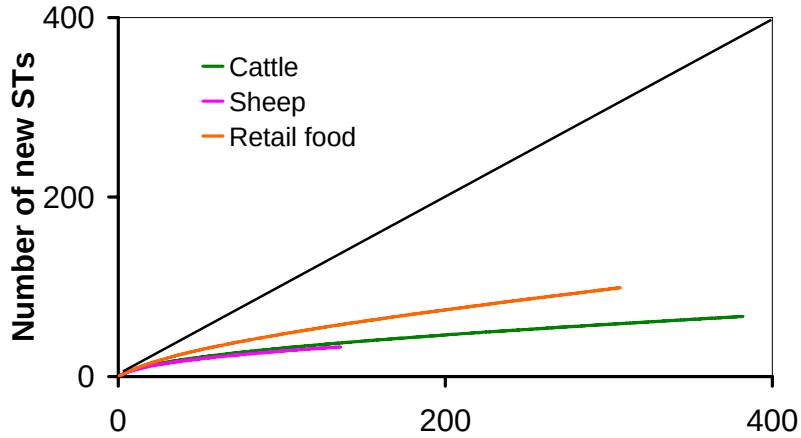
ST diversity was quantified as the Hunter-Gaston diversity index (DI): a measure of the diversity of types in a group that takes account of differences in sample size among groups. DI values range from 0 (all types are the same) to 1 (all types are different). Red bars indicate 95% confidence intervals

Figure 15. Rarefaction curves for ST isolates from reservoir hosts.

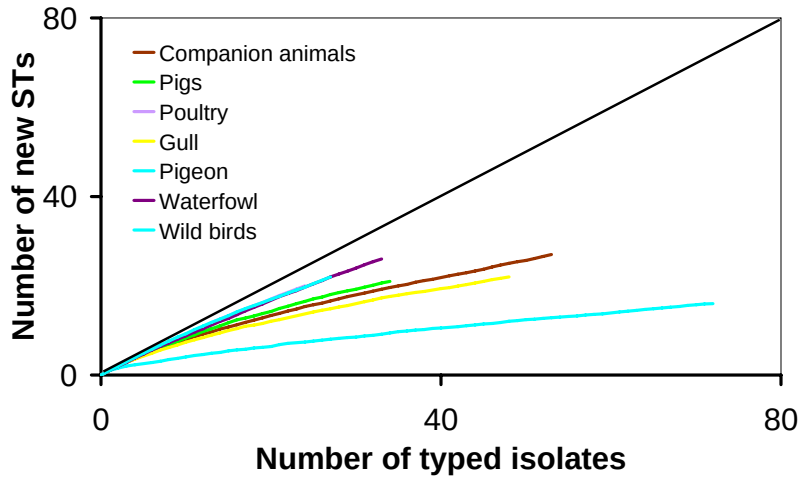
(a) Clinical cases



(b) Cattle, sheep and retail foods



(c) Seven further reservoir hosts



The diagonals indicate the maximum possible level of diversity where each new isolate is a new ST.

4.3 Differentiation of clinical *Campylobacter* strains

Differentiation across Health Boards

Differentiation of *Campylobacter* strains across the 12 mainland Health Boards was analysed at the levels of species and ST. Because of small sample sizes, the three island Health Boards (OR, SH, WI) were excluded from this and subsequent analyses of differentiation.

Differentiation in *C. coli vs*. *C. jejuni* composition among Health Boards was evident by two different analyses (see also Table 4). First, the species composition of the isolates from each Health Board was compared with that of every other Health Board, *i.e*., all pairwise comparisons among Health Boards were tested, using the exact test of population differentiation available in the ARLEQUIN package (test details: 100,000 Markov chain steps, 100,000 dememorisation steps). Most pairs of Health Boards yielded no evidence for a difference in species composition. The four exceptions all involved Grampian Health Board, which had a significantly lower proportion of *C. coli* than Dumfries and Galloway, Greater Glasgow, Lanarkshire and Lothian (P=0.00005-0.00056). In the second analysis, all 12 mainland Health Boards were tested together and evidence for differences in *C. coli vs*. *C. jejuni* composition within this overall group was evaluated (GENEPOP package, Fisher's exact test implemented using the strucFile setting). The group of 12 Health Boards yielded evidence for a difference in species composition (P=0.0015) but this evidence disappeared when Grampian Health Board was removed from the dataset (P=0.34). Both analyses yielded the same conclusion: only Grampian Health Board stood out in having a significantly lower proportion of *C. coli* than other Health Boards.

Differences in ST composition among Health Boards were also analysed. The 12 mainland Health Boards had visually similar proportions of the 25 most common STs (Figure 16). When all pairwise comparisons of ST composition among Health Boards were tested, only one pair (Dumfries & Galloway *versus* Forth Valley) yielded a significant difference (ARLEQUIN, exact test of population differentiation, details as above, P=0.0097). The differentiation was quantified as the index F_{ST}, which was found to be very low (ARLEQUIN, F_{ST}=0.003) and to be due to minor cumulative differences involving several rare STs.

The clinical *Campylobacter* strains thus showed minor evidence for differentiation among the 12 mainland Health Boards according to species composition, and almost no evidence at all

for differentiation according to ST composition. There was therefore no evidence that the incomplete submissions from four Health Boards (Table 4) had created any bias in the ST composition of clinical isolates.

Differentiation across month of sampling

Differentiation of *Campylobacter* strains over the 15 months of the project was analysed at the levels of species and ST. No comparison between any pair of months yielded evidence for a difference in *C. coli vs*. *C. jejuni* composition (all pairwise comparisons *P*=0.006-1.000, overall *P*=0.20). In contrast, most comparisons between pairs of months of the project yielded evidence for a difference in ST composition. Only five comparisons yielded no evidence for ST differentiation (*P*=0.049-0.563), 13 comparisons yielded weak evidence (*P*=0.001-0.025), and the remaining 87 comparisons (83% of all the pairwise comparisons) yielded strong evidence for ST differentiation (*P*=zero-0.001).

The 120 most common STs were ranked according to their degree of monthly variation of occurrence using the coefficient of variation (CV) of the monthly totals of each ST. Three groups of 40 STs were distinguished according to high (CV=0.58-1.07), intermediate (CV=0.43-0.58) and low (CV=0.28-0.43) variation of monthly occurrence, and three STs from each group were used as visual examples of this variation (Figure 17a-c). Among the high-variation group, ST45 peaked during mid- to late summer in both 2005 and 2006, ST53 peaked during August in both 2005 and 2006, and ST2030 peaked during May 2006 (May 2005 was not sampled). Among the intermediate-variation group, ST21 peaked during August 2005 and July 2006, ST48 peaked during August 2005 and during April-August 2006, without showing a single monthly peak, and ST827-*C. coli* peaked during September-December 2005, without showing a single monthly peak, and peaked during August 2006. Among the low-variation group, ST257 peaked during September 2005 and June-September 2006, and neither ST50 nor ST354 showed any strong seasonal pattern.

The extent to which the 25 commonest STs (N>40) occurred in step with overall fluctuations in clinical isolates was analysed in relation to the total occurrence of each ST during the whole project (Figure 18). Six STs had monthly occurrences that were highly correlated with total monthly numbers of clinical isolates (ST21, ST45, ST48, ST137, ST354 and ST572; red diamonds in Figure 18). Some of these STs were common but others were relatively rare (N=58-399) so their strong correlation with the overall monthly clinical fluctuations was not simply due to their comprising a large proportion of total clinical isolates. Three STs (ST50,

ST53 and ST827-*C. coli*; N=132-174; orange diamonds in Figure 18) showed lower correlations, four STs (ST42, ST61, ST257 and ST262; N=70-468; yellow diamonds in Figure 18) showed weak correlations, and the remaining 12 STs (N=41-202; grey diamonds in Figure 18) showed no significant correlations with the overall monthly clinical fluctuations. Thus, 13 of the 25 most common STs showed at least some evidence of occurring in step with the overall fluctuations in clinical isolates.

The clinical *Campylobacter* strains thus showed no evidence for differentiation in species composition but did show evidence for significant differentiation in ST composition across the 15 months of the project.

Spatio-temporal stratification of ST diversity

Clinical *Campylobacter* strains defined by ST can be viewed as having a total diversity made up of a between-Health Board component, a between-month component and a within Health Board-month component. The above analysis shows that clinical strains are differentiated among different months of sampling but not among Health Boards, suggesting that the between-month component is significant whereas the between-health Board component is not. It is of further interest to estimate *how much* of the total ST diversity each component accounts for. This question was addressed using an analysis of molecular variance (AMOVA) in the ARLEQUIN package. Two analyses were done. The first used the hierarchical levels (from the highest) “Among Health Boards”, then “Among months within Health Board”, then “Within Health Board-months” (Figure 19). The second analysis used the hierarchical levels (from the highest) “Among months”, then “Among Health Boards within month”, then “Within Health Board-months” (as in Figure 19 but with the upper two grouping levels reversed).

The first analysis (Table 11; AMOVA 1) confirmed the above result on ST differentiation by Health Board. The highest level (Among Health Boards) did not account for any significant proportion of ST diversity. The second-highest level (Among Months within Health Board) accounted for a significant proportion of ST diversity but this was less than 1% of the total ST diversity, and over 99% of ST diversity remained within Health Board-month. The second analysis (Table 11; AMOVA 2) confirmed the result of the above analysis of ST differentiation by month. The highest level (Among months) and second-highest level (Among Health Board within months) each accounted for a significant proportion of ST diversity but these

proportions averaged less than 0.5% of the total ST diversity, and over 99% of ST diversity remained within Health Board-month.

Thus, the main conclusion of both analyses is that over 99% of the ST diversity of clinical *Campylobacter* occurs within isolates reported by each Health Board in each month of the year, and that less than 1% of this diversity is stratified between months.

Differentiation across patient age

Differentiation of *Campylobacter* strains among patient age classes (5 year bands) was analysed at the levels of species and ST.

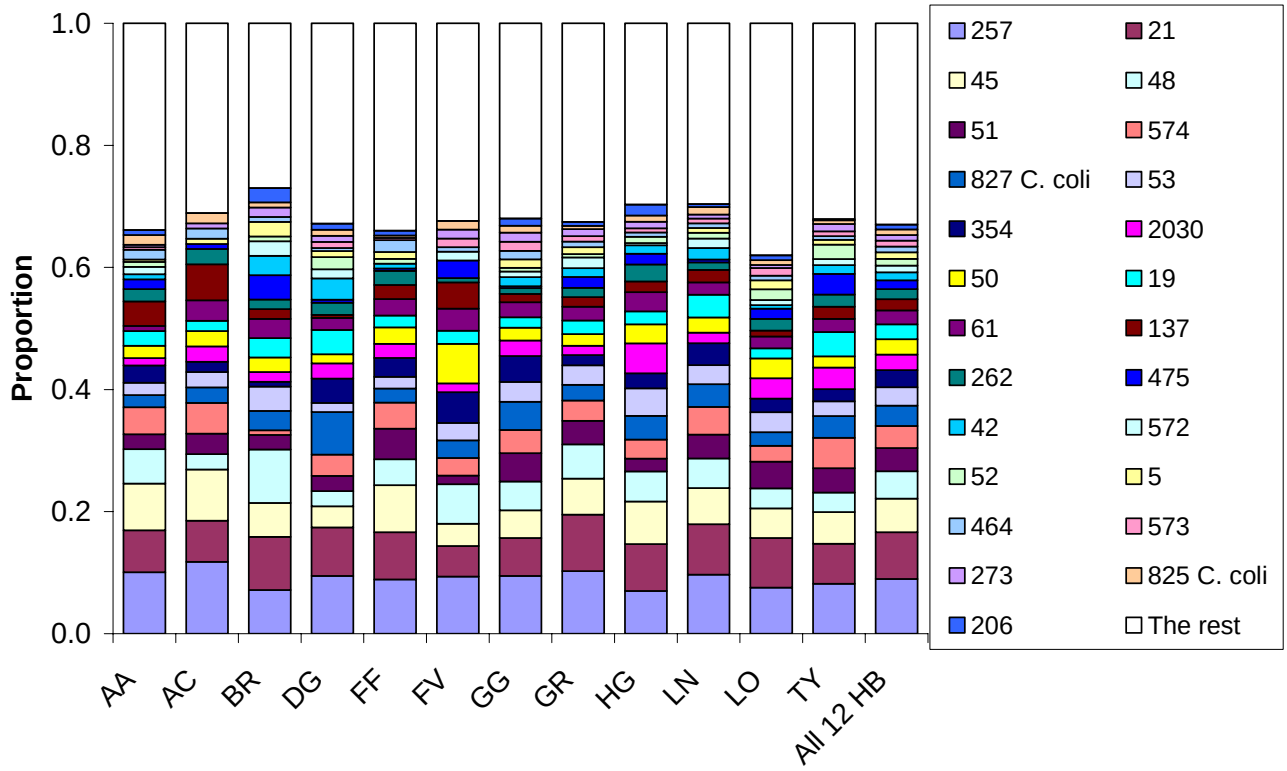
Species composition showed a marked visual trend with patient age: the percentage of *C. coli* strains approximately tripled from 5-6% in patients up to 10 years old to 15-21% in patients over 80 years old (Figure 20). This trend was statistically significant: the species composition of strains showed significant differentiation among the 19 age classes (GENEPOP package, strucFile setting, *P*=0.005), and this remained true after progressive removal of the four highest age classes with small sample sizes (same test, *P*=0.004-0.014).

ST composition showed no obvious visual differences across patient age classes, except for the three oldest age classes with the smallest sample sizes (Figure 21). The 14 commonest STs (ST257-ST137 in Figure 21 legend) occurred in most of the age classes, and the main difference involved an increase in ST827-*C. coli* (most commonly found in sheep, see section 4.6) from 66 years and older, consistent with the species analysis. ST composition was compared among all pairs of age classes after excluding the *C. coli* STs (ARLEQUIN, exact test of population differentiation, details as above). ST composition differed in 32 of the 171 pairwise comparisons (*P*=zero-0.01). These differences were spread across all of the age classes except 56-60 years. The age classes differing most from the others were 0-5 years, 61-65 years and 76-80 years. Age classes 6-10 years, 16-20 years and the three classes in 26-40 years showed fewer differences, and 10 of the remaining 11 classes differed from only one or two of the others. These differences were not due to one or a few STs, they were cumulative across several STs.

The clinical *Campylobacter* strains separated into different patient age classes were thus mainly differentiated according to species composition, with the proportion of *C. coli* strains increasing approximately three-fold from young children to old adults. The age classes also

differed in the composition of *C. jejuni* STs, and these differences were spread across most of the age classes, not associated with a particular age class.

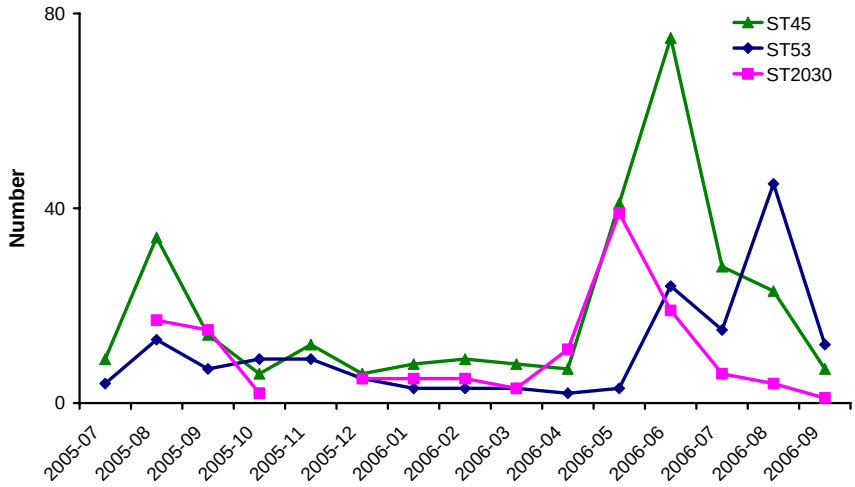
Figure 16. Percentages of the 25 commonest STs in clinical isolates from the 12 mainland Scottish Health Boards.



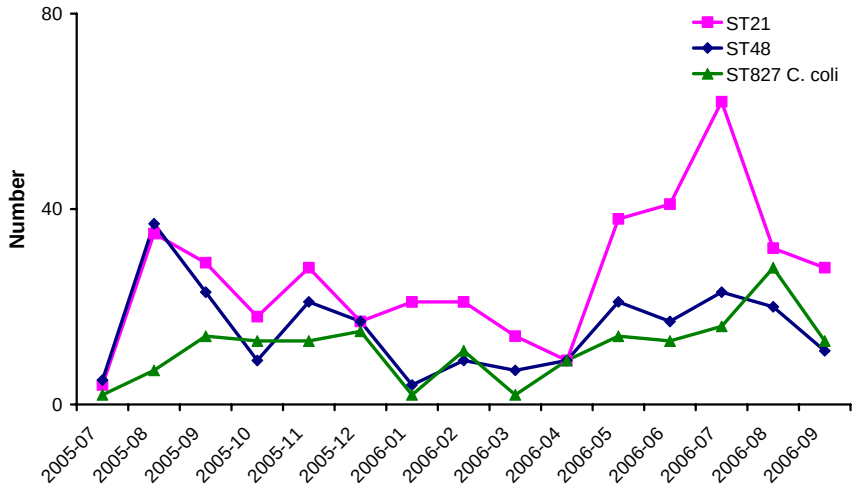
Orkney, Shetland and Western Isles were excluded because these Health Boards submitted far fewer isolates than the mainland Health Boards.

Figure 17. Monthly occurrence of clinical strains identified according to ST.

(a) Examples of three STs with high monthly variation in occurrence



(b) Examples of three STs with intermediate monthly variation in occurrence



(c) Examples of three STs with low monthly variation in occurrence

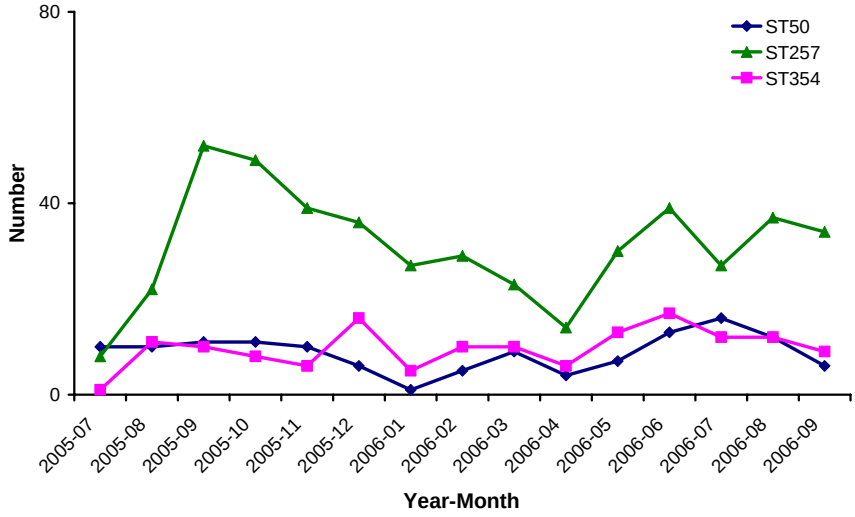
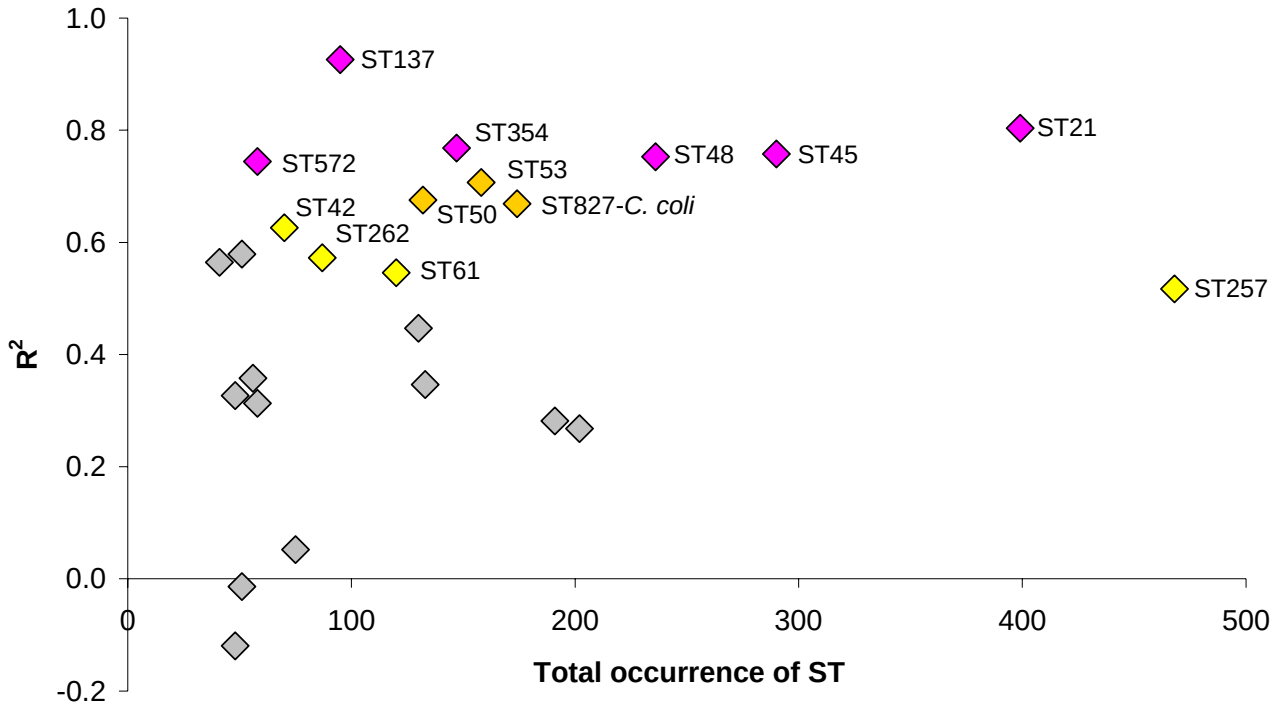


Figure 18. Relationship between the total occurrence of an ST and the correlation between its monthly occurrence and monthly total of clinical strains.



Calculated for the 25 most common STs in clinical strains. The significance of the correlation coefficient for each ST is indicated as red (P< 0.001), gold (0.001 < P < 0.01), yellow (0.01 < P < 0.05) and grey (P > 0.05).

Figure 19. Hierarchical grouping of isolates in AMOVA 1.

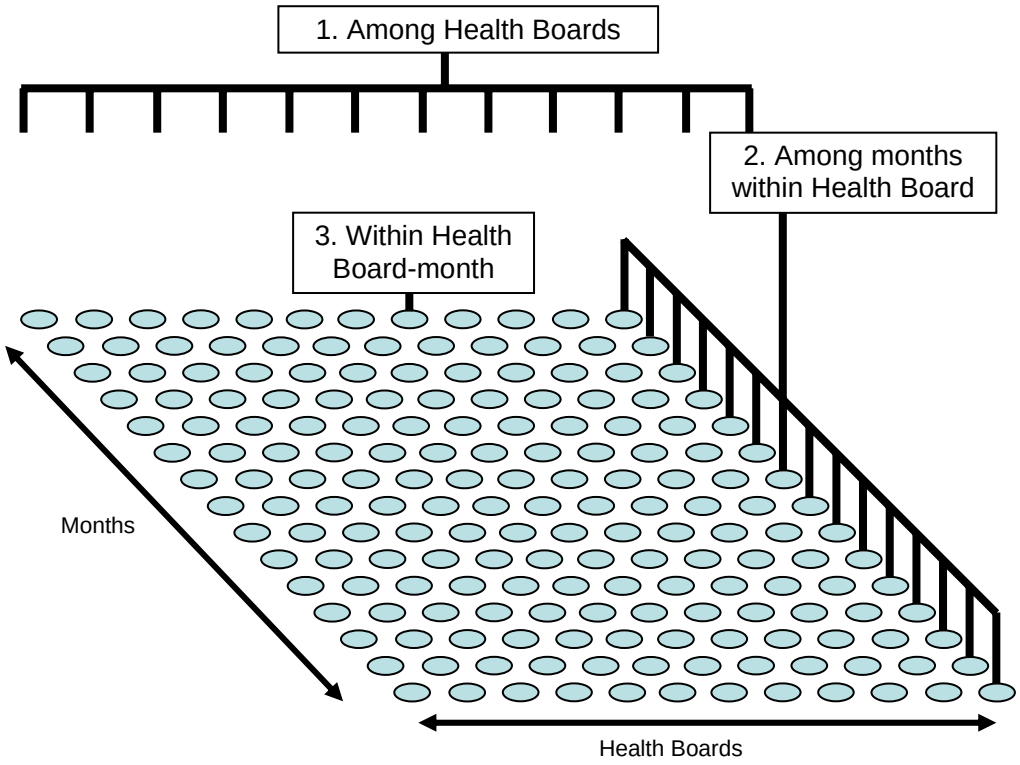


Table 11. Hierarchical stratification of ST diversity in clinical isolates according to AMOVA.

Source of variation	Variance component	Percentage of variation
AMOVA 1		
1. Among Health Boards	0.00258	-0.02 NS
2. Among months within Health Board	0.00427	0.88***
3. Within Health Board-month	0.48125	99.14***
Total	0.48544	100.00***
AMOVA 2		
1. Among months	-0.00009	0.53***
2. Among Health Boards within month	0.00178	0.37***
3. Within Health Board-month	0.48125	99.10***
Total	0.48562	100.00***

Figure 20. Number of clinical *Campylobacter* cases and proportion of *C. coli* isolates by patient age class during July 2005-Sept. 2006.

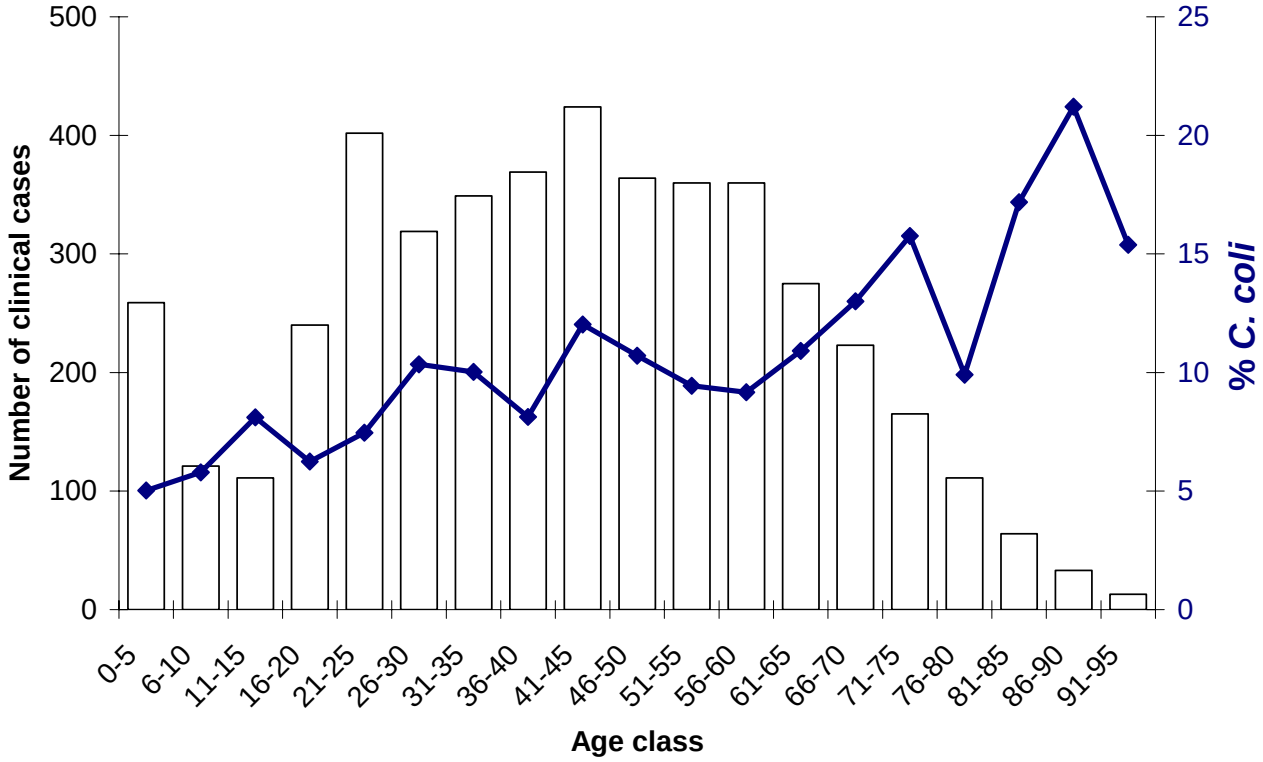
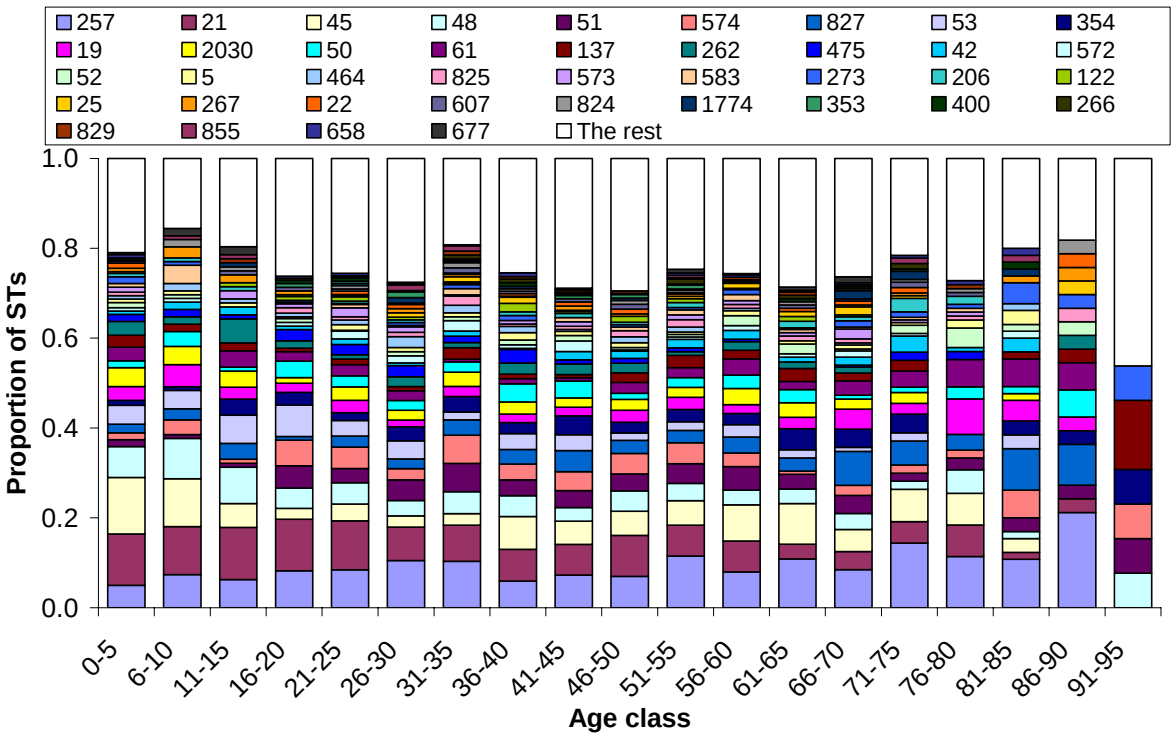


Figure 21. Variation in ST proportion by patient age in clinical *Campylobacter* infections during July 2006-Sept. 2006 in Scotland.



4.4 Strain diversity and clustering: known *Campylobacter* outbreaks

Two reported *Campylobacter* outbreaks that occurred in Scotland during the project were analysed with respect to strain diversity, strain clustering and co-infection in patients.

The first outbreak involved five of a group of seven people who had attended a restaurant in North Ayrshire in October 2005. The only food linking the five was chicken liver pâté. Both Glasgow City Public Analysts and UoA tested the suspect pâté but *Campylobacter* was not isolated, probably due to the two-week interval between preparation and sampling. *Campylobacter* was identified from stool samples in four of the five cases (92).

The second outbreak affected 165 people attending an annual farmers' dance in Montrose, Scotland in November 2005. Epidemiological investigation, including a cohort study, identified chicken liver pâté as the most likely source of the outbreak (relative risk of developing gastroenteritis symptoms = 3.94; 95% C.I. = 2.28-6.09). The pâté preparation involved deliberate undercooking of chicken livers by flash-frying followed by processing in a blender. The outbreak was probably due to several livers contributing *Campylobacter* that survived undercooking (129) and that were dispersed throughout the pâté.

As usual for clinical isolates, cultures of the *Campylobacter* isolated were sent to UoA, with four isolates from the Ayrshire outbreak and 35 isolates from the Montrose outbreak (from Dundee and Aberdeen diagnostic laboratories), all from different patients. The Ayrshire isolates yielded two strains, and the Montrose isolates, seven strains, two of which came from one mixed specimen, according to ST and *flaA* (Table 12). Both of the Ayrshire strains also occurred in the Montrose isolates. The DI values for both outbreaks based on ST frequencies are much lower than the DI values for all clinical isolates from Ayrshire & Arran, or from Grampian and Tayside Health Boards (Montrose), where these outbreaks occurred (section 5.2, Figure 12). This result implies that a reduction in the DI value might provide a useful indicator of the presence of a local outbreak.

Three of the Montrose specimens yielded two different colony morphologies. Five single colonies from each of these specimens, and from specimens identified with mixed ST profiles, were retyped to ST. The single colonies from the mixed specimen continued to yield mixed sequences. The three specimens with different colony morphologies each yielded two different STs. In each pair, one of the STs was the same as originally identified for that

isolate. In two of the pairs, the other ST (ST262) had already been identified in four other Montrose isolates. In the third pair, the other ST (ST1301) had not previously been identified in the outbreak. Thus, four of the 35 patients from the Montrose outbreak were co-infected with at least two strains each.

The occurrence of the large, known outbreak at Montrose provided the opportunity to assess whether indices based on ST composition can be useful as an indicator of the presence of a local outbreak. One index that was assessed was the proportion of all pairs of isolates that had a matching ST, and this was calculated for the Montrose outbreak isolates.

Baseline values for comparison were calculated as follows. The same index was calculated for 10,000 replicates of 33 randomly-chosen isolates from Tayside Health Board: these were assumed to represent mostly sporadic cases. The distribution of 10,000 values from the randomly-chosen isolates was then compared to the original value for the Montrose isolates.

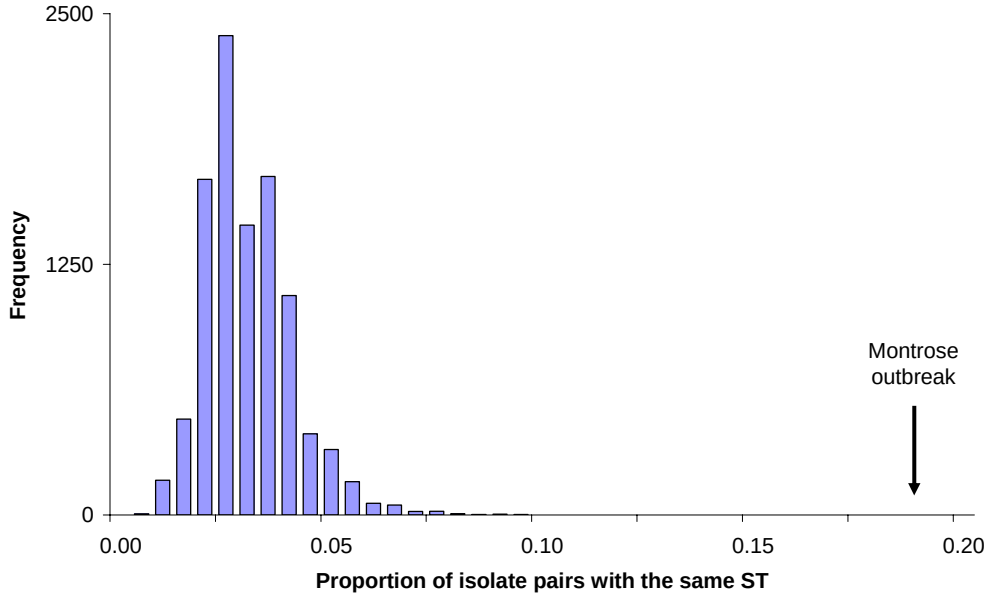
The Montrose outbreak isolates yielded an index value distinct from and much higher than the distribution of baseline values (Figure 22). This result implies that indices of ST composition, such as the ST-matching proportion or possibly the DI value, can indicate the presence of a local outbreak of *Campylobacter* infection, even in circumstances when the epidemiological information normally required for outbreak identification is incomplete.

Table 12. Numbers of isolates from two reported Campylobacter outbreaks with different molecular strains.

Outbreak	DI for ST	Number of isolates [1]	ST	CC	*flaA*
Ayrshire	0.67±0.00	2	51	443	21
		2	257	257	16
Montrose	0.75±0.04	2	21	21	245
		5	**51**	443	8
		5	**51**	443	21
		4	257	257	16
		4	**262**	21	37
		14	**574**	574	8
		1 [2]	1301		

Notes: all isolates were *C. jejuni*; STs in bold were identified as clustered in two analyses of unrecognised outbreaks (see Tables 13 and 15); 1: one isolate from each patient was supplied; 2: isolate obtained after replicate plating.

Figure 22. Comparison of ST-matching proportion in the Montrose outbreak.



Montrose outbreak isolates are indicated by the arrow in comparison to 10,000 replicates of randomly-chosen isolates from Tayside Health Board (histograms).

4.5 Strain diversity and clustering: unrecognised *Campylobacter* outbreaks and clusters.

The clinical *Campylobacter* cases collected in this study (apart from the Montrose outbreak and two other small outbreaks) were all apparently sporadic. Evidence that some of these cases are potentially part of outbreaks was assessed by three different methods of analysing the incidence and typing data. These are exploratory analyses of potential outbreaks, more definitive identification of outbreaks would require additional epidemiological data on linkage among cases, and such data only became available after the end of the project.

In Method 1, incidence data were analysed for the presence of unusually high incidence peaks, then the detected peaks were analysed for significantly high values of ST-matching, as was done for the Montrose outbreak.

The observed incidences of clinical cases were tabulated for each week of the project in two versions: one based on the cases reported to HPS during the period of the CaMPS project and the other based on the cases successfully typed by MLST in CaMPS. An expected incidence was generated using Fourier time-series models based on *Campylobacter* incidence data reported to HPS during 2003 and 2004. Briefly, incidence data were smoothed (double three-point averaging) and then fitted using least-squares to a Fourier model consisting of combined sine waves. The model with 95% confidence intervals was then extrapolated for the duration of the study (83). The observed and expected incidences were compared, and periods when the observed incidence lay outside the expected 95% confidence intervals were identified.

This analysis was conducted first at the level of the whole of Scotland (Figure 23). The expected incidence curve demonstrates the seasonal pattern of human *Campylobacter* incidence. The low points for CaMPS observed cases in July 2005 are due to low submission rates at the start of the study. Two points on the CaMPS observed incidence curve can be considered as epidemiological deviations from expected incidence: a small peak in December 2005 and a very high summer peak in July 2006, neither of which was due to reported outbreaks. CaMPS observed incidence also showed a peak around 1st March 2006 that coincided with an expected peak. This peak was absent from HPS observed incidence one year earlier but obviously occurred in the HPS observed incidences for 2003-2004. This analysis was also conducted within each of the 12 mainland Health Boards (the island Health Boards were excluded because of low sample sizes). Unexpectedly high peaks were observed in 10 Health Boards, with 1-4 peaks in each Health Board and 23

peaks in total (Figure 24; Table 13). The ‘extra’ cases in these peaks accounted for 13.1% of all clinical cases from the 12 mainland Health Boards.

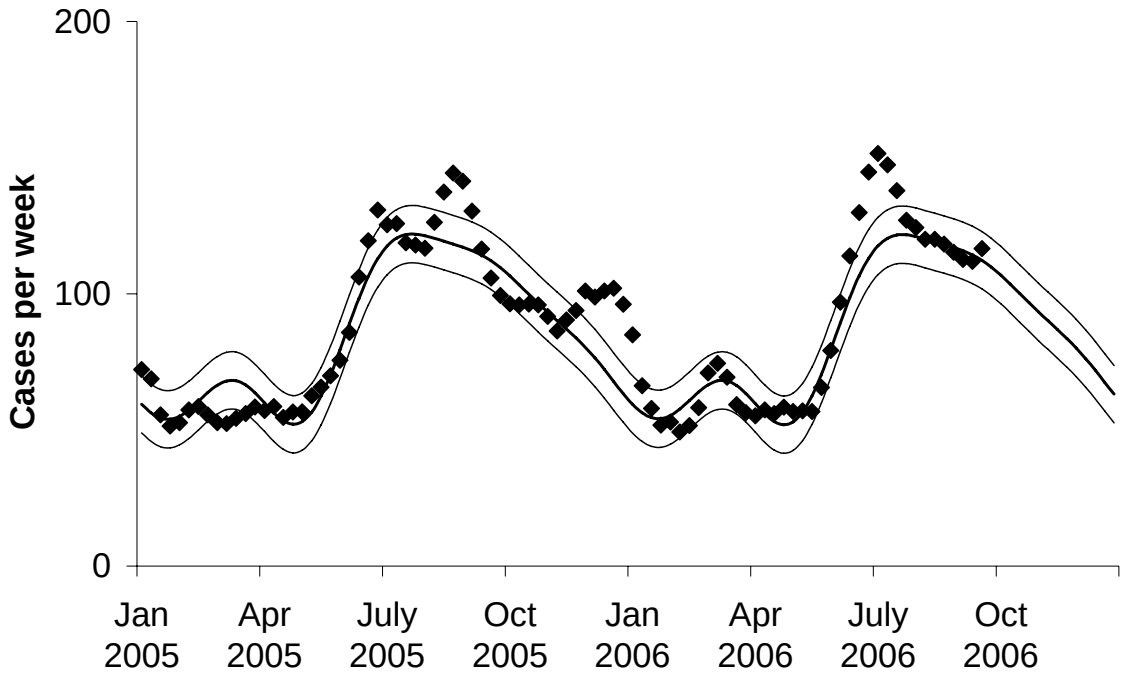
The observed and expected values of ST-matching for the 23 incidence peaks were compared. The observed values were calculated for the cases from the week with the highest incidence, and the expected values were based on randomised samples from the same Health Board. Twelve of the peaks, occurring in eight Health Boards, had significantly high ST-matching values. The 12 peaks contained 16 STs contributing to the high ST-matching values (Table 13). These STs represented some of the commonly found STs, *e.g*., ST21, ST45, ST257, ST827-*C. coli*, and some rarer STs, *e.g*., ST262 and ST436. These peaks accounted for 4.1% of all ST-typed clinical cases from the 12 mainland Health Boards.

Further epidemiological data would allow the determination of whether the cases in these peaks were likely to be linked.

Figure 23. Observed and expected incidences of *Campylobacter* infection in Scotland.

Expected incidence (thick line) with 95% confidence intervals (thin lines) are compared to observed weekly incidences (dots).

(a) cases reported to HPS



(b) cases submitted to CaMPS

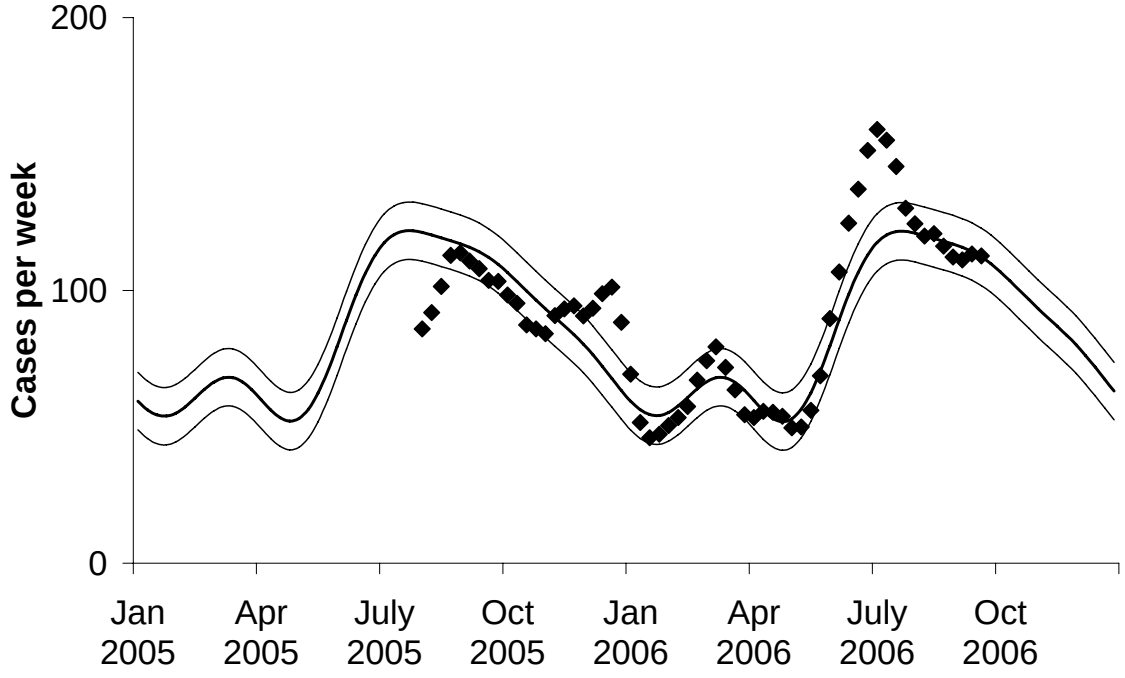
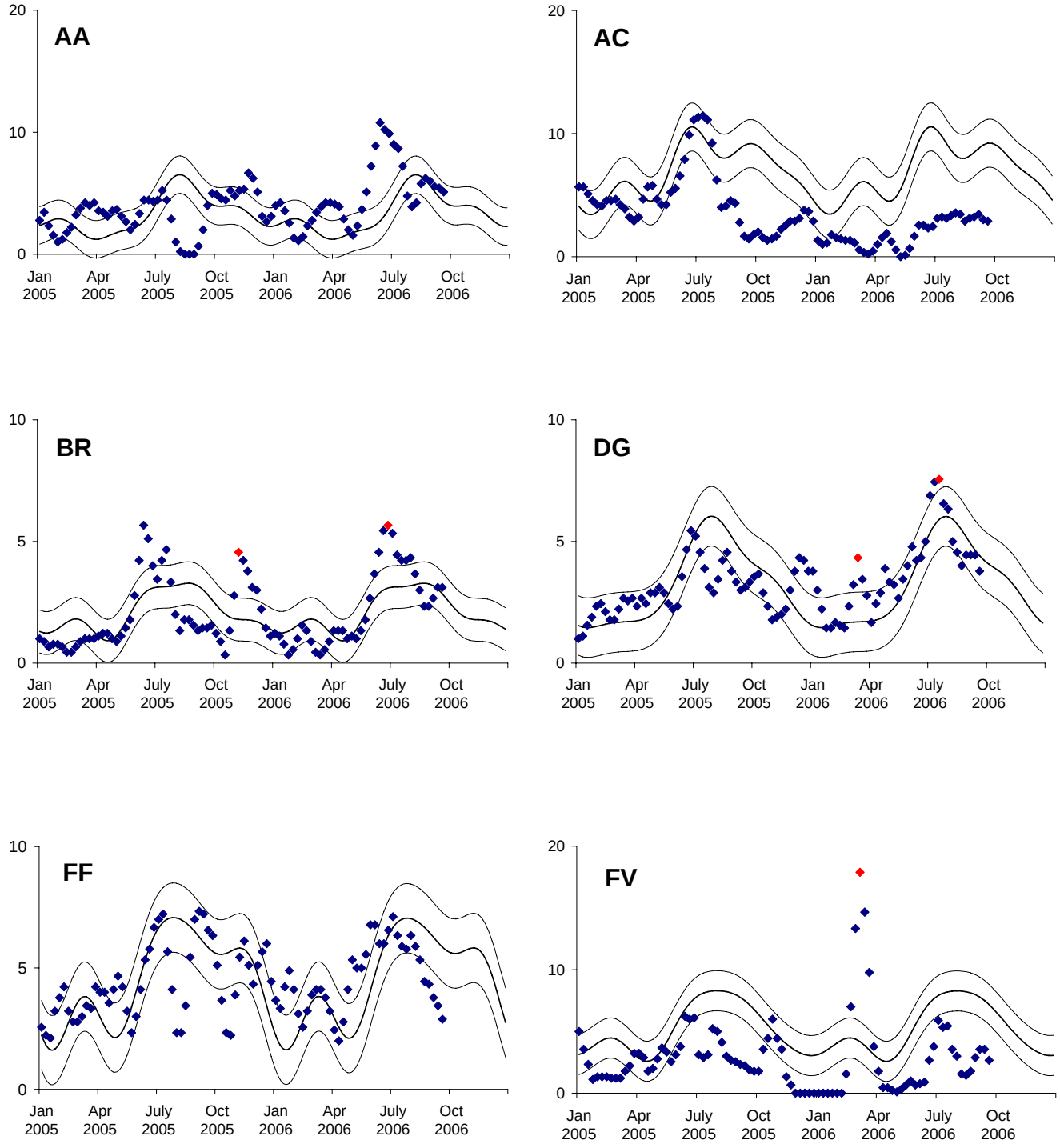


Figure 24. Observed and expected incidence of *Campylobacter* infection in each mainland Health Board in Scotland.



Expected incidence (thick line) with 95% confidence intervals (thin lines) are compared to observed weekly incidences (dots). The Y axes give the number of weekly cases: observed incidence is cases submitted to CaMPS and expected incidence is cases reported to HPS. The red dots indicate the weeks of peak incidence with significantly high levels of ST-matching among cases.

Figure 24. (cont.)

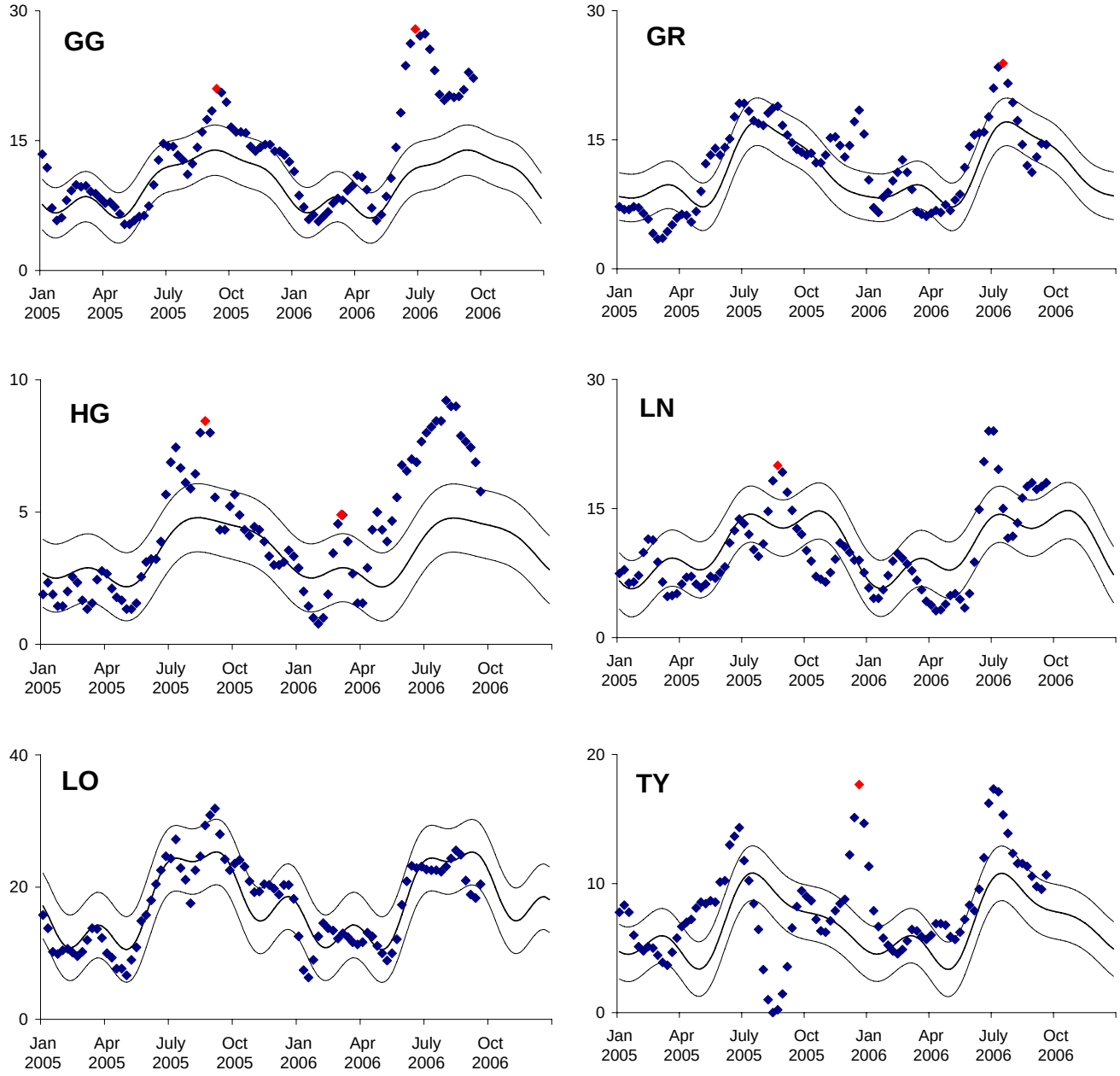


Table 13. Peaks and other observations relating to *Campylobacter* incidence in each mainland Health Board.

Health Board	Incidence peaks	No of cases	STs showing matches	Proportion of isolate pairs with matching ST
AA [1]	1. Nov 2005	7	257	0.047
	2. Mar 2006	6	50	0.067
	3. June 2006	10	21, 52	0.044
AC [2]	None.	-	-	-
BR	1. Nov 2005	11	21, **475**	0.127**
	2. June 2006	7	**21**, **45**	0.095*
DG	1. Dec 2005	8	827	0.036
	2. Mar 2006	9	19, 257, 436	0.083*
	3. April 2006	8	25	0.071
	4. July 2006	9	**21**, 257	0.111*
FF [3]	None			
FV	1. Mar 2006	33	19, **48**, 50, 61, 257, **354**, **607**	0.072**
GG	1. Sept 2005	24	21, 48, **257**, 354	0.065*
	2. July 2006	28	45, 53, 257, 827	0.053*
GR	1. Dec 2005.	16	61, 257	0.058
	2. Feb 2006	10	257, 574	0.044
	3. July 2006	31	**21**, 45, 257, 574, 827	0.084**
HG [1]	1. Aug 2005	13	21, 48	0.077*
	2. Mar 2006	3	574	0.333**
	3. Aug 2006	14	50, 2786	0.022
LN	1. Aug 2005	17	48, 855	0.081*
	2. July 2006	8	75, 829	0.071
LO [3]	1. Sept 2005	25	257, 273, 824	0.030
TY	1. Dec 2005 [4]	33	**51**, 257, **262**, **574**	0.188***
	2. July 2006	15	19, **21**, 257, 827	0.038

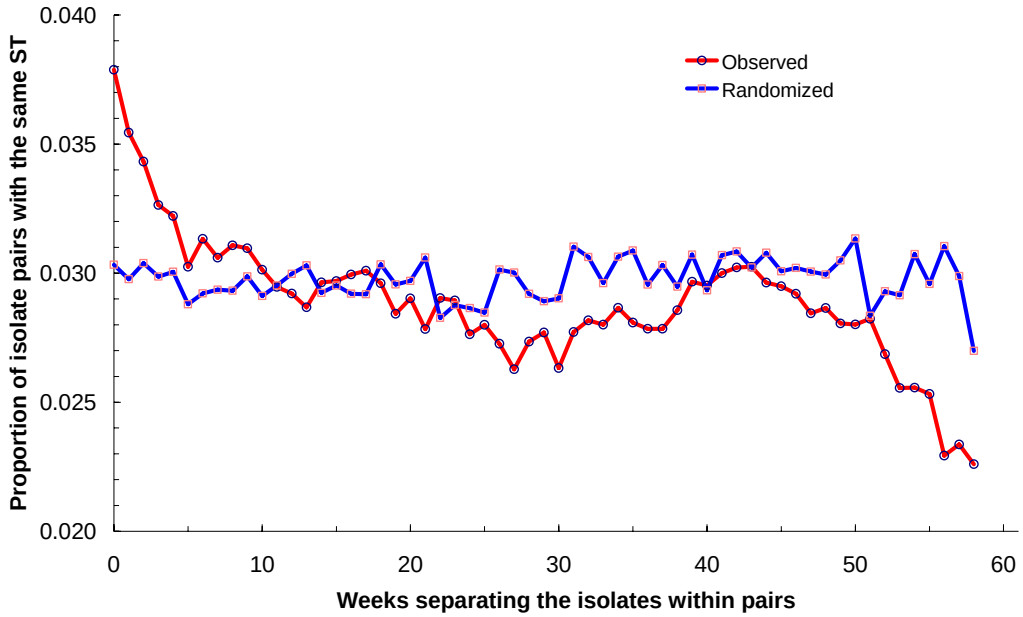
1: incidences in AA and HG are noisy; 2: AC has sparse data because of underreporting; 3: incidences in FF and LO fit the model very well; 4: peak is the Montrose outbreak. The probability of finding a given value for the proportion of isolates pairs with matching ST is indicated as *** (P< 0.001), ** (0.001 < P < 0.01), * (0.01 < P < 0.05) and - (P > 0.05). The STs in bold were also identified as being part of potential outbreaks in a subsequent analysis (see Table 15).

In Method 2, the extent to which clinical *Campylobacter* isolates with the same ST were clustered in time during the project was assessed as follows. The proportion of all pairs of isolates with the same ST was calculated for all time intervals for the observed data. A pair of isolates was randomly chosen from the clinical dataset, the time interval in weeks between the isolates was calculated and whether or not the pair had the same ST was noted. This procedure was repeated 2.5 million times. The same proportion was then calculated for randomised data. The first procedure was followed except that the week of one of the isolates in the pair was exchanged with a randomly-chosen week. The observed and randomised distributions of the proportion of ST-matching pairs of isolates were then compared.

The observed proportion of ST-matched pairs was highest for isolates from the same week (Figure 23, red line), and was higher than expected for isolates from 0-10 weeks apart (excess of red line over blue line). The excess was approximately 2.25% of all pairs of isolates (the area between the blue and red lines up to week 10). The observed distribution eventually tails off because longer time intervals are underrepresented in such a fixed-duration study.

This analysis shows that pairs of isolates with reporting dates up to at least five weeks apart are significantly more likely to match for ST than would be expected by chance.

Figure 25. Proportion of pairs of human isolates with the same ST in relation to the time interval separating the isolates.



In Method 3, the degree of spatio-temporal clustering of single STs in the clinical isolates was assessed for the 12 mainland Scottish Health Boards. Clustering was assessed using SATSCAN analysis (65, 66), with the following settings: Poisson Model, space-time analysis, high and low cluster detection, maximum size of spatial clusters equal to 50% of the population at risk, the cut-off probability value used to declare a cluster was p=0.01, and maximum size of temporal clusters equal to 30 days. Differences in relative risk of infection were taken into account by adjustment using expected values of overall incidence of *C. jejuni* and *C. coli* infection combined for each Health Board-month, and by using population data for the mid-2004 Census for each Health Board (32). In this method, the expected incidence of each ST is thus adjusted using the overall observed geographical and seasonal differences in *Campylobacter* incidence.

The 64 most common STs (82% of clinical isolates) were assessed for spatio-temporal clustering (Table 14). Over half of the STs analysed (N=39) showed no clustering, 17 STs showed one cluster, six showed two clusters and two showed three clusters. The proportion of isolates of each ST that were allocated to a cluster ranged from zero to 60% (ST334). The clusters accounted for 7.4% of all ST-typed isolates from the 12 mainland Health Boards. Clusters were located in all Health Boards Areas and during all months except April 2006 (Table 15).

In summary, the results of the three methods were in agreement in that 2.2-7.4% of clinical isolates from the 12 mainland Health Boards showed evidence of spatiotemporal clustering. The results of methods 1 and 3 that identified specific STs as being clustered showed weak correspondence. Method 1 identified 37 STs and method 3 identified 30 STs but only 13 were identified by both methods (Tables 13 and 15, STs in bold). These discrepancies probably arise because methods 1 and 3 both analyse the dataset in incomplete ways.

The analyses are incomplete for three main reasons. First, method 1 assesses evidence for clustering only in incidence peaks (however these are defined) whereas method 3 does so across the whole period of sampling. Method 1 therefore cannot detect outbreaks occurring outwith incidence peaks whereas method 3 can. Second, method 1 uses data on incidence peaks directly whereas method 3 uses such data to standardise the expected incidence of each ST being analysed. Method 1 can

therefore detect a large outbreak that consists of one or a few STs and that causes an overall incidence peak whereas method 3 might fail to detect such a peak through over-normalisation. Third, method 1 was implemented as a strictly within-Health Board analysis whereas method 3 analyses an increasing spatial scale up to the whole dataset. Method 1 is therefore unlikely to detect a “diffuse” outbreak, *e.g.*, routed through widespread food chains, that occurs across more than one unit of analysis.

Table 14. Inferred spatio-temporal clusters in human *Campylobacter* cases stratified by ST

ST	N	Cluster (Obs - Exp)	Area (HBs)	**Period**
ST257	466	39 - 14.6	AA AC DG FV GG LN	**13/9–12/10/05**
ST21	397	14 - 1.6	FF GR HG TY	**3–7/7/06**
		8 - 0.5	BR DG	**30/6–24/7/06**
		8 - 0.5	AC GG LN	**4/7/06**
ST45	287	31 - 5.1	AA BR DG LN	**29/5–27/6/06**
		28 - 6.6	FF FV GR HG TY	**12/6–5/7/06**
ST48	233	6 - 0.2	FF GR HG TY	**11/7/06**
		4 - 0.04	FV	**16–22/2/06**
ST51	199	14 - 0.4	FF GR HG TY	**5–7/12/05**
		19 - 4.3	AA BR DG FV LN LO	**6–27/10/05**
ST574	189	12 - 0.04	FF TY	**6–7/12/05**
		8 - 0.5	LN	**17–31/1/06**
ST827	172	5 - 0.7	BR DG	**30/8–11/9/06**
C. coli		5 - 0.14	HG	**11/5–26/5/06**
ST53	157	23 - 4.9	AA GG LN	**11/8–5/9/06**
ST354	146	3 - 0.0	FV	**22/2/06**
ST2030	132	19 - 2.1	FF GR HG TY	**25/4–23/5/06**
ST50	131	7 - 0.2	FV LO	**21–26/7/05**
ST19	127	10 - 0.9	BR FF FV LO TY	**12–20/9/06**
		8 - 0.7	AC GG LN	**14–20/9/06**
ST61	118	4 - 0.6	HG	**31/8–12/9/06**
ST137	94	3 - 0.0	FV	**10/10/05**
		5 - 0.12	AA AC LN	**29/5–31/5/06**
		13 - 2.2	FF GR HG TY	**20/6–17/7/06**
ST262	87	7 - 0.2	FF LO TY	**6–8/12/05**
ST475	75	3 - 0.0	BR	**25–28/10/05**
ST42	68	5 - 0.1	BR DG	**18/7–14/8/06**
ST572	58	none		
ST52	57	3 - 0.01	TY	**15/3/06**
ST5	55	2 - 0.0	BR	**23/11/05**
ST464	51	none		
ST573	50	none		
ST273	48	none		
ST825	48	none		
ST206	41	none		
ST122	39	none		
ST583	39	none		
ST25	37	5 - 0.14	AC GG	**29/8–2/9/05**
ST22	33	none		
ST267	32	none		
ST607	28	2 - 0.0	FV	**22/2/06**
ST824	27	none		
ST1774	25	none		
ST353	23	none		
ST400	23	none		
ST266	22	none		
ST855	21	none		
ST2033	20	none		
ST334	19	4 - 0.1	AA BR DG	**22/6–11/7/06**
ST658	19	8 - 0.9	All	**21/11–12/12/05**
ST829	18	none		
ST1614	18	none		
ST44	17	none		
ST677	17	2 - 0.0	HG	**8/8/06**
ST814	17	none		
ST962	17	none		
ST436	16	none		
ST872	15	3 - 0.02	LO	**28–29/9/05**
ST883	15	none		
ST49	14	none		
ST104	14	none		
ST904	14	none		
ST38	13	none		
ST270	13	none		
ST661	13	none		
ST828	13	none		
ST969	13	none		
ST82	12	none		
ST403	12	none		
ST441	12	none		
ST227	11	none		
ST1044	11	none		
ST356	10	none		
ST581	10	none		

ST, the 64 STs occurring 10 or more times in the clinical isolates; N, total number of isolates of each ST; HBs, Scottish Health Board Areas where each cluster occurred; Period, start and end dates when each cluster occurred; Obs, Observed number of each ST; Exp, Expected number of each ST according to a SatScan analysis.

Table 15. Spatio-temporal distribution of inferred ST-clusters in human *Campylobacter* cases.

	AA	AC	BR	DG	FF	FV	GG	GR	HG	LN	LO	TY	No. HBs in cluster	Total clusters
2005														
July						50					50		2	1
Aug		25					25						2	1
Sept	257	257		257		257	**257**			257	872		1, 6	2
Oct	51		51 **475**	51		51 137				51	51		1, 1, 6	3
Nov			5										1	1
Dec					51 262 574			51 [1]	51		262	**51** [1] **262** **574**	2, 3, 4	3
2006														
Jan										574			1	1
Feb						**48** **354** **607**							1, 1, 1	3
March												52	1	3
April													-	-
May	137	137			2030			2030	827 2030	137		2030	1, 3, 4	3
June	45		**45**	45	45	45		45	45	45		45	9	1
July	334	21	**21** 334	**21** 334	21 48 137		21	**21** 48 137	21 48 137	21		**21** 48 137	3, 4, 4, 9	4
Aug	53		42 827	42 827			53		667	53			1, 2, 3	3
Sept		19	19		19	19	19		61	19	19	19	1, 2, 8	3

The STs indicated in bold were also identified as being part of potential outbreaks in the first analysis (see Table 13); 1: the clustering of ST51, 262 and 574 in Grampian and Tayside Health Boards corresponds to the Montrose outbreak.

4.6 Host associations of *Campylobacter* strains and the origins of human infection

Host associations

The six most intensively sampled host species and food sources showed obvious visual differences in the composition of *Campylobacter* strains categorised according to ST (Figures 26 and 27a-f). When these differences were analysed quantitatively, ST composition differed significantly between all pairs of the six *Campylobacter* sources (ARLEQUIN, exact test of population differentiation, 100,000 Markov chain steps, 100,000 dememorisation steps, *P*=zero for all 15 comparisons). Thus, each of the six sources possesses a characteristic spectrum of STs, with some STs being specific to a source and other STs differing in frequency across sources. Nonetheless, previous analyses (Figure 14b) showed that all host species and food sources except pigeon generally contained very high levels of ST diversity, prompting the question of how much of the total ST diversity in the six sources is distributed between sources compared to how much occurs within sources. This question was addressed using an analysis of molecular variance or AMOVA (Table 16; AMOVA, see legend for details). Approximately 5% of the total ST diversity in the six host or food sources was distributed among sources (Table 16; “2. Among hosts within groups” under “Source of variation”), and the remaining 95% occurred within each source (Table 16; “3. Within hosts”). Thus, the distinctiveness of the six *Campylobacter* sources in terms of ST composition is real but represents only approximately 5% of the total ST diversity in these sources.

The six *Campylobacter* sources had obvious differences in the patterns of occurrence of several STs or *Campylobacter* species, and these differences are now discussed in detail.

Cattle and sheep shared more STs than any other pair of sources (Figure 27a, b). Despite this, some STs yielded evidence for host specificity. Nineteen STs occurred more often in cattle (67-100%) than in any other source (zero-33%), 5 STs occurred more often in sheep (64-100%) than in any other source (zero-23%), and four STs occurred approximately equally in cattle (40-61%) and sheep (12-53%) while being rare (zero-14%) in the three other sources. Regarding specific STs, the three most common STs in cattle were, in descending order: ST61, ST42 and ST48. ST61 was also one of the two most common STs in sheep (with ST827-*C. coli*). These patterns were consistent with previous studies that identified CC61 (includes ST61), CC42 (includes ST42) and CC48 (includes ST48) as associated with farm ruminants (13, 29,76).

The fourth most common ST in cattle (ST2217) was a new discovery: it occurred uniquely in cattle isolates, was absent in clinical isolates, and was novel to the PubMLST global list of STs in October 2006. The ST2217 isolates originated from six farms within a linear 35 km transect in NE Scotland and from a single farm in Dumfries & Galloway, and they were obtained during June-October 2005 and June-August 2006.

The four most common STs in sheep were (in descending order): ST61, ST827-*C. coli*, ST825-*C. coli* and ST962-*C. coli*, and these accounted for just over half of all the isolates from sheep (Figure 27b). The high percentage of *C. coli* (39.7%) in the *Campylobacter* isolates from sheep in Scotland found in the present study is in accordance with the results of a previous study based in NW England (8).

Pigs yielded (with one exception) *C. coli* isolates (Figure 27c), consistent with many previous studies. The pig isolate STs were highly diverse (DI=0.952 ±0.017) and they showed limited overlap with isolates from other host and food sources. Only three of the 21 STs found in pigs in this project occurred in isolates from other host and food categories. ST1068 is common in pigs in the USA (84) but was not found in the present project.

Cats and dogs (cats: 2 healthy and 5 sick, dogs: 2 healthy and 43 sick) yielded six STs that occurred more than once in this source (Figure 27d). The two most common STs were ST45, which was actually more common in wild bird and retail chicken than in cats and dogs, and ST48, which was equally common in sheep and retail chicken and most common in cattle. Two further STs (ST403 and ST508) did not occur in the other source groups, and the last two STs (ST21 and ST137) occurred in other sources.

Wild birds yielded one ST (ST220) that did not occur in any other source (Figure 27e). Its species origin in ‘unidentified wild birds’ was most likely to have been from pigeon because ST220 was the most common ST in identified pigeon faeces. Regarding specific wild bird species, 75% of pigeon isolates comprised three STs from CC179, with 64% of these isolates being ST220, thus explaining the exceptionally low ST diversity in this host (Section 4.2, Figure 14b). In addition, just over half the isolates from gulls (56%) comprised six STs from CC1275, with 29% of these isolates being a single ST (ST637).

Retail chicken yielded a distinctive spectrum of STs (Figure 27f). Chicken yielded 30 STs that occurred more than once in this source, of which 13 occurred only in chicken and four occurred more often in chicken (73-95%) than in the other five sources (0-20%). Of the remaining 13 STs from retail chicken, three were most common in cattle (ST21, ST42,

ST61), two were most common in sheep (ST825, ST962), three were approximately equally common in cattle and sheep (ST19, ST48, ST827) and five had no strong pattern of host association (ST11, ST45, ST52, ST53, ST137).

Retail liver portions yielded *Campylobacter* isolates representing four of the six source groups: cattle (calf or ox liver, 12 isolates), sheep (lamb's liver, 11 isolates), pig (5 isolates) and chicken (5 isolates). The 12 cattle liver isolates yielded 12 different STs that included three cattle-associated STs (ST19, ST21, ST48), ST827, which was approximately equally common in cattle and sheep, but also ST574, which was otherwise found only in retail chicken, where it was the second most common ST. The 11 sheep liver isolates yielded 10 different STs that included two sheep-associated STs (ST273 and ST962), two STs that were approximately equally common in cattle and sheep (ST206 and ST827), but also ST52, which occurred in cattle, sheep and retail chicken. The five pig liver isolates yielded five different STs that included ST19 (cattle and sheep-associated), ST58 (cattle-associated) and three *C. coli* STs that had not been previously detected in the six sources. The five chicken liver isolates yielded four different STs that included ST257 (chicken-associated), ST19 (cattle and sheep-associated), ST45 (no host association), and ST2687, which had been found once in the six sources (in retail chicken).

The overall picture that emerged from analysing ST composition of *Campylobacter* isolates from the retail liver portions was two-fold. First, levels of ST diversity were as high as were found in the most diverse clinical and environmental sources (Figure 14a,b), with the 33 isolates with complete STs yielding 27 different STs (DI=0.987, 95% CI: 0.981-0.993). Second, some evidence emerged for cross-contamination among species, with chicken-associated STs occurring in cattle and sheep liver portions, and cattle- or sheep-associated STs occurring in chicken and pig liver portions.

Figure 26. *Campylobacter* ST composition in strains from the most intensively sampled host and food categories. The histograms show the 51 most common STs overall so the STs shown in each category is not necessarily the commonest in that category.

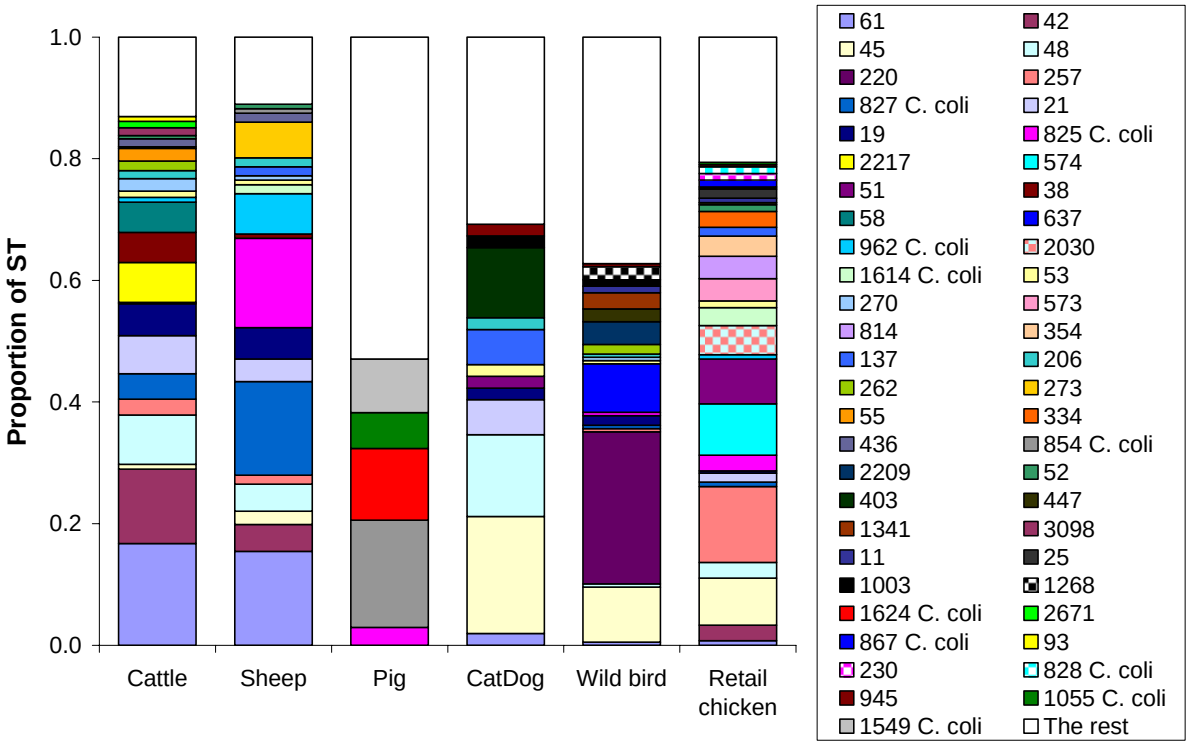


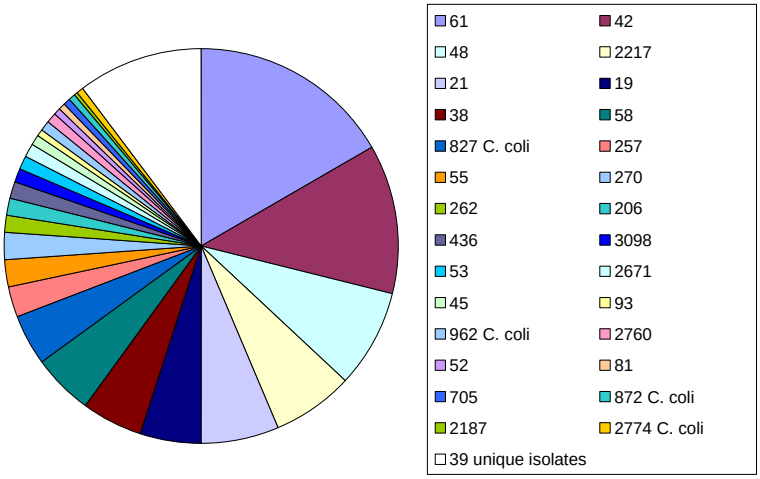
Table 16. Hierarchical stratification of ST diversity among the six types of host and food according to AMOVA.

Source of variation	Variance component	Percentage of variation
1. Between groups [1]	-0.00461	-0.94NS
2. Among hosts within groups	0.02739	5.58***
3. Within hosts	0.46782	95.36***
Total	0.48544	100.00***

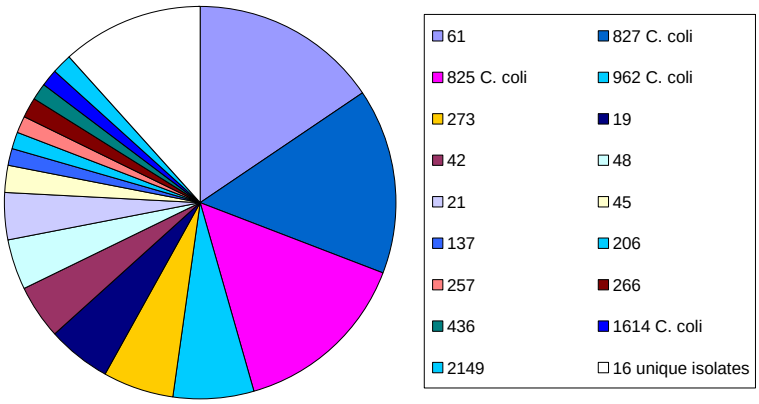
1: The two groups used were (Cattle, Sheep, Pig, Cat and Dog, Wild Bird) versus (Retail chicken). ***, evidence for differentiation at $p<0.0001$.

Figure 27. ST frequency in isolates from the five most intensively-sampled animal and bird hosts.

(a) Cattle (383 isolates)



(b) Sheep (136 isolates)



(c) Pig (34 isolates)

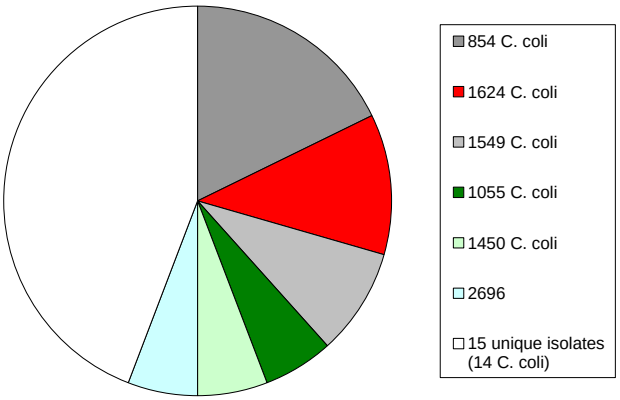
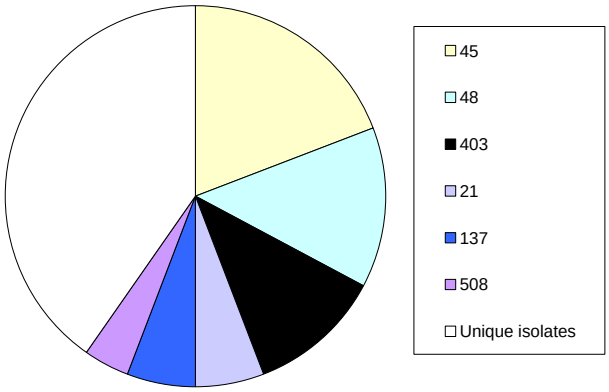
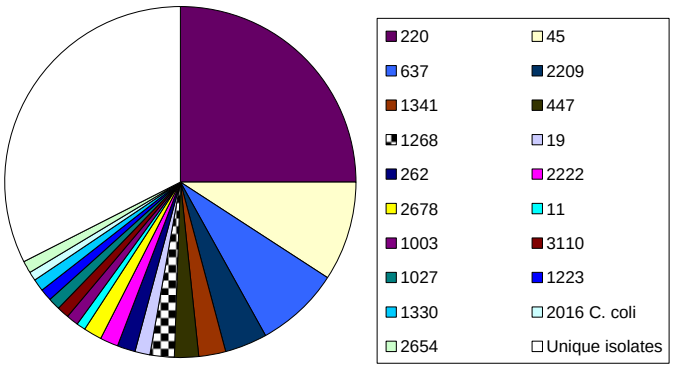


Figure 27 (cont.). Frequencies of STs of isolates from the five most intensively-sampled animal and bird hosts.

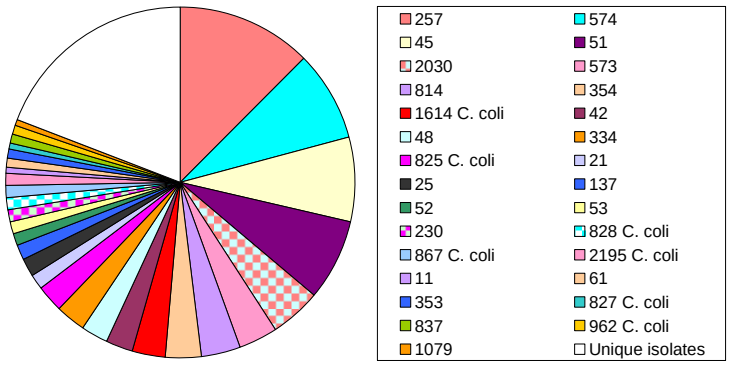
(d) Cat & dog (52 isolates)



(e) Wild bird (188 isolates)



(f) Retail chicken (272 isolates)



Origins of human infection

The previous section showed that potential infection sources differed to varying degrees according to the ST composition of their *Campylobacter* isolates, such that ST identity can yield some information about the source origin of strains. Most of the STs found in potential infection sources also occurred in clinical isolates, and this can allow human *Campylobacter* cases to be attributed to these sources.

Clinical *Campylobacter* isolates were attributed to infection sources using ST composition in isolates from the six infection sources most intensively sampled in CaMPS: cattle, sheep, pig, cat & dog, wild bird and retail chicken. The STs of isolates from these sources were obtained from two databases: the CaMPS dataset generated in this project (originating from July 2005–Sept 2006) and the PubMLST database (1179 isolates from the six source categories, that originated in mainland UK during 1988-2007 inclusive, with four more isolates from 1982, that excluded isolates already submitted from CaMPS, and that were listed on the download date of 6 May 2008). These are the only available, comprehensive databases of *Campylobacter* isolates from infection sources typed to ST.

The fraction of attributable clinical isolates, *i.e*., clinical isolates with STs that also occurred in source isolates, was approximately three-quarters in both datasets (Figure 28 a, b). The remaining quarter of clinical isolates are discussed on the next page. The STs in clinical isolates ranged from being common to being rare or non-existent across the six source categories. The 64 most common STs in clinical isolates (representing 81% of all clinical isolates) demonstrate this range of occurrence in the six sources (Figure 29a). At one extreme, the most common ST in clinical isolates (ST257) was most common in retail chicken, rarer in cattle, sheep and wild birds, and was absent from pigs and cats & dogs in both the CaMPS and the CaMPS+PubMLST datasets. At the other extreme, ST220, which only occurred once in clinical isolates (not shown in Figure 29 a, b), was the most common ST in pigeon and occurred in no other source.

Using ST257 as an example, clinical STs were attributed proportionally to the six sources as follows. The clinical ST257 isolates were attributed to each of the six sources according to the proportion of ST257 in each source. In visual terms, this is equivalent to dividing the height of the white (clinical) bar for ST257 in Figure 29a into segments according to the relative heights of the coloured (six-source) ST257 bars. These proportions were then

multiplied by the proportion of ST257 in total clinical isolates to yield an assignment of clinical isolates to each source for one ST. This process was repeated for the rest of the attributable clinical STs. Finally, the proportions attributed to each source were summed across STs to yield overall attributions.

The CaMPS and CaMPS+PubMLST datasets yielded very similar levels of overall attribution to the six sources (Figure 29a). Starting with the lowest attribution, less than 1% of human infection was attributable to pigs, 5-6% was attributable to wild birds, 12-16% was attributable to each of cattle, sheep and cats & dogs, and 31% was attributable to retail chicken. A five-source version (Figure 29b) of the attribution was also done after excluding cats and dogs as potential sources because of the uncertainty over whether these animals are reservoirs of human infection. In this case, less than 1% of human infection was still attributed to pigs, 7-8% was attributed to wild birds, 15-18% each was attributed to cattle and sheep, and 35-36% was attributed to retail chicken.

The clinical STs that were either absent or rare in the infection source datasets were also analysed using the whole of the PubMLST database. Depending on which of the two source datasets was used, either 1164 (22%) or 1266 (24%) of the clinical isolates were absent from the six source categories (Figure 28 a, b) and thus could not be attributed to a source in the above ST-based analysis. Further clinical isolates were rare in the six-source categories. The 27 clinical STs that occurred at least 10 times in the clinical isolates and were either absent (Table 17a) or rare (Table 17b) in the six-source categories in the CaMPS+PubMLST dataset were searched for in the total PubMLST database. All but two of them (ST824 and ST855) were recorded in PubMLST as clinical isolates, and 13 of them were recorded as environmental or food isolates, with 11 isolated from chicken or chicken offal or meat, two from pig, and one each from calf and sheep.

The 27 clinical STs that were rare or absent in the CaMPS and PubMLST databases of environmental and food strains might reflect (i) superficially sampled or unsampled infection sources, especially sources that transmit *Campylobacter* easily to people, (ii) transmission that occurs mainly within human populations with limited dependence on external infection sources, or (iii) transmission resulting from travel outwith the sampling areas, especially overseas travel, which is often highlighted in case-control studies as a risk factor for human *Campylobacter* infection. Epidemiological information on patients would be essential for specific attribution of these cases to foreign travel.

Examples were found where STs were approximately equally common in source isolates but showed a much wider range of occurrence in clinical isolates. For example, ST334, ST354, ST814 and ST2030 were restricted to retail chicken, where they each comprised 2.5-4.7% of isolates. In clinical isolates, by contrast, ST354 and ST2030 were approximately equally frequent (2.5-2.8%) whereas ST334 and ST814 were approximately 10-fold rarer (both 0.3%). Such examples are interesting because they potentially indicate that ST strains within the same infection source differ in their epidemiological properties, *e.g*., shedding rates, survival in free environments, transmissibility or infectiveness to humans. Alternatively, different spatio-temporal collections of strains from a given host might have different ST frequencies, leading to biased estimates of ST composition. For example, most *Campylobacter* infections of broiler chicken consist of an outbreak of one or a few strains within a flock of 1000-15000 birds (100), such that different infected flocks will normally have different ST compositions, as shown (3). Depending on the structure of the food chain, such differences might persist until retail sale, such that sampling retail chicken without knowledge of the food chain structure might have yielded biased estimates of ST composition. A similar argument might apply to other hosts.

The above attribution analysis estimates foodborne routes of infection (represented by retail chicken) to account for 31-36% of human *Campylobacter* infections, and environmental routes of infection (represented mainly by farm ruminants) to account for 32-42% of human *Campylobacter* infections with 22-24% being unattributable. This analysis suggests that human *Campylobacter* cases are due to the approximately equal contributions of infection from retail chicken in the food chain and infection from farm ruminants in the environment.

Attribution based on ST is less ambiguous when the ST being attributed is restricted to a single source where it is common and more ambiguous when the ST being attributed is rare in a particular source or occurs across different sources. STs restricted to single sources were therefore analysed as tags of source origin. All the STs that occurred more than once in the six sources and that were restricted to a single source were tabulated according to whether they also occurred in human isolates (Table 18). Between 3% and 15% of the STs that occurred in each of the six sources was restricted to a single source. The single ST that was restricted to cats and dogs was absent from clinical isolates. Between 1 in 6 and 1 in 10 of the STs restricted to cattle, sheep, pig or wild bird also occurred in clinical isolates. In contrast, almost half of the STs restricted to retail chicken occurred in clinical isolates. This result suggests that the foodborne route predominates over the various environmental routes of infection but to what extent is unclear.

The proportional attributions to five potential source categories (excluding cats and dogs) were also displayed as pie charts for each of the 37 most common STs and the 14 most common CCs (Figure 30 a, b). These display the predominance of chicken, with second highest contributions from cattle and sheep, as the main sources of STs and CCs from human infections. The CC distribution shows the over-attribution of CC-828 to pigs that stems from virtually all *C. coli* STs being members of CC-828.

The present analyses show that *Campylobacter* isolates from foodborne sources and farm ruminant sources overlap in ST composition to an approximately equal extent with clinical isolates. Does overlap in ST composition provide comparable evidence for foodborne and environmental infection routes? Retail chicken is a plausible source of *Campylobacter* infection: high proportions of carcasses are infected, the bacterial counts are often high enough to provide infective doses, and processing in the food chain and handling of raw chicken in domestic kitchens are obvious infection routes. Farm ruminants are a less plausible infection source: prevalence was lower than in retail chicken and liver, and while bacterial counts in the retail foods and animal faeces tested were comparable, the route of infection from farm ruminants to humans through direct exposure is less obvious. Retail meats of farm ruminants on sale in the UK have a *Campylobacter* prevalence range much lower (4.7-7.4%) than in retail chicken so the relative levels of transmission from red meat and retail chicken cannot be comparable (70).

There are at least two scenarios for the high level of attribution of human *Campylobacter* infections to farm ruminants.

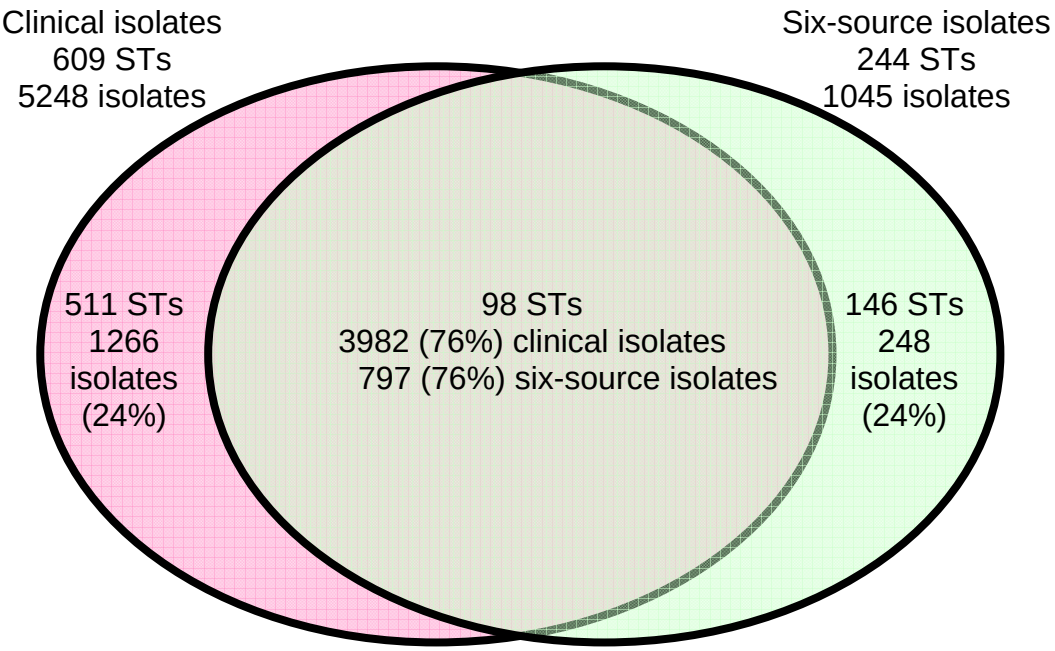
In scenario 1, the attribution analysis is correct: both farm ruminants and retail chicken transmit *Campylobacter* directly to humans (Figure 31a). The infection route from retail chicken is easy to envisage but the infection route from farm ruminants is more difficult to envisage, especially for people who spend most of their time in urban locations. Furthermore, outbreaks of *E. coli* O157 transmitted from cattle or sheep faeces are known but are not typically accompanied by *Campylobacter* outbreaks. In scenario 2, all *Campylobacter* infecting humans is transmitted from retail chicken but some STs are falsely attributed to farm ruminants because they both also occur in farm ruminants and are undetected in retail chicken (Figure 31b).

Two hypothetical mechanisms for the non-detection of STs involve sparse sampling of retail chicken in combination with either (i) outbreak dynamics or (ii) different infection rates. According to the “outbreak dynamics” mechanism, single broiler flocks become infected

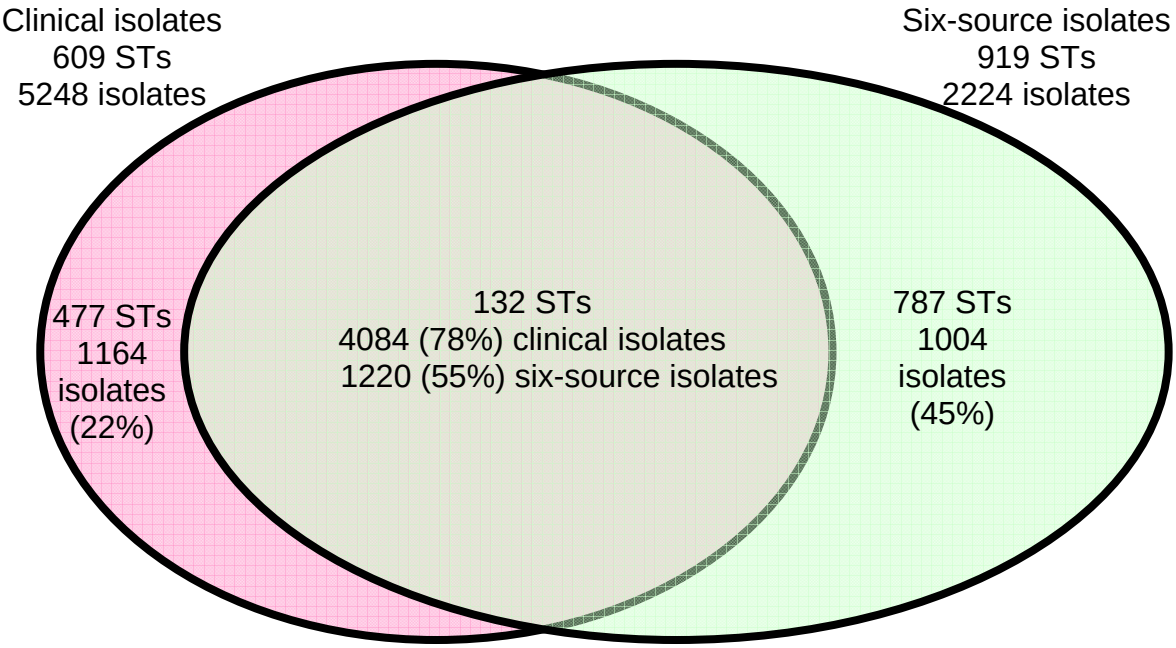
through a flock-specific outbreak that consists of a bottleneck of a few STs, such that the carcasses from each flock acquire a distinct, biased spectrum of STs. The carcasses from a single flock tend to remain together as they travel through the food chain, even when subdivided into batches destined for different branches of a single supermarket, such that retail chicken available for sampling in a given outlet at a given time tends to reflect at least part of the original biased ST spectrum in each flock, leading to the *random* non-detection of STs in retail chicken. According to the “different infection rates” mechanism, some STs infect broilers less often than others, makes them harder to detect and leading to the *systematic* non-detection of STs in retail chicken. Both mechanisms could play a role: which one is more common remains to be determined.

Figure 28. Numbers of STs and percentages of isolates shared between clinical and environmental isolates from six potential sources of human infection.

(a) Source isolates from CaMPS

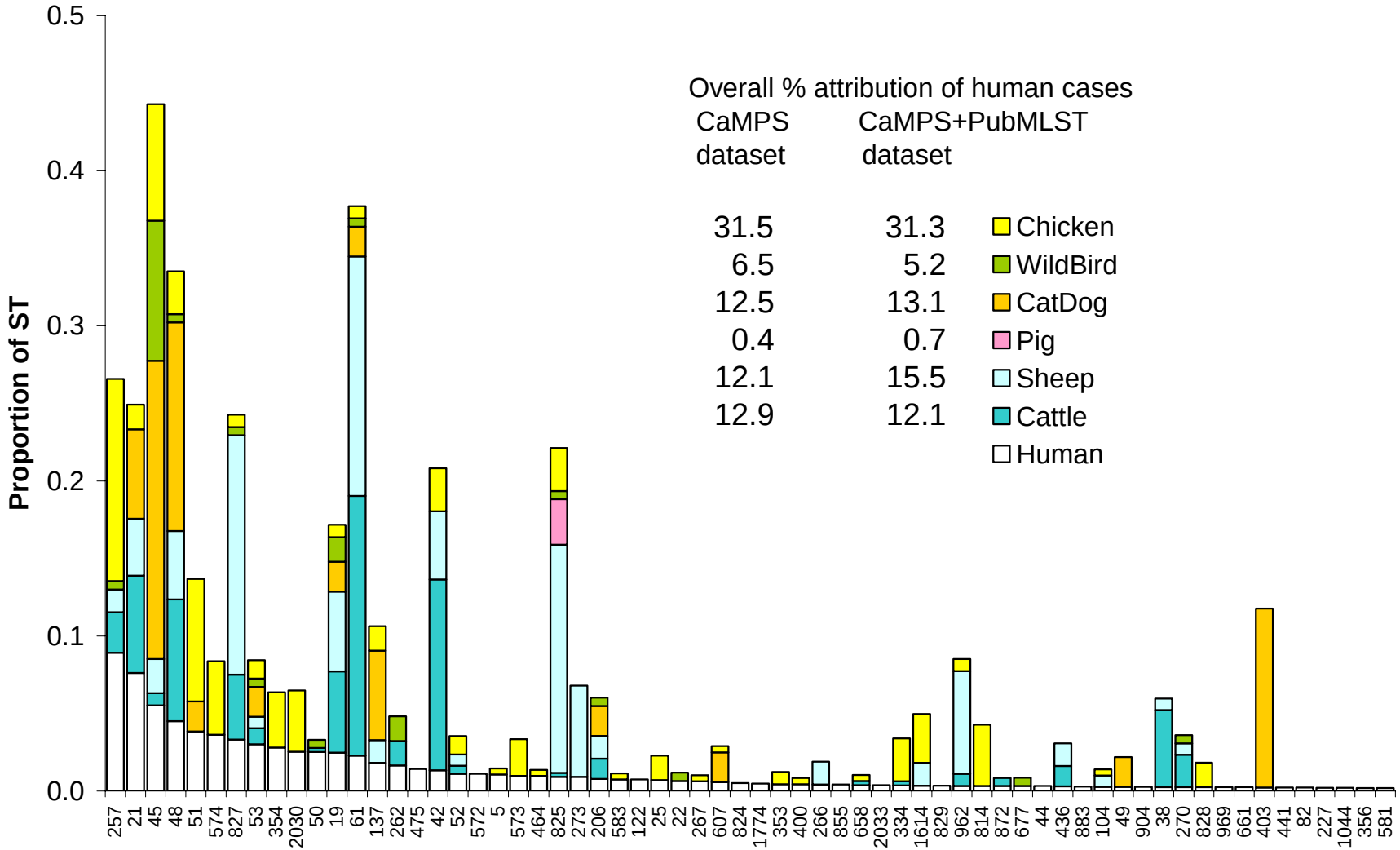


(b) Source isolates from CaMPS and PubMLST



The six source categories account for 94% of all isolates from environmental sources and retail foods.

Figure 29. Proportions of *Campylobacter* STs in clinical isolates and potential sources of human infection, and overall attribution of clinical cases to sources. The histograms shown are for the CaMPS sources dataset. (a) six potential infection sources.



The X axis shows the 64 most common clinical STs in decreasing order of occurrence (all clinical STs with N³10; 81% of all clinical isolates). The legend shows the overall percentages of clinical isolates attributed to each source.

Figure 29 (b) five potential infection sources, excluding cats and dogs.

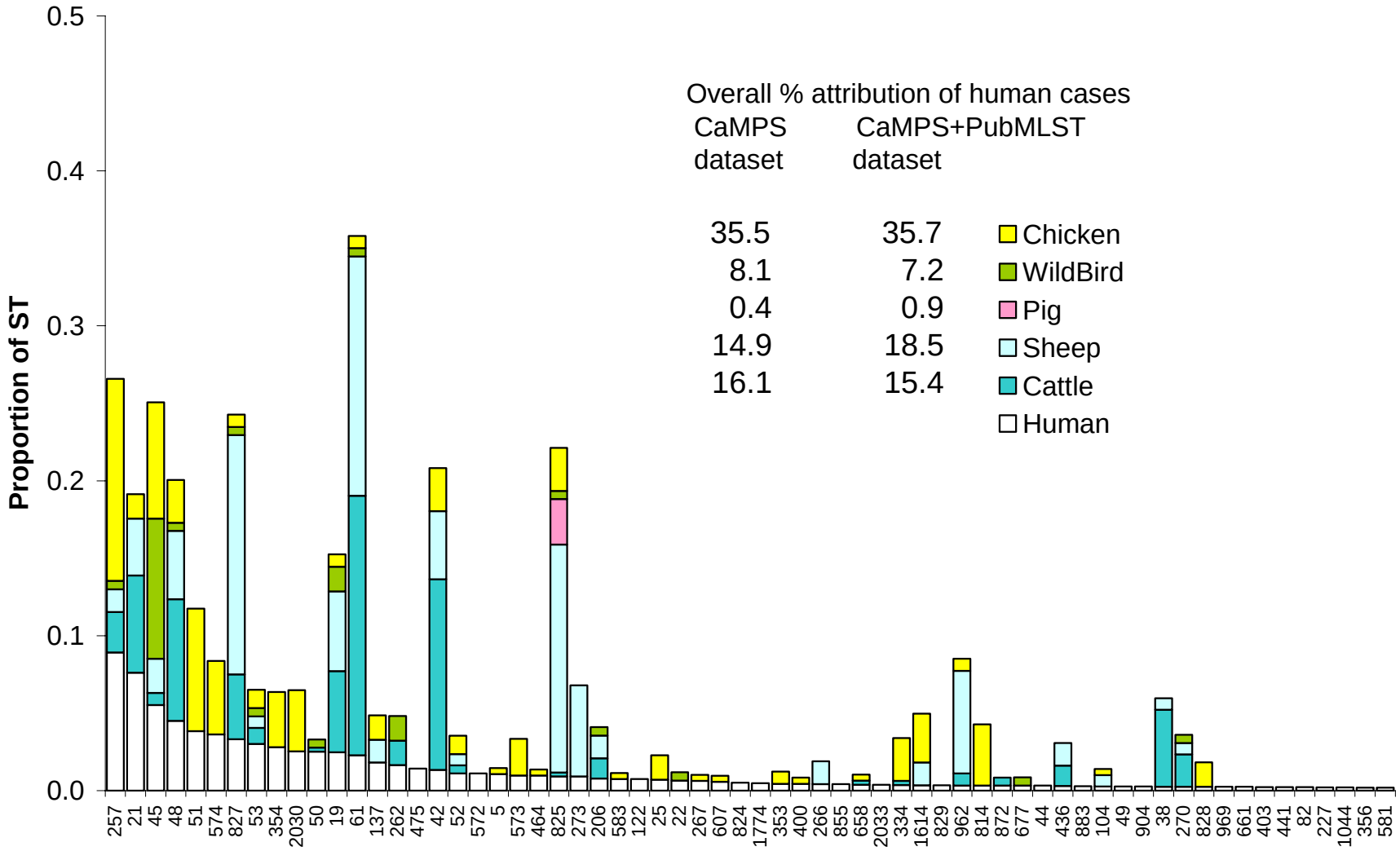


Figure 30. (a) Abundance of clinical isolates by ST and relative importance of likely reservoirs for human infection.

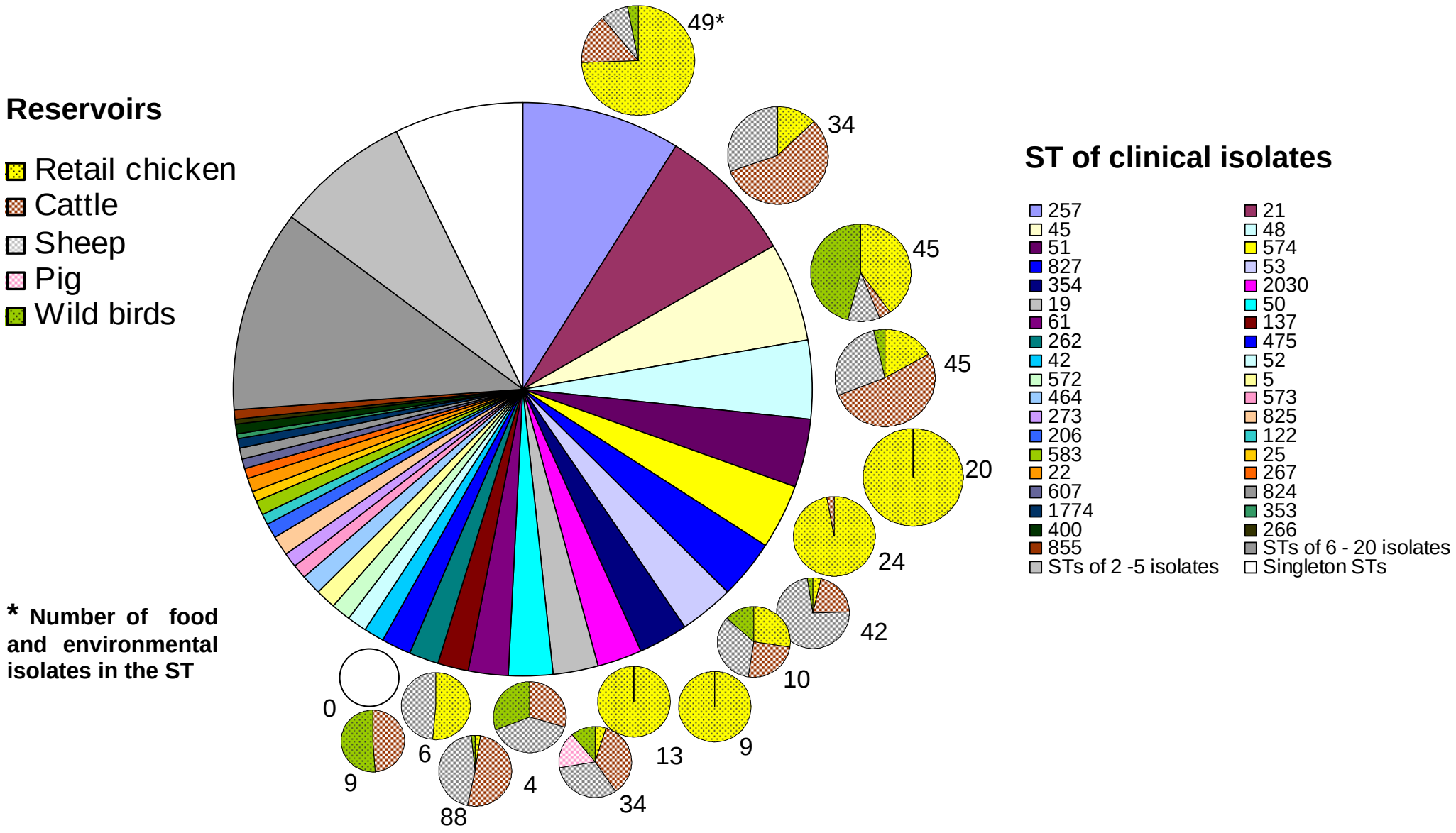


Figure 30. (b) Abundance of clinical isolates by CC and relative importance of likely reservoirs for human infection

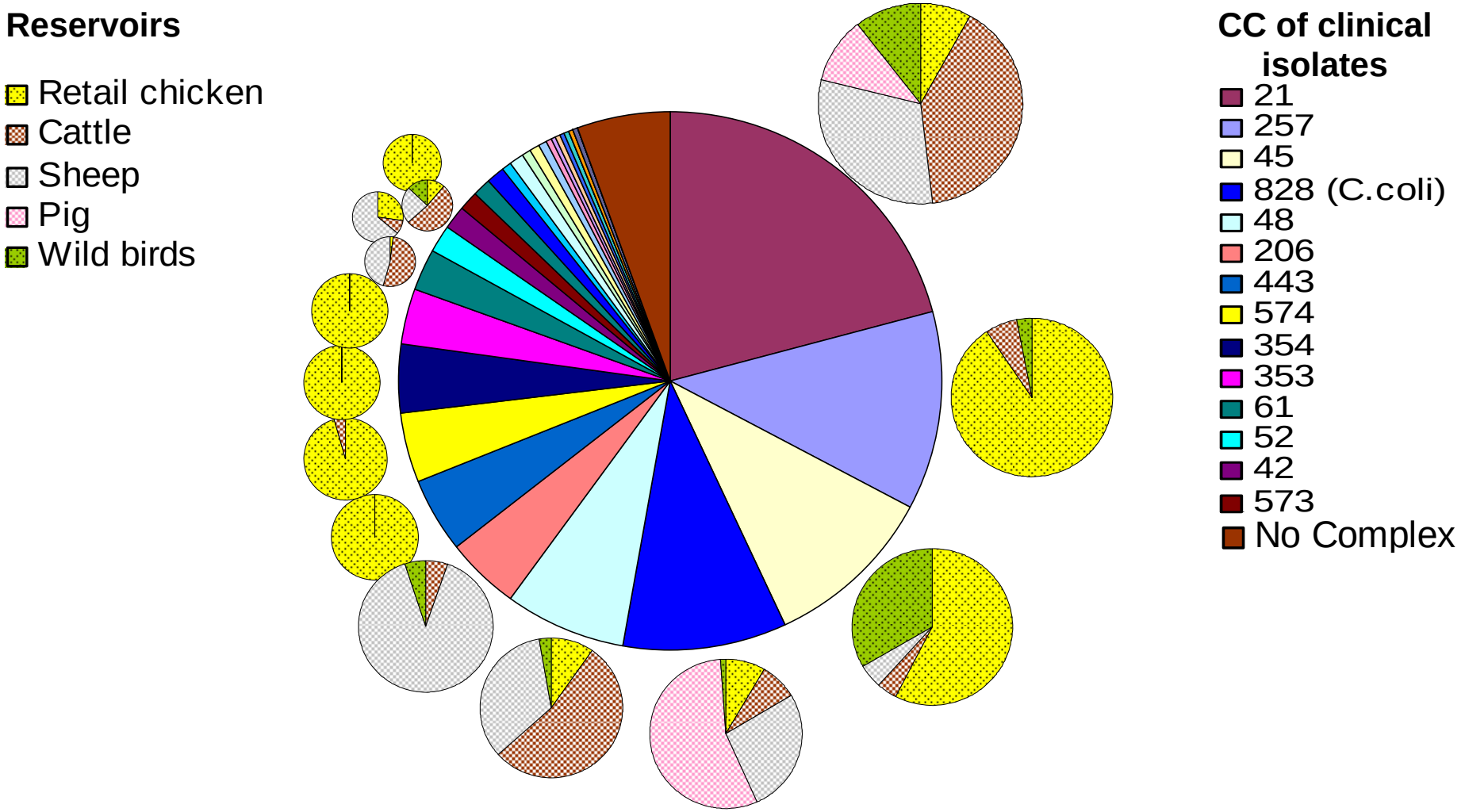


Table 17. Occurrence of STs common in clinical isolates but absent or rare in the six-host isolates.

			Occurrence in the total PubMLST dataset, excluding CaMPS			
ST	**No. of clinical isolates**	**No. of six-host isolates**	**No. of human isolates**	**Human origin**	**No./type of source isolates [1]**	**Source origin**
(a)						
44	17	-	2	Netherlands, UK	2/chicken	Netherlands
82	12	-	1	UK	-	
122	39	-	4	Netherlands, USA	1/chicken	Netherlands
356	10	-	5	Netherlands, UK	-	
441	12	-	4	Canada, UK	1/chicken o/m 1/lake	Canada Spain
475	75	-	22	Curaçao	1/chicken	Denmark
572	58	-	2	Belgium, UK	1/chicken o/m 1/chicken 1/lake	Belgium Spain Spain
581	10	-	1	UK	-	
661	13	-	1	UK	-	
824	27	-	-		-	
829	18	-	1	UK	2/pig	USA
883	15	-	1	UK	1/chicken o/m	Belgium
904	14	-	1	UK	-	
969	13	-	2	Belgium, UK	-	
1044	11	-	1	UK	-	
1774	25	-	1	UK	-	
2033	20	-	2	UK	-	
(b)						
5	56	1	6	Australia, Canada, UK	1/pig	Unknown
227	11	1	9	Australia, Canada, UK	1/chicken	UK
267	33	3	9	Belgium, Sweden, UK	1/chicken o/m 1/chicken 1/lake	UK
354	147	12	12	Australia, Belgium, Canada, UK	1/calf 1/sheep 1/chicken o/m	UK
400	23	1	4	Belgium, Netherlands, UK	-	
464	51	1	2	Canada, Japan	1/chicken o/m	Belgium
607	30	2	1	UK	-	
658	20	2	2	UK	-	
855	22	2	-		1/chicken	UK
2030	133	13	1	UK	-	

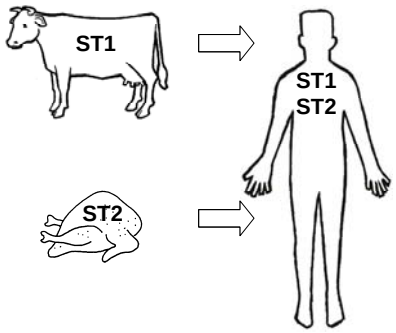
(a) all the STs that were absent from the combination of the CaMPS and selected PubMLST six-source datasets and that occurred 10 times or more in the clinical isolates; (b) all the STs that were at least 10-fold rarer in the CaMPS+PubMLST six-source dataset than in the clinical isolates. 1: o/m, offal or meat.

Table 18. Occurrence of STs restricted to single hosts in clinical isolates.

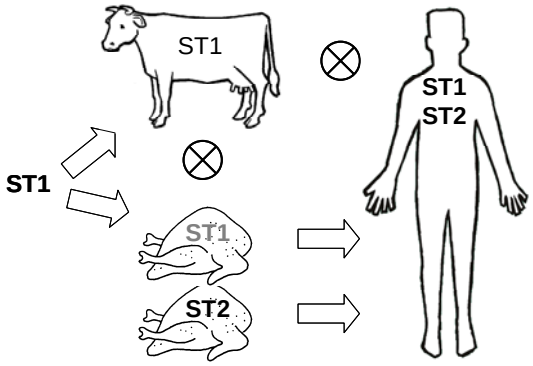
	Cattle	Sheep	Pig	Cat Dog	Wild Bird	Retail chicken
No. of source-specific STs (% of total STs in source)	36 (13.5)	6 (5.6)	10 (11.1)	1 (3.0)	34 (15.1)	33 (10.6)
N (%) of the source-specific STs occurring in clinical isolates	5 (13.9)	1 (16.7)	1 (10.0)	-	4 (11.8)	16 (48.5)

Figure 31. Two scenarios for the attribution of human *Campylobacter* infection to farm ruminants.

(a) Human infection comes from both farm ruminants (ST1) and retail chicken (ST2).



(b) Flock stratification for STs and under-sampling lead to non-detection of ST1 in retail chicken and its false attribution to farm ruminants.



Arrows indicate transmission of resident STs, crossed circles indicate no transmission, grey text indicates an undetected ST, ST1 indicates a set of *Campylobacter* strains associated with farm ruminants, and ST2, a set of strains associated with retail chicken, according to the present study.

5 Overall Conclusions

5.1 Novelty and significance of the study

National datasets on *Campylobacter* strains, and integration with further projects

This study is the world's largest national-scale, contemporaneous comparison to date of *Campylobacter* strain types from clinical cases and a broad range of environmental and food sources of infection. The study's material outputs are an archived collection of over 7000 *Campylobacter* isolates from clinical, environmental and food sources and (at the date of writing) the world's largest database of MLST-typed isolates (almost 6400 isolates) of any bacterial species.

This project's overall goal was to use the transferability and high-throughput characteristics of MLST to overcome the limitations of existing studies of *Campylobacter* epidemiology: their small-scale scope and their lack of comparability. The outcomes of this project show that these limitations can be overcome. The MLST isolate datasets are not only large-scale when considered in isolation: they are also suitable for further integration with epidemiological datasets in FSAS-funded projects ("*Campylobacter* Case Control Study" – S01023; "The role of private water supply in *Campylobacter* infection" - S14024; "Spatial and temporal distribution of *Campylobacter* in Scotland - S14004"). This combination of molecular typing and epidemiological information is unique and will provide a priceless resource for understanding *Campylobacter* epidemiology in Scotland.

Prevalence, bacterial concentration and *Campylobacter* species composition in environmental and food sources

Most studies on prevalence, concentration and species composition of *Campylobacter* in environmental and food sources focus on farm ruminants or retail meats. Furthermore, the results of the attribution analyses (see below) show that pigs and wild birds (the main environmental source studied) are minor or non-existent

sources of human *Campylobacter* infections so a discussion of prevalence, *etc*., in these species seems irrelevant to the goals of the present study. The comparative discussion of prevalence, concentration and species composition will therefore focus on *Campylobacter* in cattle and sheep (farm ruminants) and retail chicken and offal (retail meats).

Reported values of *Campylobacter* prevalence, concentration and species composition in cattle and sheep vary, probably reflecting many differences among studies, including differences in specimen type, microbiological methodology, seasonality, animal age, animal species, husbandry type and local climate (Table 19). Reported values of *Campylobacter* prevalence in sampled cattle faeces (13-51%, Table 19) are comparable to the values found in the present study (24% for the structured study and 36% for all samples). Reported values of *Campylobacter* prevalence in sampled sheep faeces (28 and 29%, Table 19) are very similar to the value found in the present study (28%). Reported prevalence values obtained from the intestines of animals at abattoirs in cattle (29-89%) and sheep (44-92%) tend to be higher than values obtained using faeces (Table 19), perhaps reflecting reduced *Campylobacter* survival after faecal shedding.

The majority of published studies characterise prevalence and species composition but not concentration, and studies that characterise concentration generally do not report tabulated distributions of concentration values from individual animals. The comparative discussion of the CaMPS findings on *Campylobacter* concentrations in farm ruminants is therefore limited. Stanley *et al*. (115) found concentrations shed by beef cattle at slaughter were $6x10^2$/g with loads in dairy cattle and calves approximately 100-fold greater. These authors also suggested that 25% of animals were high shedding (>10^4/g) which is significantly greater than our figure of 6%. This may in part be due to the seasonality of the reporting periods. Bacterial counts in sheep (intestinal carriage) averaging 10^3/g have also been reported (114). The present study found higher bacterial counts in sheep specimens (faecal carriage), with an overall value of 26% of specimens in the high-shedder category. We defined previously (91) high-shedding animals as those excreting target bacteria >10^4/g in faeces and in cattle reported 5-9% of animals shedding *E. coli* O157 above this value. The data presented here suggests that *Campylobacter* shedding from farm ruminants follows a similar trend to *E. coli* O157, and high shedders are more

common in sheep than in cattle. The impact of high-shedding animals on *Campylobacter* transmission to humans is discussed below in the section on future research.

Reported values of *Campylobacter* species composition vary widely in cattle (16-100% *C. jejuni*) and sheep (19-88% *C. jejuni*), undermining comparisons with the findings of the present study (Table 19). Nonetheless, all reported values except the lowest one in each species for the percentage of *C. jejuni* in cattle (66-100%) and sheep (60-88%) were high, as were the values found in the present study (92% in cattle, 60% in sheep). In CaMPS and most published studies, *C. coli* comprised the balance of the *Campylobacter* species present in cattle and sheep (Table 19). The overall implication is that cattle and sheep together constitute the largest of the sampled sources of the two most clinically relevant *Campylobacter* species shed into the farm environment.

Reported values of *Campylobacter* prevalence and concentration in broiler chickens at slaughter or on retail sale vary widely (Table 20), probably reflecting differences in national history of *Campylobacter* prevalence in broilers, relative proportions of freezing *versus* chilling of carcasses in the food chain, seasonality (80), stage of carcass processing, specimen type and microbiological methodology. The reported prevalence range (16-83%) encompassed the prevalence values found in the present study in 2005-06 (75%) and in a previous FSA survey (81%) of raw chicken on retail sale across Scotland during April-June 2001 (27). Gormley *et al*. found no evidence that prevalence had changed significantly between these two studies (35). The reported distributions of Campylobacter concentrations (Table 20) are also highly variable, presumably reflecting similar factors that influence prevalence, including methodological differences determining the lowest detectable concentration. Taking the level of log 4 CFU/carcass as a threshold and ignoring one study that reported values per g, the percentage of carcasses above the threshold varied from 5.0 to 62.7% in reported studies and encompassed the value for the CaMPS study (13.4%). A recently published model-based risk analysis study suggests that log 4 CFU/carcass is the level at which consumer mishandling of chicken starts to noticeably increase infection rates (68). The combination of enumeration data from CaMPS and reported studies and the risk assessment suggests that retail chicken poses a risk of infection and that the level of risk will be highly variable depending on

the percentage of carcasses with high bacterial counts. The threshold of bacterial load corresponding to increased infection risk is poorly known and should be studied further.

Twenty-seven of the 30 *Campylobacter*-positive offal portions had bacterial counts below the lowest detectable level of $<10^3$ CFU/portion, with only three portions from lamb and ox having counts of 10^4-10^5 CFU/portion, suggesting that levels of cross-contamination post-slaughter, or rates of invasion during the lifetime of the animal, are low. Nonetheless, liver as a vehicle of human *Campylobacter* infection must be considered seriously. The recent outbreak in Montrose was attributed to chicken liver pâté, which was prepared by flash-frying livers. In addition, 37% of a small collection (N=30) of retail chicken livers from New Zealand had bacterial counts of 10^3-10^4 CFU /g, and *Campylobacter* was detectable after 4 minutes cooking (129).

Incidence and stratification in clinical infections

The annual incidence of human *Campylobacter* infection during the study was consistent with the increasing trend in Scotland and the UK during the last few years. *Campylobacter* species composition in human infection was consistent with expectations based on surveillance data from England and Wales (10). The approximate three-fold increase in the proportion of *C. coli* from youngest to oldest patient age class was demonstrated for the first time on a national scale while differences in incidence among patient age classes were consistent with previous findings. The incidence peak during summer months was confirmed, with a particularly steep rise in overall incidence being evident during May-June 2006. Annual incidence values and seasonal patterns of incidence in each of the 12 mainland Health Boards were also in line with expectations based on cases reported to HPS in 2003 and 2004, except for a low incidence in Argyll and Clyde that possibly reflected the split and merger of this Health Board with Highland and Greater Glasgow part-way through the collection of clinical isolates in March 2006.

The 12 mainland Health Boards yielded *Campylobacter* isolates that showed few significant differences in species composition, ST diversity or ST composition, despite differing by up to two-fold in *Campylobacter* incidence. In addition, levels of

ST diversity were very high, with new MLST alleles and new STs being detected at the end of sampling the clinical isolates.

The groups of isolates from each of the 15 consecutive months of clinical sampling showed no significant differences in species composition but most groups differed in ST composition. Most of the STs fluctuated in their monthly occurrence, and this was evident even for the most consistent STs. The heterogeneity in monthly ST composition was partly due to some STs fluctuating in step with overall *Campylobacter* incidence, *e.g*., ST21, ST45 and ST48, and partly due to specific STs being common during specific periods, *e.g*., ST19 during September 2006, ST50 during July 2005 and ST2030 during April-May 2006. ST45 occurred predominantly during June 2005 and 2006 in Scotland, consistent with the occurrence of CC45 strains during spring months in NW England during 2003-2004 (111). Despite this evidence for monthly differences in ST composition, less than 1% of the total ST diversity occurred among months and almost all the remainder occurred within each Health Board-month combination. The latter finding emphasises the “large pool” view of *Campylobacter jejuni*/*C. coli* molecular diversity.

The isolates from each of the 15 patient age classes showed some minor differences in ST composition. The incidence peak typical of very young children was not obviously associated with any particular group of STs, but age classes from 66 years and older were associated with higher proportions of *C. coli* strains, as previously reported in England (33).

These findings imply that the processes causing transmission of *Campylobacter* to people of all age classes in Scotland yield very similar outcomes across Health Boards, at least at species and ST levels, despite the known differences in incidence. They also imply that *Campylobacter* transmission in Scotland cannot be regarded as a closed system with a fixed, stable spectrum of subtypes. Scotland is contiguous with the rest of the UK, and immigration from overseas, *e.g*., travel-related cases, is typical of a European country. It would be interesting to reassess the concept of spatial homogeneity in clinical infections by comparison with clinical ST datasets from the rest of the UK or continental Europe.

Outbreaks and linked cases

This project demonstrated spatio-temporal linkage at a national scale among clinical *Campylobacter* cases. There were three lines of evidence that some apparently sporadic clinical cases were linked, given that the Montrose outbreak yielded evidence for increased ST-matching among known outbreak-linked strains. First, 2.25% of all clinical cases were linked according to higher-than-expected ST-matching. Second, 4.1% of clinical cases were linked according to increased ST-matching in cases from incidence peaks. Third, 7.4% of clinical cases were linked according to spatio-temporal clustering within each ST.

The percentages of clinical cases showing evidence of linkage in the present project were intermediate between the value expected according to reported outbreaks in Scotland during 2005 and 2006 (0.4%) (51) and the value (17%) expected according to cases with evidence for temporal linkage at household level in England and Wales (12). The latter value presumably includes unreported cases, and such cases were not analysed in this project.

The results reported here are of particular significance since *Campylobacter* is weakly clonal and presents much higher levels of strain diversity at restricted levels of sampling, *e.g*., within the Montrose outbreak or within isolates occurring in a single Health Board during a single month. This contrasts with previous linkage and cluster-detection studies which have been applied to isolates from meningococcal disease (22), methicillin-resistant *Staphylococcus aureus* (81), *E*. *coli* O157 (93) and *Mycobacterium tuberculosis* (38) which all have strong clonal strain structures.

Attribution of clinical infections to sources

The data collected in this project has enabled for the first time the attribution of clinical cases to host species on a national scale. Previously, the lack of agreement over typing methods has made this impossible to attempt for *Campylobacter*. This scientific indeterminacy has caused great difficulty for policy makers wishing to determine the most appropriate interventions for reducing human infection. The attributions of clinical cases to hosts were in the following ascending order.

Less than 1 in 100 human *Campylobacter* infections were attributed to pigs, and between 1 in 12 and 1 in 20 human infections were attributed to wild birds. These results are consistent with the conclusions of local, small-scale studies using other strain typing methods that *Campylobacter* strains in pigs and wild birds are not major contributors to human disease. These hosts carry distinctive strains that are either rare or do not occur in clinical isolates. Wild birds could nonetheless be short-term vectors transferring strains from farm animals or other sources to humans, either directly or through the broiler chicken food chain.

Approximately 1 in 8 human infections were attributed to cats and dogs. *Campylobacter* prevalence in the presumably healthy cats and dogs that produced the faecal specimens collected from public places in city centres was very low, and most of the isolates typed in this study originated from sick animals. Such animals might represent outcomes of *Campylobacter* transmission, from environmental or foodborne sources, and not reservoir hosts for human infection. Hence, it is expected that the 1 in 8 figure is an overestimate.

Between 1 in 4 and 1 in 3 human infections were attributed to cattle and sheep. These ruminants have approximately equal levels of attribution, and, with the exception of ST273 and ST825-*C. coli*, the clinical STs attributed to sheep were also attributed to cattle. This result is consistent with cattle and sheep being the most similar hosts according to their *Campylobacter* STs in the PubMLST database (77). This attribution to farm ruminants is the most controversial finding of this study, and, as discussed above, is ambiguous.

Approximately 1 in 3 human infections were attributed to retail chicken. The same ST (ST257) was most common in human infections and retail chicken. A single-locus variant of ST257 (ST2030) was 10th most-common in clinical isolates and fourth most-common in retail chicken. Of the 64 most common clinical STs, 17 were either more common in retail chicken than in other hosts or only found in retail chicken. This major attribution to retail chicken is consistent with the findings of most case-control studies that include chicken consumption as a risk factor, and with the reductions in the incidence of human *Campylobacter* infections that follow either natural experiments that temporarily remove chicken from the human food chain

(121) or interventions that reduce contamination levels on chicken carcasses (89, 99,123).

Table 19. Comparison of estimates of prevalence, enumeration and species composition of *Campylobacter* in farm ruminants from CaMPS and 10 published studies.

					Prevalence		Enumeration			
Study	Country	Years	Source	Specimens	N	% (group range)	N	Mean (group range)	Units	% of *C. jejuni*
CaMPS	Scotland	2005-2006	Beef and dairy cattle: farms and pasture	Faeces	1015	36.3	1015	($<10^3$ - $>10^7$)	CFU/g	91.9 [4]
citation			Sheep and lambs on pasture	Faeces	477	28.5	477	($<10^3$ - $>10^7$)	CFU/g	60.3 [4]
115	England	1993-1994	Beef cattle at abattoir	Intestines	360	89.4 (53-100)	1080	$6.1x10^2$	MPN/g	nd
			Dairy cattle on pasture	Faeces	nd	nd	1080	(0-$2.4x10^7$)		
114	England	1993-1994	Lambs at abattoir	Intestines	360	91.7 (57-100)	1080	$1.0x10^4$	MPN/g	88.2 [3]
			Sheep on pasture	Faeces	420	29.3 (21-36)	nd			87.7 [6]
55	Canada	ns	Beef cattle on feedlots	Faeces	299	13.4 (8.2-16.7) [1]	299	($<10^1$ - $>10^6$) [1]	Cells/g	15.6 [2]
8	England	2000	Dairy cattle on pasture	Faeces	1014	32	nd	nd	nd	75.6 [3]
4	USA	2002-2003	Beef cattle and veal calves on feedlots or pasture, and dairy cattle on farms	Faeces	686	38.2	nd	nd	nd	81.5 [4]
6	USA	2000	Beef cattle on a feedlot	Faeces	2000	44.6 (1.6-62.2)	nd	nd	nd	100 [5]
1	Turkey	2004	Sheep at abattoir	Faeces	150	28.0	nd	nd	nd	19.0 [4]
				Intestines	210	49.5				60.2 [4]
57	Norway	1999-2001	Beef and dairy cattle at abattoir	Intestines	804	29	nd	nd	nd	90.4 [4]
23	USA	2002	Dairy cattle on pasture	Faeces	1435	51.2	nd	nd	nd	88.9 [4]
86	All UK	2003	Beef cattle at abattoirs	Intestines	667	54.6	nd	nd	nd	83.9 [6]
			Sheep at abattoirs		713	43.8				65.5 [4]

Notes: N, number of specimens analysed; ns, years not stated; nd, values not determined; 1: values are for *C. jejuni* only; 2-6: the other named Campylobacter species detected were [2] *C. lanienae*, *C. hyointestinalis* and *C. fetus*; [3] *C. coli*, *C. hyointestinalis* and *C. lari*; [4] *C. coli*; [5] none; [6] *C. coli* and *C. hyointestinalis*. (1,4,6,23,55,57,86)

Table 20. Comparison of estimates of prevalence and enumeration of *Campylobacter* in broiler or retail chicken made in CaMPS and in five published studies.

Study	Country	Years	Specimen type	N	Method	Prevalence (%)	Percentages of all specimens in different classes of log(10) CFU/carcass or portion, except where noted						
							<LDL	1-2	2-3	3-4	4-5	5-6	>UDL
CaMPS citation	Scotland	2005-2006	Carcasses and portions (raw: chilled and frozen) on retail sale	208	Carcass or portion rinse in CEB	75.0	72.1 <3.0 log			14.5	6.7	4.8	1.9 >6.0 log
39	Belgium [1]	2007	Portions from carcass processing plants	656	Portion rinse in BB	48.0	59.0 <1.0 log	29.4	11.6 >2.0 log				
59	England	1998-2000	Carcasses (raw: chilled and frozen) on retail sale	241	Carcass rinse in BPW [3]	82.6	60.0 <2.8 log			7.5	13.0	14.0	5.5 >6.0 log
88	USA	2001-2003	Carcasses (raw: chilled) on retail sale	420	Carcass rinse in BPD	79.3	21.0 <0.9 log	16.0	32.0	26.0	5.0		
67	Sweden [2]	2002-2003	Carcasses (post-chill) from slaughterhouses	636	Carcass rinse in BPW	16.4	10.0 <3.0 log			90.0			10.0 >6.0 log
116	Iceland	2003-2004	Carcasses (post-chill) from slaughterhouses	615	Carcass rinse in SDW	74.4	27.7 negative			10.2	17.5	23.8	21.4 >6.0 log

Notes: N, number of specimens analysed; BB, Bolton Broth; BPD, Butterfield's phosphate diluent; BPW, buffered peptone water; CEB, Campylobacter Enrichment Broth; SDW, sterile distilled water; <LDL, less than the lower detection limit; >UDL, greater than the upper detection limit. 1: enumeration values are per g; 2: the percentiles of enumeration values are for the positive samples only; 3: other types of sample were also analysed: this type was the most comparable to the chicken samples analysed in the present project. (39,59,67,88,116)

5.2 Implementation of findings and future research

Research findings and implementation

The implementation of the present study's findings focus mainly on the source attribution of human infection because the infection routes linking sources to humans are the intervention targets. The single largest attributed source of human *Campylobacter* infection in Scotland was retail chicken. Many other scientific studies worldwide have reached the same conclusion, and this is the majority view of *Campylobacter* epidemiology researchers. The infection route is clear: broiler flocks become infected during rearing, contaminated carcasses enter the food chain and people eat contaminated chicken. Interventions consist of preventing or controlling infection in broiler flocks and reducing *Campylobacter* prevalence and concentration in the broiler chicken food chain.

Approximately one-third of *Campylobacter* strains infecting humans were attributed to farm ruminants. Other studies based in Denmark, New Zealand and the UK also reach this conclusion (Table 1), and some *Campylobacter* researchers view farm ruminants as a source of human infection in specific contexts, such as people living in rural locations with high farm ruminant densities in New Zealand. In contrast to the broiler chicken infection route, however, infection routes from farm ruminants to humans are poorly known and likely to be much more diverse.

Some strains infecting humans were found in both ruminants and retail chicken whereas other strains were apparently restricted to farm ruminants. The "ruminant-specific" strains might also infect retail chicken but were undetected in the present project because of some combination of limited sampling, subdivision of retail chicken into batches with different strains and transient occurrence of certain strains in broilers. We speculate that the broiler chicken food chain is the broadest niche for *Campylobacter*, such that virtually all strains infecting humans that occur in farm ruminants also occur to some extent in broilers. Accordingly, human infections attributed to farm ruminants in the present project actually come from the broiler chicken infection route, in which case interventions aimed at the broiler chicken food chain would also be effective for reducing human infection. Alternatively, a

proportion of human infection attributed to farm ruminants could actually come from farm ruminants through a variety of environmental exposure routes, such as recreational or sporting activities in the countryside, in which case interventions in the broiler chicken food chain would have less effect on human infection. Analysis of patient epidemiological data (age class, rural versus urban habitation, recent rural excursions) would provide additional evidence on infection routes for the apparently ruminant-associated strains, especially in cases where the human infections show co-incidence or strain-matching evidence of statistical linkage.

Lastly, the present study showed that *Campylobacter* strains resident in wild birds rarely cause human infection and strains in pigs almost never do so.

Despite being to some extent speculative, the present study's findings on attribution of human infection provide an evidence base for current activities concerning interventions to reduce human *Campylobacter* infection in the UK.

The interventions with the highest consensus as to their effectiveness target the start of the broiler chicken food chain: either to prevent broiler flocks becoming infected or, if that proves impractical, to limit *Campylobacter* growth to low bacterial counts. According to the view of the ACMSF, "robust biosecurity regimes" can theoretically prevent flock infection. This view is supported by national-scale experience of using biosecurity interventions to reduce *Campylobacter* prevalence in broiler flocks in Sweden, where the percentage of infected flocks fell from 20% in 2001 to 13% in 2005 (43), and Norway, where the percentage of infected flocks fell from 6% in 2002 to 3% in 2004 (47). Percentages of infected flocks are higher in other European countries, ranging from 20% in the Netherlands during 1999-2002 (119) to approximately 40% in Germany during 2004-2005 (96) and Northern Ireland during 2001-2002 (78). Baseline levels of *Campylobacter* prevalence in broiler flocks are currently being estimated in surveys in all EC Member States as part of an EC food safety programme (Defra /FSA UK flock survey (OZ0613) will inform the EU baseline survey of *Campylobacter* (2007/516/EC) which is likely to report September 2009).

FSA initiatives are currently in place including: Research on reducing *Campylobacter* on broiler farms (http://www.food.gov.uk/ science/research/ researchinfo/ foodborneillness/ eggsresearch/b15programme/ b15projects/); Cleaner farms better flocks Initiative (http://www.food.gov.uk/ safereating/microbiology/ flocks/). The FSA-

accepted baseline for *Campylobacter* prevalence in UK-produced chicken is 70%, and a recent FSA account of the initial results from the EC baseline survey in the UK suggests that current prevalence is “very high”. The FSA initiatives might therefore prove ineffective at preventing infection, broiler farmers might lack sufficient incentives to implement them, or both. Nonetheless, the attribution results of the present study highlight the importance of the broiler chicken food chain as a source of human *Campylobacter* infection, and these results emphasise the need to overcome any undesirable features of the present biosecurity measures for broiler chicken farms, such as technical deficiencies or implementation obstacles.

These conclusions need to be communicated to UK government policy makers, NHS epidemiologists, food industry representatives and the public. This can be achieved by presentations of this work to stakeholders, peer-reviewed publication in scientific journals and wider dissemination by the internet and media.

Future research

The results and conclusions of this project raise several questions and topics for future research and these are listed below under three headings.

Infection sources and transmission routes

- Comprehensive sampling of *Campylobacter* strain diversity in the broiler chicken food chain is required to resolve the ambiguity in the attribution of human strains to farm ruminants and pets. This could be done in conjunction with the current UK baseline survey of *Campylobacter* prevalence.
- The broiler chicken food chain, encompassing individual flocks, broiler farms, slaughter houses, cutting plants, buyers, distributors and sellers, should be more comprehensively characterised in terms of flock clustering. The codes on retail labels only allow tracing back to the slaughterhouse or cutting plant whereas infection of single flocks occurs further back in broiler houses. This would require the co-operation of supermarkets and other food retailers.

- Further analysis of the broiler chicken food chain might confirm that some human *Campylobacter* strains come from farm ruminants. In that case, it will become necessary to understand how such strains are maintained and transferred, especially among cattle, sheep and the farm environment. This would involve modelling survival trajectories (108) and collecting further datasets in prevalence, bacterial counts and molecular strain types.
- The theoretical effects of implementing improved biosecurity on broiler farms should be modelled using Quantitative Microbiological Risk Assessment, and the actual consequences should be assessed by performing microbiological analyses (to identify conditions yielding high counts) and strain genotyping (to verify that chicken-associated human strains are declining). The present study shows that clinical cases from a single mainland Health Board would provide a representative picture, and the current clinical dataset provides an excellent basis for monitoring changes in source attribution following the introduction of new farm biosecurity and food chain interventions.
- The sampling of foodstuffs for *Campylobacter* by Local Authority environmental health officers and their analysis by Public Health Analysts could be extended and could include more food groups.

Human disease

- Methods for attributing human *Campylobacter* strains to infection sources and inferring outbreaks from spatio-temporal strain clusters should take account of human exposure to sources and other relevant epidemiological data, as has been done with *Salmonella* (41). This will require integration of data from questionnaire surveys to quantify individuals' exposure to infection sources.
- The present project and concurrent FSAS projects are jointly creating a large resource for studies of *Campylobacter* epidemiology, with extensive epidemiological data on patients, spatio-temporal and strain type data on clinical cases, and strain associations with environmental and food origins. These resources should be curated for future studies.

- All comparisons using *Campylobacter* strain types can be carried out over a range of scales of strain definition. These are to some extent hierarchical: clonal complex, sequence type, allele number, concatenated allele sequences, addition of *flaA* and *porA* sequences. Comparison at different scales can yield valuable insights into underlying processes.

- The role of immunity in protecting people from *Campylobacter* infection is unclear. Humoral responses involve different antibody classes: these decay at different rates post-infection (Peter Teunis, pers comm), and cell-mediated immune responses also occur but less is known about them. Whether antigen responses are generic (conferring broad protection) or have species- or strain-specificity (conferring narrower protection) is unknown. Immune responses could be studied using Western blots with antigens from different strains tested against patient sera and probing for each of the different antibody classes. Patients of different ages and from urban or rural residence could be screened for any differences in immune response. Patients could be screened during post-infection against the above 'standard panel' of strains and against their own disease isolate. Most people probably acquire immunity from the time of first infection (58). If future improvements in broiler farm biosecurity cause people to have fewer or later exposures to *Campylobacter*, acquired immunity might be postponed, causing the burden of disease in the population to shift to older people. This trend could be evaluated using HPS data on incidence, and any strain-immune response links identified in the above studies could be used to screen patients or their isolates for chicken-associated immune responses or *Campylobacter* strains.

Epidemiological properties of molecular strains

- The current dataset offers the opportunity to understand the distribution and potentially the transmission and inheritance of antibiotic resistance in *Campylobacter*.

- Genetic comparison of strains with different traits would lead to an understanding of the genetic, heritable bases of these traits; or might indicate that some traits are environmentally driven. Genotype-phenotype links are

essential for understanding how to respond to future threats from emerging *Campylobacter* species, strains and phenotypes. Previous *Campylobacter* 'genome mining' has been carried out in a laboratory environment on laboratory isolates, however the knowledge from this study now allows comparative genetic studies of more biologically appropriate characteristics. Recent advances in (next generation) genome sequencing ('the sub-£500 genome') and in SNP screening open up exciting new avenues of study.

6 Study Outputs

6.1 Scientific Publications

In print:

1. Fraser J Gormley, Marion MacRae, Ken J. Forbes , Iain D. Ogden, John F. Dallas and Norval J.C. Strachan. (2008) “Has Retail Chicken Played a Role in the Decline of Human Campylobacteriosis?” Appl. Environ. Microbiol., 74, 383- 390.

In press:

1. Samuel K Sheppard, John F Dallas, Norval JC Strachan, Marian Macrae, Noel D McCarthy, Daniel Falush, Iain D Ogden, Martin CJ Maiden, Ken J Forbes. “The origin of human campylobacteriosis in Scotland 2005/6.” Clinical Infectious Diseases (in press).
2. Ken J Forbes, Fraser J Gormley, John F Dallas, Olga Labovitiadi, Marion Macrae, Robert J Owen, Judith Richardson, Norval JC Strachan, John M Cowden, Iain D Ogden, Chris C. Mcguigan. “*Campylobacter*: immunity and co-infection following a large outbreak in a farming community.” J. Clin. Microbiol. (in press).
3. Norval JC Strachan, Fraser J Gormley, Ovidiu Rotariu, Iain D Ogden, Gordon Miller, Geoff M. Dunn, Samuel K. Sheppard, John F. Dallas, Thomas M.S. Reid, Helen Howie, Martin CJ Maiden, Ken J Forbes. “Attribution of *Campylobacter* infections in northeast Scotland to specific sources using multi-locus sequence typing (MLST).” J. Infect. Disease (in press).

Submitted:

1. Samuel K Sheppard, John F Dallas, Marian Macrae, Noel D McCarthy, Emma L Sproston, Fraser J Gormley, Norval JC Strachan, Iain D Ogden, Ken J Forbes, Martin CJ Maiden. “*Campylobacter* genotypes from food animals, environmental sources and clinical disease in Scotland 2005/6.” Int. J. Food Microbiol.

In preparation:

1. “Molecular evidence for strain homogeneity and diffuse outbreaks in a national-scale human *Campylobacter* infection study.“
2. “ST257: A genomic perspective on an emerging clonal complex of *Campylobacter jejuni* in human cases of Campylobacteriosis.”
3. “Antimicrobial resistance profiles support chicken as a major source of human campylobacteriosis but are a poor indicator of population structure.”
4. “MLST type elucidates host specificity and reservoirs of *Campylobacter* excreted into the environment by animal sources.”
5. “The prevalence and diversity of *Campylobacter* sequence types in a longitudinal farm study.”
6. “Do flies and slugs act as vectors of *Campylobacter* in the farming environment.”
7. “Spatial and temporal differences in *Campylobacter* strain types in cattle and sheep”
8. “Myocarditis complication following *Campylobacter jejuni* enteritis.”
9. “A transect study of strain diversity and prevalence in adjacent farms.”
10. “Do MLST and Penner Serotyping of *Campylobacter* correlate?”
11. “The molecular epidemiology of clinical *Campylobacter coli* enteritidis.”
12. “The seasonality of clinical and environmental isolates of *Campylobacter*”
13. “Attribution of clinical isolates of *Campylobacter* from urban and rural communities to likely animal reservoirs”

6.2 Presentations at Scientific and Stakeholder Meetings

1. “The Campylobacter MLST Project in Scotland” [Zoonoses Pub. Health, 54, suppl. 1] 14th Into Workshop on CHRO September 2007.
2. “Local outbreaks of campylobacteriosis in Scotland involving more than one strain – a CaMPS study.” [Zoonoses Pub. Health, 54, suppl. 1] 14th Into Workshop on CHRO September 2007.
3. “Prevalence, enumeration and molecular subtyping of *Campylobacter* from environmental and retail food sources in Scotland – a CaMPS study.” [Zoonoses Pub. Health, 54, suppl. 1] 14th Into Workshop on CHRO September 2007.
4. “The decline of human campylobacteriosis and retail poultry in NE Scotland - a CaMPS study.” [Zoonoses Pub. Health, 54, suppl 1] 14th Int. Workshop on CHRO September 2007.
5. “Incidence, diversity and vector transmission of *Campylobacter* sequence types in a longitudinal study of farm ruminants – a CaMPS study.” [Zoonoses Pub. Health, 54, suppl. 1] 14th Int. Workshop on CHRO September 2007.
6. “The importance of environmental sources of *Campylobacter* for human infection – a CaMPS study.” [Zoonoses Pub. Health, 54, suppl. 1] 14th Int. Workshop on CHRO September 2007.
7. “CaMPS: The *Campylobacter* MLST project in Scotland project” FSA-Scotland Microbiological Safety of Food Research Day, Edinburgh, May 2007.
8. “*Campylobacter* MLST project in Scotland; an FSA-funded project”. Quarterly meeting of the Scottish Consultants in Public Health Medicine, Edinburgh, August 2005
9. “The Campylobacter MLST project in Scotland project Emerging *Campylobacter* spp. ” The Food Chain Workshop, Feb 2006.

10. "The *Campylobacter* MLST project in Scotland project" 13th International Workshop on *Campylobacter*, *Helicobacter* and related organisms (CHRO 2005), Queensland, Australia, Sept 2005.
11. One day workshop to recruit the 28 Scottish NHS diagnostic microbiology laboratories and the 14 Scottish Health Board public health departments into "*Campylobacter* MLST project in Scotland project", Perth, June 2005.
12. "A co-ordinated, multidisciplinary research programme to understand Campylobacteriosis in Scotland." 13th Int. Workshop on CHRO, September 2005.
13. "Has retail poultry played a key role in the fall of human campylobacteriosis?" 20th International Food Microbiology Symposium, September 2006.
14. "A multi-strain outbreak of *Campylobacter* following a farmer's dinner dance." 20th International Food Microbiology Symposium, September 2006.
15. "Antimicrobial resistance and MLST of *Campylobacter* isolated from humans and retail chicken in NE Scotland." 21st International Food Microbiology Symposium, September 2008.
16. "*Campylobacter* from retail poultry: MLST analysis and the origin of human infection." 21st International Food Microbiology Symposium, September 2008.
17. "Population genetics, evolution and disease attribution of *Campylobacter* in Scotland 2005 -06." 21st International Food Microbiology Symposium, September 2008.

6.3 Public Awareness

Two newsletters about the project were sent out to all participants submitting specimens to the project (Appendix G).

7 Citations

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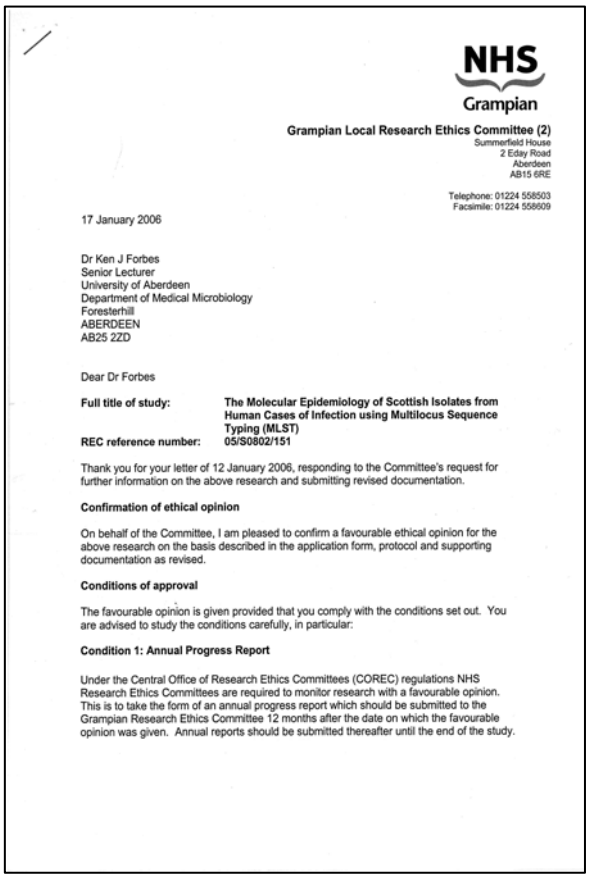
8 Appendices

Appendix A. Ethical Approval Application



NHS
Grampian

Grampian Local Research Ethics Committee (2)
Summerfield House
2 Eday Road
Aberdeen
AB15 6RE

Telephone: 01224 558503
Facsimile: 01224 558609

17 January 2006

Dr Ken J Forbes
Senior Lecturer
University of Aberdeen
Department of Medical Microbiology
Foresterhill
ABERDEEN
AB25 2ZD

Dear Dr Forbes

Full title of study: **The Molecular Epidemiology of Scottish Isolates from Human Cases of Infection using Multilocus Sequence Typing (MLST)**
REC reference number: **05/S0802/151**

Thank you for your letter of 12 January 2006, responding to the Committee's request for further information on the above research and submitting revised documentation.

Confirmation of ethical opinion

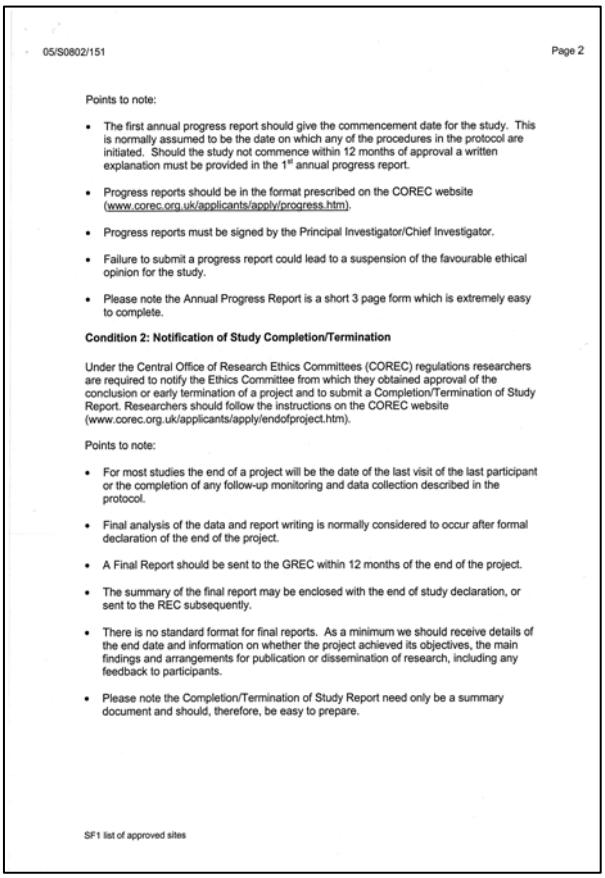
On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out. You are advised to study the conditions carefully, in particular:

Condition 1: Annual Progress Report

Under the Central Office of Research Ethics Committees (COREC) regulations NHS Research Ethics Committees are required to monitor research with a favourable opinion. This is to take the form of an annual progress report which should be submitted to the Grampian Research Ethics Committee 12 months after the date on which the favourable opinion was given. Annual reports should be submitted thereafter until the end of the study.



Points to note:

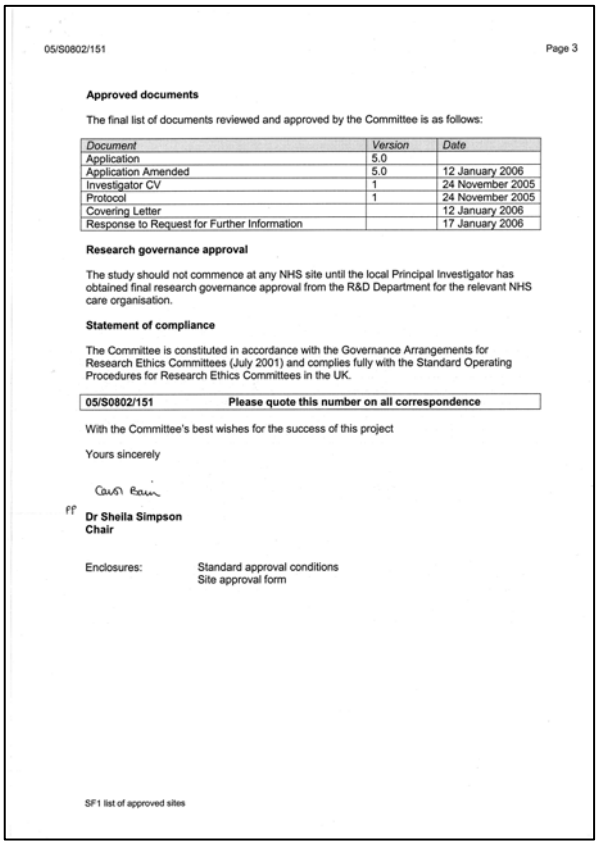
- The first annual progress report should give the commencement date for the study. This is normally assumed to be the date on which any of the procedures in the protocol are initiated. Should the study not commence within 12 months of approval a written explanation must be provided in the 1st annual progress report.
- Progress reports should be in the format prescribed on the COREC website (www.corec.org.uk/applicants/apply/progress.htm).
- Progress reports must be signed by the Principal Investigator/Chief Investigator.
- Failure to submit a progress report could lead to a suspension of the favourable ethical opinion for the study.
- Please note the Annual Progress Report is a short 3 page form which is extremely easy to complete.

Condition 2: Notification of Study Completion/Termination

Under the Central Office of Research Ethics Committees (COREC) regulations researchers are required to notify the Ethics Committee from which they obtained approval of the conclusion or early termination of a project and to submit a Completion/Termination of Study Report. Researchers should follow the instructions on the COREC website (www.corec.org.uk/applicants/apply/endofproject.htm).

Points to note:

- For most studies the end of a project will be the date of the last visit of the last participant or the completion of any follow-up monitoring and data collection described in the protocol.
- Final analysis of the data and report writing is normally considered to occur after formal declaration of the end of the project.
- A Final Report should be sent to the GREC within 12 months of the end of the project.
- The summary of the final report may be enclosed with the end of study declaration, or sent to the REC subsequently.
- There is no standard format for final reports. As a minimum we should receive details of the end date and information on whether the project achieved its objectives, the main findings and arrangements for publication or dissemination of research, including any feedback to participants.
- Please note the Completion/Termination of Study Report need only be a summary document and should, therefore, be easy to prepare.



Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	5.0	
Application Amended	5.0	12 January 2006
Investigator CV	1	24 November 2005
Protocol	1	24 November 2005
Covering Letter		12 January 2006
Response to Request for Further Information		17 January 2006

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/S0802/151 **Please quote this number on all correspondence**

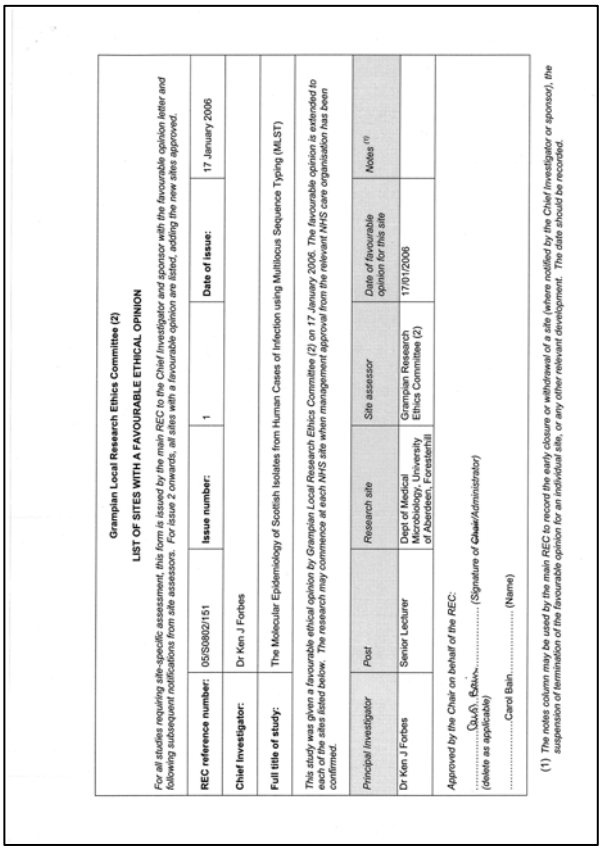
With the Committee's best wishes for the success of this project

Yours sincerely

Carol Bain

pp **Dr Sheila Simpson**
Chair

Enclosures: Standard approval conditions
Site approval form



Grampian Local Research Ethics Committee (2)
LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	05/S0802/151	**Issue number:**	1	**Date of issue:** 17 January 2006
Chief Investigator:	Dr Ken J Forbes			
Full title of study:	The Molecular Epidemiology of Scottish Isolates from Human Cases of Infection using Multilocus Sequence Typing (MLST)			

This study was given a favourable ethical opinion by Grampian Local Research Ethics Committee (2) on 17 January 2006. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

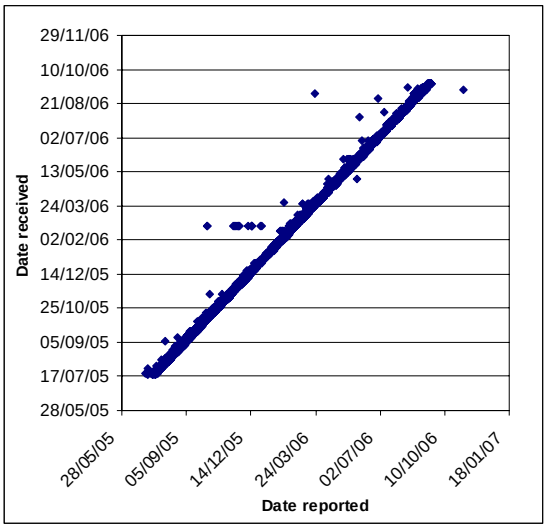
Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes*[1]
Dr Ken J Forbes	Senior Lecturer	Dept of Medical Microbiology, University of Aberdeen, Foresterhill	Grampian Research Ethics Committee (2)	17/01/2006	

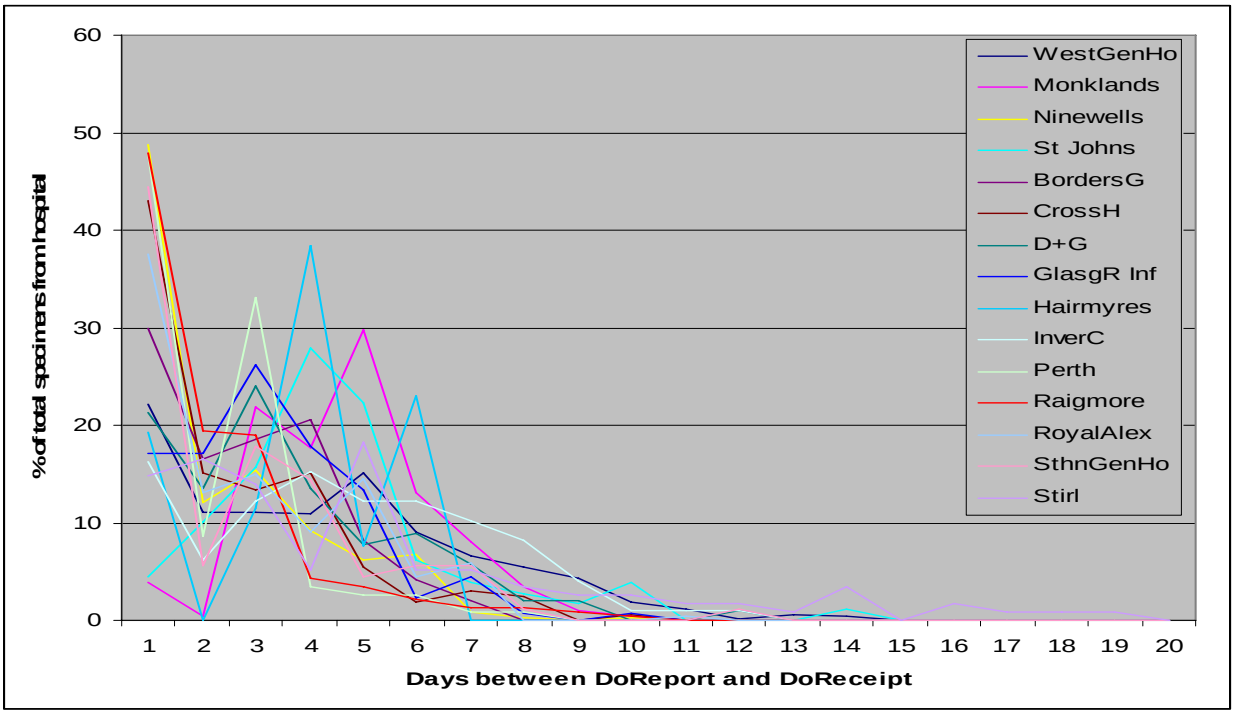
Approved by the Chair on behalf of the REC:
.....Carol Bain.....*(Signature of Chair/Administrator)*
(delete as applicable)
.....Carol Bain.....(Name)

(1) *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

Appendix B. Date of Reporting vs Date of Receipt

Since Date of Infection is unknown, Date of Reporting is the most appropriate date available. 15% of clinical specimens received from the diagnostic laboratories did not record the date of reporting of the case. There was a strong correlation between the Date of Reporting and the Date of Receipt of specimens at UoA by regression analysis (see graph). There was no dramatic difference in the spread in the number of days between Date of Reporting and the Date of Receipt of specimens for different hospitals (see graph). For those specimens without a known Date of Reporting an estimate of this was made by subtracting 4d from the Date of Receipt; this being the mean number of days for all cases where both dates were known.





Appendix C. Environmental Sampling

Table. Numbers of environmental specimens collected in Ayrshire and Arran Health Board by postcode district and year-week, and numbers of sites visited in each postcode district.

Year week	KA 1	KA 2	KA 3	KA 4	KA 5	KA 6	KA 7	KA 9	KA 10	KA 11	KA 13	KA 15	KA 16	KA 17	KA 18	KA 19	KA 22	KA 23	KA 24	KA 25	KA 26	KA 29	KA 30
2005-37																	5	5		9			8 [2]
2005-39			4							4			8	9									
2005-46					4		8	8								9							
2006-02																		[c]1					
2006-03											8	4								4			10
2006-10		4		8					5	4													
2006-16	4					8									8						8		
2006-18												4						8	4				4 [2]
2006-21		4	8	12 [1]																			
2006-27	4						8	4								8							
2006-32																						1 [3]	
2006-37																	1 [3]						
No. of sites visited	2	2	2	3	1	1	2	2	1	2	1	2	1	1	1	2	1	2	1	2	1	0	2

Notes: each value represents a unique farm or other environmental site except in the cases indicated. 1: two farms were included in the same visit; 2: the same farm was included in different visits; 3: specimens from other unique locations in the same postcode district were donated.

Table. Numbers of environmental specimens collected in Grampian Health Board by postcode district and year-week, and numbers of sites visited in each postcode district.

Year week	AB 12	AB 14	AB 15	AB 21	AB 23	AB 30	AB 31	AB 32	AB 33	AB 34	AB 35	AB 36	AB 39	AB 41	AB 42	AB 43	AB 45	AB 51	AB 52	AB 53	AB 54	AB 55	AB 56	IV 32
2005-26							5																	
2005-27							2																	
2005-28		2					1																	
2005-29		2	10	11											6		12							
2005-30							11																	
2005-31					10		1	10								11		14 [2]						
2005-32		12 [1]					2																	
2005-33							1																	
2005-34							7																	
2005-35							35			15 [1]		16 [1]												
2005-36			1 [3]																					
2005-38																				14 [1]		21 [1]	10	8
2005-39							8																	
2005-42	1																							
2005-43			2 [3]																					
2005-44			12	13										6 [1]	9 [1]									
2005-45							10												2					
2005-46																		9						
2005-50	8					12	8				8		7	1 [1]			1 [1]	1						
2005-51																5		5						
2006-03																		1						
2006-05				5																				
2006-08									11 [1]								10		12		10			
2006-11					4			9 [1]						4				10						
2006-15																		5						
2006-17						8	8			8			4											

Table (contd).

Year week	AB 12	AB 14	AB 15	AB 21	AB 23	AB 30	AB 31	AB 32	AB 33	AB 34	AB 35	AB 36	AB 39	AB 41	AB 42	AB 43	AB 45	AB 51	AB 52	AB 53	AB 54	AB 55	AB 56	IV 32
2006-20							3		9	10							11		6		9 [1]			
2006-21							5																	
2006-22	18 [2]						6																	
2006-23							81																	
2006-25							9																	
2006-26			11	1 [3]			4																	
2006-27							5																	
2006-28							23																	
2006-29							49																	
2006-30							42																	
2006-31							48																	
2006-32							52																	
2006-33	16 [2]						45				32 [1]	24 [1]												
2006-34							40																	
2006-35							45																	
2006-36							1																	
2006-37							2																	
2006-38			2 [3]				100																	
2006-39																				21 [1]				
2006-40																		13 [1,2]						
2006-41						2		1			2			23					14					
No. of sites visited	3	4	3	3	2	3	21	4	3	4	4	4	2	4	3	2	3	7	4	4	3	2	1	1

1-3: notes as in Table 16. Most of the large values under AB31 represent visits to 1-4 farms from a core group of six Deeside farms.

Table. Numbers of environmental specimens collected in Dumfries and Galloway Health Board by postcode district and year-week, and numbers of sites visited in each postcode district.

Year week	DG 1	DG 2	DG 3	DG 4	DG 5	DG 6	DG 7	DG 8	DG 9	DG 10	DG 11	DG 12	DG 13	DG 14	DG 16
2005-36		10		10	10	9									
2005-41	13										4	4			
2005-45			10				8	8	8						
2006-04										4			8	8	8
2006-09			8	4	9	8									
2006-12										12 [1]				8	4
2006-17	4														
2006-19		4					4	4	4						
2006-22	4										8	4	9		
2006-28		4		8				7							8
No. of sites visited	3	3	2	3	2	2	2	3	2	3	2	2	2	2	3

1: note as in Table 16.

Figure. Information sheet used to record animal and farm details.

Post code area	**Animal type**	**Collection date**	**Animal numbers in field [1]**	**Housed? [2]**	**Feed [3]**	**Presence of other animals? [4]**	**AU Lab no. [5]**
AB xx	Sheep	6 Mar 06					
DG xx	Dairy / beef						
KA xx	pigeon						

Data in the shaded area was required on each specimen bag. The remainder was provided on a separate printed sheet.

1: number of sheep in flock, cattle in herd; 2: whether the cattle or sheep were housed at time of sampling; 3: grass in summer or turnips/silage in winter (feed may affect *Campylobacter* types and/or numbers); 4: in same field or adjacent field, *e.g.*, goats with sheep; 5: leave blank.

Table. Specimen numbers, *Campylobacter* prevalence and percentage of *C. jejuni* in isolates from reservoir hosts.

	NE Scotland	SW Scotland	Aberdeen	Edinburgh	Glasgow	Other areas [1]	% of *C. jejuni* [2]	Total
Farm species								
Cattle	722 39.6	263 24.7	13 38.5			17 70.6 [4]	91.9	1015 36.3
Sheep	286 29.0	184 25.0				7 100.0	60.3	477 28.5
Pig	101 29.7					1 100.0	8.8	102 30.4
Poultry	54 44.4	2 50.0	1 0.0				66.6	57 43.9
Wild birds								
Feral pigeon	13 15.4	2 0.0	66 25.8	18 27.8	154 31.8		98.6	253 28.9
Gull [3]	2 100.0		89 20.2	72 31.9	51 37.2		91.7	214 29.0
Unidentified	22 40.9	15 20.0	27 18.5	30 16.7	18 22.2		100.0	112 23.2
Goose	14 35.7	1 0.0	2 50.0	27 11.1	22 40.1		94.1	66 27.3
Duck	3 33.3		7 42.9	17 41.2	19 21.1		58.3	46 32.6
Swan			4 25.0	17 23.5	18 0		50.0	39 12.8
Passerine	7 28.6	1 100.0	2 0.0				100.0	10 30.0
Pheasant	7 57.1						100.0	7 57.1
Companion animals								
Cat	8 0.0		38 10.5			15 33.3	100.0	61 14.8
Dog-healthy	10 10.0		14 14.3	21 14.3	31 0.0		100.0	76 7.9
Dog-sick [4]		1 0.0				97 49.5	95.3	98 49.0
Horse	4 0.0	6 16.7	1 0.0	1 0.0			100.0	12 8.3
Miscellaneous sources [5]								
Animal	8 25.0	3 0.0	5 0.0	2 0.0			100.0	19 10.5
Unknown SAC-VET						8 100.0	75.0	8 100.0
Total	**1261 35.8**	**478 24.5**	**271 20.7**	**206 24.3**	**313 27.2**	**157 52.9**	**82.9**	**2686 31.3**

Table entries give the number of specimens collected followed by the percentage of specimens positive for *Campylobacter* (defined as *C. jejuni* or *C. coli)*. 1: areas outside the structured sampling plan; 2: *C. coli* accounted for the rest of the isolates in each case (three other species were also provisionally identified: eight *C. lari* isolates from gulls, eight *C. upsaliensis* isolates from dogs, and two *C. fetus* isolates, one from cattle and one from pigs); 3: mixed gull species; 4: specimens from vets; 5: specimens that were not used in the main analysis.

Appendix D. Laboratory Methods

Campylobacter enrichment broth

Nutrient broth (DM180D, Mast Group Ltd.) made up in distilled water according to the manufacturer's instructions.

5% (v/v) defibrinated horse blood (BHB100, E&O Laboratories Ltd,)

Selectavial growth supplement (SV61, Mast Group Ltd.): one 10 ml vial added to 1.25 l of nutrient broth.

2 µg/ml amphotericin B

15 µg/ml cefoperazone

10 µg/ml trimethoprim

2.5 IU/ml polymixin B

5 µg/ml rifampicin.

DNA extraction - PBS method

Suspend freshly grown bacterial cells in 125 µl phosphate buffered saline (Sigma Aldrich Company Ltd, Dorset, UK).

Vortex briefly and immediately incubate at 100ºC for 10 min.

Centrifuge the tube at 13,000 x *g* for 10 minutes then transfer the supernatant to a new tube and store it at -80ºC.

DNA extraction - CHELEX method:

1. Scrape *Campylobacter* cells (1-3 sq. cm) from a plate using a yellow loop without also lifting pieces of black CCDA agar.

2. Resuspend the cells in 0.5 ml of cold PBS buffer in a 0.6 ml tube by twirling the loop quickly. Close the tube and vortex it at low speed until visible lumps of cells have been resuspended (~10 s). You can vortex a few tubes at a time.

3. Centrifuge the tubes 12 at a time for 1 min at 13,000 g to pellet the cells. Discard the supernatant using a P1000/ blue tip, add 200 µl of just-mixed Chelex 100 suspension (10% wt/vol, see below) using a P200/ yellow tip.

4. Incubate the tube in a dry-block heater or PCR block at 56°C for 15-30 min. Then vortex the tube at high speed for 10 s, and pulse spin.

5. Incubate the tube in a dry-block heater or PCR block at 100°C for 8 min.

6. Vortex the tube at high speed for 10 s then centrifuge it for 3 min at 13K g.

7. Store the tube at -80°C. Use 0.1 x PCR volume of supernatant in PCR, e.g. 2.5 µl in a 25 µl PCR. Repeat step 6 when re-using the DNA prep for PCR.

Notes: The Chelex resin settles rapidly so mix the suspension by inversion after adding every 3rd lot of 200 µl to a series of tubes, and aspirate the 200 µl volume from half-way down the Chelex solution in the Universal, not from the top. Prepare a 10% (w/v) suspension of Chelex 100 (BIO-RAD catalogue no. 142-1253) by adding 2.5 g of Chelex 100 resin using a flamed metal spatula to a 25 ml sterile Universal Container. Add Sigma water by pouring to a total volume of 25 ml (use the mark on the container as a guide) and mix by inversion.

Campylobacter speciation by multiplex PCR

The PCR assay that was reliable for speciation of *C. coli and C. jejuni* is based on detection of the *lpxA* gene (Klena *et al*. 2004). The forward primers were (all primers 5'-to-3'): the published version of lpxA*C. jejuni* (ACAACTTGGTGACGATGTTGTA), and a modified version of lpxA*C. coli* with a higher annealing temperature and one degenerate site (GATAGTAGACAAATAAGAGAGAATMAG). The published reverse primer lpxARKK2m was not used because it is highly (54-fold) degenerate. Instead, one 2-fold degenerate reverse primer, lpxA-R1 (CAATCATGTGCGATATGACAATAYGCCAT) and one non-degenerate reverse primer lpxA-R2 (CAATCATGAGCAATATGACAATAAGCCAT) were designed to anneal to the same site of *lpxA* in *C. coli* and *C. jejuni*, and were used in a 50:50 mixture. The composition of each speciation PCR was: 0.2 mM of each dNTP (BioLine, London, UK), 1x reaction buffer (Qiagen Ltd., Crawley, UK), 2.5 mM $MgCl_2$, 0.025 units/µl of HotStar *Taq* polymerase (Qiagen), 0.25 µM of each primer, 0.4 µl bacterial DNA preparation and molecular biology

grade water (Sigma Aldrich Company Ltd.) to a final volume of 10 µl. The thermal profile was: 95 ºC for 7 min, followed by 35 cycles of 94ºC for 30s, 55ºC for 30s, 72ºC for 1 min, then a final extension of 72ºC for 5 min and a hold at 4ºC. PCR products were subjected to electrophoresis on a 2% agarose gel, and amplicon sizes were estimated by comparison with Hyperladder IV molecular weight marker (Bioline). Amplicon sizes were used for speciation as follows: c390 bp indicated *C. coli* and c330 bp indicated *C. jejuni*.

Campylobacter MLST PCR

The original system for *Campylobacter* MLST, based on 7 housekeeping genes (Dingle et al. 2001; 2005), is now extended to a more universal version (Miller et al. 2005), which was used for this project (Appendix D). Initial results showed that the success rates for typing two of these genes, *tkt* and *uncA*, were lower than for the other five genes. Primers were therefore designed from the flanking sequences of the first 39 (*tkt*) and 20 (*uncA*) different alleles obtained during this project, and the redesigned primers yielded higher success rates. The two extra genes analysed, *flaA* and *porA*, were typed using the same procedures as for the standard 7 MLST genes. The *flaA* reverse primer used initially (FLA625R) was found to yield flanking sequences too short for consistent automatic processing during allele assignment so a new primer yielding 51 bp longer flanking sequences (FLA676R) was designed. The *flaA* forward primer was also redesigned to take account of polymorphisms among *flaA* sequences in GenBank and to include an extra *flaA*-specific, *flaB*-mismatching base at the 3’ end. In all cases, the primers used for gene amplification were also used for DNA sequencing.

Table. MLST primers used in this project

Gene	Sequence 5' - 3'	Primer name
aspA	GAGAGAAAAGCWGAAGAATTTAAAGAT	[a] aspA-F1
	TTTTTTCATTWGCRSTAATACCATC	[a] aspA-R1
glnA	TGATAGGMACTTGGCAYCATATYAC	[a] glnA-F
	ARRCTCATATGMACATGCATACCA	[a] glnA-R
gltA	GARTGGCTTGCKGAAAAYAARCTTT	[a] gltA-F
	TATAAACCCTATGYCCAAAGCCCAT	[a] gltA-R
glyA	ATTCAGGTTCTCAAGCTAATCAAGG	[a] glyA-F
	GCTAAATCYGCATCTTTKCCRCTAAA	[a] glyA-R
pgm	CATTGCGTGTDGTTTTAGATGTVGC	[a] pgm-F1
	AATTTTCHGTBCCAGAATAGCGAAA	[a] pgm-R1
tkt	GCAAAYTCAGGMCAYCCAGGTGC	[a] tkt-F1
	TTTTAATHAVHTCTTCRCCCAAAGGT	[a] tkt-R
	GCWGATATTTTAASKGTTTTAAGTTATC	[b] tkt-JD-F
	TGACTKCCTTCAAGCTCTC	[b] tkt-JD-R
uncA	GWCAAGGDGTTATYTGTATWTATGTTGC	[a] atpA-F
(atpA)	TTTAADAVYTCAACCATTCTTTGTCC	[a] atpA-R
	TGTTGCMATWGGWCAAAAGC	[b] uncA-JD-F
	CTTTGTCCRCGTTCAAGTTG	[b] uncA-JD-R
flaA	GGATTTCGTATTAACACAAATGGTGC	[c] FLA-4F
	CAAGWCCTGTTCCWACTGAAG	[c] FLA-625RU
	CGTATTAACACMAATGKTGCA	[d] FLA10F
	GCKCKAACWCCKGTTTTATC	[d] FLA676R
porA	GATGGTTTAGTWGGMACAGG	[e] momp-3F
	TGAGAAGTTAAGTTTTGGAGAG	[e] momp-2R

A; published primers for *Campylobacter* MLST using 7 standard genes (Miller et al. 2005); B: primers designed during this project from allele-flanking sequences obtained using published *tkt* and *uncA* primers; C: published primers for *flaA* analysis (79); D: primers redesigned for *flaA* analysis during this project; E: unpublished primers for *porA* analysis (Kate Dingle, personal communication).

The composition of each MLST PCR was: 0.2 mM of each dNTP (BioLine, London, UK), 1x reaction buffer (BioLine), 2.5 mM $MgCl_2$, 0.025 units/µl of BioTaq polymerase (BioLine), 0.25 µM of each primer, 1 µl bacterial DNA preparation and molecular biology grade water (Sigma Aldrich Company Ltd.) to a final volume of 12.5 µl. The thermal profile was: 95 ºC for 2 min, followed by 35 cycles of 94ºC for 15s, 55ºC for 30s, 72ºC for 30s, then a final extension of 72ºC for 5 min and a hold at 4ºC. *Campylobacter* DNA extracts were typed in batches of 95 in 96-well microplates, and one row or column of each plate for each locus was used to verify successful PCR amplification. A 1 µl sample of the PCR products was subjected to electrophoresis on a 2% agarose gel, and amplicon sizes were estimated by comparison with Hyperladder IV molecular weight marker (Bioline). The amplicon from each PCR was purified by 20% polyethylene glycol/2.5 M NaCl precipitation and resuspended in 40 µl of molecular biology grade water. All processing was done in 96-well plates.

Amplicons were sequenced on both strands by dideoxy sequencing. The composition of each sequencing reaction was: 20x dilution of BigDye™ ReadyReaction Mix Version 3.1 (Applied Biosystems, Foster City, CA, USA), 1x reaction buffer (Applied Biosystems), 0.25 µM of each primer, .2 µl purified PCR amplicon and molecular biology grade water (Sigma Aldrich Company Ltd.) to a final volume of 10 µl. The thermal profile was: 96 ºC for 2 min, followed by 35 cycles of 96ºC for 10s, 60ºC for 2 min, then a final hold at 4ºC. Sequencing reaction products were purified by ethanol/sodium acetate precipitation in 96-well plates then sent to the DNA sequencing core facility at Oxford University where the DNA sequences were determined using an ABI 3730 automated sequencer (PE Biosystems). Sequence electropherograms were assembled to double-stranded sequence, trimmed to standard allele lengths and assigned allele numbers using STARS software accessed remotely from Oxford University. A new STARS project template was developed for automatic processing of sequence electropherograms generated using (i) the “Miller” primers for the 7 standard MLST loci, (ii) the FLA10-F & FLA-676-R primers for flaA, and (iii) the primers 3F and 2R for porA. The details of this template are not listed here because it is now available to all users of STARS. Novel alleles and sequence types (STs) were submitted to the *Campylobacter* MLST database at http://pubmlst.org/*Campylobacter*/ submission.shtml

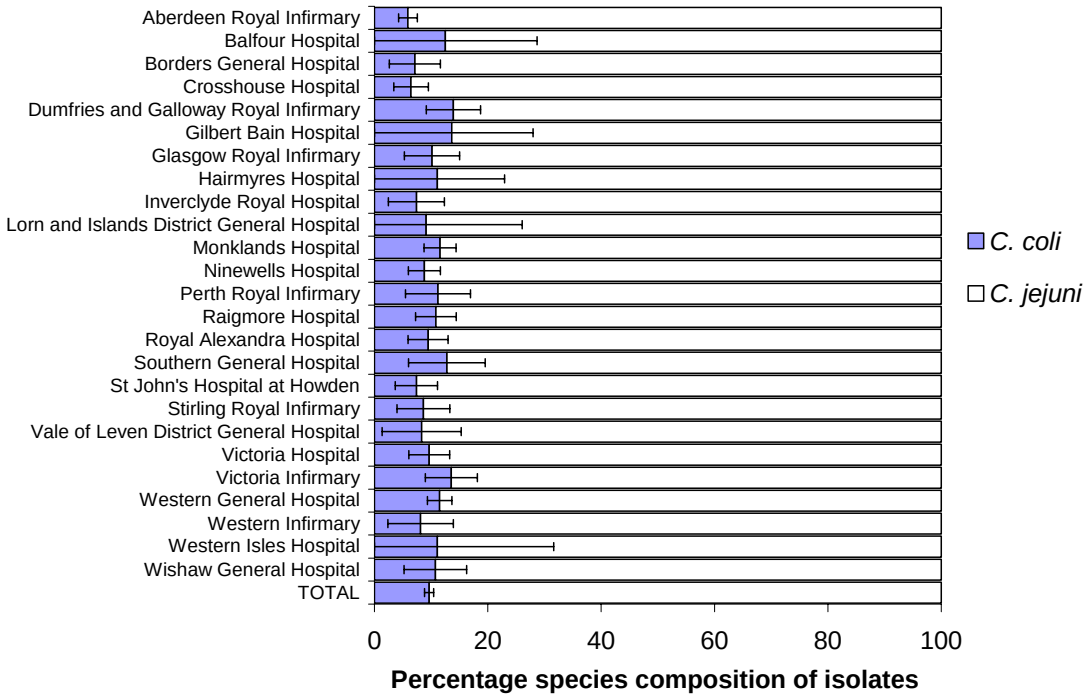
Table. The CaMPS Access database tables and fields.

TABLES									
Isolates: clinical	Isolates: environmental food	Isolates: ST, flaA, porA	Dates	NHS hospitals	Outbreaks	Locations	[4]ST alleles, CC species	[5]FlaA allele: peptide	[6]porA allele: peptide
FIELDS									
Sort no.	Sort no.	Isolate ID	Date	Hospital ID	Epidemiology ID	[3]OS Grid Ref	ST	*flaA* allele	*porA* allele
AU code	AU code	AU code	Week-HPS	Hospital	Epidemiology type	[3]OS easting	*aspA* allele	*flaA* peptide	*porA* peptide
Date received	Date collected	ST	Year-Week	Hospital code	Outbreak location	[3]OS northing	*glnA* allele		
Hospital ID	Collector	flaA allele	Year-Month-Day	Department	Date	Location 1	*gltA* allele		
Patient ID	Submitting lab code	porA allele	Year-Month	Address	Comments	Location 2	*glyA* allele		
[1]Date of birth	Environmental (1) or food (2)			Town		[3]Postcode	*pgm* allele		
Age patient	[3]OS Grid Ref			Postcode		Health Board Area	*tkt* allele		
Specimen ID	Source species			[3]OS easting		HB code	*uncA* allele		
Date reported	Specimen type			[3]OS northing			Clonal complex		
[2]Date reported corrected	No of farm animals present			Health Board Area			Clonal complex number		
Duplicated?	Chicken fresh or frozen			HB code			Species		
Comments: patient	Comments								
Comments: collection	Camps presence								
Comments: microbiology	Counts per g								
Comments: data	Counts per specimen								
Multiple camps	Comments on counts								
Epidemiology ID									

The primary key field in each table is shown in bold. [1]obtained by translation of "patient ID" code, [2]"Date received" minus 4 days was used in cases where the "Date reported" was not provided, [3]obtained from the website Streetmap.co.uk. Molecular data were obtained from: [4]the *C. jejuni*/*C. coli* MLST website (pubmlst.org/perl/mlstdbnet/mlstdbnet.pl?file=pub-cj_isolates.xml), [5]the *Campylobacter flaA* variable region database (hercules.medawar.ox.ac.uk/flaA/), and [6]the *Campylobacter porA* database (hercules.medawar.ox.ac.uk/momp/ - ID & password access).

Appendix E. Speciation of clinical isolates by Hospital

Hospital	Number			%	
	C. coli	C. *jejuni*	Total	*C. coli*	C. *jejuni*
Aberdeen Royal Infirmary	46	734	780	5.9	94.1
Balfour Hospital	2	14	16	12.5	87.5
Borders General Hospital	9	117	126	7.1	92.9
Crosshouse Hospital	16	232	248	6.5	93.5
Dumfries and Galloway Royal Infirmary	28	173	201	13.9	86.1
Gilbert Bain Hospital	3	19	22	13.6	86.4
Glasgow Royal Infirmary	15	133	148	10.1	89.9
Hairmyres Hospital	3	24	27	11.1	88.9
Inverclyde Royal Hospital	8	100	108	7.4	92.6
Lorn and Islands District General Hospital	1	10	11	9.1	90.9
Monklands Hospital	57	436	493	11.6	88.4
Ninewells Hospital	34	352	386	8.8	91.2
Perth Royal Infirmary	13	103	116	11.2	88.8
Raigmore Hospital	31	255	286	10.8	89.2
Royal Alexandra Hospital	25	239	264	9.5	90.5
Southern General Hospital	12	82	94	12.8	87.2
St John's Hospital at Howden	14	175	189	7.4	92.6
Stirling Royal Infirmary	12	127	139	8.6	91.4
Vale of Leven District General Hospital	5	55	60	8.3	91.7
Victoria Hospital	25	234	259	9.7	90.3
Victoria Infirmary	29	185	214	13.6	86.4
Western General Hospital	97	747	844	11.5	88.5
Western Infirmary	7	79	86	8.1	91.9
Western Isles Hospital	1	8	9	11.1	88.9
Wishaw General Hospital	13	108	121	10.7	89.3
TOTAL	506	4741	5247	9.6	90.4



Horizontal bars indicate 95% confidence intervals.

Appendix F. Newsletters

Campylobacter MLST project in Scotland (CaMPS)

Newsletter – March 2006

Tracing clinical outbreaks and environmental sources of *Campylobacter*

The Food Standards Agency-Scotland (FSAS) project to identify linked clinical cases of *Campylobacter* infection and its environmental sources in Scotland is now well underway.

This 2.5-year project (July 2005 – Dec. 2007) is based in the Department of Medical Microbiology at the University of Aberdeen, with collaborators at the University of Oxford, and is led by Dr Ken Forbes.

Malcolm Leitch and Marion MacRae in the *Campylobacter* microbiology laboratory

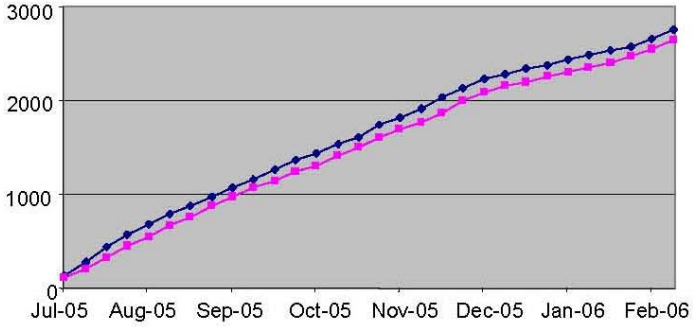
We are using multilocus sequence typing (MLST) to characterise clinical isolates from NHS diagnostic laboratories, retail food isolates from Public Health laboratories and environmental isolates from our own collections and those received from Vet Schools and Scottish Agricultural College stations throughout Scotland.

MLST can be used to classify *Campylobacter* isolates according to sequence type (ST), and similar STs can be clustered into closely related groups called clonal complexes. Isolates that belong to the same ST are also likely to share the same epidemiological history, so MLST generates information useful for epidemiological analysis.

Campylobacter samples

Isolates from clinical cases of *Campylobacter* occurring during the period 15th July 2005 – 15th October 2006 are being submitted from all the relevant NHS laboratories in Scotland. The nearly 3000 isolates received so far represent most of the *Campylobacter* cases reported to HPS, and almost all have yielded *Campylobacter* growth on selective plates. Most of the clinical isolates (93%) were *C. jejuni* and a minority (7%) were *C. coli* according to species-diagnostic PCR.

During the same period, over 1250 environmental isolates were collected or received. Approximately 34% of these yielded *Campylobacter*, of which 73% were *C. jejuni*, 9% were *C. coli* and 18% were either other *Campylobacter* species or other genera.



Cumulative *Campylobacter* cases reported to HPS (blue) and clinical isolates submitted to CaMPS (pink).

Ethical issues

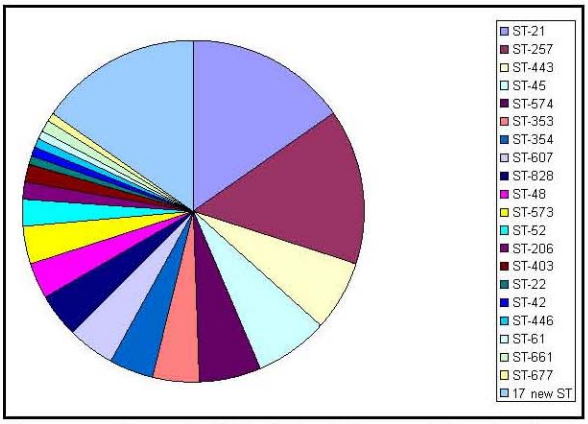
To minimise the information from laboratories, and to ensure patient confidentiality, we are now requesting only patient date of birth (rather than CHI number). This is sufficient to link isolates with background information on risk factors. The project was awarded full ethical approval at the end of 2005, and a copy is available on request.

CaMPS Newsletter – March 2006

Fraser Gormley setting up MLST-PCR on the robot in the *Campylobacter* genetics laboratory

Sequence typing

All clinical isolates currently being submitted to the project are being typed by MLST using high-throughput robotic PCR and DNA sequencing, and online sequence analysis for automated identification of MLST alleles.



Sequence type in 120 clinical *Campylobacter* from 17 NHS laboratories (clockwise from top)

The sequence types of the first batch of 120 clinical isolates were highly diverse, representing 41 known STs belonging to 20 clonal complexes and 17 new STs. We therefore expect MLST to be highly informative for discriminating among *Campylobacter* strains from different epidemiological events.
Typing results for specific clinical isolates will be made available to project participants in the near future.

Outbreaks

We are particularly keen to analyse any *Campylobacter* infections that are known or suspected to be linked. We have already analysed some of these: one involved an outbreak of over thirty clinical cases and another involved a clinical-companion animal linkage. We are also analysing such isolates using more traditional typing methods (Penner HS serotyping, phage typing, resistotyping and PFGE).
If you suspect that particular cases of *Campylobacter* infection are linked or if you would like to discuss any issues relating to such cases then please contact us, as below.

Modelling *Campylobacter* epidemiology

An FSAS project "Factors associated with geographic and temporal variation in campylobacteriosis in humans" will be starting shortly. The project aims to elucidate the puzzling epidemiology of *Campylobacter* infections using a combined analysis of MLST data from the above project and new epidemiological data. Issues to be studied include spatial and temporal trends of infection, urban versus rural infection rates, cases associated with travel, outbreaks and surges of infection, and linkage with infection reservoirs. This project is led by the University of Glasgow in collaboration with Health Protection Scotland and the University of Aberdeen.

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Iain Ogden (food and environmental)
01224 551132
Ken Forbes (clinical)
01224 554953

Many thanks to everyone who has sent us isolates.
This project would not be possible without your help.

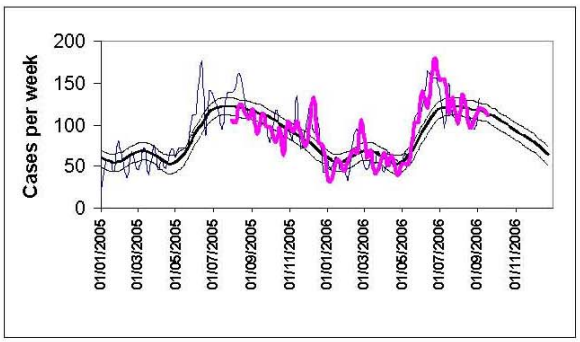
Campylobacter MLST project in Scotland (CaMPS)

Newsletter – Nov 2006

Tracing clinical outbreaks and environmental sources of *Campylobacter*

The Food Standards Agency-Scotland project to identify linked clinical infections of *Campylobacter* and environmental sources of infection has now reached its target for clinical isolates.

Clinical sampling was originally due to last 15 months (15th July 2005 – 15th Oct 2006) with a target of 5500 isolates. In fact, clinical submissions to the end of Sept 2006 totalled nearly 5700 isolates. The excess occurred mainly during June-July 2006 (see below), and was evident across several laboratories according to comments received. **All labs - please stop submitting to the project.**



Trends in *Campylobacter* clinical cases during the CaMPS project.
Pink: isolates submitted to CaMPS.
Blue: cases reported to HPS.
Black: annual mean with 95% confidence limits modelled on cases reported to HPS Jan. 2003 - July 2005.

In parallel, collection of almost 2700 environmental samples in most of the 15 NHS-Scotland Health Board regions has yielded nearly 1200 *Campylobacter* isolates from a wide variety of animals and birds, both domesticated and wild. Microbiological growth and storage of all isolates is being done at the University of Aberdeen.

Iain Ogden collecting pig faeces for environmental isolates

Sequence typing

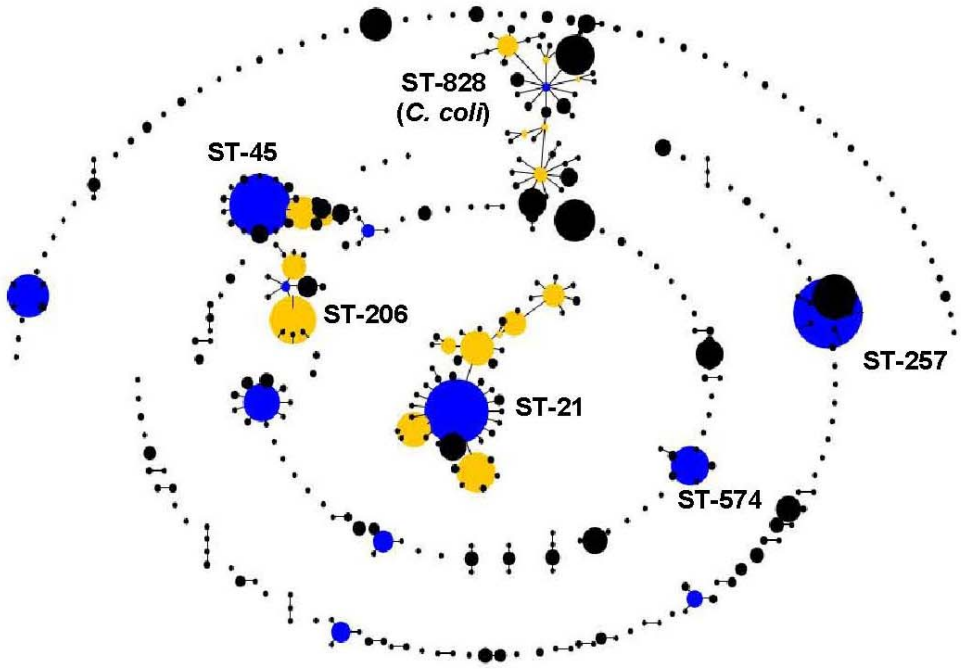
Campylobacter isolates are being characterised by multilocus sequence typing (MLST) in the PCR laboratories at the Universities of Aberdeen and Oxford. These facilities provide high-throughput robotic PCR and DNA sequencing, and online sequence analysis for automated identification of MLST profiles at 7 genes. The main clinically significant species were *C. jejuni* (90%) and *C. coli* (9%), and the rest (1%) were other species found mainly in environmental samples. Over 2500 clinical and 300 environmental isolates have been typed so far, and these have turned out to be highly diverse. Almost 550 different sequence types (STs) have been found, comprising both known, common STs (N=208) and new, rare ones (N=340).

Clonal complexes

Relationships among the STs found in the CaMPS project are being studied by clonal complex analysis, which draws relationships among identical and highly similar STs.

Target reached – Many thanks to all participants

CaMPS Newsletter – Nov 2006



Clonal complexes in 2841 clinical and environmental *Campylobacter*.

Most of the CaMPS isolates belonged to existing clonal complexes in the public database, with almost two-thirds falling into six common complexes (labelled above). Most of the *C. coli* isolates clustered as a single, complex (ST-828 above) distinct from the other *C. jejuni* isolates, so MLST data can separate these clinically relevant species. Most of the common clonal complexes in the CaMPS dataset were also common in the public database of UK isolates. ST-257 clonal complex, by contrast, was three times commoner in the CaMPS dataset although it was found across Scotland.

Modelling *Campylobacter* epidemiology

The University of Glasgow, Health Protection Scotland and the University of Aberdeen have joined forces in a new FSAS-funded project to study environmental and behavioural associations of *Campylobacter* transmission in Scotland. Analyses of MLST and epidemiological data will be used to study associations with geographic location, season, urban and rural residence, travel overseas and contact with reservoir hosts. Public health departments and diagnostic laboratories will also be contacted later this year about this new study and with preliminary analyses from the CaMPS study. We also hope to organize a workshop on *Campylobacter* epidemiology next year.

New staff

Dr Sam Sheppard has joined the CaMPS project; he will be working at the University of Oxford.

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Appendix I. Acknowledgements

We gratefully acknowledge the help of the following.

For provision of isolates, specimens and information:

NHS diagnostic laboratories

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Veterinary laboratories (Scottish Agricultural College, University of Glasgow)

Staff of University of Aberdeen and NHS Grampian for collecting *ad hoc* faecal specimens.

Animal Rescue Centres and Wildlife Reserves

Postgraduate Research students

Visiting Research students

CPHMs

To farmers, particularly in Grampian and in SW Scotland for access to private land and providing information on animal stocking

The CaMPS team