Epidemiological study of *Campylobacter spp.* colonisation of wild game pheasants (*Phasianus colchicus*) processed in Approved Game Handling Establishments in Scotland and its relevance to public health

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Declaration

I declare that the work presented in this thesis is my own original work, except where specified, and it does not include work forming part of a thesis presented successfully for a degree in this or another university.

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Abstract

Campylobacteriosis in humans due to *Campylobacter jejuni* and *C. coli* is the most common bacterial diarrhoeal disease worldwide. Control measures currently focus on the reduction of *Campylobacter* in chickens, as 60-80% of human cases can be attributed to the poultry reservoir as a whole. However, *C. jejuni* and *C. coli* have also been reported in a range of livestock and wildlife species, including live pheasants. Pheasants reach the consumer's table as a by-product of the shooting industry. Approximately 3.5 million game birds are shot in Scotland every year; however, only 700,000 (20%) are received at Scottish Approved Game Handling Establishments (AGHEs) for veterinary inspection. Despite this volume of wild game entering the food chain, there is a lack of information concerning the risk of campylobacteriosis in humans arising from consumption of wild game meat and the role wild game birds may have as a reservoir of infection.

This study's aims were to determine the prevalence of *Campylobacter* in wild game pheasants processed in AGHEs in Scotland, to identify the main sequence types (STs) present and to evaluate their impact on public health.

Scotland was divided into five geographical regions. Five sampling sites and 13 estates were selected to collect a total of 287 caecal and 59 skin samples from pheasant carcases during the hunting season 2013/2014. Laboratory isolation of *Campylobacter* was performed using standard culture methods and positive caecal samples were subjected to PCR and High Throughput Multi Locus Sequence Typing (HiMLST).

36.5% of 287 caecal samples (CI 13.9% - 61.2%) were *Campylobacter* positive while all 59 skin samples were negative. Using PCR, *C. coli* and *C. jejuni* accounted for 62.7% and 37.3% of positive samples tested (n=99), respectively. Nineteen STs of *Campylobacter* were recovered from MLST (n=80). Sequence type 828 (n=19) was the most common, followed by ST827 (n=12) and ST19 (n=7).

Overall, the STs found in pheasants are more common in livestock than chickens, raising the possibility of cross-infection between pheasants, cattle and sheep in the field. STs 827 and 19 are common in humans and primarily associated with livestock, however, ST828 is primarily chicken-associated so this also implies direct involvement of poultry in the transmission of infection to pheasants.

This study suggests that wild game birds are a possible source of *Campylobacter* infection in humans and helps in the understanding of risk to humans of pheasant meat consumption.

Lay Summary

Campylobacteriosis in humans due to *C. jejuni* and *C. coli* is the most common bacterial diarrheal disease worldwide. Control measures currently focus on the reduction of *Campylobacter* in chickens, as 60-80% of human cases can be attributed to the poultry reservoir as a whole. However, *C. jejuni* and *C. coli* have also been reported in a range of livestock and wildlife species, including live pheasants. Pheasants reach the consumer's table as a by-product of the shooting industry. Approximately 3.5 million game birds are shot in Scotland every year; however only 700,000 are received at Scottish Approved Game Handling Establishments (AGHEs) for veterinary inspection. Despite this volume of wild game entering the food chain, there is a lack of information concerning the risk of campylobacteriosis in humans arising from consumption of wild game meat and the role wild game birds may have as a reservoir of infection.

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This study suggests that wild game birds are a host of *Campylobacter* and a potential risk to humans through consumption of pheasant meat.

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Abbreviations

AGHE	Approved Game Handling Establishment
BASC	British Association for Shooting and Conservation
CC	Clonal Complex
CDT	Cytolethal Distending Toxin
CFA	Campy Food Agar
CFU	Colony Forming Unit
CI	Confidence Interval
EU	European Union
FSA	Food Standards Agency
FSS	Food Standards Scotland
GFA	Game Farmers Association
HACCP	Hazard Analysis Critical Control Points
HiMLST	High Throughput Multi Locus Sequence Typing
HPS	Health Protection Scotland
mCCDA	Modified Cefperazone Charcoal Deoxycholate Agar
MHS	Meat Hygiene Service
MLST	Multi Locus Sequence Typing
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
R(D)SVS	Royal (Dick) School of Veterinary Studies
ROI	Reactive Oxygen Intermediates
SOD	Superoxide dismutase
ST	Sequence Type

Chapter 1 Introduction

1.1 Campylobacter

Campylobacter is a genus of Gram-negative spirally curved rods that comprises 15 species, 12 of which are associated with human disease (Lastovica and Skirrow, 2000). They have a single polar flagellum at one or both ends of the cell and are strictly microaerophilic, requiring between 3 and 6% of oxygen and 10% of carbon dioxide to grow. They are catalase and oxidase positive and will not grow at temperatures below 30°C, or in the presence of 3.5% of NaCl, or at a pH below 4.9 (Park, 2002). The optimum temperature for growth is 42°C at a pH range of 5.5 to 8.0 (Park, 2002).

Campylobacter species are commonly found in nature and can contaminate drinking water but are more often associated with warm-blooded animals as commensal gastrointestinal organisms in livestock, domestic and wild animals or as pathogens in humans (EFSA, 2012). They do not generally cause disease in animals, but *C. fetus fetus* can be an abortifacient agent in cattle and sheep and may occasionally cause serious systemic disease in humans.

The two species of primary importance to public health are *C. jejuni* and *C. coli*, responsible for over 95% of *Campylobacter* infections in humans (Park, 2002). *Campylobacter jejuni* and *C. coli* can readily contaminate various foodstuffs, including meat, raw milk and dairy products, and, less frequently, fish and fishery products, mussels and fresh vegetables. Among sporadic human cases, contact with live poultry, consumption of poultry meat, drinking water from untreated water sources, and contact with pets and other animals have been identified as the major sources of infections (EFSA, 2013). Cross-contamination during food preparation has also been described as an important transmission route. Raw milk and contaminated drinking water have been implicated in both small and large outbreaks (EFSA, 2013). Other *Campylobacter* species, such as *C. upsaliensis*, *C. sputorum*, *C. hyointestinalis* and *C. lari* are present in mammals and birds in the UK, but are not generally considered of public health importance (DEFRA, 2013).

1.1.1 Poultry as reservoir of human campylobacteriosis

Poultry are recognised as the most important reservoir of *Campylobacter* infection for humans (Park, 2002). They can contract infection from their environment, via contaminated water or following breaches of biosecurity (e.g. poor cleaning and disinfection of poultry houses). Infection in poultry is mainly through the oral-faecal route or via vertical transmission from

parent flocks (Humphrey *et al.*, 2007). *Campylobacter* spp. mainly colonise the caeca, large intestine and cloaca of poultry; in particular, they target the intestinal mucous layer coating the crypts in these locations (Beery *et al.*, 1988). This is a highly specialised environment but *C. jejuni* has evolved features that enhance bacterial colonisation of this restricted ecological niche, in particular:

- Bacterial motility: This is necessary for the intestinal colonisation of animals by *Campylobacter* spp. since non-motile mutants are incapable of establishing infection (Morooka *et al.*, 1985). The polar flagellum and the spiral shape of *Campylobacter* spp. give the cells a typical rapid, darting, corkscrew-like pattern of motility that enables them to colonise competitively the mucous layer of the gastrointestinal tract of animals. This environment will otherwise rapidly paralyse other motile rod-shaped bacteria (Wassenaar and Blaser, 1999).
- Chemotactic mechanisms: These allow *Campylobacter* spp. to be attracted to mucin and, more specifically, fucose, a constituent of mucin, and consequently colonise the mucous layer of the gastrointestinal tract of animals (Hugdahl *et al.*, 1988).

The main factors that enhance the role of poultry as the main natural host for *Campylobacter* are:

- The body temperature of poultry, around 41°C, is very close to the optimal growth temperature of the organism (42°C) and differs considerably from that encountered in the mammalian intestinal tract (37 to 39°C) (Park, 2002).
- The ability of *C. jejuni* to produce the outer membrane protein CadF that allows the binding of the bacterium to fibronectin that is present on the gastrointestinal tract of chickens (Ziprin *et al.*, 1999).

1.1.2 Pathogenesis of human disease

Much of the world's poultry meat production is contaminated with *Campylobacter* spp. and this is reflected in the reportedly high isolation rate for these pathogens in poultry products sold in major supermarket outlets (Park, 2002; EFSA, 2013). In the USA, 69% of chickens bought from a local supermarket were found to be contaminated with *C. jejuni* (Willis and Murray, 1997). In the UK, 70% of chickens tested positive for the presence of *Campylobacter* in a year-long survey published in November 2014, with 18% of them harbouring loads greater than 10^4 colony forming units per gram (CFU/g) (FSA, 2014). *Campylobacter jejuni* infection in humans has been associated with handling and eating of raw or undercooked poultry, a risk

that is enhanced by the widespread distribution of infection in the poultry meat industry and the high levels of poultry consumption meat per-capita (EFSA, 2010).

Transmission of *Campylobacter* to humans is usually by the consumption of contaminated food or water. In particular, a scientific opinion published by EFSA in 2010 indicated that "Handling, preparation and consumption of broiler meat may account for 20% to 30% of human cases of campylobacteriosis, while 50% to 80% may be attributed to the poultry reservoir as a whole" (EFSA, 2010).

On the basis of experimental evidence, at least two mechanisms have been identified by which *Campylobacter* Spp. induce gastrointestinal disease in humans. These involve intestinal adherence and toxin production, and bacterial invasion and proliferation within the intestinal mucosa (Park, 2002).

Campylobacter jejuni strains produce at least one cytotoxin, the cytolethal distending toxin (CDT) (Pickett, 2000). From *in-vitro* experiments it has been hypothesised that if the CDT toxin is produced in the presence of rapidly dividing and differentiating intestinal crypt cells, it could lead to loss of function or erosion of the epithelial layer, ultimately leading to diarrhoea (Pickett *et al.*, 1996; Purdy *et al.*, 2000). This is not the only way *Campylobacter* spp. express their pathogenic effect in humans and it is possible that additional toxigenic activities are present in some strains of *C. jejuni*. This may explain why disease manifestation can differ with strain (Park, 2002). Equally, it is also possible that certain strains of *C. jejuni* or *C. coli* may not be capable of causing disease in humans since the relative distributions of genotypes from poultry and humans are not necessarily the same (Korolik *et al.*, 1995; Clow *et al.*, 1998). In this context, the determination of the sequence type of *C. jejuni* and *C. coli* from different animal sources can give an indication of their potential to cause disease in humans.

The infective dose of these bacteria is generally low (500 to 800 CFU) (Janssen *et al.*, 2008) and the average incubation period in humans ranges from one to seven days (Blaser *et al.*, 1987: Wood *et al.*, 1992). Patients may experience mild to severe symptoms, most common ones including watery (sometimes haemorrhagic) diarrhoea, abdominal pain, fever, headache and nausea (Humphrey *et al.*, 2007). Usually infections are self-limiting and last only between 5 and 7 days. Extra-intestinal infections or post-infection complications, such as reactive arthritis and neurological disorders, can also occur. *Campylobacter jejuni* has become the most commonly recognised antecedent cause of Guillain–Barré syndrome, a polio-like form of paralysis that can result in respiratory failure, severe neurological dysfunction and even death (Humphrey *et al.*, 2007). Not all strains of *C. jejuni* seem capable of causing these sequelae

and there are even differences between those strains associated with the two syndromes of respiratory failure and neurological dysfunction (Takahashi *et al.*, 2005).

1.1.3 *Campylobacter* as a foodborne pathogen

As described in section 1.1 *Campylobacter* spp. possess strict growth requirements and are sensitive to environmental stress, making them appear to be unlikely foodborne pathogens compared to salmonellae and *Escherichia coli*, organisms that are considered to be relatively robust and capable of surviving common food processing practices such as refrigeration and cooking (Humphrey *et al.*, 2007). Factors influencing *Campylobacter* survival in food, and therefore its pathogenicity to humans, are briefly explained below:

Resistance to low temperature: The factor that limit the growth of *Campylobacter* spp. at low temperatures is currently unknown. Many bacteria produce characteristic cold shock proteins that allow them to replicate at temperatures below their optimum growth temperature (Phadtare *et al.*, 1999). An analysis of the *C. jejuni* genome sequence suggests that *Campylobacter* spp. do not produce this type of cold shock protein (Parkhill *et al.*, 2000). This may contribute to explain why these pathogens fail to grow at temperatures below 30°C. Although at this temperature replication is not possible, the organism is still fully motile and retains its aptitude to move towards more favourable environments (Hazeleger *et al.*, 1998). At lower temperatures however, viability is rapidly lost and, although *Campylobacter* spp. can still be recovered from frozen meats and poultry products (Fernandez and Pison, 1996), freezing significantly reduces its survival (Humphrey and Cruickshank, 1985; Harrison *et al.*, 2013). Several factors, like ice nucleation and dehydration, have been associated with the freeze-thaw induced injury of bacterial cells, and oxidative stress has also been shown to be detrimental to *Campylobacter* survival (Stead and Park, 2000).

Resistance to oxidative stress: Exposure to oxygen is inevitable for bacterial pathogens but it leads to the formation of reactive oxygen intermediates (ROIs), such as superoxide radicals. If these highly reactive agents are not deactivated, they can induce lethal damage to nucleic acids, proteins and cell membranes. As a response against ROIs excesses, many bacterial cells are able to induce the synthesis of anti-oxidant enzymes. Although it is possible to grow *Campylobacter* spp. in the presence of air under certain conditions (Jones *et al.*, 1993), these organisms are generally considered to be microaerophilic, indicating an intrinsic sensitivity towards oxygen and its reduction products. Thus, cellular defences against the damaging effects of oxidative stress play an important role in the survival of these bacteria during exposure to air. Superoxide dismutase (SOD) is an enzyme that, amongst others, plays a key

role in the oxidative defence system of *Campylobacter* (Purdy and Park, 1994; Pesci *et al.*, 1994; Purdy *et al.*, 1999). The deleterious effects of exposure to superoxide radicals are counteracted by the SOD that both *C. jejuni* and *C. coli* possess (Purdy and Park, 1994; Pesci *et al.*, 1994). SOD-deficient *Campylobacter* mutants are less able to survive on poultry meat or freezing, suggesting that SOD is an important factor of *Campylobacter* survival in food (Purdy *et al.*, 1999; Stead and Park, 2000).

Resistance to heat treatment: Although thermophilic in growth requirement, *Campylobacter* spp. are sensitive to heat and readily inactivated by pasteurization and domestic cooking processes (Humphrey *et al*., 2007). Bacterial cells exposed to temperatures above that which is optimal for growth generally respond by producing a heat shock response involving the synthesis of proteins able to act as ATP-dependent proteases (Park, 2002). These contribute to the degradation or stabilization of abnormal proteins and this is considered to be an important homeostatic mechanism that enables bacterial cells to survive heating and a variety of environmental stresses (Arsene *et al.*, 2000). *Campylobacter* spp. are able to produce a heat shock response similar to that observed in other bacteria, however, specific heat shock regulatory genes that are present in other bacteria are absent from *C. jejuni* (Parkhill *et al.*, 2000).

Campylobacter in foodstuff of animal origin

Campylobacter spp. are commonly found on red meat and dairy products as well as on poultry meat as shown in Table 1.1.

Food or Animal Tested	Mean % positive samples	% Range
Chicken flocks	58.7	2.9-100
Chicken at retail	57.4	23-100
Turkey flocks	78.0	20-100
Turkey at retail	47.8	14-94
Dairy cows	30.0	6-64
Raw milk	3.2	0-9.2
Beef cattle	62.1	42-83
Beef at retail	2.7	0-9.8
Sheep	31.1	18-44
Lamb at retail	6.0	0-12.2
Pigs	61.0	50-69
Pork at retail	2.0	0-5.1

Table 1.1: (adapted from Humphrey *et al.*, 2007)**:** Isolation of *Campylobacter* spp. from raw foods and food producing animals.

Although red meat animals can be heavily contaminated with *Campylobacter* spp. on arrival at the abattoir, due to increased excretion of bacteria subsequent to the transport stress (Beach *et al.*, 2002), the level of contamination at retail is substantially lower than poultry meat possibly due to the longer time red meat spends in chill before entering the food chain and possibly related to the deleterious effect of dehydration on *Campylobacter* (Humphrey *et al.*, 1995). A dry environment also appears to be the reason why *Campylobacter* spp. doesn't survive for long on egg shells (Humphrey *et al.*, 2007). Milk pasteurisation can easily destroy *Campylobacter* contamination (Humphrey *et al.*, 2007).

1.2 Epidemiology of campylobacteriosis in humans

Campylobacter was first confirmed as a cause of human illness in 1972 (Dekeyser *et al.*, 1972) and by 1986 it was recognised as the most commonly reported gastrointestinal pathogen in the UK, ahead of *Salmonella* spp. (Skirrow, 1987). The species of greatest public health importance are *C. jejuni* and *C. coli* (thermophilic *Campylobacter* spp.) that account for over 95% of *Campylobacter* infections in humans (Park, 2002). While in livestock and wild animals *Campylobacter* spp. rarely cause disease, in humans they are the most common cause of bacterial gastrointestinal infection worldwide (Humphrey *et al.*, 2007). According to WHO estimates, *Campylobacter*-related illness affects around 1% of populations in developed countries every year (WHO, 2013).

1.2.1 Campylobacteriosis in humans in the EU

In 2013, *Campylobacter* was the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union (EU) and has been so since 2005 (EFSA and ECDC, 2013). The number of confirmed cases of human campylobacteriosis was 214,779 with an EU notification rate of 64.8 per 100,000 population, which was at the same level as in 2012 (Figure 1.1).

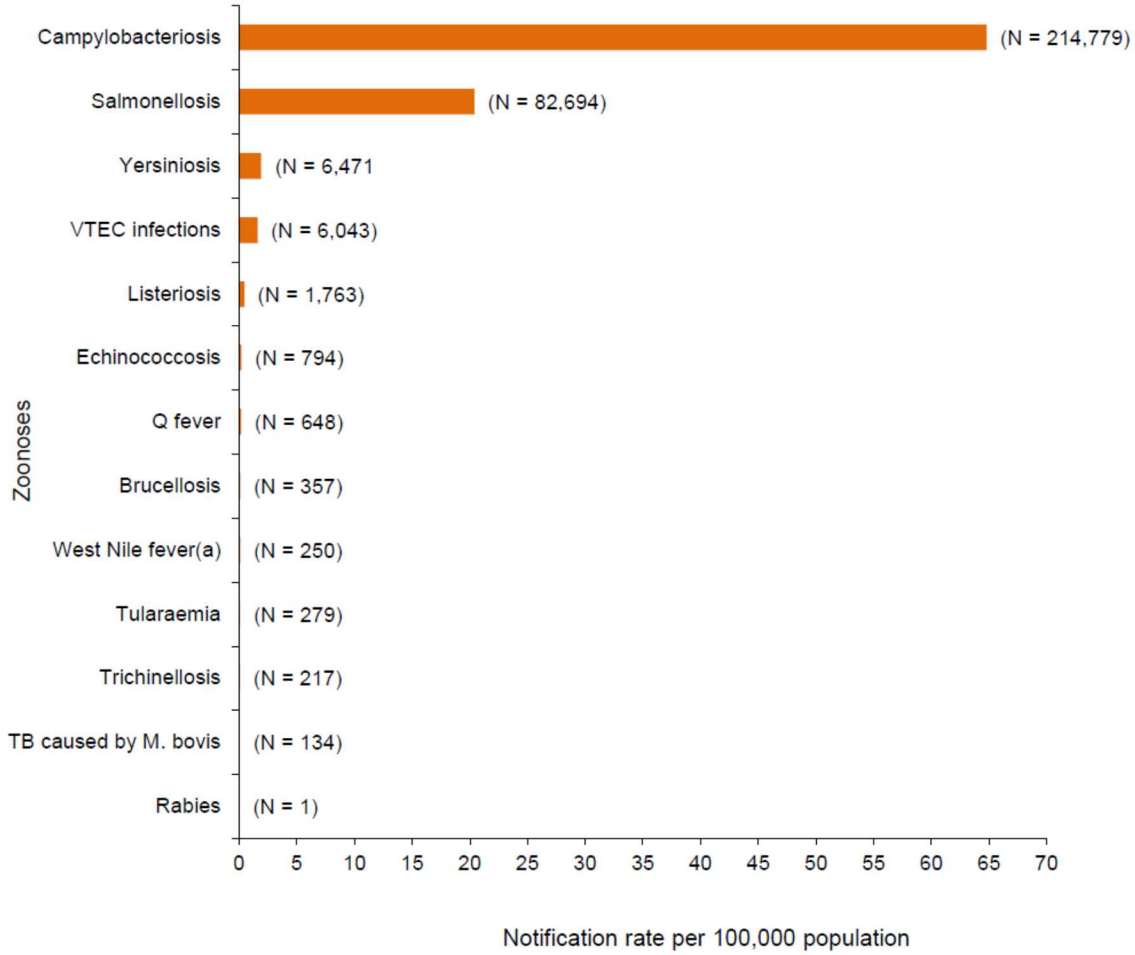


Figure 1.1: (from EFSA and ECDC, 2013)**:** Reported notification rates of zoonoses in confirmed human cases in the EU, 2013.

In 2013, 414 *Campylobacter* outbreaks were reported in the EU. The sources of the outbreaks were, in decreasing order of importance reported as, broiler meat, mixed or unspecified poultry meat, milk and mixed food (EFSA and ECDC, 2013).

Campylobacter spp. information was available for 48% of confirmed cases reported in the EU. Of these, 81% were reported to be *C. jejuni*, 7% *C. coli*, 0.2% *C. lari*, 0.1% *C. foetus* and 0.1% *C. upsaliensis*. Other *Campylobacter* spp. accounted for 12% of human cases but the large majority of those cases were reported at the national level as: "*C. jejuni* / *C. coli* not differentiated" (EFSA and ECDC, 2013).

A seasonal trend can be identified in confirmed campylobacteriosis cases reported in the EU in 2009-2013, with peaks in the summer months. The 12-month moving average was fairly stable over the 5-year period with no statistically significant increase or decrease in trend when analysed by month (p=0.334 with linear regression) (Figure 1.2) (EFSA and ECDC, 2013).

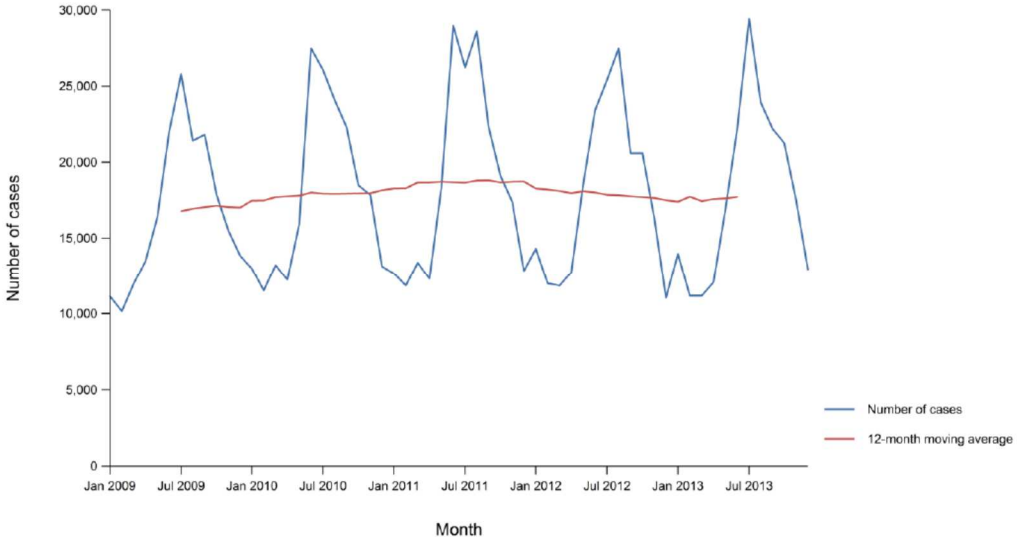


Figure 1.2: (from EFSA and ECDC, 2013)**:** Seasonal trend in reported confirmed cases of human campylobacteriosis in the EU, 2009-2013.

1.2.2 Campylobacteriosis in humans in the UK

Foodborne disease in the UK affects about 1 million people with 19,000 hospitalisations and 500 deaths at an approximate cost of £1.5 billion each year (FSA, 2011). Campylobacteriosis accounts for a third of the total cost to the UK (Humphrey, 2007). *Campylobacter* spp. are the most commonly reported bacterial cause of Infectious Intestinal Disease in the UK (FSA, 2012).

In 2012, there were 72,592 laboratory reports of *Campylobacter* in the UK. This is an increase of 0.5% from 2011. However, whilst reports increased by 3% in Northern Ireland and 0.5% in England and Wales, they fell by 0.3% in Scotland (Table 1.2).

Year	England & Wales	Scotland	Northern Ireland	UK
2010	62,686	6,601	1,040	70,327
2011	64,726	6,365	1,175	72,266
2012	65,032	6,349	1,211	72,592

Table 1.2: (from Zoonoses report UK, 2012)**:** Number of *Campylobacter* reports in humans in the UK, 2010-2012.

The Second Study of Infectious Intestinal Disease in the Community established that the ratio of unreported to reported human *Campylobacter* infections is one in nine cases (FSA, 2012). This suggests that there were approximately 650,000 *Campylobacter* cases in the UK in 2012.

Seven foodborne *Campylobacter* outbreaks were reported in the UK in 2012, six of which were associated with the consumption of chicken liver and chicken liver parfait, and one was associated with the consumption of lamb (DEFRA, 2013).

During 2014 in Scotland, the Health Protection Scotland (HPS) reported 6,636 laboratory cases of *Campylobacter* in humans, an increase of 472 (7.7%) compared to 2013 and the highest number of *Campylobacter* cases reported in the past ten years (Figure 1.3) (HPS, 2015).

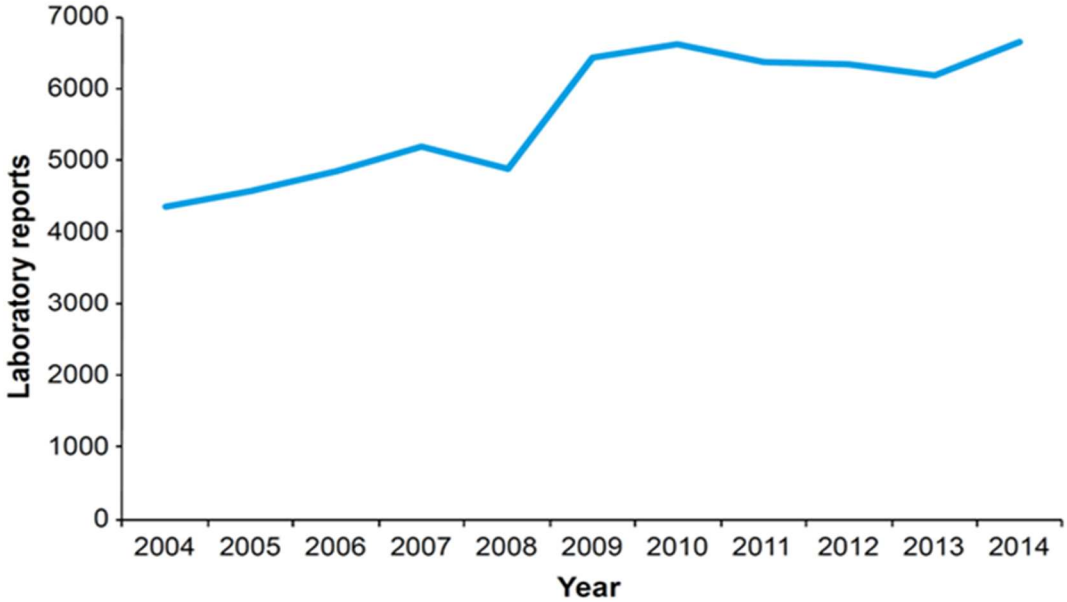


Figure 1.3: (from HPS Weekly Report, 2015): Laboratory isolates of *Campylobacter* reported to HPS 2004-2014.

During the same year, in mainland Scotland, the annual incidence rates of *Campylobacter* ranged from 81.2 to 162.8 per 100,000 population (HPS, 2015). However, there are usually consistent regional differences in the reported incidences of campylobacteriosis cases in humans where Northern islands, the Eastern coast of the central belt of Scotland and the Southwest have the higher notification rates (Figure 1.4).

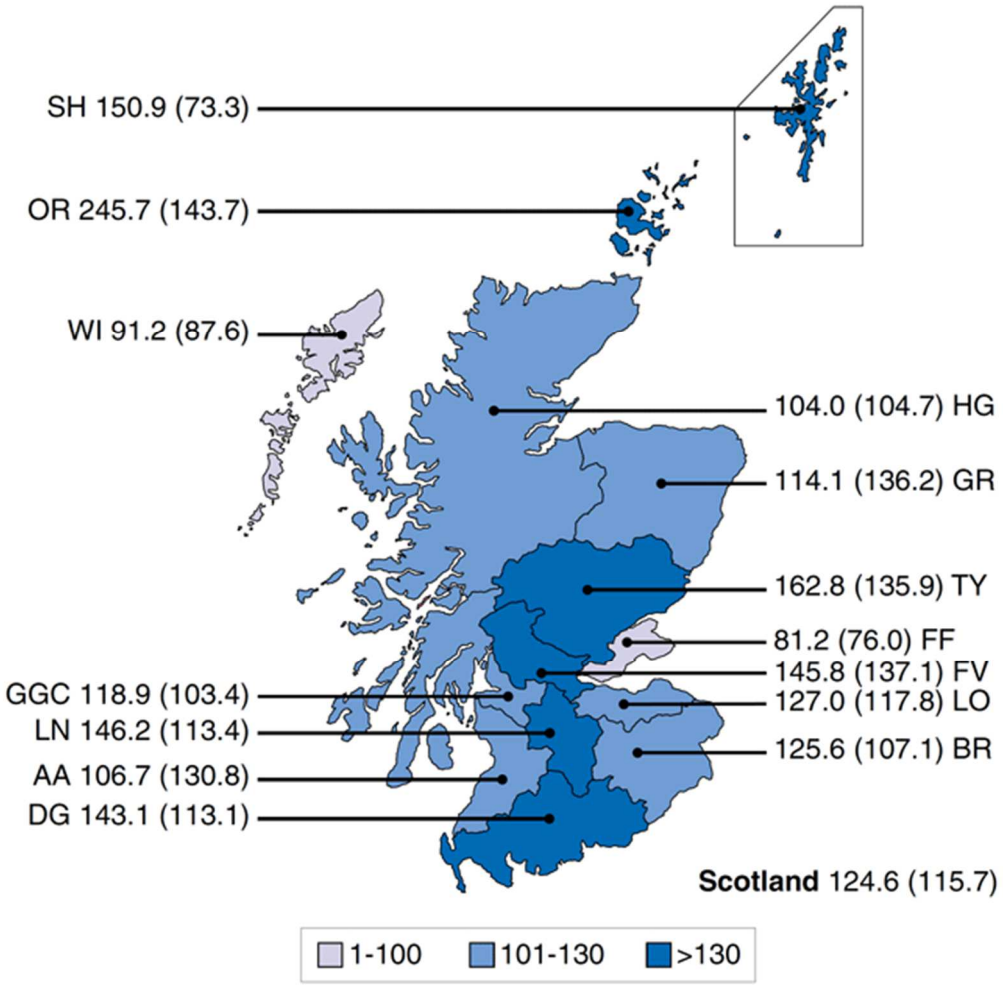


Figure 1.4: (from HPS Weekly Report, 2015): Rates per 100,000 population of reports of *Campylobacter* to HPS 2014 (2013). (BR=Borders, FV=Forth Valley, GR=Grampian, GGC=Greater Glasgow & Clyde, HG=Highland, LN=Lanarkshire, LO=Lothian, OR=Orkney, TY= Tayside, WI Western Isles, AA=Ayrshire & Arran, DG= Dumfries & Galloway, FF=Fife and SH=Shetland)

1.2.3 Final remarks on *Campylobacter* epidemiology in humans

Most human *Campylobacter* infections appear to be sporadic as only a few household or localised outbreaks are identified each year in the UK (O'Brien *et al.*, 2002). Some attempts have been made in the literature to try to correlate seasonality, age of people affected, and peaks of human disease with carriage of *Campylobacter* in poultry and other farm animals, but no definitive conclusions have been reached (Humphrey *et al*., 2007).

A characteristic of *Campylobacter* epidemiology in humans is its marked seasonality since, in the UK and other European countries, incidence peaks in late spring/early summer (FSA, 2009). In NorthWest England surveillance of campylobacteriosis indicated a peak of cases in

May (Sopwith *et al.*, 2003). In Scotland, the annual peak is in late June-early July (Miller et *al.*, 2004). There are discordant reports in the literature describing a possible correlation between ambient temperature and number of human cases but the general consensus is that there may be a weak association (Humphrey *et al.*, 2007).

Data from England and Wales from 1990 to 2007 indicate that the proportion of cases reported for patients of different age groups has slowly changed over time, with a general decline in campylobacteriosis in young children under the age of 9 years, while older groups, in particular the over 60 year olds, have experienced an increase in infection over the last 10 years (Gillespie *et al.*, 2009). Strachan (2009) reported that in the Northeast of Scotland *Campylobacter* infection in young children living in rural areas was greater than in urban areas and it was linked to the direct contact with farm animals and contaminated water rather than consumption of poultry meat (Strachan *et al.*, 2009). In the same study, the foodborne route was considered to be of primary importance in *Campylobacter* infection in the adult population rather than contact with animals and water (Strachan *et al.*, 2009).

The peak in human cases has been related to the fluctuations in carriage in poultry and other food-producing animals. Some studies reported that *Campylobacter* carriage rates in broiler chicken flocks (Wallace *et al.*, 1997) and dairy cattle (Stanley *et al.*, 1998a) peak in the spring and late summer, in contrast to lamb and beef cattle where such marked seasonal variation in carriage rates have not been observed (Stanley *et al.*, 1998b). However, available evidence does not consistently support this hypothesis and the level of infection in humans and poultry seems to be associated with what Meldrum (2005) defines as "a common, but unidentified, environmental source" (Meldrum *et al.*, 2005).

1.3 Epidemiology of campylobacteriosis in animals

Thermotolerant *Campylobacter* spp. are widespread in nature (Kwan *et al.*, 2008a). The principal reservoirs are the alimentary tract of wild and domesticated birds and mammals. These bacteria are prevalent in food-producing animals such as poultry, cattle, pigs and sheep, companion animals (including cats and dogs), wild birds and in environmental water sources (Humphrey *et al.*, 2007). Animals acquire infection mainly through the faecal-oral route from the contaminated environment and rarely show signs of disease caused by these organisms (Blaser *et al.*, 1980). *Campylobacter* infection in companion animals will not be covered in this thesis and the focus will be on food producing animals and wild game birds.

1.3.1 *Campylobacter* infection in food producing animals

Campylobacter jejuni and *C. coli* are commonly found in cattle, sheep and pigs (Stanley and Jones, 2003; Nielsen, 2002; Payot *et al.*, 2004; Boes *et al.*, 2005) as shown in Table 1.1. Intestinal carriage of *Campylobacter* in cattle can range from 0.8 to 89% and in lambs can be as high as 91% (Stanley and Jones, 2003). This marked variation in the carriage of *Campylobacter* in the farm animal gastrointestinal tract is not well understood and many factors can contribute to it. There may be differences in the level of immunity or *Campylobacter* spp. may not be natural gut commensals in these animals, in contrast to *E. coli* (Humphrey *et al.*, 2007). Most cattle and sheep are reared in outdoor systems where there will be frequent contact with the external environment and they may become infected with *Campylobacter* in those circumstances (Schaffner *et al.*, 2004); however infection can also be sustained within the herd by cycling between individuals (Humphrey *et al.*, 2007).

In 2000, *Campylobacter* infection in poultry was estimated to reach 60% of broiler flocks slaughtered in the UK (Berrang *et al.*, 2000). A more recent analysis of the EU baseline survey of the prevalence of *Campylobacter* in broiler batches estimated that the UK prevalence in broilers at slaughter (based on caecal contents) was 75.3% (EFSA, 2008). The prevalence of *Campylobacter* carriage in poultry during the summer months can reach 100% (DEFRA, 2009). As mentioned in Section 1.1.1, poultry can contract infection from their environment, via contaminated water or following breaches of biosecurity (e.g. poor cleaning and disinfection of poultry houses). Infection in poultry is mainly through the oral-faecal route or via vertical transmission from parent flocks (Humphrey *et al.*, 2007). Chickens that are reared under extensive (free-range) systems are more likely to be *Campylobacter*-positive than housed animals (Heuer *et al.*, 2001).

1.3.2 *Campylobacter* infection in wild game birds

Wild game birds, and in particular pheasants and partridges, are commonly reared in outdoor farms and may be exposed to *Campylobacter* infection from the environment and/or at a later stage when they are released in the field where they may share their immediate surroundings with other livestock (Dampney, 2009; Heuer *et al.*, 2001). In live wild game birds the presence and prevalence of *C. jejuni* and *C. coli* has been reported in both pheasants and partridges from studies conducted in Germany, Russia, Italy and the Czech Republic (Atanassova and Ring, 1999; Stern *et al.*, 2004; Dipineto *et al.*, 2008a; Dipineto *et al.*, 2009; Nebola *et al.*, 2007) but current scientific knowledge is still scarce. One study conducted on live healthy pheasants sampled on a pheasant farm in the South of Italy reported a prevalence of 43.3% (n=240) with

C. coli and *C. jejuni* found in 100% and 13.5% of the positive samples taken, respectively (Dipineto *et al*., 2008a). In the same study, the prevalence was significantly higher in adult pheasants compared to younger pheasants. This finding is consistent with *Campylobacter* spp. infection in chickens where younger birds in the second to the fourth week of life are less likely to be affected (Newell & Fearnley, 2003; Shreeve *et al.*, 2000). There was no significant gender difference (Dipineto *et al.*, 2008a). In another study Dipineto (2008b) reported a prevalence of 86.7% (n=60) from cloacal swabs of live farmed pheasants; all positive samples were identified as *C. coli* and 19.2% of positive samples were also positive for *C. jejuni*.

A study from the Czech Republic reported isolation of *Campylobacter* spp. from 502 caecal samples collected from adult farmed and wild pheasants (n=302 farm / n=200 wild). The prevalence of *Campylobacter* spp. in the intestinal contents of pheasants from the farm was 70.2% with 50.5% of isolates identified as *C. coli* and 41.4% as *C. jejuni* (Nebola *et al.*, 2007). Farmed pheasants had a higher prevalence than birds shot in the wild (27.5% of cases) and this was linked to the fact that samples from wild pheasants were not taken immediately after they had been shot, while the samples from farmed pheasants were gathered within two hours of their death (Nebola *et al.*, 2007). *C. jejuni* was more prevalent (58.2%) than *C. coli* (36.4%) in the wild birds. On the other end, studies from Germany and Russia reported an estimated prevalence of *Campylobacter* spp. in wild pheasants of 26% and 25% respectively (Atanassova and Ring, 1999; Stern *et al.*, 2004). There are no data available in the literature on *Campylobacter* spp. intestinal carriage in pheasants.

1.4 The game meat supply chain and microbiological hazards to public health

1.4.1 Pheasants and the shooting industry

Phasianus colchicus is the most common species of pheasant in the UK (ADAS, 2005). Males are striking, with chestnut, golden brown and black markings on the body and tail, a green head and red face. The female has paler brown, mottled plumage and is usually smaller than the male (Figure 1.5).


Figure 1.5: Male pheasant on the left and female on the right (image from Meat Hygiene Service – Small Wild Game Training Course).

Pheasants are not innate to Britain. As reported by Pennycott (2001): ”They originated in parts of Asia, such as the Himalayas, Manchuria, Korea, Vietnam and Japan. They were introduced to the British Isles in the distant past by the Romans or the Normans but were certainly present in Britain by the 14th century”. There have been more recent additions, introducing specific types and breeds for sport shooting. Pheasants like a habitat that includes woodland or copses and hedgerows. There is a resident population of approximatively 8 million wild pheasants in the UK across the whole of England, Scotland, Wales and Ireland, except for the far north and west of Scotland and on the very high ground in England and Wales (Dampney, 2009).

Pheasants can be farmed for meat in a similar way to broiler chicken production although they are more difficult to rear intensively and are prone to welfare problems. Despite this, there are producers offering farm-reared partridge and pheasant meat that are more attractive to some restaurants and supermarket chains, compared to wild game meat, as there is no risk to diners of damaging their teeth on any shot left in the bird and also because they will be slaughtered in an approved abattoir, as normally happens for broilers (ADAS, 2005).

Wild pheasant meat reaches the consumer’s table as a by-product of the shooting industry. Shooting is a sport that is worth approximately £1.6 billion to the UK economy and it is estimated that 600,000 people are involved in the provision of sporting shooting in the UK (PACEC, 2006). The vast majority of the income from shooting is gained from the actual sporting activity, itself worth £240 million in Scotland (PACEC, 2006). The meat from wild game species is thus a by-product of the shooting industry and the value of game birds sold is minimal. The shooting industry focuses mainly on pheasants, partridges and grouse. Within the UK in 2004 approximately 15 million pheasants, 3.6 million pigeons, 2.6 million partridges, 970,000 ducks, 400,000 grouse, and 250,000 woodcock and snipe were shot. Within Scotland, up to 2 million pheasants, 500,000 pigeons, 370,000 partridges, 140,000 ducks, 200,000 grouse, and 37,500 woodcock and snipe are shot per annum (PACEC, 2006).

1.4.2 Pheasant management on farm

The pheasant shooting season closes at the end of January and approximatively 15 million pheasants are shot for sport in the UK each year (PACEC, 2006). This number of birds cannot be provided by the population of wild pheasants, of which there are approximately three million breeding birds each spring (Gibbons *et al.*, 1993), so the numbers are supplemented by artificially reared pheasants. On average, four-fifths (83%) of all shooting providers rely on released pheasants or partridges (PACEC, 2006). It is a common practice for pheasant-rearing sites to catch pheasants from the wild each year in February and March and transfer the birds to static or moveable breeding pens (Anon, 1993). Breeding pheasants will lay eggs from early March and the last eggs are placed in incubators in the middle of June. Incubation lasts 24 days. Hatching commences in the first week of May and finishes in the first week of July. Chicks will be transferred to brooder houses that provide heat, light and ventilation in controlled conditions. Heat is gradually reduced and space increased as the birds grow so that, by the time their feathers have developed, the birds can be given access to outside runs and become acclimatised to the outdoors. At 3 to 4 months old they will be mature enough to be released in the field until the shooting season starts at the beginning of October. Some gamekeepers by-pass this stage by buying birds at 6-8 weeks of age so they can be placed in outdoor release pens immediately. Others will buy at day-old and rear on. 35 million pheasants and 6.5 million partridges were reared and released for shooting in 2004 in the UK (PACEC, 2006).

1.4.3 Pheasant game meat processing and supply chain

The hunting season for pheasants in England, Scotland and Wales extends from 1 October to 1 February. In practice, most shoots do not actually commence shooting until 1 November as birds reared in Britain are not normally ready to shoot until the end of October. On the other hand commercial shoots will want to maximise the number of days shooting and are more likely to start at the beginning of October.

Pheasant carcases from large shoots are usually collected in larders and then given to game dealers, consumed locally or sent to AGHEs.

The EU Hygiene Regulations require that wild game meat for human consumption must be supplied to AGHEs and subjected to veterinary inspection (OJEU, 2004b), however some derogations to the legislation are in force in the UK that allows pheasants from small shoots to be consumed locally by hunters, beaters and local householders, including restaurants, butcher shops and pubs. Retailers that operate on a national level (e.g. supermarkets or

restaurants chains) can only source their game from AGHEs. Similarly, game bird carcases in feathers or “oven-ready” for the export market can only be sourced from AGHEs. A schematic representation of the pheasant game meat supply chain is shown in Figure 1.6.

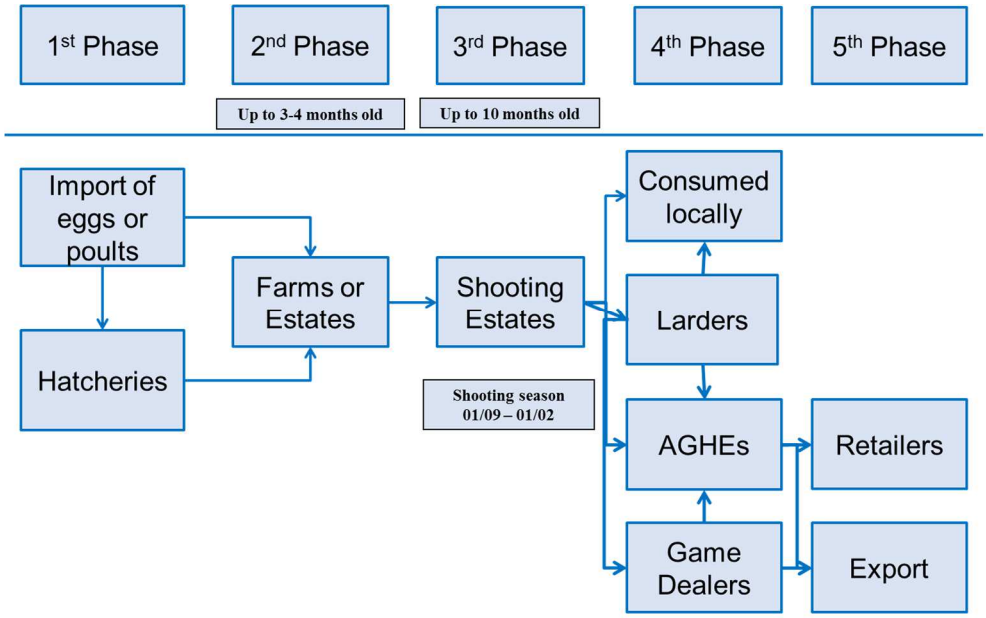


Figure 1.6: Schematic representation of the pheasant game meat supply chain.

Pheasant carcases delivered to AGHEs are stored in batches in intake chillers at a temperature below 4°C, waiting to be processed. The first step of the process is dry feather-plucking. Scalding of carcases; as used for broilers in poultry abattoirs, is not common practice for small wild game; however some AGHEs may immerse pheasants in hot wax after dry plucking to facilitate the removal of feathers. At this stage damaged carcases will proceed for breast and thigh meat removal and the rest of the carcass will be discarded. Well-presented carcases will usually be manually eviscerated and then packed as “oven-ready” product. Breast and thigh meat will be vacuum-packed in portions of different sizes according to customer specifications. “Oven-ready” product and breast meat can be stored in refrigerated conditions below 4°C or frozen to extend their shelf-life.

1.4.4 Official controls in Approved Game Handling Establishments (AGHEs) and identification of microbiological hazards in wild game meat related to public health

EU food hygiene legislation is applied across the UK and has been implemented in Scotland by The Food Hygiene (Scotland) Regulations 2006 that came into force on 11 January 2006

(SSI, 2006/3). This legislation includes the three EU food Hygiene Regulations that are: Regulation 852/2004 on basic hygiene requirements for production of foodstuffs (OJEU, 2004a); Regulation 853/2004 laying down specific hygiene rules - these apply to food business operators producing food of animal origin (OJEU, 2004b); and, Regulation 854/2004 that relates to the organisation of official controls on products of animal origin intended for human consumption (OJEU, 2004c). The legislation considers the hunting of wild game as primary production but private domestic consumption of wild game is unregulated (OJEU, 2004a). However, the EU Hygiene Regulations also require that wild game meat for human consumption must be supplied to AGHEs for veterinary inspection and must be passed fit for human consumption (OJEU, 2004b).

The British Association for Shooting and Conservation (BASC) estimates that 90% of the wild game birds shot in Scotland are supplied directly from the shooting estates to the AGHEs (SRUC, 2012). However, for the shooting season 2010/11, the Meat Hygiene Service (former executive agency of the Food Standards Agency) recorded a throughput of small game birds and ground game by Scottish AGHEs of approximately 700,000 (SRUC, 2012) which is actually a much smaller proportion (approximatively one fifth) of the estimated 3.5 million game birds shot annually. Game meat can be offered directly to consumers without veterinary inspection because of extensive derogations from the EC Hygiene Regulations that have been applied in the UK allowing the direct supply of game meat from hunters or retail outlets to the final consumer (SRUC, 2012).

However, concerns are raised by the fact that more than two thirds of game bird meat reach the consumer's table without veterinary inspection and without being passed fit for human consumption. A Veterinary Laboratory Agency report on a qualitative risk assessment of wild game meat published in 2003 (VLA, 2003) stated that post mortem veterinary inspection in small wild game does not have any additional beneficial effect in identifying foodborne diseases and an effective Hazard Analysis and Critical Control Point (HACCP) system should be able to detect and discard unfit meat. *Salmonella* spp., *C. jejuni* and *E. coli* O157:H7 were considered the most important zoonotic hazards from small wild game species and they can pass undetected at veterinary inspection in AGHEs because they may not produce visible lesions in the carcass (VLA, 2003). The risk associated with these pathogens in small wild game meat inspected at AGHEs is summarized in Table 1.3 below (VLA, 2003 and Coburn *et al.*, 2005). *Campylobacter jejuni* was the pathogen that was considered to be the greater risk to public health from game bird meat.

Hazard	Risk	Comments
Campylobacter jejuni	Moderate. High prevalence of *Campylobacter* spp. in wild game meat.	Survives well at refrigeration. Very susceptible to cooking temperature of 70°C for a minimum of 2 minutes.
Salmonella spp.	Low. There is a low prevalence of *Salmonella* spp. in wild game meat.	The absence of salmonella in small wild game meat is not unusual and reported by other authors (Paulsen et al., 2008). Susceptible to cooking temperature of 70°C for a minimum of 2 minutes.
E. coli O157:H7	Low. Prevalence is considered low.	Susceptible to cooking temperature of 70°C for a minimum of 2 minutes.

Table 1.3: Summary of hazards and risk associated with small wild game species.

1.5 Aims of the study

The reduction of foodborne disease caused by *Campylobacter* spp. is a key aim of the FSA Strategic plan 2010-2015. This is focused on the reduction of *Campylobacter* in chicken, as 60-80% of cases of campylobacteriosis in humans can be attributed to poultry meat. However, *C. jejuni* and *C. coli* have also been reported in a range of livestock and wildlife species, including live pheasants. Pheasants reach the consumer's table as a by-product of the shooting industry and wild game meat has recently increased in popularity among consumers on the grounds of sustainability, healthy eating, and support to local production. Approximately 3.5 million game birds are shot in Scotland every year; however, only 700,000 are received at Scottish AGHEs for veterinary inspection. Despite this volume of wild game entering the food chain, there is a lack of information concerning the risk of campylobacteriosis in humans arising from consumption of wild game meat and the role wild game birds may have as a reservoir of infection. However, there is evidence that the greatest hazard from consumption of wild game meat relates to handling procedures after killing, where hygienic standards are difficult to sustain in the wild and *Campylobacter* can play an important role as a foodborne hazard from small wild game meat.

As a contribution to the surveillance of *Campylobacter* infection in wild game birds in Scotland, this study proposes to explore the role that pheasants play in *Campylobacter*

infection in Scotland and to expand our knowledge base on wild game campylobacteriosis. This study's aims were:

1. To determine the prevalence of *Campylobacter* spp. in wild game pheasants processed in AGHEs in Scotland.

2. To identify the main sequence types (ST) of *Campylobacter* spp. isolated from pheasants.

3. To determine if the ST of *Campylobacter* spp. from pheasants are the same as in broilers and humans and evaluate the impact on public health.

Chapter 2 Materials and Methods (study site and sample collection)

2.1 Introduction

The research project ran over a two-year period, starting in September 2013 and finishing in August 2015. It was composed of two stages and the main steps followed are outlined below:

Stage 1:

- Stage 1a: During the hunting season, caecal and neck skin samples were collected from pheasant carcases in Approved Game Handling Establishments (AGHEs) in Scotland and sampled birds were traced to their originating shooting estates.

- Stage 1b: *Campylobacter* spp. were isolated using traditional culture methods (based on ISO 10272) at the Roslin Institute; molecular species identification of the isolated *Campylobacter* spp. was performed using Polymerase Chain Reaction (PCR) at the Roslin Institute and High-Throughput Multilocus Sequence Typing (HiMLST) was carried out at the Regional Laboratory for Public Health Kennemerland, Haarlem, the Netherlands.

Stage 2:

- Stage 2a: The results obtained from stage 1 were analysed to estimate the prevalence and characterise sequence types (ST).

- Stage 2b: Sequence types identified from pheasants were compared and combined with the molecular epidemiology of *Campylobacter* isolates from humans and other food producing animals archived in the PubMLST Database to perform host association and source attribution.

2.2 Geographical stratification and sample size selection

Caecal and skin samples were collected from pheasant carcases in selected AGHEs in Scotland during the hunting season 2013/2014.

The FSA records 11 AGHEs in Scotland (SRUC, 2012):

- One in the Western Isles
- Three in the Highlands and Moray (Region 1)
- One in Aberdeenshire (Region 2)
- Two in Perth, Kinross and Angus (including Argyle and Bute) (Region 3)
- Two in Edinburgh and the Scottish Borders (Region 4)
- Two in Glasgow, Ayrshire, Dumfries and Galloway (Region 5)

Based on this information and for the purpose of this study Scotland was divided into five geographical regions that excluded the Western Isles which were not easily accessible for sampling purposes. Five sampling sites were selected to collect birds from different estates across Scotland, one from each region. The selection was made based on the size of the business, the ability to receive pheasants consistently during the hunting season and the capacity to receive birds from several estates within the catchment area (Table 2.1).

AGHEs Name	Region	Remarks
Tarradale Game Ltd.	1	Not operating on a regular basis
Ardgay Game Ltd.		Chosen for sampling
Simpson Game Ltd.		Not operating on a regular basis
Aberdeenshire Larder	2	Chosen for sampling
Hubertus Game Ltd.	3	Chosen for sampling
J C Mitchell Poultry and Game Dealers		Not operating on a regular basis
Kezie (UK) Ltd.	4	Randomly chosen for sampling
Burnside Farm Foods		
Braehead Foods Ltd.	5	Biggest AGHEs for small wild game in Scotland – chosen for sampling.
Craigadam Country Larder		Small size AGHE

Table 2.1: List of approved small wild game AGHEs in Scotland (excluding Western Isles) by region and reason for selection for sampling.

Figure 2.1 below shows the boundaries of the five Scottish regions with the location of the sampling sites and estates sampled.

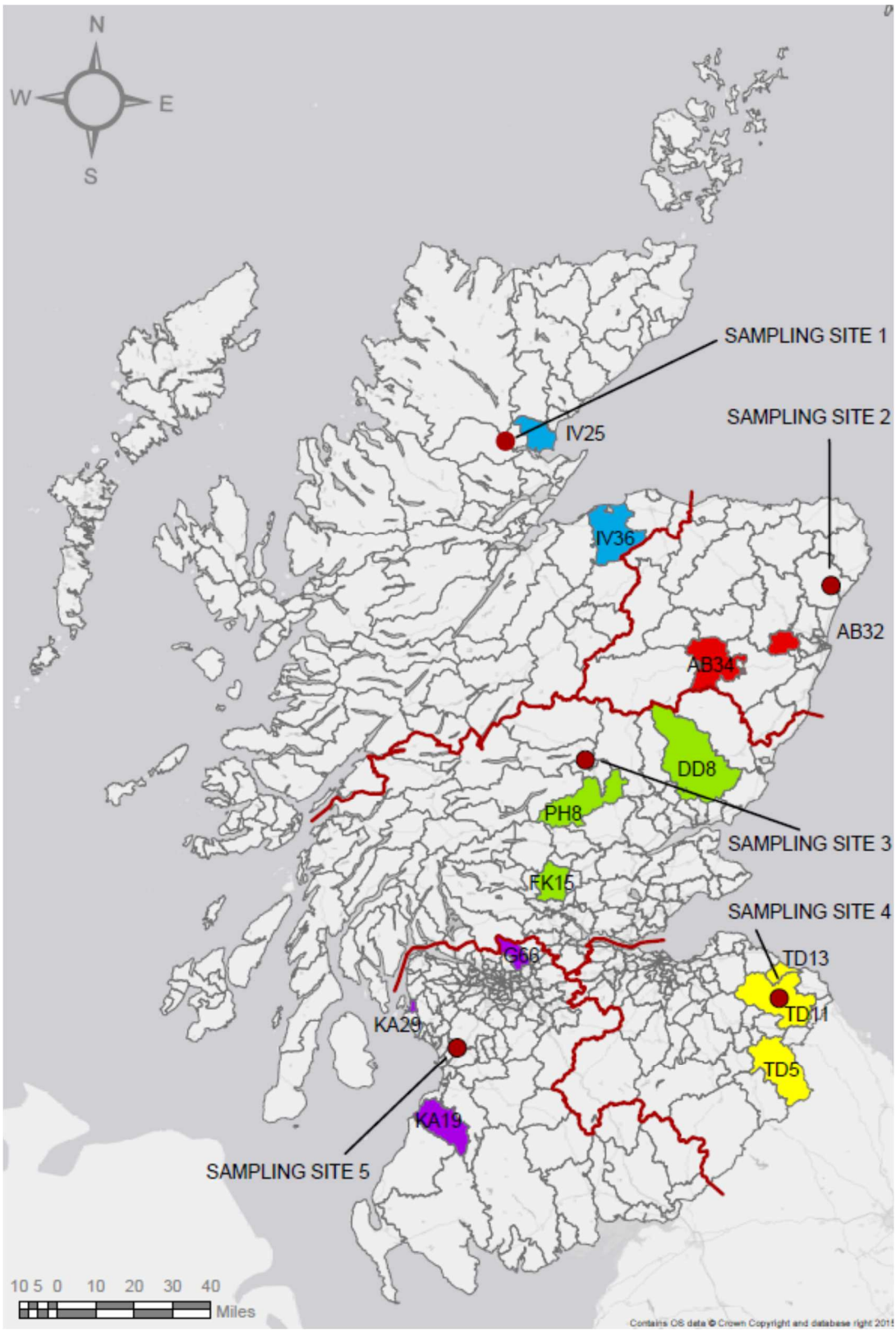


Figure 2.1: The five Scottish regions for sampling are delimitated by a red line, the location of the five sampling sites is identified by a red dot and estate sampled are highlighted in colours (IV=Inverness, AB=Aberdeenshire, FK=Falkirk, PH=Perth, DD=Dundee, TD=Galashiels, G=Glasgow and KA=Kilmarnock).

The AGHEs selected, estates of origin and number of samples collected from each site are shown in Table 2.2 below:

Sampling Site	Name of AGHE sampled, date and total number of samples collected	Estates sampled	Total number of pheasants in each batch sampled	Samples collected (% of birds sampled)	Region estate contributes to
1	Ardgay Game (Bonar Bridge) (38) ¥08/11/2013	†IV25	40	38 (95%)	Region 1
2	Aberdeenshire Larder (Aberdeen) (56) 22/10/2013	AB32	148	23 (15.5%)	Region 2
		AB34	273	33 (12%)	Region 2
3	Hubertus Game (Pitlochry) (73) 29/10/2013	IV36	110	18 (16.3%)	Region 1
		FK15	228	10 (4.3%)	Region 3
		PH8	196	35 (17.8%)	Region 3
		DD8	145	10 (6.8%)	Region 3
4	Kezie Ltd. (Duns) (42) 18/11/2013	TD11	102	12 (11.7%)	Region 4
		TD13	31	30 (96.7%)	Region 4
5	Braehead Foods Ltd. (Kilmarnock) (78) 29/01/2014	TD5	230	18 (7.8%)	Region 4
		KA19	83	22 (26.5%)	Region 5
		KA29	32	16 (50%)	Region 5
		G66	65	22 (33.8%)	Region 5
Total			**1683**	**287 (17%)**	

Table 2.2: Summary of samples collected (n) from each estate (†), date of collection (¥), their contribution to the number of samples collected per region and total number of pheasants present in each batch sampled. Estates are identified by the digits in the first half of the postcode as follows: IV=Inverness, AB=Aberdeenshire, FK=Falkirk, PH=Perth, DD=Dundee, TD=Galashiels, KA=Kilmarnock, G=Glasgow).

A simple random sampling estimate was used to determine the sample size as proposed by Thrusfield (2005) for a large, theoretically infinite population since the pheasant population in Scotland is approximatively 2.5 million (PACEC, 2006). Assuming an expected prevalence of 25% in wild pheasants, inferred from relevant literature (Atanassova and Ring, 1999; Stern *et al.*, 2004; Nebola *et al.*, 2007), and a desired confidence level of 95% with an absolute precision of 5%, it was necessary to sample approximately 58 birds per region. The total number of pheasants sampled for this project was 287.

The sampling date was agreed with the sampling site depending on the availability of birds. On the agreed date, a subset of all pheasants processed that day was sampled, the sampled birds generally originating from more than one estate. Estates were selected from different areas within the same region based on the digits in the first half of their postcode. For logistical reasons, however (e.g. not enough pheasants present in the chiller on the sampling date), it was not possible to collect all the required samples for regions 1 and 4 on the sampling date. To compensate for this, the rest of the samples required for region 1 were sampled from sampling site 3 and those remaining for region 4 were sampled from sampling site 5, as shown in Table 2.2. This was possible because AGHEs receive pheasants from estates located in different Scottish regions. In addition, some batches were already being partially processed by the Food Business Operator at the time of sampling. For logistic reasons, a uniformly standardised approach to sampling was not possible. The sampling approach is detailed in Table 2.2.

Time of year and date of kill, where known, were recorded at the time of sampling and were taken into consideration when analysing the data. The sex of sampled birds was not recorded as this was not considered relevant in terms of *Campylobacter* infection in pheasants (Dipineto *et al.*, 2008a).

2.3 Sample collection

Pheasants are received at AGHEs from several shooting estates. The same estate can deliver pheasant carcases from different shooting days all at the same time. Usually pheasant carcases from the same shooting day are considered to be a batch, identified by a unique serial batch number and kept separate from other batches in crates (Figure 2.2).

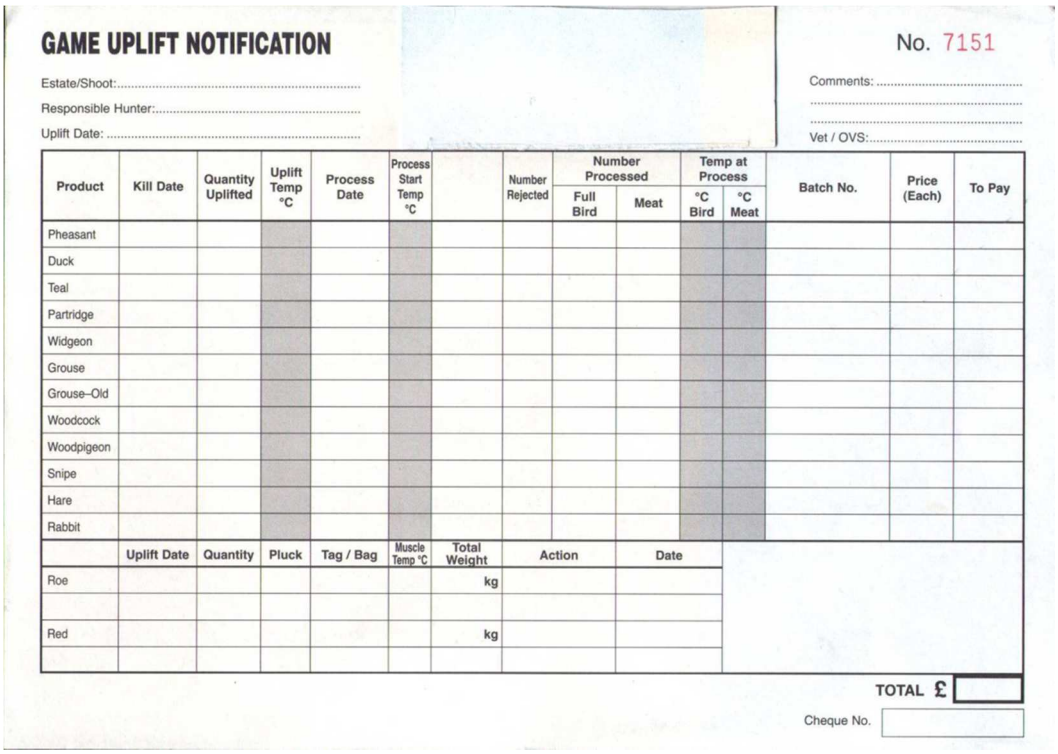
Figure 2.2: Pheasants in crates: the red arrow shows the batch number on the label attached to the crates.

Every batch is delivered with documentation (Figure 2.3) that will state:

- Name and address of the shooting estate
- Date of shooting
- Unique batch number (related to the crate)
- Type and number of wild game birds in the batch
- Name of the Trained Hunter

Optional information for small wild game is:

- Trained Hunter Declaration
- Temperature of carcases at collection
- Uplift date



GAME UPLIFT NOTIFICATION

No. 7151

Estate/Shoot:.....

Responsible Hunter:.....

Uplift Date: .....

Comments: .....

.....

.....

Vet / OVS:.....

Product	Kill Date	Quantity Uplifted	Uplift Temp °C	Process Date	Process Start Temp °C		Number Rejected	Number Processed		Temp at Process		Batch No.	Price (Each)	To Pay
								Full Bird	Meat	°C Bird	°C Meat			
Pheasant														
Duck														
Teal														
Partridge														
Widgeon														
Grouse														
Grouse–Old														
Woodcock														
Woodpigeon														
Snipe														
Hare														
Rabbit														

	Uplift Date	Quantity	Pluck	Tag / Bag	Muscle Temp °C	Total Weight	Action	Date
Roe						kg		
Red						kg		

TOTAL £

Cheque No.

Figure 2.3: Example of a document which gamekeepers complete for every batch of wild game birds collected at the shooting estate.

After reception at the AGHE pheasant carcases await processing in "intake" chillers, as the legislation requires the temperature of pheasant carcases to fall below 4°C and to be kept under refrigerated conditions along the food chain.

For this project, batches to be sampled were traced to the shooting estate through the relevant documentation. Pheasants were then selected and samples collected at intake prior to processing (Figure 2.4) to avoid the risk of cross-contamination during plucking and evisceration.

Pheasants were individually sampled. A skin sample the size of a one pound coin (approximately 1 cm in diameter) was taken from the neck region. Pheasant carcases were opened (Figure 2.5) and caeca were detached from the rest of the intestine (Figure 2.6). Skin and caecal samples were individually placed in sampling pots, labelled with the bird number, in sequential numerical order and stored at refrigeration temperature in the chiller. Gloves and blades were changed between samples and the sampling surface cleaned and disinfected after discarding each carcase, to avoid cross-contamination.

Figure 2.4: Pheasant carcases ready for sample collection.

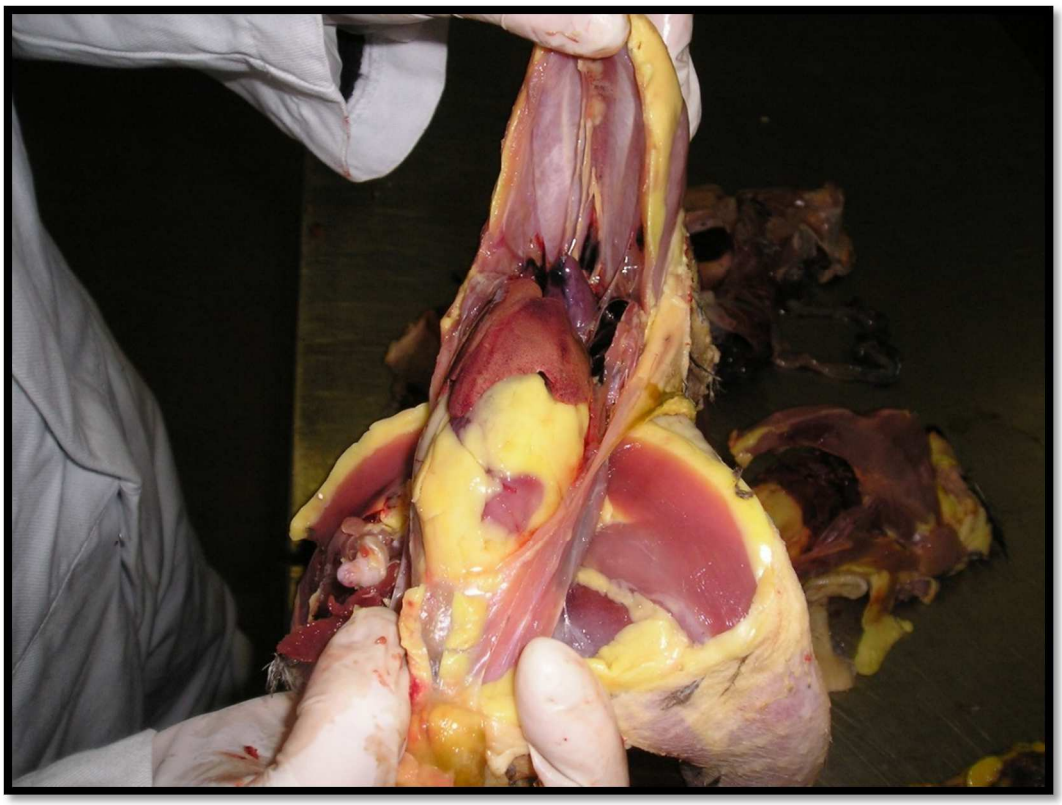


Figure 2.5: Opening pheasant carcase to expose the gastrointestinal tract.

Figure 2.6: The red arrow shows the caeca still attached to the rest of the gastrointestinal tract.

Samples were kept at refrigeration temperature using ice packs in refrigerated boxes during transport, then were stored in a chilling room at the Royal (Dick) School of Veterinary Studies (R(D)SVS). Samples were transferred to the Roslin Institute laboratories the day after the collection day and processed as follows:

- Day 0 – Sample collected at AGHE.
- Day 1 – Sample plating: 1g of caecal content from each bird diluted 1:10 in phosphate buffered saline (PBS) and then four further 10-fold dilutions were prepared from each sample; each dilution was plated on modified charcoal cefoperazone deoxycolate agar (mCCDA) and incubated for 48 hours at 37°C, under microaerophilic conditions (5%CO_2, 5%O_2, 90%N_2) (PHE, 2014).
- Day 3 – Plate reading: mCCDA plates were examined and colonies counted. From each sample, 1 to 8 colonies were isolated and plated individually on mCCDA plates to obtain single pure colonies and incubated for 48 hours at 37°C, under microaerophilic conditions (PHE, 2014).
- Day 6 – Pure colonies from mCCDA plates were transferred into 16.67% glycerol in Bolton broth and frozen to -80°C. This process ensured long-term survival of recovered colonies (PHE, 2014).

Details of the laboratory technique used are given in Chapter 3.

Chapter 3 *Campylobacter* isolates, prevalence and bacterial loads

3.1 Introduction

As detailed in the main introduction (section 1.3.2), there are few prevalence studies relating to *Campylobacter* infection in pheasants in the literature. Studies conducted in Germany, Russia, Italy and the Czech Republic (Atanassova and Ring, 1999; Stern *et al.*, 2004; Dipineto *et al.*, 2008a; Dipineto *et al*., 2009; Nebola *et al.*, 2007) vary in terms of the sampled population, the sampling method and the resulting prevalence. Based on cloacal swabs collected from farmed pheasants, the Italian researchers detected a prevalence that ranged from 43.3% to 86.7% (Dipineto *et al*., 2008a; Dipineto *et al*., 2009). In Germany and Russia, caecal content was collected from hunted wild pheasants and the prevalence was lower, at 26% and 25%, respectively (Atanassova and Ring, 1999; Stern *et al.*, 2004). The study from the Czech Republic sampled intestinal contents and reported a prevalence of 70.2% in farmed birds and 27.5% in birds shot in the wild (Nebola *et al.*, 2007). The prevalence of infection in farmed pheasants was consistently higher across these studies compared to wild pheasants. This has been attributed to the difference in the kill-to-process time that is generally shorter (usually within 2 hours of death) for farmed birds compared to wild (Nebola *et al.*, 2007). A specific kill–to-process time was not defined in the aforementioned studies for the wild birds sampled. A prolonged kill-to-process time has been thought to be detrimental for *Campylobacter* survival due to the organism's restricted growth requirements, however, other studies report that the method of pheasant carcase storage and the location of the shooting wound can greatly influence bacterial growth and survival in wild game birds (Paulsen *et al.*, 2008). Survival of *C. jejuni* and *C. coli* on skin also seems challenging as there are no reported cases of either of these species being recovered from pheasant skin samples; however, they have been recovered at a low prevalence (5.3%: n=57) from pigeon skin (Soncini *et al.*, 2006). There are no data available in the literature on *Campylobacter* spp. intestinal carriage in pheasants.

3.2 Materials and methods

Isolation of *Campylobacter* from caecal samples was carried out at the Roslin Institute bacteriology laboratory while skin samples were processed at the R(D)SVS microbiology

laboratory. Laboratory techniques to recover *Campylobacter* are standardised and refer to internationally recognised methods for the detection and enumeration of *Campylobacter* species (PHE, 2014). Modified Cefperazone Charcoal Deoxycholate Agar (mCCDA) is the preferred isolation medium for *Campylobacter* spp. and on this medium *Campylobacter* colonies form grey-tinged, flat and moist colonies, often with a metallic sheen and a tendency to spread (Figure 3.1). Other chromogenic agars may be used instead of mCCDA; options include Campy Food Agar (CFA), Brilliance Campy Count Agar (BCCA) and Campylobacter Selective agar (CASA).

Figure 3.1: Typical appearance of *Campylobacter* colonies on mCCDA

The method used to isolate *Campylobacter* spp. from caecal content and skin was based on the BS EN ISO 10272-1:20063 (detection) and BS EN ISO/TS 10272-2:20064 (enumeration) standards (PHE, 2014). Detection of *Campylobacter* in the bacteriology laboratory is only able to identify typical *Campylobacter* colony formation; species identification and sequence typing of *Campylobacter* spp. is carried out by PCR and MLST techniques described later in Chapter 4 of this dissertation.

The detection of *Campylobacter* spp. from organic samples usually involves initial enrichment in a selective liquid medium at 37ºC for 5 hours (h) followed by incubation in a microaerophilic atmosphere (5%CO_2, 5%O_2, 90%N_2) at 37ºC for 48h to allow recovery and growth. This is sub-cultured onto selective solid medium followed by examination for colonies considered to be typical of *Campylobacter*. Confirmation of the colonies as *Campylobacter* is

performed using morphological (Gram staining), biochemical (oxidase and catalyse tests) and growth property tests.

The enumeration of *Campylobacter* species by this method requires the inoculation of the surface of mCCDA plates with a defined volume of an appropriate decimal dilution of the test sample. Calculation of the number of colony forming units (CFU) per gram (g) of sample for *Campylobacter* spp. is determined from the number of typical colonies obtained on the selective medium. The number of CFU of *Campylobacter* spp. per 1g of caecal content is calculated as follows:

$$CFU/g = 1 \times \text{colony count} \times \text{dilution factor}$$

Counts greater than 100 are reported with one figure before and one after the decimal point multiplied by the appropriate power of 10. Decimal counts are rounded up if the last figure was 5 or more, or down if the last figure was 4 or less. When no colonies are detected then this is reported as *Campylobacter* spp. <10 CFU/g based on 1:10 dilution. Due to the large range of *Campylobacter* loads recovered from positive samples for the purpose of statistical analysis and graphical representation, CFU/g values were transformed and expressed in a logarithmic scale (log_{10}).

Statistical analysis

Statistical analysis of results was performed using Minitab 17 statistical software (© 2013 Minitab Inc). Presence of infection across regions, estates and sampling sites was compared using a Binary Logistic Regression calculation. Results were considered to be statistically significant if the overall *P* value was <0.05 and the 95% Confidence Interval (CI) Odds Ratio between groups did not cross 1. Odds Ratios that are greater than 1 indicated that the condition was more likely in the first group, conversely Odd Ratios that were less than 1 indicated that the condition was less likely in the first group. Comparison of the level of *Campylobacter* load, expressed as Log_{10}CFU/g, across regions, estates and sampling sites was calculated with One-Way ANOVA using the Tukey method and 95% CI to allow pairwise comparison "post-hoc". Results were considered to be statistically significant if the overall *P* value of the test result was <0.05 and the 95% CI of the different groups did not cross each other. WinPepi software Version 11.35 (© J.H. Abramson, 2013) was used to calculate prevalence and CIs for clusters of different sizes because of the variation in the number of samples collected from each estate. Microsoft Excel 2013 was used for raw data handling.

3.2.1 *Campylobacter* isolation – Pilot studies

Two pilot studies were carried out in February and September 2013 to test the reliability of the culture methods in the microbiology labs. The trial run in February was carried out on caecal content obtained from six birds all belonging to the same estate and on 14 neck skin samples from birds collected from four different estates. The September study was carried out by sampling five pheasants for skin and caecal content belonging to the same estate. The laboratory technique used in February was the same as outlined in paragraph 3.2.2 and 3.2.3 for *Campylobacter* isolation in skin and caecal samples, respectively, the only difference being that the microaerophilic conditions for *Campylobacter* growth were achieved by using CampyGen (Oxoid) paper sachets in jars and not microaerophilic cabinets. As a consequence of further literature review, the isolation technique was refined in the September pilot by using microaerophilic cabinets instead of paper sachets in jars (PHE, 2014).

3.2.2 Skin Samples

Skin samples were prepared for swabbing by removing excessive fat, if present. Sterile swabs moistened in Bolton broth were used to swab the skin samples then bathed in 500ml of enrichment Bolton Broth (Oxoid) for 1h, with occasional agitation. The swabs were then incubated for 48h at 37^{o}C in a microaerophilic atmosphere. For direct plating, a decimal dilution was prepared for each sample in phosphate buffered saline (PBS) solution, followed by four further 10-fold dilutions; 0.1ml of each dilution was plated on mCCDA (Oxoid) and CFA (Biomerieux) and incubated for 48h at 37^{o}C under microaerophilic conditions. Positive control samples were also prepared by contaminating the skin samples with known *Campylobacter* colonies taken from the microbiology laboratory stock isolates. These were confirmed as *Campylobacter* spp. by morphological, biochemical and growth property tests. Positive control samples were processed the same way as the other samples.

The presence or absence of *Campylobacter* colonies was determined by examination of mCCDA and CFA plates for their typical appearance, according to the plate manufacturers' instructions. On CFA plates C*ampylobacter* bacteria form burgundy-red to orange-red colonies on the light beige agar (Figure 3.2). Suspect isolates were subjected to morphological examination. *Campylobacter* colonies were counted visually on the plate in decimal solution series up to 250 colonies and were standardised as CFU/g.

Figure 3.2: Typical appearance of *Campylobacter* colonies on CFA

3.2.3 Caecal samples

Two hundred and eighty seven caecal samples were collected for *Campylobacter* isolation; 1g of caecal content (0.5g from each caecum) from each sample was diluted 1:10 in PBS and then four further 10-fold dilutions were prepared from each sample; 0.1ml of each dilution was plated on mCCDA and incubated for 48h at 37ºC, under microaerophilic conditions. Campy Food Agar plates were not inoculated with caecal samples because from the skin samples cultures there was no difference in efficiency compared to the mCCDA plates.

The presence or absence of *Campylobacter* colonies was determined by examination of mCCDA plates for their typical appearance according to the manufacturer's instructions and *Campylobacter* colonies were counted visually on the plate in decimal solution series up to 250 colonies and were standardised as CFU/g.

Eight pure colonies from mCCDA isolates were transferred into Bolton broth, diluted 1:3 with 16.67% glycerol and frozen to -80ºC. This process ensured the long-term survival of recovered colonies.

3.3 Results and Data Analysis

3.3.1 *Campylobacter* isolation – Pilot studies

In the February pilot study, the 14 neck skin samples no *Campylobacter* colonies were detected (*Campylobacter* spp. <10 CFU/g based on 1:10 dilution). Only one pheasant caecal sample

out of the six tested was positive for *Campylobacter* spp., no count was performed on positive sample.

In the September pilot study, using the modified laboratory technique, three of the five pheasants tested were positive for *Campylobacter* spp. but no *Campylobacter* count was performed. Pure colonies were isolated and frozen at -80°C for future reference. The skin samples showed all *Campylobacter* spp. <10 CFU/g based on 1:10 dilution, so were considered negative. A summary of the results of the two pilot studies is shown in Table 3.1.

	Caecal content	**Skin**
Feb '13	1/6	0/14
Sept '13	3/5	0/5

Table 3.1: Proportion of samples positive for *Campylobacter* in two pilot studies undertaken in February and September 2013.

3.3.2 Main survey, skin samples

Forty neck skin samples were collected in this study and plated to isolate *Campylobacter* colonies but there was no growth, expressed as *Campylobacter* spp. < 10 CFU/g based on 1:10 dilution (Upper 95% C.I. <7.2%). There was no difference in colonies growth results by using mCCDA and CFA plates.

3.2.3 Main survey, caecal samples

Prevalence of infection

The 287 samples indicated an overall prevalence of infection of 37.6% (CI 13.9% - 61.2%), with the lowest prevalence of 6.6% (CI 0% - 22.5%) recorded in region 5. The prevalence of infection by region and estate is shown in Table 3.2. The prevalence of infection by sampling site is shown in Table 3.3 and was determined because, as detailed in section 2.2, for logistical reasons it was not possible to collect all the required samples for regions 1 and 4 on the sampling date. The rest of the samples required for region 1 were sampled from sampling site 3 and those remaining for region 4 were sampled from sampling site 5.

	Region 1	Region 2	Region 3	Region 4	Region 5	Totals
Samples collected	**56**	**56**	**55**	**60**	**60**	**287**
Samples collected by estate	IV25 ‡(38)	AB32 (23)	PH8 (35)	TD11(12)	KA19 (22)	
	IV36 (18)	AB34 (33)	DD8 (10)	TD13 (30)	KA29 (16)	
			FK15 (10)	TD5 (18)	G66 (22)	
Positive samples	**29**	**25**	**20**	**30**	**4**	**108**
Prevalence by estate	IV25Ω (26) *(68.4%) †CI 51.3% - 82.4%	AB32 (9) (39.1%) CI 19.7% - 61.4%	PH8 (10) (28.6%) CI 14.6% - 46.3%	TD11 (6) (50%) CI 21% - 78.9%	KA19 (2) (9%) CI 1.1% - 29.1%	
	IV36 (3) (16.6%) CI 3.5% - 41.4%	AB34 (16) (48.4%) CI 30.7% - 66.4%	DD8 (4) (40%) CI 12.1% - 73.7%	TD13 (22) (73.3%) CI 54.1% - 87.7%	KA29 (2) (12.5%) CI 1.6% - 38.3%	
			FK15 (6) (60%) CI 26.2% - 87.8%	TD5 (2) (11.1%) CI 1.3% - 34.7%	G66 (0) (0%) CI <12.7%	
Overall Prevalence	**51.7%** **CI 0% - 100%**	**44.6%** **CI 0% - 95.8%**	**36.3%** **CI 1.8% - 70.8%**	**50%** **CI 0% - 100%**	**6.6%** **CI 0% to 22.5%**	**37.6%** **CI 13.9% - 61.2%**

Table 3.2: Summary of samples collected‡ (n) and positive results Ω (n) from each region and estate. Overall prevalence* (%) and 95% Confidence Intervals† (CI) were estimated using different-size clusters calculation. Prevalence and CI for single estates was estimated using standard CI calculation.

Sampling Site	1	2	3	4	5	Totals
Samples collected	38	56	73	42	78	287
Samples collected by estate	IV25 ‡(38)	AB32 (23)	PH8 (35)	TD11(12)	KA19 (22)	
		AB34 (33)	DD8 (10)	TD13 (30)	KA29 (16)	
			FK15 (10)		G66 (22)	
			IV36 (18)		TD5 (18)	
Positive samples	26 Ω	25	23	28	6	108
Overall Prevalence	***68.4%** †**CI 51.3% - 82.4%**	**44.6%** **CI 0% - 95.8%**	**31.5%** **CI 10.8% - 52.83%**	**66.7%** **CI 0% - 100%**	**7%** **CI 0% - 17%**	**37.6%** **CI 5.8% - 69.5%**

Table 3.3: Summary of caecal samples collected‡ (n) and positive results Ω (n) by sampling site. Overall prevalence* (%) and 95% Confidence Intervals† (CI) were estimated using different-size clusters calculation. Prevalence and CI for sampling site 1 was estimated using standard CI calculation.

Using a binary logistic regression calculation it was possible to estimate if there was any statistically significant difference in terms of prevalence of infection between regions and sampling sites (Table 3.4 and 3.5, respectively).

Regions (Chi-Square 40.90; P<0.001)		Odds Ratio	95% CI
2	1	0.750	(0.357, 1.578)
3	1	0.532	(0.249, 1.136)
4	1	0.931	(0.449, 1.929)
5	**1**	**0.066**	**(0.021, 0.208)**
3	2	0.708	(0.331, 1.516)
4	2	1.240	(0.597, 2.574)
5	**2**	**0.088**	**(0.028, 0.277)**
4	3	1.750	(0.829, 3.693)
5	**3**	**0.125**	**(0.039, 0.396)**
5	**4**	**0.071**	**(0.023, 0.221)**

Table 3.4: Binary logistic regression analysis to estimate any statistical differences in the level of infection between regions. Statistically significant differences are highlighted in bold.

Sampling Sites (Chi-Square 68.99; P<0.001)		Odds Ratio	95% CI
1	**2**	**2.686**	**(1.133, 6.370)**
3	**1**	**0.212**	**(0.091, 0.493)**
4	1	0.923	(0.361, 2.357)
5	**1**	**0.038**	**(0.013, 0.113)**
3	2	0.570	(0.277, 1.174)
4	**2**	**2.480**	**(1.081, 5.688)**
5	**2**	**0.103**	(0.038, 0.276)
4	**3**	**4.347**	**(1.935, 9.769)**
3	**5**	**5.520**	**(2.096, 14.535)**
4	**5**	**24.000**	**(8.387, 68.671)**

Table 3.5: Binary logistic regression analysis to estimate any statistical differences in the level of infection between sampling sites. Statistically significant differences are highlighted in bold.

The statistical analysis indicated that there was a significant difference in terms of prevalence of infection (P<0.001) between regions. This was particularly prominent in region 5 when compared to the other regions. When the same statistical test was applied excluding region 5, there was no significant difference (P=0.352) in prevalence of infection across the remaining regions. Region 5, due to its very low prevalence, could thus be considered a potential confounder in terms of statistical analysis of the results. Not including region 5, the overall prevalence of infection increased to 45.8% (CI 34.9% - 56.6%). The same pattern was observed when prevalence of infection was compared across sampling sites, where sampling site 5 was significantly different compared to all the other sampling sites.

Campylobacter bacterial load

The average *Campylobacter* bacterial load of positive samples (n=108) was 5.8 x 10^6 CFU/g (median 3 x 10^4 CFU/g and range of <10 CFU/g to 4 x 10^8 CFU/g). A breakdown of the *Campylobacter* bacterial counts in the positive samples by region is depicted in Figure 3.3. Statistical analysis by One-way ANOVA showed no significant difference in the means of *Campylobacter* carriage across and between regions (P=0.441) (Figure 3.4).

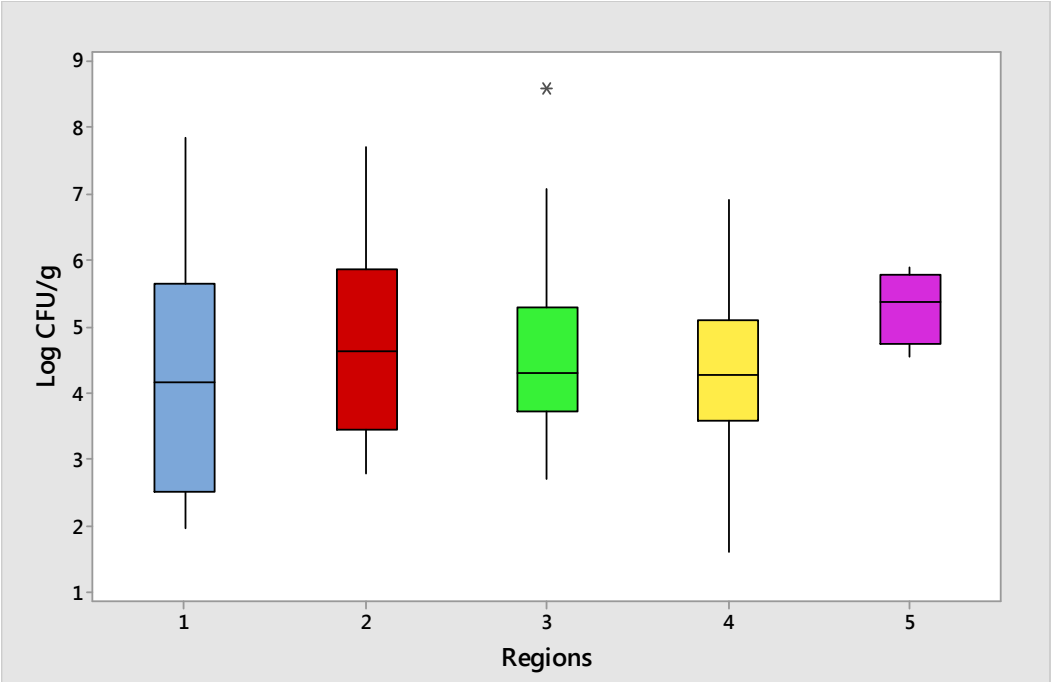


Figure 3.3: Median and range of *Campylobacter* load in positive caecal samples by region expressed as log CFU/g.

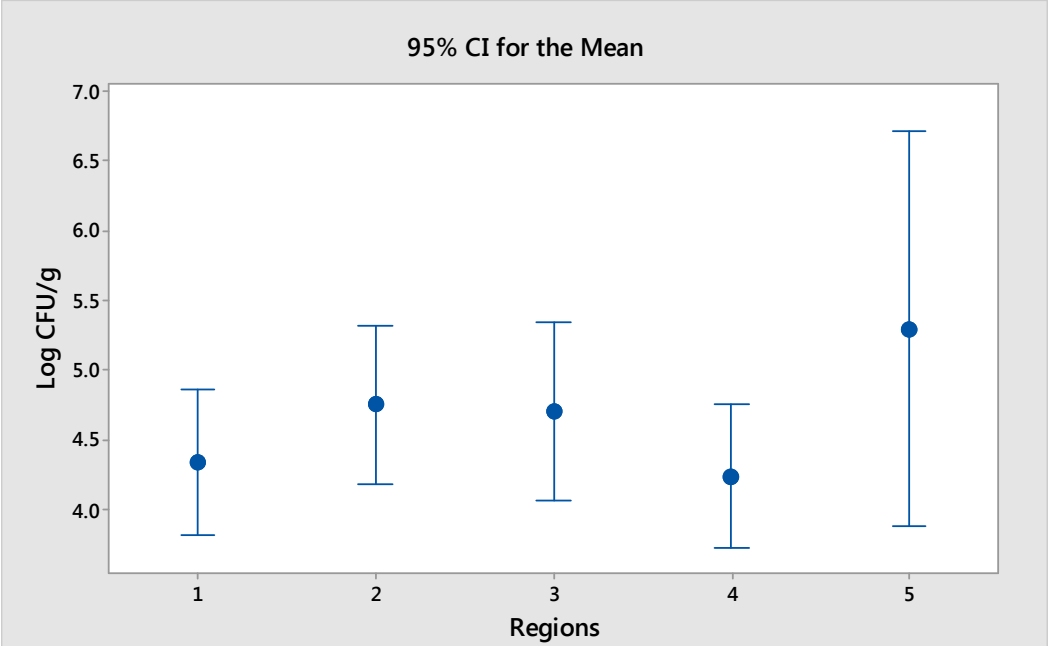


Figure 3.4: Means of *Campylobacter* load in positive caecal samples by region expressed as log CFU/g.

The distribution of the concentrations of *Campylobacter* bacterial load count in caecal content of pheasants (n=287) by region was $>10^3$ CFU/g in 33.4% (CI 28% - 39.2%) of samples and

5.6% (CI 3.2% - 8.9%) were >10^6 CFU/g. Negative samples with *Campylobacter* spp. <10 CFU/g based on 1:10 dilution counted for 61.3% (CI 55.4% - 67%) (Figure 3.5).

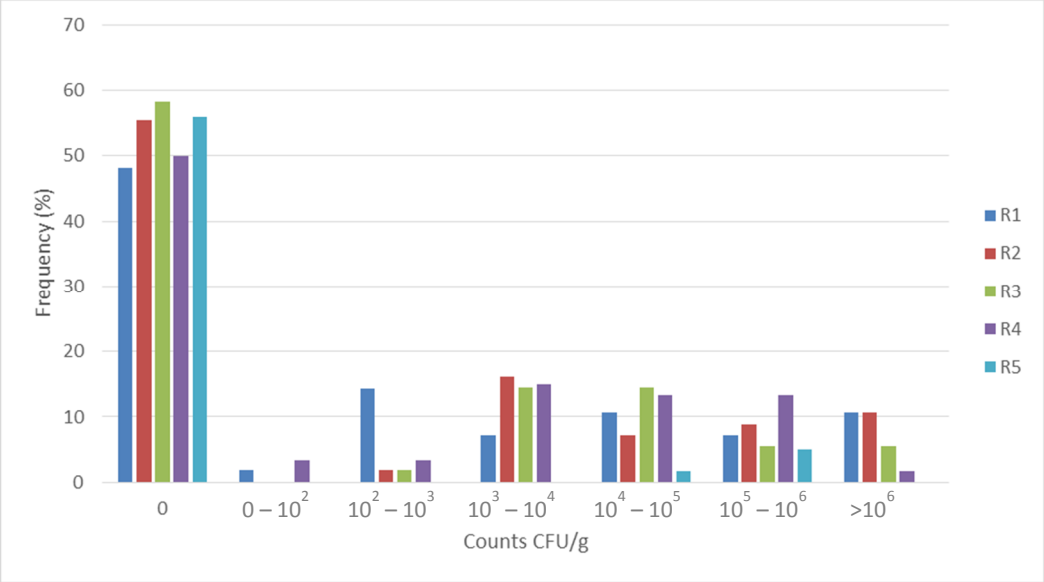


Figure 3.5: Frequency distribution (%) of the *Campylobacter* load counts in pheasants by region (R) (0=negative samples).

An overall summary of *Campylobacter* load by estate is shown in Figure 3.6.

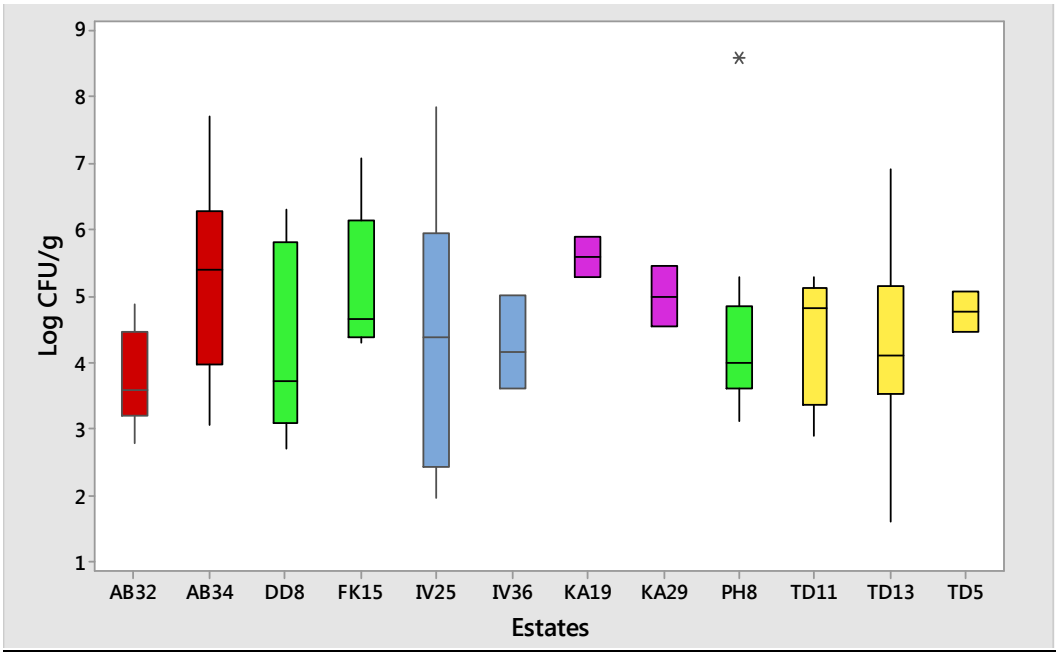


Figure 3.6: Median and range of *Campylobacter* load in positive sample by estate expressed as log CFU/g.

The same statistical analysis also showed no significant difference in the mean of *Campylobacter* carriage across and between estates (P=0.342) (Figure 3.7) and sampling sites (P=0.461).

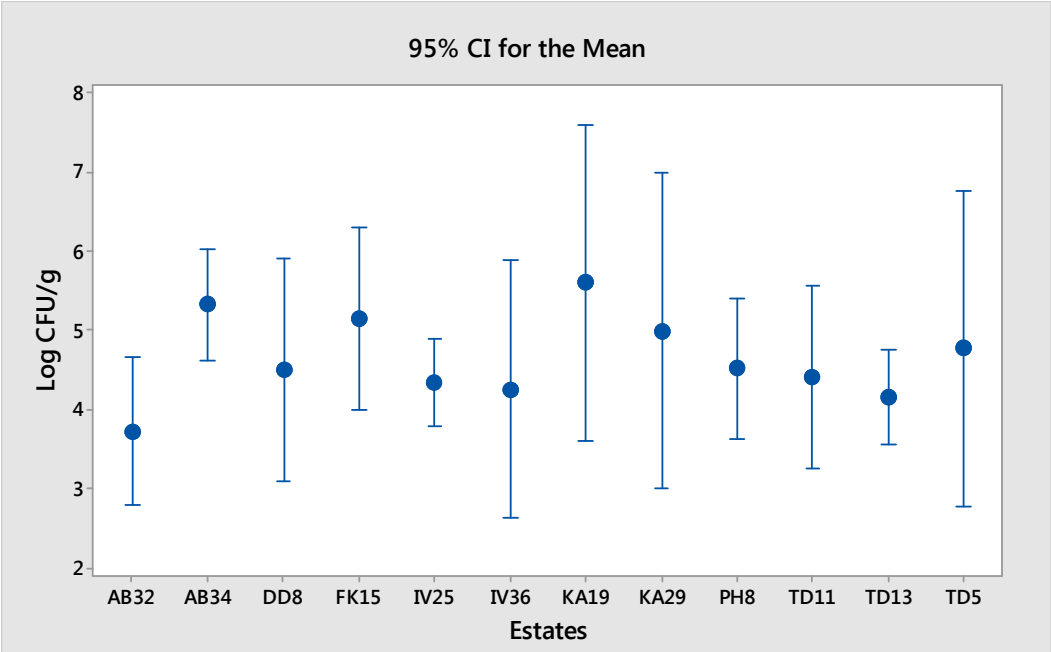


Figure 3.7: Means of *Campylobacter* load and 95% Confidence Interval (CI) of positive samples by estate expressed as log CFU/g.

Regional analysis

Region 1: Two estates were sampled from region 1, estates IV25 and IV36. Estate IV25 had a prevalence of infection of 68.4% (CI 51.3% - 82.4%) with an average *Campylobacter* load of 4.7 x 10^6 CFU/g. A prevalence of 16.6% (CI 3.5% - 41.4%) was observed in estate IV36 with an average *Campylobacter* load of 3.9 x 10^3 CFU/g. Combined, these results indicate that the overall prevalence for region 1 is 51.8% (C.I. 0% - 100%) but this is driven by the vast majority of positive samples recovered from estate IV25 so it may not be an entirely true representation of the level of infection across the region (P<0.001). In terms of *Campylobacter* carriage, even though samples from estate IV25 had a wider range of *Campylobacter* load compered to estate IV36 (Figure 3.6), overall there was no statistical difference (P=0.930) in the means of bacterial carriage between the two estates (Figure 3.7).

Region 2: Two estates were sampled from region 2, estates AB32 and AB34. The prevalence of positive samples from estate AB32 was 39.1% (CI 19.7% - 61.4%) with an average *Campylobacter* load of 1.7 x 10^4 CFU/g. The prevalence in estate AB34 was 48.4% (CI 30.7%

- 66.4%) with an average *Campylobacter* load of 4.9 x 10^6 CFU/g. The results for region 2 showed a consistent spread of *Campylobacter* infection in positive samples across the two estates (*P*=0.488) with an overall prevalence of 44.6% (CI 0% - 95.8%). However, there was a significant difference in the level of *Campylobacter* load between the two estates (*P*=0.004), with a lower mean *Campylobacter* count and load range in AB32 compared to AB34 (Figure 3.6).

Region 3: Region 3 comprised three estates, PH8, DD8 and FK15. Based on these three estates, the overall prevalence of infection was 36.3% (CI 1.8% - 70.8%). The average *Campylobacter* load of positive samples from estate DD8 was 5.7 x 10^5 CFU/g. In estate FK15 there was an average *Campylobacter* load of 2.2 x 10^6 CFU/g and in estate PH8 the average *Campylobacter* load was 4 x 10^7 CFU/g. The results for Region 3 showed a difference in prevalence across the three estates that varied from 28.6% (CI 14.6% - 46.3%) in estate PH8 to 40% (CI 12.1% - 73.7%) in estate DD8 and 60% (CI 26.2% - 87.8%) in estate FK15 although this difference was not statistically significant (*P*=0.192). There was a wide range in *Campylobacter* loads in positive samples from all three estates but there was no statistically significant difference (*P*=0.677) in the *Campylobacter* load means (Figure 3.7).

Region 4: Three estates were sampled from this region, TD11, TD13 and TD5. The overall prevalence of infection was 50% (CI 0% - 100%) with an average *Campylobacter* load of 3.5 x 10^5 CFU/g. The prevalence in estate TD11 was 50% (CI 21% - 78.9%), 73.3% in estate TD13 (CI 54.1% - 87.7%) and 11.1% (CI 1.3% - 34.7%) in estate TD5. The average *Campylobacter* load in the positive samples from estate TD11 was 7.6 x 10^4 CFU/g; in TD13 it was 4.5 x 10^5 CFU/g; and in estate TD5 it was 7.4 x 10^4 CFU/g. There was a statistically significant difference in the *Campylobacter* load (*P*<0.001) but only for TD11 and TD13 when compared to TD5. The difference in *Campylobacter* infection between TD13 and TD11 was not significant (Table 3.6).

Estates		**Odds Ratio**	**95% CI**
TD13	TD11	2.750	(0.684, 11.053)
TD5	**TD11**	**0.125**	**(0.019, 0.799)**
TD5	**TD13**	**0.045**	**(0.008, 0.243)**

Table 3.6: Binary logistic regression analysis to estimate any statistically significant differences in the *Campylobacter* load between estates in region 4. Statistically significant differences are highlighted in bold.

There was no statistically significant difference (*P*=0.727) observed in the means of *Campylobacter* load between estates in region 4 (Figure 3.7).

Region 5: This region comprised three estates, KA19, KA29 and G66. The overall prevalence of infection in region 5 was 6.6% (CI 0% to 22.5%). All samples from estate G66 contained < 10 *Campylobacter* CFU/g based on 1:10 dilution (Upper 95% CI <12.7%) so were considered negative; estate KA19 had a prevalence of 9% (CI 1.1% - 29/1%) and estate KA29 had a prevalence of 12.5% (CI 1.6% - 38.3%). The average *Campylobacter* load in the positive samples from estate KA19 was 5 x 10^5 CFU/g while in KA29 it was 1.6 x 10^5 CFU/g. As mentioned above, region 5 had a consistently low prevalence of infection, at least based on the three estates surveyed, and this was statistically different to the other regions. A binary logistic regression to compare the prevalence of infection among estates could not be undertaken because of the lack of infection in estate G66. The bacterial load means were not statistically significant (*P*=0.376) between the two estates (Figure 3.7). However, the small number of positive samples (n=4) from this region limits statistical analysis.

Relationship between bacterial carriage and kill–to-process time

The period of time between shooting batches of pheasants on the estates and laboratory processing of caecal samples varied between 2 and 7 days. Statistical analysis of kill-to-process time did not indicate any significant difference in terms of *Campylobacter* carriage (*P*=0.338) (Figure 3.8 and 3.9) even when sampling site 5 was excluded from the analysis (*P*=0.322).

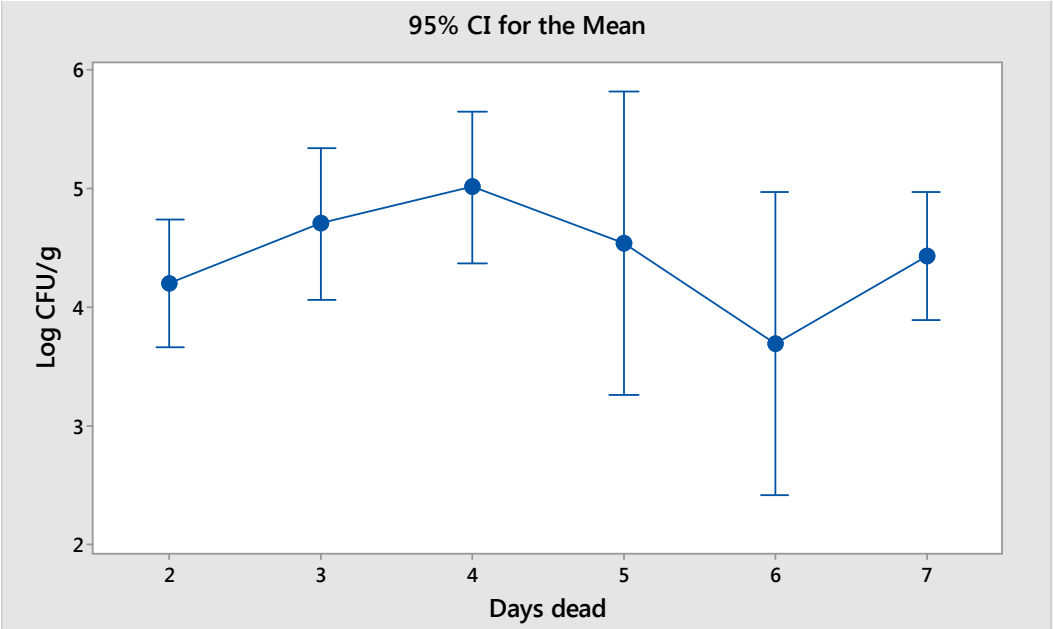


Figure 3.8: *Campylobacter* load means (Log_{10} CFU/g) and 95% Confidence Interval (CI) of positive caecal samples in relation to the kill-to-process time.

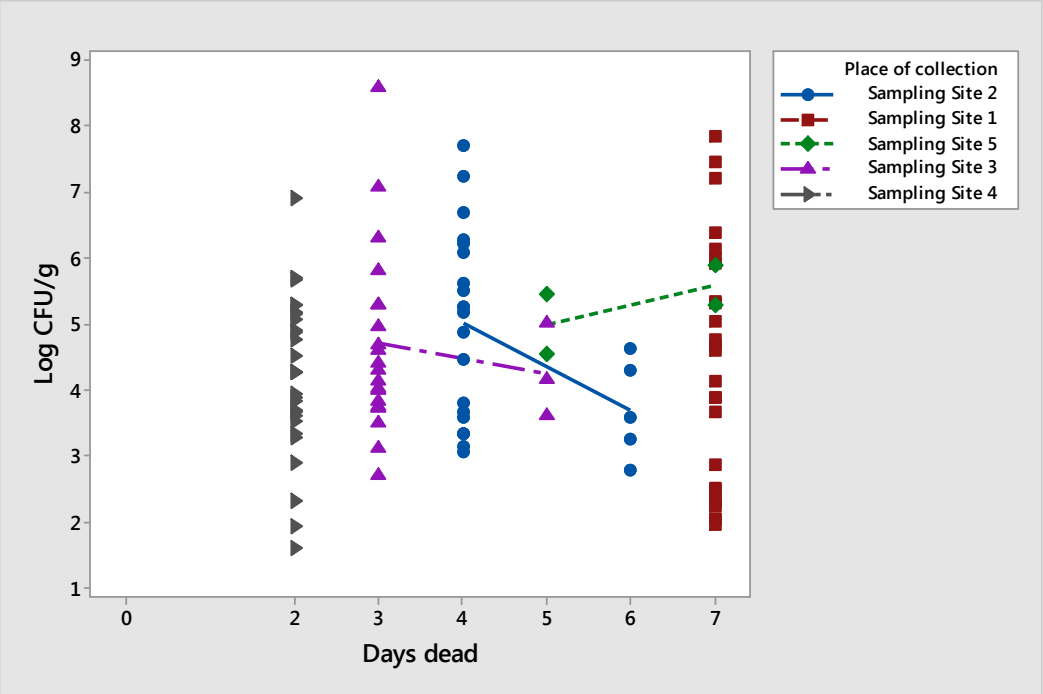


Figure 3.9: Variation in *Campylobacter* load count (Log_{10} CFU/g) in positive caecal samples in relation to the sampling site and the kill-to-process time.

3.4 Discussion

This is the first UK based study estimating not only the prevalence of *Campylobacter* infection but also the bacterial carriage in caecal content from pheasants based on a confidence level of 95% with a desired absolute precision of 5%. It contributes to the epidemiological knowledge base and surveillance data relating to *Campylobacter* infection in wild game birds in Scotland. The survey indicated an overall prevalence of infection in pheasants of 37.6% (based on 287 birds) (CI 13.9% - 61.2%). This is in line with previously reported prevalence levels which were based on analyses of caecal content in hunted wild pheasants elsewhere in Europe, levels which ranged from 25 to 27% (Nebola *et al.*, 2007; Atanassova and Ring, 1999; Stern *et al.*, 2004). The main study was preceded and informed by two small scale pilot studies that allowed some refinement of established *Campylobacter* culture methods.

Prevalence was not uniform across all the regions but this was likely influenced by two factors, one relating to time of sampling, the other to the fact that prevalence was particularly low in region 5. Excluding the results from region 5, the overall prevalence of infection of 45.8% (CI 34.9% - 56.6%) was higher than that reported by previous studies. It was still lower compared to farmed pheasants slaughtered on farm, a pattern previously reported by Nebola

(2007), where the prevalence was as high as 70% (Nebola *et al.*, 2007). It is also lower than previous studies based on cloacal swabs from live birds where the prevalence reached almost 90% (Dipineto *et al.*, 2008b). The discrepancy in bacterial carriage between farmed and hunted pheasants has been attributed to the fact that samples from farmed pheasants are generally processed more quickly (Nebola *et al.*, 2007). In previous studies, while a kill-to-process time was not always specified, wild pheasant samples were not taken immediately after shooting, potentially compromising *Campylobacter* survival in the caeca (Nebola *et al.*, 2007). In the study reported herein, the time from shooting in the field to processing in the laboratory varied from two to seven days but there was no statistically significant relationship between the bacterial load in positive caecal samples and the kill-to-process time (P=0.338). The main factors that influence microbiological growth in uneviscerated pheasants are storage temperature, days stored and location of shot wounds, especially if perforation of the intestine is present and these can possibly explain the difference in results found in this study from the literature (Paulsen *et al.*, 2008). More specific studies need to be carried out to study the survival of *Campylobacter* spp. in caecal samples in relation to the time of kill-to-process.

The low prevalence in samples collected from sampling site 5 could be at least partially explained by a genuinely low prevalence of infection in the estates sampled but it is also possible that the time of the year had an influence on *Campylobacter* carriage. Seasonal fluctuation of *Campylobacter* carriage in food producing animals has been observed in multiple previous studies (Wallace *et al.*, 1997; Stanley *et al.*, 1998a; Stanley *et al.*, 1998b). In this study there was a statistical difference (P<0.001) in terms of *Campylobacter* infection across sampling sites whereby samples collected at the end of January 2014 from sampling site 5 had a significantly lower prevalence compared to samples collected from the beginning of October to the end of November 2013 (sampling sites 1, 2, 3 and 4). This apparent seasonal influence was mirrored by the pilot study where the samples collected in February 2013 (n=6) had a prevalence of 16.6% (CI 0.4% - 64.1%) while those collected in September (n=5) had a prevalence of 60% (CI 14.7% - 94.7%). More studies would be required to verify if a seasonal pattern of *Campylobacter* carriage is actually present in pheasants, as reported in other farm animal species.

The statistically significant difference in prevalence of infection between sampling sites 1 and 3 may be due to the fact that samples from estate IV36 were collected from site 3 (region 3) and not site 1 (region 1). The same could be postulated for the significant differences between sampling sites 1 and 2 and between sampling sites 3 and 4. Therefore, these latter results may not be a true reflection of the prevalence differences at the regional level. There was also a

statistically significant difference in the *Campylobacter* load ($P<0.001$) of estates TD11 and TD13 compared to TD5. This may be explained by the fact that samples from estates TD11 and TD13 were collected in November 2013 while samples from estate TD5 were collected in January 2014 when the overall prevalence in these samples was significantly lower than the others.

The average bacterial load of positive samples (n=108) was 5.8×10^6 CFU/g (6.7 Log_{10}/g) and this was broadly in line with the bacterial load of extensively reared British-based poultry flocks surveyed in 2011 (Allen *et al.,* 2011). This study herein also found no significant difference in bacterial carriage means across and between Scottish regions and estates ($P=0.441$), even in region 5 where the prevalence of infection was very low. Based on the enumeration of *Campylobacter* spp. in positive samples, most pheasants had high *Campylobacter* counts across all five regions, so that only 5.2% of birds harboured $<10^3$ CFU/g (Figure 3.5).

Super-shedders can be defined as those animals that for a period of time shed high counts of infective organisms of a particular type than most other individuals of the same host species (Chase-Topping *et al.,* 2008). It also involves persistent colonisation of a specific section of the gastrointestinal tract where proliferation of that particular organism is enhanced. It has been calculated that the cut-offs for super-shedding are bacterial counts of $>10^4$ CFU/g in faeces (Chase-Topping *et al.,* 2008). When these counts are present above the specified cut-offs, even in a small number of animals in the population they will increase the risk of disease spread to other in-contact animals (Chase-Topping *et al.,* 2008). A typical example of bacteria that have been identified with super-shedders features is *E. coli* O157:H7 in cattle, but super-shedding has been reported in other bacterial infections like *Mycobacterium avium* subsp. *Paratuberculosis* (Mitchell *et al.,* 2008) and *Salmonella enterica* subsp. enterica serovar Typhimurium (Lawley *et al.,* 2008).

In this study, 23% of pheasants had a *Campylobacter* count $>10^4$ CFU/g (CI 18.3% - 28.3%) and 5.6% (CI 3.2% - 8.9%) were $>10^6$ CFU/g. Although these results relate to caecal and not faecal content they may still support super-shedding of *Campylobacter* in the environment which would potentially increase the risk of infection to other pheasants and to humans. The consumer may be exposed through an increased risk of meat contamination during evisceration at AGHEs while employees of pheasant farms may also be at increased risk through exposure to pheasant faeces. In a study from the USA, an outbreak of campylobacteriosis in workers on a pheasant farm was associated with occupational exposure to pheasants (Heryford and Seys, 2004). Although *Campylobacter* counts from faecal swabs from live pheasants have not been

reported in the literature, as mentioned above, the prevalence of *Campylobacter* spp. in live farmed pheasants can be as high as 86.7% (Dipineto *et al.*, 2008b). More studies need to be carried out to determine if *Campylobacter* spp. may have super-shedding features as previously reported in *E. coli* and *Salmonella* spp.

In both pilot studies none of the 19 skin samples tested were positive for *Campylobacter* spp. In the first pilot only one out of six caecal samples were positive, so the negative results were mainly attributed to the laboratory technique used because from the literature review it was expected to have a higher prevalence of infection in pheasants. Some refinements of the laboratory technique led to more rewarding results, so these were applied for the isolation of *Campylobacter* spp. in the main study. Despite this, all five skin samples in pilot two and all forty from the main study were negative. In combination, the pilot study and main survey analysed 59 neck skin samples from which no *Campylobacter* spp. were isolated. There is a number of potential reasons for these negative findings. In particular, they could be due to the storage of carcasses in cold, dry environment (e.g. chillers in larders and AGHEs) and under normal atmospheric conditions, both of which are detrimental to *Campylobacter* survival (Humphrey *et al.*, 1995). It was considered unlikely that all 59 samples were truly negative, although this is a theoretical explanation. A third possibility is that the culture method was unreliable. The recovery of only a single isolate from the six caecal samples analysed in the February 2013 pilot study may also support this as an explanation, at least for the skin samples analysed in the first pilot study. However, since the refinements in culture technique outlined in the materials and methods ultimately led to more rewarding yields in the September trial, the culture method was considered to be sufficiently dependable. Ultimately, the negative findings from the neck skin samples were not considered unusual as this is consistent with the literature where negative results were confirmed by more sensitive molecular tests (Soncini *et al.*, 2006). Therefore, the decision was made to focus solely on detection, isolation and sequencing from caecal samples.

The molecular diversity of *Campylobacter* strains recovered from positive caecal samples was investigated in Chapter 4.

Chapter 4 Molecular diversity of *Campylobacter* strains

4.1 Molecular epidemiology of *Campylobacter*

Two genotyping methods, Polymerase Chain Reaction (PCR) and High Throughput Multi Locus Sequence Typing (HiMLST), were used in this project to define the molecular diversity of *Campylobacter* isolates obtained from caecal samples of wild pheasants. In particular, PCR was used to determine the *Campylobacter* genus and species (*C. jejuni* or *C. coli*) while HiMLST was used to identify the strain of the isolates. *Campylobacter lari* has also been identified in wild game birds and has known zoonotic potential (Waldenstrom *et al.*, 2006) but, because of its much lesser importance in terms of human infection compared to *C. jejuni* and *C. coli*, it was not included in this study (DEFRA, 2013).

Polymerase Chain Reaction is commonly used for genus and species determination because this method is able to detect specific fragments of DNA that originate from highly conserved regions of the genome, such as that encoding 16S rRNA, that are characteristic of the genus *Campylobacter* (Bang *et al.*, 2002). Following the same principle, more specific loci are used for the detection of different species. A general outline of the PCR process is as follows. After DNA denaturation of the test genomic material, specific primers are added to the reaction that will attach to the single stranded fragment of DNA to replicate (annealing). DNA polymerase enzymes will then attach nucleotides to the primers following the DNA template to create a copy of the original DNA (extension). By repeating this cycle several times it is possible to amplify the genetic material so that it can be visualised by electrophoresis in an agar gel (Stoflet *et al.*, 1988). Genus and species determination is achieved by comparing the size of the PCR products with a DNA ladder (a molecular weight marker), which contains DNA fragments of a known positive control that are run on the gel alongside the test sample.

Multi Locus Sequence Typing (MLST) is the most common molecular method for subtyping *Campylobacter* spp. and is applicable to all strains of *C. jejuni* and *C. coli* (EFSA, 2013). *Campylobacter* spp. are characterised by a high evolutionary diversity with a non-clonal population structure that allows extensive horizontal genetic exchange. However, this technique, following PCR amplification, is able to compare DNA sequence differences in seven *Campylobacter* house-keeping genes that are an essential and well conserved part of the genome: aspA (aspartase A), glnA (glutamine synthase), gltA (citrate synthase), glyA (serine hydroxymethyltransferase), pgm (phosphoglucomutase), tkt (transketolase) and uncA (ATP synthase alpha subunit) (Dingle *et al.*, 2001, 2005). Multi Locus Sequence Typing detects the

allele profile of each isolate across the set of genes and identifies it as a sequence type (ST). As explained by Forbes (2009): "Isolates matching across the whole set of genes are categorised as being the same ST; isolates mismatching for one gene within the set are defined as single-locus variants 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 expected to be more recent for isolates in the same ST than for isolates in the same CC". Clonal complexes are the units of analysis used to determine characteristics of interest such as host association, survival in the environment and in the food chain (EFSA, 2013).

4.2 Materials and methods

One *Campylobacter* pure colony per positive caecal sample was selected for plating on mCCDA and incubating for 48h in microaerophilic conditions. Nine (8.3%) positive samples out of 108 isolates failed to recover when defrosted and plated on mCCDA plates. The 99 successfully retrieved colonies were packed at room temperature and sent by next day delivery to the Regional Laboratory for Public Health Kennemerland, Haarlem, the Netherlands, for PCR and HiMLST sequencing. DNA extraction was carried out in the Dutch laboratory according to their internal specification (Boers *et al.*, 2012) and primers obtained from the PubMLST website http://pubmlst.org/campylobacter/info/primers.shtml were used to perform the PCR and HiMLST (Table 4.1). After species identification by PCR, MLST was performed to determine the STs of the isolates using the seven house-keeping genes based on the method outlined by Dingle *et al.*, 2001 and modified by Miller *et al*., 2005.

Statistical analysis

The statistical analysis of results to determine the distribution of *C. coli* and *C. jejuni* across regions and estates was performed using the same approach as described in Chapter 3 section 3.2. *Campylobacter* STs recovered from pheasant caecal samples were assigned to CCs by comparing the lists of STs and assigned CCs found in human, poultry, farmed animals, wild life and pet isolates downloaded from the PubMLST database http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_campylobacter_isolates&page=query (last access on February 2015).

Primer Name	Primer Sequence	Species
aspA S3 SP6 tail	GACACTATAGCCAACTGCAAGATGCTGTACC	*C. jejuni*
aspA S6 T7 tail	CACTATAGGGTTCATTTGCGGTAATACCATC	*C. jejuni*
glnA S3 SP6 tail	GACACTATAGCATGCAATCAATGAAGAAAC	*C. jejuni*
glnA S6 T7 tail	CACTATAGGGTTCCATAAGCTCATATGAAC	*C. jejuni*
gltA S3 SP6 tail	GACACTATAGCTTATATTGATGGAGAAAATGG	*C. jejuni*
gltA S6 T7 tail	CACTATAGGGCCAAAGCGCACCAATACCTG	*C. jejuni*
glyA S3 SP6 tail	GACACTATAGAGCTAATCAAGGTGTTTATGCGG	*C. jejuni*
glyA S4 T7 tail	CACTATAGGGTGATTATCCGTTCCATCGC	*C. jejuni*
pgm S5 SP6 tail	GACACTATAGGTTTTAGATGTGGCTCATG	*C. jejuni*
pgm S2 T7 tail	CACTATAGGGTCCAGAATAGCGAAATAAGG	*C. jejuni*
tkt S5 SP6 tail	GACACTATAGCTTAGCAGATATTTTAAGTG	*C. jejuni*
tkt S6 T7 tail	CACTATAGGGAAGCCTGCTTGTTCTTTGGC	*C. jejuni*
uncA S3 SP6 tail	GACACTATAGAAAGTACAGTGGCACAAGTGG	*C. jejuni*
uncA S4 T7 tail	CACTATAGGGTGCCTCATCTAAATCACTAGC	*C. jejuni*
Aspcoli S1 SP6 tail	GACACTATAGCAACTTCAAGATGCAGTACC	*C. coli*
Aspcoli S2 T7 tail	CACTATAGGGATCTGCTAAAGTATGCATTGC	*C. coli*
Glncoli S1 SP6 tail	GACACTATAGTTCATGGATGGCAACCTATTG	*C. coli*
Glncoli S2 T7 tail	CACTATAGGGCTTTGGCATAAAAGTTGCAG	*C. coli*
Gltcoli S1 SP6 tail	GACACTATAGATGTAGTGCATCTTTTACTC	*C. coli*
Gltcoli S2 T7 tail	CACTATAGGGAAGCGCTCCAATACCTGCTG	*C. coli*
Glycoli S1 SP6 tail	GACACTATAGTCAAGGCGTTTATGCTGCAC	*C. coli*
Glycoli S2 T7 tail	CACTATAGGGCCATCACTTACAAGCTTATAC	*C. coli*
Pgmcoli S1 SP6 tail	GACACTATAGTTATAAGGTAGCTCCGACTG	*C. coli*
Pgmcoli S2 T7 tail	CACTATAGGGTTCCGAATAGCGAAATAACAC	*C. coli*
Tktcoli S1 SP6 tail	GACACTATAGAGGCTTGTGTTTTCAGGCGG	*C. coli*
Tktcoli S2 T7 tail	CACTATAGGGTGACTTCCTTCAAGCTCTCC	*C. coli*
Unccoli S1 SP6 tail	GACACTATAGAAGCACAGTGGCTCAAGTTG	*C. coli*
Unccoli S2 T7 tail	CACTATAGGGCTACTTGCCTCATCCAATCAC	*C. coli*

Table 4.1: Table of primers used by the laboratory in the Netherlands for PCR and sequencing of *Campylobacter* isolates.

4.2.1 Polymerase chain reaction

The same 99 isolates that were sent for sequencing at the laboratory in the Netherlands were subjected to diagnostic multiplex PCR at the Roslin Institute laboratory by the author of this project for internal validation of the *Campylobacter* isolates. Pure colonies were plated on mCCDA and incubated in microaerophilic conditions for two days. PCRs were performed using the Phuson DNA Polymerase (Thermo Scientific) in a reaction volume of 25µl, according to the manufacturer's instructions. 1µl of template DNA solution in water was added to each reaction. This was obtained by heating a loop of bacteria collected from each mCCDA

plate. Primers used for species identification of *C. coli* and *C. jejuni* were based on detection of the lpxA gene (Klena *et al*, 2004). The forward primer of the lpxA gene for *C. jejuni* was (ACAACTTGGTGACGATGTTGTA). For the detection of the lpxA gene for *C. coli* the forward primer used was (GATAGTAGACAAATAAGAGAGAATMAG) (FSA, 2009). Reverse primers, lpxA-R1 (CAATCATGTGCGATATGACAATAYGCCAT) and lpxA-R2 (CAATCATGAGCAATATGACAATAAGCCAT) were used to detect *C. coli* and *C. jejuni* and were used in a 50:50 mixture (FSA, 2009). The cycling conditions were as follows:

- Initial denaturation for 45s at 94ºC
- Thirty cycles of denaturation for 45s at 94ºC, annealing for 30s at 55ºC and extension for 90s at 72ºC
- Final extension for 10 minutes at 72ºC
- Holding of the reaction at 4ºC until use.

Agarose gel electrophoresis was undertaken in 1% agarose gels, with 1:10,000 dilution of SybrSafe (Invitrogen, UK) and run at 80-100V for 45-90 minutes. Amplicon sizes were estimated by comparison with Thermo Scientific 1KD DNA ladder markers (Figure 4.1).

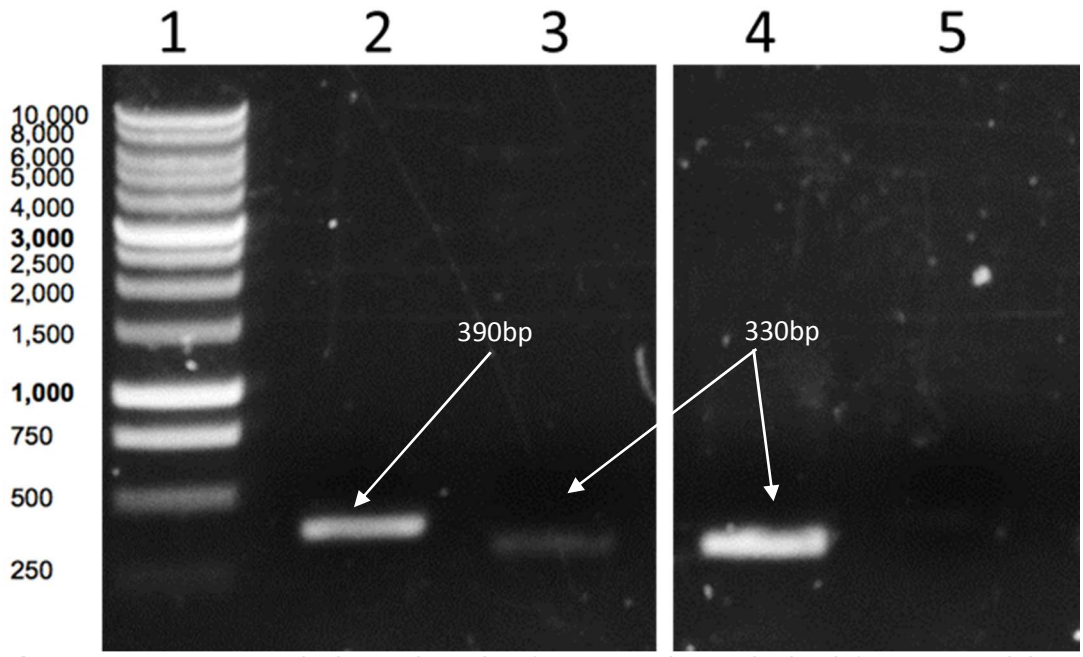


Figure 4.1: Agarose gel electrophoresis of PCR products obtained from *Campylobacter* isolates. Lane 1 contains the 1KD DNA ladder, lane 2 shows *C. coli* (390bp) and lanes 3 and 4 contain *C. jejuni* (330bp). Lane 5 contains the negative control.

4.3 Results and Data Analysis

4.3.1 PCR results

PCR yielded positive results for all 99 isolates confirming the detection of positive samples as *Campylobacter* spp. in the bacteriology laboratory and also that they belonged to either *C. jejuni* or *C. coli* species. Overall *C. coli* and *C. jejuni* accounted for 62.7% (CI 14.6% - 100%) and 37.3% (CI 0% - 85.3%) of positive samples tested, respectively. Region 5 yielded only three isolates for PCR detection, two of which were positive for *C. coli* and one of which was positive for *C. jejuni*. Table 4.2 summarises the PCR results by region, estate and *Campylobacter* spp. identified.

	Region 1	**Region 2**	**Region 3**	**Region 4**	**Region 5**	**Totals**
Samples collected	56	56	55	60	60	287
Positive samples	29	25	20	30	4	108
Number of isolates for PCR	‡**22 out of 29**	**25 out of 25**	**20 out of 20**	**29 out of 30**	**3 out of 4**	**99**
C. coli	Ω **17** IV25 ‡(15) IV36 (2) ***77.3%** †**CI 54.6% - 92.2%**	**3** AB32 (2) AB34 (1) **12%** **CI 25.5% - 31.2%**	**14** DD8 (2) FK15 (5) PH8 (7) **70%** **CI 45.7% - 88.1%**	**26** TD11 (6) TD13 (18) TD5 (2) **89.7%** **CI 72.6% - 97.8%**	**2** KA19 (1) KA29 (1) **66.6%** **CI 9.4% - 99.2%**	**62** **62.6%** **CI 14.6% - 100%**
C. jejuni	**5** IV25 (4) IV36 (1) **22.7%**	**22** AB32 (7) AB34 (15) **88%**	**6** DD8 (2) FK15 (1) PH3 (3) **30%**	**3** TD11 (0) TD13 (3) TD5 (0) **10.3%**	**1** KA19 (0) KA29 (1) **33.4%**	**37** **37.3%**

Table 4.2: Summary of PCR results showing number of isolates‡ (n) by region and estate and number of isolates Ω identified as *C. coli* or *C. jejuni*. Overall distribution* (%) and 95% Confidence Intervals† (CI) calculated using binary logistic regression. Distribution and CI by region was estimated using a standard CI calculation.

Figure 4.2 below illustrates the prevalence of positive *Campylobacter* spp. isolates by estate, as identified using PCR.

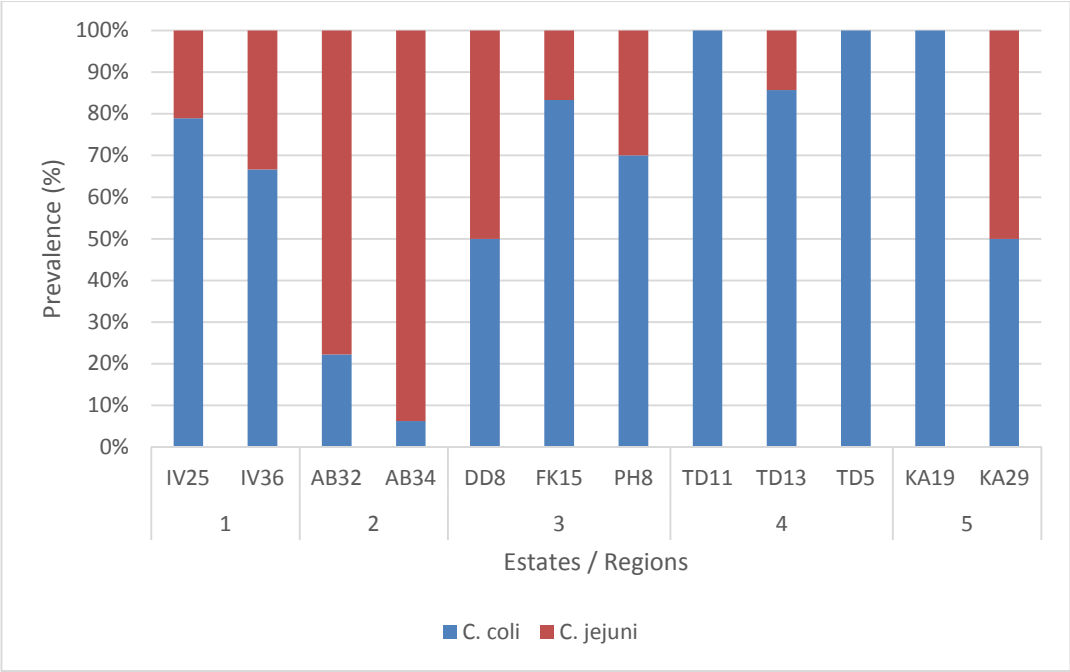


Figure 4.2: Prevalence (%) of *C. coli* and *C. jejuni* species by estate, as identified using PCR.

The percentage of *C. coli* was higher than that of *C. jejuni* across all regions, with the exception of region 2. A higher percentage of *C. coli* was also found in all estates sampled other than those in region 2, with the exception of estates DD8 and KA29, where the distribution was 50% (CI 11.8% - 88.2%) for each species. Conversely, in region 2 *C. jejuni* was the more common species across the sampled estates. Binary logistic regression analysis across regions confirmed a statistically significant difference in the distribution of *C. coli* and *C. jejuni* (P<0.001) between region 2 and regions 1, 3 and 4 (Table 4.3). These results were confirmed by removing region 5 that, due to the low number of samples tested, can be considered a potential confounder.

Region Chi-Squared 41.39 P<0.001		**Odds Ratio**	**95% CI**
2	**1**	**0.040**	**(0.008, 0.191)**
3	1	0.686	(0.172, 2.732)
4	1	2.549	(0.537, 12.087)
5	1	0.588	(0.043, 7.914)
3	**2**	**17.111**	**(3.670, 79.767)**
4	**2**	**63.555**	**(11.633, 347.217)**
5	2	14.666	(0.999, 215.309)
4	3	3.714	(0.803, 17.164)
5	3	0.857	(0.064, 11.356)
5	4	0.230	(0.015, 3.370)

Table 4.3: Binary logistic regression analysis to estimate statistically significant differences in the level of infection among regions. Statistically significant differences are highlighted in bold.

An analysis of the results based on the sampling sites also indicated statistically significant differences ($P<0.001$) in the *Campylobacter* spp. recovered. The differences mirrored those observed across the regions in that the presence of *C. jejuni* recovered from sampling site 2 was consistently higher than that from the other sampling sites. Statistical analysis of the distribution of *Campylobacter* spp. recovered across estates within the same region confirmed the prominence of one of the two species in each region (Region 1 $P=0.650$; Region 2 $P=0.249$; Region 3 $P=0.531$; Region 4 and 5 P value not available because of the lack of *C. jejuni* infection in estates TD11, TD5 and KA19.

4.3.2 MLST results

Sequence types analysis

Of the 99 isolates sent for DNA sequencing, a ST was only assigned in 80 because, for 19 (19.2%) isolates, one or more alleles failed to amplify when subjected to the MLST test. Nineteen STs were detected by MLST across the data set (n=80). Eleven (57.9%; CI 33.5% - 79.7%) were consistent with *C. jejuni* and eight (42.1%; CI 20.3% - 66.5%) were consistent with *C. coli* species. Sequence Type 828 (n=19; 23.75%; CI 14.9% - 34.6%) was the most common in the 80 samples tested, followed by ST827 (n=12; 15%; CI 8% - 24.7%) and ST19 (n=7; 8.75%; CI 3.6% - 17.2%) which collectively represented 47.5% (CI 36.2% - 59%) of the entire data set. Five STs (6.25%; CI 0.3% - 6.8%) appeared only once and six STs (15%; CI 0.3% - 8.7%) appeared twice (Figure 4.3).

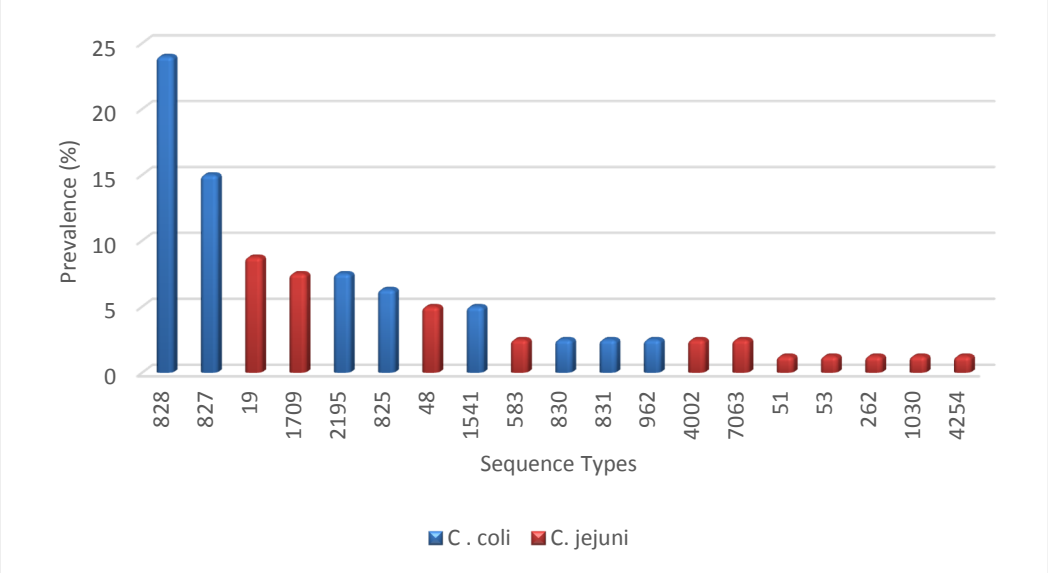


Figure 4.3: Prevalence (%) of the 19 Sequence Types found across the data set (n=80), illustrating whether they belonged to *C. jejuni* or *C. coli* species.

The distribution of STs by region and by estate is shown in Figures 4.4 and 4.5, respectively.

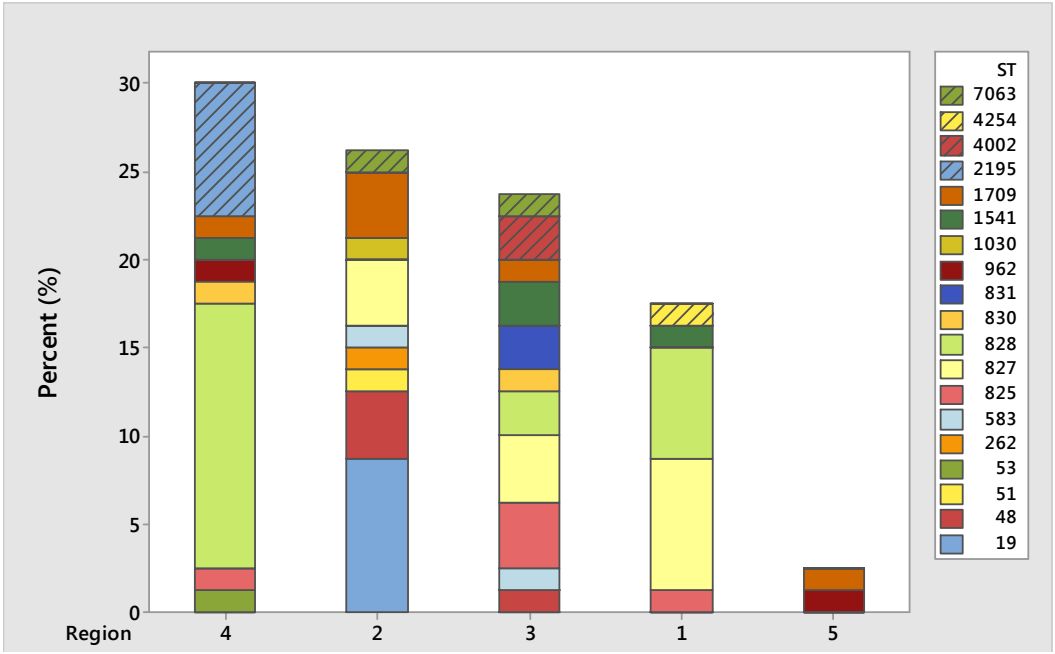


Figure 4.4: Distribution (%) of Sequence Types (ST) by region.

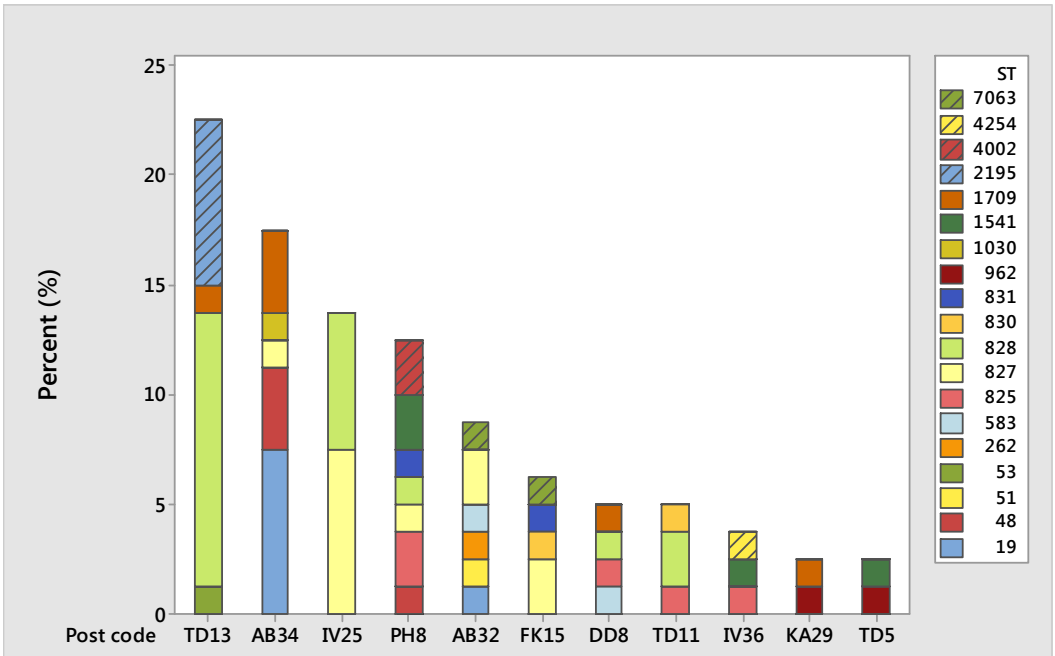


Figure 4.5: Distribution (%) of Sequence Types (ST) by estate.

Three out of five STs that appeared only once were recovered from region 2; two from estate AB32; and one from AB34. All five were *C. jejuni*.

Clonal Complexes analysis

Fourteen of the 19 STs (73.6%; CI 48.8% - 90.9%) found were grouped into six Clonal Complexes (CCs) while the remaining five STs (26.3%; CI 9.1% - 51.2%) were unassigned to any CCs (orphan STs) (STs 1030, 2195, 4002, 4254 and 7063). Furthermore, orphan STs 4002, 4254 and 7063 were not present in human, poultry, farmed animals, wild life or pet isolates uploaded on the PubMLST database. Clonal complex ST-828 alone represented 57.5% (n=46; CI 45.9% - 68.5%) of the 80 assigned isolates and together with the other two most frequent CCs, ST-21 (n=9; 11.2%; CI 5.3% - 20.3%) and ST-1034 (n=6; 7.5%; CI 2.8% - 15.6%), represented 76.3% (CI 65.4% - 85.1%) of the data set. Sequnce Types that were not assigned to any CC represented 15% (n=12; CI 8% - 24.7%) of the isolates (Figure 4.6).

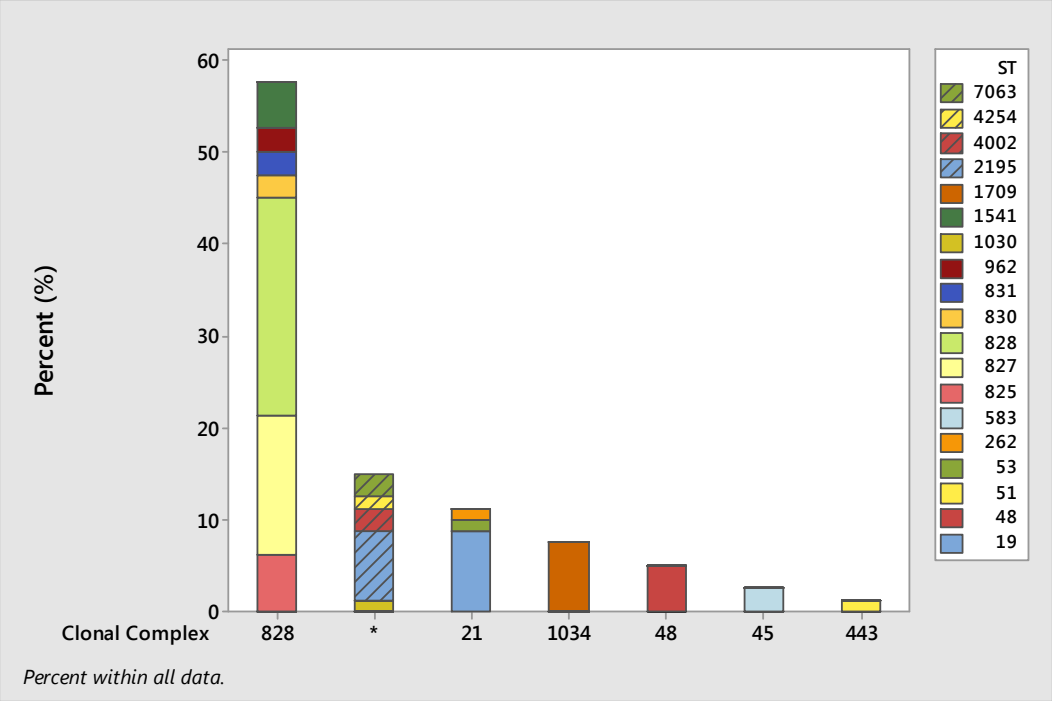


Figure 4.6: Distribution (%) of Clonal Complexes in 80 *Campylobacter* isolates from pheasant caecal samples with a summary of Sequence Types (ST) (*= ST not assigned to a CC).

Figure 4.7 shows the distribution of CCs by region and estate.

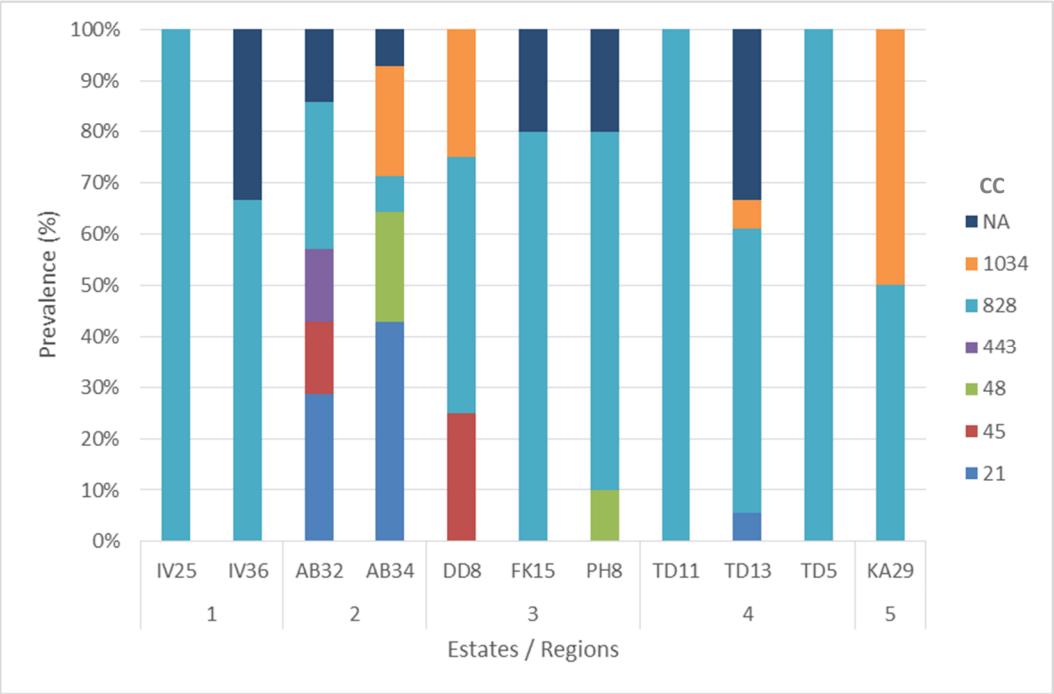


Figure 4.7: Distribution (%) of Clonal Complexes (CC) by region and estate (NA= Not assigned to a CC).

Regional analysis

There was a statistically significant difference (P<0.001) in the CCs represented in region 2 compared to those in regions 1, 3 and 4 (Table 4.4). Similar results were obtained by removing region 5 that, due to the low number of isolates (n=3) was considered a potential confounder. Binary logistic regression performed on regions 1, 3 and 4 confirmed statistically that CC ST-828 was the most represented (P=0.120) in these regions.

Region Chi-Squared 27.64 P<0.001		**Odds Ratio**	**95% CI**
2	**1**	**0.012**	**(0.001, 0.137)**
3	1	0.166	(0.017, 1.584)
4	1	0.153	(0.017, 1.393)
5	1	0.076	(0.002, 2.394)
3	**2**	**13.000**	**(2.735, 61.786)**
4	**2**	**12.000**	**(2.709, 53.139)**
5	2	6.000	(0.290, 124.099)
4	3	0.923	(0.254, 3.342)
5	3	0.461	(0.024, 8.693)
5	4	0.500	(0.027, 9.076)

Table 4.4: Binary logistic regression analysis to estimate any statistical difference in CCs represented among regions assuming that CC ST-828 was the most frequently represented. Statistically significant differences are highlighted in bold.

Region 1: 92.9% (n=13; CI 66.1% - 99.8%) of isolates (n=14) belonged to ST-828. Sequence Type 4254, recovered from estate IV36 and not assigned to a CC, belonged to the species *C. jejuni*.

Region 2: Isolates were very varied, consisting of nine STs in six different CCs and two orphan STs (1030 in AB34 and 7063 in AB32) both belonging to the species *C. jejuni*. Clonal Complex ST-21 (n=8; 38.1%; CI 18.1% - 61.6%) was the most frequently represented followed by CC ST-48 (n=3; 14.3%; CI 3% - 36.3%) and ST-1034 (n=3). Clonal complex ST-828 (n=3) accounted for all the *C. coli* species isolated in this region.

Region 3: CCs ST-45 (n=1), 1034 (n=1) and 48 (n=1) represented 15.7% (CI 3.8% - 39.6%) of the isolates. Two orphan STs, ST4002 (n=2) and ST7063 (n=1), had an overall prevalence of 15.7%. Both isolates belonging to ST4002 were found in estate PH8, while ST7063 was recovered from estate FK15. 68.4% (n=13; CI 43.4% - 87.4%) of isolates in this region belonged to CC ST-828. This was found in all three estates sampled, with 80% (n=4; CI 28.4% - 99.5%) presence in estate FK15, 70% (n=7; CI 34.8% - 93.3%) in estate PH8 and 50% (n=2; CI 6.8% - 93.2%) in estate DD8.

Region 4: Region 4 also had a high percentage of CC ST-828 (n=16, 66.7%; CI 44.7% - 84.4%) with 100% presence in estates TD11 (n=4; 95% Lower CI >47.3%) and TD5 (n=1; 95% Lower CI >0.5%). The orphan ST2195 accounted for 33.3% (n=6; CI 13.3% - 59%) of isolates from estate TD13 and this ST contributed 25% (CI 9.8% - 46.7%) of the total isolates from this region. Clonal complex ST-21, found in estate TD13, accounted for 4.2% (n=1; CI 0.1% - 21.1%) of isolates recovered in this region.

Region 5: Region 5 yielded only two isolates, one belonging to CC ST-828 and the other to CC ST-1034, both originating from estate KA29.

4.4 Discussion

Polymerase Chain Reaction results confirmed a higher presence of *C. coli* (62.7%) compared to *C. jejuni* (37.3%) in caecal content of pheasants in Scotland. In their Italian survey, Dipineto (2008a) reported that 100% (n=104) of cloacal swab isolates subjected to PCR were identified as *C. coli*, with 13.5% also positive for *C. jejuni*. In contrast, Nebola (2007) reported that *C. jejuni* was more prevalent (n=54: 58%) than *C. coli* (36%) in wild pheasants in the Czech Republic, with mixed infection in 5% of birds examined. However, the same study also

reported that in farmed pheasants (n=211) 51% of isolated strains were *C. coli* and 41% were *C. jejuni*. This discrepancy in results may reflect the varying sources of infection to which pheasants on different estates or farms are exposed, since cattle, sheep and chickens are major reservoirs and shedders of *Campylobacter* spp. and they are associated with different *Campylobacter* spp. (Sheppard *et al.*, 2010). The next chapter will expand on host association of *Campylobacter* spp. found in pheasants in Scotland.

Campylobacter coli was more widespread than *C. jejuni* in all Scottish regions surveyed, with the exception of region 2. Similarly, *C. coli* was the predominant species across all estates and sampling sites when region 2 was excluded from the analysis. Forbes (2009) sampled seven pheasants from the North East of Scotland (region 2) and found that 57% were positive for *C. jejuni* (FSA, 2009). However, a study carried out across north-eastern and south-western of Scotland on *Campylobacter* isolates from cattle and sheep faeces did not find a statistically significant regional difference between the two species (Rotariu *et al.*, 2009). Chapter 5 will investigate the regional inference of different animal hosts on pheasant infection.

The statistical analysis of this study also confirmed that, across regions, there is a significant prevalence of either *C. coli or C. jejuni* over the other. In particular *C. coli* dominates in regions 1 and 3 while *C. jejuni* prevails in region 2. The same pattern is evident at estate level. Rotariu (2009) reported that cattle and sheep isolates were more likely to be genetically similar if they originated within rather than between farms. This result supports the supposition that recycling of *Campylobacter* spp. in the farm or estate is an important way of sustaining infection between individual animals (Humphrey *et al.*, 2007).

Nineteen STs were isolated from positive caecal samples of pheasants with STs 828, 827 and 19 representing 47.5% of the isolates tested. Fourteen STs were assigned to six CCs and five STs were not assigned to any CC when compared to isolates uploaded on the PubMLST database. Three out of five orphan STs were novel to the PubMLST global list of STs in February 2015. This confirms observations in the study by Forbs (2009) that suggest there is a large pool of *Campylobacter* strains present in Scotland that are not only continually evolving by mutation and recombination, but are being expanded by externally derived strains from migrating wildlife, human travel and trade. However, wild game birds are thought to be less likely to generate new STs compared to gull and other waterfowl (FSA, 2009).

Clonal Complex ST-828 was the most represented (57.5%) and, together with ST-21 and ST-1034, represented 76.3% of all isolates. Seven out of eight *C. coli* STs belonged to CC ST-828. The eighth was an orphan ST, designated ST2195. Clonal complex ST-21 included three

STs, while the other CCs contained only one ST each. 15% of STs recovered were not assigned to any CC and, taken collectively, were exceeded in frequency only by CC ST-828 (Figure 4.6). With the exception of ST2195 all other orphan STs belonged to *C. jejuni* species. As already reported in the literature, this study suggests that *C. coli* strains are quite conserved genetically compared to *C. jejuni*. Phylogenetic studies on *C. coli* have identified only three clades and the variation of genotypes within the same clade usually results from a re-assortment of existing alleles rather than from the formation of new ones by point mutation and there is little evidence of genetic recombination among clades (Sheppard *et al.*, 2008 and 2010).

Clonal complexes present in clade 1 are usually associated with animal sources while clades 2 and 3 are associated with CCs recovered from environmental waters (Sopwith *et al.*, 2009). Clonal complex ST-828 belongs to clade 1 and although STs included in this CC are genetically very similar, mismatching for one allele only, there is still enough variation to identify possible correlation with different animal hosts (Miller *et al.*, 2006). Conversely, *C. jejuni* is characterised by a deep branching phylogenetic structure that is typical of higher genetic variability compared to *C. coli* due to extensive recombination that generates a wide assortment of STs belonging to different CCs.

Further phylogenetic study on orphan STs, especially those not recovered from isolates uploaded on the PubMLST database, may shed more light on their origins, possibly highlighting additional sources of contamination for pheasants and enabling us to predict the likelihood of a threat to public health in the future.

The next chapter will explore the host association of CCs found in pheasants with the main farm animal species, in order to determine if there is any risk of cross contamination between species at estate and/or regional level and also to investigate the contribution of pheasant campylobacteriosis to human infection.

Chapter 5 Host association and source attribution

5.1 Introduction

Host association is the process by which sequence typing of isolates can provide evidence for an association with specific hosts (FSA, 2009). For *Campylobacter* spp., several studies carried out in the UK and Northern Europe have shown an association between *Campylobacter* CCs and various animal hosts; a summary of the literature review findings is provided in Table 5.1.

In these previous studies the host association analysis was carried out at a CC level because the limited number (few hundreds) of samples collected from environmental sources did not allow statistical analysis to be carried out at ST level (FSA, 2009). They also suggested that some CCs are strongly associated with a particular animal species, for example CC ST-828 is often associated with sheep, CC ST-61 with cattle and CC ST-45 with poultry, but other CCs can be found in multiple animal hosts (e.g. CCs ST-21, ST-42 and ST-45). As such, host association of *Campylobacter* CCs is the first step in attributing human clinical cases to different sources of infection.

Source attribution is an important tool in scanning surveillance and outbreak investigations but it is also valuable in developing effective food safety interventions (Havelaar *et al.*, 2007; EFSA, 2013). It has been defined by Pires (2009) as: "the portioning of the human disease burden of one or more foodborne infections to specific sources, where the term source includes animal reservoirs and vehicles, e.g. foods". It also covers exposure of humans to different pathways of infection, for example from the environment or arising from different types of consumer behaviour (EFSA, 2013). Source attribution is part of the exposure risk assessment, one of the key steps in the risk analysis process. In particular, it gives a qualitative and/or quantitative evaluation of the likely exposure to hazards via different sources (CAC, 2011). Different methods can be used for source attribution. In this project we used the microbial subtyping approach that compares the distribution of *Campylobacter* STs in pheasant caecal samples with the ST distribution in humans and other farm animal species and quantifies the association and contribution of the STs found in animal sources to human infections. This method has been used for source attribution of *Campylobacter* infection in humans in Scotland (Strachan *et al.*, 2009) and New Zealand (Muellner *et al.*, 2013). *Campylobacter* subtyping by MLST has high discriminatory power for identifying strong associations between STs in human clinical cases and those found in different sources of infection (EFSA, 2013). The

discriminatory power is the average probability that a particular laboratory test will assign a different type to two unrelated strains randomly sampled in the microbial population of a given taxon (Hunter, 1990). In particular, the high discriminatory power of MLST for detecting *Campylobacter* STs is based on the seven genetic loci that are highly conserved within the *Campylobacter* genome and that allows for a high degree of discrimination between STs.

Species	CC	Host	Reference
C. jejuni	45	Broilers	Colles *et al.*, 2003
			Manning *et al.*, 2003
			Strachan *et al.*, 2009
		Wild birds, rabbits	Strachan *et al.*, 2009
			French *et al*., 2005
			Kwan *et al.*, 2008a
	42	Cattle, sheep	Colles *et al.*, 2003
			Strachan *et al.*, 2009
		Sheep	Manning et al., 2003
		Cattle	Kwan *et al.*, 2008b
			FSA, 2009
			Rotariu *et al.*, 2009
	48	Cattle, sheep	Colles *et al.*, 2003
		Cattle, sheep, broilers	Strachan *et al.*, 2009
		Cattle	Manning *et al.*, 2003
			Kwan et al., 2008a
			FSA, 2009
		Cattle, sheep	Rotariu *et al.*, 2009
	61	Cattle, sheep	Colles *et al.*, 2003
			FSA, 2009 and Strachan *et al.*, 2009
		Cattle	Manning *et al.*, 2003
			Kwan *et al.*, 2008a,b
			French et al., 2005
			Rotariu *et al.*, 2009
	21	All sources	Colles *et al.*, 2003 and Strachan *et al.*, 2009
		Cattle	Kwan *et al.*, 2008a,b
	220	Wild birds (pigeon)	FSA, 2009 and Strachan *et al.*, 2009
	257	Broilers	Manning *et al.*, 2003
			Strachan *et al.*, 2009
	283	Broilers	Manning *et al.*, 2003
	403	Pigs	Manning *et al.*, 2003
	1034	Sheep	Rotariu *et al.*, 2009
	1275	Wild birds (gulls)	FSA, 2009
C. coli	828	Sheep	Strachan *et al.*, 2009
		Sheep, pigs	FSA, 2009
		Sheep, cattle	Rotariu *et al.*, 2009

Table 5.1: Summary of literature review of host association of the main C*. jejuni* and *C. coli* clonal complexes (CC) with farm animal species and wild birds in the UK and Northern Europe.

Several studies have been conducted in different countries including the UK, New Zealand, Finland and the US that confirm the overlapping of *Campylobacter* STs found in poultry, cattle, sheep and pigs with STs found in human clinical cases; a detailed overview is available from Forbes (2009) and EFSA (2013). Overall, these studies point out that cattle, sheep and poultry are likely to be the main sources of human campylobacteriosis, while pigs play a minor role as a source for human *C. coli* infection. These studies also suggest that consumption of poultry meat is strongly associated with human infection in urban and suburban areas, while direct contact with cattle and sheep is the main source of human infection in rural areas.

Strachan (2009) investigated the attribution of *Campylobacter* infection in humans in the Northeast of Scotland (region 2) to different animal sources. This study confirmed that young children are mostly affected by *Campylobacter* infection, as already reported by Gillespie (2009) in England and Wales. The source of infection in rural areas, although not clearly understood, was thought to be environmental (e.g. contact with farm animals, birds or contaminated water) rather than direct consumption of poultry meat. In adults, *Campylobacter* infection appeared to be food-related, though resulting from the consumption of contaminated fresh produce (e.g. vegetables) or raw milk, rather than poultry meat. The study also confirmed findings from previous work suggesting that, although there was a high number of pigs reared in rural areas (n=278,000), their contribution to human campylobacteriosis was minimal (Strachan *et al.*, 2009).

For the purpose of this study a host association between CCs found in pheasants and other farm animal species could not be performed due to the lack of contemporary data. However, it was possible to perform a host association analysis of the prevalence of *Campylobacter* in pheasants and relate it to the density of the main farm animal species present in the same post code areas. Source attribution of *Campylobacter* STs recovered from pheasant caecal samples in Scotland was performed to quantify the contribution to human campylobacteriosis.

5.2 Materials and methods

5.2.1 Host association

Host association of the *Campylobacter* spp. in pheasants in Scotland was performed by comparing the geographical distribution of the number of cattle, sheep and goats, pigs, broilers and poultry other than chickens (i.e. duck, geese and turkeys) in relation to the post code areas of the estates where pheasant samples were collected.

The number of cattle, sheep and goats, pigs, broilers and other poultry, hereafter "farm animals", was obtained by accessing the EDINA agcensus website http://edina.ac.uk/agcensus/ in February 2015. From the agcensus website it was possible to download the number of farm animal species of interest present in Scotland in 2013 in a 2 x 2km grid square. The number of farm animals was exported to an Excel spreadsheet that also included the easting and northing of all the points in the grid from which farm animal numbers were collected.

The ArcGIS 10.3.1 (©1999-2015 Esri Inc.) software was used to illustrate and elaborate upon the density of the farm animals in the postcode areas from which pheasant samples were collected. Prior to importing the data from the agcensus website into the ArcGIS software, the base map of Scotland was imported from the Ordinance Survey (OS) Open background (©Crown Copyright and Database Right 2015). At this point the Excel spreadsheet from the agcensus website was imported into the software and the ArcGIS was able to visualise all the data points by coordinates on the map of Scotland. Figure 5.1 (a, b, c, d, and e) maps the density of farm animals in Scotland, as of 2013.

To enable resolution at post code level, the nationalrecordofscotland.gov.uk website was accessed in August 2015 and the file encompassing the map of post code areas in Scotland, current as of 2014, was downloaded. This file was then uploaded to the ArcGIS software to enable visualisation of postcode districts. The intersected data option on the ArcGIS software gave the opportunity to merge and extract the information on the number of farm animals from the agcensus spreadsheet in relation to the postcode areas of interest for this study (Figure 5.2).

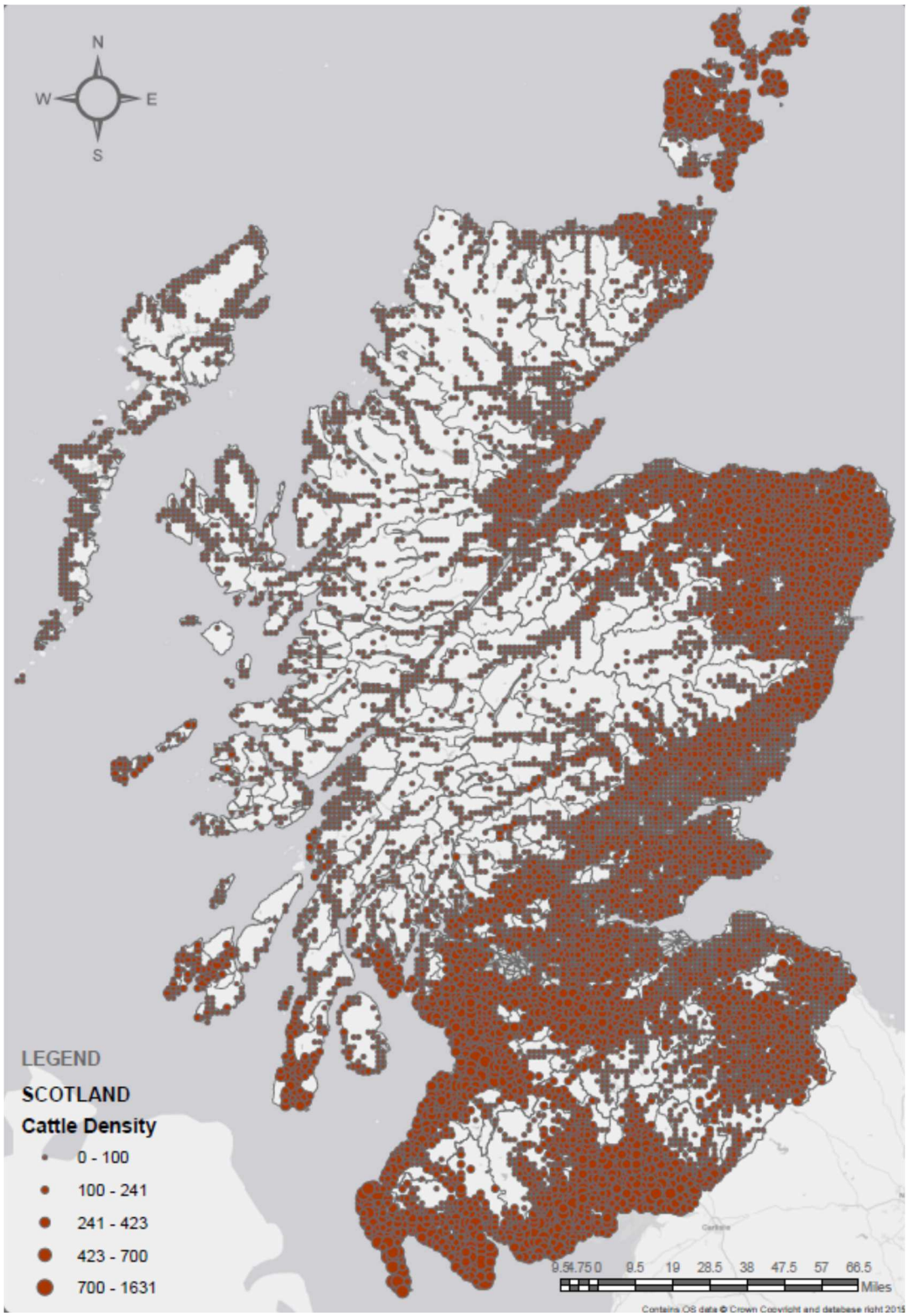


Figure 5.1a: Cattle density in Scotland

Cattle are mainly concentrated in the Northeast (region 2) and Southwest of Scotland (region 5).

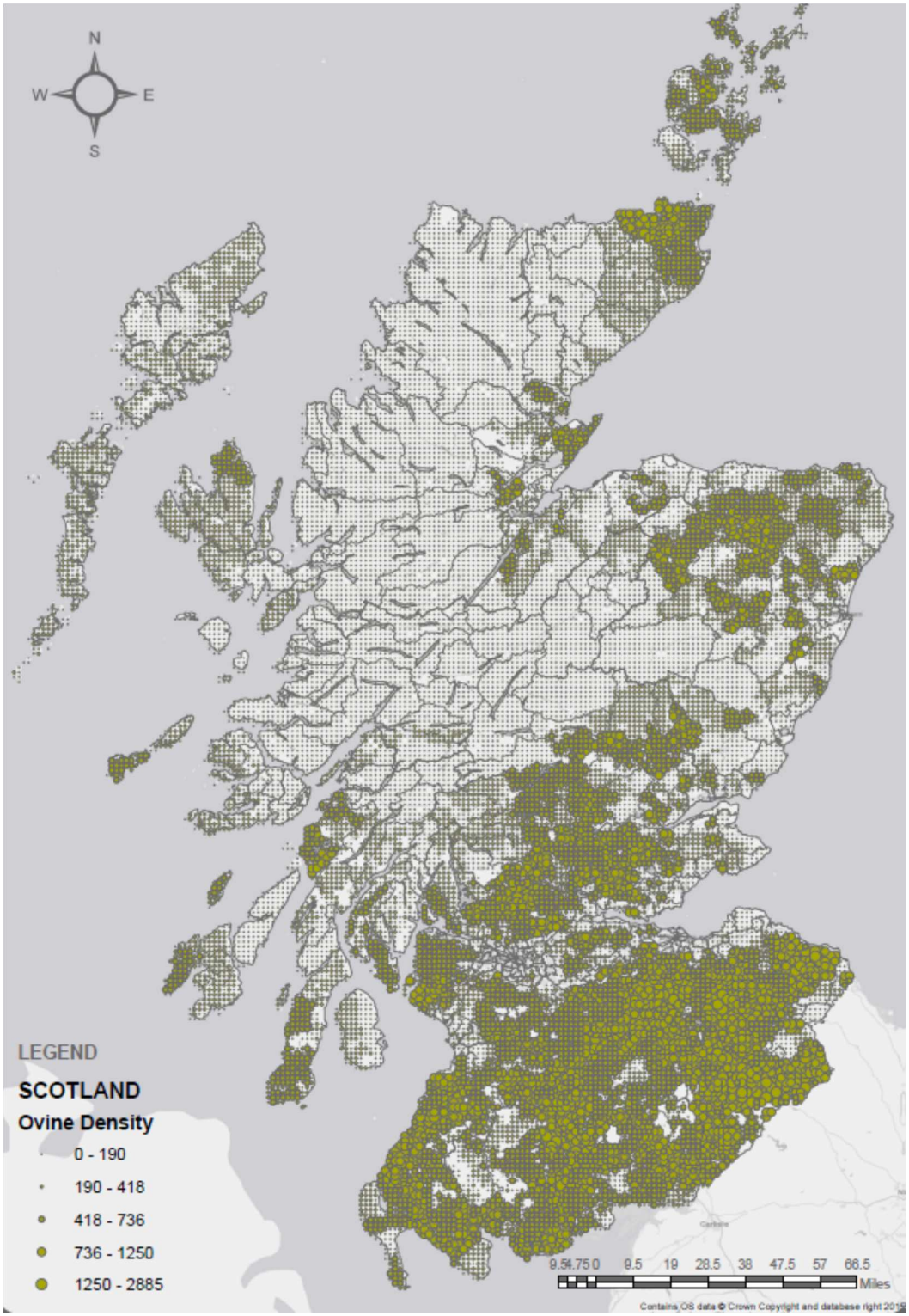


Figure 5.1b: Sheep and goats density in Scotland

Sheep are widely spread all over the country but are mainly concentrated in the Northeast (region 2), the Central and Eastern parts of the central belt (region 3) and the South of Scotland (region 4 and 5).

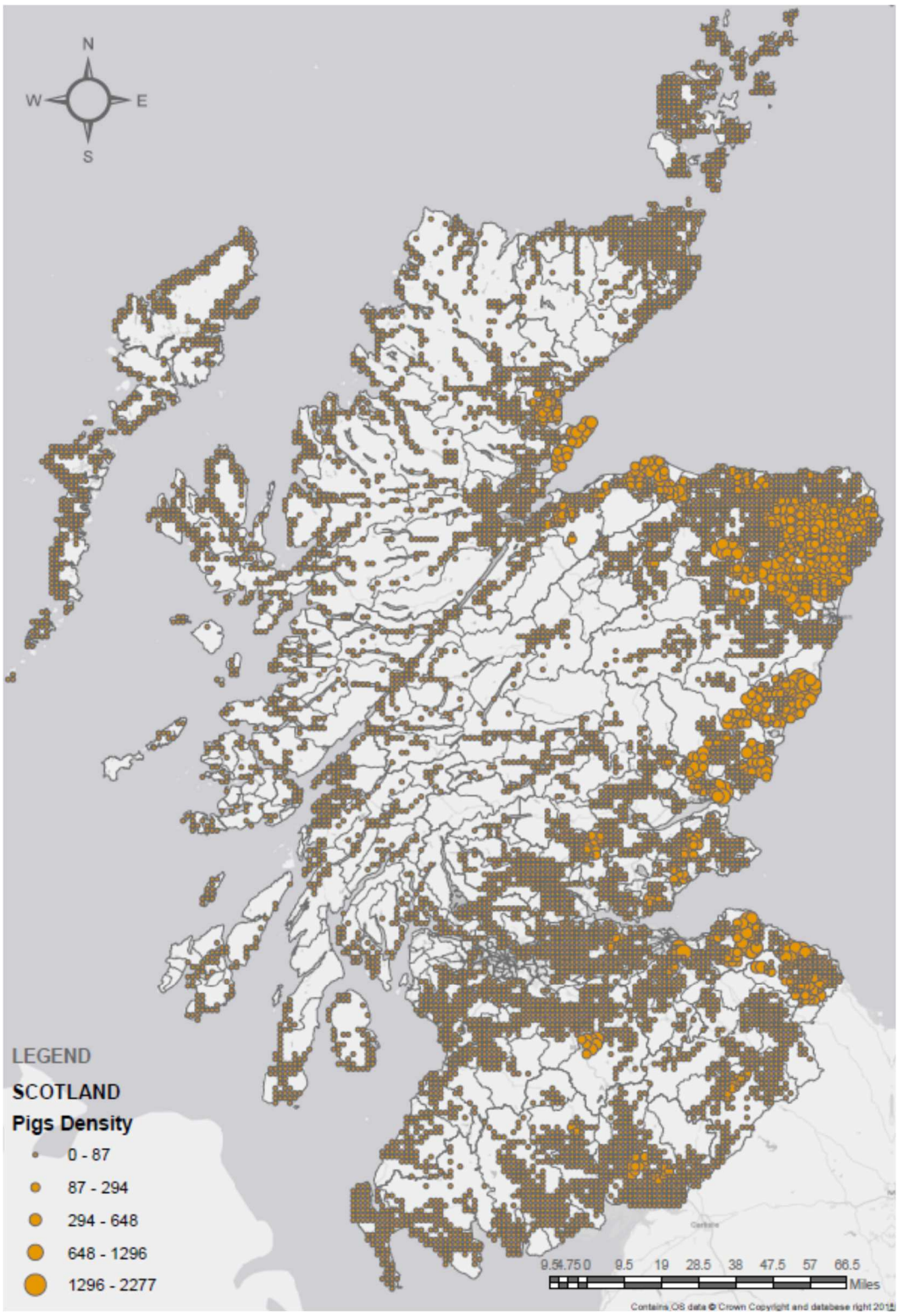


Figure 5.1c: Pigs density in Scotland

Pig farms are scattered in low numbers across Scotland but the higher density can be found along the Eastern coast (region 2, 3 and 4).

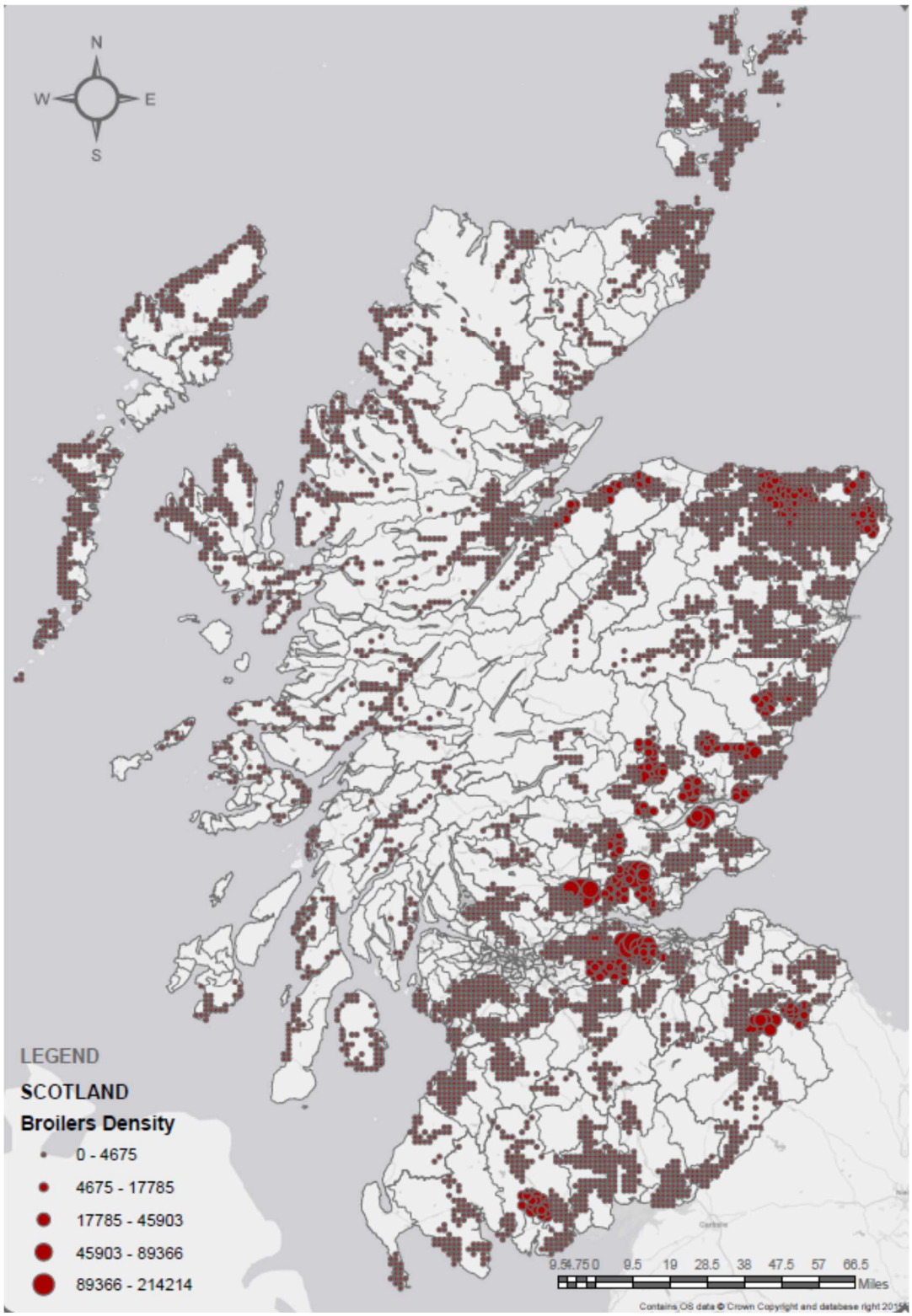


Figure 5.1d: Broilers density in Scotland

Main broiler farms also can be found along the Eastern coast of Scotland (region 2, 3 and 4).

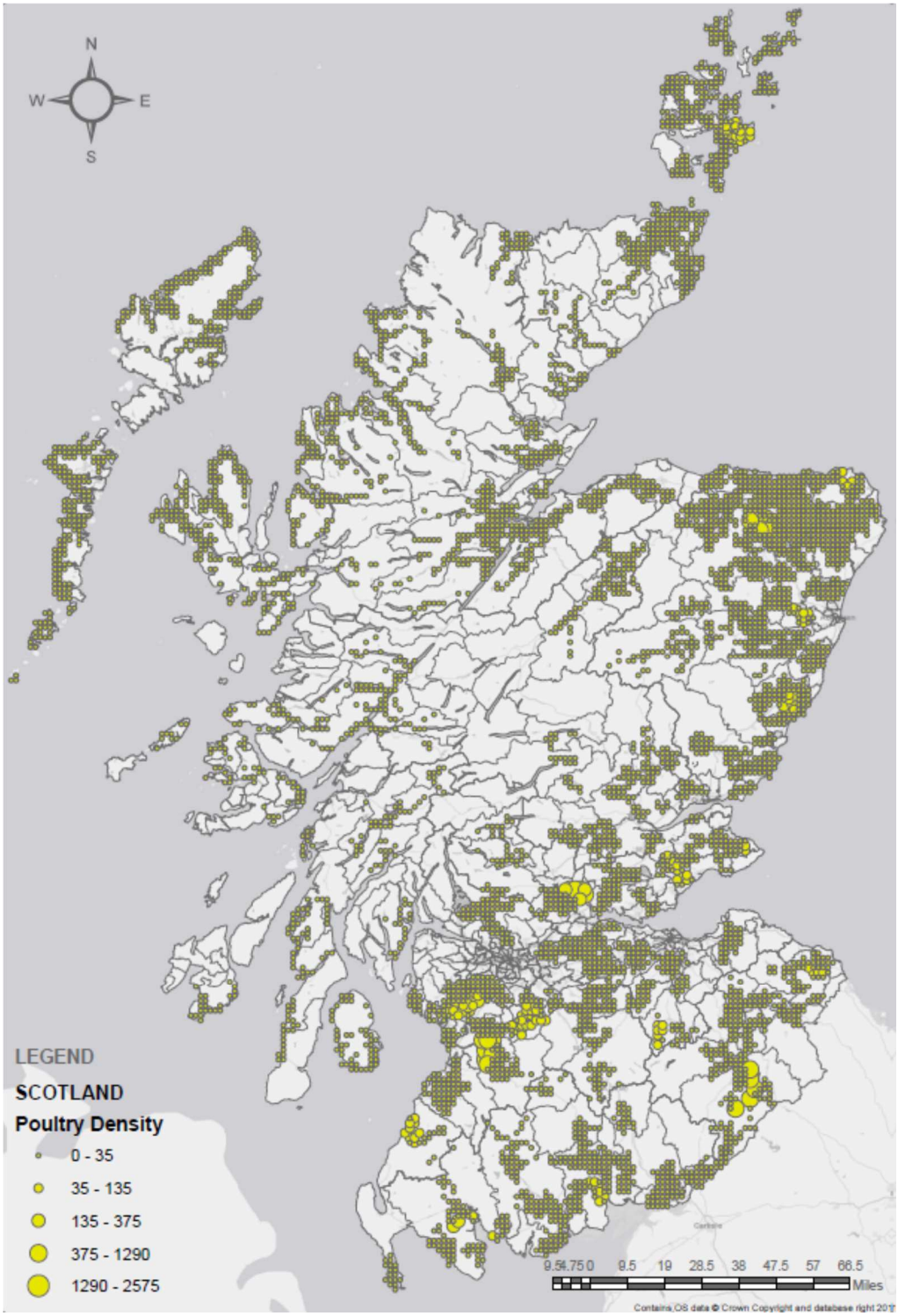


Figure 5.1e: Poultry (other than chickens) density in Scotland

Poultry farms other than chickens can be found in low numbers mainly in the Northeast and South of Scotland.

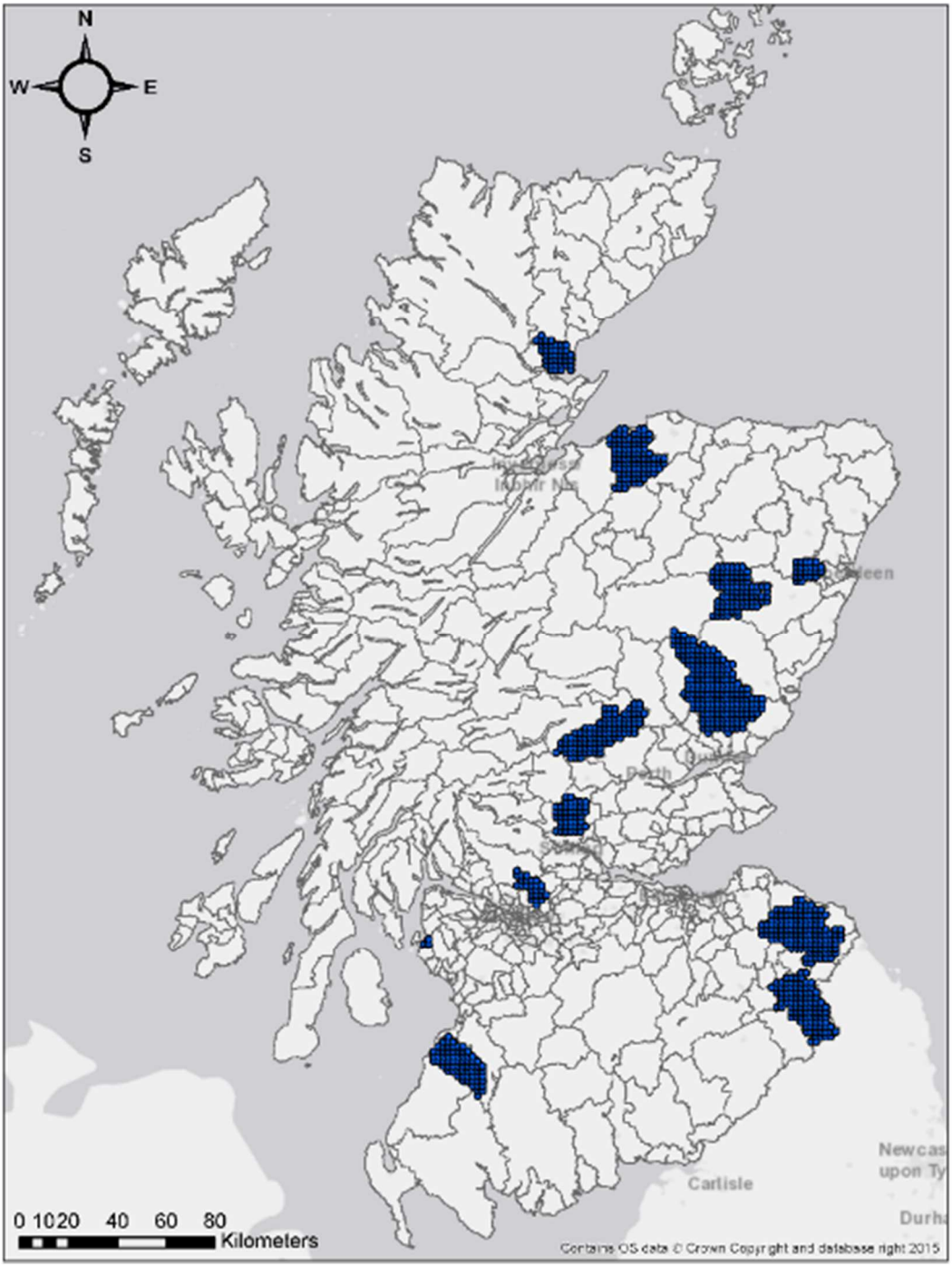


Figure 5.2: Distribution of the data points of farm animals within the postcode areas from which pheasant samples were collected.

The number of farm animals per square kilometre (km) was exported into an Excel file for further statistical analysis.

The statistical analysis aimed to investigate the host association between farm animal density and prevalence of *Campylobacter* spp. in pheasants in the same postcode areas and was performed using a binary logistic regression calculation in Minitab. Results were considered to be statistically significant if the overall *P* value was <0.05 and the 95% Confidence Interval Odds Ratio between groups did not cross 1.

5.2.2 Source attribution

Attribution of human clinical *Campylobacter* isolates to animal sources of infection was undertaken by downloading the lists of STs found in humans, farm animals and wildlife isolates from the PubMLST on-line database (last access on February 2015) http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_campylobacter_isolates&page=query. The list of STs found in the pheasant samples from this study was added to the PubMLST lists and source attribution was performed using arithmetic and graphical functions in Microsoft Excel.

As indicated in section 5.1, source attribution is used as a means of investigating any associations between the presence of *Campylobacter* contaminants in various sources with human infections. This is done by evaluating the distribution of STs (not CCs) in humans and in different potential sources of infection in such a way that enough discriminatory power is generated to identify a strong association between human campylobacteriosis and their sources of infection (Pires, 2009). Use of STs was possible instead of CCs because there was no sample size limitation as described in section 5.1 for host association statistical analysis. In fact the PubMLST databases provided a total of 12743 human (n=9646), farm animal (n=3021) and wild bird (n=76) isolates. There were 1665 STs identified from human clinical cases and reported on the PubMLST website in February 2015. For the purpose of this study, only 56 of the most common STs were selected from those 1665 isolates for further consideration as they included 12 STs found in pheasants. These 56 STs accounted for 69% of all the human cases. Human cases attributed to STs 831 (n=8), 2195 (n=7), 1541 (n=6) and 1030 (n=2) were also taken into consideration because, although not very common in humans, they were the remaining STs recovered from pheasants in this study. Source attribution of farm animals and pheasants to human infections, based on the isolates reported to the PubMLST database, was performed by examining only the STs that were common to humans, farm animals, pheasants and wild game species. The number of clinical isolates from humans and animals that shared the same STs was extracted for each species group. Each animal species contribution was calculated as a percentage of the overall number of human and animal cases that shared the same STs. A similar calculation was performed to determine the contribution of each animal

species to the overall number of *Campylobacter* cases. An example of the calculations performed to determine the contribution of each animal source to human campylobacteriosis is as follows:

Cattle and humans shared 22 STs from the PubMLST database. This translated into 4036 human and 276 cattle isolates attributed to the shared STs. This resulted in a total of 4312 human and cattle isolates. The percentage of the proportion of cases attributed to cattle was calculated by multiplying the number of isolates in cattle by 100 and dividing this number by the total number of isolates found in humans and cattle (276 x 100 / 4312 = 6.4%). To determine the contribution of cattle to the overall infections in humans, the total number of human isolates (n=6712) from the 60 STs considered was added to the number of isolates in cattle then the percentage of the proportion of cases attributed to cattle was calculated as above (276 x 100 / 6988 = 3.9%).

5.3 Results and Data Analysis

5.3.1 Host association

After plotting the density of farm animals in Scotland, it was possible to extract their counts per square km in the post code areas from which samples from pheasants were collected (Table 5.2.)

Post Code Area	Area (Square Km)	Cattle	Sheep and Goats	Pigs	Broilers	Other Poultry
AB32	101	56	89	14	0	4
AB34	345	26	61	0	0	0
DD8	817	25	65	11	430	1
FK15	178	17	135	0	0	0
G66	95	29	141	0	0	0
IV25	155	8	73	17	0	1
IV36	394	20	48	5	370	0
KA19	281	65	194	0	0	0
KA29	121	1	12	0	0	0
PH8	407	6	99	0	29	0
TD11	478	34	230	30	19	1
TD13	60	42	284	74	0	1
TD5	422	39	194	0	224	0

Table 5.2: Number of farm animals per square kilometre (km) in post code areas from which pheasant samples were collected.

A binary logistic regression calculation was performed to determine statistically if there was an association between the prevalence of *Campylobacter* infection in the pheasants sampled in this study and the density of different farm animal species in the same post code areas. Results are shown in Figure 5.3 (a, b, c, d and e).

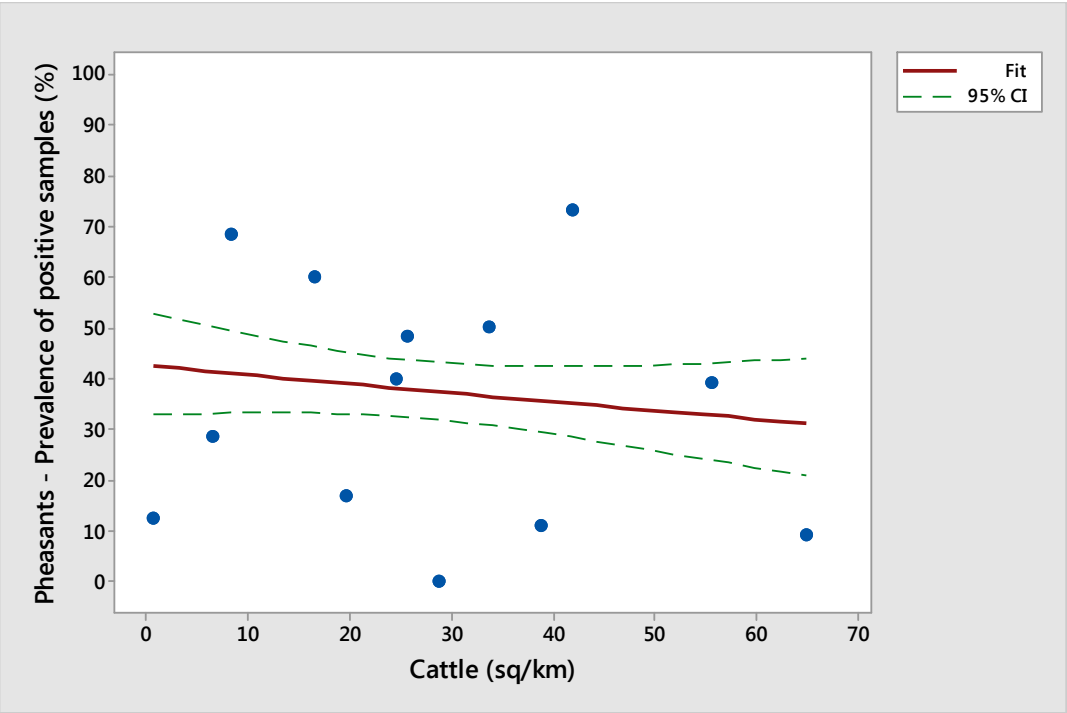


Figure 5.3a: Cattle

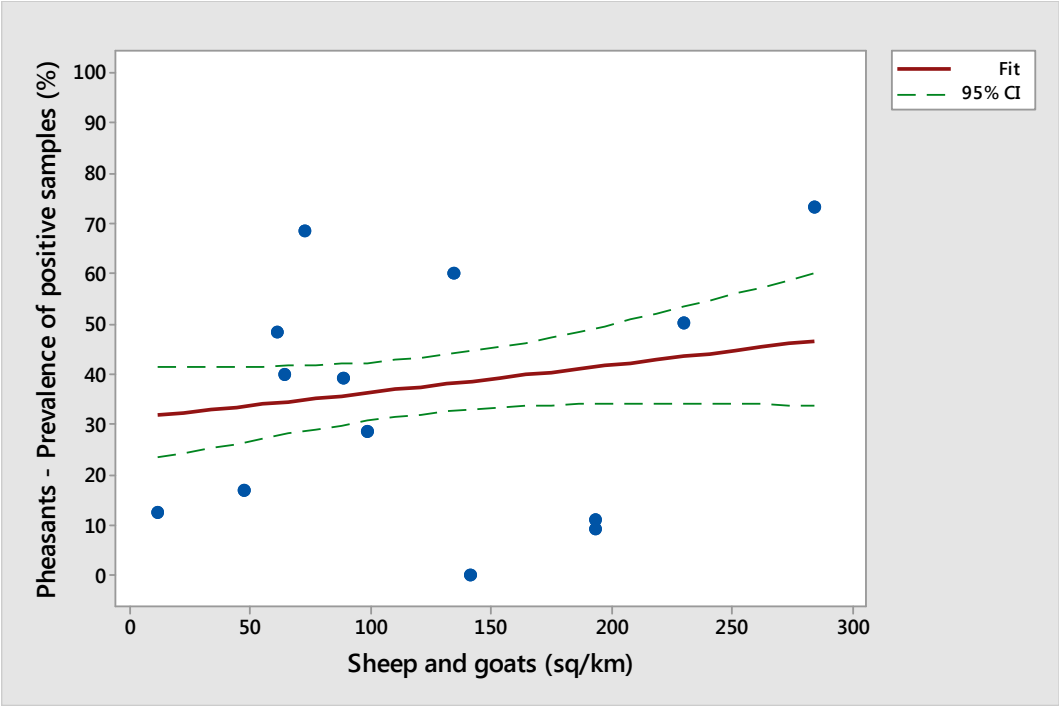


Figure 5.3b: Sheep and goats

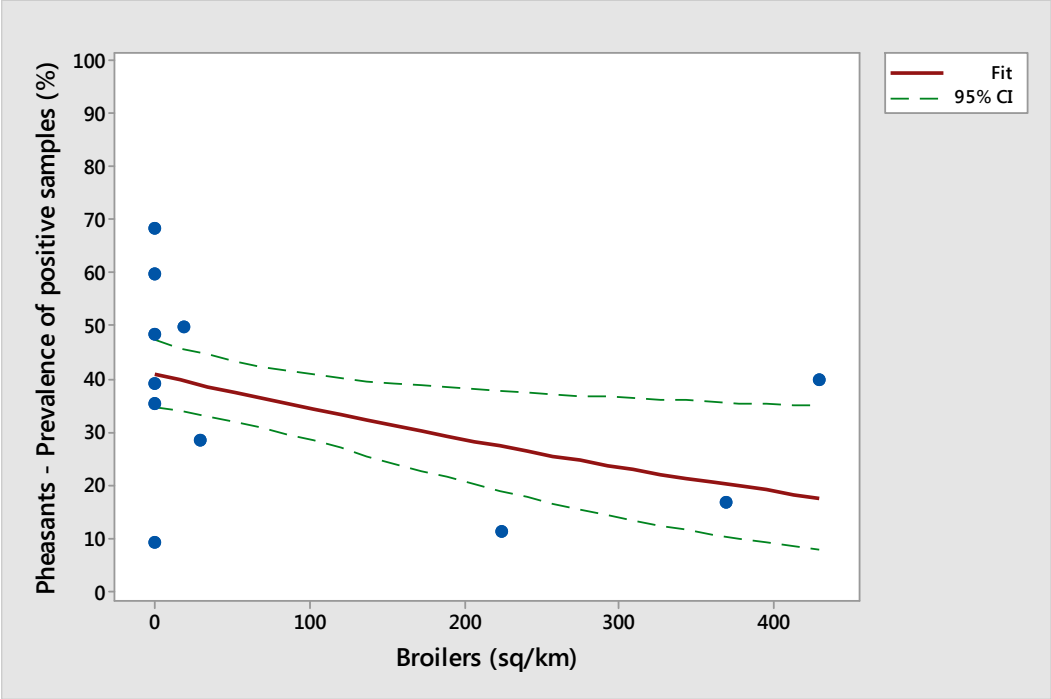


Figure 5.3c: Broilers

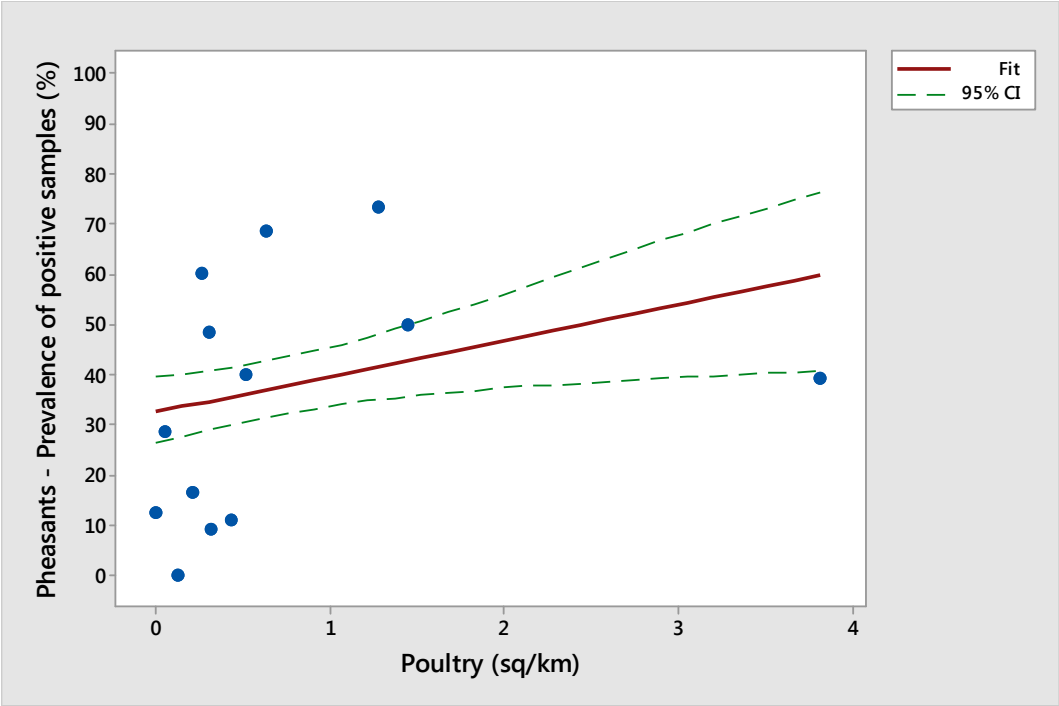


Figure 5.3d: Poultry (other than chickens)

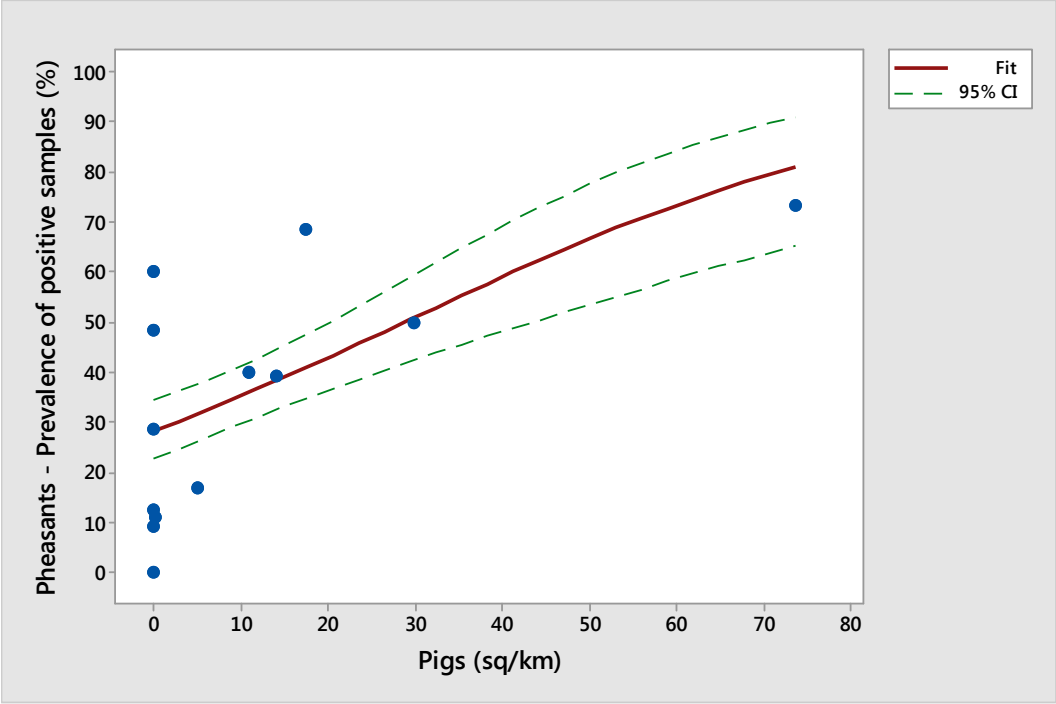


Figure 5.3e: Pigs

Figure 5.3 (a, b, c, d and e): Binary logistic regression analysis to estimate any statistical association between prevalence of *Campylobacter* infection in pheasants (%) and number per square kilometre (sq/km) of cattle (Figure 5.3a), sheep and goats (Figure 5.3b), broilers (Figure 5.3c), poultry other than chickens (Figure 5.3d) and pigs (Figure 5.3e) in the post code areas where pheasants were sampled.

The statistical analysis indicated no significant association between the densities of cattle (*P*=0.245) and sheep and goats (*P*=0.137) and *Campylobacter* infection in pheasants sampled in the same post code areas. However, the density of pigs (*P*<0.001), broilers (*P*=0.012) and poultry other than chickens (*P*=0.015) showed a weak but statistically significant association with *Campylobacter* infection in pheasants (Table 5.3).

Farm Animal Species	Odds ratio	95% CI
Pheasants vs Cattle	0.992	(0.979, 1.005)
Pheasants vs Sheep and Goats	1.002	(0.999, 1.005)
Pheasants vs Pigs	**1.033**	**(1.020, 1.046)**
Pheasants vs Broilers	**0.997**	**(0.995, 0.999)**
Pheasants vs Poultry	**1.341**	**(1.057, 1.702)**

Table 5.3: Binary logistic regression analysis to estimate any statistical association between prevalence of *Campylobacter* infection in pheasants and density of farm animals per square kilometre. Statistically significant associations are highlighted in bold.

A binary logistic regression analysis to estimate any association between the Odds Ratios of *C. jejuni* to *C. coli* and farm animal species in the same post code areas could not be performed because *C. jejuni* predominated in the post code areas of region 2.

5.3.2 Source attribution

The 60 STs used for source attribution in this study accounted for 70% of human cases reported on the PubMLST database. The relative proportions of the 60 STs found in the human clinical isolates database was plotted in descending order. To illustrate how common these STs were in farm animals and wild bird sources, their relative proportions as obtained from the PubMLST database was staked in the same graph (Figure 5.4).

Sequence type 21 was the most common ST (n=729) found in human clinical cases and accounted for 7.6% of the total human infections. Sequence type 257 (n=595; 6.2%), ST48 (n=447; 4.6%), ST50 (n=407; 42%) and ST45 (n=356; 3.7%) followed in descending order and together accounted for 26% of human clinical cases.

Sequence type 21 was recovered from cattle, sheep, chickens, wild birds and poultry other than chickens. However, cattle and sheep contributed 8.3% (n=48) and 8.8% (n=22), respectively, to all infections attributed to ST21 while chickens only contributed 2.1% (n=36) of those infections. Conversely, ST257 was quite common in chickens (6.1%; n=103) and less so in cattle (1.6%; n=9) and sheep (1.2%; n=3).

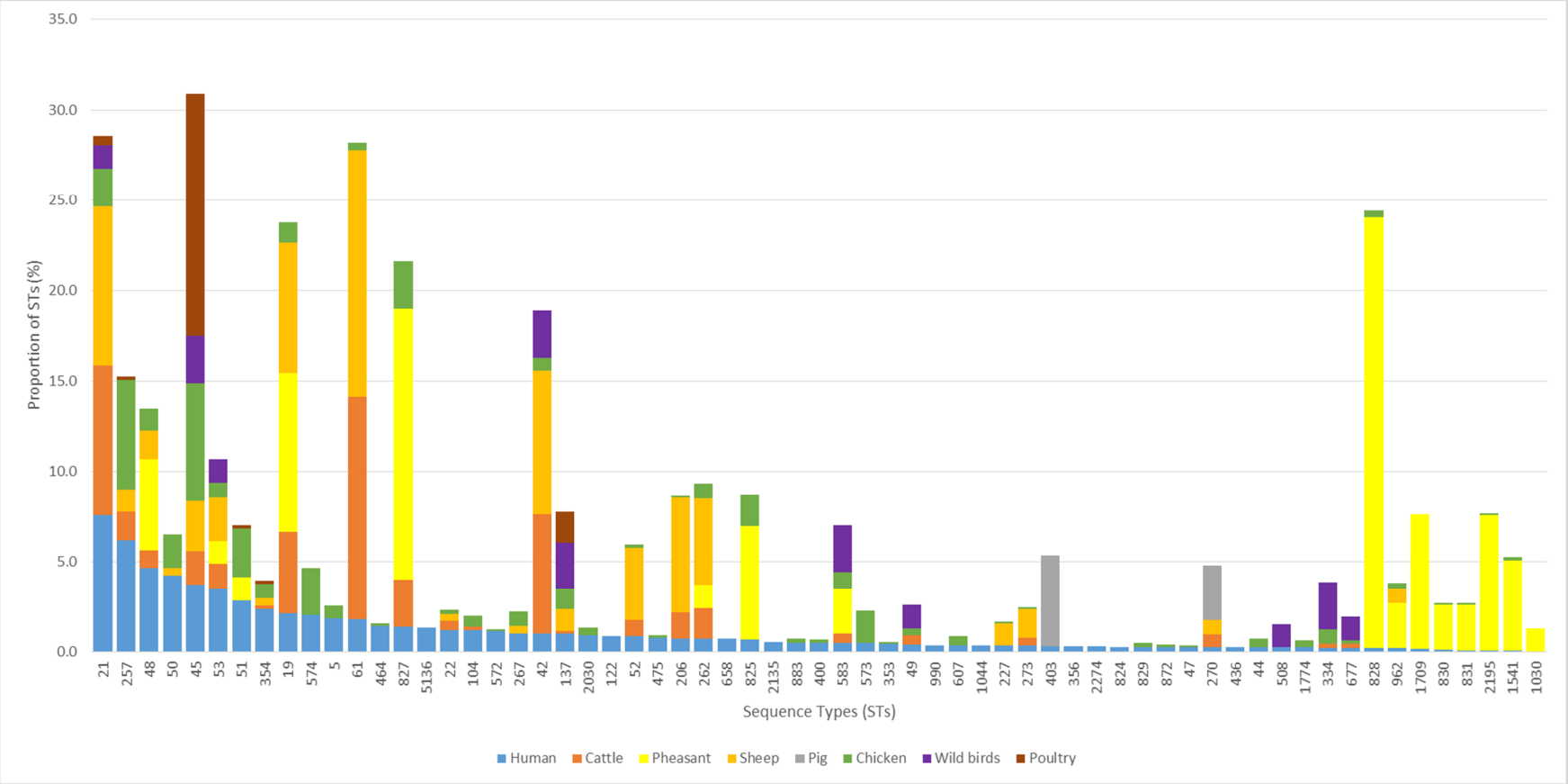


Figure 5.4: The X-axis lists the 56 most common *Campylobacter* sequence types (STs) found in human isolates and four STs found in pheasants but not common in humans. The Y-axis shows in proportion (%) how common each ST is in human isolates (n= 9646), farm animals and wild bird sources (cattle (n=578), pheasants (n=80), sheep (n=249), pigs (n=88), chickens (n=1696), wild birds (n=76) and poultry other than chickens (n=410)) as sourced from the PubMLST database including STs isolated from pheasant samples.

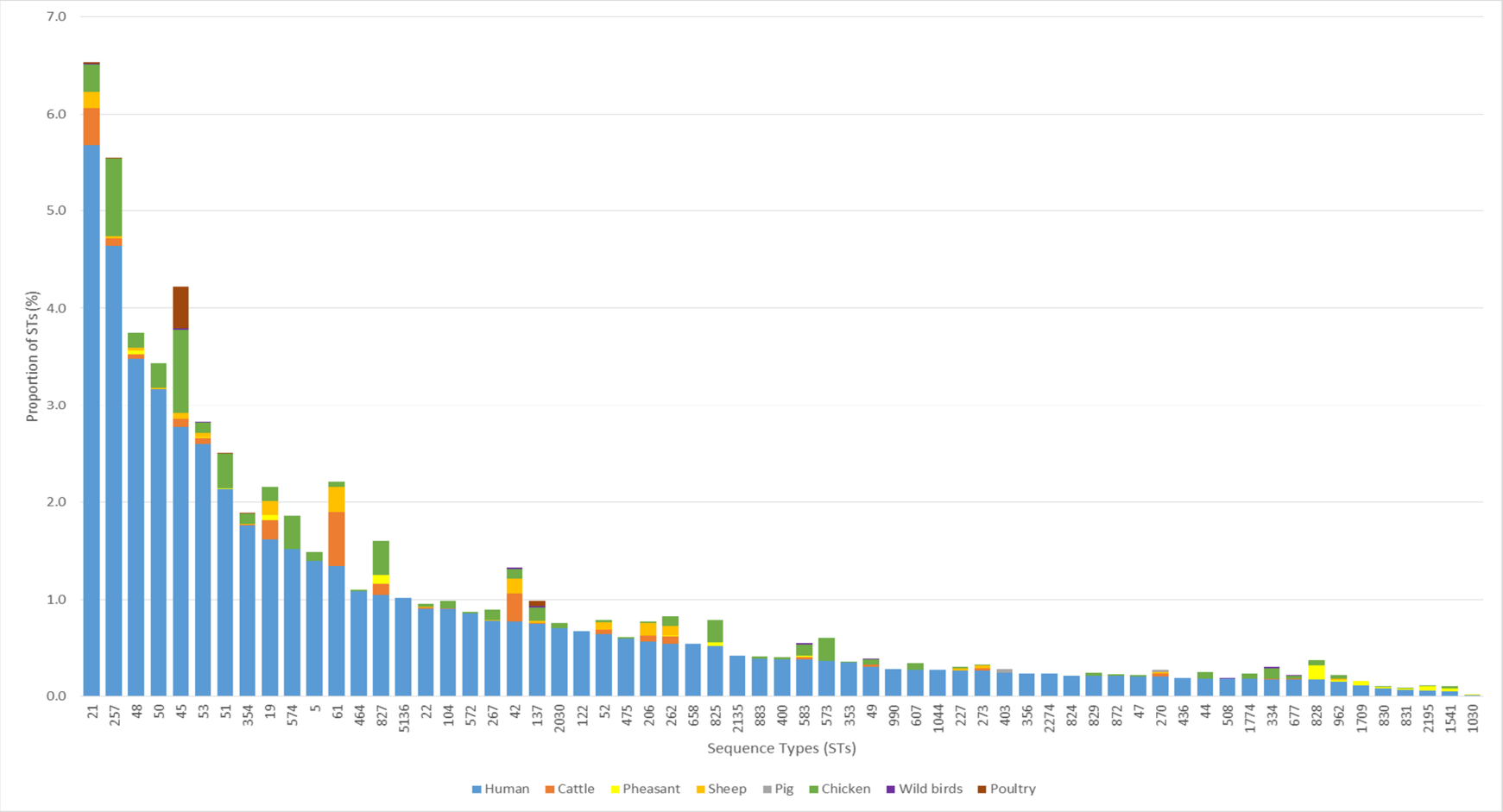


Figure 5.5a: The X-axis lists the 56 most common *Campylobacter* sequence types (STs) found in human isolates and four STs found in pheasants but not common in humans. The Y-axis shows each ST as a proportion (%) of all cases of *Campylobacter* infection taken as a whole (in humans, cattle, pheasants, sheep, pigs, chickens, wild birds and poultry other than chickens (n=12823)), as sourced from the PubMLST database including STs isolated from pheasant samples.

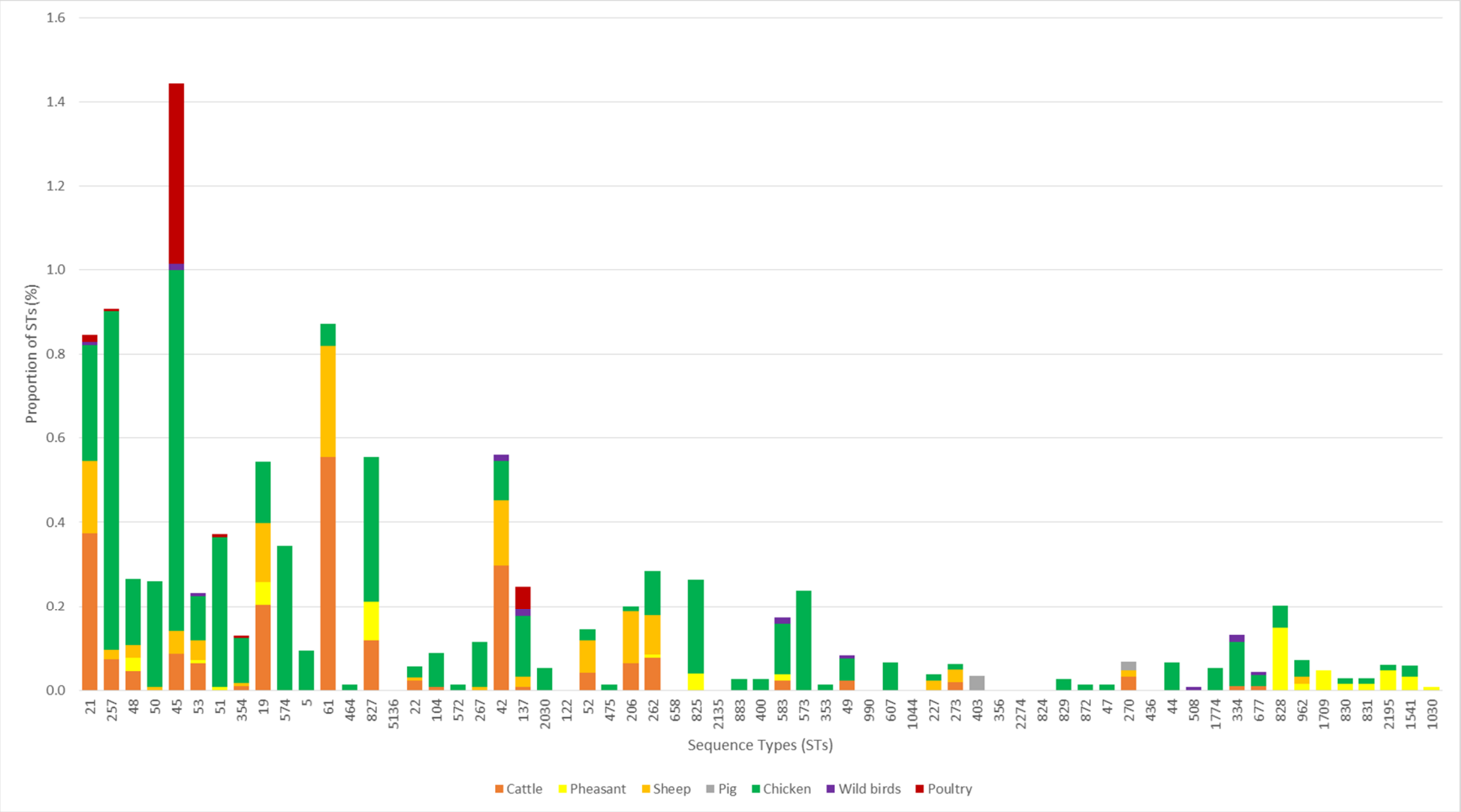


Figure 5.5b: The X-axis lists the 56 most common *Campylobacter* sequence types (STs) found in human isolates and four STs found in pheasants but not common in humans. The Y-axis shows each ST as a proportion (%) of all cases of *Campylobacter* infection taken as a whole (in cattle, pheasants, sheep, pigs, chickens, wild birds and poultry other than chickens (n=3177)) but excluding humans, as sourced from the PubMLST database including STs isolated from pheasant samples.

Sequence type 48 is present in similar proportions in cattle (1%; n=6), sheep (1.6%; n=4) and chicken isolates (1.2%; n=20) but is also present in pheasants (5%; n=4).

Sequence type 45 is common in chickens (6.5%; n=110) and even more frequent in poultry other than chickens (13.4%; n=55) but has also been recovered from cattle (1.9%; n=11), sheep (2.8%; n=7) and wild bird (2.6%; n=2) isolates.

Although eight STs recovered from pheasants are also among the most common in human infections (e.g. STs 19, 48, 51, 53, 262, 583, 825 and 827), eight STs are less common in humans and other animal species, with the exception of sheep and chickens (STs 828, 830, 831, 962, 1030, 1541, 1709 and 2195). Three STs recovered from pheasants (STs, 4002, 4254 and 7063) have not been isolated from human clinical cases at all on the PubMLST database.

Figure 5.5a shows the overall proportions of STs recovered from all cases of *Campylobacter* infection in human, farm animal, pheasant and wild bird isolates from the PubMLST database and the pheasant samples from this study (n=12823). The vast majority of isolates are from human infections (n=9646) but it is also possible to identify how animal sources of human infection contribute to the STs reported to the PubMLST database. A more detailed summary of the contribution of farm animal sources of infection to humans, excluding human isolates, is illustrated in Figure 5.5b. Chicken, cattle and sheep isolates (n=2523) collectively contributed 79% of the non-human isolates.

Table 5.4 below summarises the source attribution performed on human (n=6712), farm animal (n=1269), pheasant (n=75) and wild game (n=15) isolates from the 56 more common *Campylobacter* STs found in humans and the additional four STs found in pheasants.

Animal source	Number of human isolates sharing the same STs with animal sources	Number of animal isolates sharing the same STs with humans	Attribution (%) of animal sources to human infection from shared STs	Overall attribution (%) of animal sources to human infection
Cattle	(22) 4036	276	6.4	3.9
Sheep	(19) 4215	170	3.8	2.5
Pigs	(2) 57	7	11	0.1
Chickens	(45) 6097	750	11	10
Wild birds	(10) 1765	15	0.8	0.2
Poultry	(6) 2275	66	2.8	1
Pheasants	(16) 1666	75	4.3	1.1

Table 5.4: Source attribution (%) of human isolates to animal sources based on the PubMLST database including STs isolated from pheasant samples. (n)=number of shared STs between humans and animal source.

As a species group, cattle accounted for 22 of 60 STs that are a potential source of human campylobacteriosis. Those 22 STs accounted for 6.4% of all isolates common to humans and cattle and 3.9% to human infections overall.

Sheep shared 19 of 60 STs with human clinical isolates and 15 STs with cattle. Those 19 STs accounted for 3.8% of all isolates common to humans and sheep and 2.5% to human infections overall.

In the PubMLST database, pig STs were rarely found in humans, since they were only recovered from isolates containing STs 403 and 270. They accounted for only 0.1% of human infections overall, however, as a proportion of human and pig cases caused by STs 403 and 270, pigs accounted for 11%.

Chickens shared 45 of 60 STs with human cases and these were responsible for more than 6000 human cases. Chickens contributed to 11% of human and chicken infections resulting from shared STs and contributed to 10% of human infections overall.

Wild birds shared 10 of 60 STs with human cases and contributed to 0.8% of human and wild bird campylobacteriosis cases due to these shared STs. They only accounted for 0.2% of human infections overall.

Poultry other than chickens had six STs responsible for human infections and these contributed to 1% of the human cases overall.

Pheasants are a potential source of 16 out of 60 STs that are responsible for human infections. They contributed to 4.3% of human and pheasant cases caused by these shared STs and they accounted for 1.1% of the human infections overall.

5.4 Discussion

This study did not find a statistically significant host association between the prevalence of infection in pheasants and the density of cattle, sheep and goats in Scotland. However, previous studies (Strachan *et al.*, 2009; FSA, 2009; Rotariu *et al.*, 2009) have suggested a strong association between the CC ST-828 (*C. coli*), the most prevalent in pheasants sampled in this project, and sheep. Analysis of the PubMLST database including pheasant samples also indicates that pheasants and sheep can both be infected by four *C. jejuni* STs (STs 19, 48, 53 and 262). Transmission of *Campylobacter* infection between these species is possible because

they are usually reared in the same estates, sharing contaminated grassland and/or water sources. Host association of *Campylobacter* infection between pheasants and sheep is evident in all Scottish regions where this study found a statistically significant predominance of CC ST-828 in pheasants with the exception of region 2. In region 2 the abundance of *C. jejuni* infection in pheasants suggests a shared association with cattle and sheep. Although cattle and pheasants are common hosts for six STs (STs 19, 48, 53, 262, 583 and 827), all belonging to the CC ST-21, four of them are not exclusive to cattle but also occur in sheep (STs 19, 48, 53 and 262). Sequence types recovered from pheasants in region 2 from this study were STs 19, 48, 262, 583 and 827. Therefore, the most likely route of transmission of *Campylobacter* infection from STs 19, 48 and 262 to pheasants is either from cattle or sheep, while ST583 and 827 seem more likely to originate from cattle. However, more studies are needed to clarify the routes of transmission between cattle, sheep and pheasants. Other STs recovered from pheasants in region 2 were STs 1030, 1709 and 7063. Sequence type 1030 has been recovered from human and chicken isolates; ST1709 is only present on the list of STs recovered from human isolates and not from other farm animal species; ST7063 is so far unique to pheasants on the PubMLST database. Unpublished results from a study carried out by Forbes in 2006 on five pheasants collected in Aberdeenshire (region 2) detected the presence of STs 45 (n=2) and 262 (n=3) belonging to CCs ST-45 and ST-21, respectively. CC ST-45 is commonly found in chickens and wild birds, while CC ST-21 is mainly linked to cattle and sheep. These findings indicate that CC ST-21 in cattle and sheep in region 2 seems to play an important role in the circulation of *C. jejuni* between farm animals, pheasants and humans, as reported by Strachan (2009).

The results of the statistical analysis from this study indicated an association ($P<0.001$) between *Campylobacter* infection in pheasants and density of pigs and poultry other than chickens ($P=0.015$) but the reasons for these associations remain unknown. Pigs can contaminate the environment if reared outdoors but they are very unlikely to be present in great numbers in the same estates that are used for shooting pheasants. Furthermore, pigs are mostly infected by *C. coli* species, as observed in pheasants, but the specific STs recovered from pheasants, although belonging to the same CC ST-828, have not been recovered from pig isolates. As reported by Strachan (2009), even in rural areas with a high pig density, the presence of pigs does not seem to be associated with human infection. According to the PubMLST database, poultry other than chickens do not share any STs with pheasants, however, there may be the possibility of cross-contamination between these species, on-farm or at the estate.

This study also found a weak statistically significant association (P=0.012) between the prevalence of *Campylobacter* infection in pheasants and chicken density in Scotland. Chickens host 14 out of 16 STs found in pheasants, particularly ST828 that was the most common ST (24%) in pheasant isolates in this study. Furthermore, ST828 so far has been only recovered from chicken isolates on the PubMLST database as also reported in other studies conducted in Scotland (FSA 2009; Strachan et al., 2009; Rotariu et al., 2009) but has never been recovered from sheep. These findings seem to confirm those described by Sheppard (2010), who reported that poultry and ruminants (and not pigs and turkeys) are the most likely source of *C. coli* infection for other animal species and for humans in Scotland.

Source attribution, as outlined in Table 5.3, has re-confirmed the prominent role that chickens, cattle and sheep have in human campylobacteriosis when infection from pets and human-to-human transmission are not taken into consideration, as also reported in other studies (Sheppard *et al*, 2010; EFSA 2010; Humphrey *et al.,* 2007). STs found in chickens in particular contributed to 10% of human clinical cases from the PubMLST database analysis but the contribution of chicken meat to human infection is thought to be as high as 30%, while 50-80% may be attributed to the poultry reservoir as a whole (EFSA, 2010). This may be linked to the fact that most of the *Campylobacter* STs that are responsible for campylobacteriosis in humans are also carried by chickens. Furthermore, human infections are mainly due to *C. jejuni*, which may reflect the prevalence of *C. jejuni* over *C. coli* in chickens. For instance, a European level survey carried out in 2008 reported that two-thirds of *Campylobacter* isolates from pooled caecal contents of chickens and broiler carcasses were identified as *C. jejuni*, while one-third was *C. coli* (EFSA, 2008). Another important factor to take into consideration in terms of human exposure to *Campylobacter* infection from chickens is the higher rate of poultry meat consumption per-capita that, in the UK in 2013, was almost double that of beef (31.2 Kgs compared to 17.3 Kgs) (EBLEX, 2014). As mentioned in section 1.1.3, the level of contamination of red meat at retail is substantially lower than poultry meat, possibly due to the longer time red meat spends chilled before entering the food chain and due to the deleterious effects of dehydration on *Campylobacter* (Humphrey *et al.*, 2007). These two factors together tend to support the hypothesis that cattle and sheep are responsible for *Campylobacter* infection in humans more through direct contact with farm animals or their faeces or through consumption of contaminated water, rather than via food-borne sources.

Farmed poultry other than chickens are a possible source of *Campylobacter* infection to humans. In the PubMLST database they were mainly carriers of ST45, but STs 21, 51, 137 and 257 were also represented. The overall contribution to human infection was 1% so they

can be considered a lower risk to public health compared to chickens and other farm animal species. However, as also confirmed from a study carried out by ADAS (2007), they are carriers of CC ST-45 and ST-21 that are frequently associated with human campylobacteriosis. Consumer consumption of poultry meat other than chickens is increasing, therefore their role in human infections may become more prominent in the future (FSA, 2007).

In this study it has been calculated that pheasants may contribute to 1% of human campylobacteriosis cases. Pheasants share 16 STs responsible for human clinical cases, although only eight STs are relatively frequent in human infections. The remaining STs are not very common in human clinical cases. This includes ST828 that was the most prevalent (24%) in positive pheasant samples and also recovered from chicken isolates on the PubMLST database yet only accounts for 0.2% of human cases.

A more detailed analysis of the risk posed to humans from live pheasants and pheasant meat is undertaken in chapter 6.

Chapter 6 Conclusions and future studies

Campylobacteriosis in humans, due to *C. jejuni* and *C. coli*, is the most common bacterial diarrhoeal disease worldwide (WHO, 2012). There are many studies investigating the role of poultry, cattle and sheep as reservoirs of *Campylobacter* infection in humans (EFSA, 2013) but, so far, very little has been done to investigate the role of other animal species that, although not a major source of infection to humans, may contribute to the maintenance of *Campylobacter* infection in wildlife and on farm land, and possibly even act as super-shedders of infection.

The aim of this research project was to explore the role of wild pheasants as hosts of *Campylobacter* infection in Scotland and investigate the contribution that contact with live pheasants and pheasant meat may have on human infections. The presence and prevalence of *Campylobacter* spp. in pheasants has been reported in the literature but the sample size is often small in such reports and pheasants tend to originate from a single farm or shooting estate. This project was a much wider study and expanded to the whole of Scotland by sampling different Scottish regions and estates across the country with the purpose of not only estimating the prevalence of *Campylobacter* infection in wild pheasants but also performing a *Campylobacter* bacterial count from pheasant caecal content and skin, something not yet reported in the literature. The reason for choosing AGHEs as sampling sites and not farms or shooting estates was to try to establish if infection was present at that particular stage, since then it could serve as a possible source of meat contamination during processing and storage, thus potentially posing a risk to public health similar to that which occurs following consumption of *Campylobacter* contaminated poultry meat. A larger cross-sectional study extended to the entire pheasant supply chain (e.g. including pheasant farms and shooting estates) could help clarify the role played in terms of animal and human health by *Campylobacter* infection in pheasants in Scotland.

Taxonomy classification places pheasants in the order of Galliformes, an order that also comprises chickens, turkeys, partridges and grouse. As such, one might conclude that pheasants behave like chickens as hosts of *Campylobacter* infection but this research project helped to demonstrate that this is not always the case. By performing an enumeration of *Campylobacter* colonies in positive caecal samples it was possible to confirm that pheasants, like chickens, can harbour high counts of *Campylobacter* CFU/g, comparable to those found in positive broiler flocks (Allen *et al.*, 2011). As such, it was also possible to hypothesise a

possible role for pheasants as "super-shedders" of *Campylobacter*, helping in the recycling and persistence of *Campylobacter* infection on farm land and among farm animal species. More studies need to be carried out to determine if *Campylobacter* spp. in pheasants may have super-shedding features, such as those previously reported for *E. coli* and *Salmonella* spp. (Chase-Topping *et al.*, 2008).

Another aspect that this study partially helped to clarify is the effect of kill-to-process time on *Campylobacter* loads. Review of previous literature suggested that, due to the strict growth requirements of *Campylobacter* spp., there was a decline in *Campylobacter* carriage in pheasants as the kill-to-process time increased (Nebola *et al.*, 2007). In this study there was no statistically significant difference in the relationship between *Campylobacter* load in positive caecal samples and kill-to-process time and some non-eviscerated pheasants carried high *Campylobacter* loads even at 7 days from the day of kill when compared to the average load of infection. However, this findings are only based on samples taken in one point in time and not over time. Storage temperature, number of days stored and location of shot wounds, especially if the intestine is perforated, are important factors that can influence *Campylobacter* growth and survival in non-eviscerated pheasants (Paulsen *at al.*, 2008). More specific studies need to be performed to study the survival of *Campylobacter* spp. in caecal samples over time and in the context of the kill-to-process time period.

A comparison of the prevalence of *Campylobacter* infection in chickens and pheasants is difficult to make, mostly because of the different husbandry systems of these species where chickens are usually intensively reared while wild pheasants are not. However, it has been reported that free range chicken flocks that have access to the outdoors have a higher level of *Campylobacter* infection than flocks kept exclusively indoors (Heuer *et al.*, 2001). As such, the prevalence of *Campylobacter* infection in broiler flocks has been reported to vary from 0% (*Campylobacter* free flocks) over 80% (Allen *et al.*, 2007). Considering the results of this study and previously reported surveys in combination, the prevalence of *Campylobacter* infection in wild pheasants seems to be consistently in the range of 25 to 38%. In this study, however, the level of *Campylobacter* infection in pheasants could have been even higher (46%) if the results from sampling site 5 had been excluded. Sampling site 5 had a significantly lower prevalence compared to the other sampling sites, considered to be due to a seasonal influence on infection levels. More studies would be required to verify if a seasonal pattern of *Campylobacter* carriage is actually present in pheasants, as reported in other farm animal species (Stanley *et al.*, 1998a).

Molecular epidemiology of *Campylobacter* spp. identified another important difference between pheasants and chickens: Two thirds of pheasants seem to be carriers of *C. coli* rather than *C. jejuni*, while in chickens it is the other way round. This is important not only in terms of host association but also in terms of possible transmission of infection to humans. Polymerase Chain Reaction and MLST were not performed on all pheasant isolates recovered from this study due to funding limitations. Evaluation of all samples may have given a better indication of the prevalence of *C. coli* and *C. jejuni* in pheasants and also the level of co-infections, as previously reported in the literature (Dipineto *et al*., 2008a). This study also identified three STs that were not associated with human, farm animal or wild bird isolates on the PubMLST database. A phylogenetic analysis of these STs was not performed as it was outside the scope of this project but such an analysis could perhaps promote our understanding of their evolutionary origin and their association with different animal species, as well as helping to predict the likelihood of their posing a future threat to public health (EFSA, 2013).

Molecular typing of *Campylobacter* spp. showed that CC ST-828 is the most common in pheasants but in Scotland this is not the case in all geographical areas. In fact, in Aberdeenshire *C. jejuni* CCs seem to be the more prominent as also reported by Forbes (2009). It also showed that it is very likely that pheasants will acquire *Campylobacter* infection mainly from cattle, sheep and chickens rather than from pigs, turkeys or wild birds, as supported by other studies (Sheppard *et al.*, 2010). This also suggests at present that it is unlikely that pheasants can be considered a reservoir of infection but rather a host with potentially super-shedder features. Chickens in particular, are the exclusive host and carry several STs that are associated with human infections, according to the PubMLST database. They can be considered to be the specific host since it is very unlikely that the transmission of infection to chickens could originate from humans due to the overall very low level of exposure of live chickens to humans. The same could be said for pheasants, where their level of exposure to *Campylobacter* infection from cattle, sheep and chickens is much greater than vice versa. More specific studies need to be carried out to clarify the role of pheasants as a reservoir or as a host of *Campylobacter* infection for other farm animals and for humans.

The vast majority of human campylobacteriosis cases are associated with *C. jejuni* and this tends to give an indication that pheasants are a lower risk for transmission of *Campylobacter* infection to humans. Other factors could also contribute to reduce the risk of transmission of *Campylobacter* infection to humans from pheasants and they are briefly outlined below:

- Human exposure to live pheasants and their meat products is restricted to gamekeepers and pheasant farm workers that are in direct contact to these animals, and consumers

who enjoy game meat. Although an outbreak of campylobacteriosis in pheasant farm workers has been reported in the literature (Heryford and Seys, 2004), it can be considered as an occupational disease and could be prevented by applying strict biosecurity and hygiene rules, as in all other farm animal activities.

- The consumption per-capita in the UK of pheasant meat can be considered very low compared to chicken and beef and can be estimated to be 5g per person per year. However, results from this study suggest that consumption of pheasant meat, together with direct contact transmission of infection from live birds, can potentially account for 1% of human campylobacteriosis cases. This can be translated to a total cost to the UK of approximately £5,000,000 per annum, affecting around 6,500 people. At present there is no record of any foodborne disease case that can be definitively traced back to consumption of pheasant meat (PACEC, 2006) and only one outbreak was reported in the US in humans from direct contact with live pheasants on a pheasant farm (Heryford and Seys, 2004). However, there is evidence that this food is meeting an increasing consumer demand so a risk assessment of human exposure to *Campylobacter* infection through pheasant game meat should be reviewed in the future as for meat from farmed poultry other than chickens (FSA, 2007).
- Although this study demonstrated in section 3.4 that *Campylobacter* can survive at high counts up to seven days from the date of kill in pheasant caecal content, the chances of contamination of meat during processing and survival of *Campylobacter* spp. on pheasant meat could be reduced by applying strict HACCP controls on hygienic production (VLA, 2003; ACMSF, 2005) and a close control of the cold chain since dry and cold conditions are deleterious to *Campylobacter* survival (Allen *et al.*, 2006). In this study, no *Campylobacter* growth was observed in all skin samples tested as also reported previously in the literature (Soncini *et al.*, 2006), suggesting that dry and cold conditions are not ideal for *Campylobacter* survival outside the gastrointestinal tract. Dry plucking of pheasants also helps to reduce the moisture content on pheasant skin during processing compared to broilers where birds are submerged in hot water in scalding tanks before plucking (Allen *et al.*, 2006; Hayama *et al.*, 2011).
- Pheasant meat is available to consumers in restaurants, butcher shops and supermarkets mainly in winter time during the hunting season (October to February) and this does not coincide with the peak in human campylobacteriosis cases (Figure 1.2); usually the notification rates in these months is decreasing or very low, giving an indication that the higher consumption of pheasant meat in these months, even if it

is posing a potential risk to public health, does not statistically contribute to an increase of *Campylobacter* infection in humans. Pheasant meat that is available to consumers all year around is generally stored frozen and, since there is evidence in the literature that freezing is detrimental to *Campylobacter* survival in food (Harrison *et al.*, 2013), the risk to public health is reduced. Regardless, consumers, restaurants and pubs should always be advised to cook meat thoroughly, in order to prevent any risk of infection through food.

Based on these findings, the risk to public health from live pheasants and pheasant meat at the present time can be considered to be low. However, consumer consumption of pheasant meat is increasing, therefore its role in human infections may become more prominent in the future, with the potential to have a substantial impact as a foodborne source of *Campylobacter* infection for humans.

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