

i-CaMPS4

Employing Source Attribution and Molecular  
Epidemiology to measure the impact of  
interventions on human campylobacteriosis  
in Scotland.

An extension focused on the role of  
Scottish broiler production on human  
campylobacteriosis cases.

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# Lay Summary

## **The Background**

*Campylobacter* is the number one cause of bacterial gastroenteritis in the UK. In Scotland in 2016, there was a decrease of 15.5% of *Campylobacter* infections reported to Health Protection Scotland (HPS) compared to the previous year. In Grampian, the incidence was high during 2011-2014 and decreased statistically significantly in 2015 and 2016. Campylobacteriosis in the Grampian region has been studied in depth since 2005 and a declining trend of human cases has been observed since 2015 (Forbes, Horne 2009, Lopes, Forbes et al. 2016). Food Standards Scotland (FSS) commissioned this study to understand the key transmission pathways through which this pathogen contributes to the burden of infectious intestinal disease (IID) in the Scottish population by examination of the disease in Grampian. This study focused on obtaining evidence on the importance of retail ready chicken in Scotland as a source of human campylobacteriosis. It also assessed the impact of targeted interventions at Scottish farms and abattoirs. This study assessed the farm-to-fork chain by looking at *Campylobacter* in chicken caeca, a pouch connected to the junction of the small and large intestines (representative of on-farm colonisation) and whole bird samples from the end of abattoir line (representative of retail ready birds) and the link of these to campylobacteriosis cases in Grampian. It also highlighted the circulation of the *Campylobacter* strain types that are present in the farm environment, in the abattoir environment and from human cases. This study used methods which attribute clinical strains to major host reservoirs of *Campylobacter* (chicken, cattle, sheep, wild birds and, pigs), in order to understand what are the important sources of *Campylobacter* infection. The study also looked at whether case clusters can be detected using whole genome sequencing approaches by looking at genetic variations (changes in DNA) in the genome and phylogenetic trees (family trees).

## **The Study**

*Campylobacter* isolates were collected from clinical cases from Apr 2015 to Dec 2016 along with those from abattoir sampled whole birds (representing retail ready chicken) and caecal samples (representing the farm environment). Different strains were identified from the isolates obtained and molecular attribution models were used to attribute clinical isolates to specific host reservoirs. The information obtained from a questionnaire sent to all human cases allowed greater understanding of the exposures, routes of infection, seasonal trends and the risk factors associated with human campylobacteriosis in Grampian. Sensitivity analyses were performed in order to test the robustness of attribution models used. Molecular phylogeny methods (family trees) were used to detect case clustering of subtypes of *Campylobacter* in order to establish whether a direct link between cases and sources might be discernible.

## The Findings

The chicken strains from Grampian supermarkets and the studied Scottish abattoirs had some overlap with high carriage of *Campylobacter* on both whole birds and from farms. All of the abundant strains found in whole birds at the abattoir and caeca were also commonly found in human cases. These consisted of ST50 (27/34 farms), ST257 (19/34 farms), ST5136 (19/34 farms), ST45 (13/34 farms), ST122 (10/34 farms) and ST53 (9/34 farms). Around half of the flocks were treated with at least one antibacterial agent. As observed in the previous study, the strains from chickens were most common in humans, followed by those from ruminant (cattle and sheep) sources. In 2016, the STRUCTURE attribution model allocated clinical isolates to the following reservoirs: chicken 52%, cattle 11%, sheep 26%, pigs 2% and wild birds 8%. The Asymmetric Island attribution model allocation was: chicken 68%, cattle 9%, sheep 14%, pigs 1% and wild birds 7%. This was a decline in chicken attributed cases compared to the previous year although not statistically significant; however chicken was still the largest attributed source of *Campylobacter* infection. Some strains such as ST61 and ST827 were strongly associated with the rural environment. The sensitivity of these molecular epidemiological attribution models for detecting changes was assessed. The source attribution data did not confirm that the drop in human clinical cases was solely due to a drop in *Campylobacter* levels in the chicken reservoir, however, both the attribution models would be able to test this further in the future if the fall in *Campylobacter* cases continues or if a larger dataset was used.

## The Conclusions

This report provides a continuing baseline for monitoring the farm-to-fork trends associated with *Campylobacter* infection and specifically of the principal role that retail chicken plays in this. A number of specific observations as they relate to FSS were made, which include:

- Virtually all broiler farms were positive for *Campylobacter* indicating more needs to be done on-farm in order to minimise risk of human campylobacteriosis.
- 21% of the caecal strains (representative of farm environment) were different to the strains from that flock's whole birds, suggesting multiple strain colonisation on the farm or cross contamination between the flocks during processing in the abattoir.
- More than half of the paired bird samples had the same strain with one or both whole bird isolates being the same as the caecal isolate, implying predominant strain colonisation at the farm level.
- Half of the flocks were treated with at least one antimicrobial agent, which can lead to emergence of resistant strains and their persistence in the poultry chain or the farm environment.

- There was commonality of strains from chickens and human cases which indicates strong evidence of a link between these. SNP types were found which were identical between chicken and human isolates further strengthening this link.
- Cases from rural areas continue to have higher infection rates than from urban and declines in recent years in under-fives infected by non-chicken associated strains in rural areas warrants further study to improve our understanding of human disease.
- Genetic clustering of isolates sampled within temporally tight windows can be detected. Along with other whole genome phylogeny studies, this should provide better understanding of the epidemiology of human infection and their direct association with *Campylobacter* from particular sources. Outbreak detection and linkage to sources may be possible.
- The recent fall in human cases appears not to be due solely to a fall in *Campylobacter* cases attributable to chicken. Should the decline in human cases continue, then with more robust data, sensitivity analysis on the source attribution models should be able to detect this.

# Glossary and Abbreviations

**AgdbNet:** An antigen sequence database for web-based bacterial typing. The software facilitates simultaneous BLAST querying of multiple loci using either nucleotide or peptide sequences.

**Allele:** A variant form of a gene with each allocated a unique number.

**ARI:** Aberdeen Royal Infirmary Medical Microbiology Diagnostic Laboratory

**Attribution:** Inference of the source of human *Campylobacter* infection using strain type.

**BPC:** British Poultry Council

**cfu:** Colony forming units. Typically a measure of the number of live cells in a sample.

**Clonal complex (CC):** A group of sequence types (STs) whose members are linked to at least one other member by being identical for six of the seven **MLST** genes.

**Contigs:** A contiguous genomic sequence that is assembled from smaller DNA sequencing reads. Draft genomes comprise many such contigs of non-overlapping sequence.

**DEFRA:** Department for Environment, Food and Rural Affairs

**EBI:** European Bioinformatics Institute

**FASTQ:** A text-based format for storing nucleotide sequence

**FSA:** Food Standards Agency

**FSAS:** Food Standards Agency Scotland

**FSS:** Food Standards Scotland

**Host reservoir:** Domesticated and wild animal and bird species, in which *Campylobacter* is maintained as a source of human infection.

**HPS:** Health Protection Scotland

**IID:** Infectious Intestinal Disease

**Isolate:** A *Campylobacter* organism isolated and cultured from a specimen by microbiological methods.

**MCMC:** Markov Chain Monte Carlo

**MLST: Multi Locus Sequence Typing.**

**mlstdbNet:** Web based site which drives a large number of publicly available MLST databases.

**MEGA:** Molecular Evolutionary Genetics Analysis is computer software for conducting statistical analysis of molecular evolution and for constructing phylogenetic trees.

**MUSCLE:** Multiple Sequence Comparison by Log-Expectation is a nucleotide or protein sequence alignment tool

**NFU:** National Farmers Union

**ObSurv:** Outbreak surveillance system for infectious intestinal disease outbreaks in Scotland

**PanSeq:** Pan-genome sequence analysis tool for rapid analysis of core and accessory genomic regions

**PHE:** Public Health England

**Prevalence:** number of isolates belonging to the same species within a population. It is calculated as, total number of strains belonging to a specific species from a specific host ÷ total number of specimens collected from a specific host

**PWS:** Private water supply

**RESAS:** Rural and Environment Science and Analytical Services

**Retail chicken:** Chicken available for purchase from retail shops.

**Retail ready chicken:** Chicken processed and prepared at the abattoir to be transported to retail shops.

**Sequence type (ST):** For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate the alleles at each of the seven loci (location) of the housekeeping genes define the sequence type (ST). Example:

ST	<i>aspA</i>	<i>glnA</i>	<i>gltA</i>	<i>glyA</i>	<i>pgm</i>	<i>tkl</i>	<i>uncA</i>
1	2	1	54	3	4	1	5

**Specimen:** A swab containing *Campylobacter* growth from clinical, food or veterinary sources, a sample of animal or bird faeces, or a portion of retail food.

**Strain:** A collection of genotypically indistinguishable isolates. Usually of a common ST.

**Type / typing:** Often genotyping or phenotyping. The process of characterising an **isolate** at the level of its DNA or at the level of its expressed biological characters (biochemical, physiological, antigenic etc.)

**WGS:** Whole genome sequencing. Genome sequencing is a technique that allows figuring out the order of DNA nucleotides, or bases in a genome- the order of As', Cs', Gs' and Ts' that make up an organism's DNA



# 1. Introduction

## 1.1. Background

*Campylobacter* species are Gram-negative spiral, rod-shaped, or curved bacteria with small genomes (1.6–2.0 megabases) and can establish long-term associations with their hosts, sometimes with pathogenic consequences (Young, Davis et al. 2007). *Campylobacter* spp. predominantly live as a commensal in the gastrointestinal tracts of a wide range of birds and animals, including farmed and companion animals (Sahin, Kassem et al. 2015). Outbreaks are uncommon, but recent reports highlight that outbreaks of gastroenteritis in the UK can often be linked to *Campylobacter*. One such outbreak involved eating chicken liver pâté at a wedding in East England in 2014 (Edwards, Milne et al. 2014). The study found a statistically significant association between dose (amount of chicken liver pâté eaten) and the risk of campylobacteriosis. Six cases of campylobacteriosis were linked to the sale of raw milk in an outbreak in Kendal, Cumbria in 2016 (BBC News 2016). Human campylobacteriosis shows a marked seasonality with a peak starting during the early summer months. Sporadic *Campylobacter* cases comprise by far the majority of cases of *Campylobacter* infection. Case control studies have identified risk factors, including the handling of raw poultry and eating undercooked poultry, which play a major role in human campylobacteriosis (Sheppard, Dallas et al. 2009). Other risk factors accounting for a smaller proportion of sporadic illnesses include traveling abroad, eating barbecued foods and contact with dogs and cats (particularly juvenile pets or pets with diarrhoea) (Smith-Palmer, Cowden et al. 2010, Altekruise, Stern et al. 1999). Human exposure to *Campylobacter* via retail chicken is, however, considered to be the most important route of transmission of campylobacteriosis and so any changes in the population structure of campylobacters in the poultry reservoir must be taken into account when investigating human infection (Gormley, Macrae et al. 2008).

Campylobacteriosis typically lasts for about a week with symptoms in humans including self-limiting bouts of diarrhoea, cramping, abdominal pain, fever, headache, nausea and vomiting; rarer long-term conditions include arthritis and Guillain-Barré Syndrome, a demyelinating disorder resulting in acute neuromuscular paralysis (Acheson, Allos 2001).

The health and economic burden of IID in high-income countries is high. There are 17 million IID cases annually in the UK, of which 6 million are caused by the 12 most common pathogens. *Campylobacter* is the cause of almost half of all bacterial IID cases with 90% of cases attributed to *Campylobacter jejuni* and 10% to *Campylobacter coli* (Tam, O'Brien et al. 2003, Acheson, Allos 2001, Roux, Sproston et al. 2013). The Food Standards Agency (FSA) commissioned the IID-2 study to estimate, overall and by organism, the incidence of IID in the community, presenting to general practice (GP) and reported to national

surveillance. The study found that *Campylobacter* was the most common bacterial pathogen, with a rate of 9.3 cases per 100 person-years in the community, and 1.3 GP consultations per 1000 person-years and *Campylobacter* is responsible for 500,000 cases and 80,000 GP consultations annually (Tam, Rodrigues et al. 2012). As the number one cause of bacterial gastroenteritis in the UK, *Campylobacter* presents an economic burden of over £1 billion per annum (Winstanley, Haldenby et al. 2015).

The sources of *Campylobacter* on poultry farms were reviewed previously (Newell, Fearnley 2003), but there has been no comprehensive review of the effectiveness and feasibility of on-farm interventions against *Campylobacter*. The ways by which farms can be colonised is through horizontal and vertical transmission, contaminated water, ineffective broiler house cleansing and disinfection, aerosols through vents that take in air from potentially contaminated areas, human traffic in and out of a broiler house and contaminated environments with wild and domesticated animals and insect vectors around the broiler house (Newell, Fearnley 2003). The sources of *Campylobacter* and routes of transmission on the poultry farm were reviewed critically in a study with the options for different types of intervention (Newell, Elvers et al. 2011). It was concluded that, in most instances, biosecurity on conventional broiler farms can be enhanced and this should contribute to the reduction of flock colonisation but complementary, non-biosecurity-based approaches were also required in order to maximise the reduction of *Campylobacter*-positive flocks at the farm level (Newell, Elvers et al. 2011).

Further along the farm-to-fork chain, at abattoir, changes in the carriage of *Campylobacter* strains on poultry carcasses during processing was assessed and it was concluded that some *Campylobacter* strains survive poultry processing better than others, perhaps due to greater tolerance to environmental stresses (Newell, Shreeve et al. 2001). The more robust subtypes contaminated the abattoir environment, surviving through carcass chilling, and even carrying over onto subsequent flocks. It was also observed that some *Campylobacter*-negative flocks reached the abattoir but the carcasses were subsequently contaminated by *Campylobacter* during processing. *Campylobacter* belonging to the same subtype as those recovered from the carcasses were isolated from the crates used in bird transport and sometimes crates were contaminated even before the birds were loaded (Newell, Shreeve et al. 2001).

The *Campylobacter* Risk Management programme (CRMP) was implemented as part of the FSA's 2010-2015 Foodborne Disease Strategy as a result of the continuing rise in the number of laboratory confirmed cases of *Campylobacter*. Control of campylobacteriosis continues to be a high priority in the UK, as highlighted by the scientific evidence and FSA's 2015-20 delivery plan and FSS strategic plan and strategy to reduce foodborne illness (FSA 2016a, FSS 2016a, FSS 2016b). *Campylobacter* research funded under the FSA's foodborne disease research programme aimed to provide robust information on the presence, growth, survival and elimination of micro-organisms throughout the food chain

and to provide the extent, distribution, causes and costs of foodborne disease. Research was commissioned in support of the FSA's strategy to achieve a reduction in the incidence of foodborne disease by 20% over a five-year period. Within Scotland, Food Standards Scotland (FSS) and its predecessors, Food Standards Agency Scotland (FSAS), have been commissioning research since 2005 to improve understanding of human campylobacteriosis in the Scottish population. These projects were aimed at understanding the cause of human campylobacteriosis in the Scottish population and include CaMPS (2005-2007), iCaMPS1 (2010-2011), iCaMPS2 (2011-2012) and iCaMPS3 (2012-2015).

### **Campylobacter strategy: Food Standards Scotland**

The purpose of FSS is to ensure that information and advice on food safety and standards, nutrition and labelling is evidence-based, independent and consistent and focused towards public health and consumer protection with engagement of consumers and stakeholders (FSS 2016b). The promotion of health through the reduction of human campylobacteriosis is one of the major responsibilities shared by the UK and Scottish governments. *Campylobacter* reduction has been a key food safety priority for the FSS since 2000, and continues to be an important objective in its strategic plan (McElhiney 2016b). By elucidating the burden of foodborne illness in Scotland, FSS has not only helped in understanding the consumption habits and behaviours of Scottish consumers but played an important role in protecting consumers who are at the greatest risk (FSS 2016a). There are on-going consultations on the food surveillance strategy developed by FSS to understand the prevalence of contaminants in the food chain and the risks associated with foods produced in Scotland compared with imported products which may be implemented as the years progress (FSS 2017a, FSS 2017b). With the advent of next generation sequencing, FSS continues to focus on the application of approaches which can improve our understanding of transmission of foodborne pathogens within the environment and its implications on the food chain with the purpose of developing appropriate performance measures that are capable of evaluating impact (FSS 2016a). Food safety regulatory bodies and organisations in the food production sector are constantly identifying and implementing interventions in attempts to reduce campylobacter in chicken and other foods. A working group on *Campylobacter* was established in August 2009 as a joint industry and government group with bodies that included the British Poultry Council (BPC), the National Farmers Union (NFU) the British Retail Consortium (BRC), the FSA, and DEFRA, which aimed at identifying interventions that can reduce campylobacteriosis through a Joint Action Plan during the 2009-2014 period. The key activities of the action plan relate to on-farm, transport, processing, retail, consumer and catering sector trials and interventions, as well as surveillance and monitoring. The overarching aim of FSS's strategy to 2021 is to create a beneficial and trustworthy food and drink environment in Scotland that is aimed at protecting consumers (FSS 2016b).

### **UK-wide survey of *Campylobacter* on retail broilers**

An on-going study conducted by the FSA since February 2014 is looking at the prevalence and levels of *Campylobacter* contamination in whole chilled chickens and their packaging. It is thought that chickens with >1000 cfu/g of *Campylobacter* contamination are the most likely to infect consumers (FSA 2014).

In Year 1 (2014-2015), more than 4000 whole chicken bought from UK retail outlets and other stores were tested. It was observed that 73% of chickens tested positive for *Campylobacter* with 19% of chickens positive for *Campylobacter* at the highest level of contamination (>1000 cfu/g). Of the packaging tested 0.1% (n=5) was positive at the highest level of contamination and 7% of packaging tested positive for the presence of *Campylobacter* (FSA 2015). Over the period of Year 1 survey none of the retailers achieved the joint industry target for reducing *Campylobacter* (FSA 2015).

In Year 2 (2015-2016), more than 3000 whole chicken bought from UK retail outlets and other stores were tested. A newly introduced abattoir intervention was to trim back the chicken neck skin (neck flap) which is typically a highly contaminated part of the carcass. This is because at evisceration, faecal matter and *Campylobacter* present on the rest of the skin of the carcass can all drip down to collect on this neck flap area as the bird is hung upside down. It can also contaminate the evisceration equipment. The smaller neck flaps required an increase in the amount of breast skin used in analyses (FSA 2016b); hence like-for-like comparisons could not be made with previous data. Overall, 7% of chickens tested positive for *Campylobacter* within the highest band of contamination compared with 19% in year 1.

Interventions, including improved biosecurity, use of a secondary scald tank, SonoSteam, and the trimming of neck skins introduced by some retailers to reduce levels of *Campylobacter*, may be helping to deliver the lower incidence of campylobacteriosis seen recently (FSA 2016b). The SonoSteam process typically achieved a *Campylobacter* reduction on breast skin of 0.85 to 0.95 log over two trials conducted by Faccenda but it was more difficult to optimise to achieve a consistent *Campylobacter* reduction on neck skin (Keeble 2015). The trimming of the neck skin, the most highly contaminated skin area, means chickens are carrying less *Campylobacter* and impact of this intervention will be reviewed to ensure survey results remain robust (FSA 2016b).

### **Campylobacteriosis in England and Wales**

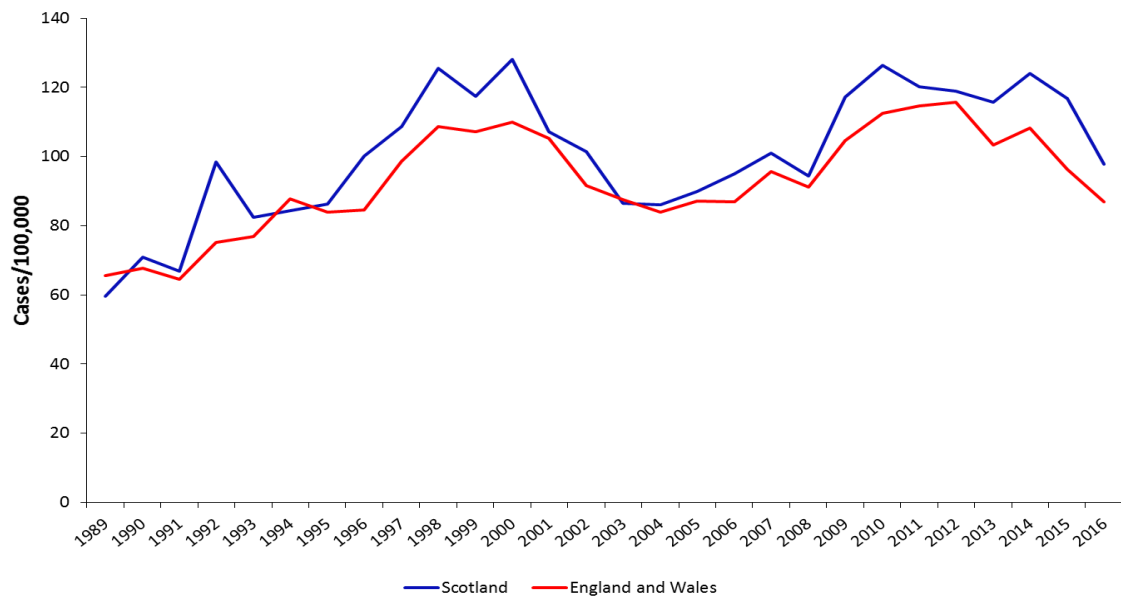
A descriptive study reviewing 1 million cases in England and Wales between 1989 and 2011 found an increase in campylobacteriosis in all age groups between 2004 and 2011, where the prevalence was higher in young children than in other ages (Nichols, Richardson et al. 2012). The summer peaks in young children were more prominent than those in other ages (Nichols, Richardson et al. 2012). There was a marked long-term increase in the

percentage of cases in people older than 50 years, in areas with a low population density, of low deprivation and with a lower percentage of people from a non-white background (Nichols, Richardson et al. 2012). The study also found that several mechanisms influencing seasonality, age distribution, population density, socioeconomic and long-term differences are diverse with their relative contributions yet to be established (Nichols, Richardson et al. 2012). The prevalence in children could be higher as they are more likely than adults to attend a physician and have a specimen taken as a result of triaging referrals associated with NHS Direct, and only a small proportion of people with diarrhoea respond to NHS Direct (Gillespie, O'Brien et al. 2009, Tam, Rodrigues et al. 2012). There has been increase in campylobacteriosis in the older generation (>50 years) with cases are more likely to use proton pump inhibitors drugs which may make them susceptible to infection with *Campylobacter* and/or indicate an underlying problem in gut health (Nichols, Richardson et al. 2012). The increase in population size and the decreased mortality rate among senior adults may also influence the year on year trends. Improved isolation methods of culturing with improved media and conditions may also have led to better isolation of cases with infection (Nichols, Richardson et al. 2012). Another important influencing factor is that isolation by culture alone for community cases was better than those with GP presentation when compared with culture and PCR (Nichols, Richardson et al. 2012).

### **Campylobacteriosis in Scotland**

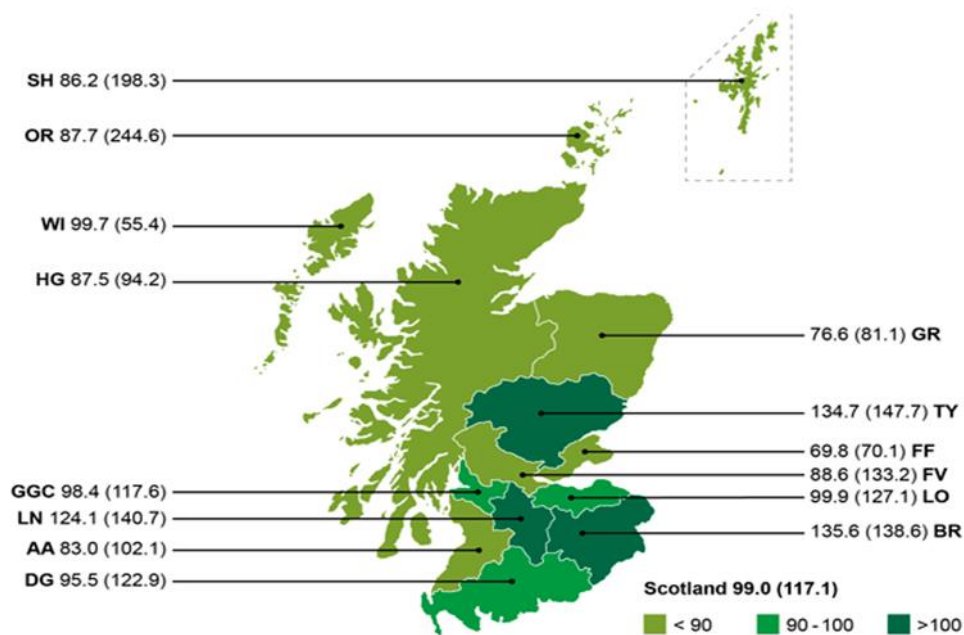
There are typically 43,000 cases, 5,800 GP presentations and 500 hospitalisations due to foodborne illnesses in Scotland each year (McElhiney 2016a, HPS 2015). *Campylobacter* remains the main cause of bacterial gastroenteritis in Scotland (McElhiney 2016a). In 2016, 5296 laboratory reports of *Campylobacter* infection were notified to HPS. This was a decrease of 968 (15.5%) cases compared to 2015 when 6262 isolates were reported (Figure 1, 2) (HPS 2017). This was the second consecutive year in which there had been a decline in reports of human campylobacteriosis, with the reports in 2016 representing a decline of 1340 (20.2%)(HPS 2017). As seen in Figure 1, the annual incidences across the countries of the UK tend to track each other, suggesting close underlying origins. It has been shown that laboratory reports probably underestimate the incidence of campylobacteriosis by a factor of 9.3 due to reporting bias (95% CI, 6-14.3) so the actual number of cases in the community is much greater (HPS., FSAS 2013).

Figure 1. Annual incidence of campylobacteriosis based on cases reported in Scotland and in England & Wales.



Annual incidence of *Campylobacter* infection per 100,000 population in Scotland (blue; data from HPS) and England and Wales (red, data from PHE). Extent of study periods indicated.

Figure 2. Annual incidence of campylobacteriosis in 2016 (2015) by Scottish region.



Annual incidence of *Campylobacter* infection per 100,000 population. Data from HPS campylobacteriosis report for 2016.

In Scotland, the overall rate of campylobacteriosis in 2016 was 99 per 100,000, compared to 117 in 2015 (Figure 1). The decline in campylobacteriosis was observed in all mainland NHS boards compared to the rates in 2015 (Figure 2). As in previous years rates were higher in children under one year of age compared with older children and young adults and increased among those 50 years and older (HPS 2017). Overall rates are higher among males, with 109 per 100,000 compared to 89 per 100,000 for females (HPS 2017). This higher rate among males was observed in all apart from one age band (35-39 years) (HPS 2017). In females under one year of age the rate was 59/100,000 compared to 115/100,000 observed in males (HPS 2017).

A total of 35 *Campylobacter* outbreaks have been reported since ObSurv (surveillance system for all general outbreaks of infectious intestinal disease in Scotland) was established in 1996 (HPS 2017). There were no general outbreaks of campylobacteriosis in 2016 as reported to ObSurv. Likewise no general outbreaks had been reported in 2015 and one small outbreak in 2014 associated with chicken liver pate.

### **Campylobacteriosis in Grampian**

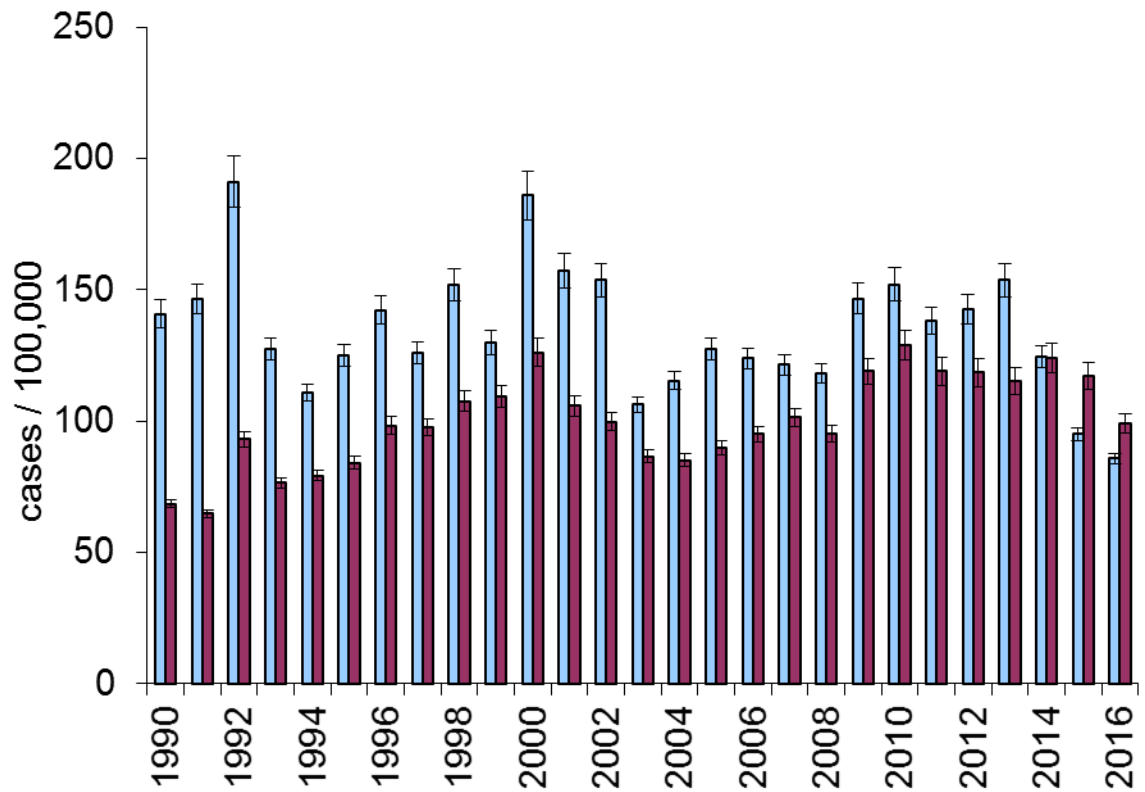
Previous studies funded by FSS on campylobacteriosis in Scotland (CaMPS (2005-2007), iCaMPS1 (2010-2011), iCaMPS2 (2011-2012) and iCaMPS3 (2012-2015)) have improved our understanding on the causes of human campylobacteriosis in the Scottish population. Most of these studies have focused on cases in the Grampian region where the epidemiological picture has been considered to be representative of Scotland as a whole (Figures 3, 4, 5). It is worthy to note that the incidence in Grampian was high during 2011-2014 and decreased in 2015 and 2016 (Figures 2, 3, 4). In Scotland as a whole there was a slight decline in 2016 compared to the period 2009-2015 (Figure 3).

The decline in cases can be seen in all months of the year, but with the exception that the Welsh cases declined only in the Jan-Apr period. A significant increase ( $p < 0.05$ ) was seen in 15-64 age group from 2011-2012 followed by decrease in 2013 and significant drop in 2014 and 2015 ( $p < 0.05$ ). Over the summer, the trend in the 65+ age group during 2011-2013 remained fairly constant followed by a decrease from 2014-2016 (data not shown). These studies highlighted that retail chicken was more important than cattle and sheep as the main source of human infection. Source-attribution modelling in iCaMPS3 using chicken, cattle, sheep, pig and wild birds sources showed that chicken was the largest source for human infection at 55% to 75%, followed by 10% to 22% for sheep, 10% for cattle, 0% to 8% for pigs and 4% to 8% for wild birds (Lopes, Forbes et al. 2016).

This current study commissioned by FSS is an extension of iCaMPS3 (FSS00017) and as such, it builds on the findings made in these previous reports. It employs source attribution and molecular epidemiology to measure the impact of interventions on human campylobacteriosis in Scotland. It also focuses on the

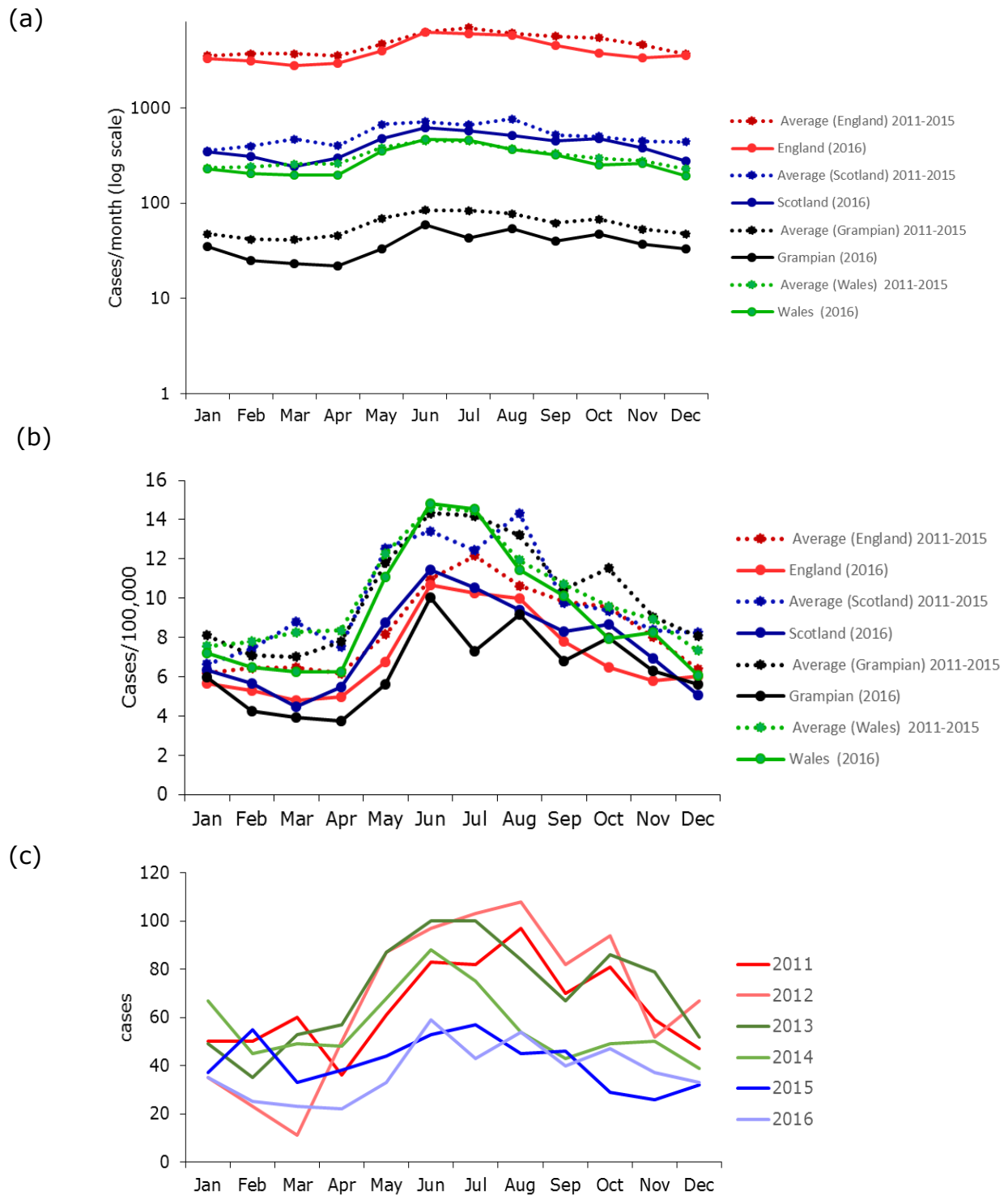
role of Scottish broiler production on human campylobacteriosis cases in Grampian.

Figure 3. Annual incidence of campylobacteriosis in Scotland and in Grampian from 1990 to 2016.



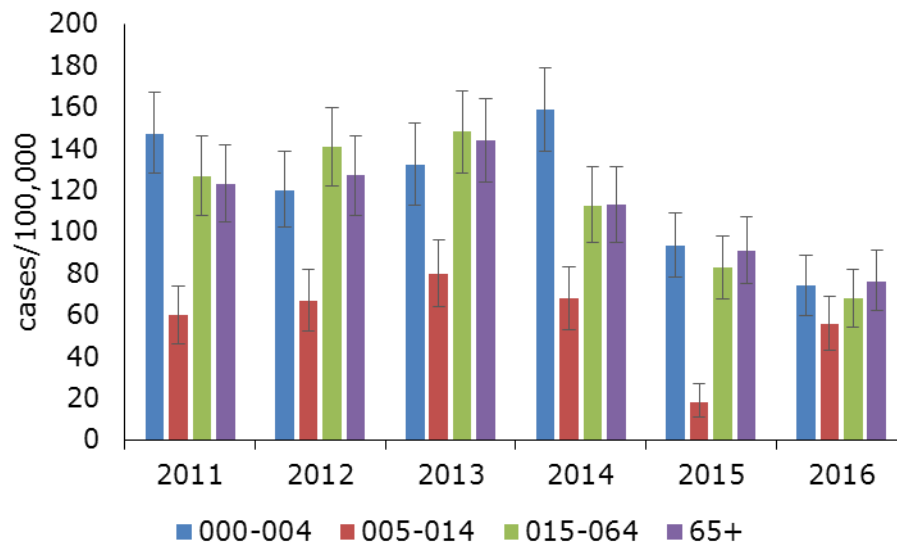
Annual incidence of campylobacteriosis per 100,000 population. Scottish data (purple) from HPS, Grampian data (blue) from ARI. Error bars are 95% CI calculated by bootstrapping.

Figure 4. Cases of campylobacteriosis in England, Wales, Scotland and Grampian.



(a) *Campylobacter* cases per month (Log scale). (b) Incidence of campylobacteriosis (cases/100,000). (c) *Campylobacter* cases per month in Grampian from 2011-2016.

Figure 5. Age structured incidence of campylobacteriosis in Grampian for 2011- 2016.



Data from ARI. Error bars are 95% CI calculated by bootstrapping.

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## 1.2. Aims

The contemporaneous sampling and collection of *Campylobacter* isolates from human, food and environmental sources, in combination with the molecular genotyping of isolates and use of state of the art molecular attribution modelling allows an integrated and quantitated determination of the importance of different *Campylobacter* sources in human campylobacteriosis.

This present study characterised the role of Scottish broiler production on human campylobacteriosis cases and assessed any changes in source attribution of campylobacteriosis in Grampian from April 2015 to December 2016. This was based on contemporaneous sampling and collection of clinical isolates obtained from cases in Grampian and chicken isolates from a Scottish abattoir. Previously typed isolates from food and environmental sources have also been used for comparison and in source attribution analyses.

The two broad themes of this current study, with specific topics within these were:

***Campylobacter* at farm and abattoir.** To consider:

1. whether the abattoir's *Campylobacter* strains are distinct from chicken strains from the rest of the UK.
2. the evidence of transmission between farms.
3. the usage of antimicrobials on farms.
4. cross contamination between batches (flocks) at abattoir.
5. the impact of abattoir interventions on *Campylobacter* on retail ready broilers.

**Human campylobacteriosis in Grampian.** To consider:

1. the *Campylobacter* strains in human campylobacteriosis.
2. *Campylobacter jejuni* and *C. coli* epidemiology.
3. the sources of human campylobacteriosis.
4. seasonal trends and the risk factors for human campylobacteriosis.
5. the sensitivity of molecular analyses for detecting a drop in the proportion of cases attributable to chicken.
6. case clustering detection between human and chicken isolates.

The implications of this study's findings for FSS are discussed.

## 2. Materials and Methods

### 2.1. Collection of isolates

All available presumptively identified clinical isolates in Grampian for the period Apr 2015 – Dec 2016 (n=821) were received from Aberdeen Royal Infirmary diagnostic microbiology laboratory of which 785 were identified as either *C. jejuni* or *C. coli* and were typed (Table 1). Contemporaneous *Campylobacter* isolates from chickens were also collected from a Scottish abattoir which is a slaughtering, cutting and whole bird retail packaging site which specialises in processing of whole birds, skin on/skinless fillets and standard welfare, free range birds (Table 1).

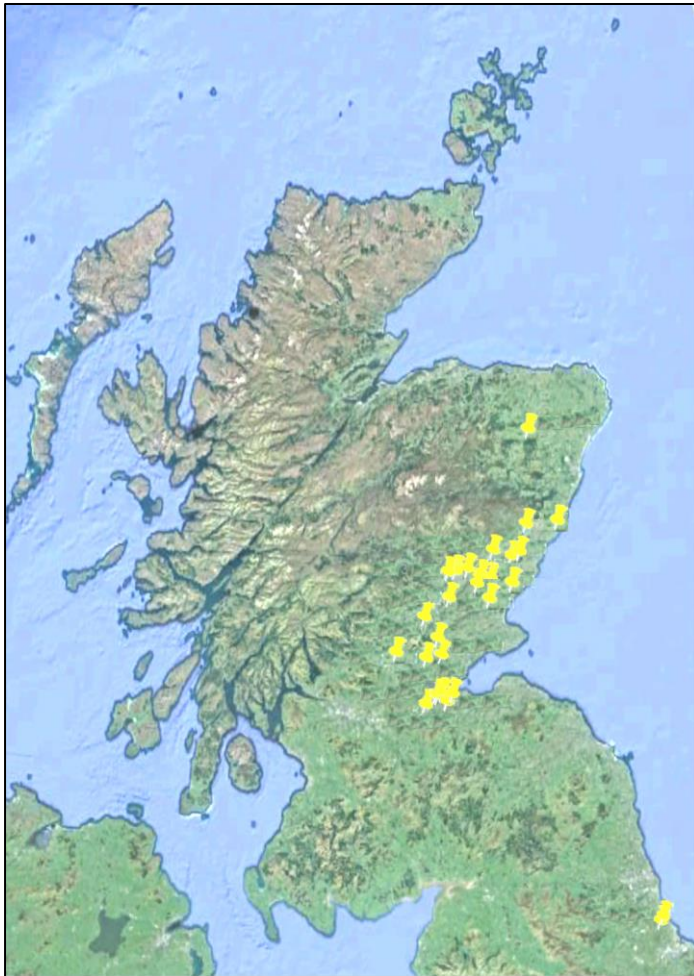
Table 1. Specimens collected: *Campylobacter* spp. isolated with complete 7-locus MLST genotype.

	Samples	Isolates Typed as <i>C. jejuni/C. coli</i>
Clinical (Jan 2016-Dec 2016)	451	439
Clinical (Apr 2015-Dec 2015)	370	346
Abattoir sourced chicken	304	252
Abattoir sourced caeca	152	136

Chicken and caecal isolates from Nov 2015-Dec 2016.

From each tested flock two whole birds and five pairs of full (i.e. with contents intact) caeca were supplied by staff at the Scottish abattoir. Three flocks per week were tested using birds processed on a Sunday or Monday as this allowed appropriate time for the laboratory processing of the samples for the isolation of *Campylobacter*. Where possible, farms were not resampled within a three month period and if this was not possible then different sheds/houses were selected. A completed food chain information sheet (FCI) accompanied the samples in transit. The source farms, 34 in total, of these broilers were located in East Scotland and the North of England (Figure 6).

Figure 6. Location of broiler farms tested.



The *Campylobacter* isolates from whole birds and caeca were cultured at the University of Aberdeen by microbiological culture methods as described in previous studies (Forbes, Horne 2009). Each whole bird was incubated at ambient temperature in 500 mL enrichment broth for 1 hr with occasional agitation. Caecal specimens (25g) were homogenised in 300 mL of *Campylobacter* enrichment broth. Further details on how the isolation and culture was carried out has been published in earlier reports (Forbes, Horne 2009).

Enumeration of *Campylobacter* from whole birds and caecal sample was by direct plating, and further isolations were made after extended incubation in enrichment broth. The neat culture (0.1 mL) and a 10-fold dilution for both whole bird washes and caecal homogenates were plated onto modified charcoal-cefoperazone-deoxycholate (mCCDA) plates, and the plates incubated as below. For enrichment growth, the remaining volume of enrichment broth was incubated under microaerobic conditions at 37°C for 2 days after which 0.1 mL of broth was removed and plated onto mCCDA plates and in incubated further for 48 hrs. The colonies were enumerated as cfu/gram.

The presence or absence of *Campylobacter* colonies was determined visually and confirmed by visible agglutination with Microscreen *Campylobacter* latex confirmation assay (product code M46, Microgen Bioproducts). Colony growth from both isolation procedures was harvested from blood agar plates, archived and DNA extraction was performed as described in section 2.2.

Excreted wild bird faeces were collected on charcoal swabs by several volunteers, mostly as part of on-going bird ringing studies from Grampian and were couriered or posted to the University of Aberdeen during the iCaMPS3 and the current study. The swabs were streaked directly onto both mCCDA and blood agar plates and were also put into universal tubes containing 20 mL of enrichment broth. Both plates and enrichment were incubated for 48 hours under microaerobic conditions. After 2 days, plates were checked for visible colonies of *Campylobacter*. If plates were negative, 0.1 mL of enrichment was then plated to check for the presence of *Campylobacter* following incubation at 37°C for 48 hrs under microaerobic conditions.

## 2.2. Genomic DNA extraction, Whole genome sequencing and Strain typing

Genomic DNA was extracted from a loopful of fresh colonies on blood agar plates using the Promega Wizard Genomic DNA Purification Kit (Catalogue #A1125). It was checked for quality by running on 1.5% agarose gels and submitted to the Wellcome Trust Centre for Human Genetics (WTCHG) in Oxford for Whole Genome Sequencing (WGS).

All of the DNA samples went through an automated genomic library preparation designed for high numbers of low complexity genomic samples submitted in a 96 well format. The samples were normalised to ~30 ng/ $\mu$ L in 100  $\mu$ L volume. The libraries were sequenced on Illumina HiSeq4000 on 150bp paired end runs. Typically, a single lane which has 90 to 96 multiplexed samples produced 50 Gb data (in the form of 150bp reads). Assuming the data is distributed evenly between all the samples, each isolate will produce about 400Mb. The paired read files were *de novo* assembled using the Velvet assembler via an established pipeline by Centre for Genome-Enabled Biology and Medicine (CGEBM) at the University of Aberdeen.

### **Bacterial Isolate Genome Sequence Database (BIGSdb)**

The assemblies were matched with the required meta-data and the sequences were uploaded to BIGSdb (Bacterial Isolate Genome Sequence Database) (Jolley, Maiden 2010), a bioinformatic pipeline developed at University of Oxford. BIGSdb is software designed to store and analyse sequence data for bacterial isolates. Any number of sequences can be linked to isolate records - these can be small contigs assembled from dideoxy sequencing through to whole genomes (complete or multiple contigs generated from parallel sequencing technologies such as 454 or Illumina Solexa). All the functionality of mlstdbNet and AgdbNet software has been incorporated into BIGSdb and this software will be used to eventually host all the databases on pubmlst.org. BIGSdb extends the principle of multilocus sequence typing (MLST) to genomic data, where large numbers of loci can be defined, with alleles assigned by reference to sequence definition databases. Loci can be grouped into schemes so that types can be defined by combinations of allelic profiles, a concept analogous to MLST.

### **Multilocus sequence typing (MLST)**

MLST is a technique for characterising isolates of bacterial species using the sequences of internal fragments of seven house-keeping genes (Maiden, Bygraves et al. 1998). The variation in bacterial house-keeping genes provides many alleles per locus, allowing billions of distinct allelic profiles to be distinguished using seven house-keeping loci (Urwin, Maiden 2003). MLST and the related method of multilocus sequence analysis (MLSA) (Gevers, Cohan et al. 2005) are advantageous as they have a high resolving power. The great advantage of MLST is that sequence data are unambiguous and the allelic

profiles of isolates can easily be compared to those in a large central database using the internet and precise characterisation can be made even if they cannot be cultured (Urwin, Maiden 2003).

### **Strain typing of clinical and chicken isolates**

The whole genome sequences of the isolates were used to classify them to species level, and for *C. jejuni* and *C. coli* into strain types using MLST. Allele numbers and sequence types (ST) were assigned using the public *Campylobacter* PubMLST database <http://publmst.org/Campylobacter/>. MLST was carried out on all isolates and this is summarised in Table 1. Not all presumptive isolates were confirmed to be *Campylobacter jejuni/coli* by MLST and this was due to the challenge in confirmation by visual inspection of colonies and false positive results by latex sero-agglutination.

## 2.3. Data archiving

The Microsoft Access database at the University of Aberdeen is central to the functionality of this study, and its linkage to the previous studies. The database includes metadata associated with the original specimens, the resultant microbiology, the genomic metadata, the typing data and also the associated case questionnaire data. This relational database allows the linkage of disparate fields enabling subsequent analyses. The database is backed up regularly and images are archived prior to the addition of new data. All of the sequencing raw read files (FASTQ format) are uploaded to the publicly available database at the European Bioinformatics Institute (EBI).

## 2.4. Patient questionnaires

Questionnaires have continued to be sent to all cases in Grampian by the Health Protection Team of Grampian Health Board. A total of 19 questions were asked out of which 11 were close-option questions. A sample of the questionnaire can be found in Appendix 1. This study was approved by North of Scotland Research Ethics Committee (REC reference number 11/S0802/1). The information collected by the questionnaire is as outlined below:

1. Basic information on who was ill: age, gender, first 5 digits of the post-code and employment status.
2. Basic information on illness: start date and end date of symptoms, type of symptoms experienced (diarrhoea, bloody stools, vomiting, abdominal pain or other symptoms), if the subject was admitted to the hospital and if anyone else in the household was ill during that time.
3. Foreign travel: Information on whether the subject had an overnight stay abroad and if anyone else in that area experienced similar illness.

4. Exposure: If the case, in the 5 days prior to onset of illness had contact with animals, had used a private water supply, if they or anyone else in the household had handled raw chicken, eaten chicken or chicken liver pate, if chicken was purchased and prepared at home and which supermarket it was bought from, in the five days before onset of illness.

Aberdeen Royal Infirmary laboratory provided information on patient age, gender, post-code and sample received date. The questionnaire information had the date of birth of the patient, age, gender, start and end date of the illness and date on which the form was completed. This information was used to link the questionnaire (case) with a specific isolate.

The information obtained from the paper questionnaires was input into an Access database and then processed in Excel for performing empirical analyses.

## 2.5. Analyses

### **Statistical analyses and aggregation of data**

Data aggregation is a process in which information is gathered and expressed in a summary form for purposes such as statistical analysis. A common aggregation purpose is to get more information about particular groups based on specific variables such as age, sex, gender etc. The statistical analyses performed in this report are by aggregating different ages within an age group band consisting of 0-4 (pre-school/children), 5-14 (school children), 15-64 (adults) and 65+ (senior adults). This is done in order to maintain uniformity with the previous report and because risk factors associated with particular groups vary depending on age. Some examples of these are listed below:

- In pre-school children infection can occur as a result of touching *Campylobacter* contaminated surfaces as children tend to put their fingers in their mouths.
- School children may contract infection due to farm animal contact as a result of school trips for recreation and learning purposes
- Adults can acquire infection as a result of washing/handling of raw chicken or from eating undercooked meat
- Senior adults can acquire infection as a result of as a result of washing/handling of raw chicken or from eating undercooked meat and infection can have more severe consequences due to age and/or compromised immune status.

Data are generally aggregated by year and by a range of factors as described above. It should be noted that if the aggregations result in very small groups then there will be lower statistical power. Confidence intervals (95%) are determined by boot strapping (10,000 iterations). If the confidence intervals do not overlap (e.g. between two years for a particular factor) then the comparison is significantly different ( $p < 0.05$ ).

## **Molecular characterisation of source and clinical *Campylobacter* isolates: Host reservoir isolate datasets**

In previous reports issued on projects commissioned by FSS and FSA, food and animal isolates from the 2005-06 Scottish study and the iCaMPS 2010-12 period were used in the molecular attribution analyses. This was because there were insufficient isolates to appreciably change the resultant attribution scores and enable comparison of attribution results with the previous report. Strain prevalences change over time (Ogden, Dallas et al. 2009) however incorporating data that is recent but not contemporaneous is not expected to alter findings. This report makes use of the iCaMPS3 host dataset which includes pig, chicken, cattle, sheep and wild bird isolates. Additionally wild bird isolates from 2015-2016 (n=38) have also been included (Table 2).

Table 2. Isolate datasets used in molecular attribution calculations.

<b>Host dataset post-2012 Grampian-wide hosts</b>		
Cattle	2012-2015	257
Sheep	2012-2015	296
Chicken	2012-2015	635
Wild Birds	2005-2009 2015-2016	197 38
Pigs	2012-2015	110
Clinical	Apr 2015- Dec 2016	785

Host dataset 2012-2015 are as used in iCaMPS3 study. Clinical isolates for 2015-2016 are this iCaMPS4 study. 38 wild bird isolates from 2015-2016 are also included.

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### **Attribution of human cases to the sources**

Attribution by microbial strain typing is now a well-recognised epidemiological tool. The term "source attribution" has been defined as: "...the partitioning of the human disease burden of one or more foodborne infections to specific source, where the term *source* includes animal reservoirs and vehicles (e.g. foods)" (Pires, Evers et al. 2009).

Furthermore, the microbial strain typing methodology uses the distribution of different types of strains in each of the sources and compares this with the distribution found in humans. This can be done in terms of simple proportions (e.g. the Dutch model (French 2008)) or using Bayesian stochastic methods (e.g. STRUCTURE)(Pritchard, Stephens et al. 2000). Currently, there are five main techniques for attributing disease on a population level using microbial sub-typing (EFSA 2010). From previous reports, two representative self-attribution methods were selected for use here and are detailed below.

Although the attribution statistics are different, the results are consistent, which enhances the robustness of the conclusion that chicken is the principal source of human disease associated with *Campylobacter*.

**STRUCTURE** (Pritchard, Stephens et al. 2000). This is a Bayesian clustering model designed to infer population structure and to attribute individuals to population groups. The program can use MLST genotyping data. Each isolate is attributed on the basis of a training dataset consisting of isolates from known populations. The algorithm calculates the frequency of each particular sequence type in each population taking into account the uncertainty due to the sample size. Based on these frequencies, the probability to belong to a population group/reservoir is calculated following multiple iterative steps using the Markov chain Monte Carlo (MCMC) method for the estimation of frequencies. The programme has the option to consider the allele independently (no-admixture model – independent alleles) and starts with equal frequencies for each isolate type. Following an initial number of MCMC burn-in steps (e.g. 1000), further iterations (e.g. 10000) are used for estimation of the probabilities that an isolate belongs to each particular population being considered (e.g. cattle, sheep, chicken, turkey etc.). To enable the largest reference dataset to be used (often datasets are small due to the cost of typing many isolates) only one ST is selected at a time from the unknown dataset by using the jackknife method, which is a resampling technique especially useful for variance and bias estimation. Jackknife predates other common resampling methods such as the bootstrap. The jackknife estimator of a parameter is found by systematically leaving out each observation from a dataset and calculating the estimate and then finding the average of these calculations. This process is repeated to enable multiple estimations of the same sequence type so that uncertainty in the attribution scores can be determined.

STRUCTURE can be used at ST or allele level (Table 3) as it incorporates uncertainty and takes account of sample size. But gives a more realistic estimation of the attribution to a specific reservoir than the Dutch Model. Also, like the Dutch Model, at allele level it can assign human cases that have STs that are not found in the animal reservoirs. However, it is highly time consuming and does not consider any exposure to risk factors or the viability of pathogens.

Table 3. Molecular attribution models used in the current study.

Model	Average correct self-attribution (%)
STRUCTURE (alleles)	65.7
Asymmetric Island (alleles)	54.4

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Average correct self-attribution (%) scores are from iCaMPS3 report.

**The Asymmetric Island (AI) Model** (Wilson, Gabriel et al. 2008). This model incorporates a Bayesian approach and uses the allelic profile of the sequence subtypes to reconstruct the genealogical history of the isolates. The host populations are considered to exist on separate “islands” (e.g. the sheep island). Mutations and recombination occur on each island. Migrations from between each reservoir (island) into the human population are used to estimate the degree of attribution to each source. This model has previously been applied to MLST data from England (Wilson, Gabriel et al. 2008), Scotland (Sheppard, Dallas et al. 2009) and New Zealand where 56%, 78% and 75% of human cases were attributed to poultry respectively.

The AI model incorporates recombination and mutation, uses MLST data at the allele level and achieves relatively high values for self-attribution. However, the model appears to be complicated and the current explanations of its operation difficult to comprehend. The Asymmetric Island model assigns each human case to the potential source populations on the basis of DNA sequence similarity. By comparing human isolates to a panel of reference sequences of known source (e.g. cattle, sheep, chickens, pigs, wild birds and turkey), each human case can be assigned a probability of originating in each source population. The source attribution probabilities are calculated using a statistical model of the way the DNA sequences evolve in the populations of bacteria. In the statistical model, there are parameters representing the processes of mutation, DNA exchange between bacteria (recombination or horizontal gene transfer) and zoonotic transmission between populations. These processes lead to differences in gene frequencies between the source populations, facilitating source attribution. The model can be modified, by estimating the parameters exclusively from the sequences of known sources, before using it to calculate source attribution probabilities for *Campylobacter* obtained from human clinical samples. The iCaMPS 2011-2012 report established that the use of allelic, rather than the coarser ST typing of strains, gave the most useful attributions; and has therefore been employed in this report.

### **Self-attribution**

Self-attribution or correct attribution is a key performance measure for the models used in this study (Smid, Mughini Gras et al. 2013, Sheppard, Dallas et al. 2009). This is the average percentage accuracy that any given isolate from a reservoir can be correctly attributed back to its own reservoir. This can be performed in a number of ways. By chance you would expect a correct self-attribution of 20% and 14% for 5 and 7 sources respectively. The average correct attribution percentages for each model shows that STRUCTURE-alleles has the highest (~66%) average correct attribution score (Table 3). STRUCTURE-alleles gives an output typical for the other tests, whilst for Asymmetric Island (AI) the underlying assumptions and methodology are rather different – and so this gives different outcomes. Ranges are reported as between 62-97% for between 5-7 hosts (Sheppard, Dallas et al. 2009, Wilson, Gabriel et al. 2008) and 38-70% for STRUCTURE (Sheppard, Dallas et al.

2009). Taking into account all the various models we found that Dutch alleles, STRUCTURE alleles and AI gave the best scores in self-attribution of isolates and so these latter two have been used to give a balanced overview of source attribution. The classical Dutch model was excluded because it considers only the overlap between clinical and animals as a whole group (thus a clinical isolate can be attributed only to one host and not to other host reservoirs). In classical Dutch model clinical STs that are not found in animals should be removed. AI and STRUCTURE use Bayesian stochastic methods which are advantageous over the Dutch model. It is important to note that modelling provides insights into what would ideally happen but it may not reflect exactly what is happening. In self-attribution model-validation tests, STRUCTURE consistently gave a higher probability (65.7%) than Asymmetric Island model (54.4%) of correct assignment of an isolate to origin at the allelic level (Table 3).

## 3. Results and Discussion

### 3.1. Retrospective analysis of *Campylobacter* strains from retail chicken purchased in Grampian, with a focus on Scottish abattoirs

A review of the *Campylobacter* strains from Scottish abattoirs, which had been collected over the period 2005 – 2015 from chicken purchased at retail in Grampian, was undertaken to identify whether there were strains more geographically restricted to this region compared to the rest of the UK (Table 4).

Over the period 1,395 retail chicken samples had been tested with 20% of these originating from Scottish abattoirs (Table 4, a). From these samples the overall prevalence of *Campylobacter* was found to be similar, 78%, at the Scottish abattoirs as from the rest of the UK (Table 4, b).

Typing of the 998 isolates from the contiguous seven year period from 2009 (Table 5) did not indicate a statistically significant geographical bias in strain distribution; ST50 is apparently of lower prevalence from the Scottish abattoirs, whilst ST5136 was higher, however sample sizes are too small to confirm statistically significant differences. ST5136 is used as an exemplar as it is a strain that was previously unknown and only emerged in the UK (Oxfordshire) in 2010 and so its distribution throughout the UK has occurred within the timeframe of this study. This strain is being studied as it is one of the most common strains of *Campylobacter* that infects humans. Moreover this strain also has several resistances associated with it that may provide it with a selective advantage over the sensitive counterparts. Hence monitoring the trends associated with this strain is important as increase or decrease in abundances can be paralleled to human infection associated with this strain. The abundance of this strain (Table 6) across the UK is seen to increase then decline. For the Scottish abattoirs however no clear trend is apparent due to paucity of data.

Table 4. Retail chicken samples collected from 2005 - 2015 and *Campylobacter* positivity by abattoir.

(a) Period	Scottish	Other	TOTAL	%	
				Scottish	% Other
2005Jul - 2006Jul	16	130	<b>146</b>	11%	89%
2007					
2008					
2009	5	39	<b>44</b>	11%	89%
2010	33	57	<b>90</b>	37%	63%
2011	79	163	<b>242</b>	33%	67%
2012	34	57	<b>91</b>	37%	63%
2013	54	200	<b>254</b>	21%	79%
2014	42	279	<b>321</b>	13%	87%
2015	21	186	<b>207</b>	10%	90%
2016					
<b>TOTAL</b>	<b>284</b>	<b>1111</b>	<b>1395</b>	<b>20%</b>	<b>80%</b>

(b) Period	# +ve at Scottish	# +ve at Other	TOTAL	% +ve at Scottish	% +ve at Other	% +ve
						Average
2005Jul - 2006Jul	11	76	<b>87</b>	69%	58%	60%
2007						
2008						
2009	5	30	<b>35</b>	100%	77%	80%
2010	22	35	<b>57</b>	67%	61%	63%
2011	65	137	<b>202</b>	82%	84%	83%
2012	26	38	<b>64</b>	76%	67%	70%
2013	34	154	<b>188</b>	63%	77%	74%
2014	39	227	<b>266</b>	93%	81%	83%
2015	19	167	<b>186</b>	90%	90%	90%
2016						
<b>TOTAL</b>	<b>221</b>	<b>864</b>	<b>1085</b>	<b>78%</b>	<b>78%</b>	<b>78%</b>

Annual retail chicken samples (a) collected and (b) *Campylobacter* positivity by Scottish abattoirs vs. other UK abattoirs.

Table 5. Ranked abundance of stains in retail chicken samples by abattoir from 2009 - 2015.

(a)				(b)		
Unique ST	# at Scottish	# at Other	TOTAL	% at Scottish	% at Other	% at Average
50	4	86	<b>90</b>	1.9%	10.9%	9.0%
5136	27	55	<b>82</b>	12.9%	7.0%	8.2%
45	20	59	<b>79</b>	9.5%	7.5%	7.9%
48	26	53	<b>79</b>	12.4%	6.7%	7.9%
51	13	66	<b>79</b>	6.2%	8.4%	7.9%
257	3	50	<b>53</b>	1.4%	6.3%	5.3%
21	12	25	<b>37</b>	5.7%	3.2%	3.7%
583	6	19	<b>25</b>	2.9%	2.4%	2.5%
2030	0	25	<b>25</b>	0.0%	3.2%	2.5%
53	11	11	<b>22</b>	5.2%	1.4%	2.2%
122	5	17	<b>22</b>	2.4%	2.2%	2.2%
19	6	15	<b>21</b>	2.9%	1.9%	2.1%
354	1	19	<b>20</b>	0.5%	2.4%	2.0%
464	1	16	<b>17</b>	0.5%	2.0%	1.7%
267	4	10	<b>14</b>	1.9%	1.3%	1.4%
881	0	14	<b>14</b>	0.0%	1.8%	1.4%
1044	5	8	<b>13</b>	2.4%	1.0%	1.3%
5	5	7	<b>12</b>	2.4%	0.9%	1.2%
42	1	11	<b>12</b>	0.5%	1.4%	1.2%
990	4	7	<b>11</b>	1.9%	0.9%	1.1%
574	0	11	<b>11</b>	0.0%	1.4%	1.1%
61	6	3	<b>9</b>	2.9%	0.4%	0.9%
827	4	5	<b>9</b>	1.9%	0.6%	0.9%
945	7	1	<b>8</b>	3.3%	0.1%	0.8%
other ST	39	195	<b>234</b>	18.6%	24.7%	23.4%
<b>TOTAL</b>	<b>210</b>	<b>788</b>	<b>998</b>	<b>100.0%</b>	<b>100.0%</b>	<b>100.0%</b>

Strains present in retail chicken ranked by overall abundance. (a) abundance of strains in *Campylobacter* positive isolates by abattoir. (b) % abundance of strain within Scottish or Other abattoirs.

Table 6. ST5136 abundance in retail chicken samples by abattoir from 2009 - 2015.

Year	# +ve at		TOTAL	% ST5136 of +ve at Scottish	% ST5136 of +ve at Other	% ST5136 of +ve Average
	Scottish	Other				
2009	0	0	<b>0</b>	-	-	-
2010	1	1	<b>2</b>	20%	3%	4%
2011	9	5	<b>14</b>	41%	4%	7%
2012	1	4	<b>5</b>	2%	11%	8%
2013	8	17	<b>25</b>	24%	11%	13%
2014	6	14	<b>20</b>	15%	6%	8%
2015	2	13	<b>15</b>	17%	8%	9%
2016						
<b>TOTAL</b>	<b>27</b>	<b>54</b>	<b>81</b>			

(a) abundance of ST5136 isolates by abattoir, (b) % abundance of ST5136 isolates each year within each abattoir and as % abundance amongst all isolates.

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### 3.2. *Campylobacter* on Farm and at Abattoir

Sampling for *Campylobacter* was from five pooled caeca and two whole birds from the end of line per sampled flock. 152 flocks from 34 different farms were tested over the period from November 2015 to December 2016. Isolates from the caeca, which are internal to the bird, were taken as representative of the *Campylobacter* of the birds on the farm. Isolates from the whole birds, which will predominantly be from the skin, were taken as representative of the *Campylobacter* of the birds on the farm and potentially also of subsequent cross contamination at the abattoir.

#### **Campylobacter on farm**

Table 7 shows the isolation rate of *Campylobacter* from whole birds and caeca on 34 farms. 90% of the whole birds tested and 88% of caecal samples yielded isolates of either *C. jejuni* or *C. coli*. Of the 90% of whole birds' isolates, 39% were obtained from enrichment and from the 88% caecal isolates, 15% were obtained from sample enrichment (data not shown).

Thus for whole bird samples *Campylobacter* was detected more commonly from enrichment positive samples, whilst *Campylobacter* from caeca was detected more readily in samples with a heavy load of *Campylobacter*. It reaffirms that *Campylobacter* infected birds carry higher loads of the organism in their gastrointestinal tract especially the caeca where it multiplies (Hermans, Pasmans et al. 2012) compared to than on the skin of the bird where the organism is essentially a contaminant from faeces.

Detection of *Campylobacter* using an enrichment step helps in the detection of positive samples where *Campylobacter* may be stressed in a niche such as the pores of chicken skin (Hansson, Nyman et al. 2015).

The most abundant strain types observed on the farms were ST50 (27/34 farms), ST257 (19/34 farms), ST5136 (19/34 farms), ST45 (13/34 farms) ST122 (10/34 farms) and ST53 (9/34 farms) (Table 8). More sequence types of *Campylobacter* were found to be circulating on farms 20 (n=13), 13 (n=10) and 32 (n=11) which were larger in size compared to other farms (Table 8).

Table 7. *Campylobacter* presence in whole birds and caeca linked to different farms.

Farm #	Whole birds			Caeca			Total samples per farm
	<i>Campylobacter</i> absent	Cj/Cc isolated	Mixed including non Cj/Cc present	<i>Campylobacter</i> absent	Cj/Cc isolated	Mixed including non Cj/Cc present	
1		7	1		4		12
2		1			2		3
3		11	1		4	2	18
4		4			1	1	6
5		6			2	1	9
6		7	3		5		15
7		3	1		2		6
8		6			2	1	9
9	2	4		1	2		9
10		2			1		3
11		2	1	1	5		9
12		5			9	1	15
13		28			13	1	42
14	1	9		1	4		15
15		8			4		12
16		5	1		3		9
17	1	9			5		15
18		6			3		9
19		2			1		3
20	1	29	2		16		48
21		7	1		4		12
22		6			3		9
23		6			3		9
24		10	2		6		18
25		10			4	1	15
26		6			2	1	9
27		5	1		3		9
28		5	1		3		9
29		6			3		9
30		6			2	1	9
31		5	1		2	1	9
32	2	18	2	3	7	1	33
33		4	2		3		9
34		4	2		3		9
TOTAL	7	252 (89.7%)	22	6	136 (88.3%)	12	435

*Campylobacter* presence/absence was tested in whole birds from abattoir and caeca linked to 34 individual farms. Cj= *C. jejuni*, Cc= *C. coli*

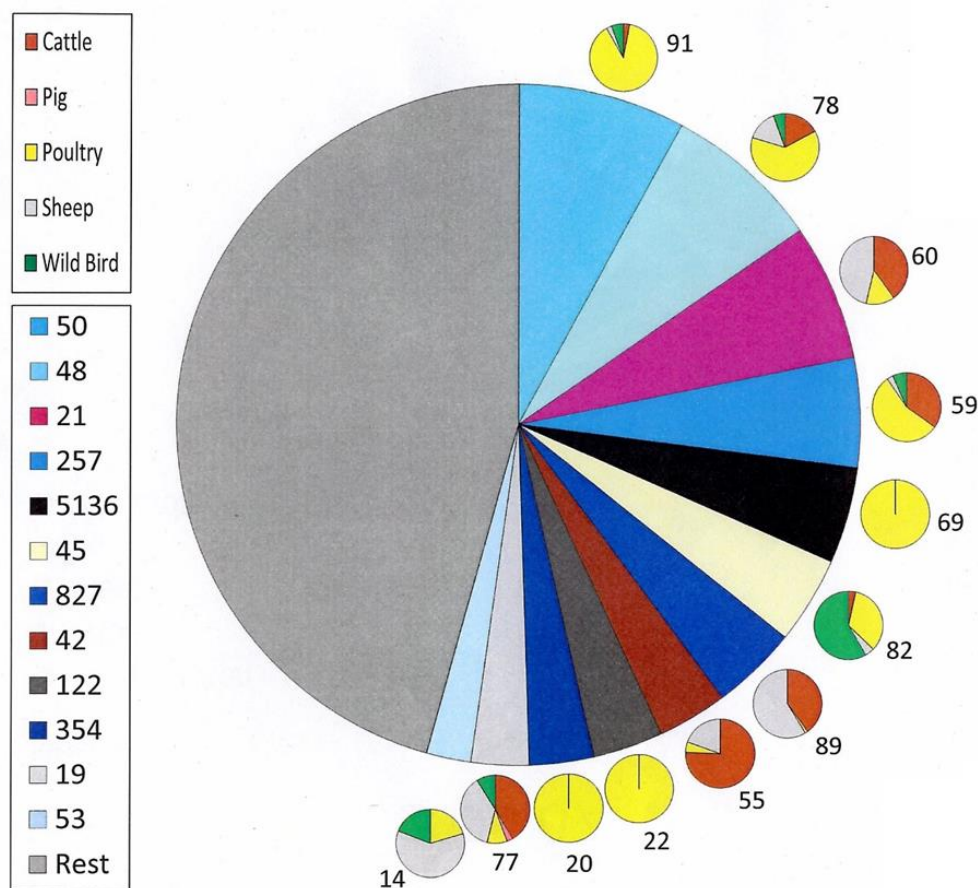
Table 8. Comparison of strains found on whole birds and caeca.

Farm #	No. of diff ST	ST																																													
		50	257	5136	45	53	122	1614	48	436	825	19	583	573	814	945	2120	7435	354	8545	21	137	230	262	828	1367	8207	8552	8554	61	637	677	1326	1489	3515	3567	8555										
1	5			5														2				1		1					2																		
2	1						3																																								
3	9	1	1		1	4			3			2												1														1	1								
4	3	1		1	3																																										
5	4	2		1	4																																1										
6	4	1	3		5						3																																				
7	3	2				1					2																																				
8	3	1	6	1																																											
9	4	2	1		2																	1																									
10	2	1	2																																												
11	3	3		3																		1																									
12	5	3		5		1	3																2																								
13	10	14	6	1	6	6	2		2			2		1																														1			
14	6	3	1	1	3	3																																									
15	5	3	3	1	2						3																																				
16	5	1	3		1		1		2																																						
17	6	3	3	3	1				3			1																																			
18	4		3	3	2		1																																								
19	1				3																																										
20	13	11	7	5	2		4	3	2		1				4						1	1				2	2																				
21	5	4	3				2															1																								1	
22	3		3	3	3																																										
23	3	5		3																		1																									
24	6	4	3			2	1				3				3																																
25	5	4	5	2		2									1																																
26	5	2		3	1	1						1																																			
27	4			1	3								2	2																																	
28	4	2	3		2										1																																
29	4		3																							2																			1		
30	4	1	1	1		5																																									
31	4	3	1			2					1																																				
32	11	5	3	3	7		1	1			1																																				
33	2	3		4																																											
34	4	2	2		2	1																																									
Total		87	66	49	35	25	24	12	10	8	7	6	6	5	4	4	4	4	4	4	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2		

Farms were allocated a random identification number. For each farm the number of different strains is indicated and for each strain the number of isolates detected is indicated. 36 different types of STs were detected in a total of 388 isolates.

The prevalence of ST50 in clinical isolates has risen since 2011 and this sequence type was the most commonly found in both birds and caeca in the current study. Historically, ST50 strains were almost exclusive to ruminant host reservoirs but now show a broader host range and occur mostly in chickens which is a 'specialist to generalist transition of this strain' (Figure 7). There are no recent reports of ST50 isolates circulating in broilers in the UK other than our DEFRA study (contract number OZ0625) which reported that ST50 was the most abundant strain found in broilers in the UK. ST50 has been isolated from humans and retail chickens in Finland, New Zealand and the Netherlands (Mullner, Collins-Emerson et al. 2010, Smid, Mughini Gras et al. 2013, de Haan, Kivisto et al. 2010). Recently, ST50 strains have been reported in cattle faecal samples in Austria linking association of this strain with cattle host reservoir as observed in the previous Scottish studies on *Campylobacter* (Klein-Jöbstl, Sofka et al. 2016).

Figure 7. Strain abundance in host reservoirs and in clinical isolates (2012-15).



*Campylobacter* isolates from 2012-15 (iCaMPS3 study report). Clinical (n=2378) and animal sources (n=1538). The outer, smaller, source pies have segments coloured by source and are based on a proportional contribution of each source assuming an equal sampling of sources; the actual number of isolates collected from food and environmental sources indicated. The strain key on the left indicates sequence types (ST).

Isolates of ST257 are found in broilers across UK and are commonly associated with a chicken host (Cody, McCarthy et al. 2012). ST257 isolates were the second most commonly found strains in the farms sampled. ST257 strain had the highest prevalence in humans during 2000-2005 in England (Sopwith, Birtles et al. 2008, McCarthy, Gillespie et al. 2012). It was one of the four most prevalent strains to be identified in humans, but was also identified in water samples which could indicate an environmental transmission route of flock contamination (Sopwith, Birtles et al. 2008). ST257 strains have also been found in the cattle host reservoir (Kwan, Birtles et al. 2008). Furthermore, ST257 strains have also been isolated in Australia and Luxembourg from isolates predominantly from clinical samples and chickens (Mickan, Doyle et al. 2007, Mossong, Mughini-Gras et al. 2016). In the iCaMPS3 study, ST257 strains were mostly associated with chicken and cattle host reservoirs.

ST5136 was a strain which emerged from ST464 and was first recorded in 2010 from a clinical stool sample in Oxford. ST5136 was the third most commonly found strain in the bird and caecal samples. In the previous study (iCaMPS3, 2012-15), ST5136 isolates were recovered from retail chicken samples originating from 18 different abattoirs. These strains are exclusive to chicken and turkey and have been reported in the UK but not from elsewhere.

ST45 isolates were the fourth most prevalent in the farm environment and can probably be linked to flock colonisation via transmission through the environment as it has been frequently isolated from environmental water sources and from wild birds (Bronowski, James et al. 2014, Llarena, Huneau et al. 2015, Mullner, Collins-Emerson et al. 2010).

ST53 isolates were the fifth most commonly found on farms. The iCaMPS3 study showed that these isolates are common to chicken, wild birds and sheep host reservoirs with a large proportion heavily associated with the sheep reservoir (Figure 7)(Lopes, Forbes et al. 2016). ST53 was significantly associated with isolates from cattle in a Finnish study (Karenlampi, Rautelin et al. 2007). Another study reported that ST53 isolates were present among calves, sheep, chicken, and starlings (Colles, Jones et al. 2003, Mullner, Collins-Emerson et al. 2010).

ST122 isolates were only found in humans and poultry host reservoirs according to the previous iCaMPS3 study (Lopes, Forbes et al. 2016). These isolates were the sixth most commonly found isolates on farms. ST122 was first detected in appreciable numbers in 2012 and continued to increase in proportion as observed in the iCaMPS3 study. There is no epidemiological data related to ST122 in the UK but this strain has been isolated from clinical samples, dog faeces and from caeca & carcasses of slaughtered broiler flocks in Switzerland (Kittl, Heckel et al. 2013).

In this study ST48 and ST21 were less commonly present in the chicken farm environment. In the previous iCaMPS3 study, ST48 isolates were the second most predominant strain associated with chickens and humans (Figure 7, Table

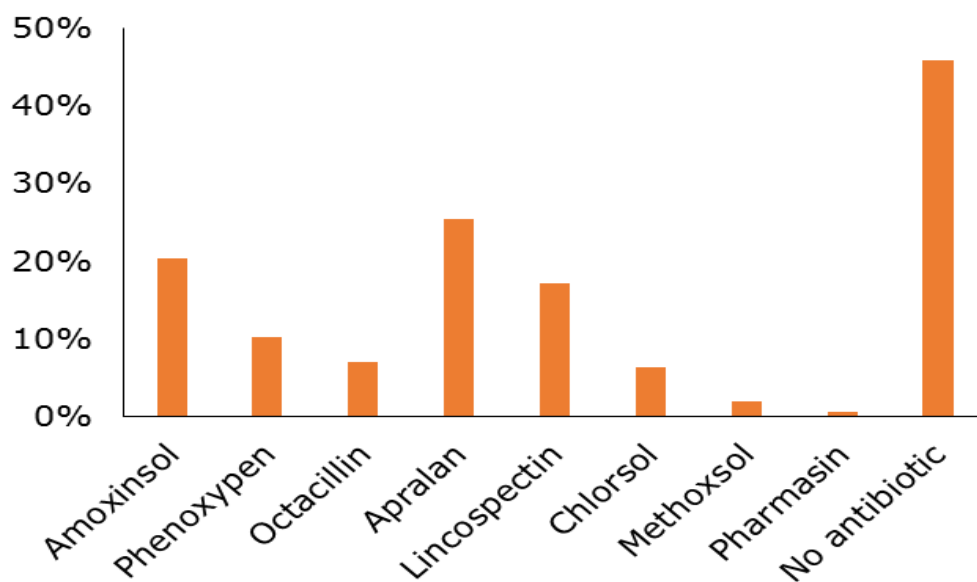
8). ST48 isolates are often cattle associated and are commonly isolated from beef offal and human disease cases but have also been found in the poultry chain environment in New Zealand and The Netherlands (Smid, Mughini Gras et al. 2013, Mullner, Collins-Emerson et al. 2010, Dingle, Colles et al. 2002). This may suggest a generalist trait for ST48. ST21 isolates were found in significant numbers in chicken and humans during CaMPS, iCaMPS1, 2 (2005-2012) studies but have since decreased in abundance. ST21 isolates were rare in the farms of this study (Table 8). It is worthy to note that they have been isolated from various sources such as chicken, cattle, human and other food sources. They possess phenotypic flexibility and high genetic micro-diversity, revealing properties of a generalist (Gripp, Hlahla et al. 2011). Previous studies reported that ST21, ST42, ST48, ST61 and ST45 are also associated with cattle host reservoir (Kwan, Birtles et al. 2008, French, Barrigas et al. 2005, Mickan, Doyle et al. 2007).

### **Antibacterial usage on farm**

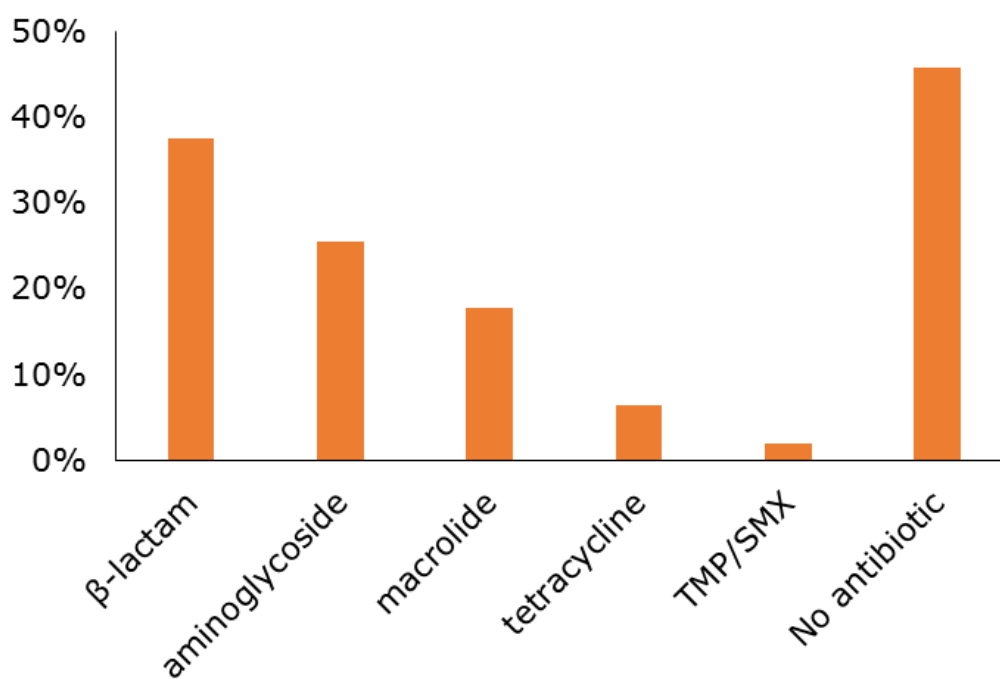
The usage of antibacterials was recorded for all of the flocks tested. Eight different antibacterials from five different drug classes were used (Figure 8). In this study 54% (85/157) of flocks were treated with at least one antibacterial agent. 38% of the flocks were administered at least one of the 3 different types of  $\beta$ -lactam antibiotics, 26% were treated with aminoglycoside class of antibiotic, 18% were administered two types of macrolide class of drugs, 6% were treated with tetracycline and 2% of the flocks were treated with trimethoprim-sulphamethoxazole.

Figure 8. Antibacterial agent usage on flocks sent to abattoir.

(a)



(b)



(a) Drugs used (b) antibacterial class. Percentage of 157 flocks tested which had utilised an antibacterial agent.

Amoxicillin, spectinomycin, trimethoprim, sulfamethoxazole and tetracycline antibiotics used in the poultry industry are on the World Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system (World Health Organization 2015). Apramycin treatment is shown to affect spread of resistant bacteria in pig production as its usage leads to enhanced spread of gentamicin-resistant *E. coli* (Herrero-Fresno, Zachariassen et al. 2016). As gentamicin is listed in WHO Model List of Essential Medicines and is the first choice drug for human bacteraemia, this can be of concern (World Health Organization 2015, Herrero-Fresno, Zachariassen et al. 2016). Details of these antimicrobials are given in Appendix 2.

The usage of antibiotics in agriculture in the UK has increased from 387 tonnes in 2008 to 429 tonnes in 2014 (Grace, Harris et al. 2014). Although, there was a decrease in 2015 usage (388 tonnes) (Broadfoot, Brown et al. 2015), it is important to note that the average usage since 1993 was 430 tonnes of active ingredient of antibiotic sold for all species of food producing animals (Broadfoot, Brown et al. 2015). The trend of antibiotic usage is fairly uniform throughout the years 1993-2015 and ranges between 533 tonnes (1996) to 346 tonnes (2011) (Broadfoot, Brown et al. 2015).

Resistance to antibiotics in bacteria caused by their usage in agriculture and for human use. Antibiotic resistance in bacteria not only limits the use of drugs in clinical practice but has a direct impact on patient health. Use of antimicrobials in agriculture is now restricted in the EU to the treatment of animals rather than its previous use as a prophylactic or growth enhancer. The use of antimicrobials in farmed animals and birds has the potential to generate strains of *Campylobacter* with new resistances and maintain pre-existing resistant strains. Using wgMLST analysis and 136 representative isolates from the UK we identified that ST5136, now a UK wide clone, had emerged from ST464 through substantial genetic recombination and mutation (Lopes et al, unpublished data). Further work on antimicrobials and the genetic basis of *Campylobacter* resistances is being carried out by us in a project currently funded by the Scottish government through RESAS.

### **Cross contamination at abattoir**

In this study, *Campylobacter* was sought from flocks by testing two whole bird samples and caecal samples. The rationale behind this being that caeca represent the campylobacter present on the originating farm whereas whole bird isolates may also represent later acquisition, e.g. at the abattoir.

Table 7 shows the isolation rate of *Campylobacter* from whole birds and caeca on 34 farms with 88% (n=154) caecal samples and 90% (n=281) of whole bird isolates were either *C. jejuni* or *C. coli*. Table 9 shows comparison of strains within flocks from both caecal and whole bird samples. 75% of the paired bird samples were of the same strain. 73% of flocks had one or both whole bird

isolates the same as the caecal isolate, implying that most flocks typically had one predominant strain type.

21% (30/145) of flocks had a caecal strain different to either one or both of the whole bird strains (Table 9). This might indicate cross contamination between the flocks during processing in the abattoir environment, but could be a reflection of multiple strain colonisation on the farm. Further study would be required to clarify this and would need to include sampling of consecutive flocks at processing to detect spill over from one flock to the next.

Table 9. Comparison of strains within flocks from caecal and whole bird samples.

	same ST	different ST	no pairing data <sup>a</sup>	Total number of flocks
Paired birds	90 (62%)	28 (19%)	27 (17%)	145
Caeca and either whole bird	85 (59%)	30 (21%)	30 (21%)	145
Caeca and both birds	60 (41%)	48 (33%)	37 (26%)	145

For each flock tested comparison was made between the strains found from the two whole bird samples and the strain isolated from the caecal sample.

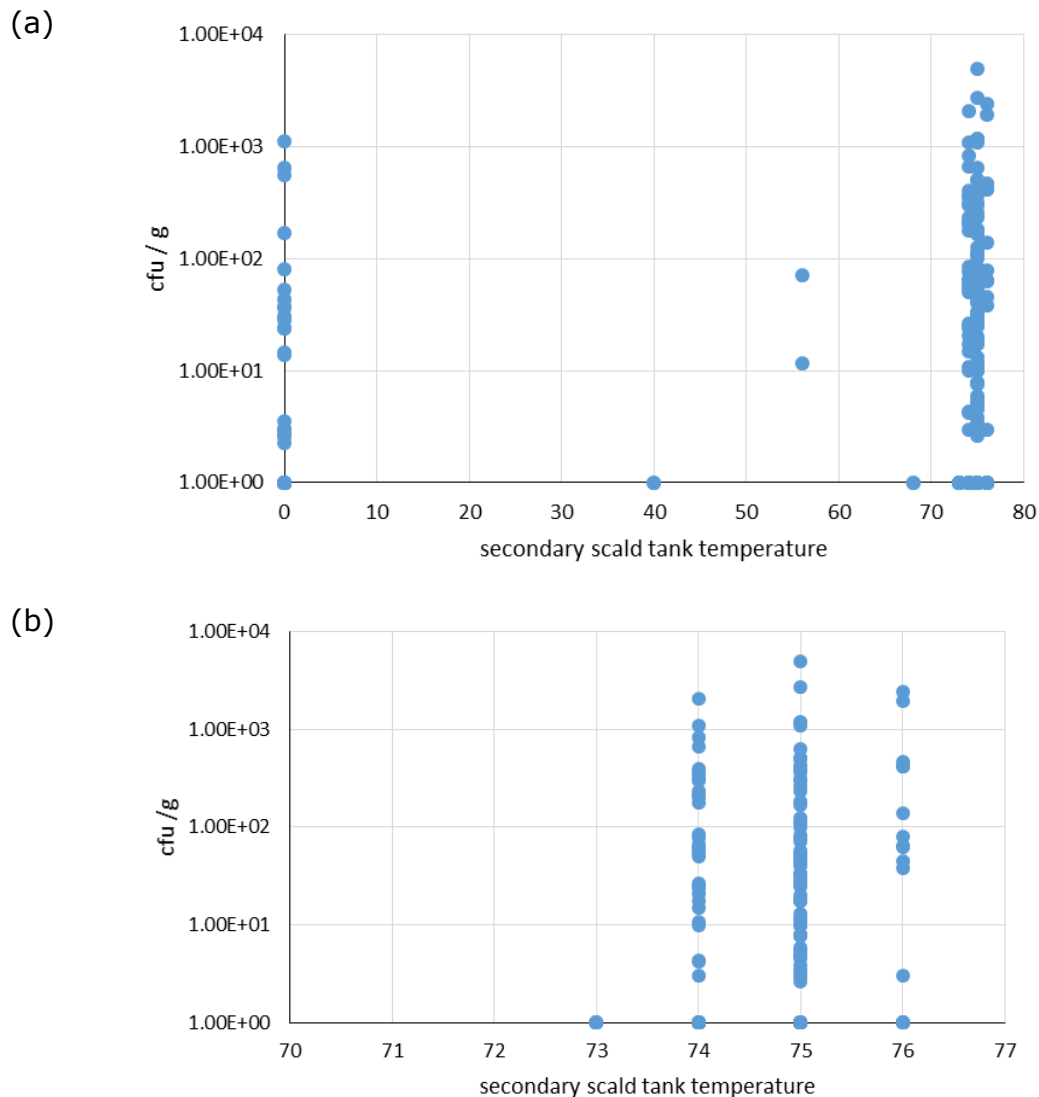
<sup>a</sup>no pairing data means that the data were unavailable.

### **Interventions at the Scottish abattoir**

The abattoir introduced two interventions designed to reduce the *Campylobacter* load on the retail ready birds. The trimming of neck flaps (where contaminated water run-off collects on the hanging bird) had been introduced prior to this study and a secondary scalding tank was in intermittent use for the first few months of this study and was subsequently mostly active.

*Campylobacter* load on the whole birds at the end of line was graphed against the temperature of the secondary scalding tank (Figure 9). The loads were similar when the tank was not in operation (plotted as "0°C" in Figure 9) and scald tank temperatures ranging from (73-76°C). Data comparing the absence vs. presence and the different operating temperatures does not allow a conclusion to be drawn on its effectiveness. Mainly this is due to an absence of comparable samples for the absence vs. presence of the measure, but is also compounded by the variation in flock *Campylobacter* loads and quite possibly in seasonality of contamination.

Figure 9. Effect of secondary scald tank temperature on load of *Campylobacter* on whole abattoir birds.



Counts (cfu/g whole bird) for all whole birds where the secondary scald tank temperature was known and where the tank was either not yet installed or was not operating (scored as 0 °C). (a) All temperatures, (b) Temperatures 70 – maximum (76 °C).

Tank off, n= 50, mean=5.83E+01  
 Tank= 73 °C, n= 12, mean=1.00E+00  
 Tank= 74 °C, n= 66, mean=1.24E+02  
 Tank= 75 °C, n= 126, mean=1.42E+02  
 Tank= 76 °C, n= 22, mean=2.77E+02

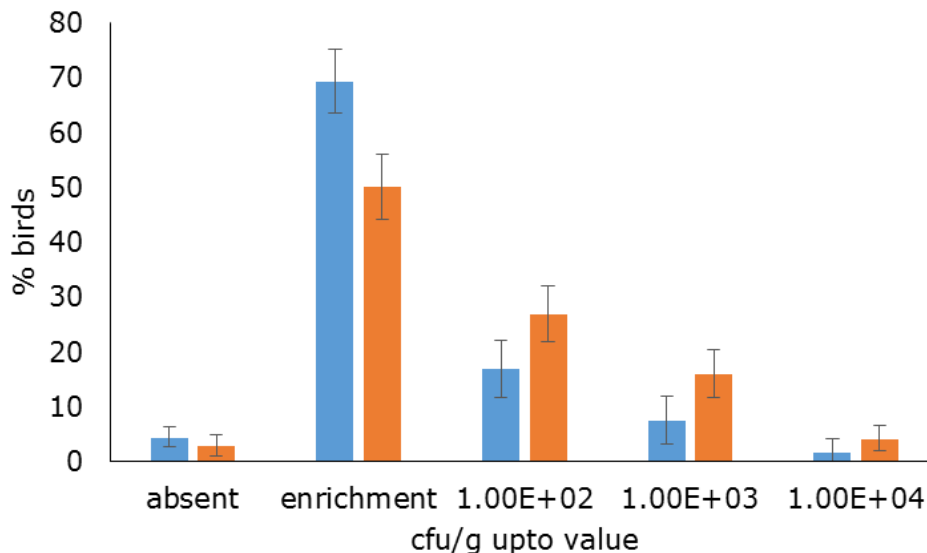
It was reported previously that studies that sampled before and after scalding showed that the prevalence of *Campylobacter* decreased immediately, whereas the prevalence increased after de-feathering and evisceration (Guerin, Sir et al. 2010). The testing of the bird samples in this study was after they had undergone all processing and were retail ready; we did not measure loads directly after scalding but only when other steps such as de-feathering, evisceration and chilling were complete.

Further work would be required to better understand the stages along the processing line and the impact on both the load and the prevalence of contamination of the birds, either from indigenous or cross contamination.

**Campylobacter loads on retail ready birds at abattoir and at retail**

*Campylobacter* counts on whole birds from the abattoir were compared to those from birds sampled from retail during the previous iCaMPS3 study (2012-2015) (Figure 10). Over both studies there was a clear trend of few birds with no *Campylobacter*. 17% of retail and 27% whole birds had load of 100 cfu/g. Of positive birds the majority had low loads and of the birds with highest levels of greater than or equal to 1000 cfu/g (highest level of contamination band category listed by the FSA), 20% of the abattoir birds were positive and 10% of the retail chicken. The bird carcasses at the Scottish abattoir had a heavy load of *Campylobacter* compared to that of retail birds (Figure 10). This may be because the Scottish abattoir birds had a shorter time since slaughter so were likely to have higher loads of *Campylobacter* as there was less time for the organism to have become less viable or have died. This is also reflected in the higher levels of enrichment positive samples obtained from retail birds (70%) compared to abattoir birds (50%); again reflecting a lower load in retail birds. There may also be confounding because the two studies are not contemporaneous and it is also possible that there might be different factors that influence *Campylobacter* levels along the production chain. Further work could be done to investigate this.

Figure 10. *Campylobacter* load on whole birds.



Whole birds from this study from the end of abattoir line (orange bar; n=289) and retail chicken from iCaMPS-3 [2012-15] (blue bar; n=1280). Quantitative enumeration was by scoring direct plating of serial dilutions from the birds washed in enrichment broth. 'Enrichment positive' was a qualitative result following incubation of 25 mL of enrichment broth, and 'absent' was an enrichment negative result.

The FSA ran a broiler survey in which in year 1 (Jul 2014 – Feb 2015), 2,618 samples of chicken were tested with 7.5% of neck skin samples positive for *Campylobacter* and 20% of these positive for the highest category load of 1000 cfu/g. During the year 2 (Jul 2015 – Feb 2016), 2,685 samples of chicken were tested of which 5.6% were positive for *Campylobacter* and 12% of these were positive for the highest category load of 1000 cfu/g. Over these two years, there has been a reduction in the loads seen on the birds. These FSA survey results are not, however, directly comparable to our study as testing was of neck flaps compared to whole birds.

### **Nei's genetic distance**

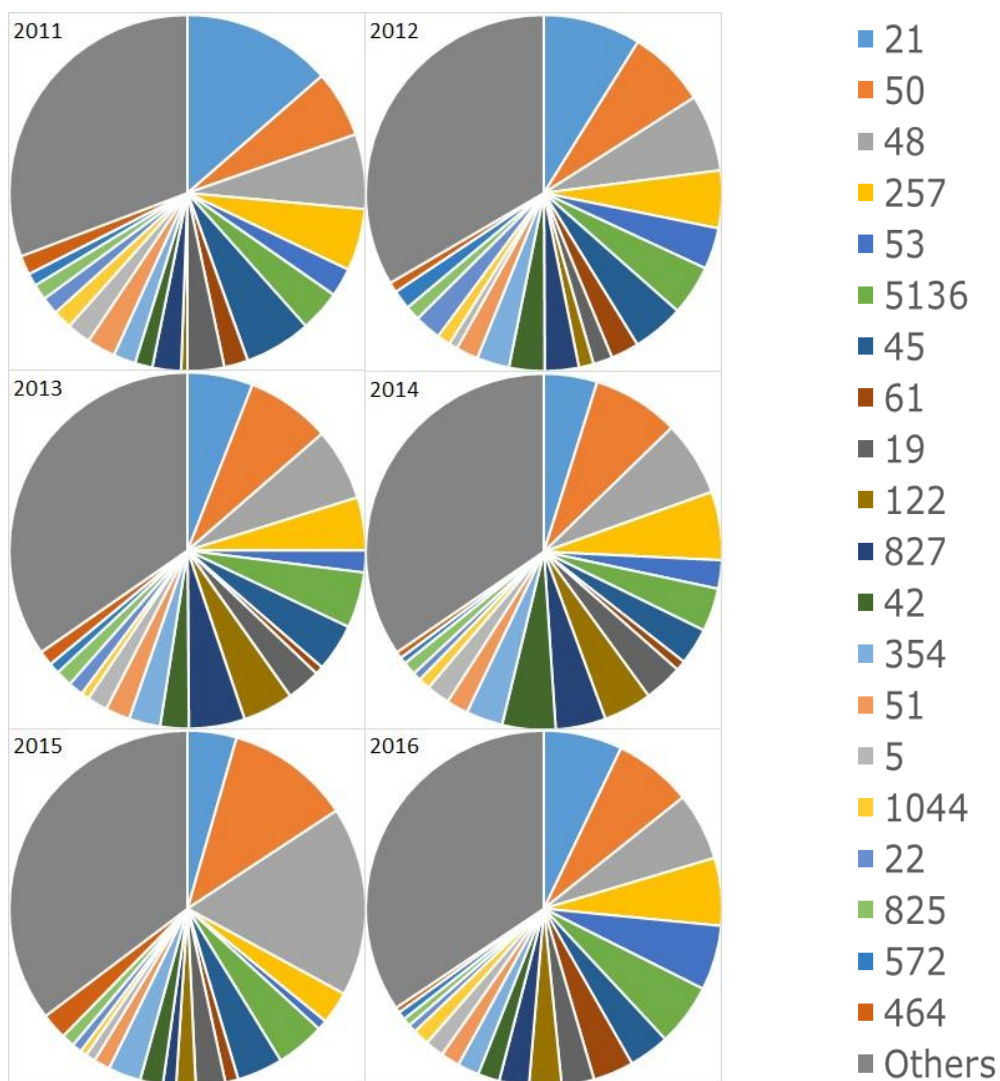
Nei's genetic distance is a measure of the overlap in the genetic content of populations and was measured at the strain level (a single measure of similarity using ST number) for retail and Scottish abattoir whole birds. Nei's genetic distance comparing retail birds (iCaMPS3) with abattoir birds iCaMPS4 (2016) was  $d_1=0.2493$ . The populations of strains in retail chicken (in 2012-15) and at abattoir (in 2016) were found to be significantly different ( $p<0.001$ ). But it is worthy to note that even though the populations were different, there were many strain types in common.

Our previous iCaMPS3 report revealed that chicken isolates were significantly more diverse from each other than cattle, sheep or pigs, implying a more rapid turnover of chicken strains with time, compared to other sources. It also highlighted that the greatest similarity of isolates was between chicken and clinical isolates, suggesting a link between the strains of chicken and human host reservoir.

### 3.3. *Campylobacter* strain diversity from human cases in Grampian

The annual clinical strain abundances from 2011-2016 are shown in Figure 11. Figure 12 illustrates the changing abundance of clinical strains circulating from 2011-2016 (3 month rolling mean) with ST21, ST48, ST50 and ST257 ranking consistently in the top four strains (Figure 11). The trends for the top seven clinical strains which were most abundant since 2005 - ST21, ST50, ST48, ST257, ST5136, ST45 and ST827 - for 2011-2016 are shown in Figure 13 along with their abundance in chickens. The conclusions drawn for each of the STs is shown below:

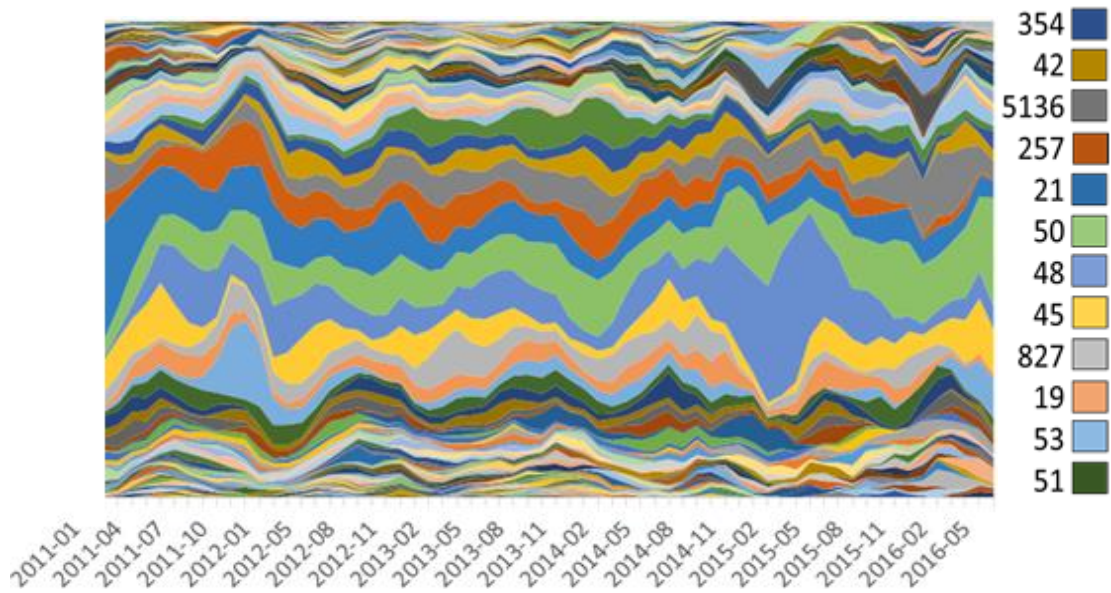
Figure 11. Pie charts of annual clinical strain abundances.



Order of strains is the same for all years thus order in any one year is not ranked by that year's abundance. ST numbers are indicated on the right of the figure.

Figure 12. Changing abundance of clinical strains between 2011 and 2016.

(a)

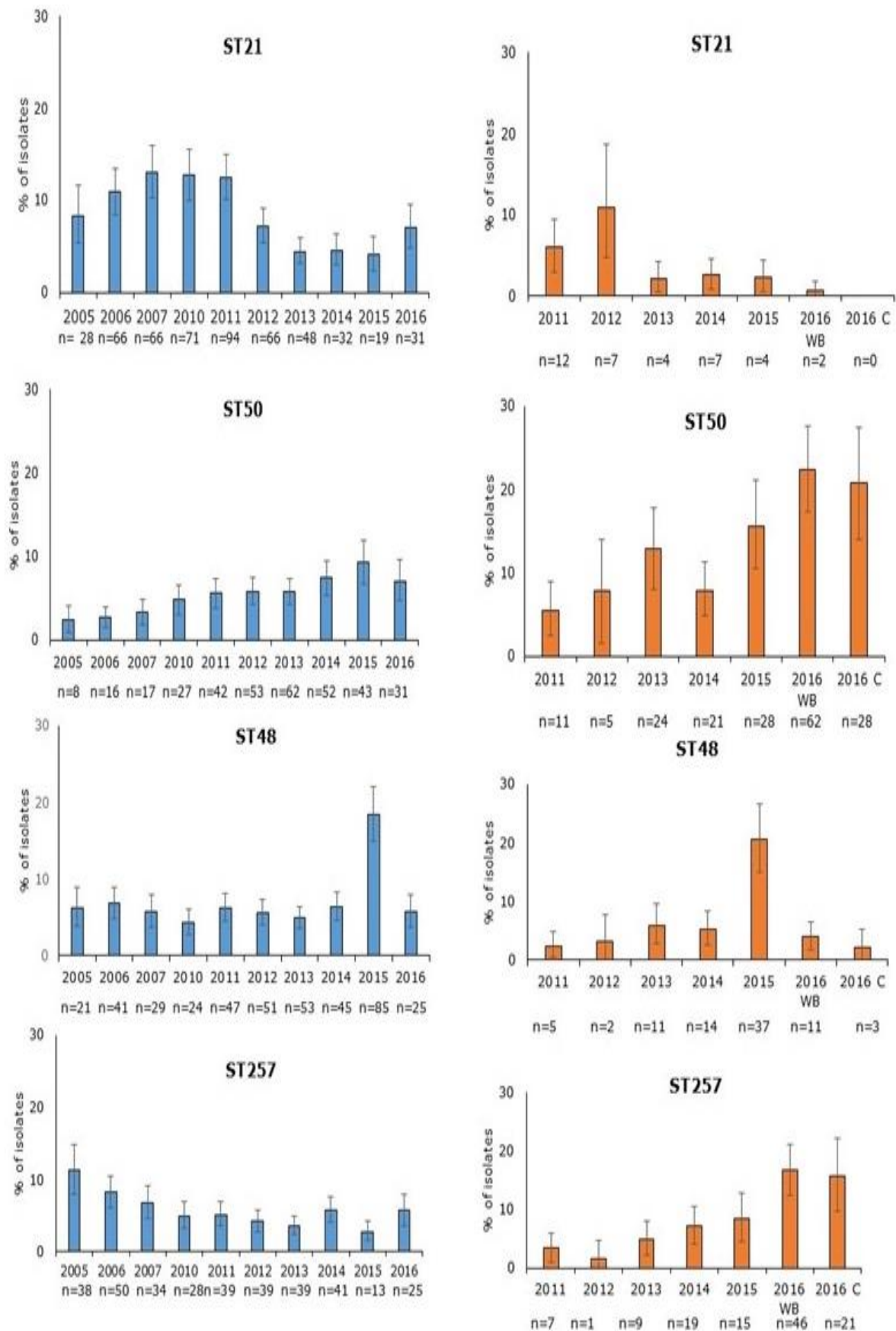


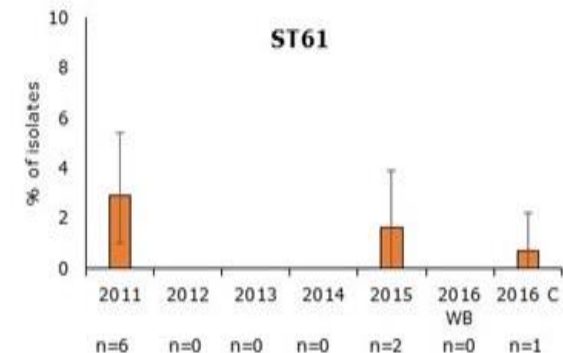
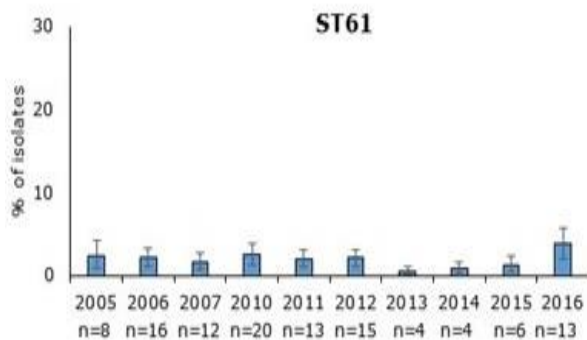
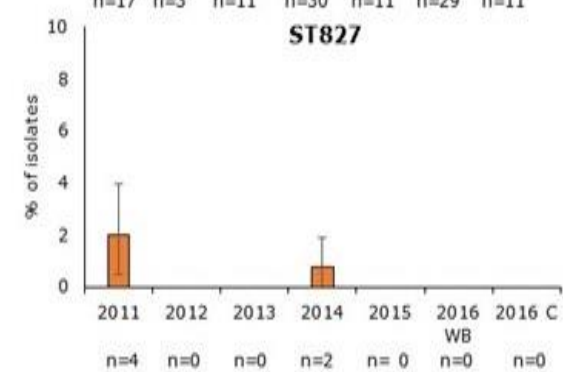
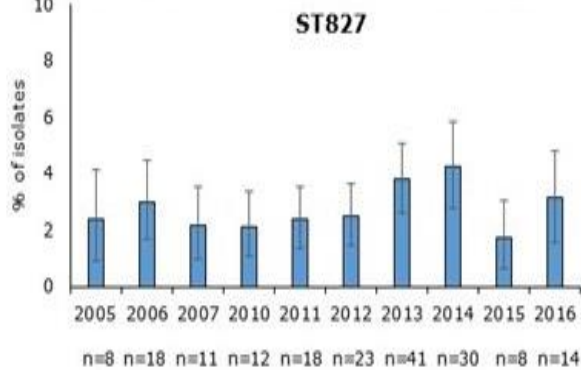
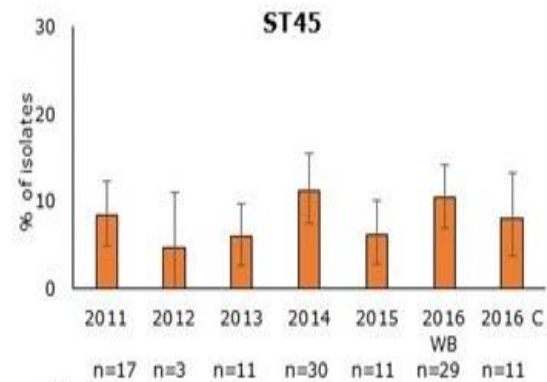
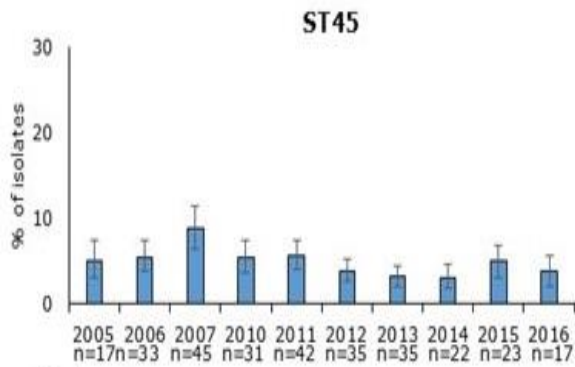
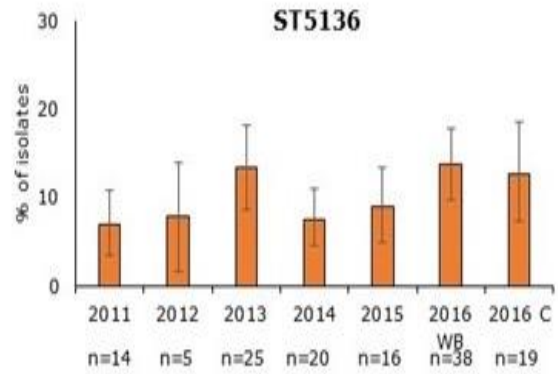
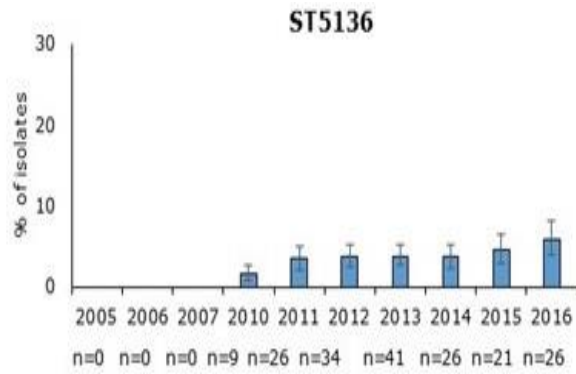
The abundance of isolates each month in the more common STs is plotted as their % contribution in each month. (a) All ST with Key to the most common 12 STs.

Abundances were smoothed using the formula:

$(0.1 * M - 2) + (0.2 * M - 1) + (0.4 * M_0) + (0.2 * M + 1) + (0.1 * M + 2)$  where M is the number of isolates of a particular ST in a particular month. STs with lower overall abundances are placed further out from the central line of the graph.

Figure 13. Annual % abundance of clinical and chicken strains.





Error bars are 95% CI calculated by bootstrapping. WB: Whole bird, C: Caeca, n indicates number of isolates positive for a specific ST. Blue bars are clinical isolates, orange are chicken isolates. Percentages are calculated by taking into account the total sample size during the year.

**ST21:** The percentage of isolates belonging to ST21 is decreasing in humans as well as in chickens (Figure 12, 13). This continues a trend reported in an earlier Scottish study which reported a decline in ST21 chicken strains in 2006 from higher levels in 2001 (Gormley, Macrae et al. 2008). Although this was not reflected in human cases associated with ST21 (Gormley, Macrae et al. 2008). In this current study very few ST21 isolates were detected in retail ready whole birds (and were not detected in caeca) which indicates that in the farms studied, there is a lower circulation of ST21 isolates. Whether the clinical ST21 isolates in 2016 are associated with retail chicken from producers elsewhere in the UK, or/ and from environmental exposures is unclear. ST21 are generalist which means that they can be found in different host reservoirs such as cattle, sheep and poultry (Gripp, Hlahla et al. 2011). Indeed more than three-quarters of ST21 isolates were associated with ruminant hosts in iCaMPS3 (Figure 7).

**ST50:** The proportion of ST50 isolates has been increasing since 2011 and was very high in the whole bird and caecal samples collected in 2016 (Figure 13). ST50 has not been much reported in studies from the UK other than the iCaMPS3 study. This strain was historically associated with ruminants and wild birds in Grampian (CaMPS study, 2005-09) and with cattle in Austria (Klein-Jöbstl, Sofka et al. 2016). By the iCaMPS3 study (2012-2015), ST50 was almost exclusive to chicken with a small proportion found in cattle (Figure 7). ST50 isolates are commonly found in poultry in other European countries (Smid, Mughini Gras et al. 2013, de Haan, Kivisto et al. 2010). This strain has apparently evolved from being predominant in ruminants and wild birds to one now found in chicken. Molecular phylogeny and comparative genomic studies are underway (RESAS project) to elucidate whether this is the result of the evolution of a new lineage which is more adapted to a chicken host.

**ST48:** ST48 abundance in both humans and chickens has remained rather constant other than in 2015 when it spiked significantly (cause unknown) (Figure 13). ST48 was the second most common strain in the iCaMPS-3 study and is the third most abundant strain found in this 2016 study along with ST257. Both strains are commonly found in poultry with a proportion also associated with cattle as reported in the previous iCaMPS3 study (Figure 7).

**ST257:** Historically ST257 strains were mostly chicken associated but have become increasingly common in cattle (Kwan, Birtles et al. 2008, Gormley, Macrae et al. 2008, Manning, Dowson et al. 2003). Figures 11 and 13 show that ST257 has remained common in clinical samples since 2011 with an increase in isolation from chickens over the same period (Figure 13).

**ST5136:** The strains belonging to ST5136 evolved from ST464 around the beginning of this decade. ST5136 was the 6<sup>th</sup> most commonly reported in clinical cases in the current study and was the 3<sup>rd</sup> most common strain reported in chicken isolates at farm and abattoir (Table 8). Its appearance in chicken and increase in clinical isolates can be seen in Figure 13. It will be interesting to track this strain over the coming years: will it continue to increase; it is now known in turkey (iCaMPS3), but will it spill over into other reservoirs? It is a

clone expanding all over the UK and seems to have a fitness advantage (better survival) over other strains (Lopes et al, unpublished data).

**ST45:** Previous studies show that isolates belonging to ST45 are frequently related to environmental water sources (Bronowski, James et al. 2014, Llarena, Huneau et al. 2015). However, in the iCaMPS3 2012-2015 study ST45 was associated with poultry, humans and wild birds where more than half of ST45 strains were associated with wild birds with a small proportion found in ruminants (Figure 7). Since 2011 the abundance of this strain seems to have been pretty constant (Figure 13). A previous large cross-sectional survey of wild bird populations in northern England was undertaken to investigate the epidemiology of *Campylobacter* infection which found ST45 strain association with wild birds with 19% of the isolates belonging to ST45 (Hughes, Bennett et al. 2009). As the samples were collected from birds present in the farm environment and there was an absence of typical *Campylobacter* wild bird strains, it suggested that transmission was most likely from livestock to wild birds (Hughes, Bennett et al. 2009).

**ST827:** This is a *C. coli* strain and is the overwhelmingly predominant *C. coli* strain, representing the main agricultural lineage in this species (Sheppard, Didelot et al. 2013). It was rarely found in chickens during 2011-2016, yet contributes to a good proportion of human cases (Figure 13). ST827 was the second most predominant ST found in the ruminant host reservoir in a previous Scottish study (Rotariu, Dallas et al. 2009). The strains belonging to ST827 were very common to the ruminant host reservoir in the previous iCaMPS-3 study: in chickens 0.3%, whereas for cattle and sheep it was 12.4% and 18.2% respectively, or some 50 times more in ruminants. During 2011-2015, ST827 was rarely found in chicken samples and was only detected in 2011 (2%) and 2014 (1%) being absent in 2012, 2013, 2015 and 2016. In Grampian, most clinical cases are presumably a result of infection from environmental sources. Grampian has intensive cattle and sheep farming with around 8,000 properties with private water supplies (Strachan, Gormley et al. 2009, Aberdeenshire council 2017). On the contrary a positive correlation between increase in ST827 in both human and chicken isolates has been observed in Switzerland (Kittl, Heckel et al. 2013).

**ST61** is a strongly ruminant associated strain. In the current study ST61 (4%) was the 8<sup>th</sup> most common ST found in clinical cases and has doubled since 2005 (2%) (Figure 11). ST61 was a strain that was constant at 2% from 2005-2012 and declined during 2013-2015 but increased in abundance in 2016. The prevalence for 2013-15 ranged from 0-1%. During 2011-2016, ST61 was rarely found in chicken samples and was only detected in 2011 (3%) and 2015-2016 (0-1%). The previous iCaMPS3 study highlighted that ST61 strains are very common in the ruminant reservoirs. Isolates of ST61 were virtually absent on the farms sampled in the current study. Strains belonging to ST61 clonal complexes have been associated with those from cattle and humans in the US and were significantly over-represented in cattle (French, Barrigas et al. 2005,

Sanad, Kassem et al. 2011). ST61 was most commonly associated with cattle and sheep across North-East and South-West of Scotland with strong evidence that they are transmitted to humans via ruminant sources (Rotariu, Dallas et al. 2009). All other reports also suggest that ST61 isolates are very commonly associated with cattle and beef liver (Kwan, Birtles et al. 2008, Noormohamed, Fakhr 2014).

The appearance, rise and fall of strains in reservoir hosts and from human cases is clearly very complex and variation can be due to noise of sampling. Hence, it is implausible that there will be a one-size-fits-all answer to the increase and decline in strain types and host associations. There does seem to be quite good tracking between incidences in sources and in humans under particular situations. Strains that are host restricted to poultry tend to have their abundances reflected in the human cases, for example the poultry associated ST50 and ST5136 which are both rising in abundance. ST61 and ST827 are ruminant and environmentally associated strains and seem to yield quite constant proportions of clinical cases. ST21, ST48, and ST257 are rather more generalist strains with more complex epidemiologies reflecting this: ST257 has been increasing in chickens in recent years but declining in clinical importance. Greater understanding of these relationships will be central in conquering the campylobacteriosis epidemic in the long run.

### 3.4. *Campylobacter jejuni* and *Campylobacter coli*.

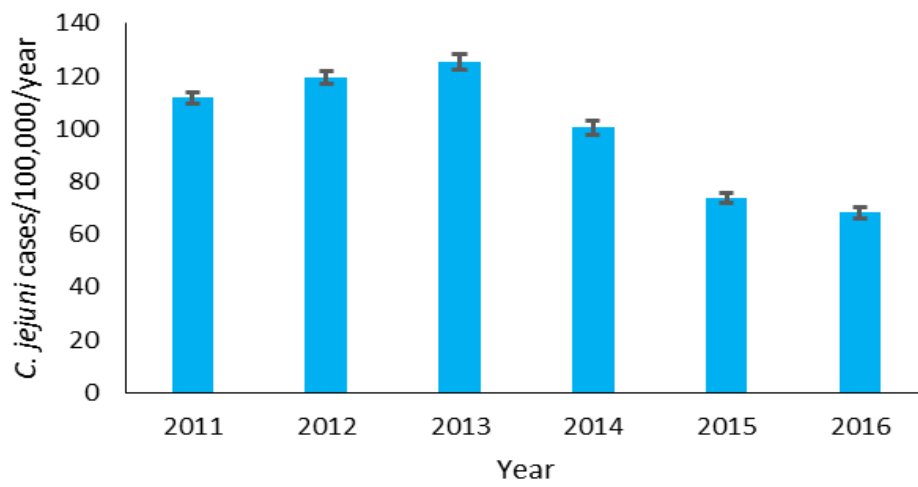
Typically in the UK and elsewhere, studies of campylobacteriosis have treated the two dominant causative species, *C. jejuni* and *C. coli*, as having common biology and epidemiology. With the help of extensive and long time frames of the datasets of the iCaMPS Grampian studies it is possible to examine these species in more detail. Clinical isolates of *C. jejuni* and *C. coli* are usually identified at a ratio of 10:1 (Tam, O'Brien et al. 2003, Acheson, Allos 2001, Roux, Sproston et al. 2013) and so larger datasets are required to be able to meaningfully interpret the epidemiology of the rarer *C. coli*.

As seen elsewhere, *C. jejuni* is the major cause of human campylobacteriosis in Grampian with an incidence of 114 cases/100,000 (2011-2014) which has recently fallen to 71 cases/100,000 (2015-2016) (Figure 14a). A significant drop in *C. jejuni* cases can be seen during 2014-2016 ( $p < 0.05$ ). Figure 14b shows that the incidence of *C. coli* has also fallen in the recent years from 11 cases/100,000 (2011-2014) to 6 cases/100,000 (2015-2016). The fall in *C. jejuni* cases in 2015 and 2016 is significant ( $p < 0.05$ ) compared to the previous years (2011-2014). Thus as elsewhere, in Grampian *C. jejuni* incidence is about ten-fold higher than for *C. coli*.

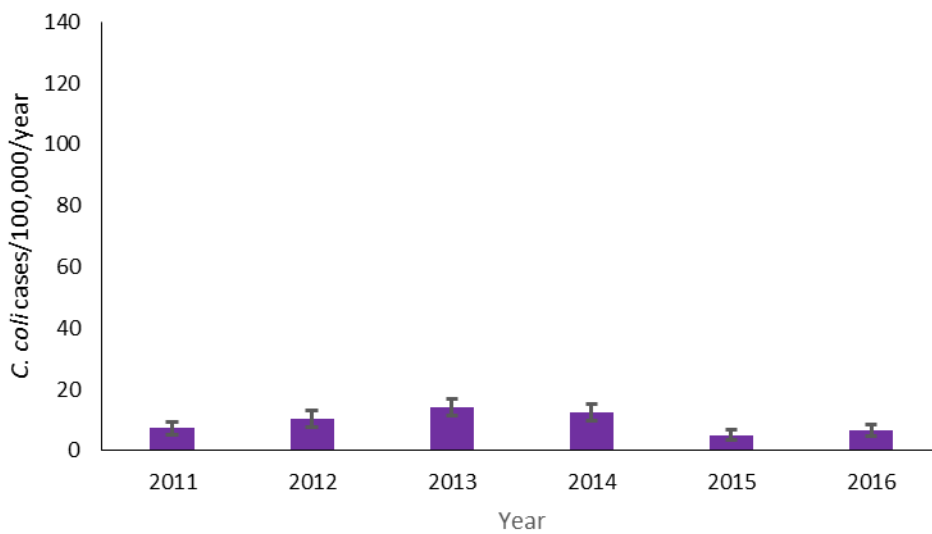
The monthly trends of *C. jejuni* and *C. coli* in humans over the period 2011-16 are presented in Figure 15. The incidence of *C. coli* is low over the 2011-16 period, whether this is because *C. jejuni* is a better coloniser than *C. coli* or just simply because *C. jejuni* is more abundant than *C. coli* or indeed because *C. coli* is better at colonising older birds, is not known. Hence, more follow up studies are required to understand the epidemiology of *C. coli* in detail.

Figure 14. Annual incidence of *C. jejuni* and *C. coli* clinical isolates in Grampian 2011 - 2016.

(a)



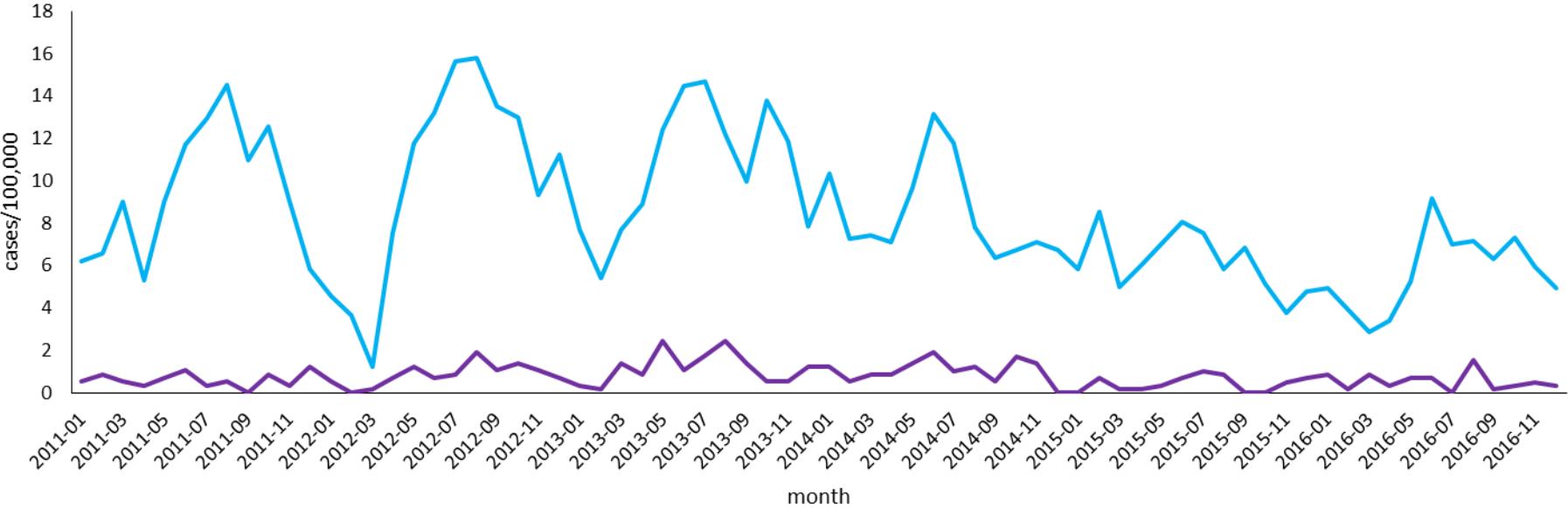
(b)



Data from current and previous iCaMPS reports. Error bars are 95% CI calculated by bootstrapping.

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Figure 15. Monthly incidence *C. jejuni* and *C. coli* clinical isolates in Grampian 2011 – 2016.

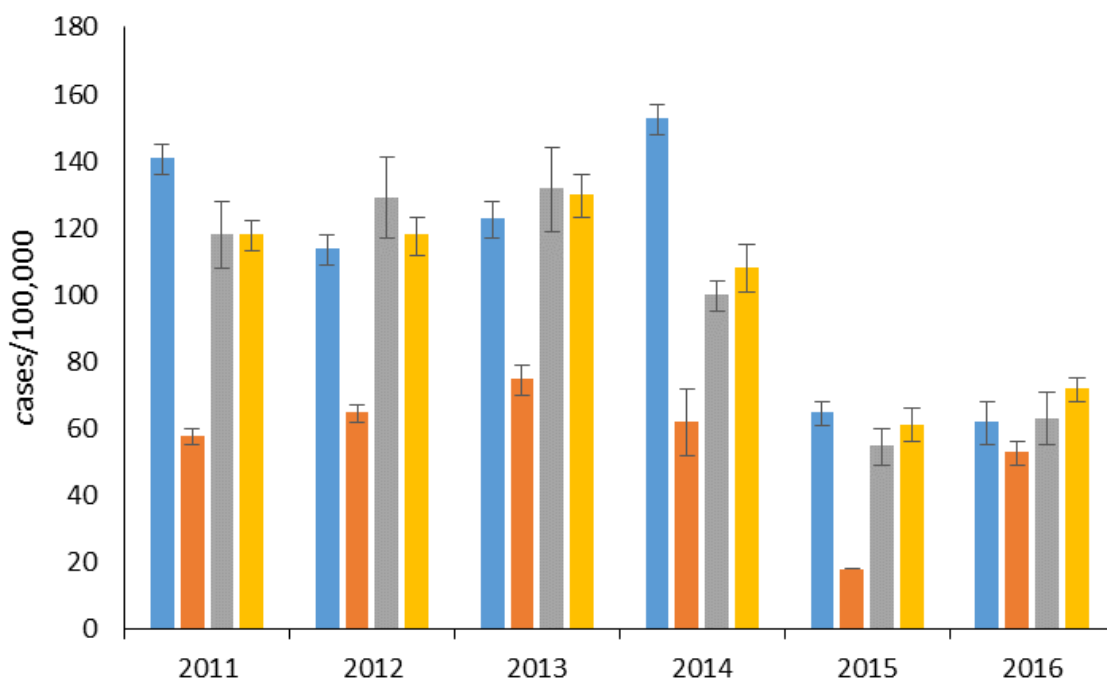


Data from current and previous iCaMPS reports. (Only 8 isolates were linked and typed during March 2012) Light blue: *C. jejuni*, purple: *C. coli*

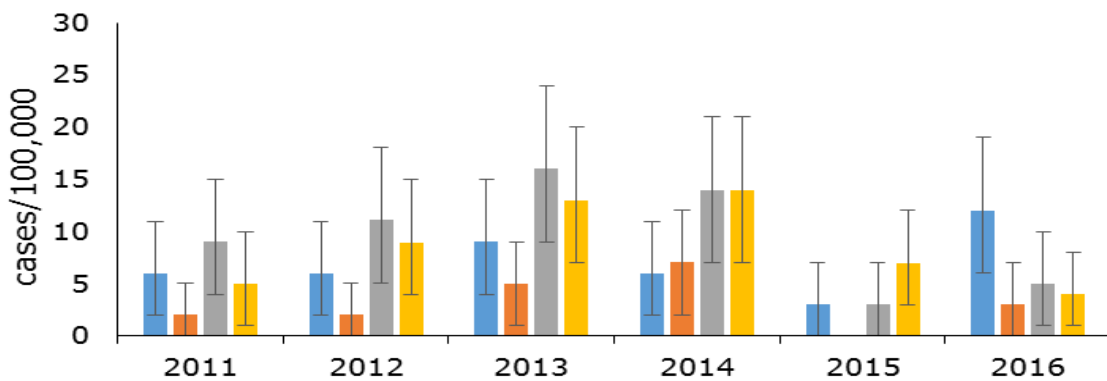
The age distribution of cases attributable to *C. jejuni* and *C. coli* (Figure 16) does not seem to be different for the two species, but the results must be interpreted with caution as error bars for *C. coli* are too big to make any meaningful comparisons. The 5-14 age group has lower incidences for both species. A decrease in *C. jejuni* incidence for 5-14 age group was seen in 2015 compared to the previous years (2011-2014) but 2016 incidence is comparable to that observed between 2011 and 2014. Also the trends between years with respect to age are comparable, except that a non-statistical increase ( $p>0.05$ ) in incidence of *C. coli* for the 0-4 age group was seen in 2016 compared to the previous years (Figure 16). In Grampian studies of *Campylobacter* in retail chicken there was typically *C. jejuni* in c.85% of samples with *C. coli* detected in the remaining c.15% (Figure 17). Our results concur with findings reported previously which indicate that *C. jejuni* is abundantly present on chicken carcasses whereas *C. coli* is found less commonly (Perdoncini, Sierra-Arguello et al. 2015). Studies of older, free-range, organic, breeder birds, as well as turkeys, have been shown to harbour multiple strains of *Campylobacter*, with a change in the dominant strain at around 30-35 days of age as a result of competitive advantage or due to host related changes (Colles, McCarthy et al. 2015, El-Shibiny, Connerton et al. 2005) *C. coli* is sometimes shown to succeed *C. jejuni* in broiler flocks, particularly those that are free range or slightly older with the change occurring at a critical time point of 35 days of age for the change in dominant *Campylobacter* strain type (Colles, McCarthy et al. 2015). A study comparing characteristics of patients infected with *C. jejuni* or *C. coli* showed that slightly older patients and those who travelled abroad were at a greater risk of *C. coli* infection than with *C. jejuni* (Bessède, Lehours et al. 2014).

Figure 16. Annual incidence of *C. jejuni* and *C. coli* in clinical isolates by patient age.

(a)

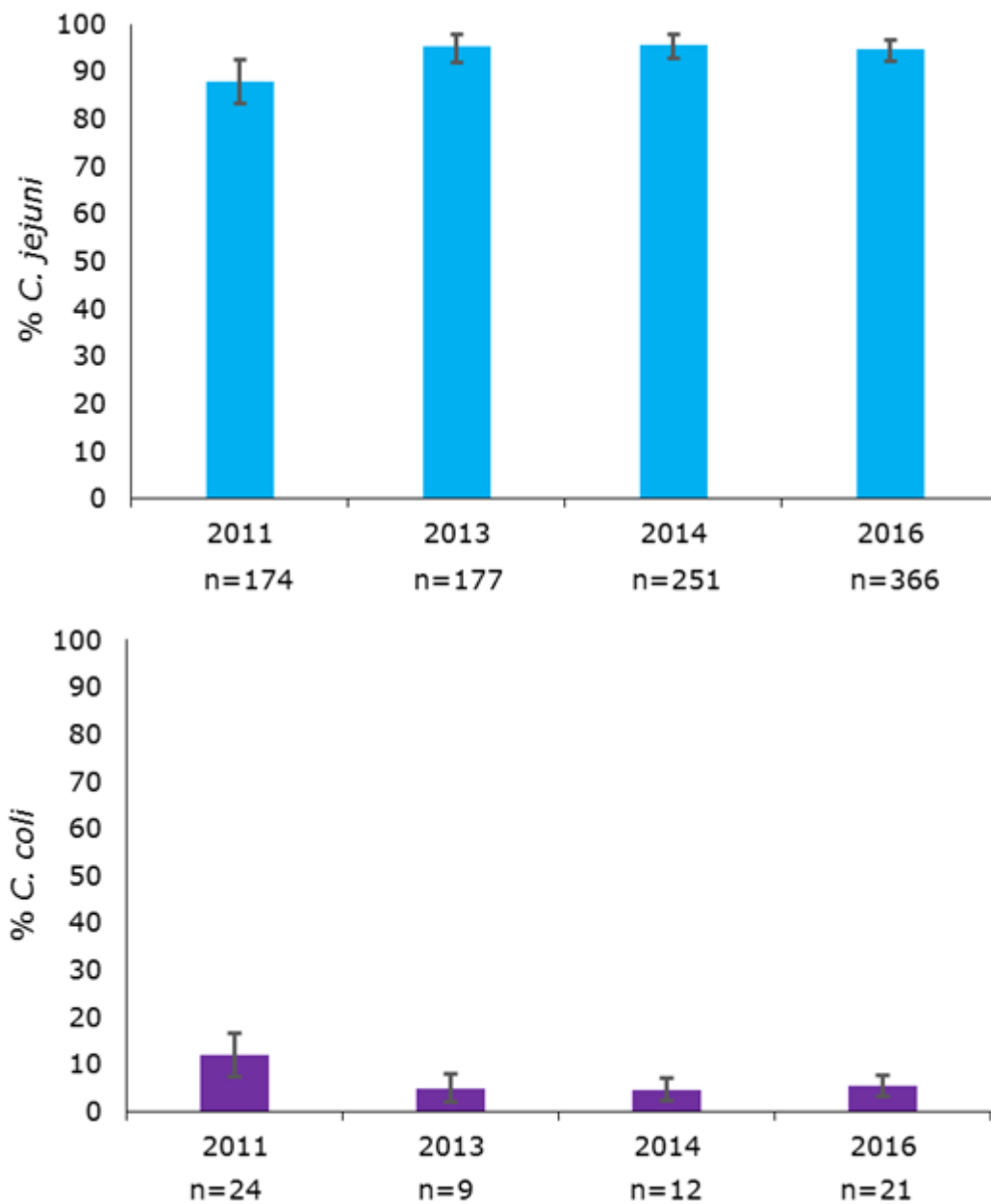


(b)



(a) *C. jejuni*, (b) *C. coli*. Age ranges ■ 000-004 ■ 005-014 ■ 015-064 ■ 65+ Error bars are 95% CI calculated by bootstrapping.

Figure 17. Annual proportion of *C. jejuni* and *C. coli* isolates in chicken.



Isolates from 2011, 2013 and 2014 are from retail birds sampled in iCaMPS3. For 2012 isolates were sampled in first 2 months only and hence are not included. The 2016 isolates are from abattoir birds sampled in this study. 'n' is the number of isolates positive during the year. Percentages are calculated for number of *C. jejuni* or *C. coli* by taking into account the total sample size normalised at 100%. Error bars are 95% CI calculated by bootstrapping.

### 3.5. Sources of human infection

#### **Temporal trends in source attribution**

Source attribution is defined as the partitioning of the human disease burden of one or more foodborne infections to specific source, where the term 'source' includes animal reservoirs and vehicles (e.g. foods) (Pires, Evers et al. 2009).

In this study of the source attribution of clinical isolates of *Campylobacter*, samples were linked to the five host reservoirs of cattle, sheep, chicken, wild birds and pigs using the STRUCTURE (alleles) and Asymmetric Island models; both models generated a similar annual pattern (Figure 18). Neither pigs nor wild birds contributed significantly to the burden of human campylobacteriosis in Grampian. On average cattle and sheep attributed cases comprised of one third of the clinical cases whereas chicken sources were attributed to two thirds of all clinical cases. The attribution ranges (%) are calculated referring to the mean clinical attribution to five host reservoirs (chicken, cattle, sheep, pigs, wild birds) using the models AI and STRUCTURE.

For the year 2015 the modelling allocated clinical isolates to the following reservoirs: chicken 59-76%, cattle 6-9%, sheep 10-21%, pigs 0-3% and wild birds 6-7% (Figure 18). In 2016 the modelling allocated clinical isolates to the following reservoirs: chicken 52-68%, cattle 9-11%, sheep 14-26%, pigs 1-2% and wild birds 7-8% (Figure 18).

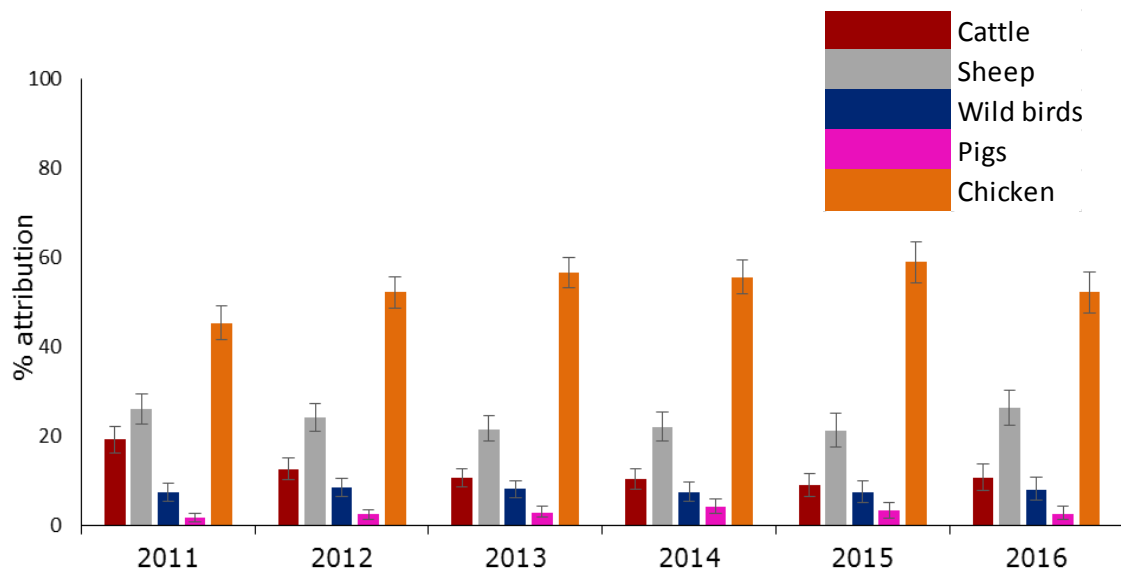
In the STRUCTURE model, a significant decline ( $p < 0.05$ ) in cattle attributed cases and a non-significant rise ( $p > 0.05$ ) in pig attributed cases has been observed from 2011 to 2016 (Figure 18a). The sheep and wild bird attributed cases have remained constant (Figure 18a). There was an increasing trend of chicken attributed clinical cases from 2011 to 2016 using the STRUCTURE model but this is not statistically significant (Figure 18a).

In the AI model, a significant rise ( $p < 0.05$ ) was seen in wild bird attributed clinical isolates in 2016, compared to 2011 (Figure 18b). The attribution to ruminant sources and pigs has remained fairly constant but a non-significant increase has been observed in 2016 ( $p > 0.05$ ) (Figure 18b). A statistically significant decline was seen in chicken attributed cases in 2016 compared to 2011 in the AI model (Figure 18b). The AI model attributed 68% of clinical isolates to chicken in 2016 compared to 81% in 2011.

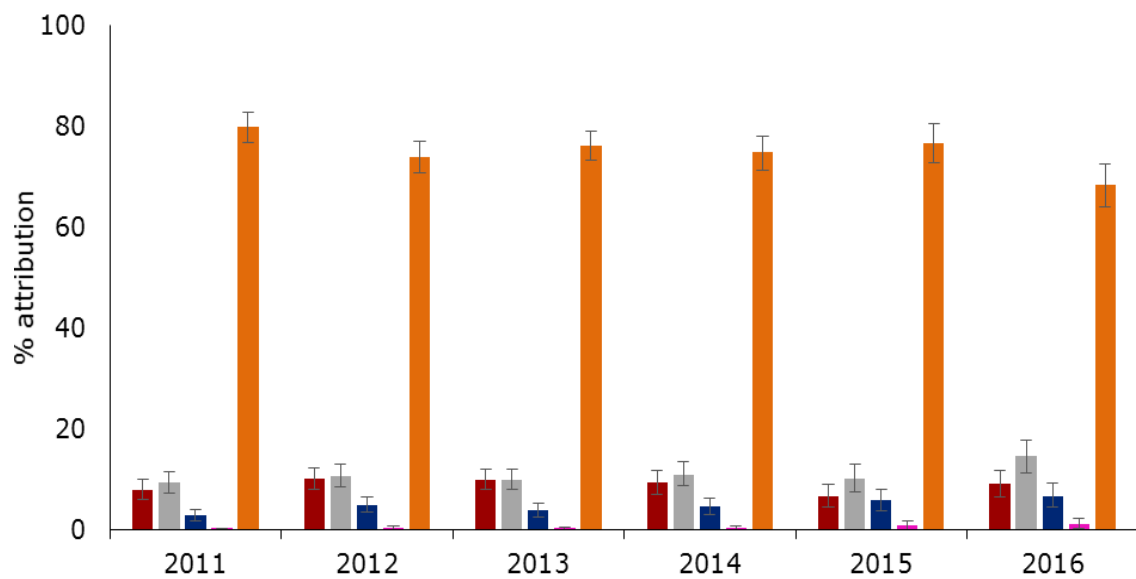
Chicken still remains the largest attributed source of clinical *Campylobacter* strains. This decline is discussed further in Chapter 3.7.

Figure 18. Annual source attribution of Grampian clinical isolates.

(a)



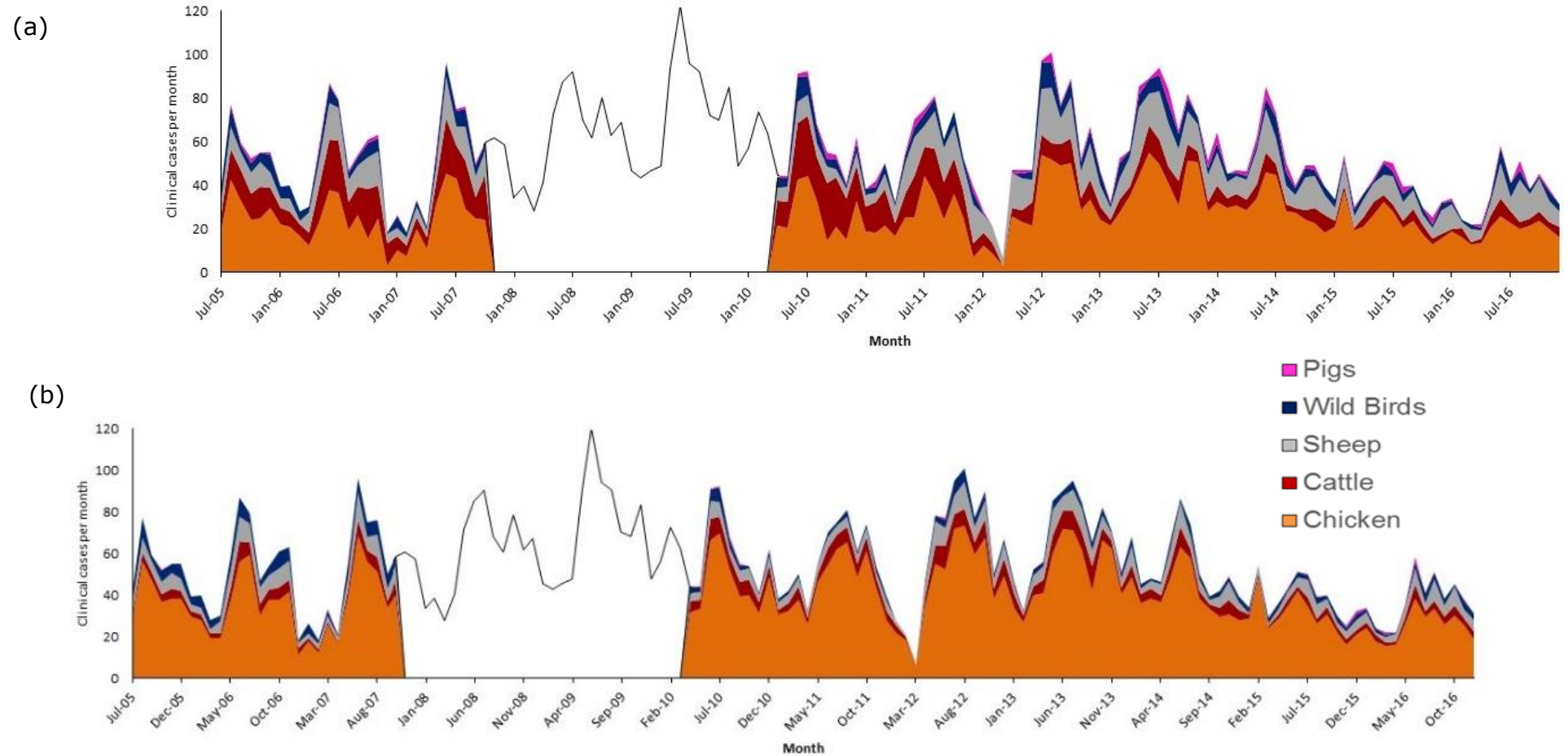
(b)



(a) STRUCTURE. (b) Asymmetric Island. Error bars are 95% CI calculated by bootstrapping.

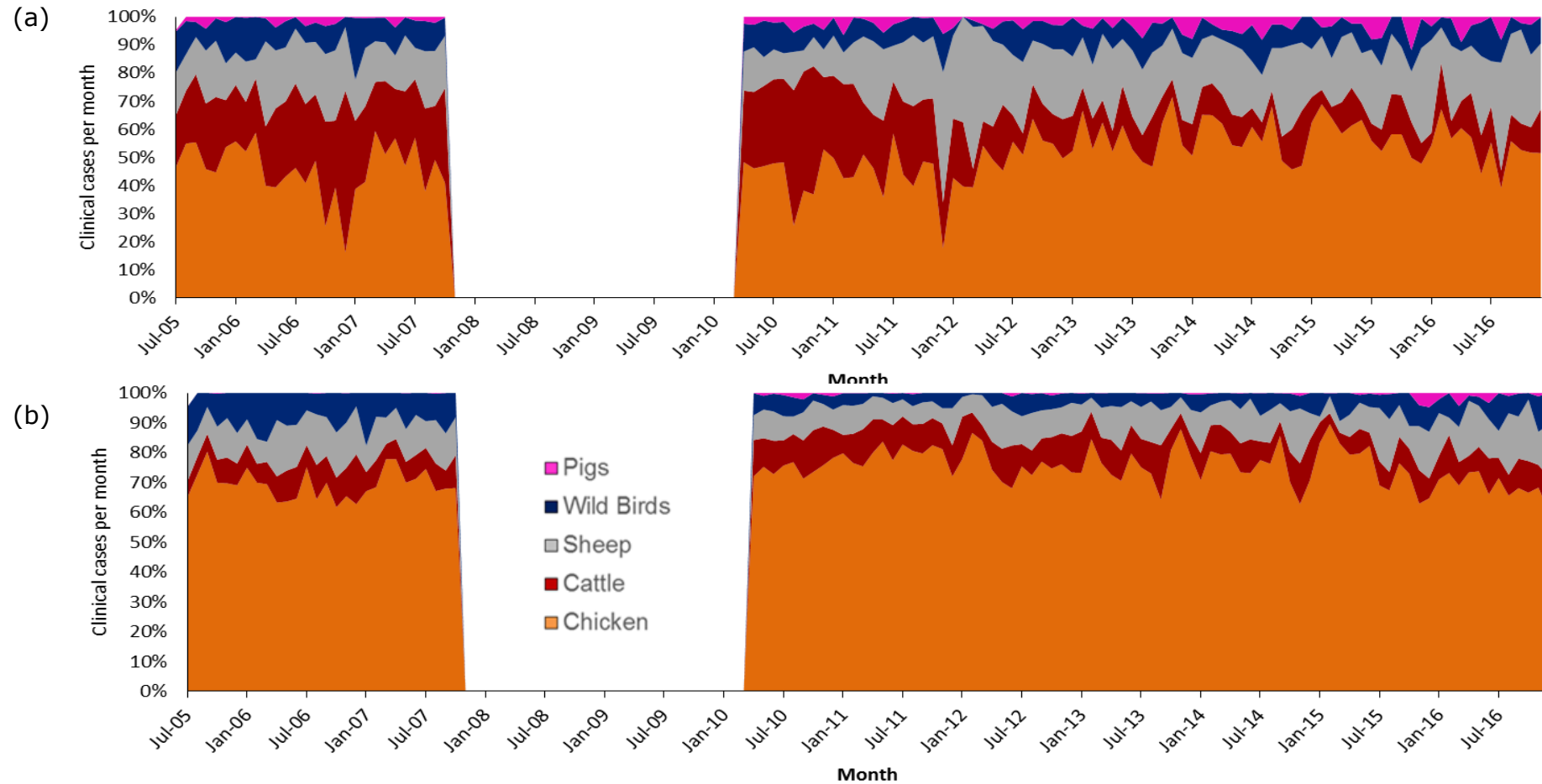
Figure 19 and figure 20 show the monthly attribution to the five host reservoirs of cases with *Campylobacter* infection in Grampian using the STRUCTURE (alleles) and Asymmetric Island models. Figure 19 illustrates the regular and prominent summer peak of cases. In Grampian, there were 36% more summer cases (May–Aug) than winter cases (Nov–Mar) for 2011–2016. The increase in rate of infection between weeks 18 (early May) and 22 (early June) is widely acknowledged (Nichols, Richardson et al. 2012). The causes of the summer peak are still largely an enigma, although greater travel away from home is a contributor (Ekdahl, Andersson 2004).

Figure 19. Attribution to five potential host reservoirs of clinical *Campylobacter* cases in Grampian per month.



(a) STRUCTURE with alleles Model, (b) Asymmetric Island Model. Graph stacked to total number of clinical cases per month.

Figure 20. Attribution to five potential host reservoirs of clinical *Campylobacter* cases in Grampian per month stacked to 100%.



(a) STRUCTURE with alleles Model, (b) Asymmetric Island Model. Graph of total number of clinical cases per month stacked to 100% per month.

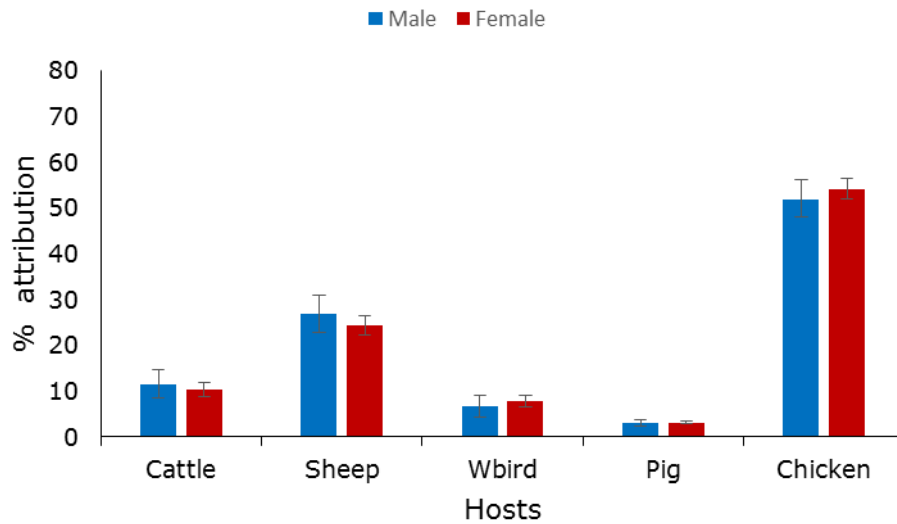
### **Source attribution by gender and age**

Our study did not find an association between the gender of cases and the attribution of their isolate to the five reservoirs by either the STRUCTURE (alleles) or Asymmetric Island models. Clinical attribution for both males and females was highest to the chicken reservoir and was fairly constant in both groups without any statistically significant differences (Figure 21). For 2016, HPS reported campylobacteriosis rates of 108.7 per 100,000 in males compared to 88.9 per 100,000 for females indicating a higher incidence in males (HPS 2017). The greatest difference of 56 per 100,000 was observed in children (<1 year) where the rates in males were 115 per 100,000 compared to 59 per 100,000 females. A previous study on age-specific prevalence, showed a 30% higher prevalence in men compared with women across most age groups with an overall 14% more reported *Campylobacter* cases in men than in women and a 1.14 M/F ratio (Nichols, Richardson et al. 2012). Another study also reported higher rates in males than in females, regardless of age, with incidence rates highest in children 0-4 years of age (Louis, Gillespie et al. 2005). These higher rates in males have been noted since 1985 but the cause for this remains to be established (HPS 2017, Hopkins, Olmsted 1985). It has been shown that for human campylobacteriosis, physiological factors play an important role in the higher incidence in males although behavioural differences are suggested to play a role as well; there is however a paucity of data available (Strachan, Watson et al. 2008).

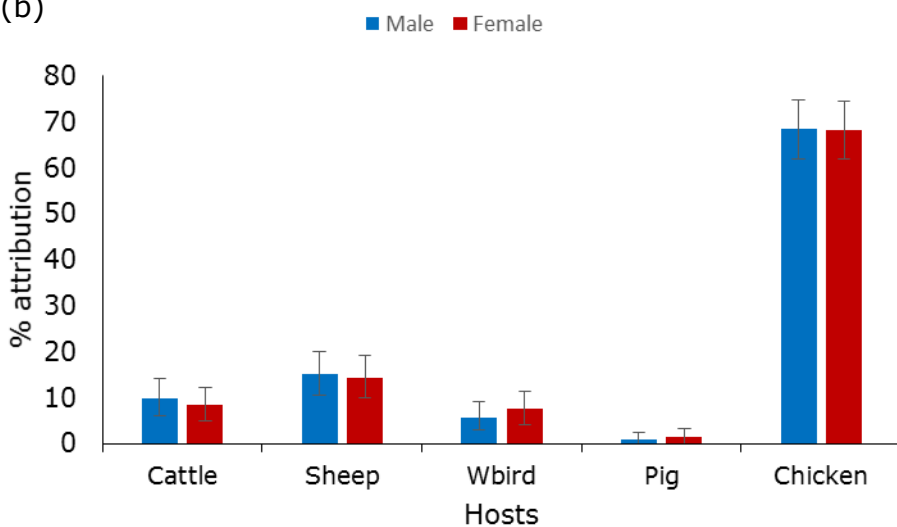
Previously, attribution of clinical isolates across all case ages in Grampian was highest to chicken sources, followed by attribution to ruminant sources (cattle and sheep) (Figure 22b) and this held true in 2016 (Figure 22a). To minimise the extent of the confidence intervals the smaller 2016 dataset was analysed with broader age bands, but despite this, the confidence intervals are still large, and conclusions from this data should be treated with caution. For wild birds and pigs there was not a significant difference in attribution across the different age groups between the current and previous study (Figure 22). The proportion of cases in the 5-14 age group attributed to a cattle reservoir was high (although not statistically significant) but could indicate that contact between farm animals (as a result of recreational school activities or educational farm visits) or drinking from a private water supply are risk factors for this age group (Evans, Roberts et al. 1996, Osbjer, Boqvist et al. 2016, Conrad, Stanford et al. 2017).

Figure 21. Gender of cases by host source attribution in 2016.

(a)



(b)



Attribution by (a) STRUCTURE, (b) Asymmetric Island. Error bars are 95% CI calculated by bootstrapping. Blue (male), red (female)

### **Source attribution and urban-rural cases**

Figure 23 illustrates the partitioning of cases by three factors: chicken vs. non-chicken; urban vs. rural residence, and age. The datasets for the periods 2016, 2012-15 and 2005-2011 allow trends over this timeframe to be examined. Aggregated age bands have been employed, as above, to maximise the number of isolates in a group whilst still stratifying into epidemiologically useful groups:

0-4 (pre-school/children), 5-14 (school children), 15-64 (adults) and 65+ (senior adults).

A decline in both chicken and non-chicken attributed cases was observed across all the age groups in 2016 compared with 2012-2015 but this was not statistically significant for every group (Figure 23).

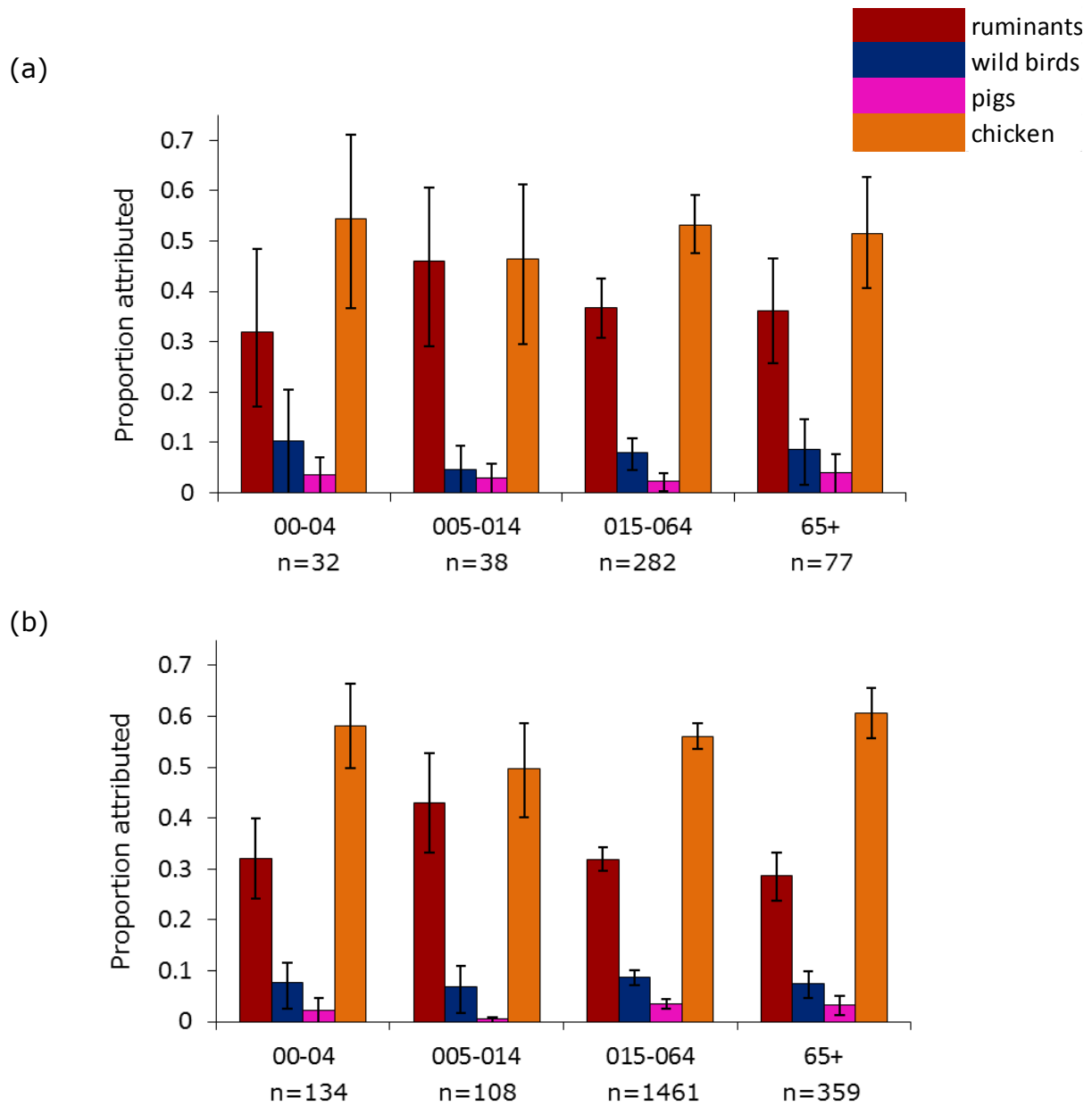
*Rural/Urban comparison:* Across all three time periods the incidence of *Campylobacter* cases was higher in rural areas compared to urban areas but not statistically significant for all age groups. The proportion of cases in the 5-14 age group attributed to the cattle reservoir was high (although was not statistically significant) (Figure 22). This could be due to contact between farm animals as a result of recreational school activities or educational farm visits or due to drinking from a private water supply which are known risk factors for this age group (Figure 22). There was no difference in incidence between chicken attributed and non-chicken attributed cases at the level of each individual age group in the urban population in this study or in iCaMPS3 (2012-2015).

*Age comparisons:* For both chicken attributed and non-chicken attributed cases the incidence was higher in the rural population than the urban and is uniform across all ages for the current study and for iCaMPS3 (Figure 23a, b) but is not statistically significant. Upon data aggregation (2005-2011), 0-4 and 15-64 age groups were significantly more likely to have infection from a non-chicken source (Figure 23c).

The aetiology of *Campylobacter* is quite complex and human infection can be caused by multiple infection pathways besides the consumption of *Campylobacter* from contaminated chicken, for example, drinking contaminated water, contact with pets or farm animals (Ogden, Dallas et al. 2009, Conrad, Stanford et al. 2017).

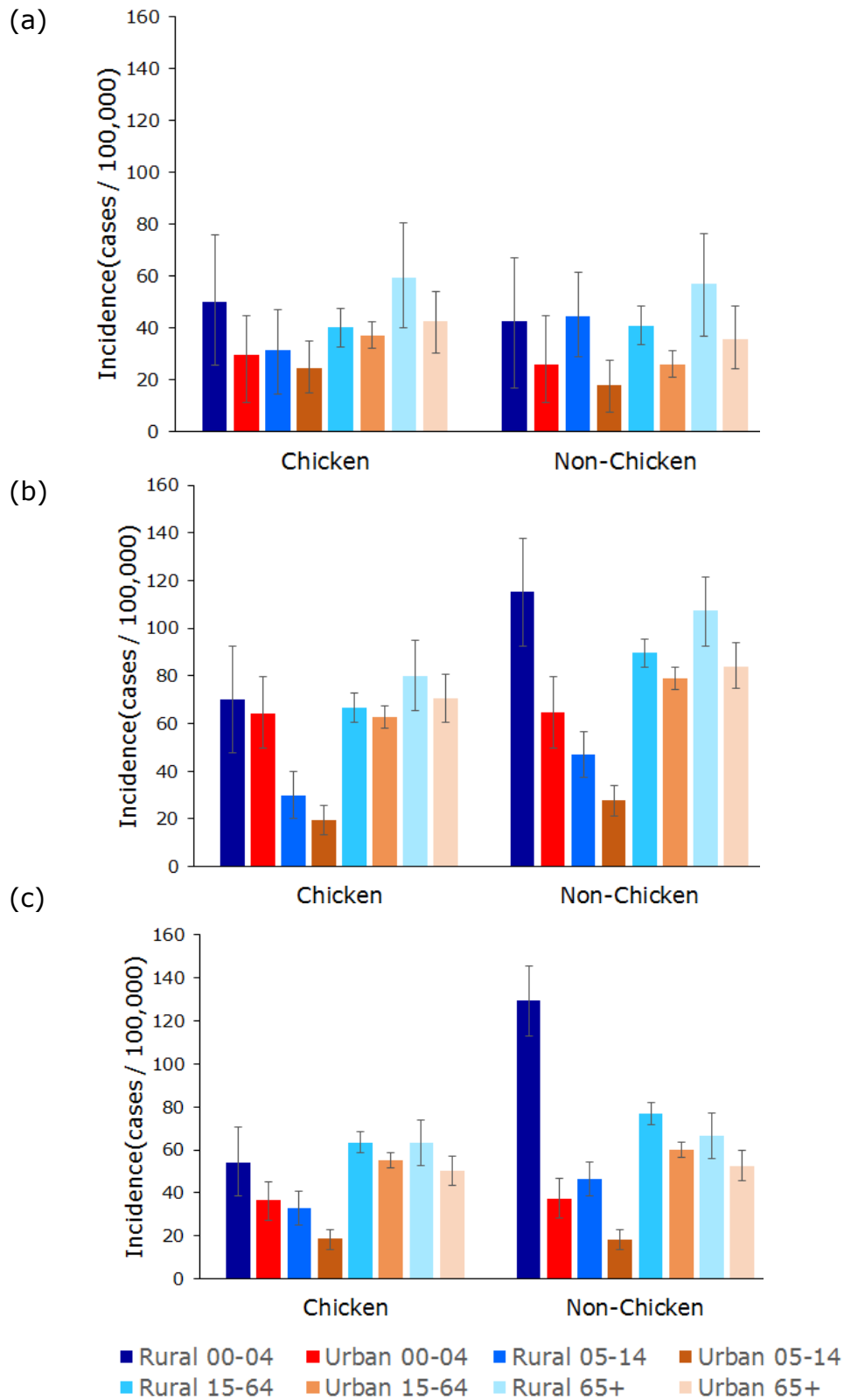
Historically, cases in the non-chicken attributed, rural, 0-4 age group have been significantly higher than any of the other groups, however, this higher incidence seems to have been declining over the timeframes of these studies such that the incidence of these cases is now comparable to all of the other groups (Figure 23). It has been previously reported that rural children are more likely to have an infection from a non-chicken attributable isolate compared to urban children through their greater exposures to *Campylobacter* in the rural environment (Smith-Palmer, Cowden et al. 2010). Notwithstanding this, the reason(s) for this specific decline are not apparent. Direct contact with farm animals is an important risk factor for infections in children visiting a farm as indicated by previous source attribution studies (Mullner, Collins-Emerson et al. 2010); children's hygiene – less effective hand washing, will play a role here. The less developed immunity to pathogens in children is also a contributing factor for campylobacteriosis (Domingues, Pires et al. 2012).

Figure 22. Attributed host sources of clinical isolates partitioned by patient age.



Clinical isolates from Grampian for period (a) 2016, (b) 2012 -15. Age banding is narrower for the larger 2012-15 dataset. STRUCTURE alleles model attributed source of chicken (yellow), ruminant (brown), pig (pink) or wild bird (blue). Error bars are 95% CI calculated by bootstrapping

Figure 23. Chicken and Non-Chicken attributed cases from Rural or Urban cases, stratified by age in Grampian.

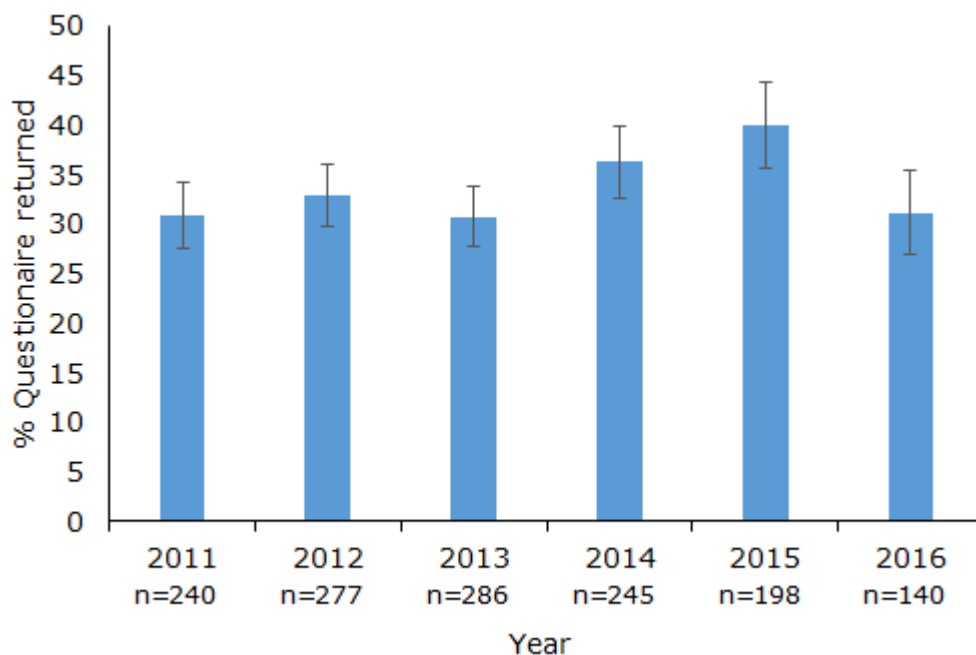


(a) 2016. (b) 2012-15. (c) 2005-2011. Isolates attributed to source by STRUCTURE alleles model. Error bars are 95% CI calculated by bootstrapping.

### 3.6. Questionnaire identified behaviours and exposures of cases.

A three page questionnaire has been sent to all cases with *Campylobacter* infection in Grampian Health Board since 2011. This questionnaire provides information on cases' lifestyle and exposure to potential sources of *Campylobacter*. The return rate for 2011-2016 period was 34% (n=1367/4055); a response rate which is considered to be high for such studies (Schwille-Kiuntke, Enck et al. 2011). The annual rates of people returning questionnaires ranged from 31 - 40% (Figure 24). The return rate was slightly higher in females (53%) compared to males (47%). The age of the subjects which responded to the questionnaire ranged from 0 to 93 years. Out of the 1,367 returned questionnaires 1,299 were linked to the corresponding cases' *Campylobacter* isolate (Table 10). This linkage permitted comparison of patient characteristics and behaviours to the genotype of strain isolated from the clinical specimen.

Figure 24. Questionnaire return rate.



'n' is the number of returned questionnaire during the year. Percentages are calculated for number of questionnaires returned by taking into account the total number of cases during the year with error bars are 95% CI calculated by bootstrapping.

Table 10. Cases with both typed isolate and questionnaire.

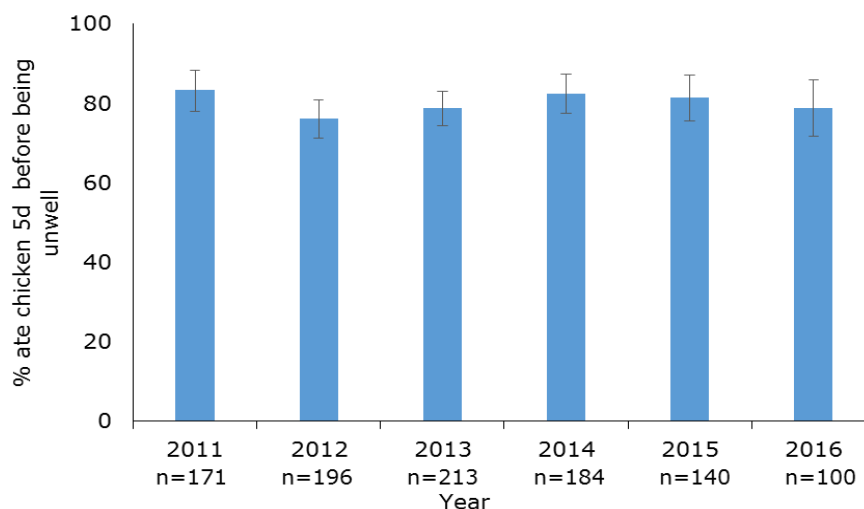
Year	2011	2012	2013	2014	2015	2016
Questionnaires received	240	277	286	245	198	140
Questionnaires with linked typed isolate (%)	220 (92%)	257 (93%)	269 (94%)	238 (97%)	182 (91%)	133 (94%)

## **Consumption and handling raw chicken before illness**

A high percentage of cases (ranging between 76-86%) reported eating chicken 5 days before becoming ill from 2011-2016 (Figure 25). No statistically significant increase or decrease was seen during these years. The strains found to be reported in these cases belonged to ST48 (8%), ST50 (7%), ST21 (6%), ST5136 (5%) and 4% each for ST45, ST257, ST42. ST5136 is exclusively associated with poultry host reservoir. ST50 showed a transition from ruminant to chicken host reservoir over the years since the CaMPS study. ST21, ST48 and ST257 are commonly found in poultry with a proportion also associated with cattle as reported in the previous iCaMPS3 study (Figure 7). ST42 has a strong ruminant association and ST45 is strongly associated with wild birds.

ST50 strains were reported in the previous iCaMPS3 report to have not been systematically described in the UK in respect of chicken borne campylobacteriosis, and this remains the case. ST48 and ST50 strains are reported in other countries such as Finland and New Zealand where they are common to the poultry chain environment (Mullner, Collins-Emerson et al. 2010, de Haan, Kivisto et al. 2010, Llarena, Huneau et al. 2015). ST5136 has been exclusive to the UK and to the poultry host reservoir. ST21 strains are generalist and are widely reported from the farm environment (Gripp, Hlahla et al. 2011). In poultry, chicken is the predominant species used for meat production (70%–80%) and global poultry meat production has increased from 58.5 million tonnes in 2000 to 95.5 million tonnes in 2014 (Sarp, Hänninen et al. 2016). In the UK, the annual consumption of chicken is about 30kg/capita (Tam, Larose et al. 2014, Clements 2015). Consuming chicken is a risk factor for campylobacteriosis caused by chicken-associated STs (Mughini Gras, Smid et al. 2012).

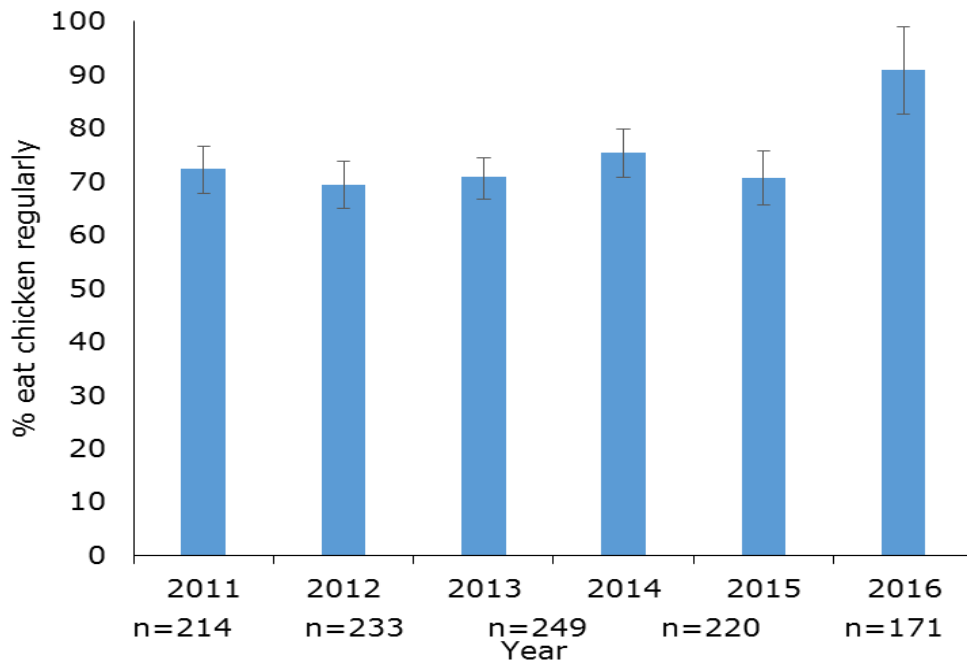
Figure 25. Cases that ate chicken within 5d prior to being unwell.



'n' is the number cases during the year that reported eating chicken 5 days prior to being unwell. Percentages are calculated for number of cases by taking into account the total number of cases based on the questionnaire data collected. Error bars are 95% CI calculated by bootstrapping.

A high percentage of cases over the study period 2011-2016 reported eating chicken regularly (69-90%) (Figure 26). A statistically significant rise was observed in 2016 compared to the previous years. The same strains associated with consumption of chicken 5 days prior to illness were also found to be associated with cases who reported eating chicken regularly: ST48 (7%), ST50 (6%), ST21 (6%), ST45 (5%) and ST5136 (5%).

Figure 26. Cases that eat chicken regularly.

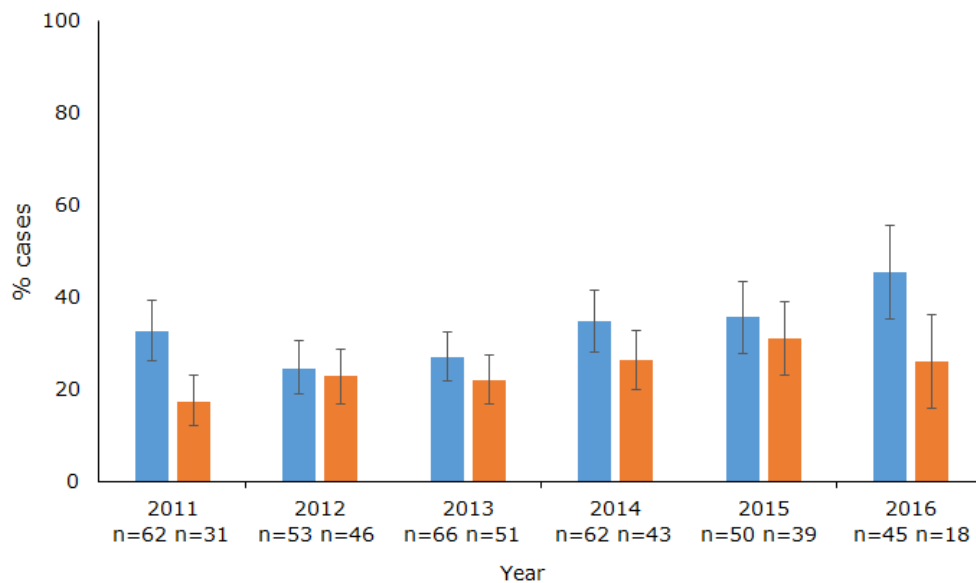


'n' is the number of cases that eat chicken regularly during a year. Percentages are calculated for these cases by taking into account the total number of cases during the same year based on the questionnaire data collected. Error bars are 95% CI calculated by bootstrapping

Cases that handled raw chicken or cases where another householder did so harboured the typical chicken associated strains: ST50 (8%), ST48 (8%), ST21 (6%), ST45 (6%) and ST5136 (6%). Over the period 2011-16 there seems to have been an increase in the proportion of cases, or others in the household, handling raw chicken (Figure 27).

Further work would be merited in elucidating whether there is a trend over the study periods from 2011 -16 of increasing chicken consumption by cases (at least in 2016) and of cases and of others in the household handling raw chicken.

Figure 27. Cases that handled raw chicken.



Cases that handled raw chicken in 5 days before onset illness (blue bar), others in household handled raw chicken in 5 days before onset illness (orange bar). Error bars are 95% CI calculated by bootstrapping. 'n' is the number of positive cases. Percentages are calculated for these cases by taking into account the total number of cases that returned questionnaire during the year.

### **Farm animal contact**

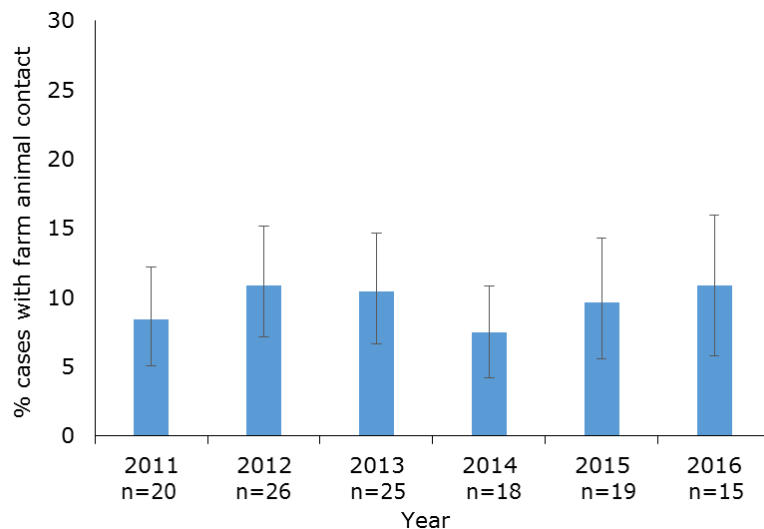
The percentage of cases that reported farm animal contact ranged between 7-11% during 2011-2016 (Figure 28). As the error bars overlap for most of the years any change (increase or decrease) is not statistically significant. The strains that were found in cases that had had contact with farm animals during this period belonged to ST45 (12%), ST21 (10%), ST48 (6%) and ST827 (6%). Previous studies report that these strains are commonly found in cattle and in sheep (Kwan, Birtles et al. 2008, Rotariu, Dallas et al. 2009). The previous iCaMPS3 study showed association of ST45 with wild birds (Figure 7). A previous large cross-sectional survey of wild bird populations in North of England found that ST45 strain was associated with wild birds (Hughes, Bennett et al. 2009). It suggested that transmission was most likely from livestock to wild birds as samples were collected from birds present in the farm environment with previous reports showing association of ST45 with the ruminant reservoir (Hughes, Bennett et al. 2009).

It is estimated that 14% of illnesses from common zoonotic pathogens are due to direct contact with farm animals, of which *Campylobacter* is responsible for 42% of cases (Hale, Scallan et al. 2012). Several studies have highlighted contact with farm animals as a mechanism for infection by *Campylobacter* (Conrad, Stanford et al. 2017, Gallay, Bousquet et al. 2008) and it has been identified as a significant risk factor for *Campylobacter* infection in Scotland (Smith-Palmer, Cowden et al. 2010). Another study reported association with

illness at home and a farm visit two weeks prior to onset of campylobacteriosis (Gillespie, O'Brien et al. 2003).

There is a consistent finding in our studies of ~10% of campylobacteriosis cases reporting farm animal contact suggesting that a maximum of 10% of cases may actually be due to animal contact – though the actual percentage will inevitably be less. This will put a base figure on the disease in Scotland, even if all directly foodborne sources of infection are removed.

Figure 28. Cases with farm animal contact.



Error bars are 95% CI calculated by bootstrapping. 'n' is the number of positive cases that reported farm animal contact. Percentages are based on cases that reported farm animal contact taking into account the total number of cases that returned questionnaire during the year.

### **Private water supply**

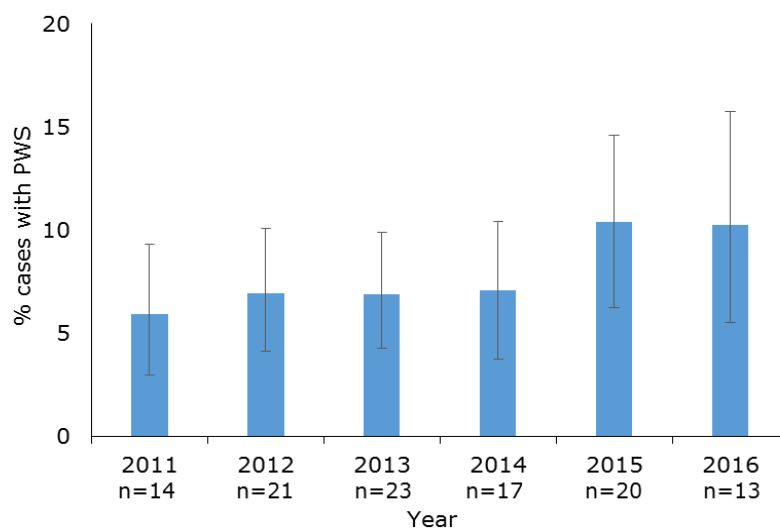
The percentage of cases that reported having a private water supply ranged from 5-10% from 2011-2016 (Figure 29). There was an increase the number of cases seen in 2015 and 2016 however the change is not statistically significant for most as error bars are overlapping. The number of cases that had a private water supply and reported animal contact ranged from 3-10 (21%-48%). The strains reported from private water supply cases typically belonged to ST21 (9%), ST45 (6%), ST827 (6%) and ST42 (5%).

These strains are associated with cattle and sheep as described in the section on farm animal contact above. There is an association between both private water supply and farm animals with the risk of infection being higher in those, such as visitors, not normally exposed to these *Campylobacter* sources possibly due to lower immuno-protection (Havelaar, van Pelt et al. 2009, Smith-Palmer, Cowden et al. 2010). This is because private water supplies are significantly more likely to be contaminated with a range of bacteria with ~10% of households in rural areas having private water supplies (mainly springs or wells) which are not part of public water treatment schemes and are often located adjacent to fields with

farms animals (Strachan, Gormley et al. 2009). Problems associated with private water supplies include old piping, proximity of livestock, inadequate knowledge of the layout and limited resources for monitoring and maintenance, lack of disinfection or failure of disinfection. An outbreak of *Campylobacter* infection was reported in the UK, as a result of drinking water that was untreated and was not boiled (Duke, Breathnach et al. 1996). Investigations found three lambs in a collection chamber connected with the private water supply or run-off of slurry from surrounding fields which were the main source of contamination (Duke, Breathnach et al. 1996). In Scotland, 3.6% and in Grampian 13.7% of the population is on a private water supply (DWQR 2016). Approximately 8000 private water supplies serve both domestic and commercial properties in Aberdeenshire (DWQR 2016, Aberdeenshire council 2017). The cases in 2016 (n=13) linked to private water supplies was the lowest of the 6 years that this questionnaire has run.

The consistent finding of up to 10% of campylobacteriosis cases reporting the use of a private water supply will put a base prevalence on the disease in Scotland, even if all directly foodborne sources of infection removed.

Figure 29. Cases with private water supply.



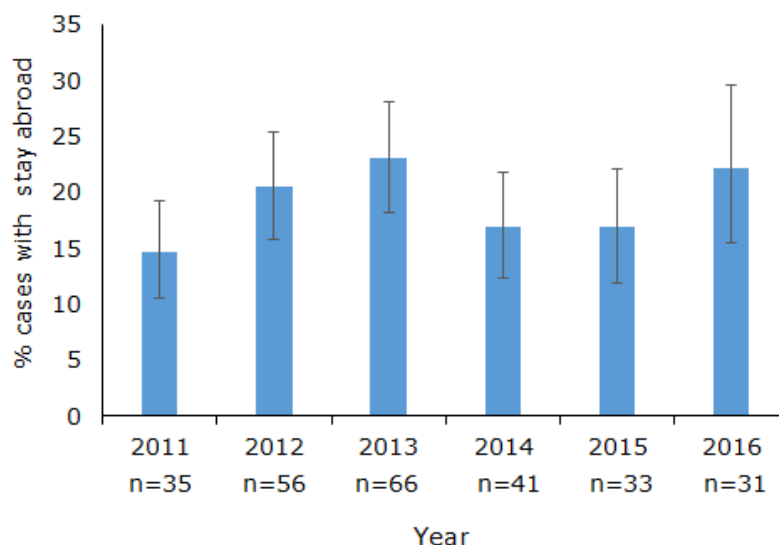
Error bars are 95% CI calculated by bootstrapping. 'n' is the number of positive cases that had private water supply. Percentages are based on cases that reported private water supply taking into account the total number of cases that returned questionnaire during the year.

### **Stay abroad**

Figure 30 shows cases with an overnight stay abroad varied from 14-22%, however no statistically significant rise or fall could be detected as the error bars overlap. Over the study period, one-in-five cases had been abroad in the period during which they were likely to have been infected. Questionnaires reported foreign travel in the five days before illness comprised 33 cases in 2015 and 31 cases in 2016. Over this period visits were made to 17 countries in Europe (10 in

2015, 14 in 2016) and 5 countries elsewhere (4 in 2015, 1 in 2016). The countries visited were Austria, Croatia, Cyprus, Czech Republic, France, Germany, Greece, Hungary, Italy, Mallorca, Netherlands, Norway, Poland, Portugal, Spain, Tenerife, Turkey, Dominican Republic, Morocco, Peru, South Korea, Zambia, multiple countries (3 cases in 2015, 3 cases in 2016). The highest number of reported cases were from Spain (5 in 2015, 6 in 2016) and France (3 in 2015, 3 in 2016), but of course these are both very popular destinations. There was no data recorded for travel within the UK and eating out of home but previous studies have shown a positive correlation with overnight stay in the UK and abroad and eating out, with cases having a significant association with eating chicken whilst eating out when compared to controls (OR = 1.52 (1.14 to 2.01),  $p = 0.0026$ ) (Smith-Palmer, Cowden et al. 2010). This positive correlation between overnight stay and eating out may demonstrate a possible link between affluence and factors such as travel abroad and eating chicken outside the home and affordability of chicken products linked to the number of times fresh chicken is consumed during the week (Smith-Palmer, Cowden et al. 2010). A previous study reported that travel amongst the elderly population from Switzerland was high, with 29% of cases associated with consumption of poultry as the source of infection and 82–89% having at least one trip with overnight stay (Buettner, Wieland et al. 2010).

Figure 30. Cases with overnight stay abroad



Error bars are 95% CI calculated by bootstrapping. 'n' is the number of positive cases that had an overnight stay abroad. Percentages are based on cases that had and an overnight stay abroad taking into account the total number of cases that returned questionnaire during the year.

The strains that were most commonly found in travel associated cases belonged to ST48 (4%), ST21 (5%) and ST50 (5%) which have been isolated mainly from chicken host reservoir (Mullner, Collins-Emerson et al. 2010, Smid, Mughini Gras et al. 2013). An unpublished MSc study at University of Aberdeen identified that

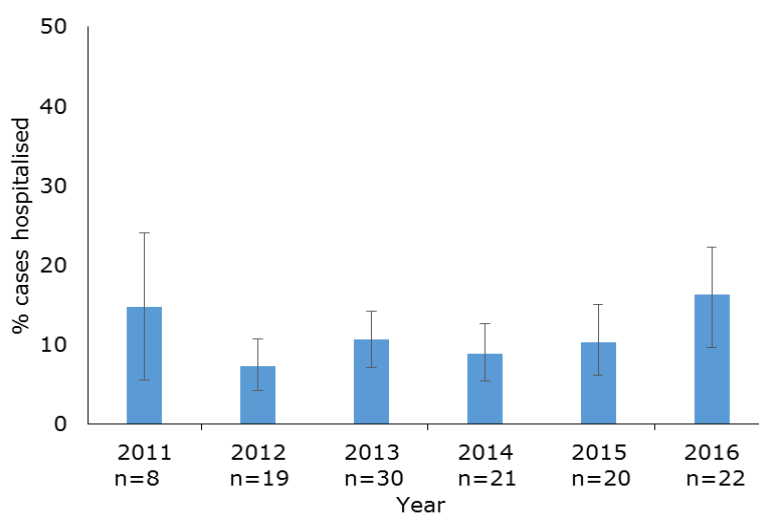
there was a positive correlation between the genetic distance (Nei's genetic distance) and the geographical distance of isolates from the UK and from other countries (foreign travel cases). Also, isolates from some of the rarer foreign travel cases' strains were found to cluster such STs to specific countries. The consistent finding of 20% of campylobacteriosis cases reporting foreign travel will put a base prevalence on the disease in Scotland, even with all other domestic sources of infection removed.

### **Hospitalised cases**

The percentage of cases that were hospitalised ranged from 7-16% from 2011-2016 (Figure 31). An increase in hospitalised cases was seen in 2016 but as the error bars overlap, this was not significant ( $p > 0.05$ ). There were no cases hospitalised for 0-4 age group in the returned questionnaires. Upon normalisation (cases/100,000), the hospitalisation rate was 18% for 5-14 age group, 17% for 15-64 age group and 31% for 65+ age group. This shows that the rate in 65+ is higher than the other age groups.

The hospitalised cases' strains were the same as those in the non-hospitalised cases: ST48 (11%), ST50 (10%), ST21 (8%), ST5136 (5%) and ST45 (5%). The hospitalisation rates may be due to individual susceptibilities or as a result of strain level differences in isolates due to genetic mutations in functional genes or the presence or absence of virulence genes which would influence virulence in strain types. This is being currently investigated in the RESAS project funded by the Scottish government. The factor that is likely to be most important in virulence of infections is prior infection with the same ST which may confer some immunoprotection. Hospitalised cases have changed little in the previous five years according to the data in Figure 31.

Figure 31. Cases that were hospitalised.



Error bars are 95% CI calculated by bootstrapping. 'n' is the number of cases that were hospitalised. Percentages are based on cases that were hospitalised taking into account the total number of cases that returned questionnaire during the year.

### 3.7. Detection of changes in campylobacteriosis incidence caused by changes in exposure.

#### **Sensitivity of attribution analyses to changes in campylobacteriosis incidence caused by changes in exposure.**

The purpose is to determine if an intervention that produces an x% reduction of human cases associated with chicken can be detected.

There are a number of sources of variation that need to be considered:

- The attribution scores do not attribute 100% to a particular host. It is known from the attribution method that some isolates from chicken will be classified as originating from another host. Also some of the isolates found in non-chicken hosts will be attributed to chicken. This is also true for instances where cross contamination of *Campylobacter* from chicken to salad can occur. Here, the source attribution would be to chicken, not the final food vehicle. Basically there is not 100% self-attribution.
- This uncertainty can be incorporated by resampling using the Monte Carlo method.
- There will be seasonal variation – this can be eliminated if comparing year to year or comparing the same period of time between years (e.g. summer 2016 with summer 2015).
- There will be year on year variation in distribution of types.

A total of 815 clinical isolates from 1<sup>st</sup> April 2012 to 31<sup>st</sup> March 2013 were collated and included their 7 locus MLST and 5 host attribution scores. Both STRUCTURE and Asymmetric Island methods were used. These clinical isolates were assigned to an animal source based on their attribution scores using the iCaMPS3 dataset (see Chapter 2.5). This was expressed as a *percentage* and boot-strapped confidence intervals were obtained using the Monte Carlo method comprising 10,000 iterations. This is the *base result*. Since a hypothetical intervention to reduce cases associated with chicken is being studied – the percentage attribution to chicken and its confidence intervals were stored for further analysis. To simulate the effect of an intervention that reduces cases in chickens (e.g. by 10%), 10% of randomly selected clinical isolates attributed to chicken were removed. The attribution to source of all the remaining clinical isolates was then determined as was the bootstrapped confidence intervals as described above. This was repeated for interventions reducing 20%, 30%, 40% and 50% of chicken attributed clinical isolates. The attribution to chicken for each of these reductions was then compared with the *base result* to determine what attribution change could be detected. This protocol takes into account the variation in attribution scores (i.e. where an isolate's scores are split amongst a number of host sources). Seasonal variation is also accounted for as the analysis is carried out over a period of 12 months, however, it does not take into account the errors associated with self-attribution.

For STRUCTURE, source attribution assigns 53.8% of human cases to chicken with the full dataset. Removal of 10%, 20%, 30%, 40% and 50% of the human cases assigned to chicken corresponds to an overall drop of 5.4%, 10.7%, 16.1%, 21.5% and 26.9% in human cases. This was repeated 10,000 times to generate bootstrapped confidence intervals. Figure 32a shows that it is possible to detect an intervention that reduces chicken cases by 30%. This effects a corresponding drop of 16.1% of cases in the human population.

For AI, source attribution assigns 73.8% of human cases to chicken with the full dataset. Removal of 10%, 20%, 30%, 40% and 50% of the human cases assigned to chicken corresponds to an overall drop of 7.4%, 14.8%, 22.2%, 29.6% and 36.9% in human cases. This was repeated 10,000 times to generate bootstrapped confidence intervals. Figure 32b shows that it is possible to detect an intervention that reduces chicken cases by 40%. This has a corresponding drop of 29.6% of cases in the human population.

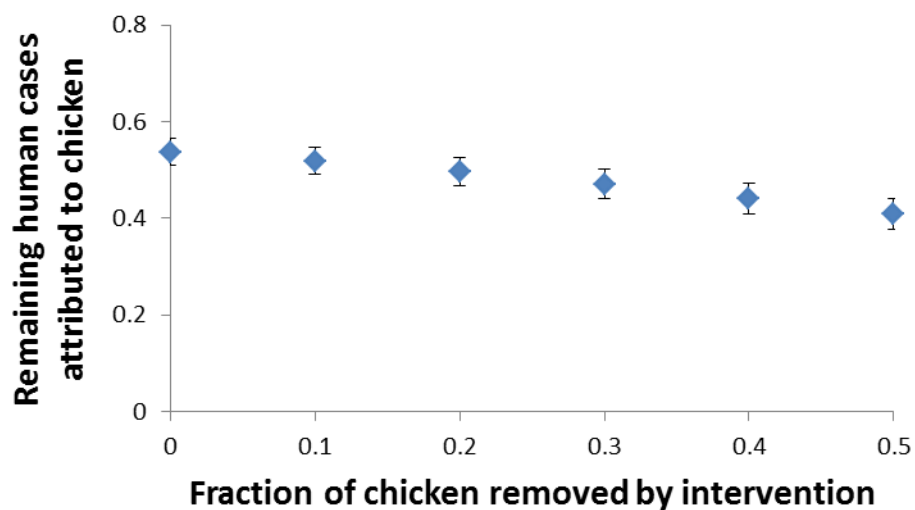
### **Was the recent fall in campylobacteriosis cases associated with a fall in the proportion of human cases associated with chicken?**

In New Zealand when there was a fall of 54% of human cases, it was claimed to be due to a number of interventions applied in the poultry industry as well as increased consumer awareness. There was also a fall in the proportion of cases attributed to chicken of 74% (Sears, Baker et al. 2011) and other sources (ruminant etc.) became more important. In Scotland there has been a fall in the number of cases recently (20.2% between 2014 and 2016) and in Grampian (31% between 2014 and 2016). There has been some evidence that this could be due to work done by the poultry industry in reducing the load of *Campylobacter* on broiler carcasses (see Chapter 1.1 above). It is therefore pertinent to identify whether the proportion of cases attributed to chicken has reduced. In Grampian between 2014 and 2016 the attribution of human cases to chicken by STRUCTURE had a fractional decrease of 9.5% (see also Figure 18). Using the Asymmetric Island model the corresponding drop was 8.5%. Both methods show a drop in chicken attributed cases during this period but the confidence intervals show the change was not significant. For STRUCTURE from Figure 32a if the whole 31% drop of clinical cases in Grampian was due to chicken then it should have been possible to detect it. However for Asymmetric Island this is only just above the detection limit (Figure 32b).

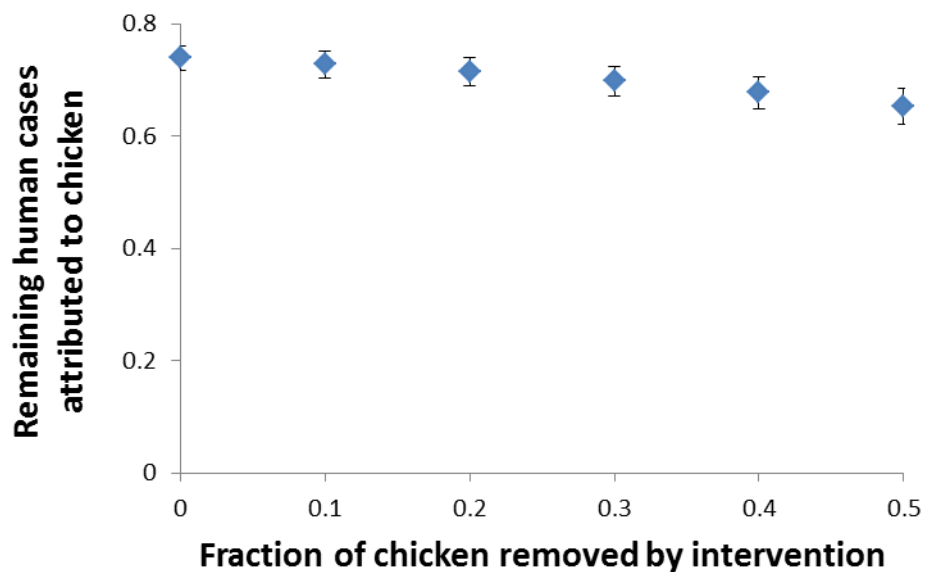
In conclusion, it is not possible using the source attribution data to confirm whether the drop in human clinical cases was due to reduction of *Campylobacter* exposure from the chicken reservoir. If the fall in *Campylobacter* cases continues, then it is likely that the attribution methods will be able to confirm if this is the case.

Figure 32. Remaining human cases attributed to chicken after a fraction of chicken attributed cases have been removed by an intervention.

(a)



(b)



(a) STRUCTURE and (b) Asymmetric Island models. Error bars are 95% CI calculated by bootstrapping.

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### **Visualising accuracy in attribution of animal and food isolates**

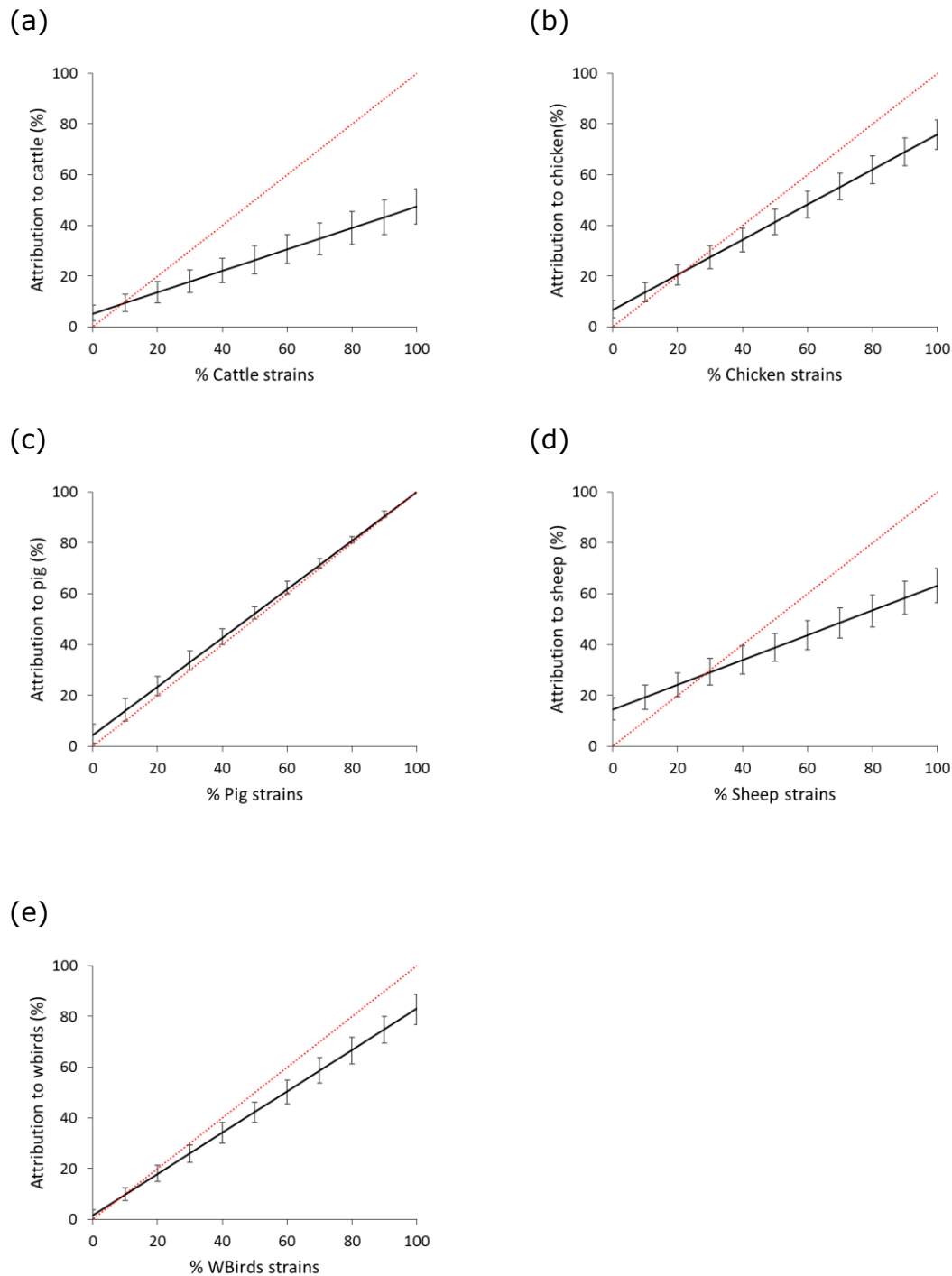
Attribution methods are not 100% correct in assigning (self-attribution) animal and food isolates to the appropriate host source. To visualise the errors that are present the following process was carried out: 200 isolates from a source (e.g. cattle) from the iCaMPS3 database were selected and their attribution to cattle was calculated using the full, five-host dataset. This was then repeated for 90% cattle isolates but with the balance of 10% isolates as 2.5% for each of the other four sources. Again the percentage to cattle was calculated. This was then repeated in 10% decrements until cattle was 0% and the other four sources comprised 25% each of the data. Confidence intervals were generated by bootstrapping. This was repeated for both STRUCTURE (Figure 33) and Asymmetric Island (Figure 34).

Correct attribution is defined as the percentage attribution to source X where all isolates originate from source X. For example, for cattle analysed with STRUCTURE there is a correct attribution to cattle of 47.5% (Figure 33a). Wrong attribution is defined as the percentage attribution to source X where all isolates originate from sources other than X. Since there are four other sources, the other sources comprise 25% of each. There is a wrong attribution to cattle of 5.23% (Figure 33a).

Looking across all five hosts for both STRUCTURE (Figure 33) and Asymmetric Island (Figure 34) gives an indication of the robustness of the attributions for each host. The biggest error in attribution is between cattle and sheep in both models. For STRUCTURE the smallest error is for pig strains, whilst for AI this is less obvious. Overall the correct attribution is 73.9% for STRUCTURE and 62.8% for AI, respectively. The overall wrong attribution is 6.5% for STRUCTURE and 9.3% for AI, respectively, the former model performing better.

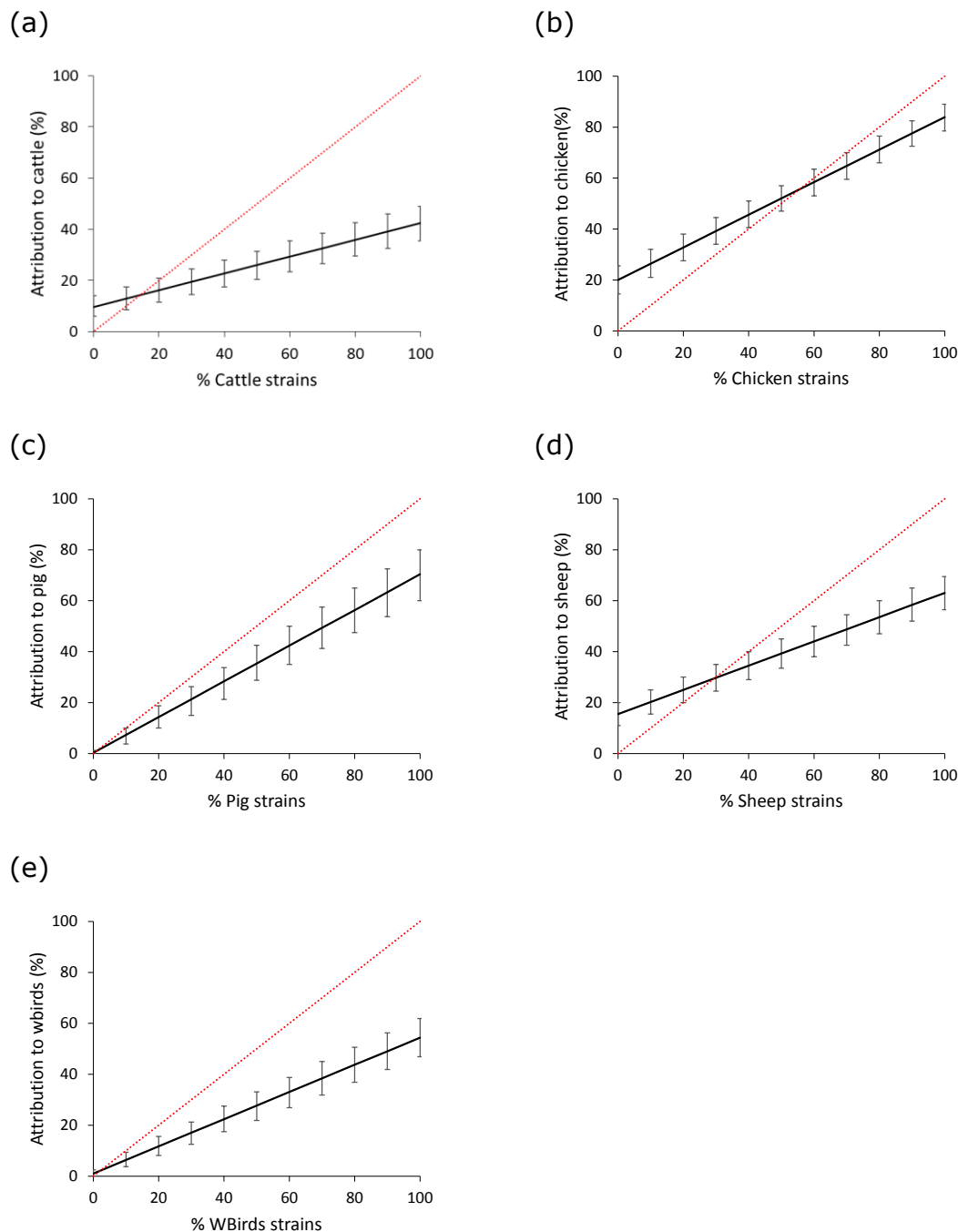
Future analytical work on how to utilise the self-attribution results to quantitatively revise the attribution results from the clinical cases will provide better insights into understanding the association of strains with different host reservoirs.

Figure 33. Estimating the accuracy of attribution to animal and food sources using STRUCTURE.



(a) cattle: correct attribution 47.5% and wrong attribution 5.2%, (b) chicken: correct attribution 75.9% and wrong attribution 6.7%, (c) pig: correct attribution 99.9% and wrong attribution 4.5%, (d) sheep: correct attribution 63.2% and wrong attribution 14.5% and (e) wild birds: correct attribution 83.1% and wrong attribution 1.6%. Error bars are 95% CI calculated by bootstrapping. Red line is the ideal fit fine, black line represents variation occurring in attribution at the expense of 10% decrements of the host source (but substitution with other 4 hosts) on Y axis.

Figure 34. Estimating the accuracy of attribution of animal and food sources using Asymmetric Island.



(a) cattle: correct attribution 42.5% and wrong attribution 9.7%, (b) chicken: correct attribution 83.9% and wrong attribution 19.9%, (c) pig: correct attribution 70.4% and wrong attribution 0.3%, (d) sheep: correct attribution 63.0% and wrong attribution 15.5% and (e) wild birds: correct attribution 54.4% and wrong attribution 1.0%. Error bars are 95% CI calculated by bootstrapping. Red line is the ideal fit line; black line represents variation occurring in attribution at the expense of 10% decrements of the host source (but substitution with other 4 hosts) on Y axis.

### 3.8. Linking *Campylobacter* from cases to sources using genomics

Source attribution enables linkage of a human isolate to a particular source (chicken, cattle etc.). However, it would be very helpful to link clinical isolates with higher precision epidemiologically. For example, to identify groups of isolates that form an outbreak and to track an isolate from farm to shop to clinical case. Whole genome sequencing offers the potential to achieve this and this has been demonstrated for the identification of outbreaks of *E. coli* O157 (Dallman, Byrne et al. 2015).

*Campylobacter* has a high degree of genetic diversity and the lifestyles of the different sequence types can vary considerably. Here, two sequence types are studied. ST50 which is predominantly chicken associated and ST61 that is predominantly from ruminant sources.

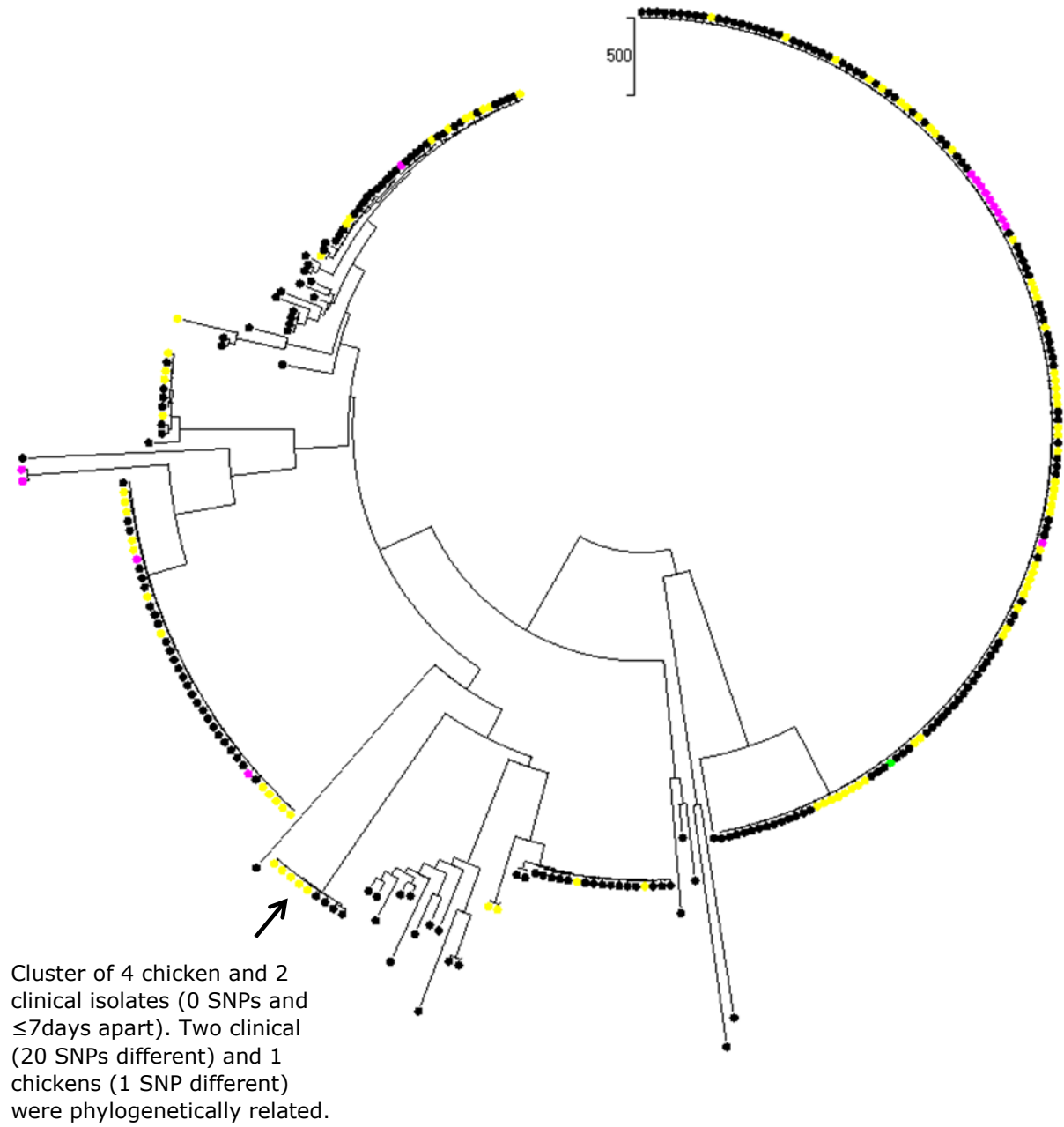
*Isolates:* All available clinical isolates (n=268) of sequence types ST50 (n=222) and ST61 (n=46) were collected between Apr 2011 to Mar 2015. All available food and environmental isolates (n=244) of sequence types ST50 (n=102) and ST61 (n= 142) were collected between Jun 2011 to Apr 2015. These were distributed as follows: ST50 (cattle - n=1, chicken - n=85, sheep - n=1, turkey - n=15), ST61 (cattle - n=60, chicken - n=9, sheep - n=72, turkey - n=1).

*Pan-genomic SNP analysis:* PANSEQ was used to construct a non-redundant pan-genome from the 512 animal, food and clinical genomes. The pan-genome was constructed by using a seed genome and identifying regions of  $\geq 1000$  bp not found in the seed but present in any other genome at a 99 percent sequence identity cut-off. The pan-genome was subsequently fragmented into 1000 bp segments, and the presence/absence of each locus in every genome determined at a 99 percent sequence identity threshold was documented. Loci present in all genomes underwent multiple sequence alignment using Muscle (Edgar 2004), and were concatenated together. This aligned pan-genome was used to identify SNPs in the core genome of all isolates. A neighbour joining tree was generated and visualised in MEGA (Kumar, Nei et al. 2008).

The core genome for ST61 comprised 1.497 Mb which incorporated 13,448 SNP's. The core genome for ST50 comprised 1.348 Mb which incorporated 21,596 SNP's. Figure 35 shows the neighbour joining trees for ST50 and ST61 strains. The difference in hosts between the two STs and that human cases are scattered throughout the phylogenies can be readily seen. As expected, ST50 had a preponderance of isolates from chicken and ST61 had a preponderance of isolates from ruminants. Since there are more ST50 than ST61 associated clinical cases, ST50 is more common in the human population which appears to be originating from chicken host reservoir. Our analysis is based on "all available" food/environmental isolates. The generalist phylogenies are being looked into in the RESAS project funded by the Scottish government.

Figure 35. High resolution SNP based phylogenetic tree for ST50 and ST61.

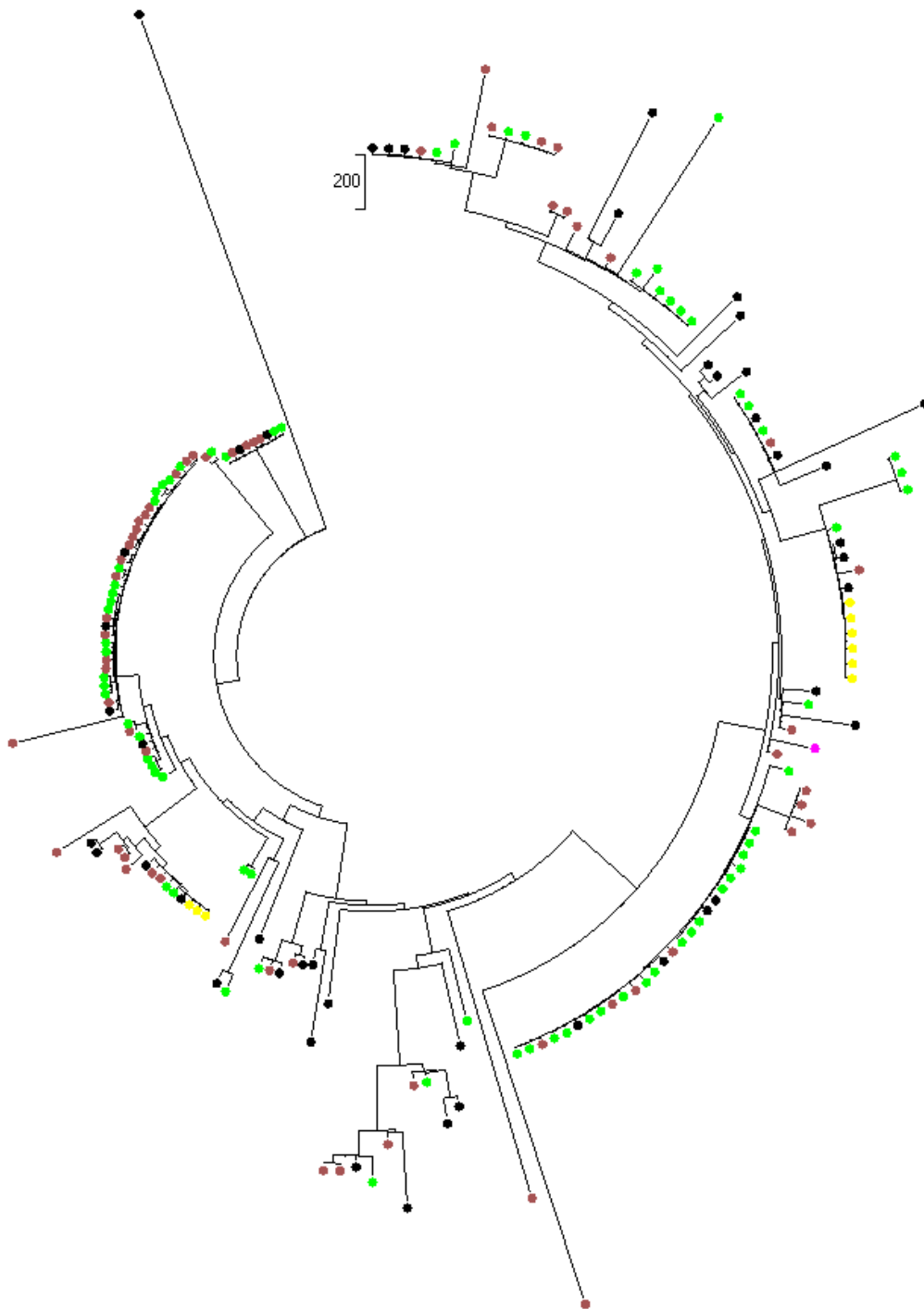
(a)



High resolution SNP based phylogenetic tree for (a) ST50 to detect clustering between clinical isolates and animal and food sources.

Sources: Clinical (●); Cattle (●); Sheep (●); Chicken (●); Turkey (●)

(b)



High resolution SNP based phylogenetic tree for ST61 to detect clustering between clinical isolates and animal and food sources.

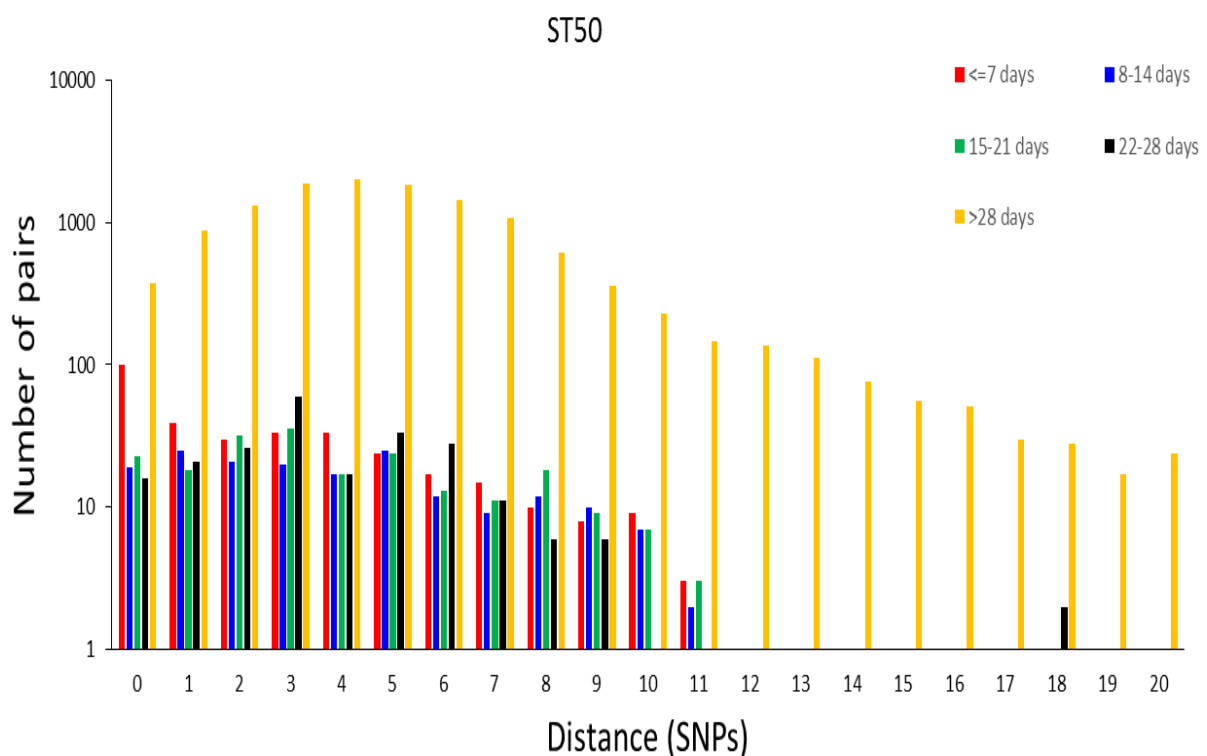
Sources: Clinical (●); Cattle (●); Sheep (●); Chicken (●); Turkey (●)

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**Genetically related in time:** pairwise SNP distances were calculated for all ST50 isolates and then stratified by time (0-<=7 days, 8-14 days, 15-21 days, 21 – 28 days and >28 days).

Figure 36 shows that there are many pairs of isolates (n = 372) that have a zero SNP distance even when they are isolated >28 days apart. In the current study the maximum time between a pair of ST50 isolates with 0 SNP distance was found to be 847 days. So SNP distance alone does not have sufficient resolving power to detect clusters of isolates that are constrained in time as has been done for *E. coli* O157 (Dallman, Byrne et al. 2015). Hence, there is the need to use time information in the analysis.

Figure 36. Frequency of pairwise SNP distances for ST50 isolates stratified by time



To determine whether there was potential clustering of isolates a randomisation test was performed. This test first counted the actual number of pairs with a specific SNP distance (e.g. 0 SNP's) occurring within one of the timeframes mentioned above. The SNP distances were then shuffled (just like a pack of cards) and the number of "randomised" pairs with the same SNP distance and within the same specified timeframe was calculated. This was repeated 10,000 times using the Monte Carlo methods so that an average of the randomised pairs could be calculated, the range determined as well as a p value. Table 11 shows actual number of pairs of isolates found within a particular SNP distance and time frame for ST50.

Table 11. Actual number of pairs of isolates found within a particular SNP distance and time frame for ST50.

Distance (SNPs)	Time Difference between isolates (Days)	Actual number of pairs	Randomised number of pairs (Range)	p-value
0	≤7	101	8.2 (3 – 14)	<0.0001
	8 - 14	19	5.8 (2 – 11)	<0.0001
	15 - 21	23	5.5 (1 – 10)	<0.0001
	22 - 28	16	5.8 (2 – 11)	0.0002
	>28	372	506.0 (496 – 515)	1.0000

Actual number of pairs of isolates found within a particular SNP distance and time frame for ST50 with results of the randomisation test and associated p – value.

For ST50 there were 50,721 pairs that were compared. Table 11 shows that the number of pairs of isolates found to have 0 SNP distance are significantly higher than would be expected by chance for all of the time periods ≤28 days. Hence, providing evidence of clustering. For the time period >28 days the opposite is found to be the case. Analysis has not been done for >0 SNPs but it may well be that similar results could be obtained for 1 or 2 SNPs for example.

Referring to the 101 pairs of ST50 isolates described in Table 11 with 0 SNP distance and within ≤7 days it was attempted to find whether these were clustered in time. It was found that only 81 of the isolates were in clusters. In total there were 30 clusters (17 contained 2 isolates, 11 contained 3 isolates, 1 contained 6 isolates and 1 contained 9 isolates).

The 9 isolate cluster comprised solely turkey isolates obtained from 3 different postal districts.

The cluster that contained 6 isolates (Figure 35a) comprised two clinical and 4 chicken isolates. The 4 chicken isolates were obtained from the same postal sector and on the same day and were likely collected from the same shop. Associated phylogenetically with this cluster was 1 further chicken isolate that had 1 SNP difference, was obtained from a different postal sector and isolated approximately 2 weeks after the other isolates. The 2 other human isolates on the same branch of the tree were 20 SNPs different, however since they are 20 SNPs different their relationship is not that close. These 2 isolates were isolated approximately 3 years after the other clinical and human isolates.

Four of the remaining 28 clusters (containing 3 isolates or less) comprised a mixture of chicken and clinical isolates. Of the other 24 clusters, 13 comprised

clinical only and the rest comprised chicken only except for one which was turkey only.

This section shows that there is the potential to identify clusters of isolates using SNP based whole genome sequencing techniques. It is worth noting that "identical" SNP profiles were obtained from isolates up to 847 days apart. This indicates that for detection of outbreaks that SNP based information may be insufficient on its own and that other epidemiological information including isolation time will be required.

## 4. Conclusions

This study has addressed the following:

### **Campylobacter at farm and abattoir**

#### **1. Whether the Scottish abattoir's *Campylobacter* strains are distinct from chicken strains from the rest of the UK (Section 3.1, 3.2).**

The strain distribution from whole birds from the Scottish abattoir were statistically significantly different from those found in retail birds sampled during the iCaMPS3 (2012 -15) study, however, the sampling periods are not contemporaneous. A retrospective analysis of isolates from retail chicken purchased in Grampian shops over the period from 2005 -2015 observed common strain types from Scottish abattoirs compared to birds from abattoirs from the rest of the UK. In conclusion, there is much commonality between *Campylobacter* strains found from Scottish produced broilers and those from abattoirs elsewhere in the UK.

#### **2. The evidence of transmission of *Campylobacter* between farms (Section 3.2).**

The carriage of *Campylobacter jejuni* and *Campylobacter coli* by flocks was found to be 88% (based on caeca collected at abattoir) with very few flocks having the organism absent. All of the abundant sequence types found in these flocks were typical of those found elsewhere in the UK and are also commonly found in human cases. In conclusion, there is much commonality in the *Campylobacter* strains found across Scottish farms, suggesting that these sequence types are endemic to the industry and would be maintained by on-going transmission of strains between farms.

#### **3. The usage of antimicrobials on farms (Section 3.2).**

54% of flocks were treated with at least one antibacterial agent. Overall, eight different antibacterials from five different drug classes were used. The use of antimicrobials has the potential to generate *Campylobacter* strains with new resistances and to maintain pre-existing resistant strains. The transmission of these resistance genes and the organisms into the human food chain is of concern. Antimicrobial resistance mechanisms of selected *Campylobacter* strains from the current and previous studies are being investigated in more detail in an on-going study funded by Scottish Government through RESAS.

#### **4. Cross contamination between chicken batches (flocks) at abattoir (Section 3.2).**

21% of flocks had a caecal strain different to those found on whole birds from the same flock. This might indicate cross contamination between the flocks during processing in the abattoir environment, but could also be a reflection of multiple strain colonisation on the farm. Further study would be required to clarify this and would need to include sampling of consecutive flocks at

processing to detect spill over from one flock to the next and also deeper sampling to determine the distribution of strains carried within each flock.

### **5. The impact of abattoir interventions on *Campylobacter* levels on retail ready broilers (Section 3.2).**

The abattoir introduced two interventions designed to reduce the *Campylobacter* load on retail ready birds: trimming of neck flaps and a secondary scald tank. The former was not assessed as it was introduced prior to this study. *Campylobacter* absence vs. presence on whole birds at the end of line compared with the different operating temperatures of the scalding tank did not detect a difference, however a conclusion as to its effectiveness could not be drawn due to several difficulties such as the lack of availability of control birds, seasonality, time, initial strain carriage and isolate differential responses to heat stress. However, it is worthy to note that, of positive birds the majority had low loads and only 20% of abattoir and 10% of retail birds were positive with highest levels of greater than or equal to 1000 cfu/g. A greater number of retail birds were enrichment positive compared to abattoir birds. The bird carcasses at the Scottish abattoir had a heavier load of *Campylobacter* compared to that of retail birds. However, this may be because the Scottish abattoir birds had a shorter time since slaughter with less time for the organism to have become less viable or have died. It is hard to eliminate confounding factors when interventions are piloted at both farm and abattoir. This needs to be considered when designing and interpreting results of such studies.

## **Human campylobacteriosis in Grampian**

### **1. The *Campylobacter* strains in human campylobacteriosis cases and disease severity (Section 3.3).**

As reported in the previous studies of *Campylobacter* in Grampian, there continue to be changes in the abundances of the different strains seen in human cases (Forbes, Horne 2009, Lopes, Forbes et al. 2016). These changes reflect the dynamic turnover of retail poultry strains compared to the apparently slower turnover of strains in other sources of *Campylobacter*. Over longer timeframes some strains are seen to increase, or indeed appear *de novo*, or decline in relative abundance. For example, recent years have seen an increase in clinical abundance of ST50 which ten years ago was an uncommon human clinical strain found only in ruminants and wild birds, but is now the most commonly found strain in human clinical isolates and is also now common in retail chicken. Similarly strain ST5136 was first identified in retail chicken and in human cases, has now spread to turkey, but has not been identified elsewhere. Strains associated with ruminants, such as ST61, and with pigs appear to be much more stable. The percentage of cases that were hospitalised ranged from 7-16% from 2011-2016 (Figure 31). A non-significant ( $p>0.05$ ) increase in hospitalised cases was seen in 2016. The hospitalised cases' strains were the same as those in the non-hospitalised cases and belonged to ST48, ST50, ST21, ST5136 and ST45. The hospitalisation rate was higher in the 65+ age group. This may be due to

individual susceptibilities or as a result of strain level differences in isolates due to genetic mutations in functional genes or the presence or absence of virulence genes that would influence virulence in stain types.

## **2. *Campylobacter jejuni* and *C. coli* epidemiology (Section 3.4).**

*C. coli* are typically the cause of some 10% of campylobacteriosis cases with *C. jejuni* comprising the bulk of the remainder. Our analysis suggests that *C. coli* infection of people may be less seasonal than is the case for *C. jejuni* however this needs further study. Both species seem to be following the same year-on-year trend in incidence of human infection. The incidence of both *C. jejuni* (significant decrease in 2015-2016,  $p < 0.05$ ) and *C. coli* has decreased in recent years. The incidence of *C. coli* is always lower, whether this is because *C. jejuni* is a better coloniser than *C. coli* or just simply because *C. jejuni* is more abundant than *C. coli* or indeed because *C. coli* is better at colonising older birds, is not known. More follow up studies are required to understand the epidemiology of *C. coli* in detail. Figure 16 suggests that both *C. jejuni* and *C. coli* have similar incidences of infection within each of the age groups examined but the sample size for *C. coli* is small. Further examination of the epidemiological similarities and differences between these two species will help in understanding the place of these species in human infection.

## **3. The sources of human campylobacteriosis (Section 3.5, 3.6, 3.7).**

Molecular attribution analyses during the period from 2005 – 2016 continue to show a preponderance of cases attributable to chicken sources. With the STRUCTURE model, a significant decline ( $p < 0.05$ ) in cattle attributed cases and a rise in pig attributed cases has been seen from 2011 to 2016 (Figure 18). The sheep and wild bird attributed cases have remained constant (Figure 18). There was an increasing trend of chicken attributed clinical cases from 2011 to 2015 using the STRUCTURE model but this was not statistically significant. In the AI model, a significant rise ( $p < 0.05$ ) was seen in wild bird attributed clinical isolates in 2016, compared to 2011 (Figure 18). The attribution to ruminant sources and pigs has fairly remained constant but a non-significant increase has been observed in 2016 ( $p > 0.05$ ) (Figure 18). A significant decline is seen in chicken attributed cases in 2016 compared to 2011 ( $p < 0.05$ ) in the AI model (Figure 18). It is important to note that modelling provides insights into what has happened but it may not reflect exactly what has happened. Chicken still remains the largest attributed source of clinical *Campylobacter* infection.

The percentage of cases that reported eating chicken regularly during the 2011-2016 period ranged from 69-90% (Figure 26) with a significant increase ( $p < 0.05$ ) seen in 2016 compared to the previous years. The strains found to be reported in these cases belonged to ST50 (previous reports show strong ruminant association but now more common in poultry), ST48, ST257 and ST21 (generalist strains), ST45 (associated with ruminants and reported commonly in

wild birds), ST5136 (exclusively associated with poultry), ST42 (strongly associated ruminant strain).

The percentage of cases that reported farm animal contact ranged between 7-11% during 2011-2016 (Figure 28) but as the error bars overlap for most of the years therefore any apparent change (increase or decrease) is not statistically significant.

In Scotland, 3.6% of the population is on private water supply (DWQR 2016). The 13.7% of the Grampian population served by a private water supply comprises 8000 such supplies to both domestic and commercial properties in Aberdeenshire (DWQR 2016, Aberdeenshire council 2017). The percentage of cases that reported having a private water supply ranged from 5-10% from 2011-2016 (Figure 29). There was an increase the number of cases seen in 2015 and 2016 however the change was not statistically significant for most as error bars are overlapping.

During the 2011-2016 period, the strains that were found in cases reporting contact with farm animals and those having private water supply belonged to ST45 (associated with ruminants and reported commonly in wild birds), ST21 and ST48 (generalist strains that occur in chicken as well as cattle) and ST42, ST827 (strongly associated ruminant strains).

Cases with an overnight stay abroad varied from 14-22%, however no statistically significant rise or fall could be detected as the error bars overlap ( $p > 0.05$ ; Figure 30). Over the period one-in-five cases had been abroad in the period during which they were likely to have been infected. Spain and France were the most popular destinations in 2015 and 2016. The strains that were most commonly found in travel associated cases belonged to ST48, ST21 and ST50 which have been isolated mainly from chickens.

Returned questionnaires with information on cases' exposures and activities identified that up to 40% of cases reported contact with farm animals, having PWS or foreign travel; all of which are known risk factors for infection.

#### **4. Seasonal trends and risk factors for human campylobacteriosis (Section 3.5).**

No seasonal variation in source of infection was detected. Gender did not influence the likelihood of infection from different sources ( $p > 0.05$ ; Figure 21). Age may have an effect though through what cause is unclear: older cases are more prevalent than younger cases, and 5-14 year old children are less likely to have a chicken attributable isolate (Figure 22). There was no difference ( $p > 0.05$ ) in incidence between chicken attributed and non-chicken attributed cases at the level of each individual age group in the urban population in this study or in iCaMPS3 (2012-2015) (Lopes, Forbes et al. 2016). For both chicken attributed and non-chicken attributed cases the incidence was higher, but not significant ( $p > 0.05$ ) in the rural population than the urban and is uniform across all ages for the current study and for iCaMPS3 (Figure 23a, b). Upon data

aggregation (2005-2011), 0-4 and 15-64 age groups were significantly more likely to have infection from a non-chicken source when compared to a chicken source (Figure 23c). A non-significant decline in both chicken and non-chicken attributed cases was observed in 2016 (Figure 23). Rural cases continue to have higher infection rates in general compared to urban (though not significant,  $p > 0.05$ ), but are less likely to have infection from a chicken source (although not significant,  $p > 0.05$ ) but show statistically significant risk associated with infection from non-chicken sources in all except 65+ age groups with the iCaMPS 3 dataset (2012-2015) and 2005-2011 datasets which are larger in size. For 2016 dataset both the chicken and non-chicken associated urban cases are lower than rural but it is not statistically significant as the error bars overlap. Of note, is the apparent significant decline in recent years in cases in rural under-fives infected by non-chicken associated strains.

#### **5. The sensitivity of molecular analyses for detecting a drop in the proportion of cases attributable to chicken (Section 3.7).**

Investigating the sensitivity of the attribution models used to ascribe a source to a fall in clinical cases determined that an intervention that reduced chicken cases by 30-40% would result in a corresponding drop of 15-30% of cases in the human population, depending on the model. The recent 31% fall in human cases in Grampian between 2014 and 2016 resulted in a reduction in the percentage of cases attributed to chicken by both models but this was not statistically significant. These results, together with the sensitivity analysis, suggest that a reduction in exposure to *Campylobacter* from chicken by humans in Grampian was not the sole causative factor for the reduction in human cases. This warrants further study particularly if the current fall in human cases continues.

#### **6. Case clustering detection between human and chicken isolates (Section 3.7).**

Single nucleotide polymorphisms (SNPs) were used to "fingerprint" the core genome of ST50 which was selected as it is now a common strain in both clinical cases and chickens.

However, there is evidence of clustering of ST50 isolates that are zero SNPs apart and isolated within 28 days. There is some bias in that some strains from food and animal isolates will have been collected at the same time and from the same location. Five clusters comprised mixtures of chicken and human isolates. Further epidemiological details on the source of the chicken consumed by the humans (ideally traced back to abattoir and farm) would offer the potential of providing an epidemiological link between clinical case and source. The analysis done so far is preliminary and further work needs to be conducted for different SNP distances and timeframes (e.g. humans will take some days to fall ill and a sample to be received in the lab). Application of this method to other STs could prove useful and a comparison of the performance of SNP based methods with whole genome MLST is required.

## 5. Implications for FSS

Listed below are how the main findings are of relevance to FSS:

- As there are many strains of *Campylobacter* that cause disease in humans that are also found in poultry farms and retail chicken it is likely that the industry need to develop a common approach needs to be in place to tackle human campylobacteriosis.
- Given the critical role of poultry colonised at farm reported here (and elsewhere) an improved understanding of the farming practices that enable *Campylobacter* to colonise chickens is required in order to inform interventions strategies at the farm level.
- Understanding the biology of *Campylobacter* strains and their apparent categorisation into specialist or generalist strains will be helpful in implementing strategies that can be targeted at the most abundant strain types that occur in poultry (or indeed in secondary reservoirs such as ruminants). Whether this is achieved by using natural probiotic bacteria (*Lactobacillus spp.*) that are classified as QPS (Qualified Presumption of Safety) by European Food Safety Authority (EFSA) or phage that can selectively target and inhibit *Campylobacter*, or by a vaccine, the method must be effective against all of the major strains.
- Assessing the effectiveness of interventions at abattoir is difficult. It requires close cooperation with industry and proper planning and design to reduce confounding and to have appropriate statistical power. Industry and researchers sharing their results in conjunction with FSS/FSA offers the potential of sharing best practice in this area which is vital for future progress.
- Greater attention needs to be paid to the emergence of antimicrobial resistant clones of *Campylobacter*. In this study 54% of flocks were treated with at least one antibacterial agent. Little is known about antibiotic resistance in *Campylobacter*, its impact on the chicken gut microbiome, or on human health. In the first instance, attention should be focused on those classes of antimicrobials that are also used in humans. For example fluoroquinolone resistant *Campylobacter* is designated as a high priority pathogen by the WHO.
- Keeping track of the proportion of human cases attributable to chicken will require contemporaneous genotyping of both human and chicken isolates. Regular sampling from other sources appears to be less critical as the turnover of strains here is less than it is for chicken.
- Contact with farm animals, having a private water supply and foreign travel are all risk factors for human *Campylobacter* infection. They are also risk factors for a number of the other gastrointestinal pathogens.

Hence an approach that for example reduces bacterial contamination in private water supplies will likely be important for *E. coli* O157 as well.

- A methodology to determine the sensitivity of molecular attribution methods to detect a statistically significant fall in chicken associated cases has been made here. This will be helpful for future source attribution studies that aim to detect whether interventions in the poultry chain result in reducing the number of human cases of disease.
- Whole genome sequencing offers the opportunity to help trace *Campylobacter* between the farm and the human case. This is important for identifying, confirming and investigating outbreaks, determining the origin of sporadic cases of disease and providing further evidence to demonstrate to poultry farmers that it is *Campylobacter* from their farms that end up causing disease in humans.
- The incidence of cases or other people connected with a case in a household that handled raw chicken appears to be increasing (of those who returned the questionnaire). If confirmed through more extensive sampling, this could be an increasing risk for campylobacteriosis. It would be important therefore to understand kitchen practices and awareness of the risk as well as how the public should be informed to reduce the risk.

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- Data for cases in Wales: Amy Phillips, PHW

## 7. Recent outputs and related studies

### 7.1. Publications

- Miller WG, Yee E, Lopes BS, Chapman MH, Huynh S, Bono JL, Parker CT, Strachan NJC and Forbes KJ. (2017) Comparative genomic analysis identifies a *Campylobacter* clade deficient in selenium metabolism. *Genome Biology and Evolution*, 9: 1843-58.
- Jones NR, Millman C, van der Es M, Hukelova M, Forbes KJ, Glover C, Haldenby S, Hunter PR, Jackson K, O'Brien SJ, Rigby D, Strachan NJC, Williams N, Lake IR, Enigma consortium. (2017) A novel sampling method for assessing human-pathogen interactions in the natural environment using boot socks and citizen scientists, with an application to the seasonality of *Campylobacter*. *Applied Env Microbiol*, in press.
- van der Graaf-van Bloois L, Duim B, Miller WG, Forbes KJ, Wagenaar JA, Zomer AL (2016) "Whole genome sequence analysis indicates recent diversification of mammal-associated *Campylobacter fetus* and implicates a genetic factor associated with H<sub>2</sub>S production." *BMC Genomics*, 17: 713
- Graaf-van Bloois, LVD., Miller, WG., Yee, E., Gorkiewicz, G., Forbes, KJ., Zomer, AL., Wagenaar, JA. & Duim, B. 2016. '*Campylobacter fetus* Subspecies Contain Conserved Type IV Secretion Systems on Multiple Genomic Islands and Plasmids'. *PloS one*, vol 11, no. 4, e0152832.
- Gilbert, MJ., Miller, WG., Yee, E., Zomer, AL., van der Graaf-van Bloois, L., Fitzgerald, C., Forbes, KJ., Méric, G., Sheppard, SK., Wagenaar, JA. & Duim, B. 2016. 'Comparative Genomics of *Campylobacter fetus* from Reptiles and Mammals Reveals Divergent Evolution in Host-Associated Lineages'. *Genome biology and evolution*, vol 8, no. 6, pp. 2006-2019.

### 7.2. Data release

Title: iCaMPS-4: Employing Source Attribution and Molecular Epidemiology to measure the impact of interventions on human campylobacteriosis in Scotland.

Accession number is: PRJEB19206. Release date: 25-01-2017

Link: <http://www.ebi.ac.uk/ena/data/view/PRJEB19206>

### 7.3. Talks, Presentations and Posters

- Strachan NJC (2016) A pot-pourri of GI pathogen anecdotes: *Campylobacter*, Anisakiasis and STEC. University of Aberdeen, Roslin, Edinburgh

#### 7.4. Research Projects which have been assisted by this study

- Forbes K, Scott K (2017-20) "Evolution of antimicrobial resistances in *Campylobacter* from farm and clinical sources" UoA Elphinstone Scholarship.
- Forbes K, Strachan N, Stevens M, Psifidi A, Vervelde L (2016-19) "A systems-wide approach to the control of *Campylobacter* in the food chain: exploiting genetic variation." £806,000 from Scottish Government RESAS.
- Sparks N, Forbes K, Strachan N (2016-17) "Improving the efficiency of Scottish chicken production while reducing the carriage of *Campylobacter*" £190,409 from RESAS
- Strachan N, Forbes K, Rotariu O, McGuigan C (2014-18) "Factors affecting variations in *Campylobacter* disease rates in Scotland" £399,849 from Food Standards Scotland.
- Forbes K, Strachan N (2014-17) "*Campylobacter* disease in Nigeria" £70,000 from University of Aberdeen Elphinstone PhD Scholarship.
- Holden N, Brennan F, Forbes K, Strachan N (2013-16) "Pre-harvest contamination of fresh produce with VTEC" PhD Studentship, £60,000 from BBSRC EASTBIO
- Strachan N, Watts D, Forbes K, Perez-Reche F (2013-16) "Addressing food security by controlling the risk of food poisoning: an exemplar case study of listeriosis in the Scottish smoked salmon industry" PhD Studentship, £60,000 from University of Aberdeen Environment and Food Security Theme.
- O'Brien SJ, Bennett M, Diggle PJ, Forbes KJ, Griffith R, Humphrey T, Hunter P, Lake I, Rigby D, Rushton S, Strachan NJC, Wadsworth R, Winstanley C, Wren B (2012 -2017) "Sources, Seasonality, Transmission and Control: *Campylobacter* and human behaviour in a changing environment." £3,419,121 from MRC.

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## 9. Appendices

9.1. Appendix 1: Questionnaire sent to campylobacteriosis cases.

# The sources of *Campylobacter* infection



If you have any queries relating to any aspect of this study or the questionnaire please contact Ken Forbes (01224 437023; [k.forbes@abdn.ac.uk](mailto:k.forbes@abdn.ac.uk)) or Norval Strachan (01224 272699; [n.strachan@abdn.ac.uk](mailto:n.strachan@abdn.ac.uk)) at The University of Aberdeen.

Please return this Questionnaire in the addressed, reply-paid envelope provided to:

Professor Ken Forbes  
School of Medicine, Medical Sciences and Nutrition

0:015 Polwarth Building  
University of Aberdeen  
Foresterhill  
Aberdeen AB25 2ZD

**Thank you for helping us with our study and for completing this questionnaire.**

## **Section 1: Basic information about the person who was ill**

1.1 Age \_\_\_\_\_ years

1.2 Gender Male  Female

1.3 Today's date (dd/mm/yyyy):

1.4 Is the person answering these questions the person who was ill?

Yes  No

If no, please state your relationship to the person who was ill? (Parent, carer etc)

*For the rest of the questionnaire 'you' refers to the person who is ill.*

*Please record their experience as closely as possible.*

1.5 Post code (first 5 digits)

1.6 At present are you: Employed  Unemployed  Retired

Full time education

Other, please specify:

If you are employed,  
what is your occupation?

## Section 2: Basic information about your illness

2.1 When did the symptoms started

2.2 Are you still suffering symptoms that you associate with the illness?

Yes

Please specify:

No  When did the illness finish?

2.3 What symptoms did you experience?

(Tick each symptom experienced but *don't report any long-term symptoms unrelated to this episode of illness*)

<i>Symptoms</i>	How long did the symptom persist?
Diarrhoea <input type="checkbox"/>	days
Bloody stools <input type="checkbox"/>	days
Vomiting <input type="checkbox"/>	days
Abdominal pain <input type="checkbox"/>	days
Other (specify): <input type="checkbox"/>	days

2.4 Were you admitted to hospital?

Yes

No

If yes,

how long were you in hospital?  
days

2.5 How many people live in your household?  
people

What is their relationship to you?	Did they have the same symptoms as you?	What date did their illness start?

1)	Yes <input type="checkbox"/> No <input type="checkbox"/>	dd/mm/yyyy
2)	Yes <input type="checkbox"/> No <input type="checkbox"/>	dd/mm/yyyy
3)	Yes <input type="checkbox"/> No <input type="checkbox"/>	dd/mm/yyyy
4)	Yes <input type="checkbox"/> No <input type="checkbox"/>	dd/mm/yyyy
5)	Yes <input type="checkbox"/> No <input type="checkbox"/>	dd/mm/yyyy

### **Section 3: Foreign travel**

3.1 Did you have an overnight stay abroad in the 14 days before you first started feeling unwell?

Yes  No

If yes, which countries did you visit:

3.2 Dates of travel: from  /  /  to  /  /

3.3 Were you aware of anyone else with a similar illness where you were staying?

Yes  No

If yes please provide details

### **Section 4: Exposure**

4.1 Did you have any contact with farm animals in the 5 days before you first started feeling unwell?

Yes  No

If yes please specify:

4.2 Do you use a private water supply (e.g. spring, well)?

Yes  No

If your household has a private water supply is it treated? Yes  No  Not sure

If yes, is it: Chlorinated Yes  No  Not sure

UV Filter Yes  No  Not sure

Other  
(please specify)

4.3 Did you have any contact with chicken 5 days before you first started feeling unwell:  
by eating chicken? Yes  No  Not sure

by eating chicken liver pate? Yes  No  Not sure

by handling raw chicken? Yes  No  Not sure

by anyone in the house handling raw chicken? Yes  No  Not sure

4.4 Do you eat chicken regularly? Yes  No

4.5 In the 5 days before you first started feeling unwell was fresh raw chicken purchased  
which was then prepared at home? Yes  No

4.6 If YES, what shop /supermarket did you buy it from? (please specify below)

Shop/Supermarket	Location

## 9.2. Appendix 2: Antimicrobials used on farms

Amoxinsol, Octacillin: Amoxicillin (**class:  $\beta$ -lactam**). Amoxicillin is a 3<sup>rd</sup> generation extended spectrum penicillin antibiotic used in the treatment of a number of human bacterial infections. It is the first line treatment for middle ear infections. It may also be used for treating strep sore throat, pneumonia, skin infections, and urinary tract infections among others (Kaur, Rao et al. 2011). Amoxicillin is used in treating a variety of systemic infections in poultry (Anadón, Martínez-Larrañaga et al. 1996). 21% of flocks were administered amoxinsol and 7% Octacillin.

Phenoxyphen: Phenoxymethylpenicillin (**class:  $\beta$ -lactam**). Phenoxymethylpenicillin, a 1st generation narrow spectrum antibiotic, is known to exert a bactericidal action against penicillin-sensitive microorganisms during the stage of active multiplication. It inhibits the biosynthesis of bacterial cell-wall peptidoglycan. In this study, 9% of the flocks were treated with Phenoxyphen.

Lincospectin: Lincomycin (**class: macrolide**) (22%), Spectinomycin (44%)(class: aminoglycoside). It comprises of lincosamide which inhibit bacterial replication by interfering with the synthesis of proteins. They bind to bacterial ribosomes and cause premature dissociation of the peptidyl-tRNA from the ribosome (Tenson, Lovmar et al. 2003). Spectinomycin is an aminocyclitol class of drugs and works by inhibiting protein synthesis in certain bacteria. It can be used for people allergic to penicillin or cephalosporins. In chickens, spectinomycin is administered orally in drinking water and feed at doses to prevent mortality due to Chronic respiratory disease associated with susceptible *Mycoplasma gallisepticum* (ASPCA 2008). Lincospectin was used on 17% of flocks in this study.

Pharmasin, Tylosin (**class: macrolide**). Tylosin is a bacteriostatic antibiotic as well as a feed additive used in broilers and in veterinary medicine (Soliman, Sedek 2016). It has a broad spectrum of activity against Gram-positive organisms and a limited range of Gram-negative organisms (Soliman, Sedek 2016). Tylosin acts by inhibition of protein synthesis through binding to the 50S subunit of the bacterial ribosome (Suchodolski, Dowd et al. 2009). 0.6% of flocks were treated with Pharmasin.

Apralan, Apramycin (**class: aminoglycoside**). Apramycin is an aminoglycoside antibiotic used in veterinary medicine. It is produced by *Streptomyces tenebrarius* (Ryden, Moore 1977). It can be used to treat bacterial infections in animals caused by *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Wang, Jung et al. 2011). Apralan was used on 25% of flocks in the current study.

Chlorsol: Chlortetracycline (**class: tetracycline**). Chlortetracycline is a tetracycline antibiotic, the first tetracycline to be identified. Tetracyclines are broad-spectrum antibiotics whose general usefulness has been reduced with the onset of antibiotic resistance. Tetracycline antibiotics are protein synthesis

inhibitors. They do so mainly by binding to the 30S ribosomal subunit in the mRNA translation complex (Chopra, Roberts 2001). In the current study, 6% of the flocks were treated with Chlorsol.

Methoxasol: **Trimethoprim, Sulfamethoxazole** (TMP / SMX; 1:5). Sulfamethoxazole inhibits bacterial synthesis of dihydrofolic acid by competing with para-aminobenzoic acid (PABA). Trimethoprim blocks the production of tetrahydrofolic acid from dihydrofolic acid by binding to and reversibly inhibiting the required enzyme, dihydrofolate reductase (Hitchings 1973). Thus blocking folate production resulting in bacterial death. The level of resistance to trimethoprim, an antibiotic frequently used for treatment of UTI, has increased significantly (Jakobsen, Hammerum et al. 2010). A study found that a clone of *E. coli* found in both broiler chickens and chicken meat may be responsible for UTI in humans (Jakobsen, Hammerum et al. 2010). Feed has been implicated as a source for antibiotic resistant broiler-derived *E. coli* and broilers as a source for drug resistant *E. coli* (Thorsteinsdottir, Haraldsson et al. 2010). It is needless to say that usage of antibiotics not only affects resistance levels in *Campylobacter* but also leads to co-selection of antibiotic resistance in micro-organisms that constitute the chicken gut microbiome. The usage of Methoxasol in this study was low as 2% of the flocks were treated with this antibiotic.