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Public health significance of *Campylobacter* spp. colonisation of wild game pheasants (*Phasianus colchicus*) in Scotland.

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Abstract

Campylobacter is the most common cause of bacterial food-borne diarrhoeal disease worldwide. Chicken meat is considered the main source of human infection; however, *C. jejuni* and *C. coli* have also been reported in a range of livestock and wildlife species, including pheasants. Wild pheasant meat reaches the consumer's table because of hunting but there is a lack of information concerning the risk of *Campylobacter* infection in humans. This study aimed to determine the prevalence of *Campylobacter* in wild game pheasants in Scotland, to identify the main sequence types (STs) present and to evaluate their impact on public health. A total of 287 caecal samples from five Scottish regions were collected during the hunting season 2013/2014. *Campylobacter* was detected and enumerated using standard culture methods. PCR and High Throughput Multi Locus Sequence Typing (HiMLST) were used for species identification and sequence typing. In total, 36.6% of 287 caecal samples (n=105; 95% CI: 14-59.2) were *Campylobacter* positive. Using PCR, 62.6% of samples (n=99) were identified as *C. coli* and 37.4% as *C. jejuni*. HiMLST (n=80) identified 19 different STs. ST-828 (n=19) was the most common, followed by ST-827 (n=12) and ST19 (n=7). Sixteen of the 19 STs isolated are present in humans and eight are *C. coli* STs that account for 6.96% of human infections, although the overall risk to public health from pheasant meat is still considered to be low.

Keywords: *Campylobacter*, pheasants, source attribution, food-borne infections, gastrointestinal disease, zoonoses, wild game meat.

1. Introduction

Campylobacter is the main cause of food-borne gastrointestinal disease world-wide (WHO, 2015) and the two primary species of public health importance are *C. jejuni* and *C. coli*, responsible for over 95% of *Campylobacter* infections in humans (Park, 2002). Typically, it causes mild to severe diarrhoea lasting 5 to 7 days (Humphrey et al., 2007). In a proportion of patients it can result in debilitating sequelae, such as the Guillain–Barré syndrome or reactive arthritis (Tam et al., 2006). In the UK alone, *Campylobacter* caused 70,353 clinically diagnosed infections in 2014 (DEFRA, 2015), with a further nine undiagnosed cases estimated to occur for every diagnosed case (IID2 Study, 2012). The economic burden was recently estimated to be £50 million per year (Tam and O’Brien, 2016).

Poultry are recognised as the most important animal reservoir of infection for humans, with 60-80% of cases attributable to this reservoir as a whole (EFSA, 2013) but *C. jejuni* and *C. coli* have also been reported in a range of livestock and wildlife species, including pheasants. In Scotland, shooting game is a sport that contributes to the country’s economy. Although meat production is not a primary aim, the game meat produced from this activity typically ends up on the consumer’s table. Game meat has recently increased in popularity among consumers on the grounds of sustainability, healthy eating, and support for local production (ADAS, 2007). This is particularly prominent for the Scottish rural economy, where approximately 3.5 million game birds are shot annually. Despite this volume of wild game entering the food chain, there is a lack of information concerning the risk its consumption poses to humans in terms of exposure to *Campylobacter* and the role game birds may have as a reservoir of infection.

Pheasants can be farmed for meat in a similar way to broiler chickens or they can be reared in free range farming conditions up to 3 to 4 months of age, then released in the field prior to the hunting season. At this stage pheasants may be in contact with other farm animals, primarily cattle and sheep, but also with wildlife and a contaminated environment where transmission of infection can occur (Schaffner et al., 2004). In wider European studies, the prevalence of infection in pheasants varies

substantially depending on sampling site (cloacal swab versus caecal content), whether pheasants are alive or dead at time of sampling, and whether they are hunted or farmed (Nebola et al., 2007; Atanassova and Ring, 1999; Stern et al., 2004; Dipineto et al., 2008b). Study results also vary with country in terms of which species of *Campylobacter* predominates (Dipineto, 2008b; Nebola et al., 2007). However, no published data are available on sequence types (STs) of *Campylobacter* present in pheasants.

This study aimed to determine the prevalence and bacterial load of *Campylobacter* spp. in wild game pheasants processed in Approved Game Handling Establishments (AGHEs) in Scotland. Furthermore, to evaluate the impact on public health, it also aimed to identify the main *Campylobacter* spp. and STs present and compare these STs to those that are common in food producing animals, wild birds and humans.

2. Methods

2.1 Geographical stratification and sample size selection

A total of 287 caecal samples were collected from pheasant carcasses in Scotland during the hunting season 2013/2014. Scotland was divided into five geographical regions and an AGHE was selected as a sampling site in each region. A simple random sampling estimate was used to determine the sample size (Thrusfield, 2005) based on a pheasant population in Scotland of approximately 2 million birds (PACEC, 2006). Assuming an expected prevalence of 25% in wild pheasants, inferred from relevant literature (Atanassova and Ring, 1999; Stern et al., 2004; Nebola et al., 2007), and a desired confidence level of 95% with an absolute precision of 5%, it was necessary to sample 58 birds per region. The estates of origin and number of caecal samples collected from each region are shown in Table 1. Time of year and date of kill, where known, were recorded at time of sampling. The sex of sampled birds was not recorded as this was not considered relevant to *Campylobacter* infection in pheasants (Dipineto et al., 2008a). Caecal samples were collected at intake prior to processing to avoid cross-contamination during evisceration. Pheasants were individually sampled. Pheasant carcasses were opened and caeca were detached from the rest of the intestine. Caecal samples were individually placed in sampling pots, labelled with the bird number, in sequential numerical order and stored on ice in

insulated boxes during transport, then overnight at 4°C before being processed the following day.

2.2 *Campylobacter* isolation and molecular diversity

The BS EN ISO 10272-1:20063 and BS EN ISO/TS 10272-2:20064 standards (PHE, 2014) were followed for isolation and enumeration of *Campylobacter* spp; 1g of caecal contents was weighed and a 10-fold (w/v) dilution was made in PBS. A further six 10-fold dilutions were prepared. One hundred microliters of each dilution were plated on modified charcoal-cefoperazone-deoxycholate agar (mCCDA) and incubated at 41°C for 40 hours, in a microaerobic atmosphere (5% O₂, 5%CO₂ and 90% N₂). The detection limit was 10 CFU/g and bacterial counts were logarithmically transformed for statistical analyses. Eight single colonies that had typical morphology of *C. jejuni* or *coli* were picked off the mCCDA plates using sterile 10 ul plastic loops from each positive sample. Each colony was then spread onto a separate mCCDA plate and the resulting growth was harvested and stored in 16.67% glycerol at -80°C. Although the BS EN ISO 10272-1:20063 standards suggest confirmation of at least five colonies from each plate as *Campylobacter* using metabolic tests or PCR, only one pure colony from each positive pheasant was used for PCR testing, due to funding limitations.

DNA extraction, PCRs for species identification and HiMLST for ST identification were undertaken by the Regional Laboratory for Public Health Kennemerland, Haarlem, the Netherlands. DNA extraction was performed on bacteria harvested from mCCDA plates inoculated with one glycerol stock of a single colony from each positive bird, according to previously published protocols (Boers et al., 2012). DNA was extracted from bacterial cultures using the High Pure PCR Template Preparation Kit (Roche, Almere, The Netherlands) according to the manufacturer's instructions. Primer sequences for HiMLST were obtained from the PubMLST website (<http://pubmlst.org/campylobacter/info/primers.shtml>). *Campylobacter* STs recovered from pheasant caecal samples were compared to the lists of STs found in humans, poultry, farm animals and wildlife downloaded from the PubMLST database (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_campylobacter_isolates&page=query) (last access on February 2015).

2.3 Source attribution

The STs used for source attribution accounted for all human cases reported on the PubMLST database. The relative proportions of STs from each *Campylobacter* species found in the human clinical isolates database were plotted in descending order to show the potential sources of human infection from farm animals (cattle, sheep, pigs, chickens and poultry other than chickens), wild birds and pheasants using R (v3.3.2, © 2016 The R Foundation for Statistical Computing). Source attribution of human infection, based on the isolates reported to the PubMLST database (accessed in February 2015), was performed by examining the STs that were common to humans, farm animals, wild birds and pheasants. The number of clinical isolates from human and animal sources that shared the same STs was extracted for each species group. The attribution of animal sources to human infection from shared STs was expressed as a percentage, calculated by dividing the number of animal isolates sharing the same STs with humans by the total number of isolates (human and animal) sharing the same STs. The overall attribution of animal sources to human infection was also expressed as a percentage, this time calculated by dividing the number of animal isolates sharing the same STs with humans by the total number of human (n=9573) and animal isolates.

2.4 Statistical Analysis

Statistical analysis was performed using R (v3.3.2, © 2016 The R Foundation for Statistical Computing). Overall differences in the prevalence of infection between regions and estates within individual regions were analysed by general linear models with binomial errors (*GLM_b*). Post-hoc pairwise comparisons of detected differences were considered using the Tukey method using the *multcomp* package (v 1.4-6). *GLM_b* and post-hoc Tukey tests were also employed to examine the proportion of *C. coli* in positive samples. Comparisons of the level of *Campylobacter* load in positive samples between regions and estates within individual regions were analysed with one-way analyses of variance and post-hoc Tukey tests. Differences between regions and in estates within individual regions in the ratio of *C. coli* to *C. jejuni* in PCR positive samples were also analysed with *GLM_b* and post-hoc Tukey tests. P<0.05 was taken to indicate statistical significance throughout. WinPepi software Version 11.35 (© J.H. Abramson, 2013) was used to calculate prevalence and 95% confidence intervals (CI) for clusters of different sizes because of the variation in the number of samples collected from each estate. Prevalence and CI for single estates was estimated using standard CI calculation.

3. Results

3.1 Caecal samples: prevalence of infection

The mean prevalence of infection was 36.6% (CI: 14-59.2), with the lowest prevalence of 6.8% recorded in region 5 and overall statistically significant differences between regions ($P<0.001$, Table 1, with region 5 lower than the other four regions ($P<0.004$)). Excluding region 5, the overall infection prevalence increased to 44.5% (CI: 35.3-53.6) and there was no statistical difference in prevalence across the remaining regions ($P=0.518$). Within these remaining regions, there were differences between the two region 1 estates ($P=0.004$), and between estates in TD5 and TD13 ($P=0.001$), but no other inter-regional differences were observed ($P>0.153$, Table 1).

(Insert Table 1 here)

3.2 Caecal samples: *Campylobacter* bacterial load

The geometric mean (GM) *Campylobacter* bacterial load in positive samples ($n=105$) was 2.7×10^4 CFU/g (CI: 1.5×10^4 – 4.9×10^4 ; Figure 1). There was no statistically significant difference in bacterial load between all five regions; nor was there a difference when region 5 was excluded (which only had four positive samples) ($P>0.257$). Within the other four regions, the only difference between inter-regional estates was observed in region 2 ($P=0.006$), where the bacterial load in positive samples from the estate in AB32 was lower compared to the estate in AB34 (GM= 5.3×10^3 CFU/g (7.8×10^2 – 3.7×10^4) and GM= 1.9×10^5 CFU/g (4.4×10^4 – 7.9×10^5), respectively).

(Insert Figure 1 here)

3.3 PCR and HiMLST results

Ninety-nine samples were recovered for species identification using PCR, with six samples failing to recover after freezing at -80°C . All 99 samples were positive for *Campylobacter* spp. Overall *C. coli* and *C. jejuni* accounted for 62.6% and 37.4% of samples, respectively (Table 1).

Region 2 was the only region where the proportion of *C. coli* was lower than that of *C. jejuni* (12%); in the remaining regions $>66\%$ of positive PCR samples were *C. coli* (Table 1). As region 5 yielded only three isolates for PCR detection it was excluded from the statistical analysis. Post-hoc Tukey analysis confirmed that region 2 was

statistically different, in terms of *C. jejuni* infection, from regions 1, 3 and 4 ($P < 0.003$) while regions 1, 3 and 4 did not differ from each other ($P > 0.274$). There were also no statistically significant differences between estates within these four regions ($P > 0.265$, Table 1).

Of the 99 isolates subjected to DNA sequencing, a ST was only assigned in 80 because, for 19 (19.2%) isolates, one or more alleles failed to amplify. Nineteen STs were detected by HiMLST. Eleven (57.9%) were consistent with *C. jejuni* and eight (42.1%) with *C. coli*. Sequence Type 828 ($n=19$; 23.75%) was the most common in the 80 samples tested, followed by ST-827 ($n=12$; 15%) and ST-19 ($n=7$; 8.75%) which collectively represented 47.5% of all samples (Figure 2). Five STs appeared only once and were all *C. jejuni*; six STs appeared twice. Three of the five *C. jejuni* STs that appeared only once were recovered from region 2.

(Insert Figure 2 here)

3.4 Source attribution

The relative proportions of the STs found in all the human and animal clinical isolates in the PubMLST database were calculated to illustrate how common these STs were in animal sources. Also included were the pheasant samples from the present study. Sequence Types from human cases ($n=9573$) accounted for 75.1% of the total isolates while STs from poultry cases ($n=2103$) accounted for 16.5% and STs found in pheasants ($n=80$) accounted for 0.6% of isolates. Similarly, the relative proportions of STs of each *Campylobacter* species from human ($n=735$ *C. coli* and $n=8838$ *C. jejuni*) and animal isolates accounted for 53.3% of human *C. coli* and 77.7% of *C. jejuni* STs, 36.1% of poultry *C. coli* and 14.1% of *C. jejuni* STs, and 3.8% of pheasant *C. coli* and 0.2% of *C. jejuni* STs (Figure 3a and 3b).

(Insert Figure 3a and b here)

With regard to the 19 STs found in pheasants in the current study, all eight *C. coli* STs and 8 of 11 *C. jejuni* STs have previously been isolated from human samples (Figure 3a, b). They represented 84.2% of the STs isolated from these pheasants that could be responsible for human infection (Table 2). Pheasant STs contributed to 4.35% of human cases caused by shared STs and they accounted for 0.77% of overall human infections. However, when only *C. coli* STs were considered, these rose to 16.46% of human cases attributed to STs common to pheasants and humans and 6.96% of overall human infections.

So far, for *C. coli* isolates from pheasants, ST-830 has only been recovered from humans, while STs 825, 828, 831, 1541 and 2195 have also been found in humans and poultry, and STs 827 and 962 in humans, poultry and cattle or sheep, respectively (Figure 3a). *Campylobacter jejuni* STs originating from pheasants contributed 1.63% of the STs common to pheasants and humans, and 0.26% to overall human infection (Table 2). For *C. jejuni* STs isolated from pheasants, the host source appears broader, with all eight STs common to pheasants and humans (STs 19, 48, 51, 53, 262, 583, 1030 and 1709) also isolated from poultry. Sequence Types 19, 48, 53 and 262 have also been found in sheep and cattle, while ST 583 has only been found in cattle and not sheep. Finally, STs 53 and 583 have also been detected in wild birds. In contrast to pheasants, poultry accounted for 10.29% of overall human infections, followed by cattle at 3.08% (Table 2).

(Insert Table 2 here)

4. Discussion

There are relatively few prevalence studies relating to *Campylobacter* infection in pheasants in Europe. Studies conducted in Germany, Russia, Italy and the Czech Republic (Atanassova and Ring, 1999; Stern et al., 2004; Dipineto et al., 2008a; Dipineto et al., 2009; Nebola et al., 2007) vary in terms of the sampled population, the sampling method and the resulting prevalence. For example, based on cloacal swabs collected from farmed pheasants, the Italian researchers detected a prevalence that ranged from 43.3% to 86.7% (Dipineto et al., 2008a; Dipineto et al., 2009). In Germany and Russia, caecal content was collected from hunted wild pheasants and the prevalence was lower, at 26% and 25%, respectively (Atanassova and Ring, 1999; Stern et al., 2004). There are no data available in the literature on intestinal load of *Campylobacter* spp. in pheasants.

This is the first UK based study estimating both the prevalence of *Campylobacter* infection and bacterial load in pheasant caecal contents. The survey indicated an overall infection prevalence of 36.6% (n=287) (CI: 14-59.2) which is in line with previously reported prevalence levels based on analyses of caecal content in hunted wild pheasants elsewhere in Europe (Nebola et al., 2007; Atanassova and Ring, 1999; Stern et al., 2004). Prevalence was not uniform across all the regions, in particular region 5 had a very low prevalence. Excluding region 5, the overall infection

prevalence of 44.5% (CI: 35.3-53.6) was higher than that reported by previous studies. The low prevalence in region 5 could reflect a genuinely low prevalence of infection in the estates sampled but it is also possible that the time of year influenced *Campylobacter* carriage (Weber et al., 2014). Seasonal fluctuation of *Campylobacter* carriage in food producing animals has been observed in previous studies (Wallace et al., 1997; Stanley et al., 1998a;b).

The average bacterial load of positive samples (n=105) was 2.7×10^4 CFU/g (CI: 1.5×10^4 – 4.9×10^4), broadly in line with the bacterial load of extensively reared British-based poultry flocks surveyed in 2011 (Allen et al., 2011). Our study also found no significant difference in bacterial carriage means across and between Scottish regions and estates ($P > 0.257$), even in region 5 where infection prevalence was very low. Based on the enumeration of *Campylobacter* spp. in positive samples, most pheasants had high counts across all five regions; in fact 23% (CI: 18.3-28.3) of pheasants had a *Campylobacter* count $> 10^4$ CFU/g and 5.6% (CI: 3.2-8.9) harboured $> 10^6$ CFU/g. Although these results relate to caecal and not faecal content they may still support super-shedding of *Campylobacter* into the environment, which would potentially increase the risk of infection to other pheasants and to humans, as reported for *E. coli* O157:H7 in cattle (Chase-Topping et al., 2008).

PCR results indicated a higher level of *C. coli* (62.6%) in caecal content of the sampled Scottish pheasants (n=99) when compared to *C. jejuni* (37.4%). In their Italian survey, Dipineto (2008a) reported that 100% (n=104) of cloacal swab isolates subjected to PCR were identified as *C. coli*, with 13.5% co-infected with *C. jejuni*. In contrast, Nebola (2007) reported that *C. jejuni* was more prevalent (n=54: 58%) than *C. coli* (36%) in wild pheasants in the Czech Republic, with mixed infection in 5% of birds examined. In the UK, pheasants of the same age are reared in free range farming conditions prior to release in time for the hunting season, so the discrepancy in results may reflect the varying sources of infection to which pheasants on different estates are exposed. For instance, cattle, sheep and chickens are not only major reservoirs and shedders of *Campylobacter* spp. but they vary in the sequence types and species that they carry (Figure 3a, b) (Sheppard et al., 2010). Age of pheasants may also be an important factor, as older birds tend to have a higher *C. coli* prevalence compared to younger ones, possibly due to longer exposure to sources of contamination (Nather, 2006; El-Shibiny et al., 2005). *Campylobacter coli* was more widespread than *C. jejuni* in four of the five Scottish regions surveyed, with the

exception being region 2. Similarly, *C. coli* was the predominant species across all estates when region 2 was excluded from the analysis. However, a study carried out across north-eastern Scotland on *Campylobacter* isolates from cattle and sheep faeces did not find a statistically significant regional difference between the two species (Rotariu et al., 2009).

Nineteen STs were isolated from positive caecal samples from pheasants (n=80); STs 828, 827 and 19 represented 47.5% of the isolates tested and 16 of the 19 had been previously isolated from humans. According to previous studies, *C. jejuni* infection in pheasants suggests a shared association with cattle and sheep (Strachan et al., 2009; FSA, 2009; Rotariu et al., 2009). Although cattle and pheasants are common hosts for five STs (STs 19, 48, 53, 262 and 583), four of them also occur in sheep (STs 19, 48, 53 and 262). Therefore, the most likely source of *Campylobacter* STs 19, 48 and 262 is cattle or sheep, while ST-583 seems more likely to originate from cattle. Transmission of *Campylobacter* between these species is possible because they are usually reared in the same estates, sharing contaminated grassland and/or water sources (Shaffner et al., 2004). However, more studies are needed to clarify routes of transmission between cattle, sheep and pheasants.

Poultry host 14 out of 19 STs found in pheasants, in particular ST-828 (the most common ST (24%) in pheasant isolates in this study) and all the STs belonging to *C. coli*. Furthermore, with the exception of STs 827 and 962, all other *C. coli* STs have only been recovered from chicken isolates on the PubMLST database thus far, which suggests that pheasants, like chickens, can be natural hosts for some *C. coli* strains. Sequence Type 830 has only been recovered from pheasant and human isolates which opens the possibility of transmission of infection from humans to birds since it is the sixth most common *C. coli* ST in human cases, although a common environmental source of infection for both humans and pheasants may be an alternative explanation (Meldrum et al., 2005).

In this study we calculated that pheasants may contribute to 0.77% of human *Campylobacter* cases. Pheasants share 16 STs responsible for human clinical cases, although only eight are relatively frequent. ST-828 was the most prevalent in positive pheasant samples and was also recovered from chicken isolates on the PubMLST database, yet only accounted for 0.2% of human cases. The vast majority of human *Campylobacter* cases are associated with *C. jejuni*, tending to indicate that pheasants pose a lower *Campylobacter* transmission risk to humans. Despite this, *C. coli* should

not be underestimated in terms of possible transmission from animal sources to humans, since it could be accounting for 6.96% of human cases from pheasants and 18.02% from poultry, while *C. jejuni* could be accounting for 0.26% and 9.35%, respectively. This raises the hypothesis that recirculation of *C. jejuni* infection among humans or from other environmental or food sources is a more important factor in the spread of infection than are farm animals (ACMSF, 2005). This is further supported by the finding that the 21 most common *C. jejuni* STs in human isolates on the PubMLST database, thus far, have only been isolated from humans, collectively causing 45% of cases.

The level of consumption of poultry meat is an important risk factor in the epidemiology of *Campylobacter* infection in humans (ACMSF, 2005). With respect to pheasant meat, the consumption per-capita in the UK is very low compared to chicken and beef (estimated at 15g per person per year assuming that all consumed pheasant meat originates from AGHEs) and this further reduces the risk of exposure to infection from pheasants. At present there is no record of any foodborne disease case that can be definitively traced back to consumption of pheasant meat (PACEC, 2006).

Human exposure to pheasant meat is also seasonal. Pheasant meat is available to consumers mainly in winter, during the hunting season (October to February) and this does not coincide with the peak in human *Campylobacter* cases in the UK (Louis et al., 2005 and Millers et al., 2004); usually the notification rates in these months is decreasing or very low, suggesting that higher consumption of pheasant meat in these months does not contribute to an increase in *Campylobacter* infection in humans. Pheasant meat that is available to consumers all year around is generally stored frozen and, since there is evidence in the literature that freezing is detrimental to *Campylobacter* survival in food (Harrison et al., 2013), the risk to public health is reduced. Regardless, consumers and eating outlets should always be advised to cook meat thoroughly to prevent any risk of food-borne infection.

In conclusion, the risk to public health from live pheasants and pheasant meat is currently considered to be low. However, consumer consumption of pheasant meat is increasing (FSA, 2007) so it would be prudent to maintain a continued awareness of its potential role as a source of *Campylobacter* infection in humans.

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Conflict of interest

None declared.

Authors' contributions

Specimen sampling: AS. Microbiological diagnostics: AS, CC. Statistical analysis DJS, AS. Wrote the manuscript: AS, DJS. SHS. All authors have read and approved the final version of the manuscript.

Figure legends

Figure 1. *Campylobacter* load in pheasant caecal content samples: Mean (green lines) of positive samples and median (black lines) of all samples by region are expressed in log₁₀ CFU/ gram (n=number of samples collected per region).

Figure 2. Percentage (%) of the 19 STs found across the dataset (n=80), illustrating whether they belonged to *C. jejuni* (black columns) or *C. coli* (grey columns) species.

Figure 3(a, b). (a) Percentage (%) of *C. coli* STs found in human and animal isolates from the PubMLST database including the STs from pheasant samples. (b) Percentage (%) of *C. jejuni* STs found in human and animal isolates from the PubMLST database including the STs from pheasant samples.

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