TITLE: Anthropogenic environmental drivers of antimicrobial resistance in wildlife

AUTHORS: Swift, B M C; Bennett, M; Waller, K; Dodd, C; Murray, A; Gomes, R L; Humphreys, B; Hobman, J L; Jones, M A; Whitlock, S E; Mitchell, L J; Lennon, R J; Arnold, K E

JOURNAL: Science of the Total Environment

PUBLISHER: Elsevier

PUBLICATION DATE: 16 August 2018 (online)

DOI: https://doi.org/10.1016/j.scitotenv.2018.08.180

Anthropogenic Environmental Drivers of Antimicrobial Resistance in Wildlife

Benjamin M. C. Swift[a,b], Malcolm Bennett[b], Katie Waller[b], Christine Dodd[c], Annie Murray[d] Rachel L. Gomes[e] , Bethan Humphreys[f], Jon Hobman[c], Michael A. Jones[b], , Sophia E. Whitlock[d], Lucy J. Mitchell[d], Rosie J. Lennon[d], and Kathryn E. Arnold[d]

[a] Current address: Pathobiology and Population Sciences, Royal Veterinary College, UK

[b] School of Veterinary Medicine and Science, University of Nottingham, UK

[c] School of Bioscience, University of Nottingham, UK

[d] Environment Department, University of York, UK

[e] Food, Water, Waste Research Group, Faculty of Engineering, University of Nottingham, UK

[f] School of Pharmacy, University of Nottingham, UK

Emails: bswift@rvc.ac.uk, m.bennett@nottingham.ac.uk, svyklw@nottingham.ac.uk, Christine.Dodd@nottingham.ac.uk, am1400@york.ac.uk, rachel.gomes@nottingham.ac.uk, mbybmh@nottingham.ac.uk, Jon.Hobman@nottingham.ac.uk, michael.a.jones@nottingham.ac.uk, sew553@york.ac.uk, ljr540@york.ac.uk, rjl529@york.ac.uk, kathryn.arnold@york.ac.uk

Corresponding Author: Kathryn Arnold, Environment Department, University of York, YO10 5NG, UK

Tel: 01904 322997 Email: Kathryn.Arnold@york.ac.uk

Word count including references, figure legends and tables: 8528

Conflict of Interest: The authors have no conflicts of interest to declare.

Abstract

The isolation of antimicrobial resistant bacteria (ARB) from wildlife living adjacent to humans has led to the suggestion that such antimicrobial resistance (AMR) is anthropogenically driven by exposure to antimicrobials and ARB. However, ARB have also been detected in wildlife living in areas without interaction with humans. Here, we investigated patterns of resistance in *Escherichia coli* isolated from 408 wild bird and mammal faecal samples. AMR and multi-drug resistance (MDR) prevalence in wildlife samples differed significantly between a Sewage Treatment Plant (STP; wastes of antibiotic-treated humans) and a Farm site (antibiotic-treated livestock wastes) and Central site (no sources of wastes containing anthropogenic AMR or antimicrobials), but patterns of resistance also varied significantly over time and between mammals and birds. Over 30% of AMR isolates were resistant to colistin, a last-resort antibiotic, but resistance was not due to the *mcr-1* gene. ESBL and AmpC activity were common in isolates from mammals. Wildlife were, therefore, harbouring resistance of clinical relevance. AMR *E. coli*, including MDR, were found in diverse wildlife species, and the patterns and prevalence of resistance were not consistently associated with site and therefore different exposure risks. We conclude that AMR in commensal bacteria of wildlife is not driven simply by anthropogenic factors, and, in practical terms, this may limit the utility of wildlife as sentinels of spatial variation in the transmission of environmental AMR.

Key words: E. coli, Antimicrobial resistance, wildlife, birds, multi-drug resistance, wastewater treatment

Running head: Wildlife and AMR

1. Introduction

Antimicrobial resistance (AMR) has existed for millions of years, and is an inevitable evolutionary consequence of microbial competition in the environment (D'Costa et al 2011, Davies and Davies 2010, Martinez 2009). While the increasing prevalence of AMR in clinically important and commensal bacteria in both humans and livestock can be attributed largely to selection through the use of antimicrobials (Ibrahim et al 2016, Karesh et al 2012), AMR has also been reported in the commensal bacteria of wildlife (Arnold et al 2016). Commensal bacteria have the potential to act as reservoirs of resistance genes, contributing to the development of AMR in pathogens by horizontal transmission (Arnold et al 2016, Taylor et al 2011, von Wintersdorff et al 2016). AMR is a problem in human and veterinary medicine worldwide, inhibiting the treatment of bacterial infections and is estimated to be responsible for 25,000 preventable human deaths in Europe annually (Marston et al 2016) and an estimated global economic cost of 100 trillion USD by 2050 if not addressed (O'Neill 2016). Thus, there is increasing interest in the environment, including wildlife, as both a source of clinically relevant AMR and in order to better understand the effects of anthropogenically-derived antimicrobial pollution and resistance in ecosystems (Arnold et al 2016, Carroll et al 2015, Huijbers et al 2015).

It is often assumed that antimicrobial-resistant bacteria (ARB) in wildlife result from contact with anthropogenic sources such as farms and human waste that pollute the environment with AMR bacteria and/or with antimicrobials (Allen et al 2010, Clarke and Smith 2011, Radhouani et al 2011). Farms on which manure and slurry can be contaminated with ARB, antibiotics (or their metabolites) and other selective drivers of AMR are important habitats for many small mammals and birds, as are sewage treatment plants (STPs) where some birds and mammals feed directly from the bioprocessers (reviewed in Arnold et al 2016). Run-off from farms, slurry tanks and manure-fertilised fields, along with sewage effluent, can result in antimicrobial drug and ARB contamination of local water courses and land (Fahrenfeld et al 2013). Consequently, it is unsurprising that ARB have been found in wild

67 animals in close contact with humans (Allen et al 2011, Bondo et al 2016, Furness et al 2017, Gilliver
68 et al 1999).

69 Assigning the source and directionality of AMR dissemination is challenging. Even within
70 wildlife populations living in close contact with humans or livestock, or at least their wastes, there is
71 little evidence directly linking an anthropogenic source of AMR with specific patterns of AMR and/or
72 resistance genes. For example, few overlaps in resistance patterns and AMR genes were found
73 between *E. coli* isolated from wildlife living on or near dairy farms and dairy cattle in England (Arnold
74 et al 2016, Wu et al 2018). Whereas wild rodents nearer to a river receiving sewage effluent excreted
75 more resistant *E. coli* than inland animals (Furness et al 2017), this was an association lacking evidence
76 of a clear transmission pathway. Moreover, other highly mobile taxa such as birds also carry ARB that
77 have not been attributed to any particular anthropogenic source (Guenther et al 2017, Schaufler et al
78 2016). Moreover, AMR has been detected in wildlife living in remote and isolated locations with no
79 obvious contact with the wastes of antimicrobial-treated humans or livestock (Cristobal-Azkarate et
80 al 2014). Thus, although transmission of AMR from humans or livestock to wildlife via direct contact
81 with sewage, slurry or faeces, has been suggested, the empirical evidence is lacking or contradictory.
82 Species or ecological guilds with different dispersal patterns, resource requirements and foraging
83 behaviours are likely to have different roles in the evolution and dispersal of AMR (Arnold et al 2016).
84 We argue that the efficacy of wildlife species as sentinels of environmental transmission of AMR will
85 vary depending on the spatial and temporal scales of interest.

86 In this study, three nearby communities of small wild rodents and birds were investigated for
87 evidence of AMR in faeces. The antimicrobials used to screen for resistance were chosen as they
88 represent a range of antibiotic classes of medical and veterinary interest. For example, cefpodoxime
89 resistance is seen as an indicator of extended spectrum beta-lactamase (ESBL) or AmpC beta-
90 lactamase producing bacteria which cause significant problems in human medicine especially with
91 urinary tract infections (Rawat and Nair 2010). Colistin resistance is also of relevance due to colistin
92 being an antibiotic of last resort. The sites for sampling were chosen to represent different exposures

to wastes and thus potentially different selection pressures for AMR: a dairy farm with antimicrobial-treated livestock, a STP containing waste from humans treated by antimicrobials and an area of parkland and neighbouring arable field edge with no obvious sources of waste containing antimicrobials or ARB. We sampled wildlife species typical for small woodlands, farmland and hedgerow habitats in the UK; small rodents including wood mice *Apodemus sylvacticus*, bank voles *Myodes glareolus* and a number of bird species.

The overall aim of this study was to investigate the role of environmental contamination in the patterns of AMR found in wildlife. We addressed whether the spatial location where wild birds and mammals were sampled, including proximity to human and livestock wastes, explained variation in: 1) prevalence and genomic diversity of AMR *E. coli* in birds and mammals; 2) patterns of AMR and MDR prevalence in *E. coli* isolates; and 3) prevalence of phenotypic resistance to medically important antimicrobials and the resistance genes responsible.

2. Material and Methods

2.1 Study sites

Three nearby study sites in the East Midlands of England, on a 1200m transect, were selected (Figure S1), based on their differing potential exposure to human and livestock sources of AMR and antimicrobial drugs. The 'Farm site' was a small woodland and hedgerows immediately adjacent to a dairy farm that received run-off from farm buildings and livestock faeces potentially contaminated with AMR bacteria and antimicrobials. The 'Central site', around 600m from the Farm site, comprised an arboretum and neighbouring hedgerow edging an arable field. It was not adjacent to known sources of human or livestock waste. The 'STP site' was a small sewage treatment plant around 450-600m from the Central-site, comprising the land and hedgerows surrounding all the tanks and trickling filters making up the STP and hedgerows adjacent to the pipe where treated water outflowed into a local stream. All the sites were close enough to share common environmental traits and weather.

Conversely, the three sites were far enough apart, with physical barriers to dispersal (roads and a railway line), such that most of the species sampled would not regularly move between the sites.

2.2 Sampling wildlife

All sampling took place between July and August ('Summer'), and October and November ('Autumn') 2016 and was subject to full ethical review (see Supplementary Material). Sampling occurred each week per month per site, but mammals and birds were not captured simultaneously to avoid excessive disturbance. Small mammals were trapped in Longworth or similar live, small mammal traps with shrew escape holes. The traps were sterilised between sites, filled with sterile hay as bedding and mixed grain and carrot or apple as food and water sources. Traps were placed at 5m intervals and checked daily. Faeces were collected with a sterile swab into a sterile sampling tube for transport to the laboratory. The species of each rodent caught, the date and trap location were recorded.

Wild birds were caught in mist nets, under licence from the British Trust for Ornithology (BTO), located along and across hedgerows and patches of woodland within each study site. Each capture location was selected to overlap with trapping sites for small mammals (above) and was pre-baited for at least 3 days with bird feeders containing mixed seed. After capture, each bird was placed on its own into a single use brown paper bag for up to 20 min in order to collect a faecal sample. The bird was then fitted with a BTO leg ring, before being released. Sterile swabs were used to remove faeces from the bags into sterile sampling tubes. If the same bird was caught more than once on the same day the faecal samples were pooled. In addition, feral pigeons, which formed a large flock at the Farm-site, were sampled for faeces post-mortem after shooting as part of pest control. Table S3 shows the range of species caught. The foraging ecology of the species did not explain any of the patterns of AMR or MDR observed (see Supplementary Material).

142 **2.3 Isolation and AMR characterisation of presumptive *E. coli* isolates**

143 Phenotypic resistance to eight antibiotics was determined first by plating on antibiotic-
144 supplemented media or by disk diffusion. Faecal samples (0.5 g) were incubated in buffered peptone
145 water (BPW) at 37 °C for 18 h and 100 µl was spread onto Tryptone Bile X-Glucuronide Medium (TBX;
146 Oxoid, UK) agar supplemented with; ampicillin (10 µg/ml), apramycin (30 µg/ml), colistin (4 µg/ml) or
147 ciprofloxacin (1 µg/ml) or without antibiotics and incubated at 37°C for 18h. Presumptive *E. coli*
148 (blue/green) colonies were taken forward for further characterisation.

149 One presumptive antibiotic resistant *E. coli* colony per plate obtained from the initial
150 screening was then tested for resistance to other antibiotics using disc diffusion assays. Briefly, isolates
151 were cultured in BPW at 37 °C for 18 h. Samples (100 µl) were spread plated onto Muller-Hinton agar
152 (MH; Oxoid, UK) and left to dry. Six antibiotic discs impregnated with ampicillin (10 µg/ml), tetracycline
153 (3 µg/ml), apramycin (15 µg/ml), trimethoprim (2.5 µg/ml), imipenem (10 µg/ml) and cefpodoxime
154 (10 µg/ml), were placed on the agar and the plates were incubated for 18 h at 37 °C. After incubation
155 the diameter of the zone of clearance around each disc was measured and isolates were classified as
156 resistant if the zone was less than or equal to published breakpoints (EUCAST 2016).

157

158 **2.4 Characterisation and ERIC-PCR genotyping of *E. coli* isolates**

159 A representative subsample of presumptive E. coli isolated from mammals from each site and
160 every presumptive E. coli isolated from birds were subject to rRNA PCR and sequencing (Srinivasan et
161 al 2015). BLAST searches confirmed all were *Escherichia*, and the vast majority clearly *E. coli.* In order
162 to identify any patterns of genotypic similarity among *E. coli* by spatial location or host (mammal/bird),
163 we used ERIC-PCR. Twenty-four resistant *E. coli* isolates from mammals at each sample site and all the
164 resistant *E. coli* isolates from birds (total 91 samples) were subjected to ERIC-PCR (Ibrahim et al 2016,
165 Versalovic et al 1991). DNA (diluted 1:100) extracted from the *E. coli* isolates, 12.5 µl of PCR Master

Mix Plus (Qiagen, UK), 5 µM of the each ERIC primer (Table S1), 2 µl of Coral Load Dye (Qiagen, UK) and sterile molecular grade water to 25 µl. The PCR parameters for the ERIC-PCR are found in Table S1.

2.5 Analysis of ESBL and AmpC resistance in cefpodoxime-resistant *E. coli*

Cefpodoxime resistant isolates were tested for ESBL or AmpC activity using the AmpC & ESBL Detection Set (Mast Group, UK). Briefly, overnight liquid cultures of cefpodoxime resistant isolates were spread plated onto MH agar and left to dry before discs containing cefpodoxime 10 µg (A), cefpodoxime 10 µg + ESBL inhibitor (B), cefpodoxime 10 µg + AmpC inhibitor (C) ad cefpodoxime 10 µg + ESBL and AmpC inhibitor (D) were added. Comparison of the zones of clearance enabled ESBL and/or AmpC resistant bacteria to be identified using the manufacturer's calculator (Mast Group, UK).

2.6 DNA extraction and PCR parameters

DNA was extracted from *E. coli* by heat-lysis. One colony was placed in 10 µl of sterile molecular grade water and heated at 95° for 10 min. Samples were centrifuged (13000 *x g*; 3 min) and the supernatant removed. The supernatant was stored at -20 ^{o}C until used as template DNA for subsequent PCR reactions. PCR amplifications (apart from ERIC-PCR) were carried out in 20 µl reaction mixtures comprising of 10 µl of PCR Master Mix Plus (Qiagen, UK): 0.5 µM of each primer, 2 µl of Coral Loading Dye (Qiagen, UK) and molecular grade sterile water to 20 µl. See Table S1 for primers and PCR cycling parameters.

2.7 Molecular characterisation of colistin and ciprofloxacin resistant *E. coli*

E. coli isolates with phenotypic colistin and ciprofloxacin resistance were further characterised. DNA from ciprofloxacin and colistin-resistant colonies was diluted 1:100 and used as template DNA for PCR to amplify the *gyrA* and if present the transposable *mcr-1* gene (Liu et al 2016). For ciprofloxacin resistant isolates DNA was purified from agarose gels using a Gel DNA Extraction Kit (ZymoResearch, UK) and sequenced. The sequences were aligned and compared against *E. coli* K12 using CLC SequenceCe Viewer (Qiagen) to identify specific point mutations in *gyrA* associated with ciprofloxacin resistance. As a positive control for colistin resistance, DNA harbouring the mcr-1 gene was used.

2.8 Statistical analyses

Binomial logistic regression models were used to ascertain the effects of site (Farm, Central and STP), season (Summer = Jul/Aug, Autumn = Oct/Nov,) and taxa (bird or mammal) on the prevalence of *E. coli* in faecal samples and prevalence of resistance, i.e. if *E.coli* were resistant to one or more antibiotic ('AMR ≥1 antibiotic') or MDR (resistant to three or more antibiotics). All of these analyses were carried out using SPSS v.24.

The ERIC-PCR gel image was analysed using a Gel-Doc XR system (Bio-Rad, UK)(Ibrahim et al 2016). Using GelCompar II (Applied Maths) a dendrogram was generated from the comparison of ERIC-PCR profiles, using the Dice coefficient, and clustered by the unweighted pair group method with arithmetic averages (UPGMA) with 1.5% of optimization and 1.5% of tolerance. Molecular variance framework analysis (AMOVA) (Excoffier et al 1992) was used to analyse the confidence of the selected similarity threshold and the significance of clusters. The AMOVA calculation was carried out using GenAlEx v 6.5b5 software (Peakall and Smouse 2006). The significance was examined with the calculation of ΦPT, a measure of population differentiation that suppresses intra-individual variation. In the case of AMOVA, the null hypothesis (H0; ΦPT = 0) meant that there was no genetic difference

among the populations and the alternative hypothesis (H1; ΦPT > 0) meant there were genetic differences amongst the populations.

3. Results

3.1 *E. coli* in rodent and avian samples

In total, 125 faecal samples from bank voles, 15 from field voles and 89 from wood mice were collected. A further 96 faecal samples were collected from traps in which small rodents had escaped, and were recorded as 'unknown' (see Table S2). We collected 84 avian faecal samples from 18 different species, but one sample did not yield an isolate.

Overall *E. coli* were isolated from 66 % (269/408) of faecal samples (Figure 1). The prevalence of *E. coli* was explained by site, season and taxa (Table 1a). Samples collected from the Central (63%; n= 145) and STP sites (64%; n= 125) did not differ significantly. Samples collected from the Farm Site (prevalence = 71 %; n = 138) were significantly more likely to contain *E. coli* than those from the Central Site (Table 1a; Figure 1). Mammalian samples were significantly more likely to contain *E. coli* (prevalence = 74%; n = 325) than avian samples (33%; n = 83)(Table 1a). Samples collected in Summer (prevalence = 73%; n = 227) were significantly more likely to contain *E.coli* than those collected in Autumn (57%; n = 181)(Table 1a).

3.2 Genotyping of *E. coli* isolates by ERIC-PCR

A selection of AMR *E. coli* representing different hosts and sites were compared by ERIC-PCR (Figure 2). Cluster analysis suggested five main groups of isolates at a 50 % similarity threshold (indicated as 1-V in Figure 2). Cluster significance analysis demonstrated these were non-overlapping and hence genomically independent groups (cluster significance ΦPT = 0.036; $p < 0.001$). Each larger cluster (II-V) contained *E. coli* from a range of hosts and sites with no obvious association between

their AMR pattern and which cluster the isolates resided in. However, there was a tendency towards certain clusters containing isolates from predominantly one site: cluster II with Farm Site, cluster III with Central Site and cluster V with STP Site. Given an expected probability of 0.33, binomial tests indicated that the proportion (0.69) of Farm Site samples in Cluster II was significantly higher than expected (p = 0.0002), as was the proportion of Central Site samples (0.62) in Cluster III (p = 0.033) and the proportion of Farm Site samples (0.75) in Cluster V (p = 0.0006).

3.3. Antimicrobial resistance

The prevalence of AMR was expressed as the percentage of samples from which *E. coli* was isolated (on the TBX plate without antibiotics) that also contained at least one isolate resistant to at least one of the antibiotics tested (AMR ≥ 1). The overall prevalence of AMR *E. coli* was 54 % (n = 262) and was significantly explained by a model that included season, taxa and site (Table 1b). AMR prevalence in samples from the STP was 61.3 % (n = 80) which was significantly higher than the prevalence of resistance in samples from the Central Site (50.0 %; n = 86) (Table 1b; Figure 3a). Prevalence in samples from the Farm site was 52.1 % (n = 96) and did not significantly differ from that in Central Site samples (Table 1b).

E. coli from samples collected in Summer (prevalence = 65.4 %; n = 159) were significantly more likely to be resistant than those collected in Autumn (36.9 %; n = 103) Table 1b). There was a tendency (p = 0.056; Table 1b) for mammalian faecal samples to have a higher prevalence (55.7 %; n = 235) of resistant *E. coli* than avian samples (40.7 %; n = 27).

3.4 Multi-drug resistance (MDR)

For the purpose of this study MDR was defined as resistance to three or more of the eight classes of antibiotics tested. Overall, 80.3 % (n = 142) of the AMR *E. coli* were MDR. A model including taxa and site significantly explained MDR prevalence (Table 1c). Prevalence in samples from the Farm site

(66.0 %; n = 50), was significantly lower than from the Central site (83.7 %; n = 43). Prevalence of MDR in samples from the Central and STP sites (91.8 %; n = 49) did not differ significantly (Fig. 3b; Table 1c). *E. coli* from samples collected from mammals (prevalence = 84.7 %; n = 131) were significantly more likely to be MDR than those collected from birds (27.3%; n = 11) (Table 1c). Season (MDR prevalence in Summer = 77.9 %; n = 104 and in Autumn = 86.8 %; n=38) was non-significant so was excluded from the model.

Individual *E. coli* isolates were resistant to up to seven different antibiotics (Figure 3c).There was no obvious difference in MDR profiles between the different sites tested (Table 2).

3.5 Prevalence of ESBL or AmpC producing *E. coli*

All isolates resistant to cefpodoxime were further investigated for ESBL or AmpC production. From the 53 cefpodoxime resistant *E. coli*, six were ESBL, 22 were AmpC and six were positive for both ESBL and AmpC production (Table 3). Across all samples, there was a significant difference between the sites in the number of isolates testing positive for AMPC and/or ESBL, with the highest number at the STP site (χ^2 (2) = 6.59, p = 0.034; Table 3).

3.6 Genotypic analysis of ciprofloxacin and colistin resistant isolates

Ciprofloxacin resistant *E. coli* were further characterised by sequence comparison with a known sensitive strain of *E. coli* (K-12) and four of amino acid changes were observed (Figure 4). All colistin resistant isolates were subjected to *mcr-1* PCR and none were found to be positive for this gene, suggesting resistance is derived from other ARGs.

4. Discussion

AMR, including MDR, was common among the commensal *E. coli* of the wildlife studied, but clear patterns in resistance were not seen in terms of spatial proximity to anthropogenic sources of waste containing antimicrobials and ARB. Previous studies have suggested that wildlife could be used as sentinels of environmental AMR (Furness et al 2017, Vittecoq et al 2016). Our study supports this to some extent, although as with previous work by ourselves and others (Arnold et al 2016, Bondo et al 2016, Gilliver et al 1999, Literak et al 2010, Williams et al 2011), factors other than geographic distance from the wastes of antibiotic treated animals or humans clearly influence AMR. This is also demonstrated by the wide variations in MDR profiles within and between sites suggesting other factors affecting AMR in these animals (Table 2). Host taxonomic differences, as well as spatial and temporal factors, seemed to influence AMR prevalence. Moreover, our models explained about 20% of the variance in AMR and MDR, indicating that other, unmeasured factors, were also important in determining prevalence. Thus, there are significant caveats to using wildlife as sentinels of environmental transmission of AMR due to antimicrobials and ARB in anthropogenic wastes.

Some studies have reported relatively high AMR prevalence in wildlife collected near AMR sources such as water bodies receiving sewage effluent or agricultural wastes, compared with more pristine sites (Bonnedahl et al 2009, Furness et al 2017). In our study, a significantly higher prevalence of AMR was observed at the STP (61%) compared with the other two sites (<53%). That site and site-specific environments might be drivers of exposure is supported by the ERIC analysis that found that genotypes of *E. coli* showed spatial- rather than host-specific clustering (VanderWaal et al 2014). Multidrug resistance prevalence showed somewhat different patterns with the STP (92%) again having a significantly higher MDR prevalence than the farm (66%), but a similar prevalence to the Central site (84%). If the prevalence and patterns of resistance were driven by exposure to either anthropogenic antimicrobials or ARB from humans and/or livestock, a higher prevalence of resistance would have been expected at the Farm Site as well as the STP Site, and the

prevalence at the Central site might have been expected to be lower than both of the other two sites. However, this was not the case (see also (Carter et al 2018).

4.1 Host taxa and temporal variation

Taxonomic differences in both the prevalence of samples containing *E. coli* and the prevalence of AMR and MDR were observed. Mammals (74%) were significantly more likely to be carrying *E. coli* than birds (33%), with a prevalence of 66% overall. Host taxonomic differences in *E. coli* may reflect the relatively small size of faecal samples from birds and their tendency to dry out, but might also simply reflect the relative contribution of *E. coli* to the normal gut biota of very different taxa. The prevalence of phenotypic AMR (expressed as the percentage of samples that contained resistant *E. coli*) was 54% overall, with a marginally higher prevalence in mammalian (56%) than avian (41%) samples ($p = 0.056$). Our prevalence of ARB in mammals was similar to that previously reported in the UK (35% and 79% for inland and coastal populations respectively of small mammals (Furness et al 2017), but higher than that reported in similar species from mainland Europe (for example 5.5% AMR in *E. coli* from rural small mammals in Germany (Guenther et al 2010) and 2 – 12% in a range of wild mammals the Czech Republic (Literak et al 2010). Reported AMR prevalence in wild birds is similarly diverse, varying both by species and geography (Carter et al 2018). For example, a study of AMR in *E.coli* from gulls across Europe found a prevalence of 32% overall, but with considerable geographic variation, from 61% in Spain to 8% in Denmark (Stedt et al 2014). Notably, a larger number of avian than mammal species were sampled, so differences in ecology and diet among species might obfuscate comparisons of the relative roles of mammals and birds in AMR dispersal.

Furthermore, in our study, as in others (Ahammad et al 2014, Bondo et al 2016, Sun et al 2012, Williams et al 2011), *E. coli*, AMR and MDR patterns and prevalence varied over time. Temporal variation in *E. coli* and resistance patterns might reflect changing environmental conditions (temperature and rainfall), selective drivers (e.g. patterns in antibiotic usage) and/or food

availability (and changing gut biota) for wildlife as well as differences between the species' population dynamics (Waite and Taylor 2014, Williams et al 2011). Since sampling took place during only two seasons, temporal and seasonal patterns in AMR evolution and dispersal need further study. Despite some limitations, our study lays the foundations for future studies looking a larger numbers of animals at a wider variety of sites and, ideally, longitudinally, along with direct sampling of the environment for antibiotics and ARB.

4.2 MDR prevalence and resistance profiles

As described in other studies (Arnold et al 2016, Williams et al 2011), many AMR isolates from mammalian wildlife were multidrug-resistant (MDR). This was likely an outcome of prevalent mobile genetic elements such as plasmids and transposons (Carroll et al 2015), but chromosomal mutations are also common. The prevalence of MDR (AMR ≥3), like overall AMR (AMR ≥1) was higher in mammal (85%) than in bird samples (27%). On the other hand, the large diversity of MDR profiles found (Table 2) suggests only limited MDR transmission between individuals. Some of these resistances (ciprofloxacin) were found to be derived from point mutations and therefore are not necessarily linked to the other resistances carried by the individual bacterium. Moreover, MDR prevalence was highest at the STP. It is tempting, therefore, to speculate that animals at the STP Site were exposed to a wider range of MDR bacteria, plasmids, or antimicrobials, than animals at other sites. This in turn would fit well with a hypothesis that these animals had exposure to sewage derived from many different people, with different histories of antimicrobial exposure, whereas wildlife at the Central and Farm Sites would have exposure to less varied sources and drivers. This would still, however, leave unanswered the questions of what might be the drivers that led to such high MDR prevalence overall, why different animals in the same population might have such different exposure histories and why the Farm Site and not the Central Site had the lowest MDR prevalence.

The most common MDR resistance profile encountered in this study was combined resistance to ampicillin, colistin and ciprofloxacin (Table 2). A high prevalence of resistance to ampicillin was expected as this beta-lactam antibiotic is frequently used in both human and veterinary medicine and resistance is common not only in clinical samples (Briñas et al 2002) but has also been described previously in wild rodents (Arnold et al 2016, Williams et al 2011). It is commonly plasmid-encoded and associated with MDR, as in this study where 83% of the ampicillin resistant isolates were resistant to three or more antibiotics and 23% to five or more antibiotics (Table 2). A high prevalence of phenotypic resistance to colistin was neither expected nor has been described previously in wild rodents, although colistin-resistant *E. coli* strains have been isolated from waterbird faeces (Wu et al 2018). Colistin resistance genes have been demonstrated in waste-impacted river water (Wu et al 2018), and especially at STPs (Hembach et al 2017). Although chromosomally-encoded colistin resistance has been described for many years, its prevalence was historically generally low. The recent discovery of the *mcr-1* gene, that confers colistin resistance and is plasmid encoded, enabling rapid horizontal transmission of resistance, (Liu and Wong 2013) is of great clinical concern as colistin is now a 'last line' antibiotic used for treating MDR infections in humans (Velkov et al 2013). The high prevalence of colistin resistance found in our study (35-40%), along with most colistin resistant isolates being MDR (87% resistant to three or more antibiotics and 26% to five or more antibiotics) is suggestive of horizontal transmission although screening for the mcr-*1* gene by PCR was negative. However, other plasmid-encoded genes for colistin resistance have been subsequently described (Xavier et al 2016), and further characterisation of the underlying mechanism of the colistin resistance found in in our study is underway. Seven out of the nine ciprofloxacin resistant isolates contained four nonsynonymous mutations in the *gyrase A* gene (Figure 4), which had been reported previously, and two had mutations that have not previously been reported in *E. coli.* Wildlife can. Therefore, harbour and disperse novel and/ or clinically important ARGs in the environment.

385 In terms of other clinical important resistances, cefpodoxime resistance is a common
386 indicator of ESBL production (Oliver *et al.*, 2002), also of major concern in human medicine. From
387 the 53 cefpodoxime resistant *E. coli* isolated from wildlife, six were ESBL producers, 22 were AmpC
388 and six were positive for both ESBL and AmpC production (Table 3). ESBLs have previously been
389 detected in *E. coli* isolates from a range of wildlife taxa, for example, 32% of *E. coli* isolates obtained
390 from gulls' faeces (Simões et al 2010), and such findings have been ascribed to contact with human
391 waste. In our study, significantly more ESBL and/or AmpC – producing *E. coli* were found in wildlife
392 samples collected from the STP Site, which suggests that human waste may be a factor driving
393 ESBL/AmpC resistance in the environment.
394
395 **4.3. Conclusions**
396 Taken together, the results of this study support those of previous studies in that they
397 confirm that wildlife commonly harbour ARB. Whether or not wildlife might be a source for onward
398 transmission to domestic animals or to humans has not been directly examined. Our study was more
399 concerned with beginning to investigate the drivers of AMR in wildlife, and in particular the role that
400 anthropogenic waste, whether of directly human or domestic animal origin, might play in developing
401 and maintaining that resistance. Diverse patterns of resistance were found in *E. coli* from wildlife in
402 this study, suggesting variation within and between host taxa, between individuals, and over time.
403 Overall, study site was not associated clearly with AMR, MDR or resistance patterns. However,
404 resistance to antibiotics used only in human medicine was more prevalent at the STP site than the
405 Farm and Central sites. Thus, the drivers of AMR in wildlife appear to be more complex than simple
406 anthropogenic causes. Consequently, care needs to be taken if wildlife are to be used as sentinels of
407 environmental AMR or pollution.
408
409

Acknowledgements

We would like to thank K. Hemsley and the South Notts Ringing group for help with capturing birds.

Funding

The project was funded by the University of Nottingham, the University of York and the EPSRC Bridging the Gaps between the Engineering and Physical Sciences and Antimicrobial Resistance funds to the University of York and University of Nottingham.

References

Ahammad ZS, Sreekrishnan TR, Hands CL, Knapp CW, Graham DW (2014). Increased Waterborne bla(NDM-1) Resistance Gene Abundances Associated with Seasonal Human Pilgrimages to the Upper Ganges River. *Environmental Science & Technology* **48:** 3014-3020.

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews Microbiology* **8:** 251-259.

Allen SE, Boerlin P, Janecko N, Lumsden JS, Barker IK, Pearl DL *et al* (2011). Antimicrobial Resistance in Generic Escherichia coli Isolates from Wild Small Mammals Living in Swine Farm, Residential, Landfill, and Natural Environments in Southern Ontario, Canada. *Applied and Environmental Microbiology* **77:** 882-888.

Arnold KE, Williams NJ, Bennett M (2016). 'Disperse abroad in the land': the role of wildlife in the dissemination of antimicrobial resistance. *Biology Letters* **12**.

Bondo KJ, Pearl DL, Janecko N, Boerlin P, Reid-Smith RJ, Parmley J *et al* (2016). Epidemiology of Antimicrobial Resistance in Escherichia coli Isolates from Raccoons (Procyon lotor) and the Environment on Swine Farms and Conservation Areas in Southern Ontario. *Plos One* **11**.

Bonnedahl J, Drobni M, Gauthier-Clerc M, Hernandez J, Granholm S, Kayser Y *et al* (2009). Dissemination of Escherichia coli with CTX-M Type ESBL between Humans and Yellow-Legged Gulls in the South of France. *Plos One* **4**.

Briñas L, Zarazaga M, Sáenz Y, Ruiz-Larrea F, Torres C (2002). β-Lactamases in Ampicillin-Resistant Escherichia coli Isolates from Foods, Humans, and Healthy Animals. *Antimicrobial Agents and Chemotherapy* **46:** 3156-3163.

Carroll D, Wang J, Fanning S, McMahon BJ (2015). Antimicrobial Resistance in Wildlife: Implications for Public Health. *Zoonoses and Public Health* **62:** 534-542.

Carter DL, Docherty KM, Gill SA, Baker K, Teachout J, Vonhof MJ (2018). Antibiotic resistant bacteria are widespread in songbirds across rural and urban environments. *Science of the Total Environment* **627:** 1234-1241.

Clarke BO, Smith SR (2011). Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. *Environ Internat* **37:** 226-247.

Cristobal-Azkarate J, Dunn JC, Day JMW, Amabile-Cuevas CF (2014). Resistance to Antibiotics of Clinical Relevance in the Fecal Microbiota of Mexican Wildlife. *Plos One* **9**.

D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C *et al* (2011). Antibiotic resistance is ancient. *Nature* **477:** 457-461.

Davies J, Davies D (2010). Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews* **74:** 417-+.

EUCAST (2016). "The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 6.0, 2016. http://www.eucast.org.".

Excoffier L, Smouse PE, Quattro JM (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131:** 479-491.

Fahrenfeld N, Ma Y, O'Brien M, Pruden A (2013). Reclaimed water as a reservoir of antibiotic resistance genes: distribution system and irrigation implications. *Frontiers in Microbiology* **4:** 130-130.

Furness LE, Campbell A, Zhang L, Gaze WH, McDonald RA (2017). Wild small mammals as sentinels for the environmental transmission of antimicrobial resistance. *Environmental Research* **154:** 28-34.

Gilliver M, Bennett M, Begon M, Hazel S, Hart C (1999). Enterobacteria: Antibiotic resistance found in wild rodents. *Nature* **401:** 233 - 234.

Guenther S, Grobbel M, Heidemanns K, Schlegel M, Ulrich RG, Ewers C *et al* (2010). First insights into antimicrobial resistance among faecal Escherichia coli isolates from small wild mammals in rural areas. *Science of the Total Environment* **408:** 3519-3522.

Guenther S, Semmler T, Stubbe A, Stubbe M, Wieler LH, Schaufler K (2017). Chromosomally encoded ESBL genes in Escherichia coli of ST38 from Mongolian wild birds. *Journal of Antimicrobial Chemotherapy* **72:** 1310-1313.

Hembach N, Schmid F, Alexander J, Hiller C, Rogall ET, Schwartz T (2017). Occurrence of the mcr-1 Colistin Resistance Gene and other Clinically Relevant Antibiotic Resistance Genes in Microbial Populations at Different Municipal Wastewater Treatment Plants in Germany. *Frontiers in Microbiology* **8**.

Huijbers PMC, Blaak H, de Jong MCM, Graat EAM, Vandenbroucke-Grauls CMJE, Husman AMdR (2015). Role of the Environment in the Transmission of Antimicrobial Resistance to Humans: A Review. *Environmental Science & Technology* **49:** 11993-12004.

Ibrahim DR, Dodd CER, Stekel DJ, Ramsden SJ, Hobman JL (2016). Multidrug resistant, extended spectrum β-lactamase (ESBL)-producing Escherichia coli isolated from a dairy farm. *FEMS Microbiology Ecology* **92:** fiw013-fiw013.

Karesh WB, Dobson A, Lloyd-Smith JO, Lubroth J, Dixon MA, Bennett M *et al* (2012). Zoonoses 1 Ecology of zoonoses: natural and unnatural histories. *Lancet* **380:** 1936-1945.

Literak I, Dolejska M, Radimersky T, Klimes J, Friedman M, Aarestrup FM *et al* (2010). Antimicrobial-resistant faecal *Escherichia coli i*n wild mammals in central Europe: multiresistant Escherichia coli producing extended-spectrum beta-lactamases in wild boars. *Journal of applied microbiology* **108:** 1702-1711.

Liu JL, Wong MH (2013). Pharmaceuticals and personal care products (PPCPs): A review on environmental contamination in China. *Environ Internat* **59:** 208-224.

Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J *et al* (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *The Lancet Infectious Diseases* **16:** 161-168.

Marston HD, Dixon DM, Knisely JM, Palmore TN, Fauci AS (2016). Antimicrobial resistance. *Journal of the American Medical Association* **316:** 1193-1204.

Martinez JL (2009). The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proceedings of the Royal Society B-Biological Sciences* **276:** 2521-2530.

O'Neill J (2016). Tackling Drug Resistant Infections Globally: Final Report and Recommendations.

Peakall ROD, Smouse PE (2006). genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6:** 288-295.

Radhouani H, Igrejas G, Carvalho C, Pinto L, Gonçalves A, Lopez M *et al* (2011). Clonal Lineages, Antibiotic Resistance and Virulence Factors in Vancomycin-Resistant Enterococci Isolated from Fecal Samples of Red Foxes (Vulpes Vulpes). *Journal of Wildlife Diseases* **47:** 769-773.

Rawat D, Nair D (2010). Extended-spectrum β-lactamases in Gram Negative Bacteria. *Journal of Global Infectious Diseases* **2:** 263-274.

Schaufler K, Semmler T, Pickard DJ, de Toro M, de la Cruz F, Wieler LH *et al* (2016). Carriage of Extended-Spectrum Beta-Lactamase-Plasmids Does Not Reduce Fitness but Enhances Virulence in Some Strains of Pandemic E-coli Lineages. *Frontiers in Microbiology* **7**.

Simões RR, Poirel L, Da Costa PM, Nordmann P (2010). Seagulls and Beaches as Reservoirs for Multidrug-Resistant Escherichia coli. *Emerging Infectious Diseases* **16:** 110-112.

Srinivasan R, Karaoz U, Volegova M, MacKichan J, Kato-Maeda M, Miller S *et al* (2015). Use of 16S rRNA Gene for Identification of a Broad Range of Clinically Relevant Bacterial Pathogens. *PLOS ONE* **10:** e0117617.

Stedt J, Bonnedahl J, Hernandez J, McMahon BJ, Hasan B, Olsen B *et al* (2014). Antibiotic resistance patterns in Escherichia coli from gulls in nine European countries. *Infection Ecology & Epidemiology* **4:** 10.3402/iee.v3404.21565.

Sun L, Klein EY, Laxminarayan R (2012). Seasonality and Temporal Correlation between Community Antibiotic Use and Resistance in the United States. *Clin Infect Dis* **55:** 687-694.

Taylor NGH, Verner-Jeffreys DW, Baker-Austin C (2011). Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? *Trends in Ecology & Evolution* **26:** 278-284.

VanderWaal KL, Atwill ER, Isbell LA, McCowan B (2014). Linking social and pathogen transmission networks using microbial genetics in giraffe (Giraffa camelopardalis). *J Anim Ecol* **83:** 406-414.

Velkov T, Roberts KD, Nation RL, Thompson PE, Li J (2013). Pharmacology of polymyxins: new insights into an 'old' class of antibiotics. *Future Microbiology* **8:** 10.2217/fmb.2213.2239.

Versalovic J, Koeuth T, Lupski R (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19:** 6823-6831.

Vittecoq M, Godreuil S, Prugnolle F, Durand P, Brazier L, Renaud N *et al* (2016). REVIEW: Antimicrobial resistance in wildlife. *J App Ecol* **53:** 519-529.

von Wintersdorff CJH, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB *et al* (2016). Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Frontiers in Microbiology* **7**.

Waite DW, Taylor MW (2014). Characterizing the avian gut microbiota: membership, driving influences, and potential function. *Frontiers in Microbiology* **5**.

Williams NJ, Sherlock C, Jones TR, Clough HE, Telfer SE, Begon M *et al* (2011). The prevalence of antimicrobial-resistant Escherichia coli in sympatric wild rodents varies by season and host. *Journal of applied microbiology* **110:** 962-970.

Wu J, Huang Y, Rao D, Zhang Y, Yang K (2018). Evidence for environmental dissemination of antibiotic resistance mediated by wild birds. *Frontiers in Microbiology* **9**.

Xavier BB, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H *et al* (2016). Identification of a novel plasmid-mediated colistin-resistance gene, mcr-2, in Escherichia coli, Belgium, June 2016. *Eurosurveillance* **21:** 30280.

D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C *et al* (2011). Antibiotic resistance is ancient. *Nature* **477:** 457-461.

Davies J, Davies D (2010). Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews* **74:** 417.

EUCAST (2016). "The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 6.0, 2016. http://www.eucast.org.".

Excoffier L, Smouse PE, Quattro JM (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131:** 479-491.

Fahrenfeld N, Ma Y, O'Brien M, Pruden A (2013). Reclaimed water as a reservoir of antibiotic resistance genes: distribution system and irrigation implications. *Frontiers in Microbiology* **4:** 130-130.

Furness LE, Campbell A, Zhang L, Gaze WH, McDonald RA (2017). Wild small mammals as sentinels for the environmental transmission of antimicrobial resistance. *Environmental Research* **154:** 28-34.

Gilliver M, Bennett M, Begon M, Hazel S, Hart C (1999). Enterobacteria: Antibiotic resistance found in wild rodents. *Nature* **401:** 233 - 234.

Guenther S, Grobbel M, Heidemanns K, Schlegel M, Ulrich RG, Ewers C *et al* (2010). First insights into antimicrobial resistance among faecal *Escherichia coli* isolates from small wild mammals in rural areas. *Science of the Total Environment* **408:** 3519-3522.

Guenther S, Semmler T, Stubbe A, Stubbe M, Wieler LH, Schaufler K (2017). Chromosomally encoded ESBL genes in Escherichia coli of ST38 from Mongolian wild birds. *Journal of Antimicrobial Chemotherapy* **72:** 1310-1313.

Hembach N, Schmid F, Alexander J, Hiller C, Rogall ET, Schwartz T (2017). Occurrence of the mcr-1 Colistin Resistance Gene and other Clinically Relevant Antibiotic Resistance Genes in Microbial

618 Populations at Different Municipal Wastewater Treatment Plants in Germany. *Frontiers in*
619 *Microbiology* **8**.

620
621 Huijbers PMC, Blaak H, de Jong MCM, Graat EAM, Vandenbroucke-Grauls CMJE, Husman AMdR
622 (2015). Role of the Environment in the Transmission of Antimicrobial Resistance to Humans: A Review.
623 *Environmental Science & Technology* **49:** 11993-12004.

624
625 Ibrahim DR, Dodd CER, Stekel DJ, Ramsden SJ, Hobman JL (2016). Multidrug resistant, extended
626 spectrum β-lactamase (ESBL)-producing *Escherichia coli i*solated from a dairy farm. *FEMS Microbiology*
627 *Ecology* **92:** fiw013-fiw013.

628
629 Karesh WB, Dobson A, Lloyd-Smith JO, Lubroth J, Dixon MA, Bennett M *et al* (2012). Zoonoses 1
630 Ecology of zoonoses: natural and unnatural histories. *Lancet* **380:** 1936-1945.

631
632 Literak I, Dolejska M, Radimersky T, Klimes J, Friedman M, Aarestrup FM *et al* (2010). Antimicrobial-
633 resistant faecal *Escherichia coli i*n wild mammals in central Europe: multiresistant Escherichia coli
634 producing extended-spectrum beta-lactamases in wild boars. *Journal of Applied Microbiology* **108:**
635 1702-1711.

636
637 Liu JL, Wong MH (2013). Pharmaceuticals and personal care products (PPCPs): A review on
638 environmental contamination in China. *Environment International* **59:** 208-224.

639
640 Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J *et al* (2016). Emergence of plasmid-mediated
641 colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and
642 molecular biological study. *The Lancet Infectious Diseases* **16:** 161-168.

643
644 Marston HD, Dixon DM, Knisely JM, Palmore TN, Fauci AS (2016). Antimicrobial resistance. *Journal of*
645 *the American Medical Association* **316:** 1193-1204.

646
647 Martinez JL (2009). The role of natural environments in the evolution of resistance traits in pathogenic
648 bacteria. *Proceedings of the Royal Society B-Biological Sciences* **276:** 2521-2530.

649
650 O'Neill J (2016). Tackling Drug Resistant Infections Globally: Final Report and Recommendations.

651
652 Peakall ROD, Smouse PE (2006). genalex 6: genetic analysis in Excel. Population genetic software for
653 teaching and research. *Molecular Ecology Notes* **6:** 288-295.

654
655 Radhouani H, Igrejas G, Carvalho C, Pinto L, Gonçalves A, Lopez M *et al* (2011). Clonal Lineages,
656 Antibiotic Resistance and Virulence Factors in Vancomycin-Resistant Enterococci Isolated from Fecal
657 Samples of Red Foxes (*Vulpes vulpes*). *Journal of Wildlife Diseases* **47:** 769-773.

658
659 Rawat D, Nair D (2010). Extended-spectrum β-lactamases in Gram Negative Bacteria. *Journal of Global*
660 *Infectious Diseases* **2:** 263-274.

661

662 Schaufler K, Semmler T, Pickard DJ, de Toro M, de la Cruz F, Wieler LH *et al* (2016). Carriage of
663 Extended-Spectrum Beta-Lactamase-Plasmids Does Not Reduce Fitness but Enhances Virulence in
664 Some Strains of Pandemic E-coli Lineages. *Frontiers in Microbiology* **7**.

665
666 Simões RR, Poirel L, Da Costa PM, Nordmann P (2010). Seagulls and Beaches as Reservoirs for
667 Multidrug-Resistant Escherichia coli. *Emerging Infectious Diseases* **16:** 110-112.

668
669 Srinivasan R, Karaoz U, Volegova M, MacKichan J, Kato-Maeda M, Miller S *et al* (2015). Use of 16S
670 rRNA Gene for Identification of a Broad Range of Clinically Relevant Bacterial Pathogens. *PLOS ONE*
671 **10:** e0117617.

672
673 Stedt J, Bonnedahl J, Hernandez J, McMahon BJ, Hasan B, Olsen B *et al* (2014). Antibiotic resistance
674 patterns in *Escherichia coli* from gulls in nine European countries. *Infection Ecology & Epidemiology* **4:**
675 10.3402/iee.v3404.21565.

676
677 Sun L, Klein EY, Laxminarayan R (2012). Seasonality and Temporal Correlation between Community
678 Antibiotic Use and Resistance in the United States. *Clinical Infectious Diseases* **55:** 687-694.

679
680 Taylor NGH, Verner-Jeffreys DW, Baker-Austin C (2011). Aquatic systems: maintaining, mixing and
681 mobilising antimicrobial resistance? *Trends in Ecology & Evolution* **26:** 278-284.

682
683 VanderWaal KL, Atwill ER, Isbell LA, McCowan B (2014). Linking social and pathogen transmission
684 networks using microbial genetics in giraffe (*Giraffa camelopardalis*). *J Anim Ecol* **83:** 406-414.

685
686 Velkov T, Roberts KD, Nation RL, Thompson PE, Li J (2013). Pharmacology of polymyxins: new insights
687 into an 'old' class of antibiotics. *Future Microbiology* **8:** 10.2217/fmb.2213.2239.

688
689 Versalovic J, Koeuth T, Lupski R (1991). Distribution of repetitive DNA sequences in eubacteria and
690 application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19:** 6823-6831.

691
692 Vittecoq M, Godreuil S, Prugnolle F, Durand P, Brazier L, Renaud N *et al* (2016). REVIEW: Antimicrobial
693 resistance in wildlife. *Journal of Applied Ecology* **53:** 519-529.

694
695 von Wintersdorff CJH, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB *et al* (2016).
696 Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer.
697 *Frontiers in Microbiology* **7**.

698
699 Williams NJ, Sherlock C, Jones TR, Clough HE, Telfer SE, Begon M *et al* (2011). The prevalence of
700 antimicrobial-resistant Escherichia coli in sympatric wild rodents varies by season and host. *Journal of*
701 *Applied Microbiology* **110:** 962-970.

702
703 Xavier BB, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H *et al* (2016). Identification of a
704 novel plasmid-mediated colistin-resistance gene, mcr-2, in *Escherichia coli*, Belgium, June 2016.
705 *Eurosurveillance* **21:** 30280.

Figure Legends

Figure 1: Inter-site variation in the percentage prevalence of faecal samples testing positive (solid blue bars) or negative (orange hatched bars) for a) *E. coli*. Boxes on the bars show the number of samples in each category.

Figure 2. ERIC profile of *E. coli* isolated from both small mammals and birds at Farm site (light green, mammals; dark green birds) Central site (red, mammals; dark red, birds) and STP site (light purple, mammals; dark purple, birds). Horizontal lines demonstrate significant clusters (I - V) based on 50 % cut-off (vertical line). Red cells demonstrate resistance to each antibiotic: Amp – ampicillin; Cef – cefpodoxime; Col – colistin; Apra – apramycin; Imi – imipenem; Trim – trimethoprim; Tet – tetracycline; Cip – ciprofloxacin

Figure 3: Site-specific patterns of resistance in *E. coli* isolates*:* a) AMR: The percentage of faecal samples which contained *E. coli* susceptible to ≥1 antimicrobial (negative = orange hatched bars) or resistant to one or more antimicrobial drugs (positive = solid blue bars); b) MDR - The percentage of samples containing *E. coli* that were resistant to ≥3 antibiotics (positive = resistant = solid blue bars); c) Prevalence of resistance to 1 – 7 different antibiotics. The sites were Farm, Central and STP.

Figure 4. Mutations of ciprofloxin-resistant *E. coli* isolated from small mammals (blue boxes). Translated sequences of *gyraseA* gene from ciprofloxacin resistant *E. coli* isolates compared to the known sensitive reference strain K-12.

728 Table 1: Final binomial logistic regression model outputs explaining prevalence of a) E. coli; b)
729 AMR ≥1 antibiotic; c) MDR (AMR ≥3 antibiotics). The coefficients for the Site variable are compared to
730 the Central Site, for the Taxa variable was compared to birds and for the Season variable was
731 compared to Autumn.

	Nagelkerke R^2	**χ2 (df)**	**Wald (df)**	**p-value**	**Odds ratio**	**95% C.I.**
a) *E. coli*	21%	67.50 (4)		< 0.0001		
Site:			16.21 (2)	< 0.0001		
Farm			15.07 (1)	< 0.0001	3.51	1.86 – 6.60
STP			0.23 (1)	0.63	1.14	0.67 – 1.93
Taxa			45.75 (1)	< 0.0001	9.26	4.86 - 17.66
Season			3.89 (1)	0.048	1.57	1.00 - 2.46
b) AMR	14.4%	29.97 (4)		< 0.0001		
Site:			4.75 (2)	0.093		
Farm			1.17 (1)	0.28	1.44	0.74 - 2.79
STP			4.742 (1)	0.029	2.11	1.08 - 4.73
Taxa			3.64 (1)	0.056	2.48	0.98 - 6.32
Season			23.93 (1)	< 0.0001	3.96	2.28 - 6.89
c) MDR	25.9%	40.91 (4)		< 0.0001		
Site:			8.02 (2)	0.018		
Farm			0.05 (1)	0.82	1.09	0.51 - 2.34
STP			7.07 (1)	0.008	3.37	1.38 - 8.26
Taxa			14.30 (1)	< 0.0001	12.53	3.38 – 46.43
Season			0.57 (1)	0.45	1.34	0.63 - 2.84

732

733 **Table 2:** Frequencies of MDR profiles for combinations of antibiotics to which *E. coli* isolates were
734 resistant for faecal samples collected from birds and mammals captured at the STP, Central and Farm
735 sites. Only profiles that were found at two or more individuals are presented. Amp – ampicillin; Cef –
736 cefpodoxime; Col – colistin; Apra – apramycin; Imi – imipenem; Trim – trimethoprim; Tet –
737 tetracycline; Cip – ciprofloxacin

738

Antibiotics	**Farm**	**Central**	**STP**	**Totals**
Amp Tet Col	7	8	8	23
Apra Col Tet	2	3	2	7
Amp Cip Tet	5	0	0	5
Amp Tet Cef	0	1	3	4
Amp Tet Trim	0	2	2	4
Amp Apra Tet	1	2	1	4
Col Cef Tet	0	1	2	3
Apra Trim Col	0	0	2	2
Amp Apra Cef	1	1	0	2
Amp Apra Col Tet	2	5	2	9
Amp Tet Trim Col	2	1	3	6
Col Trim Cef Tet	0	1	2	3
Amp Tet Cef Col	0	0	3	3
Amp Cef Trim Col	1	0	2	3
Apra Tetra Cef Col	1	1	0	2
Amp Apra Trim Col	0	2	0	2
Amp Apra Cef Trim Col	3	2	3	8
Amp Col Trim Cef Tet	1	2	4	7
Amp Apra Tet Trim Col	2	0	0	2
Amp Apra Tet Cef Trim Col	1	2	1	4

739

740

Table 3: Number of AmpC and ESBL producing *E. coli* isolates for bird and mammal samples collected at the Farm (livestock waste dominated), Central (no waste source) and STP (human waste dominated) sites. The percentages in brackets were calculated across all 53 cefpodoxime resistant isolates that were tested for AmpC and ESBL activity.

Site	**Mammal**				**Bird**			
	AmpC	**ESBL**	**AmpC & ESBL**	**Negative**	**AmpC**	**ESBL**	**AmpC & ESBL**	**Negative**
Farm	4 (8%)	0	2 (4%)	5 (9%)	0	0	0	2 (4%)
Central	6 (11%)	2 (4%)	1 (2%)	4 (8%)	1 (2%)	0	0	0
STP	7 (13%)	4 (8%)	3 (6%)	7 (13%)	4 (8%)	0	0	1 (2%)
Total	**17 (32%)**	**6 (12%)**	**6 (12%)**	**16 (30%)**	**5 (9%)**	**0**	**0**	**3 (6%)**