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Anthropogenic environmental drivers of antimicrobial resistance in wildlife

Benjamin M.C. Swift [a,1], Malcolm Bennett [a], Katie Waller [a], Christine Dodd [b], Annie Murray [c], Rachel L. Gomes [d], Bethan Humphreys [e], Jon L. Hobman [b], Michael A. Jones [a], Sophia E. Whitlock [c], Lucy J. Mitchell [c], Rosie J. Lennon [c], Kathryn E. Arnold [c,*]

[a] School of Veterinary Medicine and Science, University of Nottingham, UK
[b] School of Bioscience, University of Nottingham, UK
[c] Department of Environment and Geography, University of York, UK
[d] Food, Water, Waste Research Group, Faculty of Engineering, University of Nottingham, UK
[e] School of Life Sciences, University of Nottingham, UK

HIGHLIGHTS

- Does AMR in wildlife reflect exposure to human or livestock wastes?
- AMR patterns in *E. coli* from birds and mammals varied with site, season and host.
- Colistin resistance and ESBL/AmpC activity high in wildlife exposed to human waste.
- Our data question the use of wildlife as sentinels of anthropogenic environmental AMR.

GRAPHICAL ABSTRACT
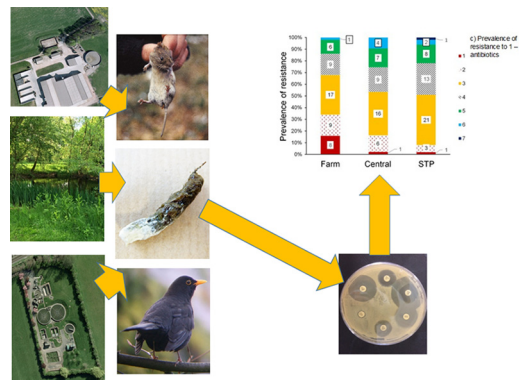
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ABSTRACT

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

* Corresponding author: Department of Environment and Geography, University of York, YO10 5NG, UK.
  E-mail addresses: bswift@rvc.ac.uk (B.M.C. Swift), m.bennett@nottingham.ac.uk (M. Bennett), svyklw@nottingham.ac.uk (K. Waller), Christine.Dodd@nottingham.ac.uk (C. Dodd), am1400@york.ac.uk (A. Murray), rachel.gomes@nottingham.ac.uk (R.L. Gomes), mbybmh@nottingham.ac.uk (B. Humphreys), Jon.Hobman@nottingham.ac.uk (J.L. Hobman), michael.a.jones@nottingham.ac.uk (M.A. Jones), sew553@york.ac.uk (S.E. Whitlock), ljr540@york.ac.uk (L.J. Mitchell), rjl529@york.ac.uk (R.J. Lennon), kathryn.arnold@york.ac.uk (K.E. Arnold).
  [1] Current address: Pathobiology and Population Sciences, Royal Veterinary College, UK.

Birds
Multi-drug resistance
Wastewater treatment

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.

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

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

In this study, three nearby communities of small wild rodents and birds were investigated for evidence of AMR in faeces. The antimicrobials used to screen for resistance were chosen as they represent a range of antibiotic classes of medical and veterinary interest. For example, cefpodoxime resistance is seen as an indicator of extended spectrum beta-lactamase (ESBL) or AmpC beta-lactamase producing bacteria which cause significant problems in human medicine especially with urinary tract infections (Rawat and Nair, 2010). Colistin resistance is also of relevance due to colistin 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 sylvaticus*, 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 1200 m transect, were selected (Fig. 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 600 m 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 450–600 m 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 5 m 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 postmortem 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).

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

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

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

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

A representative subsample of presumptive E. coli isolated from mammals from each site and every presumptive E. coli isolated from birds were subject to rRNA PCR and sequencing (Srinivasan et al., 2015). BLAST searches confirmed all were Escherichia, and the vast majority clearly E. coli. In order to identify any patterns of genotypic similarity among E. coli by spatial location or host (mammal/bird), we used ERIC-PCR. Twenty-four resistant E. coli isolates from mammals at each sample site and all the resistant E. coli isolates from birds (total 91 samples) were subjected to ERIC-PCR (Ibrahim et al., 2016; 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 (13,000 ×g; 3 min) and the supernatant removed. The supernatant was stored at −20 °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; $\Phi PT = 0$) meant that there was no genetic difference among the populations and the alternative hypothesis (H1; $\Phi PT > 0$) meant there were genetic differences among 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 from which small mammals had escaped, and were recorded as 'unknown' (see Table S2). We collected 83 avian faecal samples from 18 different species.

Overall *E. coli* were isolated from 66% (269/408) of faecal samples (Fig. 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; Fig. 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 (Fig. 2). Cluster analysis suggested five main groups of isolates at a 50% similarity threshold (indicated as I–V in Fig. 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 ($p = 0.0006$).
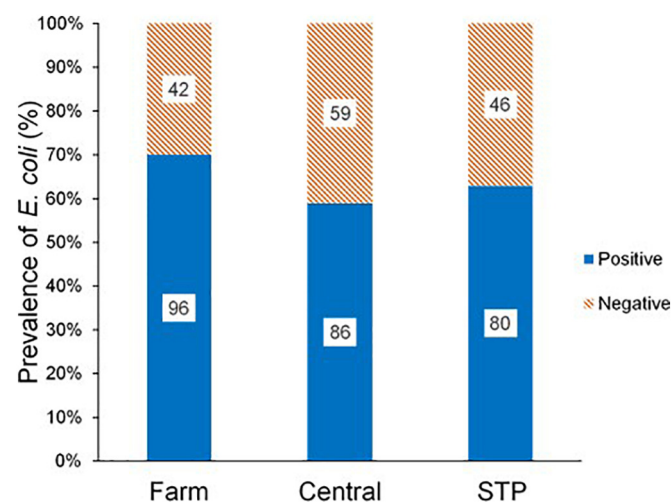

Fig. 1. Inter-site variation in the percentage prevalence of faecal samples testing positive (solid blue bars) or negative (orange hatched bars) for a) *E. coli*. Boxes on the bars show the number of samples in each category.

Table 1

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

	Nagelkerke R^2	χ^2 (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

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; Fig. 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 (Fig. 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).
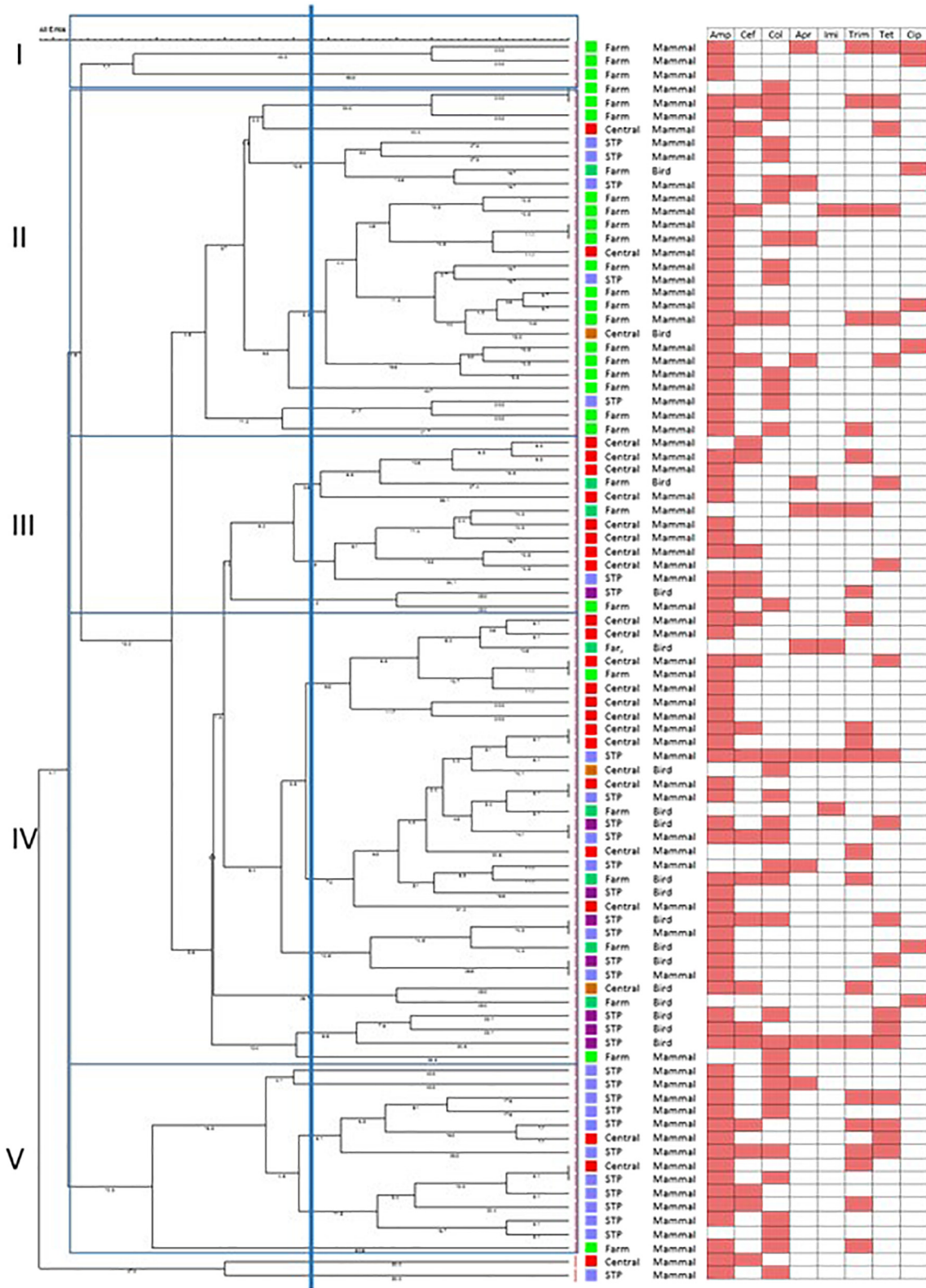

Fig. 2. ERIC profile of *E. coli* isolated from both small mammals and birds at Farm site (light green, mammals; dark green birds) Central site (red, mammals; dark red, birds) and STP site (light purple, mammals; dark purple, birds). Horizontal lines demonstrate significant clusters (I–V) based on 50% 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.
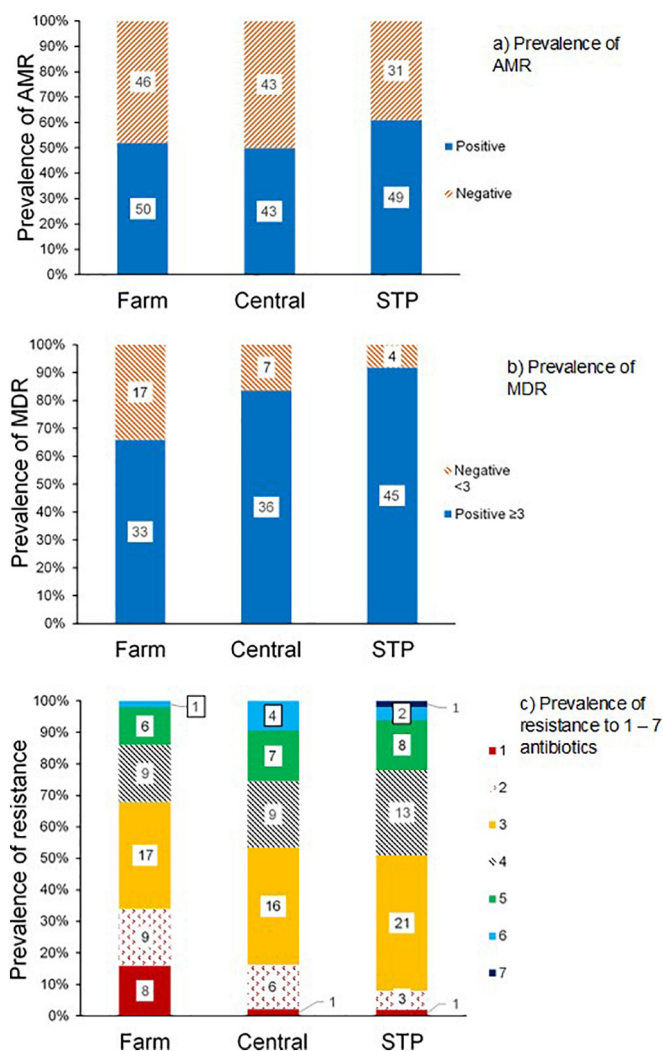
Fig. 3. Site-specific patterns of resistance in *E. coli* isolates: a) AMR: The percentage of faecal samples which contained *E. coli* susceptible to ≥1 antimicrobial (negative = orange hatched bars) or resistant to one or more antimicrobial drugs (positive = solid blue bars); b) MDR - the percentage 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.

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 (Fig. 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

Table 2

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

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

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 ($p = 0.056$).

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%)

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. However, we did not find that our, albeit relatively crude, indices of foraging ecology explained any of the variance in AMR or MDR, but this remains an area requiring further research.

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 chromosomal 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

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

found in in our study is underway. Seven out of the nine ciprofloxacin resistant isolates contained four nonsynonymous mutations in the *gyrase A* gene (Fig. 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.

In terms of other clinical important resistances, cefpodoxime resistance is a common indicator of ESBL production (Oliver et al., 2002), also of major concern in human medicine. From the 53 cefpodoxime resistant *E. coli* isolated from wildlife, six were ESBL producers, 22 were AmpC and six were positive for both ESBL and AmpC production (Table 3). ESBLs have previously been detected in *E. coli* isolates from a range of wildlife taxa, for example, 32% of *E. coli* isolates obtained from gulls' faeces (Simões et al., 2010), and such findings have been ascribed to contact with human waste. In our study, significantly more ESBL and/or AmpC – producing *E. coli* were found in wildlife samples collected from the STP Site, which suggests that human waste may be a factor driving ESBL/AmpC resistance in the environment.

4.3. Conclusions

Taken together, the results of this study support those of previous studies in that they confirm that wildlife commonly harbour ARB. Whether or not wildlife might be a source for onward transmission to domestic animals or to humans has not been directly examined. Our study was more concerned with beginning to investigate the drivers of AMR in wildlife, and in particular the role that anthropogenic waste, whether of directly human or domestic animal origin, might play in developing and maintaining that resistance. Diverse patterns of resistance were found in *E. coli* from wildlife in this study, suggesting variation within and between host taxa, between individuals, and over time. Overall, study site was not associated clearly with AMR, MDR or resistance patterns. However, resistance to antibiotics used only in human medicine was more prevalent at the STP site than the Farm and Central sites. Thus, the drivers of AMR in wildlife appear to be more complex than simple anthropogenic causes. Consequently, care needs to be taken if wildlife are to be used as sentinels of environmental AMR or pollution.

Conflict of interest

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.08.180.

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