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Genomic analysis of antimicrobial resistant *Escherichia coli* isolated from manure and manured agricultural grasslands

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Antimicrobial resistance (AMR) is a multifactorial issue involving an intertwining relationship between animals, humans and the environment. The environment can harbour *Escherichia coli* that are pathogenic or commensal. *Escherichia coli* is used as an indicator of environmental faecal contamination. Through culture dependent approaches this study identified 46 *E. coli* isolates in porcine and bovine manure, non-manured and manured soil, and manured grass. The grass isolation highlights grass as an environmental reservoir for *E. coli*. We also identified a diverse plasmidome with 23 different plasmid replicon types. The *E. coli* isolates were phenotypically antimicrobial resistant, predominantly multidrug resistant. Whole genome sequencing identified 31 antimicrobial resistance genes, and mutations in the *gyrA*, *parC*, and *parE* genes, conferring fluoroquinolone resistance. This study demonstrates grass as an understudied environmental niche of AMR *E. coli*, which directly links the environment to the grass grazing animal and vice-versa via the circular economy of manure application.

The One Health concept is critically important to understand the dissemination of antimicrobial resistance (AMR). One Health involves understanding the link between humans, animals and the environment and, in the case of AMR, is particularly relevant due to the ubiquitous nature of antimicrobial resistant bacteria (ARB)[1]. Manure is utilised to enable the circular economy of resources on farms to transfers additional nutrients to grass and soil[2,3]. Furthermore, as these environments yield markedly different conditions to the intestinal tract, evolutionary adaptation can be reasonably expected in isolates observed in these environments[4]. The role of the environment in the spread of AMR and on the occurrence of AMR in environmental bacteria is of interest, particularly in environments associated with human use, such as agricultural land[5–11]. In recent years, the impact manure application has on the occurrence of AMR of agricultural land has received attention due to the AMR selection pressure caused by manure application, and the associated introduction of antimicrobial resistance genes (ARGs), mobile genetic elements (MGEs) and antimicrobial residues into the soil[12]. Additionally, bacteria that are clinically important nosocomial pathogens can also be found in the environment, such as the WHO Bacterial Priority Pathogens List (BPPL): carbapenem or third generation cephalosporin resistant *Klebsiella pneumoniae* and *Escherichia coli*, and carbapenem resistant *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. In terms of AMR occurring in these pathogens, carbapenem resistant strains of these species and extended spectrum β-lactamase (ESBL) producing *Enterobacterales* are of critical priority. The plasticity of the *Escherichia coli* genome has been well documented[2,13] displaying high amenability to recombination and mobile genetic element uptake[14–16]. A large degree of this richness can be attributed to the enormous plasmid diversity observed in *E. coli*[17–20].

In this study, *E. coli* were isolated and compared from bovine and porcine manure, and soil and grass receiving these manures, demonstrating differential gene profiles on both the chromosome and plasmid sequences. We aimed to genomically characterise antimicrobial resistant *E. coli* in agricultural grassland and to shed light on their potential pathogenicity.

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Table 1 | Cluster and sequence types of all isolates

E. coli isolate	Serogroup	Sequence type	Clonal cluster	Chromosome group	Updated plasmid group	Set	Timepoint
1	O8:H25	58	ST155 complex	CG1	PG1B	Porcine	pre application
2	O8:H26	156	ST156 complex	CG3	PG3	Porcine	pre application
3	none:H26	156	ST156 complex	CG3	PG3	Porcine	pre application
4	None:H26	156	ST156 complex	CG3	PG3	Porcine	pre application
5	Onovel32:H10	617	ST10 complex	CG5	PSG3	Porcine	pre application
6	O8:H30	1431	NaN	CG2	PG2	Bovine	pre application
7	O8:H30	1431	NaN	CG2	PG2	Bovine	pre application
8	O8:H30	1431	NaN	CG2	PG2	Bovine	pre application
9	O8:H30	1431	NaN	CG2	PG2	Bovine	pre application
10	O17:H18	106	ST69 complex	CSG1	PG1C	Bovine	pre application
11	O17:H25	1126	ST155 complex	CG1	PG1C	Bovine	pre application
12	O8:H25	1126	ST155 complex	CG1	PG1C	Bovine	pre application
13	O8:H25	58	ST155 complex	CG1	PG1B	Bovine	pre application
14	O8:H30	1431	NaN	CG2	PG2	Bovine	pre application
15	O8:H30	1431	NaN	CG2	PSG1	Bovine	pre application
16	O8:H30	1431	NaN	CG2	PG2	Bovine	pre application
17	O8:H30	1431	NaN	CG2	PG2	Bovine	pre application
18	O8:H30	1431	NaN	CG2	PG2	Bovine	pre application
19	O8:H25	1126	ST155 complex	CG1	PG1C	Bovine	pre application
20	O8:H25	58	ST155 complex	CG1	PG1B	Soil	Week 1
21	O8:H25	58	ST155 complex	CG1	PG1B	Soil	Week 1
22	O8:H25	58	ST155 complex	CG1	PG1B	Soil	Week 1
23	O8:H25	58	ST155 complex	CG1	PG1B	Soil	Week 1
24	O8:H25	58	ST155 complex	CG1	PG1B	Soil	Week 1
25	O8:H25	58	ST155 complex	CG1	PG1B	Soil	Week 1
26	O88:H8	446	ST446 complex	CG4	PG4	Soil	Week 10
27	O88:H8	446	ST446 complex	CG4	PG4	Soil	Week 10
28	O88:H8	446	ST446 complex	CG4	PG4	Soil	Week 10
29	O88:H8	446	ST446 complex	CG4	PG4	Soil	Week 10
30	O163:H6	189	ST165 complex	CG6	PG6	Grass	Week 1
31	O100:H32	10	ST10 complex	CG7	PG7	Grass	Week 1
32	O100:H32	10	ST10 complex	CG7	PG7	Grass	Week 1
33	O163:H6	189	ST165 complex	CG6	PG6	Grass	Week 1
34	O23:H16	453	ST86 complex	CSG2	PSG4	Grass	Week 3
35	O8:H25	58	ST155 complex	CG1	PG1A	Grass	Week 5
36	O8:H25	58	ST155 complex	CG1	PG1A	Grass	Week 5
37	O8:H25	58	ST155 complex	CG1	PG1A	Grass	Week 5
38	O8:H25	58	ST155 complex	CG1	PG1A	Grass	Week 5
39	O8:H25	58	ST155 complex	CG1	PG1A	Grass	Week 5
40	O8:H25	58	ST155 complex	CG1	PG1A	Grass	Week 5
41	O8:H25	58	ST155 complex	CG1	PG1A	Grass	Week 5
42	O8:H25	58	ST155 complex	CG1	PG1A	Grass	Week 5
43	O8:H25	58	ST155 complex	CG1	PG1A	Grass	Week 5
39286	O187:H52	1248	NaN	CSG3	PSG5	Soil	Week 3
39287	O187:H10	617	ST10 complex	CG5	PSG2	Porcine	pre application
39288	Onovel32:H26	156	ST156 complex	CG3	PG3	Porcine	pre application

NaN = no ST complex assigned.

Results

Escherichia coli were isolated only from manure treated grass ($n = 14$) i.e. no grass without manure contained *E. coli*. The *E. coli* were isolated from bovine and porcine manure ($n = 21$), but not poultry manure (Table 1). They were also isolated from soil across timepoints T1, T3 and T7, to which no manure was added ($n = 9$) or porcine manure was added ($n = 2$).

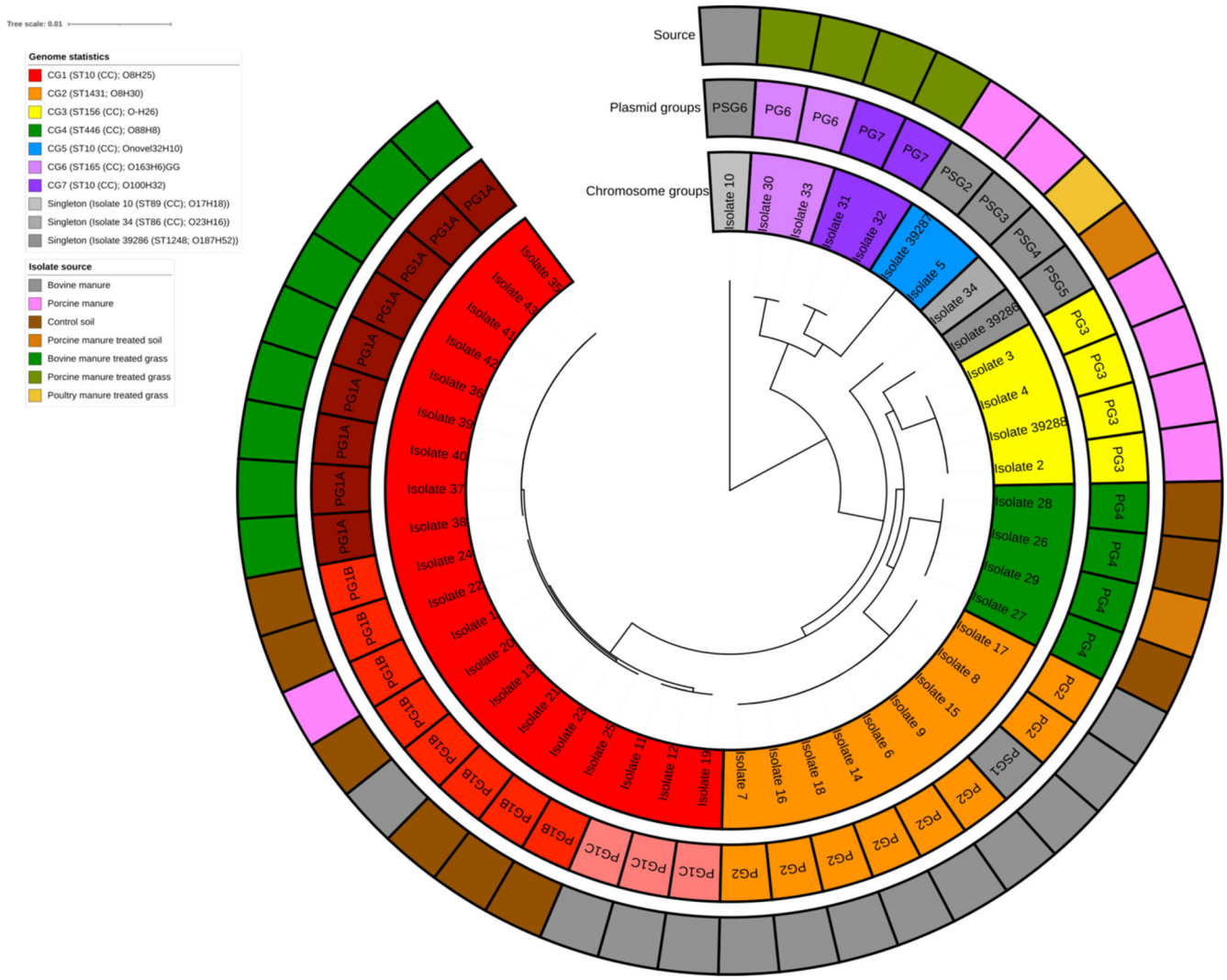

Fig. 1 | Chromosome and plasmid phylogeny of manure and manured agricultural grasslands *Escherichia coli*. The phylogeny tree of all *E. coli* is used to describe the levels of relatedness across source, plasmid and chromosome. The outer ring describes the source from which the *E. coli* were isolated. The same source is denoted by the same colour. The middle ring classifies the plasmids into related groups. The same plasmid group is denoted by the same colour and the same name. The inner ring contains the isolate name and the chromosome group. The phylogenetic tree shows the relatedness of each isolate based on the total genome. The flat lines denotes that the isolates are highly related.

Determination of clonal genomes

Isolates within the same chromosomal groups (CG) were clonal (Table 1, Fig. 1). Except for CG1 and CG4, all other isolates in the same cluster were sampled from the same source in this study; this may be due the relatively small subset sizes ($n = 1$ to 4), with the exception of CG2 ($n = 9$). Chromosomal group 4 (CG4) isolates were sampled from T7 and comprised control soil i.e. no manure added and soil with porcine manure added. Chromosomal group 1 (CG1) isolates were isolated from bovine manure, bovine manure treated grass, porcine manure, and control soil. The CG1 isolates were isolated in both bovine and porcine manure pre-field application and the control soil in T0. In addition, at T5 they were isolated from grass with bovine manure applied. Although porcine manure contained CG1 isolates no E. coli of CG1 were detected in the receiving grass or soil. There were also no isolates from the control grass. The sequence type of isolates from each grouping was ST58 and the plasmid group comprised two groups, suggesting that these are very highly related isolates. This suggests that the isolates on the grass originated from either the soil or the bovine manure and required five weeks to detectable by culture from grass but were not maintained on grass in the subsequent weeks.

When plasmid derived sequences from this study were compared, eight distinct plasmid groups (PG) were observed (Table 1; Fig. 1). Plasmid groups largely followed chromosomal groups except for CG1. For CG1, isolates were distributed between PG 1 A, 1B or 1 C with all CG1 bovine manure treated grass assigned to in PG1A ($n = 9$), all control soil isolates, the single porcine manure isolate, and one bovine manure isolate assigned to PG1B ($n = 8$), and the remaining bovine manure treated isolates were assigned to PG1C ($n = 3$). For CG2 (all bovine manure isolates), eight of nine isolates were assigned to PG2 ($n = 8$) and one isolate presented a unique plasmid set (PSG1). All CG3 isolates (all porcine manure isolates) were assigned to PG3 ($n = 4$). All CG4 isolates (three control soil isolates and one porcine treated soil isolate) were assigned to PG4. Both CG5 isolates (porcine manure isolates) displayed unique plasmid sets (PSG2 and PSG3). CG6 and CG7 isolates (porcine manure treated grass isolates) were assigned to PG7 and PG8, respectively. The individual isolates in CGs 6, 24 and 378 contained unique plasmids assigned to PSG6, PSG4 and PSG5, respectively.

Multilocus sequence typing of isolates sequenced for this study

A total of 11 sequence types (STs) were observed across isolates sequenced for this study (Table 1, SI Table Genomics). These STs could be further categorised to seven clonal complexes (CC). Neither ST1248 nor 1431 could be assigned to a CC. All CG1 isolates belonged to CC-ST155, with all isolates

possessing PG1A and PG1B belonging to ST58 and PG1C belonging to ST1126. All other CGs returned a single ST per group.

O-antigen H-fimbriae typing

A total of eight O-antigens (O8, O17, O23, $O_{novel}32$, O88, O100, O163, and O187; two isolates (isolates 3 and 4; CG3) did not possess an O-antigen) and ten H-fimbriae (H6, H8, H10, H16, H18, H25, H26, H30, H32, and H52) were observed across the 46 sequenced isolates, culminating to 14 serotypes (Table 1, SI Table Genomics). Except for isolate 11, all CG1 isolates presented the O8:H25 serotype, isolate 11, by contrast, presented the O17:H25 serotype. All CG2 isolates were of serotype O8:H30. All CG3 isolates possessed H26 but three different O-antigen combinations were present resulting in three serotypes (H26, $O_{novel}32$:H26, and O8:H26. All CG4 isolates were of serotype O88:H8. Both CG5 isolates had H10 fimbriae but different O-antigens. Both CG6 isolates contained the O163:H6 serotype and both CG7 isolates presented the O100:H32 serotype. The serotypes for CSG1, CSG2, and CSG3 were each unique.

Plasmid typing of isolates sequenced for this study

When plasmid families were considered, all plasmid sets within each PG (except PG1A, PG1B, and PG7) displayed the same plasmid replicon types (SI Table PlasmidFinder). All PG1A contained Col440II and IncFIB, however only five isolates contained Col156. All PG1B isolates possessed Col8282 and IncFIB, and all but one (isolate 24) possessed ColpVC, six of eight isolates possessed Col(BS512) and four isolates possessed Col156. One isolate contained the IncFIB replicon and the other within the PG7 group did not. Both contained the IncN and Col440II replicons. All PG1C isolates possessed Col440II or ColRNAI and IncFIB. All PG2 isolates possessed IncY only. All PG3 isolates possessed IncFII and p0111. All PG4 isolates possessed IncFIB only. Both PG6 isolates contained IncX2 and IncX5. The PSG1 and PGS2 contained no replicon gene. The remaining PSG plasmids were all IncFIB and PSG5 also contained Col440II_1.

Antimicrobial, metal and biocide resistance and virulence genes

A range of antimicrobials were utilised in the detection of AMR, including those of relevance to human medicine e.g. imipenem and those most frequently administered to livestock e.g. tetracycline. The antimicrobial resistance profiles of the *E. coli* ($n = 46$) comprised, 35 tetracycline resistant, nine were cefotaxime resistant, 24 were kanamycin resistant, and 16 ciprofloxacin resistant. There were no amikacin or imipenem resistance. One isolate displayed intermediate resistance to imipenem. Multi-drug resistance, resistance to three of more different antimicrobial classes, was present in 31 isolates, with 18 conferring resistance to two antimicrobial classes and 13 resistant to three antimicrobial classes. *Escherichia coli* isolates displaying resistance to two classes were resistant to tetracycline and kanamycin or tetracycline and ciprofloxacin. Two MDR phenotypes were detected (1) resistance to tetracycline, kanamycin and ciprofloxacin or (2) resistance to tetracycline, cefotaxime and ciprofloxacin (Table 2). The cefotaxime resistant *E. coli* ($n = 9$) were isolated from bovine manure and tested negative for AmpC β-lactamase production and positive for extended spectrum beta-lactamase (ESBL) production.

Antimicrobial resistance genes were detected on the chromosomes and plasmids (SI Table). The mobile resistance genes detected conferred resistance to chloramphenicol, tetracyclines, aminoglycosides, quinolones and cephalosporins. The *qnrS* and $bla_{CTX\text{-}M\text{-}15}$ AMR genes were the most clinically relevant. The $bla_{CTX\text{-}M\text{-}15}$ positive isolates were from bovine manure samples but were not detected on any manured soil or grass. They all also contained the *qnrS* gene. Four additional isolates containing the *qnrS* genes lacked $bla_{CTX\text{-}M\text{-}15}$ but contained the extended spectrum beta-lactamase gene $bla_{TEM\text{-}150}$ and were isolated only from pig manure treated grass.

Tetracycline resistance was widespread in this study with 35 isolates phenotypically resistant, predominantly conferred by the *tetA* or *tetB* gene. The lack of these genes in other tetracycline resistant strains however suggests the possibility of an unscreened mutation or other mechanism.

The ciprofloxacin resistance was conferred by chromosomal mutations in all ciprofloxacin resistant isolates except isolate 39286, which contained no chromosomal mutation in any target gene. All ciprofloxacin resistant isolates had point mutations in *gyrA* and *parC*. From a plasmid perspective, all isolates containing the *qnrS1* were bovine manure isolates, which were co-selected with an ESBL ($bla_{CTX\text{-}M\text{-}15}$ or $bla_{TEM\text{-}150}$) and tetracycline resistance gene. The $bla_{CTX\text{-}M\text{-}15}$ positive isolates were only detected in bovine manure.

Resistance to kanamycin but not to amikacin was observed. Kanamycin was administered to bovines in this study when required to treat infection and were not administered as a prophylactic[2]. Aminoglycosides are recommended for use with caution in Irish veterinary practice and therefore are not the first line of treatment. As isolates from control soil and manure treated grass (within CG1) were also aminoglycoside resistant, the reservoir may be from the environment. The three CG1 plasmid groups all displayed a similar aminoglycoside resistance genotype (*APH(6)-Id, APH(3")-Ib*, and *APH(3')-Ia*), with PG1C also possessing *AAC(3)-IV*, suggesting these genes to be aetiological of resistance. Interestingly, both *APH(6)-Id* and *APH(3")-Ib* were observed in PG2 which were also isolated from bovine manure treated grass but selected on cefotaxime or ciprofloxacin media. The control soil isolates also contained *APH(3')-Ia*, which confers resistance to kanamycin but not amikacin. These results suggest that *APH(3')-Ia* played the most active role in resistance and as the CG1 bovine manure treated grass isolates were extracted five weeks after initial spread, PG1A may have acquired this gene from PG1B isolates. As isolate 10 (bovine manure) also possessed *APH(3')-Ia, APH(6)-Id* and *APH(3")-Ib* but a different plasmid group (PSG6) may further highlight this acquisition theory. The porcine manure isolates with kanamycin resistance all possessed *AAC(6')-Ib7*, suggesting this to be the genetic mechanism of resistance in these isolates.

A total of 65 biocide resistance, 83 metal resistance genes, and 23 biocide and metal resistance genes were observed across the chromosomes of isolates sequenced for this study. Biocide resistance genes comprised a wide range of genes conferring resistance to clinically significant detergents e.g. quaternary ammonium compounds (QACs) and acridines. Co-selection of AMR through the use of metal compounds in animal feed may occur by the presence of metal resistance genes on plasmids conferring AMR. Zinc, copper and a range of metal resistance genes were co-detected on plasmids with AMR genes.

Between 74 and 111 virulence factor genes were observed in each chromosome (SI Table VFDB Chromosome). Of these, 48 were ubiquitously distributed which, in broad terms (as assigned by VFDB), confer the following predicted capabilities: adherence, biofilm, effector delivery system, immune modulation, motility, and nutritional or metabolic factor (enterobactin siderophores). Of the most clinically relevant virulence factors all isolates except CG5 and CG7 possessed *fimAFGI*, and the *fimCDH* were present in one copy for all isolates and two copies in most isolates; the *papBCDEFHIJK* gene cluster was observed in all CG1 isolates with a partial cluster (*papCD*) also observed in isolate 34 (CSG2). Regarding biofilm forming virulence factors, only one was observed (*algA*), which was contained in all CG1 bovine manure treated grass isolates (PG1A). Only the *icsP/sopA* exoenzymes were detected in all CG1, CG4, isolates 39287 (CG5), 10, and 39286. Aerobactin synthesis genes (*iutA* and *iucABCD*) were observed in isolate 10, all PG1B, and CG5. Salmochelin synthesis genes (*iroCDEN*) were only observed in isolate 39287 (CG5). Finally, the *chu* and *shu* heme utilisation clusters (*chuSUWY* and *shuATVX*) were observed in isolate 10 only. Regarding stress survival, only one gene (*clpP*) was observed in isolate 34, isolate 39286, and in CG2-CG7. Plasmids in 28 isolates contained a virulence factor (SI Table VFDB: Plasmid). The exoenzyme *icsP/sopA* was observed in all PG1A, PG1B, PG4, and PSG3-6 isolates. For siderophores, the salmochelin synthesis gene cluster *iroBCDEN* was observed in all PG1A, PG1B, PSG2, and PSG4 isolates. The aerobactin gene *iutA* and gene cluster *iucABCD* were observed in all PG1A isolates.

Chromosomal point mutations associated with antimicrobial resistance

Chromosomal point mutations conferring resistance to nalidixic acid and ciprofloxacin were observed in 16, were phenotypically

Table 2 | Antimicrobial resistance phenotypes of all isolates

Isolates	Tetracycline	Cefotaxime	Kanamycin	Amikacin	Ciprofloxacin	Imipenem
1	R	S	R	S	S	S
2	R	S	R	S	R	S
3	R	S	R	S	R	S
4	R	S	R	S	R	S
5	R	S	S	S	R	S
6	R	R	S	S	R	S
7	R	R	S	S	R	S
8	R	R	S	S	R	S
9	R	R	S	S	R	S
10	S	S	R	S	S	S
11	S	S	R	S	S	S
12	S	S	R	S	S	S
13	R	S	R	S	S	S
14	R	R	S	S	R	S
15	R	R	S	S	R	S
16	R	R	S	S	R	S
17	R	R	S	S	R	S
18	R	R	S	S	R	S
19	S	S	R	S	S	S
20	R	S	R	S	S	S
21	R	S	R	S	S	S
22	R	S	R	S	S	S
23	R	S	R	S	S	S
24	R	S	R	S	S	S
25	R	S	R	S	S	S
26	S	S	S	S	S	S
27	S	S	S	S	S	S
28	S	S	S	S	S	S
29	S	S	S	S	S	S
30	R	S	S	S	S	S
31	R	S	S	S	S	S
32	R	S	S	S	S	S
33	R	S	S	S	S	S
34	R	S	S	S	R	S
35	R	S	R	S	S	S
36	S	S	S	S	S	I
37	R	S	R	S	S	S
38	R	S	R	S	S	S
39	R	S	R	S	S	S
40	R	S	R	S	S	S
41	R	S	R	S	S	S
42	R	S	R	S	S	S
43	R	S	R	S	S	S
39286	S	S	S	S	S	S
39287	R	S	S	S	R	S
39288	I	S	R	S	R	S

S susceptible, *I* intermediate, *R* resistant.

ciprofloxacin resistant. In addition, isolate 10, which contained only one point mutation in the *parC* was susceptible (SI Table Pointfinder, Table 2). Mutations in the *gyrA* gene corresponding to amino acid 87 was observed in 16 isolates with all CG4 isolates with D87H and CG2, CG5, and isolate 34 with D87N. An additional *gyrA* mutation corresponding to the amino acid 83 (S83L) was observed in isolate 34 and all CG2, CG3, and CG5. A mutation conferring an amino acid change S80I in *parC* was observed in isolate 34, and all CG2 and CG5 isolates.

Finally, a mutation conferring an amino acid change S57T in *parC* was detected in isolate 10.

Discussion

We aimed to shed light on antimicrobial resistance and potential pathogenicity in *E. coli* in agricultural manure and the receiving manured grassland over time. Transfer of *E. coli* via the fecal oral route is a major cause of diarrhoea. If AMR *E. coli* can survive on grassland they can transfer to animals and proliferate either within the animal or transfer into water sources, which may be used for recreation or drinking purposes. Isolates in this study were mainly of the O8:H25 or O8:H30 serogroups. While other serogroups and sequence types (including ST10) were observed, the prevalence and persistence of this serogroup was of interest from a One Health perspective, especially due to the relative stability of chromosomal gene content and differential plasmid gene content (CG1 and CG2). The presence of the O8 in CG1, CG2, and CG4 with a lack of an O antigen in CG3 and different O antigen in 39286 may suggest that O8 acquisition is ancestral to the divergence of CG1-4 from CG5-7. The chromosomal proximity of CGs may offer an insight as to why certain chromosome groups (*e.g.*, CG5 and CG7) repeatedly displayed the same genotypes as these were sister taxa. It is important to note that any database of genomic data is biased towards clinical *E. coli* and isolates rather than environmental *E. coli*. Thus, *E. coli* are analysed in a clinical view even when isolated from non-clinical biomes.

The tetracycline resistance results suggest that a bias towards tetracycline resistance exists in the agriculture as tetracycline was not used as a selective agent in the isolation of the bacteria. This could reflect previously observed per-weight antimicrobial bias in Irish veterinary practice, with tetracyclines comprising 55.8%[21].

While zinc was incorporated into pig feed, no zinc resistance genes were detected in any isolate. Zinc may co-select for AMR genes where the metal resistance gene is located on the same mobile element as the AMR genes. Thus, where zinc is used as an alternative to antimicrobials in the hope of reducing AMR it may exacerbate the AMR problem. From a virulence genotype perspective, the presence of *icsP/sopA* across the phylogeny but particularly in CG1 (PG1A and PG1B) was of interest. This gene is associated with actin mediated intracellular locomotion in *Shigella* spp., which may suggest that these isolates may be enteroinvasive[22,23]. Further to this, the diverse siderophore production genes in CG1 compared to other isolates suggests that it may be adaptive to low iron environments (*e.g.*, grass, most soils, or blood)[24]. From an evolutionary perspective, the chromosomal acquisition of *algA* (a *Pseudomonas* associated alginate-rich biofilm forming gene) on the chromosomes of all CG1 isolates from bovine manure treated grass was particularly interesting. To our knowledge, this is the first report of *algA* in *E. coli*, with *E. coli* most commonly forming biofilms using β-1,6-N-acetyl-D-glucosamine polymers, colanic acid, or cellulose[25]. As the human lung is a hostile environment subject to free radical DNA damage like what may be induced by UV, this gene may offer a survival strategy to cells on sun facing leaves[26,27]. Furthermore, *E. coli* presenting O8 and O17 are commonly associated with biofilm formation in clinical samples, suggesting a potential role in adaptability to diverse environments[28,29]. Finally, the presence of *astA* (a heat stable enterotoxin) within isolate 34 three weeks after application suggests that toxicogenic strains may survive in exposed environments but as this was only observed in one isolate, more work is required to determine the validity of this observation.

There are relatively few genomic studies of *E. coli* isolated from soil and none from grass. A study of on *E. coli* from Brazilian soil demonstrated the presence of plasmid mediated AMR. The AMR profile of the isolate was similar to several in this study i.e. tetracycline, aminoglycoside, beta-lactams, phenicols, trimethoprim and sulphonamide resistance conferred by plasmid mediated genes[30]. However, the genes differed to those in this study. In addition, similar chromosomal mutations conferring fluoroquinolone resistance were identified. This *E. coli* was ST189 of serotype O80:H26-fimH54 with IncF, IncN and IncP plasmids. Two ST189 isolates in this study were isolated from pig manured grass, but they were serotype O163H6 and an IncX plasmid. Thus, while similar the isolates in this study are not identical to those identified in the Brazilian soil. A Canadian study analysed the genomes of 96 AMR *E. coli* from racoons ($n = 20$), manure ($n = 31$) and soils ($n = 45$) on a pig farm[31]. Similar to our study the Canadian E. coli collection did not contain any serovars of the major shiga-toxin producing *E. coli* in human infections (O157, O26, O45, O103, O111, O121, O145) and the only sequence type associated with *E. coli* infections e.g. urinary tract infections was ST10. Two isolates in this study and 11 in the Canadian study were ST10. In our study they were isolated from pig manured grass and in the Canadian study while it was not specifically described it was at least associated with the pig farm as all samples were taken there. Thus, a potential link to pig farming and ST10 AMR *E. coli*. Our study demonstrates an additional potential transmission from pig manure to grass. These studies raise the question of whether these E. coli can cause infections in humans and how we define risk from environmental or farm located *E. coli* to the human population.

Due to the predominance towards biofilm forming virulence factors and the relatively large abundance of O-antigen types associated with increased biofilm formation, manure spreading may facilitate introducing persistent and potentially pathogenic bacteria to grassland environments. Depending on how long the antimicrobial resistance genes survive, they may be reintroduced to grazing animals at a later stage, potentially weakening the effect of prescribed antimicrobials, and posing a One Health threat. Greater analysis of persistence of these bacteria and research into their transmission from primary elements of the food chain are required to understand their risk to animal and human health.

Methods

Bacterial isolation from manure, grass, and soil samples

The details of the field trial sampling and sample processing were previously described in detail and do not diverge from this method in this paper[2]. A summary of the sampling is provided for an overview: A grassland field trial was conducted on a research field site in the Southeast of Ireland. Bacterial isolation samples from the following seven timepoints were used: Background (BM) prior to manure application, one week following manure spreading (T1), three weeks following manure spreading (T3), five weeks following manure spreading (T5), ten weeks following manure spreading (T7), 14 weeks following manure spreading (T8) and 18 weeks following manure spreading (T9). The field trial resulted in the collection of 176 grass and 176 soil samples. In addition, four biological replicates of each manure type (bovine, pig and poultry) were sampled prior to application. The *E. coli* were derived from nine samples over the course of the field trial: pig manure, bovine manure, control (untreated) soil (T0/NM, T7), pig manure treated soil (T3, T7), pig manure treated grass (T1), poultry manure treated grass (T3), and bovine manure treated grass (T5). A total of 46 *E. coli* were isolated. Grass samples were prepared for isolation by sonicating 100 g of material in 250 ml of PBS for 5 min using a modified method[2]. Following filtering the liquid through a sterile sieve, 10 ml of the sonication liquid was filtered aseptically using a 0.2 µm nitrocellulose membrane (Sartorius, Merck). This membrane was then placed into a 50 ml falcon tube containing 20 ml of nutrient broth (Oxoid) and incubated at 37 °C in a shaker at 225 rpm (New Brunswick Scientific C25) for 24 h for bacterial isolation. One gram of manure or soil were added to 20mls of nutrient broth (Oxoid) and incubated for 24 h at 37 °C at 225 rpm (New Brunswick Scientific C25). Following the 24-hour enrichment step, the soil and manure samples were left to stand for 5 min to allow solid particles to settle. The enriched soil, manure and grass samples underwent tenfold serial dilutions (dilution factor = 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) in sterile PBS. For each enriched manure, grass and soil sample 100 µl was used to inoculate Eosin Methylene Blue (EMB) Agar (Oxoid) for the isolation of *E. coli* supplemented with each respective antimicrobial at breakpoint concentrations[32]. The antimicrobials used were

kanamycin (4 mg/L), cefotaxime (4 mg/L), colistin (4 mg/L) and ciprofloxacin (1 mg/L). Plates were incubated at 37 °C for 24 to 48 h. Following incubation, presumptive colonies were sub-cultured and purified on low-salt Luria-Bertani (LB) agar (Duchefa) and incubated overnight at 37 °C. A maximum of six colonies were picked per agar plate. Glycerol stocks of isolates were prepared and were stored at −80 °C.

Identification of isolates using MALDI-TOF

A bacterial colony of pure cultures was transferred by direct smearing in duplicate onto spots of the MALDI-TOF mass spectrometry (MS) target (MTP ground steel, Bruker Daltonics) with a tooth-pick. To the dried spots, 1 µL matrix solution (10 mg α-cyano-4-hydroxycinnamic acid, Bruker Daltonics) dissolved in 1 mL acetonitrile-water-trifluoroacetic acid (50:47.5:2.5, (vol/vol/vol), Sigma-Aldrich) was added, and this solution was air-dried. Sample spectra were acquired using a microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics) and the flexControl software *v*.3.4 (Bruker Daltonics). Spectra were classified using the Bruker Taxonomy main spectra database (MBT Compass *v*.4.1. with 8468 spectra present, Bruker Daltonics). Bacterial were identified to the species level if the score value was above 2.00 or to the genus level if the score was between 1.70 and 1.99.

Antimicrobial susceptibility testing

The *E. coli* ($n = 46$) underwent antimicrobial susceptibility testing using the EUCAST or CLSI disk diffusion method for susceptibility to cefotaxime (5 µg), imipenem (10 µg), amikacin (30 µg), kanamycin (30 µg), tetracycline (30 µg) and ciprofloxacin (5 µg) (Oxoid). As there are no EUCAST breakpoints for kanamycin and tetracycline CLSI (2020) guidelines and breakpoints were used[32,33]. The cefotaxime resistant isolates were investigated for the production of AmpC lactamases and extended spectrum beta-lactamases using EUCAST guidelines[32].

DNA extraction and whole genome sequencing

Bacterial total DNA was extracted from *E. coli* ($n = 46$) using the NucleoSpin Microbial DNA Mini kit for DNA from microorganisms (Machery Nagel) according to manufacturer's instructions. Three *E. coli* samples (39286, 39287, 39288) were sequenced using short read Illumina sequencing by MicrobesNG and the remainder were sequenced by Novogene.

Dataset construction

Each genome was subjected to MLST using mlst *v*.2.19.0 (https://github.com/tseemann/mlst). Each genome was separated into chromosomal and plasmid components using PlasClass *v*.0.1.1 with default settings[34]. Each chromosomal genome was annotated using Prokka *v*.1.14.6 with default settings and assessed for completeness using CheckM *v*.1.2.2 under default settings and using the *E. coli* marker gene set[35]. Genomes were confirmed to have a completeness score ≤97.5%. The N50 score for each genome was calculated using Quast *v*.5.2[25]. Chromosomal genomes were used to construct a pairwise similarity network using Mash *v*.2.2.2 with a distance cut-off ($D \leq 0.005$) and clustered using MCL *v*.14-137 using default settings[36,37]. Chromosomal sequences from isolates sequenced for this study were also used in this clustering step.

Plasmid partitioning

Each genome sequenced for this study was partitioned into chromosomal and plasmid sequences using platon *v*.1.5.0 using database *v*.1.5 with default settings[38].

Genome clustering

A phylogeny was constructed using FastTree *v*.2.1 using the "-nt" (nucleotide specific) flag and the GTR + I + G model[39]. This phylogeny was rooted at the branch representing the divergence of *Citrobacter fruendii* from *E. coli*. The earliest branching *E. coli* lineage according to this phylogeny was selected as the root of the 931-genome phylogeny. This rooting strategy was confirmed using FastRoot (MinVar) *v*.1.5 under default settings[40]. Finally, to allow greater exploration into isolates sequenced for this analysis, a pangenome graph was constructed for these 46 genomes using Roary and each gene family was aligned and trimmed using Prank and TrimAl as above; ubiquitous, single copy gene alignments ($n = 3,386$) were extracted and concatenated into a super-alignment (length = 3,251,889 bp). Again, this superalignment was used to construct a consensus phylogeny with 10,000 bootstrap replicates using IQTree2 as above, where the GTR + I + R4 model was selected as the most appropriate model of nucleotide evolution. The root, as selected by FastRoot, was determined to be Isolate 10, in agreement with the other two phylogenies. The phylogeny was visualised using iToL as above. The genome phylogeny was partitioned into phylogenetic groups using FastBAPS *v.1.08* using the super alignment as support[41]. These results were used to annotate the previously constructed phylogeny.

Genome annotation

Each chromosomal genome was annotated for antimicrobial resistance using (https://github.com/tseemann/abricate) with the comprehensive antimicrobial resistance database for biocide and heavy metal resistance using ABRicate with a backtranslated version of BacMet *v*2.0, for virulence factors using the virulence factor database (VFDB), for O- and H- antigen types using the EcOH *v*.2 database[41]. The CARD v3.2.4, VFDB, and EcOH, databases are provided as standard with ABRicate and the backtranslated version of BacMet was obtained from a clinical microbiology study on vancomycin resistant *Enterococcus faecium*[42]. Resistance genotypes associated with point mutations were derived using PointFinder *v*.3.1.1. with the *E. coli* reference dataset provided with PointFinder[43]. Previously assigned MLSTs were used to assign genomes to clonal complexes with PubMLST[44]. Additionally, each chromosome and plasmid sequence from isolates sequenced for this study were annotated using Bakta *v*.1.5.1 using database *v*.1.5[38,45]. This secondary analysis was performed to restrict the ambiguity arising from hypothetical proteins as assigned by Prokka. Plasmid sequence typing was performed on isolates sequenced for this study using ABRicate with the PlasmidFinder database[45,46].

Data availability

The genome sequences have been deposited in Genbank. Bioproject number PRJNA1080214 and SRP491607 in the sequence read archive https://www.ncbi.nlm.nih.gov/sra/?term=SRP491607.

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Author contributions

C.T.: Investigation, Writing—Original Draft, Methodology, Data Curation; C.M.B.: Project Administration, Resources, Supervision; FPB: Supervision, Project Administration, Resources; D.M.: Investigation, Data Curation; D.D.: Data Curation, Resources, Supervision; R.J.L.: Data Curation, Writing—Original Draft, Formal Analysis, Methodology, Visualization; F.W.:

Conceptualization, Funding Acquisition, Project Administration, Validation, Writing—Review & Editing, Supervising.

Competing interests
The authors declare no competing interests.

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