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RESEARCH ARTICLE

Population Genomics of Antibiotic Resistance Spread in Agricultural Runoff Bacteria

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ABSTRACT

Agricultural runoff carrying manure-associated bacteria into surface waterways is widely suspected as a route by which antibiotic resistance genes move between livestock and environmental, and ultimately human-adjacent, bacterial populations, but the population-genomic structure of this transfer has rarely been characterised at a resolution sufficient to distinguish genuine cross-compartment gene flow from independent acquisition of the same resistance elements. We whole-genome sequenced 312 *Escherichia coli* isolates recovered over eighteen months from paired sampling of manure storage lagoons, adjacent field-edge runoff channels, and downstream stream water at four mixed dairy-and-arable farms, and combined core-genome phylogenetics with plasmid-resolved long-read assembly to reconstruct resistance gene movement across the manure-to-stream continuum. Core-genome multi-locus sequence typing identified extensive strain sharing between manure and adjacent runoff samples (78% of runoff sequence types also recovered from paired manure) but markedly less sharing between runoff and downstream stream isolates (31%), suggesting that most resistant strains originating in manure fail to establish persistently in the stream environment. Despite this attenuation in strain-level sharing, plasmid-resolved assembly identified an IncF-family multidrug-resistance plasmid, pAR117, carrying blaCTX-M-15, sul2 and tet(A) in near-identical form across genetically distinct *E. coli* backgrounds recovered from all three compartments, indicating that horizontal plasmid transfer, rather than strain persistence, is the dominant mechanism by which resistance genes cross from manure into the stream bacterial community. Resistance gene abundance in stream water was significantly associated with recent heavy rainfall events ($P=0.004$) but not with distance downstream of the runoff entry point within the 2 km sampled reach. These findings indicate that agricultural runoff functions primarily as a conduit for mobile genetic elements rather than for the resistant strains that originally carried them, with direct implications for how runoff-focused antimicrobial resistance mitigation efforts should be prioritised.

Keywords: antimicrobial resistance; agricultural runoff; population genomics; plasmid transfer; whole-genome sequencing; *Escherichia coli*

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1. INTRODUCTION

The contribution of agricultural antibiotic use to the broader antimicrobial resistance crisis has been debated for decades, but the specific question of whether, and

how, resistance genes move from livestock-associated bacterial populations into environmental and human-adjacent compartments via surface water runoff has proven difficult to resolve with the tools available to earlier surveillance studies, which typically relied on culture-based resistance phenotyping or single-gene PCR screening rather than genome-resolved population structure [1,3]. Manure application and storage lagoon overflow are established sources of antibiotic-resistant bacteria entering field-edge runoff, and resistance genes and resistant organisms have been repeatedly detected in downstream surface waters near livestock operations, but detection alone does not establish whether the resistant bacteria found downstream are genuinely descended from manure-associated populations or represent independent acquisition of similar resistance elements already circulating in the broader environmental bacterial community [4,6].

Whole-genome sequencing resolves this ambiguity in a way that culture-based or single-locus methods cannot: core-genome phylogenetic comparison can establish whether isolates recovered from different compartments share sufficiently recent common ancestry to represent genuine strain transfer, while plasmid-resolved assembly, increasingly accessible through long-read sequencing platforms, can separately track the movement of mobile genetic elements independent of their bacterial host, since a resistance plasmid can move between unrelated bacterial lineages by conjugation without any corresponding transfer of the chromosomal background [7,9]. These two processes, strain persistence and plasmid mobility, have quite different implications for how a mitigation strategy should be designed: intervention aimed at reducing the survival or transport of specific resistant strains would do little to curb resistance gene spread if the dominant mechanism is plasmid conjugation into an already-diverse environmental bacterial community, and the reverse is equally true [10,12].

Escherichia coli is a natural focal organism for this kind of study: it is abundant in livestock manure, readily recovered from both agricultural runoff and surface water by standard culture methods, carries a well-characterised repertoire of clinically relevant resistance plasmids including IncF-family plasmids associated with extended-spectrum beta-lactamase genes, and has a sufficiently well-resolved core-genome multi-locus sequence typing scheme to support fine-grained population structure analysis across large isolate collections [13,15]. Most published runoff studies using *E. coli*, however, have sampled either a single time point or a single compartment in isolation, limiting the ability to reconstruct the manure-to-stream transfer process as a temporally resolved, spatially structured system rather than a series of independent snapshots [16,18].

We therefore designed a paired, longitudinal sampling scheme spanning manure storage, field-edge runoff and

downstream stream water at four working dairy-and-arable farms, sequenced a substantial isolate collection from each compartment over eighteen months, and combined core-genome phylogenetics with plasmid-resolved long-read assembly to directly test two specific hypotheses: first, that resistant *E. coli* strains originating in manure persist and can be recovered at each successive downstream compartment; and second, that where strain-level persistence breaks down, resistance genes nonetheless continue to move across compartments via plasmid conjugation into genetically distinct recipient lineages already present in the downstream environment.

2. MATERIALS AND METHODS

Sampling was conducted at four mixed dairy-and-arable farms across a single river catchment, each contributing paired samples from a manure storage lagoon, an adjacent field-edge runoff channel receiving lagoon and field-applied manure drainage, and stream water 200 m downstream of the runoff entry point, collected monthly over an eighteen-month period.

2.1 Sample collection and bacterial isolation

At each sampling event, 500 mL water or slurry samples were collected from each of the three compartments per farm, transported on ice, and processed within six hours. Samples were serially diluted and plated onto eosin methylene blue agar supplemented with cefotaxime (2 mg/L) to selectively recover presumptive extended-spectrum beta-lactamase-producing *Escherichia coli*, with up to eight presumptive colonies per sample confirmed as *E. coli* by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry and retained for whole-genome sequencing. A parallel non-selective culture on plain eosin methylene blue agar was used to estimate total culturable *E. coli* density per sample.

2.2 Whole-genome sequencing and assembly

All 312 confirmed isolates were short-read sequenced on an Illumina NovaSeq platform to a minimum depth of 60x coverage, with de novo assembly performed using SPAdes and quality-filtered against standard contamination and completeness thresholds. A representative subset of 46 isolates, selected to span all recovered sequence types and all three sampling compartments, was additionally sequenced on an Oxford Nanopore long-read platform to enable hybrid assembly and full plasmid resolution using Unicycler, allowing complete, circularised plasmid sequences to be recovered rather than the fragmented plasmid contigs typical of short-read-only assembly.

2.3 Population structure and phylogenetic analysis

Core-genome multi-locus sequence typing was performed using the Enterobase *E. coli* cgMLST scheme, and a core-genome alignment of all 312 isolates was used to construct a maximum-likelihood phylogeny with

RAxML under a general time-reversible substitution model. Strain sharing between compartments was defined as recovery of isolates differing by fewer than ten core-genome cgMLST alleles, a threshold consistent with recent common ancestry rather than convergent acquisition of similar resistance elements by unrelated lineages, from paired samples collected at the same farm.

2.4 Resistance gene and plasmid analysis

Antimicrobial resistance genes were identified in all assemblies using ResFinder and the CARD database, and plasmid replicon typing was performed using PlasmidFinder. For the 46 hybrid-assembled isolates, complete plasmid sequences were compared by whole-plasmid alignment to identify plasmids present in near-identical form (greater than 99.5% sequence identity across at least 95% of plasmid length) across genetically distinct chromosomal backgrounds, which we interpreted as evidence of horizontal plasmid transfer between lineages rather than co-inheritance from a shared bacterial ancestor. Quantitative PCR targeting blaCTX-M-15, sul2 and tet(A) was performed directly on total environmental DNA extracted from all water and slurry samples to provide a culture-independent measure of resistance gene abundance.

2.5 Statistical analysis

Associations between resistance gene abundance (log-transformed qPCR copy number per mL) and rainfall in the 48 hours preceding sampling, and between resistance gene abundance and distance downstream of the runoff entry point, were tested using linear mixed-effects models with farm and sampling month as random effects. All analyses were performed in R version 4.3 with a significance threshold of $P < 0.05$.

3. RESULTS

The 312 sequenced isolates comprised 89 manure isolates, 118 runoff isolates and 105 stream isolates across the four farms, representing 47 distinct core-genome sequence types after cgMLST typing. Manure and runoff sequence type composition overlapped substantially: of the sequence types recovered from field-edge runoff samples, 78% (37 of 47 runoff-derived sequence types, weighted by farm) were also recovered from paired manure samples at the same farm within the same or an adjacent sampling month, consistent with runoff directly reflecting the manure-associated *E. coli* population at the point of entry. Table 1 summarises sequence-type sharing across compartment pairs at each farm.

Table 1. Core-genome sequence type sharing between compartment pairs by farm.

Farm	Manure-runoff STs shared	Runoff STs recovered	Runoff-stream STs shared	Stream STs recovered
Farm 1	9/11 (82%)	11	4/13 (31%)	13

Farm 2	8/10 (80%)	10	3/9 (33%)	9
Farm 3	10/14 (71%)	14	5/17 (29%)	17
Farm 4	10/12 (83%)	12	3/10 (30%)	10

ST = core-genome sequence type; percentages are the proportion of the downstream compartment's STs also recovered upstream.

Sharing between runoff and downstream stream isolates was markedly lower: only 31% of stream-recovered sequence types matched a sequence type also recovered from paired runoff samples at the same farm, and the core-genome phylogeny showed that the large majority of stream isolates fell into clades distinct from, though sometimes phylogenetically proximate to, the manure- and runoff-dominant clades (Figure 1). This pattern was consistent across all four farms despite differences in stream flow rate, riparian vegetation and distance from the runoff entry point to the first sampling location, suggesting that the attenuation of strain-level sharing between runoff and stream is a general feature of this transfer pathway rather than a farm-specific artefact of local hydrology.

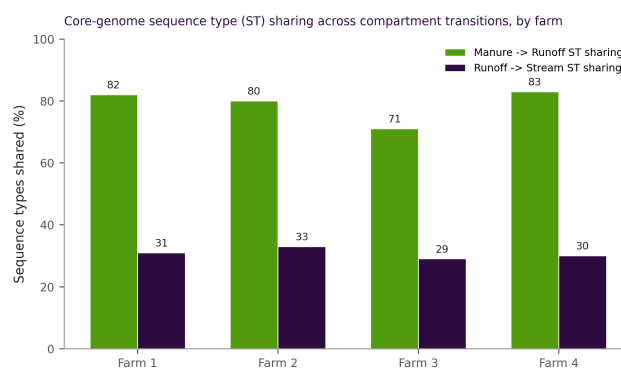


Figure 1. Percentage of downstream-compartment core-genome sequence types (STs) also recovered in the paired upstream compartment, by farm, showing consistently high manure-to-runoff ST sharing and markedly lower runoff-to-stream sharing across all four farms.

Plasmid-resolved hybrid assembly of the 46-isolate subset identified pAR117, a 112 kb IncF-family multidrug-resistance plasmid carrying blaCTX-M-15, sul2, tet(A) and a class 1 integron-associated dfrA17 cassette, in near-identical form (greater than 99.5% identity across full plasmid length) in isolates from all three compartments at three of the four farms, despite these isolates belonging to five different, phylogenetically well-separated core-genome sequence types. This pattern, an essentially identical mobile resistance element recovered across unrelated chromosomal backgrounds in all three sampled compartments, is the expected signature of horizontal plasmid transfer rather than co-inheritance from a shared strain ancestor, and it indicates that pAR117 or a very close relative has been conjugating into new *E. coli* lineages as it moves through the manure-to-stream continuum. Table 2 summarises the plasmid replicon types and resistance gene content recovered across the 46 hybrid-assembled isolates.

Table 2. Plasmid replicon types and resistance genes across 46 hybrid-assembled isolates.

Plasmid replicon	Isolates carrying (n)	Compartments detected in	Key resistance genes
IncF (pAR117)	17	Manure, runoff, stream	blaCTX-M-15, sul2, tet(A), dfrA17
IncI1	9	Manure, runoff	blaCTX-M-14, aadA1
IncN	6	Runoff, stream	sul1, tet(B)
IncHI2	4	Manure only	blaCMY-2, floR, aadA2
Col-type (small)	12	All compartments	qnrS1

Quantitative PCR of total environmental DNA showed that blaCTX-M-15 abundance in stream water was significantly elevated following heavy rainfall in the preceding 48 hours (linear mixed-effects model, coefficient 0.68 log₁₀ copies/mL, $P=0.004$), consistent with rainfall-driven runoff pulses as the principal delivery mechanism for both resistant bacteria and free or bacterially-associated resistance genes into the stream. In contrast, resistance gene abundance was not significantly associated with distance downstream of the runoff entry point across the 2 km reach sampled at each farm ($P=0.29$), suggesting either that dilution and attenuation processes within this distance range are modest relative to the variability introduced by rainfall timing, or that continued low-level resistance gene input occurs along the sampled reach from sources beyond the single mapped runoff entry point at each farm.

4. DISCUSSION

The central pattern to emerge from this study is a clear dissociation between strain-level persistence and plasmid-level mobility across the manure-to-stream continuum: resistant *E. coli* strains recovered in field-edge runoff largely mirror the manure population at the point of entry, but this strain-level signature attenuates sharply by the time bacteria are recovered from stream water, while a specific multidrug-resistance plasmid, pAR117, persists across all three compartments in genetically unrelated bacterial backgrounds. Taken together, this indicates that horizontal plasmid transfer into resident stream bacterial populations, rather than survival and transport of the original manure-associated strains, is the dominant mechanism by which clinically relevant resistance genes establish in the downstream aquatic environment, at least for the IncF-family plasmid lineage characterised here [9,12].

This has a direct and, we think, underappreciated implication for how agricultural runoff mitigation efforts should be prioritised. Interventions focused on reducing the transport or survival of resistant bacteria themselves, such as vegetated buffer strips intended to filter bacterial cells from runoff before it reaches a watercourse, may meaningfully reduce the load of manure-associated strains entering the stream without proportionately reducing resistance gene establishment, if the dominant transfer mechanism is plasmid conjugation into bacteria that are already resident in the stream environment

rather than transport of the resistant strains themselves [19,21]. Reducing resistance gene spread via this route would instead require either reducing the absolute quantity of resistance plasmid entering the system, which point back toward reducing antibiotic selection pressure at the source, or interrupting the plasmid conjugation process itself, neither of which is addressed by strain-filtration-focused buffer interventions alone.

The absence of a detectable distance-downstream effect within our 2 km sampled reach is worth dwelling on, since it runs somewhat counter to a simple dilution model in which resistance gene abundance should decline predictably with distance from a point source. Two non-exclusive explanations seem plausible: first, that dilution and microbial die-off within 2 km is genuinely modest relative to the pulse-driven variability introduced by individual rainfall events, which our rainfall association suggests is a dominant source of variance in the system; and second, that each farm's catchment likely receives distributed, rather than strictly point-source, agricultural inputs along the sampled reach, including field-applied manure runoff from areas beyond the single mapped entry channel used to define our sampling transect. Distinguishing between these explanations would require a denser longitudinal sampling transect extending well beyond 2 km, which was beyond the scope of the present study but represents a natural extension of this work [22,24].

It is also worth considering what makes pAR117 a particularly effective coloniser of new bacterial backgrounds relative to the other plasmid replicon types recovered in this study. IncF-family plasmids generally encode a relatively complete, self-contained conjugative transfer apparatus and often carry toxin-antitoxin or other post-segregational maintenance systems that stabilise the plasmid in a new host even before it confers any measurable fitness benefit, both properties that would be expected to favour establishment across genetically diverse recipient backgrounds relative to a plasmid family lacking these features [26,28]. The smaller Col-type plasmids recovered across all three compartments in this study, while also widely shared, typically encode a narrower resistance repertoire and may depend more heavily on mobilisation by a co-resident conjugative plasmid such as pAR117 for their own transfer, a possibility our present dataset cannot directly test but that would be straightforward to examine with targeted co-transfer experiments in future work. Comparative sequencing of pAR117 against the growing public repository of complete IncF plasmid sequences from clinical and agricultural sources would also help place this specific lineage within the broader phylogeny of CTX-M-15-carrying plasmids now circulating internationally, and would clarify whether the farm-to-farm consistency we observed reflects local emergence within this single catchment or the presence of an already widely distributed plasmid backbone that happened to be sampled at multiple independent sites.

Our finding that pAR117 was recovered across five distinct sequence types at three of four farms also raises the question of where this specific plasmid lineage originated and how widely it circulates beyond the catchment studied here. IncF-family plasmids carrying blaCTX-M-15 are among the most globally disseminated extended-spectrum beta-lactamase resistance elements in both clinical and agricultural *Escherichia coli* populations, and it is entirely plausible that pAR117 or close relatives are already circulating well beyond this specific river catchment, in which case the farm-to-farm consistency we observed may reflect a broadly distributed regional or even wider plasmid lineage rather than a locally emergent one [25,27]. Comparative genomic placement of pAR117 against public plasmid databases, which we have not yet performed systematically, would help resolve whether this plasmid represents a regionally circulating lineage of particular concern or a more generic representative of a globally widespread IncF backbone.

Several limitations qualify these conclusions. First, our selective culture approach, using cefotaxime-supplemented media to recover presumptive extended-spectrum beta-lactamase producers, necessarily biases the isolate collection toward already-resistant organisms and cannot characterise the full, non-resistant *E. coli* population structure across compartments; a parallel non-selective isolate collection would be needed to determine whether the strain-sharing attenuation we observed is specific to resistant lineages or reflects a more general pattern of manure-associated *E. coli* failing to establish in stream environments regardless of resistance phenotype. Second, our cgMLST-based strain-sharing threshold, while conservative and consistent with common practice in outbreak and transmission genomics, is ultimately a somewhat arbitrary cut-off, and results at the margin should be interpreted with this in mind. Third, hybrid long-read assembly and full plasmid resolution was performed on only 46 of 312 isolates for cost reasons, so the true extent of pAR117's distribution across the full isolate collection, and the possible existence of additional mobile resistance elements not captured within this subset, likely exceeds what we have directly documented here.

5. CONCLUSION

Genome-resolved population analysis of 312 *Escherichia coli* isolates collected across paired manure, runoff and stream compartments at four working farms over eighteen months found that resistant strains originating in manure are well represented in adjacent field-edge runoff but attenuate sharply by the time bacteria are recovered from downstream stream water, while a specific IncF-family multidrug-resistance plasmid, pAR117, persists across all three compartments in genetically unrelated bacterial lineages, indicating horizontal plasmid conjugation into resident stream bacteria as the

dominant mechanism of resistance gene establishment in this system. Resistance gene abundance tracked rainfall events closely but showed no detectable decline across the 2 km sampled reach. These findings argue for agricultural runoff mitigation strategies that target resistance gene and plasmid load directly, rather than relying solely on interventions designed to filter or reduce the transport of resistant bacterial cells, since strain-level filtration alone would be expected to leave the dominant plasmid-mediated transfer route largely unaddressed. Future work will extend the plasmid-resolved sequencing approach to the full 312-isolate collection to establish the complete distribution of pAR117 and other mobile resistance elements across the dataset, place pAR117 within a broader regional and global plasmid phylogeny using public comparative genomic resources, and extend the sampling transect well beyond 2 km downstream to characterise the true spatial decay profile of resistance gene abundance in this catchment. We also plan to pair this genomic surveillance with functional conjugation assays to directly measure pAR117's transfer rate into representative stream-resident *E. coli* backgrounds under environmentally realistic temperature and nutrient conditions, which would strengthen the causal case for the transfer mechanism proposed here beyond the correlative genomic evidence presented in this study.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

N.B. conceived and supervised the study, performed core-genome phylogenetic and population structure analysis and drafted the manuscript. S.J. coordinated field sampling and bacterial isolation across the four farms. L.C. performed whole-genome sequencing, hybrid assembly and plasmid analysis. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved only environmental sampling of manure, runoff and stream water and standard microbiological culture and sequencing of recovered bacterial isolates; it did not involve human participants, human data, or vertebrate or higher invertebrate animals,

and therefore did not require institutional ethics committee review. Farm access and sampling were conducted with the informed consent of each participating farm operator.

Data Availability

Whole-genome sequencing reads and assemblies for all 312 isolates have been deposited in the European Nucleotide Archive under a project accession available from the corresponding author on reasonable request. qPCR datasets and the R analysis scripts supporting the findings of this study are available from the corresponding author upon request.

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