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Assessing Antimicrobial and Metal Resistance Genes in Escherichia Coli from Domestic Groundwater Supplies in Rural Ireland

Luisa Andrade
University College Dublin, Ireland

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Assessing antimicrobial and metal resistance genes in *Escherichia coli* from domestic groundwater supplies in rural Ireland☆

Luisa Andrade [a,b,c,*], Michael P Ryan [d], Liam P Burke [e,f], Paul Hynds [b,g], John Weatherill [a,b,c], Jean O'Dwyer [a,b,c]

[a] School of Biological, Earth and Environmental Sciences, University College Cork, Cork, Ireland
[b] Irish Centre for Research in Applied Geosciences, University College Dublin, Dublin, Ireland
[c] Environmental Research Institute, University College Cork, Cork, Ireland
[d] Department of Applied Sciences, Technological University of the Shannon Midwest, Moylish, Ireland
[e] Antimicrobial Resistance and Microbial Ecology Group, School of Medicine, University of Galway, Galway, Ireland
[f] Centre for One Health, Ryan Institute, University of Galway, Galway, Ireland
[g] Environmental Sustainability and Health Institute, Technological University Dublin, Dublin 7, Ireland

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ABSTRACT

Natural ecosystems can become significant reservoirs and/or pathways for antimicrobial resistance (AMR) dissemination, with the potential to affect nearby microbiological, animal, and ultimately human communities. This is further accentuated in environments that provide direct human exposure, such as drinking water. To date, however, few studies have investigated AMR dissemination potential and the presence of co-selective stressors (e.g., metals/metalloids) in groundwater environments of human health significance. Accordingly, the present study analysed samples from rural (drinking) groundwater supplies (i.e., private wells) in the Republic of Ireland, where land use is dominated by livestock grazing activities. In total, 48 *Escherichia coli* isolates tested phenotypically for antimicrobial susceptibility in an earlier study were further subject to whole genome sequencing (WGS) and corresponding water samples were further analysed for trace metal/metalloid concentrations. Eight isolates (i.e., 16.7%) were genotypically resistant to antimicrobials, confirming prior phenotypic results through the identification of ten antimicrobial resistance genes (ARGs); namely: *aph(3″)-lb* (*strA*; n=7), *aph(6)-Id* (*strA*; n = 6), *blaTEM* (n = 6), *sul2* (n = 6), *tetA* (n = 4), *floR* (n = 2), *dfrA5* (n = 1), *tetB* (n = 1), and *tetY* (n = 1). Additional bioinformatic analysis revealed that all ARGs were plasmid-borne, except for two of the six *sul2* genes, and that 31.2% of all tested isolates (n = 15) and 37.5% of resistant ones (n = 3) carried virulence genes. Study results also found no significant relationships between metal concentrations and ARG abundance. Additionally, just one genetic linkage was identified between ARGs and a metal resistance gene (MRG), namely *merA*, a mercury-resistant gene found on the same plasmid as *blaTEM, dfrA5, strA*, *strB*, and *sul2* in the only isolate of inferred porcine (as opposed to bovine) origin. Overall, findings suggest that ARG (and MRG) acquisition may be occurring prior to groundwater ingress, and are likely a *legacy issue* arising from agricultural practices.

1. Introduction

The continuing global rise of antimicrobial resistance (AMR) has been seen through multiple reports of increased morbidity and mortality from resistant infections (Murray et al., 2022; Cassini et al., 2019), and is now widely recognised as a global public health threat (World Health Organization, 2022a). Briefly, the principal known causes of AMR are bacterial mutation and, more commonly (Bennett, 2008; Von Wintersdorff et al., 2016), acquisition of new resistance determinants (i.e., antimicrobial resistance genes; ARGs). Both can occur and are amplified in response to selective environments, such as in the presence of elevated concentrations of antimicrobial drugs (i.e., antimicrobials) (Bennett, 2008; Christaki et al., 2020). Conjugation is arguably the most important mechanism of AMR acquisition (Christaki et al., 2020), it can occur when a bacterium comes into direct physical contact with other ARG-carrying bacteria, particularly when the ARGs are located in

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* Corresponding author. School of Biological, Earth and Environmental Sciences, University College Cork, Cork, Ireland.
E-mail address: luisa.andrade@ucc.ie (L. Andrade).

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mobile genetic elements (MGEs – e.g., plasmids, transposons, integrating conjugative elements etc.) (Partridge et al., 2018) as opposed to chromosomal placement. Upon acquisition, newly ARG-carrying bacteria can, when pathogenic, cause harder-to-treat (resistant) infections, requiring alternative therapeutic options which may not always be available. Thus, "One Health" mitigative efforts to enforce the rational use of antimicrobials in human and veterinary medicine and increase development (and widespread access to) novel antimicrobial classes are key priorities (Infectious Diseases Society of America, 2011; Laxminarayan et al., 2020, WHO, 2022a).

The occurrence of AMR pre-dates the discovery and commercialisation of antimicrobials (D'Costa et al., 2011), however historic over-prescription and excessive/inappropriate antimicrobial use have led to it heightened dissemination. This culminates in the increased detection of antimicrobial resistant bacteria and genes within natural environments (Larsson and Flach, 2022), particularly those heavily impacted by human activities such as agriculture, aquaculture, and ineffective waste and wastewater treatment/disposal (Kraemer et al., 2019). Compounding this, anthropogenic and geogenic contaminants which are present in the environment (in concurrence with antimicrobials) can promote/facilitate processes that indirectly select (i.e., co-select) for AMR. Potentially toxic metals, for example, some of which also possess antimicrobial properties (Poole, 2017; Gharpure et al., 2020), have been extensively reported as co-selective for AMR (Seiler and Berendonk, 2012; Pal et al., 2017; Imran et al., 2019; Nguyen et al., 2019), including in environments unaffected by large-scale antimicrobial use (Wardwell et al., 2009; Hobman and Crossman, 2015). This poses further challenges in reducing the environmental dissemination of AMR, as metals are ubiquitous in the natural environment (occurring as mineral phases, mobile cations, and surface complexes) with concentrations of μg/L to mg/L and mg/kg, while antimicrobials are typically detected at trace to ultra-trace levels (ng/L to μg/L and μg/kg); even in areas adjacent to potential sources, such as agriculture (Zhao et al., 2018; Veiga-Gómez et al., 2021).

The key mechanisms by which co-selection can happen have been defined as cross- and co-resistance. Cross-resistance occurs when a single (physiological) mechanism such as an 'efflux pump' provides resistance to both the co-selective compound and the antimicrobial drug (Chapman, 2003; Baker-Austin et al., 2006), while co-resistance describes genes conferring resistance to a co-selective compound (e.g., metal resistance genes; MRGs) and antimicrobial(s) (i.e., ARGs) being located next to each other on a single MGE or chromosome (Chapman, 2003). In both cases, presence of co-selective compounds, such as metals, disinfectants, biocides, etc., at toxic concentrations may pose extended pressures for the evolution (i.e., acquisition and/or persistence) of antibiotic resistance in the local microbiome (Nguyen et al., 2019). Metals have been shown to also increase the level of tolerance to antimicrobials via resistance gene co-regulation (Baker-Austin et al., 2006). It is important to note, however, that not all metals are equally toxic to bacteria. Metals (including metalloids) such as silver (Ag), arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb), for example, have limited biological function and are considered toxic to most bacteria and other microorganisms, even at (very) low concentrations (Nies, 1999; Naik and Dubey, 2013). Conversely, cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), and zinc (Zn) are metals which can be vital to bacterial physiological functions, only becoming toxic at higher concentrations - and further subject to the element's bioavailability (Nies, 1999).

Multiple studies focused on surface water, soil, and effluents report associations between increased metal concentrations and heightened environmental abundance of ARGs (McKinney et al., 2010; Graham et al., 2011; Knapp et al., 2011; Ji et al., 2012; Hu et al., 2016; Knapp et al., 2017; Zhao et al., 2017; Zhao et al., 2019; Komijani et al., 2021) and, to a lesser extent, increases in ARG-carrying (Martins et al., 2014) and phenotypically resistant bacteria (Stepanauskas et al., 2005; Wright et al., 2006). Additionally, associations have been reported between MRG and ARG abundance in livestock waste lagoons (McKinney et al., 2010) and between specific MRG-ARG combinations encoded by environment-derived bacteria (Martins et al., 2014; Pal et al., 2015; Chen et al., 2019). Notably, however, studies tend to focus on more "exposed" and heavily (metal) contaminated environments as noted by large-scale reviews carried out by Imran et al. (2019) and Nguyen et al. (2019), such as surface water and soils directly impacted by manure application, industrial waste, and urbanization. This results in less knowledge availability on presumably 'cleaner' groundwater environments, where exposure to metal and antimicrobial contamination is limited, but which can provide direct dissemination pathways for AMR to humans (and animals) via consumption. Additionally, most co-selection groundwater-based studies examine ARG occurrence at a community level (i.e., present in total DNA extracted from groundwater) (viz. Li et al., 2018; Zainab et al., 2020; Zou et al., 2021; Liu et al., 2022; Kampouris et al., 2022), which may limit their ability to quantify the overarching human (and animal) health threats. The current study sought to address both gaps in the literature by (i) investigating co-selective pressures in rural groundwater resources used for drinking in the Republic of Ireland (RoI), and (ii) incorporating the analyses of groundwater-derived *Escherichia coli* through whole genome sequencing (WGS), while also quantifying isolates' pathogenic potential (e.g., presence of virulence genes).

2. Methods

2.1. Study sites and groundwater sample collection

This was an exploratory study of rural (domestic) groundwater supplies (i.e., private wells) in the RoI, a country characterised by high rural-reliance on domestic groundwater abstractions (i.e., private wells) (EPA, 2021), and with a rural profile marked by widespread livestock grazing activities and high populational reliance on domestic wastewater treatment systems (DWWTSs; e.g., septic tanks) (CSO, 2016). Overall, 250 samples were collected from 132 private wells, selected based on responses to a nationwide recruitment effort, and sampled during two distinct periods: autumn 2019 (Round 1; n = 132) and summer 2021 (Round 2; n = 118). Site locations were geographically distributed across the country and located >1 km away from hospital, industrial (including mining areas), and large-scale domestic waste and wastewater disposal points. See Andrade et al. (2023) for a complete description of study sites and their geographic locations.

Groundwater samples were collected prior to any microbiological treatment, following ethanol disinfection of sample taps and after being allowed to run at high flow for a minimum of 60 s in order to purge any stagnant water within the distribution network. Samples intended for *E. coli* enumeration and subsequent WGS analyses were collected into 100 ml sterile vessels (IDEXX Laboratories Inc., USA) and transported to the laboratory at 4 °C for analysis within 6 h. Samples for trace metal analysis were filtered onsite using 0.45 μm pore size inline syringe filters (Sartorius Stedim Biotech, Germany) and collected into 60 ml factory clean HDPE bottles (Thermo Fisher, UK) containing 0.5 ml of 20% ultrapure nitric acid (67–70%, Optima™, Ultra Trace Elemental Analysis; Fisher Chemical, UK) to buffer samples' pH to <4. Temperature, pH, and specific electrical conductivity were measured immediately prior to sample collection using a pre-calibrated multiparameter in-situ water quality sonde (Manta+20, Eureka Water Probes, USA) with readings recorded after stabilisation to <5%. The specific well type (i.e., drilled versus hand-dug) was recorded following sample collection.

2.2. Laboratory procedures

2.2.1. Bacterial enumeration, whole genome sequencing, and bioinformatic analyses

The most probable number (MPN) of *E. coli* in each water sample was determined using standard ISO approved (ISO, 1998) commercial

culture kits (Colilert; IDEXX Laboratories Inc., Westbrook, Maine) and 51-'well' IDEXX quanti-trays (IDEXX Laboratories Inc., Westbrook, Maine) according to the manufacturer's instructions (see Andrade et al., 2023 for full description). WGS of recovered *E. coli* isolates was carried out by MicrobesNG (University of Birmingham, UK). Briefly, standard Nextera protocols (Adey et al., 2010) were used for strain recovery and 2 × 250 bp paired-end reads HiSeq Illumina Miseq technology, allowing for a 30x genome coverage. Reads were adapter-trimmed using Trimmomatic 0.30 with a sliding window quality cut-off of Q15 (Bolger et al., 2014) and a de-novo assembly of reads was performed using SPAdes (Bankevich et al., 2012). The reads were then mapped back to the resultant contigs using the Burrows-Wheeler Aligner mem (Li and Durbin, 2010), with automated annotation performed using Prokka (Seemann, 2014).

Genome sequence FASTA (.faa) files produced were analysed using several open-source bioinformatic platforms. Firstly, the EZBioCloud ANI calculator tool (https://www.ezbiocloud.net/tools/ani) was employed to confirm isolate species by comparing isolate genomes to that of an established reference strain (i.e., *E. coli* K-12), with 'identity' ≥95% used to designate a match (Yoon et al., 2017). Subsequently, the Galaxy platform (ABRicate tool v1.0.1 - https://usegalaxy.org/; Afgan et al., 2018) was utilised, applying a minimum 'identity' and 'coverage' of 80%, to identify antibiotic resistance genes (ARGs) (ResFinder and CARD), *E. coli* virulence genes (Ecoli.VF), and plasmids (PlasmidFinder) present, as well as the isolate's serotype (EcOH). Additionally, the IntegronFinder tool (v2.0.2; Néron et al., 2022) was used through the Galaxy Europe platform (https://usegalaxy.eu/) to detect integrons, with an applied e-value cut-off of 1e−5.

The metal resistance genes (MRGs) present were identified by subjecting genome sequences to a BLASTx search (v2.2.26; Altschul et al., 1997) against the BacMet 'Experimentally Confirmed' database (v2.0 - http://bacmet.biomedicine.gu.se/index.html; Pal et al., 2014), with an applied e-value cut-off of 1e−5 and minimum 'identity' and 'positives' of 80%. The Clermont Typing (v21.03 - http://clermontyping.iame-research.center/index.php; Beghain et al., 2018) and Multi Locus Sequence Typing (MLST v2.0.9 - http://www.cbs.dtu.dk/services/mlst; Larsen et al., 2012) pipelines were used to ascertain isolates' phylotypes and sequence types (STs), respectively. Finally, the 'Platon' tool (Schwengers et al., 2020) was utilised to detect likely chromosome and plasmid contigs. These were then scanned using ResFinder/CARD and subjected to BLASTx BacMet searches, as described above, to infer which ARGs and/or MRGs are located on plasmids versus on the bacterial chromosome. The presence of transposons (and their genetic context) was searched for using TnCentral (https://tncentral.ncc.unesp.br/; Ross et al., 2021) and MGEfinder (https://cge.food.dtu.dk/services/MobileElementFinder/; Durrant et al., 2020) and, where detected, the genomic context of ARGs and MRGs found on plasmid-related contigs in the same isolate were further investigated using BLASTn (Altschul et al., 1990).

2.2.2. Trace metal analysis

In groundwater samples where *E. coli* were detected, total dissolved trace metals (including metalloids) analysis was carried out in the School of Chemistry (University College Cork, Ireland) using inductively coupled argon plasma-mass spectrometry (ICP-MS) (Agilent 7700x, Agilent Technologies, United Kingdom). The typical limit of quantification was <0.001 μg/L and the isotopes used to quantify the selected elements were: ^{107}Ag, ^{75}As, ^{59}Co, ^{111}Cd, ^{52}Cr, ^{63}Cu, ^{56}Fe, ^{55}Mn, ^{60}Ni, ^{208}Pb, ^{121}Sb, and ^{66}Zn.

2.3. Statistical analyses

Associations between each ARG and the specific MRGs detected in the groundwater-derived *E. coli* (i.e., all bivariate ARG-MRG combinations) were examined using the Fisher's exact test, except in cases where just one 'positive' outcome was detected. Additionally, Mann–Whitney U tests were employed to assess associations between median metal concentrations and presence of the relevant MRG(s), and Kruskal–Wallis H tests were used to identify individual metal concentrations in their associations with ARG and MRG abundance (i.e., using the quantities of ARGs and MRGs found in each isolate as dependent variables, respectively). All statistical analyses were undertaken using IBM SPSS version 28, with confidence level set at 95% (i.e. *p*-value <0.05).

3. Results

3.1. Descriptive characteristics of groundwater supplies and samples

As described elsewhere (Andrade et al., 2023), *E. coli* isolates were identified in 54 groundwater samples, with 48 isolates recovered and re-occurrence (i.e., repeat *E. coli* presence) identified in 13 wells. Thus, the *E. coli* analysed in the current study originated from 35 distinct groundwater wells (See Fig. 1 for their locations). Most *E. coli*-positive wells were drilled (77.1%; n = 27), with the remainder classified as shallow hand-dug wells (22.9%; n = 8).

Further analyses of the 48 *E. coli*-positive groundwater samples indicated temperatures of 11.5–18.6 °C, pH of 5.8–7.5, and electrical conductances of 142.0–943.6 μS/cm, respectively, with no outliers found. A median of 5.30 MPN/100 ml (interquartile range = 2.0 to 26.4) was identified for *E. coli*; see Table 1 for quantity distributions. As also shown in Table 1, highest median trace metal concentrations were found for zinc (median = 32.52 μg/L, IQR = 7.68–109.86), followed by copper (6.57 μg/L; 2.70–21.50), manganese (1.38 μg/L, <0.00–15.21), nickel (0.24 μg/L, <0.001–0.64), arsenic (0.19 μg/L, 0.07–0.44), and chromium (0.14 μg/L, 0.08–0.27), the remaining elements (i.e., cobalt, cadmium, silver, iron, and lead) all had median concentrations lower than 0.05 μg/L. Dissolved trace metal concentrations remained within safe drinking water levels except for manganese, which exceeded the recommended 80 μg/L in four samples (WHO, 2022b).

3.2. Description of the groundwater-derived E. coli

3.2.1. Antimicrobial resistance

AMR was genotypically identified in 16.7% of the *E. coli* isolates (n=8), with 12.5% (n = 6) classified as multidrug resistant (i.e., resistant to ≥3 antimicrobials belonging to different classes; Magiorakos et al., 2012) confirming phenotypic findings in Andrade et al. (2023) (Fig. 2). No repeated instances (i.e., re-occurrence) of resistant-*E. coli* contamination were detected. As shown in Fig. 2 genes were identified which conferred resistance to antimicrobials from the Aminoglycosides (14.%; n = 7), Tetracyclines (12.5%; n = 6), Beta-Lactams (12.5%; n = 6), Sulphonamides (12.5%; n = 6), Amphenicols (4.2%; n = 2), and Diaminopyrimidine (2.1%; n = 1) antimicrobial classes. Namely: *aph(3″)-lb* (n = 7; encoded by *strA*), *aph(6)-Id* (n = 6; encoded by *strB*), *blaTEM* (n = 6), *tetA* (n = 4), *floR* (n = 2), *dfrA5* (n = 1), *tetB* (n = 1), and *tetY* (n = 1). When comparing genotypic findings with and phenotypic data from Andrade et al. (2023) a single discrepancy was found whereby one isolate was phenotypically classified as intermediately (not categorically) resistant to piperacillin despite presence of the *blaTEM* gene (see Fig. 2).

3.2.2. Metal resistance

As shown (Table 2), eleven MRGs were identified among the sequenced *E. coli* isolates, of which the three most abundant were the zinc-resistance gene *zntA* (97.9%; n = 47), the nickel- and cobalt-resistance gene *rcnA* (93.8%; n = 45), and the arsenic-resistance gene *arsB* (81.3%; n = 39). Other identified MRGs included the arsenic-resistance genes *arsC*, and *arsA* found in 4.2 and 2.1% of isolates, respectively, the copper-resistance genes *pcoA*, *pcoB*, *pcoC*, and *pcoD* carried by 6.3, 4.2, 2.1 and 2.1% of isolates, respectively, the mercury-resistance gene *merA* (2.1%; n = 1), and the zinc-resistance gene *rcnB* (2.1%; n = 1). All tested isolates carried at least one MRG, with *arsB*-

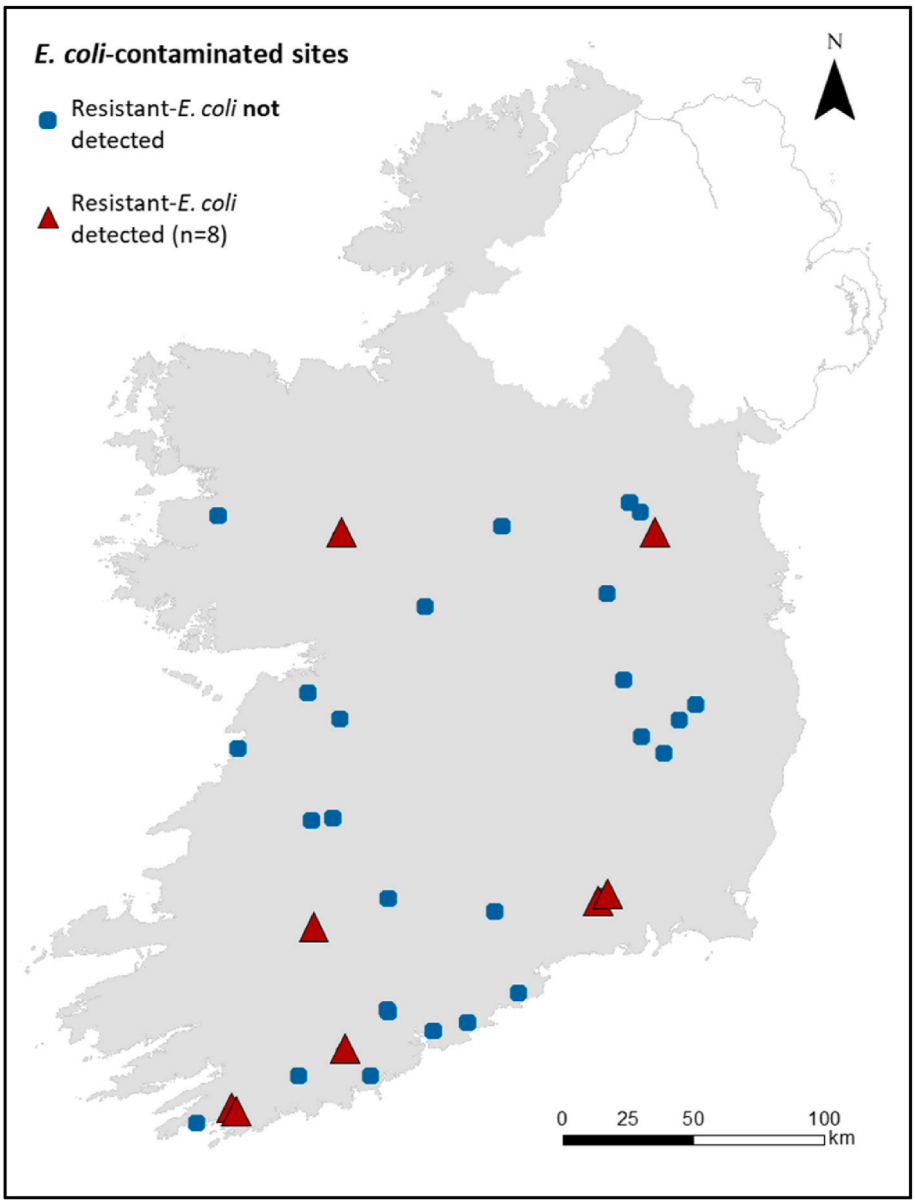

Fig. 1. Map of Ireland showing the 35 groundwater wells where *E. coli* was detected and cultured for subsequent genotypic analyses (n = 48) and highlighting the wells harbouring antimicrobial-resistant *E. coli* (n = 8).

rcnA-zntA being the most common combination (68.8%; n = 33), followed by *rcnA-zntA* (14.6%; n = 7); all other MRG combinations occurred just once (see **Supplementary data**).

3.2.3. Genetic elements

Approximately 85% of sequenced *E. coli* isolates harboured one or more plasmids (85.4%; n = 41 – see Table 2), of which 90.2% carried IncF (n = 37/41) and 46.3% carried Col (n = 19/41) replicon types, while just one isolate was identified as harbouring a Class 1 integrase: *intI1* (see isolate 23 in Table 3). Isolate 23 was collected from a hand-dug well in a pig farm; the only one in the current study. Among the antimicrobial-resistant isolates, all of which carried plasmid(s), the most frequently detected replicons were *IncFIB* (n = 7/8), *IncI1* (n = 5/8) and *ColRNAI* (n = 4/8). With regards to the genomic locations of ARGs in the eight antimicrobial-resistant isolates, Platon findings show that most were in plasmid contigs, except for *sul2,* which were located in chromosome contigs in two of the six isolates where it was present (as shown in Table 3). Conversely, all MRGs from the resistant-*E. coli*, except one (i.e., *merA* in isolate 23), were in chromosome contigs. Further investigation of the composite transposon identified in isolate 23 found that it was the same (100% identity score using Blastn analysis) as a transposon found on an *E.coli* plasmid (pRes13-Lact-PEB17-18) from pig faeces (Poulin-Laprade et al., 2021). Comparison of the plasmid sequence to the sequence of isolate 23 shows high levels of similarity (including a contig containing the mercury resistance operon). This indicates that in Isolate 23, the five identified ARGs (i.e., *dfrA5_1*, *bla-TEM-1B_1*, *aph(6)-Id_1*, *aph(3")-Ib_5*, and *sul2_3*) and *merA* MRG are co-located on the same plasmid, such was not the case for any of the other isolates in this study. Further investigation also indicates that multiple ARGs may be located on transposon like elements associated with IS*Vsa*3 on *IncI* plasmids in four of the five remaining multidrug resistant isolates (i.e., 6, 11, 32 and 33).

3.2.4. Sequence types, phylotypes, and virulence

Overall, 36 serotypes (STs) were detected among the *E. coli* isolates in the current study. The most prevalent STs were ST155 (n = 3), ST906 (n = 3) and ST1079 (n = 3), followed by ST10 (n = 2), ST58 (n = 2), ST187 (n = 2) and ST196 (n = 2); the 29 remaining types were not

Table 1
Statistical summary of metal (μg/L) and *E. coli* (MPN/100 ml) concentrations in all *E. coli* isolates (n = 48) and those in which antimicrobial resistance was detected (n = 8).

	Metals (μg/L)											*E. coli* (MPN/100 ml)	
	Ag	As	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Sb	Zn	
(A) All *E. coli* isolates													
Minimum value	0*	0*	0*	0*	0.02	0.65	0*	0*	0*	0*	0.01	0*	1.0
1st quartile	0*	0.07	0*	0.02	0.08	2.70	0*	0*	0*	0*	0.04	7.68	2.0
Median	0*	0.19	0.01	0.04	0.14	6.57	0*	1.38	0.24	0*	0.07	32.52	5.3
3rd quartile	0*	0.44	0.03	0.14	0.27	21.50	0*	15.22	0.64	0.33	0.13	109.86	26.5
95th percentile	0.03	1.59	0.27	0.74	0.35	72.89	16.46	98.07	4.37	0.69	0.37	186.75	>200.5[#]
Maximum value	0.07	9.97	0.45	5.11	0.56	289.62	4602^	319.56	7.61	1.92	0.53	693.25	>200.5[#]
(B) Resistant-*E. coli*													
Minimum value	0*	0*	0*	0.01	0.05	0.65	0*	0*	0*	0*	0.01	0*	1.0
1st quartile	0*	0.06	0.01	0.02	0.07	5.12	0*	0.76	0*	0*	0.07	12.80	3.1
Median	0*	0.15	0.02	0.03	0.12	6.61	0*	4.84	0.19	0.01	0.10	66.61	4.8
3rd quartile	0.01	0.32	0.11	0.06	0.17	15.49	0.22	23.18	0.36	0.08	0.19	131.09	62.4
95th percentile	0.06	1.30	0.36	0.19	0.33	22.86	1.32	108.27	2.98	0.28	0.48	178.21	>200.5[#]
Maximum value	0.07	1.62	0.37	0.23	0.36	24.05	1.57	152.85	4.28	0.33	0.53	191.17	>200.5[#]

* metal concentration below the limit of quantification; [#]*E. coli* enumeration above the maximum detection limit of 200.5 MPN/100 ml; ^ outlier; Ag = silver; As = arsenic; Co = cobalt; Cu = copper; Cr = chromium; Cd = cadmium; Fe=Iron; Mn = manganese; Ni = nickel; Pb = lead; Sb = antimony; Zn = zinc.

repeated (see Table 2). Three isolates of different unknown STs were also detected. STs identified for the eight resistant isolates were ST10, ST58 (n = 2), ST95, ST155, ST164, ST685, and ST1079 (Table 3). Isolates belonging to phylogroup B1 were most prevalent across all *E. coli* (62.5%; n = 30), followed by 10.4% in phylogroup E (n = 5) and 8.3% (n = 4) in A, B2 and D lineages, with a single isolate exhibiting an ambiguous classification (i.e., belonging to both A and B2 phylogroups). In the case of antimicrobial-resistant isolates, phylogroup B1 was once again the most prevalent (62.5%; n = 5/8), followed by groups A (25%; n = 2/8) and B2 (12.5%; n = 1/8).

One or more virulence genes were detected in 31.2% of the 48 tested isolates (n = 15) and 37.5% of the resistant-isolates (n = 3). As shown (Table 2), the most prevalent of these were *astA* (20.8%; n = 10), followed by *ctd* (8.3%; 4) *cnf2* (6.3%; 3), *hlyA* (4.2%; n = 2), and *subA*, *stx2* and *espL2* (each corresponding to 2.1%; n = 1). Notably, the shiga toxin virulence gene *stx2* was present in a single isolate belonging to phylogroup B1 (ST8621), this shiga toxin-producing *E. coli* (STEC) was susceptible to all antimicrobials tested and did not harbour ARGs. Another (also susceptible) B1 isolate (ST765) carried the *espL2* virulence gene and is thus considered an enterohemorrhagic *Escherichia coli* (EHEC; Bugarel et al., 2011). Both the STEC and EHEC isolates were found in drilled wells and carried *arsB*, *rcnA*, and *zntA* MRGs.

3.3. Assessing co-selective pressures for antimicrobial resistance

3.3.1. ARG and MRG co-occurrence in groundwater E. coli

A single isolate contained ARGs and an MRG (*merA*) co-located on the same plasmid (i.e., one co-resistant plasmid; Chapman, 2003). Additionally, as shown (Table 4), significant associations were identified between co-occurrence of the *arsC* MRG and *aph(3″)-Ib* (*strA*; *p*-value = 0.019), *aph(6')-Id* (*strB*; $p = 0.013$), *blaTEM* ($p = 0.013$), *dfrA5* ($p = 0.042$) and *sul2* ($p = 0.013$). Bivariate associations containing *drfA5*, *tet(Y)*, *pcoC*, *pcoD*, *merA*, and/or *rcnB* were not presented in Table 4 as these genes were only detected once across all studied isolates.

3.3.2. Associations between trace metal concentrations and ARG/MRG abundance in groundwater E. coli

Bivariate statistical test results (Table 5) were shown to approach significance in the associations between ARG abundance and arsenic concentrations ($p = 0.084$). No significance or approaching significance was found for relationships between trace metal levels and the respective MRGs (Mann-Whitney, data not shown) or overall MRG abundance (Kruskal-Wallis, see Table 5).

4. Discussion

AMR has been recognised as a key global health issue by the WHO (WHO, 2014; WHO, 2022a) and primary emerging issue of environmental concern by the United Nations Environment Programme (UNEP, 2017). With that, extensive research has been undertaken to better understand the conditions facilitating/permitting the occurrence and dissemination of AMR, such as presence of co-selective contaminants (e.g., metals/metalloids) in soil (Knapp et al., 2011; Hu et al., 2017), surface (Wright et al., 2006; Martins et al., 2014; Xu et al., 2017) and groundwater environments (Zou et al., 2021; Liu et al., 2022). However, recent reviews highlight the need for more studies which investigate 'cleaner' environments of human health significance and that measure co-selective pressures on specific bacterial isolates (Pal et al., 2017; Imran et al., 2019; Nguyen et al., 2019). The present study sought to fill the first gap by studying domestic groundwater resources in the RoI, which are present in agricultural areas with no noteworthy point-sources of antimicrobial/metal pollution (e.g., hospitals, wastewater treatment plants, mines, and industrial sites). The *E. coli* isolates detected were tested for encoded ARGs and MRGs, and water samples were analysed for levels of potentially co-selective elements (i.e., metals/metalloids). Statistical analyses were further employed to identify potential relationships between metal concentrations and abundance of MRGs and ARGs in the groundwater-derived *E. coli*, and between ARG-MRG co-occurrence.

Of the 48 *E. coli* isolates examined, 16.7% (n=8) and 12.5% (n = 6) harboured ≥1 and ≥3 ARGs, respectively, amounting to ten distinct ARGs: *aph(3″)-lb* (*strA*; n = 7), *aph(6)-Id* (*strB*; n = 6), *blaTEM* (n = 6), *sul2* (n = 6), *tetA* (n = 4), *floR* (n = 2), *dfrA5* (n = 1), *tetB* (n = 1), and *tetY* (n = 1). Conversely, all tested isolates (n = 48) harboured one or more MRGs (e.g., *zntA* = 47, *rcnA* = 45, *arsB* = 39, and others). This is unsurprising, as metal concentrations are typically much higher than that of antimicrobial residues in natural environments associated with agricultural activities (Guo et al., 2018; Veiga-Gómez et al., 2021). Of all MRGs found among the resistant-*E. coli,* just one was located in a plasmid (i.e., *merA*), implying that, for the most part, MRG-carrying resistant isolates have adapted to metal-contaminated environments (Remenar et al., 2018). This is not reflected, however, in the dissolved metal concentrations reported here (see Table 1B), with maximum values (not taking bioavailability or particulate mass into account) being lower than what are considered toxic to *E. coli* (i.e., Ag = 5 μg/L, Cd = 100 μg/L, Co = 350 μg/L, Cu = 400 μg/L, Ni = 550 μg/L, Zn = 200 μg/L; Hoegler and Hecht, 2018). Compounding this, no statistical significance was found between metal concentrations and presence of the relevant MRG(s) or overall MRG abundance in the tested isolates. As such,

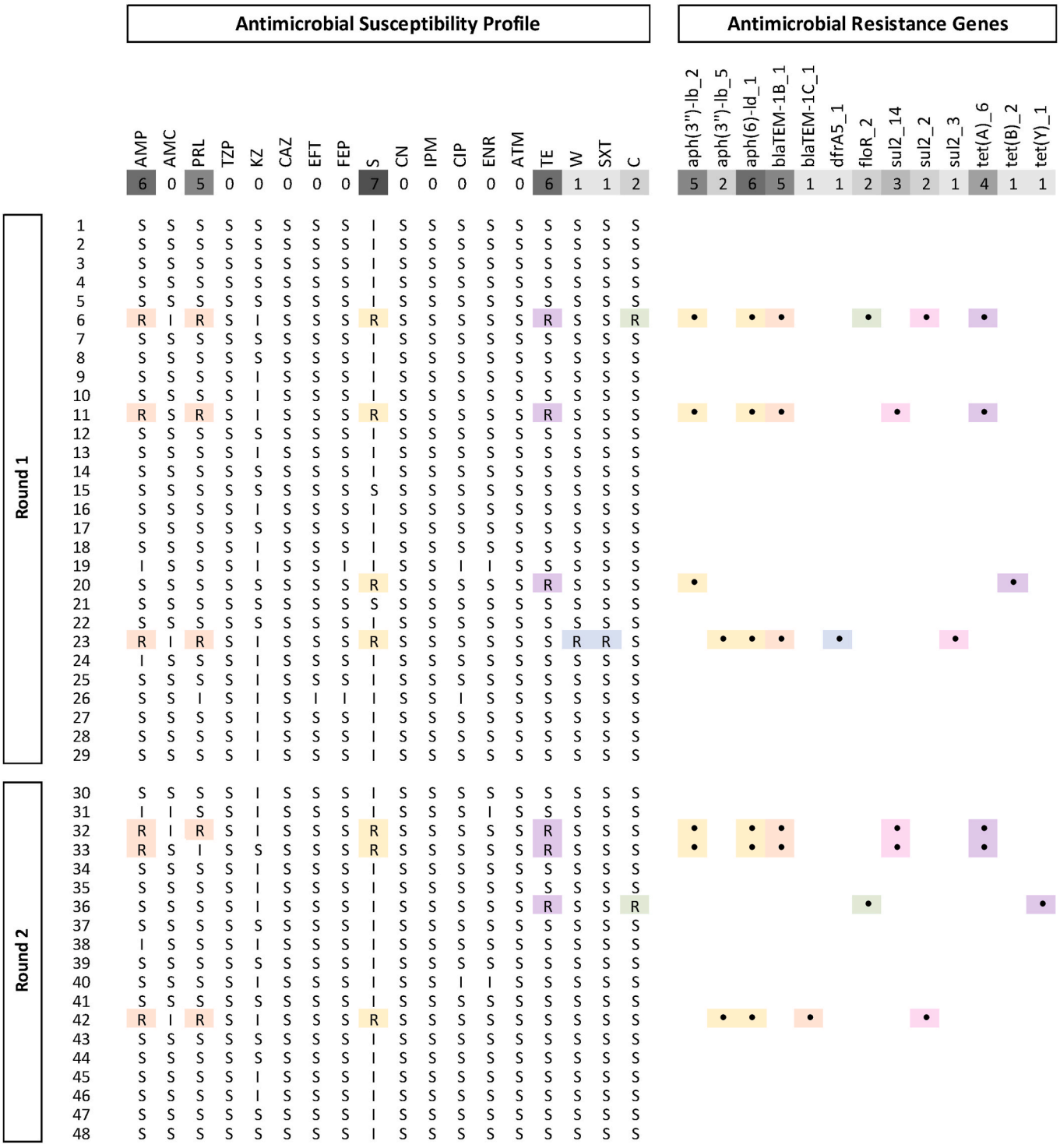

Fig. 2. Antibiogram and resistance gene profiles for all groundwater-derived *E. coli* (n = 48) (after Andrade et al., 2023). Colour groupings indicate genotypic and phenotypic resistance to corresponding antimicrobials and antimicrobial classes. R = resistant; I=Intermediately resistant; S = susceptible; AMP = ampicillin; AMC = amoxicillin-clavulanic acid; PRL = piperacillin; TZP = piperacillin-tazobactam; KZ = cefazolin; CAZ = ceftazidime; EFT = ceftiofur; FEP = cefepime; S = streptomycin; CN = gentamycin; IPM = imipenem; CIP = ciprofloxacin; ENR = enrofloxacin; ATM = aztreonam; TE = tetracycline; W = trimethoprim; SXT = trimethoprim-sulfamethoxazole; C = chloramphenicol. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

findings suggests that MRG acquisition is likely taking place outside of these groundwater environments. Within agricultural settings manure-treated soils and grasslands, for example, can provide sufficiently selective environments to drive metal resistance (Peng et al., 2019; Shi et al., 2019).

Two studies have previously described minimum cadmium, cobalt, copper, lead, nickel, and zinc concentrations correlated with increased detection of antimicrobial resistance in bacteria (i.e., minimum co-selective concentration; MCC) within surface water environments (Wright et al., 2006; Stepanauskas et al., 2005). Based on these, Seiler

Table 2
Summary of whole genome sequence results for all tested isolates (n = 48).

Sequence types (n)	Phylotypes (n)	Plasmid replicons (n)	Integrase (n)	Virulence genes (n)	ARGs (n)	MRGs (n)
155 (**3**), 906 (**3**), 1079 (**3**), 10 (**2**), 58 (**2**), 187 (**2**), 196 (**2**), 95 (**1**), 101 (**1**), 129 (**1**), 164 (**1**), 297 (**1**), 345 (**1**), 401 (**1**), 642 (**1**), 681 (**1**), 685 (**1**), 753 (**1**), 765 (**1**), 1056 (**1**), 1086 (**1**), 1122 (**1**), 1131 (**1**), 1152 (**1**), 1170 (**1**), 1621 (**1**), 1643 (**1**), 2002 (**1**), 2536 (**1**), 2538 (**1**), 2853 (**1**), 4382 (**1**), 5597 (**1**), 5662 (**1**), 8621 (**1**), unknown (**3**)	B1 (**30**), E (**5**), A (**4**), B2 (**4**), D (**4**), A/B1 (**1**)	*IncFIB* (**34**), *ColRNAI* (**16**), *IncFIA* (**13**), *IncFII* (**8**), *Col156* (**8**), *IncI1* (**7**), *IncFIC* (**5**), *IncB/O/K/Z* (**4**), *IncY* (**4**), *Col(MG828)* (**1**), *IncP1* (**1**), *IncR* (**1**), *Col(Ye4449)* (**1**), *IncHI1A* (**1**), *IncHI1B* (**1**), *Col440I* (**1**), *IncX1* (**1**), *IncX3* (**1**), *p0111* (**1**), NONE (**7**)	*IntI1* (**1**)	*astA* (**10**), *ctd* (**4**), *cnf2* (**3**), *hylA* (**2**), *subA* (**1**), *espL2* (**1**), *stx2* (**1**)	*strA* (**7**), *strB* (**6**), *blaTEM* (**6**), *sul2* (**6**), *tetA* (**4**), *floR* (**2**), *tetB* (**1**), *tetY* (**1**), *dfrA5* (**1**)	*zntA* (**47**), *rcnA* (**45**), *arsB* (**39**), *pcoA* (**3**), *pcoC* (**2**), *arsC* (**2**), *arsA* (**1**), *pcoC* (**1**), *pcoD* (**1**), *rcnB* (**1**), *merA* (**1**)

ARG = antimicrobial resistance gene; MRG = metal resistance gene.

Table 3
Summary of whole genome sequence results related to the antimicrobial resistant *E. coli* isolates (n = 8).

N	ST	Phylotype	Plasmid Replicons	Integrase	VGs	ARGs		MRGs	
						chromosome	plasmid	chromosome	plasmid
6	155	B1	*IncI1*, *IncFIA*, *IncFIB*	–	–	–	*floR_2*, *aph(6)-Id_1*, *aph(3″)-Ib_2*, *sul2_2*, *tet(A)_6*, *blaTEM-1B_1*	*rcnA*, *arsB*, *arsC*, *zntA*	–
11	164	B1	*IncFIA*, *IncFIB*, *IncI1*, *ColRNAI*	–	–	*su2_14*	*tet(A)_6*, *aph(3″)-Ib_2*], *aph(6)-Id_1*, *blaTEM-1B_1*	*rcnA*, *arsB*, *zntA*	–
20	58	B1	*Incl1*, *IncFIB*	–	*astA*, *cdt*, *cnf2*	–	*tet(B)_2*, *aph(3″)-Ib_2*	*arsB*, *rcnA*, *zntA*	–
23	58	B1	*IncFII*, *IncFIB*, *Col156*, *ColRNAI*, *Col(MG828)*	*intI1*	–	–	*dfrA5_1*, *blaTEM-1B_1*, *aph(6)-Id_1*, *aph (3″)-Ib_5*, *sul2_3* [50]	*arsB*, *arsC*, *zntA*, *rcnA*	*merA*
32	1079	B1	*Incl1*, *IncY*, *IncFIB*	–	–	*su2_14*	*tet(A)_6*, *aph(3″)-lb_2*, *aph(6)-Id_1*, *blaTEM-1B_1*	*rcnA*, *arsB*, *zntA*	–
33	10	A	*Incl1*, *IncFIB*, *ColRNAI*	–	*cdt*, *cnf2*	–	*tet(A)_6*, *sul2_14*, *aph(6)-Id_1*, *aph(3″)-lb_2*, *blaTEM-1B_1*	*rcnA*, *zntA*, *arsB*	–
36	685	A	*IncFIB*, *IncB/O/K/Z*, *ColRNAI*	–	–	–	*floR_2*, *tet(Y)_1*	*zntA*, *rcnA*, *arsB*	–
42	95	B2	*IncB/O/K/Z* [13]	–	*cdtABC*	–	*sul2_2*, *aph(3″)-lb_5*, *blaTEM-1C_1*, *aph (6)-Id_1*	*rcnA*, *zntA*	–

N = isolate number (see also Fig. 2); ST = serotype; ARG = antimicrobial resistance gene; MRG = metal resistance gene; VG = virulence gene

Table 4
Fisher's exact test results for each ARG-MRG pair in the groundwater-derived *E. coli*

MRGs	ARGs						
	strA [a]	strB [b]	blaTEM	floR	sul2	tetA	tetB
arsA	0.854	0.875	0.875	0.958	0.875	0.917	0.979
arsB	0.608	0.688	0.688	0.657	0.688	0.423	0.813
arsC	**0.019***	**0.013***	**0.013***	0.082**	**0.013***	0.161	0.958
pcoA	0.616	0.664	0.664	0.878	0.664	0.766	0.938
pcoB	0.727	0.763	0.763	0.918	0.763	0.839	0.958
rcnA	0.616	0.664	0.664	0.878	0.664	0.766	0.938
zntA	0.854	0.875	0.875	0.958	0.875	0.917	0.979

*$p < 0.05$ (highlighted in bold); ** $0.05 < p < 0.1$; ARG = antimicrobial resistance gene, MRG = metal resistance gene; *drfA5*, *tet(Y)*, *pcoC*, *pcoD*, *merA*, and *rcnB* not included as they were carried by a single isolate; [a] encodes *aph(3″)-lb*; [b] encodes *aph(6)-Id*.

and Berendonk (2012) determined MCCs for these metals in water (i.e., Cd = 0.03 μg/L, Co = 0.05 μg/L, Cu = 1.5 μg/L, Ni = 0.29 μg/L, Pb = 0.15 μg/L; Zn = 19.61 μg/L). Notably, median concentrations found in the 8 water samples containing resistant-*E. coli* exceeded MCCs for all metals except Cadmium (Table 1B). However, as MCC exceedances were also found in 39/40 water samples harbouring susceptible-*E. coli* (see supplementary materials) and in the absence of significant associations between metal/metalloid concentrations and encoded ARG abundance (Table 5) findings suggests that metal co-selection is unlikely to be taking place. Statistical analyses (Table 3) also indicate that *arsC*, an arsenate reductase gene (Hobman and Crossman, 2015) detected in two isolates, was significantly associated with the presence of five separate ARGs: *aph(3″)-lb* (*strA*; p = 0.019), *aph(6)-Id* (*strB*; p = 0.013), *blaTEM* (p = 0.013), *sul2* (p = 0.013), and *dfrA5* (p = 0.045). It is important to highlight, however, that association does not mean causation, and may merely indicate that *arsC* and the listed ARGs have a shared origin, with arsenic identified as an unintentional livestock feed pollutant in the RoI (Mac Monagail et al., 2018), in addition to geogenic soil sources in recharge waters. Moreover, a single isolate (isolate 23) displayed genetic associations between encoded mercury-resistance gene *merA* and five ARGs. The low sample numbers and very low number of positive AMR outcomes preclude definitive conclusions, however, and further investigations are required to examine these relationships. Undeniably, focusing solely on specific bacterial species can provide a limited picture of the local resistome (e.g., other bacterial species may be present which carry ARGs even in the absence of resistant-*E. coli*). Thus, a combined analytical approach employing (1) targeted quantitative polymerase chain reaction (qPCR) of ARGs within groundwater samples in addition to (2) WGS of local bacterial isolates (following phenotypic testing) is recommended to provide an increasingly holistic assessment of AMR and existing (co-)selective pressures in these environments. Briefly, qPCR results can provide a complete picture of the local resistome, while

Table 5
Kruskal-Wallis test results assessing metal magnitude associations with abundance of ARGs and MRGs in the groundwater-derived *E. coli*

Trace metals	ARG abundance		MRG abundance	
	Variable Coefficient	*p*-value	Variable Coefficient	*p*-value
Silver (Ag)	4.46	0.347	3.83	0.574
Arsenic (As)	8.22	0.084**	2.46	0.783
Cadmium (Cd)	3.18	0.528	4.61	0.465
Cobalt (Co)	2.83	0.587	2.36	0.797
Chromium (Cr)	5.89	0.207	5.53	0.355
Copper (Cu)	0.70	0.951	4.36	0.498
Iron (Fe)	1.05	0.902	9.16	0.103
Manganese (Mn)	7.21	0.125	2.46	0.783
Nickel (Ni)	0.66	0.956	4.18	0.524
Lead (Pb)	1.62	0.805	2.86	0.722
Antimony (Sb)	7.48	0.112	5.72	0.335
Zinc (Zn)	2.09	0.720	3.52	0.621

** $0.05 < p < 0.1$; ARG = antimicrobial resistance gene; MRG = metal resistance genes.

bacterial-specific genome sequences allow for the identification of co-resistance. It is worth mentioning that while metagenomics is a useful tool for determining the gene types and relative abundances in environmental samples, it may not be the optimal method in this case due to the lower DNA yields expected from drinking (and supposedly cleaner) groundwater samples. Additionally, the authors highlight the importance of establishing MCCs specific to *E. coli*, as this species is the most widely used in water quality monitoring and surveillance, including in the field of groundwater contamination with AMR (Andrade et al., 2020).

Andrade et al. (2023) recently identified significant positive associations between presence of phenotypic AMR in tested *E. coli* and increased cattle density at the local level and, from this, hypothesised cattle farming as the likely origin of resistant-*E. coli*. This is further supported by the genotypic results for the same isolates presented here (Fig. 2), with prevalent ARGs being among the most abundant in previous studies of cattle-derived *E. coli* in the RoI (Karczmarczyk et al., 2011a, 2011b), namely: *aph(3″)-lb* (*strA*), *aph(6)-Id* (*strB*), *blaTEM*, *sul2*, and *tetA*. Occurrence of similar multidrug resistance profiles conferring resistance to veterinary antimicrobials were also common (i.e., *aph--sul-bla*$_{TEM}$ and *aph-tet-bla*$_{TEM}$ groupings found in 6/6 and 4/6 multidrug-resistant isolates respectively; Fig. 2) and may reflect combined therapy practices, with ampicillin, streptomycin, tetracycline, and sulphonamide antimicrobials in particular among the most commonly prescribed in bovine agriculture in RoI (Gibbons et al., 2014). Additionally, multiple ARGs appear to be located on transposon-like elements associated with IS*Vsa*3 on *IncI* plasmids in four isolates (i.e., 6, 11, 32, and 33) with similar multidrug-resistance transposons found on *InC* plasmids from cattle-isolated *Salmonella enterica* (Lewis et al., 2023). Thus, and in light of the apparent absence of metal co-selection, it is plausible to suppose that ARG-acquisition is taking place inside these mammalian hosts and ARG-carrying isolates are transported to groundwater via faecal ingress (e.g., direct wellhead ingress of contaminated surface run-off; Andrade et al., 2023) owing, for example, to inappropriate faeces storage, manure/slurry spreading, and animal excretion while grazing. However, specific investigations quantifying and examining the impact of both trace metal/metalloids and antimicrobial residue concentrations in *E. coli*-borne ARG abundance are needed to confirm this.

Examining the antimicrobial resistant STs (see Table 3), it is apparent that two of the seven have been previously associated exclusively with animals (including livestock), namely: ST164 (Wang et al., 2020), and ST1079 (Barth et al., 2016; Apostolakos et al., 2017), while the other five have been found in both humans and farm animals (i.e., poultry, pigs, horses, and/or cattle) – i.e., ST10 (Manges et al., 2015), ST58 (Reid et al., 2022), ST95 (Manges et al., 2015), ST155 (Aworh et al., 2021), and ST685 (Souverein et al., 2016; Apostolakos et al., 2017). Additionally, 86.5% of resistant-*E. coli* isolates (n = 7/8) belonged to A or B1 phylogenetic classification groups, which were also the most common in *E. coli* from Irish cattle – accounting for 90, 86, and 79% in cattle from healthy farm herds (Karczmarczyk et al., 2011a), those with clinical mastitis symptoms (Keane, 2016), and from veterinary hospitals (Karczmarczyk et al., 2011b), respectively. *E. coli* belonging to phylogroup B1 have also been most frequently detected from surface waters affected by livestock agriculture internationally (Berthe et al., 2013; Tymensen et al., 2015). Although phylogenetic groups B2 and D have a greater frequency and diversity of virulence traits (Johnson et al., 2001), phylogroups A and B1 have also been isolated from humans, accounting for 46, 40 and 33% of human-derived *E. coli* in France (Escobar-Páramo et al., 2004), Sweden (Nowrouzian et al., 2005) and Canada (Tropea et al., 2021), respectively, as well as other animals (Tenaillon et al., 2010; Tropea et al., 2021). Indeed, isolate number 23, which belonged to phylogroup B1 (ST58), was collected from a hand-dug well on a pig farm (the only one in the present study) and, thus, is likely of porcine (not bovine) origin. In line with this finding, it is worth noting that antimicrobial prescription rates for pig farming is the highest of all livestock activities in the RoI (despite cattle being the predominant livestock sector), with data from 2016 research and surveillance (combined) showing that the pig sector used over 42% of all veterinary antimicrobials sold that year (Martin et al., 2020).

Resistant-*E. coli* presence within groundwater sources used for human consumption, particularly in the absence of microbiological treatment such as is the case here, can lead to potential adverse health consequences. These are even more pronounced when considering that 31.2% of all *E. coli* isolates (n = 15) and 37.5% of the resistant-*E. coli* (n = 3) carried virulence genes (i.e., have the potential to cause infections). This similar distribution of virulence genes between AMR and non-AMR strains may relate to the low relative incidence of virulence in so-called commensal intestinal *E. coli* strains from phylogenetic groups A and B1 (Clermont et al., 2011; Cocchi et al., 2007), which comprised 7 of 8 (87.5%) AMR isolates. Susceptible pathogens included one ETEC (ST765 carrying the *espL2* virulence gene) and one STEC (ST8621 carrying the *stx2* virulence gene) isolate, with STEC recently shown to be associated with human sources of contamination (i.e., septic tanks) in a recent study of private wells in the RoI (Burke et al., 2023). Notedly, ST10, ST95 and ST58 seen in four resistant isolates (i.e., 50%) are among the most reported extraintestinal pathogenic *E. coli* globally (Manges et al., 2019). Such highly successful clones are adapted to colonisation and infection and thus can contribute significantly to the dissemination and increased health burden of AMR to humans and animals.

A previous study carried out in Canada reported that those using groundwater supplies harbouring resistant-*E. coli* were 1.26 times more likely to become colonised with resistant-*E. coli* (i.e., carry them in their gastrointestinal tract; Coleman et al., 2012), thus furthering the spread of AMR beyond the groundwater environment, as once in the human gut the ARG-carrying plasmids can be shared with other commensal, and potentially pathogenic, bacteria (Christaki et al., 2020). Human and animal hosts are also at an increased risk of resistant infection(s) upon colonisation with antimicrobial resistant bacteria (Tacconelli et al., 2009). This further highlights the importance of 'One Health' studies which investigate environments of human significance regardless of their contamination level, as they may still pose significant health risks to individuals and be noteworthy contributors to the spread of AMR (Larsson and Flach, 2022). Such is also true for countries/regions with presumably larger sources of AMR and/or metals, as purposely focusing sampling solely in high susceptibility areas may lead to skewed "worst case scenario" findings and, as such, provide unrealistic baseline data.

With this being an exploratory study of a lesser-contaminated environment a key limitation stems from the low number of *E. coli*-positive samples (i.e., 48 in total, and just 8 ARG-carrying isolates) which restricted statistical analyses and interpretation of findings. Another

limitation is the absence of antimicrobial residue analyses owing to resource constraints which precluded the analyses of selective pressures. Finally, it must be acknowledged, that further limitations stem from lack of information regarding ARG abundance/diversity at a community level (i.e., present in total DNA extracted from groundwater) such as could be obtained through targeted qPCR, and which would have allowed for a more complete picture of the local resistome. It is evident that heavily contaminated environments have a more pronounced impact in the overall dissemination of AMR, however the current study highlights that even presumably 'cleaner' groundwater environments, when of human significance, are still relevant reservoirs for antimicrobial resistant bacteria.

5. Conclusion

The present study analysed data from 48 *E. coli*-harbouring domestic groundwater supply samples in rural areas of the RoI. Findings show no significant association between (overarchingly low) metal concentrations and abundance of ARG (or MGE) carried by the groundwater *E. coli* and suggest that the bacteria may have acquired the resistance genes prior to groundwater ingress. Genomic analysis results also show just one case of co-resistance (i.e. ARGs and MRGs located on the same plasmid) and no instances of re-occurring resistant-*E. coli* contamination. Therefore, these environments may be less likely to become long-term (i.e., continuous) AMR reservoirs, which is a key concern for more exposed fresh and marine water environments. However, while co-selective pressures may not be present, rural groundwater supplies are still noteworthy pathways for the dissemination of AMR insofar as they are commonly used for human consumption both in the RoI and abroad. A key recommendation from the current study is that future research combines qPCR with WGS of specific bacterial isolates, where possible, to better quantify AMR outcomes and (co)-selective relationships in these environments. The authors also emphasise the importance of carrying out such investigations in groundwater environments of human significance worldwide and regardless of their presumed level of exposure to antimicrobial drugs, antimicrobial resistant bacteria, and/or co-selective contaminants. Gathered information can help inform holistic/'One Health' AMR action plans and safeguard human (and animal) health.

Credit author statement

Luisa Andrade: Conceptualization, Methodology, Investigation, Formal analysis, Data Curation, Visualisation, Writing - original draft, Writing – review & editing. Michael P. Ryan: Formal analysis, Validation, Resources, Writing – Review & Editing. Liam Burke: Data Curation, Formal analysis, Resources, Writing – Review & Editing. Paul Hynds: Conceptualization, Writing - review & editing, Funding acquisition, Supervision. John Weatherill: Conceptualization, Writing - review & editing, Supervision, Funding acquisition. Jean O'Dwyer: Conceptualization, Methodology, Writing - review & editing, Funding acquisition, Supervision.

Declaration of competing 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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