

# The characterisation of antimicrobial resistant *Escherichia coli* from dairy calves

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## Abstract

**Introduction.** Dairy calves, particularly pre-weaned calves have been identified as a common source of multidrug resistant (MDR) *Escherichia coli*.

**Gap statement.** *E. coli* strains isolated from dairy calves and the location of their resistance genes (plasmid or chromosomal) have not been well characterised.

**Aim.** To characterise the phenotypic and genotypic features as well as the population structure of antimicrobial-resistant *E. coli* isolated from calves located on dairy farms that feed waste-milk to their replacement calves.

**Methodology.** Recto-anal swab enrichments from 40 dairy calves ( $\leq 14$  days old) located on four dairy farms were examined for tetracycline, streptomycin, ciprofloxacin, and third-generation cephalosporin resistant *E. coli*. Whole genome sequencing was performed using both short- and long-read technologies on selected antimicrobial resistant *E. coli*.

**Results.** Fifty-eight percent (23/40) of calves harboured antimicrobial resistant *E. coli*: 43% (17/40) harboured tetracycline resistant, and 23% (9/40) harboured chromosomal mediated AmpC producing *E. coli*. Whole genome sequencing of 27 isolates revealed five sequence types, with ST88 being the dominant ST (17/27, 63% of the sequenced isolates) followed by ST1308 (3/27, 11%), along with the extraintestinal pathogenic *E. coli* lineages ST69 (3/27, 11%), ST10 (2/27, 7%), and ST58 (2/27, 7%). Additionally, 16 isolates were MDR, harbouring additional resistance genes that were not tested phenotypically. Oxford Nanopore long-read sequencing technologies enabled the location of multiple resistant gene cassettes in IncF plasmids to be determined.

**Conclusion.** Our study identified a high incidence of tetracycline and streptomycin-resistant *E. coli* in dairy calves, and highlighted the presence of multidrug-resistant strains, emphasising the need for further investigation into potential associations with farm management practices.

## DATA SUMMARY

All genome data for this study have been deposited in GenBank. Illumina sequence reads and assemblies were deposited under accession PRJNA938096. The ST10, ST69, and ST88 isolates with their accession details are given in Table S3, available in the online version of this article. The supporting external data included in this study can be found under Bioprojects PRJNA656363, PRJNA292667, PRJNA338676, PRJNA357722, PRJNA398288, PRJNA510384, PRJNA531554, PRJNA566319, PRJNA576546, PRJNA600954, PRJNA659030, PRJNA716674, PRJNA767367, PRJNA837629, PRJNA844174, PRJNA944375. The authors confirm all supporting data and protocols have been provided within the article or through supplementary data files.

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**Keywords:** dairy; calves; *Escherichia coli*; antimicrobial resistance.

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All genome data for this study have been deposited in GenBank under the Bioproject accession PRJNA938096.

One supplementary figures and five supplementary tables are available with the online version of this article.

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### Impact Statement

This study highlights the prevalence of multidrug resistant (MDR) *Escherichia coli* in pre-weaned dairy calves. Whole genome sequencing revealed five sequence types of *E. coli*, with ST88 being the dominant ST. Comparative phylogenetic analysis determined that the ST69 and ST10 calf isolates were distinct from other New Zealand human, animal, and environmental isolates. The use of long-read sequencing enabled the location of multiple resistance gene cassettes to be determined. Overall, this study provides valuable insights into the prevalence and genetic characteristics of MDR *E. coli* in dairy calves and emphasizes the need for effective strategies to mitigate the spread of antimicrobial resistance in animal populations.

## INTRODUCTION

Antimicrobial resistance (AMR) has become a global human, animal and environmental health concern threatening current effective prevention and treatment options. Although current evidence indicates that human reservoirs are the main sources for the spread of resistant bacteria within human populations, the emergence and transmission of resistant strains does occur via other pathways including contact with animals and ingestion of food products.

The use of antimicrobials in livestock has come under critical review, due to the association of ‘routine’ antimicrobial use in livestock with the emergence and spread of AMR. Antibiotic use in food-producing animals is low in New Zealand compared to the United States, Canada, Australia, and many European countries [1]. Generally, low antibiotic use is reported in cattle, compared to other food animals such as swine or poultry [2]. In the dairy sector, antibiotics are used to treat a variety of infections including endometritis, mastitis, foot-rot, and respiratory infections [3]. Of particular concern are the third and fourth generation cephalosporins which have been associated with a dairy farm being positive for ESBL and AmpC producing *Enterobacteriaceae* [4–6]. The use of other antimicrobials may also be important for the selection of other AMR determinants, including multidrug resistant (MDR) *E. coli*. Other farm management practices, such as waste milk feeding and housing, may also be associated with the emergence and spread of antimicrobial resistant bacteria [7, 8].

Young calves (up to 2 weeks in age) are more likely to shed antimicrobial resistant *E. coli*, compared with older calves or lactating cows [9–12]. Additionally, the abundance of resistance genes decreases in the gut of calves over time [13]. Feeding waste milk to calves has been associated with changes in the calf microbiome, and an increase in antimicrobial resistant bacteria and their genes [14–16]. Waste milk is milk from cows that have been treated with antibiotics or other types of drugs before the withholding period is over. Waste milk also refers to milk with increased somatic cell counts (over 150000 cells ml<sup>-1</sup>) [17]. Studies have found that waste milk containing fourth generation or third generation cephalosporins, was associated with the presence of third generation cephalosporin resistant *E. coli* [18–20]. Additionally, other types of antimicrobial resistant *E. coli* have been associated with waste milk feeding such as chloramphenicol, aminoglycoside, and beta-lactam resistance [14, 21]. However, the genetic basis for these resistance types of *E. coli* has not been well described. In this study, our aim was to characterise the genotypic features and population structure of antimicrobial-resistant *E. coli* isolated from calves located on dairy farms that fed waste-milk to their replacement calves. We used whole genome sequencing, to investigate the genetic relationship of these *E. coli* with other calf and dairy cattle isolates as well as New Zealand human, environmental and companion animal isolates. Additionally, we aimed to explore the role of plasmids in mediating antibiotic resistance.

## METHODS

### Sampling

Sampling and enrichment were carried out as part of a previous study [22], where calf recto-anal mucosal swabs were collected from dairy farms across New Zealand and the swabs were enriched in modified Tryptone Soy Broth. The calves were predominantly Friesian and as previously described, healthy calves were randomly selected [22]. Our study utilised samples from four of these farms from the Canterbury region. (Table 1 and Table S1).

**Table 1.** Description of farms

Farm sample ID	Date sampled	Milking herd size	Breed	Other livestock	Replacement calves isolated	Colostrum
VCF77	11 Sep 2014	950	Friesian	No	Yes	Yes
VCF79	15 Sep 2014	1100	Friesian/Jersey or Jersey	Pigs and sheep	No	Yes
VCF80	15 Sep 2014	1000	Friesian	Pigs	Yes	Yes
VCF89	17 Sep 2014	1700	Friesian or Holstein	Pigs and sheep	Yes	Yes

## Isolation of *E. coli* and antibiotic susceptibility testing

*E. coli* was isolated from previously collected recto-anal mucosal swabs from calves (2–14 days old) enriched in modified Tryptone Soy Broth [22], by streaking onto four agar plates: MacConkey agar, two selective MacConkey agar plates (each containing 1 mg l<sup>-1</sup> cefotaxime sodium (Sigma Aldrich, St. Louis, MO, USA) or 1 mg l<sup>-1</sup> ceftazidime pentahydrate (Sigma Aldrich) as well as CHROMagar ESB (CHROMagar, Paris, France) and incubated at 35 °C overnight. Presumptive *E. coli* isolates were purified and identified as previously described [23]. Confirmed *E. coli* isolates were screened against the six antibiotics cefoxitin (30 µg), cefpodoxime (10 µg), cefotaxime (5 µg), tetracycline (30 µg), streptomycin (10 µg) and ciprofloxacin (5 µg) using the Kirby-Bauer disc diffusion tests with both EUCAST and CLSI guidelines. AmpC confirmation using a three-disc diffusion test (D69C AmpC disc test; Mast Group Ltd., Liverpool, UK) was carried out on those *E. coli* that were resistant to cefoxitin and an ESB confirmation disc diffusion test (D62C cefotaxime paired discs and D64C ceftazidime paired discs, Mast Group Ltd., Liverpool, UK) according to EUCAST guidelines, was carried out on those *E. coli* that were resistant to cefpodoxime and/or cefotaxime.

## Molecular characterisation

Crude DNA extractions were prepared using the heat lysis method where 3–4 colonies were suspended in 1 ml of nuclease-free water in an Eppendorf, heated at 100 °C for ten minutes, and the supernatant was stored at –20 °C for later PCR reactions. Confirmed antimicrobial resistant *E. coli* isolates were phylotyped by quadruplex PCR as described by Clermont *et al.* [24]. Confirmed AmpC producing isolates were further analysed by PCR to determine if they harboured genes encoding the plasmid-mediated AmpC enzymes (types MOX, CIT, DHA, ACC, and FOX) [25], or if they harboured a mutation in the promoter region of the *ampC* chromosomal gene as previously described [26]. A list of primers and annealing temperatures are described in Table S2.

## DNA extraction, whole genome sequencing and assembly

*E. coli* isolates that were resistant to at least one antibiotic (tetracycline, streptomycin, and/or cefoxitin) were selected for short-read whole genome sequencing. Six (one from at least one farm) of these isolates that were resistant to at least two classes of antibiotics, were also sequenced using long-read Oxford Nanopore Technologies (ONT). For short-read sequencing genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as per the manufacturers' instructions. The DNA was eluted in 50 µl of sterile PCR-grade water. The library preparations and next-generation sequencing were performed by either Massey Genome Service (Massey University, Palmerston North, New Zealand) using the Illumina MiSeq (San Diego, California, U.S.A) or Beijing Genomics Institute (BGI, Qingdao, China) using the BGI DNBSEQ. The Illumina Nextera XT library preparation kit (San Diego, California, U.S.A) was used to prepare the libraries as per the manufacturers' instructions and sequencing was performed on an Illumina MiSeq (San Diego, California, U.S.A). For sequencing using the BGI DNBSEQ platform, DNA was sheared into 250- to 300 bp fragments using a Covaris M220 Focused-Ultrasonicator (ThermoFisher Scientific, Waltham, MA, United States), and libraries were then prepared using the MGIEasy Universal DNA Library Prep Set (MGI, Shenzhen, China).

Raw DNA sequence reads were quality assessed using FastQC (v. 0.11.9, [www.bioinformatics.babraham.ac.uk/projects/fastqc](http://www.bioinformatics.babraham.ac.uk/projects/fastqc)), then trimmed and quality filtered using fastp (v. 0.11.9) [27], prior to assembly using SKESA (v. 2.3.0) as part of the Nullarbor pipeline (v. 2.0.20191013, <https://github.com/tseemann/nullarbor>) [28], using *E. coli* J1887 (accession CP014316) as the reference [29].

For long-read sequencing, genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and the libraries were prepared using the Rapid Barcoding Sequencing kit (SQK-RBK004; ONT, UK). The libraries were loaded onto a SpotON flow cell R9 version FLO-MIN106D and sequenced using a MinION Mk1B device (ONT, Oxford, England). Base-calling was carried out using Guppy (v. 6.1.2) with the super high accuracy model. Reads were demultiplexed using qcat (v. 1.1.0; <https://github.com/nanoporetech/qcat>), trimmed using Porechop (v. 0.2.4; <https://github.com/rrwick/Porechop>) and filtered using Filtlong (v. 0.2.1; <https://github.com/rrwick/Filtlong>), keeping a minimum length of 1000 bp. The reads were then assembled using Flye (v. 2.9) [30] or Unicycler (v. 0.4.4) [31]. The most robust assembly, based on genome completeness and accuracy [32, 33], was used for further analysis. Flye assemblies were polished with short-reads, using three rounds of Pilon (v. 1.24) [34]. All genome data for this study have been deposited in GenBank under the Bioproject accession PRJNA938096.

For all the genome assemblies the resistance genes, plasmid type and serotype were determined using ABRicate (v. 1.0.0, <https://github.com/tseemann/abricate>) with the databases (all updated 11 September 2022): ResFinder [35], CARD [36], PlasmidFinder [37], and EcoH [38].

## Genome sequences and bioinformatics

The sequence reads of other New Zealand ST10, ST69 strains as well as ST88 cattle and calf isolates previously sequenced by other institutes (Table S3) [39–46], were downloaded from the NCBI Sequence Read Archive database and processed using the Nullarbor pipeline (v. 2.0.20191013). Whole genome multi-locus sequence typing (wgMLST) was undertaken using Fast-GeP (v. 1.0) [47] and Single Nucleotide Polymorphism (SNP) analyses using Snippy (v. 4.3.6, <https://github.com/tseemann/snippy>).

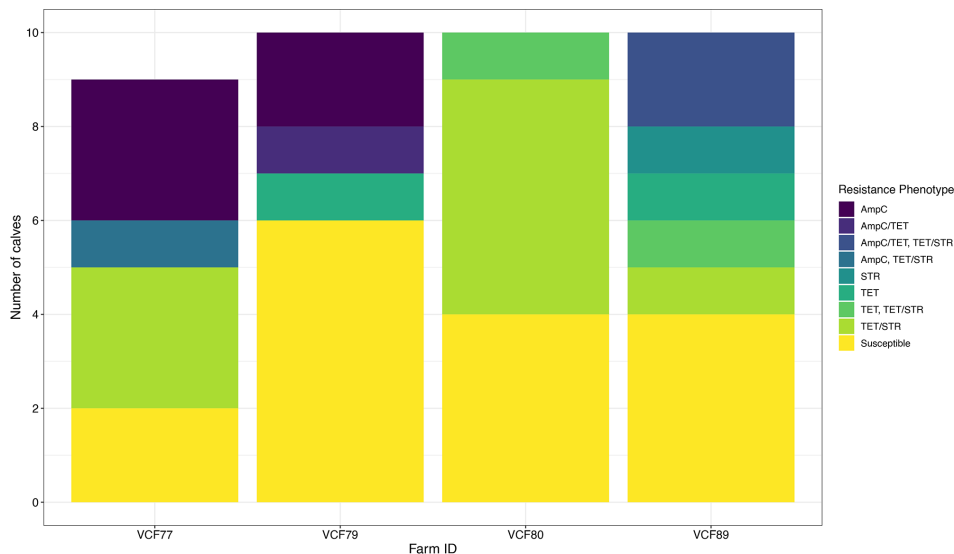


Fig. 1. Number of calves harbouring isolates that showed various resistance phenotypes from the four farms.

Neighbour-joining trees were generated using the resulting Snippy alignments in SplitsTree (v. 4.1.7.1) [48] and the final trees were visualised using the Interactive Tree of Life (iTOL v. 6.7) [49].

### Plasmid analysis

The closest plasmid relatives were determined using the plasmid database, PLSDB (v. 2021\_06\_32), using the default settings [50]. Plasmid and resistance cassette comparisons were carried out in EasyFig (v.2.2.2) [51]. Plasmid incompatibility and sequence types were determined using pMLST (v. 2.0) and PlasmidFinder (v. 2.0) [37].

## RESULTS

Forty recto-anal mucosal swab-enrichments previously collected from 2 to 9 day old calves (dairy replacement, bobby, and beef calves; Table S1) off four farms (Table 1) were screened for ESBL- and AmpC-producing, as well as tetracycline-, streptomycin- and ciprofloxacin-resistant *E. coli*. No *E. coli* were isolated from one calf from farm VCF77. Overall, 58% (23/40) of the calves harboured antibiotic resistant *E. coli*. Of these 23 calves, 17 harboured tetracycline resistant *E. coli*, 15 harboured streptomycin resistant *E. coli* and nine harboured AmpC-producing *E. coli* (Fig. 1). Additionally, 15 calves harboured *E. coli* that were co-resistant, to tetracycline, streptomycin and/or AmpC-producing (Fig. 1). None of the calves harboured ESBL-producing or ciprofloxacin resistant *E. coli*. The remaining 16 calves harboured *E. coli* that were not resistant to tetracycline, streptomycin, or the third generation cephalosporins.

### Genotypic characterisation of the antimicrobial resistant *E. coli*

A total of 57 resistant *E. coli* isolates from 23 calves were phylotyped using the Clermont method [24]. Results showed that the resistant *E. coli* isolates were distributed among phylogroups B1, C and D (Table 2). All the AmpC-producing *E. coli* were confirmed to have mutations in the promoter and attenuator regions of the chromosomal *ampC* gene at positions -42, -18, -1 and +58, and originated from farms VCF77, VCF79, or VCF89.

### Population structure of AmpC-producing, tetracycline resistant and streptomycin resistant *E. coli*.

Twenty-seven *E. coli* isolates displaying various AMR phenotypes were selected for whole genome sequencing to explore their genetic relatedness and diversity (Table 3, Fig. 2). The whole genome sequence data were used to determine sequence type (ST), serotype, plasmid type, and AMR genes. Most of the isolates belonged to ST88 (17/27, 63%) with the remaining belonging to ST69 (3/27, 11%), ST1308 (3/27, 11%), ST10 (2/27, 7%), and ST58 (2/27, 7%). There was variability in the ST of strains isolated from each farm.

The AMR genotype was concordant with the phenotypic results. Noticeably no plasmid mediated AmpC beta lactamase and ESBL genes were found. However, some additional AMR genes, *catA1* for chloramphenicol resistance, *dfrA1* and *dfrA5* for trimethoprim resistance, *sul1* and *sul2* for resistance to sulphonamides and *qacEdelta1* for resistance to quaternary ammonium were detected.

**Table 2.** Distribution of *E. coli* phylogroups among resistant *E. coli* isolates

	no. of isolates by phylogroup (No. of calves)						Total
	AmpC only		AmpC and TET	TET and/or STR resistant			
	B1	C	C	B1	C	D	
VCF77	12 (4)				2 (2)	3 (3)	17 (9)
VCF79		3 (2)	8 (2)		1 (1)		12 (5)
VCF80				2 (2)	8 (6)	1 (1)	11 (9)
VCF89			8 (2)	2 (2)	7 (5)		17 (9)
<b>Total</b>	<b>12 (4)</b>	<b>3 (2)</b>	<b>16 (4)</b>	<b>4 (4)</b>	<b>18 (14)</b>	<b>4 (4)</b>	

These were not tested for phenotypically. Based on their genotype, 16 of 27 isolates were MDR (defined as having ARGs conferring resistance to three or more classes of antibiotics). The ST69 isolates harboured the greatest number of resistance genes (8–10 ARGs). No ARGs, except for *mdf(A)* which encodes a multidrug efflux pump, were detected in the ST1308 genomes and in two ST88 genomes. In general, each clonal group harboured the same resistance determinants. Exceptions were the ST69 isolates CE0007a, CE009a and CE0010a which had differences in the number and type of genes conferring resistance to tetracycline as well as the ST88 isolate CE0033b where only two resistance genes [*mdf(A)* and *tet(A)*] were detected.

To give insights into the transmission of these resistant *E. coli* from a One Health perspective a core SNP analysis was undertaken on the ST10 and ST69 isolates (Figs 3 and S1) [39]. The New Zealand dairy calf isolates from this study were distinct from the other New Zealand derived isolates but were closely related to each other, with the ST10 isolates from farms VCF77 and VCF80 differing by six SNPs suggesting they were derived from a shared source or that there was farm to farm transmission (Table S4).

*E. coli* ST88 from New Zealand have rarely undergone whole genome sequence analysis, but this sequence type has frequently been isolated from cattle, therefore, we compared the 17 ST88 isolates with other ST88 adult cattle and calf isolates (Fig. 4). Our New Zealand calf derived isolates were distributed throughout the phylogeny but generally clustered by farm. There was one exception where isolates from farms VCF89 and VCF79 (CE0026f, CE0027c, CE0032, and CE0033) differed by 3–46 SNPs.

None of the isolates could be classified as STEC and 23 were classified as ExPEC, containing at least two of the genes *afaA*, *iutA*, *kpsM*, and *papC* [52, 53]. Eleven isolates contained the gene *astA*, which encodes the enteroaggregative *E. coli* enterotoxin EAST1 and 23 isolates had at least one gene associated with siderophores (*fyuA*, *iutA*, and/or *iucC*).

### Plasmid analysis

Contigs harbouring plasmid replicons were identified from 25 isolates, of which five were fully assembled using long-read ONT sequencing. IncF was the most common plasmid replicon identified in the 25 isolates, followed by IncI (5/27 isolates), IncB/O/K/Z (3/27 isolates) and IncY (2/27 isolates). The IncF plasmids only harboured resistance genes.

Six isolates, originating from the four different farms, were selected for long-read sequencing to enable the genetic location of the AMR genes to be determined and the structure of the plasmids to be compared (Fig. 5). No plasmid was detected in isolate CE0011b. A similar backbone (including the genes required for conjugation) was shared across the five plasmids with differences in the resistance gene cassettes, mobilome and defence mechanisms. Plasmids pCE0026f and pCE0032f were near identical. Three different resistance gene cassettes were identified (Fig. 5b). The closest relatives of the five IncF complete plasmids from the long-read assemblies, originated from *E. coli*, *Salmonella enterica* and *Klebsiella pneumoniae* (Table S5) [54, 55].

## DISCUSSION

### Presence of resistance phenotypes and genotypes

The main aim of this study was to phenotypically and genotypically characterise antimicrobial resistant *E. coli* isolated from dairy calves located on farms where waste milk feeding is practiced. *E. coli* displaying resistance to two or more classes of antibiotics were isolated from 15/40 calves. In our study phenotypic resistance was characterised for only four classes of antibiotics (beta-lactams, tetracyclines, aminoglycosides and fluoroquinolones). For isolates selected from MacConkey agar with no antibiotics, tetracycline resistance was the most common resistance phenotype displayed. Tetracycline resistant *E. coli* have been frequently isolated from young calves [56, 57], and to our knowledge no farm management practices, including antimicrobial use, have been shown to be associated with the presence of these resistant bacteria [56, 58].

Tetracycline resistance genes have frequently been detected in both the gut microbiome of dairy calves and their environment [59, 60]. In our study, tetracycline resistance genes (*tetA* and *tetB*) were the most prevalent. On New Zealand dairy farms

**Table 3.** Genomic characteristics of the 27 *E. coli* isolates

Isolate	Farm	Phylo-group	ST	Serotype*	Genome size (bp)	Assembly method	No. contigs	Plasmid type	AMR genes
CE0002c	77	B1	ST1308	ONT:H20	5026889	SKESA	101	IncFIC	<i>mdf(A)</i>
CE0006c	77	B1	ST1308	ONT:H20	5032406	SKESA	96	IncFIC	<i>mdf(A)</i>
CE0007a†	77	C	ST69	O17/O44: H18	5155817	Flye‡	2	IncFIA/IncFIB/ IncFII	<i>sul1</i> , <i>ant(3'')-Ia</i> , <i>dfrA1</i> , <i>catA1</i> , <i>mdf(A)</i> , <i>aph(3'')-Ia</i> , <i>aph(6)-Id</i> , <i>aph(3'')-Ib</i> , <i>tet(B)</i> , <i>sul2</i>
CE0007c	77	B1	ST1308	ONT:H20	5036665	SKESA	92	IncFIC	<i>mdf(A)</i>
CE0008b	77	D	ST10	O32:H9	5049054	SKESA	221	N/D	<i>mdf(A)</i> , <i>sul2</i> , <i>tet(A)</i> , <i>aph(6)-Id</i> , <i>aph(3'')-Ib</i>
CE0009b	77	C	ST69	ONT: H18	5223496	SKESA	467	IncFIA/IncFIB	<i>tet(B)</i> , <i>sul1</i> , <i>ant(3'')-Ia</i> , <i>dfrA1</i> , <i>catA1</i> , <i>aph(3'')-Ib</i> , <i>aph(6)-Id</i> , <i>aph(3'')-Ia</i> , <i>sul2</i> , <i>mdf(A)</i>
CE0010a	77	D	ST69	O17/O44/O77:H18	5365196	SKESA	173	IncFIA/IncFII/ IncFIB	<i>aadA1</i> , <i>aph(3'')-Ib</i> , <i>aph(3'')-Ia</i> , <i>aph(6)-Id</i> , <i>catA1</i> , <i>dfrA1</i> , <i>sul1</i> , <i>sul2</i> , <i>tet(A)</i> , <i>tet(B)</i>
CE0011b†	80	D	ST10	O101: H9	5044749	Unicycler‡	2	N/D	<i>aph(3'')-Ib</i> , <i>aph(6)-Id</i> , <i>sul2</i> , <i>tet(A)</i>
CE0012a	80	B1	ST58	O8:H25	4890068	SKESA	99	IncFII/IncFIB	<i>dfrA5</i> , <i>tet(A)</i> , <i>mdf(A)</i> , <i>bla<sub>TEM-1B'</sub></i> , <i>aph(6)-Id</i> , <i>aph(3'')-Ib</i> , <i>sul2</i>
CE0013a	80	C	ST88	O9: H19	5204498	SKESA	128	IncFIA/IncFIB	<i>aph(3'')-Ia</i> , <i>aph(6)-Id</i> , <i>aph(3'')-Ib</i> , <i>sul2</i> , <i>tet(B)</i> , <i>mdf(A)</i>
CE0014b	80	C	ST88	O9: H19	5161136	SKESA	117	IncFII/IncFIB	<i>sul1</i> , <i>dfrA5</i> , <i>aph(6)-Id</i> , <i>aph(3'')-Ib</i> , <i>sul2</i> , <i>tet(A)</i> , <i>mdf(A)</i> , <i>aph(3'')-Ia</i> , <i>bla<sub>TEM-1B</sub></i>
CE0015a	80	C	ST88	O9: H19	5169766	SKESA	95	IncFII/IncFIB	<i>tet(A)</i> , <i>mdf(A)</i> , <i>aph(3'')-Ia</i> , <i>bla<sub>TEM-1B'</sub></i> , <i>sul1</i> , <i>dfrA5</i> , <i>aph(6)-Id</i> , <i>aph(3'')-Ib</i> , <i>sul2</i>
CE0015b	80	C	ST88	O78: H4	5313154	SKESA	138	IncFII/IncB/O/ K/Z/IncFIB	<i>tet(A)</i> , <i>mdf(A)</i>
CE0017a	80	C	ST88	O11: H9	5239367	SKESA	154	IncFII/IncB/O/ K/Z/IncFIB/ Incl	<i>mdf(A)</i> , <i>bla<sub>TEM-1B'</sub></i> , <i>aph(6)-Id</i> , <i>aph(3'')-Ib</i> , <i>sul2</i> , <i>tet(A)</i> , <i>aph(3'')-Ia</i>

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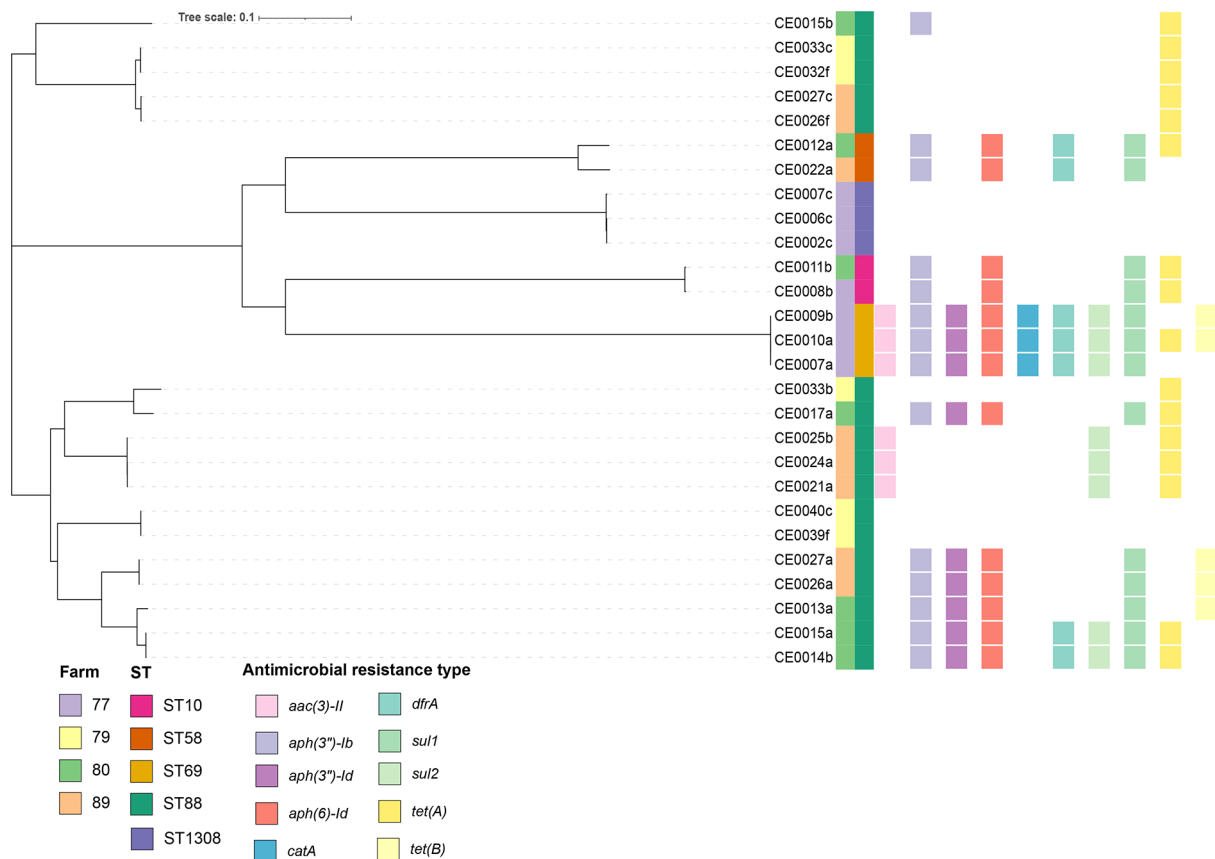
Table 3. Continued

Isolate	Farm	Phylo-group	ST	Serotype*	Genome size (bp)	Assembly method	No. contigs	Plasmid type	AMR genes
CE0021a†	89	C	ST88	O21: H21	5216590	Flye‡	2	IncFIB/IncFII	<i>mdf(A)</i> , <i>sul1</i> , <i>ant(3'')-Ia</i> , <i>tet(A)</i>
CE0022a	89	B1	ST58	O9:H9	4994399	SKESA	121	IncFIB/IncFII	<i>mdf(A)</i> , <i>bla<sub>TEM-1B'</sub></i> , <i>aph(3'')-Ib</i> , <i>sul2</i> , <i>dftrA5</i>
CE0024a	89	C	ST88	O21: H21	5200757	SKESA	136	IncFIB	<i>mdf(A)</i> , <i>sul1</i> , <i>ant(3'')-Ia</i> , <i>tet(A)</i>
CE0025b	89	C	ST88	O21: H21	5200223	SKESA	137	IncFIB	<i>sul1</i> , <i>ant(3'')-Ia</i> , <i>tet(A)</i> , <i>mdf(A)</i>
CE0026a†	89	C	ST88	O8: H4	5150963	Flye‡	3	IncFIA/IncFII/IncY/IncFIB	<i>mdf(A)</i> , <i>aph(3'')-Ib</i> , <i>aph(6)-Ia</i> , <i>aph(6)-Ia</i> , <i>bla<sub>TEM-1</sub></i> , <i>sul2</i> , <i>tet(B)</i>
CE0026f†	89	C	ST88	O32: H19	5240654	Flye‡	3	IncFIB/IncFII/IncII	<i>mdf(A)</i> , <i>tet(A)</i> , <i>bla<sub>TEM-1C</sub></i>
CE0027a	89	C	ST88	O8: H4	5156626	SKESA	122	IncFIA/IncFIB/IncY	<i>bla<sub>TEM-1B'</sub></i> , <i>tet(B)</i> , <i>aph(3'')-Ia</i> , <i>aph(6)-Ia</i> , <i>aph(3'')-Ib</i> , <i>sul2</i> , <i>mdf(A)</i>
CE0027c	89	C	ST88	O8:H19	5227125	SKESA	170	IncFIB/IncFII/IncII	<i>bla<sub>TEM-1C'</sub></i> , <i>mdf(A)</i> , <i>tet(A)</i>
CE0032f†	79	C	ST88	O32: H19	5229645	SKESA	3	IncFIB/IncFII/IncII	<i>mdf(A)</i> , <i>bla<sub>TEM-1C'</sub></i> , <i>tet(A)</i>
CE0033b	79	C	ST88	O128H9	5303646	SKESA	170	IncFIB/IncFII/IncB/O/K/Z	<i>mdf(A)</i> , <i>tet(A)</i>
CE0033c	79	C	ST88	O8/O32: H19	5229408	SKESA	106	IncFIB/IncFII/IncII	<i>tet(A)</i> , <i>mdf(A)</i> , <i>bla<sub>TEM-1C</sub></i>
CE0039f	79	C	ST88	O8:H19	5064751	SKESA	133	IncFIA/IncFIB/IncFIC	<i>mdf(A)</i>
CE0040c	79	C	ST88		5062176	SKESA	134	IncFIA/IncFIB/IncFIC	<i>mdf(A)</i>

\*O NT – where serotype O was non-typeable.

†Isolate sequenced using both Illumina and Oxford Nanopore Technologies

‡The most robust assembly method was used.



**Fig. 2.** Neighbour joining tree of 27 *E. coli* isolated from New Zealand dairy calves, generated from 3294 shared alleles identified using the wgMLST tool Fast-Gep. The first coloured strip represents the farm, the second strip the sequence type, followed by the presence (coloured squares) or absence of antimicrobial resistance genes.

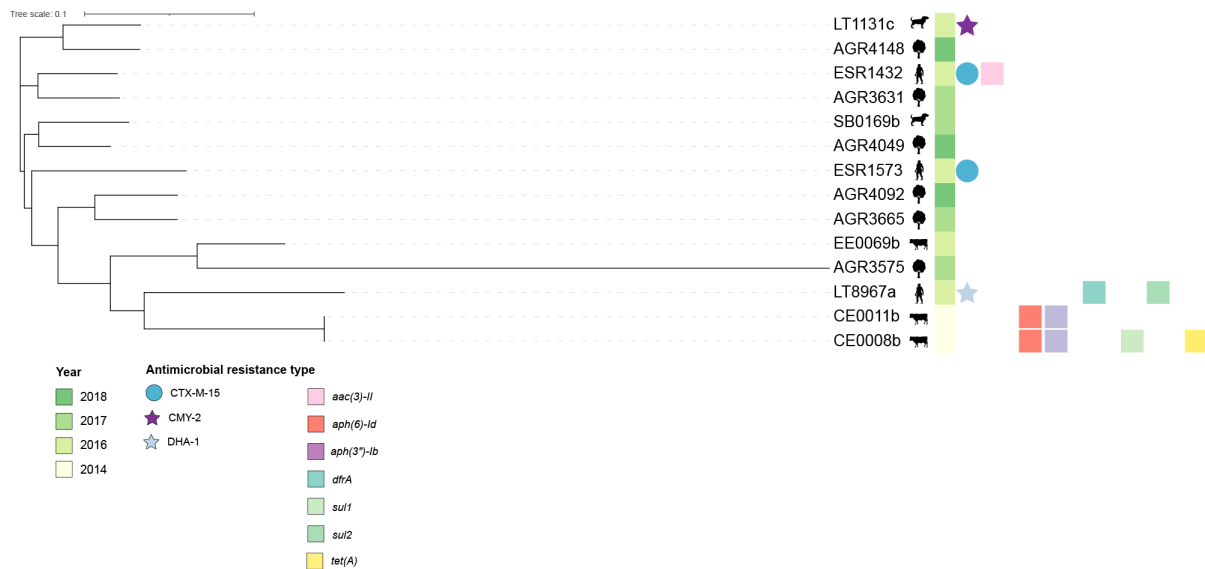
tetracyclines are the fourth most used antibiotic class (after penicillins, macrolides and cephalosporins), whereas aminoglycosides (including streptomycin, oleandomycin and neomycin) are rarely used [61, 62].

Whole genome sequencing enabled the detection of multiple resistant determinants covering six classes of antibiotics, including resistance genes for trimethoprim, sulphonamides and chloramphenicol were found that were not tested for phenotypically. Although our study identified a variety of different plasmid replicons, only the IncF plasmid type was associated with antimicrobial resistant genes. This contrasts with other studies which have identified a range of plasmid types associated with AMR in *E. coli* isolates from livestock [55]. Given trimethoprim/sulphonamides are rarely used on NZ dairy farms and chloramphenicols are prohibited for food-producing animal use, we hypothesise that the resistance genes for these antibiotics (*dfrA*, *sul1*, *sul2*, and *catA*) were co-selected with other resistance genes. As previously found in other studies these genes were co-located on the same plasmid as aminoglycoside and tetracycline resistance genes as well as the *qacEdelta1* gene, which encodes resistance to quaternary ammonium compounds. Disinfectants are used on dairy farms, including quaternary ammonium compounds which are sometimes used in the acid wash for cleaning milking equipment [63].

One *E. coli* strain in our study also harboured the *mer* operon on the same plasmid as the *tetA*, *sul1*, and *ant(3'')-Ia* resistance genes. The co-location of tetracycline, sulphonamide and aminoglycoside encoding resistance genes with the mercury operon on IncF plasmids has been previously described [54, 64, 65] and the presence of the *mer* operon was positively correlated with sulphonamide and aminoglycoside resistance genes, but not tetracycline resistance genes [65].

### Farm management practices and their association with antimicrobial resistance

Heavy metals and disinfectants have previously been shown to co-select for resistance genes [66, 67]. Other farm management practices, such as waste milk feeding, may also be associated with the presence of antimicrobial resistant *E. coli* [14, 21, 37, 68]. In the study by Maynou, Migura-Garcia [14] feeding waste milk was associated with beta-lactam and florfenicol resistance, but not tetracycline or aminoglycoside resistance. Waste milk has also been identified as a risk factor for ESBL/AmpC-producing



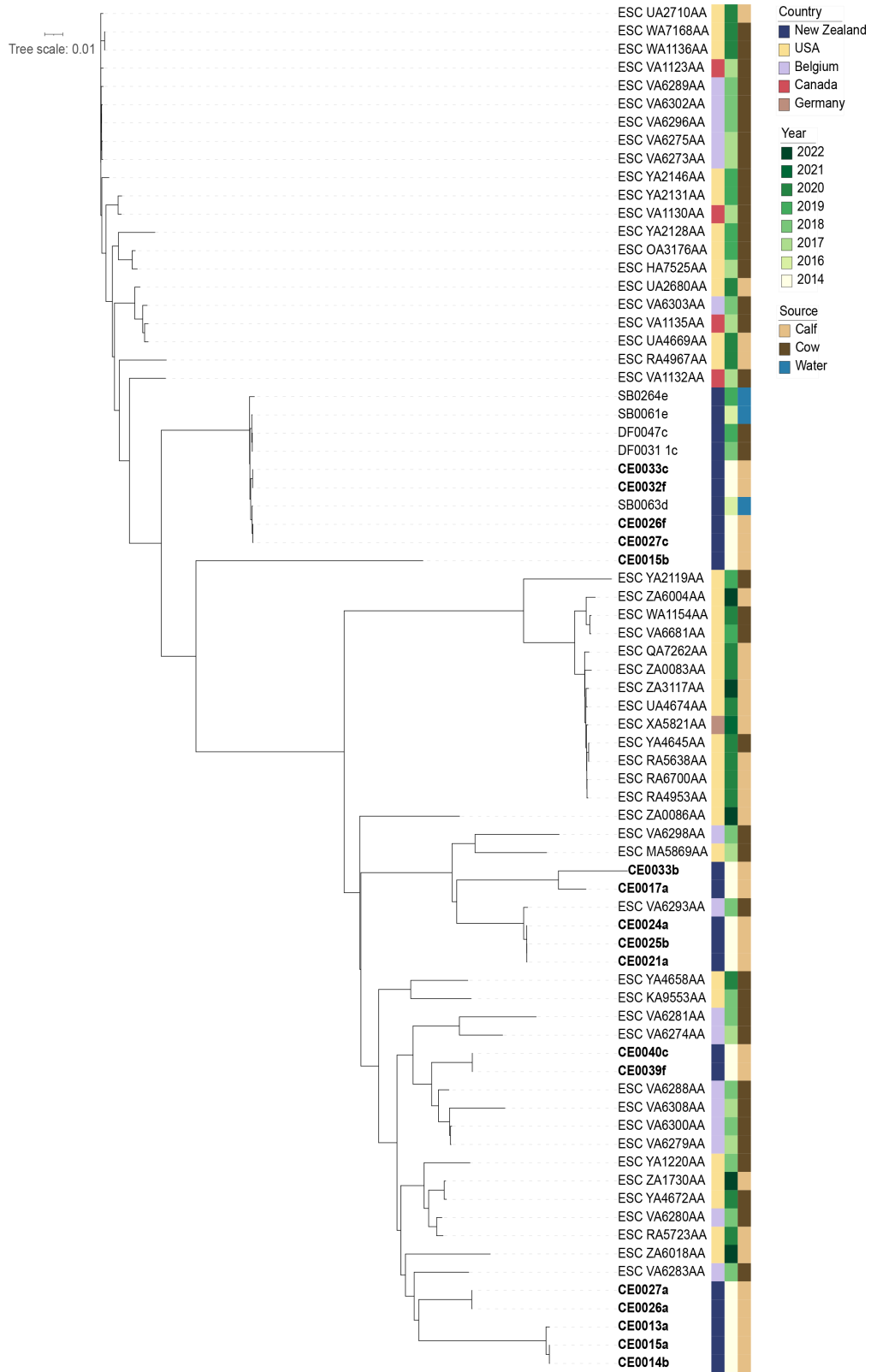
**Fig. 3.** Neighbour joining phylogeny of New Zealand *E. coli* ST10 isolates generated using 30,198 core SNPs. CE0011b was used as the reference. The symbols represent the source (dog, natural environment, human, or cattle/calf) of the isolate, the coloured strip represents the year of isolation, the circles represent the presence of the *bla*<sub>CTX-M</sub> gene, the stars the presence of the *bla*<sub>CMY</sub> or *bla*<sub>DHA-1</sub> gene and the squares the presence of other antimicrobial resistance genes.

*E. coli* positive calves [11]. Studies have shown that by adding antimicrobials (penicillin) to waste milk an increased antibiotic concentration is associated with an increased number of antimicrobial resistant faecal bacteria [16, 69, 70]. Our study investigated calves from farms who fed waste milk to their keeper calves but did not include farms that do not use waste milk as a feed, therefore associations between the use of waste milk and the presence of antimicrobial resistant bacteria could not be made. As with our study many other studies have hypothesised that the presence of antimicrobial resistant bacteria is due to waste milk feeding. However, there are limited studies (due to the lack of controls) that have determined a relationship between waste milk feeding and the presence of specific resistant bacteria or their determinants [20, 60]. Although quantitative data was and is still lacking on the impact of feeding waste milk to calves, the European Food Safety Authority carried out a qualitative risk assessment and found that there was an increased risk that the feeding of waste milk to calves ‘will lead to increased faecal shedding of antimicrobial-resistant bacteria’ [17]. The genomic epidemiology of antimicrobial resistant *E. coli* in waste milk and their potential transfer to calves has not been well studied. Although, ESBL-producing *E. coli* have previously been isolated from waste milk [20] and a recent study found both the abundance and diversity of AMR determinants was greater in waste milk compared with bulk milk.

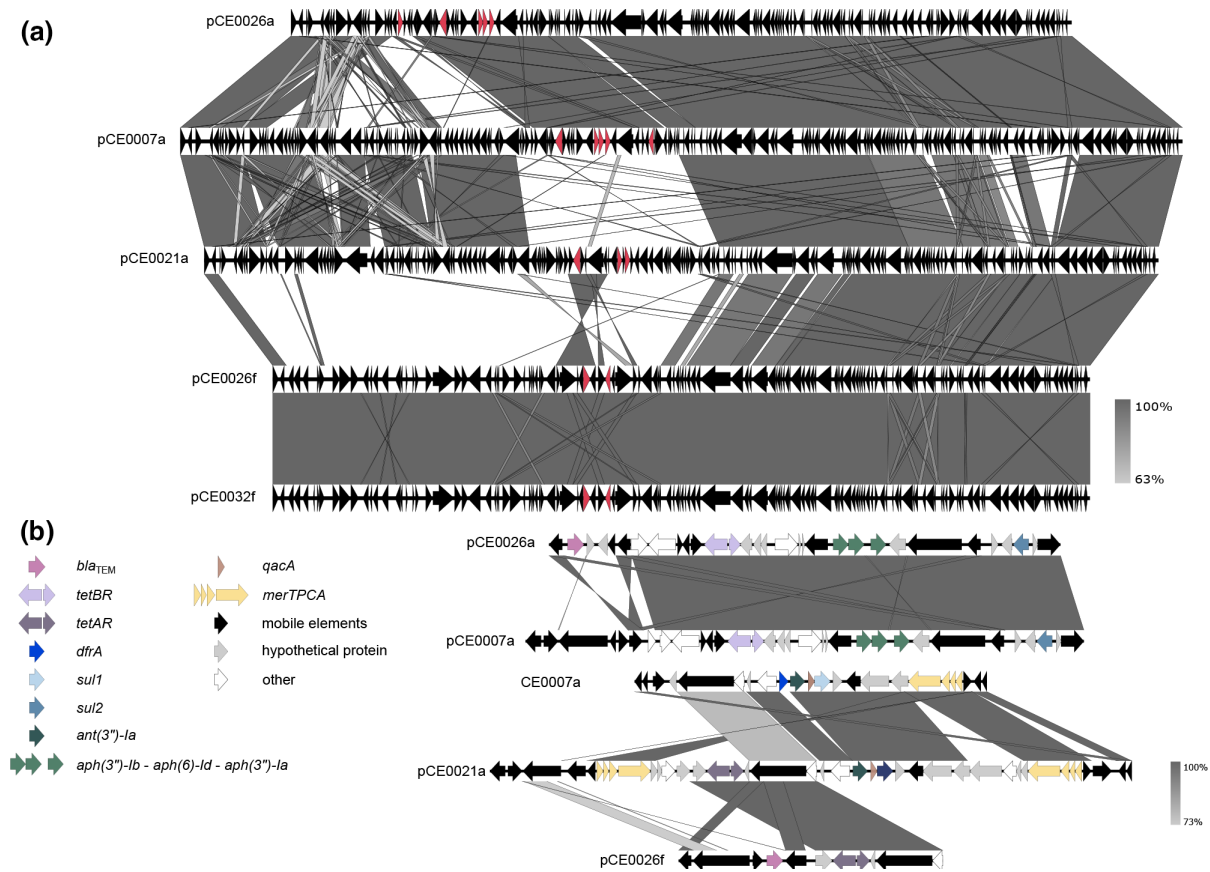
Sixteen *E. coli* isolates from three of the four farms were found to carry *E. coli* with a MDR genotype. Data on antibiotic use on our study farms was not available; therefore, we were unable to determine if low antimicrobial use was associated with no MDR *E. coli* being isolated from farm VCF79. The prevalence of MDR *E. coli* from healthy and sick calves has previously been reported to be high, ranging from 9.8–100% (of isolates from non-selective agar) in healthy calves [56, 71]; and 34–68% in diarrhoeic calves [56, 72] from Sweden and the USA. Previous studies have found that the prevalence of antimicrobial resistant *E. coli* and their genes is greater in calves compared with adult cattle [11, 58, 73]. Although our study did not directly compare calves with adult cattle; MDR *E. coli* have rarely been isolated from New Zealand adult cattle [7, 23, 62]. It has been proposed that MDR (to tetracycline, streptomycin and sulfadiazine) *E. coli* have adapted to the calf environment and are therefore more likely to persist and to be excreted compared with susceptible *E. coli* [58].

### Carriage of ESBL-/ACBL-producing *E. coli* within calves and dairy cattle

No ESBL or plasmid mediated ACBL producing *E. coli* were isolated from any of the calves, supporting findings from other studies that New Zealand dairy farms have a low prevalence of these resistant bacteria [23, 62, 74]. The low prevalence of ESBL/plasmid mediated ACBL producing *E. coli* could be due to New Zealand dairy farms being low users of antibiotics, particularly third and fourth generation cephalosporins [61, 62, 75]. Globally the prevalence of ESBL positive samples from calves range from 13–30% in the USA to 30% in Europe [71, 73, 76]. In our study those ACBL producing *E. coli* isolated were putative chromosomal mediated, with the mutations found in the promoter region of the *ampC* gene previously described in *E. coli* isolates from both calves and adult cattle [77, 78]. This present study also found that none of the putative AmpC hyperproducing isolates were MDR. This is in line with previous studies that have showed ESBLs and plasmid mediated AmpC, but not overexpressed chromosomal mediated AmpC, being associated with multidrug resistance [41, 79].



**Fig. 4.** Neighbour joining phylogeny of *E. coli* ST88 isolated from calves and adult cattle, generated using 19, 422 core SNPs. CE0026f was used as the reference. The first coloured strip represents the country of isolation, the second strip represents the year of isolation, and third strip represents the source.



**Fig. 5.** Comparison of IncF plasmids and their resistance gene cassettes. (a) Genetic maps and degree of homology between the IncF plasmids from five strains of *E. coli* isolated from New Zealand dairy calves. (b) Genetic maps and degree of homology between the resistance gene cassettes.

### Genetic diversity of MDR *E. coli*

In this study, using the seven-allele house-keeping genes MLST scheme five STs were observed with ST88 being the most common. Various studies have reported ST88 being common in both calves and adult cattle, additionally ST88 has been associated with the enterotoxigenic *E. coli* (ETEC) pathotype and AMR [77, 80, 81]. The other STs: ST10, ST69, ST58 and ST1308 have previously been isolated from both humans and livestock [81–84]. ST10, ST58, and ST69 are commonly associated with extraintestinal infections in humans [85, 86]. Our study demonstrated that those ST10 and ST69 strains that were previously isolated from the environment or animals (dairy cattle and calves, birds, and dogs) in New Zealand were distributed amongst the human isolates, but with no evidence of any sharing of strains between sources.

### CONCLUSION

In conclusion, our study found a high incidence of tetracycline and streptomycin resistant *E. coli* in dairy calves as has been found in other studies, but no ESBL/pACBL-producing *E. coli* in contrast to other European and US studies where these resistant bacteria are commonly isolated from both calves and adult cattle. Whole genome sequencing enabled the genetic location of the tetracycline and aminoglycoside resistance genes to be determined and demonstrated that 16 of 27 (60%) strains sequenced had a MDR genotype. Further investigations are required to determine whether these MDR *E. coli* are associated with the use of waste milk or other farm management practices.

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#### Author contributions

Conceptualisation and investigation, M.M., A.C., J.B., and S.B. Methodology and formal analysis, M.M., S.Z., A.F., and S.B. Data curation, A.F. and S.B. Supervision A.C., A.F., J.B., and S.B. Resources, S.Z. and J.B. Funding acquisition, S.B., A.C., and S.Z. Writing – original draft, M.M. and S.B. Writing– review and editing, A.C., A.F., S.Z., and J.B. All authors read and approved the final manuscript.

#### Conflicts of interest

The author(s) declare that there are no conflicts of interest.

#### Ethical statement

The Animal Ethics Committee of Massey University, Palmerston North, New Zealand approved the collection of recto-anal mucosal swabs from calves on 17 April 2014, under protocol number 14/29.

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