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The epidemiology of AmpC-producing *Escherichia coli* isolated from dairy cattle faeces on pasture-fed farms

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Abstract

Introduction. Antibiotic use, particularly amoxicillin-clavulanic acid in dairy farming, has been associated with an increased incidence of AmpC-hyperproducing *Escherichia coli*.

Gap statement. There is limited information on the incidence of AmpC-hyperproducing *E. coli* from seasonal pasture-fed dairy farms.

Aim. We undertook a New Zealand wide cross-sectional study to determine the prevalence of AmpC-producing *E. coli* carried by dairy cattle.

Methodology. Paddock faeces were sampled from twenty-six dairy farms and were processed for the selective growth of both extended-spectrum beta-lactamase (ESBL)- and AmpC-producing *E. coli*. Whole genome sequence analysis was carried out on 35 AmpC-producing *E. coli*.

Results. No ESBL- or plasmid mediated AmpC-producing *E. coli* were detected, but seven farms were positive for chromosomal mediated AmpC-hyperproducing *E. coli*. These seven farms were associated with a higher usage of injectable amoxicillin antibiotics. Whole genome sequence analysis of the AmpC-producing *E. coli* demonstrated that the same strain (<3 SNPs difference) of *E. coli* ST5729 was shared between cows on a single farm. Similarly, the same strain (≤15 SNPs difference) of *E. coli* ST8977 was shared across two farms (separated by approximately 425 km).

Conclusion. These results infer that both cow-to-cow and farm-to-farm transmission of AmpC-producing *E. coli* has occurred.

INTRODUCTION

Antibiotic usage for animal production and the potential contamination of food with antimicrobial resistant bacteria is an established consumer concern [1, 2]. Antimicrobial resistance is a true 'One Health' problem where infections associated with antimicrobial resistant bacteria result in poorer treatment outcomes and potentially death in both human and animal populations. The third generation cephalosporin (3GC) resistant *E. coli* are an important group of resistant bacteria associated with infections, but also

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Keywords: antimicrobial resistance; AmpC; dairy; *Escherichia coli*.
Abbreviations: amc, amoxicillin-clavulanic acid; CLSI, Clinical & Laboratory Standards Institute; ESBL, extended-spectrum beta-lactamase; F, Friesian- Holstein; 3GC, third generation cephalosporin; I, intermediate; inj, injectable; intra, intramammary; J, Jersey; MLST, multi-locus sequence typing; PCU, population correction unit; R, resistant; S, sensitive; SNP, single nucleotide polymorphism; ST, sequence type; XB, cross-breed.
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Six supplementary tables are available with the online version of this article.

asymptomatic carriage in both humans and animals [3–8]. Extended-spectrum beta-lactamases, plasmid mediated AmpC beta-lactamases and hyperproducing chromosomal encoded AmpC enzymes are all able to confer resistance to 3GCs. The CTX-M and CMY-2 types are the most common plasmid mediated extended-spectrum beta-lactamase (ESBL) and AmpC variants, respectively [3, 9]. The chromosomal *ampC* gene is usually constitutively expressed at low levels in *E. coli*. However, mutations in the promoter and attenuator regions results in overproduction of the AmpC enzyme, resulting in resistance to the second and third generation cephalosporins and sometimes also fourth generation cephalosporins [10–12].

Antibiotic use is one of the drivers for the spread of antimicrobial resistance. Although dairy farming is less reliant on antibiotics compared with pig and poultry farming, the prophylactic use of antibiotics (for dry cow therapy) is common practice to prevent intramammary infections during the dry period prior to calving. Additionally, both intramammary and systemic injectable antibiotics are important for the treatment of mastitis, metritis, endometritis, respiratory disease and foot-rot [13]. The use of 3GCs and beta-lactams have been associated with the presence of 3GC resistant *E. coli* in dairy cattle [14–16]. The carriage of these resistant bacteria may have implications for the spread of antimicrobial resistance to other sources as well as the treatment of mastitis-associated *E. coli*.

In New Zealand, dairy farms are predominantly seasonal pasture-based (ryegrass-clover) systems, with calving occurring late winter through to early spring (August – September) and cows dried off in late autumn (April – May). Antibiotics are primarily using for the prevention and treatment of mastitis [17]. Globally, New Zealand is a low user of antibiotics in food production systems, including dairy [18, 19]. Despite there being a low use of antibiotics, there is some evidence for an increase in mastitis associated resistant *Streptococcus uberis* [20]. However, there is little data on the use of antibiotics and prevalence of resistant Gram-negative bacteria. We recently carried out a cross-sectional survey across one New Zealand region and found a higher prevalence of overexpressed chromosomal *ampC E. coli* compared with plasmid mediated AmpC or ESBL producing *E. coli* [21]. It is unknown whether this observation was associated with the use of specific antibiotics and if this characteristic *E. coli* resistance phenotype occurred in dairy farms from other New Zealand regions. The objectives of this study were to assess the prevalence of AmpC-producing *E. coli* across New Zealand dairy farms and whether the presence of AmpC-producing *E. coli* were associated with the use of specific antibiotics.

METHODS

Sampling

Twenty-six dairy farms (Table 1), from four regions across New Zealand were visited in autumn or winter (between 15 May – 21 July 2017) as part of a parent study investigating the effectiveness of dry cow therapy [22]. On each farm, paddock faeces were collected, from 12 different pats using a sterile scoop and collection container, across a diagonal transect of a freshly grazed paddock. The samples were stored at 4 °C until they were transported on icepacks to the Hopkirk Research Centre, Palmerston North for processing.

Sample processing and bacterial culture

Four faecal samples were pooled by transferring approximately 1 g of faecal matter from each sample into 15 ml phosphate-buffered peptone and incubated for 18–20 h at 35 °C. Three pooled enrichments were generated for each farm and these were each given a sample number (ED##). The enrichments were streaked onto four agar plates: CHROMagar ESBL (CHROMagar, Paris, France), MacConkey agar (Fort Richard Laboratories, Auckland, New Zealand), MacConkey agar with 1 mg l^{-1} cefotaxime sodium (Sigma-Aldrich, St. Louis, U.S.A) or 1 mg l^{-1} ceftazidime pentahydrate (Sigma-Aldrich) and these were incubated for approximately 18 h at 35 °C. Two characteristic *E. coli* colonies from each agar (in total 2–6 colonies from each enrichment) were purified and identified as described previously [21]. ESBL- and AmpC-producing *E. coli* confirmation disc diffusion assays were carried out on all the presumptive ESBL- and AmpC-producers using a double-disc (for ESBL) and triple-disc (for AmpC) assessment methods, as per the EUCAST guidelines, described previously [23]. The reference strains *E. coli* NZRM4402, *Klebsiella pneumoniae* NZRM3681 and *E. coli* NZRM916 were used as controls in the confirmatory disc diffusion assays. Selected AmpC-producing *E. coli* were screened against six beta-lactams (ampicillin, amoxicillin plus clavulanic acid, cefoxitin, cefpodoxime, ceftazidime, cefotaxime, and cefepime) according to CLSI guidelines using Kirby-Bauer disc diffusion tests (Table S1, available in the online version of this article).

Whole genome sequencing and analysis

Genomic DNA was extracted from bacterial growth recovered off a Columbia horse blood agar plate, with a QIAamp DNA mini kit (Qiagen, Hilden, Germany). Libraries were made using the Nextera XT DNA library preparation kit (Illumina Inc., San Diego, USA) and sequencing was carried out using the Illumina HiSeq X platform (Novogene, Hong Kong, China) with 2×150 bp paired-end reads.

Raw reads were processed using previously described bioinformatics protocols [21], with the Nullarbor bioinformatics pipeline (v.2.20181010) [24]. Resistance genes were determined using ResFinder (v.3.1) [25], multi-locus sequence typing using MLST (v. 2.16.1) [26], *in silico* phylotyping using ClermonTyper (v. 1.4.0) [27] and serotypes using SerotypeFinder (v. 2.0) [28]. Core single nucleotide polymorphism (SNP) analysis was carried out using Snippy (v. 4.3.6) [29] using isolate ED0041f as the reference (Biosample accession SAMN18132542). A Neighbour-joining phylogenetic tree was generated using SplitsTree (v.4.14.8) [30], which was uploaded to the Interactive Tree of Life (iTOL v.5.7) for annotation [31]. Virulence genes were identified using VirulenceFinder (v.2.0, 2019-04-23) [32], with the virulence genes included

Table 1. Description of farms included in study. AmpC-hyperproducing *E. coli* were isolated from grey shaded farms

Farm	Region	Herd size	Breed (% of herd)			Antimicrobial use (mg PCU*$^{-1}$)			No. of AmpC positive-	
			F	J	XB	Total	Intra amc	Inj amc	pooled samples	isolates
DF001	Waikato	440	50.6	1.2	48.1	7.03	0	0	0/3	0
DF002	Waikato	450	47.2	5.6	47.2	4.43	0	0	0/3	0
DF003	Waikato	266	33.8	6.3	60.0	6.40	0	0	1/3	1
DF004	Waikato	288	46.3	0.0	53.7	13.82	0	0	0/3	0
DF005	Canterbury	680	65.1	0.0	34.9	11.80	0	0.73	0/3	0
DF006	Otago	1444	0.0	41.5	58.5	12.65	0	0.46	0/3	0
DF007	Canterbury	702	48.8	1.3	50.0	6.01	0	0.35	1/3	4
DF008	Canterbury	702	58.1	1.4	40.5	17.43	0	0.59	1/3	2
DF009	Waikato	1032	31.3	1.3	67.5	5.80	0	0.13	0/3	0
DF010	Waikato	332	0.0	94.1	5.9	15.35	0	0.10	0/3	0
DF011	Waikato	855	27.9	4.9	67.2	12.41	0.15	0.08	0/3	0
DF012	Waikato	378	36.5	1.4	62.2	20.65	0	0	0/3	0
DF013	Waikato	711	91.5	0.0	8.5	12.91	0	0	0/3	0
DF014	Southland	663	36.4	5.5	58.2	Unknown	Unknown	Unknown	0/3	0
DF015	Otago	1582	23.5	9.9	66.7	7.55	0.15	0.17	3/3	12
DF016	Waikato	275	97.1	0.0	2.9	20.92	0.19	0.36	0/3	0
DF017	Otago	730	58.2	1.3	40.5	9.66	0.22	0.23	0/3	0
DF018	Canterbury	549	67.5	0.0	32.5	14.42	0	1.2	1/3	1
DF019	Canterbury	745	13.4	11.0	75.6	4.39	0	0	0/3	0
DF020	Canterbury	509	84.0	0.0	16.0	12.21	0	0.18	0/3	0
DF021	Canterbury	813	48.8	0.0	51.3	7.16	0	0.29	3/3	14
DF022	Southland	573	85.5	0.0	14.5	Unknown	Unknown	Unknown	0/3	0
DF023	Otago	1907	14.5	0.0	85.5	6.72	0.08	0.05	1/3	2
DF024	Southland	1095	41.9	2.7	55.4	Unknown	Unknown	Unknown	0/3	0
DF025	Southland	696	85.5	0.0	14.5	Unknown	Unknown	Unknown	0/3	0
DF026	Southland	Unknown		Unknown		Unknown	Unknown	Unknown	0/3	0

*PCU, population correction unit is the kilogram of live weight per annum
amc, amoxicillin-clavulanic acid; F, Friesian-Holstein; inj, injectable; intra, intramammary; J, Jersey; XB, cross-breed.

in the VirulenceFinder database listed in Table S2. Sequence reads originating from this study have been deposited in the sequence reads archive under the BioProject number PRJNA706437. Accession numbers are listed in Table S3.

Data analysis

Farmers consented to antimicrobial use data taken from sales data obtained through the veterinary clinic which collected the samples. The fisher.test function in the R studio statistics package (v. 1.1.463) was used to investigate the association between the presence of AmpC-producing *E. coli* and the use of specific antibiotics.

RESULTS

Paddock faecal samples were collected from 26 dairy farms across four regions in New Zealand (ten farms in Waikato, seven farms in Canterbury, four farms in Otago and five farms in Southland). Three pooled faecal enrichments from each farm were screened for ESBL- and AmpC-producing *E. coli*; no ESBL producers were detected in any of the 78 enrichments, but 11/78 (14%) enrichments from 7/26 (27%) farms were found to contain AmpC-producing *E. coli* (Table 1). Although not statistically significant, a higher proportion of AmpC-positive enrichments (6/11, 0.55 [95% CI: 0.28–0.79])

Table 2. Promoter and attenuator nucleotide point mutation profiles of AmpC-hyperproducing *E. coli* obtained from this study

Isolate	Mutation profile					
	−82	−73	−42	−18	+1	+58
ED0006b	A->G	T->C	C	G->A	C->T	C->T
ED0058g ED0058h ED0058i	A->G	T->C	C->T	G->A	C->T	C->T
All other isolates (*n*=31)	A->G	T->C	C->T	G->A	C->T	C->T

*Numbering denotes position in the promoter or attenuator region as used by [49].

originated from four Canterbury farms, compared with the other regions (Otago: 4/11, 0.36 [95% CI: 0.12–0.68] enrichments from two farms; Waikato: 1/11, 0.09 [95% CI: 0.005–0.43] enrichments from one farm; Southland: 0 enrichments from five farms). In total 35 AmpC-producing *E. coli* were isolated from seven farms, with multiple isolates generally being purified from the same enrichment (Table S3). All the presumptive AmpC-producing *E. coli* (*n*=31) were isolated from MacConkey agar with ceftazidime or cefotaxime and were confirmed as being AmpC-positive using the AmpC confirmatory disc diffusion assay. The three presumptive ESBL producers (ED0058g, ED0058h, ED0058i) isolated from ESBL CHROMagar were not confirmed as being ESBL positive but were confirmed as being AmpC-positive. The remaining confirmed AmpC-positive isolate (ED0006b) originated from plain MacConkey.

Characterisation of the AmpC phenotype and genotype

Whole genome sequencing was carried out on all the AmpC-producing *E. coli*. All 35 isolates were phylogroup B1 with six different sequence types identified: ST45, ST351, ST1079, ST1730, ST5729 and ST8977 (Table S3). None of the isolates carried acquired resistance genes, but they all harboured mutations in the promoter region of the *ampC* gene (Table 2) including non-synonymous changes impacting the AmpC amino acid sequence (Table 3). Three different mutation profiles were identified compared with the non-AmpC *E. coli* control strain ATCC 25922 (Table 2). Isolates ED0058g, ED0058h and ED0058i, which were isolated from ESBL CHROMagar, all had an ALA13SER change in the signal peptide sequence. Next, we examined whether seven of the AmpC-producing isolates with a different mutation profile or sequence type had different beta lactam resistance phenotypes (Table 4). Six of the seven isolates were resistant to ampicillin, which is characteristic of an AmpC hyperproducing phenotype, and the remaining isolate ED006b which was isolated from plain MacConkey was resistant to cefoxitin. All the isolates originating from antibiotic agar were resistant to cefpodoxime and three isolates (ED0020c, ED0040c and ED0058c) were non-susceptible to ceftazidime and/or cefotaxime. The beta lactam resistance profile of strain ED0058g, which was isolated from ESBL CHROMagar, was similar to those strains isolated from MacConkey agar containing cefotaxime or ceftazidime.

The genetic relatedness of AmpC-producing *E. coli*

To determine whether strains of AmpC-producing *E. coli* were shared between farms, a core SNP comparison was carried out and a phylogenetic tree generated (Fig. 1a). *E. coli* ST8977 from Farm 21 and Farm 23 had SNP distances of 11–15 SNPs suggesting recent farm to farm transmission or a common source (Fig. 1b, Table S4). These two farms are separated by approximately 425 km. There was no other ST sharing between farms. However, multiple ST5279 isolates from different enrichment samples were obtained from farm 15 with 1–2 SNPs difference suggesting cow to cow transmission.

None of the isolates could be classified as ExPEC (presence of ≥2 genes characteristic of this pathotype) [33] or STEC [34]. Isolates ED023c and ED023d had the most genes (*n*=38 genes) associated with virulence, followed by isolate ED006b (*n*=34 genes) (Fig. 1a, Table S5). ED006b was the only isolate that had virulence genes associated with toxin production (*hylABCD* operon and *astA*). Isolates ED023c and ED023d carried multiple genes important for siderophore activity (*fyuA*, *irp*, *iutA, iucABCD*).

Antimicrobial use and its association with AmpC-positive *E. coli*

Over the 2016/2017 season penicillins were the most frequently used group of antibiotics as inferred from the sales data, followed by the macrolides, tetracyclines and aminopenicillins (Table S6). AmpC *E. coli* positive farms were associated with a larger herd size and greater antibiotic use (calculated as mg per population correction unit (PCU)) based on their sales data (Fig. 2). Six of the seven (86%) AmpC *E. coli* positive farms had purchased injectable amoxicillins inferring previous exposure to these antibiotics (OR 4.2, 95% CI: 0.3–240.5, *P*=0.3) compared with 1/7 (14%) AmpC-positive farms which had purchased intramammary amoxicillin (OR 0.43, 95% CI: 0.007–5.9, *P*=0.6) and similarly 6/7 (86%) of the AmpC-positive farms used ceftiofur. Of the AmpC *E. coli* negative farms, 8/14 (57%), 6/14 (43%) and 7/14 (50%) purchased injectable amoxicillins, intramammary amoxicillins, and ceftiofur respectively.

Table 3. Amino acid mutation profiles of AmpC-hyperproducing *E. coli* obtained from this study

	Position of amino acid (includes the signal peptide)																
Strain	13	191	209	210	231	235	236	248	251	254	255	257	261	298	304	312	316	366
ATCC 25922	A	K	S	A	A	E	A	R	Q	L	K	L	E	I	D	R	P	V
ED0006b	–	Q	P	–	–	–	–	–	R	M	N	R	D	S	G	H	A	–
ED0058g ED0058h ED0058i	S	Q	P	–	–	K	–	C	R	M	N	R	D	S	G	H	A	–
All other isolates (n=31)	–	Q	P	P	V	–	A	–	R	M	N	R	D	S	G	H	A	–
EC18 [50]	–	Q	S	P	–	–	–	–	R	M	N	R	D	S	G	H	A	F

DISCUSSION

In this study the prevalence of 3GC resistant *E. coli* was assessed across 26 New Zealand dairy farms, with seven farms (27%) found to be positive for AmpC-hyperproducing *E. coli*. This is in comparison with our previous regional cross-sectional study carried out in both spring and autumn, where 5/75 (7%) of enriched pooled faecal samples from across 4/15 (27%) farms were positive for AmpC-hyperproducing *E. coli* [21]. To our knowledge only one study in Israel determined that the prevalence of ESBL-producing *E. coli* were lower on pasture-based dairy farms compared with intensive dairy farms [35]. However, their study did not assess the prevalence of AmpC-producing *E. coli*. Few studies have found chromosomal mediated AmpC-hyperproducing *E. coli* to be more prevalent in dairy cattle compared with plasmid mediated AmpC [14, 36]. In previous cross-sectional studies carried out in Europe, of those *E. coli* isolates with an AmpC phenotype, 18–20% harboured a mutation in the *ampC* promoter and the remainder carried the $bla_{\text{CMY-2}}$ or $bla_{\text{CMY-4}}$ gene [15, 37]. The mutations found in the promoter and attenuator regions of the *E. coli* isolates reported from this work were the same as those previously found in both human and dairy cattle isolates [14, 38, 39]. However, although two of the AmpC isolates were sub-cultured from ESBL CHROMagar, they did not have an ESBL phenotype; whole genome sequencing revealed mutations in the signal peptide and amino acid sequence (changes ALA13SER, GLU235LYS, ARG248CYS) that to our knowledge have not been previously documented. However, these mutations did not result in an increased range of beta-lactam resistance phenotype compared with the other AmpC-producing isolates.

In vitro studies have shown that exposure to amoxicillin was associated with the development of AmpC hyperproduction in *E. coli* through mutations in the promoter/attenuator region of the *ampC* gene [40, 41]. This is in concordance with observations herein where an association between AmpC-positive farms and the use of injectable amoxicillin was noted, but not intramammary administered amoxicillin. This is not unexpected given intramammary administration of antibiotics would be predicted to have less or no impact on the gut and faecal microbiota compared with systemic injectable antibiotics. Other studies have demonstrated that the use of amoxicillin is a risk factor for the increased incidence of AmpC-hyperproducing *E. coli* [14].

Other AmpC-hyperproducing *E. coli* isolated from cattle belong to a diverse range of STs [14, 21, 42]. In a study from South West England [14], *E. coli* ST88 was found to be the dominant ST, in contrast to our study where ST8977 followed by ST5729 predominated. These STs have been rarely reported (Enterobase, https://enterobase.warwick.ac.uk/species/index/ecoli accessed 30 August 2021) [43], with three ST8977 strains reported to have been isolated from human urine, water, and soil, and 22 ST5729 strains from cattle, sheep, forage plants and soil. Our whole genome sequence analyses inferred that there was recent within farm transmission of ST5729 and both within and between farm transmission of ST8977. Previous

Table 4. Susceptibility* to extended-spectrum beta-lactams and cephalosporins by selected AmpC-hyperproducing *E. coli* isolates obtained from this study

Isolate	ST	Ampicillin	Amoxicillin and clavulanic acid	Cefoxitin	Cefpodoxime	Ceftazidime	Cefotaxime	Cefepime
ED0006b	ST351	S	S	R	S	S	S	S
ED0020c	ST8977	R	R	R	R	S	I	S
ED0023c	ST56	R	R	S	R	S	S	S
ED0040c	ST5729	R	R	S	R	I	I	S
ED0050c	ST1730	R	R	I	R	S	S	S
ED0058c	ST8977	R	R	R	R	S	I	S
ED0058g	ST1079	R	R	S	R	S	S	S

*Isolates were determined to be S, I or R as per CLSI guidelines, Table S1.
I, intermediate; R, resistant; S, sensitive; ST, sequence type.

studies have also used a core SNP approach to infer that farm-to-farm transmission of both AmpC-hyperproducing *E. coli* and mastitis associated *E. coli* has occurred; however as with our study no metadata was collected to explain the pathway of transmission, for example, through movement of cattle from one farm to another [14, 44].

A limitation to our study is that it is cross-sectional and does not take seasonal changes into consideration. The absence of ESBL-producing *E. coli* could be attributed to a low prevalence on New Zealand dairy farms, a small farm sample size and the collection of samples in autumn only. In our previous cross-sectional study, ESBL-producing *E. coli* were detected

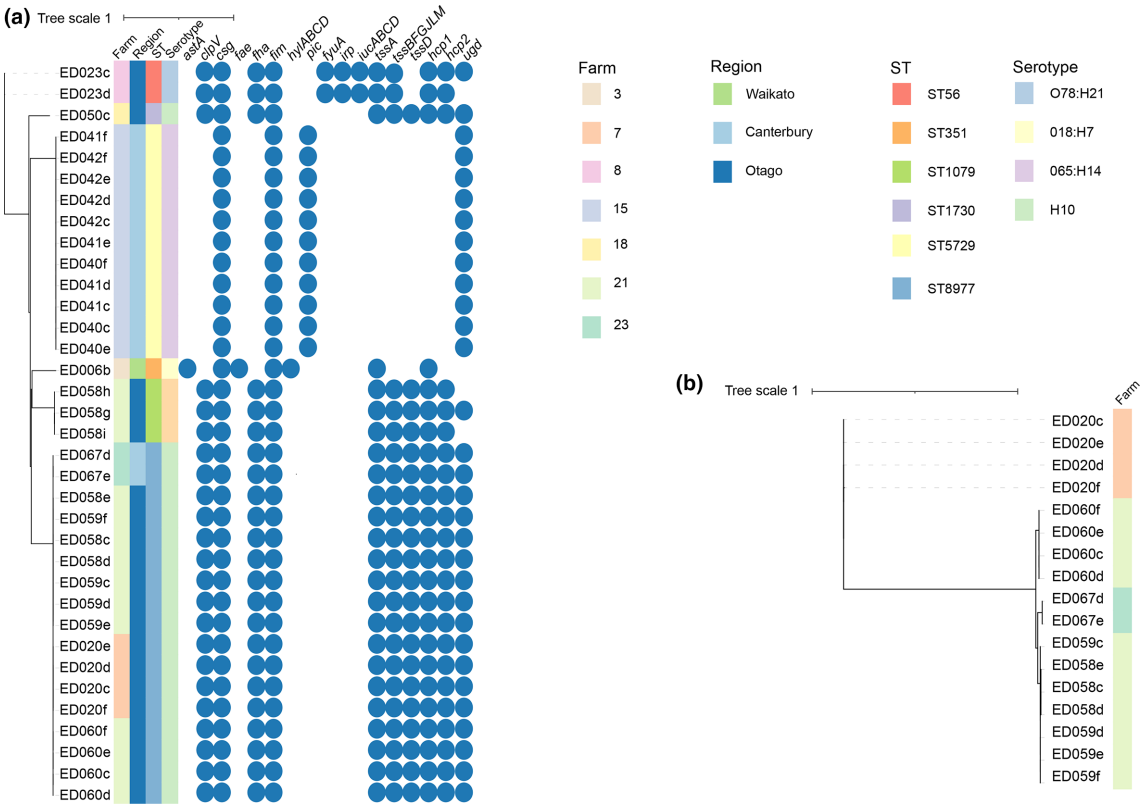

Fig. 1. Neighbour-joining phylogenetic trees of AmpC-producing *E. coli* obtained from this study. Core SNPs were generated using Snippy (v. 4.3.6). (a) Thirty-four *E. coli* isolated from across seven farms and constructed using 62 545 core SNPs. The tree was annotated with, from left to right, farm, region, ST, serotype and putative virulence factors using iTOL as illustrated in the legend with coloured bands. (b) Core SNPs of *E. coli* ST8977 alone constructed using 321 core SNPs. The coloured band represents the farm.

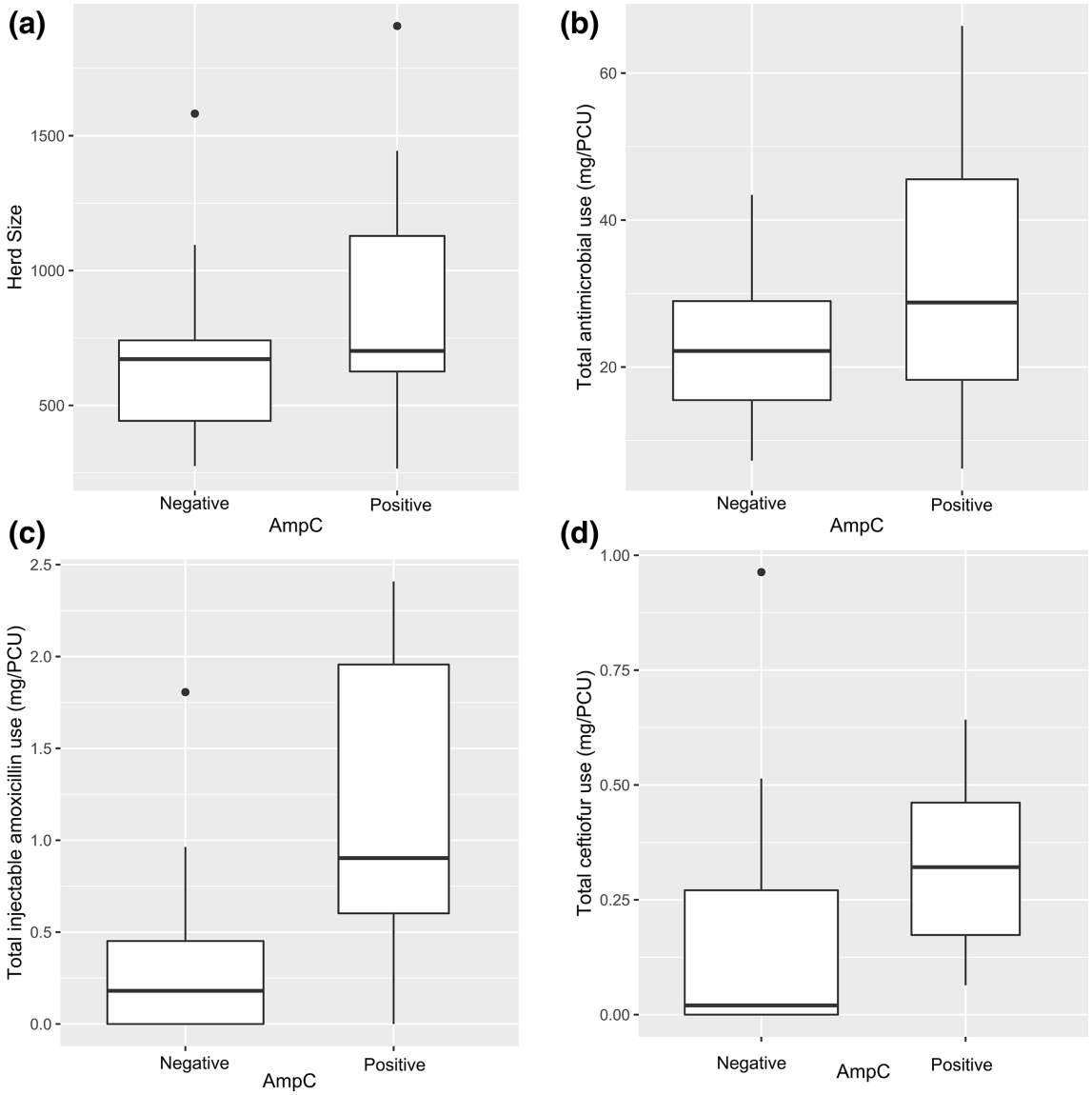

Fig. 2. Boxplots showing (a) farm size, (b) total antimicrobial use (mg per kg liveweight adult cows), (c) total injectable amoxicillin use, (d) total ceftiofur use for AmpC negative and positive herds.

in spring only from one of fifteen farms [21]. Previous longitudinal studies in France and the UK suggest the incidence of ESBL producing *E. coli* is greater in spring, immediately after calving [45, 46], and that increased temperature is associated with increased numbers of ESBL-producing *E. coli* [47]. New Zealand dairy farming is seasonally based with calving generally occurring in late winter or early spring and drying off in late autumn. Additionally, New Zealand dairy farms are low users of 3GCs [17], which have previously been identified as a risk factor for ESBL producing *E. coli* [15].

On New Zealand dairy farms aminopenicillins, such as amoxicillin, are administered as both intramammary and injectables for the treatment and prevention of mastitis as well as secondary bacterial infections [13]. Whilst penicillins are the most frequently used group of antibiotics on New Zealand dairy farms [17], there is little data on amoxicillin use. In this current study penicillins accounted for 79% of the total estimated antimicrobial usage and aminopenicillins (including amoxicillin) accounted for 3% of total sales. In 2017 the New Zealand Veterinary Association (NZVA) announced the vision statement 'By 2030 New Zealand Inc. will not need antibiotics for the maintenance of animal health and wellness'. As part of this vision the NZVA supports the move to use less critically important antibiotics as defined by the World Health Organisation. It is yet to be established whether this may result in the increased use of aminopenicillins including amoxicillin [48].

In conclusion, our study found a low incidence of AmpC-producing *E. coli* (11/78 enrichments from 7/26 farms) compared with European studies [14, 15, 37, 47], and no ESBL- or plasmid-mediated AmpC-producing *E. coli* were detected. This may be a due to a combination of samples being collected in autumn, when there was a lower use of injectable antibiotics compared with in spring during calving, and New Zealand dairy farms having a low prevalence of 3GC resistant *E. coli*. AmpC-positive farms were associated with injectable amoxicillin use. However, because of the small sample size we were unable to determine whether injectable amoxicillin use was a significant factor. Genomic analyses suggested there may have been transmission of AmpC-hyperproducing *E. coli* between two farms. Further investigation is needed to determine any seasonal variation in the prevalence of these bacteria and the risk factors which drive the development and clonal spread of AmpC-hyperproducing *E. coli*.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Ethics approval from the Ruakura Animal Ethics Committee of AgResearch (application number 14046) was obtained as part of the parent study [22]. A written informed consent was provided by the recruited farms, acknowledging that farm management data could be accessed through electronic or paper means, provided that the data is anonymised to protect their identity.

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