Extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase (AmpC) producing *Escherichia coli* in dairy calves from the Canterbury region

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Abstract

Antimicrobial resistance (AMR) has become a global public health concern threatening current effective prevention and treatment options. Infections caused by antimicrobial resistance strains are costly, often harder to treat and sometimes fatal. One group of bacteria that contributes to AMR are the extended beta lactamase (ESBL) and AmpC beta lactamase (AmpC) producing *E. coli*. These are on the critical list of important antibiotic-resistant bacteria of human importance compiled by the World Health Organisation and are a public health concern due to their resistance to an extended range of beta lactams.

The main driver for the spread of AMR is the use of antimicrobials in both human and animals. One potential spread is by the feeding of waste milk to calves. Waste milk is the milk that contains antibiotics or other drugs. Waste milk is not recommended as feed for calves due to its association with the development of antibiotic resistance bacteria.

Using the culture depended methods and whole genome sequencing, this study aimed to determine the prevalence of ESBL and AmpC producing *E. coli* isolated from recto-anal mucosal swabs (RAMS) from waste milk fed dairy calves, and to phenotypically and genotypically characterise ESBL and AmpC producing isolates. Recto-anal mucosal swabs samples (n = 40) from waste milk fed dairy calves collected from Canterbury region, New Zealand were screened for antimicrobial resistant *E. coli*.

Fifty-eight percent (23/40) of the calves harboured antibiotic resistant *E. coli*. 25% (10/40) calves were positive for AmpC producing *E. coli* and none of the calves were positive for ESBL producing *E. coli*. The highest prevalence of resistance was observed for tetracycline. PCR and Sanger sequencing revealed that all the AmpC positive *E. coli* were chromosomal mediated with four mutations in the promoter region of the *ampC* gene. Whole genome sequencing of eight isolates resistant to both tetracycline and streptomycin revealed additional resistance genes that were not tested phenotypically. Using the Clermont phylogrouping method of *E. coli*, the AmpC positive, the tetracycline and streptomycin resistant *E. coli* isolates were distributed among phylogroups B1, C and D.

In conclusion, this study revealed the presence of AmpC producing *E. coli* and other resistance genes in *E. coli* isolated from waste milk fed calves. Further epidemiological studies are required to determine whether these antibiotic resistant *E. coli* are associated with waste milk.

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Abbreviations and Acronyms

AMR	: Antimicrobial resistance
ARB	: Antibiotic resistance bacteria
CDC	: Centre for Disease Control and Prevention
CLSI	: Clinical and laboratory Standard Institute
EARS-Net	: European Antimicrobial Resistance Surveillance Network
EUCAST	: European Committee on Antimicrobial Susceptibility Testing
ECDC	: European Centre for Disease Prevention and Control
EEA	: European Economic Area
ECOFF	: Epidemiological cut-off
EFSA	: European Food Safety Authority
EMA	: European Medicines Agency
ESBL	: Extended spectrum beta lactamase
ESR	: Institute of Environmental Science and Research
EU	: European Union
HGT	: Horizontal gene transfer
NZVA	: New Zealand Veterinary Association
NRI	: Normalised resistance interpretation
ST	: Sequence type
WHO	: World Health Organisation

1 Literature review

1.1 Introduction

Antimicrobial resistance (AMR) has become a global public health concern threatening current effective prevention and treatment options. Infections caused by AMR strains are costly, often harder to treat and more likely to have fatal outcomes (WHO, 2014). In Europe, a joint report by the European Centre for Disease Prevention and Control (ECDC) and the European Medicines Agency (EMA) estimated that annually there are approximately 25 000 deaths and a €1.5 billion cost, associated with infection caused by antibiotic resistant bacteria (ARB) (ECDC & EMA, 2009). In addition, in 2015, it was estimated that in the European Union, 671 689 (95% uncertainty interval [UI] 583 148–763 966) infections were due to antibiotic resistance bacteria and these contributed to 33 110 (28 480–38 430) attributable deaths (Cassini et al., 2019). In USA, a report from Centres for Disease Control (CDC) estimated that approximately 2 million people suffer from infections caused by ARB, leading to not less than 23,000 deaths every year, with an estimated annual cost of US$ 55 Billion for hospital care and production losses (CDC, 2013; Prestinaci et al., 2015). There is limited accurate and reliable data on AMR in most African countries even though AMR occurs and deaths due to infections caused by ARB have been documented in some countries such as Kenya, Uganda, South Africa, Egypt, Tanzania and Rwanda (Ampaire et al., 2016; Kimang'a, 2012; B. T. Tadesse et al., 2017). On the other hand, New Zealand had low rates of AMR. However, the situation has considerably changed over the past two decades, with the emergence and spread of antibiotic resistance pathogens such as the extended-spectrum β-lactamase-producing Enterobacteriaceae due to overuse of antimicrobials, importation of ARB from antibiotic resistance endemic areas and spreading of ARB from hospital settings to the communities (Thomas et al., 2014; Williamson & Heffernan, 2014).

The development of resistance in bacteria has been associated with the use of antimicrobials in both human and animal health settings (Holmes et al., 2016). In addition, exposure to heavy metals, biocides and disinfectants may also contribute to AMR (Berendonk et al., 2015; Horner et al., 2012). However, resistance mechanisms also occur naturally and without external forces in bacteria facilitating adaptation for their survival (Etebu & Ukpong, 2016; Holmes et al., 2016). Resistance develops either through inherent or acquired mechanisms with the latter being the major concern as multiple resistance genes can be transferred from one bacteria cell to the other (Levin & Rozen, 2006). Bacterial resistance mechanisms include; 1) efflux pumps,

2) modification of cellular targets, 3) inactivation of enzymes, 4) overproduction of enzymes and 5) triggering of an alternative pathway that bypasses the drug action (Alekshun & Levy, 2007; Christaki et al., 2020; Tenover, 2006).

One bacterial group that contributes to AMR are the extended-spectrum β-lactamase producing Enterobacteriaceae (ESBL-E) and AmpC β-lactamase producing Enterobacteriaceae (AmpC-E). The ESBL and AmpC β-lactamases confer resistance by means of inactivation of enzymes. ESBL-E are resistant to β-lactam antibiotics including the first, third and some fourth generation cephalosporins, as well as monobactams. AmpC-E are resistant to cephamycins (second generation cephalosporins) in addition to the above antibiotics but not resistance to the fourth generation cephalosporins (Ewers et al., 2012; Pfaller & Segreti, 2006). Their resistance to a wide range of commonly used antibiotics is worrisome and has complicated treatment options in hospitalised patients in both humans and animals.

1.2 *Escherichia coli*: the bacterial pathogen

Escherichia coli is a gram-negative bacteria belonging to the Enterobacteriaceae family (Croxen et al., 2013; Lukjancenko et al., 2010). *E. coli* is part of the normal microflora of the gastrointestinal tract of warm-blooded animals, including humans, and may persist in the environment (Van Elsas et al., 2011). However, gene gain and loss has led to increased pathogenicity of certain strains of *E. coli,* causing serious illness in humans and animals worldwide (Kaper et al., 2004). *E. coli* can be classified in multiple ways according to, 1) pathogenicity, there is pathogenic and non-pathogenic *E. coli,* 2) site of infection or clinical manifestations in humans, such as enteropathogenic or uropathogenic pathotypes, and 3) virulence mechanism, for instance enterohaemorrhagic *E. coli* (EHEC) or enterotoxigenic *E. coli* (ETEC). Traditionally, *E. coli* have also been serotyped based on the somatic (O) and flagella (H) antigens. Another commonly used method is multilocus sequence typing (MLST) using the concatenated sequence of seven housekeeping genes *adk, fumC, gyrB, icd, mdh, purA,* and *recA* (Kaper et al., 2004; Lukjancenko et al., 2010; Wirth et al., 2006).

As a population (pathogenic and non-pathogenic), *E. coli* isolates can be assigned into 7 phylogroups: A, B1, B2, C, D, E and F through a multiplex PCR process described by Clermont et al. (2013). Most commensal isolates belong to phylogroup A or B1, while extraintestinal isolates mainly belong to group B2 or D. Various studies have reported group A and B1 being the most common in calves, while B2 is commonly found in humans. (Barzan et al., 2017;

Escobar et al., 2006; Johnson et al., 2003; Johnson et al., 2002). However, it should be noted that an *E. coli* pathotype may be represented by more than one phylogroup.

ESBL/AmpC producing *E. coli* are on the critical list of important antibiotic-resistant bacteria compiled by WHO. This priority list of 20 bacteria of human importance was compiled using ten criteria related to health and antibiotic resistance such as mortality and prevalence of resistance respectively, categorising bacteria as critical, high or medium importance (Tacconelli et al., 2018). Antibiotic resistant ESBL/AmpC producing *E. coli* has been reported in both humans and animals worldwide (Ahmed et al., 2009; Gagliotti et al., 2011; Khachatryan et al., 2004; Okeke et al., 2005; D. A. Tadesse et al., 2012; Vieira et al., 2011)

Horizontal gene transfer (HGT) is an important evolutionally force in the survival of bacteria (Soucy et al., 2015). HGT can occur through transformation, transduction and conjugation. The loss or gain of new mobile genetic elements has a pivotal role in the survival of pathogenic bacteria in a hostile environment. Mobile genetic elements include transposons, integrons, and insertion sequence common region (ISCR) elements. This method is important as many new traits, including antimicrobial resistance genes are transferred between various strains of *E. coli* using this mechanism (Christaki et al., 2020; von Wintersdorff et al., 2016).

1.3 Beta lactamases

E. coli, previously called *Bacillus coli,* was the first organism to be associated with the production of β-lactamase enzymes (Abraham & Chain, 1940). Since then, the production of β-lactamase enzymes has been the most common resistant mechanism observed in gram-negative bacteria (Bush, 2018). These enzymes protect the bacteria by inactivating β-lactam antibiotics through a hydrolysis reaction (Zeng & Lin, 2013). However, the resistance of these β-lactamase enzymes can be inhibited by β-lactamase inhibitors, such as clavulanic acid, tazobactam, avibactam and sulbactam (Bush, 2018; Drawz & Bonomo, 2010). β-lactamases have been classified based on two major schemes. The Ambler classification scheme (A to D) is based on amino acid sequence (Ambler, 1980), while the Bush-Jacoby-Medeiros scheme (1 to 4) is based on molecular structure and later changed to substrate and inhibitors profiles (Bush et al., 1995) **(Table 1)**.

Table 1. Important β-lactamase enzymes of *E. coli* and the affected β-lactam antibiotics (Drawz & Bonomo, 2010; Olsen, 2015)

β-lactamase classes		**Enzymes**	**Enzyme type**	**Inhibitor**	**Affected β-lactam antibiotics**
Ambler class	**Bush-Jacoby-Medeiros class**				
A	2b	Penicillinases, narrow spectrum β-lactamase (NSBL)	TEM-1, SHV-1 (parent enzymes), TEM-2	Clavulanate	Penicillin,1st generation cephalosporins
	2be	Penicillinases, extended spectrum β-lactamase (ESBL)	Mutated variants of SHV-1, TEM-1, CTX-Ms	Clavulanate	Penicillin, cephalosporins[1], monobactams except cephamycin and carbapenems
B	3	Metallo β- lactamases carbapenamase	IMP-1, VIM-1, NDM	EDTA, dipicolinic acid	Penicillin, cephalosporins, carbapenems except monobactams

C	1	Ampicillin hydrolysing β-lactamase AmpC	AmpC (Chromosomal encoded AmpC)	Avibactam, boronic acids	Penicillin, cephalosporins, monobactams cephamycin except carbapenems
			CMY, FOX, ACC, LAT MIR, ACT, MOX, DHA (Plasmid mediated AmpC)	Avibactam, boronic acids	Penicillin, cephalosporins, monobactams cephamycin except carbapenems
D	2d	Oxacillinases	OXA	NaCl	Penicillin, cephalosporins, carbapenems, monobactams

[1] cephalosporins 1st, 2nd, 3rd and 4th generation

1.4 Extended Spectrum β-lactamase enzymes (ESBL)

Extended Spectrum β-lactamase enzymes (ESBL) are a class of β-lactamases that confer bacterial resistance to penicillins, cephalosporins and monobactams but not cephamycins and carbapenems. ESBL can be inhibited by clavulanic acid (Bradford, 2001; Paterson & Bonomo, 2005; Pitout & Laupland, 2008). In the 1960s increased bacterial resistance to penicillin and 1st generation cephalosporins due to penicillinase and ampicillin hydrolysing β-lactamase, led to the discovery of 3rd generation cephalosporins and other oxyimino-cephalosporins in 1980s, that were stable in the presence of these β-lactamases. However, that stability did not last long, as resistance was discovered in the 3rd generation cephalosporins and the oxyimino cephalosporins due to their overuse and mutation was observed on the parent enzyme (TEM-1 and SHV-1) (Paterson & Bonomo, 2005). TEM-1 (based on patient name Temoniera) from *E. coli* and SHV-1 (sulfhydryl variable) from *Klebsiella pneumoniae* and *E. coli* (Datta & Kontomichalou, 1965; Philippon et al., 1989) are parent ESBL enzyme types and are plasmid mediated. The first ESBL enzyme type was reported in 1983 from a German patient and the encoding gene indicated there was a mutation of the parent enzyme SHV-1. Other β-lactamases were later discovered that were closely related to TEM-1 and TEM-2 and conferred resistance to 3rd generation cephalosporins (Knothe et al., 1983). These are called extended spectrum β-lactamase enzymes due to their expanded ways of hydrolysing a wider range of β-lactams, including 3rd generation cephalosporins, penicillin and 1st generation cephalosporins. To date there are at least 167 TEM and 132 SHIV β-lactamase types that have emerged globally due to mutation of the parent enzyme TEM-1 and SHV-1 http://www.laced.uni-stuttgart.de/.

CTX-M (based on greater hydrolytic activity against cefotaxime than ceftazidime) is an ESBL enzyme type that has emerged as a result of horizontal gene transfer, probably from a chromosomally encoded β-lactamase gene of *Kluyvera spp.* (Bonnet, 2004; Humeniuk et al., 2002). CTX-M types have been the most common ESBL enzymes reported globally and there are over 172 CTX-M enzymes classified into 5 clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25) (Ramadan et al., 2019). In humans the most common variant reported is CTX-M-15, which belongs to cluster CTX-M-1, followed by CTX-M-14 which belongs to the CTX-M-9 group (Iovleva & Bonomo, 2017; Saravanan et al., 2018). In livestock CTX-M-1, is the most prevalent, followed by CTX-M-15 and CTX-M-14 **(Table 2)**. Other ESBL enzymes PEM, CME, GES, FEC, TLA, SFO and VEB, are of less significance (Bradford, 2001).

1.5 AmpC β-lactamase

In contrast to ESBLs as described in the previous section, AmpC can hydrolyse cephamycins such as cefoxitin and cefotetan, in addition to the penicillin, cephalosporins and monobactams and are not inhibited by clavulanic acid (Jacoby, 2009). Genes expressing ampicillin hydrolysing β-lactamase enzyme (AmpC β-lactamase) can be found either on the bacterial chromosome or plasmid. These cannot be differentiated phenotypically but rather through multiplex and promoter polymerase chain reaction (PCR) (Pérez-Pérez & Hanson, 2002). Chromosomal AmpC, from *E. coli,* was the first bacterial enzyme recorded to destroy penicillin (Abraham & Chain, 1940). The first plasmid mediated AmpC was reported in 1989 in *K. pneumoniae,* with an enzyme type designated as CMY-1 (based on greater activity on cephamycin) (Philippon et al., 2002). Currently over 139 variants of CMY have been reported worldwide (Bush, 2018). Plasmid mediated AmpC have been found in both nosocomial and community isolates around the world. Apart from CMY, other important enzyme varieties including FOX, ACC, LAT, MIR, ACT, MOX, DHA, CFE **(Figure 1)** have also been reported although CMY is the most prevalent (Jacoby, 2009).

Expression of the chromosomal gene *ampC* is low in many Enterobacteriaceae but can be overexpressed when exposed to β-lactams (Jacoby, 2009). This process is induced by the action of the *ampC* genes *ampD*, *ampG*, *ampR*, and intermediates in peptidoglycan recycling. This process does not occur in *E. coli* as it lacks the *ampR* gene. However, expression of *ampC* and overproduction can still occur after mutation in the promoter or attenuator region (Philippon et al., 2002). It has been observed that overproduction of chromosomal AmpC occurs most in phylogroup A *E. coli* isolates (Corvec et al., 2007).

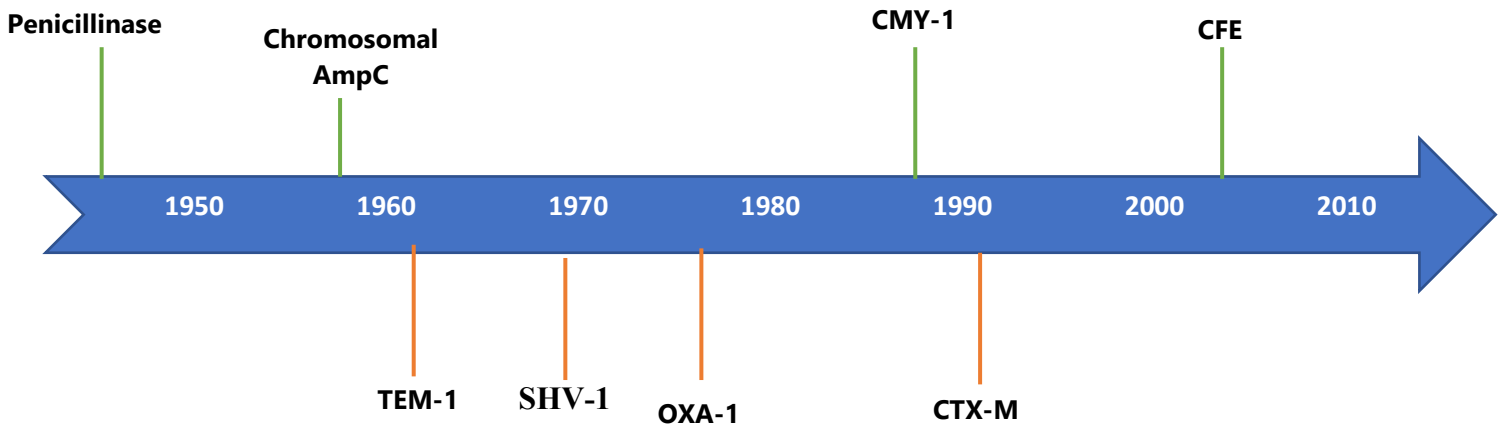


Figure 1. Reporting timeline of important ESBLs and AmpC β-lactamase evolution in *E. coli,* CMY, CFE are AmpC enzymes while TEM, SHV OXA and CTX-M are ESBL.

1.6 ESBL/AmpC producing *E. coli* in New Zealand

The prevalence of human infections associated with ESBL-E/AmpC producing *E. coli* has increased rapidly in recent years in New Zealand (Dyet et al., 2014; Thomas et al., 2014). National annual surveys conducted by the Institute of Environmental Science and Research Limited (ESR) since 2005 has reported an increase in the prevalence of ESBL/AmpC producing *E. coli*. Results from a 2006-2008 survey, showed that about 2.6% of *E. coli* from community and hospital-based isolates were ESBL positive, while 3.8% was recorded in 2009-2011, a 46% increase (Heffernan et al., 2013). By 2016, there was a national average prevalence rate of 11.1 per 100 000 population, from whom ESBL producing Enterobacteria were isolated, based on clinical samples. *E. coli* was more prevalent 74.1% (386/521) than other Enterobacteriaceae species and The Manukau Counties District Health Board registered the highest prevalence rate of 21.3 people per 100 000 (Heffernan et al., 2018). Furthermore, in 2012 it was reported that about 4000 people had suffered infections caused by ESBL producing pathogens with *E.coli* being the main cause (Thomas et al., 2014).

In New Zealand human clinical studies of ESBL-E isolates, the most predominant ESBL genes are *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-14}}$. In a 2006 study of human urine samples (community-acquired), it was found that the most common enzyme type was CTX-M-15 76% (63/83), followed by CTX-M-14 14% (11/83). From this study, a novel CTX-M ESBL was identified from a *K. pneumoniae* isolate (laboratory number ARS06/441). This was designated CTX-M-68 by the Lahey Clinic (H. M. Heffernan et al., 2009). A subsequent study using non-duplicate isolates of ESBL *E. coli* at the Auckland hospital laboratory found that 55% (59/108) were CTX-M-15, followed by 27% (29/102) CTX-M-14 (Freeman et al., 2012). In addition, a prospective unmatched case control study conducted in 2017 in Auckland and Northland regions on human urine samples from people that had UTI and kept at least 1 pet, found a prevalence of 68% (90/132) of ESBL/AmpC producing *E. coli* with *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CMY-2}}$ as the predominant gene types (Toombs-Ruane et al., 2019). Co-resistance with other classes of antibiotics was observed in all these studies.

A cross-sectional study conducted by Karkaba et al. (2017) in companion animals reported a prevalence of 60% (36/60) for carriage ESBL/AmpC producing *E. coli* in cats and dogs. This study reported *bla*$_{\text{CTX-M-14}}$ as the most prevalent gene type (4/10) and co-resistance with other classes of antibiotics was observed (Karkaba et al., 2017). Another study conducted 10 years ago (2009-2010), a surveillance study of various food products (pig, poultry and calf) across

New Zealand, showed that none of the 1000 isolates recovered were ESBL/AmpC positive (H. Heffernan et al., 2011).

1.7 Detection of AmpC/ESBL producing *E. coli*

Accurate identification of ESBL/AmpC producing pathogens is crucial in the selection of appropriate antibiotic therapy. It also helps in adopting proper control measures to reduce cross transmission of bacteria between patients, and for surveillance purposes (Pfaller & Segreti, 2006). The standardised methodologies for phenotypic detection of AmpC/ESBL producing *E. coli* are available from both Clinical and Laboratories Standards Institute (CLSI) (CLSI, 2017) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2020). Both CLSI and EUCAST recommend a 2-step method, first by screening, followed by a confirmatory test. For ESBL, reduced susceptibility to at least two of the indicator 3rd generation cephalosporins (cefpodoxime, cefotaxime, ceftriaxone or ceftazidime) is recommended for screening, followed by a double-disk diffusion synergy test of the indicator cephalosporins (cefotaxime, ceftazidime) and clavulanic acid (Bajaj et al., 2016; Stürenburg & Mack, 2003). Unlike ESBLs, there are no recommended guidelines for the detection of AmpC producing *E. coli*. Nevertheless, a reduced susceptibility to cefoxitin may be used for screening, followed by a confirmatory test of cefoxitin–cloxacillin or boronic acid combination disk method (Bajaj et al., 2016).

It is important to distinguish ESBL-producing *E. coli* from AmpC-producing *E. coli*, as failure to do so may result in serious public health impacts, ranging from treatment failure to emergence of new resistant bacteria.

Despite the availability of various phenotypic methods, molecular biology techniques such as PCR and whole genome sequence analysis serve as gold standards for detection, identification and differentiation of different genes encoding for ESBL/AmpC, even though they are less applied in many clinical laboratories because of their cost and lack of expertise (Bradford, 2001). However, the use of whole genome sequencing is becoming more common in co-ordinated studies investigating ESBL-E from a national prevalence perspective (Heffernan et al., 2018).

Beside the molecular techniques mentioned above, another method Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) has been utilised in the detection and identification of various pathogens including the ESBL/AmpC producing

E. coli (Angeletti, 2017; Dekker & Branda, 2011). This method is more rapid than the traditional lengthy phenotypic identification method. MALDI-TOF MS has a higher sensitivity, making it a preferred method to use in hospital-based and other research laboratories. MALDI-TOF MS rapid and accurate diagnosis leads to fast administration of effective antimicrobials and this contributes to a reduced mortality and hospitalization time of patients and consequently has a significant impact on cost reduction and public health (Angeletti, 2017).

1.8 Role of dairy farms in dissemination of resistant bacteria

The use of antimicrobials in livestock has come under critical review, due to the association of "routine" antimicrobial use in livestock with the development of antimicrobial resistance. As a result, there has been a global call to develop guidelines that safeguard the use of antibiotics. These include restricting their usage, develop proper legislative action and vigorous research to produce other antibiotics (WHO, 2015). In line with this call, the New Zealand Veterinary Association (NZVA) has stated that by 2030, antibiotics will be reserved for treatment of diseases only, rather than for animal health maintenance and welfare (NZVA, 2018).

Guidelines for AMU vary in many countries. While some countries such as USA and China allow the use of antibiotics as growth promoters in addition to therapeutic treatment, this is prohibited in New Zealand and some European countries where antibiotics are used solely to treat animal diseases and not as growth promoters (FDA, 2010; Maron et al., 2013). AMU also differs between conventional and organic dairy farms. Unlike conventional dairy farms, organic dairy farms do not permit the use of antibiotics, unless the animal is sick, in which case it is isolated from the rest of the herd to be treated. In the USA it will not be returned to the herd, while in European countries, it will be returned but there is longer milk withholding period (Maron et al., 2013). In New Zealand, on the other hand, the use of antibiotics in organic farming is banned (with some exceptions) in order to meet the export market requirements (AsureQuality, 2018).

Generally, low antibiotic use is reported in cattle, compared to other food animals such as swine or poultry (Marshall & Levy, 2011). In dairy cattle, antibiotics are used for various reasons. These include; treating infections related to calving e.g. endometritis, metritis, retained foetal membranes, mastitis and infections not related to calving, such as Dry Cow Therapy (DCT). A study carried out in New Zealand by Compton et al., (2014) found that 86% of antibiotics were

used mainly for DCT and treatment for mastitis. It should be noted that antibiotic use in animals is lower in New Zealand compared to other countries according to a study conducted by Hillerton et al. (2017). In this study, New Zealand was third lowest in the use of antibiotics in food animals. This is due to the extensive pasture-based system practiced on most dairy farms in the country that could mean low risk of exposure to infections from the environment and thus healthier animals (Hillerton et al., 2017). To show more prudent AMU, the NZVA has provided guidelines on the use of antibiotics in dairy cattle and have grouped the antibiotics according to the criteria set by WHO and OIE (WHO, 2019). A traffic light system is used to rank classes of the antimicrobials. Those that should be used as first line therapy have been grouped as green. Yellow are restricted antibiotics that should only be used as second line therapy and the final group has red colour which are considered critical important and should only be used when there is a need to do so under prescription from a veterinarian. 3rd and 4th generation cephalosporins, Macrolides and Fluoroquinolones belong to the red colour group (NZVA, 2018)

Different classes of antibiotics are used in dairy livestock for various clinical reasons, some of which have been highlighted in the previous paragraph. Of concern are the 3rd and 4th generation cephalosporins which have been associated with the emergence of ESBL-E and AmpC producing pathogens. A cross-sectional study conducted in the Netherland found that the positive ESBL/AmpC status of the dairy herds was linked to the use of 3rd and 4th generation cephalosporins (Gonggrijp et al., 2016). Similarly, Snow et al. (2012) reported a higher likelihood of positive ESBL/AmpC status in dairy herds that used 3rd and 4th generation cephalosporins. Furthermore, Randall et al. (2014) observed greater shedding of CTX-M positive *E. coli* in calves that were fed waste milk containing cefquinome a fourth generation cephalosporin.

The prevalence of ESBL/AmpC producing *E. coli* in dairy cattle (milking herd, heifers, calves) is unknown in New Zealand. To date, only one published study has investigated AMR surveillance of various food products from pig, poultry and calves across New Zealand and none of the 999 *E. coli* isolates were ESBL/AmpC positive (H. Heffernan et al., 2011). However, unpublished data of a cross-sectional study on lower North Island dairy farms found 6% (1/15) farms were positive for ESBL-producing *E. coli* (Aplin et al., 2018; unpublished data). Another unpublished study on calves fed either waste or non-waste milk from 12 Waikato dairy farms found that none of the *E. coli* isolates (n = 191) were ESBL/AmpC

producing (Kelly et al, 2016, unpublished data). Globally, numerous studies have reported the prevalence and incidence of ESBL/AmpC-producing *E. coli* and the associated enzyme type in dairy cattle **(Table 2)**. However, the results varied widely depending on the sample size, sample type and detection methods.

Table 2. Prevalence of ESBL/AmpC producing *E. coli* in dairy farms

Sample type	**Country**	**Detection method**	**Prevalence**	**ESBL/AmpC gene type**	**Reference**
Faeces of dairy calves	Canada	Culture-based selective media with antibiotics (ceftazidime,) after enrichment with buffered peptone water (BPW)	4 *E. coli* isolates positive for Extended spectrum cephalosporins (ESC) selected from a previous study whose prevalence was 81% (396/488).	*bla*$_{\text{CMY-2}}$	Awosile et al. (2020)
Faeces of dairy cattle	Netherlands	Culture-based: Selective media (cefotaxime) with and without enrichment in Luria–Bertani (LB) broth	40% (8/20) of the farms were positive for ESBL/AmpC producing *E. coli*	*bla*$_{\text{CTX-M-1}}$ *bla*$_{\text{CTX-M-14}}$ *bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{CTX-M-32}}$ *bla*$_{\text{CMY-2}}$	Hordijk et al. (2019)

Faeces from dairy cattle and veal calves	Netherlands	Culture-based: Selective media (cefotaxime) after enriched with BPW and non-selective (without cefotaxime and BPW)	ESC *E. coli* isolates from veal calves increased from 17.9% (54/301) in 2014, to 37% (114/302) in 2017 while in dairy cattle prevalence increased from 8.7% (26/300) in 2014, to 12.3% (36/292) in 2017	*bla*$_{\text{CTX-M-1}}$ *bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{CTX-M-14}}$ *bla*$_{\text{CMY-2}}$ Were common in both Veal calves and cattle	(Ceccarelli et al., 2019)
Rectal swabs from dairy calves	Latvia	Culture-based: Selective agar	11.1% (20/180) of *E. coli* isolates were ESBL/AmpC producing	*bla*$_{\text{CTX-M}}$ *bla*$_{\text{CMY}}$	Terentjeva et al. (2019)
Faeces from calves, young stock and dairy cows	Netherlands	Culture-based: Selective media (cefotaxime)	48.6% (89/183) of calves, 15.3% (29/183) young stock and 23% (42/183 cows harboured ESBL/AmpC producing *E. coli.*	*bla*$_{\text{CTX-M-1}}$ *bla*$_{\text{CTX-M-2}}$ *bla*$_{\text{CTX-M-32}}$ *bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{CTX-M-22}}$	Heuvelink et al. (2019)

				bla$_{\text{CMY-2}}$	
Faeces from dairy cows	China	Culture based: Selective media (ChromID ESBL agar)	43.6% (284/651) of *E. coli* isolates were ESBL positive.	*bla*$_{\text{CTX-M-14}}$ *bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{CTX-M-17}}$ *bla*$_{\text{CTX-M-55}}$	Zheng et al. (2019)
Environmental faecal matter	United Kingdom	Culture based: Selective media (cephalexin, cefotaxime)	79.2% (42/53) of the farms were positive for cefotaxime-resistant *E. coli* and 5.4% (224/4145) of the isolates were cefotaxime-resistant	*bla*$_{\text{CTX-M}}$	Schubert et al. (2019)
Bulk Tank milk	Turkey	Culture-based: Selective media (ChromID ESBL)	22.6% (14/62) of the isolates were ESBL producing *E. coli*	*bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{CTX-M-55}}$	Kürekci et al. (2019)
Rectal faecal swabs	Canada	Culture-based:	81.2% (396/488) of the *E. coli* isolated over the study period were	*bla*$_{\text{CMY-2}}$ *bla*$_{\text{CTX-M-2}}$	Awosile et al. (2018)

		Selective media (ceftazidime) after enrichment with (BPW)	positive for ESC. 52% (205/488) of the calves were positive at both neonatal and weaning stage	*bla*$_{\text{CTX-M-9}}$ *bla*$_{\text{CTX-M-1}}$	
Slurry from organic dairy farms	Netherlands	Culture-based: Selective media (cefotaxime)	13% (12/90) of the herds were ESBL/AmpC positive	Not determined	Santman-Berends et al. (2017)
Feaces from stable floor, dust samples and boot swabs from feed alley of the dairy farms	Germany	Culture-based: Enriched with (LB) broth, then selective media (cefotaxime).	48% (156/323) of the combined samples were ESBL/AmpC positive.	Not determined	Hille et al. (2017)
Rectal swabs from dairy cattle	Turkey	Culture based: Selective media (cefotaxime)	5.8% (10/172) of the *E. coli* isolates were ESBL/AmpC producing.	*bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{CTX-M-1}}$ *bla*$_{\text{CTX-M-3}}$ *bla*$_{\text{CMY-2}}$	Aslantaş et al. (2017)

Milk	Indonesia	Culture based: Enrichment then selective media (cefotaxime)	3.1% (4/129) of the milk samples harbored ESBL/AmpC producing *E. coli*	*bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{CTX-M- 155}}$	Sudarwantoa et al. (2017)
Slurry	Netherlands	Culture based: Enrichment then selective media (cefotaxime)	41% (41/100) of the herds were positive for ESBL/AmpC producing *E. coli*.	*bla*$_{\text{CTX-M-1}}$ *bla*$_{\text{CTX-M-2}}$ *bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{CTX-M-14}}$ *bla*$_{\text{CMY-2}}$ Chromosomal encoded *ampC* gene	Gonggrijp et al. (2016)

Bulk tank milk	Germany	Culture-based: Enrichment then selective agar (cefotaxime)	75.6% (62/82) *E. coli* isolates were ESBL/AmpC producing	*bla*$_{\text{CTX-M-1}}$	Odenthal et al. (2016)
Rectal swabs from dairy cattle	Egypt	Culture based: Enriched with BPW then slective agar	46.6% (98/210) *E. coli* isolates were positive for ESBL	*bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{CTX-M-1}}$ *bla*$_{\text{CTX-M-9}}$	Braun et al. (2016)
Faeces from veal calves	France	Culture-based: selective agar (ChromID ESBL)	29.4% (144/489) of *E. coli* isolates were positive for ESBL	*bla*$_{\text{CTX-M-1}}$ *bla*$_{\text{CTX-M-9}}$ *bla*$_{\text{CTX-M-2}}$	Haenni et al. (2014b)
Faeces from veal calves	Netherlands	Culture-based: Enrichment then selective media	18-26% of the farms were positive for ESBL/AmpC at the time of calves arrival, this decreased during the study period	*bla*$_{\text{CTX-M-1}}$ *bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{CTX-M-14}}$	Hordijk et al. (2013a)

Pooled fecal samples from veal calves	Netherlands	Culture-based: Enrichment then selective agar	In 1998 4% (4/49) of the *E. coli* isolates were ESBL/AmpC producing while 39% (71/182) was observed in 2010.	*bla*$_{\text{CTX-M-1}}$ *bla*$_{\text{CTX-M-15}}$ *bla*$_{\text{CTX-M-14}}$ *bla*$_{\text{CMY}}$	Hordijk et al. (2013c)
Faeces from veal calves	Netherlands	Culture-based: Enrichment then selective agar	66% (66/100) of the herds harbored ESBL/AmpC producing *E. coli* and within- herd prevalence ranging from 0% to 90%	*bla*$_{\text{CTX-M-1}}$ *bla*$_{\text{CTX-M-14}}$ *bla*$_{\text{CTX-M-15}}$	Hordijk et al. (2013b)

Besides AMU, other farm management practices such as feed, housing system and hygiene have been associated with AMR on dairy farms (Gonggrijp et al., 2016; Snow et al., 2012). A study by Gonggrijp et al. (2016) found floor scrappers which are used for cleaning pens, was associated with positive ESBL/AmpC herd status. Lacy-Hulbert et al. (2002) found higher incidences of AMR in cows fed total mixed ration feed (silage and concentrates) than those fed all- pasture diet.

Potential pathways for transmission of ESBL/AmpC producing *E. coli* from the dairy farm environments to humans have been hypothesised (Collis et al., 2019; Horigan et al., 2016). These include: 1) direct contact with the infected animal or contaminated faeces, 2) through food chains such as drinking raw milk or undercooked meat or 3) through contaminated water (Dahms et al., 2015; Valentin et al., 2014; Van Elsas et al., 2011) (**Figure 2)**. However, various studies have reported low to no evidence of transmission between livestock and humans, and interventions to avoid transmission are recommended (Alzayn et al., 2020; Findlay et al., 2020; Hordijk et al., 2019; Liu et al., 2018; Ludden et al., 2019)

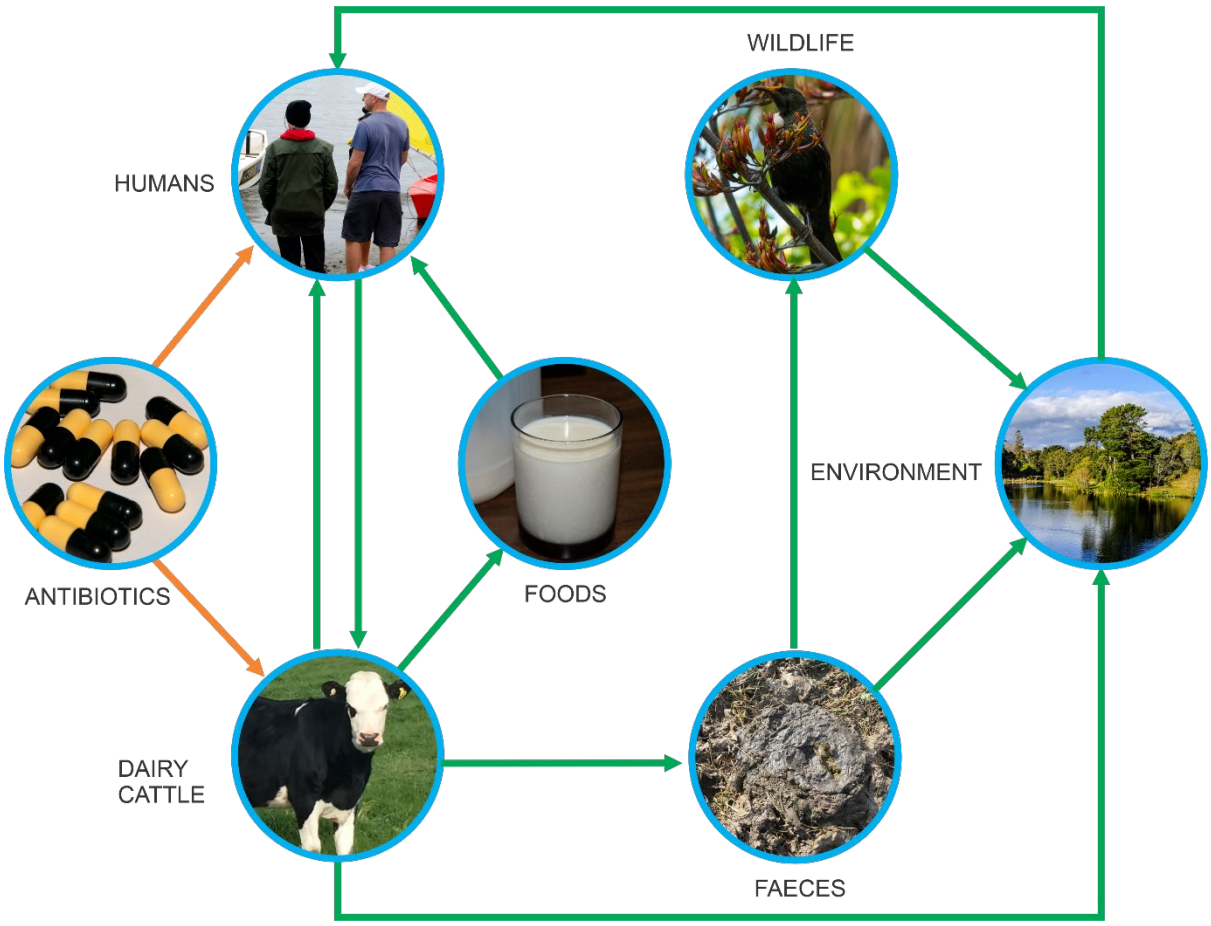


Figure 2. Potential transmission pathways of the ESBL/AmpC producing *E. coli*. Orange colour represent the main drivers of AMR, green colour represents the various transmission pathways of ESBL/AmpC producing *E. coli* while arrow direction shows the relationship. Adopted from (Collis et al., 2019).

1.9 Role of waste milk use in dairy calves in disseminating ESBL/AmpC producing *E. coli*

Waste milk is milk that is unfit for human consumption. It includes milk from cows that have been treated with antibiotics, other types of drugs or with increased somatic cell counts (over 150,000 cells/ml) before withholding period is over (EFSA, 2017). Some studies have reported the use of waste milk as feed in dairy farms, for different groups of calves (raised for veal, replacement stock or beef) as an alternative to milk replacers or non-waste milk (Duse et al., 2013; Tempini et al., 2018). However, this is not recommended, as waste milk has been linked to the development of ARB (Foutz et al., 2018). In addition to that, it has been hypothesised that milk containing antibiotic residues is unpalatable to calves, and this might lead to reduced intake, resulting in poor calf growth, poor performance and less weight gain, however this hypothesis has not been proven (Aust et al., 2013; Brunton et al., 2014; Thames et al., 2012).

The development of ESBL/AmpC-producing *E. coli* in calves, due to feeding with waste milk is of concern. A study conducted in England observed an increased number of CTX-M-producing *E. coli* isolates (25/45) from calves that were fed waste milk containing cefquinome, a 4th generation cephalosporin, compared to the control group (18/45) even though statistically there was no significant difference (Brunton et al., 2014). Subsequent studies conducted in USA and Germany reported isolation of ceftiofur and cefotaxime resistant *E. coli* in calves that were fed waste milk containing 3rd generation cephalosporins (Aust et al., 2013; Berge et al., 2006).

To the authors' knowledge, no study has investigated the prevalence of ESBL/AmpC producing *E. coli* in waste milk fed calves in New Zealand, although both NZVA and NZ Dairy Industry does not recommend the use of waste milk as feed in calves https://www.dairynz.co.nz/news/waste-milk-is-it-calf-feed/. However, studies undertaken overseas have reported prevalence ranging from 18 to 94% **(Table 3)** with results varying widely depending on the sample size, sample type and detection methods.

Table 3. Prevalence of ESBL/AmpC producing *E. coli* in waste milk fed calves

Sample type	**Country**	**Detection method**	**Prevalence**	**ESBL/AmpC gene type**	**Phylogroup**	**References**
Faeces	Czech Republic	Culture-based: Selective media (cefotaxime)	94% (87/126) of the *E. coli* isolates were positive for AmpC and none for ESBL.	*bla*$_{CMY-2}$	A (3/10), B1 (1/10), B2 (1/10), C (3/10), D (2/10)	Manga et al. (2019)
Faeces	Germany	Culture-based: Selective media (Chromagar ESBL)	Not determined	Not determined	Not determined	Tetens et al. (2019)
Fecal swabs	France	Culture based: Enriched with Brain heart infusion broth then selective media	42.2% (75/178) of *E. coli* isolates were resistant to ceftiofur in calves fed waste milk while 21.5% (27/178) of *E. coli* isolates resistant to ceftiofur	*bla*$_{CMY-2}$ (58%) *bla*$_{CTX-M}$ (30%) *bla*$_{TEM}$ (16%)	Not determined	Maynou et al. (2017b)

			were from calves fed milk replacer.			
Faeces	UK	Culture based: Enrichment with BPW then selective media (Chromagar, cefotaxime)	50% (32-64%) and 77% (60-90%) of *E. coli* isolates were positive for CTX-M in the control and treatment group respectively.	*bla*$_{\text{CTX-M}}$	Not determined	Brunton et al. (2014)
Faeces	UK	Culture based: Enrichment with BPW then selective media	75% (12/16) of CTX-M producing *E. coli*	*bla*$_{\text{CTX-M}}$	Not determined	Randall et al. (2014)
Faeces	Germany	Culture based: Selective media	18% (9/50) of *E. coli* isolates were resistant to cefotaxime in waste milk fed calves, 20.8%	Not determined	Not determined	Aust et al. (2013)

			(10/40) from calves fed pasteurized waste milk and 4.8% (2/42) were from bulk milk.			

1.10 Relevance of this study

Every year more than two million bobby calves are slaughtered in New Zealand https://www.mpi.govt.nz/. Most bobby veal meat is exported to Asian countries (mainly Thailand, China and Hong Kong) for human consumption and the remainder is used to make pet food. Therefore, presence of ESBL/AmpC *E. coli* in bobby calves poses a public health concern if good hygiene practices are not followed at slaughter and during veal processing which may lead to carcass contamination and this may subsequently be transferred into food chain and the environment. This problem can be exacerbated when food safety practices such as proper cooking and proper food storage are not observed.

A lack of data on the prevalence of ESBL/AmpC producing *E. coli* in dairy calves fed with waste milk, prompted this observational study to be conducted by analysing culture enrichments from a previous national cross-sectional study that estimated the prevalence of Shiga toxin-producing *E. coli* in dairy calves (Browne, 2018) . We hypothesize that ESBL/AmpC producing *E. coli* will be present in the faeces of waste-milk fed calves. Using both culture-based isolation methods and whole genome sequencing, this study aims to; 1) determine the prevalence of ESBL and AmpC producing *E. coli* isolated from recto-anal mucosal swabs from waste milk fed dairy calves and 2) phenotypically and genotypically characterise ESBL and AmpC producing isolates.

2 Materials and methods

This study used samples and information collected by Springer Browne for his PhD study (Browne, 2018). Springer's study was approved by The Animal Ethics Committee of Massey University, Palmerston North, New Zealand on 17thApril 2014, under protocol number 14/29.

2.1 Sample Collection

Recto-anal mucosal swab samples (RAMS) were collected using Amies transport swabs (Copan Diagnostics Inc., Brescia, Italy), by Springer Browne for his PhD study from calves 2 to 21 days old. Sampling was carried out during the spring calving season, 28th July to 24th September 2014 across six regions (Northland, Waikato, Taranaki, Manawatu-Wellington, Canterbury and Southland) in New Zealand. All RAMS collected were shipped on ice overnight to the ^{m}EpiLab, Massey University, Palmerston North, and enriched with modified Tryptone Soy Broth (mTSB, Oxoid Limited, Hampshire, United Kingdom) at 42°C for 15-21 hours in preparation for further processing. All samples were later suspended in glycerol (4:1 ratio) and stored in a -80°C freezer for future use (Browne, 2018). This present study only used samples collected from the Canterbury region from waste milk user farms **(Appendix A).**

Forty samples from four farms that used waste milk in the Canterbury region were randomly selected, each contributing 10 calves aged 2 to 9 days old. The RAMS were from mixed-sex calves meant either for bobby (veal meat), heifer replacement, or for beef **(Appendix B).**

2.2 Screening for *Escherichia coli*

MacConkey agar supplemented with antibiotics was prepared for screening of ESBL/AmpC producing *E. coli*. Cefotaxime and ceftazidime (Sigma-Aldrich New Zealand Co., Auckland, NZ) antibiotics were previously prepared by dissolving in Milli-Q water to a stock concentration of 10mg/ml and filter sterilized using a 0.22µm filter syringe (Merck MF-Millipore Membrane filter). The prepared antibiotic stocks were stored at -80°C. Each working stock of cefotaxime and ceftazidime was added into a different bottle of the molten MacConkey agar (Difco™, BD) to make a final concentration of 1mg/ml and poured into petri dishes. These were dried and stored in the cold room. The enriched frozen RAMS were then removed from the freezer and streaked on to the four different types of media: Plain MacConkey (Fort Richard Laboratories, Auckland, NZ), MacConkey with cefotaxime (MAC + CTX), MacConkey with ceftazidime (MAC + CAZ) and CHROMagar ESBL (Fort Richard, Auckland, NZ). The plates

were incubated at 35°C for 16 to 24 hours. After incubation, two single lactose positive colonies that had different morphologies (dark pink colonies) from each MacConkey (with and without antibiotics) plate were chosen randomly, as well as two purple, pink or blue coloured colonies from the ESBL CHROMagar. Each colony was purified on Columbia Horse Blood agar (Fort Richard, Auckland, NZ) and incubated overnight. Finally, presumptive *E. coli* isolates were collected with sterile cotton swabs and added into 15% glycerol broth for cryopreserving at -80°C.

Presumptive *E. coli* isolates were subjected to confirmation using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS; MALDI Biotyper® System, Bruker Daltonics Inc, Billerica, MA, USA). Using a sterilised toothpick, a small amount from a single colony was picked and spotted on to the biotyper target plate and overlaid with 70% formic acid. The spots were air-dried and overlaid with 1µl of alpha-Cyano-4-hydroxycinnamic acid (HCCA) matrix (Bruker Daltonics Inc, Billerica, MA, USA). The samples were analysed in duplicate using the MALDI Biotyper and MBT Compass (v4.4.100) software package (Bruker Daltonics Inc, Billerica, MA, USA). The confirmed *E. coli* and *Klebsiella pneumoniae* isolates were further screened for ESBL production and multi-drug resistance using an antibiotic susceptibility test (AST).

2.3 Screening for ESBL/AmpC producing *E. coli*

The Mastdiscs® AST (Mast Group Ltd., Merseyside, UK) antibiotic discs were used for the screening of ESBL and/or AmpC producing isolates. Following guidelines from EUCAST, cefotaxime (CTX) 30µg, cefpodoxime (CPD) 30µg were used for screening of ESBL production (EUCAST, 2020). Cefoxitin (FOX) 30µg was used for screening of potential AmpC producers (Bajaj et al., 2016). To test for multidrug resistance; streptomycin (STR) 10µg, tetracycline (TET) 30µg and ciprofloxacin (CIP) 5µg antibiotic discs were used along with the ESBL/AmpC screening discs **(Figure 3).** The ASTs were performed according to guidelines from both CLSI and EUCAST (CLSI, 2017; EUCAST, 2020). *E. coli* reference strain ATCC 25922 (NZRM 916) was used as a control strain. Purified isolates were streaked on Columbia Horse Blood Agar (Fort Richard, Auckland, NZ) and incubated at 35°C for 16–24 hours. Using a sterile cotton swab, colonies were transferred to sterile saline solutions to prepare the suspension with an optical density of 0.5 McFarland. The suspensions were spread onto Mueller-Hinton agar (Fort Richard, Auckland, NZ) in three directions using a sterile cotton swab, and the antimicrobial discs mentioned above were applied using the dispenser (Mast®

Disc master Dispenser). After incubation at 35°C for 20 hours, the inhibition zones were measured using a caliper. The antibiotic discs used in this study and their breakpoints are shown in **Table 4**. The isolates which were resistant to either cefotaxime, cefpodoxime or cefoxitin were considered as potential ESBL and/or AmpC producing *E. coli.*

Table 4. Antibiotic discs and their breakpoint

Antimicrobial Agent	**Class**	**Concentration (µg)**	**Breakpoints (mm)**		**Reference**
			Susceptibility (S) >	**Resistance (R) ≤**	
Cefotaxime (CTX)	3rd generation cephalosporin	5	20	17	EUCAST
Cefpodoxime (CPD)	3rd generation cephalosporin	10	21	21	EUCAST
Cefoxitin (FOX)	Cephamycin	30	19	19	EUCAST
Ciprofloxacin (CIP)	Fluoroquinolone	5	22	22	EUCAST
Streptomycin (STR)	Aminoglycoside	10	15	11	CLSI
Tetracycline (TET)	Tetracycline	30	11	15	CLSI

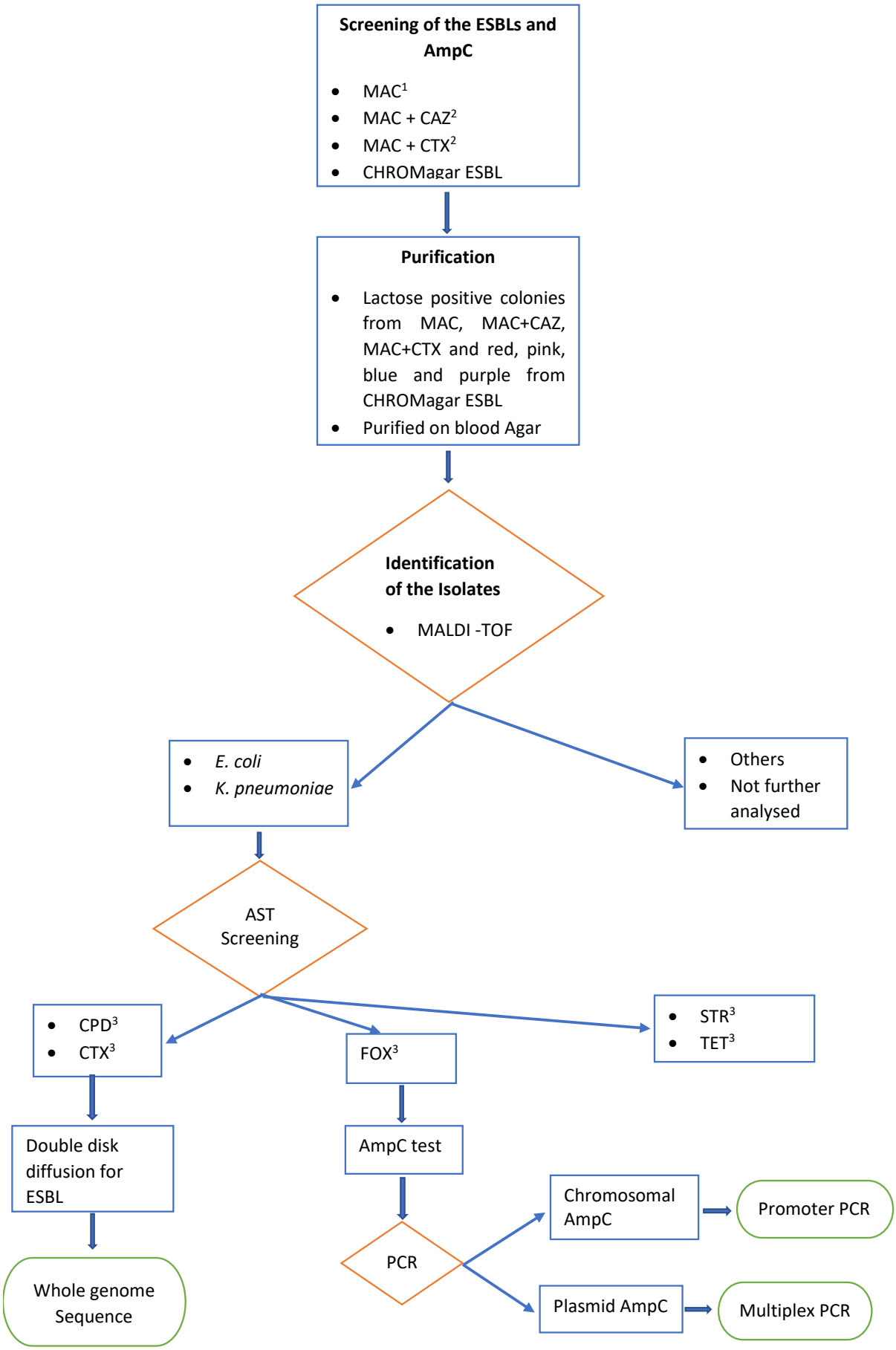
S: Susceptible, R: Resistant

2.4 Epidemiological cut-Off values (ECOFFs)

Epidemiological cut-off values (ECOFFs) of the *E. coli* population were defined using the normalised resistance interpretation (NRI) method, as described by Kronvall and Smith (2016). Disc diffusion zone diameter results of the 6 antibiotics (CTX, CPD, FOX, STR, TET,CIP) were used in an already developed spreadsheet file http://www.bioscand.se/nri/Automatic_NRI-zone_2019.xlsm. Normal distribution curves for each antibiotic were generated after entering the number of isolates under the given zone diameters. The means and standard deviations generated from these normal distribution curves were used to produce the breakpoints (ECOFFs), categorising the strains as wild-type (fully susceptible) and non-wildtype (reduced susceptibility). The ECOFFs were compared with the EUCAST/CLSI breakpoints.

2.5 ESBL and AmpC Confirmation

Potential ESBL and/or AmpC producing isolates were confirmed by the disc diffusion method using Mastdisc™ Combi ESBL (D64C and D62C)and AmpC (D69C) detection sets (Mast Group Ltd., Merseyside, UK) according to EUCAST guidelines (EUCAST, 2020) . For the detection of ESBL production, zone diameters were determined for cefotaxime; 30µg (disc A), ceftazidime; 30µg (disc B), cefotaxime; 30µg + clavulanic acid; 10µg (disc C) and ceftazidime; 30µg + clavulanic acid; 10µg (disc D). Zone size differences between either disc A and C, or disc B and D, larger than 5mm were considered ESBL positive. For the detection of AmpC producing *E. coli*, zone diameters were determined for cefpodoxime; 10µg + AmpC inducer (disc A), cefpodoxime; 10µg + AmpC inducer + ESBL inhibitor (disc B), and cefpodoxime; 10µg + AmpC inducer + ESBL inhibitor + AmpC inhibitor (disc C). The zone size of disc C was then compared with those of both discs A and B. If both zone size differences were larger than 5mm, the isolate was confirmed phenotypically as AmpC positive.


Screening of the ESBLs and AmpC
MAC[1]
MAC + CAZ[2]
MAC + CTX[2]
CHROMagar ESBL
Purification
Lactose positive colonies from MAC, MAC+CAZ, MAC+CTX and red, pink, blue and purple from CHROMagar ESBL
Purified on blood Agar
Identification of the Isolates
MALDI -TOF
E. coli
K. pneumoniae
Others
Not further analysed
AST Screening
CPD[3]
CTX[3]
FOX[3]
STR[3]
TET[3]
Double disk diffusion for ESBL
AmpC test
Whole genome Sequence
PCR
Chromosomal AmpC
Promoter PCR
Plasmid AmpC
Multiplex PCR

[1]MacConkey agar

[2]MacConkey agar with antibiotics cefotaxime (CTX) and ceftazidime (CAZ), 1mg/ml concentration

[3]Antibiotic sensitivity disc; cefotaxime (CTX), cefpodoxime (CPD), cefoxitin (FOX), ciprofloxacin (CIP), tetracycline (TET) and streptomycin (STR)

Figure 3. Flow process of detection of ESBL/AmpC producing *E. coli*

2.6 AmpC Genotypic Confirmation

Confirmed AmpC producing isolates were further analysed by PCR to determine if they were chromosomal or plasmid mediated AmpC. Crude DNA extractions were prepared using the heat lysis method. Three or four colonies of confirmed AmpC producing *E. coli* isolates were collected from the Columbia Horse Blood Agar plates (Fort Richard, Auckland, NZ), using disposable loops and suspended in 1ml of nuclease-free water in an Eppendorf tube. The Eppendorf tubes were incubated at 102°C with 1,200 rpm shaking speed for ten min using a Provocell™ Microplate Shaker/Incubator (ESCO Micro Pte Ltd, Singapore). The Eppendorfs were then cooled down for 10 min at room temperature and centrifuged at 12,000 rpm for 3 min in a microcentrifuge (Sigma 1-14, Sigma Laborzentrifugen GmbH, Germany). The supernatants were transferred into new Eppendorf tubes to be used for PCR. Isolates were analysed for the presence of genes encoding the plasmid-mediated AmpC enzymes (MOX, CIT, DHA, ACC, FOX), and the promoter region of the *ampC* chromosomal gene as described by Caroff et al. (1999) and Pérez-Pérez and Hanson (2002).

The AmpC multiplex PCR reaction mix was made using 4µl DNA polymerase mastermix (Hot fire Pol®), 1µl of each 10µM forward and reverse primer and 5µl PCR-grade water. Each PCR reaction was prepared in a total volume of 20µl containing 19µl of the PCR mix and 1µl of the template and was performed in a SensoQuest PCR machine. The multiplex PCR program consisted of 3 min at 95°C, followed by 25 cycles of 95°C for 30 s, 64°C for 30 s and 72°C for 60 s, followed by one final cycle of 72°C for 7 min. The *ampC* promoter region PCR reaction mix was made of 4µl DNA polymerase mastermix (Hot fire Pol®), 1µl of each 10µM forward and reverse primer and 13µl PCR-grade water. Each PCR reaction was prepared in a total volume of 20 µl containing 19 µl of the PCR mix and 1 µl of the template. and performed in a SensoQuest PCR machine. The PCR program was the same as for AmpC multiplex PCR apart

from the annealing temperature which was 57°C in a SensoQuest PCR machine. The list of primers used in this study is shown in **Table 5**.

The PCR amplicons were then analysed using gel electrophoresis by running 5 ul of each PCR product on a 2% agarose gel containing 10µl nucleic acid gel stain (Gelred®, Biotium, Fremont, California, USA) for 90 min at 80V. The gel was visualized using a UV trans-illumination Geldoc (Bio-Rad, California, USA).

Table 5. Primers used for AmpC detection in *E. coli* isolates

Enzyme	Target	Primer name	Primer sequence (5' - 3')	Product size (bp)	References
Plasmid-mediated AmpC	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	MOXM-F MOXM-R	GCTGCTCAAGGAGCACAGGAT CACATTGACATAGGTGTGGTG	520	Pérez-Pérez and Hanson (2002)
	LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CIMT-F CIMT-R	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462	
	DHA-1, DHA-2	DHAM-F DHAM-R	CCGTACGCATACTGGCTTTGC AACAGCCTCAGCAGCCGGTTA	405	
	ACC	ACCM-F ACCM-R	AACAGCCTCAGCAGCCGGTTA TTCGCCGCAATCATCCCTAGC	346	
	FOX-1 to FOX-5b	FOXM-F	CAAAGCGCGTAACCGGATTGG AACATGGGGTATCAGGGAGATG	190	

		FOXM-R			
AmpC Promoter	AmpC Promoter	AmpC171 AmpC2120	AATGGGTTTTCTACGGTCTG GGGCAGCAAATGTGGAGCAA		Caroff et al. (1999)

2.7 Sequencing of the *ampC* promoter region

Confirmed chromosomal AmpC producing isolates were further sequenced to determine mutations in the promoter and attenuator regions. The promoter PCR amplicons were purified using the QIAquick® PCR purification kit (Qiagen, Hilden, Germany) as per the manufacturers' instructions. The purified PCR product was eluted in 50ul of water. The quality of the purified PCR product was checked with a Nanodrop® (ThermoFisher Scientific, Massachusetts, United States) spectrophotometer.

The purified PCR product was prepared for sequencing by adding 17µl of PCR-grade water, 1µl of forward AmpC promoter primer (AmpC171) and 2 µl of the purified PCR sample to make a total volume of 20µl. This was repeated with the reverse primer (AmpC2120). These were sent to Massey Genome Service (Massey University, Palmerston North, New Zealand) for Sanger sequencing. Sequencing results were analysed using Geneious software v10.2.6 (Biomatters, Ltd. Auckland, New Zealand), and compared with the reference strain *E. coli* ATCC 25922 (Accession number CP009072) as described by Tracz et al. (2007).

2.8 Phylogrouping

Confirmed resistant *E. coli* isolates were phylotyped by quadraplex PCR as described by (Clermont et al., 2013). DNA extraction was carried out using the heat lysis method. The quadraplex PCR reaction mix was made using 10 µl of DNA polymerase mastermix (Kapa Hifi, Kapa Biosystems, Cape Town, South Africa), 0.6 µl of each of the primers and 4.2µl of PCR-grade water. Each PCR reaction was prepared in a total volume of 20µl containing 19µl of the PCR mix and 1µl of the template and performed in a SensoQuest PCR machine. The quadraplex PCR programme consisted of four minutes of initial denaturation at 94°C, followed by 30 cycles of denaturation at 98°C for 20 s, 20 s of annealing at 61°C, and 5 min of extension at 72°C. The PCR amplicons were then analysed with gel electrophoresis by running 5 µl of each PCR product on a 2% agarose gel containing 10µl nucleic acid gel stain (Gelred®, Biotium, Fremont, California, USA) for 2 hours at 80V. The gel was visualized using a UV trans-illumination Geldoc (Bio-Rad). The list of primers used in this study are shown in **Table 6.**

Table 6. primers used in phylogrouping of *E. coli* isolates (Clermont et al., 2013)

Primer name	Primer ID	Primer sequence	Target gene	Product size (bp)
arpA	AceK.f	5′-AACGCTATTCGCCAGCTTGC-3′		
	ArpA1.r	5′-TCTCCCCATACCGTACGCTA-3′	*arpA*	400
chuA	chuA.1b	5′-ATGGTACCGGACGAACCAAC-3′		
	chuA.2	5′-TGCCGCCAGTACCAAAGACA-3′	*chuA*	288
yjaA	yjaA.1b	5′-CAAACGTGAAGTGTCAGGAG-3′		
	yjaA.2b	5′-AATGCGTTCCTCAACCTGTG-3′	*yjaA*	211
TspE4C2	TspE4C2.1b	5′-CACTATTCGTAAGGTCATCC-3′		
	TspE4C2.2b	5′-AGTTTATCGCTGCGGGTCGC-3′	*TspE4C2*	152

2.9 Whole genome sequencing, assembly and analysis

12 resistant (tetracycline, streptomycin, cefpodoxime, cefoxitin) *E. coli* isolates across the 4 farms were selected for whole genome sequencing. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as per the manufacturers' instructions. The DNA was eluted in 50µl of sterile PCR-grade water.

The DNA quantity and quality were determined using fluorometry, the dsDNA HS Assay (Qubit 2.0, Invitrogen, Carlsbad CA, USA), spectrophotometry using the Nanodrop® (ThermoFisher Scientific, Massachusetts, USA) as per the manufacturers' instructions. The integrity of the DNA was checked using gel electrophoresis. 5µl of each DNA extraction was loaded onto a 0.8% agarose gel (Bioline®), stained with a nucleic acid gel stain (Gelred®, Biotium) and run for 3 hours at 80V. The gel was visualized using a UV trans-illuminator (BioRad).

The genomic DNA was diluted in PCR-grade water to a total volume of 15µl. The library preparations and next-generation sequencing were performed by Massey Genome Sequence (Massey University, Palmerston North, New Zealand). 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).

Raw sequences were processed and assembled using the Nullarbor pipeline (v. 2.0.20181010) by using the raw pair-ended FASTQ read file as input and using the reference accession CP014316 (Seemann et al., 2018). A core single nucleotide polymorphism (SNP) analysis was carried out using the internal reference CE0015a and a neighbour-joining tree generated using SplitsTree (v.4.16.1) (Huson & Bryant, 2006) which was annotated using the Interactive Tree of Life (iTOL) software (Letunic & Bork, 2019) and Inkscape open source software (v.1.0.1)(https://inkscape.org).

2.10 Statistical Analysis

The package ggplot from R studio software (v.3.6.3) was used to visualise the frequency of isolates that were resistant, and the calves that harboured the antibiotic resistant isolates in the four farms.

3 Results

3.1 Prevalence of *E. coli* from the four dairy farms

A total of 40 rectoanal mucosal swab enrichment samples from waste milk-fed dairy calves from four dairy farms were screened for *E. coli*. A total of 109 isolates recovered from 39 calf samples (no isolates were recovered from calf sample VC1128/CE0001 from farm VCF77), of which 78/109 were isolated from plain MacConkey, 17/109 from MacConkey with ceftazidime (MacConkey + CAZ), 14/109 from MacConkey with cefotaxime (MacConkey +CTX) and none from CHROMagar ESBL **(Appendix C)**. From the 109 isolates recovered, 95.4% (104/109) were *Escherichia coli* and 4.6% (5/109) were *Klebsiella pneumoniae*. The number of suspected ESBL/AmpC positive isolates from MacConkey with antibiotics (ceftazidime or cefotaxime) were almost similar across farms VCF77, 79 and 89 **(Figure 4)**. No isolates were recovered from MacConkey with antibiotics from farm VCF80 as indicated in **Figure 4**. Further details on the recovered isolates are in **Appendix C**.

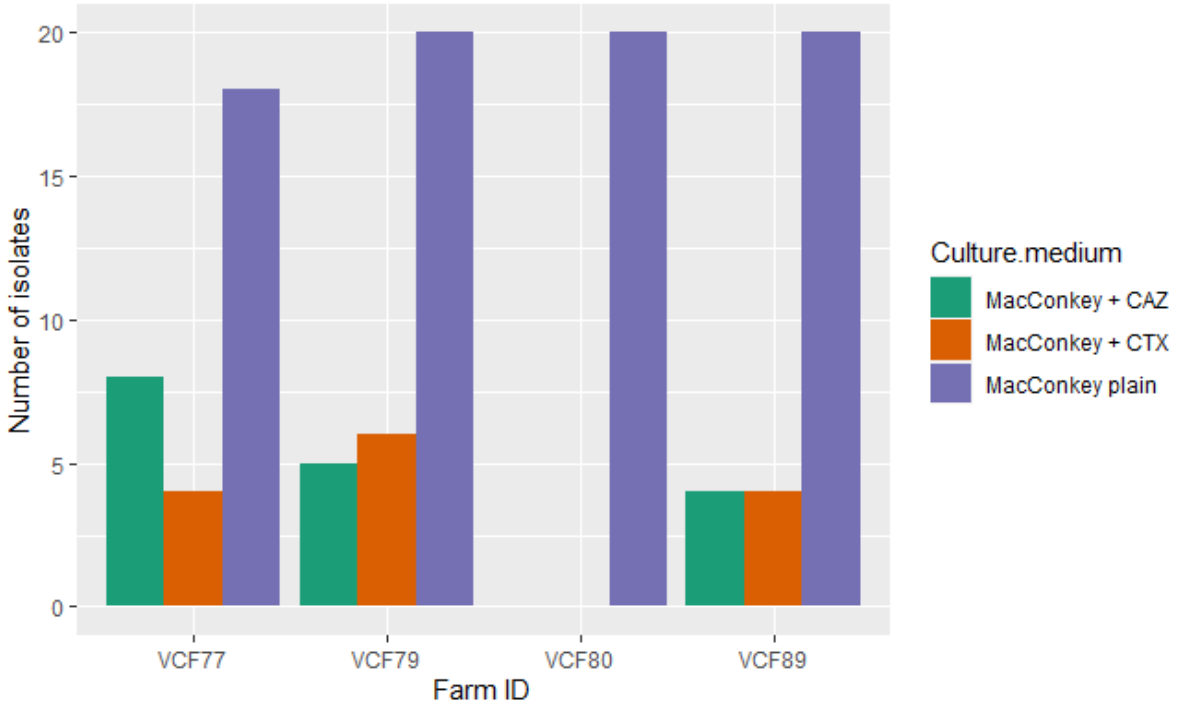


Figure 4. Number of *E. coli* isolates recovered from different medium from each of the four farms. MacConkey plain: MacConkey without antibiotic, MacConkey + CTX: MacConkey with 1mg/ml cefotaxime antibiotic, MacConkey + CAZ: MacConkey with 1mg/ml ceftazidime antibiotic

3.2 Antimicrobial resistant profiles

The 109 recovered isolates were tested for antimicrobial resistance using six different antibiotics. The antibiotic susceptibility test results together with their zone diameters are reported in **Appendix C** and summarised in **Table 7**. Among the 104 *E. coli* isolates, 45% (47/104) were sensitive to all the six antibiotics used and 55% (57/104) showed resistance to at least one of the antibiotics. More of the *E. coli* isolates, 39% (41/104), were resistant to tetracycline than any other antibiotic, whilst 18% (19/104) showed resistance to streptomycin. Co-resistance to tetracycline and streptomycin was observed in 17% (18/104) of the *E. coli* isolates. 30% (31/104) of the isolates showed resistance to cefpodoxime and cefoxitin. These were suspected of being ESBL/AmpC producing and were subjected to further ESBL/AmpC confirmation test. No isolates showed resistance to three or more classes of antibiotics; hence no multidrug resistance was observed. In addition, none of the isolates showed resistance to cefotaxime and ciprofloxacin **(Table 7)** and none of the *K. pneumoniae* isolates showed resistance to any of the antibiotics.

Overall, 58% (23/40) (95% CI 41% - 73%) of the calves harboured antibiotic resistant *E. coli.* Of these 23 calves 18 harboured *E. coli* isolates that were resistant to tetracycline, 13 harboured *E. coli* isolates that showed resistance to streptomycin and 10 harboured *E. coli* isolates that showed resistance to cefpodoxime and cefoxitin. There were 12 calves that harboured *E. coli* isolates that were co-resistant to tetracycline and streptomycin, and none of the calves harboured isolates that were resistant to cefotaxime or ciprofloxacin **(Figure 7).**

Table 7. Number and percentage of *E. coli* isolates showing resistance to the six antibiotics based on the diffusion test

Farm ID	**Total number of *E. coli* isolates**	**Number of resistant *E. coli* isolates (%)**						
		CTX[1]	**CPD[1]**	**FOX[1]**	**CIP[2]**	**TET[3]**	**STR[4]**	**R2**
VCF77	27	0	12	12	0	5	5	5
VCF79	31	0	11	11	0	9	0	8

VCF80	20	0	0	0	0	11	10	10
VCF89	26	0	8	8	0	16	4	11
Total	**104**	**0(0)**	**31(30)**	**31(30)**	**0(0)**	**41(39)**	**19(18)**	**34(33)**

CTX[1]: cefotaxime; CPD[1]: cefpodoxime; FOX [1]: cefoxitin (cephalosporins)

CIP[2]: ciprofloxacin (fluoroquinolones)

TET[3]: tetracycline (tetracyclines)

STR[4]: streptomycin (aminoglycosides)

R2: resistant to two classes of antibiotics

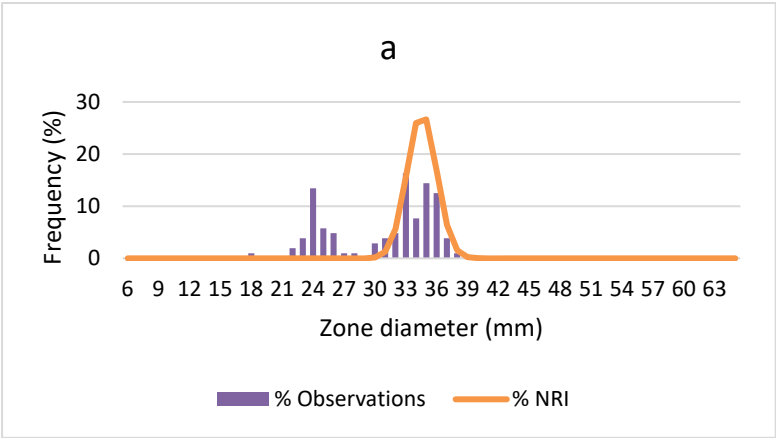
3.3 Normalised resistance interpretation (NRI)

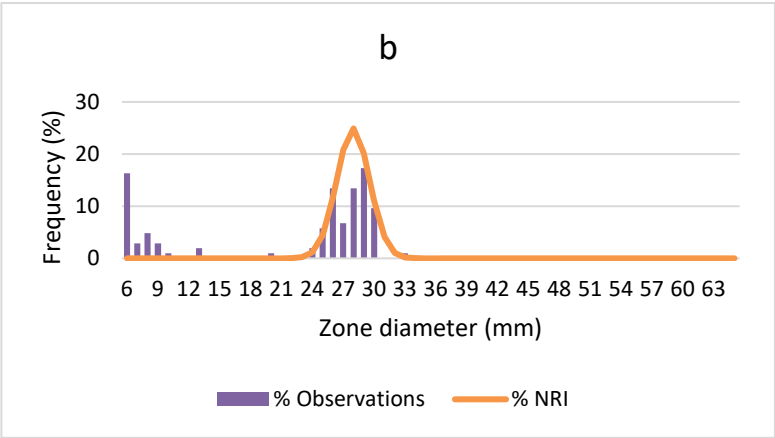
Epidemiological Cut-Off values (ECOFFs) of the *E. coli* population were defined by using the normalised resistance interpretation (NRI) method. Disc diffusion zone diameter results of the 6 antibiotics (CTX, CPD, FOX, STR, TET, CIP) from Appendix 1 were used. A normal distribution curve for each antibiotic **(Figure 5)** was generated by entering the number of isolates according to their zone diameters. The ECOFFs generated were used to categorise the strains as wild-type (fully susceptible) and non-wildtype (reduced susceptibility). The NRI ECOFFs were compared with the EUCAST/CLSI clinical breakpoints **(Table 8).**

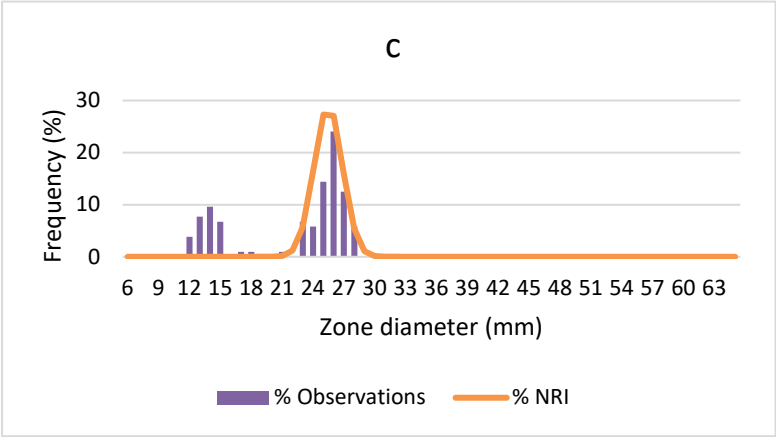
The highest percent of 41% (43/104) of non-wild type *E. coli* strains, were observed under tetracycline, followed by cefotaxime which had 36% (37/104) isolates displaying non-wild type. Noticeably, two populations of isolates were observed under cefotaxime **(Figure 5a)**. Two separate curves were used to represent this, a and b **(Figure 6)** with a single isolate and three isolates displaying non-wild type in population a and b respectively. No isolate displayed reduction in susceptibility to ciprofloxacin as indicated in **Figure 5d.**

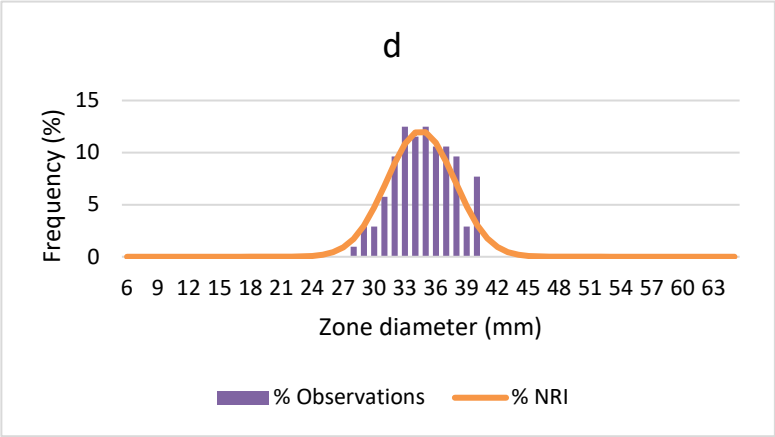
Table 8. Antibiotics used in this study with NRI-ECOFFs values compared with the EUCAST/CLSI clinical breakpoints

Antibiotic	**Concentration (µg)**	**EUCAST/CLSI breakpoints (mm) ≥**	**NRI-ECOFFS (mm) ≥**
Cefotaxime (CTX)	5	20	31
Cefpodoxime (CPD)	10	21	24
Cefoxitin (FOX)	30	19	22
Ciprofloxacin (CIP)	5	22	26
Streptomycin (STR)	10	15	16
Tetracycline (TET)	30	11	16


a
Frequency (%)
30
20
10
0
6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63
Zone diameter (mm)
% Observations
% NRI


b
Frequency (%)
30
20
10
0
6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63
Zone diameter (mm)
% Observations
% NRI


c
Frequency (%)
30
20
10
0
6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63
Zone diameter (mm)
% Observations
% NRI


d
Frequency (%)
15
10
5
0
6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63
Zone diameter (mm)
% Observations
% NRI

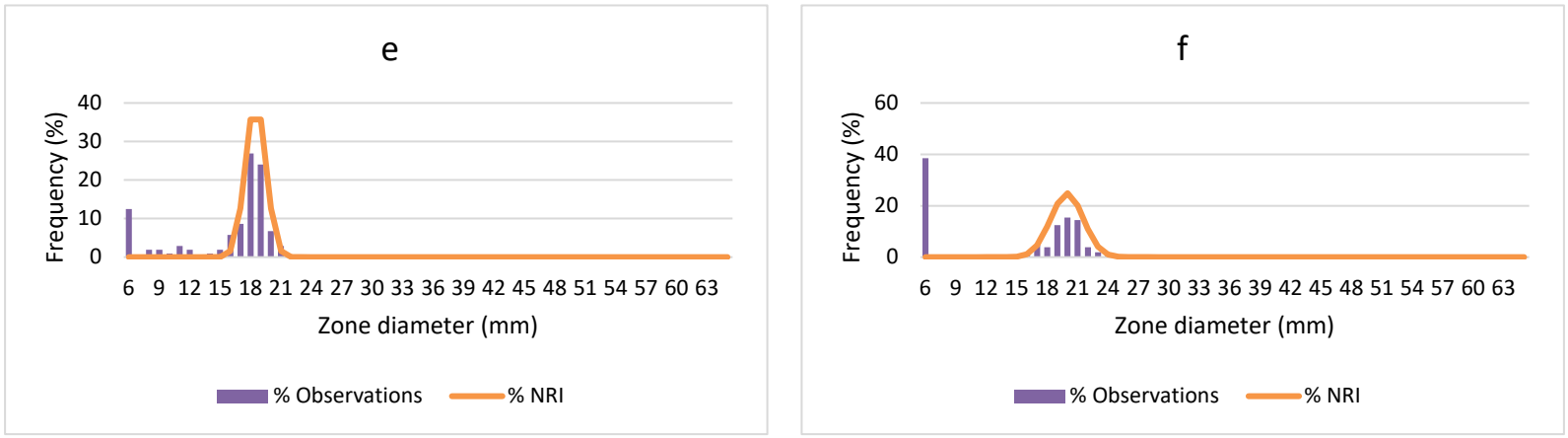


Figure 5. Normalised curves of the antibiotics used on the *E. coli* study population using the NRI method

a: cefotaxime, b: cefpodoxime, c: cefoxitin, d: ciprofloxacin, e: streptomycin and f: tetracycline

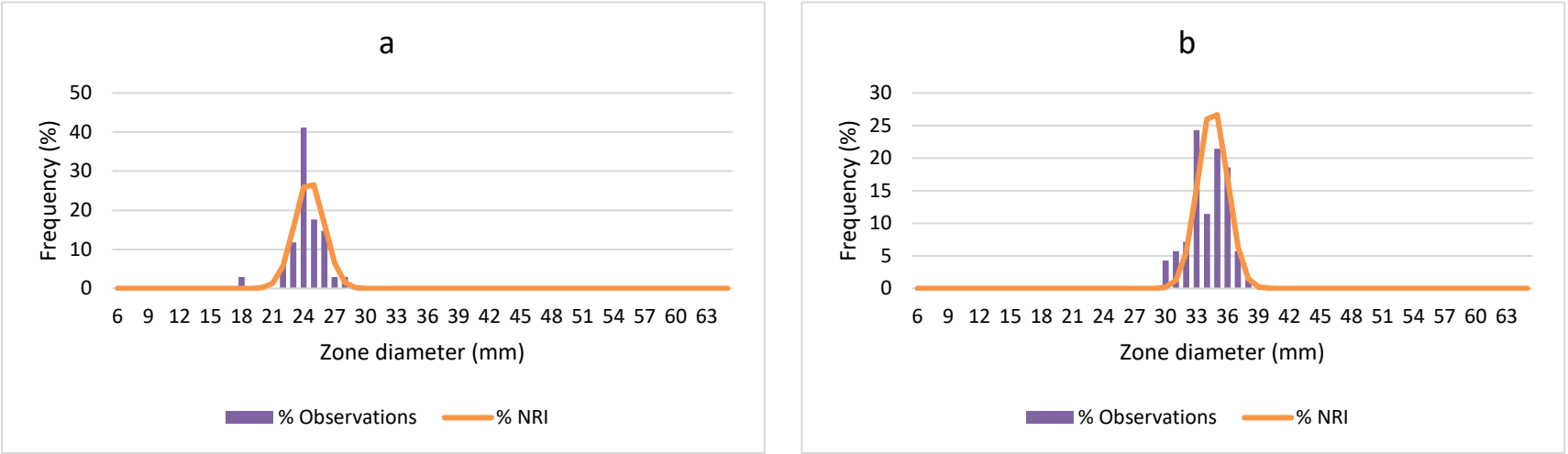


Figure 6. Normalised curves of the antibiotic cefotaxime used on the *E. coli* population using the NRI method

a: cefotaxime (first population), b: cefotaxime (second population)

3.4 Prevalence of ESBL/AmpC producing *E. coli*

Isolates that were resistant to cefotaxime, cefpodoxime or cefoxitin from the antibiotic susceptibility testing were suspected to be ESBL/AmpC producers. In total, 31 out of 104 isolates were presumptive ESBL/AmpC producing *E. coli*. These were tested using the double diffusion test for the presence of an ESBL/AmpC phenotype. All 31 isolates were confirmed to be AmpC producers and none of the isolates displayed the ESBL phenotype. Detailed results are reported in **Appendix D** and summarised in **Table 9**. Overall prevalence of AmpC producing *E. coli* was 30% (31/104) (95% CI 21% - 40%). Farm VCF77 had the highest number of AmpC producing isolates. No isolates were positive for AmpC from farm VCF80 **(Table 9)**.

At the calf level, 25% (10/40) (95% CI 13% - 41%) of calves were positive for AmpC producing *E. coli*. **(Figure 7).**

Table 8. *E. coli* isolates with an ESBL/AmpC phenotype derived from milk-fed calves in the Canterbury region

Farm ID	**Total number of isolates**	**Number of isolates showing an AmpC phenotype (%)**
VCF77	27	12 (44)
VCF79	31	11 (35)
VCF80	20	0 (0)
VCF89	26	8 (31)
Total	**104**	**31 (30)**

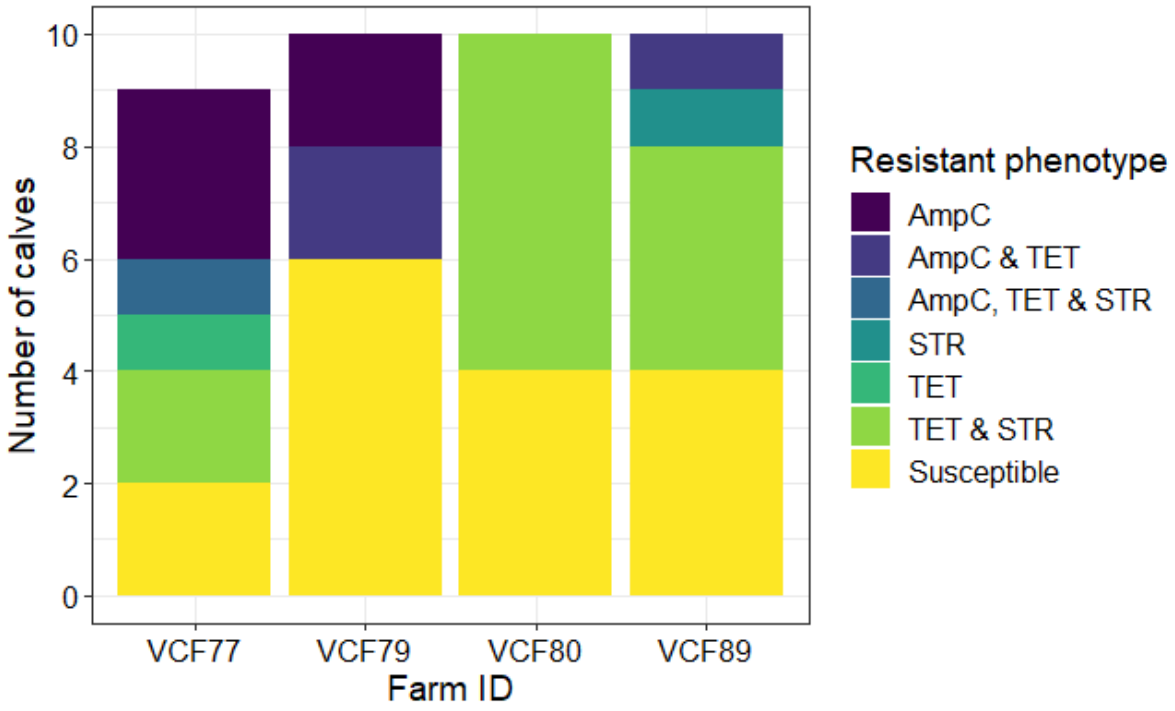


Figure 7. Number of calves harbouring isolates that showed various resistance phenotypes from the 4 farms.

All 31 confirmed AmpC producers were further analysed using Multiplex and Promoter PCR to determine whether the AmpC production was chromosomal or plasmid mediated. Notably, all 31 were chromosomal mediated.

3.5 AmpC sequence results

The promoter region from the *ampC* gene of all 31 AmpC positive isolates, was sequenced, to determine whether there were mutations in the promoter and attenuator regions. The sequence results were analysed and compared with that of *E. coli* ATCC 25922 wild type that expresses low levels of AmpC and thus has negative AmpC phenotype. The functional elements, which are the -35 box, -10 box and the attenuator regions of the reference wild type, were compared to the isolates. Nucleotide changes (mutations) were observed in 28/31 isolates at position -42, -18, -1 and +58 **(Figure 8)**. The same base change was present in all 28 isolates. The sequence files for the other three isolates was unable to be analysed due to their chromatograms being noisy and lacking peaks, hence no further analysis was done.

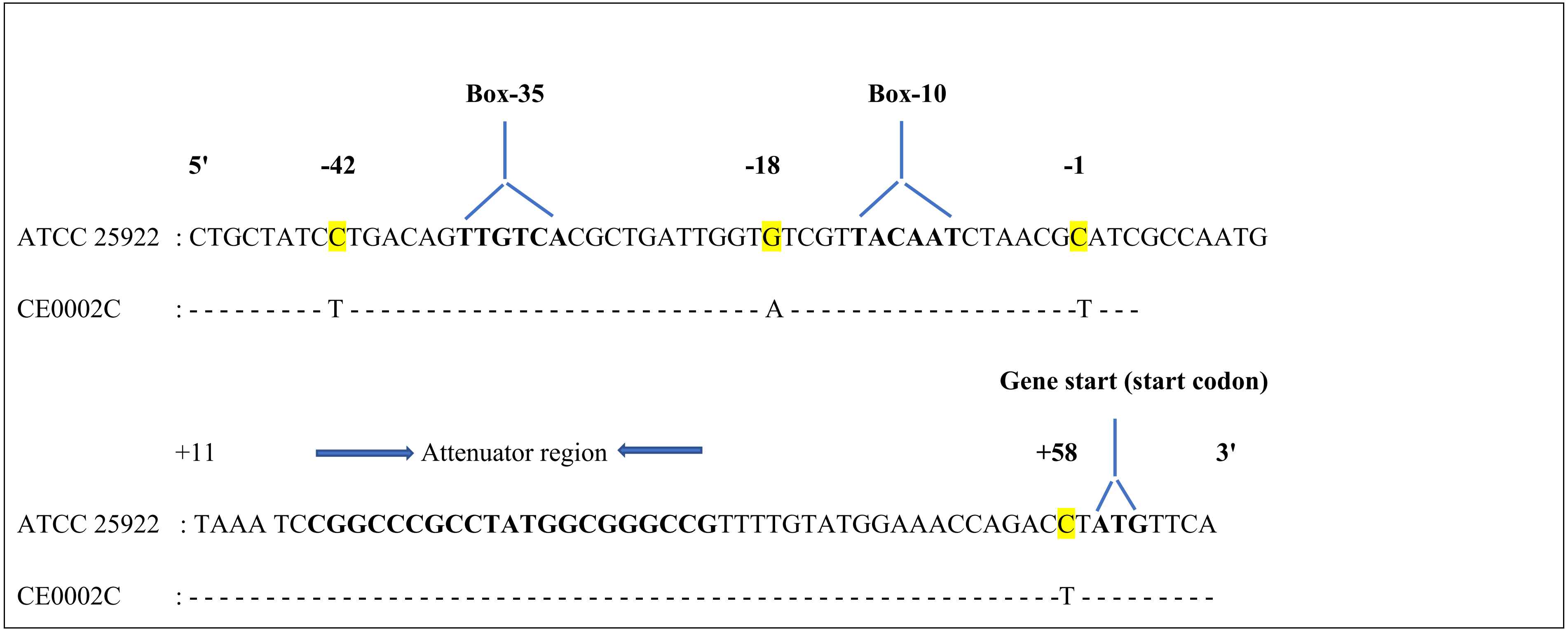


Figure 8. Nucleotide sequence of the *amp*C gene showing the promoter region (box -35, box -10), the attenuator region and the nucleotide change at position -42, -18, -1 and +58. ATCC 25922 is the wild type

3.6 Phylogrouping results

A total of 57 resistant *E. coli* isolates were phylotyped using the Clermont method (Clermont et al., 2013). Phylotyping results are reported in **Appendix E** and summarised in **Table 10**. Results showed that *E. coli* isolates were distributed among phylogroup B1, C and D. None of the resistant isolates belonged to phylogroup A, B2, E or F. Two isolates were unassigned according to Clermont et al. (2013)

Table 9. Distribution of *E. coli* phylogroups among the resistant and the susceptible *E. coli* isolates

Phylogroup	**AmpC positive isolates**	**Tetracycline and/or Streptomycin resistant isolates**	**Total resistant isolates (%)**
B1	10	4	14 (25)
C	19	34*	37 (65)
D	0	4	4 (7)
Unknown	2	0	2 (4)
Total	**31**	**42**	**57**

*Combined with AmpC positive isolates that were co-resistant with tetracycline

3.7 Whole genome sequence results

Twelve *E. coli* isolates displaying various antimicrobial resistance phenotypes were selected for whole genome sequencing to explore their genetic relatedness and diversity. The whole genome sequence data were used to determine sequence type (ST), antimicrobial resistance and virulence genes. Most of the isolates belonged to ST88 (8/12) with the remaining four belonging to ST10 (2/12) and ST69 (2/12). The antimicrobial resistance genotype was concordant with the phenotypic results. However, some additional antimicrobial resistance genes, *catA1* for chloramphenicol, *dfrA1* and *dfrA5* for trimethoprim, *sul1* and *sul2* for

sulphonamides and *qacEdelta* for quaternary ammonium were detected. These were not tested for phenotypically. Noticeably no plasmid mediated AmpC beta lactamase and ESBL genes were found, agreeing with the phenotypic results. The most common virulence genes found for adhesion were *fimA* (encoding type 1 fimbrae protein) found in 11/12 isolates, followed by *afaA* (encoding afimbrial adhesin transcriptional regulator) found in 8/12 isolates. The most prevalent virulence genes found affecting iron uptake were, *irp1* and *irp2* (encoding iron regulatory protein 1 and 2) found in 10/12 isolates and *iroN* (encoding a siderophore receptor) found in 6/12 isolates. In addition, virulence genes affecting outer membrane protein were also found, with *ompA* being found in all the 12 isolates. Virulence genes conferring an increased survival in serum *kpsM* were detected in 2/12 isolates. The summarized genomic characteristics of the 12 isolates are reported in **Table 11.** The virulence factors, as well as phenotype of the resistance genes, are reported in **Appendix F and G** respectively.

The genetic relatedness of these 12 isolates was further investigated using a core SNP approach, (**Figure 9)**. The pairwise distance between isolates is presented in **Appendix H**

Table 10. Genomic characteristic of the 12 *E. coli* isolates

Farm ID	**Isolate ID**	**Genome size (Mb)**	**GC (%)**	**Number of contings**	**Sequence type**	**Virulence genes**	**Resistance genotype**	**Resistance phenotype**
VCF77	CE0007a	5.16	50.8	148	69	*afaA, fimA, kpsD, kpsM, ompA,*	*aadA1, bla*$_{EC}^{*-8}$ *catA1, dfrA1, sul1, qacEdelta1*	STR[1], TET[2]
VCF77	CE0008b	5.05	52.7	221	10	*fimA, fyuA, irp1, irp2, ompA, pap*	*apha(6)-1d, sul2, bla*$_{EC}$*, tet(A)*	STR, TET
VCF77	CE0009b	5.22	52.2	467	69	*afaA, fimA, iucD, kpsD, kpsM, ompA*	*aadA1, aph(3'')-1b, aph(3')-1a, aph(6)-1d, bla*$_{EC-8}$ *, catA1, dfrA1, sul1, sul2, qacEdelta1, tet(B)*	STR, TET
VCF80	CE0011b	5.04	52.1	171	10	*fimA, fyuA, iroN, irp1, irp2, ompA, pap*	*aph(6)-1d, bla*$_{EC-8}$ *, sul2, tet(A)*	STR, TET

VCF80	CE0014b	5.16	51.1	117	88	*afaA, fimA, fyuA, iroN, irp1, irp2 ompA, pap*	*aph(3")-1b, aph(3')-1a, aph(6)-1d,* bla_{EC-13}*,* bla_{TEM-1}*, dfrA5, sul1, sul2, qacEdelta1, tet(A)*	STR, TET
VCF80	CE0015a	5.17	50.7	95	88	*afaA, fimA, fyuA, irp1, irp2, ompA, pap*	*aph(3")-1b, aph(3')-1a, aph(6)-1d,* bla_{EC-13}*,* bla_{TEM-1}*, dfrA5, sul1, sul2 qacEdelta1, tet(A)*	STR, TET
VCF89	CE0021a	5.22	51.6	164	88	*afaA, fyuA, irp1, irp2, ompA*	*aadA1,* bla_{EC-13}*, sul1, qacEdelta1, tet(A)*	STR, TET
VCF89	CE0026a	5.15	50.6	177	88	*afaA, fimA, fyuA, iroN, irp1, irp2, ompA, pap*	*aph(3")-1b, aph(3')-1a, aph(6)-1d,* bla_{EC-13}*,* bla_{TEM-1}*, sul2, tet(B)*	AmpC[3], TET
	CE0026f	5.24	50.9	117	88	*fimA, fyuA, iroN, irp1, ompA*	bla_{EC-13}*,* bla_{TEM-40}*, tet(A)*	AmpC, TET

VCF89	CE0027c	5.23	51.1	170	88	*fimA, fyuA, iroN, irp1, irp2, ompA*	*bla*$_{\text{EC-13}}$, *bla*$_{\text{TEM-40}}$, *tet(A)*	AmpC, TET
VCF79	CE0032f	5.23	50.7	97	88	*afaA, fimA, fyuA, iroN, irp1, irp2, ompA*	*bla*$_{\text{EC-13}}$, *bla*$_{\text{TEM-40}}$, *tet(A)*	AmpC, TET
VCF79	CE0033c	5.23	51.1	106	88	*afaA, fimA, fyuA, iroN, irp1, irp2, ompA*	*bla*$_{\text{EC-13}}$ *bla*$_{\text{TEM-40}}$, *tet(A)*	AmpC, TET

**ampC* gene

Resistance phenotype; STR[1]: streptomycin, TET[2]: tetracycline, AmpC[3]: AmpC phenotype

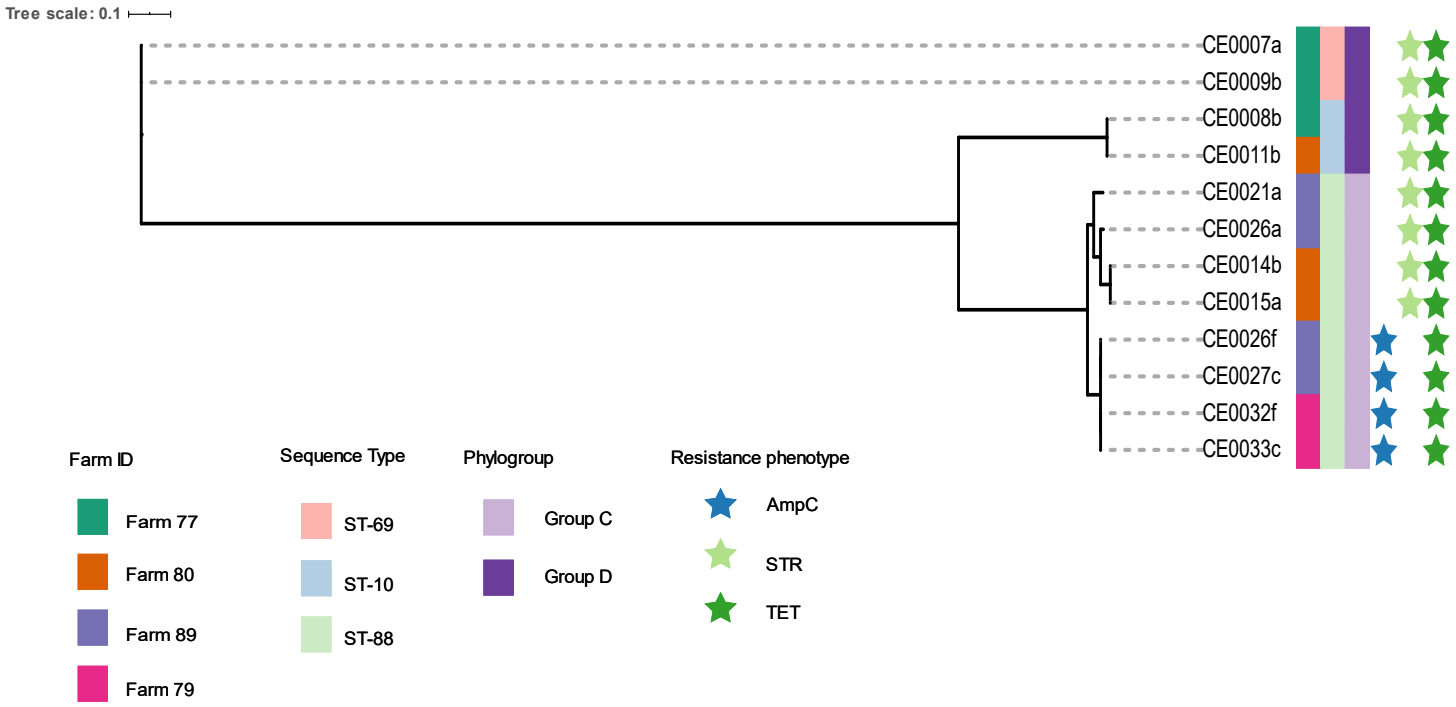


Figure 9. Neighbour joining phylogeny tree of the 12 *E. coli* isolates generated using 141221 core SNPs and annotated with the farm ID, sequence type, phylogroup (colour strip) and resistance phenotype (stars) using the Interactive Tree of Life (IToL) and Inkscape software.

4 Discussion

This study aimed to investigate the prevalence of ESBL and AmpC producing *E. coli* isolated from recto-anal mucosal swabs (RAMS) from waste milk fed dairy calves. Using both culture dependent isolation methods and whole genome sequencing, the study also aimed to phenotypically and genotypically characterise ESBL and AmpC producing isolates. The prevalence of AmpC producing *E. coli* at the calf level was 25% (10/40) (95% CI 13%-41%) and at the isolate level was 30% (31/104) (95% CI 21%-40%). None of the isolates displayed an ESBL phenotype.

The AmpC prevalence was lower than previous studies conducted overseas from waste milk fed dairy calves. A study conducted in the Czech Republic from 13 dairy calves with documented antimicrobial usage including cefoperazone and cefquinome, a 3rd and 4th generation cephalosporin respectively, reported all 13 calves shedding cefotaxime-resistant *Escherichia coli* with 94% (82/87) of *E. coli* isolates displaying an AmpC phenotype and none displaying an ESBL phenotype (Manga et al., 2019). Another study from the United Kingdom (UK) reported an ESBL/AmpC prevalence of 75% (12/16) *E. coli* isolates from waste milk fed dairy calves (Randall et al., 2014). Another UK study conducted by Brunton et al. (2014) reported a prevalence of 77% (20/25) of dairy calves shedding cefotaxime resistant *E. coli* isolate in calves that were fed waste milk containing cefquinome a 4th generation cephalosporin. In Germany Aust et al. (2013) reported a prevalence of 44% (22/50) of calves that shed extended spectrum cephalosporins resistant *E. coli* (ESC-E) isolates that were fed waste milk containing cefoperozone, cefotaxime and cefquinome. A New Zealand based study, which compared the prevalence of antibiotic resistant *E. coli* from waste and non-waste milk fed dairy calves across 12 Waikato dairy farms, found no 3rd generation cephalosporin resistant *E. coli*. However, 5.2% (10/191) of *E. coli* isolates were resistant to tetracycline from that study (Jarvis & McDougall, 2016).

The low prevalence of ESBL/AmpC producing *E. coli* in both our study and that of the Jarvis and McDougall (2016) study could be due to New Zealand dairy farms being low users of antibiotics, particularly 3rd and 4th generation cephalosporins (Bryan & Hea, 2017; Compton & McDougall, 2014). However, in our study data on antibiotic use in study farms was not available, hence it is difficult to determine whether the low prevalence could have been due to low antibiotic use. Other farm management practices such as ryegrass grazing and outdoor

housing used in pasture-based farming system that is practiced in New Zealand may also be associated with low incidences of AMR compared to the intensive farming systems practiced in Europe, Asia and the USA which has been hypothesised to have higher incidences of AMR (Hillerton et al., 2017; Maron et al., 2013).

This present study also found that the AmpC producing *E. coli* isolates were chromosomal mediated. The phenotypic and genotypic results also indicated that none of the AmpC hyperproducing isolates were multidrug resistant (not resistant to more than three classes of antibiotics). This is in line with data presented by Pitout and Laupland (2008) that show ESBLs and plasmid mediated AmpC, but not overexpressed chromosomal mediated AmpC being associated with multidrug resistance. This is due to the ability of plasmids picking up different resistance genes which could lead to accumulation of various resistant genes and hence emergence of multi-drug resistance isolates (Bush, 2018).

This study also found mutations in the promoter and attenuator region of the *ampC* gene that could influence the hyperproduction of the AmpC. Expression of the chromosomal *ampC* gene is not induced in *E. coli* when exposed to β-lactams. This is due to lack of the *ampR* gene that suppresses the expression of the *ampC* gene when the bacterium is not exposed to the inducer (β-lactams antibiotics); contrary to other Enterobacteriaceae. However, expression and overproduction of AmpC can still occur after mutation in the promoter (-35 box and -10 box) or attenuator region (Jacoby, 2009; Philippon et al., 2002). The promoter and the attenuator are the functional elements of the *ampC* gene. They regulate the mRNA transcription, hence any change in these regions affects the production of the AmpC enzyme (Jaurin & Grundström, 1981). Analysis of the Sanger sequence data indicated mutation at positions -42, -18, -1 and +58 in the promoter and attenuator region of the *ampC* gene. The same mutations were evident in all isolates. These mutations were the same as those found in *E. coli* isolates from veal calves (Ceccarelli et al., 2019) and clinical *E. coli* isolates (Caroff et al., 1999; Mulvey et al., 2005; Olsson et al., 1982; Tracz et al., 2007). Studies have shown that mutations at positions -42, -18 and -1 lead to overproduction of the AmpC enzyme by enhancing transcription, while changes at the +58 position modifies the attenuator loop structure, thereby increasing the transcription process leading to overexpressed AmpC enzyme (Caroff et al., 1999). In addition, studies suggest that mutations at the -35 box are the most important for increased transcription while the attenuator is the less important (Caroff et al., 1999; Mulvey et al., 2005).

In this study, 18/104 *E. coli* isolates were co-resistance to tetracycline and streptomycin from 12/40 calves. This was in line with the results reported in previous studies which reported high co-resistance between tetracycline and streptomycin in *E .coli* isolates from dairy farms, despite these antibiotics not being used on the farm (Kyselková et al., 2015; Maynou et al., 2017a; Maynou et al., 2017b). Kyselková et al. (2015) hypothesised that these resistant bacteria emerged due to the mobilization and transfer of resistant genes from bacteria or genes found in the farm environment to fecal derived isolates. Similarly, a study by Srinivasan et al. (2008) detected co-resistance between tetracycline and streptomycin in *E. coli* isolates from dairy farm soil suggesting there could be clonal spread of tetracycline and aminoglycoside resistant *E. coli* or horizontal gene transfer from dairy cattle to the soil or vice versa. However, no sequencing of the isolates or their plasmids were done to support this hypothesis. In our study we did not have information on tetracycline or streptomycin use on the farms. However, tetracycline and aminoglycosides are the next commonly used antibiotics after the penicillins in New Zealand dairy farms (Bryan & Hea, 2017; McDougall et al., 2018). None of the isolates were resistant to ciprofloxacin. This could be due to ciprofloxacin being rarely used in dairy farms in New Zealand (Bryan & Hea, 2017). However, this antibiotic was included in the susceptibility testing as it is a critically important antibiotic, according to the classification by WHO (2019) and NZVA (2018).

In this present study, bacterial resistance to antibiotics was investigated using the EUCAST/CLSI clinical breakpoints and the ECOFFs generated by the NRI methods. This approach was chosen because NRI Epidemiological Cut-Off values determine whether a collection of bacterial isolates has a non-wild type (less susceptible) or wild type (fully susceptible) phenotype, in contrast to determining the likelihood of treatment failure as is the case with the clinical breakpoints. Results of the resistance profiles were close between these two methods; for example, tetracycline resistance was 39% (41/104 *E. coli* isolates) and 41% (43/104 *E. coli* isolates) using EUCAST/CLSI and NRI methods respectively. However, for cefotaxime, a higher percentage of non-wildtype isolates were noted but none of the isolates were classified as cefotaxime resistant according to the EUCAST/CLSI breakpoints. This could be because the cefotaxime breakpoints are based on human clinical isolates which are different from veterinary isolates due to their difference in physiology, pharmacokinetics and pharmacodynamic between human and livestock hence underestimation of the resistance rate. A study in Portugal that assessed antibiotic resistance in *E. coli* and *Salmonella* in wild ungulates using both the NRI and EUCAST/CLSI breakpoints found that NRI ECOFFs were

generally lower than the EUCAST/CLSI breakpoints (Dias et al., 2015). The results were contrary to ours, NRI ECOFFs were higher than the EUCAST/CLSI breakpoints, this could be due to *E. coli* isolates from calves being exposed to more selection pressure of antibiotics, biocides and disinfectants compared with the isolates from wild ungulates whose exposure is limited. Another study in Scotland and Norway that investigated *E. coli* and *S. aureus* resistance in sheep, using both EUCAST/CLSI breakpoints and NRI ECOFFs, found disparities between the two methods with the later able to pick up non-wild type isolates that were missed by the EUACAST/CLSI method (Silva et al., 2020). This highlights the importance of developing host-species specific ECOFFs.

Phylogenetic analysis showed that the *E. coli* isolates were distributed among groups B1, C and D, with none of the resistant isolates belonging to A, B2, E and F. Phylogrouping of *E. coli* can be achieved through the sequencing of four genes (*chuA, yjaA , TspE4.C2* and *arpA*) by using the Clermont method grouping the *E. coli* into 7 groups A, B1, B2, C, D, E and F (Clermont et al., 2013). The basis is that the genomic structure of *E. coli* isolates that belong to a particular phylogroup are not randomly distributed, but rather are associated with a common source or sources. Therefore it is important to phylogenetically group *E. coli* to provide a better understanding of their population and potential disease causing abilities (Tenaillon et al., 2010). Due to time constraints, only resistant isolates (n = 57) were phylotyped using the Clermont quadruplex PCR method. Phylogroup C was the most common 65% (37/57), followed by B1 25% (14/57). Most AmpC producing isolates belonged to phylogroup C (19), followed by B1 (10). Similar results were observed among tetracycline and/or streptomycin resistant isolates with most isolates belonging to phylogroup C (34) and (4) to B1. Our results were different from previous studies, that used non-selective media, found that phylogroup A and B1 were the most common in healthy and diarrheic calves respectively (Barzan et al., 2017; Coura et al., 2017). In the study by Barzan et al. (2017) B1 was the second most abundant phylogroup, as found in our study. Our results also indicate that most of the isolates were commensal (phylogroup C and B1) while only four isolates belonged to phylogroup D, which is commonly associated with extraintestinal characteristics. In addition, two isolates could not be assigned to any group according to the Clermont method, this could be due to the strain being extremely rare, or due to having variable gene content as a result of gene loss which is common in *E. coli* (Touchon et al., 2009). It should be noted that most of the B1 and D isolates were from calves of the same farm (VCF77), while group C were distributed between 3 farms (VCF79, 80 and 89).

Whole genome sequencing has become a gold standard in the surveillance, typing and diagnosis of pathogens. It allows comparisons between the phenotypic and genotypic characteristics of a pathogen and how different genes associate with each other as well as determining transmission pathways (Awosile et al., 2020). In this study, using the 7-allele house-keeping genes (*adk, fumC, gryB, icd, mdh, purA* and *recA),* three sequence types were observed with ST88 being the most common. Various studies have reported ST88 being common in calves and it has been associated with an enterotoxigenic *E. coli* (ETEC) pathotype and antimicrobial resistance (Awosile et al., 2020; Haenni et al., 2014a). Two isolates belonged to ST69. Studies have demonstrated that ST69 display an extra-intestinal pathogenic *E. coli* (EXPEC) pathotype associated with urinary tract infections (UTI) in humans, and are mostly phylogroup D (Denamur et al., 2020; Ramchandani et al., 2005). In our study both isolates belonged to phylogroup D which was concordant with the findings of other studies. Clinically significant virulence genes, e.g. those associated with Shiga toxins were not found, but *afaA, espP, irp, fyuA, pap* and other genes associated with EXPEC were present (Bélanger et al., 2011; Dezfulian et al., 2003).

The phenotypic resistance results were concordant with the genotypic results. In addition, further resistance genes for trimethoprim, sulfonamides and chloramphenicol were found that were not tested phenotypically, which suggests that the tetracycline and streptomycin resistance isolates are multidrug resistance. In addition, *qacEdelta* genes coding for quaternary ammonium were detected. Quaternary ammonium products are used as disinfectants in dairy farms. However, they are under critical review due to their association with AMR (Davies & Wales, 2019; Hegstad et al., 2010). We did not have information on the use of antibiotics, from the study farms, however sulfonamide use has been reported in dairy farms in New Zealand while chloramphenicol and trimethoprim are rarely used (Bryan & Hea, 2017). In this case these resistance genes may be linked (found on the same plasmid) to the *tet* genes, where the use of tetracycline may select for both the *tet* genes and other resistance genes. This shows the importance of whole genome sequencing providing genetic information that is not readily available using phenotypic methods.

Analysis of core genome SNP profiles also demonstrated the phylogenetic relationship between isolates. It was observed that isolates were clustered by resistance phenotype, sequence type and phylogroups, but not by farm. Noticeably isolates CE0026f, CE0027c, CE0032f and CE0033c were closely related and clustered by the AmpC and tetracycline

resistant phenotype even though they were from different farms. This indicated that there may be clonal spread between farms VCF79 and VCF89 which where about 15 kilometers apart. Results from a Dutch study indicated that clonal spread of *E. coli* may have occurred between dairy farms in the Netherlands which received calves from Dutch farms and other European countries. Analysis revealed that the source was from one of the current receiving farms that was not thoroughly disinfected prior to receiving of the new batch of calves that distributed the calves to other farms (Hordijk et al., 2013a). Using WGS Findlay et al. (2020) also reported clonal spreads of *E. coli* isolates between dairy farms in a range of 1500 square kilometers. Isolates CE0007a and CE0009b from the same farm VCF77 were observed to be distinct from the rest of the isolates but showed that transmission between calves within the farm may have occurred.

5 Limitations of the study

This study investigated waste-milk fed calves only, therefore associations between the use of waste milk and the presence of antibiotic resistance bacteria could not be made. In addition, we did not have on-farm antibiotic use data to make any conclusions on the presence of antimicrobial resistant *E. coli.* Furthermore, there was sampling bias in the selection of farms and calves for screening of *E. coli* as well as the selection of isolates for the whole genome sequencing. Lastly the study sample size was inadequate to generalize results as a representative of all farms that use waste milk in New Zealand. Nevertheless, it's a starting point in providing information about AMR in farms that use waste milk.

6 Recommendations

There is lack of data on ESBL/AmpC producing *E. coli* in dairy calves in New Zealand, therefore, we recommend further epidemiological studies to be carried out across New Zealand to determine the prevalence AMR in both waste milk fed calves and non-waste milk fed calves. We also recommend surveillance data on the use of antibiotics in dairy farms. Having up to date information on how antibiotics are used will provide an understanding on how antibiotic use may influence AMR.

7 Conclusion

This study has taken the first step in characterising ESBL/AmpC producing *E. coli* in waste milk fed dairy calves from the Canterbury region, New Zealand. Our results indicate the presence of AmpC hyperproducing *E. coli* in calves but not plasmid mediated AmpC or ESBLs. The results also indicated the presence of other resistance phenotypes; tetracycline and streptomycin. Whole genome sequencing of selected resistant isolates provided some genomic context on the additional resistance genes displayed by the tetracycline and streptomycin resistant isolates making them multidrug resistance but not AmpC positive isolates. Whole genome sequence also revealed clonal transmission of *E. coli* within farm and between farms. Further investigations are required to determine whether these antibiotic resistance *E. coli* are associated with the use of waste milk.

8 References

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9 Appendices

Appendix A. Table of detailed information of the Four dairy farms from Canterbury region and their metadata

Farm ID	Farm sample ID	Date visited	Farming system	Milking Herd size	Spring calving size	Shed type	Shed animal count	Floor of the shed	Substrate	Water source	Effluent method
77	VCF77	2014-9-11	Dairy only	950	650	Open	22	earth	straw	bore	spread
						Open	25	earth	straw		
						Open	80	earth	straw		
80	VCF80	2014-9-15	Dairy only	1000	1000	Open	8	earth	woodchips	bore	spread
						Open	9	earth	woodchips		
						Open	10	earth	woodchips		
89	VCF89	2014-9-17	Dairy only	1700	1700	Conversion	5	earth	woodchips	city	spread
						open	40	metal stone	woodchips		
						Conversion	5	earth	woodchips		

79	VCF79	2014-9-15	Dairy only	1100	1100	Open	7	slats	straw	bore	spread
						open	8	metal stone	sawdust		
						Conversion	11	metal stone	sawdust		

Appendix B. Table of detailed information of the dairy calves from Canterbury region and their metadata

Farm sample ID	**Calf ID**	**Calf sample ID**	**Date sampled**	**Age**	**Sex**	**Breed**	**Class**
VCF77	1128	VC1128	2014-09-11	Young[1]	Male	Friesian	Bobby
VCF77	1129	VC1129	2014-09-11	Young	Male	Friesian	Bobby
VCF77	1130	VC1130	2014-09-11	Young	Male	Friesian	Bobby

VCF77	1131	VC1131	2014-09-11	Young	Male	Friesian	Bobby
VCF77	1132	VC1132	2014-09-11	Young	Male	Friesian	Bobby
VCF77	1133	VC1133	2014-09-11	Young	Female	Friesian	Replacement
VCF77	1134	VC1134	2014-09-11	Young	Female	Friesian	Replacement
VCF77	1135	VC1135	2014-09-11	Young	Female	Friesian	Replacement
VCF77	1136	VC1136	2014-09-11	Young	Female	Friesian	Replacement
VCF77	1137	VC1137	2014-09-11	Young	Female	Friesian	Replacement

VCF80	1173	VC1173	2014-09-15	Young	Male	Friesian	Bobby
VCF80	1174	VC1174	2014-09-15	Young	Male	Friesian	Bobby
VCF80	1175	VC1175	2014-09-15	Young	Female	Friesian	Bobby
VCF80	1176	VC1176	2014-09-15	Young	Male	Friesian	Bobby
VCF80	1177	VC1177	2014-09-15	Young	Male	Friesian	Bobby
VCF80	1178	VC1178	2014-09-15	Young	Female	Friesian	Replacement

VCF80	1179	VC1179	2014-09-15	Young	Female	Friesian	Replacement
VCF80	1180	VC1180	2014-09-15	Young	Female	Friesian	Replacement
VCF80	1181	VC1181	2014-09-15	Young	Female	Friesian	Replacement
VCF80	1182	VC1182	2014-09-15	Young	Female	Friesian	Replacement
VCF89	1299	VC1299	2014-09-17	Young	Male	Friesian	Beef
VCF89	1300	VC1300	2014-09-17	Young	Male	Friesian	Beef
VCF89	1301	VC1301	2014-09-17	Young	Male	Friesian	Beef

VCF89	1302	VC1302	2014-09-17	Young	Male	Friesian	Beef
VCF89	1303	VC1303	2014-09-17	Young	Male	Friesian	Beef
VCF89	1304	VC1304	2014-09-17	Young	Female	Friesian	Replacement
VCF89	1305	VC1305	2014-09-17	Young	Male	Friesian	Beef
VCF89	1306	VC1306	2014-09-17	Young	Male	Friesian/Jersey mix	Bobby
VCF89	1307	VC1307	2014-09-17	Young	Male	Friesian	Beef

VCF89	1308	VC1308	2014-09-17	Young	Female	Holstein	Beef
VCF79	1158	VC1158	2014-09-15	Young	Male	Friesian/Jersey mix	Bobby
VCF79	1159	VC1159	2014-09-15	Young	Male	Friesian/Jersey mix	Bobby
VCF79	1160	VC1160	2014-09-15	Young	Male	Friesian/Jersey mix	Bobby
VCF79	1161	VC1161	2014-09-15	Young	Male	Friesian/Jersey mix	Bobby
VCF79	1162	VC1162	2014-09-15	Young	Male	Friesian/Jersey mix	Bobby
VCF79	1163	VC1163	2014-09-15	Young	Male	Jersey	Replacement

VCF79	1164	VC1164	2014-09-15	Young	Male	Friesian/Jersey mix	Replacement
VCF79	1165	VC1165	2014-09-15	Young	Male	Friesian/Jersey mix	Replacement
VCF79	1166	VC1166	2014-09-15	Young	Male	Jersey	Replacement
VCF79	1167	VC1167	2014-09-15	Young	Male	Jersey	Replacement

[1]Young: age range 2–9 days old

Appendix C. Results of screening, identification and antibiotic zone diameters of the isolates recovered from waste milk fed dairy calves from four dairy farms.

FARM ID	CALF SAMPLE ID	ISOLATE ID	PATHOGEN SPECIES	ZONE DIAMETER OF ANTIMICROBIALS (MM)					
				CTX	CPD	FOX	CIP	STR	TET
VCF77	VC1128/CE0001	NG	-	-	-	-	-	-	-
VCF77	VC1129/CE0002	CE0002a	*E. coli*	26.3	25.4	22.5	33.0	17.8	17.4
		CE0002b	*E. coli*	27.2	26.4	20.9	29.1	16.0	19.0
		CE0002c	*E. coli*	18.4	7.6*	13.9*	28.0	17.5	18.0
		CE0002d	*E. coli*	23.8	8.2*	13.2*	30.2	17.6	19.3
		CE0002e	*E. coli*	23.6	8.2*	13.5*	30.2	18.6	19.6
		CE0002f	*E. coli*	23.5	9.1*	13.2*	29.6	18.7	18.1
VCF77	VC1130/CE0003	CE0003a	*E. coli*	30.1	20.2	23.6	28.6	17.4	19.6
		CE0003b	*E. coli*	30.6	25.5	24.4	33.6	17.9	19.8

VCF77	VC1131/CE0004	CE0004a	*E. coli*	29.9	25.1	27.0	29.4	15.9	15.3
		CE0004b	*E. coli*	30.6	25.3	23.3	32.5	17.4	18.5
		CE0004c	*E. coli*	22.6	7.3*	12.7*	32.6	18.2	16.2
		CE0004d	*E. coli*	24.9	8.6*	12.6*	30.8	17.4	18.5
		CE0004e	*E. coli*	23.9	9.7*	12.9*	32.2	18.1	17.2
		CE0004f	*E. coli*	22.7	9.1*	13.1*	31.6	18.0	16.5
VCF77	VC1132/CE0005	CE0005a	*E. coli*	30.4	24.1	25.8	32.2	17.1	17.0
		CE0005b	*K. pneumoniae*	35.0	27.6	25.6	30.6	17.9	21.7
VCF77	VC1133/CE0006	CE0006a	*E. coli*	33.4	25.5	23.3	32.8	18.7	18.9
		CE0006b	*K. pneumoniae*	32.5	28.3	24.2	32.4	18.2	19.3
		CE0006c	*E. coli*	25.0	7.1*	12.3*	34.6	19.5	19.6
		CE0006d	*E. coli*	23.4	7.7*	11.7*	34.7	19.1	18.7
	VC1134/CE0007	CE0007a	*E. coli*	34.4	27.0	25.4	38.1	8.1*	6.0*

VCF77		CE0007b	*E. coli*	31.6	25.8	23.0	33.2	10.7*	6.1*
		CE0007c	*E. coli*	21.9	7.7*	11.8*	34.6	19.3	18.0
		CE0007d	*E. coli*	22.0	7.0*	11.6*	31.8	19.1	17.3
VCF77	VC1135/CE0008	CE0008a	*E. coli*	32.2	26.4	25.5	32.2	18.0	20.1
		CE0008b	*E. coli*	36.6	28.4	26.2	39.6	7.9*	6.0*
VCF77	VC1136/CE0009	CE0009a	*E. coli*	35.2	27.8	27.0	34.0	18.1	18.9
		CE0009b	*E. coli*	32.8	27.7	24.6	33.1	6.2*	6.0*
VCF77	VC1137/CE0010	CE0010a	*E. coli*	36.2	29.4	25.5	39.8	9.0*	6.0*
		CE0010b	*K. pneumoniae*	35.4	30.6	23.2	29.7	19.6	19.9
VCF80	VC1173/CE0011	CE0011a	*E. coli*	36.4	29.6	25.6	36.2	6.0*	6.0*
		CE0011b	*E. coli*	36.4	30.4	26.4	37.2	6.0*	6.0*
VCF80	VC1174/CE0012	CE0012a	*E. coli*	32.6	27.6	25.7	36.0	6.0*	6.0*
		CE0012b	*E. coli*	35.4	26.3	24.8	37.6	6.0*	6.0*

VCF80	VC1175/CE0013	CE0013a	*E. coli*	33.0	25.3	26.2	35.2	9.2*	6.0*
		CE0013b	*E. coli*	36.6	26.2	26.5	37.4	6.0*	6.0*
VCF80	VC1176/CE0014	CE0014a	*E. coli*	35.2	28.6	26.6	39.0	17.5	20.2
		CE0014b	*E. coli*	32.8	26.6	26.6	37.2	6.0*	6.0*
VCF80	VC1177/CE0015	CE0015a	*E. coli*	32.6	26.8	25.2	33.2	6.0*	6.0*
		CE0015b	*E. coli*	32.8	27.0	27.5	32.6	16.8	6.0*
VCF80	VC1178/CE0016	CE0016a	*E. coli*	35.3	30.0	26.4	34.8	16.6	22.5
		CE0016b	*E. coli*	36.2	25.3	23.1	32.0	16.0	18.7
VCF80	VC1179/CE0017	CE0017a	*E. coli*	34.0	28.8	26.4	37.2	6.0*	6.0*
		CE0017b	*E. coli*	35.0	28.6	26.4	36.2	6.1*	6.0*
VCF80	VC1180/CE0018	CE0018a	*E. coli*	37.3	30.2	24.7	39.6	16.8	20.6
		CE0018b	*E. coli*	33.0	25.9	27.9	33.7	17.6	20.8
VCF80	VC1181/CE0019	CE0019a	*E. coli*	32.6	27.8	24.0	31.2	15.7	19.2

		CE0019b	*E. coli*	34.4	28.2	26.4	30.5	15.7	19.9
VCF80	VC1182/CE0020	CE0020a	*E. coli*	34.5	28.4	26.0	30.7	16.6	21.0
		CE0020b	*E. coli*	31.8	28.8	27.3	34.6	16.2	18.2
VCF89	VC1299/CE0021	CE0021a	*E. coli*	36.0	28.6	26.2	34.8	6.0*	6.0*
		CE0021b	*E. coli*	36.2	28.8	25.6	36.4	6.0*	6.0*
VCF89	VC1300/CE0022	CE0022a	*E. coli*	34.4	26.4	28.0	34.7	6.0*	19.7
		CE0022b	*E. coli*	35.2	26.4	27.5	33.7	18.2	20.4
VCF89	VC1301/CE0023	CE0023a	*E. coli*	32.7	26.3	26.8	32.9	17.6	21.3
		CE0023b	*E. coli*	33.9	25.8	25.5	35.5	19.3	20.2
VCF89	VC1302/CE0024	CE0024a	*E. coli*	35.6	29.1	25.3	39.7	11.8	6.0*
		CE0024b	*E. coli*	35.7	28.2	25.6	38.9	11.2	6.0*
VCF89	VC1303/CE0025	CE0025a	*E. coli*	32.8	29.1	26.3	38.0	17.7	19.2
		CE0025b	*E. coli*	36.2	28.6	25.2	39.0	11.7	6.0*

VCF89	VC1304/CE0026	CE0026a	*E. coli*	32.9	25.5	25.7	36.4	10.1*	6.0*
		CE0026b	*E. coli*	34.3	28.6	25.0	38.4	17.8	21.3
		CE0026c	*E. coli*	24.0	6.1*	13.6*	32.4	18.4	6.0*
		CE0026d	*E. coli*	24.4	6.2*	13.8*	34.3	18.0	6.1*
		CE0026e	*E. coli*	24.6	6.2*	14.0*	34.4	18.3	6.1*
		CE0026f	*E. coli*	24.2	6.4*	14.5*	34.6	18.4	6.2*
VCF89	VC1305/CE0027	CE0027a	*E. coli*	34.7	27.2	25.0	36.3	11.2	6.0*
		CE0027b	*E. coli*	33.4	28.2	25.0	33.2	14.1	12.2*
		CE0027c	*E. coli*	27.8	13.0*	17.9*	32.0	15.4	6.0*
		CE0027d	*E. coli*	26.4	13.0*	17.2*	31.2	15.1	6.0*
		CE0027e	*E. coli*	24.2	6.0*	14.2*	32.4	18.3	6.0*
		CE0027f	*E. coli*	23.4	6.0*	14.2*	32.2	18.5	6.0*
VCF89	VC1306/CE0028	CE0028a	*E. coli*	35.8	29.0	24.1	40.0	20.4	20.2

		CE0028b	*E. coli*	37.0	30.0	25.3	39.6	20.8	21.7
VCF89	VC1307/CE0029	CE0029a	*E. coli*	35.2	30.2	26.8	35.6	18.2	23.7
		CE0029b	*K. pneumoniae*	36.4	32.8	27.1	32.5	18.6	21.1
VCF89	VC1308/CE0030	CE0030a	*E. coli*	32.0	29.2	24.6	36.8	18.0	20.0
		CE0030b	*K. pneumoniae*	32.7	31.0	27.7	34.4	19.0	21.4
VCF79	VC1158/CE0031	CE0031a	*E. coli*	35.0	30.0	27.0	40.0	19.4	21.0
		CE0031b	*E. coli*	35.4	30.0	26.3	37.0	18.4	21.4
VCF79	VC1159/CE0032	CE0032a	*E. coli*	31.4	25.1	25.6	36.7	18.9	19.0
		CE0032b	*E. coli*	31.3	28.2	26.7	35.0	18.2	19.3
		CE0032c	*E. coli*	24.0	6.1*	14.7*	34.4	18.5	6.0*
		CE0032d	*E. coli*	25.6	6.1*	15.0*	35.0	19.5	6.0*
		CE0032e	*E. coli*	24.4	6.1*	14.8*	33.3	19.0	6.0*
		CE0032f	*E. coli*	24.9	6.2*	14.1*	34.4	18.5	6.0*

VCF79	VC1160/CE0033	CE0033a	*E. coli*	32.3	23.7	22.6	36.1	20.3	20.9
		CE0033b	*E. coli*	36.0	28.6	27.7	40.0	21.4	6.0*
		CE0033c	*E. coli*	26.1	6.2*	14.6*	33.5	18.8	6.0*
		CE0033d	*E. coli*	25.7	6.0*	14.2*	32.8	18.7	6.0*
		CE0033e	*E. coli*	24.2	6.1*	14.6*	33.8	18.1	6.0*
		CE0033f	*E. coli*	24.2	6.2*	14.7*	30.5	18.1	6.0*
VCF79	VC1161/CE0034	CE0034a	*E. coli*	32.7	28.6	26.8	34.6	20.8	20.8
		CE0034b	*E. coli*	34.8	28.6	26.3	38.1	19.6	20.5
		CE0035a	*E. coli*	34.2	30.2	26.4	33.5	17.1	22.0
VCF79	VC1162/CE0035	CE0035b	*E. coli*	34.0	28.0	26.4	33.0	16.5	22.6
VCF79	VC1163/CE0036	CE0036a	*E. coli*	36.0	27.6	25.3	36.9	18.6	20.8
		CE0036b	*E. coli*	38.4	32.8	25.4	37.2	20.1	21.5
VCF79	VC1164/CE0037	CE0037a	*E. coli*	24.0	28.1	24.9	37.0	20.3	22.2

		CE0037b	*E. coli*	35.2	26.6	26.5	38.0	18.5	21.3
VCF79	VC1165/CE0038	CE0038a	*E. coli*	35.4	28.9	22.8	35.5	17.6	20.6
		CE0038b	*E. coli*	36.0	29.6	27.2	38.0	19.0	20.9
VCF79	VC1166/CE0039	CE0039a	*E. coli*	32.7	25.9	23.6	36.0	18.5	20.0
		CE0039b	*E. coli*	33.3	28.1	26.0	34.9	18.7	24.7
		CE0039e	*E. Coli*	24.7	6.0*	13.1*	35.8	18.9	19.7
VCF79	VC1167/CE0040	CE0040a	*E. coli*	32.5	27.1	24.4	37.2	19.4	21.4
		CE0040b	*E. coli*	34.6	28.9	27.2	38.2	18.5	20.4
		CE0040c	*E. coli*	24.2	6.0*	13.6*	38.0	18.9	19.7
		CE0040e	*E. coli*	24.6	6.2*	13.2*	38.2	18.8	19.8

a: MacConkey, b: MacConkey, c: MacConkey + CTX, d: MacConkey + CTX, e: MacConkey + CAZ, f: MacConkey + CAZ

CTX: cefotaxime, CPD: cefpodoxime, FOX: cefoxitin, STR: streptomycin, CIP: ciprofloxacin, TET: tetracycline, CAZ: ceftazidime

NG: No growth

*: Possible resistance

Appendix D. Phenotypic confirmation tests for ESBL or AmpC producing *E. coli* isolates

Isolate ID	**Cultured from**	**Pathogen specie**	**Zone diameters (mm)**											**ESBL/AmpC phenotype**
			ESBL *CTX*[1]			**ESBL *CAZ*[2]**			***AmpC*[3]**					
			Z1	**Z2**	**Z2-Z1**	**Z1**	**Z2**	**Z2-Z1**	**Z1**	**Z2**	**Z3**	**Z3-Z1**	**Z3-Z1**	
CE0002c	CAZ	*E. coli*	27.9	27.6	-0.3	22.6	20.4	-2.2	10.9	10.9	27.8	16.9	16.9	AmpC
CE0002d	CAZ	*E. coli*	25.8	24.8	-1	21.3	18.3	-3	10.8	11.0	23.8	13	12.8	AmpC
CE0002e	CTX	*E. coli*	26.2	25.2	-1	22.0	19.0	-3	10.8	10.9	26.5	15.7	15.6	AmpC

CE0002f	CTX	*E. coli*	24.9	25.2	0.3	20.6	18.0	-2.6	11.3	11.3	26.0	14.7	14.7	AmpC
CE0004c	CAZ	*E. coli*	27.1	26.2	-0.9	22.4	19.3	-3.1	11.1	11.6	26.4	15.3	14.8	AmpC
CE0004d	CAZ	*E. coli*	26.5	26.1	-0.4	21.6	19.1	-2.5	10.6	11.1	26.9	16.2	15.8	AmpC
CE0004e	CTX	*E. coli*	26.1	27.0	0.9	22.0	19.7	-2.3	11.1	11.1	27.4	16.3	16.3	AmpC
CE0004f	CTX	*E. coli*	26.7	26.9	0.2	21.2	19.2	-2	11.0	11.2	27.0	16.0	15.8	AmpC
CE0006c	CAZ	*E. coli*	26.5	25.6	-0.9	21.5	19.3	-2.2	11.2	11.1	26.6	15.4	15.5	AmpC
CE0006d	CTX	*E. coli*	27.0	26.1	-0.9	22.3	19.8	-2.5	13.6	13.7	27.8	14.2	14.3	AmpC

CE0007c	CAZ	*E. coli*	26.9	25.6	-1.3	22.2	19.9	-2.3	11.7	11.7	27.7	16	16	AmpC
CE0007d	CAZ	*E. coli*	26.0	25.4	-0.6	22.5	20.6	-1.9	13.2	14.0	27.6	14.4	13.6	AmpC
CE0026c	CAZ	*E. coli*	27.9	27.2	-0.7	22.9	19.8	-3.1	15.1	15.7	27.7	12.6	12	AmpC
CE0026d	CAZ	*E. coli*	25.6	27.0	1.4	20.5	18.9	-1.6	16.0	16.0	28.1	12.1	12.1	AmpC
CE0026e	CTX	*E. coli*	28.3	27.9	-0.4	22.6	19.9	-2.7	15.4	16.7	27.8	12.4	11.1	AmpC
CE0026f	CTX	*E. coli*	26.8	26.2	-0.6	20.2	17.8	-2.4	11.6	11.7	27.1	15.5	15.6	AmpC

CE0027c	CAZ	*E. coli*	27.2	27.6	0.4	19.7	17.9	-1.8	11.0	11.8	26.0	15.0	14.2	AmpC
CE0027d	CAZ	*E. coli*	26.3	25.8	-0.5	19.6	17.1	-2.5	10.9	11.0	26.0	15.1	15	AmpC
CE0027e	CTX	*E. coli*	26.4	25.9	-0.5	19.9	16.7	-3.2	11.3	11.6	27.0	15.7	15.4	AmpC
CE0027f	CTX	*E. coli*	27.3	26.7	-0.6	19.6	17.6	-2.0	11.1	11.1	26.8	15.7	15.7	AmpC
CE0032c	CAZ	*E. coli*	26.9	25.7	-1.2	20.1	17.4	-2.7	10.6	10.8	27.3	16.7	16.5	AmpC
CE0032d	CAZ	*E. coli*	26.9	26.0	-0.9	20.1	17.0	-3.1	10.4	10.5	26.9	16.5	16.4	AmpC
CE0032e	CTX	*E. coli*	26.9	26.2	-0.7	20.7	17.3	-3.4	10.7	10.7	27.4	16.7	16.7	AmpC

Ce0032f	CTX	*E. coli*	26.6	26.1	-0.5	20.2	16.8	-3.4	10.4	11.0	26.4	16.0	15.4	AmpC
CE0033c	CAZ	*E. coli*	26.3	25.3	-1.0	20.2	17.5	-2.7	10.7	11.4	26.7	16.0	15.3	AmpC
CE0033d	CAZ	*E. coli*	26.3	26.1	-0.2	20.2	17.2	-3.0	10.5	11.6	25.5	15.0	13.9	AmpC
CE0033e	CTX	*E. coli*	26.5	26.2	-0.3	19.4	16.5	-2.9	11.3	1.6	26.2	14.9	14.6	AmpC
CE0033f	CTX	*E. coli*	26.6	25.3	-1.3	20.6	16.7	-3.9	10.1	11.4	26.0	15.9	14.6	AmpC
CE0039e	CTX	*E. coli*	26.8	26.1	-0.7	20.0	15.9	-4.1	9.6	10.1	26.7	17.1	16.6	AmpC

CE0040c	CAZ	*E. coli*	26.4	25.1	-1.3	19.7	16.1	-3.6	9.2	10.1	26.8	17.6	16.7	AmpC
CE0040e	CTX	*E. coli*	26.4	26.0	-0.4	19.3	16.3	-3.0	9.4	10.4	26.4	17.0	16.0	AmpC

[1] ESBL CTX = clavulanate double disc synergy test with cefotaxime, [2] ESBL CAZ = clavulanate double disc synergy test with ceftazidime,

[3] AmpC = AmpC double disc diffusion test, Z = zone

Appendix E. Distribution of *E. coli* phylogroups among the resistant and the susceptible *E. coli* isolates

Isolate ID	**arpA (400bp)**	**chuA (288bp)**	**yjaA (211bp)**	**TspE4C2 (152bp)**	**Extra (476bp)**	**Phylo-group**	**Resistant phenotype**
CE0002c	+	-	-	+	-	B1	AmpC[1],
CE0002d	+	-	-	+	-	B1	AmpC
CE0002e	+	-	-	+	-	B1	AmpC
CE0002f	+	-	-	+	-	B1	AmpC

CE0004c	+	-	-	+	-	B1	AmpC
CE0004d	+	-	-	+	-	B1	AmpC
CE0004e	+	-	-	+	-	B1	AmpC
CE0004f	+	-	-	+	-	B1	AmpC
CE0006c	-	-	-	+	-	Unknown	AmpC
CE0006d	-	-	-	+	-	Unknown	AmpC
CE0007a	+	-	+	-	-	C	STR[2], TET[3]
CE0007b	+	+	-	-	-	D	STR, TET
CE0007c	+	-	-	+	-	B1	AmpC
CE0007d	+	-	-	+	-	B1	AmpC
CE0008b	+	+	-	-	-	D	STR, TET
CE0009b	+	-	+	-	-	C	STR, TET
CE0010a	+	+	-	-	-	D	STR, TET

CE0011a	+	-	+	-	-	C	STR, TET
CE0011b	+	-	+	-	-	D	STR, TET
CE0012a	+	-	-	+	-	B1	STR, TET
CE0012b	+	-	+	-	-	C	STR, TET
CE0013a	+	-	+	-	-	C	STR, TET
CE0013b	+	-	+	-	-	C	STR, TET
CE0014b	+	-	+	-	-	C	STR, TET
CE0015a	+	-	-	+	-	B1	STR, TET
CE0015b	+	-	+	-	-	C	TET
CE0017a	+	-	+	-	-	C	STR, TET
CE0017b	+	-	+	-	-	C	STR, TET
CE0021a	+	-	+	-	-	C	STR, TET
CE0021b	+	-	+	-	-	C	STR, TET

CE0022a	+	-	-	+	-	B1	STR
CE0024a	+	-	+	-	-	C	TET
CE0024b	+	-	+	-	-	C	TET
CE0025b	+	-	+	-	-	C	TET
CE0026a	+	-	+	-	-	C	STR, TET
CE0026c	+	-	+	-	-	C	AmpC, TET
CE0026d	+	-	+	-	-	C	AmpC, TET
CE0026e	+	-	+	-	-	C	AmpC, TET
CE0026f	+	-	+	-	-	C	AmpC, TET
CE0027a	+	-	+	-	-	C	TET
CE0027b	+	-	-	+	-	B1	TET
CE0027c	+	-	+	-	-	C	AmpC, TET
CE0027d	+	-	+	-	-	C	AmpC, TET

CE0027e	+	-	+	-	-	C	AmpC, TET
CE0027f	+	-	+	-	-	C	AmpC, TET
CE0032c	+	-	+	-	-	C	AmpC, TET
CE0032d	+	-	+	-	-	C	AmpC, TET
CE0032e	+	-	+	-	-	C	AmpC, TET
CE0032f	+	-	+	-	-	C	AmpC, TET
CE0033b	+	-	+	-	-	C	TET
CE0033c	+	-	+	-	-	C	AmpC, TET
CE0033d	+	-	+	-	-	C	AmpC, TET
CE0033e	+	-	+	-	-	C	AmpC, TET
CE0033f	+	-	+	-	-	C	AmpC, TET
CE0039e	+	-	+	-	-	C	AmpC
CE0040c	+	-	+	-	-	C	AmpC

CE0040e	+	-	+	-	-	C	AmpC

Resistant phenotypes; AmpC[1]: STR[2]: Streptomycin, TET[3]: Tetracycline

Appendix F. Virulence genes and what they encode for

Virulence gene	**description**
afaA	Transcriptional regulator
afaD	Afimbrial adhesion
astA	EAST-1 heat stable toxin
chuA	Outer membrane hemin receptor
espP	Extracellular serine protease
fimA	Adhesin
fyuA	Siderophore receptor
iroN	Siderophore receptor protein
irp2	High molecular weight protein 2 non-ribosomal peptide synthetase
iucD	Iron uptake
kpsD	Polysialic acid transport protein; group 2
kpsM	Polysialic acid transport protein; group 2
ompA	Outer membrane protein
papC	Outer membrane usher P fimbriae

Appendix G. Resistance genes and their phenotypic description

Resistance gene	**Gene encodes**	**Phenotype description**
aadA1	Aminoglycoside adenylyltransferase,	streptomycin
aph(3")-1b	Aminoglycoside 3'-phosphotransferase	streptomycin
aph(3)-1a	Aminoglycoside 3'-phosphotransferase,	neomycin, kanamycin
aph(6)-1d	Aminoglycoside 3'-phosphotransferase	streptomycin
bla_{EC-13}	Beta-lactamase	ampicillin, amoxicillin, cephalosporin
bla_{EC-8}	Beta-lactamase	ampicillin, amoxicillin, cephalosporin
bla_{TEM-1}	Beta-lactamase	ampicillin, amoxicillin, cephalosporin
bla_{TEM-40}	Beta-lactamase	ampicillin, amoxicillin, cephalosporin
catA1	Chloramphenicol acetyltransferase	chloramphenicol
dfrA1, dfrA5	Dihydrofolate reductase	trimethoprim
qacEdelta1	Antiseptic-resistance protein	quaternary ammonium
sul1, sul2	Dihydropteroate synthase	sulphonamides
tet(A), tet(B)	Tetracycline resistance protein	tetracycline

Appendix H. Pairwise core SNP distances between the 12 *E. coli* isolates

	CE0007a	**CE0008b**	**CE0011b**	**CE0014b**	**CE0015a**	**CE0021a**	**CE0026a**	**CE0026f**	**CE0027c**	**CE0032f**	**CE0033c**	**CE0039b**	**Reference**
CE0007a	0	74319	74311	73914	73913	74507	74110	74492	74486	74489	74487	18	87229
CE0008b	74319	0	22	39862	39861	39506	38904	39258	39266	39254	39254	74313	86772
CE0011b	74311	22	0	39856	39855	39500	38898	39252	39260	39248	39248	74305	86776
CE0014b	73914	39862	39856	0	3	5868	3116	7470	7478	7470	7468	73918	86702
CE0015a	73913	39861	39855	3	0	5867	3115	7469	7475	7469	7467	73917	86701
CE0021a	74507	39506	39500	5868	5867	0	4399	6310	6318	6310	6308	74511	86819

CE0026a	74110	38904	38898	3116	3115	4399	0	6303	6311	6303	6301	74114	86564
CE0026f	74492	39258	39252	7470	7469	6310	6303	0	10	46	44	74494	86964
CE0027c	74486	39266	39260	7478	7475	6318	6311	10	0	54	52	74488	86958
CE0032f	74489	39254	39248	7470	7469	6310	6303	46	54	0	2	74491	86958
CE0033c	74487	39254	39248	7468	7467	6308	6301	44	52	2	0	74489	86960
CE009b	18	74313	74305	73918	73917	74511	74114	74494	74488	74491	74489	0	87219
Reference	87229	86772	86776	86702	86701	86819	86564	86964	86958	86958	86960	87219	0

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